Endothelin: Cardiovascular Pharmacology, Physiology & Pathophysiology

Dr James Christopher Samuel Spratt

BSc MB ChB MRCP

Submitted for the Degree of Doctor of Medicine

University of Edinburgh April 2002

Table of Contents

Dedication

To Fiona, Erin & Ailsa.

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. By the nature of the unit these studies were completed in, this work is collaborative. Several studies contained within this thesis have therefore been previously submitted as part of a higher degree. These are as follows: Study 1, "Inhibition Of Neutral Endopeptidase Causes Vasoconstriction Of Human Resistance Vessels In Vivo" was submitted by Dr Charlie Ferro for the degree of MD; Study 3 "Systemic blockade of the ET_B receptor increases peripheral vascular resistance in healthy men" was submitted by Ms Fiona Strachan for the degree of PhD. It is also probable that Dr Jane Goddard (studies 2 & 4) and Dr Steve Leslie (study 5) will also have part of this work in their respective submissions for the degree of PhD.

Ethics

All studies were conducted with the approval of the Lothian Ethics of Medical Research Committee and 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 forearm vasoconstriction. *Circulation* 1998;**97**: 2323-30.

Strachan FE, **Spratt JC**, Wilkinson IB, Webb DJ. Systemic blockade of the ET_A receptor increases peripheral vascular resistance in healthy volunteers in vivo. *Hypertension* 1999:**33:**581-585.

Spratt JCS, Goddard J, Patel N, Strachan FE, Rankin AJ & Webb DJ. Systemic ET_A selective receptor antagonism with BQ-123 blocks ET-1 induced forearm vasoconstriction and decreases peripheral vascular resistance in healthy men. *BJ Pharm* 2001;**134:**648-654.

Submitted Papers

Spratt JCS, Goddard J, Wilkinson I B, MacCallum H, Wammes W, Webb, DJ. The pressor effects of angiotensin II in healthy men are not acutely mediated by endothelin-1 (submitted to *Hypertension*).

Leslie SJ, **Spratt JCS**, McKee S, Strachan FE, Northridge DN, Denvir M & Webb DJ. The effects of systemic ET_A / $_B$ receptor antagonism in conventionally treated patients with congestive heart failure (submitted to *JACC*).

Presentations arising from this work:

2nd Symposium on Endothelin Antagonism: Oral presentation: *Inhibition of neutral endopeptidase causes vasoconstriction in human forearm vasoconstriction.* Zurich, Switzerland. March 1998.

International Congress on Vascular Medicine: Key-note lecture: Endothelin antagonists, experience from healthy volunteers and clinical trials. Graz, Austria. June 1998.

British Pharmacological Society: The haemodynamic effects of systemic endothelin A receptor antagonism in healthy humans in vivo. Brighton, England. January 1999.

ET-6: Oral presentation: Systemic blockade of the ET_A receptor blocks agonist-induced vasoconstriction and decreases peripheral vascular resistance in healthy men. Montreal, Canada, October 1999.

ET-6: Poster presentation: The pressor effects of angiotensin II in healthy men are not acutely mediated by endothelin-1. Montreal, Canada, October 1999.

Acknowledgements

All the work presented in this thesis was carried out at the Clinical Research Centre and Department of Clinical Pharmacology at the University Department of Medicine, Western General Hospital, Edinburgh. I would like to thank all the staff, past and present for their help and support during my time there.

The transition from Clinical Medicine to research can be quite a culture shock and I am grateful to those scientists and technical staff that helped to expand my perspective. In particular Ian Megson was always helpful, obliging and friendly; Big Neil was full of useful information on Hearts FC. and Fiona Strachan always willing to help.

Colleagues who undertook that transition with me were always a reliable source of help, but also served a useful siphon for day-to-day frustrations. Catherine Labinjoh deserves particular mention as a fellow "stuck-in-that-boat" feeling. I must also offer particular thanks to Steve Leslie and Jane Goddard who in their dealings with me both received plenty of opportunities to demonstrate their patience and good nature.

Of course no dedication to time spent in the Clinical Research Centre would be complete without mention of Professor David Webb and his (at that time) long suffering secretary Elspeth Sheils. It is a credit to David's exceptional powers of perserverance that much of this work was completed. Elspeth's all-encompassing attention to all the staff in the CRC helped at times to serve as a barrier when David's well intentioned attention to detail threatened to create excessive frustration. I would hope that my time in research has taught me something of these skills which make David such a formidable scientist.

Abbreviations

ACE	angiotensin converting enzyme
ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
AIx	Augmentation index
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
CTF	C-terminal fragment of big endothelin
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
HR	heart rate
HSA	human serum albumin
5-HT	5-hydroxytryptamine

Hz	Hertz
ia	intra-arterial
IC_{50}	inhibitory constant
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
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
PAP	Pulmonary artery pressure(s)
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
RBF	renal flood flow
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

1.1 Background to the vascular endothelium

The early impression of the vascular endothelium as an inert monolayer of cells acting as a diffusion barrier has dramatically changed recently, with the realisation that the endothelium plays a central role in vascular homeostasis (Vane *et al.* 1990). Indeed, it is tenable to suggest that most of the recent major advances in therapeutic pharmacology have had their origin in the vascular endothelium, including aspirin, nitrates and angiotensin-converting enzyme (ACE) inhibitors. Landmark work in this area includes the discovery of prostacyclin (Moncada et al. 1976), a substance, which inhibits platelet aggregation and causes vascular relaxation and the identification of an endothelium-derived relaxing factor (Furchgott & Zawadzki. 1980) later identified as nitric oxide (NO) (Palmer et al. 1987). The vascular endothelium has been further shown to release a constricting factor (Hickey et al. 1985; Gillespie et al. 1986), a discovery, which has formed the basis for further study into endothelial-based constricting factors.

1.2 Endothelin

Endothelin (ET) was first identified by Yanagisawa in the culture medium of porcine aortic endothelial cells in 1988, who described it as the most potent vasoconstrictor peptide isolated to that date (Yanagisawa et al. 1988). Later work has identified it as part of 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 1.1 & 1.2, Inoue et al. 1989). ET-1 is the most potent mammalian vasoconstrictor peptide isolated to date (Inoue et al. 1989; Yanagisawa et al. 1988) with veins 3 to 10 times more sensitive to the effects of ET-1 than arteries, both *in vitro* (Cocks et al. 1989a) and *in vivo* (Cocks et al. 1989b). ET-1 is the major isoform produced by endothelial cells (Bloch et al. 1989) and probably the most important in the cardiovascular system. The endothelins show remarkable structural similarities to the sarafotoxins, a family of snake venoms (Gray & Webb 1996). They both have 21 amino-acids, 2 intra-chain disulphide bonds constraining overall structure and a conserved C-terminal sequence necessary for biological activity and act through common receptors to elicit a multitude of effects, so much so that isoforms of the sarafotoxins have been used to characterise ET receptors.

1.2.1 ET-1 - properties

ET-1 is the major peptide produced by human endothelial cells and is present in the greatest concentration in the blood. When infused exogenously it causes intense vasoconstriction, which is characteristically slow in onset, sustained and resistant to washout from isolated arteries, veins and microcirculatory vessels of experimental animals and humans (Clarke et al. 1989). Although endogenous levels of ET-1 are very low, in the picomolar range, ET-1 is locally active and predominately released in an abluminal direction towards underlying smooth muscle (Wagner et al. 1992), allowing tissue concentrations high enough to activate local receptors.

1.2.1.1 Pressor response

The original description of ET-1 (Yanagisawa et al. 1988) described an increase in BP, following i.v. administration of ET-1 in chemically denervated rats. In contrast with most other endogenous vasoconstrictors this effect is sustained for > 60 minutes. This effect has also been demonstrated with ET-2 and ET-3, with ET-3 having the least response and for the shortest duration. As well as being demonstrated in other mammals, (Braquet et al. 1989; Pernow et al. 1996) this effect is consistent in humans.

Furthermore this pressor effect in humans is not affected by pre-treatment with cyclosporin, calcium antagonism with nifedipine or cyclo-oxygenase inhibition with indomethacin (Vieerhapper et al. 1992) and occurs despite rapid clearance of ET-1 from the circulation (Anggard et al. 1989). This prolonged effect is probably due to slsow dissociation of ET-1 from its vascular receptors.

More chronic-dosing studies have shown that prolonged infusion of ET-1 results in sustained hypertension in rats. An effect that appears to be independent of sodium balance and mediated through an increase in total peripheral vascular resistance (Mortensen & Fink 1990; Mortensen & Fink 1992). A similar pressor effect is seen with big ET-1 infusion (Kashiwabara et. al 1989; Gardiner et al. 1991), and is in contrast to a 100-fold reduced pressor potency when studied directly on blood vessels *in vitro* (Kashiwabara et. al 1989). This might suggest that the action of big ET-1 is mediated via conversion to the peptide ET-1, rather than through direct binding.

1.2.1.2 Depressor response

Although all 3 isoforms of ET show a transient hypotension, prior to the more sustained pressor response, it is most marked with ET-3, the most ET_B selective. This may be due to temporary activation of endothelial ET_B receptors, which mediate vasodilatation through production of nitric oxide and prostacyclin. A hypothesis that is substantiated by the finding that inhibitors of NO synthase attenuate

this transitory hypotension (Whittle et al. 1989; Gardiner et al. 1990; Granstam et al. 1993) and by studies showing potentiation by co-administration of ET_A receptor antagonists (Cirino et al. 1994).

1.3 Endothelin genes

Regulation of endothelin synthesis is thought to take place mainly at the level of gene transcription, with *de novo* production and release occurring in response to endothelial cell synthesis. Within the human genome, the endothelins are represented by a separate gene encoding a specific precursor for the mature isoform (Innoue et al. 1989). Three genes encoding 'ET-like' sequences in mammalian genomes (Innoue et al. 1989) were identified soon after the original description of ET-1 and have subsequently 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, (Bloch et al.1989b) the ET-2 gene on chromosome 1 (Bloch et al.1991) and the ET-3 gene on chromosome 20 (Bloch et al.1989a).

ET-1 generation can be modified, either positively or negatively by extracellular factors acting through liberation of intracellular mediators that modulate gene transcription; these include vasoactive hormones, inflammatory mediators and physio-chemical factors such as altered shear stress and hypoxia. Several agents enhancing ET-1 generation (Gray & Webb 1996) 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 (Curran & Franza. 1988; Lee et al. 1991). In the 5' flanking sequence there are binding sites for AP-1 and nuclear factor 1 through which angiotensin II and transforming growth factor ß act respectively to induce ET-1 expression. There are also binding sites for acute phase proteins, which may mediate the effects of acute physiological stress.

1.3.1 Processing of endothelin precursors

The initial product of the human endothelial-1 gene is preproendothelin-1, a 212 amino-acid peptide (Figure 1.3). After removal of the signal sequence, preproendothelin-1 undergoes the first proteolytic step that cleaves between Lys (Berger et. al .1948) –Arg (Bergland et. al .1965) and Arg (Clouthier et. al .1998) -Arg (Clozel 1989) to release the 38 amino acid precursor big ET-1 (Gray & Webb 1996). This step is similar to the processing of other peptide hormones and is probably dependent on a dibasic pair specific endopeptidase, of which furin, a proprotein convertase of the constitutive secretory pathway, is the most likely candidate (Laporte et al. 1993).

Subsequent conversion to the mature, biologically active, peptide requires selective cleavage of the Trp (Arai et al. 1990)-Val (Arai et al. 1993) bond in the carboxy terminal of big ET-1. This step occurs through the action of endothelin-converting enzymes (ECE; Figure 1.5). Several ECE-like enzymes have been identified (Opgenorth et al. 1992; Turner & Murphy. 1995), including serine proteases (Yanagisawa et al. 1988), aspartic proteases (Takaoka et al. 1990), cathepsin D (Sawamura et al. 1990), and soluble thiol protease (Deng et al. 1992). Although inhibitors of these enzymes can prevent conversion of big ET-1, their contribution to ET-1 biosynthesis is not thought to be of major physiological significance (Gray & Webb 1996).

The physiologically relevant ECE is a membrane-bound, zinc-containing metalloprotease inhibited by the combined ECE and neutral endopeptidase inhibitor (NEP), phosphoramidon (Opgenorth et al. 1992). The activity of this ECE is not affected by thiorphan, another NEP inhibitor, or by inhibitors of the neutral metalloprotease ACE.

1.3.2 Characteristics of cloned endothelin-converting enzymes

The physiologically active ECE exists in 2 different isoforms – ECE-1a and ECE-1b, which have functionally distinct roles and tissue distributions. ECE-1a is an intracellular enzyme expressed in the Golgi apparatus of cells such as the endothelial cells that synthesise ET-1, whereas responder cells, such as vascular smooth muscle cells, express extracellular ECE-1b that can convert extracellular big ET-1 to mature ET-1. Both ECE-1, which has a neutral pH optimum and ECE-2, which has an acidic pH optimum (Emoto et al. 1992) are inhibited by phosphoramidon. ECE-1 is widely distributed but not found in neural tissues, which are known to produce mature endothelins (Xu et al. 1992). ECE-2, in contrast is abundantly expressed in neural tissues (Emoto & Yanagisawa 1995).

ECE-1 is a Type II integral membrane protein composed of 754 or 758 amino acids, with a short N-terminal cytoplasmic tail, a hydrophobic transmembrane domain and a large extracellular domain containing a zinc-binding motif (Gray & Webb 1996). It is highly glycosylated (Shimada et al. 1995), with up to 10 potential glycosylation sites. There are also a number of highly conserved cysteine residues, with studies suggesting that ECE-1 might exist as a disulphide-linked dimmer (Schmidt et al. 1994; Turner & Murphy. 1995). The 2 isoforms that do exist probably result from alternative splicing of the ECE gene during transcription (Yorimutso et al. 1995). Despite this, 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.

ECE-2 is also a Type II integral membrane protein, composed of 787 amino acids, with 59% overall sequence homology with ECE-1 (Emoto & Yanagisawa. 1995). The structure of ECE-2, is similar to ECE-1, having a short N-terminal, a single trans-membrane domain and a large C-terminal containing a zinc-binding motif in the catalytic domain. It is also highly glycosylated and preferentially converts big ET-1 (Emoto & Yanagisawa. 1995). This substrate selectivity suggests that there may be yet another ECE(s) selective for big ET-2 or big ET-3 still to be discovered.

Although the major site for big ET-1 conversion has not been conclusively determined (Gray & Webb 1996), 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 (Gray & Webb 1996). This conclusion is consistent with immunohistochemical staining for ET-1 in the cytoplasm of endothelial cells and the reported ability of a low-density intracellular fraction to convert big ET-1 to the mature peptide (Gui et al. 1993; Harrison et al. 1993). The pH in the secretory granules of the trans-Golgi network is ~5.5, within optimum pH range for ECE-2 conversion (Emoto et al. 1995). Formation of ET-1 within secretory vesicles that can recognise transport pathways would also provide a suitable mechanism to explain the directional release of ET-1 towards the abluminal surface of endothelial cells (Wagner et al. 1992). ECE-1, however, works most efficiently in a neutral pH optimum, and therefore would not efficiently convert big ET-1 under the acidic conditions, which are found within secretory granules, leaving the intracellular location of ECE-1 as yet undetermined.

Although endogenous big ET-1 is converted efficiently by intracellular ECE to ET-1, a proportion is still secreted unconverted, accounting for the big ET-1 detectable in plasma (Matsumoto et al. 1994). Although circulating big ET-1 concentrations are below the K_m of ECE-1, it is likely that some big ET-1, like exogenously administered big ET-1 (McMahon et al. 1991; Auguet et al. 1992; Haynes & Webb. 1994; Xu et al. 1994) can be converted by plasma membrane-bound ECE-1. It is possible that ECE is localised in invaginations of the plasma membrane, called caveoli and abundant in endothelial and smooth muscle cells (Turner & Murphy 1995; Barnes et al. 1995). This could concentrate secreted big ET-1 for more efficient conversion by ECE-1.

1.3.3 Endothelin-converting enzyme inhibitors

Although phosphoramidon effectively inhibits ET-1 formation, its therapeutic potential is limited by its low potency and lack of selectivity for ECE (Gray & Webb 1996). 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 (Ohnaka et al. 1993; Shimada et al. 1994). To date there are no commercially available ECE-inhibitors developed. Even if selective and potent ECE-inhibitors were discovered, the problem of accessibility would still exist if intracellular ECE proves to be of greater physiological importance.

1.3.4 Clearance of endothelin

Due to efficient extraction by the splanchnic, pulmonary and renal vascular beds, the plasma half-life of ET-1 in humans is less than 1.5 min (Gasic et al. 1992; Stewart et al. 1991). Extraction of ET-1 follows binding to cell surface receptors, which are then internalised, allowing degradation to be carried out within the cell (Gandhi et al. 1993). To date levels of ET-1 have been increased by both non-selective $ET_{A/B}$ receptor antagonism as well as ET_B receptor antagonism (Strachan *et al.*, 1999). This would support other evidence suggesting a clearance role for the ET_B receptor (Fukuroda *et al.*, 1994) and indicates that the ET_A receptor does not play a major role in clearance of ET-1. Low affinity ET_B binding sites that might serve this purpose have been found in arteries and veins (Gray et al. 1994; Teerlink et al. 1994). Intracellular ET-1 degradation may be carried out by soluble proteases found in platelets, endothelial and vascular smooth muscle cells (Deng et al. 1994; Jackman et al. 1993). NEP can also degrade ET's, and is found in association with venous and arterial endothelial cell plasma membranes (Lloren-Cortes et al. 1992).

1.4 Endothelin Receptors

The ET's act on 2 receptor subtypes, ET_A and ET_B, classified according to their relative affinities to the endothelin isopeptides. ET-1 has a similar binding affinity for the ET_A and ET_B receptors – in the nanomolar range – and a much higher affinity for the ET_A receptor than ET-3. In contrast the ET_B receptor has high and equal affinity for all three isopeptides (Sakurai et al. 1990). These respective characteristics are closely reflected in differences in agonist potency of the isopeptide in isolated tissues, demonstrating functionality of the binding sites (Maggi et. al, 1989)

1.4.1 ET_A and ET_B receptors – general comments

After cloning the human ET_A and ET_B receptor have been demonstrated to exhibit ~ 60% homology (Adachi et al. 1991; Arai et al. 1991; Elshourbagy et al. 1993). The cDNAs encoding the human ET_A and ET_B receptors predict 427 and 442 amino acids, respectively. The ET_A and ET_B receptor genes, located on chromosome 4 (Hosada et al. 1992) and 13,(Arai et al. 1991) respectively have similar structural organisation suggesting that they originated from a common ancestral gene.

As with the endothelin genes, the non-transcribed 5' flanking regions of the endothelin receptor genes contain a number of regions involved in regulation of gene transcription. Receptor transcription can be modified by many exogenous factors, e.g., ET_A receptor mRNA is up-regulated by insulin (Frank et al. 1993) and ET_B receptor mRNA by angiotensin II (Kanno et al. 1993). Mechanisms such as these may influence responsiveness of ETs in pathophysiological states. For example ET_B receptor mRNA is selectively increased in marmosets fed a high cholesterol diet (Elshourbagy et al. 1993), whereas ET_A receptor expression is reduced in atherosclerotic human arteries (Winkles et al. 1993). Prolonged exposure to ET-1 is a major factor in reducing endothelin receptor number, due to down-regulation and / or feedback inhibition of receptor expression (Hirata et al. 1993).

All the cloned endothelin receptor genes are classical heptahelical rhodopsin-like G-protein coupled receptors that activate phospholipase C leading to hydrolysis of phosphatidyl inositol and generation of cytosolic inositol triphosphate and membrane bound diacylglycerol (Rubanyi & Polokoff 1994). Inositol triphosphate causes an early rise in $[Ca^{2+}]$, through its release from intracellular stores. A more sustained rise of intracellular calcium occurs through opening of membrane Ca^{2+} channels. Diacylglycerol activates protein kinase C, increasing sensitivity of the contractile apparatus to Ca^{2+} and activating nuclear signalling mechanisms causing a rise in the intracellular pH through an effect on the sodium-hydrogen ion exchange membrane pump. ET-1 many also interact with the ATP-sensitive potassium channel, so contributing to the rise in $[Ca^{2+}]$. In addition it may activate phospholipase A_2, increasing production of arachidonic acid, and hence of prostacyclin (PGI_2) and thromboxane A_2 (Gray & Webb, 1996). The transcribed region of both receptor genes encodes sites for post-translational modification that influence the structure of the receptor and therefore linkage to intracellular messenger systems.

1.4.2 Distribution and function of endothelin receptors in the cardiovascular system

ET_A receptor mRNA can be detected in many tissues, with the highest expression in aorta, heart and kidney. The ET_A receptor predominates in vascular smooth muscle (Arai et al. 1990), while ET_B receptor mRNA is most abundant in endothelial cells (Hosada et al. 1990; Molenaar et al. 1993). This would support the view that constriction of vascular smooth muscle is mediated predominantly by ET_A receptors and that this constriction is modified by release of relaxing factors from the endothelium, partly through stimulation of ET_B receptors (Figure 1.4). However, ET_B receptor mRNA is also detectable in vascular smooth muscle cells (Winkles et al. 1993) and ET_B selective agonists can evoke constriction *in vitro* (Shetty et al. 1993; Sumner et al. 1992) and pressor responses *in vivo* (Clozel et al. 1992; Williams et al. 1991), suggesting the presence of ET_B receptors that mediate constriction of vascular smooth muscle cells (Figure 1.4).

In isolated human blood vessels, the ET_A receptor subtype primarily mediates constriction in large calibre arteries (Davenport et al. 1994), but the relative functional role of ET_B receptors is greater in small calibre arteries (Takase et al. 1995; Tschudi, & Lüscher 1994). This balance of receptors may be altered under pathophysiological conditions.

1.5 Endothelin agonists

Synthetic ET-1, ET-2, ET-3 and sarafotoxin 6c (SX6c), an ET_B agonist, are the main endothelin receptor agonists available commercially. ET_A receptors have a high affinity for ET-1 and ET-2 and a lower affinity for ET-3, whereas the ET_B receptors have equal affinity for ET-1, ET-2 and ET-3. ET-3 and especially SX6c have been used as tools to investigate the role of the ET_B receptor, as ET-3 is 2000 fold selective for the ET_B receptor and SX6c is 30,000 fold selective for the ET_B receptor.

Although agonist studies are useful in defining target organs and receptor subtypes involved in physiological responses to endothelins, endothelins act predominantly via autocrine and paracrine mechanisms (Hocher et al. 1997), meaning that administration of exogenous agonist is unlikely to reproduce physiological responses and subsequently results from such studies may be misleading.

1.5.1 Effects on Resistance Vessels

ET-1 (5 pmol/min) into the brachial artery causes a slow onset, but long-lasting dose-dependent reduction in blood flow of ~ 40% when infused into human forearms (Clark et al 1989). ET-1 also causes early vasodilatation (Haynes et al. 1995), with sustained vasodilatation described in one study with ET-1 at low concentrations (0.2 pmol/min/100 ml forearm tissue) (Kiowski et al. 1990).

SX6c and ET-3, both ET_B agonists, also cause constriction of forearm resistance vessels *in vivo* but to a lesser degree than ET-1 (Haynes et al 1995a), thus implicating the ET_B receptor in vasoconstriction. However, ET-1, ET-3 and SX6c also caused a transient vasodilatation prior to a sustained vasoconstriction. The transient vasodilatation is more marked with ET-3 and SX6c suggesting that this is mediated via endothelial ET_B receptors (Haynes et al. 1995b), suggesting that ET_B receptors can mediate both vasodilatation and vasoconstriction. It is postulated that the ET_B receptors on the vascular smooth muscle cells cause vasoconstriction (Williams et al. 1991, Moreland et al. 1992, Sumner et al.

1992) while the endothelial cell receptors mediate vasodilatation (Takayanagi et al. 1991), possibly also acting as clearance receptors for ET-1.

The exact mechanism of ET-3 and SX6c mediated vasoconstriction is unclear. It may be caused by direct binding to the ET_B receptors on the vascular smooth muscle cells resulting in vasoconstriction. However, it may be that ET_B binding causes displacement of ET-1 thus increasing stimulation at the ET_A receptor resulting in vasoconstriction. Indeed, there is animal evidence that SX6c may act through non-endothelin dependent mechanisms, and the pressor responses may be independent of the endothelin system (Flynn et al 1995). Local infusions of big ET-1 (50 pmol/min) produce vasoconstriction, which can be blocked by phosphoramidon, a combined endothelin converting enzyme (ECE) and neutral endopeptidase (NEP) inhibitor (Haynes et al 1994b). As there is limited plasma ECE activity (Watanabe et al 1991) this would suggest that forearm resistance vessels have local ECE activity.

1.5.2. Effects on Capacitance Vessels

Constriction of dorsal hand veins is seen with ET-1 infusions of 5 pmol/min (Clark et al. 1989, Haynes et al. 1993, 1995a), but not with local infusion of big ET-1 (50 pmol/min), suggesting no local ECE activity in hand veins (Haynes et al. 1995b). The mechanism of action of ET-1 in hand veins is thought to mediated via both the opening of voltage operated Ca^{2+} channels and the closure of ATP sensitive K^+ channels thus offering other targets for therapeutic intervention (Haynes et al. 1993b). Sarafotoxin (SX6c) and ET-3 (ET_B agonists) causes constriction of hand capacitance vessels *in vivo* but to a lesser degree than ET-1 (Haynes et al. 1995a), thus implicating the ET_B receptor in venoconstriction. In pre-constricted human hand veins, as in arteries, ET-1, ET-3 and SX6c also caused a transient vasodilatation prior to a sustained vasoconstriction. The transient vasodilatation was more marked with

ET-3 and SX6c suggesting that it is mediated via endothelial ET_B receptors (Haynes et al. 1995b), this effect may be prostanoid dependent, as it is blocked by Aspirin.

1.5.3. Effects on Microcirculation

Intra-dermal injection of ET-1 results in vasoconstriction of the microcirculation (Wenzel et al 1994), but intra-dermal injection of ET-3 does not, suggesting that vasoconstriction in the skin microcirculation is an ET_A mediated response. More recently, studies have demonstrated vasodilatation, 1-2 cm from the site of injection (Wenzel et al 1998a). It appears that this is an ET_A receptor mediated effect through stimulation of polimodal nociceptor fibres leading to nitric oxide release because this effect is blocked by BQ-123 and pre-treatment with L-NMMA, lignocaine and capsaicin. This potentially, implicates endothelins in the process of neurogenic inflammation, suggesting ET's may possibly have a role to play in the treatment of inflammatory conditions.

The effect of ET-1 at the ET_B receptor will therefore be dependent on the balance of effects between its actions at the endothelial and vascular smooth muscle ET_B receptors. As discussed, the results of agonist studies should be interpreted with caution as the endothelins act in a paracrine and autocrine fashion and the administration of supra-physiological concentrations of exogenous agonists may not mimic *in vivo* physiology.

1.5.4 Cardiovascular Effects of Systemic Activation in Healthy Volunteers

The administration of ET-1 (5ng/kg/min for 15 min) to healthy volunteers results in an increase in mean BP of 5-10 mmHg and a reduction in HR, probably reflex-mediated. This dose of ET-1 increased plasma concentrations by 50-fold (Vierhapper et al. 1990). In more recent studies, both ET-1 (8 pmol/kg/min for 10 min) and big ET (8 pmol/kg/min) infusions, sufficient to cause increases in plasma ET-1 of 30 and 2.4 fold respectively, caused similar increases in BP and a reduction in HR

persisting for 30 and 90 min respectively after stopping the infusion. These doses of ET-1 and big ET-1 also reduce coronary sinus blood flow, by ~25%, and increase coronary vascular resistance by ~50% and 100% respectively (Pernow et al. 1996). Furthermore doses of ET-1 insufficient to cause systemic or pulmonary pressor effects (0.75 pmol/kg/min) can cause diastolic dysfunction and are negatively inotropic (Kiely et al. 1997).

1.6 Endothelin receptor antagonists

Several endothelin receptor antagonists have been developed of varying selectivity. Some are peptides and therefore orally inactive, such as BQ-123, which has 2000-fold selectivity for the ET_A receptor (Ihara et al. 1992) and BQ-788, which demonstrates 200-fold selectivity for the ET_B receptor (Ishikawa et al. 1994) and TAK-044, a mixed $ET_{A/B}$ antagonist (Kikuchi et al. 1994). Other, orally active endothelin receptor antagonists include bosentan, a mixed $ET_{A/B}$ receptor antagonist.

1.6.1 Effects on Resistance Vessels

Local infusion of BQ-123 (Haynes et al. 1994, Berrazueta et al. 1997, Verhaar et al. 1998) and TAK-044 (Haynes et al. 1996) causes vasodilatation in forearm arteries of healthy volunteers. BQ-123 causes greater vasodilatation than TAK-044, suggesting vasoconstriction to ET-1 is primarily mediated through ET_A receptors located on vascular smooth muscle cells. Inhibition of the ET_B receptors results in a net effect of vasoconstriction. The role of the ET_B receptor has been further clarified by studies with the ET_B receptor selective antagonist, BQ-788 (Ishikawa et al 1994). BQ-788 causes vasoconstriction in the forearm vessels of healthy volunteers persisting despite ET_A antagonism (Verhaar et al 1998),

reinforcing the hypothesis that ET-1 causes dilatation at the endothelial ET_B receptor and vasoconstriction at the vascular smooth muscle ET_B receptor.

1.6.2. Effects on Microcirculation

In the skin microcirculation of healthy volunteers, intra-dermal injection of a mixed $ET_{A/B}$ antagonist causes vasodilatation, similar to that seen with selective ET_A antagonism suggesting vasoconstriction to endothelin is solely ET_A receptor mediated (Wenzel et al. 1994). In patients with coronary artery disease, however, there is increased vasodilatation with mixed $ET_{A/B}$ antagonism versus ET_A antagonism, suggesting ET_B receptor mediated vasoconstriction in these patients (Wenzel et al. 1996). In addition, intravenous administration of bosentan reverses the vasoconstrictor effect of ET-1 measured in the skin microcirculation (Weber et al. 1996). In a more recent study, pre-treatment with intra-dermal ET_A receptor antagonist prevents ET-1 induced vasoconstriction and vasodilatation in the surrounding area. There is no additional effect with mixed $ET_{A/B}$ antagonist suggesting the flare reaction is ET_A receptor mediated (Wenzel et al. 1998). Thus, in the skin microcirculation ET_A receptors appear to be involved in endothelin-mediated vasoconstriction and to mediate distal vasodilatation.

1.6.2 Cardiovascular Effects of Systemic Activation in Healthy Volunteers

The results of systemic studies with endothelin receptor antagonists have, in general confirmed predictions made from local vascular studies.

1.6.2.1 Non-selective Inhibition

TAK-044, a mixed $ET_{A/B}$ antagonist reduces systolic BP, diastolic BP and SVR by 4%, 18% and 26% respectively and increases HR and cardiac index and plasma ET concentrations. These results suggest an effect predominantly in resistance vessels (Haynes et al. 1996). The increase in plasma ET is probably due to displacement of bound ET-1 and reduced ET-1 clearance, as there is no associated increase in big ET-1 or its C-terminal fragment (Plumpton et al. 1996). Similar results are found in healthy volunteers given oral and intra-venous bosentan (Weber et al. 1996).

1.6.2.2 Selective inhibition

To date there have been no studies examining the effects of either selective ET_A receptor antagonism or ET_B receptor antagonism. The results of the forearm studies mentioned earlier may suggest a haemodynamic benefit (either in health or in disease states, such as CHF) of selective ET_A receptor antagonism compared with non-selective receptor antagonism – a hypothesis that underlay the design of Study 5. Prior to this it was necessary to confirm, by systemic administration, studies in the forearm resistance vessels showing vasodilatation to ET_A receptor antagonism - a hypothesis that underlay the design of Study 2.

Earlier results in the forearm would also suggest that systemic ET_B receptor antagonism would result in a rise in SVR and MAP a hypothesis that underlay the design of Study 3.

1.7 *Endothelin and cardiovascular pathology*

1.7.1 Hypertension

In patients with essential hypertension there is an increased venoconstrictor response to local ET-1 (5 pmol/min) and sympathetically mediated venoconstriction of capacitance vessels is potentiated by ET-1 (Haynes et al. 1994). Vasodilatation in patients with essential hypertension following intra-arterial administration of BQ-123 is no different to that seen in normal healthy volunteers, suggesting no major dysfunction of endothelium dependent vasodilatation (Ferro et al. 1996b).

Results of systemic studies performed with hypertensive patients have recently been reported. Bosentan (4 weeks) causes significant lowering of BP without reflex neurohormonal stimulation of the sympathetic nervous system or renin-angiotensin system. Bosentan caused a similar reduction in BP to that seen with 20 mg of the ACE inhibitor enalapril (Krum et al 1998).

1.7.2 Coronary artery disease (CAD)

There have been few studies performed specifically in patients with coronary artery disease. In healthy volunteers, intra-dermal injection of mixed $ET_{A/B}$ receptor antagonist causes a similar vasodilatation to selective ET_A receptor antagonism in the skin microcirculation. However, in patients with CAD, mixed inhibition causes a greater vasodilatation (Wenzel et al. 1996) suggesting that the ET_B receptor may have increased functional significance in patients with CAD.

When bosantan is given to patients with angiographically documented coronary artery disease a decrease in systolic BP and a small increase in HR is seen. In addition, there is a maximal increase in coronary artery diameter, with no further increase was noted after treatment with glycerol trinitrate suggesting basal coronary artery vasoconstrictor tone, *in vivo* is mediated by endogenous ET (Wenzel et al. 1998b).

1.7.3 Congestive cardiac failure

The syndrome of CHF, which is most commonly caused by ischaemic heart disease, is characterised by diminished cardiac output (CO) and reserve. The reduction in CO is associated with reflex activation of neurohumoral responses involving the renin-angiotensin system (RAS) and the sympathetic nervous system (SNS), which promote vasoconstriction, tachycardia, sodium and water retention and expansion of the extracellular fluid compartment (Levine *et al* 1982). Although these responses act in the short term to maintain CO, BP and the blood flow to vital organs, they also increase cardiac pressures and workload. In the longer term neuroendocrine activation contributes to progressive 'remodelling' of the left ventricle and an associated decline in cardiac function. To date, only drugs that block the RAS (Cohn *et al* 1986, CONSENSUS 1987, SOLVD 1991) and those that modify the harmful actions of the SNS (CIBIS I 1994, Packer *et al* 1996) have been conclusively demonstrated to improve the survival of CHF patients, supporting the central importance of neuroendocrine activation in the poor outcomes associated with CHF. Indeed, recent studies continue to confirm this hypothesis (RALES 1998, CIBIS II 1999).

The function of the endothelium is disturbed in CHF leading to reduced responsiveness to endothelium-dependent vasodilators in both the peripheral (Kubo *et al* 1991; Katz *et al* 1992) and coronary vessels (Holdright *et al* 1994). The increase in vasomotor tone seen in CHF represents the net effect of the interaction of locally produced factors with systemically activated vasoconstrictor reflexes, especially the SNS and RAS. Circulating concentrations of big ET-1, the precursor of ET-1, and ET-1 itself are significantly increased in CHF and relate strongly to morbidity and mortality (McMurray *et al* 1992, Packer *et al* 1996). These changes may contribute to the characteristic increase in SVR and left ventricular afterload in CHF. Drugs infused locally to block ET-1 production or actions cause

significant peripheral vasodilatation in CHF patients even in the presence of ACE inhibition (Love *et al* 1996), suggesting that local ET-1 release makes a significant contribution to the increased SVR. Local brachial artery infusion of ET-1 (5 pmol/min for 60 min) causes less vasoconstriction compared with matched controls in resistance (Love et al. 1996b) and capacitance vessels (Love et al. 1996c). In turn local brachial artery infusion of BQ-123 causes vasodilatation. Although there is increased vasoconstriction to the ET_B receptor agonist, S6Xc (5 pmol/min for 60 min), in CHF patients when compared with matched controls, BQ-788 also causes vasoconstriction, suggesting the net effect of stimulation of the ET_B receptor is vasodilatation.

In animal models of CHF long-term survival is increased after both selective ET_A receptor blockade (Sakai *et al* 1996) and mixed $ET_{A/B}$ receptor blockade (Mulder *et al.* 1997). The vasodilatation seen with ET_A antagonism may, in part, be mediated by NO acting via the endothelial ET_B receptor (Verhaar *et al.* 1998; Figure 2). This may have added importance because NO has anti-proliferative, anti-mitogenic and anti-platelet actions (Moncada *et al.* 1991) that may be beneficial in CHF and related conditions. If these findings with ET_A and ET_B antagonists in the forearm circulation were extended to the systemic circulation, one might conclude that selective ET_A receptor antagonists would offer distinct haemodynamic, and probably additional clinically relevant advantages (Haynes & Webb, 1998), over non-selective $ET_{A/B}$ receptor antagonists for CHF patients.

Kiowski and colleagues first investigated the *systemic* haemodynamic effects of bosentan, a 'mixed' $ET_{A/B}$ antagonist (though actually 100-fold more active against ET_A than ET_B receptors), in patients with CHF withdrawn from ACE inhibitor treatment (Kiowski *et al.* 1995). Bosentan decreased SVR and MAP, while substantially reducing pulmonary vascular resistance and increasing cardiac index, without a significant effect on HR and importantly no increase in plasma angiotensin II or noradrenaline (Kiowski et al. 1995). These findings were confirmed in patients with coronary artery disease (Wenzel *et al* 1998).

More recently bosentan has been shown to cause substantial haemodynamic benefits in CHF patients, already treated with conventional therapy including ACE inhibitor therapy, that were sustained for 14 days (Sutsch *et al.* 1998). It was, therefore, on this basis that we designed Study 5 to investigate the effects of short-term infusions of BQ-123 compared with the combination of BQ-123 and BQ-788. Our initial working hypothesis was that selective ET_A receptor antagonism would have additional haemodynamic benefit over non-selective $ET_{A/B}$ receptor antagonism.

1.8 Conclusions

Endothelin antagonists have proved extremely useful in extending our understanding of cardiovascular physiology and for providing new insights into the pathophysiology of cardiovascular disease. A broad body of experimental and clinical evidence now exists to support the clinical development of drugs that block the production or actions of endothelin for use in cardiovascular medicine. There is particularly good evidence to support their development in conditions associated with chronic vasoconstriction, such as hypertension and heart failure. It is still not clear whether mixed ET_A and ET_B receptor antagonists have therapeutic advantages over selective ET_A receptor antagonists, as both receptors can contribute to ET-1 induced vasoconstriction in humans. Currently, combined ET_A and ET_B receptor antagonists appear to have the widest potential for clinical application and are currently under investigation. These drugs represent a novel therapeutic approach to a fundamental and newly discovered vasoconstrictor mechanism and the results of the clinical trials are awaited with considerable interest.

1.9 Figure Legends

Figure 1.1 Endothelin & isoforms

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

Figure 1.2 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 and then mature ET-1 is formed through cleavage of big ET-1 at Try^{21}-Val^{22} by a specific endothelin converting enzyme.

Figure 1.3 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 1.4 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 endothelial cells and mediates smooth muscle vasodilatation by releasing NO and vasodilator prostanoids such as prostacyclin.

