THE ENDOTHELIN SYSTEM IN HUMAN CARDIOVASCULAR PHYSIOLOGY AND PATHOPHYSIOLOGY

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ABSTRACT (REGULATION 1.1.3)

The experiments presented here arose from an interest in endothelial function and, particularly, a wish to better understand the pharmacology and physiology of the endothelin (ET) system in human blood vessels in health, and the influence of cardiovascular disease on the ET system. This work followed from the discovery of ET-1 as a peptide endothelial mediator of vascular tone in 1988. Publications are grouped into sections representing different aspects of the work.

Section 1 is concerned with exploring pharmacological responses to the ET family of peptides, the sarafotoxin analogue peptides, and ET antagonists, in human blood vessels *in vivo*. This was amongst the first work with ET-1 in humans, and certainly the first to use the sarafotoxins, ET receptor antagonists and ET converting enzyme (ECE) inhibitors. After characterisation of the pharmacological tools, it was possible to show clearly that endogenous ET-1 plays a physiological role in the control of peripheral resistance and blood pressure in healthy humans, suggesting important clinical applications for these agents. It was also shown that the ETA receptor is the major vasocontrictor receptor and that the major role in health of the ETB receptor is endothelium-dependent vasodilatation, enhancement of which may contribute to the beneficial clinical attributes of ETA receptor antagonism. In addition, local ET-1 infusion in the forearm circulation was shown to be a system whereby the clinical efficacy of systemically administered ET receptor antagonists could be modelled pharmacodynamically.

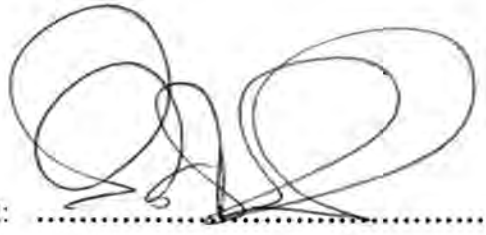
Sections 2-4 cover work confirming the substantial clinical utility of ET receptor antagonists and ECE inhibitors as vasodilators, particularly in essential hypertension, heart failure and renal failure. Other work, following congenital heart surgery, suggests that a cautious approach may be needed in some cases of pulmonary hypertension. Studies with neutral endopeptidase (NEP) inhibitors show unequivocally, but unexpectedly, that these agents are peripheral vasoconstrictors, and the evidence presented is consistent with this effect occurring because endogenously generated vascular ET-1 is an important substrate for NEP.

Section 5 contains some miscellaneous but related studies, together with a series of review articles written from 1991-98 synthesising the literature at each stage and drawing conclusions about potential areas of major clinical interest in cardiovascular disease.

STATEMENT (REGULATION 1.1.4)

(a) Part of the work in Paper 1 was presented in my MD thesis (University of London, 1990). Work in Papers 4, 6-8, and 22 was presented in Dr William Haynes' thesis (University of Sheffield, 1995). Work in Papers 13, 18 and 25 was presented in Dr Charles Ferro's MD thesis (University of Edinburgh, 1998).

(b) This work was performed in close collaboration with a number of young physicians who came under my research supervision between 1990 and 1999. By the nature of complex clinical research, this work was multidisciplinary, often in collaboration with specialist physicians whose patients we were grateful to have the opportunity to study. I was leader of the research team responsible for all the work presented here. In particular, I was responsible for the conception and design of the studies, applications for grant funding, teaching of the research techniques, development of new methods, initiation of the experiments, clinical supervision of the work during its performance, and preparation of the work for publication. I also had the final responsibility of vouching for the content of the submitted manuscripts and of making the decisions on the journals to which the work should be sent.



Signed: ..... (David J. Webb)

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CARDIOVASCULAR PHYSIOLOGY

Papers 1-19

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Papers 28-30

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SECTION 5

MISCELLANEOUS TOPICS AND REVIEWS

Papers 31-34 and 35-46

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SECTION 1

CARDIOVASCULAR PHYSIOLOGY

Papers 1-19

Endothelin is a potent long-lasting vasoconstrictor in men

JOHN G. CLARKE, NIGEL BENJAMIN, SIMON W. LARKIN, DAVID J. WEBB, GRAHAM J. DAVIES, AND ATTILIO MASERI
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CLARKE, JOHN G., NIGEL BENJAMIN, SIMON W. LARKIN, DAVID J. WEBB, GRAHAM J. DAVIES, AND ATTILIO MASERI. *Endothelin is a potent long-lasting vasoconstrictor in men.* Am. J. Physiol. 257 (Heart Circ. Physiol. 26): H2033–H2035, 1989.—Endothelin, a 21-amino acid peptide synthesized by cultured porcine aortic endothelial cells, has recently been identified and shown to produce a potent and prolonged constriction of mammalian blood vessels in vitro. We have studied the effect of local infusion of this peptide on resistance and capacitance vessels of normal volunteers. Infusion of endothelin (5 pmol/min) reduced forearm blood flow by 39 ± 7% from control observations. The maximum response was seen after ~55 min of infusion. After stopping the infusion, return of flow to basal values took ~120 min. This contrasts with the short onset and duration of action observed when angiotensin II was infused. During coinfusion studies, reversal by nicardipine (0.3–10 μg/min) occurred at similar concentrations in both endothelin-induced and angiotensin-induced flow reduction. This observation suggests that nicardipine nonspecifically antagonizes the flow-reducing effects of endothelin. A pattern of slow onset of constriction was found on local infusion of endothelin (5 pmol/min) into dorsal hand veins. During coinfusion of nicardipine (1.5 μg/min), no reversal of endothelin-induced (5 pmol/min) constriction of dorsal hand veins occurred. The pharmacological profile of this peptide in the peripheral circulation of humans suggests that it may be involved in long-term regulation of vascular tone.

vascular; artery; vein; human; endothelium; nicardipine

FOLLOWING REPORTS THAT endothelial cell culture supernatant exhibits vasoconstrictor activity (5, 9, 16), Yanagisawa et al. (23) have isolated and characterized a novel constrictor peptide, endothelin, from the culture supernatant of porcine aortic endothelial cells. They have shown in vitro that the potency of endothelin as a vasoconstrictor is greater than that of other known constrictor agents, including angiotensin II.

Initially, the structural similarity with other peptides that affect membrane ion channels and its sensitivity to nicardipine and extracellular calcium suggested a role for endothelin as an endogenous ligand for voltage-dependent dihydropyridine-sensitive calcium channels (11). However, contractile responses to endothelin, obtained in calcium-free media, unaffected by nifedipine, suggest that endothelin may modulate intracellular calcium (8). Endothelin also developed a slow and sustained contraction in rat aorta in a calcium free medium. When calcium was replaced, a tonic contraction was obtained, which was insensitive to potential-dependent calcium blockers (1). Nicardipine attenuated the sustained but not the initial rise of calcium in cultured vascular smooth muscle cells (10). The contractile properties of a dihydropyridine agonist (BAY K 8644) were compared with endothelin and were found to differ markedly (1). Endothelin failed to displace the binding of a dihydropyridine ([^{3}H]PN 200/110), a phenylakylamine ([^{3}H]desmethoxyverapamil), and a benzothiazepine (*cis*-[^{3}H]diltiazem) in cardiac membranes (7). Calcium channel blockers did not affect the binding of endothelin to cultured vascular smooth muscle cells (10).

Using forearm plethysmography, we have compared the constrictor response of resistance and capacitance vessels to endothelin with the response to angiotensin II and have studied the effect of both peptides in the presence of a dihydropyridine calcium channel blocking agent, nicardipine.

METHODS

Subjects. Twelve healthy male volunteers between 20 and 41 years of age participated in these studies, which were conducted with the approval of the Institutional Ethics Committee and with the informed consent of each volunteer. Studies were performed after subjects had rested supine in a quiet clinical laboratory for a minimum of 30 min. Room temperature (between 25 and 28°C) was maintained within ±1°C for each study.

Forearm arterial studies. Forearm blood flow was measured in both arms using venous occlusion plethysmography with temperature-compensated mercury-in-Silastic strain gauges (20). Collecting cuff pressure was 40 mmHg, and wrist cuff occlusion pressure was 200 mmHg. A 27 standard wire gauge unmounted steel cannula was inserted into the left brachial artery using 1% lignocaine hydrochloride to provide local anesthesia. Drugs were dissolved in saline and were infused at a constant rate of either 0.5 or 1.0 ml/min throughout the experiment by means of constant-rate infusion pumps (Harvard 944A). In experiments where two drugs were infused simultaneously, a Y-connector delayed mixing until the solutions entered the cannula. Flows were recorded for 10 s in every 15 s. The mean of the final five

measurements of each recording period was used for analysis. The percentage change in forearm blood flow after drug administration was calculated according to the method of Greenfield (6). Blood flow was measured during infusion of sterile, toxin-free endothelin (Peninsula Laboratories) or angiotensin II (Calbiochem) for 60 min each at 5 pmol/min. The infusions were followed in nine studies (5 endothelin, 4 angiotensin II) by saline alone for a further 120 min and in eight studies (4 endothelin, 4 angiotensin II) by nicardipine (0.3, 1.0, 3.0, 10 μg/min, each for 5 min).

Venous studies. Endothelin (5 pmol/min) was infused alone for 60 min at a constant rate (0.25 ml/min) into dorsal hand veins of nine normal volunteers, and percentage change in vein size was measured by a modification of the method of Nachev et al. (15). A lightweight lever connected to a transducer was rested on the summit of the vein, 1 cm downstream from the tip of the infusing needle. Vein size was taken as the vertical displacement of this lever on deflating an upper arm cuff inflated to 30 mmHg. In four subjects, after 60 min of endothelin infusion, nicardipine (1.5 μg/min) was coinfused for a further 5 min.

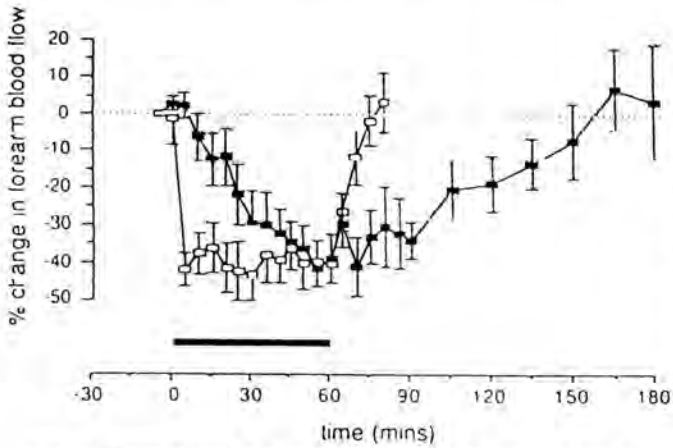

FIG. 1. Endothelin (■, $n = 9$) or angiotensin II (□, $n = 8$) was infused into left brachial artery for 60 min (horizontal bar) each at 5 pmol/min. Endothelin produced a slowly developing reduction in flow, which persisted on stopping infusion, whereas effect of angiotensin II was of rapid onset and offset. Maximum reduction in flow was similar with both peptides.

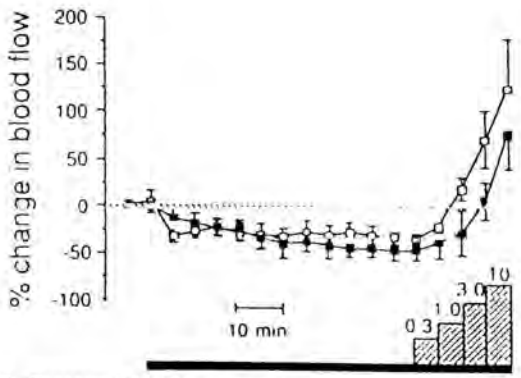

FIG. 2. Forearm blood flow was measured as in Fig. 1. Nicardipine was infused (hatched bars) in addition to either endothelin (■, 5 pmol/min, $n = 4$) or angiotensin II (□, 5 pmol/min, $n = 4$). Reversal of flow reduction with nicardipine was similar with both peptides.

RESULTS

The reduction in forearm blood flow resulting from brachial arterial infusion of 5 pmol/min of endothelin (39 ± 7%) was similar to that induced by 5 pmol of angiotensin II (40 ± 7%) but was slower in onset, taking ~55 min to reach maximum (Fig. 1), and reversed only slowly on stopping the infusion (Fig. 1). Addition of nicardipine (0.3–10 μg/min) to either endothelin or angiotensin II infusion resulted in a similar reversal of flow reduction in both cases (Fig. 2).

A similar constrictor response was seen when endothelin (5 pmol/min) was infused into dorsal hand veins, the maximal mean reduction in size (83 ± 12%) being progressive over 60 min. Nicardipine failed to reverse the endothelin-induced venoconstriction ($n = 4$).

DISCUSSION

The demonstration that cultured mammalian vascular endothelial cells synthesize a potent pressor peptide, endothelin, has provided further evidence of the importance of the endothelium in control of vascular smooth muscle tone. The kinetics of endothelin-induced constriction in human vessels in this study infer that this may be the same constrictor agent originally isolated by Hickey et al. (9) from endothelial cell culture supernatant.

In this study we have demonstrated a potent and long lasting constriction of the forearm resistance vessels and the dorsal hand veins of men. These findings are in agreement with previous in vitro and in vivo observations. However, there are recent reports of vasodilator responses to relatively low doses of endothelin (12, 14, 21, 22). We too observed transient increases of forearm blood flow during the first 5 min of the endothelin infusion, but the changes were small and statistically insignificant (Fig. 1).

The effects of other constrictor peptides (2) may be partly due to enhancement of sympathetically induced constriction. In isolated peripheral vascular tissue, endothelin may interact with sympathetic nerve activity in addition to having a direct vasoconstrictor action, and this may contribute to its effect on forearm resistance vessels (19). Because the dorsal hand veins of warm, relaxed volunteers have no basal sympathetic tone (4), this mechanism is unlikely to be important in the venoconstriction seen in this study.

With the use of the forearm plethysmographic technique, it is difficult to differentiate between reversal of endothelin-induced and reversal of basal flow reduction after administration of calcium channel blocking agents. However, the observation that nicardipine reverses flow reduction caused by both endothelin and angiotensin II at similar concentrations suggests that it is not a specific antagonist of the constrictor effects of endothelin. Given the large multisubunit structure of the Ca^{2+} channel complex (3), endothelin might interact with the L-type Ca^{2+} channel, perhaps by utilizing other binding sites. Alternatively, endothelin may act by increasing free cytosolic calcium, as has been demonstrated in vascular smooth muscle (10); endothelin produces a rapid increase

in $^{45}Ca^{2+}$ efflux from aortic smooth muscle cells even when no extracellular calcium is present, implying that endothelin probably mobilizes Ca^{2+} from intracellular storage sites (13). A third possible mechanism could relate to the effect of endothelin on protein kinase C affinity for calcium, since protein kinase C inhibition can reverse endothelin-induced contraction (18).

The failure of nicardipine to reverse the venoconstrictor action of endothelin at a dose that we (unpublished observations) and others (17) have found to completely abolish K^+-induced constriction suggests that the mechanism of action in veins is independent of voltage-dependent calcium channels.

The pattern of slow onset and offset of action might suggest a role for this novel peptide in the long-term control of peripheral vascular resistance and venous tone.

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The effect of intra-arterial endothelin on resting blood flow and sympathetically mediated vasoconstriction in the forearm of man

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1 The hypothesis that endothelin (ET) influences sympathetically mediated vasoconstriction was investigated in 13 healthy, male subjects.
2 ET (1 pmol min^{-1}) was infused for 60 min into the left brachial arteries of seven healthy male subjects. Resting forearm blood flow, and sympathetic vasoconstriction produced by lower body negative pressure (LBNP; 15 mm Hg), was measured in both arms by strain gauge plethysmography. In a further six subjects, noradrenaline (NA) was infused intra-arterially at doses of 150–600 pmol min^{-1}, with and without co-infusion of ET (1 pmol min^{-1}), with blood flow measured in both forearms.
3 ET produced a small but significant reduction of blood flow in the infused forearm from 3.9 ± 0.6 ml 100 ml^{-1} min^{-1} during infusion of saline, to 3.3 ± 0.7 ml 100 ml^{-1} min^{-1} during infusion of ET at 60 min ($P < 0.05$). Blood flow in the non-infused forearm was not altered by ET infusion.
4 NA produced a significant and dose-dependent reduction of blood flow in the infused forearm, from 3.13 ± 0.5 ml 100 ml^{-1} min^{-1} during saline infusion, to 1.49 ± 0.2 ml 100 ml^{-1} min^{-1} with NA at 600 pmol min^{-1} ($P < 0.001$). During co-infusion of ET, blood flow was reduced similarly in the infused arm from 3.15 ± 0.7 ml 100 ml^{-1} min^{-1} during saline infusion to 1.55 ± 0.2 ml 100 ml^{-1} min^{-1} with NA at 600 pmol min^{-1}. Blood flow in the non-infused arm was not altered by ET and NA infusion.
5 During saline infusion, LBNP reduced blood flow in the infused and non-infused forearms from 3.9 ± 0.6 to 3.0 ± 0.4, and from 4.5 ± 0.6 to 3.2 ± 0.5 ml 100 ml^{-1} min^{-1} respectively. Following ET for 60 min, blood flow in the infused and non-infused forearms was similarly reduced by LBNP, from 3.3 ± 0.7 to 2.5 ± 0.4, and from 4.7 ± 1.2 to 3.6 ± 0.7 ml 100 ml^{-1} min^{-1} respectively. Infusion of ET did not affect responses to LBNP.
6 We conclude that ET is a potent vasoconstrictor of resistance vessels in the forearm circulation in man, producing a slowly progressive effect which is unique among the known constrictor agents and supports a role for ET in long-term maintenance of vascular tone. Intra-arterial ET does not, however, appear to affect NA or sympathetically (LBNP) mediated vasoconstriction in the human forearm.

Keywords endothelin sympathetic nervous system forearm blood flow lower body negative pressure

Introduction

Endothelin (ET) is a 21 amino acid peptide first isolated from the medium of porcine aortic endothelial cells in culture (Yanagisawa *et al.*, 1988). It is a potent vasoconstrictor *in vivo* both in animals (Yanagisawa *et al.*, 1988) and in man (Clarke *et al.*, 1989), with a slow onset, and prolonged duration of action.

Evidence from animal studies suggests that there may be an interaction between ET and the sympathetic nervous system. ET attenuates sympathetic activity in the perfused guinea pig femoral artery (Wiklund *et al.*, 1988), at least in part by reducing noradrenaline (NA) release presynaptically. ET increases catecholamine release from bovine adrenal cells in culture (Boarder & Marriott, 1989), and enhances the response to infused

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NA in the perfused rat mesenteric artery (Tabuchi *et al.*, 1989).

In the present experiments, we have investigated the effects of prolonged arterial infusion of ET (60 min) on resting blood flow of the upper limb in man, and on the vasoconstriction produced by lower body negative pressure (LBNP) and NA infusion. The dose of ET used (1 pmol min^{-1}) was selected to produce only a moderate reduction in resting forearm blood flow, based on earlier studies in man (Clarke *et al.*, 1989).

Methods

Thirteen healthy male volunteers, aged between 21 and 24 years, participated in these studies which were conducted with the approval of the St George's Hospital Ethics Committee and with the informed consent of each volunteer. Studies were performed after subjects had rested supine in a quiet clinical laboratory for a minimum of 30 min. Room temperature (between 25 and 27° C) was maintained constant ± 1° C for each study.

Blood flow was measured in both forearms using venous occlusion plethysmography with temperature compensated mercury-in-silastic strain gauges (Whitney, 1953). Collecting cuff pressure was 40 mm Hg and wrist cuff occlusion pressure was 200 mm Hg. Flows were recorded for 10 s in every 15 s, and the mean of the final five measurements of each recording period was used for analysis. A 27 gauge (SWG) steel cannula was inserted into the left brachial artery using lignocaine hydrochloride (1%; Antigen Ltd, Ireland) to provide local anaesthesia. Saline (0.9%; Travenol, UK), ET (1 pmol min^{-1} in 0.9% saline; Penninsula Labs, Europe), and NA (150–600 pmol min^{-1} in 0.9% saline (with ascorbic acid as antioxidant); Winthrop, UK) were given at a rate of 0.5 ml infusate min^{-1} throughout the experiments by means of a constant-rate infusion pump (Harvard 944A).

Lower body negative pressure (LBNP) was applied using the method described by Brown and colleagues (Brown *et al.*, 1966). Subjects rested supine in a plastic-covered steel cage enclosing the lower limbs and hips and sealed above the level of the anterior superior iliac spines. Suction was applied using an industrial vacuum cleaner to produce a constant negative pressure of 15 mm Hg below atmospheric pressure. The alteration from atmospheric pressure was both applied and relieved rapidly.

In the first series of experiments six subjects received saline for 12 min, followed by three incremental doses of NA (150, 300, then 600 pmol min^{-1}), with each dose given for 12 min. After a further 12 min period of saline infusion, subjects received ET (1 pmol min^{-1}) for 12 min before, and then throughout a second period of incremental infusion of NA (150, 300, then 600 pmol min^{-1}), with each dose again given for 12 min. Forearm blood flow was measured for the second 6 min of each 12 min infusion period throughout the study.

In the second series of experiments, seven subjects received saline for 12 min, followed by ET (1 pmol min^{-1}), given for 60 min. Forearm blood flow was measured for the second 6 min of each 12 min period throughout the study. LBNP (15 mm Hg) was applied for the second 3 min of each measurement period.

The effect of ET on responses to LBNP and NA, was assessed by repeated measures analysis of variance followed by application of the Wilcoxon signed rank test where appropriate. Results are presented as mean ± s.e. mean.

Results

NA alone produced a significant and dose related reduction in blood flow in the infused forearm from 3.1 ± 0.5 ml 100 ml^{-1} min^{-1} during saline infusion, to 1.5 ± 0.2 ml 100 ml^{-1} min^{-1} with NA at 600 pmol min^{-1} ($P < 0.001$). During co-infusion of ET (1 pmol min^{-1}), NA reduced blood flow in the infused forearm from 3.2 ± 0.7 ml 100 ml^{-1} min^{-1} during ET alone to 1.6 ± 0.2 ml 100 ml^{-1} min^{-1} with NA at 600 pmol min^{-1} ($P < 0.001$). The absolute and percentage reductions in forearm blood flow (Figure 1) produced by NA were, however, unaffected by co-infusion of ET. Blood flow in the non-infused forearm was not affected by infusion of either NA or ET.

Infusion of ET for 60 min produced a 15% reduction in blood flow in the infused forearm, from 3.9 ± 0.6 ml 100 ml^{-1} min^{-1} during saline infusion to 3.3 ± 0.7 ml 100 ml^{-1} min^{-1} after 60 min ET ($P < 0.05$). Blood flow in the non-infused forearm, during the same period, increased from 4.5 ± 0.6 to 4.7 ± 1.2 ml 100 ml^{-1} min^{-1} (NS) (Figure 2, Table 1).

Blood flow was consistently reduced in both forearms in all subjects by LBNP. During saline infusion, LBNP reduced blood flow from 3.9 ± 0.6 to 3.0 ± 0.4 ml 100 ml^{-1} min^{-1}, and from 4.5 ± 0.6 to 3.2 ± 0.5 ml 100 ml^{-1} min^{-1} in the infused and non-infused forearms respectively. After 60 min ET infusion, LBNP reduced blood flow from 3.3 ± 0.7 to 2.5 ± 0.4 ml 100 ml^{-1} min^{-1}, and from 4.7 ± 1.2 to 3.6 ± 0.7 ml 100 ml^{-1} min^{-1} in the infused and non-infused forearms respectively. The reduction in forearm blood flow produced by LBNP was unaffected by infusion of ET, either in terms of absolute or percentage change in flow (Figure 2, Table 1).

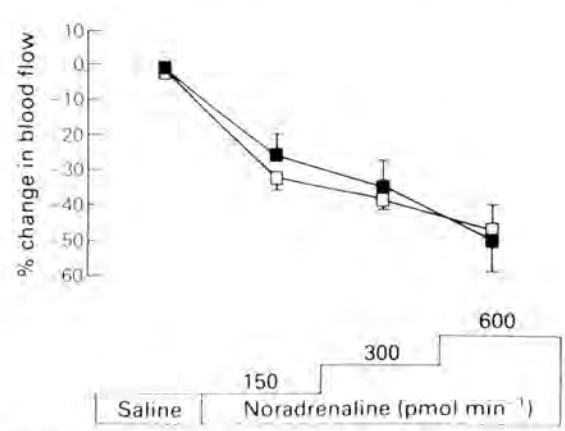

Figure 1 Effect on forearm blood flow of infusion of incremental doses of noradrenaline (150–600 pmol min^{-1}) with (■) and without (□) co-infusion of endothelin (1 pmol min^{-1}).

Table 1 Effect of endothelin (1 pmol min^{-1}) on resting forearm blood flow (mean ± s.e. mean; ml $100\ ml^{-1}\ min^{-1}$) and on the response to lower body negative pressure (15 mm Hg; LBNP)

	Time (min)					
	0	12	24	36	48	60
Blood flow: infused arm						
Resting	3.9 ± 0.6	3.8 ± 0.6	3.5 ± 0.7	3.4 ± 0.6	3.3 ± 0.6	3.3 ± 0.7
During LBNP	3.0 ± 0.4	2.8 ± 0.5	2.7 ± 0.5	2.7 ± 0.5	2.6 ± 0.5	2.5 ± 0.4
Blood flow: non-infused arm						
Resting	4.5 ± 0.6	4.5 ± 0.7	4.3 ± 0.9	4.6 ± 1.0	4.7 ± 1.1	4.7 ± 1.2
During LBNP	3.2 ± 0.5	3.2 ± 0.6	3.4 ± 0.6	3.8 ± 0.8	3.6 ± 0.8	3.6 ± 0.7

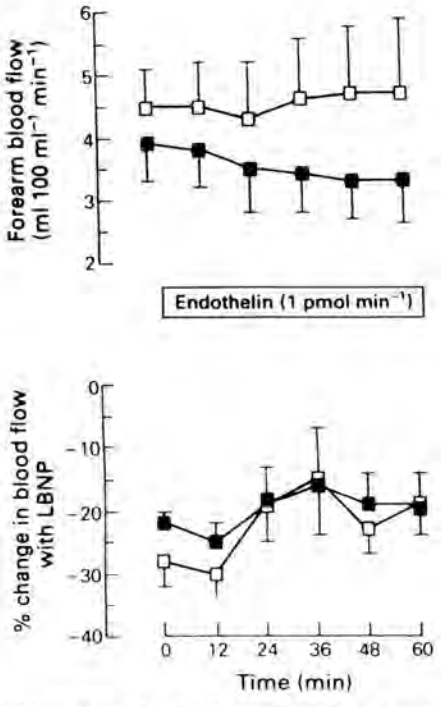

Figure 2 Effect of endothelin (1 pmol min^{-1}), given for 60 min, on resting forearm blood flow (upper panel), and on percentage change in blood flow during lower body negative pressure (lower panel), in the infused (■) and non-infused (□) forearms.

Discussion

Endothelin (ET) is a recently discovered peptide vasoconstrictor (Yanagisawa *et al.*, 1988). Although the potency of ET in the forearm of man is similar to that of angiotensin II (Clarke *et al.*, 1989), the slowly progressive onset and offset of its action (Clarke *et al.*, 1989; Hughes *et al.*, 1989) is unique, differing from the actions of other constrictor agents in the forearm. In the present study in man, as in previous experiments employing a higher dose of ET (5 pmol min^{-1}), there was a slowly progressive reduction in forearm blood flow during ET infusion, maximal at the end of the 60 min infusion period. The maximum 15% reduction of blood flow associated with infusion of ET at 1 pmol min^{-1} compares with a 39% reduction at 5 pmol min^{-1} given for 60 min (Clarke *et al.*, 1989), showing this response to be dose dependent. Experiments with shorter infusions of ET at 5 pmol min^{-1} suggest that the maximum effect does not develop when ET infusion is stopped before 60 min (unpublished observations). The characteristics of the response to ET are compatible with progressive and tight binding of the peptide to its receptor in vascular smooth muscle. These experiments provide no evidence for the tachyphylaxis to ET which has been demonstrated *in vitro* (Hughes *et al.*, 1989) and the characteristics of the response to ET would provide support for its role in long term, rather than short term regulation of vascular tone (Editorial, 1988).

Immunoreactive ET has recently been detected in the plasma of uraemic patients undergoing haemodialysis, with the majority of patients having circulating plasma levels greater than 4×10^{-12} mol l^{-1}, and some as high as 20×10^{-12} mol l^{-1} (Koyama *et al.*, 1989). Infusion of ET (1×10^{-12} mol min^{-1}) in the present study, into a forearm blood flow of approximately 50 ml min^{-1}, would be predicted to produce local concentrations of ET in blood in the order of 20×10^{-12} mol l^{-1}, similar to dialysed patients with the highest plasma levels of ET. If, as our experiments suggest, the response to circulating ET in man is dose dependent, and if the effect on forearm resistance vessels is common to other resistance vessels in man, similar circulating concentrations of ET might be expected to exert a direct effect on arteriolar tone. This view is supported by the results of a recent report of responses to systemic infusion of ET in man (Vierhapper *et al.*, 1990). Infusion of ET at a dose sufficient to provide a circulating plasma ET concentration of 50×10^{-12} mol min^{-1} produced a significant increase in arterial pressure. Assuming that measurement of plasma immunoreactive ET provides a true reflection of its biological activity, our findings are consistent with the hypothesis that ET could act as a circulating pressor agent in patients with renal failure.

In the present experiments in man, ET had no effect on vasoconstriction in forearm resistance vessels produced either by NA or LBNP. The latter has been shown to be a reliable stimulus for reflex sympathetic vasoconstriction in the upper limb (Ardill *et al.*, 1967) and a moderate degree of LBNP, as with the 15 mm Hg used in our experiments, produces constriction of the resistance vessels of forearm muscle through activation of low pressure cardiopulmonary baroreceptors (Abboud *et al.*, 1979; Johnson *et al.*, 1974), with no change in heart rate or arterial pressure (Johnson *et al.*, 1974). The

absence of an effect of ET on responses to exogenous NA in human resistance vessels *in vivo* indicates that ET does not affect postsynaptic responses to NA in man, contrasting with its enhancement of the response to NA in the rat mesenteric artery (Tabuchi *et al.*, 1989). In the absence of a postsynaptic action, the lack of an effect of ET on the response to LBNP suggests that ET does not affect presynaptic neurotransmitter release in human resistance vessels *in vivo*. This contrasts with its effect on guinea pig femoral artery (Wiklund *et al.*, 1988), and with the enhancement of responses to LBNP produced by the pressor peptide angiotensin II via a presynaptic action (Webb *et al.*, 1988). It is possible, however, that concentrations of ET close to sympathetic nerves in vascular smooth muscle, and generated locally by vascular endothelial cells, may be much higher than those in plasma. If ET acts predominantly as a local rather than circulating mediator, it is not possible to entirely exclude an effect of ET on peripheral sympathetic function in man.

In conclusion, the infusion of ET causes constriction of the resistance vessels in the forearm circulation in man, producing a slowly progressive effect which is unique among the known constrictor agents. The concentrations of ET producing vasoconstriction intra-arterially are similar to those detected in the plasma of patients with renal failure, suggesting that circulating ET may contribute to elevated peripheral vascular tone in this circumstance. Intra-arterial ET does not, however, appear to affect NA or sympathetically (LBNP) mediated vasoconstriction in the human forearm.

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THE POTASSIUM CHANNEL OPENER BRL 38227 INHIBITS BINDING OF [^{125}I]-LABELLED ENDOTHELIN-1 TO RAT CARDIAC MEMBRANES

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Binding of [^{125}I]-labelled endothelin-1 (ET-1) to rat cardiac membranes and the effects of endothelin-1 (ET-1), endothelin-3 (ET-3), the calcium channel antagonist nifedipine, and both enantiomers of the potassium channel opener cromakalim (BRL 34915) on binding have been examined. Specific binding of [^{125}I]-ET-1 was inhibited in a concentration dependent manner by both unlabelled ET-1 (10^{-12} - 10^{-7} M) and ET-3 (10^{-12} - 10^{-6} M). Nifedipine (10^{-11} - 10^{-5} M) did not affect [^{125}I]-ET-1 binding. However, BRL 38227 (10^{-11} - 10^{-5} M), the biologically active isomer of cromakalim, significantly inhibited [^{125}I]-ET-1 binding. The inactive isomer, BRL 38226 (10^{-11} - 10^{-5} M) had no effect. These findings provide the first evidence for a stereospecific interaction between BRL 38227 and an ET-1 binding site in rat cardiac membranes.

The endothelins (ETs) are a recently discovered group of vasoactive peptides. ET-1 is a 21-amino acid peptide which was first identified in the culture medium of porcine endothelial cells, and has potent vasoconstrictor properties (1). Binding sites for ET-1 have been identified, in high density, in areas related to regulation of the cardiovascular system. Pharmacological studies suggest the existence of at least three distinct ET receptor subtypes based on their relative affinities for ET isoforms: those which bind ET-1 > ET-3 (the ET_A subtype); those which bind ET-1 and ET-3 with equal affinity (ET_B), and those which bind ET-3 > ET-1 (the proposed ET_C subtype). Two ET receptor subtypes (ET_A and ET_B) have now been cloned (2, 3). One group have identified a single population of specific, high affinity binding sites for [^{125}I]-labelled porcine ET-1 ([^{125}I]-ET-1) in rat cardiac membranes, binding to which is unaffected by a variety of vasoactive compounds: the Ca^{2+} agonist Bay K8644; the Ca^{2+}

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antagonists nicardipine and cis-diltiazem; Na^+-H^+ exchange inhibitors; α_1- and β_1-adrenoceptor antagonists; angiotensin II; vasopressin; glyceryl trinitrate; and ergometrine (4). In guinea pig and rat aorta, trachea and pulmonary artery, potassium (K^+) channel openers such as cromakalim (5) and pinacidil (6, 7) inhibit ET-1-induced contractions in a concentration dependent manner. *In vitro* studies have also shown that ET-1 attenuates the vasodilator responses to BRL 38227, the active isomer of cromakalim (8). Cromakalim has also been shown to prevent ET-1-induced venoconstriction *in vivo* in human subjects (9). *In vivo* studies in the feline pulmonary vascular bed have shown that vasodilatation in response to ET isopeptides is mediated by K^+ channel activation (10). Since ET-1 receptor binding is closely linked to its biological actions (11), the present study was undertaken to examine the effects of the K^+ channel opener BRL 38227, its inactive stereoisomer BRL 38226, and the Ca^{2+} channel antagonist nifedipine on [^{125}I]-ET-1 binding to rat cardiac membranes.

Materials and Methods

Cardiac membranes were prepared by an adaptation of methods previously described (4, 12). Briefly, ventricles from adult male Sprague Dawley rats (250-300 g) were minced into 1 mm pieces, washed in 50 ml of ice-cold saline, filtered through 20 μm gauze and then homogenised in 10 ml ice-cold 20 mM $NaHCO_3$, 0.1 mM phenylmethylsulphonylfluoride (PMSF), pH 7.4, while contained in a tube surrounded in ice, using two 15 s bursts of a Polytron homogeniser operating at 4/5 maximum speed. The homogenate was centrifuged at 1000 x g for 10 min at 4°C, and the supernatant diluted in ice-cold Tris buffer (50 mM Tris-HCl containing 0.1 mM PMSF; pH 7.4) to provide a final concentration of 2 mg protein ml^{-1}.

Binding was performed in 12 x 75 mm glass tubes, in duplicate for 2 h at 37°C, using 0.5 mg protein per tube. The reaction mixture contained 0.5 nM [^{125}I]-ET-1, prepared by the chloramine T method (13) with specific activity 500 Ci $mmol^{-1}$, and increasing concentrations of unlabelled ET-1, unlabelled ET-3, nifedipine, BRL 38227 or BRL 38226 in a final volume of 0.5 ml. Nifedipine was dissolved in dimethylsulphoxide (DMSO), whereas BRL 38227 and BRL 38226 were dissolved in ethanol. The final concentration of DMSO or ethanol in the reaction mixture did not exceed 1.0% (v/v). Non-specific binding was defined in the presence of 10^{-6}M ET-1, and was subtracted from the total bound activity to give specific binding.

After incubation for 2 h at 37°C, binding was terminated by the addition of 3 ml of ice-cold 10mM Tris-HCl buffer containing 6.6% polyethyleneglycol 6000, pH 7.4. Bound and free [^{125}I]-ET-1 were separated by rapid filtration across Whatman GF/C glass microfibre filters (1.2μm retention), thus removing any cytosolic components included in the homogenate after the low speed spin. The filters were washed 3 times with 10 ml additions of the above buffer, and the radioactivity of the filters counted (67% efficiency) in a Nuclear Enterprises NE1612 multiwell γ-counter. Results are expressed as a percentage of the specific binding in the presence of [^{125}I]-ET-1 alone (%B/Bo). Statistical significance was determined by two-way analysis of variance performed using the untransformed data.

ET-1 and ET-3 were obtained from NovaBiochem Ltd. PMSF and nifedipine were obtained from Sigma Chemical Co. BRL 38227 and BRL 38226 were kind gifts from SmithKline Beecham Pharmaceuticals. Sephadex G-25 was purchased from Pharmacia Ltd. All other reagents were obtained from BDH Ltd.

Results

Rat cardiac membranes incubated with 10^{-12} - 10^{-7} M unlabelled ET-1 (Fig 1) showed a concentration dependent inhibition of [^{125}I]-ET-1 binding ($P = 0.0001$; n = 8). Binding was almost completely inhibited in the presence of 10^{-7} M ET-1. Analysis of data for inhibition of [^{125}I]-ET-1 binding by unlabelled ET-1 using the non-linear logistic expression $Y = MX^P / (X^P + IC_{50}^P)$ (14), yielded values for M, P, and IC_{50} of 93.6 ± 6.1, -0.49 ± 0.10 and 1.56 ± 0.78 nM respectively (Fig 1).

Rat cardiac membranes incubated with 10^{-12} - 10^{-6} M ET-3 showed a concentration dependent inhibition of [^{125}I]-ET-1 binding ($P = 0.001$; n = 3) with 50% inhibition of binding at 200 nM (Fig 1).

Incubation of [^{125}I]-ET-1 with BRL 38227 (10^{-11} - 10^{-5} M) also caused a concentration dependent inhibition of binding ($P = 0.042$; n = 5) and %B/Bo reduced to 48.9 ± 9.4 % at the highest concentration of BRL 38227. BRL 38226 and nifedipine (both 10^{-11} - 10^{-5} M) had no effect on binding of [^{125}I]-ET-1 (Fig 2).

Control experiments showed that neither DMSO nor ethanol at 1.0% (v/v) affected [^{125}I]-ET-1 binding. Incubation of [^{125}I]-ET-1 for 2 h at 37°C followed by separation through a 49 x 1

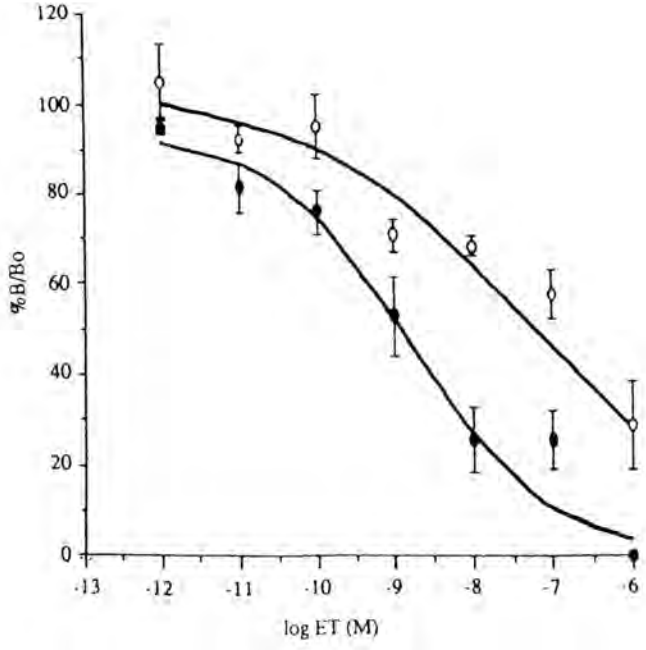

Figure 1. [^{125}I]-ET-1 binding to rat cardiac membranes in the presence of ET-1 (●) and ET-3 (○). Mean values; n = 8 for ET-1, n = 3 for ET-3. Vertical bars show s.e. mean. Inhibition of [^{125}I]-ET-1 binding to rat cardiac membranes in the presence of ET-1 is fitted to the logistic expression $Y = MX^P / (X^P + IC_{50}^P)$.

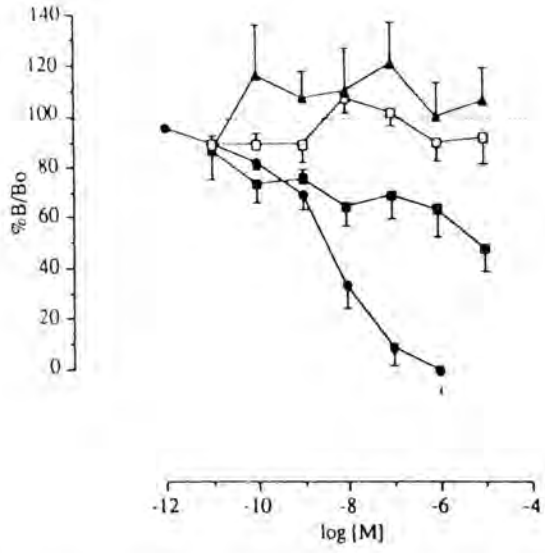

Figure 2. [125I]-ET-1 binding to rat cardiac membranes in the presence of endothelin (●) nifedipine (▲), BRL 38227 (■) and BRL 38226 (□). Mean values; n = 5. Vertical bars show s.e. mean.

cm column of Sephadex G-25 showed that there was no significant degradation of [^{125}I]-ET-1. When membrane preparations were examined under light microscopy no reformed membrane vesicles were detected.

Discussion

These results demonstrate that binding of [^{125}I]-ET-1 to rat cardiac membranes is inhibited by unlabelled ET-1 and unlabelled ET-3 (Fig 1); but not by the dihydropyridine Ca^{2+} channel blocker nifedipine (Fig 2), in agreement with the previous studies (4). Inhibition of [^{125}I]-ET-1 binding by unlabelled ET-1 yielded an IC_{50} of 1.56 ± 0.78 nM, a value similar to those obtained by others (15, 16). The potency of inhibition of [^{125}I]-ET-1 binding by unlabelled ET-3 was approximately 130 times less than that shown by unlabelled ET-1, a similar order of potency to that reported previously (17). These results indicate that rat cardiac membranes preferentially bind ET-1 > ET-3, suggesting the presence of an ET_A receptor subtype.

Since analysis of the inhibition of [^{125}I]-ET-1 binding by unlabelled ET-1 (Fig 1) gives a value for the slope of the logistic fit of substantially less than unity (-0.49; P in the logistic expression above), this may indicate binding site heterogeneity in the membrane. Functional evidence for more than one type of endothelin binding site in coronary tissues has been reported recently (15, 18). Our findings contrast with previous studies (4), which found a

single population of high affinity sites. However, multiple ET receptors have been described in rat heart (15), and a recent study which investigated the existence of ET receptor subtypes using similar binding competition experiments and peptide mapping techniques (16) showed direct evidence for multiple ET receptors in bovine atrial membranes.

Here, we show for the first time that the K^+ channel opener BRL 38227, but not its inactive isomer BRL 38226, inhibits [^{125}I]-ET-1 binding to rat cardiac membranes in a concentration dependent manner (Fig 2). This effect contrasts with the lack of effect of a wide range of other vasoactive substances on binding (4). As no reformed vesicles were found on examination of the membrane preparations, our findings cannot be explained on the basis of membrane hyperpolarisation associated with K^+ channel opening (19). Furthermore, as BRL 38227 does not fully inhibit binding, BRL 38227-sensitive and -insensitive [^{125}I]-ET-1 binding sites may be present in rat cardiac membranes.

The inhibition of [^{125}I]-ET-1 binding by BRL 38227 may be competitive in nature, and reflect a direct interaction with a subpopulation of ET-1 binding sites. Alternatively, BRL 38227 may have an indirect effect, for example, by converting the binding site for ET-1 from a high to a low affinity state. Our findings are consistent with the previously demonstrated antagonism of ET-1-induced vasoconstriction *in vitro* by cromakalim (5) and with our *in vivo* studies in humans (9), where cromakalim inhibited venoconstriction at similar concentrations to those which inhibit [^{125}I]-ET-1 binding in rat cardiac tissue (1-4 μM). These observations suggest that there may be a relationship between ET-1 binding to its receptors and the activity of K^+ channels.

Acknowledgment

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Endothelium-dependent modulation of responses to endothelin-1 in human veins

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1. We have investigated whether local vascular production of nitric oxide or prostacyclin regulates venoconstriction induced by the endothelium-derived peptide, endothelin-1, *in vivo* in man.
2. Six healthy subjects received local dorsal hand vein infusion of endothelin-1 for 60 min alone or, on two separate occasions, co-infused with the donator of nitric oxide, glyceryl trinitrate, or the vasodilator prostaglandin, prostacyclin. In further studies, endothelin-1 was co-infused with an inhibitor of nitric oxide production, N^G-monomethyl-L-arginine, or after oral administration of the irreversible inhibitor of prostaglandin production, acetylsalicylic acid (aspirin).
3. At a low dose (5 pmol/min), endothelin-1 alone caused slowly developing and long-lasting venoconstriction (maximal constriction: $66 \pm 4\%$). Although glyceryl trinitrate partially prevented endothelin-1-induced venoconstriction (maximum: $33 \pm 5\%$), inhibition of nitric oxide production did not affect endothelin-1-induced venoconstriction (maximum: $55 \pm 4\%$).
4. Prostacyclin was more effective at blocking the venoconstriction in response to endothelin-1 than glyceryl trinitrate (maximum: $12 \pm 3\%$), and there was substantial potentiation of endothelin-1-induced venoconstriction after pretreatment with aspirin (maximum: $90 \pm 3\%$).
5. Despite the capacity of nitric oxide to attenuate responses to endothelin-1, N^G-monomethyl-L-arginine did not potentiate endothelin-1-induced venoconstriction, suggesting little or no stimulated production of nitric oxide in human veins. However, the potentiation of responses to endothelin-1 by aspirin indicates that endothelial production of prostacyclin attenuates responses to endothelin-1 in human veins *in vivo*.

INTRODUCTION

Endothelin-1 is a 21-amino acid peptide [1], and is a member of a family of related peptides [2, 3]. Endothelin-1 appears to be the predominant isoform generated by the vascular endothelium [2], and is a potent long-lasting vasoconstrictor and pressor agent in animals [1] and humans [4, 5]. Endothelin-1 is more potent and effective as a constrictor of isolated veins than it is of isolated arteries [6, 7], and increased venous sensitivity to endothelin-1 can also be indirectly observed in rat pulmonary and mesenteric vascular beds [8, 9].

Endothelin-1 causes transient vasodilatation *in vitro*, and hypotension *in vivo* [10], before the development of prolonged vasoconstriction and hypertension. Endothelial denudation of the isolated rat mesenteric bed abolishes the initial dilatation caused by endothelin-1, and potentiates the subsequent constriction, suggesting a role for endothelium-derived dilators in the modulation of these responses [10]. These dilators may include prostacyclin [11] and non-prostanoid relaxing factors, such as nitric oxide [12].

Evidence from studies *in vitro* and *in vivo* in animals has shown that both nitric oxide [13, 14] and prostaglandins [8, 15, 16] may have a role in modulation of responses to endothelin-1. However, the role of the endothelium in the regulation of responses to endothelin-1 has not been investigated in man. We have therefore examined, *in vivo* in man, whether nitric oxide or prostaglandins modulate the responses of human dorsal hand veins to local doses of endothelin-1. We have studied responses in human subjects because the known interspecies heterogeneity of endothelium-dependent vascular responses limits the value of extrapolating from results in animals to man [17], and have used local doses of drugs because, when given systemically, any direct vascular action may be obscured by direct effects on other organs, such as the heart and kidney. Furthermore, if systemic doses of a drug were to alter blood pressure, stimulation of cardiac, hormonal and neurally mediated reflexes would make any vascular effects difficult to interpret. We have studied responses in veins because of the potential clinical relevance of the effects of endothelin-1 on the venous system, in particular in chronic heart failure [18] and ischaemic heart disease [19, 20].

In order to examine whether nitric oxide or dilator prostaglandins can influence responses to

Key words: aspirin, endothelium, N^G-monomethyl-L-arginine, nitric oxide, prostacyclin, prostaglandins, veins.
Abbreviations: GTN, glyceryl trinitrate; L-NMMA, N-monomethyl-L-arginine.
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endothelin-1, we have used glyceryl trinitrate (GTN), an organic nitrate that is converted to nitric oxide in vascular smooth muscle [2], and the vasodilator prostaglandin, prostacyclin. We have then investigated whether endogenous generation of nitric oxide, or prostaglandins, modulates responses to endothelin-1, by examining whether inhibitors of nitric oxide synthase and cyclo-oxygenase affect endothelin-1-induced venoconstriction.

METHODS

Experimental procedures

Six healthy male subjects, between 20 and 28 years of age, participated in each of the studies, which were conducted with the approval of the local Ethics Review Committee and with the written, informed consent of each volunteer. Subjects rested semi-recumbent in a quiet room maintained at a constant temperature for each study (between 25 and 27°C). The left hand was supported above the level of the heart by means of an arm rest. A dorsal hand vein was then selected, as described previously [21], and a 23 SWG cannula (Abbott) was sited in this vein in the direction of flow. Local anaesthesia was not employed. The same dorsal hand vein was used in each study.

The internal diameter of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mmHg, was measured by the technique of Aellig [22]. In brief, a magnetized lightweight rod rested on the summit of the infused vein approximately 1 cm downstream from the tip of the infusion cannula. This rod passed through the core of a linear variable differential transformer supported above the hand by a small tripod, the legs of which rested on the dorsum of the hand, distant from any dorsal hand vein. If venoconstriction occurs during cuff inflation, or if the upper arm cuff is deflated with consequent emptying of the vein, there is a downward displacement of the lightweight rod. This displacement causes a linear change in the voltage generated by the linear variable differential transducer, and thus allows determination of the internal diameter of the vein, after calibration against standard displacements. Vein size was measured at 5 min intervals throughout each study.

Blood pressure was measured in the non-infused arm using a well-validated semi-automated technique (Takeda UA-751 sphygmomanometer) [23]. Recordings were made three times, at 10 min intervals, before infusion of endothelin-1, with the last measurement used as basal, and then every 30 min after starting endothelin-1 infusion.

Drugs

Endothelin-1 (NovaBiochem, Nottingham, U.K.) was administered in a dose of 5 pmol/min, based on results from previous studies [24]. A single dose was used because the slow onset and long-lasting action of endothelin-1 precludes the use of repeated doses in a single study to examine conventional dose-response relationships.

GTN (American Hospital Supply, Newbury, Berks, U.K.) was administered in a dose of 2 nmol/min. GTN exerts its venodilator actions through vascular generation of nitric oxide, independent of nitric oxide synthase [2]. A dose of 2 nmol/min should maximally reverse constriction of dorsal hand veins, as it is 100-fold higher than that necessary to abolish constriction induced by noradrenaline [25], and is sufficient to cause vasodilatation of the forearm vasculature when given intra-arterially [26]. However, GTN at 2 nmol/min is at least 10-fold lower than a systemically active dose [27].

N^G-Monomethyl-L-arginine (L-NMMA; NovaBiochem), a specific substrate analogue inhibitor of nitric oxide synthase in animals [12] and man [28], was administered in a dose of 100 nmol/min. This dose of L-NMMA has no effect on basal hand vein size, but prevents venodilatation of noradrenaline-preconstricted veins by the nitric oxide-releasing agent acetylcholine [25]. A dose of 100 nmol/min L-NMMA is approximately 10 times lower than the intra-arterial doses needed to constrict the forearm vasculature [28], and is 1000 times lower than doses used systemically [29].

The vasodilator prostaglandin, prostacyclin (Wellcome, Beckenham, Kent, U.K.), was administered in a dose of 100 pmol/min. Prostacyclin is the most potent vasodilator prostaglandin produced by blood vessels [11], and is a selective venodilator of the human dorsal hand vein as compared with the forearm resistance bed [30]. The dose used was shown in pilot experiments to maximally reverse noradrenaline-induced venoconstriction, and is sufficient to cause vasodilatation of the forearm vasculature when given intra-arterially [30]. However, it is at least 10-fold lower than a systemically active dose [31].

Aspirin (600 mg, as soluble tablets; Boots, Nottingham, U.K.) was dissolved in 200 ml of water and was administered 30 min before the start of the endothelin-1 infusion. Aspirin irreversibly acetylates, and thus inhibits, fatty acid cyclo-oxygenase (prostaglandin-endoperoxide synthase, EC 1.14.99.1) [32], which is responsible for the production of prostaglandins and thromboxanes. When given at a dose of 600 mg, aspirin inhibits bradykinin-stimulated endothelial production of prostacyclin by at least 85%, with recovery occurring over the next 6 h [33]. Platelet thromboxane generation is also inhibited by this dose of aspirin, although for much longer periods [33].

All drugs, except for aspirin, were dissolved in saline (0.9% NaCl; Travenol) and infused through the venous cannula. The toal rate of infusion was maintained constant throughout all studies at 0.25 ml/min. Where two agents were administered

simultaneously, the two infusions mixed close to the cannula.

Study design

Comparison between the effects of different agents on responses to endothelin-1 was facilitated by use of the same subjects in five separate studies, each at least 2 weeks apart. In each of these studies, after initial saline infusion for 30 min, endothelin-1 was infused for 60 min, and was followed by saline infusion for 30 min. On each of four separate occasions saline placebo, GTN, prostacyclin and L-NMMA were co-infused with endothelin-1, with this infusion maintained for 90 min (see Fig. 1). In the remaining study, subjects received a systemic dose of aspirin orally 30 min before the start of the endothelin-1 infusion. The order of the studies was as follows: in the first study, subjects received endothelin-1 with saline placebo; in the second and third studies, subjects received endothelin-1 with GTN and L-NMMA, with the order governed by balanced randomization; in the remaining studies, subjects received endothelin-1 with prostacyclin and aspirin, with the order again governed by balanced randomization.

Data presentation and statistics

Basal vein size was calculated, in mm, as the mean of the last three measurements taken before starting endothelin-1 infusion. Vein size during drug administration was calculated for each 10 min period by averaging the two recordings in each period, and expressed as the percentage change in vein size from basal. Unless otherwise stated, all results are expressed as means ± SEM. Data were analysed using a repeated measures analysis of variance, except when comparing basal vein size and blood pressures, where Student's paired *t*-test was used. Statistical significance was accepted at the 5% level.

RESULTS

All six subjects completed each part of the study. Internal vein diameter, before infusion of endothelin-1 and drugs, did not differ significantly between studies. Basal vein diameter was 1.1 ± 0.1, 1.0 ± 0.2, 0.9 ± 0.1, 1.1 ± 0.1 and 1.0 ± 0.1 mm before infusion of endothelin-1 with saline placebo, GTN, L-NMMA, prostacyclin and aspirin, respectively. Endothelin-1 alone caused a slowly developing venoconstriction (Fig. 1), with a maximum 66 ± 4% reduction in basal internal vein diameter at 60 min ($P = 0.0001$ versus basal). Venoconstriction was slow to reverse, with 55 ± 8% venoconstriction still present 30 min after stopping endothelin-1.

GTN reduced, but did not prevent, the response

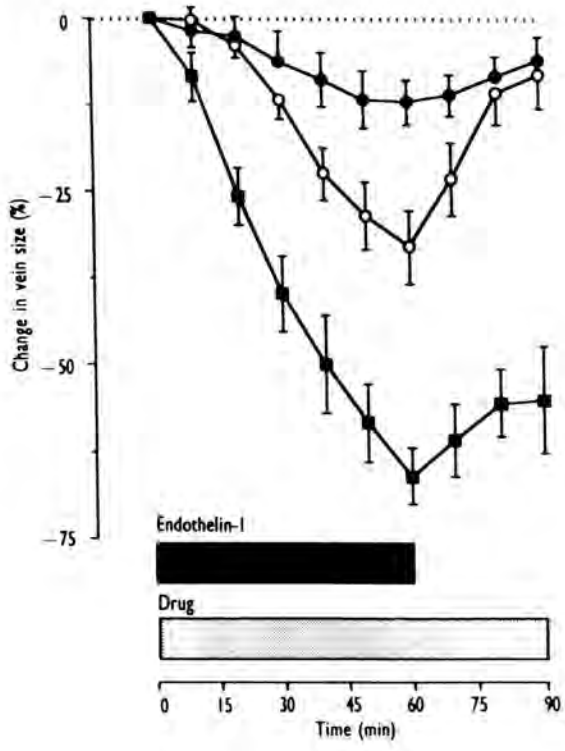

Fig. 1. Responses of veins to endothelin-1 alone and with GTN or prostacyclin. Endothelin-1 was infused intravenously in six subjects with saline placebo (■, 5 pmol/min), and, on separate occasions, with co-infusion of GTN (○, 2 nmol/min) or prostacyclin (●, 100 pmol/min). There was significantly less venoconstriction during co-infusion of prostacyclin with endothelin-1 than with GTN.

to endothelin-1 ($P = 0.0001$ versus basal; $P = 0.004$ versus endothelin-1 alone), with a maximum 33 ± 5% reduction in vein size at 60 min (Fig. 1). By contrast, the decrease in vein size during infusion of prostacyclin with endothelin-1 was not significant, with a 12 ± 3% reduction in vein size at 60 min ($P = 0.06$ versus basal; $P = 0.0001$ versus endothelin-1 alone). The response to endothelin-1 during co-infusion of prostacyclin was significantly less than that during co-infusion of GTN ($P = 0.04$).

Co-infusion of L-NMMA with endothelin-1 (Fig. 2) led to a response that was not significantly different from that to endothelin-1 alone ($P = 0.124$), with a 55 ± 4% venoconstriction at 60 min ($P = 0.0001$ versus basal). By contrast, infusion of endothelin-1 after oral aspirin caused a significantly greater venoconstriction than endothelin-1 alone ($P = 0.001$ versus endothelin-1), with a 91 ± 2% venoconstriction at 60 min ($P = 0.0001$ versus basal).

There was no significant difference between any of the basal values for the mean systolic and diastolic blood pressures, and blood pressure did not change significantly after administration of any drug (Table 1). There were no significant differences in trends for blood pressure after administration of endothelin-1 alone compared with endothelin-1 given with each of the agents investigated.

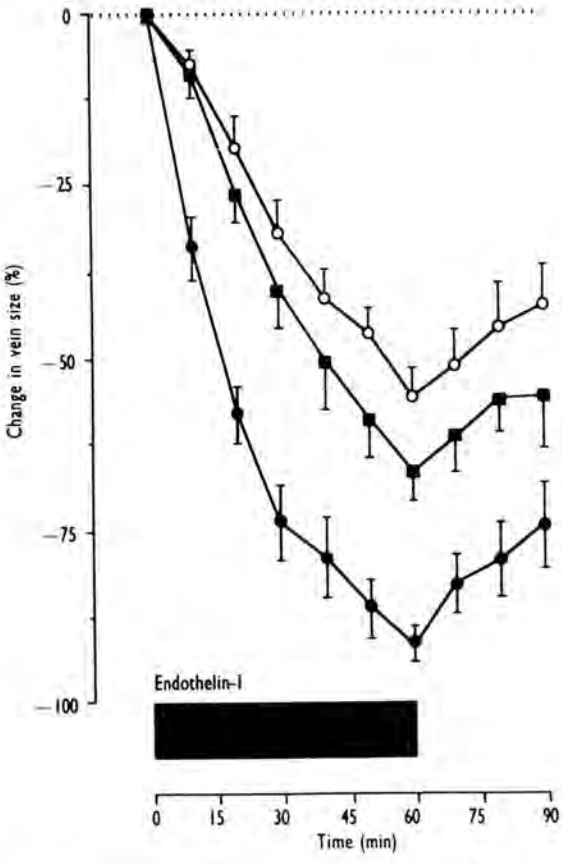

Fig. 2. Responses of veins to endothelin-1 alone and with L-NMMA or after aspirin. Endothelin-1 was infused intravenously in six subjects with saline placebo (■, 5 pmol/min), and, on separate occasions, with co-infusion of L-NMMA (○, 100 nmol/min) or after oral aspirin (●, 600 mg). Aspirin, but not L-NMMA, significantly potentiated endothelin-1-induced venoconstriction.

Table 1. Mean systolic and diastolic blood pressures before and during administration of drugs for each of the studies. Values are means ± SD. There was no significant difference between any of the basal values (paired *t*-test) and blood pressure did not change significantly after administration of any drug (paired *t*-test).

	Blood pressure (mmHg)			
Time...	Basal	30 min	60 min	90 min
Endothelin-1 alone				
Systolic	120 ± 4	120 ± 6	120 ± 6	124 ± 9
Diastolic	69 ± 4	69 ± 7	67 ± 3	69 ± 5
Endothelin-1 with GTN				
Systolic	117 ± 16	119 ± 15	119 ± 12	120 ± 13
Diastolic	72 ± 12	70 ± 12	73 ± 10	72 ± 11
Endothelin-1 with L-NMMA				
Systolic	123 ± 12	123 ± 10	123 ± 13	122 ± 11
Diastolic	74 ± 8	78 ± 9	78 ± 7	76 ± 9
Endothelin-1 with prostacyclin				
Systolic	114 ± 13	112 ± 13	116 ± 14	116 ± 15
Diastolic	71 ± 7	70 ± 10	67 ± 7	69 ± 6
Endothelin-1 with aspirin				
Systolic	121 ± 12	120 ± 8	126 ± 17	122 ± 9
Diastolic	77 ± 4	70 ± 8	72 ± 7	71 ± 4

DISCUSSION

In these studies, in healthy humans, we have investigated local modulation of responses to endothelin-1 in hand veins by the endothelium-derived mediators nitric oxide and prostacyclin. We have shown that although donation of nitric oxide by an organic nitrate partially prevents endothelin-1-induced venoconstriction, inhibition of nitric oxide synthase does not potentiate venoconstriction in response to endothelin-1. In contrast with the findings for nitric oxide, the vasodilator prostaglandin, prostacyclin, almost completely inhibited responses to endothelin-1, and inhibition of endogenous prostaglandin generation markedly potentiated responses to endothelin-1. These findings suggest a role for vascular generation of vasodilator prostaglandins in the modulation of venoconstriction to endothelin-1 in man.

In warm, comfortable, unstressed, semi-recumbent subjects, human dorsal hand veins have no tone [21, 26] and, in previous studies, have been shown to exhibit highly reproducible responses to agonists such as noradrenaline at room temperatures of between 22 and 26°C [22, 34]. The lack of basal tone contrasts with human forearm resistance vessels, which have also been used to investigate local vascular responses. Intrinsic vascular tone is a disadvantage when investigating inhibition by dilator agents of the effects of constrictors. For example, brachial artery administration of nicardipine, into a forearm where the resistance vessels have been preconstricted with endothelin-1, causes an increase of forearm blood flow to above basal [4]. This implies that at least some, and perhaps all, of the effect of nicardipine is mediated against basal arteriolar tone, rather than specifically against vasoconstriction to endothelin-1. Other workers have used an alternative approach to overcome the problem of basal tone in the forearm vasculature, by eliciting maximum vasodilatation in response to intra-arterial nitrates before administration of endothelin-1 [35]. However, this does not remove all underlying tone, as the maximum forearm blood flow during post-ischaemia hyperaemia is substantially greater than that achieved using vasodilator agents [36]. Also, in the prolonged studies needed to examine the effects of endothelin-1, the doses of vasodilators required for these types of forearm studies may be close to those exerting systemic effects, with potential for stimulation of reflex neurohumoral responses.

The use of a constant rate of infusion helped to avoid the possibility that changes in flow through a vein might alter local release of endothelium-derived mediators. In any case, changes in flow are unlikely to have biased our results, as previous studies have

shown that increasing the rate of infusion by up to 100%, but keeping the dose of drug infused constant, does not alter dorsal hand vein responses to a number of agents [22, 26]. The order of studies performed was not fully randomized, leaving open the possibility that an order effect may have occurred. However, tachyphylaxis in response to endothelin-1 seems unlikely in view of the potentiated responses to endothelin-1 observed with aspirin pretreatment late in the study.

In these studies, endothelin-1 alone caused venoconstriction of slow onset, confirming earlier observations [24]; a long biological half-life is confirmed by the presence of substantial venoconstriction 30 min after stopping endothelin-1 infusion (Fig. 1). This prolonged effect of endothelin-1 contrasts with the known short-lasting actions of noradrenaline, 5-hydroxytryptamine and angiotensin II in human dorsal hand veins [34].

The attenuation of endothelin-1-induced venoconstriction by GTN in this study suggests that nitric oxide can, at least partially, prevent responses to endothelin-1 in humans. It is of interest that GTN did not completely prevent responses to endothelin-1, even at a maximally effective dose, as nitric oxide-releasing agents are capable of reversing venoconstriction induced by most other constrictor agents [26]. It is unlikely that our failure to observe complete prevention of responses to endothelin-1 was due to an inadequate dose, as GTN is maximally effective against noradrenaline-induced venoconstriction even at doses 100-fold lower than those used in this study [26]. However, our results are in accord with studies *in vitro*, which have demonstrated less attenuation of responses to endothelin-1 with the nitric oxide donator sodium nitroprusside in the saphenous vein than in the human internal mammary artery [37]. The only studies *in vivo* in man have examined endothelin-1-induced vasoconstriction of the forearm resistance bed, and have shown no effect of nitric oxide-releasing agents on arteriolar constriction to endothelin-1 [35].

L-NMMA, at the dose used in this study, has no effect alone on human hand veins, but maximally prevents acetylcholine-induced venodilatation [25]. Hand veins, therefore, do not seem to produce nitric oxide basally, but are capable of generating nitric oxide when stimulated. Our finding, that L-NMMA does not potentiate endothelin-1-induced venoconstriction, suggests that human dorsal hand veins do not generate nitric oxide when stimulated by endothelin-1. This contrasts with previous observations using cultured endothelial cells [38], and studies in the rat pulmonary and mesenteric vascular beds [10]. Furthermore, inhibition of nitric oxide synthase potentiates responses to endothelin-1 *in vitro* in the rat mesenteric bed [13] and *in vivo* in the rat [14]. However, there is evidence of heterogeneity between species, with rabbit aorta producing nitric oxide in response to endothelin-1, whereas pig coronary artery generates prostacyclin [38]. In man, there is evidence to suggest heterogeneity between arterial and venous beds in the production of nitric oxide. Brachial artery administration of L-NMMA leads to substantial forearm vasoconstriction [28], whereas L-NMMA alone has no effect on dorsal hand vein size [25]. This suggests that there is differential basal production of nitric oxide in the resistance and capacitance beds of man. As human saphenous veins *in vitro* are much less capable of generating nitric oxide when exposed to platelets than are internal mammary arteries [39], there may also be differences between arteries and veins in the stimulated generation of nitric oxide.

We have demonstrated that prostacyclin markedly attenuates venoconstriction induced by endothelin-1. Previously, studies *in vitro* have shown that exogenous prostacyclin can reverse endothelin-1-induced constriction in human hand vessels, although preferentially in arteries [40]. In our study, the relatively greater efficacy of prostacyclin, as compared with GTN, suggests that the mechanisms of action of endothelin-1 in veins may be more sensitive to increases in cyclic AMP than to increases in cyclic GMP.

The potentiation of endothelin-1-induced venoconstriction in our study by aspirin suggests that endothelin-1 stimulates vascular generation of vasodilator prostaglandins by the venous endothelium in man. Previous studies of the effects on responses to endothelin-1 of cyclo-oxygenase inhibition have mainly examined the resistance vasculature of animals, and have given conflicting results. Although aspirin pretreatment potentiates renal vasoconstriction to endothelin-1 in dogs, it has no effect on endothelin-1-induced femoral vascular bed constriction [16]. In rats, indomethacin potentiates endothelin-1-induced mesenteric vasoconstriction, although it also prevents renal vasoconstriction, indicating that the peptide may release both dilator and constrictor eicosanoids *in vivo* [15]. Endothelin-1-stimulated release of constrictor eicosanoids is supported by the demonstration that generation of thromboxane is responsible, in part, for the endothelin-1-induced venoconstriction of guinea-pig lung [8]. In the only previous study in human veins, indomethacin did not potentiate responses to endothelin-1, but these experiments were performed *in vitro* [40].

Our results are consistent with data showing that endothelin-1 stimulates prostacyclin generation *in vitro* by cultured endothelial cells [38], dog kidney [16] and guinea-pig lung [8]. Endothelin-1 also stimulates production of prostaglandin E_2 [16], the other major vasodilator prostaglandin produced by the vasculature [41], and of the vasoconstrictor eicosanoid, thromboxane A_2 [10]. That aspirin potentiated, rather than inhibited, venoconstrictor responses in this study suggests that, on balance, there is relatively greater production of dilator than of constrictor eicosanoids in endothelin-1-constricted veins. Of the vasodilator prostaglandins,

prostacyclin is the most likely candidate for the effects we have observed, since it is the major prostaglandin product of large blood vessels [42], whereas prostaglandin E_2 is produced predominantly in the microvasculature [41, 43]. It would be of interest to examine whether prostacyclin also modulates responses to endothelin-1 in the human resistance vasculature. Unlike L-NMMA [28], aspirin does not influence basal forearm resistance vessel tone in man *in vivo* [44], and therefore results should be easier to interpret.

There are at least two distinct receptors for endothelins [3]. The ET_A receptor is the predominant subtype on vascular smooth muscle, selectively binding endothelin-1, and accounting for the majority of the vasoconstrictor actions of the peptide. Vascular expression of the ET_B receptor seems to be confined to endothelial cells, and stimulation of this receptor may be responsible for the endothelin-1-mediated production of vasodilator prostaglandins that we have demonstrated. The stimulation of generation of prostacyclin, but not nitric oxide, by endothelin-1 suggests that there are differences in the mechanisms of signal transduction for endothelial production of these two dilator agents. Indeed, although generation of both nitric oxide and prostacyclin are dependent on increases in intracellular free Ca^{2+}, studies *in vitro* have shown differences in the pattern of elevation of intracellular free Ca^{2+} required for their production [45].

Elevated plasma concentrations of endothelin-1 in patients with chronic heart failure [18], together with our demonstration that endothelin-1 is a potent long-lasting constrictor of human veins, suggests that endothelin-1 may contribute to the raised cardiac filling pressures observed in heart failure. Circulating endothelin-1 concentrations are also raised in patients with atherosclerosis, in proportion to the severity of the disease, with strong immunostaining for endothelin-1 in atherosclerotic arteries [19]. These findings, taken with its potent constrictor [6] and mitogenic [46] effects, may indicate a role for endothelin-1 in atherosclerosis. As atherosclerotic saphenous veins that have been used for coronary artery bypass grafting have particularly high immunostaining for endothelin-1 [20], it may contribute to the high rates of late occlusion of these grafts [47]. Saphenous veins have a relative deficiency, *in vitro*, of stimulated nitric oxide production as compared with internal mammary arteries [39]. Thus, with only the production of prostacyclin to attenuate constrictor influences, and with the known increased sensitivity of large veins to endothelin-1 [6], grafted saphenous veins may be more likely to constrict and thus occlude. It is interesting to speculate that our findings may offer a further mechanism whereby prostacyclin-sparing doses of aspirin may prevent saphenous vein graft stenosis after coronary artery surgery [48].

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Endothelin-1 and Aggregation of Human Platelets In Vitro

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Summary: Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by endothelial cells. We investigated whether ET-1, like other potent endothelium-derived vasoactive agents, interacts directly with human platelets in vitro. Platelet-rich plasma was obtained from healthy male volunteers and incubated with ET-1 (1 μM) or vehicle (sodium chloride 154 mM) for 10 min at 37°C. Platelet aggregation was measured by the Born method, using light transmittance through the plasma sample as an index of activation. Although a significant increase in light transmittance was observed when plasma was incubated with ET-1 compared with vehicle, (3.8 ± 0.4% versus 2.7 ± 0.2%; $n = 24$; $p = 0.038$), this effect was small and is unlikely to be of biologic significance. To investigate the possibility that ET-1–stimulated platelet nitric oxide (NO) synthesis might be masking a direct aggregatory effect of ET-1, in a second study in six subjects N^G-monomethyl-L-arginine (L-NMMA, 10 and 100 μM), an inhibitor of NO synthase, was preincubated with the plasma before the addition of ET-1 (1 nM and 1 μM). No significant difference was observed whether samples were incubated with L-NMMA alone or with L-NMMA and ET-1. The results of this study suggest that ET-1 does not have a major direct effect as a platelet aggregating agent. **Key Words:** Human—Platelets—Endothelin-1—Nitric oxide.

Endothelin-1 (ET-1) is a potent 21–amino acid vasoconstrictor peptide produced by endothelial cells (1). Bolus administration of ET-1 causes transient vasodilation (2) followed by prolonged vasoconstriction (1). Whereas the prolonged vasoconstrictor effects of ET-1 are paracrine and are believed to be mediated by an ET_A receptor subtype situated on vascular smooth muscle (3), the brief vasodilator effects appear to be autocrine and mediated by an ET_B receptor situated on endothelial cells (4). Vasodilation can be caused by production of either nitric oxide (NO), prostacyclin (PGI_2), or a combination of the two, depending on the species and the vascular bed studied (5,6).

Previous studies have shown that ET-1 inhibits platelet aggregation in vivo and ex vivo (7,8). As this is associated with a rise in platelet cAMP, it has been attributed to endothelial PGI_2 production, although the effect is only partially attenuated by indomethacin (7). We examined the direct effects of ET-1 on human platelets in vitro in the absence of the influence of other endothelial factors, using Born aggregometry. To investigate whether platelet aggregation induced by ET-1 is masked by stimulation of platelet NO synthase, we also performed experiments in the presence and absence of the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA).

METHODS

Subjects were healthy men aged 21 to 44 years ($n = 24$). None of the participants had taken vasoactive drugs or drugs known to alter platelet activity, including nonsteroidal anti-inflammatory drugs, in the preceding 10 days. All subjects had abstained from tobacco and alcohol for a minimum of 2 h, and rested supine for 30 min before venesection.

The aggregation protocol was based on that described by Gow et al. (9). Venous blood (54 ml) was drawn through a 19 SWG steel needle into a polypropylene syringe and immediately transferred into six 10-ml tubes, each containing 1 ml acid citrate dextrose (8 mg/ml citric acid, 22 mg/ml sodium citrate, 20 mg/ml glucose). Blood was centrifuged at 110 g for 10 min at room temperature to obtain platelet-rich plasma (PRP). PRP was removed and the residue was spun for a further 20 min at 3,000 g to obtain platelet-poor plasma (PPP). PRP was stored under 5% CO_2:95% O_2 in sealed polypropylene tubes at room temperature until required. Aggregation was assessed at 37°C on a six-channel aggregometer (Malin) linked to a Macintosh computer through a MacLab digital analogue converter.

Experiments were performed according to the method

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of Born (10). Briefly, the extent of aggregation was calculated by the change in light transmittance, where light transmittance through PRP was taken as 0% aggregation and that through PPP as 100% aggregation. Aggregometer recordings were examined for the three classical signs of platelet activation and aggregation: shape change, primary aggregation, and secondary irreversible aggregation.

In the first study in 24 subjects, 900 μl of sample was incubated for 3 min before the addition of 50 μl vehicle (sodium chloride 154 m*M*), followed 1 min later by 50 μl ET-1 (final concentration 1 μ*M*) or vehicle. In the second study in six subjects, 900 μl of sample was incubated for 3 min before the addition of 50 μl L-NMMA, (final concentrations 10 and 100 μ*M*) or vehicle, followed 1 min later by the addition of 50 μl ET-1 (final concentrations 1 n*M* and 1 μ*M*) or vehicle. Responses were followed for 10 min after the addition of ET-1. Both L-NMMA (Sigma Chemical Co. Ltd, Poole, U.K.) and ET-1 (NovaBiochem, Nottingham, U.K.) were dissolved in sodium chloride 154 m*M*. Results are expressed as means ± SEM. Statistical analysis was by analysis of variance followed by Student's paired *t* test where applicable, with a value of $p \leq 0.05$ accepted as significant.

RESULTS

Aggregation was slightly but significantly greater in the 10-min period after the addition of ET-1 than after saline (3.8 ± 0.4% and 2.7 ± 0.2%, respectively; $n = 24$; $p = 0.038$). There were no significant differences in the responses to L-NMMA whether or not ET-1 was present, showing that L-NMMA did not unmask aggregation to ET-1 (Table 1). No shape change or secondary aggregation was observed in any of these studies.

DISCUSSION

A slight but significant difference in aggregation, as observed by light transmittance, was noted between the control sample and the sample incubated with ET-1 (1 μ*M*) in the first study. Such a difference is unlikely to be of biological significance and may not be related to platelet aggregation. It is unlikely that a substantial aggregatory effect would emerge with higher ET-1 concentrations because the concentration employed was substantially higher than the K_d for either the ET_A or ET_B receptor (3,4), which is in the nanomolar range, and higher than concentrations associated with biologic activity (5).

TABLE 1. *Aggregation, as measured by change in light transmittance, from coincubation of* L-*NMMA with ET-1 or vehicle (n = 6)*

	Vehicle	ET-1 (1 n*M*)	ET-1 (1 μ*M*)
Vehicle	2.3 ± 0.29%	—	—
L-NMMA 10 μ*M*	3.2 ± 0.42%	3.0 ± 0.85%	2.8 ± 0.79%
L-NMMA 100 μ*M*	3.3 ± 0.31%	3.8 ± 0.53%	2.8 ± 0.77%

From these data it can be concluded that ET-1 has, at most, very limited aggregating properties and is unlikely to act directly as an aggregating agent at physiologic concentrations.

Light transmittance after incubation of platelets with L-NMMA at concentrations of 10 and 100 μ*M* was not significantly different in the presence or absence of ET-1 at either 1 n*M* or 1 μ*M*. This result excludes the possibility that ET_B-mediated stimulation of NO generation masks a direct aggregatory effect of ET-1.

Although ET-1 may not stimulate platelet aggregation directly, there is evidence that it modulates platelet aggregation initiated by other agonists. ET-1 can modify epinephrine- (11,12) and thrombin-induced aggregation (13). Dequeker et al. (13) also demonstrated that endothelin-3 produces a small but significant decrease in Ca^{2+} mobilization when coincubated with thrombin. Stimulation of the ET_A receptor might be expected to mediate phosphatidylinositol hydrolysis, resulting in platelet aggregation and a concomitant rise in intracellular calcium $[Ca^{2+}]_i$. By activating NO synthase, stimulation of the ET_B receptor might reduce $[Ca^{2+}]_i$ and inhibit platelet aggregation. ET-1 may act like other potent vasoactive agents, angiotensin II (14) and epinephrine (15), via specific membrane-bound receptors to modify the actions of other aggregating agents without directly initiating platelet aggregation. This requires further investigation.

From our results, we conclude that ET-1 causes significant but not physiologically relevant platelet aggregation and that aggregatory effects are not masked by ET_B-mediated NO production.

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Venoconstriction to endothelin-1 in humans: role of calcium and potassium channels

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Haynes, William G., and David J. Webb. Venoconstriction to endothelin-1 in humans: role of calcium and potassium channels. *Am. J. Physiol.* 265 (*Heart Circ. Physiol.* 34): H1676–H1681, 1993.—Recent studies in vitro have suggested that there may be an interaction between endothelin-1 and ATP-sensitive K^+ channels in vascular smooth muscle. Here we have investigated whether agents acting on membrane Ca^{2+} and K^+ channels modulate endothelin-1-induced venoconstriction in vivo in human subjects. In a series of studies, six healthy subjects received, on separate occasions, local infusions into dorsal hand veins of endothelin-1 coinfused with *1*) the ATP-sensitive K^+ channel opener, cromakalim; *2*) the dihydropyridine Ca^{2+} antagonist, nicardipine; *3*) a control vasodilator, hydralazine; and *4*) saline placebo. Endothelin-1 caused local venoconstriction with a maximum reduction in vein size of 66 ± 4% at 60 min (P = 0.0001 vs. basal). Cromakalim prevented endothelin-1-induced venoconstriction (9 ± 10% maximum constriction; P = 0.68 vs. basal). By contrast, nicardipine, in a dose sufficient to block depolarization-induced constriction caused by K^+ infusion, had only a partial effect on endothelin-1-induced venoconstriction (35 ± 8% maximum constriction; P = 0.001 vs. basal; P = 0.02 vs. endothelin-1), whereas a 10-fold higher dose of nicardipine had no additional effect and hydralazine had no effect. In further studies, cromakalim, but not nicardipine, reversed endothelin-1-induced venoconstriction. Cromakalim did not prevent constriction induced by norepinephrine. Although calcium entry through dihydropyridine-sensitive Ca^{2+} channels may account in part for the vasoconstrictor action of endothelin-1 in humans, the abolition of endothelin-1 responses by a K^+ channel opener suggests additional mechanisms of action for endothelin-1.

human; in vivo; cromakalim; nicardipine

ENDOTHELIN-1 is a 21-amino acid peptide produced by vascular endothelial cells (32) and is a member of a family of related peptides, the actions of which have been widely reviewed (14, 27, 29). Endothelin-1 appears to be the major isoform generated by the vascular endothelium (14), causing long-lasting constriction of resistance and capacitance vessels in animals (6) and humans (5). There is substantial experimental evidence to suggest that endothelin-1 is involved in the pathophysiology of vasospastic conditions, acute renal failure, and myocardial infarction (14). Inhibitors of endothelin-1 generation and antagonists of endothelin type A (ET_A) receptors reduce blood pressure in both normotensive and hypertensive rats, indicating a role for the peptide in regulation of blood pressure (14).

In view of the characteristically prolonged vasoconstrictor effects of endothelin-1, much interest has focused on the mechanisms underlying the sustained rise in free intracellular Ca^{2+} caused by endothelin-1. It was initially proposed that endothelin-1 might be an endogenous agonist of the dihydropyridine-sensitive voltage-operated Ca^{2+} channel (12, 32). This hypothesis was based on the similarity in structure of endothelin to peptides that influence membrane ion channels, the antagonism of endothelin contractions by dihydropyridine Ca^{2+} antagonists, an apparent dependence of endothelin-induced contractions on extracellular Ca^{2+}, and increases in the Ca^{2+} current in whole cell patch clamping studies. However, it has since been shown that *1*) the effect of dihydropyridine Ca^{2+} antagonists on endothelin-induced contractions in vitro is noncompetitive (24); *2*) contractions to endothelin can still develop, though to a lesser extent, in the absence of extracellular Ca^{2+} (2); *3*) dihydropyridine Ca^{2+} channel antagonists are unable to inhibit the sustained rise in intracellular free Ca^{2+} (25); and *4*) that endothelin-1 binding is unaffected by dihydropyridine calcium antagonists (13, 28). On this basis, it is unlikely that endothelin-1 is an endogenous agonist at this channel or that entry of Ca^{2+} into cells occurs solely through indirect activation of these Ca^{2+} channels.

However, there is increasing evidence that endothelin-1 interacts closely with membrane K^+ channels. The ATP-sensitive K^+ channel opener, cromakalim, and the K^+ ionophore, valinomycin, antagonize endothelin-1-induced contractions of rat aortic strips, whereas the Ca^{2+} channel antagonists, verapamil and nicardipine, are less effective (15). Pinacidil, another K^+ channel opener, reverses endothelin induced contractions of rat pulmonary artery (20). Also, endothelin-1 attenuates vasodilator responses to K^+ channel openers but not dihydropyridine Ca^{2+} antagonists (17). In addition, cromakalim has been shown to prevent the systemic pressor and coronary vasoconstrictor effects of endothelin-1 in dogs (26). The balance of published evidence from in vitro and in vivo animal studies suggests that openers of ATP-sensitive K^+ channels may be more effective than other vasodilators, particularly Ca^{2+} antagonists, at blocking responses to endothelin-1.

So, with continuing uncertainty regarding the mechanisms of action of endothelin-1, and its potential pathophysiological role in vascular disease, we have investigated the interaction between endothelin-1 and membrane ion channels, using an in vivo model in humans. Using a well-validated technique (1, 11, 30), we have examined the effects of endothelin-1 on the venous tone of human dorsal hand veins, blood vessels in which endothelin-1 is known to produce vasoconstriction (5). Studies were performed with endothelin-1 administered alone or coadministered with vasoactive agents acting on voltage-operated Ca^{2+} channels (nicardipine) (23) and ATP-sensitive K^+ channels (cromakalim) (10). Each of these drugs was given in a dose chosen to be maximally effective in dorsal hand veins. As a negative control, we performed separate studies with hydralazine, which, like dihydropyridine Ca^{2+} antagonists (22) and

openers of ATP-sensitive K^+ channels (30), is a relatively arterioselective vasodilator (8) but does not appear to have effects on membrane ion channels.

METHODS

Sixteen healthy male subjects, between 20 and 38 yr of age, participated in each of the studies, which were conducted with the approval of the local Ethics Review Committee and the written informed consent of each volunteer. Subjects rested semirecumbent in a quiet room maintained at a constant temperature for each study (between 25 and 27°C). The left hand was supported above the level of the heart by means of an arm rest. A dorsal hand vein was then selected as described previously (30), and on each occasion a 23 SWG cannula (Abbott) was sited in this vein in the direction of flow. Local anesthesia was not used. Internal diameter of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mmHg, was measured by the technique of Aellig (1). In brief, a magnetized lightweight rod rested on the summit of the infused vein ~1 cm downstream from the tip of the infusion cannula. This rod passed through the core of a linear variable differential transformer supported above the hand by a small tripod, the legs of which rested on areas of the dorsum of the hand that were free of veins. If venoconstriction occurs during cuff inflation, or if the upper arm cuff is deflated with consequent emptying of the vein, there is a downward displacement of the lightweight rod. This displacement causes a linear change in the voltage generated by the linear variable differential transformer, and thus allows determination of the internal diameter of the vein after calibration against standard displacements. Unless otherwise stated, vein size was measured at 5-min intervals throughout each study.

Blood pressure was measured in the noninfused arm using a well-validated semiautomated technique (UA 751 sphygmomanometer, Takeda) (31). Recordings were made three times before infusion of endothelin-1, with the last pressure used as basal and then at 30-min intervals after starting endothelin-1 infusion.

The following agents were administered via the local venous cannula: endothelin-1 (5 pmol/min, Nova Biochem, Nottingham, UK); hydralazine (100 μg/min, Ciba-Geigy, Horsham, UK), the dihydropyridine Ca^{2+} antagonist, nicardipine (1.5 and 15 μg/min, Syntex, Maidenhead, UK); the benzopyran ATP-sensitive K^+ channel opener, cromakalim (1 μg/min; Smith-Kline Beecham, Harlow, UK); norepinephrine (4–32 ng/min; Winthrop, Guildford, UK); K^+ chloride (100 mM; Travenol); and saline (0.9%; Travenol). Endothelin-1 and drugs were dissolved in saline. Ascorbic acid (Evans Medical, Horsham, UK) was added to norepinephrine solutions, at a final concentration of 10 μg/ml, to prevent degradation by oxidation (8). The total rate of infusion was maintained constant throughout all studies at 0.25 ml/min.

Five protocols were followed, with the same six subjects completing all parts of the first three protocols, four further subjects completing the fourth protocol, and an additional six subjects completing the fifth protocol. The first and second protocols were designed to examine whether the mechanisms underlying the action of any one of the vasodilators used would prevent or reverse development of venoconstriction to endothelin-1. The third protocol examined whether cromakalim prevents the development of venoconstriction to an alternative venoconstrictor agent, norepinephrine. The fourth protocol examined whether nicardipine, at the dose used in the first two protocols, reverses venoconstriction induced by depolarization produced by infusion of K^+ chloride. The final protocol examined the effects on endothelin-1-induced vasoconstriction of a dose of nicardipine 10-fold higher than that used earlier.

Protocol 1: prevention of endothelin-1-induced venoconstriction. Subjects participated in four separate studies, each at least 2 wk apart. In each of the four studies, after initial saline infusion for 30 min, endothelin-1 was infused for 60 min and followed by saline infusion for 30 min. One of the vasodilators, either hydralazine, nicardipine (1.5 μg/min), cromakalim, or saline placebo was then coinfused from the start of endothelin-1 infusion, with this second infusion maintained for 90 min (Fig. 1). The two infusions mixed close to the cannula. The order of the studies was as follows: in the first study, subjects received endothelin-1 with saline placebo; in the second and third studies, subjects received hydralazine and cromakalim, with the order governed by balanced randomization; in the last study, subjects received endothelin-1 coinfused with nicardipine. In this protocol, hydralazine served primarily as a control dilator because it is not understood to act on membrane ion channels or to act as a venodilator.

Protocol 2: reversal of endothelin-1-induced venoconstriction. Subjects participated in two separate studies, at least 2 wk apart. As in the prevention experiments, saline was infused for 30 min, followed by endothelin-1 for 60 min, and then by saline for 30 min. Nicardipine (1.5 μg/min) or cromakalim was then coinfused from 45 min after the start of endothelin-1 infusion and given for 45 min (Fig. 2). The order of these studies was governed by balanced randomization.

Protocol 3: prevention of norepinephrine-induced venoconstriction. Subjects were studied on one occasion only. Initially, after saline infusion for 30 min, norepinephrine was infused in incremental doses (2, 4, 8, 16, and 32 ng/min) until a stable venoconstriction of ~50% was achieved. Each dose was given for 6 min, with vein size measured at the end of this period, because the effect of norepinephrine has been shown to be maximal at this time (8). Norepinephrine was then stopped and, when vein size had returned to basal, norepinephrine, at the dose producing 50% constriction, was reinfused with cromakalim for 60 min.

Protocol 4: nicardipine and K^+-induced venoconstriction.

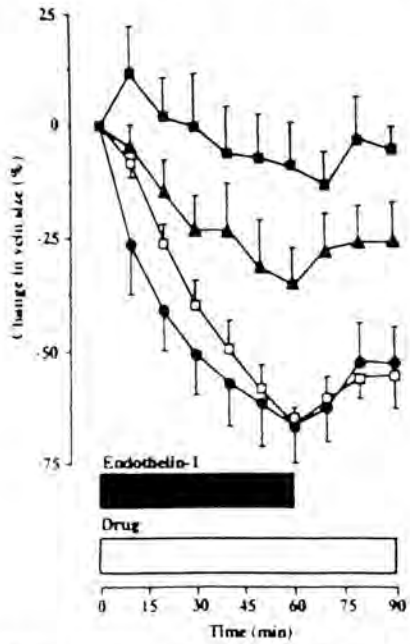

Fig. 1. Endothelin-1 (5 pmol/min) was infused intravenously in 6 subjects with saline placebo (○) and, on separate occasions, coinfused with cromakalim (■, 1 μg/min), nicardipine (▲, 1.5 μg/min), and hydralazine (●, 100 μg/min). Nicardipine reduced and cromakalim prevented endothelin-1-induced venoconstriction.

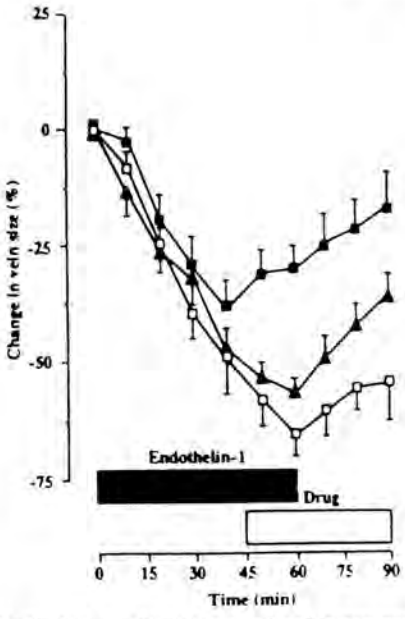

Fig. 2. Endothelin-1 (5 pmol/min) was infused intravenously in 6 subjects with saline placebo (□) and, on separate occasions, with coinfusion of cromakalim (■, 1 μg/min) or nicardipine (▲, 1.5 μg/min) starting at 45 min. Cromakalim, but not nicardipine, prevented further development of endothelin-1-induced venoconstriction after 45 min.

Four subjects were studied on one occasion only. Initially, after saline infusion for 30 min, K^+ chloride was infused at a concentration of 100 mM for 30 min. Vein size was measured twice at the end of this period. Nicardipine, at a dose of 1.5 μg/min, was then coinfused with K^+ for 40 min, with vein size measured twice at the end of this period.

Protocol 5: high-dose nicardipine and endothelin-1-induced venoconstriction. Subjects participated in two separate studies, at least 2 wk apart. As in the prevention experiments, saline was infused for 30 min, followed by endothelin-1 for 60 min, and then by saline for 30 min. On the separate study days, high dose nicardipine (15 μg/min) and saline placebo were then coinfused with endothelin-1 and given for 90 min. The order of these studies was governed by balanced randomization.

Data presentation and statistics. Basal vein size was calculated by taking the mean of the last three measurements before endothelin-1 infusion and was expressed in millimeters. Vein size during drug administration was calculated for each 10-min period by averaging the two recordings in each period and expressed as percentage change in vein size from basal. Unless otherwise stated, all results are expressed as means ± SE. Data were examined using analysis of variance with statistical testing by Scheffé's F test, except when comparing basal blood pressures with those after treatment and vein size in the norepinephrine and K^+ studies, where Student's paired t test was used. Statistical analyses were performed using STATVIEW 512+ software for the Macintosh (Brainpower, Calabasas, CA).

RESULTS

Internal vein diameters, before infusion of endothelin-1 and drugs, did not differ significantly between studies. In *protocol 1*, basal vein diameter was 1.1 ± 0.1, 1.0 ± 0.2, 1.2 ± 0.2, and 1.0 ± 0.2 mm before coinfusion of endothelin-1 with saline, hydralazine, nicardipine, and cromakalim, respectively. In *protocol 2*, basal vein diameter was 1.1 ± 0.1, 1.1 ± 0.2, and 1.2 ± 0.2 mm before infusion of endothelin-1 with saline, nicardipine, and cromakalim, respectively. In *protocols 3* and *4*, basal vein size was 1.1 ± 0.2 and 1.0 ± 0.1 mm, respectively; in *protocol 5* vein size was 0.9 ± 0.2 and 0.8 ± 0.2 mm before coinfusion of endothelin-1 with saline and high dose nicardipine, respectively. Endothelin-1 alone caused a slowly developing venoconstriction ($P = 0.0001$ vs. basal), with a maximum 66 ± 4% reduction of basal internal vein diameter at 60 min (Fig. 1). Venoconstriction was slow to reverse, with 55 ± 8% venoconstriction still present 30 min after stopping endothelin-1.

Protocol 1: prevention of endothelin-1-induced venoconstriction. In these studies (Fig. 1), hydralazine did not affect endothelin-1-induced venoconstriction ($P = 0.0001$ vs. basal; $P = 0.55$ vs. endothelin-1 alone), with a maximum 66 ± 8% reduction in vein size at 60 min. Nicardipine reduced but did not prevent the response to endothelin-1 ($P = 0.01$ vs. basal; $P = 0.02$ vs. endothelin-1 alone), with a maximum 35 ± 8% reduction in vein size at 60 min. Cromakalim prevented venoconstriction, with a maximum reduction in vein size of only 9 ± 10% at 60 min ($P = 0.68$ vs. basal; $P = 0.001$ vs. endothelin-1 alone).

Protocol 2: reversal of endothelin-1-induced venoconstriction. In these studies (Fig. 2) there was no difference in venoconstriction to the 45-min infusion of endothelin-1 alone before coinfusion of either nicardipine (47 ± 6%) or cromakalim (39 ± 6%; $P = 0.39$) nor was there a difference between these responses and those to endothelin-1 with saline placebo at 45 min (50 ± 7%; $P = 0.80$ vs. nicardipine; $P = 0.23$ vs. cromakalim). Coinfusion of nicardipine did not affect the further development of endothelin-1-induced venoconstriction, with a reduction in vein size of 56 ± 2% at 60 min ($P = 0.07$ vs. endothelin-1). By contrast, coinfusion of cromakalim reversed endothelin-1-induced venoconstriction, with a reduction in vein size of only 31 ± 5% at 60 min ($P = 0.0006$ vs. endothelin-1). Interestingly, the rate at which reversal of endothelin-1 induced constriction occurred when cromakalim infusion was started was similar to that seen after stopping the infusion of endothelin-1 when given alone.

Protocol 3: prevention of norepinephrine-induced venoconstriction. The mean dose of norepinephrine required to induce a stable venoconstriction of ~50% was 20 ± 5 ng/min (means ± SE; range 8–32 ng/min). In these studies, cromakalim did not prevent the development of venoconstriction induced by norepinephrine, with a vein size of 47 ± 4% of basal with norepinephrine alone, compared with a vein size of 49 ± 4% of basal ($P = 0.23$) after 60 min of norepinephrine coinfused with cromakalim (Fig. 3).

Protocol 4: nicardipine and K^+-induced venoconstriction. Infusion of K^+ chloride (Fig. 4) caused a marked venoconstriction (82 ± 5%). Coinfusion of nicardipine, at a dose of 1.5 μg/min, abolished this effect, with only a 5 ± 2% constriction present after 40 min of nicardipine ($P = 0.01$ vs. K^+ chloride alone).

Protocol 5: high-dose nicardipine and endothelin-1-induced venoconstriction. Constriction to endothelin-1 was still present when nicardipine was infused at a high local dose (15 μg/min), with a maximum 29 ± 3% reduction in vein size at 60 min compared with a 53 ± 6% constriction

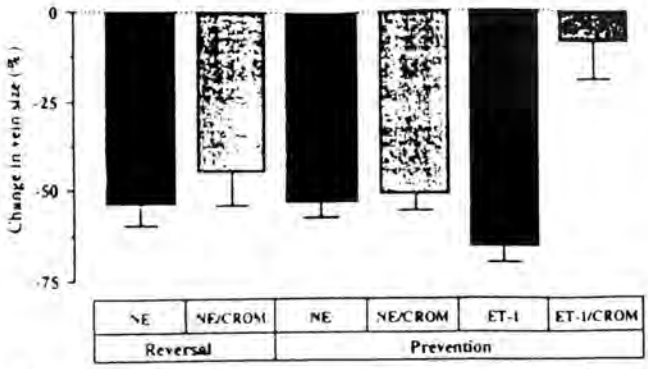

Fig. 3. In reversal experiments, ascending doses of cromakalim (CROM; 0.001-1 μg/min) were infused into norepinephrine (NE) preconstricted veins; response to maximum dose (1 μg/min) is shown (data from Ref. 30). In prevention experiments, NE was coinfused with CROM (1 μg/min) for 60 min (see METHODS for details). CROM did not reverse or prevent NE-induced venoconstriction, in contrast to its effect on endothelin-1 (ET-1) responses after 60 min coinfusion.

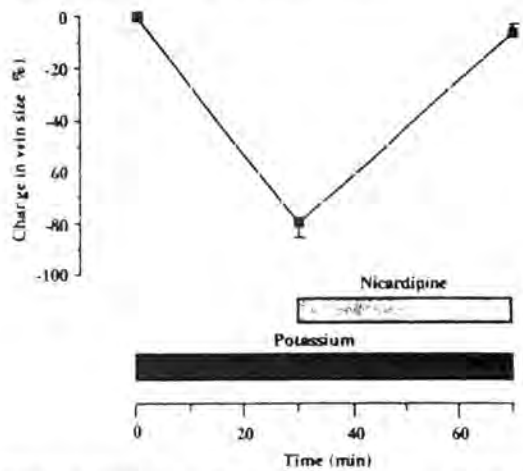

Fig. 4. Effect of nicardipine on K^+-induced venoconstriction. K^+ ions (100 mM as KCl) were infused into dorsal hand vein of 4 subjects for 30 min with development of marked venoconstriction. Coinfusion of nicardipine (1.5 μg/min) for 40 min caused reversal of venoconstriction.

to endothelin-1 alone ($P = 0.03$ vs. basal; $P = 0.0003$ vs. endothelin-1), this response was not significantly different from that obtained with nicardipine at 1.5 μg/min (35 ± 8%; $P = 0.32$).

Blood pressure. Basal systolic and diastolic blood pressures were not significantly different between studies and did not change consistently during any of the studies (Table 1).

DISCUSSION

In these studies in healthy humans, we have investigated modulation of responses to endothelin-1 by drugs that influence membrane ion channels and have shown that the ATP-sensitive K^+ channel opener cromakalim prevents venoconstriction to endothelin-1. We examined responses in human subjects because the known interspecies heterogeneity of endothelium-dependent vascular responses limits the value of extrapolating from results in animals to humans (18). We used local doses of drugs because, when they are given systemically, the direct vascular action of these agents may be obscured by stimulation of cardiac, hormonal, and neurally mediated reflexes, making vascular responses difficult to interpret. We chose to examine responses in human dorsal hand veins because these vessels have no intrinsic tone (8, 30), unlike the human forearm resistance vessels that have been examined in other studies.

Brachial arterial administration of nicardipine into a forearm where the resistance vessels have been preconstricted with endothelin-1 causes an increase of forearm blood flow to levels substantially above basal (5). This implies that at least some of the effect of nicardipine is mediated against basal arteriolar tone, rather than specifically against vasoconstriction to endothelin-1. A lack of specificity against endothelin-1 is also supported by the observation that nicardipine causes greater reversal of forearm vasoconstriction induced by angiotensin II (5). Other workers have used an alternative approach to overcome the problem of basal tone by eliciting maximum vasodilatation to intra-arterial Ca^{2+} antagonists before administration of endothelin-1 (16). However, this by no means removes all of the underlying tone, as the maximum forearm blood flow during postischemia hyperemia is substantially greater than that achieved using Ca^{2+} antagonists (3). Also, in the prolonged studies needed to examine the effects of endothelin 1, the doses of vasodilators used intra arterially are close to those exerting systemic effects, with potential for stimulation of reflex responses. By contrast, in hand veins, effective doses are at least an order of magnitude lower, and since there is no

Table 1. *Mean systolic and diastolic blood pressures before (basal) and after administration of drugs for each protocol*

Treatment	Basal	30 Min	60 Min	90 Min
ET-1 (*protocols 1* and *2*)				
Systolic	120±4	120±6	120±6	124±9
Diastolic	69±4	69±7	67±3	69±5
ET-1/cromakalim (*protocol 1*)				
Systolic	117±13	115±9	116±12	118±10
Diastolic	68±5	68±4	68±7	73±3
ET-1/nicardipine (*protocol 1*)				
Systolic	117±8	121±7	121±5	123±5
Diastolic	71±6	73±6	71±7	72±5
ET-1/hydralazine (*protocol 1*)				
Systolic	123±12	121±11	118±13	116±11
Diastolic	75±8	75±7	72±5	73±9
ET-1/cromakalim (*protocol 2*)				
Systolic	118±7	115±7	116±10	118±8
Diastolic	67±4	68±7	65±5	67±6
ET-1/nicardipine (*protocol 2*)				
Systolic	119±6	116±9	118±11	116±12
Diastolic	70±10	70±9	69±8	69±7
NE/cromakalim (*protocol 3*)				
Systolic	116±8	119±6	118±9	116±4
Diastolic	68±5	69±9	68±6	71±8
K^+/nicardipine (*protocol 4*)				
Systolic	121±9	122±8	124±10	120±12
Diastolic	73±7	76±9	80±7	74±9
ET-1 (*protocol 5*)				
Systolic	114±8	117±9	115±11	119±10
Diastolic	71±6	69±8	71±7	73±9
ET-1/nicardipine (*protocol 5*)				
Systolic	118±9	119±9	121±9	123±8
Diastolic	73±6	71±7	70±6	68±6

Values are means ± SE. ET-1, endothelin-1; NE, norepinephrine.

basal tone, any drug-induced dilatation of endothelin-1-induced venoconstriction is likely to be specific.

A single dose of endothelin-1 (5 pmol/min) was used because the slow onset and long-lasting action of endothelin-1 precludes the use of repeated doses in a single study to examine conventional dose-response relationships (5). The long biological half-life of endothelin-1 was confirmed by the presence of substantial venoconstriction 30 min after stopping infusion of the peptide (Fig. 1). This prolonged effect of endothelin-1 contrasts with the known short-lasting actions of angiotensin II (7) and norepinephrine (8) in human dorsal hand veins. In view of the prolonged action of endothelin-1, it was also impractical to use a wide range of doses of cromakalim or nicardipine against constriction elicited by endothelin-1, so single maximally effective doses were used. Comparison between the effects of different agents on responses to endothelin-1 was facilitated by use of the same subjects in all studies.

The K^+ channel opener cromakalim (1 μg/min) both prevented and reversed endothelin-1-induced venoconstriction. This effect appears to be selective for endothelin-1, as the same dose of cromakalim neither reverses (29) nor prevents norepinephrine-induced venoconstriction (Fig. 3). In earlier experiments, this dose of cromakalim was sufficient to cause forearm vasodilatation when infused into the brachial artery (29). Because it is known that vasodilator doses one to two orders of magnitude greater than those having effects in the dorsal hand vein are needed to have effects via the brachial artery (8), the dose of cromakalim we used should be sufficient to exert maximal K^+ channel opening properties.

The results with cromakalim contrast with the partial prevention and lack of reversal of endothelin-1-induced venoconstriction by nicardipine, at a dose which completely abolishes K^+-induced venoconstriction (1.5 μg/min; Fig. 4) and at a 10-fold higher dose (15 μg/min). It appears, therefore, that there is a qualitative difference between responses to nicardipine and cromakalim. Hydralazine, at a dose previously shown to have modest venodilator activity in norepinephrine preconstricted hand veins (8), had no effect on endothelin-1-induced venoconstriction. Because there was slightly greater venoconstriction to endothelin-1 in the presence of hydralazine than to endothelin-1 alone (Fig. 1), the decreased response to endothelin-1 with cromakalim cannot be attributed to development of tachyphylaxis after repeated exposure to the peptide. The results of these studies provide evidence supporting an interaction between endothelin-1 and ATP-sensitive K^+ channels and may also have implications for the mechanisms of action of drugs that open these channels. These possibilities are detailed below.

Mechanisms of action of endothelin-1. These studies show that endothelin-1 responses in human veins depend only in part on Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels because the K^+ channel-opening drug cromakalim is more effective than the dihydropyridine Ca^{2+} antagonist nicardipine in preventing endothelin-1-induced venoconstriction. Endothelin-1 may additionally act to open voltage-operated Ca^{2+} channels of the T or N type, which are insensitive to dihydropyridines but are closed by the membrane hyperpolarization induced by K^+ channel opening (9). However, as nicardipine completely abolishes K^+ induced venoconstriction, dihydropyridine-sensitive L type channels are likely to be the predominant voltage-operated Ca^{2+} channels in the vascular smooth muscle of human hand veins. Alternatively, endothelin-1 may act through a receptor-operated Ca^{2+} channel that is closed by the hyperpolarization induced by opening of K^+ channels (9). Our results would also be consistent with endothelin-1 acting as a direct antagonist at ATP-sensitive K^+ channels, with membrane depolarization then causing vasoconstriction by mechanisms additional to opening of voltage-operated Ca^{2+} channels. Mechanisms that may be linked to depolarization and contribute to endothelin-1-induced venoconstriction include changes in other ion transport mechanisms and activation of protein kinase C (14). Indeed, endothelin-1 has recently been shown to close ATP-sensitive K^+ channels, and thus cause membrane depolarization, in electrophysiological studies of porcine coronary arterial smooth muscle cells (19).

Mechanisms of action of cromakalim. Drugs that open ATP-sensitive K^+ channels include the established hypotensive agents minoxidil and diazoxide, as well as newer drugs such as pinacidil and cromakalim (10). Opening of these channels allows the efflux of K^+ ions, leading to membrane hyperpolarization and consequent closure of dihydropyridine-sensitive voltage-operated Ca^{2+} channels (21). It is generally believed that the vasodilator effects of K^+ channel openers are mediated through the closure of these Ca^{2+} channels. However, these studies in dorsal hand veins suggest that the vasorelaxant actions of K^+ channel openers do not depend solely on closure of dihydropyridine-sensitive Ca^{2+} channels and that other actions must be invoked (10). ATP-sensitive K^+ channel openers have been shown to impair both the release of Ca^{2+} from intracellular stores and the replenishment of these stores (4, 21), so K^+ channel openers may modulate intracellular Ca^{2+} fluxes and so prevent endothelin-1 induced rises in cytosolic Ca^{2+}. This possibility is rendered less likely by our demonstration that cromakalim has no effect on constriction induced by norepinephrine, which exerts at least some of its contractile actions through release of Ca^{2+} from intracellular stores. Recent studies have shown that binding of endothelin-1 to rat cardiac membrane preparations is inhibited by the active enantiomer of cromakalim, levcromakalim but not by the inactive enantiomer, D-cromakalim (28). Such displacement of endothelin-1 receptor binding might occur through a direct interaction with the K^+ channel opening drug or by an allosteric interaction between the endothelin-1 receptor and the ATP-sensitive K^+ channel. Hence, an alternative additional action of cromakalim may involve inhibition of endothelin-1 binding to the vascular smooth muscle of human dorsal hand veins. The finding that the rate of reversal of constriction by cromakalim was similar to that seen after halting the infusion of endothelin-1 alone (Fig. 2), would be consistent with an effect on endothelin-1 binding.

We have demonstrated an important interaction between ATP-sensitive K^+ channels and endothelin-1 in vivo in humans. Our findings suggest that the mechanisms of action of endothelin-1 may include closure of ATP-sensitive K^+ channels and offer insights into differences between K^+ channel-opening drugs and Ca^{2+} antagonists. Because endothelin-1 may play a role in the pathogenesis of a variety of cardiovascular disorders, it is possible that K^+ channel-opening drugs may offer novel therapeutic benefits in these diseases.

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Contribution of endogenous generation of endothelin-1 to basal vascular tone

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Summary

Endothelin-1 is an endothelium-derived vasoconstrictor peptide, possibly involved in the pathophysiology of cardiovascular disease. We examined the contribution of endogenously generated endothelin-1 to maintenance of peripheral vascular tone in healthy subjects by local intra-arterial administration of an inhibitor of endothelin converting enzyme, phosphoramidon, and of a selective endothelin receptor A antagonist, BQ-123.

Brachial artery infusion of local doses of proendothelin-1, the precursor to endothelin-1, caused a slow-onset dose-dependent forearm vasoconstriction which was abolished by co-infusion of phosphoramidon. Phosphoramidon did not affect responses to endothelin-1. Phosphoramidon caused slow-onset vasodilatation when infused alone, with blood flow increasing by 37% at 90 min ($p = 0.03$). Vasoconstriction to endothelin-1 was abolished by co-infusion of BQ-123 ($p = 0.006$), with forearm blood flow tending to increase. Infusion of BQ-123 alone caused progressive vasodilatation, with blood flow increasing by 64% after 60 min ($p = 0.007$).

These results show that endogenous production of endothelin-1 contributes to the maintenance of vascular tone. Endothelin converting enzyme inhibitors and receptor antagonists may have therapeutic potential as vasodilators.

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Introduction

Endothelin-1 is an endothelium-derived 21-aminoacid peptide with sustained vasoconstrictor actions.[1] It is generated from a 38-aminoacid precursor, proendothelin-1, through the action of endothelin-converting enzyme (ECE), a membrane-bound neutral metalloprotease inhibited by phosphoramidon.[2,3] Endothelin-1 binds to at least two receptors:[4] the ET_A receptor appears to be the major receptor causing vasoconstriction in arteries, the ET_B receptor mediates release of endothelium-dependent vasodilator substances and is also present in some resistance and capacitance vessels where it contributes to vasoconstriction.

Endothelin-1 may play a part in the pathophysiology of several conditions associated with vasoconstriction, including chronic heart failure, hypertension, Raynaud's disease, and renal failure.[4,5] Inhibitors of ECE, and recently described antagonists of endothelin receptors, may therefore have potential as vasodilator drugs.[5] However, the contribution of endogenous endothelin generation to resistance vessel tone in human beings is not known. We examined the effects of local inhibition of ECE and antagonism of the ET_A receptor on forearm blood flow.

Methods

18 healthy men, 21–37 years old, participated in these studies, which were conducted with the approval of the Lothian ethics review committee and with the written, witnessed, informed consent of each volunteer. All subjects abstained from vasoactive drugs for 1 week, from alcohol for 24 hours, and from food, caffeine-containing drinks, and tobacco for 3 hours before each study. Subjects rested recumbent in a quiet room maintained at a constant temperature of 24–26°C. A 27 G cannula was inserted into the brachial artery of the non-dominant arm under local anaesthesia for drug infusion. Local doses of endothelin-1 (Clinalfa AG, Läufelfingen, Switzerland), proendothelin-1 (Clinalfa AG), the ECE and neutral endopeptidase inhibitor phosphoramidon (Clinalfa AG), the neutral endopeptidase inhibitor thiorphan (NovaBiochem, Läufelfingen, Switzerland), and the ET_A antagonist BQ-123 (Cyclo[—D-Asp—L-Pro—D-Val—L-Leu—D-Trp—]; American Peptide Company, Sunnyvale, CA, USA) were dissolved in 0·9% saline and infused intra-arterially at a constant rate of 1 mL/min. Blood flow was measured in both forearms by venous occlusion plethysmography before and at 5 min interval after dosing.[6] Blood pressure was measured in the non-infused arm before and at 10 min intervals after dosing with a Takeda UA-751 semi-automated sphygmomanometer (Takeda Medical Inc, Tokyo, Japan).

6 subjects received, on six separate occasions and in random order, proendothelin-1 (5, 15, or 50 pmol/min) given alone for 90 min, proendothelin-1 (50 pmol/min) given for 90 min with phosphoramidon (30 nmol/min) for the first 60 min, endothelin-1 (5 pmol/min) given alone for 90 min, and endothelin-1 (5 pmol/min) given for 90 min with phosphoramidon (30 nmol/min) for the first 60 min (table). A second group of 6 subjects received on two separate occasions phosphoramidon alone and thiorphan

Intervention	% change in average blood flow	
	0–60 min mean (95% CI)	60–90 min mean (95% CI)
Proendothelin-1 (5 pmol/min)	−6 (−18 to 7)	−2 (−17 to 14)
Proendothelin-1 (15 pmol/min)	−8 (−18 to 2)	−13 (−21 to −5)*
Proendothelin-1 (50 pmol/min)	−33 (−49 to −17)*	−42 (−55 to −29)*
Proendothelin-1 (50 pmol/min) and Phosphoramidon (30 nmol/min)§	0 (−11 to 10)†	−20 (−36 to −4)††
Endothelin-1 (5 pmol/min)	−28 (−40 to −17)*	−41 (−58 to −24)*
Endothelin-1 (5 pmol/min) and Phosphoramidon (30 nmol/min)§	−24 (−43 to −6)†	−36 (−51 to −21)*

*p < 0·01 *vs* proendothelin-1 (50 pmol/min) alone; †p < 0·01 *vs* basal; ††p < 0·05 *vs* basal; §phosphoramidon halted at 60 min in these studies

Table: **Effects of proendothelin-1 and endothelin-1, alone or coinfused with phosphoramidon, on average forearm blood flow**

alone, both at 30 nmol/min for 90 min. A further 6 subjects received, on three separate occasions and in random order, endothelin-1 (5 pmol/min) given alone for 90 min, endothelin-1 (5 pmol/min) given for 90 min with BQ-123 (100 nmol/min) for the first 60 min, and BQ-123 (100 nmol/min) given alone for 60 min followed by saline for 30 min. The doses of phosphoramidon and BQ-123 were chosen to achieve local concentrations in the forearm that were equivalent to the IC_{50} of phosphoramidon for ECE and 10-fold higher than the pA_2 at the ET_A receptor respectively.[3,7]

Percentage change from basal values in the ratio of blood flow between infused and non-infused arms was calculated with blood flow in the non-infused arm as a concurrent control.[8] These results are shown as means with 95% CI in the table, and with standard errors in the figures. Because serial measurements were made with each treatment, mean responses over each infusion period were calculated as a summary measure to avoid making multiple comparisons.[8] Data were analysed by paired *t* tests.

Results

Systolic, diastolic, and mean arterial pressure, heart rate, and blood flow in the non-infused arm did not change significantly after infusion of any study agent, confirming that drug effects were confined to the infused arm.

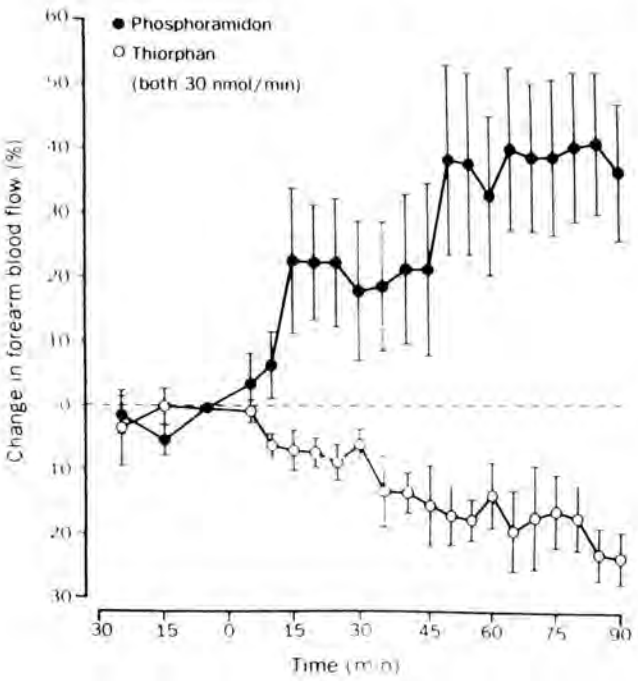

Figure 1: **Forearm blood flow after intra-arterial infusion of phosphoramidon and thiorphan**

Phosphoramidon produces progressive vasodilatation (p = 0·03), whereas thiorphan causes vasoconstriction (p = 0·01; p = 0·007 *vs* phosphoramidon)

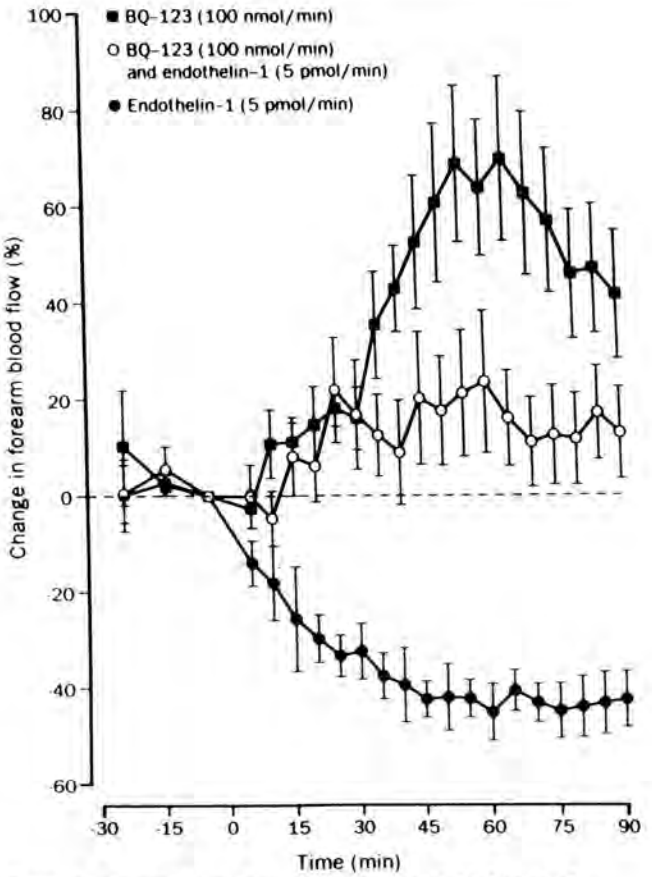

Figure 2: **Forearm blood flow after intra-arterial infusion of endothelin-1 and BQ-123**

Vasoconstriction to intra-arterial endothelin-1 is abolished by intra-arterial infusion of BQ-123 (0 = 0·006 *vs* endothelin-1). BQ-123 alone causes progressive forearm vasodilatation (p = 0·007).

Blood flow in the infused arm was progressively reduced by endothelin-1 and proendothelin-1, reaching a maximum at 60–90 min (table). The effect of proendothelin-1 was dose dependent. Phosphoramidon prevented vasoconstriction by proendothelin-1. Stopping phosphoramidon infusion after 60 min resulted in vasoconstriction. Comparing individuals, endothelin-1 was 11-fold more potent as a constrictor of the forearm bed than proendothelin-1 (95% CI; 4 to 18 fold). Infusion of phosphoramidon alone resulted in progressive forearm vasodilatation (figure 1). In contrast, infusion of the same dose of thiorphan caused vasoconstriction (figure 1). Coinfusion of BQ-123 with endothelin 1 prevented vasoconstriction, even after halting BQ-123 at 60 min, and infusion of BQ-123 alone caused forearm vasodilatation (figure 2).

Discussion

We have shown that local inhibition of the generation or actions of endothelin-1 causes forearm vasodilatation; thus demonstrating, for the first time, a physiological role for endogenous generation of endothelin-1 in regulation of vascular tone.

Proendothelin-1 caused a dose-dependent forearm vasoconstriction that was blocked by phosphoramidon, suggesting that the effects of the precursor are mediated through conversion to the mature peptide by ECE. Because circulating blood does not show ECE activity,[9] conversion in the forearm probably occurs through the action of ECE within the forearm blood vessels. The approximately 10-fold lower potency of proendothelin-1 as a constrictor

than endothelin-1 suggests that local ECE converts about 10% of luminally presented proendothelin-1 to endothelin-1.

The finding that phosphoramidon alone caused forearm vasodilatation suggests that generation of endothelin-1 provides an important contribution to the maintenance of basal vascular tone. Thiorphan, a relatively selective neutral endopeptidase inhibitor,[3,10] caused vasoconstriction, perhaps because of impaired breakdown of constrictor peptides, such as angiotensin II and endothelin-1. Thus, inhibition of neutral endopeptidase cannot explain the vasodilatation we observed to phosphoramidon, which is probably due to inhibition of ECE. Indeed, vasodilatation to phosphoramidon through ECE inhibition may be offset to some degree by vasoconstriction through inhibition of neutral endopeptidase.

Vasodilatation caused by local infusion of the specific and ET_A-selective receptor antagonist BQ-123[11,12] confirms and extends the findings with the ECE inhibitor. The results with BQ-123 show that endogenous generation of endothelin-1 maintains vascular tone through activation of ET_A receptors. However, ET_B receptors may also mediate vasoconstriction. Our results extend the findings of animal studies, in which infusions of phosphoramidon or BQ-123 decrease blood pressure by about 10% in both normotensive and hypertensive rats.[3,12-14]

Our study shows that a phosphoramidon-sensitive ECE is present in resistance vessels and that endogenous generation of endothelin-1 contributes to maintenance of basal vascular tone in human beings, acting (at least in part) through ET_A receptors. Orally active ECE inhibitors and endothelin-receptor antagonists may therefore have potential as vasodilators in the treatment of hypertension and heart failure.[5,15]

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Endothelin ET_A and ET_B Receptors Cause Vasoconstriction of Human Resistance and Capacitance Vessels In Vivo

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Background The role of endothelin ET_B receptors in mediating vasoconstriction in humans is unclear. As yet, there have been no in vivo studies in resistance vessels, and in vitro data have been contradictory. We therefore investigated the function of ET_B receptors in vivo in human forearm resistance and hand capacitance vessels using endothelin-1 as a nonselective agonist at ET_A and ET_B receptors and endothelin-3 and sarafotoxin S6c as selective agonists at the ET_B receptor.

Methods and Results A series of single-blind studies were performed, each in six healthy men. Brachial artery infusion of endothelin-1 and endothelin-3 caused slow-onset dose-dependent forearm vasoconstriction. Although endothelin-3 caused significantly less forearm vasoconstriction than endothelin-1 at low doses, vasoconstriction was similar to the two isopeptides at the highest dose (60 pmol/min). Endothelin-3 caused transient forearm vasodilatation at this dose, whereas endothelin 1 showed only a nonsignificant trend toward causing early vasodilatation. Intra-arterial sarafotoxin S6c caused a progressive reduction in forearm blood flow, although less than that to endothelin-1 ($P=.04$). Dorsal hand vein infusion of sarafotoxin S6c caused local venoconstriction that was also less than that to endothelin-1 ($P=.002$).

Conclusions Selective ET_B receptor agonists cause constriction of forearm resistance and hand capacitance vessels in vivo in humans, suggesting that both ET_A and ET_B receptors mediate vasoconstriction. Hence, antagonists at both ET_A and ET_B receptors, or inhibitors of the generation of endothelin-1, may be necessary to completely prevent vasoconstriction to endogenously generated endothelin-1. **(*Circulation*. 1995;92:357-363.)**

Key Words • endothelin • vasoconstriction • vessels

The endothelins are a family of 21-amino-acid peptides with potent and characteristically sustained vasoconstrictor and vasopressor actions.[1] Endothelin-1 is the predominant isopeptide generated by the vascular endothelium.[2] Endothelin-2 and endothelin-3 are more difficult to detect in humans and are probably less important in their cardiovascular effects.

Two specific receptors for the endothelins have been isolated by in vitro expression of cloned human cDNA.[3,4] The ET_A receptor has a high affinity for endothelin-1, with a K_i of 0.6 nmol/L for endothelin-1 compared with 140 nmol/L for endothelin-3.[5] ET_A receptor mRNA was initially reported to be highly expressed in human aorta but not cultured human endothelial cells, suggesting selective vascular expression of this receptor in smooth muscle cells.[3] The ET_B receptor has equal affinity for all three endothelins, with K_i values for endothelin-1 and endothelin-3 of 0.12 and 0.06 nmol/L, respectively.[5] The ET_B receptor has been reported to be highly expressed in cultured endothelial cells[4] but not vascular smooth muscle cells.[6]

On the basis of the greater vasoconstrictor potency of endothelin-1 than endothelin-3 and the apparently exclusive expression of ET_A receptors in vascular smooth muscle, vasoconstriction to endothelin-1 was initially thought to be mediated solely by vascular smooth muscle cell ET_A receptors. Vascular ET_B receptors located on endothelial cells were thought only to mediate generation of endothelium-derived dilator substances. More recent evidence suggests that ET_B receptor mRNA is expressed in human vascular smooth muscle obtained from the aorta, pulmonary artery, and coronary artery,[7] consistent with a potential vasoconstrictor role for this receptor. Indeed, in animals, there is functional evidence for ET_B receptor–mediated vasoconstriction in vitro, particularly in venous tissue.[8-13] In addition, selective ET_B receptor agonists have pressor effects in animals in vivo.[12,14-16] However, the contribution of ET_B receptors to vasoconstriction is variable and appears to depend markedly on species, vessel type, and vessel size.[17] Furthermore, the functional significance of such vascular smooth muscle ET_B receptors in humans is unclear, with in vitro studies reporting that ET_B receptors make either a minimal[11,17-24] or, at most, a moderate contribution[25-27] to vasoconstriction, depending on the types of vessel studied.

The relevance of this issue is emphasized by the recent development of both selective and nonselective antagonists at ET_A and ET_B receptors. For example, selective ET_A receptor antagonists will block vasoconstriction mediated by ET_A receptors but may not block all constriction to endothelin-1 if there are also vasoconstrictor ET_B receptors. However, if the putative constrictor ET_B receptor is relatively unimportant in humans, then blocking both ET_A and ET_B receptors may cause less vasodilatation than blocking the ET_A receptor alone, because such receptor antagonists will also block the endothelial dilator ET_B receptor.

In view of the inconsistent results with and the potential disadvantages of in vitro studies, we investigated the function of endothelin ET_A and ET_B receptors in blood vessels in vivo in humans. We used endothelin-1 as a nonselective agonist at ET_A and ET_B receptors and endothelin-3 and sarafotoxin S6c as selective ET_B receptor agonists; these peptides have about 2000- and 300,000-

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fold selectivity, respectively, for the ET_B over the ET_A receptor.[5,28] Using locally active doses of these agents, we assessed responses both of resistance vessels, using brachial artery administration,[29] and of capacitance vessels, using dorsal hand vein administration.[30-32] We used local doses of peptides so that interpretation of the results would not be confounded by direct effects of systemic administration on kidney, heart, or brain or by reflex effects consequent to changes in blood pressure.

Methods

Subjects

Twenty-four healthy male subjects between 22 and 38 years of age participated in these studies, which were conducted with the approval of the Lothian Medicine and Clinical Oncology Ethics of Medical Research Subcommittee and with the written informed consent of each subject. No subject received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 hours and from food, caffeine-containing drinks, and cigarettes for at least 3 hours before any measurements were made. All studies were performed in a quiet room maintained at a constant temperature of between 22°C and 25°C.

Drugs

Pharmaceutical-grade endothelin-1 (Clinalfa, NovaBiochem), endothelin-3 (Clinalfa), and sarafotoxin S6c (Sigma Chemical Co Ltd) were administered. A single dose of each peptide was used in individual studies because the slow onset and long-lasting action of the endothelin isopeptides preclude the use of repeated doses in a single study to examine conventional dose-response relations.[33] The peptides were dissolved in physiological saline (0.9%; Baxter Healthcare Ltd).

Intra-arterial Administration

The left brachial artery was cannulated under local anesthesia (1% lignocaine; Astra Pharmaceuticals) with a 27-standard wire gauge steel needle (Coopers Needle Works) attached to a 16-gauge epidural catheter (Portex Ltd). Patency was maintained by infusion of 0.9% physiological saline via a Welmed P1000 syringe pump (Welmed Clinical Care Systems). The total rate of intra-arterial infusion was maintained constant throughout all intra-arterial studies at 1 mL/min.

Intravenous Administration

A vein on the dorsum of the left hand was cannulated in the direction of flow with a 23-gauge butterfly needle (Abbott) attached to a 16-gauge epidural catheter, without use of local anesthesia. The same vein was used in each subject for each individual study. Patency was maintained by infusion of 0.9% physiological saline via a Welmed P1000 syringe pump. The total rate of intravenous infusion was maintained constant throughout all studies at 0.25 mL/min.

Measurements

Forearm Blood Flow

Blood flow was measured in the infused and noninfused forearms by venous occlusion plethysmography[34] using indium/gallium-in-Silastic strain gauges[29] that were securely applied to the widest part of each forearm. The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mm Hg. Upper-arm cuffs were intermittently inflated to 40 mm Hg for 10 in every 15 seconds to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Recordings of forearm blood flow were made repeatedly over 3-minute periods unless otherwise stated. Voltage output from a dual-channel Vasculab SPG 16 strain-gauge plethysmograph (Medasonics Inc) was transferred to a Macintosh personal computer (Classic II, Apple Computer Inc) with a MacLab analog-to-digital converter and CHART software (v. 3.2.8; both from AD Instruments). Calibration was achieved by use of the internal standard of the Vasculab plethysmography unit.

Dorsal Hand Vein Diameter

The left hand was supported above the level of the heart by means of an arm rest. The ID of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mm Hg, was measured by the technique of Aellig.[30] In brief, a magnetized lightweight rod rested on the summit of the infused vein ≈1 cm downstream from the tip of the infusion cannula. This rod passed through the core of a linear variable differential transformer (LVDT; model 025 MHR, Lucas Schaevitz Inc) supported above the hand by a small tripod, the legs of which rested on areas of the dorsum of the hand free of veins. If venoconstriction occurred while this cuff was inflated or if the cuff was deflated with consequent emptying of the vein, there was a downward displacement of the lightweight rod that caused a linear change in the voltage generated by the LVDT. The voltage output from the LVDT was transferred to a Macintosh personal computer by use of a MacLab analog-to-digital converter and CHART software. Standard displacements were used to calibrate the LVDT to determine the ID of the vein.

Blood Pressure

A well-validated semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc) was used to make duplicate measurements of blood pressure in the noninfused arm.[35]

Study Design

Four single-blind studies were performed, with the experimental subjects but not the investigators blinded to the peptide and dose administered in each study.

Forearm Resistance Bed Protocols

Subjects rested recumbent throughout each study. Strain gauges and arm cuffs were applied, and the left brachial artery cannula was sited. Saline was infused for 30 minutes, during which two measurements of forearm blood flow were made (at −20 and −10 minutes). Blood pressure was measured immediately after each forearm blood flow measurement, thereby avoiding any effect on forearm blood flow measurements of the venous congestion caused by this procedure.[36] Three protocols were then followed, each in separate groups of subjects, as follows.

Protocol 1: Low-dose intra-arterial endothelin-1 and endothelin-3. On four separate occasions, in random order, six subjects received brachial artery infusion of endothelin-1 and endothelin-3 at 1 and 5 pmol/min, each for 60 minutes. The choice of doses was based on previous work showing, in vivo, that 5 pmol/min of endothelin-1 causes slow-onset vasoconstriction in human forearm resistance vessels, reducing blood flow by ≈40%.[29,33] Forearm blood flow was recorded from 3 minutes before to 5 minutes after the endothelin infusion was begun. Thereafter, measurements were made at 5-minute intervals for 60 minutes. Blood pressure was measured 60 minutes after the infusion was begun.

Protocol 2: High-dose intra-arterial endothelin-1 and endothelin-3. On two separate occasions, in random, balanced order, six subjects received endothelin-1 and endothelin-3 via the brachial artery at 60 pmol/min for 5 minutes, followed by physiological saline for 55 minutes. Because no significant vasodilatation had been observed in previous studies using intra-arterial endothelin-1 at 5 pmol/min,[29,33] we chose a dose of 60 pmol/min with the intention of stimulating sufficient endothelial generation of dilator substances to cause vasodilatation before the development of vasoconstriction. Forearm blood flow was recorded from 3 minutes before to 10 minutes after the endothelin infusion was begun. Thereafter, measurements were made at 5-minute intervals for 60 minutes. Blood pressure was measured 10 and 60 minutes after the infusion was begun.

TABLE 1. Mean Arterial Pressure, Heart Rate, and Forearm Blood Flows Before and After Brachial Artery Administration of Peptides in the Two Study Protocols (1 and 2) Comparing Endothelin-1 and Endothelin-3

	Protocol 1				Protocol 2	
Parameter	ET-1 (1 pmol)	ET-3 (1 pmol)	ET-1 (5 pmol)	ET-3 (5 pmol)	ET-1 (60 pmol)	ET-3 (60 pmol)
MAP, mm Hg						
Basal	89±5	88±6	90±5	88±4	87±5	83±4
10 min	...	...	...	...	89±6	87±3
60 min	91±6	89±7	89±4	86±4	89±6	90±4
HR, bpm						
Basal	65±4	66±3	67±5	66±4	66±2	66±2
10 min	...	...	...	...	63±4	65±3
60 min	71±6	67±4	68±6	62±5	64±5	65±2
Infused FBF, mL·100 $mL^{-1}\cdot min^{-1}$						
Basal	3.9±0.9	4.0±0.7	3.9±0.9	4.4±1.3	3.7±0.6	3.0±0.3
60 min	3.4±0.9	3.7±0.8	3.1±1.0	3.3±0.5	3.2±0.4	2.4±0.3
Noninfused FBF, mL·100 $mL^{-1}\cdot min^{-1}$						
Basal	3.0±0.5	3.2±0.5	3.0±0.5	4.1±0.6	3.4±0.7	3.3±0.2
60 min	2.8±0.5	3.1±0.6	3.9±0.9	4.0±1.0	3.6±0.6	3.7±0.6

ET indicates endothelin; MAP, mean arterial pressure; HR, heart rate; bpm, beats per minute; and FBF, forearm blood flow. Values are mean±SEM. There were no significant differences in basal MAP, HR, and FBF between study days. MAP, HR, and FBF in the noninfused arm did not change significantly on any study day after infusion of peptides.

Protocol 3: Intra-arterial endothelin-1 and sarafotoxin S6c. On two separate occasions, in random, balanced order, six subjects received endothelin-1 and sarafotoxin S6c via the brachial artery at 5 pmol/min for 60 minutes. Forearm blood flow was recorded from 3 minutes before to 5 minutes after peptide infusion was begun. Thereafter, measurements were made at 5-minute intervals for 60 minutes. Blood pressure was measured at 60 minutes, just before the infusion was terminated.

Hand Vein Studies

Protocol 4: Intravenous endothelin-1 and sarafotoxin S6c. Six subjects were studied on two separate occasions, in random, balanced order. Subjects rested semirecumbent throughout. The dorsal hand vein cannula and the LVDT were sited. Saline was infused for 30 minutes, during which vein diameter was measured every 5 minutes. Endothelin-1 and sarafotoxin S6c were infused at 5 pmol/min for 60 minutes, with measurements of vein diameter every 5 minutes. The choice of this dose was based on previous work that showed, in vivo, that endothelin-1 at 5 pmol/min causes slow-onset venoconstriction of ≈60% in human skin capacitance vessels.[29-31] Blood pressure was measured twice before the dose was given and at 60 minutes, just before the infusion was terminated.

Data Analysis and Statistics

Plethysmographic data listings were extracted from the CHART data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (EXCEL 4.0; Microsoft Ltd). Because wrist cuff inflation results in a transient forearm vasoconstriction,[37] recordings made in the first 60 seconds after wrist cuff inflation were not used for analysis. Usually, the last five flow recordings in each 3-minute measurement period were calculated and averaged for the infused and noninfused arms. However, to detect early transient changes in blood flow, every recording made immediately before and after the start of peptide infusion was analyzed. Basal blood flow was taken as the average of all flow recordings made in the 2 minutes before infusion of peptides was begun. The intersubject, intrasubject (interstudy), and intrasubject (intrastudy) coefficients of variation for basal (interstudy), and intrasubject (intrastudy) coefficients of variation for the basal ratio of blood flow between infused and noninfused arms in our laboratory are 22%, 15%, and 8%, respectively. Therefore, to reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point, in effect using the noninfused arm as a contemporaneous control for the infused arm.[38] Forearm blood flow results are shown as a percentage change from basal in the ratio of blood flow between infused and noninfused arms.[29]

Basal vein diameter was calculated as the mean of the last three measurements before the start of the peptide infusion, expressed in millimeters. The intersubject, intrasubject (interstudy), and intrasubject (intrastudy) coefficients of variation for basal hand vein diameter measurements in our laboratory are 43%, 26%, and 5%, respectively. Given the high intersubject and interstudy variability in hand vein diameter, responses after infusion of peptides are expressed as percentage change in vein diameter from basal.[32] Duplicate blood pressure measurements were averaged at each time point. Basal blood pressure was taken as the average of the second set of measurements made before infusion of peptides.

To obtain an estimate of the contribution of ET_B receptors to vasoconstriction, the ratio of constriction to the ET_B agonist compared with constriction to endothelin-1 was calculated for each subject at the 60-minute time point. Because these data had a skewed distribution, ratios were logarithmically transformed for statistical analysis. Data are shown as mean values, with 95% confidence intervals (CI) shown in the text and SEM in the figures. Data were examined by a repeated-measures ANOVA with statistical testing of overall significance by Scheffé's F test (ANOVA) using STATVIEW 512+ software (Brainpower Inc) for the Apple Macintosh personal computer.

Results

Basal blood pressure, heart rate, forearm blood flow, and vein diameter were similar on the different study days, and there was no significant difference in basal forearm blood flow between the infused and noninfused arms (Tables 1 and 2). Blood pressure, heart rate, and blood flow in the noninfused arm did not change significantly after infusion of any study agent, confirming that

TABLE 2. Mean Arterial Pressure, Heart Rate, Forearm Blood Flow, and Hand Vein Size Before and After Administration of Peptides in the Two Study Protocols (3 and 4) Comparing Endothelin-1 and Sarafotoxin S6c

	Protocol 3		Protocol 4	
Parameter	ET-1 (5 pmol IA)	S6c (5 pmol IA)	ET-1 (5 pmol IV)	S6c (5 pmol IV)
MAP, mm Hg				
Basal	82±3	82±3	89±5	84±3
60 min	85±3	85±4	89±4	84±2
HR, bpm				
Basal	62±8	63±3	70±4	65±4
60 min	55±3	64±4	68±5	59±5
Infused FBF, mL · 100 mL^{-1} · min^{-1}				
Basal	3.1±0.4	3.1±0.5	...	...
60 min	1.4±0.3	1.8±0.3	...	...
Noninfused FBF, mL · 100 mL^{-1} · min^{-1}				
Basal	2.7±0.4	2.7±0.4	...	...
60 min	2.3±0.2	2.3±0.5	...	...
Vein size, mm				
Basal	...	...	0.37±0.08	0.44±0.08

IA indicates intra-arterial; IV, intravenous. Other abbreviations as in Table 1. There were no significant differences in basal MAP, HR, FBF, and hand vein diameter between study days. MAP, HR, and FBF in the noninfused arm did not change significantly after infusion of peptides.

Protocol 1: Low-Dose Intra-arterial Endothelin-1 and Endothelin-3

Endothelin-1 at 1 pmol/min caused a modest but significant forearm vasoconstriction, with an 11% reduction in forearm blood flow at 60 minutes (CI, −22% to −1%; ANOVA, $P=.02$; Fig 1). Endothelin-3 at 1 pmol/min tended to decrease forearm blood flow, with a 5% reduction in blood flow at 60 minutes, but this was not significant (CI, −14% to +3%; ANOVA, $P=.163$; Fig 1). There was no significant difference between the responses to endothelin-1 and endothelin-3 at 1 pmol/min (ANOVA, $P=.454$). There was no significant vasodilatation early in the course of infusion of either peptide. The average ratio of forearm vasoconstriction to endothelin-3 and endothelin-1 was 0.16, although this estimate had wide CIs (CI, 0.03 to 0.98).

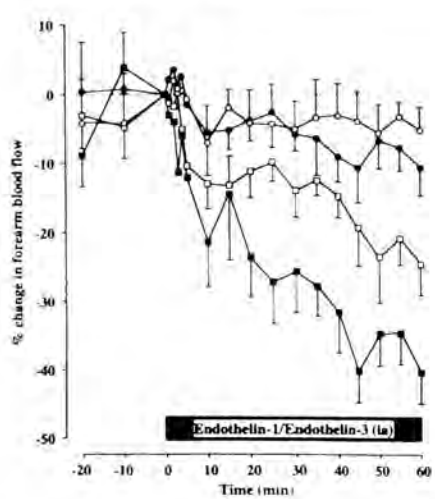

FIG 1. Graph. Six subjects received brachial artery infusion of endothelin-3 (○, 1 pmol/min; □, 5 pmol/min) and on a separate occasion, endothelin-1 (●, 1 pmol/min; ■, 5 pmol/min), each for 60 minutes. Shaded bar indicates period of infusion of endothelin isopeptides. Endothelin-1 caused significant forearm vasoconstriction at both doses, whereas the effect of endothelin-3 was significant only at 5 pmol/min. For clarity, error bars have been removed from that part of the figure showing results for the first 5 minutes of peptide infusion. ia indicates intra-arterial.

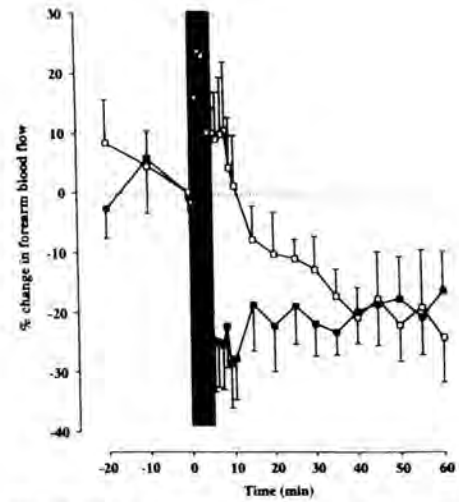

FIG 2. Graph. Six subjects received brachial artery infusion of endothelin-1 (■, 60 pmol/min) and on a separate occasion, endothelin-3 (□, 60 pmol/min), each for 5 minutes. Shaded area indicates period of infusion of endothelin isopeptides. Forearm vasodilatation occurred initially with endothelin 3 but not with endothelin-1. Both isopeptides then caused vasoconstriction of similar degree. ia indicates intra-arterial.

Endothelin-1 at 5 pmol/min caused substantial forearm vasoconstriction, with a 40% reduction in forearm blood flow at 60 minutes (CI, −52% to −28%; ANOVA, $P=.0002$; Fig 1). The same dose of endothelin-3 also significantly reduced forearm blood flow, with a 25% reduction in blood flow at 60 minutes (CI, −36% to −13%; ANOVA, $P=.001$; Fig 1). There was significantly greater vasoconstriction after endothelin-1 than endothelin-3 (ANOVA, $P=.04$). There was no significant vasodilatation early in the course of infusion of either peptide. The average ratio of forearm vasoconstriction to endothelin-3 and endothelin-1 was 0.58 (CI, 0.39 to 0.87).

Protocol 2: High-Dose Intra-arterial Endothelin-1 and Endothelin-3

Endothelin-1, at 60 pmol/min for 5 minutes, caused a trend to transient nonsignificant forearm vasodilatation in the first 2 minutes of infusion, with a maximum increase of 16% (CI, −7% to +23%; Fig 2) at 2 minutes. Thereafter, vasoconstriction occurred, with the maximum decrease in blood flow occurring at 10 minutes (−28%; CI, −48% to −9%), although flow was still reduced after 60 minutes (−17%; CI, −30% to −4%). Endothelin-3 caused significant early forearm vasodilatation, with a maximum increase in flow of 24% at 3 minutes (CI, +4% to +43%; Fig 2). Forearm vasoconstriction occurred after 10 minutes, with a maximum reduction in blood flow of 24% at 60 minutes (CI, −43% to −5%). There was a significant difference between the overall responses to endothelin-1 and endothelin-3 over the 60 minutes after bolus administration of isopeptide (ANOVA, $P=.04$). However, maximum vasoconstriction to the isopeptides was similar (Fig 2). The average ratio of forearm vasoconstriction to endothelin-3 and endothelin-1 was 0.82, although this estimate had wide CIs (CI, 0.13 to 5.07).

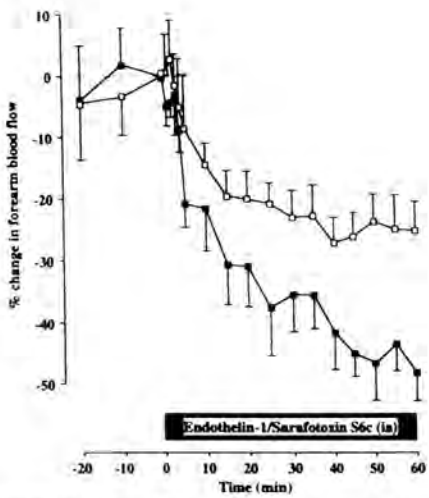

FIG 3. Graph. Six subjects received brachial artery infusion of endothelin-1 (■, 5 pmol/min) and on a separate occasion, sarafotoxin S6c (□, 5 pmol/min), each for 60 minutes. Shaded bar indicates period of infusion of peptides. Both peptides caused significant forearm vasoconstriction, although the effect of sarafotoxin S6c was less than that of endothelin-1. ia indicates intra-arterial.

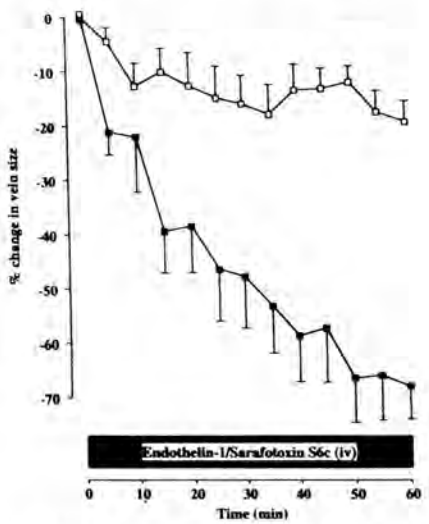

FIG 4. Graph. Six subjects received dorsal hand vein infusion of endothelin-1 (■, 5 pmol/min) and on a separate occasion, sarafotoxin S6c (□, 5 pmol/min), each for 60 minutes. Shaded bar indicates period of infusion of peptides. Both peptides caused significant venoconstriction, although the effect of sarafotoxin S6c was less than that of endothelin-1. iv indicates intravenous.

Protocol 3: Intra-arterial Endothelin-1 and Sarafotoxin S6c

Endothelin-1 at 5 pmol/min did not cause early vasodilatation but did produce slow-onset forearm vasoconstriction, with a maximum reduction in forearm blood flow of 48% at 60 minutes (CI, −60% to −37%; ANOVA, $P=.0001$; Fig 3). There was no significant vasodilatation to sarafotoxin S6c early in the course of the infusion, although there may have been a trend for this to occur (Fig 3). Like endothelin-1, sarafotoxin S6c caused slow-onset forearm vasoconstriction (ANOVA versus basal, $P=.002$; Fig 3). However, the maximum change in blood flow with sarafotoxin S6c at 60 minutes (−25%; CI, −37% to −13%) was significantly less than that to endothelin-1 (ANOVA, $P=.04$). The average ratio of forearm vasoconstriction to sarafotoxin S6c and endothelin-1 was 0.48 (CI, 0.30 to 0.75).

Protocol 4: Intravenous Endothelin-1 and Sarafotoxin S6c

Endothelin-1 caused a slow-onset and marked decrease in hand vein diameter, with a maximal reduction at 60 minutes (−68%; CI, −84% to −52%; ANOVA, $P=.001$; Fig 4). Sarafotoxin S6c also caused venoconstriction, although the maximum decrease in hand vein size at 60 minutes (−19%; CI, −29% to −9%; ANOVA versus basal, $P=.003$; Fig 4) was significantly less than that to endothelin-1 (ANOVA, $P=.002$). The average ratio of venoconstriction to sarafotoxin S6c and endothelin-1 was 0.25 (CI, 0.14 to 0.44).

Discussion

These studies show that selective agonists at endothelin ET_B receptors constrict forearm resistance and hand capacitance vessels in vivo in humans. In addition, high doses of endothelin-3, and perhaps of endothelin-1, cause transient forearm vasodilatation. These results suggest an important role for ET_B receptors in mediating the vascular effects of endothelin-1. It is possible that different findings might have been obtained if other vascular beds had been studied. However, responses in human forearm resistance vessels and dorsal hand veins are thought to be broadly representative of responses in other resistance and capacitance beds.[39] Given that resting forearm blood flow is ≈50 mL/min,[39] doses of 1, 5, and 60 pmol/min of peptide should achieve local concentrations of ≈0.02, ≈0.1, and ≈1 nmol/L, respectively. Endothelin-1 would be expected to act equally on both ET_A and ET_B receptors at these concentrations, while endothelin-3 would be expected to be relatively selective for the ET_B receptor, because this isopeptide has a K_i at ET_A receptors of about 140 nmol/L.[5] Sarafotoxin S6c at 5 pmol/min should have been highly selective for the ET_B receptor, because the calculated concentration in forearm blood (0.1 nmol/L) is at least 70 000-fold lower than its K_i at ET_A receptors (>7300 nmol/L).[5]

Administration of endothelin-3 at 60 pmol/min caused significant early forearm vasodilatation, and there was also a tendency for similar transient vasodilatation to occur with endothelin-1, although this was not statistically significant. Vasodilatation is likely to have been due to activation of ET_B receptors on endothelial cells, causing generation of endothelium-derived dilator substances.[31] The apparent absence of significant vasodilatation to high-dose endothelin-1 may have been due to additional early vasoconstriction mediated by ET_A receptors masking dilatation, although it should be noted that the CIs at these time points were sufficiently wide for an ≈20% vasodilatation to endothelin-1 to have been missed by chance. Lower doses of endothelin-1 and sarafotoxin S6c failed to cause early vasodilatation. The lack of vasodilatation to endothelin-1 contrasts with previous findings,[40] possibly because of differences in doses used and experimental design. In view of the relatively high doses required to cause vasodilatation, it is likely that vasodilatation to the endothelins represents a pharmacological rather than a physiological phenomenon. Because human dorsal hand veins have no basal tone, it is not possible to demonstrate whether endothelin-1 or sarafotoxin S6c causes venodilatation without

preconstriction of the vein. Previous work has shown no venodilatation to endothelin-1 or endothelin-3 in preconstricted dorsal hand veins.[41] Nonetheless, inhibition of prostaglandin but not nitric oxide generation potentiates venoconstriction to endothelin-1 in vivo in humans.[31] Thus, the venous endothelium may generate vasodilator substances in response to endothelin, but the vasodilator effects of such substances appear to be masked by the simultaneous direct venoconstriction caused by the peptide and serve only to modulate venoconstriction.

Given that both endothelin-3 and sarafotoxin S6c caused vasoconstriction, our results suggest the presence of vasoconstrictor ET_B receptors. However, constriction to the ET_B agonists was almost always less than that to the nonselective ET_A and ET_B agonist endothelin-1, implying that both ET_A and ET_B receptors contribute to vasoconstriction. The 95% CIs of the ratio of forearm vasoconstriction to sarafotoxin S6c and endothelin-1 are consistent with ET_B receptors contributing substantially to constriction, accounting for between 30% and 75% of the response to endothelin-1. Although the magnitude of the ET_B contribution in vitro appears to differ between vessels,[17] the similarity of responses in forearm resistance vessels and cutaneous capacitance vessels of the hand suggests that ET_B receptors may be of widespread functional importance in human blood vessels.

Our finding of ET_B receptor–mediated vasoconstriction of resistance vessels contrasts with some in vitro studies that suggest little contribution of ET_B receptors to constriction of human arteries.[11,17-24] This difference may reflect the fact that we examined responses in an intact resistance bed, because ET_B receptor–mediated vasoconstriction appears to play a relatively greater role in smaller vessels, particularly those responsible for determining resistance.[17,42] All of the in vitro studies in which human vessels exhibited little or no ET_B–mediated arterial vasoconstriction examined vessels >400 μm in diameter.[11,17-24] In addition to the influence of vessel size on the contribution of ET_B receptors, there may be regional differences. Local injection of the ET_A antagonist PD147953 has been shown to completely prevent vasoconstriction of human skin vessels caused by intradermal injection of endothelin-1, suggesting that vasoconstriction is mediated mainly by ET_A receptors in this microvascular bed.[43] The effects of sarafotoxin S6c, compared with those of endothelin-1, were less in hand veins than in forearm resistance vessels, despite in vitro evidence from animal vessels that responses to ET_B agonists are greater in veins than arteries.[8,9,12,13] This may reflect a true species difference, because endothelin-1 is about eightfold more potent as a venoconstrictor than endothelin-3 in human hand veins,[41] which also suggests that ET_A receptors predominate in these vessels.

Although vasoconstriction to the ET_B agonists endothelin-3 and sarafotoxin S6c is most likely to be caused by stimulation of vascular smooth muscle ET_B receptors, there are alternative explanations. First, ET_B receptors may be confined to endothelial cells but cause late-onset vasoconstriction through stimulation of the generation of endothelium-derived vasoconstrictor agents. These substances might include constrictor prostanoids or even endothelin-1, because endothelin-3 is known to stimulate production of endothelin-1 in vitro.[44] Second, some of the effects of endothelin-3 could have been mediated by a putative ET_C (endothelin-3–selective) receptor situated on endothelial cells. However, although there is evidence from binding[44] and functional[45] studies to support the existence of an endothelin-3–selective receptor in the vasculature, and a potential candidate has been identified in *Xenopus laevis* melanophores,[46] this receptor has not been identified in humans. Any contribution from the putative ET_C receptor will depend on its isolation and pharmacological characterization. Third, there may be receptor-mediated clearance of endogenously generated endothelin-1 by ET_B receptors, as has been shown in animals.[47] If this were the case, ET_B agonists might prevent local clearance of endothelin-1, which would then act on ET_A receptors to cause vasoconstriction. However, this possibility appears highly unlikely, given that ET_A antagonists do not influence vasoconstriction to sarafotoxin S6c in vitro.[8,10,13,26,48] In future, studies with selective ET_B receptor antagonists should clarify this issue, because such agents would be expected to potentiate responses to endothelin-1 if ET_B receptor–mediated clearance of endothelin-1 does occur.

