Regulation of vascular tone and renal function by endothelin in health and renal disease

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Abstract

Since its discovery in 1988 the powerful vasoconstrictor endothelin-1 (ET-1), has been widely implicated in the pathophysiology of renal and cardiovascular disease. ET antagonists have favourable effects in experimental models of these conditions and orally acting ET antagonists appear very promising in clinical trials of pulmonary and systemic hypertension. However, there is a paucity of human data regarding the role of ET-1 and its receptors both on renal function and in renal disease. In this thesis, using selective ET receptor antagonists, I have therefore explored the systemic and renal haemodynamic and renal tubular effects of ET-1, in a series of acute clinical studies.

After confirming, in a dose ranging study, that ETA receptor blockade produces vasodilatation in healthy subjects, I have shown, in patients with chronic renal failure (CRF), that selective ETA but not combined ETA/B receptor antagonism produces substantial reductions in blood pressure that are associated with renal vasodilatation, a reduction in EFF and a reduction in proteinuria, suggesting a renoprotective action. These data show that ET-1 plays a major role in regulating blood pressure and renal vascular resistance in CRF, consistent with activation of the ET system in this condition. By contrast, selective ETB receptor antagonism alone produced substantial systemic and renal vasoconstriction in both healthy subjects and CRF. Thus the effects of ETB receptor blockade, whether in the presence of ETA receptor blockade or not, suggest the net effect of ETB receptor activation on the circulation, in health and renal disease, is to produce vasodilatation. Clearance of ET-1 from the circulation is also reduced during ETB receptor blockade and studies of urinary ET-1
excretion under conditions of ET receptor blockade suggest that renal tubular excretion of ET-1 is at least partly mediated through the ETB receptor.

I have also explored the interaction between the ET and renin-angiotensin systems, demonstrating that, while ETA receptor antagonism does not alter the pressor effect of angiotensin II, prior ACE inhibition will enhance the effect of ETA receptor blockade in healthy subjects to produce a picture similar to that seen in CRF, an action mediated by nitric oxide, and abolished by concomitant ETB receptor blockade.

In summary, these studies confirm a role for ET-1 in the pathophysiology of systemic and renal vasoconstriction in CRF and suggest that ETA, rather than combined ETA/B receptor antagonism is potentially the most useful stratagem for ET system blockade in the treatment of CRF, particularly in combination with ACE inhibitors. Chronic studies are now needed in patients with CRF to confirm this potential.
Declaration

I declare that all the work presented in this thesis is my own except where stated below, and it has been entirely composed by myself.

1. Studies

All clinical studies were performed by myself with the exception of Studies 1 & 2 (Chapters 3 & 4) which were performed as joint studies in conjunction with Dr JC Spratt and Dr IB Wilkinson.

2. Assays

Because of the very large number of samples produced by clearance studies requiring analysis, these were performed in conjunction with the laboratory staff of the Clinical Pharmacology Unit*. All immediate processing of samples was undertaken by me. The subsequent analyses can be divided into those I undertook completely (flame photometer measurements, inulin measurements, haematocrit, osmolality), those I performed to gain experience of the assay but which were largely analysed by the laboratory staff (PAH, BQ-123, ET-1, ANG II) and those which I observed but were all analysed by the laboratory staff (PRA, Aldosterone, serum ACE activity, urinary protein). Cyclosporin was performed in the main hospital laboratory.

*Mr NR Johnston, Miss FE Howe, Miss L Coppard
Acknowledgements:

Special thanks must go to:

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Professor Allan Cumming for his ongoing encouragement and renal back-up in a clinical pharmacology setting.

Finally, Professor David Webb, who afforded me the opportunity to undertake this research in the first place, who has been unstinting in his backing, advice and support.
# Abbreviations

<table>
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<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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<tr>
<td>ANG II</td>
<td>Angiotension II</td>
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<tr>
<td>ARAS</td>
<td>Atherosclerotic renal artery stenosis</td>
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<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
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<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
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<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
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<tr>
<td>CI</td>
<td>Cardiac index</td>
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<td>CO</td>
<td>Cardiac output</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CRF</td>
<td>Chronic renal failure</td>
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<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
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<tr>
<td>CyA</td>
<td>Cyclosporin</td>
</tr>
<tr>
<td>ECE</td>
<td>Endothelin converting enzyme</td>
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<tr>
<td>EFF</td>
<td>Effective filtration fraction</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ERBF</td>
<td>Effective renal blood flow</td>
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<tr>
<td>ERPF</td>
<td>Effective renal plasma flow</td>
</tr>
<tr>
<td>ERVR</td>
<td>Effective renal vascular resistance</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
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<tr>
<td>FeNa</td>
<td>Fractional excretion of sodium</td>
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<tr>
<td>FWC</td>
<td>Free water clearance</td>
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<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>Hct</td>
<td>Haematocrit</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischaemic heart disease</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>IMCD</td>
<td>Inner medullary collecting duct</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>L-NG-monomethyl-arginine</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>NE</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PAH</td>
<td>Para-aminohippurate</td>
</tr>
<tr>
<td>PHT</td>
<td>Pulmonary hypertension</td>
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<tr>
<td>PIH</td>
<td>Pregnancy induced hypertension</td>
</tr>
<tr>
<td>PET</td>
<td>Pre-eclampsia</td>
</tr>
<tr>
<td>PVD</td>
<td>Peripheral vascular disease</td>
</tr>
<tr>
<td>RBF</td>
<td>Renal blood flow</td>
</tr>
<tr>
<td>SAH</td>
<td>Sub-arachnoid haemorrhage</td>
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<tr>
<td>SVRI</td>
<td>Systemic vascular resistance index</td>
</tr>
<tr>
<td>UFR</td>
<td>Urinary flow rate</td>
</tr>
<tr>
<td>UNaV</td>
<td>Urinary sodium excretion</td>
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Chapter 1

Introduction
1.1 Introduction
Since its discovery in 1988, endothelin (ET) has been widely implicated in the pathophysiology of renal and cardiovascular disease. ET receptor antagonists have favourable effects in experimental models of these conditions and have proved useful in elucidating the role of the ET system. Orally acting ET receptor antagonists appear promising in clinical trials in pulmonary and systemic hypertension, but there is a paucity of human data regarding the role of ET and its receptors both on renal function and in renal disease. This thesis therefore explores the systemic and renal haemodynamic and renal tubular effects of ET, using selective ET receptor antagonists.

1.2 Biology of Endothelins
The endothelins are a family of 21 amino acid peptides with powerful vasoconstrictor and pressor properties that were first described by Yanagisawa in 1988 [1]. Three different isopeptides, ET-1, ET-2 and ET-3, have so far been identified, each with distinct genes and tissue distributions [1-3]. Each has a common structure of a hydrophobic C terminal end and two disulphide bonds within the peptide that form a loop [2, 3]. ET-2 differs from ET-1 by 2 amino acids and ET-3 from ET-1 by 6 amino acids. Endothelins are highly conserved peptides that show a similarity to sarafotoxins, cardiotoxic constrictor snake venom peptides produced by the Israeli burrowing asp Actractaspis engaddensis [4] (Fig 1.1).

Figure 1.1 Structure of endothelins and sarafotoxin S6b
Of the three peptides, ET-1 is the major endothelial isoform and is thus likely to be the most important in the regulation of vascular function. Its main site of production is the vascular endothelial cell but it is also produced by other cell types including renal tubular and mesangial cells, vascular smooth muscle cells and epicardial cells. (Table 1.1).

1.2.1 ET-1 biosynthesis

The gene product is the 212 amino acid pre-pro-ET-1. Regulation of the production of ET is thought to be at the level of gene transcription. Enhanced gene transcription occurs with a wide range of stimuli including other vasoactive hormones such as angiotensin and vasopressin [20-22] cytokines such as interleukin-1, platelet derived growth factor, transforming growth factor beta [23, 24], endotoxin [25, 26] hypoxia [27], insulin [28] oxidized low density lipoprotein [29] and cyclosporin (CyA) [30]. In contrast, prostacyclin [31], nitric oxide [32], the natriuretic peptides [33], oestragen [34] and heparin all inhibit gene transcription.

<table>
<thead>
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<tbody>
<tr>
<td><strong>ET-1</strong></td>
</tr>
<tr>
<td>Vasculature</td>
</tr>
<tr>
<td>- vascular smooth muscle</td>
</tr>
<tr>
<td>cells [6]</td>
</tr>
<tr>
<td>Heart (epicardium) [9]</td>
</tr>
<tr>
<td>Lung (bronchus) [15]</td>
</tr>
<tr>
<td>Central and peripheral</td>
</tr>
<tr>
<td>nervous system [17, 18]</td>
</tr>
<tr>
<td>Macrophages [16]</td>
</tr>
</tbody>
</table>
Pre-pro-ET-1 is cleaved by intracellular furin-like proteases to proET-1 then big ET-1 (38 amino acids) which is largely, but not wholly, biologically inactive [35]. Endothelin converting enzyme (ECE), a metalloproteinase [36], then splits big ET-1 to the biologically active ET-1 and C-terminal fragment (CTF). Two ECEs have been identified, ECE-1, existing in four distinct isoforms [37-39] and ECE-2 [40], both of which preferentially catalyse the cleavage of big ET-1. Each ECE has a different tissue distribution and different pH at which it acts [37, 41]. ET-1 can also be produced from big ET-1 by chymases [42], which can also cleave big ET-1 to produce the vasoconstrictor ET-1$_{1.31}$ [43], and non-ECE metalloproteases. ET-1 secretion from endothelial cells is largely abluminal, towards the adjacent vascular smooth muscle [44] (Fig 1.2).

### 1.2.2 ET receptors

Two ET receptors have been identified and cloned in mammals. Both are seven domain trans-membrane proteins. ETA receptors [45] have a high affinity for ET-1 compared to ET-2 and ET-3, whereas ETB receptors [46] have an equal affinity for all three ET peptides. Within the circulation, ETA receptors are found on vascular smooth muscle cells [45, 47] and their activation results in vasoconstriction. ETB receptors are also found on vascular smooth muscle cells [48], where they can mediate vasoconstriction, but are primarily found on the vascular endothelium [49] where their activation results in vasodilatation via the nitric oxide (NO) system [50] and prostacyclin [51] (Fig 1.2). Within the kidney, in man, ETA receptors are localised to vascular smooth muscle, notably in the glomeruli, vasa recta and arcuate arteries. ETB receptors are more numerous (ETB to ETA ratio 2:1) and more widespread with a high concentration in the collecting system [11, 52].

### 1.2.3 ET receptor signalling

Signal transduction is via coupling to a G protein [53] and activation of intracellular phospholipase-C with subsequent hydrolysis of phosphatidyl inositol [54, 55], activation of protein kinase C [56] and changes in intracellular calcium [57, 58]. Binding of ET-1 to its receptors is irreversible, largely due to the internalisation of the receptor-ligand complex, and produces slow onset, sustained actions (Fig 1.2).
Figure 1.2  ET-1 biosynthesis

**ENDOTHELIAL CELL**
- Prostaglandin Synthase
- NOS

**ETB Receptor**
- NO
- Prostacyclin

**ENDOTHELIN-1 (21AA)**
- Clearance
  - Renal
  - Neutral endopeptidases
  - ET-B binding & internalisation

**ETA (ETB) Receptor**
- ET-receptor dissociation slow

**SMOOTH MUSCLE CELL**
- G protein
  - Phospholipase C
    - Hydrolysis of phosphatidylinositol
    - ↑ Membrane
      - Diacyl glycerol
    - ↑ Inositol triphosphate
    - Protein kinase C
      - Activation
        - Sensitises contractile apparatus of cell to Ca^{2+}
        - Release from intracellular stores
        - Voltage-dependent channels

**SMOOTH MUSCLE CONTRACTION**

**SMOOTH MUSCLE RELAXATION**
1.2.4 Actions of ET-1

Endothelin was originally identified as a vascular endothelial cell derived peptide acting on vascular smooth muscle cells to produce a biphasic response on blood vessels of a transient ETB receptor mediated vasodilatation [59] followed by a sustained ETA receptor mediated vasoconstrictor and pressor response [60]. However, in addition to its vasoconstrictor properties, it is clear now that it has many other functions in vascular and other tissues.

ET-1 can produce contraction of other cell types eg. airway smooth muscle cells producing bronchoconstriction [61], detrusor smooth muscle cells causing bladder contraction [62] and mesangial cells reducing the filtration coefficient [63]. ET-1 also functions as a powerful mitogen promoting cell growth in a wide variety of cell types [63-65] and interacting with other growth factors (eg. vascular endothelial growth factor in vascular smooth muscle and endothelial cells [66]). It is pro-inflammatory [67, 68] modulating cytokine production (eg. TNF-α and interleukin-6 in mast cells [69], interleukin-1β in astrocytes [70]) and pro-fibrotic [71]. It modulates other hormone systems, (eg. atrial natriuretic peptide [72], angiotensin [73], noradrenaline (NE) [74], arginine vasopressin (AVP) [75], and aldosterone [76]) and also influences ion and fluid transport in the gut [77], lungs [78] and kidneys [79-81]. ET-1 can also act as a neuropeptide in the brain [82] and mediate pain nociception [83].

Knockout models have also demonstrated that ET is vital in normal embryonic development of tissues derived from the neural crest. ET-1 knockout mice die shortly after birth from craniopharyngeal malformations [84]. ETB receptor knockout mice have aganglionic megacolon and an abnormal coat colour demonstrating abnormal development of myenteric ganglion neurones and epidermal melanocytes [85].
1.2.5 ET clearance

The half-life of exogenously administered ET-1 in the circulation is ~1 min [25, 86]. Clearance of ET-1 from the circulation is by receptor and non-receptor mediated mechanisms. ETB receptor binding and internalisation with intracellular degradation accounts for the majority of clearance particularly in the pulmonary circulation, with splanchnic and renal circulations also contributing [86]. In rats ~70% of ET-1 is removed in the lungs, in man this is ~50% [87, 88]. Renal clearance [86] and enzymatic degradation by neutral endopeptidases on endothelial cell membranes [89] also occurs.

1.3 Plasma ET-1 concentrations

In studying the activity of the ET system, a marker of that activity should be sought. Throughout this thesis, plasma ET-1 concentration is given. However, in interpretation of its significance, it must be borne in mind that, though big ET and its cleavage products can all be detected in the circulation, most ET-1 is released from endothelial cells abluminally [44] where it acts on smooth muscle cells. Because of ET-1’s paracrine action, plasma ET-1 concentrations may not be an appropriate measure of endothelial synthesis.

Also, plasma ET level will represent a balance between production and clearance. ET-1 is rapidly cleared from the circulation. Though it will exert effects for up to 60 minutes, the plasma half-life in normal subjects is about 1 minute [25]. Reductions in ETB receptor numbers or in renal function may reduce this clearance increasing plasma ET-1 concentrations without altering production, though there is no evidence yet as to whether these elevated concentrations consequent upon reduced clearance are biologically active as opposed to simply immuno-competent and thus detected by current assays. Measurement of big-ET and CTF have been promoted as more accurate reflectors of endothelial synthesis because their removal from the circulation is slower and more consistent [90, 91]. ETB inhibition (i.e. reduced receptor clearance) results in a rise in plasma ET-1 but not big ET-1 or CTF i.e. no increase in production.
Finally, in considering the kidney, because less than 1% of injected radiolabelled ET-1 is recovered in the urine [92], neither glomerular filtration nor tubular secretion of plasma ET-1 accounts for urinary ET-1, which is therefore assumed to be primarily of renal origin. Thus urinary ET-1 excretion rate may be used as a marker of renal ET-1 production.

1.4 ET receptor antagonists

ET receptor agonists and antagonists have been used to define the role of the endogenous ET system and confirm its importance in cardiovascular function and dysfunction. Attention is now focusing increasingly on drugs that may have a role in clinical medicine. Though ECE inhibitors and monoclonal antibodies have been used experimentally, and the former may be a potential future therapeutic option, most clinical work to date has been with ET receptor antagonists.

ET receptor antagonists can be divided, according to their receptor selectivity, as ETA or ETB selective, and non-selective ETA/B receptor antagonists. The term ETA selective is generally used for compounds with >1000 fold selectivity for the ETA over the ETB receptor. It is important to appreciate that so-called ‘non-selective’ antagonists are still generally selective for the ETA receptor but the ratio of ETA to ETB effect is less than for the selective agents (Table 1.2). In practical terms, more work with these selective vs. non-selective receptor antagonists is needed to delineate the respective roles of ETA and ETB receptors in normal physiology and, more critically, in pathophysiology before conclusions can be drawn regarding which are likely to be most beneficial in a given clinical situation.

A further division, in terms of their clinical potential, must be made into orally active compounds and those available only as parenteral preparations. There will be certain situations where an intravenous preparation is preferred, notably to counter an acute, isolated insult (such as the cerebral ischaemia following subarachnoid haemorrhage or stroke, unstable angina, acute myocardial infarction, acute renal failure and post-operative pulmonary hypertension). However, many of the anticipated indications for the ET receptor antagonists currently in development (including hypertension,
chronic heart failure and coronary restenosis) will require chronic treatment and, thus, oral agents are more suitable (Table 1.2).