Figure 1.1

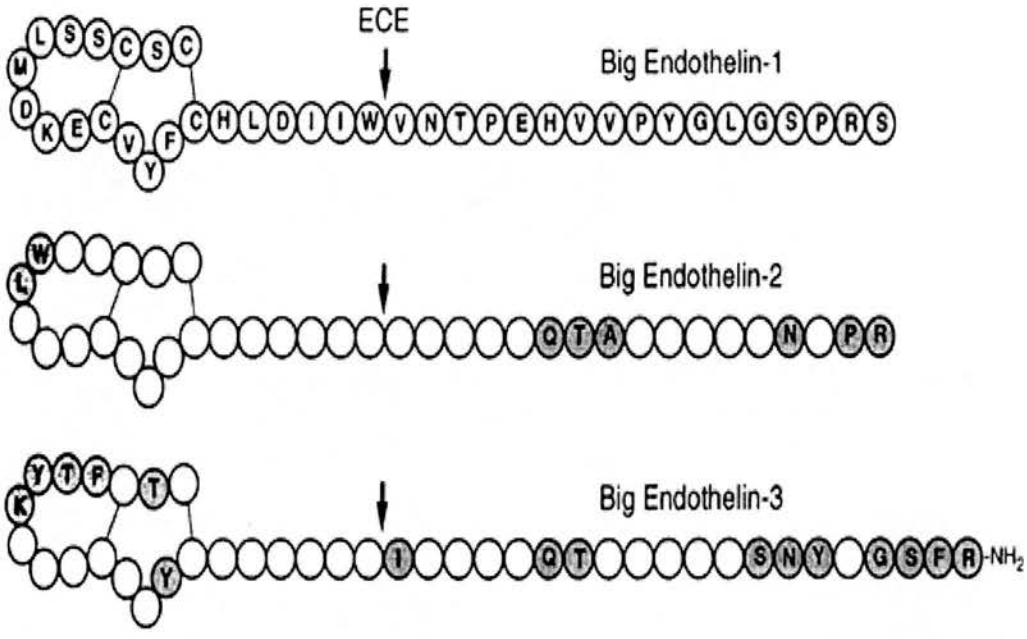


Figure 1.2

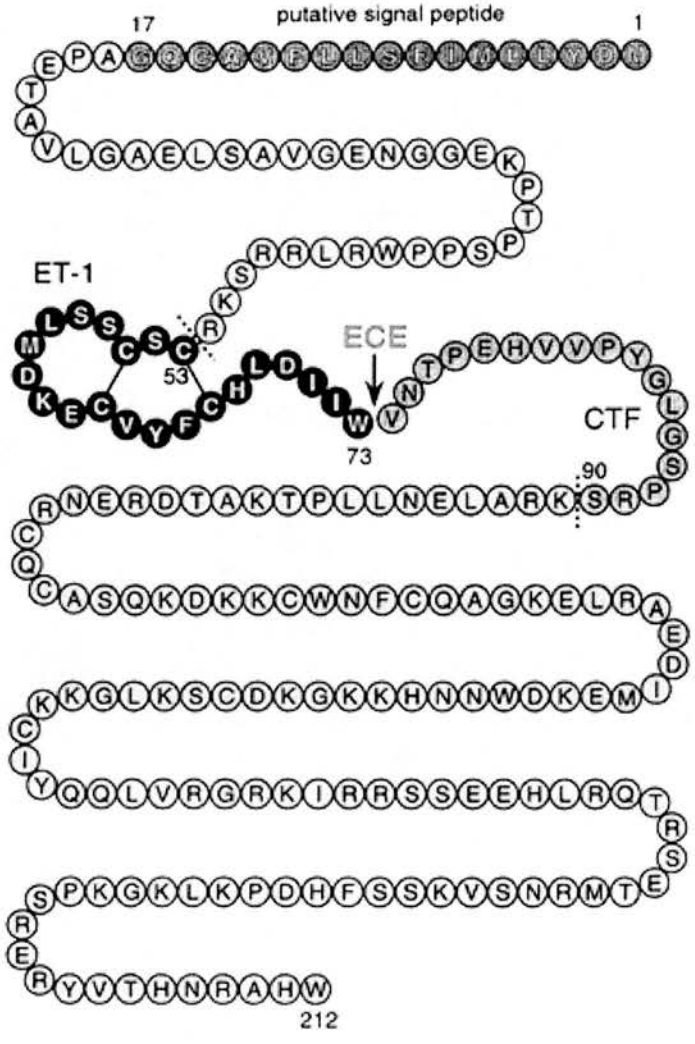


Figure 1.3

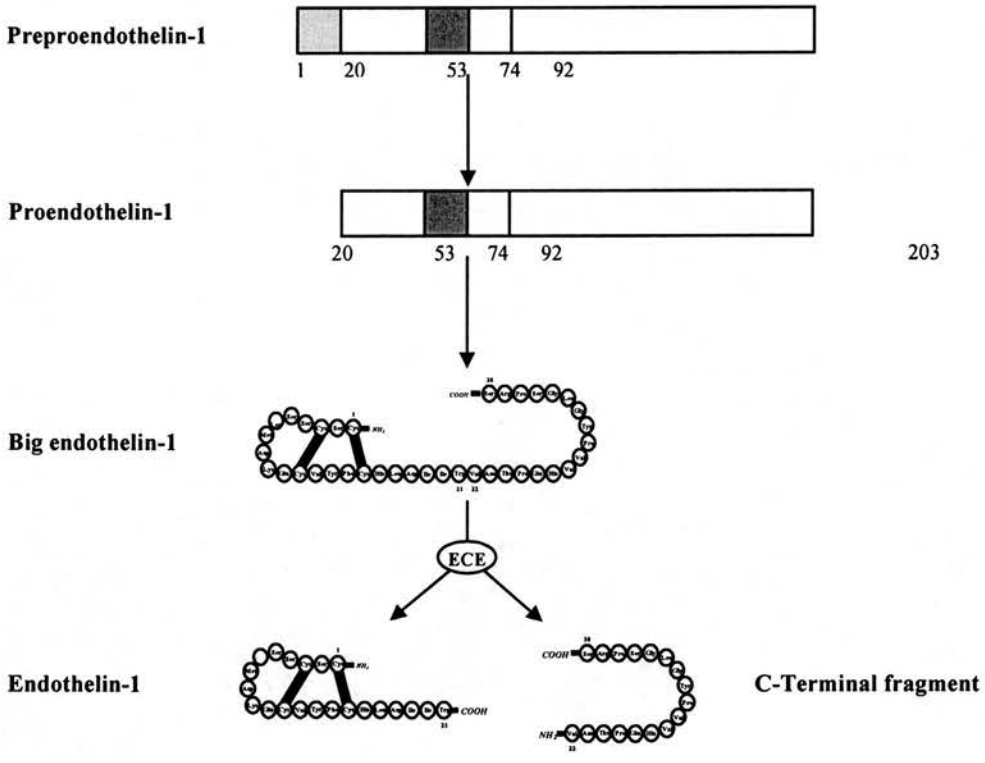
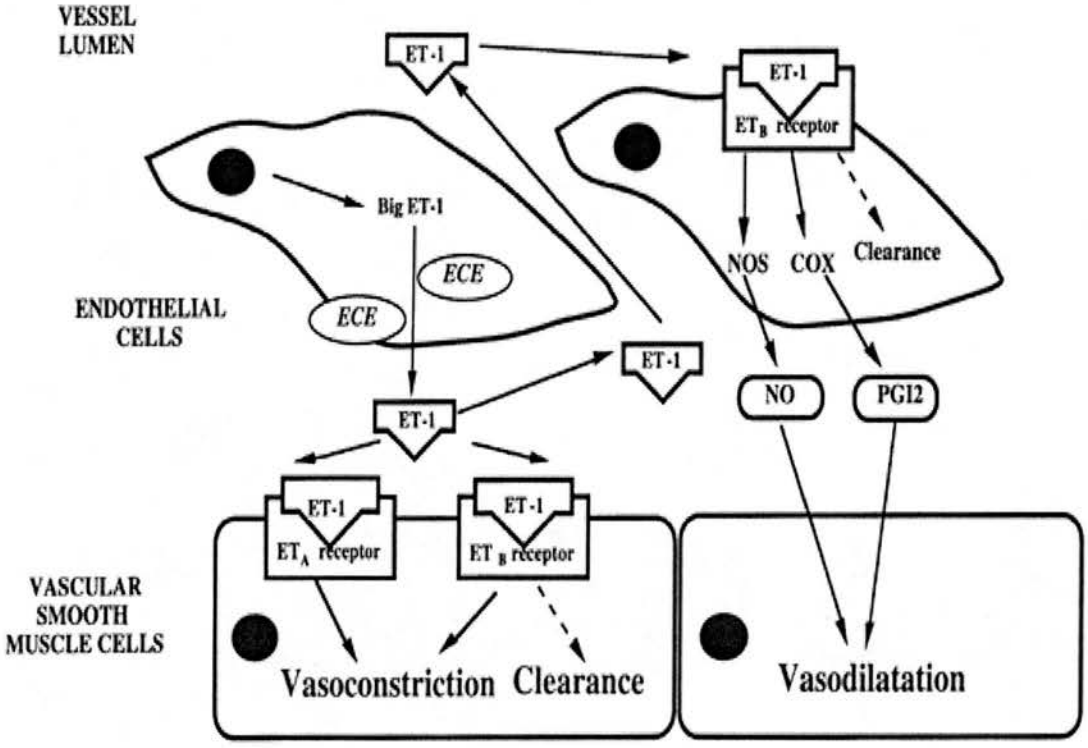

Preproendothelin-1
1
20
53
74
92
Proendothelin-1
20
53
74
92
203
Big endothelin-1
ECE
Endothelin-1
C-Terminal fragment

Figure 1.4


VESSEL LUMEN
ET-1
ET-1
ET$_B$ receptor
Big ET-1
ECE
ECE
NOS
COX
Clearance
ENDOTHELIAL CELLS
ET-1
NO
PGI2
ET-1
ET-1
ET$_A$ receptor
ET-1
ET$_B$ receptor
VASCULAR SMOOTH MUSCLE CELLS
Vasoconstriction
Clearance
Vasodilatation

2. Methods

2.1 Venous occlusion plethysmography

2.1.1 Principle

2.1.2 Technique

2.1.3 Arterial administration

2.1.4 Infusate

2.1.5 Analysis of Responses

2.2 Blood pressure measurement

2.3 Impedance cardiography

2.3.1 Principles

2.3.2 Technique

2.1 Venous Occlusion Plethysmography

Human studies of blood vessels *in vivo* using systemic doses of drug can cause effects on other organs and stimulate neurohumoral reflexes by changes in systemic haemodynamics. This makes the direct vascular effects of study drugs difficult to interpret. To avoid these confounding influences, locally active doses of drugs can be infused into single regional vascular beds enabling the study of vascular pharmacology in resistance and capacitance vessels in humans (Aellig 1981; Whelan 1967).

2.1.1 Principle

Venous occlusion plethysmography has been used to measure limb blood flow for over 80 years (Hewlett & Van Zwaluwenburg 1909). Although air and water plethysmographs have been replaced by externally applied gallium/indium-in-Silastic and mercury-in-Silastic strain gauge devices (Haynes & Webb 1994; Newby et. al. 1997) the underlying principle remains the same. A proximal limb cuff is inflated rapidly to greater than venous pressure but lower than arterial pressure, halting venous return but not affecting arterial inflow. The increase in limb volume with time thus gives a measure of arterial 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 ($ml.min^{-1}.100\ ml^{-1}$ 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 (Ensink & Hellige 1981). Although early studies often involved the lower limb, the studies in this thesis use only the forearm vascular bed.

2.1.2 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 to minimise variability in blood flow and BP and therefore maximise reproducibility of 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 (figure 2.3). 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 (Wilkins & Bradley 1946) and generally provides analysable linear tracings (Figure 2.4) (Greenfield et. al. 1963). 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 (FBF) 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 (v. 3.2.8; both from AD Instruments, Castle Hill, NSW, Australia). Calibration is achieved using the internal standard of the Vasculab plethysmography units.

During measurements, hand blood flow is excluded by wrist cuffs inflated to 220 mm Hg. As flow only stabilises after 60 seconds of cuff inflation (Kerslake 1949) cuffs must be inflated for 60 seconds longer than the period of measurement. Hand ischaemia limits the time of any one period of measurement to ~10 minutes. Venous occlusion plethysmography has been shown to be a reliable (Ensink 1981) and reproducible technique in individual subjects (Roberts 1986)

Compared with muscle blood flow, skin blood flow varies more markedly with temperature and emotion, (Abramson 1940) 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 FBF measurements can result in non-linear and immeasurable flows. Therefore, hand blood flow is excluded during measurements of FBF. Even with hand exclusion, it should be recognised that blood flow to skin contributes to total FBF (Cooper et. al. 1955), accounting for ~25% of flow <6 $ml.100ml.^{-1}min^{-1}$ and as much as 50% of higher flows.

2.1.3 Arterial administration

Barcroft and colleagues (Allen et. al. 1946) first described arterial cannulation to assess responses of skeletal muscle to local drug administration in 1946. They infused adrenaline into the femoral artery (although now most workers use the brachial artery route), and noted that this technique could 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 are 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, and / or actions 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, 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 (Benjamin et. al. 1989). 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 (Webb 1995).

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). Very fine steel needles (27 SWG: Cooper's Needle Works, Birmingham, UK) mounted on a 16-gauge epidural cannula (Portex Ltd) are used. They are 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 (Moran et. al. 1988; Gardner et. al. 1974). 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 (Haynes & Webb 1994). Some researchers use cannulae of a size sufficient to allow direct measurement of arterial blood pressure. However, these are less well tolerated, and therefore 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, data from animal pharmacology studies are of great importance. 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, this has not been observed in practice even with major reductions in local blood flow caused by ANG II (Benjamin et. al. 1989) and ET-1 (Haynes et. al. 1991). 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.

2.1.4 *Infusate*

Separate issues relate to the infusate. It is important to use the appropriate 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 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 (Overbeck et. al. 1970). Where possible, infusate flow of $>1 ml.min^{-1}$ 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 can be helpful.

2.1.5 *Analysis of Responses*

In a fasted subject resting comfortably in the supine position in a quiet, warm, temperature-controlled environment, BP and FBF remain stable for several hours (Webb 1995). 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 (Benjamin et. al. 1989).

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 smooth 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 (Webb 1995).

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 (Robinson 1990) and no simple way of distinguishing the autoregulatory response from the direct effect of a drug.

2.2 Blood Pressure Measurements

A semi-automated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) was used to make duplicate measurements of BP in the non-infused arm, which were then averaged. This system has been well validated against intra-arterial BP measurements, ordinary auscultation and semi-automatic devices using the microphone detection of Korotkoff sounds for the registration of BP (Wiinberg et. al. 1988).

2.3 Impedance Cardiography

2.3.1 Principles

Impedance cardiography (bioimpedance) is a simple, accurate and non-invasive method for measuring cardiac output (CO) and function. A constant sinusoidal current is applied between electrodes placed on the neck and lower chest and changes in the current bioimpedance 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 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 (Mohapatra 1981). By measuring the intervals between 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, relate to left ventricular performance and have been used non-invasive assessment of drug effects (Caplan et. al. 1987; Weissler 1977).

The impedance cardiogram can be 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 approximate assumptions about the relationship between the impedance cardiogram and aortic blood flow, Kubicek *et al (*Kubicek et al 1976*),* devised a plethysmographic formula for estimating stroke volume from systolic thoracic impedance change. While the formula produced acceptable estimates of stroke volume and CO at rest and with exercise in healthy volunteers, it was sometimes inaccurate, especially in patients with cardiovascular disease (Donovan et. al. 1986).

More recently Sramek and Bernstein have described an empirical modification to Kubicek's original formula (Bernstein 1986) combined with a computer algorithm to produce a simple and cheap method

for measuring stroke volume. Several studies have shown that the new method is more accurate in the clinical setting than Kubicek's (Appel et. al. 1986; Northridge et. al. 1990; Spinale et. al. 1988; Thomas 1992).

2.3.2 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 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 CO are calculated on-line by the microprocessor using the Sramek-Bernstein formula:

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

Were L is the thoracic length, estimated from the patient's height and weight using a normogram (Bernstein 1986).

Using this method, both absolute CO and changes in CO measured by bioimpedance agree closely with thermodilution measurements, and the within-subject coefficient of variation is lower with bioimpedance (Thomas 1992).

2.4 Right-heart Catheterisation

2.4.1 Principle

In the majority of situations non-invasive cardiovascular monitoring provides enough information about haemodynamic change for further invasive monitoring to be regarded as an unnecessary risk. In the assessment of pharmaceutical intervention on the pulmonary circulation, or on left atrial filling pressures, there is little in the way of alternative. Of the techniques available for right heart catheterisation, balloon-flotation catheters are the simplest and most widely used. Right-heart catheterisation allows measurement, albeit indirectly, of left atrial pressures as well as pulmonary artery and right ventricular pressures.

Swan-Ganz Catheterisation involves insertion of a flexible, balloon-tipped catheter into right side of the circulation and pulmonary artery for haemodynamic monitoring. Intravascular pressures are measured by a fluid-filled catheter, attached to a pressure transducer. The pressure wave is transmitted from the catheter tip to the transducer by the fluid column within the transducer. The pressure wave than distorts the wire within the transducer. This energy is then converted to an electrical signal, which is proportional to the pressure applied. The signal is then amplified and recorded as an analogue signal (Grossman & Baim 1991).

2.4.2 Technique

The pressure transducer must be first calibrated against a known pressure or 'zeroed' prior to commencing the procedure. To 'zero' the transducer, it is placed at the level of the heart with the transducer. The skin and subcutaneous tissue is then infiltrated with lignocaine (1%) via a small gauge needle. A large gauge needle (16- or 18-gauge) is attached to the syringe and placed into the vessel following the course of the "finder needle." When blood is easily aspirated, the syringe is disconnected from the needle and a flexible guide-wire threaded through the needle into the vein. Once the guide-wire passes into the vessel, the needle is removed and a dilator advanced over the guide-wire and through the skin, facilitating passage of the venous introducer. The introducer is flushed with heparinised saline prior to insertion, put together as a unit (dilator within introducer) and slid over the guidewire into the vein. The guidewire and dilator are removed, leaving only the venous introducer sheath within the patient. The introducer is then secured to the patient's skin with sutures.

Prior to catheter insertion, the balloon tip is checked under sterile water for leaks and the catheter flushed. The catheter is then connected to the monitoring lines and flushed again via the pressure tubing ensuring that the catheter is bubble-free and that a column of uninterrupted fluid exists from the tubing through the tip of the catheter. The catheter is then guided via the introducer, under direct fluoroscopic guidance, via the right femoral vein up the inferior vena cava, through the right atrium (RA), right ventricle (RV), pulmonary artery (PA), and into the wedge position. The catheter position is ascertained by a combination of pressure waveforms and fluoroscopy.

When an indirect measurement of left atrial filling pressure is required the catheter is "wedged", by inflating the balloon with 5cm of air and thereby briefly occluding a small pulmonary artery branch. Direct pressure measurements are obtained in the pulmonary artery. The catheter can also be used to measure CO, SVR and mixed venous oxygen saturation.

2.4.3 Calculation of cardiac output

The Swan-Ganz catheter is designed with a proximal and distal thermistor and calculates cardiac output based on the thermodilution procedure. This requires the proximal thermistor coil to deliver a set amount of energy automatically every 30 seconds, which is transmitted into a rise in temperature and measured by the distal thermistor. The CO is then calculated as being inversely proportional to the area under the temperature-time curve, using the following formula:

$$Q = 60(V_I S_I C_I CF(T_B - T_I) / [S_B C_B \int_O^{\infty} \Delta T_B(t)dt]$$

CO is then adjusted for body surface area as follows:

$$CI = CO / BSA\ (L / min / m^2)$$

Where CI = cardiac index; CO = cardiac output, $Lmin^{-1}$; BSA = body surface area, m^2.

2.4.4 Calculation of systemic vascular resistance

SVR is a derived value, which can be useful in describing the interaction between the systemic and pulmonary circulation. Pousuille's law states that the ratio of pressure gradient to flow is a function of dimension and viscosity flowing through the tube. The ratio of mean pressure gradient to mean flow is, thus, a measure of the extent to which a system opposes or resists flow. In the context of the circulatory system, this ratio is called vascular resistance (R) and is analogous to the definition of electrical resistance in Ohm's Law:

R = mean pressure gradient / mean flow

Systemic vascular resistance is further defined as:

SVR = {(MAP – CVP) x 80} / CO (dynes-sec / cm^5)

Where MAP = mean arterial pressure, mmHg; CVP = central venous pressure, mmHg; CO = cardiac output, $Lmin^{-1}$

This is further adjusted for body size and expressed as systemic vascular resistance index, as follows:

SVRI = SVR / BSA (dynes-sec-m^2) / cm^5

It should be noted that using the above calculations, only the opposition to mean flow is calculated, which largely neglects the pulsatile components of cardiac loading.

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 (Düsterdieck & McElwee. 1971).

The method for radioimmunoassay of ANG II is described in described in detail in Morton, 1985 (Morton & Webb. 1985). In brief, samples were extracted by passage through Sep-pak C18 cartridges (Waters Associates, Milford, MA). The cartridges were pre-treated 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 re-dissolved 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 for Endothelin, Big Endothelin-1 and C- of Big Endothelin-1

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 min 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 CTF was eluted with a subsequent 2 ml of 80% methanol, 0.1% TFA and immunoractive endothelin and big ET-1 were separately eluted with a subsequent 2 ml of 80& methanol, 0.1% TFA. Eluates were evaporated to dryness in polypropylene tubes using a Savant sample concentrator (Life Sciences International (UK) Ltd. Basingstoke, Hants).

Radioimmunoassay

Plasma IR endothelin, big ET-1 and CTF were determined by radioimmunoassay using rabbit antisera raised against the C-termini of endothelin (ET-1 (Ängaard & G. Rae 1991) and big ET-1 (Bakris et. al. 1993). 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, pH7.4) 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 s.d. above zero standard) were <1.25 fmol/tube. The recoveries of ET-1, big ET-1 and CTF were 57.5%, 39.8% and 76.6%, respectively (n=4).

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, big ET-1, 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, thus allowing the quantification of CTF following fractionation. 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.

2.5.4 BQ-123 Assay

BQ-123 levels in plasma were measured by high performance liquid chromatography (HPLC) with fluorescent detection. One volume of plasma had 10ul of TAK-044 (Takeda, Takeda Medical Inc) added as internal standard, was precipitated with 4 volumes of ethanol, centrifuged at 4 °C for 15 min at 10000g, and the resulting supernatant injected into the HPLC column. The HPLC system consisted of a Waters 510 HPLC pump, WISP intelligent sample processor and Spherisorb S5 ODS column (Waters Ltd, Watford, Herts. UK) with detection by LS-5 fluorometric detector (Perkin-Elmer Ltd, Beaconsfield, Bucks. UK) with excitation and emission wavelengths of 284nm and 348nm respectively. The mobile phase consisted of 60:40 Acetonitrile: de-ionised water with tri-fluoroacetic acid at a

concentration of 0.1%. Recovery of BQ-123 from plasma was found to be 107% and the intra- and inter-assay variations were 5.8% and 9.6% respectively.

2.5.5 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} (Richards et. al. 1987).

The method for radioimmunoassay of ANP is described in detail in Richards, 1987 (Richards et. al. 1987). 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^{-1} 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 h. Separation of free and bound ligand was achieved by mixing with 1 ml dextran-coated charcoal. The mixture was immediately centrifuged for 20 min 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%.

Figure 2.1 Plethysmograph set-up

The subject is shown with brachial artery canula in-situ and pressure cuffs attached above the elbow and at the wrist.

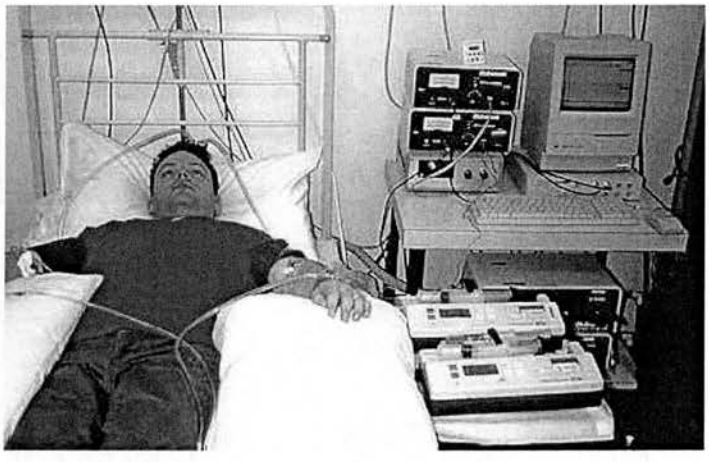
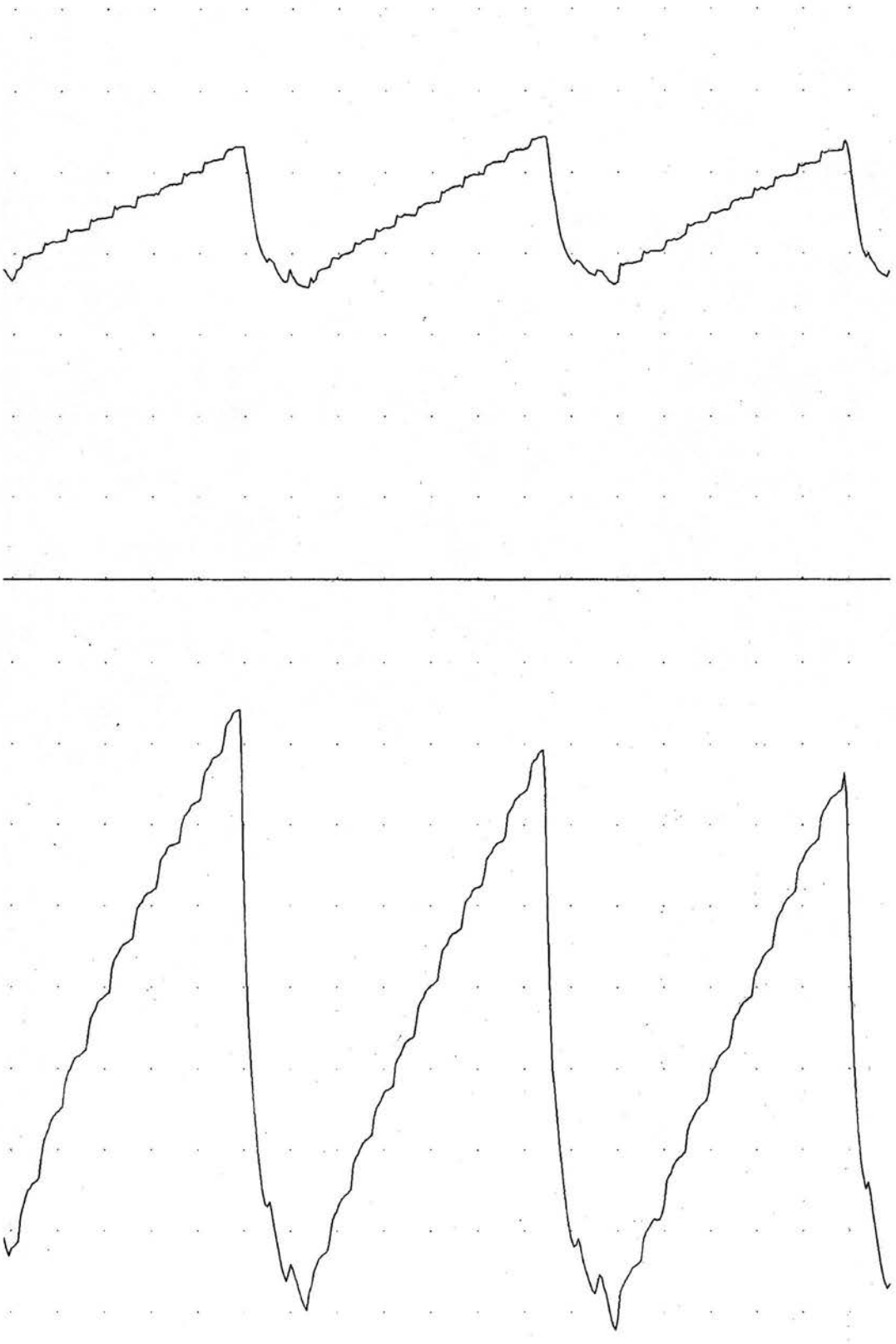


Figure 2.2



Study 1: Inhibition of neutral endopeptidase causes vasoconstriction of human resistance vessels *in vivo*

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 (Erdos & Skidgel 1989). It catalyses the degradation of a number of endogenous vasodilator peptides, including atrial natriuretic peptide (ANP) (Stephenson & Kenny 1987), brain natriuretic peptide (Lang et. al. 1992), C-type natriuretic peptide (Kenny et. al. 1993), substance P (Skidgel et. al. 1984) and bradykinin (Erdos & Skidgel 1989), as well as vasoconstrictor peptides including endothelin-1 (ET-1) (Abassi et. al. 1992) and angiotensin II (ANG II) (Erdos & Skidgel 1989). In addition to degrading vasoactive peptides to inactive breakdown products, NEP can also convert big ET-1 to the active peptide, ET-1 (Murphy et. al. 1994). 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 (Danilewicz et. al. 1989), thiorphan (Schwartz et. al. 1990) and its prodrug, sinorphan (Gros et. al. 1989), and phosphoramidon (Erdos & Skidgel 1989). ANP has potent natriuretic (De Bold et. al. 1981) and vasodilator properties (Currie et. al. 1983; Webb et. al. 1988), and inhibits activity of the renin-angiotensin-aldosterone system by reducing both renin (Brands et. al. 1988) and aldosterone (Atarashi et. al. 1984) 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 (Smith et. al. 1988). However, although NEP inhibitors increase circulating ANP concentrations in man, and cause the expected

natriuresis (Bevan et. al. 1992; Gros et. al. 1992; Northridge et. al. 1989; Richards et. al. 1991), they do not generally lower blood pressure in normotensive subjects (Richards et. al. 1991; Gros et. al. 1989; O'Connell et. al. 1992; Richards et. al. 1990). Indeed, both candoxatril (Ando et. al. 1995) and candoxatrilat (Motwani et. al. 1995) 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 (Lefrancois et. al. 1990; Ogihara et. al. 1994; Richards et. al. 1993a; Richards et. al. 1993b; Richards et. al. 1993c; Tunny et. al. 1993), this finding has not been universal (Bevan et. al. 1992; Favrat et. al. 1995; O'Connell et. al. 1992; Richards et. al. 1992). 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 (Kahn et. al. 1990; Northridge et. al. 1989).

We hypothesised that if the predominant substrates for vascular NEP were vasodilator peptides, then local inhibition of this enzyme should cause peripheral vasodilatation. However, in previous studies using brachial artery administration of the NEP inhibitor, thiorphan, a modest vasoconstriction had been observed (Haynes & Webb 1994; Love et. al. 1996) 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 angiotensin converting enzyme (ACE) inhibition. Furthermore, whether accumulation of ET-1 was the cause of the forearm vasoconstriction by thiorphan was examined by co-infusing an endothelin receptor A (ET_A) antagonist, BQ-123, together

with thiorphan. The effect of brachial artery administration of thiorphan in a group of hypertensive patients was also examined to confirm the clinical relevance of our 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°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) (Barclay et. al. 1990) 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* (Barclay et. al. 1990). 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 (Haynes & Webb 1994). 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* (Barclay et. al. 1990), 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 (Haynes & Webb 1994).

The ACE inhibitor, enalapril (Merck, Sharp & Dohme Ltd), was administered orally in ascending, single, daily doses of 2.5, 5, 10 and 15 mg over a period of 4 days. This ascending dose design was used to minimize the already low risk of hypotension. On the fifth day, subjects were admitted to the clinical research center and, after lying supine for 30 minutes, 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 to a level close to the detection limit of radioimmunoassay 4 hours after administration (Juillerat et. al. 1990).

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 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 (Plumpton et. al. 1995). 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.

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 center 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) (Matthews et. al. 1990) and as individual maximum responses (max). Haemodynamic and assay measures where analysed by ANOVA and Student's *t*-test were appropriate (Altman & Bland. 1996) using Statview 512^{+} software.

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).

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 non-infused 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 ± 6%; max 33 ± 7%; p=0.05) and placebo phase (mean 13 ± 3%; max 24 ± 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 ± 3%; max 47 ± 9%; p=0.0001) whereas thiorphan caused a slowly progressive vasoconstriction (mean -

14 ± 1%; max -22 ± 4%; p=0.0001). Co-infusion of BQ-123 and thiorphan caused a vasodilatation (mean 32 ± 2%; max 48 ± 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 ± 2%; max -20 ± 3%; p=0.0001). This was not significantly different from that observed in the healthy volunteers in the third study (p=0.39; Figure 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. Our findings are unlikely to be due to other actions of these agents because both candoxatrilat (Danilewicz et. al. 1989; Northridge et. al. 1989) and thiorphan (McMahon et al. 1991) 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 (Webb 1995). Our findings have potential implications both for the physiological role of NEP and for the therapeutic use of NEP inhibitors.

Although it was initially thought that the most important site of natriuretic peptide metabolism by NEP was the kidney (Stephenson & Kenny 1987), candoxatrilat is just as effective in reducing clearance of ANP in nephrectomized animals (Barclay et. al. 1991), implying other, non-renal, sites of action. NEP is now known to be expressed in blood vessels, by both endothelial (Graf et. al. 1995) and vascular smooth muscle cells (Dussaule et. al. 1993). Despite the clear evidence for vascular generation and metabolism of natriuretic peptides, these studies have demonstrated that 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, our finding that brachial artery administration of thiorphan produces forearm vasoconstriction in the

presence of substantial systemic ACE inhibition implies that ANG II accumulation is not responsible for the observed vasoconstriction. In addition, ANP blocks activity of the renin-angiotensin system by reducing renin release and blocking aldosterone secretion, 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 (Brands & Freeman 1988). 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 (Ando et. al. 1995). In another recent study, systemic administration of candoxatrilat in healthy subjects produced a significant increase in systolic blood pressure (Motwani et. al. 1995). However, because this rise was prevented by pre-treatment with enalapril, it was suggested that the increase in blood pressure was caused by potentiation of ANG II. Our findings do not support this conclusion. Indeed, in our study thiorphan produced arterial vasoconstriction in the presence of systemic ACE inhibition, despite 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 our 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, our 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 (Doorembos & van Brummelen 1989; Motwani et. al. 1995) 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 (Ando et. al. 1995; Favrat et. al. 1995; O'Connell et. al. 1993). ANP may also be metabolised by an aminopeptidase which is insensitive to thiorphan (Olins et. al. 1987). 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* (Barclay et. al. 1990).

Consistent with earlier work (Uemasu et. al. 1993), 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 (Wagner et. al. 1993) 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 (Plumpton et. al. 1995). 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 (Davenport & Maguire. 1994). 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 (Gros et. al. 1989; Northridge et. al. 1989). However, a reduction in blood pressure has not been clearly demonstrated in normotensive subjects (O'Connell et. al. 1992; Gros et. al. 1989; Richards et. al. 1991) and two studies have even reported an increase in blood pressure (Ando et. al. 1995; Motwani et. al. 1995) despite the potent vasodilator actions of the natriuretic peptides (Struthers 1994; Webb et. al. 1988). Also, several studies on hypertensive patients (Bevan et. al. 1992; Favrat et. al. 1995; O'Connell et. al. 1992; Richards et. al. 1992 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 (Kentsch et. al. 1996). Our 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 vasoconstriction 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.

Our study shows that the vasoconstriction produced by NEP inhibitors may be mediated by ET-1 or other vasoconstrictor peptides. Given that systemic NEP inhibition has been shown to increase venous endothelin concentrations (Ando et. al. 1995), 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 (Haynes & Webb. 1994; Love et. al. 1996), and can lower blood pressure in normotensive and hypertensive rats (McMahon et. al. 1991). 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 (Ando et. al. 1995). In addition, NEP inhibitors appear to possess favourable anti-mitogenic effects in models of left ventricular hypertrophy (Monopoli et. al. 1992) and atherosclerosis (Kugiyama et. al. 1993). Such effects would need to be counterbalanced against potential mitogenic actions of ET-1 (Gray & Webb. 1996).

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

Header:

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.

Footer:

CI indicates confidence intervals.