Thus, the most likely explanation for our results is that there are functionally active ET_A and ET_B receptors on vascular smooth muscle cells causing vasoconstriction, to both of which endothelin-1 would have access. These findings have implications for the future development of antiendothelin therapies, because they suggest that full inhibition of vasoconstriction to endogenously generated endothelin-1 may be obtained only by use of either combined $ET_{A/B}$ endothelin receptor antagonists[49] or inhibitors of endothelin generation.[29]

Acknowledgments

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Endothelium-Dependent Modulation of Venoconstriction to Sarafotoxin S6c in Human Veins In Vivo

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Summary: We investigated the vascular effects mediated by ET_A and ET_B receptors in human dorsal hand veins in vivo, using sarafotoxin S6c (SFTX6c) as a selective agonist of ET_B receptors and endothelin 1 (ET-1) as a non selective agonist of ET_A and ET_B receptors. The cyclooxygenase inhibitor aspirin and the nitric oxide synthase inhibitor L-NMMA were used to examine the modulating role of endothelial vasodilators on the response to SFTX6c. Drugs were all infused into the hand veins, at locally but not systemically active doses, via a 23 SWG butterfly cannula, with the exception of aspirin, which was administered orally. Hand vein size was measured by the Aellig technique. The study was performed in six healthy male subjects. Data (mean ± SEM) were examined by ANOVA. Results are expressed as percent change from baseline at 60 min. ET-1 (5 pmol/min for 60 min) caused venoconstriction of 68 ± 6% ($p = 0.0001$). SFTX6c at the same dose caused venoconstriction of 19 ± 4% ($p = 0.003$). The response to SFTX6c was significantly less than to ET-1 ($p = 0.002$). Constriction to SFTX6c tended to increase when this agent was co administered with aspirin (25 ± 7%) or L-NMMA (24 ± 10%) and was significantly potentiated when these agents were co-administered (45 ± 4%; $p = 0.01$ vs. SFTX6c alone). We have demonstrated that the selective ET_B agonist SFTX6c produces venoconstriction in human hand veins in vivo and that this venoconstriction is modulated by the generation of endothelium-derived vasodilators. In this vascular bed, venoconstriction rather than venodilatation appears to be the predominant effect of stimulation of ET_B receptors with SFTX6c. Key Words: Human—Venoconstriction—Endothelin-1—Sarafotoxin S6c—Endothelium.

The endothelins are a family of 21-amino-acid peptides with potent and characteristically sustained vasoconstrictor and vasopressor actions (1). Endothelin-1 (ET-1) is the predominant isopeptide generated by the vascular endothelium (2) and therefore appears to be the most important isoform mediating cardiovascular effects. Two specific receptors for the endothelins have been isolated by in vitro expression of cloned human cDNA (3,4). The ET_A receptor has a high affinity for ET-1 compared with endothelin-3 (ET-3), whereas the ET_B receptor has equal affinity for both endothelins.

Vasoconstriction to ET-1 was initially believed to be mediated solely by vascular smooth-muscle cell ET_A receptors, and endothelial cell ET_B receptors were believed only to mediate generation of endothelium-derived dilator substances. Indeed, there is considerable evidence that stimulation of endothelial ET_B receptors can cause production of nitric oxide and dilator prostanoids, both to cause the initial depressor effect of systemic bolus administration of ET-1 and to modulate the sustained vasoconstriction associated with ET-1 (5). More recently, it has been shown that ET_B receptor mRNA is expressed in human vascular smooth muscle (6), consistent with a potential vasoconstrictor role for the ET_B receptor. Indeed, in animals and in humans, there is functional evidence for ET_B receptor-mediated vasoconstriction in vitro, particularly in veins (7–9). However, the contribution of ET_B receptors to ET-1–mediated constriction can vary depending on species, vessel type, and vessel size.

In human hand veins, venoconstriction to ET-1 is attenuated by dilator prostanoids but not by nitric oxide (10). Here, we have examined the role of the endothelium-derived dilators nitric oxide and prostacyclin in modulating venoconstriction to locally but not systemically active doses of sarafotoxin S6c

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(SFTX6c), an endothelin receptor agonist with 30,000-fold selectivity for the ET_B receptor in vitro (11).

MATERIALS AND METHODS

Experimental procedures

Six healthy men (age range 25–39 years) participated in the study, with local Ethics Review Committee approval. Subjects rested semirecumbent in a quiet room maintained at a constant temperature for each study (25–27°C). Drugs were infused via a 23 SWG cannula (Abbott) sited in a selected dorsal hand vein in the direction of flow, as described previously (10). Local anesthesia was not employed. The same dorsal hand vein was used in each study. Internal diameter of the dorsal hand vein was measured by the Aellig displacement technique (12) at 5-min intervals throughout each study period. Blood pressure was measured in the noninfused arm at 30-min intervals.

Drugs

On separate occasions, ET-1 (NovaBiochem, Nottingham, U.K.) and SFTXS6c (Sigma Chemical Co Ltd., Nottingham, U.K.) were administered for 60 min at a constant rate of 5 pmol/min, based on results from previous studies (10,13). A single dose was used because the slow onset and long-lasting action of the endotheiins precludes the use of repeated doses in a single study to examine conventional dose-response relationships. $L\text{-}N^G$-monomethyl-arginine (L-NMMA; NovaBiochem, Nottingham, U.K.), a specific substrate analogue inhibitor of nitric oxide synthase in humans (14), was administered in a dose of 100 nmol/min (10). This dose of L-NMMA has no effect on basal hand vein size (15). Aspirin (600 mg, soluble: Reckitt & Coleman, Hull, U.K.) was dissolved in 200 ml water and administered 30 min before local peptide infusions. Aspirin irreversibly inhibits cyclo-oxygenase (EC 1.14.99.1) and, when given at this dose, inhibits bradykinin-stimulated endothelial production of prostacyclin by at least 85%, with recovery developing over the next 6 h (16). All drugs, with the exception of aspirin, were dissolved in saline (0.9%; Travenol). In each study, saline was infused for 30 min before infusion of the study agent. The *total* rate of infusion was maintained constant throughout all studies at 0.25 ml/min.

Study design

The study involved five separate study periods in the same subjects, each study period separated by at least 1 week. Responses to ET-1 and SFTX6c alone were examined. In addition, responses to SFTX6c were investigated in the presence of aspirin and L-NMMA, administered both independently and together. The order of the study periods was randomized, with the exception of the sarafotoxin and combined L-NMMA and aspirin study, which was the final study period for each subject.

Data presentation and statistics

Basal vein size was calculated in millimeters as the mean of the last two measurements taken during saline infusion. Vein size during drug administration was expressed as percentage change in vein size from basal, at 60 min. All results are expressed as mean ± SEM. Data were examined by repeated measures analysis of variance. Statistical significance was accepted at the 5% level.

RESULTS

In the six subjects, basal vein size was similar for each study and blood pressure did not alter significantly throughout any of the study periods. ET-1 alone caused a slowly developing venoconstriction, with a maximum 68 ± 6% reduction of basal internal vein diameter at 60 min ($p = 0.0001$ vs. basal). SFTX6c also caused significant venoconstriction (19 ± 4%; $p = 0.003$ vs. basal). The response to ET-1 was substantially greater than that to SFTX6c ($p = 0.002$) (Fig. 1). Venoconstriction to SFTX6c tended to increase after administration of aspirin (25 ± 7%) and during co-infusion of L-NMMA (24 ± 10%), but was not significantly different than that to SFTX6c alone (Fig. 2). Venoconstriction to SFTX6c was substantially and significantly increased in the presence of aspirin and L-NMMA together (46 ± 9%; $p = 0.01$ vs. SFTX6c; $p = 0.0001$ vs. basal). Venoconstriction to SFTX6c in the presence of both aspirin and L-NMMA was less prominent than, but did not differ significantly from, that to ET-1 when infused alone ($p = 0.1$) (Figs. 1 and 2).

DISCUSSION

In these studies we have investigated the venoconstrictor effects of SFTX6c and their modulation by the endothelium-derived dilators nitric oxide and prostacyclin. We have confirmed the earlier findings that ET-1 (10) and SFTX6c (13) constrict human hand veins. As before (13), an equivalent dose of SFTX6c causes less venoconstriction than ET-1.

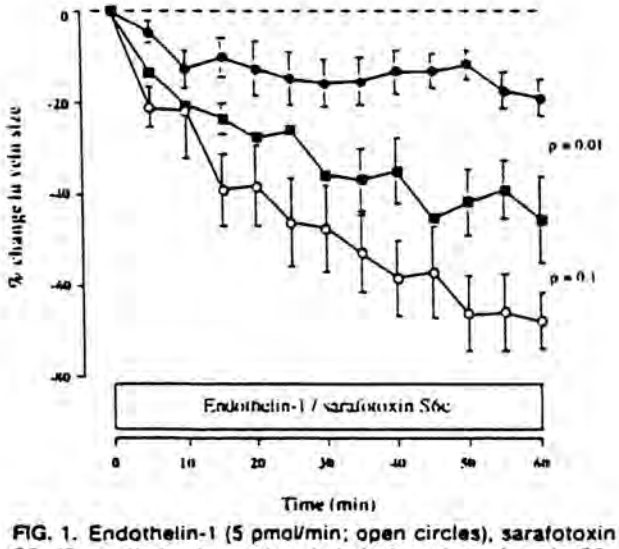

FIG. 1. Endothelin-1 (5 pmol/min; open circles), sarafotoxin S6c (5 pmol/min alone; closed circles), and sarafotoxin S6c (5 pmol/min with L-NMMA 100 nmol/min and after aspirin 600 mg; closed squares).

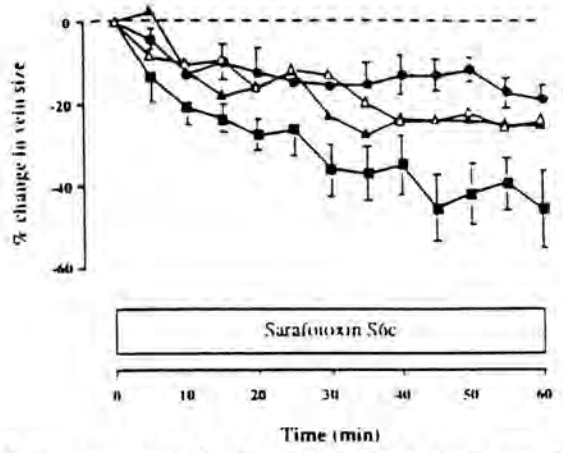

FIG. 2. Sarafotoxin S6c (5 pmol/min alone; closed circles), co-infused with L-NMMA (open triangles), after aspirin 600 mg (closed triangles), and with both L-NMMA and aspirin (closed squares).

These findings suggest that ET_B receptors contribute to but do not wholly account for ET-1-mediated venoconstriction.

In addition, we have demonstrated that the venoconstriction to SFTX6c is significantly and substantially increased when generation of both nitric oxide and dilator prostanoids, most likely prostacyclin, is blocked. Combined inhibition of nitric oxide and prostacyclin appeared to produce a greater effect on venoconstriction to SFTX6c than the addition of the individual effects of inhibition of generation of either nitric oxide or prostacyclin alone. This may reflect a capacity for the endothelium to compensate for inhibition of one dilator mediator by increased production of another. We have shown previously that responses to ET-1 are potentiated by aspirin administration but not by co-infusion of L-NMMA (10). It would be important to further investigate responses to ET-1 in human hand veins in vivo in the presence of L-NMMA and aspirin, both alone and given together. It appears from our results with SFTX6c that the endothelial ET_B receptor modulates the constrictor effects produced by stimulation of the vascular smooth muscle ET_B receptors, through generation of vasodilator substances by the endothelium. However, in these experiments the vasoconstrictor action predominates. In diseases such as chronic heart failure, in which endothelium-dependent vasodilatation is impaired, vasoconstrictor effects of ET-1 may be enhanced as a result of unopposed vasoconstrictor effects mediated by both ET_A and ET_B receptors on vascular smooth muscle. Given that the overall effect of combined stimulation of the vascular smooth muscle and endothelial cell ET_B receptors is venoconstriction, it may be necessary to block both ET_A and ET_B receptors to completely block venoconstriction to ET-1. Drugs that would act specifically on the vascular smooth-muscle receptors (17) may be more effective vasodilators, because they would fully block ET-1 mediated vasoconstriction while allowing the potentially desirable effects of the endothelial ET_B receptors to be preserved.

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The Effect of Cooling on the Contractile Response to Endothelin-1 in Small Arteries from Humans

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Summary: The pathogenesis of the prolonged cold-induced vasospasm of Raynaud's disease (RD) is unknown. Cooling has been shown to decrease the sensitivity of cutaneous rabbit ear arteries to endothelin-1 (ET-1), perhaps by increasing the availability of nitric oxide (NO). If the endothelium in RD lacks this NO-mediated inhibitory function during cooling, increased production and enhanced constriction to ET-1 could cause vasospasm. This study examined the effect of cooling on the contractile response to ET-1 in human microvessels. Small arteries were dissected from biopsy specimens of human fat and were cannulated in a small vessel arteriograph. Cumulative concentration–response curves to ET-1 (10^{-12} to 3×10^{-7} *M*) were obtained in vessels at 37°C ($n = 8$) and 24°C ($n = 7$). Cooling to 24°C resulted in an eightfold decrease in sensitivity to ET-1 (EC_{50} 6.6 ± 2.5×10^{-10} *M* at 37°C vs. 5.5 ± 2.5×10^{-9} *M* at 24°C; $p < 0.05$, unpaired *t* test). The E_{max} was not significantly different at 37°C and 24°C (114 ± 9 at 37°C vs. 138 ± 14 at 24°C; $p = 0.18$). These results suggest that cooling decreases the sensitivity of human microvessels to ET-1. Further investigation is needed to establish the mechanism of this effect, including studies in vessels obtained from patients with RD. **Key Words:** Endothelin—Nitric oxide—Cooling—Human—Resistance arteries—Raynaud's disease.

Raynaud's disease (RD) is characterized by intense vasospasm of the extremities, particularly the digits, in response to cold exposure or marked emotion. It causes numbness and pallor, followed by cyanosis, and can produce extreme local discomfort on reperfusion. The pathogenesis of RD remains unknown and available treatments are not particularly effective. The vascular endothelium plays a major role in the regulation of vascular tone. Endothelial dysfunction occurs in a number of vascular conditions, including hypertension, diabetes mellitus, and atheromatous disease (1,2). Clinical studies implicate the endothelium-derived constrictor endothelin-1 (ET-1) in mediating the prolonged cold-induced vasospasm of RD (3). Using cutaneous arteries from rabbit ear, Monge et al. (4) have shown that cooling decreases sensitivity to ET-1, perhaps owing to increased availability of nitric oxide (NO). If the endothelium in Raynaud's patients lacks this NO-mediated inhibitory function during cooling, increased production and enhanced constriction to ET-1 might lead to the development of vasospasm. This study examined the effect of cooling on the response to ET-1 in human microvessels isolated from biopsy specimens of subcutaneous fat obtained at surgery.

MATERIALS AND METHODS

Subcutaneous fat biopsy specimens obtained from patients undergoing elective gastrointestinal surgery at the Western General Hospital were placed in a dissecting dish containing physiologic salt solution (PSS: in m*M*, 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose). Small arteries with a mean luminal diameter of 300 ± 28 μm ($n = 15$) were excised under a dissection microscope and cannulated in a small vessel arteriograph (Living Systems Instrumentation Inc., Burlington, VT, U.S.A.). A pressure servo unit maintained intraluminal pressure, without flow, at 50 mm Hg. Luminal diameter was measured using a video dimension analyzer. After a 60–90 min period of equilibration, during which the vessel chamber was superfused with PSS, continuously gassed with 95% O_2/5% CO_2, and the temperature raised to 37°C, the contractility of the arterioles was assessed separately using phen-

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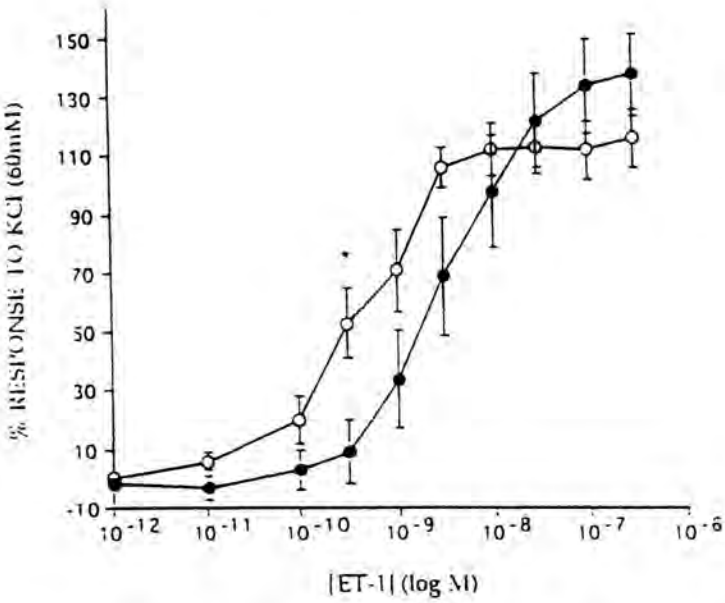

FIG. 1. The effect of cooling on the contractile response to ET-1 in human arterioles: concentration–response curves to ET-1 (expressed as percentage response to KCl, 60 m*M*) at 37°C (○; $n = 8$) and 24°C (●; $n = 7$). All values are mean ± SEM. $^*p < 0.05$.

ylephrine 10^{-5} *M* (PE: Sigma) and potassium chloride 60 m*M* (KCl: Sigma). Acetylcholine 10^{-5} *M* (ACh: Sigma) was given during maximal contraction to PE to assess endothelial integrity. Cooling was achieved by passing the superfusate through a rapid heat-exchange Peltier element (Moor Instruments Ltd., Millwey, Axminster, Devon, U.K.) before it entered the arteriograph. Cumulative concentration–response curves to ET-1 10^{-12} to 3×10^{-7} *M* (Novabiochem) were obtained in vessels at 37°C ($n = 8$) and 24°C ($n = 7$). Mean resting lumal diameter, percentage contraction to KCl, and percentage relaxation to ACh at 37°C did not differ significantly between groups, and the age and sex ratio of the patients in each group were not significantly different (Table 1).

Data analysis

Responses were expressed as percentage of the contraction to KCl at 37°C. EC_{50} (the concentration producing half-maximum KCl contraction) and E_{max} values (the maximal contraction relative to KCl) are shown as mean ± SEM. Student's unpaired *t* test was used to compare the responses in vessels studied at 37°C and 24°C. Statistical significance was achieved at $p < 0.05$.

RESULTS

ET-1 produced a concentration-dependent constriction in all vessels. Cooling to 24°C caused a decrease in sensitivity to ET-1, as seen by a rightward shift of the concentration–response curve (Fig. 1) and an eightfold increase in the EC_{50} (6.6 ± 2.5×10^{-10} *M* at 37°C vs. 5.5 ± 2.5×10^{-9} *M* at 24°C; $p < 0.05$). There was a tendency for the maximal contraction to be higher during cooling, but the difference was not statistically significant (114 ± 9 at 37°C vs. 138 ± 14 at 24°C; $p = 0.18$).

TABLE 1. *Patient details and baseline data for vessels studied at 37°C and 24°C*

	37°C	24°C
Age (years)	58 ± 6	57 ± 10
Sex ratio (M:F)	6:2	5:2
LD	278 ± 26	328 ± 56
% Contraction to KCl	39 ± 6	51 ± 7
% Relaxation to ACh	86 ± 8	77 ± 14

LD, resting luminal diameter (μm); KCl, potassium chloride (60 m*M*); ACh, acetylcholine (10^{-5} *M*). Values are mean ± SEM.

DISCUSSION

This study shows that cooling decreases the sensitivity to ET-1 in small arteries from humans. In a similar study using the rabbit ear artery, Monge et al. (4) examined the effect of L-NAME, an inhibitor of NO synthase, and also de-endothelialization, on responses to ET-1 at 37°C and 24°C. Their findings suggest that the decrease in ET-1 sensitivity during cooling is caused either by an increased availability of NO, which could be due to increased generation or reduced clearance or, alternatively, is caused by increased sensitivity to NO. The role of NO in modulating the actions of ET-1 during cooling in human arterioles requires further investigation. If the endothelium in patients with RD lacks this apparent increase in NO availability during cooling, possibly through injury to the endothelial cells, increased production and enhanced constriction to ET-1 could cause vasospasm. Evidence that the endothelium may be injured in RD comes from studies showing that factor VIII and von Willebrand factor antigen levels are raised in Raynaud's patients (5), and higher levels are associated with greater severity of the disease (6). An alternative explanation for our present results is a decrease in vascular smooth-muscle sensitivity or responsiveness to ET-1 at 24°C. This is presently the subject of investigation in our laboratory.

In conclusion, this study suggests that cooling decreases the sensitivity to ET-1 of small arteries from humans. Further experiments are required to examine the mechanism of this effect, including studies comparing responses in vessels obtained from patients with RD with those from healthy subjects.

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Phosphoramidon inhibition of the *in vivo* conversion of big endothelin-1 to endothelin-1 in the human forearm

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1 The vasoconstrictor peptide, endothelin-1 (ET-1) and a biologically inactive C-terminal fragment (CTF) are generated from an intermediate big ET-1 by a putative ET converting enzyme, sensitive to phosphoramidon. We have developed a procedure using selective solid-phase extraction and specific radioimmunoassays to measure the levels of immunoreactive (IR) big ET-1 and the products of conversion (ET-1 and CTF) in human plasma. These techniques have been used to determine the levels of the three peptides in venous plasma following local infusions of ET-1 and big ET-1, both alone and together with phosphoramidon.

2 Infusion of ET-1 into the brachial artery (5 pmol min^{-1}) significantly increased ($P<0.05$) IR ET levels from a basal level of 2.3 pM to 55.2 pM in plasma from the infused arm after 60 min of infusion. This corresponded with a marked decrease in forearm blood flow from a basal level of 2.6 ml dl^{-1} min^{-1} to 1.7 ml dl^{-1} min^{-1}. The levels of IR big ET-1 and CTF were unchanged. Co-infusion of phosphoramidon (30 nmol min^{-1}) with ET-1 had no significant effect on the plasma IR levels of ET, big ET-1, CTF, or blood flow.

3 Big ET-1 (50 pmol min^{-1}) significantly increased ($P<0.05$) venous concentrations of all three IR peptides after 60 min compared to basal (ET: from 2.2 to 7.7 pM, big ET-1: from 0 to 386.0 pM, CTF: from 0.2 to 37.0 pM). Forearm blood flow decreased significantly ($P<0.05$) from a basal level of 3.0 ml dl^{-1} min^{-1} to 1.6 ml dl^{-1} min^{-1}.

4 When phosphoramidon was co-infused with big ET-1, both the rise in IR ET and associated vasoconstriction were abolished. However, IR CTF was still detected, suggesting that either some conversion by phosphoramidon-insensitive enzyme(s) was occurring, and/or that CTF was being protected from further degradation by phosphoramidon.

5 These data show that in the human forearm the activity of a phosphoramidon-sensitive ET converting enzyme is at least in part responsible for the vasoconstrictor properties of exogenous big ET-1. Furthermore, because measurable levels of newly synthesized ET-1 are likely to be rapidly reduced in the blood/plasma through receptor binding, assay of IR big ET-1 and CTF may be a more sensitive measure of ET-1 generation in disease.

Keywords: Endothelin; big endothelin; endothelin converting enzyme; phosphoramidon; selective solid-phase extraction; radioimmunoassay; human brachial artery; vasoconstriction

Introduction

The vasoconstrictor peptide, endothelin-1 (ET-1, Yanagisawa *et al.*, 1988) is thought to be synthesized from a 212 amino acid precursor preproET-1, initially by removal of the signal sequence to form proET-1. Subsequently dibasic pair proteolysis, carboxypeptidase and possibly furin activity (Laporte *et al.*, 1993) forms the intermediate 38 amino acid peptide, big ET-1. The final stage of synthesis is an unusual cleavage catalysed by a putative ET converting enzyme (ECE) between residues 21 and 22 (tryptophan and valine) to form the biologically active, mature ET-1 and a C-terminal fragment (CTF) of big ET-1 (big $ET\text{-}1_{(22\text{-}38)}$) in an equimolar ratio. In support of this biosynthetic pathway, both mature ET and big ET-1 have been localized to the cytoplasm of endothelial cells from a range of human vascular beds (Howard *et al.*, 1992) and ET-1, big ET-1 and CTF have been identified in the conditioned medium from cultured endothelial cells (Sawamura *et al.*, 1989; Hexum *et al.*, 1990; Ikegawa *et al.*, 1990).

Big ET-1 shows little vasoconstrictor activity compared with ET-1 in human isolated blood vessels (Howard *et al.*, 1992). However, local brachial artery infusion of big ET-1 causes a slow onset, long-lasting vasoconstriction, with a potency about one tenth that of mature ET-1, suggesting an approximately 10% conversion of the luminally presented big ET-1 (Haynes & Webb, 1994). In addition, systemic intravenous infusion of big ET-1 in healthy human volunteers results in significant, long-lasting cardiovascular effects (Ahlborg *et al.*, 1991). Vasoconstrictor responses elicited by big ET-1 in animals and man can be blocked or inhibited by the metalloprotease inhibitor, phosphoramidon (Gardiner *et al.*, 1991; McMahon *et al.*, 1991; Bennett & Gardiner, 1994; Haynes & Webb, 1994). Moreover, in cultures of human endothelial cells, treatment with phosphoramidon simultaneously reduces the secretion of immunoreactive (IR) ET-1, whilst increasing that of the big ET-1, consistent with the inhibition of a phosphoramidon-sensitive ECE (Plumpton *et al.*, 1994). In further support of a phosphoramidon-sensitive enzyme, metalloprotease ECEs have recently been cloned from rat, bovine and human sources (Ikura *et al.*, 1994; Schmidt *et al.*, 1994; Shimada *et al.*, 1994; Xu *et al.*, 1994).

Conversion of big ET-1 to ET-1 appears to be essential to elicit the full haemodynamic effects observed *in vivo* with big ET-1; therefore human ECE may represent a useful therapeutic target in clinical conditions where ET-1 generation is increased. However, because ET-1 binds with high affinity to its receptors, we hypothesized that using circulating ET-1 concentrations as an index of the formation of ET-1 from exogenous big ET-1 may be misleading. A more complete picture would be obtained by measuring the precursor and both products of conversion. Our aim in the present study was to quantify plasma IR ET-1, big ET-1 and CTF following

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brachial artery infusion of ET-1 or big ET-1 in human volunteers and to compare the levels with the corresponding forearm blood flow responses. Secondly, we tested the effects of coinfusion of phosphoramidon with ET-1 or big ET-1 in the same system. A preliminary account of part of this work has been presented to the British Pharmacological Society (Plumpton *et al.*, 1995).

Methods

Clinical procedures

Six healthy males, aged 27 – 34 years, participated in this study with the approval of the local ethical committee, as previously described (Haynes & Webb, 1994). Briefly, all volunteers abstained from vasoactive drugs for one week, from alcohol for 24 h, and from food, caffeine-containing drinks and tobacco for 3 h before each infusion. Subjects rested recumbent in a quiet room maintained at a constant temperature of 24 – 26°C. A 27G cannula was inserted into the brachial artery of the non-dominant arm, under local anaesthesia, for drug infusion, and into the antecubital vein of both arms for the withdrawal of blood samples. ET-1, big ET-1 and phosphoramidon were dissolved in 0.9% saline and infused locally at a constant flow rate of 1 ml min^{-1}. Blood flow was measured in both forearms by venous occlusion plethysmography before and 60 and 90 min after dosing. Venous blood samples were taken before and 60 and 90 min after dosing where appropriate. Blood was collected into EDTA tubes and separated immediately. The resulting plasma was stored at −70°C until assayed.

On separate occasions, each subject received in random order, ET-1 (5 pmol min^{-1}) given alone for 60 min, ET-1 (5 pmol min^{-1}) given for 90 min with phosphoramidon (30 nmol min^{-1}) being co-infused for the first 60 min, big ET-1 (5, 15, or 50 pmol min^{-1}) given alone for 60 min, and big ET-1 (50 pmol min^{-1}) given for 90 min with phosphoramidon (30 nmol min^{-1}) being co-infused for the first 60 min. Drug doses were designed to have local activity in the infused forearm, but not systemically. The dose of phosphoramidon was chosen to achieve local concentrations in the forearm equivalent to the IC_{50} for ECE as determined by McMahon *et al.* (1991). None of the agents had any effect on systemic haemodynamics (forearm blood flow in the contralateral arm, arterial pressure or heart rate).

Sample preparation

After thawing, 4 ml EDTA-plasma samples were acidified by adding 1 ml hydrochloric acid 2 M, and clarified by centrifuging for 15 min at 2000 *g* at 4°C. The resulting supernatants were applied to activated 500 mg Spe-ed C_{18} (14% carbon coverage) disposable mini-columns using a vacuum manifold (Applied Separations, Laboratory Impex Ltd, Middx.). Unbound materials were washed from the mini-columns with 5 ml 0.1% trifluoroacetic acid and discarded. Immunoreactive CTF was eluted with 5 ml of 50% methanol, 0.1% TFA and immunoreactive (IR) ET and big ET-1 were separately eluted with a subsequent 2 ml of 80% methanol, 0.1% TFA. Eluates were evaporated to dryness in polypropylene tubes using a Savant sample concentrator (Life Sciences International (U.K.) Ltd, Basingstoke, Hants).

Radioimmunoassay

Plasma IR ET, big ET-1 and CTF were determined by radioimmunoassay as previously described (Plumpton *et al.*, 1993) using rabbit antisera raised against the C-termini of ET (ET-$1_{(15-21)}$) and big ET-1 (big ET-$1_{(31-38)}$). Briefly, plasma extracts were reconstituted in assay buffer (50 mM sodium phosphate, 0.25% bovine serum albumin (BSA), 0.01% Tween 20, 0.05% sodium azide, pH 7.4) and incubated in duplicate with diluted antisera overnight at 4°C. Following a further overnight incubation with ~10,000 c.p.m./tube tracer ([^{125}I]-ET-1 or [^{125}I]-big ET-1, Amersham International plc, Amersham, Bucks), bound counts were separated using Amerlex-M reagent (Amersham International plc, Amersham, Bucks) and radioactivity determined in a gamma counter (Canberra Packard, Pangbourne, Berks). Immunoreactivity was calculated by reference to standard curves (0.5 – 1000 fmol/tube) of authentic ET-1 (Peptide Institute, Scientific Marketing Associates, Barnet, Herts) or Novabiochem Ltd, Nottingham), or big ET-1 (Peninsula Laboratories Ltd, St. Helens, Lancs). For both assays, ED_{50} values were 20 – 25 fmol/tube, inter- and intra-assay coefficients of variation were < 13% in the range 6 – 30 fmol/tube and the sensitivities of detection (defined as two s.d. above zero standard) were < 1.25 fmol/tube (equivalent to sample sensitivities of < 1.56 pM under the current extraction procedure). The recoveries of ET-1, big ET-1 and CTF were 57.5%, 39.8% and 76.6%, respectively ($n = 4$). Plasma IR peptide concentrations are shown uncorrected for recovery.

The mature ET RIA cross-reacted 100% with ET-1, ET-2 and ET-3 as expected since the immunogen contained the seven C-terminal residues of ET-1 common to all three mature ET isoforms. Cross-reactivities with ET-$1_{(1-20)}$, big ET-$1_{(22-38)}$, big ET-1, big ET-2 and big ET-3 were < 0.02%. The big ET-1 RIA showed < 0.007% cross-reactivity with the mature ETs, big ET-2 and big ET-3 and cross-reacted 143% with big ET-$1_{(22-38)}$ thus allowing the quantification of CTF following fractionation. Neither of the assays showed any detectable cross-reactivity (< 0.000014%) with phosphoramidon. Furthermore phosphoramidon did not interfere with either the ET or big ET-1 assays as indicated by superimposable standard curves at concentrations an order of magnitude higher than that of the infusate. No cross-reactivity was detected (< 0.005%) at the highest concentrations tested with unrelated vasoactive peptides such as angiotensin II, atrial natriuretic factor and α-calcitonin gene-related peptide.

Drugs

The ET-1, big ET-1 and phosphoramidon for infusion were obtained from Clinalfa AG, Laufelfingen, Switzerland.

Statistical analysis

Results are given as the mean of six individuals ± s.e.mean. There was no evidence for non-normality of the data using the Shapiro-Francia test (Shapiro *et al.*, 1968) and so results were analysed by analysis of variance.

Results

Sample extraction

The extraction characteristics for synthetic ET-1, big ET-1 and CTF were determined by applying each peptide to a range of disposable reverse-phase minicolumns with differing packing chemistries and from various manufacturers. Immunoreactive peptides, determined by RIA, were eluted with a stepwise gradient of increasing concentrations of methanol. The C_{18} minicolumns obtained from Applied Separations gave the best resolution of the three peptides and were therefore chosen for further optimisation of conditions. The final extraction procedure described in the methods resulted in 96.0% of IR CTF eluting in the 50% methanol fraction and 99.7% and 96.9% of IR ET-1 and big ET-1, respectively eluting in the 80% methanol fraction ($n = 6$).

ET-1 infusions

Infusion of ET-1 at 5 pmol min^{-1} resulted in a marked increase in the levels of IR ET above basal in the infused arm (Figure 1b) and was accompanied by a significant decrease in forearm blood flow again only in the infused

arm (Figure 1a). Coinfusion of phosphoramidon had no effect on the blood flow or IR ET levels both after 60 min coinfusion and 30 min after withdrawal of phosphoramidon (Figure 1e and f). The endogenous levels of IR big ET-1 and CTF were below the detection limits of the RIA in almost all cases.

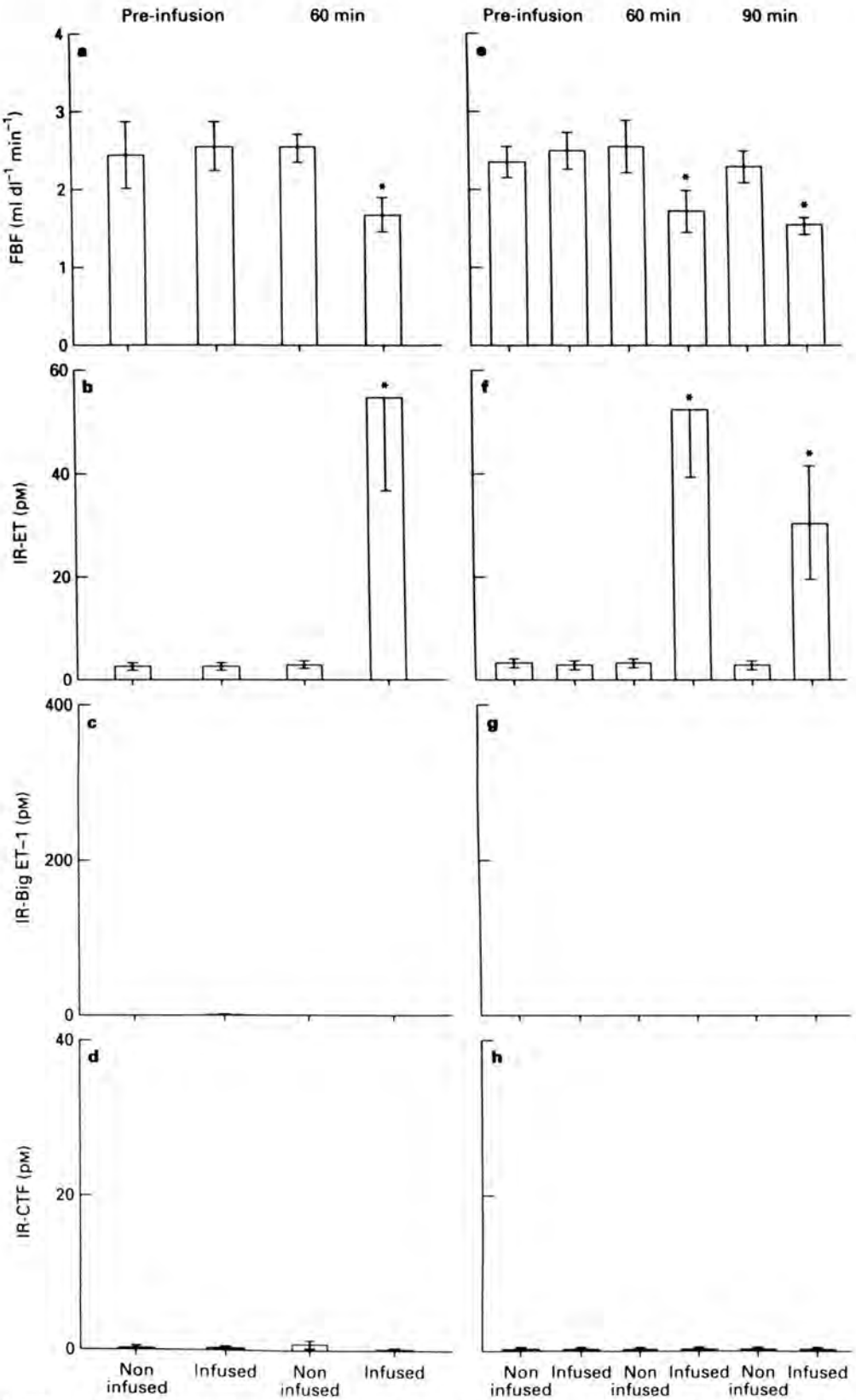

Figure 1 Effect of brachial artery infusion of ET-1 (5 pmol min^{-1}) for 60 min (a,b,c,d) and ET-1 (5 pmol min^{-1}) for 90 min with co-infusion of phosphoramidon (30 nmol min^{-1}) for the first 60 min (e,f,g,h). Forearm blood flow, plasma immunoreactive ET, big ET-1 and CTF are shown in (a,e), (b,f), (c,g) and (d,h), respectively. Mean values ($n = 6$) are shown ± s.e.mean. *$P < 0.05$ compared to basal.

Big ET-1 infusions

When big ET-1 was infused at 5 or 15 pmol min^{-1}, only basal levels of ET were detected and there was no detectable change in forearm blood flow (Figure 2a,b,e and f). At 50 pmol min^{-1} significantly increased levels of ET were detected from the infused arm (Figure 3b) concomitant with a decrease in forearm blood flow comparable with that elicited by the 5 pmol min^{-1}

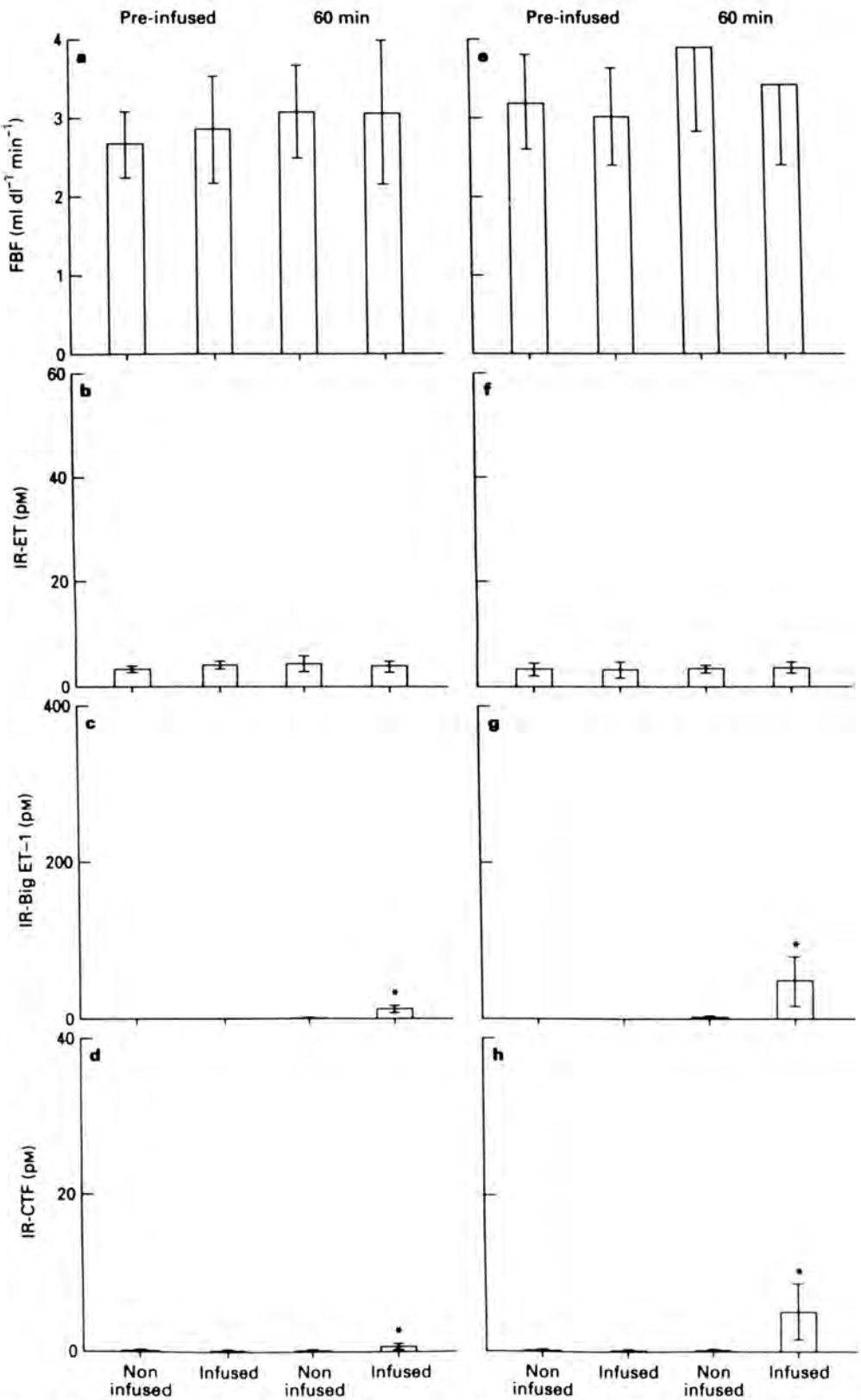

Figure 2 As Figure 1, except infusates were big ET-1 (5 pmol min^{-1}) for 60 min (a,b,c,d) and big ET-1 (15 pmol min^{-1}) for 60 min (e,f,g,h)

infusion of ET-1 (Figure 3a). The levels of IR big ET-1 and CTF increased in a dose-dependent fashion from both arms (Figures 2c,d,g,h and 3c and d), although the concentrations in the non-infused arm were negligible and accordingly were not associated with any detectable vasoconstriction (Figure 3a).

In the presence of phosphoramidon, there was no significant increase in IR ET from the infused arm after 60 min (Figure 3f) and the associated vasoconstriction observed with big ET-1

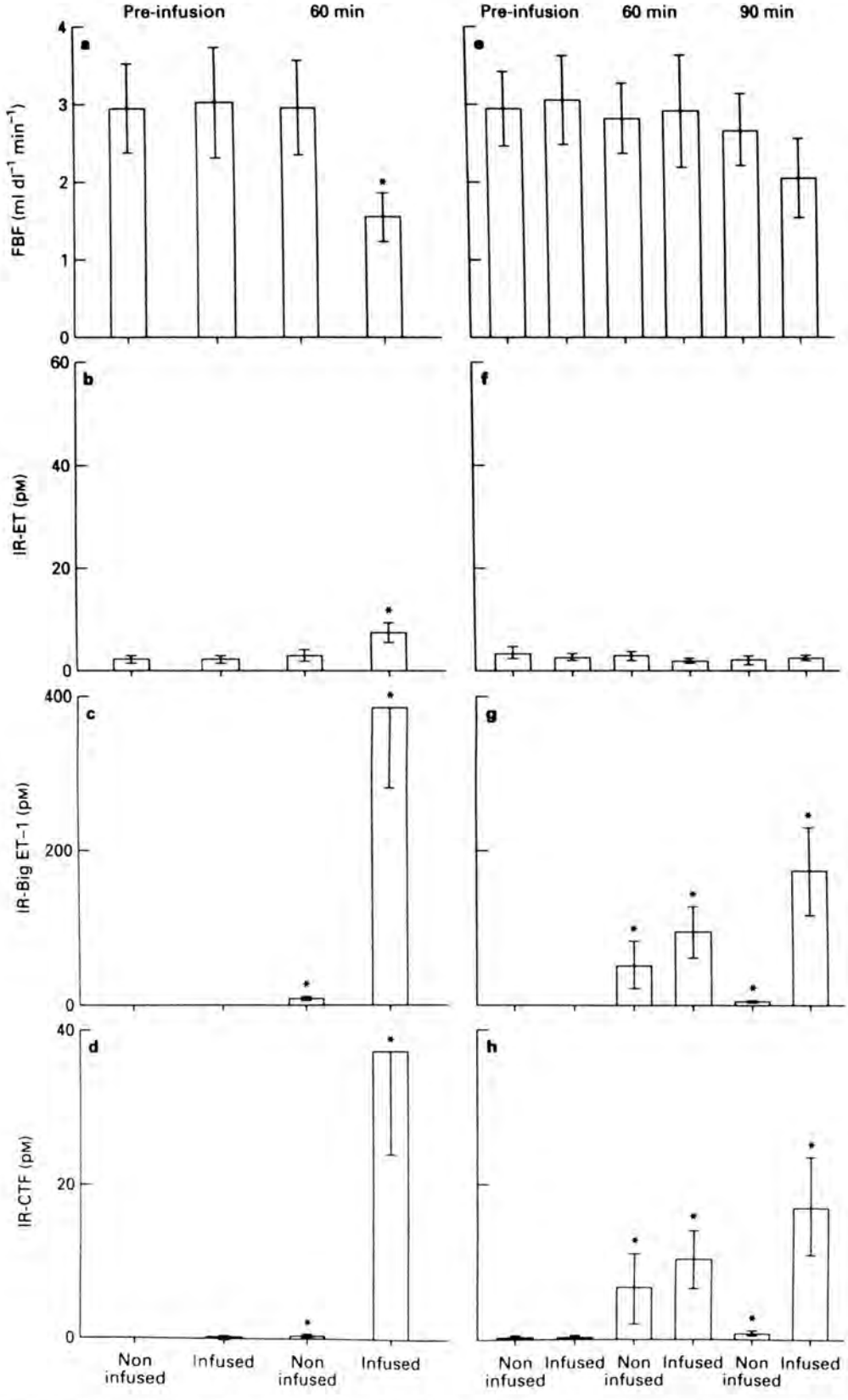

Figure 3 As Figure 1, except infusates were big ET-1 (50 pmol min $^{-1}$) for 60 min (a,b,c,d) and big ET-1 (50 pmol min $^{-1}$) for 90 min with co-infusion of phosphoramidon (30 nmol min $^{-1}$) for the first 60 min (e,f,g,h).

alone was also abolished (Figure 3e). The levels of IR big ET-1 from the infused arm were lower and the proportion detected from the non-infused arm higher than those in the absence of phosphoramidon (Figure 3g). However, 30 min after the withdrawal of phosphoramidon, the forearm blood flow and levels of IR big ET-1 show a return to the levels detected after 60 min without phosphoramidon (Figure 3e and g). The levels of IR CTF essentially mirrored those of IR big ET-1 despite the lack of vasoconstriction after 60 min (Figure 3h).

Discussion

This study has shown that in healthy human volunteers, brachial artery infusion of ET-1 resulted in a marked increase in local venous plasma IR ET. A significant increase in IR ET was also detected when the precursor big ET-1 was infused. Local brachial artery infusions of ET-1 have been shown to cause significant vasoconstriction (Clarke *et al.*, 1989; Pernow *et al.*, 1991; Haynes & Webb, 1994) and systemic intravenous infusion of ET-1 elicits a sustained pressor response (Weitzberg *et al.*, 1991). In addition, ET-1 is a potent constrictor of human isolated blood vessels *in vitro*, with an EC_{50} of 3–18 nM in a range of human isolated blood vessels (Davenport *et al.*, 1989; Maguire & Davenport, 1995). Infusion of big ET-1 also results in vasoconstriction, presumably by conversion to biologically active ET-1 (Ahlborg *et al.*, 1994; Haynes & Webb, 1994).

High-performance liquid chromatography coupled with immunoassays have identified ET-1, big ET-1 and CTF in extracts of cultured animal endothelial cells (Sawamura *et al.*, 1989; Hexum *et al.*, 1990; Ikegawa *et al.*, 1990) and demonstrated that CTF is the major form of IR big ET-1 in porcine lung (Kitamura *et al.*, 1990). In addition, in human subjects, we have detected significant IR CTF in conditioned medium from cultured endocardial endothelial cells and umbilical vein endothelial and smooth muscle cells (unpublished observations). Therefore in the present study we measured the plasma IR levels of all three peptides by RIAs. Measurements were made following local infusions of either ET-1 or big ET-1 both with and without phosphoramidon which blocks the functional responses to big ET-1 (Haynes & Webb, 1994). Two-site enzyme-linked immunosorbent assays previously used for quantifying ET-1 and big ET-1 could not be used to measure CTF because this peptide contains only one of the two epitopes necessary to generate a signal in these assay systems (Plumpton *et al.*, 1994). However, the big ET-1 RIA used in the present study showed a marked cross-reactivity with CTF and could be used to determine plasma IR CTF following a fractionation step. This is the first report of CTF measurement with this technique, which may be useful for assessing the effects of orally active ET antagonists on the levels of ET synthesis. The levels of IR ET detected in the basal samples were in good agreement with previously reported normal human plasma levels using a similar RIA technique (Davenport *et al.*, 1990a). Previously reported basal levels for plasma IR big ET-1 (Suzuki *et al.*, 1990) are below the detection limit of the RIA under these conditions, possibly explaining why endogenous IR big ET-1 was not detected.

Infusion of ET-1 into the brachial artery of the human forearm resulted in the expected significant rise in the levels of IR ET from the infused arm, with concomitant vasoconstriction. Since ET-1 has been shown to be a substrate for neutral endopeptidase -24.11 (EC. 3.4.24.11, NEP) (Sokolovsky *et al.*, 1990; Turner, 1993) causing rapid inactivation, co-infusion of phosphoramidon might have been expected to increase the levels of IR ET. However, no significant differences were observed indicating that other mechanisms controlling the levels of ET may be involved.

Big ET-1 infusion caused a dose-dependent increase in the levels of IR big ET-1, and decrease in blood flow in the infused arm. Furthermore there was a dose-dependent increase in the levels of IR CTF from the infused arm and at the highest dose, a small but significant increase in the IR ET. These data are consistent with local conversion of exogenous big ET-1 in the infused arm, in accord with animal studies (Gardiner *et al.*, 1991; Bennett & Gardiner, 1994; Corder & Vane, 1994). Although the vasoconstrictor effects were confined to the infused arm, there were detectable increases of IR big ET-1 and CTF from the contralateral arm indicating systemic increases in the levels of these peptides. Since the vasoconstrictor responses to both ET-1 and big ET-1 reached significance by 5 min (unpublished observations), the effects are unlikely to be due to *de novo* synthesis, although the processing of stored precursors cannot be excluded. We have been unable to detect binding sites for [^{125}I]-big ET-1 in human cardiovascular tissues at concentrations up to 1 nM (Davenport *et al.*, 1990b) suggesting that there are no specific high affinity receptors for big ET 1. In addition, 1 μM big ET-1 does not compete for the binding of [^{125}I]-ET-1 binding sites. In human isolated saphenous vein, CTF, at concentrations up to 1 μM, has no detectable vasoconstrictor action nor does it antagonize the actions of ET 1 (unpublished observations). Taken together, these results strongly suggest that the vasoconstrictor effects are due to the formation of biologically active ET-1 from exogenous big ET-1 rather than a direct effect of CTF or big ET-1 itself.

The small rise in IR ET is in good agreement with animal studies where the levels of ET-1 have been measured (Hemsen *et al.*, 1991; Corder & Vane, 1994). In particular the apparent discrepancy between the levels of IR ET following local infusion of ET-1 and big ET-1 which resulted in similar fuctional responses are concordant with the results of systemic infusion of these peptides in human subjects (Weitzberg *et al.*, 1991; Ahlborg *et al.*, 1994). We propose that the detected increases in plasma IR ET were modest following infusion of big ET-1 owing to ET binding to its receptors with a high affinity immediately following synthesis (Davenport *et al.*, 1995) in support of a 'stoichiometric' model for ET-1 binding and action (Frelin & Guedin, 1994). Furthermore, once bound, the peptide dissociates slowly from its receptors in the vasculature (Molenaar *et al.*, 1993). These data question the ability of plasma IR ET estimations to provide relevant information. It is likely that measurement of big ET-1 and CTF in addition to ET will be of greater value in monitoring the generation of ET.

Co-infusion of phosphoramidon with big ET-1 results in the abolition of vasoconstriction and increase in IR ET in the infused arm. This is consistent with the inhibition of a phosphoramidon sensitive ECE and in agreement with *in vitro* data from cultured animal (Ohnaka *et al.*, 1991; Sawamura *et al.*, 1991) and human endothelial cells (Plumpton *et al.*, 1994), and porcine (Fukuroda *et al.*, 1990) and human isolated blood vessels *in vitro* (Mombouli *et al.*, 1993). The presence of CTF following coinfusion of phosphoramidon suggests that other ECEs may still be active, for example an aspartyl protease that has been identified in human endothelial cell cultures (Plumpton *et al.*, 1994). In addition, phosphoramidon may protect CTF, generated from endogenous and exogenous big ET-1, from proteolysis by NEP as CTF has been shown also to be a substrate for this enzyme (Murphy *et al.*, 1994). Phosphoramidon caused an increase in the levels of big ET-1 from the non-infused arm suggesting a systemic protection against degradation and/or conversion. In contrast, the levels in the infused arm decreased when compared to the infusion of big ET-1 alone. We propose that this effect was due to the increased dilution effect of the relatively higher blood flow. This is supported by the return of blood flow and IR big ET-1 levels towards those found after big ET-1 infusion following the withdrawal of phosphoramidon.

The precise location of big ET-1 conversion in the present study is not clear. Watanabe *et al.* (1991) have demonstrated that whole human blood is not a major site of conversion. In addition, Fukuroda *et al.* (1990) and Mombouli *et al.* (1993) have shown that in porcine and human isolated blood vessels, phosphoramidon-sensitive conversion of big ET-1 is independent of the endothelium. The concentrations of phosphoramidon used in the present study were low compared with

reported IC_{50} values for ECE inhibition (Opgenorth *et al.*, 1992) and the inhibitor does not enter cells efficiently (Turner, 1993). It is therefore most likely that the ECE is located on the extracellular surface of the vascular smooth muscle cells of the resistance vessels of the forearm, proximal to target vasoconstrictor ET_A receptors (Davenport & Maguire, 1994; Davenport *et al.*, 1995; Maguire & Davenport, 1995). Specific antisera or other selective probes for ECE will undoubtedly aid the location of the ECE involved. We predict that this ECE is physiologically relevant as the local infusion of phosphoramidon alone resulted in vasodilatation presumably due to inhibition of endogenous ET synthesis (Haynes & Webb, 1994). However, it is possible that other ECEs contribute to endogenous ET synthesis.

In conclusion, we have shown that big ET-1-induced forearm vasoconstriction is associated with a significant increase in IR ET and CTF after 60 min. The increases in IR ET and vasoconstriction are blocked by coinfusion of phosphoramidon, suggesting that a phosphoramidon-sensitive ECE is at least in part responsible for the vasoconstrictor properties of big ET-1. This enzyme is, therefore, a valid target for therapeutic agents in human subjects. The development of more potent, specific and orally active ECE inhibitors may lead to specific vasodilator drugs use which may be of clinical benefit perhaps in addition to specific antagonists at ET receptors. Such drugs may have potential in lowering the levels of circulating ET-1 in cardiovascular diseases where the levels of ET-1 are raised. Measurable levels of newly synthesized ET-1 are likely to be reduced through rapid receptor binding, therefore assay of IR big ET 1 and CTF may be a more sensitive measure of the overexpression of ET-1 in disease.

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An investigation into the direct and indirect venoconstrictor effects of endothelin-1 and big endothelin-1 in man

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1 Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide that is generated through cleavage of its precursor big endothelin-1 by 'endothelin converting enzyme' (ECE) in resistance vessels, including those of the forearm vascular bed. In some animal tissues, but not in resistance vessels of healthy human subjects, endothelin-1 appears to potentiate the actions of the sympathetic nervous system. We examined whether ECE activity is present in human hand veins and whether endothelin-1 or big endothelin-1 potentiate sympathetically mediated venoconstriction.

2 Six healthy subjects received dorsal hand vein infusion of local, non-systemic doses of endothelin-1 (5 pmol min^{-1}), big endothelin-1 (50 pmol min^{-1}) and, as a control, sodium chloride (0.9%; w/v) for 90 min. Vein diameter was measured using the Aellig displacement technique. Sympathetically mediated venoconstriction was elicited using the single deep breath reflex.

3 Endothelin-1 caused a progressive decrease in hand vein diameter, by 49% at 90 min (95% confidence intervals [CI]: −68 to −30%; $P = 0.0001$). Vein diameter did not change significantly after 90 min infusion of big endothelin-1 (+3%; CI: −11 to +17%; $P = 0.0007$ *vs* endothelin-1; $P = 0.40$ *vs* baseline) or sodium chloride (+2%; CI: −12 to +16%; $P = 0.0002$ *vs* endothelin-1; $P = 0.60$ *vs* baseline). Venoconstriction to deep breath was not potentiated by endothelin-1.

4 These results suggest that, in contrast to the situation in forearm resistance vessels, there is little or no local ECE activity in human hand veins and that endothelin does not potentiate sympathetic responses in these cutaneous capacitance vessels.

Keywords vasoconstrictor peptide blood pressure endothelium veins sympathetic nervous system

Introduction

The endothelins are a family of peptides with extremely potent and characteristically sustained vasoconstrictor and vasopressor actions [1]. Endothelin-1 is the predominant isoform in the vascular endothelium [2], where it is generated from its precursor, big endothelin-1 by the action of 'endothelin converting enzyme' (ECE) [3]. In humans, brachial artery infusion of big endothelin-1 causes a dose-dependent forearm vasoconstriction that can be blocked by the ECE inhibitor phosphoramidon [4]. Because circulating blood contains no ECE activity [5], these findings suggest that the forearm resistance vessels contain an ECE able to cleave luminally presented big endothelin-1 to the mature peptide. It is not known whether human capacitance vessels also exhibit ECE activity.

In addition to its direct vascular effects [1], endothelin-1 may mediate vasoconstriction indirectly by potentiating the activity of the sympathetic nervous system, either centrally [6] or peripherally [7, 8]. However, in man, brachial artery administration of endothelin-1 does not potentiate forearm vasoconstriction to application of lower body negative pressure [9], a model which has been used to show that angiotensin II facilitates sympathetically mediated vasoconstriction [10].

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Therefore, the aims of this study were to assess whether ECE activity is present in human hand veins and whether endothelin-1 potentiates local activity of the sympathetic nervous system in these vessels. In order to investigate whether ECE was present, we examined the effect of local intravenous infusion of big endothelin-1, endothelin-1 and, as a control, sodium chloride on dorsal hand vein diameter. The interaction between endothelin-1 and the sympathetic nervous system in veins was assessed using the single deep breath manoeuvre [11], a reflex that causes brief sympathetically mediated venoconstriction. This reflex has been used to demonstrate that sub-constrictor doses of angiotensin II facilitate sympathetically mediated venoconstriction in humans [12]. We examined responses in veins because the venous system is an important influence on cardiac output. In addition, abnormalities of venous tone occur in chronic heart failure, essential hypertension and chronic renal failure, and may contribute to the pathophysiology of these conditions. We chose the dorsal hand vein because responses to vasoactive drugs in these veins are similar to those predicted from the pharmacological profile of action following systemic dosing *in vivo* [13]. In addition, there is clear evidence that cutaneous limb veins are under sympathetic venomotor control, whereas skeletal muscle veins do not participate in these reflexes [14, 15]. Thus, cutaneous capacitance vessels would appear to play an important role in the physiological regulation of venous capacitance and cardiac preload. Finally, these studies do not require systemically active doses of drugs that may obscure any direct vascular action by direct effects on other organs, such as the heart and kidney, or activate reflex mechanisms secondary to changes in blood pressure.

Methods

Subjects

Six healthy male subjects between 19 and 22 years of age participated in these studies, which were conducted with the approval of the Lothian Medicine and Clinical Oncology Medical Research Ethics Sub-Committee and with the written informed consent of each subject. No subject received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from food for 4 h, and from alcohol, caffeine containing drinks and cigarettes for at least 12 h before any measurements were made. All studies were performed in a quiet room maintained at a constant temperature of between 24 and 26° C.

Drugs

A 23 SWG cannula (Abbott, Sligo, Republic of Ireland) was sited in a selected dorsal hand vein, without use of local anaesthesia, in the direction of flow. Patency was maintained by infusion of sodium chloride (0.9% w/v; Baxter Healthcare Ltd, Thetford, UK). The total rate of infusion was maintained constant throughout all studies at 0.25 ml min^{-1}. Endothelin-1 (Clinalfa AG, Läufelfingen, Switzerland) was dissolved in sodium chloride (0.9% w/v) to a final strength of 20 pmol ml^{-1}. Big endothelin-1 was dissolved in sodium chloride (0.9% w/v) to a final strength of 200 pmol ml^{-1}. The dose of endothelin-1 (5 pmol min^{-1}) was based on that shown in previous hand vein studies to cause an ~50% venoconstriction [16]. The dose of big endothelin-1 (50 pmol min^{-1}) was based on the apparent ten-fold lower potency of big endothelin-1 than endothelin-1 in the forearm resistance bed [4].

Measurements

Dorsal hand vein size The left hand was supported above the level of the heart by means of an arm rest. Internal diameter of the cannulated dorsal hand vein, distended by inflation of an upper arm cuff to 30 mmHg, was measured by a displacement technique using a linear variable differential transformer (LVDT; Model 025 MHR, Lucas Schaevitz Inc, Pennsauken, NJ, USA), as previously described [16,17]. Standard displacements were used to calibrate the LVDT in order to determine the internal diameter of the vein. The background 'noise' of the signal from the LVDT whilst recording hand vein size, expressed as the standard deviation of the baseline signal, is ± 5 μm. The sensitivity of this method, defined as a signal to noise ratio of more than 3, is 15 μm.

Single deep breath venoconstrictor stimulus The deep breath stimulus was performed as previously described [16]. When vein size was stable, subjects were asked to breath out fully before breathing in as deeply as possible. They were asked to hold this inspiration for 10 s and avoid any tendency to breath out. The technique was practised before the study to ensure that subjects did not perform a Valsalva manoeuvre. This manoeuvre usually causes a 5–20% venoconstriction within ~30 s [12, 16].

Blood pressure A well-validated semi-automated technique (Takeda UA 751 sphygmomanometer, Takeda Medical Inc, Tokyo, Japan) was used to measure blood pressure in the non-infused arm [18].

Study design

This was a three phase, single-blind, randomised crossover study. Subjects rested recumbent during each phase. The dorsal hand vein cannula and LVDT apparatus were sited. Sodium chloride (0.9% w/v) was infused for 30 min during which vein size was measured every 2 min. Blood pressure was measured 20, 15 and 5 min before dosing. Subjects were asked to take single deep breaths, to elicit sympathetically mediated venoconstriction, 25 and 10 min before dosing. On three separate occasions, in random order, subjects received intravenous infusion of sodium

chloride (0.9% w/v; control), endothelin-1 (5 pmol min^{-1}) or big endothelin-1 (50 pmol min^{-1}), each for 90 min. Vein size was measured every 5 min and blood pressure every 30 min. Single deep breaths were taken 28, 58 and 88 min after starting the study drug infusion.

Data presentation and statistics

Basal vein size was calculated by taking the mean of the last five measurements before the start of the study infusion, and is expressed in millimeters. Because basal vein size varies between subjects, responses to big endothelin-1 and endothelin-1 are expressed as percentage change in vein size from basal in order to reduce the inter subject variability. The deep breath response was taken as the maximum decrease in vein size in the 30 s after the manoeuvre and expressed as a percentage change from the last measurement of vein size. Because endothelin-1 caused venoconstriction, deep breath responses are also analysed in terms of the absolute change in vein size. In order to obtain a summary measure of the change in deep breath responses from baseline on each day, we calculated the mean post-treatment deep breath response in each individual study session by averaging the 28, 58 and 88 min responses. The effect of the treatment is expressed as a ratio (mean post treatment response/baseline response). These ratios had a skewed distribution and were therefore log transformed for analysis. The mean of the last two blood pressure measurements during baseline sodium chloride infusion was used as baseline. Data are shown as mean values, with 95% confidence intervals (CI) shown in the text and s.e. mean in Figure 1 and Table 1. All data were examined by a repeated measures analysis of variance (ANOVA), with significance testing by Scheffe's *F*-test, using StatView 512+ software (Brainpower Inc, Calabasas, CA, USA) for the Apple Macintosh personal computer.

Results

Baseline vein sizes were similar on the three study days. No study agent significantly altered blood pressure or heart rate, confirming the non systemic nature of the doses used (Table 1).

There was no significant change in vein size in the control session, with a change from basal in vein diameter, after infusion of sodium chloride for 90 min, of only +2% (CI: −12 to +16%; $P = 0.60$ *vs* basal) (Figure 1). Intravenous infusion of big endothelin-1 did not cause venoconstriction, with a change in vein size at 90 min of +3% (CI: −11 to +17%; $P = 0.40$ *vs* basal; $P = 0.65$ *vs* control) (Figure 1). In contrast, a 10-fold lower dose of endothelin-1 caused progressive venoconstriction, with a maximal reduction at 90 min of 49% (CI: −68 to −30%;

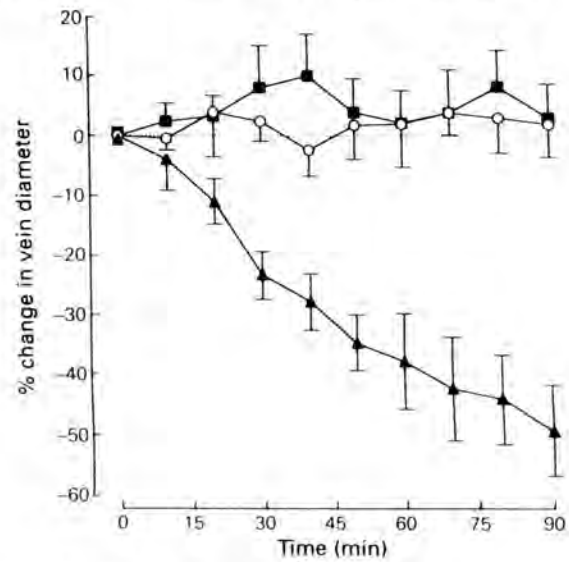

Figure 1 Changes in hand vein diameter following infusion of sodium chloride (0.9% w/v; ○), endothelin-1 (5 pmol min^{-1}; ▲) and big endothelin-1 (50 pmol min^{-1}; ■). Significant venoconstriction occurs only during infusion of endothelin-1 ($P = 0.0001$).

Table 1 Mean arterial pressure (MAP), heart rate (HR), hand vein diameter, and venoconstriction (% and mm) to deep breath before and after brachial artery administration of sodium chloride (SAL), big endothelin-1 (big ET-1) and endothelin-1 (ET-1).

Parameter	*Time*	*SAL*	*Big ET-1*	*ET-1*
MAP	Basal	85 ± 2	84 ± 2	84 ± 3
(mm Hg)	90 min	87 ± 4	87 ± 4	89 ± 2
HR	Basal	57 ± 3	62 ± 3	63 ± 4
(beats min^{-1})	90 min	58 ± 3	64 ± 5	62 ± 4
Hand vein size (mm)	Basal	1.5 ± 0.2	1.3 ± 0.1	1.6 ± 0.1
Venoconstriction	Basal	16 ± 6	11 ± 4	20 ± 9
to deep breath (%)	28 min	13 ± 5	11 ± 4	9 ± 3
	58 min	12 ± 3	11 ± 4	12 ± 4
	88 min	10 ± 4	9 ± 4	19 ± 6
Venoconstriction	Basal	0.17 ± 0.07	0.14 ± 0.05	0.25 ± 0.12
to deep breath	28 min	0.13 ± 0.04	0.14 ± 0.05	0.11 ± 0.03
(mm)	58 min	0.15 ± 0.04	0.13 ± 0.05	0.11 ± 0.03
	88 min	0.10 ± 0.03	0.11 ± 0.05	0.14 ± 0.04

P = 0.0001 *vs* basal; P = 0.0002 *vs* control; P = 0.0007 *vs* big endothelin-1) (Figure 1).

The single deep breath manoeuvre caused significant venoconstriction from baseline before and during infusion of sodium chloride (P = 0.01), big endothelin-1 (P = 0.002) and endothelin-1 (P = 0.01; Table 1). The ratio of post-treatment to baseline responses was 0.98 (95% CI: 0.69 to 1.36) following sodium chloride and was not significantly different from control following infusion of endothelin-1 (ratio = 0.97; 95% CI: 0.80 to 1.17; P = 0.72 *vs* control) and big endothelin-1 (ratio = 0.97; 95% CI: 0.71 to 1.32; P = 0.79 *vs* basal). When venoconstriction to deep breath is expressed in absolute terms, absolute responses decreased following endothelin-1 (ratio = 0.69; 95% CI: 0.54 to 0.89), but this was not significantly different from control (ratio = 1.02; 95% CI: 0.73 to 1.40; P = 0.25 *vs* endothelin 1) (Table 1).

Discussion

In these studies, we have shown that big endothelin-1, the precursor to endothelin-1, does not constrict human hand veins. It is unlikely that any significant ECE activity is present in these veins because we found no venoconstriction despite using a dose of big endothelin-1 that was ten-fold higher than a dose of endothelin-1 sufficient to cause 50% venoconstriction. In addition, we have demonstrated that big endothelin-1 and endothelin-1 are unlikely to substantially potentiate sympathetically mediated venoconstriction in healthy subjects. We were only able to test responses to a single dose of endothelin 1 because the slow onset and sustained actions of endothelin-1 preclude the use of repeated doses in a single study to examine conventional dose-response relationships [16]. Hand vein size did not alter during sodium chloride infusion, confirming that these veins are fully relaxed under our experimental conditions.

The lack of effect of big endothelin-1 in human hand veins contrasts with the forearm vasoconstrictor actions of the same dose of big endothelin-1 administered via the brachial artery [4]. Big endothelin-1 is ~10-fold less potent as a vasoconstrictor than endothelin-1 in the forearm, implying that ~10% of exogenously administered big endothelin-1 is cleaved by ECE to the mature peptide in resistance vessels. This functional estimate of forearm ECE activity is supported by assay of plasma concentrations of endothelin peptides in venous blood draining the infused arm [19]. It could be argued that this difference between hand veins and forearm resistance vessels is due to the fact that the segment of the hand vein under study is too small (~1 cm), to allow adequate biochemical conversion of big endothelin-1. However, similar studies have shown that angiotensin I constricts human hand veins, this effect being blocked by an angiotensin converting enzyme (ACE) inhibitor, demonstrating the presence of ACE in these vessels [20, 21]. There is only a small difference in constrictor potency between angiotensin I and II in hand veins, similar to the observations with angiotensin peptides in the forearm resistance bed [20]. The latency of response for angiotensin I is only about 30 s longer than that for angiotensin II [20], suggesting that precursors to vasoconstrictor peptides can be converted relatively quickly within small segments of hand veins if the relevant enzyme is expressed. Complementary DNA for one membrane-associated isoform of ECE (ECE-1) has recently been identified and the enzyme characterised [22]. It appears that ECE-1 is expressed predominantly in endothelial cells, both intracellularly and extracellularly, the latter site most likely being responsible for conversion of exogenous big endothelin-1 in sites where it occurs.

There is experimental evidence to suggest that endothelin-1 has central actions in the nervous system to increase sympathetic nerve outflow [6], and thus cause venoconstriction [23]. However, the interactions between endothelin-1 and the peripheral sympathetic nervous system are less clear. *In vitro*, threshold concentrations of endothelin-1, insufficient to exert a direct vasoconstrictor effect, have been shown to markedly potentiate vasoconstriction to sympathetic nerve stimulation in rabbit ear arteries [7]. However, endothelin-1 may have a biphasic effect on peripheral sympathetic function because higher concentrations appear to inhibit noradrenaline release from nerve terminals and thus attenuate sympathetically mediated vasoconstriction *in vitro* in guinea pig femoral artery [24]. In human vessels *in vitro*, endothelin-1 potentiates vasoconstriction to noradrenaline in human isolated mammary arteries [8]. However, forearm resistance vessel constriction to lower body negative pressure and noradrenaline is not potentiated by low doses of endothelin-1 administered via the brachial artery [9]. Given that the Aellig LVDT methodology is sufficiently sensitive to have detected even small changes in venoconstriction to deep breath, the results of the present study suggest that endothelin 1 does not potentiate peripheral sympathetic nervous system activity in human capacitance vessels. Indeed, although percentage venoconstriction to deep breath appeared not to change during endothelin-1 infusion, absolute responses to deep breath tended to be lower after endothelin 1, although they were not significantly different from control. The possibility that peripheral sympathetic responses are inhibited by endothelin-1 in man needs further investigation. These results are in marked contrast to the situation in patients with untreated essential hypertension, in whom venoconstriction to deep breath is potentiated ~5-fold by locally administered endothelin-1 [16].

In conclusion, our results show that human hand veins lack the capacity to convert exogenous big endothelin-1 and suggest that these capacitance vessels exhibit little or no ECE activity. Under physiological circumstances, endothelin-1 does not appear to potentiate the activity of the sympathetic nervous system in these veins.

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Systemic Endothelin Receptor Blockade Decreases Peripheral Vascular Resistance and Blood Pressure in Humans

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Background Although local inhibition of the generation or actions of endothelin-1 has been shown to cause forearm vasodilatation, the systemic effects of endothelin receptor blockade in healthy humans are unknown. We therefore investigated the cardiovascular effects of a potent peptide endothelin $ET_{A/B}$ receptor antagonist, TAK-044, in healthy men.

Methods and Results Two randomized, placebo-controlled, crossover studies were performed. In nine subjects, TAK-044 (10 to 1000 mg IV over a 15-minute period) caused sustained dose-dependent peripheral vasodilatation and hypotension. Four hours after infusion of the highest dose (1000 mg), there were decreases in mean arterial pressure of 18 mm Hg and total peripheral resistance of 665 AU and increases in heart rate of 8 bpm and cardiac index of 0.9 $L \cdot min^{-1} \cdot m^{-2}$ compared with placebo. TAK-044 caused a rapid, dose-dependent increase in plasma immunoreactive endothelin (from 3.3 to 35.7 pg/mL within 30 minutes after 1000 mg). In a second study in eight subjects, intravenous administration of TAK-044 at doses of 30, 250, and 750 mg also caused peripheral vasodilatation, and all three doses abolished local forearm vasoconstriction to brachial artery infusion of endothelin-1. Brachial artery infusion of TAK-044 caused local forearm vasodilatation.

Conclusions The endothelin $ET_{A/B}$ receptor antagonist TAK-044 decreases peripheral vascular resistance and, to a lesser extent, blood pressure; increases circulating endothelin concentrations; and blocks forearm vasoconstriction to exogenous endothelin-1. These results suggest that endogenous generation of endothelin-1 plays a fundamental physiological role in maintenance of peripheral vascular tone and blood pressure. The vasodilator properties of endothelin receptor antagonists may prove valuable therapeutically. (***Circulation.*** **1996;93:1860-1870.)**

Key Words • endothelin • receptors • vasculature • blood pressure • drugs • hemodynamics

The endothelins are a family of three isopeptides with extremely potent and characteristically sustained vasoconstrictor and pressor actions.[1] They also have mitogenic and neuroendocrine properties that act to increase blood pressure.[2] Endothelin-1 is the predominant isopeptide in the vascular endothelium[3] and is therefore likely to be the most physiologically relevant of the three isopeptides in regulation of vascular tone. Endothelin-1 is generated from a precursor, big endothelin-1, through the action of a unique neutral metalloprotease, ECE.[4] Two distinct endothelin receptors have been identified and characterized.[5,6] The ET_A receptor has a high affinity for endothelin-1 and is selectively expressed in vascular smooth muscle cells.[5] The ET_B receptor has equal affinity for all three endothelins. Although vascular expression of the ET_B receptor has been thought to be limited to vascular endothelial cells,[6] recent evidence suggests that ET_B receptors are expressed in vascular smooth muscle cells[7] and are functionally active.[8,9]

The physiological relevance of endogenous generation of endothelin-1 in control of blood pressure has been unclear. If basal generation of endothelin-1 contributes to resistance-vessel tone, then drugs that inhibit the generation or actions of endothelin would be expected to cause vasodilatation and decrease blood pressure and might have potential therapeutic value in diseases associated with sustained peripheral vasoconstriction, such as hypertension and chronic heart failure. However, results of animal studies using ECE inhibitors and endothelin receptor antagonists have been contradictory. Some have shown no apparent effect of antiendothelin therapy on blood pressure in normotensive animals.[10-15] However, most of these studies had not been designed primarily to test this hypothesis, with the result that they may have lacked statistical power to confidently exclude a hypotensive effect. In addition, in some, blood pressure was not measured for sufficient time after dosing to detect the expected slow-onset hypotensive effect of antiendothelin therapy. Other studies have shown that endothelin blockade apparently reduces blood pressure significantly only in hypertensive animals,[16,17] leading to suggestions that endothelin-1 has a pathological rather than a physiological role. However, there were similar percentage decreases in blood pressure in normotensive and hypertensive animals in these and other studies,[16-18] suggesting that endothelin plays a similar role in hypertensive and normotensive animals. The lack of a significant effect of antiendothelin

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Selected Abbreviations and Acronyms

AU = arbitrary units
AUC = area under the concentration-time curve
C_{max} = maximum drug concentration
ECE = endothelin-converting enzyme
ET_A, ET_B = endothelin receptors
HPLC = high-performance liquid chromatography

therapy on blood pressure in normotensive animals may be due to the relative imprecision of measurement of small changes in blood pressure. Other studies have shown that ECE inhibitors and endothelin receptor antagonists do decrease blood pressure in normotensive animals.[18-22] These positive studies have usually examined hemodynamic responses for several hours after drug administration, thereby taking into account the known slow reversal of endothelin-1–induced vasoconstriction by antiendothelin therapy.[23]

In the first such study in humans, we recently demonstrated that brachial artery infusion of the ECE inhibitor phosphoramidon and the ET_A receptor antagonist BQ-123 causes progressive forearm vasodilatation.[24] These effects of phosphoramidon and BQ-123 on forearm blood flow indicate a physiological role for basal generation of endothelin-1 in maintenance of vascular tone. However, homeostatic mechanisms often obscure the blood pressure effects of quite large changes in resistance vessel tone, with the result that changes in blood pressure may be quite small in relation to the effects of a drug on peripheral resistance.[25] Thus, the magnitude of any potential effect of an endothelin antagonist on blood pressure is difficult to predict without systemic administration. As far as we are aware, the effects of systemic endothelin receptor blockade on hemodynamics in healthy human subjects have not previously been reported.

Therefore, we examined the hemodynamic effects of systemic administration of a potent combined ET_A and ET_B receptor peptide antagonist, TAK-044,[26-29] in healthy male subjects. Because animal data show that endothelin receptor antagonists increase circulating endothelin concentrations,[30,31] we also measured plasma immunoreactive endothelin concentrations. In addition, in a second study, we investigated whether systemic pretreatment with TAK-044 blocked forearm vasoconstriction to brachial artery infusion of endothelin-1 and whether local administration of TAK-044 caused direct vasodilatation of forearm resistance vessels.

Methods

Subjects

Eighteen healthy male subjects between 21 and 60 years of age and within 15% of ideal body weight were recruited. Studies were conducted with the approval of the Lothian Healthy Volunteer Research Ethics Subcommittee and the Inveresk Clinical Research Ethics Committee and with the written, informed consent of each subject. No subject received vasoactive or nonsteroidal medication in the week before or during the study. In addition, subjects abstained from alcohol for 48 hours, from caffeine-containing drinks and cigarettes for at least 24 hours, and from food for at least 10 hours before any measurements were made.

Drugs

TAK-044 is a cyclic hexapeptide (cyclo[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)-glycyl-L-leucyl-D-tryptophyl] disodium salt; molecular weight, 972) that potently antagonizes ^{125}I–endothelin-1 binding at both ET_A (IC_{50}=0.08 nmol/L) and ET_B (IC_{50}=120 nmol/L) receptors in vitro.[26] TAK-044 also blocks constriction of isolated coronary vessels to endothelin-1 (ET_A and ET_B receptor agonist) and sarafotoxin S6c (ET_B receptor agonist).[26] Specificity of TAK-044 for endothelin receptors has been shown in vitro in porcine coronary arteries, in which it does not affect vasoconstriction to histamine, serotonin, acetylcholine, U-46619, and potassium and in which it is without direct vasoactive effects even at concentrations of 100 μmol/L.[29] Specificity has also been shown in vivo, where systemic pretreatment of rats with TAK-044 at 10 mg/kg does not alter pressor or depressor responses to phenylephrine, angiotensin II, nitroglycerin, and acetylcholine.[28] In contrast, TAK-044 dose-dependently blocks the pressor response to bolus doses of endothelin-1 and sarafotoxin S6c in rats, with 90% blockade apparent at a dose of 10 mg/kg, the effects of which persist for 3 hours.[28,29] For the clinical studies in humans, we chose to use doses of TAK-044 ranging from 10 to 1000 mg. This dose range was based on the animal evidence of specificity and efficacy at doses from 0.1 to 10 mg/kg and also on the safety profile of TAK-044 in toxicological studies at higher doses (information on file, Takeda Euro R&D Centre GmbH). Pharmaceutical grade TAK-044 for parenteral use was obtained from Takeda Euro R&D Centre GmbH and was dissolved in physiological saline (0.9%; Baxter Healthcare Ltd). The placebo was dextrose (50 mg), also dissolved in physiological saline.

Endothelin-1 was administered intra-arterially at a dose of 5 pmol/min, based on previous work showing that this dose of endothelin-1 causes slow-onset vasoconstriction of human forearm resistance vessels in vivo.[9,24] Pharmaceutical grade endothelin-1 was obtained from Clinalfa AG (NovaBiochem) and dissolved in physiological saline (0.9%; Baxter Healthcare Ltd) to a final concentration of 5 pmol/mL.

Drug Administration

For intravenous administration of TAK-044, an antecubital vein was cannulated at least 1 hour before dosing. TAK-044 or placebo was dissolved in physiological saline and infused at 200 mL/h over a period of 15 minutes (total volume, 50 mL). This cannula was not used for blood sampling. For intra-arterial infusion of endothelin-1 or TAK-044, the left brachial artery was cannulated under local anesthesia (1% lidocaine; Astra Pharmaceuticals Ltd) with a 27-standard wire gauge steel needle attached to a 16-gauge epidural catheter (Portex Ltd). Patency was maintained by infusion of 0.9% physiological saline via a Welmed P1000 syringe pump (Welmed Clinical Care Systems). The total rate of intra-arterial infusion was maintained constant throughout all intra-arterial studies at 1 mL/min.

Measurements

Systemic hemodynamics. Blood pressure and heart rate were measured with semiautomated oscillometric monitors (study 1, Hewlett-Packard M1165A, Hewlett-Packard GmbH; study 2, Takeda UA 751, Takeda Medical Inc). Cardiac function (stroke volume, cardiac output, and heart rate) was measured with a noninvasive bioimpedance methodology (BoMed NCCOM3, BoMed Medical Manufacturer Ltd). Absolute cardiac output measured by bioimpedance has been validated against thermodilution measurements with correlation coefficients ranging from 0.83 to 0.90 and mean differences ranging from 2% to 12%.[32-35] In addition, bioimpedance measures of changes in cardiac output after drug intervention are in close agreement with simultaneous thermodilution measurements.[35] Furthermore, within-subject coefficient of variation is lower with bioimpedance than with thermodilution (4.7% versus 7.8%).[33] The safety and reproducibility of the bioimpedance

technique confers specific advantages in studies of drug action in healthy subjects.[25]

Side-effect assessments. The following assessments were performed to detect potential adverse effects: 12-lead ECGs, visual analogue scale (for sedation), urinalysis, clinical chemistry screen (liver enzymes, electrolytes, creatinine, blood urea, protein), and hematology screen (full blood cell count, white blood cell differential count).

Forearm blood flow. Blood flow was measured simultaneously in both forearms by venous occlusion plethysmography using indium/gallium-in-Silastic strain gauges as previously described,[9,24] except that single-channel Hokanson EC 4 plethysmographs (DE Hokanson Inc) were used. Recordings of forearm blood flow were made repeatedly over 3-minute periods.

Pharmacokinetic and endothelin assays. Fifteen-milliliter venous blood samples were obtained at intervals for assay of serum TAK-044 and plasma immunoreactive endothelin concentrations. TAK-044 was extracted from sodium acetate (pH 5) buffered serum by methanol/acetic acid–conditioned Varian Certify II cartridges and was measured by HPLC. Eluates were evaporated to dryness under vacuum at 40°C, and the dry residues were taken up in 200 μL of 39% acetonitrile. Chromatographic separation was achieved by a column-switching technique using two Alltech C18 HPLC columns with Gilson model 307 HPLC pumps. The first mobile phase comprised 40% acetonitrile and 60% 0.01 mol/L KH_2PO_4/0.005 mol/L tetrabutylammonium bromide, pH 3.8. The second mobile phase comprised 45% acetonitrile/1% acetic acid/54% water. Detection was achieved by fluorimetry (excitation, 286 nm; emission, 348 nm) with Hitachi F-1050 fluorescence detectors. The limit of quantification of the assay, defined as the lowest quantifiable amount of compound at which the loss of precision was <20% and the accuracy was between ±20%, was 5 ng/mL of TAK-044.

Plasma immunoreactive endothelin was measured by radioimmunoassay (ITS Production BV) as previously described.[36] The sensitivity of this assay is 2 pg/mL immunoreactive endothelin. The assay does not cross-react with TAK-044. Cross-reactivity of the assay with endothelin-1, endothelin-2, endothelin-3, and big endothelin-1 is 100%, 52%, 96%, and 7%, respectively.

Study Design

Study 1: Dose-Ranging Hemodynamic Study

Two groups of five subjects were recruited to a double-blind, ascending-dose, crossover study with a randomized placebo phase. Group 1 subjects were studied on five occasions, receiving placebo and 10, 100, 500, and 1000 mg of TAK-044, with 7 days between phases. Group 2 subjects were studied on four occasions, receiving placebo and 30, 250, and 750 mg of TAK-044, with 7 days between phases. The ascending-dose design enabled us to evaluate safety and tolerability of TAK-044 at lower doses before proceeding to higher doses and entailed that the study days for groups 1 and 2 occur on different days of the same week for the first 4 weeks of dosing. For example, in the first week, subjects in group 1 received either placebo or 10 mg on Monday and group 2 subjects received placebo or 30 mg on Wednesday.

In each study phase, subjects were admitted to the research unit the day before dosing and fasted from 11 PM. At least 1 hour before dosing, an antecubital venous cannula was sited in each arm for administration of TAK-044 and blood sampling. Subjects received a 15-minute intravenous infusion of TAK-044 or placebo at ≈9 AM and, apart from voiding, were not permitted to stand until 4 hours after dosing. Hemodynamic measurements were made and blood samples were obtained for TAK-044 and endothelin concentrations before and after dosing (see Figs 1 through 4). Sedation was assessed and 12-lead ECGs were recorded before and after dosing. Blood and urine samples were obtained for safety assessments before and 24 hours after dosing. Subjects were fasted until 4 hours after dosing, when they received a light meal. An evening meal was provided 10 hours after dosing. Subjects were discharged 24 hours after dosing.

Study 2: Effect of TAK-044 on Forearm Vasoconstriction to Endothelin-1

Eight subjects were recruited to a five-phase, double-blind, randomized, placebo-controlled crossover study, with at least 7 days between phases. These studies using forearm plethysmography were performed in a quiet clinical research ward maintained at a constant temperature between 22°C and 25°C. In each phase, subjects were admitted to the research unit at 7 AM, and blood and urine samples were obtained for safety assessments before dosing. At least 1 hour before dosing, an antecubital venous cannula was sited in each arm for administration of TAK-044 and blood sampling. In the first four phases, subjects received, in random order, placebo and 30, 250, and 750 mg of TAK-044 IV over 15 minutes, at ≈9 AM. Brachial artery cannulation was performed after the infusion of TAK-044 or placebo had finished, and intra-arterial infusion of endothelin-1 (5 pmol/min) commenced 60 minutes after the start of TAK-044 dosing and continued for 120 minutes thereafter (ie, until 180 minutes after TAK-044 dosing). Measurements were made of forearm blood flow (see Fig 5) and blood pressure and cardiac output (−25, −15, −5, +15, +30, +45, +60, +90, +120, +150, and +180 minutes). Blood samples were obtained at −15, +15, +60, +120, and +180 minutes for assay of TAK-044 and endothelin concentrations. In the fifth phase, TAK-044 was infused intra-arterially via the brachial artery, with subjects receiving 10 mg over 1 hour followed by 100 mg over 1 hour. Measurements were made of forearm blood flow (see Fig 6), blood pressure, and cardiac function (−10 and +120 minutes), and blood samples were obtained for assay of endothelin (−15, +15, +60, and +120 minutes). In each phase, subjects remained supine until 3 hours after dosing and were fasted until 4 hours after dosing, when they received a light meal. Subjects were discharged 6 hours after dosing.

Data Presentation and Statistical Analysis

Mean arterial pressure was calculated as diastolic blood pressure plus one third pulse pressure. Data for stroke volume and cardiac output were corrected for body surface area, calculated according to a standard nomogram, to provide measures of stroke and cardiac indexes. Total peripheral resistance index was calculated as mean arterial pressure divided by cardiac index and expressed in AU. For the systemic hemodynamic data, the change from the last measurement before dosing was calculated at each time point and corrected for the changes that occurred at the same time point after placebo. Plethysmographic data listings were extracted from computer data files and forearm blood flows (mL · dL forearm tissue^{-1} · min^{-1}) calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 4.0; Microsoft Ltd). The last five flow recordings in each 3-minute measurement period were averaged. To reduce the variability of blood flow data, the ratio of flows in the infused and noninfused arms was calculated for each time point and expressed as a percentage change from the last baseline measurement (+55 minutes for phases A through D; −10 minutes for phase E), in effect using the noninfused arm as a contemporaneous control for the infused arm.[37,38]

Pharmacokinetics of TAK-044 were analyzed by use of SIPHAR software (version 4, SIMED). The following parameters were calculated: AUC, C_{max}, and elimination half-life. Plasma immunoreactive endothelin concentrations were analyzed in a similar manner, with AUC and C_{max} being calculated.

Absolute values are presented as mean±SEM. Placebo-corrected hemodynamic changes from baseline were arithmetically averaged over the relevant measurement period (0 to 24 hours for study 1; 0 to 3 hours for study 2), with uniform weighting given to each time point, and are shown in the tables

TABLE 1. Baseline Hemodynamic Values in Groups 1 and 2 of Study 1

	SBP, mm Hg	DBP, mm Hg	Heart Rate, bpm	Stroke Index, mL/m²	Cardiac Index, L · min⁻¹ · m⁻²	TPRI, AU
Placebo (group 1)	110±4	50±9	56±6	65±6	3.4±0.4	1480±301
Placebo (group 2)	112±7	53±7	57±7	66±3	3.8±0.5	1400±237
10 mg (group 1)	115±6	53±6	59±7	67±12	3.7±1.0	1496±334
30 mg (group 2)	111±4	57±6	59±11	59±3	3.5±0.8	1628±426
100 mg (group 1)	114±7	53±6	60±7	71±10	4.2±1.0	1304±260
250 mg (group 2)	113±4	57±10	56±12	60±4	3.4±1.0	1744±540
500 mg (group 1)	111±5	52±6	59±3	64±8	3.7±0.7	1428±368
750 mg (group 2)	110±6	57±6	60±5	57±6	3.4±0.8	1686±564
1000 mg (group 1)	110±6	54±11	54±5	62±5	3.3±0.4	1605±320

There were no significant differences between baseline values on the different study days for either group 1 or 2. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; and TPRI, total peripheral resistance index.

with 95% CIs. Data were analyzed statistically by repeated-measures ANOVA. Factors included in the ANOVA were subject, dose of TAK-044, time point, and dose–time point interaction. In none of the analyses was there evidence of a statistically significant dose–time point interaction. Therefore, the adjusted dose group means from the ANOVA were compared with the null hypothesis, for all time points combined, by a two-sided *t* test. In addition, dose-response trends were assessed statistically by the technique of linear contrast. Linear contrast analyzes trends between groups of subjects that are categorized quantitatively, using variances derived from an ANOVA. Each linear contrast was calculated as the sum of the mean of each group multiplied by a coefficient that represented that group's dose (adjusted so that the sum of all coefficients equals zero).[39] Statistical testing of a linear contrast involved calculation of its SEM using the pooled estimate of variance from the ANOVA, with a *t* statistic given by the linear contrast divided by this SEM. Simple regression analysis was used to explore whether there was a correlation between plasma endothelin and hemodynamic changes. Statistical analyses were performed by use of the software package SAS (version 6.07, SAS Institute Inc).

Results

General

In study 1, one subject withdrew from group 1 after the second phase for non–study-related reasons and was not replaced; he did not receive placebo and was therefore not included in the analysis. All other subjects completed the study protocols. TAK-044 was well tolerated, with no difference between placebo and TAK-044 phases in the prevalence of minor symptoms. There were no serious adverse events in either study, and no clinically significant abnormalities were detected on safety monitoring (urinalysis, hematology, clinical chemistry, ECG, and sedation scores).

Study 1: Dose-Ranging Hemodynamic Study

Baseline hemodynamic parameters did not differ between study days (Table 1). Compared with placebo, all doses of TAK-044 reduced blood pressure, with the hypotensive effect apparent within 30 minutes, maximal between 1 and 6 hours, and persisting to 24 hours at the higher doses (Figs 1 and 2). For example, after the 30- and 1000-mg doses, mean arterial pressure was reduced at 4 hours by 8 and 18 mm Hg from baselines of 75 and 72 mm Hg, respectively. Diastolic and mean arterial pressures were reduced by all doses; systolic pressure was significantly decreased by all doses except 750 mg.

Heart rate was significantly increased by TAK-044 at all doses except 30 and 100 mg; this increase persisted to ≈8 hours for doses >250 mg (Figs 1 and 2). Most doses of TAK-044 significantly increased stroke and cardiac indexes (Fig 2). Total peripheral resistance index was significantly and substantially reduced at all doses, and this effect was sustained for up to 24 hours (Figs 1 and 2). For example, after the 30- and 1000-mg doses, total peripheral resistance index was reduced at 4 hours by 378 and 665 AU from baselines of 1628 and 1605 AU, respectively. There were significant dose-related trends on linear contrast testing for heart rate, stroke index,

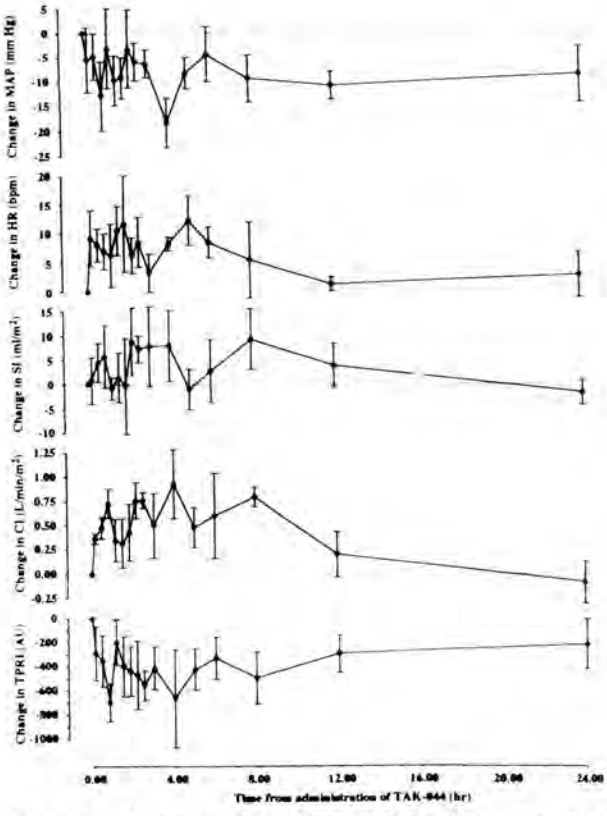

FIG 1. Time course of the effects of the highest dose of TAK-044 (1000 mg) on mean arterial pressure (MAP), heart rate (HR), stroke index (SI), cardiac index (CI), and total peripheral resistance index (TPRI) in group 1 from study 1. TAK-044 significantly decreased MAP ($P<.001$) and TPR ($P<.001$) and increased HR ($P<.001$), SI ($P=.034$), and CI ($P<.001$); these effects were maximal at 4 hours and sustained for at least 12 hours. Data shown represent placebo-corrected changes from predose (change from predose [active] minus mean change from predose [placebo]).

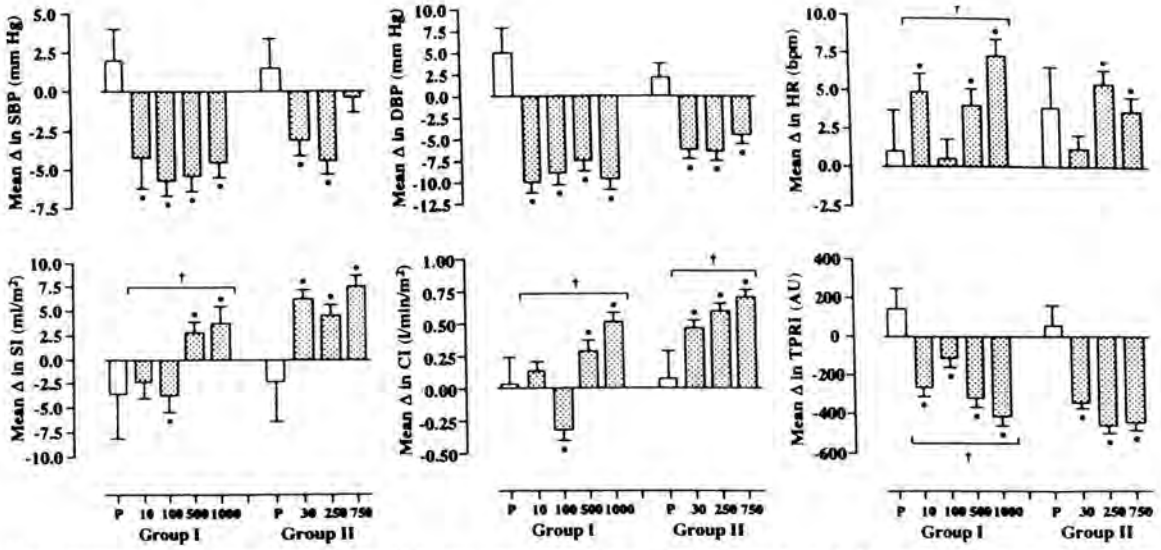

FIG 2. Mean hemodynamic changes (Δ) over 24 hours after dosing with TAK-044 in study 1. For the placebo columns (open), mean change from predose is shown. For the active treatment columns (stippled), placebo-corrected changes from predose are shown (change from predose [active] minus mean change from predose [placebo]). *$P \leq .05$ for comparison with predose; †$P \leq .05$ for linear contrast test of trend with dose. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; other abbreviations as in Fig 1.

cardiac index, and total peripheral resistance, although not for blood pressure (Fig 2).

TAK-044 increased plasma immunoreactive endothelin concentrations in a dose-dependent manner, with significant increases at all doses except for 10 mg (Table 2). For example, after 1000 mg, plasma endothelin concentrations increased from 3.3 to 35.7 pg/mL within 30 minutes. Compared with the sustained hemodynamic effects of TAK-044, increases in plasma endothelin were maximal within 30 minutes and waned rapidly, even at the highest doses (Fig 3). Even so, there was a significant correlation between the increase in plasma endothelin and the change in total peripheral resistance in both group 1 ($r=-.15$; $P=.032$) and group 2 ($r=-.156$; $P=.021$). In group 2 only, plasma endothelin was also correlated with change in systolic ($r=-.189$; $P=.005$) and mean arterial ($r=-.160$; $P=.018$) pressures. TAK-044 plasma concentrations increased dose-dependently (Fig 4); the terminal half-life was short (30 to 60 minutes) and tended to increase with dose (Table 2).

Study 2: Effect of TAK-044 on Forearm Vasoconstriction to Endothelin-1

As in the first study, all intravenous doses of TAK-044 significantly decreased diastolic blood pressure, increased heart rate and cardiac index, and caused peripheral vasodilatation (Table 3), with the effects sustained over the measurement period. There were significant dose-related trends for systolic blood pressure, mean arterial pressure, and total peripheral resistance (Table 3). As in study 1, plasma immunoreactive endothelin concentrations were increased in a dose-dependent manner, with significantly higher C_{max} values after 250 mg (22.9 pg/mL; $P<.0001$) and 750 mg (37.2 pg/mL; $P<.0001$) compared with placebo (7.8 pg/mL). Similarly, there were significant correlations between plasma endothelin concentrations and changes in cardiac index ($r=.226$; $P=.012$), diastolic pressure ($r=-.225$; $P=.011$), mean arterial pressure ($r=-.206$; $P=.021$), and total peripheral resistance ($r=-.249$; $P=.006$).

In the first four phases, forearm blood flow in the infused arm was not significantly different from that in

TABLE 2. Summary Pharmacokinetic Parameters for Plasma Immunoreactive Endothelin Concentrations and TAK-044 Concentrations in Study 1

	Plasma Endothelin		Plasma TAK-044		
	C_{max}, pg/mL	AUC, pg · h/mL	C_{max}, ng/mL	AUC, ng · h/mL	$t_{1/2}$, h
Placebo (group 1)	4.3±0.1	88±7	...	...	
Placebo (group 2)	4.8±0.3	83±3	...	...	...
10 mg (group 1)	7.4±0.8*	90±4	581±183	NA	NA
30 mg (group 2)	9.6±1.0	112±8*	1679±268	557±89	0.51±0.09
100 mg (group 1)	13.3±1.4	128±10*	7912±1810	2279±263	0.72±0.07
250 mg (group 2)	29.3±4.3	136±12†	16 228±2994	5764±1025	0.73±0.09
500 mg (group 1)	33.2±1.7†	160±14†	34 425±2402	11 687±1013	0.69±0.13
750 mg (group 2)	42.8±1.9†	166±11†	66 760±13 658	24 571±4175	1.04±0.07
1000 mg (group 1)	38.4±3.2†	212±13†	123 000±10 916	40 748±1248	1.01±0.11

C_{max} indicates maximal endothelin concentrations after dosing; $t_{1/2}$, terminal half-life of compound; NA, parameters not available because data were insufficient for calculation.

*$P<.05$ vs placebo; †$P<.005$ vs placebo.

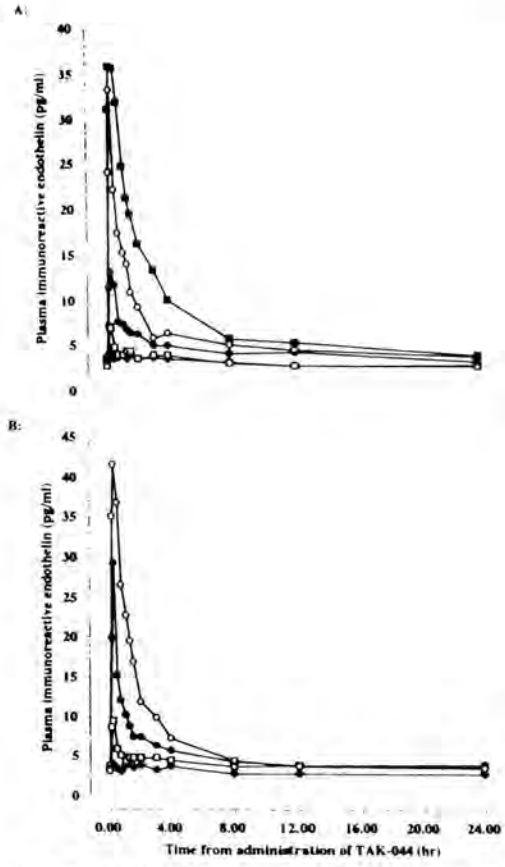

FIG 3. Effect of TAK-044 on plasma immunoreactive endothelin levels in study 1. A, Group 1 results after infusion of placebo (♦) and TAK-044 at 10 mg (□), 100 mg (●), 500 mg (○),and 1000 mg (■). B, Group 2 results after infusion of placebo (♦) and TAK-044 at 30 mg (□), 250 mg (●), and 750 mg (○). TAK-044 dose-dependently increased circulating endothelin concentrations.

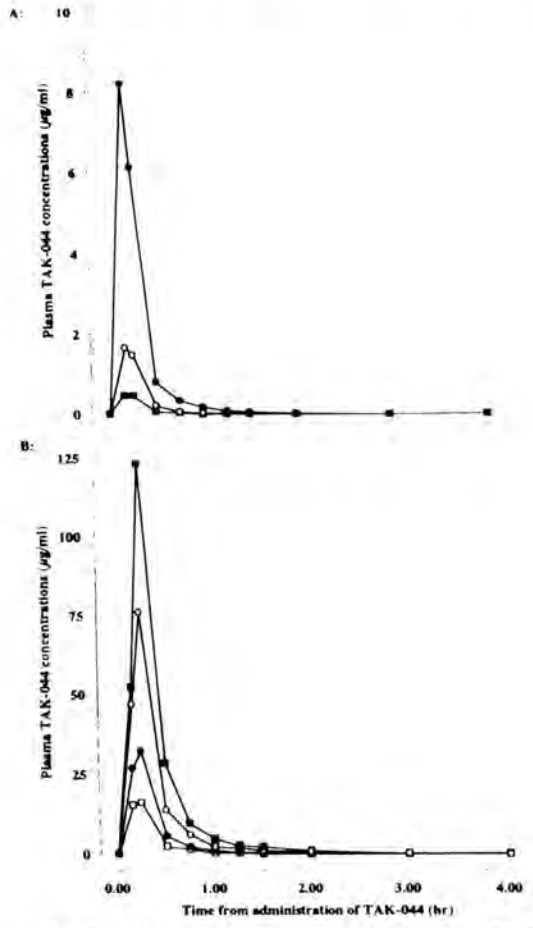

FIG 4. Graphs showing pharmacokinetic profiles of TAK-044 in study 1. A, Results after infusion of TAK-044 at 10 mg (■), 30 mg (○), and 100 mg (●). B, Results after infusion of TAK-044 at 250 mg (□), 500 mg (●), 750 mg (○), and 1000 mg (■). Results are shown here only for the first 4 hours after dosing, although pharmacokinetic calculations were based on all time points up to 24 hours (see Table 2). Plasma levels after 4 hours were <0.025 μg/mL for all doses.

the noninfused arm at baseline on any phase, and baseline blood flows were similar between the different treatment days (Table 4). Blood flow in the noninfused arm did not change significantly after placebo. Brachial artery infusion of endothelin-1 in the placebo phase caused a significant slow-onset local forearm vasoconstriction, reaching ≈30% after 60 minutes (P=.0031 versus basal; Fig 5). Systemic administration of TAK-044 did not significantly change blood flow in the noninfused arm compared with placebo. However, forearm vasoconstriction to endothelin-1 was completely blocked by TAK-044 at doses of 30 mg (P=.34 versus basal; P=.02 versus placebo), 250 mg (P=.60 versus basal; P=.01 versus placebo), and 750 mg (P=.89 versus basal; P=.01 versus placebo), with no significant difference between doses (Fig 5, Table 4).

In the fifth phase, brachial artery infusion of TAK-044 at 10 mg caused significant local vasodilatation, with an increase in the ratio of blood flow between infused and noninfused arms of ≈20% (P=.0062; Fig 6, Table 4). At the higher dose (100 mg/h), blood flow remained elevated in the infused arm but also increased in the noninfused arm, with the result that the percentage increase in the ratio of blood flow between the infused and noninfused arms fell toward baseline (P=.10; Fig 6, Table 4). A possible systemic effect of TAK-044 at the higher dose is supported by the fact that total peripheral resistance decreased by 347±113 AU at 2 hours, compared with an increase of 158±134 AU at 2 hours in the placebo phase. In addition, although circulating endothelin concentrations did not differ from placebo at baseline or 1 hour after the start of intra-arterial dosing, endothelin concentrations at 2 hours (17.3±1.6 pg/mL) were significantly greater than at 2 hours in the placebo phase (4.6±0.2 pg/mL; P=.01).

Discussion

These studies are the first report of the effects of systemic endothelin receptor blockade in healthy humans. We have shown that a 15-minute infusion of the endothelin $ET_{A/B}$ receptor antagonist TAK-044 decreased systolic blood pressure (by ≈4%), diastolic blood pressure (by ≈18%), and total peripheral resistance (by ≈26%) over a 24-hour period. Systemic $ET_{A/B}$ receptor blockade also increased circulating immunoreactive endothelin (by up to 1000%) and blocked peripheral vasoconstriction to exogenous endothelin-1. In addition, local administration of TAK-044 caused forearm vasodilatation. These findings have implications for the physiological role of endothelin-1 generation, the phar-

TABLE 3. Mean Hemodynamic Changes Over 24 Hours After Dosing With TAK-044 in Study 2

	Placebo	TAK-044, mg		
		30	250	750
SBP*, mm Hg				
Baseline value	132±24	128±16	122±15	127±12
Mean Δ over 3 h	−3.0	+3.0	+2.1	−2.5
95% CI	−22.1 to +16.4	+0.7 to +5.3	−0.2 to +4.3	−4.8 to −0.2
P	...	0.010	0.078	0.034
DBP, mm Hg				
Baseline value	75±7	73±6	75±8	73±6
Mean Δ over 3 h	+0.7	−5.8	−6.7	−6.5
95% CI	−5.1 to +6.5	−7.2 to −4.5	−8.0 to −5.3	−7.9 to −5.2
P	...	<0.001	<0.001	<0.001
Heart rate, bpm				
Baseline value	57±7	60±9	61±11	62±11
Mean Δ over 3 h	+2.7	+1.8	+2.0	+1.4
95% CI	−1.6 to +7.0	+0.6 to +3.0	+0.7 to +3.3	+0.2 to +2.6
P	...	0.004	0.003	0.027
Stroke index, mL/m^2				
Baseline value	56±4	51±3	55±3	47±4
Mean Δ over 3 h	−3.1	+4.4	+2.3	+5.2
95% CI	−6.3 to +0.2	+3.3 to +5.5	+1.1 to +3.6	+4.0 to +6.3
P	...	<0.001	<0.001	<0.001
Cardiac index, L·min^{-1}·m^{-2}				
Baseline value	3.2±0.7	3.0±0.4	3.2±0.4	2.8±0.6
Mean Δ over 3 h	−0.09	+0.39	+0.34	+0.43
95% CI	−0.36 to +0.18	+0.33 to +0.45	+0.27 to +0.41	+0.37 to +0.49
P	...	<0.001	<0.001	<0.001
TPRI*, AU				
Baseline value	2484±838	2472±330	2212±288	2719±701
Mean Δ over 3 h	+70	−329	−286	−485
95% CI	−274 to +414	−395 to −263	−359 to −213	−552 to −419
P	...	<0.001	<0.001	<0.001

Abbreviations as in Table 1. For the placebo column, mean change from predose is shown. For the active treatment columns, placebo corrected changes from predose are shown (change from predose [active] minus mean change from predose [placebo]).
*$P \leq .05$ for linear contrast test of trend with dose.

macology of endothelin receptor antagonists, and their ultimate therapeutic relevance.

Physiological Role of Endothelin-1 in Regulation of Blood Pressure

As noted earlier, animal data on the hemodynamic effects of systemic endothelin receptor antagonism are apparently contradictory. We have previously shown that brachial artery administration of an ECE inhibitor or ET_A antagonist causes local forearm vasodilation, suggesting that basal vascular generation of endothelin-1 contributes to vascular tone.[24] Our demonstration here that systemic administration of an $ET_{A/B}$ antagonist causes peripheral vasodilation and hypotension confirms that endogenous generation of endothelin plays a fundamental physiological role in the maintenance of blood pressure in humans.

Pharmacology of Endothelin Receptor Antagonists

TAK-044 decreased mean arterial pressure and increased heart rate and cardiac index, resulting in a substantial decrease in calculated peripheral resistance. The greater reduction in diastolic as opposed to systolic pressure is consistent with a primary action of TAK-044 on peripheral resistance. TAK-044 also increased forearm blood flow when administered via the brachial artery, although this local action was obscured at the higher dose by systemic vasodilatation. Taken together, these effects indicate that the resistance vessels are the major site of action after endothelin $ET_{A/B}$ receptor blockade with TAK-044. In spontaneously hypertensive rats, a 6-hour infusion of an $ET_{A/B}$ endothelin receptor antagonist (SB 209670) also decreases blood pressure through an effect on total peripheral resistance.[40] However, heart rate tends to decrease in these animals, suggesting other sites of action for endothelin receptor antagonists. The difference between our results and these animal data may reflect differences in species, resting blood pressure, or mode of administration of the antagonist.

Vasodilatation and hypotension caused by TAK-044 occurred within 15 minutes and persisted for 12 to 24 hours. In contrast to its sustained hemodynamic actions, the marked increase in plasma endothelin concentrations caused by TAK-044 was relatively short in duration, and TAK-044 itself appeared to have a short half-life. In animals, the blood pressure–lowering effects of ECE inhibition or endothelin ET_A receptor blockade usually take several hours to reach maximum,[16,22] and forearm vasodilatation to these agents is also slow in onset.[24] This gradual effect is thought to be related to the slow dissociation of endothelin-1 from its receptor, resulting in persistent vasoconstriction even after new receptor binding is inhibited. There are two speculative explanations for the rapid onset

TABLE 4. Forearm Blood Flows and Ratio of Blood Flows Between Infused and Noninfused Arms in Study 2

Time From IV TAK-044	Phases 1 to 4				Phase 5	
		IV TAK-044				
	Placebo	30 mg†	250 mg†	750 mg†	Time From IA TAK-044	IA TAK-044 10/100 mg
−10 min (before IV TAK-044)					−10 min (before IA TAK-044)	
Infused	3.4±1.0	3.2±0.3	3.4±0.6	2.8±0.3		2.9±0.2
Control	3.1±0.9	2.6±0.3	3.2±0.5	2.9±0.4		2.3±0.2
Ratio	1.08±0.08	1.25±0.10	1.01±0.08	0.97±0.09		1.27±0.10
+55 min (before IA endothelin-1)					+60 min (after 10 mg IA TAK-044)	
Infused	4.3±1.4	4.7±0.8	4.3±0.8	3.3±0.4		3.9±0.4
Control	3.0±0.6	3.4±0.4	3.9±0.5	3.5±0.4		2.8±0.3
Ratio	1.29±0.17	1.35±0.10	1.05±0.10	0.99±0.09		1.43±0.08‡
+120 min (60 min after IA endothelin-1)					+120 min (after 100 mg IA TAK-044)	
Infused	4.3±1.5	4.7±0.8	4.4±0.6	3.8±0.4		4.1±0.3
Control	3.9±0.9	3.6±0.4	4.2±0.5	4.3±0.6		3.5±0.5
Ratio	0.93±0.13*	1.29±0.10	1.08±0.10	0.96±0.10		1.24±0.10
+180 min (120 min after IA endothelin-1)						
Infused	4.1±1.2	4.8±0.7	4.3±0.6	4.0±0.4		...
Control	4.0±0.7	3.6±0.4	4.3±0.6	4.6±0.6		...
Ratio	0.94±0.14*	1.32±0.06	1.02±0.07	0.94±0.12		...

IV indicates intravenous; IA, intra-arterial. Forearm blood flow is expressed as mL · 100 dL forearm tissue^{-1} · min^{-1}. For all columns, −10 min is the baseline measurement before dosing with TAK-044. For the columns containing data from the intravenous systemic TAK-044 phases, the +55 min row contains the last measurements before IA infusion of endothelin-1 was started (at +60 min). For the column containing data from the IA TAK-044 phase, the +60 min row contains measurements made at the end of the 10 mg/h treatment period.

*$P \leq .05$ vs baseline ratio before endothelin-1 (+55 min); †$P \leq .05$ vs placebo for change in ratio during endothelin-1 infusion (+60 min to +180 min); ‡$P \leq .05$ vs baseline ratio before TAK-044 (−10 min).

of vasodilatation observed here. First, the rapid effects of TAK-044 may be related to its potency as an endothelin receptor antagonist, with plasma concentrations being achieved that were sufficient to reverse, rather than prevent, endothelin-1 receptor binding. Second, TAK-044 is active at ET_B as well as ET_A receptors[26-29]; there is some evidence that vasoconstrictor ET_B receptors may have a more rapid onset of action than ET_A receptors.[41]

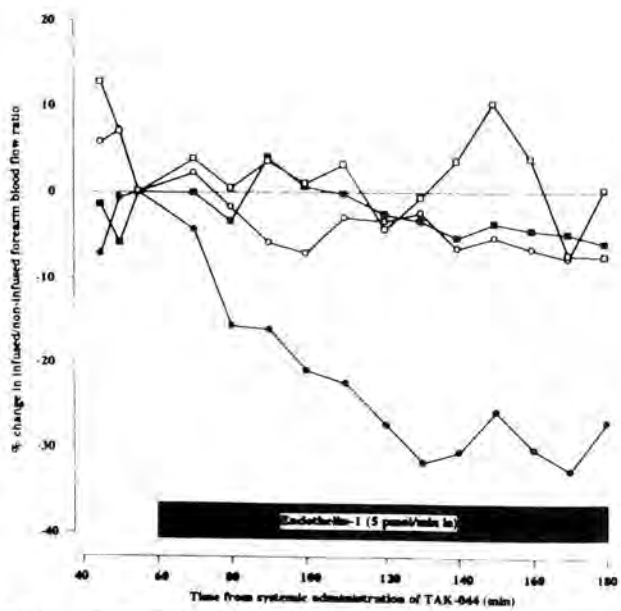

FIG 5. Effect of TAK-044 on forearm vasoconstriction to locally administered endothelin-1 in study 2. On four separate occasions, subjects received a 15-minute intravenous infusion of placebo (●) and TAK-044 at 30 mg (○), 250 mg (■), and 750 mg (□), followed 60 minutes later by brachial artery infusion of endothelin-1 (5 pmol/min for 120 minutes). Endothelin-1 caused a slow-onset forearm vasoconstriction after infusion of placebo. All three doses of TAK-044 significantly ($P < .05$) blocked forearm vasoconstriction to endothelin-1.

Endothelin-1 has a slow onset of action, and this may partially explain the sustained vasodilatation caused by $ET_{A/B}$ receptor blockade with TAK-044. In addition, although TAK-044 had a short half-life (30 to 60 minutes), TAK-044 concentrations were substantially greater than the IC_{50} for binding to ET_A receptors (0.08 nmol/L or 0.08 ng/mL) for at least 12 hours at doses >500 mg. Furthermore, it is possible that TAK-044 concentrations were above this level for longer periods or at lower doses; however, the limit of quantification for the TAK-044 assay was 5 ng/mL, ≈50-fold higher than the IC_{50} at ET_A receptors. Finally, the dissociation between pharmacokinetic and pharmacodynamic parameters may reflect entry into and activity of TAK-044 in another tissue compartment. This might be within the vasculature or in the central or peripheral nervous system. Entry into and actions in other tissue compartments appear to explain the similar dissociation between actions and plasma concentrations observed for inhibitors of the renin-angiotensin system.[42,43]

The trend analysis shows that vasodilatation to TAK-044 was dose dependent. However, given that vasodilatation occurred at almost all doses, including the lowest (10 mg), it is probable that doses <10 mg may be effective. Indeed, the pharmacokinetic results, together with the in vitro pharmacology data discussed earlier, suggest that the initial plasma levels were probably sufficiently high even after 10 mg to block endothelin ET_A receptors for at least 2 hours. This is confirmed by the complete blockade of fore-

arm vasoconstriction to endothelin-1 at 3 hours by doses as low as 30 mg in the second study. We did demonstrate a dose response for the elevation of circulating immunoreactive endothelin by TAK-044. In addition, peripheral vasodilatation was related to plasma endothelin concentrations, further supporting a dose-dependent effect on peripheral resistance.

The increase in plasma immunoreactive endothelin after TAK-044 may have several components. The radioimmunoassay we used detected both endothelin-1 and endothelin-3. Although it also cross-reacted with big endothelin-1, this was to a limited degree (7%) and therefore is unlikely to explain the substantial increases in circulating endothelin concentrations. The increase in circulating endothelin may have been due to increased generation or decreased receptor-mediated clearance of endothelin isopeptides. Decreased clearance of endothelin by ET_B receptors appears to be the most likely explanation, for several reasons. First, in animals, blockade of endothelin receptors of the ET_B subtype but not of the ET_A subtype increases plasma endothelin-1 and endothelin-3 concentrations[30] and prolongs the half-life of exogenous ^{125}I-endothelin-1.[31] Second, blockade of endothelin receptors increases plasma endothelin-1 within 15 minutes,[30] whereas de novo generation is thought to take several hours.[1] Third, endothelin receptor blockade does not increase big endothelin-1 concentrations.[30] The substantial increase in total immunoreactive endothelin in this study, together with the animal findings above, suggests that ET_B receptor binding is an important mechanism in clearance of endogenous endothelin peptides.

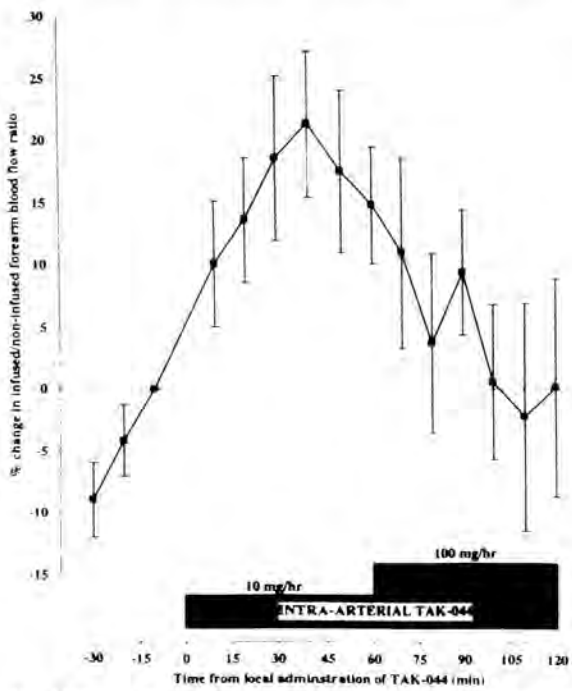

FIG 6. Graph showing effect of brachial artery administration of TAK-044 on forearm blood flow in the last phase of study 2. TAK-044 was infused at 10 and 100 mg/h sequentially, each for 60 minutes. TAK-044 caused significant local vasodilatation of the cannulated arm during infusion at 10 mg/h ($P = .0062$). At 100 mg/h, local vasodilatation appears to diminish ($P = .10$); this is probably related to an increase in blood flow in the noninfused arm, which decreases the blood flow ratio between infused and noninfused arms (see text and Table 5).

It would be useful for the clinical development of endothelin receptor antagonists to have a simple and reproducible index of endothelin receptor blockade. Although increases in plasma immunoreactive endothelin correlated with decreases in total peripheral resistance, this association was relatively weak, with correlation coefficients of ≈0.2. Changes in circulating endothelin concentrations probably only reflect antagonism at the ET_B receptor, which, in addition to its functional roles, appears to mediate clearance of circulating endothelin-1.[30,31] For a drug with ET_A receptor blocking properties, such as TAK-044, pharmacodynamic effects may be apparent at concentrations that do not substantially increase circulating endothelin concentrations, as was the case here. This may help to explain the different timings of changes in circulating endothelin and peripheral resistance, as well as the rather weak correlation between these parameters. In the second study, we used forearm vasoconstriction to endothelin-1 1 to 3 hours after dosing with TAK-044 to test endothelin receptor blockade. Vasoconstrictor responses to locally infused endothelin-1 were completely inhibited by all three doses, consistent with the similar hemodynamic responses to these doses. This model is safer than using intravenous infusion of systemic doses of endothelin-1 to increase blood pressure, particularly given the sustained and potent nature of vasoconstriction to endothelin-1. Given that both ET_A and ET_B receptors mediate vasoconstriction to endothelin-1 in the forearm,[9] blockade of vasoconstriction to endothelin-1 is likely to reflect antagonism at both ET_A and ET_B receptors. Antagonism at ET_B receptors could be tested by brachial artery administration of a selective ET_B receptor agonist, such as sarafotoxin S6c.[9]

Potential Therapeutic Role of Endothelin Receptor Antagonists

Experimental evidence supports a pathophysiological role for endothelin-1 in several diseases thought to be associated with acute vasoconstriction or vasospasm. These include acute renal failure,[21] coronary vasospasm,[44] unstable angina,[45] myocardial infarction,[46] and cerebral vasospasm associated with subarachnoid hemorrhage.[21] The potent inhibition of peripheral vasoconstriction to exogenous endothelin-1, as a model of vasospasm, by TAK-044 in this study suggests that it could be of benefit in such conditions. Indeed, in experimental animal models, TAK-044 has been shown to prevent postischemic acute renal failure[27] and limit myocardial infarction size.[29] The sustained vasodilator actions of TAK-044 in healthy subjects suggest that orally available endothelin receptor antagonists with a similar profile of action may have a valuable therapeutic role in diseases associated with chronic peripheral vasoconstriction, such as essential hypertension, chronic heart failure, and chronic renal failure.

In conclusion, we have shown that systemic endothelin ET_A and ET_B receptor blockade with the peptide TAK-044 causes sustained and substantial peripheral vasodilatation and, to a lesser extent, hypotension. This response suggests a fundamental physi-

ological role for endogenously generated endothelin-1 in cardiovascular regulation. Circulating immunoreactive endothelin concentrations were increased dose-dependently, and TAK-044 blocked forearm vasoconstriction to intra-arterial endothelin-1. Blockade of forearm vasoconstriction to brachial artery infusion of endothelin-1 appears to be a sensitive model for detecting endothelin receptor antagonism in humans. These findings support the development of endothelin receptor antagonists as therapies for diseases associated with acute and sustained peripheral vasoconstriction.

Acknowledgments

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Proceedings Paper

Activation of the endothelin B receptor causes a dose-dependent accumulation of cyclic GMP in human platelets

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Endothelins modulate *in vitro* aggregation of human platelets in a bi-directional manner. Thus endothelin-1 has been shown to act as a potentiator of primary aggregation and an inhibitor of secondary aggregation. The endothelin receptors and corresponding second messengers which cause these effects have not yet been characterised. This study investigated the effect of endothelin-1, an agonist at both the ET_A and the ET_B receptors and sarafotoxin (SRTX) S6c, a selective ET_B agonist, on human platelet cyclic nucleotide levels. Neither endothelin-1 (10^{-11}–10^{-7} M) nor SRTX S6c (10^{-11}–10^{-7} M) significantly altered platelet cAMP levels. In contrast, both agonists produced a dose-dependent increase in platelet cGMP. From these data, we conclude that activation of the ET_B receptor in human platelets is responsible for an increase in platelet cGMP and may contribute to the inhibition of platelet aggregation caused by the endothelins.

Key words: Platelet, endothelin, sarafotoxin S6c, cAMP, cGMP.

Introduction

The endothelins are a family of three 21-amino acid vasoactive peptides, endothelin-1, endothelin-2 and endothelin-3, of which endothelin-1 is considered to be the predominant isoform in the human vasculature. First identified in 1988, endothelin-1 is produced by the vascular endothelial cells and is the most powerful vasoconstrictor known.[1] Local infusion of endothelin-1 into the human brachial artery induces a characteristically sustained vasoconstriction, while infusion of the ET_A receptor antagonist BQ-123 induces vasodilatation, suggesting a role for endothelin in the maintenance of basal vascular tone.[2] The cellular actions of endothelin are mediated by two G-protein-linked cell-surface receptors; the ET_A receptor, that has a greater affinity for endothelin-1 than for endothelin-3, and the ET_B receptor, which has an equal affinity for the endothelin isoforms. Both receptors are present on vascular smooth muscle cells mediating contraction. However, relaxation can be induced indirectly through activation of an endothelial ET_B receptor leading to release of the endothelium derived dilators prostacyclin and nitric oxide. In addition to the stimulation of phosphoinositide hydrolysis, in many cell types endothelin-1 can modulate the levels of cyclic nucleotide second messengers. Studies in isolated preparations of bovine vascular smooth muscle cells have shown that endothelin-1 increases cyclic AMP (cAMP) levels via the ET_A receptor, presumably linked to a Gs protein, while in bovine endothelial cells the ET_B receptor attenuates forskolin stimulation of adenylate cyclase.[3] In transfected Chinese hamster ovary cells a similar pattern is observed.[4] In rat aortic strip preparations, however, ET_B receptor activation caused an increase in cyclic GMP (cGMP) which was abolished by pre-incubation with the nitric oxide synthase inhibitor N-monomethyl-L-arginine.[5] This suggests that ET_B receptor activation could lead to an increase in nitric oxide production, which in turn increases cellular cGMP levels, the second messenger for nitric oxide.

In studies on human platelets endothelin-1 appears to have two potentially opposing effects; potentiating

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adrenaline induced primary aggregation[6] but inhibiting secondary aggregation.[7] The contrasting effects of endothelin-1 may, at least in part, be due to the presence of different endothelin receptor subtypes exerting opposing effects. However, to date, the endothelin receptor or receptors involved in signal transduction in platelets have not been conclusively characterised.

Inhibitory pathways including both cAMP and cGMP are well characterised in the platelet. To investigate the possible modulation of cyclic nucleotide second messengers in platelets by endothelin receptor activation we have examined the effect of *in vitro* incubation of endothelin-1 with human platelet-rich plasma on platelet cAMP and cGMP concentrations. The snake venom peptide sarafotoxin S6c (SRTX S6c) has a close structural similarity to the endothelins and 30 000-fold selectivity for the ET_B receptor. Therefore, in order to elucidate the possible receptor type involved we have examined the effect of SRTX S6c on platelet cAMP and cGMP concentrations.

Materials and methods

Subjects

Six healthy male subjects, aged 27–35 years, participated in this study, with ethics approval. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each study, or alcohol or caffeine on the day of the study. Venous blood was collected into acid citrate dextrose (final concentrations; citric acid 0.8 mg ml^{-1}, sodium citrate 2.2 mg ml^{-1}, glucose 2.0 mg ml^{-1}), and promptly centrifuged at 120 × *g* for 20 min at room temperature to obtain platelet-rich plasma. Plasma was divided into sub-aliquots and stored at room temperature under 95%O_2:5%CO_2 until required. Plasma was stirred at 37°C for 4 min prior to the addition of endothelin-1 (10^{-11} 10^{-7} M), SRTX S6c (10^{-11} 10^{-7} M) or vehicle (0.9% saline). Dose-response studies to each agonist were performed in separate parallel aliquots. After 5 min, the platelet-rich plasma was immediately centrifuged at 10 000 × *g* for 2 min at 4°C, the plasma was removed, the platelet pellet resuspended in 2 ml ethanol and incubated at room temperature for 15 min. The ethanol was centrifuged at 1200 × *g* for 15 min and the supernatant decanted and dried down. The samples were resuspended in assay buffer and stored at −20°C until required for assay.

Materials

Citric acid, sodium citrate and glucose used in the anticoagulant were all obtained from Sigma (Poole, Dorset). Endothelin-1 was obtained from Novabiochem (Nottingham, UK), SRTX S6c from Alexis Corporation (Nottingham, UK) and sodium chloride for the vehicle and activated charcoal were obtained from Sigma (Poole, Dorset).

Cyclic nucleotide assays

Cyclic nucleotide concentrations were measured by in house radioimmunoassay as previously described.[8] Briefly, 500 µl samples were acetylated by the addition of 10 µl triethylamine and 5 µl acetic anhydride; 50 µl aliquots of samples were incubated for 18 h at 4°C with 100 µl of antibody and 150 µl of iodinated tracer. Antibody-bound tracer was separated from free by centrifugation with activated charcoal at 4°C for 30 min at 1700 × *g*. Free radioactivity was measured on a LKB multiwell gamma counter. Cyclic nucleotide levels are expressed as pmol ml^{-1} of platelet-rich plasma.

Statistics

Statistical analysis was by repeated measure analysis of variance. Differences were considered to be statistically significant at the 5% level. All statistical analysis was performed using the computer software package Statview 512+ (BrainPower Inc., Calabasas, CA, USA).

Results

Neither endothelin-1 ($P = 0.11$, $n = 6$) nor SRTX S6c ($P = 0.15$, $n = 6$) caused a significant change in platelet cAMP concentrations (Figure 1). However, both endothelin-1 and SRTX S6c caused a significant dose-dependent increase in platelet cGMP concentrations, ($P < 0.05$ and $P < 0.01$ respectively, $n = 6$; Figure 2).

Discussion

Incubation of platelet rich plasma with the non-selective ET_A/ET_B receptor agonist endothelin-1 or with the ET_B selective agonists SRTX S6c did not significantly alter platelet cAMP levels. However, both agonists significantly increased platelet cGMP. This would suggest that

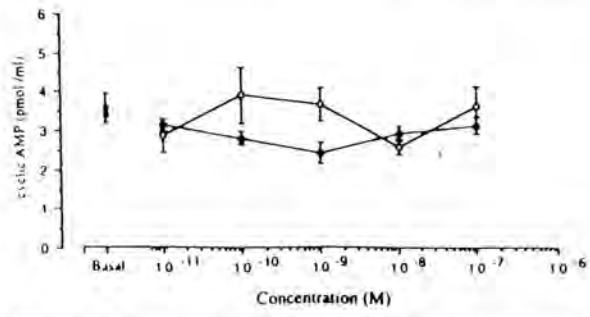

Figure 1. Change in platelet cAMP, mean ± SEM, in response to treatment with endothelin-1 (open circles; $P = 0.11$) and SRTX S6c (closed circles; $P = 0.15$) in pmol ml^{-1} of plasma. Statistical analysis by repeated measures ANOVA.

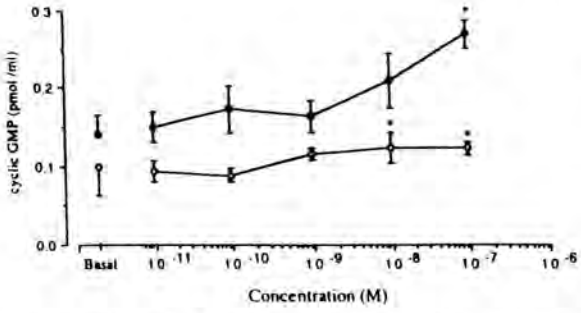

Figure 2. Change in platelet cGMP, mean ± SEM, in response to treatment with endothelin-1 (open circles; $P < 0.05$) and SRTX S6c (closed circles; $P < 0.01$) in pmol ml^{-1} of plasma. Statistical analysis was by repeated measures ANOVA.

human platelets contain an endothelin receptor which is responsible for increasing platelet cGMP concentrations either through stimulation of guanylate cyclase or inhibition of a specific cGMP phosphodiesterase. On the basis of the findings with SRTX S6c, this is probably an ET_B receptor. The marked elevation of cGMP by SRTX S6c contrasts with the slight effect seen with endothelin-1. This may reflect an ET_A mediated inhibition of guanylate cyclase stimulation or of an increase in cGMP degradation.

Selective activation of the ET_B receptor in rat aorta by IRL 1620, in the presence of an intact endothelium, stimulates an increase in cGMP and leads to relaxation of the aorta by a mechanism which can be blocked by the nitric oxide synthase inhibitor N-monomethyl-L-arginine.[5] This demonstrates that the ET_B receptor is able to induce an increase in cGMP in neighbouring vascular smooth muscle cells by stimulation of nitric oxide production from the endothelium. In cultured porcine epithelial cells both endothelin-1 and endothelin-3 were able to cause an increase in cGMP in the target cell, by a mechanism which was also blocked by N-monomethyl-L-arginine,[9] demonstrating that ET_B stimulated nitric oxide can have an autocrine as well as paracrine role.

The platelet L-arginine:nitric oxide pathway was characterised in 1990,[10] demonstrating that platelet derived nitric oxide inhibited platelet aggregation through elevation of intra-platelet cGMP. It is, therefore, likely that activation of the platelet ET_B receptor stimulates platelet nitric oxide, and by activation of soluble guanylate cyclase, increases cGMP.

However, there is evidence for an insulin receptor linked mechanism for increasing platelet cGMP which is independent of nitric oxide synthase,[11] and recently the benzylindazole derivative YC-1 has also been shown to activate soluble guanylate cyclase through a nitric oxide independent mechanism in human platelets.[12] This not only indicates that platelet guanylate cyclase may be activated by several different pathways but also confirm that the elevation of cGMP *per se* inhibits platelet aggregation. Therefore, we are not able to conclude that the platelet ET_B receptor is linked to nitric oxide synthase. This could be investigated either by inhibition of the enzyme or direct measurement of nitric oxide.

In conclusion, we have provided evidence for the existence of an endothelin ET_B receptor on human platelets. Activation of this receptor stimulates cGMP accumulation, which is known to be involved in the inhibition of platelet aggregation. This not only provides a useful model for investigating regulation of this receptor but may also be important in the wide range of disease states where abnormalities in platelet activity has been reported, such as myocardial infarction and cirrhosis of the liver.

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The increase in human plasma immunoreactive endothelin but not big endothelin-1 or its C-terminal fragment induced by systemic administration of the endothelin antagonist TAK-044

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1 We examined the effects of systemic infusion, in healthy human volunteers, of the endothelin antagonist TAK-044 on the plasma concentrations of mature endothelin, big endothelin-1 and the C-terminal fragment of big endothelin-1, by selective solid-phase extraction and specific radioimmunoassays.

2 Unlabelled TAK-044 competed with specific [^{125}I]-endothelin-1 binding to human left ventricle tissue in a biphasic manner giving K_D values of 0.11 nM and 26.8 nM at the ET_A and ET_B receptor subtypes, respectively, indicating a 244 fold selectivity for the ET_A receptor subtype.

3 A 15 min intravenous infusion of placebo or 30 mg TAK-044 (giving a serum concentration of 2 nM, calculated to block >95% of ET_A but <5% ET_B receptors) had no effect on the immunoreactive plasma concentrations of the three peptides.

4 At the higher dose of 750 mg TAK-044 (giving a serum concentration of 80 nM, calculated to block >99% of ET_A and >75% ET_B receptors), the immunoreactive plasma endothelin concentrations were increased 3.3 fold over basal levels ($P<0.01$). The concentrations of big endothelin-1 or C-terminal fragment of big endothelin-1 were unchanged.

5 At both doses of TAK-044, there were significant decreases in diastolic blood pressure, and peripheral vascular resistance, with corresponding increases in cardiac index and stroke index. There were no changes in systolic or mean arterial blood pressures or heart rate.

6 Since only the concentrations of the mature peptide were increased, we conclude that the most likely sources of endothelin contributing to the observed rise were displacement of receptor-bound peptide and reduction in plasma clearance rather than peptide synthesis.

Keywords: Endothelin; big endothelin; C-terminal fragment; human vasodilatation; selective solid-phase extraction; radioimmunoassay

Introduction

The endothelins are a family of three potent vasoconstrictor peptides (endothelin-1 (ET-1), ET-2 and ET-3, Inoue *et al.*, 1989). ET-1 and a biologically inactive C-terminal fragment (CTF, big ET-$1_{(22-38)}$) are generated from an intermediate big ET-1, following an unusual cleavage, by one or more endothelin-converting enzymes (Battistini *et al.*, 1995). In functional assays the endothelins exert their actions through two distinct receptor subtypes, ET_A and ET_B, distinguished by the rank order of potency of the three endothelin isoforms (Arai *et al.*, 1990, Sakurai *et al.*, 1990).

We have previously demonstrated that local infusion of the ET_A-selective antagonist BQ123 causes vasodilatation suggesting that endothelin contributes to basal vascular tone in man (Haynes & Webb, 1994). In addition, we have previously shown that local infusion of exogenous big ET-1 results in an increase in immunoreactive (IR) plasma CTF as well as IR endothelin (Plumpton *et al.*, 1995a, b). All three peptides can be detected in the conditioned medium from cultured endothelial cells (Plumpton *et al.*, 1996a), suggesting that any *de novo* synthesis of endothelin would be accompanied by a rise in CTF.

Animal and human studies have suggested that endothelin is important in a range of clinical conditions and have demonstrated the potential therapeutic beneficial effects of endothelin receptor antagonism (Ferro & Webb, 1996). However, plasma concentrations of IR endothelin are increased following non-selective endothelin receptor antagonism (Löffler *et al.*, 1993; Donckier *et al.*, 1995; Kiowski *et al.*, 1995; Teerlink *et al.*, 1995). Our aim was to determine whether the systemic infusion of the cyclic hexapeptide endothelin receptor antagonist TAK-044 (cyclo[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl) carbonyl]-L-analyl-L-α-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl] disodium; Kikuchi *et al.*, 1994) altered the concentrations of IR endothelin in human plasma. Secondly, to distinguish between possible sources of IR plasma endothelin, we also measured the concentrations of the precursor big ET-1, and by-product CTF. A preliminary account of this work has been presented to the British Pharmacological Society (Plumpton *et al.*, 1996b).

Methods

Competition binding experiments

The selectivity of TAK-044 was determined for native human endothelin receptors as previously described (Davenport *et al.*, 1995). The human left ventricle was used as this expresses both endothelin receptor subtypes and allows the selectivity of competing ligands to be determined in the same assay. Frozen sections of left ventricle (10 μM, $n=3$) were thaw mounted onto gelatine coated microscope slides and pre-incubated for

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15 min at 23°C in 50 mM HEPES, 5 mM $MgCl_2$, 0.3% BSA pH 7.4. Sections were then incubated for 2 h at 23°C in the same buffer containing 0.1 nM [^{125}I]-ET-1 (2000 Ci mmol^{-1}) and increasing concentrations of TAK-044 (20 pM - 100 μM). Total ET-1 binding was determined in the absence of TAK-044 and non-specific binding was defined by inclusion of 1 μM unlabelled ET-1. Sections were washed three times in ice-cold Tris-HCl (pH 7.4) for a total of 15 min then wiped from the slides and counted using a gamma counter. Protein estimations were made by use of a modified Lowry assay (Bio-Rad) by reference to bovine serum albumin (BSA) standards. Pseudo Hill coefficients were determined by use of EBDA (McPherson, 1983) from which the final values of equilibrium dissociation constant (K_D) were calculated with the iterative curve fitting program LIGAND (Munson & Rodbard, 1980).

Clinical procedures

Healthy male volunteers (25–60 years of age, mean age 40) participated in the study with the approval of the local ethical committee. Each subject had an antecubital vein of the left and right arm cannulated for administration of infusate and withdrawal of blood samples, respectively. TAK-044 was synthesized and supplied by Takeda Chemical Industries Ltd, Osaka, Japan. Blood pressure and heart rate were measured by semi-automated oscillometric monitors, cardiac function (stroke volume, cardiac output and heart rate) was measured by a non-invasive bioimpedance methodology (Haynes *et al.*, 1996). Eight subjects received either 50 ml sucrose placebo, 30 mg or 750 mg TAK-044 dissolved in saline over 15 min on separate occasions in a single-blind, three-phase, randomised, placebo-controlled, crossover study. The two doses of TAK-044 resulted in serum concentrations of 2 and 80 nM TAK-044 after 15 min of infusion, respectively as determined by high performance liquid chromatography (h.p.l.c.). In six subjects, venous blood samples were taken before and 15 min after dosing. Blood was collected into EDTA tubes and separated immediately. The resulting plasma was stored at −70°C until assayed.

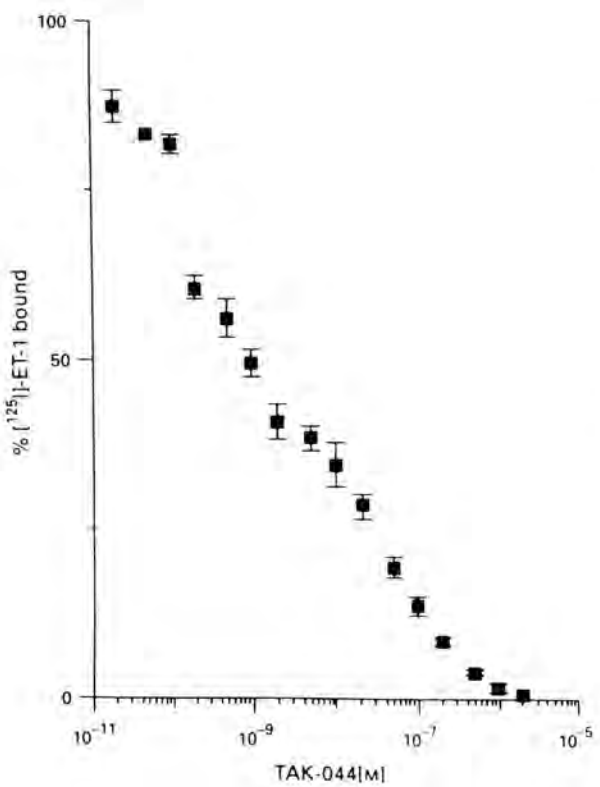

Figure 1 Competition assays showing the inhibition of [^{125}I]-endothelin-1 (ET-1) binding to sections of human left ventricle by TAK-044. Results are expressed as percentages of the specific binding. Mean values ($n = 3$) are shown and vertical lines indicate s.e mean.

Radioimmunoassays for endothelin peptides

After being thawed, 5 ml plasma samples were assayed for IR endothelin, big ET-1 and CTF by selective solid-phase extraction and radioimmunoassay as previously described (Plumpton *et al.*, 1995a; 1996a) using rabbit antisera raised against the C-termini of endothelin (ET-$1_{(15-21)}$) and big ET-1 (big ET-$1_{(31-38)}$). For both assays, ED_{50} values were 20–25 fmol/tube, inter- and intra-assay coefficients of variation were <13% in the range 6–30 fmol/tube and the sensitivities of detection (defined as two s.d. above zero standard) were <1.25 fmol/tube. The recoveries of ET-1, big ET-1 and CTF were 57.5%, 39.8% and 76.6%, respectively ($n = 4$). Plasma IR peptide concentrations are shown uncorrected for extraction recovery.

Under the present conditions, the mature endothelin RIA cross-reacted 100% with ET-1, ET-2 and ET-3. Cross-reactivity with ET-$1_{(1-20)}$, big ET-$1_{(22-38)}$, big ET-1, big ET-2 and big ET-3 were <0.02%. The big ET-1 RIA showed <0.007% cross-reactivity with the mature endothelins, big ET-2 and big ET-3, and cross-reacted 143% with big ET-$1_{(22-38)}$ thus allowing the quantification of CTF following fractionation. Neither of the assays showed any detectable cross-reactivity (<0.000002%) with TAK-044. Furthermore, TAK-044 did not interfere with either assay as indicated by superimposable standard curves at concentrations five orders of magnitude greater than the serum TAK-044 levels achieved. No cross-reactivity was detected (<0.005%) at the highest concentrations tested with unrelated vasoactive peptides such as angiotensin II, atrial natriuretic factor, and α-calcitonin gene-related peptide.

Results

TAK-044 competed biphasically for the binding of [^{125}I]-ET-1 giving K_D values of 0.11 nM and 26.8 nM at the ET_A and ET_B receptor subtypes, respectively (Figure 1). Based on these affinities, the concentration of TAK-044 measured in the serum following the low dose was calculated to block >95% of the ET_A but <5% of the ET_B receptors. However, at the high dose, the majority (>75%) of the ET_B receptors were calculated to be blocked in addition to >99% of the ET_A receptors.

At both doses of TAK-044, there were significant decreases in diastolic blood pressure, and peripheral vascular resistance, with corresponding increases in cardiac index and stroke index. There were no changes in systolic or mean arterial blood pressures or heart rate in any of the phases (Table 1). These findings are in agreement with an action of TAK-044 as an arteriolar vasodilator. Although most of the changes in the haemodynamic parameters appeared greater after 750 mg than 30 mg TAK-044, none of these differences reached statistical significance. TAK-044 was well tolerated in all volunteers as assessed by direct questions, visual analogue scales, routine electrocardiography, biochemistry and haematology. In particular, no serious or clearly drug-related adverse effects were detected.

When placebo or TAK-044 at the 30 mg dose was infused there were no significant changes in the concentration of IR peptide compared with basal levels (Figure 2). However, at the higher dose of 750 mg TAK-044, although there were no detectable changes in the concentrations of IR big ET-1 or CTF, the IR endothelin increased from 4.0 ± 1.5 pM to 13.3 ± 2.2 pM ($P < 0.01$, compared to pre-infusion).

Table 1 Baseline values and mean changes (Δ) over 15 min for systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate, stroke index, cardiac index and peripheral vascular resistance (PVR) after dosing with TAK-044

	Placebo			*TAK-044*					
				30 mg			*750 mg*		
	Baseline value	*Mean Δ over 15 min*	*95% CI*	*Baseline value*	*Mean Δ over 15 min*	*95% CI*	*Baseline value*	*Mean Δ over 15 min*	*95% CI*
SBP (mmHg)	127±5	4±2	−3 to +10	128±5	1±1	−2 to +5	129±5	−8±4	−18 to +2
DBP (mmHg)	75±2	1±1	−2 to +5	73±2	−3±1*	−5 to −1	74±2	−4±2*	−8 to 0
MAP (mmHg)	92±3	2±1	−2 to +6	91±1	−2±1	−4 to +1	92±3	−6±2	−9 to +1
Heart rate (beats min^{-1})	58±3	1±1	−2 to +3	57±4	1±2	−5 to +6	59±2	1±1	−2 to +4
Cardiac index ($l\,min^{-1}\,m^{-2}$)	3.2±0.3	−0.1±0.04	−0.2 to +0.1	3.0±0.2	0.3±0.1*	+0.1 to +0.5	2.8±0.2	0.4±0.1*	+0.1 to +0.6
Stroke index ($ml\,m^{-2}$)	56±5	0±1	−2 to +2	51±3	3±1**	+1 to +5	47±5	5±1**	+2 to +8
PVR ($dyn\,s\,cm^{-5}\,m^{-2}$)	2392±237	77±57	−66 to +220	2472±117	−259±61**	−413 to −105	2719±248	−452±41***	−554 to −350

For the placebo column, mean change from predose is shown. For the active treatment columns, placebo corrected changes from predose are shown. Mean values ($n=8$) are shown ± s.e.mean and 95% confidence intervals (CI). Statistical differences were analysed by ANOVA followed by paired Student's t tests. *$P<0.05$; **$P<0.01$; ***$P<0.0001$ compared to basal.

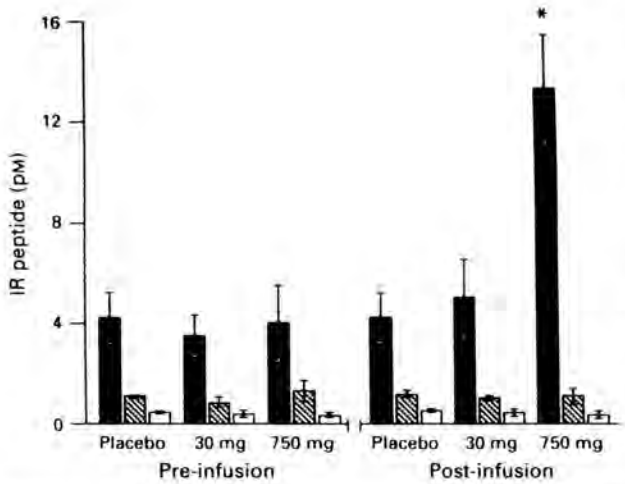

Figure 2 Effect of intravenous infusion of placebo or TAK-044 (30 mg or 750 mg) for 15 min on immunoreactive plasma endothelin (solid columns), big endothelin-1 (hatched columns) and C-terminal fragment of big endothelin-1 (open columns). Mean values ($n=6$) are shown and vertical lines indicate s.e.mean. Statistical differences were analysed by ANOVA followed by Student's t tests. *$P<0.01$ compared to basal.

Discussion

This study shows that in healthy human volunteers, systemic infusion of 750 mg TAK-044, but not placebo or 30 mg TAK-044, results in a significant increase in venous plasma IR endothelin compared to basal levels, with no detectable changes in the corresponding concentrations of either big ET-1 or CTF.

Local infusion of big ET-1 in healthy human volunteers results in significant increases of IR plasma endothelin and CTF compared to basal (Plumpton *et al.*, 1995a, b). Therefore, if the present rise in IR endothelin were due to an increased synthesis, we would predict altered concentrations of big ET-1 and/or CTF, but no change from basal levels was detected. Taken together with the rapid rate of increase in plasma IR endothelin, the effects are unlikely to be due to *de novo* synthesis, although the processing of stored precursors cannot be excluded. These data support other endothelin peptide measurements obtained following endothelin receptor antagonism in animals (Löffler *et al.*, 1993; Donckier *et al.*, 1995; Teerlink *et al.*, 1995) and patients with chronic heart failure (Kiowski *et al.*, 1995).

At the serum concentrations achieved with both doses of TAK-044, it was calculated that virtually all of the ET_A receptors are blocked and may therefore produce the observed haemodynamic changes, since ET-1 predominantly mediates vasoconstriction via ET_A receptors (Davenport & Maguire, 1994; Maguire & Davenport, 1995). In contrast, only at the higher dose are the majority of the ET_B sub-type antagonised. This suggests that the increase in human plasma IR endothelin following the infusion of 750 mg TAK-044 could be associated with displacement of ET_B receptor-bound peptide and/or blockade of clearance receptors. This is consistent with the identification of a high density of non-vascular ET_B receptors, possibly clearance receptors, in human kidney (Karet *et al.*, 1993) and their efficient regional extraction of mature endothelins (Weitzberg *et al.*, 1991; Gasic *et al.*, 1992).

We conclude that the increase in IR plasma endothelin following infusion of TAK-044 under the present conditions, is most likely due to the displacement of mature endothelin from binding sites and/or blockade of clearance from the circulation.

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Physiological role of nitric oxide in regulation of renal function in humans

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[1]*Clinical Pharmacology Unit and Research Centre, Western General Hospital, University of Edinburgh, Edinburgh EH4 2XU;* [2]*Department of Clinical Pharmacology, Glaxo-Wellcome, Beckenham BR3 3BS; and* [3]*Department of Clinical Pharmacology, St. Bartholomew's Hospital, London EC1A 7BE, United Kingdom*

Haynes, William G., Malcolm F. Hand, Mark E. C. Dockrell, David W. Eadington, Michael R. Lee, Ziad Hussein, Nigel Benjamin, and David J. Webb. Physiological role of nitric oxide in regulation of renal function in humans. *Am. J. Physiol.* 272 (*Renal Physiol.* 41): F364–F371, 1997.—The physiological role of endogenous nitric oxide in regulation of renal function in humans is unclear. Eight healthy men received an inhibitor of nitric oxide synthase, N^G-monomethyl-L-arginine (L-NMMA, 3 mg/kg), and saline placebo intravenously on two occasions. L-NMMA significantly increased mean arterial pressure (+7%) and total peripheral resistance (+36%). However, because renal plasma flow did not decrease significantly, the increase in renal vascular resistance (+21%) was significantly less than the increase in total peripheral resistance. Glomerular filtration rate (−19%), filtration fraction (−10%), urine flow rate (−18%), sodium (−28%), and free water excretion (−25%) all decreased significantly. Fractional distal, but not proximal, sodium reabsorption increased. L-NMMA also significantly decreased plasma nitrate and urinary excretion of nitrate and dopamine. There were no significant changes in plasma renin activity, plasma endothelin, and aldosterone or in platelet number and ex vivo aggregation. L-NMMA had a plasma half-life of 75 min. Basal generation of nitric oxide appears to contribute less to vascular tone in the kidney than systemically but may alter afferent arteriolar tone. Decreased fractional sodium excretion supports an important physiological role for nitric oxide in inhibition of tubular sodium reabsorption, possibly mediated by the renal dopaminergic system.

endothelium-derived relaxing factor; kidney; endothelins; dopamine; platelet

NITRIC OXIDE is a free radical molecule that has many biological actions, including smooth muscle relaxation, inhibition of platelet aggregation, and neurotransmission (18). Nitric oxide is generated from L-arginine by a family of synthase enzymes and has a very short half-life, being rapidly oxidized in blood to nitrite and nitrate (30). Animal studies suggest that endogenously produced nitric oxide may play an important role in regulation of renal hemodynamics and tubular function (17, 20). However, several contradictory results have been obtained. For example, there is debate about whether nitric oxide acts predominantly on the afferent (10) or efferent renal arteriole (13, 20). In addition, sodium excretion has been reported to be increased (3), unchanged (20), or decreased (13, 19) following nitric oxide synthase inhibition. Furthermore, the role of nitric oxide in regulation of proximal and distal tubular sodium reabsorption is uncertain (17, 19).