A series of peptide antagonists have been developed from the modification of the ET peptides to produce compounds that bind to, but do not activate the receptors. Cyclic peptides mimic the hairpin loop of the endothelins; BQ-123 is a cyclic pentapeptide, ETA selective receptor antagonist developed from BE 18257B (a natural product of *Streptomyces misakiensis*) [93, 94] and TAK-044, a cyclic hexapeptide, non-selective ETA/B receptor antagonist [95]. Modification to produce a series of linear peptides can generate ETB selective receptor antagonists such as BQ-788 [96]. As peptides, these agents are broken down in the gut and are thus only active parenterally, and then with only relatively short half-lives. Experimentally, the half-life of BQ-123 can be increased by side-chain modifications that reduce hepatic extraction [97], but the clinical applicability of this group of compounds remains limited by the lack of oral analogues.

Screening libraries for compounds with ET blocking activity, and molecular modelling, has resulted in a series of non-peptide antagonists with greater bioavailability. Ro 47-0203, bosentan [98], is a sulphonamide compound with non-selective receptor blocking activity that has the benefit of being available as both an oral and intravenous preparation. Practically, this would allow treatment to be started in the acute situation, say, an exacerbation of undiagnosed heart failure, with the intravenous preparation and continuing treatment thereafter with the oral formulation. SB 209670, a carboxylic acid derivative, is a more potent non-selective receptor antagonist but is only available as an intravenous preparation [99]. SB 217242 (enrasentan) is an analogue of this compound with improved bioavailability, currently in clinical studies as an oral drug [100]. Orally active and selective ETA receptor antagonists have also been developed. ABT 627 is a carboxylic acid derivative with 2000-fold selectivity for the ETA vs. the ETB receptor and a half-life of 24 hours in humans [101] making it theoretically a very attractive choice where chronic selective receptor antagonism is required. Minor modifications to this structure produce compounds highly selective for the ETB receptor [101]. TBC 11251 and TA 0201 are
sulphonamide derivatives with high bioavailabilities which are also highly selective for the ETA receptor [102]. TA 0201 has a half-life of only 0.9 hours in pharmacokinetic studies but generates an active metabolite with demonstrable inhibitory effect at 8 hours [103].

Table 1.2: Endothelin receptor antagonists in development

1.2a: ETA selective endothelin receptor antagonists

<table>
<thead>
<tr>
<th>Name</th>
<th>Route</th>
<th>ETA selectivity*</th>
<th>Stage of Development</th>
<th>Anticipated indication</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBC 11251</td>
<td>O+IV</td>
<td>x7000</td>
<td>Phase III</td>
<td>PHT</td>
<td>Encysive/Texas Biotechnology</td>
</tr>
<tr>
<td>(Sitaxentan)</td>
<td></td>
<td></td>
<td>Phase II</td>
<td>CHF</td>
<td></td>
</tr>
<tr>
<td>LU 135252</td>
<td>O+IV</td>
<td>x130</td>
<td>Phase III</td>
<td>CHF</td>
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### 1.2b: Non-selective endothelin receptor antagonists

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<tr>
<th>Name</th>
<th>Route</th>
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<th>Anticipated indication</th>
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**Key:** O - Oral, IV - intravenous, NK - not known, * based on binding and functional assays

BPH - benign prostatic hypertrophy, CHF - Congestive heart failure, COPD - Chronic obstructive pulmonary disease, CRF - chronic renal failure, DM - diabetes, HT - hypertension, IHD - ischaemic heart disease, PHT - pulmonary hypertension, PVD - peripheral vascular disease, SAH - sub-arachnoid haemorrhage

A potentially interesting preclinical development is the description of L-747072, an angiotensin receptor antagonist with non-selective ET receptor antagonist activity, providing a possible dual target approach for the treatment of hypertension and other cardiovascular conditions [104].
1.5 Endothelin and the kidney

1.5.1 Physiology

Within the kidney, ET-1 is synthesised by endothelial, mesangial, glomerular epithelial and medullary collecting duct cells [13, 105-107] and may act as a paracrine/autocrine regulator of renal and intrarenal blood flow, glomerular haemodynamics and sodium and water homeostasis [80, 108]. Though the tubular effects are probably intra-renal and paracrine in nature, and ET-1 probably also acts locally to regulate glomerular haemodynamics, there is some evidence that circulating ET may also be important in renal functional changes in cardiorenal states of ET activation such as heart failure [109].

In animals, exogenous ET-1 causes renal vasoconstriction, sometimes preceded by transient vasodilatation [110-113]. Indeed, the renal vasculature is more sensitive to the vasoconstricting effects of ET-1 than other vascular beds [114]. Studies suggest that this vasoconstriction is primarily cortical [115, 116] with an initial medullary vasodilatation [115, 117]. In man, the effect of exogenous ET-1 on the renal vasculature is to cause vasoconstriction, a fall in glomerular filtration rate (GFR), and an increase in filtration fraction [118-120].

There are abundant ET receptors in the kidney but the roles of the ET-A and ET-B receptors may show a species difference. In pigs, rabbits and dogs, ET-A inhibition blocks the vasoconstricting effects of exogenous ET-1 [59, 121-123], while, in dogs, administration of an ET-B agonist causes vasodilatation [124] and ET-A inhibition unmasks a presumably ET-B mediated vasodilatation in response to ET-1 [123]. Conversely, in rats there is some evidence to support ETB as well as ETA receptor mediated renal vasoconstriction. ET-B agonist administration has been shown to causes renal vasoconstriction [125] and combined ETA/B inhibition to abolish whereas ETA receptor blockade only attenuate ET-1 induced vasoconstriction [126, 127].
Studies of intrarenal changes in blood flow suggest that the observed cortical vasoconstriction is mediated by ETA receptors \([115, 116]\) whereas the medullary vasodilatation is ETB receptor mediated and NO dependent \([115, 117]\).

In respect of tubular functions, the highest concentrations of ET-1 are found in the medulla. \([128, 129]\), and there is now a substantial body of evidence supporting a role for ET-1 in the renal regulation of volume homeostasis. ET-1 is produced by inner medullary collecting duct cells (IMCD) where it inhibits the AVP stimulated retention of water \([80]\), and extra-cellular sodium concentrations may regulate IMCD ET-1 production \([108]\). Additionally, the ETB receptor appears to have a natriuretic role at least in animals. ET-1, acting via ETB and NO can inhibit chloride transport, thus promoting natriuresis, in the medullary thick ascending limb of Henlé \([130, 131]\). Additionally, *in vitro* low dose picomolar concentrations ET-1, binding to ETB receptors, will activate amiloride-sensitive sodium channels in distal tubular cells, though higher, nanomolar doses inhibit this channel by a non-ETB receptor dependent mechanism \([132]\). *In vivo*, dogs subjected to high-grade ETA blockade demonstrate a vasodilatation and natriuresis in response to administration of exogenous low dose ET-1 presumed to be an unmasking of an ETB receptor effect \([123]\) and ETB receptor blockade shifts the pressure diuresis and natriuresis response to the right in rats (ie. a greater renal perfusion pressure is needed to excrete the same salt and water) \([133]\). Finally, ETB knockout mice, and ETB antagonist treated rats, develop a sodium dependent hypertension \([134, 135]\), and collecting duct-specific ET-1 knockout mice develop salt retention and hypertension on a high salt diet \([136]\).

In man, the effect of exogenous ET-1 on the renal vasculature is to cause vasoconstriction, a fall in glomerular filtration rate (GFR), an increase in filtration fraction and a profound retention of salt and water, probably as a consequence of the renovascular changes rather than any direct ET-1 effect. \([118-120]\). Within the human kidney, ET-A receptors are localised to vascular smooth muscle, notably in the glomeruli and vasa recta and arcuate arteries. ET-B receptors are more numerous (ET-B to ET-A ratio 2:1) and more widespread with a high concentration in the collecting
system [11, 52]. The distribution of the receptors in man would suggest a vasoactive role for ET-A receptors and a role in sodium and water handling for ET-B receptors.

Human studies comparing exogenous ET-1 and ET-3, as ET-A and ET-B receptor agonists respectively, suggests that ET induced renal vasoconstriction is ET-A receptor mediated [137]. However, renal vasoconstriction with ET-3 has also been demonstrated, although at high dose, suggesting a possible constrictor role for ET-B receptors [138]. ET-A blockade can counter the effects of exogenously administered ET-1 on renal blood flow (RBF) and GFR but has no effects alone on renal function [120, 139-141] suggesting again that ETA receptors mediate ET-1 induced renal vasoconstriction, but that endogenous ET-1 has little role in the maintenance of normal renovascular tone. However, administration of the non-selective ETA/B receptor antagonist SB209670 does produce a significant increase in RBF compared to placebo [142]. No studies in man to date have demonstrated significant effects on salt and water homeostasis.

Thus, the paucity of work with antagonists in the human kidney has left significant uncertainties regarding the importance of endogenous ET and the roles of the receptor subtypes in the kidney in human physiology.

1.5.2 Pathophysiology

ET-1 has been widely implicated in renal disease, both in acute renal failure eg. ischaemia induced renal injury, CyA nephrotoxicity and radiocontrast nephrotoxicity [143-147] and progressive renal dysfunction [148].

**Radiocontrast nephrotoxicity**

As contrast induced nephrotoxicity is characterised by intense renal vasoconstriction, ET-1 has been proposed as a potential mediator of this condition. Contrast media increase ET-1 gene transcription in endothelial cells [149] and increases in both plasma and urinary ET-1 have been observed after contrast media administration in animals [149, 150] and in patients with CRF [151]. Additionally, non-selective ET receptor antagonism abolishes contrast induced renal vasoconstriction in dogs [152],
as does selective ETA receptor antagonism in rats [153]. Selective ETA receptor antagonism also prevents contrast induced renal medullary hypoxia in rats [154]. However, in patients with CRF undergoing cardiac angiography, ET receptor antagonism with SB 209670 was associated with a worse outcome than hydration alone [155].

**Ischaemic acute renal failure**

Hypoxia can increase ET-1 production in renal tubules [156]. Increased ET-1 peptide expression is seen in the renal vasculature and in the cortical tubular epithelium of kidneys after ischaemia reperfusion [157]. Specifically, ET-1 increases in renal medullary interstitial cells, damaged tubules at the corticomedullary junction and peritubular capillaries surrounding these damaged tubules. An increase in renal cortical and medullary ETA and cortical ETB receptors can also be demonstrated within 24hrs of ischaemia, with changes being maximal at 8 days. By contrast, medullary ETB receptors decrease [158].

ET receptor antagonists have ameliorated ischaemia-reperfusion injury in animal models [147, 157, 159-162] and can improve the warm [163] and cold [164] ischaemic injury that occurs during transplant retrieval. As selective ETB antagonists are not protective in ischaemia-reperfusion [165], it appears that ET-1 mediated vasoconstriction via the ETA receptor is the pathogenic mechanism abrogated. There is also some evidence from knockout models that the ETB receptor is involved in the renal recovery after ischaemia reperfusion [166], an observation supported by a study demonstrating that non-selective ETA/B, but not selective ETA receptor blockade during ischaemia reperfusion is associated with increased tubulointerstitial fibrosis and proliferation at 6 months [167].

**Cyclosporin nephrotoxicity**

CyA is associated with both acute and chronic renal dysfunction, the former probably consequent upon changes in renal haemodynamics, the latter associated with irreversible structural changes. CyA is well documented as causing acute renal vasoconstriction in animal models with a reduction in RBF and GFR. [168]. *In vitro,*
CyA has been shown to increase gene transcription and secretion of ET-1 from endothelial and smooth muscle cells [169-171]. CyA treatment increases ET-1 in proximal tubules in rats [172] and CyA causes down regulation of ET receptors in the kidney [173]. Increased activity of the ET system is also indicated by the ability of ET receptor antagonism to attenuate CyA induced smooth muscle cell contraction [171] and CyA induced renal vasoconstriction [145].

*In vitro,* ET receptor antagonism attenuates CyA induced smooth muscle cell contraction [171]. *In vivo,* ET receptor antagonists can improve acute CyA induced renal vasoconstriction in animal models [144, 145, 174-176], and a similar attenuation of CyA induced fall in RBF, but not blood pressure rise, has been demonstrated in healthy men with bosentan [177]. Plasma ET-1 concentrations [178] and big ET-1 concentrations [179] are elevated in patients on CyA, but, as yet, no studies with ET receptor antagonists have been performed in patients on chronic treatment.

**Chronic renalfailure**

Besides its haemodynamic effects ET-1 has mitogenic and pro-fibrotic properties that may play a role in CRF. In support of a role for ET-1 in CRF, low renal mass models are associated with increased production intra-renally, both in vascular and renal tissues, of ET-1 with a positive correlation between ET-1 levels and glomerulosclerosis [148, 180-182] apparent indirectly in increased urinary ET-1 excretion [180, 182]. Also combined ETA and ETB blockade, drops the filtration fraction whilst maintaining the GFR [134] raising the possibility that ET promotes hyperfiltration with its consequent potential for renal injury.

ET receptor antagonists have been shown to prevent or attenuate the progression of renal insufficiency in a rat remnant kidney model [182-185]. While non-selective ETA/B receptor antagonists have produced positive results [183], the effect is less than that seen with selective ETA receptor antagonism [185], and concomitant administration of a selective ETB receptor antagonist abolishes the beneficial effects of ETA receptor antagonism [184]. A reduction in cortical ETB mRNA receptor
expression by contrast with an increase in ETA receptor and ET-1 expression may explain this differential response [184], and have implications for the use of non-selective ET receptor antagonists in CRF.

Human data supporting a role for ET-1 in CRF progression is more circumstantial. In patients with autosomal dominant polycystic kidney disease, intra-renal ETA mRNA is increased 5-10-fold compared to healthy controls [186]. Plasma and urinary ET-1 concentrations in patients with nephrotic syndrome due to focal segmental glomerulosclerosis are significantly higher than in normal controls [187] and nephrotic patients who show a reduction in proteinuria with immunosuppressive therapy also show reductions in urinary ET-1 excretion [188]. Urinary excretion of ET-1 is doubled in patients with CRF compared to healthy controls. This occurs alongside a reduction in prostaglandin I₂ urinary metabolites suggesting that a vasoconstrictor-dilator balance present in healthy kidneys is disrupted in CRF [189].

While the above points to the therapeutic potential of ET receptor antagonists in CRF, to date only one interventional study has been performed in patients. Type I diabetics with proteinuria but essentially normal renal function treated for 6 weeks with an ETA receptor antagonist showed no alterations in RBF but did have a reduction in proteinuria, a potentially renoprotective effect [190].

1.6  Endothelin and the cardiovascular system

1.6.1  Physiology

The importance of ET-1 in cardiovascular function is suggested by the actions of exogenous ET-1. In respect of the vasculature, in vitro, activation of vascular smooth muscle ETA receptors causes smooth muscle contraction and blood vessel constriction, and the administration of exogenous ET-1 is associated with a sustained increase in blood pressure and peripheral vascular resistance [59, 60, 119]. In animals, this is preceded by a transient period of hypotension and vasodilatation [59, 60] mediated by ETB receptors [59].
In man, forearm models in healthy volunteers have shown that ETA receptor antagonism causes local vasodilatation [191-195] consistent an important role for ET-1 in the maintenance of normal vascular tone via the ETA receptor. These forearm findings are disputed [196, 197], but differences may be attributable to concentrations of antagonists used [198]. However, acute systemic studies in healthy men [139, 199, 200] have failed to demonstrate a significant effect of ETA receptor antagonism on basal haemodynamics. Similarly, while non-selective ETA/B receptor antagonism with TAK-044 has been shown to cause vasodilatation and a reduction in blood pressure in healthy volunteers [201], studies using the non-selective antagonists bosentan [202], and SB 209670 [142] have demonstrated little effect on systemic haemodynamics in healthy subjects. Thus, while the ETA receptor mediates ET-1 induced vasoconstriction, the role of the ETA receptor in the maintenance of vascular tone is not proven.

ETB receptors can be both vasodilator (endothelial) and vasoconstrictor (vascular smooth muscle) in function. The balance of these two functions in health has yet to be fully elucidated. Selective ETB receptor agonists can cause constriction \textit{in vitro} and increase blood pressure \textit{in vivo} [203-205], but the relative importance of vasoconstrictor ETB receptors appears to vary between species and vessel type [206].

In man, ETB agonists cause constriction of forearm resistance and hand capacitance vessels in vivo in healthy subjects [207, 208], suggesting that ETB receptors also mediate ET-1 induced vasoconstriction. However, the vasoconstriction produced by exogenous ET-1 can be abolished by ETA receptor blockade alone both locally [209], and systemically [139]. Forearm studies have also demonstrated vasoconstriction in response to ETB receptor blockade in healthy volunteers [194, 196] and an attenuation of the vasodilator actions of ETA receptor blockade when ETB receptor antagonists are concurrently administered [194]. Similarly, systemic administration of ETB receptor antagonists produces increases in systemic vascular resistance [210] consistent with the net result of ETB receptor activation being vasodilatation in normal physiology.
The direct effects of ET-1 on the heart are probably species specific but a positive inotropic response to ET-1 is seen in isolated cardiac tissue from rats and humans [211, 212], and in in vivo models where coronary vasoconstriction is countered with a vasodilator [213]. The integrated haemodynamic response is, however, much more complicated. ET-1 associated increased peripheral vascular resistance and blood pressure produce reflex reductions in HR and cardiac output and coronary vasoconstriction can cause myocardial ischaemia and cardiodepression.