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

† p = 0.05 v. placebo phase

‡ p = 0.005 v. placebo phase

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 endothelin-1 (ET-1) and angiotensin II (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 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 co-infusion 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		Enalapril phase	
	Time		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 BP (mm Hg)	126 ± 6 (110 to 141)	130 ± 6 (113 to 146)	124 ± 7 (104 to 142)	124 ± 7 (104 to 142)
Diastolic BP (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 (μU/ml)	22 ± 4 (12 to 25)	19 ± 2 (12 to 25)	75 ± 9 ‡ (52 to 99)	136 ±12*‡ (106 to 166)
Aldosterone Concentration (ng/ml)	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)

Figure 3.1

Actions of NEP

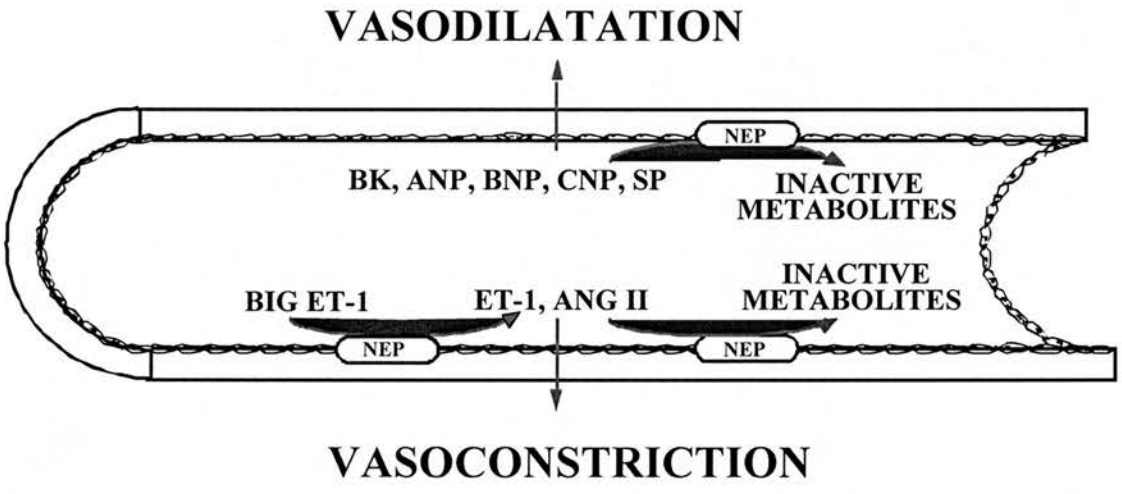


Figure 3.2

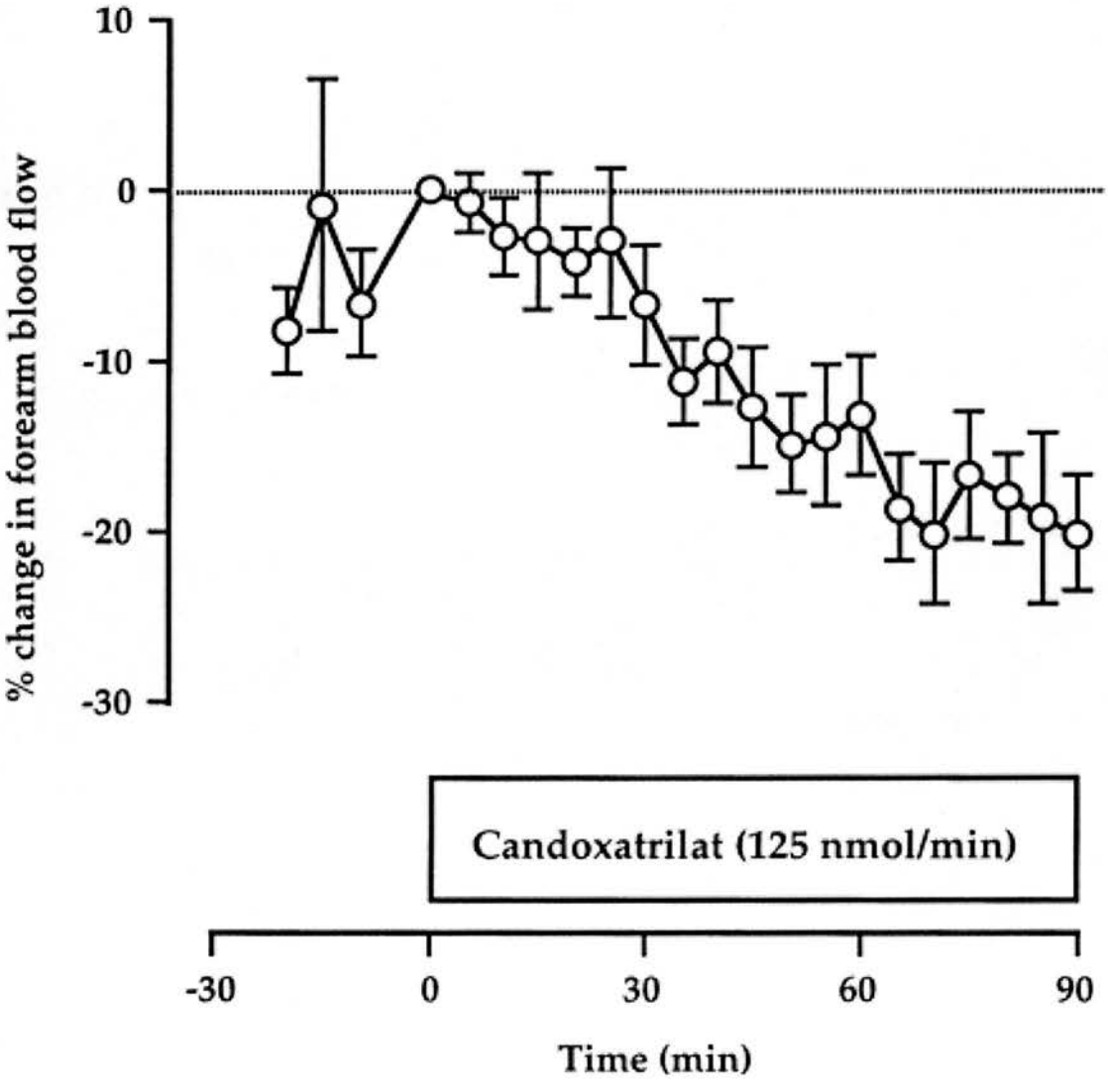

10
0
-10
-20
-30
% change in forearm blood flow
Candoxatrilat (125 nmol/min)
-30
0
30
60
90
Time (min)

Figure 3.3

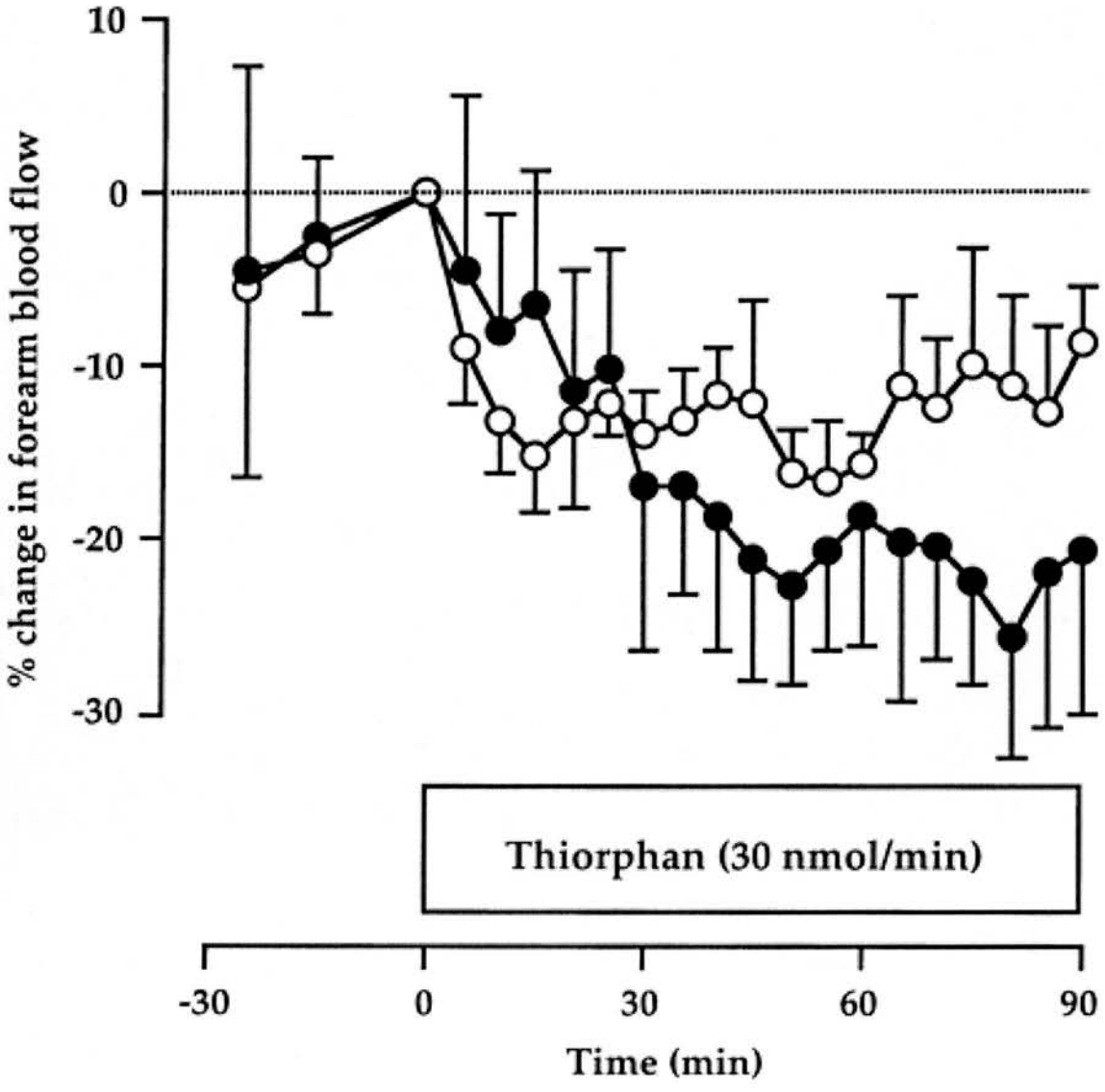

10
0
-10
-20
-30
% change in forearm blood flow
Thiorphan (30 nmol/min)
-30
0
30
60
90
Time (min)

Figure 3.4

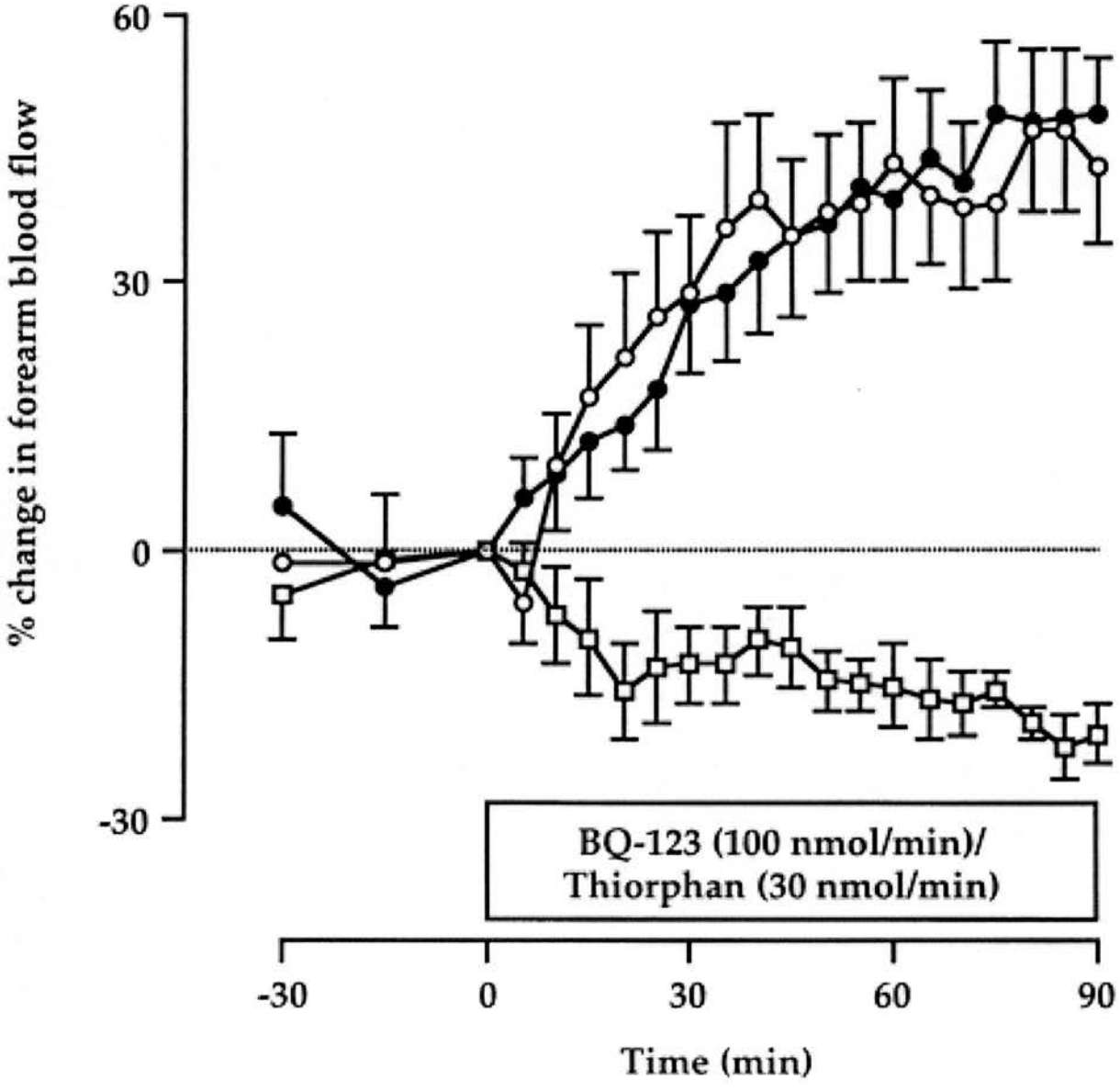
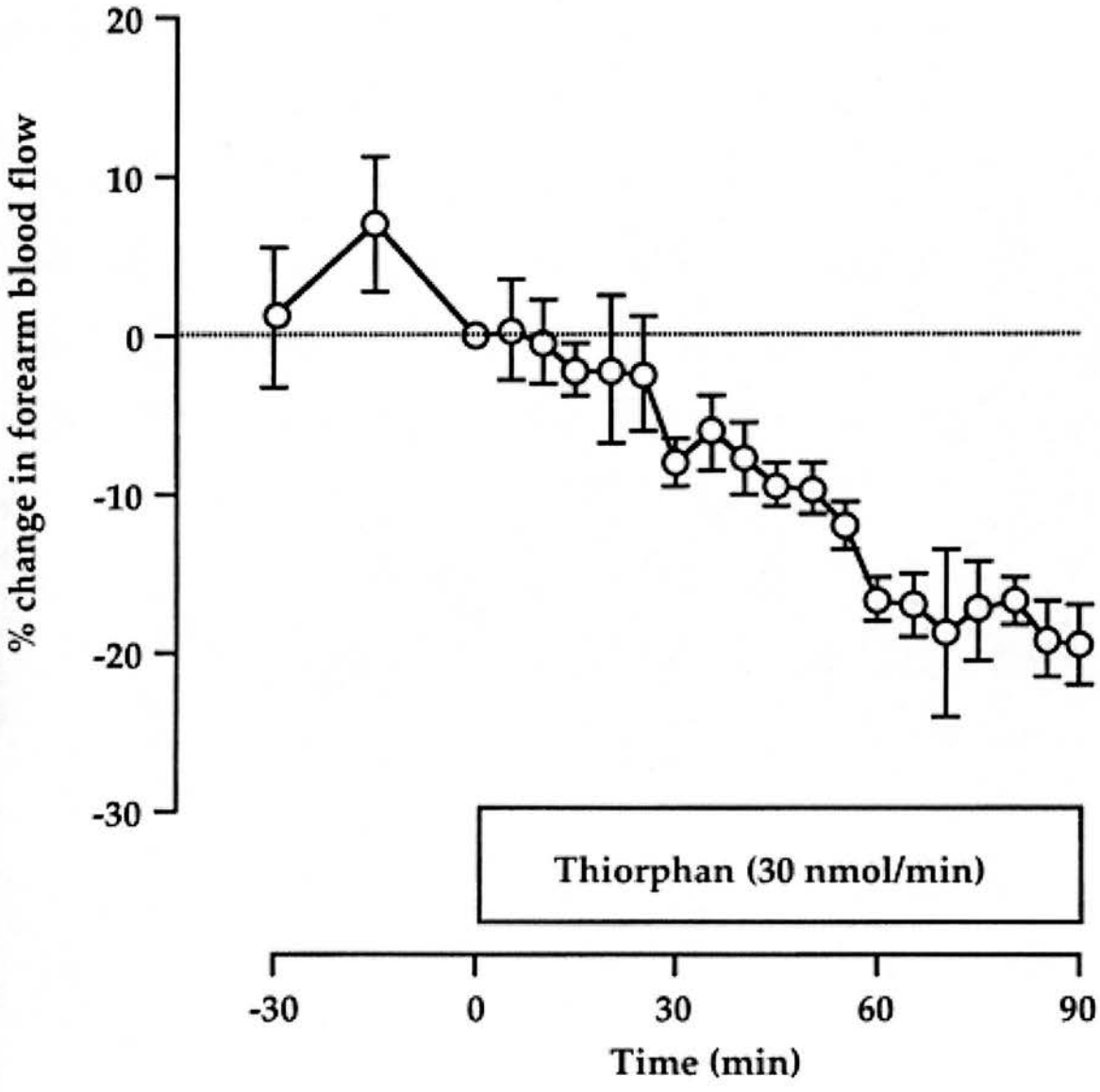

60
30
0
-30
% change in forearm blood flow
BQ-123 (100 nmol/min)/
Thiorphan (30 nmol/min)
-30
0
30
60
90
Time (min)

Figure 3.5


20
10
0
-10
-20
-30
% change in forearm blood flow
Thiorphan (30 nmol/min)
-30
0
30
60
90
Time (min)

Study 2: Systemic ET_A receptor antagonism with BQ-123 blocks ET-1 induced forearm vasoconstriction and decreases peripheral vascular resistance in healthy men.

4.4 Discussion

4.5 Figure Legends

4.6 Table 4.1

4.6.1 Table 4.2

4.7 Figure 4.1

4.7.1 Figure 4.2

4.1 Introduction

Endothelin-1, which was first identified by Yanagisawa and colleagues (Yanagisawa *et al.* 1988), is a well characterised, potent and sustained vasoconstrictor and pressor agent involved in the endothelium-mediated regulation of vascular tone (Haynes & Webb, 1998). Two ET receptor subtypes have been identified at a molecular level and characterised pharmacologically in blood vessels. ET_A receptors (Arai *et al.* 1990) have higher affinity for ET-1 than ET-3, are found on vascular smooth muscle cells, and mediate vasoconstriction. ET_B receptors have equal affinity for ET-1 and ET-3 (Sakurai *et al.* 1990) and are found on vascular endothelial cells, where they mediate endothelium dependent vasodilatation (De Nucci *et al.* 1988; Tsukahara *et al.* 1994). ET_B receptors are also present on vascular smooth muscle cells, where they may contribute to vasoconstriction (Clozel *et al.* 1992; Reizebos *et al.* 1994; Seo *et al.* 1994; Tschudi & Luscher, 1994).

Local studies in human forearm resistance vessels using phosphoramidon, an endothelin converting enzyme inhibitor, and BQ-123, a selective ET_A receptor antagonist, first demonstrated the importance of ET-1 in maintaining basal resistance vessel tone, in large part through an action on the ET_A receptor (Haynes & Webb, 1994). These observations have since been confirmed by others (Berrazueta *et al.* 1997; Verhaar *et al.* 1998). Responses in the forearm resistance vessels are usually predictive of those in the systemic circulation (Webb, 1995), so these data suggested that systemic ET_A receptor antagonism would produce systemic vasodilatation. Recently, however, acute systemic administration of the selective ET_A antagonist, BQ-123, was reported to have no effect on systemic haemodynamics (Schmetterer *et al.* 1998; Montanari *et al.* 2000).

We hypothesised that a systemic haemodynamic effect of ET_A receptor blockade was not seen in these studies, in which subjects received co-infusions of ET-1 (2.5 ng kg(-1) min(-1) for 120 min) or placebo

and BQ-123 (15 microg min(-1) for 60 min and subsequently 60 microg min(-1) for 60 min) or placebo (Schmetterer *et al.* 1998) or 0.125 nmol. Kg (Montanari *et al.* 2000), because the doses of BQ-123 used provided insufficient ET_A receptor blockade to affect blood pressure. In addition, because healthy subjects have a number of reflex mechanisms that serve to defend blood pressure, we hypothesised that an important effect on systemic vascular resistance might have been missed by measurement of blood pressure alone. We also hypothesised that systemically effective ET_A antagonism with BQ-123 might be associated with inhibition of the vasoconstriction to exogenous doses of infused ET-1 sufficient to cause modest effects on basal forearm vascular resistance. We, therefore, undertook two studies. First, we assessed the haemodynamic effects of increasing doses of BQ-123, using bioimpedance cardiography, with the aim of achieving a high degree of ET_A selective receptor blockade. Second, we examined whether haemodynamically active doses of BQ-123 would antagonise the response to exogenous ET-1, by infusion of local doses of ET-1 into the forearm circulation, after administration of BQ-123 systemically, and measuring responses using forearm plethysmography.

4.2 *Methods*

4.2.1 Subjects

Five healthy men (age range 18 - 30 years) were recruited to each of the 2 studies, which were performed in the Clinical Research Centre at the Western General Hospital, Edinburgh, with the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed to the principles outlined in the Declaration of Helsinki. No subject received vasoactive medication in the week before each phase of the study, and all subjects were asked to abstain from alcohol, nicotine and caffeine-containing products for 24 h and from food for at least 4 h before any measurements were made. All studies were performed from 8.30 am, in a quiet room kept at a controlled temperature (22-24°C).

4.2.2 Drugs

BQ-123 (Clinalfa AG, Laufelfingen, Switzerland, molecular weight 632.7) at doses ranging from 100 - 3000 nmol min^{-1} was used as a selective ET_A receptor antagonist, with 2,500 lower affinity for the ET_B receptor (IC_{50} ET_A of 7.3 nM against IC_{50} ET_B of 18 μM) (Ihara *et al.* 1992). The dose range was selected from previous studies investigating the local effects of BQ-123, which suggested that 100 nmol min^{-1} is the threshold around which systemic effects might be observed (Haynes & Webb, 1994). BQ-123 was dissolved in physiological saline (0.9%, Baxter Healthcare Ltd, Thetford, UK). Saline was also used as placebo. BQ-123 and placebo were administered in a double-blind manner and infused intravenously at a constant rate of 1 ml min^{-1} for 15 min via an 18 standard wire gauge (SWG) cannula sited in the right antecubital fossa (Study 1) or right distal forearm (Study 2).

ET-1 (Clinalfa AG, molecular weight 2492) was dissolved in physiological saline to a concentration of 5 pmol min^{-1} (Haynes & Webb, 1994) and infused into the left brachial artery via a 27 SWG steel cannula at a constant rate of 1 ml min^{-1} for a total of 90 min as described in the methods section 2.1.

All solutions were prepared from sterile stock solutions on the day of the study. For the forearm studies 1% lignocaine (Astra Pharmaceuticals, Stockholm, Sweden) was used as a local anaesthetic.

4.2.3 Systemic haemodynamics

Haemodynamic measurements were made at 10 min intervals from 30 min pre-dose until 60 min post-dose, then at 30 min intervals until 2 h, then hourly until 4 h post-dose. BP was recorded in duplicate in the right arm as described in the methods section 2.2. CO and heart rate (HR) were recorded using bioimpedance technique as described in the methods section 2.3.

4.2.4 Forearm Blood Flow

Blood flow was measured in both forearms by venous occlusion plethysmography as described in the methods section 2.3.

4.2.5 Plasma ET-1, big ET-1 and BQ-123

During Study 1, 10 ml of venous blood was obtained from a cannula inserted in the left antecubital vein before and at 5, 15 and 240 min after BQ-123 infusion for measurement of plasma ET-1 and big ET-1. In addition, during the 300 and 1000 nmol min^{-1} phases, sub-aliquots of the samples were used for

plasma BQ-123 assay. Plasma ET-1 and big ET-1 concentrations were measured as described in the methodology section (2.6.4).

4.2.4 Study design

4.2.4.1 Systemic haemodynamic study

This was a double-blind, placebo-controlled, balanced 5-way crossover study in 5 subjects, investigating the responses to four doses of BQ-123 (100, 300, 1000 and 3000 nmol min^{-1}) and placebo (0.9% saline). An ascending dose regimen was followed, allowing safety and tolerability of lower doses to be assessed before proceeding. Total doses of BQ-123 administered were 1.5, 4.5, 15 and 45 μmol (or 0.95, 2.84, 9.5 and 28.4 mg). The order of the placebo dose was randomly allocated so that each subject received it on a different visit. Each visit was separated by at least 5 days. Subjects rested supine for 20 min before any haemodynamic measurements, and baseline measures were then made in the 30 min before study drug administration.

4.2.4.2 ET-1 challenge study

This was a double-blind, placebo-controlled, 3-way crossover study in 5 subjects (3 of whom participated in the systemic study), investigating the effects of intra-arterial ET-1 on forearm blood flow (FBF), after treatment with either 300 or 1000 nmol min^{-1} of BQ-123 or placebo. After baseline infusion of saline for 30 min, subjects received a 15 min intravenous infusion of BQ-123 (300 or 1000 nmol min^{-1}) or placebo via a cannula in the right forearm, followed immediately by an intra-arterial infusion of ET-1 at a dose of 5 pmol min^{-1} for a total of 90 min via a left brachial artery cannula.

4.2.5 Data analysis

4.2.5.1. Systemic haemodynamics

Data were stored and analysed using the Microsoft Excel data analysis package (Excel 5.0, Microsoft Ltd). Blood pressure data at each time point were calculated as the mean of two recordings and represented as mean arterial pressure (MAP), calculated as diastolic BP + 1/3 pulse pressure. Bioimpedance data at each time point were calculated as the mean of four recordings. Data were corrected using body surface area to give cardiac index (CI) for direct comparison of subjects. Systemic vascular resistance index (SVRI) was calculated by dividing MAP by CI and expressed in arbitrary units. Baseline data were calculated as the mean of -10 min and 0 min recordings. Haemodynamic data are expressed as placebo-corrected percentage change from baseline ± SEM. Statistical analysis was performed on untransformed data. Responses were examined by repeated measures analysis of variance (ANOVA) and Bonferroni correction was applied to examine significance at each time point. Statistical significance was taken at the 5% level.

Using MAP & SVRI measurements from a previous placebo-controlled study over 4 h (Strachan *et al.* 1999), the study was calculated to have a power of ~90% to detect a 15% change in MAP and 20% change in SVRI ($p=0.05$) with 5 subjects. The number of subjects was agreed with the local ethics committee on that basis.

4.2.5.2 ET-1 challenge

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. FBF results are shown as the percentage change from basal values in the ratio of blood flow between infused and non-infused arm. Data were examined by repeated measures analysis of

variance (ANOVA) and Bonferroni correction was applied to examine significance at each time point. Statistical significance was taken at the 5% level.

From FBF measurements in a previous study using ET-1 at 5 pmol min^{-1} (Newby *et al.* 1998b), the study was calculated to have a power of 99% to detect abolition of the vasoconstriction response to ET-1 by BQ-123, and a power of ~80% to detect a 66% attenuation of this response ($p=0.05$) with 5 subjects.

4.3 Results

4.3.1 Systemic haemodynamics

All 5 subjects (mean age 26 ± 2 years) completed all parts of the study. No adverse effects of treatment were reported.

Plasma ET-1 and big ET-1

Baseline values of plasma ET-1 and big ET-1 concentrations ranged from 4.4 to 5.2 pg ml^{-1} and 25 to 42 pg ml^{-1} respectively. There were no significant differences between baseline plasma ET-1 or big ET-1 concentrations in any phase of the study. Neither ET-1 nor big ET-1 changed significantly following treatment with any dose of BQ-123 or placebo (Table 1A & 1B).

Plasma BQ-123 concentrations

Plasma concentrations of BQ-123 were undetectable with both doses at baseline. For 300 nmol min^{-1} BQ-123, mean plasma concentrations were 126 ± 11 nmol l^{-1} at 5 min rising to 174 ± 20 nmol l^{-1} at 15 min. For 1000 nmol min^{-1} BQ-123, they were 424 ± 33 nmol l^{-1} and 510 ± 64 nmol l^{-1} respectively (ET_A receptor IC_{50} 7.3 nM; ET_B receptor IC_{50} 18 nM) (Ihara *et al.* 1992). BQ-123 was no longer detectable in the plasma by 4 h at either dose.

Haemodynamic parameters

Baseline measurements for haemodynamic parameters were similar during all treatment periods (Table 2). After BQ-123 administration, changes were apparent in all parameters by the first measurement at 10

min. Maximal changes occurred between 40 and 60 min, with a prolonged effect occurring at the 2 highest doses, excepting changes of heart rate, which were maximal at 15 min.

MAP decreased in a dose-dependent fashion. This was statistically significant at 300, 1000 and 3000 nmol min^{-1} BQ-123 (300 nmol min^{-1}: ANOVA $p<0.05$ vs. placebo, 1000 & 3000 nmol min^{-1}: $p<0.01$ vs. placebo) with a maximum mean placebo-corrected reduction of 12.4 ± 3.5% after 3000 nmol min^{-1}. Placebo corrected SVRI also decreased in a dose dependent fashion. This decrease was significant for all doses of BQ-123 (ANOVA $p<0.01$ vs. placebo). The maximum decrease in SVRI (23.3 ± 4.3%) occurred with 3000 nmol min^{-1} of BQ-123 (Table 3 & Figure 1).

CI and HR increased significantly at all doses ($p<0.01$; ANOVA, Table 3 & Figure 1).

4.3.2 ET-1 challenge

Subjects who received placebo followed by local infusion of ET-1 developed a slow onset progressive vasoconstriction in the infused arm compared to the non-infused arm (maximum reduction in FBF: -48 ± 10% at 90 min). This response was attenuated by 300 nmol min^{-1} BQ-123 (-27 ± 8% at 90 min $p>0.5$ vs. placebo) and abolished by 1000 nmol min^{-1} BQ-123 (-8% ± 3%, $p<0.01$ vs. placebo, Figure 2).

4.4 Discussion

We have shown, in healthy humans that BQ-123 causes substantial systemic vasodilatation, associated with a small but significant reduction in arterial BP. A dose-dependent effect was observed, with little additional effect occurring above 1000 nmol min^{-1}. This work confirms the importance of the endothelin system, and of the vascular ET_A receptor in controlling vascular tone and BP (Haynes & Webb, 1998), and is in accord with the results of previous local forearm infusion studies (Haynes & Webb, 1994; Berrazueta *et al.* 1997; Verhaar *et al.* 1998).

We have several reasons for concluding that the effects on vascular tone and BP are mediated by the ET_A receptor. Measured BQ-123 concentrations in plasma at both 300 and 1000 nmol min^{-1} were substantially greater than the IC_{50} for BQ-123 at the ET_A receptor. Even so, at 1000 nmol min^{-1} the plasma concentration of 510 nmol l^{-1}, was more than 35 fold lower than the IC_{50} for the ET_B receptor (18 uM), consistent with effective but selective ET_A receptor blockade (Ihara *et al.* 1992). In addition, there is a substantial body of evidence that the ET_B receptor is a clearance receptor for ET-1 (Fukuroda *et al.* 1994; Ozaki *et al.* 1995; Dupuis *et al.* 1996) and that agents that block the ET_B receptor in vivo cause increases in plasma ET-1 concentrations (Haynes *et al.* 1996; Weber *et al.* 1996; Sutsch *et al.* 1998; Strachan *et al.* 1999). In contrast, in this study, there was no significant increase in either big ET or ET-1 plasma concentration at any dose of BQ-123 (Tables 1A & 1B). Finally, we have previously shown that the net effect of systemic ET_B receptor blockade is to cause systemic vasoconstriction, and therefore, we would anticipate that any ET_B blockade would attenuate the vasodilatation associated with ET_A blockade. Indeed, this effect may contribute to the lack of further vasodilatation at the highest dose of BQ-123, which was associated with a tendency for a rise in plasma ET-1 concentration, also consistent with a threshold effect on the ET_B receptor at this dose. Doses sufficient to lower BP (300 & 1000

nmol min^{-1}) also antagonised the forearm vasoconstriction to brachial artery administration of ET-1 and in keeping with its sub-maximal effect on SVRI, the lower dose of BQ-123 (300 nmol min^{-1}) only partially antagonised forearm vasoconstriction to ET-1. Of note, however, in the presence of a higher degree of ET_A blockade, exogenous ET-1 failed to produce vasodilatation. This likely reflects the local balance of dilator and constrictor effects mediated by endothelial and vascular smooth muscle ET_B receptors.

Although previous studies in healthy men (Schmetterer *et al.* 1998; Montanari *et al.* 2000) failed to demonstrate a significant haemodynamic effect of BQ-123, their doses were substantially lower at 23.7 nmol/min for 60 min, followed by 94.8 nmol/min for 60 min (Schmetterer *et al.* 1998) and ~9 nmol/min for 90 min (Montanari *et al.* 2000). These should be compared with 100 nmol/min BQ-123 for 15 min as the threshold dose for a systemic haemodynamic effect in our studies. In addition, both other studies measured BP but not SVRI, whereas, from the current study, the latter was a more powerful measure of the vascular effect of BQ-123, underlining the importance of this measurement in detecting modest haemodynamic influences. In this regard, other published studies in humans with endothelin antagonists do appear to show modest (~10 mmHg) reductions in BP, with both bosentan (mixed ET_A/ET_B) (Weber *et al.* 1996) and ABT-627 (ET_A selective) (Verhaar *et al.* 2000). In the latter study, although systemic haemodynamics were only recorded at 30 min and 8 hours after dosing, SVR was measured and significant effects on this parameter were found in both acute and chronic dosing.

In the current study, there was an increase in heart rate similar to that observed in other acute studies (Weber *et al.* 1996; Wenzel *et al.* 1998). These effects are not generally seen in chronic dosing studies with endothelin antagonists in patients with either hypertension or heart failure (Krum *et al.* 1998; Sutsch *et al.* 1998). For this reason, the effects are probably mediated through the activation of a

cardiopulmonary reflex response to systemic vasodilatation rather a direct chronotropic effect on the heart.

Although the total number of subjects studied was low (n=5), the power of the study was sufficient to allow clear conclusions to be drawn. Given the limited experience with BQ-123 at these systemic doses, there were safety reasons for keeping the number of subjects to a minimum. In this regard, it is reassuring to note that, despite substantial systemic vasodilatation, and significant lowering of the MAP, no side effects were observed or reported by the subjects.

In conclusion, this study with BQ-123 demonstrates that systemic ET_A receptor antagonism causes substantial peripheral vasodilatation and modest lowering of BP, consistent with an important role for the endothelin system in the maintenance of vascular tone in man. It remains to be seen, in direct comparison between selective ET_A and mixed $ET_{A/B}$ receptor antagonists, which of the therapeutic approaches will offer the greater haemodynamic benefit in specific clinical indications.

Table 4.1 - Baseline data - absolute values

BQ-123 nmol/min	MAP (mmHg)	SVRI	CI (L/min/m^2)	HR (bpm)
Placebo	78.9 ± 1.5	22.7 1.3	3.52 ± 0.21	64.2 ± 6.6
100	79.2 ± 2.3	21.9 ± 1.7	3.69 ± 0.25	57.1 ± 5.2
300	80.8 ±3.5	21.8 ±1.8	3.80 ±0.31	63.9 ±6.6
1000	78.2 ± 2.1	22.5 ± 2.9	3.63 ± 0.38	54.5 ± 4.8
3000	78.6 ± 3.3	23.5 ± 2.2	3.45 ± 0.35	57.3 ± 5.9
Anova BL data	*P*=0.96	*P*=0.98	*P*=0.89	*P*=0.69

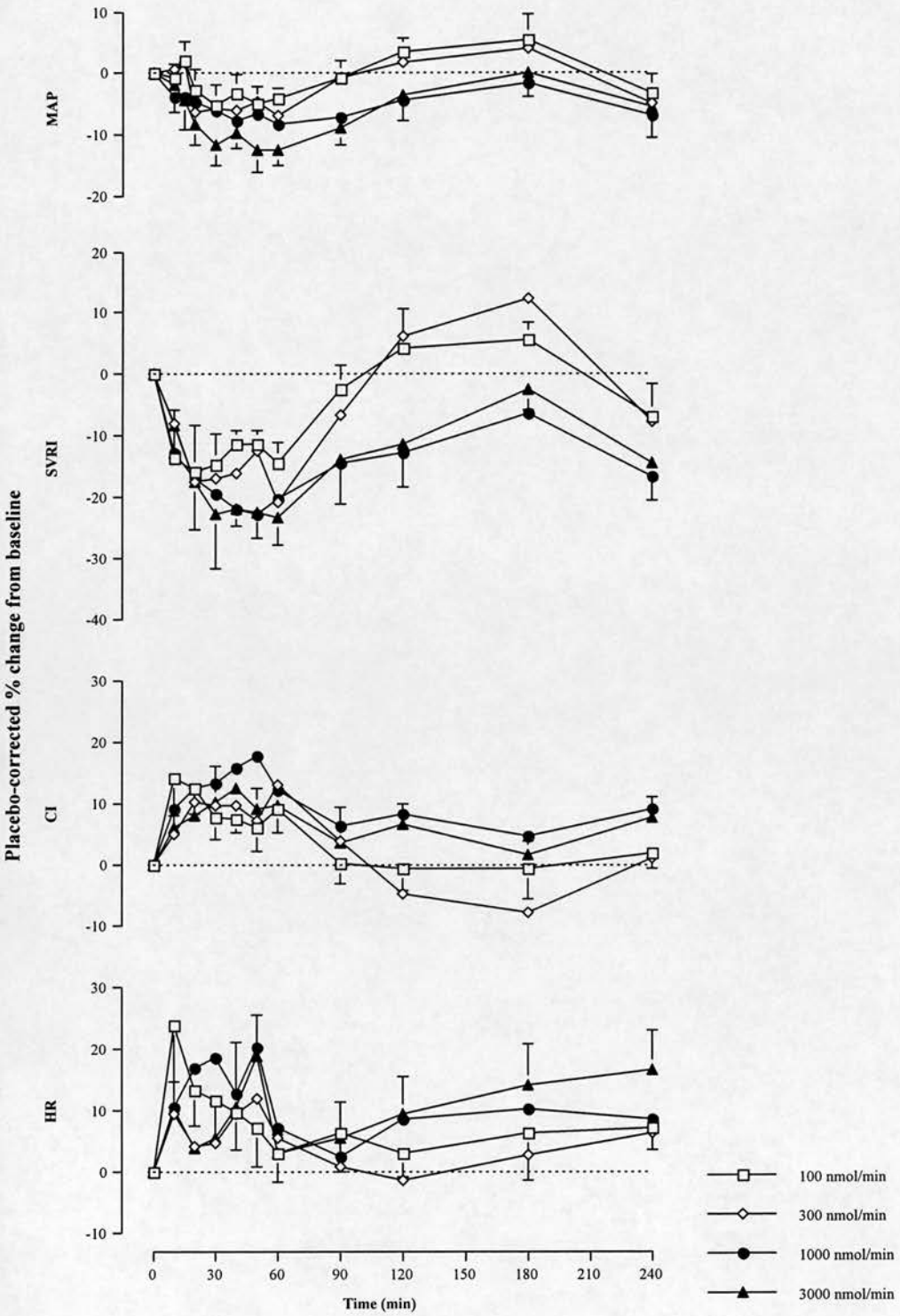
Table 4.2 - Haemodynamic changes after BQ-123 administration.

Results given are maximum placebo corrected percentage change from baseline ± SEM

$^{*}p<0.05$ vs placebo, $^{\dagger}p<0.01$ vs placebo: ANOVA + Bonferroni correction

BQ-123 (nmol/min)	MAP (mmHg)	SVRI	CI (L/min/m^2)	HR (bpm)
100	-4.8 ± 2.6%	-15.8 ± 7.6%	14.3 ± 8.9%	23.8 ± 13.4%†
	(-4.0 ± 2.5)	(-3.9 ± 2.1)	(0.49 ± 0.32)	(12.1 ± 6.5)
300	-6.8 ± 3.6%	-20.6± 3.0%*	13.0 ± 2.2%*	11.8± 4.4%
	(-5.4 ± 2.8)	(-4.5 ± 0.7)	(0.47 ± 0.07)	(6.8 ± 2.4)
1000	-8.2 ± 3.1%†	-22.7 ± 5.2%†	17.9 ± 5.7%†	20.0 ± 5.9%†
	(-6.4 ± 2.4)	(-5.4 ± 1.9)	(0.59 ± 0.16)	(11.6 ± 3.0)
3000	-12.4 ± 3.5%†	-23.3 ± 4.3%†	12.7 ± 1.6%	18.7 ± 6.8%*
	(-10.1 ± 3.2)	(-5.6 ± 2.6)	(0.43 ± 0.04)	(10.7± 3.1)

Figure 4.1



Change in MAP against time for ascending doses of BQ-123; change in SVRI against time for ascending doses of BQ-123; change in CI against time for ascending doses of BQ-123; change in HR against time for ascending doses of BQ-123.

Figure 4.2

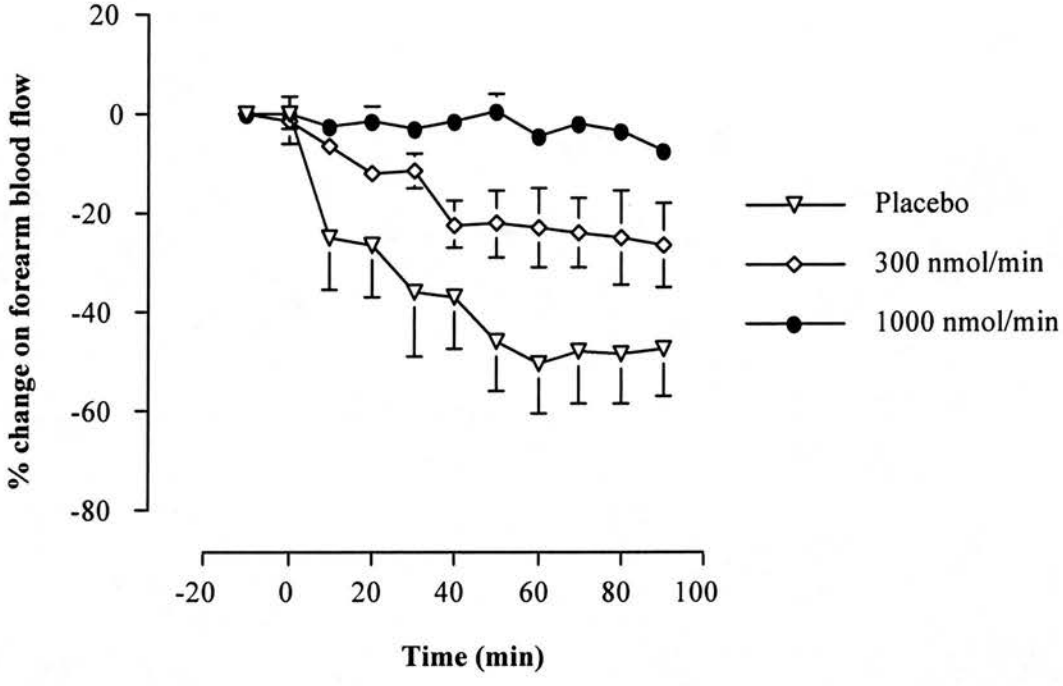


Figure 4.2 - Graph illustrating response in forearm blood flow to intra-arterial ET-1, when pre-treated with placebo or BQ-123 (either 300 or 1000 nmol min^{-1} for 15 min).