We and others have shown that systemic administration of the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) to humans causes vasoconstriction and increases arterial pressure, confirming that nitric oxide is generated physiologically to oppose vasoconstrictor influences in resistance vessels (7, 9, 25). We also reported that L-NMMA increased urine sodium excretion but did not change creatinine clearance over a 2.5 h period in healthy male subjects (9). However, we did not perform frequent urine collections in these studies, nor did we measure the effects of L-NMMA on indexes of renal hemodynamics and tubular function. Given the animal data supporting an important role for endogenous nitric oxide in regulation of renal function, we have now performed a more rigorous evaluation of the renal effects of nitric oxide synthase inhibition in humans. We tested the adequacy of nitric oxide synthase blockade by measuring plasma and urine nitrate concentrations (2). Because it is not clear whether the hemodynamic and renal actions of endogenous nitric oxide are solely due to direct effects, we also assessed plasma renin activity, plasma aldosterone and endothelin concentrations, and urinary free dopamine excretion. In addition, in view of the potent anti-aggregatory properties of nitric oxide (18), we examined whether L-NMMA altered platelet number or ex vivo platelet aggregation.

METHODS

Subjects. Eight healthy male subjects (age range, 19–38 yr) participated in this study, which was conducted with the approval of the local Ethics Review Committee and with the written informed consent of each volunteer. No subject received vasoactive or nonsteroidal anti-inflammatory drugs in the week prior to each study day, and all were asked to abstain from alcohol for 24 h and from food, caffeine-containing drinks, and cigarettes for at least 16 h before baseline measurements were made. A 24-h collection of urine was performed by all subjects beginning at 7:00 AM on the day before each study day. Subjects received a physiologically inert dose of oral lithium carbonate (Camcolit, 250 mg sustained release; Norgine, Harefield, UK) before each study day (~14 h before baseline measurements) to assess proximal tubular reabsorptive capacity of sodium (5).

Assessments. Blood pressure was measured in the right arm in duplicate using a well-validated semi-automated oscillometric technique (Takeda UA 751 sphygmomanometer; Takeda Medical, Tokyo, Japan) (31). Cardiac function (stroke

volume, cardiac output, and heart rate) was measured using a noninvasive bioimpedance method (BoMed NCCOM3, BoMed Medical Manufacturer, Irvine, CA). Both absolute cardiac output and changes in cardiac output measured by bioimpedance agree closely with thermodilution measurements, and the within-subject coefficient of variation is lower with bioimpedance (26). Effective renal plasma flow (ERPF) and glomerular filtration rate (GFR) were estimated by measuring the renal clearances of *p*-aminohippurate sodium (PAH; Merck, Hoddesdon, UK) and polyfructosan-S (Inutest; Laevosan, Linz, Austria), respectively. Priming doses of PAH (0.45 g) and polyfructosan-S (3.5 g) were diluted in 100 ml of 0.9% saline and infused over 15 min, followed by a maintenance infusion of PAH (8.3 g/l) and polyfructosan-S (10 g/l) in 0.9% saline at 120 ml/h. An equilibration period of 90 min allowed plasma concentrations of PAH and polyfructosan-S to stabilize before baseline collections started.

Sample collection. Venous blood samples were collected into heparinized tubes for assay of plasma polyfructosan-S, PAH, sodium, potassium and aldosterone concentrations, and plasma osmolality; into EDTA tubes for assay of plasma immunoreactive endothelin, plasma renin activity, hematocrit (Hct), and platelet counts; into plain glass tubes for assay of serum lithium concentrations; and into acid citrate dextrose tubes for platelet aggregation studies. Plasma was separated and frozen within 15 min of sampling. Urine was collected into plain plastic containers, except for those for dopamine estimations, where 20 ml urine was added to 0.5 ml of 4 M hydrochloric acid. All samples were stored at −80°C before assay.

Assay techniques. Polyfructosan-S and PAH concentrations were assayed using established semi-automated chemical methods (5). Electrolytes were assayed by emission-flame photometry with lithium as the internal standard for sodium and potassium estimations and cesium as the internal standard for lithium estimations. Total plasma and urine nitrite/nitrate concentrations were assayed using the Griess reaction (6). Briefly, passage of urine or deproteinized plasma samples at high pressure through a cadmium column reduced nitrate to nitrite, which then reacted with Griess reagent (0.05% napthylethylenediamine and 0.05% sulfanilamide) to form a purple azo dye, the absorbance of which at 546 nm was detected by a spectrophotometer. The procedure for determining plasma L-NMMA concentrations utilized pre-column derivatization with *o*-phthaladehyde, separation of the isoindole derivative by reverse-phase high-performance liquid chromatography (HPLC) and fluorescence detection. This method had an intra-assay precision of 10.2, 7.4, 6.6, and 7.9 (coefficient of variance in %) at nominal concentrations of 0.4, 2, 4, and 100 μmol/l, respectively. The accuracy (expressed as %bias) at the same L-NMMA concentrations was 0.0, 2.0, 0.0, and 4.0, respectively. Urine dopamine was measured using HPLC with electrochemical detection as previously described (15). Plasma aldosterone concentrations and renin activity were measured by radioimmunoassay as previously described (5). Plasma immunoreactive endothelin was measured by radioimmunoassay (ITS Production, Utrecht, The Netherlands) as previously described (8); the sensitivity of this assay is 2 pg/ml immunoreactive endothelin, and cross reactivities with endothelin-1, endothelin-2, endothelin-3, and big endothelin-1 are 100, 52, 96, and 7%, respectively.

Platelet aggregation studies. Blood collected for platelet aggregation studies was promptly centrifuged at room temperature for 10 min at 120 *g* to obtain platelet-rich plasma; the remaining blood was centrifuged for a further 20 min at 3,000 *g* to obtain platelet-poor plasma. Platelet aggregability was measured at 37°C using the light transmittance method of Born (1) in a Malin 6 channel aggregometer. Light transmittance through platelet-rich plasma prior to the addition of the aggregating agent was taken as 0% aggregation and through platelet-poor plasma as 100% aggregation. Separate aliquots of platelet-rich plasma were incubated with vehicle or different doses of ADP (0.1–10 μM) for 10 min.

Study design. This was a randomized, single-blind, crossover study comparing L-NMMA with vehicle placebo. The two phases were separated by at least 6 days. Studies were performed in a quiet clinical research laboratory maintained at a constant temperature of between 22 and 25°C. On the morning of each study day, subjects were asked to drink 450 ml of water at 7:00 AM (~3 h before the first baseline measurements). After arrival at the laboratory, subjects voided urine and then drank 450 ml water at 8:00 AM. Subjects were then asked to void at intervals of ~30 min, with water intake adjusted before dosing with L-NMMA or placebo to induce a diuresis of ~10 ml/min. Except when voiding, subjects rested in a recumbent position. A plastic cannula was inserted under local anesthesia into a superficial antecubital vein of each arm, one for drug administration and one for blood sampling. Intravenous infusion of the priming doses of PAH and inulin commenced at ~8:30 AM. During the 90-min equilibration period, the subject was acclimatized to the hemodynamic assessments by measuring blood pressure and cardiac function every 15 min. Baseline assessments started at ~10:00 AM, when blood samples were obtained before and after two accurately timed 30-min urine collections. At ~11:00 AM, L-NMMA (3 mg/kg; Clinalfa, Laufelfingen, Switzerland) or vehicle placebo (0.9% saline) was infused intravenously over 5 min. An additional four accurately timed 30-min urine collections were then made. After dosing, to maintain strict fluid balance, water intake was varied only to match fluid losses (urine and blood), taking into account intravenous infusion volumes. Blood pressure and cardiac function were measured at 5-min intervals before and after dosing with L-NMMA. Blood samples were obtained at 30-min intervals throughout, except for samples for estimation of plasma L-NMMA concentrations, which were also obtained 5, 10, and 15 min after starting infusion of L-NMMA. Blood for estimation of platelet aggregation was obtained just before and 15 min after L-NMMA.

Data analysis. Mean arterial pressure (MAP) was calculated as diastolic blood pressure plus one-third pulse pressure. Data for stroke volume and cardiac output were corrected for body surface area, calculated according to a standard nomogram, to provide measures of stroke and cardiac index. Total peripheral resistance was calculated as (MAP × 72.269)/cardiac index, and expressed in dyn·s·cm^{-5}. Data for arterial pressure and cardiac index were averaged over each 30-min clearance period. Renal clearance was calculated using the formula UV/P, where U is the urinary concentration, V is the urinary flow rate, and P is the mean of the plasma concentrations at the beginning and end of a clearance period. Effective renal vascular resistance (ERVR) was calculated using a formula that assumed a constant renal venous pressure of 10 mmHg

$$\text{ERVR} = 72{,}269 \times (\text{MAP} - 10) \times \frac{(1 - \text{Hct})}{\text{ERPF}}\ \text{dyn}\cdot\text{s}\cdot\text{cm}^{-5}$$

Filtration fraction was calculated as (GFR/ERPF) × 100%. Lithium ions are reabsorbed almost exclusively in the proximal tubule and to the same degree as sodium and water. Thus clearance of lithium (C_L) is thought to be representative of proximal tubular sodium clearance. The following formulae

were used to estimate proximal and distal sodium reabsorption: fractional proximal tubule sodium reabsorption = (1 − C_{Li}/GFR) × 100%; and fractional distal tubule sodium reabsorption = (1 − C_{Na}/C_{Li}) × 100%, where C_{Na} is sodium clearance.

Data are presented as means ± SE. Data from the two baseline clearance periods were never significantly different, and the mean of these periods was used in the analyses. Absolute changes from baseline are shown in the text. To aid interpretation of the relative magnitude of changes in different parameters in the figures, the percentage change from baseline after L-NMMA or placebo was calculated and the percentage change after placebo was subtracted from the percentage change after L-NMMA. Baseline data for the placebo and L-NMMA study days were compared using Student's paired *t*-test. All other comparisons were made using a two-way, repeated-measures analysis of variance, with testing of statistical significance by Scheffé's *F*-test. Significance levels were adjusted for multiple comparisons using Tukey's test. Statistical analyses were performed using Microsoft Excel Statistical Analysis ToolPak software (GreyMatter International, Cambridge, MA) on an Apple Macintosh personal computer.

RESULTS

Baseline data. There were no significant differences between basal values for any parameter on the placebo and L-NMMA study days (Table 1). There were no significant changes in any parameter following administration of placebo.

Systemic hemodynamics. Compared with placebo, infusion of L-NMMA caused a modest but significant increase in MAP ($P < 0.001$), with an increase of 6 ± 2 mmHg over the first 30 min (Fig. 1). Both systolic and diastolic blood pressure increased, although this was only significant for diastolic pressure ($P < 0.0001$), with an increase of 8 ± 1 mmHg in the first 30 min. There were significant reductions in both heart rate ($P < 0.05$) and stroke index ($P < 0.0001$), leading to a substantial fall in cardiac index ($P < 0.0001$), with a decrease of 0.6 ± 0.1 l/min after 30 min (Fig. 1). There was, therefore, a substantial increase (649 ± 119 dyn · s · cm^{-5}, $P < 0.0001$) in total peripheral resistance index, which persisted for at least 60 min (Fig. 1).

Renal hemodynamics. In contrast to the substantial effects on cardiac index, infusion of L-NMMA did not significantly alter ERPF ($P = 0.836$), with a decrease of only 62 ± 55 ml/min in the first 30 min (Fig. 2). Although renal vascular resistance did increase significantly following infusion of L-NMMA ($P < 0.05$; 1,281 ± 430 dyn · s · cm^{-5} at 30 min; Fig. 2), in percentage terms (+21 ± 9%), this was significantly less than the change

Table 1. *Baseline values before administration of L-NMMA and placebo*

	L-NMMA	Placebo
Mean arterial pressure, mmHg	84 ± 2	84 ± 2
Heart rate, beats/min	58 ± 2	60 ± 3
Stroke index, ml/m^2	60 ± 6	64 ± 5
Cardiac index, l · min^{-1} · m^2	3.5 ± 0.3	3.7 ± 2
Total peripheral resistance, dyn · s · cm^{-5}	1,843 ± 152	1,680 ± 94
Effective renal plasma flow, ml/min	684 ± 93	677 ± 75
Effective renal vascular resistance, dyn · s · cm^{-5}	6,149 ± 488	5,225 ± 517
Glomerular filtration rate, ml/min	97 ± 8	88 ± 9
Filtration fraction, %	17 ± 4	14 ± 2
Sodium excretion in previous 24 h, mmol/day	199 ± 13	204 ± 20
Baseline sodium excretion, μmol/min	261 ± 25	269 ± 18
Urine flow rate, ml/min	13 ± 1	12 ± 1
Fractional sodium excretion, %	1.9 ± 0.2	2.2 ± 0.3
FNa^+R_{prox}, %	76 ± 3	70 ± 3
FNa^+R_{dist}, %	91 ± 1	92 ± 1
Plasma lithium concentration, μmol/l	80 ± 6	80 ± 4
Dopamine excretion, nmol/min	1.60 ± 0.08	1.76 ± 0.18
Plasma NO_3^-, μmol/l	24 ± 4	25 ± 4
Urine NO_3^- excretion, μmol/min	0.98 ± 0.11	1.07 ± 0.13
Plasma endothelin, pg/ml	4.2 ± 0.8	4.8 ± 0.7
Plasma renin activity, ng · ml^{-1} · h^{-1}	0.44 ± 0.12	0.54 ± 0.13
Plasma aldosterone, pmol/l	115 ± 21	138 ± 44
Platelets, × 10^9 cells/l	196 ± 19	191 ± 14

Values are means ± SE in 8 subjects. L-NMMA, N^G-monomethyl-L-arginine; FNa^+R_{prox}, proximal tubule fractional sodium reabsorption; FNa^+R_{dist}, distal tubule fractional sodium reabsorption.

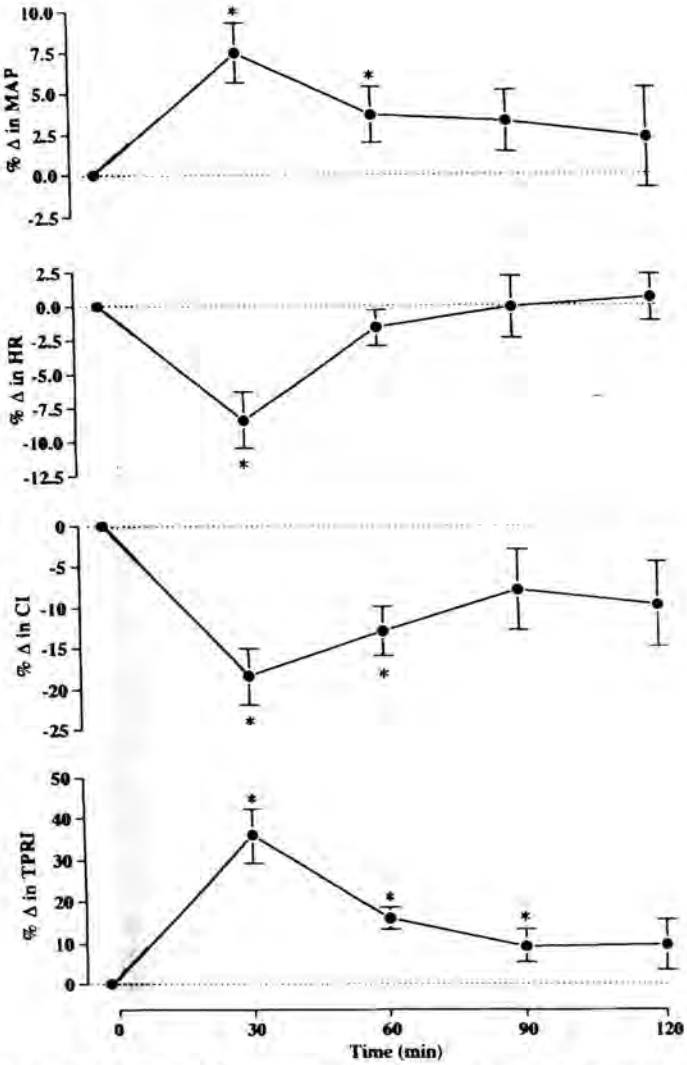

Fig. 1. Effects of N^G-monomethyl-L-arginine (L-NMMA, 3 mg/kg) given intravenously over 5 min (solid area) on mean arterial pressure (MAP), heart rate (HR), cardiac index (CI), and total peripheral resistance index (TPRI). Percentage changes (%Δ) from basal are shown corrected for changes with placebo. Values are means ± SE in 8 subjects. *$P < 0.05$ vs. baseline.

in total peripheral resistance (+36 ± 7%; $P < 0.05$ vs. renal vascular resistance). However, GFR fell significantly ($P < 0.01$), with a decrease of 18 ± 8 ml/min in the first 30 min (Fig. 2). The combination of a decrease in GFR with an unchanged ERPF meant that filtration fraction decreased significantly (Fig. 2).

Tubular function. Infusion of L-NMMA significantly reduced urine flow rate by 2.3 ± 0.8 ml/min at 30 min ($P < 0.05$; Fig. 3). Sodium excretion was significantly reduced by 73 ± 31 μmol/min ($P < 0.05$). The reduction in sodium excretion was not solely due to a decrease in filtered load following the fall on GFR, because fractional sodium excretion also decreased by 0.27 ± 0.07% ($P < 0.05$). Lithium clearance did not change significantly. Proximal tubule fractional sodium reabsorption tended to decrease, but this did not achieve statistical significance ($P = 0.10$). Distal tubule fractional sodium reabsorption increased significantly by 2.2 ± 0.7% at 30 min ($P < 0.05$) (Fig. 4). Potassium clearance did not change significantly after L-NMMA.

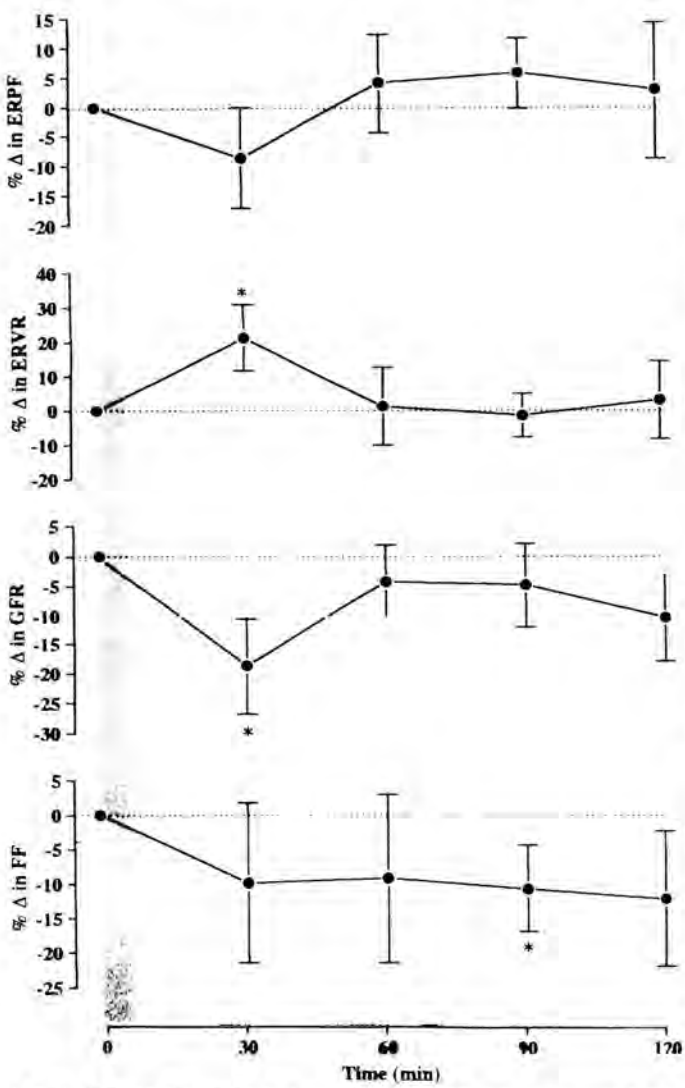

Fig. 2. Effects of L-NMMA (3 mg/kg) given intravenously over 5 min (solid area) on effective renal plasma flow (ERPF), effective renal vascular resistance (ERVR), glomerular filtration rate (GFR), and filtration fraction (FF). Percentage changes from basal are shown corrected for changes with placebo. Values are means ± SE in 8 subjects. *$P < 0.05$ vs. baseline.

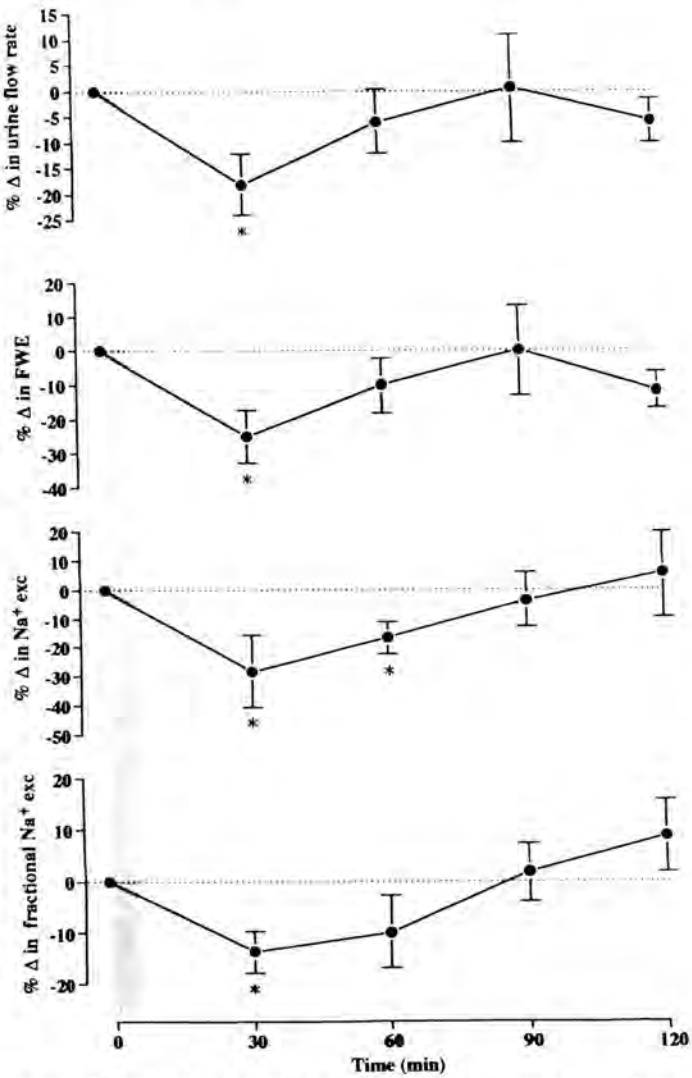

Fig. 3. Effects of L-NMMA (3 mg/kg) given intravenously over 5 min (solid area) on urine flow rate, free water excretion (FWE), Na^+ excretion (exc), and fractional Na^+ excretion. Percentage changes from basal are shown corrected for changes with placebo. Values are means ± SE in 8 subjects. *$P < 0.05$ vs. baseline.

Nitrate, L-NMMA, and hormone assays. Infusion of L-NMMA significantly and progressively reduced plasma nitrate concentrations ($P < 0.01$), with a maximum decrease of 9.8 ± 2.6 μmol/l at 120 min (Fig. 5). Urinary nitrate excretion rate also reduced significantly by 0.38 ± 0.07 μmol/min at 30 min ($P < 0.01$). Unlike plasma nitrate, urine nitrate excretion rapidly returned to baseline after 30 min (Fig. 5). Plasma concentrations of L-NMMA were maximal at 9 μg/ml immediately after the end of the 5 min infusion and declined in a two phase manner to a concentration of ~1 μg/ml after 60 min. L-NMMA plasma clearance and elimination half-life averaged 9.5 ± 0.6 $ml \cdot min^{-1} \cdot kg^{-1}$ and 75 ± 9 min, respectively. Initial and steady-state volumes of distribution were 281 ± 32 and 634 ± 65 ml/kg, respectively. Urine dopamine excretion rate fell significantly after L-NMMA ($P < 0.05$), with a decrease

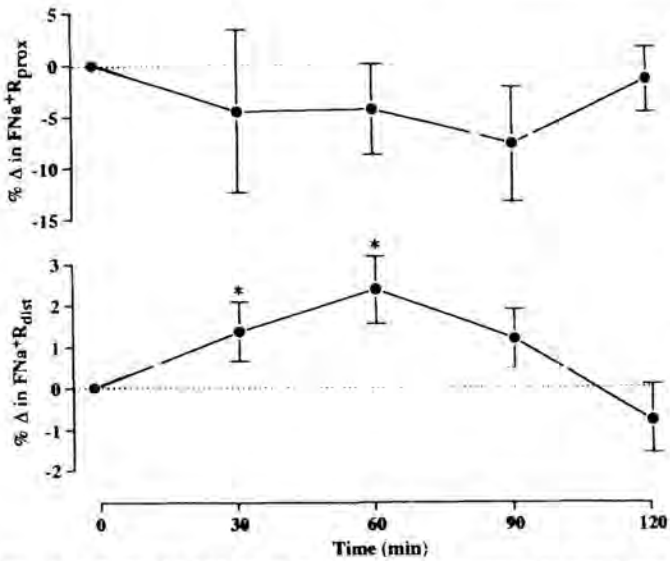

Fig. 4. Effects of L-NMMA (3 mg/kg) given intravenously over 5 min (solid area) on proximal tubule fractional sodium reabsorption (FNa^+R_{prox}) and distal tubule fractional sodium reabsorption (FNa^+R_{dist}). Percentage changes from basal are shown corrected for changes with placebo. Values are means ± SE in 8 subjects. *$P < 0.05$ vs. baseline.

from basal of 0.3 ± 0.06 nmol/min at 30 min (Fig. 5). L-NMMA did not cause significant changes in plasma renin activity (-0.09 ± 0.13 ng·ml^{-1}·h^{-1} at 30 min) or in plasma concentrations of immunoreactive endothelin (+0.63 ± 0.38 pg/ml at 30 min) or aldosterone (+1 ± 29 pmol/l at 30 min).

Platelet numbers and aggregation. There was a tendency for total platelet number to decrease after L-NMMA ($-7 \pm 8 \times 10^9$ cells/l at 30 min), which did not achieve statistical significance ($P = 0.322$). Similarly, there was no significant difference between placebo and L-NMMA study days in the extent of ex vivo platelet aggregation to ADP (Fig. 6).

DISCUSSION

This study confirms previous findings (7, 9, 25) that the nitric oxide synthase inhibitor L-NMMA increases arterial pressure and systemic vascular resistance in healthy human subjects. We report here several important new results. First, despite a marked increase in systemic vascular resistance, renal vascular resistance only increases modestly after L-NMMA. Second, L-NMMA increases tubular sodium and water reabsorption, possibly because it also inhibits urinary dopamine excretion. Third, L-NMMA decreases plasma nitrite/nitrate concentrations and urine nitrite/nitrate excretion. Fourth, we provide the first description of the pharmacokinetics of L-NMMA. L-NMMA does not appear to alter plasma renin activity, plasma endothelin and aldosterone concentrations, or platelet number and ex vivo aggregability to ADP.

Hemodynamic effects. Although it has been suggested that the renal circulation is particularly sensitive to the effects of nitric oxide synthase inhibition (10, 13), we found that L-NMMA had significantly less effect on renal vascular resistance than on total peripheral resistance. L-NMMA did not significantly decrease renal plasma flow, yet substantially reduced GFR and thus filtration fraction. These effects of L-NMMA are consistent with a role for endogenous nitric oxide in control of afferent arteriolar tone in humans, in line with some animal studies (10, 13).

Tubular effects. We found that L-NMMA substantially decreased urine flow rate and sodium and water excretion in human subjects, although arterial pressure was increased. The decrease in urinary sodium excretion differs from the results from our earlier study (9). Because subjects in the earlier study had only one urine collection performed over the whole experimental period (including baseline), baseline differences may have contributed to the apparent increase in sodium excretion in that study. Although some of the decrease in sodium excretion in the current study was due to a reduction in filtered load of sodium, the decrease in fractional sodium excretion implies that nitric oxide had effects on tubular sodium handling. No effect on proximal tubule handling of sodium was apparent, as evidenced by the absence of a change in lithium clearance, but distal tubule fractional sodium reabsorption increased by 2.4% (equivalent to an ~25% decrease in distal tubule sodium excretion). There are several

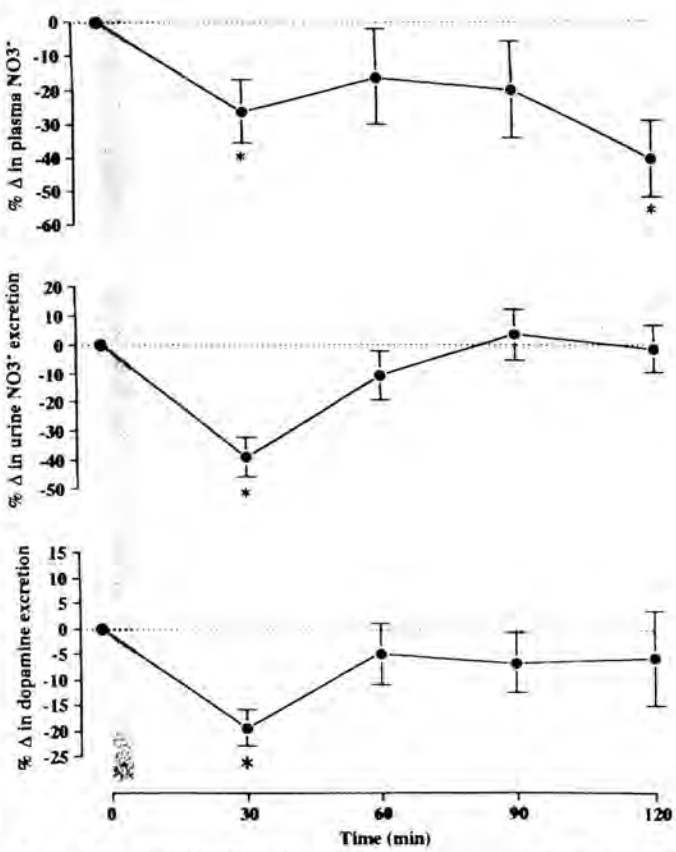

Fig. 5. Effects of L-NMMA (3 mg/kg) given intravenously over 5 min (solid area) on plasma nitrate (NO_3^-) concentrations, urine nitrate excretion rate, and dopamine urinary excretion rate. Percentage changes from basal are shown corrected for changes with placebo. Values are means ± SE in 8 subjects. *$P < 0.05$ vs. baseline.

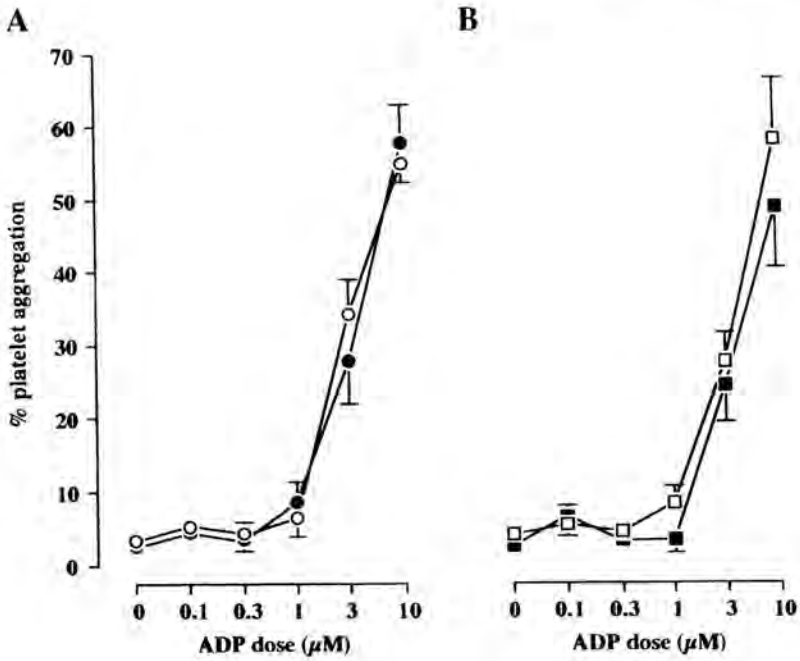

Fig. 6. Dose response for platelet aggregation to ADP before (○) and after (●) placebo (*A*) administration and before (□) and after (■) L-NMMA (*B*) administration.

potential mechanisms for the increase in fractional sodium reabsorption, including changes in renal interstitial pressure (19), medullary blood flow (16), and tubular sodium transport (17).

Plasma nitrate. Administration of L-NMMA caused a progressive decrease in plasma nitrite/nitrate concentrations and a less sustained decrease in urinary nitrite/nitrate excretion rate. The decrease in urinary nitrite/nitrate excretion was not solely due to a decrease in GFR, because urinary nitrate concentrations also decreased. Although human studies have previously shown that nitrate is the predominant metabolite of exogenously administered nitric oxide gas (30) and that labeled arginine is metabolized to labeled nitrate (2), this is the first demonstration that blockade of endogenously produced nitric oxide decreases plasma nitrate in humans. Because nitrate is cleared from plasma with a half-life of ~5 h (2), the magnitude of the decrease in plasma nitrate may not have fully reflected the degree of inhibition of endogenous nitric oxide generation.

Endocrine. The renal effects of L-NMMA may be mediated indirectly through alterations in other neurohumoral systems. Although inhibition of nitric oxide synthesis did not alter circulating endothelin concentrations, endothelin-1 is a locally acting paracrine substance, and plasma endothelin concentrations provide only an approximation of local tissue endothelin-1 generation. Inhibition of nitric oxide generation has been shown to increase plasma endothelin concentrations in anesthetized dogs (20), although not in sheep (27). Renal vasoconstriction induced by L-NMMA would be expected to decrease clearance of endothelin-1 (11). However, this should have increased, rather than decreased, circulating endothelin concentrations and thus make it less likely that overall endothelin-1 generation increased. We found no alteration in plasma renin activity or aldosterone by L-NMMA. In animal studies, inhibition of nitric oxide synthesis has been shown to have contradictory effects on plasma renin activity (21, 23), perhaps reflecting differences in renal perfusion pressures. We were unable to maintain a constant renal perfusion pressure and may therefore have missed an effect of L-NMMA on renin release. Dopamine is generated within the proximal tubule by the action of the enzyme aromatic L amino acid decarboxylase on its substrate L-dopa, which is actively transported into renal epithelial cells (14). We found that L-NMMA significantly decreased urinary dopamine excretion rate. Previous animal data suggest that it is unlikely that nitric oxide acts tonically to promote aromatic-L-amino-acid decarboxylase activity (24). However, nitric oxide could affect the transport of L-dopa into renal epithelial cells. Dopamine increases renal blood flow and GFR, inhibits proximal tubular sodium reabsorption, and blocks the effects of antidiuretic hormone on the collecting duct (14). Thus some of the hemodynamic and tubular effects we observed may have been attributable to withdrawal of endogenous dopamine production.

Platelet effects. Nitric oxide is generated in platelets, and exogenous nitric oxide inhibits platelet aggregability in vitro (18). However, L-NMMA did not alter platelet number or ex vivo aggregation to ADP. This suggests that human endothelial and platelet nitric oxide production is not sufficiently high to influence spontaneous platelet aggregation and aggregability to ADP under physiological conditions. This finding is consistent with previous work showing that brachial artery infusion of L-NMMA does not potentiate local whole blood ex vivo platelet aggregation (28).

Limitations. Several potential limitations should be considered when interpreting this study. First, we did not test higher doses of L-NMMA. We may, therefore, have underestimated the renal and other effects of

endogenously produced nitric oxide. However, previous dose-ranging studies by ourselves suggest that maximal systemic vasoconstriction is achieved at threefold lower doses than we used here (9). In addition, there were substantial reductions in plasma and urine nitrate concentrations after administration of L-NMMA. Even so, our data do not allow us to exclude the possibility that a higher dose of L-NMMA may have had a greater effect on renal function or platelet aggregability. L-NMMA had a significantly greater effect on total peripheral resistance than on renal vascular resistance. Nonetheless, although the renal vasculature may be less sensitive to L-NMMA than other beds, it is still likely that nitric oxide contributes importantly to modulation of renal vascular tone. Second, renal vasoconstriction to L-NMMA is proportional to sodium intake (4), and we did not study subjects at different levels of sodium intake. However, because our subjects had relatively high sodium excretion, sodium status cannot account for the lesser degree of renal vasoconstriction to L-NMMA. Third, L-NMMA induced changes in arterial pressure may have altered renal hemodynamics and tubular function. For example, in animals, low doses of L-NMMA cause sodium retention without changing arterial pressure; higher doses of L-NMMA that elevate arterial pressure cause natriuresis (13). Thus a rise in renal perfusion pressure may induce a pressure natriuresis that opposes a putative direct tubular effect of L-NMMA to increase sodium reabsorption. However, such an effect would tend to have underestimated the increase in sodium reabsorption that we observed after L-NMMA. Fourth, concerns have been raised regarding the validity of lithium clearance as an estimate of proximal nephron sodium excretion in humans, because other renal sites may handle lithium and because lithium may not be inert at certain doses (12). It is therefore possible that lithium reabsorption at more distal sites obscured a proximal tubular effect of L-NMMA. At the low dose we used, pharmacological effects of lithium are unlikely to have confounded our results.

Perspectives. In contrast to some animal studies, inhibition of nitric oxide generation in humans caused only modest renal vasoconstriction, possibly reflecting more rigorous homeostatic control of the renal circulation in humans. Alternatively, it is possible that nitric oxide contributes less to control of vascular tone in renal vessels than in the rest of the peripheral circulation. The relative effects of L-NMMA on renal plasma flow and glomerular filtration rate suggest that endogenous nitric oxide has a greater effect on the afferent than the efferent renal arteriole. Even so, the magnitude of the effect on GFR suggests that endogenously generated nitric oxide may also influence mesangial cell contractile tone, as has been reported in vitro (22). The substantial decrease in fractional distal sodium excretion after L-NMMA suggests an important physiological role for nitric oxide in regulation of distal tubular sodium reabsorption. Given our data suggesting that endogenous nitric oxide stimulates renal dopamine formation, it is possible that some of these tubular actions of nitric oxide are mediated by dopamine. Metabolites of L-arginine, such as L-NMMA, are endogenous circulating substances, and levels are increased in chronic renal failure (29). Plasma concentrations of L-NMMA 60 min after infusion, when total peripheral resistance was still increased, were similar to those of L-arginine metabolites seen in renal failure (29), implying that circulating L-NMMA may have a role in the peripheral vasoconstriction and sodium retention associated with renal impairment.

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The peptide endothelin receptor antagonist, TAK-044, produces sustained inhibition of endothelin-1 mediated arteriolar vasoconstriction

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Aims Endothelin-1 (ET-1) has been implicated in the pathophysiology of a number of cardiovascular diseases for which endothelin receptor antagonists are currently under clinical development. We have previously reported that systemic administration of the combined endothelin A/B receptor antagonist, TAK-044, abolishes the forearm vasoconstriction caused by intrabrachial ET-1 infusion for at least 3 h. In this study we investigated whether TAK-044 can inhibit ET-1 mediated forearm vasoconstriction for longer periods.

Methods Eighteen subjects were recruited to a randomized, placebo-controlled, single-blind, three-way, crossover study. Subjects were divided into three groups of six. Groups received 25 mg, 50 mg or 100 mg TAK-044 on two separate occasions, 6 and 10 h before the start of a 2 h intrabrachial infusion of ET-1 (5 pmol min^{-1}). On a third occasion subjects received only placebo before intra-arterial ET-1 infusion. Forearm vasoconstriction to ET-1 was measured by venous occlusion plethysmography.

Results In the placebo phase, ET-1 caused significant, slowly-progressive local forearm vasoconstriction of ~30% ($P<0.01$) in all three groups. All three doses of TAK-044, administered at both timepoints, tended to blunt the vasoconstriction caused by ET-1. When the responses from all three groups were combined, TAK-044 significantly reduced ET-1 mediated vasoconstriction compared with placebo −9% (95% CI −15 to −3; $P=0.01$) at 8 h and by −9% (95% CI −17 to −2; $P=0.01$) 12 h after dosing.

Conclusions TAK-044 attenuated, but did not abolish, local ET-1 mediated vasoconstriction, for up to 12 h after administration. Vasoconstriction to local intra-arterial administration of ET-1 appears to represent a safe and reproducible pharmacodynamic index of systemic endothelin receptor antagonism in humans.

Keywords: endothelin, endothelin antagonists, pharmacodynamics, forearm blood flow, humans

Introduction

The endothelium derived vasoconstrictor peptide endothelin-1 (ET-1) has been implicated in the pathophysiology of a number of conditions associated with sustained elevation of vascular tone, such as hypertension and congestive cardiac failure, as well as in vasospastic disorders, such as subarachnoid haemorrhage and acute ischaemic renal failure [1]. Two endothelin receptor subtypes, ET_A [2] and ET_B [3], both of which have been identified and cloned in man, can mediate vasoconstriction [4]. Antagonists at these receptors may, therefore, have therapeutic potential in these conditions.

We have previously shown that systemic intravenous administration of the cyclic hexapeptide combined $ET_{A/B}$ receptor antagonist, TAK-044 [5], at doses of 10 to 1000 mg, is well tolerated and produces dose-dependent, long-lasting vasodilatation resulting in decreases of blood pressure and systemic vascular resistance persisting, at the highest dose, for at least 24 h [6]. In a separate study, systemic administration of TAK-044, at doses of 30, 250 and 750 mg abolished vasoconstriction to locally infused ET-1, as a model of ET-1 induced 'vasospasm', for up to 3 h [6].

Endothelin receptor antagonists are currently under clinical development [1]. Peptide antagonists such as TAK-044 requiring intravenous administration are better suited for the treatment of acute vasospastic conditions in which endothelin has been implicated in the pathophysiology. These conditions include subarachnoid haemorrhage, acute ischaemic renal failure and myocardial infarction. In these clinical situations intermittent dosing might have both practical and economic advantages over continuous infusion. We, therefore, studied the potential of TAK-044 to inhibit the forearm vasoconstriction produced by brachial artery infusion of ET-1 for up to 12 h after dosing.

Methods

Subjects

Eighteen healthy male subjects between 19 and 41 years of age participated in these studies, which were conducted

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with the approval of the Lothian Research Ethics Committee and with the written informed consent of each subject. All had normal baseline results on routine biochemical and haematological screening tests. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the 2 weeks before the study or during the study period. All of the subjects abstained from alcohol for 48 h, from caffeine containing drinks and cigarettes for at least 24 h, and from food for 12 h before the start of the ET-1 infusion. All studies were performed in a quiet room maintained at a constant temperature of 24–26° C.

Drugs

TAK-044, cyclo[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl) carbonyl]L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl] disodium salt (Takeda Chemical Industries Ltd, Japan), is a potent hexapeptide endothelin receptor antagonist that inhibits the binding of ET-1 to endothelin receptors on rabbit ventricular (mainly ET_A) and cerebellar membrane fractions (mainly ET_B) with IC_{50} values of 3.8 nM and 130 nM respectively *in vitro* [5]. TAK-044 inhibits, with equal efficacy, both ET_A and ET_B mediated responses *in vivo* [5, 7]. The initial dose of TAK-044 studied, 25 mg, was chosen on the basis that 30 mg TAK-044 completely inhibited ET-1 mediated vasoconstriction for up to 3 h after administration [6]. Depending on whether or not 25 mg TAK-044 abolished the vasoconstriction to ET-1, it was intended to study either lower (10 and 5 mg) or higher (50 and 100 mg) doses of TAK-044 respectively. TAK-044 and 50 mg sucrose placebo were dissolved in physiological saline (0.9%; Baxter Healthcare Ltd, Thetford, UK) and administered as a 50 ml intravenous infusion over 15 min using two Welmed P1000 syringe pumps (Welmed Clinical Care Systems, Bramley, Hampshire, UK) via a right antecubital fossa vein cannulated with an 18G intravenous cannula (Venflon; Viggo-Spectramed, Helsinborg, Sweden). This cannula was not used for blood sampling.

The left brachial artery was cannulated under local anaesthesia (1% lignocaine; Astra Pharmaceuticals, Kings Langley, UK) with a 27 standard wire gauge steel needle (Cooper's Needle Works, Birmingham, UK) attached to a 16G epidural catheter (Portex Ltd, Hythe, Kent, UK). Patency was maintained by infusion of physiological saline via a Welmed P1000 syringe pump. Pharmaceutical grade ET-1 (Clinalfa, Nottingham, UK) dissolved in physiological saline was infused at a rate of 5 pmol min^{-1}. The rate of intra-arterial infusion was maintained constant throughout all studies at 1 ml min^{-1}.

Measurements

Side-effect assessments The following assessments were performed to detect potential adverse effects: 12-lead electrocardiographs, repeated questioning for symptoms, urinalysis, clinical chemistry screen (liver enzymes, electrolytes, creatinine, blood urea, protein), and haematology screen (full blood count, white blood cell differential count).

Systemic haemodynamics A well-validated semi-automated non invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) was used to make duplicate measurements of blood pressure and heart rate in the non-infused arm, which were then averaged [8].

Forearm blood flow Blood flow was measured in both arms by venous occlusion plethysmography [9] using mercury-in-Silastic strain gauges that were securely applied to the widest part of each forearm. The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mmHg. Upper arm cuffs were intermittently inflated to 40 mmHg for 10 s in every 15 s to temporarily prevent venous outflow from the forearm and, thus, obtain plethysmographic recordings. Recordings of forearm blood flow were made every 10 min over 3 min periods. Voltage output from two single channel Hokanson EC 4 strain gauge plethysmographs (DE Hokanson Inc, Bellevue, WA) was transferred to a Macintosh personal computer (Performa 475, Apple Computer Inc, Cupertino, CA) using a MacLab analogue digital converter and Chart software (v. 3.3.3; both from AD Instruments, Castle Hill, NSW, Australia). Calibration was achieved using the internal standard of the Hokanson plethysmography units.

Plasma assays Venous blood samples were taken from a right antecubital fossa vein cannulated with an 18G intravenous cannula attached to a manometer connecting tube (Portex Ltd, Hythe, Kent, UK). All assays were performed as single batches. Plasma endothelin was measured by radioimmunoassay (New England Nuclear Endothelin 1,2 kit) as previously described [10]. The sensitivity of this assay is 2.2 pg ml^{-1} immunoreactive ET. Cross-reactivity of this assay with ET-1, ET-2, ET-3 and big ET-1 is 100%, 53%, 4% and 70% respectively. The normal range for this assay is 12–28 pg ml^{-1}.

TAK-044 was extracted from buffered (Merck 9437; pH 5) plasma by methanol/buffer-preconditioned Bakerbond SPE cartridges and was measured by high performance liquid chromatography (h.p.l.c.). Eluate was evaporated to dryness under nitrogen at 40° C, and the residue reconstituted in 100 μl water. Chromatographic separation was achieved using two Merck LiChrospher columns with Hewlett Packard 1090 h.p.l.c. pumps. The first mobile phase comprised 40% acetonitrile and 60% 6 mmol l^{-1} KH_2PO_4/3 mmol l^{-1} tetrabutylammonium bromide. The second mobile phase comprised 52% acetonitrile/0.5% acetic acid/47.5% water. Detection was achieved by fluorimetry (excitation, 286 nm; emission 348 nm) with Jasco 821 fluorescence detectors. The limit of quantification of this assay, defined as the lowest quantifiable amount of compound at which the loss of precision was ≤15% and the accuracy was ±15%, was determined to be 2.1 ng ml^{-1}.

Study design

Eighteen subjects took part in a randomized, placebo-controlled, single-blind, three-way, crossover study. Subjects were studied on three occasions, each 1 week or more apart. They were admitted to the clinical research centre at 20.00 h on the day before the study and were discharged at

13.00 h on the study day. On two occasions subjects received TAK-044 either at 24.00 h or 04.00 h. Sucrose placebo was substituted for TAK-044 at the other timepoint. On a third occasion subjects received only sucrose placebo at both timepoints. The first group of six subjects (group 1) was studied using 25 mg TAK-044. Because this dose did not appear to abolish ET-1 mediated vasoconstriction, groups 2 and 3 were studied using 50 and 100 mg TAK-044 respectively as previously determined. ET-1 was infused intra-arterially starting at 10.00 h and ending at 12.00 h, the end of the infusion corresponding to either 8 or 12 h after administration of TAK-044. Venous blood samples (10 ml) were obtained at 10.00 h and 12.00 h for assay of plasma endothelin and venous samples (5 ml) were obtained at 08.00 h, 10.00 h and 12.00 h for assay of serum TAK-044. Blood and urine samples were collected for safety assessments before the start of the study and at the end of the third study phase. Electrocardiographs were recorded before dosing, at 09.30 h and before discharge on every study day.

Data presentation and statistical analysis

From previous studies using intrabrachial ET-1 infusions done in our unit (data on file), studying six subjects at each dose would have 95% power to detect a 50% reduction in the vasoconstriction produced by ET-1 at the 5% level. However, studying six subjects would lack the power to detect smaller differences in ET-1 vasoconstriction. Therefore, the *a priori* decision to pool the data from all 18 subjects was taken. Combining the responses from all 18 subjects, would give 90% power of detecting a 25% decrease at the 5% level. Power calculations were done using Graphpad Instat software (GraphPad Software, San Diego, CA, USA).

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 5.0; Microsoft). Recordings from the first 60 s after wrist cuff inflation were not used because of the reflex vasoconstriction this causes [11]. Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm [9]. The analysis of all of the forearm blood flows was performed by one of the investigators (CJF) who was blinded to the study phases.

Data are described as mean ± s.e. mean, with 95% confidence intervals where appropriate. Mean arterial pressure was calculated as diastolic pressure plus one third of the pulse pressure. Haemodynamic data are presented as absolute values and as placebo-corrected changes from baseline (24.00 h). Data were examined by repeated measures analysis of variance (ANOVA) using Stat View 512+ software (Brainpower Inc, Calabasas, CA, USA) for the Apple Macintosh computer. Where the F value obtained by ANOVA was significant ($P<0.05$), the Fisher PLSD (protected least significant difference) multiple comparison test was used to compare pairs of mean values.

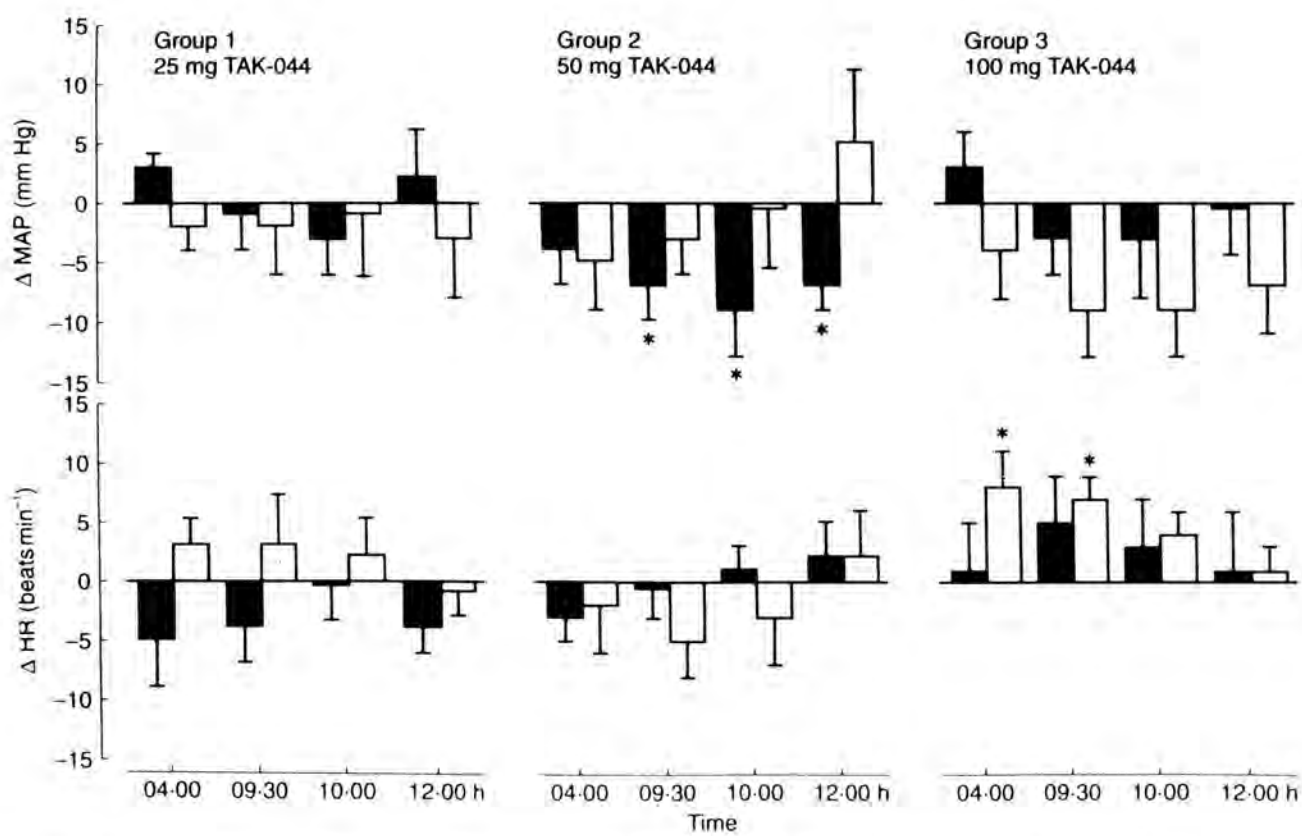

Figure 1 Placebo corrected changes (Δ) in mean arterial pressure (MAP) and heart rate (HR) from baseline (24.00 h) following intravenous administration of TAK-044 at 24.00 h (open bars) and 04.00 h (shaded bars) for group 1, 2 and 3. *$P \leq 0.05$ from baseline

Results

TAK-044 was well tolerated, with no difference between placebo and TAK-044 phases in the prevalence of minor symptoms. There were no serious adverse events during the study, and no clinically significant abnormalities were detected on safety monitoring (urinalysis, haematology, clinical chemistry and electrocardiograph).

Mean arterial pressure showed a tendency to decrease after infusion of TAK-044 (Figure 1). However, this decrease was only significant in group 2 after 50 mg TAK-044 (maximum decrease: -10 ± 4 mm Hg; $P=0.005$). Similar trends were noted for systolic and diastolic pressures. Heart rate (Figure 1) increased only after dosing with 100 mg TAK-044 at 24.00 h (maximum increase: 8 ± 3 beats min^{-1}; $P=0.03$). There were no differences between phases in any of the haemodynamic parameters measured at the start of the intra-brachial ET-1 infusion at 10.00 h (Table 1). Mean arterial pressure and heart rate did not change during infusion of ET-1 (Figure 1). Administration of TAK-044 did not cause an increase in immunoreactive endothelin concentrations after 8 or 12 h compared with placebo (Table 2).

In group 1, TAK-044 plasma concentrations were not higher than the limit of quantification of the assay (2.1 ng ml^{-1}) at any time in any of the subjects. In group 2, TAK-044 was detectable in >50% of subjects only at 08.00 h and only after administration of TAK-044 at 04.00 h.

Table 1 Haemodynamics in groups 1, 2 and 3 before intra-arterial ET-1 infusion.

	Systolic blood pressure (mm Hg)	*Diastolic blood pressure (mm Hg)*	*Heart rate (beats min^{-1})*	*Forearm blood flow (ml min^{-1} dl^{-1})*
Group 1 (TAK-044 25 mg)				
Placebo	109 ± 6 (95 to 123)	62 ± 4 (52 to 72)	51 ± 2 (46 to 55)	2.8 ± 0.3 (2.0 to 3.6)
TAK-044 at 24.00 h	118 ± 6 (103 to 132)	58 ± 3 (51 to 66)	52 ± 1 (49 to 55)	2.8 ± 0.3 (2.0 to 3.5)
TAK-044 at 04.00 h	113 ± 2 (107 to 119)	58 ± 3 (49 to 66)	57 ± 3 (50 to 64)	4.0 ± 0.5 (2.7 to 5.3)
Group 2 (TAK-044 50 mg)				
Placebo	121 ± 6 (104 to 137)	64 ± 3 (55 to 72)	53 ± 4 (44 to 62)	3.5 ± 0.3 (2.7 to 4.3)
TAK-044 at 24.00 h	124 ± 7 (107 to 142)	63 ± 3 (55 to 71)	56 ± 1 (52 to 60)	3.4 ± 0.4 (2.3 to 4.5)
TAK-044 at 04.00 h	115 ± 4 (105 to 125)	57 ± 3 (51 to 63)	56 ± 3 (48 to 64)	4.0 ± 0.4 (3.0 to 5.0)
Group 3 (TAK-044 100 mg)				
Placebo	109 ± 6 (115 to 137)	68 ± 3 (60 to 75)	52 ± 2 (46 to 57)	3.4 ± 0.7 (1.6 to 5.1)
TAK-044 at 24.00 h	118 ± 6 (113 to 133)	59 ± 4 (49 to 69)	54 ± 2 (48 to 61)	3.7 ± 0.7 (2.0 to 5.4)
TAK-044 at 04.00 h	113 ± 3 (115 to 138)	62 ± 2 (56 to 67)	56 ± 3 (48 to 63)	4.0 ± 0.9 (1.7 to 6.3)

There were no significant differences between baseline values on the different study days for any of the three groups. 95% confidence intervals are shown in brackets.

Table 2 Plasma TAK-044 and plasma endothelin concentrations in groups 1, 2 and 3.

	Plasma TAK-044 concentration (ng ml^{-1})			*Plasma ET concentration (pg ml^{-1})*	
	08.00 h	*10.00 h*	*12.00 h*	*10.00 h*	*12.00 h*
Group 1 (TAK-044 25 mg)					
Placebo	<2.1	<2.1	<2.1	12.3 ± 1.1 (9.5 to 15.0)	12.9 ± 0.6 (11.3 to 14.5)
TAK-044 at 24.00 h	<2.1	<2.1	<2.1	16.1 ± 1.3 (12.9 to 19.4)	13.6 ± 0.7 (11.7 to 15.5)
TAK-044 at 04.00 h	<2.1	<2.1	<2.1	13.4 ± 1.0 (10.8 to 16.0)	14.1 ± 0.7 (12.4 to 15.8)
Group 2 (TAK-044 50 mg)					
Placebo	<2.1	<2.1	<2.1	11.6 ± 0.8 (9.5 to 13.7)	12.8 ± 0.5 (11.6 to 13.9)
TAK-044 at 24.00 h	<2.1	<2.1	<2.1	11.7 ± 0.5 (10.3 to 13.1)	13.1 ± 1.0 (10.5 to 15.6)
TAK-044 at 04.00 h	3.9 ± 0.9	<2.1	<2.1	10.9 ± 0.3 (10.3 to 11.6)	13.2 ± 1.1 (10.4 to 16.0)
Group 3 (TAK-044 100 mg)					
Placebo	<2.1	<2.1	<2.1	11.4 ± 0.8 (9.4 to 13.4)	11.6 ± 0.4 (10.7 to 12.6)
TAK-044 at 24.00 h	3.7 ± 1.3	4.5 ± 1.6	<2.1	11.7 ± 0.5 (10.4 to 13.0)	11.9 ± 0.6 (10.4 to 13.5)
TAK-044 at 04.00 h	8.9 ± 2.5	<2.1	<2.1	13.2 ± 0.5 (12.0 to 14.4)	13.7 ± 0.7 (11.9 to 15.4)

Descriptive statistics for plasma TAK-044 concentrations were calculated only for the timepoints where >50% of the subjects showed concentrations >2.1 ng ml^{-1}, the limit of quantification of the assay. There were no significant differences between plasma endothelin concentrations in any of the groups.

In group 3, TAK-044 was detectable at 08.00 h after administration of TAK-044 at 24.00 h and 04.00 . TAK-044 was also detectable in plasma at 10.00 h after administration of TAK-044 at 24.00 h (Table 2). The inter-subject variation for this assay was high at >50%.

There were no differences between any of the phases in absolute forearm blood flows at the start of the intra-brachial ET-1 infusions (Table 1). Blood flow in the non-infused arm did not change significantly during infusion of ET-1 in any of the phases. Brachial artery infusion of ET-1 caused significant slowly-progressive local forearm vasoconstriction. Infusion of ET-1 reduced forearm blood flow over 120 min by 30±5% ($P=0.0001$ *vs* basal), 30±4% ($P=0.0001$) and 28±5% ($P=0.013$) after placebo groups 1, 2 and 3 respectively (Figure 2, Table 3). TAK-044 at all three doses and administered at both timepoints tended to blunt the vasoconstriction caused by ET-1 but these effects failed to achieve statistical significance (Figure 2, Table 3). However, when the responses from all three groups were combined, vasoconstriction to ET-1 was reduced by TAK-044 administered 8 and 12 h previously (Figure 3, Table 3), when compared with placebo.

Discussion

The combined endothelin $ET_{A/B}$ receptor antagonist, TAK-044, given as a 15 min intravenous infusion, attenuated peripheral vasoconstriction to exogenous ET-1 by ~30% for up to 12 h after administration. This inhibition occurred at a time when plasma concentrations of TAK-044 were below the limit of quantification of the assay and plasma concentrations of endothelin were not elevated. These findings have important implications for the clinical development of endothelin receptor antagonists.

A simple and reliable pharmacodynamic index of endothelin receptor blockade would be useful for the clinical development of endothelin receptor antagonists. Forearm vasoconstriction to intra-brachial administration of ET-1 is highly reproducible [4, 6, 12] and this model may be safer than using systemic intravenous infusions of ET-1 to increase blood pressure, given the sustained and potent nature of vasocontriction to ET-1, especially in the coronary, renal and cerebral circulations [1, 13, 14]. It is possible that endothelin receptor antagonism may produce different effects in other blood vessels. However, responses in forearm resistance vessels are generally thought to be broadly representative of those in other vascular beds [9, 15].

The vasoconstriction produced by intra-arterial ET-1 in this study was consistent with other published reports [4, 6, 12]. The results demonstrate that bolus doses of TAK-044 up to 100 mg can still inhibit ET-1 mediated forearm vasoconstriction by ~30% for up to 12 h after administration. However, this contrasts with complete inhibition for up to 3 h of ET-1 mediated vasoconstriction by TAK-044 30 mg [6] and suggests a marked time dependence for this inhibitory action. This was a small study with insufficient power to exclude a dose dependent effect and, therefore, studies with larger doses would be needed to show whether greater inhibition of ET-1 induced vasoconstriction could be achieved. However, the finding that 25 mg TAK-044 seemed to be as effective as 50 and 100 mg

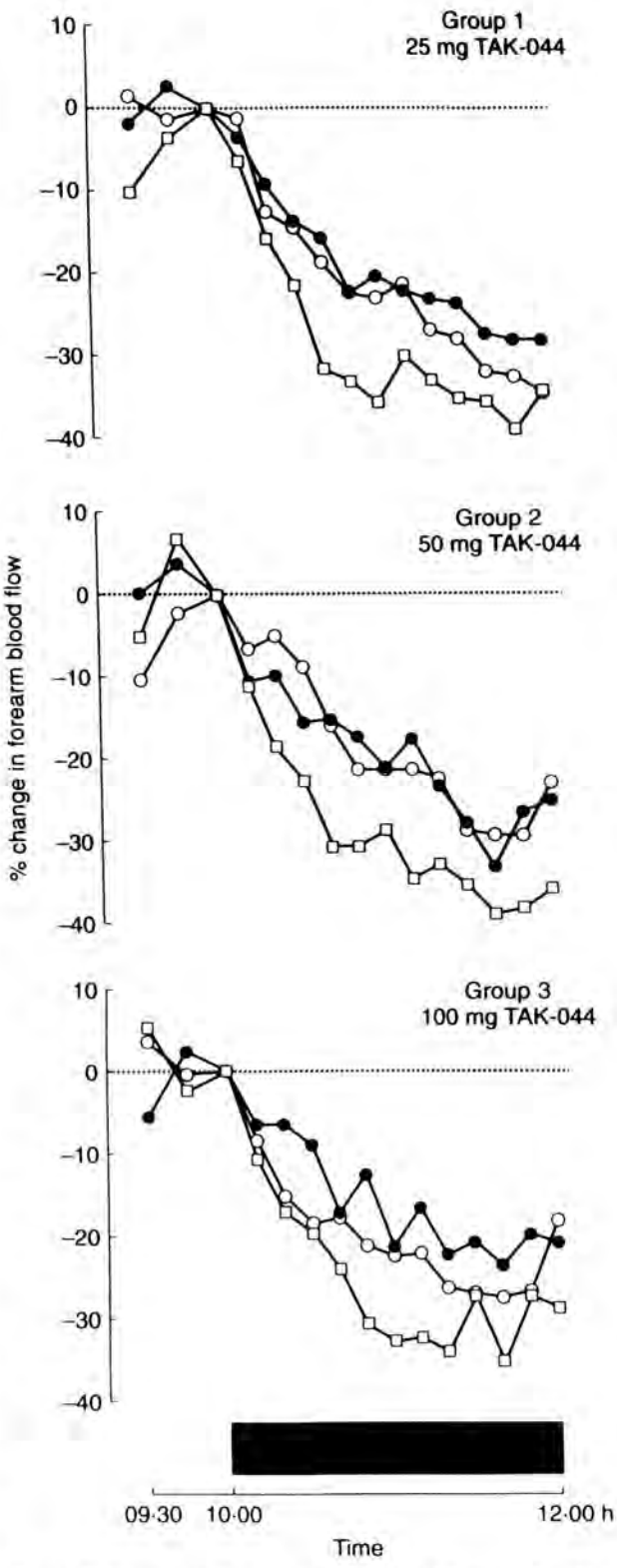

Figure 2 Percentage change in forearm blood flow produced by brachial artery infusion of endothelin-1 (5 pmol min^{-1} for 2 h) following intravenous administration of TAK-044 at 24.00 h (open circles) and at 04.00 h (closed circles) and during the placebo phase (open squares) for groups 1, 2 and 3. Standard errors have been omitted for sake of clarity.

TAK-044 suggests that 25 mg may achieve maximum inhibition at this late stage after systemic administration and that increasing the dose of TAK-044 further might not produce greater inhibition of ET-1 mediated vasoconstric-

Table 3 Mean percentage vasoconstriction to 120 min intra-arterial ET-1 infusion after placebo and TAK-044 8 and 12 h earlier.

	Placebo	*TAK-044 at 24.00 h*	*Difference from placebo*	*TAK-044 at 04.00 h*	*Difference from placebo*
Group 1 ($n=6$) (TAK-044 25 mg)	-30 ± 5	-20 ± 4	-10 (-20 to $+13$)	-22 ± 3	-8 (-22 to $+5$)
Group 2 ($n=6$) (TAK-044 50 mg)	-30 ± 4	-20 ± 3	-11 (-26 to $+5$)	-20 ± 7	-10 (-30 to $+10$)
Group 3 ($n=6$) (TAK-044 100 mg)	-28 ± 4	-21 ± 4	-7 (-22 to $+7$)	-19 ± 3	-9 (-26 to $+6$)
All ($n=18$)	-29 ± 2	-20 ± 2	-9 (-17 to -2)*	-20 ± 3	-9 (-15 to -3)*

*$P=0.01$ 95% confidence intervals for differences for placebo are shown in brackets.

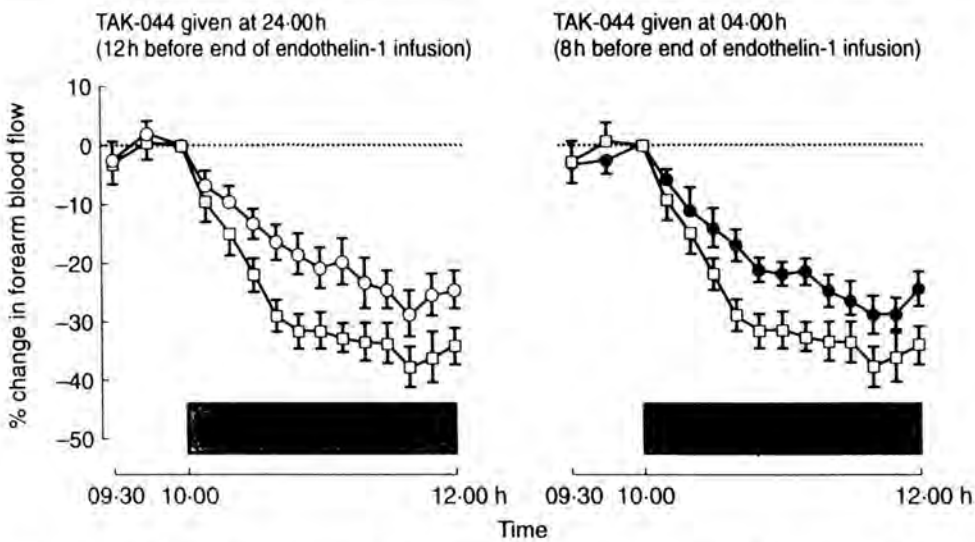

Figure 3 Percentage changes in forearm blood flow for all three groups of subjects combined, following brachial artery infusion of endothelin-1 (5 pmol min^{-1} for 2 h from 10.00 h to 12.00 h) following intravenous administration of placebo (open squares) and TAK-044 at 04.00 h (closed circles) and at 24.00 h (open circles). Endothelin-1 caused a slowly progressive forearm vasoconstriction during the placebo phase. TAK-044 (25–100 mg) administered at either 04.00 h and 24.00 h (corresponding to 8 and 12 h before the end of the endothelin-1 infusion) significantly inhibited this vasoconstriction ($P=0.01$).

tion. Therefore, if an inhibition $>30\%$ is required, more frequent dosing (3 to 6 hourly) or a continuous infusion, may be needed. Clinical trials with endothelin receptor antagonists, including TAK-044, are currently in progress. These should indicate the doses required for clinical effect and the forearm model can then be used to determine effective doses of other endothelin receptor antagonists. The effects of repeated dosing with TAK-044 on ET-1 mediated vasoconstriction are not yet known and it is possible that a cumulative inhibition might be achieved in this manner if the clearance mechanisms for TAK-044 were to become saturated. These issues remain to be addressed.

Although increases in plasma endothelin concentrations have been shown to correlate with some of the haemodynamic changes observed, these associations were relatively weak, with correlation coefficients of ~ 0.2 [6]. Furthermore, changes in circulating endothelin concentrations are likely to reflect only antagonism of the ET_B receptor [6, 16] which, in addition to its functional roles, appears to mediate clearance of circulating ET-1 [17, 18]. For a drug with ET_A and ET_B receptor blocking activity, such as TAK-044, effects on systemic haemodynamics may be apparent at concentrations that do not substantially increase circulating endothelin concentrations, as was the case in our previous study [6]. Similarly, here we found that plasma endothelin concentrations were not raised 12 h after administration of TAK-044 despite continuing inhibition of ET-1 mediated vasoconstriction.

TAK-044 was not detected in any of the volunteers 12 h after administration of any of the doses. These findings are in close agreement with our previously reported study in which the plasma half-life of TAK-044 was 30 to 60 min [6]. However, the IC_{50} for TAK-044 at ET_A receptors is 0.08 ng ml^{-1} [6] ~ 25 fold below the limit of quantification of the assay (2.1 ng ml^{-1}). It is, therefore, possible that circulating TAK-044 remains present in plasma at concentrations sufficient to inhibit the vasoconstriction produced by exogenous ET-1 at 12 h. It is also conceivable that TAK-044 binds tightly to endothelin receptors and remains bound for several hours in a similar manner to ET-1 [19]. Indeed, in intact cells ET-1 appears only to become dissociated from its receptors following receptor internalisation [20]. Thus, prolonged receptor binding may explain the sustained inhibition of ET-1 mediated vasoconstriction by TAK-044. Another possible explanation for the observed inhibition could be the entry of TAK-044 into another

tissue compartment, probably within the vasculature. A similar situation arises with inhibitors of the renin-angiotensin system, where entry into and actions in other tissue compartments appear to explain the dissociation between actions and plasma concentrations observed [21, 22]. These possibilities require further investigation.

Our previous study showed that TAK-044 lowers systemic vascular resistance and blood pressure [6]. The current study was not designed primarily to assess these measures and factors such as diurnal variation of blood pressure, disturbed sleep and the measurement of forearm blood flow may all have interfered with their optimal assessment. Thus, although the study was placebo controlled, small changes in blood pressure and heart rate may have been obscured by these factors. Nevertheless, systolic, diastolic and mean arterial pressure tended to decrease after administration of TAK-044, to a similar extent to that reported previously [6], and heart rate was increased after administration of the highest dose of TAK-044. However, in this study we did not measure systemic vascular resistance, the most sensitive index of peripheral vasodilatation in our previous study [6].

In conclusion, the cyclic hexapeptide, combined $ET_{A/B}$ receptor antagonist, TAK-044, inhibited local ET-1 mediated vasoconstriction by ~30% for up to 12 h after administration. This inhibition occurred at a time when plasma concentrations of TAK-044 were below the limit of quantification of the assay and plasma concentrations of endothelin were not elevated. Therefore, in this study, the most sensitive index of effect of endothelin receptor antagonism was inhibition of ET-1 mediated vasoconstriction. Although TAK-044 is a peptide, these features may be common to non-peptide endothelin receptor antagonists and this study sets up a marker against which other endothelin receptor antagonists can now be compared. The long lasting effects of the short lived peptide, TAK-044, are generally encouraging for the clinical development of endothelin receptor antagonists and emphasise the valuable contribution that the combination of local intra-arterial administration of ET-1 and forearm plethysmography can make to the early clinical evaluation of this novel class of vasoactive drugs.

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Endothelin-A Receptor Antagonist–Mediated Vasodilatation Is Attenuated by Inhibition of Nitric Oxide Synthesis and by Endothelin-B Receptor Blockade

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Background—The role of endothelin (ET)-1 in maintenance of basal vascular tone has been demonstrated by local and systemic vasodilatation to endothelin receptor antagonists in humans. Although the constrictor effects mediated by the vascular smooth muscle ET_A receptors are clear, the contribution from endothelial and vascular smooth muscle ET_B receptors remains to be defined. The present study, in human forearm resistance vessels in vivo, was designed to further investigate the physiological function of ET_A and ET_B receptor subtypes in human blood vessels and determine the mechanism underlying the vasodilatation to the ET_A-selective receptor antagonist BQ-123.

Methods and Results—Two studies were performed, each in groups of eight healthy subjects. Brachial artery infusion of BQ-123 caused significant forearm vasodilatation in both studies. This vasodilatation was reduced by 95% ($P=.006$) with inhibition of the endogenous generation of nitric oxide and by 38% ($P<.001$) with coinfusion of the ET_B receptor antagonist BQ-788. In contrast, inhibition of prostanoid generation did not affect the response to BQ-123. Infusion of BQ-788 alone produced a 20% reduction in forearm blood flow ($P<.001$).

Conclusions—Selective ET_A receptor antagonism causes vasodilatation of human forearm resistance vessels in vivo. This response appears to result in major part from an increase in nitric oxide generation. ET_B receptor antagonism either alone or on a background of ET_A antagonism causes local vasoconstriction, indicating that ET_B receptors in blood vessels respond to ET-1 predominantly by causing vasodilatation. **(*Circulation*. 1998;97:752-756.)**

Key Words: endothelin ■ nitric oxide ■ flow ■ receptors ■ prostaglandins

The endothelin (ET) family of peptides (ET-1, ET-2, ET-3) are generated in a variety of tissues and act primarily as paracrine and autocrine factors. The major isoform in the cardiovascular system, ET-1, is generated in the endothelium from a precursor, big ET-1, through cleavage by a specific endothelin-converting enzyme (ECE). Its actions are mediated by two receptors, the ET_A and the ET_B receptor, which have been characterized and cloned[1,2] and are pharmacologically distinct. The ET_A receptor has a higher affinity for ET-1 (ET-1≫ET-3), whereas the ET_B receptor is nonisopeptide selective (ET-1=ET-3). ET_A receptors are expressed on vascular smooth muscle cells, and their activation by ET-1 leads to vasoconstriction. The physiological importance of endogenous ET-1 in the maintenance of basal vascular tone and blood pressure in humans has been demonstrated by local[3,4] and systemic[4] vasodilatation in response to inhibitors of the endothelin system. An important role for the ET_A receptor in mediating this response is suggested by the substantial forearm vasodilatation to local administration of the selective ET_A receptor antagonist BQ-123[5] in healthy subjects.

Initially, it was thought that ET_B receptors were present only on endothelial cells, where they cause vasodilatation through release of endothelium-derived vasodilators, including nitric oxide (NO) and prostacyclin.[6,7] However, it is now recognized that ET_B receptors are also present on the smooth muscle of human arteries[8] and can mediate vasoconstriction,[9-11] although their contribution to ET-1–mediated constriction in humans remains to be defined.[12] Therefore, although ET_A receptor–mediated vasoconstriction is undisputed, it is unclear whether the balance of the effects of endogenous ET-1 on the endothelial and vascular smooth muscle ET_B receptors results predominantly in a vasodilator or constrictor tone.

In addition to mediating vasodilator effects of endothelial ET_B receptor activation, endothelium-derived dilators can in turn modulate the production and actions of ET-1.[6,13-15] In the short term NO inhibits production of ET-1[13] whereas chronic

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exposure causes upregulation of ET_A receptors.[16] In addition, endothelin receptor antagonists attenuate the pressor response to NO inhibition,[17,18] suggesting that this response may not simply be due to loss of basal NO-mediated dilator tone. These interactions indicate the existence of a complex relationship between the endothelin and NO systems.

As a consequence of its potent vasoconstrictor[19] and growth-promoting properties,[20] ET-1 has also been implicated in the pathophysiology of diseases such as hypertension, heart failure, and renal failure.[21] The recognition of the endothelin system as a new therapeutic target in the treatment of cardiovascular disease has lead to the rapid development of pharmacological agents that inhibit either the production of ET-1 or its actions. Recently, potent intravenous and orally active endothelin receptor antagonists with different pharmacological profiles have become available for clinical studies.[21,22] We are now in a position where it would be valuable to explore the contribution of the ET_B receptor to the vascular effects of ET-1.

The present study, in human forearm resistance vessels in vivo, was designed to further investigate the physiological role of ET_A and ET_B receptor subtypes and their possible interactions in mediating the vasodilator response to selective ET_A receptor antagonism. The first part of the study aimed to investigate whether increased release of the endothelium-dependent relaxant factors NO and prostacyclin contributes to the vasodilator response to selective ET_A receptor antagonism. We therefore compared the response to the selective ET_A receptor antagonist BQ-123 during local inhibition of NO synthase and during systemic inhibition of prostanoid generation with the response to BQ-123 alone. In the second part of the study, to investigate the role of the ET_B receptor in BQ-123–induced vasodilatation, we examined the effects of simultaneous ET_A and ET_B receptor blockade compared with ET_A or ET_B receptor blockade alone.

Methods

Subjects

Twenty-two healthy subjects (1 woman) ranging in age from 20 to 43 years participated in two studies that were performed in the University Hospital Utrecht (study 1) and the University Department of Medicine, Western General Hospital, Edinburgh (study 2), with the approval of the local research ethics committees of each hospital and the written informed consent of each subject. The investigations conformed with the principles outlined in the Declaration of Helsinki. No subjects had received vasoactive medication or nonsteroidal anti-inflammatory drugs within the week before each phase of a study, and all subjects abstained from alcohol for 24 hours and from food, caffeine-containing drinks, and tobacco for at least 4 hours before any measurements were made. All studies were performed in a quiet room maintained at a controlled temperature between 22°C and 24.5°C.

Drug Administration

The brachial artery of the nondominant arm was cannulated with a 22 (study 1) or 27 SWG cannula (study 2) under lidocaine local anesthesia (lidocaine 2%, Astra Pharmaceuticals Ltd). Drugs, with the exception of aspirin, were dissolved in physiological saline (0.9%, Baxter Healthcare Ltd) and infused intra-arterially at locally active doses. The infusion rate was kept constant at 80 mL/h (study 1) or 60 mL/h (study 2). All solutions were prepared aseptically from sterile stock solutions or ampules on the day of the study.

Drugs

BQ-123 (100 nmol/min, study 1; 10 nmol/min, study 2), was used as a selective ET_A receptor antagonist (study 1: American Peptide Co; study 2: Clinalfa AG). We have demonstrated previously local forearm vasodilatation to intra-arterial infusion of BQ-123 (100 nmol/min).[3] In study 2, we used a 10-fold lower dose of BQ-123 (10 nmol/min) because more recent studies have shown that this causes vasodilatation of equal magnitude to that seen with the higher dose.[23] BQ-788 (1 nmol/min) was used as a selective ET_B receptor antagonist[24] (American Peptide Co). This dose has been shown to completely inhibit venoconstriction to the selective ET_B receptor agonist sarafotoxin S6c.[25]

The endogenous NO system in the forearm was inhibited by use of an "NO clamp," as described previously.[26] The NO synthase inhibitor L-N^G monomethyl-arginine (L-NMMA; Institut fur Pharmazie, Universitat Leipzig) was continuously infused at a rate of 200 μg/100 mL forearm volume per minute to achieve maximal inhibition of local NO synthase.[27–29] Sodium nitroprusside (SNP), an exogenous NO donor (Merck) was then coinfused at titrated doses (12 to 30 ng/min). After 8 minutes of L-NMMA infusion, when steady state forearm blood flow was obtained, SNP was coinfused in incremental doses and titrated until baseline forearm blood flow had been restored. L-NMMA and SNP were then coinfused, at these rates, for the remainder of the study. This allowed simulation of normal basal NO activity during continuous inhibition of endogenous NO synthesis.

Aspirin (600 mg calcium acetylsalicylic acid; Carbasalatum Calcium, Dagra Pharma BV) was administered orally 30 minutes before measurements in one phase of study 1. Aspirin irreversibly inhibits cyclooxygenase (EC 1.14.99.1), which is responsible for the production of prostaglandins and thromboxanes. When given at a dose of 600 mg, aspirin inhibits bradykinin-stimulated endothelial production of prostacylin by at least 85% with recovery occurring over the next 6 hours.[30]

Measurements

Forearm Blood Flow

Forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography using calibrated mercury-in-Silastic strain-gauges applied to the widest part of the forearm.[3,27,31] The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mm Hg. Upper arm cuffs were intermittently inflated to 40 mm Hg for 10 seconds every 15 seconds to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Recordings of forearm blood flow were made over 2.5-minute periods at 5-minute intervals (study 1) and over 3-minute periods at 10-minute intervals (study 2). Venous occlusion plethysmography was performed using a dual-channel strain-gauge plethysmograph (Hokanson), and calibration was achieved using the internal standard of the Hokanson plethysmography unit. In study 1, a microcomputer-based R-wave–triggered system for online semicontinuous monitoring was used,[32] whereas in study 2, voltage output was transferred to a Macintosh personal computer (Classic II; Apple Computer) using a MacLab analog-digital converter and Chart software (version 3.2.8; both from AD Instruments).

Blood Pressure

Blood pressure was monitored during each study using either continuous intra-arterial measurements in the infused arm (study 1) or a semiautomated noninvasive oscillometric method in the noninfused arm (study 2).[33] Blood pressure in study 2 was measured immediately after each forearm blood flow measurement, thereby avoiding any effect of venous congestion caused by this procedure on blood flow.

General Study Design

Subjects rested recumbent throughout each study with both forearms resting slightly above the level of the heart. Strain gauges and arm cuffs were applied, and the left brachial artery cannula was sited. Before the administration of drugs, saline was infused for at least 30 minutes,

Baseline Hemodynamic Values During Saline Infusion Before Infusion of Drugs

Parameter	Study 1			Study 2		
	BQ-123 (100 nmol/min)	BQ-123+ NO-Clamp	BQ-123+ Aspirin	BQ-123 (10 nmol/min)	BQ-123+ BQ-788	BQ-788 (1 nmol/min)
Forearm blood flow (mL/100 mL per minute)						
Infused arm	3.7±0.4	3.7±0.3	4.1±0.5	3.6±0.6	4.5±1.0	4.5±0.7
Control arm	4.6±0.5	3.4±0.2	3.4±0.5	3.4±0.6	3.3±0.8	3.1±0.4
Mean arterial pressure, mm Hg	78±2	82±3	79±2	83±5	90±3	93±4

Forearm blood flow in infused and control arm and mean arterial pressure in each phase of studies 1 and 2 at baseline before infusion of, respectively, BQ-123; BQ-123 SNP, and L-NMMA; BQ-123; BQ-123; BQ-123 and BQ-788; and BQ-788. There were no significant differences in baseline forearm blood flow or mean arterial pressure between the phases of each study.

n=8 in each phase of studies 1 and 2.

during which baseline measurements of forearm blood flow were made.

Study 1: Inhibition of NO Synthase and Prostanoid Generation With ET_A Receptor Blockade

Eight subjects were studied on three separate occasions, each separated by at least 1 week. After baseline infusion of saline, BQ-123 was infused for 90 minutes: on one occasion, during saline coinfusion; on another, after stabilization of the NO-clamp; and on another, after systemic inhibition of prostanoid generation. The effects of the NO-clamp on forearm blood flow were studied during a 2-hour period in 3 subjects (time control NO-clamp).

Study 2: Separate and Combined Blockade of ET_A and ET_B Receptors

On 2 separate study days, in 8 subjects, the ET_A receptor antagonist BQ-123 was infused for 120 minutes alone or during coinfusion of BQ-788, also for 120 minutes. On a separate occasion, BQ-788 was infused alone for 120 minutes in 8 subjects (2 of whom also participated in the earlier parts of study 2).

Analysis

Blood flow in both forearms was obtained from the mean of the last five consecutive recordings of each measurement period. Because wrist cuff inflation results in a transient forearm vasoconstriction, recordings made in the first 60 seconds after wrist cuff inflation were not used for analysis. The ratio of flows in the infused and noninfused arms was calculated for each time point and expressed as percentage change from baseline or, in the NO-clamp experiments, as percentage change from the average of the last four recordings during NO-clamping, before the administration of BQ-123. In both studies, plethysmographic data listings were extracted from data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 5.0; Microsoft). All results are expressed as mean±SEM. Data were examined by repeated measures ANOVA (study 1, SigmaStat; Jandel Corp; study 2, Excel 5.0; Microsoft). Statistical significance was taken at the 5% level.

Results

There were no significant changes in baseline hemodynamics between phases of each study (Table) and no change in blood pressure or blood flow in the noninfused forearm during the course of the studies.

Study 1

Baseline forearm blood flow was restored during the NO-clamp (basal infused forearm blood flow; 3.7±0.3; during basal NO clamp; 3.4±0.2; P= 15) and kept stable for at least 40 minutes before BQ-123 infusion was started. Blood flow in the infused forearm in the time control NO clamp protocol varied by <5% between baseline (pre–NO clamp) and with 120 minutes of NO clamping in 3 subjects.

BQ-123 caused progressive vasodilatation during coinfusion of saline and after inhibition of prostanoid generation (P<.01 for both). The response appeared to plateau at 60 minutes, and no differences were observed in these responses (38±9% versus 42±7% at 90 minutes; P=.5). The vasodilator response to BQ-123 was markedly reduced during NO-clamping (2±5% at 90 minutes, P=.006 versus saline coinfusion) (Fig 1).

Study 2

Both BQ-123 alone and coadministration of BQ-123 and BQ-788 caused progressive vasodilatation (P<.001) that appeared to plateau at 60 minutes (Fig 2). The vasodilatation to BQ-123 alone was significantly greater than that during coinfusion with BQ-788 (76±13% versus 47±14% at 120 minutes, P<.001). BQ-788 alone caused a small but consistent reduction in forearm blood flow (20±3% at 120 minutes, P<.001) (Fig 2).

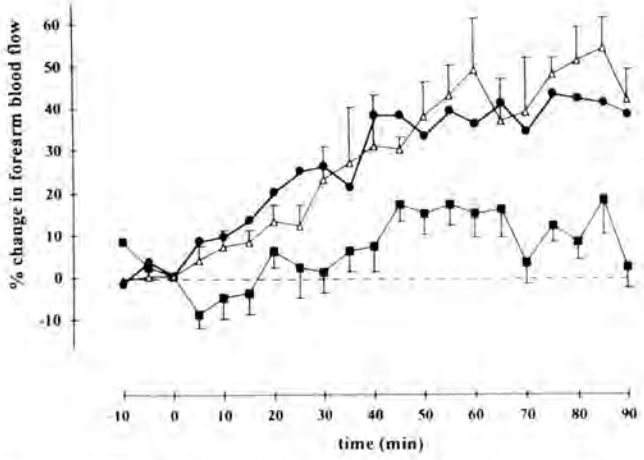

Figure 1. Eight subjects received brachial artery infusion of BQ-123 (100 nmol/min) during coinfusion of saline (●), BQ-123 (100 nmol/min) during inhibition of prostanoid generation (△), or BQ-123 (100 nmol/min) during inhibition of NO generation (■). Slow-onset vasodilatation occurred in response to BQ-123; this response was attenuated during NO clamp but not during inhibition of prostanoid generation.

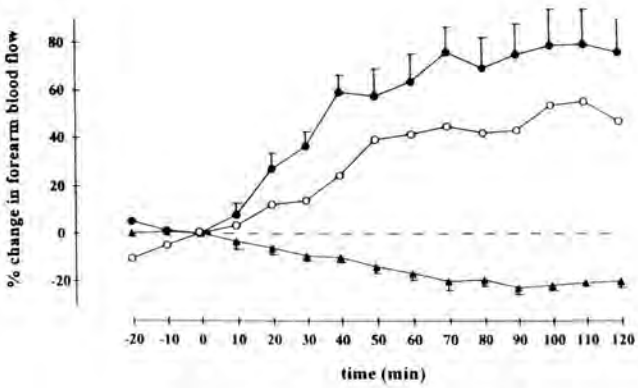

Figure 2. Eight subjects received brachial artery infusion of BQ-123 (10 nmol/min) alone (●), BQ-788 (1 nmol/min) alone (▲), or BQ-123 (10 nmol/min) coinfused with BQ-788 (1 nmol/min) (○). Slow-onset vasodilatation occurred in response to BQ-123; this response was attenuated during coinfusion of BQ-788. BQ-788 infusion alone caused a small but significant vasoconstriction.

Discussion

In two centers, we have demonstrated slow-onset forearm vasodilatation in response to local arterial infusion of the selective ET_A receptor antagonist BQ-123, confirming the importance of endogenous ET-1 in the mediation of vascular tone. From these data, it appears that this vasodilator response is caused in large part by increased generation of NO, which could be mediated by stimulation of the endothelial ET_B receptor. Indeed, our observation that the vasodilator response to combined ET_A and ET_B receptor antagonism was significantly less than that to selective ET_A receptor antagonism alone probably reflects the presence of an endogenous ET_B-mediated vasodilator tone. This is further supported by the local vasoconstrictor effect of ET_B receptor antagonism in the forearm resistance vessels.

In the present study, to exclude the influence of the endogenous NO system in mediation or modulation of the effects of ET-1, L-NMMA was infused to inhibit endogenous local generation of NO. SNP was coinfused with L-NMMA to restore baseline blood flow[35] because local inhibition of NO would otherwise result in vasoconstriction. In this situation, endogenous NO is replaced with exogenous NO, in effect applying a clamp to the local endogenous NO system. Using this technique we have shown, for the first time in humans in vivo, that the vasodilatation to BQ-123 is in large part related to NO generation. Inhibition of endogenous prostanoid generation by oral administration of aspirin has no effect on basal forearm blood flow or systemic hemodynamics and, more importantly, had no effect on the response to BQ-123, indicating that the dilator prostanoids do not provide an important contribution to the vasodilator response to BQ-123. Almost all of the response to BQ-123 appeared to be blocked by NO clamping. However, on the basis of vasodilatation to the ECE inhibitor phosphoramidon[34] in previous studies,[3] we think it is likely that at least part of the response to BQ-123 is directly due to withdrawal of endogenous ET_A-mediated vasoconstriction.

Selective ET_A antagonism inhibits the actions of ET-1 at the ET_A receptor while allowing its actions at the ET_B receptor to be unopposed. ET-1 can stimulate both the endothelial ET_B receptor to cause dilatation and the vascular smooth muscle ET_B receptor to cause vasoconstriction. Therefore, the overall effect depends on a balance between these two actions. Unfortunately, there are no available pharmacological tools that have been shown clearly to distinguish between the endothelial and vascular smooth muscle ET_B receptors. We have shown that coinfusion of the ET_B receptor antagonist BQ-788 reduces the vasodilator response to BQ-123, suggesting that the balance of effects of ET-1 favors vasodilatation via the endothelial ET_B receptor. This is further supported by the vasoconstriction in these vessels to BQ-788 alone and by the lesser degree of vasodilatation to the combined ET_A/ET_B endothelin receptor antagonist TAK-044[4] than to the ET_A-selective agent BQ-123.[3] It is possible that the predominant effects of intra-luminal infusion of BQ-788 selectively affect the endothelial ET_B receptor because the drug has better access to the endothelial than to the smooth muscle receptors. However, we believe this is unlikely because ET-1 and BQ-123 find ready access to the smooth muscle. The response to BQ-788 may indicate either displacement of ET-1 from, or failure of clearance of ET-1 by, ET_B receptors.[35] However, our present results cannot distinguish between these effects.

The observation that selective ET_A receptor blockade not only antagonizes direct ET_A receptor–mediated constriction but also preserves beneficial ET_B receptor–mediated vasodilator tone and enhances endogenous NO generation may have important implications in the use of endothelin antagonists as treatments in cardiovascular disease. For example, the increased NO generation caused by ET_A receptor antagonists is potentially beneficial in ischemic heart disease. However, the clinical relevance of our findings in various pathophysiological conditions cannot be fully determined from the present study because endothelin receptors may be modified under these circumstances. Indeed, in ischemic heart disease, there appears to be upregulation of human coronary ET_B receptors,[36] and this is associated, in heart failure, with enhanced vasoconstrictor responses to sarafotoxin S6c in both the forearm[37] and coronary circulation,[38] whereas the response to BQ-788 appeared similar to that of controls.[39] Clearly, at some stage, it will be necessary to examine the integrated physiology of systemic ET_A and ET_B blockade in physiological and pathophysiological conditions to fully understand the relative importance of the receptor subtypes.

In summary, we have demonstrated that the local vasodilator response to selective ET_A receptor antagonism in human forearm resistance vessels is derived in large part from increased NO-mediated vasodilatation, most probably mediated by the endothelial ET_B receptor. Although our observations were made in the forearm resistance vessels, these vessels are generally representative of other vascular beds[40,41] and, importantly, reflect the interaction of these systems in vivo. Our results may indicate new therapeutic uses for ET_A receptor antagonists because increased NO synthesis may be a desirable effect in, for example, ischemic heart disease. One could also postulate that enhanced endogenous NO generation may be responsible for the headaches that are a recognized side effect of ET receptor antagonists.

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Systemic Blockade of the Endothelin-B Receptor Increases Peripheral Vascular Resistance in Healthy Men

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Abstract—Endothelin-1 (ET-1) is an important mediator of vascular tone in humans, and a number of endothelin receptor antagonists are currently in clinical development as vasodilator agents. While the vasoconstrictor role of the ET_A receptor is undisputed, the role of the ET_B receptor remains unclear. Hemodynamic effects of systemic doses of the ET_B-selective antagonist BQ-788 were investigated in 5 healthy male volunteers (age range, 33 to 48 years) in a placebo-controlled, four-way crossover study. After a 15-minute infusion of BQ-788 (3, 30, or 300 nmol/min) or placebo, plasma ET-1 and big ET-1, blood pressure, heart rate, cardiac index, and stroke index were measured. Total peripheral vascular resistance was calculated from cardiac index and mean arterial pressure. Hemodynamic data are expressed as maximum, placebo-corrected, percentage change from baseline following BQ-788 (300 nmol/min) and were examined by ANOVA. Plasma ET-1 increased by 3.7±1.2 pg/mL (maximum at 15 minutes, $P=0.02$), whereas there was no significant change in plasma big ET-1. Although BQ-788 had no effect on mean arterial pressure, there was a reduction in heart rate (13±7% at 50 minutes; $P=0.002$), cardiac index (17±5% at 40 minutes; $P<0.0001$), and stroke index (8±4% at 40 minutes; $P=0.002$) and an increase in total peripheral vascular resistance (24±5% at 40 minutes; $P<0.0001$). The selective ET_B receptor antagonist BQ-788 causes peripheral vasoconstriction in healthy volunteers, suggesting that the overall balance of effects of endogenous ET-1 at the vascular ET_B receptor favors vasodilatation. Further investigation is now clearly required to address whether selective ET_A or combined ET_A/ET_B receptor blockade will be more effective in the clinical setting. (***Hypertension*. 1999;33[part II]:581-585.**)

Key Words: endothelin ■ vasoconstriction ■ blood pressure ■ receptors, endothelin ■ endothelin receptor antagonist

The importance of endothelin-1 (ET-1) as a mediator of basal vascular tone in vivo in humans has been demonstrated by local[1-3] and systemic[2] vasodilatation in response to endothelin receptor antagonism. The potent vasoconstrictor effects of ET-1,[4,5] combined with the increased plasma concentrations of ET-1 associated with cardiovascular diseases, including heart failure[6] and renal failure,[7] provide strong evidence to support a functional role for ET-1 in the development and maintenance of the increased peripheral vascular resistance associated with these conditions.

The vascular effects of ET-1 are mediated by two distinct receptors: the ET-1–selective ET_A receptor[8] and the nonisopeptide-selective ET_B receptor.[9] The sustained vasoconstrictor effects of ET-1 are predominantly mediated by the ET_A receptor, although vascular smooth muscle ET_B receptors have also been described[10] and may, under some circumstances, contribute to ET-1–mediated vasoconstriction in animal models[11] and humans in vivo.[12] ET_B receptors were first described on endothelial cells, where they act to modulate the vasoconstrictor effects of ET-1 through generation of nitric oxide[13] and prostacyclin.[14] The ET_B receptor also has a role in the clearance of ET-1 from the circulation,[15] although the exact site of the clearance receptor remains to be confirmed. The contribution of the vascular ET_B receptor to the recognized endogenous ET-1–mediated constrictor tone depends on the balance between the ET_B receptor–mediated effects of vasodilatation, vasoconstriction, and ET-1 clearance.

Local vasoconstriction to ET_B receptor agonists has been described in healthy volunteers[12,16] and in patients with heart failure.[17] However, more recently, vasoconstriction after local administration of the selective ET_B receptor antagonist BQ-788[18] has been described in healthy volunteers[3] and in patients with heart failure.[19] The results with antagonists are particularly important as they indicate that the endogenous effect of vascular ET_B receptor stimulation in vivo favors vasodilatation. Indeed, hypertension has been described after administration of systemic doses of the selective ET_B receptor antagonists A192621 in rats and BQ-788 in rabbits in vivo, as well as in rescued ET_B knockout mice.[20,21] The vasoconstrictor effects of ET_B antagonism may result directly from blockade of an endothelial ET_B receptor–mediated dilator

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tone or indirectly from displacement of endogenously generated ET-1 to vasoconstrictor ET_A receptors, or as a result of reduced clearance of ET-1 by vascular ET_B receptors. Confirmation of the balance of the vascular effects mediated by the ET_B receptor in different circumstances is important in understanding the physiology of the endothelin system and in determining whether selective ET_A receptor antagonists or combined ET_A/ET_B receptor antagonists are likely to be more effective vasodilator agents in the clinical setting. Although both selective and nonselective endothelin receptor antagonists have demonstrated vasodilator effects in healthy subjects,[1,2] in patients with heart failure[22,23] and in patients with hypertension,[24,25] the question of whether selective ET_A or combined ET_A/ET_B receptor antagonism will be of more benefit as vasodilator therapy remains to be clarified.

As a first step in understanding the contribution of the ET_B receptor to the maintenance of vascular tone in vivo, we investigated the systemic hemodynamic effects of BQ-788 in healthy male volunteers.

Methods

Subjects

Five healthy male subjects between 18 and 50 years of age were recruited to the study, which was performed in the Clinical Research Center at the Western General Hospital, Edinburgh, with the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed with the principles outlined in the Declaration of Helsinki. No subject received vasoactive medication or nonsteroidal anti-inflammatory drugs in the week before each phase of a study, and all subjects abstained from alcohol for 24 hours and from food, caffeine-containing drinks, and tobacco for at least 4 hours before any measurements were made. All studies were performed in a quiet room kept at a controlled temperature between 22°C to 24°C.

Drugs

BQ-788 (Clinalfa AG) was used as a selective ET_B receptor antagonist on the basis of both a 1000-fold selectivity of BQ-788 for the ET_B receptor, in the nanomolar range, in human cell lines[18] and inhibition of ET-3 binding to recombinant human ET_B receptors expressed in Chinese hamster ovary cells, also in the nanomolar range.[26] The dose range (3 to 300 nmol/min) used in the current study was selected from previous work investigating the local effects of BQ-788 in the forearm circulation[3] and from a dose ranging pilot study in which 2 volunteers were studied at each dose level (data not shown). Selected doses (1 to 300 nmol/min) were administered in the pilot study to identify a no-effect dose and select an appropriate maximum dose for the main study.

BQ-788 was dissolved in physiological saline (0.9%, Baxter Healthcare, Ltd). Saline (0.9%, Baxter Healthcare, Ltd) was administered as placebo. BQ-788 and placebo were administered in a single-blind manner and infused intravenously at a constant rate for 15 minutes via an 18 standard wire gauge (SWG) cannula sited in the left antecubital vein. All solutions were prepared from sterile stock solutions on the day of the study.

Measurements

Plasma ET-1 and Big ET-1

Blood samples were obtained before dose and at 5, 15, 60, and 240 minutes after dose via an 18 SWG cannula sited in the noninfused arm. In brief, 10-mL samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Becton Dickinson Vacutainer Systems), centrifuged immediately at 2000*g* for 20 minutes, and stored in plain tubes at −80°C before assay. ET-1 and big ET-1 (Peninsula Laboratories Europe) were determined by standard radioimmunoassay, as previously described.[27,28]

Blood samples were also taken on admission and before discharge for routine biochemistry and hematology blood tests (sodium, potassium, creatinine, urea, alkaline phosphatase, γ-glutamyl transpeptidase, hemoglobin, and white cell count).

Hemodynamic Recordings

Hemodynamic recordings were made at 10-minute intervals from 30 minutes before dose until 1 hour after the start of the infusion, with an additional blood pressure measurement at 15 minutes corresponding with the end of the infusion. Recordings were again made at 30-minute intervals until 2 hours and hourly until 4 hours after the start of the infusion.

Blood pressure and heart rate (HR) were recorded in duplicate at each time point using a semiautomated noninvasive oscillometric method in the noninfused arm (Takeda UA 751 sphygmomanometer, Takeda Medical Inc)[29]; values were averaged for each time point. Blood pressure is presented as mean arterial pressure (MAP; diastolic blood pressure + 1/3 pulse pressure, in millimeters of mercury).

Cardiac output and stroke volume were recorded by a well-validated noninvasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd).[30] These parameters were corrected for body surface area and described as cardiac index (CI, liters per minute per meters squared) and stroke index (SI, milliliters per meter squared).[2] Total peripheral vascular resistance index (TPVRI) was calculated as MAP divided by CI and expressed in arbitrary units (AU).

Study Design

Responses to BQ-788 (3, 30, and 300 nmol/min) and placebo were investigated in a placebo-controlled, four-way crossover study. Study drugs were administered in a single-blind manner. The order of treatments was randomized. Five subjects attended for 4 separate study visits, each separated by at least 5 days. Subjects were resident in the research center for at least 6 hours. Subjects rested supine for at least 20 minutes before hemodynamic measures, and baseline measures were made in the 30 minutes before study drug administration.

Analysis

Plasma ET-1 and big ET-1 are represented as absolute change from predose (picograms per milliliter), with statistical significance assessed by paired *t* test. Hemodynamic results are expressed as maximum placebo-corrected percentage changes from baseline ±SEM.[2] Statistical analysis was performed on untransformed data. Responses were examined by repeated-measures ANOVA. Statistical significance was taken at the 5% level, and analysis was performed using an Excel data analysis package (Excel 5.0, Microsoft Ltd).

Results

All 5 healthy male subjects (age range, 33 to 48 years) completed the study. No adverse events were reported, and there were no clinically relevant changes in routine biochemistry and hematology blood tests.

Plasma ET-1 and Big ET-1

Predose plasma ET-1 concentrations did not differ significantly for any of the treatments (range of baseline mean values, 4.4 to 4.9 pg/mL). Plasma ET-1 concentration increased significantly after administration of BQ-788 (from 4.6±0.8 to 8.4±1.8 pg/mL at 15 minutes with 300 nmol/min, $P=0.02$) but not during treatment with the lower doses of BQ-788 or placebo (Figure 1). In contrast, concentrations of big ET-1 did not change significantly with treatment.

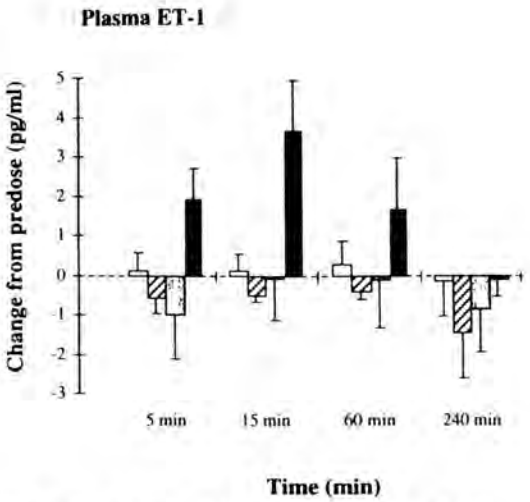

Figure 1. Change in plasma ET-1 concentrations after 15-minute intravenous infusion of BQ-788 or saline placebo in 5 subjects. Solid columns indicate BQ-788 at 300 nmol/min; shaded columns, BQ-788 at 30 nmol/min; hatched columns, BQ-788 at 3 nmol/min; and open columns, placebo. Plasma ET-1 concentrations increased significantly after administration of BQ-788 at 300 nmol/min.

Hemodynamic Parameters

Baseline measurements for hemodynamic parameters during the placebo treatment period were as follows: MAP, 79±3 mm Hg; HR, 79±3 bpm; CI, 2.6±0.2 (L/min)/m^2; SI, 49±3 mL/m^2; and TPVRI, 31.1±1.8 AU. Baseline values were similar for each of the other treatment periods. MAP did not alter significantly after administration of BQ-788 at any dose (3±2% at 90 minutes with 300 nmol/min; P=0.4) (Figure 2). After administration of BQ-788, there were changes in all other hemodynamic parameters when compared with placebo that appeared to be dose-related and that were significant at 300 nmol/min; HR decreased (13±7% at 50 minutes after dose; P=0.002), CI decreased (17±5% at 40 minutes; P<0.0001), and there was a small reduction in SI (8±4% at 40 minutes; P=0.002). TPVRI increased (24±5% at 40 minutes; P<0.0001).

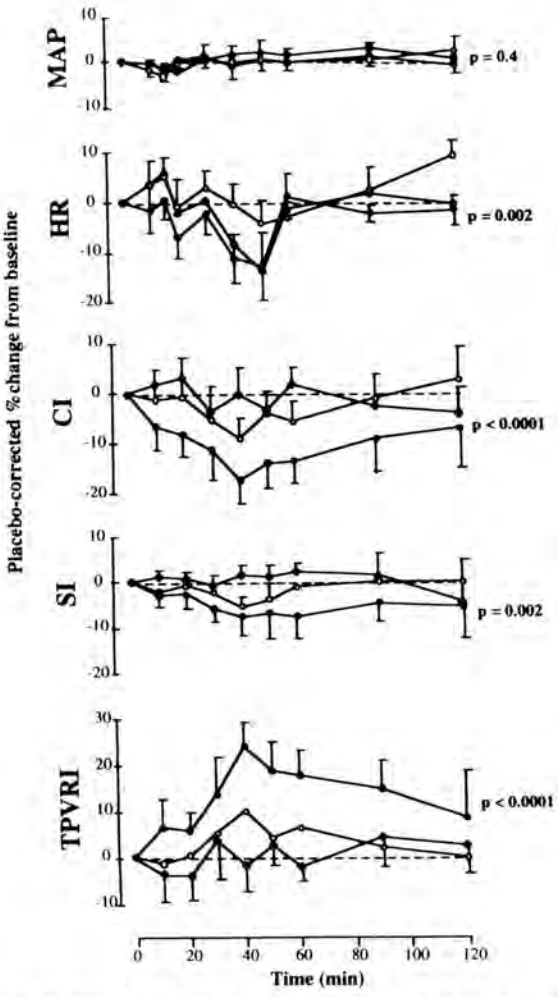

Figure 2. Placebo-corrected mean percentage change in MAP, HR, CI, SI, and TPVRI after 15-minute intravenous infusion of BQ-788 or saline placebo in 5 subjects. Closed circles indicate BQ-788 at 300 nmol/min; open circles, BQ-788 at 30 nmol/min; and shaded diamonds, BQ-788 at 3 nmol/min. There was no change in MAP, but there was a reduction in HR, CI, and SI and an increase in TPVRI after administration of BQ-788 at 300 nmol/min.

Discussion

We have demonstrated substantial systemic vasoconstriction, associated with a reduction in HR and CI but no change in MAP, in response to administration of the selective ET_B receptor antagonist BQ-788 in healthy men. Consistent with our earlier work in the forearm circulation,[3] these observations are highly suggestive of the overall effect of endogenous ET_B receptor–mediated vascular tone favoring vasodilatation. An alternative explanation for the hemodynamic effects is that BQ-788 is directly negatively chronotropic and that peripheral effects are indirect. However, this is unlikely given our earlier work[3] and the lack of evidence of an important positive chronotropic and inotropic role of the cardiac ET_B receptor.[20] Although peripheral resistance was substantially increased, blood pressure was unaffected because of a decrease in HR that was probably reflex in origin. We have also demonstrated increases in plasma ET-1, but not big ET-1 concentrations after ET_B receptor blockade, consistent with reduced clearance of ET-1 by the ET_B receptor.[15] All of these effects were prominent with BQ-788 at the highest dose but were not clearly seen at lower doses.

The vasoconstrictor effects of ET_B receptor antagonism may result directly from blockade of the vasodilator effects of the endothelial ET_B receptor or indirectly from displacement of endogenously generated ET-1 from ET_B receptors to unoccupied ET_A receptors. It is unlikely that these effects are mediated by nonselective ET_A/ET_B receptor blockade because they are the opposite of those found with selective ET_A receptor antagonists in healthy subjects (unpublished data, 1998) and patients with heart failure[22] and of those found with combined ET_A/ET_B receptor antagonists in healthy subjects.[2] Clearly, the indirect effects of ET-1 on ET_A receptors are more relevant with administration of selective ET_B antagonists than with nonselective ET_A/ET_B receptor antagonists, because in this latter situation the constrictor ET_A receptor is also blocked. Indeed, vasodilator effects have been demonstrated with both selective[3,20] and nonselective[2,21]

endothelin receptor antagonists in humans, and the nonselective ET_A/ET_B antagonist bosentan has recently been shown to effectively lower blood pressure in patients with hypertension.[25] However, direct comparison of the effects of selective and nonselective endothelin receptor antagonism will be important in assessing the relative contribution of each receptor subtype to the vascular effects of ET-1.

We and others have previously demonstrated forearm vasodilatation in response to local ET_A receptor antagonism with BQ-123.[1,3,32] In the presence of BQ-788 in healthy volunteers, this effect was attenuated,[3] suggesting that the overall effect of vascular ET_B receptor stimulation by endogenous ET-1 is vasodilatation. This attenuation of BQ-123–mediated vasodilatation by BQ-788 suggests that the vasoconstrictor effect of ET_B receptor blockade is not mediated by displacement of ET-1 onto the ET_A receptor but is due to direct blockade of ET_B–mediated vasodilator tone. We have also shown, using a "nitric oxide clamp" technique, that the vasodilator response to BQ-123 is in part mediated by nitric oxide[3] and, therefore, probably mediated by the endothelial ET_B receptor. Loss of endothelial cell ET_B–mediated vasodilator tone may occur in cardiovascular diseases, such as essential hypertension and hypercholesterolemia, in which there is associated endothelial dysfunction.[33,34] Here, because of a reduced capacity for ET_B receptor–mediated, nitric oxide–dependent dilatation, selective ET_A receptor antagonists may be less effective.

In summary, we have demonstrated systemic vasoconstriction in response to acute ET_B receptor blockade with the selective ET_B receptor antagonist BQ-788 in healthy men in vivo, indicating that the predominant endogenous effect of stimulating vascular ET_B receptors is vasodilatation. One exciting possibility is that tonic endogenous ET-1 release, acting via the endothelial ET_B receptor, is responsible for the physiological basal release of nitric oxide. This now needs to be addressed in clinical studies. Further investigation of the influence of ET_B receptor antagonism on the sympathetic nervous system and renal function are also warranted. In addition, direct comparison of the effects of chronic administration of selective ET_A and combined ET_A/ET_B receptor antagonists is required in patients with cardiovascular disease, with and without endothelial dysfunction, in order to confirm which of these approaches is likely to be more effective in the clinical setting.

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SECTION 2

HYPERTENSION AND RENAL DYSFUNCTION

Papers 20-24

Direct and Sympathetically Mediated Venoconstriction in Essential Hypertension

Enhanced Responses to Endothelin-1

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Abstract

Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide. Although circulating concentrations are not increased in essential hypertension, enhanced sensitivity to endothelin-1 has been observed in animal models of hypertension. We investigated dorsal hand vein responses to local infusion of endothelin-1 and norepinephrine in 12 patients with essential hypertension who had never received treatment and in 12 age and sex matched normotensive control subjects.

The maximal venoconstriction and the geometric mean of the dose of norepinephrine that caused 50% of maximal venoconstriction were similar in hypertensive (mean±SE; 80±4%; 31±8 pmol/min) and normotensive subjects (87±5%; 22±9 pmol/min). In contrast, mean venoconstriction to endothelin-1 was significantly greater in hypertensive (49±5%) than in normotensive subjects (27±2%; $P = 0.004$). Sympathetically mediated venoconstriction elicited by deep breath was substantially potentiated by endothelin-1 in hypertensive (67±7% at 90 min) but not normotensive subjects (11±3% at 90 min; $P = 0.001$). Venoconstriction to endothelin-1 correlated positively with mean arterial pressure in the hypertensive subjects ($r = 0.82$; $p = 0.001$) but negatively in the normotensive subjects ($r = -0.58$; $p = 0.047$).

Endothelin-1 may contribute to the reduction of venous compliance occurring in the early stages of essential hypertension and to the altered systemic hemodynamics in this condition. (*J. Clin. Invest.* 1994. 94:1359–1364.) Key words: vasoconstrictor peptide • blood pressure • endothelium • veins • sympathetic nervous system

Introduction

The endothelins are a family of peptides with extremely potent and characteristically sustained vasoconstrictor and vasopressor actions (1). Endothelin-1 is the predominant isoform in the vascular endothelium, where it is generated from its precursor, proendothelin-1 or 'big endothelin-1' (2). In addition to its direct vascular effects (3), endothelin-1 has inotropic (4) and mitogenic properties (5), influences salt and water homeostasis (6) and stimulates generation of renin, angiotensin II, aldosterone and epinephrine (6). Furthermore, centrally (7, 8) and peripherally (9, 10) administered endothelin-1 alters peripheral sympathetic activity. In view of these actions, there has been interest in the potential role that endothelin-1 may play in the pathophysiology of hypertension (11, 12).

Several investigators have invoked elevated concentrations of circulating immunoreactive endothelin-1 as evidence of increased production in essential hypertension (13, 14). However, endothelin-1 is cleared from the blood by the kidneys (14, 15) and the very high concentrations found in severe and accelerated phase hypertension are probably secondary to impaired renal clearance. Studies in hypertensive patients with normal renal function have shown similar concentrations of endothelin-1 to those in normotensives (16, 17). Indeed, in one study a negative correlation between blood pressure and plasma endothelin-1 was observed in the hypertensive group (16), making a global increase in generation of endothelin-1 unlikely as a cause of essential hypertension.

The results of studies examining vascular sensitivity to endothelin in hypertension are complex. In animal studies comparing WKY and SHR, both conduit (renal artery and aorta) and mesenteric resistance vessels from the hypertensive rat have been shown to be more sensitive to the effects of endothelin-1 by some investigators (18–21). Other investigators have reported decreased sensitivity to endothelin in the aorta and isolated mesenteric resistance arteries from SHR (22), DOCA-salt (23) and renovascular hypertensive rats (24). In vivo, endothelin-1 has been shown to have a greater pressor effect in SHR than WKY rats (25). In patients with essential hypertension, in vitro efficacy of endothelin in subcutaneous resistance arteries appears to be reduced (26). To date, in vivo responses to endothelin-1 have not been examined in patients with essential hypertension.

With continuing uncertainty regarding the vascular actions of endothelin-1 in hypertension, we have investigated whether venoconstriction to endothelin-1 is enhanced in patients with essential hypertension as compared with normotensive control subjects. We examined responses in veins for two reasons. First, the venous system is an important influence on cardiac output in its own right, and has been reported to be abnormal in early hypertension (27, 28). This raises the possibility that abnormal venous responses may contribute directly to the pathophysiology of essential hypertension. Second, studies of pressor responses or vasoconstriction in resistance beds in vivo may be confounded by the presence of vascular hypertrophy in resistance vessels (29), whereas this process does not appear to occur in veins (30).

We examined the effect of local intravenous infusion of endothelin-1 and norepinephrine on dorsal hand vein diameter,

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using norepinephrine as a control constrictor to detect any potential confounding effect produced by alterations in venous structure or function in hypertension. In view of the potential interaction between endothelin and activity of the sympathetic nervous system, and the known increase in sympathetic nerve activity in hypertension (31), we used the single deep breath response (32, 33) to assess the effect of endothelin-1 on sympathetically mediated venoconstriction in hypertensive and normotensive subjects. We chose the dorsal hand vein because responses to vasoactive drugs in these veins are very similar to those predicted from the pharmacological profile of action after systemic doses in vivo (34). In addition, there is clear evidence that cutaneous limb veins are under sympathetic venomotor control, whereas skeletal muscle veins do not participate in these reflexes (35, 36). Thus, responses in hand veins should reflect responses of the component of the venous system that is most important in physiological regulation of venous capacitance and cardiac preload. Finally, these studies do not require systemically active doses of drugs that may obscure any direct vascular action by direct effects on other organs, such as the heart and kidney, or activate reflex mechanisms due to changes in blood pressure.

Methods

Subjects

Consecutive patients with hypertension (BP > 160/100 mmHg) attending the Cardiovascular Risk Clinic at the Western General Hospital were considered for the study. Patients were only eligible for recruitment if there was no evidence of a secondary cause for hypertension; if mean daytime awake blood pressure was more than 140/90 mmHg on ambulatory monitoring (measurements every 30 min using Spacelabs 90207) (37); if there were no significant concurrent illnesses; and if they had never received antihypertensive therapy. Normotensive (BP < 140/90 mmHg) control subjects matched for age, sex, weight and height were recruited by advertisement. On the basis of power calculations (see *Data presentation and statistics*), we recruited 12 hypertensive and 12 normotensive control subjects. No subject received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from food, caffeine containing drinks, and cigarettes for at least 3 h before any measurements were made. The studies were conducted with the approval of the Lothian Medicine and Clinical Oncology Ethics of Medical Research Sub-Committee and with the written, witnessed, informed consent of each subject.

Drugs

A 23 SWG cannula (Abbott, Sligo, Republic of Ireland) was sited in a selected dorsal hand vein, without use of local anesthesia, in the direction of flow, for infusion of endothelin-1 (5 pmol/min; Clinalfa AG, Läufelfingen, Switzerland) and norepinephrine (6–768 pmol/min; Sterling-Winthrop, Guildford, United Kingdom). These doses, based on an estimated dorsal hand vein flow of 1 ml/min (34), would be expected to achieve local concentrations, within the infused hand vein, approximating to 10^{-8} M for endothelin-1 and to between 10^{-8} and 10^{-6} M for norepinephrine. Drugs were dissolved in saline. Ascorbic acid (Evans Medical, Horsham, UK) was added to norepinephrine solutions, at a final concentration of 10 μg/ml, to prevent degradation by oxidation (34). The total rate of infusion was maintained constant throughout all studies at 0.25 ml/min.

Measurements

Dorsal hand vein size. The left hand was supported above the level of the heart by means of an arm rest. Internal diameter of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mmHg, was measured by the technique of Aellig (38). In brief, a magnetized lightweight rod rested on the summit of the infused vein ~ 1 cm downstream from the tip of the infusion cannula. This rod passed through the core of a linear variable differential transformer (LVDT) supported above the hand by a small tripod, the legs of which rested on areas of the dorsum of the hand free of veins. If venoconstriction occurs while this cuff is inflated, or if the cuff is deflated with consequent emptying of the vein, there is a downward displacement of the lightweight rod. This displacement causes a linear change in the voltage generated by the LVDT, and thus allows determination of the internal diameter of the vein, after calibration against standard displacements. Voltage output from the LVDT was transferred to a Macintosh personal computer file using a MacLab analogue-digital converter and Chart software (v. 3.2.8; both from AD Instruments, Castle Hill, NSW, Australia).

Single deep breath venoconstrictor stimulus. When vein size was stable, subjects were asked to breath out fully before breathing in as deeply as possible (32). They were asked to hold this inspiration for 10 s and avoid any tendency to breath out. The technique was practiced before the study to ensure that subjects did not perform a Valsalva maneuver. The deep breath stimulus usually causes a 5–20% venoconstriction in the 30 s after the maneuver (33).

Blood pressure. A well-validated semi-automated technique (Takeda UA 751 sphygmomanometer, Takeda Medical Inc., Tokyo, Japan) was used to measure blood pressure in duplicate in the non-infused arm (39).

Endothelin assay. Plasma immunoreactive endothelin was measured by radioimmunoassay (40). Immunoreactive endothelin was extracted from acidified plasma using SepPak C18 silica columns (Waters Associates, Milford, MA). Duplicate extracted samples and standards were incubated with rabbit polyclonal antibody raised against endothelin-1 (ITS Production B.V., Wijchen, The Netherlands; in 100 μl distilled water) and ^{125}I-endothelin-1 (ITS; in 100 μl distilled water). After incubation for 18 h at 4°C, donkey anti-rabbit gamma globulin bound on solid phase (ITS; 100 μl) was added, and tubes were incubated for 30 min at room temperature. The amount of radioactivity in the antibody-bound fraction was determined by gamma counting for 3 min. The recovery of added endothelin-1 was 84%. Intra- and inter-assay coefficients of variation were 2.4% ($n = 6$) and 4.2% ($n = 5$), respectively. The sensitivity of this assay is 2 pg/ml endothelin. Cross reactivity of the assay with endothelin-1, endothelin-2, endothelin-3 and proendothelin-1 is 100, 52, 96, and 7%, respectively.

Study design

Subjects rested recumbent during each phase, in a quiet room maintained at a constant temperature of between 22 and 25°C. An intravenous cannula was placed in the right antecubital vein under local anesthesia for blood sampling and the dorsal hand vein cannula and the LVDT sited. Saline was infused for 30 min during which vein size and blood pressure were measured every 5 min. Subjects were asked to take single deep breaths, to elicit sympathetically mediated venoconstriction, after 5 and 20 min of saline infusion. A venous blood sample was obtained from the non-infused arm for assay of circulating endothelin concentrations. Norepinephrine was then infused at incremental doubling doses of between 6 and 768 pmol/min for 10 min each to obtain a full dose response curve. Vein size was measured 5 and 10 min after starting infusion of each dose of norepinephrine. Blood pressure was measured 10 min after starting each dose of norepinephrine. Once a maximal response to norepinephrine was obtained saline was infused until vein size returned to basal values. Endothelin-1 was then infused at 5 pmol/min for 90 min with vein size measured every 5 min and blood pressure every 30 min. The sustained duration of action of endothelin-1 in human veins precluded randomizing the order of infusions of norepinephrine and endothelin-1 (41). Single deep breaths were taken at 28, 58, and 88 min. In a subset of subjects ($n = 8$ from each group), a further blood sample was obtained from the non-infused arm at the end of the endothelin infusion for assay of circulating endothelin concentration.

Data presentation and statistics

The power of the study to detect a 20% difference in endothelin-1–induced venoconstriction between 12 hypertensive and 12 normotensive

Table 1. Subject Characteristics

	Hypertensives	Normotensives
Number (n)	12	12
Age (yr)	47 ± 3	48 ± 4
Sex (M/F)	8/4	8/4
Supine systolic blood pressure (mmHg)	159 ± 5*	120 ± 3
Supine diastolic blood pressure (mmHg)	103 ± 2*	75 ± 2
Mean arterial blood pressure (mmHg)	122 ± 3*	90 ± 2
Daytime ambulatory systolic pressure (mmHg)	158 ± 6	
Daytime ambulatory diastolic pressure (mmHg)	103 ± 4	
Creatinine (μmol/l)	106 ± 5	90 ± 4
Cholesterol (mmol/l)	5.3 ± 0.2	5.1 ± 0.2
Basal vein diameter (mm)	0.8 ± 0.1	0.9 ± 0.2

* Indicates $P < 0.05$ for difference from normotensive subjects.

subjects was 90% at the 0.01 significance level. This calculation is based on the standard deviation of venoconstriction to endothelin-1 (10%) observed in a previous study (41). Basal vein size was calculated by taking the mean of the last three measurements before the start of the norepinephrine infusion, and is expressed in millimeters. Because basal vein size varies between subjects, responses to deep breath, norepinephrine and endothelin-1 are expressed as percentage change in vein size from basal in order to reduce the inter-subject variability. Venoconstriction with each dose of norepinephrine was calculated by averaging the two measurements for each dose. Individual norepinephrine dose–response curves were then analyzed using an iterative non-linear curve fitting program (Kaleidagraph, Abelbeck Software, CA) to obtain estimates of maximal responses (E_{max}) and norepinephrine dose producing a half-maximal response (ED_{50}). Individual ED_{50} values were log transformed for statistical analysis and results shown as geometric means. Because serial measurements were made in each subject following infusion of endothelin-1, mean constriction to endothelin-1 over 90 min was calculated as a summary measure for each individual in order to avoid making multiple comparisons of data (42). The mean of the last two duplicate blood pressure measurements during saline infusion was used as baseline. All results are expressed as mean±standard error of the mean. Data were examined using Student's unpaired *t* test and by simple regression analysis. Statistical analyses were performed using StatView 512+ software for the Macintosh (Brainpower Inc., Calabasas, CA).

Results

There was no significant difference between hypertensive and normotensive subjects in age, sex distribution, basal hand vein size, or renal function (Table I). Hypertensive and control subjects did not differ significantly in their venous sensitivity ($P = 0.46$ for ED_{50}) or responsiveness ($P = 0.30$ for E_{max}) to norepinephrine (Fig. 1). Vein diameter was no different from baseline following washout of the venoconstriction induced by norepinephrine before infusion of endothelin-1 in the hypertensive (0.8±0.1 mm; $P = 0.80$) and normotensive subjects (0.9±0.1 mm; $P = 0.69$).

Venoconstriction to endothelin-1 was substantially greater in hypertensive subjects (49±5%) than control subjects (27±2%; $P = 0.004$) (Fig. 2). Basal venoconstriction to a single deep breath was not different between hypertensive (17±3%) and control subjects (13±3%; $P = 0.37$). However, sympathetically mediated venoconstriction to deep breath was greater in hypertensive (67±7% at 90 min) but not normotensive subjects (11±3% at 90 min) after infusion of endothelin-1 ($P = 0.001$; Fig. 3). This was true even when venoconstriction

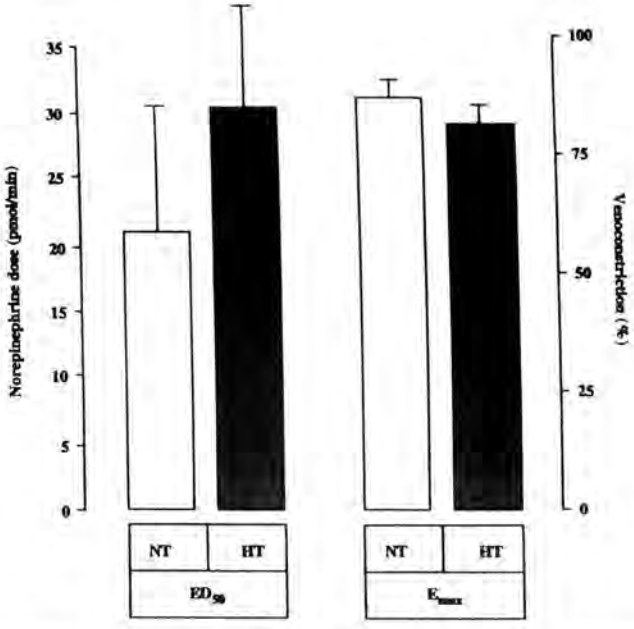

Figure 1. The norepinephrine dose producing a half maximal response (ED_{50}; a measure inversely proportional to sensitivity) and the maximum responsiveness to norepinephrine (E_{max}). ED_{50} is expressed as a geometric mean dose. There is no significant difference between normotensive (*NT*) and hypertensive (*HT*) subjects, either for ED_{50} ($P = 0.46$) or E_{max} ($P = 0.30$).

to deep breath was expressed as the absolute change in vein size rather than percentage change in order to account for the differences in underlying venoconstriction to endothelin-1 in hypertensive (0.14±0.03 mm at 90 min) and normotensive subjects (0.06±0.02 mm; $P = 0.03$).

Circulating plasma endothelin concentrations were similar in hypertensive (5.0±0.6 pg/ml) and control subjects (5.4±0.8 pg/ml; $P = 0.65$). Circulating endothelin concentrations did not change significantly from baseline following local infusion of endothelin in the hypertensives (baseline = 5.3±1.0; postendothelin = 6.2±1.1; $P = 0.32$; $n = 8$) or the normotensives (baseline = 5.5±1.2; postendothelin = 6.9±1.4; $P = 0.26$; $n = 8$). Blood pressure and heart rate did not alter significantly during infusion of norepinephrine or endothelin-1.

Venoconstriction to endothelin-1 was positively correlated on regression analysis with baseline systolic ($r = 0.85$; $P = 0.0004$), diastolic ($r = 0.69$; $P = 0.01$) and mean arterial pressure ($r = 0.82$; $P = 0.001$) in the hypertensive subjects. In contrast, in the normotensive subjects, venoconstriction to endothelin-1 was negatively correlated with baseline systolic ($r = -0.50$; $P = 0.10$), diastolic ($r = -0.51$; $P = 0.09$) and mean arterial pressure ($r = -0.58$; $P = 0.047$; Fig. 4). Venoconstriction to endothelin-1 did not correlate with basal vein size, ED_{50} or E_{max} to norepinephrine or plasma endothelin concentrations in either the hypertensive or normotensive subjects. There was no correlation, in either group, between blood pressure and plasma endothelin, or blood pressure and the ED_{50} or E_{max} to norepinephrine.

Discussion

In these studies, we have shown that patients with essential hypertension have enhanced venoconstriction to endothelin-1

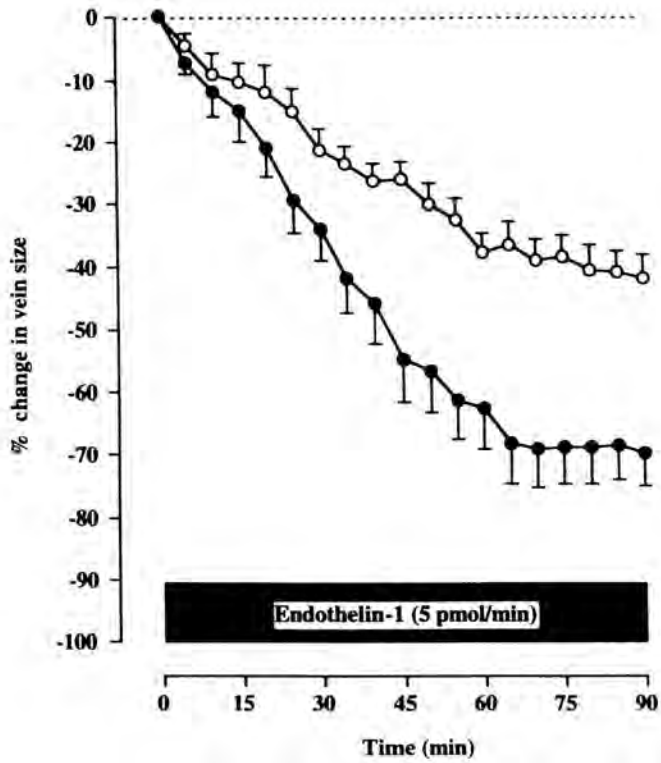

Figure 2. Venoconstriction to endothelin-1 in normotensive (○) and hypertensive (●) subjects. There is a significantly greater response in the hypertensive subjects ($P = 0.004$ vs. normotensives).

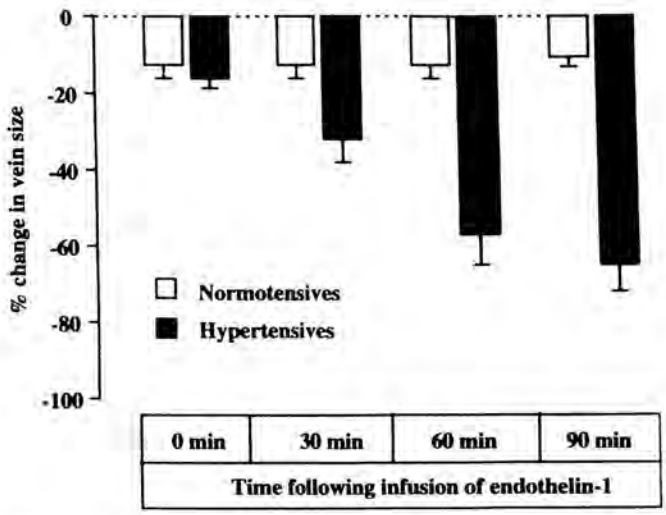

Figure 3. Sympathetically mediated venoconstriction induced by single deep breath before and during infusion of endothelin-1 in hypertensive and normotensive subjects. Endothelin-1 potentiates sympathetically mediated venoconstriction in the hypertensive subjects only ($P = 0.001$ v. normotensives).

that is positively correlated with blood pressure. In addition, we have demonstrated that endothelin-1 substantially potentiates sympathetically mediated venoconstriction in hypertensive but not in normotensive subjects. We have also confirmed earlier work showing that essential hypertension is not associated with increased plasma endothelin concentrations (16) or with altered dorsal hand vein responses to norepinephrine (30).

We were only able to test responses to a single dose of endothelin-1 because the slow onset and long lasting action of endothelin-1 precludes the use of repeated doses in a single study to examine conventional dose–response relationships (41). Thus we cannot say whether the enhanced venous responsiveness to endothelin-1 in hypertension is due to increased sensitivity or responsiveness to the peptide. Different basal vein blood flow may have resulted in different concentrations of agonist reaching venous smooth muscle. However, total forearm blood flow is not decreased in essential hypertension (43), and there is no evidence for a selective redistribution of blood flow from the superficial to deep hand veins in hypertension. In addition, venous diameter was similar in the two groups, implying that any change in blood flow in the veins under consideration would have to be accounted for solely by an increase in velocity of blood flow. Furthermore, the almost identical responses to norepinephrine would suggest that delivery of agonist was similar in hypertensives and normotensives. The use of a constant rate of infusion helped to minimize the possibility that changes in flow through a vein might alter local release of endothelium derived mediators. In any case, such changes in flow are unlikely to have biased our results, as previous studies have shown that increasing the rate of drug infusion by up to 100%, but keeping the dose infused constant, does not alter dorsal hand vein responses to a number of agents (34, 38). The interval of at least 3 h between the last meal and first measurement minimized the possibility that high insulin levels may have altered responses. It is possible, however, that caffeine, which has a long half-life, may have been present in quantities sufficient to alter cardiovascular responses at the time of the study. It is very unlikely that the presence of vascular hypertrophy resulted in amplification of responses to endothelin because we examined responses in vessels which are not thought to undergo hypertrophy in hypertension and because responses to norepinephrine were not altered.

Our results are different from previous in vitro work that showed an apparent diminished efficacy of endothelin-1 in isolated small arteries of hypertensive patients after correction for media hypertrophy (26). This difference may be accounted for by methodological differences, such as the in vivo nature of the dorsal hand vein technique, the avoidance of local anesthesia, which might influence sympathetic responses, and the absence of vessel wall hypertrophy. Alternatively, the difference may be due to the type of vessel studied. However, in diseases associated with abnormalities in resistance or conduit vessels, such as essential hypertension and Raynaud's disease, similar abnormalities are found in hand veins (44, 45).

There are several potential mechanisms for enhanced venous responsiveness to endothelin-1 in essential hypertension. First, there may be decreased local venous endothelin-1 generation. Plasma concentrations of immunoreactive endothelin, which are thought to reflect local concentrations at the interface between endothelial and vascular smooth muscle cells (2), were no different between hypertensive and control subjects, suggesting that there was no global increase in endothelin generation in the hypertensive subjects. However, because endothelial generation of endothelin-1 is directed mainly abluminally (2), it is conceivable that local endothelin generation differs between hypertensive and normotensive subjects. Thus, it is possible, though perhaps unlikely, that our findings may have been due to increased endothelin receptor number or affinity caused by decreased vascular generation of endothelin.

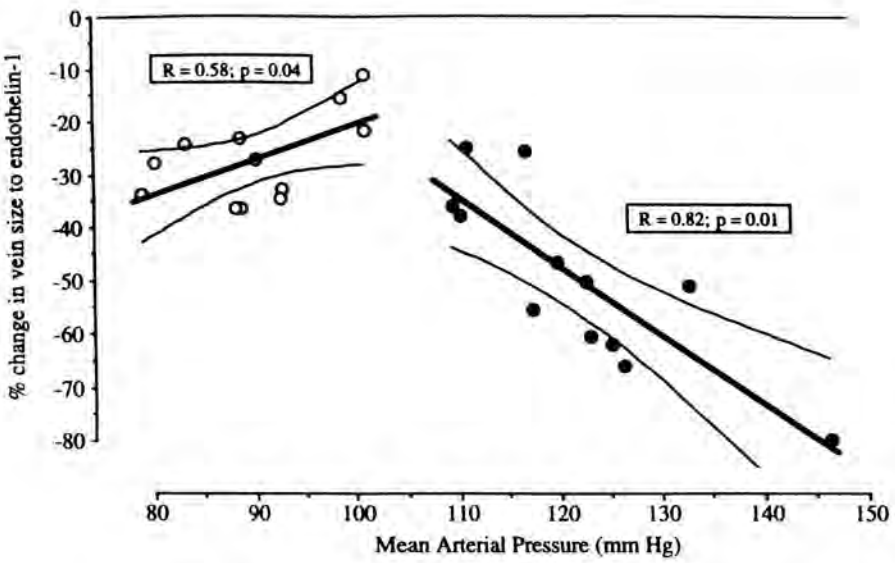

Figure 4. Regression curves (with 95% confidence intervals) for the correlation between blood pressure and change in vein size to endothelin-1 in normotensive (○) and hypertensive (●) subjects. There is a negative correlation in normotensive subjects but a positive correlation in hypertensive subjects.

Second, the modulating influence of endothelium derived vasodilator substances on endothelin-1 induced venoconstriction may be abnormal in essential hypertension (46), resulting in enhanced responses without changes in generation or sensitivity. This would presuppose that impaired endothelial function in hypertension is not the result of elevated pressure per se; recent studies in resistance vessels suggest that impaired endothelial function is not normalized with lowering of blood pressure by anti-hypertensive treatment (47). There is no published work examining endothelium dependent responses in veins of hypertensive subjects, although insulin-mediated venodilatation, which may be endothelium dependent, is clearly abnormal in mild hypertension (44). However, the unaltered response to norepinephrine in hypertensives, which has endothelial effects, would suggest that endothelial abnormalities are unlikely to fully account for our findings.

Third, it is possible that these findings represent an abnormality of venous smooth muscle in hypertension. The underlying abnormality may be a change in receptor sub-type expression, receptor number or receptor affinity. Alternatively, there may be an abnormality in second messenger systems resulting in a specific amplification of responses to endothelin-1, for example in G-proteins.

Finally, our results may be due to enhanced facilitation of sympathetic vasoconstriction by endothelin in hypertension. It has been suggested from in vitro studies that endothelin may increase peripheral sympathetic activity through postsynaptic potentiation of the effects of norepinephrine (10). However, these findings have not been confirmed in vivo in the forearm resistance bed of healthy subjects (48). The potentiation of sympathetically induced venoconstriction by endothelin-1 only in hypertensive subjects would be consistent with this explanation for our findings. However, at least in normal subjects, dorsal hand veins have no underlying sympathetic tone, so facilitation of sympathetic activity is unlikely to be the sole or initiating mechanism involved.

Although enhanced venoconstriction to endothelin-1 in hypertension may be an epiphenomenon, not causally related to the elevation of blood pressure, this appears unlikely given the positive correlation with blood pressure in hypertensive subjects (Fig. 4). Even so, enhanced venoconstriction to endothelin 1 may occur only secondarily to the increase in blood pressure. However, if this was the case one might also expect a positive correlation between blood pressure and endothelin-induced venoconstriction in normotensive subjects. That there is a negative correlation in these subjects suggests that enhanced venoconstriction to endothelin 1 may be a causative factor in patients with essential hypertension.

These findings in capacitance vessels suggest that exaggerated responsiveness to endothelin 1 may contribute to reduced venous compliance in hypertension. This may, in turn, contribute to the raised cardiac preload and cardiac output observed in the early stages of essential hypertension (27, 28). Further in vivo studies in resistance vessels will help to show whether there is a global abnormality in endothelin responsiveness in essential hypertension, although such studies will need to take account of structural changes in these vessels. The role of endothelin in hypertension may be further clarified with the use of endothelin receptor antagonists, which are currently entering clinical investigation (49).

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Serial changes in blood pressure, renal function, endothelin and lipoprotein (a) during the first 9 days of cyclosporin therapy in males

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Objective: To elucidate the sequential mechanisms underlying cyclosporin-induced hypertension and nephrotoxicity.

Design: A study of healthy males over the first 9 days of drug ingestion to permit the detection of serial changes in renal function and blood pressure in a situation free from the confounding variables of concomitant disease or drugs.

Methods: Double-blind, placebo-controlled, randomized crossover study with cyclosporin (5 mg/kg twice a day) or placebo. Blood pressure and urinary sodium excretion were measured each day, and glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were measured on days 1, 4, 7 and 9. Cholesterol, lipoprotein (a) and endothelin were measured on days 1 and 9.

Results: GFR decreased by 9% with cyclosporin and was significantly lower than with placebo on day 4 of therapy. ERPF fell by 24%. The fall in GFR correlated significantly with suppressed plasma renin activity ($P<0.0001$). Cyclosporin-induced hypertension occurred in the absence of any change in urinary sodium output or in plasma endothelin. Cyclosporin did not affect lipoprotein (a) levels during 9 days of cyclosporin therapy.

Conclusions: Cyclosporin-induced hypertension and renal vasoconstriction are well established after 9 days of cyclosporin 5 mg/kg twice a day. We found no evidence to implicate either circulating endothelin or renal sodium retention in the onset of cyclosporin-induced hypertension. Cyclosporin-induced renal vasoconstriction appeared to occur when the protective mechanism of plasma renin activity suppression became exhausted.

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Keywords: Cyclosporin, hypertension, kidney, renin, endothelin, lipoprotein (a), man.

Introduction

Cyclosporin is an important therapeutic agent with a wide range of applications, not only in transplantation but also in numerous autoimmune diseases. Unfortunately, its well-recognized side effects of nephrotoxicity and hypertension preclude its more-widespread use. Strategies to combat these side effects have produced variable results, but are significantly hampered by a basic lack of understanding of the pathogenetic mechanisms involved in its development. Animal studies of these side effects are difficult to interpret and their findings cannot be directly transcribed to humans because animals are generally resistant to the side effects of cyclosporin even at very high doses [1–3]. In humans most of the data concerning cyclosporin come from patients who have been treated with cyclosporin for several months, by which time nephrotoxicity and hypertension

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are well established [4–7]. Furthermore, these patients have the contaminating factors of concomitant disease and coincidental drug therapy.

Clearly, the best scenario for studying the pathogenesis of the side effects of cyclosporin is to follow closely the early effects of cyclosporin in normal humans to the point where hypertension and renal changes occur. To do this, single-dose effects of cyclosporin have previously been assessed by two groups of workers. Weir *et al.* [8] administered the intravenous preparation of cyclosporin to healthy females and found that a small but significant fall in glomerular filtration rate (GFR) occurred within a few hours of injection, whereas renal blood flow (RBF) remained unchanged. However, the preparation of cyclosporin used contained the potent vasoconstrictor Cremophor-EL, which is known to exert major renal haemodynamic effects itself [9]. Conte *et al.* [10] administered a single oral dose of cyclosporin to eight healthy males at a dose of 12 mg/kg, which achieved a peak drug level of 1500–2000 ng/ml, and they reported a 27% decline in GFR by 100 min and a similar fall in RBF. We have misgivings concerning these dramatic acute findings for three reasons. First, that study was not placebo-controlled. Secondly, Conte *et al.* repeated the study on another occasion and found no consistent change in GFR when using the same protocol [11]. Thirdly, we have performed several detailed studies of the effects of cyclosporin over the first 4 days of therapy in normal humans [12,13], and in those studies cyclosporin usually increased blood pressure after the first dose. However, administering cyclosporin to more than 50 different healthy human subjects, we have consistently found that even various different doses of cyclosporin consistently produced no significant change in renal haemodynamics up to 4 days of therapy.

From our findings [12,13] we have concluded that the healthy kidney has inherent homeostatic mechanisms which, at least in the short term (up to 4 days), protect against cyclosporin-induced renal vasoconstriction. To support this, an earlier patient study [14] suggested that the renal effects of cyclosporin become manifest after 7–14 days of treatment. We have therefore now extended our studies of the initial effects of cyclosporin from 4 to 9 days of therapy, in order to establish the point at which cyclosporin-induced renal vasoconstriction occurs and to examine possible pathogenetic mechanisms. We report here on the neurohormonal, renal and blood pressure effects of cyclosporin administered daily over a 9-day period in healthy males. We have also included the definitive study of two issues which are of current interest for cyclosporin. First, endothelin might contribute to the ability of cyclosporin to vasoconstrict according to animal work and to the finding of elevated endothelin levels in transplant patients [15]. There are many complicating factors in that previous work, and the present study provides a cleaner experimental model to answer finally the question of whether cyclosporin itself increases endothelin levels. Secondly, transplant recipients are susceptible to coronary atherosclerosis, and an increase in lipoprotein (a) was found in one study of transplant patients [16]. We therefore took this opportunity to investigate whether cyclosporin itself alters lipoprotein (a) levels, at least in the first 9 days of administration.

Methods

Nine normal, healthy, salt-replete male volunteers [mean age 32.3 years (95% confidence interval 26.9–37.7), mean weight 77.7 kg (60.0–89.1)] were recruited into the study. They were screened by a full medical history and examination, as well as by haematological and biochemical parameters before inclusion. They had no illnesses and were taking no medications (prescribed or proprietary). Written informed consent was obtained before randomization. The study was approved by the Tayside Committee on Medical Ethics. Subjects were given dietary advice to follow in the form of a diet sheet and general recommendations, aimed at maintaining dietary sodium intake at approximately 150 mmol/day. Subjects were not admitted to hospital for the present study, therefore there was not absolute control over their dietary intake.

The study was randomized, double-blind, placebo-controlled and crossover. Subjects were treated with either cyclosporin (Sandimmun; Sandoz Pharmaceuticals, Camberley, Surrey, UK) at a dose of 5 mg/kg twice a day or placebo (provided by our pharmacy) for 9 days. Treatment periods were separated by a washout period of at least 14 days. The half-life of cyclosporin is approximately 8 h [17] and we found no cyclosporin in the bloodstream during the placebo phase (if the latter was given first), as evidenced by whole-blood cyclosporin levels on the first day of each treatment period.

Renal haemodynamic assessments were performed on days 1, 4, 7 and 9 of each treatment period. The protocol for this was as follows [18]. On days 1, 4, 7 and 9 subjects attended the laboratory at 0800 h after having fasted from midnight. They were waterloaded over a 30-min period using a previously reported method and given their morning dose of trial medication [16]. In essence, the subjects were given 20 ml/kg water to drink. A 90-min period of equilibration was then allowed, after which subjects voided their urinary bladders completely every 20 min and were given the equivalent volume of water to drink. A (bolus of *para*-aminohippurate sodium; Merch Sharp & Dohme, West Point, Pennsylvania, USA) and inulin (Inutest; Laevosan-Gesellschaft, mbH, Linz, Austria), followed by infusion, was administered via or through an intravenous cannula situated in the right antecubital vein at doses calculated to obtain steady plasma levels of 200 and 25 mg/l for *para*-aminohippurate and inulin, respectively. A 2-h equilibration period was then allowed, during which 20-min renal clearance periods were performed such that by the end

of this period urine output was steady and varied by <5% for each individual. Formal 20-min renal clearance periods were then observed for the subsequent 100 min. These consisted of subjects voiding their urinary bladder completely every 20 min and being given the equivalent volume of water to drink. Aliquots of urine were saved for estimation of urine sodium, *para*-aminohippurate and inulin. Blood was sampled at the midpoint of each period for plasma concentration of *para*-aminohippurate and inulin. Data from the last three clearances of *para*-aminohippurate and inulin were meaned for each individual, the values being used for subsequent statistical analysis. The reasoning for this was that our previous experience has shown that the peak drug level and drug effect of cyclosporin was between 3 and 4 h after ingestion. Clearance data are taken from this period for analysis. The urine collected during day 9 of both placebo and cyclosporin treatments was also pooled for assay of urinary endothelin-1.

At the beginning and end of each study session, and after 30 min supine, blood was taken for hormonal analysis of plasma renin activity, aldosterone and endothelin, as well as for cholesterol and lipoprotein. Whole-blood cyclosporin levels were measured hourly on these study days. Supine blood pressure was also measured at the midpoint of each period by a semi-automated sphygmomanometer (Dinamap; Critikon Inc., Tampa, Florida, USA).

On days 2, 3, 5, 6 and 8 subjects attended the Department of Clinical Pharmacology approximately 4 h after the morning dose of their trial drug. Blood pressure was recorded as described above, and 24-h urine collections from days 1–9 were performed, aliquots being saved for electrolyte and water excretion measurements. The total sodium output during the waterloading study was added to the 24-h urine collection data to provide day-to-day sodium output data plus a total for each subject over each 9-day study period.

Laboratory methods

Urinary sodium was measured by flame photometry (Instrumentation Laboratory, Milan, Italy). Commercially available radioimmunoassay kits were used to measure whole-blood cyclosporin levels (Cyclo-Trac; Incstar Corporation, Stillwater, Minnesota, USA), plasma aldosterone (Diagnostic Products Corporation, Abingdon, Oxfordshire, UK) and plasma renin activity (by generation of angiotensin I at 37°C for 90 min; Incstar Corporation). Plasma and urine *para*-aminohippurate and inulin concentrations were analysed as described previously [19], as were plasma and urine endothelin levels [20]. Cholesterol and lipoprotein (a) were kindly assayed by M. Ryan (Biochemical Medicine, Ninewells Hospital and Medical School, Dundee, UK). Cholesterol was measured by the cholesterol oxidase method (AXON system method SM4-2139F90; Technicon Instruments Co. Ltd, Basingstoke, Hampshire, UK) on a Technicon AXON analyser in a laboratory accredited by CPA (UK) Ltd. Lipoprotein (a) was measured by an enzyme-linked immunosorbent assay technique (Tint Elize Catalogue No. 610120; Porton Products Ltd, Oxford, UK).

Calculations

Mean arterial pressure was calculated by adding one-third of the pulse pressure to the diastolic blood pressure. GFR and effective renal plasma flow (ERPF) were estimated from the clearances of inulin and *para*-aminohippurate respectively, and were corrected for a body surface area of 1.73 m^2. Clearance was defined as urinary concentration multiplied by urine volume divided by plasma concentration.

Statistical analysis

Values are expressed as means (95% confidence interval). All renal parameters were analysed by repeated-measures analysis of variance. Student's t test was used to detect changes in hormonal and lipid parameters. Pearson's linear correlation coefficient and linear regression analysis were used to assess the relationships between PRA and GFR and between endothelin and mean arterial pressure. $P<0.05$ was considered statistically significant.

Results

Whole-blood cyclosporin levels on day 9 were 187 ng/ml (119–255) at trough and 941 ng/ml (651–1231) at 4 h. No cyclosporin was detected on placebo days. Cyclosporin induced a fall in GFR and ERPF by day 4 compared with placebo, although when compared with baseline day 1 cyclosporin values there was no significant difference in GFR until day 7 (Fig. 1). Cyclosporin also exerted a hypertensive effect by day 4 (Fig. 2). Although absolute values of the plasma renin activity and aldosterone did not change significantly (Table 1), there was a strong correlation between plasma renin activity and GFR on cyclosporin treatment, with plasma renin activity suppression being associated with the lowest GFR ($P<0.0001$; Fig. 3). There was no evidence whatsoever of sodium retention occurring during cyclosporin therapy (Table 2).

The effects of cyclosporin were not associated with any change in plasma endothelin levels (Table 1) over the 9-day course, neither was there any correlation between blood pressure and plasma endothelin levels (Pearson's correlation coefficient 0.27, $r^2=0.07$, $P=0.01$). Urinary endothelin levels on day 9 of therapy were not significantly different between placebo [16.8 ng/h (12.1–21.5)] and cyclosporin [21.5 ng/h (15.2–27.8)]. Lipoprotein (a) and cholesterol levels were not affected by cyclosporin (Table 1).

Discussion

The present study allowed new insights into the development of cyclosporin-induced nephrotoxicity and

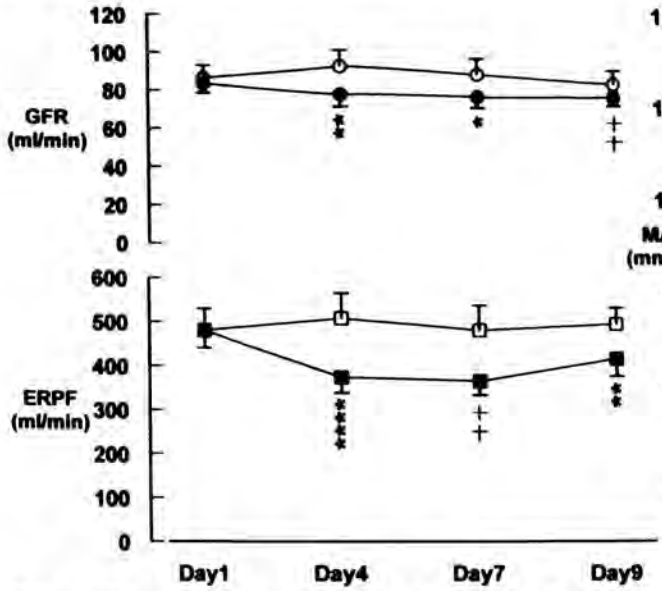

Fig. 1. The effects of cyclosporin (■, ●) and placebo (□, ○) on glomerular filtration rate (GFR) and effective renal plasma flow (ERPF). Values are expressed as means ± 95% confidence interval. *$P<0.05$, **$P<0.01$, ††$P<0.005$, ****$P<0.0001$, versus placebo.

