ENDOTHELIN IN MAN:

STUDIES IN PHARMACOLOGY, PHYSIOLOGY AND

PATHOPHYSIOLOGY

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ABSTRACT OF THESIS

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The endothelins (ET-1, ET-2 and ET-3) are a family of 21-amino acid peptides with extremely potent and characteristically sustained vasoconstrictor and vasopressor actions. ET-1 is the predominant isoform in the vascular endothelium and is, therefore, likely to be the isoform of most importance in regulation of vascular tone and blood pressure. The endothelins are synthesised from inactive precursors (big endothelins) by an endothelin-converting enzyme. Two endothelin receptor subtypes, ET_A and ET_B have been identified. The ET_A receptor is mainly expressed in smooth muscle cells and mediates vasoconstriction. The ET_B receptor is mainly expressed on endothelial cells where it mediates vasodilatation. However, the ET_B receptor is also expressed on vascular smooth muscle cells where it mediates vasoconstriction.

In this thesis I review the biology of endothelin as well as discuss the evidence supporting a pathophysiological role for endothelin in a number of cardiovascular and renal diseases and consequently, the potential for therapeutic intervention in this sytsem. In the studies described, I have further investigated the physiology of the endothelin system as well as examined the pharmacology of endothelin receptor antagonists in man. Finally, I have also examined the potential contribution of endothelin to the pathophysiology of chronic renal failure and essential hypertension.

Study 1:   Local inhibition of the enzyme neutral endopeptidase causes forearm vasoconstriction probably by preventing endothelin breakdown.

Study 2:   Big ET-3 is converted to the mature peptide ET-3 in the forearm circulation, but not in capacitance vessels, of healthy volunteers.

Study 3:   Sytemic infusions of TAK-044, a non-selective endothelin receptor antagonist, lower systemic vascular resistance and blood pressure in man.

Study 4:   Systemic doses of the endothelin receptor antagonist, TAK-044, completely blocks the local vasoconstriction produced by intrabrachial artery infusion of ET-1 for up to 3 hours.

Study 5:   Systemic doses of TAK-044 only partially block ET-1 mediated vasoconstriction for 12 hours with important implications for dosing schedules in potential therapeutic interventions.

Study 6:   TAK-044 causes renal vasodilatation and lowers effective filtration fraction by a relative decrease in glomerular filtration rate and increase in effective plasma flow in healthy volunteers.

Study 7:   TAK-044 lowers systemic vascular resistance and blood pressure in patients with chronic renal failure, with a reduction in effective filtration fraction. Glomerular filtration rate is not significantly affected.

Study 8:   Forearm vasodilatation to BQ-123, a selective ET_A receptor antagonist is not different in patients with essential hypertension compared with normotensive controls. However, vasoconstriction to ET-1 is impaired in hypertensive patients, whereas vasoconstriction to the selective ET_B receptor agonist, sarafotoxin S6c is not.

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Studies

Inhibition of the enzyme neutral endopeptidase 24.11 causes vasoconstriction in humans *in vivo* by inhibiting the degradation of endothelin

The inactive precursor big endothelin-3 is converted to active ET-3 *in vivo* in human resistance vessels but not in veins

Dedication

To my wife, Jo.

Declaration

I declare that I have participated in the planning, design and execution of all the studies presented in this thesis as well as in the analysis and interpretation of the results obtained. No part of this thesis has been presented as part of a thesis for the award of a higher degree from Edinburgh University or any other centre of higher education.

Ethics

All studies were conducted with the approval of the Lothian Ethics of Medical Research Committee and the Inveresk Clinical Research Ethics Committee and with the written, informed consent of each subject. All studies were performed in accordance with the guidelines set out in the revised Declaration of Helsinki 1964.

Publications arising from this work

Ferro CJ, Spratt JC, Haynes WG, Webb DJ. Inhibition of neutral endopeptidase causes vasoconstriction in human resistance vessels in vivo. *Circulation* 1998; **97**: 2323-2330.

Ferro CJ, Haynes WG, Johnston NR, Lomax CC, Newby DE, Webb DJ. The peptide endothelin receptor antagonist, TAK-044, produces sustained inhibition of endothelin-1 mediated arteriolar vasoconstriction.*Br J Clin Pharmacol* 1997; **44**: 377-383.

Ferro CJ, Webb DJ. The clinical potential of endothelin receptor antagonists in cardiovascular medicine. *Drugs* 1996; **51**: 12-27.

Ferro CJ, Webb DJ. Endothelial dysfunction in hypertension. *Drugs* 1997; **53** (Suppl. 1): 30-41.

Haynes WG, **Ferro CJ**, O'Kane KPJ, Somerville D, Lomax CC, Webb DJ. Systemic endothelin receptor blockade decreases peripheral vascular resistance and blood pressure in man. *Circulation* 1996; **93**: 1860-1870.

Plumpton C, **Ferro CJ**, Haynes WG, Webb DJ, Davenport AP. 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. *Br J Pharmacol* 1996; **119**, 311-314.

Presentations arising from this work

Fourth International Congress on Endothelin, London, April 1995.
Role of endothelin in maintenance of vascular tone in normotensive and hypertensive subjects.

British Hypertension Society Annual Scientific Meeting, Glasgow, September 1995.
The endothelin antagonist, TAK-044, causes vasodilatation in humans.

British Pharmacological Society Winter Meeting, Brighton, December 1995.
Role of neutral endopeptidase in control of peripheral vascular tone in man.

Thirtieth Annual Meeting of The European Society for Clinical Investigation, Interlaken, Switzerland, April 1996.
Systemic administration of the endothelin receptor antagonist, TAK-044, causes vasodilatation and reduces blood pressure in man.

Thirtieth Annual Scientific Meeting of the European Society for Clinical Investigation, Interlaken Switzerland, April 1996.
Inhibition of vascular neutral endopeptidase causes vasoconstriction in man.

Thirtieth Annual Scientific Meeting of the European Society for Clinical Investigation, Interlaken Switzerland, April 1996.
Are the vascular endothelin and nitric oxide systems involved in the pathophysiology of essential hypertension?

British Hypertension Research Group 10th Anniversary Meeting, Edinburgh, May 1996.
Vascular actions of neutral endopeptidase inhibition.

Sixteenth Scientific Meeting of the International Society of Hypertension, Glasgow, June 1996.
Neutral endopeptidase inhibitors cause vasoconstriction in man.

Sixteenth Scientific Meeting of the International Society of Hypertension, Glasgow, June 1996.
The vascular endothelin and nitric oxide systems in essential hypertension.

Sixteenth Scientific Meeting of the International Society of Hypertension, Glasgow, June 1996.
The endothelin receptor antagonist, TAK-044, reduces systemic vascular resistance and blood pressure in man.

Annual Research Meeting of the Caledonian Clinical Pharmacology Society, Dundee, October 1996.
The vascular endothelin system in essential hypertension.

British Hypertension Group Autumn Meeting, Hexham, October 1996.
Endothelin in essential hypertension.

Fifth International Congress on Endothelin, Osaka, September 1997.
Actions of systemic endothelin receptor antagonism in patients with chronic renal failure.

British Hypertension Research Group, Glasgow, October 1997
Endothelin receptor antagonism in chronic renal failure.

Thirtyfifth Congress of the European Renal Association – European Dialysis and Transplantation Association, Rimini, June 1998
Actions of the endothelin A/B receptor antagonist TAK-044 in chronic renal failure

Thirtyfifth Congress of the European Renal Association – European Dialysis and Transplantation Association, Rimini, June 1998
The vascular endothelin system in essential hypertension

Prizes presented to the author for this work

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Abbreviations

ACE	angiotensin converting enzyme
ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
ANG II	angiotensin II
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
AP-1	activation protein-1
ARF	acute renal failure
AU	arbitrary units
AUC	area under the concentration-time curve
Big ET-1	big endothelin-1
Big ET-2	big endothelin-2
Big ET-3	big endothelin-3
BNP	B-type natriuretic peptide
bpm	beats per minute
BSA	bovine serum albumin
cGMP	cyclic GMP
cDNA	complementary deoxyribonucleic acid
CHF	congestive heart failure
CI	confidence intervals
CI	cardiac index
C_{max}	maximum drug concentration
CNP	C-type natriuretic peptide

cpm	counts per minute
CRF	chronic renal failure
CSF	cerebrospinal fluid
CV	coefficient of variation
DBP	diastolic blood pressure
DHBA	dihdroxybenzylamine
DNA	deoxyribonucleic acid
DOCA	deoxycorticosterone acetate
ECE-1	endothelin-converting enzyme-1
ECE-2	endothelin-converting enzyme-2
ECG	electrocardiograph
EC_{50}	enzyme inhibition constant
EDHF	endothelium derived hyperpolarising factor
EDTA	ethylenediamine-tetraacetic acid
ERPF	effective renal plasma flow
ERVR	effective renal vascular resistance
ET_A, ET_B	endothelin receptors
ET-1	endothelin-1
ET-2	endothelin-2
ET-3	endothelin-3
FHR	Fawn hooded rat
GFR	glomerular filtration rate
HPLC	high performance liquid chromatography
HR	heart rate
HSA	human serum albumin

5-HT	5-hydroxytryptamine
Hz	Hertz
ia	intra-arterial
IC_{50}	inhibitory constant where an antagonist causes an inhibitory response, the IC_{50} is the molar concentration which produces 50% of the maximum possible inhibition
iv	intravenous
K_m	steady state concentration at which 50% of maximum enzyme function is reached
LBNP	lower body negative pressure
LDL	low-density lipoprotein
Li	lithium
L-NAME	L-monomethyl aginine
L-NMMA	N^G-nitro-L-arginine methyl ester
LVDT	linear variable differential transformer
M	molar
MAP	mean arterial pressure
mol	moles
mRNA	messenger ribonucleic acid
Na	sodium
NEP	neutral endopeptidase
NF-1	nuclear factor-1
NO	nitric oxide
NOS	nitric oxide synthase

PAH	*p*-amoinohippurate
pA$_2$	the negative log of the concentration of an antagonist that would produce a 2-fold shift in the concentration response curve for an agonist
PEP	pre-ejection period
PGI$_2$	prostacyclin
PGE$_2$	prostaglandin E$_2$
pH	hydrogen ion concentration equal to $-\log\{H^+\}$
PKC	protein kinase C
PLC	phospholipase C
PTCA	percutaneous transluminal coronary angioplasty
PPH	primary pulmonary hypertension
RIA	radioimmunoassay
RMS	root mean square
SAH	subarachnoid haemorrhage
SBP	systolic blood pressure
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
SI	stroke index
SNP	sodium nitroprusside
STI	systolic time interval
SWG	standard width gauge

SVRI	systemic vascular resistance index
TBF-β	transforming growth factor-β
TFA	trifluoroacetic acid
VET	ventricular ejection time

1. Introduction

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1.1 The Vascular Endothelium: Historical Perspective

Historically, the vascular endothelium was thought only to be a semi-selective barrier to the diffusion of macromolecules from the blood lumen to the interstitial space. Indeed, Marcello Malpighi (1628-1694), a founding father of modern anatomic pathology,[485, 487, 502] considered the endothelium to be no more than an inert 'wallpaper' lining blood vessels. In 1976, Moncada et al[361] demonstrated that endothelial cells were a source of prostacyclin, a substance which inhibits platelet aggregation and causes vascular relaxation. However, full recognition of the endothelium as an important cardiovascular regulator did not come until Furchgott's seminal paper in 1980 describing an endothelium-derived relaxing factor,[179] currently thought to be nitric oxide (NO).[422] In 1983 de Mey and Vanhoutte predicted the existence of an endothelium-derived constricting factor.[122] This prediction culminated in 1988 with the discovery of endothelin by Masashi Yanagiawa and colleagues.[635] The discovery of endothelin has resulted in an explosion of research activity with over 4000 publications in endothelin research during 1996 alone.[603] It is now clear that the endothelium plays a key role in the regulation of vascular tone producing a number of vasoactive mediators, and has been the target of several of the most succesful pharmacological treatments in the treatment of cardiovascular disease, including aspirin, nitrates and angiotensin-converting enzyme (ACE) inhibitors (Figure 1.1).[320]

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1.2 Endothelin

The endothelins[635], comprise a family of three related peptides (ET-1, ET-2 & ET-3), each of 21 amino acids, with two intra-chain disulphide bridges linking paired cysteine residues (Figures 1.2 & 1.3).[242] Since 1988, there has been a major focus of research based on their potential pathophysiological role in a number of cardiovascular diseases.[603] ET-1 is the most potent mammalian vasoconstrictor peptide known,[242, 635] with veins being 3 to 10 times more sensitive to the effects of ET-1 than arteries, both *in vitro*[98] and *in vivo*.[99] ET-1 is also the major isoform produced by endothelial cells[60] and is probably the most important isoform in the cardiovascular system. The endothelins also show striking structural similarities with a family of snake venoms, the sarafotoxins (Figure 1.3).[197]

1.2.1 Endothelin genes and regulation

The first description of ET-1[635] was rapidly followed by the identification of at least three genes encoding 'ET-like' sequences in mammalian genomes.[242] These sequences have since been shown to encode the precursors prepro ET-1, ET-2 and ET-3. In the human genome, the ET-1 gene is found on chromosome 6,[61] the ET-2 gene on chromosome 1[62] and the ET-3 gene on chromsome 20.[60]

Like other eukaryotic genes, the genes that encode the endothelin precursors have promoter regions through which external factors are able to modulate transcription.[48, 225] Selective gene modification has demonstrated regions necessary for high level transcription[303, 305, 624] and also regions that might determine the tissue selectivity of ET-1 expression.[47] Extracellular factors can influence ET-1 generation both positively and negatively through liberation of a series of intracellular mediators that modulate gene transcription (Table 1.1). Several agents enhancing ET-1 generation[197] do so 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 (AP-1) transcription regulatory element of the ET-1 promoter.[111, 304]

Recent studies have shown that different isoforms of the endothelin precursor mRNA can arise due to the presence of alternative transcription initiation sites in the ET-1 gene that are controlled by different promoter regions[47] and also by alternative splicing of the ET-2 and ET-3 genes during transcription.[416, 417] The consequences for translation efficiency and for subsequent conversion of the endothelin precursors remain to be shown. Future investigations should also demonstrate whether insertion polymorphisms identified in the non-coding region of the ET-1 gene[534] might, like polymorphisms of the ACE gene,[74] prove to be related to risk of developing cardiovascular disease.

1.1.2 Processing of endothelin precursors

All of the endothelins are formed through a two-step processing pathway from their respective precursor peptides. In the case of human ET-1 (Figure1.4), removal of the signal sequence on secretion of the 212 amino acid prepro ET-1 from the 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.[197] 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.[497, 530] Furin, a proprotein convertase of the constitutive secretory pathway, has been proposed as a likely candidate.[299]

Big ET-1 is several orders of magnitude less active than ET-1 for displacement of binding to endothelin receptors and also in stimulating vascular constriction.[226] Final processing of big ET-1 to release the biologically active ET-1 requires selective cleavage of the Trp^{21}-Val^{22} bond in the carboxy terminal of big ET-1, cataysed by activity referred to as endothelin-converting enzyme (ECE; Figure 1.5). Several ECE-like enzymes have been identified[415, 579]. These include serine proteases,[635] aspartic proteases[554] and cathepsin D,[486] and soluble thiol protease.[125] 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.[197]

The physiologically relevant ECE is thought to be a membrane-bound, zinc-containing metalloprotease that is inhibited by the neutral endopeptidase (NEP) inhibitor, phosphoramidon.[415] The activity of this ECE is not affected by thiorphan, another NEP inhibitor, or by inhibitors of the neutral metalloprotease ACE.

1.1.3 Characteristics of cloned endothelin-converting enzymes

Purification of rat and bovine ECE[405, 553] was rapidly followed by molecular cloning and characterisation of the enzyme from rat,[633] bovine[495] and human tissue.[495] This enzyme, ECE-1, has a neutral pH optimum and is inhibited by phosphoramidon. A second ECE, ECE-2, has also been cloned.[152] ECE-2 is also inhibited by phosphoramidon, but has an acidic pH optimum. The distribution of these enzymes also appears to be different. ECE-1 is widely distributed but not found in neural tissues which are known to produce mature endothelins.[633] ECE-2, in contrast is abundantly expressed in neural tissues.[152]

ECE-1 is an integral membrane protein composed of 754 or 758 amino acids. These two isoforms probably result from alternative splicing of the ECE gene during transcription.[641] No differences in the activity of the two isoforms have yet been discovered, with both forms converting big ET-1 in preference to big ET-2 or big ET-3, although Northern blot analysis suggests that the tissue distribution of these isoforms may be

different.[582] ECE-1 has a short N-terminal cytoplasmic tail, a hydrophobic transmembrane domain containing a zinc binding motif that is common to the catalytic domains of many metalloproteases,[197] is highly glycosylated[506] and has a number of highly conserved cysteine residues, and recent studies have suggested that ECE-1 might exist as a disulphide-linked dimer.[495, 579]

ECE-2 is also an integral membrane protein, composed of 787 amino acids, with 59% overall sequence homology with ECE-1.[152] 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. ECE-2 also preferentially converts big ET-1.[152] 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.

The major site for big ET-1 conversion still remains to be conclusively determined.[197] 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.[197] 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.[204, 209] The pH in the secretory granules of the trans-Golgi network is ~5.5, within optimum pH range for ECE-2.[152] Formation of ET-1 in the

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.[593] 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. Therefore, the intracellular location of ECE-1 still 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.[339] Circulating big ET-1 concentrations are below the K_m of ECE-1, but it seems likely that some of the big ET-1, like exogenously administered big ET-1,[29, 218, 345, 633] can be converted by plasma membrane-bound ECE-1. It has been suggested that ECE is localised in caveoli, invaginations of the plasma membrane that are abundant in endothelial and smooth muscle cells.[40, 579] This could provide a mechanism whereby secreted big ET-1 is concentrated for more efficient conversion by ECE-1. Further studies will show whether big ET-1 can be secreted directly into these caveoli for physiological ET-1 synthesis.

1.1.4 Endothelin-converting enzyme inhibitors

While phosphoramidon effectively inhibits ET-1 formation, its therapeutic potential is limited by its low potency and by its lack of selectivity for ECE.[197] IC_{50} values for inhibition of purified ECE-1 by phosphoramidon

range from 0.35 mM to 0.8 mM, several orders of magnitude higher than the IC_{50} for inhibition of NEP.[405, 507] Several strategies have been used to develop inhibitors of ECE that are more potent than phosphoramidon. However, they have not yet met with great success and the development of ECE-inhibitors as potential therapeutic agents is at least several years behind the development of endothelin receptor antagonists. Furthermore, even if selective and potent ECE-inhibitors were to be discovered, they would still have to overcome the problem of accessability should the intracellular ECE prove to be of more importance physiologically.

1.1.5 Clearance and degradation of endothelin

The plasma half-life of ET-1 in humans is less than 1.5 minutes because of its efficient extraction by the splanchnic, pulmonary and renal vascular beds.[188, 536, 614] Extraction of ET-1 follows binding to cell surface receptors, which are then internalised, allowing degradation to be carried out within the cell[16, 182] perhaps in lysosomes.[317] The observation that circulating concentrations of ET-1 are increased by a combined $ET_{A/B}$ receptor antagonist[316] or by an ET_B receptor antagonist,[178] but not by ET_A selective antagonists suggests that ET_B receptors may be involved in the clearance of ET-1. Low affinity ET_B binding sites that might serve this purpose have been found in arteries and veins.[196, 558] Soluble proteases found in endothelial and vascular smooth muscle cells have been implicated in intracellular ET-1 degradation.[124, 247] The endothelins can

also be degraded by NEP, which is associated with venous and arterial endothelial cell plasma membranes.[314]

1.2   Endothelin Receptors

Specific binding sites for endothelin are classified according to their relative affinities to the endothelin isopeptides. The ET_A is characterised by its very high (subnanomolar) affinity for ET-1 and ET-2 and its ~100-fold lower affinity for ET-3.[21] The ET_B receptor has high and equal affinity for all three isopeptides.[481] These binding site characteristics are reflected in the agonist potency of the isopeptide in isolated tissues, demonstrating that the binding sites represent functional receptors.[327, 598]

1.2.1 ET_A and ET_B receptors

Within 2 years of the initial description of endothelin,[635] the genes encoding the ET_A and ET_B receptor subtypes that mediate its actions were cloned and characterised.[21, 481] 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.[7, 22, 150, 222] The ET_A and ET_B receptor genes, located on chromosome 4[234] and 13,[22] respectively have similar structural organisation suggesting that they originated from a common ancestral gene.

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As with the endothelin genes, the nontranscribed 5' flanking regions of the endothelin receptor genes contain a number of regions involved in regulation of gene transcription. Exogenous factors can act through these regions to increase receptor transcription. For example, ET_A receptor mRNA is upregulated by insulin[172] and ET_B receptor mRNA by ANG II.[256] These mechanisms may be important in regulation of responsiveness to the endothelins in pathophysiological states. ET_B receptor mRNA is selectively increased in marmosets fed a high cholesterol diet[150] and following glycerol-induced acute renal failure in rats.[469] In contrast, endothelin receptor expression is reduced in atherosclerotic human arteries[625] and in the lungs of rats with pulmonary hypertension.[640] One of the major factors that reduces endothelin receptor number is prolonged exposure to ET-1 itself, because of down-regulation or feedback inhibition of receptor expression,[227] 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.[480]

All the cloned endothelin receptor genes predict a heptahelical membrane spanning structure, common to members of the G-protein-coupled receptor superfamily and similar to many neuroreceptors.[70] The transcribed region 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.[150, 233, 380, 400] Phosphorylation may play a role in the down-regulation of endothelin receptors that follows prolonged exposure to the endothelin isopetides.[227, 348, 467]

1.2.2 Pharmacological evidence suggesting the existence of additional receptors

Based on studies of cultured rat anterior pituitary cells,[334] rat PC12 phaeochromocytoma cells,[483] and cultured human endothelial cells,[638] the existence of a receptor specific for ET-3 has been suggested. Indeed, a putative ET_C receptor subtype, relatively selective for ET-3, has been cloned from *Xenopus* dermal melanophores.[260] However, Southern blot analysis of human DNA revealed the existence of only two endothelin receptor genes, probably corresponding to the ET_A and ET_B genes.[477] Indeed, an analysis of human endothelin genes revealed eight and seven exons for ET_A and ET_B genes, respectively, and suggested only one product from each gene.[22, 234] Additional mammalian endothelin receptors, if they exist, must be markedly different from both ET_A and ET_B receptors.

1.2.3 Agonists at endothelin receptors

All the endothelin and sarafotoxin 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, Figure 1.3). Examination of the binding characteristics of these peptides reveals that the ET_A receptor has much more rigid structural requiremnts for ligand binding than the ET_B receptor.[156, 236, 523] 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 carboxy terminal and the Trp^{21} are essential for high affinity binding to the ET_B receptor.[268] ET-3 and sarafotoxin S6c can be considered ET_B selective ligands, ET-3 having ~2000-fold and sarafotoxin S6c ~30 000-fold selectivity for binding to ET_B rather than the ET_A receptor.[623] 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.

1.2.4 Endothelin receptor antagonists

Since the first description of compounds that could inhibit the binding or actions of ET-1 in 1991,[238, 526] a large number of endothelin receptor antagonists, peptide and nonpeptide, selective and nonselective, have become available (Table 1.2).[198, 320, 597]

Peptide antagonists have been obtained by chemical modification of ET-1 itself, or of microbial products with endothelin receptor binding activity.[238, 526] BQ-123 is a cyclic pentapeptide derived from microbial broth that has relatively high potency for binding to the ET_A receptor.[239] Although several ET_A antagonists are now available, studies using BQ-123 first confirmed the role of endothelin in a number of pathologies.[368] BQ-788,[243] is a peptide compound that is more selective for inhibition of ET-1 binding to the ET_B receptor. The first nonselective endothelin receptor antagonists to be described were also peptides.[101, 296] TAK-044, is a cyclic hexapeptide with ~20-fold higher affinity at the ET_A compared with the ET_B receptor.[267] This compound, unlike many of the other peptide antagonists, has a relatively long duration of action following intravenous administration *in vivo*.[241] 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.

Potent nonpeptide antagonists have been developed through optimisation of compounds isolated from plant extracts,[173, 349] and microbial broths[403] or screened from chemical libraries.[92, 93, 372, 404, 529] SB 209670, a nonselective antagonist, is amongst the most potent of these and inhibits the actions of ET-1 whether administerd intravenously or orally.[140, 404]

1.2.5 Distribution and function of endothelin receptors in the cardiovascular system

In vascular tissue, ET_A receptor mRNA is expressed predominantly in smooth muscle,[21, 232, 636] while ET_B receptor mRNA is most abundant in endothelial cells.[233, 360, 400, 625] 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 (Figure 1.6). However, ET_B receptor mRNA is detectable in vascular smooth muscle cells[43, 118, 328, 625] and ET_B selective agonists can evoke constriction *in vitro*[369, 503, 540] and pressor responses *in vivo*.[58, 94, 623] These observations suggest the presence of ET_B receptors that mediate constriction of vascular smooth muscle cells (Figure 1.6).

The relative contributions of ET_A and ET_B receptors to vasoconstriction is variable and depends on species and the vessel type studied.[116] ET_B receptors are generally more important in the low pressure venous circulation.[368] In isolated human blood vessels, it is the ET_A receptor subtype that primarily mediates constriction in large calibre arteries,[116] but recent studies show that the relative functional role of ET_B receptors is greater in small calibre arteries.[127, 556, 577] The balance of receptors may 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 synthetic phenotype,[147] in hypertension[43] and under the influence of ANG II.[256]

1.2.6 Distribution and function of endothelin peptides and receptors in the kidney

The kidney is an important organ for endothelin synthesis, and ET-1 is produced by endothelial,[333] mesangial,[32, 237, 278, 280, 478] glomerular epithelial[261, 407] and medullary collecting duct cells.[272-275, 279] Although studies in rats and cell cultures also demonstrated ET-3 production in mesangial cells and glomerular epithelial cells,[274] no ET-3 production in the human kidney has been demonstrated.[257, 259]

Mesangial endothelin synthesis is regulated by factors and mechanisms similar to endothelial cells[276, 277, 511] and is stimulated by various humoral factors involved in glomerular injury, such as thrombin, transforming growth factor ß (TGF- ß) and ANG II.[479] In general, mesangial cell endothelin production is increased by activation of PKC and reduced by activation of protein kinase A or cGMP.[276] Interestingly, endothelin can autoinduce its own production by stimulation of ET_B receptors on mesangial cells.[246] The regulation of endothelin synthesis by medullary cells is still largely unknown, although osmolarity and water balance are thought to play a significant role.[279, 500]

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The human kidney, as in the dog, is rich in ET_B receptors and these predominate at least two-fold over ET_A receptors with the ET_A receptor being mainly confined to the vasculature.[68, 258, 383] However, the ET_A receptor predominates in the rat kidney with renal vasoconstriction predominantly mediated by ET_B receptors.[121, 616] As a consequence of the inter-species variations in renal endothelin receptor function, caution should be taken when extrapolating the results from animal experiments to humans.

Endothelin may act both in an endocrine (endothelin derived from the systemic circulation) as well as in an autocrine/paracrine manner (endothelin derived from the kidney itself). Infusions of ET-1 may mimic the former mode of action, while the autocrine/paracrine actions can only be evaluated by studying tissue expression of endothelin and the effects of intrarenal endothelin receptor antagonists.

Administration of low dosages of ET-1 in humans that increase plasma concentrations two-fold produce sustained renal vasoconstriction.[442, 524, 614] From *in vitro* studies, it appears that the reduction in renal blood flow is caused by constriction of the glomerular afferent and efferent arterioles, and the arcuate and interlobar arteries.[30, 145, 269, 288] In addition, ET-1 can reduce ultrafiltration coefficient by mesangial contraction.[31]

Administration of ET-1 in humans also consistently induces sodium retention[59, 252, 442, 524] even at very low doses of ET-1 that do not affect

renal blood flow, GFR or the renin-angiotensin system.[442] In contrast, studies in the isolated perfused rat kidney have consistently shown a natriuretic effect.[160, 433] Similarly, several rat studies report a natriuretic effect of low dose systemic administration of ET-1, despite a fall in GFR and renal blood flow.[208, 432] This natriuresis may be due to stimulation of atrial natriuretic peptide (ANP)[373] or pressure natriuresis secondary to a higher blood pressure during endothelin infusion.[269] These findings suggest that the actions of endothelin on sodium excretion depend on a balance between sodium retaining and natriuretic factors. In humans, it appears that the predominate effect of small increases in plasma endothelin concentration is sodium retention.

The physiological importance of possible autocrine actions of endothelin in the kidney are still unclear. Studies using intrarenal administration of endothelin receptor antagonists have shown no significant effect on renal blood flow and sodium excretion[119, 437, 559] under basal conditions while one study demonstrated an increase in renal blood flow and sodium excretion.[67] As yet, there have been no studies of the actions of endothelin receptor antagonism on renal sodium handling in humans.

1.3 Endothelin in the Pathophysiology of Cardiovascular and Renal Disease and the Clinical Potential of Endothelin Receptor Antagonists

There are many endothelin receptor antagonists either in, or shortly to be entering, clinical development (Table 1.2). 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 cardiovascular 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[217, 577] there may be some advantages from blocking both of these receptors. However, there may be some benefits from leaving the endothelial ET_B receptor, which is known to mediate vasodilatation, unaffected. Indeed, there is some evidence that the endothelial and smooth muscle ET_B receptors can be distinguished pharmacologically[595] 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[219, 471]. This section specifically examines the evidence implicating ET-1 in the pathophysiology of cardiovascular disease and discusses the potential of endothelin receptor antagonists in cardiovascular medicine.

1.3.1 Hypertension

Over the last 20 years, it has become well established that the vascular endothelium plays a fundamental role in the regulation of cardiovascular function.[588] Endothelial cells produce both vasodilating substances such as prostacyclin,[361, 363] NO,[421, 422] C-type natriuretic peptide (CNP)[284, 285] and endothelium-derived hyperpolarising factor[589] as well as vasoconstricting substances such as thromboxane A_2[363] and endothelin.[635] Hypertension is characterised by increased peripheral vascular resistance[430] and may, therefore, be associated with a generalised dysfunction of either an endothelium-dependent vasodilator[17, 362, 583] or vasoconstrictor system[635] or both. Both NO[216, 448, 449, 584] and endothelin[218] have been shown to contribute to the maintenance of basal vascular tone and have, so far, been the major focus of research activity into endothelial dysfunction in hypertension. In this section I will consider the evidence supporting a generalised dysfunction of the endothelium in hypertension and why modulation of endothelial function may be an attractive therapeutic concept in the treatment of hypertension.

NO is synthesised from L-arginine by a family of nitric oxide synthases (NOS).[421] The isoform present in endothelial cells is calcium-dependent,[297] constitutively expressed[297] and is modulated by several stimuli including shear stress[69, 501] and hormones,[612] as well as by receptor activation by agonists such as acetylcholine (ACh), bradykinin and substance P (Figure 1.7).[494] Basally produced NO is important in the

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regulation of vascular tone[584] and blood pressure[215, 448, 449] as well as in inhibition of platelet aggregation.[443] NO also inhibits mitogenesis and proliferation of vascular smooth muscle cells (Figure 1.8).[187]

It has been proposed that a deficiency of endogenous NO would lead to impaired vasodilatation and a rise in peripheral vascular resistance leading to hypertension.[17, 362, 585] The antiplatelet[443] and antiproliferative actions[187] of NO may also be connected with the consequences of sustained hypertension such as vascular hypertrophy, atherosclerosis, stroke and myocardial infarction. The evidence for this hypothesis is based on experiments using animal models of hypertension as well as in studies in patients with essential hypertension.

Several studies using both large conduit and resistance arteries taken from different animal models of hypertension have demonstrated impaired endothelium-dependent dilatation to ACh, but not to endothelium-independent NO donors such as sodium nitroprusside (SNP) or glyceryl trinitrate, *in vitro*.[79, 131-133, 235, 315, 321, 322, 508, 626, 632, 645] Chronic administration of inhibitors of NO synthesis, such as L-monomethylarginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME), produce a hypertensive response in both normotensive animals[23, 448, 449] and humans.[215] Conversely, treatment with L-arginine prevents the development of hypertension in salt-sensitive Dahl rats and spontaneously hypertensive rats.[86] Studies in patients with essential hypertension have demonstrated a blunted decrease in forearm

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blood flow induced by L-NMMA compared with normotensive controls.[73, 323] Furthermore, several studies have also demonstrated impaired endothelium-dependent vasodilatation to ACh in the forearm circulation of patients with essential[228, 312, 423-428, 546-550] and secondary hypertension.[550] Paradoxical vasoconstrictor responses to ACh are enhanced in epicardial coronary arteries and vasodialatation to ACh is impaired in the coronary microcirculation (especially if left ventricular hypertrophy is present).[146, 573, 574, 648] It has been suggested that the response to NO in hypertensive animals and patients may be masked by the concomitant release of vasoconstrictor prostaglandins as an apparently impaired relaxation to ACh is normalised by cotreatment with inhibitors of cyclooxygenase.[130, 547] However, different mechanisms may be simultaneously involved since L-NMMA does not inhibit the vasodilatation to ACh in hypertensive patients suggesting a defect in the L-arginine-NO pathway.[425]

This evidence appears to present a strong case for a generalised defect in endothelial NO production. However, not all *in vitro* studies using vessels taken from hypertensive animals[18, 19, 575] and humans[19] have demonstrated impaired relaxation to ACh. Also, not all studies in have demonstrated impaired vasodilatation to ACh in the forearm circulation of hypertensive patients.[78, 96] Some of these differences may be in part explained by the use of different vessels taken from different animal models of hypertension and the selection of hypertensive patient populations. Nevertheless, several potential problems with the use of ACh

as a tool for investigating endothelial function have been highlighted and caution must be taken when interpreting the results of studies using ACh as an endothelium-dependent agonist.[19] However, the existence of a dysfunctional endothelium in hypertension is further supported by the demonstration of impaired relaxation to other endothelium-dependent agonists, such as substance P and bradykinin, in both hypertensive animals[235] and humans.[146, 263, 424]

Recent work has shown that the activity of NO synthase is increased in mesenteric arteries taken from spontaneously hypertensive rats (SHR) compared with normotensive controls[384] and the plasma concentration of nitrate, the oxidative product from of NO, is higher in stroke-prone SHR than in normotensive rats,[385] suggesting that there is no deficiency of NO synthesis in this animal model of hypertension. However, the capacity of the vascular smooth muscle to respond to NO is not impaired as shown by the normal response to organic nitrates.[130] These findings suggest that there must be some factor preventing endothelium-derived NO from eliciting normal vasodilator responses in this animal model. Oxidative stress has been implicated in the pathophysiology of some cardiovascular conditions[381, 402, 560] and interestingly, it has recently been demonstrated that the release of NO from isolated resistance vessels is improved in the stroke-prone SHR in the presence of superoxide dismutase.[576] Thus an increase in the production of oxidative radicals, such as superoxide, or a fall in the superoxide dismutase activity may cause an increase in the degradation of NO. These findings suggest the exciting possibility that

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endothelial dysfunction in hypertension may be reversed by administration of antioxidatants, raising the prospect of novel therapeutic approaches to the treatment of essential hypertension. Indeed, antioxidants have already been found to reverse endothelial dysfunction in hypercholesterolaemic patients[192] as well as lowering the incidence of cardiovascular events and mortality in patients with ischaemic coronary artery disease.[532]

Antihypertensive drugs which have a beneficial effect on endothelial function by direct action on the L-arginine-NO system, or indeed antihypertensive agents with antioxidant actions, may have additional benefits in the treatment of hypertension. Such agents could be more effective in further reducing the vascular consequences of high blood pressure, such as atherosclerosis and smooth muscle hypertrophy, and could potentially further reduce the incidence of associated cardiovascular events, such as stroke and myocardial infarction.

In their original paper, Yanagisawa and colleagues[635] 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[244] and mitogenic[512] properties, has an antinatriuretic action,[442, 524] increases central[325, 390] and peripheral sympathetic activity,[545, 629] and stimulates generation of renin, ANG II,[444] aldosterone[110] and adrenaline.[63] Furthermore, ET-1 potentiates, at threshold and subthreshold concentrations, contractile responses to other vasoconstrictor substances such as noradrenaline and

serotonin.[637] 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,[294] ET-3,[44] ET_A[90] and ET_B[44] knockouts all cause severe developmental disturbances, which shows the importance of the endothelins in growth and development, but is not helpful concerning their physiological effects in cardiovascular regulation.

Plasma endothelin concentrations are not raised in animal models of hypertension unless malignant hypertension or renal dysfunction are present.[282] However, because ET-1 is preferentially secreted abluminally[593, 643] locally increased production may not necessarily result in raised plasma endothelin concentrations. Indeed, increased immunoreactive endothelin concentrations have been reported in both aortic and mesenteric arteries of DOCA-salt hypertensive rats, despite normal plasma endothelin concentrations, suggesting that increased vascular generation of ET-1 may be involved in some forms of hypertension.[301]

In vitro studies using resistance arteries taken from animal models of hypertension have shown either increased[91] or decreased[132] vascular sensitivity to ET-1. Systemic doses of ET-1 *in vivo* have greater pressor effects in SHR than in Wistar-Kyoto rats[356] and in renovascular hypertensive than normotensive rabbits[459]. The interpretation of these

results is difficult given the propensity for vascular hypertrophy to non-specifically enhance responses to vasoconstrictors.

Endothelin-specific antibodies[406] and the selective ET_A antagonists BQ-123[138] and FR139317[174] have been shown to lower blood pressure in animal models of hypertension. Chronic administration of BQ-123 has been reported to prevent the development of stroke and renal abnormalities in stroke-prone SHR[389]. Non-peptide combined ET_A/ET_B receptor antagonists SB 209670[404] and Ro 46-2005[92] lower blood pressure in SHR and conscious normotensive salt depleted monkeys respectively. Although some studies 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 concentrations are not raised in patients with essential hypertension and normal renal function,[114, 218] although very high concentrations are found in severe and malignant hypertension[642] probably as a result of impaired renal clearance. Increased plasma endothelin has been reported in the presence of normal renal function in patients with pre-eclampsia.[167] Plasma endothelin was also increased in two patients who developed hypertension with the skin tumour haemangio-endothelioma.[639] Tumour cells showed increased expression of ET-1

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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.

Sensitivity to ET-1 *in vitro* appears to be decreased in resistance arteries taken from hypertensive patients[490] but increased in the capacitance vessels of hypertensive patients *in vivo*.[213] 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 post-receptor level.

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 (Figure 1.8). Indeed, plasma endothelin concentrations are raised in advanced atherosclerosis[308], and expression of ET-1 mRNA is increased in the vascular smooth muscle of atherosclerotic human arteries.[625] Furthermore, increased tissue endothelin immunoreactivity has been reported in the active atherosclerotic lesions associated with unstable angina.[649] 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.[106] In a recent multicentre study, bosentan, a combined ET_A/ET_B receptor antagonist, has been reported to lower blood pressure in patients with essential hypertension.[290] Further longer term studies with outcome data are now eagerly awaited.

1.3.2 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.[555] Exogenously administered ET-1 also produces myocardial ischaemia[293] by causing coronary vasoconstriction. Plasma endothelin concentrations[590] and myocardial ET-1 binding sites[313] are increased during reperfusion following ischaemia in animals. Interestingly, in animal models of myocardial infarction, ECE inhibitors[203], monoclonal antibodies against ET-1[601], selective ET_A antagonists[202] and combined ET_A/ET_B antagonists[600] have been reported to reduce myocardial infarct size.

In humans, plasma endothelin is raised in acute myocardial infarction and unstable angina,[358] suggesting a possible pathophysiological role for ET-

1, whereas patients with stable angina do not have raised plasma endothelin.[446] The higher the plasma endothelin in myocardial infarction[413] and unstable angina[619] the worse the prognosis. Plasma endothelin concentrations on the third day after myocardial infarction significantly related to mortality,[413] and plasma endothelin concentrations at 9 weeks after hospitalisation with unstable angina or non Q-wave myocardial infarction significantly related to the incidence of further cardiovascular events.[619] Patients undergoing fibrinolysis during the acute phases of myocardial infarction have been shown to have reduced plasma endothelin compared with patients who did not have early reperfusion.[302] As previously discussed, the increased tissue endothelin immunoreactivity in active atherosclerotic plaques causing unstable angina[649] 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. Second, they may reduce the incidence of further ischaemic events or the need for revascularisation. Third, they may prevent remodelling after infarction, possibly 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, companies may be wary of developing endothelin receptor antagonists in this indication.

Coronary sinus ET-1 levels are raised during and immediately after percutaneous transluminal coronary angiography (PTCA)[551]. 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 later risk of restenosis. Clinical restenosis occurs in up to 30% of patients within the first year following the procedure[347] 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.

1.3.3 Varaint angina

Prinzmetal's or variant angina, first described in 1959,[440] 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 fixed stenotic lesions.[129]

Patients with variant angina are known to have endothelial dysfunction affecting the L-arginine-NO system[441] and, as a powerful vasoconstrictor

of human[637] and canine[293] coronary arteries, ET-1 has been implicated in the pathophysiology of this condition.[319] ET-1 also potentiates the coronary vasoconstriction induced by serotonin and noradrenaline in isolated human arteries.[637] Patients with variant angina have elevated plasma endothelin concentrations during provocation of coronary vasospasm[341] and one study has also found basal plasma concentrations of endothelin to be elevated.[24] Interestingly, there is an increased prevalence of primary Raynaud's disease and migraine in patients with variant angina[351, 409] 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.