Study 3: Systemic blockade of the ET_B receptor increases peripheral vascular resistance in healthy men.

5.1.1 Introduction

5.2 Methods

5.2.1 Subjects

5.2.2 Drugs

5.2.3 Measurements

5.2.4 Study Design

5.2.5 Data analysis and statistics

5.3 Results

5.4 Discussion

5.5 Table Legends

5.6 Figure Legends

5.1 Introduction

The importance of endothelin-1 (ET-1) as a mediator of basal vascular tone *in vivo* in man has been demonstrated by local (Haynes & Webb; Haynes et al. 1996; Verhaar et al. 1998) and systemic (Haynes et al. 1996) vasodilatation in response to endothelin receptor antagonism. The potent vasoconstrictor effects of ET-1 (Yanagisawa et al. 1988; Clarke et al. 1989), combined with the increased plasma concentrations of ET-1 associated with cardiovascular diseases including heart failure (Pacher et al. 1993) and renal failure (Koyama et al. 1989), provide strong evidence to support a functional role for ET-1 in the development and maintenance of the increased peripheral vascular resistance associated with these conditions.

The vascular effects of ET-1 are mediated by two distinct receptors; the ET-1 selective ET_A receptor (Arai et al. 1990) and the non-isopeptide selective ET_B receptor (Sakurai et al. 1990). The sustained vasoconstrictor effects of ET-1 are predominantly mediated by the ET_A receptor, although vascular smooth muscle ET_B receptors have also been described (Davenport et al. 1993) and may, under some circumstances, contribute to ET-1 mediated vasoconstriction in animal models (Clozel et al. 1992) and humans *in vivo* (Haynes et al. 1995). ET_B receptors were first described on endothelial cells, where they act to modulate the vasoconstrictor effects of ET-1 through generation of nitric oxide (Tsukahara et al. 1994) and prostacyclin (De Nucci et al. 1988) The ET_B receptor also has a role in the clearance of ET-1 from the circulation (Fukuroda et al. 1994), although the exact site of the clearance receptor remains to be confirmed. The contribution of the vascular ET_B receptor to the recognised endogenous ET-1 mediated constrictor tone depends on the balance between the ET_B receptor mediated effects of vasodilatation, vasoconstriction and ET-1 clearance.

Local vasoconstriction to ET_B receptor agonists has been described in healthy volunteers (Haynes et al. 1995; Strachan et al. 1995) and in patients with heart failure (Love et al. 1996). However, more recently, vasoconstriction following local administration of the selective ET_B receptor antagonist BQ-788 (Ishikawa et al. 1994) has been described in healthy volunteers (Verhaar et al. 1998) and in patients with heart failure (Love et al. 1996b). The results with antagonists are particularly important as they indicate that the endogenous effect of vascular ET_B receptor stimulation *in vivo* favours vasodilatation. Indeed, hypertension has been described following administration of systemic doses of the selective ET_B receptor antagonists A192621 in rats and BQ-788 in rabbits *in vivo*, as well as in rescued ET_B knockout mice (Gratton et al. 1997; Webb et al. 1998). The vasoconstrictor effects of ET_B antagonism may result from direct blockade of an endothelial ET_B receptor mediated dilator tone or indirectly from displacement of endogenously generated ET-1 to vasoconstrictor ET_A receptors, or as a result of reduced clearance of ET-1 by vascular ET_B receptors. Confirmation of the balance of the vascular effects mediated by the ET_B receptor in different circumstances is important in understanding the physiology of the endothelin system, and in determining whether selective ET_A receptor antagonists or combined $ET_{A/B}$ receptor antagonists are likely to be more effective vasodilator agents in the clinical setting. Although both selective and non-selective endothelin receptor antagonists have demonstrated vasodilator effects in healthy subjects (Haynes & Webb; Haynes et al. 1996), in patients with heart failure (Cowburn et al. 1998; Kiowski et al. 1995) and in patients with hypertension (Ferro et al. 1996; Krum et al. 1998), the question of whether selective ET_A or combined $ET_{A/B}$ receptor antagonism will be of more benefit as vasodilator therapy remains to be clarified.

ET_A receptor mRNA can be detected in many tissues, with the highest expression in aorta, heart and kidney. The ET_A receptor predominates in vascular smooth muscle (Arai et al. 1990), while ET_B receptor mRNA is most abundant in endothelial cells (Hosada et al. 1990; Molenaar et al. 1993). Vascular smooth muscle constriction is mediated predominantly by ET_A receptors, modified by release of relaxing factors from the endothelium, partly through stimulation of ET_B receptors. ET_B receptor

mRNA is also detectable in vascular smooth muscle cells (Winkles et al. 1993) and ET_B selective agonists can evoke constriction *in vitro* (Shetty et al. 1993; Sumner et al. 1992) and pressor responses *in vivo* (Clozel et al. 1992; Williams et al. 1991), suggesting the presence of ET_B receptors that mediate constriction of vascular smooth muscle cells (Figure 1.4).

In isolated human blood vessels, the ET_A receptor subtype primarily mediates constriction in large calibre arteries (Davenport et al. 1994), but the relative functional role of ET_B receptors is greater in small calibre arteries (Takase et al. 1995; Tschudi, & Lüscher 1994). This balance of receptors may be altered under pathophysiological conditions.

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

5.2 Methods

5.2.1 *Subjects*

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

5.2.2 *Drugs*

BQ-788 (Clinalfa AG, Laufelfingen, Switzerland) was used as a selective ET_B receptor antagonist, based on a 1000 fold selectivity of BQ-788 for the ET_B receptor, in the nanomolar range, in human cell lines (Ishikawa et al. 1994) and on inhibition of ET-3 binding to recombinant human ET_B receptors expressed in Chinese Hamster ovary cells, also in the nanomolar range (Reynolds et al. 1995). The dose range (3-300 nmol/min) used in the current study was selected from previous work investigating the local effects of BQ-788 in the forearm circulation (Verhaar et al. 1998) and from a dose ranging pilot study in which 2 volunteers were studied at each dose level (data not shown). Selected doses (1-300 nmol/min) were administered in the pilot study to identify a no effect dose and select an appropriate maximum dose for the main study.

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

5.2.3 Measurements

Plasma ET-1 and Big ET-1

Blood samples were obtained; pre-dose and at 5, 15, 60 and 240 minutes post dose, via an 18 SWG cannula sited in the non-infused arm. In brief, 10 ml samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Becton Dickinson Vacutainer Systems, Europe) centrifuged immediately at 2000 *g* for 20 min and stored in plain tubes at -80°C prior to assay. Plasma ET-1 and big ET-1 concentrations were measured as described in the methodology section (2.6.4).

Blood samples were also taken on admission and prior to discharge for safety tests (sodium, potassium, creatinine, urea, alkaline phosphatase, gamma-glutamyl transpeptidase, hemoglobin and white cell count).

Haemodynamic recordings

Haemodynamic recordings were made at 10 minute intervals from 30 minutes pre-dose until 1 hour following the start of the infusion, with an additional blood pressure measurement at 15 minutes corresponding with the end of the infusion, and then at 30 minute intervals until 2 hours and hourly until 4 hours following the start of the infusion.

BP and heart rate were recorded in duplicate and averaged for each time-point as described in the methods section 2.2. BP is presented as mean arterial pressure (MAP; diastolic blood pressure + 1/3 pulse pressure, mm Hg).

CO and HR were recorded using bioimpedance technique as described in the methods section 2.3. These parameters were corrected for body surface area and described as cardiac index (CI, l/min/m^2) and stroke index (SI, ml/m^2) [2]. Total peripheral vascular resistance index (TPVRI) was calculated as MAP divided by CI and expressed in arbitrary units (AU).

5.2.4 Study Design

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

5.2.5 Data analysis and statistics

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

5.3 Results

All five healthy male subjects (age range 33-48 years) completed the study. No adverse events were reported and there were no clinically relevant changes in safety blood samples.

Plasma ET-1 and Big ET-1

There was no significant difference between pre--dose plasma ET-1 concentrations for any of the treatments (range of baseline mean values 4.4-4.9 pg/ml). Plasma ET-1 concentration increased significantly following administration of BQ-788 (from 4.6±0.8 to 8.4±1.8 pg/ml at 15 minutes with 300 nmol/min, p=0.02) but not during treatment with the lower doses of BQ-788 or placebo [Figure 1]. In contrast, concentrations of big ET-1 did not change significantly with treatment.

Haemodynamic parameters

Baseline measurements for haemodynamic parameters on the placebo treatment period were as follows: MAP, 79±3 mmHg; HR, 79±3 bpm; CI, 2.6±0.2 l/min/m^2; SI, 49±3 ml/m^2; TPVRI, 31.1±1.8 AU. Baseline values were similar for each of the other treatment periods. MAP did not alter significantly following administration of BQ-788 at any dose (3±2% at 90 min with 300 nmol/min; p=0.4) [Figure 2]. Following administration of BQ-788, there were changes in all other haemodynamic parameters when compared with placebo which appeared to be dose related and which were significant at 300 nmol/min; HR decreased (13±7% at 50 minutes post dose: p=0.002), CI decreased (17±5% at 40 min; p<0.0001) and there was a small reduction in stroke index (8±4% at 40 min: p=0.002). TPVRI increased (24±5% at 40 min; p<0.0001).

5.4 Discussion

We have demonstrated substantial systemic vasoconstriction, associated with a reduction in HR and CI but no change in BP, in response to administration of the selective ET_B receptor antagonist BQ-788 in healthy men. Consistent with our earlier work in the forearm circulation (Verhaar et al. 1998), these observations are highly suggestive of the overall effect of endogenous ET_B receptor mediated vascular tone favouring vasodilatation. An alternative explanation for the haemodynamic effects is that BQ-788 is directly negatively chronotropic and that peripheral effects are indirect. However, this is unlikely given our earlier work (Verhaar et al. 1998) and the lack of evidence of an important positive chronotropic and inotropic role of the cardiac ET_B receptor (Zhu et al. 1997). Although peripheral resistance (TPVRI = MAP / CI) was substantially increased, BP was unaffected because of a decrease in HR, which was probably reflex in origin. It would be expected that this reflex bradycardia would be more pronounced in younger, heallthy subjects. It is possible that if BQ-788 was administered to older or hypertensive subjects a rise in blood pressure would be seen. Earlier work, as discussed above in isolated vascular beds, such as the forearm (Verhaar et al. 1998), has demonstrated vasoconstriction consistent with the rise in TPVRI that we observed. Taken in conjunction with other work on the role of the cardiac ET_B receptor (Zhu et al. 1997), which failed to demonstrate an inotropic effect with stimulation of this receptor, out results are entirely consistent with a direct effect on peripheral vascoconstriction. We have also demonstrated increases in plasma ET-1, but not big ET-1, concentrations following ET_B receptor blockade, consistent with reduced clearance of ET-1 by the ET_B receptor (Fukuroda et al. 1994). All of these effects were prominent with BQ-788 at the highest dose but were not clearly seen at lower doses.

The vasoconstrictor effects of ET_B receptor antagonism may result directly from blockade of the vasodilator effects of the endothelial ET_B receptor or indirectly from displacement of endogenously generated ET-1 from ET_B receptors to unoccupied ET_A receptors. It is unlikely that these effects are

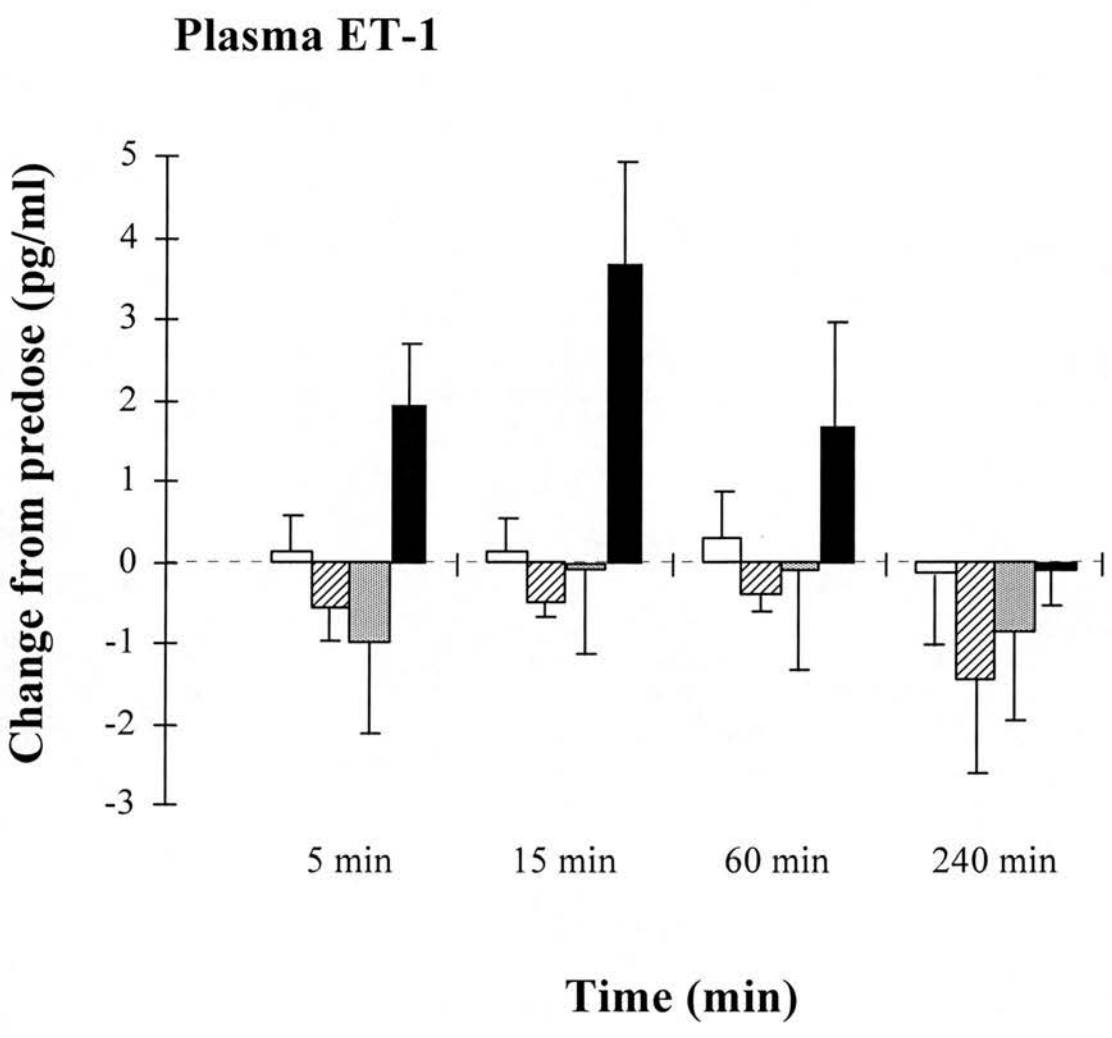
mediated by non-selective $ET_{A/B}$ receptor blockade because they are the opposite of those found with selective ET_A receptor antagonists in healthy subjects (Spratt et al. 2001) and patients with heart failure (Cowburn et al. 1998), and of those found with combined $ET_{A/B}$ receptor antagonists in healthy subjects (Haynes et al. 1996). Clearly, the indirect effects of ET-1 on ET_A receptors are more relevant with administration of selective ET_B antagonists than with non-selective $ET_{A/B}$ receptor antagonists, because in this latter situation the constrictor ET_A receptor is also blocked. Indeed, vasodilator effects have been demonstrated with both selective (Haynes & Webb; Verhaar et al. 1998; Cowburn et al. 1998) and non-selective (Haynes et al. 1996; Kiowski et al. 1995) endothelin receptor antagonists in humans and the non-selective $ET_{A/B}$ antagonist, bosentan, has recently been shown to effectively lower BP in patients with hypertension (Krum et al. 1998). However, direct comparison of the effects of selective and non-selective endothelin receptor antagonism will be important in assessing the relative contribution of each receptor subtype to the vascular effects of ET-1.

We and others have previously demonstrated forearm vasodilatation in response to local ET_A receptor antagonism with BQ-123 (Haynes & Webb; Verhaar et al. 1998; Berrazueta et al. 1997). In the presence of BQ-788 in healthy volunteers this effect was attenuated (Verhaar et al. 1998), suggesting that the overall effect of vascular ET_B receptor stimulation by endogenous ET-1 is vasodilatation. This attenuation of BQ-123 mediated vasodilatation by BQ-788 suggests that the vasoconstrictor effect of ET_B receptor blockade is not mediated by displacement of ET-1 onto the ET_A receptor, but is due to direct blockade of ET_B mediated vasodilator tone. We have also shown, using a 'nitric oxide clamp' technique, that the vasodilator response to BQ-123 is in part mediated by nitric oxide (Verhaar et al. 1998) and, therefore, probably mediated by the endothelial ET_B receptor. Loss of endothelial cell ET_B mediated vasodilator tone may occur in cardiovascular diseases, such as essential hypertension and hypercholesterolaemia, in which there is associated endothelial dysfunction (Casino et al. 1995; Panza et al. 1995). Here, because of a reduced capacity for ET_B receptor mediated, nitric oxide dependent

dilatation, selective ET_B receptor antagonists may be less effective. ET_B mediated vasodilatation is also in part mediated by generation of prostacyclin (de Nucci et al 1988), which is less likely to be affected by changes in endothelial function, although the relative balance of vasodilatory effects are unclear.

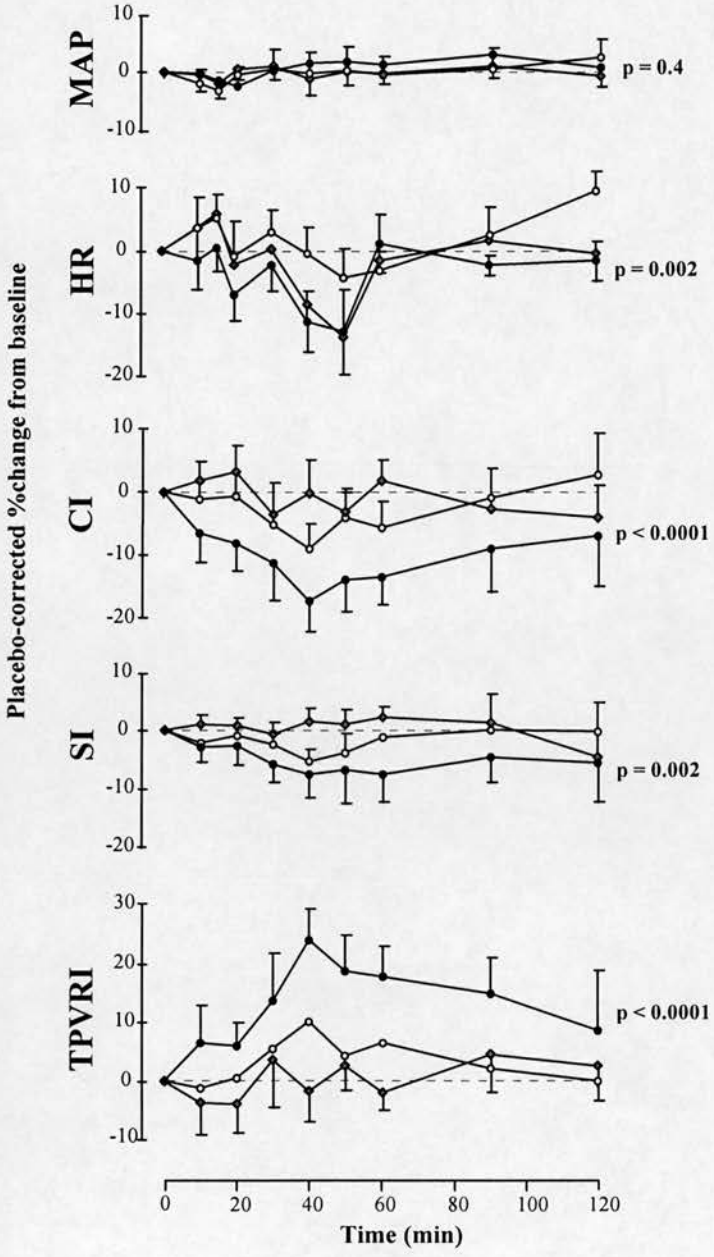
In summary, we have demonstrated systemic vasoconstriction in response to acute ET_B receptor blockade with the selective ET_B receptor antagonist BQ-788 in healthy men *in vivo*, indicating that the predominant endogenous effect of stimulating vascular ET_B receptors is vasodilatation. One exciting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

Figure 5.1



The change in plasma ET-1 concentrations (pg/ml) following a 15 minute intravenous infusion of BQ-788 or saline placebo in five subjects: solid columns, BQ-788 (300 nmol/min); shaded columns, BQ-788 (30 nmol/min); hatched columns, BQ-788 (3 nmol/min); open columns, placebo. Plasma ET-1 concentrations increased significantly following administration of BQ-788 (300 nmol/min).

Figure 5.2



Placebo corrected mean percentage change in hemodynamic parameters; mean arterial pressure (MAP), heart rate (HR), cardiac index (CI), stroke index (SI), total peripheral vascular resistance (TPVRI) following a 15 minute intravenous infusion of BQ-788 or saline placebo in five subjects: closed circles, BQ-788 (300 nmol/min); open circles, BQ-788 (30 nmol/min); shaded diamonds, BQ-788 (3 nmol/min). There was no change in MAP, but there was a reduction in HR, CI and SI, and an increase in TPVRI following administration of BQ-788 (300 nmol/min).

Study 4: The pressor effects of angiotensin II in healthy men are not acutely mediated by endothelin-1

6.1 Introduction

Angiotensin II (ANG II) is a powerful vasoconstrictor substance formed from angiotensin I (ANG I) by the actions of the endothelial cell ecto-enzyme, angiotensin converting enzyme (ACE). The success of ACE inhibitors in treating conditions such as hypertension and CHF underlines the importance of ANG II in pathophysiological situations involving vasoconstriction (SOLVD investigators 1992; SOLVD investigators 1991). ET-1 is one of the most potent vasoconstricting substances so far discovered (Yanagisawa et al 1988). As with ANG II, the beneficial effects of endothelin receptor antagonists (ERAs) in studies in animals and patients with cardiovascular disease suggest an important role for ET-1 in such conditions (Goddard & Webb 1999). The overall effect of ET-1 on BP in man is the result of a complex interplay between vasoconstriction mediated by vascular smooth muscle cell endothelin subtype A (ET_A) receptors and possibly vascular smooth muscle cell ET_B receptors, and vasodilatation and receptor-mediated clearance by endothelial cell ET_B receptors (Haynes & Webb 1998; Strachan et al 1999; Spratt et al 2001).

Recently, several lines of evidence have suggested that the effects of ANG II on vascular structure and function are at least in part mediated by ET-1. Studies in rats have demonstrated that pre-treatment with, or chronic administration of, ERAs can attenuate or even abolish the pressor response to chronic ANG II infusion (Herizi et al 1998; d'Uscio et al 1997; Moreau et al 1997; Barton et al 1998; Ortiz et al. 2001; Alexander et al 2001). In contrast, acute administration of ERAs show no effect on (Gardiner et al. 1999; Boemke et al. 2001) or, at best, only partial blunting of (Balakrishnan et al 1996; Heinemann et al 1997; Riggleman et al 2001) the pressor response to acute ANG II infusions.

In this study, we have investigated the effects of acute ET_A receptor blockade with the peptide BQ-123 (Ihara et al 1992) on the acute systemic haemodynamic effects of ANG II in man, using a dose of BQ-123 known to have maximal haemodynamic actions and to block the vasoconstrictor effects of

exogenous ET-1 (Spratt et al 2001). To allow for any non-specific hypotensive and vasodilatory effects of BQ-123 on the response to ANG II, norepinephrine (NE) was used as a control pressor agent.

6.2 Methods

6.2.1 Subjects

Eight healthy men (mean age 28 ± 4 years) were recruited to the study, which was performed in the Clinical Research Centre at the Western General Hospital, Edinburgh. The study had the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed to the principles outlined in the Declaration of Helsinki. Subjects taking prescription or over-the-counter medicines in the previous two weeks were excluded from the study. All subjects were asked to abstain from alcohol, nicotine and caffeine-containing products for 24 hours, and from food for at least 4 hours, before measurements were made. All studies were performed in a quiet room kept at a controlled temperature between 22-24 °C with the subject supine throughout.

6.2.2 Drugs

BQ-123, a selective ET_A receptor antagonist (Ihara et al 1992) (Clinalfa AG, Laufelfingen, Switzerland), was infused at 1000 nmol/min for 15 min. This dose was selected from a previous systemic dose-ranging study showing it to exert a maximal haemodynamic effect, abolish vasoconstriction to exogenous ET-1 (Spratt et al 2001), not increase plasma ET-1, and have a peak plasma concentration exceeding the K_i for ET_A receptor inhibition but twenty-fold lower than the K_i for ET_B receptor inhibition (Ihara et al 1992). ANG II (Clinalfa AG) was administered in ascending doses of 1, 3 and 6 ng/kg/min for 15 min at each dose. NE (Sanofi Winthrop, Guildford, UK) was administered in ascending doses of 60, 120 and 210 ng/kg/min for 15 min at each dose as a control pressor agent. The doses of ANG II (Collier et al 1973; Hollenberg et al 1976) and NE (Bramnert et al 1994) were chosen from previously published studies to achieve a maximum increase in MAP of ~20 mmHg. Physiological saline (0.9%, Baxter Healthcare Ltd, Thetford, UK) was administered as placebo. All drugs were

prepared as described previously (Section 2.1.4), and infused intravenously at a constant rate of 1ml/min *via* an 18 standard wire gauge (SWG) cannula sited in a left antecubital fossa vein.

6.2.3 Measurements

BP was recorded using a validated semi-automated non-invasive oscillometric device (section 2.2).

6.2.4 Plasma ET-1 and ANG II

Ten ml of venous blood for the measurement of ET-1 was obtained from an 18 SWG cannula inserted into a left forearm vein, before administration of BQ-123/placebo, at 30 min after BQ-123/placebo infusion and after each dose increment of ANG II/NE. Samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Becton Dickinson Vacutainer Systems, Europe). During the studies in which ANG II was infused, an additional 10 ml blood for the measurement of ANG II was collected at each time point and plasma ET-1 and ANG II concentrations determined as previously described (Section 2.6.2).

6.2.5 Study design

This was a double-blind, placebo-controlled study. Subjects attended for a total of 4 studies. On two study days they received ANG II, and on two NE. The subjects were randomly assigned to receive ANG II or NE first and, for each pressor agent, subjects received BQ-123 or placebo in random order. Each visit was separated by at least 3 days.

Subjects rested supine for 45 min on each occasion before beginning the study. BQ-123 or placebo were then administered intravenously via the ante-cubital cannula at a constant rate for 15 min, followed by saline for 15 min. ANG II or NE were then administered in incremental ascending doses. The timing of the pressor infusions was chosen to correspond to the period of maximal vasodilatation of this dose

of BQ-123 (Spratt et al 2001). Haemodynamic recordings were made in duplicate at 15 min intervals from 45 min before administration of BQ-123 or placebo until completion of the ANG II or NE infusions.

6.2.6 Data Analysis & statistics

Data were stored and analysed using a statistical analysis package (Excel 5.0; Microsoft Ltd, Wokingham UK). BP data at each time point were calculated as the mean of the two recordings.

Study baseline data were calculated as the mean of the two time points immediately preceding the administration of BQ-123 or placebo. Because BQ-123 reduced BP, changes in MAP during pressor infusions were calculated from the time point, immediately before the pressor infusions were started, 30 min after BQ-123 infusion commenced. Haemodynamic results are expressed as mean change from this time point ± SEM. Responses were examined by repeated measures analysis of variance (ANOVA) and Bonferroni correction was applied to examine significance identical time points. Statistical significance was taken at the 5% level.

6.3 Results

All 8 subjects completed the study. No adverse events were reported. Baseline haemodynamic measurements were similar on all four study days (Table 1).

6.3.1 Haemodynamic parameters

After placebo, ANG II and NE both increased MAP in a dose-related manner. This rise in MAP at each time point was similar for the two pressor agents (ANOVA P=0.13). Administration of BQ-123 was associated with a small reduction in MAP at 30 min (Table 1) and a subsequent significant attenuation of the observed pressor effect of both ANG II and NE (maximum attenuation, -6.3 ± 3.0 mmHg, ANOVA, P=0.02 vs. placebo for ANG II, -8.4 ± 4.0 mmHg, P=0.05 vs. placebo for NE). This attenuation was of a similar magnitude for both agents (P =0.22) (Figure 1).

6.3.2 Plasma ET-1 and ANG II

In both ANG II and NE studies, plasma concentrations of ET-1 were not significantly different at any time point. Plasma ANG II increased with each successive ANG II dose increment. There were no significant differences in the plasma ANG II levels between the BQ-123 and placebo phases, although there was a trend to higher levels in the BQ-123 phase (Table 3).

6.4 Discussion

Our studies demonstrate that the effect of acute ET_A antagonism on the pressor actions of ANG II in man is modest and non-specific, having a similar effect on the pressor response to NE. This suggests that ET-1, acting through the ET_A receptor, does not account for the acute pressor effects of ANG II.

In rats, the evidence for such an interaction between the ANG II and ET systems *in vivo* has largely rested on the results of chronic studies. Selective ET_A and non-selective $ET_{A/B}$ receptor antagonists, administered over 10 to 14 days to rats, have, however, produced varying results. Non-selective antagonism with bosentan abolished the pressor effects of continually infused ANG II at 200 ng/kg/min (Herizi et al 1998) but not 400 ng/kg/min (Casellas et al 1997). Chronic ET_A antagonism with ABT-627 abolished the pressor effects of 50 ng/kg/min of ANG II (Alexander et al 2001) but the ET_A antagonist LU 135 252 only partially attenuated the pressor effects of ANG II at a dose of 200 ng/kg/min (d'Uscio et al 1997; Barton et al 1998). However, none of these studies included an ET independent pressor agent as a control. In acute studies, by contrast, at best, only partial blunting of the pressor response has been shown with both selective ET_A (Riggleman et al 1998) and non-selective $ET_{A/B}$ antagonists (Balakrishnan et al 1996; Heinemann et al 1997). Other acute studies with the non-selective antagonist SB209670 (Gardiner et al 1999) have failed to demonstrate a significant effect on equivalent ANG II induced rises in BP. Similarly, in dogs, acute administration of LU135 252 has no effects on the pressor response to 20 ng/kg/min of ANG II (Boemke et al 2001).

Because of evidence supporting the role of ET_A receptors in the maintenance of vascular constrictor tone (Haynes & Webb 1998; Spratt et al 2001), and, because non-selective ET receptor antagonists will block nitric oxide and prostaglandin mediated vasodilatation via the ET_B receptor, and thus, theoretically reduce any effect on ANG II pressure response, we elected to use a selective ET_A

antagonist for this study. We have previously shown that our dose of 1000 nmol/min of BQ-123 for 15 min is at the top of the dose response curve in terms of the effects of BQ-123 on BP in healthy volunteers, thus is likely to give maximal ET_A receptor blockade (Spratt et al 2001). Pilot studies in the department failed to demonstrate an effect of a lower dose 300 nmol/min of BQ-123 on ANG II induced BP rise. However, in view of the above, it was felt this could equally be interpreted as incomplete ET receptor blockade rather than the absence of an interaction between ANG II and ET.

Our chosen dose of BQ-123 has significant depressor actions in man (Spratt et al 2001). We, therefore, elected to use NE as a control pressor agent to allow us to assess any non-specific hypotensive and vasodilatory effects of BQ-123 at this dose. Previous animal studies have shown that ERAs, while inhibiting ANG II induced contractions, had no effect on NE induced aortic ring contractions (Webb et al 1992). Additionally, while ET and ANG II have been shown to be synergistic in elevating BP *in vivo*, there is a lack of such synergism between ET and NE (Yoshida et al 1992). These data tend to support our use of NE as an ET-independent pressor agent. As such, the similar efficacy of BQ-123 in attenuating equivalent rises in BP induced by ANG II and NE leads us to conclude that the modest effect of acute ET_A blockade on the pressor actions of ANG II is non-specific. Additionally, although the essentially paracrine nature of the ET system may makes interpretations of changes in plasma concentrations difficult, the failure of ANG II or NE to change plasma ET-1 concentrations also suggests that neither ANG II nor NE achieve their acute pressor effect by stimulating ET-1,

Our observation is consistent with the finding that both Selective ET_A and non-selective $ET_{A/B}$ have an added haemodynamic benefit in patients with CHF already treated with ACE inhibitors (Sutsch et al 1998) and supports the development of ERAs as adjunctive treatment in such patients. However, it does not preclude an interaction between ANG II and ET-1 via ET_B receptors. Nor does it preclude a

chronic effect of ET_A blockade on attenuating ANG II mediated cardiovascular remodelling or more chronic adaptive changes.

In vitro evidence does suggest that ANG II can upregulate the ET system. ANG II can stimulate ET-1 gene transcription (Sung et al 1994; Imai et al 1992) and peptide secretion (Kohno et al 1992; Scott-Burden et al 1991) in a variety of cell types, including endothelial and vascular smooth muscle cells, probably via the AT-1 receptor (Ferri et al 1999). It is possible that we failed to detect an effect of blocking this ANG II stimulated increase in ET-1 because such up-regulation, if occurring at the transcription level, would not fully develop within the time course of this acute study. It remains possible that such an interaction could exist chronically and that, in particular, some alterations to vascular structure induced by ANG II may be mediated by ET-1.

In conclusion, we have found a moderate and non-specific effect of BQ-123 on the pressor response to ANG II in man suggesting that ANG II does not achieve its acute pressor effect via ET-1. This supports the development of ERA's as adjunctive treatments to ACE inhibitors of ET-1 receptor antagonists.

Table 1: Baseline haemodynamic data on each of the 4 study days.

	ANG II + Placebo	ANG II + BQ-123	NE + Placebo	NE + BQ-123
Mean arterial pressure at baseline (mmHg)	81.5 ± 2.5	83.5 ± 2.5	84.5 ± 2.8	83.1 ± 3.5
Mean arterial pressure at 30 min post BQ-123 (mmHg)	81.5±2.2	81.1±2.7	82.7±3.1	81.3±2.7

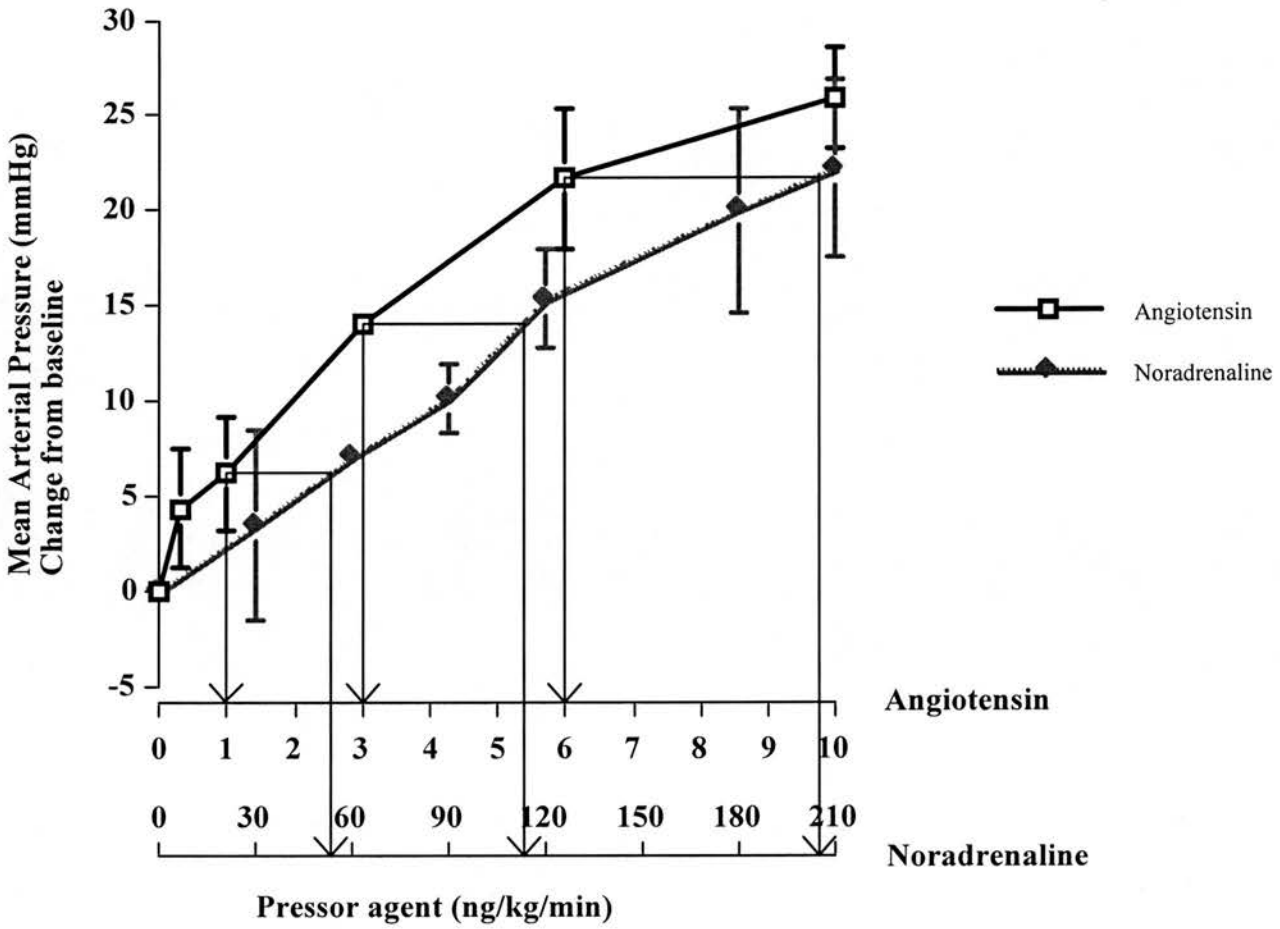
Table 2: Plasma ET-1 concentrations (pg/ml) (mean ± SEM).

	ANG II + Placebo	ANG II + BQ-123	NE + Placebo	NE + BQ-123
Baseline	2.41 ± 0.35	3.81 ± 0.51	2.72 ± 0.41	2.32 ± 0.23
30 min post BQ-123	3.07 ± 0.12	3.05 ± 0.28	2.67 ± 0.55	2.40 ± 0.44
Post ANG II (1 ng/Kg/min)/ NE (60 ng/Kg/min)	2.74 ± 0.42	3.47 ± 0.49	2.88 ± 0.38	3.06 ± 0.28
Post ANG II (3 ng/Kg/min)/ NE (120 ng/Kg/min)	3.10 ± 0.27	3.21 ± 0.33	3.38 ± 0.68	3.13 ± 0.43
Post ANG II (6 ng/Kg/min)/ NE (210 ng/Kg/min)	3.04 ± 0.26	3.60 ± 0.43	3.89 ± 0.79	3.12 ± 0.44

Table 3: Plasma ANG II concentrations (pg/ml) (mean ± SEM).