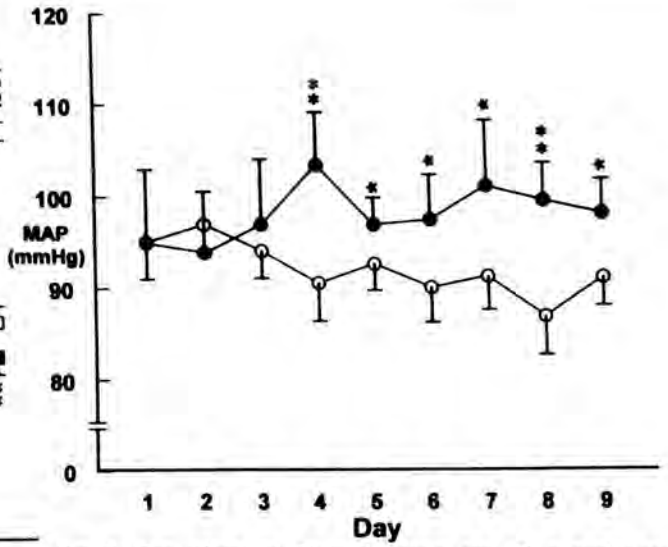

Fig. 2. The effects of (●) cyclosporin and (○) placebo on mean arterial pressure (MAP) measured 4 h after dosing on days 1–9. Values are expressed as means ± 95% confidence interval. *$P<0.05$, **$P<0.005$, versus placebo.

hypertension in humans. We found that the healthy kidney can initially counteract cyclosporin-induced renal vasoconstriction and thus maintain renal perfusion and glomerular filtration. However, between days 4 and 7 these homeostatic mechanisms are overcome and an 8.4% fall in GFR occurs, accompanied by a 24% fall in ERPF. By day 4 hypertension also occurs, yet urinary sodium excretion remains unchanged.

It is well established that cyclosporin induced renal vaso constriction is the initial process in the kidney leading ultimately to nephrotoxicity with ischaemic and fibrotic histological changes [5]. However, the pathogenetic mechanisms which underlie the initial renal vasoconstriction remain uncertain. Clearly, a model for cyclosporin induced changes in humans is desirable for studying in detail the abnormalities which cyclosporin produces. The present experimental design of 9 days of clinically relevant doses of cyclosporin to healthy males

Table 1. The effects of cyclosporin compared with placebo on plasma renin activity, aldosterone, endothelin, lipoprotein (a) and cholesterol.

	Plasma renin activity (ng/ml per h)		Aldosterone (pg/ml)		Endothelin (pg/ml)		Lipoprotein (a) (mg/l)		Cholesterol (mmol/l)	
	Placebo	Cyclosporin	Placebo	Cyclosporin	Placebo	Cyclosporin	Placebo	Cyclosporin	Placebo	Cyclosporin
Day 1										
Pre	0.94 (0.40–1.49)	1.23 (0.60–1.86)	113 (86–140)	148 (112–184)	4.87 (3.17–6.58)	7.33 (3.79–10.87)	100 (57–142)	103 (65–142)	3.9 (3.3–4.4)	4.2 (3.7–4.7)
Post	0.46 (0.18–0.75)	0.75 (0.34–1.16)	84 (53–115)	135 (103–167)	6.57 (5.29–7.85)	7.29 (5.88–8.70)				
Day 4										
Pre	1.04 (0.30–1.78)	0.65 (0.28–1.02)	128 (91–165)	146 (114–178)	6.07 (4.80–7.34)	6.71 (5.38–8.04)	113 (66–159)	85 (53–116)		
Post	0.66 (0.29–1.03)	0.41 (0.19–0.63)	84 (54–114)	93 (79–107)	6.34 (4.92–7.76)	7.65 (5.49–9.81)				
Day 7										
Pre	1.13 (0.76–1.50)	0.69 (0.14–1.24)	134 (95–173)	127 (99–155)	7.06 (5.31–8.81)	7.34 (5.40–9.28)	94 (51–138)	75 (40–111)		
Post	0.63 (0.34–0.92)	0.49 (0.18–0.80)	80 (58–102)	111 (97–125)	6.71 (4.31–9.11)	8.09 (6.02–10.16)				
Day 9										
Pre	0.98 (0.53–1.43)	0.71 (0.32–1.10)	123 (87–159)	131 (103–159)	6.50 (5.32–2.68)	6.60 (4.93–8.27)	109 (60–158)	94 (61–128)	4.0 (3.6–4.4)	4.3 (3.8–4.9)
Post	0.76 (0.33–1.19)	0.50 (0.25–0.75)	58 (41–75)	93 (76–110)	7.18 (5.01–9.35)	8.56 (6.27–6.85)				

Values are expressed as means (95% confidence interval).

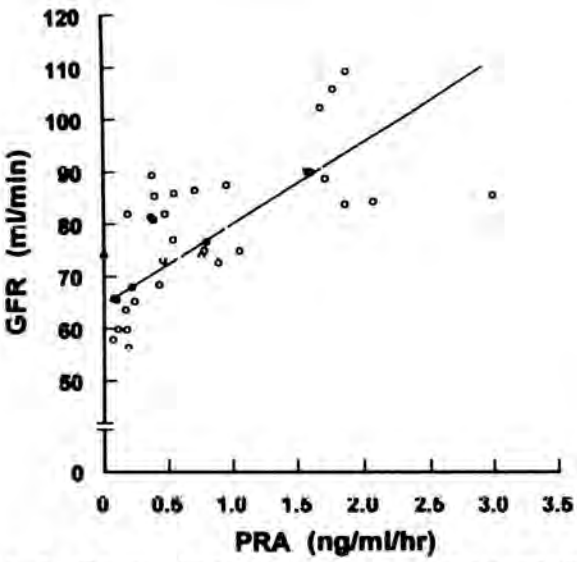

Fig. 3. The correlation between plasma renin activity (PRA) and glomerular filtration rate (GFR) on cyclosporin therapy with linear regression analysis. $r = 0.602$, $n = 36$, $y = 15.25x + 64.68$ ($P < 0.0001$).

has proved safe and suitable for such studies. This study is a natural progression from our previous work [12,13], in which we found that neither single doses of cyclosporin nor 4 days of therapy had any effect on renal haemodynamic parameters. We have not been able to plot serially the changes in renal function which occur between days 4 and 9.

We have confirmed our previous findings that renal function is maintained initially after acute cyclosporin dosing, which again contrasts with the results of other groups [8,10]. We have demonstrated an initial resilience of the healthy kidney to cyclosporin. This homeostatic mechanism broke down between days 4 and 7. We had previously found that cyclosporin had no effects on renal function by day 4 [13]. We do not believe there is a fundamental difference in the results of the present and the previous study [13] for the following reasons. First, significance was not attained until day 7, when day 1 results for cyclosporin were used as baseline instead of comparing placebo and cyclosporin on each day. Statistically significant results were obtained on day 4 primarily because of the change in placebo values which showed simply natural variation. Thirdly, the present subjects were different from in our previous study, and their ability to compensate by suppressing their renin angiotensin aldosterone system may have been different, which might account for the variation found between the present and our previous study. With regard to renal change, we found a striking correlation between reduced GFR and plasma renin activity suppression after cyclosporin. In another previous study (Sturrock NDC, Lang CC and Struthers AD, unpublished results, 1994) we artificially stimulated the renin–angiotensin system, and this amplified cyclosporin induced renal vasoconstriction. Because of this knowledge, the correlation between plasma renin activity and GFR in the present study can be interpreted as suggesting that cyclosporin induced renal vasoconstriction occurs when the protective mechanism of PRA suppression becomes exhausted. The time course of renal effects found in the present study mirrors events in patient studies. Balletta *et al.* [21] found that cyclosporin did not induce a fall in GFR on day 2 after therapy, but by 1 month it had produced a significant reduction. Similarly, other workers have found that changes in renal function begin to appear in week 2 of therapy [14] or in a dose-dependent manner by 2 months [22]. Such changes in GFR, at least initially, may be transient and related to the time of dosing [23]. Therefore, there appears to be a time within the first week of cyclosporin therapy when renal function is maintained, which is also when nephroprotective treatments should be started. Certainly workers have reported [24] that for vasodilator drugs such as nifedipine to be most effective at counteracting the renal effects of cyclosporin they must be given as early as possible and, indeed, at the time of onset of the cyclosporin therapy. One group

Table 2. (a) Twenty-four-hour urinary sodium data (mmol/day) for cyclosporin and placebo.

	Day 1–2	Day 2–3	Day 3–4	Day 4–5	Day 5–6	Day 6–7	Day 7–8	Day 8–9
Placebo	135 (90–180)	121 (88–154)	153 (141–165)	125 (92–153)	124 (95–153)	147 (101–193)	171 (145–197)	167 (116–218)
Cyclosporin	129 (109–149)	105 (95–115)	118 (100–136)	108 (71–145)	130 (123–137)	193 (130–256)	213 (171–255)	188 (127–249)

(b) Individual total sodium output (mmol) for 9 days.

	Subject									
	1	2	3	4	5	6	7	8	9	Mean
Placebo	1309	999	925	1507	984	1176	1256	1173	1306	1182 (1066–1297)
Cyclosporin	1296	1314	963	1486	1429	1079	1395	1665	1036	1185 (1019–1350)

Values are expressed as means (95% confidence interval).

has found nifedipine to be effective even though it had no effect on systemic blood pressure [25].

In the present study we found that hypertension did not occur until day 4, in contrast to our earlier data using higher doses of cyclosporin [12,13]. It is plausible that the different doses explain this discrepancy. We have again been unable to demonstrate any link between blood pressure rise and sodium retention. Urinary sodium excretion did not change, confirming our previous conclusions that alterations in sodium balance play no role in the early hypertensive effect of cyclosporin. Clearly, this leaves open the possibility that changes in sodium balance could contribute to the hypertension in the longer term, especially once renal function is frankly abnormal. We therefore sought other hormonal mechanisms to account for the presumed systemic vasoconstriction. We and others have been unable to demonstrate a role for the sympathetic nervous system [12,26], despite earlier data to the contrary [27]. We therefore measured levels of the vasoconstrictor hormone endothelin in order to investigate whether this changed after cyclosporin, and to assess whether there is any correlation between endothelin levels and cyclosporin-induced hypertension. Animal studies had initially suggested a role for endothelin, with the finding that cyclosporin induces endothelial disruption, elevates endothelin levels and subsequently induces renal vasoconstriction, and that these effects could be attenuated by antibodies against endothelin [28–33]. In humans endothelial destruction has been reported in renal biopsies with cyclosporin nephrotoxicity [32] and transplant recipients have been found to have raised endothelin levels [15]. However, cyclosporin does not augment endothelin-induced changes in blood pressure in humans [33]. In the present study we found no change in plasma endothelin levels over the 9 days of the study, despite cyclosporin producing marked cyclosporin-induced renal and blood pressure changes; furthermore, endothelin levels did not correlate with blood pressure changes. The present study does not discount the possibility that endothelin could act locally within tissues. Our observation that cyclosporin does not alter urinary endothelin significantly excludes a major effect of cyclosporin on urinary endothelin, but does not exclude a small effect.

There are major reasons to be interested in the effect of cyclosporin on lipoprotein (a), because the combination of raised lipoprotein (a) and hypertension would be potently atherogenic, and a worrying feature of cardiac transplant patients is that they rapidly develop coronary artery disease. A recent study [15] found an increase in lipoprotein (a) in renal transplant recipients, but we could not confirm this in healthy subjects, and, indeed, others have found suppression of lipoprotein (a) by cyclosporin [34]. The present model has the advantage of no confounding variables but the disadvantage of being a relatively short-term assessment.

In summary, the present study has demonstrated the progressive effect of cyclosporin on renal function and has delineated the point at which homeostatic mechanisms in the healthy kidney are overcome. The study also suggests that suppression of plasma renin activity is one such homeostatic mechanism which is eventually overcome. We have also virtually excluded a role for circulating endothelin or renal sodium retention in initiating cyclosporin-induced hypertension in humans. Finally, this paper describes a human model which can be used in the future for comparative studies to define which is the best nephroprotective or antihypertensive therapy against cyclosporin.

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Erythropoietin enhances vascular responsiveness to norepinephrine in renal failure

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Erythropoietin enhances vascular responsiveness to norepinephrine in renal failure. The mechanism of hypertension induced by recombinant human erythropoietin (rHuEPO) is unclear but may include an increase in peripheral vascular resistance. We studied changes of arterial pressure and plasma endothelin in nine consecutive hemodialysis patients before, and 6 and 12 weeks after, starting rHuEPO. In six patients, changes in cardiac index (CI), stroke index (SI) and total peripheral resistance index (TPRI) were measured by bioimpedance, and forearm vascular responsiveness to intra-arterial norepinephrine (30 to 240 pmol/min) and endothelin-1 (5 pmol/min) were assessed. Six healthy age and sex matched subjects also underwent assessment of forearm vascular responsiveness to norepinephrine and endothelin-1. Treatment with rHuEPO significantly increased hemoglobin and mean arterial pressure (MAP). TPRI also increased by 35 ± 11%. Plasma endothelin, although elevated basally, remained unchanged. Intra-arterial infusion of norepinephrine caused a maximal increase in forearm vascular resistance (FVR) of 17 ± 9% before rHuEPO, significantly less than the 32 ± 5% increase in healthy control subjects ($P = 0.04$). The response increased to 65 ± 15% ($P = 0.03$) after 12 weeks rHuEPO treatment ($P = 0.51$ vs. controls). Endothelin-1 caused a maximal increase of FVR at 60 minutes of 45 ± 24% before rHuEPO, which was not significantly different from controls, and tended to decrease with rHuEPO therapy. The response to endothelin-1, but not norepinephrine, correlated inversely with MAP ($r = -0.52$; $P = 0.03$) and TPRI ($r = -0.51$; $P = 0.04$). In conclusion, these studies show that anemia in chronic renal failure is associated with depressed vascular responsiveness to norepinephrine which is restored by rHuEPO therapy. In contrast, vascular responses to endothelin-1 tend to reduce with rHuEPO therapy. The change in vascular responsiveness to norepinephrine may contribute to the increase in arterial pressure associated with rHuEPO therapy.

Recombinant human erythropoietin (rHuEPO) corrects the anemia of chronic renal failure [1, 2]. It is recognized, however, that rHuEPO may increase arterial pressure in previously normotensive subjects, or exacerbate pre-existing hypertension [3]. Although the mechanism for this hypertension is unknown, it is likely to be multifactorial [4].

Hypertension following rHuEPO is associated with an increase in peripheral vascular resistance [5] and may be exacerbated by failure of an elevated cardiac output to decrease in response to increases in hemoglobin [6, 7]. Although increases of hematocrit and viscosity secondary to rHuEPO therapy may contribute to the rise in vascular resistance [8], and although the development of hypertension may occur in parallel with the increase in hematocrit, one fifth of individuals who become hypertensive do so some months after the hematocrit has stabilized at its new higher level [6]. Also, studies in animals suggest that when the hematocrit is increased to a similar extent with rHuEPO or transfusion, arterial pressure increases in the rHuEPO, but not the transfused, group [9]. In addition, although increasing the hematocrit in humans from 20 to 40% by transfusion increases arterial pressure [10], hypertension does not appear to occur when the hematocrit is increased only to 30% [11]. Furthermore, several studies have failed to find a relationship between changes in arterial pressure and changes in hematocrit or viscosity [4, 6, 12]. Finally, although rHuEPO increases whole blood viscosity, peripheral vascular resistance increases to a much greater extent than would be predicted from this effect [13].

Increased circulating concentrations of vasoactive hormones may contribute to the increase in arterial pressure in patients treated with rHuEPO [14]. However, reports of the effects of rHuEPO have been conflicting: renin activity and atrial natriuretic peptide concentrations do not change [11, 14, 15] or decrease [16–18], and changes in the renin-angiotensin system appear to be secondary to alterations in red cell mass and plasma volume [19]. Norepinephrine concentrations may increase [20], decrease [13] or, like epinephrine and dopamine, remain unchanged [11]. Plasma concentrations of the locally active hormone endothelin-1 have been reported as unchanged [15] or increased [12, 17, 21] in response to treatment with rHuEPO.

Although animal studies and *in vitro* studies in humans have been undertaken, it is not known whether *in vivo* vascular reactivity to norepinephrine or endothelin-1 is altered in patients with chronic renal failure receiving rHuEPO. Hence, we have investigated the effect of treatment with rHuEPO on systemic hemodynamics and responsiveness of the forearm resistance vessels to local doses of norepinephrine and endothelin-1 given via the brachial artery.

Methods

Subjects

We studied nine consecutive patients (5 men, 4 women; 24 to 67 years) with established end-stage renal disease (Table 1), who had been maintained on hemodialysis given three times each week for at least three months before starting rHuEPO. Subjects had all

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Table 1. Patient characteristics

Patient	Diagnosis	Age	Sex	Antihypertensive medication	Ranitidine	Iron sulphate	Phosphate binders	1-Alpha calcidol	NSAIDs
1	Obstructive uropathy	67	M	None	−	+	+	+	+
2	Chronic pyelonephritis	39	F	None	−	−	−	−	−
3	Chronic pyelonephritis	34	M	None	−	+	+	+	−
4	Bilateral nephrectomy	59	F	None	−	+	+	+	−
5	Mesangiocapillary glomerulonephritis	32	M	Enalapril, Nifedipine SR	+	−	+	+	−
6	Focal necrotizing glomerulonephritis	26	F	None	−	−	+	+	−
7	Chronic pyelonephritis	58	F	None	+	+	−	+	−
8	Crescentic glomerulonephritis	42	M	Nifedipine SR	+	+	−	−	+
9	Mesangiocapillary glomerulonephritis	32	M	None	+	+	+	−	+

Subjects 1–6 underwent both intra-arterial and hemodynamic studies; subjects 7–9 underwent blood pressure monitoring and blood sampling only. Non-steroidal anti-inflammatory drugs (NSAIDs) were avoided for 1 week before each intra-arterial study. Abbreviations are: M, male; F, female; +, drug used; −, drug not used.

maintained a stable arterial pressure for the preceding three months, had not previously received rHuEPO, and had a stable hemoglobin of 8 g/dl or less. All studies were conducted with the approval of the local Ethics Review Committee and patients gave their written informed consent to participate. Patients avoided non-steroidal anti-inflammatory drugs for the week before, and caffeine-containing drinks or cigarettes on the day of, each study. In addition, patients were fasted for at least three hours before each study.

Six subjects (3 men, 3 women; 24 to 67 years: mean age 43 ± 5 years) completed the full study protocol. The other three subjects (2 men, 1 woman, 32 to 58 years), because of poor vascular access, underwent blood sampling and arterial pressure monitoring but not intra-arterial drug infusion. All patients were self-medicating (Table 1) and their diet was high in calories but limited to a daily intake of 1 g/kg body wt protein, 80 to 100 mmol sodium and 1 mmol/kg body wt potassium, with a daily fluid restriction of 500 ml plus a volume equal to the previous 24 hours urine output.

Six healthy age and sex matched subjects (4 men, 2 women; 29 to 63 years, mean age 43 ± 5 years) were studied at a later date to establish vascular responsiveness to norepinephrine and endothelin-1 in a healthy control group. These subjects had normal renal function (plasma creatinine 83 ± 8 μmol/liter), did not receive rHuEPO, and underwent intra-arterial drug infusion on one occasion only.

Procedures

A 21 SWG cannula was inserted under local anesthesia using 1% lignocaine hydrochloride (Astra Pharmaceuticals Limited, Kings Langley, UK) into a vein on the fistula arm, but not into the fistula, for the purpose of blood sampling in the hemodialysis patients, or into the dominant arm in controls. A 27 SWG steel cannula (Cooper's Needle Works, Birmingham, UK) was inserted into the brachial artery of the non-fistula arm, or non-dominant arm in controls, under local anesthesia for the purpose of intra-arterial infusion of drugs. Forearm blood flow was measured only in the infused arm, using venous occlusion plethysmography [22] adapted for use with indium-and-gallium-in-silastic strain gauges [23]. Measurements were made for 10 seconds in every 15 seconds over a three minute period. Blood flow to the hand was excluded during each three minute measurement period.

Arterial pressure was measured using a well-validated semi-automated oscillometric method (Takeda UA 751 [24, 25]). Cardiac output and stroke volume were measured using a well-validated non-invasive bioimpedance method (model NCCOM3; BoMed Medical Manufacturer Ltd, Irvine, CA, USA) [26, 27].

Table 2. Hematological effects of rHuEPO

	Pre-rHuEPO	6 Weeks rHuEPO	12 Weeks rHuEPO
Hemoglobin *g/dl*	7.0 ± 0.3	7.8 ± 0.6	9.1 ± 0.6*
Hematocrit %	20.4 ± 0.9	22.8 ± 1.8	26.5 ± 1.7*
Mean cellular volume *fl*	92 ± 2	94 ± 2	91 ± 2
Platelets $\times 10^9$/liter	212 ± 30	219 ± 26	228 ± 25
White blood count $\times 10^9$/liter	6.4 ± 0.8	5.7 ± 0.6	6.3 ± 0.6
Plasma viscosity *cp*	1.59 ± 0.05	1.56 ± 0.04	1.58 ± 0.04

* $P < 0.02$ (pre vs. 12 weeks)

Drugs

All patients received subcutaneous recombinant human erythropoietin-beta (rHuEPO; Recormon-Boehringer Mannheim UK, Livingston, UK), three times weekly after dialysis, at a dose of 50 U/kg. Our local protocol dictated that this dose should be adjusted to allow no more than a 4% rise of hematocrit every two weeks. Intra-arterial norepinephrine (30, 60, 120 and 240 pmol/min; Sanofi Winthrop Limited, Guildford, UK), endothelin-1 (5 pmol/min; Clinalfa, Laufelfingen, Switzerland) and physiologic saline (0.9%; Baxter Healthcare Limited, Thetford, UK) were administered at locally but not systemically active doses via the brachial artery cannula. The dose of endothelin-1 was based on previous work showing that it produced progressive vasoconstriction, with a maximum reduction in forearm blood flow of ~40% after 60 minutes [23]. The doses of norepinephrine were based on previous studies [28], and intended to cause a similar reduction in local blood flow. Vasoconstriction to endothelin-1 is sustained whereas that to norepinephrine is short-lasting. Therefore, it was necessary to give norepinephrine first rather than randomize the order of infusions. All solutions were made up in physiologic saline containing 100 μg/ml ascorbic acid (Evans Medical Ltd, Dunstable, UK) to avoid oxidation. The total rate of intra-arterial infusion was kept constant at 1 ml/min.

Design

Studies were performed before, and at 6 and 12 weeks after, starting rHuEPO therapy, on a mid-week non-dialysis day in a quiet room maintained at a constant temperature of between 25

Table 3. Hemodynamic effects of rHuEPO

	Pre-rHuEPO	6 Weeks rHuEPO	12 Weeks rHuEPO	Control
Basal FBF before norepinephrine *ml/100 ml/min*	3.2 ± 0.5	3.6 ± 0.7	3.5 ± 0.5	2.6 ± 0.3
Basal FVR before norepinephrine *AU*	35 ± 5	34 ± 7	32 ± 6	32 ± 5
FBF before endothelin-1 *ml/100 ml/min*	4.5 ± 0.5	3.0 ± 0.6	3.6 ± 0.5	3.2 ± 0.3[c]
FVR before endothelin-1 *AU*	26 ± 5	43 ± 5[a]	36 ± 9	27 ± 2
Baseline systolic pressure *mm Hg*	120 ± 12	130 ± 13[b]	131 ± 12[c]	121 ± 5
Baseline diastolic pressure *mm Hg*	66 ± 5	77 ± 7	77 ± 5[c]	69 ± 3
Baseline mean arterial pressure *mm Hg*	84 ± 7	95 ± 9[b]	95 ± 7[c]	86 ± 3
Total peripheral resistance index *dyn/min/cm^5/m^2*	24 ± 5	32 ± 7[a]	29 ± 6	
Cardiac index *liter/min/m^2*	3.8 ± 0.3	3.3 ± 0.3[c]	3.8 ± 0.7	
Stroke index *ml/m^2*	48 ± 5	45 ± 3	52 ± 9	
Heart rate *beats/min*	81 ± 3	76 ± 4	73 ± 3	62 ± 3[e]

Abbreviations are: FBF, forearm blood flow; FVR, forearm vascular resistance.
[a] $P = 0.03$ (compared to basal FVR before norepinephrine)
[b] $P = 0.06$, [c] $P \leq 0.05$, [d] $P \leq 0.01$: all versus pre-rHuEPO. [e] $P \leq 0.04$ versus pre, 6 weeks and 12 weeks rHuEPO

and 28°C. The time and day of each study was the same for each patient.

Subjects initially rested supine for 30 minutes after which, in the patients only, cardiac output and stroke volume were measured. Brachial artery and venous cannulation were then performed. Saline was infused via the brachial artery cannula for 30 minutes, after which blood was withdrawn from the venous cannula to measure plasma endothelin-1, plasma renin activity, and serum sodium, potassium, urea, creatinine, calcium, albumin and phosphate. Hemoglobin, hematocrit, platelet count, white blood count and plasma viscosity were also measured. Norepinephrine was then infused at incremental doses, each dose for 10 minutes, via the brachial artery cannula, after which saline was infused for 20 minutes to allow washout of vasoconstriction to norepinephrine, and was followed by infusion of endothelin-1 for one hour. Forearm blood flow was recorded at 10 minute intervals during infusion of saline and endothelin-1, and at the end of each 10-minute incremental infusion of norepinephrine. The mean of the final five measurements of each recording period was used for analysis. Studies were performed in a similar way in the control subjects, although venous blood was sampled only to measure creatinine and hemoglobin concentrations. Arterial pressure was measured in the infused arm immediately after each blood flow recording.

Analytical

Venous plasma samples for radioimmunoassay of endothelin-1 and renin were separated within 10 minutes and stored at −20°C. Plasma electrolytes, hemoglobin, platelet count and plasma viscosity were assayed immediately after each study. Plasma biochemistry was measured using a Kodak Ectachem System E700 XRC analyzer (Kodak Diagnostics Ltd, UK) in the Department of Clinical Chemistry, Western General Hospital, Edinburgh. Full blood count and plasma viscosity were measured in the Department of Hematology, Western General Hospital, Edinburgh, using a Coulter STKS analyzer (Coulter Electronics Ltd, UK) and a Luckhams viscometer (Denley, UK), respectively.

Plasma immunoreactive endothelin was measured using an established method [29] with a previously validated assay [30]. The recovery of added endothelin-1 was 84%. The sensitivity of the assay was 2 pg/ml endothelin and the mean intra- and inter-assay coefficient of variations in our laboratory are 2.4% and 4.2%, respectively. Cross reactivity of the assay with endothelin-1, endothelin-2, endothelin-3 and proendothelin-1 was 100%, 52%, 96% and 7%, respectively. Plasma renin activity was measured using a commercially available radioimmunoassay method (RIANEN, Europath Ltd, Cornwall, UK). The minimal detectable activity was 0.3 ng/ml/hr and the mean intra- and inter-assay coefficient of variations in our laboratory was 12.5%.

Data presentation and statistics

Mean arterial pressure was calculated as diastolic arterial pressure + ⅓ pulse pressure. Forearm blood flow was calculated in ml/100 ml of forearm tissue/min. Changes in forearm blood flow are expressed as the percentage change in blood flow at a particular timepoint from the basal recording. Forearm vascular resistance was calculated as mean arterial pressure divided by forearm blood flow. Data for cardiac output and stroke volume were corrected for body surface area, calculated according to a standard nomogram, to provide measures of cardiac (CI) and stroke index (SI) and are expressed as liter/min/m^2 and ml/m^2, respectively. Total peripheral resistance index (TPRI) was calculated as mean arterial pressure divided by cardiac index, and expressed as dyne/min/cm^5/m^2.

All results are expressed as mean ± SEM. The maximal response to the norepinephrine and endothelin-1 infusions, as well as biochemical, hematological and hemodynamic data, were analyzed by Student's paired *t*-test and by simple regression analysis, as appropriate. Dose-response relationships were compared using analysis of variance (ANOVA). The relationships between percentage change in responsiveness of forearm blood flow and vascular resistance to norepinephrine and endothelin associated with rHuEPO, and the percentage change in blood pressure, peripheral resistance and hemoglobin with this treatment were also examined. Comparisons between control subjects and patients for dose-response relationships were examined by analysis of variance (ANOVA) whereas other comparisons with controls were analyzed using Student's unpaired *t*-test. Statistical analysis was performed using the STATVIEW 512+™ software (Brainpower Inc., Calabasas, CA, USA) for the Apple Macintosh microcomputer. Values of $P < 0.05$ were considered statistically significant. Simple regression analysis was used primarily for hypothesis generation and the *P* values accepted for statistical significance were, therefore, not corrected for multiple comparisons.

Results

Hematology

rHuEPO caused a progressive rise in hemoglobin and hematocrit (Table 2). In no case was it necessary to change the dose of rHuEPO according to the requirements of our local schedule. Two patients (patients 3 and 8) failed to have a sustained response to rHuEPO, both as a result of iron deficiency, but also, in patient 8, because of an associated hereditary elliptocytosis. Other hematological parameters, white blood count, platelet count and plasma viscosity remained unchanged throughout the study (Table 2). The hemoglobin of the control subjects (12.9 ± 0.5 g/dl) was significantly higher than that of patients ($P < 0.001$) throughout the study (Table 2).

Arterial pressure and hemodynamics

There was a progressive increase in resting systolic, diastolic and mean arterial pressure with rHuEPO therapy (Table 3). In one patient (patient 5), there was a rapid increase in the diastolic pressure from 80 to 130 mm Hg requiring aggressive medical intervention. It was necessary to increase the dose of nifedipine to 40 mg twice daily and enalapril to 20 mg twice daily, and to add doxazosin 2 mg three times daily and metoprolol 50 mg twice daily, to control the arterial pressure. Over the period of the study, resting diastolic, but not systolic or mean arterial pressure, correlated with hemoglobin ($r = 0.45$; $P = 0.02$) and hematocrit ($r = 0.44$; $P = 0.02$). Also, % change in hemoglobin with treatment correlated with % change in resting systolic ($r = 0.60$; $P = 0.05$), diastolic ($r = 0.75$; $P = 0.008$) and mean arterial ($r = 0.76$; $P = 0.007$) pressure. TPRI increased markedly and significantly ($P = 0.01$) after six weeks of treatment, but then decreased slightly by 12 weeks, though still tending to be higher than before treatment (Table 3), consistent with the major effect of rHuEPO being on peripheral vascular resistance. There was a reduction in CI at six weeks ($P = 0.05$), returning to pre-treatment levels by 12 weeks, whereas SI did not change (Table 3).

Forearm blood flow responses

There was no effect of rHuEPO on resting forearm blood flow or vascular resistance (Table 3). In the studies performed six weeks after starting rHuEPO, but not in the other studies, forearm vascular resistance had not returned to basal levels ($P = 0.03$) after norepinephrine and before endothelin-1, and may have tended to mask subsequent changes in forearm vascular resistance to endothelin-1. Local infusion of norepinephrine and endothelin-1 had no effect on pulse rate or arterial pressure.

Norepinephrine caused a dose-dependent reduction in forearm blood flow. This response was increased significantly by rHuEPO. The maximum dose of norepinephrine (240 pmol/min) reduced blood flow by 13 ± 4% before rHuEPO, by 44 ± 8% ($P = 0.01$ vs. pre-treatment: $P = 0.07$; ANOVA) at six weeks, and by 32 ± 7% ($P = 0.07$ vs. pre-treatment: $P = 0.24$; ANOVA) at 12 weeks of rHuEPO therapy (Fig. 1). There was no significant relationship between the % change in forearm blood flow with norepinephrine (240 pmol/min) and resting systolic ($r = -0.36$; $P = 0.15$), diastolic ($r = -0.14$; $P = 0.59$) or mean arterial pressure ($r = -0.27$; $P = 0.30$), total peripheral resistance ($r = -0.19$; $P = 0.45$) or hemoglobin ($r = 0.03$; $P = 0.92$). Forearm vascular resistance increased significantly with rHuEPO in response to norepinephrine (240 pmol/min); by 17 ± 9% before rHuEPO, by 123 ± 51% ($P = 0.08$ vs. pre-treatment: $P = 0.07$; ANOVA) at six weeks, and by 65 ± 15% ($P = 0.03$ vs. pre-treatment: $P = 0.08$; ANOVA) at 12 weeks of rHuEPO therapy. There was no significant relationship between the % change in forearm vascular resistance with norepinephrine (240 pmol/min) and resting systolic ($r = -0.35$; $P = 0.17$), diastolic ($r = -0.24$; $P = 0.35$) or mean arterial pressure ($r = -0.31$; $P = 0.23$), total peripheral resistance ($r = -0.24$; $P = 0.35$) or hemoglobin ($r = -0.27$; $P = 0.30$). There was no significant relationship between the % change in vascular responsiveness to norepinephrine (240 pmol/min) associated with

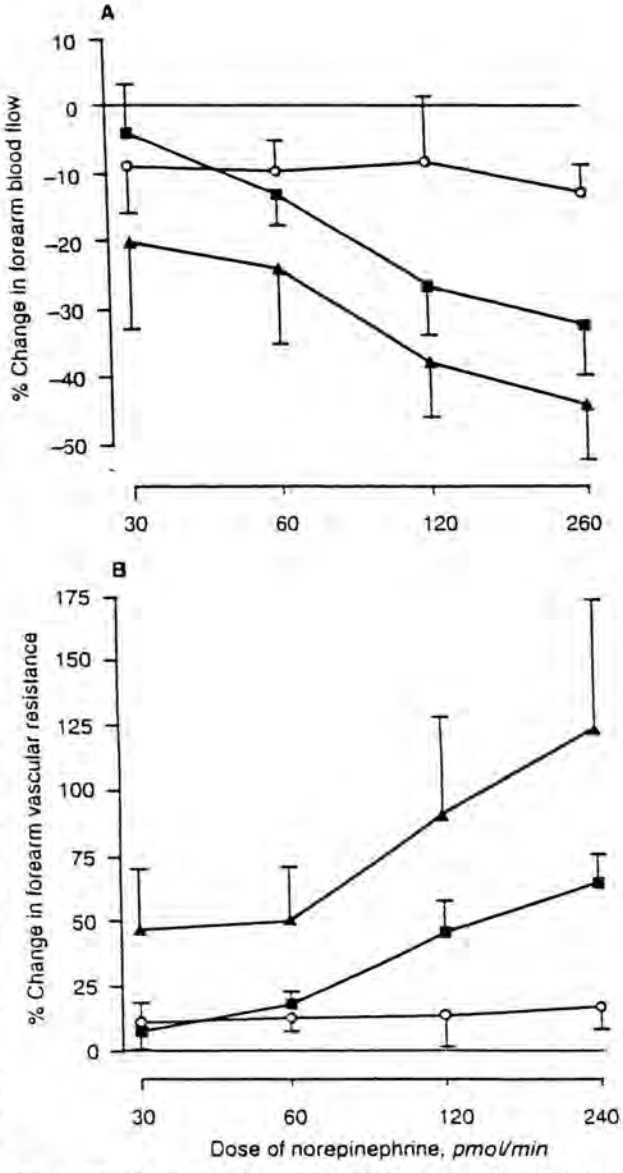

Fig. 1. *Effect of intra-arterial norepinephrine on forearm blood flow* (**A**) *and forearm vascular resistance* (**B**). Symbols are: patients before EPO therapy (○), after six weeks of EPO (▲), and after 12 weeks of EPO (■).

rHuEPO therapy and the % change in arterial pressure, TPRI or hemoglobin with this treatment.

The control subjects were more responsive to norepinephrine than the patients. The maximum dose of norepinephrine (240 pmol/min) reduced blood flow by 38 ± 5% in the controls, an effect that was significantly greater than in patients before (P = 0.01; ANOVA) but not after 6 (P = 0.70; ANOVA) or 12 weeks (P = 0.20; ANOVA) of rHuEPO therapy. The maximum dose of norepinephrine (240 pmol/min) increased forearm vascular resistance by 32 ± 5% in the controls, an effect that was also significantly greater than the response in patients before (P = 0.04; ANOVA) but not after 6 (P = 0.31; ANOVA) or 12 weeks (P = 0.51; ANOVA) of rHuEPO therapy.

Endothelin-1 caused a progressive reduction in forearm blood flow, maximal by 60 minutes. Forearm blood flow at 60 minutes reduced by 15 ± 15% before rHuEPO, by 14 ± 12% at six weeks (P = 0.93 vs. pre-treatment: P = 0.74; ANOVA), and by 7 ± 10% at 12 weeks (P = 0.59 vs. pre-treatment: P = 0.42; ANOVA) (Fig. 2). The % change in forearm blood flow at 60 minutes correlated inversely with resting systolic arterial pressure (r = −0.55; P = 0.02) and total peripheral resistance (r = −0.51; P = 0.04) but not diastolic (r = −0.33; P = 0.20) or mean arterial pressure (r = −0.46; P = 0.06), or hemoglobin (r = −0.30; P = 0.25). Forearm vascular resistance at 60 minutes increased by 45 ± 24% before rHuEPO, by 33 ± 18% (P = 0.63 vs. pre-treatment: P = 0.72; ANOVA) at six weeks, and by 20 ± 14% at 12 weeks (P = 0.20 vs. pre-treatment: P = 0.29; ANOVA) of rHuEPO therapy (Fig. 2). The % change in forearm vascular resistance to endothelin-1 at 60 minutes correlated inversely with resting systolic (r = −0.53; P = 0.03) and mean arterial pressure (r = −0.52; P = 0.03), and total peripheral resistance (r = −0.51; P = 0.03; Fig. 3), but not with diastolic pressure (r = −0.45; P = 0.07) or hemoglobin (r = −0.43; P = 0.08). There was no significant relationship between the % change in vascular responsiveness to endothelin-1 that occurred with rHuEPO therapy, based on the 60 minute responses, and the % change in arterial pressure, TPRI or hemoglobin.

Forearm blood flow with 60 minutes of endothelin-1 was reduced by 23 ± 10% in the control subjects which was not significantly different to before (P = 0.87; ANOVA) or after 6 (P = 0.56; ANOVA) or 12 weeks (P = 0.23; ANOVA) of rHuEPO therapy. Forearm vascular resistance at 60 minutes was increased by 27 ± 2% in the control subjects which was not significantly different to before (P = 0.92; ANOVA) or after 6 (P = 0.57; ANOVA) or 12 weeks (P = 0.13; ANOVA) of rHuEPO therapy.

If subject 5 was excluded, on the assumption that the change in antihypertensive medication may have influenced his results, the maximum dose of norepinephrine reduced forearm blood flow by 10 ± 4% before rHuEPO, by 46 ± 10% (P = 0.007 vs. pre-treatment: P = 0.05; ANOVA) at six weeks, and by 35 ± 8% (P = 0.04 vs. pre-treatment: P = 0.18; ANOVA) at 12 weeks of rHuEPO therapy. Forearm vascular resistance increased by 10 ± 7% before rHuEPO, by 139 ± 59% (P = 0.08 vs. pre-treatment: P = 0.05; ANOVA) after six weeks, and by 69 ± 18% (P = 0.02 vs. pre-treatment: P = 0.06; ANOVA) after 12 weeks of rHuEPO therapy. After infusing endothelin-1 for 60 minutes, forearm blood flow reduced by 26 ± 12% before rHuEPO, by 14 ± 15% (P = 0.50 vs. pre-treatment: P = 0.50; ANOVA) at six weeks, and by 3 ± 12% (P = 0.004 vs. pre-treatment: P = 0.15; ANOVA) at 12 weeks of rHuEPO therapy. Forearm vascular resistance increased by 56 ± 26% before rHuEPO, by 38 ± 21% (P = 0.55 vs. pre-treatment: P = 0.68; ANOVA) after six weeks, and by 17 ± 17% (P = 0.02 vs. pre-treatment: P = 0.21; ANOVA) after 12 weeks of rHuEPO therapy.

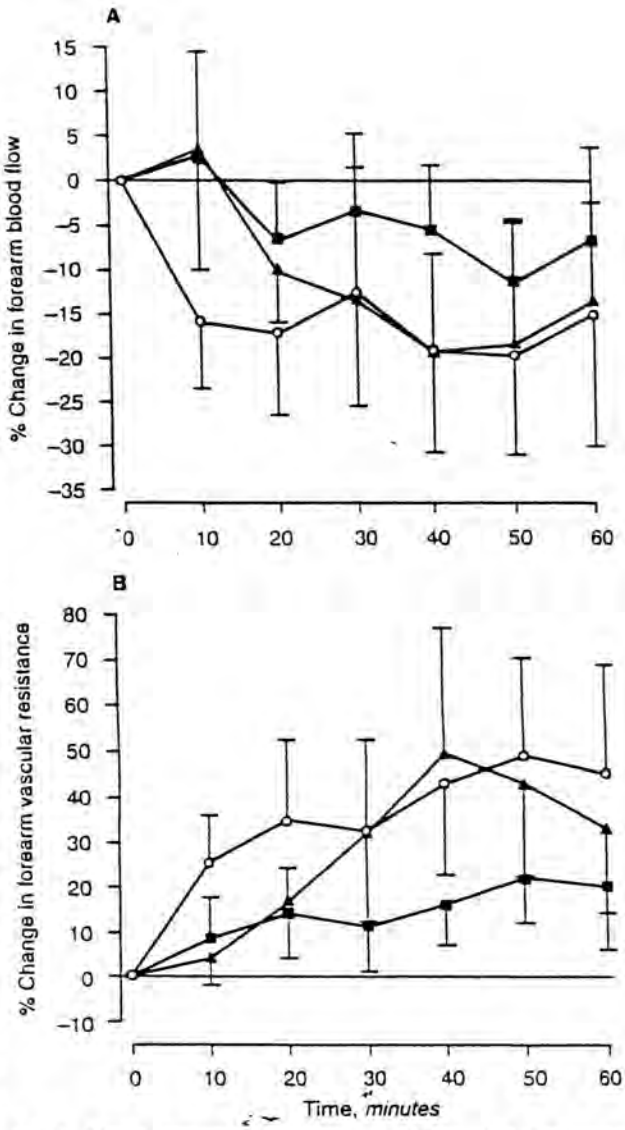

Fig. 2. Effect of intra arterial endothelin-1 on forearm blood flow (A) and forearm vascular resistance (B). Symbols are: patients before EPO therapy (○), after six weeks of EPO (▲) and after 12 weeks of EPO (■).

Plasma endothelin and plasma renin activity

Plasma endothelin concentration, although substantially elevated at 16.2 ± 4.1 pg/ml before rHuEPO (normal as measured in our laboratory 5.4 ± 0.8 pg/ml) [29], did not change significantly across the study, being 11.1 ± 0.9 and 11.6 ± 0.6 pg/ml at 6 and 12 weeks of treatment with rHuEPO, respectively. There was no significant relationship between plasma endothelin concentration

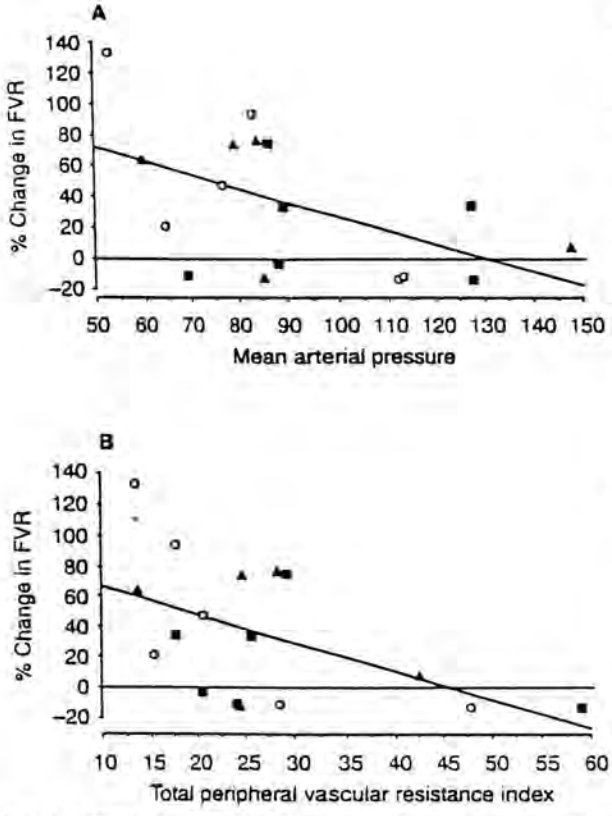

Fig. 3. *Correlation between the % increase in forearm vascular resistance (FVR) with endothelin-1 at 60 minutes and* (A) *mean arterial pressure (mm Hg) and* (B) *total peripheral vascular resistance index (dyn/min/cm^5/m^2).* Symbols are: patients before EPO therapy (○), after six weeks of EPO (▲) and after 12 weeks of EPO (■).

and arterial pressure or % change in vasoconstriction to endothelin-1 or norepinephrine across the period of the study. Plasma renin activity did not change significantly across the study; at 1.7 ± 0.9 ng/ml/hr before rHuEPO, and 1.3 ± 0.6 and 1.2 ± 0.5 ng/ml/hr at 6 and 12 weeks treatment with rHuEPO, respectively. There was no significant relationship between plasma renin activity and changes in arterial pressure or % change in vasoconstriction to endothelin-1 or norepinephrine across the period of the study.

Plasma biochemistry

Apart from small changes in plasma urea and albumin concentrations, the measured biochemical parameters did not alter. The number of hours of dialysis for each patient and the clinically assessed dry weight did not change during the study. Plasma endothelin concentrations correlated positively with creatinine (r = 0.56; P = 0.003), urea (r = 0.51; P = 0.008) and the number of hours dialyzed per week (r = 0.50; P = 0.01).

Discussion

In anemic hemodialysis patients with chronic renal failure selected to start treatment with rHuEPO, we have demonstrated an impaired responsiveness to norepinephrine which is corrected by chronic administration of a dose of rHuEPO sufficient to cause an increase in arterial pressure and hematocrit. In contrast, in the same subjects, the responsiveness of forearm resistance vessels to endothelin-1 is unaffected, or even decreased, by rHuEPO therapy.

The increase in total peripheral resistance with rHuEPO in our study confirms previous observations [5]. Although failure of the cardiac output to decrease with rHuEPO may contribute to rHuEPO-associated hypertension [6, 7], the significant decrease in cardiac index after treatment with rHuEPO for six weeks, but not 12 weeks, in our study suggests that the relationship may be more complex. These results are likely to be reliable, within the limitations of bioimpedance technology [26, 27], but need to be confirmed using other techniques.

In contrast to previous studies demonstrating either impaired [31, 20] or normal vascular responsiveness [32] to norepinephrine in hemodialysis patients, our study did not use systemically active doses of drugs that might obscure any direct vascular action by direct effects on other organs, such as the heart and kidney, or activate reflex mechanisms due to changes in arterial pressure. As in our previous studies [23, 28], the lack of a systemic effect is indicated by the absence of a change in arterial pressure or pulse rate during infusion of either norepinephrine or endothelin-1. Thus, our study directly and specifically examines the responsiveness of forearm resistance vessels in rHuEPO-treated hemodialysis patients.

Changes of vascular responsiveness during rHuEPO therapy might occur as a result of a change in vascular structure or of a non-specific effect of treatment on the vascular smooth muscle cells. In studies of a similar nature in patients with essential hypertension, vascular responsiveness to norepinephrine is either unaltered [33] or increased [34, 35] compared with healthy control subjects, probably as a consequence of vascular structural changes including hypertrophy [35]. In our study, correction of the impaired responses to norepinephrine in hemodialysis patients by rHuEPO treatment are unlikely to be explained by vascular structural or non-specific functional effects of rHuEPO given the contrasting changes in response to norepinephrine and endothelin-1.

Our results are consistent with those of Bode-Böger et al [36], who showed rHuEPO caused enhancement of the vascular response to norepinephrine in rabbit aorta, and of Müller et al [13], who found that anemia caused a disturbance of post-synaptic alpha$_2$-receptor function in end-stage renal disease, leading to diminished vascular responsiveness to sympathetic stimuli that was corrected by treatment of the anemia. In addition, Berglund and Ekblom [37] demonstrated an increase in arterial pressure with exercise in healthy men treated with rHuEPO, suggesting an accentuation of the arterial pressure reaction to sympathetic stimulation. The increase in arterial vessel responsiveness with rHuEPO therapy to locally active doses of norepinephrine in our study, however, contrasts with the observation of a decreased pressor effect of systemic norepinephrine after rHuEPO therapy [20], although this effect may occur through a central mechanism.

High plasma concentrations of norepinephrine occur in end-stage renal disease [38], and may contribute to impaired responsiveness to norepinephrine because alpha$_2$-adrenoceptor numbers are reduced on exposure to high levels of physiological agonists.

Receptor down-regulation is supported indirectly through evidence of impaired pressor responses to norepinephrine [31], and more directly by reductions in platelet alpha$_2$-receptor density and affinity [13], in hemodialysis patients. Interestingly, there was an inverse correlation between hemoglobin and both platelet alpha$_2$-receptor density and affinity in the latter study, suggesting that renal anemia may contribute to this effect. This would certainly be consistent with our finding of reduced responsiveness to norepinephrine before rHuEPO, and the subsequent increase in responsiveness with partial correction of the anemia by rHuEPO. It may also explain the higher standing blood pressures that have been reported when the associated anemia of primary autonomic failure is treated with rHuEPO [39, 40]. The absence of a correlation between vasoconstriction to norepinephrine and either total peripheral resistance or arterial pressure in our study suggests that enhanced adrenergic responsiveness may not fully account for the pressor effects of rHuEPO. On the basis of sympathetic microneurography, nerve activity does not appear to contribute because, although basally increased in hemodialysis patients, it is not further enhanced by rHuEPO [41].

In contrast with the responses to norepinephrine, the vascular responsiveness to endothelin-1 tended to decrease rather than increase with rHuEPO therapy. With some variability within the data, the lack of statistical significance may reflect a type II statistical error; indeed, the responsiveness to endothelin-1 did decrease significantly when analyzed after exclusion of the patient who had uncontrolled hypertension requiring a substantial change in treatment during the study. Endothelin-1 induced vasoconstriction correlated significantly but negatively with systolic arterial pressure, mean arterial pressure and total peripheral resistance by simple regression analysis, as has been observed previously in normotensive but not hypertensive subjects [29]. This may have several possible explanations. First, there may be a specific reduction in responsiveness to endothelin-1 caused by an effect on signal transduction mechanisms coupled to endothelin receptors, but not to those for norepinephrine. This has been shown in the blood vessels of hypertensive patients [42]. Second, there may be true down-regulation of receptor expression. Third, there may be decreased endothelin-1 receptor number owing to prior receptor occupation by increased local concentrations of endothelin. This might be expected if increased endothelin-1 production occurs. Increased local production of endothelin-1 in response to rHuEPO could account for the decreased blood vessel responsiveness to exogenous endothelin, while still being a factor in causing the increase in arterial pressure and total peripheral resistance. Conversely, increased systolic arterial pressure may itself cause down-regulation of the endothelin receptor such as has been described in some animal models [43, 44]. Such receptor down-regulation would yield similar responses to exogenous endothelin, but would occur as a result of changes in arterial pressure and not as a cause. Further studies, using local infusion of endothelin receptor antagonists, may serve to differentiate between receptor down-regulation and increased receptor occupancy.

Although plasma endothelin concentrations were elevated in our patients, they did not increase further following rHuEPO, in contrast with some other studies [12, 17, 21]. This may have been because rHuEPO was given subcutaneously and did not achieve the same maximum plasma concentrations as when given by the intravenous route [45]. Experimental evidence that rHuEPO induces an increase in endothelin-1 production has usually required plasma concentrations of rHuEPO similar to those found with intravenous rather than subcutaneous administration [36, 45]. However, because 80% of endothelin-1 derived from the vascular endothelial cell is released towards the vascular smooth muscle [46], an unaltered plasma concentration of endothelin with rHuEPO treatment does not exclude increased local vascular production [47]. Increased generation of endothelin-1 might contribute to the increasing vascular responsiveness to norepinephrine because low concentrations of endothelin-1 have been shown in organ bath experiments to potentiate contractions to norepinephrine [48].

Plasma endothelin did not correlate with the increase in hemoglobin, arterial pressure or total peripheral resistance, but did correlate with plasma urea, creatinine and the number of hours dialyzed per week. These associations are probably multifactorial in origin, involving such variables as protein and carbohydrate intake, body mass, the catabolic/anabolic state and clearance of urea and creatinine by dialysis. How these indices of protein metabolism relate to the concentration of endothelin-1 is at present unclear.

In conclusion, this study has demonstrated that rHuEPO increases blood pressure, and corrects the anemia and the impaired vascular responsiveness to norepinephrine, in hemodialysis patients. In contrast, there was a trend for responsiveness to endothelin-1 to be reduced by rHuEPO, and responsiveness to endothelin-1 correlated inversely with the changes in arterial pressure. The mechanisms underlying the changes in vascular responsiveness to norepinephrine and endothelin-1 with rHuEPO treatment merit further investigation in studies using selective antagonists at adrenergic and endothelin receptors [23].

Acknowledgments

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Inhibition of Neutral Endopeptidase Causes Vasoconstriction of Human Resistance Vessels In Vivo

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Background—Neutral endopeptidase (NEP) degrades vasoactive peptides, including the natriuretic peptides, angiotensin II, and endothelin-1. Systemic inhibition of NEP does not consistently lower blood pressure, even though it increases natriuretic peptide concentrations and causes natriuresis and diuresis. We therefore investigated the direct effects of local inhibition of NEP on forearm resistance vessel tone.

Methods and Results—Four separate studies were performed, each with 90-minute drug infusions. In the first study, 10 healthy subjects received a brachial artery infusion of the NEP inhibitor candoxatrilat (125 nmol/min), which caused a slowly progressive forearm vasoconstriction (12±2%; P=0.001). In a second two-phase study, 6 healthy subjects received, 4 hours after enalapril (20 mg) or placebo, an intra-arterial infusion of the NEP inhibitor thiorphan (30 nmol/min). Thiorphan caused similar degrees of local forearm vasoconstriction (P=0.6) after pretreatment with both placebo (13±1%, P=0.006) and enalapril (17±6%, P=0.05). In a third three-phase study, 8 healthy subjects received intra-arterial thiorphan (30 nmol/min), the endothelin ET_A antagonist BQ-123 (100 nmol/min), and both combined. Thiorphan caused local forearm vasoconstriction (13±1%, P=0.0001); BQ-123 caused local vasodilatation (33±3%, P=0.0001). Combined thiorphan and BQ-123 caused vasodilatation (32±1%, P=0.0001) similar to BQ-123 alone (P=0.98). In a fourth study, 6 hypertensive patients (blood pressure >160/100 mm Hg) received intra-arterial thiorphan (30 nmol/min). Thiorphan caused a slowly progressive forearm vasoconstriction (10±2%, P=0.0001).

Conclusions—Inhibition of local NEP causes vasoconstriction in forearm resistance vessels of both healthy volunteers and patients with hypertension. The lack of effect of ACE inhibition on the vasoconstriction produced by thiorphan and its absence during concomitant ET_A receptor blockade suggest that it is mediated by endothelin-1 and not angiotensin II. These findings may help to explain the failure of systemic NEP inhibition to lower blood pressure. (***Circulation.* 1998;97:2323-2330.**)

Key Words: natriuretic peptides ■ vasoconstriction ■ endothelin ■ angiotensin II ■ human

Neutral endopeptidase (EC 3.4.24.11, enkephalinase) is a plasma membrane-bound zinc metalloprotease that was initially isolated from renal epithelial brush border cells and cleaves peptide substrates at the amino side of hydrophobic amino acids.[1] It catalyzes the degradation of a number of endogenous vasodilator peptides, including ANP,[2] brain natriuretic peptide,[3] C-type natriuretic peptide,[4] substance P,[5] and bradykinin,[1] as well as vasoconstrictor peptides, including ET-1[6] and Ang II.[1] In addition to degrading vasoactive peptides to inactive breakdown products, NEP can also convert big ET-1 to the active peptide ET-1.[7] Therefore, the physiological actions of NEP in vivo will be the balance of its effects on the breakdown of vasodilators and vasoconstrictors and on the synthesis of ET-1 from big ET-1 (Figure 1).

NEP is inhibited by several agents, including candoxatrilat[8]; thiorphan[9] and its prodrug, sinorphan[10]; and phosphoramidon.[1] ANP has potent natriuretic[11] and vasodilator properties[12,13] and inhibits activity of the renin-angiotensin-aldosterone system by reducing both renin[14] and aldosterone[15] release. Therefore, increasing the circulating concentrations of ANP through inhibition of NEP is an attractive therapeutic approach to a number of cardiovascular diseases such as hypertension and heart failure.[16] However, although NEP inhibitors increase circulating ANP concentrations in humans and cause the expected natriuresis,[10,17-19] they do not generally lower blood pressure in normotensive subjects.[10,18,20,21] Indeed, both candoxatril[22] and candoxatrilat[23] have been reported as raising blood pressure in normotensive subjects. Although NEP inhibitors have been reported to lower blood pressure in patients with essential hypertension,[24-31] this finding has not been universal.[19,32-35] Thus, the therapeutic value of NEP inhibitors in hypertension remains uncertain. In patients with heart failure, these agents do not reduce afterload although they do reduce pulmonary capillary wedge pressure, presumably because of natriuresis.[17,36]

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Selected Abbreviations and Acronyms

Ang II = angiotensin II
ANP = atrial natriuretic peptide
CV = coefficient of variation
ET = endothelin
ET_A = endothelin receptor A
NEP = neutral endopeptidase

We hypothesized that if the predominant substrates for vascular NEP were vasodilator peptides, then local inhibition of this enzyme should cause peripheral vasodilatation. However, in previous studies using brachial artery administration of the NEP inhibitor thiorphan, we observed a modest vasoconstriction,[37,38] suggesting accumulation of vasoconstrictor peptides such as Ang II or ET-1. Therefore, in the present study, we examined the effects of brachial artery administration of a structurally different NEP inhibitor, candoxatrilat, on forearm blood flow to determine whether the vasoconstriction produced by thiorphan is a class effect of NEP inhibitors. We also investigated whether an accumulation of Ang II was the cause of the forearm vasoconstriction produced by thiorphan by infusing thiorphan into the brachial artery in the presence or absence of concurrent systemic ACE inhibition. Furthermore, we investigated whether accumulation of ET-1 was the cause of the forearm vasoconstriction in response to thiorphan by coinfusing an ET_A antagonist, BQ-123, together with thiorphan. We also examined the effects of brachial artery administration of thiorphan in a group of hypertensive patients to confirm the clinical relevance of our findings in healthy subjects.

Methods

Subjects

Twenty-four healthy male subjects and 6 hypertensive male patients (mean age, 45±3 years; 24-hour ambulatory mean arterial pressure, 116±3 mm Hg) who had not yet received any treatment participated in these studies, which were conducted with the approval of the local ethics committee and with the written informed consent of each subject. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 hours and from food, caffeine-containing drinks, and cigarettes for at least 3 hours before any measurements were made. All studies were performed in a quiet room maintained at a constant temperature of between 22°C and 25°C.

Drugs

Candoxatrilat (Pfizer) and thiorphan (Sigma) were administered intra-arterially dissolved in physiological saline (0.9%; Baxter Healthcare Ltd). We used (+)candoxatrilat (UK-73,967) in this study; this eutomer has twice the potency as an NEP inhibitor than the racemate, (±)candoxatrilat (UK-69,578),[39] and is the active metabolite of the orally available prodrug candoxatril. The dose of candoxatrilat (125 nmol/min) was chosen to achieve forearm blood concentrations >50-fold higher than the IC_{50} (40 nmol/L) of (+)candoxatrilat in vitro.[39] The dose of thiorphan (30 nmol/min) used in this study has been shown to produce ≈20% reduction in forearm blood flow when infused via the brachial artery.[37] This dose is known to achieve local concentrations in forearm blood after brachial artery administration, >10-fold higher than the IC_{50} of thiorphan (35 nmol/L) for NEP in vitro.[39]

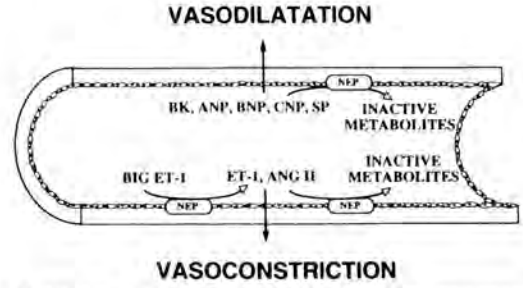

Figure 1. NEP catalyzes the metabolism of the vasoconstrictor peptides ET-1 and Ang II, as well as the metabolism of several vasodilator peptides, including bradykinin (BK), ANP, brain and C-type natriuretic peptides (BNP and CNP, respectively), and substance P (SP). NEP is also involved in the enzymatic conversion of big ET-1 to its active form, the vasoconstrictor peptide ET-1. The balance of effects of NEP inhibition on vascular tone, therefore, will depend on whether the predominant substrate(s) degraded by NEP are vasodilators or vasoconstrictors and on the extent of NEP involvement in the processing of big ET-1.

The peptide ET_A antagonist BQ-123 (Cyclo[—D-Asp—L-Pro—D-Val—L-Leu—D-Trp—]; American Peptide Co) was administered intra-arterially (100 nmol/min) dissolved in physiological saline. This dose achieves local concentrations in the forearm >10-fold higher than the PA_2 at the ET_A receptor and is known to produce ≈40% increase in blood flow when infused via the brachial artery.[37]

The ACE inhibitor enalapril (Merck, Sharp & Dohme Ltd) was administered orally in ascending, single, daily doses of 2.5, 5, 10, and 15 mg over a period of 4 days. This ascending dose design was used to minimize the already low risk of hypotension. On the fifth day, subjects were admitted to the clinical research center and, after lying supine for 30 minutes, received 20 mg enalapril orally at 8:30 AM. The final dose of 20 mg was chosen because it reduces plasma concentrations of Ang II to a level close to the detection limit of radioimmunoassay 4 hours after administration.[40]

Intra-arterial Administration

The left brachial artery was cannulated under local anesthesia (1% lidocaine) with a 27-standard wire gauge needle attached to a 16-gauge epidural catheter. Patency was maintained by infusion of physiological saline via a Welmed P1000 syringe pump. The total rate of intra-arterial infusion was maintained constant throughout at 1 mL/min. In all studies, physiological saline was infused for 30 minutes before infusion of vasoactive agents.

Measurements

Forearm Blood Flow

Blood flow was measured in both forearms by venous occlusion plethysmography[41] by use of indium/gallium-in-Silastic gauges as previously described.[37] Recordings of forearm blood flow were made repeatedly over 3-minute periods. Voltage output from a dual-channel Vasculab SPG 16 strain-gauge plethysmograph (Medasonics, Inc) was transferred to a Macintosh personal computer by use of a MacLab analogue digital converter and Chart software (AD Instruments). Calibration was achieved by use of the internal standard of the Vasculab plethysmography units. In all studies, forearm blood flow was recorded in both arms every 5 minutes during infusion of the study agents.

Blood Pressure

A well-validated semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751) was used to make duplicate measurements of blood pressure in the noninfused arm, which were then averaged.[42] In all studies, blood pressure was measured at 10-minute intervals during infusion of the study agents.

Plasma Assays

Venous blood samples (40 mL) were obtained at intervals for assay of concentrations of plasma active renin, Ang II, aldosterone, ANP,

and ET from both arms. This technique of bilateral venous sampling, from deep veins in the antecubital fossae and with intra-brachial artery infusion of locally active agents, has been reported previously.[41] Samples were collected into chilled tubes, centrifuged at 1500*g* for 20 minutes at 4°C, and stored at −80°C until assay. Samples for renin were collected into tubes coated with potassium EDTA, and plasma active renin concentration was measured by an antibody trapping technique.[44] The intra-assay CV for this assay is 3.4%. Samples for Ang II were collected into plain tubes containing potassium EDTA/*o*-phenanthroline, and plasma concentration of Ang II was measured by radioimmunoassay.[45] The intra-assay CV is 10%. Venous blood samples for plasma aldosterone concentrations were collected into lithium heparin tubes, and plasma aldosterone was measured by use of a solid-phase (coated tube) radioimmunoassay with a commercially available kit (Diagnostic Products UK Ltd). The intra-assay CV is <8.3%. Samples for ANP were collected into EDTA/trasylol tubes, and plasma ANP concentration was measured by radioimmunoassay.[46] The intra-assay CV is 3.9%. Samples for ET assay were collected into tubes coated with EDTA, and plasma ET was assayed with a commercially available kit (Endothelin-1 Radioimmunoassay, Peninsula Laboratories) as previously described,[47] except samples were extracted with acetic acid.[48] This method gives an extraction recovery of ET-1 of 89%. The intra-assay CV is <6%, and the cross-reactivities of this assay with ET-1, ET-2, ET-3, and big ET-1 are 100%, 7%, 7%, and 10%, respectively. All assays were done in single batches.

Study Design

Four single-blind studies were performed.

Protocol 1: Intra-arterial Candoxatrilat

Ten subjects participated in this single-phase, single-blind study. Candoxatrilat (125 nmol/min) was infused for 90 minutes.

Protocol 2: Intra-arterial Thiorphan and Systemic ACE Inhibition

Six subjects participated in this two-phase, single-blind, crossover study. In each phase, subjects were administered orally either increasing single daily doses of enalapril (as detailed above) or matching placebo. On the fifth day, subjects were admitted to the clinical research center at 8:00 AM, and deep veins in both antecubital fossae were cannulated with 18-gauge intravenous cannulas for blood sampling. After the subject lay recumbent for 30 minutes (8:30 AM), a venous blood sample was taken from the right (noninfused) arm for assays of renin, Ang II, aldosterone, ANP, and ET concentrations. Blood pressure was measured, and enalapril 20 mg or placebo was administered at 8:30 AM. Blood pressure was then measured at 30-minute intervals for 3.5 hours with the subjects remaining recumbent. At midday, physiological saline was infused via the left brachial artery for 30 minutes. At 12:30 PM, thiorphan (30 nmol/min) was infused for 90 minutes, 4 hours after administration of the final dose of enalapril or placebo. Before the start of the thiorphan infusion, a venous blood sample was taken for aldosterone, renin, and clinical biochemistry from the right (noninfused) arm. Blood samples were also taken from both arms at the beginning and end of the period of thiorphan infusion for measurement of plasma Ang II, ANP, and ET concentrations.

Protocol 3: Intra-arterial Thiorphan and Intra-arterial BQ-123

Eight subjects participated in this randomized three-phase, single-blind study. In random order and on separate occasions at least 1 week apart, either thiorphan (30 nmol/min) or BQ-123 (100 nmol/min) alone or both in combination was infused for 90 minutes.

Protocol 4: Intra-arterial Thiorphan in Hypertensive Patients

Six hypertensive patients participated in this single-phase, single-blind study. Thiorphan (30 nmol/min) was infused for 90 minutes.

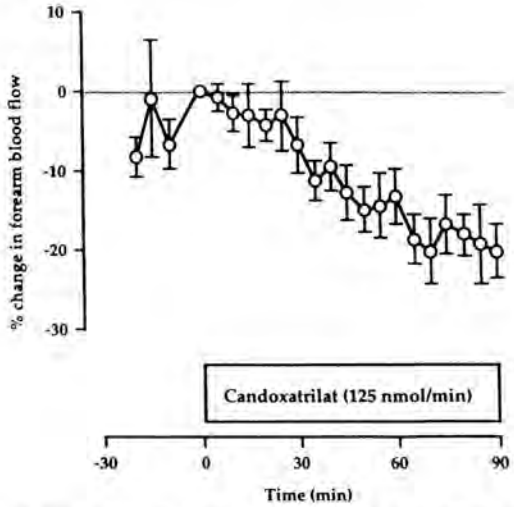

Figure 2. Effect of brachial artery administration of candoxatrilat (125 nmol/min) on forearm blood flow in 10 healthy, male volunteers. Candoxatrilat produced a slowly progressive vasoconstriction confined to the infused forearm ($P=0.001$).

Data Analysis and Statistics

Plethysmographic data listings were extracted from the Chart data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations. Because flow stabilizes only after 60 seconds of wrist cuff inflation,[49] recordings made in the first 60 seconds were not used for analysis. The last five flow recordings in each measurement period were calculated and averaged for the infused and noninfused arms. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point, in effect using the noninfused arm as a contemporaneous control for the infused arm.[41] Forearm blood flow results are shown as a percentage change from basal in the ratio of blood flow between the infused and noninfused arm.

Data are shown as mean±SEM in the figures and as mean±SEM with 95% confidence intervals in the tables. Forearm blood flows were examined by repeated-measures ANOVA with Statview 512+ software (Brainpower Inc, USA). The overall forearm blood flow responses are described in the text as the area under the curve[50] and as individual maximum responses. Hemodynamic and assay measures were analyzed by ANOVA and Student's *t* test as appropriate.[51]

Results

Protocol 1: Intra-arterial Candoxatrilat

Brachial artery infusion of candoxatrilat did not alter systolic, diastolic, or mean arterial pressure (86±2 to 90±2 mm Hg) or heart rate (63±3 to 64±3 bpm). Also, blood flow in the noninfused arm did not alter significantly after infusion of candoxatrilat, confirming that drug effects were confined to the infused arm. Brachial artery infusion of candoxatrilat caused a slowly progressive forearm vasoconstriction, with blood flow decreasing by a mean (area under the curve) of 12±2% and maximum of −28±3% ($P=0.001$; Figure 2) during the 90-minute infusion.

Protocol 2: Intra-arterial Thiorphan and Systemic ACE Inhibition

There were no significant differences between plasma urea, electrolytes, and creatinine concentrations at the start of the thiorphan infusion during the placebo and enalapril phases. Heart rate and mean arterial pressure were not significantly different at the start of thiorphan infusion in either phase and

TABLE 1. Heart Rate, Blood Pressure, Plasma Active Renin, and Aldosterone Concentrations Before (8:30 AM, Basal) and 4 Hours After (12:30 PM) Oral Administration of Placebo or Enalapril 20 mg

	Placebo Phase Time		Enalapril Phase Time	
	8:30 AM	12:30 PM	8:30 AM	12:30 PM
Heart rate, bpm	58±3 (51–65)	57±3 (47–63)	56±3 (49–63)	61±3 (52–69)
Systolic blood pressure, mm Hg	126±6 (110–141)	130±6 (113–146)	124±7 (104–142)	124±7 (104–142)
Diastolic blood pressure, mm Hg	63±2 (57–69)	69±5 (55–84)	57±2 (51–62)	65±2 (59–71)
Mean arterial pressure, mm Hg	84±3 (75–92)	89±5 (55–84)	79±3 (70–87)	85±4 (75–94)
Plasma active renin, μU/mL	22±4 (12–25)	19±2 (12–25)	75±9‡ (52–99)	136±12*‡ (106–166)
Aldosterone concentration, ng/mL	15.8±2.5 (8.9–21.9)	11.4±1.3 (8.0–14.9)	12.3±1.5† (8.3–16.1)	6.9±0.7*‡ (5.0–8.8)

Values are mean±SEM. Values in parentheses are 95% confidence intervals.
*$P \leq 0.05$ vs basal (8:30 AM); †$P \leq 0.05$ vs placebo phase; ‡$P \leq 0.005$ vs placebo phase.

did not change during the intra-arterial infusion of thiorphan in either phase (Table 1).

Plasma active renin concentrations were higher after 4 days of treatment with enalapril than with placebo. Plasma active renin concentration increased further 4 hours after administration of 20 mg enalapril, with no change during the placebo phase (Table 1). Plasma Ang II concentration tended to be lower after 4 days of enalapril, although this difference between phases did not reach statistical significance ($P=0.09$; Table 2). Plasma Ang II concentrations did not change significantly during the placebo phase in either the infused or noninfused arm. Four hours after administration of 20 mg enalapril, there was a substantial reduction in plasma Ang II concentration (Table 2). Plasma Ang II concentration did not change further during the 90-minute thiorphan infusion in the enalapril phase in either the infused or noninfused arm (Table 2). Venous aldosterone concentration was lower after 4 days of enalapril than after 4 days of placebo (Table 2). During both phases, aldosterone concentration tended to decrease after 4 hours of supine posture. However, this decrease was significant only after 20 mg enalapril compared with basal (Table 2).

Neither oral enalapril nor intra-arterial thiorphan had any effect on plasma ANP or plasma ET concentrations in either the infused or noninfused arm (Table 2).

Basal forearm blood flow in the infused arm tended to be lower during the enalapril phase than the placebo phase, although this was not statistically significant (2.9±0.4 and

TABLE 2. Plasma ANP, Ang II, and ET Concentrations Taken From the Infused and Noninfused Arms Before (Basal, 8:30 AM) and 4 Hours After (12:30 PM) Oral Administration of Enalapril 20 mg and After 90-Minute Intra-arterial Infusion of Thiorphan 30 nmol/min (2:00 PM; 5.5 Hours After Enalapril 20 mg Orally)

Concentration, mg/mL	Phase	Time	Arm	
			Noninfused	Infused
ANP	Placebo	8:30 AM	13.0±3.4 (4.4–21.7)	NA
		12:30 PM	14.8±3.6 (4.7–24.9)	21.5±7.9 (1.2–41.8)
		2:00 PM	21.8±5.5 (7.8–35.8)	18.6±3.6 (9.3–28.0)
	Enalapril	8:30 AM	15.2±2.3 (9.2–21.1)	NA
		12:30 PM	13.0±3.1 (5.0–21.0)	17.1±3.1 (9.2–25.0)
		2:00 PM	17.2±2.6 (10.6–23.8)	21.5±5.3 (7.9–35.0)
Ang II	Placebo	8:30 AM	12.7±2.5 (6.2–19.2)	NA
		12:30 PM	12.6±2.6 (6.0–19.1)	10.0±1.9 (5.2–14.8)
		2:00 PM	7.8±1.8 (3.4–12.2)	8.2±3.3 (4.7–11.7)
	Enalapril	8:30 AM	7.4±1.2 (4.2–10.6)	NA
		12:30 PM	1.7±0.3*† (0.8–2.5)	1.6±0.4*† (0.6–2.7)
		2:00 PM	1.5±0.4*† (0.5–2.6)	1.5±0.4*† (0.6–2.4)
ET	Placebo	8:30 AM	3.8±0.3 (3.0–4.6)	NA
		12:30 PM	3.7±0.4 (2.6–4.6)	4.3±0.3 (3.6–4.9)
		2:00 PM	4.3±0.5 (2.9–5.7)	3.6±0.5 (2.3–4.1)
	Enalapril	8:30 AM	4.1±0.2 (3.5–4.7)	NA
		12:30 PM	3.8±0.2 (3.3–4.4)	3.9±0.4 (3.7–4.6)
		2:00 PM	4.5±0.8 (2.8–5.1)	3.4±0.5 (2.2–4.7)

Values are mean±SEM. Values in parentheses are 95% confidence intervals.
*$P \leq 0.05$ vs basal (8:30 AM); †$P \leq 0.005$ vs placebo.

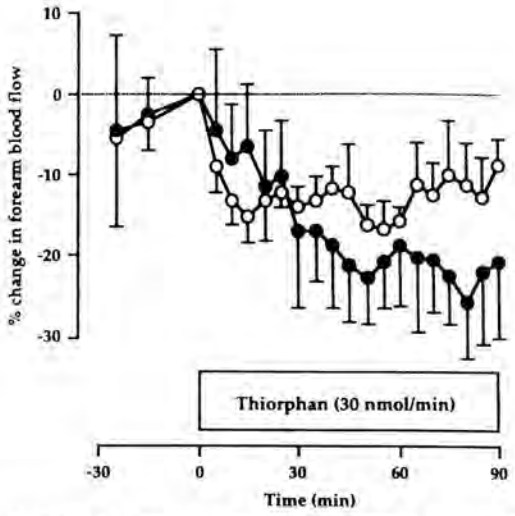

Figure 3. Effect of brachial artery administration of thiorphan (30 nmol/min) on forearm blood flow after oral placebo (○) or oral enalapril (●) in six healthy, male volunteers. Thiorphan produced a slowly progressive vasoconstriction during both the placebo ($P=0.05$) and enalapril ($P=0.01$) phases, with no significant difference between the two phases ($P=0.6$).

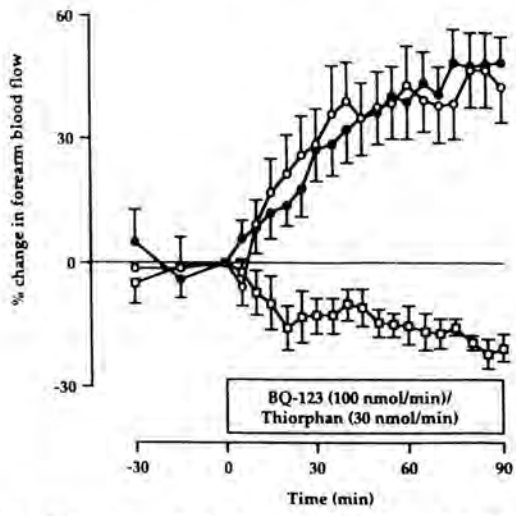

Figure 4. Effect of brachial artery administration of thiorphan (□, 30 nmol/min) and BQ-123 (○, 100 nmol/min) and coinfusion of both agents (●) on forearm blood flow. Thiorphan produced a slowly progressive vasoconstriction ($P=0.0001$), whereas BQ-123 caused a slowly progressive vasodilatation ($P=0.0001$). Coinfusion of BQ-123 and thiorphan produced a vasodilatation ($P=0.0001$) not significantly different from that produced by BQ-123 alone ($P=0.98$).

3.7±0.4 mL · 100 mL^{-1} · min^{-1}, respectively; $P=0.12$). Blood flow in the noninfused arm did not change significantly after infusion of thiorphan, confirming that drug effects were confined to the infused arm. Brachial artery administration of thiorphan caused a slowly progressive forearm vasoconstriction, with blood flow decreasing during both the enalapril phase (mean, 17±6%; maximum, 33±7%; $P=0.05$) and placebo phase (mean, 13±3%; maximum, 24±2%; $P=0.006$). The reductions in blood flow were similar during either phase ($P=0.6$; Figure 3).

Protocol 3: Intra-arterial Thiorphan and Intra-arterial BQ-123

Brachial artery administration of BQ-123 alone caused a progressive forearm vasodilatation (mean, 33±3%; maximum, 47±9%; $P=0.0001$), whereas thiorphan caused a slowly progressive vasoconstriction (mean, −14±1%; maximum, −22±4%; $P=0.0001$). Coinfusion of BQ-123 and thiorphan caused a vasodilatation (mean, 32±2%; maximum, 48±6%; $P=0.0001$) that was not different from that observed with BQ-123 alone ($P=0.98$; Figure 4).

Protocol 4: Intra-arterial Thiorphan in Hypertensive Patients

In hypertensive patients, brachial artery administration of thiorphan caused a slowly progressive forearm vasoconstriction (mean, −10±2%; maximum, −20±3%; $P=0.0001$). This was not significantly different from that observed in the healthy volunteers in the third study ($P=0.39$; Figure 5).

Discussion

We have shown that the specific NEP inhibitors candoxatrilat and thiorphan cause slowly progressive vasoconstriction when given by direct brachial artery infusion to healthy subjects and patients with essential hypertension. The vasoconstriction caused by thiorphan was not reversed by systemic ACE inhibition but was abolished by ET receptor antagonism. Our findings are unlikely to be due to other actions of these agents because both candoxatrilat[8,9,17] and thiorphan[52,53] are highly specific for NEP. Furthermore, the finding that two structurally independent inhibitors of NEP produce vasoconstriction strongly suggests that this is a class effect of NEP inhibition on human resistance vessels. It is possible that different effects may be obtained in other blood vessels, although responses in forearm resistance vessels are generally thought to be broadly representative of those in other vascular beds.[41,54] Our findings have potential implications both for the physiological role of NEP and for the therapeutic use of NEP inhibitors.

Although it was initially thought that the most important site of natriuretic peptide metabolism by NEP was the kidney,[2] candoxatrilat is just as effective in reducing clear-

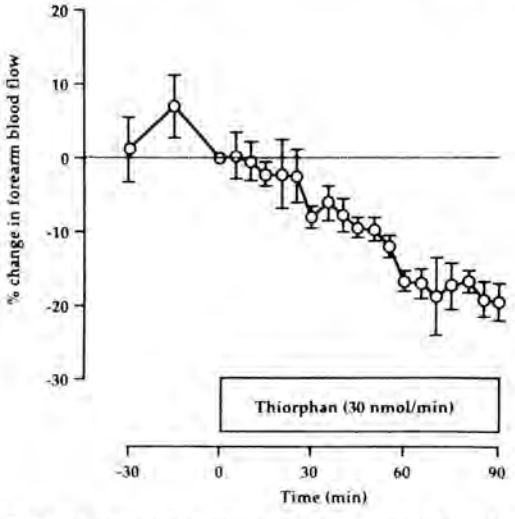

Figure 5. Effect of brachial artery administration of the NEP inhibitor thiorphan (30 nmol/min) on forearm blood flow in six hypertensive patients. Thiorphan produced a slowly progressive vasoconstriction confined to the infused forearm ($P=0.0001$).

ance of ANP in nephrectomized animals,[55] implying other, nonrenal sites of action. NEP is now known to be expressed in blood vessels by both endothelial[56] and vascular smooth muscle cells.[57] Despite the clear evidence for vascular generation and metabolism of natriuretic peptides, we found that local NEP inhibitors caused vasoconstriction rather than vasodilatation. This finding implies that under physiological conditions, vasoconstrictor peptides, such as Ang II and ET-1, are more important substrates for vascular NEP than dilator substances, such as the natriuretic peptides and bradykinin (Figure 1). However, our finding that brachial artery administration of thiorphan produces forearm vasoconstriction in the presence of substantial systemic ACE inhibition implies that Ang II accumulation is not responsible for the observed vasoconstriction. In addition, ANP blocks activity of the renin-angiotensin system by reducing renin release[14] and blocking aldosterone secretion,[15] so Ang II generation is likely to be decreased by NEP inhibition.

The vasoconstriction to candoxatrilat and thiorphan was slowly progressive, which is more in keeping with an effect of ET-1 than Ang II, on the basis of the known rate of onset of forearm vasoconstriction after brachial artery infusion of these peptides.[58] This is supported by a recent study in which systemic oral doses of candoxatril in healthy men produced an increase in both systolic blood pressure and venous plasma ET concentration.[22] In another recent study, systemic administration of candoxatrilat in healthy subjects produced a significant increase in systolic blood pressure.[23] However, because this rise was prevented by pretreatment with enalapril, it was suggested that the increase in blood pressure was caused by potentiation of Ang II. Our findings do not support this conclusion. Indeed, in our study, thiorphan produced arterial vasoconstriction in the presence of systemic ACE inhibition despite the very low Ang II concentrations. Furthermore, we did not detect any increase in Ang II concentrations in venous blood draining the infused arm during the placebo phase of our study, suggesting that NEP inhibition does not cause an accumulation of Ang II.

We did not demonstrate a significant decrease in blood pressure after 20 mg enalapril orally despite the very low concentrations of Ang II produced. However, our study was not designed to specifically measure changes in systemic hemodynamics. The hypotensive effect of enalapril would be expected to have been greatest when subjects were being prepared for the intra-arterial stage of the study. This involved subjects standing to pass urine and having the blood pressure cuff repositioned over the rapid inflation cuffs required for forearm plethysmography, as well as insertion of an intra-arterial needle.

In this study, enalapril had no effects on plasma ANP concentrations. This is in agreement with other published reports.[23,59,60] Intra-arterial thiorphan did not produce a detectable increase in ANP concentrations in venous blood draining the infused arm. However, any changes in local ANP concentrations are likely to be small and may have been below the sensitivity of the assay. In addition, not all studies of acute NEP inhibition have demonstrated an increase in ANP concentrations.[21,22,35] ANP may also be metabolized by an aminopeptidase, which is insensitive to thiorphan.[61] Although incomplete local NEP inhibition is possible, it is highly unlikely because the doses of both candoxatrilat and thiorphan used were chosen to achieve local blood concentrations in the forearm >50-fold and >10-fold higher than the IC_{50} of (+)candoxatrilat and thiorphan, respectively, for ANP in vitro.[39]

Consistent with earlier work,[62] systemic ACE inhibition with enalapril had no effect on plasma ET concentrations. In addition, intra-arterial thiorphan did not increase plasma ET concentrations in samples collected from the infused arm. However, ET produced by endothelial cells is preferentially secreted abluminally,[63] and inhibition of local ET degradation may not have resulted in increased plasma ET concentrations. Furthermore, any measurable increase in plasma ET concentrations is likely to be rapidly reduced through tissue receptor binding.[43] Therefore, the absence of any detectable increase in plasma ET does not exclude local accumulation of the peptide, and it is still possible that decreased ET-1 breakdown is the cause of the vasoconstriction produced by NEP inhibitors.

ET-1 mediates vasoconstriction primarily by effects on the vascular smooth muscle ET_A receptor.[64] We find that the selective ET_A receptor antagonist BQ-123 abolishes the vasoconstriction produced by thiorphan. This provides strong evidence that accumulation of ET-1, resulting from an inhibition of its degradation, mediates the vasoconstriction caused by local NEP inhibition. Nevertheless, it is also possible that accumulation of an as-yet undiscovered vasoconstrictor may contribute to the observed vasoconstriction, although its abolition by BQ-123 makes this unlikely.

In clinical trials, NEP inhibitors have been shown to cause a natriuresis and diuresis.[10,17] However, a reduction in blood pressure has not been clearly demonstrated in normotensive subjects,[10,18,20,21] and two studies have even reported an increase in blood pressure[22,23] despite the potent vasodilator actions of the natriuretic peptides.[12,13,16] Also, several studies on hypertensive patients[19,32–35] have not demonstrated a reduction in blood pressure. Interestingly, and perhaps relevant to our own findings, a recent study in patients with chronic heart failure showed that candoxatril increases systemic vasoconstriction and decreases cardiac index.[65] Our results help to explain these apparent contradictions. The hemodynamic effects of systemic NEP inhibition will depend on the balance between its cardiac, renal, and vascular actions. We have shown that local NEP inhibition causes forearm vasoconstriction in healthy subjects and, of greater clinical relevance, that this effect occurs in untreated essential hypertensive patients. Thus, peripheral vasoconstriction may play an important role in counteracting the antihypertensive actions of NEP inhibition.

Our study shows that the vasoconstriction produced by NEP inhibitors may be mediated by ET-1 or other vasoconstrictor peptides. Given that systemic NEP inhibition has been shown to increase venous ET concentrations,[25] it is possible that the combination of NEP inhibition and ET antagonism may be useful therapeutically. Indeed, phosphoramidon, a combined ET-converting enzyme and NEP inhibitor, is known to produce substantial vasodilatation when infused intra-arterially in humans[37,38] and can lower blood pressure in

normotensive and hypertensive rats.[52] Nevertheless, even without reducing blood pressure, NEP inhibition may offer therapeutic benefits in hypertension and heart failure. For example, infusion of ANP causes sympathoinhibition in humans.[22] In addition, NEP inhibitors appear to possess favorable antimitogenic effects in models of left ventricular hypertrophy[66] and atherosclerosis.[67] Such effects would need to be counterbalanced against potential mitogenic actions of ET-1.[68]

In conclusion, local inhibition of NEP causes slowly progressive vasoconstriction in healthy subjects and essential hypertensive patients, suggesting that the predominant physiological substrates for vascular NEP are vasoconstrictor peptides. The slowly progressive nature of the vasoconstriction, together with the finding that it is not blocked by systemic ACE inhibition but is abolished by ET antagonism, supports accumulation of ET-1 as the cause. Vasoconstriction produced by NEP inhibitors may help to explain some of the apparently contradictory hemodynamic results obtained after systemic dosing with NEP inhibitors.

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Reduced endogenous endothelin-1–mediated vascular tone in chronic renal failure

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Reduced endogenous endothelin-1–mediated vascular tone in chronic renal failure.

Background. Endothelin-1 generated by the vascular endothelium contributes to basal vascular tone and blood pressure in healthy humans. Plasma concentrations of endothelin-1, which are elevated in chronic renal failure (CRF), may contribute to increased vascular tone.

Methods. We investigated the contribution of endogenous and exogenous endothelin-1 to the maintenance of vascular tone in patients with CRF (creatinine ≥ 200 μmol/liter) and in age- and sex-matched healthy subjects. In a series of experiments, we measured forearm vascular responses to intra-arterial norepinephrine (30 to 240 pmol/min), endothelin-1 (5 pmol/min), the selective endothelin A (ET_A) receptor antagonist BQ-123 (3 mg/hr), the mixed endothelin-converting enzyme and neutral endopeptidase inhibitor phosphoramidon (30 nmol/min), and the selective neutral endopeptidase inhibitor thiorphan (30 nmol/min).

Results. The maximum reduction in forearm blood flow (FBF) to norepinephrine in CRF (33 ± 7%) was similar to that in controls (43 ± 7%, $P = 0.53$). Endothelin-1 also produced a similar reduction in FBF in CRF (35 ± 6%) and controls (36 ± 5%, $P = 0.81$). BQ-123 increased FBF in CRF (11 ± 4%) but significantly less than in controls (44 ± 10%, $P = 0.02$). Phosphoramidon increased FBF in CRF (68 ± 20%), again significantly less than in controls (181 ± 41%, $P = 0.001$). Thiorphan reduced FBF similarly in CRF (22 ± 6%) and controls (14 ± 6%, $P = 0.39$). Responses to phosphoramidon were substantially greater than to BQ-123.

Conclusions. These studies show that endogenous generation of endothelin-1 contributes to the maintenance of resting vascular tone in patients with CRF, as well as in healthy subjects. Although the contribution of endogenous endothelin-1 to resting vascular tone appears to be reduced in CRF, ET_A receptor antagonism, and particularly endothelin-converting enzyme inhibition, should be explored as means by which to reduce vascular tone and blood pressure in patients with CRF.

Key words: endothelin receptors, BQ-123, blood pressure, vasoconstriction, hypertension, renal failure.

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Endothelin-1 is an endothelium-derived 21-amino acid peptide with sustained vasoconstrictor properties [1]. Endothelin-1 is generated from an inactive 38-amino acid precursor, big endothelin-1, through the action of endothelin-converting enzyme (ECE), a unique membrane-bound neutral metalloprotease [2]. Endothelin-1 binds to at least two receptors [3]: The endothelin A (ET_A) receptor is present on vascular smooth muscle cells in which it plays a major role in causing vasoconstriction [4]; the ET_B receptor is present on endothelial cells in which it mediates release of endothelium-derived vasodilator substances [5], and also on vascular smooth muscle cells in which it mediates vasoconstriction [6]. Endogenous generation of endothelin-1 has been shown to contribute to the maintenance of basal vascular tone [4] and blood pressure [7] in healthy humans.

Plasma concentrations of immunoreactive big endothelin-1 and endothelin-1 are elevated in renal failure [8, 9]. Because these high plasma endothelin concentrations may be within a range sufficient to cause vasoconstriction [10], plasma endothelin-1 may play a role in the development, or maintenance, of hypertension in chronic renal failure (CRF). Indeed, plasma endothelin concentrations are positively correlated with blood pressure in hemodialysis patients [11]. This contrasts to findings in patients with mild to moderate essential hypertension in whom no such relationship was found [12]. However, it is not known whether endothelin-1 contributes to elevated basal resistance vessel tone in patients with CRF.

Therefore, we have investigated the responses of forearm resistance vessels to brachial artery infusion of endothelin-1 and a control constrictor, norepinephrine, in patients with stable CRF who were independent of dialysis and in healthy age- and sex-matched control subjects. We also examined the contribution of endothelin-1 acting via the ET_A receptor to basal vascular tone by brachial artery infusion of a maximally effective dose [13, 14] of the specific ET_A receptor antagonist, BQ-123 [4, 15]. We further examined the contribution of endogenous generation of endothelin-1 to the maintenance of forearm vas-

cular tone by comparing responses to brachial artery infusion of the combined ECE and neutral endopeptidase (NEP) inhibitor, phosphoramidon [4, 16, 17], with those to the selective NEP inhibitor, thiorphan [4, 17].

METHODS

Subjects

A total of 11 patients (10 male, 1 female) with established chronic renal disease (creatinine ≥ 200 μmol/liter and stable blood pressure for 6 months or more) caused by IgA nephropathy ($N = 3$), obstructive uropathy ($N = 2$), reflux nephropathy ($N = 2$), nephrosclerosis ($N = 2$), interstitial nephritis ($N = 1$), and Alport's syndrome ($N = 1$) were recruited. All patients were independent of dialysis. Three subjects underwent all of the study protocols. Five subjects underwent the protocols involving infusion of norepinephrine, endothelin-1, and BQ-123 only, and three subjects underwent infusion of phosphoramidon and thiorphan only. If patients were taking antihypertensive or other vasoactive medication, this was withdrawn at least one week before each study. Ten age- and sex-matched healthy control subjects (nine male, one female) who were not taking any vasoactive medication were also recruited, of whom four undertook all the study protocols, and four the protocols involving infusion of norepinephrine, endothelin-1, and BQ-123 only. Two subjects underwent infusion of phosphoramidon and thiorphan only. All studies were conducted with the approval of the local ethics review committee, and all subjects gave their written informed consent to participate. Subjects avoided nonsteroidal anti-inflammatory drugs for the week before and caffeine-containing drinks or cigarettes on the day of each study. In addition, subjects fasted for at least three hours before each study.

Procedures

A 21 SWG cannula was inserted into a vein on the dominant arm under local anesthesia using 1% lidocaine hydrochloride (Lidocaine; Astra Pharmaceuticals Ltd., Kings Langley, UK) for the purpose of sampling blood. A 27 SWG steel cannula (Cooper's Needle Works, Birmingham, UK) was inserted into the brachial artery of the nondominant arm, under local anesthesia, for the purpose of intra-arterial infusion of drugs. Forearm blood flow (FBF) was measured in both arms using venous occlusion plethysmography [18] adapted for use with indium-and-gallium-in-silastic strain gauges [4]. Measurements were made for 10 seconds in every 15 seconds over a three-minute period. Blood flow to the hands was excluded during each three-minute measurement period.

Arterial pressure was measured using a well-validated semiautomated oscillometric method (Takeda UA 751; Takeda, Tokyo, Japan) [19, 20].

Drugs

Norepinephrine (30, 60, 120, and 240 pmol/min; Sanofi Winthrop Ltd., Guildford, UK), endothelin-1 (5 pmol/min; Clinalfa, Laufelfingen, Switzerland), BQ-123 (3 mg/hr; American Peptide Company, Sunnyvale, CA, USA), phosphoramidon (30 nmol/min; Clinalfa), thiorphan (30 nmol/min; Sigma Chemicals, Poole Dorset, UK), and physiologic saline (0.9%; Baxter Healthcare Ltd., Thetford, UK) were administered at locally but not systemically active doses via the brachial artery cannula. These doses were based on previous studies [4, 21]. All solutions were made up in physiologic saline. The norepinephrine solutions were made up in physiologic saline containing 100 μg/ml ascorbic acid (Evans Medical Ltd., Dunstable, UK) to avoid oxidation. The total rate of intra-arterial infusion was kept constant at 1 ml/min.

Design

Studies were performed in a quiet room maintained at a constant temperature of between 22°C and 25°C. After brachial artery and venous cannulation, saline was infused via the brachial artery cannula for 30 minutes, and blood was withdrawn from the venous cannula to measure plasma creatinine, endothelin 1, big endothelin 1, and he moglobin. In eight patients with CRF and in eight control subjects, norepinephrine was then infused at incremental doses (30, 60, 120, and 240 pmol/min, each dose for 6 min) via the brachial artery cannula. After infusion of norepinephrine, saline was infused for 20 minutes to allow for the reversal of vasoconstriction. Endothelin-1 was then infused at a single dose of 5 pmol/min for one hour. FBF was recorded at 10-minute intervals during infusion of saline and during the last three minutes of each increment of the norepinephrine infusion and at 10-minute intervals during the endothelin-1 infusion. On a separate day, after the 30-minute infusion of saline, BQ-123 was infused in these subjects at a single dose of 3 mg/hr for one hour, and FBF was measured at five-minute intervals. In a further subgroup of three CRF patients and seven control subjects, the BQ-123 was infused at 3 mg/hr and was continued for two hours.

In a separate study in six patients with CRF and in six control subjects, after a 30-minute infusion of saline, phosphoramidon or thiorphan was infused, on separate days, at a single dose of 30 nmol/min for 90 minutes, and FBF was measured at 10-minute intervals.

In all studies, the mean of the final five blood flow measurements of each recording period was used for analysis. Arterial pressure was measured in the noninfused arm immediately after each blood flow recording. In each subject, infusions were on separate days, at least one week apart. The order in which the experiments were undertaken was randomized. However, vasoconstriction to endothelin-1 is sustained, whereas that to

norepinephrine is short lasting. Therefore, in the combined norepinephrine/endothelin-1 study, it was necessary to give norepinephrine first rather than randomize the order of infusions.

Analytical

Venous plasma samples for radioimmunoassay of endothelin-1 and big endothelin-1 were separated within 10 minutes and were stored at −80°C. Plasma biochemistry was measured using a Kodak Ectachem System E700 XRC analyzer (Kodak Diagnostics Ltd., Hemel Hempstead, Herts., UK) in the Department of Clinical Chemistry, Western General Hospital (Edinburgh, UK). Hemoglobin was measured in the Department of Hematology, Western General Hospital, using a Coulter STKS analyzer (Coulter Electronics Ltd., Luton, Beds., UK).

Plasma immunoreactive endothelin-1 and big endothelin-1 concentrations were measured using an acetic acid extraction technique [22] and a modified commercial radioimmunoassay using rabbit antihuman endothelin-1 or big endothelin-1 (Peninsula Laboratories Europe, St. Helens, UK) [23]. Briefly, sample extract was incubated with either endothelin-1 or big endothelin-1 antibody for 24 hours at 4°C. Following incubation, ^{125}I-labeled endothelin-1 (NEN Life Science Products, Boston, MA, USA) or big endothelin-1 (Peninsula Laboratories Europe) was added, and incubation was continued for an additional 20 hours at 4°C. Complexes were precipitated with Amerlex™ donkey antirabbit antibody (Amersham Life Sciences Ltd., Little Chalfont, Bucks., UK) and were counted for radioactivity. All endothelin values were expressed as picograms per milliliter. Recovery of endothelin-1 and big endothelin-1 was 89% and 91%, respectively. Intraassay and interassay coefficients of variations in our laboratory are 6.3% and 7.2%, respectively; the sensitivity of the assay for endothelin-1 was 0.25 pg/ml, and for big endothelin-1, sensitivity was 1 pg/ml.

Cross-reactivities of the endothelin-1 assay were endothelin-1 (100%), endothelin-2 (0%), endothelin-3 (0%), and big endothelin-1 (10%). The cross-reactivities of the big endothelin-1 assay were endothelin-1 (0%), endothelin-2 (0%), endothelin-3 (0%), and big endothelin-1 (100%).

Data presentation and statistics

Mean arterial pressure was calculated as diastolic arterial pressure plus one-third pulse pressure. FBF was calculated in milliliters per 100 ml of forearm tissue per minute. The ratio of FBF in the infused arm compared with that in the control arm was calculated for each measurement period. The ratio of FBF (infused:control arm) was measured in response to drugs and was expressed as a percentage of the ratio (infused:control arm) measured during the control period [24]. This method uses the noninfused arm as a contemporaneous control and compensates for the continual small adjustments affecting the circulation of both arms that occur even at rest and for other external systemic factors such as the level of arousal that may otherwise act as confounding factors [24].

All results are expressed as mean ± SEM. Dose–response relationships were compared using analysis of variance. All other parameters were analyzed by Student's paired or unpaired t-test as appropriate. Statistical analysis was performed using the STATVIEW 512+™ software (Brain-power Inc., Calabasas, CA, USA) for the Apple Macintosh microcomputer. Values of P of less than 0.05 were considered statistically significant.

RESULTS

There was no significant difference in age, sex, hemoglobin, or resting FBF between the patients with CRF and the healthy control subjects when receiving norepinephrine, endothelin-1, or BQ-123 (Tables 1 and 2). However, resting FBF was higher in patients with CRF who underwent the phosphoramidon/thiorphan studies compared with control subjects (Table 2). FBF in the noninfused arm did not change during any infusion period apart from when endothelin-1 and thiorphan was infused in control subjects (Table 2) when the FBF in the control arm increased, whereas the blood flow in the infused forearm decreased. Plasma creatinine and mean arterial pressure were elevated in the CRF groups when compared with control subjects (Table 1). The plasma immunoreactive endothelin-1 concentration was higher in CRF patients (3.9 ± 0.2 pg/ml, $N = 9$) than in control subjects (2.9 ± 0.2 pg/ml, $N = 10$, $P = 0.0001$). Similarly, the plasma immunoreactive big endothelin-1 concentration was higher in the CRF patients (43.5 ± 4.0 pg/ml, $N = 9$) than in control subjects (31.4 ± 3.5 pg/ml, $N = 10$, $P = 0.03$).

Infusion of norepinephrine and endothelin-1

Norepinephrine caused a dose-dependent reduction in FBF (Fig. 1 and Table 2) with a change in blood flow at the highest dose of −33 ± 7% in patients with CRF and −43 ± 7% in control subjects, which was similar between the two groups ($P = 0.53$). Saline infusion following the norepinephrine infusion allowed FBFs to return to baseline flows of 2.8 ± 0.3 ml/100 ml/min before endothelin-1 and 3.1 ± 0.6 ml/100 ml/min before norepinephrine ($P = 0.40$) for patients and, similarly, 2.9 ± 0.2 ml/100 ml/min before endothelin-1 and 3.4 ± 0.4 ml/100 ml/min before norepinephrine ($P = 0.24$) for control subjects. Endothelin-1 caused a progressive reduction in FBF (Fig. 2 and Table 2) with a change in blood flow after 60 minutes of infusion of −35 ± 6% in patients with CRF and −36 ± 5% in control subjects ($P = 0.81$).

Table 1. Subject characteristics

	Norepinephrine/ endothelin-1		BQ-123		Phosphoramidon		Thiorphan	
	Control	CRF	Control	CRF	Control	CRF	Control	CRF
Number	8	8	8	8	6	6	6	6
Age *years*	46 ± 5	47 ± 6	46 ± 5	47 ± 6	40 ± 8	41 ± 8	40 ± 8	41 ± 8
Sex *M/F*	7/1	7/1	7/1	7/1	6/0	6/0	6/0	6/0
Hemoglobin *g/dl*	12.7 ± 0.5	11.1 ± 0.8	12.7 ± 0.5	11.1 ± 0.8	13.0 ± 0.6	11.6 ± 0.7	13.0 ± 0.6	11.6 ± 0.7
Creatinine *μmol/liter*	87 ± 5	391 ± 54[a]	87 ± 5	391 ± 54[a]	84 ± 7	449 ± 66[a]	84 ± 7	449 ± 66[a]
Mean arterial pressure *mm Hg*	88 ± 3	104 ± 2[a]	85 ± 3	100 ± 4[a]	86 ± 4	110 ± 6[a]	87 ± 6	108 ± 4[b]

Results are expressed as mean ± SEM.
[a] $P < 0.002$, [b] $P \leq 0.05$ when compared to control subjects

Table 2. Forearm blood flows (FBF) before and during infusions

	Norepinephrine		Endothelin-1		BQ-123		Phosphoramidon		Thiorphan	
	Control	CRF	Control	CRF	Control	CRF	Control	CRF	Control	CRF
Control arm FBF *ml/100 ml/min*										
Basal	2.4 ± 0.2	3.0 ± 0.6	2.7 ± 0.3	2.8 ± 0.5	2.4 ± 0.3	3.2 ± 0.4	2.9 ± 0.4	3.4 ± 0.4	1.7 ± 0.2	3.9 ± 0.7[a]
End of infusion	2.9 ± 0.3	2.8 ± 0.6	3.2 ± 0.4[b]	3.0 ± 0.7	2.1 ± 0.3	3.5 ± 0.6[a]	3.6 ± 0.7	3.5 ± 0.4	2.5 ± 0.3[b]	2.8 ± 0.7[c]
Infused arm FBF *ml/100 ml/min*										
Basal	3.0 ± 0.3	3.1 ± 0.6	2.9 ± 0.2	2.8 ± 0.3	2.6 ± 0.3	3.7 ± 0.5	2.5 ± 0.4	4.6 ± 0.8[a]	1.9 ± 0.2	5.4 ± 1.3[a]
End of infusion	2.0 ± 0.2[b]	1.8 ± 0.2[b]	2.4 ± 0.3[b]	1.8 ± 0.3[b]	3.2 ± 0.4[b]	4.5 ± 0.7[b]	7.4 ± 0.8[b]	6.9 ± 0.7[b]	2.4 ± 0.3[b]	3.9 ± 0.7[b,c]

[a] $P < 0.05$ for resting blood flow when compared to control subjects (t-test)
[b] $P < 0.05$, compared to basal value (paired t-test)
[c] $P < 0.05$, compared to control response to infusion (ANOVA)

Infusion of BQ-123

Infusion of BQ-123 caused a progressive increase in FBF (Fig. 3 and Table 2), maximal at 60 minutes, of 11 ± 4% in patients with CRF and 44 ± 10% in control subjects; the response in patients with CRF was significantly reduced (Fig. 3) when compared with that of the control subjects ($P = 0.02$). In those subjects undergoing the two-hour infusion, there was no further vasodilation after 60 minutes of BQ-123 in either patients (13 ± 11% at 60 min, −3 ± 3% at 120 min, $P = 0.35$) or control subjects (46 ± 11% at 60 min, 48 ± 9% at 120 min, $P = 0.76$), respectively.

Infusion of phosphoramidon and thiorphan

Infusion of phosphoramidon caused a progressive increase in FBF (Fig. 4 and Table 2), maximal at 90 minutes, of 68 ± 20% in patients with CRF and 181 ± 41% in control subjects. The response of the patients with CRF to phosphoramidon was reduced (Fig. 4) when compared with that of the control subjects ($P = 0.001$). Infusion of thiorphan caused a progressive reduction in FBF (Table 2), maximal at 90 minutes, of −22 ± 6% in patients with CRF and −14 ± 6% in control subjects and was similar when comparing the two groups ($P = 0.39$; Fig. 4).

DISCUSSION

We have shown, using the ECE inhibitor, phosphoramidon, and the ET_A antagonist, BQ-123, that endogenous endothelin-1 contributes to the maintenance of resting forearm vascular tone in patients with CRF. We have also demonstrated, using BQ-123, that the contribution of endothelin-1 to resting vascular tone mediated through the ET_A receptor is decreased in patients with CRF compared with healthy controls. In addition, using phosphoramidon, the reduced contribution of endothelin-1 to the maintenance of vascular tone in patients with CRF appears to be due at least in part to reduced generation of endothelin-1. These functional responses are in contrast to the observed elevated concentrations of endothelin-1 and big endothelin-1 in CRF.

These studies involved infusion of locally but not systemically active doses of vasoactive agents into the forearm vascular bed. Indeed, FBF in the noninfused arm did not change during any infusion period apart from when endothelin-1 and thiorphan were infused in control subjects (Table 2), when the FBF in the control arm increased and the blood flow in the infused forearm decreased. The use of locally active doses of vasoactive agents is important in these studies, as systemic doses may obscure any direct vascular action through effects on other organs, such as the heart and kidney, or through

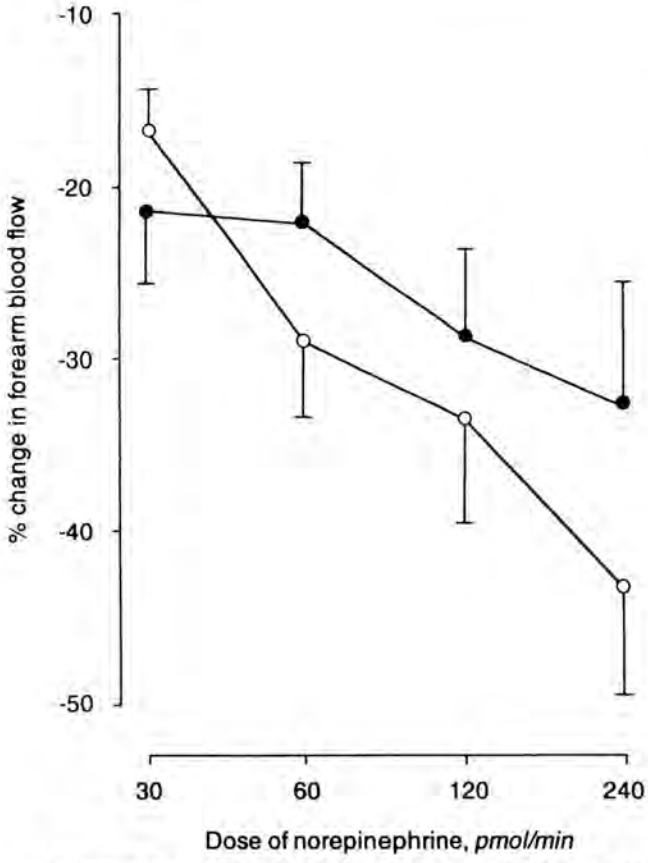

Fig. 1. Effect of norepinephrine infusion on forearm blood flow (FBF). Symbols are: (●) patients with CRF and (○) healthy control subjects ($P = 0.53$ by ANOVA).

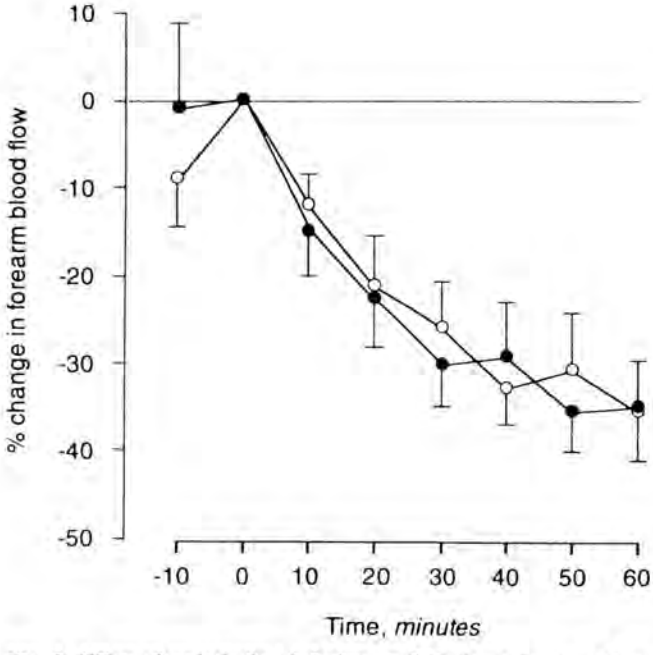

Fig. 2. Effect of endothelin-1 infusion on FBF. Symbols are: (●) patients with CRF and (○) healthy control subjects ($P = 0.81$ by ANOVA).

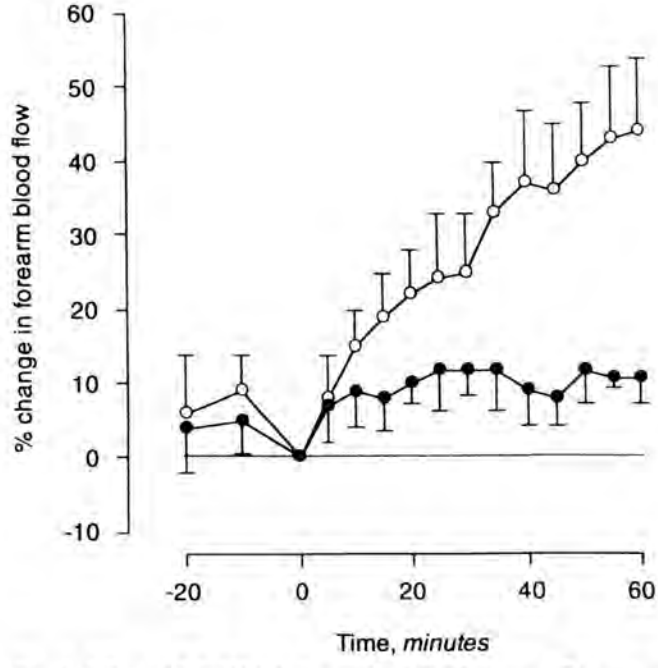

Fig. 3. Effect of BQ-123 infusion on FBF. Symbols are: (●) patients with CRF and (○) healthy control subjects ($P = 0.02$ by ANOVA).

activation of reflex mechanisms caused by changes in blood pressure [25]. The forearm vascular bed was chosen because the responses to vasoactive agents within this vascular bed are thought to be broadly representative of responses in other functionally important resistance beds [25, 26].

In contrast to the impaired responsiveness of the forearm resistance vessels to norepinephrine in patients with CRF who are dialysis dependent and anemic [21], there was no difference in the responsiveness to norepinephrine of the forearm resistance vessels between the dialysis-independent CRF patients and control subjects in this study. Also, in response to infusion of endothelin-1, there was a progressive vasoconstriction that was of a similar magnitude in the patients and control subjects, suggesting that responsiveness to endothelin-1 is not reduced in CRF in the way it is in chronic heart failure [27]. In addition, similar vascular responsiveness to infusion of norepinephrine and endothelin-1 in CRF patients and control subjects implies vascular functional integrity with respect to these two endogenous vasoactive mediators.

The specific ET_A receptor antagonist BQ-123 produced a progressive vasodilation in both patients with CRF and the control subjects, implying that endothelin-1, acting through the ET_A receptor, has a role in the maintenance of resting vascular tone. Furthermore, we have demonstrated, in both patients and controls, that the response to BQ-123 was maximal at 60 minutes. However, the response to BQ-123 in the patient group was significantly impaired when compared with control subjects. This im-

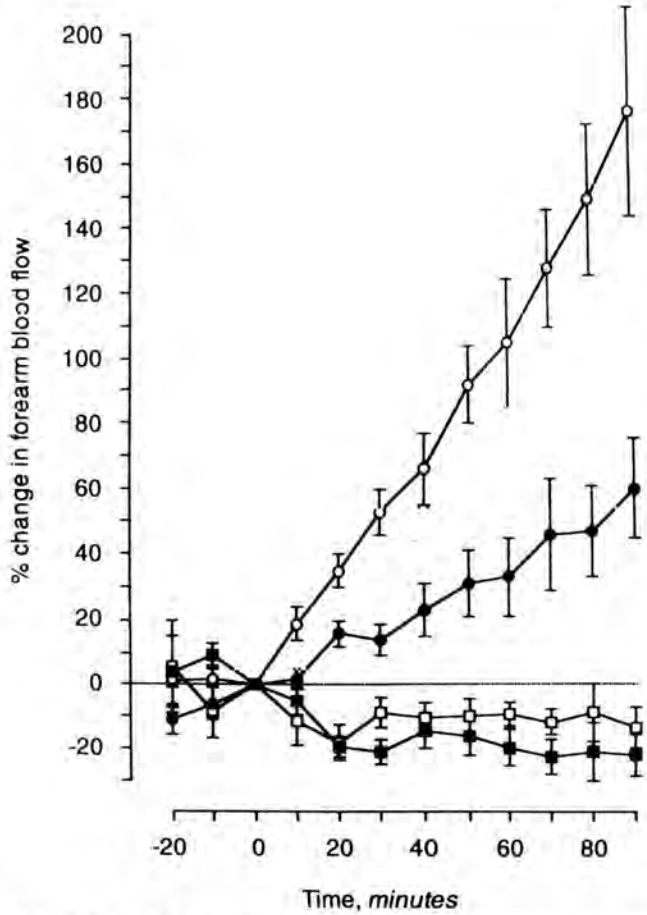

Fig. 4. Effect of phosphoramidon and thiorphan infusion on FBF. Symbols are: patients with CRF receiving phosphoramidon (●) and thiorphan (■) and healthy control subjects receiving phosphoramidon (○, $P = 0.001$ by ANOVA) and thiorphan (□, $P = 0.39$ by ANOVA).

paired response may have occurred for several reasons. First, elevated circulating concentrations of biologically active endothelin-1, either as a result of increased generation or decreased clearance, may have been sufficiently high that the dose of BQ-123 used was not sufficient to block the ET_A receptor. However, because the dose of BQ-123 infused was at least 10-fold higher than that previously shown to cause a maximal effect in the forearm in healthy subjects [13, 14], this is unlikely to be the explanation.

Second, in renal failure, endothelin-1 may cause vasoconstriction by acting on the ET_B receptor. ET_B agonists can cause vasoconstriction [6]. Our observations in renal failure may be consistent with those in chronic heart failure where there appears to be a relative up-regulation of the constrictor ET_B receptor [27, 28]. ET_B-mediated vasoconstriction in CRF may be of greater importance than in heart failure because ET_B-mediated vasodilation may be reduced, perhaps as a result of endothelial dysfunction, in CRF patients as compared with healthy subjects. However, our studies were not designed to fully investigate potential changes in ET_B receptor function in CRF.

Third, the altered response to infused BQ-123 in CRF may occur as a consequence of diminished responsiveness of the vascular smooth muscle cell in CRF. However, our findings of similar responsiveness to exogenous endothelin-1 between patients with CRF and control subjects are against this hypothesis.

Fourth, despite the increased concentrations of immunoreactive endothelin 1 and big endothelin 1 observed by our experiments and others [9], concentrations of biologically active endothelin-1 may be decreased in patients with CRF as a consequence of impaired generation of endothelin-1. Fractionation of plasma samples reveals that the elevation in immunoreactive endothelin-1 in CRF may be due to marked increases in immunoreactive big endothelin-1 degradation products [29] rather than biologically active endothelin-1. Furthermore, Shichiri reported similar concentrations of immunoreactive endothelin-1 in patients with renal failure and healthy control subjects when using an assay that does not cross-react with either big endothelin-1 or the C-terminal fragment [30].

Fifth, the increased blood pressure observed in the patients with CRF may contribute to the different responses between the CRF group and the control subjects rather than the altered renal function. However, this is unlikely because a study similar to ours found no difference in response to BQ-123 when comparing hypertensive subjects with normal renal function and control subjects [13]. Also against this is the inverse relationship between blood pressure and forearm vascular responsiveness to endothelin 1 in patients with dialysis dependent renal failure using human recombinant erythropoietin [21].

Sixth, BQ-123–induced vasodilation may not only occur as a consequence of abolition of a constrictor stimulus but may, in part, be mediated by nitric oxide–dependent vasodilator tone [31]. The reduced response to BQ-123 observed in the CRF subjects may reflect an abnormality in nitric oxide-mediated vascular tone, possibly as a consequence of impaired endothelial function as described in patients with end-stage renal failure [32].

Infusion of the NEP and ECE inhibitor, phosphoramidon, caused forearm vasodilation. In contrast, infusion of the selective NEP inhibitor thiorphan caused a slow-onset vasoconstriction that was similar between the patients with CRF and control subjects. The vasodilator effect of the infused phosphoramidon was therefore likely to be an action of inhibiting ECE rather than NEP [4]. Vasodilation of the forearm vasculature in response to phosphoramidon suggests that endothelin-1 generation by the vascular endothelium is important in the maintenance of resting vascular tone in healthy subjects and shows that this is also the case in patients with CRF, although to a lesser extent. Given that constriction to

endothelin-1 was identical in CRF patients and controls, the implication is that there may be reduced generation of endothelin-1 in patients with CRF. However, this interpretation must be accepted with some caution because our renal failure patients had higher resting FBFs than control subjects, which may have reduced the concentration, and responses, to phosphoramidon. Also, the phosphoramidon responses observed in this study were greater than responses observed previously [4, 27]. In support of our conclusion, however, responses in patients to phosphoramidon and BQ-123 were reduced using the percentage change in FBF as a ratio of the infused to noninfused arm, this measure being less sensitive to differences in baseline blood flow [33]. In addition, the resting FBFs in the renal failure patients receiving BQ-123 and phosphoramidon were similar (infused arms, $P = 0.36$; noninfused arms, $P = 0.80$; Table 2), allowing direct comparison of inhibition of endothelin generation with phosphoramidon to blockade of the ET_A receptor with BQ-123. The greater increase in FBF in renal failure patients caused by phosphoramidon is consistent with an important vasoconstrictor role for the ET_B receptor in the maintenance of resting vascular tone in patients with CRF, although further studies with selective ET_B antagonists are required to confirm this observation.

In conclusion, we have confirmed elevated concentrations of immunoreactive endothelin-1 and big endothelin-1 in CRF. Furthermore, we have demonstrated that endogenous generation of endothelin-1 in the forearm plays an important role in the maintenance of resting vascular tone in healthy subjects and in patients with CRF who are independent of renal replacement therapy. Also, we have demonstrated that the contribution of endothelin-1 to resting vascular tone is decreased in CRF as a consequence of reduced generation of endothelin-1. However, our findings also suggest that the use of ECE inhibitors may be of greater potential for control of hypertension associated with CRF than selective ET_A receptor antagonists. Further studies with systemic and chronic dosing are needed to explore these issues.

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SECTION 3

ISCHAEMIC HEART DISEASE

Papers 25-27

Br Heart J 1994;72:436-441

Raised plasma endothelin in unstable angina and non-Q wave myocardial infarction: relation to cardiovascular outcome

Iwona Wieczorek, William G Haynes, David J Webb, Christopher A Ludlam, Keith A A Fox

Abstract

***Background*—Among patients with independent evidence of coronary disease and recent onset unstable angina or non-Q wave myocardial infarction the incidence of subsequent cardiovascular events is high. Markers predictive of adverse cardiac outcome in unstable angina and non-Q wave myocardial infarction need to be defined more accurately. Endothelin-1 is a potent endothelium derived vasoconstrictor peptide that may play a part in the pathophysiology of acute myocardial ischaemia.**

Aim and study design **In a study that specifically identified high risk patients a group of 16 consecutive patients with either unstable angina at rest or non-Q wave myocardial infarction were prospectively investigated to establish whether these conditions are associated with high plasma immunoreactive endothelin and whether endothelin concentration at presentation is related to cardiovascular events within the next 12 weeks. Controls consisted of a group of 40 healthy subjects.**

***Results*—Patients had significantly higher mean (SD) plasma endothelin at presentation than did healthy controls (7·4 (1·1) *v* 5·0 (1·2) pg/ml, P < 0·0001). At nine weeks plasma endothelin was still significantly higher in those patients who had subsequent cardiovascular events, (n = 9, acute myocardial infarction or refractory angina with electrocardiographic changes and revascularisation procedures, 8·5 (2·6) pg/ml, P < 0·005 *v* controls) whereas its concentration returned to normal in those patients who had a favourable outcome (n = 7, 5·9 (0·7) pg/ml). Compared with those patients who had an uneventful course, patients with subsequent events had significantly higher plasma endothelin, both at presentation and at nine weeks (P < 0·05 on both occasions).**

***Implications*—Endothelin may contribute to the pathophysiology of acute coronary syndromes and may relate to subsequent cardiovascular outcome.**

(*Br Heart J* 1994;72:436-441)

Patients presenting with recent onset unstable angina or non Q wave myocardial infarction constitute a group that is at high risk of progression to further myocardial infarction or sudden death.[1] These patients have not been shown to benefit from thrombolysis,[2] and a proportion of them fail to stabilise on antianginal treatment and aspirin and may experience recurrent episodes of myocardial ischaemia prompting revascularisation.[1] In the antithrombotic therapy in acute coronary syndromes (ATACS) study there was no difference in the cardiovascular outcome at three months after presentation between the group with unstable angina and that with non Q wave infarction.[3] The search continues for reliable markers to facilitate early identification of those patients with acute coronary syndromes who are at a high risk of developing subsequent cardiovascular events, in whom early intervention may be indicated.

Intracoronary thrombosis as a result of a disrupted atherosclerotic plaque is regarded as the pivotal mechanism in acute coronary syndromes and the angiographic and pathological findings are not dissimilar in unstable angina and non-Q wave infarction.[4-6] Clinically, the two diagnoses may not be distinguishable at presentation and both are associated with the release of sensitive markers of cardiac muscle injury, such as troponin T.[7] Abnormalities of haemostasis[8-10] and increased coronary reactivity to constrictor stimuli[11] have been suggested as possible pathogenetic factors in acute coronary syndromes. Previous studies have shown the importance of activation of platelets and of the coagulation system in the pathophysiological mechanism of acute myocardial ischaemia,[8-10] whereas the role of endogenous vasoconstrictor substances has so far received less attention.

Endothelin is a potent endothelium derived vasoconstrictor peptide in animals[12] and humans.[13] Raised plasma concentrations of endothelin have recently been reported in patients with acute myocardial infarction and in a heterogeneous group of patients with symptomatic atherosclerosis.[14,15] In these the plasma endothelin concentration was positively correlated with the number of sites of atherosclerotic involvement but not with age. Patients with stable angina have, on the other hand, been shown to have normal plasma endothelin concentrations.[14,16]

Our aim was to investigate prospectively whether unstable angina and non-Q wave myocardial infarction are associated with changes in plasma immunoreactive endothelin and whether raised endothelin concentrations are associated with indicators of adverse cardiovascular prognosis.

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Patients and methods

PATIENT SELECTION

This study was performed on patients recruited for a multicentre trial of ATACS, which compared the effectiveness of combination antithrombotic treatment with aspirin and anticoagulation *v* aspirin alone in patients with unstable angina and non-Q wave myocardial infarction.[1] The inclusion criteria were (*a*) age > 21; (*b*) ischaemic chest pain due to unstable angina or non-Q wave myocardial infarction, with the qualifying episode of chest pain within the 48 hours before recruitment to ATACS, and (*c*) additional evidence of ischaemic heart disease was required. This evidence consisted of at least one of the following: (*a*) electrocardiographic changes during pain on admission suggesting ischaemia—for example, ST segment depression or elevation, or T wave inversion; (*b*) previous myocardial infarction; (*c*) history of typical exertional angina, with chest pain precipitated by effort and relieved by rest or glyceryl trinitrate, or a previously positive exercise test; (*d*) previous coronary angiography showing $\geqslant 50\%$ luminal narrowing in any coronary artery. Patients with Q wave infarction at presentation were excluded from ATACS. As stipulated by the protocol, no patient was treated with thrombolysis, and coronary angiography was performed on clinical indications only.

Based on screening forms, 4·5 patients were screened for each patient identified for the study. Among 51 patients recruited into the ATACS study in our centre, prospective endothelin measurements were undertaken in the last 16 consecutive patients. Our control group consisted of 40 healthy volunteers, mean (SD) age 34 (17), range 20–78. Eleven control subjects were in the same age range as the patients (59 (13), range 40–74).

STUDY DESIGN

The patients were prospectively followed up for a period of 12 weeks with assessments at least every three weeks in the cardiology clinic. Blood samples were taken on the first morning after admission and at nine weeks of follow up from all patients, except for two patients from whom follow up samples could not be obtained for technical reasons. The controls were sampled on one occasion only. Venous blood was taken from an antecubital vein into chilled potassium EDTA tubes, put immediately on ice and centrifuged at 4000 rpm for 30 min at 4°C. Plasma was stored at −70°C until assay. Clinicians were blinded to the results of endothelin measurements.

ANALYTICAL PROCEDURES

Plasma immunoreactive endothelin was measured by radioimmunoassay. SepPak C18 silica columns (Waters Associates, Milford, MA, USA) were equilibrated by washing with methanol (5 ml), distilled water (5 ml), and then 4% acetic acid (5 ml). Each 2 ml plasma sample was diluted with 3 ml of 4% acetic acid and loaded on to a column; these were washed with 25% ethanol (3 ml), and eluted with 4% acetic acid in 86% ethanol (2 × 1 ml). The eluates were evaporated under nitrogen in a water bath at 37°C, and reconstituted in borate buffer of pH 8·4 (2 ml). Duplicate extracted samples and standards containing 1 to 48 pg/ml of endothelin-1 (each 200 μl) were incubated with rabbit polyclonal antibody raised against endothelin-1 (ITS Production BV, Wijchen, The Netherlands; in 100 μl distilled water) and ^{125}I-endothelin-1 (ITS; in 100 μl distilled water). After vortexing, tubes were incubated for 18 h at 4°C. Donkey antirabbit γ globulin bound on solid phase (ITS; 100 μl) was added to all tubes, except those for total counts, and the tubes were incubated for 30 min at room temperature. The amount of radioactivity in the antibody bound fraction was determined by γ counting for 2 min. The recovery of added endothelin-1 was 84%. Within and between assay coefficients of variation were 2·4% (n = 6) and 4·2% (n = 5). The sensitivity of this assay was 1 pg/ml endothelin. Cross reactivity of the assay with endothelin-1, endothelin-2, endothelin-3, and proendothelin-1 was 100%, 52%, 96%, and 7%.

STATISTICAL ANALYSIS

Initial statistical analyses were performed on the whole group of patients with unstable angina and non-Q wave infarction (primary analysis, n = 16). Subsequent more detailed comparisons were carried out separately for the two diagnostic categories (subsidiary analysis: unstable angina, n = 10, and non-Q wave myocardial infarction, n = 6).

Statistical analyses were performed with Minitab statistical software (Minitab, Release 7). All values are expressed as mean (SD). Individual comparisons between groups were carried out with unpaired *t* test. Categorical data were analysed by χ^2 test, with Yates' correction, when appropriate. Correlation coefficients were calculated with Pearson's test. Two tailed tests were performed and P values $< 0{\cdot}05$ were considered to be significant.

Results

STUDY PATIENTS

The table shows the main characteristics of the 16 patients. None was hypertensive at the time of the study. Coronary artery disease was verified in all patients by at least one of the criteria: electrocardiographic changes on admission (ST segment depression or elevation or T wave inversion, n = 14 (88%)), cardiac catheterisation performed during the 12 weeks of follow up or before admission (n = 8 (50%)) and a history of myocardial infarction (n = 7 (44%)). At presentation all patients were in Killip class I, and at nine weeks all patients but one were free of left ventricular failure.

UNSTABLE ANGINA V NON-Q WAVE INFARCTION

Although all 16 patients fulfilled criteria for unstable angina on admission (all class IIIB

Demographic and epidemiological data

	All	Unstable angina	Non-Q wave MI
No	16	10	6
Age (yr):			
Mean (SD)	62 (10)	62 (8)	62 (12)
Range	44–77	50–76	4–78
Sex ratio (F/M)	4/12	3/7	1/5
Duration of angina (yr):			
Mean (SD)	5·1 (7·5)	7·3 (8·7)	1·4 (2·1)
Median	2	4	0·3
Range	0–25	0–25	0–5
Previous MI	7 (44%)	6	1
Previous CABG/PTCA	2 (13%)	1	1
Coronary angiography:			
Total	8 (50%)	7	1
1 vessel	3 (38%)	2	1
2 vessel	1 (12%)	1	0
3 vessel	4 (50%)	4	0
Ischaemic electrocardiogram at presentation	14 (88%)	9	5
Medical treatment:			
Heparin	10 (63%)	9	1
Aspirin	16 (100%)	10	6
Beta-blockers	14 (88%)	8	6
Calcium antagonists	12 (72%)	10	2
Nitrates	13 (81%)	9	4
Mean (SD) creatine kinase (u/ml)	347 (631)	58 (26)	901 (812)
Range	28–2339	28–101	335–2339

CABG, coronary artery bypass grafting; MI, myocardial infarction; PTCA, percutaneous transluminal coronary angioplasty.

according to Braunwald,[17]—for example, chest pain at rest within preceding 48 h), six patients were subsequently shown to have enzymatic evidence of an evolving non-Q wave myocardial infarction (increase in cardiac enzymes, no electrocardiographic evidence of new Q waves). Patients with unstable angina did not differ significantly from those with non-Q wave infarction with respect to age, sex distribution, smoking habits, history of hypertension and acute myocardial infarction, incidence of ischaemic electrocardiogram at presentation, and treatment with aspirin, β-blockers, calcium channel blockers, and nitrates (table). They tended to have a longer history of previous angina and a higher rate of performed angiography, but these trends did not reach significance. Significantly more patients with unstable angina (n = 9) were treated with heparin or anticoagulation regimens on admission and during follow up, compared with patients with non-Q wave myocardial infarction (n = 1, $\chi^2 = 8{\cdot}6$, $P < 0{\cdot}005$).

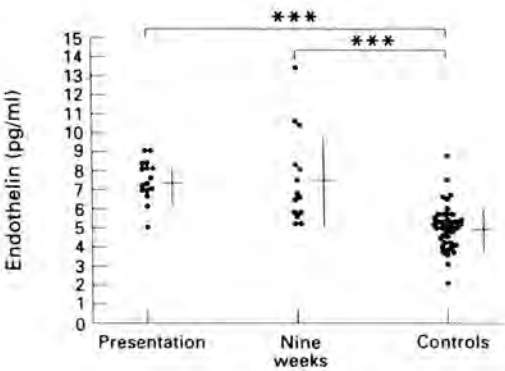

*Figure 1 Plasma endothelin concentrations were significantly higher in patients with unstable angina or non-Q wave myocardial infarction (n = 16), both at presentation and at nine weeks than in the controls (n = 40). ***P < 0·0001.*

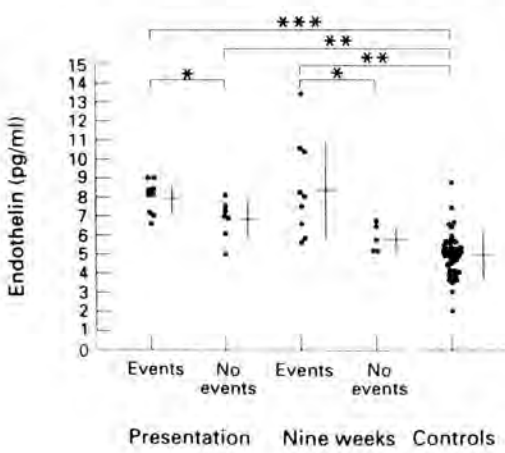

*Figure 2 Cardiovascular outcome analysis. Plasma endothelin concentrations were significantly higher in patients with subsequent cardiac events (n = 9) than in those who remained event free (n = 7), both at presentation and at nine weeks. * P < 0·05; ** P < 0·005; *** P < 0·0001.*

CARDIOVASCULAR OUTCOME

In the primary analysis of all 16 patients with acute coronary syndromes, nine (56%) patients had cardiovascular events during 12 weeks of follow up, whereas seven patients remained event free or had only mild angina. The cardiac events were as follows: four patients had episodes of refractory angina with electrocardiographic changes despite maximal medical treatment, one developed acute Q wave myocardial infarction, and four underwent surgical revascularisation on account of evidence of continuing ischaemia (three coronary artery bypass grafting (CABG) and one percutaneous transluminal coronary angioplasty (PTCA)). In none of the cases did a myocardial infarction or a revascularisation take place within four weeks of the nine week sample being taken. Of the four patients with episodes of refractory pain one patient refused angiography, two had inoperable coronary artery disease, and in one the clinical symptoms resolved and he did not proceed to angiography during the study period.

Subsidiary analysis showed that the subsequent event rate among patients with unstable angina was six of 10 (three CABG, one PTCA, two angina with electrocardiographic changes), whereas three of six patients with subendocardial infarction had subsequent events (one myocardial infarction, two angina with electrocardiographic changes). The event rate at 12 weeks did not differ between unstable angina and non-Q wave infarction groups ($\chi^2 = 0{\cdot}15$, P = NS).

ENDOTHELIN CONCENTRATIONS

In the primary analysis of all 16 patients (fig 1), plasma endothelin was significantly raised both at presentation (7·4 (1·1) pg/ml) and at nine weeks (7·6 (2·4) pg/ml), compared with healthy controls (5·0 (1·2) pg/ml, $P < 0{\cdot}0001$ on both occasions). At presentation, those patients who developed subsequent events (8·0 (0·9) pg/ml) and those who remained event free (6·9 (1·0) pg/ml) had higher endothelin concentrations than the controls ($P < 0{\cdot}0001$ and $P < 0{\cdot}005$; fig 2). At nine weeks, when compared with the controls, endothelin remained significantly raised in those patients who had an unfavourable progress (8·5 (2·6) pg/ml, $P < 0{\cdot}005$ *v* controls), but it returned to normal values in those patients who remained asymptomatic (5·9 (0·7) pg/ml). Patients with subsequent events had significantly higher endothelin than those who remained event free, both at presentation ($P < 0{\cdot}05$) and at nine weeks

($P < 0.05$, fig 2). One of the patients had signs and symptoms of mild left ventricular failure nine weeks after presentation, but the exclusion of this patient did not affect the results from the nine week analysis.

In the analysis based on diagnosis patients with unstable angina (7·9 (0·9) pg/ml) and those with non-Q wave myocardial infarction (6·8 (1·1) pg/ml) had significantly higher endothelin concentrations at presentation than healthy controls ($P < 0.0001$ and $P < 0.01$). There was a small but significant difference in endothelin concentrations at presentation between patients who presented with unstable angina and those who presented with a subendocardial infarction (7·9 (0·9) *v* 6·8 (1·1) pg/ml, $P < 0.05$). At nine weeks, endothelin remained higher both in the unstable angina (7·8 (2·5) pg/ml) and the non-Q wave infarction groups (7·3 (2·5) pg/ml) than in the control population ($P = 0.0002$ and $P < 0.005$). At nine weeks there was no difference in the endothelin concentrations between patients who initially presented with either unstable angina or subendocardial infarction.

All comparisons between patients and controls were subsequently repeated with the age matched control group ($n = 11$). None of the conclusions were altered nor were the measures of significance abrogated by this analysis.

In view of the highly significant P values obtained for the above comparisons, the differences remained significant even after adjustments for multiple comparisons. Comparison of patients with and without events was performed only once at each of the analysed time points and in this instance the results were of borderline significance ($P = 0.042$ at presentation and $P = 0.018$ at nine weeks).

CORRELATION WITH CLINICAL CHARACTERISTICS

There was no difference in endothelin concentrations on admission between smokers, non-smokers, and ex-smokers (7·3 (1·1) *v* 7·8 (1·0) pg/ml) or between patients who received heparin and those who did not (7·8 (0·9) *v* 7·0 (1·2) pg/ml). Endothelin concentrations did not differ between men and women (7·3 (1·1) *v* 8·1 (0·8) pg/ml). There was no correlation between endothelin concentrations and age, either in the patients ($r = 0.23$, $P = 0.38$) or in the controls ($r = 0.06$, $P = 0.72$). We showed no correlation between endothelin concentrations and either systolic ($r = 0.38$, $P = 0.14$) or diastolic blood pressure ($r = 0.07$, $P = 0.8$) or the number of involved coronary arteries, as found by coronary angiography ($r = 0.03$, $P = 0.94$).

Discussion

This is the first prospective investigation of the relation between plasma endothelin and cardiovascular outcome in acute coronary syndromes. We have shown a persistent increase of plasma immunoreactive endothelin, lasting for at least nine weeks after admission, in a group of high risk patients presenting with unstable angina or non-Q wave myocardial infarction. Also, we have shown an association between endothelin concentrations at presentation and subsequent cardiovascular outcome.

Previous studies of endothelin in acute myocardial infarction have reported an early rise in plasma endothelin, with a subsequent progressive fall to normal values, completed within 3–14 days of the infarct.[14 18-20] Our results, which show that unstable angina and non-Q wave myocardial infarction are associated with high circulating endothelin at presentation, are consistent with those previous findings. Persistently increased circulating endothelin, present only in those patients who developed subsequent cardiovascular events, has not previously been reported.

There have been few previous studies of endothelin concentrations in unstable angina and these have provided conflicting results.[16 21 22] Ray *et al* and Stewart *et al* found no difference in endothelin concentrations in plasma and coronary sinus samples between patients with unstable angina, stable angina, and controls.[21 22] Also, in the study by Ray *et al* endothelin concentrations in unstable angina, although comparable with those in stable angina, were significantly lower than in a group of patients with acute myocardial infarction.[21] In contrast, in a study of 29 patients with unstable angina, Qiu *et al* reported that endothelin concentrations were high at presentation compared with healthy controls, and decreased progressively to normal at six hours.[16] In their study, the size of the initial increase of endothelin concentration in unstable angina was comparable with that found in 29 patients with acute myocardial infarction.

None of the previous studies of endothelin in acute coronary syndromes has provided cardiac outcome data. In our high risk population (56% event rate at 12 weeks) we have shown that endothelin concentrations at presentation may relate to subsequent cardiac events. The event rate in our population was higher than that usually described for unstable angina. It is conceivable that the populations studied by Ray *et al* and Stewart *et al* were of relatively lower risk and this may explain why they had no evidence of increased endothelins at the time of presentation. It is not clear, however, why in our study patients with unstable angina tended to have higher endothelin concentration at presentation than the non-Q wave infarction group. Qiu *et al* also reported a different time course of the increase in endothelins in unstable angina and myocardial infarction: in unstable angina the concentrations decreased progressively to normal values by six hours, whereas in myocardial infarction there was a further increase at 6 h, then a decrease at 12 hours. In the absence of multiple endothelin measurements, we cannot speculate as to whether different dynamics of the endothelin rise

could have contributed to the reported differences in endothelin concentrations in our patients. It has also to be considered whether these differences could have been related to more extensive use of heparin in our patients with unstable angina than in those who presented with subendocardial infarction. Although the evidence of an effect of heparin on the generation of endothelins is limited, heparin induced inhibition rather than stimulation of endothelin-1 expression and production by cultured endothelial cells has been reported.[25] In view of these findings, the pattern of heparin use would, if anything, tend to diminish the observed differences in endothelin concentrations between patients with unstable angina and non-Q wave infarction in our population.

In agreement with previous studies[15,16] we did not show a correlation between endothelin concentrations and common clinical characteristics, including age, systolic and diastolic blood pressure, smoking, and the extent of coronary artery disease.

We cannot differentiate which specific endothelin isoforms contribute to the rise in total plasma immunoreactivity in the studied population, because our assay cross reacts substantially with all three isoforms. As endothelin-2 is usually undetectable in plasma[23] this isoform is unlikely to constitute a large proportion of total immunoreactivity. Thus endothelin-1 and/or endothelin-3 may have contributed to the rise in circulating endothelin. Proendothelin-1 also cross reacts with our antibody, although only to a minor degree (7%), and is unlikely to have accounted for the rise in endothelin immunoreactivity.

Both increased generation and decreased clearance of endothelin could have caused the rise in plasma concentrations in patients with coronary syndromes. Plasma creatinine remained normal in all these patients, however, and thus increased generation seems more likely to have accounted for the rise. There is evidence from experimental studies that endothelin production is enhanced by shear stress, impaired release of nitric oxide, and physiological stress.[24-26] Both thrombin and transforming growth factor-β are also known to stimulate endothelin production in cell culture.[27,28] As unstable angina is linked with markers of a hypercoagulable state,[8-10] it is conceivable that excessive generation of thrombin or release of transforming growth factor-β from activated platelets may contribute to increased endothelin concentrations in acute coronary syndromes. This hypothesis would be indirectly supported by previous reports that endothelin concentrations were positively correlated with both thrombin-antithrombin III complex and β thromboglobulin concentrations in patients presenting with acute myocardial infarction.[14]

Any increase in generation of endothelins in our patients may have been a localised phenomenon or it may have occurred as a systemic process. If the increase occurred in the heart, high local concentrations of endothelin may have been reached. When present in sufficiently high concentrations endothelin may cause direct local or systemic vasoconstriction and potentiate platelet aggregation.[29,30] In cases of a chronic increase, endothelin may stimulate proliferation of vascular smooth muscle cells in atherosclerotic plaques.[31] Ultimately, endothelin could be implicated in the pathophysiological mechanism of acute coronary syndromes and its biological actions may actively contribute to myocardial ischaemia. Indeed, there is evidence that antibodies to endothelin limit the infarct size in animal models.[32] Alternatively, high endothelin concentrations may be a consequence of intermittent episodes of myocardial ischaemia. In either case endothelin could prove to be a useful prognostic marker for adverse outcome in acute coronary syndromes. If confirmed in larger prospective studies, the findings of this study raise a possibility of influencing the cardiovascular outcome in acute coronary syndromes with use of the recently described specific inhibitors to the generation of endothelin.[24]

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RESUSCITATION

Plasma endothelin following cardiac arrest: differences between survivors and non-survivors

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Abstract

Cardiac arrest is associated with major metabolic disturbances, including severe hypoxia and large increases in circulating catecholamines, both of which are known to stimulate generation of the potent endothelium-derived vasoconstrictor peptide endothelin-1. We have, therefore, examined plasma immunoreactive endothelin concentrations following cardiac arrest. Blood was sampled at 10-min intervals from a central venous catheter inserted at onset of resuscitation in 38 patients (13 female; mean age, 67 years) presenting with cardiac arrest to the Accident and Emergency Department at the Royal Infirmary of Edinburgh. Plasma immunoreactive endothelin concentrations (mean ± S.D.) in patients following cardiac arrest (5.4 ± 2.3 pg/ml) were no different from those in healthy subjects (5.1 ± 1.2 pg/ml). There was no significant difference between endothelin concentrations at presentation in survivors and non-survivors of cardiac arrest. However, non-survivors had a significant fall in endothelin concentrations with time from onset of resuscitation from 5.4 ± 2.2 pg/ml to 3.5 ± 1.8 pg/ml ($P = 0.002$), while survivors had a non-significant increase in concentrations. On multiple regression analysis there was a significant association between higher plasma endothelin concentration and survival ($r = 0.37$; $P = 0.009$). The failure of plasma endothelin to increase after cardiac arrest is unexpected. Although the fall in plasma endothelin with time in non-survivors may reflect the adverse physiological milieu that occurs during cardiac arrest, it is also possible that low endothelin concentrations contribute to the poor prognosis in this condition.

Key words: Resuscitation; Ventricular fibrillation; Asystole; Ischaemic heart disease; Endothelin; Endothelium; Vasoconstrictor peptides; Catecholamines

1. Introduction

The vascular endothelium, which forms the inner lining of all blood vessels, produces both vasodilator agents, such as nitric oxide and prostacyclin [1], and vasoconstrictors, such as angiotensin II [2] and endothelin-1 [3]. Endothelin-1 is a potent vasoconstrictor and pressor peptide with a uniquely prolonged action in both animals [3] and man [4,5]. Endothelin-1 is a member of a family of related isoforms, endothelin-1, -2 and -3, and endothelin-1 is the major isoform produced within

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the vasculature [6]. Although very high concentrations have been measured in patients on haemodialysis [7], sufficient to exert effects on vascular tone [8], in most circumstances endothelin-1 appears to be a locally acting autocrine and paracrine rather than a circulating hormone. Plasma concentrations of endothelin are likely to represent overspill from much higher concentrations at the interface between endothelium and vascular smooth muscle cell, similar to the situation for catecholamine release from nerve terminals.

Increased circulating concentrations of endothelin are found in diseases associated with regional vasoconstriction, including Prinzmetal's angina [9], Raynaud's disease [10] and cardiac failure [11,12]; in the last, plasma endothelin correlates with the severity of pulmonary hypertension. Plasma endothelin is also elevated in cardiogenic [13,14] and septic shock [15,16], possibly as a homeostatic response to maintain arterial pressure.

Cardiac arrest results in circulatory standstill and, even with adequate resuscitation, arterial blood pressure, oxygenation and pH are greatly reduced. During cardiac arrest there is a marked increase in sympathetic discharge, producing substantially higher plasma catecholamine concentrations than recorded in any other condition [17]. We hypothesised that plasma endothelin would increase during cardiac arrest, not only because factors that stimulate endothelin-1 production, such as catecholamines [3,18] and hypoxia [19], increase in cardiac arrest, whatever the cause, but also because impaired renal function would decrease clearance of endothelin-1 [20]. We have, therefore, measured plasma concentrations of immunoreactive endothelin in patients following cardiac arrest, and assessed their relationship with arterial blood oxygenation and survival.

2. Methods

2.1. Patients and protocol

All patients presenting with cardiac arrest to the Accident and Emergency Department at the Royal Infirmary of Edinburgh between 0900 and 1700 h in the period October 1991 to February 1992 were prospectively enrolled into this study, which was approved by the local Ethics Review Committee. The Accident and Emergency Department treats approximately 400 cardiac arrests each year. Resuscitation was carried out strictly according to the current UK Resuscitation Council guidelines [21,22]. Where indicated, patients were intubated, received DC countershock, and standard cardiac resuscitation drugs in doses directed by treatment protocols. A mechanical compression/ventilation device (Thumper™) was used in all patients as an aid to cardiopulmonary resuscitation.

Soon after the onset of resuscitation a central venous catheter was inserted for administration of drugs. Blood was withdrawn from this line at 10-min intervals while circulatory standstill persisted. In a subset of patients, a simultaneous arterial sample was obtained on one occasion for blood gas analysis. For each sample, 9 ml of blood was added to 1 ml ethylenediamine-tetra-acetate (EDTA potassium salt; final concentration 10 mmol/l). Each sample was separated at 4°C (within 10 min) and then stored at −20°C for 24 h before being transferred to a −70°C freezer.

2.2. Analytical procedures

Plasma immunoreactive endothelin was measured by radioimmunoassay [23]. SepPak C18 silica columns (Waters Associates, Milford, MA) were equilibrated by washing with methanol (5 ml), distilled water (5 ml) and then 4% acetic acid (5 ml). Each 2-ml plasma sample was diluted with 3 ml of 4% acetic acid and loaded onto a column; these were washed with 25% ethanol (3 ml), and eluted with 4% acetic acid in 86% ethanol (2 × 1 ml). The eluates were evaporated under nitrogen in a waterbath at 37°C, and reconstituted in borate buffer of pH 8.4 (2 ml). Duplicate extracted samples and standards containing 1–48 pg/ml of endothelin-1 (each 200 μl) were incubated with rabbit polyclonal antibody raised against endothelin-1 (ITS Production B.V., Wijchen, Netherlands; in 100 μl distilled water) and [^{125}I]endothelin-1 (ITS; in 100 μl distilled water). After vortexing, tubes were incubated for 18 hours at 4°C. Donkey anti-rabbit gamma globulin bound on solid phase (ITS; 100 μl) was added to all tubes,

except to the total count tubes, and tubes were incubated for 30 min at room temperature. After adding distilled water (1 ml), tubes were centrifuged for 15 min at 2000 × *g* at room temperature. The amount of radioactivity in the antibody-bound fraction was determined by gamma counting for 2 min. The recovery of added endothelin-1 was 84%. Intra- and inter-assay coefficients of variation were 2.4% ($n = 6$) and 4.2% ($n = 5$), respectively. The sensitivity of this assay is 1 pg/ml endothelin. Cross reactivities of the assay with endothelin-1, endothelin-2, endothelin-3 and proendothetin-1 are 100, 52, 96 and 7%, respectively. The normal range (mean ± 2 S.D.) for plasma immunoreactive endothelin with this assay, derived from blood obtained from 19 healthy control subjects (six female; age, 20–38 year) maintained recumbent for 30 min, is 2.7–7.5 pg/ml (mean = 5.1).

2.3. Statistical analysis

Statistical analysis was performed using Student's paired and unpaired *t*-tests, together with simple and multiple regression analysis of plasma immunoreactive endothelin against time from resuscitation, arterial hydrogen ion concentration and oxygen tension (P_aO_2) in survivors and non-survivors, taking significance at the 5% level. Results are expressed as mean ± S.D., with ranges provided where relevant.

3. Results

During the study period, 38 patients fulfilled the entry criterion; 13 were female and 25 were male with mean age 67 years (range, 49–91). Fifteen patients left the resuscitation room alive and 23 died. The mean time from arrest to commencement of resuscitation was significantly shorter in survivors (5 ± 7 min) than in non-survivors (10 ± 8 min; $P = 0.03$). The primary documented rhythm was ventricular fibrillation in 20, asystole in 14 and electromechanical dissociation in four. All 15 of the survivors had ventricular fibrillation. All patients received intravenous adrenaline (2.1 ± 1 mg), with non-survivors (2.9 ± 1 mg) tending to receive higher doses than survivors (1.3 ± 0.9 mg; NS). Non-survivors also received more atropine (0.6 ± 0.2 mg) than survivors (0.2 ± 0.2 mg; $P = 0.01$). The mean time from onset of resuscitation to obtaining the first sample for endothelin assay was 20 ± 14 min in survivors, and 25 ± 12 min in non-survivors ($P = 0.27$), with between one and four samples taken per patient (mean = 2.1).

The mean plasma concentration of endothelin was 5.4 ± 2.3 pg/ml in the first sample obtained from all patients presenting with cardiac arrest. This was not significantly different from our normal range. Endothelin concentrations in the first sample from survivors (5.9 ± 2.6 pg/ml) were not significantly different from those in non-survivors (5.1 ± 2.1 pg/ml). However, plasma endothelin fell significantly between the first (5.4 ± 2.2 pg/ml) and last (3.5 ± 1.8 pg/ml; $P = 0.002$) samples in those non-survivors who had serial samples taken, but tended to increase in those survivors who had serial samples taken (from 5.6 ± 2.4 pg/ml to 7.0 ± 2.8 pg/ml; $P = 0.16$; Fig. 1). In addition, while there was no significant correlation between endothelin concentrations and time from institution of resuscitation in survivors ($r = 0.10$; $P = 0.68$), there was a significant negative correlation in non-survivors ($r = -0.45$; $P = 0.002$; Fig. 1).

Nineteen patients had simultaneous samples obtained for arterial blood gas analysis, with a mean time to arterial sampling of 19 ± 11 min in survivors and 26 ± 10 min in non-survivors (not significantly different; $P = 0.15$). Arterial P_aO_2 was not significantly different between survivors (mean = 21 ± 16 kPa) and non-survivors (mean = 20 ± 22 kPa; $P = 0.89$), and P_aO_2 did not correlate with plasma endothelin concentration in either survivors ($r = 0.13$; $P = 0.74$) or non-survivors ($r = 0.09$; $P = 0.81$). Hydrogen ion concentration was significantly ($P = 0.003$) lower in survivors (mean = 55 ± 18 mmol/l) than non-survivors (mean = 87 ± 22 mmol/l). There was a positive correlation between the degree of acidosis and endothelin concentration in the survivors ($r = 0.75$; $P = 0.02$), but not in the non-survivors ($r = 0.26$; $P = 0.46$; Fig. 2).