1.3.4 Heart Failure

Many definitions of congestive heart failure (CHF) exist but highlight only selected features of this complex syndrome.[563] Patients with CHF are typically breathless or fatigued, either at rest or during exertion, develop ankle swelling and have objective evidence of major cardiac dysfunction at rest.[563] Neuroendocrine activation occurs in patients with CHF[171, 563] and elevation of plasma concentrations of noradrenaline, renin, ANG II and aldosterone are related to the severity and prognosis of heart failure.[543]

However, in individual patients these predictors are inaccurate and difficult to interpret because diuretics, vasodilator agents and ACE inhibitors alter plasma concentrations in a complex fashion.[563, 587] Indeed, plasma renin activity is not raised in patients with left ventricular dysfunction without CHF not treated with diuretics.[171] Furthermore, plasma renin activity is stimulated in patients with CHF treated with diuretics despite symptomatic improvement, decreased sympathetic activity and decreased plasma natriuretic peptide concentrations.[587]

Intravenous ET-1 raises blood pressure, increases peripheral and coronary vascular resistance, and decreases cardiac output,[352] despite having positive inotropic[166] and chronotropic[244] actions. ET-1 also reduces renal blood flow and GFR, in association with a reduction in sodium excretion, an increase in plasma renin-aldosterone activity, and increased plasma concentrations of ANP, vasopressin and aldosterone.[352] ET-1 may, therefore, be involved in the pathophysiology of CHF as part of the neurohumoral response to cardiac failure[535] and its actions would certainly contribute to the vicious circle of haemodynamic decline associated with this condition.

Plasma endothelin concentrations are raised in animal models of CHF[80] and in patients with CHF.[346] In patients, the increase in plasma endothelin concentration correlates closely with the degree of haemodynamic and functional impairment,[420, 611] with higher concentrations predicting a greater likelihood of death or need for cardiac

transplantation.[420] Although impaired renal function and decreased clearance of ET-1 may be involved in generating high plasma endothelin concentrations, raised plasma concentrations of big ET-1[420, 611] suggest that increased production may also contribute. Also, ET-1 release by cultured cells is increased in the presence of ANG II and vasopressin, plasma concentrations of which are elevated in CHF.[151]

The ET_A receptor antagonist, BQ-123, and the ECE inhibitor, phosphoramidon, cause arterial 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.[318] In comparison to healthy subjects, vasodilatation to BQ-123 tended to be reduced, and to phosphoramidon increased in patients with CHF, consistent with upregulation of ET_B mediated vasoconstriction. More recent studies with ET-1 and the ET_B specific agonist, sarafotoxin S6c, are also consistent with this hypothesis[318]. ANG II concentrations are increased in CHF and, interestingly, ANG II has been shown to down-regulate total endothelin binding sites[466] but up-regulate ET_B receptor mRNA[256]. Given that ET_B receptors can mediate vasoconstriction,[217] it may be that smooth muscle ET_B receptors have greater functional significance in CHF. Encouragingly, recent studies have shown that administration of systemic doses of the combined ET_A/ET_B antagonist, bosentan, to patients with severe chronic heart failure produces sustained systemic, pulmonary and peripheral venous vasodilatation, and improved cardiac performance, without causing reflex tachycardia.[271] These haemodynamic effects show

a similar pattern to those seen with ACE inhibitors and so justify the further clinical 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[102, 562] and, therefore, the results of longer term clinical studies of endothelin receptor antagonists in CHF patients must be awaited with interest.

1.3.5 Primary pulmonary hypertension

Primary pulmonary hypertension (PPH) is a rare condition of unknown aetiology. It mainly affects young people, causes progressive shortness of breath, and most of those affected are dead within 4 years of diagnosis.[72, 484] The condition is characterised by endothelial injury and by proliferation of pulmonary arterial smooth muscle.[72] Although smooth muscle proliferation may be the major cause of luminal narrowing in this condition,[387] a role for vasoconstriction has also been proposed.[630] Indeed, some patients do have favourable haemodynamic and symptomatic responses to vasodilators such as isoproterenol, hydralazine and nifedipine.[484]

Depending on the state of vasomotor tone, endothelin isopeptides can cause either pulmonary vasoconstriction or vasodilatation.[41] Under basal conditions ET-1, -2 and -3 contract pulmonary vascular rings[570, 628] and increase pulmonary vascular resistance in the perfused vascular beds of several species.[231, 570] However, when vascular tone is already high, as

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in the foetus, or has been raised pharmacologically, these peptides induce a dose-related pulmonary vasodilatation.[570, 628] The constrictor response is mediated by the ET_A receptor, whereas the dilator response is ET_B mediated.[41] ET-1 also stimulates DNA synthesis and proliferation of pulmonary artery smooth muscle cells.[249] In fawn-hooded rats (FHR), a strain which spontaneously develops pulmonary hypertension, plasma endothelin concentrations are not elevated.[531] However, tissue endothelin concentration are two-fold higher and preproET-1 mRNA expression is three-fold higher in whole lung homogenates from FHR compared with controls. Interestingly, preproET-1 mRNA expression is increased in FHR even before the development of pulmonary hypertension. Furthermore, neither endothelin nor preproendothelin mRNA concentrations are raised in adult control rats with an equivalent amount of pulmonary hypertension due to chronic hypoxia.[531] These findings suggest that raised endothelin production may be directly involved in the pathogenesis of PPH and not just a secondary phenomenon.

ET-1 produces concentration-dependent vasoconstriction under basal conditions of human pulmonary arteries[210] and stimulates the proliferation of pulmonary artery smooth muscle cells.[646] This proliferation appears to be mediated by the ET_A receptor, because it is inhibited by the selective ET_A receptor antagonist, BQ-123.[646] Expression of ET-1 mRNA is increased in vascular endothelial cells of patients with PPH, with a strong positive correlation between the intensity of ET-1-like immunoreactivity and pulmonary vascular resistance.[191]

Unlike the FHR, patients with PPH, and those with pulmonary hypertension due to other causes, have increased circulating endothelin concentrations which might reflect changes in production or clearance of endothelin by the lung.[191] However, only patients with PPH have high arterial, compared with venous concentrations, suggesting that only these patients have increased pulmonary production of endothelin.[191] Therefore, increased endothelin synthesis may contribute to the elevated pulmonary vascular resistance observed in this condition.

Direct assessment of the functional importance of endothelin in PPH awaits studies with endothelin receptor antagonists or ECE inhibitors. These agents could, potentially, reduce the progression of both structural and functional narrowing of pulmonary arteries. Given that ET-1 can cause ET_B receptor mediated vasodilatation in conditions of raised pulmonary vascular tone it may be that selective ET_A antagonists will prove more useful than combined $ET_{A/B}$ antagonists in this condition.

1.3.6 Raynaud's disease

Raynaud's disease[447] is a common condition in colder climates. It is characterised by the development of episodic ischaemia, usually in exposed extremities and associated with exposure to cold, hormones, drugs and emotional stimuli.[108] Although many treatments are available for patients with Raynaud's disease, none is effective in the majority of subjects.[141, 627] Currently, the pathophysiology of Raynaud's disease remains

unresolved. However, there is an association between Raynaud's disease and other vasospastic conditions, including migraine and variant angina,[351, 409] suggesting that there may be a vascular defect common to these conditions. In the rabbit ear artery, responses to ET-1 are temperature dependent and, under physiological conditions, the vasoconstriction to ET-1 is attenuated by an increase in endothelial NO production during cooling.[364] A defect in endothelium-dependent dilatation in the veins of patients with Raynaud's disease has been reported,[46] suggesting that the defect is widely expressed in blood vessels. 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 endothelin increases rapidly during the cold-pressor test in healthy subjects, peaking at four minutes after immersion of the arm in cold water.[180] Cold provocation tests in patients with Raynaud's disease have shown an exaggerated increase in endothelin concentrations in venous blood draining from the cold-challenged arm, compared with both the control arm and with responses in healthy control subjects.[647] Elevated basal plasma endothelin concentrations have been reported in Raynaud's patients between vasospastic episodes consistent with increased production.[647] During basal conditions, resistance arteries taken from patients with Raynaud's do not appear to have abnormal sensitivity to ET-1.[521] This suggests that any abnormality in the endothelin system in Raynaud's disease is a result of

increased ET-1 production or increased sensitivity to ET-1 during a vasospastic event.

These results, taken as a whole, appear to support a role for ET-1 in the pathophysiology of Raynaud's disease. However, there have so far been no confirmatory studies using endothelin antagonists. Endothelin antagonists would need to be more effective or better tolerated than the current drugs of choice, the calcium channel blockers nifedipine and diltiazem,[141] if they are to achieve widespread use in the treatment of Raynaud's disease.

1.3.6 Subarachnoid haemorrhage

The development of delayed cerebral vasospasm within the first two weeks after subarachnoid haemorrhage (SAH) is responsible for much of the morbidity and mortality associated with this condition.[25, 499] ET-1 has been implicated in mediating SAH-induced vasospasm. ET-1 is a potent vasoconstrictor of isolated cerebral arteries[26] and its effects are potentiated after SAH.[11] Plasma endothelin levels are raised in a canine model of SAH[634] and injection of exogenous ET-1 into the cerebrospinal fluid (CSF) of dogs reproduces the vasospasm that occurs following SAH.[26] Furthermore, both thrombin and oxyhaemoglobin, which are present in high concentrations in SAH, are known to induce ET-1 release.[344, 493, 635]

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Inhibition of the enzymatic conversion of big ET-1 to ET-1 by phosphoramidon reduces the vasospasm seen in a dog model of SAH,[340] as does administration of monoclonal antibodies to ET-1[634]. Selective ET_A antagonists BQ-123,[92] BQ-485[245] and FR139317[388] have also been shown to reduce vasospasm in animal models of SAH. The mixed ET_A and ET_B antagonist, Ro 46-2005, markedly reduces the cerebral vasoconstriction seen in a rat model of SAH without lowering systemic blood pressure.[92] This finding is of great significance because the major potential complication of nimodipine, the currently preferred drug treatment for SAH,[137] and for any other vasodilator used in the treatment of SAH, is hypotension sufficient to compromise cerebral perfusion.

Plasma and CSF endothelin concentrations are significantly raised in patients after SAH[336, 499] and the plasma levels of endothelin are highest in those patients who develop vasospasm.[542] Thus, it appears that ET-1 may be implicated in the pathophysiology of delayed vasospasm following SAH in humans. Endothelin antagonists may have great potential in the management of SAH and clinical studies with these agents must be awaited with great interest.

1.3.7 Ischaemic stroke

The evidence of a pathophysiological role for ET-1 in ischaemic stroke is much more limited than in SAH. Increased plasma and CSF concentrations of endothelin are seen in animal models of stroke.[42, 622] Although there

have been few studies with endothelin receptor antagonists, both the selective ET_A and the combined ET_A/ET_B receptor antagonists, BQ-123[162] and SB 209670,[404] have been shown to protect against ischaemia-induced neuronal degeneration in a gerbil stroke model. Plasma endothelin is also raised in humans after ischaemic stroke,[652] suggesting a possible pathophysiological 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.

1.3.8 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.[223] The prevalence of migraine ranges widely, depending on the diagnostic criteria used. However, using the International Headache Society Classification,[223] the 1-year-period prevalence of migraine is ~10%.[445] 5-Hydroxytryptamine (5-HT) appears to play an important role in the pathophysiology of migraine[170] and 5-HT agonists have proved of major therapeutic value in its treatment.[193, 510, 615]

Studies have shown local cerebral hypoperfusion during migraine attacks, especially those associated with an aura.[300, 392, 631] 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 has been found to be elevated during migraine headaches.[157, 181] Plasma endothelin has not been found to be raised between attacks, or in patients with episodic or chronic tension headaches[181], 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[652] or changes in vascular shear stress[353] and hence be a secondary phenomenon.

It has been proposed that dural blood vessels play a central role in headache pathogenesis.[431] Indeed, both the ergot alkaloids[474] and sumatriptan[71] block peripheral small fibre-dependent neurogenic inflammation within the dura mater in a rat model. Endothelin receptor antagonists have also been shown to block neurogenic inflammation,[65] providing additional support for the view that these agents may be useful in the treatment of migraine headaches. However, a small trial with the combined $ET_{A/B}$ receptor antagonist, bosentan, has failed to show any improvement in migraine symptoms[343] and enthusiasm for the clinical development of endothelin receptor antagonists in this condition appears to have waned.

1.4.1 Acute 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.[522, 580] The renal vasculature is very sensitive to the actions of endothelin. When infused at doses that have little vasoconstrictive effect, ET-1 inhibits sodium reabsorption by its actions on the ET_A receptor in the rat,[189] and by its actions on the ET_B receptor in the dog[67]. A possible autocrine role for endothelin in the regulation of body volume status and water reabsorption has been proposed.[279] At higher doses, exogenous ET-1 causes potent and long lasting vasoconstriction similar to that seen in ARF,[164, 504] together with a reduction in renal blood flow, GFR and urine production.[30, 262] In the rat, the renal vasoconstriction to ET-1 is mediated by the ET_B receptor,[189] whereas in the dog, renal vasoconstriction appears to be mediated by the ET_A receptor.[67]

As a consequence of the inter-species variations in renal endothelin receptor function, caution should be taken when extrapolating the results from animal experiments to humans. The human kidney, as in the dog, is rich in ET_B receptors and these predominate at least two-fold over ET_A receptors, which are limited mainly to the vasculature.[258] On this basis, the dog may be a better model of human ARF than the rat.

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Renal ischaemia and reperfusion increases immunoreactive ET-1 binding affinity,[95] ET_A and ET_B receptor numbers on the renal vasculature,[382, 470] ET-1 mRNA expression[165] and plasma and urine immunoreactive ET-1 in rats.[504] In the rat, anti-endothelin monoclonal antibodies prevent renal vasoconstriction following ischaemia induced ARF.[504] This has been confirmed with the selective ET_A antagonist, BQ-123[83, 189, 354] and the combined ET_A/ET_B antagonists Ro 46-2005[92] and TAK-044.[295] In the dog, BQ-123 has no effect on the reduction in GFR produced by ischaemia.[67, 537] Although renal vasoconstriction in the dog is mainly ET_A receptor mediated[258], the combined ET_A/ET_B antagonist, SB 209670, does attenuate this reduction in GFR.[67] 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.[67]

In humans, plasma immunoreactive ET-1 concentrations are significantly elevated in ARF.[571] There is currently no effective drug therapy for ARF[580] 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[168, 287] and X-ray contrast media.[331] 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.

1.4.2 Chronic renal failure

Under physiological conditions, the actions of endothelin on the glomerular microcirculation may help to maintain perfusion pressure. However, the sustained reductions of GFR and renal blood flow observed during administration of ET-1 in humans[442, 524] may endanger oxygen supply within the kidney.[410] Such vascular effects may contribute to renal injury in chronic renal failure (CRF), a condition characterised by markedly raised plasma endothelin concentrations in both animal models[376, 644] and humans.[128, 289, 329, 439, 462, 475, 505, 538, 599] The mechanism by which plasma endothelin concentrations are raised is still unclear with both increased production and decreased clearance of endothelin having been implicated. Furthermore, it has been suggested that these high endothelin concentrations may contribute to the increased vascular tone and the high incidence of cardiovascular mortality in this condition.[123, 607]

In addition to impairing renal haemodynamics, endothelin may also cause renal injury by stimulating excessive cell proliferation and/or extracellular matrix formation. There is accumulating evidence that endothelin is involved in the pathogenesis of proliferative glomerulonephritis. ET-1 is a potent mitogen and partly mediates the proliferative effects of several cytokines,[33, 281, 391] as well as activating and being chemotactic for monocytes which can in turn secrete ET-1.[6, 335] Furthermore, endothelin receptor antagonists reduce mesangial cell proliferation in experimental mesangial proliferative glomerulonephritis[175] and decrease renal injury in murine lupus nephritis.[378] In addition, renal ET-1 mRNA levels are also elevated in kidneys from mice with an autosomal recessive form of polycystic kidney disease.[375]

There are also several lines of evidence suggesting that endothelin may contribute to the excessive accumulation of extracellular matrix components and fibrosis. Endothelin can increase renal cell fibronectin and collagen production, tissue inhibitor of metalloproteinases levels, and release of cytokines that stimulate matrix accumulation.[414, 472] Glomerular ET-1 and endothelin receptor mRNA levels correlate with the degree of glomerular sclerosis in rats with puromycin nephrosis.[379] Endothelin receptor antagonists have also been shown to decrease matrix accumulation in experimental models of nephritis.[175, 379] Several studies have noted increased renal endothelin production in streptozocin diabetic rats.[176, 177, 366] Furthermore, chronic treatment with an endothelin receptor antagonist attenuates increases in glomerular mRNA levels of

collagen, laminin, tumour necrosis factor-α, TGF-β, platelet-derived growth factor, and basic fibroblast growth factor in diabetic rats.[377] However, endothelin receptor blockade does not appear to affect the degree of fibrosis in chronic cyclosporin nephrotoxicity.[286] It would therefore appear that the extent of involvement of endothelin in matrix accumulation and fibrosis depends on the underlying disease process.

Once substantial renal scarring exists, there is typically an inevitable progression towards end-stage kidney disease.[309, 450] This process involves gradual glomerular sclerosis and interstitial fibrosis, and occurs regardless of the initial renal insult. There is now evidence that endothelin contributes to this progressive renal deterioration. Most studies, but not all,[572] have reported increased renal endothelin production in experimental animals or humans with reduced renal mass.[418, 552, 557] Furthermore, ET_A receptor blockade has been shown to reduce glomerular injury in progressive renal failure in rats.[49] However, interpretation of this study is confounded by the blood pressure lowering effect of endothelin antagonism, an effect that has been shown to reduce renal fibrosis in severely hypertensive rats.[408] Hence, endothelin may contribute directly or indirectly (through blood pressure elevation) to fibrosis when renal function is severely and irreversibly compromised.

There are now sufficient theoretical reasons as well as accumulating experimental animal evidence to warrant the further evaluation of endothelin receptor antagonists in the treatment of chronic renal failure.

However, ACE inhibition has been shown in several studies, in both diabetic[183, 207, 240, 255, 311] and non-diabetic patients,[337, 561] to slow the progression of renal impairment in CRF and endothelin antagonists would have to demonstrate additional benefits to these agents. As yet there are no studies examining the actions of endothelin antagonism in CRF or in healthy subjects.

1.5   Conclusions

Endothelin antagonists have proved extremely useful in extending the 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 subarachnoid haemorrhage and Raynaud's phenomenon. 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 they 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 will be awaited with considerable interest.

1.5 Table Legends

Table 1.1   Factors that influence ET-1 biosynthesis

Table 1.2   Endothelin receptor antagonists

1.6   Figure Legends

Figure 1.1   Endothelium-derived vasoactive substances

The vascular endothelium is a source of relaxing and contracting factors and has been the target of several of the most succesful pharmacological interventions in cardiovascular medicine, including nitrates, aspirin and ACE inhibitors.

Figure 1.2   Endothelin

Endothelin-1 is a 21-amino acid peptide with 2 intra-chain disulfide bridges linking paired cysteine residues.

Figure 1.3   Endothelins and sarafotoxin S6c

Structures of the endothelin isopeptides and sarafotoxin S6c. The filled circles indicate the amino acids that differ from those of ET-1.

Figure 1.4   Endothelin synthetic pathway

The gene encoding ET-1 on chromosome 6 contains 5 exons and 4 intervening intron sequences. Once translated, an amino terminal 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 Try^{21}-Val^{22} by a specific endothelin converting enzyme.

Figure 1.5   Big endothelin-1 and C-terminal fragment

Big ET-1 is cleaved at Try^{21}-Val^{22} by a specific endothelin converting enzyme to form ET-1 and the C-terminal fragment of big ET-1. In certain conditions associated with raised plasma concentrations of ET-1, measuring plasma concentrations of big ET-1 and/or C-terminal fragment may help distinguish increased production of ET-1 or decreased clearance of ET-1 as the cause of the raised ET-1 concentrations.

Figure 1.6   Vascular effects of endothelin and its receptors

Endothelin produced by endothelial cells acts on two receptors: ET_A and ET_B. Both these receptors are located on smooth muscle cells and mediate smooth muscle contraction by increasing intracellular calcium. The ET_B receptor is also found on on endothelial cells and mediates smooth muscle vasodilatation by releasing NO and vasodilator prostanoids such as prostacyclin.

Figure 1.7   Constitutive release of NO

Agonists such as acetylcholine, shear stress, bradykinin, adenosine diphosphate (ADP) or substance P increase intracellular calcium activating NO synthase. NO activates guanylate cyclase in smooth muscle cells increasing intracellular cGMP and lowering cytosolic calcium resulting in smooth muscle relaxation. NO also inhibits platelet aggregation by similar actions on platelet guanylate cyclase.

Figure 1.8   Actions of nitric oxide and endothelin in the pathogenesis of atherosclerosis

Endothelial injury plays an initial role in the increased adherence and migration of monocytes into the subendothelial space. Once in the arterial intima monocytes are activated to macrophages. Uptake of oxidised low density lipoprotein by the macrophages leads to foam cell formation and together with smooth muscle cell proliferation and platelet adhesion leads to the formation of lipid-laden plaques. The rupture of these plaques, together with the associated thrombosis and vasospasm, leads to vascular occlusion and myocardial infarction or stroke. The main actions of ET appear to facilitate, whereas the actions of endothelial NO mainly inhibit, the atherogenic process. High plasma concentrations of endothelin are associated with a worse outcome after myocardial infarction.

Table 1.1

Factor	Effect	Cellular signal	Gene promoter element
Thrombin	Increase	PLC/PKC	AP-1
	Decrease	NO/cGMP	
Heparin	Decrease	NO/cGMP	
PGI$_2$/PGE$_2$	Decrease	cGMP	
ANP/BNP	Decrease	cGMP	
LDL	Increase	PLC/PKC	AP-1
Insulin	Increase	PLC/PKC	AP-1
ANG II	Increase	PLC/PKC	AP-1
Vasopressin	Increase	PLC/PKC	AP-1
Shear stress	Increase	Disruption of cytoskeleton	
	Increase	PKC	
	Decrease	NO/cGMP	
Hypoxia	Increase		
Cyclosporin	Increase	TGF-β	NF-1

Table 1.2

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
	PD151242	Parke-Davis	pseudo-tripeptide
	50-235	Shionogi	caffeoyl ester
	97-139	Shionogi	modification of 50-235
	BMS-182874	Bristol-Myers Squibb	benzenesulfonamide*
ET_B	BQ-788	Banyu	tripeptide
	RES-701-1	Kyowa Hakko Kogyo Co	16 amino acid cyclic peptide endothelial ET_B selective
ET_A & ET_B	PD 142893	Parke-Davis	linear hexapeptide
	PD 145065	Parke-Davis	linear hexapeptide
	TAK-044	Takeda	cyclic hexapeptide
	Ro 46-2005 Ro 47-0203 (bosentan)	Hoffman-La Roche	sulphonamide* sulphonamide*
	CGS 27830	Ciba-Geigy	irreversible binding, unstable
	SB 209670	SmithKline Beecham	carboxylic acid derivative*

*.orally active

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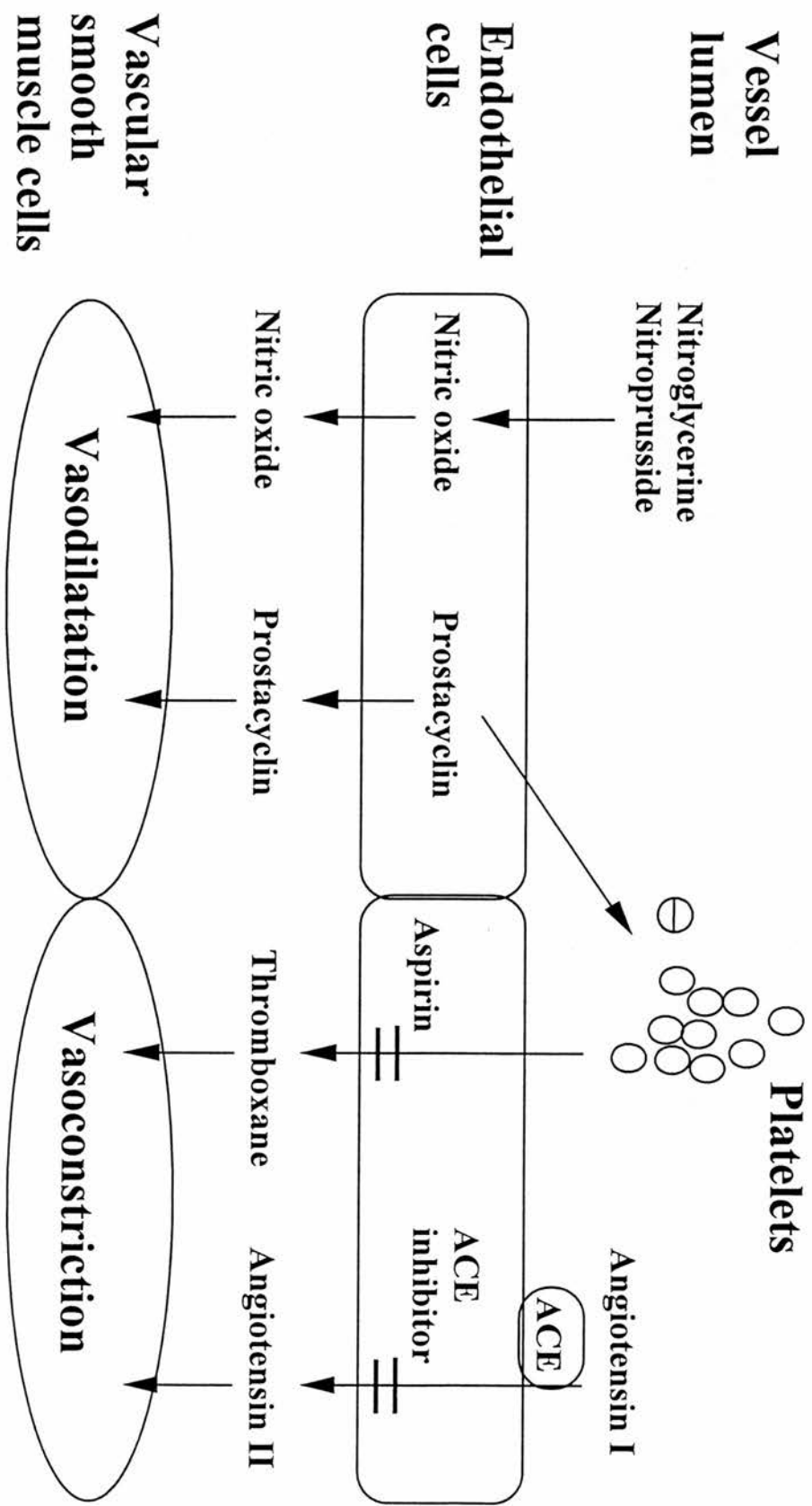
Figure 1.1

Figure 1.2

Endothelin-1
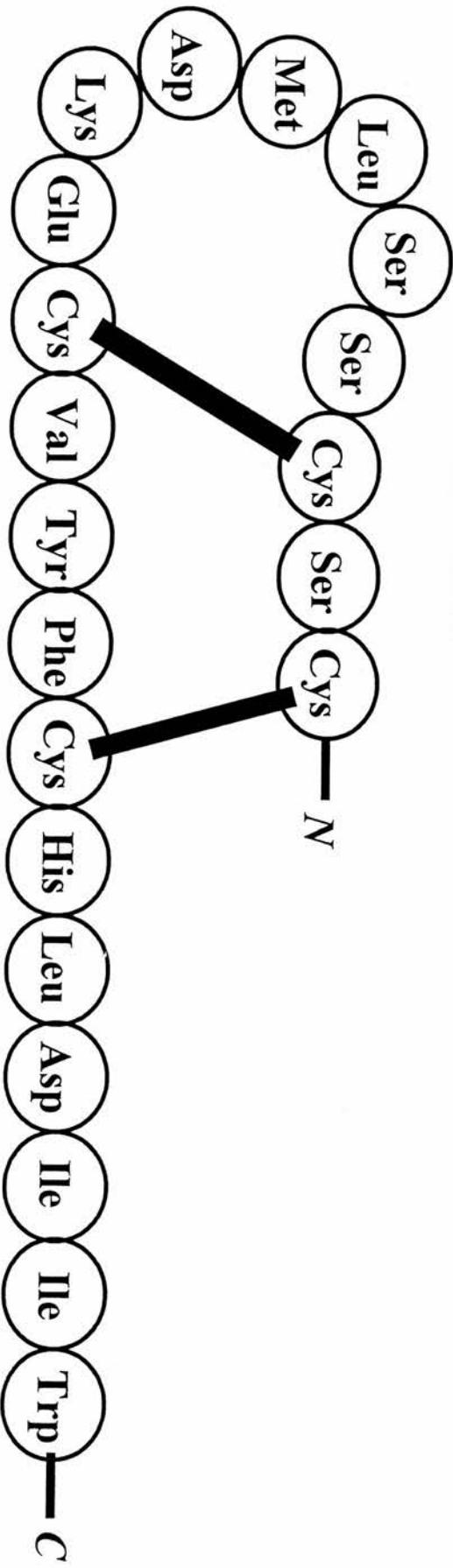
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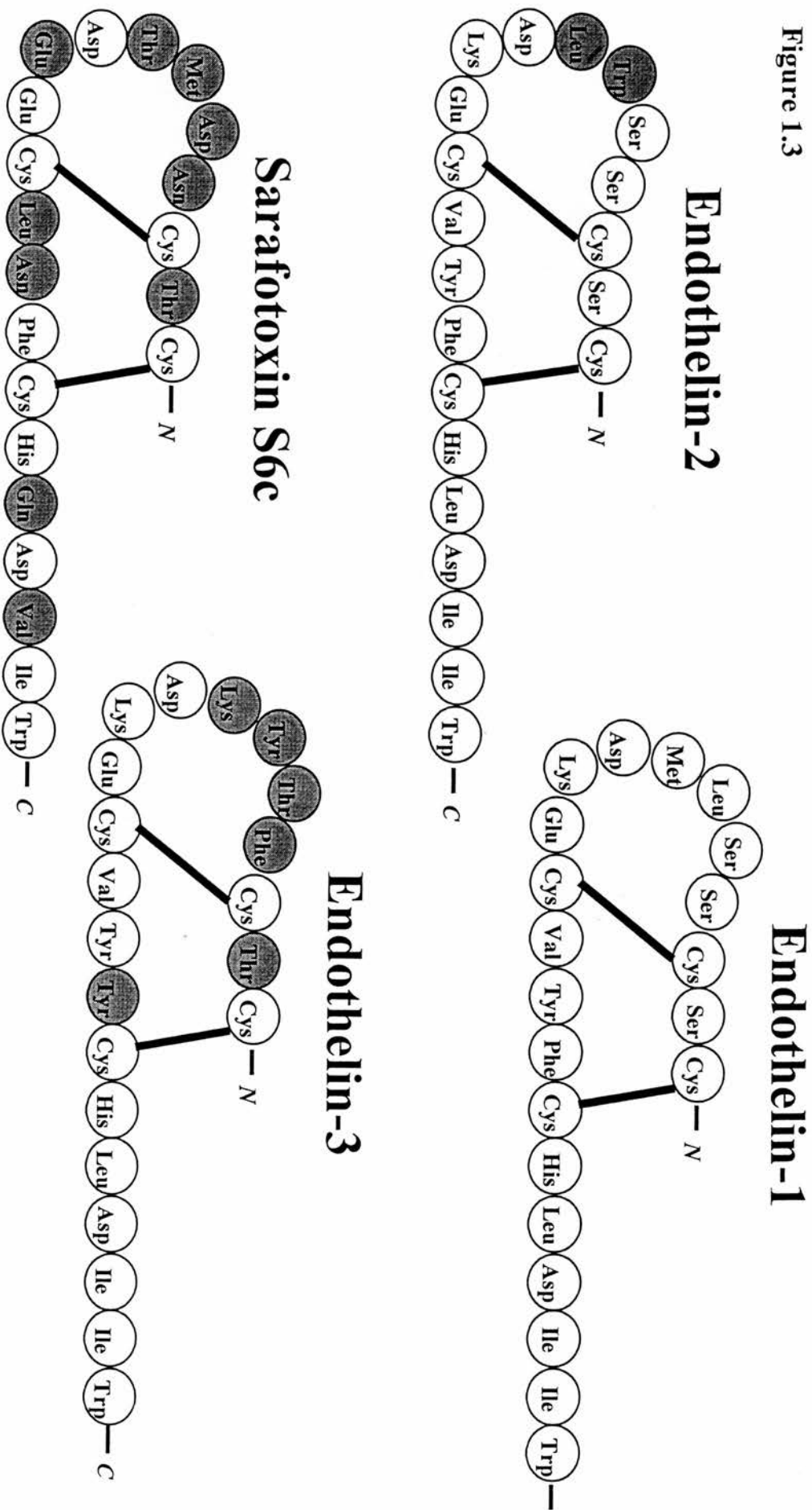
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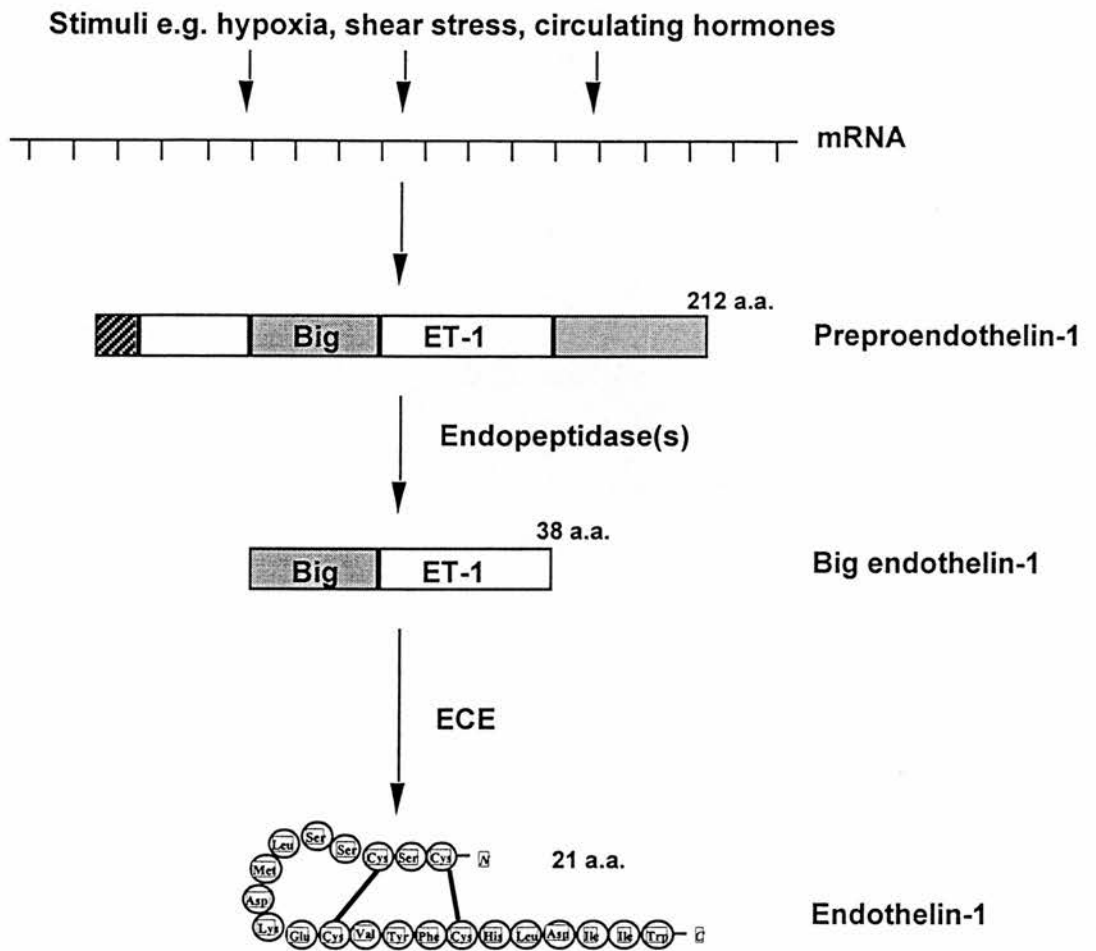
Figure 1.5

Figure 1.6

Figure 1.7

VESSEL LUMEN

Acetylcholine
Bradykinin
ADP
Substance P
Shear stress

L-arginine

ENDOTHELIAL
CELL

$\oplus$
Nitric oxide synthase

L-citrulline

nitric oxide

CELL
MEMBRANE

$\oplus$
Guanylate cyclase

↑ Cyclic GMP

Smooth muscle relaxation

Inhibition of platelet aggregation and adhesion

Inhibition of monocyte adhesion

Inhibition of smooth muscle proliferation and migration
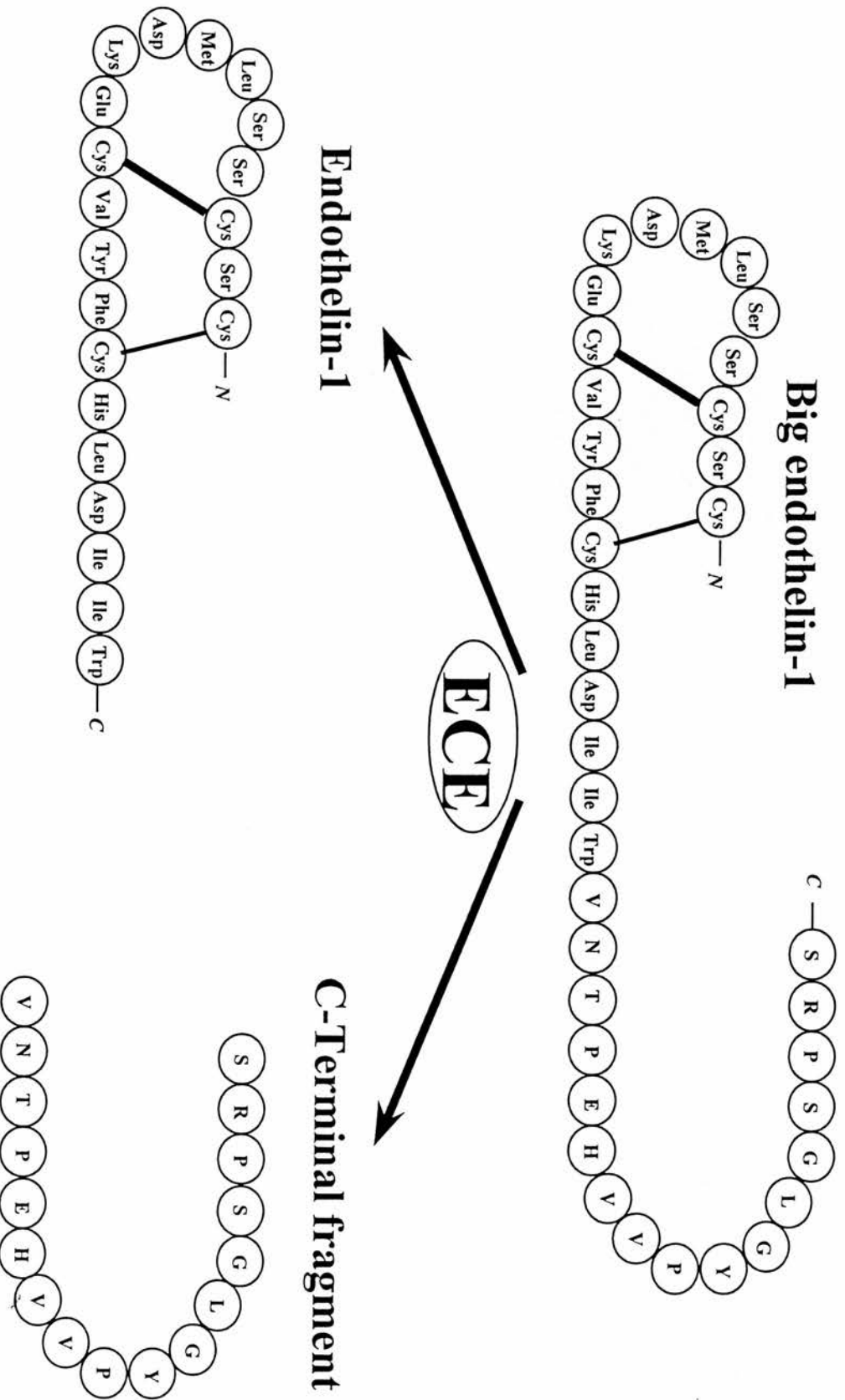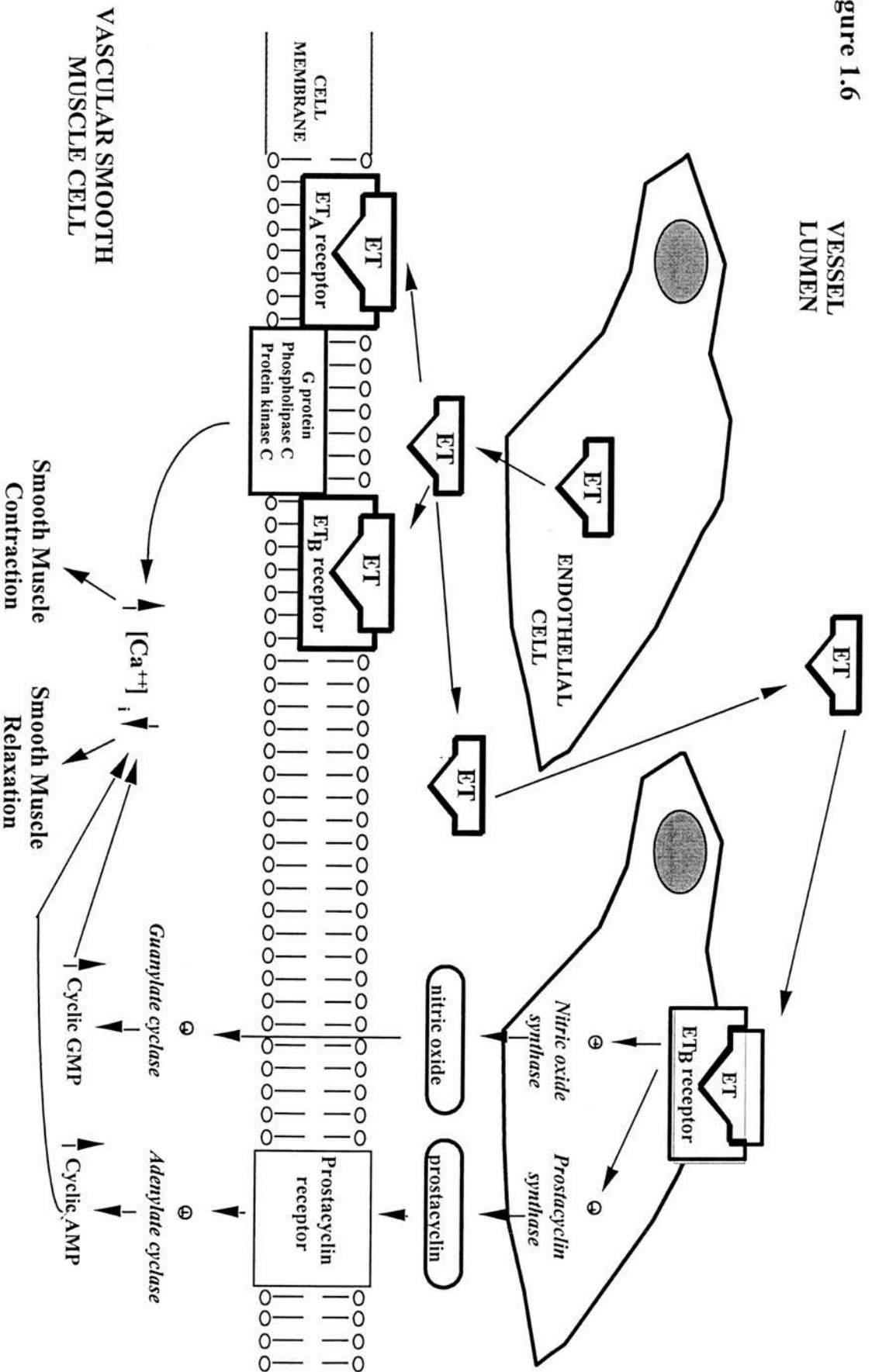
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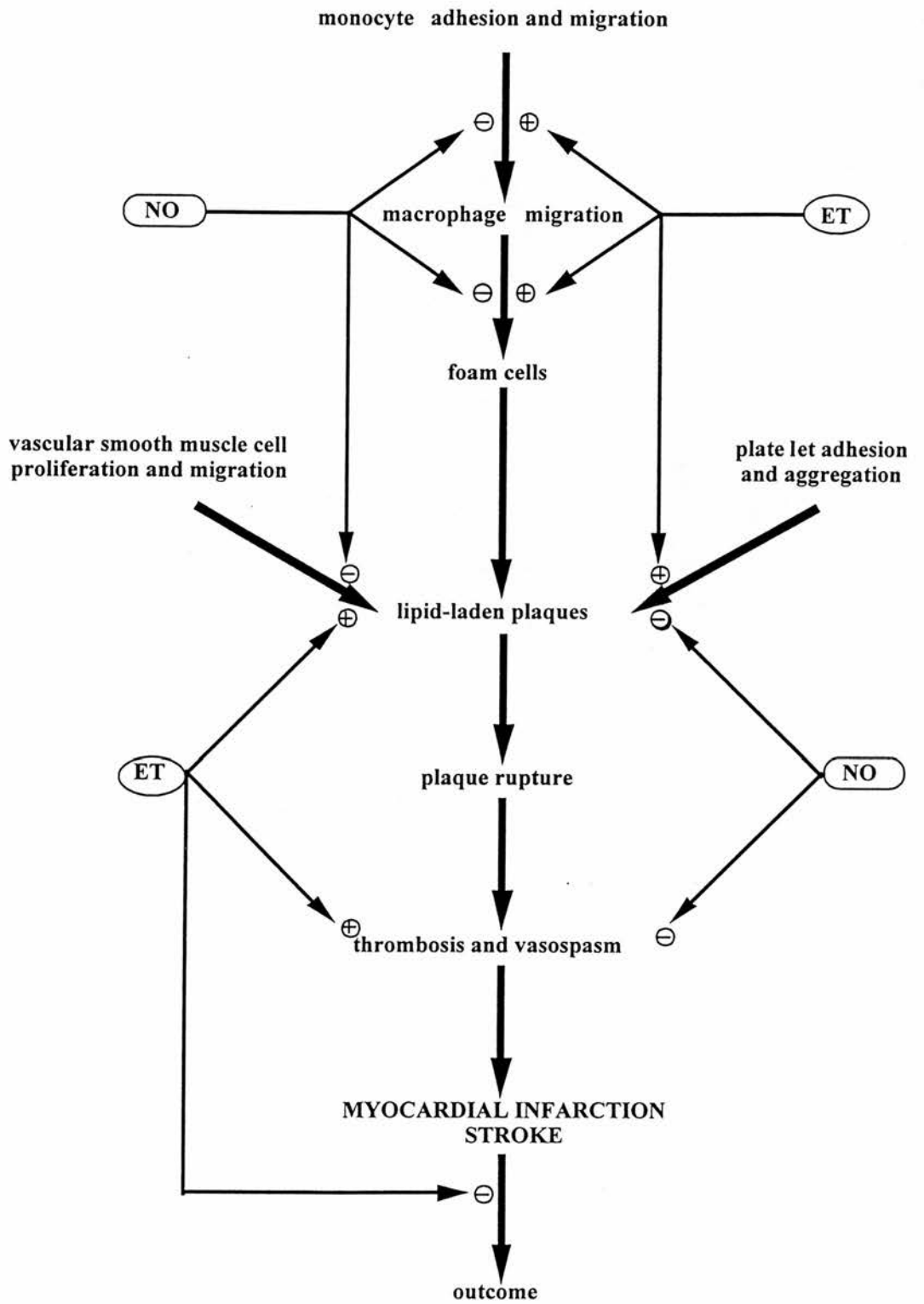
2.   Methods

2.1   Local Techniques

    2.1.1   Venous occlusion plethysmography and arterial administration of vasoactive agents

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2.1 Local Techniques

When studying the physiology of human blood vessels, *in vivo* techniques offer distinct advantages over *in vitro* techniques: *in vivo*, the vessels are exposed to physiological pressure and have physiological dimensions; they are bathed in the physiological medium of blood and exposed to local and circulating vasoactive and growth factors; they have intact nervous mechanisms; and they have not been exposed to a general anaesthetic agent or to potentially significant vessel trauma or hypoxia.