1.6.2 Pathophysiology

1.6.2a Hypertension

With respect to pathophysiology, in addition to its haemodynamic actions, exogenous ET-1 has been demonstrated in vitro to be mitogenic, thus causing vascular remodelling and cell proliferation, and causes renal sodium retention [118], features of hypertension. Production of ET-1 is increased in some (such as the Dahl salt-sensitive and the stroke-prone spontaneously hypertensive rat), but not all rat models of hypertension. Those models where ET-1 production is increased (mostly, but not exclusively salt-dependent types) are associated with increased vascular growth and a response to both selective and non-selective ET receptor antagonists. This response comprises not only a modest reduction in blood pressure but also a marked regression of vascular growth [214]. Altered intrarenal ET-1 production may contribute to essential hypertension. Animal studies indicate that tubular ET-1 has an important role in the physiological regulation of sodium excretion and volume status and hence blood pressure. Knockout mice with no collecting duct ET-1 retained sodium and developed salt sensitive hypertension [136] and ET-1 produced by IMCD also inhibits the AVP stimulated retention of water [80]. Urinary ET-1 excretion is reduced in hypertensives compared to normal controls suggesting that either renal ET-1 synthesis is reduced or breakdown is enhanced [215, 216]. Hence ET nephron production or handling may be deranged in essential hypertension, there may be inappropriate sodium and water retention aiding the development or maintenance of hypertension. Spontaneously hypertensive rats provide an
experimental model of this in that they have reduced medullary ET levels after the development of hypertension [217].

In man, elevated plasma ET concentrations are found in hypertension, though this is not consistent [218]. These high concentrations would appear, mostly, to be a feature of severe hypertension or indicative of the presence of complications or co-existing disease. Prepro ET-1 and ECE mRNA is increased in the vascular smooth muscle cells of hypertensive patients [219], and there is also evidence to suggest that endothelial function, specifically endothelium-dependent vascular relaxation, is impaired both in hypertension, and in groups at risk of hypertension [220, 221].

Forearm studies with ETA and ETB receptor antagonism suggest increased vascular ET activity in patients with essential hypertension compared to normotensive controls, (though this has not been confirmed by other groups [222]) and a greater response to non-selective antagonism compared to selective ETA antagonism [196, 223], suggestive of a possible increase in the importance of vascular smooth muscle vasoconstrictor ETB receptors in disease.

To date, one major study has examined the anti-hypertensive effects of ET receptor antagonism in man. In 293 hypertensive patients, bosentan at a dose of 500 to 2000 mg/day for 4 weeks produced a reduction in blood pressure equivalent to that with 20 mg of enalapril [224]. This reduction was achieved without a reflex increase in activity of the sympathetic nervous system (as measured by plasma NE levels and HR) or renin-angiotensin system. Further confirmatory work is needed but, given the number of different anti-hypertensive drugs already available, a new class of drug would have to demonstrate greater efficacy, a better side-effect profile, or actions additional to its blood pressure lowering abilities. It is here that potential benefits in terms of reduction in long-term structural changes in the blood vessel (vascular hypertrophy or atherogenesis) or myocardium, an amelioration in endothelial dysfunction, or a beneficial effect on salt and water balance, might accrue. Hypertensive sub-groups such as salt sensitive patients, or black hypertensives (with
higher plasma ET levels and impaired endothelium-dependent relaxation [221, 223, 225]) might potentially be targeted.

**Chronic renal failure and hypertension**

Hypertension in CRF is a major cause of morbidity and mortality. ET-1 has been implicated as a mediator of this hypertension by a number of mechanisms. Firstly, as a consequence of reduced renal clearance, plasma ET concentrations are certainly consistently elevated in CRF [216, 226-228]. The effect of exogenous ET-1 on the renal vasculature is to cause vasoconstriction (which will activate the renin-angiotensin system) and profound salt and water retention, both having the potential to raise blood pressure [118]. It is not proven yet whether the elevated concentrations of ET-1 seen in CRF represent biologically or just immunologically competent peptides but in an animal model where exogenous ET-1 was administered to bilaterally nephrectomised rats, there was a prolonged rise in blood pressure compared to sham-operated rats associated with an increased plasma half-life of ET-1 [229] suggesting that there is potential for elevated plasma ET concentrations to cause hypertension.

Secondly, renal ET-1 production [181] and urinary excretion [216] are increased in CRF. Additionally, in experimental nephritis associated with mesangial proliferation, the renal vasculature is more sensitive to the constricting effects of ET than in normal kidneys [230] hence an amplification of the renovascular effects of ET-1 could be envisaged in CRF, an effect that could be exacerbated by the higher plasma ET concentrations due to reduced renal clearance. ETB receptors appear to be important in protecting against hypertension in CRF. In a 5/6 nephrectomy remnant kidney model of CRF, ETB receptor deficient rats have higher renal ET-1 content, a higher blood pressure and faster progression in renal failure than wild type animals [231].
Erythropoietin induced hypertension

Recombinant human erythropoietin (EPO) is widely used to improve the haematocrit of patients with CRF and is associated with a rise in blood pressure [232, 233]. In vitro, EPO has been shown to increase production of ET-1 from endothelial cells [234]. In transgenic mice overproducing EPO, increasing haematocrit to 80% is associated with increased ET-1 mRNA levels in aorta, liver, heart, and kidney, despite systemic vasodilatation [235]. In man, intra-arterial infusion of ET-1 causes less forearm vasoconstriction after EPO therapy than before [233]. If plasma ET were to be increased by EPO therapy, this might cause a down-regulation in receptors and thus a reduced response to exogenous ET. Thus a link between EPO associated hypertension and plasma ET has been sought.

Patient studies, however, are again conflicting. Some do not show an elevation in plasma ET after EPO treatment [233, 236, 237] but in two studies, chronic administration was associated with an eventual rise in plasma ET concentration, correlating additionally with blood pressure [232, 238].

Atherosclerotic renal artery stenosis (ARAS)

Lerman et.al. [239] surveyed patients with symptomatic atherosclerosis and found a significant correlation with plasma ET levels and the number of sites affected by atherosclerosis. More recently, ET-1 mRNA and ET-1 have been demonstrated not only in endothelial cells but also in cell rich areas of atherosclerotic plaques particularly foamy macrophages [240, 241]. Mechanical injury to vascular endothelium with a balloon angioplasty catheter has been shown to cause accumulation of ET within the injured vessel in animal studies and occlusion during angioplasty procedures released ET distally in human coronary arteries in vivo [241, 242]. Similarly, after aortic cross-clamping for atheromatous aortic aneurysm repair, increased levels of plasma ET have been observed [243].

In ARAS, atherosclerosis and ischaemia co-exist; the supplying vessel to the kidney will be atheromatous and the kidney ischaemic because of the stenosis. Thus, the possibility of a role for ET in the secondary hypertension of ARAS arises, a theory...
supported by the observation that, in a rat model, ET antagonism will attenuate the rise in blood pressure that occurs after single renal artery clipping [244].

The elevation of plasma ET does not need to be systemic; an elevation in the post-stenotic renal vessels might suffice to have a sodium retaining effect via renal vasoconstriction that will generate hypertension, but one might expect to see changes in renal vein ET levels. Unfortunately, while some studies in man have shown at least a trend towards higher plasma ET concentrations in ARAS and an elevated renal vein ET ratio ( [Renal vein ET - inferior vena cava (IVC) ET] / IVC ET ) on the stenotic side [245-247], others cannot demonstrate any significant difference in either systemic or renal vein ET concentrations in ARAS compared to essential hypertension or normal controls [248, 249]. Given the local nature of ET activity, receptor antagonist studies in man are required to establish a role for ET here; plasma ET probably does not have a role in diagnosis or prognosis of ARAS.

**Pregnancy induced hypertension (PIH) and pre-eclampsia (PET)**

With a few exceptions [250-252], plasma ET has repeatedly been demonstrated to be elevated in PIH/PET when compared to normal pregnancies of similar gestational age and to pregnant patients with pre-existing hypertension, the latter suggesting that the increased plasma ET is not due to a blood pressure effect per se [253-260] Also, while the elevated plasma ET concentrations do correlate negatively with creatinine clearance, they are probably not simply due to reduced renal clearance consequent upon renal dysfunction as elevated plasma ET concentrations are seen in PIH patients with normal renal function [261].

However, though elevated ET levels may correspond to disease severity, with a positive correlation between plasma ET and serum urate levels [255], and return to normal after parturition [253, 257-259] no clear relationship between ET levels and blood pressure has been demonstrated [256, 257]. PET is a disease characterised by vascular endothelial damage and thus the elevated ET levels might simply be a marker of generalised endothelial injury. However, increased ET-1 mRNA has been demonstrated in the placental villous tissue of pre-eclamptic pregnancies suggesting
an increased local production [262]. This could conceivably contribute to local blood flow redistribution and thus the placental ischaemia central to the pathogenesis of PET. A significant negative correlation exists between plasma ET levels and birth weight, low birth weight, in this respect, being a response to placental ischaemia [260, 263]. Thus, in respect of the hypertension of PET, the role of ET may be at the level of the placenta effecting a rise in systemic blood pressure by more general secondary mechanisms. Plasma ET does, however, appear to be a potentially useful correlate of disease severity in this condition.

**Pulmonary hypertension (PHT)**

ET-1 is elevated in experimental models of PHT [264] and in man, possibly consequent upon increased pulmonary production as the pulmonary venous concentration of ET-1 exceeds the arterial concentration [265]. Also, tissue levels of mRNA for ET-1 appear to correlate with the pulmonary vascular resistance [266]. Thus PHT, a disease characterised by endothelial injury, vascular smooth muscle cell proliferation and pulmonary vasoconstriction, is another potential target for ET receptor antagonists.

Non-selective and selective ET receptor antagonism are both able to prevent NG-monomethyl-L-arginine (L-NMMA) induced pulmonary vasoconstriction in a rat monocrotaline model of PHT [264]. The lack of effect of ETB antagonism suggests this is an ETA effect. In a pig hypoxic model, again, selective ETA and non-selective receptor antagonists were equally effective in countering hypoxia induced increases in pulmonary artery pressure with ETB selective drugs having no effect [267].

Consistent with these animal studies, two recent studies in 32 [268] and 213 patients [269] with PHT treated with bosentan over 12-16 weeks produced a clear improvement in clinical status. Similar initial encouraging results have been seen with the ETA receptor antagonist sitaxentan [270].
Systemic sclerosis

Systemic sclerosis is a disease characterised by vascular injury, fibrosis and, occasionally, malignant phase hypertension (hypertensive renal crisis) or pulmonary hypertension. While plasma levels of ET-1 are elevated in diffuse disease compared to limited cutaneous disease, there is no further rise in plasma levels in the subset of patients with diffuse disease who have hypertensive renal crisis or PHT [271, 272] suggesting that ET is not the trigger provoking transformation of the disease into these phases, nor is plasma ET a useful marker of the impending transformation. In vitro work has shown, however, that stimulated alveolar macrophages from scleroderma patients secrete higher amounts of ET-1 and big ET-1 than normal controls [273] and that there is a down regulation of ETA receptors in scleroderma fibroblasts [274]. ET is a powerful fibroblast and smooth muscle cell mitogen and its role systemic sclerosis may be a more central one in the pathogenesis of the disease with the elevated plasma levels representing an overproduction and subsequent overspill of this fibrotic mediator. Indeed, increased levels of ET-1 receptors have been demonstrated in scleroderma lung tissue, localised to the alveolar epithelium and the pulmonary interstitium, composed of mainly fibroblastic cells [275].

Additionally, the recent trials in PHT included patients with systemic sclerosis [268, 269], demonstrating clinical improvement in this group.

1.6.2b Congestive Heart Failure (CHF)

There is considerable evidence for a role of ET-1 in CHF. Experimentally, ET-1 causes hypertrophy of cardiac myocytes [276] and has a direct toxic effect on these cells [277]. In animal models of CHF, increased cardiac and pulmonary ET-1 synthesis has been documented [278-281]; In a Dahl salt-sensitive hypertensive rat model, the initial compensated left ventricular hypertrophy (LVH) was not associated with an increase in cardiac ET-1 but a five fold increase was noted after progression to the stage of left ventricular (LV) dilatation and global hypokinesis [282]. ETA receptors are more numerous than ETB receptors in both the normal and failing heart, but there may be an upregulation of ETA receptors in the failing heart [212, 278] suggesting that ETA receptors may be the major therapeutic target. As
with coronary artery disease, however, some evidence points toward the enhancement of vascular smooth muscle ETB mediated vasoconstriction in CHF [283-285]. Also, although an attenuation of myocyte contractility in response to ET-1 in cells from CHF animals and humans has been demonstrated [212, 286, 287], there is some evidence that endogenous ET-1 is involved in the maintenance of cardiac function in CHF [278], making cautious blockade of the ET system necessary.

Animal models of CHF have shown substantially improved survival with both selective ETA and non-selective ETA/B receptor antagonists. In CHF rats (coronary artery ligation model) although acute ETA receptor blockade with BQ-123 reduced myocardial contractility [279], long term treatment resulted in a 90% survival rate, compared to 40% for the non-treated animals [278]. This improved survival was associated with an amelioration of LV dysfunction and prevention of ventricular remodelling. Treatment with the non-selective receptor antagonist bosentan for 9 months in a coronary artery ligation model of CHF resulted in beneficial alterations in haemodynamics, cardiac geometry and function, and an 18% increase in survival in the treated group [288]. In the Dahl salt-sensitive hypertensive model of CHF, chronic treatment with bosentan, started at the stage of compensated LVH, did not alter LV mass but improved survival by 36% [282]. Of note, in a pacing CHF model, chronic (6 weeks) non-selective receptor antagonism also increased coronary sinus NO concentrations and improved endothelium-dependent relaxation in coronary arteries [289].

In man, the increase in circulating big ET-1 or ET-1 seen in patients with CHF correlates with the severity of symptoms and with prognosis [290, 291]. A series of trials point towards potential usefulness of ET receptor antagonists in this condition (Table 1.3), though it is unclear whether selective or non-selective receptor antagonism would offer the greatest benefit.

Initial studies with the non-selective ET receptor antagonist bosentan reported marked haemodynamic benefits both acutely and in the short term (2 weeks) [292,
On the basis of these results, larger, longer-term trials have been undertaken with bosentan. The REACH study (370 patients followed for 6 months) was terminated early because of asymptomatic, reversible increases in hepatic transaminases, but reported improvements in symptoms, and reduced hospitalisations, in the treatment group in those completing the 6 month protocol [294]. However, in >1500 patients enrolled into the ENABLE study, bosentan conferred an early risk of worsening heart failure [295]. Acute studies with the combined ETA/B receptor antagonist tezosentan suggest that a much lower dose is required than originally to achieve benefit without significant side-effects [296-298].

The possible theoretical benefits of selective ETA antagonism in CHF have been explored in small acute studies. BQ-123 [299] and TBC 11251 [300] have produced significant systemic and pulmonary haemodynamic benefits respectively. Acute ETB receptor antagonism with BQ-788, caused systemic vasoconstriction in CHF patients, an effect reversed by the addition of BQ-123, suggesting that ETB receptors contribute to vasodilator tone in CHF [301]. The HEAT study demonstrated for the first time in a large patient population that 3 weeks of selective ETA receptor blockade improved CI in patients with CHF [302]. Larger, longer-term studies with ETA receptors have not, however, confirmed this potential. In a 24 week study, darusentan did not improve cardiac remodelling, clinical symptoms or outcome [303].

### 1.6.2c Coronary Artery Disease (CAD)

**Ischaemia and myocardial infarction**

Inducing coronary ischaemia in CAD patients results in a significant release of ET-1 of cardiac origin [304]. Plasma ET-1 concentrations have been shown to be high in CAD, particularly in patients with myocardial infarction or coronary vasospasm [305-308], and elevations of plasma ET-1 and big ET-1 in unstable angina and after myocardial infarction are correlated with a worse prognosis [309, 310] suggesting that an activated ET system may contribute to a poor outcome.
Both ETA and ETB receptors in the coronary circulation contribute to vasoconstriction [205, 311] and in isolated porcine and canine coronary arteries, TAK-044 inhibits ET-1 induced coronary vasoconstriction to a greater extent than BQ-123 [311]. These data might suggest that non-selective receptor blockade would be more appropriate than selective. In man, acute administration of the non-selective receptor antagonist bosentan to patients with CAD resulted in an increase in vessel diameter in coronary epicardial arteries, that was not further augmented by nitrates. This effect was, however, only clear in normal or mildly diseased arteries and was shown to correlate inversely with plasma LDL-cholesterol levels [312].

With respect to myocardial infarction, both ETA receptor and non-selective ETA/B receptor antagonists have been shown to reduce infarct size in coronary ligation models [313, 314]. Though these animal studies are promising, work on man in this respect has not yet been published.

**Atherosclerosis**

Plasma ET-1 concentrations are elevated in patients with atherosclerosis [239] and increased ET-1 had been demonstrated in atherosclerotic lesions [315] and throughout the vessel walls of patients with CAD [219] suggesting upregulation of the ET system in atherosclerosis.

In hypercholesterolaemic hamsters, ETA receptor inhibition decreases the progression of atherosclerosis mainly by reducing the number and size of macrophage-foam cells [316]. In addition, apolipoprotein E-deficient mice, genetically susceptible to severe atheroma and endothelial dysfunction, are protected from atherosclerosis when treated with ETA receptor antagonists and show an increase in NO-mediated endothelium-dependent relaxation [317]. Similarly, hypercholesterolaemic pigs treated with chronic ET receptor antagonists, both selective and non-selective, have improved coronary vascular function [318]. In respect of the failure of bosentan to significantly vasodilate severely diseased coronary arteries in man [312], it is possible that the lack of effect may relate to
greater endothelial dysfunction and that this might be respond to longer term ET receptor antagonism.