In a multiple regression analysis of endothelin concentrations in all samples against outcome,

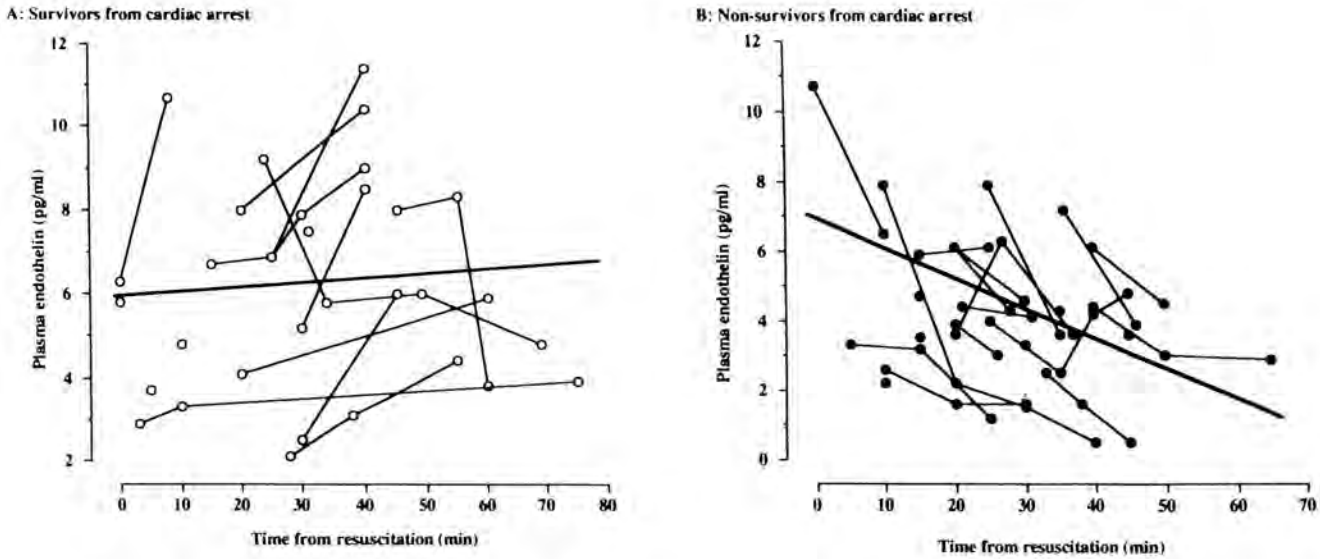

Fig. 1. Plasma immunorcactive endothelin concentrations plotted against time from institution of resuscitation in individual survivors (A) and non-survivors (B) from cardiac arrest. Endothelin concentrations fall with time in non-survivors ($P = 0.002$), but not survivors ($P = 0.16$), of cardiac arrest. Correlation between plasma endothelin and time is also shown and is significant for non survivors ($r = -0.45$; $P = 0.002$), but not for survivors ($r = 0.10$; $P = 0.68$).

time from resuscitation, arterial hydrogen ion concentration and P_aO_2, there was a significant positive association with outcome ($r = 0.37$; $P = 0.0009$), but not with the other factors.

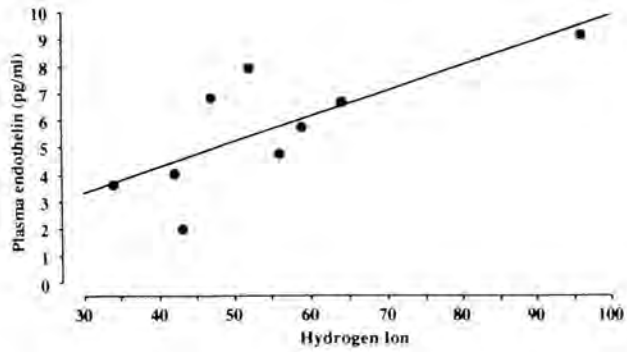

Fig. 2. Correlation between plasma immunoreactive endothelin concentration and arterial hydrogen ion concentration in survivors ($r = 0.75$; $P = 0.02$) from cardiac arrest.

4. Discussion

In this study, plasma immunoreactive endothelin was not elevated in patients with cardiac arrest. This is an unexpected finding, given the severe metabolic disturbance that occurs following cardiac arrest. This includes severe hypoxaemia, metabolic acidosis and massive release of catecholamines [17]; factors which stimulate generation of endothelin-1 [3,18,19]. Further, it might be expected that plasma endothelin concentrations would increase due to decreased renal clearance during circulatory standstill and resuscitation. It is unlikely that our negative findings could be accounted for by the difference in ages between the patients and the control group, because plasma endothelin concentrations do not appear to be affected by age [24].

Plasma endothelin may fail to rise early after cardiac arrest because endothelin-1 is not stored within endothelial cells and de novo synthesis of the peptide takes several hours to occur. This is certainly the case for stimulation of generation of endothelin-1 in vitro by hypoxia and catechol-

amines [3,18,19]. However, plasma endothelin can increase within minutes in vivo in man, in response to stimuli such as orthostasis and the cold pressor test [6]. Generation of endothelin-1 by isolated endothelial cells is stimulated by shear stress [25]. Therefore, endothelin concentrations may fail to increase because of decreased exposure of endothelial cells to shear stress during cardiac arrest, particularly pulsatile shear stress. In addition, the adverse physiological conditions that occur during cardiac arrest may prevent DNA transcription, protein production from mRNA or the activity of proendothelin-1 converting enzyme. Such conditions may help to explain why endothelin concentrations fall with time in non-survivors.

Although such factors may account for the lack of elevation of endothelin-1 concentrations early in resuscitation, they do not explain why endothelin concentrations are not increased later in the course of the arrest. It is possible that other mechanisms come into play to prevent increases in endothelin generation. These may include an increase in the generation of nitric oxide, and of A- and C-type natriuretic peptides, as well as rapid destabilisation of the mRNA for endothelin-1 [6,26].

Although there was no difference in plasma endothelin concentrations at presentation between survivors and non-survivors, there was a significant positive association between outcome and plasma endothelin concentrations in multiple regression analysis. This relation with outcome may help to explain the associations we observed with time and hydrogen ion concentrations in the non-survivors and survivors, respectively.

As opposed to blood flow to the heart and brain, blood flow to peripheral tissues is very poor during cardiopulmonary resuscitation. This leads to local tissue hypoxia, even in the presence of apparently adequate arterial P_aO_2. This in turn causes a local metabolic acidosis, which may stimulate production of endothelin-1, which will tend to accumulate in pooled blood in these tissues. The positive correlation of endothelin concentrations with hydrogen ion only in survivors, may therefore be most readily explained by higher peripheral tissue blood flows during resuscitation in these patients, with greater venous return of peripheral blood, containing high concentrations of endothelin-1. The failure of plasma endothelin to correlate with hydrogen ion in non-survivors is not explained by survivors having a longer delay to arterial sampling, allowing more time for generation of endothelin, because survivors tended to have a shorter time from resuscitation to arterial sampling than non-survivors. Neither can the association be attributed to a greater degree of acidosis in survivors, reflecting a more potent stimulus for generation of endothelin, because this group had significantly lower hydrogen ion concentrations.

Because plasma endothelin concentrations are not elevated at presentation in patients with cardiac arrest, it is likely that endothelin has no major role as a circulating pressor hormone in this condition. However, the failure of circulating endothelin concentrations to increase does not exclude a role for endothelin in the cardiovascular response to cardiac arrest. Sustained endothelin generation may occur without an increase in circulating endothelin, because generation is directed away from the lumen of the blood vessel. Here, experimental evidence may be helpful, using specific receptor antagonists or inhibitors of proendothelin-1 converting enzyme in animal models to determine whether local generation of endothelin contributes to the restoration of vascular tone following cardiac arrest.

During cardiac arrest and cardiopulmonary resuscitation, vasopressor agents such as adrenaline are used to increase systemic vascular resistance in order to improve myocardial and cerebral blood flow [27]. As endothelin-1 has potent long lasting vasoconstrictor, pressor and positive inotropic effects [6], high endothelin concentrations may be beneficial during cardiac arrest. Although the correlation of plasma endothelin with outcome may reflect the adverse physiological milieu that occurs during cardiac arrest, it is also possible that the fall in endothelin concentrations in non-survivors contributes to their poor prognosis.

5. Acknowledgement

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Reduced Responsiveness to Endothelin-1 in Peripheral Resistance Vessels of Patients With Syndrome X

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Objectives. **This study sought to assess the contribution and action of nitric oxide and endothelin-1 in peripheral resistance vessels of patients with syndrome X.**

Background. **Patients with syndrome X may have a generalized disorder of vascular and endothelial function, promoting vasospasm.**

Methods. **Changes in blood flow responses to intrabrachial infusion of the endothelium-dependent vasodilators substance P and acetylcholine, the endothelium-independent nitric oxide donor sodium nitroprusside and the endothelin type A (ET_A) receptor antagonist BQ-123 were assessed using venous occlusion plethysmography in 10 patients with syndrome X and 10 matched control subjects. Vasoconstrictor responses to the nitric oxide synthase inhibitor L-N^G-monomethyl arginine (L-NMMA) and endothelin-1 were also determined.**

Results. **There were no significant differences in the responses to acetylcholine, substance P, sodium nitroprusside or BQ-123 between patients and control subjects. However, despite similar degrees of vasoconstriction in response to L-NMMA in both groups, endothelin-1 caused a reduction in forearm blood flow of only 20 ± 2% in patients with syndrome X compared with 35 ± 3% in matched control subjects at 90 min ($p < 0.001$). Although plasma endothelin-1 concentrations were not significantly higher in patients with syndrome X (4.8 vs. 4.0 pg/ml, $p = 0.17$), the vasoconstriction caused by endothelin-1 infusion correlated inversely with plasma endothelin-1 concentrations ($r = -0.51$, $p = 0.04$).**

Conclusions. **Patients with syndrome X had normal basal and stimulated nitric oxide activity and basal endogenous ET_A receptor-mediated vascular tone. However, despite otherwise normal vascular function, there was reduced responsiveness to exogenous endothelin-1, possibly reflecting overactivity of this system and ET_A receptor downregulation.**

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Over 100 years ago, Sir William Osler described a condition of "hysterical angina" or "pseudoangina" in patients who had typical anginal pain but were found to have normal coronary arteries after death (1). With the emergence of electrocardiography and coronary angiography, this condition became known as syndrome X (2). The precise definition of this syndrome varies among centers and clinicians. However, a relatively homogeneous group of patients can be defined on the basis of typical anginal chest pain, a positive exercise tolerance test and a normal coronary angiogram (3,4).

Results from several major studies suggest that within syndrome X there is a substantial subgroup of patients with abnormal vascular responses. Various investigators have documented impaired coronary vascular reserve with abnormal responses to vasodilators (5–8) and to atrial pacing (9,10). Some investigators (11) have postulated that in this subgroup of patients with syndrome X, the chest pain is a consequence of increased coronary resistance vessel tone leading to ischemic pain—so-called "microvascular angina"—and that this may be due to endothelial dysfunction (6,8,12). Evidence in favor of an ischemic origin of the chest pain in syndrome X arises from histologic abnormalities seen in myocardial biopsies (13), the reduction of myocardial blood flow (by positron emission tomography and thallium scanning) (7,13,14) and the identification of elevated coronary sinus lactate concentrations (10). Moreover, the vascular abnormalities of syndrome X are not confined to the coronary circulation because impaired reactive hyperemia to forearm ischemia (12) and structural alterations in the small arteries of the skin and subcutaneous tissue (15) have been detected. Therefore, there may be a generalized dysfunction of vascular or endothelial function, as opposed to a local coronary abnormality, in syndrome X.

Both the nitric oxide and endothelin systems contribute to the maintenance of basal peripheral vascular tone in humans (16,17). The aim of the current study was to assess the contribution and action of nitric oxide and endothelin-1 in

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Abbreviations and Acronyms

ANOVA = analysis of variance
ECG = electrocardiogram
ET_A receptor = endothelin type A receptor
ET_B receptor = endothelin type B receptor
L-NMMA = L-N^G-monomethyl arginine
vWF = von Willebrand factor

peripheral resistance vessels of patients with syndrome X. The peripheral vascular actions of endogenous and exogenous nitric oxide and endothelin-1 were determined using the nitric oxide synthase inhibitor, L-N^G-monomethyl arginine (L-NMMA), the nitric oxide donor, sodium nitroprusside, the endothelin type A (ET_A) receptor antagonist, BQ-123, and endothelin-1 peptide. In addition, endothelial cell function was assessed using the endothelium-dependent, nitric oxide–generating vasodilators, substance P and acetylcholine (18).

Methods

Patients. Patients with syndrome X were recruited according to the criteria in Table 1 and were matched by age and gender with healthy control subjects. The patients had undergone coronary angiography and exercise treadmill tests within 12 months of the study and were considered to have normal smooth coronary arteries on angiography by two independent cardiologists. All subjects attended a screening visit and received a clinical examination, rest electrocardiogram (ECG), echocardiogram and oral glucose tolerance test. Control subjects did not have a history of chest pain or clinically significant disease and had a normal rest ECG and echocardiogram. Studies were undertaken with the approval of the Lothian Research Ethics Committee and were in accordance with the Declaration of Helsinki. Each subject gave written informed consent before entry into the study.

Assays. Plasma endothelin-1 (Peninsula Laboratories Europe Ltd., St. Helens, United Kingdom) and big endothelin-1 (Peninsula Laboratories Europe Ltd.) were determined by radioimmunoassay (19); von Willebrand factor (vWF) antigen (Dako A/S, Glostrup, Denmark) and insulin (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) by an enzyme-linked immunosorbent assay; and nonesterified fatty acid (Wako, Neuss, Germany), triglyceride (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) and cholesterol (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) by an enzymatic colorimetric method. Low density lipoprotein cholesterol was determined by the method of Friedewald et al. (20).

Study design. Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-Silastic strain gauges applied to the widest part of the forearm, as described previously (17,21). Blood pressure was monitored in the noninfused arm at intervals throughout each study using a semiautomated, noninvasive oscillometric sphygmomanometer (22) (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan). The brachial artery of the nondominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd., Birmingham, United Kingdom) under 1% lidocaine (xylocaine; Astra Pharmaceuticals Ltd., Kings Langley, United Kingdom) local anesthesia. The total rate of intraarterial infusions was maintained constant throughout all studies at 1 ml/min. Substance P (Clinalfa AG, Läufelfingen, Switzerland), sodium nitroprusside (Nipride; Roche, Welwyn Garden City, United Kingdom), acetylcholine (Miochol; Iolab, Brack-

Table 1. Inclusion and Exclusion Criteria for Study

Inclusion Criteria	Exclusion Criteria
Age between 21 and 75 years	Hypertension (systolic blood pressure >160 mm Hg or diastolic blood pressure >90 mm Hg)
Typical anginal chest pain	Diabetes mellitus
	Echocardiography
≥2-mm horizontal or downsloping ST segment depression (60 ms after the J point) on exercise treadmill testing using the Bruce protocol	Left ventricular hypertrophy (>1.3-mm thickness of posterior wall of left ventricle)
	Regional wall motion abnormalities
	Valvular heart disease (trivial mitral incompetence was permitted)
Angiographically normal smooth coronary arteries	Impaired ventricular function
	Electrocardiography
	Left ventricular hypertrophy (>35 mm from summation of amplitude of S wave in lead V_1 and R wave in lead V_5 or V_6)
	Left bundle branch block
	Pathologic Q waves or T wave inversion
	Any evidence of coronary artery atherosclerosis
	Previous myocardial infarction
	History of dyspepsia, peripheral vascular disease or cerebrovascular disease
	Other significant cardiac disease

Table 2. Characteristics of Study Group

	All Subjects		Nitric Oxide Studies		Endothelin Studies	
	Pts With Syndrome X	Control Subjects	Pts With Syndrome X	Control Subjects	Pts With Syndrome X	Control Subjects
Age (years)	56 ± 1	55 ± 3	56 ± 2	54 ± 3	54 ± 3	55 ± 4
Gender (M/F)	4/11	4/7	4/6	4/6	3/7	3/7
Body mass index (kg/m^2)	28 ± 1	25 ± 1*	28 ± 2	25 ± 1*	28 ± 2	25 ± 1*
Blood pressure (mm Hg)						
Systolic	138 ± 4	127 ± 3	139 ± 5	127 ± 3	137 ± 4	128 ± 3
Diastolic	78 ± 1	77 ± 2	79 ± 2	77 ± 2	79 ± 2	78 ± 2
Heart rate (beats/min)	71 ± 1	70 ± 1	70 ± 1	70 ± 1	70 ± 1	70 ± 1
ST segment depression during treadmill exercise testing (mm)	2.7 ± 0.2	—	2.6 ± 0.2	—	2.7 ± 0.1	—
Plasma endothelin-1 (pg/ml)	4.8 ± 0.5	4.0 ± 0.2	4.5 ± 0.6	4.0 ± 0.2	4.8 ± 0.5	4.0 ± 0.3
Plasma big endothelin-1 (pg/ml)	20 ± 2	18 ± 2	17 ± 0.7	18 ± 1.7	20 ± 1.5	19 ± 1.9
Plasma vWF (IU/ml)	1.17 ± 0.12	0.98 ± 0.05	1.13 ± 0.16	0.97 ± 0.05	1.3 ± 0.2	0.98 ± 0.05
Total cholesterol (mg/dl)	258 ± 15	268 ± 23	260 ± 21	257 ± 23	258 ± 23	276 ± 24
LDL cholesterol (mg/dl)	174 ± 13	197 ± 22	175 ± 18	186 ± 22	178 ± 19	202 ± 24
HDL cholesterol (mg/dl)	55 ± 4	47 ± 5	58 ± 5	47 ± 5	50 ± 5	49 ± 5
Triglycerides (mg/dl)	141 ± 15	120 ± 22	134 ± 18	121 ± 24	146 ± 19	124 ± 25
Oral glucose tolerance						
Fasting glucose (mmol/liter)	4.9 ± 0.2	4.9 ± 0.2	5.0 ± 0.2	4.9 ± 0.1	5.0 ± 0.1	4.9 ± 0.2
2-h glucose (mmol/liter)	5.1 ± 0.4	5.2 ± 0.4	5.0 ± 0.4	4.9 ± 0.4	5.2 ± 0.5	5.4 ± 0.4
Fasting insulin (IU/ml)	8.9 ± 1.5	10.9 ± 2.7	7.2 ± 1.3	9.7 ± 2.7	9.9 ± 2.1	11.6 ± 2.9
2-h insulin (IU/ml)	45.3 ± 14.5	25.8 ± 5.9	36.5 ± 15	22.6 ± 5.6	59.0 ± 22.5	28.0 ± 6.1
Fasting NEFA (mEq/liter)	584 ± 71	572 ± 76	541 ± 60	550 ± 81	626 ± 108	604 ± 77
2-h NEFA (mEq/liter)	26 ± 12	33 ± 13	29 ± 17	36 ± 14	32 ± 18	36 ± 14
Thyroid stimulating hormone (mU/liter)	2.0 ± 0.6	1.3 ± 0.3	2.0 ± 0.6	1.4 ± 0.3	2.5 ± 0.8	1.2 ± 0.2
Thyroxine (pmol/liter)	15.2 ± 0.9	15.3 ± 0.8	15.1 ± 1.0	15.3 ± 0.8	14.9 ± 1.0	15.6 ± 0.8
Urea (mmol/liter)	5.8 ± 0.3	5.2 ± 0.3	5.8 ± 0.3	5.2 ± 0.3	5.8 ± 0.4	5.1 ± 0.4

*p = 0.02 (unpaired *t* test). Data are presented as mean value ± SD. F = female; HDL = high density lipoprotein; LDL = low density lipoprotein; M = male; NEFA = nonesterified fatty acids; Pts = patients; vWF = von Willebrand factor.

nell, United Kingdom), L-NMMA (Clinalfa AG), endothelin-1 (Clinalfa AG) and BQ-123 (American Peptide Company) were administered after dissolution in 0.9% saline (Baxter Healthcare Ltd., Thetford, United Kingdom). Before the subjects underwent each of the studies, aspirin was discontinued for 10 days and vasoactive or nonsteroidal anti-inflammatory drugs for at least 5 half-lives. All subjects abstained from alcohol for 24 h and from food and caffeinated drinks for at least 5 h before each study. Subjects rested recumbent in a quiet, temperature-controlled room maintained at 23.5 to 24.5°C. Before participating in one of the following protocols, saline was infused for the first 30 min to allow time for equilibration, with forearm blood flow measured every 10 min and the final measurement taken as basal blood flow.

Nitric oxide system. In 10 patients with syndrome X and 10 age- and gender-matched control subjects, intrabrachial substance P was administered at 1, 2 and 4 pmol/min (18,23); sodium nitroprusside at 5, 15 and 30 nmol/min (23); and acetylcholine at 27.5, 55 and 110 nmol/min (16,18,23) for 6 min at each dose. Administration of the three agents was separated by 20-min saline infusions and given in random order. Finally, after a further 20-min saline infusion, L-NMMA was administered at 4 μmol/min (16,18) for 10 min. Forearm blood flow measurements were made for the last 3 min of each infusion period.

Endothelin system. In 10 patients with syndrome X and 10 age- and gender-matched control subjects, intrabrachial BQ-123 was administered at 10 nmol/min (17) for 90 min. On a separate study day, at least 1 month later, endothelin-1 was administered at 5 pmol/min (17) for 90 min. Forearm blood flow measurements were made for 3 min every 6 min.

All subjects were not able to attend both the nitric oxide and endothelin studies; five patients and nine control subjects were common to both phases.

Data analysis and statistics. Study group size, based on reproducibility data derived from forearm resistance vessel responses to endothelin-1, gave a 90% power to detect a 24% difference in blood flow responses at a significance level of 5%. Data were examined, as appropriate, by two-way analysis of variance (ANOVA) with repeated measures, the two-tailed unpaired Student *t* test and regression analysis using Excel version 5.0 (Microsoft). All results are expressed as the mean value ± SEM. Statistical significance was set at the 5% level.

Results

Subject characteristics. Patients with syndrome X were well matched with control subjects for age, gender and serum lipid and thyroid profiles (Table 2). The rest ECG was normal in both patients and control subjects, with no baseline ST

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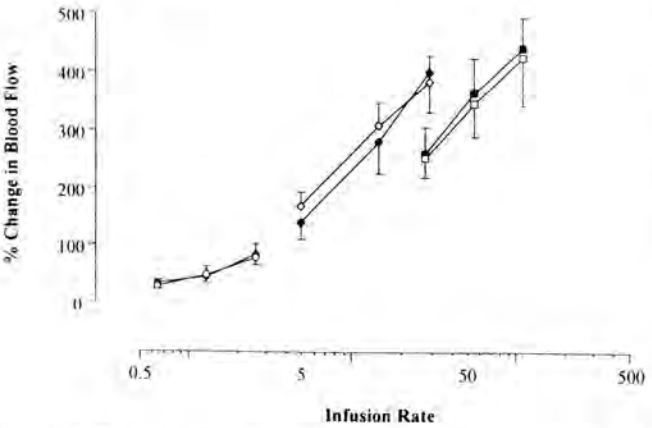


Figure 1. Responses in forearm blood flow to incremental doses of substance P **(circles [pmolmin])**, acetylcholine **(squares [nmol/min])** and sodium nitroprusside **(triangles [nmol/min])** in patients with syndrome X **(solid symbols)** and control subjects **(open symbols)**. There were no significant differences between patients and control subjects.

segment changes. Body mass index was significantly higher in the patients with syndrome X ($p = 0.02$), with a trend ($0.10 > p > 0.05$) toward higher systolic blood pressures and 2-h plasma insulin concentrations. There were no significant differences in other hemodynamic or metabolic variables. There were no significant differences in mean plasma vWF ($p = 0.16$), endothelin-1 ($p = 0.17$) or big endothelin-1 concentrations ($p = 0.58$).

Nitric oxide system. There were no significant differences in length (25.4 ± 0.7 vs. 25.2 ± 0.7 cm) or basal blood flow (3.8 ± 0.4 [range 2.9 to 9.2] vs. 3.3 ± 0.5 [range 1.0 to 6.3] ml/100 ml per min) of the infused forearm in patients with syndrome X versus control subjects (24). There were no significant changes in blood pressure, heart rate or blood flow in the noninfused forearm during the course of the studies.

Substance P, acetylcholine and sodium nitroprusside all caused dose-dependent vasodilation in the infused forearm ($p < 0.001$ for all) (Fig. 1). L-NMMA caused a 36.7 ± 3.4% ($p < 0.001$) and 38.4 ± 3.1% ($p = 0.007$) reduction in forearm blood flow in patients with syndrome X and control subjects, respectively. There were no significant differences in the blood flow responses to substance P, acetylcholine, sodium nitroprusside or L-NMMA between patients and control subjects.

Endothelin system. There were no significant differences in basal blood flow of the infused forearm on the endothelin-1 (3.5 ± 0.8 [range 2.3 to 6.2] vs. 4.2 ± 0.5 [range 1.8 to 10.2] ml/100 ml per min) and BQ-123 (3.2 ± 0.9 [range 1.7 to 6.4] vs. 4.1 ± 0.5 [range 1.0 to 10.0] ml/100 ml per min) study days in patients with syndrome X versus control subjects. There were no significant changes in blood pressure, heart rate or blood flow in the noninfused forearm during the course of the BQ-123 and endothelin-1 study days.

BQ-123 caused a progressive vasodilation ($p < 0.001$ by ANOVA for both groups), which appeared to reach a maximum by 72 min (Fig. 2). At 90 min, forearm blood flow was increased by 39 ± 6% in the patients with syndrome X and 37 ± 9% in the control subjects. There was no significant difference between patients and control subjects.

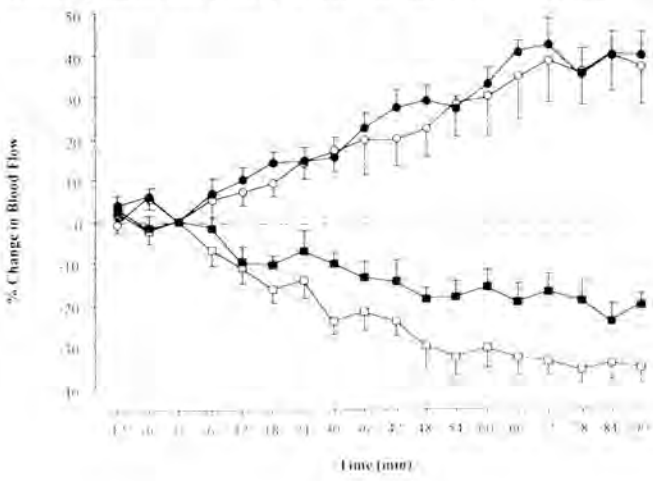
Endothelin-1 caused a progressive vasoconstriction ($p < 0.001$ by ANOVA for both groups), which appeared to reach a maximum by 66 min (Fig. 2). The reduction in forearm blood flow was significantly ($p < 0.001$ by two-way ANOVA) less in the patients with syndrome X (20 ± 2% [range 9% to 29%] at 90 min) than in the control subjects (35 ± 3% [range 25% to 53%]) at 90 min. Using data from all subjects, the degree of vasoconstriction produced by endothelin-1 infusion was inversely correlated with plasma endothelin-1 concentrations ($r = -0.51$, $p = 0.04$), but the correlation with mean arterial pressure was not significant ($r = 0.19$, $p = 0.41$).

Discussion

A number of previous studies have used broader criteria for defining syndrome X, including patients with ≥1 mm ST segment depression (8,25–27) and those with minor, "hemodynamically nonsignificant" atherosclerotic plaques in the coronary arteries (27,28). In an attempt to define a more precise group of patients with syndrome X, we employed criteria of ≥2 mm ST segment depression on exercise testing in combination with typical anginal chest pain and normal smooth coronary arteries on angiography. Patients with other potential causes of chest pain or with coexisting conditions associated with microangiopathy were excluded. In agreement with previous studies (26,29), our patients with syndrome X tended to be insulin-resistant, although they also had a larger body mass index and tended to have a higher systolic blood pressure.

Syndrome X and endothelin-1. For the first time, to our knowledge, we report that patients with syndrome X, as compared with control subjects, have a reduced responsiveness of peripheral resistance vessels to endothelin-1 despite normal vasodilation to the ET_A receptor antagonist BQ-123. In an earlier study with a larger number of patients (n = 40), Kaski

Figure 2. Responses in forearm blood flow to 90-min infusions of BQ-123 **(circles)** and endothelin-1 **(squares)** in patients with syndrome X **(solid symbols)** and control subjects **(open symbols)**. $p < 0.001$ between patients and control subjects for endothelin 1 response.



et al. (25) reported a significant elevation in plasma endothelin-1 concentrations (3.8 ± 1.3 vs. 2.9 ± 0.7 pg/ml) in patients versus control subjects. We did not demonstrate a significant elevation in plasma endothelin-1 or big endothelin-1 concentrations in our patients with syndrome X, although the magnitude was similar to the previous study and probably reflects the fact that the study was not powered to detect such a difference. However, interestingly, we did find an inverse correlation between endothelin-1–induced vasoconstriction and circulating plasma endothelin-1 concentrations. This suggests that in the presence of higher endothelin-1 concentrations, possibly related to increased endothelin generation, there may be ET_A receptor downregulation such that the overall contribution of ET_A receptor-mediated vascular tone remains unchanged. Although ET_A receptor downregulation may explain the reduction in vasoconstriction to exogenous endothelin-1, endothelin type B (ET_B) receptor function may also be important and merits further investigation. Indeed, abnormalities of ET_B receptor function have been demonstrated in the coronary vessels of an animal model of heart failure (30), in the peripheral resistance vessels of patients with chronic heart failure (31) and in human atherosclerotic vessels (32).

We have recently shown that the majority of the vasodilation seen with selective ET_A receptor antagonism results from nitric oxide release (33). Given our findings of normal nitric oxide–mediated responses in syndrome X, it may not be surprising that the BQ-123 responses were similar in the two groups. Thus, detecting a reduction or augmentation of endogenous endothelin-1–mediated vasoconstriction may be obscured by the nitric oxide release seen with BQ-123.

Syndrome X and nitric oxide. Despite evidence in support of a nitric oxide–mediated endothelial dysfunction involving the coronary resistance vessels of patients with syndrome X (6,8,28,34), we were unable to detect a significant abnormality affecting normal basal and stimulated release of, and sensitivity to, nitric oxide in the resistance vessels of the forearm circulation in vivo. This is in agreement with the findings of normal endothelium-dependent and -independent nitric oxide–mediated vasorelaxation in structurally abnormal peripheral resistance arteries of patients with syndrome X studied ex vivo (15). However, in a post hoc analysis, we did find that plasma concentrations of vWF were above the normal range (0.42 to 1.22 IU/ml) in six patients with syndrome X, but in none of the control subjects ($p < 0.01$ by chi-square test). Given our findings and those of Kaski et al. (25), it would appear that patients with syndrome X may have a generalized endothelial dysfunction that does not universally affect the nitric oxide system.

Study limitations. The failure to detect differential responses to endothelium-dependent nitric oxide–mediated peripheral vascular responses may reflect the relative hypercholesterolemia of the study groups. Both the patient and control groups had relatively high mean serum cholesterol concentrations, although this is consistent with the average prevailing serum cholesterol concentrations in the Scottish population (35). This may have conferred some degree of endothelial dysfunction on the subjects (36) and obscured the contribution of further dysfunction. In addition, the slow onset and offset of action of endothelin agonists and antagonists mean that only one dose of each agent can be administered on each study day. Thus, a full dose-response relation cannot easily be determined. Moreover, further characterization of responses mediated by the ET_B receptors is now needed to further clarify this response. There also remains the possibility that the observed differences are related to the disparity in body mass index between the two groups.

Conclusions. Our study suggests that despite normal overall peripheral resistance vessel function, there appears to be a reduced responsiveness to endothelin-1 in patients with syndrome X, consistent with increased endothelin-1 production and ET_A receptor downregulation. Further studies are now required to fully characterize the role of the endothelin system in both the peripheral and coronary circulations in this condition.

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SECTION 4

HEART FAILURE AND PULMONARY HYPERTENSION

Papers 28-30

Heart 1996;75:576–581

Hormonal and renal differences between low dose and high dose angiotensin converting enzyme inhibitor treatment in patients with chronic heart failure

Neil C Davidson, Wendy J Coutie, David J Webb, Allan D Struthers

Abstract

***Objective*—To assess the differential effects of low dose (5 mg) and high dose (20 mg) lisinopril treatment on cardiovascular hormones, renal function, and blood pressure over 24 hours in patients with heart failure.**

***Design*—Double-blind crossover study.**

***Setting*—Department of Clinical Pharmacology, Ninewells Hospital and Medical School, Dundee.**

***Patients*—19 patients with chronic heart failure and left ventricular ejection fraction ⩽ 45%.**

***Results*—Plasma concentrations of aldosterone and endothelin were lower on the 20 mg dose (plasma aldosterone mean at peak drug effect: 90·7 *v* 152·0 pg/ml, P < 0·001; mean at trough effect: 124·7 *v* 174·4 pg/ml, P < 0·01; plasma endothelin at trough effect 4·70 *v* 6·04 pmol/l, P = 0·03). Creatinine clearance was lower on 20 mg lisinopril (68·7 *v* 82·1 ml/min, P < 0·05). The area under the curve for diastolic blood pressure over 24 hours was significantly lower on 20 mg (mean difference 3·0 mm Hg, P = 0·04); for systolic blood pressure there was a similar trend (mean difference 5·7 mmHg, P = 0·05). Plasma concentrations of atrial natriuretic peptide (ANP) and B-type natriuretic peptide were similar for both doses; urinary excretion of ANP was lower on 20 mg (12·2 *v* 13·6 pmol, P < 0·05).**

Conclusions—These results indicate that within the usual therapeutic range, high doses of lisinopril cause greater suppression of selected cardiovascular hormones than low doses in heart failure, but are associated with lower creatinine clearance in some patients.

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Keywords: heart failure; ACE inhibition; lisinopril; hormones

The role of angiotensin-converting enzyme (ACE) inhibitors in the treatment of heart failure is well-established but the optimal dose is unknown. Each of the large studies that showed improved survival with ACE inhibitors in heart failure used relatively high doses of enalapril: in CONSENSUS the mean dose was 18·4 mg,[1] in VeHFT II it was 15 mg,[2] and in the SOLVD treatment limb it was 16·6 mg.[3] Often the doses of ACE inhibitors prescribed for patients with heart failure in clinical practice are much lower, although there is little evidence to suggest that these low doses are effective.[4] The effects of low dose (5 mg once a day) and high dose (35 mg once a day) lisinopril on mortality in over 3000 patients with severe heart failure will be determined by the international ATLAS trial which is due to be completed in 1997.

It is evident from heart failure research that the hormonal response to drug treatment is a major determinant of its efficacy. ACE inhibitors have a range of hormonal effects in addition to inhibiting the conversion of angiotensin I to angiotensin II; ACE itself is a non-specific enzyme with several substrates, including bradykinin—a peptide with a range of cardiovascular and renal effects. ACE inhibitors may therefore increase the activity of bradykinin[5] and may also indirectly affect the turnover of other vasoactive peptides, such as the endothelins that are released from endothelial cells in response to angiotensin II.[6] The haemodynamic effects of ACE inhibitors may also influence the release of the cardiac natriuretic peptides, atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP).[7] Recent studies have shown that raised plasma concentrations of these peptides are important adverse prognostic indicators in patients with ischaemic heart disease,[8,9] suggesting that a reduction in their secretion rates from the heart with treatment may predict a long-term benefit.

Though it is likely that these diverse hormonal effects of ACE inhibitors, and their biological consequences, will have different dose-response relations, there are few data available on the dose-related effects of ACE inhibitors. In this study we compared directly the effects of two doses of an ACE inhibitor, lisinopril, in a crossover trial in patients with heart failure who were already on long-term ACE inhibitor treatment. The doses used (5 mg once a day and 20 mg once a day) were chosen to represent the bottom end and the top end of the usual therapeutic range in heart failure. Furthermore in this study we measured blood pressure and plasma and urine electrolyte and hormonal concentrations over a 24 hour period to explore possible differences between the doses at both peak and trough drug effect.

Patients and methods

We performed a double blind, randomised, crossover study to compare the effects of low dose and high dose lisinopril in patients with heart failure over a 24 hour period. The study protocol was approved by the local committee on medical research ethics. Twenty one patients with symptomatic heart failure secondary to ischaemic heart disease (NYHA class II-III) and left ventricular ejection fraction (LVEF) $\leq 45\%$ were recruited. All patients gave informed consent to participate in the study. Two patients were unable to complete the study protocol for personal reasons and were therefore excluded from the efficacy analyses. All patients had been treated with a diuretic and ACE inhibitor for at least one month before the study. LVEF was measured by radionuclide ventriculography within three months of the study.

During a two week run-in period all patients were treated with lisinopril 10 mg once a day in place of their usual ACE inhibitor medication and in combination with their usual diuretic treatment. After two weeks, patients were randomised to change their dose of lisinopril to either 5 mg once a day or 20 mg once a day in addition to their usual dose of diuretic (fig 1). This new dose lasted for two weeks; on the final day of the two week period on this treatment the patients were admitted to the research unit to be studied over 24 hours. After a clinical assessment the dose of lisinopril was given under supervision with all concomitant medication. Blood sampling and measurements of heart rate and blood pressure were made at regular intervals throughout the 24 hours (fig 1). All urine passed during the study period was collected for analysis of creatinine, electrolytes, and ANP concentration. Urine collections from three patients were incomplete and all urine results from these patients were excluded from the analysis.

After this study period, patients took 10 mg lisinopril once a day in addition to all usual medication for two weeks, after which they received another two weeks of treatment with either lisinopril 5 mg once a day or 20 mg once a day according to the randomisation plan. On the last day of this second treatment period the patients were again studied over 24 hours as described above. The order of administration of low dose and high dose lisinopril was governed by balanced randomisation. Patient compliance was assessed by a tablet count.

Blood pressure measurements and all blood samples were taken after 30 minutes supine bed rest. Throughout the rest of the study period the patients were free to walk around; mealtimes and bedtimes were standardised as shown in figure 1. Blood pressure was measured with a semi-automated sphygmomanometer (Dinamap) placed around the left arm. All of the measurements and laboratory analyses were performed by individuals who were blinded to the treatment.

NATRIURETIC PEPTIDE ASSAYS

Venous blood was taken into chilled tubes containing EDTA and aprotinin (Trasylol, Bayer, 4000 kallikrein inactivation units per tube). Plasma was separated immediately and stored at −70°C until the measurement of ANP-like immunoreactivity (ANP-li) and BNP-li. Each peptide was measured in all of the samples from a patient in a single assay run. Plasma was applied to C8 solid phase extraction columns which had been pretreated with 4 ml methanol, 4 ml distilled water, and 4 ml 1% trifluoroacetic acid. The columns were washed with 9 ml of 1% trifluoroacetic acid and samples were eluted with 4 ml of 95% methanol and 1% trifluoroacetic acid. Samples were dried and the radioimmunoassay was performed with commercial kits supplied by Peninsula: ANP 1–28 (human, canine) and BNP 32 (human). Recovery of added peptides was 88% for ANP and 86% for BNP. The coefficients of variability for each assay were: ANP inter-assay = 11·8%, intra-assay = 12·6%; BNP inter-assay = 14·8%, intra-assay = 9·9%. Urinary ANP was measured, without any prior extraction procedure, by radioimmunoassay.

ENDOTHELIN ASSAYS

Samples were taken and stored as described above for the natriuretic peptide assays. Plasma immunoreactive endothelin was measured by radioimmunoassay as described previously.[10] The recovery of added endothelin was 84%. Intra and inter assay coefficients of variability were 2·4% and 4·25 respectively. The sensitivity of this assay is 2 pg/ml endothelin. Cross reactivity of the assay with endothelin-1, endothelin-2, endothelin-3, and proendothelin is 100, 52, 96, and 7% respectively

ALDOSTERONE ASSAYS

Blood samples for measurement of plasma aldosterone activity were taken into lithium-heparin tubes; plasma was separated immediately and stored at −20°C until analysis. Radioimmunoassay was performed using a standard commercial kit (Sorin, Italy). The intra-assay coefficient of variability was 7·8% and the inter-assay coefficient of variability was 9·6%.

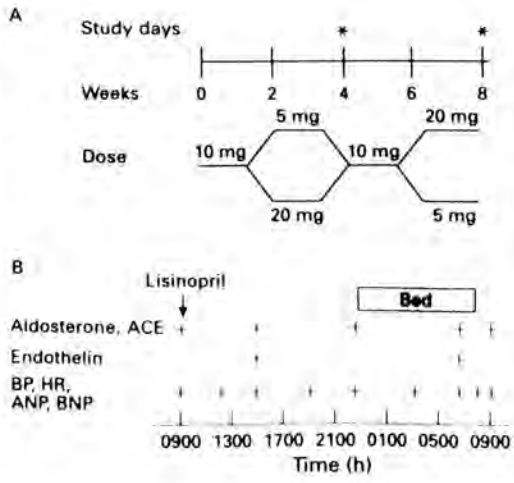

Figure 1 (A) Study plan showing daily dose of lisinopril and timing of study days. (B) Schedule of study days showing timing of supervised lisinopril medication; blood sampling for aldosterone angiotensin converting enzyme activity (ACE), endothelin, atrial natriuretic peptide (ANP), and B-type natriuretic peptide (BNP); and measurement of heart rate (HR) and blood pressure (BP).

Table 1 Patient characteristics

Variable	
Male	16 (84) %
Age (mean (SD))	60·3 (7·6) y
LVEF (mean (SD))	31·2 (16·0) %
NYHA class II	12 (63) %
NYHA class III	7 (37) %
Duration of ACE inhibitor treatment (mean (SD))	17·1 (14·8) months
Diuretic dose – equivalent dose of frusemide (mean (SD))	67·4 (45·6) mg

LVEF, left ventricular ejection fraction; NYHA, New York Heart Association.

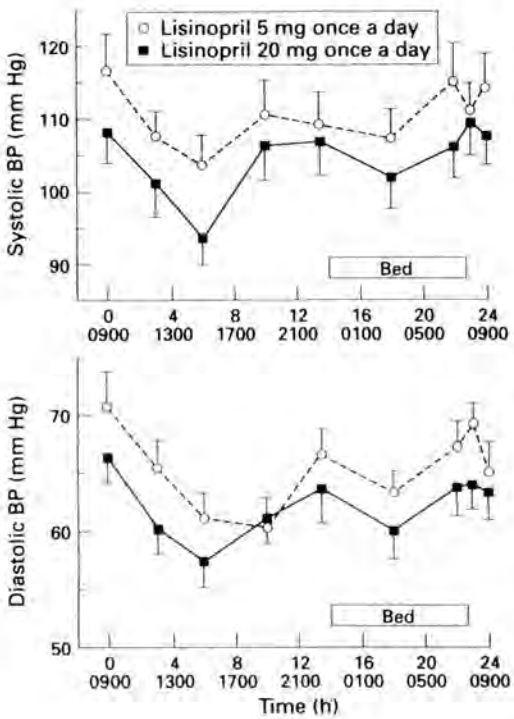

Figure 2 (A) Systolic and (B) diastolic blood pressure over 24 hours in patients taking 5 mg and 20 mg of lisinopril. Results are expressed as mean (SEM).

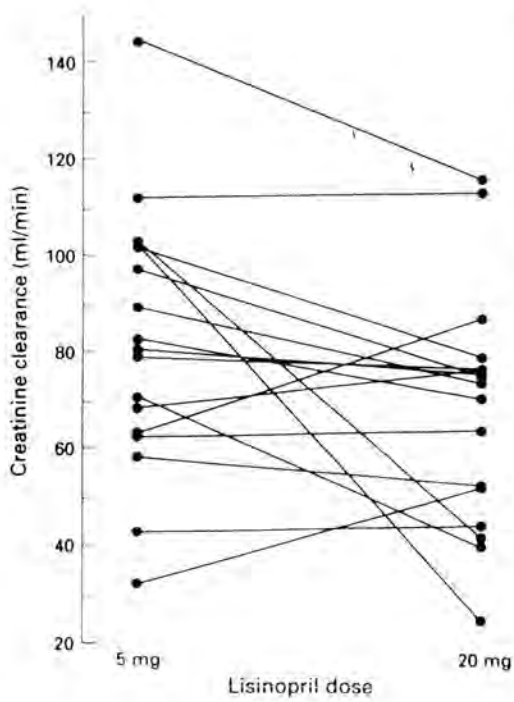

Figure 3 Creatinine clearance in patients taking 5 mg and 20 mg of lisinopril. Values for individual patients are shown.

PLASMA ACE ACTIVITY

Blood samples for measurement of plasma ACE activity were taken into lithium-heparin tubes; plasma was separated immediately and stored at −20°C until analysis. Plasma ACE activity was determined by the spectrophotometric kinetic rate method, using the synthetic substrate N-3-(2-furyl) acryloyl-L-phenylalanylglycylglycine.[11]

ACE GENOTYPE

Before the study, blood samples were taken for detection of the insertion/deletion polymorphism of the gene for angiotensin-converting enzyme. This was determined from leucocyte DNA using the polymerase chain reaction.[12]

STATISTICAL ANALYSIS

For plasma concentrations of ANP, BNP, and aldosterone the results were summarised as mean at peak effect (mean of 6 and 13·5 hour values for aldosterone; mean of 6 and 10 hour values for ANP and BNP), mean at trough effect (mean of 22 and 24 hour values for aldosterone; mean of 22, 23, and 24 hour values for ANP and BNP), and area under the curve for the values over 24 hours (calculated according to the trapezoidal method and divided by the time period). For blood pressure and heart rate, values were expressed as area under the curve. These summary measures were compared between treatments using the analysis of variance methods (ANOVA) of Grizzle,[13] which incorporate effects due to period and carry-over of the previous treatment. The analysis for these variables is presented using differences between adjusted means for the two treatments (adjusted for treatment period effects). All other variables, which had one or two values for each dose were compared using two-tailed paired *t*-tests. The assessment of carry-over effects is a between-patient evaluation and the test for carry-over was therefore performed at the 10% level of significance. All other tests were performed at the 5% level of significance.

Results

Baseline characteristics of the patients are shown in table 1. The compliance rate was > 85% for each patient in all parts of the study. There were no significant differences in symptomatic status, clinical findings, or patient weight between the two doses of lisinopril. There was no evidence of a significant period or carry-over effect for any of the variables measured.

BLOOD PRESSURE AND HEART RATE

Blood pressure values on each dose of lisinopril are shown in figure 2. The mean area under the curve for systolic blood pressure was lower during the high-dose phase than during the low-dose phase, although this effect was of borderline statistical significance (P = 0·05). The difference between the adjusted treatment means (5 mg dose minus 20 mg dose) was 5·7 (95% CI 0·0 to 11·5) mm Hg. The mean area under the curve for diastolic blood pressure

Table 2 Plasma electrolytes 24 hour urinary electrolytes and atrial natriuretic peptide (ANP) excretion and creatinine clearance in patients taking 5 mg and 20 mg of lisinopril (mean (SD))

Variable	Lisinopril dose	
	5 mg	20 mg
Plasma (Na) (mmol/l)	137·3 (2·45)	138·1 (2·43)
Plasma (K) (mmol/l)	4·11 (0·47)	4·18 (0·38)
Plasma (creatinine) (mmol/l)	111·7 (22·7)	113·4 (25·7)
24 hour urine Na excretion (mmol)	207·6 (82·8)	212·0 (83·6)
24 hour urine K excretion (mmol)	88·4 (27·8)	80·2 (22·1)
Creatinine clearance (ml/min)	82·2 (27·1)	68·7 (24·4)*
24 hour urinary ANP excretion (pmol)	13·6 (4·3)	12·2 (4·5)†

*P < 0·05 for 5 mg *v* 20 mg; †P = 0·02 for 5 mg *v* 20 mg.

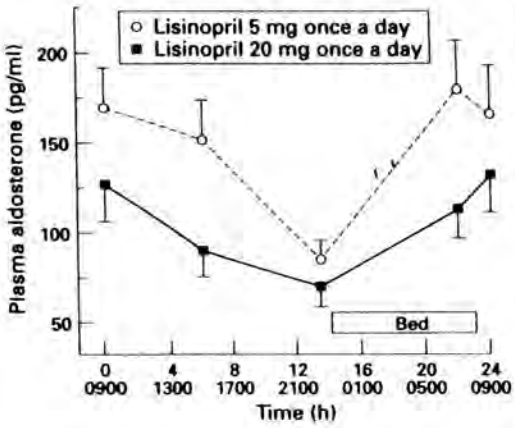

Figure 4 Plasma aldosterone concentrations over 24 hours in patients taking 5 mg and 20 mg lisinopril. Results are expressed as mean (SEM).

Table 3 Plasma ACE activity in groups divided according to ACE genotype (mean (SD))

ACE genotype	Lisinopril dose	
	5 mg	20 mg
DD (n = 7)	14·38 (2·79)	10·27 (3·76)
ID (n = 7)	11·49 (8·87)	8·87 (2·62)
II (n = 5)	9·80 (4·67)	7·95 (2·62)
Total (n = 19)	12·11 (3·83)	9·14 (3·07)*

*Area under curve over 24 hours expressed as standard international units. P < 0·01 for 5 mg *v* 20 mg.
ACE, angiotensin converting enzyme.

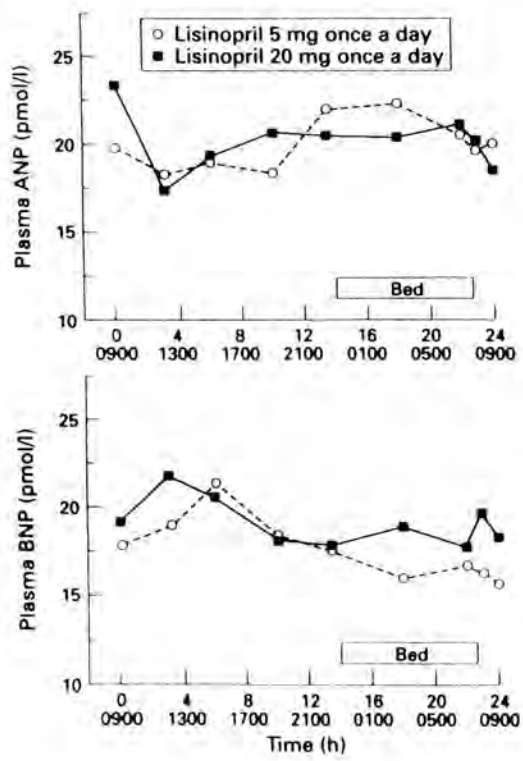

Figure 5 Mean plasma concentrations over 24 hours of (A) atrial natriuretic peptide and (B) B-type natriuretic peptide in patients taking 5 mg and 20 mg of lisinopril.

was significantly lower during the high-dose phase than during the low-dose phase (P = 0·04). There was no significant difference in heart rate between doses.

PLASMA /URINARY ELECTROLYTES AND CREATININE CLEARANCE

Creatinine clearance was significantly lower on the 20 mg dose than on the 5 mg dose (fig 3). There were no significant differences between plasma and urinary electrolyte concentrations between doses (table 2).

ALDOSTERONE CONCENTRATIONS

Plasma aldosterone concentrations were significantly lower on the 20 mg dose than on the 5 mg dose (fig 4). Mean at peak effect 90·7 *v* 152·0 pg/ml, P < 0·001; mean at trough effect 124·7 *v* 174·4 pg/ml, P < 0·01. For the area under the curve, the difference between the treatment means (5 mg dose minus 20 mg dose) was 44·0 (95% CI 24·0 to 63·9) pg/ml (P < 0·001).

NATRIURETIC PEPTIDE CONCENTRATIONS

Plasma concentrations of natriuretic peptides are shown in figure 5. There were no significant differences in the area under the curve or the concentrations at peak or trough drug effect for either ANP or BNP on the two doses. Urinary excretion of ANP over 24 hours was significantly lower on 20 mg lisinopril than on the 5 mg dose.

ENDOTHELIN CONCENTRATIONS

Plasma concentrations of endothelin at six hours (peak drug effect) were 4·74 *v* 3·27 pmol/l on 5 mg lisinopril and 5·01 *v* 2·98 on 20 mg lisinopril (mean (SD), P = NS). At 22 hours (trough drug effect) concentrations were 6·04 *v* 3·58 pmol/l on 5 mg lisinopril and 4·70 *v* 2·72 pmol/l on 20 mg lisinopril (P = 0·03).

PLASMA ACE ACTIVITY

Plasma ACE activity was significantly lower on high-dose than on low-dose lisinopril (table 3). There was a non-significant trend towards lower ACE activity on both doses of lisinopril in patients with the II genotype than in patients with either the ID or DD genotype.

Discussion

The results of this study demonstrate significant dose-related effects of lisinopril within the usual therapeutic range in patients with chronic heart failure. It is important to note that because all of the patients were established on ACE inhibitor treatment for at least one month before the study, they had all received a minimum of six weeks treatment before the first study day. In fact, as figure 1 shows, most patients had been treated with ACE inhibitors for considerably longer periods; the results therefore represent the response to chronic ACE inhibition. As expected, the higher dose of lisinopril caused a greater degree of inhibition of ACE than the lower dose. There was no evidence of a large difference in the dose-response relation for any

of the variables measured between the three groups of insertion/deletion polymorphisms of the ACE gene, but this study was not designed to detect such a difference and the numbers of patients with each of the three genotypes were small.

The superior suppression of aldosterone produced by the higher dose of lisinopril is striking. The early morning rise in plasma aldosterone which was seen on both doses of lisinopril occurred before the patients got out of bed. It is uncertain whether this was due primarily to the diurnal pattern of adrenocorticotrophic hormone (ACTH) release or due to reduced drug effect at this time; the large difference in early morning aldosterone concentrations between the two doses suggests an important drug effect, but even on the higher dose the suppression of aldosterone was not sustained over the 24 hour period. The importance of aldosterone in heart failure is that it has been shown experimentally to cause a wide range of potentially detrimental effects. These include increased potassium and magnesium excretion (which may promote arrhythmias),[14] impairment of baroreceptor function,[15] and stimulation of myocardial fibrosis.[16] We found that during long-term treatment with an ACE inhibitor suppression of aldosterone can be improved by increasing the dose of the ACE inhibitor but this suppression was incomplete, especially in the early morning. Further suppression of plasma aldosterone may be achieved by using even higher doses of an ACE inhibitor, nocturnal dosing with an ACE inhibitor, the addition of a receptor antagonist, or a combination of these approaches.

The role of the endothelins in the pathophysiology of cardiovascular disease is not clearly established. It is known that the plasma concentrations of these peptides are increased in heart failure[17] and the concomitant increase in plasma concentrations of big endothelin-1 is consistent with increased production of endothelins in heart failure.[18] It has recently been reported that blockade of endothelin receptors causes vasodilatation in patients with heart failure,[19] which suggests that endothelin contributes to the increase in vascular tone associated with this condition. Previously there have been conflicting reports of the effects of short-term ACE inhibition on plasma endothelin in experimental and clinical heart failure[20,21]; the data from the current study provide the first evidence that endothelin concentrations can be suppressed more by high dose than by low strengthened by our observation that the higher dose of lisinopril reduced endothelin concentrations despite a fall in creatinine clearance, suggesting that reduced secretion of endothelins, rather than increased renal clearance, was reponsible for the effect. ACE inhibitors may reduce the secretion of endothelin by reducing the concentration of angiotensin II, a potent stimulant for endothelin release,[22] or by increasing bradykinin activity, resulting in increased production of nitric oxide, an inhibitor of endothelin release.[23] On the basis of current knowledge it is uncertain whether the observed difference in endothelin concentrations is of clinical relevance, but it suggests an intriguing alternative mechanism for some of the beneficial effects of ACE inhibitors.

The observed differences in blood pressure between the two doses of lisinopril are small overall, but six hours after dosing (that is, peak drug effect) the mean systolic blood pressure was about 10 mm Hg lower and the mean diastolic blood pressure was about 1 mm Hg lower on high dose than on low dose lisinopril (fig 2). Though most patients had very little change in creatinine clearance between doses, in some patients it dropped considerably on the higher dose. This was not associated with a rise in serum creatinine during the two weeks of the study, but it is possible that such alterations in renal function might be detrimental in the long term.

The similar plasma concentrations of atrial and B-type natriuretic peptide on each dose are surprising in view of the described differences in blood pressure. It seems likely that the reduction in afterload produced by the higher dose would result in a reduced secretion of these peptides from the myocardium, a process that is thought to depend mainly on cardiac stretch.[24] Previous studies have reported a reduction in natriuretic peptide concentrations during acute ACE inhibition in heart failure[7] but other investigators showed that the relation between plasma ANP concentrations and pulmonary capillary wedge pressure is weakened during ACE inhibition,[25] a phenomenon which has not been fully explained. It is likely that in the present study any favourable cardiac effect of high dose lisinopril is offset by reduced renal clearance of ANP and BNP, due to reduced renal blood flow. This explanation is supported by the lower urinary concentrations of ANP on high dose than on low dose lisinopril.

In conclusion, suppression of plasma aldosterone and endothelin concentrations was greater on the 20 mg dose of lisinopril than on the 5 mg dose, but the observed falls in creatinine clearance associated with the higher dose may outweigh these beneficial effects in clinical practice. The issue of the optimal dose of ACE inhibitor should be settled by the results of the ATLAS (Assessment of Treatment with Lisinopril and Survival) trial which will ultimately provide data on clinical endpoints. In the meantime it is essential that renal function is monitored as the dose of ACE inhibitor is titrated upwards in patients with heart failure.

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Vasodilator Effects of Endothelin-Converting Enzyme Inhibition and Endothelin ET_A Receptor Blockade in Chronic Heart Failure Patients Treated With ACE Inhibitors

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Background The importance of endothelin-1 in chronic heart failure (CHF) is unclear. We therefore investigated the effects of endothelin-converting enzyme (ECE) inhibition and endothelin ET_A receptor blockade in CHF patients treated with ACE inhibitors. We also compared the function of ET_A and ET_B receptors in healthy subjects and patients with CHF.

Methods and Results Locally active doses of study drugs were infused into the nondominant brachial artery while forearm blood flow (FBF) was measured by venous occlusion plethysmography. In CHF patients (n=10), phosphoramidon (a combined ECE and neutral endopeptidase inhibitor) and BQ-123 (an ET_A receptor antagonist) increased FBF by 52±10% (P=.0006) and 31±6% (P=.002), respectively, and thiorphan (a selective neutral endopeptidase inhibitor) reduced FBF by 15±5% (P=.0007). Forearm vasoconstriction to endothelin-1 (an ET_A and ET_B receptor agonist) was significantly blunted in CHF patients compared with control subjects (both n=10; CHF versus control subjects, P<.001), whereas vasoconstriction to sarafotoxin S6c (an ET_B receptor agonist) was significantly enhanced in CHF patients compared with control subjects (both n=10; CHF versus control subjects, P<.05).

Conclusions ECE inhibitors and ET_A receptor antagonists may be useful as vasodilator agents in CHF patients already receiving treatment with an ACE inhibitor. Both ET_A and ET_B receptors can mediate agonist-induced vasoconstriction in healthy subjects and patients with CHF, but further studies are required to clarify the contribution of each receptor subtype in mediating the effects of endogenous endothelin-1. (***Circulation.*** **1996;94: 2131-2137.)**

Key Words • endothelin • heart failure • vasodilatation • vasoconstriction • receptors

Chronic heart failure is an important and increasingly common healthcare problem. Even with full conventional medical therapy, CHF impairs quality of life more than most other chronic medical illnesses and carries an extremely poor prognosis.[1] The development of novel and more effective therapeutic strategies for CHF is therefore an important priority in cardiovascular medicine.

In terms of symptoms and survival, the greatest impact on the medical management of CHF has been made by the ACE inhibitors[2]; angiotensin II receptor antagonists have recently shown similar therapeutic promise.[3] This has led to the view that vasodilatation through neuroendocrine modulation may be the most appropriate and effective therapeutic strategy in CHF. Novel therapies capable of achieving a greater reduction in vascular resistance than is possible with current treatment modalities are urgently required.

The endothelins are a family of endogenous peptides with potent vasoconstrictor, antinatriuretic, and mitogenic properties that may be important in the pathophysiology of CHF.[4] Endothelin-1, the principal endothelin isoform generated in the human vasculature,[5] has uniquely sustained vasoconstrictor actions and has been shown to contribute to basal vascular tone in humans.[6] Plasma levels of the peptide are increased twofold to threefold in CHF of all causes in proportion to the symptomatic and hemodynamic severity of the syndrome.[7-15] Big endothelin-1, the biologically inactive precursor of mature endothelin-1, may be preferentially increased in CHF,[13,15] suggesting enhanced endothelial synthesis or secretion of the peptide. Plasma big endothelin-1 also predicts prognosis, with a higher concentration predicting a greater likelihood of death or need for cardiac transplantation.[13]

We have addressed the contribution of endothelin-1 to peripheral vasoconstriction in CHF by pursuing two possible therapeutic interventions suggested by the cellular processing and actions of endothelin-1.[4] In study A, we investigated the effects of blocking the generation (via ECE inhibition) and action (via selective endothelin ET_A receptor blockade) of endothelin-1 in a heterogeneous group of patients with conventionally treated CHF. We have also tried to clarify the role of the two principal

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Selected Abbreviations and Acronyms

CHF = chronic heart failure
ECE = endothelin-converting enzyme
FBF = forearm blood flow
NEP = neutral endopeptidase

endothelin receptor subtypes, ET_A and ET_B, in mediating vasoconstriction in vivo.[16,17] Vascular smooth muscle ET_A receptors were previously believed to be solely responsible for mediating endothelin-1–induced vasoconstriction, whereas endothelial ET_B receptors were believed to mediate vasodilatation through release of prostacyclin and/or nitric oxide. More recently, it has been suggested that vascular smooth muscle ET_B receptors might also mediate vasoconstriction.[18-21] Knowledge of the contribution of each receptor subtype to vasoconstriction has obvious implications for the potential therapeutic use of selective or nonselective endothelin receptor antagonists in CHF. In study B, we attempted to clarify the role of constrictor ET_A receptors by comparing the vascular effects of the selective ET_A receptor antagonist BQ-123 in healthy subjects and patients with CHF. In study C, we tried to assess the relative importance of ET_A and ET_B receptors by comparing the vascular effects of endothelin-1 (a nonselective [ET_A and ET_B] receptor agonist) and sarafotoxin S6c (a selective ET_B receptor agonist) in healthy subjects and patients with CHF.

Methods

Subjects

Studies were conducted with the approval of the local ethics committee and the written, informed consent of each CHF patient and healthy control subject.

Fourteen patients with chronic (>3 months) stable heart failure participated in study A. No specific cause of CHF was excluded, but no patients had concomitant hypertension, diabetes mellitus, or chronic renal failure. Each was receiving maintenance treatment with a diuretic and a maximal dose of an ACE inhibitor (either captopril 50 mg TID, enalapril 10 mg BID, or lisinopril 10 mg UID). Medication was continued as usual before and during the day of studies because we wished to evaluate the effects of ECE inhibition and ET_A receptor blockade over and above the effects of conventional medical therapy for CHF. Patient characteristics are summarized in Table 1.

Six patients with chronic stable heart failure and 10 healthy control subjects participated in study B. Ischemic heart disease was the underlying cause of heart failure in each case, and no patients had concomitant hypertension, diabetes mellitus, or chronic renal failure. All CHF patients were receiving maintenance treatment with a diuretic, digoxin, and ACE inhibitor, and no control subject was taking any regular medication. The medication of CHF patients was withheld for 24 hours before studies. Patient characteristics are summarized in Table 2.

Ten patients with chronic stable heart failure and 10 healthy control subjects participated in study C. The underlying cause of heart failure was ischemic heart disease in 9 patients and alcoholic cardiomyopathy in 1; none had concomitant hypertension, diabetes mellitus, or chronic renal failure. All CHF patients were receiving maintenance treatment with a diuretic and ACE inhibitor, and no control subject was taking any regular medication. The medication of CHF patients was withheld for 24 hours before studies. Patient characteristics are summarized in Table 3.

Drug Infusion and FBF Measurement

Studies were performed with subjects resting supine in a quiet clinical laboratory maintained at a constant temperature between 23°C and 25°C. Subjects were asked to abstain from caffeine-containing drinks, alcohol, and cigarette smoking on study days and to fast for at least 2 hours before studies commenced.

With 1% lidocaine used for local anesthesia, an unmounted 27–standard wire gauge steel cannula (Coopers Needle Works) was inserted into the brachial artery of the nondominant arm and

TABLE 1. Patient Characteristics, Study A (Phosphoramidon, Thiorphan, and BQ-123 Protocols)

	Protocol		
	Phosphoramidon	Thiorphan	BQ-123
Sex, n	9 M/1 F	10 M	9 M/1 F
Age, y (mean±SD)	66±4	68±3	64±4
Primary diagnosis, n			
Ischemic heart disease*	6	8	7
Dilated cardiomyopathy†	1	1	1
Alcoholic cardiomyopathy†	2	1	2
Valvular heart disease‡	1	...	...
NYHA functional class, n			
II	6	4	6
III	4	6	3
IV	...	...	1
Drug therapy, n			
ACE inhibitor	10	10	10
Diuretic	10	10	10
Digoxin	6	7	6
Amlodipine	3	4	3
Oral nitrate	3	4	1
β-Blocker	0	1	3

*Documented previous myocardial infarction or confirmed by coronary angiography.
†Normal coronary arteries at coronary angiography.
‡Severe mitral regurgitation.

TABLE 2. Patient and Control Subject Characteristics, Study B (BQ-123 Protocol)

	Patients	Control Subjects
Sex, n	6 M	10 M
Age, y (mean±SD)	67±4	60±6
Primary diagnosis, ischemic heart disease,* n	6	...
NYHA functional class, n		
II	2	...
III	4	...
Mean ejection fraction,† %	23±2	65±3‡
Baseline FBF,§ ‖ mL·min^{-1}·100 mL^{-1}	3.3±0.3	4.0±0.3
Baseline mean arterial pressure, ‖ mm Hg	86±3	93±2
Baseline heart rate, bpm	66±4	66±2
Drug therapy, n		
ACE inhibitor	6	...
Diuretic	6	...
Digoxin	6	...
Oral nitrate	2	...
Amlodipine	0	...

*Documented previous myocardial infarction or confirmed by coronary angiography.
†Calculated from short- and long-axis M-mode echocardiogram at mitral leaflet tips.
‡Two control subjects were insufficiently echogenic to allow quantification of their ejection fractions but had subjectively normal left ventricular systolic function.
§Expressed as mean of infused and noninfused arms.
‖The small baseline differences in FBF and mean arterial pressure between patients and control subjects were not statistically significant.

TABLE 3. Patient and Control Subject Characteristics, Study C (Endothelin-1 and Sarafotoxin S6c Protocols)

	Patients	Control Subjects
Sex, n	10 M	10 M
Age, y (mean±SD)	61±3	59±5
Primary diagnosis, n		
Ischemic heart disease*	9	
Alcoholic cardiomyopathy†	1	...
NYHA functional class, n		
II	4	
III	5	...
IV	1	...
Mean ejection fraction,‡ %	29±2	63±3
Baseline forearm blood flow,§‖ mL·min^{-1}·100 mL^{-1}		
ET-1 protocol	3.1±0.3	4.0±0.4
S6c protocol	3.5±0.3	4.1±0.6
Baseline mean arterial pressure,‖ mm Hg		
ET-1 protocol	98±2	91±2
S6c protocol	96±2	91±2
Baseline heart rate,‖ bpm		
ET-1 protocol	71±3	67±2
S6c protocol	70±3	65±2
Drug therapy, n		
ACE inhibitor	10	...
Diuretic	10	...
Digoxin	6	...
Amlodipine	3	...
Oral nitrate	3	...

ET-1 indicates endothelin-1; S6c, sarafotoxin S6c.

*Documented previous myocardial infarction or confirmed by coronary angiography.

†Normal coronary arteries at coronary angiography.

‡Calculated from short- and long-axis M-mode echocardiogram at mitral leaflet tips.

§Expressed as mean of infused and noninfused arms.

‖ None of the small baseline differences in FBF, mean arterial pressure, and heart rate between patients and control subjects were statistically significant.

connected to a constant-rate infusion pump (Wellmed P1000) via a 16-gauge epidural catheter (Portex Ltd). Physiological saline (0.9%) was infused at 1 mL/min for an equilibration period of ≥30 minutes before infusion of study drugs. Locally active doses of drugs were dissolved in physiological saline and administered at 1 mL/min.

FBF was measured by venous occlusion plethysmography with indium (gallium)-in-Silastic strain gauges applied to the widest aspect of each forearm.[22] The hand circulation was excluded during periods of blood flow measurement by inflation of wrist cuffs to 220 mm Hg. Upper arm cuffs were inflated to 40 mm Hg for 10 in every 15 seconds to prevent venous outflow and obtain plethysmographic recordings. Voltage output from a Vasculab SPG 16 strain-gauge plethysmograph (Medasonics Inc) was transferred to an Apple Macintosh computer (LC III, Apple Computer Inc) for analysis with a MacLab analog-to-digital converter and Chart software (version 3.2.8; both from AD Instruments).

In all studies, FBF was measured in both arms at 10-minute intervals during saline equilibration and at 5-minute intervals during study drug infusion. In study A only, saline was infused for a further 30 minutes after discontinuation of drug infusion with blood flow measured every 10 minutes. The last five measurements of FBF during each ≈3-minute recording period were averaged, and the mean percentage change from baseline in the ratio of flow between the infused and noninfused arms was calculated. This method of analysis uses the noninfused arm as a contemporaneous control and ensures that the effects of study drugs are distinguished from any other external or environmental factors.[22]

Blood pressure and heart rate were recorded in the noninfused arm at 10-minute intervals throughout all studies with a validated semiautomated sphygmomanometer (Takeda UA-751, Takeda Medical Inc).

Data Analysis

All results are expressed as mean values with 95% CIs in the text and SEM in figures. The significance of the effect of each study agent on FBF was examined with Statview 4.02 software (Abacus Concepts Inc) for the Apple Macintosh computer to perform repeated-measures ANOVA. An unpaired t test was performed to test the significance of the difference in peak antagonist and agonist effects between healthy subjects and patients with CHF in studies B and C. Differences were considered statistically significant at a value of $P<.05$.

Detailed Protocols

Study A: ECE Inhibition, NEP Inhibition, and Selective ET_A Receptor Blockade

Three single-blind protocols were performed, each in 10 patients with CHF. Phosphoramidon (Clinalfa AG) was infused at 30 nmol/min for 60 minutes, a dose sufficient to achieve local forearm concentrations equivalent to the IC_{50} of phosphoramidon for ECE assuming resting FBF of ≈50 mL/min.[23] Phosphoramidon, however, also inhibits NEP, the enzyme responsible for the degradation of various biologically active peptides, including bradykinin, substance P, natriuretic peptides, angiotensin II, and endothelin-1 itself.[24,25] We therefore compared its effect with that of thiorphan (Sigma Chemical Co Ltd), a selective inhibitor of NEP.[26] Thiorphan was also infused at 30 nmol/min for 60 minutes because it is effectively equipotent with phosphoramidon as an inhibitor of NEP.[24] Concentrations of thiorphan >100-fold greater than achieved at this rate of infusion have no inhibitory effect on ECE.[23] The selective ET_A receptor antagonist BQ-123 (American Peptide Co) was infused at 100 nmol/min for 60 minutes, a dose sufficient to achieve local forearm concentrations 10-fold higher than the pA_2 for BQ-123 at the ET_A receptor.[27]

Study B: Selective ET_A Receptor Blockade

Ten healthy control subjects received brachial artery infusion of the selective ET_A receptor antagonist BQ-123 at 100 nmol/min for 90 minutes, again a dose sufficient to achieve local forearm concentrations 10-fold greater than the pA_2 for BQ-123 at the ET_A receptor.[27] We also infused BQ-123 for 90 minutes in 6 more patients with CHF because it was not clear whether a maximal effect had been obtained after 60 minutes of infusion in study A.

Study C: Nonselective (ET_A and ET_B) Receptor Agonism and Selective ET_B Receptor Agonism

On separate days at least 1 week apart and in single-blind fashion, 10 healthy control subjects and 10 patients with CHF received brachial artery infusion in random, balanced order of pharmaceutical grade endothelin-1 (Clinalfa AG) and sarafotoxin S6c (Sigma Chemical Co Ltd) at 5 pmol/min for 60 minutes. This dose of both agonists has previously been shown to cause slow-onset vasoconstriction in human forearm resistance vessels.[21] Assuming resting FBF of ≈50 mL/min, infusion of 5 pmol/min of endothelin-1 and sarafotoxin S6c would achieve a local concentration of each peptide in the infused forearm of about 0.1 nmol/L. Endothelin-1 would be expected to act equally on both ET_A and ET_B receptors at this concentration, given that the K_i values for endothelin-1 at ET_A and ET_B receptors are 0.6 and 0.12 nmol/L, respectively.[28] Sarafotoxin S6c would be expected to be highly selective for the ET_B receptor (K_i, 0.25 nmol/L) because its local forearm concentration would be at least 70 000-fold lower than its K_i at the ET_A receptor (>7300 nmol/L).[28]

Results

Heart rate, blood pressure, and FBF in the noninfused arm did not change significantly in any study protocol, confirming that drugs had only local actions on the forearm

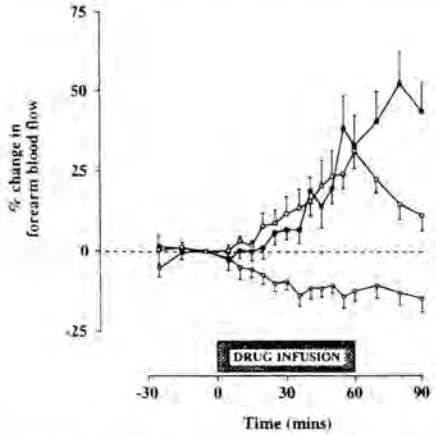

FIG 1. Effect of brachial artery infusion of phosphoramidon 30 nmol/min (■), thiorphan 30 nmol/min (○), and BQ-123 100 nmol/min (□), each for 60 minutes in 10 patients with CHF. Hatched bar represents period of study drug infusion. Phosphoramidon (P=.0006) and BQ-123 (P=.002) caused progressive forearm vasodilatation, whereas thiorphan caused progressive forearm vasoconstriction (P=.0007).

vasculature of the infused arm and had no systemic hemodynamic effects.

Study A: ECE Inhibition, NEP Inhibition, and Selective ET_A Receptor Blockade

Phosphoramidon caused slow-onset vasodilatation, with a peak increase in FBF of 52±10% at 80 minutes (CI, 32% to 72%; ANOVA versus baseline, P=.0006; Fig 1), ie, 20 minutes after discontinuation of phosphoramidon infusion. Thiorphan caused slow-onset vasoconstriction, with a peak reduction in FBF of 15±5% at 90 minutes (CI, −6% to −24%; ANOVA versus baseline, P=.0007; Fig 1), ie, 30 minutes after discontinuation of thiorphan infusion. BQ-123 caused slow-onset vasodilatation, with a peak increase in FBF of 31±6% at 60 minutes (CI, 20% to 42%; ANOVA versus baseline, P=.002; Fig 1), ie, at the end of BQ-123 infusion.

Study B: Selective ET_A Receptor Blockade

BQ-123 caused slow-onset vasodilatation in control subjects, with a peak increase in FBF of 54±10% at 90 minutes (CI, 34% to 74%; ANOVA versus baseline, P<.0001; Fig 2). As in study A, BQ-123 caused slow-onset vasodilatation in CHF patients, with a peak increase in FBF of 33±4% at 90 minutes (CI, 26% to 40%; ANOVA versus baseline, P<.0001; Fig 2). Vasodilatation to BQ-123 tended to be blunted in CHF patients compared with control subjects (CHF versus control subjects, P=.14); the maximal increase in FBF at 90 minutes was not statistically significantly different between CHF patients and control subjects unless the solitary control subject whose FBF was unaffected by BQ-123 infusion was excluded from the analysis (CHF versus control subjects, P=.04).

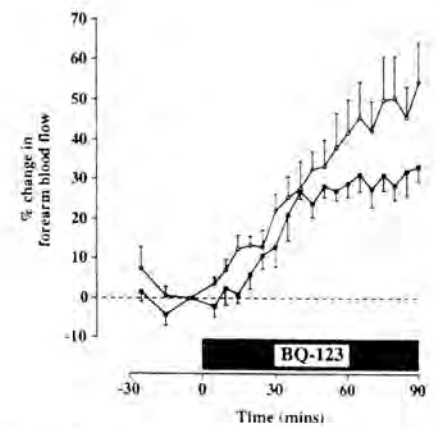

FIG 2. Effect of brachial artery infusion of BQ-123 100 nmol/min for 90 minutes in 6 patients with CHF (■) and 10 age-matched healthy control subjects (○). Hatched bar represents period of BQ-123 infusion. Vasodilatation to BQ-123 tended to be blunted in CHF patients compared with control subjects (P=.14).

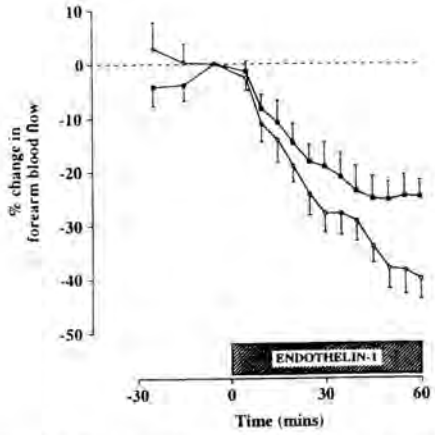

FIG 3. Effect of brachial artery infusion of endothelin-1 5 pmol/min for 60 minutes in 10 patients with CHF (■) and 10 age-matched healthy control subjects (○). Hatched bar represents period of peptide infusion. Vasoconstriction to endothelin-1 was significantly blunted in CHF patients compared with control subjects (P<.001).

Study C: Nonselective (ET_A and ET_B) Receptor Agonism and Selective ET_B Receptor Agonism

Endothelin-1 caused slow-onset vasoconstriction in control subjects and patients with CHF (Fig 3). Endothelin-1 reduced FBF by 41±4% in control subjects (CI, −34% to −49%; ANOVA versus baseline, P<.0001) and by 25±3% in patients with CHF (CI, −19% to −31%;

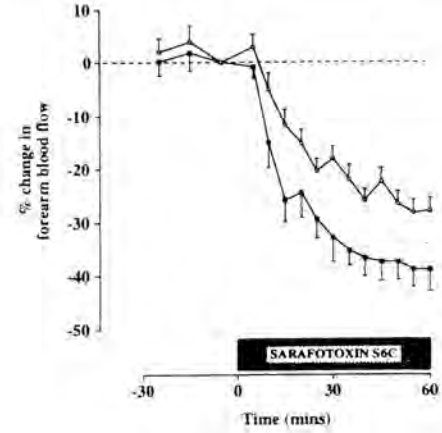

FIG 4. Effect of brachial artery infusion of sarafotoxin S6c 5 pmol/min for 60 minutes in 10 patients with CHF (■) and 10 age-matched healthy control subjects (○). Hatched bar represents period of peptide infusion. Vasoconstriction to sarafotoxin S6c was significantly enhanced in CHF patients compared with control subjects (P<.05).

ANOVA versus baseline, $P<.0001$) after 60 minutes of infusion. Vasoconstriction to endothelin-1 was significantly blunted in CHF patients compared with control subjects (CHF versus control subjects, $P<.001$). Sarafotoxin S6c also caused slow-onset vasoconstriction in control subjects and patients with CHF (Fig 4). Sarafotoxin S6c reduced FBF by 29±3% in control subjects (CI, −24% to −34%; ANOVA versus baseline, $P<.0001$) and by 39±4% in patients with CHF (CI, −31% to −47%; ANOVA versus baseline, $P<.0001$) after 60 minutes of infusion. Vasoconstriction to sarafotoxin S6c was significantly greater in CHF patients than control subjects (CHF versus control subjects, $P<.05$).

Discussion

These studies demonstrate that endothelin-1 contributes importantly to peripheral vascular resistance in patients with CHF and suggest that ECE inhibitors and endothelin ET_A receptor antagonists may be useful as vasodilator agents, even in patients who are already receiving treatment with an ACE inhibitor. We have also shown that both ET_A and ET_B receptors can mediate vasoconstriction in the peripheral vasculature of healthy subjects and patients with CHF, a finding that may have important implications for the potential therapeutic use of endothelin receptor antagonists.

Studying the effects of agents that block either the generation or action of endothelin-1 in vivo is the only way to clarify the putative pathophysiological role of the peptide in CHF. A major advance in the field of endothelin research has been the cloning and characterization of ECE,[23] but at present no specific and selective inhibitors of the enzyme exist. Several selective and nonselective endothelin receptor antagonists potentially suitable for human therapeutic use are at various stages of clinical development.[29]

The best available pharmacological tool at present for studying the effects of blocking the generation of endothelin-1 is the combined ECE and NEP inhibitor phosphoramidon.[23] Phosphoramidon caused a substantial (≈50%) increase in FBF when a locally active dose was infused into the brachial artery of patients with CHF (Fig 1). Given the broader substrate specificity of NEP than of ECE,[24,25] this vasodilatation could theoretically have been due to NEP rather than ECE inhibition, causing local accumulation of one of the many dilator substances metabolized by NEP. However, brachial artery infusion of the selective NEP inhibitor thiorphan caused forearm vasoconstriction in patients with CHF (Fig 1), indicating that the vasodilatation observed with phosphoramidon was most likely due to inhibition of ECE, with simultaneous NEP inhibition probably offsetting the magnitude of its overall vasodilator effect. The specific mechanism underlying the effect of thiorphan is likely to be complex, given the many vasoactive factors known to be metabolized by NEP, but the most probable explanation is that of local accumulation of constrictor substances, such as angiotensin II or endothelin-1 itself. Further studies will be necessary to elucidate the mediators underlying the vasoconstrictor response to NEP inhibition.

Further evidence that endothelin-1 contributes to peripheral vascular resistance in CHF is provided by our studies with the selective ET_A receptor antagonist BQ-123. Brachial artery infusion of a locally active dose of BQ-123 for 60 minutes increased FBF by ≈30% in patients with CHF in study A. Whereas maximal vasodilatation to phosphoramidon was observed 20 minutes after its infusion was discontinued (suggesting persisting inhibition of ECE), the vasodilatory effect of BQ-123 declined immediately after discontinuation of its infusion, in keeping with its action as a competitive antagonist at the ET_A receptor. The maximum response to BQ-123 was probably achieved after 60 minutes of infusion in study A (Fig 1) because the effect of BQ-123 appeared to reach plateau by 60 minutes in study B without any significant incremental vasodilatation after 60 minutes (Fig 2). ECE inhibition appeared to cause greater vasodilatation than selective ET_A blockade in study A, but further studies with varying doses and durations of infusion of both phosphoramidon and BQ-123 would be required to validate any direct comparison of their effects. Our principal aim was to determine whether antiendothelin drugs have therapeutic potential as vasodilator agents in patients with CHF already receiving conventional medical therapy rather than to compare the relative effects of ECE inhibition and ET_A receptor blockade.

Though we investigated only the short-term effects of ECE inhibition and ET_A receptor blockade in a single vascular bed, responses in human forearm resistance vessels are thought to be broadly representative of responses in other resistance beds. Indeed, our observations support and extend the recent findings of Kiowski et al,[30] who reported that acute bolus administration of a mixed ET_A and ET_B receptor antagonist, bosentan, significantly reduced systemic and pulmonary vascular resistance in patients with CHF. A crucial difference from our own studies was that these hemodynamic effects were observed in patients whose ACE inhibitors had been discontinued for >4 plasma half-lives. Importantly, our observations suggest that antiendothelin drugs have the potential to offer hemodynamic benefit in CHF patients over and above that already obtained with an ACE inhibitor.

Collectively, our agonist and antagonist studies suggest that both ET_A and ET_B receptors can mediate vasoconstriction in forearm resistance vessels of healthy subjects and patients with CHF. Our agonist studies in healthy control subjects are consistent with similar recently reported studies in healthy volunteers.[21] The lesser mean constriction to sarafotoxin S6c than to endothelin-1 in control subjects (Figs 3 and 4) implies that both ET_A and ET_B receptor subtypes mediate vasoconstriction, but it is difficult to extrapolate our results to quantify the relative contribution of each receptor subtype in mediating the effects of endogenous endothelin-1; further comparative studies with selective ET_A and ET_B receptor antagonists, when available for use in humans, will clarify this issue.

We found that the forearm vasodilator effect of the selective ET_A receptor antagonist BQ-123 tended to be blunted in CHF patients compared with healthy control subjects (Fig 2). Although this raises the possibility of ET_A receptor downregulation in CHF, any comparison of the effects of BQ-123 between CHF patients and control subjects must be made with extreme caution. The time course of the effect of BQ-123 and the need for brachial artery cannulation do not lend themselves to repeated dose-response studies; consequently, we cannot be certain that we have demonstrated the maximal effect of BQ-123 in the forearm vasculature. Additionally, because we were reluctant to withhold drug treatment from our CHF patients for

any longer than 24 hours before studies, it is possible that persisting vascular effects of their medication (in particular of ACE inhibition) may have contributed to the difference observed between patients and control subjects.

Our finding that the vasoconstrictor effect of endothelin 1 was attenuated and that of sarafotoxin S6c was enhanced in the forearm vasculature of CHF patients compared with control subjects (Figs 3 and 4) suggests changes in ET_A and ET_B receptor sensitivity similar to those observed by Cannan et al[31] in the coronary vasculature of dogs with experimental CHF. However, the functional significance of these apparent alterations in receptor sensitivity is unclear; further studies comparing the hemodynamic effects of selective ET_A and ET_B receptor antagonists are necessary to delineate the relative importance of each receptor subtype in regulating vascular tone. The divergent nature of the agonist responses we observed in patients with CHF, ie, the blunted constrictor effect of endothelin-1 coupled with the enhanced constrictor effect of sarafotoxin S6c, makes it unlikely that the differences observed were simply due to any persisting vascular effect of the patients' medication. Similarly, although the small baseline differences in FBF, mean arterial pressure, and heart rate between patients and control subjects (Table 3) might have influenced vascular responsiveness to each agonist, these differences on their own would not explain the divergent variation in responses in patients with CHF. It is, however, important to acknowledge that our studies cannot definitively exclude the possible existence of a dilator subtype of ET_A receptor sensitive to endothelin-1 or another species of constrictor receptor sensitive to sarafotoxin S6c in patients with CHF.

Our studies with BQ-123 support an important role for ET_A receptors in mediating the constrictor effects of endogenous endothelin-1 in patients with CHF, but the role of ET_B receptors in this regard needs further clarification. Whether selective ET_B receptor blockade would result in a net vasoconstrictor or vasodilator effect in healthy subjects or patients with CHF is not currently known and cannot be determined from our present data. Our observation that forearm vasoconstriction to sarafotoxin S6c was enhanced in patients with CHF might be partly related to endothelial dysfunction,[32] resulting in diminished endothelial ET_B receptor–mediated release of dilator substances, but whether this is indicative of a shift in the relative functional importance of endothelial and vascular smooth muscle ET_B receptors in CHF requires further investigation.

In summary, our findings have potentially important therapeutic implications. Our central and most important finding from a clinical perspective was that ECE inhibition and ET_A receptor blockade produced a significant additional reduction in forearm vascular resistance in patients with CHF already treated with conventional medical therapy, including an ACE inhibitor. For a new treatment modality to be of real value in CHF, it will have to offer hemodynamic benefit over and above that obtained with an ACE inhibitor; antiendothelin drugs appear to have this potential. If both ET_A and ET_B receptors do indeed mediate vasoconstriction in patients with CHF, then this suggests that use of a nonselective receptor antagonist or a selective ECE inhibitor would be necessary to achieve optimal inhibition of the constrictor effects of endogenous endothelin-1. The ideal receptor antagonist would probably be one that blocked constrictor ET_A and ET_B receptors but preserved endothelial ET_B receptor–mediated vasodilatation; such antagonists with differential selectivities for constrictor and dilator ET_B receptor subtypes have not yet been developed. Much is still to be learned about the role of the endothelins in human physiology and pathophysiology, but if orally active endothelin receptor antagonists and ECE inhibitors produce sustained systemic hemodynamic changes of the type we have observed in the forearm, then they may represent a further therapeutic advance in the treatment of CHF.

Acknowledgments

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Early therapeutic experience with the endothelin antagonist BQ-123 in pulmonary hypertension after congenital heart surgery

B Prendergast, D E Newby, L E Wilson, D J Webb, P S Mankad

Abstract

***Objective*—To assess the effect of endothelin type A (ET_A) receptor antagonism in infants with pulmonary hypertension following corrective surgery for congenital heart disease.**

***Design*—Open label, preliminary study.**

***Setting*—Tertiary paediatric cardiothoracic surgical centre.**

***Patients*—Three infants (aged 3 weeks, 7 weeks, and 8 months) with postoperative pulmonary hypertension unresponsive to conventional treatment, including inhaled nitric oxide.**

***Interventions*—Patients received incremental intravenous infusions (0.1 to 0.3 mg/kg/h) of the ET_A receptor antagonist BQ-123.**

***Main outcome measures*—The response to BQ-123 administration was determined using continuous invasive monitoring of cardiorespiratory variables.**

***Results*—BQ-123 infusion caused a reduction in the ratio of pulmonary to systemic pressures (0.62 (0.01) to 0.52 (0.03), mean (SEM)) with an accompanying decrease in right ventricular stroke work index (4.6 (0.4) to 2.5 (0.3) g/m) and a tendency for the cardiac index to rise (2.1 (0.2) to 2.7 (0.6) l/min/kg/m^2). This was associated with a well tolerated fall in the arterial partial pressure of oxygen (16.5 (4.1) to 12.4 (3.3) kPa) and mean systemic arterial pressure (57 (3) to 39 (3) mm Hg).**

***Conclusions*—ET_A receptor antagonism in infants with postoperative pulmonary hypertension after corrective surgery for congenital heart disease led to significant improvement in pulmonary haemodynamic indices. However, these benefits were associated with reductions in systemic blood pressure and arterial oxygen saturation, the latter consistent with a ventilation–perfusion mismatch. On the basis of these results, studies in pulmonary hypertension will need to proceed with caution.**

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Keywords: endothelin-1; pulmonary hypertension; receptor antagonism; congenital heart disease

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Postoperative pulmonary hypertension is a common clinical problem following successful surgical correction of congenital heart defects and may lead to significant morbidity and mortality.[1] Its occurrence relates both to pre-existing pulmonary hypertension and the acute effects of surgery and cardiopulmonary bypass. Dysfunction of the pulmonary vascular endothelium appears to be a major contributing factor for the development of pulmonary hypertension in this group of patients.

Endothelin-1 is an extremely potent endothelium derived vasoconstrictor peptide[2] which is released and cleared in the pulmonary circulation.[3] Plasma concentrations of endothelin-1 are increased in subjects going to high altitude,[4] in patients with chronic heart failure,[5] and in patients with pulmonary hypertension.[6] Moreover, in these conditions, the degree of pulmonary hypertension and pulmonary vascular resistance correlates closely with plasma endothelin-1 concentrations.[4 6] Children with pulmonary hypertension[7] and persistent pulmonary hypertension of the newborn[8] also have raised plasma endothelin-1 concentrations that correlate with disease severity,[8] are particularly marked after cardiopulmonary bypass,[9] and may play a role in its pathogenesis.[10]

Studies in animal models of pulmonary hypertension have reported reversal of pulmonary hypertension with endothelin receptor antagonists[11 12] and endothelin converting enzyme inhibition.[13] Indeed, in a sheep model of pulmonary hypertension induced by aortopulmonary shunting in utero, endothelin antagonism eliminated the postoperative increase in pulmonary vascular resistance following cardiopulmonary bypass.[14] Reddy and colleagues[14] concluded that endothelin antagonism warrants further study in children at risk of pulmonary hypertension after surgical repair with cardiopulmonary bypass. There have been no published clinical studies to date assessing the therapeutic benefits of endothelin antagonism in postoperative pulmonary hypertension.

We report our preliminary experience with the therapeutic use of the endothelin type A (ET_A) receptor antagonist, BQ-123, in three infants with postoperative pulmonary hypertension following corrective surgery for congenital heart disease.

Methods

Written informed parental consent was obtained for each child and the study was approved by the local research ethics committee.

BQ-123 (American Peptide Company, Sunnyvale, California, USA) was given under a Department of Health (UK) Doctors and Dentists Exemption Certificate.

Table 1 Patient characteristics

Patient	Age (weeks)	Sex	Weight (g)	Diagnosis	Procedure	Inotropes and vasodilators
1	3	Female	3100	Anomalous aortic origin of left pulmonary artery, patent foramen ovale, persistent arterial duct	Mobilisation of left pulmonary artery with formation of pulmonary artery bifurcation, closure of patent foramen ovale, division of persistent arterial duct	Dopamine 3 μg/kg/min
2	7	Male	3500	Obstructed partial anomalous pulmonary venous drainage, left pulmonary artery stenosis, atrial septal defect	Right pulmonary vein to right atrium anastamosis, atrial diverting patch, left pulmonary artery patch	Dobutamine 20 μg/kg/min Glyceryl trinitrate 2 mg/kg/min
3	25	Female	6100	Ventricular septal defect, persistent arterial duct (Aicardi and Poland syndromes)	Patch closure of ventricular septal defect, closure of persistent arterial duct	Dobutamine 20 μg/kg/min Dopamine 3 μg/kg/min

The three infants (aged 3 weeks, 7 weeks, and 8 months) were anaesthetised according to our standard protocol. Phenoxybenzamine (1 mg/kg) was given before establishing cardiopulmonary bypass. Corrective surgery was performed after inducing systemic hypothermia and cold crystalloid cardioplegic arrest. A thermodilution pulmonary artery flow catheter (3 F; Baxter Health Care, Thetford, UK) and a left atrial line were inserted before discontinuing cardiopulmonary bypass.

After chest closure, the infants were returned to the intensive care unit and maintained on a standard regimen of vecuronium (0.1 mg/kg/h), fentanyl (5.0 μg/kg/h), and midazolam (0.1 mg/kg/h). Following rewarming, the ratio of pulmonary to systemic arterial pressure (P/S ratio) was determined using invasive monitoring. Infants were entered into the study if they did not have a residual left to right shunt on echocardiography and had a P/S ratio greater than 0.5 which did not respond to standard treatment, including inhaled nitric oxide (10 ppm increasing to 20, 30, and 40 ppm for 30 minutes at each dose). During the study period, the amount of sedation and inotropic support was maintained constant and atrial pressures kept stable using packed red blood cells or human albumin solution. Following stabilisation for three hours, BQ-123 was dissolved in 0.9% saline and given intravenously at 0.1, 0.2, and 0.3 mg/kg/h, for 30 minutes at each dose.

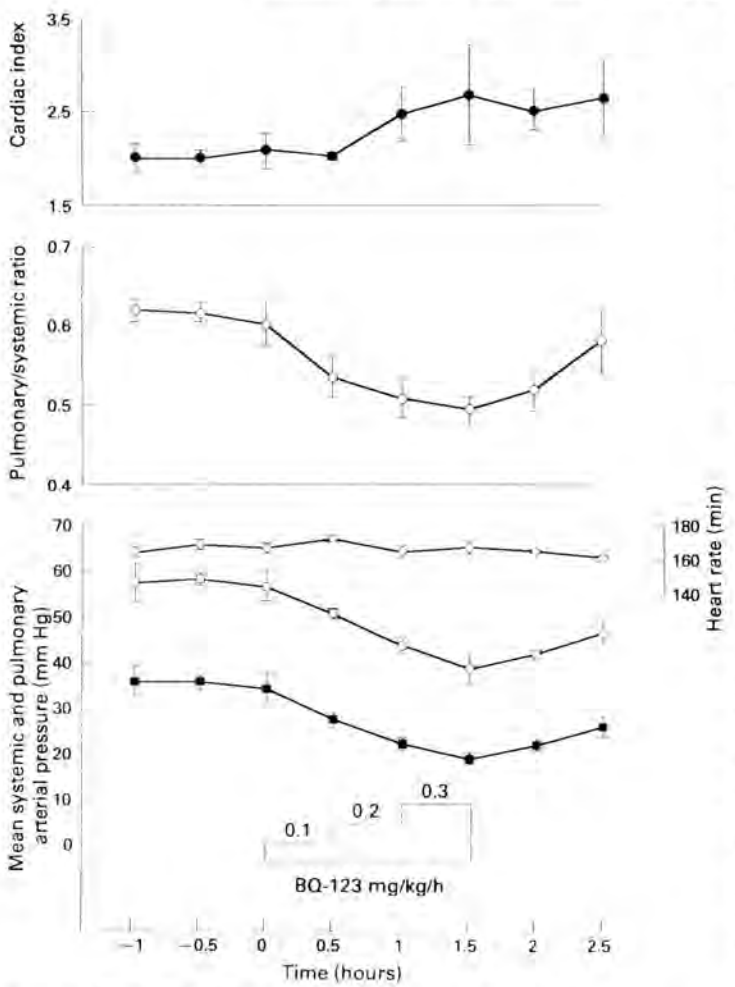

Figure 1 Effect of BQ-123 (0.1 to 0.3 mg/kg/min) on cardiac index (●), pulmonary/systemic ratio (▽), heart rate (○), and mean systemic (□) and pulmonary (■) arterial pressure in infants with postoperative pulmonary hypertension following corrective surgery for congenital heart disease. (Error bars are SEM, n = 3.)

Data are presented as mean (SEM). Haemodynamic variables were measured in triplicate at each time point and the mean taken. Recognising the small sample size and inherent variation between haemodynamic variables, non-parametric analyses (Wilcoxon rank sum) were used to compare variables before, during, and after BQ-123 infusion. Statistical significance was assumed at the 5% level.

Results

Characteristics of the patients are shown in table 1.

Baseline left and right atrial pressures were 9.6 (1.2) and 7.3 (0.3) mm Hg, respectively, and did not change during or after BQ-123 infusion. However, the P/S ratio fell in all patients during BQ-123 administration ($p < 0.001$; fig 1) and returned to baseline about 90 minutes after discontinuation of the infusion. Concomitant with the changes in the P/S ratio, the systemic arterial pressure fell (fig 1), although the fall was proportionately less than for the pulmonary arterial pressure and was well tolerated. Right ventricular stroke work index mirrored the changes in P/S ratio and fell significantly in response to BQ-123 infusion, from 4.56 (0.31) to 2.90 (1.90) g.m/m^2 ($p < 0.001$). The cardiac index tended to increase and left ventricular stroke work index fell from 8.0 (0.9) to 6.2 (0.9) g.m/m^2 ($p < 0.001$) but there were no changes in heart rate, acid–base balance, or urine output.

Despite haemodynamic improvements, the arterial partial pressure of oxygen fell in all three infants during BQ-123 infusion, from 16.5 (4.1) to 12.4 (3.3) kPa. Because of the

reduction in arterial oxygen partial pressures, nitric oxide (20 ppm) was reintroduced into the ventilatory circuit of two infants two hours after starting the BQ-123 infusion (patients 1 and 3). Both infants then responded promptly to nitric oxide administration, with the partial pressures rising from 7.4 to 8.2 kPa and from 8.4 to 10.5 kPa. The third infant (patient 2) did not receive inhaled nitric oxide because in that infant the partial pressure of oxygen was 18.6 kPa at its nadir. Qualitatively, this did not appear to correlate with cardiopulmonary bypass time.

No adverse effects were seen on withdrawal of BQ-123 and all three infants survived to the 30th postoperative day.

Discussion

Although potentially life threatening, postoperative pulmonary hypertension following corrective surgery for congenital heart disease is usually reversible. When conventional treatment, including inhaled nitric oxide, fails further measures such as extracorporeal circulatory support and membrane oxygenation may be required. We have conducted the first preliminary study to examine the therapeutic effects of ET_A receptor antagonism under such circumstances. Although we were able to show a significant improvement in pulmonary haemodynamic indices, this was associated with arterial hypoxaemia and systemic hypotension.

Endothelin-1 is continuously released by the endothelium and contributes to the maintenance of basal vascular tone[15 16] and blood pressure.[17 18] It is therefore not surprising that BQ-123 caused a reduction in systemic as well as pulmonary arterial pressure and this is consistent with the haemodynamic effects seen with the acute administration of BQ-123 in patients with heart failure.[19] However, in these three infants, ET_A receptor antagonism appeared to be more selective for the pulmonary vascular bed, with a proportionately greater effect in comparison with the systemic circulation. This suggests that endothelin-1 provides a greater contribution to the maintenance of vascular tone in the pulmonary circulation in postoperative pulmonary hypertension.

In animal models of pulmonary hypertension induced by aortopulmonary shunting, not only have raised plasma endothelin-1 concentrations been found, but also an increased pulmonary vasoconstrictor response to endothelin-1 infusion.[20] These findings may, in part, relate to the upregulation of endothelin-1 and endothelin converting enzyme expression, as well as the 10-fold downregulation of the ET_B receptor within the pulmonary vasculature.[21] The balance of receptor expression is therefore largely shifted to the vasoconstrictor ET_A receptor and this may exacerbate the pulmonary hypertension. Thus it would be anticipated that selective ET_A receptor antagonism would produce a greater reduction in pulmonary vascular resistance than combined ET_A and ET_B receptor antagonism.

A degree of systemic hypotension and impaired oxygenation is the inevitable consequence of effective systemic vasodilatation and these are the limiting factors in the clinical use of conventional agents. Intrapulmonary ventilation/perfusion matching is dependent upon local hypoxic vasoconstrictive reflexes and so is impaired by pulmonary vasodilatation. Although they were initially unresponsive to inhaled nitric oxide, improved oxygenation was seen in the two patients who received inhaled nitric oxide (20 ppm) after ET_A receptor antagonism. This effect may be related to the recently described improvement in pulmonary vascular responsiveness to nitric oxide following ET_A receptor antagonism.[22] The mechanisms of this effect remain to be established, but in an animal model of pulmonary hypertension, ET_A receptor antagonism was associated with both an improvement in endothelium dependent vasodilatation and an increase in pulmonary vascular smooth muscle sensitivity to nitric oxide.[22]

This first preliminary report of the use of ET_A receptor antagonism in infants with postoperative pulmonary hypertension following corrective surgery for congenital heart disease suggests an improvement in the pulmonary to systemic ratio and right ventricular stroke-work index. However, these benefits were counterbalanced by potentially adverse reductions in arterial oxygenation and systemic blood pressure. These findings suggest that endothelin antagonism, particularly in combination with inhaled nitric oxide, may represent a valuable new approach to the treatment of refractory postoperative pulmonary hypertension which merits further but cautious investigation.

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SECTION 5

MISCELLANEOUS TOPICS AND REVIEWS

Papers 31-34 and 35-46

Intensive Care Med (1996) 22: 52–56

BRIEF REPORT

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Endothelin production in sepsis and the adult respiratory distress syndrome

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Abstract *Objective:* Septic shock is characterised by a decrease in systemic vascular resistance. Nevertheless, regional increases in vascular resistance can occur which may predispose to organ dysfunction, including the adult respiratory distress syndrome (ARDS). Because endothelial damage is a major feature of acute lung injury, we examined whether the potent endothelial vasoconstrictor peptide endothelin-1 plays a pathophysiological role in sepsis or ARDS.
Design: Plasma endothelin was measured in mixed venous, pulmonary capillary and arterial blood, and the relationship with outcome measures was determined.
Setting: The intensive care unit of a university teaching hospital.
Patients and participants: A consecutive series of well-characterised patients with sepsis syndrome, both with ($n = 11$) and without ($n = 15$) ARDS, and ventilated controls without sepsis or ARDS ($n = 7$).
Measurements and results: Plasma endothelin was significantly elevated in patients with sepsis alone and in patients with sepsis and ARDS. Plasma endothelin did not differ among mixed venous, pulmonary capillary and systemic arterial blood. On multiple regression analysis, plasma endothelin correlated positively with organ failure score and with oxygen consumption, and negatively with the $P_aO_2:FiO_2$ ratio. There was no correlation with plasma creatinine, suggesting that decreased renal clearance did not account for the high plasma endothelin concentrations.
Conclusions: Although the lung does not appear to be the major site of endothelin production in critically ill patients with sepsis, increased endothelin production may contribute to regional increases in vacular resistance, hypoperfusion, and the development of organ failure, including ARDS, in patients with sepsis.

Key words: Adult respiratory distress syndrome · Endothelin · Endothelium · Intensive care · Lung · Sepsis

Introduction

Endothelin-1 is a 21-amino-acid peptide, first isolated in 1988 [1] and subsequently shown to be a potent and long-lasting vasoconstricting agent in humans [2]. In most circumstances, endothelin-1 is a local paracrine or autocrine, rather than a circulating hormone. Plasma endothelin is elevated in cases of cardiogenic and septic shock, possibly as part of a homeostatic response to maintain arterial pressure [2].

The adult respiratory distress syndrome (ARDS), which often complicates the clinical course of patients with severe sepsis, is characterised clinically by hypoxia, diffuse infiltrations on chest X-ray and decreased pulmonary compliance, and carries a poor prognosis. ARDS is associated with pulmonary vasoconstriction and increased microvascular permeability [3]. Because these features may be caused by endothelin [2], this peptide may be implicated in the pathogenesis of ARDS.

In this study, we measured circulating plasma concentrations of edothelin in well-characterised patients with sepsis syndrome, with and without ARDS. We also examined whether there is net clearance or production of endothelin across the pulmonary circulation.

Subjects and methods

Thirty-three patients admitted to the Intensive Therapy Unit at the Western General Hospital between May and December 1992 were enrolled in the study, which was approved by the local ethics committee. Patients were subdivided into three groups: those with sepsis without ARDS ($n = 15$); those with both sepsis and ARDS ($n = 11$); and a 'control' group of head-injured patients with neither sepsis nor ARDS and with no evidence of systemic or pulmonary pathology ($n = 7$). All patients were mechanically ventilated, and all but 5 of the control group had pulmonary artery catheters in place for clinical reasons. Sepsis and ARDS were defined in accordance with published criteria [4, 5].

The severity of illness was scored in three ways: by an Acute Physiology and Chronic Health Evaluation (APACHE II) score, recorded 24 h after admission; a Murray Acute Lung Injury Score [3]; and an Organ Failure Score. The latter score ranges from zero (best) to ten (worst-), depending on the severity of dysfunction in five organ systems (pulmonary, cardiovascular, renal, hepatic and hematological), determinations that are based on the criteria described by Goris [6]. In addition, the doses of vasoactive drugs administered to each patient to maintain optimal blood pressure and cardiac output were recorded.

Following categorisation, and within 48 h of either admission to the ITU or the development of sepsis or ARDS, full haemodynamic measurements were made, including thermodilution cardiac output (Table 1). Immediately after these measurements, simultaneous arterial and mixed venous (pulmonary artery) samples were taken to determine haemoglobin, oxygen tension and oxyhaemoglobin saturation, from which oxygen delivery and oxygen consumption were calculated, and to estimate plasma immunoreactive endothelin concentrations. In the five control subjects in whom a pulmonary artery catheter was not present, arterial and central venous samples were used for plasma endothelin estimations; cardiac output was not measured and no derived measures were recorded. Immediately after arterial and mixed venous sampling, a further sample for plasma endothelin assay was obtained from the distal port of the pulmonary artery catheter with the balloon inflated to occlude the pulmonary artery. This was assumed to represent pulmonary capillary blood. Plasma endothelin concentrations were measured by radioimmunoassay [7]. Cross-reactivity of the assay with endothelin-1, endothelin-2, endothelin-3 and proendothelin-1 is 100%, 52%, 96% and 7%, respectively. The normal range (mean ± 2 SD) for plasma

Table 1 Characteristics of patient groups (*SVR* systemic vascular resistance; *MAP* mean arterial pressure; *PVR* pulmonary vascular resistance)

	Head injuries	Sepsis alone	Sepsis and ARDS
Number of patients	7	15	11
Number of survivors	7	13	6
APACHE II score	13.0 ± 1.9	26.0 ± 2.4*	25.0 ± 2.0*
Organ Failure Score	0.3 ± 0.9	3.3 ± 0.6*	4.6 ± 0.6*
Lung Injury Score	0.6 ± 0.2	1.9 ± 0.1*	3.0 ± 0.1*†
P_aO_2 (kPa)	18.7 ± 1.1	16.5 ± 1.1	12.3 ± 1.2*†
P_aO_2:FiO_2 (kPa)	54.0 ± 7.2	31.0 ± 2.4*	19.0 ± 2.0*†
Oxygen consumption ($ml^{-2}\ min^{-1}\ m^2$)	110 ± 10[a]	121 ± 7	147 ± 15
Oxygen delivery ($ml^{-2}\ min^{-1}\ m^{-2}$)	581 ± 28[a]	599 ± 42	586 ± 39
SVR ($dyne.s/cm^5$)	1070 ± 413[a]	850 ± 93	741 ± 55
MAP (mmHg)	97 ± 6	82 ± 4*	79 ± 3*
PVR ($dyne/s/cm^5$)	109 ± 6[a]	226 ± 29	207 ± 35
Noradrenaline dose ($\mu g\ kg^{-1}\ h^{-1}$)	0.6 ± 0.6	8.7 ± 4.0	7.5 ± 4.7
Adrenaline dose ($\mu g\ kg^{-1}\ h^{-1}$)	0	6.9 ± 2.2	4.7 ± 1.9
Dopamine dose ($\mu g\ kg^{-1}\ min^{-1}$)	0	1.4 ± 0.5	0.7 ± 0.5
Dobutamine dose ($\mu g\ kg^{-1}\ min^{-1}$)	0	1.5 ± 0.5	0
Plasma creatinine (μmol/l)	74 ± 5	242 ± 38*	236 ± 69
Mixed venous endothelin (pg/ml)	9.4 ± 1.5	22.0 ± 3.3*	40.8 ± 14*
Pulmonary capillary endothelin (pg/ml)	6.6 ± 0.4[a]	22.6 ± 2.9	45.1 ± 14.4
Arterial endothelin (pg/ml)	9.1 ± 1.2	21.3 ± 2.7*	44.5 ± 15.8*

* $p < 0.05$ vs. controls; † $p < 0.05$ vs. sepsis alone

[a] $n = 2$ in the head injury group for these parameters

immunoreactive endothelin with this assay in our hands is 2.7–7.5 pg/ml (mean = 5.1).

Unless otherwise stated, all results are expressed as mean ± standard error of the mean. Statistical analyses were performed using StatView 512 + software for the Macintosh microcomputer (Brainpower, Calabasas, Calif.). Data were examined using Student's unpaired *t*-test, together with multiple regression analyses of plasma immunoreactive endothelin against the other recorded factors.

Results

Details of patients and outcome are shown in Table 1. The control group had normal cardiovascular and pulmonary function. Patients with sepsis alone and with sepsis and ARDS had significantly higher Apache II, organ failure and lung injury scores than the control subjects, indicating more severe illness. As compared with controls, both these groups also demonstrated haemodynamic compromise, characterised by hypotension necessitating vasoactive drug therapy and impaired pulmonary function with lower ratios of P_aO_2 to FiO_2. The main differences between the patients with sepsis alone and with sepsis and ARDS were that the latter had worse lung function, as shown by higher Murray lung injury scores, lower P_aO_2 and a lower P_aO_2:FiO_2 ratio. The two groups of patients with sepsis did not differ according to any other indices of illness severity, haemodynamic function or oxygen transport.

There were no significant differences between mixed venous, pulmonary capillary and arterial immunoreactive endothelin concentrations in any group (Table 1). Plasma endothelin was higher in patients with sepsis, both with and without ARDS, than in controls. Although plasma endothelin was higher in septic patients with ARDS than in those without ARDS, this difference was not statistically significant. Endothelin concentrations tended to be higher in non-survivors (35.1 ± 13 pg/ml) than in survivors (21.3 ± 4.8 pg/ml; $P = 0.23$).

Upon multiple regression analysis performed to determine which factors were independently correlated with plasma endothelin, there were positive associations with both organ failure score and oxygen consumption, and a negative association with the P_aO_2:FiO_2 ratio (Fig. 1): the overall regression coefficient was 0.92 ($P = 0.0001$). There was no correlation with any of the other factors given in Table 1.

Discussion

Our results, consistent with those of others [8, 9], show that plasma immunoreactive endothelin concentrations are markedly increased in patients with sepsis, multiple organ failure and ARDS, as compared with ITU controls or healthy subjects. We also demonstrate, for the first time, that the degree of organ failure, efficiency of pulmonary oxygen exchange and rate of oxygen consumption all correlate independently with plasma endothelin. Although we cannot specify which of the endothelin isoforms produced the rise in plasma immunoreactivity in our studies, endothelin-2 is undetectable in plasma [2]. Furthermore, an earlier study using an endothelin-1 specific assay appears to implicate this endothelially derived isoform [9].

Both increased generation and decreased clearance of endothelin may have caused the rise in plasma concentrations in patients with severe sepsis. Interestingly, despite the presence of impaired renal function in most patients with sepsis and contrary to the findings in patients wtih chronic renal failure [2], there was no correlation between plasma endothelin and plasma creatinine. This strengthens the case for the occurrence of increased generation of endothelin in sepsis, although other vascular beds, including the liver and skeletal muscles, may also contribute to the clearance of endothelin.

Plasma endothelin concentrations did not differ among mixed venous, pulmonary capillary wedge and systemic arterial samples from any of the patient groups, including controls; increased pulmonary generation of endothelin thus does not seem to occur in sepsis and ARDS. In previous studies examining pulmonary arteriovenous ratios of plasma endothelin, healthy subjects were shown to clear endothelin across the pulmonary vascular bed, with a ratio of arterial to venous endothelin that was substantially less than unity [8–10]. We found no evidence for clearance of endothelin across the pulmonary circulation in our 'controls'. Rather than drawing a comparison with healthy subjects, we studied critically ill, head-injured patients undergoing mechanical ventilation, considering that those constituted a more closely matched control group for the patients with sepsis and ARDS. Because our control subjects did not extract endothelin across the lung, this may be a feature of mechanical ventilation or of the critically ill. Our findings emphasise the need for an appropriate control group in order to demonstrate reliably that sepsis or ARDS is associated with altered endothelin metabolism. It has been reported that patients with ARDS have pulmonary arteriovenous ratios for endothelin substantially greater than unity [8]. This was not the case in our patients, nor in those reported in another study [9], most of whom had co-existing sepsis, which is the usual clinical situation in which ARDS occurs.

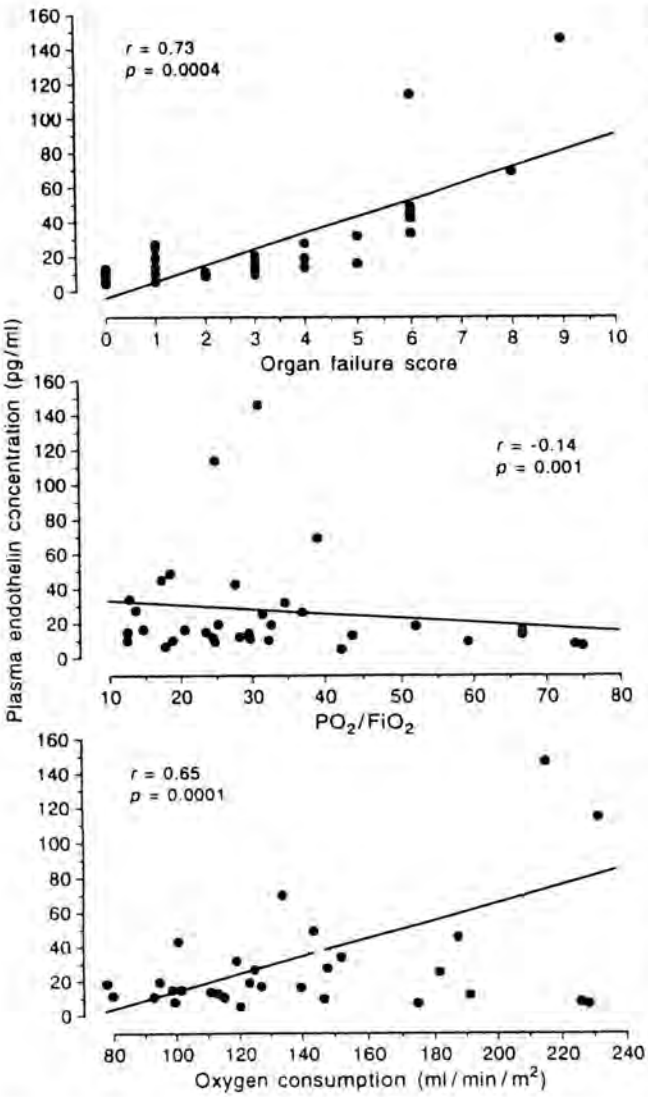

Fig. 1 Scattergrams showing the relationship between mixed venous endothelin concentrations and the three factors (Organ Failure Score, $P_aO_2:FiO_2$ and oxygen consumption) which correlated independently with endothelin on multiple regression analysis. Relevant regression coefficients (r) and significance values (p) are given on each scattergram.

Because endothelin-1 causes pulmonary vasoconstriction and enhances microvascular permeability [2], and because ARDS is characterised by increased pulmonary vascular resistance and microvascular permeability, the very high circulating concentrations of endothelin in septic patients with ARDS may contribute to the pathophysiology of lung damage. This possibility is strengthened by the independent, though weak ($r = -0.17$), negative association of plasma endothelin with the $P_aO_2:FiO_2$ ratio on multiple regression analysis. The absence of a correlation between plasma endothelin and pulmonary vascular resistance suggests that the major pulmonary effect of endothelin in sepsis and ARDS may be on microvascular permeability. The association of organ failure score with endothelin suggests that regional vasoconstriction in sepsis may be due, at least in part, to increased plasma endothelin.

The association between plasma endothelin and oxygen consumption is unexpected, as endothelin is not generally regarded as a hormone that plays a major role in control of metabolic rate. It is possible that this association reflects other unmeasured factors, such as increased concentrations of endogenous and exogenous catecholamines and of growth factors, which act to stimulate both metabolic rate and endothelin production [2]. One may speculate that, of the three variables independently associated with endothelin concentration, one (oxygen consumption) may reflect alterations in the regulation of endothelin generation, while the others ($P_aO_2:FiO_2$ ratio and organ failure score) may reflect pathophysiological activities of endothelin. However, the question of whether local generation of endothelin is a cause or a consequence of ARDS and multiple organ failure in sepsis syndrome will only be answered by studies with inhibitors of endothelin synthesis or endothelin receptor antagonists [2].

Our findings of very high plasma endothelin concentrations in patients with severe sepsis and ARDS, and their correlation with organ dysfunction, suggest that local increases in endothelin generation may compromise vital organ perfusion. Thus, there may be a place for the newly described endothelin receptor antagonists in the treatment of patients with sepsis and ARDS.

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Activation of endothelin ET_A receptors masks the constrictor role of endothelin ET_B receptors in rat isolated small mesenteric arteries

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1 Endothelin-1 (ET-1) produces constriction of the rat mesenteric vascular bed *in vivo* via ET_A and ET_B receptor subtypes. The aim of this study was to investigate the relative roles of these receptor subtypes in rat isolated, endothelium-denuded, small mesenteric arteries, under pressure, by use of ET-1; the ET_A receptor antagonist, BQ-123; the ET_B receptor selective agonist, sarafotoxin S6c (SRTX S6c); the ET_B receptor selective antagonist, BQ-788; and the ET_A/ET_B antagonist, TAK-044.

2 In 3rd generation mesenteric arteries, ET-1 ($10^{-13}-10^{-7}$ M) produced concentration-dependent contractions (pD_2 9.86). SRTX S6c ($10^{-12}-10^{-7}$ M) also induced concentration-dependent contractions in 53% of arteries studied, although the E_{max} was much less than that obtained with ET-1 ($10.7\pm2.9\%$ vs $101.9\pm2.6\%$ of the 60 mM KCl-induced contraction).

3 Neither ET_B receptor desensitization, by a supra-maximal concentration of SRTX S6c (10^{-7} M), nor incubation with BQ-788 (3×10^{-8} M), had any significant effect on the ET-1 concentration-response curve, although both treatments tended to enhance rather than inhibit responses to ET-1.

4 In the presence of BQ-123 (10^{-6} M), responses to low concentrations of ET-1 (up to 10^{-10} M) were unaffected but responses to concentrations of ET-1 above 10^{-10} M were significantly inhibited.

5 SRTX S6c desensitization followed by incubation with BQ-123 (10^{-6} M) or co-incubation with BQ-788 (3×10^{-8} M) and BQ-123 caused inhibition of responses to all concentrations of ET-1, resulting in a rightward shift of the ET-1 concentration-response curve. The same effect was obtained by incubation with TAK-044 (10^{-8} M and 3×10^{-7} M).

6 Thus, responses of rat small mesenteric arteries to ET-1 are mediated by both ET_A and ET_B receptors. The relative role of ET_B receptors is greater than that predicted by the small responses to SRTX S6c or by resistance of ET-1-induced contraction to ET_B receptor desensitization or BQ-788. The effect of ET_B receptor desensitization or blockade is only revealed in the presence of ET_A receptor blockade, suggesting the presence of a 'crosstalk' mechanism between the receptors. These results support the concept that dual receptor antagonists, like TAK-044, may be required to inhibit completely constrictor responses to ET-1.

Keywords: Endothelin-1; sarafotoxin S6c; ET_A receptors; ET_B receptors; BQ-123; BQ-788; TAK-044

Introduction

It is now well established that the vasoactive effects of the peptide endothelin-1 (ET-1) are mediated via both ET_A (Arai *et al.*, 1990) and ET_B receptors (Sakurai *et al.*, 1990). Administration of ET-1 to anaesthetized or conscious rats leads to a brief decrease, followed by a long lasting increase, in blood pressure (Yanagisawa *et al.*, 1988) that is accompanied by increased resistance in virtually all vascular beds studied (Gardiner *et al.*, 1994; Allcock *et al.*, 1995). Prior administration of an ET_A receptor antagonist, e.g. BQ-123 or FR 139317, enhances the initial depressor effect of ET-1 (an ET_B receptor-mediated effect) and reduces the pressor effect (McMurdo *et al.*, 1993; Gardiner *et al.*, 1994). However, the pressor and regional constrictor effect of ET-1 is not fully inhibited by ET_A receptor antagonists, even with high doses, implying that ET_B receptors may also have a vasoconstrictor role (McMurdo *et al.*, 1993). Consistent with this possibility, the ET_B receptor selective agonist, sarafotoxin S6c (SRTX S6c) was found to produce vasoconstriction in pithed rats (Williams *et al.*, 1991; Clozel *et al.*, 1992).

In vitro experiments have also demonstrated ET_A receptor antagonist-resistant responses to ET-1 (Ihara *et al.*, 1992; Sumner *et al.*, 1992; Fukuroda *et al.*, 1994b) and constrictions to SRTX S6c (Moreland *et al.*, 1992; Sumner *et al.*, 1992; La Douceur *et al.*, 1993; Gray *et al.*, 1994). As a consequence of these *in vitro* data, it has been suggested that constrictor ET_B receptors have a role only in large calibre vessels and in the venous circulation (Moreland *et al.*, 1992; Davenport & Maguire, 1995). However, in the conscious rat (Gardiner *et al.*, 1994) and the anaesthetized ganglion-blocked rat (Allcock *et al.*, 1995), ET-1-induced reduction of blood flow to the mesenteric resistance bed is partly resistant to ET_A receptor inhibition. Reduction of regional blood flow in response to SRTX S6c is also most marked in the mesenteric bed of the pithed rat (Clozel *et al.*, 1992). In man, ET-1 constrictions in upper limb blood vessels are also partly resistant to BQ-123 and constrictions to SRTX S6c can be seen (Haynes *et al.*, 1995; Strachan *et al.*, 1995). Thus, there may be an important role for constrictor ET_B receptors in mediating vascular resistance and blood pressure. Indeed, the recently described non-peptide ET_B receptor antagonist, Ro 46-8443, causes a reduction in blood pressure in anaesthetized, normotensive rats (Clozel & Breu, 1996).

In contrast to the evidence for ET_B receptor-mediated constriction of the rat mesenteric bed *in vivo*, *in vitro* studies of perfused mesenteric beds or human and rat isolated mesenteric arteries mounted in wire or perfusion myographs have led to the conclusion that constrictor ET_B receptors have little (Tschudi & Luscher, 1994; Takase *et al.*, 1995; Deng *et al.*, 1995; Touyz *et al.*, 1995) or no role (D'Orleans-Juste *et al.*, 1993) in this vascular bed. All of these studies have based their conclusions on inhibition of ET-1-induced contraction by ET_A

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receptor antagonists, or responses to ET_B selective agonists. The aim of the present study was to investigate further the role of ET_B receptors in mediating constriction in pressurized rat mesenteric arteries by use of ET-1, the ET_A receptor antagonist, BQ-123 (Ihara *et al.*, 1992), the ET_B selective agonist SRTX S6c (Williams *et al.*, 1991), the ET_B receptor selective antagonist, BQ-788 (Ishikawa *et al.*, 1994) and the ET_A/ET_B antagonist, TAK-044 (Kikuchi *et al.*, 1994).

Some of this work has been presented to the British Pharmacological Society (Mickley *et al.*, 1995).

Methods

Male Wistar rats (10–16 weeks old) were killed by exsanguination and the mesenteric bed immediately excised and placed into cold, oxygenated Krebs-Henseleit solution. Third order branches of the mesenteric artery (internal diameter 150–350 μm) were dissected (~3 mm length) and mounted between two glass microcannulae in a small vessel arteriograph (Living Systems Instrumentation Inc., Burlington, U.S.A.). The vessel was constantly superfused with warmed (37°C), oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution (composition, in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.5). The intraluminal pressure of the vessel was raised to 60 mmHg and maintained at this pressure with a pressure servo unit without further intraluminal perfusion. Luminal diameter was measured with a video dimension analyser (Living Systems Instrumentation Inc., U.S.A.) and by hand, with a calibrated micrometer, when the optical dimension analyser was unable to detect differences in optical density at smaller lumen diameters. After an equilibration period of 60 min, the vessels were exposed twice to modified Krebs-Henseleit solution containing 60 mM KCl (equimolar replacement of NaCl by KCl) in order to produce maximum constriction. KCl induced a reduction in lumen diameter but never to the level where the lumen was completely occluded (see Table 1). The endothelium was removed by passing an air bubble through the lumen of the vessel (Falloon *et al.*, 1993; Smith, 1996) and complete denudation was confirmed by addition of acetylcholine (ACh 10^{-6} M) to vessels pre-constricted with phenylephrine (PE 10^{-5} M). In all vessels, the relaxation induced by ACh before the passage of an air bubble (usually back to resting diameter), was completely abolished after endothelial denudation. After washing, a closed system with a total volume of 30 ml of Krebs-Henseleit solution was constantly superfused at a constant flow rate of 5 ml min^{-1}. It was this reservoir of Krebs-Henseleit solution to which the agonists and antagonists were applied, keeping the volume at 30 ml by removing one ml of Krebs and adding one ml of the drug in a stepwise fashion (as previously described, Smith *et al.*, 1995). Responses were recorded 5 min after addition of each agonist concentration, which was sufficient time for an equilibrium response. All of the following studies were carried out in random order and only one concentration response curve to ET-1 or SRTX S6c was performed per tissue. None of the drug treatments resulted in complete occlusion of the vessel lumen within the concentration range studied (see Table 1).

ET-1 and SRTX S6c study

In the first set of experiments cumulative concentration-response curves to ET-1 (10^{-13}–3×10^{-8} M, $n=10$) or SRTX S6c (10^{-12}–10^{-7} M, $n=17$) were obtained as described above.

Receptor antagonism study

In the second set of experiments, vessels were exposed to either BQ-123 (10^{-6} M, $n=8$), BQ-788 (3×10^{-8} M, $n=8$), TAK-044 (10^{-8} and 3×10^{-7} M, $n=4$ and 8 respectively), BQ-123 + BQ-788 (concentrations as before, $n=8$) or vehicle ($n=8$) for 30 min, before concentration-response curves to ET-1 (10^{-13}–3×10^{-8} M) were obtained. For these experiments, agonists were prepared in a solution of antagonist so that addition to the perfusion circuit did not dilute the antagonist solution superfusing the tissue. In some experiments, the vessels were exposed for 30 min to SRTX S6c (10^{-7} M) twice (with a wash out period of 10 min between each exposure), in order to desensitise the ET_B receptor before commencement of the ET-1 concentration-response curve. This was carried out both in the absence and in the presence of BQ-123 ($n=8$ each). In all experiments, the time-course of the protocol was the same; 2 h after verification of the removal of the endothelium, the concentration-response curve to ET-1 was begun.

Data analysis

The results are calculated as a percentage of maximum constriction obtained with the second exposure to 60 mM KCl Krebs solution and are expressed as mean ± s.e.mean. Where a maximum response to the agonist was obtained, the negative log of the concentration causing half-maximal contraction (pD_2) was calculated by linear regression analysis and compared by unpaired one-tailed *t* test. The concentration-response curves were compared by one-way ANOVA followed by Fisher's least significant difference test. Significance was taken at $P<0.05$.

Materials

ET-1 and SRTX S6c were purchased from Novabiochem (Nottingham, U.K.) and BQ-788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-MeLeu-D-Trp($COOCH_3$)-D-Nle, sodium salt) from Neosystems (Strasbourg, France), all were reconstituted in 50:50 methanol:distilled water. BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Vel-L-Leu]) from Neosystems (France) and TAK-044 (cyclo[D-α-Asp-3-[(phenylpiperazin-1-yl)carbonyl]-L-Ala-α-Asp-D-2-(2-thienyl)-Gly-L-Leu-D-Trp] disodium salt) synthesised by Takeda Chemical Industries (Osaka, Japan) were reconstituted in 0.9% saline, placed in aliquots and stored frozen at −20°C until use. All peptide agonists and antagonists were diluted in Krebs-Henseleit solution containing 0.1% bovine serum albumin (BSA, Sigma, Poole, U.K.). In

Table 1 Mean resting lumen diameters and lumen diameters after exposure to 60 mM KCl solution or after the maximum concentration of endothelin-1 (ET-1) or sarafotoxin S6c (SRTX S6c) in each experimental group

	ET-1 control	*SRTX S6c*	*+ BQ-123*	*+ BQ-788*	*+ SRTX S6c desens*	*+ BQ-123 + BQ-788*	*+ BQ-123 + SRTX S6c desens*	*+ TAK-044 (10^{-8} M)*	*+ TAK-044 (3×10^{-7} M)*
Resting diameter	277 ± 15	300 ± 9	261 ± 13	287 ± 15	281 ± 7	273 ± 21	304 ± 19	300 ± 12	301 ± 13
+ 60 mM KCl diameter	51 ± 3	48 ± 2	53 ± 3	51 ± 1	48 ± 3	55 ± 2	50 ± 3	45 ± 3	50 ± 2
Max ET-1/SRTX S6c diameter	47 ± 3	273 ± 12	56 ± 7	50 ± 2	48 ± 3	64 ± 8	118 ± 27	118 ± 39	234 ± 31

Data shown are mean ± s.e.mean

all antagonist experiments the ET-1 concentrations were diluted in 0.1% BSA Krebs-Henseleit solution with the appropriate antagonist. ACh (chloride salt, Sigma, Poole, U.K.) and PE (hydrochloride salt; Fisons, U.K.) were prepared in saline at stock concentration of 10^{-2} M, placed in aliquots, and stored at −20°C until use when diluted in Krebs-Henseleit solution.

Results

Effects of 60 mM KCl

In all experiments 60 mM KCl superfusion constricted the arteries, an effect which was reversible, back to initial resting diameter, on washout (Table 1). The initial diameter remained constant until agonist-induced constriction was generated.

Effects of ET-1 and SRTX S6c

ET-1 constricted the arteries in a concentration-dependent manner (Figure 1, pD_2 9.86, E_{max} 101.9 ± 2.6% KCl induced contraction at 10^{-8} M ET-1, $n = 10$). SRTX S6c also produced a concentration-dependent contraction (Figure 1), but the response was extremely variable, the maximum response obtained with 3×10^{-8} M SRTX S6c ranging from 0 to 39% of KCl contraction (mean response = 10.7 ± 2.9%, $n = 17$). In fact, only 9 of the 17 vessels (53%) responded to SRTX S6c.

Effect of ET_A receptor blockade

Incubation with BQ-123 (10^{-6} M) before and during exposure to ET-1 (Figure 2) had no effect on contractile responses to low concentrations of ET-1 (10^{-13} to 10^{-10} M) but resulted in inhibition of responses to concentrations of ET-1 between 10^{-10} and 3×10^{-8} M. Incubation with BQ-123 significantly inhibited the constrictions to 10^{-9} and 3×10^{-9} M ET-1 ($P = 0.006$ and 0.01, respectively) when compared by ANOVA. However, the effect of BQ-123 on the overall pD_2 of the ET-1 concentration-response curve did not reach statistical significance (pD_2 9.15 ($n = 8$) vs 9.86 ($n = 10$) NS, $P = 0.094$).

Effect of ET_B receptor desensitization or blockade

Exposure to a supra-maximal concentration of SRTX S6c (10^{-7} M), to achieve ET_B receptor desensitization, produced an initial constriction in 4 out of the 8 vessels studied (mean response = 8.1 ± 3.5% KCl constriction). The vessel diameter returned to the initial resting value during the first 30 min exposure to SRTX S6c. No constriction was seen, in any of the vessels studied, during the second exposure to SRTX S6c confirming that tachyphlaxis had occurred. The ET-1 concentration-response curve was not significantly altered by either ET_B receptor desensitisation (Figure 3a, $pD_2 = 9.88$, $n = 8$) or following incubation with the selective ET_B receptor antagonist, BQ-788 (3×10^{-8} M, Figure 3b, $pD_2 = 10.02$, $n = 8$), although both treatments tended to shift the ET-1 concentration-response curve to the left ($P = 0.5$ and 0.34, respectively).

Effect of combined ET_A and ET_B receptor blockade

Co-incubation of vessels with BQ-123 (10^{-6} M) and BQ-788 (3×10^{-8} M) resulted in a parallel shift of the ET-1 concentration-response curve to the right (Figure 4, $n = 8$). Incubation with BQ-123 (10^{-6} M) following desensitization of ET_B receptors with 10^{-7} M SRTX S6c caused a similar rightward shift (Figure 4, $n = 8$). Incubation of vessels with the ET_A/ET_B receptor antagonist, TAK-044 (Figure 5, 10^{-8} M, $n = 4$ and 3×10^{-7} M, $n = 8$) also caused a parallel concentration-dependent shift to the right of the ET-1 concentration-response curve. As the maximum response to ET-1 was not reached within the concentration range studied it was not possible to calculate pD_2 values for ET-1 in experiments with BQ-123 plus either BQ-788 or SRTX S6c desensitization, or with TAK-044 (both concentrations).

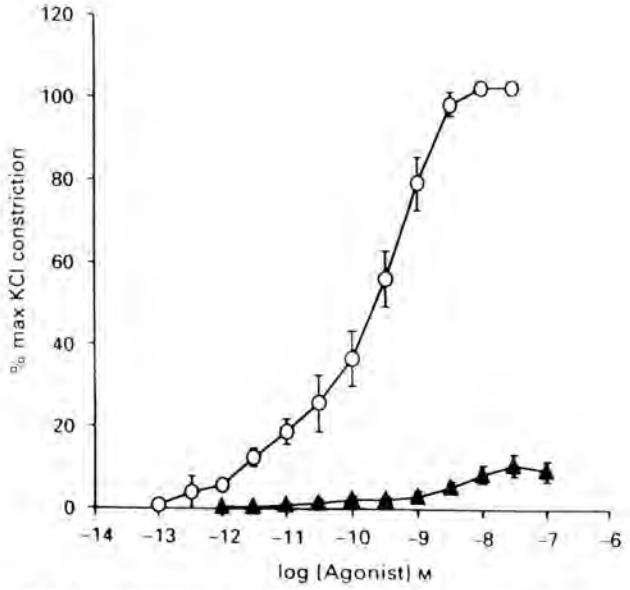

Figure 1 Comparison of the contractile responses to endothelin-1 (ET-1, ○) and sarafotoxin S6c (SRTX S6c, ▲) in rat small mesenteric arteries. ET-1 ($n = 10$) produced a maximal constriction of similar proportion to 60 mM KCl at 3×10^{-9} M SRTX S6c ($n = 17$) induced small constrictions at the highest concentrations, suggesting a small population of ET_B receptors present on the smooth muscle of the resistance arteries. All values are mean and vertical lines show s.e. mean.

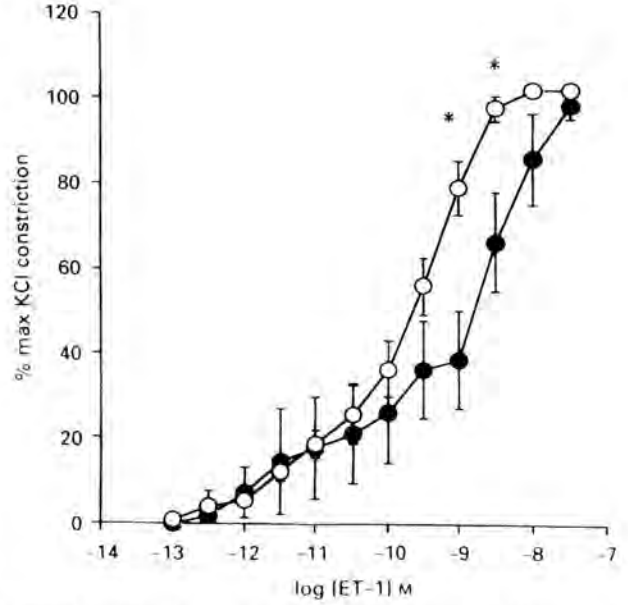

Figure 2 Effect of the ET_A receptor antagonist BQ-123 on the endothelin-1 (ET-1) concentration-response curve in rat small mesenteric arteries. Pre-incubation with BQ-123 (10^{-6} M) for 30 min (●, $n = 8$) shifted the responses to the higher concentrations of ET-1 in a parallel fashion to the right. All values are mean and vertical lines show s.e. mean. *$P < 0.05$ compared to control (○) ET-1 responses.

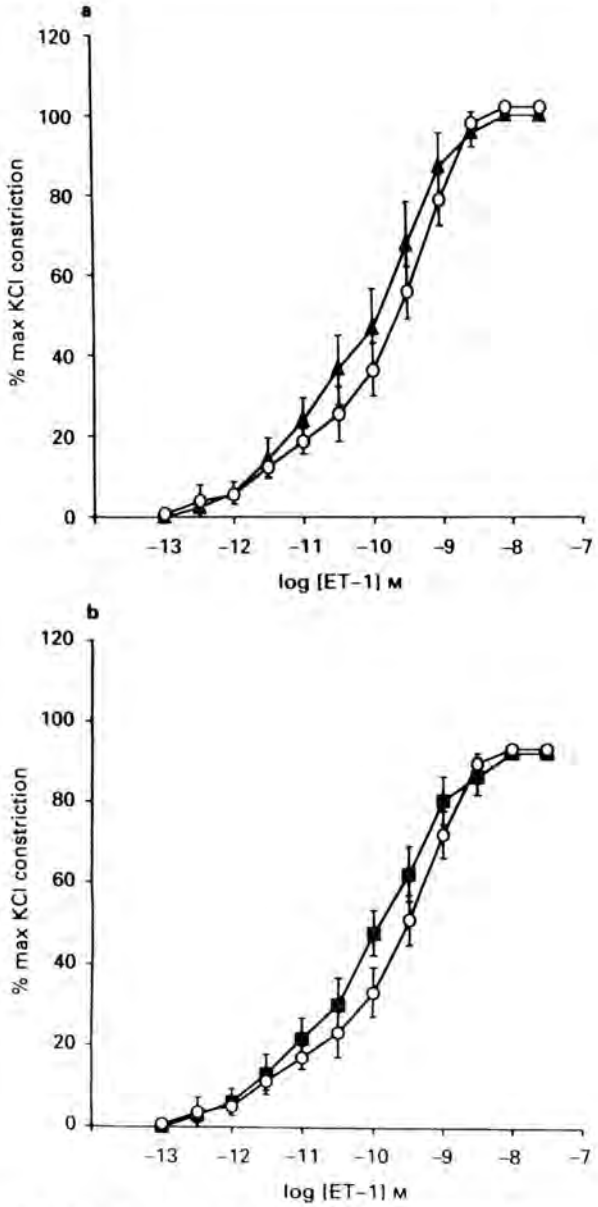

Figure 3 The effects of selective ET_B receptor blockade on endothelin-1 (ET-1)-induced constrictions in rat small mesenteric arteries. The vessels were exposed to either (a) sarafotoxin S6c (SRTX S6c; 10^{-7} M, ▲, $n=8$) twice before addition of ET-1 or (b) BQ-788 (3×10^{-8} M, ■, $n=8$) pre-incubated for 30 min before the start of the ET-1 concentration-response curve. In both treatments the ET-1 concentration-response curves tended to be shifted slightly to the left as compared to control (○), (though not significant, $P=0.54$ and 0.42, respectively, as compared by ANOVA). All values are mean and vertical lines show s.e.mean.

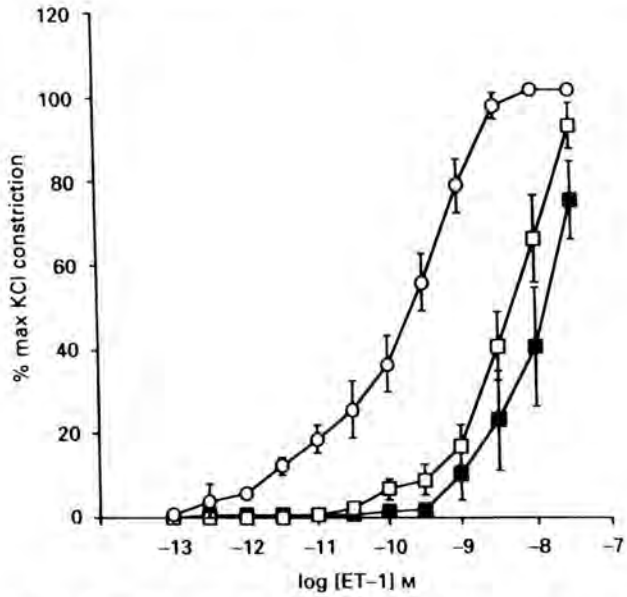

Figure 4 The effects of non-selective ET_A/ET_B combination treatment on endothelin-1 (ET-1)-induced constrictions in rat small mesenteric arteries. The vessels were exposed to either vehicle (○), BQ-123 plus BQ-788 (10^{-6} M and 3×10^{-8} M, □, $n=8$) or pre-incubated with sarafotoxin S6c twice (each 10^{-7} M) plus BQ-123 (10^{-6} M, ■, $n=8$). Both treatments significantly shifted the ET-1 concentration-response curve to the right in a parallel fashion ($P=0.0001$ for both). All values are mean and vertical lines show s.e.mean.

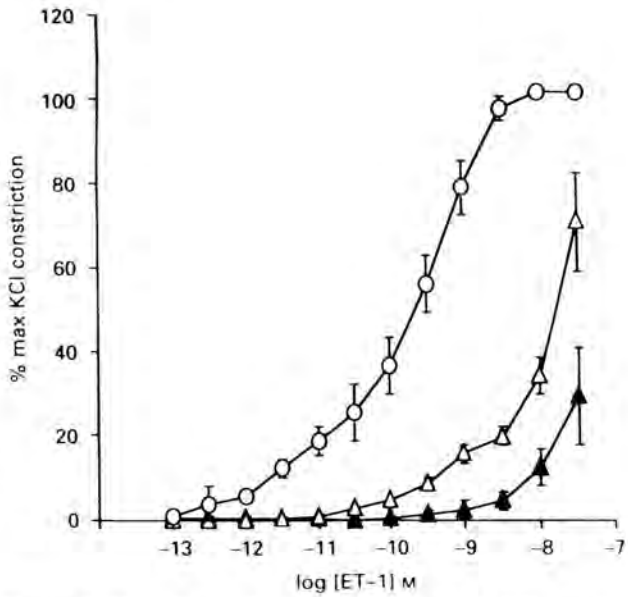

Figure 5 The effects of the non-selective ET_A/ET_B receptor antagonist TAK-044 on endothelin (ET-1)-induced constrictions in rat small mesenteric arteries. The vessels were pre-incubated for 30 min with either 10^{-8} M (△, $n=4$) or 3×10^{-7} M (▲, $n=8$) TAK-044. Both treatments significantly inhibited the ET-1 concentration-response curve ($P=0.0002$ and 0.0001 respectively) as compared to control (○). All values are mean and vertical lines show s.e.mean.

Discussion

Previous *in vivo* studies have clearly indicated a role for ET_B receptors in mediating vasoconstriction in resistance beds, but their role has been difficult to demonstrate in isolated resistance vessels. In the present study, we show that a role for ET_B receptors in rat isolated mesenteric arteries emerges when both ET_A and ET_B receptors are blocked, whereas blockade of ET_A receptors alone only partially inhibited ET-1-induced contraction and inhibition of ET_B receptors alone had no effect. This phenomenon is similar to previous observations in rabbit pulmonary artery (Fukuroda *et al.*, 1994c), rat trachea (Clozel & Gray, 1995) and human bronchus (Fukuroda *et al.*, 1996), and may be explained by the existence of a 'crosstalk' mechanism between the ET_A and ET_B receptors.

In initial experiments we used the highly selective ET_B receptor agonist SRTX S6c (Williams *et al.*, 1991) to investigate the presence of ET_B receptors in pressurised mesenteric arteries. SRTX S6c produced concentration-dependent con-

striction but the maximum constriction reached only ~10% of that routinely seen with ET-1, much less than would have been predicted from previous *in vivo* experiments (Clozel *et al.*, 1992). However, the magnitude of responses to SRTX S6c is in agreement with responses obtained by Takase *et al.* (1995) and Deng *et al.* (1995), in rat mesenteric arteries studied in the perfusion and wire myograph, respectively. Interestingly, in all three studies, the contractions of SRTX S6c occurred at relatively high concentrations (10 nM). The ET_B receptor agonists, BQ-3020 and IRL 1620, were equally ineffective in the rat perfused mesenteric bed at concentrations up to 1 nM (D'Orleans-Juste *et al.*, 1993). This is quite different to the ET_B agonist responses induced in large blood vessels, which are generally larger and occur at lower concentrations (Moreland *et al.*, 1992, Sumner *et al.*, 1992; LaDouceur *et al.*, 1993; Gray *et al.*, 1994). Another interesting feature of our results, not mentioned by previous investigators, is the variability in responsiveness to SRTX S6c. While some vessels failed to respond, others gave up to ~40% of the maximum contraction obtained with ET-1. This might be explained by differential distribution of ET_B receptors in the mesenteric bed, although 3rd generation branches of the main mesenteric artery were routinely used for these studies. Another possibility is variation in intrinsic myogenic tone that these vessels can develop when under pressure. In a separate experiment, in which vessels mounted in the wire myograph were studied, we found that no responses were obtained to STRX S6c until some tone was introduced by a low concentration of the stable thromboxane analogue, U46619 (Mickley *et al.*, 1995).

An alternative approach for the investigation of the role of ET_B receptors is to remove the influence of ET_B receptors, either by desensitization (LaDouceur *et al.*, 1993) or by use of a selective ET_B receptor antagonist, like BQ-788 (Ishikawa *et al.*, 1994). In the present study, neither of these interventions inhibited ET-1 induced contraction, a result which would support the view that ET_B receptors have little or no role in rat mesenteric arteries. Interestingly, both desensitization and BQ-788 treatment seemed to potentiate responses to ET-1 slighty, although this effect was not significant. Seo (1996) recently found a similar potentiation of ET-1-induced constriction by the ET_B receptor antagonist, Res 701-1 in human gastroepiploic arteries. There are several possible explanations for these observations. Potentiation of contractions by ET_B receptor antagonists would be expected in the presence of the vascular endothelium due to blockade of endothelial ET_B receptor-mediated release of relaxing factors by ET-1. However, this is an unlikely explanation for the present results as the endothelium was effectively removed by passing of an air bubble through the lumen of the vessels, as evidenced by the loss of relaxant responses to acetylcholine. Previous histological studies in our laboratory have also shown complete removal of the endothelium by this method (Smith, 1996). The experiments of Seo (1996) were also conducted in endothelium-denuded vessels. Alternatively, potentiation might have been caused by displacement of ET-1 from low affinity ET_B clearance receptors (Fukuroda *et al.*, 1994a) by BQ-788, but this would not account for the similar effect of receptor desensitisation. Another alternative, suggested by Seo (1996), is the presence of sensitive ET_B receptors on smooth muscle which inhibit or negatively modulate ET_A receptor-mediated constrictions to ET-1.

From the results obtained with SRTX S6c, BQ-788, and desensitization alone, one would predict that blockade of ET_A receptors, by use of a selective competitive antagonist, like BQ-123 (Ihara *et al.*, 1992), would cause a parallel rightward shift of the ET-1 concentration-response curve. However, in the presence of BQ-123 the ET-1 concentration-response curve in mesenteric arteries under pressure was biphasic, only responses to high concentrations of ET-1 being shifted to the right in a parallel manner by BQ-123, consistent with competitive antagonism at the ET_A receptor. Interestingly, the BQ-123-resistant, possibly ET_B-mediated, responses to ET-1 were at the lower end of the dose-response curve, consistent with the presence of a high affinity ET_B receptor. Takase *et al.* (1995) obtained similar results with the ET_A receptor antagonist, FR139317 in rat mesenteric arteries, although in that case the ET_A-resistant component was smaller than seen here. Takase *et al.* perfused the vessels at a pressure of 30 mmHg, half of that used in the present study. Given our observation that increased tone may reveal constrictor ET_B receptors, as implied by the responses to SRTX S6c (Mickley *et al.*, 1995), the lower pressure used by Takase *et al.* (1995) may account for the smaller ET_A receptor antagonist-resistant element of the ET-1 curve. The results of the present study are consistent with the ET_A receptor antagonist resistant reduction in mesenteric blood flow induced by ET-1 *in vivo* found by Gardiner *et al.* (1994) and Allcock *et al.* (1995).

In order to investigate whether the residual ET_A antagonist resistant portion of the ET-1 response is mediated by ET_B receptors, we used combined treatment with BQ-123 and either desensitization or BQ-788. Both of these combination treatments resulted in a parallel shift of the ET-1 concentration-response curve. In fact, the BQ-123-sensitive portion was moved further to the right than with BQ 123 alone, in agreement with Fukuroda *et al.* (1996) who described a similar phenomenon in human bronchi. Responses to ET-1 were also inhibited, in a concentration-dependent manner, by TAK-044, a peptide antagonist with similar potency at both ET_A and ET_B receptors (Kikuchi *et al.*, 1994).

These results demonstrate a clear role for ET_B receptors in mediation of constrictor responses to ET-1 in small mesenteric arteries that is only revealed when ET_A receptors, in addition to ET_B receptors, are blocked. The lack of effect of ET_B receptor blockade or desensitization alone seems to indicate that ET_A receptors can somehow compensate for the inactivation of ET_B receptors. Similar observations have been obtained in vascular (Fukuroda *et al.*, 1994c) and non-vascular (Clozel & Gray, 1995; Fukuroda *et al.*, 1996) tissues. The concept of receptor 'crosstalk' has been proposed to explain these observations. The mechanism is not fully understood, although interactions at the second messenger level have been suggested, such that blockade of the ET_B receptor releases an inhibitory mechanism acting at the ET_A receptor (Fukuroda *et al.*, 1996). Allosteric interactions between ET receptors have been suggested to account for the results of radioligand binding studies in rat heart (Sokolovsky, 1993). Further biochemical studies are required to elucidate the interactions between ET receptors co-existing in the same tissue and the mechanism of the apparent crosstalk phenomenon. Interestingly, similar interactions have been described between α_1- and α_2-adrenoceptors activated by noradrenaline (Daly *et al.*, 1988).

In the rat, the mesenteric bed receives a high proportion of cardiac output and thus resistance in this bed is an important determinant of total peripheral resistance and of blood pressure. The present results show that simultaneous blockade of both ET_A and ET_B receptors is required for complete inhibition of constrictor responses to ET-1 in the rat mesentery *in vitro*. This agrees with observations that blockade of both receptors is required to inhibit ET-1-induced increases in blood pressure *in vivo* (McMurdo *et al.*, 1993). The role of ET_B receptors in regulating constrictor responses to ET-1 might be even greater in human resistance vessels, where ET_B agonists have a greater direct effect than in other species *in vitro* (Takase *et al.*, 1995, Mickley, unpublished observations) and *in vivo* (Haynes *et al.*, 1995).

In some pathophysiological states associated with increased peripheral resistance and increased plasma concentrations of ET-1, there is evidence for an upregulation of smooth muscle ET_B receptors; most notably in heart failure in dogs (Cannan *et al.*, 1996) and man (Love *et al.*, 1996); in atherosclerosis (Winkles *et al.*, 1993; Dagassan *et al.*, 1996) and in hypertension (Kanno *et al.*, 1993; Batra *et al.*, 1993). The results of the present study suggest that blockade of both ET_A and ET_B receptors may be required for effective inhibition of ET-1-induced constriction in these diseases. This study was conducted

in vessels without endothelium. However, in the presence of endothelium, ET_B receptor blockade can actually enhance responses to ET-1 by blocking the release of nitric oxide and prostacyclin through endothelial ET_B receptor stimulation (De Nucci *et al.*, 1988). Thus, the effectiveness of endothelin receptor blockade therapeutically will depend on the level of endothelial ET_B receptor stimulation and on the relative selectivity of the antagonist for endothelial and smooth muscle ET_B receptors, the ideal antagonist allowing ET-1 to act at the endothelial ET_B receptor while blocking its effects at smooth muscle ET_A and ET_B receptors.

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A Nonradioactive Method for Localization of Endothelin Receptor mRNA In Situ

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Summary: To investigate relationships between the distribution of endothelin (ET) receptor expression and histopathology of heart and blood vessels, we developed a method of nonradioactive in situ hybridization in paraffin sections. Rat mesenteric bed, rat heart, and human uterine artery were fixed in formalin and embedded in paraffin. ET_A and ET_B receptor cDNAs were subcloned into plasmid vectors for synthesis of sense and anti-sense probes. Digoxigenin (DIG)-UTP was incorporated into every twentieth to twenty-fifth nucleotide of the newly transcribed cRNA. mRNA was detected in situ using an anti-DIG alkaline phosphatase antibody and an alkaline phosphatase substrate. In blood vessels, ET_A receptor mRNA was localized to the medial smooth muscle layer and ET_B receptor mRNA to the endothelial and adventitial layers. Hearts from rats that had undergone coronary artery ligation for induction of CHF showed intense staining for ET_B receptor mRNA in the scarred and infarcted zone of the left ventricle. This method provides a suitable alternative to radioisotope-labeled probes for detection of ET receptor mRNA. It allows better preservation of tissues, shorter detection time, and improved morphology for microscopic analysis. **Key Words:** ET– Receptors mRNA—In situ hybridization—Digoxigenin.

Since the description of endothelin (ET) in 1988 (1) and subsequent cloning of the genes for the ET receptor subtypes ET_A and ET_B and endothelin-converting enzyme (ECE), there has been a wealth of interest in the ET system in a number of pathophysiologic conditions, such as hypertension, atherosclerosis, and heart failure (2). The most commonly used methods to investigate the role of ET and its receptors at the molecular level in tissues such as heart and blood vessels are limited to techniques that require either cultures of cells, homogenates of tissue for mRNA extraction, or the use of potentially hazardous radioactive isotopes. Although cell culture methods provide preliminary evidence of receptor activity and yield valuable information about intracellular and extracellular events (3), the cells are removed from their physiologic environment and therefore little information is gained about where the receptors are expressed within the whole tissue and how the receptors are organized in relation to other cell types. Moreover, Northern blotting techniques require tissues to be homogenized for blotting of RNA onto nitrocellulose membranes and, because most tissues are heterogeneous, it can be difficult to localize ET receptor expression to a particular cell type.

In situ hybridization is a technique in which tissue sections can be probed with cRNA. Normally, cRNA is labeled with radioactive isotopes such as ^{35}S, ^{3}H, or ^{32}P for use as cRNA probes. However, not only does this method require a specially designated laboratory for radioisotope use but frozen tissue sections also tend to be used to facilitate detection of the hybridized mRNA by autoradiography. The use of cryotomy often means that morphology and microscopic resolution of the tissues are compromised by ice crystal damage during the freezing process (4), and sections tend to be fragile, resulting in tissue degradation and loss by the rigorous posthybridization process. Furthermore, the development process for autoradiographs is arduous and can take several months before results are available for analysis.

The aim of the present study, therefore, was to simultaneously correlate expression studies with histopathologic changes in rat and human heart and in blood vessels by developing a sensitive nonradioactive method of localizing ET receptors in situ, in which results can be obtained within days with minimum loss of cell detail, for examination by brightfield microscopy.