One substantial disadvantage of most human studies of blood vessels *in vivo* is that systemic doses of drug are used. This can cause effects on other organs such as the brain, heart and kidneys, as well as influence neurohumoral reflexes through change in systemic haemodynamics. Using such methods, the direct vascular effects of drugs cannot readily be interpreted in humans. In animals, to avoid these confounding influences, locally active doses of drugs have been infused into single regional vascular beds, such as the mesentery or hindlimb. Similar local infusion techniques have been available for some years to study the vascular pharmacology of resistance and capacitance vessels in humans,[10, 12, 104, 463, 617] but their power as tools in the study of cardiovascular physiology and pharmacology has only recently been widely appreciated.

2.1.1 Venous Occlusion Plethysmography

Principle

Venous occlusion plethysmography has been used to measure limb blood flow for over 80 years.[224] Although air and water plethysmographs have been replaced by externally applied gallium/indium-in-Silastic and mercury-in-Silastic strain gauge devices,[218, 386] the underlying principle remains the same. A proximal limb cuff is inflated rapidly to greater than venous pressure but lower than arterial pressure, so that initially, when venous return is halted but arterial inflow continues unimpeded, the increase in limb volume with time gives a measure of blood flow. In strain gauge plethysmography, changes in limb circumference, and hence limb volume, are detected as a change in electrical resistance of the gauge. Because limb circumference and not volume is measured by the strain gauge technique, a measure of blood flow per unit volume of tissue ($ml^{-1}min^{-1}100$ ml tissue) is obtained. Limb volume must be measured separately in order to obtain an absolute measure of blood flow, though for many studies this is not necessary. The mathematical principles underlying this technique have been reviewed.[154, 618] Although early studies often involved the lower limb, the studies described in this thesis are confined to the forearm vascular bed.

Technique

Subjects are made to feel relaxed resting supine in a comfortable, quiet, draught-free environment maintained at a constant temperature between 22

and 26^0C so as to minimise variability in blood flow and blood pressure and maximise the reproducibility of the results. The arms are maintained resting comfortably, above the level of the central venous pressure and with venous emptying unimpeded, so that venous pressure cannot rise sufficiently to affect arterial inflow during measurements. The upper arm cuff is inflated to 40 mm Hg for 10 seconds and then deflated for 5 seconds, a manoeuvre which does not affect arterial inflow or pressure[621] and generally provides analysable linear tracings (Figure 2.1).[200, 618] At high flow rates, a shorter inflation period and a longer deflation period are needed to ensure adequate venous emptying. Recordings of forearm blood flow are made repeatedly over 3-minute periods. Voltage output from a dual-channel Vasculab SPG 16 strain gauge plethysmograph (Medasonics Inc) is transferred to a Macintosh personal computer (Performa 475, Apple Computer Inc, Cupertino, CA) using a MacLab analogue digital converter and Chart software (version 3.2.8; both from AD Instruments, Castle Hill, NSW, Australia). Calibration is achieved using the internal standard of the Vasculab plethysmography units.

During measuements, hand blood flow is excluded by wrist cuffs inflated to 220 mm Hg. Flow only stabilises after 60 seconds of cuff inflation[266] so thses cuffs must be inflated for 60 seconds longer than the period of measurement. Hand ischaemia limits the time of any one period of measuremenmt to ~10 minutes. Venous occlusion plethysmography has been shown to be a reliable[153] and reproducible technique in individual ·subjects.[458]

Compared with muscle blood flow, skin blood flow varies more markedly with temperature and emotion.[5] It also has a different physiology and exhibits different responses to drugs. Compared with the forearm, the hand is primarily made up of skin. In addition, the tissues of the hand cannot stretch in the same way as those of the forearm, and inclusion of the hand during forearm blood flow measurements can result in non-linear and unmeasurable flows.[618] Therefore, hand blood flow is excluded during measurements of forearm blood flow. Even with hand exclusion, it should be recognised that blood flow to skin contributes to total forearm blood flow,[107, 651] accounting for ~25% of flow <6 ml.100ml.$^{-1}$min^{-1} and as much as 50% of higher flows.

Arterial administration

The first descriptions of arterial cannulation to assess responses of skeletal muscle to local drug administration were provided by Barcroft and colleagues[12, 39] in 1946. They infused adrenaline into the femoral artery, although now most workers use the brachial artery route. They noted the major advantage of this technique over systemic administration, which is that it can be used to examine the direct effects of a drug on the resistance vessels without eliciting effects mediated through actions on other organs or by stimulation of neurohumoral reflexes. These unwanted effects can be avoided by giving doses that do not have systemic actions, which is feasible because the blood flow to the forearm at rest is low (~50 ml.min^{-1}) compared with the cardiac output (5000 ml.min^{-1}). Hence, doses 100- to 1000-fold lower than those active systemically are effective within the

upper limb circulation. Assuming that drugs are used for short periods, or are short-lived, their effects are restricted to the infused limb. The opposite arm can then act as a contemporaneous control for the experimental arm receiving drug (Figure 2.1), taking account of any minute-to-minute changes in blood flow that affect both arms, such as due to emotion or minor changes in basal state.[50, 199] In general, although the forearm is obviously only one of several resistance circuits, effects in the circulation to forearm muscle are commonly predictive of those found in other major systemic resistance beds, such as the mesentery and the kidney.[604]

Technique

Drugs are infused via a Welmed P1000 syringe pump (Welmed Clinical Care Systems), with the total rate of infusion maintained constant throughout at 1 ml.min^{-1}, through a cannula sited in the brachial artery at the elbow under local anaesthesia with lignocaine (Astra Pharmaceuticals Ltd, Herts, UK). The discomfort associated with this procedure is usually no greater than that associated with placement of a venous cannula. Very fine steel needles (27 SWG: Cooper's Needle Works, Birmingham, UK) mounted on a 16-gauge epidural cannula (Portex Ltd) were used in these studies. They were regularly checked for 'flashback' of blood at intervals during the studies to ensure correct placement. These needles have been found to be safe and atraumatic, consistent with the safety of long-term siting of larger brachial and radial artery cannulae in clinical practice.[186, 367] The technique is also extremely well tolerated allowing studies to be repeated at 1- to 2- week intervals up to 6 times in the same subjects.[218]

Some researchers use cannulae of a size sufficient to allow direct measurement of arterial blood pressure. However, these are less well tolerated, and difficult to justify ethically. Except in unusual circumstances, the combination of intermittent non-invasive measurement of blood pressure, either at the brachial or digital artery, with measurement of blood flow in the control arm is sufficient for safety purposes and to exclude a systemic drug effect.

In these local infusion studies, particularly with potent vasoconstrictors, it is critical to carefully consider, and be guided by, the data from animal pharmacology studies. Although critical closure is theoretically possible in resistance vessels maintaining constant transmural pressure in the face of an increasing tension secondary to administration of a vasoconstrictor agent,[27] this has not been observed in practice even with major reductions in local blood flow caused by ANG II[50] and ET-1.[211] It should be noted that, where pharmacological considerations have been taken into account, vasoconstrictors have proved remarkably safe in the forearm, avoiding the potential hazards that might be associated with studies undertaken in other vascular beds, such as the coronary circulation, or with systemic administration.

Separate issues relate to the infusate. It is important to use the appropiate vehicle as the time control for the active drug, particularly if the pH of the infusate has to be non-physiological in order to dissolve the drug or if one component of the vehicle might might have vasoactive properties. To

avoid these problems, physiological (0.9%) saline is used to dissolve agents for arterial infusion. It is also critical to avoid the use of hyperosmolar solutions for infusion as these can cause substantial vasodilatation.[35, 419] Where posssible, infusate flow of >1ml.min^{-1} and changing infusate flow rate should be avoided, because this can make a measurable contribution to total blood flow, especially during the infusion of vasoconstrictors. Clearly, some drugs cannot be given, even at locally active doses, in humans because of the potential for toxicity. However, if particular concerns exist about local vascular inflammatory effects of the infusate from intravenous studies in animals or humans, specific testing in the rabbit ear artery[270] can be helpful.

Analysis of Responses

In a fasted subject resting comfortably in the supine position in a quiet, warm, temperature-controlled environment, blood pressure and forearm blood flow remain stable for several hours.[604] Although there may be small fluctuations in blood flow associated with changing levels of alertness, these affect both arms simultaneously, so blood flow in the control arm can serve as a contemporaneous control for drug effects in the infused arm. In general, the effects of external stimuli on drug responses, and the inter-subject variability in drug effects, are minimised by describing the drug effect as the percentage change from baseline of blood flow in the infused arm as a ratio of the same percentage change in the control arm. This method uses all the information obtained from flow

measurements, and serves to minimise the effects of variations in blood flow caused by minor external factors.[50, 200]

The validity of this method for handling the data is dependent on arterial pressure remaining stable during the experiment, in which case changes in blood flow provide a reliable measure of drug effect on the contractile state of the smmoth muscle. Some workers use flows and pressures to calculate changes in vascular 'resistance', although with a non-newtonian fluid, a distensible vascular system and pulsatile flow, this derived parameter offers no particular advantage.[604]

If significant changes in arterial pressure do occur, the results must be interpreted with considerable caution because the contractile state of the smooth muscle is not independent of distending pressure, depending on the balance between passive stretch caused by the increased pressure and the evoked contraction of the circular smooth muscle (autoregulation). There is much variability between subjects in the response of the forearm vascular bed to changes in arterial pressure[460] and no simple way of distinguishing the autoregulatory response from the direct effect of a drug.

Lower Body Negative Pressure (LBNP): Sympathetic Function in Resistance Vessels

Application of a small degree of negative pressure to the lower body (10-15 mm Hg) causes venous pooling within the legs leading to unloading of low-pressure cardiopulmonary baroreceptors.[3] This generates a selective

increase in sympathetic activity[541] of the efferent nerves serving the upper limbs, and produces ~20% reduction in forearm blood flow without affecting arterial pressure or heart rate.[498] This technique can be used to examine the influence of drugs on peripheral sympathetic function and, where required, can be coupled with assessment of venous noradrenaline overspill or with direct measurements of sympathetic nerve activity.

2.1.2 The Aellig Technique

Principle

Several methods exist for studying venous responses in the upper limb.[8] It is possible to study compliance in deep forearm veins using venous occlusion plethysmography and such studies have been performed during intra-arterial infusion to assess simultaneously the effects of drugs on the arteries and veins.[85, 135, 338] Venous compliance can also been assessed in single dorsal hand veins, a technique which has been widely applied using the method of Aellig.[10] This technique measures the internal diameter of the vein while maintaining its distension through inflation of an upper arm cuff to a constant low level of pressure. Contraction of the venous smooth muscle can be detected by a reduction in internal diameter. One limitation of this methodology, but also a strength, is that under resting conditions in healthy subjects these veins have no intrinsic tone.[103, 606] They, therefore, have to be pre-constricted to study dilator agents and thus, responses may depend on the constrictor agent chosen. Noradrenaline is generally preferred, being the physiological mediator of

sympathetic tone in these vessels.[51] It should also be remembered that there are may differences between the responses in the deep and superficial veins. However, only the cutaneous limb veins participate in venomotor reflexes,[4, 650] so responses in hand veins should reflect responses of that component of the venous system which is most important in physiological regulation of venous capacitance and cardiac preload. Given also the relative ease with which hand veins can be studied, these are now widely used in preference to the deep veins.

Technique

For reproducible results in hand vein studies, subjects are studied under the same general conditions as for forearm studies, except that they can rest semi-recumbant, with the hand to be studied supported above the level of central venous pressure by means of an arm rest. A dorsal hand vein without branching over a length of at least 2 cm is then selected and a small steel cannula (23-gauge butterfly needle; Abbott, Sligo, Republic of Ireland) sited in the direction of flow without use of local anaesthesia. Internal diameter of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mm Hg can then be measured by the technique of Aellig.[10] A magnetised lightweight rod rests on the summit of the infused vein ~1 cm down-stream from the tip of the infusion cannula. This rod passes through the core of a linear variable differential transformer (LVDT; Model 025 MHR,Lucas Schaevitz Inc, Pennsauken, NJ, USA) supported above the hand by a small tripod, the legs of which rest on areas of the dorsum of the hand which are 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. The displacement causes a linear change in the voltage generated by LVDT, and thus allows determination of the internal diameter of the vein, after calibration against standard displacements.

As with forearm studies, this technique has the advantage that locally active doses can be used. The blood flow in a single dorsal hand vein is ~1 ml.min^{-1}, so the doses administered are ~1000-fold lower than are systemically active. Given the lack of potential for systemic dosing and the relative constancy of vein size, most investigators do not use a control vein as with the control arm in forearm studies.

Value of Hand Vein Studies

There are a number of reasons for wishing to study veins. First, the venous system has an important influence on cardiac output in its own right, and has been reported to be abnormal in several conditions, including borderline[149, 473] and established hypertension,[213] acute myocardial infarction,[461] and chronic renal failure.[206] This raises the possibility that abnormal venous responses may contribute directly to the pathophysiology of a number of vascular diseases. Second, in diseases primarily associated with abnormalities in resistance or conduit vessels, such as essential hypertension,[159] and Raynaud's disease,[46] similar abnormalities are found in the hand veins. Third, studies of responses to vasoconstrictors in resistance beds may be confounded by the presence of

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vascular hypertrophy,[169] whereas the process does not appear to occur, at least in hypertension,[148, 213] within the hand veins. Fourth, because human dorsal hand veins have no intrinsic tone in rested subjects,[103, 606] tone can be induced with any number of venoconstrictor agents and then the effects of dilators examined. If another drug is co-infused and reverses induced tone then one can presume it is acting against the venoconstrictor mechanism involved in generating tone.

Exact concentrations of drug are not obtained in vein studies because vein size, but not blood flow, is measured. However, concentrations can be roughly calculated, at least to the nearest order of magnitude, based on a flow of ~1 ml.min^{-1}. Differences in basal vein flow between patients and a control group might affect responses in hand veins. However, unless forearm blood flow is also different between the groups, there is no reason to believe that this should influence responses. In any case, 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.[10, 103]

2.1.3 Combining Systemic Drug Administration with Local Studies

Although the forearm and hand vein techniques are generally at their most powerful in a mechanistic sense when drugs are administered locally, they can usefully be combined with systemic drug administration in studies.

The situation is simplest, as with aspirin, where the systemic drug does not have haemodynamic effects, although the techniques can also be used with vasoactive drugs.

2.2   Blood Pressure Measurements

2.2.1 Single Blood Pressure Measurements

A semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) was used to make duplicate measurements of blood pressure in the noninfused arm, which were then averaged. This system has been well-validated against intra-arterial blood pressure measurements, ordinary auscultation and semi-automatic devices using the microphone detection of Korotkoff sounds for the registration of blood pressure.[620]

2.2.2 Twenty-four Hour Blood Pressure Monitoring

Twenty-four hour ambulatory blood pressure monitoring was performed using Spacelabs 90207 monitors. The Spacelabs were set to take readings every 30 minutes and to deflate in 8 mm Hg bleed steps. These monitors have been evaluated according to the protocol of the British Hypertension Society[396] and are recommended for the measurement of twenty-four hour blood pressure.[395]

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2.3   Impedance Cardiography

Principles

Impedance cardiography (bioimpedance) is a simple, accurate and non-invasive method for measuring cardiac output and function. Essentially, a constant sinusoidal current is applied between electrodes placed on the neck and lower chest and changes in the bioimpedance to the current are related to cardiac events and blood flow. The major change involved is reduction in impedance (Z) during left ventricular ejection. The first derivative of this impedance cardiogram (dZ/dt) includes inflections which are timed with cardiac events such as aortic valve opening and closure and peak systolic aortic flow and the maximum change in dZ/dt (dZ/dt{max}) is related to peak aortic flow.[359] By measuring the intervals between the impedance reflections and those on the ECG it is possible to derive systolic time intervals (STIs) such as the pre-ejection period (PEP) and ventricular ejection time (VET). These and the ratio PEP/VET, are related to left ventricular performance and have often been used in the non-invasive assessment of drug effects.[34, 76, 613]

The impedance cardiogram has also been used to determine stroke volume. Using a model in which the chest is considered to consist of two parallel conductors (blood and thoracic tissues), and making several loose assumptions about the relationship between the impedance cardiogram and aortic blood flow, Kubicek *et al,*[291] devised a plethysmographic formula for estimating stroke volume from the extent of the systolic thoracic

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impedance change. While the formula produced acceptable estimates of stroke volume and cardiac output both at rest and with exercise in healthy volunteers, the method was sometimes inaccurate, especially in patients with cardiovascular disease.[134, 359]

More recently Sramek and Bernstein have described an empirical modification to Kubicek's original formula[56] and combined this with a sophisticated computer algorithm to produce a very simple and cheap method for measuring stroke volume. Several studies have shown that the new method is more accurate in the clinical setting than was Kubicek's.[20, 55, 194, 248, 394, 482, 525, 564]

Technique

Impedance measurements were made using a Non-invasive Computerised Cardiac Output Monitor (NCCOM) 3, series 6 (Biomed Medical Manufacturing, Irving, California) impedance cardiograph. A constant sinusoidal alternating current (2.5 mA RMS, 70 kHz) was applied between electrode pairs placed on the lateral aspects of the neck and lower chest., and the voltage associated with this was detected by two inner sensing electrode pairs placed 5 cm from the corresponding current injecting electrodes and parallel to the current path. Self adhesive Ag/AgCl electrodes (Red Dot 3M, Minneapolis USA) were used. This voltage was relayed to an amplifier within the apparatus. An impedance (Z) signal was produced using Ohms law and differentiated to give the dZ/dt signal. A microprocessor incorporated in the apparatus uses algorithms to produce on-line measurements of basal impedance (Z_0), peak rate of change of

impedance (dZ/dt{max}), and VET. Stroke volume and cardiac output are calculated on-line by the microprocessor using the Sramek-Bernstein formula:

$$\text{Stroke volume} = L^3.dZ/dt\{max\}.VET / Z_0.$$

where L is the thoracic length, estimated from the patient's height and weight using a normogram.[56]

Using this method, 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.[482, 564]

2.4   Measurement of Renal Function

2.4.1   Effective Renal Plasma Flow

Principles

The classic clearance technique for the determination of effective renal plasma flow (ERPF) is based on the application of the Fick principle to the disappearance of an indicator substance from blood passing through the kidneys and its subsequent appearance in the urine.[520] If the indicator is neither synthesised or nor metabolised in the kidney, its rate of appearance in the urine equals its rate of extraction from the blood.

p-Aminohippurate (PAH) is thought to undergo essentially quantitative renal tubular excretion at plasma concentrations below 25 mg.l^{-1}, and its renal clearance under these conditions has been established as the standard measure of the ERPF for ~50 years.[52, 84, 519] The original method for estimating the renal clearance of PAH[519] has stood the test of time well and it is calculated from the urinary excretion rate and plasma concentration at steady state following an appropriate intravenous loading dose and constant infusion. However, significantly reduced values of PAH extraction have been observed in patients with renal disease.[54, 64, 586] Therefore, determination of the actual extraction ratio' by measuring simultaneous renal arterial and venous concentrations of PAH is necessary whenever a precise value for renal plasma flow is required. In these studies, PAH clearance was estimated both after active treatment and placebo, with the data expressed as placebo-corrected changes from baseline in order to overcome this problem.

2.4.2 Glomerular Filtration Rate

Principles

The gold standard for the assessment of GFR is the urinary clearance of inulin[518] or polyfructosan.[53] These agents are inert and do not bind to plasma proteins. They distribute in extracellular fluid, are freely filtered by the glomerulus, and are neither reabsorbed nor secreted by renal tubules.[310] The rate of appearance of these substance in the urine, therefore, equals its rate of filtration by the glomerulus.

The original method for estimating GFR[520] has stood the test of time well and it is calculated from the urinary excretion rate and plasma concentration at steady state following an appropriate intravenous loading dose and constant infusion.

2.4.3 Renal Sodium Handling

Principles

Lithium clearance has been proposed as a valuable method for estimating the proximal reabsorption of sodium.[565] This approach is based on the assumption that lithium is reabsorbed in the proximal renal tubule to the same extent as sodium and water, and that lithium is neither reabsorbed nor secreted in the distal tubule.[565] This conclusion is is supported by experimental data obtained in animals with micropuncture technique,[221, 509, 567] and in humans by non-invasive methods.[528, 568] Although under certain experimental conditions such as extremely low sodium intake[566] or frusemide-induced diuresis,[528] a small fraction of lithium reabsorption may take place within the distal nephron, lithium clearance appears to represent under most clinical situations a useful indicator of proximal tubular reabsorption.[565] This technique has been largely used to analyse the renal tubular handling of sodium in patients with normal renal function.[77, 144, 212, 230, 527, 610]

In patients with impaired renal function, a reduction of GFR is accompanied by an equivalent reduction in lithium clearance.[89, 254, 569]

This is consistent with the delivery of tubular fluid from the end of the proximal tubule falls in parallel with the decrease in GFR.[89, 254, 569] Thus, in the presence of mild to moderate renal failure (GFR>50 ml.min^{-1}) the fractional proximal and distal reabsorption of sodium are unchanged[89] and lithium clearance can be used to investigate tubular sodium handling in these patients.

2.4.4 Protocol

In each study, subjects received oral lithium carbonate (Camcolit: 250 mg sustained release) before each study day (~14 hours before baseline measurements), to assess proximal tubular reabsorptive capacity of sodium. This dose of lithium is physiologically inert.[144] A 24 hour collection of urine was performed by all subjects from 7:00 AM on the day before each study day. At 7:30 AM on the study day, subjects drank 450 ml of water before attending the clinical research centre at 8:00 AM. On arrival subjects were given 5 ml.kg^{-1} water to to drink and two cannulas were placed in the antecubital fossa of the non-dominant arm for infusion of PAH (Merck, Hoddeston, UK) / polyfructosan-S (Inutest; Laevosan GmbH, Linz, Austria) and the study agent. A third cannula was placed in the antecubital fossa of the opposite arm for blood sampling and the subjects remained supine thereafter except when passing urine. 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 minutes (8:30 AM), followed by a maintenance infusion of PAH (8.3 g.l^{-1}) and polyfructosan-S (10g.l^{-1}) in

0.9% saline at 120 ml.hr^{-1}. After an equilibration period of 75 minutes, blood samples were drawn at the beginning and at the end of accurately timed 30 minute clearance periods. An appropriate volume of water was drunk by the subjects after all urine collections to maintain a stable water balance throughout the rest of the study. Two baseline periods preceded intravenous infusion of the study agents dissolved in 50 ml 0.9% saline over 15 minutes, followed by 4 further collection periods.

2.5   Plasma Assays

All venous blood samples were collected into chilled tubes, centrifuged at 1500 g for 20 minutes at 4°C and stored at -80°C until assay. All assays were done in single batches.

2.5.1 Measurement of Plasma Aldosterone

Venous blood samples for plasma aldosterone concentrations were collected into lithium heparin tubes and plasma aldosterone was measured using a solid-phase (coated tube) radioimmunoassay from unextracted serum using a commercially available kit (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA). The intra-assay CV is <8.3%.

2.5.2 Measurement of Plasma Angiotensin II

Venous blood samples were taken into EDTA/o-phenanthroline to inhibit converting enzyme and angiotensinase enzymes.[143]

The method for radioimmunoassay of ANG II is described in described in detail in Morton, 1985.[370] In brief, samples were extracted by passage through Sep-pak C18 cartridges (Waters Associates, Milford, MA). The cartridges were pretreated with methanol (5 ml) and then water (5 ml). Plasma (5 ml) was then passed through the cartridge under gentle vacuum. After washing with water (5 ml), ANG II was then eluted from the column with aqueos 20% methanol (2 ml). The extracts were dried and redissolved in Tris buffer (50 mmol.l^{-1}; pH 7.5) for assay. The recovery from plasma of added ANG II was 95%. The intra-assay CV is 10%.

2.5.3 Radioimmunoassay of Atrial Natriuretic Peptide

Blood samples (10 ml) were collected in chilled tubes containing EDTA as anticoagulant and enough Trasylol to give a final concentration of 50 Kallikrein inhibitor units.ml^{-1}.[454]

The method for radioimmunoassay of ANP is described in detail in Richards, 1987.[454] In brief, ANP was extracted from 4 ml plasma on Sep-pak C18 reverse phase columns. Sep-Paks were pre-activated with 5 ml methanol and washed with 5 ml distilled water prior to application to

the acidified (0.25 ml 2N HCl.ml^{-1} of plasma) plasma. Acidified plasma was centrifuged (1000 g, 4°C, 10 minutes) prior to its application to the Sep-paks. The Sep-pak cartridges were then washed with 0.1% trifluoroacetic acid (TFA; 3 x 5 ml) and the adsorbed peptide was eluted with 2 ml 60% acetonitrile (v/v) 0.1% TFA into plastic tubes. The extracts were dried down under compressed air, and reconstituted in 0.5 ml buffer (100 mmol.l^{-1} sodium phosphate, pH 7.4, containing 50 mmol.l^{-1} NaCl, 0.1% w/v BSA, 0.1% w/v Triton x-100 and 50 kallikrein inhibitor units/ml Trasylol). Recovery of peptide form the Sep-paks was >80%.

Antibodies to α-hANP were raised in New Zealand white rabbits (Peninsula Laboratories). Reconstituted plasma extract (100 ml), antibody in buffer (100 ml)at a dilution of 1/10 000, and 2 pg ^{125}I-α-hANP in 50 ml of the buffer were incubated at 4°C for 24 hours. Separation of free and bound ligand was achieved by mixing with 1 ml dextran-coated charcoal. The mixture was immediately centrifuged for 20 minutes at 2°C and the free label was counted. Cross-reaction of the antibody with a variety of synthetic ANP sequences (5-28 hANP, 7-28 hANP, atriopeptins I, II, III and rat ANP) was >90%. No significant cross-reactions with bradykinin, arginine vasopressin, angiotensins I and II or adrenocorticotrophic hormone (ACTH) have been reported with this method. The intra-assay CV was 3.9%.

2.5.4 Endothelin (Studies 1 & 8)

Samples for endothelin assay were collected into tubes coated with EDTA and plasma endothelin was assayed using a commercially available kit (Endothelin-1 Radioimmunoassay, Peninsula Lab, Belmont, CA) as previously described,[100] except samples were extracted using acetic acid.[464] This method gives an extraction recovery of ET-1 of 89%. The intra-assay CV is <6% and the cross-reactivity of this assay with ET-1, ET-2, ET-3 and big ET-1 is 100, 7, 7 and 10% respectively.

2.5.5 Radioimmunoassay for Endothelin (Studies 3 & 4)

Immunoreactive endothelin was extracted from acidified plasma using Sep-pak C18 silica columns. Duplicate extracted samples and standards were incubated with rabbit polyclonal antibody raised against ET-1 (ITS Production B.V., Wijchen, The Netherlands; in 100 ml distilled water and ^{125}I-ET-1 (ITS; 100 ml) was added, and tubes were incubated for 18 hours at 4°C. Donkey anti-rabbit gamma globulin bound on solid-phase (ITS; 100 ml) was added, and tubes were incubated for 30 minutes at room temperature. The amount of radioactivity in the antibody-bound fraction was determined by gamma counting for 3 minutes. The recovery of added ET-1 was 84%. Intra- and inter-assay CVs were 2.4% (n=6) and 4.2% (n=5), respectively. The sensitivity of this assay is 2 pg.ml^{-1} endothelin. Cross-reactivity of the assay with ET-1, ET-2, ET-3 and big endothelin-1

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is 100, 52, 96, and 7%, respectively. This assay does not cross-react with TAK-044.

2.5.6 Endothelin (Study 5)

Plasma endothelin was measured by radioimmunoassay (New England Nuclear Endothelin 1,2 kit) as previously described.[464] 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}.

2.5.7 Radioimmunoassay for Endothelin, Big Endothelin-1 and the C-terminal fragment of Big Endothelin-1 (Studies 6 & 7)

Sample preparation

After being thawed, 5 ml plasma samples were acidified by adding 1 ml hydrochloric acid 2 M, and clarified by centrifuging for 15 minutes at 2000 g at 4°C. The resulting supernatants were applied to activated supernatants 500 mg Spe-ed C18 (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 a vacuum manifold with a 5 ml 0.1% TFA and discarded. Immunoreactive C-terminal fragment was eluted with a subsequent 2 ml of 80% methanol, 0.1% TFA and immunoractive endothelin and big ET-1 were separately

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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 (UK) Ltd. Basingstoke, Hants).

Radioimmunoassay

Plasma immunoreactive endothelin, big ET-1 and C-terminal fragment were determined by radioimmunoassay using rabbit antisera raised against the C-termini of endothelin (ET-1$_{(15-21)}$ and big ET-1$_{(31-38)}$. 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 (^{125I}-ET-1 or ^{125I}-big ET-1, Amersham International plc, Amersham, Bucks), bound counts were separated using Amerlex-M reagent (Amersham International plc) 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 2 standard deviations above zero standard) were <1.25 fmol/tube. The recoveries of ET-1, big ET-1 and C-terminal fragment were 57.5%, 39.8% and 76.6%, respectively (n=4).

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The mature endothelin RIA cross-reacted 100% with ET-1, ET-2 and ET-3 as expected as the immunogen contained the 7 C-terminal residues of ET-1 common to all 3 mature ET isoforms. 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 C-terminal fragment 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 ANG II, ANP and α-calcitonin gene-related peptide.

2.5.8 Measurement of Plasma Active Renin by an Antibody Trapping Technique

The antibody trapping technique relies on incubating plasma with renin substrate at concentrations sufficient to ensure zero-order kinetics. Reaction velocity is therefore proportional to renin concentration. The ANG I generated is 'trapped' with ANG I antibodies present in high concentration. External calibration against the International Standard Renin[350] allows results to be expressed in enzyme concentration units rather than velocities.

This technique is described in detail in Millar et al.[350] In brief, 35 ml of plasma was incubated at 37°C for 30 minutes with 55 ml of a premixed solution consisting of ox or sheep renin substrate, buffer (3 mmol.l^{-1} Tris/HCl), pH 6.9, 0.005 mmol.l^{-1} EDTA) and antibody at appropriate (predetermined by titration) trapping concentration. The ratio of the premixed components was 8:2:1. The enzyme reaction was terminated by dilution and cooling, obtained by the addition of 1.4 ml of ice-cold buffer (0.25 mol.l^{-1} Tris/HCl, pH 7.4, 0.01% human serum albumin; HSA), followed at once by trace ^{125}I-ANG I (20 pg, 5000 - 10 000 cpm). The radioimmunoassay for ANG I was completed by further incubation at 4°C for 48 hours. Separation of free and bound ligand was achieved by addition of Dextran-coated charcoal (150 ml). The intra-assay CV for this assay is 3.4%.

Standard curves for ANG I were prepared by serial dilution of Ile^5-ANG I in 35 ml buffer (0.25 mol.l^{-1} Tris/HCl, 7% HSA) from 1000 - 16 pg per tube in duplicate. Tubes containing no ANG I were also included. Fifty-five ml of antibody-substrate-buffer was added and tubes were then treated identically as unkowns.

2.5.9 TAK-044 (Studies 3 & 4)

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 ml of 39% acetonitrile. Chromatographic separation was achieved by 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^{-1} KH$_2$PO$_4$/0.005 mol.l^{-1} 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 this assay, defined as the lowest quantifiable amount of compound at which the loss of precision was 10% and the accuracy was between ± 20%, was 5 ng.ml^{-1} of TAK-044.

2.5.10 TAK-044 (Studies 5, 6 & 7)

TAK-044 was extracted from buffered (Merck 9437; pH 5) plasma by methanol/buffer-preconditioned Bakerbond SPE cartridges and was measured by HPLC. 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 HPLC pumps. The first mobile phase comprised 40% acetonitrile and 60% 6 mmol.l^{-1} KH$_2$PO$_4$/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}.

2.5.11 Plasma Adrenaline and Noradrenaline

Plasma adrenaline and noradrenaline concentrations were measured by dual-electrode coulometric detection after separation on a reverse phase HPLC column as previously described.[517] A simple solvent extraction system was used for the selective and quantitative isolation of adrenaline and noradrenaline.[517] The limits of detection were.<5 pg per injection for both adrenaline and noradrenaline with a coefficient of variation of 2% for adrenaline and 1.6% for noradrenaline. The average recovery of dihdroxybenzylamine (DHBA), which was used as the internal standard, was 88.3% (n=100).

2.6   Figure Legends

Figure 2.1   Forearm blood flow measurements

Simultaneous measurements of blood flow in both cannulated and control arms, during brachial artery infusion of physiological saline followed by angiotensin II in the cannulated arm. Flows are given by the gradient of the increase in forearm volume with time during inflation of upper arm cuffs to 40 mm Hg. Flow is markedly reduced in the cannulated arm by angiotensin II.

Figure 2.1
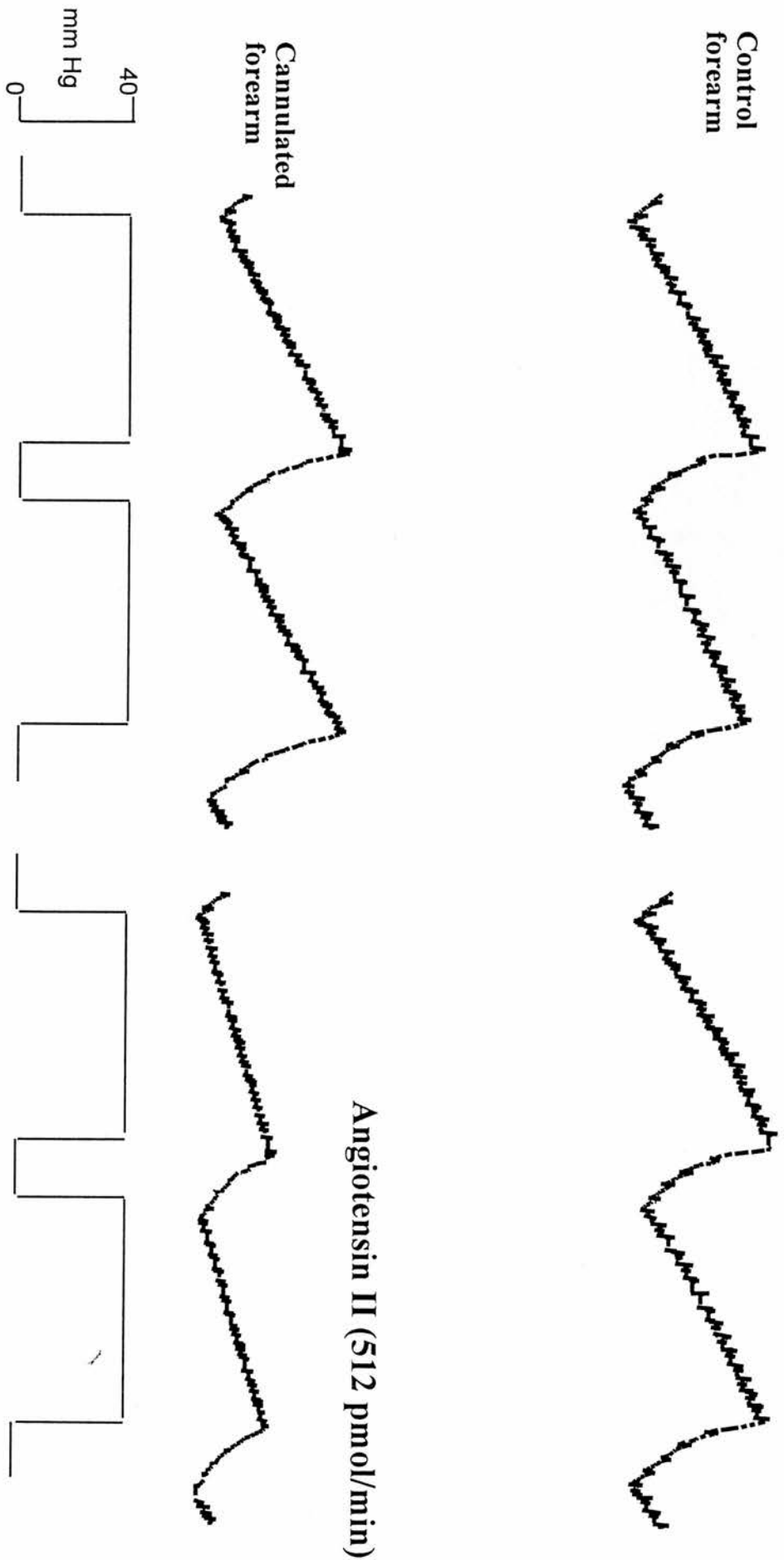
3. Study 1.