	ANG II + Placebo	ANG II + BQ-123
Baseline	21.8±7.1	12.0±1.6
30 min post BQ-123	23.4±8.6	11.8±2.2
Post ANG II (1 ng/Kg/min)/ NE (60 ng/Kg/min)	34.7±4.5	27.2±4.7
Post ANG II (3 ng/Kg/min)/ NE (120 ng/Kg/min)	67.8±8.5	83.2±20.4
Post ANG II (6 ng/Kg/min)/ NE (210 ng/Kg/min)	97.8±9.5	121.6±20.8

Figure 1:



MAP during ANG II and NE infusions following placebo (□) or BQ-123 (◆). Data are expressed as mean change from baseline ± SEM. *P<0.05 placebo vs. BQ-123 (ANOVA + Bonferroni correction).

Study 5: The systemic haemodynamic effects of selective ET_A blockade versus non-selective ET_A/ET_B blockade in patients with chronic heart failure (CHF) on standard treatment.

7.1 *Introduction*

Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor in man and acts via two receptors; the ET_A and ET_B. Both receptor subtypes are expressed on vascular smooth muscle cells where they mediated vasoconstriction whilst only the ET_B receptor is located on the endothelium producing a prostanoid and nitric oxide mediated vasodilatation. Therefore the ET_A receptor mediates vasoconstriction whilst the actions mediated via the ET_B receptor are more complex, and include vasodilatation, vasoconstriction and a role in the clearance of plasma ET-1.

The ET system contributes to the maintenance of basal vascular tone and blood pressure and blockade of the ET_A receptor causing vasodilatation and vasodepressor effects while ET_B receptor blockade has vasoconstrictor and pressor actions. Thus the balance of effects at the ETB receptor appears to be in favour of vasodilatation.

The renin-angiotensin and sympathetic nervous systems in part mediate neurohumoral reflexes that lead to peripheral vasoconstriction and increased systemic vascular resistance. However, the ET system also contributes to the pathophysiology with increased plasma ET-1 and big ET-1 concentrations (Hiroe *et al* 1991, McMurray *et al* 1992, Cody *et al* 1992, Wei *et al* 1994) that correlate with a poor prognosis (Pousset *et al* 1997). The use of drugs to block the renin-angiotensin and sympathetic nervous systems and cause a reduction in systemic vascular resistance have been demonstrated to improve outcome in patients with CHF. The potentially detrimental haemodynamic effects of ET-1 and the finding of elevated plasma concentrations suggest that the ET system may provide a therapeutic target in CHF.

Short-term systemic selective ET_A blockade in stable patients with CHF reduces systemic vascular resistance and increases cardiac output (Cowburn *et al* 1998, Givertz *et al* 2000, Spieker *et al* 2000, Ruschitzka *et al* 2001). In addition, dual $ET_{A/B}$ blockade reduces systemic vascular resistance and increases cardiac output in patients with CHF (Kiowski *et al* 1995, Packer *et al* 1998, Sutsch *et al* 1998, Schalcher *et al* 2000, Torre-Amione *et al* 2001, Abraham *et al* 2001). However, short-term systemic selective ET_B blockade increases SVR suggesting that the balance of action at the ET_B receptor is in favour of systemic vasodilatation in patients with CHF (Cowburn *et al* 1999).

To date there have been no direct head-to-head comparisons between selective ET_A blockade and dual $ET_{A/B}$ blockade. The aims of this study were to determine, in patients with stable CHF, the effects of selective ET_A blockade compared with dual $ET_{A/B}$ blockade on systemic and pulmonary haemodynamics and to study the effects on plasma concentrations of ET-1 and its precursor big ET-1.

7.2 Methods

7.2.1 *Subjects*

Nine patients with CHF (New York Heart Association Grade II – III) due to left ventricular dysfunction were recruited if they had an ejection fraction of less than 35% (by echocardiography Simpson's rule) and had been stable on therapy, including angiotensin converting enzyme inhibitors or angiotensin receptor antagonists, for at least 3 months. Patients were excluded if they had insulin dependent diabetes mellitus, abnormal liver function tests, recent myocardial infarction, coronary artery bypass grafting or coronary artery intervention in the previous 3 months, renal impairment (creatinine >200 μmol/L for men; >180 μmol/L for women) or had a systolic BP >190 or <90 mmHg.

The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject before entry into the study.

7.2.2 *Drugs*

A venous cannula (Venflon®; Ohmeda, BOC Ohmeda AB, Helsingborg, Sweden) for drug adminstration was inserted under local anaesthesia (Emla™ cream 5%; Astra Pharmaceuticals Ltd, Kings Langley, UK). Pharmaceutical grade BQ123 and BQ788 (Clinalfa AG, Laufelfingen, Switzerland) were dissolved in 0.9% saline (Baxter Healthcare Ltd, Thetford, UK). Patients received on different study days and in random order saline placebo, BQ123 (100 and 1000 nmol/min) or BQ123 (100 and 1000 nmol/min) and concomitant BQ788 (30 and 300 nmol/min). On all study days patients received low dose

infusion at t = 0 for 15 min followed by high dose infusion at t = 60 min for 15 min. Because of safety issues the principle investigator was not blinded but other investigators were to the treatments given.

7.2.3 Measurements

BP was recorded using a validated semi-automated non-invasive oscillometric device (section 2.2). CO, mean pulmonary artery pressure (PAP), pulmonary capillary wedge pressure (PCWP) and CVP were measured via a thermodilution cardiac output pulmonary artery catheter (section 2.5).

7.2.4 Drug assays

Venous blood for routine biochemistry and hormone assay was taken from the femoral vein. Plasma was assayed for ET-1 and big ET-1 (section 2.6).

7.2.5 Study Design

All patients attended fasted at 7.30 am on 3 occasions 1 week apart. Haemodynamic measurements were made in a quiet, draught free room maintained at a constant temperature (22 – 24 °C). After catheter insertion, patients underwent an equilibration period of at least 90 min until BP, HR and CO were stable with 3 consecutive measurements within 10% before commencing the study. Study drugs were administered by 15 min infusion in 2 incremental doses 60 min apart. The parameters were recorded in a double blind manner although for safety reason the principle physician present was un-blinded.

7.2.6 Data analysis and statistics

Data are expressed as mean percentage change from baseline +/- SEM unless otherwise specified. Mean area under the curve +/- SEM for responses was calculated. Data were examined by repeated measures ANOVA and Student's t-test with Bonferroni correction (Excel v5.0, Microsoft). Statistical significance was taken at the 5% level.

7.3 Results

Patients' characteristics and medication are shown in Table 1. There were no significant adverse events and the study was well tolerated by all patients. There were no significant differences in baseline haemodynamic variables between study visits.

7.3.1 Cardiac output and heart rate

BQ123 infusion caused an increase in CO ($p = 0.03$ vs placebo) with a maximum increase of 33% at 75min. Combined BQ123 and BQ788 infusion significantly attenuated these effects ($p < 0.05$ vs BQ123) (Figure 2). There was a small increase in HR with both BQ123 and combined BQ123 and BQ788 infusion of ~10% (ANOVA, $p = 0.002$ and 0.007 respectively vs placebo; Figure 1).

7.3.2 Left ventricular filling pressure and systemic haemodynamics

Both BQ123 and combined BQ123 and BQ788 infusion reduced PCWP by ~ 20% ($p = 0.016$ and 0.0004 respectively; Figure 2). MAP was reduced by BQ123 ($p = 0.001$ vs placebo) with a maximal decrease of 13% at 150 min. Combined BQ123 and BQ788 also significantly

reduced BP ($p = 0.008$ vs placebo) and this was not significantly different from BQ123 alone ($p = 0.2$) (Figure 1). SVR was therefore significantly lowered by BQ123 compared with placebo ($p = 0.01$) and compared with combined BQ123 and BQ788 ($p = 0.04$) (Figure 3).

7.3.3 Right ventricular filling pressure and pulmonary haemodynamics

Although there was a small statistically significant rise in CVP with concomitant BQ123 and BQ788, this was not a consistent finding (Figure 3). Mean PAP was significantly reduced by both BQ123 and combined BQ123 and BQ788 compared with placebo by a maximum of 25% at 90 min ($p = 0.01$ and 0.005 respectively) (Figure 4). Both treatments resulted in a reduction in PVR ($p = 0.006$ and 0.005) but because of differential effects on CO selective ET_A blockade with BQ123 resulted in a larger reduction in PVR ($p = 0.02$ vs BQ123 + BQ788) (Figure 4).

7.3.4 Plasma ET-1 and big ET-1

There was no significant increase in plasma concentrations of big ET-1 with placebo, BQ123 or combined BQ123 and BQ788 infusion. There was no significant increase in plasma concentrations of ET-1 with placebo or BQ123, however, combined BQ123 and BQ788 caused a dose dependent increase in plasma ET-1 concentrations after both low dose ($39 \pm 14\%$, $p = 0.02$) and high dose antagonism ($75 \pm 24\%$, $p < 0.02$) (Figure 5).

7.4 Discussion

We have shown for the first time, in a randomised placebo controlled trial, that there are differences between selective ET_A blockade compared with dual blockade in patients with CHF. In our study selective ET_A blockade and dual blockade reduced SVR, pulmonary vascular resistance and increase in CO, however there were potentially important differences between these two treatment strategies. In our study, dual $ET_{A/B}$ blockade resulted in significant a reduction in PAP, but although there was a trend towards a greater effect than with selective ET_A receptor blockade it failed to reach significance.

There is increasing evidence that the ET_B has a role in the clearance of plasma ET-1 with increases in plasma ET-1 following systemic ET_B blockade in both healthy volunteers (Haynes *et al* 1996, Plumpton *et al* 1996, Strachan *et al* 1999, Goddard *et al* 2001) and patients with CHF (Weber *et al* 1996, Kioski *et al* 1995, Sutsch et al 1998, Krum *et al* 1998). However the effects of systemic ET_A blockade are less consistent with little net increase, if any, in plasma concentrations of ET-1 in either group (Verharr *et al* 1998 Spieker *et al* 2000, Givertz *et al* 2000, Cowburn et al 1998). These results are confirmed in our study as selective ET_A blockade did not increase plasma ET-1 concentration whereas concomitant ET_B blockade increased plasma ET-1 after both low dose ($39 \pm 14\%$, $p = 0.02$) and high dose antagonism ($75 \pm 24\%$, $p < 0.02$). Thus, selective ET_A blockade has the benefit of leaving the ET_B receptor, with its ET-1 clearance role, unblocked and does not increase plasma ET-1. This will avoid not only the adverse effects of raised ET-1 concentrations on systemic haemodynamics primarily mediated through the ET_A receptor, but also the non-haemodynamic adverse effects of ET-1 such as mitogenesis etc.

However there may be situations where blockade of the ET_B receptor is desirable. There is a higher density of ET_B receptors in the pulmonary vasculature and there is evidence suggesting that these are up-regulated in patients with pulmonary hypertension. Raised PAP is an independent risk factor in patients with CHF and responses poorly to conventional therapies. Dual $ET_{A/B}$ blockade may be of more benefit in patients with raised PAP. Indeed, Bosentan, a dual $ET_{A/B}$ antagonist, has recently received a licence for the treatment of primary pulmonary hypertension in the USA (Rubin et al 2002). The effects in patients with pulmonary hypertension secondary to CHF are not known.

Our results are in general agreement with results from other, single drug studies, however, the literature is somewhat complicated by the often arbitrary distinction between selective and dual receptor blockers. There is considerable variation between the available ET receptor blockers in term of receptor specificity and binding affinity for the two receptor subtypes and indeed this may differ between tissues, in vivo.

The use of accurate invasive monitoring techniques such as pulmonary artery catheterisation limits the duration of each study visit safety reasons and thus only the short-term effects of these treatments could be assessed. Although a longer run in period would have been preferable we addressed this issue by inclusion of a placebo limb. Indeed there were small changes in the placebo. This is a short-term haemodynamic study and whether the potentially beneficial haemodynamic changes confer benefit in terms of morbidity and mortality will require large scale trials.

While our study suggests that selective ET_A blockade may provide greater, systemic haemodynamic changes over dual $ET_{A/B}$ blockade the effects were less clear in the pulmonary circulation and there are good reasons to support the use of dual $ET_{A/B}$ blockade in further trials.

7.5 Table legend

Baseline characteristics of study subjects, including age, smoking history, ejection fraction, history of coronary artery disease and grade of heart failure.

7.6 Figure legends

Figure 7.1

This illustrates changes in HR & MAP with time, demonstrated graphically on the left for placebo infusion; BQ-123= ; BQ-788 = . The bar charts on the right of the figure demonstrate compartitive changes with placebo, BQ-123 and BQ-788.

Figure 7.2

This illustrates changes in CO and PAW with time, demonstrated graphically on the left; BQ-123= , BQ-788= . The bar charts on the right of the figure demonstrate comparititive changes with placebo, BQ-123 and BQ-788.

Figure 7.3

This illustrates changes in SVRI & CVP with time, demonstrated graphically on the left; BQ-123 =, BQ-788= . The bar charts on the right of the figure demonstrate comparititive changes with placebo, BQ-123 and BQ-788.

Figure 7.4

This illustrates changes in PVR & MPAP with time, demonstrated graphically on the left; BQ-123=; BQ-788= . The bar charts on the right of the figure demonstrate comparititive changes with placebo, BQ-123 and BQ-788.

Figure 7.5

This illustrates changes in big ET and ET-1 with time for each individual subject, for placebo infusion, BQ-123 and BQ-788.

Table 1

Patient	Sex	Age	BMI	EF%	Smoker?	BP	P	Coronaries	NYHA
1	M	75	26	35	N	186/79	52	CABG'87	III
2	M	63	23	35	N	120/74	64	Normal	II
3	M	60	27	35	N	122/58	62	MI'78	III
4	M	60	23	34	N	120/84	93	MI'87	II
5	M	43	30	20	N	98/64	77	MI'82	II
6	F	61	21	23	N	92/62	88	3 VES	III
7	M	56	29	35	N	139/88	96	MI'95	III
8	M	61	26	30	N	136/77	66	MI'99	III
9	M	52	36	25	N	184/106	94	CABG'93	III
10	M	74	33	35	N	143/75	58	CADx1	III
11	M	67	21	35	N	154/78	65	CADx3	II
AVE		61	27	31		136/77	74		
SEM		3	1	2		9/4	5		

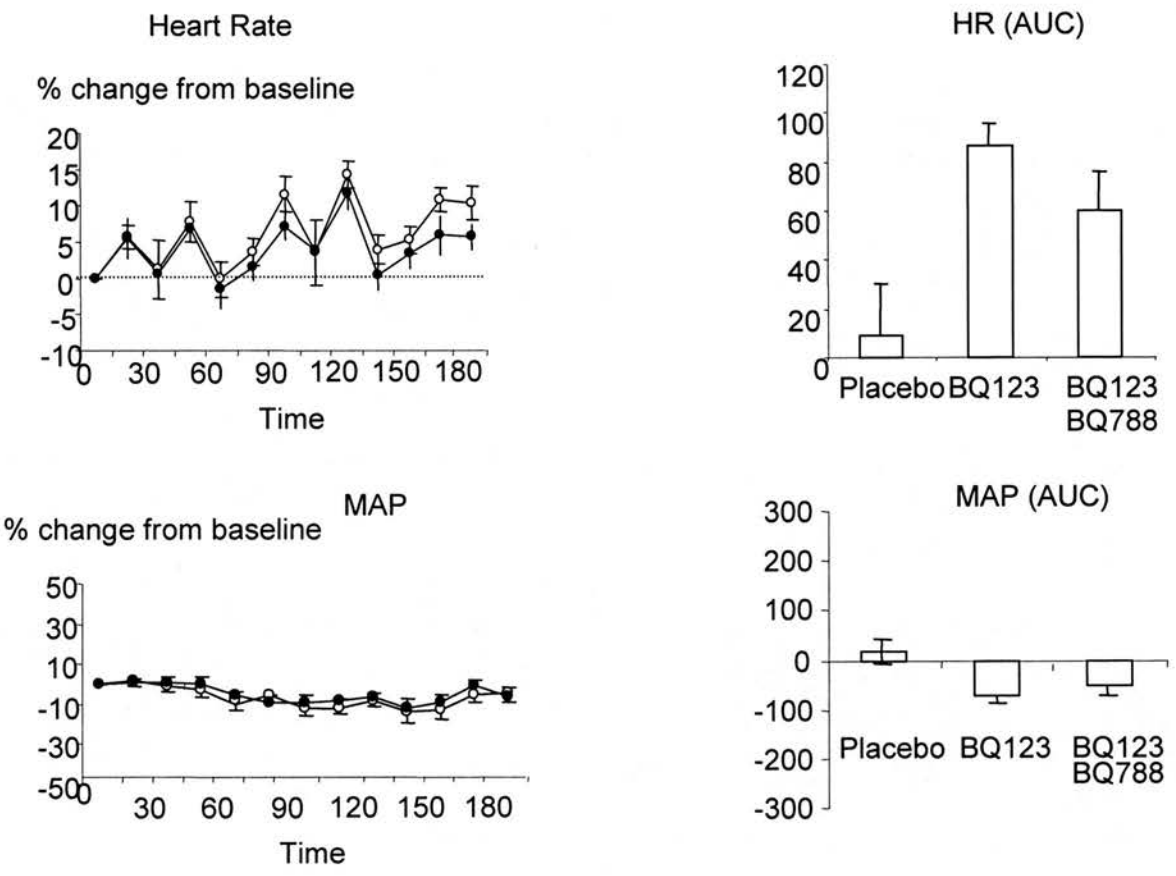

Heart Rate
% change from baseline
20
15
10
5
0
-5
-10
0 30 60 90 120 150 180
Time
HR (AUC)
120
100
80
60
40
20
0
Placebo BQ123 BQ123 BQ788
MAP
% change from baseline
50
30
10
-10
-30
-50
0 30 60 90 120 150 180
Time
MAP (AUC)
300
200
100
0
-100
-200
-300
Placebo BQ123 BQ123 BQ788

Figure 1

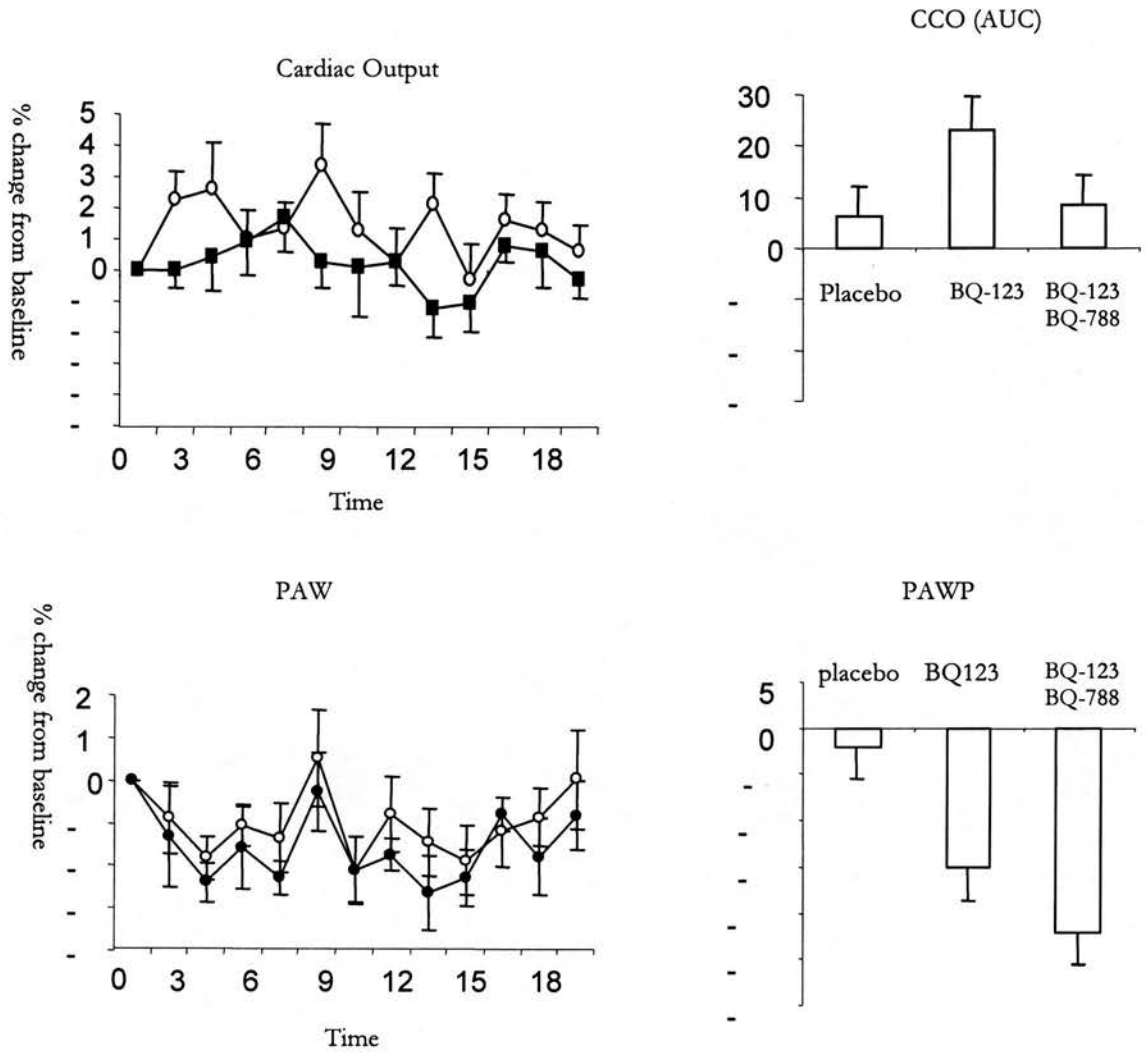

Cardiac Output
% change from baseline
5
4
3
2
1
0
0 3 6 9 12 15 18
Time
CCO (AUC)
30
20
10
0
Placebo
BQ-123
BQ-123
BQ-788
PAW
% change from baseline
2
1
0
0 3 6 9 12 15 18
Time
PAWP
placebo
BQ123
BQ-123
BQ-788
5
0

Figure 2

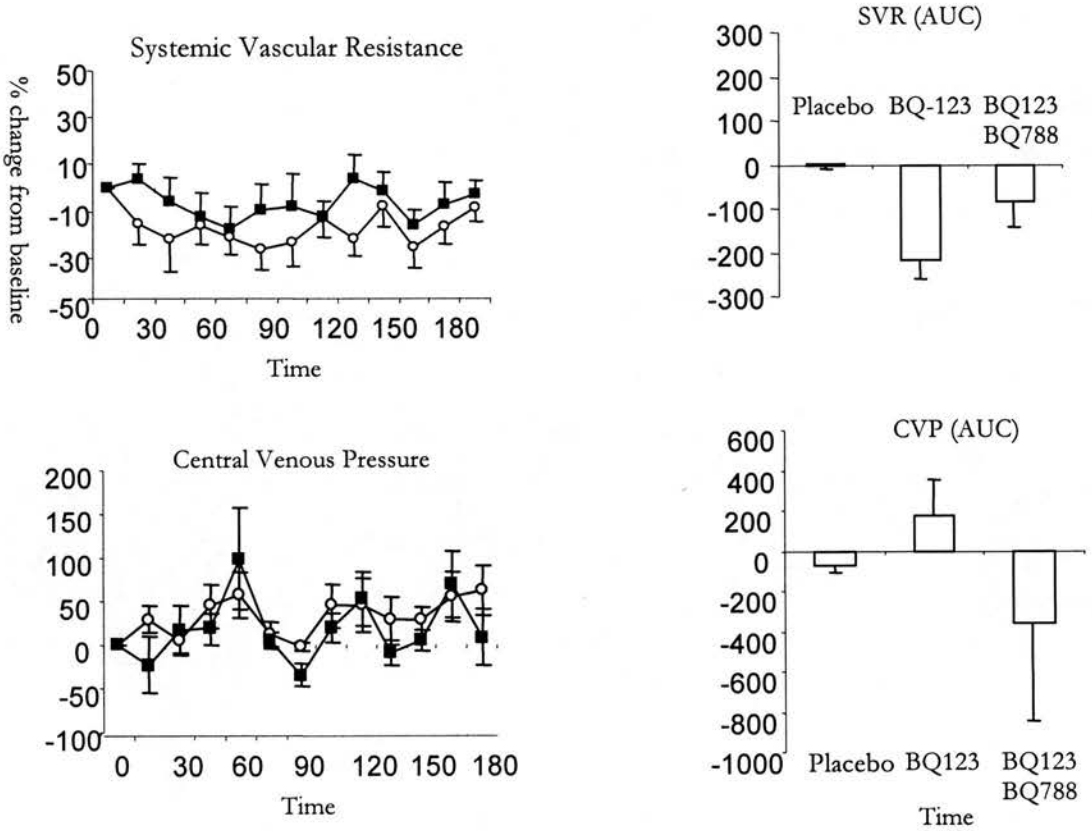

Systemic Vascular Resistance
% change from baseline
50
30
10
-10
-30
-50
0 30 60 90 120 150 180
Time
SVR (AUC)
300
200
100
0
-100
-200
-300
Placebo BQ-123 BQ123 BQ788
Central Venous Pressure
200
150
100
50
0
-50
-100
0 30 60 90 120 150 180
Time
CVP (AUC)
600
400
200
0
-200
-400
-600
-800
-1000
Placebo BQ123 BQ123 BQ788
Time

Figure 3

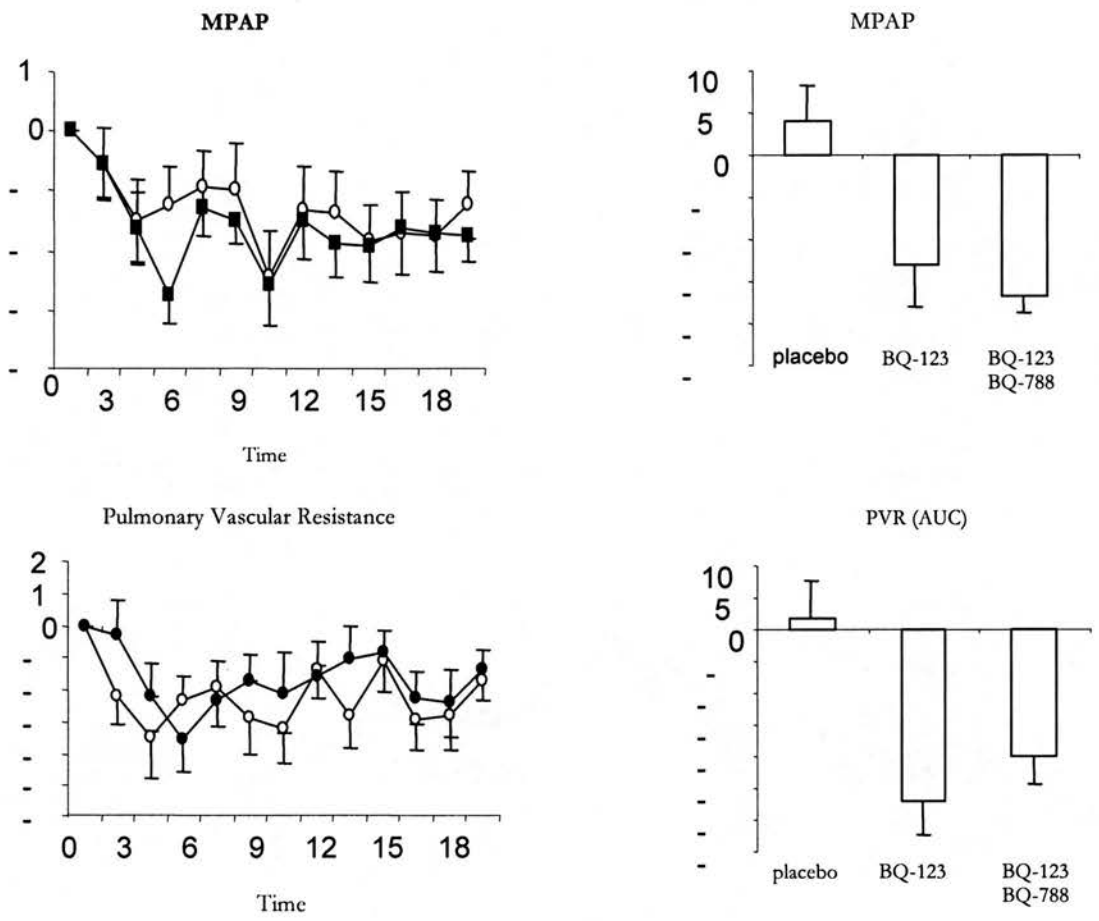

MPAP
1
0
0 3 6 9 12 15 18
Time
MPAP
10
5
0
placebo
BQ-123
BQ-123
BQ-788
Pulmonary Vascular Resistance
2
1
0
0 3 6 9 12 15 18
Time
PVR (AUC)
10
5
0
placebo
BQ-123
BQ-123
BQ-788

Figure 4

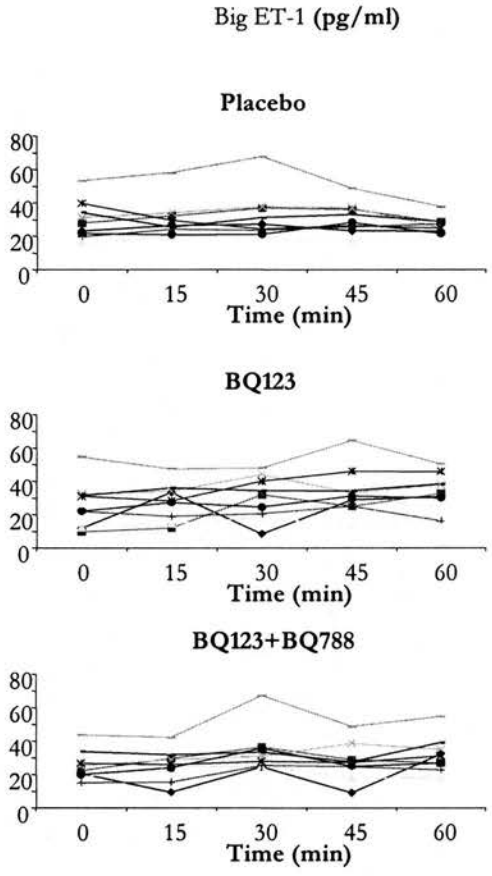


Figure 5a

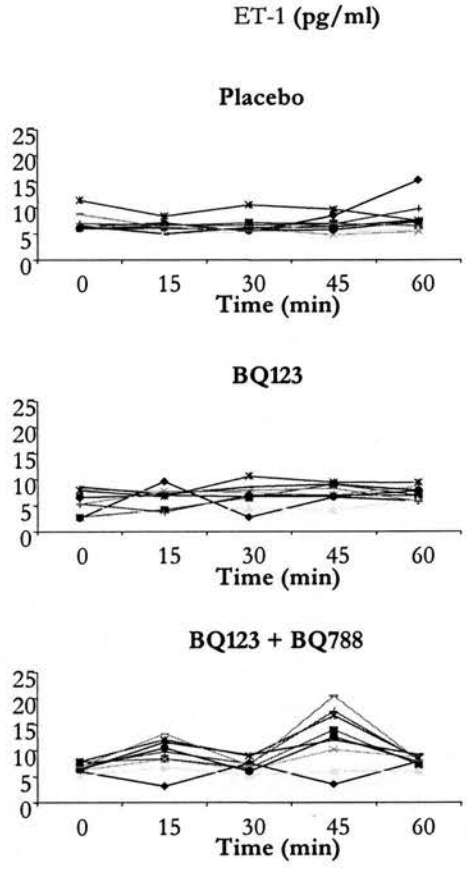


Figure 5b

Discussion & conclusions

8.1 General comments and background

This MD thesis originally began as an extension of a colleague's work started in 1996 and the latest paper from the work presented therein is expected to be published in 2003. This timespan goes a long way to explain the diversity of work contained within. This work focuses on the mechanism behind the role of endothelin on the cardiovascular system, a system that was being rapidly explored in a search for medications to favourably impact on cardiovascular disease.

8.2 Comments re mechanistic studies

There is a general similarity in the ways that these studies were carried out, which carries both strengths and weaknesses; although these largely relate to the way they are interpreted. Without exception these are mechanistic studies which use receptor antagonists to unmask the role of the agent being studied either in a physiological or a pathophysiological situation. As mechanistic studies they have been designed to explain mechanisms and degrees of actions of the agents investigated, thus providing a basis for further directed drug development. As alluded to, mechanistic studies are generally carried out in small subject groups with the results designed to be interpretated on this basis. Important information can be obtained about interactions between compounds and with local studies direct actions on isolated arterial beds.

8.3 Comments re forearm blood flow studies- study 1; study 2

Whilst the results from local arterial studies can be extrapolated to give information on systemic haemodynamics, they should be regarded primarily as an impetus for further discussion. The strengths of forearm studies are also, in some cases, their weakness. Due to isolation of the studied arterial bed, in this case the forearm, the effects of the study drug on vasoreactivity can be determined. This isolation has several advantages: much lower doses of drugs can be used, greatly reducing or eliminating the possiblity of systemic side-effects – which can be particularly important in newly discovered drugs with a relatively unknown side-effect profile; no activation of baroreceptors occurs, which is particularly evident in young healthy subjects and may obscure the true effects of the study medication; as the other forearm acts as a contemparaneous control even relatively small numbers of subjects can give accurate, reproducible results, indeed the same subject can return on several occasions for dose-ranging studies due to the short-lived nature of the studies. This is particularly relevant in study 1, where with relatively small subject numbers we demonstrated vasoconstriction in the forearm with the NEP inhibitorss candoxtrilat and thiorphan, results later confirmed by systemic studies showing that oral doses of candoxatril in healthy men produced an increase in both systolic blood pressure and venous plasma endothelin concentration (Ando et. al. 1995; Motwani et. al. 1995). Yet on the other hand forearm studies are by defination acute studies, which examine only the vascular effects of a short-term infusion of the study medication; it is also assumed that the forearm vascular bed will provide an accurate reflection of the effects of the study medication on the other vascular beds in the body – although this is generally an accurate assumption.

In study 1, the conclusion reached, that NEP inhibitors produced systemic vasoconstriction, due to the balance of their effects on vasoconstrictors such as endothelin and vasodilators such as bradykinin helped to explain why, despite causing significant naturesis NEP inhibitors failed to lower blood pressure. In retrospect this study has proved influential in influencing the development of some of the newer anti-cardiac failure treatment, such as omapatrilat.

8.4 Comments re short-term systemic antagonist studies – studies 2, 3, 4 & 5

In all the studies intravenous agents are used, therefore limiting these studies to short duration in resting subjects. This limits the conclusions that can be drawn, as it does not allow for the effects of tolerance to develop or indeed changes in receptor actions or density. Furthermore it provides "snapshot" information of the effects of the studied medication, which does not allow for changes in physiological state.

All of the studies (2-5) in which we used short-term administration of agents to examine their haemodynamic effects have similar limitations: the patient numbers were low – these were calculated beforehand based on the expected haemodynamic changes, for safety reasons as well as financial it was felt necessary to perform these studies with the minimum number of subjects. In retrospect, with particular relevance to study 5, this limited the conclusion that we were able to draw and in some circumstances we failed to reach significance due to insufficient numbers. I feel that these compromises are inevitable with grant-driven research which aims to get the maximum "bang per buck"! Furthermore with respect to studies 2 to 4 one of the major limitations was the method of measuring the haemodynamic changes – cardiac bioimpedance, which is in essence a derived measure subject to a number of assumptions. This compromise was made as we were working with

non-patient groups, i.e. healthy subjects recruited and paid according to study length. It was felt that the additional risk of invasive monitoring with Swan-Ganz catheterisation and radial artery blood pressure monitoring could not be justified, there is no doubt it would have considerably added to the expense as well. In retrospect I feel that this was probably not a justified concern and although the integrity of the studies were not significantly compromised, more inclusive monitoring may have helped particularly with systemic infusion of BQ-788. In this study it was particularly difficult to tease out the relative effects of the study medication, where we observed an increase in TPVRI along with a decrease in CI and HR. It was necessary to draw conclusions from several studies – those examining the effects of ET_B receptor stimulation on isolated cardiac tissue as well as forearm studies showing vasoconstriction with BQ-788. With appropriate invasive monitoring, including right heart pressures we could have clearly delineated these effects. The same comments also apply to study 4. Yet both our systemic studies, infusing BQ123 and BQ-788 were the first in man to demonstrate significant Haemodynamic changes and as such have formed an important basis for future work.

8.5 Conclusions & future directions

In general I believe that the studies contained within this thesis were well constructed and answered the questions they were designed for, within the confines of the limitations described above. In particular they have helped to answer important questions about the role of the ET_A & ET_B receptors in both health and disease. Although a large scale randomised study investigating the effects of the mixed ET_A / ET_B receptor antagonist, bosentan, have failed to demonstrate benefit in congestive heart failure (Kalra et al. 2002), bosentan is now licensed for the treatment of primary pulmonary hypertension (Rubin et al

2002) proving that endothelin antagonists still have a future in the management of cardiovascular conditions. Indeed our study in CHF, conceived prior to either of the previous 2 studies, suggested 2 things which may yet prove to be prophetic:: firstly that selective ET_A receptor antagonism offers additional haemodynamic benefit over mixed ET_A / ET_B receptor antagonism in CHF and secondly that the haemodynamic benefit of selective ET_A receptor antagonism may not extend to the pulmonary circulation. It may be that the future of research in this area lies in this direction, finding the balance between selective and mixed receptor antagonism in pulmonary hypertension and CHF. Other areas which may prove of promise are the development of mixed antagonists (angiotensin / endothelin) to treat remodelling following myocardial infarction or aggressive myocardial hypertrophy secondary to hypertensive heart disease or the aggressive smooth muscle, almost tumour-like growth seen following angioplasty-induced injury which manifests as restenosis.

References

Aalkjaer, C., A.M. Heagerty, K.K. Petersen, J.D. Swales, and M.J. Mulvany. 1987. Evidence for increased media thickness, increased neuronal amine uptake, and depressed excitation-contraction coupling in isolated resistance vessels from essential hypertensives. *Circ Res.* 61:181-186.

Abassi, Z., E. Golomb, and H.R. Keiser. 1992. Neutral endopeptidase inhibition increases the urinary excretion and plasma levels of endothelin. *Metabolism.* 41:683-685.

Abboud, F.M., D.L. Eckberg, U.J. Johannsen, and A.L. Mark. 1979. Carotid and cardiopulmonary baroreceptor control of splanchnic and forearm vascular resistance during venous pooling in man. *J Physiol (Lond).* 286:173-184.

Abboud, F.M., and J.W. Eckstein. 1966. Comparative changes in vascular resistance in response to nerve stimulation and to norepinephrine. *Circ Res.* 18:263-269.

Abraham et al Effects of enrasentan, a non-selective endothelin receptor antagonist in class II-III heart failure: results of the ENCOR trial. J Am Coll Cardiol 2001

Abramson, D.I., and E.B. Ferris Jr. 1940. Responses of blood vessels in the resting hand and forearm to various stimuli. *Am Heart J.* 19:541-553.

Achmad, T., and G. Rao. 1992. Chemotaxis of human blood monocytes toward endothelin-1 and the influence of calcium channel blockers. *Biochem Biophys Res Commun.* 189:994-1000.

Adachi, M., Y.-Y. Yang, Y. Furuichi, and T. Watanabe. 1991. Cloning and characterization of cDNA encoding human A-type endothelin receptor. *Biochem Biophys Res Commun.* 180:1265-1272.

Aellig, W.H. 1994. Clinical pharmacology, physiology and pathophysiology of superficial veins-1. *Br J Clin Pharmacol.* 38:181-196.

Aellig, W.H. 1994. Clinical pharmacology, physiology and pathophysiology of superficial veins-2. *Br J Clin Pharmacol.* 38:289-305.

Aellig, W.H. 1981. A new technique for recording compliance of human hand veins. *Br J Clin Pharmacol.* 11:237-243.

Alafaci, C., I. Jansen, M.A.R. Arbab, Y. Shiokawa, N.A.A. Svengaard, and L. Edvinsson. 1990. Enhanced vasoconstrictor effect of endothelin in cerebral arteries from rats with subarachnoid haemorrhage. *Acta Physiol Scand.* 138:317-319.