MATERIALS AND METHODS

Rat mesenteric blood vessels, rat heart, and human uterine arteries were dissected free of fat and fixed in 10% formalin. Tissues were dehydrated in alcohol, embedded in paraffin, 3-μm sections were mounted on 3-aminopropyltriethoxysilane-

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(TESPA)-coated slides, and sections were dewaxed in xylene, alcohol, and PBS. Sections were treated with 0.2 *M* hydrochloric acid for 20 min, washed in 0.3% Triton X 100, incubated with proteinase K (40 μg/ml Tris EDTA buffer, pH 8) at 37°C for 30 min, and washed in 0.1 *M* triethanolamine with 0.5% acetic anhydride.

In situ hybridization

PCR products of human ET_A (600 bp) and rat ET_B receptor (800 bp) were subcloned into Bluescript SK (Stratagene, La Jolla, CA, U.S.A.) or pCRII (Invitrogen) plasmid vectors. Single-stranded digoxigenin (DIG)-labeled (Boehringer Mannheim, Germany) sense and anti-sense probes were synthesized using linearized templates and SP6, T3, and T7 RNA polymerases (Promega, Madison, WI, U.S.A.). Probe concentration and integrity were determined by dot-immunoblotting and formaldehyde gel electrophoresis. Sections were incubated with prehybridization (prehyb) solution (5 *M* NaCl, 1 *M* Tris, 50 m*M* Denhardt's, 250 m*M* EDTA, 10 mg/ml salmon sperm DNA, 50 mg/ml yeast tRNA) for 2 h at 50°C, then treated with sense or anti-sense cRNA diluted in 1:1 hybridization buffer (prehyb and 0.2 g/ml dextran sulfate) and deionized formamide overnight at 42°C. Sections were washed in 2× standard sodium citrate (SSC) buffer at 22°C and 60°C with 0.2× SSC/deionized formamide, then treated with RNase (50 μg/ml) for 30 min at 37°C, rinsed in maleic acid buffer, treated with 10% sheep serum at 22°C for 1 h, and then incubated with an anti-DIG alkaline phosphatase-conjugated antibody (diluted 1:2,000 in 1% sheep serum) for 2 h at 22°C. mRNA was detected using alkaline phosphatase substrate and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) diluted 1:40 in Tris-HCl/MgCl, pH 9.5. Sections treated with sense probes were counterstained with hematoxylin and mounted in aquamount (BDH, Poole, U.K.). Messenger RNA appeared black. Other tissue sections were treated with elastic van Gieson's stain for elastic and collagen.

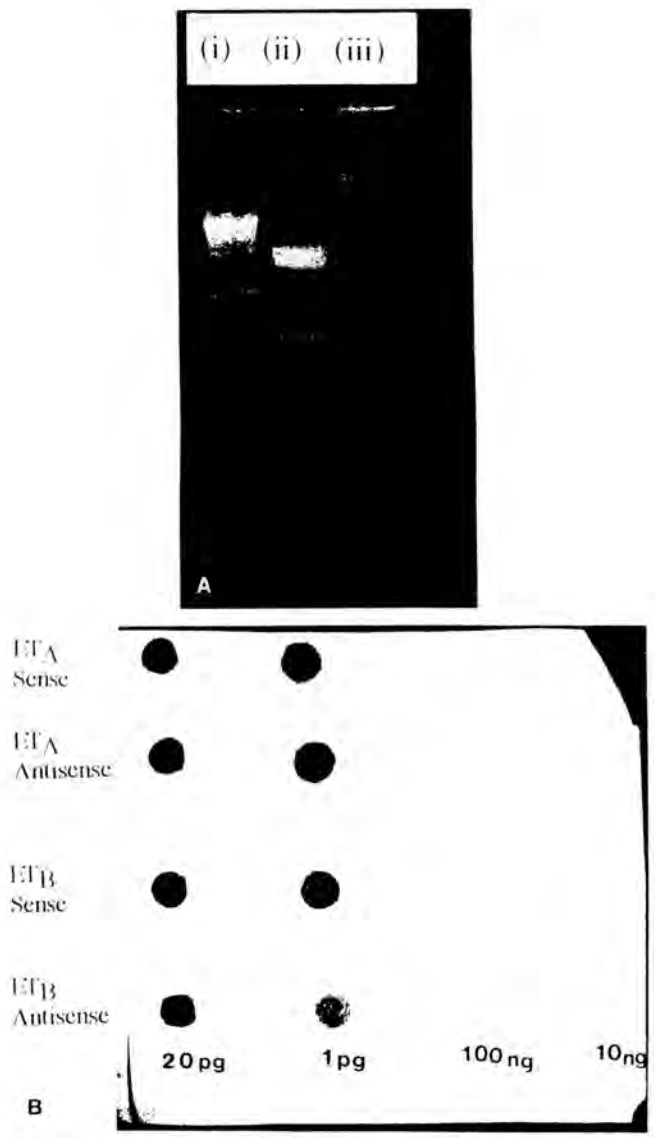

FIG. 1. A: A 1% agarose gel showing (Lane i) 1-kb DNA ladder, (Lane ii) rat ET_B receptor 800-bp sequence, and (Lane iii) human ET_A receptor 600-bp sequence cut from PCR II with ECoR I restriction enzyme. **B:** RNA dot-blot of human ET_A and rat ET_B receptor sense and anti-sense probes showing 20 pg, 1 pg, 100 ng, and 10 ng concentrations of DIG-labeled RNA.

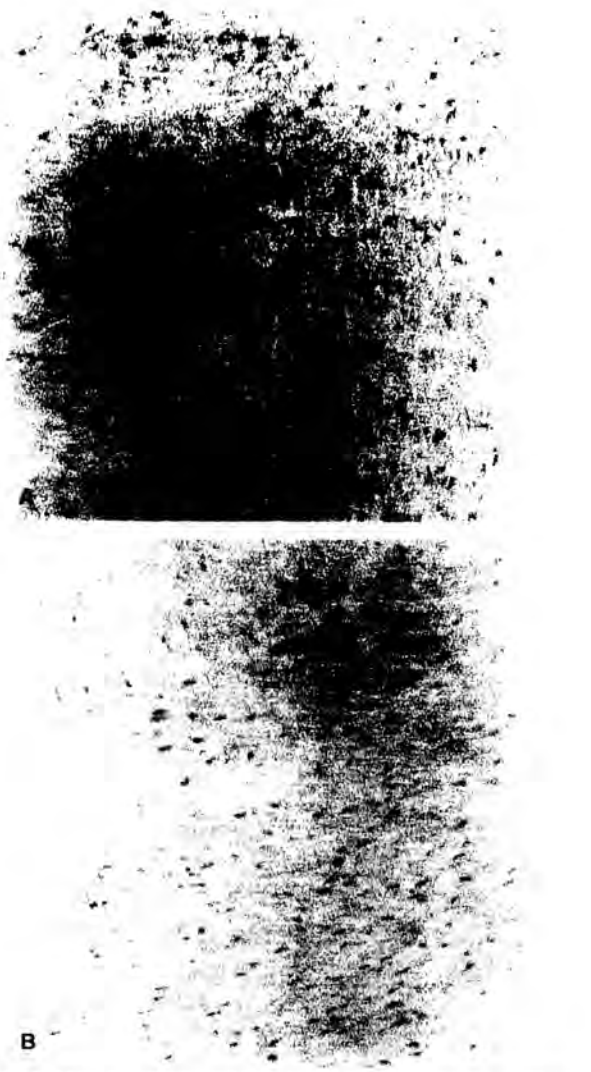

FIG. 2. A: Human uterine artery treated with anti-sense probe to ET_A receptors. ET_A receptor mRNA (*black*) is distributed in the arterial media (M). **B:** ET_A receptor sense control in human uterine artery, showing no evidence of ET_A receptor mRNA. The nuclei (*dark*) are counterstained with hematoxylin. ×240.

in cardiovascular diseases such as atherosclerosis and heart failure (2). Although the main source of ET-1 is presumed to be the endothelial cells, some evidence suggests that, in pathophysiologic states, cells other than the endothelium are able to produce ET (6). It is therefore, possible, that autocrine or paracrine ET systems exist in tissues of the cardiovascular system. Although a role for the ET receptors in disease is suggested by studies with ET receptor antagonists (7,8), little is known about the role of the ET receptors at the molecular level in disease, and this is an area of ET research that remains to be investigated. The use of DIG-labeled probes is ideal for assessing changes in expression of the components of the ET system in situ. Changes in receptor expression can be localized to particular cell types and correlated with morphologic and pathophysiologic changes in tissues.

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Functional Studies in Small Arteries Do Not Support a Primary Role for Endothelin in the Pathogenesis of Raynaud's Disease

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Summary: Endothelin-1 (ET-1) has been implicated in the pathogenesis of Raynaud's disease (RD). This study examined the effect of cooling on the response to ET-1 in human microvessels. Subcutaneous small arteries were dissected from gluteal fat biopsies taken from patients with RD ($n = 20$) and from age- and sex-matched control subjects ($n = 17$) and were cannulated in a small vessel arteriograph. Cumulative concentration–response curves to ET-1 (10^{-12} to 3×10^{-7} M) were obtained in vessels at 37°C and 24°C, with the endothelium either intact or removed ($n = 6$ per group). There were no significant differences in responses to ET-1 between RD patients and controls in either intact or denuded vessels, at either 37°C or at 24°C. There was, however, a significant endothelium-dependent interaction between the groups when the effect of temperature on the response to ET-1 was examined ($p = 0.01$; two-way ANOVA). Whereas cooling tended to reduce the sensitivity in RD, the opposite effect was observed in controls. Measurements of plasma ET-1 did not reveal any significant difference between patients with RD and healthy controls. These results suggest that ET-1 does not play a primary pathophysiologic role in RD. ET-1 might be responsible for mediating the prolonged vasospasm in RD, but secondary to another factor(s), such as impaired endothelium-dependent vasodilatation. **Key Words:** Raynaud's disease—Endothelium—Endothelin—Cooling—Small arteries—Human.

A temperature-dependent disorder of the endothelium involving overproduction of the vasoconstrictor endothelin-1 (ET-1) might be the critical factor underlying the pathogenesis of the prolonged cold-induced vasospasm of Raynaud's disease (RD). In 1990, Zamora and colleagues (1) performed cold provocation studies in RD patients and found an exaggerated increase in ET-1 concentration in venous blood draining the cold-challenged arm compared to the control arm or to responses in healthy control subjects. Basal ET-1 levels were also elevated in RD patients, a finding that has been supported by others (2). In addition, a rapid increase in plasma ET-1 during the cold-pressor test in healthy subjects has been reported (3). Although these results are consistent with a role for ET-1 in mediating the prolonged cold-induced vasospasm of RD, others have found that ET-1 concentrations do not increase during cold exposure in RD (4,5), creating some uncertainty as to whether or not this peptide has a primary role in RD. Of course, plasma concentrations of ET-1 are not necessarily a good predictor of its pathogenetic role in disease, because ET is preferentially secreted abluminally and is therefore unlikely to be acting as a circulating hormone, with elevated levels merely reflecting spillover from its release in the vasculature.

In this study we examined constrictor responses to ET-1 in subcutaneous small arteries isolated from gluteal fat biopsies taken from RD patients and from age- and sex-matched control subjects to investigate the pathogenetic role of this peptide in RD. We hypothesize that any defect present in the digital circulation will be manifest in arteries taken from the gluteal region because there is evidence to suggest that RD may be part of a generalized vasospastic disorder, in that there is a higher incidence of RD in patients with migraine and/or variant angina (6–8). The temperatures of 37°C and 24°C were chosen in the present study because they are directly comparable with many published reports and represent body temperature and moderate cooling, respectively.

MATERIALS AND METHODS

The protocol of this study was approved by the Lothian Research Ethics Committee and written, witnessed, informed consent was obtained from each subject. Seventeen control subjects (29–64 years; three men, 14 women) and 20 RD patients (28–62 years; three men, 17 women) were recruited in this study (Table 1). All subjects has an alcohol intake of <14 (women) and <21 (men) units per week and were normotensive [systolic blood pressure <140 mm Hg; diastolic blood pressure <90 mm Hg; mean arterial pressure 92 ± 2 mm Hg for controls

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TABLE 1. *Subject and patient details and baseline data for vessels studied at 37°C and 24°C*

	Control subjects		Raynaud's patients	
	37°C	24°C	37°C	24°C
Age (years)	41 ± 4	37 ± 3	48 ± 3	46 ± 3
Sex ratio (M:F)	1:8	2:6	2:9	1:8
Lumen diameter	348 ± 39	328 ± 21	321 ± 30	304 ± 20
% Contraction to KCl	65 ± 4	68 ± 3	65 ± 2	69 ± 2
% Relaxation to ACh	72 ± 7	65 ± 12	72 ± 11	81 ± 5

Values are mean ± SEM. Mean duration of RD was 18 ± 3 years. One patient had Raynaud's phenomenon associated with Sjögren's syndrome. Three control subjects and one RD patient were smokers. Except for one control subject and one RD patient who were taking hormone replacement therapy, none of the volunteers was receiving any medication at the time of the study and all were in general good health. Values represent $n = 12$ for lumen diameter and % contraction to KCl (intact and denuded vessels), and $n = 6$ for % relaxation to ACh (intact vessels only). Resting lumen diameter (μm); potassium chloride (KCl) (60 m*M*); acetylcholine (ACh) (10^{-6} *M*).

vs. 94 ± 2 mm Hg for RD patients ($p = 0.48$)]. Subjects abstained from aspirin-containing drugs for 1 week, and from caffeine-containing drinks or alcohol for 12 h before the biopsy was taken.

Skin biopsies approximately 2 cm long, 0.75 cm wide, and 0.75 cm deep were taken from the gluteal region under local anesthesia (9) (1% lignocaine; Astra Pharmaceuticals Ltd., U.K.) and were placed directly into physiologic salt solution (PSS) (m*M*: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose). Small arteries with a mean luminal diameter of 324 ± 13 μm ($n = 48$) were excised under a dissection microscope and cannulated in a perfusion myograph (Living Systems Instrumentation, Burlington, VT, U.S.A.). If two arteries were taken from the same biopsy, each was randomized to a different experimental group, i.e., 37°C or 24°C. A pressure servo unit maintained intraluminal pressure, without flow, at 50 mm Hg. Luminal diameter was measured using a video dimension analyzer. After a 60–90-min equilibration period during which the vessel chamber was superfused with PSS, continuously gassed with 95% O_2/5% CO_2, and the temperature raised to 37°C, the contractility of the microvessels was assessed separately using norepinephrine 10^{-5} *M* (NE; Sigma, U.K.) and potassium chloride 60 m*M* (KCl; Sigma). Acetylcholine 10^{-6} *M* (ACh; Sigma) was given during maximal contraction to NE to assess endothelial integrity. Removal of the endothelium was achieved by passing air through the lumen of the vessel and was confirmed by the loss of ACh-induced relaxation during constriction to NE. Cooling was achieved by passing the superfusate through a rapid heat-exchange Peltier element (Moor Instruments, Millwey, Axminster, Devon, U.K.) before it entered the arteriograph. Cumulative concentration–response curves to ET-1 (10^{-12} to 3×10^{-7} *M*; Novabiochem, U.K.) were generated in either intact or denuded vessels at 37°C or 24°C ($n = 6$ per group).

Plasma separated from whole blood samples was stored at −70°C until ET-1 levels were measured by radioimmunoassay. Immunoreactive ET was extracted from acidified plasma using 200 mg C18 preparative columns (Bond Elut; Phenomenex, U.K.). Extracted samples and duplicate standards (100-μl aliquots) were incubated with rabbit polyclonal antibody raised against ET-1 (100 μl; Peninsula Labs, U.K.). After incubation for 24 h at 4°C, [^{125}I]ET-1 (100 μl; Peninsula Labs) was added and incubated for a further 24 h at 4°C. Goat anti-rabbit γ-globulin (100 μl; Peninsula Labs) and normal rabbit serum (100 μl; Peninsula Labs) were then added and incubated at room temperature for 90 min. Immune complexes were precipitated and centrifuged at 4°C to remove the supernatant. The amount of radioactivity in the precipitate was determined by γ-counting for 1 min. The recovery of added ET-1 was 89%. The sensitivity of this assay is 0.5 pg/ml, and crossreactivity with ET-1, ET-2, ET-3, and big ET-1 is 100, 7, 7, and 10%, respectively.

Data analysis

Kruskall–Wallis nonparametric tests were performed to compare the responses between 37°C and 24°C groups and/or intact and denuded groups, because the variances between the groups differed significantly ($p < 0.05$; Bartlett's test). Two-way ANOVA was used to examine the influence of temperature and subject group on responses. Student's unpaired *t* test was used to compare plasma ET-1 levels between RD patients and controls. Statistical significance was accepted when $p < 0.05$.

RESULTS

Resistance artery studies

Mean resting lumen diameter, percent contraction to KCl, and percent relaxation to ACh did not differ significantly between the control subjects studied at 37°C and 24°C, and the RD patient group at 37°C and 24°C (Table 1). ET-1 produced a concentration-dependent constriction in all vessels. There were no significant changes in sensitivity or maximal contraction to ET-1 after cooling or denudation of vessels from control subjects or RD patients (Table 2). Figure 1 shows concentration–response curves to ET-1 in intact arteries from RD patients and controls at 37°C (Fig. 1A) and 24°C (Fig. 1B). Comparison of responses between control and RD arteries showed no significant difference in EC_{50} or E_{max} values ($p = 0.15$; Kruskall–Wallis test) (Table 2). However, in intact arteries there was a significant interaction between RD patients and control subjects when the effect of temperature on the sensitivity to ET-1 was examined ($p = 0.01$; two-way ANOVA). Vessels from RD patients showed a trend of reduced sensitivity to

TABLE 2. *EC_{50} and E_{max} values for endothelin-1 concentration–response curves in human resistance arteries obtained from gluteal biopsies*

	Control subjects		Raynaud's patients	
	37°C	24°C	37°C	24°C
EC_{50} intact	$4.1 \pm 1.0 \times 10^{-10}$ *M*	$1.9 \pm 0.4 \times 10^{-10}$ *M*	$2.1 \% 0.3 \times 10^{-10}$ *M*	$3.1 \pm 0.7 \times 10^{-10}$ *M*
EC_{50} denuded	$3.2 \pm 1.1 \times 10^{-10}$ *M*	$5.4 \pm 1.5 \times 10^{-10}$ *M*	$4.7 \% 1.3 \times 10^{-10}$ *M*	$4.2 \pm 1.1 \times 10^{-10}$ *M*
E_{max} intact	117 ± 10	122 ± 5	122 ± 4	124 ± 4
E_{max} denuded	124 ± 7	114 ± 3	117 ± 9	120 ± 4

ET-1 responses were expressed as a percentage of the contraction to potassium chloride (KCl) (60 m*M*) at 37°C. EC_{50} (the concentration producing half-maximal KCl contraction) and E_{max} (the maximal contraction relative to KCl) values are shown as mean ± SEM; $n = 6$ for each group. EC_{50} values were calculated from individual log concentration–response curves via Graphpad Prism 2.1.

ET-1 during cooling ($EC_{50} = 2.1 \pm 0.3 \times 10^{-10}$ *M* at 37°C vs. $3.1 \pm 0.7 \times 10^{-10}$ *M* at 24°C) in contrast to control vessels, in which cooling tended to increase sensitivity to ET-1 ($EC_{50} = 4.1 \pm 1.0 \times 10^{-10}$ *M* at 37°C vs. $1.9 \pm 0.4 \times 10^{-10}$ *M* at 24°C).

Plasma ET-1 levels

Plasma ET-1 levels were not significantly different between RD patients and control subjects (4.02 ± 0.20 pg/ml for controls vs. 4.47 ± 0.24 pg/ml for RD; $p = 0.18$; Student's unpaired *t* test) (Fig. 2).

DISCUSSION

In this study, using small arteries obtained from gluteal fat biopsies, we did not find a significant difference in the responsiveness to ET-1 between RD patients and control subjects, either at 37°C or at 24°C, in intact or denuded arteries. If ET-1 is implicated in the pathogenesis of RD, one might expect to find either an enhanced response to exogenous ET-1 compared to controls, owing to increased smooth muscle sensitivity, or a depressed response, secondary to smooth muscle desensitization through receptor downregulation if endogenous ET levels are increased during cooling in RD patients (see below). The fact that no significant difference was found implies that ET is not a primary candidate in the pathogenesis of RD, assuming that gluteal microvessels share the same responsiveness to ET-1 as those of the digital circulation.

Our present data reveal that, despite the lack of significant influence of temperature or disease on ET-1 responses, arteries from RD patients behave differently in response to cold stimulu than those from control subjects, as evidenced by the significant interaction between the groups. Whereas cooling tended to reduce the sensitivity to ET-1 in vessels from patients with RD, the opposite was observed in control arteries. This phenomenon was seen only in arteries with an intact endothelium, suggesting that endothelium-dependent mechanisms were responsible. Recent studies in our laboratory demonstrate that endothelium-dependent dilatation is significantly attenuated in arteries from RD patients at 37°C compared to 24°C (Smith, unpublished data). This may contribute to the small, nonsignificant trend of greater sensitivity to ET-1 at 37°C compared to 24°C in vessels from RD patients.

Vasospasm in RD may result from increased production and/or reduced metabolism of ET-1 as opposed to enhanced contractile responsiveness. There are conflicting reports of plasma ET-1 levels in RD: whereas some groups have shown increased basal levels in RD patients (1,2), others find no difference compared to the control group (4,5). We did not find elevated basal ET-1 levels in patients with RD compared to controls. Plasma levels

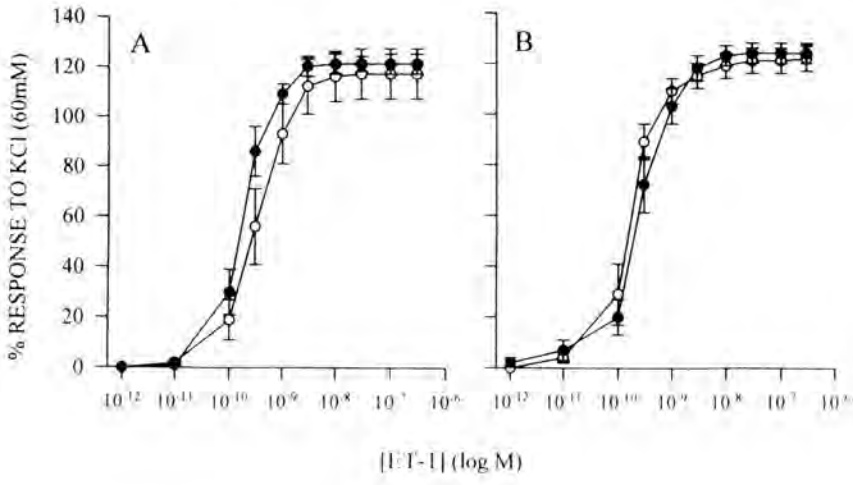

FIG. 1. Comparison of the contractile response to ET-1 in subcutaneous resistance arteries isolated from control subjects and RD patients: concentration–response curves to ET-1 (expressed as % response to KCl, 60 m*M*) at **(A)** 37°C and **(B)** at 24°C in intact vessels from control subjects (○; $n = 6$) and RD patients (●; $n = 6$). All values are mean ± SEM.

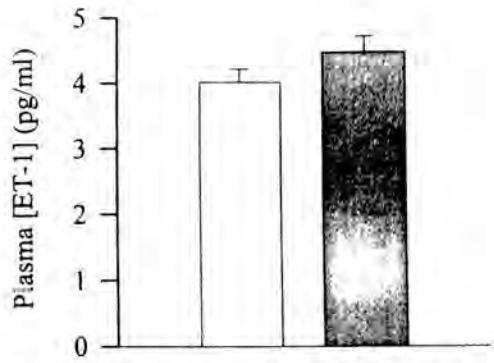

FIG. 2. Plasma endothelin concentrations (pg/ml) in healthy controls (*open column; n* = 15) and patients with Raynaud's disease (*shaded column; n* = 18).

are not necessarily a good predictor of pathogenetic roles because ET-1 is predominantly secreted abluminally and, in addition, differences between studies may be partly due to the number of vasospastic episodes experienced by the patient group before blood sampling, with raised ET-1 levels reflecting residual circulating peptide. Perhaps more informative data come from measurement of plasma ET-1 concentrations during cold challenge, although there is still a discrepancy in the results among published studies. A rapid increase in plasma ET-1 during the cold pressor test in healthy subjects has been reported (3), which is exaggerated in patients with RD (1). In contrast, others found no significant difference in cold-challenged plasma ET-1 levels in RD patients compared to controls (5).

To our knowledge, this is the first study to examine constrictor responses to ET-1 in isolated vessels from RD patients. Our results suggest that ET-1 does not play a *primary* pathophysiologic role in RD because a difference in responsiveness to ET-1 was not observed between vessels from RD patients and controls and because plasma levels of ET-1 did not differ between the groups. ET-1 might be responsible for mediating vasospasm in RD, the prolonged nature of which favors the involvement of a long-lasting constrictor such as ET-1, but it is likely to be *secondary* to other factors. Impaired endothelium-dependent vasodilatation could be such a factor.

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MEETING REPORT

Endothelin receptors cloned, endothelin converting enzyme characterized and pathophysiological roles for endothelin proposed

In 1988, Yanagisawa and colleagues[1] isolated from the culture medium of vascular endothelial cells a novel 21 amino-acid peptide, endothelin, which is the most potent vasopressor compound yet discovered[1–4]. A family of mammalian endothelin isopeptides (ET-1, ET-2 and ET-3; Fig. 1) has since been identified[5] of which only ET-1 is produced by endothelial cells, through formation of a 38 amino acid precursor, 'big ET-1'. This is subsequently processed by an unusual cleavage achieved by a putative 'ET-1 converting enzyme' (ECE).

Developments in endothelin research were brought up to date at a symposium held recently in Tsukuba*, home of the team responsible for discovery of the peptide. These included the further characterization of ECE, cloning of cDNA encoding two distinct endothelin receptors, and evidence supporting a role for endogenous ET-1 in health and disease.

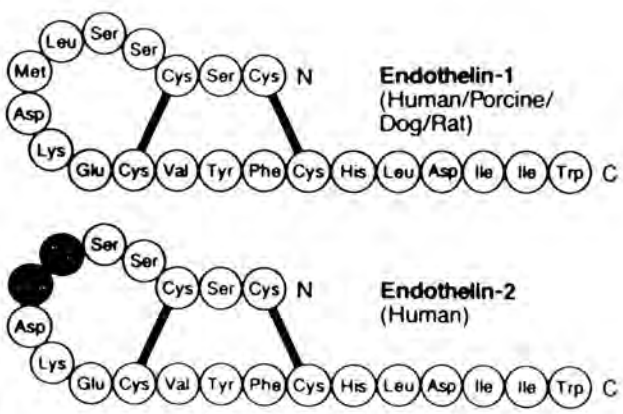
**Second International Conference on Endothelin, University of Tsukuba, Japan, 9–12 December 1990.*

Endothelin converting enzyme

In porcine aortic endothelial cells, the 39 amino acid intermediate, big ET-1, is hydrolysed at the Trp21–Val22 bond by the putative endothelin converting enzyme (ECE) to generate ET-1 and a C-terminal fragment. Although big ET-1 and ET-1 have similar pressor activity in whole animals, big ET-1 generates less than 1% of the contractile activity of ET-1 in isolated preparations (P. D'Orléans-Juste, William Harvey Research Institute). Hence, conversion of big ET-1 by ECE is essential for induction of physiological responses *in vivo*. There was widespread consensus at the meeting that physiological ECE activity resides with a novel metal-dependent neutral protease, and not (as had previously been suggested) with the cathepsin D-like protease which also degrades ET-1 but which is kinetically slow and active only at acid pH.

Membrane preparations derived from cultured vascular endothelial cells (Y. Matsumura, Osaka University of Pharmaceutical Sciences; M. Yano, Banyo Pharmaceutical Co., Tokyo) were shown to produce substantial conversion of big ET-1 to ET-1 at neutral pH. This effect was abolished by metal chelators (EDTA and 1,10-phenanthroline), and inhibited by the neutral metalloprotease inhibitor, phosphoramidon, but not by other protease inhibitors. Phosphoramidon also reduced secretion of ET-1 and increased secretion of big ET-1 by endothelial cells in culture, and reduced the conversion of big ET-1 to ET-1 by polymorphonuclear leukocytes (W. Sesse, William Harvey Research Institute). In anaesthetized ganglion-blocked rats, E. McMahon (Monsanto Co., St Louis) confirmed[6–8] that the pressor response to big ET-1, but not ET-1, is blocked by the metalloprotease inhibitor phosphoramidon (ID_{50} 5 mg kg^{-1}), but not by inhibitors of serine protease (leupeptin), thiol protease (E-64), or of other metalloproteases (captopril and kelatorphan). A further metalloprotease inhibitor, thiorphan, also blocked responses to big endothelin, but with much lower potency (ID_{50} 60 mg kg^{-1}).

Phosphoramidon was originally identified as a potent inhibitor of the bacterial metalloprotease thermolysin, although it also inhibits other metalloproteases, most notably neutral endopeptidase-24.11 (Ref. 9). However, phosphoramidon, and kelatorphan and thiorphan (also metalloprotease inhibitors) are essentially equipotent as inhibitors of endopeptidase-24.11 (Ref. 10) so that the lack of effect of kelatorphan on big ET-1 conversion suggests that ECE is a novel and distinct phosphoramidon-sensitive neutral metalloprotease. As circulating



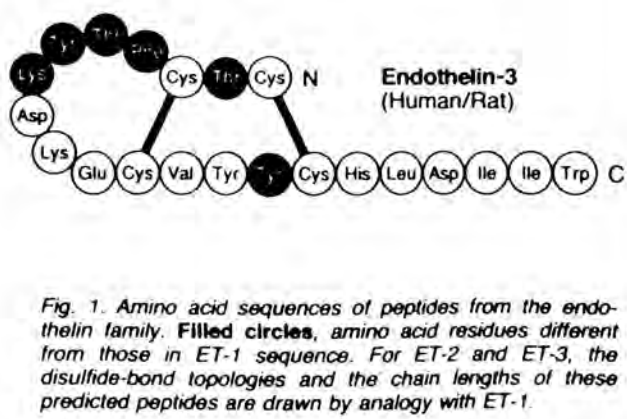


Fig. 1. Amino acid sequences of peptides from the endothelin family. **Filled circles**, *amino acid residues different from those in ET-1 sequence. For ET-2 and ET-3, the disulfide-bond topologies and the chain lengths of these predicted peptides are drawn by analogy with ET-1.*

blood does not appear to be a major site of big endothelin conversion (Y. Watanabe, Mitsubishiyuka Bioclinical Labs, Tokyo), it appears that the most likely candidate for conversion of circulating big ET-1 *in vivo* is the membrane-bound phosphoramidon-sensitive ECE. However, cytosolic preparations from endothelial cells also exhibit ECE activity, insensitive to phosphoramidon (Matsumura), and other ECEs may be responsible for intracellular processing of big ET-1.

McMahon described responses to intravenous administration of metalloprotease inhibitors in conscious rats. Infusion of phosphoramidon over four hours lowered mean arterial pressure in both Wistar–Kyoto and spontaneously hypertensive rats (SHR) compared with saline control infusion (see Fig. 2). Kelatorphan, at the same dose, had no effect on blood pressure, confirming that phosphoramidon is not acting on neutral endopeptidase-24.11. These findings suggest that endothelin may play a physiological role in blood pressure regulation and that, with the development of specific inhibitors, ECE may represent an important therapeutic target for pharmacological intervention in hypertension, and other vascular diseases.

Endothelin receptors

Many contributors presented additional support for the multiple endothelin receptor subtypes previously identified on the basis of agonist affinities (ET-1 > ET-3; ET-1 = ET-3; ET-3 > ET-1)[3,11]. In addition, direct evidence for at least two distinct receptors, using molecular biological techniques, was presented by two groups (H. Arai, Kyoto University Faculty of Medicine; T. Sakurai, University of Tsukuba). These findings have subsequently been published in *Nature*[12,13].

Arai and colleagues employed an expression cloning strategy. Using a bovine lung cDNA library they isolated a cDNA clone which encoded a receptor shown to be functional in their *Xenopus* oocyte assay system. The endothelin receptor sequence encoded by this clone consisted of 427 amino acid residues (48.5 kDa). After transfection of the cloned cDNA into monkey COS cells there was saturable binding of ^{125}I-labelled ET-1 to membranes prepared from these cells (K_d 0.18 nM). Displacement experiments showed ET-1 to be the most potent inhibitor of radioligand binding to this receptor (K_i 0.9 nM), with order of potency ET-1 > ET-2 >> ET-3 > sarafotoxin S6b.

Sakurai and colleagues, using a somewhat different expression cloning approach, also constructed a cDNA library from poly(A)$^+$ RNA prepared from rat lung, a tissue that is rich in endothelin binding sites. Sub-pools of cDNA were transfected into COS-7 cells, which have no detectable endothelin receptor, and screened for expression of endothelin binding sites by incubation with ^{125}I-labelled ET-1 followed by autoradiography. A single clone conferring ET 1 binding was isolated, and an encoded receptor sequence, consisting of 415 amino acid residues (46.9 kDa), was identified. Subsequent displacement studies indicated similar affinities for the endothelin isoforms (ET-1 = ET-2 = ET-3). This receptor was shown, in COS-7 cells, to be coupled through a G protein to phospholipase C with production of inositol phosphates and a transient increase in intracellular Ca^{2+}.

Fortuitously, the receptors isolated by the two groups are structurally and functionally distinct, though they share several features. Each contains seven membrane spanning domains, and exhibits significant sequence and topographical similarity with the photoreceptor rhodopsin and other G protein-coupled receptors. Both receptors also contain two potential *N*-glycosylation sites within the N-terminal region, and serine residues in the third cytoplasmic loop and the cytoplasmic C-terminal tail which may be phosphorylated by serine/threonine kinases and therefore are potential target sites for receptor regulation. Cloning of endothelin receptor cDNAs will facilitate investigation of endothelin receptor distribution, regulation and function, as well as their potential role in pathophysiological processes.

Northern blot analysis showed that mRNA for both receptors can be detected in a variety of tissues, including heart, lung, kidney and brain. An important difference, however, is that the ET-1-'selective' receptor is expressed in aorta, while the 'nonselective receptor' is not. Speaking on behalf of the Committee on Receptor Nomenclature and Drug Classification constituted by IUPHAR, P. Vanhoutte (Baylor College of Medicine) recommended that the ET-1-'selective' receptor described by Arai, and which may be the vascular smooth muscle receptor, be named the ET_A receptor, and the nonselective receptor described by Sakurai, be named the ET_B receptor. In the context of the confusion which might otherwise arise, an early agreement on the classification of endothelin receptors must be welcomed, although an alphabetical classification is a departure from the usual numerical systems (perhaps E_1). It is to be hoped that the identification of other receptors (including the ET 3-'selective' receptor) will soon follow. Definitive pharmacological confirmation of the subtypes of endothelin receptor, however, awaits the development of selective antagonists. None were described at the meeting, though it is clear that with the therapeutic potential of such agents, much energy is being channelled in this direction.

Endothelin production

Contributions described additional sites of ET-1 expression, and the interaction of ET-1 with EDRF/NO. B. Dardik (Ciba-Geigy Corp., Summit, USA) showed sustained release of endothelin-like immunoreactivity (ET-LI) by rat aortic smooth muscle cell in culture, and inhibition by cycloheximide indicates the involvement of *de novo* protein synthesis. ET-LI was also found in human monocytes and human tissue macrophages, and ET-1 mRNA was shown to be induced by phorbol ester in macrophage culture (H. Ehrenreich, NIH, Bethesda). Polymorphonuclear leukocytes (PMN) and lymphocytes do not generate ET-LI, but activated PMN appear to act indirectly to increase production and metabolism of ET-1 generated by endothelial cells (M. Yoshizumi, University of Tsukuba). In light of this, endothelin regulation at sites of inflammation is likely to be complex.

The pressor activity of ET-1 is strongly limited by release of

prostacyclin and EDRF/NO (Ref. 4). E. Bassenge (Freiburg University) examined the influence of EDRF/NO on endothelin release in cultured endothelial cells. The phosphodiesterase inhibitor IBMX and NO donator SIN-1 alone increased cGMP levels twofold, and together sevenfold, but did not affect ET-1 production or release. In addition, 8-bromo-cGMP did not suppress basal or thrombin-stimulated endothelin release. These findings indicate that EDRF/NO does not exert feedback inhibition of endothelin release.

Interestingly, cerebral microvessel endothelial cells grown on filters were shown by S. Yoshimoto (University of Tokyo) to release ET-1, mainly across the 'basal membrane', suggesting that endothelin release may be directed towards vascular smooth muscle and away from the vascular lumen.

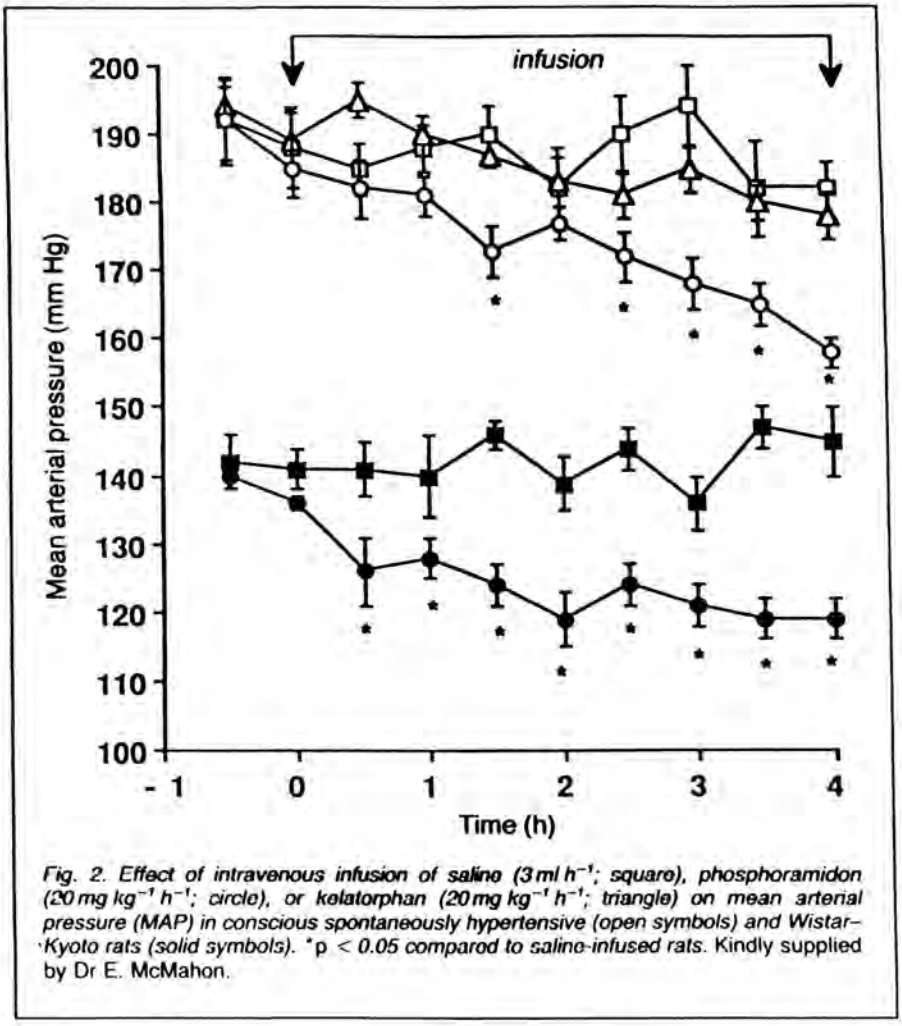


*Fig. 2. Effect of intravenous infusion of saline (3 ml h^{-1}; square), phosphoramidon (20 mg kg^{-1} h^{-1}; circle), or kelatorphan (20 mg kg^{-1} h^{-1}; triangle) on mean arterial pressure (MAP) in conscious spontaneously hypertensive (open symbols) and Wistar–Kyoto rats (solid symbols). *p < 0.05 compared to saline-infused rats.* Kindly supplied by Dr E. McMahon.

Physiology and pathophysiology

Evidence for modulation of adrenergic and cholinergic neuroeffector transmission by ET-1 was presented by P. Wiklund (Karolinska Institute). Localization of very dense endothelin binding in the carotid body, in the nodose and superior cervical ganglia, and in nucleus of the tractus solitarius, where chemoreceptor afferents terminate, all suggest involvement of endothelin in the arterial chemoreceptor reflex (D. McQueen, University of Edinburgh).

Low concentrations of ET-1 were shown by H. Lippton (Louisiana State University) to dilate pulmonary vessels through activation of ATP-sensitive K^+ channels. During pulmonary alveolar hypoxia, ET-LI in rat lung is increased (G. Shirakami, Kyoto University School of Medicine) and with prolonged hypoxia, constrictor sensitivity to ET-1 increases and dilatation is lost (S. Adnot, Institut Henri Beaufour, Les Ulis). These findings raise the possibility that ET-1 may be involved in sustaining chronic hypoxic pulmonary hypertension. ET-LI was also found by Ehrenreich in samples at bronchopulmonary lavage, and to correlate with disease activity in patients with Wegener's granulomatosis.

Elevated levels of plasma ET-1 were found in patients with myocardial infarction, vasospastic angina, cardiogenic and septic shock, sub-arachnoid haemorrhage, renal failure and a variety of connective tissue disorders. There remains some controversy over whether plasma ET-1 is increased in hypertension or diabetes, and in this regard the importance of adequate numbers of subjects and appropriate controls is crucial. J. Lundberg (Karolinska Institute, Stockholm) described elevated levels of ET-LI in umbilical arterial plasma (15 pM, increasing to 94 pM after establishment of fetal breathing), compared with maternal plasma (2 pM), and ET-1 immunostaining is heavy in umbilical vessels (J. Wharton, Hammersmith Hospital), raising the interesting possibility that endothelin may be responsible for umbilical cord closure.

Perhaps the most compelling evidence in support of a pathophysiological role for ET-1 was described by K. Yokokawa (Osaka City University Medical School). He reported two cases of haemangio-endothelioma of the scalp, a rare malignant tumour, associated with hypertension and elevated plasma ET-1 levels. Blood pressure and plasma ET-1 fell in both cases after surgical excision of the tumour and, in one patient, recurrence was associated with redevelopment of hypertension and raised endothelin levels. In one case tumour content of ET-1 and ET-1 mRNA was shown to be higher than in normal skin. Plasma endothelin levels in these patients were 15–20-fold higher than in normal subjects which, considering the rapid clearance of ET-1 from the circulation[14] suggests profound secretion of ET-1 by the tumour. Selective venous sampling would have strengthened the argument.

□ □ □

There are now many indications that the endothelins are involved as local factors in regulation of the cardiovascular system, and evidence to suggest that ET-1 may

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Editorial Review

The endothelin family of peptides: local hormones with diverse roles in health and disease?

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INTRODUCTION

The vascular endothelium forms the inner lining of all blood vessels and acts, not simply as a barrier between blood and vascular smooth muscle, but rather as a modulator of vascular function. The endothelium is now known to play a key role in the regulation of coagulation, lipid transport, immunological reactivity and vascular tone. Over the last 20 years it has become clear that a number of important vasodilator and constrictor substances are produced by endothelial cells [1]. The first identified was prostacyclin, which acts both as a potent vasodilator and as an inhibitor of platelet aggregation [2]. In 1980, Furchgott & Zawadzki [3] demonstrated the presence of a second, locally acting, non-prostaglandin, endothelium-dependent vasodilator factor that was named endothelium-derived relaxing factor (EDRF) and which has since been identified as nitric oxide [4].

The isolation of EDRFs prompted a search for counterbalancing constricting factors (or EDCFs). The pulmonary vascular conversion of angiotensin I to angiotensin II was already known [5], and endothelial angiotensin-converting enzyme (ACE) activity has since been demonstrated in most blood vessels [6]. By 1985, the vascular endothelium had been shown to generate at least two other vasoconstrictor substances, one of which produced prolonged vasoconstriction lasting more than 60 min [7, 8], in contrast to the brief actions of other mediators of vascular tone. This long-acting agent, which appeared to be a peptide, was isolated and sequenced in 1988 by Yanagisawa et al. [9] from endothelial cell cultures, and called endothelin. It is a 21-amino acid peptide of unusual structure, and is the most potent vasoconstrictor agent yet identified. Its long-lasting vasoconstrictor and pressor actions have since been confirmed in human subjects [10, 11], and there is now increasing evidence to suggest that endothelin may be important in cardiovascular regulation [1, 12, 13]. In this article we review the physiology of endothelin, with particular emphasis on its putative role in cardiovascular, renal and respiratory disease. For more detailed review of the intracellular and neuroendocrine actions of the endothelins, see elsewhere [14, 15].

THE ENDOTHELIN FAMILY

The endothelins are a family of three related peptides [16], each of 21 amino acids (Fig. 1). Each isoform contains two intra-chain disulphide bridges linking paired cysteine amino acid residues, and producing an unusual semi-conical structure. Endothelin-2 exhibits the closest structural similarity to endothelin-1, differing by only two amino acid residues, while endothelin-3 differs by six amino acids. The disulphide bridges and C-terminal domain appear to be necessary for the actions of the peptide as their removal leads to substantial loss of biological activity [17, 18]. Endothelin-1, the peptide originally identified by Yanagisawa et al. [9], and endothelin-2 are the more potent vasoconstrictors [16].

In their original description, Yanagisawa et al. [9] noted similarities in structure between endothelin and several peptide neurotoxins, such as the bee venom, apamin, and α-scorpion toxin, both of which contain multiple disulphide bridges. Endothelin was subsequently found to have a close structural similarity (Fig. 1) to sarafotoxin S6b, a snake venom toxin from *Actractaspis engaddensis*, the Israeli burrowing asp [19], which causes death from myocardial ischaemia and infarction through development of coronary vasoconstriction [20, 21].

GENERATION

Regulation of production

Yanagisawa et al. [9] demonstrated that endothelin-1 mRNA may be induced in endothelial cells through exposure to adrenaline, thrombin and the Ca^{2+} ionophore A23187. Adrenaline-stimulated endothelin-1 production appears to be mediated through α_1-adrenoceptors, because it is inhibited by

Key words: blood pressure, blood vessel, endothelium, peptide, mitogen, vascoconstrictor.

Abbreviations: ACE, angiotensin-converting enzyme; ADH, antidiuretic hormone; ANP, atrial natriuretic peptide; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; ECE, endothelin-converting enzyme; EDRF, endothelium-derived relaxing factor; GFR, glomerular filtration rate; SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats.

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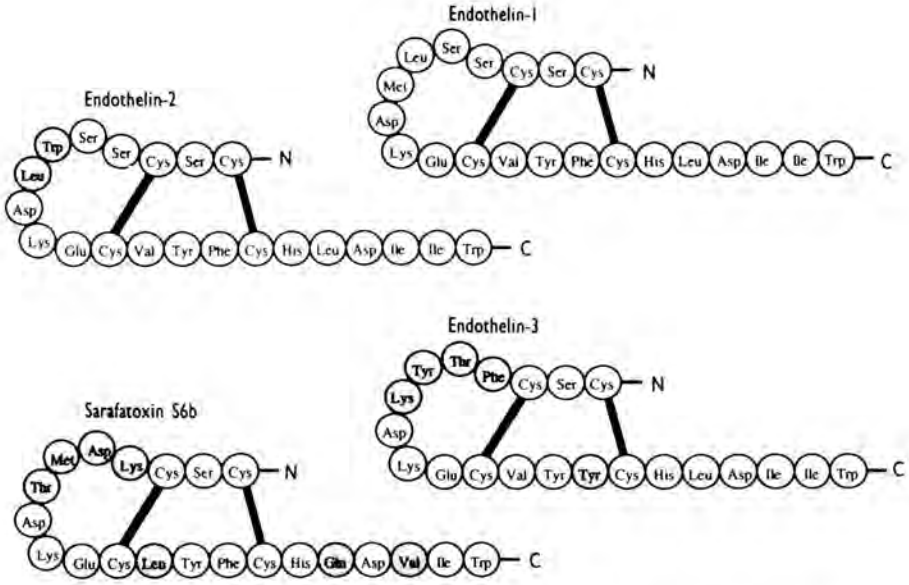

Fig. 1. Amino acid sequences of the three members of the endothelin family, and of the structurally related snake venom toxin sarafotoxin S6b. Shaded circles indicate where amino acids differ from endothelin-1.

the selective α_1-adrenoceptor antagonist prazosin, but not by the selective α_2-adrenoceptor antagonist yohimbine [22] or the β-adrenoceptor antagonist propranolol [23]. Endothelin-1 production, on the basis of induction of mRNA or elevated immunoreactive endothelin-1 concentrations, is also stimulated by other vasoactive hormones, including angiotensin II and arginine vasopressin [24, 25], by the isoform endothelin-3 [26], by cytokines and growth factors, including transforming growth factor β [27] and interleukin-1 [28], by physical stimuli, including hypoxia [29, 30] and shear stress [31], and by exposure to free radicals [22] and endotoxin [32]. Interestingly, glucocorticoids increase production of endothelin-1 by cultured vascular smooth muscle by up to 500% but have no effect on endothelial cell endothelin-1 production [33].

Production of endothelin-1 is inhibited by endothelium-derived nitric oxide [34] and by the nitrovasodilators, which donate nitric oxide [35]. It is interesting to speculate that decreased generation of nitric oxide may account for the stimulatory effects of glucocorticoids on vascular smooth muscle cell endothelin production, since these agents are known to inhibit nitric oxide synthase in vascular smooth muscle cells [36]. Atrial natriuretic peptide (ANP), which, like nitric oxide, acts by increasing intracellular cyclic GMP concentrations, also inhibits generation of endothelin-1 [35] at concentrations that are similar to those found in patients with chronic heart failure [37]. A key role for cyclic GMP in the control of endothelin-1 production is supported by the fact that 8-bromo-cyclic GMP, a cyclic GMP mimetic, inhibits production of endothelin-1, whereas a guanylate cyclase inhibitor, Methylene Blue, potentiates it [34].

Molecular genetics

Each member of the endothelin family is represented by a separate gene that encodes a specific precursor for the mature isoform [16]. The human gene for preproendothelin-1, the precursor of endothelin-1, is located on chromosome 6 [38], whereas that for preproendothelin-3 [39] is on chromosome 20. There are several regulatory DNA sequences in the non-transcribed regions of the gene [40]. At the 5' flanking region there are binding sites for nuclear factor 1 and acute-phase reactant regulatory elements, which may mediate the induction of mRNA for endothelin-1 by transforming growth factor β and acute physiological stress, respectively (Fig. 2).

The 5' region also contains a base pair sequence (-109 to -102) that is identical with the well-characterized AP-1 site, which is known to mediate hormone and growth factor responsiveness for other eukaryotic genes [41, 42]. This AP-1 site is necessary for transcription of the preproendothelin-1 gene, and is sensitive to the tumour promoter 12-*O*-tetradecanoyl-phorbol 13-acetate, which acts to increase intracellular inositol trisphosphate and Ca^{2+} concentrations. This process activates protein kinase C, and induces expression of the proto-oncogenes c-*fos* and c-*jun*. The products of these genes then bind to the AP-1 site to stimulate preproendothelin-1 gene expression [40]. Angiotensin II and arginine vasopressin activate protein kinase C to induce endothelin gene expression via the AP-1 site [25]. In addition, this site probably contributes to the tissue specificity of preproendothelin-1 gene transcription, as a result of endothelial cell-specific expression of proteins that bind to the AP-1 site [41, 42].

The 3′ flanking region of the gene contains a region which codes for poly AUUUA mRNA sequences, similar to those of transiently expressed cytokines and growth factors [40]. These AU-rich sequences may mediate selective destabilization of preproendothelin-1 mRNA, accounting for its relatively short half-life of 15 min [13].

Sites of production

Immunoreactive endothelin-1, or expression of mRNA for preproendothelin-1, can be detected in a wide variety of tissues, including blood vessels [34], heart, lung, pancreas, spleen [39], kidney [43], posterior pituitary [44] and cerebral neurons [45]. In the vasculature, endothelin-1 is the major isoform expressed within endothelial cells [39, 40], and so is probably the most important for local regulation of vascular tone. Vascular smooth muscle cells also produce endothelin-1 *in vitro*, although the rate of production is about 100-fold less than by endothelial cells [33, 46]. Despite the lower level of production, the relatively greater mass of vascular smooth muscle cells in larger blood vessels suggests that smooth muscle may here contribute substantially to local endothelin-1 production. Although endothelin-2 immunoreactivity cannot be detected in human plasma [47], immunostaining of vascular endothelial cells, using highly selective antisera to proendothelin-2, can demonstrate the presence of this isoform of endothelin [48]. This raises the possibility that local production of endothelin-2 may play a role in control of vascular tone. Immunoreactivity to endothelin-3, or expression of mRNA for proendothelin-3, can be detected in the central nervous system, anterior pituitary, lung, pancreas and spleen, but not in endothelial cells or cardiac tissue [39, 45, 48, 49].

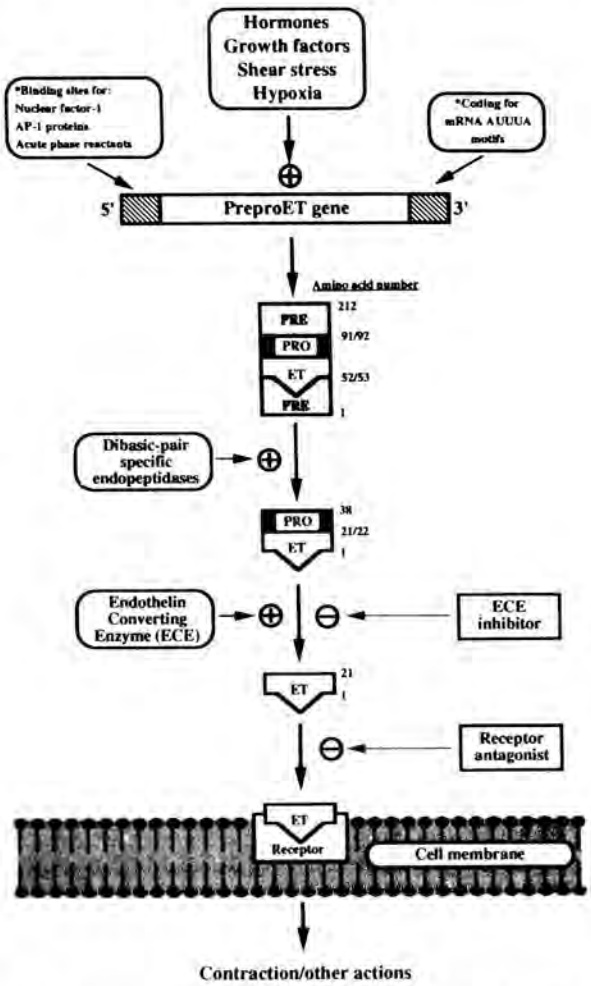

Fig. 2. Synthetic pathway for production of the endothelins (ET). The major potential sites for therapeutic modulation are shown on the right. *See text for details of the regulatory elements of the endothelin-1 gene. Abbreviation: preproET, preproendothelin.

Biosynthesis

Human preproendothelin-1 consists of 212 amino acids, containing a characteristic hydrophobic secretory sequence at the N-terminal, suggesting that the prepro-form is transported across the nuclear membrane for further processing. Preproendothelin-1 is cleaved (Fig. 2) at Lys-52–Arg-53 and at Lys-91–Arg-92 by dibasic-pair specific endoproteases to form proendothelin-1, a 38-amino acid precursor peptide [9]. Since the bathing medium of cultured vascular endothelial cells contains proendothelin-1 as well as endothelin-1 [50], and since proendothelin-1 is found in plasma at higher concentrations than mature endothelin-1 [47], it appears that proendothelin-1 is secreted by endothelial cells before conversion to endothelin-1. Nevertheless, mature endothelin-1 has been detected within the cytoplasm of human vascular endothelial cells [48], suggesting that some conversion occurs within the cell.

The generation of endothelin-1 from proendothelin-1 occurs through an unusual enzymic cleavage, at the Trp-21–Val-22 site, by the action of an 'endothelin-converting enzyme' (ECE) [9]. Three fractions of endothelial cells have been shown to display ECE activity: two intracellular and one membrane-bound. One of the intracellular ECE fractions is an aspartic protease, most active at pH 3.5 and with the immunoreactivity of cathepsin D [51]. This enzyme is, however, unlikely to account for endothelial cell production of endothelin-1, as it cleaves proendothelin-1 at Asn-18–Ile-19 as well as at Trp-21–Val-22 [52], and pepstatin, an inhibitor of cathepsin D, does not affect endothelial cell production of endothelin-1 [53]. The second intracellular ECE fraction is an intracellular metalloendopeptidase, probably sensitive to phosphoramidon [13]. This enzyme may be responsible for the intracellular processing of proendothelin-1, particularly in tissues, such as the central nervous system, where proendothelin-1 is not released from cells [13].

The physiologically most important ECE appears

to be membrane-bound [54], active at neutral pH, sensitive to metal ions and inhibited by phosphoramidon [55]. This neutral metalloendopeptidase does not circulate in blood [56] and is different from ACE [57, 58]. It has been suggested that ECEs display isoform specificity, with endothelial ECE converting proendothelin-1 ten times more readily than proendothelin-3 [59]. However, because phosphoramidon completely inhibits the functional effects of proendothelin-2 and proendothelin-3 in the rat [60], the enzymes that convert the precursors of the other endothelin isoforms may have similar properties to the endothelial ECE described above. Endothelial ECE is not the same enzyme as neutral endopeptidase-24.11, which degrades a number of peptides, including ANP, and is also inhibited by phosphoramidon, since ECE activity is not affected by the potent neutral endopeptidase inhibitors thiorphan or kelatorphan [13, 57]. Recently, SQ 28,603, a further neutral endopeptidase-24.11 inhibitor, has been shown to block the functional effects of proendothelin-1 in the rat, as well as to potentiate the effects of ANP, implying that this compound is also an inhibitor of ECE [61]. There are qualitative differences between the cardiovascular responses to proendothelin-1 after ECE inhibition with SQ 28,603 and phosphoramidon, raising the possibility that several different ECEs may be involved [61]. In addition, there appear to be regional variations in ECE activity, because, at doses with similar systemic pressor effects, proendothelin-1 and endothelin-1 differ in their effects *in vivo* on some vascular beds, such as the kidney [58] and mesenteric arcade [62].

Release

Like prostacyclin and nitric oxide, endothelin-1 does not seem to be stored intracellularly in most tissues. Generation *de novo* was first suggested by Yanagisawa et al. [9], using as evidence the fact that production of endothelin-1 by cultured endothelial cells cannot be detected until at least 30 min after stimulation with thrombin. In addition, the protein synthesis inhibitor cycloheximide can prevent release of endothelin by cultured vascular endothelial cells [63] and intact aortic strips [34]. However, immunoreactive endothelin-1 concentrations can increase in response to certain stimuli more rapidly than would be expected if endothelin-1 were always synthesized *de novo*. These stimuli include shear stress on rabbit cultured endothelial cells [31], upright tilt in patients with vasovagal syncope [64] and a cold pressor test on human subjects, with a sevenfold increase in endothelin-1 concentrations within 2 min [65]. Moreover, endothelin-1 immunoreactivity can be detected in granular form within the posterior pituitary [44], where the granules are depleted by water deprivation.

Plasma concentrations

Circulating concentrations of endothelin-like immunoreactivity in venous plasma have been reported to vary between 0.25 and 20 pg/ml [1, 66], and are very dependent upon assay conditions [66]. This immunoreactivity comprises proendothelin-1 (65%), endothelin-1 (25%), and endothelin-3 (10%) [47, 67]. The source of endothelin-3 is unknown, although it may be either neural or endocrine in origin. Endothelin-2 has not been detected in human plasma. Circulating concentrations of endothelin-1 are approximately tenfold lower than those which cause vascular contraction *in vitro* [9] or *in vivo* [68], although concentrations at the interface between endothelial cell and vascular smooth muscle are likely to be much higher. The presence of endothelin in lymphatic fluid suggests bidirectional release of the peptide, particularly as systemically infused endothelin does not appear in lymph [69]. In addition, cultured endothelial cells release substantially more endothelin to the basement membrane side (i.e. abluminally) than luminally [70]. Thus, endothelin-1 appears to be primarily a locally acting paracrine hormone rather than a circulating endocrine hormone. However, the level of endothelin-1 in the venous plasma may be useful as a marker for endothelial synthesis of the peptide, as it is likely to reflect overspill from local production. Additionally, in certain pathophysiological circumstances, such as renal failure (see below), circulating endothelin-1 concentrations may be sufficient to exert biological effects [68, 71].

Clearance

Radiolabelled endothelin-1 is rapidly cleared from the circulation after bolus injection into animals, with a half-life of less than 1 min. High tissue uptake by the lungs and kidneys reflects the large number of high-affinity binding sites in these organs [72]. In these experiments, over 90% of radioactivity was concentrated in the cell membrane/internal organelle fraction, suggesting that the clearance of endothelin-1 is by binding and then internalization. Plasma endothelin-1 concentrations vary inversely with renal function, suggesting either increased production or reduced clearance of endothelin-1 by the diseased kidney [73, 74]. Prolonged plasma and biological half-lives of endothelin-1 in bilateral nephrectomized rats suggest that the major effect of renal disease is through impaired clearance [75]. Enzymic degradation of endothelin by neutral endopeptidase-24.11 also occurs [76], although the biological significance of this action is unclear, because inhibitors such as thiorphan do not potentiate the systemic effects of exogenous endothelin-1 [58]. In humans, intravenously administered endothelin-1 has a calculated half-life of less than 3.6 min, with clearance by both the splanchnic and

renal vascular beds [77]. However, the cardiovascular effects persist for considerably longer [11, 68].

SIGNAL TRANSDUCTION

Binding sites and receptors

There are specific high-affinity binding sites for the endothelins [78] in blood vessels, heart, adrenal glands, kidneys and brain, their distribution lending support to a role in cardiovascular regulation [79, 80]. Dissociation of endothelin from these sites is extremely slow [81], and endothelin–receptor complexes are rapidly internalized into vascular smooth muscle cells [82]. Persistence of binding may mediate the uniquely prolonged contractile effects of endothelin although indirect evidence suggests that these effects may be due to an, as yet unidentified, late intracellular signalling event [83]. Differing functional responses to endothelin isopeptides and evidence from radiolabelled endothelin binding experiments suggest the existence of at least three receptor types for the endothelins [16, 84, 85, 86]. One type seems to bind endothelin-1 preferentially, the second is non-selective between endothelin-1 and -3, while the third binds endothelin-3 preferentially; two of these receptors have been isolated by expression of cloned cDNA *in vitro*.

One receptor (ET_A) was first isolated from a bovine lung cDNA library, and has a high affinity for endothelin-1 (binding potency: endothelin-1 > endothelin-2 ≫ endothelin-3), with a K_D of 0.2 nmol/l [87]. Recently, the human ET_A receptor has been cloned and localized to chromosome 4 [88, 89]; this consists of 427 amino acids and has 94% sequence identity with the bovine receptor. ET_A receptor mRNA is expressed in many human tissues, with the highest expression in the aorta, heart, lung and kidney. No mRNA can be detected in the liver or endothelial cells, suggesting selective vascular expression of this receptor in smooth muscle cells [88]. A cyclic pentapeptide (BE-18257B), which is a selective antagonist at the ET_A receptor, has been isolated from *Streptomyces misakiensis* [90]. BE-18257B displaces endothelin-1 but not endothelin-3 from binding sites in vascular smooth muscle, and antagonizes the vasoconstrictor effects *in vitro* and the pressor effects *in vivo* of endothelin-1 [9]. Other, more potent, ET_A antagonists have since been isolated, including BQ-123 [91], FR 139317 [92] and CGS 26343A [93].

A second endothelin receptor (ET_B) was first cloned from a rat cDNA library [94], having equal affinity for all three endothelins (binding potency: endothelin-1 = endothelin-2 = endothelin-3). This ET_B receptor has also been cloned from human DNA, having 442 amino acids [95, 96] and 88% sequence identity with the rat ET_B receptor, but only 55% sequence identity with the human ET_A receptor. ET_B receptor mRNA is expressed in human tissues but, in contrast to the ET_A receptor, largely in cerebral cortex and cerebellum, with moderate expression in lung, kidney, adrenal, aorta and cultured vascular endothelial cells [96].

The deduced structure for both ET_A and ET_B receptors has much in common with the superfamily of G-protein-coupled receptors, having seven hydrophobic membrane-spanning domains, and a relatively long extracellular N-terminal, although there is only a 25% sequence identity with other peptide receptors. The third postulated endothelin receptor type, having greater affinity for endothelin-3 than for the other isoforms [85, 86], has not yet been cloned. Endothelin receptor number, as measured by binding of radiolabelled endothelin-1, is regulated by a variety of factors. Ischaemia [97] and cyclosporin A [98] appear to increase the number of endothelin receptors, whereas elevated endothelin-1 concentrations [99], angiotensin II and phorbol esters [100] decrease receptor number.

Intracellular events

Endothelin-1 exerts its actions through a complex series of intracellular events that include phospholipase C activation, increased gene transcription and interactions with membrane ion channels. An increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is the final common event thought to mediate the vasoconstrictor and other actions elicited by the endothelins. After binding to receptors, endothelin-1 activates phospholipase C via a pertussis toxin-insensitive G-protein [101]. This causes a rapid increase (within 30 s) in the intracellular concentration of water-soluble inositol trisphosphate [102], which releases Ca^{2+} from intracellular stores [103]. Phospholipase C activation also increases membrane diacylglycerol [102], thus activating protein kinase C [104].

$[Ca^{2+}]_i$ is increased in a biphasic manner by endothelin-1 [81, 101, 105]. Initially there is a rapid peak in $[Ca^{2+}]_i$ with concentrations elevated up to ten-fold above normal [81], due to inositol trisphosphate-mediated release of Ca^{2+} from intracellular stores. $[Ca^{2+}]_i$ then falls to a plateau concentration approximately double the resting concentration. The plateau phase can be sustained for up to 20 min [105], but does not occur in the absence of extracellular Ca^{2+} [81], suggesting that entry of extracellular Ca^{2+} is responsible. The rapid rise in $[Ca^{2+}]_i$ appears to occur only at high endothelin-1 concentrations (> nanomolar), whereas the sustained increase can occur alone at lower concentrations [106].

Endothelin-1 can also induce vasoconstriction of Ca^{2+}-depleted tissues kept in a Ca^{2+}-free medium. This is abolished by the protein kinase C inhibitor H-7 [107], suggesting that protein kinase C activation may account in part for the contractile activity of the endothelins, by sensitizing the contractile apparatus of the cell to Ca^{2+}, as described for noradrenaline [108]. Protein kinase C-mediated

sensitization of the contractile apparatus may occur by activation of the Na^+/H^+ antiporter, because endothelin-1 causes intracellular alkalinization [105, 109], thus sensitizing contractile myofilaments to Ca^{2+}. This suggestion is supported by inhibition of endothelin-1 induced alkalinization by protein kinase C inhibitors [110]. In addition, activation of protein kinase C by endothelin-1 increases the transcription of growth-promoting genes [105].

In view of the characteristically prolonged vasoconstrictor effects of endothelin-1, much interest has focused on the mechanisms underlying the sustained rise in $[Ca^{2+}]_i$. It was initially suggested that endothelin-1 might be an endogenous agonist of the dihydropyridine-sensitive voltage-operated Ca^{2+} channel [9, 111], because endothelin-1-induced contractions were dependent on extracellular Ca^{2+} and antagonized by dihydropyridine Ca^{2+} antagonists. However, it has since been shown that the effect of dihydropyridine Ca^{2+} antagonists on endothelin-induced contraction *in vitro* is non-competitive [112], and that contractions in response to endothelin can still develop, although to a lesser extent, in the absence of extracellular Ca^{2+} [113]. It is now thought unlikely that endothelin-1 is an endogenous agonist of the dihydropyridine-sensitive voltage-operated Ca^{2+} channel, although it may act to open these channels indirectly. There is, however, increasing evidence that endothelin-1 may interact with ATP-sensitive K^+ channels [114–117].

ACTIONS OF THE ENDOTHELINS

Vascular effects

Endothelin-1 causes long-lasting contraction of large arteries, and in the isolated porcine coronary artery has a potency 10-fold higher than any other known constrictor substance [9]. Endothelin-2 and endothelin-3 also constrict isolated large arteries, although endothelin-3 is less potent than the other isoforms [16]. All endothelins cause transient endothelium-dependent vasodilatation before the development of constriction (see below) [118]. Resistance vessels are very sensitive to the effects of endothelin-1, as indicated by increases in perfusion pressure of the isolated rat perfused mesenteric bed [17, 119], but veins may be even more sensitive [118, 120]. In man, brachial artery infusion of endothelin-1 leads to dose-dependent vasoconstriction of the forearm resistance bed, of slow onset and prolonged duration, lasting more than 2 h after infusion is stopped [10, 121]. Endothelin-1 also causes venoconstriction in humans, with characteristics similar to those in arteries [116].

The endothelins are potent pressor agents *in vivo* in animals, with endothelin-3 evoking the least response [9, 16]. The effect lasts for more than 60 min after bolus injection, and is preceded by transient hypotension, lasting a few minutes, which is most marked for endothelin-3 [16]. This hypotension may reflect a pharmacological, rather than physiological, response to the briefly sustained high concentrations of endothelins that occur after bolus administration [122]. Under more physiological conditions, in which endothelin concentrations rise more slowly, such as occur after administration of proendothelins, hypotension does not occur [123]. Nevertheless, the hypotensive response to bolus administration may be useful in demonstrating the endothelial actions of the endothelins (see below). Coronary and renal vascular beds are more sensitive to systemic endothelin-1 than the splanchnic and hindquarters beds, which may vasodilate at low doses [124–126]. Infusion of endothelin-1 over 7 days leads to sustained hypertension in rats, mediated through an increase in total peripheral resistance [127], which is salt-dependent [128]. In healthy human subjects, systemic administration of low doses of endothelin-1, mimicking the effects of a generalized increase in vascular generation, produces a modest increase in blood pressure [11].

Interactions with other local mediators of vascular tone. The endothelins stimulate nitric oxide generation by cultured endothelial cells [129, 130] and isolated vessels [131]. The early and brief vasodilator and hypotensive actions of bolus doses of the endothelins appear to be due, in part, to the release of nitric oxide from endothelial cells, as nitric oxide synthase inhibitors, such as N^G-nitro-L-arginine methyl ester, substantially attenuate these effects [132, 133]. Perhaps more relevant physiologically, nitric oxide synthase inhibitors also potentiate the constrictor and pressor effects of endothelin-1, suggesting that there is an autocrine feedback mechanism to modulate vasoconstriction to endothelin, by stimulation of the endothelial generation of nitric oxide [132, 133]. Endothelin-1 also increases prostacyclin generation by cultured endothelial cells [129], and cyclo-oxygenase inhibitors potentiate endothelin-1-induced constriction [134], suggesting that vascular generation of vasodilator prostaglandins attenuates constriction induced by endothelin-1. In human veins *in vivo*, prostacyclin appears to be more important than nitric oxide in modulating responses to endothelin-1 in veins [135]. However, prostaglandins may not mediate the hypotensive response to the endothelins, since this is unaffected by cyclo-oxygenase inhibition [136]. The hypotensive response to the endothelins does not appear to be due to stimulation of β_2-adrenoceptors as selective β_2-adrenoceptor antagonists do not prevent the dilator response [137]. It is, however, possible that activation of K^+ channels contributes to the dilator response to endothelin by causing membrane hyperpolarization [137]. Platelet-activating factor, a further endothelium-derived vasodilator, is also released by endothelin-1 and may mediate or modulate some of its actions [138].

Therefore, stimulation of endothelial cell generation of relaxant factors by endothelins attenuates, but does not prevent, their long-lasting vaso-

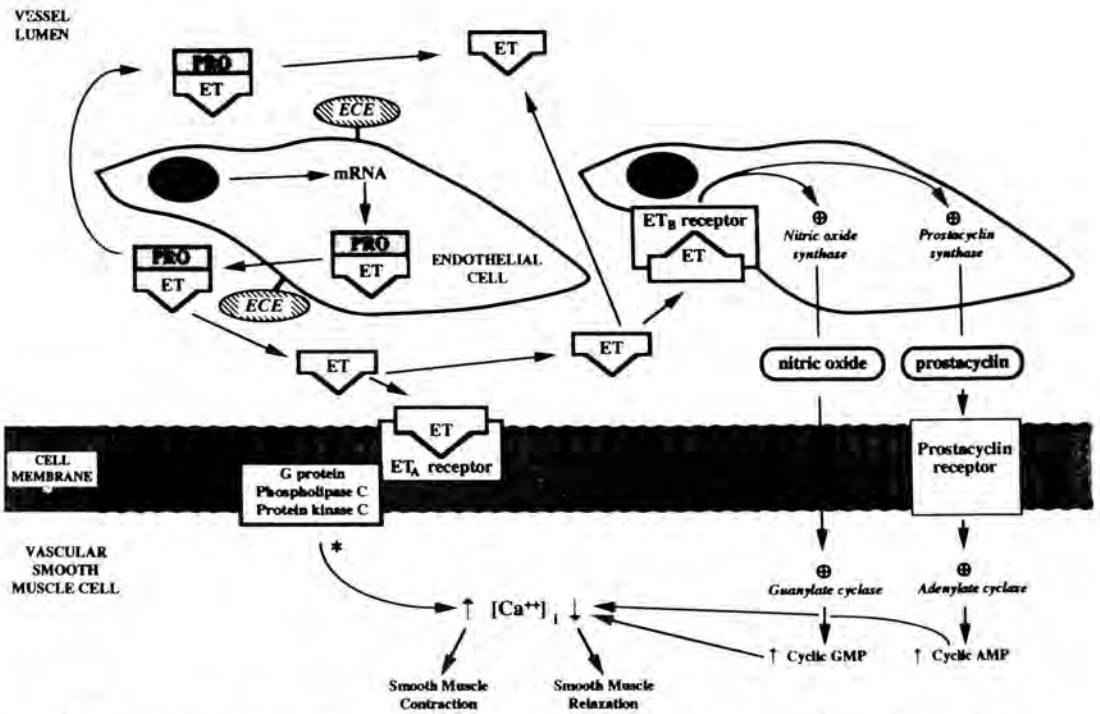

Fig. 3. Generation and actions of endothelin-1 in the blood vessel wall. Endothelin-1 is generated via cleavage of its precursor proendothelin-1 by membrane-bound ECE. The mature peptide can then act locally either at vascular smooth muscle cell ET_A receptors to directly cause vasoconstriction, or at endothelial cell ET_B receptors to stimulate production of endothelium-derived vasodilators. Note that ET_B receptors may also be present on vascular smooth muscle cells, where they cause vasoconstriction. *See the text for details of the mechanisms by which endothelin-1 increases $[Ca^{2+}]_i$. Abbreviations: PRO ET, proendothelin-1; ET, endothelin-1.

constrictor effects. This autocrine effect is probably mediated through the non-isopeptide-selective ET_B receptor expressed on endothelial cells (Fig. 3). There is, however, recent evidence to suggest that this receptor is also present on vascular smooth muscle cells, as selective agonists of ET_B receptors, such as [Ala^{1,3,11,15}]endothelin-1 and sarafotoxin S6c, cause vasoconstriction of isolated animal vessels [139, 140]. This ET_B-receptor-dependent vasoconstriction varies between vessels and species, being more apparent in veins than arteries [140]. Variations in the distribution of receptor subtypes may explain why endothelin-3 is equipotent to endothelin-1 as a vasodilator (endothelial cell, ET_B-mediated), but less potent as a vasoconstrictor (vascular smooth muscle, mainly ET_A-mediated) [131].

In addition to acting as a direct vasoconstrictor, endothelin-1 may potentiate the actions of the sympathetic nervous system in threshold doses [141, 142], although this effect has not been demonstrated in man [121].

Growth effects

Endothelin-1 is a potent mitogen for cultured vascular smooth muscle cells [143, 144], Swiss 3T3 fibroblasts [145], cardiocytes [146] and glomerular mesangial cells [105, 147]. This occurs, in part, because of increased expression of mRNA for the growth promoting proto oncogenes c *fos* and c *myc* [143]. Endogenous endothelin-1 may play an autocrine role in controlling endothelial cell growth, as anti-endothelin γ-globulin inhibits DNA synthesis by cultured endothelial cells [148].

Cardiac effects

Endothelin-1 is a potent constrictor of coronary vessels after local administration *in vivo* to animals, causing myocardial ischaemia [149] and fatal ventricular arrhythmias [150]. Systemic administration causes prolonged coronary vasoconstriction in animals, and the coronary resistance bed is more sensitive to the effects of endothelin than other resistance beds, excepting the kidney [125]. Endothelin-1 has potent positive chronotropic [151] and inotropic [152] effects *in vitro*, with the positive inotropic effects apparent at lower doses than those which cause coronary vasoconstriction [153]. At high doses, positive inotropism is opposed by ischaemia. Endothelin-1 impairs diastolic relaxation of the left ventricle *in vitro*, leading to a reduction in cardiac filling [154]. *In vivo*, although positively inotropic at low doses, higher doses cause cardiac output to fall [124, 155], probably due to a combination of systemic vasoconstriction, increasing after-

load, and coronary vasoconstriction, causing myocardial ischaemia.

Renal effects

Endothelin-1 contracts afferent and efferent arterioles equally *in vitro* [156], and thus reduces both renal plasma flow and glomerular filtration rate (GFR) in the isolated perfused kidney [157]. This contrasts with the selective effect of angiotensin II on the efferent arteriole. The renal circulation *in vivo* is also exquisitely sensitive to the effects of endothelin-1, with marked reduction in renal blood flow and GFR [124], even at sub-pressor doses of the peptide [147]. Intraglomerular capillary hydraulic pressure shows little change, as expected from effects on both afferent and efferent arterioles. In contrast, the glomerular capillary ultrafiltration coefficient is markedly reduced, probably due to endothelin-1-induced mesangial cell contraction leading to a reduction in glomerular capillary surface area, accounting in part for the fall in GFR. Surprisingly, despite decreases in renal blood flow and GFR, at low doses endothelin-1 increases urinary Na^+ excretion [147, 158]. This natriuresis may be due to enhanced production of ANP [159], to inhibition of the renal response to antidiuretic hormone [160], to pressure natriuresis or to a combination of these effects.

Respiratory effects

Endothelin-1 is a potent constrictor of pulmonary resistance vessels [161] and bronchioles, causing long-lasting bronchoconstriction when administered by aerosol to animals [162]. Endothelin-1 is synthesized by bronchial epithelial cells [163], and stimulates bronchiolar subepithelial fibrosis and the production of chemoattractants for leucocytes [164].

Central nervous system effects

Binding sites for endothelin-1 are present in the thalamus, hypothalamus, basal ganglia and brainstem [79]. The endothelins constrict cerebral arteries both *in vitro* and *in vivo*, and the effects of endothelin-1 on the basilar artery of the dog last for at least 3 days after intracisternal injection [165]. Topical application of exogenous endothelin-3 to the hypothalamus exerts profound effects on salt and water balance in rats, with potent inhibition of thirst in water-deprived animals [49]. In addition, binding sites for endothelin-1 are present in the carotid bifurcation, and topical application of the peptide inhibits baroreceptor, and stimulates chemoreceptor, responses at this site [166]. Intraventricular administration of low dose endothelin-1 increases blood pressure and heart rate through stimulation of central sympathetic outflow [167], supporting a role for the endothelins in cardiovascular homoeostasis.

Endocrine effects

Endothelin-1 has complex effects on the renin–angiotensin–aldosterone system, inhibiting release of renin from isolated rat glomeruli [168], but stimulating endothelial ACE activity [169]. In the rat isolated mesenteric bed, endothelin-1 stimulates the tissue renin–angiotensin system, increasing generation of renin and angiotensin II [170]. In the adrenal gland, endothelin-1 stimulates release of aldosterone from isolated cortical zona glomerulosa cells [171], and adrenaline from medullary chromaffin cells [172]. Administration of endothelin-1 *in vivo* to animals increases renin, aldosterone and adrenaline concentrations [173], renin concentration apparently rising because of endothelin-1-induced renal vasoconstriction.

Endothelin-3, in non-pressor doses, stimulates the rat hypothalamic–pituitary–adrenal axis, increasing release of corticotrophin-releasing hormone, adrenocorticotrophic hormone and, thereby, of corticosterone [174]. In cultured anterior pituitary cells, endothelin-1 and endothelin-3 directly inhibit prolactin secretion, while stimulating gonadotrophin secretion [175]. The endothelins may also play a role in the posterior pituitary, as they are present in hypothalamic paraventricular and supraoptic nuclei, and their projections into the posterior pituitary, and concentrations are reduced by water depletion [44]. Concentrations of both endothelin-1 and antidiuretic hormone (ADH) rise in parallel during upright tilt in normal subjects and are unchanged during tilt in patients with diabetes insipidus [64]. Although the suggestion has been made that endothelin-1 is acting as a classical circulating hormone in these circumstances, this seems unlikely, as its concentration is less than that necessary to cause direct vasoconstriction. However, endothelin-1 may have an autocrine or paracrine role in control of ADH release as infusion of the peptide increases ADH concentrations in dogs [173].

Endothelin-1 stimulates ANP production and release by cultured atrial myocytes *in vitro* [176, 177], and circulating concentrations of ANP are increased by infusion of endothelin-1 in rats [178]. ANP attenuates the effects of endothelin-1, as it dose-dependently relaxes endothelin-1 precontracted, aortic strips [179, 180]. However, the vasodepressor effects of ANP are qualitatively different from those of endothelin-1, suggesting that ANP does not mediate the transient hypotensive responses of endothelin-1 [181].

PATHOPHYSIOLOGY

In the absence of human studies using inhibitors of endothelin generation, or antagonists of endothelin receptor binding, much of the work on the role of endothelin in pathophysiology has been based on changes in circulating plasma concentrations of immunoreactive endothelins. These are dependent

not only on generation, but also on receptor-mediated clearance and enzyme-mediated metabolism of the peptide. In some studies, sensitivity to endothelin-1 has been examined, but as receptor number can be downregulated by increased endothelin-1 concentrations [99], these studies may also prove difficult to interpret.

Hypertension

Some investigators have used elevated concentrations of circulating immunoreactive endothelin-1 to suggest increased production in essential hypertension [182]. However, clearance of endothelin-1 is dependent on renal function [73, 75], and the very high concentrations found in severe and accelerated phase hypertension are probably due to impaired renal clearance. This is supported by results from animal models of accelerated hypertension, where endothelin-1 concentrations are raised and correlate positively with creatinine [183]. There seems to be no difference in generation of endothelin-1 between normotensive Wistar–Kyoto (WKY) and spontaneously hypertensive (SHR) rats, as assessed by hypotensive responses to phosphoramidon, an inhibitor of ECE [57]. Studies in hypertensive patients with normal renal function have shown similar concentrations of endothelin-1 to those in normotensive subjects [184]. Indeed, in this study the negative correlation between blood pressure and plasma endothelin-1 concentration in the hypertensive group makes a global increase in generation of endothelin-1 unlikely as a cause of essential hypertension.

In animal studies comparing WKY and SHR, both conduit and resistance vessels from the hypertensive rats are more sensitive to the effects of endothelin-1 [185–188]. *In vivo*, endothelin-1 has a greater pressor effect in SHR than WKY [189]. The mechanism for this enhanced sensitivity to endothelin-1 is unclear, as the number of binding sites for endothelin-1 in aortic smooth muscle [186] and heart [190] are lower in SHR, suggesting increased post-receptor sensitivity, although these studies may be confounded by altered vascular structure in established hypertension. There is, however, a relative increase in the number of binding sites for endothelin-1 in the brain of SHR as compared with WKY [190], so there may be increased central nervous system sensitivity to the peptide in hypertension.

Recent studies with endothelin receptor antagonists support for a role for endothelin-1 in experimental animal hypertension. The ET_A antagonist BQ-123 acutely lowers blood pressure in salt-loaded stroke-prone SHR, but not in salt-deprived controls or in WKY [191]. When administered chronically, BQ-123 lowers blood pressure, and prevents the developments of stroke and renal abnormalities in stroke-prone SHR [191]. CGS 26343A, another ET_A receptor antagonist, lowers blood pressure in low-renin animal models of hypertension (SHR and deoxycorticosterone acetate–salt rats), but not in control animals or in high-renin animals [93]. These results do not indicate the mode (increased generation or sensitivity) by which endothelin-1 may contribute to hypertension in these animal models.

The vasodilator function of the endothelium is impaired in essential hypertension, and responses to endothelium-dependent relaxant agents are reduced [192]. This may be due to impaired release of endothelium-derived dilators (prostacyclin and nitric oxide). Alternatively, there may be greater production of, or sensitivity to, endothelium-derived vasoconstrictors, such as endothelin-1. Even if the impaired dilator response is solely due to decreased production of nitric oxide, the balance between endothelium-derived dilator and constrictor factors may alter to favour vasoconstriction.

The potent mitogenic effects [143] of endothelin-1 might potentially contribute to hypertension-induced hypertrophy of vascular smooth muscle, thus amplifying any vasoconstrictor influences [193, 194]. In addition, endothelin-1 might promote the development of left ventricular hypertrophy in hypertension, a factor that adversely affects prognosis [195]. The growth-promoting properties of endothelin-1 may also contribute to the development of atherosclerosis. Atherosclerotic human blood vessels show strong immunostaining for endothelin-1, and patients with atherosclerosis have raised plasma concentrations of the peptide, with the highest concentrations in patients with the largest number of affected vessels [196].

Increased production of endothelin-1 certainly appears to be associated with one secondary form of hypertension, albeit rare. Yokokawa et al. [197] have described two cases of the skin tumour, haemangio-endothelioma, in which hypertension was associated with increased plasma endothelin-1 concentrations (Fig. 4). Biopsies of tumour cells displayed increased expression of mRNA for endothelin-1, and strong immunohistochemical staining for the peptide. Blood pressure and endothelin-1 concentrations returned to normal in both cases after surgical resection of the tumours and, in one patient, recurrence of the tumour led to further increases in both blood pressure and plasma endothelin-1 concentration.

Digital and coronary vasospasm

There are a number of vascular conditions that are characterized by instability of tone in small arteries, such as Raynaud's disease and Prinzmetal's angina, associated with vasospasm in digital and coronary vessels, respectively. Patients with the latter have an increased incidence of Raynaud's phenomenon, migraine and ocular vasospasm [198], suggesting that such instability of vascular tone may be a more widespread phenomenon, perhaps due to an imbalance between endothelium-derived dilator

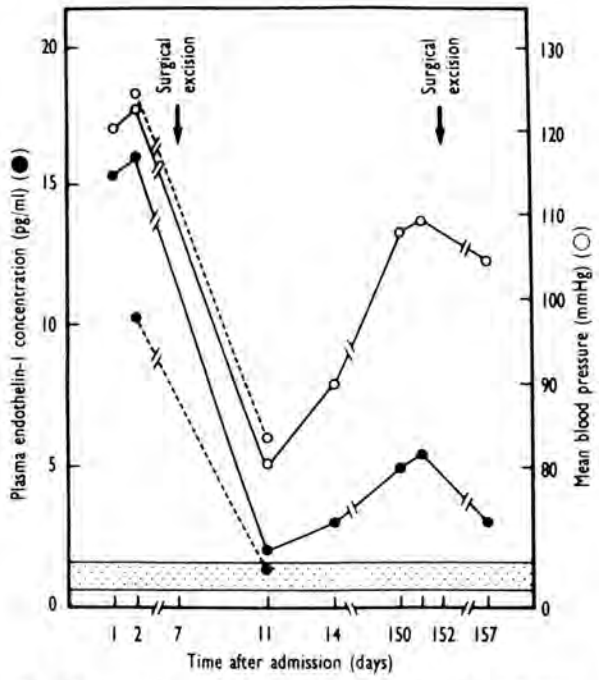

Fig. 4. Sequential plasma endothelin concentrations (●) and mean blood pressure (○) in two patients with malignant haemangio-endothelioma (——, case 1; ----, case 2). See the text for details. The stippled area indicates the normal range of plasma endothelin-1 concentrations in humans with this assay. Reproduced with permission from [197].

and constrictor factors. Indeed, circulating endothelin-1 concentrations are elevated in patients with Raynaud's disease and coronary vasospasm, between episodes of spasm, consistent with a generalized endothelial abnormality associated with increased endothelin-1 production [199, 200]. In Raynaud's disease, circulating concentrations increase further after a cold challenge to the hand [199], and endothelin-1 concentrations are higher still in venous blood draining the cold-challenged hand. By contrast, in coronary vasospasm, endothelin-1 concentrations do not increase further during ischaemic episodes [200], suggesting that another factor, such as decreased nitric oxide production, may be responsible for initiating the attack. By contrast, plasma concentrations of endothelin-1 are normal in patients with either stable [201] or mild unstable [202] angina. Endothelial dysfunction, associated with increased generation of endothelin-1, may therefore predispose to vasoconstriction in vasospastic states.

Myocardial infarction and heart failure

Circulating concentrations of endothelin-1 and proendothelin-1 rise rapidly after myocardial infarction, with concentrations raised two- to three-fold on the day of infarction, and only returning to basal over the next 7–10 days [203]. The duration and degree of elevation is proportional to the severity of infarction, as judged by Killip class, with the highest concentrations found in cardiogenic shock [201]. These findings are consistent with the elevated endothelin-1 concentrations described in other forms of shock [204], and suggest that in such severe hypotensive states, endothelin-1 may act as a 'mechanism of last resort' to maintain blood pressure and perfusion of vital organs. However, this interpretation of the findings may be open to question since impaired renal function in hypotensive shock will reduce renal clearance of endothelin-1.

In addition to elevated plasma concentrations, immunoreactive endothelin-1 also increases substantially (six-fold) in cardiac tissue after experimental myocardial infarction in rats [205], with this increase lasting longer than that in plasma [206]. Interestingly, the number of cardiac binding sites for endothelin-1 increase after either ischaemia alone (by 62%) or ischaemia with reperfusion (by 140%) [97]. Interestingly, pretreatment with anti-endothelin γ-globulin in a rat model of myocardial infarction causes a 40% decrease in infarct size [205, 206]. Thus a combination of increased cardiac endothelin-1 content and additional binding sites for the peptide may contribute to extension of infarct size by compromising blood flow in reperfused areas.

It has been speculated that endothelin-1 may also play a role in the neurohumoral maladaption to chronic heart failure and, like the renin–angiotensin system, contribute to the morbility and mortality associated with this condition. In animal models of heart failure, endothelin-1 concentrations rise three-fold, and correlate closely with pulmonary capillary wedge pressure [207]. Endothelin-1 concentrations are also elevated in patients with chronic heart failure [208, 209] and are correlated closely with the degree of pulmonary hypertension [210].

Renal failure

Plasma immunoreactive proendothelin-1, endothelin-1 and endothelin-3 concentrations are elevated in renal failure, and are raised two–four-fold in patients on haemodialysis, probably reflecting impaired renal clearance of these peptides [73, 74]. However, renal production of endothelin-1 may also contribute, as there are increases in both renal cortical endothelin-1 production [211] and urinary endothelin excretion [212] in animal models of reduced renal mass. Urinary endothelin and protein excretion are positively correlated in these animals [212], suggesting that endothelin may contribute to the rate of progression of chronic renal failure. Elevated plasma concentrations of endothelin may also play a role in the development or maintenance of hypertension in chronic renal failure. In contrast to essential hypertension, endothelin-1 concentrations in haemodialysis patients are positively correlated with blood pressure [213]. Interestingly,

concentrations of endothelin-3, but not those of endothelin-1, rise further during haemodialysis; this may reflect production or release of endothelin-3 by the central nervous system in response to dialysis-induced hypotension [213].

Acute renal failure due to post-ischaemic acute necrosis is characterized by intense renal vasoconstriction. This may be mediated by endothelin-1, which is a potent renal vasoconstrictor [157], since production of, and sensitivity to, the peptide is increased by hypoxia [29, 97]. Circulating plasma concentrations of endothelin are increased in patients with post-ischaemic renal failure [214]. A role for endothelin-1 is supported by the finding that, in animals, renal immunoreactive endothelin increases, for at least 24 h, after 45 min of bilateral renal artery occlusion [215]. Pretreatment of these animals with a monoclonal antibody against endothelin ameliorates renal failure. In addition, infusion of anti-endothelin γ-globulin into a branch artery of a rat kidney 48 h after ischaemic insult will reverse renal vasoconstriction [216].

Gram-negative septic shock is associated with a reduction in peripheral vascular resistance and blood pressure, which is thought to be due to endotoxin, and other mediators, stimulating nitric oxide production [217]. Paradoxically, renal vasoconstriction occurs during septic shock, leading to acute renal failure [218]. As endotoxin stimulates endothelin-1 release, both *in vitro* and *in vivo* [32], and local administration of anti-endothelin γ-globulin completely reverses endotoxin-induced renal vasoconstriction [159], endothelin may be responsible for acute renal failure in this situation.

Endothelin-1 may also contribute to the hypertension and renal impairment caused by cyclosporin A. Production of endothelin-1 *in vitro* [219] and *in vivo* [220] is stimulated by cyclosporin A, which also increases renal endothelin-1 receptor number [98]. Also, cyclosporin A-induced renal vasoconstriction is substantially attenuated by anti-endothelin γ-globulin [220].

Respiratory disease

Plasma concentrations of endothelin-1 in venous blood are elevated in patients with primary and secondary pulmonary hypertension [221]. Also, the pulmonary circulation seems to generate more endothelin-1 than it clears in primary pulmonary hypertension, as the ratio of arterial to venous concentrations of endothelin-1 is significantly greater than unity (~2.2) [221]. This is not the case for healthy control subjects, who have an arterial to venous ratio substantially less than unity (~0.6). In addition, a role for endothelin-1 in the adult respiratory distress syndrome, which is associated with elevated pulmonary vascular resistance in the face of normal pulmonary artery occlusion pressures, is suggested by marked increases in mixed venous and wedged pulmonary artery concentrations of endothelin-1 [222].

Asthma is a disease characterized by episodes of reversible bronchoconstriction. Substantially increased amounts of endothelin-1, as measured by immunoreactive staining, have been demonstrated in the bronchial epithelium of asthmatic patients as compared with control subjects [164]. In addition, during severe attacks of asthma there are increased concentrations of endothelin-1 in both plasma and bronchoalveolar lavage fluid [223]. These findings, taken in conjunction with the ability of the peptide to cause marked bronchoconstriction [162], make endothelin-1 a strong candidate for further investigation as a mediator in asthma.

THERAPEUTIC POTENTIAL

Selective ECE inhibitors [55] and endothelin-1 receptor antagonists [90–93] are only beginning to be made available for basic research, but should provide powerful insights into the physiology and pathophysiological role of the endothelins. Prolonged intravenous infusion of a relatively non-selective agent, phosphoramidon, which inhibits both ECE and neutral endopeptidase-24.11, lowers the blood pressure of both WKY and SHR by approximately 15% after 4 h [57]. In contrast, kelatorphan, an inhibitor of neutral endopeptidase-24.11, does not alter blood pressure, implying that the effect of phosphoramidon is due to inhibition of ECE. These results are consistent with a role for endothelin in regulation of blood pressure, and suggest that the therapeutic potential of ECE inhibitors should be investigated at a later stage in man. Endothelin receptor antagonists are at a very early stage of development [90–93], but may allow selective interruption of the actions of particular isoforms of endothelin. For example, an antagonist at the ET_A receptor would block constrictor responses to endothelin-1, while leaving any ET_B-receptor-mediated vasodilator effects of the endothelins unaffected. There is recent evidence that ET_A receptor antagonists cause the expected decrease in blood pressure in rats with experimental hypertension [93, 191]. Vasodilator drugs which act by opening K^+ channels also markedly inhibit endothelin-1-induced vasoconstriction [114, 116], although the therapeutic role of these drugs remains unclear.

The use of ACE inhibitors, such as captopril and enalapril, and angiotensin II receptor antagonists, such as saralasin and losartan (Dup 753), have helped to define the role of the renin–angiotensin system in health and disease, and ACE inhibitors have become established as effective treatment for patients with hypertension and heart failure. As endothelin-1 is an extremely potent constrictor, pressor and mitogenic agent, it is possible that ECE inhibitors and endothelin receptor antagonists may,

Table 1. Role of endothelin in pathophysiology. The diseases in which endothelin may play a role are shown.

Relatively strong experimental evidence
Myocardial infarction
Acute renal failure
Cyclosporin nephrotoxicity
Maladaptation to chronic heart failure
Raynaud's phenomenon
Coronary vasospasm
Asthma
Primary pulmonary hypertension
Relatively weak experimental evidence
Essential hypertension
Vascular hypertrophy
Left ventricular hypertrophy
Renal hypertension

in future, offer substantial therapeutic benefits in these and other vascular conditions.

CONCLUSIONS

The endothelins are potent long-acting endogenous vasoconstrictor and growth-promoting peptides, first isolated from vascular endothelial cells. The widespread expression of mRNA for the endothelins, and the distribution of their receptors, suggests that these peptides may play an important role in local regulation of the cardiovascular, respiratory, endocrine and central nervous systems. Endothelin-1 is the predominant peptide generated by vascular endothelial cells, and is the most potent vasoconstrictor and pressor agent yet identified. Endothelin-1 may serve as a long-acting physiological antagonist to the short-lived effects of the endothelium-derived vasodilators, prostacyclin and nitric oxide, and may also act as a vasoconstrictor 'of last resort' under circumstances of extreme cardiovascular stress.

There is substantial experimental evidence to suggest that endothelin-1 may be involved in the pathophysiology of vasospastic conditions, renal failure and asthma (Table 1). In some conditions, such as chronic renal failure or myocardial infarction, it is not clear whether the high circulating concentrations of endothelin-1 are a cause or consequence of the underlying condition. Even if endothelin is not the primary cause of these diseases, the peptide may contribute to their progression. Development of agents that interfere with the production or actions of the endothelins may increase knowledge of the physiological and pathological roles of the endothelins, and generate drugs with potentially novel benefits in the treatment of cardiovascular and respiratory disease.

Note added in proof

Orally active non-peptide endothelin receptor antagonists were recently reported at the Third International Conference on Endothelin in Houston Texas, U.S.A. (15–17 February 1993), and are shortly expected to enter clinical development.

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MEETING REPORT

Endothelin: progress in pharmacology and physiology

The discovery of the potent vasoconstrictor family of peptides, endothelin-1 (ET-1) in 1988 (Ref. 1), and ET-2 and ET-3 in 1989 (Ref. 2), led rapidly to the cloning of two receptor subtypes, ET_A (agonist potency: ET-1 > ET-2 >> ET-3) and ET_B (ET-1 = ET-2 = ET-3)[3]. There has subsequently been considerable progress in characterizing both the pharmacology and physiology of endothelin, particularly with the development of specific receptor agonists and antagonists, as reported at a recent conference*.

Generation and metabolism

Progress towards isolation of the endothelin-converting enzyme remains disappointing. Although the enzyme appears to be membrane bound, the question remains whether it is intracellular. Substantial endothelin-converting enzyme activity in endothelial cell membranes appears to be located within the endoplasmic reticulum (G. Gui, University of Texas, Dallas). V. J. Harrison (William Harvey Research Institute, London), however, presented evidence that intracellular vesicles might be an important site of proET-1 processing.

Stimulation of endothelin production by shear stress and hypoxia is well recognized. However, J. A. Frangos (Pennsylvania State University) showed that the effect of shear stress may be biphasic at high levels of shear, with initial protein kinase C-dependent stimulation of endothelin production, and later nitric oxide-dependent inhibition. Interestingly, an *in vivo* model of acute hypoxia in dogs is associated with increases in both plasma and urinary endothelin (A. Nir, Mayo Clinic, Rochester). Two groups have demonstrated that heparin inhibits ET-1 expression and generation by cultured endothelial cells (T. Imai, Tokyo Medical and Dental University; K. Yokokawa, Osaka City University), possibly via nitric oxide.

Although ET-1 and ET-2 are equipotent as constrictors, ET-2 has not been detected previously in human tissues. Using immunocytochemistry with highly specific antibodies to precursors of the different endothelin isoforms, C. Plumpton (University of Cambridge) demonstrated the presence of ET-2, in addition to ET-1 and ET-3, in human pulmonary, cardiac and vascular tissue. This group also demonstrated that the preproET-2 gene may generate several alternative spliced variants in different human tissues, suggesting that splicing may play an important regulatory role, possibly by altering the post-transcriptional processing of ET-2 or by generation of alternative functional variants (G. O'Reilly, University of Cambridge). M. Paul (University of Heidelberg) described transgenic rats overexpressing the human ET-2 gene, insertion of the human ET-1 gene having produced a lethal effect during development. Blood pressure was not increased, despite a fourfold elevation in concentrations of circulating immunoreactive endothelin.

Although impaired renal function is associated with reduced clearance of endothelin, the relative contributions from urinary excretion, receptor mediated clearance and enzyme mediated metabolism are unclear. S. Kaw (William Harvey Research Institute, London) showed the substantial capacity of the kidney for inactivating ET-1. A. Y. Jeng (Ciba-Geigy, New Jersey) isolated a candidate ET-1- (and ET-3-) degrading protease from rat kidney, inactive against proET-1 and proET-3, which cleaved the C-terminal tryptophan from ET-1 to yield a peptide with vasoconstrictor potency three orders of magnitude less than ET-1.

Receptors

Endothelin receptors are unusual in that the ET_A and ET_B subtypes were isolated and cloned before the development of subtype-selective ligands. To define the molecular basis of ligand-binding selectivity, A. Sakamoto (University of Kyoto) constructed chimeric endothelin receptors and showed that regions containing transmembrane domains I–III and VII were necessary for ET_A, whereas IV–VI were necessary for ET_B-specific binding. Mutation of Cys148 and Cys229 to Ala in the first and second extracellular loops of the ET_B receptor abolished ET-1 binding (B. Haendler, Schering, Berlin). C. Miyamoto (Nippon-Roche, Kamakura) showed that the 13 C-terminal amino acids, and the amino acids of the C-terminal end of the third intracellular loop, are involved in signal transduction.

Molecular biology studies[4] suggest that only the ET_A receptor is expressed in vascular smooth muscle, with vascular expression of the ET_B receptor confined to endothelial cells, where it causes release of vasodilator prostaglandins and nitric oxide. However, at least in animals, studies with ET_B-selective agonists, such as sarafotoxin S6c, suggest that ET_B-like receptors may also mediate vasoconstriction. There is now increasing evidence that the venous circulations of the dog and rabbit have vasoconstrictor ET_B receptors (N. J. Lodge and S. Moreland, Bristol-Myers Squibb, Princeton; I. Watts, Glaxo, Ware).

The ratio of ET_A and ET_B receptors may vary within and between vascular beds, and between species. J. L. Balwierczak and D. F. Rigel (Ciba-Geigy, Summit) found evidence for both ET_A- and ET_B-mediated coronary vasoconstriction; constriction of large conduit arteries and arrythmogenesis was attributable to ET_A receptors in the dog, while constriction of the coronary resistance bed additionally involves ET_B receptors in the dog and rat. D. P. Brooks (SmithKline Beecham, King of Prussia) confirmed that vasoconstriction in rat kidney is mediated by ET_B receptors. By contrast, in the dog, renal vasoconstriction is mediated exclusively by the ET_A receptor (Brooks), and reduction of renal

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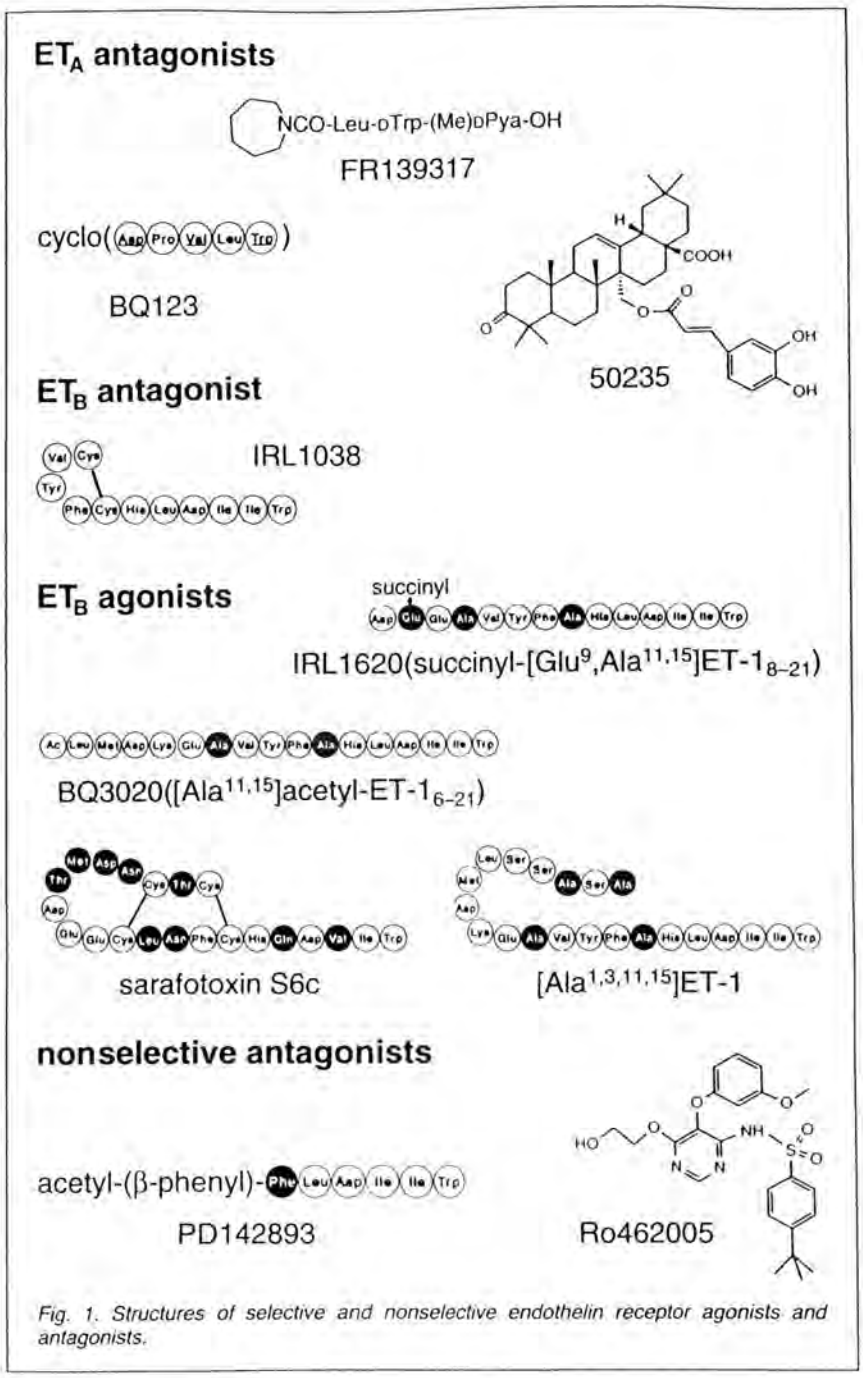

Fig. 1. Structures of selective and nonselective endothelin receptor agonists and antagonists.

vascular resistance following intra-renal infusion of BQ123 (A. L. Clavell, Mayo Clinic) suggests a role for these receptors in maintenance of basal tone. The dog may be a better model than the rat for the human kidney, in which only ET_A receptors can be localized to the vasculature (F. E. Karet, University of Cambridge).

The importance of ET_B-mediated vasoconstriction in man is unclear. Although ET_B receptor protein (by ligand binding) and ET_B mRNA (by reverse transcriptase polymerase chain reaction) have been detected in the media of a number of human blood vessels, the ET_B-selective compounds have little or no agonist activity, and Schild plots using the ET_A-selective antagonist BQ123 show a slope close to one in these preparations (A. P. Davenport, University of Cambridge).

BQ123 (Ref. 5) and FR139317 (Ref. 6) have become established as highly selective ET_A antagonists, while IRL1038 appears to be a selective antagonist at the ET_B receptor (H. Karaki, University of Tokyo). An ET_A-selective agonist has not yet been reported, in contrast to the diversity of ET_B agonists (Fig. 1). M. Clozel (Hoffman-La Roche, Basel) provided the first report of a nonpeptide, orally active endothelin receptor antagonist, Ro462005, detected by random screening. This sulphonamide derivative is a competitive nonselective antagonist at ET_A and ET_B receptors, has no partial agonist activity, and is inactive against other vasoconstrictors. Ro462005 antagonizes ET-1-induced constriction of rat aorta and small mesenteric arteries (ET_A receptors; pA_2 6.5) and sarafotoxin S6c-induced constriction of rat trachea and dilatation of small mesenteric arteries (ET_B receptors; pA_2 6.1) (G. A. Gray; Hoffman-La Roche Basel). In the rat, Ro462005 inhibits the pressor response to proET-1 and both the depressor and pressor responses to ET-1. Ro462005 prevents renal vasoconstriction after 45 min renal ischaemia, and cerebral vasoconstriction in a model of subarachnoid haemorrhage. Interestingly, Ro462005 doubled plasma ET-1 concentrations (with no effect on plasma proET-1) in conscious rats, while BQ123 and FR139317 had no such effect. B-M. Loffler (Hoffman-La Roche, Basel) postulated that Ro462005 may be displacing ET-1 from ET_B receptors or, alternatively, reducing receptor-mediated clearance.

Several groups described pharmacological responses that could not be attributed to activation of the recognized subtypes of ET_A or ET_B receptors, as defined by existing antagonists. T. D. Warner (William Harvey Research Institute, London) and H. Karaki presented functional evidence for non-isopeptide-selective endothelin receptors, insensitive to ET_B (and ET_A) antagonists, in rabbit pulmonary artery and isolated rabbit stomach strips (Warner), and porcine pulmonary veins and rabbit saphenous veins (Karaki).

Intracellular actions

Endothelins are known to activate phospholipase C through a process involving a G protein-coupled receptor. S. Eguchi (Tokyo Medical and Dental University) demonstrated that endothelin receptors may also be coupled to adenylyl cyclase and cAMP formation, activation occurring through ET_A receptors in cultured

vascular smooth muscle cells, and inhibition through ET_B receptors in endothelial cells. Inositol trisphosphate formation does not appear to account for the prolonged contractile effects of ET-1, which are largely dependent on extracellular Ca^{2+}, and may result from coupling of endothelin receptors with ion channels. S. Hu (Ciba-Geigy, Summit) demonstrated that ET-1 and ET-3 open Cl^- channels, and close ATP-insensitive K^+ channels, in cultured rat mesangial cells, thus causing membrane depolarization. W. G. Haynes (University of Edinburgh) reported that cromakalim, an ATP-sensitive K^+ channel opener, was more effective at preventing venoconstriction to ET-1 *in vivo* in humans than the dihydropyridine Ca^{2+} antagonist nicardipine, suggesting that ET-1 interacts with ATP-sensitive K^+ channels, independent of dihydropyridine-sensitive Ca^{2+} channels, in human vascular tissue.

Endothelial interactions

ET-1 stimulates the generation of endothelium-derived dilators, such as prostacyclin and nitric oxide, probably by activation of an endothelial cell ET_B receptor. P. D'Orleans-Juste (University of Sherbrooke) showed, in perfused rat lung and rabbit kidney, that ET_A receptors mediate prostacyclin release. S. Taddei (Baylor College of Medicine, Houston), reported that endothelial ET_A receptors may mediate release of the constrictor prostanoid thromboxane A_2 in spontaneously hypertensive rats. ET_B receptors may also be linked to release of an endothelium-dependent hyperpolarizing factor, distinct from nitric oxide (M. Nakashima, Baylor College of Medicine; I. Sakuma, Hokkaido University), that acts in part by opening apamin-sensitive small-conductance Ca^{2+}-activated K^+ channels (Sakuma). In humans, constriction of hand veins by ET-1 is modulated *in vivo* by the production of prostacyclin, but not nitric oxide (D. J. Webb, University of Edinburgh).

ET-1 stimulates atrial natriuretic peptide release, which inhibits the production and actions of ET-1, and attenuates ET-1-stimulated proliferation of vascular smooth muscle cells (D. Neuser, Bayer, Wuppertal). C-type natriuretic peptide (CNP), which has similar actions to atrial natriuretic peptide, is co-localized with ET-1 in endothelial cells (D. M. Heublein, Mayo Clinic). Intracoronary administration of CNP inhibited coronary production of endothelin in dogs (R. S. Wright, Mayo Clinic), and systemic CNP prevented the rise in plasma endothelin occurring in a dog model of heart failure (R. R. Brandt, Mayo Clinic).

Pathophysiology

Evidence in support of a role for endothelin in cardiovascular, renal and cerebrovascular disease was also provided. T. Tønnessen (University of Oslo) reported that endothelin is released from the heart following 10 min of myocardial ischaemia in anaesthetized pigs. C. C. Zaugg (University Hospital, Basel) demonstrated that on reperfusion of the isolated rat heart there is exaggerated coronary vasoconstriction to ET-1, compared to the thromboxane A_2 analogue U46619.

F. C. Wilkins (University of Mississippi, Jackson) described a conscious dog model for the increased circulating endothelin concentrations found in some diseases. ET-1 infusion at $2.5\,ng\,kg^{-1}\,min^{-1}$ for 7 days increased plasma ET-1 by two- to threefold, increasing peripheral vascular resistance and producing a sustained elevation of blood pressure. This suggests that moderately elevated circulating ET-1 concentrations can affect vascular tone, either directly or indirectly. Nevertheless, to attain these same plasma levels with endogenous production, concentrations at the interface between the endothelium and vascular smooth muscle are likely to be considerably higher, so the true contribution of ET-1 to vasoconstriction in such diseases may be underestimated by the model. Inhibition of the action of ET-1 at ET_A receptors, using BQ123, was reported to lower blood pressure in conscious spontaneously hypersensitive rats but not Wistar-Kyoto rats (E. H. Ohlstein, SmithKline Beecham, King of Prussia). Clozel showed that Ro462005 also failed to lower blood pressure in normotensive rats. These findings contrast with a published report of administration of intravenous bolus BQ123 to anaesthetized Sprague-Dawley rats, in which blood pressure was reduced[7].

Endothelin has potent mitogenic actions, and has been implicated in the pathogenesis of atherosclerosis. Infusion of ET-1 increased the severity of myointimal hyperplasia following balloon angioplasty to the rat common carotid artery (J. Trachtenberg, Washington University, St. Louis; S. A. Douglas, SmithKline Beecham, King of Prussia), and increased receptor binding of [^{125}I]ET-1 (M. R. Dashwood, Royal Free Hospital, London) occurs in sections of saphenous vein grafts with atheroma and neovascularization. This increased binding may be growth factor mediated, since epidermal, platelet-derived and fibroblast growth factors increase [^{125}I]ET-1 binding to cultured human vascular smooth muscle cells (P. Bonin, Upjohn, Kalamazoo). Atheromatous vein grafts from hyperlipidaemic rabbits failed to vasodilate to low (physiological) concentrations of ET-1 (H. G. Davies, Duke University, Durham), possibly reflecting relative loss of endothelial ET_B receptors.

The inner medullary collecting duct of the kidney produces substantial amounts of ET-1, and contains a high density of endothelin receptors. Exogenous ET-1 potently inhibits water reabsorption and Na^+/K^+-transporting ATPase activity. Spontaneously hypertensive rats (SHR) produce less ET-1 from the inner medullary collecting duct than Wistar-Kyoto rats[8]; this possibly underlies a tendency to salt and water retention in the SHR. The idea that locally generated ET-1 plays a tonic physiological role in regulation of salt and water transport in the terminal nephron is supported by evidence that vasopressin produces a greater increase in cAMP when cultured duct cells are pre-incubated with specific ET-1 antisera, and by the finding that renal medullary ET-1 increases during salt loading (D. Kohan, University of Utah, Salt Lake City). Interestingly, endothelin generation is increased by both ciclosporin and FK506, potent immunosuppressive agents (Y. Takeda, Kanazawa University). At clinically relevant doses, the effect is smaller with FK506 than

ciclosporin, which may help to explain why it is less nephrotoxic.

Many of these observations now require confirmation in studies using specific receptor antagonists or endothelin converting enzyme inhibitors. Extrapolating results from animal models to human diseases is still problematic, and clinical studies and human models are eagerly awaited.

□ □ □

The past two years have seen substantial advances in the field of endothelin research. Although endothelin receptors distinct from ET_A and ET_B, and the putative converting enzymes, have not yet been isolated and cloned, there has been progress in the development of specific agonists and antagonists at the known endothelin receptors. There is now considerable evidence that endothelins play a role in regulation of cardiovascular and renal function, and these are clear indications of diseases in which they may be implicated. Considering the benefits of angiotensin-coverting enzyme inhibition in heart failure, interruption of the actions of endothelin may also be important in diseases where it acts as a contributory, rather than a causative, factor. Given the known inter-species differences, and the recent identification of vascular ET_B receptors, it is difficult to predict whether ET_A-selective or nonselective agents will have the greater clinical utility. With the development of orally active endothelin receptor antagonists, we are now within sight of clinical studies to answer these questions.

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U46619: 9,11-dideoxy-11α-apoxymethanoprostaglandin $F_{2\alpha}$

CURRENT AWARENESS

5-HT_1-like receptors: six down and still counting

The application of molecular cloning techniques in the field of pharmacology has uncovered a diversity of neurotransmitter receptors that may be exploited to lead to targeted, selective drug development. There is no better example of this multiplicity than 5-HT receptors in general and 5-HT_1-like receptors in particular, and to date six 5-HT_1-like receptors have been cloned: 5-HT_{1A}, 5-HT_{1C}, 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$ (5-HT_{1B}), 5-HT_{1E} and 5-HT_{1F} (5-$HT_{1E\beta}$).

The genomic clone encoding the 5-HT_{1A} receptor was the first 5-HT_1-like receptor clone to be isolated in 1987 (Ref. 1), although it was not fully characterized until 1988 (Ref. 2). The characterization of a functional cDNA encoding the 5-HT_{1C} receptor followed in the same year[3]. The higher sequence homology shared between the rat 5-HT_{1C} and 5-HT_2 receptors (69%) compared to that between the 5-HT_{1C} and 5-HT_{1A} receptors (29%) was reflected in their similar pharmacological profiles and second-messenger systems (stimulation of phospholipase C-mediated phosphoinositide turnover) and this has led to a suggestion that the 5-HT_{1C} receptor should be reclassified into the 5-HT_2 subclass.

Another member of the 5-HT_1-like class of 5-HT receptors, the 5-HT_{1B} receptor, has, for some years, been thought to be a species homologue of the 5-HT_{1D} receptor and to be confined to the rat and mouse[4]. This notion, however, was questioned in the early 1990s when a seemingly confusing array of 5-HT_{1D}-like receptor clones from different species were reported by several independent laboratories[5–7].

The picture became clearer when two distinct genes encoding two members of the 5-HT_{1D} subfamily were cloned in the human, and the receptors were named 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ (Refs 8,9). The proteins encoded by these two genes exhibit 63% identity with each other but retain a similar pharmacology. In contrast, the previously named rat 5-HT_{1B} receptor is 93% identical with the human 5-$HT_{1D\beta}$ receptor yet has a distinctly different pharmacology. In fact, the differences between these receptors were shown to be due to a single amino acid substitution of Thr to Asn at position 355 in transmembrane domain (TM) VII (Ref. 10). Although both amino acids are uncharged polar residues, the Asn allows β-adrenoceptor antagonists such as pindolol and propranolol to bind to the rat homologue with a greater affinity than to the 5-$HT_{1D\beta}$ receptor.

The existence of a further 5-HT_1-like receptor was indicated by the emerging story of the 5-HT_{1D} receptor. The 5-HT_{1D} recognition site was first reported by Heuring and Peroutka[11] in 1987. This group described a binding site in bovine brain membranes that could be labelled with low nanomolar concentrations of [^{3}H]5-HT in the presence of 100 nM 8-OH-DPAT and 100 nM mesulergine, blocking out 5-HT_{1A} and 5-HT_{1C} receptors, respectively. Several other groups subsequently confirmed these findings but consistently reported that displacement studies, under

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Review

Endothelins as regulators of growth and function in endocrine tissues

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Endothelin-1 (ET-1) was originally isolated in 1988 as a secretory product of cultured porcine aortic endothelial cells (Yanagisawa *et al.*, 1988) and was shown to be a potent vasoconstrictor and pressor peptide. This effect has subsequently been confirmed in healthy human subjects (see Haynes & Webb, 1993). The pressor response is often preceded by a short phase of vasodilatation which may be due to ET-stimulated release of vasodilator prostaglandins and nitric oxide (see Simonson & Dunn, 1991). Three isopeptides have been identified (ET-1, ET-2 and ET-3) each of which is encoded by a separate gene (Inoue *et al.*, 1989). The gene products (preproETs) are cleaved within the cell to generate inactive 38–39 amino acid peptides (proETs) which are subsequently cleaved to active isoforms by the action of putative ET converting enzymes (ECEs). The isopeptides all comprise 21 amino acids with two intra-chain disulphide bonds, ET-2 and ET-3 differing from ET-1 by two and six amino acids respectively. ET-1 and ET-2 are more potent than ET-3 as vasoconstrictors.

ET-1 gene expression in vascular endothelium is increased by exposure of cells to adrenaline, thrombin, angiotensin II, transforming growth factor-β (TGF-β), interleukin-1, the calcium ionophore A23187, hypoxia and shear stress (see Haynes & Webb, 1993). ET-1 production is inhibited by endothelium-derived nitric oxide. Secretory granules containing ET have been identified in the central nervous system but not in the vascular endothelium where the major regulation of ET production may occur at the level of gene expression. Immunoreactive ET is produced in a variety of tissues including blood vessels, lungs, kidney and nervous system. ET-1 is the major isoform found in vascular endothelium while ET-3 may be the predominant form in neural tissue. Vascular smooth muscle cells also produce ET-1 *in vitro*, although at a rate 100-fold less than endothelial cells (Resink *et al.*, 1990). Despite the lower level of production, the relatively greater mass of vascular smooth muscle cells in larger blood vessels suggests that smooth muscle may contribute substantially to local ET-1 production.

Circulating concentrations of ET are typically between 0·25 and 20 ng/l, below the concentration generally associated with biological effects. It seems likely, therefore, that ETs function predominantly as paracrine or autocrine agents rather than as circulating hormones. The plasma half-life is short and ETs are cleared from the circulation mainly by the kidney. Two classes of ET receptor were originally identified using molecular cloning techniques, the bovine ET_A (Arai *et al.*, 1990) and rat ET_B receptor (Sakurai *et al.*, 1990). These receptors have since been cloned from human tissues (see Haynes & Webb, 1993). The ET_A receptor (binding potency ET-1 = ET-2 > ET-3) mediates vasoconstriction to ET-1. The ET_B receptor (binding potency ET-1 = ET-2 = ET-3), which was originally thought only to mediate endothelium-dependent vasodilatation, has recently also been shown to cause vasoconstriction (Bigaud & Pelton, 1992), more prominent in veins than in arteries (Moreland *et al.*, 1992; Sumner *et al.*, 1992). There is relatively strong pharmacological evidence (Warner *et al.*, 1989; Douglas & Hiley, 1990; Harrison *et al.*, 1992) in favour of an additional ET_C receptor (ET-3 > ET-1). Although conclusive evidence of its isolation has not yet been provided, suggesting that it shares limited homology with the known sub-types, a candidate receptor has been reported from *Xenopus* melanophores. ET receptors, like many other hormone receptors, have seven transmembrane loops and their intracellular action is mediated through signal-transducing G proteins. Interaction of ET-1 with its receptor on vascular smooth muscle results in activation of phospholipase C with release of the second messengers diacylglycerol and inositol trisphosphate (IP_3). The former activates protein kinase C leading to the phosphorylation of key intracellular proteins while the latter increases free intracellular calcium.

There is intense research interest in the role of ETs in the regulation of vascular tone and in the pathogenesis of hypertension and vascular disease. ETs act as mitogens for a variety of cell types including cancer cells (Shichiri *et al.*, 1991). Because of the widespread distribution of ET receptors, the production of ET in many cell types, and their powerful actions at a cellular level, it is likely that ETs are a

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component of the complex network of locally acting regulatory factors which exists in all organs. This review focuses on the emerging role of ETs in regulating the endocrine system.

Endothelins and the anterior pituitary

The best documented endocrine actions of ETs, to date, are on the hypothalamic–pituitary axis. In the rat, ET and ET receptors have been identified in the hypothalamus and pituitary gland (Matsumoto *et al.*, 1989; Samson *et al.*, 1991a; Takahashi *et al.*, 1991a; Domae *et al.*, 1992). Dispersed rat pituitary cells release ET-3, an effect which is markedly stimulated by insulin-like growth factor-I (IGF-I) and inhibited by TGF-β (Matsumoto *et al.*, 1990). The effect of ET on prolactin release depends on concentration and experimental design. At higher concentrations (in the nanomolar range) there is transient stimulation of prolactin release (Stojilkovic *et al.*, 1990; 1991; Samson *et al.*, 1991b). This effect is mediated through activation of phospholipase C with consequent increased intracellular calcium. However, the predominant, and most sustained, effect of ET is to inhibit prolactin secretion (Samson *et al.*, 1991b; Kanyicska *et al.*, 1991a, b; Domae *et al.*, 1992). Although ET-3 is the major isoform in the pituitary, ET-1 is more potent at inhibiting prolactin release (Kanyicska *et al.*, 1991a, b) while, at least in the rat, ET-2 may be the most potent isoform with inhibition of prolactin release at 1 pM (Samson & Skala, 1992). Inhibition by staurosporine suggests that this action is mediated by protein kinase C. The inhibitory action of ET is not mediated via the dopamine receptor (Samson *et al.*, 1990; Samson & Skala, 1992) and is not sensitive to dihydropyridine calcium channel blockade (Samson *et al.*, 1991b; Samson & Skala, 1992). It is, however, abolished with pertussis toxin and presumably, therefore, involves a G protein (Burris *et al.*, 1991).

ETs stimulate the release of LH, FSH, growth hormone and TSH (Stojilkovic *et al.*, 1990; Samson *et al.*, 1991b; Kanyicska *et al.*, 1991a, b). These actions are inhibited by dihydropyridine calcium channel blockers (Samson *et al.*, 1991b). The stimulation of pituitary hormone release appears to be a local effect since it is not reproduced by either intracerebroventricular or peripheral venous injection of ET (Samson *et al.*, 1991b). However, Moretto *et al.* (1993) have recently shown ET-3 to stimulate GnRH release from arcuate nucleus–median eminence fragments and from a GnRH-secreting neuronal cell line. ET-3 also stimulated release of prostaglandin E_2. The stimulation of GnRH release was blocked by inhibitors of prostaglandin synthesis. The above observations have all been made using rat pituitary, and the actions of ET on human pituitary have not, thus far, been investigated. There is potential for ET to act as a modulator of growth and function both physiologically and in pituitary tumours.

Effects of endothelins on sodium and water balance

These effects are complex. Immunoreactive ET has been identified in the paraventricular and supraoptic nuclei of the hypothalamus and in the terminals of these neurones in the posterior pituitary of the rat (Yoshizawa *et al.*, 1990). The endothelin containing secretory granules of these cells are depleted when the animal is deprived of water. In normal human subjects, plasma ADH and ET-1 concentrations rise in parallel during upright tilt (Kaufmann *et al.*, 1991). This response is lost in patients with diabetes insipidus. There is evidence from studies in dogs that ET may be involved in regulation of ADH release since infusion of ET increases plasma ADH concentration (Nakamoto *et al.*, 1989). Intracerebroventricular injection of ET-1 stimulates release of ADH (Matsumura *et al.*, 1991), an effect which is antagonized by the simultaneous administration of brain natriuretic peptide (Makino *et al.*, 1992).

In the kidney, ET-1 appears to have at least two separate actions. The first is vasoconstriction, affecting both afferent and efferent arterioles, and leading to reduced glomerular filtration and, at high doses, sodium retention (Lopez-Farre *et al.*, 1989). The second is natriuresis. The inner medullary collecting duct (IMCD) of the kidney produces substantial amounts of ET-1 (Kohan, 1991), and contains a high density of endothelin receptors (Kohan *et al.*, 1992). Exogenous ET-1 blocks sodium reabsorption by inhibiting Na^+-K^+-ATPase activity (Zeidel *et al.*, 1989), and water reabsorption by inhibiting the effects of ADH on tubular osmotic permeability (Oishi *et al.*, 1991). More recently (Kohan & Padilla, 1993), it has been shown that ET- release and ET-1 mRNA expression is reduced by exposure of cultured rat IMCD, but not endothelial or vascular cells, to increased osmolality. Urinary ET-1 excretion and inner medullary ET-1 mRNA were also shown to be reduced by volume depletion *in vivo* in rats. Taken together, these findings suggest that locally generated ET-1 plays a tonic physiological role in regulation of salt and water transport in the terminal nephron, with changes in local generation influencing both natriuresis and diuresis. Interestingly, spontaneously hypertensive rats have less IMCD production of ET-1 than Wistar Kyoto rats (Hughes *et al.*, 1992), and associated sodium retention may contribute to hypertension in this strain of rats.

The effects of ET-1 on other vasoregulatory hormones are less clear. In rat kidney cortical slices, isolated glomeruli, and isolated juxtaglomerular cells, ET-1 inhibits basal and

stimulated renin release by a calcium dependent mechanism (Rakugi *et al.*, 1990; Moe *et al.*, 1991). However, ET-1 directly stimulates angiotensin II generation in endothelial cells and in the isolated rat mesenteric bed (Rakugi *et al.*, 1990; Kawaguchi *et al.*, 1991). ET stimulates aldosterone release from isolated bovine and rat adrenal (Cozza *et al.*, 1989; Rosolowski & Campbell, 1990; Hinson *et al.*, 1991a). In the intact animal, ET-1 administration increases plasma renin and aldosterone concentrations, probably because of renal vasoconstriction (Nakamoto *et al.*, 1989). ET-1 is a potent stimulus for atrial natriuretic peptide mRNA accumulation and peptide release from cultured atrial myocytes (Fukuda *et al.*, 1989) and circulating concentrations of atrial natriuretic peptide are increased by ET-1 infusion in rats (Stasch *et al.*, 1989; Garcia *et al.*, 1990). Atrial natriuretic peptide reduces the basal and stimulated secretion of ET from cultured endothelial cells, raising the possibility of a feedback regulatory mechanism (Saijonmaa *et al.*, 1990; Kohmo *et al.*, 1991). The vasoconstrictor action of ET-1 is antagonized by atrial natriuretic peptide (Suzuki *et al.*, 1991). Thus, ETs have a variety of effects on sodium regulatory hormones but their precise effect, if any, in regulation of sodium balance is not known.

Other actions of endothelins on the endocrine glands

Many of the observations reported here remain unconfirmed and their exact significance is not at this stage certain.

The recent identification of ET-1 immunoreactivity in porcine thyroid cells is of interest and may be relevant in goitrogenesis (Colin *et al.*, 1992) although the actions of ET on thyroid cell growth and function remain to be fully evaluated. Recently, Tsushima *et al.* (1992) have shown that ET enhances IGF-I stimulated DNA synthesis in rat FRTL-5 thyroid cells. ET receptors have been identified on human erythrocytes (Jackson *et al.*, 1992) and incubation of thyrocytes with ET inhibits thyroglobulin release by a cyclic nucleotide-independent mechanism. The binding of ET to human thyrocytes is increased by exposure of cells to TGF-β (Tseng *et al.*, 1993). Human thyrocytes also release ET and this release may be modulated by TGF-β and by TSH (Tseng *et al.*, 1993). It has long been known that endothelial proliferation and capillary enlargement are early features in goitre development (Wollman *et al.*, 1978; Ericson & Wollman, 1980; Many *et al.*, 1984). Vascular endothelium is also a source of interleukin-1 (Miyazaki *et al.*, 1989) which is not only a potent mitogen for thyroid cells but also increases ET-1 release from vascular endothelium (Yoshizumi *et al.*, 1990). A further relevant recent observation is that patients with hypo or hyper-thyroidism have increased plasma concentrations of fibronectin and von Willebrand factor, both of which are derived from endothelial cells (Arnaout *et al.*, 1992).

A role for ET in maintenance of calcium balance has been postulated. Rat parathyroid cells express mRNA for pre-proET, secrete ET-1 peptide and express ET_A receptors (Fujii *et al.*, 1991). Cloned rat parathyroid cells release ET-1 in response to atrial and brain natriuretic peptides (De Feo *et al.*, 1991). This is the opposite effect to that of atrial natriuretic peptide on endothelial cells. There are no documented effects of ET on parathyroid hormone secretion. It is tempting to speculate that ET could be involved in the pathogenesis of the hypertension which accompanies hyperparathyroidism. Bone cells, like other endocrine tissues, are in close contact with extensive vascular networks and may, therefore, be exposed to high concentrations of ET. Recently, Alam *et al.* (1992) have shown that ET-1 inhibits osteoclastic bone resorption and cell motility of osteoclasts, effects which are not mediated by changes in intracellular calcium.

It is not known whether ET is secreted by, or has actions on, the endocrine pancreas. Endothelial proliferation and damage contributes to the micro and macro-vascular complications of diabetes. Plasma ET concentrations are increased in patients with diabetes (Takahashi *et al.*, 1990) and in some animal models of diabetes (Takahashi *et al.*, 1991b). Impaired conversion of ET precursors to active peptide has recently been described in patients with diabetes (Tsunoda *et al.*, 1991).

Endothelins may be involved in the regulation of reproductive function. Thus, immunoreactive ET has been identified in the corpus luteum of superovulated rats (Usuki *et al.*, 1991) and in the medium of cultured porcine granulosa cells (Iwai *et al.*, 1991). Recently, ET_A receptors were identified on porcine granulosa cells (Kamada *et al.*, 1992). Incubation of these cells with ET stimulated accumulation of water soluble inositol phosphates and increased intracellular calcium. These authors also reported that ET enhanced progesterone secretion. Previously ET has been shown to inhibit luteinization of granulosa cells, inhibiting LH-induced cAMP accumulation, progesterone secretion and morphological changes (Iwai *et al.*, 1991). Ergul *et al.* (1993) have shown that ET-1 enhances steroidogenesis and proto-oncogene expression in a cell line derived from transformed murine Leydig cells although ET-1 was not mitogenic for these cells.

ET-1 is secreted by endometrial cells (Orlando *et al.*, 1990) increasing intracellular calcium and inducing myometrial contraction (Word *et al.*, 1990). Messenger RNA for all three isotypes of ET and for ET_A and ET_B receptors is present in human endometrium (O'Reilly *et al.*, 1992). ET_A receptor mRNA was the more abundant in the proliferative phase of the cycle while ET_B was predominant in the secretory and

menstrual phases. High affinity ET receptors are present in human myometrium and appear to be regulated by ovarian steroids, being more abundant in premenopausal women (Schiff *et al.*, 1993). Human placenta expresses ET gene and secretes ET-1 and ET-3 peptides (Benigni *et al.*, 1991). ET receptors have been identified on human trophoblastic cells as well as on placental vascular smooth muscle (Mondon *et al.*, 1993). Placental production of ET is almost certainly responsible for the increased plasma concentrations of ET seen in pregnancy and may contribute to systemic and renal haemodynamic changes. Plasma ET is particularly increased in women with pre-eclampsia (Kamoi *et al.*, 1990; Taylor *et al.*, 1990). Branch *et al.* (1991) showed that serum from women with pre-eclampsia actually inhibited ET production by endothelial cells although this observation was not confirmed by others (Brown *et al.*, 1991). The mechanism which controls ET production in pregnancy requires further investigation. ET is found at high concentrations in murine seminal plasma (Casey *et al.*, 1992), although its function is unknown at present.

There are effects of ET on both the adrenal cortex and medulla. Femtomolar concentrations of ACTH stimulate ET release from isolated, perfused rat adrenal and ET stimulates glucocorticoid secretion by dispersed rat adrenal cells (Hinson *et al.*, 1991b). ET-1 and ET-3 are also potent stimulators of steroid secretion from human adrenal cortex cells (Hinson *et al.*, 1991c). Administration of ET increases catecholamine secretion in intact animals (Goetz *et al.*, 1988; Miller *et al.*, 1989). ET also potentiates the responses to catecholamines (Wong-Dusting *et al.*, 1990). Specific ET receptors have been identified on PC12 phaeochromocytoma cells (Martin *et al.*, 1990) and ET-1 stimulates catecholamine release from medullary chromaffin cells (Boarder & Marriott, 1989). A rare form of secondary hypertension is that associated with malignant haemangioendothelioma. These vascular neoplasms secrete large amounts of ET-1 into the circulation (Yokokawa *et al.*, 1991).

Conclusions

ET peptide secretion and ET receptors are widespread in the endocrine system. In most cases, the specific stimulus to ET production and the effects of ET on the growth and function of endocrine cells have not been fully characterized. We can thus largely only speculate as to the physiological and pathophysiological roles of these peptides in endocrinology. Furthermore, most of the work on ET production and action in endocrine tissues has been in rats and ET effects on human endocrine tissues have not been investigated. However, these peptides are likely to be produced in, and have actions on, endocrine tissues in all species. Recent work has demonstrated that ETs and ET receptors are expressed in human thyroid and reproductive tissues. The potent effects of ETs on cellular second messenger systems makes further investigation of their endocrine effects mandatory. Like other locally acting cytokines and growth factors their precise actions and the relative importance of these actions will be difficult to disentangle. The recent development of specific ET receptor antagonists (see Haynes & Webb, 1993) will allow further progress.

In the rat pituitary there is good evidence that ET inhibits prolactin secretion while stimulating the secretion of other anterior pituitary hormones. The interaction of ET with hypothalamic releasing factors and the production or action of ET on different cell types in the pituitary remain to be investigated. Endothelins may be involved in salt and water balance having actions on ADH release as well as on the renin–angiotensin–aldosterone system. Many of the other observations on ET in endocrine tissues are, as yet, unconfirmed and further studies are required.

The effects of ET on growth of endocrine cells are of interest since endocrine tissues are highly vascular and may thus be exposed to high local concentrations of ET. This may well be important in relation to goitre development. There is now much evidence to suggest that ET is a potent growth factor in many cell types. Its role in cell proliferation in endocrine tumours needs to be explored.

Thus, while the effects of ETs on endocrine tissues remain unclear in many cases, evidence is emerging to suggest they may play an important part in a complex network of autocrine and paracrine factors which modulate the actions and production of the classical endocrine hormones.

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Moreover, inhalation of excreta from infected animals is needed for human infection.

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Endothelins come of age

The reports by Clozel and colleagues[1-3] of a pathophysiological role for endothelin, based on the first studies with orally active endothelin antagonists, emphasise the rapidity of progress in this area since the isolation of endothelin-1 in 1988.[4] Moreover, this work, in conjunction with the results of other studies on the role of endothelin in health and disease, indicates the exciting clinical potential of endothelin antagonists.

The endothelins are a family of three peptides with extremely potent and characteristically sustained vasoconstrictor and vasopressor actions. They also have mitogenic and neuroendocrine properties.[5] Endothelin-1 predominates in the vascular endothelium, where it is generated from proendothelin-1 through the action of a unique neutral metalloprotease, "endothelin converting enzyme". Two distinct endothelin receptors were cloned in 1990.[6,7] The ET_A receptor is preferentially activated by endothelin-1 and is highly expressed in vascular smooth muscle cells. It is the main receptor subtype causing vasoconstriction. The ET_B receptor is activated equally by all three endothelin isoforms and is present on the luminal surface of endothelial cells, where it mediates release of endothelium-dependent vasodilator substances, and on the smooth muscle of some tissues, where it causes constriction. The presence of an ET_C receptor subtype, preferentially activated by endothelin-3, remains controversial.

Many studies have shown raised plasma concentrations of immunoreactive endothelin in conditions associated with systemic vasoconstriction, including chronic heart failure, pulmonary hypertension, and vasospastic disorders such as Raynaud's disease and Prinzmetal's angina.[5] However, endothelin-1 acts in a paracrine fashion rather than as a circulating hormone, because vascular endothelin-1 release is mainly abluminal. Consequently, plasma concentrations are a poor reflection of local production. Increased tissue concentrations of, or binding sites for, endothelin may be more useful; both are found in renal disease and myocardial infarction, providing stronger evidence of a local role for endothelin. Persuasive evidence likewise exists for the sustained vasospasm following subarachnoid haemorrhage. Here, plasma immunoreactive endothelin concentrations are highest in patients with cerebral vasospasm, and cerebrospinal fluid endothelin concentrations are raised only in this subgroup. However, to confirm that production of endothelin-1 fulfils a function in health and contributes to pathophysiology studies with specific inhibitors of its generation or action were needed.

The first such studies, in rats, showed that the endothelin converting enzyme inhibitor, phosphoramidon, lowered blood pressure,[8] indicating that endothelin may contribute to cardiovascular homoeostasis. However, these results had to be confirmed with specific receptor blockers, because phosphoramidon is also a weak inhibitor of the neutral endopeptidase that degrades atrial natriuretic peptide. Endothelin antibodies have been used in several experimental preparations in the rat, where they ameliorate the acute renal failure after arterial occlusion and prevent the renal dysfunction associated with cyclosporin. In addition, they limit the extent of myocardial infarction, even when they are given before coronary artery occlusion.

More recent studies with the ET_A-specific antagonist, BQ123, and the combined ET_A and ET_B antagonists, Ro 46–2005 and Ro 47–0203, broadly confirm these findings. In a rat model of chronic renal failure with reduced renal mass, dependent more on abnormal growth and fibrosis than on vasoconstriction, BQ123 prevents renal dysfunction and structural changes.[9] In a rat model of subarachnoid haemorrhage, in which autologous blood is given into the cisterna magna, pretreatment with Ro 46–2005 profoundly reduces cerebral vasospasm.[1] In a similar model in the rabbit, Ro 47–0203 reverses established vasospasm[2] without affecting blood pressure. Studies with antagonists also support a role for endothelin-1 in the maintenance of hypertension in spontaneously hypertensive and deoxycorticosterone-salt treated rats,[3,10] and for regulation of blood pressure in conditions of sodium depletion.[3]

Thus, there is now considerable experimental and some clinical evidence for involvement of endothelin in cardiovascular and renal disease,[5] where it represents a novel target for therapeutic intervention. There are no effective drug treatments for acute or chronic renal failure, and there is still a substantial need for better treatment in subarachnoid haemorrhage, myocardial infarction, and congestive heart failure. Endothelin antagonists should be explored in these conditions, and may also prove useful in hypertension. Orally active inhibitors have now been developed and these questions will shortly be put to the test in clinical trials.

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Adverse reaction reporting and new antipsychotics

Remoxipride, clozapine, and risperidone number among a new generation of antipsychotics. The most cautious conclusions from the clinical trial data are that these compounds are as effective as the older antipsychotic agents but with far fewer extrapyramidal side-effects.[1] Clozapine is probably better than the classic drugs but, because of the risk of neutropenia and agranulocytosis, is restricted to treatment-resistant or intolerant patients, with strict haematological monitoring.[2] These new agents also have interesting modes of action that may point the way to a better understanding of schizophrenia and more efficient drug development in the future. Thus clozapine, for a highly effective antipsychotic, has peculiarly low occupancy at D_2 receptors in vivo,[3] whereas remoxipride has high selectivity for the mesolimbic-specific D_{2b} receptor.[4]

The Committee on Safety of Medicines (CSM) in the UK lately issued a warning of 8 reports of aplastic anaemia among 50 000 patients world wide associated with remoxipride and 4 cases of myocarditis in 5000 UK patients exposed to clozapine.[5] The CSM rightly urges caution—but what do we know about the still widely used classic antipsychotics, especially in view of their less favourable side-effect profile?

Notwithstanding the basic difficulties that classic drugs are only partly effective and liable to cause several neurological, endocrine, and autonomic side-effects, phenothiazines and similar drugs are additionally associated with arrhythmias, hepatic abnormalities, agranulocytosis, thrombocytopenia, convulsions, neuroleptic malignant syndrome, myocarditis, severe tardive dyskinesia, and sudden death.[6] The frequency of agranulocytosis with phenothiazines is 1/1300,[7] which is probably an underestimate since adverse events with standard agents are likely to be under-reported. Thus both myocarditis and life-threatening blood dyscrasias are associated with the older compounds. So, is the burden of risk with remoxipride and clozapine any greater than for phenothiazines? With respect to myocarditis, clozapine use is restricted to treatment-resistant schizophrenia; such patients will almost certainly have had long exposure to phenothiazines which are themselves a potential cause of the cardiac lesions. Moreover, myocarditis is a very difficult condition to define, and is an incidental finding in 10% of routine necropsies.[8] Although inflammatory changes were reported in the clozapine cases (CSM, personal communication) it is not clear if these abnormalities were associated with the myocyte degeneration or necrosis required in the Dallas classification of myocarditis.[9]

Second, much is made of the risk-benefit analysis of the new drugs. At worst they are equieffective and better tolerated then classic neuroleptics. Without proper quantifiable comparisons with other drugs it is difficult to compare the risk profile, but careful elimination of high-risk patients and regular monitoring, to which schizophrenic patients are entitled, will lessen the risk. Resorting to central monitoring, as some suggest, takes the important burden of clinical responsibility away from psychiatrists and runs the risk of pricing the new drugs off the market altogether.

Schizophrenia is a common and devastating illness. I believe the new generation of antipsychotics represents an improvement, and pharmacological research into their mechanisms of action is likely to yield even better agents. The call for closer medical monitoring of these drugs is a welcome step in bringing about overall better care for schizophrenics, but just because the new drugs are being made to walk the plank by the drug regulatory agencies we cannot presume that older and potentially dangerous agents are "safe".

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Evidence for Endothelin-1–Mediated Vasoconstriction in Severe Chronic Heart Failure

Endothelin Antagonism in Heart Failure

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Endothelin-1, generated by vascular endothelium, causes sustained vasoconstriction, mainly through an action on the ET_A receptor subtype; in some tissues, vascular ET_B receptors contribute. As well as affecting tone in resistance and capacitance vessels, endothelin augments the activity of the renin-angiotensin and sympathetic nervous systems, stimulates mitogenesis, and can cause renal vasoconstriction and sodium retention. Since these are features of heart failure, the endothelin system is an attractive target for therapeutic intervention. Plasma endothelin concentrations in heart failure are two to four times normal and correlates with symptomatic and hemodynamic indexes of severity. A crucial question is whether endothelin contributes to the pathophysiology of heart failure or simply acts as a marker of its severity.

Kiowski et al[1] now report evidence that endothelin-1 contributes to vasoconstriction in severe chronic heart failure. Twenty-four patients with New York Heart Association class III chronic heart failure and ejection fractions ≤30% received either the combined ET_A and ET_B receptor antagonist bosentan (100 mg, then 200 mg, each over a period of 15 minutes and separated by 1 hour) or vehicle, both given intravenously, randomly, and double-blind. Angiotensin-converting enzyme inhibitors were discontinued for four half-lives before each study. Baseline endothelin-1 and big endothelin-1 concentrations were increased and correlated directly with the extent of pulmonary hypertension, with right and left heart filling pressures, and with pulmonary vascular resistance as well as inversely with cardiac output. Compared with placebo, bosentan reduced mean arterial pressures by 8%, pulmonary artery pressures by 14%, and pulmonary artery wedge pressures by 9%; cardiac index was increased by 14%, and systemic and pulmonary vascular resistances were reduced by 17% and 33%, respectively. Interestingly, heart rate did not change, and plasma concentrations of angiotensin II and norepinephrine were unaltered.

That endothelin-1 contributes to basal vascular tone and blood pressure was already known, but Kiowski's group provided the first evidence that this mechanism operates in patients with heart failure. Since there was no control group without heart failure, their study does not tell us the extent to which bosentan acted against pathophysiologically raised tone, nor do we know yet whether the benefits add to those of angiotensin-converting enzyme inhibitors or are sustained. Finally, we did not learn from this study whether the increased vasoconstrictor tone is mediated primarily by ET_A or ET_B receptors.

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The Clinical Potential of Endothelin Receptor Antagonists in Cardiovascular Medicine

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Summary

The endothelin family of peptides are extremely potent endogenous vasoconstrictor and pressor agents. Of the 3 isoforms, endothelin-1 is the major isoform produced by the vascular endothelium and is, therefore, likely to be of most importance for regulation of vascular function. Two endothelin receptor subtypes have so far been cloned in mammalian species; ET_A and ET_B. Both receptor subtypes are found on smooth muscle cells and mediate the vasoconstrictor and pressor actions of endothelin. The ET_B receptor is also found on vascular endothelial cells and mediates endothelin-dependent vasodilatation through release of nitric oxide and prostacyclin.

Since their discovery in 1988, the endothelins have been the subject of intense research on their physiological function and potential pathophysiological role in cardiovascular disease. There is now good evidence that endothelin regulates vascular tone and blood pressure, and studies to support the development of endothelin receptor antagonists in conditions associated with chronic vasoconstriction, such as hypertension and heart failure, as well as in vasospastic disorders, such as subarachnoid haemorrhage and Raynaud's disease.

There are now a number of selective ET_A and combined $ET_{A/B}$ receptor antagonists available for preclinical studies. However, it is still not clear which of these will prove to be of most therapeutic value. Some of these agents are cur-

rently being assessed in early phase clinical trials. Endothelin receptor antagonists represent a novel therapeutic approach to a fundamental and newly discovered endogenous vasoconstrictor mechanism. The results of the current clinical trials are awaited with considerable interest.

The endothelins (ETs), first discovered by Yanagisawa and colleagues in 1988,[1] comprise a family of 3 related peptides (ET-1, ET-2 and ET-3), each of 21 amino acids, with 2 intrachain disulphide bridges linking paired cysteine residues (fig. 1).[3] Since 1988, researchers have focused on the potential pathophysiological role of these peptides in a number of cardiovascular diseases.

ET-1 is the most potent mammalian vasoconstrictor peptide known,[1,3] with veins being 3 to 10 times more sensitive to the effects of ET-1 than arteries, both *in vitro*[4] and *in vivo*.[5] ET-1 is also the major isoform produced by endothelial cells[6] and is probably the most important isoform in the cardiovascular system.

Human ET-1 is generated as preproET-1 (fig. 1), a 212 amino acid peptide that is enzymatically cleaved in 2 stages to form a 38 amino acid precursor, big ET-1.[2] Big ET-1 is further processed to form the active peptide by the action of endothelin converting enzyme (ECE).[1,7]

ECE-1 is a unique membrane-bound metalloprotease that has recently been recognised. It cleaves both intra- and extracellular big ET-1 to ET-1 at neutral pH, and is structurally related to neutral endopeptidase 24.11.[8] It may provide a useful target for pharmacological intervention. However, this ECE is relatively selective for big ET-1 and pharmacological studies indicate that there are several other ECEs with different characteristics.

An additional ECE, ECE-2, has also been identified.[9] ECE-2 has an acidic pH optimum, and probably acts as an intracellular enzyme cleaving endogenously synthesised big ET-1 to ET-1 at the trans-Golgi network, where the vesicular fluid is acidified.

Circulating levels of ET-1 are low[10] and approximately 80% of ET-1 synthesised by endothelial cells is secreted abluminally,[11,12] suggesting that ET-1 has primarily paracrine and autocrine actions. A neurohumoral role for the endothelins has also been proposed.[13]

On the basis of molecular studies, 2 endothelin receptors have so far been characterised in mammalian species.[14,15] The ET_A receptor is relatively selective for ET-1,[14] whereas the ET_B receptor has equal affinity for all 3 ET isoforms.[15] Results from early studies[16,17] suggested that the ET_A receptor is restricted to vascular smooth muscle and mediates vasoconstriction, whereas the ET_B receptor is restricted to the endothelium and mediates vasodilatation through the production of endothelium-dependent vasodilators, such as nitric oxide and prostacyclin.[2,3] However, it is now recognised that this concept is an oversimplification because ET_B receptors are also expressed on vascular smooth muscle cells[18] and contraction of human smooth muscle can be generated through either ET_A or ET_B receptor stimulation.[19,20] However, in a wide range of human vessels *in vitro*, ET-1–mediated vasoconstriction occurs predominantly via the ET_A receptor[21] and the importance of the smooth muscle ET_B receptor in humans remains to be clarified.

A putative ET_C receptor, selective for ET-3, has been cloned from a toad melanophore cDNA library,[22] but has not yet been identified within the mammalian genome.

Following brief vasodilator and vasodepressor actions mediated by endothelial ET_B receptors,[1] ET-1 causes sustained vasoconstrictor[23] and pressor effects[24] in humans. Endogenous generation of ET-1 has also been shown to contribute to the maintenance of basal vascular tone and blood pressure.[25-27]

There are many ET receptor antagonists either in, or shortly to be entering, clinical development (table I). Some are peptidic in nature and only likely to be suitable for short term use, whereas others are orally active and may, therefore, have wider applications in the treatment of cardio-

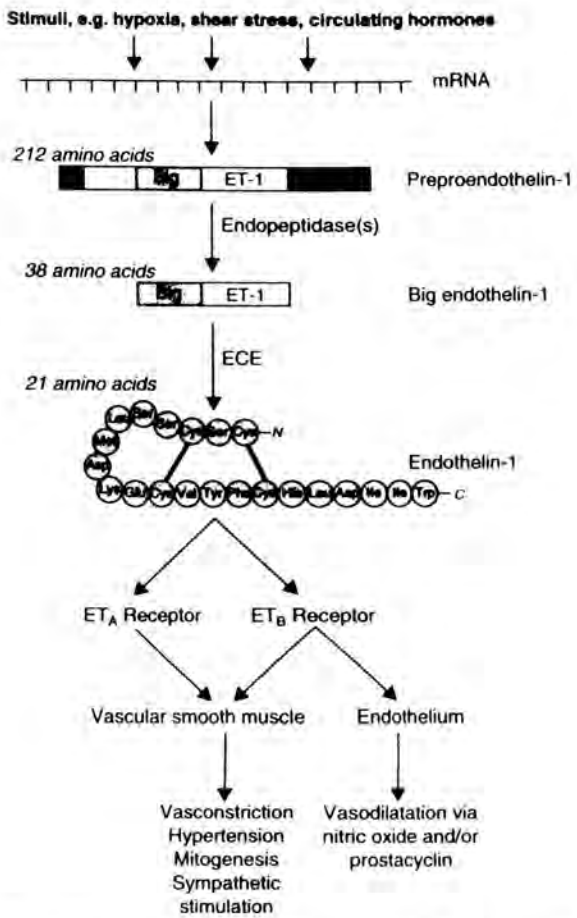

Fig. 1. Endothelin synthesis and pathways. Each endothelin isoform is a product of a separate gene that codes for a large precursor protein mRNA. The human endothelin-1 gene is on chromosome 6 and codes for a 212 amino acid precursor protein known as preproendothelin-1. Synthesis of this protein is stimulated by hypoxia, shear stress and circulating hormones such as angiotensin II, insulin and bradykinin, as well as by growth factors and cytokines. Preproendothelin-1 is processed by dibasic pair specific endopeptidases to form a 38 amino acid protein referred to as big endothelin-1, which is further cleaved by an endothelin converting enzyme (ECE) to the mature peptide, endothelin-1. Endothelin-1 acts on both endothelin A and B receptor subtypes located on vascular smooth muscle cells. These receptors mediate the vasoconstrictor, pressor and mitogenic effects of endothelin-1. The endothelin B receptor is also found on vascular endothelial cells and mediates the vasodilator actions of endothelin-1 (see Haynes & Webb[2] for further details).

vascular disease. Some are ET_A-selective, whereas others are combined ET_A/ET_B antagonists. As vascular smooth muscle ET_A and ET_B receptors can both mediate vasoconstriction in humans,[19,20] there may be some advantages from blocking both of these receptors. However, there may be some benefits from leaving the endothelial ET_B receptor unaffected, as it is known to mediate vasodilatation. Indeed, there is some evidence that the endothelial and smooth muscle ET_B receptors can be distinguished pharmacologically,[28] so it may be possible to develop agents with appropriate selectivity.