Inhibition of neutral endopeptidase causes vasoconstriction of human resistance vessels *in vivo*

3.1   Introduction

3.2   Methods

    3.2.1   Subjects

    3.2.2   Drugs

    3.2.3   Intra-arterial administration

    3.2.4   Measurements

        Forearm blood flow

        Blood Pressure

        Plasma assays

    3.2.5   Study Design

    Protocol 1:   Intra-arterial candoxatrilat

    Protocol 2:   Intra-arterial thiorphan and systemic ACE inhibition

    Protocol 3:   Intra-arterial thiorphan and intra-arterial BQ-123

    Protocol 4:   Intra-arterial thiorphan in hypertensive patients

    3.2.6   Data analysis and statistics

3.3   Results

3.4   Discussion

3.5   Table Legends

3.6   Figure Legends

3.1 Introduction

Neutral endopeptidase (EC 3.4.24.11; enkephalinase; NEP) 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.[155] It catalyses the degradation of a number of endogenous vasodilator peptides, including atrial natriuretic peptide (ANP),[533] brain natriuretic peptide,[298] C-type natriuretic peptide,[264] substance P[515] and bradykinin,[155] as well as vasoconstrictor peptides including ET-1[2] and ANG II.[155] In addition to degrading vasoactive peptides to inactive breakdown products, NEP can also convert big ET-1 to the active peptide, ET-1.[374] 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 3.1).

NEP is inhibited by several agents, including candoxatrilat[113], thiorphan[496] and its prodrug, sinorphan,[201] and phosphoramidon.[155] ANP has potent natriuretic[120] and vasodilator properties,[112, 605] and inhibits activity of the renin-angiotensin-aldosterone system by reducing both renin[66] and aldosterone[28] 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.[539] However, although NEP inhibitors increase circulating ANP concentrations in man, and cause the expected

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natriuresis,[57, 201, 393, 455] they do not generally lower blood pressure in normotensive subjects.[201, 397, 455, 457] Indeed, both candoxatril[15] and candoxatrilat[371] 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,[161, 306, 401, 452, 453, 456, 544, 578] this finding has not been universal.[57, 158, 397, 451, 513] 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 due to natriuresis.[253, 393]

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, a modest vasoconstriction had been observed[218, 318] suggesting accumulation of vasoconstrictor peptides such as ANG II or ET-1. Therefore, in the present study, the effects of brachial artery administration of a structurally different NEP inhibitor, candoxatrilat, on forearm blood flow were examined to determine whether the vasoconstriction produced by thiorphan is a class effect of NEP inhibitors. Whether an accumulation of ANG II was the cause of the forearm vasoconstriction produced by thiorphan was also investigated by infusing thiorphan into the brachial artery, in the presence or absence, of concurrent systemic ACE inhibition. Furthermore, whether accumulation of ET-1 was the cause of the forearm vasoconstriction by thiorphan was

examined by co-infusing an ET_A antagonist, BQ-123, together with thiorphan. The effects of brachial artery administration of thiorphan in a group of hypertensive patients was also examined to confirm the clinical relevance of these findings in healthy subjects.

3.2   Methods

3.2.1 Subjects

Twenty-four healthy male subjects, and 6 hypertensive patients (BP > 160/100 mmHg) who had not yet received any treatment, participated in these studies. None of the subjects received vasoactive or non-steroidal 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 and $25^{\circ}C$.

3.2.2 Drugs

Candoxatrilat (Pfizer Central Research, Sandwich, UK) and thiorphan (Sigma, Poole, UK) were administered intra-arterially dissolved in physiological saline (0.9%; Baxter Healthcare Ltd). (+)Candoxatrilat (UK-73,967) was used in this study; this eutomer has twice the potency as a NEP inhibitor than the racemate, (±)candoxatrilat (UK-69,578)[38] and is

the active metabolite of the orally available prodrug candoxatril. The dose of candoxatrilat (125 nmol.min^{-1}) was chosen to achieve forearm blood concentrations >50-fold higher than the IC$_{50}$ (40 nmol.l^{-1}) of (+)candoxatrilat *in vitro*.[38] The dose of thiorphan (30 nmol.min^{-1}) used in this study has been shown to produce ~20% reduction in forearm blood flow when infused via the brachial artery.[218] This dose is known to achieve local concentrations in forearm blood, following brachial artery administration, >10-fold higher than the IC$_{50}$ of thiorphan (35 nmol.l^{-1}) for NEP *in vitro*,[38] based on a forearm blood flow of 50 ml.min^{-1}.

The peptide ET$_A$ antagonist, BQ-123 (Cyclo{−D-Asp−L-Pro−D-Val−L-Leu−D-Trp−}; American Peptide Company, Sunnyvale, CA, USA) was administered intra-arterially (100 nmol.min^{-1}) dissolved in physiological saline. This dose chosen 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.[218]

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 centre and, after lying supine for 30 minutes, they 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

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to a level close to the detection limit of radioimmunoassay 4 hours after administration.[251]

3.2.3 Intra-arterial administration

The left brachial artery was cannulated under local anesthesia (see Section 2.1.1). The total rate of intra-arterial infusion was maintained constant throughout all studies at 1 ml.min^{-1}.

3.2.4 Measurements

Forearm Blood Flow

Blood flow was measured in both forearms by venous occlusion plethysmography using indium/gallium-in-Silastic gauges (see Section 2.1.1).

Blood Pressure

Duplicate measurements of blood pressure in the non-infused arm were taken, which were then averaged (see Section 2.2.1).

Plasma assays

Forty ml venous blood samples were obtained at intervals for assay of concentrations of plasma active renin, ANG II, aldosterone, ANP and

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endothelin from both arms. This technique of bilateral venous sampling, from deep veins in the antecubital fossae, together with intra-brachial artery infusion of locally active agents has been reported previously.[436] Samples were collected into chilled tubes, centrifuged at 1500 g for 20 minutes at 4°C and stored at -80°C until assay. All assays were performed as single batches (see Section 2.5).

3.2.5 Study Design

Four single-blind studies were performed.

Protocol 1: Intra-arterial candoxatrilat

Ten subjects participated in this single-phase, single-blind study. Subjects rested recumbent throughout. Physiological saline was infused via the left brachial artery for 30 minutes. Candoxatrilat (125 nmol.min^{-1}) was then infused for 90 minutes. Forearm blood flow was recorded in both arms every 5 minutes. Blood pressure was measured at 10 minute intervals.

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 centre at 8:00 AM, and deep veins in both antecubital fossae were cannulated with 18G

intravenous cannulae (Venflon; Viggo-Spectramed) for blood sampling. After lying 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 endothelin 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 12:00 AM, physiological saline was infused via the left brachial artery for 30 minutes. At 12:30 PM, thiorphan (30 $nmol.min^{-1}$) 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 endothelin concentrations.

Protocol 3: Intra-arterial thiorphan and intra-arterial BQ-123

Eight subjects participated in this randomised three-phase, single-blind study. Subjects rested recumbent throughout. Physiological saline was infused via the left brachial artery for 30 minutes. In random order and in separate occasions, at least one week apart, either thiorphan (30 $nmol.min^{-1}$) or BQ-123 (100 $nmol.min^{-1}$) alone, or both in combination were then infused for 90 minutes. Forearm blood flow was recorded in both arms every 5 minutes. Blood pressure was measured at 10 minute intervals.

Protocol 4: Intra-arterial thiorphan in hypertensive patients

Six hypertensive patients participated in this single-phase, single-blind study. Subjects rested recumbent throughout. Physiological saline was infused via the left brachial artery for 30 minutes. Thiorphan (30 nmol.min^{-1}) was then infused for 90 minutes. Forearm blood flow was recorded in both arms every 5 minutes. Blood pressure was measured at 10 minute intervals.

3.2.6 Data Analysis and Statistics

Plethysmographic data listings were extracted from the Chart data files and forearm blood flows calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 4.0; Microsoft Ltd) as described in Section 2.1.1.

Data are shown as mean ± standard error of the mean (SEM) in the figures and as mean ± SEM with 95% confidence intervals in the tables for the effects of NEP inhibition. Forearm blood flows were examined by repeated measures analysis of variance (ANOVA) using Statview 512$^+$ software (Brainpower Inc, Calabasas, Ca, USA) for the Apple Macintosh personal computer. The overall forearm blood flow response to intra-arterial candoxatrilat and thiorphan are described in the text as the area under the curve (AUC)[342] and as individual maximum responses (max). Haemodynamic and assay measures where analysed by ANOVA and

Student's *t*-test were appropriate[13] using Statview 512$^+$ software (Brainpower Inc, Calabasas, CA, USA) for the Apple Macintosh personal computer.

3.3 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 beats per minute). Also, blood flow in the noninfused arm did not alter significantly following 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 (AUC) of 12 ± 2% and maximum of -28 ± 3% (p=0.001; Figure 3.2) 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 did not change during the intra-arterial infusion of thiorphan in either phase (Table 3.1).

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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 3.1). Plasma ANG II concentration tended to be lower after 4 days of enalapril, although this difference between phases did not reach statistical significance (Table 3.2; p=0.09). Plasma ANG II concentrations did not change significantly during the placebo phase in either the infused or non-infused arms. Four hours after administration of 20 mg enalapril, there was a substantial reduction in plasma ANG II concentration (Table 3.2). Plasma ANG II concentration did not change further during the 90 minute thiorphan infusion in the enalapril phase in either the infused or non-infused arms (Table 3.2). Venous aldosterone concentration was lower after 4 days of enalapril than after 4 days of placebo (Table 3.2). During both phases, aldosterone concentration tended to decrease after four hours of supine posture. However, this decrease was only significant after 20 mg enalapril when compared to basal (Table 3.2).

Neither oral enalapril nor intra-arterial thiorphan had any effect on plasma ANP or plasma endothelin concentrations in either the infused or non-infused arms (Table 3.2).

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 3.7 ± 0.4 ml.100 ml^{-1}.min^{-1} respectively; p=0.12). Blood flow in the noninfused arm did not change significantly following

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 $17 \pm 6\%$; max $33 \pm 7\%$; p=0.05) and placebo phase (mean $13 \pm 3\%$; max $24 \pm 2\%$; p=0.006). The reductions in blood flow were similar during either phase (p=0.6; Figure 3.3).

Protocol 3: Intra-arterial thiorphan and intra-arterial BQ-123

Brachial artery administration of BQ-123 alone caused a progressive forearm vasodilatation (mean $33 \pm 3\%$; max $47 \pm 9\%$; p=0.0001) whereas thiorphan caused a slowly progressive vasoconstriction (mean $-14 \pm 1\%$; max $-22 \pm 4\%$; p=0.0001). Coinfusion of BQ-123 and thiorphan caused a vasodilatation (mean $32 \pm 2\%$; max $48 \pm 6\%$; p=0.0001)which was not different from that observed with BQ-123 alone (p=0.98; Figure 3.4).

Protocol 4: Intra-arterial thiorphan in hypertensive patients

In hypertensive patients, brachial artery administration of thiorphan caused a slowly progressive forearm vasoconstriction (mean $-10 \pm 2\%$; max $-20 \pm 3\%$; p=0.0001). This was not significantly different from that observed in the healthy volunteers in the third study (p=0.39; Figure 3.5).

3.4   Discussion

These studies 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 endothelin receptor antagonism. These findings are unlikely to be due to other actions of these agents because both candoxatrilat[113, 393, 496] and thiorphan[345, 411] 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.[103, 604] These 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,[533] candoxatrilat is just as effective in reducing clearance of ANP in nephrectomized animals,[37] implying other, non-renal, sites of action. NEP is now known to be expressed in blood vessels, by both endothelial[195] and vascular smooth muscle cells.[142] Despite the clear evidence for vascular generation and metabolism of natriuretic peptides, these studies have demonstrated that

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local NEP inhibition causes 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 3.1). However, the 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[66] and blocking aldosterone secretion,[28] 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, based on the known rate of onset of forearm vasoconstriction after brachial artery infusion of these peptides.[88] 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 endothelin concentration.[15] In another recent study, systemic administration of candoxatrilat in healthy subjects produced a significant increase in systolic blood pressure.[371] 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. These findings do not support this conclusion. Indeed, in this study thiorphan produced arterial vasoconstriction in the presence of systemic ACE inhibition, despite ANG

II concentrations being very low. Furthermore, no increase in ANG II concentrations was detected in venous blood draining the infused arm during the placebo phase of this study suggesting that NEP inhibition does not cause an accumulation of ANG II.

No significant fall in blood pressure after 20 mg enalapril orally was detected despite the very low concentrations of ANG II produced. However, this study was not designed to specifically measure changes in systemic haemodynamics. 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.[81, 136, 371] 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.[15, 158, 399] ANP may also be metabolised by an aminopeptidase which is insensitive to thiorphan.[412] Although incomplete local NEP inhibition is possible, this 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 higher than the IC_{50} of (+)candoxatrilat and thiorphan respectively for ANP *in vitro*.[38]

Consistent with earlier work[581], systemic ACE inhibition with enalapril had no effect on plasma endothelin concentrations. In addition, intra-arterial thiorphan did not increase plasma endothelin concentrations in samples collected from the infused arm. However, endothelin produced by endothelial cells is preferentially secreted abluminally[593] and inhibition of local endothelin degradation may not have resulted in increased plasma endothelin concentrations. Furthermore, any measurable increase in plasma endothelin concentrations is likely to be rapidly reduced through tissue receptor binding.[436] Therefore, the absence of any detectable rise in plasma endothelin 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.[116] 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.[201, 393] However, a reduction in blood pressure has not been clearly demonstrated in normotensive subjects[201, 399, 455, 457] and two studies have even reported an increase in blood pressure[15, 371] despite the potent vasodilator actions of the natriuretic peptides.[112, 539, 605] Also, several studies on hypertensive patients[57, 158, 398, 451, 514] have not demonstrated a reduction in blood pressure. Furthermore, a recent study in patients with CHF showed that candoxatrilat further increased systemic vascular resistance in these patients.[265] These results help to explain this apparent contradiction. The haemodynamic effects of systemic NEP inhibition will depend on the balance between its cardiac, renal and vascular actions. These studies have shown that local NEP inhibition causes forearm vasocontriction in healthy subjects and, of greater clinical relevance, that this effect is also occurs in untreated essential hypertensive patients. Thus, peripheral vasoconstriction may play an important role in counteracting the anti-hypertensive actions of NEP inhibition.

This 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 endothelin concentrations,[15] it is possible that the combination of NEP inhibition and endothelin antagonism may be useful therapeutically. Indeed, phosphoramidon, a combined endothelin-converting enzyme and NEP inhibitor, is known to produce substantial vasodilatation when infused intra-arterially in humans,[218, 318] and can lower blood pressure in

normotensive and hypertensive rats.[345] 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 man.[15] In addition, NEP inhibitors appear to possess favourable anti-mitogenic effects in models of left ventricular hypertrophy[365] and atherosclerosis.[292] Such effects would need to be counterbalanced against potential mitogenic actions of ET-1.[197]

In conclusion, local inhibition of NEP causes slowly progressive vasoconstriction in healthy subjects and essential hypertensive patients, suggesting that the predominant physiologic 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 endothelin antagonism, supports accumulation of ET-1 as the cause. Vasoconstriction produced by NEP inhibitors may help to explain some of the apparently contradictory haemodynamic results obtained following systemic dosing with NEP inhibitors.

3.5   Table Legends

Table 3.1   Systemic haemodynamics after oral enalapril and intra-arterial thiorphan

Heading   Heart rate, blood pressure, plasma active renin and aldosterone concentrations (mean ± SEM) before (8:30 AM; basal) and 4 hours after (12:30 PM) oral administration of placebo or enalapril 20 mg.

Footnote   CI indicates confidence intervals.

*p = 0.05 vs. basal (8:30 AM)

† p = 0.05 vs. placebo phase

‡ p = 0.005 vs. placebo phase

Table 3.2   Plasma concentrations of vasoactive hormones after oral enalapril and intra-arterial thiorphan

Heading   Plasma ANP, ANG II and endothelin (ET) concentrations (mean ± SEM) taken from the infused and non-infused arms (see main text for details) before (basal; 8:30 AM) and 4 hours (12:30 PM) after oral administration of enalapril 20 mg, and after 90 minutes intra-arterial infusion of thiorphan 30 nmol.min^{-1} (2:00 PM; 5.5 hours after enalapril 20 mg orally). 95% confidence intervals are shown in brackets.

Footnote   *p = 0.05 vs. basal (8:30 AM)

†p = 0.005 vs. placebo

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3.6   Figure Legends

Figure 3.1   Actions of neutral endopeptidase

The enzyme neutral endopeptidase 24.11 (NEP) catalyses the metabolism of the vasoconstrictor peptides ET-1 and ANG II, as well as the metabolism of several vasodilator peptides, including bradykinin (BK), atrial, brain and C-type natriuretic peptides (ANP, 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 will, therefore, depend on whether the predominant substrate(s) degraded by NEP are vasodilators or vasoconstrictors and in the extent of NEP involvement in the processing of big ET-1.

Figure 3.2   Intra-arterial candoxatrilat

Effect of brachial artery administration of the NEP inhibitor, candoxatrilat ($125 \ nmol.min^{-1}$ for 90 minutes), on forearm blood flow in 10 healthy, male volunteers. Candoxatrilat produced a slowly progressive vasoconstriction confined to the infused forearm (p=0.001).

Figure 3.3   Intra-arterial thiorphan and systemic ACE inhibition

Effect of brachial artery administration of the NEP inhibitor, thiorphan (30 $nmol.min^{-1}$), on forearm blood flow after oral placebo (o) or oral enalapril (•) in 6 healthy, male volunteers (see text for details). Thiorphan produced

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a slowly progressive vasoconstriction both during the placebo (p=0.05) and enalapril phases (p=0.01), with no significant difference between the two phases (p=0.6).

Figure 3.4   Intra-arterial thiorphan and BQ-123

Effect of brachial artery administration of the NEP inhibitor, thiorphan (o; 30 nmol.min^{-1}), the endothelin receptor antagonist, BQ-123 (•; 100 nmol.min^{-1}) 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). Co-infusion of BQ-123 and thiorphan produced a vasodilatation (p=0.0001) not significantly different from that produced by BQ-123 alone (p=0.98).

Figure 3.5   Intra-arterial thiorphan in hypertensive patients

Effect of brachial artery administration of the NEP inhibitor, thiorphan (30 nmol.min^{-1}), on forearm blood flow in 6 hypertensive patients. Thiorphan produced a slowly progressive vasoconstriction confined to the infused forearm (p=0.0001).

Table 3.1

	Placebo phase Time		Enalapril phase Time	
	8:30 AM	**12:30 PM**	**8:30 AM**	**12:30 PM**
Heart rate (beats per min)	58 ± 3 (51 to 65)	57 ± 3 (47 to 63)	56 ± 3 (49 to 63)	61 ± 3 (52 to 69)
Systolic blood pressure (mm Hg)	126 ± 6 (110 to 141)	130 ± 6 (113 to 146)	124 ± 7 (104 to 142)	124 ± 7 (104 to 142)
Diastolic blood pressure (mm Hg)	63 ± 2 (57 to 69)	69 ± 5 (55 to 84)	57 ± 2 (51 to 62)	65 ± 2 (59 to 71)
Mean arterial pressure (mm Hg)	84 ± 3 (75 to 92)	89 ± 5 (55 to 84)	79 ± 3 (70 to 87)	85 ± 4 (75 to 94)
Plasma Active Renin ($\mu U.ml^{-1}$)	22 ± 4 (12 to 25)	19 ± 2 (12 to 25)	75 ± 9 ‡ (52 to 99)	136 ± 12*‡ (106 to 166)
Aldosterone Concentration ($ng.ml^{-1}$)	15.8 ± 2.5 (8.9 to 21.9)	11.4 ± 1.3 (8.0 to 14.9)	12.3 ± 1.5† (8.3 to 16.1)	6.9 ± 0.7*‡ (5.0 to 8.8)

Table 3.2

	Time	Arm	
		non-infused	**infused**
ANP (pg.ml^{-1})			
Placebo phase	8:30 AM	13.0 ± 3.4 (4.4 to 21.7)	N/A
	12:30 PM	14.8 ± 3.6 (4.7 to 24.9)	21.5 ± 7.9 (1.2 to 41.8)
	2:00 PM	21.8 ± 5.5 (7.8 to 35.8)	18.6 ± 3.6 (9.3 to 28.0)
Enalapril phase	8:30 AM	15.2 ± 2.3 (9.2 to 21.1)	N/A
	12:30 PM	13.0 ± 3.1 (5.0 to 21.0)	17.1±3.1 (9.2 to 25.0)
	2:00 PM	17.2 ± 2.6 (10.6 to 23.8)	21.5±5.3 (7.9 to 35.0)
ANG II (pg.ml^{-1})			
Placebo phase	8:30 AM	12.7 ± 2.5 (6.2 to19.2)	N/A
	12:30 PM	12.6 ± 2.6 (6.0 to 19.1)	10.0 ± 1.9 (5.2 to 14.8)
	2:00 PM	7.8 ± 1.8 (3.4 to 12.2)	8.2 ± 3.3 (4.7 to 11.7)
Enalapril phase	8:30 AM	7.4 ± 1.2 (4.2 to 10.6)	N/A
	12:30 PM	1.7 ± 0.3*† (0.8 to 2.5)	1.6 ± 0.4*† (0.6 to 2.7)
	2:00 PM	1.5 ± 0.4*† (0.5 to 2.6)	1.5 ± 0.4*† (0.6 to 2.4)
ET (pg.ml^{-1})			
Placebo phase	8:30 AM	3.8 ± 0.3 (3.0 to 4.6)	N/A
	12:30 PM	3.7 ± 0.4 (2.6 to 4.6)	4.3 ± 0.3 (3.6 to 4.9)
	2:00 PM	4.3 ± 0.5 (2.9 to 5.7)	3.6 ± 0.5 (2.3 to 4.1)
Enalapril phase	8:30 AM	4.1 ± 0.2 (3.5 to 4.7)	N/A
	12:30 PM	3.8 ± 0.2 (3.3 to 4.4)	3.9 ± 0.4 (3.7 to 4.6)
	2:00 PM	4.5 ± 0.8 (2.8 to 5.1)	3.4 ± 0.5 (2.2 to 4.7)

Figure 3.1
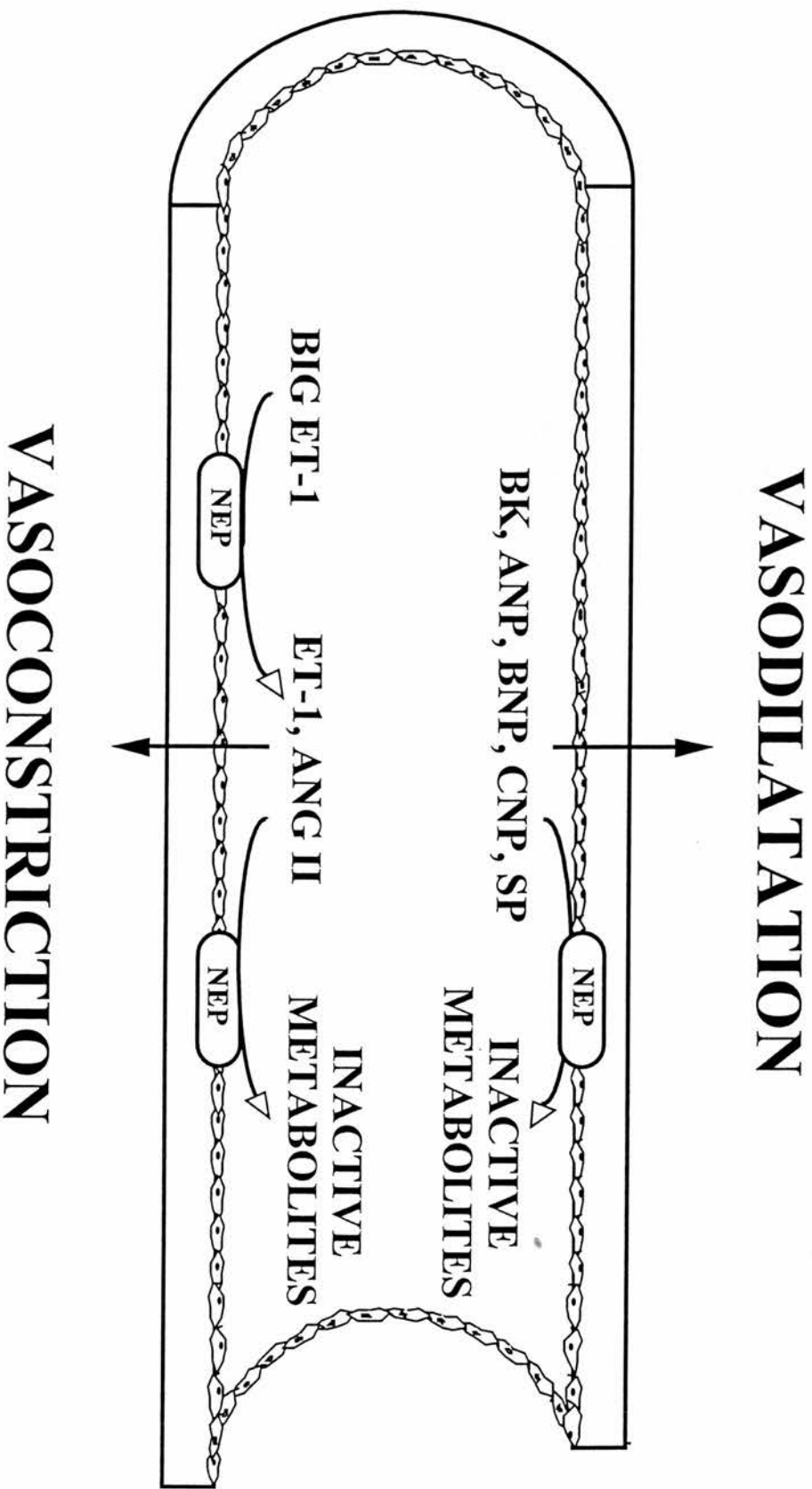
Figure 3.2

Figure 3.3

Figure 3.4

Y-axis: % change in forearm blood flow (60, 30, 0, -30)

X-axis: Time (min) (-30, 0, 30, 60, 90)

BQ-123 (100 nmol.min^{-1})/
Thiorphan (30 nmol.min^{-1})
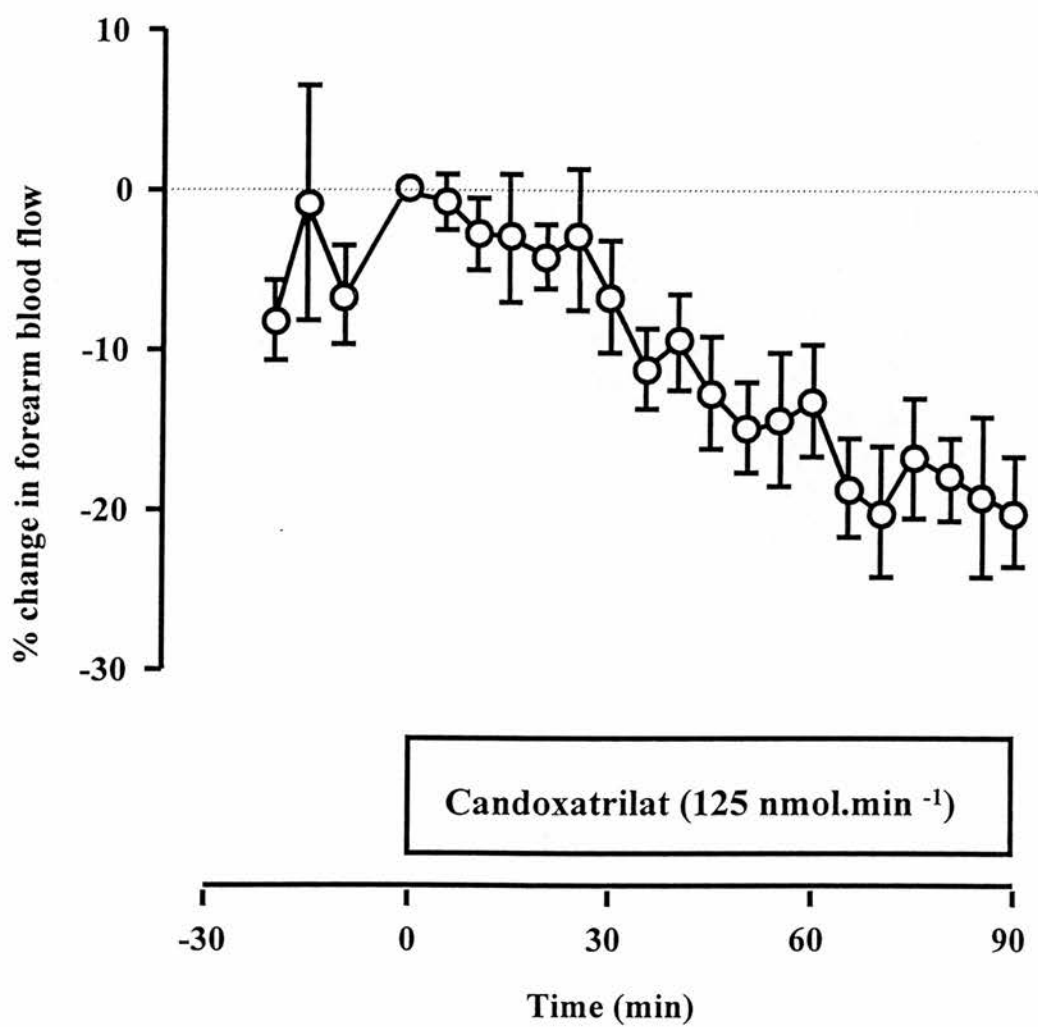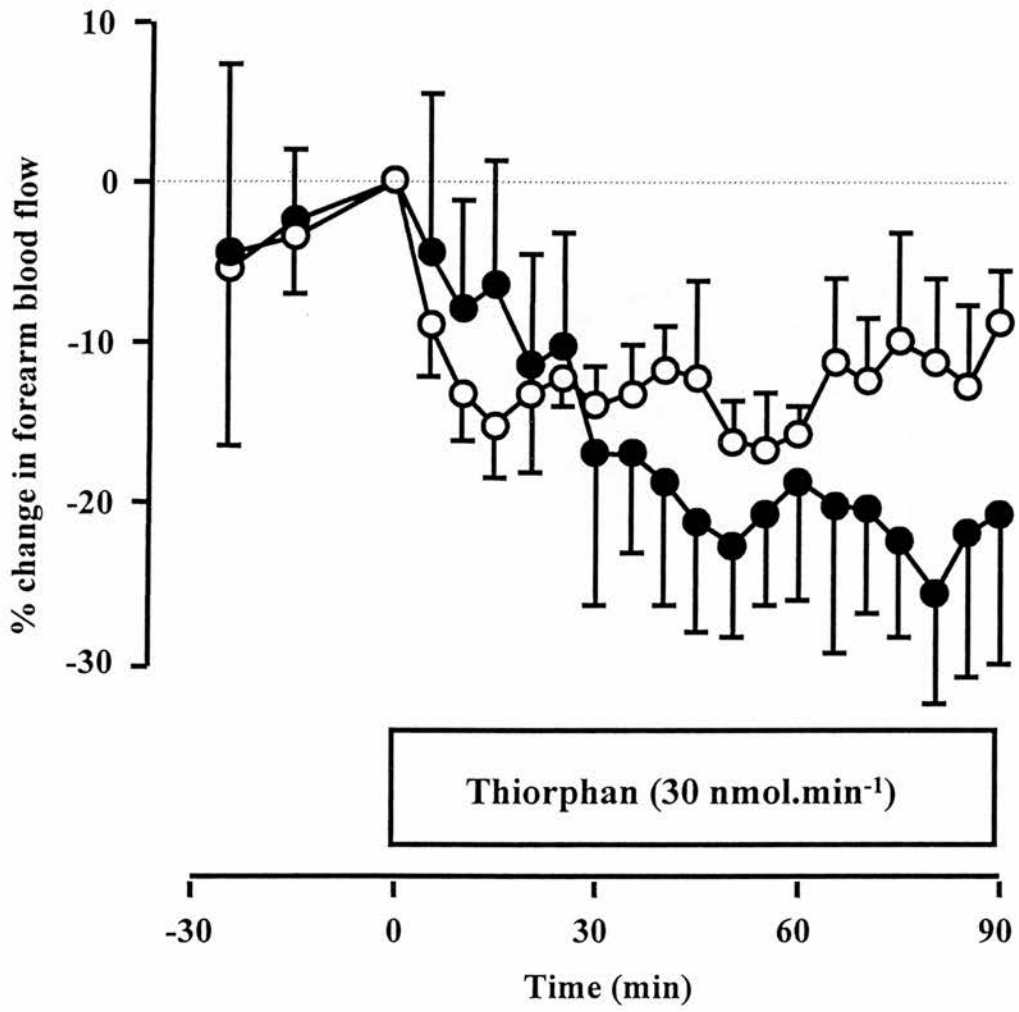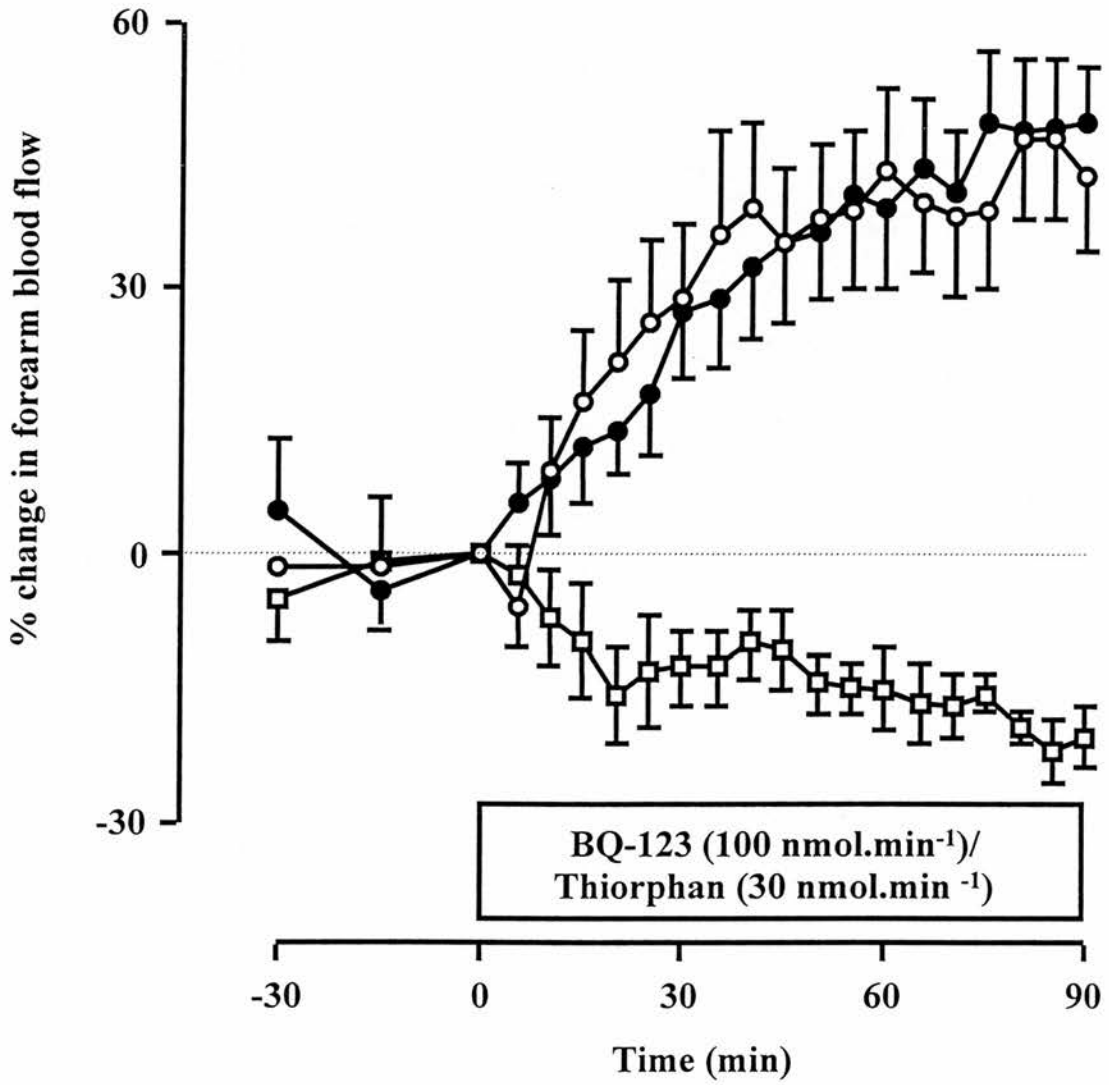
Figure 3.5
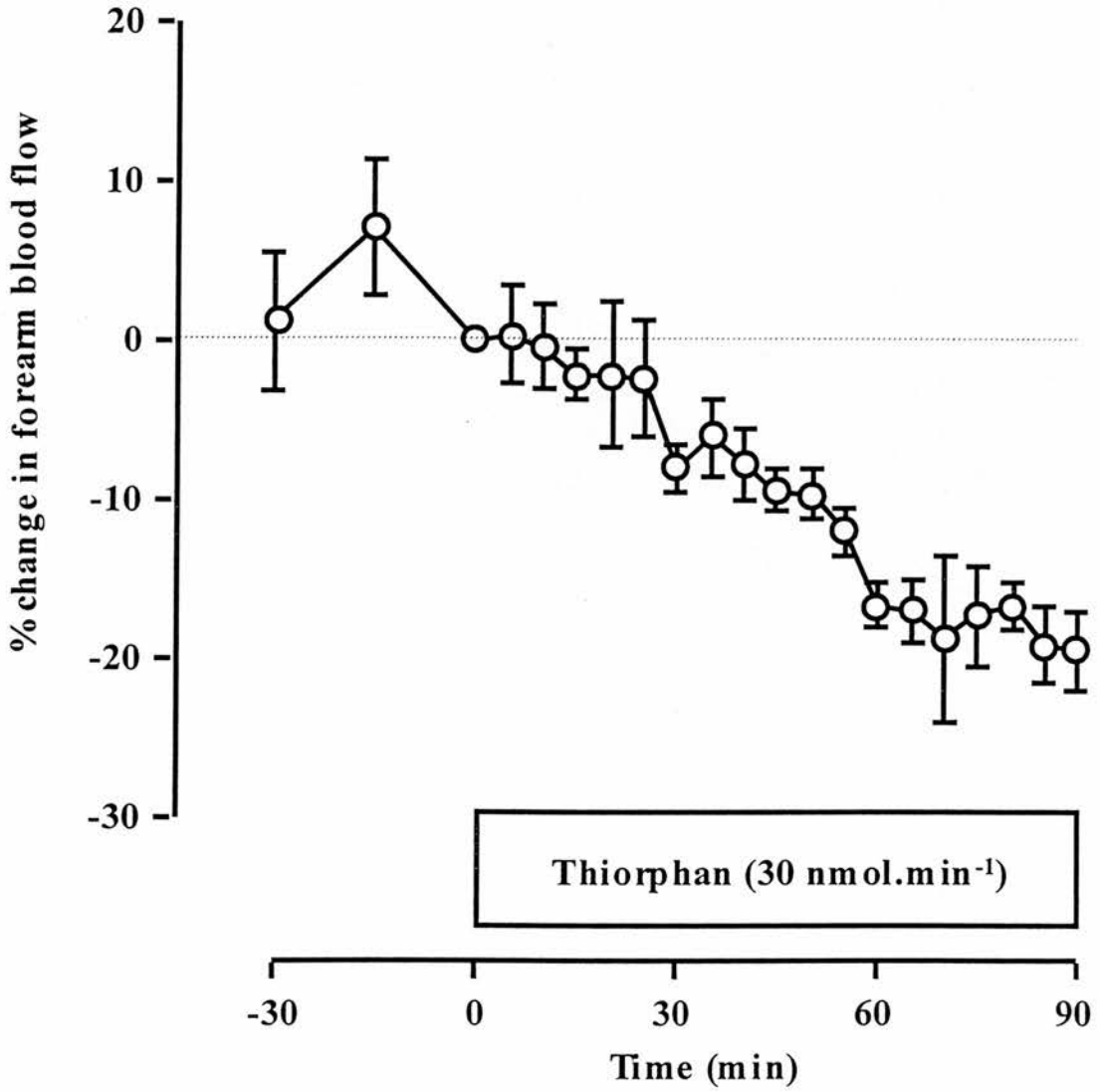
4.   Study 2

Big endothelin-3 constricts forearm resistance vessels but not hand veins in man

4.1   Introduction

4.2   Methods

    4.2.1   Subjects

    4.2.2   Drugs

    4.2.3   Measurements

    4.2.4   Study design

    4.2.5   Data analysis and statistics

4.3   Results

4.4   Discussion

4.5   Table legends

4.6   Figure legends

4.1   Introduction

Of the three endothelins, ET-1 appears is the major isoform produced by the vascular endothelium and is therefore likely to be the most important isoform in terms of cardiovascular function. ET-1 is produced from an inactive 38-amino acid intermediate form, big ET-1, by selective cleavage of the Trp^{21}-Val^{22} bond in the carboxy-terminal of big ET-1 catalysed by an endothelin-converting enzyme (ECE).[471] In humans, ET-2 and ET-3 are also formed by corresponding intermediates, big ET-2 and big ET-3.[471] Immunoreactive big ET-2, ET-3 and big ET-3 have also been detected in human plasma[190, 339] and prepro ET-3 mRNA has been detected in blood vessels,[594] suggesting that blood vessels might synthesize and respond to other isopeptides in addition to ET-1.