**Post-angioplasty restenosis**

ET-1 is known to have a mitogenic effect on vascular smooth muscle cells in vitro [319, 320]. In animal studies, expression of mRNA for all of the components of the ET system is increased at balloon angioplasty sites [321], and intra-arterial administration of ET-1 post-angioplasty increases neointima formation in vivo [322]. In humans, an increase in plasma ET-1 concentration is seen distal to the angioplasty site correlating with the duration and pressure of the balloon inflation [241, 323]. In animals, non-selective receptor antagonism with bosentan [324], SB 209670 [322, 325] or its analogue SB 217242 [326] reduces neointima formation after endothelial injury by balloon angioplasty whereas ETA receptor antagonism alone with BQ-123 is ineffective [325]. This may not indicate a need for combined receptor antagonism, however, as ETA receptor blockade with the potent, highly selective, oral agent ABT-627 does reduce restenosis [327]. Thus, targeting restenosis for prevention by ET antagonists might seem appropriate, although many other potentially useful agents have failed to fulfil their promise in this condition.

**Arrhythmias**

In animals, both combined receptor antagonism with SB 209670 and selective ETA receptor antagonism with LU 135 252 reduced the incidence of arrhythmias induced by ischaemia or exogenous ET-1 [328-330]. Of note, however, though BQ-123 did reduce ischaemic arrhythmias in rats at a low dose, a high dose increased mortality, due to an increase in refractory ventricular fibrillation [331].

**1.7 Summary**

ET receptor antagonists have been, and continue to be, crucial experimental tools in elucidating of the role of the ET system in renal and cardiovascular physiology and pathophysiology. Few studies have yet been performed in man to clarify the role of ET in renal function in health and disease, and on vascular function in chronic renal
disease. With the increasing availability of oral antagonists, both selective and non-selective, a knowledge of the actions of ET and its receptors is crucial to fully establish the clinical role of these drugs in renal and cardiovascular disease. If they live up to their promise, ET receptor antagonists could offer a wide range of benefits including a renoprotective effect, reduction of systemic and pulmonary blood pressure, a reduction of cardiac pre- and after-load, an improvement in coronary blood flow, and, in the longer term, a reduction in abnormal ventricular and vascular remodelling, and atherosclerosis. Through these effects, they might be useful in the management of CRF, hypertension, PHT, CAD and CHF.

1.8 Aims and Hypotheses
In a series of acute studies in healthy subjects and renal patients, this thesis explores the role of ET-1 and its receptors on systemic haemodynamics and renal function in health and renal disease.

**Study 1: (Chapter 3)** This study sought to clarify the role of ET-1 acting via the ETA receptor in the maintenance of normal vascular tone, hypothesising that previous systemic studies in healthy subjects with ET receptor antagonists have failed to demonstrate a haemodynamic effect as the doses of antagonists used have been insufficient to achieve significant ETA receptor blockade. The effect of incremental doses of ETA receptor antagonists to plasma concentrations sufficient to significantly block the ETA receptor was studied to ascertain a haemodynamic dose response curve for ETA receptor blockade. The information from this study was then used in the choice of ETA receptor antagonist dose in further studies.

**Study 2: (Chapter 4)** This study examined the interaction between angiotensin II (ANG II) and ETA receptor antagonism, hypothesising that the acute effects of ANG II would be mediated through the ETA receptor in healthy subjects.

**Study 3: (Chapter 5)** This study examined the effects of selective ETA and ETB, and combined ETA/B receptor antagonism on systemic and renal haemodynamics
and on renal tubular function in healthy subjects and patients with CRF. It explored the role of ET-1 in the maintenance of systemic and renal vascular tone in health and disease, and in renal tubular function. It also provided a direct comparison between selective ETA and combined ETA/B receptor antagonism. The hypothesis was that ETA receptor antagonism would produce vasodilatation, ETB receptor antagonism would produce vasoconstriction, and the addition of ETB to ETA receptor antagonism would attenuate the vasodilator effects of ETA blockade. It was also hypothesised that, based on forearm studies, the systemic haemodynamic response to ET receptor antagonism would be less in CRF patients compared to healthy controls, and finally that ETA receptor blockade should promote ET-1 induced, ETB receptor mediated natriuresis.

**Study 4: (Chapter 6)** This study examined the interaction between ET receptor antagonism and angiotensin converting enzyme (ACE) inhibition in healthy subjects. Based on animal data, the hypothesis was that there would be a synergism between these two agents.

**Study 5: (Chapter 7)** This study explored the mechanism of the synergy observed between ETA receptor antagonism and ACE inhibition in healthy subjects, hypothesising that this synergism would be mediated through NO, and possibly vasodilator prostaglandins.

**Study 6: (Chapter 8)** This study explored the actions of the ETB receptor by examining the effects of exogenous ET-1 in the presence of ETA receptor antagonism on systemic and renal haemodynamics and tubular function in healthy volunteers. The hypothesis was that, with the ETA receptor blocked, ET-1 would produce systemic and renal vasodilatation and natriuresis via the unblocked ETB receptor.

**Study 7: (Chapter 9)** This study examined the influence of a high salt diet on the effects of ETA receptor antagonism in healthy volunteers. The hypothesis was that,
in individuals whose blood pressure exhibited a high degree of salt sensitivity, the relative reduction in endothelial NO activity on a high salt diet would result in a reduced systemic haemodynamic response to BQ-123 compared to salt-resistant individuals.

**Study 8: (Chapter 12)** This study examined the effects of a single high dose of CyA on systemic and renal haemodynamics and tubular function in healthy volunteers. The purpose of this study was to establish a model of renal vasoconstriction that could then be used to explore the effects of intervention with ET receptor antagonists.

**Study 9: (Chapter 13)** This study sought to explain the systemic vasodilatation and lack of renal effects of high dose CyA in healthy volunteers by examining the differences in response to a standard dose of CyA in renal transplant patients chronically on the drug compared with those naïve to it. The hypothesis was that chronic exposure to CyA is required to produce the endothelial dysfunction, which then produces vasoconstriction to CyA.
Chapter 2

Materials and Methods
2 Methods

All studies were performed in the University of Edinburgh’s Clinical Research Centre except for two patient studies (Chapter 5), which were performed in Monkland’s Hospital, Airdrie, Lanarkshire, with the approval of the local research ethics committees and the written informed consent of each subject. The investigations conformed to the principles outlined in the Declaration of Helsinki.

All subjects abstained from alcohol, nicotine and caffeine-containing products for 24 hours, and had a light breakfast before attending on each study day. All studies were carried out in a quiet, temperature-controlled room, at 22-24°C, with the subject recumbent throughout, except when voiding urine.

Healthy subjects taking any medications in the previous 2 weeks were excluded from the study. Patients continued taking their normal medication up to and including each study day with the exception of diuretics, which they omitted that morning. During renal clearance studies, (Chapter 5 - 13) subjects were asked to adhere to a standard diet containing 150 mmol sodium for 3 days before each study.

2.1 Drug administration

2.1.1 Locally active doses - intra-arterial administration

The brachial artery of the non-dominant arm was cannulated under local anaesthesia (1% lignocaine; Astra Pharmaceuticals, Stockholm, Sweden) with a 27 SWG steel needle (Cooper’s Needle Works, Birmingham, UK) attached to a 16G epidural catheter (Portex Ltd, Hythe, Kent, UK), and patency was maintained by infusion of physiological saline (0.9%; Baxter Healthcare Ltd, Thetford, UK) at 1 ml/min. Saline was infused for 30 min prior to the infusion of ET-1.

2.1.2 Systemically active doses - intravenous administration

For systemic intravenous administration, study drugs were infused via an 18 SWG cannula sited in an antecubital vein. PAH and Inulin were diluted in dextrose (5%; Baxter Healthcare Ltd, Thetford, UK) and infused intravenously at a constant rate of
2 ml/min. All other drugs were dissolved in physiological saline and infused intravenously at a constant rate of 1 ml/min. Saline was administered as placebo.

2.2 Drugs

2.2.1 Endothelin-1

ET-1 (Clinalfa, Laufelfingen, Switzerland) was used as a non-selective ET receptor agonist. It is a 21 amino acid polypeptide that is highly selective for both the ETA and the ETB receptor (IC$_{50}$: ETA = 0.12 nM, ETB = 0.02 nM [96]) Its binding to its receptors is non-competitive.

In forearm studies (Chapter 3), ET-1 was dissolved in saline 0.9% and infused at a locally active dose of 5 pmol/min via the brachial artery for 90 min. This dose was selected from previous studies showing that ET-1 at this dose causes slow onset vasoconstriction of up to 40% in human resistance vessels [191].

In systemic studies (Chapter 8), it was infused at a dose of 2.5 ng/Kg/min for 60 min [118, 332]. The systemic effects of this dose are minimal [118]. The renal haemodynamic effects of this dose (-20% fall in RBF) return to baseline within 30 min of ceasing the infusion [118, 139] and are blocked by low dose BQ-123 [139].

2.2.2 BQ-123

BQ-123 (Clinalfa AG, Laufelfingen, Switzerland) was used as a selective ETA receptor antagonist (Chapters 3 - 11). It is a synthetic derivative of BE 18257B, a product of *Streptomyces misakiensis* and is a cyclic pentapeptide that is highly selective for the ETA receptor (IC$_{50}$: ETA = 7.3 nM, ETB = 18 μM [93]). Studies with radiolabelled BQ-123 demonstrate that it binds competitively to the ETA receptor, achieving steady state within 7 minutes of injection and dissociates with a half-life of 1.4 min [94]. It is extracted from the circulation by the hepatic anion transport system [97]. It does not inhibit the binding of ANG II, calcitonin gene-related peptide or peptide YY to their respective receptors [93], and has been specifically shown not to bind to ANG II receptors [333].
In study 1 (Chapter 3), I sought to establish a dose response curve for BQ-123 in healthy humans. Doses ranged from 100 - 3000 nmol min\(^{-1}\) for 15 min. The starting dose was selected from a previous study investigating the local effects of BQ-123, which suggested that 100 nmol min\(^{-1}\) is the threshold around which systemic effects might be observed [191].

On the basis of this dose response curve, in subsequent studies (Chapter 4 - 11), a dose of 100 nmol/min for 15 min was used as a threshold dose for systemic ETA blockade, and 1000 nmol/min over 15 min as that achieving maximal haemodynamic effects. The haemodynamic effects of 1000 nmol/min for 15 min are demonstrable for ~2 hours after injection. BQ-123 is undetectable in the plasma 45 min after the 100 nmol/min dose and 150 min after the 1000 nmol/min dose.

### 2.2.3 BQ-788

BQ-788 (Clinalfa AG, Laufelfingen, Switzerland) was used as a selective ETB receptor antagonist (Chapters 5, 6, 10). It is a linear tripeptide that is highly selective for the ETB receptor (IC\(_{50}\): ETA = 280 nM, ETB = 1.2 nM [96]). It is specific, (it does not inhibit the binding of ANG II, calcitonin gene-related peptide or peptide YY to their respective receptors [96]), and is a competitive inhibitor of the ETB receptor.

BQ-788 was infused at 30 and 300 nmol/min for 15 min, doses shown to be haemodynamically active in a previous systemic dose-ranging study [210].

### 2.2.4 Angiotensin II

ANG II (Clinalfa AG, Laufelfingen, Switzerland) (Chapter 4) was administered in ascending doses of 1, 3 and 6 ng/kg/min for 15 min at each dose. The doses were chosen from previously published studies [334, 335], and pilot studies in the department, to achieve a maximum increase in MAP of ~20 mmHg.
2.2.5 Noradrenaline

Noradrenaline (Sanofi Winthrop, Guildford, UK) (Chapter 4) ANG II (Clinalfa AG, Laufelfingen, Switzerland) was administered in ascending doses of 60, 120 and 210 ng/kg/min for 15 min at each dose as a control pressor agent. Doses of NE were selected from a previously published study [336], and confirmed by pilot studies in the department to give an equivalent pressor response to that achieved with the chosen doses of ANG II.

2.2.6 Enalapril

Enalapril (Dexcel Pharma Ltd, Daventry, UK) used as an ACE inhibitor (Chapter 6 - 8). It is a non-sulphydryl derivative of the amino acids L-proline and L-alanine. It reduces concentrations ANG II by inhibiting the conversion of ANG I to ANG II by ACE. ACE is also identical to kininase II that breaks down bradykinin. Some of the effects of ACE inhibitors may therefore be mediated by increased concentrations of bradykinin.

After oral dosing, ~60% of the dose of enalapril is absorbed, reaching peak plasma concentrations at 1 hour. It has low ACE inhibitory activity but is hydrolysed by liver esterases to form enalaprilat, the active drug (enalaprilat is poorly absorbed from the GI tract). Enalaprilat reaches peak plasma concentrations ~3-4 hours after administration of enalapril [337] and has a half-life of ~11 hours in the circulation. Excretion is primarily renal, and consequently delayed in renal failure. Steady state is achieved after 3-4 days administration in healthy volunteers [338].

Haemodynamically, enalapril causes an increase in arterial compliance, a reduction in systemic vascular resistance and a reduction in blood pressure. In healthy volunteers, RBF increases and renal vascular resistance falls without significant changes in GFR. [338]. Enalapril also produces a moderate increase in urinary sodium excretion 6-10 hours after oral administration [339]. In terms of hormonal effects, acutely, plasma renin activity increases while ANG II and aldosterone concentrations are reduced [340].
Plasma ACE inhibition is maximal between 2-10 hours after oral dosing, with 20 mg producing 80-95% ACE inhibition [339, 341, 342]. This ACE inhibition has been shown to be sustained over extended periods of up to 6 months of chronic dosing [338]. However, while a single dose of enalapril will still produce substantial ACE inhibition after 24 hours, ANG II concentrations have returned to baseline by this time [340]. Maximal increases in urinary sodium excretion, and renal haemodynamics do not occur until 72 hours of treatment [343].

Thus, I chose to use a dose of 20 mg twice daily for 5 days before each study because 20 mg ensures maximal achievable ACE inhibition, twice daily dosing ensures continued ANG II suppression, pre-treatment allows steady state to be reached and maximal renal changes to develop, and pre-treatment does not result in an attenuation of ACE inhibition. The final dose of 20 mg was given at 0830 in each study day. Baseline measurements are therefore 2.5 - 3.5 hrs after administration, and the study measurements are made from 3.5 - 7.5 hr after administration. Thus, ACE inhibition was maximal throughout the period under study.

### 2.2.7 L-NMMA

L-\(\text{NG}^\text{G}\)-monomethyl-arginine (L-NMMA) (Clinalfa AG, Laufelfingen, Switzerland) was used as an inhibitor of nitric oxide synthase (NOS) activity (Chapter 7). It is a specific substrate analogue that acts as a competitive inhibitor NOS in humans.

A dose of 3 mg/Kg over 5 min was used in the studies. Based on previous studies, this dose is sufficient to cause a small transient increase in blood pressure (7-10%) and decrease in RBF (-10%) with no adverse effects noted. After a 5 min infusion, blood pressure returns to baseline by 30 min and RBF returns to baseline by 60 min. The plasma half life of L-NMMA given in this form is 75 min [191, 193, 344].

### 2.2.8 Indomethacin

Indomethacin (Cox Pharmaceuticals, Devon, UK) is a cycloxygenase (COX) inhibitor that prevents prostaglandin formation (Chapter 7). It was administered as a
single 100 mg dose at study start. This is sufficient to significantly reduce urinary prostaglandin formation [345]. Subjects were specifically instructed to eat breakfast on each study day, therefore, it has not been given on an empty stomach. None of the subjects recruited had dyspepsia or a history of peptic ulcer disease.

2.2.9 Slow sodium

Slow Sodium tablets (Novartis Pharmaceuticals, Frimley, UK), each containing 10 mmol of sodium, were used to supplement dietary sodium intake in high salt studies (Chapter 9).

2.2.10 PAH

Para-aminohippurate sodium (PAH, Clinalfa AG) was used for the measurement of renal plasma flow by standard clearance techniques [346] (Chapter 5 - 9, 12, 13). It is an inert and non-toxic compound that is both filtered at the glomerulus and actively secreted by the proximal tubules, reaching the kidney only via the bloodstream. The extraction by the kidneys in a single transit is not complete (the full criteria for a marker of RBF by clearance) but about 80-90%, thus measurements are quoted as "effective" renal plasma flow (ERPF). This extraction rate is not affected by BQ-123, BQ-788 or ET-1 in man [141].

PAH was administered as a bolus loading dose of 0.4 g in 100 ml dextrose 5 over 15 min, and a maintenance infusion of 6.6 g/L at a rate of 2 ml/min. For subjects with a calculated GFR <40 ml/min, doses were reduced by a third.

2.2.11 Inulin

Inutest (Fresenius Pharma, Austria GmbH) was used for the measurement of GFR by standard clearance techniques [346] (Chapter 5 - 10, 12, 13). Inulin is an inert and non-toxic complex polyfructose with a molecular weight of 5,200 daltons. It is not protein bound, is freely filtered at the glomerulus, is neither secreted not reabsorbed within the tubules, nor metabolised within the kidney and hence fulfills the criteria for the measurement of GFR by clearance measurements. Its problems with
solubility have been overcome by the introduction of Sinistrin (Inutest) a related polysaccharide with identical clearance.

Inutest was administered as a bolus loading dose of 3.5 g in 100 ml dextrose 5 over 15 min, and a maintenance infusion of 10 g/L at a rate of 2 ml/min. For subjects with a calculated GFR <40 ml/min, doses were reduced by a third.