Alexander BT, Cockrell KL, Rinewalt AN, Herrington JN, Granger JP. Enhanced renal expression of preproendothelin mRNA during chronic angiotensin II hypertension. Am J Physiol 2001;280:R1388-92.

Allen, W.J., H. Barcroft, and O.G. Edholm. 1946. On the action of adrenaline on the blood vessels in human skeletal muscle. *J Physiol (Lond).* 105:255-267.

Altman, D., and M. Bland. 1996. Comparing several groups by analysis of variance. *Br Med J.* 312:1472-1473.

Anderson, E.A., C.A. Sinkey, W.J. Lawton, and A.L. Mark. 1989. Elevated sympathetic nerve activity in borderline hypertensive patients: direct evidence from intraneural recordings. *Hypertension.* 14:177-183.

Ando, S., M.A. Rahman, G.C. Butler, B.L. Senn, and J.S. Floras. 1995. Comparison of candoxatril and atrial natriuretic factor in healthy men; effects on haemodynamics, sympathetic activity, heart rate variability, and endothelin. *Hypertension.* 26:1160-6.

Ängaard, E., S. Galton, and G. Rae. 1989. The fate of radioiodinated endothelin-1 and endothelin-3 in the rat. *J Cardiovasc Pharmacol.* 13 (Suppl. 5):S46-S49.

Änggard, E. 1994. Nitric oxide: mediator, murderer, and medicine. *Lancet.* 343:1199-1206.

Angus, J.A., A.C. Dyke, G.L. Jennings, P.I. Korner, K. Sudhir, J.E. Ward, and C.E. Wright. 1992. Release of endothelium-derived relaxing factor from resistance arteries in hypertension. *Kidney Int.* 41 (Suppl. 37):S73-8.

Angus, J.A., and M.J. Lew. 1992. Interpretation of the acetylcholine test of endothelial dysfunction in hypertension. *J Hypertens.* 10 (Suppl. 7):S179-S186.

Appel, P.L., H.B. Kram, J. Mackabee, A.W. Fleming, and W.C. Shoemaker. 1986. Comparison of measurements of cardiac output by bioimpedance and thermodilution in severely ill surgical patients. *Crit Care Med.* 14:933-935.

Arai, H., H. Hori, I. Aramori, H. Ohkubo, and S. Nakanishi. 1990. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature.* 348:730-732.

Arai, H., K. Nakao, K. Takaya, K. Hosoda, Y. Ogawa, S. Nakanishi, and H. Imura. 1993. The human endothelin B receptor gene. Structural organization and chromosomal assignment. *J Biol Chem.* 268:3463-3470.

Artigou, J.Y., J. Salloum, A. Carayoni, P.H. Lechat, G. Maistre, R. Isnard, M. Komajda, J.C. Legrand, and Y. Grosgogeat. 1993. Variations in plasma endothelin levels during coronary spasm. *Eur Heart J.* 14:780-784.

Asano, T., I. Ikega, S. Satoh, Y. Suzuki, M. Shibuya, Y. Sugita, and H. Hidaka. 1990. Endothelin: a potential modulator of cerebral vasospasm. *European Journal of Pharmacology.* 190:365-372.

Asano, T., I. Ikega, Y. Suzuki, S. Satoh, and M. Shibuya. 1989. Endothelin and the production of cerebral vasospasm in dogs. *Biochem Biophys Res Commun.* 159:1345-1351.

Ashton, H. 1963. Peripheral circulation in man: critical closure in human limbs. *Br Med Bull.* 19:149-153.

Auguet, M., S. Delaflotte, P.E. Chabrier, and P. Braquet. 1992. The vasoconstrictor action of big endothelin-1 is phosphoramidon-sensitive in rabbit saphenous artery, but not in saphenous vein. *Eur J Pharmacol.* 190:101-102.

Badr, K., J. Murray, M. Breyer, K. Takahashi, T. Inagami, and R.C. Harris. 1989. Mesangial cell, glomerular and renal vascular responses to endothelin in the rat kidney. *J Clin Invest.* 83:336-342.

Badr, K.F., J.J. Murray, M.D. Breyer, K. Takahashi, T. Inagami, and R.C. Harris. 1989. Mesangial cell, glomerular and renal vascular responses in the rat kidney: elucidation of signal transduction pathways. *J Clin Invest.* 83:336-342.

Bakris, G.L., and R.N. Re. 1993. Endothelin modulates angiotensin II-induced mitogenesis of human mesangial cells. *Am J Physiol.* 264:F937-F942.

Balakrishnan SM, Wang HD, Gopalakrishnan V, Wilson TW, McNeill JR. Effect of an endothelin antagonist on haemodynamic responses to angiotensin II. *Hypertension* 1996;28:806-9.

Balasubramanian, V., O.P. Mathew, A. Behl, S.C. Tewari, and S.W. Hoon. 1986. Electrical impedance cardiogram in derivation of systolic time intervals. *Br Heart J.* 40:268-275.

Bank, A.J., T.S. Rector, N. Burke, L.K. Tschumperlin, and S.H. Kubo. 1992. Impaired forearm vasodilatation to hyperosmolar stimuli in patients with congestive heart failure secondary to idiopathic dilated cardiomyopathy or to ischaemic cardiomyopathy. *Am J Cardiol.* 70:1315-1319.

Barclay, P.L., J.A. Bennet, G.M.R. Samuels, and N.B. Shepperson. 1991. The atriopeptidase inhibitor (±) candoxatrilat reduces the clearance of atrial natriuretic factor in both intact and nephrectomized rats: evidence for an extra-renal site of action. *Biochem Pharmacol.* 41:841-4.

Barclay, P.L., J.C. Danilewicz, and G.M.R. Samuels. 1990. Inhibitory properties of UK-69 578 and thiorphan. *Lancet.* 336:754-5.

Barcroft, H., and H. Konsett. 1949. On the actions of noradrenaline, adrenaline and isopropyl noradrenaline on the arterial blood pressure, heart rate and muscle blood flow in man. *J Physiol (Lond).* 110:194-204.

Barnes, K., L.J. Murphy, M. Takahashi, K. Tanzanawa, and A.J. Turner. 1995. Localisation and biochemical characterisation of endothelin-converting enzyme. *J Cardiovasc Pharmacol.* 26 (Suppl. 3):S37-S39.

Barnes, P.J., and S.F. Liu. 1995. Regulation of pulmonary vascular tone. *Pharmacol Rev.* 47:87-131.

Barton M, Shaw S, d'Uscio LV, Moreau P, T.F. L. Differential modulation of the renal and myocardial endothelin systems by angiotensin II in vivo. Effects of chronic selective ET_A receptor blockade. J Cardiovasc Pharmacol 1998;31 Suppl 1:S265-8.

Batra, V.K., J.R. McNeill, Y. Xu, T.W. Wilson, and V. Gopalakrishnan. 1993. ET_B receptors on aortic smooth muscle cells of spontaneously hypertensive rats. *Am J Physiol.* C479-C484.

Baynash, A.G., K. Hosoda, A. Giaid, J.A. Richardson, N. Emoto, R.E. Hammer, and M. Yanagisawa. 1994. Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell.* 79:1277-1285.

Bazil, M.K., R.W. Lappe, and R.L. Webb. 1992. Pharmacologic characterization of an $endothelin_A$ (ET_A) receptor antagonist in conscious rats. *J Cardiovasc Pharmacol.* 20:940-948.

Bedarida, G.V., D. Kim, T.F. Blaschke, and B.B. Hoffman. 1993. Venodilatation in Raynaud's disease. *Lancet.* 342:1451-1454.

Benatti, L., M.S. Fabrini, and C. Patrono. 1994. Regulation of ET-1 biosynthesis. *Ann NY Acad Sci.* 714:109-121.

Benigni, A., C. Zoja, D. Corna, S. Orisio, L. Longaretta, T. Bertani, and G. Remuzzi. 1993. A specific endothelin subtype A receptor antagonist protects against injury in renal disease progression. *Kidney Int.* 44:440-444.

Benjamin, N., J.R. Cockcroft, J.G. Collier, C.D. Dollery, J.M. Ritter, and D.J. Webb. 1989. Local inhibition of converting enzyme and vascular responses to angiotensin and bradykinin in the human forearm. *J Physiol (Lond).* 412:543-555.

Benjamin, N., J.G. Collier, and D.J. Webb. 1988. Angiotensin II augments sympathetically induced venoconstriction in man. *Clin Sci.* 75:337-340.

Berger, E.Y., S.J. Farber, and D.P. Earle. 1948. Comparison of the constant infusion and urine collection techniques for the measurement of renal function. *J Clin Invest.* 27:710-716.

Bergström, J., H. Bucht, J. Ek, B. Josephson, H. Sundell, and L. Werkö. 1959. The renal extraction of para-aminohippurate in normal persons and in patients with diseased kidneys. *Scandinav J Clin Lab Invest.* 11:361-375.

Bernstein, D.P. 1986. Continuous non-invasive real-time monitoring of stroke volume and cardiac output by thoracic electrical bioimpedance. *Critical Care Med.* 14:898-901.

Bernstein, D.P. 1986. A new stroke volume equation for thoracic electrical bioimpedance: theory and rationale. *Critical Care Med.* 14:904-909.

Bevan, E.G., J.M.C. Connell, J. Doyle, H.A. Carmichael, D.L. Davies, A.R. Lorimer, and G.T. McInnes. 1992. Candoxatril, a neutral endopeptidase inhibitor: efficacy and tolerability in essential hypertension. *J Hypertens.* 10:607-13.

Bijlsma, J.A., A.J. Rabelink, K.A.H. Kaasjager, and H.A. Koomans. 1995. L-arginine does not prevent the renal effects of endothelin in humans. *J Am Soc Nephrol.* 5:1508-1516.

Bland JM and Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;i:307-10.

Bloch, K.D., R.L. Eddy, T.B. Shows, and T. Quertermous. 1989. cDNA cloning and chromosomal alignment of the gene encoding endothelin-3. *J Biol Chem.* 264:18156-18161.

Bloch, K.D., S.P. Freidrich, M.-E. Lee, R.L. Eddy, T.B. Shows, and T. Quertermous. 1989. Structural organization and chromosome assignment of the gene encoding endothelin. *J Biol Chem.* 264:10851-10857.

Boarder, M.R., and D.B. Marriott. 1989. Characterisation of endothelin-1 stimulation of catecholamine release from adrenal chromaffin cells. *J Cardiovasc Pharmacol.* 13 (Suppl. 5):S223-S224.

Boemke W, Hocher B, Schleyer N, Krebs MO, Kaczmarczyk G. Hemodynamic, renal, and endocrine responses to acute ET_A blockade at different ANG II plasma levels. Am J Physiol 2001;280:R1322-31.

Braquet, P., Touvey, C., Lagente, V., Vilian, B., Pons, F., Hosford, D., Chabrier, P.E., Mencia-Huerta, J.M. 1989. Effect of ET-1 on blood pressure and broncho-pulmonary system of the guinea pig. *J. Cardiovasc. Pharmacol.* 13 (Suppl. 5):S143-S146.

Bradley, S.E., G.P. Bradley, C.J. Tyson, J.J. Curry, and W.D. Blake. 1950. Renal function in renal diseases. *Am J Med.* 9:766-773.

Bramnert M, Hallengren B, Lecerof H, Werner R, Manhem P. Decreased blood pressure response to infused noradrenaline in normotensive as compared to hypertensive patients with primary hypothyroidism. Clin Endocrinol 1994;40:317-21.

Brandil, P., B.M. Loffler, V. Breu, R. Osterwalder, J.P. Maire, and M. Clozel. 1996. Role of endothelin in mediating neurogenic plasma extravasation in rat dura mater. *Pain.* 64:315-322.

Brands, M.W., and R.H. Freeman. 1988. Aldosterone and renin inhibition by physiological levels of atrial natriuretic factor. *Am J Physiol.* 254:R1011-R1016.

Brooks, D.P., P.D. DePalma, M. Gellai, P. Nambi, E.H. Ohlstein, J.D. Elliott, J.G. Gleason, and R.R. Ruffolo. 1994. Nonpeptide endothelin antagonists. III. Effect of SB 209679 and BQ-123 on acute renal failure in anaesthetized dogs. *J Pharmacol Exp Ther.* 271:769-775.

Buga, G.M., M.E. Gold, J.M. Fukuto, and I. Ignarro. 1991. Shear stress-induced release of nitric oxide from endothelial cells grown on beads. *Hypertension.* 17:187-193.

Burbach, J.P.H., and O.C. Meijer. 1992. The structure of neuropeptide receptors. *Eur J Pharmacol Mol Pharmacol.* 227:1-18.

Buzzi, M.G., and M.A. Moskowitz. 1990. The antimigraine drug, sumatriptan (GR43175), selectively blocks neurogenic plasma extravasation from blood vessels in dura mater. *Br J Pharmacol.* 99:202-206.

Cacoub, P., R. Dorent, G. Maistre, P. Nataf, A. Carayon, J. Piette, P. Godeau, C. Cabrol, and I. Gandjbakhch. 1993. Endothelin-1 in primary pulmonary hypertension and the Eisenmenger Syndrome. *Am J Cardiol.* 71:448-450.

Calver, A., J. Collier, S. Moncada, and P. Vallance. 1992. Effect of local intra-arterial NG-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator system appears abnormal. *J Hypertens.* 10:1025-1031.

Cao, L., and R.O. Banks. 1990. Cardiorenal actions of endothelin, part I: effects of converting enzyme inhibition. *Life Sci.* 46:577-583.

Caplan, L.M., D.P. Bernstein, K.P. Patel, J. Sange, and H. Turndorf. 1987. Measurement of ejection fraction by bioimpedance method. *Crit Care Med.* 15:402.

Cappuccio, F.P., and P. Strazzullo. 1993. Determinants of the renal clearance of exogenous lithium in a large sample of a white male working population. *Clin Sci.* 85:479-485.

Carvalho, M.H.C., R. Scivoletto, Z.B. Fortes, D. Nigro, and S. Cordellini. 1987. Reactivity of aorta and mesenteric microvessels to drugs in spontaneously hypertensive rats: role of the endothelium. *J Hypertens.* 5:377-382.

Casellas D, Bouriquet N, Herizi A. Bosentan prevents pre-glomerular alterations during angiotensin II hypertension. Hypertension 1997;30:1613-20.

Cavero, P.G., W.L. Miller, D.M. Heublein, K.B. Marguilies, and J.C. Burnett Jr. 1990. Endothelin in experimental heart failure in the anaesthetized dog. *Am J Physiol.* 259:F312-F317.

Chalmers, J.P., L.M.H. Wing, M.J. West, A.J.C. Bune, J.M. Elliott, M.J. Morris, M.D. Cain, J.R. Graham, and D.O. Southgate. 1986. Effects of enalapril and hydrochlorothiazide on blood pressure, renin-angiotensin system, and atrial natriuretic factor in essential hypertension: a double blind factorial cross-over study. *Aust NZ J med.* 16:475-480.

Chan, D.P., A. Clavell, J. Keiser, and J.C. Burnett. 1994. Effects of renin-angiotensin system in mediating endothelin-induced renal vasoconstriction. *J Hypertens.* 12(Suppl. 4):S43-S49.

Chan, L., A. Chittinandana, J.I. Shapiro, P.F. Shanley, and R.W. Schrier. 1994. Effect of an endothelin-receptor antagonist on ischaemic acute renal failure. *Am J Physiol.* 266:F135-F138.

Cheesman, A.R., and N. Benjamin. 1994. Lack of tolerance in forearm blood vessels in man to glycerol trinitrate. *Br J Clin Pharmacol.* 37:441-444.

Cirino, M., B. Battisini, M. Yano, and I.W. Rodger. 1994. Dual cardiovascular effects of endothelin-1 dissociated by BQ-153, a novel ET_A receptor antagonist. *J Cardiovasc Pharmacol.* 24:587-594.

Clarke, J.G., N. Benjamin, S.W. Larkin, D.J. Webb, G.J. Davies, and A. Maseri. 1989. Endothelin is a potent long-lasting vasoconstrictor in men. *Am J Physiol.* 257:H2033-H2035.

Clericetti, N., and C. Beretta_Piccoli. 1991. Lithium clearance in patients with chronic renal diseases. *Clin Nephrol.* 36:281-289.

Clouthier, D.E., K. Hosoda, J.A. Richardson, S.C. Williams, H. Yanagisawa, T. Kuwaki, M. Kumuda, R.E. Hammer, and M. Yanagisawa. 1998. Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. *Development.* 125:813-824.

Clozel, M. 1989. Endothelin sensitivity and receptor binding in the aorta of spontaneously hypertensive rats. *J Hypertens.* 7:913-917.

Clozel, M., V. Breu, K. Burri, J.M. Cassal, W. Fischill, G. Gray, G. Hirth, B.M. Loffler, M. Muller, W. Neidhart, and H. Ramuz. 1993. Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. *Nature.* 365:759-761.

Clozel, M., V. Breu, G. Gray, B. Kalina, B. Loffler, K. Burri, J. Cassal, G. Hirth, M. Muller, W. Neidhardt, and H. Ramuz. 1994. Pharmacological characterisation of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J Pharmacol Exp Ther.* 270:228-235.

Clozel, M., G. Gray, W. Breu, B.M. Löffler, and R. Osterwalder. 1992. The endothelin ET_B receptor mediates both vasodilatation and vasoconstriction in vivo. *Biochem Biophys Res Commun.* 186:873-876.

Cockcroft, J.R., J.G. Clarke, and D.J. Webb. 1991. The effect of intra-arterial endothelin on resting blood flow and sympathetically mediated vasoconstriction in the forearm of man. *Br J Clin Pharmacol.* 31:521-524.

Cockcroft, J.R., P.J. Chowienczyk, N. Benjamin, and J.M. Ritter. 1994. Preserved endothelium-dependent vasodilatation in patients with essential hypertension. *New Engl J Med.* 330:1036-40.

Cocks, T.M., N.L. Faulkner, and K. Sudhir. 1989. Reactivity of endothelin-1 on human and canine large veins compared with large arteries in vivo. *Eur J Pharmacol.* 171:17-24.

Cody, R.J., G.J. Haas, P.F. Binkley, Q. Capers, and R. Kelley. 1992. Plasma endothelin correlates with the extent of pulmonary hypertension in patients with chronic congestive heart failure. *Circulation.* 85:504-509.

Cody, W.L., A.M. Doherty, J.X. He, P.L. De Pue, S. Rapundalo, G.A. Hingorani, T.C. Major, R.L. Panek, D.T. Dudley, S.J. Haleen, D. La Douceur, K.E. Hill, M.A. Flynn, and E.E. Reynolds. 1992. Design of a functional hexapeptide antagonist of endothelin (1). *J Med Chem.* 35:3301-3303.

Cohn, J., G. Johnson, and S. Ziesche. 1991. A comparison of enalapril with hydralazine-isosorbide dinitrate in the treatment of chronic congestive heart failure. *N Engl J Med.* 325:303-310.

Collier, J.G., and B.F. Robinson. 1974. Comparison of effects of locally infused angiotensin I and II on hand veins and forearm arteries in man: evidence for converting enzyme activity in limb vessels. *Clin Sci Mol Med.* 47:189-192.

Collier JG, Robinson BF, Vane JR. Reduction of the pressor effects of Angiotensin I in man by synthetic nonapeptide B.P.P.5a or SQ 20,881 which inhibits converting enzyme. Lancet 1973;i (7794):72-4.

Collins, R., R. Peto, S. MacMahon, P. Hebert, N.H. Fiebach, K.A. Eberlain, J. Godwin, N. Qizilbash, J.O. Taylor, and C.H. Hennekens. 1990. Blood pressure, stroke and coronary artery disease. Part 2, short term reductions in blood pressure: an overview of randomised drug trials in their epidemiological context. *Lancet.* 335:827-838.

Cooper, K.E., O.G. Edholm, and R.F. Mottram. 1955. The blood flow in skin and muscle of the human forearm. *J Physiol (Lond).* 128:258-267.

Cotton, L.T., and O. Khan. 1986. Raynaud's phenomenon: a review. *Inter Angio.* 5:215-236.

Cowburn PJ, Cleland JGF, McArthur JD, MacLean MR, McMurray JJV, Dargie HJ. Short-term haemodynamic effects of BQ-123, a selective endothelin ET_A receptor antagonist, in chronic heart failure.*Lancet.*1998;**352**:201-2

Cowburn PJ, Cleland JGF, McDonagh A, MaArthur JD, MacLean MR, Dargie HJ. Adverse haemodynamic effects of a selective endothelin ETB receptor antagonist in patients with chronic heart failure: reversal with a selective endothelin ET_A receptor antagonist (abstract). Circulation 1998;98:718

Cozza, E.N., S. Chiou, and C.E. Gomez-Sanchez. 1992. Endothelin-1 potentiation of angiotensin II stimulation of aldosterone production. *Am J Physiol.* 262:R85-R89.

Currie, M.G., D.M. Geller, B.R. Cole, J.G. Boylan, W. YuSheng, S.W. Holmberg, and P. Needleman. 1983. Bioactive cardiac substances: potent vasorelaxant activity in mammalian atria. *Science.* 221:71-3.

Danilewicz, J.C., P.L. Barclay, I.T. Barnish, D. Brown, S.F. Campbell, K. James, G.M.R. Samuels, N.K. Terret, and M.J. Wythes. 1989. UK-69,578, a novel inhibitor of EC 3.4.24.11 which increases endogenous ANF levels and is natriuretic and diuretic. *Biochem Biophys Res Commun.* 164:58-65.

Dargie HJ, McMurray JJV. Diagnosis and management of heart failure. *Br Med J* 1994;**308**:321-8

Davenport, A.P., M.J. Ashby, P. Easton, S. Ella, J. Bedford, C. Dickerson, D.J. Nunez, S.J. Capper, and M.J. Brown. 1990. A sensitive radioimmunoassay measuring endothelin-like immunoreactivity in human plasma: comparison of levels in patients with essential hypertension and normotensive control subjects. *Clin Sci.* 78:261-264.

Davenport, A.P., R.E. Kuc, S.L. Hoskins, F.E. Karet, and F. Fitzgerald. 1994. [^{125}-I]-PD151242: a selective ligand for endothelin ET_A receptors in human kidney which localises to renal vasculature. *Br J Pharmacol.* 113:1303-1310.

Davenport, A.P., and J.J. Maguire. 1994. Is endothelin-induced vasoconstriction mediated only by ET_A receptors in humans? *Trends Pharmacol Sci.* 15:9-11.

Davenport, A.P., G. O'Reilly, and R.E. Kuc. 1995. Endothelin ET_A and ET_B mRNA and receptors expressed by smooth muscle in the human vasculature: majority of the ET_A subtype. *Br J Pharmacol.* 114:1110-1116.

De Bold, A.J., H.B. Borenstein, A.T. Veress, and H. Sonnenberg. 1981. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.* 28:89-94.

De Nucci G, Thomas R, D'Orleans-Juste P et al. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. 1988. *Proc Natl Acad Sci U S A.*;85:9797-800

De Leon, H., and R. Garcia. 1995. Characterization of endothelin receptor subtypes in isolated rat renal preglomerular microvessels. *Regul Pept.* 60:1-8.

Demuth, K., J. Blacher, A.P. Guerin, M.-O. Benoit, N. Moatti, M.E. Safar, and G.M. London. 1998. Endothelin and cardiovascular remodelling in end-stage renal disease. *Neprol Dial Transplant.* 13:375-383.

Deng, A.Y., L.L. Martin, J.L. Balwiercak, and A.Y. Jeng. 1994. Purification and characterization of an endothelin degradation enzyme from rat kidney. *J Biochem.* 115:120-125.

Deng, L.-Y., J.-S. Li, and E.L. Schiffrin. 1995. Endothelin receptor subtypes in resistance arteries from human and rats. *Cardiovasc Res.* 29:532-535.

Deray, G., A. Carayon, G. Maistre, M. Benhmida, F. Masson, C. Barthlemy, T. Peticlerc, and C. Jacobs. 1992. Endothelin in chronic renal failure. *Nephrol Dial Transplant.* 7:300-305.

Dhurandhar, R., D. Watt, M. Silver, A. Trimble, and A. Adelman. 1972. Prinzmetal's variant form of angina with arteriographic evidence of coronary arterial spasm. *Am J Cardiol.* 30:902-905.

Diedrich, D., Z. Yang, F.R. Bühler, and T.F. Lüscher. 1990. Impaired endothelium-dependent relaxations in hypertensive resistance arteries involve cyclooxygenase pathways. *Am J Physiol.* 258:H445-H451.

Dohi, Y., and T.F. Lüscher. 1991. Endothelin-1 in hypertensive resistance arteries: intraluminal and extraluminal dysfunction. *Hypertension.* 18:543-549.

Dohi, Y., M.A. Thiel, F.R. Bühler, and T.F. Lüscher. 1990. Activation of the endothelial L-arginine pathway in pressurized mesenteric resistance arteries: effect of age and hypertension. *Hypertension.* 15:170-179.

Donovan, K.D., G.J. Dobb, W.P. Woods, and B.G. Hockings. 1986. Comparison of transthoracic electrical bioimpedance and thermodilution methods for measuring cardiac output. *Crit Care Med.* 14:1038-1044.

Doorembos, C.J., and P. van Brummelen. 1989. The effect of acute ACE inhibition on atrial natriuretic peptide. *Br J clin Pharmacol.* 27:243S-7S.

Dorsch, N. 1995. Cerebral arterial spasm: a clinical review. *Br J Neurosurg.* 9:403-412.

Douglas, S., M. Gellai, M. Ezekiel, and E.H. Ohlstein. 1994. BQ-123, a selective endothelin subtype A-receptor antagonist, lowers blood pressure in different rat models of hypertension. *J Hypertens.* 12:561-567.

Douglas, S.A., J.D. Elliot, and E.H. Ohlstein. 1992. Regional vasodilatation to endothelin-1 is mediated by a non-ET_A receptor subtype in the anaesthetised rat: effect of BQ-123 on haemodynamic responses. *Eur J Pharmacol.* 221:315-324.

Douglas SA. Meeting Report - Clinical development of endothelin receptor antagonists. *TiPS* 1997;**18**:408-12

Dowd, P., P.G. Goldsmith, H.A. Bull, G. Burnstock, J.C. Foreman, and I. Marshall. 1995. Raynaud's phenomenon. *Lancet.* 346:283-290.

d'Uscio LV, Moreau P, Shaw S, Takase H, Barton M, Luscher TF. Effects of chronic ET_A-receptor blockade in angiotensin II-induced hypertension. Hypertension 1997;29:435-41.

Dussaule, J.-C., A. Stefanski, M.-L. Bea, P. Ronco, and R. Ardaillou. 1993. Characterization of neutral endopeptidase in vascular smooth muscle cells of rabbit renal cortex. *Am J Physiol.* 264:F45-52.

Eadington, D.W., S. Freestone, C.J. Waugh, C.P. Swainson, and M.R. Lee. 1992. Lithium pre-treatment affects renal and systemic responses to angiotensin II infusion in normal man. *Clin Sci.* 82:543-549.

Edwards, M., W. Trizna, and E.H. Ohlstein. 1990. Renal microvascular effects of endothelin. *Am J Physiol.* 259:F217-F221.

Egashira, K., S. Suzuki, Y. Hirooka, H. Kai, M. Sugimachi, T. Imaizumi, and A. Takeshita. 1995. Impaired endothelium-dependent vasodilatation of large epicardial and resistance coronary arteries in patients with essential hypertension: different responses to acetylcholine and substance P. *Hypertension.* 25:201-206.

Eguchi, S., Y. Hirata, T. Imai, K. Kanno, and F. Marumo. 1994. Phenotypic change of endothelin receptor subtype in cultured rat vascular smooth muscle cells. *Endocrinology.* 134:222-228.

Eichler, H.-G., G.A. Ford, T.F. Blaschke, A. Swislocki, and B.B. Hoffman. 1989. Responsiveness of superficial hand veins to phenylephrine in essential hypertension: alpha-adrenergic blockade during prazosin therapy. *J Clin Invest.* 83:108-112.

Elshourbagy, N.A., D.R. Korman, H.L. Wu, D.R. Sylvester, L.J. R, P. Nuthalaganti, D.J. Bergsma, C.S. Kumar, and P. Nambi. 1993. Molecular characterization and regulation of the human endothelin receptors. *J Biol Chem.* 268:3873-3879.

Emori, T., Y. Hirata, K. Ohta, K. Kanno, S. Eguchi, T. Imai, M. Shichiri, and F. Marumo. 1991. Cellular mechanism of endothelin 1 release by angiotensin and vasopressin. *Hypertension.* 18:165-170.

Emoto, N., and M. Yanagisawa. 1995. Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J Biol Chem.* 270:15262-15268.

Ensink, F.B.M., D. Baller, H.G. Wolpers, J. Zipfel, and G. Hellige. 1981. The reliability of venous capacity and blood flow determination by plethysmography. *In* Non-invasive methods on cardiovascular haemodynamics. A.H.M. Jageneau, editor. Elsevier/North-Holland, Amsterdam.

Erhardt, P.W. 1992. Endothelin structure and structure-activity relationships. *In* Endothelin. G.M. Rubanyi, editor. Oxford University Press, New York.

Farkkila, M., J. Palo, O. Saijonmaa, and F. Fyhrquist. 1992. Raised plasma endothelin during an acute migraine attack. *Cephalalgia.* 12:383-384.

Favrat, B., M. Burnier, J. Nussberger, J.M. Lecomte, R. Brouard, B. Waeber, and H.R. Brunner. 1995. Neutral endopeptidase versus angiotensin converting enzyme inhibition in essential hypertension. *J Hypertens.* 13:797-804.

Ferri C, Desideri G, Baldoncini R, Bellini C, Valenti M, Santucci A, De Mattia G. Angiotensin II increases the release of endothelin-1 from human cultured endothelial cells but does not regulate its circulating levels. Clin Sci 1999;96:261-70.

Ferro, C.J., W.G. Haynes, N.R. Johnston, C.C. Lomax, D.E. Newby, and D.J. Webb. 1997. The peptide endothelin receptor antagonist, TAK-044, produces sustained inhibition of endothelin-1 mediated arteriolar vasoconstriction. *Br J Clin Pharmacol.* 44:377-383.

Ferro, C.J., and D.J. Webb. 1996. The clinical potential of endothelin receptor antagonists in cardiovascular medicine. *Drugs.* 51:12-27.

Ferro, C.J., and D.J. Webb. 1997. Endothelial dysfunction in hypertension. *Drugs.* 53 (Suppl. 1):30-41.

Ferro CJ, Haynes WG, Hand MF, Webb DJ (1996) The vascular endothelin and nitric oxide systems in essential hypertension. *J Hypertens* 14:S50

Fettner, S.H., S. Pai, G.-R. Zhu, T. Kosoglou, C.R. Banfield, and V. Batra. 1995. Pharmacokinetic-pharmacodynamic (PK-PD) modeling for a new antihypertensive agent (neutral metallopeptidase inhibitor SCH 42354) in patients with mild to moderate hypertension. *Eur J Clin Pharmacol.* 48:351-9.

Firth, J.D., A.E.G. Raine, P.J. Ratcliffe, and J.G.G. Ledingham. 1988. Endothelin: an important factor in acute renal failure? *Lancet.* ii:1179-82.

Firth, J.D., A.F. Roberts, and A.E. Raine. 1990. Effect of endothelin on the function of the isolated perfused working rat heart. *Clin Sci.* 79:221-226.

Florijn, K.W., F.H.M. Derkx, W. Visser, J.A. Hofman, F.M. Rosmalen, H.C. Wallenburg, and M.A. Schalekemp. 1991. Plasma immunoreactive endothelin-1 in pregnant women with and without pre-eclampsia. *J Cardiovasc Pharmacol.* 17 (Suppl. 7):S446-448.

Fogo, A., S.E. Hellings, T. Inagami, and V. Kon. 1992. Endothelin receptor antagonism is protective in in-vivo acute cyclosporine toxicity. *Kidney Int.* 42:770-774.

Folkow, B. 1978. Cardiovascular structural adaptation: its role in the initiation and maintenance of primary hypertension. *Clin Sci Mol Med.* 55:3S-22S.

Francis, G.S., S.R. Goldsmith, B. Levinr, M.T. Olivari, and J.N. Cohn. 1984. The neurohumoral axis in congestive cardiac failure. *Ann Int Med.* 101:370-377.

Frank, H.J.L., E.R. Levin, R.M. Hu, and A. Pedram. 1993. Insulin stimulates endothelin binding and action on cultured vascular smooth muscle cells. *Endocrinology.* 133:1092-1097.

Fujimoto, M., S. Mihara, S. Nakajima, M. Ueda, M. Nakamura, and K. Sakurai. 1992. A novel-non-peptide endothelin antagonist isolated from bayberry, Myrica cerifera. *FEBS Lett.* 305:41-44.

Fujita, K., Y. Matsumura, S. Kita, Y. Miyazaki, K. Hisaki, M. Takaoka, and S. Morimoto. 1995. Role of endothelin-1 and the ET_A receptor in the maintenance of deoxycorticosterone acetate-salt-induced hypertension. *Br J Pharmacol.* 114:925-930.

Fukuroda, T., T. Fujikawa, S. Ozaki, K. Ishikawa, M. Yano, and M. Nishikibe. 1994. Clearance of circulating endothelin-1 by ET_B receptors in rats. *Biochem Biophys Res Commun.* 199:1461-1465.

Furchgott, R.F., and J.V. Zawadzki. 1980. The obligatory role endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* 288:373-376.

Fyhrquist, F., O. Saijonmaa, K. Metsarinne, I. Tikkanen, K. Rosenlof, and T. Tikkanen. 1990. Raised plasma endothelin-1 concentration following cold pressor test. *Biochem Biophys Res Commun.* 169:217-221.

Gallai, V., P. Sarchielli, C. Firenze, A. Trequattrini, M. Paciaroni, F. Usai, and R. Palumbo. 1994. Endothelin 1 in migraine and tension-type headache. *Acta Neurologica Scandinavica.* 89:47-55.

Gandhi, C.R., S.A.K. Harvey, and M.S. Olson. 1993. Hepatic effects of endothelin: metabolism of [^{125}I]endothelin-1 by liver-derived cells. *Arch Biochem Biophys.* 305:38-46.

Gardiner, S.M., A.M. Compton, P.A. Kemp, and T.A. Bennett. 1991. The effects of phosphoramidon on the regional haemodynamic responses to human proendothelin [1-38] in conscious rats. *Br J Pharmacol.* 103:2009-2015.

Gardiner, S.M., P.A. Kemp, J.E. March, and T. Bennett. 1994. Effects of bosentan (Ro 47-0203), an ET_A -, ET_B-receptor antagonist, on regional haemodynamic responses to endothelins in conscious rats. *Br J Pharmacol.* 112:823-830.

Gardiner SM, March JE, Kemp PA, Bennett T. Cardiovascular responses to angiotensins I and II in normotensive and hypertensive rats; effects of NO synthase inhibition or ET receptor antagonism. Br J Pharmacol 1999;128:1795-1803.

Gasic, S., O.F. Wagner, H. Vierhapper, P. Nowotny, and W. Waldhäusl. 1992. Regional haemodynamic effects and clearance of endothelin-1 in humans: renal and peripheral tissues may contribute to the overall disposal of the peptide. *J Cardiovasc Pharmacol.* 19:176-180.

Giaid, A., M. Yanagisawa, D. Langleben, R.P. Michel, R. Levy, H. Shennib, S. Kimura, T. Masaki, W. Duguid, and D.J. Stewart. 1993. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med.* 328:1732-1739.

Gillespie, M., Owasoya, J., McMurty, I., O'Brien, R. 1986. Sustained coronary vasoconstriction provoked by a peptidergic substance released from endothelial cells in culture. *J.Pharmacol. Exp. Ther.* 236:339-343.

Givertz MM, et al Acute ET_A receptor blockade causes selective pulmonary vasodilatation in patients with chronic heart failure. *Circulation* 2000; 101: 2992-7

Givertz MM, Colucci WS, Gottlieb SS, Hare JM, LeJemtel T, Slawsky MT, Leier CV, Loh E, Nicklas JM, Lewis BE (1998) Acute ET_A receptor blockade reduces pulmonary vascular resistance in patients with chronic heart failure (abstract). *Circulation* 98;Suppl 1:578

Goddard J, Webb DJ. Endothelin receptor antagonists: promising new agents in the management of cardiovascular disorders. *Drugs* R&D 1999;2:1-12.

Goli, V.D., S.M. Teague, R. Prasad, J. Harvey, W.F. Voyles, E.G. Olsen, E. Schechter, and U. Thadani. 1988. Noninvasive evaluation of aortic stenosis severity utilising Doppler ultrasound and electrical bioimpedance. *J Am Coll Cardiol.* 11:66-71.

Graf, K., P. Koehne, M. Gräfe, M. Zhang, W. Auch-Schwelk, and E. Fleck. 1995. Regulation and differential expression of neutral endopeptidase 24.11 in human endothelial cells. *Hypertension.* 26:230-5.

Gray, G.A., B.M. Löffler, and M. Clozel. 1994. Characterization of endothelin receptors mediating contraction of the rabbit saphenous vein. *Am J Physiol*:H959-H966.

Gray, G.A., and D.J. Webb. 1996. The endothelin system and its potential as a therapeutic target in cardiovascular disease. *Pharmacol Ther.* 72:109-148.

Gray, G.A., and D.J. Webb. 1995. Molecular biology and pharmacology of the endothelins. RG Landes Co, Texas.

Greenfield, A.D.M., and G.C. Patterson. 1954. Reactions of the blood vessels of the human forearm to increases in transmural pressure. *J Physiol (Lond).* 125:508-524.

Greenfield, A.D.M., R.J. Whitney, and J.F. Mowbray. 1963. Methods for the investigation of peripheral blood flow. *Br Med Bull.* 19:101-109.

Grover, G., S. Dzwonczyk, and C. Parham. 1993. The endothelin-1 receptor antagonist BQ-123 reduces infarct size in a canine model of coronary occlusion and reperfusion. *Cardiovasc Res.* 27:1613-1618.

Grover, G.J., P.G. Sleph, M. Fox, and N.C. Trippodo. 1992. Role of endothelin 1 and big endothelin 1 in modulating coronary artery vascular tone, contractile function and severity of ischaemia in rat hearts. *J Pharmacol Exp Ther.* 263:1074-1082.

Gui, G., D. Xu, N. Emoto, and M. Yanagisawa. 1993. Intracellular localization of membrane-bound endothelin-converting enzyme from rat lung. *J Cardiovasc Pharmacol.* 22:S53-S56.

Haefeli, H., L. Linder, W. Kiowski, and T.F. Lüscher. 1993. In vivo properties of endothelin-1 and endothelin-3 in human hand veins and its reversal by bradykinin and verapamil. *Hypertension.* 22:343 (Abstract).

Hand, M.F., W.G. Haynes, J.L. Anderton, R.J. Winney, and D.J. Webb. 1994. Venoconstriction to endothelin in chronic renal failure. *Nephrol Dial Transplant.* 9:930.

Hannedouche, T., P. Landais, B. Goldfarb, N. El Esper, A. Fournier, B. Godin, D. Durand, D. Chanard, F. Mignon, J.-M. Suc, and J.-P. Grünfeld. 1994. Randomised controlled trial of enalapril and β-blockers in non-diabetic chronic renal failure. *Br Med J.* 309:833-837.

Harris, P.J., J. Zhuo, F.A.O. Mendelsohn, and S.L. Skinner. 1991. Haemodynamic and renal tubular effects of low doses of endothelin in anaesthetized rats. *J Physiol.* 433:25-39.

Haynes, W.G., J. Clarke, J.R. Cockcroft, and D.J. Webb. 1991. Pharmacology of endothelin-1 in vivo in man. *J Cardiovasc Pharmacol.* 17(suppl. 7):S284-S286.