Two ECEs have so far been cloned in man, ECE-1[495] and ECE-2.[152] Both ECE-1 and ECE-2 convert big ET-1 in preference to big ET-2 and big ET-3.[152, 633] This substrate specificity suggests that there may be yet undiscovered ECE(s) selective for big ET-2 or big ET-3. Previous work has demonstrated that exogenously administered big ET-1 vasoconstricts human forearm resistance vessels[218] but not human capacitance vessels[214] *in vivo*. Because circulating blood contains no significant ECE activity,[602] these findings suggest that forearm resistance vessels contain an ECE able to cleave luminally presented big ET-1 to the mature peptide. However, it is not known whether human resistance or capacitance vessels contain an ECE able to generate ET-3 from big ET-3. These studies were

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designed to investigate the potential existence of such an ECE by infusing big ET-3 into the brachial artery and dorsal hand vein of healthy volunteers and assessing constrictor responses by measuring forearm blood flow and dorsal hand vein diameter.

4.2   Methods

4.2.1 Subjects

A total of eight healthy male subjects between 22 and 35 years of age participated in these studies. Four subjects participated in both studies. 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 hours, and from alcohol, caffeine containing drinks and cigarettes for at least 12 hours before any measurements were made. All studies were performed in a quiet room maintained at a constant temperature between 24 and 26°C.

4.2.2 Drugs

Pharmaceutical grade ET-3 (Peninsula Laboratories, Europe) and big ET-3 (Peninsula Laboratories) 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 precludes the use of repeated doses in a single study to examine conventional dose relationships.[88] The peptides

were dissolved in physiological saline (0.9%; Baxter Healthcare Ltd, Thetford, UK).

Intra-arterial Administration

The left brachial artery was cannulated under local anaesthesia as described in Section 2.1.1. The total rate of intra-arterial infusion was maintained constant at 1 ml.min^{-1}.

Intravenous Administration

A 23-gauge butterfly needle attached to a 16-gauge epidural catheter was sited in a selected dorsal hand vein, without the use of local anaesthesia, in the direction of flow as described in section 2.1.2. Patency was maintained by infusion of physiological saline. The total rate of intravenous infusion was maintained constant at 0.25 ml.min^{-1}.

4.2.3 Measurements

Forearm blood flow

Blood flow was measured in both forearms by venous occlusion plethysmography[604] using mercury-in-Silastic strain gauges as described in Section 2.1.1.

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Dorsal Hand Vein Diameter

Dorsal hand vein diameter was measured by use of the Aellig technique as described in Section 2.1.2.

Blood Pressure

A well-validated semi-automated noninvasive oscillometric sphygmomanometer was used to make duplicate measurements of blood pressure in the noninfused arm (see Section 2.2.1).

4.2.4 Study Design

Forearm Resistance Bed Protocol

Subjects were studied on four separate occasions, and rested recumbent throughout each study. Strain gauges and upper arm cuffs were applied, and the left brachial cannula was sited. Saline was infused for 30 minutes, during which three measurement of forearm blood flow were made at (-25, -15 and -5 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.[429] In random order and on separate occasions separated by at least 7 days, subjects received 90 minute brachial artery infusions of ET-3 at 5 and 10 pmol.min^{-1} and big ET-3 at 50 and 100 pmol.min^{-1}. The lower dose of ET-3 was chosen based on previous work showing *in vivo* that 5

pmol.min^{-1} of ET-3 causes slow-onset vasoconstriction in human forearm resistance vessels, reducing blood flow by ~20%.[217] The higher dose of ET-3 (10 pmol.min^{-1}) was chosen with the intention of causing a greater forearm vasoconstriction and reducing blood flow to a similar extent to ET-1 (~40%).[88, 218] The doses of big ET-3 were chosen with the intention of producing a measurable forearm vasoconstriction even with only 5-10% conversion to ET-3. Although no *in vivo* studies in human resistance vessels with big ET-3 have been reported, previous work with big ET-1 has shown that 50 pmol.min^{-1} of big ET-1 produces a vasoconstriction comparable to 5 pmol.min^{-1} of ET-1.[218] Forearm blood flow was measured at 5 minute intervals during the infusion of the study agents. Blood pressure was measured at 30, 60 and 90 minutes after the start of the infusion.

Hand Vein Protocol

Subjects were studied on three separate occasions separated by at least 7 days, in random order. Subjects rested semi-recumbent throughout each study. 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. Then, on three separate occasions, saline, ET-3 or big ET-3 were infused for 90 minutes, with measurement of vein diameter every 5 minutes. The choice of dose of ET-3 was based on previous work showing, *in vivo*, that 5 pmol.min^{-1} ET-3 causes slow-onset venoconstriction of ~5-

10%.[205] The choice of dose of big ET-3 was based on previous work with big ET-1.[214]

4.2.5 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) as described in Section 2.1.1.

Basal vein diameter was calculated as the mean of the last three measurements before the start of the study infusion, expressed in millimetres. Because basal vein size varies between subjects, responses are expressed as a percentage change in vein size from basal in order to reduce the inter-subject variability as described in Section 2.1.2.

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 analysis of variance (ANOVA) with statistical testing of overall significance by Scheffe's F test (ANOVA) using Statview 512+ software (Brainpower Inc, Calabasas, CA, USA) for the Apple Macintosh personal computer.

4.3   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 blood flow between the infused and noninfused forearms (Table 4.1). Blood pressure, heart rate, and blood flow in the noninfused arm, did not change significantly after infusion of any study agent, confirming that drug effects were confined to the infused arm (Table 4.1).

Forearm Resistance Bed Protocol

ET-3 at 5 pmol.min^{-1} caused a significant forearm vasoconstriction, with a -20% reduction in forearm blood flow at 90 minutes (CI: -40 to -1%; P=0.009 vs. baseline; Figure 4.1). ET-3 at 10 pmol.min^{-1} also significantly reduced forearm blood flow by -20% after 90 minutes (CI: -33 to -8%; P=0.001 vs. baseline; P=0.69 vs. ET-3 at 5 pmol.min^{-1}; Figure 4.1).

Big ET-3 at 50 pmol.min^{-1} caused a significant forearm vasoconstriction, with a -22% reduction in forearm blood flow at 90 minutes (CI: -38 to -5%; P=0.009 vs. baseline; P=0.88 vs. ET-3 5 pmol.min^{-1}; P=0.73 vs. ET-3 10 pmol.min^{-1}; Figure 4.1). Big ET-3 at 10 pmol.min^{-1} also significantly reduced forearm blood flow by -18% after 90 minutes (CI: -29 to -8%; P=0.001 vs. baseline; P=0.44 vs. Big ET-3 at 50 pmol.min^{-1}; P=0.65 vs. ET-3 5 pmol.min^{-1}; P=0.21 vs. ET-3 10 pmol.min^{-1}; Figure 4.1).

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There was no significant change in vein diameter in the control session, with a change from basal after infusion of saline for 90 minutes of +2% (CI: -6 to +10%; P=0.66; Figure 4.2). Intravenous infusion of big ET-3 did not cause any vasoconstriction, with a change in vein size at 90 minutes of -2% (CI: -12 to +8%; P=0.43; Figure 4.2). In contrast, a 10-fold lower dose of ET-3 caused progressive venoconstriction of -9% at 90 minutes (CI: -12 to -6%; P=0.04) which was significantly different to the response to saline (P=0.001) and big ET-3 (P=0.008).

4.4   Discussion

These clinical studies have shown that both ET-3 and its precursor big ET-3 cause forearm vasoconstriction. They have also shown that ET-3 constricts human hand veins whereas big ET-3 does not. These findings have important implications for the existence and distribution of an ECE capable of converting big ET-3 to the mature peptide and are similar to those obtained for big ET-1.

These studies have shown that intra-arterial infusion of ET-3 causes vasoconstriction of forearm resistance vessels confirming an earlier report.[217] However, the vasoconstriction to ET-3 (5 pmol.min^{-1}) is considerably less than that seen with the nonselective ET_A and ET_B agonist ET-1 (5 pmol.min^{-1}).[218] This held true even when the concentration of

ET-3 infused was doubled to 10 pmol.min^{-1}. However, it is difficult to extrapolate these results to quantify the relative contribution of each receptor subtype in mediating the vasoconstrictor effects of endogenous endothelin. Further comparative studies with selective ET_A and ET_B antagonists may help clarify this issue.

It is highly unlikely that big ET-3 had a direct vasoconstrictor action given its very low affinity for endothelin receptors.[471] Therefore the vasoconstriction caused by big ET-3 is likely to have resulted from conversion to ET-3. These studies, therefore provide direct evidence for the existence of an ECE capable of converting big ET-3 to ET-3. As both ECE-1 and ECE-2 convert big ET-1 in preference to big ET-3[197] this study also provides indirect evidence for the existence of another ECE in forearm resistance arteries. The slow onset and sustained actions of the endothelin isopeptides precluded the use of repeated doses in a single study to examine conventional dose-relationships. Therefore, the proportion of exogenously administered big ET-3 converted to ET-3 cannot be determined from this study.

There does not appear to be any ECE activity present in human hand veins because no venoconstriction to big ET-3 was found despite using a dose ten-fold higher than a dose of ET-3 sufficient to cause ~10% venoconstriction. However, it is possible that an ECE may be present in these vessels but not in a location to convert exogenously (intra-luminal) big ET-1 or big ET-3. Only responses to a single dose of ET-3 were tested

because the slow onset and sustained actions of ET-3 preclude the use of repeated doses in a single study to examine conventional dose-response relationships.[604] Hand vein size did not alter during saline infusion, confirming that these veins are completely relaxed under these experimental conditions.[214]

The lack of effect of big ET-3 in human hand veins contrasts with the forearm vasoconstrictor actions of the same dose of big ET-3 administered via the brachial artery. It could be argued that this difference between hand veins and forearm resistance vessels is a result of the reduced time infused big ET-3 is in contact with the hand vein (~1 cm in length) under study. This may be too small to allow adequate biochemical conversion of big ET-3. This would appear to be unlikely given that similar studies have shown that ANG I constricts human hand veins, this effect being blocked by an ACE inhibitor, demonstrating the presence of ACE in these vessels.[9, 105] There is only a small difference in constrictor potency between ANG I and ANG II in hand veins, similar to the observations with angiotensin peptides in the forearm resistance bed.[105]

The lack of conversion of big ET-3 coincides with finding with the lack of effect of big ET-1 in human hand veins[214] despite forearm vasoconstrictor actions of the same dose of big ET-1.[218] Thus ECE activity for big ET-1 appears to have a similar vessel distribution to ECE activity for big ET-3.

The endothelin system of peptides have been implicated in the pathophysiology of several cardiovascular diseases.[197] Although the major emphasis to date has been in the clinical development of endothelin receptor antagonists, the search for a pathophysiologically relevant ECE and potent ECE inhibitors to prevent endothelin production continues.[415, 579] There is a current controversy as to whether combined ET_A and ET_B receptor antagonists are preferable to selective ET_A antagonists which leave vasodilator ET_B receptors unblocked.[609] Given that ET-3 is a relatively selective agonist at ET_B receptors, it could be potentially beneficial for a clinically useful ECE inhibitor not to block big ET-3 conversion. The identification of the ECE responsible for big ET-3 conversion is, therefore, important.

In conclusion, these results show that human forearm resistance vessels have the capacity to convert exogenous big ET-3. In contrast, human hand veins appear to lack the capacity to convert exogenous big ET-3 and suggest that these capacitance vessels exhibit little or no ECE activity. However, the presence of ECE in these capacitance vessels cannot be completely excluded.

4.5   Table Legends

Table 4.1   Blood pressure and heart rate before and after local infusions of vasoactive agents and basal forearm blood flows and hand vein diameters

Mean arterial pressure (MAP) and heart rate (HR) before and after 90 minute infusion of sodium chloride (SAL), big endothelin-3 (big ET-3) and endothelin-3 (ET-3). Basal hand vein diameter and forearm blood flow in the infused arm are also shown.

4.6   Figure Legends

Figure 4.1   Intra-arterial ET-3 and big ET-3

Changes in forearm blood flow following infusion of endothelin-3 (5 pmol.min^{-1} $\bigcirc$; 10 pmol.min^{-1} $\bullet$) and big endothelin-3 (50 pmol.min^{-1} $\square$; 100 pmol.min^{-1} $\blacksquare$). Significant vasoconstriction occurred after infusion of both peptides at both doses.

Figure 4.2   **Intravenous ET-3 and big ET-3**

Changes in hand vein diameter following infusion of sodium chloride (0.9% w/v $\bigcirc$), endothelin-3 (5 pmol.min^{-1} $\bullet$) and big endothelin-3 (50 pmol.min^{-1} $\square$). Significant vasoconstriction occurred only during infusion of endothelin-3 (p=0.04).

Table 4.1

Intra-arterial Protocol

Parameter	Time	Big ET-3 (50 pmol.min^{-1})	Big ET-3 (100 pmol.min^{-1})	ET-3 (5 pmol.min^{-1})	ET-3 (10 pmol.min^{-1})
MAP (mm Hg)	Basal	85 ± 3	86 ± 4	84 ± 2	87 ± 4
	90 min	86 ± 3	90 ± 3	89 ± 3	91 ± 3
HR (beats.min^{-1})	Basal	60 ± 3	63 ± 5	59 ± 4	60 ± 5
	90 min	59 ± 4	58 ± 5	58 ± 3	61 ± 4
Infused forearm blood flow (ml.100ml.min^{-1})	Basal	4.8 ± 1.1	4.1 ± 1.2	4.3 ± 0.8	5.0 ± 1.5

Intravenous Protocol

Parameter	Time	Saline	Big ET-3	ET-3
MAP (mm Hg)	Basal	85 ± 4	86 ± 3	85 ± 3
	90 min	88 ± 3	88 ± 4	88 ± 5
HR (beats min^{-1})	Basal	64 ± 5	63 ± 4	63 ± 4
	90 min	63 ± 5	65 ± 6	65 ± 5
Hand vein size (mm)	Basal	2.4 ± 0.4	2.2 ± 0.2	2.2 ± 0.3

Figure 4.1
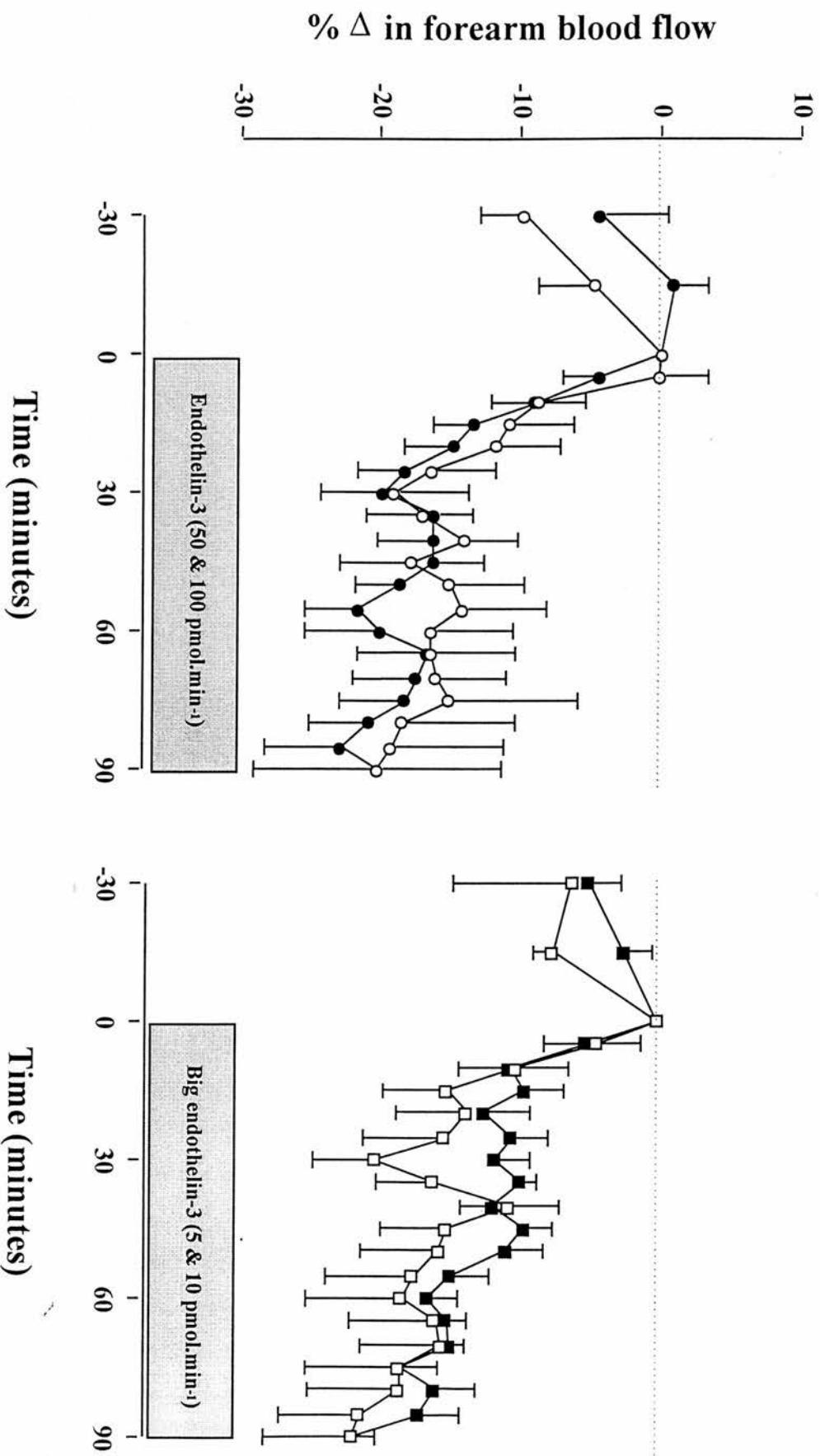
Figure 4.2
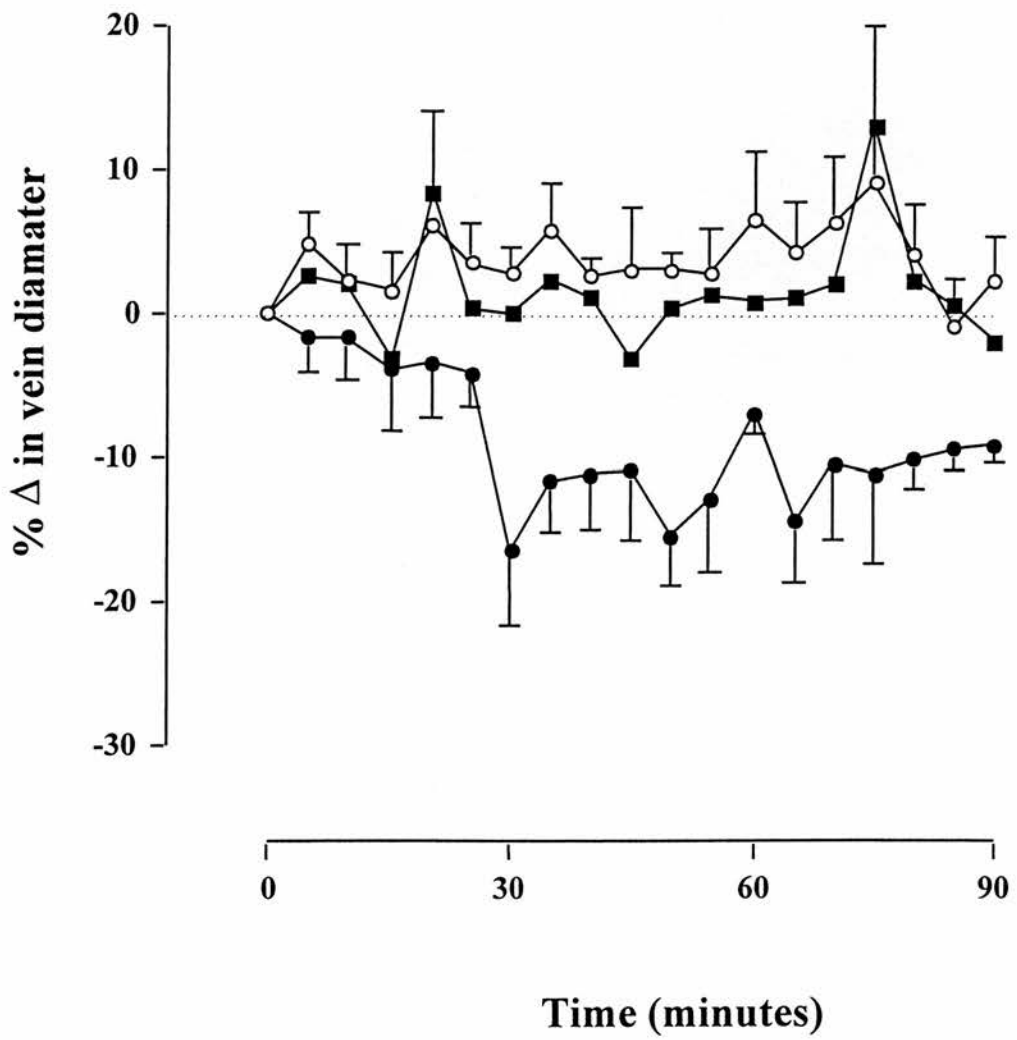
5.   Study 3

Actions of systemic endothelin receptor blockade on peripheral vascular resistance and blood pressure in humans.

5.1 Introduction

The physiological relevance of endogenous generation of endothelin in the control of blood pressure has been unclear. If basal generation of endothelin 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.[87, 139, 163, 184, 185, 239] However, most of the studies have 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 only in hypertensive animals,[45, 389] leading to suggestions that ET-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,[45, 345, 389] suggesting that endothelin plays a similar role in hypertensive and normotensive animals. The lack of a significant effect of antiendothelin therapy on blood pressure in normotensive animals may be due to the

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relative imprecision of measurements of small changes in blood pressure. Other studies have shown that ECE inhibitors and endothelin receptor antagonists do decrease blood pressure in normotensive animals.[92, 229, 345, 438, 591] These positive studies have usually examined haemodynamic responses for several hours after drug administration, thereby taking into account the known slow reversal of ET-1 induced vasoconstriction by antiendothelin therapy.[596]

Recent work has demonstrated that brachial artery infusion of the ECE inhibitor phosphoramidon and the ET_A receptor antagonist, BQ-123 causes progressive forearm vasodilatation.[218] These effects of phosphoramidon and BQ-123 on forearm blood flow indicate a physiological role for basal generation of ET-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 results that changes in blood pressure may be quite small in relation to the effects of a drug on peripheral resistance.[215] The magnitude of any potential effect of systemic endothelin blockade on haemodynamics in healthy human subjects have not previously been reported. Therefore, the haemodynamic effects of systemic administration of TAK-044, a combined $ET_{A/B}$ receptor antagonist, in healthy male subjects were examined.

5.2   Methods

5.2.1 Subjects

Two groups of five healthy male subjects, between 21 and 60 years of age and within 15% of ideal body weight were recruited. 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.

5.2.2   Drugs

TAK-044 is a cyclic hexapeptide (cyclo{D-α-aspartyl-3-{4-phenylpiperazin-1-yl)carbonyl}-L-α-aspartyl-D-2-(2-thienyl)-glycyl-L-leucyl-D-tryptophyl} disodium salt; molecular weight 972) that potently antagonises ^{125}I-ET-1 binding at both ET_A (IC_{50}=0.08 nmol.l^{-1}) and ET_B (IC_{50}=120 nmol.l^{-1}) receptors *in vitro*.[267] TAK-044 also blocks constriction of isolated coronary vessels to ET-1 (ET_A and ET_B agonist) and sarafotoxin S6c (ET_B agonist).[267] 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^{-1}.[600] Specificity has also been shown *in vivo*, where systemic pretreatment of rats with

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TAK-044 at 10 mg.Kg^{-1} does not alter pressor or depressor responses to phenylephrine, ANG II, nitroglycerine and acetylcholine.[241] In contrast, TAK-044 dose-dependently blocks the pressor response to bolus doses of ET-1 and sarafotoxin S6c in rats with 90% blockade apparent at a dose of 10 mg.Kg^{-1}, the effects of which persist for 3 hours.[241, 600] For the clinical studies in humans, doses of TAK-044 ranging between 10 and 100 mg were chosen. This dose range was based on the animal evidence of specificity and efficacy at doses from 0.1 to 10 mg.Kg^{-1} and also the safety profile of TAK-044 in toxicological studies (information on file, Takeda Euro R&D Centre GmbH). Pharmaceutical grade TAK-044 for parenteral use was obtained from Takeda Euro R&D GmbH and was dissolved in physiological saline (0.9%; Baxter Healthcare Ltd). The placebo was dextrose (50 mg), also dissolved in physiological saline. 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.hr^{-1} for a period of 15 minutes (total volume 50 ml). This cannula was not used for blood sampling.

5.2.2 Measurements

Systemic haemodynamics

Blood pressure and heart rate were measured with semiautomated oscillometric monitors (see Section 2.2.1). Cardiac function (stroke volume, cardiac output, and heart rate) were measured with a noninvasive bioimpedance methodology (see Section 2.3).

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 haematology screen (full blood cell count, white blood cell differential count).

Pharmacokinetic and endothelin assays

Fifteen ml venous blood samples were obtained at intervals for assay of serum TAK-044 (see Section 2.5.9) and plasma immnuoreactive endothelin (see Section 2.5.5).

5.2.4 Study Design

Two groups of five subjects were recruited to a double-blind, ascending dose, crossover study with a randomised placebo phase. Group 1 subjects were studied on 5 occasions, receiving placebo and 10, 100, 500 and 1000 mg TAK-044, with 7 days between phases. Group 2 subjects were studied on four occasions, receiving placebo and 30, 250 and 750 mg TAK-044, with 7 days between phases. The ascending-dose design allows the evaluation of the safety and tolerability of TAK-044 at lower doses before proceeding to higher doses and entailed that the study day for groups 1 and 2 occur on different days of the same week for the first four weeks of dosing. For example, in the first week, subjects in group 1 received either

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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. Haemodynamic measurements were made and blood samples were obtained for TAK-044 and endothelin concentrations before and after dosing (see Figures 5.1 through to 5.4). Sedation was assessed and 12-lead ECGs were recorded before and after dosing. Blood and urine samples were obtained 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.

5.2.5 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 normogram, to provide measures of stroke and cardiac indexes. Total peripheral resistance was calculated as mean arterial pressure divided by cardiac index and expresed in arbitrary units (AU). For the systemic haemodynamic 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.

Pharmacokinetics of TAK-044 were analysed 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 analysed in a similar manner, with AUC and C_{max} being calculated.

Absolute values are presented as mean $\pm$ SEM. Placebo-corrected haemodynamic changes from baseline were arithmetically averaged over the 24 hours measurements were made, with uniform weighting given to each time point, and are shown in the tables with 95% CIs. Data were analysed statistically by repeated measures ANOVA. Factors included in the ANOVA were subject, dose of TAK-044, time point, and 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 analyses trends between groups of subjects that are categorised quantitatively, using variances derived from the 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).[465] Statistical testing of the linear contrast involved calculation of its SEM using the pooled estimate of variance from

the ANOVA, with the 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 haemodynamic changes. Statistical analyses were performed by use of the software package SAS (version 6.07, SAS Institute Inc).

5.3   Results

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 the minor symptoms. There were no serious adverse events in this study, and no clinically significant abnormalities were detected on safety monitoring (urinalysis, haematology, clinical chemistry, ECG, and sedation scores).

Baseline haemodynamic parameters did not differ between study days (Table 5.1). 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 (Figures 5.1 & 5.2). 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.

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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 (Figures 5.1 & 5.2). Most doses of TAK-044 significantly increased stroke and cardiac indexes (Figure 5.2). Total peripheral resistance index was significantly and substantially reduced at all doses and this effect was sustained for up to 24 hours (Figures 5.1 & 5.2). 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, cardiac index, and total peripheral resistance, although not for blood pressure (Figure 5.2).

TAK-044 increased plasma immunoreactive endothelin concentrations in a dose-dependent manner, with significant increases at all doses except 10 mg (Table 5.2). For example, after 1000 mg, plasma endothelin concentrations increased from 3.3 to 35.7 pg.ml^{-1} within 30 minutes. Compared with the sustained haemodynamic effects of TAK-044, increases in plasma endothelin were maximal within 30 minutes and waned rapidly, even at the highest doses (Figure 5.3). 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=-0.15; P=0.03) and group 2 (r=-0.16; P=0.02). In group 2 only, plasma endothelin was also correlated with change in systolic (r=-0.19; P=0.005) and mean arterial (r=-0.16; P=0.02) pressures. TAK-044 plasma concentrations increased dose-dependently (Figure 5.4); the terminal half-life was short (30 to 60 minutes) and tended to increase with dose (Table 5.2).

5.4   Discussion

This study is the first report of the effects of systemic endothelin receptor blockade in healthy humans. This study has 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 vascular resistance (by ~26%) over a 24 hour period. Systemic $ET_{A/B}$ receptor blockade also increased circulating immunoreactive endothelin (by up to 1000%). These findings have implications for the physiological role of endothelin generation, the pharmacology of endothelin receptor antagonists, and their ultimate therapeutic relevance.

Previous work has shown that brachial artery administration of an ECE inhibitor or an ET_A receptor antagonist causes local forearm vasodilatation.[218] The finding here that systemic administration of an $ET_{A/B}$ receptor 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.

Physiological role of endothelin in regulation of blood pressure

As noted earlier, animal data on the haemodynamic effects of systemic endothelin receptor antagonism are apparently contradictory. Studies in

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humans have previously shown that brachial artery administration of of an ECE inhibitor or ET_A antagonist causes local forearm vasodilation, suggesting that basal vascular generation of endothelin contributes to vascular tone.[218] This current study demonstrates that systemic administration of an $ET_{A/B}$ antagonist causes peripheral vasodilation and hypotension confirming 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 MAP 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 in consistent with a primary action of TAK-044 on peripheral resistance. 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 endothelin $ET_{A/B}$ receptor antagonist (SB 209670) also decreases blood pressure through an effect on total peripheral resistance.[140] However, heart rate tended to decrease in these animals suggesting other sites of action for endothelin receptor antagonists. The differences between the results of this present study 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 up to 12 to 24 hours. In contrast to its sustained haemodynamic 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 inhibitors or endothelin ET_A receptor blockade usually takes several hours to reach maximum,[45, 92, 229, 345, 389, 438, 591] and forearm vasodilation to these agents is also slow in onset.[218] This gradual effect is thought to be related to the slow dissociation of endothelin from its receptor, resulting in persistent vasoconstriction even after new receptor binding is inhibited. There are two speculative explanations for the rapid onset 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 receptor binding. Second, TAK-044 is active at ET_B as well as ET_A receptors;[241, 267, 295, 600] there is some evidence that vasoconstrictor ET_B receptors may have a more rapid onset of action than ET_A receptors.[196]

ET-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 ng.ml^{-1})) for at least 12 hours after doses

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>500 mg. Furthermore, it is also 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 $ng.ml^{-1}$, ~50-fold greater than the IC_{50} at ET_A receptors. Finally the dissociation between the 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 of in other tissue compartments appear to explain the similar dissociation between actions and plasma concentrations observed for inhibitors of the renin-angiotensin system.[324, 608]

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 study also demonstrated 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 radioimmunoasay used detected both ET-1

and ET-3. Although it also cross-reacted with big ET-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 likeliest explanation for several reasons. First, in animals, blockade of ET_B receptors, but not of ET_A receptors, increases plasma ET-1 and ET-3 concentrations[316] and prolongs the half-life of exogenous ^{125}I-endothelin-1.[178] Second, blockade of endothelin receptors increases plasma endothelin concentrations within 15 minutes,[316] whereas *de novo* generation is thought to take several hours.[635] Third, endothelin receptor blockade does not increase big ET-1 concentrations.[316] 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.

In conclusion, this study has 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 physiological role for endogenously generated endothelin in cardiovascular regulation. 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. However, an intravenous agent such as TAK-044 could be therapeutically useful in conditions associated with acute vasoconstriction or vasospasm in which endothelin has been implicated in the pathophysiology. It would, therefore, be important to know whether TAK-044 can inhibit peripheral vasoconstriction to exogenous endothelin as a model of vasospasm. This was one of the aims of the next study.

5.5   Table Legends

Table 5.1   Baseline haemodynamic values

Footnote   There were no significant differences between baseline values on the different study days for either group 1 or 2.

Table 5.2   Summary pharmacokinetic parameters for plasma immunoreactive endothelin concentrations and TAK-044.

Footnote 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<0.05 vs. placebo; †P<0.005 vs. placebo.

5.6   Figure Legends

Figure 5.1   Time course of the effects of TAK-044 (1000 mg) on systemic haemodynamics

Time course of the effects of the highest dose of TAK-044 (1000 mg) on MAP, HR, SI, CI and SVRI in group 1. TAK-044 significantly decreased MAP (P<0.001) and SVRI (P<0.001) and increased HR (P<0.001), SI (P<0.034), and CI (P<0.001); 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}).

Figure 5.2   Mean haemodynamic changes over 24 hours after dosing with TAK-044.

Mean haemodynamic changes (Δ) over 24 hours after dosing with TAK-044. For the placebo columns (open), mean change from predose is shown. For the active treatment columns (stipled), placebo-corrected changes from predose are shown (change from predose {active} minus mean change from predose {placebo}). *P=0.05 for comparison with predose; †P=0.05 for linear contrast trend with dose.

Figure 5.3   Effect of TAK-044 on plasma immunoreactive endothelin

Effect of TAK-044 on plasma immunoreactive endothelin concentrations. 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.

Figure 5.4   Pharmacokinetic profile of TAK-044

Graph showing pharmacokinetic profiles of TAK-044. 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 5.2). Plasma concentrations after <0.025 µg.ml^{-1} for all doses.

Table 5.1

	SBP, mm Hg	DBP, mm Hg	Heart Rate, bpm	Stroke Index, ml.l⁻²	Cardiac Index, L.min⁻¹.m⁻²	SVRI, AU
	SBP,	DBP,	Heart Rate,	Stroke Index,	Cardiac Index,	SVRI,
	mm Hg	mm Hg	bpm	ml.l^{-2}	L.min^{-1}.m^{-2}	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

Table 5.2

	Plasma Endothelin		Plasma TAK-044		
	C_{max} pg.ml^{-1}	AUC, pg.h.ml^{-1}	C_{max} ng.ml^{-1}	AUC, ng.h.ml^{-1}	$t_{1/2}$, 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

Figure 5.1

Time from administration of TAK-044 (hr)

Figure 5.2

Figure 5.3

A

B

Time

Figure 5.4

A:

B:

Time from administration of TAK-044 (hr)
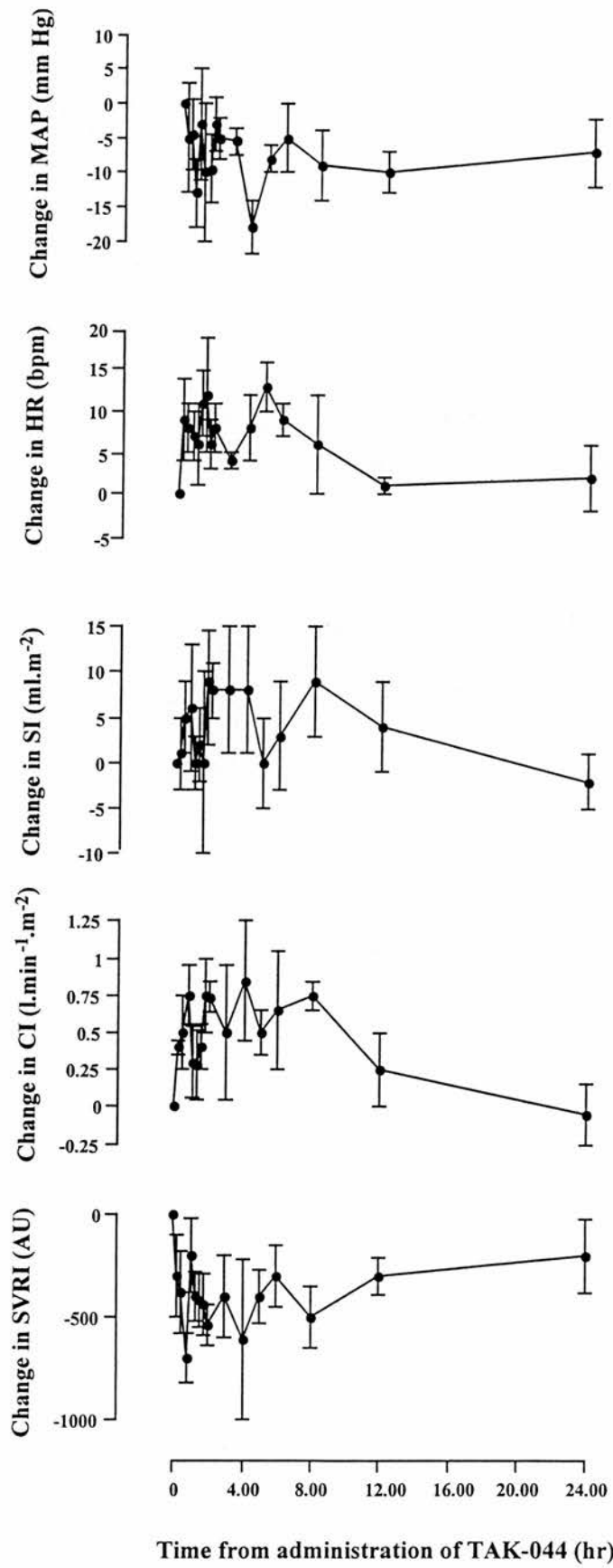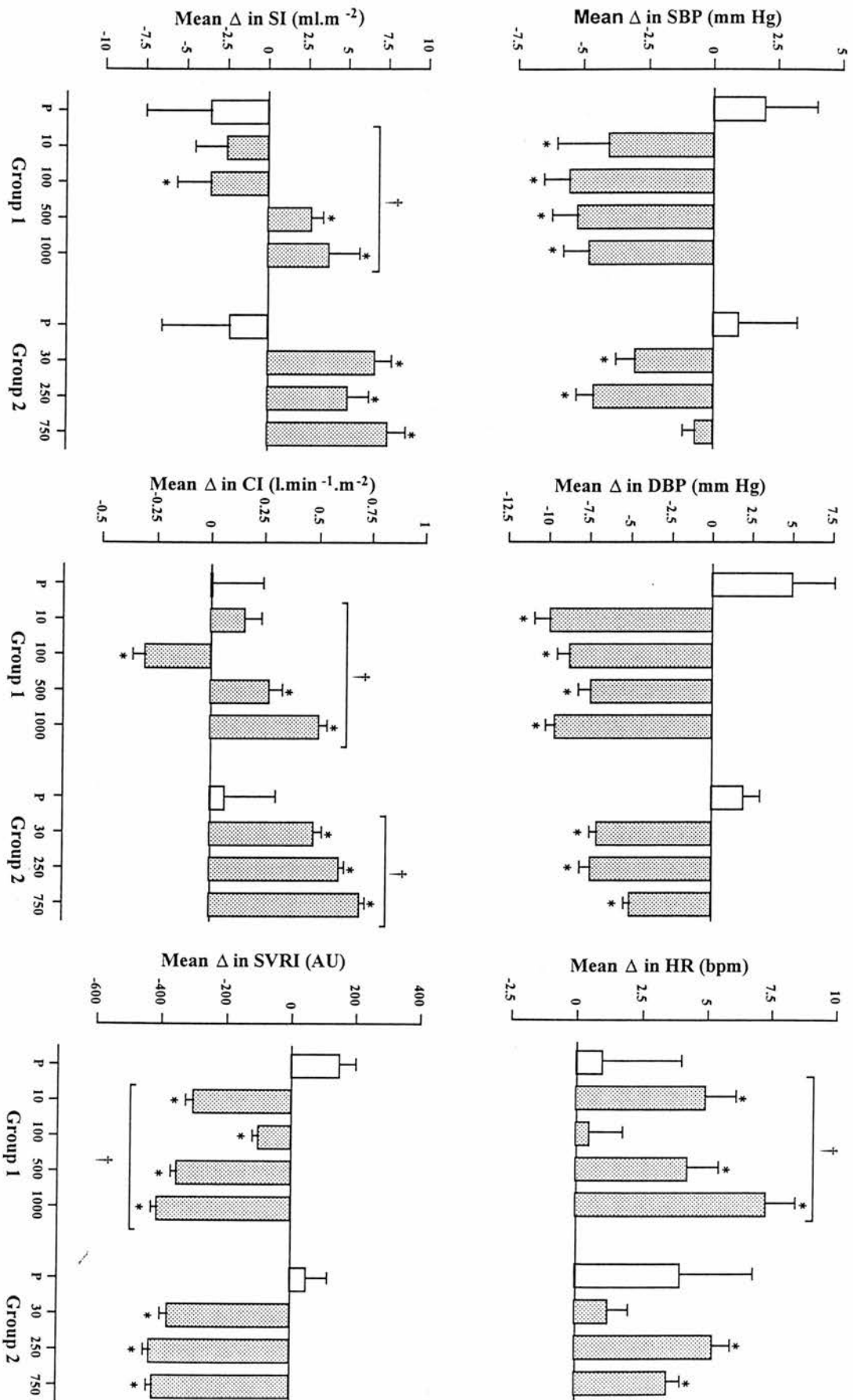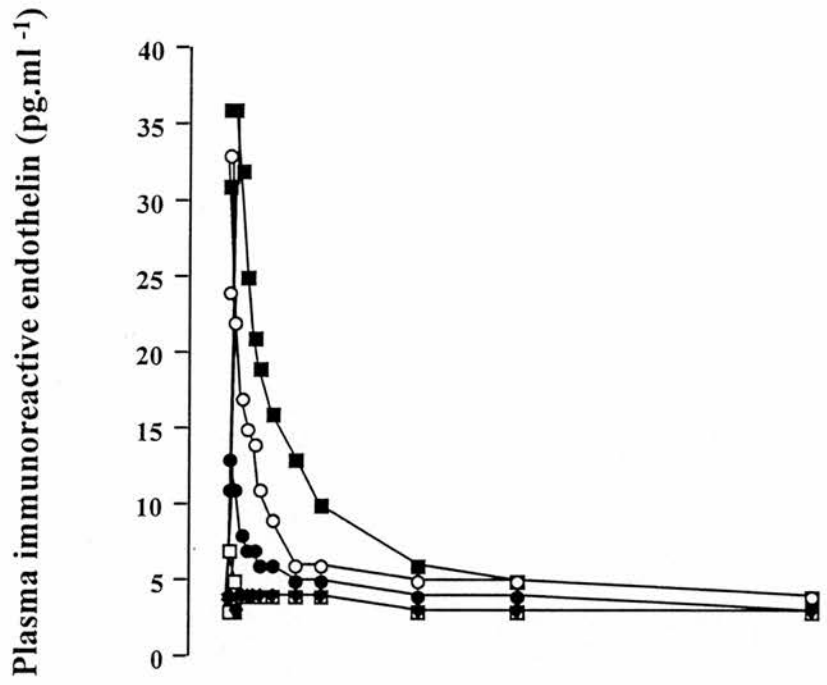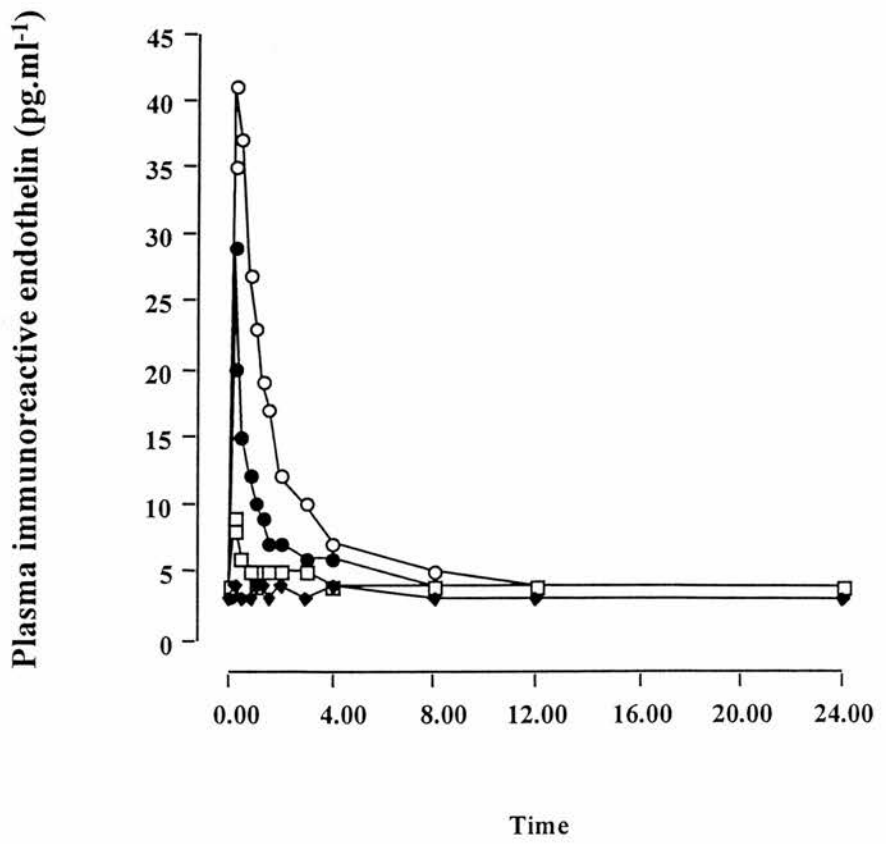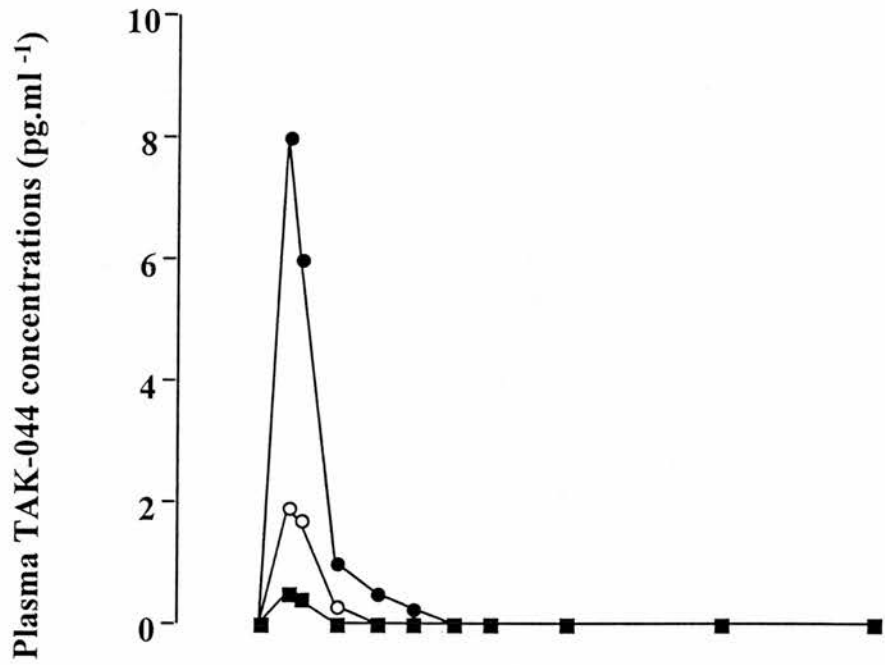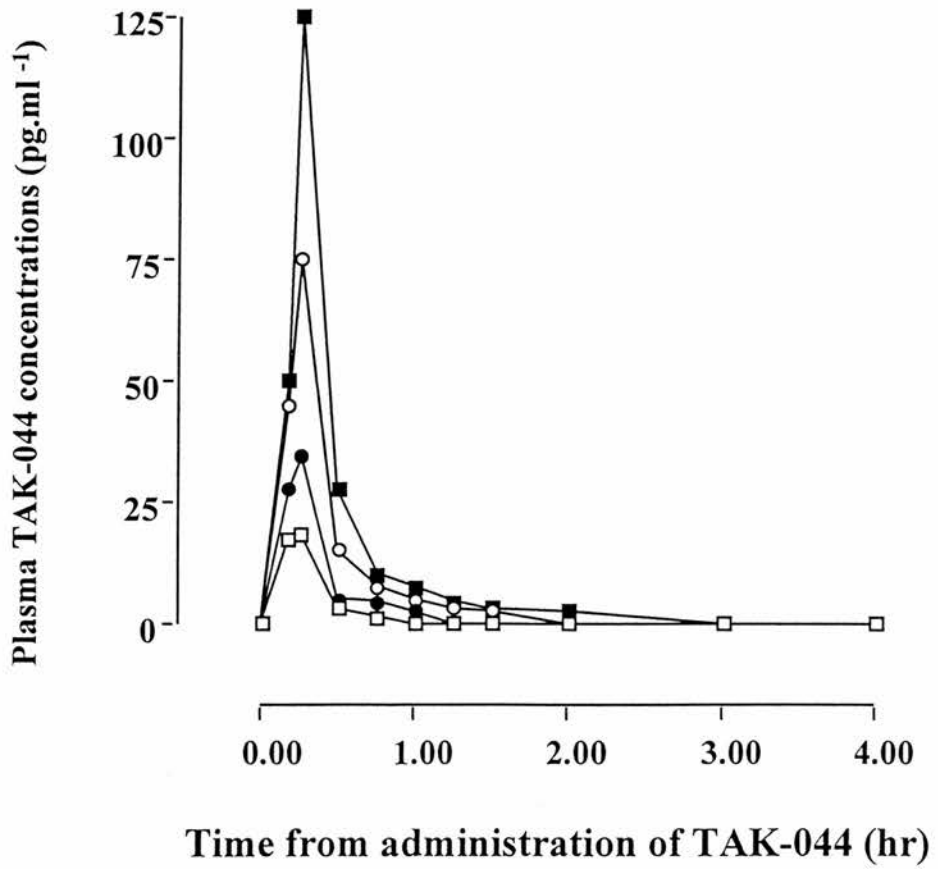
6.   Study 4