2.2.12 Cyclosporin

Cyclosporin Neoral (Novartis Pharmaceuticals, Frimley, UK) is a fungal peptide whose main target is the T lymphocyte, producing immunosuppressive activity. It is also known to have significant effects on blood pressure via actions on the sympathetic nervous system and the vascular endothelium and smooth muscle cells.

To study its vascular effects, healthy subjects CyA liquid, 10 mg/kg, or placebo (olive oil) administered in orange juice to disguise the taste (Chapter 12). This dose was chosen from a previous study demonstrating an acute, but transient decline in GFR after oral administration [347]. In patients, CyA was administered as a single dose of 100 mg (Chapter 13). This dose was chosen to be in line with standard doses taken by transplant patients on chronic therapy.

2.3 Haemodynamic measurements

2.3.1 Forearm blood flow

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic gauges [348] that were securely applied to the widest part of each forearm. The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mm Hg. Upper arm cuffs were intermittently inflated to 40 mm Hg for 10 in every 15 sec to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Recordings of forearm blood flow were made repeatedly at 10 min intervals over 3-minute periods. Voltage output from a dual-channel Vasculab SPG 16 strain gauge plethysmograph (Medasonics Inc) was transferred to a Macintosh
personal computer (Performa 475, Apple Computer Inc, Cupertino, CA, USA) using a MacLab analogue digital converter and Chart software (v. 3.2.8; both from AD Instruments, Castle Hill, NSW, Australia). Calibration was achieved using the internal standard of the Vasculab plethysmography units.

### 2.3.2 Blood pressure

Blood pressure was recorded in duplicate at each time-point using a well-validated semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA-751 sphygmomanometer, Takeda Medical Inc) [349]. Recordings were required to be within 10 mmHg of each other (systolic and diastolic). If not, blood pressure was repeated until two consecutive readings did fulfil these criteria. During forearm studies (Chapter 3), blood pressure was recorded in the dominant arm (ie. not in the arm with intra-arterial cannulation.)

### 2.3.3 Cardiac output and heart rate

Cardiac output (CO, L/min) and heart rate (HR, bpm) were recorded using a well validated non-invasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd, Irvine, California, USA) [350, 351]. This non-invasive technique measures CO by transthoracic bioimpedance. A constant sinusoidal current is applied through dual electrodes situated at the root of the neck bilaterally and to the lateral aspect of the trunk at the level of the xiphisternum. These electrodes then detect changes in bioimpedance related to the cardiac cycle and blood flow. CO is estimated from the measures of bioimpedance by the Sramek-Bernstein formula, adapted from the original formula of Kubicek [351]. HR is counted directly from detection of the cardiac electrical cycle. Each reading is the average of 15 consecutive beats. Four such readings were recorded for each measurement of CI and HR.

Bioimpedance is useful for the measurement of changes in CO in an individual in acute interventional studies [352] and is best expressed as percentage change from baseline [351]. However, bioimpedance derived CO does not correlate well with CO measured by invasive methods. I have demonstrated, against thermodilution as a
gold standard, that it is least accurate at high CO values or when the % change from baseline is high. In these circumstances, it tends to underestimate the degree of change. As such it produces conservative estimates of changes in CO (Fig 2.1).

**Fig 2.1 Comparison of cardiac output measured by thermodilution and bioimpedance**

Data presented from a study performed in the Clinical Research Centre in patients with heart failure treated with endothelin antagonists (Study by S. Leslie). Subjects had CO measured by thermodilution during right heart catheterisation and by bioimpedance. Comparison of these two measures demonstrated good correlation over a wide range of CO. Bioimpedance did, however, underestimate high CO values when compared to Swan Ganz thermodilution measurements. A similar pattern was seen when change from baseline was compared. Again bioimpedance underestimated large changes in CO. Thus, in providing a conservative estimate, bioimpedance is less likely to produce false positive results when examining changes in CO after vasoactive agents.

**2.3.4 Clearance Studies**

ERPF and GFR were measured by standard clearance techniques [346] (Chapter 5 - 10, 12, 13). On each study day, an 18 standard wire gauge (SWG) cannulae was sited in an antecubital vein in each arm. Diuresis was induced by 500 ml 5% dextrose over 30 min through the left arm cannula. After 15 min, the loading doses of PAH & inutest were administered through the same cannula. Thereafter, maintenance infusions of PAH and inutest, and 5% dextrose at 260 ml/hr continued throughout the study. Urine was collected by spontaneous voiding every 30 min. A two hour period was allowed for water, inutest and PAH equilibration before baseline measurements, made over two 30 min urine collection periods. Blood pressure, CO
and HR were recorded every 15 min. At the mid-point of each 30 min collection period, blood was sampled from the right antecubital cannula for PAH, inulin, and haematocrit measurements.

2.4 Assays

At pre-specified time points, venous blood was collected via an 18 SWG cannula for plasma measurements. Blood was collected into plain tubes (Sarstedt) for the measurement of serum sodium, and into EDTA tubes (Sarstedt) for all other plasma measurements. 20 ml aliquots of urine from each voiding were collected into plain tubes for the urinary measurements. At pre-specified time points, 20ml aliquots of urine were also collected into plain tubes containing 2.5ml of 50% acetic acid for the measurement of urinary ET-1.

Haematocrit (Hct) was measured on whole blood. All other blood samples were centrifuged immediately at 1000 g at 4°C for 20 min, and plasma and urine stored in plain tubes at −80°C until assay.

2.4.1 Plasma and urinary inulin

Inulin was determined by spectrophotometry after hydrolysis to fructose. Plasma samples were deproteinised with equal volumes of 6% perchloric acid and, after centrifugation at 1000 g for 10 min, supernatant was decanted. Urine was diluted 1/20 with 3% perchloric acid. Resorcinol (1.5 g dissolved in 1 l of ethanol) and HCl/FeCl₃ solution (7.5 mg FeCl₃ dissolved in 1 l of molar hydrochloric acid) were added in a 6:6:1 ratio to the plasma/urine. The samples were then vortexed and incubated at 80°C for 40 min. Inulin concentrations were then determined against standard curves by absorption spectrophotometry at 480 nm.

2.4.2 Plasma and urinary PAH

PAH was determined by high performance liquid chromatography (HPLC) with fluorescence detection. Plasma samples were deproteinised with equal volumes of 6% perchloric acid and, after centrifugation at 1000 g for 10 min, supernatant was
diluted by 1/40 with deionised water. Urine samples were diluted by 1/4000. Dihydroxybenzylamine hydrobromide (DHBA) was used as an internal standard. Samples were injected into the HPLC column. The HPLC system consisted of a Waters 510 HPLC pump, WISP (Waters Intelligent Sample Processor) and Spherisorb S5 ODS column (Waters Ltd, Watford, Herts. UK) with detection by an LS-5 fluorometric detector (Perkin-Elmer Ltd, Beaconsfield, Bucks, UK), with excitation and emission wavelengths of 280 nm and 360 nm respectively. The mobile phase consisted of 0.1 molar citrate acetate buffer containing 100 mg/L octane sulphonic acid.

2.4.3 BQ-123

BQ-123 concentrations in plasma were measured by HPLC with fluorescence detection. One volume of plasma was precipitated with 4 volumes of ethanol, ultracentrifuged at 4°C for 15 min at 10,000 g, and the resulting supernatant injected into the HPLC column. The HPLC system consisted of a Waters 510 HPLC pump, WISP (Waters Intelligent Sample Processor) and Spherisorb S5 ODS column (Waters Ltd, Watford, Herts. UK) with detection by an LS-5 fluorometric detector (Perkin-Elmer Ltd, Beaconsfield, Bucks, UK), with excitation and emission wavelengths of 284 nm and 348 nm respectively. The mobile phase consisted of 60:40 acetonitrile: de-ionised water with tri-fluoroacetic acid at a concentration of 0.1%. The peptide TAK-044 was found to fluoresce at identical wavelengths to BQ-123 and was eluted from the column with a retention time similar to but not identical with BQ-123, allowing its separate measurement. Hence, TAK-044 was used as a standard in this assay. 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.

2.4.4 Haematocrit

Hct was measured using a Coulter counter.
2.4.5 Hormone measurements
After extraction with acetic acid using Bond Elut® columns (Varian, harbour City, CA, USA), [353] plasma ET-1 and ANG II concentrations were determined by standard radioimmunoassay (Peninsula Laboratories Europe, St Helens, UK), as previously described [354-356]. Plasma renin activity (PRA) was measured under standard conditions through the generation of ANG I as determined by radioimmunoassay [357]. Plasma aldosterone concentrations were measured by radioimmunoassay [358].

2.4.6 Serum ACE activity
Serum ACE activity was determined spectrophotometrically using a Sigma Diagnostics kit (Sigma Diagnostics St Louis, US). ACE hydrolyses the synthetic tripeptide N-[3-(2-furyl)acryloyl]-L-phenylalanylglyglylglycine (FAPGG) to furylacryloylphenylalanine (FAP). This conversion results in a decrease in absorbance at 340 nm that can be calibrated against a standard sample to determine ACE activity. One unit of ACE activity is defined as that amount of enzyme that will catalyse the formation of one micromole of FAP from FAPGG per minute at 37°C. [359]

2.4.7 Plasma and urinary sodium
Urinary and plasma sodium concentrations were measured by flame photometry. Concentrations were determined against standard curves described by a quadratic equation. Plasma samples were diluted by 1/100 and compared against a standard curve ranging from 0 - 2 mmol/L. Urine samples were compared against a standard curve ranging from 0 - 40 mmol/L. Concentrated urine samples were diluted into this range as required.

2.4.8 Plasma and urinary osmolality
Urinary and plasma osmolality was measured by freezing point depression using a standard osmometer.
2.4.9 Urinary protein

Urinary albumin concentrations were measured using a double antibody technique (EURO/DPC Ltd, Llanberis, Gwynedd, UK) where $^{125}$I labelled albumin competes with albumin in the sample for antibody sites. Separation of bound from free albumin is then achieved with a second antibody and the antibody bound fraction precipitated and radioactivity counted. Samples were compared against standard curves ranging from 5 - 60 μg/ml. Concentrated urine samples were diluted into this range as required. The assay has a detection limit of 0.3 μg/ml.

2.5 Data analysis

All results are expressed as mean ± standard error of the mean (SEM).

2.5.1 Forearm Blood Flow

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 flow only stabilises after 60 sec of wrist cuff inflation, recordings made in the first 60 sec were not used for analysis. The last 5 flow recordings in each measurement period were calculated and averaged for the infused and non-infused arms [360]. To reduce the variability of blood flow data, the ratio of flows in the 2 arms was calculated for each time point, in effect using the non-infused arm as a contemporaneous control for the active treatment arm [348]. Forearm blood flow 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.

2.5.2 Systemic and Renal Data

Data were stored and analysed using the Microsoft Excel data analysis package (Excel 5.0, Microsoft Ltd). Blood pressure at each time point was calculated as the
mean of 2 recordings and represented as mean arterial pressure (MAP). Biompedance data at each time point were calculated as the mean of four recordings, each the average of 15 consecutive heart beats. Data were corrected using body surface area to give cardiac index (CI) for direct comparison of subjects, and systemic vascular resistance index (SVRI) derived from blood pressure and CI data (Table 2.1). GFR and ERPF were calculated from inulin and PAH clearances respectively. Effective renal blood flow (ERBF), effective renal vascular resistance (ERVR) and effective filtration fraction (EFF) were derived from these indices (Table 2.1). Urinary sodium excretion and fractional excretion were calculated from plasma and urinary sodium and inulin concentration and urinary flow rates.

Demographic data are expressed as mean ± standard error of the mean (SEM) and comparisons between groups examined by unpaired Student’s t-tests. Baseline data were calculated as the mean of the 2 time points immediately preceding administration of the first study drug. Haemodynamic results are expressed as mean ± SEM placebo-corrected maximum (%) change from baseline. Statistical analysis was performed on untransformed data. Responses were examined by repeated measures analysis of variance (ANOVA) and Bonferroni correction was used to assess significance at specific time points. Area under the curve was calculated as a summary statistic of each curve and comparisons analysed by paired Student’s t-tests. Statistical significance was taken at the 5% level.
Table 2.1  Calculations

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Calculation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Arterial Pressure (MAP)</td>
<td>dBp + (sBP-dBP)</td>
<td>mmHg</td>
</tr>
<tr>
<td>Systemic Vascular Resistance Index (SVRI)</td>
<td>MAP x 80</td>
<td>dyne.s/m²/cm²</td>
</tr>
<tr>
<td>Glomerular Filtration Rate (GFR)</td>
<td>UIn x UFR</td>
<td>ml/min</td>
</tr>
<tr>
<td>Effective Renal Plasma Flow (ERPF)</td>
<td>UPAH x UFR</td>
<td>ml/min</td>
</tr>
<tr>
<td>Effective Renal Blood Flow (ERBF)</td>
<td>PPAH</td>
<td>ml/min</td>
</tr>
<tr>
<td>Effective Renal Vascular Resistance (ERVR)</td>
<td>MAP x 80</td>
<td>mmHg/min/L</td>
</tr>
<tr>
<td>Effective Filtration Fraction (EFF)</td>
<td>GFR x 100%</td>
<td>%</td>
</tr>
<tr>
<td>Urinary Flow Rate (UFR)</td>
<td>Urinary Volume</td>
<td>ml/min</td>
</tr>
<tr>
<td>Urinary Sodium Excretion (UNaV)</td>
<td>UNa x UFR</td>
<td>(μmol/min)</td>
</tr>
<tr>
<td>Fractional Excretion of Sodium (FeNa)</td>
<td>UNa x PIn</td>
<td></td>
</tr>
<tr>
<td>Free water clearance (FWC)</td>
<td>UFR x (1 - U/Osm)</td>
<td>ml/min</td>
</tr>
</tbody>
</table>

dBp - diastolic blood pressure, sBP - systolic blood pressure, U - urine, P - plasma, In - Inulin, PAH - para-amino hippuric acid, Hct - haematocrit, Na - sodium, Osm - osmolality
Chapter 3

Investigation of the effects of systemic ETA receptor antagonism in healthy volunteers
3.1 Introduction

Local studies in human forearm resistance vessels using phosphoramidon, an ECE inhibitor, and BQ-123, a selective ETA receptor antagonist, first demonstrated the importance of ET-1 in maintaining basal resistance vessel tone, in large part through an action on the ETA receptor [191]. These observations have since been confirmed by others [192, 194]. Responses in the forearm resistance vessels are usually predictive of those in the systemic circulation [360], so these data suggested that systemic ETA receptor antagonism would produce systemic vasodilatation. However, acute systemic administration of the selective ETA antagonist, BQ-123, was reported to have no effect on basal systemic haemodynamics [139, 199].

It is possible that a systemic haemodynamic effect of ETA receptor antagonism was not seen in these studies because the doses of BQ-123 used provided insufficient ETA receptor blockade to affect blood pressure. In addition, because healthy subjects have a number of reflex mechanisms that serve to defend blood pressure, it is possible that an important effect on systemic vascular resistance might have been missed by measurement of blood pressure alone. This chapter therefore describes two studies in healthy volunteers designed to address the issue of the role of the ETA receptor in the maintenance of vascular tone in healthy human subjects. The first study examines the haemodynamic effects of increasing doses of BQ-123, using bioimpedance cardiography to allow measurement of SVRI, with the aim of achieving a high degree of ETA selective receptor blockade. The second examines 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.

3.2 Study design

3.2.1 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 for 15 min) and placebo. 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 (Table 3.1). Each visit was separated by at least 5 days. Subjects rested supine for 20 min, and baseline measures were then made in the 30 min before study drug administration. 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. Venous blood was obtained at 5, 15 and 240 min after BQ-123 infusion for measurement of plasma ET-1 and big ET-1. During the 300 and 1000 nmol/min phases, sub-aliquots of the samples were used for plasma BQ-123 assay.

Table 3.1  Systemic haemodynamic study randomisation

<table>
<thead>
<tr>
<th></th>
<th>Period 1</th>
<th>Period 2</th>
<th>Period 3</th>
<th>Period 4</th>
<th>Period 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteer 1</td>
<td>Placebo</td>
<td>100 nmol/min</td>
<td>300 nmol/min</td>
<td>1000 nmol/min</td>
<td>3000 nmol/min</td>
</tr>
<tr>
<td>Volunteer 2</td>
<td>100 nmol/min</td>
<td>Placebo</td>
<td>300 nmol/min</td>
<td>1000 nmol/min</td>
<td>3000 nmol/min</td>
</tr>
<tr>
<td>Volunteer 3</td>
<td>100 nmol/min</td>
<td>300 nmol/min</td>
<td>Placebo</td>
<td>1000 nmol/min</td>
<td>3000 nmol/min</td>
</tr>
<tr>
<td>Volunteer 4</td>
<td>100 nmol/min</td>
<td>300 nmol/min</td>
<td>1000 nmol/min</td>
<td>Placebo</td>
<td>3000 nmol/min</td>
</tr>
<tr>
<td>Volunteer 5</td>
<td>100 nmol/min</td>
<td>300 nmol/min</td>
<td>1000 nmol/min</td>
<td>3000 nmol/min</td>
<td>Placebo</td>
</tr>
</tbody>
</table>

Using MAP & SVRI measurements from a previous placebo-controlled study over 4 h [210], 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.
3.2.2 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 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) 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 for a total of 90 min via a left brachial artery cannula. Recordings of FBF by venous occlusion plethysmography were made repeatedly at 10 min intervals over 3-minute periods.

From FBF measurements in a previous study using ET-1 at 5 pmol/min [361], 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.