A pathophysiological role for the endothelins has been postulated in a wide number of diseases.[2,29] This article examines specifically the evidence implicating ET-1 in the pathophysiology of cardiovascular disease and discusses the potential of endothelin receptor antagonists in cardiovascular medicine. For a more detailed review of the role of the endothelins in cardiovascular disease the reader is referred elsewhere.[30]

1. Hypertension

In their original paper, Yanagisawa et al.[1] suggested that disturbances in the control of endothelin production could contribute to the pathogenesis of hypertension. In addition to its vasoconstrictor and pressor effects, ET-1 has positively inotropic[31] and mitogenic[32] properties and an antinatriuretic action.[33] ET-1 also increases central[34,35] and peripheral sympathetic activity,[36,37] and stimulates the generation of renin, angiotensin II,[38] aldosterone[39] and adrenaline (epinephrine).[40]

Furthermore, at threshold and subthreshold levels, ET-1 potentiates contractile responses to other vasoconstrictor substances such as noradrenaline (norepinephrine) and serotonin (5-hydroxytryptamine; 5-HT).[41] It would, therefore, appear to be entirely appropriate to examine the potential for ET-1 to be involved in the pathophysiology of hypertension.

Knockout gene experiments are unrevealing in this regard. ET-1,[42] ET-3,[43] ET_A[44] and ET_B[43] knockouts all cause severe developmental disturbances. This observation shows the importance of the endothelins in growth and develop-

Table I. Endothelin receptor antagonists

Selectivity	Compound	Company	Comments
ET_A	BQ-123	Banyu	Cyclic pentapeptide
	BQ-153	Banyu	Linear tripeptide
	BQ-485	Banyu	Linear tripeptide
	FR-139317	Fujisawa	Pseudo-tripeptide
	TTA-386	Takeda	Synthetic hexapeptide
	PD-151242	Parke-Davis	Pseudo-tripeptide
	50-235	Shionogi	Caffeoyl ester
	97-139	Shionogi	Modification of 50-235
	BMS-182874	Bristol-Myers Squibb	Benzenesulphonamide[a]
ET_B	BQ-788	Banyu	Tripeptide
	RES-701-1	Kyowa Hakko Kogyo Co.	16-amino-acid cyclic peptide endothelial ET_B selective
ET_A and ET_B	PD-142893	Parke-Davis	Linear hexapeptide
	PD-145065	Parke-Davis	Linear hexapeptide
	TAK-044	Takeda	Cyclic hexapeptide
	RO-462005	Hoffman-La Roche	Sulphonamide[a]
	Bosentan (RO-470203)		Sulphonamide[a]
	CGS-27830	Ciba-Geigy	Irreversible binding, unstable
	SB-209670	SmithKline Beecham	Carboxylic acid derivative[a]

a Orally active.

ment, but is not helpful in clarifying their physiological effects in cardiovascular regulation.

Plasma endothelin levels are not increased in animal models of hypertension unless malignant hypertension or renal dysfunction are present.[45] However, because ET-1 is preferentially secreted abluminally,[11,12] locally increased production may not necessarily result in increased plasma endothelin levels. Indeed, increased immunoreactive endothelin levels have been reported in both aortic and mesenteric arteries of DOCA-salt hypertensive rats, despite normal plasma endothelin levels, suggesting that increased vascular generation of ET-1 may be involved in some forms of hypertension.[46] Elevation of plasma levels of big ET-1 and the C-terminal fragment of big ET-1 may be useful in showing that ET-1 production is increased.[47]

In vitro studies using resistance arteries taken from animal models of hypertension have shown either increased[48] or decreased[49] vascular sensitivity to ET-1. Systemic doses of ET-1 *in vivo* have greater pressor effects in spontaneously hypertensive rats (SHR) than in Wistar-Kyoto rats[50] and in renovascular hypertensive rabbits than in normotensive ones.[51] The interpretation of these results is difficult given the propensity for vascular hypertrophy to nonspecifically enhance responses to vasoconstrictors.

Endothelin-specific antibodies[52] and the selective ET_A antagonists BQ-123[53] and FR139317[54] have been shown to lower blood pressure in animal models of hypertension. Long term administration of BQ-123 has been reported to prevent the development of stroke and renal abnormalities in stroke-prone SHR.[55] Non-peptide combined ET_A/ET_B receptor antagonists, SB-209670[56] and RO-462005[57], lower blood pressure in SHR and conscious, normotensive, salt-depleted monkeys, respectively.

Although some studies[52,55] have reported a reduction of blood pressure only in hypertensive animals, it must be remembered that the absolute effects of antihypertensive agents are proportionately greater the higher the pretreatment blood pressure. Few studies have been of sufficient power to justify the interpretation that endothelin

receptor antagonists lower blood pressure only in hypertensive animals.

Plasma endothelin levels are not increased in patients with essential hypertension and normal renal function,[58,59] although very high levels are found in severe and malignant hypertension,[60] probably as a result of impaired renal clearance. Increased plasma endothelin levels have been reported in the presence of normal renal function in patients with pre-eclampsia.[61] Plasma endothelin levels were also increased in 2 patients who developed hypertension with the skin tumour haemangioendothelioma.[62] Tumour cells showed increased expression of ET-1 mRNA and increased staining for the peptide. Blood pressure returned to normal in both cases after tumour resection, and recurrence of the tumour in one of the patients was associated with a further rise in blood pressure and plasma endothelin levels.

Sensitivity to ET-1 *in vitro* appears to be decreased in resistance arteries taken from hypertensive patients,[63] but increased in the capacitance vessels of hypertensive patients *in vivo*.[59] These latter vessels do not develop hypertrophy. Thus, it is possible that increased sensitivity to, and not necessarily increased production of, ET-1 may be involved in the pathophysiology of hypertension, indicating an abnormality at the receptor or postreceptor level.

The selective ET_A receptor antagonist BQ-123 has been shown to produce arterial vasodilatation in both healthy volunteers[25] and hypertensive patients.[64] Also, TAK-044, a combined ET_A/ET_B receptor antagonist, has recently been reported to lower blood pressure in healthy men.[27]

Hypertension is associated with the development of several cardiovascular diseases, including angina pectoris, myocardial infarction, peripheral vascular disease and cerebrovascular disease. It is possible that the vasoconstrictive properties of ET-1 could contribute to myocardial ischaemia and that the proliferative effects of ET-1 could contribute to vascular and cardiac hypertrophy and the atherosclerotic process. Indeed, plasma endothelin levels are increased in advanced atherosclerosis,[65] and expression of ET-1 mRNA is increased in the vascular smooth muscle of atherosclerotic human arteries.[66] Furthermore, increased tissue endothelin immunoreactivity has been reported in the active atherosclerotic lesions associated with unstable angina.[67]

With the multitude of drugs already available to treat hypertension, a new class of antihypertensive agents may seem unnecessary. However, endothelin receptor antagonists may prove more effective than current therapies in preventing or reversing some of the important complications that are little affected by current therapy, such as myocardial infarction.[68]

2. Unstable Angina and Myocardial Infarction

The sarafotoxins, potent vasoconstrictor peptides isolated from snake venom with close structural similarity to the endothelins, cause death from myocardial ischaemia and infarction secondary to coronary vasoconstriction.[69] Exogenously administered ET-1 also produces myocardial ischaemia[70] by causing coronary vasoconstriction. Plasma endothelin levels[71] and myocardial ET-1 binding sites[72] are increased during reperfusion following ischaemia in animals. Interestingly, in animal models of myocardial infarction, ECE inhibitors,[73] monoclonal antibodies against ET-1,[74] selective ET_A antagonists[75] and combined ET_A/ET_B antagonists[76] have been reported to reduce myocardial infarct size.

In humans, plasma endothelin levels are increased in acute myocardial infarction and unstable angina,[77] suggesting a possible pathophysiological role for ET-1. Patients with stable angina do not have increased plasma endothelin levels.[78] The higher the plasma endothelin level in myocardial infarction[79] and unstable angina[80] the worse the prognosis. Plasma endothelin levels on the third day after myocardial infarction are significantly related to mortality,[79] and plasma endothelin levels at 9 weeks after hospitalisation with unstable angina or non–Q-wave myocardial infarction are significantly related to the incidence of further cardio-

vascular events.[80] Patients undergoing fibrinolysis during the acute phases of myocardial infarction have been shown to have reduced plasma endothelin levels compared with patients who did not have early reperfusion.[81] As discussed previously, the increased tissue endothelin immunoreactivity in active atherosclerotic plaques causing unstable angina[67] suggests a possible local role for ET-1 in the associated vasospasm.

The use of endothelin receptor antagonists in acute myocardial infarction may be of clinical benefit. First, they may limit infarct size and thereby reduce or slow the progression to heart failure. Secondly, they may reduce the incidence of further ischaemic events or the need for revascularisation. Thirdly, they may prevent remodelling after infarction, possibly in a fashion similar to ACE inhibitors. However, given the wide range of drugs currently available for the treatment of myocardial infarction, and the probability of diminishing returns with additional therapy, companies may be wary of developing endothelin receptor antagonists in this indication.

Coronary sinus ET-1 levels are increased during and immediately after percutaneous transluminal coronary angiography (PTCA).[82] Although ET-1 could be involved in the ischaemia, acute vasospasm and abrupt vessel closure related to PTCA, these complications are uncommon and readily reversible with conventional therapy. The main weakness of PTCA is the relatively high risk of restenosis at a later stage. Clinical restenosis occurs in up to 30% of patients within the first year following the procedure[83] and is characteristically associated with vascular smooth muscle proliferation. Although there is currently no experimental evidence that endothelin antagonists reduce experimental restenosis, it is conceivable, given the co-mitogenic actions of ET-1, that prolonged treatment with an oral endothelin antagonist might be useful. Similar arguments are relevant to graft occlusion after coronary artery bypass grafting.

3. Variant Angina

Prinzmetal's or variant angina, first described in 1959,[84] is characterised by chest pain developing at rest, frequently in the early morning, and associated with ST elevation on the electrocardiogram. The pain is usually relieved by nitroglycerin (glyceryl trinitrate). Coronary spasm has been demonstrated at angiography in patients with this condition, as well as the absence of fixed stenotic lesions.[85]

Patients with variant angina are known to have endothelial dysfunction affecting the L-arginine–nitric oxide system[86] and, as a powerful vasoconstrictor of human[41] and canine[70] coronary arteries, ET-1 has been implicated in the pathophysiology of this condition.[87] ET-1 also potentiates the coronary vasoconstriction induced by serotonin and noradrenaline in isolated human arteries.[41] Patients with variant angina have increased plasma endothelin levels during provocation of coronary vasospasm[88] and one study has also found basal plasma levels of endothelin to be increased.[89] Interestingly, there is an increased prevalence of primary Raynaud's disease and migraine in patients with variant angina[90,91] and, as discussed later, ET-1 has also been implicated in the pathophysiology of these vasospastic disorders.

It appears, therefore, that ET-1 has a pathophysiological role in variant angina, either as a mediator of coronary vasospasm or by sensitising the vasculature to other vasoconstrictors. In either case, endothelin receptor antagonists might prove useful in the management of this condition.

4. Heart Failure

The many definitions of congestive heart failure (CHF) highlight only selected features of this complex syndrome.[92] Patients with CHF have objective evidence of major cardiac dysfunction at rest, develop ankle swelling and are typically breathless or fatigued either at rest or during exertion.[92] Neuroendocrine activation occurs in patients with CHF[92,93] and elevation of plasma levels of nor-

physiological role for endothelin in this condition. However, considerably more experimental evidence from animal models is required before clinical trials with endothelin antagonists are likely to be embarked on in this indication.

9. Migraine

Migraine headache is a very common condition characterised by a persistent unilateral headache of moderate to severe intensity frequently accompanied by nausea, vomiting and photophobia and sometimes associated with an aura.[149] The prevalence of migraine ranges widely, depending on the diagnostic criteria used. However, using the International Headache Society Classification,[149] the 1-year-period prevalence of migraine is ≈10%.[150] Serotonin appears to play an important role in the pathophysiology of migraine[151] and serotonin receptor agonists have proved of major therapeutic value in its treatment.[152-154]

Studies have shown local cerebral hypoperfusion during migraine attacks, especially those associated with an aura.[155-157] This hypoperfusion can last for several hours after the onset of the pain and can be followed by hyperperfusion. The vasoconstriction associated with the first phase of a migraine attack could be attributed, at least in part, to the release of vasoactive substances, such as the endothelins. Indeed, plasma endothelin levels have been found to be increased during migraine headaches.[158,159] Levels of endothelin have not been found to be higher between attacks, or in patients with episodic or chronic tension headaches,[159] suggesting that the rise in endothelin is specific to migraine and not merely a response to headache. Therefore, it appears possible that endothelin has a role in the pathophysiology of migraine either directly or by mediating the effects of 5-HT. Alternatively, endothelin may be produced as a result of brain ischaemia[148] or changes in vascular shear stress[160] and hence be a secondary phenomenon.

It has been proposed that dural blood vessels play a central role in headache pathogenesis.[161] Indeed, both the ergot alkaloids[162] and sumatriptan[163] block peripheral small-fibre–dependent neurogenic inflammation within the dura mater in a rat model. It also appears that endothelin receptor antagonists may block neurogenic inflammation, providing additional support for the view that these agents may be useful in the treatment of migraine headaches.

10. Acute Ischaemic Renal Failure

Acute renal failure (ARF) secondary to renal ischaemia is characterised by intense renal vasoconstriction and severe depression of renal function that may necessitate haemodialysis.[164,165] The renal vasculature is very sensitive to the actions of endothelin. When infused at doses that have minimal vasoconstrictive effects, ET-1 inhibits sodium reabsorption by its actions on the ET_A receptor in the rat,[166] and by its actions on the ET_B receptor in the dog.[167] A possible autocrine role for endothelin in the regulation of body volume status and water reabsorption has been proposed.[168] At higher doses, exogenous ET-1 causes potent and long-lasting vasoconstriction similar to that seen in ARF,[169,170] together with a reduction in renal blood flow, GFR and urine production.[171,172] In the rat, the renal vasoconstriction to ET-1 is mediated by the ET_B receptor,[173] whereas in the dog, renal vasoconstriction appears to be mediated by the ET_A receptor.[167]

As a consequence of the interspecies variations in renal endothelin receptor function, caution should be applied when extrapolating the results from animal experiments to humans. The human kidney, as in the dog, is rich in ET_B receptors. There are at least twice as many ET_B receptors as there are ET_A receptors, which are limited mainly to the vasculature.[174] On this basis, the dog may be a better model of human ARF than the rat.

Renal ischaemia and reperfusion increase immunoreactive ET-1 binding affinity,[175] ET_A and ET_B receptor numbers on the renal vasculature,[176,177] ET-1 mRNA expression[178] and plasma and urine immunoreactive ET-1 in rats.[170] In the rat, antiendothelin monoclonal antibodies prevent renal vasoconstriction following ischaemia-induced ARF.[170] This has been confirmed

with the selective ET_A antagonist BQ-123,[166,179,180] and the combined ET_A/ET_B antagonists RO-462005[57] and TAK-044.[181] In the dog, BQ-123 has no effect on the reduction in GFR produced by ischaemia.[182,183] Although renal vasoconstriction in the dog is mainly ET_A receptor-mediated,[174] the combined ET_A/ET_B antagonist SB-209670 does attenuate this reduction in GFR.[183] This suggests that endothelin may be involved in the pathophysiology of ARF in the dog by actions other than vasoconstriction, possibly through its actions on the renal tubules, mediated by the ET_B receptor.[183]

In humans, plasma immunoreactive ET-1 levels are significantly increased in ARF.[184] There is currently no effective drug therapy for ARF[165] and, from the accumulating animal evidence, endothelin receptor antagonists show great potential in this condition. From the work done on dog models of ARF and the similarities in receptor distribution between dog and human kidneys, combined ET_A/ET_B receptor antagonists may be more useful in the treatment of ARF than selective ET_A or ET_B antagonists.

Endothelin has also been implicated in the acute renal dysfunction and hypertension associated with nephrotoxic agents such as cyclosporin[185,186] and x-ray contrast media.[187] With the increasing use of cyclosporin in immunosuppressive drug regimens, endothelin receptor antagonists may have an expanding potential role in the treatment of cyclosporin nephrotoxicity.

11. Conclusions

Endothelin antagonists have proved extremely useful in extending our understanding of cardiovascular physiology and for providing new insights into the pathophysiology of cardiovascular disease. A broad body of experimental and clinical evidence now exists to support the clinical development of drugs that block the production or actions of endothelin for use in cardiovascular medicine. There is particularly good evidence to support their development in conditions associated with chronic vasoconstriction, such as hypertension and heart failure, as well as in vasospastic conditions, such as SAH and Raynaud's disease.

To date, most of the work has been with endothelin receptor antagonists. With the recent cloning of ECE-1, it is likely that selective ECE inhibitors will soon be developed, and these may also prove to have properties of benefit in the treatment of cardiovascular disease. It is still not clear whether combined ET_A and ET_B antagonists have therapeutic advantages over selective ET_A antagonists, as both these receptors can contribute to ET-1–induced vasoconstriction in humans. Theoretically, the combination of an ET_A receptor antagonist with a smooth muscle–selective ET_B receptor antagonist would be most effective because it would leave dilator ET_B receptors unblocked. However, antagonists with this selectivity are still awaited.

Currently, combined ET_A and ET_B receptor antagonists appear to have the widest potential for clinical application and several such agents are currently being assessed in phase I trials. These drugs represent a novel therapeutic approach to a fundamental and newly discovered vasoconstrictor mechanism and the results of the clinical trials are awaited with considerable interest.

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The Endothelin System and Its Potential as a Therapeutic Target in Cardiovascular Disease

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ABSTRACT. Endothelin (ET)-1, an endothelium-derived peptide, is the most potent vasoconstrictor agent described to date. ET-1 also has positive inotropic and chronotropic effects in the heart and is a co-mitogen in both cardiac and vascular myocytes. The major elements of the system involved in formation of ET-1 and its isopeptides, as well as the receptors mediating their effects, have been cloned and characterised. Antagonists of the ET receptors are now available, and selective inhibitors of the ET-converting enzymes are being developed. Early studies using receptor antagonists support the involvement of ET-1 in the pathophysiology of several cardiovascular diseases. The relative merits of ET-converting enzyme inhibitors and receptor antagonists for the treatment of cardiovascular disease are discussed. *Copyright © Elsevier Science Inc.* PHARMACOL. THER. 72(2): 109–148, 1996.

KEY WORDS. Endothelin genes, endothelin converting enzyme, endothelin receptor genes, endothelin receptor antagonists, hypertension, chronic heart failure.

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ABBREVIATIONS. ACE, angiotensin-converting enzyme; CHF, congestive heart failure; CSF, cerebrospinal fluid; cDNA, complementary DNA; DOCA, deoxycorticosterone acetate; ECE, endothelin-converting enzyme; ET, endothelin; NEP, neutral endopeptidase; NO, nitric oxide; PKC, protein kinase C; PTCA, percutaneous transluminal coronary angioplasty; SAH, subarachnoid haemorrhage; SHR, spontaneously hypertensive rat; SRTX, sarafotoxin; WKY, Wistar-Kyoto

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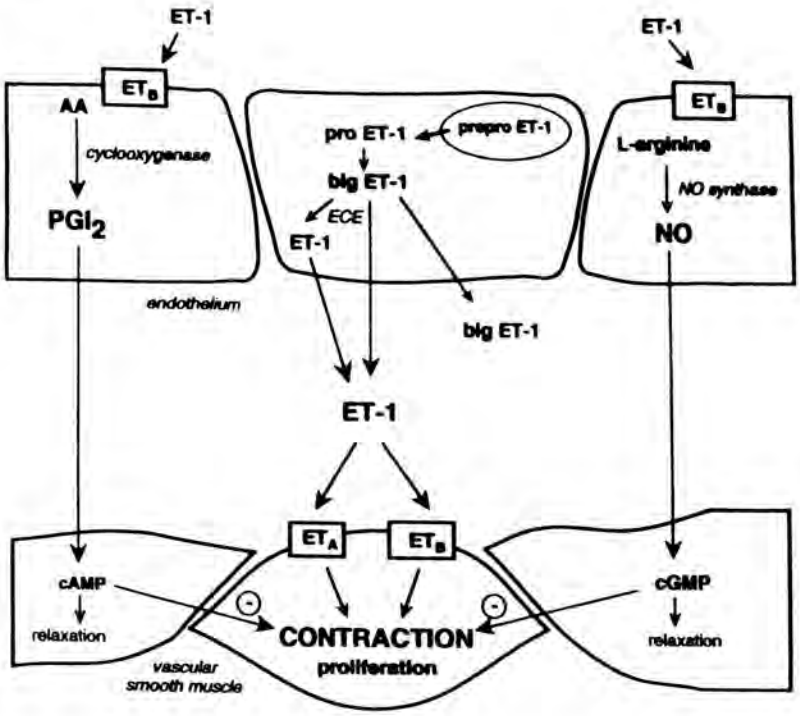


FIGURE 1. Endothelium-derived factors that regulate vascular smooth muscle tone. ET-1, formed in endothelial cells through the action of ECE from its precursor peptide prepro ET-1, promotes both constriction and proliferation of the underlying smooth muscle cells through both ET_A- and ET_B-type receptors. NO derived from L-arginine and prostacyclin (PGI_2) derived from arachidonic acid (AA) cause relaxation through cyclic GMP (cGMP) and cyclic AMP (cAMP), respectively, and also inhibit the vasoconstrictor and proliferative actions of ET-1. ET-1 can initiate relaxation through these pathways via stimulation of ET_B-type receptors located on the vascular endothelium. See text for details.

1. INTRODUCTION

Contrary to early impressions that the endothelial cell monolayer lining blood vessels simply functioned as a diffusion barrier, it is now recognised that the endothelium has a pivotal role in maintaining vascular homeostasis (Vane *et al.*, 1990). Over the past 20 years, there has been a rapid development in our understanding of the central role that locally acting endothelium-derived hormones play in the physiology of blood vessel function. Furthermore, endothelial cell dysfunction is now recognised as being a crucial factor underlying the pathophysiology of cardiovascular disease (Lüscher, 1992). Important milestones that have contributed to our current understanding (Fig. 1) include the discovery of prostacyclin (Moncada *et al.*, 1976), the classical experiments of Furchgott that revealed the role of the endothelium in mediating relaxation in response to acetylcholine (Furchgott and Zawadski, 1980), the identification of nitric oxide (NO) derived from L-arginine as an endothelium-derived relaxing factor in 1987 (Ignarro *et al.*, 1987; Palmer *et al.*, 1987), and the recognition, in 1985, that endothelial cells in culture release a constrictor factor into the bathing medium (Hickey *et al.*, 1985; Gillespie *et al.*, 1986).

In 1988, Yanagisawa *et al.* reported that this endothelium-derived factor, the most potent constrictor described to date, was a 21 amino acid peptide, which they termed endothelin (ET) (Yanagisawa *et al.*, 1988b). Subsequent studies have shown that the ET isolated from endothelial cells is one of a family of isopeptides (Fig. 2), all of which are formed through a two-step processing pathway (Fig. 3) from their respective precursor peptides that share high sequence homology, but are encoded by distinct genes. It is also now clear that the ET isopeptides share remarkable structural similarity to the sarafotoxins (SRTXs), peptides isolated from the venom of the Israeli burrowing asp *Atractaspis engaddensis* (Kloog *et al.*, 1988). The ETs and the SRTXs act through common receptors to evoke a multitude of effects. Indeed, isoforms of SRTX have been utilised extensively as tools for the characterisation of ET receptors (Sokolovsky, 1994).

ET-1 is the major isopeptide produced by human endothelial cells and is present in greatest concentration in the blood. When exogenous ET-1 is infused into the human forearm, it causes profound and long-lasting vasoconstriction, indicating its potential as an important regulator of vascular tone in humans (Clarke *et al.*, 1989a; Vierhapper *et al.*, 1990). Although endogenous ET-1 can be detected in the human circulation, the concentrations are very low (in the picomolar range). However, as ET-1 is released predominantly in an abluminal direction towards the underlying smooth muscle (Wagner *et al.*, 1992a), the tissue concentration is likely to be sufficiently high to activate local receptors. Recent studies carried out using inhibitors of ET synthesis or receptor antagonists suggest that ET 1 is released tonically to maintain basal systemic vascular resistance in humans (Haynes and Webb, 1994). In this way, ET-1 might balance the dilator actions of NO, which is also thought to be released in a tonic manner (Vallance *et al.*, 1989; Haynes *et al.*, 1993). However, these two agents do not function in an exactly parallel manner to control tone. While synthesis of NO can be increased within minutes in response to various stimuli, ET synthesis is regulated at the

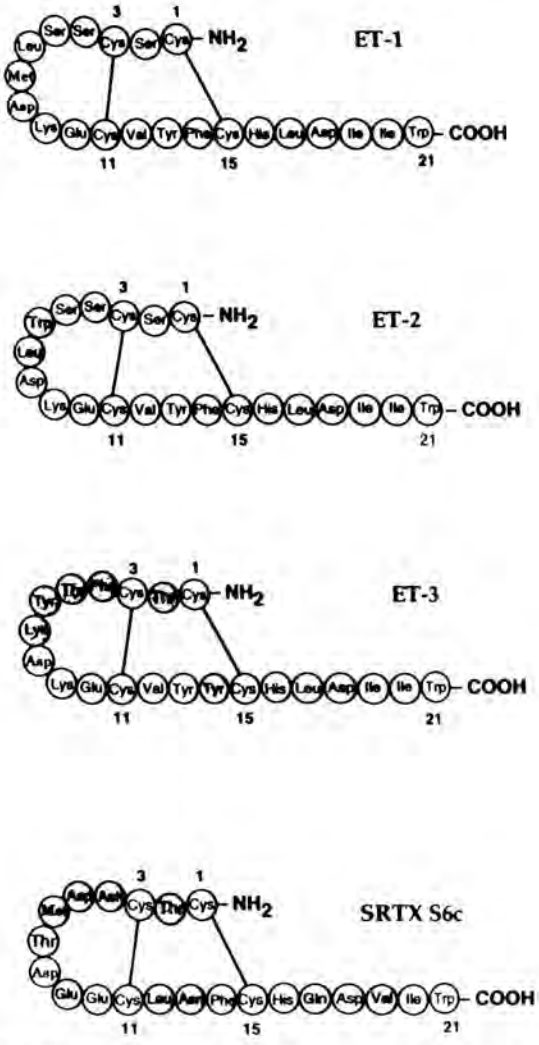


FIGURE 2. Structures of the ET isopeptides and SRTX S6c. The filled circles indicate the amino acids that differ from those in ET-1.

transcriptional level with a resultant delay in release (Yanagisawa *et al.*, 1988a; Boulanger and Luscher, 1990). The two agents also differ with respect to the duration of their action. NO has a short half-life and its effects can be terminated quickly by cessation of release. In contrast, the evidence to date suggests that endothelial ET-1 binds to its receptors on smooth muscle irreversibly and its constrictor and pressor actions are of long duration (Hirata *et al.*, 1988; Yanagisawa *et al.*, 1988b).

Intensive research carried out since the original description of ET-1 has greatly improved our understanding of the ET 'system,' from the genes that encode for ET-1 and its isopeptides, to the enzymes involved in its synthesis and the receptors and signalling pathways that mediate its actions. The purpose of this review is to summarise our current understanding of the ET system, then to consider the prospects for pharmacological intervention using ET-converting enzyme (ECE) inhibitors and ET receptor antagonists, particularly in relation to treatment of cardiovascular disease.

2. ENDOTHELIN GENERATION AND CLEARANCE

2.1. *Endothelin Genes and Regulation*

The first descriptions of ET-1 and cloning of the complementary DNA (cDNA) encoding its precursor prepro ET-1 (Itoh *et al.*, 1988; Bloch *et al.*, 1989b) were rapidly followed by identification of at least three genes encoding 'ET like' sequences in mammalian genomes (Inoue *et al.*, 1989). These sequences subsequently were shown to encode the precursors of ET-2 and ET-3, in addition to prepro ET-1. In the human genome, the ET-1 gene is found on chromosome 6 (Bloch *et al.*, 1989b; Hoehe *et al.*, 1993), the ET-2 gene on chromosome 1 (Bloch *et al.*, 1991) and the ET-3 gene on chromosome 20 (Bloch *et al.*, 1989a).

Like other eukaryotic genes, the genes that encode the ET precursors have promoter regions through which external factors are able to modulate transcription (reviewed in Hilkert *et al.*, 1992; Benatti *et al.*, 1994). Selective gene modification has demonstrated regions necessary for high level transcription (Lee *et al.*, 1990; Wilson *et al.*, 1990; Lee *et al.*, 1991b) and also regions that might determine the tissue selectivity of ET-1 expression (Benatti *et al.*, 1993). Extracellular factors can influence ET-1 generation both positively and negatively through liberation of a series of intracellular mediators that modulate gene transcription (Table 1). Several agents, including insulin, thrombin, low density lipoprotein, angiotensin II, vasopressin and ET-1 itself (Emori *et al.*, 1991; Boulanger *et al.*, 1992; Emori *et al.*, 1992; Kohno *et al.*, 1992; Benatti *et al.*, 1994) enhance ET-1 generation via activation of protein kinase C (PKC). Responsiveness to PKC is mediated by binding of the proto-oncogenes Jun and Fos to the Activator Protein-1 transcription regulatory element of the ET-1 promoter (Curran and Franza, 1988; Lee *et al.*, 1991a). PKC activation is also thought to be a mechanism by which low levels of shear stress (1.8 dyne/cm^2) enhance endothelial ET-1 release (Kuchan and Frangos, 1993; Wang *et al.*, 1993). Interestingly, higher levels of shear stress (>6 dyne/cm^2) activate another mechanism that inhibits ET-1 mRNA transcription (Kuchan and Frangos, 1993; Malek *et al.*, 1993). This effect is prevented by inhibitors of NO synthesis and by methylene blue, an inhibitor of guanylate cyclase, suggesting that endothelial cells release NO in response to shear stress and inhibit ET-1 synthesis through formation of cyclic GMP. Cyclic GMP is also implicated in inhibition of ET-1 synthesis by thrombin (Boulanger and Luscher, 1990), heparin (Yokokawa *et al.*, 1993), atrial and brain natriuretic peptides (Kohno *et al.*, 1992) and by the prostanoids prostaglandin E_2 and prostacyclin (Prins *et al.*, 1994). One action of cyclic GMP is to reduce the availability of intracellular calcium, an action that might be relevant for inhibition of ET-1 synthesis, as calcium chelation similarly reduces ET-1 liberation from endothelial cells (Emori *et al.*, 1992).

Recent studies have shown that different isoforms of the ET precursor mRNA can arise due to the presence of alternative transcription initiation sites in the ET-1 gene that

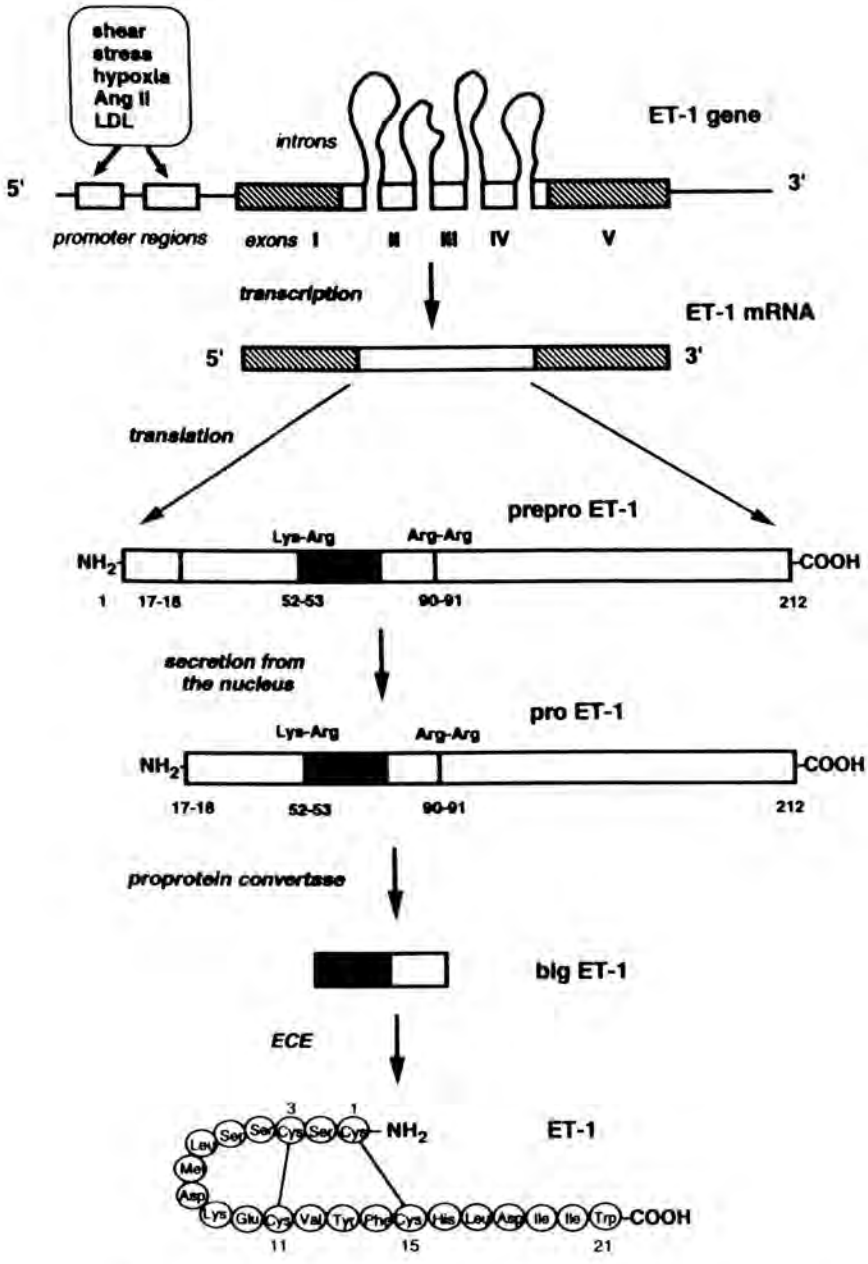


FIGURE 3. Processing pathway of ET-1. The gene encoding ET-1 on chromosome 6 contains 5 exons and 4 intervening intron sequences. The 5′ end of the gene contains promoter sequences through which external factors (including angiotensin II [Ang II] and low density lipoprotein [LDL]) can influence gene transcription. The mRNA untranslated regions at both 5′ and 3′ ends (hatched areas), the 3′ untranslated region, has a role in determination of mRNA stability. Once translated, an amino terminal signal sequence (amino acids 1–17) is cleaved on secretion of the prepro peptide from the nucleus. Big ET-1 is formed through proteolysis of pro ET-1 by dibasic pair endopeptidase enzymes, then mature ET-1 is formed through cleavage of big ET-1 at Trp^{21}-Val^{22} by a specific ECE. See text for details.

are controlled by different promoter regions (Benatti *et al.*, 1993) and also by alternative splicing of the ET-2 and ET-3 genes during transcription (O'Reilly *et al.*, 1992, 1993). The consequences for translation efficiency and for subsequent conversion of the ET precursors remain to be shown. Future investigations should also demonstrate whether insertion polymorphisms identified in the noncoding region of the ET-1 gene (Stevens and Brown, 1995) might, like the polymorphisms in the angiotensin-converting enzyme (ACE) gene (Cambien *et al.*, 1994), prove to be related to risk of developing cardiovascular disease.

2.2. *Processing of the Endothelin Precursors*

All of the ETs are formed through a two-step processing pathway from their respective precursor peptides. In the case of human ET-1 (Fig. 3), removal of the signal sequence on secretion of the 212 amino acid prepro ET-1 from nucleus to cytoplasm is followed by the first proteolytic step that cleaves between Lys^{52}-Arg^{53} and Arg^{90}-Arg^{91} to release the 38 amino acid precursor, big ET-1. This step is thought to be similar to the processing of other peptide hormones and may be dependent on one of the recently described proprotein convertases (Steiner *et al.*, 1992; Seidah *et al.*, 1993). Furin, a proprotein convertase of the constitutive secretory pathway, has been proposed as a likely candidate (Laporte *et al.*, 1993). Examination of the carboxy terminal portions of pro ET-1 remaining after cleavage of big ET-1 reveals the presence of sequences that encode 'ET-like' peptides (Yanagisawa *et al.*, 1988a; Bloch *et al.*, 1989b, 1991). Although these peptides have significant structural homology to the mature ETs, with the relative positions of

TABLE 1. Factors that Influence ET-1 Biosynthesis

Factor	Effect	Cellular signal	Gene promoter element	Reference
Thrombin	Increase	PLC/PKC	AP-1	Emori *et al.*, 1992
	Decrease	NO/cGMP		Boulanger and Lüscher, 1990
Heparin	Decrease	NO/cGMP		Yokokawa *et al.*, 1993
PGI_2/PGE_2	Decrease	cGMP		Prins *et al.*, 1994
ANP/BNP	Decrease	cGMP		Kohno *et al.*, 1992
LDL	Increase	PLC/PKC	AP-1	Boulander *et al.*, 1992
Insulin	Increase	PLC/PKC	AP-1	Oliver *et al.*, 1991
Ang II	Increase	PLC/PKC	AP-1	Emori *et al.*, 1991
Vasopressin	Increase	PLC/PKC	AP-1	Emori *et al.*, 1991
Shear stress	Increase	Disruption of Cytoskeleton		Morita *et al.*, 1993
	Increase	PKC		Kuchan and Frangos, 1993
	Decrease	NO/cGMP		Kuchan and Frangos, 1993
Hypoxia	Increase			Elton *et al.*, 1992
Cyclosporin	Increase	TGF-β	NF-1	Takeda *et al.*, 1993

PGI_2, prostacylin; PGE_2, prostaglandin E_2; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; LDL, low-density lipoprotein; Ang II, angiotensin II; PLC, phospholipase C; cGMP, cyclic GMP; TBF-β, transferming growth factor-β, NF-1, nuclear factor-1.

the four cysteine residues conserved, they do not appear to retain any biological activity (Yanagisawa *et al.*, 1988a; Cade *et al.*, 1990).

Big ET-1 is several orders of magnitude less active than ET-1 for displacement of binding to ET receptors and also in stimulating vascular constriction *in vitro* (Hirata *et al.*, 1990). Final processing of big ET-1 to release the biologically active 21 amino acid ET-1 requires selective cleavage of the Trp^{21}-Val^{22} bond in the carboxy terminal of big ET-1, catalysed by activity referred to as ECE. Several ECE-like enzyme activities representing different endopeptidase classes have been identified (reviewed in Opgenorth *et al.*, 1992; Turner and Murphy, 1995). These include serine proteases (Yanagisawa *et al.*, 1988b; McMahon *et al.*, 1989; Takaoka *et al.*, 1990a; Kaw *et al.*, 1992; Wypij *et al.*, 1992), aspartic proteases, such as pepsin (Takaoka *et al.*, 1990b) and cathepsin D (Sawamura *et al.*, 1990), and soluble thiol protease (Deng *et al.*, 1992). Although inhibitors of these enzymes can prevent conversion of big ET-1, their contribution to ET-1 biosynthesis is not thought to be of major physiological significance. Cleavage by some of them at positions in addition to Trp^{21}-Val^{22} results in simultaneous degradation of ET. Some of these pathways, however, might play a role in pathophysiological ET-1 production, for example, in states associated with mast cell degranulation (Wypij *et al.*, 1992) or neutrophil activation (Kaw *et al.*, 1992).

The physiologically relevant ECE is thought to be a membrane-bound, zinc-containing metalloprotease that is inhibited by the neutral (metallo) endopeptidase (NEP 24.11) inhibitor phosphoramidon (Opgenorth *et al.*, 1992). The activity of this ECE is not affected by thiorphan, another NEP inhibitor, or by inhibitors of the neutral metalloprotease ACE. Two other minor forms of metalloprotease enzyme with ECE activity have been described, both of which are soluble. One of them is sensitive (Takada *et al.*, 1991) and the other insensitive (Matsumura, Y. *et al.*, 1991b) to inhibition by phosphoramidon. The physiological relevance of the phosphoramidon-sensitive metalloprotease ECE is demonstrated by the ability of phosphoramidon, but not thiorphan, to inhibit the regional and systemic effects of big ET-1 *in vivo* (Fukuroda *et al.*, 1990; Matsumura *et al.*, 1990; McMahon *et al.*, 1991).

2.3. *Characteristics of Cloned Endothelin-Converting Enzymes*

Purification of rat and bovine ECE (Ohnaka *et al.*, 1993; Takahashi *et al.*, 1993) was rapidly followed by molecular cloning and characterization of the enzyme from rat (Shimada *et al.*, 1994), bovine (Ikura *et al.*, 1994; Schmidt *et al.*, 1994; Xu *et al.*, 1994) and human tissue (Schmidt *et al.*, 1994; Shimada *et al.*, 1995; Yorimitsu *et al.*, 1995). This enzyme, termed ECE-1, has a neutral pH optimum and is inhibited by phosphoramidon. More recently, a second novel enzyme, termed ECE-2, was cloned (Emoto and Yanagisawa, 1995). Like ECE-1, this enzyme is inhibited by phosphoramidon, but unlike ECE-1, it has an acidic pH optimum. These enzymes might also have differential cellular distribution. ECE-1 is widely distributed, but not found in neurons and glia in the brain, which are known to produce mature ETs (Xu *et al.*, 1994). ECE-2, in contrast, seems to be most abundantly expressed in neural tissues (Emoto and Yanagisawa, 1995).

ECE-1 is a Type II integral membrane protein composed of 754 or of 758 amino acids, with a short N-terminal cytoplasmic tail, a hydrophobic transmembrane domain and a large extracellular domain containing a zinc binding motif that is common to the catalytic domains of many metalloproteases (Fig. 4). ECE-1 has 10 potential glycosylation sites, and expression studies suggest that a high level of glycosylation is important for full enzymatic activity (Shimada

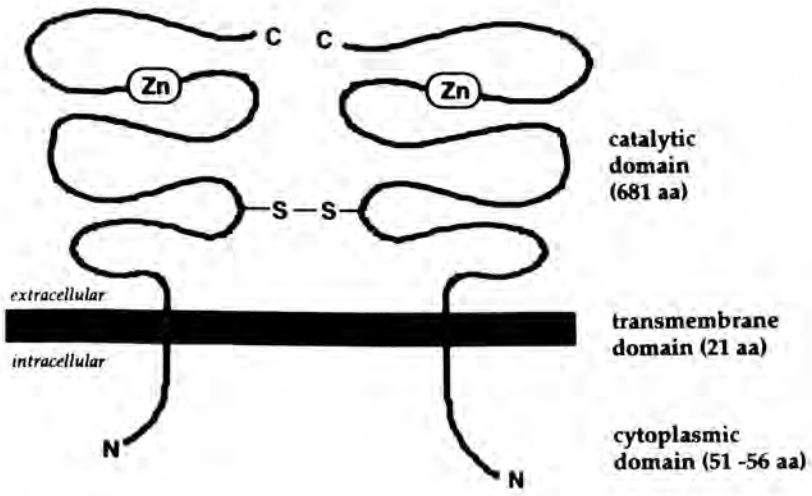


FIGURE 4. Schematic of plasma membrane bound ECE-1. From the predicted amino acid sequence, ECE-1 is thought to be a Type II integral membrane protein with a highly glycosylated, long C-terminal containing a Zn binding catalytic domain, a transmembrane domain and an N-terminal of variable length. The ECE-1 proteins can exist as dimers formed through disulphide bonds (-S-S-) between conserved cysteine residues. Although shown bound to plasma membrane, the ECE-1 protein might also exist in a form bound to the membranes of intracellular organelles. See text for details. Adapted from Turner and Murphy (1995).

et al., 1995). ECE-1 also has a number of conserved cysteine residues, and recent studies have suggested that ECE-1 might exist as a disulphide-linked dimer (Schmidt *et al.*, 1994; Turner and Murphy, 1995). While the putative extracellular domains of ECE-1 in different tissues share over 95% sequence homology, examination of the N-terminal domains reveals differences in both length and sequence homology. Two groups can be distinguished, one with 51–52 (Schmidt *et al.*, 1994; Shimada *et al.*, 1994) and one with 56 amino acids in the N-terminal (Ikura *et al.*, 1994; Xu *et al.*, 1994; Shimada *et al.*, 1995). These isoforms might arise through alternative splicing of the ECE gene during transcription (Yorimitsu *et al.*, 1995). Northern analysis reveals that the tissue distribution of these ECE-1 isoforms might be different (Valdenaire *et al.*, 1995). No differences in activity, however, have been detected to date, with both forms converting big ET-1 in preference to big ET-2 or big ET-3. Amino acid sequences in the C-terminal of the big ET isopeptides, particularly residues 27-34, seem to be important determinants of substrate recognition by ECE-1 (Okada *et al.*, 1991; Xu *et al.*, 1994). ECE-2 also converts big ET-1 preferentially (Emoto and Yanagisawa, 1995). This substrate selectivity suggests that there may be yet another ECE(s) selective for big-ET-2 or big ET-3 still to be discovered.

ECE-2 is also a Type II integral membrane protein, composed of 787 amino acids, with 59% overall identity to ECE-1 (Emoto and Yanagisawa, 1995). The structure of ECE-2 is similar to ECE-1, it has a short N-terminal, a single transmembrane domain and a large C-terminal containing a zinc-binding motif in the catalytic domain. It is also highly glycosylated.

The deduced sequences of both ECE-1 and ECE-2 have relatively high homology with NEP and human Kell blood group protein, a putative NEP (Ikura *et al.*, 1994; Schmidt *et al.*, 1994; Shimada *et al.*, 1994; Xu *et al.*, 1994; Yorimitsu *et al.*, 1995; Emoto and Yanagisawa, 1995). Despite their structural similarity, NEP and ECE have quite different cleavage site selectivity. NEP rapidly degrades many small peptides, including ET-1 itself, by cleavage at multiple internal sites, while ECE-1 cleaves only the Trp^{21}-Val^{22} bond of the big ETs (Vijayaraghaven *et al.*, 1990; Xu *et al.*, 1994). The specificity of ECE-2 for cleavage of big ET-1 is yet to be formally confirmed (Emoto and Yanagisawa, 1995).

Both ECE-1 and ECE-2 are predicted to be integral membrane proteins, but the primary site for cleavage of endogenous big ET-1 could be at the plasma membrane or at an intracellular membrane site. Exogenous big ET-1 can be converted to ET-1 *in vivo* (McMahon *et al.*, 1991), *in vitro* (Auguet *et al.*, 1992) and in COS cells transfected with the ECE-1 gene (Xu *et al.*, 1994), consistent with localisation of ECE at an accessible plasma membrane site. This would be analogous to conversion of angiotensin I to angiotensin II by plasma membrane-bound endothelial ACE (Caldwell *et al.*, 1976). However, only 5–10% of exogenous big ET-1 is converted by ECE-1 transfected COS cells (Xu *et al.*, 1994), and transformation by ECE-2 transfected COS cells is negligible (Emoto and Yanagisawa, 1995). This contrasts with the finding that 50–90% of the total ET peptide secreted by cultured endothelial cells or by COS cells cotransfected with the prepro ET-1 and ECE-1 or ECE-2 genes is mature ET-1 (Sawamura *et al.*, 1991; Xu *et al.*, 1994; Emoto and Yanagisawa, 1995). Thus, the process for endogenous formation of ET-1 appears to be much more efficient than that involved in conversion of exogenous big ET-1. The current consensus is that endogenous big ET-1 is most likely to be converted during its transit through the intracellular constitutive secretory pathways, especially within the Golgi apparatus. This conclusion is consistent with immunohistochemical staining for ET-1 in the cytoplasm of endothelial cells and the reported ability of a low density intracellular fraction to convert big ET-1 to the mature peptide (Gui *et al.*, 1993; Harrison *et al.*, 1993). The

pH in the secretory granules of the trans-Golgi network is around 5.5–5.7, within the range at which conversion of big ET-1 by ECE-2 is optimal (Emoto and Yanagisawa, 1995). Formation of ET-1 in secretory vesicles that can recognise transport pathways would also provide a suitable mechanism to explain the directional release of ET-1 towards the abluminal surface of endothelial cells (Wagner *et al.*, 1992a). However, ECE-1, which has a neutral pH optimum, would not efficiently convert big ET-1 under the acidic conditions found within the secretory granules, its intracellular location remains to be determined. Despite the fact that endogenous big ET-1 is converted relatively efficiently by intracellular ECE, a proportion of it is still secreted in the unconverted form, accounting for the big ET-1 detectable in plasma. Circulating big ET-1 concentrations are somewhat below the K_m of ECE-1, but it seems likely that some of the big ET-1, like exogenously administered big ET-1, can be converted by plasma membrane-bound ECE-1. A mechanism by which secreted big ET-1 could be adequately concentrated for more efficient conversion by plasma membrane-bound ECE-1 has been proposed recently (Barnes *et al.*, 1995; Turner and Murphy, 1995). The suggestion is that ECE is localised in caveoli, invaginations of the plasma membrane that are abundant in endothelial and smooth muscle cells. Immunohistochemical studies using antisera directed against the purified ECE-1 protein demonstrate clustering of ECE-1 on the surface of endothelial cells; this would be consistent with localisation in caveoli (Barnes *et al.*, 1995). Future studies will show whether big ET-1 can be secreted directly into these caveoli for physiological ET-1 synthesis.

2.4. *Endothelin-Converting Enzyme Inhibitors*

While phosphoramidon effectively inhibits ET-1 formation from big ET-1, its therapeutic potential is limited both by its low potency and by its lack of selectivity for ECE. IC_{50} values for inhibition of purified ECE-1 by phosphoramidon range from 0.35 μM to 0.8 μM, several orders of magnitude higher than the IC_{50} for inhibition of NEP 24.11 (Ohnaka *et al.*, 1993; Shimada *et al.*, 1994). Interestingly, the potency of phosphoramidon against purified ECE-2 is approximately 250-fold higher than against ECE-1 (4 nM; Emoto and Yanagisawa, 1995). Several strategies have been used to develop inhibitors of ECE that are more potent and more selective than phosphoramidon (Table 2). As most of these studies were conducted prior to the identification of ECE-2, the type of ECE against which the inhibitors are tested is not usually specified. Compounds based on the structure of phosphoramidon have slightly increased potency and are approximately 400-fold more selective for ECE than phosphoramidon itself, but they retain their relative selectivity for NEP 24.11 (Fukami *et al.*, 1994; Balwierczak *et al.*, 1995). It might be argued that compounds like these and the recently described orally active CGS 26303 (De Lombaert *et al.*, 1994; Trapani *et al.*, 1995), which also inhibits NEP 24.11, have therapeutic advantages over selective ECE inhibitors in that they would potentiate levels of the vasodilator atrial natriuretic peptide, which is broken down by NEP. However, as NEP also breaks down a number of constrictor peptides, including ET-1 itself and angiotensin II, concomitant NEP inhibition might actually negate any beneficial vasodilator action afforded by inhibition of ECE. [D-Val^{22}] Big ET-1(16–38), an analogue of big ET-1, inhibits big ET-1 conversion *in vitro*, although its potency is less than that of phosphoramidon (Morita *et al.*, 1994). [Phe^{22}]-big ET-1 [19–37] is more potent than phosphoramidon as an inhibitor of big ET-1 conversion in the isolated rabbit kidney, but like ET-1, is broken down by NEP and, therefore, must be co-administered with an NEP inhibitor (Claing *et al.*, 1995). The aspergillomarasmines (Matsuura *et al.*, 1993) and more recently, the compound WS 79089A

TABLE 2: Inhibitors of ECE Activity

Class	Compound	ECE inhibition vs. phosphoramidon	NEP:ECE Activity	Comment	Reference
NEP inhibitors	Phosphoramidon	—	100–1000:1	0.1–0.5 μM IC_{50} Purified ECE	Takahashi *et al.*, 1993 Tsurumi *et al.*, 1994
	Thiorphan	None up to 100 μM			Takahashi *et al.*, 1993
	CGS 26303	3×	1000:1	Active *in vivo*	De Lombaert *et al.*, 1994
	CGS 26129	2×	?		Balwierczak *et al.*, 1995
Phosphoramidon related	(2,2 napthyl ethyl)PLT	7×	25:1	Inhibits ACE	Fukami *et al.*, 1994
	S17162	0.7×	?	Active *in vivo*	Descombes *et al.*, 1995
Culture broth	Aspergillomarasmines	Similar	?	Inactive *in vivo*	Matsuura *et al.*, 1993
	WS 79089B (FR 901533)	3×	1:500		Tsurumi *et al.*, 1994
	WS 75624A + B	?	?		Tsurumi *et al.*, 1995
Big ET analogues	[D-Val^{22}] big ET-1(16–38)	0.1×	?		Morita *et al.*, 1994
	[Phe^{22}] big ET-1 (19–37)	<30×	?	Active *in vivo*	Claing *et al.*, 1995
Other	Hydroxamic acid derivative	<100×	?	0.01–6.8 nM IC_{50}	Bihovsky *et al.*, 1995
	Hydroxamic acid derivative P2'beta-Ala	?	ECE>NEP		Bihovsky *et al.*, 1995

The inhibitors are described in relation to their potency for inhibition of ECE relative to phosphoramidon in the same system where this information is available and also in relation to their selectivity for inhibition of ECE compared with NEP 24.11 (NEP).

(FR 901533; Tsurumi *et al.*, 1994) are examples of ECE inhibitors found through screening of fermentation broth. FR 901533 is more selective for ECE than NEP 24.11, while maintaining a similar potency to phosphoramidon for ECE inhibition (IC_{50} = 0.73 μM). FR 901533, unlike phosphoramidon, also has equal potency for inhibition of ECE-1 and ECE-2 (Emoto and Yanagisawa, 1995). The hydroxamic acid derivatives are the most interesting of the recently described ECE inhibitors (Bihovsky *et al.*, 1995). Among these, the P7′ β-Ala derivative is reported to be a potent inhibitor of human bronchiolar smooth muscle ECE, with lower potency for inhibition of NEP. The recent cloning of the ECE isoenzymes should improve the development of more selective and more potent ECE inhibitors. However, the problem of accessibility will also have to be faced should the intracellular ECE prove to be of more importance physiologically. Intracellular conversion of big ET-1 by ECE-1 is inhibited much less potently by phosphoramidon and FR 901533 than extracellular conversion, most likely because of the lesser accessibility of the intracellular site (Xu *et al.*, 1994).

2.5. *Sites of Endothelin Synthesis in the Cardiovascular System*

ET-1 was first isolated from medium of cultured porcine endothelial cells (Yanagisawa *et al.*, 1988b), but is also released by human endothelial cells in culture (Clozel and Fischli, 1989). In human blood vessels of varying origin, immunoreactive ET-1 is detected primarily in association with the endothelial cell layer (Howard *et al.*, 1992; Tokunaga *et al.*, 1992; Opgaard *et al.*, 1994; Properzi *et al.*, 1995), consistent with the idea that ET-1 is released by endothelial cells to act on the underlying smooth muscle cells (Fig. 1). *In situ* hybridization for ECE-1 mRNA also shows the most intense labelling over the vascular endothelial cells of most tissues (Xu *et al.*, 1994). Removal of the endothelium, however, does not completely prevent the action of big ET-1 in the perfused rat mesenteric bed (Hisaki *et al.*, 1993), suggesting that smooth muscle cells can also synthesise ET-1. Smooth muscle cells in culture express ET-1 mRNA and release ET-1 (Resink *et al.*, 1990; Yu and Davenport, 1995), but only diffuse immunohistochemical labelling for ET-1 can be detected overlying the medial smooth muscle layer of human blood vessels (Tokunaga *et al.*, 1992). Interestingly, immunohistochemical staining for ET-1 is intense over atherosclerotic plaques of human coronary arteries (Lerman *et al.*, 1991a), particularly over macrophages and intimal smooth muscle cells in active lesions (Zeiher *et al.*, 1995). It may be that only the proliferative phenotype of smooth muscle cells, such as those in culture or in atherosclerotic plaques, can actively secrete ET-1.

To date, the majority of studies that have investigated the presence of the ET isopeptides in the human circulation have focused on measurement of immunoreactive ET-1 or its precursor big ET-1. However, immunoreactive big ET-2, ET-3 and big ET-3 can also be detected in human plasma (Matsumoto *et al.*, 1994; Gerbes *et al.*, 1995), suggesting that blood vessels might synthesize other isopeptides in addition to ET-1. In human coronary arteries, big ET-2 immunoreactivity is detectable in the endothelial cytoplasm (Howard *et al.*, 1992), and prepro ET-2 mRNA can be located in the medial layer using *in situ* hybridisation (O'Reilly *et al.*, 1993). There is also evidence that human smooth muscle cells in culture can release ET-3 in addition to ET-1 (Yu and Davenport, 1995).

Radioimmunoassay of human heart extracts demonstrates the presence of immunoreactive ET-1, -2 and -3, as well ET-1 precursor pro-ET-1 (Plumpton *et al.*, 1993). Mature ET-1 is detectable in a number of human cardiac cells, including atrial and ventricular myocytes (Wei *et al.*, 1994) and endocardial endothelial cells (Giaid *et al.*, 1991), but it is unclear whether it is generated in these cells. In blood vessels, ET-1 is generated in endothelial cells to act on the underlying smooth muscle. An analogous situation might exist in the heart, with endocardial endothelial cells being the primary site of production. Cultured endocardial cells express ET-1 mRNA and also secrete ET-1 (Mebaaza *et al.*, 1993), and there is indirect evidence that ET-1 is released *in vivo* by endocardial cells lining the ventricular myocardium, particularly following ischaemia (Tonnessen *et al.*, 1993). However, prepro ET-1 mRNA is also expressed in cultured neonatal rat cardiomyocytes (Ito *et al.*, 1993) and in human cardiomyocytes (Giaid *et al.*, 1995). Northern analysis reveals the presence of ECE-1 mRNA (Xu *et al.*, 1994) and ECE-2 mRNA (Emoto and Yanagisawa, 1995) in bovine ventricular tissue, but the precise cellular location remains to be determined.

2.6. *Clearance and Degradation of Endothelin*

The plasma half-life of ET-1 in humans is less than 1.5 min because of its efficient extraction by the splanchnic and renal vascular beds (Weitzberg *et al.*, 1991; Gasic *et al.*, 1992). Although ET-1 is also reported to be taken up by the lungs (Stewart *et al.*, 1991), there are indications that pulmonary clearance is less important in humans than in other species (Ray *et al.*, 1992; Hemsen *et al.*, 1995). Extraction of ET-1 follows binding to cell surface receptors, which are then internalised, allowing degradation to be carried out within the cell (Anggård *et al.*, 1989; Gandhi *et al.*, 1993), perhaps in lysosomes (Löffler *et al.*, 1991). The observation that circulating concentrations of ET-1 are increased by a mixed ET_A/ET_B receptor antagonist (Löffler *et al.*, 1993) or by an ET_B receptor selective antagonist (Fukuroda *et al.*, 1994), but not by ET_A selective antagonists, is suggestive of a role for ET_B type receptors in clearance of ET-1. Low-affinity ET_B type binding sites that might serve this purpose have been found in arteries and veins (Gray *et al.*, 1994; Teerlink *et al.*, 1994a). A possible candidate for an intracellular degrading enzyme is a soluble protease found in human platelets, vascular smooth muscle and endothelial cells (Jackman *et al.*, 1992, 1993). A deamidase enzyme with similar characteristics recently was purified from rat kidney (Deng *et al.*, 1994; Janas *et al.*, 1994). The ETs can also be degraded by NEPs (E.C.

24.11), which are associated with venous and arterial endothelial cell plasma membranes (Llorens-Cortes *et al.*, 1992), as well as by an enzyme released from the perfused rat mesenteric bed (Perez-Vicario *et al.*, 1995). Activated polymorphonuclear lymphocytes are able to rapidly inactivate ET-1 through release of a protease, believed to be cathepsin G, which degrades ET by cleavage of His^{16}-Leu^{17} (Fagny *et al.*, 1992; Patrignani *et al.*, 1992). This process may have a role in acute inflammation following adhesion of polymorphonuclear lymphocytes to vascular endothelial cells.

3. ENDOTHELIN RECEPTORS

Specific binding sites for [^{125}I] ET-1 can be classified according to their relative affinities for the ET isopeptides. The ET_A type site is characterised by its very high (subnanomolar) affinity for ET-1 and ET-2 and its 70- to 100-fold lower affinity for ET-3, while the ET_B site has high and equal affinity for all 3 isopeptides (Table 3). These binding characteristics are reflected in the agonist potency of the isopeptides in isolated tissues, demonstrating that the binding sites represent functional receptors (Maggi *et al.*, 1989; Warner *et al.*, 1989).

3.1. *Endothelin$_A$ and Endothelin$_B$ Receptors: Cloning, Gene Regulation, and Structural Features*

Within 2 years of the initial description of ET (Yanagisawa *et al.*, 1988b), the genes encoding the ET_A and ET_B type receptors that mediate its actions were cloned and characterised (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Lin *et al.*, 1991). The cDNAs encoding the human ET_A and ET_B receptors predict 427 and 442 amino acids, respectively, and the overall identity between the two mature proteins is reported to be between 55% and 64%, depending on the tissue studied (Adachi *et al.*, 1991; Hayzer *et al.*, 1992; Arai *et al.*, 1993; Elshourbagy *et al.*, 1993). The ET_A and ET_B receptor genes, located on chromosomes 4 (Hosada *et al.*, 1992) and 13 (Arai *et al.*, 1993), respectively, have similar structural organisation, suggesting that they originated from the same ancestral gene. Although functional studies suggest the existence of further heterogeneity among ET receptors (reviewed in Bax and Saxena, 1994), analysis of human genomic DNA with probes specific for the human ET_A and ET_B receptors reveals only two hybridising fragments (Sakamoto *et al.*, 1991). Thus, if genes encoding other ET receptors exist in the mammalian genome, they must have quite low sequence similarities to the two known ET receptor genes. Screening of amphibian cDNA libraries has revealed the existence of two alternative receptor clones, neither of which have been detected yet in the mammalian genome. The first, cloned from *Xenopus* dermal melanophores, shows relative selectivity for ET-3, consistent with a putative ET_C receptor subtype (Karne *et al.*, 1993). The second, cloned from *Xenopus* heart, is termed ET_{AX} because of its high relative affinity for ET-1, like the ET_A receptor, but uncharacteristic low affinity for the ET_A receptor selective ligand BQ-123 (Kumar *et al.*, 1994).

TABLE 3. ET Receptor Agonists and Antagonists

Category	Ligand	Selectivity	Potency (IC_{50} or *, K_i) ET_A	Potency (IC_{50} or *, K_i) ET_B	Reference
Agonist	ET-1	ET_A/ET_B	160 pm	110 pm	Saeki *et al.*, 1991
	ET-3	ET_B	4.5 nM	70 pm	Saeki *et al.*, 1991
	SRTX S6c	ET_B	4.5 μM	20 pM	Williams *et al.*, 1991
	IRL 1620	ET_B	16 pM	200 pM	Takai *et al.*, 1992
	BQ-3020	ET_B	940 nM	200 pM	Ihara *et al.*, 1992b
Antagonist: peptide	BQ-123	ET_A	7.3 nM	18 μM	Ihara *et al.*, 1991
	FR 139317	ET_A	1 nM	7 μM	Aramori *et al.*, 1993
	TTA-386	ET_A	0.34 nM	<1 μM	Kitada *et al.*, 1993
	BQ-518	ET_A	1.2 nM	55 μM	Fukami *et al.*, 1995a
	BQ-788	ET_B	1300 nM	1.2 nM	Ishikawa *et al.*, 1994
	Res 701-1	ET_B	<5 μM	10 nM	Tanaka *et al.*, 1994
	BQ-017	ET_B	3.8 nM	0.8 nM	Fukami *et al.*, 1995b
	IRL 2500	ET_B	94 nM	1.3 nM	Balwierczak *et al.*, 1995
	PD 145065	ET_A/ET_B	3.5 nM	15 nM	Cody *et al.*, 1993
	TAK-044	ET_A/ET_B	0.1 nM	1.8 nM	Kikuchi *et al.*, 1994
Antagonist: nonpeptide	97-139	ET_A	1 nM*	1 μM*	Mihara *et al.*, 1994
	BMS 182874	ET_A	55 nM*	>20 μM*	Stein *et al.*, 1994
	LU 127043	ET_A	6 nM*	1 μM*	Raschack *et al.*, 1995
	PD 155080	ET_A	7.4 nM	4.5 μM	Doherty *et al.*, 1995
	Ro 46-8443	ET_B	7 μM	40 nM	Brändli *et al.*, 1996
	Ro 47-0203 (bosentan)	ET_A/ET_B	4.7 nM	95 nM	Clozel *et al.*, 1994
	CGS 27830	ET_A/ET_B	16 nM	295 nM	Mugrage *et al.*, 1993
	SB 209670	ET_A/ET_B	0.2 nM	18 nM	Ohlstein *et al.*, 1995
	PD 160672	ET_A/ET_B	0.8 nM	44 nM	Doherty *et al.*, 1995
	SB 217242	ET_A/ET_B	1 nM*	111 nM*	Barone *et al.*, 1995

As with the ET genes, the nontranscribed 5′ flanking regions of the ET receptor genes contain a number of regions involved in regulation of gene transcription (Hosada *et al.*, 1997; Arai *et al.*, 1993). Exogenous factors can act through these regions to increase receptor transcription, for example, up-regulation of ET_A receptor mRNA by insulin (Frank *et al.*, 1993) or ET_B receptor mRNA by angiotensin II (Kanno *et al*, 1993) These mechanisms may be important in regulation of responsiveness to the ETs in pathophysiological states. ET_B receptor mRNA is selectively increased in marmosets fed a high cholesterol diet (Elshourbagy *et al.*, 1993) and following glycerol-induced acute renal failure in rats (Roubert *et al.*, 1994). In contrast, ET receptor expression is reduced in atherosclerotic human arteries (Winkles *et al.*, 1993) and in the lungs of rats with pulmonary hypertension (Yorikane *et al.*, 1993). One of the major factors that reduces ET receptor number at the cell surface is prolonged exposure to ET-1 itself, because of down-regulation or feedback inhibition of receptor expression (Hirata *et al.*, 1988), or both of these in combination. In endothelial cells, ET_B receptor expression is decreased because exposure to high local concentrations of ET-1 reduces the stability of mRNA molecules rather than reducing transcription (Sakurai *et al.*, 1992). The 3′ untranslated regions of both genes contain potential polyadenylation signals that may mediate selective destabilisation of the receptor mRNA (Ogawa *et al.*, 1991; Hosada *et al.*, 1992; Arai *et al.*, 1993).

Polymorphisms in the noncoding region of the ET_A receptor gene have been identified in the human genome (Stevens and Brown, 1995), although the consequences for receptor function currently are unknown. Variants of the ET receptor mRNA can arise through alternative splicing of the ET_A and ET_B receptor genes during transcription (Miyamoto *et al.*, 1994; Shyamala *et al.*, 1994). The transcribed regions of both receptor genes encode sites for post-translational modification that influence the tertiary structure of the receptor and its linkage to intracellular messenger systems, including consensus sites for N-glycosylation, several potential sites for palmitoylation to anchor the receptor to the cell membrane, and serine residues that may be substrates for regulatory phosphorylation by serine threonine kinases (Hosada *et al.*, 1991; Nakamuta *et al.*, 1991; Ogawa *et al.*, 1991; Elshourbagy *et al.*, 1993). Phosphorylation may play a role in the down-regulation of ET receptors that follows prolonged exposure to the ET isopeptides (Hirata *et al.*, 1988; Roubert *et al.*, 1991; Miasiro and Paiva, 1992). Given the likely importance of the post-translational modification sites for receptor binding and function, it seems possible that some of the receptor heterogeneity observed in functional studies could arise through modification of these sites by alternative splicing. However, at least for the human ET_B receptor, two splice variants do not exhibit any difference in binding or linkage to intracellular signalling pathways (Shyamala *et al.*, 1994).

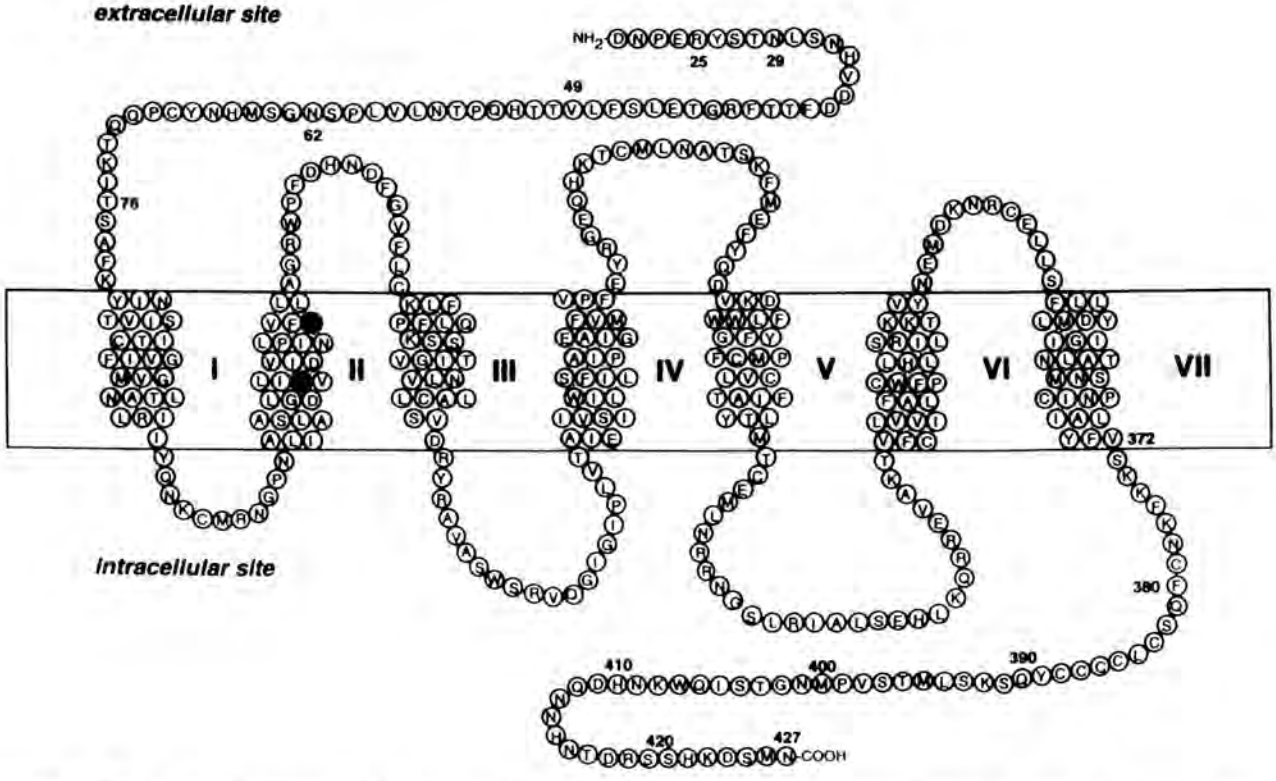


FIGURE 5. The predicted structure of the human ET_A receptor with its 7 transmembrane spanning domains. Filled circles represent amino acids (aa) in the second transmembrane domain identified as being of importance for ligand binding. See text for details. Adapted from Adachi *et al.* (1993).

All of the cloned ET receptor genes predict a heptahelical membrane spanning structure, common to members of the G-protein-coupled receptor superfamily and similar to many neuropeptide receptors (Fig. 5; Burbach and Meijer, 1992). The regions of greatest sequence conservation between the ET receptors and other G-protein-coupled receptors are concentrated within the hydrophobic transmembrane segments. Amongst the ET receptors, the 7 transmembrane domains and cytoplasmic loops of the receptors are highly conserved, but the N-terminal and other extracellular domains exhibit differences in both length and amino acid sequences (Ogawa *et al.*, 1991; Elshourbagy *et al.*, 1992).

The extracellular N-terminal regions of peptide G-protein-coupled receptors are known to be important for ligand binding (Nagayama *et al.*, 1991). Proteolytic truncation studies reveal that of the ET receptor N-terminal amino acids, only those in closest proximity to the first transmembrane domain are essential for ET binding (Hashido *et al.*, 1991; Kosuka *et al.*, 1991). Computer-assisted molecular modelling, based on the known coordinates of bacteriorhodopsin, recently has been applied in conjunction with site-directed mutagenesis, to investigate nonconserved amino acids in the ET_A and ET_B receptors that may be important in determination of ligand selectivity. These studies have identified Tyr^{129} (Krystek *et al.*, 1994; Lee *et al.*, 1994) and Lys^{140} (Adachi *et al.*, 1994) in the second transmembrane domain of the ET_A receptor (Fig. 5) and ^{181}Lys in the third transmembrane domain of the ET_B receptor (Zhu *et al.*, 1992) as being of potential importance for ligand binding. It has been proposed that the ET receptors can be divided into two distinct parts, one comprising transmembrane domains I, II, III and VII that is involved in ligand receptor binding and the other comprising transmembrane domains IV, V and VI that determines isopeptide selectivity (Sakamoto *et al.*, 1993). Consistent with this proposal, ET_B-like binding characteristics can be conferred on the ET_A receptor by substitution of transmembrane regions IV, V and VI and their intervening loops with the corresponding regions of the ET_B receptor (Adachi *et al.*, 1993; Sakamoto *et al.*, 1993). The predicted third cytoplasmic domain of the ET receptors is very short (approximately 30–50 residues), a feature common to G-protein-coupled receptors that have peptide ligands (Elshourbagy *et al.*, 1993). This region, and in particular the C-terminal end, is implicated in coupling of the human ET_A receptor to G-proteins and subsequent liberation of intracellular Ca^{2+} (Adachi *et al.*, 1993; Hashido *et al.*, 1993).

3.2. *Agonists at Endothelin Receptors*

All of the ET and SRTX peptides possess four cysteinyl residues that form two disulphide bridges, three polar charged side chains (residues 8–10) and a well-conserved hydrophobic C-terminus (residues 16–21, Fig. 2). Examination of the binding characteristics of these peptides reveals that the ET_A receptor has much more rigid structural requirements for ligand binding than the ET_B receptor (reviewed in Erhardt, 1992; Sokolovsky, 1992; Huggins *et al.*, 1993). Both the amino-terminal loop structure and the carboxy terminal linear portion with Trp in position 21 are vital for high affinity ET_A receptor binding. In contrast, only the linear carboxyl terminal and the Trp^{21} is essential for high-affinity binding to the ET_B receptor (Kimura *et al.*, 1988). A number of selective ET_B receptor ligands have been designed based on this linear portion, including BQ-3020 (N-acetyl-[$Ala^{11,15}$] ET-1 (6–21); Ihara *et al.*, 1992b) and IRL 1620 (N-succinyl-[Glu^9, $Ala^{11,15}$] ET-1 (8–21); Takai *et al.*, 1992). ET-3 and SRTX S6c can also be considered as ET_B selective ligands, ET-3 having approximately 2000-fold and SRTX S6c 30,000-fold selectivity for binding to the ET_B rather than the ET_A receptor (Williams *et al.*, 1991). Although both of these ligands contain loop and linear portions like ET-1, they have different amino acid sequences within the inner loop portion, which might account for their lower affinity at the ET_A receptor.

3.3. *Endothelin Receptor Antagonists*

Since the first description of compounds that could inhibit the binding or actions of ET-1 in 1991 (Ihara *et al.*, 1991; Spinella *et al.*, 1991), a large number of ET receptor antagonists, peptide and nonpeptide, selective and nonselective, have become available. The characteristics of some of these are shown in Table 3; for a fuller consideration, the reader is referred to several recent review articles (Warner, 1994; Gray, 1995; Lüscher and Wenzel, 1995).

Peptide antagonists have been obtained by chemical modification of ET-1 itself, or of microbial products with ET receptor binding activity (Ihara *et al.*, 1991; Spinella *et al.*, 1991). BQ-123 is a cyclic pentapeptide derived from microbial broth that has relatively high potency for binding to the ET_A receptor subtype (Ihara *et al.*, 1992a). Although several more ET_A antagonists are now available (Table 3), studies using BQ-123 first confirmed the role of ET in a number of pathologies (Moreland, 1994). Among the peptide antagonists, the hexapeptide TTA-386 (hexamethyleneiminocarbonyl-Leu-Trp-Ala-βAla-Tyr-Phe) is the most potent ET_A receptor antagonist described to date (Kitada *et al.*, 1993). BQ-788 (Ishikawa *et al.*, 1994) is a peptide compound that is more selective for inhibition of ET-1 binding to the ET_B receptor (Table 3). Res 701-1 was also initially described as an ET_B selective antagonist (Tanaka *et al.*, 1994), but recent studies show that is has approximately equal affinity at the ET_A and ET_B receptor subtypes (He *et al.*, 1995). The first nonselective ET receptor antagonists to be described were also peptides (Cody *et al.*, 1992; Lam *et al.*, 1992). TAK-044, is a cyclic hexapeptide with approximately 20-fold higher affinity at the ET_A compared with the ET_B receptor (Kikuchi *et al.*, 1994). This compound, unlike many of the other peptide antagonists, has a relatively long duration of action following i.v. administration *in vivo* (Ikeda *et al.*, 1994). Although useful as research tools, the potential of peptides as therapeutic agents may be limited by their short duration of action, as well as by their

lack of oral availability. With these problems in mind, much research effort has been applied to the development of orally active nonpeptide antagonists.

Potent nonpeptide antagonists (Table 3) have been developed through optimisation of compounds isolated from plant extracts (Fujimoto *et al.*, 1992; Mihara *et al.*, 1994) and microbial broths (Ohashi *et al.*, 1992), or screened from chemical libraries (Clozel *et al.*, 1993; Mugrage *et al.*, 1993; Clozel *et al.*, 1994; Ohlstein *et al.*, 1994; Stein *et al.*, 1994). SB 209670, a nonselective antagonist, is amongst the most potent of these. This compound inhibits the action of ET-1 *in vivo*, whether administered i.v. or p.o. (Ohlstein *et al.*, 1994; Douglas *et al.*, 1995a).

3.4. *Distribution and Function of Endothelin Receptors in the Cardiovascular System*

In vascular tissue, ET_A receptor mRNA is expressed predominantly in smooth muscle (Arai *et al.*, 1990; Hori *et al.*, 1992; Yang *et al.*, 1993), while ET_B receptor mRNA is most abundant in endothelial cells (Hosada *et al.*, 1991; Ogawa *et al.*, 1991; Molenaar *et al.*, 1993; Winkles *et al.*, 1993). These findings are consistent with the view that constriction of vascular smooth muscle is mediated predominantly by ET_A receptors and that constriction is modified by release of relaxing factors from the endothelium through stimulation of ET_B receptors (Fig. 1). However, in some isolated blood vessels, the constrictor response evoked by ET-1 has both ET_A antagonist sensitive and insensitive components (Fig. 6; reviewed in Bax and Saxena, 1994) and *in vivo*, pressor responses to ET-1 cannot be completely inhibited by ET_A receptor antagonists (Cristol *et al.*, 1993; McMurdo *et al.*, 1993). Furthermore, ET_B selective agonists can evoke constriction *in vitro* (Moreland *et al.*, 1992; Sumner *et al.*, 1992; Shetty *et al.*, 1993) and pressor responses *in vivo* (Williams *et al.*, 1991; Bigaud and Pelton, 1992; Clozel *et al.*, 1992). These observations are suggestive of the presence of ET_B receptors that mediate constriction on vascular smooth muscle cells. Indeed, ET_B receptor mRNA is detectable both in the medial smooth muscle of human arteries (Davenport *et al.*, 1993; Maguire *et al.*, 1994) and in cultured smooth muscle cells (Batra *et al.*, 1993; Winkles *et al.*, 1993). The relative contributions of ET_A and ET_B receptors to vasoconstriction is variable and depends on species and the vessel type studied (Davenport and Maguire, 1994). ET_B receptors are generally more important in the low pressure venous circulation (Moreland *et al.*, 1994). In isolated human blood vessels, it is the ET_A receptor subtype that primarily mediates constriction in large calibre arteries (Davenport and Maguire, 1994), but recent studies show that the relative functional role of ET_B receptors is greater in small calibre arteries (Tschudi and Lüscher, 1994; Deng *et al.*, 1995; Takase *et al.*, 1995). The balance of receptors might be altered under pathophysiological conditions. For example, ET_B receptor expression is increased during the change of cultured vascular smooth muscle cells from a contractile to a synthetic phenotype (Eguchi *et al.*, 1994), in hypertension

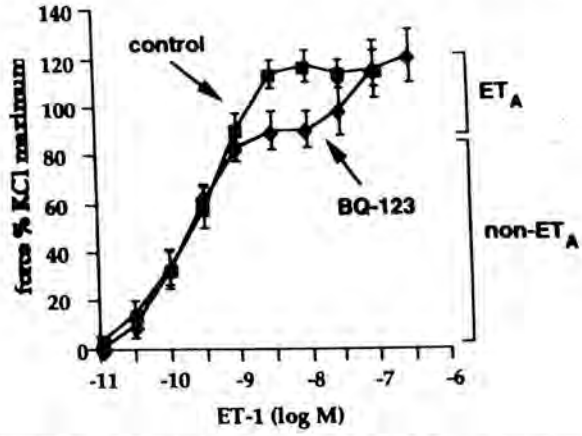


FIGURE 6. ET-1-induced constriction of the isolated rabbit saphenous vein. The dose-response curve shows a component that is sensitive and a component that is insensitive to inhibition by the ET_A receptor antagonist BQ-123 (10^{-5} M). Reproduced from Gray *et al.* (1994), with permission of the copyright holder, The American Physiological Society, Bethesda.

(Batra *et al.*, 1993) and under the influence of angiotensin II (Kanno *et al.*, 1993). Interestingly, the nonselective ET receptor antagonists bosentan (Clozel *et al.*, 1994) and PD 142893 (Warner *et al.*, 1993) have differential potency on the endothelial ET_B and smooth muscle ET_B receptors, suggesting that they might represent discrete receptor subtypes. Subtypes of vascular ET_A receptor that are insensitive to BQ-123 have also been proposed (Bodelsson and Sternquist, 1993; Moreland, 1994; Sudjarwo *et al.*, 1994).

Although ET_A receptor mRNA cannot be detected in endothelial cells derived from the peripheral vasculature (Davenport *et al.*, 1993; Winkles *et al.*, 1993), it is reported to be expressed by cultured cerebromicrovascular endothelial cells (Stanimirovic *et al.*, 1994). High-affinity binding sites typical of ET_A receptors can also be detected on rat brain microvascular endothelial cells (Frelin *et al.*, 1991). These receptors are linked to activation of phospholipase C, but their functional role *in situ* is currently unclear.

In the human heart, ET_A and ET_B receptor mRNAs have similar distribution, with both found within the atrioventricular node, the penetrating and branching bundles of His, in atrial and ventricular myocardium, and in endocardial cells (Molenaar *et al.*, 1993). ET-1 is a positive inotropic agent in human atrial and ventricular cardiac muscle strips (Schomisch Moravec *et al.*, 1989; Zerkowski *et al.*, 1992). In isolated rat cardiomyocytes, ET-1 is also reported to have positive chronotropic actions (Jones *et al.*, 1992). In addition, within the ventricular myocardium, ET-1 may have a role in mediating cardiac hypertrophy, as it potentiates the hypertrophic actions of angiotensin II in rat cardiomyocytes (Ito *et al.*, 1993; Neyses *et al.*, 1993).

4. ENDOTHELIN IN CARDIOVASCULAR PHYSIOLOGY

The widespread expression of mRNA for the ET isoforms and the wide distribution of ET_A and ET_B receptors in car-

diovascular tissues, and in pathways regulating the sympathetic nervous system, suggest that the ET system may play an important role in cardiovascular control. ET-1, the predominant endothelial isopeptide, is probably the isoform of most importance in regulation of vascular tone. ET-1 appears to be primarily a locally acting substance because release is predominantly abluminal (Wagner *et al.*, 1992a) and circulating plasma concentrations are usually not sufficient to elicit vasoconstriction directly (Haynes and Webb, 1993b). Here, the vascular physiology of the ET system is reviewed in relation to studies with ET agonists and antagonists. However, given that ET-1 is not a circulating hormone, it is not clear to what extent studies with exogenous ET can reproduce the physiological effects of the ET system. Generally, more reliance probably can be placed on studies using receptor antagonists.

4.1. *The Pressor Response*

In their original paper, Yanagisawa and colleagues (1988b) showed that i.v. administration of a bolus dose of ET-1 markedly increases blood pressure in chemically denervated rats (Fig. 7). This pressor effect is sustained for more than 60 min, in contrast to the brief effects of all other endogenous vasoconstrictor substances. ET-2 and ET-3 have also been shown to increase blood pressure in the rat (Inoue *et al.*, 1989), with ET-3 evoking the least response and having the shortest action. Similar pressor responses to the ETs have been demonstrated subsequently by other investigators in a range of other mammals (Braquet *et al.*, 1989; Pernow *et al.*, 1989; Miyamori *et al.*, 1990; Dieguez *et al.*, 1992), including humans (Vierhapper *et al.*, 1990; Weitzberg *et al.*, 1991; Sørensen *et al.*, 1994). The pressor response in humans appears not to be affected by pretreatment with cyclosporin, the Ca^{2+} antagonist nifedipine, or cyclo-oxygenase inhibition with indomethacin (Vierhapper *et al.*, 1992). The sustained increase in arterial pressure occurs despite rapid clearance of ET-1 from the circulation within a few minutes (Anggård *et al.*, 1989; Vierhapper *et al.*, 1990), mainly through receptor binding, and is consistent with the extremely slow dissociation of ET-1 from its vascular receptors (Hirata *et al.*, 1988). In rats, bilateral nephrectomy reduces the clearance of exogenous ET-1 and leads to a more prolonged pressor response to ET-1 (Kohno *et al.*, 1989), suggesting that the kidneys play an important role in its clearance.

Continuous infusion of ET-1 or ET-3 over 7 days leads to sustained hypertension in rats, mediated through an increase in total peripheral resistance (Mortensen *et al.*, 1990). Although sodium balance did not appear to change during these studies, it was interesting that the hypertension could be prevented by salt restriction (Mortensen and Fink, 1992a), perhaps by altering vascular sensitivity to ET-1 or through an interaction with the renin-angiotensin system. In the dog (Lerman *et al.*, 1991b), infusion of lower doses of ET-1, sufficient to double plasma concentrations of the peptide, do not increase blood pressure, but do produce systemic vasoconstriction.

Bolus i.v. administration of big ET-1 causes a pressor effect of similar magnitude to that of ET-1 (Kashiwabara *et al.*, 1989; Gardiner *et al.*, 1991; McMahon *et al.*, 1991; Pollock and Opgenorth, 1991). However, big ET-1 is ~100 times less potent as a direct vasoconstrictor of blood vessels *in vitro* than ET-1 (Kashiwabara *et al.*, 1989), suggesting that big ET-1 does not exert its actions through direct binding to ET receptors, but rather by conversion to the mature

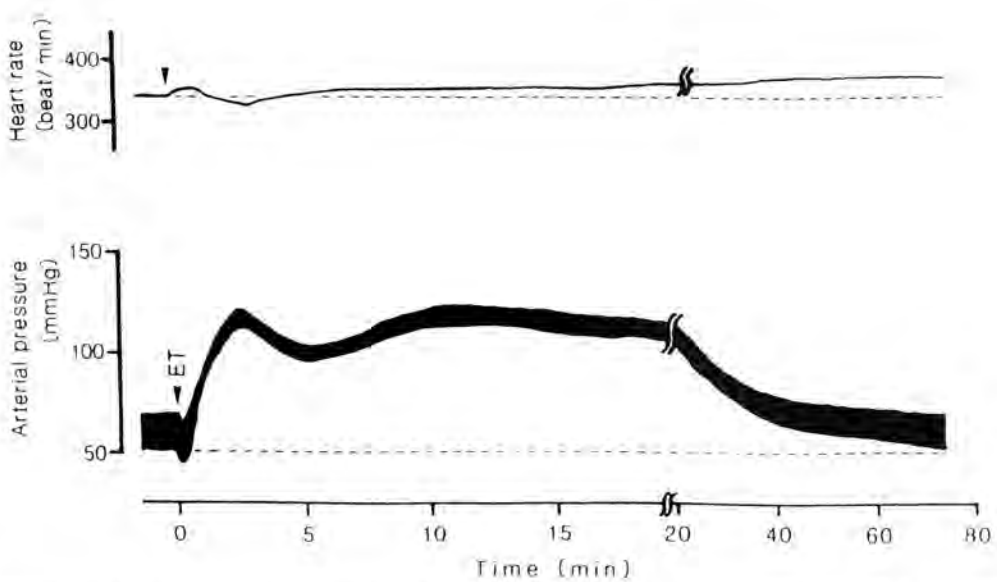


FIGURE 7. Brief depressor and sustained pressor effect following i.v. bolus injection of porcine ET-1 (ET, 1 nmol/kg) in the anaesthetised, chemically denervated rat. The baseline diastolic pressure is shown by a broken line. Reproduced from Yanagisawa *et al.* (1988b), with permission of the authors and the copyright holder, Macmillan Magazines Limited, London.

peptide. Confirmation that the pressor activity of big ET-1 is dependent on generation of ET-1 by ECE comes from the inhibition by the ECE inhibitor phosphoramidon of the pressor response to big ET-1, but not ET-1 (Gardiner *et al.*, 1991; McMahon *et al.*, 1991; Pollock and Opgenorth, 1991). In contrast, inhibitors of ACE and NEP do not block the pressor effects of big ET-1 (McMahon *et al.*, 1991; Pollock and Opgenorth, 1991). There may be regional variation in ECE activity, because mesenteric vasoconstriction to systemic big ET-1 is less sensitive to phosphoramidon than vasoconstriction in the renal and hindquarters vascular beds (Gardiner *et al.*, 1991). Big ET-2 and big ET-3 also have vasoconstrictor actions similar to their mature forms when administered *in vivo* in rats (Gardiner *et al.*, 1992; Pollock *et al.*, 1993; Matsumura *et al.*, 1993), although the magnitude of the response is smaller than that of ET-2 and ET-3. These responses also appear to involve conversion by ECE, because they can be inhibited by phosphoramidon. In contrast to studies in the rat, big ET-3 had no pressor response in the anaesthetised guinea pig (D'Orleans-Juste *et al.*, 1991). The authors suggest ECE is selective for the N- and C-terminal sequences of big ET-1. These varying results with big ET-2 and big ET-3, together with the recent *in vitro* isolation and characterisation of ECE isoforms selective for big ET-1 (Xu *et al.*, 1994; Emoto and Yanagisawa, 1995), suggest the presence of more than one ECE. The contrasting findings *in vivo* may reflect species differences in the expressions of different ECE isoforms.

At least two subtypes of vascular ET receptors exist; the ET_A and ET_B. Vasoconstriction to ET-1 was initially thought to be mediated solely by vascular smooth muscle cell ET_A receptors, with ET_B receptors restricted to the endothelium, where they mediate generation of endothelium-derived dilator substances. However, the existence of a vasoconstrictor ET_B receptor has been demonstrated subsequently *in vitro*, and selective ET_B receptor agonists have been shown to increase blood pressure in a number of species (Williams *et al.*, 1991; Douglas and Hiley, 1991; Clozel *et al.*, 1992, Gardiner *et al.*, 1994b; Moreland *et al.*, 1992). Further *in vivo* evidence for a constrictor ET_B receptor has been derived from antagonist studies, in which ET_A receptor antagonists do not fully block the pressor response to ET-1 (McMurdo *et al.*, 1993; Gardiner *et al.*, 1994b). These results suggest that both ET_A and ET_B receptors mediate the pressor effects of ET-1. The combined $ET_A/_B$ receptor antagonist bosentan effectively blocks all of the haemodynamic effects of ET-1, -2 and -3, and of big ET-1 (Gardiner *et al.*, 1994a), suggesting that non-ET_A, non-ET_B receptors play little or no role in this response. Studies using systemic administration of ET_B selective agonists and antagonists have not been reported in humans yet, but systemic administration of the combined ET_A/ET_B antagonist TAK-044 reduces blood pressure (Haynes *et al.*, 1996).

The pressor response to ET-1 may be mediated indirectly through generation of other constrictor substances. Vasoconstrictor prostanoids appear to potentiate regional vasoconstriction to ET-1, although cyclo-oxygenase inhibition does not appear to attenuate the pressor effect to ET-1 (Gardiner *et al.*, 1990a). The role of platelet-activating factor remains unclear (Kurose *et al.*, 1991; Filep *et al.*, 1991b). The sympathetic and renin-angiotensin systems may also contribute to the pressor response, but are discussed later (Section 4.4).

4.2. *The Depressor Response*

All three ET isoforms cause transient hypotension after bolus administration (shown for ET-1 in Fig. 7). This vasodepressor action precedes the sustained pressor effect and is most marked for ET-3 (Inoue *et al.*, 1989). The selective ET_B receptor agonists SRTX S6c and [$Ala^{1,3,11,15}$] ET-1 have also been shown to cause transient hypotension in rats and cynomolgus monkeys (Moreland *et al.*, 1994). However, the hypotension may reflect a pharmacological, rather than physiological, response to the briefly sustained high concentrations of ETs that occur following bolus administration (Ohlstein *et al.*, 1990). In particular, under perhaps more physiological conditions, in which ET-1 concentrations are allowed to rise more slowly (Mortensen and Fink, 1990; Gardiner *et al.*, 1993), such as following i.v. infusion of ET-1 or its precursor big ET-1, hypotension does not occur.

Nevertheless, the transient hypotension to bolus ET does demonstrate the endothelial actions of these peptides, which appear to be caused by generation of endothelium-dependent dilating factors and mediated by endothelial cell ET_B receptors. Inhibitors of NO synthase attenuate the hypotension to ET-1 (Whittle *et al.*, 1989; Gardiner *et al.*, 1990c; Fozard and Part, 1992; Rogerson *et al.*, 1993; Filep *et al.*, 1993; Granstam *et al.*, 1993), although the attenuation is often incomplete (Gardiner *et al.*, 1990c; Fozard and Part, 1992; Filep *et al.*, 1993), implying that other factors may contribute. Inhibition of cyclo-oxygenase (Rogerson *et al.*, 1993; Granstam *et al.*, 1993; Filep *et al.*, 1991a) also reduces the depressor effect, although this is not a universal finding (De Nucci *et al.*, 1988). In addition, the sustained pressor effect of ET is usually potentiated by inhibition of either NO (Gardiner *et al.*, 1990c; Filep *et al.*, 1993) or prostaglandin generation (Rogerson *et al.*, 1993; De Nucci *et al.*, 1988), indicating that the pressor effect of ET-1 is modulated by the stimulated release of endothelium-dependent vasodilators. Furthermore, the ability of ET_A receptor antagonists to enhance the vasodilator activity of ET-1 also suggests a functional antagonism between constrictor and dilator actions (Cirino *et al.*, 1994). However, in some vascular beds such as the rat hindquarters, there does not appear to be a major role for NO or prostacyclin in mediating vasodilatation to ET-1 (Ohlstein *et al.*, 1990; Gardiner *et al.*, 1989), suggesting that other mediators may be involved. Among these other mediators, endothelium-derived hyperpolarising factor is a potential candidate, but atrial natriuretic peptide does not appear to contribute (Fozard and Part, 1992).

Depressor responses to ET-1 have not been demonstrated in humans, although a parallel transient dilatation during

local brachial artery infusion has (Haynes *et al.*, 1995; and see Section 4.3), most probably because the use of high doses of ET-1 has been avoided for reasons of safety.

4.3. *Regional Vascular Responses*

The systemic haemodynamic response to exogenous ET is the sum of its actions on the heart, peripheral vasculature, nervous system, kidney and endocrine glands. There appear to be wide regional variations in the responses to ETs *in vivo*, and these may depend on the experimental circumstances, and certainly on species, vessel type and vessel size.

Infusion of ET-1 locally into the femoral artery of the anaesthetised dog (Clarke *et al.*, 1989b), or into the human brachial artery (Clarke *et al.*, 1989a), causes a sustained reduction in limb blood flow, without transient vasodilatation. After initial vasodilatation, a similar sustained response is elicited in the rat mesentery (Gardiner *et al.*, 1990a), but not in the rat hind limb (Gardiner *et al.*, 1989), when ET is administered systemically. The discrepancy between vasoconstriction produced by local administration of ET into a vascular bed and its absence during systemic administration may be merely a feature of the various resistance circuits in parallel and the greater action of ET in some vascular beds than others.

In humans, brachial artery infusion of ET-1 causes a slowly developing dose-dependent reduction in forearm blood flow, with vasoconstriction sustained for more than 2 hr after halting the infusion (Clarke *et al.*, 1989a; Cockcroft *et al.*, 1991). When given via the brachial artery (Haynes *et al.*, 1995), low doses of the ET_B selective agonists ET-3 and SRTX S6c also produce vasoconstriction in human resistance vessels *in vivo*, consistent with vascular ET_B receptors mediating at least part of the functional response to ET-1 in these vessels (Fig. 8). ET_B agonists can produce transient forearm vasodilatation, but only at high doses on bolus administration, suggesting that this may not be a physiological response. In these vessels, vasoconstriction is modulated by stimulated release of dilator prostaglandins (unpublished observations), but not NO (Kiowski *et al.*, 1991). Co-infusion of Ca^{2+} channel antagonists can overcome forearm vasoconstriction to ET-1 (Kiowski *et al.*, 1991; Andrawis *et al.*, 1992), but this effect may not be specific to ET-1 because of the high basal tone in forearm resistance vessels, against which these agents may be acting, and the finding that vasoconstriction to angiotensin II is blocked to an equal or greater extent (Clarke *et al.*, 1989a).

ET-1 (Clarke *et al.*, 1989a) and SRTX S6c (Haynes *et al.*, 1995) both cause sustained constriction of human dorsal hand veins *in vivo*, suggesting that both vascular ET_A and ET_B receptors contribute to venoconstriction to ET-1 in humans (Fig. 8). Indeed, this is further supported by the capacity for BQ-123 to attenuate venoconstriction *in vivo* to ET-1, but not SRTX S6c (Strachan *et al.*, 1995). Here, there remains a portion of the ET-1 response that is not inhibited by BQ-123 and is likely to be ET_B mediated. In addition, venoconstriction to both ET-1 (Haynes and Webb,

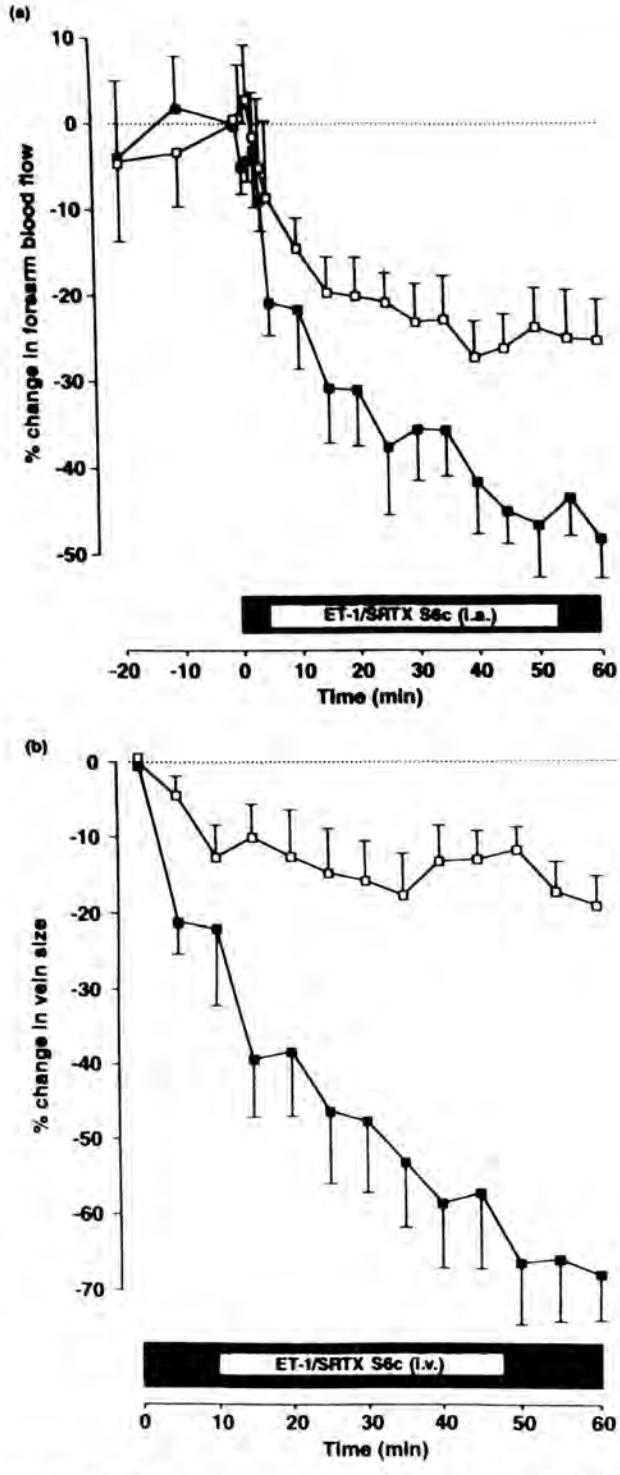


FIGURE 8. Vasoconstriction in the forearm (a) and venoconstriction in the hand vein (b) following infusion of ET-1 (■, 5 pmol/min) or SRTX S6c (□, 5 pmol/min) into the brachial artery (a) and the dorsal hand vein (b) of healthy human subjects. Values are mean ± SEM; n+6 for each group. i.a., intra-arterial. Reproduced from Haynes *et al.* (1995), with permission of the copyright holder, American Heart Association, Dallas.

1993a) and SRTX S6c (Strachan *et al.*, 1995) are markedly attenuated by endothelial ET_B-mediated production of vasodilator substances prostacyclin and NO. However, venodilatation to the ETs has not been shown in human skin capacitance vessels (Haefeli *et al.*, 1993). Venoconstriction *in vivo* is blocked more effectively by the K_{ATP} channel opener cromakalim than the Ca^{2+} channel antagonist nicardipine or by hydralazine (Haynes and Webb, 1993c), suggesting that ET-1 responses in human veins depend only in part on Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels. In addition, the greater efficacy of K_{ATP} channel opening agents is consistent with ET-1 acting to close K_{ATP} channels, causing plasma membrane depolarisation and vasoconstriction by mechanisms additional to opening of voltage-operated Ca^{2+} channels.

In humans, brachial artery administration of big ET-1 causes a dose-dependent forearm vasoconstriction that is completely blocked by phosphoramidon, suggesting that the effects of the precursor are mediated through conversion to the mature peptide by ECE (Haynes and Webb, 1994). The blockade of constriction to big ET-1 by phosphoramidon is unlikely to have been due to inhibition of ET receptor binding, because vasoconstriction to ET-1 was unaffected by phosphoramidon and because conversion to ET-1 and its C-terminal fragment was confirmed in plasma samples taken from the veins draining the infused forearm (Plumpton *et al.*, 1995). Because circulating blood exhibits little ECE activity (Watanabe *et al.*, 1991a), conversion of big ET-1 in the forearm probably occurs via vascular ECE situated within the forearm blood vessels. The difference in potency between big ET-1 and ET-1, and the ratio of C-terminal fragment to big ET-1 in venous blood, both indicate that local ECE converts about 10% of luminally presented big ET-1 to ET-1, consistent with ~10% conversion of exogenous big ET-1 by cells expressing the ECE-1 gene (Xu *et al.*, 1994).

Additional variations in regional vascular responses are indicated by the observation, in the skin microcirculation, that vasoconstriction appears to be mediated solely by ET_A receptors (Wenzel *et al.*, 1994).

4.4. *Effects on the Heart*

ET-1 is a potent and long-lasting constrictor of coronary vessels *in vivo* in many species (Clozel and Clozel, 1989; Kurihara *et al.*, 1989; Pernow *et al.*, 1989; Hirata *et al.*, 1990), producing marked reductions in coronary blood flow and causing associated myocardial ischaemia. Coronary angiograms reveal these effects are primarily due to effects on small coronary arteries (Kurihara *et al.*, 1989; Hirata *et al.*, 1990). The coronary resistance bed is more sensitive to the effects of ET than other resistance beds, excepting the kidney (Clozel and Clozel, 1989). The initial hypotension produced by ET is associated with an increase in heart rate and cardiac output, suggesting that it is secondary to systemic vasodilatation. In contrast, the pressor response to ET is associated with bradycardia and a reduction in stroke volume, causing a marked reduction in cardiac index (Miller *et al.*, 1989). The chronotropic effects of ET-1, at least *in vivo*, appear to be reflex in origin because heart rate does not change when ET-1 is administered after blockade of cardiac efferent neural mechanisms (Gardiner *et al.*, 1990b). The reduction in stroke volume is probably due to a combination of systemic vasoconstriction increasing afterload and coronary vasoconstriction causing myocardial ischaemia (Miller *et al.*, 1989; Yang *et al.*, 1991). In separate studies, both anti- and pro-arrhythmic effects have been suggested for ET-1. In isolated ventricular myocytes, ET-1, acting through ET_A receptors, inhibits a protein kinase A-dependent chloride current, an action that should help to protect the ventricle against arrhythmias (James *et al.*, 1994). However, in anaesthetised pigs, administration of ET-1 initiates fatal ventricular arrhythmias (Ezra *et al.*, 1989). Furthermore, in rat models of acute myocardial ischaemia, exogenous ET-1 reduces the threshold for ventricular fibrillation (Zhao *et al.*, 1994) and increases the severity and incidence of ischaemic arrhythmias (Garjani *et al.*, 1995). Interestingly, again in the rat acute ischaemia model, the ET_A receptor antagonist BQ-123 reduces the incidence of arrhythmias when administered at low doses, but when administered at higher doses, which may no longer be ET_A receptor selective, it is pro-arrhythmic (Garjani *et al.*, 1995). Clearly, further investigations are required to investigate the pro- and antiarrhythmic effects of ET-1.

In humans, infusion of ET-1 tends to reduce cardiac output (Wagner *et al.*, 1992b), probably through a baroreceptor-mediated decrease in heart rate, although an increase in afterload may also contribute. Coronary vasoconstriction has not been observed in clinical studies, but is recognised to occur in humans who are bitten by the burrowing asp *A. engaddensis*, the venom of which contains SRTXs (Weiser *et al.*, 1984).

4.5. *Effects on the Kidney*

ET-1 has two main direct actions on the kidney, renal vasoconstriction and increased tubular sodium and water loss. These effects probably reflect separate sites of action and perhaps separately regulated ET systems in blood vessels and renal tubules. ET-1 is a potent constrictor of both afferent and efferent glomerular arterioles *in vivo*, resulting in reduced renal plasma flow and glomerular filtration rate, and hence, reduced urine flow and sodium excretion (King *et al.*, 1989; Lerman *et al.*, 1991b). The ET receptor subtype mediating renal vasoconstriction appears to be very dependent on species, involving, for example, ET_A receptors in the dog (Brooks *et al.*, 1994) and ET_B receptors in the rat (Cristol *et al.*, 1993). The modulatory role of prostanoids remains unclear (Cao and Banks, 1990b; Munger *et al.*, 1993). ET-1 also appears to have diuretic and natriuretic effects, although it presently is unclear whether or not these are related to changes in arterial pressure (King *et al.*, 1989; Perico *et al.*, 1991). Both groups suggest that a direct action of ET-1 on the proximal tubules contributes to diuresis and

natriuresis, probably mediated by tubular ET_B receptors. In conscious rats, big ET-1 causes smaller decreases in renal blood flow and glomerular filtration rate than ET-1 (Gardiner *et al.*, 1993; Hoffman *et al.*, 1990). This difference may occur because big ET-1 is more readily converted to the mature peptide in tubular capillaries, where ET-1 mediates diuresis and natriuresis, than in the glomeruli and arterioles, where ET-1 decreases renal blood flow and glomerular filtration rate. In humans, distribution of ET receptor mRNA (Karet and Davenport, 1995) is consistent with renal vasoconstriction being mediated by ET_A receptors and the tubular actions by ET_B receptors.

In humans, systemic infusion of ET-1 causes renal vasoconstriction similar to that observed in the splanchnic bed (Weitzberg *et al.*, 1991), but more marked than in the leg (Gasic *et al.*, 1992). Infusion of ET-1 decreases renal sodium excretion (Sørensen *et al.*, 1994), even at doses insufficient to cause renal vasoconstriction (Rabelink *et al.*, 1994). Thus, to date, there is no *in vivo* evidence in humans for a renal tubular diuretic action of ET-1, although studies with selective ET receptor antagonists may prove more informative.

4.6. *Effects on the Pulmonary Circulation*

In the pulmonary circulation, ET-1 is a potent vasodilator at low doses (Lippton *et al.*, 1991; Deleuze *et al.*, 1992; Wong *et al.*, 1993) and vasoconstrictor at high doses *in vivo* (Kadowitz *et al.*, 1991). *In vitro*, the vasoconstrictor effects of ET-1 in human pulmonary resistance vessels are mediated through both ET_A and ET_B receptors (McCulloch and Maclean, 1995). The pulmonary vasodilator response, at least in newborn lambs, appears to be mediated in part by the release of NO, and may involve K_{ATP} channels, but does not involve vasodilator prostanoids (Wong *et al.*, 1993). In humans, a dose of ET-1 sufficient to increase systemic vascular resistance by about 10% does not increase pulmonary vascular resistance (Wagner *et al.*, 1992b). This area clearly merits further investigation.

4.7. *Interactions With the Nervous System*

ET-1 has central actions that may contribute to its pressor actions. Intracerebroventricular administration of low doses of ET-1 increases blood pressure through stimulation of central sympathetic outflow in the rat (Ouchi *et al.*, 1989; Yamamoto *et al.*, 1991) and rabbit (Matsumura, K. *et al.*, 1991). This pressor effect occurs with doses of ET-1 that are not sufficient to increase blood pressure when administered i.v. Also, centrally administered ET-1 appears to sensitise the baroreceptor reflex by reducing resting parasympathetic tone (Itoh and van Den Busse, 1991). In addition, chronic intracerebroventricular infusion of low dose ET-1 for 7 days progressively increases blood pressure, in association with increased urinary catecholamine and vasopressin excretion (Nishimura *et al.*, 1991). Furthermore, the presence of granules with ET-3 immunoreactivity, together with the profound effects on salt and water balance in rats, with substantial inhibition of thirst in water deprived animals, when ET-3 is topically applied to the hypothalamus (Samson *et al.*, 1991), suggests that ET may be involved in central regulation of fluid and electrolyte balance.

ET-1 may also have a role in the peripheral autonomic nervous system. Binding sites for ET-1 are present in the carotid bifurcation, and topical application of the peptide inhibits baroreceptor and stimulates chemoreceptor, responses at this site (Spyer *et al.*, 1991). In addition, ET-1 may potentiate the peripheral actions of the sympathetic nervous system in threshold doses (Wong-Dusting *et al.*, 1990; Yang *et al.*, 1990). Furthermore, ET-1 markedly elevates venous tone *in vivo* in rats via a reflex increase in sympathetic nerve activity and activation of α adrenoreceptors (Waite and Pang, 1992). However, in humans, a peripheral interaction of ET-1 with the sympathetic nervous system has not been demonstrated in forearm resistance (Cockcroft *et al.*, 1991) or cutaneous capacitance vessels (Haynes *et al.*, 1994) of healthy subjects *in vivo*.

4.8. *Interactions With Endocrine Systems*

ET-1 tends to increase the activity of the renin-angiotensin-aldosterone system, except that it inhibits renin release from isolated rat glomeruli *in vitro* (Rakugi *et al.*, 1988). ET-1 stimulates the tissue renin-angiotensin system of the rat mesenteric bed, increasing generation of renin and angiotensin II (Rakugi *et al.*, 1990). ET-1 also stimulates endothelial ACE activity (Kawaguchi *et al.*, 1991). In the adrenal gland, ET-1 stimulates release of aldosterone from isolated cortical zona glomerulosa cells (Cozza *et al.*, 1989) and adrenaline from medullary chromaffin cells (Boarder and Marriott, 1989). *In vivo*, administration of ET-1 to animals increases renin, as well as aldosterone and adrenaline concentrations (Nakamoto *et al.*, 1989; Cao and Banks, 1990a); renin secretion probably occurring as a result of ET-1-induced renal vasoconstriction. The pressor response to ET-1 is unaffected by pretreatment with the ACE inhibitor captopril (Cao and Banks, 1990a), whereas renal vasoconstriction is abolished by captopril, implying some regional variations in the interaction between ET-1 and the renin-angiotensin system. In contrast, to the acute pressor response, hypertension caused by chronic infusion of ET-1 appears to be completely prevented by concomitant administration of captopril (Mortensen and Fink, 1992b), suggesting that the chronic pressor response may be mediated via the renin-angiotensin system or that this system in some way is permissive. However, chronic infusion of ET-1 does not increase plasma angiotensin II concentrations (Mortensen and Fink, 1992b), implying either that ET-1 interacts with a tissue renin-angiotensin system or that the effect of captopril is not mediated through decreased angiotensin II generation, perhaps involving kinins. These observations may be of clinical relevance and require confirmation.

Concentrations of ET-1 and vasopressin increase in parallel during upright tilt in healthy subjects and are unchanged during tilt in patients with diabetes insipidus

(Kaufmann *et al.*, 1991). Although the suggestion has been made that ET-1 is acting as a classical circulating hormone in these circumstances, this seems unlikely, given that plasma concentrations are below that necessary to cause direct vasoconstriction. In addition, such rapid changes in plasma ET perhaps may reflect alterations in ET clearance rather than generation. Nevertheless, ET-1 may play an autocrine or paracrine role in control of vasopressin release because infusion of the peptide increases vasopressin concentrations in dogs (Nakamoto *et al.*, 1989).

Circulating concentrations of atrial natriuretic peptide are increased by infusion of ET-1 in rats (Stasch *et al.*, 1989; Garcia *et al.*, 1990), and pretreatment of rats with antiserum to atrial natriuretic peptide potentiates the pressor response to ET-1 (Valentin *et al.*, 1991). Thus, endogenously generated atrial natriuretic peptide may modulate the vasoconstriction to ET-1 *in vivo*. Brain natriuretic peptide concentrations are increased after bolus injection of ET-1 in rats, partly through a change in blood pressure and partly through direct stimulation by ET-1 (Horio *et al.*, 1992). The relevance of atrial peptides in modulating responses to ETs in humans remains to be determined.

4.9. *Endothelin and Basal Vascular Tone*

There is increasing evidence from studies using ECE inhibitors and ET receptor antagonists that basal generation of ET-1 contributes to the maintenance of basal vascular tone and to regulation of blood pressure. Many studies conclude that ET receptor antagonists have no effect on blood pressure in normotensive animals. However, carefully designed, adequately powered studies that have followed haemodynamic responses for several hours after administration of an ECE inhibitor or ET receptor antagonist do provide evidence for a role of ET-1 in regulation of basal vascular tone. Such studies are able to take into account the slow reversal of ET-1-induced vasoconstriction by anti-ET agents (Warner *et al.*, 1994). Inhibition of ET generation by administration of the ECE inhibitor phosphoramidon slowly decreases mean arterial pressure in normotensive and spontaneously hypertensive rats (SHRs) over several hours (McMahon *et al.*, 1991). Similar results have been obtained more recently using ET receptor antagonists. Intravenous infusion of the selective ET_A receptor antagonist BQ-123 into normotensive Sprague-Dawley rats decreases mean arterial pressure by ~15% over 60 min (Pollock and Opgenorth, 1993). In addition, a 6-hr infusion of BQ-123 decreases blood pressure by ~15% in Wistar-Kyoto (WKY) normotensive and spontaneously hypertensive rats (Nishikibe *et al.*, 1993). Although blood pressure did not decrease significantly in normotensive rats in this study, probably owing to the small numbers studied and the smaller absolute changes in blood pressure in these animals, they fell on the same regression line for effects of treatment as the hypertensive animals, suggesting that the relative magnitude of the hypotensive response to the ET_A antagonist is no greater in hypertensive then normotensive rats. Furthermore, the combined $ET_{A/B}$ antagonist Ro 46-2005 has been shown to dose-dependently lower blood pressure by ~30% over several hours in sodium-depleted squirrel monkeys (Clozel *et al.*, 1993). Finally the ET_A antagonist BQ-123 and the ET_A/ET_B antagonist bosentan both lower blood pressure by ~20% in anaesthetised and conscious guinea pigs (Véniant *et al.*, 1994). The hypotensive effect of bosentan is not affected by blockade of the sympathetic or parasympathetic nervous systems, or by inhibition of renin, cyclooxygenase, bradykinin or NO. Bosentan has no additional hypotensive effect to BQ-123 when co-administered with the ET_A antagonist, suggesting that ET-1 contributes to the maintenance of resting arterial blood pressure predominantly through activation of ET_A receptors in these animals. Together, these results imply that ET-1 contributes to the maintenance of blood pressure under physiological conditions, at least in some species, probably through its actions as a potent arteriolar vasoconstrictor.

It was hoped that studies with transgenic animals might improve our understanding of ET's role in blood pressure control. However, neither ET-1 overexpressing rats nor ET-1 gene-deficient mice are viable (Kurihara *et al.*, 1994; Maemura *et al.*, 1995). In the latter case, homozygous ET-1 deletion mice have serious craniofacial abnormalities that result in death from asphyxia soon after birth, demonstrating a role for ET-1 in development of pharyngeal arch-derived tissues and organs (Kurihara *et al.*, 1994). Heterozygotes, although anoxic, are viable and have reduced plasma and tissue ET-1 concentrations, but paradoxically, slightly higher blood pressure than the wild-type mice. The reasons for the increased blood pressure are not understood completely; preliminary studies have demonstrated that sensitivity to ET-1 is not increased in these mice, nor is release of endothelium-dependent relaxant factors reduced. Elevated blood pressure is unlikely to indicate a hypotensive role for ET-1, it is more likely to be explained by changes in the chemoreceptor reflex and increases in the sympathetic drive in the heterozygotic mice. Rats overexpressing the ET-2 gene are viable, but not hypertensive, perhaps because the highest gene expression is in the kidney rather than in the blood vessels and because plasma levels of ET-2 are only marginally increased (Leifeldt *et al.*, 1995).

Brachial artery infusion studies have been used to explore the role of ET-1 in maintenance of basal vascular tone in humans (Haynes and Webb, 1994). The ECE and NEP inhibitor phosphoramidon caused progressive vasodilatation of forearm resistance vessels, whereas a selective NEP inhibitor thiorphan caused vasoconstriction, suggesting that generation of ET-1 by ECE provides an important contribution to the maintenance of basal vascular tone in humans. This work is supported by the results of additional studies in which brachial artery infusion of the ET_A receptor antagonist, BQ-123 also caused progressive substantial forearm vasodilatation (Fig. 9). These results suggest that endogenous generation of ET-1 maintains forearm vascular tone in humans, at least in part through activation of ET_A receptors. However, a role for ET_B receptors cannot be ex-

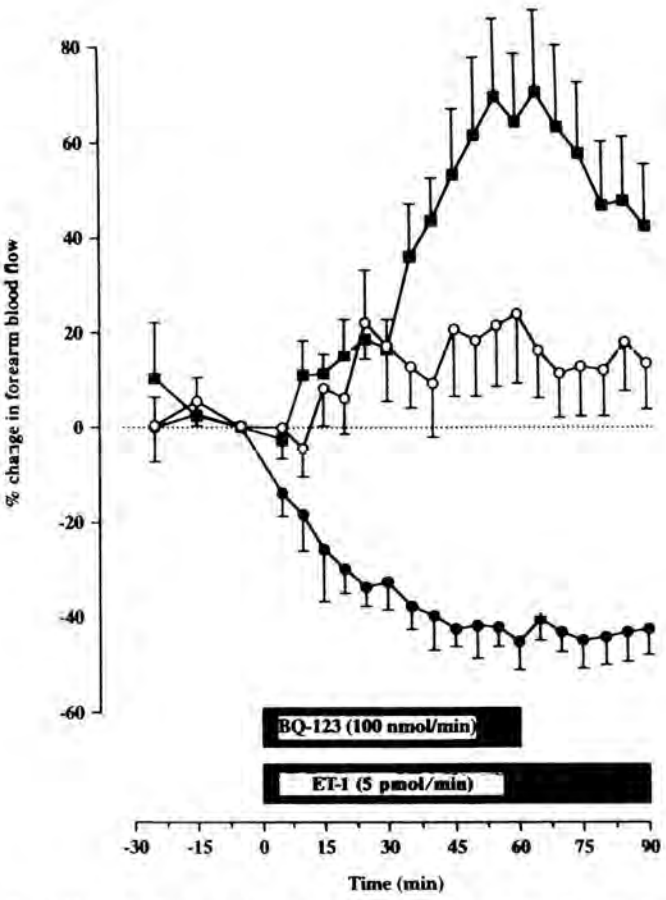


FIGURE 9. Brachial artery infusion of ET-1 to healthy human subjects causes significant forearm vasoconstriction (●) that can be abolished by co-infusion of the ET_A receptor antagonist BQ-123 (○). Infusion of BQ-123 alone causes progressive and substantial forearm vasodilatation (■). Values are mean ± SEM; n = 6 for each group. Reproduced from Haynes and Webb (1994), with permission of the copyright holder, The Lancet Ltd., London.

cluded presently, and studies with ET_B receptor antagonists are urgently needed to resolve this issue.

More recently, a major cardiovascular role for ET-1 suggested by studies in the forearm has been confirmed on systemic administration of an ET receptor antagonist in humans (Haynes *et al.*, 1996). Studies in healthy normotensive subjects have shown that i.v. administration of doses of 10–1000 mg of the peptide combined ET_A/ET_B receptor antagonist TAK-044 causes ~10% reduction of blood pressure that is sustained for at least 4 hr, predominantly through an effect on peripheral vascular resistance. The change in blood pressure occurs at doses as low as 10 mg, and is accompanied by an increase in cardiac index. Systemic doses as low as 30 mg were able to abolish the local vasoconstriction to ET-1 given into the brachial artery up to 3 hr later. As in animal studies (Löffler *et al.*, 1993; Fukuroda *et al.*, 1994), TAK-044 caused an increase in plasma ET concentration. This appears to be due to an effect on clearance, rather than production, because ET_B, but not ET_A blockade, increases plasma ET-1 and ET-3 concentrations without increasing big ET-1 concentrations (Löffler *et al.*, 1993). Additionally, only ET_B receptor blockade increases the half-life of exogenous [^{125}I]-ET-1. Furthermore, the increase in plasma ET is substantial and occurs within 15 min, which is unlikely to be related to *de novo* generation of ET-1. The rise in plasma ET-1 was not unfavourable; in fact, it provided a measure of efficacy correlating closely with the fall in blood pressure.

Few mediators, besides ET-1, have been shown to have such a fundamental physiological role in maintenance of basal vascular tone. Sustained vasoconstriction elicited by ET-1 may act in concert with the short-lived effects of other regulatory systems, such as the sympathetic nervous system and NO, to stabilise vasomotor tone, while preserving flexibility in its dynamic control. These results, and those from animals, suggest that orally active ECE inhibitors and ET receptor antagonists may have potential therapeutic uses as novel vasodilator agents in situations of chronic vasoconstriction and in conditions characterised by ET-1-mediated vasospasm.

5. ENDOTHELIN IN CARDIOVASCULAR PATHOPHYSIOLOGY

Several requirements are necessary before it is appropriate to attribute a role to ET in cardiovascular disease. These include: (i) evidence that ET mimics, or causes, the consequences of this disease; (ii) that production of ET or its receptors are increased in the disease; and (iii) that inhibitors of the production or actions of ET can ameliorate the disease process. In many conditions, some of these requirements have been fulfilled, but not all. Increased plasma ET concentrations and increased vascular sensitivity to ET can be particularly misleading: the former because ET is released abluminally (Wagner *et al.*, 1992a), is not generally a circulating hormone (Haynes and Webb, 1993b), and its plasma concentrations may be more dependent on clearance than production; and the latter because increased production may lead to receptor down-regulation and reduced sensitivity to exogenous peptide (Bloch *et al.*, 1989a), whereas reduced production may lead to receptor up-regulation and increased sensitivity. Unless responses to exogenous ET are interpreted in the light of ET receptor number and affinity, the results of such studies may suggest that ET is not involved in pathological conditions in which its production is increased. In contrast, studies with antagonists are particularly important, and these have been aided by the rapid development of selective and potent antagonists at ET_A and ET_B receptors. Another potentially confounding factor when examining responses in blood vessels is the influence of the structural changes that can occur in hypertension and heart failure and are discussed in Section 4.3. This section addresses the major cardiovascular diseases in which the ET system has been implicated and in which inhibition of the production or effects of ET might offer new therapeutic perspectives.

5.1. *Hypertension*

Established essential hypertension is a condition characterised by a high blood pressure in association with increased peripheral vascular resistance. Cardiac and vascular hypertrophy may also develop, and are associated with a worse prognosis. In the early stages, and in borderline hypertension, cardiac output appears to increase and sympathetic tone may be enhanced. This common condition, affecting around 10% of the adult population, makes a major contribution to the population burden from cardiovascular disease, predisposing particularly to myocardial infarction, stroke, and heart and kidney failure. A number of effective hypotensive drugs are available for the treatment of this condition, although none are fully effective against the commonest consequence of essential hypertension, myocardial infarction. In addition, the mechanisms causing essential hypertension are still poorly understood, and the drugs presently available may not act to oppose the important causal mechanisms.

The endothelium is an attractive target for investigation in essential hypertension, and in cardiovascular disease in general. It is clear that NO plays an important role in regulation of vascular tone and platelet aggregation (Vane *et al.*, 1990), and endothelial dysfunction related to the L-arginine/NO system occurs in hypertension, diabetes mellitus and hyperlipidaemia (Vallance and Moncada, 1994), all of which conditions predispose to an increased risk of cardiovascular disease. ET-1 is also an attractive target for investigation in hypertension because it has potent vasoconstrictor and pressor properties, is mitogenic and can cause vessel hypertrophy, and it appears to enhance sympathetic function *in vitro*.

5.1.1. Endothelin concentrations and production. In animal models of hypertension, ET-1 concentrations are not raised unless accelerated hypertension is present, in which case, they correlate positively with plasma creatinine (Yan *et al.*, 1990; Suzuki *et al.*, 1990; Vemulapalli *et al.*, 1991; Kohno *et al.*, 1991). Local mesenteric vascular generation of ET appears to be increased *in vitro* in SHR compared with WKY rats (Miyamori *et al.*, 1991). However, others have found ET immunoreactivity to be increased in blood vessels from deoxycorticosterone acetate (DOCA)-salt rats, but decreased in blood vessels from SHR as compared with normotensive WKY rats (Larivière *et al.*, 1993). In functional studies in rats, the hypotension following infusion of the ECE inhibitor phosphoramidon was similar in WKY and SHR (McMahon *et al.*, 1991). Also against a role for increased generation of ET-1 in the pathophysiology of experimental hypertension is the finding that polymorphisms of the prepro ET-1 gene do not co-segregate with blood pressure or cardiac weight in inbred Dahl rats (Cicila *et al.*, 1994). Interestingly, the ET-3 gene does appear to be linked to a locus that regulates blood pressure in these rats and in humans, variations in the prepro ET-1 and ET_A genes recently were shown to be linked to blood pressure in a population study (Stevens and Brown, 1995).

Renal effects of ET may also potentially contribute to the development of hypertension. The vascular/glomerular actions (mainly ET_A) predispose to sodium retention and may be enhanced in the SHR (Tomobe *et al.*, 1988). The renal tubular actions (mainly ET_B) act to increase urinary sodium and water excretion. Generation of ET-1 in the renal medulla, particularly the collecting duct, is reduced in SHR compared with WKY rats (Kitamura *et al.*, 1989; Hughes *et al.*, 1992) and, therefore, may contribute to sodium retention and hypertension in this species. Patients with essential hypertension excrete less immunoreactive ET in urine than normotensive controls (Hoffman *et al.*, 1990), emphasising the need for further investigation in this area.

Plasma immunoreactive ET concentrations were reported to be elevated in the first studies in hypertensive patients (Kohno *et al.*, 1990; Saito *et al.*, 1990; Shichiri *et al.*, 1990). However, clearance of ET-1 is very dependent on renal function (Kohno *et al.*, 1989; Koyama *et al.*, 1989), and the high concentrations found in severe and accelerated phase hypertension are, at least in part, secondary to impaired renal clearance of ET. Studies in well-characterised hypertensive patients with normal renal function have shown similar concentrations of ET-1 to those in well-matched normotensive subjects (Davenport *et al.*, 1990; Schiffrin and Thibault, 1991; Haak *et al.*, 1992; Haynes *et al.*, 1994). Indeed, in one study, there was a negative correlation between blood pressure and plasma ET-1 in the hypertensive group (Davenport *et al.*, 1990), making a global increase in generation of ET-1 unlikely as a cause of essential hypertension. In the hypertension associated with pre-eclampsia of pregnancy, plasma ET is elevated despite normal renal function, consistent with a role for ET in the pathophysiology of this hypertensive condition (Florijn *et al.*, 1991).

Increased production of ET-1 occurs in one secondary form of hypertension, albeit rare. In 1991, Yokokawa and colleagues described two cases of the skin tumour haemangioendothelioma in which hypertension was associated with increased plasma ET concentrations. Biopsies of tumour cells displayed increased expression of mRNA for prepro ET-1 and strong immunohistochemical staining for the peptide. Blood pressure and plasma ET concentrations returned to normal in both cases following surgical resection of the tumours, and in one patient, recurrence of the tumour led to a further increase in both blood pressure and plasma ET.

5.1.2. Sensitivity to endothelin. In studies designed to examine vascular sensitivity to ET-1 in hypertension, it is important to be aware that the development of vascular hypertrophy will tend to amplify responses in the hypertensive vessels (Folkow, 1978). In studies comparing WKY and SHR, both conduit and resistance vessels from the SHR are more sensitive to the effects of ET-1 (Tomobe *et al.*, 1988; Clozel, 1989; Maclean and McGrath, 1990; Criscione *et al.*, 1990). In the Dahl salt sensitive rat, vascular responsiveness to ET is enhanced prior to, but not after, the devel-

opment of hypertension (Goligorsky *et al.*, 1991). Other investigators have reported decreased sensitivity to ET in the aorta and mesenteric resistance arteries from SHR (Dohi *et al.*, 1991), DOCA-salt rats (Deng and Schiffrin, 1992) and renovascular hypertensive animals (Dohi *et al.*, 1991; Roberts-Thompson *et al.*, 1994). This may be a consequence of receptor down-regulation secondary either to increased local generation of ET or raised blood pressure (Larivière *et al.*, 1993). Down-regulation of receptors is suggested by the decreased Ca^{2+} response to ET-1 of isolated vascular smooth muscle cells from SHR (Touyz *et al.*, 1994). In this regard, it is interesting that sensitivity to ET in hypertensive animals can be restored by antihypertensive therapy (Dohi *et al.*, 1992).

Systemic doses of ET-1 *in vivo* have greater pressor effects in SHR than WKY rats (Miyauchi *et al.*, 1989a) and in renovascular hypertensive than normotensive rabbits (Roberts-Thompson *et al.*, 1994). The mechanism for this enhanced sensitivity to ET-1 is unclear because the number of binding sites for ET-1 in aortic smooth muscle (Clozel, 1989) and heart (Gu *et al.*, 1990) are lower in SHR, suggesting increased postreceptor sensitivity or the influence of vascular hypertrophy. There is, however, a relative increase in the number of binding sites for ET-1 in the brain of SHR, as compared with WKY rats (Gu *et al.*, 1990), so there may be increased CNS sensitivity to the peptide in hypertension.

In patients with essential hypertension, *in vitro* efficacy of ET in subcutaneous resistance arteries appears to be reduced (Schiffrin *et al.*, 1992). However, *in vivo*, the situation is rather different. A recent study (Haynes *et al.*, 1994) has investigated whether vasoconstriction to ET-1 is altered in patients with essential hypertension as compared with well-matched normotensive control subjects (Fig. 10). Responses were examined to local infusion of ET-1 into hand veins, rather than arteries, because the venous system may contribute to the high cardiac output reported to occur in early hypertension and because the confounding effects of vascular hypertrophy do not occur in hand veins (Haynes *et al.*, 1994; Eichler *et al.*, 1989). Sympathetic responses were also examined in this study because ET-1 has been shown to potentiate sympathetic responses *in vitro* (Ouchi *et al.*, 1989; Matsumura, K. *et al.*, 1991; Wong-Dusting *et al.*, 1990; Yang *et al.*, 1990). ET-1 caused a slow-onset venoconstriction in both groups of subjects, with the maximal effect reached by 90 min. However, maximal venoconstriction to ET-1 was substantially greater in hypertensive patients than in control subjects (Fig. 10). Sympathetically mediated venoconstriction was also enhanced in the hypertensive patients, but not in the control subjects (Fig. 10).

These studies (Haynes *et al.*, 1994) have shown that patients with essential hypertension have enhanced venoconstriction to ET-1. These results might be explained by decreased local venous ET-1 generation, although plasma ET concentrations were similar to those in normotensive subjects. The negative correlation with blood pressure in the normotensive subjects is against this phenomenon, occurring solely as a consequence of the increase in blood pressure, and a causal relationship with the elevation of blood pressure is supported by the positive correlation in the hypertensive subjects. Enhanced facilitation of sympathetic vasoconstriction appears to be a separate phenomenon because the lack of basal tone in these vessels precludes an explanation for enhanced venoconstriction to ET-1 by potentiated sympathetic responsiveness. In addition, the sympathetic effect appears to be confined to hypertensive patients because, as well as in this study, it was not seen in a separate study in normotensive subjects (Cockroft *et al.*, 1991). Interestingly, and very importantly, ventricular ET immunoreactivity, ventricular ET binding sites, and cardiac

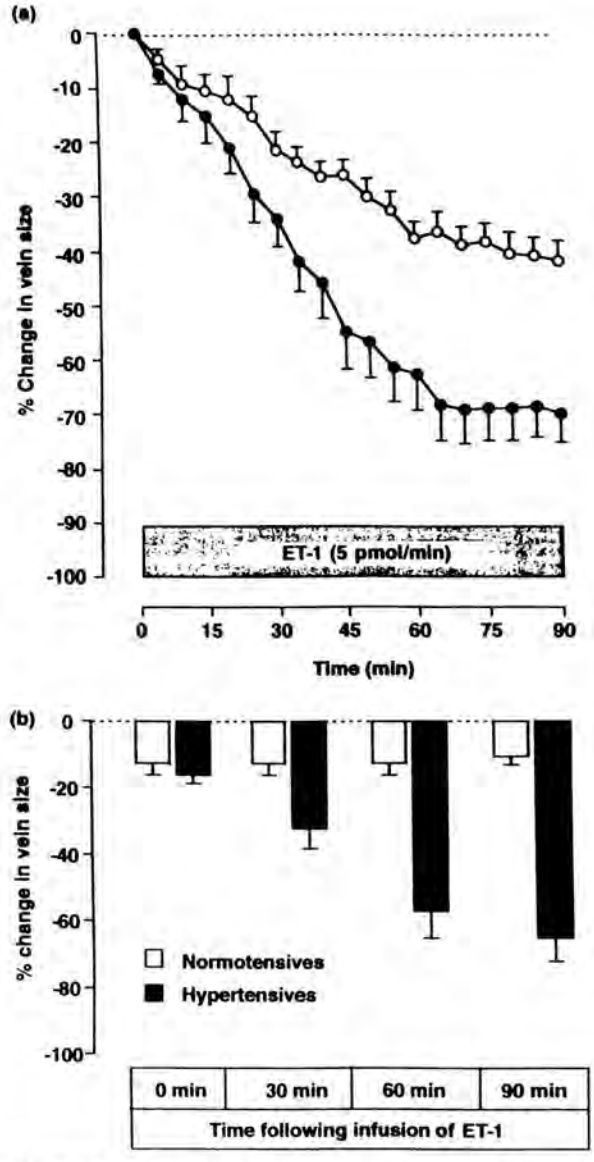


FIGURE 10. (a) Venoconstriction to ET-1 in normotensive (○) and hypertensive (●) subjects. (b) Sympathetically mediated venoconstriction induced by single deep breath before and during infusion of ET-1 in hypertensive and normotensive subjects. Values are the mean ± SEM, n = 8 for each group. Reproduced from Haynes *et al.* (1994), by copyright permission of The American Society for Clinical Investigation, New York.

myocyte prepro ET-1 mRNA are increased in the hearts of animals with cardiac hypertrophy caused by suprarenal aortic banding (Arai *et al.*, 1995). These findings are consistent with, and strongly supportive of, a role for ET-1 in the pathogenesis of cardiac hypertrophy caused by pressure overload, and suggest that ET antagonists may be capable of reversing cardiac hypertrophy.

5.1.3. Studies with inhibitors and antagonists. Both ECE inhibitors and ET receptor antagonists lower blood pressure in hypertensive rat models (McMahon *et al.*, 1991; Bazil *et al.*, 1992; Nishikibe *et al.*, 1993; McMahon *et al.*, 1993). The ET_A receptor antagonist BQ-123 acutely lowers blood pressure in stroke-prone SHR, but not in control SHR or WKY rats (Nishikibe *et al.*, 1993). When administered chronically, BQ-123 prevents the development of stroke and renal abnormalities in stroke-prone SHR (Nishikibe *et al.*, 1993). This ET_A receptor antagonist also lowers blood pressure in low-renin animal models of hypertension (the SHR and DOCA-salt treated rats), but not in control animals or in a high-renin animal model of hypertension (Bazil *et al.*, 1992).

There is now also evidence that ET receptor antagonists cause vasodilatation in hypertensive patients (Ferro *et al.*, 1996). In addition, the combined ET_A/ET_B antagonist TAK-044 lowers blood pressure in healthy normotensive humans (Haynes *et al.*, 1996). As might be expected on this basis, acute administration of the orally active combined ET_A/ET_B antagonist bosentan recently has been reported to lower blood pressure in subjects with essential hypertension.* At present, there is no evidence that drugs based on blocking the ET system will be selectively 'antihypertensive' rather than hypotensive. However, based on vasodilator, antimitogenic and sympatholytic properties, they may offer particular advantages in the treatment of hypertension and the prevention of myocardial infarction. Indeed, given the potential role of ET-1 in the atherosclerotic process, an antiatherosclerotic action may be of substantial importance (see Section 5.2). The development of ET receptor antagonists for the treatment of hypertension is likely to be a major focus for clinical investigation over the next few years.

5.2. *Ischaemic Heart Disease*

The SRTXs, potent vasoconstrictor peptides isolated from snake venom with close structural similarity to the ETs (Sokolowsky *et al.*, 1990), cause death from myocardial ischaemia and infarction secondary to coronary vasoconstriction (Wollberg *et al.*, 1987). Exogenously administered ET-1 also produces myocardial ischaemia (Kurihara *et al.*, 1989) by causing coronary vasoconstriction. Plasma ET concentrations (Valesco *et al.*, 1994) and myocardial ET-1 binding sites (Liu *et al.*, 1990) are raised following ischaemia/reperfusion in animals. However, the evidence suggesting a beneficial effect of ET antagonism upon myocardial infarct size has been contradictory. The ET_A receptor antagonists FR 139317 (McMurdo *et al.*, 1994) and BQ-123 (Krause *et al.*, 1994) had no effect on infarct size in rabbit and canine model of myocardial infarction and reperfusion. In a rabbit model, infarct size was reported to be reduced following intraventricular administration of a low dose of ET-1, suggesting that ET-1 might have a cardioprotective role (Hide *et al.*, 1995). In contrast, monoclonal antibodies against ET-1 (Watanabe *et al.*, 1991b), ECE inhibitors (Grover *et al.*, 1992), selective ET_A antagonists (Grover *et al.*, 1993) and combined ET_A/ET_B antagonists (Watanabe *et al.*, 1995) were all reported to reduce infarct size in rat and canine models, supporting the view that endogenous ET-1 worsens rather than improves the myocardial damage that follows ligation of the coronary artery.

In humans, plasma ET is raised in acute myocardial infarction and unstable angina (Miyauchi *et al.*, 1989b), suggesting a possible pathophysiological role for ET-1, whereas patients with stable angina do not have raised plasma ET (Ray *et al.*, 1992). The higher the plasma ET in myocardial infarction (Omland *et al.*, 1994) and unstable angina (Wieczorek *et al.*, 1994), the worse the prognosis. Plasma ET concentrations on the third day after myocardial infarction significantly related to mortality (Omland *et al.*, 1994), and plasma ET concentrations at 9 weeks after hospitalisation with unstable angina or non Q-wave myocardial infarction significantly related to the incidence of further cardiovascular events (Wieczorek *et al.*, 1994). Patients undergoing fibrinolysis during the acute phases of myocardial infarction have been shown to have reduced plasma ET compared with patients who did not have early reperfusion (Lechleitner *et al.*, 1993).

ET-1 could contribute to myocardial ischaemia and the proliferative effects of ET-1 could contribute to vascular and cardiac hypertrophy and the atherosclerotic process. Indeed, plasma ET concentrations are raised in advanced atherosclerosis (Winkles *et al.*, 1993), and expression of ET-1 mRNA is increased in the vascular smooth muscle of atherosclerotic human arteries (Winkles *et al.*, 1993). Furthermore, increased tissue ET immunoreactivity has been reported in the active atherosclerotic lesions associated with unstable angina (Zeiher *et al.*, 1995).

The use of ET receptor antagonists in acute myocardial infarction may be of clinical benefit. First, if they limit infarct, they would slow the progression to heart failure. Second, they may reduce the incidence of further ischaemic events or the need for revascularisation. Third, they may prevent remodelling after infarction in a similar fashion to ACE inhibitors. However, given the wide range of drugs currently available for the treatment of myocardial infarction, and the likely diminishing returns with additional therapy, pharmaceutical companies may be wary of developing ET receptor antagonists for this indication.

*Schmitt, R., Belz, G. G., Fell, D., Lehmeier, R., Prager, G., Stahnke, P. L., Sittner, W. D., Karworth, A. and Jones, C. R. (1995) Effects of the novel endothelin receptor antagonist bosentan in hypertensive patients. In Proceedings of the 7th Meeting of the European Hypertension Society, June, 1995, Milan, Italy.

Coronary sinus ET-1 levels are raised during and immediately after percutaneous transluminal coronary angioplasty (PTCA) (Tahara *et al.*, 1991). Although ET-1 could be involved in the ischaemia, acute vasospasm, and abrupt vessel closure related to PTCA, these complications are uncommon and readily reversible with conventional therapy. The main weakness of PTCA is the relatively high risk of restenosis. Clinical restenosis occurs in up to 30% of patients within the first year following the procedure (MERCATOR Study Group, 1992) and is characteristically associated with vascular smooth muscle proliferation. In experimental models of angioplasty, ET-1 levels are raised after balloon denudation of the endothelium (Azuma *et al.*, 1994) and areas of neointima formation show increased expression of prepro ET-1, ECE-1, ET_A and ET_B receptor mRNAs (Wang *et al.*, 1995). Studies with ET antagonists in these models show that smooth muscle proliferation is inhibited by an ET_A/ET_B antagonist TAK-044 (Tsujino *et al.*, 1995), but not by the ET_A antagonist BQ-123 (Azuma *et al.*, 1994; Douglas *et al.*, 1995b). Thus, it is conceivable, given the potent mitogenic property of ET-1, that prolonged treatment with an oral ET_A/ET_B or ET_B antagonist may be useful in PTCA. Similar arguments are relevant to graft occlusion after coronary artery bypass grafting.

Prinzmetal's, or variant angina, first described in 1959 (Prinzmetal *et al.*, 1959), is characterised by chest pain developing at rest, frequently in the early morning, and associated with ST elevation on the electrocardiogram. The pain is usually relieved by glyceryl trinitrate. Coronary spasm has been demonstrated at angiography in patients with this condition, as well as the absence of occlusive lesions (Dhurandhar *et al.*, 1972). Patients with variant angina are known to have endothelial dysfunction, and as a powerful vasoconstrictor of human (Yang *et al.*, 1990) and canine (Kurihara *et al.*, 1989) coronary arteries, ET-1 has been implicated in the pathophysiology of this condition (Lüscher, 1991). ET-1 also potentiates the coronary vasoconstriction induced by serotonin and noradrenaline in isolated human arteries (Yang *et al.*, 1990). Patients with variant angina have elevated plasma ET during provocation of coronary vasospasm (Matsuyama *et al.*, 1991), and one study has also found basal plasma ET concentrations to be elevated (Artigou *et al.*, 1993). Interestingly, there is an increased prevalence of primary Raynaud's disease and migraine in patients with variant angina (Miller *et al.*, 1981; O'Keefe *et al.*, 1992), and ET-1 has also been implicated in the pathophysiology of all of these vasospastic disorders (Haynes and Webb, 1993b).

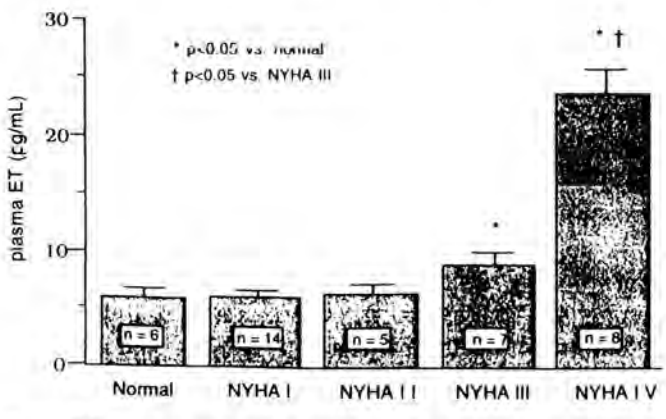


FIGURE 11 Bar graph of plasma ET concentration of healthy subjects (normal) and CHF patients, grouped according to the severity of heart failure as assessed by the New York Heart Association (NYHA) score. Values are the mean ± SEM. Reproduced from Wei *et al.* (1994), with permission of the authors and the copyright holder, American Heart Association, Dallas.

It appears, therefore, that ET-1 has a pathophysiological role in variant angina, either as a mediator of coronary vasospasm or by sensitising the vasculature to other vasoconstrictors. In either case, ET receptor antagonists might prove useful in the management of this condition.

5.3. *Heart Failure*

Congestive heart failure (CHF) is characterised by low cardiac output, sodium and water retention, peripheral vasoconstriction and activation of the renin-angiotensin-aldosterone axis (Zelis and Flaim, 1982; Francis *et al.*, 1984). ET-1 may be involved in the pathophysiology of CHF as part of the neurohumoral response to cardiac failure (Stewart, 1993), and its actions (Miller *et al.*, 1989) would certainly contribute to the vicious circle of haemodynamic decline associated with this condition.

Plasma ET concentrations are raised in animal models of CHF (Cavero *et al.*, 1990) and in patients with CHF (McMurray *et al.*, 1992). The increase in plasma ET concentration correlates closely with the degree of haemodynamic and functional impairment (Fig. 11; Wei *et al.*, 1994; Pacher *et al.*, 1993), with higher concentrations predicting a greater likelihood of death or need for cardiac transplantation (Pacher *et al.*, 1993). Although impaired renal function and decreased clearance of ET-1 may be involved in generating high plasma ET, raised plasma big ET-1 concentrations suggest that increased production may also contribute (Pacher *et al.*, 1993; Wei *et al.*, 1994). Also, ET-1 release by cultured cells is increased in the presence of angiotensin II and vasopressin, levels of which are elevated in CHF (Emori *et al.*, 1991). In patients with intractable heart failure after myocardial infarction (Tohmo *et al.*, 1994), i.v. infusion of enalaprilat (the active form of enalapril) produced a clinical improvement, as well as a reduction in plasma ET.

The ET_A receptor antagonist BQ-123 and the ECE inhibitor phosphoramidon cause peripheral vasodilatation when infused into the forearm circulation of patients with stable CHF already on treatment with a loop diuretic and a maximal dose of an ACE inhibitor (Love *et al.*, 1994). In comparison to healthy subjects, vasodilatation to BQ-123 tended to be reduced and that to phosphoramidon increased in patients with CHF, consistent with up-regulation of ET_B-mediated vasoconstriction. More recent studies

TABLE 4. Haemodynamic Effects of the ET Receptor Antagonist Bosentan in Patients with Chronic Heart Failure

		Bosentan		
	0	100 mg i.v.	200 mg i.v.	P
MAP (mmHg)	84	78	75	<0.001
CI (L/min/m²)	2.13	2.41	2.43	<0.001
SVR (dyn.sec.cm⁻⁵)	1479	1236	1187	<0.001
PCWP (mmHg)	22.5	20.8	19.8	<0.001
RAP (mmHg)	11.1	9.5	9.1	<0.001
HR (beats/min)	86	85	85	NS

Effect of bosentan on mean arterial pressure (MAP), cardiac index (CI), systemic vascular resistance (SVR), pulmonary capillary wedge pressure (PCWP), right atrial pressure (RAP) and heart rate (HR) in patients with severe (NYHA Grade IIIz–IV) heart failure. Values are shown before infusion (0), at 60 min after infusion of 100 mg bosentan and at 60 min after the subsequent infusion of 200 mg bosentan,each administered i.v. over 15 min. p values for repeated measures ANOVA.

Adapted from Kiowski *et al.*, (1995).

with ET-1 and the ET_B specific agonist SRTX S6c are also consistent with this hypothesis (Love *et al.*, 1996). Angiotensin II concentrations are increased in CHF and, interestingly, angiotensin II has been shown to down-regulate total ET binding sites (Roubert *et al.*, 1989), but up-regulate ET_B receptor mRNA (Kanno *et al.*, 1993). Given that ET_B receptors can mediate vasoconstriction (Haynes *et al.*, 1995), it may be that vasoconstrictor ET_B receptors have greater functional significance in CHF. Encouragingly, recent studies have shown that systemic doses of the combined ET_A/ET_B antagonist bosentan in patients with severe chronic heart failure produces sustained systemic, pulmonary and peripheral venous vasodilatation, and improved cardiac performance, without causing reflex tachycardia (Table 4; Kiowski *et al.*, 1995). Interestingly, in an animal model of CHF, bosentan provided additional haemodynamic benefit when combined with an ACE inhibitor (Teerlink *et al.*, 1994b).

These observations justify the development of combined ET_A/ET_B antagonists for the treatment of CHF. Vasodilatation has proved to be the most effective of the recent therapeutic approaches to CHF (Cohn *et al.*, 1991; The SOLVD Investigators, 1991), and, therefore, the results of longer-term clinical studies of ET receptor antagonists in CHF patients must be awaited with interest.

5.4. *Cerebrovascular Disease*

5.4.1. Ischaemic Stroke. The evidence for a pathophysiological role for ET-1 in ischaemic stroke is relatively weak. Increased plasma and cerebrospinal fluid (CSF) concentrations of ET have been found in animal models of stroke (Willette *et al.*, 1993). Although there have been few studies with ET antagonists, the combined ET_A/ET_B receptor antagonist SB 209670 has been shown to protect from ischaemia-induced neuronal degeneration in a gerbil stroke model (Ohlstein *et al.*, 1994). Plasma ET is also raised in humans after ischaemic stroke (Ziv *et al.*, 1992), suggesting a possible pathophysiological role for ET in this condition. However, considerably more experimental evidence from animal models is required before clinical trials with ET antagonists are likely to be embarked on in this indication.

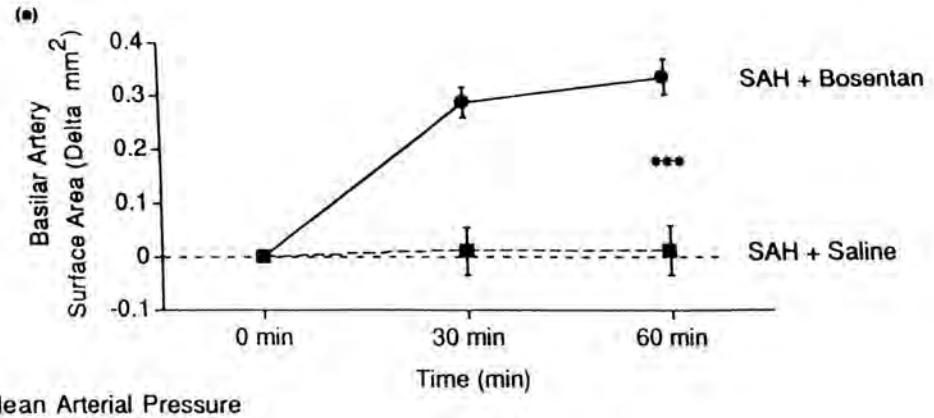
5.4.2. Subarachnoid haemorrhage. The development of cerebral vasospasm within the first 2 weeks after subarachnoid haemorrhage (SAH) is responsible for much of the morbidity and mortality associated with this condition (Asano *et al.*, 1990; Seifert *et al.*, 1995), and ET-1 has been implicated in mediating SAH-induced vasospasm. ET-1 is a potent vasoconstrictor of isolated cerebral arteries (Asano *et al.*, 1989) and its effects are potentiated after SAH (Alafaci *et al.*, 1990). Plasma ET levels are raised in a canine model of SAH (Yamamura *et al.*, 1992), and injection of exogenous ET-1 into the CSF of dogs reproduces the vasospasm that occurs following SAH (Asano *et al.*, 1989). Furthermore, both thrombin and oxyhaemoglobin, which are present in high concentrations in SAH, are known to induce ET-1 release (Yanagisawa *et al.*, 1988a; Schini *et al.*, 1989; Mayberg *et al.*, 1990).

Inhibition of the enzymatic conversion of big ET-1 to ET-1 by phosphoramidon reduces the vasospasm seen in a dog model of SAH (Matsumura, Y. *et al.*, 1991a), as do monoclonal antibodies to ET-1 (Yamamura *et al.*, 1992). The selective ET_A antagonists BQ-123 (Clozel and Watanabe, 1993), BQ-485 (Itoh *et al.*, 1993) and FR 139317 (Nirei *et al.*, 1993) have also been shown to reduce vasospasm in other models of SAH. The combined ET_A/ET_B antagonist bosentan markedly reduces the delayed cerebral vasospasm that follows injection of autologous blood into the cisterna magna in a rabbit model of SAH (Roux *et al.*, 1995). Bosentan also reverses an established spasm in this model when given i.v. without lowering systemic blood pressure (Fig. 12; Roux *et al.*, 1995). This finding may be of great significance if bosentan is able to reverse vasospasm in humans at a dose that has no systemic effects, as the major complication of nimodipine, the current preferred treatment for SAH, is hypotension with the potential risk of further cerebral hypoperfusion.

Plasma and CSF ET concentrations are significantly raised in patients after SAH (Masaoka *et al.*, 1989; Seifert *et al.*, 1995) and are highest in those patients who develop vasospasm (Suzuki *et al.*, 1992). Thus, it appears that ET-1 may be implicated in the pathophysiology of delayed vasospasm following SAH. ET receptor antagonists may provide an important addition to the limited range of drugs currently available for the management of SAH, and clinical studies must be awaited with great interest.