Inhibition of endothelin-1 mediated vasoconstriction by systemic administration of an endothelin receptor antagonist.

6.1   Introduction

6.2   Methods

     6.2.1   Subjects

     6.2.2   Drugs

     6.2.3   Measurements

     6.2.4   Study Design

     6.2.5   Data presentation and statistical analysis

6.3   Results

6.4   Discussion

6.5   Table Legends

6.6   Figure Legends

6.1 Introduction

This study was designed to examine whether it is possible to inhibit ET-1 induced vasoconstriction without causing substantial systemic haemodynamic effects. ET-1 has been implicated in the pathophysiology of conditions associated with acute vasoconstriction such as subarachnoid haemorrhage and acute renal failure (see Section 1.3). In these conditions, inhibition of ET-1 mediated vasoconstriction would be potentially therapeutically beneficial, whereas lowering of systemic arterial pressure might be disadvantageous.

This issue was addressed using a model of ET-1 mediated vasospasm in the forearm using brachial artery administration of a non-systemic, locally active dose of ET-1 over a period of 2 hours. Three different doses of TAK-044 were given systemically and their effect on systemic haemodynamics, as well as their effectiveness in reversing ET-1 mediated vasoconstriction was assessed. The direct effects of brachial artery infusion of TAK-044 were also investigated.

6.2 Methods

6.2.1 Subjects

Eight healthy male subjects, between 18-65 years of age and within 15% of their ideal body weight were recruited. 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.

6.2.2 Drugs

TAK-044 (see Section 5.2.2) at doses of 30, 250 and 750 mg were chosen. This dose range was based on the animal evidence of specificity and efficacy at doses from 0.1 to 10 $mg.Kg^{-1}$ and also on the safety profile of TAK-044 on toxicological studies at higher doses (see Section 5.2.2). Pharmaceutical grade TAK-044 was dissolved in physiological saline (0.9%; Baxter Healthcare Ltd). The placebo was dextrose (50 mg), also dissolved in physiological saline.

ET-1 was administered intra-arterially at a dose of 5 $pmol.min^{-1}$, based on previous work showing that this dose of ET-1 causes slow-onset vasoconstriction of human forearm resistance vessels *in vivo*.[217, 218] Pharmaceutical grade ET-1 was obtained from Clinalfa AG (Novabiochem) and dissolved in physiological saline (0.9%; Baxter Healthcare Ltd) to a final concentration of 5 $pmol.ml^{-1}$.

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 $ml.hr^{-1}$ over a period of

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15 minutes (total volume 50 ml). This cannula was not used for blood sampling. For intra-arterial infusion of ET-1 or TAK-044, the left brachial artery was cannulated under local anaesthesia (see Section 2.1.1). Patency was maintained by infusion of 0.9% physiological saline. The total rate of infusion was maintained constant at 1 ml.min^{-1}.

6.2.3 Measurements

Systemic haemodynamics

Blood pressure and heart rate were measured with semiautomated oscillometric monitors (see Section 2.2.1). Cardiac function (stroke volume, cardiac output, and heart rate) were measured with a noninvasive bioimpedance methodology (see Section 2.3).

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 haematology 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 (see Section 2.1.1).

Pharmacokinetic and endothelin assays

Fifteen ml venous blood samples were obtained at intervals for assay of serum TAK-044 (see Section 2.5.9) and plasma immnuoreactive endothelin (see Section 2.5.5).

6.2.4 Study Design

Eight subjects were recruited to a five-phase, double-blind, randomised, placebo-controlled crossover study, with at least 7 days between phases. These studies using forearm blood flow 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 7:00 AM, and blood and urine samples obtained for safety assessments before dosing. At least 1 hour before dosing, an antecubital fossa 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 TAK-044 IV over 15 minutes, at ~9 AM. Brachial artery cannulation was performed once the infusion of TAK-044 or placebo had finished, and intra-arterial infusion of ET-1 (5

pmol.min^{-1}) 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 Figure 6.1) 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 Figure 6.2), 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.

6.2.5 Data presentation and statistical analysis

Systemic haemodynamic data was analysed as described in Section 5.2.5. Plethysmographic data listings were extracted from computer data files and analysed as described in Section 2.1.1. Pharmacokinetics of TAK-044 and plasma immunoreactive endothelin concentrations were analysed as described in Section 5.2.5.

Absolute values are presented as mean ± SEM. Placebo-corrected haemodynamic changes from baseline were arithmetically averaged over

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the 3 hours measurements were made, with uniform weighting given to each time point, and are shown in the tables with 95% CIs. Data were analysed statistically by repeated measures ANOVA as described in Section 5.2.5. Dose-response trends were assessed statistically by the technique of linear contrast as described in Section 5.2.5. Simple regression analysis was used to explore whether there was a correlation between plasma endothelin and haemodynamic changes. Statistical analyses were performed by use of the software package SAS (version 6.07, SAS Institute Inc).

6.3   Results

As in Study 5, all intravenous doses of TAK-044 significantly decreased diastolic blood pressure, increased heart rate and cardiac index, and caused peripheral vasodilatation (Table 6.1), with effects sustained over the three hour measurement period. There were significant dose-related trends for systolic blood pressure, mean arterial blood pressure, and total peripheral resistance (Table 6.1). As in Study 5, plasma immunoreactive endothelin concentrations were increased in a dose-dependent manner, with significantly higher C_{max} values after 250 mg (22.9 pg.ml^{-1}; $P<0.001$) and 750 mg; (37.2 mg.ml^{-1}; $P<0.0001$) compared with placebo (7.8 pg.ml^{-1}). Similarly, there were significant correlations between plasma endothelin concentrations and changes in cardiac index ($r=0.23$; $P=0.01$), diastolic pressure ($r=-0.23$; $P=0.01$), mean arterial pressure ($r=-0.21$; $P=0.006$).

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 of the ET_B receptor, which in addition to its functional roles, appears to mediate clearance of circulating ET-1.[178, 316] 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 this study, forearm vasoconstriction to ET-1 at 1 to 3 hours after dosing with TAK-044 was used to test endothelin receptor blockade. Vasoconstrictor responses to locally infused ET-1 were completely inhibited by all three doses, consistent with the similar haemodynamic responses to these doses. This model is safer than using intravenous infusion of systemic doses of ET-1 to increase blood pressure, particularly given the sustained and potent nature of vasoconstriction to ET-1. Given that both ET_A and ET_B receptors mediate vasoconstriction to ET-1 in the forearm,[217] blockade of vasoconstriction to ET-1 is likely to reflect antagonism at both ET_A and ET_B receptors. Antagonism of ET_B receptors could be tested by brachial artery administration of a selective ET_B receptor agonist, such as sarafotoxin S6c.[217] There are currently no selective ET_A receptor agonists available.

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The potent inhibition of peripheral vasoconstriction to exogenous ET-1, as a model of vasospasm, by TAK-044 in this study suggests that it could be of benefit in conditions associated with acute vasoconstriction in which ET-1 has been implicated. Indeed, in experimental animal models, TAK-044 has been shown to prevent postischaemic renal failure[295] and limit myocardial infarct size.[600] The peptide nature of TAK-044, which requires it to be given intravenously, is not a problem in these acute conditions which are usually managed in association with intensive monitoring.

In conclusion, this study shows that intra-arterial infusion of TAK-044 causes local and systemic vasodilatation and that systemic dosing with TAK-044 at doses of 30, 250, and 750 mg completely inhibited the vasoconstriction produced by brachial artery infusion of ET-1 for at least three hours after administration. However, the duration of this inhibition is unknown.

Table 6.1

| | 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 -5.5	-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	2.8 ± 0.6
Mean Δ over 3 h	-3.1	+4.4	+2.3	+0.43
95% CI	-6.3 to +0.2	+3.3 to +5.5	+1.1 to +3.6	+0.37 to +0.49
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	+0.034	+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
SVRI*, 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

Table 6.2

Phases 1 to 4

Time from IV TAK-044	Placebo	IV TAK-044		
		30 mg†	250 mg†	750 mg†
-10 min (before IV TAK-044)				
Infused	3.4 ± 1.0	3.2 ± 0.3	3.4 ± 0.6	2.8 ± 0.3
Control	3.1 ± 0.9	2.6 ± 0.3	3.2 ± 0.5	2.9 ± 0.4
Ratio	1.08 ± 0.08	1.25 ± 0.10	1.01 ± 0.08	0.97 ± 0.09
+55 min (before IA endothelin-1)				
Infused	4.3 ± 1.4	4.7 ± 0.8	4.3 ± 0.8	3.3 ± 0.4
Control	3.0 ± 0.6	3.4 ± 0.4	3.9 ± 0.5	3.5 ± 0.4
Ratio	1.29 ± 0.17	1.35 ± 0.10	1.05 ± 0.10	0.99 ± 0.09
+120 min (after IA endothelin-1)				
Infused	4.3 ± 1.5	4.7 ± 0.8	4.4 ± 0.6	3.8 ± 0.4
Control	3.9 ± 0.9	3.6 ± 0.4	4.2 ± 0.5	4.3 ± 0.6
Ratio	0.93 ± 0.13*	1.29 ± 0.10	1.08 ± 0.10	0.96 ± 0.10
+180 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

Phase 5

Time from IA TAK-044	IA TAK-044 10/100 mg
-10 min (before IA TAK-044)	
Infused	2.9 ± 0.2
Control	2.3 ± 0.2
Ratio	1.27 ± 0.10
+60 min (after 10 mg IA TAK-044)	
Infused	3.9 ± 0.4
Control	2.8 ± 0.3
Ratio	1.43 ± 0.08‡
+120 min (after 10 mg IA TAK-044)	
Infused	4.1 ± 0.3
Control	3.5 ± 0.5
Ratio	1.24 ± 0.10
	...
	...
	...

Figure 6.1

Figure 6.2

7. Study 5

Duration of inhibition of endothelin-1 mediated vasoconstriction by systemic administration of an endothelin receptor antagonist.

7.1   Introduction

The previous studies have shown that systemic intravenous administration of the cyclic hexapeptide combined $ET_{A/B}$ receptor antagonist, TAK-044, 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 hours. 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 three hours.

Endothelin receptor antagonists are currently under clinical development. 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. The potential of TAK-044 to inhibit the forearm vasoconstriction produced by brachial artery infusion of ET-1 for up to 12 hours after dosing was therefore studied.

7.2   Methods

7.2.1 Subjects

Eighteen healthy male subjects between 19 and 41 years of age participated in these studies. All had normal baseline results on routine biochemical and haematolgical screening tests. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the two weeks before the study or during the study period. All of the subjects abstained from alcohol for 48 hours, from caffeine containing drinks and cigarettes for at least 24 hours, and from food for 12 hours 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.

7.2.2 Drugs

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 hours after administration. 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.

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7.2.3 Study design

Eighteen subjects took part in a randomized, placebo-controlled, single-blind, three-way, crossover study. Subjects were studied on three occasions, each one week or more apart. They were admitted to the clinical research centre at 8 PM on the day before the study and were discharged at 1 PM on the study day. On two occasions subjects received TAK-044 either at 12 PM or 4 AM. 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 6 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 AM and ending at 12 AM. The end of the infusion corresponding to either 8 or 12 hours after administration of TAK-044. Ten-millilitre venous blood samples were obtained at 10 AM and 12 AM for assay of plasma endothelin and five-millilitre venous samples were obtained at 8 AM, 10 AM and 12 AM 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 9:30 AM and before discharge on every study day.

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7.2.3 Data Presentation and Statistical Analysis

From previous studies using intrabrachial ET-1 infusions done in the Department of Clinical Pharmacology at Edinburgh University (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 6 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).

Data are described as mean $\pm$ SEM, with 95% CI where appropriate. Haemodynamic data are presented as absolute values and as placebo-corrected changes from baseline (12 PM). Data were examined by repeated measures 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.

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7.3   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 7.1). 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 7.1) increased only after dosing with 100 mg TAK-044 at 12 PM (maximum increase: 8 ± 3 beats per minute; $p=0.03$). 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 AM (Table 7.1). Mean arterial pressure and heart rate did not change during infusion of ET-1 (Figure 7.1). Administration of TAK-044 did not cause an increase in immunoreactive endothelin concentrations after 8 or 12 hours compared with placebo (Table 7.2).

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 8 AM and only after administration of TAK-044 at 4 AM. In group 3,

TAK-044 was detectable at 8 AM after administration of TAK-044 at 12 PM and 4 AM. TAK-044 was also detectable in plasma at 10 AM after administration of TAK-044 at 12 PM (Table 7.2). 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 7.1). 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 minutes 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 7.2; Table 7.3). 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 7.2; Table 7.3). However, when the responses from all three groups were combined, vasoconstriction to ET-1 was reduced by TAK-044 administered 8 and 12 hours previously (Figure 7.3; Table 7.3), when compared with placebo.

7.4   Discussion

The combined endothelin $ET_{A/B}$ receptor antagonist, TAK-044, given as a 15-minute intravenous infusion, attenuated peripheral vasoconstriction to exogenous ET-1 by ~ 30% for up to 12 hours after administration. This

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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[88, 217] 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.[434, 442] 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.[103, 604]

The vasoconstriction produced by intra-arterial ET-1 in this study was consistent with other published reports.[88, 217] 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 hours after administration. However, this contrasts with complete inhibition for up to 3 hours of ET-1 mediated vasoconstriction by TAK-044 30 mg and suggests a marked time dependence for this inhibitory action. This was a small study with

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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 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 vasoconstriction. 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. Furthermore, changes in circulating endothelin concentrations are likely to reflect only antagonism of the ET_B receptor[271] which, in addition to its functional roles, appears to mediate clearance of circulating ET-1.[178, 316] 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 the previous studies (Studies 3 & 4). Similarly, here it was found that plasma endothelin concentrations were not raised 12 hours after administration of TAK-044 despite continuing inhibition of ET-1 mediated vasoconstriction.

TAK-044 was not detected in any of the volunteers 12 hours after administration of any of the doses. These findings are in close agreement with the previous studies (Studies 3 & 4) in which the plasma half-life of TAK-044 was 30 to 60 minutes. However, the IC_{50} for TAK-044 at ET_A receptors is 0.08 ng.ml^{-1} ~ 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 hours. 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.[227] Indeed, in intact cells ET-1 appears only to become dissociated from its receptors following receptor internalisation.[332] 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.[324, 608] These possibilities require further investigation.

The previous studies (Studies 3 & 4) showed that TAK-044 lowers systemic vascular resistance and blood pressure. 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 (Studies 3 & 4) and heart rate was increased after administration of the highest dose of TAK-044. However, in this study systemic vascular resistance was not measured, the most sensitive index of peripheral vasodilatation in the previous study (Study 3).

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 hours 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

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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.

7.5 Table Legends

Table 7.1   Haemodynamics in groups 1, 2 and 3 before intra-arterial ET-1 infusion.

Footer:

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

Footer:

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.

Table 7.3   Mean percentage vasoconstriction to 120 minutes intra-arterial ET-1 infusion after Placebo and TAK-044 8 and 12 hours earlier.

Footer:

*p=0.01. 95% confidence intervals are shown in brackets.

7.6 Figure Legends

Figure 7.1   Haemodynamic changes after systemic TAK-044

Placebo corrected changes (Δ) in mean arterial pressure (MAP) and heart rate (HR) from baseline (12 PM) following intravenous administration of TAK-044 at 12 PM (open bars) and 4 AM (shaded bars) for group 1, 2 and 3.

*P=0.05 from baseline.

Figure 7.2   Effect of 3 doses of TAK-044 on forearm vasoconstriction to locally administered endothelin-1 8 and 12 hours after

Percentage change in forearm blood flow produced by brachial artery infusion of endothelin-1 (5 pmol.min^{-1} for 2 hours) following intravenous administration of TAK-044 at 12 PM (o) and at 4 AM ($\bullet$) and during the placebo phase ($\square$) for groups 1, 2 and 3. Standard errors have been omitted for sake of clarity.

Figure 7.3   Combined results of the 3 doses of TAK-044 on forearm vasoconstriction to locally administered endothelin-1 8 and 12 hours after

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 hours from 10 AM to 12 PM) following intravenous administration of placebo (□) and TAK-044 at 4 AM (•) and at 12 PM (o). Endothelin-1 caused a slowly progressive forearm vasoconstriction during the placebo phase. TAK-044 (25-100 mg) administered at either 4 AM and 12 PM (corresponding to 8 and 12 hours before the end of the endothelin-1 infusion) significantly inhibited this vasoconstriction (P=0.01).

Table 7.1

	Systolic Blood Pressure (mm Hg)	Diastolic Blood Pressure (mm Hg)	Heart Rate (beats per minute)	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 12 PM	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 4 AM	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 12 PM	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 4 AM	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 12 PM	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 4 AM	113 ± 3 (115 to 138)	62 ± 2 (56 to 67)	56 ± 3 (48 to 63)	4.0 ± 0.9 (1.7 to 6.3)

Table 7.2.

	Plasma TAK-044 Concentration (ng.ml⁻¹)			Plasma ET Concentration (pg.ml⁻¹)	
	8 AM	10 AM	12 AM	10 AM	12 AM
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 12 PM	<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 4 AM	<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 12 PM	<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 4 AM	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 12 PM	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 4 AM	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)

Table 7.3

	Placebo	TAK-044 at 12 PM	Difference from placebo	TAK-044 at 4 AM	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)*

Figure 7.1

Figure 7.2

Group 1
25 mg TAK-044

Group 2
50 mg TAK-044

Group 3
100 mg TAK-044

Endothelin-1 (5 pmol.min⁻¹)

9.30 AM 10 AM   12 PM
Time

%change in forearm blood flow

Figure 7.3

% change in forearm blood flow
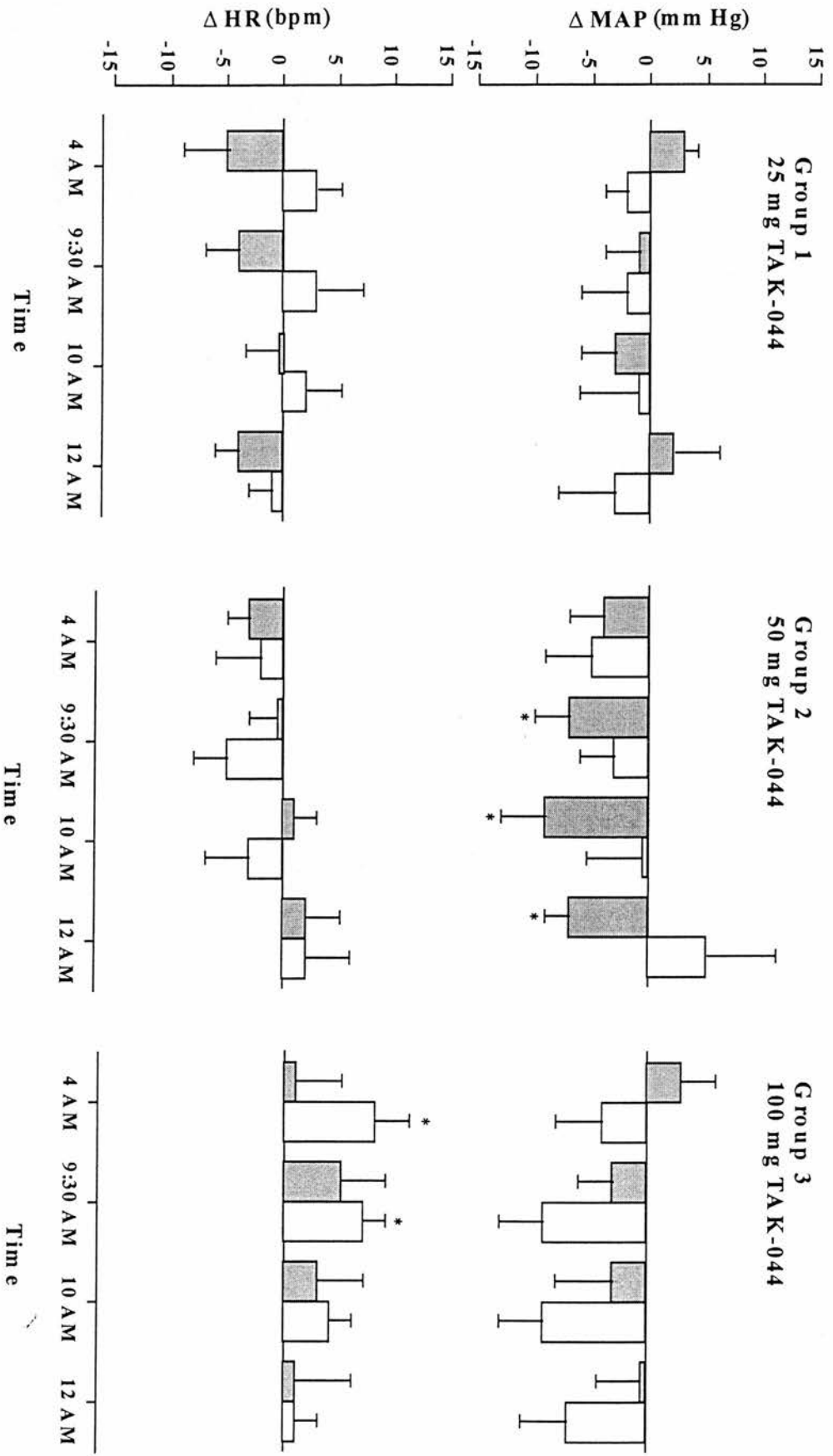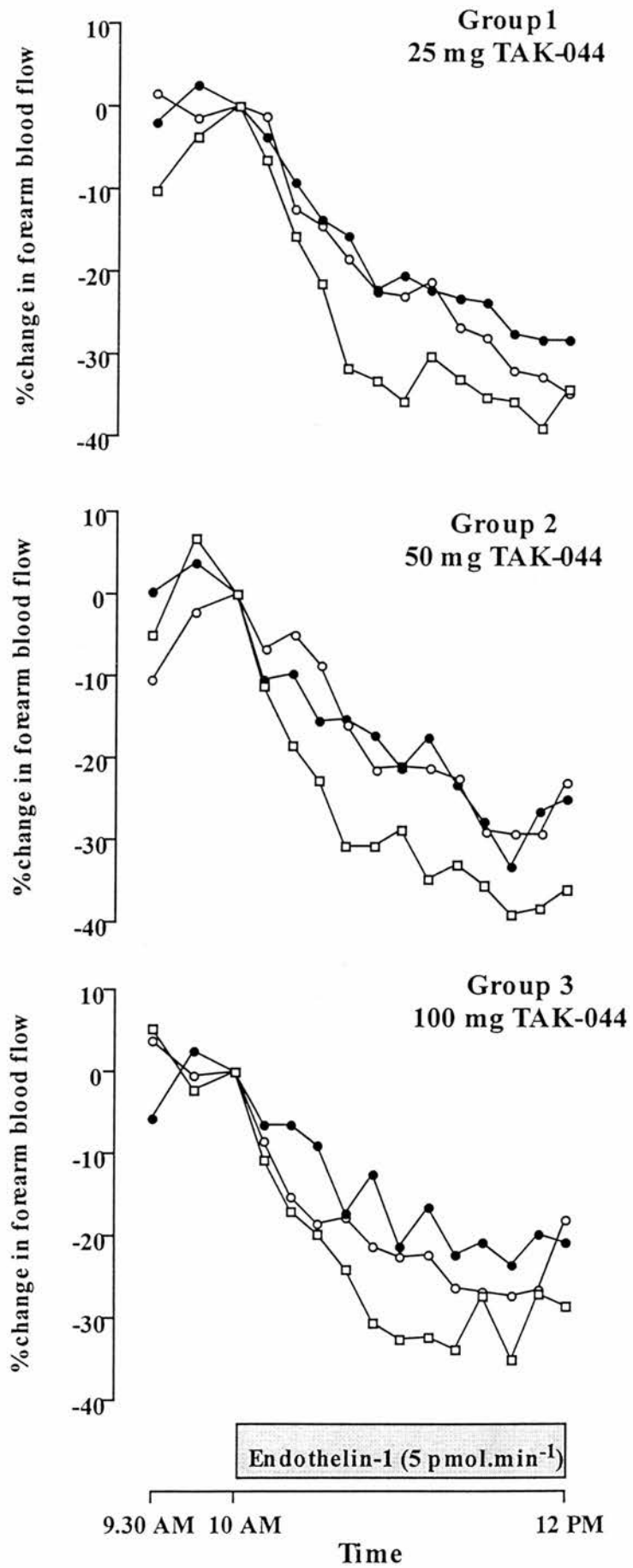
TAK-044 given at 12 PM
(12 hours before end of endothelin-1 infusion)

TAK-044 given at 4 AM
(8 hours before end of endothelin-1 infusion)

Endothelin-1 (5 pmol.min⁻¹)

Time
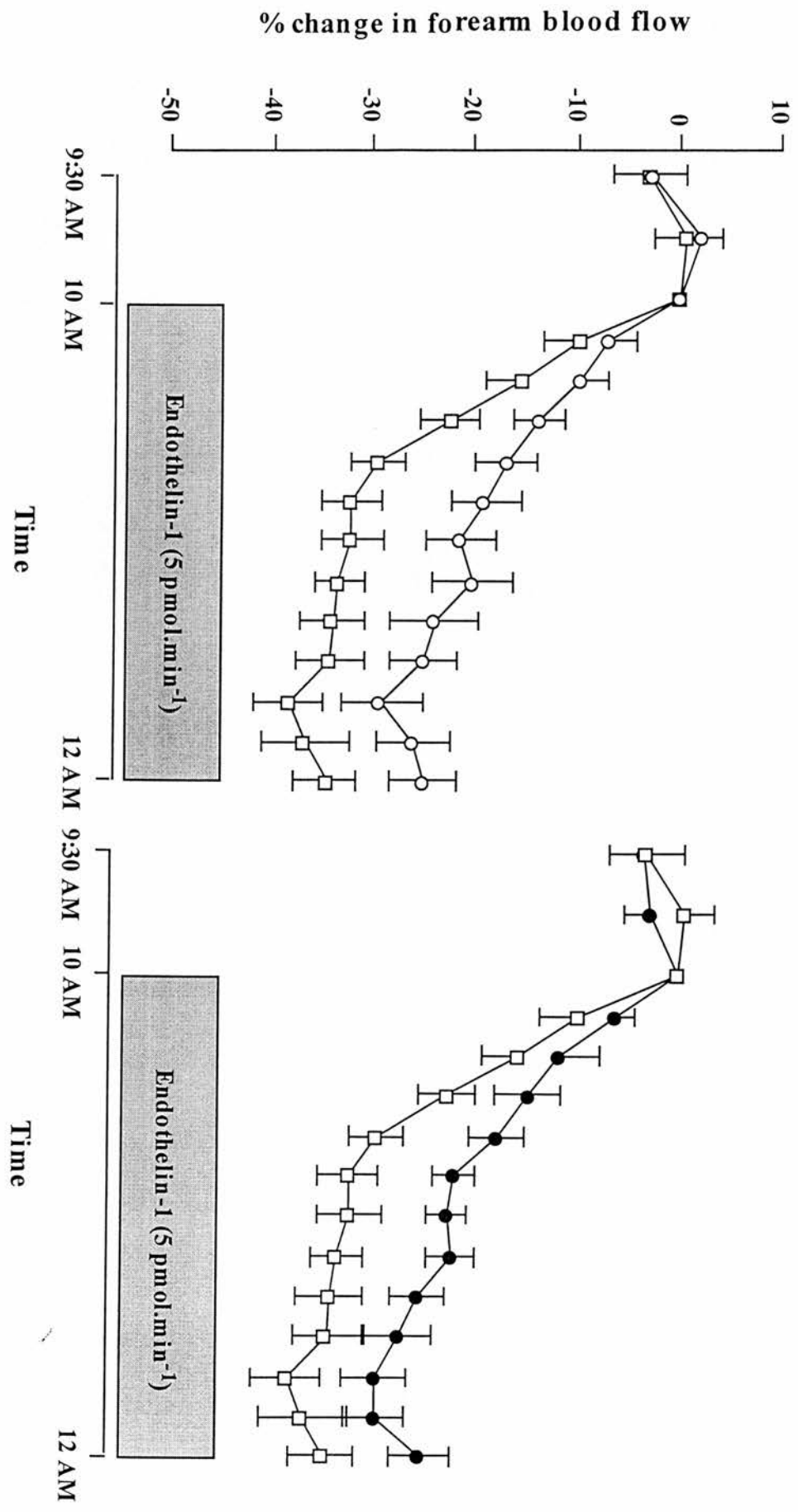
8.   Study 6

Actions of systemic endothelin receptor blockade on renal function in healthy humans.

8.1   Introduction

The kidney has been found to have high concentrations of immunoreactive endothelin,[272] mainly ET-1.[259] A variety of cell types in the kidney, other than vascular endothelial cells, including mesangial and glomerular as well as renal tubular cells are able to produce endothelin.[277] The kidney also has a high density endothelin receptors, although the distibution of receptor subtypes appears to be very variable between species.[277] In human kidney ET_B receptors predominate and are mainly localised to tubules and collecting ducts.[257] In contrast, the ET_A subtype is largely confined to the smooth muscle of the renal vasculature.[360] Systemic infusion of exogenous ET-1 in humans increases blood pressure, systemic and renal vascular resistance and sodium retention, as well as decreasing renal blood flow and GFR.[442, 524] However, the actions of endogenously produced endothelin in the human kidney have not yet been studied.

In this study, the actions of endogenously generated endothelin on renal function in healthy subjects were investigated using systemic infusions of the non-selective endothelin receptor antagonist TAK-044 (see Section 5.2.2). The actions of endothelin antagonism on other vasoactive sodium regulating hormones and on plasma catecholamines were also studied.

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8.2 Methods

8.2.1 Subjects

Eight healthy male subjects (age range, 41-49 years) participated in this study. 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 hours and from food, caffeine-containing drinks, and cigarettes for at least 16 hours before baseline measurements were made. A 24-hour 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 hours before baseline measurements) to assess proximal tubular reabsorptive capacity of sodium.[144]

8.2.2 Drugs

The doses of TAK-044 (see Section 5.2.2) studied, 100 and 750 mg mg, were chosen on the basis of Studies 3 and 4. These two doses are both known to cause significant haemodynamic effects and are at the lower and higher ends of the dose range studied.

8.2.3 Measurements

Blood pressure was measured in the right arm in duplicate using a well-validated semi-automated oscillometric technique (see Section 2.1). Cardiac function (stroke volume, cardiac output, and heart rate) was measured using a noninvasive bioimpedance method (see Section 2.2). Effective renal plasma flow (ERPF) and GFR were estimated by measuring the renal clearances of p-aminohippurate sodium (PAH; Merck Hoddesdon, UK) and polyfructosan-S (Inutest; Laevosan, Linz, Austria), respectively (see Section 2.4.1). 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 minutes, followed by a maintenance infusion of PAH (8.3 g.l^{-1}) and polyfructosan-S (10 g.l^{-1}) in 0.9% saline at 120 ml.hr^{-1}. An equilibration period of 90 minutes allowed plasma concentrations of PAH and polyfructosan-S to stabilize before baseline collections started.

8.2.4 Plasma Assays

Venous blood samples were collected into heparinized tubes for assay (see Section 2.5) of plasma polyfructosan-S, PAH, sodium, potassium and aldosterone concentrations, and plasma osmolality; into EDTA tubes for assay of plasma immunoreactive endothelin, big ET-1, C-terminal fragment, plasma active renin and hematocrit (Hct); into plain glass tube for assay of serum lithium concentrations; into EDTA/sodium metabisulphate tubes for assay of plasma adrenaline and noradrenaline;

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into EDTA/trasylol tubes for assay of plasma ANP; and into EDTA/o-phenanthroline tubes for assay of ANG II concentrations. Plasma was separated and frozen within 15 minutes of sampling. Urine was collected into plain plastic containers. All samples were stored at -80°C before assay.

8.2.5 Study Design

This was a randomized, single-blind, crossover study comparing 2 doses of TAK-044, 100 and 750 mg, with placebo (50 g dextrose). Each of the three phases were separated by at least 7 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 hours 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 TAK-044 or placebo to induce a diuresis of ~10 ml.min^{-1}. Except when voiding, subjects rested in a recumbent position. A plastic cannula was inserted under local anesthesia into a superficial antecubital vein of the right arm for blood sampling. Two plastic cannulas were inserted into superficial antecubital veins of the left arm for administration of PAH/poyfructosan-S and TAK-044/placebo. Intravenous infusion of the priming doses of PAH and poyfructosan-S started at ~8:30 AM. During the 90-minute equilibration period, the subject was acclimatised to the haemodynamic assessments by measuring blood pressure and cardiac

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function every 15 minutes. Baseline assessments started at ~10:00 AM, when blood samples were obtained before and after two accurately timed 30-minute urine collections. At ~11:00 AM, TAK-044 100 or 750 mg, or placebo in 50 ml 0.9% saline was infused intravenously over 15 minutes. An additional accurately timed 30-minute urine collection was 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 15-minute intervals before and after dosing with TAK-044. Blood samples were obtained at 30-minute intervals throughout, except for estimation of plasma renin, ANG II, aldosterone, ANP and catecholamines which were taken at hourly intervals. Blood samples for estimation of plasma immunoreactive endothelin peptides were taken at just before and 15, 60 and 120-minutes after TAK-044.

8.2.6 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 normogram, to provide measures of stroke and cardiac index. Total peripheral resistance was calculated as (MAP x 72.269)/cardiac index, and expressed in $dyn.sec.cm^{-5}$. 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

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end of a clearance period. Effective renal vascular resistance (ERVR) was calculated using a formula that assumed a constant renal venous pressure of 10 mm Hg:

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

Filtration fraction was calculated as (GFR/ERPF) x 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_{Li}) is thought to be representative of proximal tubular sodium clearance. The fractional (corrected for GFR) excretions of sodium (FE_{Na}) and lithium (FE_{Li}) were also calculated.

Data are presented as means ± SEM. Placebo-corrected changes from baseline were arithmetically averaged over the relevant measurement period (0 to 240 minutes for haemodynamic changes and 0 to 120 minutes for renal function), with uniform weighting given to each timepoint. Data were analysed statistically by repeated measures analysis of variance (ANOVA). Factors included in the ANOVA were subject, dose of TAK-044, timepoint, and dose-timepoint interaction. In none of the analyses (except plasma endothelin concentrations which were analysed as absolute values by the Kruskal-Wallis non-parametric test with Dunn's multiple comparisons post-tests) was there any evidence of a statistically significant dose-timepoint interaction. Therefore, the adjusted dose group means from the ANOVA were compared with the null hypothesis by a two-sided t-test.