3.3 Results

3.3.1 Systemic haemodynamic study

All 5 subjects (mean age 26 ± 2 yr, range 18 - 30 yr) 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 and 25 to 42 pg/ml 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 3.2 & 3.3).

Plasma BQ-123 concentrations

Plasma concentrations of BQ-123 were undetectable with both doses at baseline. For 300 nmol/min, mean plasma concentrations were 126 ± 11 nmol/l at 5 min rising to
174 ± 20 nmol/l at 15 min. For 1000 nmol/min, they were 424 ± 33 nmol/l and 510 ± 64 nmol/l respectively (ETA receptor IC<sub>50</sub> 7.3 nM; ETB receptor IC<sub>50</sub> 18 µM) [93]. BQ-123 was no longer detectable in the plasma by 4 h at either dose.

### Table 3.2 Plasma concentrations of ET-1 (pg/ml) after administration of BQ-123

<table>
<thead>
<tr>
<th>BQ-123 (nmol/min)</th>
<th>Placebo</th>
<th>100</th>
<th>300</th>
<th>1000</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>4.91 ± 0.69</td>
<td>4.57 ± 0.48</td>
<td>5.23 ± 0.52</td>
<td>4.43 ± 0.28</td>
<td>2.88 ± 0.10</td>
</tr>
<tr>
<td>5 min</td>
<td>5.16 ± 0.74</td>
<td>4.08 ± 0.39</td>
<td>5.29 ± 0.49</td>
<td>6.07 ± 0.49</td>
<td>4.08 ± 0.49</td>
</tr>
<tr>
<td>15 min</td>
<td>5.81 ± 0.50</td>
<td>3.95 ± 0.65</td>
<td>5.49 ± 0.95</td>
<td>5.56 ± 0.95</td>
<td>4.03 ± 0.63</td>
</tr>
<tr>
<td>240 min</td>
<td>5.77 ± 0.75</td>
<td>4.44 ± 0.29</td>
<td>5.88 ± 0.65</td>
<td>5.80 ± 0.65</td>
<td>4.22 ± 0.41</td>
</tr>
</tbody>
</table>

### Table 3.3 Plasma concentrations of big ET-1 (pg/ml) after administration of BQ-123

<table>
<thead>
<tr>
<th>BQ-123 (nmol/min)</th>
<th>Placebo</th>
<th>100</th>
<th>300</th>
<th>1000</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>45.7 ± 14.4</td>
<td>37.8 ± 7.4</td>
<td>40.2 ± 6.5</td>
<td>57.5 ± 9.1</td>
<td>26.6 ± 6.7</td>
</tr>
<tr>
<td>5 min</td>
<td>49.3 ± 10.7</td>
<td>29.2 ± 1.9</td>
<td>38.5 ± 6.3</td>
<td>51.9 ± 8.5</td>
<td>34.7 ± 0.6</td>
</tr>
<tr>
<td>15 min</td>
<td>66.2 ± 14.4</td>
<td>26.0 ± 2.4</td>
<td>49.5 ± 14.1</td>
<td>51.4 ± 9.4</td>
<td>30.7 ± 3.1</td>
</tr>
<tr>
<td>240 min</td>
<td>38.8 ± 7.1</td>
<td>35.8 ± 5.2</td>
<td>48.4 ± 6.0</td>
<td>51.5 ± 9.2</td>
<td>30.1 ± 4.6</td>
</tr>
</tbody>
</table>
**Haemodynamic parameters**

Baseline measurements for haemodynamic parameters were similar during all treatment periods (Table 3.4). 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 HR, which were maximal at 15 min.

### Table 3.4 Baseline haemodynamic measurements

<table>
<thead>
<tr>
<th>BQ-123 (nmol/min)</th>
<th>Placebo</th>
<th>100</th>
<th>300</th>
<th>1000</th>
<th>3000</th>
<th>ANOVA baseline data</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>78.9 ± 1.5</td>
<td>79.2 ± 2.3</td>
<td>80.8 ± 3.5</td>
<td>78.2 ± 2.1</td>
<td>78.6 ± 3.3</td>
<td>p=0.96</td>
</tr>
<tr>
<td>SVRI (dyne.sec/cm²/m²)</td>
<td>1816 ± 104</td>
<td>1752 ± 136</td>
<td>1774 ± 144</td>
<td>1800 ± 232</td>
<td>1880 ± 176</td>
<td>p=0.98</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>3.52 ± 0.21</td>
<td>3.69 ± 0.25</td>
<td>3.80 ± 0.31</td>
<td>3.63 ± 0.38</td>
<td>3.45 ± 0.35</td>
<td>p=0.89</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>64.2 ± 6.6</td>
<td>57.1 ± 5.2</td>
<td>63.9 ± 6.6</td>
<td>54.5 ± 4.8</td>
<td>57.3 ± 5.9</td>
<td>p=0.69</td>
</tr>
</tbody>
</table>

MAP decreased in a dose-dependent fashion. This was statistically significant at 300, 1000 and 3000 nmol/min BQ-123 (300 nmol/min: ANOVA p<0.05 vs. placebo, 1000 & 3000 nmol/min: p<0.01 vs. placebo) with a maximum mean placebo-corrected reduction of 12.4 ± 3.5% after 3000 nmol/min. 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 of BQ-123 (Table 3.5 & Fig 3.1). CI and HR increased significantly at all doses (p<0.01; ANOVA, Table 3.5 & Fig 3.1).
Table 3.5 Systemic haemodynamic responses to BQ-123

<table>
<thead>
<tr>
<th>BQ-123</th>
<th>100</th>
<th>300</th>
<th>1000</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>-4.8 ± 2.6%</td>
<td>-6.8 ± 3.6%</td>
<td>-8.2 ± 3.1%</td>
<td>-12.4 ± 3.5%</td>
</tr>
<tr>
<td></td>
<td>(-4.0 ± 2.5)</td>
<td>(-5.4 ± 2.8)</td>
<td>(-6.4 ± 2.4)</td>
<td>(-10.1 ± 3.2)</td>
</tr>
<tr>
<td>SVRI</td>
<td>-15.8 ± 7.6%</td>
<td>-20.6 ± 3.0%</td>
<td>-22.7 ± 5.2%</td>
<td>23.3 ± 4.3%</td>
</tr>
<tr>
<td></td>
<td>(-312 ± 168)</td>
<td>(-360 ± 56)</td>
<td>(-432 ± 152)</td>
<td>(-448 ± 208)</td>
</tr>
<tr>
<td>CI</td>
<td>14.3 ± 8.9%</td>
<td>13.0 ± 2.2%</td>
<td>17.9 ± 5.7%</td>
<td>12.7 ± 1.6%</td>
</tr>
<tr>
<td></td>
<td>(0.49 ± 0.32)</td>
<td>(0.47 ± 0.07)</td>
<td>(0.59 ± 0.16)</td>
<td>(0.43 ± 0.04)</td>
</tr>
<tr>
<td>HR</td>
<td>23.8 ± 13.4%</td>
<td>11.8 ± 4.4%</td>
<td>20.0 ± 5.9%</td>
<td>18.7 ± 6.8%</td>
</tr>
<tr>
<td></td>
<td>(12.1 ± 6.5)</td>
<td>(6.8 ± 2.4)</td>
<td>(11.6 ± 3.0)</td>
<td>(10.7 ± 3.1)</td>
</tr>
</tbody>
</table>

Results given are maximum placebo corrected percentage change from baseline ± SEM. * p<0.05 vs. placebo, † p<0.01 vs. placebo: ANOVA + Bonferroni correction
Figure 3.1 Systemic haemodynamics with ascending doses of BQ-123

- MAP
- SVRI
- CI
- HR

Doses:
- 100 nmol/min
- 300 nmol/min
- 1000 nmol/min
- 3000 nmol/min
3.3.2 ET-1 challenge study

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 BQ-123 (-27 ± 8% at 90 min p>0.5 vs. placebo) and abolished by 1000 nmol/min BQ-123 (-8% ± 3%, p<0.01 vs. placebo, Fig 3.2).

Figure 3.2 Response in forearm blood flow to intra-arterial ET-1, when pre-treated with placebo or BQ-123 (300 or 1000 nmol/min for 15 min)

3.4 Discussion

This study demonstrates that, in healthy humans, BQ-123 causes substantial systemic vasodilatation, associated with a small but significant reduction in arterial blood pressure. A dose-dependent effect was observed, with little additional effect occurring above 1000 nmol/min. This work confirms the importance of the ET system, and of the vascular ETA receptor in controlling vascular tone and blood
pressure [36], and is in accord with the results of previous local forearm infusion studies [191, 192, 194].

There are several reasons for concluding that the effects on vascular tone and blood pressure are mediated by the ETA receptor. Measured BQ-123 concentrations in plasma at both 300 and 1000 nmol/min were substantially greater than the IC$_{50}$ for BQ-123 at the ETA receptor. Even so, at 1000 nmol/min the plasma concentration of 510 nmol l$^{-1}$, was more than 35 fold lower than the IC$_{50}$ for the ETB receptor (18 μM), consistent with effective but selective ETA receptor blockade [93]. In addition, there is a substantial body of evidence that the ETB receptor is a clearance receptor for ET-1 [362-364] and that agents that block the ETB receptor in vivo cause increases in plasma ET-1 concentrations [201, 202, 210, 293]. 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. Finally, it has previously been shown that the net effect of systemic ETB receptor blockade is to cause systemic vasoconstriction [210], which would suggest that any ETB blockade would attenuate the vasodilatation associated with ETA 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 ETB receptor at this dose.

Doses sufficient to lower blood pressure (300 & 1000 nmol/min) also antagonised the forearm vasoconstriction to brachial artery administration of ET-1 and, in keeping with its submaximal effect on SVRI, the lower dose of BQ-123 (300 nmol/min) only partially antagonised forearm vasoconstriction to ET-1. Of note, however, in the presence of a higher degree of ETA blockade, exogenous ET-1 failed to produce vasodilatation via the unblocked ETB receptor. This possibly reflects the local balance of dilator and constrictor effects mediated by endothelial and vascular smooth muscle ETB receptors. However it is also possible that the locally administered ET-1 was washed out by an increase in forearm blood flow consequent upon the vasodilatation induced by systemic BQ-123. Comparison with a
constrictor agent unaffected by ET-A antagonism would be needed to clarify this further.

Previous studies in healthy men [139, 199] failed to demonstrate a significant effect of BQ-123 on basal haemodynamics. However, the doses used were substantially lower, at 23.7 nmol/min for 60 min, followed by 94.8 nmol/min for 60 min [139] and ~9 nmol/min for 90 min [199]. 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 blood pressure but not systemic vascular resistance, 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 ET antagonists do appear to show modest (~10 mmHg) reductions in blood pressure, with both bosentan (mixed ETA/ETB) [202] and ABT-627 (ETA selective) [200]. In the latter study, although systemic haemodynamics were only recorded at 30 min and 8 hours after dosing, systemic vascular resistance was measured and significant effects on this parameter were found in both acute and chronic dosing.

It should be noted that our research group has also previously failed to detect significant systemic effects, as measured by blood pressure and HR, at our lowest dose of BQ-123 (100 nmol/min) when administered into the brachial artery [191]. These current results suggest that 100 nmol/min does have a small systemic action, most apparent in its effects on systemic vascular resistance, that was not detected in forearm studies, perhaps because the major vasodilatation is in other vascular beds. In recognition of this potential problem, our research group has more recently used a 10-fold lower dose of 10 nmol/min BQ-123 [194], as a local dose for forearm studies. The study confirms the rationale for this approach.

It is also interesting to note that a 15 min infusion of BQ-123 produces haemodynamic effects for up to 4 hours at the higher doses. Although, in this study, there are measurements of BQ-123 concentrations only at 0, 15 and 240 min,
subsequent experiments, with identical dosing schedules of BQ-123, demonstrate that the peak concentration is achieved at the end of the infusion, falls to ~10% by 30 min and is undetectable by 75 min post infusion (Studies 3 & 4, Chapters 5 & 6). This suggests that the observed responses are a pharmacodynamic effect rather than a reflection of the plasma half-life of BQ-123. This is similar to our group's experience with the non-selective ET antagonist TAK 044 where the systemic haemodynamic effects of a 15 min bolus were still observable at 24 hours, whereas the peptide had a plasma half-life of 30-60 min [201].

In the current study, there was an increase in HR similar to that observed in other acute studies [202, 312]. These effects are not generally seen in chronic dosing studies with ET antagonists in patients with either hypertension or heart failure [224, 293]. 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 mean arterial pressure, no side effects were observed or reported by the subjects.

In conclusion, this study with BQ-123 demonstrates that systemic ETA receptor antagonism causes substantial peripheral vasodilatation and modest lowering of blood pressure, consistent with an important role for the ET system in the maintenance of vascular tone in man.
Chapter 4

Actions of ETA receptor antagonism on the pressor response to angiotensin II in healthy volunteers
4.1 Introduction
ANG II is a powerful vasoconstrictor substance formed from its inactive precursor ANG I by the actions of the endothelial cell ecto-enzyme, ACE [365]. The success of ACE inhibitors in treating conditions such as hypertension and congestive heart failure underlines the importance of ANG II in pathophysiological situations involving vasoconstriction [366-368]. 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 chronic administration of, [369-373] or pre-treatment with [374] ET receptor antagonists can attenuate [370-372] or even abolish [369, 373, 374] the pressor response to chronic ANG II infusion. In contrast, acute administration of ET receptor antagonists show no effect on, [375, 376] or at best only partial blunting of [377-379] the pressor response to acute ANG II infusions.

This study investigates the effects of acute ETA receptor blockade with BQ-123 on the acute pressor response to 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 (Chapter 3). 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.

4.2 Study design
This was a double-blind, placebo-controlled study in eight subjects, who attended for a total of 4 visits. 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. A pilot study was performed in 2 subjects receiving ANG II and NE in incremental doses to confirm the equivalent pressor effect of the chosen study doses of these agents (Fig 4.1).

On each study day, subjects rested supine for 15 min before beginning the study. Saline was then infused intravenously for 30 min, followed by BQ-123 or placebo
for 15 min, then saline for a further 15 min. ANG II or NE was then administered in three incremental ascending doses for 15 min at each dose. The timing of the pressor infusions was chosen to correspond to the period of maximal vasodilatation, and hence inhibition of ETA receptor function, to this dose of BQ-123 (Chapter 3). Blood pressure and HR and CI were recorded 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. Blood was taken from the left forearm cannula for the measurement of ET-1 and ANG II before administration of BQ-123/placebo, at 30 min after BQ-123/placebo infusion and after each dose increment of ANG II/NE.

**Figure 4.1 Pilot study (n=2 subjects) to confirm equivalence of pressor effect**

![Graph showing mean arterial pressure change from baseline over time with pressor agents ANG II and NE.]

**Doses for study:** ANG II - 1, 3 and 6 ng/kg/min for 15 min at each dose, NE - 60, 120 and 210 ng/kg/min for 15 min at each dose.
4.3 Results
All 8 subjects (age 28 ± 4 yr, body mass index 24.6 ±1.0 kg/m²) completed all parts of the study. No adverse events were reported.

4.3.1 Haemodynamic parameters
Baseline haemodynamic measurements were similar on all four study days (Table 4.1).

Table 4.1 Baseline haemodynamic data

<table>
<thead>
<tr>
<th></th>
<th>ANG II + Placebo</th>
<th>ANG II + BQ-123</th>
<th>NE + Placebo</th>
<th>NE + BQ-123</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>81.5 ± 2.5</td>
<td>83.5 ± 2.5</td>
<td>84.5 ± 2.8</td>
<td>83.1 ± 3.5</td>
<td>p=0.897</td>
</tr>
<tr>
<td>SVRI (dyne.s/m²/cm²)</td>
<td>1712 ± 240</td>
<td>1736 ± 248</td>
<td>1744 ± 256</td>
<td>1792 ± 272</td>
<td>p=0.997</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>60.3 ± 1.8</td>
<td>61.3 ± 2.3</td>
<td>65.7 ± 1.9</td>
<td>63 ± 1.8</td>
<td>p=0.279</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>4.3 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>4.1 ± 0.3</td>
<td>p=0.986</td>
</tr>
</tbody>
</table>

After pre-treatment with placebo, ANG II and NE both increased blood pressure in a dose-related manner and to a similar extent (maximum increase in MAP: ANG II+placebo: 25.3 ± 2.8 mmHg, NE+placebo: 31.4 ± 5.1 mmHg, p<0.01 vs. baseline for ANG II and NE, p=0.12, ANG II vs. NE), though the mechanism of the pressor effect appeared different for ANG II and NE. The increase in MAP with ANG II was associated mainly with an increase in SVRI, whereas NE significantly increased CI, as well as SVRI.