Haynes, W.G., C.J. Ferro, K.P.J. O'Kane, D. Somerville, C.C. Lomax, and D.J. Webb. 1996. Systemic endothelin receptor blockade decreases peripheral vascular resistance and blood pressure in man. *Circulation.* 93:1860-1870.

Haynes, W.G., M.F. Hand, M.E.C. Dockrell, D.W. Eadington, M.R. Lee, Z. Hussein, N. Benjamin, and D.J. Webb. 1997. Physiological role of nitric oxide in regulation of renal function in humans. *Am J Physiol.* 272:F364-F371.

Haynes, W.G., M.F. Hand, H. Johnstone, P.L. Padfield, and D.J. Webb. 1994. Direct and sympathetically mediated venoconstriction in essential hypertension. *J Clin Invest.* 94:1359-1364.

Haynes, W.G., S. Moffat, and D.J. Webb. 1995. An investigation into the direct and indirect venoconstrictor effects of endothelin-1 and big endothelin-1 in man. *Br J Clin Pharmacol.* 40:307-11.

Haynes, W.G., J.P. Noon, B.R. Walker, and D.J. Webb. 1993. Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. *J Hypertens.* 11:1375-1380.

Haynes, W.G., J.P. Noon, B.R. Walker, and D.J. Webb. 1993. L-NMMA increases blood pressure in man. *Lancet.* 342:931-932.

Haynes, W.G., F.E. Strachan, and D.J. Webb. 1995. Endothelin ET_A and ET_B receptors cause vasoconstriction of human resistance and capacitance vessels in vivo. *Circulation.* 92:357-363.

Haynes, W.G., and D.J. Webb. 1994. Contribution of endogenous generation of endothelin-1 to basal vascular tone. *Lancet.* 344:852-854.

Haynes, W.G., and D.J. Webb. 1993. The endothelin family of peptides: local hormones with diverse roles in health and disease? *Clinical Science.* 84:485-500.

Haynes, W.G., and D.J. Webb. 1993. The endothelin family of peptides: local hormones with diverse roles in health and disease? *Clinical Science.* 84:485-500.

Hayzer, D.J., P.M. Rose, J.S. Lynch, M.L. Webb, B.K. Kienzle, E.C.K. Liu, E.A. Bogosian, E. Brinson, and M.S. Runge. 1992. Cloning and expression of a human endothelin receptor subtype A. *Am J Med Sci.* 304:231-238.

Headache Classification Committee of the International Headache Society. 1988. Classification and Diagnostic Criteria for Headache Disorders, Cranial Neuralgias and Facial Pain. *Cephalgia.* 8(Suppl 7):1-96.

Hewlett, A.W., and J.G. van Zwaluwenburg. 1909. The rate of blood flow in the arm. *Heart.* 1:87-97.

Herizi A, Jover B, Bouriquet N, Mimran A. Prevention of the cardiovascular and renal effects of angiotensin II by endothelin blockade. Hypertension 1998;31:10-14.

Hickey, K.A., Rubanyi, G., Raul, R., Highsmith, R. 1985. Characterisation of a coronary vasconstrictor produced by cultured endothelial cells. *Am. J. Physiol.* 248:C550-C556.

Hilkert, R.J., M.-U. Lee, and T. Quertermous. 1992. Genetic regulation of endothelin-1 in vascular endothelial cells. *Trends Cardiovasc Med.* 2:129-133.

Hirata, K., T. Matsuda, H. Akita, M. Yokoyama, and H. Fukuzaki. 1990. Myocardial ischaemia induced by endothelin in the intact rabbit: angiographic analysis. *Cardiovasc Res.* 24:879-883.

Hirooka, Y., T. Imaizumi, H. Masaki, S. Ando, S. Harada, M. Momohara, and A. Takeshita. 1992. Captopril improves impaired endothelium-dependent vasodilatation in hypertensive patients. *Hypertension.* 20:175-180.

Hoffman, A., A. Haramati, I. Dalal, E. Shuranyi, and J. Winaver. 1994. Diuretic-natriuretic actions and pressor effects of big endothelin (1-39) in phosphoramidon treated rats. *Proc Soc Exp Med Biol.* 205:168-173.

Horgan, M., J. Pinheiro, and A. Malik. 1991. Mechanism of endothelin-1 induced pulmonary vasoconstriction. *Circ Res.* 69:157-164.

Hosada, K., K. Nakao, N. Tamura, H. Arai, Y. Ogawa, S. Suga, S. Nakanishi, and H. Imura. 1992. Organization, structure, chromosomal assignment and expression of the human endothelin-A receptor. *J Biol Chem.* 267:18797-18804.

Huggins, J.P., J.T. Pelton, and R.C. Miller. 1993. The structure and specificity of endothelin receptors: their importance in physiology and medicine. *Pharmacol Ther.* 59:55-123.

Hughes, A.K., P.K. Stricklett, E. Padilla, and D.E. Kohan. 1996. Effect of reactive oxygen species on endothelin-1 production by human mesangial cells. *Kidney Int.* 49:181-189.

Ihara, M., T. Fukuroda, T. Saeki, M. Nishikibe, K. Kojiri, H. Suda, and M. Yano. 1991. An endothelin receptor (ETA) antagonist isolated from *Streptomyces misakiensis. Biochem Biophys Res Commun.* 178:132-137.

Ihara, M., K. Noguchi, T. Saeki, T. Fukuroda, S. Tsuchida, S. Kimura, T. Fukami, K. Ishikawa, M. Nishikibe, and M. Yano. 1992. Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.* 50:247-255.

Ikeda, S., Y. Awane, K. Kusumoto, M. Wakimasu, T. Watanabe, and M. Fujino. 1994. A new endothelin receptor antagonist, TAK-044, shows long-lasting inhibition of both ET_A- and ET_B-mediated blood pressure responses in rats. *J Pharmacol Exp Ther.* 270:728-733.

Imai T, Hirata Y, Emori T, Yanagisawa M, Masaki T, Marumo F. Induction of endothelin-1 gene by angiotensin and vasopressin in endothelial cells. *Hypertension* 1992;19:753-57.

Inoue, A., M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, K. Goto, and T. Masaki. 1989. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three different genes. *Proc Natl Acad Sci USA.* 86:2863-2867.

Ishikawa, K., M. Ihara, K. Noguchi, T. Mase, N. Mino, T. Saeki, T. Fukuroda, T. Fukami, S. Ozaki, T. Nagase, M. Nishikibe, and M. Yano. 1994. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc Natl Acad Sci USA.* 91:4892-4896.

Itoh, S., T. Sasaki, K. Ide, K. Ishikawa, M. Nishikibe, and M. Yano. 1993. A novel endothelin ET_A receptor antagonist, BQ-485, and its preventative effect on experimental cerebral vasospasm in dogs. *Biochem Biophys Res Commun.* 195:969-975.

Iwasaki, S., T. Homma, Y. Matsuda, and V. Kon. 1995. Endothelin receptor subtype B mediates autoinduction of endothelin-1 in rat mesangial cells. *J Biol Chem.* 270:6997-7003.

Jackman, H.L., P.W. Morris, S.F. Rabito, G.B. Johanssen, R.A. Skidgel, and E.G. Erdos. 1993. Inactivation by endothelin-1 by an enzyme of the vascular endothelial cells. *Hypertension.* 21:II-925-II-928.

Janakidevi, K., M.A. Fisher, P.J. Del Vecchio, C. Tirrupathi, J. Figge, and A.B. Malik. 1992. Endothelin-1 stimulates DNA synthesis and proliferation of pulmonary artery smooth muscle cells. *Am J Physiol.* 263:C1295-C1301.

Johns, E.J. 1989. Role of angiotensin II and the sympathetic nervous system in the control of renal function. *J Hypertens.* 7:695-701.

Juillerat, L., J. Nussberger, J. Menard, V. Mooser, Y. Christen, B. Waeber, P. Graf, and H.R. Brunner. 1990. Determinants of angiotensin II generation during converting enzyme inhibition. *Hypertension.* 16:564-72.

Kaasjager, K.A.H., H.A. Koomans, and T.J. Rabelink. 1995. Effectiveness of enalapril versus nifedipine to antagonize blood pressure and the renal response to endothelin in humans. *Hypertension.* 25:620-625.

Kahn, J.C., M. Patey, J.L. Dubois-Rande, P. Merlet, A. Castaigne, C. Lim-Alexandre, J.M. Lecomte, D. Duboc, C. Gros, and J.C. Schwartz. 1990. Effect of sinorphan on plasma atrial natriuretic factor in congestive heart failure. *Lancet.* 335:118-9.

Kalra PR, Moon JC, Coats AJ. Do results of the ENABLE (Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart Failure) study spell the end for non-selective endothelin antagonism in heart failure? 2002 *Int J Cardiol.*;85:195-7.

Kanno, K., Y. Hirata, M. Tsujino, M. Imai, M. Shichiri, H. Ito, and F. Marumo. 1993. Up-regulation of ET_B receptor subtype mRNA by angiotensin II in rat cardiomyocytes. *Biochem Biophys Res Commun.* 194:1282-1287.

Kasinath, B.S., T.A. Fried, S. Davalath, and P.A. Marsden. 1992. Glomerular epithelial cells synthesize endothelin peptides. *Am J Pathol.* 141:279-283.

Katoh, T., H. Chang, S. Uchida, T. Okuda, and K. Kurokawa. 1990. Direct effects of endothelin in rat kidney. *Am J Physiol.* 258:F397-F402.

Kelm, M., M. Feelisch, T. Krebber, W. Motz, and B.E. Strauer. 1992. The role of nitric oxide in the regulation of coronary vascular resistance in arterial hypertension: comparison of normotensive and spontaneously hypertensive rats. *J Cardiovasc Pharmacol.* 20 (suppl. 12):S183-S186.

Kenny, A.J., A. Bourne, and J. Ingram. 1993. Hydrolysis of human and pig brain natriuretic peptides, urodilantin, C-type natriuretic peptide, and some C-receptor ligands by endopeptidase-24.11. *Biochem J.* 291:83-88.

Kentsch, M., W. Otter, C. Drummer, A. Nötges, R. Gerzer, and G. Müller-Esch. 1996. Neutral endopeptidase 24.11 inhibition may not exhibit beneficial haemodynamic effects in patients with congestive heart failure. *Eur J Clin Pharmacol.* 51:269-272.

Kerslake, D.M. 1949. The effect of application of an arterial occlusion cuff to the wrist on the blood flow in the human forearm. *J Physiol (Lond).* 108:451-457.

Kikuchi, T., T. Ohtaki, A. Kawata, T. Imada, T. Asami, Y. Masuda, T. Sugo, K. Kusumoto, K. Kubo, T. Watanabe, M. Wakimasu, and M. Fujino. 1994. Cyclic hexapeptide endothelin receptor antagonists highly potent for both receptor subtypes ET_A and ET_B. *Biochem Biophys Res Commun.* 200:1708-1712.

Kimura, S., Y. Kasuya, T. Sawamura, O. Shinmi, Y. Sugita, M. Yanagisawa, K. Goto, and T. Masaki. 1988. Structure-activity relationships of endothelin: importance of the C terminal moiety. *Biochem Biophys Res Commun.* 156:1182-1186.

King, A.J., B.M. Brenner, and S. Anderson. 1989. Endothelin: a potent renal and systemic vasoconstrictor peptide. *Am J Physiol.* 256:F1051-F1058.

Kiowski, W., G. Sütsch, P. Hunziker, P. Müller, J. Kim, E. Oechslin, R. Schmitt, R. Jones, and O. Bertel. 1995. Evidence for endothelin-1-mediated vasoconstriction in severe chronic heart failure. *Lancet.* 346:732-736.

Kohan, D. 1991. Endothelin synthesis by rabbit renal tubule cells. *Am J Physiol.* 261:F221-F226.

Kohan, D.E. 1997. Endothelins in the normal and diseased kidney. *Am J Kid Dis.* 29:2-26.

Kohno, M., K. Murakawa, T. Horio, K. Yokokawa, K. Yasunari, T. Fukui, and T. Takeda. 1991. Plasma immunoreactive endothelin-1 in experimental malignant hypertension. *Hypertension.* 18:93-100.

Kohno, M., K. Yasunari, N. Murakawa, K. Yokokawa, T. Horio, T. Fukui, and T. Takeda. 1990. Plasma immunoreactive endothelin in essential hypertension. *Am J Med.* 88:614-618.

Komatsu, S., H. Itoh, S. Suga, T. Igaki, Y. Ogawa, I. Kishimoto, O. Nakagawa, T. Yoshimasa, and K. Nakao. 1996. Regulation of secretion and clearance of C-type natriuretic peptide in the interaction of vascular endothelial cells and smooth muscle cells. *J Hypertens.* 14:585-592.

Komatsu, Y., H. Itoh, S. Suga, Y. Ogawa, N. Hama, I. Kishimoto, O. Nakagawa, T. Igaki, K. Doi, T. Yoshimasi, and K. Nakao. 1996. Regulation of endothelial production of C-type

natriuretic peptide in coculture with vascular smooth muscle cells; role of the vascular natriuretic peptide system in vascular growth inhibition. *Circ Res.* 78:606-614.

Kon, V., T.E. Hunley, and A. Fogo. 1995. Combined antagonism of endothelin A/B receptors links endothelin to vasoconstriction whereas angiotensin II affects fibrosis. *Transplantation.* 60:89-95.

Krum, H., R.J. Viskoper, Y. Lacourciere, M. Budde, V. Charlon, and for the Bosentan Hypertension Investigators. 1998. The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. *N Engl J Med.* 338:784-790.

Kubicek, W.G., J.N. Karnegis, R.P. Patterson, D.A. Witsoe, and R.H. Mattson. 1966. Development and evaluation of an impedance cardiac output system. *Aerospace Med.* 37:1208-1212.

Kugiyama, K., S. Sugiyama, M. Ohgushi, T. Matsumura, and H. Yasue. 1993. Suppression of atherosclerotic changes in cholesterol fed rabbits treated with an oral inhibitor of neutral endopeptidase 24.11 (EC 3.4.24.11; NEP). *Circulation.* 88:I-521.

Kurihara, H., K. Yamaoki, R. Nagai, M. Yoshizumi, F. Takaku, H. Satoh, J. Inui, and Y. Yazaki. 1989. Endothelin: a potent vasoconstrictor associated with vasospasm. *Life Sci.* 44:1937-1944.

Kurihara, Y., H. Kurihara, H. Suzuki, T. Kodama, K. Maemura, R. Nagai, H. Oda, T. Kuwaki, W. Cao, N. Kamada, K. Jishage, Y. Ouchi, S. Azuma, Y. Toyoda, T. Ishikawa, M. Kumada, and Y. Yazaki. 1994. Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature.* 368:703-710.

Kusumoto, K., K. Kubo, H. Kandori, T. Kitayoshi, S. Sato, M. Wakimasu, T. Watanabe, and M. Fujino. 1994. Effects of a new endothelin antagonist, TAK-044, on post-ischaemic acute renal failure in rats. *Life Sci.* 55:301-310.

Lamas, L., P.A. Marsden, G.K. Li, P. Tempest, and T. Michel. 1992. Endothelial nitric oxide synthase: molecular cloning and characterisation of a distinct constitutive enzyme isoform. *Proc Natl Acad Sci USA.* 89:6348-6352.

Lang, C.C., J.G. Motwani, W.J.R. Coutie, and A.D. Struthers. 1992. Clearance of brain natriuretic peptide in patients with chronic heart failure: indirect evidence for a neutral endopeptidase mechanism but against an atrial natriuretic peptide clearance receptor. *Clin Sci.* 82:619-23.

Lariviere, R., G. Thibault, and E.L. Schriffin. 1993. Increased endothelin-1 content in blood vessels of deoxycorticosterone acetate-salt hypertensive but not spontaneously hypertensive rats. *Hypertension.* 21:294-300.

Lechleitner, P., N. Genser, J. Mair, J. Maier, E. Artner-Dworzak, F. Dienstl, and B. Puschendorf. 1992. Plasma immunoreactive endothelin in the acute and subacute phases of myocardial infarction in patients undergoing fibrinolysis. *Clin Chem.* 39:955-959.

Lee, M.-E., K. Bloch, J. Clifford, and T. Quertermous. 1990. Functional analysis of the endothelin-1 promoter: evidence for an endothelial cell-specific cis-acting sequence. *J Biol Chem.* 10:10446-10450.

Lee, M.-E., M.S. Dhadly, D.H. Temizer, C.J. A, M. Yoshimuzi, and T. Quertermous. 1991. Regulation of endothelin-1 gene expression by Fos and Jun. *J Biol Chem.* 266:19304-19309.

Lefrancois, P., G. Clerc, J. Duchier, C. Lim, J.M. Lecomte, C. Gros, and J.C. Schwartz. 1990. Antihypertensive activity of sinorphan. *Lancet.* 336:307-308.

Lemne, C.E., T. Lundeberg, E. Theodorsson, and U. De Faire. 1994. Increased basal concentrations of plasma endothelin in borderline hypertension. *J Hypertens.* 12:1069-1074.

Lerman, A., B.S. Edwards, J.W. Hallett, D.M. Heublein, S.M. Sandberg, and J.C. Burnett Jr. 1991. Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N Engl J Med.* 325:997-1001.

Linder, L., W. Kiowski, F.R. Bühler, and T.F. Lüscher. 1990. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. *Circulation.* 81:1762-7.

Liu, J., R. Chen, D. Casley, and W.G. Nayler. 1990. Ischaemia and reperfusion increase ^{125}I-labelled endothelin-1 binding in rat cardiac membranes. *Am J Physiol.* 258:829-835.

Lloren-Cortes, C., H. Huang, P. Vicart, J.M. Gasc, D. Paulin, and P. Corvol. 1992. Identification and characterisation of neutral endopeptidase in endothelial cells from venous or arterial origins. *J Biol Chem.* 267:14012-14018.

Lockette, W., Y. Otsuka, and O. Carretero. 1986. The loss of endothelium-dependent vascular relaxation in hypertension. *Hypertension.* 8 (suppl. II):II61-II66.

Löffler, B.M., B. Kalina, and H. Kunze. 1991. Partial characterization and subcellular distribution patterns of endothelin-1, -2 and -3 binding sites in the human liver. *Biochem Biophys Res Commun.* 181:840-845.

Love, M.P., W.G. Haynes, G.A. Gray, D.J. Webb, and J.J.V. McMurray. 1996. Vasodilator effects of endothelin-converting enzyme inhibition and endothelin ET_A receptor blockade in chronic heart failure patients treated with ACE inhibitors. *Circulation.* 94:2131-2137.

Love MP, Ferro CJ, Haynes WG, Gray GA, Webb DJ, McMurray JJV. What type of Endothelin Receptor Antagonist for Patients with Chronic Heart Failure*?* *Scot. Med. J.* 1997;42:56

Love MP, Ferro CJ, Haynes WG, Webb DJ, McMurray JJ. Selective or non-selective endothelin receptor blockade in chronic heart failure? Circulation 1996; 94 (suppl I):2899-900

Lüscher, T. 1991. Endothelin, key to coronary vasospasm? *Circulation.* 83:701-703.

Lüscher, T. 1995. The endothelium in cardiovascular disease. Springer-Verlag, Berlin.

Lüscher, T.F., L. Raij, and P.M. Vanhoutte. 1987. Endothelium-dependent vascular responses in normotensive and hypertensive Dahl rats. *Hypertension.* 9:157-163.

Lüscher, T.F., and P.M. Vanhoutte. 1986. Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension.* 8:344-348.

Lyons, D., J. Webster, and N. Benjamin. 1994. The effect of antihypertensive therapy on responsiveness to local intra-arterial NG-monomethyl-L-arginine in patients with essential hypertension. *J Hypertens.* 12:1047-1053.

MacFadyen, R.J., K.R. Lees, and J.L. Reid. 1991. Tissue and plasma angiotensin converting enzyme and the response to ACE inhibitor drugs. *Br J Clin Pharmacol.* 31:1-13.

Macrae, I.M., M.A. McAuley, M.J. Robinson, J.L. Reid, and J. McCulloch. 1991. Endothelin induced hypertension: a consequence of medullary ischaemia? *J Cardiovasc Pharmacol.* 17 (Suppl.7):S496-S499.

Maggi, C.A., S. Giuliani, R. Patacchini, P. Rovero, A. Giachetti, and A. Meli. 1989. The activity of peptides of the endothelin family in various mammalian smooth muscle preparations. *Eur J Pharmacol.* 174:23-31.

Maguire, J., R.E. Kuc, G. O'Reilly, and A.P. Davenport. 1994. Vasoconstrictor endothelin receptors characterized in human renal artery and vein *in vitro*. *Br J Pharmacol.* 113:49-54.

Mallamaci, F., S. Parlongo, and C. Zoccali. 1993. Influence of cardiovascular damage and residual renal function on plasma endothelin in chronic renal failure. *Nephron.* 63:291-295.

Mancia, G., G. Grassi, G. Parati, and A. Daffonchio. 1993. Evaluating sympathetic activity in human hypertension. *J Hypertens.* 11(Suppl. 5):S13-S19.

Marguiles, K.B., F.L. Hilbebrand, D.M. Heublein, and J.C. Burnett. 1991. Radiocontrast increases plasma and urinary endothelin. *J Am Soc Nephrol.* 2:1041-1045.

Martin, E.R., B.M. Brenner, and B.J. Ballermann. 1990. Heterogeneity of cell surface endothelin receptors. *J Biol Chem.* 265:14044-14049.

Martin-Nizzard, H., H. Houssaini, S. Lestavel-Delattre, P. Duriez, and J. Fruchart. 1991. Modified low density lipoproteins activate human monocytes to secrete immunoreative endothelin. *FEBS Lett.* 293:127-130.

Masaoka, H., R. Suzuki, Y. Hirata, T. Emori, F. Marumo, and K. Hirakawa. 1989. Raised plasma endothelin in aneurysmal subarachnoid haemorrhage. *Lancet.* 2:1402.

Mason, D.T., and E. Braunwald. 1965. The effects of nitroglycerin and amyl nitrate on arteriolar and venous tone in the human forearm. *Circulation.* 32:755-766.

Matsumoto, H., N. Suzuki, C. Kitada, and M. Fujino. 1994. Endothelin family peptides in human plasma and urine: their molecular forms and concentrations. *Peptides.* 15:505-510.

Matsumura, Y., R. Ikegawa, Y. Suzuki, M. Takaota, T. Uchida, H. Kido, H. Shinyama, K. Hayashi, M. Watanabe, and S. Morimoto. 1991. Phosphoramidon prevents cerebral vasospasm following subarachnoid haemorrhage in dogs: the relationship to endothelin-1 levels in the cerebrospinal fluid. *Life Sci.* 49:841-848.

Matsuyama, K., H. Yasue, K. Okumura, Y. Saito, K. Nakao, G. Shirakami, and H. Imura. 1991. Increased plasma levels of endothelin-1-like immunoractivity during coronary spasm in patients with coronary spastic angina. *Am J Cardiol.* 68:991-995.

Matthews, J.N.S., D.G. Altman, M.J. Campbell, and P. Royston. 1990. Analysis of serial measurements in medical research. *Br Med J.* 300:230-235.

May, A., H.J. Gijsman, A. Wallnofer, R. Jones, H.C. Diener, and M.D. Ferrari. 1996. Endothelin antagonist bosentan blocks neurogenic inflammation, but is not effective in aborting migraine attacks. *Pain.* 67:375-378.

McMahon, E.G., M.A. Palomo, and W.M. Moore. 1991. Phosphoramidon blocks the pressor activity of big endothelin-1 [1-39] and lowers blood pressure in spontaneously hypertensive rats. *J Cardiovasc Pharmacol.* 17 (suppl 7):S29-33.

McMurray, J.J., S.G. Ray, I. Abdullah, H.J. Dargie, and J.J. Morton. 1992. Plasma endothelin in heart failure. *Circulation.* 85:1374-1379.

Miasiro, N., and A.C.M. Paiva. 1992. Effects of endothelin-1 and endothelin-3 on the isolated guinea pig ileum: role of Na+ ions and endothelin receptor subtypes. *Eur J Pharmacol.* 214:133-141.

Mihara, S.-I., S. Nakajima, S. Matsumura, T. Kohoike, and M. Fujimoto. 1994. Pharmacological characterization of a potent nonpeptide endothelin receptor antagonist. *J Pharmacol Exp Ther.* 268:1122-1128.

Miller, D., D.D. Waters, W. Warnica, and e. al. 1981. Is variant angina the coronary manifestation of a generalized vasospastic disorder? *N Engl J Med.* 304:736-766.

Miller, W.L., M.M. Redfield, and J.C. Burnett. 1989. Integrated cardiac, renal, and endocrine actions of endothelin. *J Clin Invest.* 83:317-320.

Mino, N., M. Kobayashi, A. Nakajima, H. Amano, K. Shimamoto, K. Ishikawa, K. Watanabe, M. Nishikibe, M. Yano, and F. Ikemoto. 1992. Protective effect of a selective

endothelin receptor antagonist, BQ-123, in ischaemic acute renal failure in rats. *Eur J Pharmacol.* 221:77-83.

Miyamori, I., Y. Takeda, T. Yoneda, and R. Takeda. 1991. Endothelin-1 release from mesenteric arteries of spontaneously hypertensive rats. *J Cardiovasc Pharmacol.* 17 (Suppl. 7):S408-S410.

Miyauchi, T., T. Ishikawa, Y. Tomobe, M. Yanagisawa, S. Kinura, Y. Sugishita, I. Ito, K. Goto, and T. Masaki. 1989. Characteristics of pressure response to endothelin in spontaneously hypertensive and Wystar-Kyoto rats. *Hypertension.* 18:543-549.

Miyauchi, T., M. Yanagisawa, K. Iida, R. Ajisaki, N. Suzuki, M. Fujino, K. Goto, T. Masaki, and Y. Sugishita. 1992. Age- and sex-related variation of plasma endothelin-1 concentration in normal and hypertensive subjects. *Am Heart J.* 123:1092-1093.

Miyauchi, T., M. Yanagisawa, T. Tomizawa, Y. Sugishita, N. Suzuki, M. Fujino, R. Ajisaka, K. Goto, and T. Masaki. 1989. Increased plasma concentrations of endothelin-1 and big endothelin-1 in acute myocardial infarct size. *Lancet.* ii:53-54.

Mohapatra, S.N. 1981. Non-invasive cardiovascular monitoring by electrical impedance technique. Pitman Medical Ltd, London.

Molenaar, P., G. O'Reilly, A. Sharkey, R.E. Kuc, D.P. Harding, C. Plumpton, G.A. Gresham, and A.P. Davenport. 1993. Characterization and localization of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium. *Circ Res.* 72:526-538.

Moncada, S., R. Gryglewski, S. Bunting, and J.R. Vane. 1976. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature.* 263:663-665.

Moncada, S., and A. Higgs. 1993. The L-arginine-nitric oxide pathway. *N Engl J Med.* 329:2002-2012.

Moncada, S., and J.R. Vane. 1979. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A_2 and prostacyclin. *Pharmacol Rev.* 30:293-331.

Monopoli, A., E. Ongini, E. Cigola, and G. Olivetti. 1992. The neutral endopeptidase inhibitor, SCH 34826, reduces left ventricular hypertrophy in spontaneously hypertensive rats. *J Cardiovasc Pharmacol.* 20:496-504.

Montanari, A., Biggi, A., Carra, N., Fasoli, E., Calzolari, M., Corsini, F., Perinotto, P. & Novarini, A. 2000. Endothelin-A blockade attenuates systemic and renal hemodynamic effects of L-NAME in humans. *Hypertension,* 35, 518-523.

Moran, K.T., D.P. Halpin, R.S. Zide, R.A. Oberfield, and E.R. Jewell. 1988. Long-term brachial artery catheterization: ischaemic complications. *J Vasc Surg.* 8:76-78.

Moreau P, d'Uscio LV, Shaw S, Takase H, Barton M, Luscher TF. Angiotensin II increases tissue endothelin and induces vascular hypertrophy. Reversal by ET_A-receptor antagonist. Circulation 1997;96:1593-97.

Moreland, S., B. Abboa-Offei, and A. Seymour. 1994. Evidence for a differential location of vasoconstrictor endothelin receptors in the vasculature. *Br J Pharmacol.* 112:704-708.

Moreland, S., D.M. McMullen, C.L. Delaney, V.G. Lee, and J.T. Hunt. 1992. Venous smooth muscle contains vasoconstrictor ET_B-like receptors. *Biochem Biophys Res Commun.* 184:100-106.

Morton, J.J., and D.J. Webb. 1985. Measurement of plasma angiotensin II. *Clin Sci.* 68:483-484.

Mortensen, L.H., Fink, G.D. 1990. Haemodynamic effect of human and rat endothelin administration into conscious rats. *Am. J. Physiol.* H362-H368.

Motwani, J.G., C.C. Lang, G. Cramb, and A.D. Struthers. 1995. Natriuretic response to neutral endopeptidase inhibition is blunted by enalapril in healthy men. *Hypertension.* 25:637-642.

Murphy, L., R. Corder, A. Mallet, and A. Turner. 1994. Generation by the phosphoramidon-sensitive peptidases, endopeptidase 24.11 and thermolysin, of endothelin-1 and C-terminal fragment from big endothelin-1. *Br J Pharmacol.* 113:137-142.

Nava, E., and T.F. Lüscher. 1995. Endothelium-derived vasoactive factors in hypertension: nitric oxide and endothelin. *J Hypertens.* 13 (Suppl 2):S39-48.

Newby, D.E., N.A. Boon, and D.J. Webb. 1997. Comparison of forearm vasodilatation to Substance P and acetylcholine: contribution of nitric oxide. *Clin Sci.* 92:133-138.

Nicod, P., and K. Moser. 1989. Primary pulmonary hypertension: the risk and benefit of lung biopsy. *Circulation.* 80:1486-1488.

Nirei, H., K. Hamada, M. Shoubo, K. Sogabe, Y. Notsu, and T. Ono. 1993. An endothelin ET_A receptor antagonist, FR139317, ameliorates cerebral vasospasm in dogs. *Life Sci.* 52:1869-1874.

Nishikibe, M., S. Tsuchida, M. Okada, T. Fukoda, K. Shimamoto, M. Yano, K. Ishika, and F. Ikemoto. 1993. Antihypertensive effect of a newly synthesised endothelin antagonist, BQ-123, in a genetic hypertensive model. *Life Sci.* 52:717-724.

Nitta, K., K. Uchida, N. Kimata, A. Kawashima, W. Yumura, and H. Nihei. 1995. Endothelin-1 mediates erythropoietin-stimulated glomerular endothelial cell-dependent proliferation of mesangial cells. *Eur J Pharmacol.* 293:491-494.

Norris, J.W., V.C. Hachinski, and P.W. Cooper. 1975. Changes in cerebral blood flow during a migraine attack. *Br Med J.* 3:676-684.

Northridge, D.B., A.G. Jardine, C.T. Alabaster, P.L. Barclay, J.M.C. Connell, H.J. Dargie, S.G. Dilly, I.N. Findlay, A.F. Lever, and G.M.R. Samuels. 1989. Effects of UK 69 578: a novel atriopeptidase inhibitor. *Lancet.* x:591-3.

Northridge, D.B., I.N. Findlay, J. Wilson, and E.D. Henderson, H J. 1990. Non-invasive determination of cardiac output by Doppler echocardiography and electrical bioimpedance. *Br Heart J.* 63:93-97.

O'Brien, E., F. Mee, N. Atkins, and K. O'Malley. 1991. Accuracy of the Spacelabs 90207 determined by the British Hypertension Society Protocol. *J Hypertens.* 9:573-574.

O'Brien, E., J. Petrie, and W. Littler. 1990. The British Hypertension Society Protocol for the evaluation of automated and semi-automated blood pressure measuring devices with special reference to ambulatory systems. *J Hypertens.* 8:607-619.

O'Connell, J.E., A.G. Jardine, A.G. Davidson, G. Davidson, and J.M.C. Connell. 1992. Candoxatril, an orally active neutral endopeptidase inhibitor, raises plasma atrial natriuretic factor and is natriuretic in essential hypertension. *J Hypertens.* 10:271-7.

O'Connell, J.E., A.G. Jardine, G. Davidson, J. McQueen, and J.M.C. Connell. 1992. UK 79,300, an orally available atriopeptidase inhibitor, raises plasma ANP and is natriuretic in essential hypertension. *J Hypertens.* 10:271-9.

Ogawa, Y., K. Nakao, H. Arai, O. Nagakawa, K. Hosada, S.-I. Suga, S. Nakanishi, and H. Imura. 1991. Molecular cloning of a non-isopeptide-selective human endothelin receptor. *Biochem Biophys Res Commun.* 178:248-255.

Ogihara, T., H. Rakugi, K. Masuo, H. Yu, M. Nagano, and H. Mikami. 1994. Antihypertensive effects of the neutral endopeptidase inhibitor SCH 42495 in essential hypertension. *Am J Hypertens.* 7:943-947.

Ohlstein, E.H., P. Nambi, S.A. Douglas, R.M. Edwards, M. Gellai, A. Lago, J.D. Leber, R.D. Cousins, A. Gao, J.S. Frazee, C.E. Peishoff, J.W. Bean, D.S. Eggleston, N.A. Elshourbagy, C. Kumar, J.A. Lee, T.L. Yue, C. Louden, and J.D. Elliott. 1994. SB 209670, a rationally designed potent non-peptide endothelin receptor antagonist. *Proc Natl Acad Sci USA.* 91:8052-8056.

Ohnaka, K., R. Takayanagi, M. Nishikawa, M. Haji, and H. Nawata. 1993. Purification and characterization of a phosphoramidon sensitive endothelin-converting enzyme in porcine aortic endothelium. *J Biol Chem.* 268:26759-26766.

Ohno, A., M. Naruse, S. Kato, M. Hosaka, K. Naruse, H. Demura, and N. Sugino. 1992. Endothelin specific antibodies decrease blood pressure and increase glomerular filtration rate and renal plasma blood flow in spontaneously hypertensive rats. *J Hypertens.* 10:781-785.

Ohta, K., Y. Hirata, T. Imai, K. Kanno, T. Emori, M. Shichiri, and F. Marumo. 1990. Cytokine-induced release of endothelin-1 from porcine renal epithelial cell line. *Biochem Biophys Res Commun.* 169:578-584.

Okada, M., M. Kobayashi, H. Maruyama, R. Takahashi, F. Ikemoto, M. Yano, and M. Nishikibe. 1994. Effects of a selective endothelin A-receptor antagonist, BQ-123, in salt-loaded stroke-prone spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol.* 22:763-768.

O'Keefe, S.T., N.P. Tsapatsaris, W.P. Beetham, and e. al. 1992. Increased prevalence of migraine and chest pain in patients with primary Raynaud's disease. *Ann Int Med.* 116:985-989.

Olins, G.M., P.A. Krieter, A.J. Trapani, K.L. Spear, and P.R. Bovy. 1989. Specific inhibitors of endopeptidase 24.11 inhibit the metabolism of atrial natriuretic peptides in vitro and in vivo. *Mol Cell Endocrinol.* 61:201-8.

Omland, T., R.T. Lie, A. Aakvaag, T. Aarsland, and K. Dickstein. 1994. Plasma endothelin determination as a prognostic indicator of 1-year mortality after acute myocardial infarction. *Circulation.* 89:1573-1579.

Opgenorth, T.J., J.R. Wu-Wong, and K. Shiosaki. 1992. Endothelin-converting enzymes. *FASEB J.* 6:2653-2659.

O'Reilly, G., D.S. Charnock-Jones, A.P. Davenport, I.T. Cameron, and K.S. Smith. 1992. Presence of messenger ribonucleic acid for endothelin-1, endothelin-2 and endothelin-3 in human endometrium and a change in the ratio of ET_A and ET_B receptor subtype across the menstrual cycle. *J Clin Endocrinol Metab.* 75:1545-1549.

O'Reilly, G., D.S. Charnock-Jones, J.J. Morrison, I.T. Cameron, A.P. Davenport, and K.S. Smith. 1993. Alternatively spliced mRNAs for human endothelin-2 and their tissue distribution. *Biochem Biophys Res Commun.* 193:834-840.

Ortiz MC, Sanabria E, Manriquez MC, Romero JC, Juncos LA. Role of endothelin and isoprostanes in slow pressor response to angiotensin II. Hypertension 2001;37:505-10.

Overbeck, H.W., and G.J. Grega. 1970. Response of the limb vascular bed in man to intrabrachial arterial infusions of hyperosmolar dextrose or hyperosmolar sodium chloride solutions. *Circ Res.* 26:717-731.

Pacher, R., J. Bergler-Klein, S. Globits, H. Teufelsbauer, M. Schuffer, A. Krauter, E. Ogris, S. Rodler, M. Nutte, and E. Hartter. 1993. Plasma big endothelin-1 concentrations in congestive heart failure patients with or without systemic hypertension. *Am J Cardiol.* 71:1293-1299.

Packer M, Caspi A, Charlon V, Cohen-Solal A, Kiowski W, Kostuk W, Krum H, Levine B, Massie B, McMurray J, Rizzon P, Swedberg K. Multicenter, double-blind, placebo controlled study of long-term endothelin blockade with bosentan in chronic heart failure - results of the REACH-1 trial (abstract). *Circulation* 1998;**98**;Suppl 1:3

Palmer, R.M., D.S. Ashton, and S. Moncada. 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature.* 333:664-666.

Palmer, R.M., D.S. Aston, and S. Moncada. 1987. Nitric oxide accounts for the biological activity of endothelium-derived relaxing factor. *Nature.* 327:524-526.

Panza, J.A., P.R. Casino, C.M. Kilcoyne, and A.A. Quyyumi. 1994. Impaired endothelium-dependent vasodilatation in patients with essential hypertension: evidence that the abnormality is not at the muscarinic receptor level. *J Am Coll Cardiol.* 23:1610-6.

Panza, J.A., P.R. Casino, C.M. Kilcoyne, and A.A. Quyyumi. 1993. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation.* 87:1468-74.

Panza, J.A., C.E. Garcia, C.M. Kilcoyne, A.A. Quyyumi, and R.O. Cannon. 1995. Impaired endothelium-dependent vasodilation in patients with essential hypertension. Evidence that nitric oxide abnormality is not localised to a single pathway. *Circulation.* 91:1732-1738.

Panza, J.L., A.A. Quyyumi, J.E. Brush jr, and S.E. Epstein. 1990. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med.* 323:22-27.

Patterson, G.C., and J.T. Shepherd. 1954. The blood flow in the human forearm following venous congestion. *J Physiol (Lond).* 125:501-507.

Peart, W.S. 1980. Concepts in hypertension: the Croonian Lecture. *J Coll Physicians Lond.* 14:141-152.

Perico, N., J. Dadan, M. Cabanelli, and G. Remuzzi. 1990. Cyclooxygenase products and atrial natriuretic peptide modulate renal response to endothelin. *J Pharmacol Exp Ther.* 252:1213-1220.

Pernow, J., L. Kaisjer, J. Lundberg, and G. Ahlborg. 1996. Comparable potent coronary constrictor effects of endothelin-1 and big endothelin-1 in humans. *Circulation.* 94:2077-2082.

Plumpton, C., C.J. Ferro, W.G. Haynes, D.J. Webb, and A.D. Davenport. 1996. The increase in 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.* 119:311-314.

Plumpton, C., W.G. Haynes, D.J. Webb, and A.P. Davenport. 1995. Phosphoramidon inhibition of the in vivo conversion of big endothelin-1 to endothelin-1 in the human forearm. *Br J Pharmacol.* 116:1821-1828.

Pollock, D.M., and T.J. Opgenorth. 1994. ET_A receptor-mediated responses to endothelin-1 and big endothelin-1 in the rat kidney. *Br J Pharmacol.* 111:729-732.