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Statistical analyses were performed by the use of the software package SAS (version 6.07, SAS Institute Inc).

8.3 Results

Baseline data

There were no significant differences between basal values for any parameter on any of the study days. There were no significant changes in any parameter following administration of placebo.

Systemic haemodynamics

Compared with placebo, both doses of TAK-044 decreased MAP, with a decrease of 5% after 100 mg and 6% after 750 mg (Table 8.1). Both systolic and diastolic blood pressure decreased after TAK-044, although this was only significant for diastolic blood pressure with a decrease of 6% after 100 mg and 8% after 750 mg (Table 8.1). These decreases in blood pressure occurred despite significant increases in heart rate and cardiac index with both doses of TAK-044 (Table 8.1). There was, therefore, a substantial decrease in total peripheral resistance index of 13% after 100 mg and 16% after 750 mg TAK-044 (Table 8.1).

Renal function

There were significant reductions in ERVR with a reduction of 5% after both 100 mg and 750 mg TAK-044 (Table 8.2). There was a trend for TAK-044 to decrease GFR. However, the decrease was only significant after 100 mg TAK-044 (Table 8.2). There was also a trend for TAK-044 to increase ERPF although this did not achieve statistical significance (Table 8.2). However, relative trends in decrease in GFR and increase in ERPF meant that the filtration fraction was significantly lowered by 14% after 100 mg and 13% after 750 mg TAK-044 (Table 8.2). Infusion of TAK-044, at both doses, had no effect on sodium and lithium clearance or on overall and fractional sodium and lithium excretions (Table 8.2).

Immunoreative endothelin, big ET-1 and C-terminal fragment

Infusion of 750 mg, but not 100 mg, TAK-044 rapidly increased plasma immunoreactive endothelin concentrations but had no effect on plasma big ET-1 or C-terminal fragment concentrations (Figure 8.1).

Vasoactive hormones

TAK-044, at both doses, tended to increase plasma active renin concentrations although this effect was not significant (Table 8.3). However, TAK-044 did increase plasma ANG II concentrations by 24% after 100 mg and 42% after 750 mg (Table 8.3). Neither dose of TAK-044 had any effect on plama aldosterone concentrations (Table 8.3). There was

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a trend for TAK-044 to increase circulating plasma ANP concentrations, although this effect was only significant after 100 mg TAK-044 (Table 8.3). TAK-044 had no effect on plasma adrenaline or noradrenaline concentrations (Table 8.3).

8.4 Discussion

This present study confirms the previously presented findings that combined $ET_{A/B}$ receptor antagonism with TAK-044 lowers blood pressure and peripheral vascular resistance, as well as increasing heart rate and cardiac index, in healthy subjects (see Studies 3, 4 and 5). This provides further evidence supporting endogenously generated endothelin plays a significant physiological role in the maintainance of blood pressure in man.

In healthy subjects, systemic infusions of ET-1 increased ERVR and decreased both GFR and ERPF.[442, 524] The decrease in ERPF proportionally exceeded the decrease in GFR, as reflected by an increase in the filtration fraction. In this study, TAK-044 decreased ERVR suggesting that endogenously generated endothelin also contributes to renal vascular resistance. However, despite the substantial decrease in ERVR in this study, TAK-044 did not increase ERPF, although there did appear to be a trend. Interestingly, GFR tended to decrease after TAK-044 although this was only significant after the 100 mg dose. As a consequence of these opposing trends in ERPF and GFR there was a significant decrease in filtratration fraction observed with both doses of TAK-044. This finding is

in agreement with exogenous infusion of ET-1 filtration fraction in man[442, 524] and suggests that endothelin acts preferentially on the efferent arteriole in healthy subjects. In laboratory animals, ET-1 contracts both afferent and efferent arterioles and the relative responsiveness of these vessels differs among species.[145, 269]

The increase in filtration fraction with endothelin agonism and increase with endothelin antagonism are similar to the changes observed with agonism and antagonism of renal ANG II receptors.[250] It is possible that the actions of endothelin on renal haemodynamics could be mediated by ANG II. In rats and dogs the decrease in GFR and ERPF caused by ET-1 were abolished by ACE inhibition.[36, 75, 326] However, ANG II anatgonists do not appear to prevent the decrease in ERPF and GFR caused by ET-1[82] suggesting that the actions of ACE inhibitors were being mediated by decreasesd breakdown of bradykinin and stimulation of nitric oxide production. In healthy human subjects, both ACE inhibition[252] and infusion of L-arginine,[59] the substrate for nitric oxide synthesis, prevented the hypertensive effects of ET-1 but neither blocked the increase in renal vascular resistance. Furthermore, in this present study, plasma concentrations of ANG II were significantly increased after administration of TAK-044. These findings strongly suggest that ANG II is not a significant mediator of the renal actions of endothelin in humans.

Endogenously generated renal endothelin is thought to promote sodium excretion and inhibit water resorption.[277] However, systemic infusion of

ET-1 in healthy subjects paradoxically reduces salt and water excretion.[442, 524] Combined $ET_{A/B}$ receptor antagonism with TAK-044 had no clear action on the clearance or fractional excretion of sodium or lithium although this study did not have the power to detect small changes. Furthermore, any potential antinatriuretic effect of antagonism of tubular endothelin receptors (mainly ET_B) may have been counteracted by the haemodynamic changes as a result of vascular endothelin receptor (mainly ET_A) antagonism. Thus, selective ET_A receptor antagonists could potentially have natriuretic actions and further studies with these agents are needed.

TAK-044 tended to increase plasma active renin and ANG II concentrations although the increase was only statistically significant for ANG II. There are several potential mechanisms for this increase, including decreased delivery of sodium to the macula densa, activation of baroreceptor sensors in the afferent arterioles and a decrease in blood pressure. The rise in ANG II observed may have, by negative feedback, inhibited further release of renin. Despite the observed increase in ANG II, there was no demonstrable increase in plasma aldosterone concentrations as might have been expected. However, it is possible that any changes may have been obscured by the normal diurnal variation in plasma aldosterone concentrations, as well as by the effects of prolonged supine posture. Furthermore, ET-1 is known to potentiate ANG II-induced aldosterone secretion[109] and, therefore, endothelin receptor antagonism with TAK-

044 may have attenuated the expected increase in aldosterone concentrations.

TAK-044 at both doses tended to increase plasma ANP concentrations, although these increases were relatively small and only just achieved statistical significance (p=0.05) after the 100 mg dose. The mechanism for this increase is not clear especially given that TAK-044 has been shown to be a potent vasodilator in humans (Studies 3 & 4) and this is likely to result in reduced atrial stretch, a primary stimulus for ANP secretion.[516] Furthermore, ET-1 is known to be a stimulator of ANP secretion by atrial myocytes[488] and, therefore, TAK-044 would be expected to have an inhibitory action on ANP release by these cells. Given that this study only demonstrated a small increase in ANP, and that this increase only occurred after the lower dose of TAK-044, it would appear that this unexpected finding requires further confirmation in larger studies with endothelin receptor antagonism.

Plasma concentrations of adrenaline and noradrenaline did not increase after TAK-044. Thus combined $ET_{A/B}$ receptor antagonism does not appear to activate the sympathetic nervous system. However, the limitations of using a single method for evaluating sympathetic activity have been previously highlighted[330] and these results should, therefore, be interpreted cautiously.

As previously reported,[435] in this study the higher dose of TAK-044, 750 mg, increased plasma immunoreactive endothelin concentrations but not big ET-1 or the C-terminal fragment. This suggests that the observed increase in plasma endothelin concentrations are a result of receptor displacement and not *de novo* synthesis and is a consistent feature of drugs with ET_B receptor antagonist properties.

In conclusion, systemic combined $ET_{A/B}$ receptor blockade with TAK-044 caused peripheral and renal vasodilatation, and lowered blood pressure in healthy subjects. In addition, TAK-044 produced a significant decrease in filtration fraction with only minor changes in GFR and ERPF. These findings suggest an important role for endogenously generated endothelin in controlling systemic and renal haemodynamics in health.

8.5 Table Legends

Table 8.1   Effects of TAK-044 (100mg or 750 mg) given intravenously over 15 minutes on systemic haemodynamics.

Footer:

Mean changes (Δ) from basal over 4 hours are shown corrected for changes with placebo. Values are mean ± SEM in 8 subjects. CI, confidence intervals; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; CI, cardiac index; SI, stroke index; TPR, total peripheral resistance. P values are for changes vs. baseline.

Table 8.2   Effects of TAK-044 (100mg or 750 mg) given intravenously over 15 minutes on renal function.

Footer:

Mean changes (Δ) from basal over 4 hours are shown corrected for changes with placebo. Values are mean ± SEM in 8 subjects. CI, confidence intervals; ERPF, effective renal plasma flow; ERVR, effective renal vascular resistance; GFR, glomerular filtration rate; Na, sodium; Li, lithium; FE_{Na}, fractional sodium excretion; FE_{Li}, fractional lithium excretion. P values are for changes vs. baseline.

Table 8.3   Effects of TAK-044 (100mg or 750 mg) given intravenously over 15 minutes on plasma renin, angiotensin II, aldosterone, atrial natriuretic peptide, adrenaline and noradrenaline.

Footer:

Mean changes (Δ) from basal over 4 hours are shown corrected for changes with placebo. Values are mean ± SEM in 8 subjects. CI, confidence intervals. P values are for changes vs. baseline.

8.6   Figure Legends

Figure 8.1   Effect of TAK-044 on plasma concentrations of endothelin, big endothelin-1 and C-terminal fragment

Effect of intravenous infusion of placebo or TAK-044 (100 mg or 750 mg) for 15 minutes on immunoreactive plasma endothelin (solid columns), big endothelin-1 (hatched columns) and C-terminal fragment of big endothelin-1 (open columns). *P<0.05 vs. baseline.

Table 8.1

	TAK-044 100 mg				TAK-044 750 mg			
	Baseline value	Mean Δ	95% CI	P	Baseline value	Mean Δ	95% CI	P
SBP, mm Hg	119 ± 4	-3.8	-9.4 to 1.7	0.16	116 ± 2	-3.0	-8.6 to 2.5	0.25
DBP, mm Hg	73 ± 3	-4.5	-6.8 to -2.2	0.001	73 ± 3	-6.0	-8.3 to -3.7	<0.001
MAP, mm Hg	89 ± 3	-4.3	-8.0 to -0.7	0.005	89 ± 3	-5.0	-9.2 to -0.9	0.002
Heart rate, bpm	55 ± 2	4.6	2.4 to 6.7	<0.001	59 ± 4	4.3	2.2 to 6.5	<0.001
Stroke index, ml.m^{-2}	50 ± 3	0.3	-1.3 to 1.9	0.68	47 ± 3	0.8	-0.8 to 2.4	0.29
Cardiac Index, L.min^{-1}.m^{-2}	2.9 ± 0.2	0.3	0.2 to 0.4	<0.001	2.7 ± 0.2	0.3	0.2 to 0.4	<0.001
SVRI, dyn.sec.cm^{-5}	2485 ± 230	-344	-442 to -247	<0.001	2462 ± 133	-415	-514 to -318	<0.001

Table 8.2

	TAK-044 100 mg				TAK-044 750 mg			
	Baseline value	Mean Δ	95% CI	P	Baseline value	Mean Δ	95% CI	P
ERPF, ml.min⁻¹	62.4 ± 4.3	13.8	-86 to 99	0.73	616 ± 57	46.1	-41 to 133	0.27
ERVR, dyn.s.cm⁻⁵	5575 ± 203	-309	-704 to -649	0.02	5545 ± 106	-301	-743 to -119	0.05
GFR, ml.min⁻¹	137 ± 9	-16.1	-28 to -4	0.01	129 ± 11	-11.2	-23 to 1	0.07
Filtration Fraction, %	22.7 ± 2.0	-2.9	-5.5 to -0.3	0.03	21.5 ± 1.8	-3.0	-5.6 to -0.4	0.03
Na Clearance, ml.min⁻¹	2.2 ± 0.3	-0.2	-0.4 to 0.1	0.20	2.1 ± 0.2	-0.2	-0.5 to 0.1	0.10
Li Clearance, ml.min⁻¹	30.6 ± 1.3	-2.4	-8.5 to 3.7	0.41	26.9 ± 1.8	5.4	-0.7 to 11.5	0.08
Na Clearance/Li Clearance, %	7.8 ± 1.2	0.7	-1.2 to 2.5	0.43	8.3 ± 1.6	-0.8	-2.6 to 1.1	0.37
Na excretion, μmol.min⁻¹	307 ± 44	-23.4	-57 to 11	0.16	294 ± 32	-28.6	-63 to 6	0.09
Li excretion, μmol.min⁻¹	2.3 ± 0.2	-0.1	-0.4 to 0.1	0.25	2.0 ± 0.1	0.1	-0.2 to 0.3	0.55
FE$_{Na}$, %	1.9 ± 0.3	0.2	-0.3 to 0.7	0.65	1.7 ± 0.3	0	-0.2 to 0.3	0.98
FE$_{Li}$, %	28.7 ± 4.5	-3.6	-8.6 to 11.6	0.43	24.3 ± 2.1	-1.4	-6 to 3	0.45

Table 8.3

	TAK-044 100 mg				TAK-044 750 mg			
	Baseline value	Mean Δ	95% CI	P	Baseline value	Mean Δ	95% CI	P
Renin, $\mu U.l^{-1}$	10.8 ± 2.5	4.4	-3.8 to 12.7	0.27	13.5 ± 6.2	3.6	-4.5 to 11.9	0.36
Aldosterone, $ng.100ml^{-1}$	6.2 ± 1.0	0.3	-1.3 to 1.9	0.67	6.0 ± 0.7	0.43	-1.2 to 2.0	0.57
Angiotensin II, $pg.ml^{-1}$	10.5 ± 1.9	2.5	1.1 to 3.9	0.002	10.1 ± 1.4	4.2	2.8 to 5.7	<0.001
ANP, $pg.ml^{-1}$	29.7 ± 4.1	4.7	0.03 to 9.33	0.05	28.8 ± 3.1	3.8	-0.8 to 8.5	0.10
Adrenaline, $nmol.l^{-1}$	0.07 ± 0.01	0.1	-0.1 to 0.2	0.19	0.10 ± 0.03	0.1	-0.1 to 0.2	0.40
Noradrenaline, $nmol.l^{-1}$	1.6 ± 0.6	0.09	-1.1 to 1.3	0.87	1.4 ± 0.3	-0.1	-1.3 to 1.1	0.85

Figure 8.1
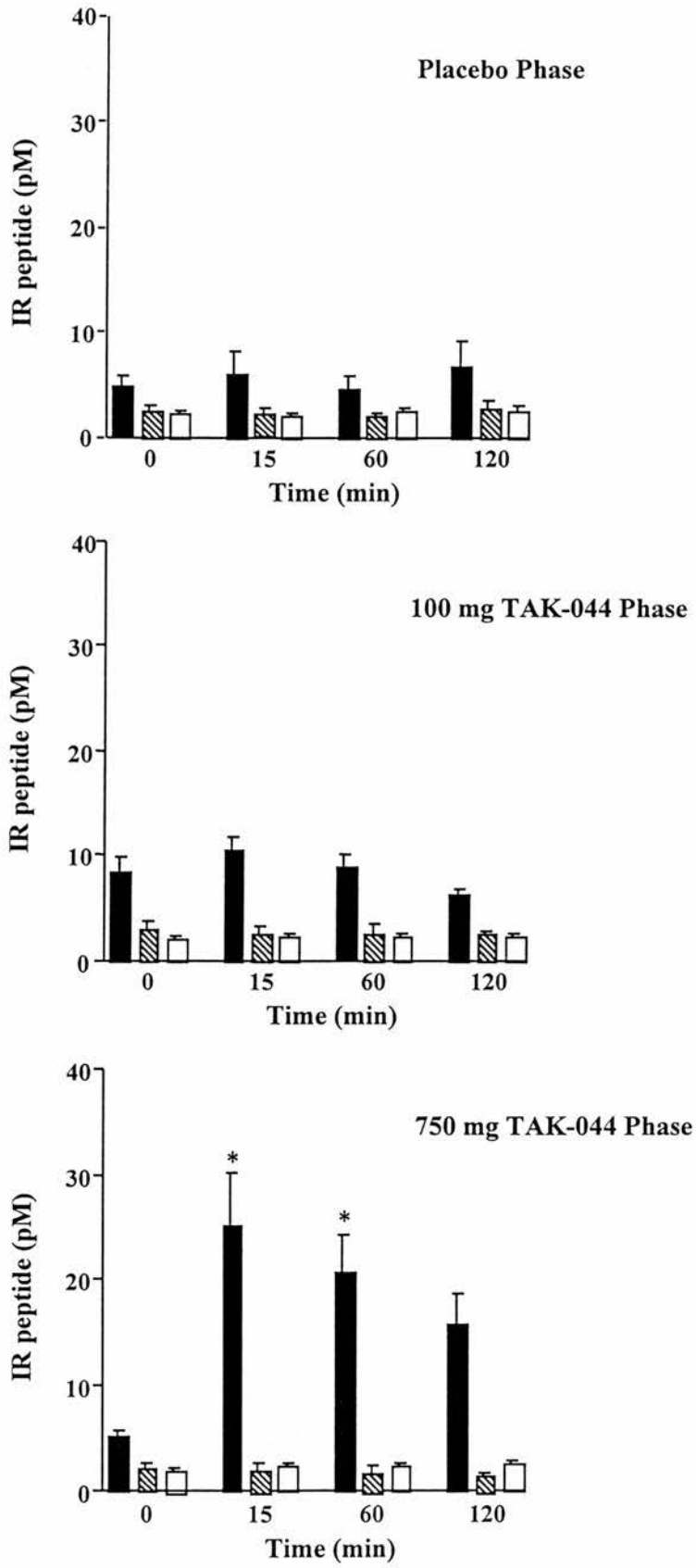
Study 7

9. Actions of systemic endothelin receptor blockade on renal function in patients with chronic renal failure

9.1   Introduction

ET-1, is the principal isoform present in the human renal[257] and cardiovascular system[259] where the peptide interacts with the two receptor subtypes, ET_A and ET_B.[115, 257] In human kidney ET_B receptors predominate and are mainly localised to tubules and collecting ducts.[257] In contrast, the ET_A subtype is largely confined to the smooth muscle of the renal vasculature[360] which is exquisitely sensitive to the vasoconstrictor action of this peptide.[328] Intravenous infusion of ET-1 in healthy humans increases blood pressure, systemic and renal vascular resistance and lowers GFR, ERPF and sodium excretion.[442, 524] Plasma concentrations of endothelin are substantially raised in chronic renal failure[289] and may have a role in the progression of this condition, contributing to the increased vascular tone and high incidence of cardiovascular mortality as well as playing a role in the progression of renal failure.[277] In support of this hypothesis, endothelin receptor antagonists have been shown to be particularly effective in animal models of renal failure.[49, 378]

This study examines the acute actions of a combined $ET_{A/B}$ receptor antagonist, TAK-044, on systemic and renal haemodynamics as well as renal tubular function in patients with chronic renal failure. The effects of acute endothelin receptor antagonism on the plasma concentrations of the precursor big ET-1 and the two products of cleavage by the endothelin

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converting enzyme, the mature peptide and the inactive C-terminal fragment were also examined.

9.2   Methods

9.2.1 Patients

Seven male patients with chronic renal failure (age 45 ± 3 years) participated in this study. Subjects had plasma creatinine concentrations >150 mmol.l^{-1} and no change in renal function in the 2 months preceding the study; and had no other significant concurrent illness other than hypertension. All medications (Table 9.1) were withheld on the morning of the studies.

9.2.2 Measurements

Blood pressure was measured using a well validated semi-automated technique (see Section 2.2.1) and cardiac function (stroke volume, cardiac output and heart rate) was measured using a non-invasive bioimpedance method (see Section 2.3). ERPF and GFR were estimated by measuring the renal clearances of p-aminohippurate sodium (PAH) and polyfructosan-S respectively (see Sections 2.4.1 & 2.4.2). Proximal tubular reabsorptive capacity of sodium was assessed using a physiologically inert dose of oral lithium carbonate (see Sections 2.4.3). Venous blood samples for separate

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assay of plasma endothelin, big ET-1 and C-terminal fragment concentrations were collected, stored and analysed (see Section 2.5.7).

9.2.3 Study Design

Subjects participated in a three-phase, double-blind, randomised, placebo-controlled crossover study, with at least 7 days between phases. The detailed protocols for administration of the combined endothelin $ET_{A/B}$ receptor antagonist, TAK-044 (see Section 5.2). and conducting clearance studies have been previously described (see Sections 2.4 & 8.2). In brief, subjects completed a 24-hour urine collection at 7:00 AM on the morning of the study and received oral lithium carbonate (Camcolit, 250 mg sustained release; Norgine, Harefield, UK) 14 hours before baseline measurements. On each study day, two intravenous cannulae were placed in the antecubital fossa of the non-dominant arm for infusion of PAH/polyfructosan-S, and TAK-044 (100 mg or 750 mg given over 15 minutes) or placebo (dextrose 50 mg). A third cannula was placed in the antecubital fossa of the opposite arm for blood sampling. Priming doses of PAH and polyfructosan-S were followed by maintenance infusions. After an equilibration period, blood samples were drawn and urine collected at the beginning and at the end of accurately timed 30 minute clearance periods. A volume of water was drunk by the subjects after all urine collections sufficient to maintain a urine output of 10 ml.min^{-1} throughout the study. Two baseline collection periods preceded, and 4 followed, infusion of TAK-044 or placebo.

9.2.4 Data Presentation and Statistical Analysis

Mean arterial pressure, stroke index, cardiac index, systemic vascular resistance index (SVRI), renal clearances, effective filtration fraction (EFF; GFR/ERPF x 100%), effective renal vascular resistance (ERVR) and fractional sodium (FE_{Na}) and lithium excretion (FE_{Li}) were calculated using standard formulae.[212, 442]

Data are presented as mean ± SEM. Baseline values were calculated as the mean of all three phases for each individual subject. Placebo-corrected changes from baseline were arithmetically averaged over the relevant measurement period (0 to 240 minutes for haemodynamic changes and 0 to 120 minutes for renal function), with uniform weighting given to each timepoint. Data were analysed statistically by repeated measures analysis of variance (ANOVA). Factors included in the ANOVA were subject, dose of TAK-044, timepoint, and dose-timepoint interaction. In none of the analyses (except plasma endothelin concentrations which were analysed as absolute values by the Kruskal-Wallis non-parametric test with Dunn's multiple comparisons post-tests) was there any evidence of a statistically significant dose-timepoint interaction. Therefore, the adjusted dose group means from the ANOVA were compared with the null hypothesis by a two-sided t-test. Statistical analyses were performed by the use of the software package SAS (version 6.07, SAS Institute Inc).

9.3   Results

The patients' diagnoses and medication are presented in Table 9.1. Five of the 7 patients were hypertensive at screening, with MAP > 105 mmHg. Patient 1 withdrew after completing the TAK-044 100 mg and placebo phases and was, therefore, included in the analysis. All the other subjects completed all 3 phases. TAK-044 was well tolerated at the doses used, with no difference between placebo and TAK-044 phases in the prevalence of minor symptoms. In particular, there was no evidence of the symptoms being dose-related and there was no excess of symptoms associated with hypotension (dizziness, headache, tachycardia) reported during the TAK-044 phases compared with placebo.

TAK-044 significantly lowered MAP by $11 \pm 4\%$ and SVRI by $24 \pm 6\%$ at the highest dose (Figure 9.1). TAK-044 750 mg increased cardiac index by $15 \pm 2\%$, stroke index by $8 \pm 3\%$ and ejection fraction by $4 \pm 1\%$. There was a trend for TAK-044 to increase heart rate, but this was only significant after 100 mg TAK-044 ($9 \pm 3\%$).

TAK-044, at both doses, had no significant effect on GFR ($-4 \pm 10\%$ after 750 mg) or ERPF ($22 \pm 17\%$). However, relative trends in decrease in GFR and increase in ERPF meant that EFF was significantly lowered after 100 mg ($22 \pm 5\%$) and 750 mg ($26 \pm 8\%$) TAK-044 (Figure 9.2). ERVR was also lowered by 100 mg ($11 \pm 4\%$) and 750 mg ($10 \pm 6\%$) TAK-044 (Figure 9.2). Both doses of TAK-044 had no significant effects on sodium

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(-3 ± 5% after 750 mg) or lithium clearance (2 ± 9%) or on FE_{Na} (0.4 ± 5.1%) or FE_{Li} (0.1 ± 8.4%).

Baseline plasma endothelin and C-terminal fragment, but not big ET-1, concentrations were significantly raised in patients with chronic renal failure when compared with sex and age matched controls (Table 9.2). Infusion of 750 mg TAK-044 rapidly increased plasma endothelin but had no effect on big ET-1 or C-terminal fragment concentrations (Figure 9.3).

9.4   Discussion

This study is the first report of the effects of systemic endothelin antagonism on the systemic haemodynamics and renal function of renally impaired subjects.

The finding that systemic administration of a combined $ET_{A/B}$ receptor antagonist causes peripheral vasodilatation and lowers blood pressure in patients with chronic renal failure strongly suggests that ET-1 is important in the maintenance of basal vascular tone and blood pressure in this condition. This is consistent with, and extends, the previous findings that endogenous generation of ET-1 plays a fundamental physiological role in the maintenance of basal vascular tone[218] and blood pressure (Study 3) in healthy subjects. Endothelin receptor antagonists may have a potential beneficial role as vasodilators and blood pressure lowering agents in the treatment of patient with chronic renal failure. It should be noted that the

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vasodilator and blood pressure lowering actions of TAK-044 occurred despite the patients antihypertensive medication having only been discontinued on the morning of the study.

In healthy subjects, systemic infusion of ET-1 increases ERVR and decreases both GFR and ERPF.[442, 524] The decrease in ERPF proportionally exceeds the decrease in GFR, as reflected by an increase in EFF. In this present study, TAK-044 significantly decreased ERVR, and tended to decrease GFR and increase ERPF. As a consequence of these opposing trends, EFF decreased significantly with both doses of TAK-044. The increase in EFF with ET-1 infusion and the currently reported decrease with endothelin antagonism are similar to the changes observed with agonists and antagonists of renal ANG II receptors.[250] Furthermore, the reduction in EFF, and the consequent reduction of glomerular hydrostatic pressure, may account for the success of ACE inhibitors in slowing the progression of renal failure.[250, 337] The reduction in EFF seen with TAK-044 occurred despite 4 of the 7 patients studied were already on treatment with an ACE inhibitor. This would tend to suggest that endothelin antagonism could potentially be used in the treatment of chronic renal failure in conjunction with ACE inhibitors.

Endogenously generated renal endothelin is thought to promote sodium excretion and inhibit water resorption.[277] However, systemic infusion of ET-1 paradoxically reduces salt and water excretion[442, 524] TAK-044 had no clear action on the clearance or fractional excretion of sodium or

lithium although this study did not have the power to detect small changes. Furthermore, any potential antinatriuretic effect of antagonism of tubular endothelin receptors (mainly ET_B) may have been counteracted by the haemodynamic changes as a result of vascular endothelin receptor (mainly ET_A) antagonism. Thus, selective ET_A receptor antagonists could potentially have natriuretic actions and further studies with these agents are needed. However, overall, the renal actions of TAK-044 do not discourage the further development of endothelin antagonists for use in chronic renal failure.

In this study, basal concentrations of immunoreactive endothelin where raised more than five-fold in patients with chronic renal failure compared with healthy controls, whereas C-terminal fragment was raised only two-fold and big ET-1 concentrations where not raised at all. These results support the hypothesis that endothelin levels are raised in patients with renal failure mainly because of decreased clearance (possibly degradation by renal neutral endopeptidase)[2] rather than increased synthesis. This contrasts with congestive heart failure, where big ET-1 is raised and increased production appears to be the major source of ET-1.[271, 611] This study has also shown that after infusion of 750 mg TAK-044, concentrations of plasma immunoreactive endothelin were significantly elevated but levels of big ET-1 and C-terminal fragment were unaltered. This is a feature of drugs with ET_B antagonist properties, probably reflecting endothelin displacement from clearance receptors and is consistent with previous studies in healthy humans.[435]

In conclusion, systemic combined $ET_{A/B}$ receptor blockade with TAK-044 caused peripheral and renal vasodilatation, and lowered blood pressure in renally impaired subjects. In addition, TAK-044 produced a significant decrease in EFF with only minor changes in GFR and ERPF. These findings suggest an important role for endogenously generated endothelin in controlling systemic and renal haemodynamics in patients with chronic renal failure. In particular, by lowering blood pressure and having potentially antiatherogenic properties,[197] endothelin receptor antagonists may also be useful in slowing the progression of renal insufficiency in various kidney diseases. This study of the acute effects a single infusion of TAK-044 are encouraging for the further development of endothelin receptor antagonists for the treatment of chronic renal failure and longer-term studies are now warranted.

9.4   Table Legends

Table 9.1   Chronic renal failure patient characteristics.

Footer:

Subject 1 completed placebo and 100 mg TAK-044 phases. Subjects 2-7 completed all three study phases. (IgA, Immunoglobin A).

Table 9.2   Mean baseline haemodynamic, renal function and immunoreactive endothelin peptide values before administration of TAK-044 or placebo in patients with chronic renal failure and in 8 sex and age matched controls.

Footer:

*p<0.05, ‡p<0.0005 and §p<0.0001.

9.5   Figure Legends

Figure 9.1   Systemic Haemodynamics

Mean changes (Δ) over 4 hours after dosing with placebo (P), TAK-044 100 mg (100) and 750 mg (750) in mean arterial pressure (MAP; mm Hg); systemic vascular resistance index (SVRI; $dyn.sec.cm^{-5}m^{-2}$) and stroke index (SI; $ml.m^{-2}$). For the placebo columns (open), mean change from predose is shown. For the active treatment columns (stipled), placebo-corrected changes from predose are shown (change from predose {active} minus mean change from predose {placebo}). *P=0.001 and §p<0.01 for comparison with predose.

Figure 9.2   Renal Haemodynamics

Mean changes (Δ) over 2 hours after dosing with placebo (P), TAK-044 100 mg (100) and 750 mg (750) in effective renal vascular resistance (ERVR; $dyn.sec.m^{-2}$) and effective filtration fraction (EFF; %). For the active treatment columns (stipled), placebo-corrected changes from predose are shown (change from predose {active} minus mean change from predose {placebo}). *P=0.05, ‡p<0.005 and §p<0.001 for comparison with predose.

Figure 9.3   Plasma Endothelin Concentrations

Effect of intravenous infusion of placebo ($\square$); 100 mg TAK-044 ($\blacksquare$) and 750 mg TAK-044 ($\bullet$) on plasma immunoreactive endothelin concentrations. *P<0.05 for comparison from predose.

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Table 9.1

Patient	Age	Diagnosis	Antihypertensive medication
1	40	Obstructive nephropathy	–
2	37	Polycystic kidneys	enalapril/frusemide/metropolol
3	47	IgA nephropathy	enalapril
4	48	IgA nephropathy	doxasosin/enalapril/metropolol
5	44	Glomerulosclerosis	doxasosin/amlodipine/bendrofluazide
6	65	Obstructive nephropathy	doxasosin/nifedipine
7	46	Hypertensive nephropathy	frusemide/metropolol/enalapril

Table 9.2

	Patients	Controls
Haemodynamics		
Heart rate (beats/min)	57 ± 3	58 ± 3
Systolic blood pressure (mm Hg)*	137 ± 21	119 ± 5
Diastolic blood pressure (mm Hg)*	86 ± 7	76 ± 5
MAP (mm Hg)*	103 ± 4	90 ± 2
SVRI (dyn.sec.cm^{-5}.m^{-2})*	3649 ± 425	2554 ± 178
Cardiac index (l.min.m^{-2})*	2.3 ± 0.2	2.8 ± 0.2
Stroke index (ml.m^{-2})	41 ± 2	48 ± 3
Ejection fraction (%)	59.7 ± 1.9	59.1 ± 1.6
Renal Function		
24 hour creatinine clearance (ml.min^{-1})§	36 ± 7	110 ± 7
Glomerular filtration rate (ml.min^{-1})‡	25 ± 4	136 ± 8.8
Effective renal plasma flow (ml.min^{-1})§	110 ± 25	598 ± 33
Effective filtration fraction (%)	26 ± 3	24 ± 2
Effective renal vascular resistance (dyn.sec.cm^{-5})*	6660 ± 302	5768 ± 126
24 hour sodium excretion (mmol.day^{-1})	94 ± 9	131 ± 22
Sodium clearance (ml.min^{-1})	2.2 ± 0.2	2.2 ± 0.2
Lithium clearance (ml.min^{-1})‡	$11.1 \pm 1.$	30.1 ± 2.4
Fractional sodium excretion (%)‡	2.0 ± 0.2	7.1 ± 0.6
Fractional lithium excretion (%)	26.7 ± 2.7	31.6 ± 2.8
Endothelin Peptides		
Endothelin (pM)‡	34.5 ± 6.5	6.0 ± 0.7
Big endothelin-1 (pM)	2.5 ± 0.3	2.5 ± 0.2
C-terminal fragment (pM)§	5.0 ± 0.7	2.0 ± 0.1

Figure 9.1
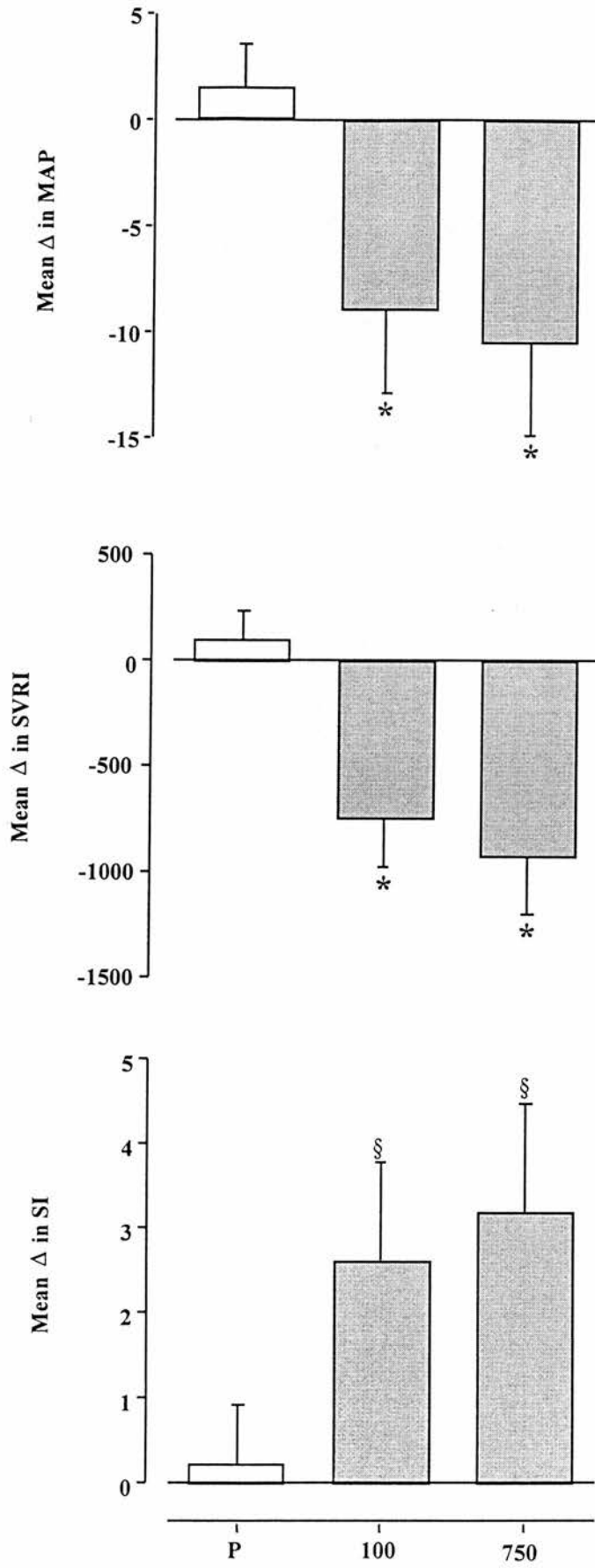
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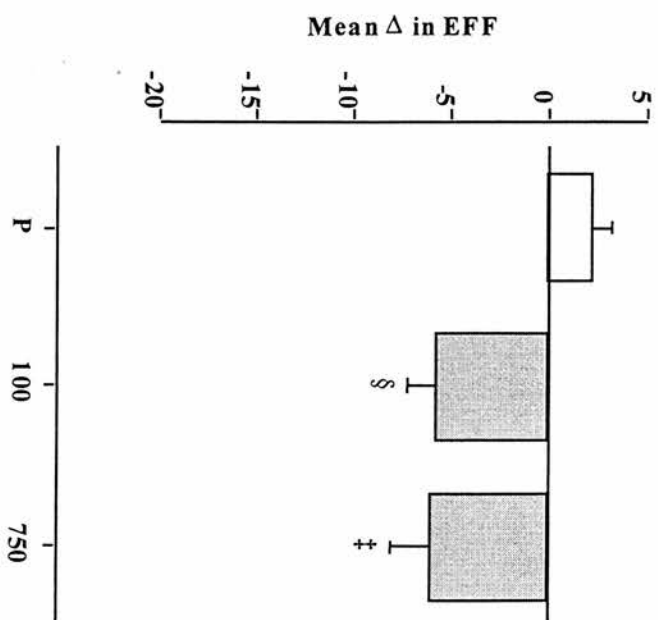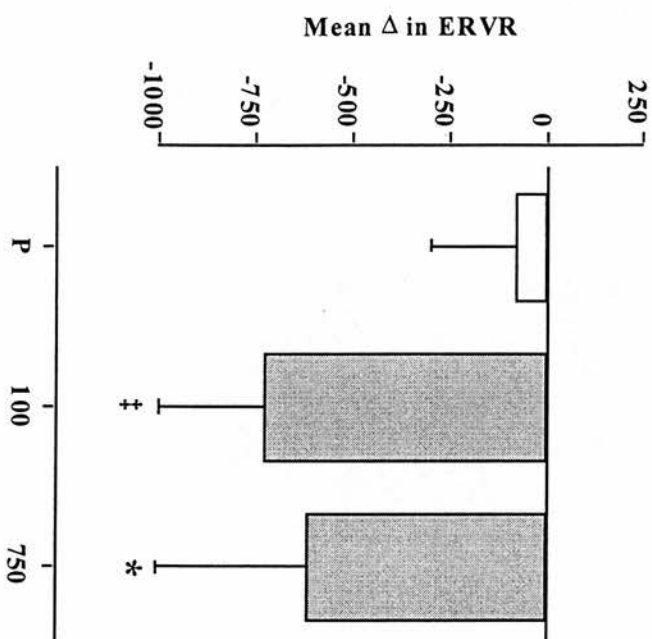
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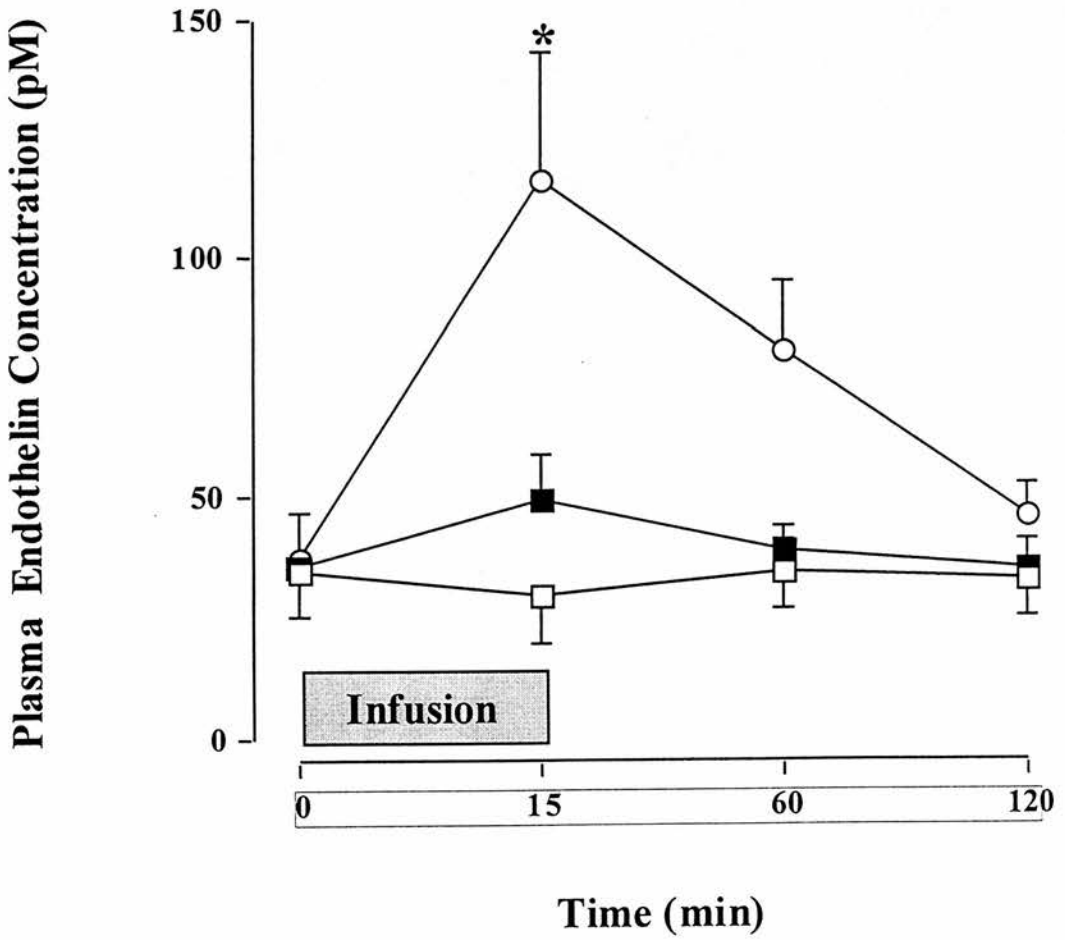
10. Forearm vasoconstriction to endothelin-1 is impaired in patients with essential hypertension

10.1   Introduction

Of the three endothelin isoforms, ET-1 is the predominant isoform produced by vascular endothelial cells[60] and has been implicated in the pathophysiology of several cardiovascular conditions including essential hypertension.[197] Circulating concentrations of ET-1 are low[439] and approximately 80% of ET-1 synthesised by endothelial cells is secreted abluminally,[593] suggesting that ET-1 has primarily paracrine and autocrine actions.