Administration of BQ-123 was associated with a small reduction in MAP at 30 min and a subsequent significant attenuation of the observed pressor effect of both ANG II and NE (maximum response to ANG II+BQ-123: 18.8 ± 1.5 mmHg, p=0.02 vs.
placebo, NE+BQ-123: 23.0 ± 4.7 mmHg, p=0.04 vs. placebo). This attenuation was of a similar magnitude for both agents (P=0.28) (Fig 4.2). BQ-123 reduced SVRI compared to placebo (% change from baseline at 30 min; BQ-123: -3.1 ± 2.8%, Placebo: +4.9 ± 1.6%, p=0.02). The increase in SVRI observed with the pressor

**Figure 4.2 Haemodynamic response to ANG II and NE ± BQ-123**

MAP (mmHg)

<table>
<thead>
<tr>
<th>Angiotensin (ng/kg/min)</th>
<th>ANOVA p=0.022</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

Noradrenaline (ng/kg/min)

<table>
<thead>
<tr>
<th>Noradrenaline (ng/kg/min)</th>
<th>ANOVA p=0.046</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

SVRI (dynase cm²/m²)

<table>
<thead>
<tr>
<th>Angiotensin (ng/kg/min)</th>
<th>ANOVA p=0.019</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>300</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Noradrenaline (ng/kg/min)</th>
<th>ANOVA p=0.090</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>0</td>
<td>300</td>
</tr>
</tbody>
</table>

- **Placebo**
- **BQ-123**
agents was also significantly attenuated by BQ-123 in the case of ANG II (-5.7 ± 2.0: \( p=0.019 \)) but not for NE (-3.2 ± 2.1: \( p=0.090 \)) (Fig 4.2). However, the magnitude of the BQ-123 effect was not significantly different for the two agents (\( p=0.875 \)). HR decreased during both ANG II and NE infusions (maximum reduction ANG II+placebo: -3.1 ± 2.1 bpm, NE+placebo: -11.0 ± 3.3 bpm), and was not altered by pre-treatment with BQ-123 (maximum reduction ANG II+BQ-123: -2.6 ± 1.9 bpm, \( p=0.57 \) vs. placebo, NE+BQ-123: -9.9 ± 3.4 bpm, \( p=0.13 \) vs. placebo).

### 4.3.2 Plasma ET-1 and ANG II

Plasma concentrations of ET-1 were not affected by ANG II or NE infusion at any time point (Table 4.2). Plasma ANG II concentrations increased with each successive ANG II dose increment, but there were no significant differences in the plasma ANG II levels between the BQ-123 and placebo phases (Table 4.3).

#### Table 4.2 Plasma ET-1 concentrations (pg/ml)

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

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Table 4.3 Plasma ANG II concentrations (pg/ml)

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

4.4 Discussion

These studies demonstrate that the blunting effect of acute ETA 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 response is similar to that seen with BQ-123 alone (Chapter 3). This suggests that ET-1, acting through the ETA receptor, does not contribute importantly to the acute pressor response to ANG II in man.

Animal evidence suggests that ANG II can interact with the ET system. ANG II stimulates ET-1 gene transcription [21, 22, 380] and peptide secretion [33, 381] in vitro in a variety of cell types, including endothelial and vascular smooth muscle cells, probably via the AT-1 receptor [382]. In vivo, (Table 4.4) a series of studies in rats have demonstrated that pre-treatment with, or concomitant administration of, either selective ETA [374] or combined ETA/B [369, 373] receptor antagonists can abolish the pressor effects of chronically administered ANG II, although this effect is blunted [370-372] or lost [383] at higher ANG II doses. By contrast, after acute administration of ANG II, concomitant administration of both selective ETA [379]
and combined ETA/B [375, 377-379] antagonists have demonstrated either no effect on, [375] or at best only partial blunting of [377-379], the pressor response.

Indeed, in dogs, selective ETA antagonism has been shown to have no effects on the rise in blood pressure during acutely administered ANG II at doses of the pressor agent ten-fold lower than those used in chronic studies [376]. However, it should be

<table>
<thead>
<tr>
<th>Study</th>
<th>ANG II dosing Acute/Chronic</th>
<th>Dose</th>
<th>ET receptor antagonist</th>
<th>ET receptor antagonist dosing Acute/Chronic</th>
<th>Outcome on pressor response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gardiner 1999[375]</td>
<td>Acute (bolus)</td>
<td>1.25-125 pmol/kg</td>
<td>SB 209670</td>
<td>Acute</td>
<td>No effect</td>
</tr>
<tr>
<td>Boemke 2001[376]</td>
<td>Acute</td>
<td>4 &amp; 20 ng/Kg/min</td>
<td>LU 135252</td>
<td>Acute</td>
<td>No effect</td>
</tr>
<tr>
<td>Balakrishnan 1996[377]</td>
<td>Acute</td>
<td>3.9,30,90 ng/Kg/min</td>
<td>Bosentan</td>
<td>Acute</td>
<td>Partial blunting lower doses only</td>
</tr>
<tr>
<td>Heinemann 1997[378]</td>
<td>Acute (bolus)</td>
<td>0.1 – 3 nmol/Kg</td>
<td>Bosentan</td>
<td>Acute</td>
<td>Partial blunting</td>
</tr>
<tr>
<td>Rigglemann 2001[379]</td>
<td>Acute</td>
<td>0.5 &amp; 2 ng/Kg/min</td>
<td>BQ-123</td>
<td>Acute</td>
<td>Partial blunting</td>
</tr>
<tr>
<td>Casellas 1997[383]</td>
<td>Chronic</td>
<td>400 ng/Kg/min</td>
<td>Bosentan</td>
<td>Chronic</td>
<td>No effect</td>
</tr>
<tr>
<td>D’Uscio 1997[370]</td>
<td>Chronic</td>
<td>200 ng/Kg/min</td>
<td>LU 135252</td>
<td>Chronic</td>
<td>Partial blunting</td>
</tr>
<tr>
<td>Moreau 1997[371]</td>
<td>Chronic</td>
<td>200 ng/Kg/min</td>
<td>LU 135252</td>
<td>Chronic</td>
<td>Partial blunting</td>
</tr>
<tr>
<td>Barton 1998[372]</td>
<td>Chronic</td>
<td>200 ng/Kg/min</td>
<td>LU 135252</td>
<td>Chronic</td>
<td>Partial blunting</td>
</tr>
<tr>
<td>Herizi 1998[369]</td>
<td>Chronic</td>
<td>200 ng/Kg/min</td>
<td>Bosentan</td>
<td>Chronic</td>
<td>Abolished</td>
</tr>
<tr>
<td>Ortiz 2001[373]</td>
<td>Chronic</td>
<td>5 ng/Kg/min</td>
<td>Bosentan</td>
<td>Chronic</td>
<td>Abolished</td>
</tr>
<tr>
<td>Alexander 2001[374]</td>
<td>Chronic</td>
<td>50 ng/Kg/min</td>
<td>ABT-627</td>
<td>Pre-treatment</td>
<td>Abolished</td>
</tr>
</tbody>
</table>
noted that none of these studies included an ET-independent pressor agent as a control, and thus it is possible that any effects observed could be non-specific, relating to the direct vasodilatory properties of ETA and ETA/B receptor antagonists.

This interaction was explored in this study using the ETA selective receptor antagonist, BQ-123, because of evidence from systemic studies supporting the role of ETA receptors in the maintenance of vasoconstrictor tone [194] (Chapter 3) and of ETB receptors in vasodilator tone [194, 210]. Study 1 (Chapter 3) has shown that the dose of BQ-123 used in this study, 1000 nmol/min for 15 min, gives maximal functional ETA receptor blockade in healthy volunteers. As this earlier study has demonstrated that this dose of BQ-123 has a significant effect on blood pressure and systemic vascular resistance, confirmed in this study by a reduction in SVRI 15 min after the end of the BQ-123 infusion of ~8% compared to placebo, a control pressor agent was used to identify any non-specific hypotensive and vasodilatory effects of BQ-123 at this dose. NE was used as a control pressor agent because previous animal studies have shown that, while ET receptor antagonists can reduce ventricular hypertrophy induced by chronic NE infusion [384], in the acute setting, while inhibiting ANG II induced aortic ring contractions, they do not affect NE induced contractions [333]. Additionally, while ET and ANG II have been shown to act synergistically to increase blood pressure in vivo, this is not so for the combination of ET-1 and NE [385]. These data tend to support the use of NE as an ET-independent pressor agent in this acute study. As such, the similar efficacy of BQ-123 in attenuating equivalent rises in blood pressure induced by ANG II and NE suggests that the modest effect of acute ETA blockade on the pressor actions of ANG II is non-specific. Wenzel et al have recently demonstrated that BQ-123 can attenuate both ANG II and NE induced vasoconstriction in the skin microcirculation of healthy volunteers and postulate an acute ET-1 interaction with both ANG I and NE [386]. As with this study, however, the dose of BQ-123 used itself produced vasodilatation, and as such, it is equally likely that the attenuation of ANG II or NE induced vasoconstriction seen after ETA receptor inhibition is non-specific. Additionally, although the essentially paracrine nature of the ET system may makes
interpretations of changes in plasma ET-1 concentrations difficult, the failure of ANG II or NE to increase plasma ET-1 concentrations in this study also suggests that neither ANG II nor NE achieve their acute pressor effect by stimulating ET-1 generation. However, it is possible that ET-1 secretion may occur with chronic elevation of plasma ANG II and that this may influence vascular structure rather than function.

In conclusion, this study demonstrates a modest and non-specific inhibition by BQ-123 of the pressor response to ANG II in man suggesting that ANG II does not achieve its acute pressor effect via ET-1. It must be noted that this study looks only at acute effects, and further chronic interaction studies are needed in man, particularly in relation to vessel structure. Nevertheless, the lack of an acute ETA-ANG II interaction on vascular tone is consistent with clinical studies showing an added haemodynamic benefit of both selective ET\textsubscript{A} and non-selective ET\textsubscript{A/B} in patients with heart failure already treated with ACE inhibitors [293, 299]. This supports the development of ET receptor antagonists as adjunctive treatments to ACE inhibitors.
Chapter 5

Actions of ET-A and ET-B receptor antagonism on systemic and renal haemodynamics and tubular function in hypertensive patients with chronic renal failure and healthy controls
5.1 Introduction

Previous ET receptor antagonist studies in man have failed to demonstrate any role for ET-1 in the maintenance of normal renal vascular tone [120, 139-141, 199]. To date, the effects of ET receptor antagonists on either basal systemic or renal haemodynamics in CRF have not been investigated. CRF is characterised by systemic and renal vasoconstriction, and commonly associated with hypertension. Given that ET receptor antagonists cause vasodilatation [191, 201], lower blood pressure [201, 224], and also ameliorate renal dysfunction in experimental models of kidney disease [387], they may be useful in the treatment of CRF.

The first aim of this study was to investigate whether ET receptor antagonists at doses known to be maximally systemically active (Study 1, Chapter 3) would have effects in the renal circulation in healthy volunteers. Secondly, it investigates whether ET receptor blockade can produce alterations in systemic or renal haemodynamics in CRF. Because of the potential opposing effects at ETA and ETB receptors, and to establish which pattern of ET receptor blockade may be the more beneficial therapeutic combination, this study also directly compared the effects of regimens involving ETA and ETB receptor blockade separately, and in combination in CRF patients and matched healthy controls.

5.2 Study design

This was a randomised, double-blind, placebo-controlled study in 8 patients with CRF and 8 matched healthy controls. Subjects attended for 4 visits, each separated by ≥7 days, receiving placebo, BQ-123, BQ-788 or the combination of BQ-123 and BQ-788. Four comparisons of interest were pre-identified as placebo vs. BQ-123, vs. BQ-788 and vs. BQ-123/788, and BQ-123 vs. BQ-123/788.

On each study day, subjects underwent a standard clearance study. After baseline measurements, the low dose of antagonist was then administered followed by three 30 min collection periods. The higher dose of antagonist was then administered followed by 5 further 30 min collection periods. (Fig 5.1)
At 0, 60 & 90 min after the start of low and high dose antagonist, and at the end of the study, blood samples were taken for the measurement of plasma ET-1, aldosterone, ANG II concentrations and PRA. BQ-123 was measured before and at 15, 45 & 90 minutes after the start of each dose of antagonist, and at the end of the study.

### 5.2.1 Subjects

8 patients with stable CRF and 8 matched controls were recruited into the study. To enhance homogeneity and avoid other influences on vascular reactivity, patients with vasculitis, other systemic inflammatory disease, polycystic kidney disease, nephrotic syndrome or obstructive uropathy were excluded. Additionally, patients with significant co-morbid disease including diabetes mellitus, heart or lung disease, peripheral vascular disease or hypercholesterolaemia were excluded. The two study
groups were matched for age, weight, serum cholesterol and blood pressure (Table 5.1). Further patient characteristics are given in Table 5.2.

**Table 5.1 Subject demographic data**

<table>
<thead>
<tr>
<th></th>
<th>Healthy Volunteers</th>
<th>Renal Patients</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>47 ± 5 (23 - 64)</td>
<td>46 ± 5 (25 - 67)</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26 ± 2 (18 - 31)</td>
<td>27 ±1 (24 - 33)</td>
<td>NS</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>92.8 ± 3.1 (83.0 - 103.6)</td>
<td>98.8 ± 3.5 (78.8 - 109.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>85 ± 5 (62 - 111)</td>
<td>255 ± 41 (122 - 434)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Urinary Na excretion (mmol/24 hr)</td>
<td>136 ±14 (56 - 199)</td>
<td>150 ± 14 (74-246)</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.3 ± 0.3 (3.9 - 6.1)</td>
<td>5.6 ± 0.2 (4.5 - 6.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary protein excretion (mg/24 hr)</td>
<td>0†</td>
<td>476 ± 110 (27 - 2033)</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

†values below limit of laboratory assay
Table 5.2 Patient characteristics

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>CrCl (ml/min)</th>
<th>Cause of renal impairment</th>
<th>Drugs</th>
<th>Other conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>18</td>
<td>Renal calculi, single kidney</td>
<td>Enalapril, bicarbonate, 1-α calcidol</td>
<td>HT, Secondary TPTH</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>16</td>
<td>NK – late presentation</td>
<td>Lisinopril, bicarbonate, omeprazole, frusemide</td>
<td>HT</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>31</td>
<td>IgA nephropathy</td>
<td>Enalapril</td>
<td>HT</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>61</td>
<td>IgA nephropathy</td>
<td>Ranitidine</td>
<td>HT, PUD</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>36</td>
<td>Proliferative GN</td>
<td>Enalapril, metoprolol, nifedipine, allopurinol, frusemide</td>
<td>HT</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>38</td>
<td>IgA nephropathy</td>
<td>Enalapril, doxazosin, bicarbonate, omeprazole</td>
<td>HT</td>
</tr>
<tr>
<td>7</td>
<td>54</td>
<td>22</td>
<td>Proliferative GN</td>
<td>Fosinipril, atenolol</td>
<td>HT</td>
</tr>
<tr>
<td>8</td>
<td>67</td>
<td>59</td>
<td>IgA nephropathy/HSP</td>
<td>Labetalol</td>
<td>HT</td>
</tr>
</tbody>
</table>

CrCl - creatinine clearance, GN - glomerulonephritis, HSP - Henoch-Schonlein Purpura, HT - hypertension, NK - not known, TPTH - hyperparathyroidism, PUD - peptic ulcer disease, TPTH - hyperparathyroidism, UPE - urinary protein excretion

5.3 Results

Twelve renal patients were initially recruited: 1 developed nausea after receiving BQ-123, and 1 was unable to void urine at 30 min intervals. Two others withdrew for reasons unrelated to the study. Eight patients, and all healthy controls, completed the study without adverse events. Baseline study data are given in Table 5.3.
### Table 5.3: Baseline data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Volunteers</th>
<th>Renal Patients</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAP (mmHg)</strong></td>
<td>94.0 ± 2.2 (86.5-104.9)</td>
<td>100.5 ± 4.0 (76.9-110.1)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>SVRI (dyne.sec/cm²/m²)</strong></td>
<td>3089 ± 269 (1558-4068)</td>
<td>3479 ± 270 (2296-4478)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CI (L/min/m²)</strong></td>
<td>2.6 ± 0.3 (1.9-4.6)</td>
<td>2.4 ± 0.2 (2.0-3.2)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>58.2 ± 2.3 (47.2-67.4)</td>
<td>56.6 ± 1.8 (49.1-62.4)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ERBF (ml/min)</strong></td>
<td>683 ± 41 (534-844)</td>
<td>295 ± 58 (81-571)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td><strong>ERVR (mmHg.min/L)</strong></td>
<td>148 ± 12 (108-191)</td>
<td>489 ± 101 (208-973)</td>
<td>p=0.01</td>
</tr>
<tr>
<td><strong>EFF (%)</strong></td>
<td>25.4 ± 2.0 (17.8-36.3)</td>
<td>20.8 ± 1.8 (13.5-30.7)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>GFR (ml/min/1.73m²)</strong></td>
<td>116 ± 11 (80-164)</td>
<td>39 ± 6 (19-67)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td><strong>FWC (ml/min)</strong></td>
<td>5.0 ± 0.5 (3.0-7.5)</td>
<td>3.1 ± 0.5 (1.4-6.0)</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td><strong>UNaV (µmol/min)</strong></td>
<td>143 ± 11 (56-215)</td>
<td>180 ± 31 (70-341)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Urinary protein (mg/L)</strong></td>
<td>0&lt;sup&gt;*&lt;/sup&gt;</td>
<td>453 ± 82 (101-1435)&lt;sup&gt;§&lt;/sup&gt;</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td><strong>Plasma ET-1 (pg/ml)</strong></td>
<td>4.2 ± 0.3 (2.9-5.3)</td>
<td>5.6 ± 0.3 (4.6-7.3)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td><strong>PRA (pg/ml/hr)</strong></td>
<td>3.6 ± 0.5 (2.2-6.4)</td>
<td>10.5 ± 2.3 (3.3-20.6)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td><strong>Plasma ANG II (pg/ml)</strong></td>
<td>7.7 ± 1.0 (4.4-12.6)</td>
<td>7.6 ± 1.1 (4.2-13.8)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Plasma aldosterone (pg/ml)</strong></td>
<td>61 ± 4 (45-81)</td>
<td>88 ± 28 (33-274)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Values are given as the mean of the two baseline periods over the four study days ± SEM with the range of values given in brackets. NS = not significant Values below limit of laboratory assay, §n= 7 one value below limit of detection.