Pousset F, Isnard R, Lechat P, Kalotka H, Carayon A, Maistre G, Escolano S, Thomas D, Komajda M. Prognostic valve of plasma endothelin-1 in patients with chronic heart failure. *European Heart Journal* 1997;**18**:254-8

Predel, H.G., H. Meyer-Lehnert, A. Backer, H. Stelkens, and H.J. Kramer. 1990. Plasma concentrations of endothelin in patients with abnormal vascular reactivity. *Life Sci.* 47:1837-1843.

Prinzmetal, M., R. Kennamer, R. Merlis, and e. al. 1959. A variant form of angina pectoris. *Am J Med.* 27:375-388.

Quyumi, A.A., R.O. Cannon, J.A. Panza, and e. al. 1989. Endothelial dysfunction in patients with chest pain and normal coronary arteries. *Circulation.* 86:1864-1871.

Rabelink, T.J., K.A.H. Kaassjager, P. Boer, E.G. Stroes, B. Braam, and H.A. Koomans. 1994. Effects of endothelin-1 on renal function in humans: implications for physiology and pathophysiology. *Kidney Int.* 46:376-378.

Radomski, M.W., R.M.J. Palmer, and S. Moncada. 1990. An L-arginine/nitric oxide pathway present in human platelets regulayes aggregation. *Proc Natl Acad Sci USA.* 87:5193-5197.

Rakugi, H., Y. Tabuchi, M. Nakamura, M. Nagano, K. Higashimoroi, H. Mikami, T. Ogihara, and N. Suzuki. 1990. Endothelin activates the vascular renin-angiotensin system in rat mesenteric arteries. *Biochem Int.* 21:867-872.

Rasmussen, B.K., and M.D. Olesen. 1994. Epidemiology of migraine and tension-type headache. *Curr Op Neurol.* 7:264-271.

Ray, S.G., J.J. McMurray, J.J. Morton, and H.J. Dargie. 1992. Circulating endothelin in acute ischaemic syndromes. *Br Heart J.* 67:383-386.

Raynaud, M. 1988. On local asphyxia and symmetrical gangrene of the extremities, 1862, and New research on the nature and treatment of local asphyxia of the extremities, 1872. *In* Selected monographs. Vol. 121. T. Barlow, editor. New Sydenham Society, London.

Rees, D.D., R.M.J. Palmer, H.F. Hodson, and S. Moncada. 1989. A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br J Pharmacol.* 96:418-424.

Rees, D.D., R.M.J. Palmer, and S. Moncada. 1989. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA.* 86:3375-3378.

Richards, A.M., I.G. Crozier, E.A. Espiner, H. Ikram, T.G. Yandle, T. Kosoglou, M. Rallings, and C. Frampton. 1992. Acute inhibition of endopeptidase 24.11 in essential hypertension: SCH 34826 enhances atrial natriuretic peptide and natriuresis without lowering blood pressure. *J Cardiovasc Pharmacol.* 20:735-741.

Richards, A.M., I.G. Crozier, E.A. Espiner, T.G. Yandle, and M.G. Nicholls. 1993. Plasma brain natriuretic peptide and endopeptidase 24.11 inhibition in essential hypertension. *Hypertension.* 22:231-236.

Richards, A.M., G. Tonolo, G.D. McIntyre, B.J. Leckie, and J.I.S. Robertson. 1987. Radioimmunoassay for plasma alpha human natriuretic peptide: a comparison of direct and pre-extracted methods. *J Hypertens.* 5:227-236.

Richards, A.M., G. Wittert, E.A. Espiner, T.G. Yandle, C. Frampton, and H. Ikram. 1991. Prolonged inhibition of endopeptidase EC 24.11 in normal man; renal, endocrine and haemodynamic effects. *J Hypertens.* 9:955-62.

Richards, M., E. Espiner, C. Frampton, H. Ikram, T. Yandle, M. Sopwith, and N. Cussans. 1990. Inhibition of endopeptidase 24.11 in humans; renal and endocrine effects. *Hypertension.* 16:269-76.

Riggleman A, Harvey J, Baylis C. Endothelin mediates some of the renal actions of acutely administered angiotensin II. Hypertension 2001;38:105-9.

Roberts, D.H., Y. Tsao, and A.M. Breckenbridge. 1986. The reproducibility of limb flow measurements in human volunteers at rest and after exercise by using mercury-in-silastic strain gauge plethysmography under standardised conditions. *Clin Sci.* 70:635-638.

Roberts-Thomson, P., R. McRitchie, and R. Chalmers. 1994. Experimental hypertension produces diverse changes in the regional vascular responses to endothelin-1 in the rabbit and the rat. *J Hypertens.* 12:1225-1234.

Robinson, B.F. 1990. Assessment of responses to drugs in forearm resistance vessels and hand veins of man: techniques and problems. *In* Dose-response relationships of drugs. J. Kuhlmann, and W.W. Wingender, editors. Zuckschwerdt, München. 40-43.

Robinson, B.F., J.G. Collier, and C. Nachev. 1972. Changes in peripheral venous compliance after myocardial infarction. *Cardiovasc Res.* 6:67-74.

Rolinski, B., I. Sadri, J. Bogner, and F.-D. Goebel. 1994. Determination of endothelin-1 immunoreactivity in plasma, cerebrospinal fluid and urine. *Res Exp Med.* 194:9-24.

Rosner, B. 1995. Multisample inference: linear contrasts. *In* Fundamentals of biostatistics. B. Rosner, editor. Duxbury Press, Boston. 310-314.

Roubert, P., V. Gillard, P. Plas, and e. al. 1989. Angiotensin II and phorbol-esters potently down-regulate endothelin (ET-1) binding sites in vascular smooth muscle cells. *Biochem Biophys Res Commun.* 164:809-815.

Roubert, P., V. Gillard, P. Plas, P.E. Chabrier, and P. Braquet. 1991. Binding characteristics of endothelin isoforms (ET-1, ET-2 and ET-3) in vascular smooth muscle cells. *J Cardiovasc Pharmacol.* 17 (Suppl. 5):S104-S108.

Roubert, P., V. Gillard, P. Plas, P.E. Chabrier, and P. Braquet. 1990. Down-regulation of endothelin-1 binding sites in rat vascular smooth muscle cells. *Am J Hypertens.* 3:310-312.

Rubanyi, G.M., and M.A. Polokoff. 1994. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev.* 46:325-415.

Rubin LJ, Badesch DB, Barst RJ et al. 2002. Bosentan therapy for pulmonary arterial hypertension. N Engl J Med.;346:896-903.

Saito, Y., N. Kazuwa, G. Shirakami, M. Mukoyama, H. Arai, K. Hosoda, S. Suga, Y. Ogawa, and H. Imura. 1991. Endothelin in patients with chronic renal failure. *J Cardiovasc Pharmacol.* 17 (Suppl. 7):S437-S439.

Saito, Y., K. Nakao, M. Mukoyama, and H. Imura. 1990. Increased plasma endothelin levels in patients with essential hypertension. *N Engl J Med.* 322:205.

Sakamoto, A., M. Yanagisawa, T. Sakurai, Y. Takuwa, H. Yanagisawa, and T. Masaki. 1991. Cloning and functional expression of human cDNA for the ET_B endothelin receptor. *Biochem Biophys Res Commun.* 178:656-663.

Sakamoto, H., S. Sasaki, Y. Hirata, T. Imai, K. Ando, T. Sukurai, M. Yanagisawa, T. Masaki, and F. Marumo. 1990. Production of endothelin-1 by cultured mesangial cells. *Biochem Biophys Res Commun.* 169:462-468.

Sakurai, T., H. Morimoto, Y. Kasuya, Y. Takuwa, H. Nakauchi, T. Masaki, and T. Goto. 1992. Level of ET_B receptor mRNA is down-regulated by endothelins through decreasing the intracellular stability of mRNA molecules. *Biochem Biophys Res Commun.* 186:342-347.

Sakurai, T., M. Yanagisawa, Y. Takuya, H. Miyasaki, S. Kimura, K. Goto, and T. Masaki. 1990. Cloning of a cDNA encoding a non isopeptide selective subtype of the endothelin receptor. *Nature.* 348:732-735.

Saladin, V., C. Zussa, G. Risica, P. Michielon, A. Paccagnella, G. Cipolotti, and G. Simini. 1988. Comparison of cardiac output estimation by thoracic electrical bioimpedance and thermodilution. *Crit Care med.* 16:1157-1158.

Samson, W.K., K.D. Skala, B.D. Alexander, and F.S. Huang. 1990. Pituitary site of action of endothelin: selective inhibition of prolactin release *in vitro. Biochem Biophys Res Commun.* 169:737-743.

Sandoval, J., O. Bauerle, A. Gomez, A. Palomar, M. Guerra, and M. Furuya. 1995. Primary pulmonary hypertension in children: clinical characterization and survival. *JACC.* 25:466-474.

Sawamura, T., S. Kimura, O. Shinmi, Y. Sugita, M. Yanagisawa, K. Goto, and T. Masaki. 1990. Purification and characterization of putative endothelin-converting enzyme in bovine adrenal medulla: evidence for cathepsin D like enzyme. *Biochem Biophys Res Commun.* 168:1230-1236.

Scarani, P., G.P. Salvioli, and V. Eusebi. 1994. Marcello Malpighi (1628-1694): A founding father of modern anatomic pathology. *Am J Surg Path.* 18:741-746.

Schiebinger, R.J., and C.E. Gomez-Sanchez. 1990. Endothelin: a potent stimulus of atrial natriuretic peptide secretion by superfused rat atria and its dependency on calcium. *Endocrinology.* 127:119-125.

Schiffrin, E.L. 1995. Endothelin: potential role in hypertension and vascular hypertrophy. *Hypertension.* 25:1135-1143.

Schiffrin, E.L., L.Y. Deng, and P. Larochelle. 1992. Blunted effects of endothelin upon small subcutaneous resistance arteries of mild essential hypertensive patients. *J Hypertens.* 10:437-444.

Schiffrin, E.L., L.Y. Deng, P. Sventek, and R. Day. 1997. Enhanced expression of endothelin-1 gene in resistance arteries in severe human essential hypertension. *J Hypertens.* 15:57-63.

Schiffrin, E.L., and A.G. Thibault. 1991. Plasma endothelin in human essential hypertension. *Am J Hypertens.* 4:303-308.

Schini, V.B., H. Hendrickson, D.M. Heublein, J.C. Burnett, and P.M. Vanhoutte. 1989. Thrombin enhances the release of endothelin from porcine aortic cells. *Eur J Pharmacol.* 165:333-334.

Schmetterer, L., Dallinger, S., Bobr, B., Selenko, N., Eichler, H.G. & Wolzt, M. 1998. Systemic and renal effects of an ET-A receptor subtype-specific antagonist in healthy subjects. *Br J Pharmacol,* 124, 930-934.

Schmidt, H.H.H.W., B. Zernikow, S. Baeblich, and E. Böhme. 1990. Basal and stimulated formation and release of L-arginine-derived nitrogen oxides from cultured endothelial cells. *J Pharmacol Exp Ther.* 254:591-597.

Schmidt, M., B. Kroger, E. Jacob, H. Seulberger, T. Subkowski, R. Otter, T. Meyer, G. Schmalzing, and H. Hillen. 1994. Molecular characterization of human and bovine endothelin converting enzyme (ECE-1). *FEBS Lett.* 356:238-243.

Scott-Burden T, Resink TJ, Hahn AW, Vanhoutte PM. Induction of endothelin secretion by angiotensin II: effects on growth and synthetic activity of vascular smooth muscle cells. *J Cardiovasc Pharmacol* 1991;17 Suppl 7:S96-100.

Seidelin, P.H., J.G. Collier, A.D. Struthers, and D.J. Webb. 1991. Angiotensin II augments sympathetically mediated arteriolar constriction in man. *Clin Sci.* 81:261-266.

Seiffert, V., B.M. Loffler, M. Zimmermann, S. Roux, and D. Stolke. 1995. Endothelin concentrations in patients with aneurysmal subarachnoid haemorrhage. *J Neurosurgery.* 82:55-62.

Serneri, G.G.N., P. Modesti, I. Cecioni, D. Biagini, A. Migliorini, A. Costoli, A. Colella, A. Naldoni, and P. Paoletti. 1995. Plasma endothelin and renal endothelin are two distinct systems involved in volume homeostasis. *Am J Physiol.* 268:H1829-H1837.

Shetty, S.S., T. Okada, R.L. Webb, D. Del Grande, and R.W. Lappe. 1993. Functionally distinct endothelin ET_B receptors in vascular smooth muscle and endothelium. *Biochem Biophys Res Commun.* 191:459-464.

Shibouta, Y., N. Suzuki, A. Shino, H. Matsumoto, Z. Terashita, K. Kondo, and K. Nishikawa. 1990. Pathophysiological role of endothelin in acute renal failure. *Life Sci.* 46:1611-1618.

Shichiri, M., Y. Hirata, K. Ando, T. Emori, K. Ohta, S. Kimoto, M. Ogura, A. Inoue, and F. Marumo. 1990. Plasma endothelin levels in hypertension and chronic renal failure. *Hypertension.* 15:493-496.

Shimada, K., Y. Matsushita, K. Wakabayashi, M. Takahashi, A. Matsubara, Y. Iijima, and K. Tanzawa. 1995. Cloning and functional expression of endothelin-converting enzyme cDNA. *Biochem Biopys Res Commun.* 207:807-812.

Singer, D.R.J., N.D. Markandu, M.G. Buckley, M.A. Miller, G.A. Sagnella, and G.A. MacGregor. 1991. Dietary sodium and inhibition of neutral endopeptidase 24.11 in essential hypertension. *Hypertension.* 18:798-804.

Skidgel, R.A., S. Engelbrecht, A.R. Johnson, and E.G. Erdös. 1984. Hydrolysis of substance P and neurotensin by converting enzyme and neutral endopeptidase. *Peptides.* 5:769-76.

Skvorak, J.P., S.J. Nazian, and J.R. Dietz. 1995. Endothelin acts as a paracrine regulator of stretch-induced atrial peptide release. *Am J Physiol.* 269:R1093-R1098.

Smith, P.J., C.J. Ferro, D.S. McQueen, and D.J. Webb. 1998. Functional studies in small arteries do not support a primary role for endothelin in the pathogenesis of Raynaud's disease. *J Cardiovasc Pharmacol.* 31 (Suppl. 1):S473-S476.

Sokolovsky, M. 1992. Structure-function relationships of endothelins, sarafotoxins, and their receptor subtypes. *J Neurochem.* 59:809-21.

The SOLVD investigators. 1992. Effect of enalapril on mortality and the development of heart failure in asymptomatic patients with reduced left ventricular ejection fractions. *New Engl J Med*;327:685-91.

The SOLVD investigators. 1991. Effects of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. *New Engl J Med*;325:293-302.

Sorensen, S.S., J.K. Madsen, and E.B. Pedersen. 1994. Systemic and renal effect of intravenous infusion of endothelin-1 in healthy human volunteers. *Am J Physiol.* 266:F411-F418.

Schalcher et al. 2000. Dose dependency of haemodynamc changes to the endothelin receptor antagonist tezosentan in patients with heart failure *Eur Heart J* 21 (suppl) 116

Schmetterer L, Dallinger S, Bobr B, Selenko N, Eichler HG and Wolzt M. 1998Systemic and renal effects of an ET_A receptor subtype-specific antagonist in healthy subjects. *Br J Pharmacol*;124:930-4

Spieker LE et al Acute haemodynamic and neurohumoral efects of selective ET_A receptor blockade in patients with congestive heart failure. 2000. ET003 Investigators. *J Am Coll Cardiol*;35:1745-52

Spinale, F.G., H.D. Reines, and F.A. Crawford Jr. 1988. Comparison of bioimpedance and thermodilutiion, methods for determining cardiac output: experimental and clinical studies. *Ann Thorac Surg.* 45:421-425.

Spinella, M.J., M.A. Malik, J. Everitt, and T.T. Anderson. 1991. Design and synthesis of a specific endothelin-1 antagonist: effect on pulmonary vasoconstriction. *Proc Natl Acad Sci USA.* 88:7443-7446.

Spratt JCS, Goddard J, Patel N, Strachan FE, Rankin AJ, Webb DJ. 2001. Systemic ET_A receptor antagonism with BQ-123 blocks ET-1 induced forearm vasoconstriction and decreases peipheral vascular resistance in healthy men. *Br J Pharmacol*;134:648-54.

Stein, P.D., J.T. Hunt, D.M. Floyd, S. Moreland, K. Dickinson, C. Mitchell, E. Liu, M.L. Webb, N. Murugesan, J. Dickey, D. McMullen, R. Zhang, V.G. Lee, R. Serafino, C. Delaney, T.R. Schaeffer, and M. Kozlowski. 1994. The discovery of sulfonamide endothelin antagonists and the development of the orally active ET_A antagonist 5-(dimethylamino-)-N-(3,4-dimethyl-5-isoxazoyl)-1-naphthalenesulfonamide. *J Med Chem.* 37:329-331.

Steiner, D.F., S.P. Smeekens, S. Ohagi, and S.J. Chan. 1992. The new enzymology of precursor processing endoproteases. *J Biol Chem.* 267:23435-23438.

Stelzner, T.J., R.F. O'Brien, M. Yanagisawa, T. Sakurai, K. Sato, S. Webb, M. Zamora, I.F. McMurtry, and J.H. Fisher. 1992. Increased lung endothelin-1 production in rats with idiopathic pulmonary hypertension. *Am J Physiol.* 262:L614-L620.

Stephens, N.G., A. Parsons, P.M. Schofield, F. Kelly, K. Cheeseman, M.J. Mitchison, and M.J. Brown. 1996. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet.* 347:781-786.

Stevens, P.A., and M.J. Brown. 1995. Genetic variability of the ET-1 and the ET_A receptor genes in essential hypertension. *J Cardiovasc Pharmacol.* 26 (Suppl. 3):S9-S12.

Stewart, D. 1993. Endothelin in cardiopulmonary disease: factor paracrine vs neurohumoral. *European Heart Journal.* 14 (Supplement 1):48-54.

Stewart, D.J., R.D. Levy, P. Cernacek, and D. Langleben. 1991. Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease? *Ann Int Med.* 114:464-469.

Stingo, A.J., A.L. Clavell, L.L. Aarhus, and J.C. Burnett. 1993. Biological role for the endothelin-A receptor in aortic cross-clamping. *Hypertension.* 22:62-66.

Stockenhuber, F., M. Gauttsauner-Wolf, L. Marosi, B. Liegish, R.W. Kurz, and P. Balcke. 1992. Plasma levels of endothelin in chronic renal failure and after renal transplantation. *Clin Sci.* 82:255-258.

Struthers, A.D. 1994. Ten years of natriuretic peptide research: a new dawn for their diagnostic and therapeutic use? *Br Med J.* 308:1615-9.

Sumner, M.J., T.R. Cannon, J.W. Mundin, D.G. White, and I.S. Watts. 1992. Endothelin ET_A and ET_B receptors mediate vascular smooth muscle constriciton. *Br J Pharmacol.* 107:858-860.

Sung CP, Arleth AJ, Storer BL, Ohlstein EH. Angiotensin type 1 receptors mediate smooth muscle proliferation and endothelin biosynthesis in rat vascular smooth muscle. J Pharmacol Therap 1994;271:429-37.

Sutsch G, Kiowski W, Yan XW, Hunziker P, Christen S, Strobel W, Kim JH, Rickenbacher P, Bertel O. Short-term oral endothelin-receptor antagonist therapy in conventially treated patients with symptomatic severe chronic heart failure. *Circulation* 1998;**98**:2262-8

Sutsch G, Bertel O, Kiowski W. Acute and short-term effects of the nonpeptide endothelin-1 receptor antagonist bosentan in humans. *Cardiovasc Drugs Therap* 1997;**10**:171-725

Suzuki, H., H. Masaoka, Y. Hirata, F. Marumo, E. Isotani, and K. Hirakawa. 1992. The role of endothelin-1 in the origin of cerebral vasospasm in patients with aneurysmal subarachnoid haemorrhage. *J Neurosurg.* 77:96-100.

Swedberg, K., P. Eneroth, and J. Kjekshus. 1990. Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality. *Circulation.* 82:1730-1736.

Sybertz, E.J., P.J.S. Chiu, S. Vemulapalli, R. Watkins, and M.F. Haslanger. 1990. Atrial natriuretic factor: potentiating and antihypertensive activity of SCH 34826. *Hypertension.* 15:152-161.

Tabuchi, Y., M. Nakamura, H. Rakugi, M. Nagano, and T. Ogihara. 1989. Endothelin enhances adrenergic vasoconstriction in perfused rat mesenteric arteries. *Biochem Biophys Res Commun.* 159:1304-1308.

Taddei, S., P. Mattei, A. Virdis, I. Sudano, L. Ghiadoni, and A. Salvetti. 1994. Effect of potassium on vasodilatation to acetylcholine in essential hypertension. *Hypertension.* 23:485-490.

Taddei, S., P. Mattei, A. Virdis, I. Sudano, L. Ghiadoni, and A. Salvetti. 1993. Forearm vasodilatation in response to acetylcholine is increased by potassium in essential hypertensive patients. *J Hypertens.* 11 (Suppl. 5):S144-S145.

Taddei, S., A. Virdis, P. Mattei, A. Natali, E. Ferrannini, and A. Salvetti. 1995. Effect of insulin on acetylcholine-induced vasodilatation in normotensive subjects and patients with essential hypertension. *Circulation.* 92:2911-8.

Tahara, A., M. Kohno, S. Yanagi, H. Itagane, I. Toda, I. Akioka, M. Teragaki, M. Yasuda, K. Takeuchi, and T. Takeda. 1991. Circulating immunoreactive endothelin in patients undergoing percutaneous transluminal coronary angioplasty. *Metabolism.* 40:1235-1237.

Takahashi, K., M. Sone, K. Totsune, F. Satoh, O. Murakami, M. Ohneda, and T. Mouri. 1995. Immunoreactive endothelin in the human kidney; comparison with natriuretic peptides. *J Cardiovasc Pharmacol.* 26(Suppl. 3):S510-S512.

Takahashi, M., Y. Matsushita, Y. Ijima, and K. Tanzawa. 1993. Purification and characterization of endothelin-converting enzyme from rat lung. *J Biol Chem.* 268:21394-21398.

Takaoka, M., Y. Takenobu, Y. Miyata, R. Ikegawa, Y. Matsumura, and S. Morimoto. 1990. Pepsin, an aspartic protease, converts porcine big endothelin to 21-residue endothelin. *Biochem Biophys Res Commun.* 166:436-442.

Takasaki, C., M. Yanagisawa, S. Kimura, K. Goto, and T. Masaki. 1988. Similarity of endothelin to snake venom toxin. *Nature.* 335:303.

Takase, H., P. Moreau, and T.F. Lüscher. 1995. Endothelin receptor subtypes in small arteries: studies with FR139317 and bosentan. *Hypertension.* 25:739-743.

Takeda, M., T. Komeyama, T. Tsutsi, T. Mizusawa, Y. Katayama, H. Go, M. Tamaki, and A. Hatano. 1994. Changes in urinary excretion of endothelin-1-like immunoreactivity before and after unilateral nephrectomy in humans. *Nephron.* 67:180-184.

Teerlink, J.R., V. Breu, M. Clozel, and J.P. Clozel. 1994. Potent vasoconstriction mediated by endothelin ET_B receptors in canine coronary arteries. *Circulation.* 74:105-114.

Telemaque, S., J.P. Gratton, A. Claing, and P. D'Orleans-Juste. 1993. Endothelin induces vasoconstriction and prostacyclin release via the activation of endothelin ET_A receptors in the perfused rabbit kidney. *Eur J Pharmacol.* 237:275-281.

Tesfamariam, B., and R.A. Cohen. 1992. Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am J Physiol.* 263:H321-326.

The GISEN Group (Gruppo Italiano di Studi Epidemiologici in Nefrologia). 1997. Randomised placebo-controlled trial of the effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy. *Lancet.* 349:1857-1863.

The Task Force on Heart Failure of the European Society of Cardiology. 1995. Guidelines for the diagnosis of heart failure. *Eur Heart J.* 16:741-751.

Thomas, S. 1992. Impedance cardiography using using the Sramek-Bernstein method: accuracy and reproducability at rest and during exercise. *Br J Clin Pharmacol.* 34:467-476.

Tomita, K., K. Ujhe, T. Nakanishi, S. Tomura, O. Matsuda, K. Ando, M. Shichiri, Y. Hirata, and F. Marumo. 1989. Plasma endothelin levels in patients with acute renal failure. *New Engl J Med.* 321:1127.

Torre-Amione G, Young JB, Durand J, et al. 2001. Hemodynamic effects of tezosentan, an intravenous dual endothelin receptor antagonist, in patients with class III to IV congestive heart failure. *Circulation*;103(7):973-80.

Treasure, C.B., J.L. Klein, J.A. Vita, S.V. Manoukian, G.H. Renwick, A.P. Selwyn, P. Ganz, and R.W. Alexander. 1993. Hypertension and left ventricular hypertrophy are associated with impaired endothelium-dependent relaxation in human coronary resistance vessels. *Circulation.* 7:86-93.

Treasure, C.B., S.V. Manoukian, J.L. Klein, J.A. Vita, E.G. Nabel, G.H. Renwick, A.P. Selwyn, R.W. Alexander, and P. Ganz. 1992. Epicardial coronary artery responses to acetylcholine are impaired in hypertensive patients. *Circ Res.* 71:776-781.

Tschudi, M.R., L. Cuiscione, and T.F. Lüscher. 1991. Effect of aging and hypertension on endothelial function in rat coronary arteries. *J Hypertens.* 9 (suppl. 6):S164-S165.

Tschudi, M.R., and T.F. Lüscher. 1995. Age and hypertension differently affect coronary contractions to endothelin-1, serotonin, and angiotensins. *Circulation.* 91:2415-2422.

Tschudi, M.R., and T.F. Lüscher. 1994. Characterization of contractile endothelin and angiotensin receptors in human resistance arteries: evidence for two endothelin and one angiotensin receptor. *Biochem Biophys Res Commun.* 204:685-690.

Tunny, T.J., M.D. Ziesak, R. Armstrong, S.A. Klemm, M. Stowasser, W.L. Finn, and R.D. Gordon. 1993. Inhibition of endopeptidase EC 3.4.24.11 by candoxatril lowered blood pressure and increased urinary but not plasma atrial natriuretic peptide in essential hypertension. *J Hypertens.* 11 (Suppl. 5):S222-3.

Turner, A.J., and L.J. Murphy. 1995. Molecular pharmacology of endothelin converting enzymes. *Biochem Pharmacol.* 51:91-102.

d'Uscio LV, Moreau P, Shaw S, Takase H, Barton M, Luscher TF. Effects of chronic ET_A-receptor blockade in angiotensin II-induced hypertension. Hypertension 1997;29:435-41.

Valdenaire, O., E. Rohrbacher, and M.G. Mattei. 1995. Organisation of the gene encoding the human endothelin-converting enzyme. *J Biol Chem.* 270:29794-29798.

Vallance, P., and J. Collier. 1994. Biology and clinical relevance of nitric oxide. *Br Med J.* 309:453-457.

Vallance, P., J. Collier, and S. Moncada. 1989. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet.* ii:998-1000.

Vallance, P., and S. Moncada. 1994. Nitric oxide: from mediator to medicines. *J Royal Coll Physicians London.* 28:209-219.

Vanhoutte, P.M. 1989. Endothelium and control of cardiovascular function. *Hypertension.* 13:658-667.

Vanhoutte, P.M. 1987. Vascular physiology: the end of the quest? *Nature.* 327:459-460.

Velasco, C., M. Turner, T. Inagami, J. Atkinson, R. Virmani, E. Jackson, J. Murray, and M. Forman. 1994. Reperfusion enhances the local release of endothelin after regional myocardial ischaemia. *Am Heart J.* 128:441-451.

Veniant, M., J.P. Clozel, P. Hess, and M. Clozel. 1994. Endothelin plays a role in the maintenance of blood pressure in normotensive guinea pigs. *Life Sci.* 55:445-454.

Verhaar, M.C., F.E. Strachan, D.E. Newby, N.L. Cruden, H.A. Koomans, T.J. Rabelink, and D.J. Webb. 1998. Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation.* 97:752-756.

Vierhapper, H. O., Wagner, O.F., Nowotny, P., Walhausal, W. 1992. Effect of endothelin-1 in man: pre-treatment with nifedipine, with indomethacin and with cyclosporine. A. Eur. J. Clin. Invest. 22:55-59.

Wagner, O.F., G. Christ, J. Wojta, H. Vierhapper, S. Parzer, P.J. Nowotny, B. Schneider, W. Waldhausl, and B. Binder. 1992. Polar secretion of endothelin-1 by cultured endothelial cells. *J Biol Chem.* 267:16066-16068.

Wang, X., S.A. Douglas, C. Louden, L.M. Vickery-Clark, G.Z. Feuerstein, and E.H. Ohlstein. 1996. Expression of endothelin-1, endothelin-3, endothelin-converting enzyme-1, and endothelin-A and endothelin-B receptor mRNA after angioplasty-induced neointimal formation in the rat. *Circ Res.* 78:322-328.

Warner, T.D., G.H. Allcock, R. Corder, and J.R. Vane. 1993. Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br J Pharmacol.* 110:777-782.

Warner, T.D., G.H. Allcock, and J.R. Vane. 1994. Reversal of established responses to endothelin-1 *in vivo* and *in vitro* by the endothelin receptor antagonists, BQ-123 and PD 145065. *Br J Pharmacol.* 112:207-213.

Warner, T.D., B. Battistini, A.M. Doherty, and R. Corder. 1994. Endothelin receptor antagonists: actions and rationale for their development. *Biochem Pharmacol.* 48:625-635.

Warrens, A.N., M.J.D. Cassidy, K. Takahashi, M.A. Ghatei, and S.R. Bloom. 1990. Endothelin in renal failure. *Nephrol Dial Transplant.* 5:418-422.

Watanabe, T., Y. Awane, S. Ikeda, S. Fujiwara, K. Kubo, T. Kikuchi, K. Kusumoto, M. Wakimasu, and M. Fujino. 1995. Pharmacology of a non-selective ET_A and ET_B receptor antagonist, TAK-044 and the inhibition of myocardial infarct size in rats. *Br J Pharmacol.* 114:949-954.

Watanabe, Y., M. Naruse, C. Monzen, K. Naruse, K. Ohsumi, J. Horiuchi, I. Yoshihara, H. Kato, N. Sugino, and H. Demura. 1991. Is big endothelin converted to endothelin-1 in circulating blood? *J Cardiovasc Pharmacol.* 17 (Suppl. 7):S503-S505.

Webb, D.J. 1997. Endothelin: from molecule to man. *Br J Clin Pharmacol.* 44:9-20.

Webb, D.J. 1995. The pharmacology of human blood vessels in vivo. *J Vasc Res.* 32:2-15.

Webb, D.J., N. Benjamin, M.J. Allen, J. Brown, M. O'Flynn, and J.R. Cockcroft. 1988. Vascular responses to local atrial natriuretic peptide infusion in man. *Br J Clin Pharmacol.* 26:245-251.

Webb, D.J., N. Benjamin, and P. Vallance. 1989. The potassium channel opening drug cromakalin produces arterioselective vasodilatation in the upper limbs of normal volunteers. *Clin Sci.* 75:35-44.

Webb, D.J., and J.R. Cockcroft. 1989. Plasma immunoreactive endothelin in uraemia. *Lancet.* i:1211.

Webb, D.J., P.J.O. Manhem, S.G. Ball, G. Inghlis, B.J. Leckie, A.F. Lever, J.J. Morton, J.I.A.S. Robertson, G.D. Murray, J. Menard, A. Hallett, D.M. Jones, and M. Szelke. 1985. A study of the renin inhibitor H142 in man. *J Hypertens.* 3:653-658.

Webb, D.J., J.C. Monge, T.J. Rabelink, and M. Yanagisawa. 1998. Endothelin: new discoveries and rapid progress in the clinic. *Trends Pharmacol Sci.* 19:5-8.

Webb ML, Dickinson KE, Delaney CL, Liu EC, Serafino R, Cohen RB, Monshizadegan H, Moreland S. 1992. The endothelin receptor antagonist, BQ-123, inhibits angiotensin II induced contractions in rabbit aorta. *Biochem Biophys Res Commun*;185:887-92.

Weber C, Schmitt R, Birnboeck H, Hopfgartner G, van Marle SP, Peeters PAM, Jonkman JHG, Jones CR. Pharmacokinetics and drug disposition: pharmacokinetics and pharmacodynamics of the endothelin-receptor antagonist bosentan in healthy human subjects. *Clin Pharmacol Ther* 1996;**60**:124-37

Wei, C., A. Lerman, R. Rodeheffer, C.G.A. McGregor, R.R. Brandt, S. Wright, D.M. Heublein, P.C. Kao, W.D. Edwards, and J.C. Burnett Jr. 1994. Endothelin in human congestive heart failure. *Circulation.* 89:1580-1586.

Weiner, C.P., I. Lizasoain, S.A. Baylis, R.G. Knowles, I.G. Charles, and S. Moncada. 1994. Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc Natl Acad Sci USA.* 91:5212-5216.

Weissler, A. 1977. Systolic time intervals. *N Engl J Med.* 296:321-324.

Weitzberg, E., G. Ahlborg, and J.M. Lundberg. 1991. Long-lasting vasoconstriction and efficient regional extraction of endothelin-1 in human splanchnic and renal tissues. *Biochem Biophys Res Commun.* 180:1298-1303.

Welch, K.M.A. 1994. Drug therapy of migraine. *New Engl J Med.* 329:1476-1483.

Wellings, R.P., R. Corder, T.D. Warner, J.-P. Cristol, C. Thiemerman, and J.R. Vance. 1994. Evidence from receptor antagonists of an important role for ET_B receptor mediated vasoconstrictor effects of endothelin-1 in the rat kidney. *Br J Pharmacol.* 111:729-732.

Wenzel RR, Fleisch M, Shaw S, Noll G, Kaufmann U, Schmitt R, Jones R, Clozel M, Meier B, Luscher TF. Hemodynamic and coronary effects of the endothelin antagonist bosantan in patients with coronary artery disease. *Circulation* 1998b;**98**:2235-40.

Whelan, R.F. 1967. Control of the peripheral circulation in man. Springfield, Thomas.

Whitney, R.J. 1953. The measurement of volume changes in human limbs. *Journal of Physiology.* 121:1-27.

Wieczorek, I., W. Haynes, D. Webb, C. Ludlam, and K. Fox. 1994. Raised plasma endothelin in unstable angina and non-Q wave myocardial infarction: relation to cardivascular outcome. *Br Heart J.* 72:436-441.

Wiinberg, N., S. Walter-Larson, C. Eriksen, and P.E. Nielsen. 1988. An evaluation of semi-automatic blood pressure manometers against intra-arterial blood pressure. *J Ambulatory Monitoring.* 1:303-309.

Williams, D.L., K.L. Jones, D.J. Pettibone, E.V. Lis, and B.V. Clineschmidt. 1991. Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochem Biophys Res Commun.* 175:556-561.

Winkles, J.A., G.F. Alberts, E. Brogi, and P. Libby. 1993. Endothelin-1 binding and endothelin immunoreactivity in normal and atherosclerotic human arteries. *Biochem Biophys Res Commun.* 191:1081-1088.

Wollersheim, H., and P.A. van Zweitten. 1993. Treatment of Raynaud's phenomenon. *European Heart Journal.* 14:147-149.

Wong, J., P.A. Vanderford, J.R. Fineman, R. Chang, and S.J. Soifer. 1993. Endothelin-1 produces pulmonary vasodilatation in the intact newborn lamb. *Am J Physiol.* 265:H318-H325.

Wong-Dusting, H.K., M. La, and M.J. Rand. 1990. Mechanisms of the effects of endothelin on responses to noradrenaline and sympathetic nerve stimulation. *Clin Exp Pharmacol Physiol.* 17:269-273.

Wood, P. 1958. Pulmonary hypertension with special reference to the vasoconstrictive factor. *Br Heart J.* 20:557-570.

Xu, D., N. Emoto, A. Giaid, C. Slaughter, S. Kaw, D. deWit, and M. Yanagisawa. 1994. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell.* 78:473-485.

Yamaura, I., E. Tani, Y. Maeda, N. Minami, and H. Shindo. 1992. Endothelin-1 of canine basilar artery in vasospasm. *J Neurosurg.* 76:99-105.

Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, and T. Masaki. 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 332:411-415.

, H., H. Tabuchi, Y. Furuichi, and C. Miyamoto. 1993. Molecular characterization of the 5'-flanking region of human genomic ET_A gene. *Biochem Biophys Res Commun.* 190:332-339.

Yang, Z., V. Richard, L. von Segresser, E. Bauer, P. Stulz, M. Turina, and T.F. Lüscher. 1990. Threshold concentrations of endothelin-1 potentiate contractions to norepinephrine and serotonin in human arteries: a new mechanism for vasospasm? *Circulation.* 82:188-195.

Yokokawa, K., M. Kohno, K. Yasunari, K. Murakawa, and T. Takeda. 1991. Endothelin-3 regulates endothelin-1 production in cultured human endothelial cells. *Hypertension.* 18:304-315.

Yorikane, R., T. Miyauchi, S. Sakai, T. Sakurai, I. Yamaguchi, Y. Sugushita, and K. Goto. 1993. Altered expression of ETB -receptor mRNA in the lung of rats with pulmonary hypertension. *J Cardiovasc Pharmacol.* 22 (Suppl. 8):S336-S338.

Yoshimoto, S., Y. Ishizaki, T. Sasaki, and e. al. 1991. Effect of carbon dioxide and oxygen on endothelin production by cultured porcine cerebral endothelial cells. *Stroke.* 22:378-383.

Yoshimura, A., S. Iwasaki, K. Inui, S. Ideura, S. Koshikawa, M. Yanagisawa, and T. Masaki. 1995. Endothelin-1 and endothelin B type receptor are induced in mesangial proliferative nephritis in the rat. *Kidney Int.* 48:1290-1297.

Young, R.-H., Y.-A. Ding, Y.-M. Lee, and M.-H. Yen. 1995. Cilazapril reverses endothelium-dependent vasodilator response to acetylcholine in mesenteric artery from spontaneously hypertensive rats. *Am J Hypertens.* 8:928-33.

Zamora, M.A., E.C. Dempsey, S.J. Walchak, and T.J. Stelzner. 1993. BQ-123, an ET_A receptor antagonist, inhibits endothelin-1 mediated proliferation of human pulmonary artery smooth muscle cells. *Am J Cell Mol Biol.* 9:429-433.

Zamora, M.R., R.F. O'Brien, R.B. Rutherford, and J.V. Weil. 1990. Serum endothelin-1 concentrations and cold provocation in primary Raynaud's phenomenon. *Lancet.* 336:1144-47.

Zeiher, A.M., H. Drexter, B. Saurbier, and H. Just. 1993. Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolaemia and hypertension. *J Clin Invest.* 92:652-662.

Zeiher, A.M., H. Goebel, V. Schachlinger, and C. Ihling. 1995. Tissue endothelin-1 immunoreactivity in the active coronary atherosclerotic plaque. *Circulation.* 91:941-947.

Zelis, R., D.T. Mason, and E. Braunwald. 1969. Partition of blood flow to the cutaneous and muscular beds of the forearm at rest and during leg exercise in normal subjects and in patients with heart failure. *Circ Res.* 24:799-806.

Ziv, I., G. Fleminger, R. Djaldetti, A. Achiron, E. Melamed, and M. Sokolowsky. 1992. Increased plasma endothelin-1 levels in acute ischaemic stroke. *Stroke.* 23:1014-101.

van Zweiten, P.A. 1994. Neurooendocrine effects of diuretics in heart failure. *Br Heart J.* 72 (Suppl.1):S51-S53