On the basis of molecular studies, two endothelin receptors have so far been characterized in mammalian species.[21, 481] The ET_A receptor is relatively selective for ET-1,[21] whereas the ET_B receptor has equal affinity for all three endothelin isoforms.[481] Results from early studies suggested that the ET_A receptor was restricted to the vascular smooth muscle and mediates vasoconstriction, whereas the ET_B receptor was restricted to the endothelium where it mediates vasodilatation through the production of endothelium-dependent vasodilators, such as nitric oxide and prostacyclin.[220, 242] However, it is now recognized that this concept is an oversimplification because ET_B receptors are also expressed on vascular smooth muscle cells[117] and contraction of human smooth muscle cells can be generated through either ET_A or ET_B receptor stimulation.[217, 577] However, in a wide range of human vessels *in vitro*, ET-1 mediated vasoconstriction occurs predominantly via the ET_A receptor[116] and the

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importance of the smooth muscle ET_B receptor in humans remains to be clarified.

In addition to its vasoconstrictor and pressor effects, ET-1 has several other actions which implicate it in the pathophysiology of essential hypertension (see Section 1.3.1). It would, therefore, appear possible that disturbances in the control of ET-1 production or sensitivity of tissues to ET-1 could participate in the mechanisms of blood pressure elevation in essential hypertension.

Studies measuring plasma concentrations of endothelin in patients with essential hypertension have produced conflicting results, with several studies reporting increased concentrations[283, 307, 476, 505] whilst others have not.[114, 213, 357, 439, 492] This apparent inconsistency has been variously attributed to the presence of vascular injury and hypertensive target organ damage.[446, 489] However, given that ET-1 appears to be mainly a paracrine agent it is possible that increased endothelial synthesis of ET-1 in essential hypertension is not necessarily reflected in raised plasma concentrations. Indeed, enhanced expression of ET-1 gene has been found in resistance arteries of rat models of hypertension[174, 355] as well as in humans with severe essential hypertension.[491]

In vitro vasoconstrictor responses to ET-1 are blunted in resistance arteries taken from patients with essential hypertension.[490] However, *in vivo* responses to ET-1 by capacitance vessels is markedly enhanced in

hypertensive patients and sympathetically mediated venoconstriction was also substantially potentiated in these patients but not in normotensive subjects.[213] Studies with endothelin receptor antagonists have been shown to lower blood pressure in both normotensive (Studies 3-6) and hypertensive subjects.[290] However, the direct contribution of the endothelin system to the raised blood pressure in essential hypertension has not yet been examined.

This study investigates whether the endothelin system contributes to the raised vascular tone associated with essential hypertension by infusing locally active doses of the selective ET_A receptor antagonist, BQ-123, into the brachial artery of patients with essential hypertension and normotensive subjects and measuring forearm blood flow (FBF) by venous occlusion plethysmography.[604] This study also examines the *in vivo* arterial responsiveness to the non-selective ET_A and ET_B agonist ET-1 and the selective ET_B agonist, sarafotoxin S6c in these subjects. In view of the potential interaction between endothelin and activity of the sympathetic nervous system, and the known increase in sympathetic nervous activity in hypertension,[14] lower body negative pressure (LBNP)[604] was used to assess the effect of ET-1 and sarafotoxin S6c on sympathetically mediated vasoconstriction in hypertensive and normotensive subjects.

10.2 Methods

10.2.1 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 minutes using Spacelabs 90207[395]; if there were no significant concurrent illnesses; and if they had never received antihypertensive therapy. Normotensive (BP < 140/90 mmHg) were recruited by advertisement. Control subjects were matched for age, sex, weight and height. A total of 27 hypertensive patients and 30 normotensive subjects participated in these studies (some subjects participated in more than one study). 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.

10.2.2 Drug Infusion and Forearm Blood Flow Measurement

Studies were performed with subjects resting supine in a quiet clinical laboratory maintained at a constant temperature 25°C.

The brachial artery of the non-dominant arm was cannulated as described in Section 2.1.1. Physiological saline (0.9% NaCl; Baxter Healthcare Ltd, Thetford, UK) was infused at 1 ml.min^{-1} for an equilibration period of at least 30 minutes prior to infusion of study agents. Locally active doses of drugs were dissolved in physiological saline and administered at 1 ml.min^{-1}. Infusions of different study agents were separated by at least 20 minutes of infusion of physiological saline or longer if FBF had not returned to baseline values.

Blood flow was measured in both forearms by venous occlusion plethysmography, using indium/gallium-in-Silastic (see Section 2.1.1). LBNP was applied using the method described by Brown and colleagues (see Section 2.1.1). A well-validated semiautomated noninvasive oscillometric sphygmomanometer (see Section 2.2.1) was used to make duplicate measurements of blood pressure in the noninfused arm, which were then averaged.

10.2.3 Data Presentation and Statistical Analysis

Data are shown as mean ± SEM in the figures and with confidence intervals (CI) in the text and tables where appropriate. Results were examined by repeated measures analysis of variance (ANOVA) and paired t-tests where appropriate using Statview 512$^+$ software (Brainpower Inc, Calabasas, Ca, USA) for the Apple Macintosh personal computer. Differences were considered statistically significant at a value of $P<0.05$.

10.2.4 Detailed Protocols

Study A: Selective ET_A receptor antagonism in healthy subjects

Six healthy male volunteers participated in a single-blind, randomised, 4-way crossover placebo-controlled study. In 3 of the 4 phases of the study, the selective ET_A receptor antagonist, BQ-123 (American Peptide Company, Sunnyvale, CA), was infused at 10, 30 and 100 nmol.min^{-1} for 120 minutes. The highest dose of BQ-123 (100 nmol.min^{-1}) used in this study has been shown to produce a slowly progressive increase in FBF of ~40% when infused via the brachial artery for 60 minutes.[218, 318] In the fourth phase, physiological saline was infused for 120 minutes instead of BQ-123.

Study B: Selective ET_B receptor agonism in healthy subjects

Six healthy male volunteers participated in a single-blind, randomised, 2-way crossover placebo-controlled study. Subjects received a brachial artery infusion of sarafotoxin S6c (Clinalfa AG, Novabiochem, Laurelfingen, Switzerland) at a dose of 5 pmol.min^{-1} and 10 pmol.min^{-1}. Intra-arterial sarafotoxin S6c at 5 pmol.min^{-1} is known to produce ~20% vasoconstriction after 60 minutes of infusion.[217] The 10 pmol.min^{-1} dose was chosen in an attempt to produce a similar vasoconstriction to ET-1 of ~40% after 60-120 minutes.[217, 218, 318]

Study C: Selective ET_A receptor antagonism in essential hypertension

Ten patients with essential hypertension and 10 matched controls participated in this study (Table 10.1). After the initial 30 minute saline infusion, sodium nitroprusside (Roche, Basel, Switzerland) was infused at incremental doses of 1, 3 and 10 $\mu g.min^{-1}$, each for 5 minutes. FBF was measured for the last 3 minutes of each 5 minute infusion. After a minimal 20 minute "washout" infusion with saline, a 90 minute infusion of BQ-123, at a dose of 100 $nmol.min^{-1}$ was started. The choice of this dose was based on the results of Study A.

Study D: Non-selective ET_A and ET_B receptor agonism in essential hypertension

Ten patients with essential hypertension and 10 matched controls participated in this study (Table 10.1). After the initial 30 minute saline infusion, noradrenaline (Sterling-Winthrop, Guildford, UK) with ascorbic acid as antioxidant (Evans Medical, Horsham, UK) was infused at incremental doses of 10, 20, 40 and 80 $ng.min^{-1}$, each for 5 minutes. FBF was measured for the last 3 minutes of each 5 minute infusion. After a minimal 20 minute "washout" infusion with saline, a 90 minute infusion of ET-1 (Clinalfa AG), at a dose of 5 $pmol.min^{-1}$ was started. This dose is known to produce ~40% vasoconstriction after 60-120 minutes.[217, 318] LBNP was applied at the end of both saline infusions, at the end of the

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highest dose of noradrenaline and after 15 and 90 minutes of ET-1 infusion.

Study E: Selective ET_B receptor agonism in essential hypertension

Ten patients with essential hypertension and 10 matched controls participated in this study (Table 10.1). After the initial 30 minute saline infusion, noradrenaline was infused at incremental doses of 10, 20, 40 and 80 ng.min^{-1}, each for 5 minutes. FBF was measured for the last 3 minutes of each 5 minute infusion. After a minimal 20 minute "washout" infusion with saline, a 90 minute infusion of sarafotoxin S6c, at a dose of 10 pmol.min^{-1} was started. This dose was chosen based on the results of Study B. LBNP was applied at the end of both saline infusions, at the end of the highest dose of noradrenaline and after 15 and 90 minutes of sarafotoxin S6c infusion.

10.3 Results

Heart rate, blood pressure and FBF in the infused arm did not change significantly in any study protocol, confirming that drugs had only local actions on the forearm vasculature of the infused arm and had no systemic haemodynamic effects.

Study A: Selective ET_A receptor antagonism in healthy subjects

BQ-123 caused slow-onset vasodilatation at all three doses (Table 10.2). Vasodilatation was maximum by 60 minutes and was not significantly increased by a further 60 minutes of infusion (Table 10.2). Infusion of saline for 120 minutes had no effect on FBF.

Study B: Selective ET_B receptor agonism in healthy subjects

Both doses of sarafotoxin S6c, 5 and 10 pmol.min^{-1}, caused a slowly progressive vasoconstriction (Table 10.2). The vasoconstriction to 10 pmol.min^{-1} was significantly greater than that caused by 5 pmol.min^{-1} (P=0.02).

Study C: Selective ET_A receptor antagonism in essential hypertension

Sodium nitroprusside caused a dose-dependent vasodilatation in both hypertensive patients and control subjects (Figure 10.1) which was not significantly different between groups (P=0.48). BQ-123 caused a slowly-progressive vasodilatation in control subjects, with an increase in FBF of 40 ± 8% at 60 minutes (CI, 22% to 59%; P=0.0001; Figure 10.1). BQ-123 also caused a slowly progressive vasodilatation in hypertensive patients, with an increase in FBF of 35 ± 5% at 60 minutes (CI, 23% to 46%;

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P=0.0001). The vasodilatation to BQ-123 was not significantly different between hypertensive patients and controls (P=0.49).

Study D: Non-selective ET_A and ET_B receptor agonism in essential hypertension

Noradrenaline caused a dose-dependent vasoconstriction in both hypertensive patients and control subjects (Figure 10.2) which was not significantly different between groups (P=0.93). ET-1 caused a slowly-progressive vasoconstriction in control subjects, with a decrease in FBF of 37 ± 3% at 90 minutes (CI, -45% to -29%; P=0.0001; Figure 10.2). ET-1 also caused a slowly progressive vasoconstriction in hypertensive patients, with a decrease in FBF of 21 ± 4% at 90 minutes (CI, -30% to -12%; P=0.001; Figure 10.2). The vasoconstriction to ET-1 was significantly blunted in hypertensive patients compared with controls (P=0.001; Figure 10.2). Application of LBNP produced a vasoconstriction of ~20% in both the infused and control arms (Table 10.3). This vasoconstriction in the infused arm was not affected by infusion of either noradrenaline or ET-1 in hypertensive patients or control subjects (Table 10.3). There were no significant differences in the vasoconstriction caused by LBNP in hypertensive patients or control subjects (Table 10.3).

Study E: Selective ET$_B$ receptor agonism in essential hypertension

Noradrenaline caused a dose-dependent vasoconstriciton in both hypertensive patients and control subjects (Figure 10.3) which was not significantly different between groups (P=0.89). Sarafotoxin S6c caused a slowly-progressive vasoconstriction in control subjects, with a decrease in FBF of 44 ± 5% at 90 minutes (CI, -55% to -33%; P=0.0001; Figure 10.3). Sarafotoxin S6c also caused a slowly progressive vasoconstriction in hypertensive patients, with a decrease in FBF of 48 ± 4% at 90 minutes (CI, -57% to -39%; P=0.0001; Figure 10.3). The vasoconstriction to sarafotoxin S6c was not significantly different between hypertensive patients and controls (P=0.95; Figure 10.3). Application of LBNP produced a vasoconstriction of ~20% in both the infused and control arms (Table 10.3). This vasoconstriction in the infused arm was not affected by infusion of either noradrenaline or sarafotoxin S6c in hypertensive patients or control subjects (Table 10.3). There were no significant differences in the vasoconstriction caused by LBNP in hypertensive patients or control subjects (Table 10.3).

10.4 Discussion

These studies demonstrate that endothelin contributes significantly to peripheral vascular resistance in patients with essential hypertension and suggest that ET$_A$ receptor antagonists may be useful as vasodilator agents

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in the treatment of this condition. They also show that both ET_A and ET_B receptors can mediate vasoconstriction in the peripheral vasculature of healthy subjects and patients with essential hypertension, a finding that may have important implications for the potential therapeutic use of endothelin receptor antagonists.

Evidence that endothelin contributes to peripheral vascular resistance in essential hypertension is provided by these 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 ~40% in patients with essential hypertension in study C (Figure 10.1). This response was probably maximal by 60 minutes, given that in study A (Table 10.1) infusion of BQ-123 for 120 minutes in healthy volunteers did not produce a greater vasodilatation. Although bigger doses of BQ-123 may have produced greater vasodilatation, this appears unlikely given that in study A doses of BQ-123 10-fold different produced similar increases in FBF (Table 10.2).

Though these studies only investigated the short-term effects of 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 vascular beds.[604] Indeed, these observations support the recent findings by Krum et al,[290] who reported that sustained oral administration of a combined ET_A and ET_B receptor antagonist, bosentan,

significantly reduced blood pressure in patients with essential hypertension.

These studies have shown that the forearm vasodilator effects of the selective ET_A receptor antagonist BQ-123, and the control vasodilator sodium nitroprusside, and were not different in patients with essential hypertension compared with normotensive controls (Figure 10.1). These findings suggest that endothelin does not contribute to the raised vascular tone associated with essential hypertension, at least not by ET_A receptor mediated actions. However, any comparison of the effects of BQ-123 between patients with essential hypertension and normotensive controls must be made with some 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 these studies may not have not demonstrated the maximal effect of BQ-123 in the forearm vasculature, although the results of study A would suggest they have, at least in healthy subjects.

These studies with BQ-123 support an important role for ET_A receptors in mediating the constrictor effects of endogenous endothelin in patients with essential hypertension, but the role of ET_B receptors in this regard needs further clarification. A recent study with BQ-788, a selective ET_B receptor antagonist, caused vasoconstriction in the forearm vasculature of healthy subjects.[592] This study suggests that endogenous activation of ET_B receptors causes vasodilatation. The result of ET_B receptor antagonism in

patients with essential hypertension is not currently known and cannot be determined from this study. Further studies with selective ET_A and ET_B antagonists in hypertensive patients are necessary to further clarify this issue.

Collectively, these 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 essential hypertension. These agonist studies in healthy volunteers are consistent with similar recently reported studies (Table 10.2, Figures 10.2 & 10.3).[217] Double the dose of sarafotoxin S6c was required to produce a similar vasoconstriction to ET-1 in control subjects, implying that both ET_A and ET_B receptors mediate vasoconstriction, but it is difficult to extrapolate these results to quantify the relative contribution of each receptor subtype in mediating the effects of endogenous endothelin.

The finding that the vasoconstrictor effects of ET-1 was blunted in the forearm vasculature of patients with essential hypertension (Figure 10.3) are very similar to those observed *in vitro* by Schiffrin et al[490] in resistance vessels taken from buttock biopsies. These findings may be related to several events. First, there could be diminished responsiveness of signal transduction mechanisms coupled to endothelin receptors. This mechanism appears unlikely given that responses to sarafotoxin S6c in hypertensive patients were not affected in study E. Second, there could be a generalised decrease in response involving either intracellular excitation-

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contraction coupling or the contractile mechanism of vascular smooth muscle cells. This latter explanation may be excluded by the finding that responses to noradrenaline were not diminished in these patients (Figures 10.2 & 10.3). Similarly, vasoconstrictor responses to noradrenaline, ANG II and arginine vasopressin *in vitro* are not diminished in arteries taken from hypertensive patients.[1, 490] Third, there could be decreased availability of endothelin receptors due to either down-regulation or prior receptor occupation. Endothelin receptor number at the smooth muscle cell surface is reduced by prolonged exposure to ET-1 itself[227, 468] and there is recent evidence suggesting that ET-1 mRNA expression is increased in resistance vessels taken from patients with severe essential hypertension.[491] In DOCA-salt hypertensive[126] and spontaneously hypertensive rats,[91] decreased density of endothelin binding sites have been demonstrated. Therefore, the finding of diminished responsiveness to ET-1 appears to provide indirect evidence of excess production of endothelin in human hypertensive blood vessels or of excess exposure of these vessels to endothelin in mild to moderate essential hypertension.

In contrast to the effects of ET-1, forearm vasculature responsiveness to sarafotoxin S6c was not diminished in patients with essential hypertension (Figure 10.3). Endothelin receptor expression and distribution may be altered in essential hypertension. As previously discussed, endothelin receptor number at the smooth muscle cell surface is reduced by prolonged exposure to ET-1 itself[227, 468]. In endothelial cells, ET_B receptor expression is also decreased by exposure to high local concentrations of

ET-1.[480] However, ET_B receptor expression by smooth muscle cells is increased in hypertension,[147] and under the influence of ANG II.[256] The preserved vasoconstriction to sarafotoxin S6c may partly be related to endothelial dysfunction in essential hypertension 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 essential hypertension requires further investigation. It is, however, important to acknowledge that these studies cannot definitively exclude the possible existence of a dilator subtype of ET_A receptor sensitive to ET-1 or another species of constrictor receptor sensitive to sarafotoxin S6c in patients with essential hypertension.

It has been suggested from *in vitro* studies that endothelin may increase peripheral sympathetic activity through postsynaptic potentiation of the effects of noradrenaline.[637] However, these findings have not been confirmed *in vivo* in resistance vessels of normotensive subjetcs in this study (Table 10.3) or in a previous report.[97] Further this study did not show a potentiation of sympathetically mediated vasoconstriction by ET-1 or sarafotoxin S6c in hypertensive subjects (Table 10.3). These findings appear to be at variance with previously reported work where sympathetically mediated dorsal hand vein constriction was potentiated by ET-1 in patients with essential hypertension but not in normotensive controls.[213] Furthermore, venoconstriction to ET-1 was also enhanced in hypertensive patients in this study.[213] However, dorsal hand veins have

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no underlying tone[604] and are probably not exposed to high concentrations of locally produced endothelin, as has been suggested are resistance vessels in essential hypertension,[491] given the normal concentrations of plasma endothelin found in patients with essential hypertension in several studies.[114, 213, 357, 439, 492] Therefore, hand veins may not be affected by down-regulatory factors as forearm resistance vessels appear to be and this could explain the apparent contradictory findings in veins and arteries. Nevertheless, the exaggerated responsiveness to ET-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.[149, 473] Thus, it is possible that the reduced reactivity of forearm resistance vessels to ET-1 is an epiphenomenon and that the enhanced venoconstriction to ET-1 in hypertensive subjects may be a causative factor in the pathophysiology of essential hypertension. This possibility would require further investigation perhaps by examining responses to ET-1 in the capacitance and resistance vessels of normotensive patients with several 'risk' factors for developing hypertension in later life.

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 endothelin could contribute to myocardial ischemia and that the proliferative effects of endothelin could contribute to

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vascular and cardiac hypertrophy and the atherosclerotic process. Indeed, plasma endothelin concetrations are increased in atherosclerosis,[308] and expression of ET-1 mRNA is increased in the vascular smooth muscle of atherosclerotic human arteries.[625] Furthermore, increased tissue endothelin activity has been reported in the active atherosclerotic lesions associated with unstable angina.[649] 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.[106]

In summary, these findings have potentially important therapeutic implications in essential hypertension. Selective ET_A receptor blockade produced a significant reduction in forearm vascular resistance in patients with essential hypertension. These studies have also shown that vasoconstriction to endothelin is mediated by both ET_A and ET_B receptors in these patients and that vasoconstricor ET_B receptors may be functionally more important in hypertensive patients than in normotensive controls. This suggests that a non-selective ET_A and ET_B receptor antagonist would be necessary to achieve optimal inhibition of the constrictor effects of endogenous endothelin. 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. An orally active non-selective ET_A and ET_B receptor antagonist has very recently been shown to

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lower blood pressure in hypertensive patients during sustained administration.[290] Given that endothelin may mediate some of the consequences of hypertension such as myocardial infarction[197] which appear to be little affected by current treatments, the results of long term studies with endothelin receptor antagonists are awaited with considerable interest.

10.5 Table legends

Table 10.1   Patient and control subject characteristics, studies C, D & E.

Footer:

*P<0.001

†Expressed as mean of infused and noninfused arms.

Table 10.2   Baseline forearm blood flow and percentage changes from baseline in Studies A and B.

Footer:

There were no significant differences between baseline values on the different study days. N/A, data not available because infusion continued for only 90 minutes; FBF indicates forearm blood flow.

*P<0.01 vs baseline, †P<0.0001 vs baseline.

‡Expressed as mean of infused and noninfused arms.

Table 10.3   Percentage vasoconstriction to lower body negative pressure in the infused and noninfused arms of patients and controls.

Footer:

There were no significant differences in the vasoconstriction caused by lower body negative pressure in the infused and noninfused arms or between patients and control subjects during infusion of the different study agents.

10.6   Figure legends

Figure 10.1   Intra-arterial sodium nitroprusside and BQ-123

Effect of brachial artery infusion of sodium nitroprusside (1, 3 and 10 μg.min^{-1} each for 5 minutes) and BQ-123 (10 nmol.min^{-1} for 60 minutes) in 10 patients with essential hypertension ($\bullet$) and 10 age-matched healthy control subjects ($\bigcirc$). There were no significant differences in the vasodilatation caused by both agents in the two groups studied.

Figure 10.2   Intra-arterial noradrenaline and endothelin-1

Effect of brachial artery infusion of noradrenaline (10, 20, 40 and 80 ng.min^{-1} each for 5 minutes) and endothelin-1 (5 pmol.min^{-1} for 90 minutes) in 10 patients with essential hypertension ($\bullet$) and 10 age-matched healthy control subjects ($\bigcirc$). There was no difference in the vasoconstriction caused by noradrenaline in the two groups studied. The vasoconstriction to endothelin-1 in patients with essential hypertension was significantly blunted compared to healthy control subjects (P=0.001).

Figure 10.3   Intra-arterial noradrenaline and sarafotoxin S6c

Effect of brachial artery infusion of noradrenaline (10, 20, 40 and 80 ng.min^{-1} each for 5 minutes) and sarafotoxin S6c (10 pmol.min^{-1} for 90 minutes) in 10 patients with essential hypertension ($\bullet$) and 10 age-matched healthy control subjects ($\bigcirc$). There were no significant differences in the vasoconstriction caused by both agents in the two groups studied (P=0.95).

Table 10.1

	BQ-123 Protocol		Endothelin-1 Protocol		Sarafotoxin S6c Protocol	
	Hypertensives	Normotensives	Hypertensives	Normotensives	Hypertensives	Normotensives
Age (years)	49 ± 3	45 ± 3	51 ± 4	50 ± 3	48 ± 3	50 ± 3
Sex (Male/Female)	9/1	9/1	7/3	7/3	8/2	8/2
Clinic MAP (mm Hg)*	124 ± 2	87 ± 3	127 ± 3	90 ± 4	130 ± 3	88 ± 3
Daytime Ambulatory MAP (mm Hg)	118 ± 2	NA	119 ± 3	NA	119 ± 3	NA
Creat (μmol.l^{-1})	93 ± 5	90 ± 4	87 ± 4	92 ± 4	88 ± 3	86 ± 3
Cholesterol (μmol.l^{-1})	6.0 ± 0.3	5.7 ± 0.3	5.5 ± 0.2	5.4 ± 0.3	5.6 ± 0.3	5.2 ± 0.3
Basal infused arm forearm blood flow (ml.100 ml^{-1} min^{-1})	3.85 ± 0.47	3.37 ± 0.24	4.02 ± 0.27	3.56 ± 0.32	3.54 ± 0.21	3.72 ± 0.33

Table 10.2

Prercentage Change in Forearm Blood Flow

	Basal FBF (ml.100 ml⁻¹.min⁻¹)	30 minutes	60 minutes	90 minutes	120 minutes
Study A					
Saline	3.82 ± 0.76	-5 ± 3 (-4 to +3)	0 ± 7 (-18 to +18)	-6 ± 4 (-17 to +4)	-6 ± 5 (-20 to +8)
BQ-123 10 nmol.min⁻¹*	2.95 ± 0.26	46 ± 12 (+5 to +77)	54 ± 8 (+33 to +79)	60 ± 8 (+40 to +79)	43 ± 14 (+6 to +79)
BQ-123 30 nmol.min⁻¹†	3.23 ± 0.53	22 ± 5 (+8 to +35)	32 ± 10 (+8 to +57)	28 ± 6 (+12 to +43)	35 ± 7 (+17 to +52)
BQ-123 100 nmol.min⁻¹†	2.71 ± 0.57	13 ± 6 (-1 to +28)	38 ± 9 (+15 to +61)	39 ± 9 (+15 to +62)	47 ± 10 (+21 to +73)
Study B					
SRFX S6c 5 pmol.min⁻¹†	3.01 ± 0.57	-18 ± 6 (-33 to -2)	-21 ± 6 (-38 to -5)	-22 ± 7 (-40 to -4)	N/A
SRFX S6c 10 pmol.min⁻¹†	3.04 ± 0.83	-25 ± 17 (-69 to +11)	-45 ± 8 (-66 to -24)	-43 ± 8 (-65 to -22)	N/A

Table 10.3

		Endothelin-1 Cohort Percentage vasoconstriction to LBNP	
Infusion	**Arm**	**Normotensives**	**Hypertensives**
Saline (30 min)	Control	-21 ± 2	-18 ± 4
	Infused	-21 ± 3	-20 ± 3
NA (80 ng.min^{-1})	Control	-20 ± 2	-25 ± 2
	Infused	-15 ± 1	-18 ± 3
Saline (90 min)	Control	-26 ± 2	-21 ± 5
	Infused	-30 ± 2	-20 ± 4
Endothelin-1 (15 min)	Control	-30 ± 4	-28 ± 7
	Infused	-25 ± 3	-19 ± 3
Endothelin-1 (90 minutes)	Control	-18 ± 2	-27 ± 4
	Infused	-19 ± 2	-24 ± 3

		Sarafotoxin S6c Cohort Percentage vasoconstriction to LBNP	
Infusion	**Arm**	**Normotensives**	**Hypertensives**
Saline (30 minutes)	Control	-21 ± 2	-21 ± 4
	Infused	-20 ± 2	-14 ± 7
NA (80 ng.min^{-1})	Control	-21 ± 3	-21 ± 2
	Infused	-15 ± 3	-16 ± 2
Saline (20 min)	Control	-29 ± 3	-21 ± 3
	Infused	-28 ± 3	-17 ± 8
Sarafotoxin S6c (15 minutes)	Control	-22 ± 3	-17 ± 3
	Infused	-23 ± 4	-17 ± 3
Sarafotoxin S6c (90 min)	Control	-23 ± 3	-24 ± 3
	Infused	-17 ± 4	-18 ± 3

Figure 10.1
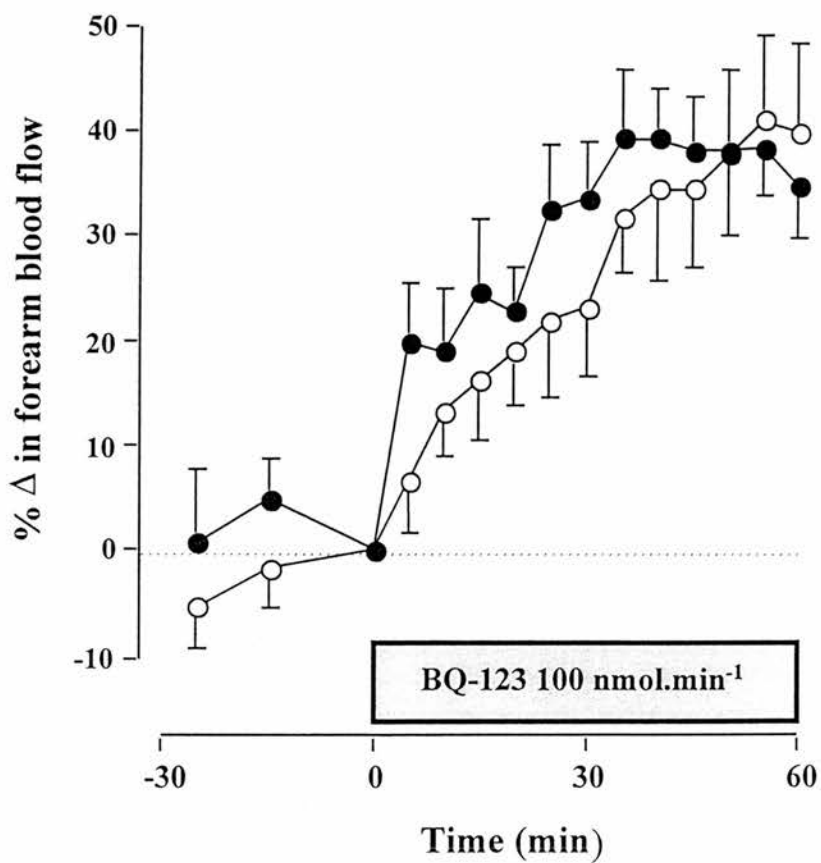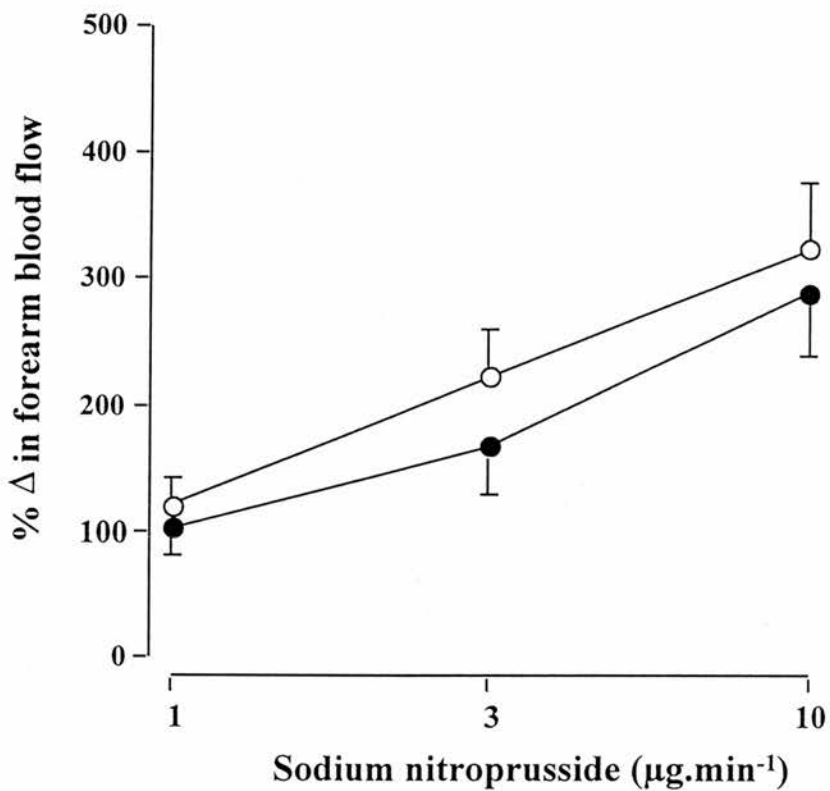
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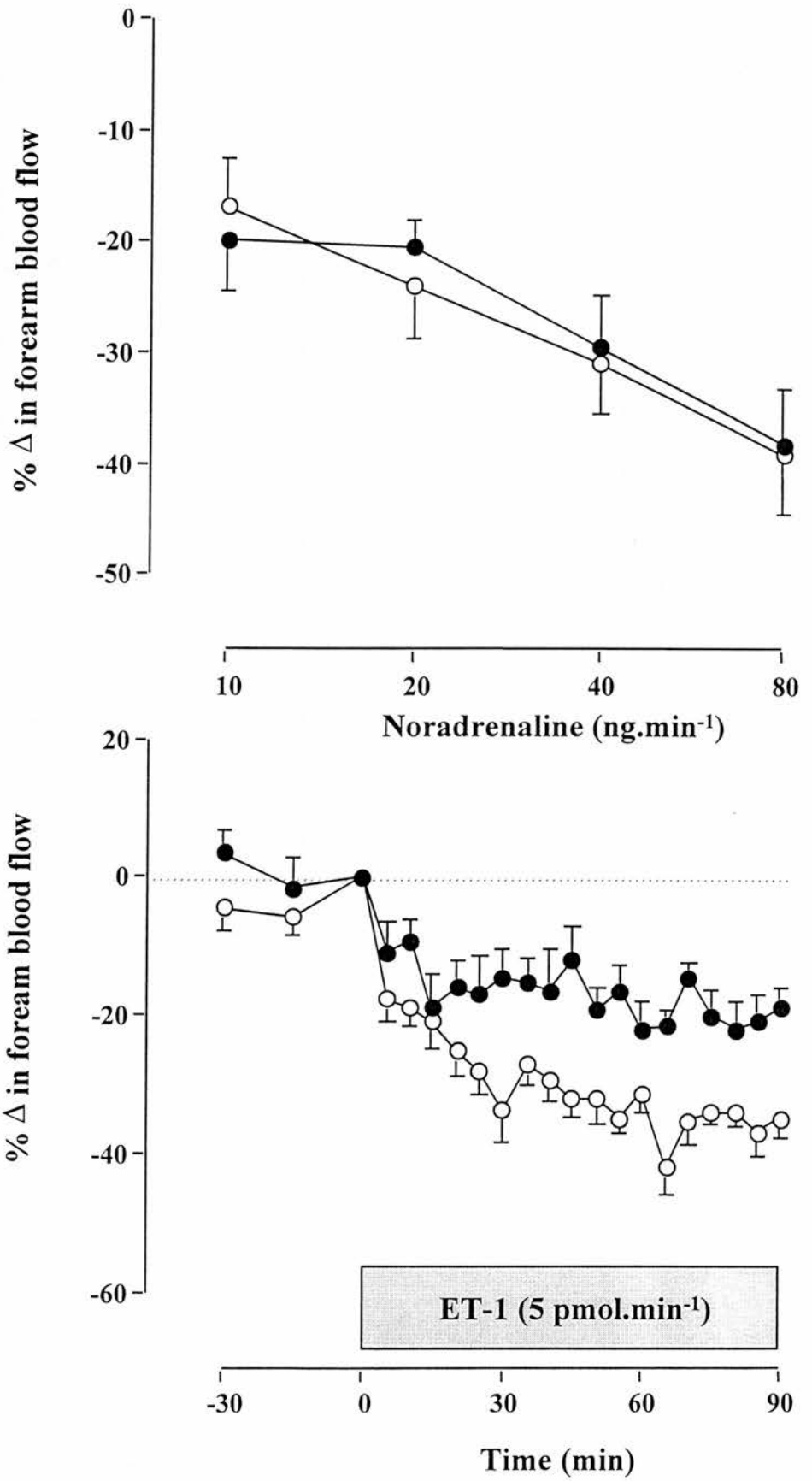
Figure 10.3
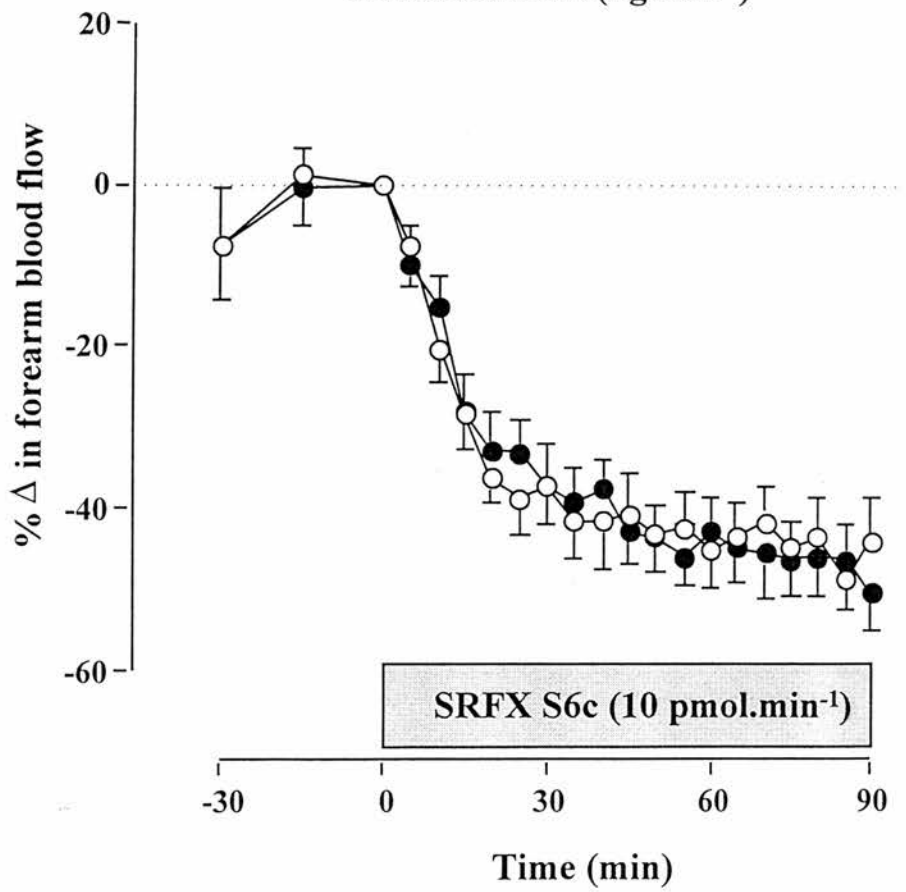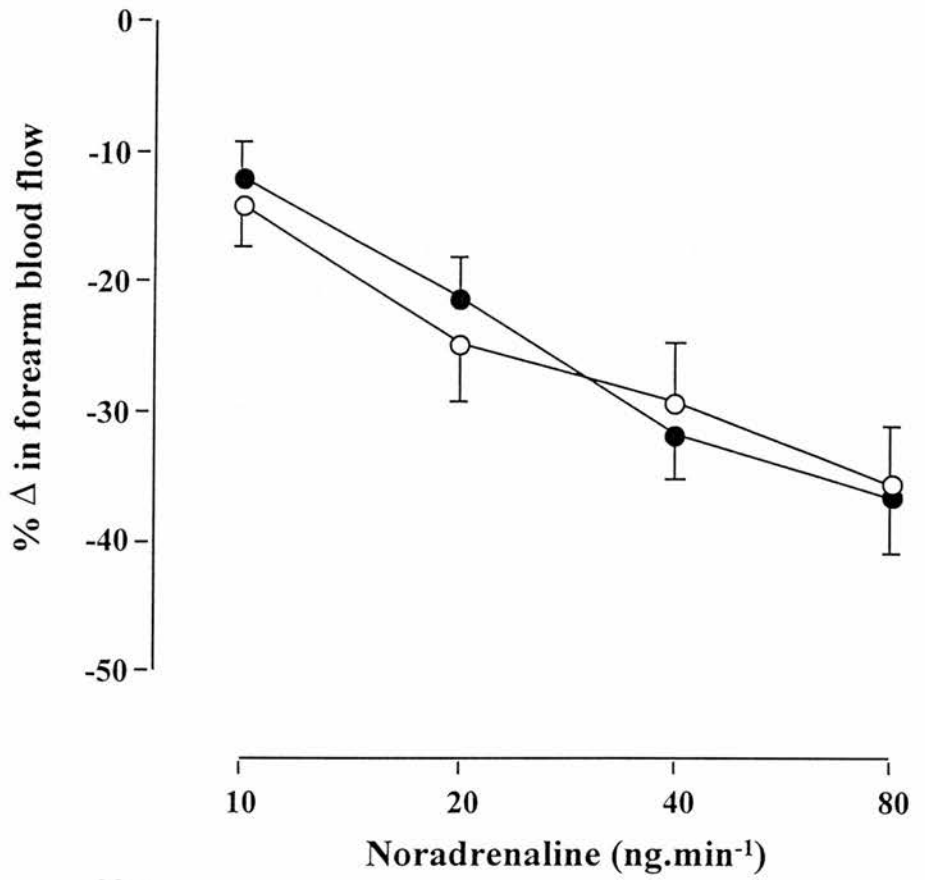
11.   Future Perspectives

The publication of the discovery of endothelin by Yanagisawa and colleagues in 1988 in that celebrated article in *Nature* was received with considerable acclaim by the scientific community. The description of this molecule's structure, actions *in vitro* and *in vivo*, DNA sequence and synthesis pathway in a single article was incredible. In fact Sir John Vane, organiser of the 4th International Congress on Endothelin, thought it was an April's Fool Joke! It was no joke. The discovery of such an agent was thought by many to have heralded a new era of discovery for the pathophysiology of several diseases and of drug development. Indeed there has been a phenomenal amount of interest and research activity into the endothelin system of peptides resulting in several thousand publications every year. By necessity, most of the initial research activity was in the preclinical sciences. However, there have now been several studies in humans examining the effects of intervention in the endothelin system in both health and disease. Some of these studies are presented in this thesis. I firmly believe we are now entering the most exciting stage in endothelin research. Drugs which block endothelin receptors are being rapidly developed and large trials are already under way. In particular, a multicentre international trial in essential hypertension was only recently published in the *New England Journal of Medicine*. I am sure this will not be the last and much more exciting developments are still to come in the not to distant future.

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