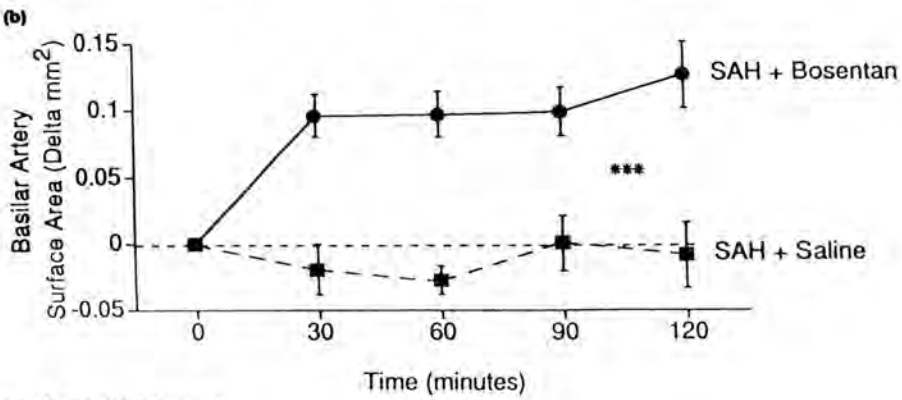
5.5. *Raynaud's Disease*

Raynaud's disease is a common condition in colder climates (Belch, 1991). It is characterised by the development of episodic ischaemia, usually in exposed extremities and associated with exposure to cold, hormones, drugs and emotional



Mean Arterial Pressure (mmHg)

	0 min	30 min	60 min
SAH + Bosentan	*103 ± 4.6*	*101 ± 4.1*	*103 ± 4*
SAH + Saline	*108 ± 4*	*103 ± 5.5*	*107 ± 4.3*



Mean Arterial Pressure (mmHg)

	0 min	30 min	60 min	90 min	120 min
SAH + Bosentan	*108 ± 4.5*	*104 ± 5.1*	*103 ± 5*	*101 ± 5.8*	*102 ± 5.6*
SAH + Saline	*116 ± 3.8*	*119 ± 3.2*	*116 ± 4.8*	*111 ± 4.8*	*112 ± 4.9*

FIGURE 12. Reversal of chronic basilar artery spasm after experimental SAH in (a) rabbit (single haemmorhage) and (b) dog (double haemmorhage), by systemic administration of an ET receptor antagonist, bosentan. SAH was induced by injection of autologous blood into the cisternal magna. Changes in cross-sectional area of the basilar artery are displayed with the corresponding arterial pressure after treatment. Reproduced from Roux *et al.* (1995), with permission of the copyright holder, Williams & Wilkins Company, Baltimore.

stimuli (Cotton and Khan, 1986). Although many treatments are available for patients with Raynaud's disease, none is effective in the majority of subjects (Cooke and Nicolaides, 1990) and the pathophysiology remains unresolved. However, there is an association between Raynaud's disease and other vasospastic conditions, including migraine and variant angina (Miller *et al.*, 1981; O'Keefe *et al.*, 1992), suggesting that there may be a vascular defect common to these conditions. A defect in endothelium dependent dilatation in the veins of patients with Raynaud's disease has been reported (Bedarida *et al.*, 1993). Overproduction of ET-1, reduced production of endothelium-dependent vasodilator substances, or a combination of these effects, may account for the vasospasm seen in Raynaud's disease.

Clinical studies have shown that plasma ET increases rapidly during the cold-pressor test in healthy subjects, peaking at 4 min after immersion of the arm in cold water (Fyhrquist *et al.*, 1990), and cold provocation tests in patients with Raynaud's disease have shown an exaggerated increase in ET concentrations in venous blood draining the cold challenged arm, compared with both the control arm and with responses in healthy control subjects (Zamora *et al.*, 1990). Elevated basal plasma ET has been reported in Raynaud's patients between vasospastic episodes consistent with increased production (Zamora *et al.*, 1990).

These results support a role for ET-1 in the pathophysiology of Raynaud's disease. However, so far there have been no confirmatory studies using ET antagonists, which would need to be more effective and better tolerated than the current 'gold standard,' nifedipine, if they are to achieve widespread clinical use.

6. CONCLUSIONS: THE THERAPEUTIC OPTIONS

The ETs are potent vasoconstrictor, pressor and co mitogenic peptides with uniquely sustained actions. They also have effects on the heart, kidney, endocrine glands and

nervous system that augment their pressor action. The ETs act on the endothelium to modulate their direct actions by stimulating generation of NO and prostaglandins. Despite these many actions, doubt had been expressed about the physiological relevance of the FTs However, recent studies now clearly show that ET-1 plays a fundamental physiological role in maintenance of basal vascular tone and blood pressure in humans. Nevertheless, the relative importance of each of the characterised pharmacological responses to the ETs require further clarification, not least because this may help in deciding which of the various ET antagonists to develop for specific clinical indications This is particularly important in regard to the selectivity of ECE for the different isoforms and in different tissues, as well as the relative importance of vascular and endothelial ET_B receptors.

There is now a wealth of evidence that ETs are implicated in the pathophysiology of cardiovascular diseases, including conditions such as hypertension and chronic heart failure, that are characterised by sustained vasoconstriction, as well as conditions such as unstable angina and SAH, characterised by development of vasospasm In animal models of these conditions, FT antagonists generally appear to improve outcome. The combined ET_A/ET_B receptor antagonists TAK-044 and bosentan recently have been shown to lower blood pressure (Haynes *et al* , 1996; Schmitt *et al.*, 1995), suggesting utility in hypertension, and bosentan has been shown to have favourable haemodynamics in patients with chronic heart failure (Kiowski *et al.*, 1995). In addition, in a human forearm model of vasoconstriction, systemic TAK-044 blocks the vasoconstriction to locally infused ET-1, suggesting that such antagonists may be valuable in the management of conditions associated with ET-mediated vasospasm. The results of such studies bode well for the clinical development of ET antagonists in states of acute and sustained vasoconstriction. For management of acute conditions, such as myocardial infarction and SAH, peptide ET antagonists without oral activity may have a place. However, even in these conditions there are still some grounds for developing orally active drugs, and it is encouraging to find that a range of these agents are now under development.

However, many questions presently remained unanswered, not least of which is whether these agents will prove safe and well tolerated in long-term use. More subtly, it is unclear whether selective ET_A receptor antagonists or combined ET_A/ET_B antagonists are needed. Certainly, where they have been used in clinical studies, combined ET_A/ET_B antagonists appear to have favourable haemodynamics. Also, there is evidence that smooth muscle ET_B receptors contribute to the vasoconstriction to ET-1 *in vivo* in humans (Haynes *et al.*, 1995), and there is a suggestion that vascular ET_B responses may be of greater importance in heart failure (Love *et al.*, 1994). Nevertheless, the loss of endothelial ET_B-mediated responses may be a disadvantage, given the beneficial effects of NO on platelet aggregation, and this matter requires clarification. Given the pharmacological evidence for a distinction between endothelial and smooth muscle ET_B receptors (Warner *et al.*, 1993), development of a combined ET_A/smooth muscle ET_B antagonist might be optimal, if perhaps currently unrealistic.

The development of ECE inhibitors to block generation of ETs is an interesting concept, analogous to the development of ACE inhibitors. To date, however, it is unclear whether there are several ECEs, and whether all of these should or could be blocked by one agent. Also, some of the drugs appear not to easily gain access to the important intracellular ECE-1 and ECE-2 enzymes (Xu *et al.*, 1994; Emoto and Yanagisawa, 1995). In addition, most of the currently available ECE inhibitors also have an action against NEP, and this may reduce their vasodilating capacity (Haynes and Webb, 1994). An alternative approach might be to aim for broader substrate specificity by inhibiting ECE, ACE and NEP, thereby combining the benefit of the ACE inhibitors with the potential additional benefits associated with ECE inhibition and raised concentrations of atrial peptides.

Many ET receptor antagonists are under development and at least some of these are already in clinical development. The clinical potential for inhibiting the ET system, indicated by animal studies, is soon to be tested in humans, and it is to be hoped, as with the renin angiotensin system, that novel and important drugs for the treatment of cardiovascular disease will be the outcome.

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The endothelin system in cardiovascular disease

Discovery to drug development in under a decade

The function of the vascular endothelium has become a major focus of research. This is partly because of the success of drugs (such as the angiotensin converting enzyme inhibitors, the nitrovasodilators, and aspirin) which act through mechanisms related to endothelial function, and partly because endothelial dysfunction is now thought to be an important early factor predisposing to atherogenesis.[1]

Endothelin-1 is a recently discovered endothelium derived vasoconstrictor and pressor peptide with mitogenic properties,[2] which is now recognised to influence basal vascular tone and blood pressure.[3] Endothelin antagonists are currently in development and may provide an important new approach to the treatment of cardiovascular disease.[4]

Endothelin-1 is generated from an inactive precursor, "big" endothelin-1, through the action of a unique endothelin converting enzyme. The mature peptide acts on endothelin type A and endothelin type B receptors. In blood vessels, endothelin-1 causes vasoconstriction largely through stimulating the endothelin type A receptor on smooth muscle cells, although type B receptors may also contribute in some vessel types. Vasoconstriction is modulated by generation of the vasodilators, nitric oxide and prostacyclin, mediated by type B receptors on endothelial cells.

The main approaches to drug treatment are inhibition of endothelin converting enzyme and antagonism of endothelin receptors. Most success has so far been achieved with receptor antagonists, either selective endothelin type A or combined type A and B receptor antagonism. Whereas endothelin type A receptors are a natural target for treatment, the benefits of inhibition of type B receptors will depend on the balance between its constrictor and dilator actions. Several drugs of both types are currently under clinical evaluation.[4]

Endothelin-1 is present in plasma and may thereby widely influence vascular tone. However, it is mainly released towards smooth muscle and functions primarily as a locally acting paracrine factor rather than a circulating hormone. The generation of endothelin-1 is increased by a wide range of vasoactive and inflammatory mediators, changes in shear stress of the vessel wall, and, importantly, by hypoxia. Evidence now suggests a role for endothelin-1 in local ischaemia (including myocardial infarction[5] and acute renal failure[6]), vasospasm (including Raynaud's disease[7] and subarachnoid haemorrhage[8]), and sustained vasoconstriction (including hypertension[3] and heart failure). But it is its role in the pathophysiology of chronic heart failure that is attracting most interest.

Chronic heart failure causes substantial morbidity and mortality and is a major consumer of healthcare resources.[9] It leads to stimulation of compensatory neurohumoral reflexes, including effects on the renin-angiotensin and sympathetic nervous systems, which also serve to increase peripheral vascular resistance, renal sodium reabsorption, and cardiac workload. This leads to a vicious circle of declining cardiac function, which provides a rationale for the use of angiotensin converting enzyme inhibitors.

Neurohumoral activation and tissue hypoxia should also increase the production of endothelin-1; and the actions of endothelin-1 (vasoconstriction and co-mitogenesis, leading to cardiac and vascular hypertrophy, enhanced activity of the renin-angiotensin and sympathetic nervous systems, and increased renal vasoconstriction and sodium retention) are all consistent with the circulatory abnormalities found in patients with chronic heart failure. Indeed, plasma endothelin concentrations are elevated in chronic heart failure, mainly through an increase in plasma concentrations of big endothelin-1, consistent with increased generation of endothelin-1. Plasma concentration of big endothelin-1 correlates well with severity of heart failure[10,11] and is the most powerful predictor of outcome.[11]

In the first clinical study bosentan, an antagonist to combined endothelin receptors A and B, was given intravenously to patients with severe chronic heart failure who had stopped angiotensin converting enzyme inhibitors.[10] This increased their cardiac output and reduced systemic and pulmonary vascular resistance without inducing reflex tachycardia or increasing plasma concentrations of angiotensin II or noradrenaline. More recent studies show that single doses of the endothelin type A receptor antagonist BQ-123 and the endothelin converting enzyme inhibitor phosphoramidon produced haemodynamic benefits in patients taking angiotensin converting enzyme inhibitors.[12] These benefits were sustained during chronic oral treatment with bosentan (W Kiowski, personal communication). The beneficial effect of angiotensin converting enzyme inhibitors on mortality was predicted from an animal model of heart failure.[9,13] It is therefore promising to find that BQ-123 substantially improved 12 week survival from 43% to 85% in this coronary occlusion model of heart failure,[14] as well as improving haemodynamic function and cardiac remodelling.

There is now substantial evidence to support further clinical investigation of endothelin receptor antagonists in several cardiovascular diseases and particularly in chronic heart failure. We need further clinical studies to show whether selective endothelin type A or combined A and B receptor antagonists are likely to produce the greatest benefit, and major clinical trials to confirm that these agents provide benefits in morbidity and mortality beyond those associated with conventional treatment of a diuretic and angiotensin converting enzyme inhibitor.

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The medical health emergency card

Not to assuage public concern, but to make users' lives easier

The idea of an emergency card carried by patients with certain conditions—for example, diabetes—is not new. A similar card for mentally ill patients is also not new: a users' group, Survivors Speak Out, first introduced a crisis card in 1989, and interest has since grown.[1] Known as a mental health emergency card, its aim is to enable patients to give advance directives about their management. As such the card poses particular problems, not least in relation to the legal status of advance directives.[2] At first sight mental health emergency cards seem to have something for everyone.[3] However, contradictions in the objectives of different groups have delayed their widespread implementation and led to an atmosphere of distrust.

Survivors Speak Out, the inventor of the card, has recently withdrawn its version. Its aim was to increase users' self determination in the event of a loss of mental capacity. But users now complain that mental health professionals are increasingly helping patients to complete their cards.[4] They fear that patients will be coerced into including potentially damaging information.

Different objectives led to trusts developing cards at the request of the public, professional carers, and the police. For these groups one of the failures of community care is that some of the most vulnerable patients are lost to follow up,[5] sometimes because of lack of communication between services. The hope was that the card would alert professionals to previous contacts with other services.

Finally, the recommendation that the Royal College of Psychiatrists should develop a card was a response to public concern about violent mentally ill offenders.[3] Thus the public may see these cards as a way of identifying potentially dangerous patients. The police and other professionals may also see them as a means of helping determine disposal—for example, through court diversion schemes.

But professionals also face difficulties in helping people with these cards. It is unclear whether, in the face of a clear advance directive on a card, their clinical judgment should be overriden. Despite a discussion document from the Law Society in 1989[6] and an enthusiastic endorsement from the Commons health select committee,[7] the legal status of these cards remains unclear. Currently both voluntary and non-voluntary bodies are awaiting the conclusions of a commons working group on the Law Commission report on mental capacity[8] before proceeding with potential card schemes.

There is no evidence from the UK or elsewhere on the success or otherwise of mental health emergency cards and on what any success may depend. In the absence of such data, practical aspects of the card are also a source of disagreement. Who, for example, should fill it out? That this has become an issue is probably more a symptom of mistrust than a fundamental problem. An obvious tension exists between privacy and information, and many fear that the cards may further stigmatise ill patients—or, worse, that the information may be used aginst the holder. One compromise might be simply to include only a contact name and number (accessible 24 hours) that would provide a bona fide caller with further information and the name of an advocate for the patient. Such a card might then be offered widely without suggesting a history of mental illness, while to some extent meeting the objectives of different groups. Such minimal information would also fit on to a necklet or bracelet, which might be more practical for some patients.

Finally, which patients should carry the cards? It is hard to imagine them being anything but voluntary, and they must certainly not be simply a knee jerk response to public concern. Whether or not a mental health emergency card can satisfy both users and professionals remains to be seen. But the cards will be successful only if patients accept and use them.

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Endothelin: from molecule to man

This article is based on a lecture given to the British Pharmacological Society at their December 1995 meeting in Brighton following the award to Professor David Webb in 1994 of the SmithKline Beecham Prize for Research

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Background

Until relatively recently, the vascular endothelium was thought to act as little more than a passive barrier to diffusion. However, in the last few years, with the discovery of prostacyclin, nitric oxide and endothelin-1, a central role has emerged for the endothelium in the regulation of vascular smooth muscle cell tonus and growth, immunological reactivity, blood coagulation and lipid metabolism. This field of research has matured rapidly (Figure 1) and, in a recent foresight exercise on behalf of the British Heart Foundation, Medical Research Council and Wellcome Trust [1], the vascular endothelium was identified as the area commanding the highest priority in cardiovascular research over the next 10 years.

My own interest in this area originally developed while working in the MRC Blood Pressure Unit in Glasgow. Observations in the first clinical trial of a renin inhibitor in man suggested the functional importance of a vascular renin-angiotensin system [2] and, later, at St George's Hospital in London I was able to show this by combining forearm plethysmography techniques with brachial artery infusions of locally active doses of angiotensin I, angiotensin II, bradykinin and angiotensin converting enzyme (ACE) inhibitors [3, 4]. Surprisingly, these studies demonstrated *in vivo* that the endothelium of the forearm resistance vessels has a considerable capacity to generate angiotensin II locally, equivalent to that of the pulmonary circulation [4, 5].

The discovery in 1988 of the novel 21 amino acid peptide, endothelin-1 [6], a more potent endothelium-derived vasoconstrictor and pressor agent even than angiotensin II, was of major interest to the cardiovascular research community, and followed closely on the identification of the endothelium-derived relaxing factor (EDRF) as nitric oxide [7]. At this early stage it was difficult to gain access to more than small quantities of synthetic endothelin-1. However, it was clear that local infusion techniques could allow delineation of the vascular effects of endothelin-1 more clearly, with greater safety, and with lower doses, than would be the case with systemic dosing. In collaboration with Dr John Clarke and Professor Attilio Maseri at the Royal Postgraduate Medical School, we began clinical pharmacology studies at St George's Hospital in London in 1988, and our first pharmacodynamic observations were published the following year [8].

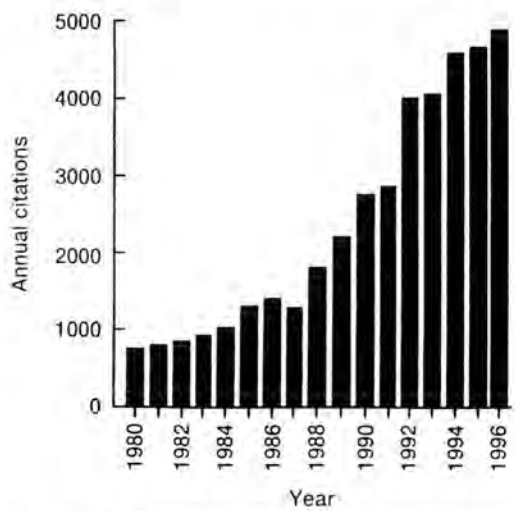

Figure 1 Annual numbers of papers in the biomedical sciences including the word 'endothelium' in the title, keywords or abstract from 1980 to 1996 inclusive, based on information from the Bath Information and Database Service (BIDS) EMBASE.

Subsequent progress in endothelin research [9, 10] has maintained an extremely rapid pace such that we were able to perform the first human pharmacology studies with an inhibitor of endothelin generation, phosphoramidon, in 1992 and with an endothelin receptor antagonist, BQ-123, in 1993. Indeed, after only 8 years, the clinical development of drugs targeting the 'endothelin system' in cardiovascular disease is now well advanced [11]. The first major clinical study in heart failure patients was reported in 1995 [12] and phase III trials should begin shortly. From a combination of fundamental and applied research it has become apparent that the 'endothelin system' is of central importance to the maintenance of normal cardiovascular function in healthy man and that endothelin-1 is likely to be a key mediator in the pathophysiology of cardiovascular disease.

The main aim of this paper is to review current knowledge of the endothelin system; particularly its physiological function in the cardiovascular system and its role in cardiovascular disease. Although I will focus on human pharmacology and physiology, and in particular on the work of our group in Edinburgh, it must be recognised that all of the major advances in clinical research have depended critically on the identification and development of selective pharmacological probes through work on animal and isolated tissues. A subsidiary aim of this review is to demonstrate that clinical pharmacology studies can provide a critical contribution to our understanding of human cardiovascular physiology and pathophysiology, and to the process of early drug development and identification of suitable therapeutic targets.

Endothelin synthesis

Endothelin 1 is the most potent vasoconstrictor and pressor peptide known. Originally identified in the culture medium

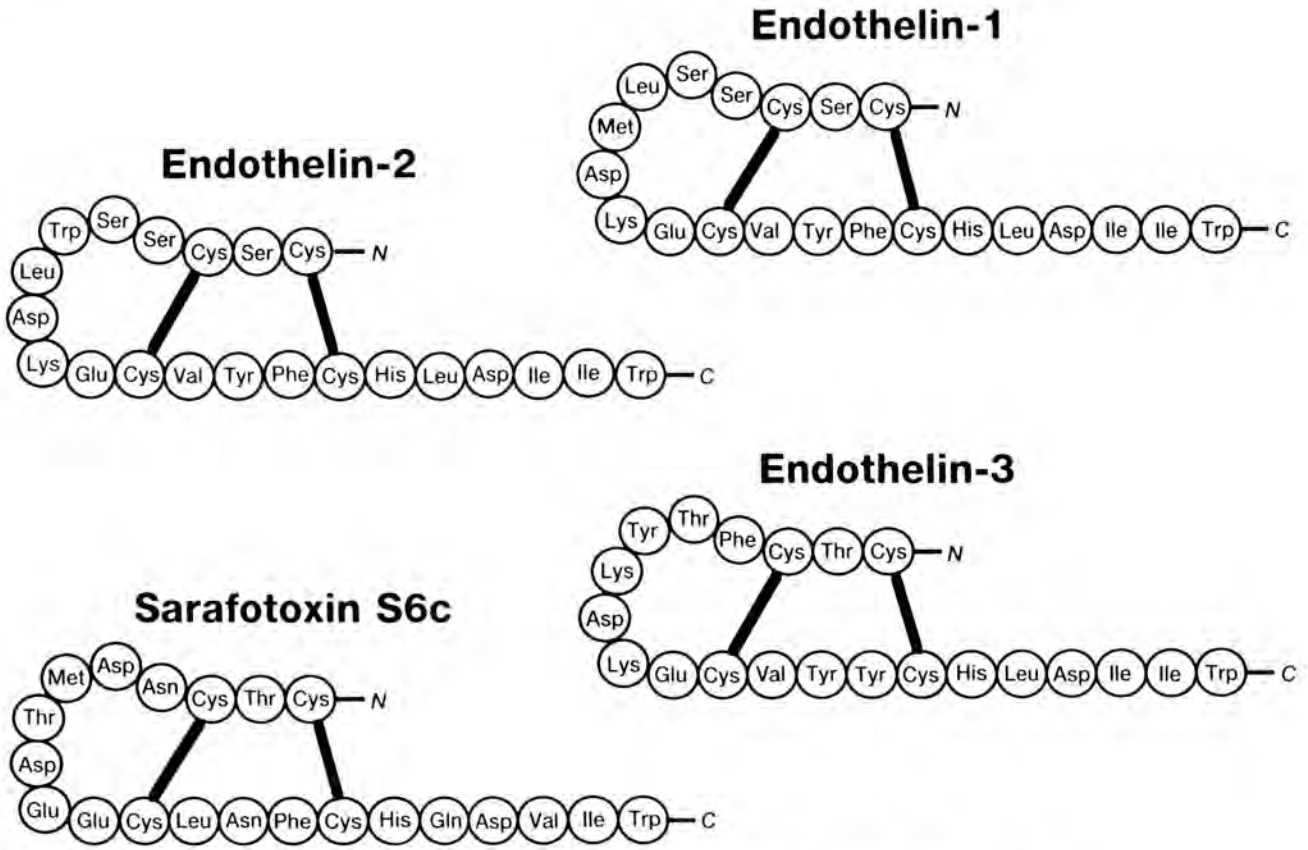

Figure 2 Structure of the endothelin family of peptides, and the related snake venom peptide sarafotoxin S6c.

of porcine aortic endothelial cells [6], it is now recognised to be a member of a family comprising three isoforms [13]: endothelin-1, endothelin-2 and endothelin-3 (Figure 2). Each isoform contains 21 amino-acids, two intra-chain disulphide bonds constraining overall structure, and a conserved C-terminal sequence necessary for biological activity [13]. This structure is unique among the mammalian peptides but is shared by the sarafotoxins, snake venom peptides from the Israeli burrowing asp, *Actractaspis engaddensis*, one of which, sarafotoxin S6c, has proved particularly valuable as a pharmacological probe. While there is evidence that endothelin-2 may possibly function as a mediator in the kidney, and endothelin-3 may act as mediator in the gut and nervous system, endothelin-1 is the major isoform generated in blood vessels and appears to be of greatest significance in cardiovascular regulation. Hence, endothelin-1 is the major focus of this review.

Within the human genome, the endothelins are each represented by a separate gene encoding a specific precursor for the mature isoform [13]. In the 5′ flanking sequence there are binding sites for activating protein-1 and nuclear factor 1 through which angiotensin II and transforming growth factor β act respectively to induce endothelin-1 expression. There are also binding sites for acute phase reactants which may mediate the effects of acute physiological stress. In the 3′ region there is a sequence regulating selective destabilisation of preproendothelin-1 mRNA, possibly accounting for its short half-life. These sites serve as potential mechanisms for regulating production of endothelin at the level of transcription and translation. Although endothelin-1 can be identified within endothelial cells, it remains unclear whether intracellular stored peptide represents an important pool available for rapid release and, currently, regulation of endothelin synthesis is thought to be primarily at the level of gene transcription, with *de novo* production and release occurring in response to endothelial cell stimulation. A large number of factors have now been shown to increase endothelin-1 synthesis (Figure 3): these include vasoactive hormones, inflammatory mediators and physico-chemical factors such as altered vascular shear stress and hypoxia. Other factors—including nitric oxide, nitric oxide donor drugs, natriuretic peptides and the dilator prostanoids—serve to inhibit endothelin-1 generation by promoting production of cyclic GMP or cyclic AMP [10].

The initial product of the human endothelin-1 gene is preproendothelin-1, a peptide of 212 amino acid residues (Figure 3). After removal of a short secretory sequence, proendothelin-1 undergoes cleavage by a dibasic pair specific endoprotease—probably furin—to generate the 38 amino-acid peptide, 'big endothelin-1' [6]. Subsequent conversion to the mature, biologically active peptide, endothelin-1, occurs through the action of endothelin converting enzymes (ECE-1 and ECE-2). This family of metalloprotease enzymes is related to neutral endopeptidase-24.11 (NEP) and the Kell protein but unrelated to angiotensin converting enzyme. ECE-1 [14] appears to be the physiologically active ECE and, by alternative gene splicing, it exists in two different isoforms—ECE-1a and ECE-1b—with functionally distinct roles and tissue distributions. ECE-1a appears to be an intracellular enzyme expressed in the Golgi apparatus of cells, such as the endothelial cells, that synthesise endothelin-1. In contrast, responder cells, such as vascular

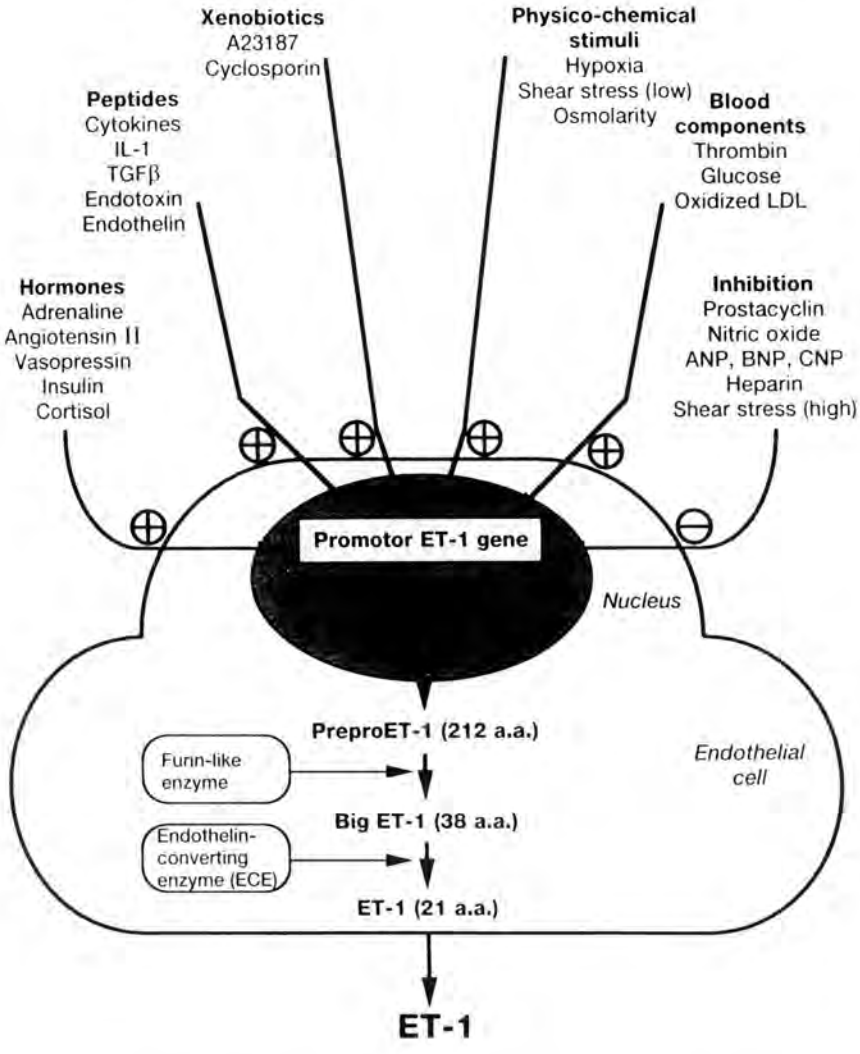

Figure 3 Factors that alter endothelin-1 (ET-1) synthesis and the pathway for endothelin-1 generation: See text for details. IL-1 = interleukin-1; TGFβ = transforming growth factor β; LDL = low-density lipoprotein; ANP, BNP, CNP = atrial, brain, and c-type natriuretic peptides. (From an original design from Professor P. Vanhoutte).

smooth muscle cells, express extracellular ECE-1b that can convert extracellular big endothelin-1 to mature endothelin-1 [15]. ECE-1 and ECE-2 are both inhibited by phosphoramidon, a combined ECE/NEP inhibitor but not by the selective NEP inhibitor, thiorphan, or by the ACE inhibitor, captopril. Both enzymes are selective for big endothelin-1, raising the possibility that other ECEs with selectivity for big endothelin-2 and -3 will be identified. Through prevention of the generation of the endothelins, ECE-1 must be a potential target for drug treatment.

The gene encoding endothelin-1 can be detected in a wide variety of tissues, including the endothelial and smooth muscle cells of blood vessels, heart, lung, brain, kidney, pancreas and spleen. The gene encoding endothelin-2 may also be found in the vascular endothelium, as well as the smooth muscle of the large and small intestine, myocardium, stomach, kidney, placenta and uterus. Endothelin-3 expression predominates in the brain but is also found in the lung, gastro-intestinal tract and kidney. Big endothelin-1, endothelin-1 and endothelin-3 are present in plasma at picomolar concentrations that are probably insufficient to exert a direct influence on vascular tone. Endothelin-1 is generally thought to be a paracrine and autocrine mediator rather than an endocrine hormone. Indeed, endothelin-1 is largely secreted abluminally by endothelial cells towards the adjacent vascular smooth muscle [16], so that concentrations are likely to be substantially higher at the interface between endothelial and vascular smooth muscle cells than in blood, consistent with a primarily local action. In addition, the half-life of endothelin-1 in blood is short, at less than 5 min, with clearance predominantly via receptor binding and metabolism in the lungs and kidneys [17].

Endothelin receptors

The endothelins act on two receptor subtypes, ET_A and ET_B, characterized on the basis of their pharmacology (Table 1). Endothelin-1 has a similar binding affinity for ET_A and ET_B receptors—in the nanomolar range—but has a much higher binding affinity for the ET_A receptor than endothelin-3. In contrast, endothelin-1 and endothelin-3 have equal affinity for the ET_B receptor. Understanding of the function of endothelin receptors has been aided by the use of specific pharmacological agonists and antagonists. Endothelin-3 and sarafotoxin S6c are respectively ~2000 fold and ~30000 fold selective as agonists at the ET_B receptor and BQ-123 and BQ-788 are selective antagonists respectively at ET_A and ET_B receptors. The human ET_A and ET_B receptors have been cloned and exhibit ~60% homology. ET_A receptor mRNA can be detected in many tissues, with the highest expression in aorta, heart and

Table 1 Endothelin receptor characterization

	Endothelin receptors		
	ET_A	ET_B	
Agonist potency	ET-1 > ET-2 ≫ ET-3	ET-1 = ET-2 = ET-3	
Tissue	Aorta	Coronary artery	Endothelium
Action	Constriction	Constriction	Dilatation
Selective agonists	None	ET-3 (600-fold specific)	
		Sarafotoxin S6c (30 000-fold specific)	
Antagonists	BQ-123	BQ-788	
	Non-selective bosentan TAK-044		

kidney. The ET_A receptor predominates on vascular smooth muscle cells and is responsible for causing vasoconstriction in both large and small blood vessels [18]. It is also the major receptor subtype in the heart [19]. In contrast, ET_A mRNA cannot be detected in the liver or endothelial cells [20]. The ET_B receptor can be detected in endothelial and vascular smooth muscle cells and is predominantly found in brain, lung, kidney and aorta [21]. The ET_B receptor on endothelial cells modulates vasoconstriction in response to endothelin-1 through the production of vasodilator substances including prostacyclin and nitric oxide. It is now widely recognised that the ET_B receptors on vascular smooth muscle cells can mediate vasoconstriction, particularly in small resistance vessels and veins. The significance of the vasoconstrictor ET_B receptor will be discussed later. There has been a recent tendency to sub-classify the ET_B receptor on the basis of responses to selective agonists and antagonists [22] but this currently cannot be justified on a molecular basis.

The ET_A and ET_B receptors are classical heptahelical rhodopsin-like G-protein coupled receptors that activate phospholipase C leading to hydrolysis of phosphatidyl inositol and generation of cytosolic inositol trisphosphate and membrane bound diacylglycerol [9]. Inositol trisphosphate causes an early rapid rise in $[Ca^{2+}]_i$ through its release from intracellular stores. A more sustained rise of intracellular calcium occurs through opening of membrane Ca^{2+} channels. Diacylglycerol activates protein kinase C, increasing sensitivity of the contractile apparatus to Ca^{2+}, activates nuclear signalling mechanisms—with possible effects on long term regulation of cellular function—and causes a rise in the intracellular pH through an effect on the sodium-hydrogen ion exchange membrane pump. Endothelin-1 may also interact with the ATP-sensitive potassium channel, so contributing to the rise in $[Ca^{2+}]_i$. In addition, it may activate phospholipase A_2, increasing production of arachidonic acid, and hence of prostacyclin (PGI_2) and thromboxane A_2 [10].

The endothelins bind tightly to their receptors in a 'pseudo-irreversible' manner and the endothelin-receptor complex is rapidly internalized. Slow dissociation from their receptors may account for prolonged actions of the endothelins. Endothelin receptor expression is itself regulated by exposure to endothelin-1 so that agents that enhance endothelin-1 production, such as angiotensin II and certain growth factors, can cause endothelin receptor downregulation [23]. In contrast, endothelin receptor number can be upregulated; for instance, by ischaemia [24], cyclosporin [25] and interleukin-1β [26].

Developmental biology

Gene defects affecting the endothelin system have been described for both animals and man. Gene knockouts for the preproendothelin-1 gene in mice cause lethal abnormalities affecting the development of craniofacial, cardiovascular and pharyngeal pouch structures [27, 28]. Interestingly, these abnormalities have also been found in teratogenicity studies during the pre-clinical development of a range of ET_A receptor antagonists indicating, at least during development, that endothelin-1 may be the natural ligand for the ET_A receptor and that endothelin-1 has an important role in the development of the pharyngeal arches, heart and great vessels. Indeed, it has been suggested that endothelin-1/ET_A receptor anomalies may contribute to the Pierre-Robin and Treacher-Collins syndromes [29]. One anomalous finding in these studies was the development of raised blood pressure in the endothelin-1 knockout mice. However, evidence is emerging that the elevation of blood pressure is related to sympatho-adrenergic overactivity caused by the severe hypoxia that is a consequence of the facial/pharyngeal anomalies [29].

Both endothelin-3 and ET_B receptor mutations lead to the formation of aganglionic megacolon and coat pigmentation anomalies in animals [30]. In this case, genetic abnormalities of either the preproendothelin-3 gene or the ET_B receptor have been documented in congenital neurocristopathies associated clinically with Hirschsprung's disease [31] and the Waardenberg-Shah syndrome [32]. Hence, the interaction of endothelin-3 with the ET_B receptor appears to be important for the development of cells within the neural crest.

Cardiovascular pharmacology

Bolus administration of endothelin-1 is known to cause a marked pressor effect lasting for more than 60 min [6] in contrast to the brief effects of all other endogenous vasoconstrictor substances. Despite rapid clearance of the peptide from blood, this sustained pressor effect has been confirmed from studies in man [33, 34], and is mediated predominantly through an increase in peripheral vascular resistance. The pressor effect tends to reduce cardiac output [35], probably through a baroreceptor mediated decrease in

heart rate, although an increase an afterload may possibly contribute. Coronary vasoconstriction is recognised to occur in humans who are bitten by the burrowing asp, *Actractaspis engaddensis*, the venom of which contains sarafotoxins [36] and the coronary vasoconstrictor effect of the endothelins has more recently been confirmed in healthy subjects [34]. It is also known that after bolus administration in animals, endothelin-1 causes transient hypotension associated with systemic vasodilatation through stimulation of the endothelial ET_B receptor. However, such studies require high doses of endothelin-1 and, because endothelin-1 preferentially causes vasoconstriction in the renal, cardiac and cerebral circulations, such studies should clearly be avoided in man.

One way to address directly the potential vasoconstrictor and dilator effects of the endothelins *in vivo* in man is to combine bilateral forearm blood flow measurements with unilateral brachial artery infusion of vasoactive drugs at subsystemic, locally active doses. Because forearm muscle blood flow is only ~ 50 ml min^{-1} compared with a cardiac output of ~ 5000 ml min^{-1} substantial local effects can be achieved without a systemic action. By avoiding confounding effects on organs such as the brain, kidney and heart, as well as potential influences on neurohumoral reflexes, vascular responses can be attributed to a direct effect of the drug, providing a powerful, reproducible and safe method of directly assessing vascular responses *in vivo* [37, 38]. Importantly, the responses obtained are also broadly predictive of those seen in the systemic and coronary circulation [38].

Continuous infusion of endothelin-1 into the brachial artery causes a slowly developing dose-dependent reduction in forearm blood flow, with vasoconstriction sustained for more than 2 h after halting the infusion [8]. When given via the brachial artery, low doses of the ET_B selective agonists, endothelin-3 and sarafotoxin S6c, also produce vasoconstriction in human resistance vessels *in vivo*, consistent with vascular ET_B receptors mediating at least part of the functional response to endothelin-1 in these vessels [39]. In human blood vessels *in vitro*, threshold concentrations of endothelin-1 potentiate contractions to noradrenaline [40]. However, a peripheral interaction of endothelin-1 with the sympathetic nervous system has not been demonstrated in forearm resistance [41] or cutaneous capacitance vessels [42] of healthy subjects *in vivo*. Endothelin-1 and endothelin-3 [39], and sarafotoxin S6c (unpublished observations), can produce transient forearm vasodilatation, the dilator response to endothelin-3 and sarafotoxin S6c being greater and more prolonged than that to endothelin-1 consistent with involvement of the endothelial ET_B receptor. However, vasodilatation to the endothelins occurs only at high doses on bolus administration, suggesting that this is not a physiological response [39]. In human hand veins [43], vasoconstriction is modulated predominantly by stimulated release of dilator prostaglandins.

Endothelin-1 and sarafotoxin S6c both cause sustained constriction of human dorsal hand veins *in vivo* [8, 39], suggesting that both vascular ET_A and ET_B receptors can contribute to venoconstriction to endothelin-1 in humans. Venoconstriction *in vivo* is blocked more effectively by the K_{ATP} channel opener, cromakalim, than the Ca^{2+} channel antagonist, nicardipine or by hydralazine [44], suggesting that endothelin-1 responses in human veins depend only in part on Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels. In addition, the greater efficacy of K_{ATP} channel opening agents is consistent with endothelin-1 acting to close K_{ATP} channels, causing plasma membrane depolarisation and vasoconstriction by mechanisms additional to opening of voltage-operated Ca^{2+} channels.

Cardiovascular physiology

Although studies with agonists are of considerable interest, when a mediator is not a classical circulating hormone the results can be frankly misleading and studies with antagonists are likely to be considerably more informative.

The first endothelin 'antagonist' described was the ECE inhibitor, phosphoramidon [45], and we decided to begin our investigation in the forearm. However, to determine a dose that would inhibit ECE we first had to examine responses to big endothelin-1. Brachial artery administration of big endothelin-1 caused a dose-dependent forearm vasoconstriction that could be blocked completely by phosphoramidon (30 nmol min^{-1}), suggesting that the effects of the precursor are mediated through conversion to the mature peptide by ECE [46]. The blockade of constriction to big endothelin-1 by phosphoramidon is unlikely to have been due to inhibition of endothelin receptor binding, because vasoconstriction to endothelin-1 was unaffected by phosphoramidon and because conversion to endothelin-1 and its C-terminal fragment was confirmed in plasma samples taken from the veins draining the infused forearm [47]. Also, because circulating blood exhibits little ECE activity [48], conversion of big endothelin-1 in the forearm presumably occurs via vascular, probably endothelial, ECE situated within the forearm resistance vessels. The difference in potency between big endothelin-1 and endothelin-1, and the ratio of C-terminal fragment to big endothelin-1 in venous blood, both indicated that local ECE converts about 10% of luminally presented big ET-1 to ET-1, consistent with $\sim 10\%$ conversion of exogenous big ET-1 by cells expressing the ECE-1 gene [14]. Big endothelin-1 does not cause venoconstriction in hand veins [49], even though these vessels respond to endothelin-1 [8], suggesting that ECE activity may not be present in all vessel types.

Administration of phosphoramidon (30 nmol min^{-1}) alone [46] resulted in a slowly progressive vasodilatation (Figure 4), consistent with a role for endothelin-1 in maintenance of basal vascular tone. Although phosphoramidon is an imperfect tool, because it also acts as an inhibitor of NEP, this latter action is unlikely to explain the vasodilatation because potent and selective inhibitors of NEP cause slowly progressive forearm *vasoconstriction* [46] (Figure 4). This effect of NEP inhibitors is likely to be caused by accumulation of a vasoconstrictor agent which may be endothelin-1, because it is a substrate for metabolism by NEP and the vasoconstriction is not blocked by an ACE inhibitor. This observation may also account for the increase in plasma endothelin by NEP inhibitors in clinical trials [50] and their failure to lower blood pressure in hypertensive subjects.

Confirmation that endogenous endothelin-1 generation

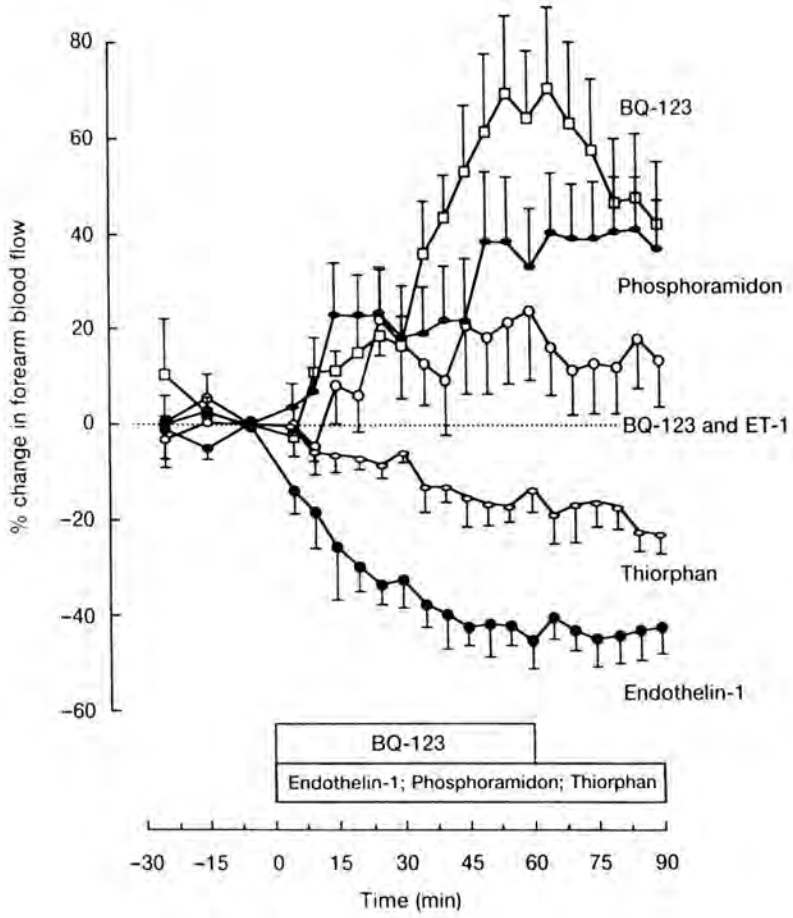

Figure 4 Forearm vasoconstriction to brachial artery infusion of endothelin-1 (5 pmol min^{-1}; ●) is abolished by the co-infusion of BQ-123 (100 nmol min^{-1}; ○). Infusion of the ET_A antagonist BQ-123 (100 nmol min^{-1}; □) or the ECE inhibitor phosphoramidon (30 nmol min^{-1}; ◎) alone produce progressive forearm vasodilatation whereas the NEP inhibitor thiorphan (30 nmol min^{-1}; ◯) causes progressive vasoconstriction. Adapted from Haynes and Webb [46], with kind permission of the *Lancet*. See text for details.

contributes to the maintenance of basal tone in forearm resistance vessels of healthy human subjects came from studies with the cyclic pentapeptide ET_A receptor selective antagonist, BQ-123 [51], and the cyclic hexapeptide combined $ET_{A/B}$ antagonist TAK-044 [52], given via the brachial artery. Both agents caused a progressive vasodilatation of the forearm vessels [46, 53]. The substantial effect of BQ-123 (Figure 4) suggests that vasoconstriction to endothelin-1 is mediated mainly through the ET_A receptor on vascular smooth muscle. Subsequent studies have confirmed this effect of BQ-123, and shown that it can be achieved with lower doses and when given for a shorter period [54]. Together, the L-arginine/nitric oxide system, the sympathetic nervous system and the endothelin system are currently the only mediator systems known to maintain basal vascular tone. It now remains to be shown how the endothelin system is regulated, though this is likely to be predominantly at a local level.

The greater effect of BQ-123 than a substantial local dose of TAK-044 would be consistent with a mainly dilator role for the ET_B receptor. Further and more direct support for this view comes from recent arterial experiments with an ET_B selective inhibitor, BQ-788 [55]. This agent causes progressive forearm vasoconstriction in healthy subjects [56], consistent with greater functional importance of the dilator than the constrictor ET_B receptor under physiological circumstances. On this basis, a proportion of the dilator response to BQ-123 is likely to be mediated by nitric oxide and prostacyclin acting through the unopposed endothelial ET_B receptor. It might also be anticipated that this component of the response might be reduced in conditions associated with endothelial dysfunction (see later). Interestingly, vasoconstriction appears to be mediated solely by ET_A receptors in the human skin microcirculation, again on the basis of studies with antagonists [57].

Responses of the forearm resistance vessels to drugs given into the brachial artery are usually predictive of the responses in the major resistance beds that serve to regulate blood pressure [38]. Indeed, for the L-arginine-nitric oxide system, the substantial—and at the time unexpected—reduction of forearm blood flow associated with administration of the nitric oxide synthase inhibitor, L-N^G-monomethyl-arginine (L-NMMA) [58], predicted the substantial increase in peripheral vascular resistance that would be associated with systemic administration [59]. Similar predictions, though for an opposite effect, were made for systemic endothelin receptor antagonism based on the data with BQ-123. These predictions were confirmed recently in healthy subjects [53] with another peptide drug, TAK-044, which is currently in clinical development.

TAK-044, which functionally inhibits both ET_A and ET_B mediated responses [52, 60], was given to healthy subjects at a range of doses from 10–1000 mg systemically and was generally well tolerated [53]. A 15 min intravenous infusion of TAK-044 at the highest dose of 1000 mg reduced systolic blood pressure by ~4%, diastolic blood pressure by ~18%, and systemic vascular resistance by ~26% over a 24 h period (Figure 5), suggesting that a major part of the effect of TAK-044 is mediated in the resistance vessels. At this dose, but not consistently at lower doses, the major haemodynamic

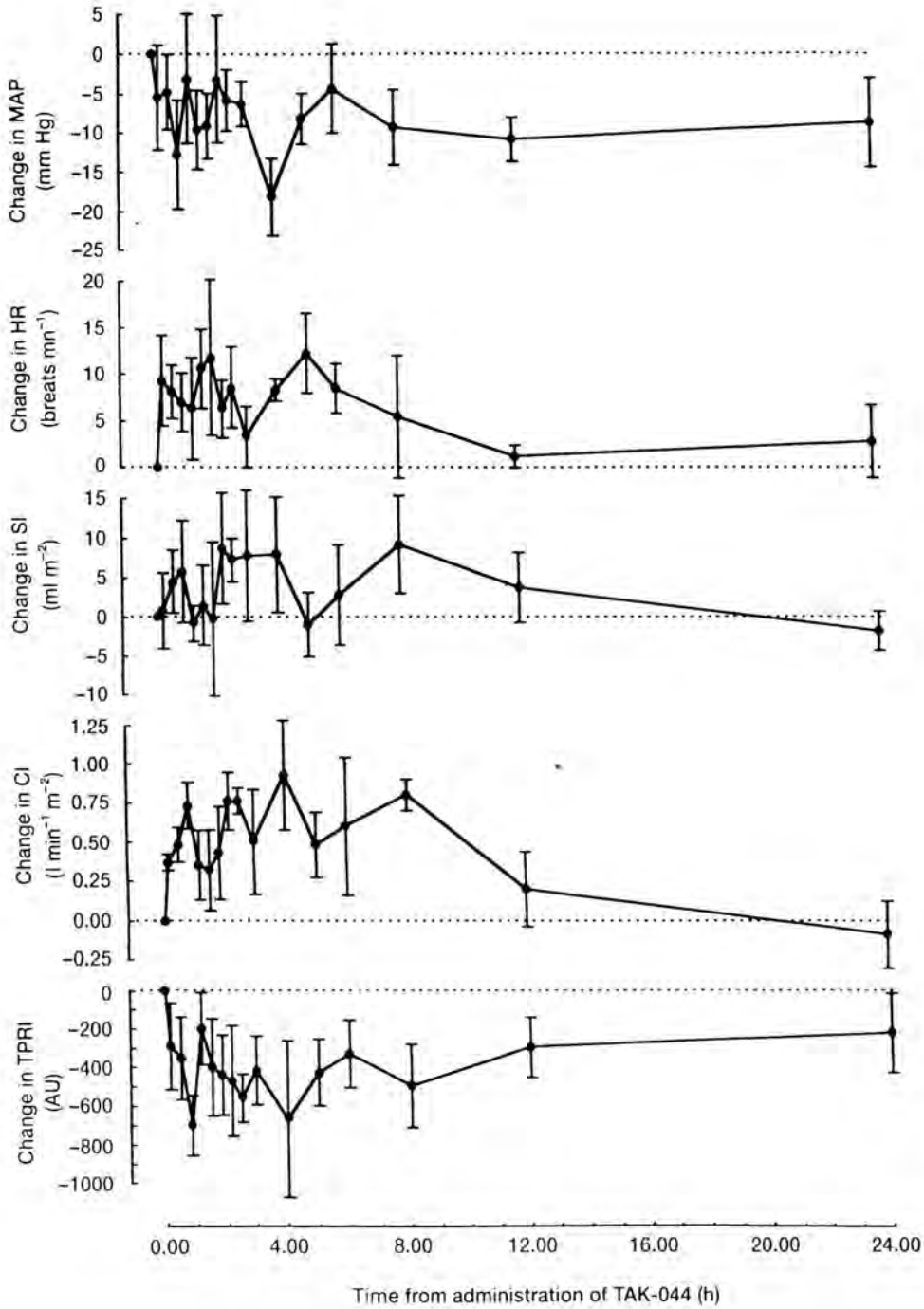

Figure 5 Graph showing time course of the effects of the highest dose of TAK-044 (1000 mg) on mean arterial pressure (MAP), heart rate (HR), stroke index (SI), cardiac index (CI), and total peripheral resistance index (TPRI). TAK-044 significantly decreased mean arterial pressure ($P<0.001$) and total peripheral resistance ($P<0.001$) and increased heart rate ($P<0.001$), stroke index ($P=0.034$) and cardiac index ($P<0.001$); these effects were maximal at 4 h and sustained for at least 12 h. Data shown represent placebo-corrected changes from predose (change from predose [active] change from predose [placebo]). (AU indicates arbitrary units). Reproduced from Haynes *et al.* [53], with kind permission of the *American Heart Association*.

effect on systemic vascular resistance was accompanied by a compensatory increase in cardiac output and heart rate. The substantial and long lasting effect of TAK-044 on systemic vascular resistance is even more remarkable considering the short half life of this peptide in the circulation and confirms that endogenous endothelin generation is critical for cardiovascular homeostasis and control of blood pressure, as originally proposed by Yanagisawa [6].

Systemically administered TAK-044 also abolished the vasoconstriction to endothelin-1 infused via the brachial artery for up to 3 h [53] but inhibited responses only partially at 8 and 12 h (unpublished data). Such studies can confirm the efficacy of endothelin receptor antagonists and determine the duration of their action. This appears to be especially important for this class of drug, where standard pharmacokinetic parameters do not always predict the pharmacodynamic activity achieved.

Cardiovascular pathophysiology

There are a number of mechanisms whereby endothelin-1 may be involved in cardiovascular disease, for instance: reduced production in congenital cardiac anomalies; enhanced production in congestive heart disease; reduced receptor number or affinity in Hirschsprung's disease; enhanced receptor number or affinity with cyclosporin treatment; reduced peptide clearance in chronic renal failure; and an unopposed action with endothelial dysfunction affecting the L-arginine/nitric oxide systems, which may be a factor in Raynaud's disease. Given the potentially beneficial

effects of nitric oxide to cause vasodilatation, and inhibit platelet aggregation and vascular growth, and the potentially adverse effects of endothelin 1 to promote vasoconstriction and vascular growth it is of considerable interest that many of the conditions associated with endothelial dysfunction causing reduced nitric oxide production, including atherosclerosis [61, 62], are further compounded by increased production of endothelin-1. Indeed, it is clear that these systems do not function independently. Endothelin-1 generation is enhanced by a range of other constrictor and growth promoting substances and inhibited by dilators including nitric oxide (Figure 3). Conversely, endothelin-1 promotes the production of nitric oxide but may also account for some of the vasoconstriction that accompanies its inhibition by L-NMMA and clinically for the development of tolerance to exogenous nitrate administration [63].

As well as having direct effects on vascular tone, endothelin-1 may enhance vascular tone indirectly: by augmenting vasoconstriction to other agents, such as angiotensin II, noradrenaline, serotonin; by enhancing central and peripheral sympathetic function; and by activating the renin-angiotensin system. Endothelin-1 is also a co-mitogen, enhancing cell division and proliferation, gene expression, protein synthesis and, ultimately, promoting hypertrophy of vascular smooth muscle, as well as cardiac myocytes and fibroblasts [64, 65]. Thus, endothelin 1 may serve to amplify vasoconstriction through the development of vascular hypertrophy.

There is a growing literature [10, 11] in support of a role for endothelin-1 in the pathophysiology of a wide range of cardiovascular diseases. These include ischaemic heart disease and atherosclerosis, as well as conditions associated with either sustained vasoconstriction—including hypertension, chronic heart failure, chronic renal failure, primary pulmonary hypertension—or with intermittent vasospasm—including Raynaud's disease, subarachnoid haemorrhage and acute renal failure. Hereafter, the review will focus on the considerable body of evidence implicating the endothelin system in chronic heart failure and suggesting that it may be a suitable target for therapeutic intervention.

Chronic cardiac failure

Chronic heart failure (CHF) is a common, disabling condition that causes substantial morbidity and mortality, and is a major consumer of health service resources [66]. This complex condition is associated with stimulation of compensatory neurohumoral reflexes, including effects on the renin-angiotensin and sympathetic nervous systems, that serve to maintain perfusion pressure but also act to increase peripheral vascular resistance, renal sodium reabsorption and cardiac workload. This leads to a 'vicious circle' of declining cardiac function and provides a rationale for the current mainstay of treatment, which is vasodilator therapy with ACE inhibitors. Although current treatment regimens are undoubtedly successful, CHF still carries a substantial morbidity and mortality [66] and there is room for additional therapeutic manoeuvres.

The now well documented reduction in mortality with ACE inhibitors in patients with CHF and left ventricular dysfunction after myocardial infarction [66] was predicted from animal models [67]. A recent report that the ET_A receptor antagonist, BQ-123, substantially improved 12 week survival from 43 to 85% in a coronary occlusion model of CHF [68] as well as haemodynamic function and cardiac remodelling is, therefore, very promising for the clinical developments in this area. Also, interestingly, raised plasma endothelin concentrations appear to be an extremely powerful predictor of 1 year mortality after acute myocardial infarction [69].

Neurohumoral activation and tissue hypoxia should increase endothelin-1 production, and the actions of endothelin-1—vasoconstriction, co-mitogenesis, leading to cardiac and vascular hypertrophy, enhancement of renin-angiotensin and sympathetic nervous system activity, and promotion of renal vasoconstriction and sodium retention—are all consistent with the circulatory abnormalities found in this condition. Indeed, plasma endothelin concentrations are elevated in CHF, mainly through an increase in plasma big endothelin-1 [70], consistent with increased synthesis, rather than decreased clearance, of endothelin-1. Plasma immunoreactive endothelin correlates with the degree of haemodynamic [71] and functional impairment [72] in CHF, is associated with a worse prognosis—irrespective of the cause of the cardiac failure—and predicts mortality or the need for cardiac transplantation [73]. Currently, measurement of plasma big endothelin 1 concentration is the best available predictor of outcome in CHF [74]. Interestingly, changes in plasma immunoreactive endothelin reflect the clinical response to the β adrenoceptor blocker carvedilol in patients with CHF [75], although it is not clear whether the drug is producing its benefits through direct inhibition of the endothelin system or by an effect on cardiac performance.

In the first clinical trial of an endothelin antagonist in CHF [12], in patients withdrawn from ACE inhibitor treatment, acute intravenous administration of the combined $ET_{A/B}$ antagonist, bosentan, increased cardiac output and reduced systemic and pulmonary vascular resistance without inducing reflex tachycardia or increasing plasma concentrations of angiotensin II or noradrenaline (Figure 6). These beneficial haemodynamic effects of inhibition of the endothelin system are similar to those associated with ACE inhibition, and beg the question whether they would add to the effects of optimal treatment with an ACE inhibitor [76]. Studies with local brachial artery administration of the ECE inhibitor, phosphoramidon, and the ET_A receptor antagonist, BQ-123, have addressed this issue in patients with CHF [77]. Even though these patients were maintained on ACE inhibitors, both agents caused substantial vasodilatation of the forearm resistance vessels, predicting that endothelin receptor antagonists might have additional value in the treatment of CHF. Indeed, from recent studies, it does appear that the beneficial haemodynamic effects of bosentan occur in the presence of ACE inhibitors and are sustained on chronic oral treatment (W. Kiowski; personal communication).

In local infusion studies, the vasoconstriction to endothelin-1 was reduced in CHF patients compared with matched control subjects in both resistance vessels [77] and hand veins [78] consistent with increased endothelin-1 generation. In contrast, vasoconstriction to the selective ET_B receptor agonist, sarafotoxin S6c, was enhanced. These

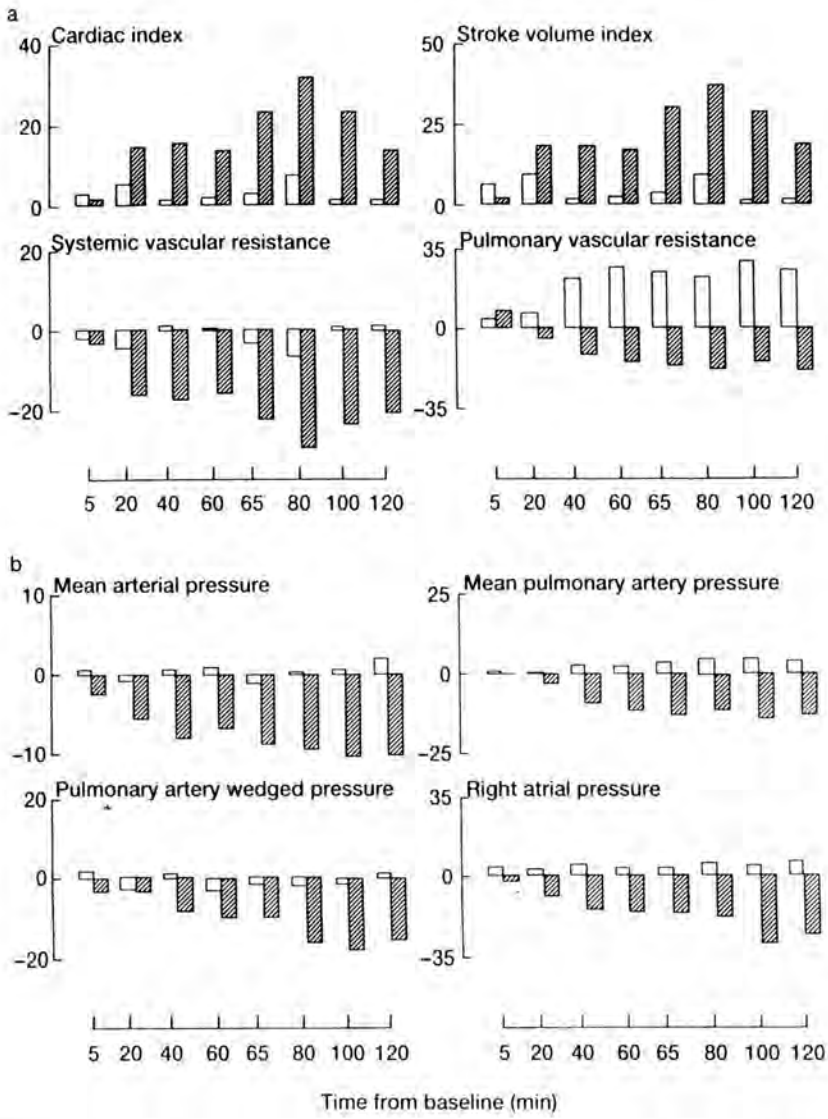

Figure 6 Changes of cardiac index, stroke volume, systemic vascular resistance and pulmonary vascular resistance (a) and of arterial, pulmonary artery, pulmonary artery wedged and right atrial pressures (b) in patients with severe congestive heart failure after intravenous placebo (unshaded columns) or bosentan (shaded columns). Bosentan 100 mg was administered intravenously at 0 min and a further 200 mg was given at 60 min. All eight parameters were significantly improved by acute administration of bosentan ($P<0.05$) without change in heart rate. Adapted and reproduced from Kiowski *et al.* [12], with kind permission of the *Lancet*.

observations are also seen in the coronary vessels in experimental CHF [79] and ET_B receptors are also upregulated in human CHF [80], consistent with widespread upregulation of the smooth muscle, and perhaps endothelial, ET_B receptor in this condition. Enhanced constriction to sarafotoxin S6c may, at least in part, be due to endothelial dysfunction affecting responses mediated through the endothelial ET_B receptor and suggests that constrictor ET_B may be of greater importance in some diseases than they are under physiological circumstances. Nevertheless, in patients with CHF, the response to arterial administration of the selective ET_B antagonist, BQ-788, is vasoconstriction, suggesting that the dilator response predominates and that selective ET_A antagonists might, therefore, offer some advantages in this condition.

Unresolved issues

A number of major issues concerning endothelin antagonists remain unresolved. The first and most important of these is the choice of the appropriate therapeutic target. On purely scientific grounds there would certainly be sufficient justification for clinical investigation of endothelin antagonists in essential hypertension, congestive heart failure, primary pulmonary hypertension, subarachnoid haemorrhage, stroke and acute ischaemic renal failure. However, there are a number of other issues for pharmaceutical companies to consider including the existence of currently effective treatment (essential hypertension), the lack of a sufficiently predictive model for the disease (angioplasty restenosis) and concerns over whether the market size is sufficiently large to justify the development (subarachnoid haemorrhage and primary pulmonary hypertension). There is obviously also a need to keep the overall budget for such compounds within reasonable bounds so companies need to be selective in their research programmes. Nevertheless, it can be expected that the role of endothelin antagonists will be explored in clinical studies in a number of these candidate diseases and it is likely—as, for instance, with the capacity of ACE inhibitors to delay the progression of renal failure—that some of the potential uses of endothelin antagonists cannot easily be anticipated at this stage. For instance, the potential antimitogenic action of endothelin antagonists may be critical in heart failure [68] and perhaps also in conditions like essential and pulmonary hypertension, and may even be relevant for cancer therapy [81].

Although the first effective 'endothelin antagonist' was an ECE inhibitor there appear to have been few recent developments in this area. All of the four or more endothelin antagonists in early clinical development, as well as at least 15 more in preclinical development [11], are endothelin receptor blockers. Most of these are orally active, although TAK-044 is a peptide and is therefore being developed for indications requiring relatively brief administration. Some of these agents are combined $ET_{A/B}$ antagonists, whereas others are selective ET_A antagonists. There are currently no selective ET_B antagonists that are clearly intended for clinical development although it might be argued that they would produce an organ-selective effect in pulmonary hypertension, avoiding systemic hypotension, given that hypoxic pulmonary vasoconstriction appears to be primarily mediated by ET_B receptors [82] whereas responses to endothelin-1 in the peripheral circulation appear to be determined primarily by effects on the ET_A receptor.

The more general issue of whether selective ET_A or combined $ET_{A/B}$ receptor antagonists will have greater utility has certainly not yet been resolved. Our current knowledge, from studies in healthy people and those with heart failure is that the major target must be the ET_A receptor. Inhibition of the ET_B receptor leads to peripheral vasoconstriction and, unlike inhibition at the ET_A receptor, causes substantial elevation of plasma concentrations of endothelin-1 [12, 53], probably by effects on clearance or displacement from ET_B receptors [83, 84]. However, the function of ET_B receptor may be more critical in some diseases or in, for instance, the cardiac and renal vascular beds, and the role of ET_B receptors in overall human cardiovascular control and pathophysiology has yet to be determined.

Summary

Endothelin-1 is an endothelium-derived vasoconstrictor and co-mitogenic agent which acts as a local paracrine and autocrine mediator, and is the most potent and sustained vasoconstrictor and pressor substance yet identified. On the basis of studies in healthy man, endothelin-1 is now known to play an important physiological role in maintaining peripheral vascular tone and blood pressure. Endothelin-1 also has actions which might influence the function of the heart, kidney and nervous system. However, their physiological importance remains to be determined.

Abnormalities of the endothelin system are now recognised to occur in a range of diseases associated with vasoconstriction, vasospasm and vascular hypertrophy and it appears that endothelin-1 may be causal, or at least contributory, in some of these pathophysiological processes. The use of endothelin receptor antagonists in experimental models of cardiovascular disease and in human clinical pharmacology studies has indicated a number of conditions—including hypertension, heart failure, acute renal failure, subarachnoid haemorrhage, and pulmonary hypertension—in which further clinical studies would be worthwhile. A number of peptide and orally-active non-peptide endothelin receptor antagonists are now under clinical investigation and further studies are now required in specific diseases to determine whether selective ET_A or combined $ET_{A/B}$ receptor antagonists would be more effective.

The discovery of endothelin-1, and the design of endothelin antagonists, has been among the most promising developments in cardiovascular medicine since the launch of ACE inhibitors 15 years ago. Major clinical trials are now needed to confirm the predicted benefits for endothelin antagonists in patients with cardiovascular disease.

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Endothelin: new discoveries and rapid progress in the clinic

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Following the discovery of endothelin-1 (ET-1) in 1988 (Refs 1, 2), research on this potent vasopressor and growth-promoting peptide has made rapid progress. The use of transgenic models and highly selective endothelin receptor antagonists has identified important developmental and physiological roles for the ET system as well as major consequences of its overactivity in a range of cardiovascular diseases[3]. Clinical studies now also support the development of ET antagonists in hypertension[4] and heart failure[5,6]. A recent conference on endothelin* addressed new developments in the field, focusing primarily on the role of ET-1 in the pathophysiology of cardiovascular disease.

Endothelin biosynthesis and ECE

The subcellular localization of endothelin converting enzymes (ECEs) and the site of physiological activation of big ET-1 remain controversial. The meeting opened with a session devoted to this topic. A. P. Davenport (University of Cambridge, UK) reported that a major site of ECE expression in humans is the vascular endothelium. He related the expression of ECE in human tissues with the distribution of big-ET and mature ET-1, and provided the first evidence of subcellular localization of ECE to the Weibel–Palade bodies, as well as to the cell surface, of human endothelial cells of both large conduit and smaller resistance vessels from heart, lung, brain and adrenal gland. ECE immunoreactivity was also present on bronchial epithelial cells, neurones and glia and, less abundantly, on the surface of vascular smooth cells.

Using site-directed mutagenesis, M. Hoang (University of Leeds, UK) and B-M. Löffler (Hoffman-La Roche, Switzerland) reported further refinements in structure–activity relations of ECE-1, describing residues important for catalytic activity and glycosylation. ECE-1 has ten potential *N*-glycosylation sites, and glycosylation was found to have a major impact on its physicochemical properties. However, single mutants (Asn to Asp) did not affect enzymatic activity. In contrast, double mutation of Asn632 and Asn651 abolished enzymatic activity whereas triple mutation of Asn residues 316, 362 and 382 had no effect. These findings suggest the functional importance of glycosylation of specific domains, and not single sites.

An elegant series of experiments by T. Kido (Kyoto University, Japan) using transfected cells provided the evidence that processing of pro ET-1 into big ET-1 by a furin-like protease is an essential step prior to its proteolytic activation by ECE-1. N. Emoto (University of Texas Southwestern Medical Center, Dallas, USA) reported that two alternatively spliced ECE-1 isoforms, termed ECE-1a and ECE-1b, differ only in the first 30 amino acids in the N-terminal cytoplasmic tails. These account for different subcellular localization in native vascular cells and in transfected Chinese Hamster Ovary (CHO) cells. ECE-1a is the predominant isoform in cultured vascular endothelial cells where it resides strictly within intracellular secretory structures and can be detected by immunostaining when the cells are permeabilized. ECE-1a is constitutively transported to the liposomes where it is rapidly degraded. In contrast, ECE-1b, the isoform found in cultured smooth muscle cells, is transported by the default pathway to the plasma membrane where it is located as an ectoenzyme and can be detected by immunostaining without permeabilization of the cells. Analyses of mutations and chimeric ECE-1/transferrin receptor constructs demonstrated that the cytoplasmic tail of ECE-1a contains signals that are necessary and sufficient for lysosomal targeting. These findings are consistent with a model in which conversion of big ET-1 in 'generator' cells, such as the endothelium, is predominantly an intracellular process but big ET-1 can also be cleaved by the cell-surface ECE-1 expressed in 'target' cells, such as vascular smooth muscle.

H. Funke-Kaiser (Benjamin Franklin University Hospital, Germany) reported that ECE-1 mRNA is expressed in two isoforms, also termed a and b, which differ only in their 5′ ends. Transfection experiments with the reporter-gene luciferase, in cultured bovine aortic endothelial cells, demonstrated that a fragment of 1.5 kb of genomic DNA immediately upstream of exon 3 of the human ECE-1 gene can act as an endothelial cell-specific alternative promoter for ECE-1a.

Endothelin receptors and antagonists

L. N. Pierre (University of Cambridge, UK) presented the virtually exclusive role of the ET_A receptor in ET-1-induced constriction of human pial arteries. D. J. Webb (University of Edinburgh) used selective ET_A and ET_B antagonists (BQ123 and BQ788, respectively) to show a vasodilatory role of the ET_B receptor in human forearm resistance vessels *in vivo*, presenting evidence that endogenous ET-1 confers basal constrictor tone via ET_A receptors on vascular smooth muscle which is modulated by endothelial cell NO-dependent ET_B-mediated dilator tone[7]. Although there has been considerable debate over the functional importance of the vascular smooth muscle ET_B receptor[8], from these studies, and others described later, the functional role of the constrictor ET_B receptor in human vessels remains in doubt.

The endothelial ET_B receptor is known to be linked to the production of the vasodilators NO and prostacyclin. M. Jougasaki (Mayo Clinic, Rochester, USA) described studies in cultured canine endothelial cells

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demonstrating that ET_B receptor stimulation by sarafotoxin 6c (S6c) can also increase the secretion of adrenomedullin, a circulating vasodilatory and natriuretic peptide of vascular endothelial and smooth muscle cell origin. Also, in anaesthetized dogs, an infusion of S6c resulted in an increase in plasma adrenomedullin concentrations. These results establish an autocrine–paracrine role of the ET_B receptor in the regulation of a further and potentially important endothelial vasoactive system.

Post-translational modifications, such as phosphorylation and palmitoylation, play an important role in the regulation of G protein-coupled receptors. C. Schroeder (Johannes Gutenberg University of Mainz, Germany) reported that the human ET_A and ET_B receptors are both palmitoylated but only the ET_B receptor is phosphorylated in a ligand-dependent manner. Evidence was also presented that phosphorylation causes rapid ET_B receptor inactivation, possibly accounting for the well-known tachyphylaxis to ET_B receptor agonists, whereas ET_A inactivation is slower and associated with receptor internalization. On the basis of earlier work[9,10], Y. Abe (University of Tsukuba, Japan) described a number of mutant ET_B alleles found in patients with Hirschsprung's disease, and used transient transfection techniques to show that, although all of these mutants bound endothelin with high affinity, binding sites were reduced in some cases and, to a varying degree, all of the mutants were significantly deficient in signal transduction as measured by phosphoinositide hydrolysis, intracellular Ca^{2+} mobilization and extracellular signal-related kinase (ERK)-2 activation. Loss of function of the receptors may, therefore, represent the molecular mechanism of Hirschsprung's disease in these kindreds. P. D'Orleans-Juste (University of Sherbrooke, Canada) and R. D. Naik (University of Saskatchewan, Canada) indicated a potentially important role for nuclear Ca^{2+} mobilization in endothelin receptor signalling.

A particularly elegant piece of medicinal chemistry was presented by T. W. von Geldern (Abbott, USA), who showed that highly ET_A-selective, mixed ET_A/ET_B, and highly ET_B-selective antagonists were derived by subtle modifications of a single chemical skeleton (ABT627, the active enantiomer of A127722). R. N. Willette (SmithKlineBeecham, USA) reported a strong correlation between the degree of plasma ET-1 elevation on administration of antagonist and the affinity of the drug towards the ET_B receptor, further confirming the 'clearance' role of the ET_B receptor for ET-1. In further key studies, T. J. Opgenorth (Abbott, USA) showed that the non-peptide ET_B-selective antagonist, A192621, causes sustained, and progressive, hypertension in rats.

Pathophysiology

Much of the meeting was devoted to pathophysiology, mainly in the cardiovascular field. Most of the work is still in animals but new and important clinical studies were reported.

Cardiovascular disease

The circulation is an important clinical target for ET antagonists. Two years ago[5], acute administration of the mixed $ET_{A/B}$ antagonist, bosentan, was shown to produce systemic and pulmonary vasodilatation in patients with chronic heart failure (CHF). However, a concern with vasodilator therapy in CHF has been that reduction in blood pressure might induce adverse counterregulatory neurohumoral activation. However, M. Ohnishi (Shiga University of Medical Science, Otsu, Japan) showed in dogs, and W. Kiowski (University of Zurich, Switzerland) showed in CHF patients, that ET receptor blockade attenuates activity of the renin–angiotensin and sympathetic nervous systems despite blood pressure lowering. Interestingly, Kiowski also showed that haemodynamic benefits with oral bosentan (1000 mg twice daily) can be sustained for at least 14 days of treatment and, on the basis of a blinded clinical assessment, that symptoms are improved by treatment. Although bosentan appeared to provide benefit, and large-scale clinical outcome studies are under way, it remains unclear whether a selective ET_A antagonist or a mixed $ET_{A/B}$ antagonist would be preferable. Recent studies in the rat coronary occlusion model suggest that selective ET_A antagonists may be effective in reducing mortality[11]. Additional work was presented at the meeting indicating in humans that vasoconstriction of coronary and resistance vessels is largely ET_A mediated in CHF. In agreement, in dogs with heart failure (which probably have a vascular endothelin receptor distribution similar to humans), selective ET_A receptor blockade with A127722 (D. Borgeson; Mayo Clinic, Rochester, USA) and with T0201 (Ohnishi) appeared to be very effective in improving left ventricular function and reducing pulmonary vascular resistance.

ET-1 also has pro-arrhythmogenic effects and arrhythmias are a major cause of death in CHF. Consequently, ET antagonism might reduce mortality through preventing arrhythmias as well as by preventing pump failure and cardiac remodelling. This concept was supported by M. Raschack (Knoll, Ludwigshafen, Germany), who showed that selective ET_A receptor blockade with LU135252 could reduce ischaemia-induced ventricular arrhythmias in pigs. Furthermore, P. Turbucz (Semmelweis University, Budapest, Hungary) reported very high ET levels in the pericardial fluid of patients with CHF and also showed, in dogs, that pericardial ET could be causally linked to ventricular arrhythmia.

ET-1 induces hypertrophy in cultured cardiac myocytes and ET receptor antagonists prevent cardiac hypertrophy and adverse remodelling in a myocardial infarction model of CHF caused by left coronary artery ligation[11]. M. Harada (Kyoto University, Japan) reported that cardiac non-myocytes (NMCs) – mostly cardiac fibroblasts – stimulate cardiomyocyte hypertrophy *in vitro* by secreting ET-1. Pure cardiomyocyte and NMC cultures prepared from

neonatal rat ventricles under cyclical stretch (20 cycles per minute) generated a hypertrophic response only in co-culture and this response could be blocked by treatment with the selective ET_A antagonist BQ123. These results indicate an ET_A-mediated paracrine action of NMCs on cardiomyocytes.

A number of participants reported the beneficial actions of ET antagonists in rat models of hypertension and its complications, including vascular remodelling, endothelial dysfunction, cerebral oedema, proteinuria and death due to fulminant hypertension. ET_A selective nonpeptide antagonists were shown by Y. Matsuo (S0139; Shinogi & Co., Osaka, Japan) to reduce brain oedema and infarction induced by transient middle cerebral artery occlusion and by E. L. A. Blezer (A127722; University of Utrecht, The Netherlands) to prevent dose-dependently cerebral oedema in stroke-prone spontaneously hypertensive rats.

Models of subarachnoid haemorrhage (SAH) have been widely shown to respond to ET receptor antagonists. Importantly, V. Breu (Hoffman-La Roche) presented evidence of reversal of post-SAH cerebral vasospasm by the mixed $ET_{A/B}$ antagonist bosentan in patients, as well as by the ET_A-selective parenteral antagonist, Ro611790 in a double-haemorrhage canine model. Interestingly, A. L. Kwan (University of Virginia, USA) showed that the alternative approach of using a nonpeptide ECE inhibitor (CGS26303) to prevent endothelin generation is effective in preventing and reversing SAH-induced spasm.

Pulmonary disease

In relation to obstructive lung disease, a number of papers reported the prevention and reversal of hypoxia-induced pulmonary hypertension with ET_A-selective antagonists, and S. Haleen (Parke-Davis) showed no rebound in pulmonary pressure on stopping therapy. Another important area of interest was lung transplantation. Here, allograft dysfunction is characterized by pulmonary hypertension and obliterative bronchiolitis and associated with strong ET-1 and ECE immunoexpression in the airways epithelium and infiltrating inflammatory cells. S. Takeda (Osaka University, Japan) reported that overexpression of ET-1 by HVJ (Sendai virus)-liposome gene transfer into the trachea of Wistar rats resulted in bronchiolitis obliterans whereas gene transfer into the pulmonary artery increased medial thickness in a manner similar to that found in pulmonary hypertension, consistent with a role for ET-1 in allograft failure. Also consistent with this view, A. Lee (McGill University, Canada) reported that the $ET_{A/B}$ antagonist, SB209670, im-proved both allograft function and survival of transplanted lungs in dogs.

It is also possible that ET antagonism will be useful in transplantation of other solid organs such as the kidney, liver and heart, where ischaemia-reperfusion and administration of cyclosporine may lead to excess activation of the ET system. Indeed, beneficial effects were presented for the $ET_{A/B}$ antagonist, TAK044 by W. Tanaka (Hyogo College of Medicine, Japan) for protection of rat liver grafts, and have previously been reported in experimental renal transplantation[12].

Renal disease

B. Hocher (Free University of Berlin, Germany) recognized glomerulosclerosis and renal interstitial fibrosis as the most prominent phenotype of human ET-1 transgenic mice, an effect linked to apoptosis. Such studies exemplify the potential role of ET-1 in renal disease. However, effects of ET antagonists in animal models are influenced both by ET-1 dependency of the model and by interspecies differences. Although studies in the rat are most common, the ET_B receptor mediates vasoconstriction in this species, whereas in dog and man renal vasoconstriction appears to be ET_A mediated. Moreover, the ET_B receptor in the kidney has been implicated in Na^+ and water homeostasis and tubular regeneration[12]. Indeed, key studies with ET-1 and an ET_A-selective (SB234551) and mixed $ET_{A/B}$ antagonist (SB209670) in chronically instrumented dogs (described by D. P. Brooks; SmithKlineBeecham, USA) clearly show that selective ET_A antagonism can prevent radiocontrast-induced renal vasoconstriction and, separately, reveal the presence of an ET_B-mediated vasodilatation and natriuresis. Nevertheless, this does not imply that mixed antagonism will be ineffective in renal disease. Indeed, in acute experiments in dogs with endotoxaemia C. Mitaka (Tokyo Medical and Dental University, Japan) demonstrated that mixed $ET_{A/B}$ blockade (TAK044) could prevent renal hypoperfusion. Comparative long-term studies in various renal disease models in dogs, and clinical studies in humans, are needed to determine the best approach.

Importantly, C. Ferro (University of Edinburgh, UK) reported the first clinical study in patients with chronic renal disease. Whilst the $ET_{A/B}$ antagonists, TAK044 and SB209670 induce only mild renal vasodilatation in healthy subjects as reported by G. Sütsch (University of Zurich, Switzerland) and D. Jorkasky (SmithKlineBeecham, Philadelphia, USA) respectively, intravenous TAK044 750 mg in patients with renal failure (baseline glomerular filtration rate, 25 ml min^{-1}) caused 10% reduction of mean arterial pressure, 24% reduction in systemic vascular resistance, and a favourable pattern of changes in renal vascular resistance and effective filtration fraction similar to those seen with ACE inhibitors. Interestingly, ACE inhibitor therapy in these patients was stopped only 24 h before TAK044 administration, implying, with this degree of renal failure, that the effects of endothelin antagonism may add to those of ACE inhibition.

Transgenic/knockout models

The meeting closed with work on genetic approaches to elucidating the function of the ET system in the whole animal. H. Morita (University of Tokyo, Japan) reported further mechanistic analysis of the small elevation of blood pressure in heterozygous

ET-1 gene-deficient mice[13]. This is not related to salt sensitivity and is likely to be due to hypoxic sympathetic activation. A. G. Baynash (University of Texas Southwestern Medical Center, Dallas, USA) reported for the first time that an ET-2 knockout mouse model is apparently normal at birth but then develops a phenotype of severe growth retardation. She showed that ET-2 is an essential molecule for postnatal growth, probably involved in the regulation of intestinal function. ECE-1 knockout mice reported by H. Yanagisawa (University of Texas Southwestern Medical Center, Dallas, USA) provided convincing genetic evidence that ECE-1 is a *bona fide* converting enzyme *in vivo*[14]. These mice showed the existence of dual ET-mediated pathways in the normal development of neural crest tissues. However, they also showed the existence of converting enzyme(s) that are not ECE-1 or ECE-2. Using isolated vascular beds from heterozygous ET_A receptor and ECE-1 knockout mice, N. Berthiaume (University of Texas Southwestern Medical Center, Dallas, USA) clearly showed that reduction of the activity of these genes by half is sufficient to cause significant alterations in vascular function.

Finally, using several genetic tricks to 'rescue' the lethal phenotype of ET_B-deficient animals, T. Ohuchi and C. Gariepy (University of Texas Southwestern Medical Center, Dallas, USA) each showed that adult ET_B-deficient mice and rats exhibit significantly elevated blood pressure under healthy, baseline conditions. In these mice, the hypertension was shown to be salt-sensitive and resistant to ET_A blockade, strongly implicating the function of the ET_B receptor as a physiologically relevant natriuretic receptor in the kidney.

Summary

This fifth international conference on ET serves to underline the rapid pace of development of our understanding of the very versatile ET system. On the one hand, the body uses ETs at several stages in embryonic development, in normal postnatal growth, and in cardiovascular homeostasis under healthy conditions. On the other hand, overwhelming evidence now exists that ET-1 plays important pathophysiological roles in conditions of decompensated vascular homeostasis. Indeed, in CHF this evidence is sufficient to justify the large-scale studies of morbidity and mortality needed to market ET antagonists as medicines. Other potentially important cardiovascular indications for ET antagonists are still emerging – including hypertension, stroke, subarachnoid haemorrhage and renal failure – and all are likely to be the subject of clinical trials over the next few years. As yet, there has been little work outside the cardiovascular and renal fields, but other areas, such as cancer treatment, may also prove promising[15].

New molecules with increasing selectivity (ET_A and ET_B) continue to emerge and may be valuable. Inhibition of ECE-1 remains as an alternative approach and nonpeptide ECE inhibitors now exist. There appears to be a consensus that ET_A blockade is beneficial in cardiovascular and renal disease. However, several strands of work presented at the meeting – the hypertensive salt-sensitive phenotype of rescued ET_B knockout mice, the sustained and progressive hypertensive effects of ET_B-selective antagonism in rats, ET_B-mediated vasodilatation and natriuresis in dogs, and nitric oxide dependent ET_B-mediated vasodilatation in humans – all suggest that ET_B-mediated vascular and renal responses may be protective. The development of selective ET_A antagonists, therefore, now seems fully justified. In the future, direct comparisons in animal models, and patients, of ET_A and $ET_{A/B}$ antagonists will be important in determining the value of additional ET_B receptor blockade in individual diseases.

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Chemical names

A127722: *trans-trans*-2-(4-methoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-[(*N*,*N*-dibutylamino)carbonylmethyl]pyrrolidine-3-carboxylate

A192621: [2R-(2α,3β,4α)]-4-(1,3-benzodioxol-5-yl)-1-[2-[(2,6-diethylphenyl)amino]-2-oxoethyl]-2-(4-propoxyphenyl)-3-pyrrolidinecarboxylic acid

ABT627: 2R-(4-methoxyphenyl)-4S-(1,3-benzodioxol-5-yl)-1-(*N*,*N*-di(*n*-butyl)-aminocarbonyl-methyl)pyrrolidine-3R-carboxylic acid, hydrochloride

BQ123: cyc(D-Trp-D-Asp-Pro-D-Val-Leu)

BQ788: *N*-*cis*-2,6-dimethylpiperidinocarbonyl-L-δ-methylleucyl-D-1-methoxy-carbonyltryptophanyl-D-norleucine

CGS26303: (*S*)-2-diphenyl-4-yl-1(1*H*-tetrazol-5-yl)ethylamino-methylphosphonic acid

LU135252: α-[(4,6-dimethoxy-2-pyrimidinyl)oxy]-methoxy-phenyl-(*S*)-benzene-propanoic acid

Ro611790: *N*-[6-[2-(hydroxyethoxy]-5-(2-methoxyphenoxy)-4-pyrimidinyl]-5-methyl-2-pyridinesulphonamide

SB209670: (±)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylene dioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid

SB234551: (*E*)-α-[[1-butyl-5-[2-[(2-carbaoxyphenyl)methoxy]-4-methoxyphenyl]-1*H*-pyrazol-4-yl]methylene]-6-methoxy-1,3-benzodioxole-5-propanoic acid

TAK044: cyclo[D-α-aspartyl-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl]disodium

Endothelin as a regulator of cardiovascular function in health and disease

William G. Haynes[a] and David J. Webb[b]

The endothelins are a family of endothelium-derived peptides that possess characteristically sustained vasoconstrictor properties. Endothelin-1 appears to be the predominant member of the family generated by vascular endothelial cells. In addition to its direct vascular effects, endothelin-1 has inotropic and mitogenic properties, influences homeostasis of salt and water, alters central and peripheral sympathetic activity and stimulates the renin–angiotensin–aldosterone system. Studies with endothelin receptor antagonists have indicated that endothelin-1 probably has complex opposing vascular effects mediated through vascular smooth muscle and endothelial ET_A and ET_B receptors. Endogenous generation of endothelin-1 appears to contribute to maintenance of basal vascular tone and blood pressure through activation of vascular smooth muscle ET_A receptors. At the same time, endogenous endothelin-1 acts through endothelial ET_B receptors to stimulate formation of nitric oxide tonically and to oppose vasoconstriction.

In view of the multiple cardiovascular actions of endothelin-1, there has been much interest in its contribution to the pathophysiology of hypertension. Results of most studies suggest that generation of, or sensitivity to, endothelin-1 is no greater in hypertensive than it is in normotensive subjects. Nonetheless, the deleterious vascular effects of endogenous endothelin-1 may be accentuated by reduced generation of nitric oxide caused by hypertensive endothelial dysfunction. It also appears likely that endothelin participates in the adverse cardiac and vascular remodelling of hypertension, as well as in hypertensive renal damage. Irrespective of whether vascular endothelin activity is increased in hypertension, anti-endothelin agents do produce vasodilatation and lower blood pressure in hypertensive humans. There is more persuasive evidence for increased endothelin-1 activity in secondary forms of hypertension, including pre-eclampsia and renal hypertension. Endothelin-1 also appears to play an important role in pulmonary hypertension, both primary and secondary to diseases such as chronic heart failure. The hypotensive effects of endothelin converting enzyme inhibitors and endothelin receptor antagonists should be useful in the treatment of hypertension and related diseases. Development of such agents will increase knowledge of the physiological and pathological roles of the endothelins, and should generate drugs with novel benefits. *J Hypertens* 16:1081–1098 © 1998 Lippincott-Raven Publishers.

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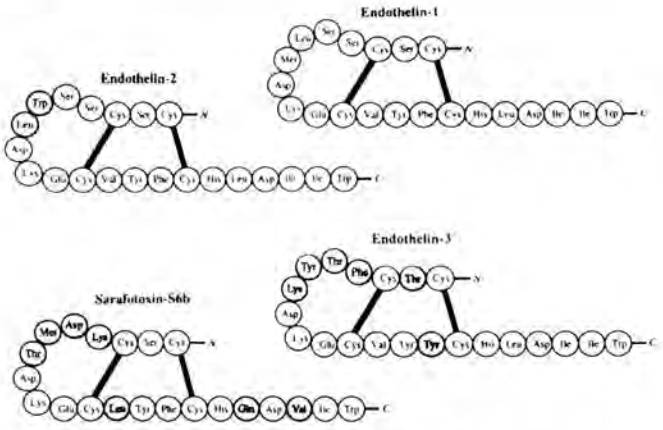
Introduction

The vascular endothelium plays a key role in the regulation of coagulation, lipid transport, immunological reactivity and vascular tone. Several important vasodilator and constrictor substances are produced by endothelial cells. The first identified was prostacyclin, which is a potent vasodilator and inhibitor of aggregation of platelets [1]. In 1980, a non-prostaglandin, endothelium-dependent vasodilator factor was postulated [2]. This endothelium-derived relaxing factor (EDRF) was subsequently identified as nitric oxide [3]. The isolation of EDRF prompted a search for counterbalancing endothelium-derived constricting factors (EDCF). By 1985, the vascular endothelium had been shown to generate a vasoconstrictor substance that produced prolonged vasoconstriction [4]. This long-acting agent appeared to be a peptide. It was finally isolated and sequenced from endothelial cell cultures in 1988 and called endothelin [5]. Three isopeptides of endothelin have since been identified, endothelin-1, endothelin-2 and endothelin-3, each containing 21 amino acids [6] (Fig. 1). Endothelin-1, the peptide originally identified by Yanagisawa, is the most potent vasoconstrictor and the predominant isoform expressed in vasculature [6,7].

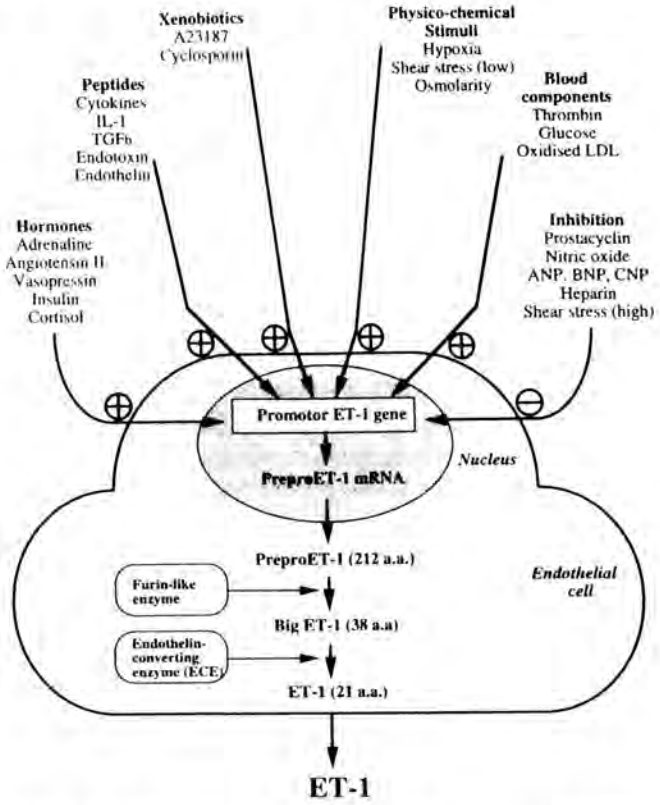
Endothelin-1 is a potent vasoconstrictor, has inotropic and mitogenic properties, influences homeostasis of salt and water and stimulates the renin–angiotensin–aldosterone and sympathetic nervous systems. Thus, the overall effect

Fig. 1



Amino acid sequences of the three members of the endothelin family and of the structurally related snake venom toxin sarafotoxin S6b. Each isoform contains two intra-chain disulphide bridges linking paired cysteine amino acid residues, thus producing an unusual semi-conical structure. Shaded circles indicate where amino acids differ from those of endothelin-1.

Fig. 2



Factors regulating synthesis of endothelin-1 (ET-1) and the pathway for generation of ET-1. See text for details of the regulatory elements of the preproendothelin-1 gene. Il-1, interleukin-1; TGFb, transforming growth factor-β; LDL, low-density lipoprotein; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptides; a.a., amino acids.

of the actions of endothelin is usually to increase vascular tone and blood pressure. Endothelin might, therefore, play an important role in the pathophysiology of cardiac, vascular and renal diseases associated with regional or systemic vasoconstriction. Anti-endothelin therapy could be beneficial in treating diseases associated with sustained vasoconstriction, such as essential hypertension, chronic heart failure (CHF) and chronic renal failure. In this article we review the biology of the endothelins and discuss in detail their putative roles in the pathophysiology of hypertension and related conditions.

Generation of endothelin

Molecular genetics and regulation of generation

Each member of the endothelin family is represented by a separate gene that encodes a specific precursor for the mature isoform [6]. In the 5′ flanking region there are binding sites for activating protein 1 and nuclear factor 1, which mediate the induction of mRNA for endothelin-1 by angiotensin II and transforming growth factor-β, respectively [7–9]. The 3′ flanking region of the mRNA contains adenine-uracil-rich sequences that mediate selective destabilization of preproendothelin-1 mRNA, accounting for its relatively short biological half life of 15 min. Generation of endothelin-1 is increased by many stimuli, including vaso-active hormones, growth factors, hypoxia, shear stress, lipoproteins, free radicals, endotoxin and cyclosporin [10] (Fig. 2). Production of endothelin-1 is inhibited by stimuli that act to increase intracellular level of cyclic guanosine monophosphate (cGMP), including endothelium-derived nitric oxide, nitrovasodilators, natriuretic peptides, heparin and prostaglandins [10] (Fig. 2).

Sites of generation

The major site of generation of endothelin-1, assessed in terms of expression of mRNA for preproendothelin-1 and the presence of intracellular converting enzyme, is in endothelial cells [6,7,11]. Endothelin-1 is also produced by the heart, kidney, posterior pituitary and central nervous system [10]. Human aortic vascular smooth muscle cells also express mRNA for endothelin-1, although its production is about 100 fold less than that in endothelial cells. Limited amounts of endothelin-2 are produced in endothelial cells, heart and kidney [12,13]. Endothelin-3 appears to be selectively expressed in the endocrine, gastro-intestinal and central nervous systems, but not in endothelial cells [10].

Biosynthetic pathway

The initial product of the human endothelin-1 gene is preproendothelin-1, a 212 amino acid peptide (Fig. 2). Proendothelin-1 is formed after removal of a short secretory sequence, and is then cleaved by furin to generate a 38 amino acid peptide, big endothelin-1 [5]. Big endothelin-1 does not appear to have any direct actions [14]. The

formation of mature endothelin-1 requires cleavage of big endothelin-1 by one of several unique endothelin converting enzymes (ECE). This family of metalloproteases is related to neutral endopeptidase-24.11 and Kell protein, but not to angiotensin converting enzyme. ECE-1 is the physiologically active ECE [15]. It possesses two splice variants, ECE-1a and ECE-1b, that have functionally distinct roles and tissue distributions [11]. ECE-1a is expressed in the Golgi apparatus of 'producer' cells, such as endothelial cells, and appears to be responsible for intracellular processing of big endothelin-1 to endothelin-1 in such cells. ECE-1b is expressed in 'responder' cells, such as vascular smooth muscle cells, and is transported to the plasma membrane where it acts to cleave extracellular big endothelin-1. A second form of ECE (ECE-2) has been cloned and characterized [16]. ECE-2 is similar to ECE-1 in that it is membrane bound, inhibited by phosphoramidon and exhibits selectivity for big endothelin-1. However, ECE-2 is active only at acidic pH (5.5) and is not expressed on the cell's surface [16]. Thus, ECE-2 could act as an intracellular enzyme responsible for the conversion of endogenously synthesized big endothelin-1 in acidic environments. ECE-1 and ECE-2 are relatively selective for big endothelin-1, having much less activity in cleaving big endothelin-2 and big endothelin-3. It is probable that there are other, as yet unidentified, ECE that are responsible for cleavage of endothelin-2 and endothelin-3. Both ECE-1 and ECE-2 are inhibited by phosphoramidon, but not by selective neutral endopeptidase and angiotensin converting enzyme (ACE) inhibitors [10].

Intra-arterial administration of big endothelin-1 to humans produces dose-dependent forearm vasoconstriction [17]. Co-infusion of the ECE inhibitor phosphoramidon completely prevents development of vasoconstriction in response to big endothelin-1. Thus, it is likely that vasoconstriction in response to big endothelin-1 reflects its vascular conversion to the mature peptide by ECE. Because circulating blood does not exhibit ECE activity [18], conversion of big endothelin-1 in the forearm probably occurs via action of vascular ECE situated in endothelial cells. Endothelin-1 is about 10-fold more potent as a constrictor of the forearm bed than is big endothelin-1, implying that local cell-surface ECE converts about 10% of luminally presented big endothelin-1 to endothelin-1. Further evidence that ECE activity is responsible for forearm vasoconstriction in response to big endothelin-1 comes from measurement of plasma concentrations of endothelin-1, big endothelin-1 and the inactive C-terminal fragment of endothelin-1 (CTF) formed by cleavage of big endothelin-1 in venous blood from infused and non-infused forearms [19]. Concentrations of big endothelin-1, endothelin-1 and CTF in venous blood from the infused arm, but not those in venous blood from the control arm, increase significantly during infusion of big endothelin-1. The ratio of CTF to big endothelin-1 is about 0.1, indicating that about 10% of the precursor is converted by ECE [19], which is consistent with functional data [17]. This 10% conversion rate for exogenously applied big endothelin-1 found in human studies is similar to that observed *in vitro* for exogenous big endothelin-1 in cells transfected with cDNA for ECE-1 [15].

Plasma concentrations of endothelin

Circulating concentrations of endothelin-like immunoreactivity in venous plasma are in the range 1–10 pmol/l in healthy subjects [20,21] and are strikingly dependent upon assay conditions [22]. This immunoreactivity comprises big endothelin-1 (~60%), endothelin-1 (~30%) and endothelin-3 (~10%) [20,21], although some investigators have not been able to detect big endothelin-1 in healthy subjects [23]. Endothelin-2 has not been detected in human plasma. Circulating concentrations of endothelin-1 are lower than those which cause vascular contraction *in vitro* and *in vivo*, although concentrations at the interface between an endothelial cell and vascular smooth muscle are likely to be much higher. Indeed, cultured endothelial cells secrete substantially more endothelin-1 towards the adjacent vascular smooth muscle than they do luminally [24]. Thus, endothelin-1 appears to be primarily a locally acting paracrine substance rather than a circulating endocrine hormone. Venous plasma endothelin 1 concentrations have been used as a marker for endothelial synthesis of the peptide, though circulating endothelin-1 is rapidly cleared from the circulation. Circulating concentrations of big endothelin-1 and CTF appear to reflect generation of endothelin-1 more accurately [19,25].

Clearance of endothelins

Endothelin-1 is rapidly cleared from the circulation after bolus intravenous injection, with a biological half life of about 1 min, although its pressor effects are sustained for up to 60 min [26,27]. A substantial proportion of clearance of endothelin-1 appears to occur through receptor binding and then internalization. Pulmonary clearance of radiolabelled endothelin-1 can be blocked by pretreatment with a large dose of unlabelled endothelin-1, supporting the hypothesis that its clearance is receptor mediated [26]. Blockade of endothelin receptors of the ET_B subtype, but not of the ET_A subtype, increases plasma concentrations of endothelin-1 and endothelin-3 [28] and prolongs the biological half life of exogenous [^{125}I]-endothelin-1 [29]. Blockade of ET_B receptors increases circulating level of endothelin-1 within 15 min [28,30] and does not affect concentrations of big endothelin-1 and C-terminal fragments [25,28], confirming that the increase is mediated by displacement of endothelin-1 from receptors rather than through generation *de novo*. Plasma concentrations of immunoreactive endothelin vary inversely with renal function [31]. Prolongation of plasma and biological half lives of endothelin-1 in bilaterally nephrectomized rats suggests that the major effect of renal disease is through impairment of its clearance [32]. Selective assays reveal that patients with chronic renal failure have marked elevations

in plasma concentration of endothelin-1 with little or no change in concentrations of big endothelin-1 [11]. Such elevations in level of mature endothelin-1, without changes in level of big endothelin-1, are most likely to be due to lower than normal renal clearance of endothelin-1. Enzymatic degradation of the endothelins by endopeptidases, particularly by neutral endopeptidase, also occurs [33].

Receptors and signal transduction

Endothelin receptors

The endothelins act on two receptor subtypes, ET_A and ET_B, characterized on the basis of their pharmacology (Table 1). The ET_A receptor is preferentially activated by endothelin-1 (K_i = 0.6 nmol/l) but not by endothelin-3 (K_i = 140 nmol/l) [34,35]. Messenger RNA for the ET_A receptor is expressed most highly in the aorta, heart and kidney but not in endothelial cells, suggesting that vascular expression of this receptor occurs selectively in smooth muscle cells [36]. Potent peptide and non-peptide ET_A antagonists have been synthesized, the prototype being the cyclic pentapeptide BQ-123 [37]. The ET_B receptor is activated equally by endothelin-1 (K_i = 0.12 nmol/l) and by endothelin-3 (K_i = 0.06 nmol/l). Messenger RNA for the ET_B receptor is most highly expressed in cultured endothelial cells [38]. Activation of this endothelial cell ET_B receptor leads to production of vasodilator substances, including nitric oxide and prostaglandins. Messenger RNA for the ET_B receptor has also been shown to be present in vascular smooth muscle [39], where it could mediate vasoconstriction [40]. Several agonists selectively activate the ET_B over the ET_A receptor. They include endothelin-3 (~2000-fold selectivity) and sarafotoxin S6c (~300 000-fold selectivity) [35]. BQ-788 is a selective peptide antagonist of the ET_B receptor [41].

The deduced structure for the ET_A and ET_B receptors has much in common with the superfamily of rhodopsin-like G-protein-coupled receptors, having seven hydrophobic membrane-spanning domains and a relatively long extracellular N terminal, although there is only a 25% sequence homology with other peptide receptors. Number of endothelin receptors is regulated by a variety of factors. Ischaemia and cyclosporin increase the number of endothelin receptors [42,43], whereas endothelin-1, angiotensin II and phorbol esters decrease number of receptors [44,45].

Intracellular events

Binding of endothelin-1 to ET_A or ET_B receptors produces G-protein dependent activation of phospholipase C. This leads to hydrolysis of phosphatidyl inositol and generation of cytosolic inositol trisphosphate and membrane-bound diacylglycerol [46,47]. Inositol trisphosphate causes a rapid increase in intracellular concentration of calcium ($[Ca^{2+}]_i$), through its release from intracellular stores [48]. A more sustained rise in $[Ca^{2+}]_i$ occurs through opening of membrane Ca^{2+} channels [48]. Diacylglycerol activates protein kinase C, increasing sensitivity of the contractile apparatus to changes in $[Ca^{2+}]_i$ [49]. Diacylglycerol also activates nuclear signalling mechanisms – with possible effects on long-term regulation of cellular function and growth – and increases intracellular pH through effects on the sodium–hydrogen ion exchange pump. In addition, there is evidence from in-vitro and in-vivo studies that endothelin-1 closes membrane K^+ channels [50–53]. Closure of these channels prevents efflux of K^+ from the cell, thereby favouring depolarization of membrane and contraction of smooth muscle. Finally, endothelin-1 can activate phospholipase A_2, increasing generation of prostacyclin and thromboxane A_2 [54].

Actions

Cardiac actions

Endothelin-1 has potent positive chronotropic and inotropic effects *in vitro* [55]. Endothelin-1 is a potent constrictor of coronary vessels, causing myocardial ischaemia and fatal ventricular arrhythmias [56]. *In vivo*, although endothelin-1 is positively inotropic at low doses, higher doses cause cardiac output to fall [57], probably due to a combination of a high afterload and myocardial ischaemia from coronary vasoconstriction. Systemic doses of endothelin-1 decrease cardiac output in humans, probably through a baroreceptor-mediated decrease in heart rate, although an increase in afterload might also contribute [58].

Direct vascular actions

Endothelin-1 causes sustained contraction of conduit arteries, with a potency 10-fold higher than those of other constrictors [5]. All three endothelins cause transient

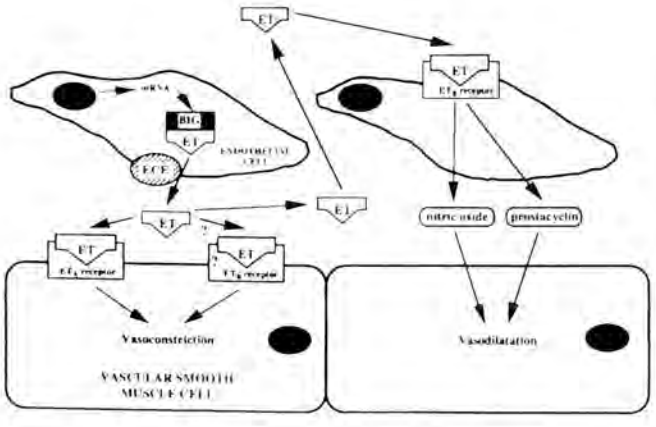
Table 1 Classification of endothelin receptors

Receptor	ET_A	$ET_{A/B}$	ET_B
Order of potency	ET-1 > ET-2 >> ET-3		ET-1 = ET-2 = ET-3
Affinity	ET-1 ~ 10^{-9} mol/l		ET-1 ~ 10^{-9} mol/l
	ET-3 ~ 10^{-6} mol/l		ET-3 ~ 10^{-9} mol/l
Tissue	Vascular smooth muscle		Endothelium Vascular smooth muscle
Vessel type	Conduit and resistance vessels		All vessels Resistance and capacitance vessels
Action	Constriction		Dilatation Constriction
Agonists	None	Endothelin-1	Endothelin-3
			Sarafotoxin S6c
Prototype antagonists	BQ-123	FAK-044, Bosentan	BQ-788

endothelium-dependent vasodilatation before the development of constriction, though this is most apparent for endothelin-3 [59]. Resistance vessels and veins are particularly sensitive to the effects of endothelin-1 [59]. Vasoconstriction in response to endothelin-1 was initially thought to be mediated solely by vascular smooth muscle cell ET_A receptors. Endothelial cell ET_B receptors were thought to mediate vasodilatation through generation of endothelium-derived dilator substances (Fig. 3). More recent evidence suggests that ET_B receptors also mediate vasoconstriction [40] (Fig. 3). Vasoconstriction in response to ET_B receptor agonists is variable and appears to depend markedly on species, vessel type and vessel size [39]. The physiological role of vascular ET_B receptors is best clarified by studies with receptor antagonists – these data are discussed later.

The endothelins increase blood pressure *in vivo* in animals for at least 60 min after a bolus dose [5]. The coronary and renal vascular beds in animals are most sensitive to the vasoconstrictor effects of systemic endothelin-1 [57]. The mesenteric bed also vasoconstricts in response to systemic endothelin-1, whereas the hindquarters skeletal muscle bed exhibits little constriction [60]. These differences among beds may be related to differences in constrictor (ET_A and ET_B) and dilator receptors (ET_B). The pressor effect of bolus doses of endothelin is usually preceded by transient hypotension that is most marked for endothelin-3 [6] and is mediated by endothelial ET_B receptors. Under more physiological conditions, in which concentrations of endothelin rise more slowly, hypotension does not occur [61]. Nevertheless, the hypotensive response to bolus administration could be useful in demonstrating the endothelial actions of the endothelins (see below).

Fig. 3



Vascular actions of endothelin 1 (ET). BIG ET big endothelin-1, ECE, endothelin converting enzyme.

Infusion of endothelin-1 into the brachial artery of humans slowly decreases forearm blood flow in a dose-dependent manner [62,63], with vasoconstriction sustained for 2 h after infusion of endothelin-1 has been halted [62]. Because vasoconstriction in response to endothelin-1 is slow to develop and reverse, most clinical investigators have used prolonged infusions rather than bolus doses of endothelin-1. When identical doses are infused over 60 and 5 min, greater vasoconstriction is observed with the more prolonged infusion [40]. Prolonged infusions also have potential safety advantages. However, this approach does limit plotting of dose–response curves, making it somewhat difficult to compare sensitivity to endothelin-1 between groups. In antagonist studies, this limitation can be addressed by using prolonged infusions of maximal doses of antagonists, to ensure that full inhibition of responses to endothelin-1 is obtained. Intra-arterial administration of a bolus endothelin-1 or endothelin-3 produces transient vasodilatation before development of vasoconstriction [40]. Endothelin-1 also causes slow-onset, sustained constriction of cutaneous veins *in vivo* [62,64]. Intravenous infusion of endothelin-1 increases blood pressure in human subjects by 5–10% at doses of about 1 pmol/kg per min administered for over 60 min [27,58]. As in animals, the haemodynamic effects are slow to come into effect and are sustained for more than 60 min. Systemic administration of endothelin-1 is also accompanied by coronary, renal and splanchnic vasoconstriction [58,65,66]. Depressor responses to systemic doses of endothelin-1 have not been shown to occur in humans, probably because endothelin-1 has been administered by slow intravenous infusion for safety reasons.

Interactions with other endothelial mediators of vascular tone

The endothelins stimulate generation of nitric oxide by vascular endothelial cells [67] (Fig. 3). The transient early vasodilator actions of the endothelins are attenuated by nitric oxide synthase inhibitors [68]. Perhaps more relevant physiologically is that nitric oxide synthase inhibitors also potentiate the constrictor and pressor effects of endothelin-1, suggesting that there is an autocrine feedback mechanism modulating vasoconstriction in response to endothelin by stimulation of the endothelial generation of nitric oxide [68]. Endothelin-1 also increases generation of prostacyclin by cultured endothelial cells [67] and cyclo-oxygenase inhibitors potentiate endothelin-1-induced constriction [68], suggesting that vasodilator prostaglandins play a similar modulatory role (Fig. 3). Venoconstriction to endothelin-1 in humans is potentiated by cyclo-oxygenase inhibition, but not by nitric oxide synthase inhibition, suggesting that prostanoids alone modulate the effects of endothelin-1 on veins [64]. In addition, endothelin-1 appears to increase endothelial generation of the potent vasodilator peptide adrenomedullin [69]. These endothelial effects of endothelin-1 that increase generation of vasodilators appear to be mediated by the

ET_B receptor, which perhaps acts by physiologically antagonizing ET_A receptor-mediated vasoconstriction.

Physiological role of endothelin-1 in maintenance of vascular tone

There has been much controversy about the physiological relevance of endogenous generation of endothelin-1 to the maintenance of basal vascular tone and blood pressure. Some of this was related to the unexpected finding that mice with one endothelin 1 gene deleted paradoxically have slightly higher blood pressure than do controls, despite their lower circulating concentration of endothelin-1 [70]. However, evidence that the elevation of blood pressure in this model is due to sympatho-adrenal overactivity caused by hypoxia secondary to abnormalities in facial/pharyngeal development is emerging [11]. Parenthetically, the occurrence of such anomalies suggests that endothelin-1 plays an important role in development of the pharyngeal arches, heart and great vessels [70]. Although it is now widely recognized that endothelin-1 does regulate arterial pressure under physiological conditions, there is still debate regarding the contributions of ET_A and ET_B receptors, particularly the latter.

The best way to address the physiological role of endogenous endothelin-1 is to examine the haemodynamic effects of drugs that selectively block the generation or actions of endothelin-1. Inhibition of ECE or ET_A receptors slowly decreases arterial pressure in normotensive animals [71–74]. This hypotensive effect of anti-endothelin therapy is not apparent in short term studies (i.e. < 10 min) [75–77]. A slow onset of vasodilatation would be consistent with the sustained vasoconstrictor effects of endothelin-1 which last for up to 2 h after an infusion has been halted [62] and also with the finding that endothelin receptor antagonists only slowly reverse the pressor effects of endothelin-1 in animals [78]. Intra-arterial administration of the ECE inhibitor and neutral endopeptidase inhibitor phosphoramidon to humans causes slow-onset forearm vasodilatation, suggesting that endogenously generated endothelin-1 plays a physiological vasoconstrictor role [17]. This effect is not due to inhibition of neutral endopeptidase, because the selective neutral endopeptidase inhibitors candoxatril and thiorphan do not cause vasodilatation [17,79]. Indeed, local infusion of neutral endopeptidase inhibitors causes a modest forearm vasoconstriction that might reflect inhibition of the metabolism of endothelin-1 [17,33,79,80]. Parenthetically, peripheral vasoconstriction secondary to inhibition of neutral endopeptidase could underlie the failure of this class of agents to decrease blood pressure in hypertensive patients [81]. Local blockade of forearm resistance vessel ET_A receptors with the peptide BQ-123 or of both ET_A and ET_B receptors with the peptide TAK-044 also causes slow-onset forearm vasodilatation, supporting the hypothesis that endogenous endothelin-1 plays a role in physiological maintenance of vascular tone [17,30]. Interestingly, at maximal doses, ET_A receptor antagonism with BQ-123 causes more vasodilatation than does combined $ET_{A/B}$ receptor antagonism with TAK 044. Systemic administration of TAK-044 to human subjects produces systemic vasodilatation and decreases arterial pressure by 10–20%, confirming the physiological importance of endogenous generation of endothelin-1 [30]. The non-peptide $ET_{A/B}$ antagonist bosentan also lowers arterial pressure in normotensive humans [82]. Taken together, these data suggest that endogenously generated endothelin 1 acts through ET_A receptors to promote vasoconstriction and maintain blood pressure.

More recently, evidence that endogenous endothelin-1 exerts additional actions causing vasodilatation and natriuresis and decreasing blood pressure, mediated through endothelial and renal ET_B receptors, has been accumulating. First, ET_B receptor antagonists, such as A192621, cause sustained and progressive hypertension in animals [11]. Second, maximal local ET_A receptor antagonism causes more vasodilatation in humans than does maximal local $ET_{A/B}$ antagonism [17,30]. Third, intra-arterial infusion of the ET_B receptor antagonist BQ-788 produces sustained vasoconstriction in humans and opposes the vasodilator action of BQ-123 [83]. Fourth, the kidney is rich in ET_B receptors that prevent tubular reabsorption of sodium and thereby cause natriuresis [84–88]. Lack of these renal ET_B receptors in ET_B knockout mice and rats causes sensitivity to salt, and hypertension that is not reversible with blockade of ET_A receptors [11]. The vasoconstrictor effects of ET_B receptor antagonists would be consistent with blockade of tonic endothelial ET_B receptor mediated stimulation of formation of nitric oxide (Fig. 3). However, it is worth remembering that ET_B receptor antagonists increase concentrations of endothelin-1 by blocking clearance receptors [28,29] and that this phenomenon could also account for the pressor effects of blockade of ET_B receptors. Indeed, there is evidence that the pressor effects of ET_B receptor antagonism are present even when formation of nitric oxide is inhibited and that these effects can be blocked by ET_A antagonism [89]. Even so, most data suggest that tonic stimulation of the ET_B receptor causes natriuresis and tends to decrease blood pressure under physiological circumstances.

In summary, there appears to be sufficient endogenous generation of endothelin-1 for it to play a physiological role in control of vascular tone and blood pressure. However, the overall cardiovascular effect of endogenous endothelin-1 depends on the balance between ET_A-mediated and ET_B-mediated effects. Activation of vascular smooth muscle ET_A receptors causes vasoconstriction and tends to elevate blood pressure. Activation of endothelial and renal ET_B receptors promotes vasodilatation and natriuresis and tends to decrease blood pressure. Interestingly, forearm vasodilatation in response to the ET_A receptor antagonist BQ-123 can be blocked either

by inhibition of nitric oxide synthesis or by blockade of ET_B receptors [83]. Thus, much of the vasodilator effects of ET_A receptor blockade may be due to unmasking of the underlying ET_B-mediated dilator tone. The hypotensive effects of combined $ET_{A/B}$ receptor antagonists on healthy subjects suggest that the overall physiological effect of endothelin-1 is to increase blood pressure [30]. Obviously, the cardiovascular effects of endogenous generation of endothelin-1 may change in cardiovascular disease if there are changes in the numbers or functions of ET_A and ET_B receptors. For example, impairment of endothelial generation of nitric oxide would be expected to attenuate dilator responses of ET_B receptors and promote constrictor responses of ET_A receptors.

Cell growth and inflammation

Endothelin-1 is a potent mitogen for vascular smooth muscle cells [90], cardiac myocytes [91] and glomerular mesangial cells [92]. Endothelin-1 increases expression of mRNA for the growth-promoting proto-oncogenes *c-fos* and *c-myc* [90]. The endothelins are also potent stimulators of monocyte production of cytokines that activate macrophages. These cytokines include tumour necrosis factor, interleukins (1, 6 and 8) and granulocyte–macrophage colony-stimulating factor [93].

Renal actions

Endothelin-1 has two main direct actions on the kidney, causing renal vasoconstriction and loss of tubular sodium and water, these actions probably reflecting separate sites of production in renal blood vessels and tubules. Endothelin-1 contracts afferent and efferent arterioles equally *in vitro* [94] and thus reduces both renal plasma flow and glomerular filtration rate (GFR) [95–97]. Endothelin-1-induced renal vasoconstriction involves ET_B receptors in the rat [72] but mainly ET_A receptors in the dog and human [98,99]. Renal vasodilatation occurs after infusion of BQ-123 into dogs, suggesting that vascular generation of endothelin-1 contributes to basal renal vascular tone [72]. Endothelin receptor antagonists decrease effective renal vascular resistance in humans by about 10%, without changing GFR [11], suggesting that the predominant effect of endogenously generated endothelin-1 is on the efferent arteriole.

Despite its potent vasoconstrictor properties, at low doses endothelin-1 increases urinary excretion of Na^+ [92,97]. There is substantial production of endothelin-1 by the inner medullary collecting duct cells [85] and renal tubular epithelial cells have a high density of endothelin receptors, mainly of the ET_B subtype [84]. Several lines of evidence suggest that this locally produced endothelin-1 plays an important role in modulation of renal excretion of sodium and water (Fig. 4). First, endothelin-1 blocks reabsorption of sodium by inhibiting tubular Na^+/K^+-ATPase activity in the proximal tubule and collecting duct [100]. Second, endothelin-1 blocks reabsorption of water in the collecting duct by inhibiting the effects of antidiuretic hormone (ADH) on tubular osmotic permeability [101,102]. Third, renal tubule generation of endothelin-1 is reduced by an increase in osmolality *in vitro* and by volume depletion *in vivo* [86,103]. Fourth, the number of endothelin receptors in glomeruli and tubules of volume-depleted rats is greater than normal [103]. Fifth, the cAMP response of inner medullary collecting duct cells to ADH is potentiated in the presence of specific endothelin-1 antisera, suggesting that endogenous production of endothelin-1 tonically inhibits responses to ADH [87]. These tubular effects also occur with ET_B receptor agonists and are not blocked by the ET_A receptor antagonist BQ-123, suggesting that they are mediated by ET_B receptors [88,104]. The hypothesis that ET_B receptors are involved is supported by the finding that ET_B knockout mice have hypertension secondary to renal retention of sodium [11]. Taken together, these findings suggest that locally generated endothelin-1 plays a tonic physiological role in regulating transport of salt and water in the terminal nephron, with increases in local generation promoting natriuresis and diuresis via activation of ET_B receptors (Fig. 4).

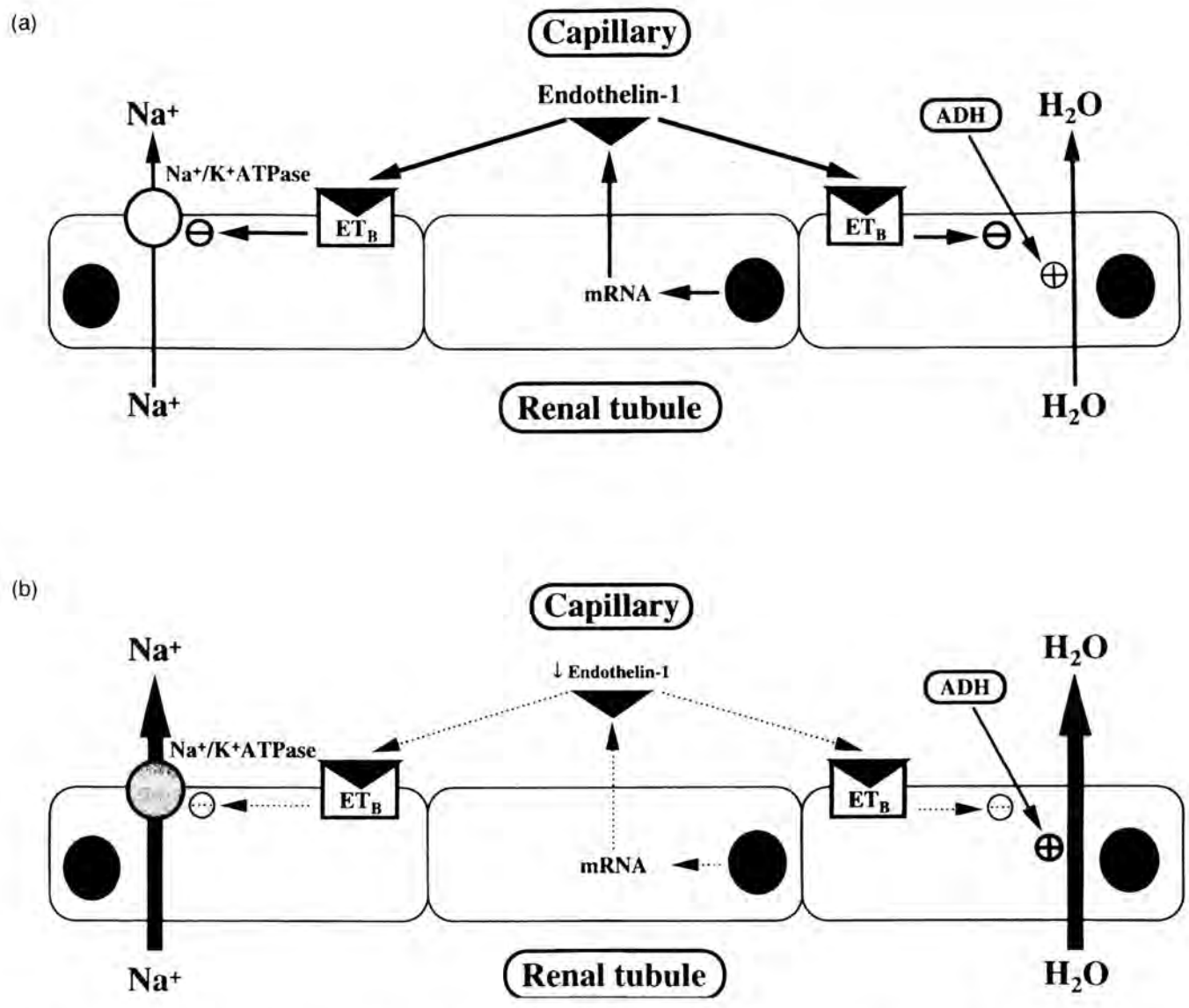
Nervous system

Intracerebroventricular administration of non-systemic doses of endothelin-1 acutely and chronically increases blood pressure through stimulation of central sympathetic outflow [105–107]. Endothelin-1 could also have a role in the peripheral autonomic nervous system. Binding sites for endothelin-1 are present in the carotid bifurcation and topical application of the peptide inhibits baroreceptor responses and stimulates chemoreceptor responses at this site [108]. In addition, endothelin-1 might potentiate the peripheral actions of the sympathetic nervous system at threshold doses [109]. However, local potentiation of peripheral sympathetic vasoconstrictor activity has not been demonstrated to occur in humans [63].

Endocrine actions

Endothelin-1 has contrasting effects on the renin–angiotensin–aldosterone system, inhibiting release of renin from isolated rat glomeruli [110] but stimulating endothelial ACE activity [111]. Endothelin-1 stimulates the tissue renin–angiotensin system in the rat isolated mesenteric bed [112]. Interestingly, angiotensin II increases endothelin-1 tissue levels and ECE activity *in vivo* and the haemodynamic and proliferative effects of angiotensin II can be prevented by blockade of ET_A receptors [113,114]. These findings raise the possibility of there being a positive-feedback loop linking endothelin-1 and angiotensin II in disease states such as heart failure. Endothelin-1 stimulates release of aldosterone from isolated cortical zona glomerulosa cells [115] and of adrenaline from medullary chromaffin cells [116]. Endothelin-1 stimulates production and release of atrial natriuretic peptide (ANP) by cultured atrial myocytes

Fig. 4



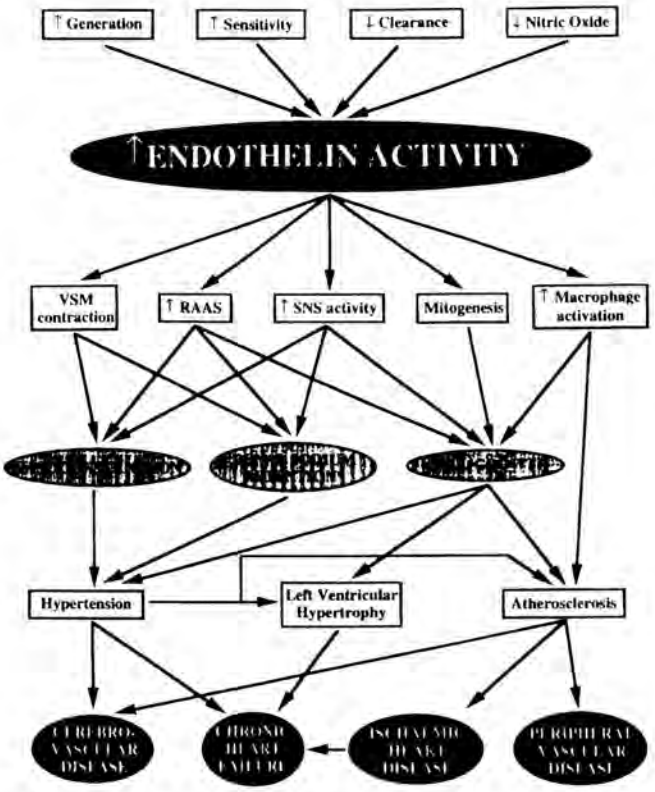
Effects of renal tubular endothelin-1 on reabsorption of sodium and water in (a) normotensives and (b) hypertensives. Under physiological circumstances, renal tubule epithelial cells generate endothelin-1 that acts on epithelial cell ET_B receptors to inhibit reabsorption of sodium (less activity of Na^+/K^+ ATPase) and reabsorption of water (less activity of ADH). This action thereby promotes excretion of sodium and water. In experimental and essential hypertension, less renal generation of endothelin-1 than normal occurs (↓ Endothelin-1). This will result in less tonic inhibition of tubular reabsorption of sodium and water and thus lead to retention of sodium.

in vitro and *in vivo* [117,118]. Pretreatment of rats with antiserum to ANP potentiates the pressor response to endothelin-1 [119]. Thus, endogenously generated ANP can modulate the vasoconstrictor actions of endothelin-1 *in vivo*. The endothelins have complicated effects on the pituitary, thyroid and parathyroid glands and on bone metabolism; these have been reviewed in detail elsewhere [120].

Endothelin and essential hypertension

The actions of endothelin-1 which increase vascular tone, activate the sympathetic nervous and renin–angiotensin–aldosterone systems and increase mitogenesis make it a plausible candidate mediator in the pathogenesis of hypertension and its complications (Fig. 5). In the absence of human studies using ECE inhibitors or endothelin receptor antagonists, much of the work on the role of endothelin-1 in human pathophysiology has been based on changes in circulating plasma concentrations of immunoreactive endothelins. However, it is important to bear in mind that these are dependent not only on generation, but also on renal and receptor-mediated clearance and enzyme-mediated metabolism of the peptide. Sensitivity to endothelin-1 has been examined in some studies, but, because number of receptors can be down-regulated by increases in concentration of endothelin-1

Fig. 5



Potential pathways by which endothelin-1 might contribute to the pathophysiology of hypertension or its complications. *An increase in vascular activity of endothelin-1 could cause retention of sodium through ET_A receptor-mediated renal vasoconstriction. There is also persuasive evidence that there is a deficiency of tubular generation of endothelin-1 in hypertension that could attenuate ET_B receptor-mediated facilitation of tubular excretion of sodium and water (see text and Fig. 4). VSM, vascular smooth muscle; RAAS, renin–angiotensin–aldosterone system; SNS, sympathetic nervous system; ↑, increase; ↓, decrease.

[14] and responses may be altered by vascular remodelling, these results may also prove difficult to interpret.

Generation of endothelin in hypertension

Concentrations of endothelin-1 in animal models of hypertension are not raised unless accelerated hypertension is present, in which case they are positively correlated to plasma level of creatinine [121,122]. Local mesenteric vascular generation of endothelin-1 *in vitro* in tissues from spontaneously hypertensive rats (SHR) appears to be greater than that in tissues from normotensive control Wistar–Kyoto (WKY) rats [123]. There appear to be strain-related differences because, although immunoreactivity to endothelin and expression of endothelin in blood vessels from deoxycorticosterone acetate (DOCA)–salt rats are greater than those in blood vessels from normotensive WKY rats, those in blood vessels from SHR are lower [124,125]. The hypothesis that alteration of generation of endothelin-1 plays no role in the pathophysiology of experimental hypertension is supported by the fact that polymorphisms of the preproendothelin-1 gene are not co-segregated with blood pressure or cardiac weight for inbred Dahl rats [126]. Interestingly, there is linkage between a locus near the preproendothelin-3 gene and blood pressure for these rats [126].

Several investigators have invoked increased concentrations of circulating immunoreactive endothelin to suggest that production of endothelin is increased in human essential hypertension [127,128]. However, because clearance of endothelin-1 depends on normal renal function [31,32], the increased concentrations of endothelin-1 found in severe and accelerated phase hypertension are probably secondary to an impairment of renal clearance. Results of studies concerning hypertensive patients with normal renal function have shown that they have similar concentrations of immunoreactive endothelin to those in normotensives [129–131]. Indeed, in one study a negative correlation between blood pressure and plasma level of immunoreactive endothelin was observed for the hypertensive group [129], making it unlikely that a global increase in generation of endothelin-1 is a cause of essential hypertension. Because African-Americans with hypertension have much higher concentrations of immunoreactive endothelin than do Caucasians, it is possible that there are racial differences in formation of endothelin-1 [132]. A polymorphism in an untranslated region of exon 1 of the preproendothelin-1 gene that abolishes a BsiY1 restriction site has been identified [133]. There are significant differences in BsiY1 preproendothelin-1 genotype between patients with essential hypertension and normotensive controls, with a strong correlation between diastolic blood pressure and the polymorphism [133].

Sensitivity to endothelin in hypertension

The results of studies examining vascular sensitivity to endothelin-1 in hypertension need to be interpreted cautiously, because of the confounding potentiating effects of vascular hypertrophy. In animal studies comparing WKY rats and SHR, both conduit (renal artery and aorta) and mesenteric resistance vessels from the SHR have been shown to be more sensitive to the effects of endothelin-1 [134,135]. However, other investigators have reported finding decreased sensitivity to endothelin-1 in the aorta and mesenteric resistance arteries from SHR [136], DOCA–salt rats [137] and renovascularly hypertensive animals [138,139]. It is possible that decreased sensitivity to endothelin-1 is related to down-regulation of endothelin receptors secondary to increased local generation of endothelin-1 or high blood pressure [124]. Systemic doses of endothelin-1 have greater pressor effects on SHR than they do on WKY rats [140] and renovascularly hypertensive rabbits [139]. There are fewer binding sites for endothelin-1 in aortic smooth muscle [141] and heart [142] in SHR than there are in WKY rats,

There are, however, relatively more binding sites for endothelin-1 in the brain of SHR than there are in the brain of WKY rats [142], so there could be a greater than normal sensitivity of the central nervous system in SHR to the peptide.

Some of the differences in responsiveness to endothelin-1 that have been observed may be related to differences in local generation of endothelin-1 and in the relative proportions of endothelin ET_A and ET_B receptor subtypes in different vessels. However, it should be remembered that studies of vascular responses in hypertension may be confounded by the presence of vascular hypertrophy in resistance vessels. In-vitro efficacy of endothelin-1 in subcutaneous resistance arteries in patients with essential hypertension appears to be less than that in normotensives [143]. Responsiveness to endothelin-1, but not norepinephrine, in cutaneous hand veins, vessels in which vascular hypertrophy does not occur, of untreated patients with essential hypertension is increased [131]. There is a positive correlation between venoconstriction in response to endothelin-1 and blood pressure in hypertensive subjects [131]. In addition, endothelin-1 appears to potentiate sympathetically mediated vasoconstriction in hypertensive but not normotensive subjects [131]. These findings suggest that endothelin-1 contributes to the elevation of preload observed during the early stages of essential hypertension. In summary, the results of studies examining sensitivity to endothelin-1 in hypertension suggest that sensitivity in resistance vessels is reduced, whereas sensitivity in capacitance and conduit vessels, particularly veins and renal arteries, is increased.

Impairment of responses of resistance vessels to endothelium-dependent dilators has been demonstrated to occur in essential hypertension [144], although this finding is by no means universal [145]. Even if impairment of endothelial dilator function in hypertension is solely due to decreased production of nitric oxide, the balance between vascular smooth muscle ET_A receptor-mediated vasoconstriction and endothelial ET_B receptor-mediated dilatation will be altered in favour of vasoconstriction. This hypothesis is supported by the finding that tonic ET_B receptor-mediated generation of nitric oxide appears to oppose the vasoconstrictor effects of endogenous endothelin-1 in healthy humans [83]. Interestingly, dysfunction of endothelial vasodilator in hypercholesterolaemic rabbits is associated with an ET_A receptor-mediated increase in coronary tone [146].

Renal effects of endothelin in hypertension

There appear to be two separate endothelin 'systems' mediating opposing actions in the kidney. The first is vasoconstriction both of afferent and of efferent glomerular arterioles, ultimately leading to retention of sodium [96], probably mediated through activation of ET_A receptors [99]. Because the renal artery of the SHR is more sensitive to endothelin-1 than is that of WKY rats [134], greater than normal renal vascular generation of endothelin-1 could promote hypertension. The second major site of generation of endothelin-1 in the kidney is the renal tubule, where it mediates excretion of salt and water [85–88,100,102], probably through activation of ET_B receptors [84]. Less endothelin-1 is generated in the renal medulla, particularly the collecting duct, of SHR than is generated in that of WKY rats [147,148]. Because tubular generation of endothelin-1 increases urinary excretion of sodium and water, any deficiency in renal tubule endothelin-1 could cause retention of sodium and thus hypertension in rats of this strain. Patients with essential hypertension have been shown to have less urinary excretion of immunoreactive endothelin than do normotensive controls [149], suggesting that local tubular deficiency of endothelin-1 predisposes hypertensive humans to retention of sodium (Fig. 4).

ECE inhibitors and endothelin antagonists in hypertension

Anti-endothelin therapy with ECE inhibitors and endothelin receptor antagonists lowers blood pressure in normotensive and hypertensive rats [71,72,74,150–156]. Although some studies have reported a more marked decrease in blood pressure in hypertensive animals [151,153], others have observed proportional decreases in blood pressure in animals of normotensive and hypertensive strains [71,152]. Studies with sufficient power have not yet been performed, so it is still not known whether anti-endothelin therapy causes a proportionately greater fall in blood pressure in hypertensive than it does in normotensive animal models. Animal models of salt-sensitive hypertension (DOCA–salt and Dahl rats) and malignant hypertension (stroke-prone SHR) appear to be especially sensitive to the hypotensive effects of endothelin receptor antagonism, suggesting that endothelin-1 plays an important role in these variant of hypertension [154,155,157–159]. In contrast, models of mild-to-moderate polygenic hypertension (young SHR) and renovascular hypertension (Goldblatt rats) are relatively insensitive to endothelin receptor blockade [160,161]. That there is a close interaction between endothelin-1 and the renin–angiotensin system [113,114] might suggest that the hypotensive effect of anti-endothelin therapy on hypertensive animals already receiving an ACE inhibitor would be blunted. However, there is convincing evidence that the roughly 20% reduction in blood pressure caused by $ET_{A/B}$ receptor antagonism with bosentan is additive to the roughly 20% decrease caused by ACE inhibition, with a total decrease in blood pressure in hypertensive dogs of 43% [162].

Administration of BQ-123 into the brachial artery of subjects with essential hypertension causes forearm vasodilatation of a similar degree to that observed with normotensive subjects [163]. Administration of the $ET_{A/B}$

receptor antagonist bosentan at a dose of 1000 mg twice daily for 4 weeks decreases 24 h ambulatory diastolic blood pressure in patients with essential hypertension by about 10 mmHg [164]. This reduction in blood pressure was similar to that achieved with a 20 mg dose of the ACE inhibitor enalapril [164]. The hypotensive effect of bosentan on hypertensive patients is similar to that observed in normotensive subjects [30], suggesting that endothelin-1 activity may not be specifically increased in essential hypertension. Nonetheless, the blood-pressure-lowering effects of anti-endothelin therapy will undoubtedly prove valuable in treating patients who are resistant to, or intolerant of, first-line antihypertensive agents. The effects of endothelin-1 on vascular growth and renal function indicate that these drugs may have specific advantages in preventing the complications of hypertension.

Endothelin and complications of hypertension

The potent mitogenic effects of endothelin-1 [90] may contribute to hypertension-induced hypertrophy of vascular smooth muscle, thus amplifying any vasoconstrictor influences [165,166]. Indeed, there is evidence that marked enhancement of vascular expression of endothelin-1 occurs in some hypertensive models, particularly salt-sensitive hypertensive rat models [125,159]. Endothelin ET_A receptor antagonists are able to normalize vascular structure in DOCA–salt SHR, although they do not normalize arterial pressure [125,167]. This reversal of vascular hypertrophy could be due to an increase in apoptosis (programmed cell death) of vascular smooth muscle cells [167]. Chronic blockade of ET_A receptor decreases arterial pressure and reduces vascular remodelling in salt-sensitive Dahl rats [159]. Combined blockade of ET_A and ET_B receptors completely prevents cerebral arteriolar hypertrophy in stroke-prone SHR, even though arterial pressure decreases only partially towards normality [154]. The abnormal distensibility and lower than normal external diameter (remodelling) of cerebral arterioles in stroke-prone SHR are not altered by blockade of endothelin receptors [154].

Endothelin-1 could also promote development of left ventricular hypertrophy, a factor that adversely affects prognosis in hypertension [168]. Blockade of endothelin receptors in SHR, stroke-prone SHR and DOCA–salt rats reduces both blood pressure and cardiac mass [155,169,170]. These results support the hypothesis that endothelin-1 plays a role in the disordered cardiac growth observed in hypertension.

In addition, renal dysfunction in hypertension can be mediated by endothelin-1. Glomerular ET_A and ET_B receptors in SHR are upregulated [156]. Acute blockade of ET_A receptors increases renal blood flow in SHR and DOCA–salt rats [156,171]. In contrast, acute combined blockade of ET_A and ET_B receptors decreases GFR, suggesting that up-regulation of ET_B receptors can act to maintain GFR in hypertensive rats [156]. Chronic blockade of endothelin receptors prevents renal dysfunction and fibrosis in genetically hypertensive rats [169,170], presumably reflecting inhibition of the growth-promoting effects of endothelin-1.

Finally, the growth-promoting properties of endothelin-1 may contribute to the development of atherosclerosis. Patients with atherosclerosis have raised plasma concentrations of endothelin, with the highest concentrations in patients with the largest numbers of affected vessels [172]. Atherosclerotic human blood vessels exhibit increased expression of mRNA and immunostaining for endothelin-1. Immunoreactivity for endothelin-1 is especially evident in hypercellular, macrophage-rich atherosclerotic lesions with many microvessels [173]. Patients with recent evidence of myocardial ischaemia and active coronary atherosclerotic lesions have very high tissue levels of endothelin-1 immunoreactivity [173]. Blockade of ET_A receptors in hyperlipidaemic hamsters inhibits formation of early atherosclerotic lesions by reducing the number and size of macrophage-foam cells [174]. Thus, in addition to its blood-pressure-lowering effects, anti-endothelin therapy could be anti-mitogenic, with potential advantages in treating hypertensive patients who exhibit vascular remodelling, left ventricular hypertrophy and atherosclerosis.

Endothelin and secondary hypertension

Haemangioendothelioma

Increased production of endothelin-1 appears to occur in patients with haemangioendothelioma, a rare skin tumour. Yokokawa *et al.* [175] have described two cases of haemangioendothelioma in which hypertension was associated with increased plasma concentrations of endothelin-1. Biopsies of tumour cells revealed increased expression of endothelin-1 mRNA and strong immunohistochemical staining for the peptide. Blood pressure and concentrations of endothelin-1 returned to normal after surgical resection of the tumours and recurrence of the tumour led to increases both in blood pressure and in plasma level of endothelin.

Pre-eclampsia

Circulating concentrations of immunoreactive endothelin in women with pre-eclampsia are greater than those in non-hypertensive pregnant women, even when their renal function is normal [176,177]. Treatment of pre-eclampsia with magnesium sulphate reduces plasma concentrations of endothelin-1 [177]. Increased generation of endothelin-1 could therefore play a role in the pathophysiology of pre-eclampsia.

Chronic renal failure

Complications of atherosclerosis are the commonest cause of death among patients with chronic renal failure and hypertension is an important contributor to this excess

mortality. Chronic renal failure, once it has become established, tends to progress to end-stage renal failure requiring therapy with dialysis. In animals with the low-renal-mass model of progressive chronic renal failure, there are increases in renal levels of preproendothelin-1 mRNA, cortical tissue levels of immunoreactive endothelin-1 and urinary excretion of endothelin [178,179]. In addition, renal generation of endothelin-1 is positively correlated to urinary excretion of protein and glomerulosclerosis in such animals [178,179]. Furthermore, glomerular expression of mRNA for ET_A and ET_B receptors is increased in experimental glomerulosclerosis [180]. Finally, administration of an endothelin ET_A receptor antagonist prevents the development of hypertension, glomerular damage and renal insufficiency in rats with low renal mass [181]. The results of these studies support that endothelin-1 plays an important role in progression of chronic renal failure.

Plasma concentrations of endothelin-1 in patients with chronic renal failure are 1–2-fold greater than normal, whereas values in those undergoing haemodialysis are 2–4-fold greater than normal [31]. Increased circulating endothelin-1 concentrations may reflect impairment of clearance; however, the fact that urinary excretion of endothelin-1 is also increased [182] suggests that renal generation of endothelin-1 in this disease is increased. It is possible that increased urinary excretion of endothelin-1 merely reflects a homeostatic effort to decrease tubular reabsorption of sodium. However, results of studies with endothelin receptor antagonists suggest that endothelin-1 plays a pathogenic role in renal failure. Administration of the $ET_{A/B}$ receptor antagonist TAK-044 to patients with chronic renal failure reduces blood pressure by 11% and renal vascular resistance by 10% [11]. Interestingly, blockade of ET_A and ET_B receptors decreases filtration fraction substantially, whilst maintaining GFR, suggesting that endogenous generation of endothelin-1 contributes to renal hyperfiltration [11].

Responsiveness to endothelin-1 of cutaneous veins of normotensive and hypertensive patients with chronic renal failure who are not yet undergoing dialysis has been examined [183]. Although there is no difference between responses in controls and normotensive patients with chronic renal failure, venoconstriction in response to endothelin-1 in hypertensive patients is attenuated. Because these patients have higher than normal concentrations of immunoreactive endothelin, their decreased responsiveness could reflect down-regulation of endothelin receptors secondary to increased generation or decreased clearance of the peptides. Results of further studies have demonstrated that patients with chronic renal failure have impairment of forearm vasodilatation in response to brachial artery infusion of the ET_A antagonist BQ-123, suggesting that vascular generation of endothelin-1 in this disease is probably decreased [184]. Alternatively, this might reflect endothelial dysfunction leading to decreased tonic ET_B-mediated formation of nitric oxide.

Erythropoietin

Erythropoietin therapy is used to reverse the anaemia of chronic renal disease, but is associated with clinically important hypertension in a substantial number of patients. In-vitro data suggest that production of endothelin-1 by endothelial cells is increased by exposure to erythropoietin [185]. However, plasma concentrations of immunoreactive endothelin are not increased by erythropoietin therapy [186]. Alteration of endothelin receptors is also not likely to underlie the association of erythropoietin therapy with hypertension, because forearm vasoconstriction in response to intra-arterial infusion of endothelin-1 decreases, rather than increases, after erythropoietin therapy has been started [186].

Cyclosporin A

Endothelin-1 could also contribute to the hypertension and renal impairment caused by cyclosporin A. Production of endothelin-1 is stimulated by cyclosporin A [187], which also increases the number of renal endothelin-1 binding sites [43]. In addition, cyclosporin-induced renal vasoconstriction is substantially attenuated by administration of the ET_A receptor antagonist BQ-123 [188,189]. Cyclosporin-induced hypertension is inhibited by blockade of ET_A and ET_B receptors [190]. However, cyclosporin A treatment does not increase plasma concentrations of immunoreactive endothelin in healthy human subjects [191]. Administration of the novel immunosuppressant tacromilus also appears to increase secretion of endothelin-1 *in vitro* [192]. Both cyclosporin A and tacromilus increase urinary excretion of endothelin-1 after liver transplantation [193].

Endothelin and pulmonary hypertension

Primary pulmonary hypertension

Pulmonary hypertension is characterized pathophysiologically by endothelial injury, proliferation of vascular smooth muscle cells and vasoconstriction of pulmonary resistance vessels. The vasoconstrictor and mitogenic effects of endothelin-1 make it a plausible contributor to the pathophysiology of pulmonary hypertension. Plasma concentrations of immunoreactive endothelin-1 in primary pulmonary hypertension are markedly increased [194]. Also, the pulmonary circulation seems to generate more endothelin-1 than it clears in primary pulmonary hypertension, because the arterial : venous concentration ratio of endothelin-1 is significantly greater than unity (~2.2) [194]. This is not the case for healthy controls, who have an arterial : venous ratio substantially less than unity (~0.6). In addition, patients with primary pulmonary hypertension exhibit increased immunoreactivity and expression of mRNA for endothelin-1 in the endothelial cells of hypertrophied pulmonary vessels, with the degree

of expression proportional to pulmonary vascular resistance [195]. These findings are consistent with the hypothesis that endothelin-1 plays a pathophysiological role in progression of primary pulmonary hypertension.

Secondary pulmonary hypertension

Pulmonary hypertension more often occurs secondarily to conditions such as chronic obstructive airway disease (*'cor pulmonale'*) and CHF. In healthy subjects, 30 min of hypoxaemia causes a twofold increase in circulating levels of immunoreactive endothelin-1, similar to those observed in patients with *cor pulmonale* [196]. Thus, it is possible that endothelin-1 is involved in this form of secondary pulmonary hypertension. Indeed, plasma concentrations of endothelin-1 in venous blood in patients with secondary pulmonary hypertension are increased [194]. There is no difference between arterial and venous concentrations of endothelin-1 in secondary pulmonary hypertension, in contrast to the apparent clearance of endothelin-1 across the pulmonary circulation in healthy subjects. Patients with secondary pulmonary hypertension also exhibit increased pulmonary vascular expression of endothelin-1 [195].

CHF

CHF is probably the commonest cause of secondary pulmonary hypertension. Neurohumoral activation occurs in CHF, with increases in sympathetic nerve activity and in circulating concentrations of adrenaline, noradrenaline, renin, angiotensin II, aldosterone and vasopressin. It has been hypothesized that these neurohumoral changes, by causing peripheral vasoconstriction and retention of sodium, contribute to the pathophysiology of CHF. The proven mortality benefits of ACE inhibitor therapy in treating CHF support the neurohumoral hypothesis. However, despite the benefits of ACE inhibitor therapy, morbidity and mortality of patients with CHF are still markedly increased.

Expression of endothelin-1 and ET_A receptors in animals with experimental heart failure is increased [197]. In addition, chronic administration of ET_A or of ET_A and ET_B antagonists reduces cardiac preload and afterload as well as preventing progressive left ventricular dilatation for several months after coronary ligation [197,198]. Such animals have markedly better long-term prognoses, the percentage of animals surviving increasing from 43 to 85% after administration of BQ-123 and from 47 to 65% after administration of bosentan [197,198]. It is possible that the higher percentage of animals surviving after administration of BQ-123 reflects an adverse effect of the additional blockade of ET_B receptors that occurs with administration of bosentan. It might be relevant that, in a comparison of selective ET_A and ET_B receptor antagonists with a canine model of heart failure, selective ET_B receptor antagonism with RES-701-1 increased intracardiac pressures and decreased cardiac output and renal flow of blood. These potentially adverse effects did not occur during blockade of ET_A receptors, which had beneficial effects [199].

Concentrations of immunoreactive endothelin in patients with CHF are greater than normal [200,201], correlated closely to the degree of haemodynamic and functional impairment [23,202] and associated with increased mortality and need for cardiac transplantation [202]. Big endothelin-1 is a substantial component of total circulating immunoreactive endothelin in CHF [23,202], perhaps reflecting increased generation of endothelin-1, rather than decreased clearance, in this condition.

Early phase clinical studies with the $ET_{A/B}$ receptor antagonist bosentan concerning patients with severe CHF have shown that sustained peripheral, pulmonary and venous vasodilatation occurs, together with improvement of cardiac performance, without reflex tachycardia [203]. These beneficial haemodynamic effects are similar to those obtained after ACE inhibition, which raises the question of whether anti-endothelin therapy would have similar effects on patients already receiving maximal ACE inhibitor therapy. This question was addressed in studies with local brachial artery administration of the ECE inhibitor phosphoramidon and the ET_A antagonist BQ-123 to CHF patients receiving ACE inhibitors [204]. Both of these agents produced substantial vasodilatation of forearm resistance vessels, suggesting that endothelin receptor antagonists have useful effects additional to those of ACE inhibition in treating CHF [204]. Indeed, results of recent studies with chronic oral administration of the $ET_{A/B}$ antagonist bosentan suggest that beneficial effects of anti-endothelin therapy do occur in CHF patients receiving ACE inhibitors [205]. Chronic therapy with oral bosentan for 14 days reduced systemic and pulmonary vascular resistances in these patients by 24 and 20% respectively [205]. Despite there having been decreases in mean arterial pressure, there was no increase in circulating noradrenaline levels and a blunting of the usual diuretic-induced activation of the renin–angiotensin system [206]. Clinical status of 35% of patients administered bosentan improved compared with 0% for placebo [205].

Although bosentan apparently is of haemodynamic and clinical benefit in treating CHF, there is intense debate regarding whether selective ET_A blockade or non-selective $ET_{A/B}$ blockade would be more beneficial in treating this disease. Interestingly, vasodilatation in response to BQ-123 appears to be reduced and that in response to phosphoramidon increased in CHF [204]. This would be consistent with occurrence of up-regulation of ET_B-mediated vasoconstriction, which has been demonstrated experimentally to occur in dogs [207]. Indeed, increased ET_B-mediated vasoconstriction has been shown to occur in forearm resistance vessels and hand veins of CHF

patients [208]. Nonetheless, patients with CHF do exhibit vasoconstriction in response to the ET_B receptor antagonist BQ-788, suggesting that the predominant vascular role of this receptor is to mediate vasodilatation under resting conditions [208]. These data, together with the results of animal studies presented above, suggest that selective ET_A blockade may be preferable in treating CHF.

Conclusions

The endothelins exert uniquely sustained vasoconstrictor actions and also activate the renin–angiotensin–aldosterone and sympathetic nervous systems and promote mitogenesis. Endothelin-1 is the predominant form generated by endothelial cells and therefore is likely to be the most important isoform in cardiovascular regulation. Inhibition of ECE or blockade of endothelin receptors produces vasodilatation and decreases blood pressure in normotensive subjects, suggesting that endogenous generation of endothelin-1 plays an important physiological role in the maintenance of blood pressure. The only other factors, besides endothelin-1, that have been shown to have a similar fundamental physiological role in maintenance of basal vascular tone are the sympathetic nervous system and nitric oxide. Results of recent studies suggest that the overall effects of endogenous endothelin-1 on blood pressure are the results of a complex interplay between tonic activation of vasoconstrictor vascular smooth muscle ET_A receptors and tonic activation of vasodilator endothelial ET_B receptors.

The role of endothelin-1 in hypertension has been contentious. There appears to be no abnormal excess vascular generation of, or sensitivity to, endothelin-1 in some animal models and in humans with mild polygenic hypertension. However, endothelial damage secondary to hypertension will decrease the usual physiological antagonism by nitric oxide of the vasoconstrictor effects of endothelin-1. There is also evidence that overall activity of the endothelin system is increased under certain circumstances, notably salt-sensitive hypertension, accelerated phase hypertension, pre-eclampsia and renal hypertension. In addition, endothelin-1 could contribute to the vascular, cardiac and renal complications of hypertension, including atherosclerosis, left ventricular hypertrophy and dysfunction and progressive renal disease. The blood-pressure-lowering effects of ECE inhibitors and endothelin receptor antagonists observed in normotensive and hypertensive subjects should provide a valuable additional option for treating patients with hypertension.

Most of the apparently deleterious effects of endothelin-1 appear to be mediated through ET_A receptors. Selective ET_A receptor antagonists block the vasoconstrictor effects of endogenous endothelin-1, whilst preserving the vasodilator and natriuretic effects mediated by ET_B receptors. Antagonists at ET_A receptors have been shown to have impressive benefits in treating several experimental models of cardiovascular disease. The role of the ET_B receptor is less clear, because it may mediate both deleterious (vasoconstriction) and beneficial (vasodilatation and natriuresis) effects. Blockade of the ET_B receptor also increases circulating concentrations of endothelin-1 by blocking clearance receptors. Nonetheless, results of early clinical studies in hypertension and CHF have shown that there are clinical benefits with combined $ET_{A/B}$ receptor antagonists. Appropriately designed clinical trials to examine whether combined $ET_{A/B}$ blockade or selective ET_A blockade is superior in the treatment of hypertension would be valuable. Further studies to elucidate the effects of anti-endothelin therapy on the complications of hypertension and on related diseases, such as CHF and chronic renal failure, are also needed.

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