Fluid balance was calculated for each subject during each phase of the study. Not accounting for insensible losses, subjects were in a small positive fluid balance by the end of the 7.5 hr study (+51±21ml/hr for healthy subjects, +69±26 ml/hr for renal patients, p=0.58 healthy subjects vs. CRF). This did not differ with different phases of the study (placebo, BQ-123, BQ-123/788 or BQ-788) and was not different between the healthy subjects and patients with renal failure.
5.3.1 Systemic haemodynamics (Fig 5.2)

In CRF patients, placebo was associated with increases in SVRI to study end (3478±240 dyne.s m²/cm⁵ vs. 3797±301 dyne.s m²/cm⁵, p<0.05) and MAP (100.7±3.8 mmHg vs. 108.0±4.1 mmHg, p<0.01), consistent with the waning effects of anti-hypertensive medication. BQ-123 and BQ-123/788 reduced SVRI to a similar extent (BQ-123 (maximum placebo corrected change from baseline) -630±145 dyne.s m²/cm⁵, BQ-123/788: -617±158 dyne.s m²/cm⁵, both p<0.01 vs. placebo). MAP was reduced in CRF patients after BQ-123/788 (-7.4±1.6 mmHg, p<0.01 vs. placebo), but to a greater extent after BQ-123 alone (BQ-123: -12.9±1.7 mmHg, p<0.01 vs. placebo and BQ-123/788). The systemic haemodynamic response observed in individual patients was not related to baseline blood pressure.

In healthy controls, placebo did not alter systemic haemodynamics. Both BQ-123 and BQ-123/788 reduced SVRI to a similar extent, and equivalent to that seen in CRF (BQ-123: -591±104 dyne.s m²/cm⁵, BQ-123/788: -498±159 dyne.s m²/cm⁵, both p<0.01 vs. placebo). The reductions in MAP after BQ-123 & BQ-123/788 were equal (BQ-123: -3.6±1.4 mmHg, BQ-123/788: -3.7±1.6 mmHg, both p<0.01 vs. placebo), and less than those seen in CRF (p<0.05).

BQ-788 alone increased MAP (CRF: +7.0±1.6 mmHg, HC: +5.8±1.5 mmHg, both p<0.01 vs. placebo) and SVRI (CRF: +454±114 dyne.s m²/cm⁵, p<0.01 vs. placebo, healthy controls: +390±76 dyne.s m²/cm⁵, p<0.05 vs. placebo) to a similar extent in CRF patients and controls.

Increases in HR and CI were observed with selective ETA and combined ETA/B receptor antagonism, while decreases in these indices were observed following ETB receptor antagonism. While HR responses were similar between healthy volunteers and patients, the increases in CI with the dilator drugs were less, particularly for ETA receptor antagonism, and the reductions in CI following ETB receptor antagonism greater in patients compared to healthy volunteers.
Figure 5.2 Systemic haemodynamics after administration of ET receptor antagonists

Healthy Volunteers
n=8

Renal Patients
n=8

MAP

SVRI

CI

HR

Time (mins)

Administration of drug

*p<0.05 vs. placebo
+p<0.01 vs. placebo
**p<0.05 vs. BQ-123

BQ-123

BQ-123/788

BQ-788
5.3.2 Renal haemodynamics (Fig 5.3)
In CRF patients, BQ-123, but not BQ-123/788, produced striking increases in ERBF, and reductions in ERVR and EFF (ERBF: +102±74 ml/min, ERVR: -243±91 mmHg.min/L, EFF: -4.2±2.9%, all p<0.01 vs. placebo and vs. BQ-123/788). GFR did not change. By contrast, in healthy controls, BQ-123 and BQ-123/788 were neutral in respect of ERBF, ERVR, EFF and GFR.

In both groups, BQ-788 reduced ERBF (CRF -77±72 ml/min, HC -134±47 ml/min; both p<0.05 vs. placebo) and increased ERVR (CRF 112±63 mmHg.min/L, HC 39±12 mmHg.min/L; both p<0.05 vs. placebo). These changes were apparent even at low dose, and associated with a reduction in GFR and an increase in EFF.

5.3.3 Urinary free water clearance and sodium excretion (Fig 5.4)
Free water clearance was modestly increased by BQ-123/788 (p<0.05 vs. placebo) in healthy controls. A similar trend was seen after BQ-123 in healthy controls and after BQ-123 and BQ-123/788 in CRF, particularly after administration of the low dose of ET receptor antagonist, though this did not reach statistical significance. FWC was, however, reduced after BQ-788 administration in 7 out of 8 CRF patients. No changes in sodium excretion were observed in either CRF or healthy controls.

5.3.4 Urinary protein excretion (Fig 5.4)
Urinary protein excretion was undetectable for one CRF subject and all healthy controls. In the remaining 7 CRF patients, after correcting for GFR, BQ-788 and BQ-123/788 did not affect proteinuria, but BQ-123 reduced protein leak by 46% (−8.1±4.9 µg/ml) at 210 min (p<0.01 vs. placebo), an effect most apparent in subjects with higher baseline urinary protein excretion.
Figure 5.3 Renal haemodynamics after administration of ET receptor antagonists

Healthy Volunteers
n=8

Renal Patients
n=8

ERBF

ERVR

GFR

EFF

Time (min)

Administration of drug

*p<0.05 vs. placebo
+p<0.01 vs. placebo
**p<0.05 vs. BQ-123

BQ-123

BQ-123/788

BQ-788
Figure 5.4 Free water clearance, urinary sodium excretion and protein excretion after administration of ET receptor antagonists

Healthy Volunteers  
n=8

Renal Patients  
n=8

- Figure shows the change in free water clearance (FWC), urinary sodium excretion (UNaV), and urinary protein excretion over time for Healthy Volunteers and Renal Patients.

- Healthy Volunteers and Renal Patients were treated with different ET receptor antagonists: BQ-123, BQ-123/788, and BQ-788.

- Administration of drug is indicated by the black bars on the x-axis.

- Statistical significance is indicated by:
  - *p<0.05 vs. placebo
  - +p<0.01 vs. placebo
  - **p<0.05 vs. BQ-123
5.3.5 Plasma BQ-123 (Fig 5.5)
BQ-123 was detectable in plasma at 15 min post low dose and at 15 & 45 min post high dose administration. There was no difference in the plasma concentrations of BQ-123 between healthy controls and renal patients (CRF 1.52±0.36 pg/ml, HC 1.21±0.17 pg/ml at 15 min post high dose), nor between study days when BQ-123 was given alone compared with when given with BQ-788 (CRF 1.62±0.20 pg/ml, HC 1.26±0.20 pg/ml).

Figure 5.5 Plasma BQ-123 concentrations after BQ-123 administration ± BQ-788

5.3.6 Plasma hormone concentrations (Fig 5.6)
Baseline ET-1 concentrations were slightly higher in renal patients compared to healthy volunteers (Table 5.3). Plasma ET-1 concentrations were increased to a similar extent after high dose BQ-788 (Healthy controls baseline: 4.1 ± 0.4 pg/ml, 120 min: 6.6 ± 1.0 pg/ml, CRF baseline: 5.6 ± 0.4 pg/ml, 120 min: 8.3 ± 0.5 pg/ml) and BQ-123/788 (Healthy controls baseline: 3.9 ± 0.5 pg/ml, 120 min: 8.5 ± 1.1 pg/ml, CRF baseline: 5.4 ± 0.3 pg/ml, 120 min: 7.9 ± 0.4 pg/ml), but unaltered by placebo or BQ-123. Plasma renin activity, aldosterone and ANG II concentrations were unchanged by the administration of placebo, BQ-123 and/or BQ-788.
Figure 5.6 Plasma hormone concentrations after ET receptor antagonists

**ET-1 (ng/ml)**

- Placebo
- BQ-123
- BQ-123/788
- BQ-788

* p<0.05 vs. placebo

**Aldosterone (pg/ml)**

**ANG II (pg/ml)**

**Renin Activity (ng/ml/hr)**

- 0
- 30
- 90
- 120
- 180

Time (min)
5.4 Discussion

This is the first clinical study to directly compare ETA, ETB and combined ETA & ETB receptor antagonism at systemic doses in man. It demonstrates that, in CRF patients, that selective ETA receptor antagonism produces substantial reductions in blood pressure that are associated with renal vasodilatation, without changes in GFR. Combined ETA/B receptor blockade was less effective in lowering blood pressure, had no effect on renal haemodynamics and reduced ET-1 clearance. Selective ETB receptor antagonism alone produced substantial systemic and renal vasoconstriction. By contrast, in healthy subjects the effects of ETA and ETA/B receptor blockade on blood pressure were similar to each other, but less than those seen in CRF, and there were no effects on renal haemodynamics.

These data confirm the physiological importance of ET-1, through activation of the ETA receptor, in the maintenance of basal systemic but not renal vascular resistance [140]. They also show that ET-1 plays a major role in regulating blood pressure and renal vascular resistance in CRF, consistent with activation of the ET system in this condition. Additionally, the reduction in EFF suggests an effect primarily on efferent arteriolar tone, which may serve to reduce glomerular pressure, and thus be renoprotective.

The effects of BQ-788, whether in the presence of BQ-123 or not, suggest the net effect of ETB receptor activation on the circulation, in health and renal disease, is to produce vasodilatation. Therefore, similar to observations in healthy subjects [194, 210] and patients with heart failure [388], any enhanced effects of constrictor ETB receptors in CRF is outweighed by ETB receptor mediated vasodilatation. Of interest, ETB receptor antagonism increased renal vascular resistance twice as much (~20-30%) as systemic vascular resistance (~10-15%), suggesting that tonic ETB receptor mediated renal vasodilatation plays a key role in maintenance of renal vascular tone. This is likely to be of particular importance in CRF, where baseline renal vascular resistance is high.
This study has demonstrated a reduction in SVRI after BQ-123 or BQ-123/788 that is similar in patients and healthy volunteers. Using a nitric oxide clamp, Verhaar et al demonstrated that the vasodilatory response of the forearm circulation to ETA receptor antagonism was dependent on NO production [194]. As many cardiovascular disease states including CRF are characterised by a reduction in NO activity [389-391], this finding would suggest that ETA receptor blockade should be less effective in disease. Consistent with this, forearm studies in renal failure patients [195], heart failure patients [388], and hypertensives [222] have demonstrated a reduced vasodilatory response to BQ-123 when compared to healthy controls. This was clearly not the case in this study.

BQ-123 concentrations were the same in the CRF patients and the healthy controls, suggesting that altered pharmacokinetics does not account for this difference. Indeed as BQ-123 is metabolised by the liver [97] one would not expect altered pharmacokinetics in renal patients (see Chapter 11 for further exploration of BQ-123 pharmacokinetics). It is possible that the medications taken by renal patients may be responsible for this finding. As the duration of the study was a minimum of four weeks plus one week washout period for each individual, withdrawal of medications was felt not only to be ethically unjustified, but it also introduced another unmatched variable, hypertension, compared to the healthy controls that might have affected the response to ET antagonists. Given the prevalence of hypertension in renal disease, it was felt that the option chosen (to maintain patients on their medication) was a clinically relevant one and in keeping with previous studies in heart failure patients [293, 294, 299]. In this respect, it is important to note that six of the eight patients were taking ACE inhibitors. These drugs, in addition to blocking the conversion of ANG I to ANG II, also increase NO activity via inhibition of bradykinin breakdown [392]. It is possible therefore that ACE inhibitors and ETA antagonists, both acting through NO, somehow interact to increase the haemodynamic response observed. An acute synergism between ETA receptor antagonism and ACE inhibitors has been demonstrated in animals in respect of systemic and renal haemodynamics [393] but has yet to be studied in man. In Chapter 6 and 7, this issue is addressed in healthy volunteers.
However, despite similar changes in SVRI, blood pressure fell significantly in renal patients, while healthy volunteers defended their blood pressure. Reductions in MAP in the healthy volunteers are small and similar to those seen in Chapter 3 and in other studies [140, 200, 202]. Our experience is that healthy subjects defend their blood pressure via reflex alterations in HR and CI, and that MAP is usually the last cardiovascular measurement to change, an observation supported by this study. It is potentially one reason why studies with ET receptor antagonists in healthy volunteers that only measure mean arterial pressure, and do not look at vascular resistance, have failed to show significant effects [139, 202]. In the renal patients, the larger reduction in blood pressure is accompanied by a lesser increase in CI in patients compared healthy volunteers, suggesting that the hypertensive renal heart less able to compensate for changes in SVRI. The greater reduction in MAP in patients after ETA receptor antagonists and corresponds with a smaller increase in CI with ETA compared to ETA/B receptor antagonism. It is interesting to speculate whether this is a direct cardiac effect of ETA receptor blockade, though this study was not designed to answer this question.

Consistent with previous studies [120, 139-141, 199], selective ETA and non-selective ETA/B receptor antagonists had little effect on ERBF or ERVR in healthy subjects. As the higher dose of BQ-123 is at the top of the dose response curve for systemic haemodynamics (Chapter 3) insufficient dose is an unlikely explanation for the lack of effect of ETA and non-selective antagonism on the renal circulation of healthy volunteers. It is possible that the ETA receptor is not an important mediator of renal vasoconstriction in health. However, the circumstances of this study mean that this is an unchallenged renal circulation and therefore likely to be maximally dilated. It is possible therefore that no effect is seen as no further dilatation can occur.

In patients however, ETA blockade produces a clear reduction in ERVR and increase in ERBF. The increased ERVR at baseline compared to healthy controls is a good indicator that, in the renal circulation in patients, even unchallenged, the balance
between constrictors and dilators is shifted towards the constrictors. Blockade of one of these constrictor systems i.e. ET-1 will, therefore, produce a dilatation. The response in renal patients to selective ETA receptor would suggest that the ETA receptor mediates ET-1 induced renal vasoconstriction. The neutral effect of non-selective receptor antagonism on ERBF and ERVR underlines the importance of the vasodilatory action of the ETB receptor in the renal circulation of CRF patients. Loss of this vasodilatory action, in contrast to the systemic circulation, reduces the effectiveness of ETA receptor blockade. Again this study provides no evidence to support an upregulation of vasoconstrictor ETB receptors in the renal circulation.

The renal vasoconstriction produced by selective ETB receptor antagonism is accompanied, not surprisingly, by a reduction in GFR. EFF is moderately increased, suggesting, in the absence of changes in filtration coefficient, an increase in efferent arteriolar tone. The reduction in EFF observed with ETA receptor antagonists in renal patients is also suggestive of a preferential efferent arteriolar effect. If this is real, it implies that the observed increase in RBF is not associated with increases in glomerular capillary perfusion. This is important as an increase in ERBF accompanied by increases in glomerular capillary perfusion pressure could be deleterious to long term renal function, conversely a fall in glomerular capillary perfusion pressure would result in a situation analogous to ACE inhibitors, and might be expected to improve long term renal outcome. Consistent with our observations, an ETA receptor selective antagonist has been shown to reduce proteinuria in type 1 diabetics [190]. However, it would mean that ETA receptor blockade would need to be approached with caution in situations of low renal perfusion pressure e.g. congestive heart failure and renovascular disease. Because of the importance of this issue to rational prescribing of ET receptor antagonists, Larger, chronic studies are needed to confirm or refute this finding.

Perhaps surprisingly, given the evidence for ETB receptor mediated natriuresis shown in animal studies [394], no changes in sodium excretion were observed in this study. However, the urinary sodium data in this study were the most variable and failed to achieve significance. Larger studies are required to adequately define the
renal tubular actions of ET receptor antagonists. Despite this, during ETA receptor blockade, in the face of substantial systemic and renal vasodilatation, sodium retention did not occur, which is important if these drugs are to be safely prescribed to patients with CRF.

Based on the data from study 1 and earlier work, this study was designed to achieve effective and selective blockade of the ETA and ETB receptor ([194, 210, 395] and Chapter 3) and not specifically to reproduce the effects of existing ET receptor antagonists, which all block ETA to a greater extent than ETB receptors [396]. Therefore, although the main effects of therapeutic interest in our study appear to derive from ETA receptor blockade, and are countered by ETB blockade, drugs causing only modest ETB receptor inhibition might produce similar effects. As a limitation, ours were acute studies. However, based on studies in heart failure, [293] haemodynamic effects would be sustained, or even greater, with continued treatment. Also, a relatively homogeneous CRF population were recruited to this study. Therefore, further work is now needed in a broader population of patients with kidney disease, including those with low renal perfusion pressure. In particular, as ACE inhibitors are most effective as renoprotective agents in patients with >3g/24hr of proteinuria, studies are needed in patients with higher degrees of urinary protein leak to address what may be a major benefit of these agents.

In conclusion, this study suggests that selective ETA receptor antagonists may be valuable anti-hypertensive drugs in patients with CRF as well as offering additional benefits, including renoprotection. ET receptor antagonists also improve endothelial function [397], reduce inflammation and fibrosis [398], and reverse cardiac [399] and vascular remodelling [400] and so may offer additional benefits to renal patients, who are at high cardiovascular risk. On this basis, longer term studies in patients with CRF are justified, paying particular attention to effects on proteinuria as a surrogate marker for the progression of renal disease.
Chapter 6

An examination of the interaction between ACE inhibition and ET receptor blockade in healthy human volunteers
6.1 Introduction

ET-1 and ANG II are both powerful vasoconstrictors involved in the regulation of vascular tone, and there is considerable evidence for an interaction between the ET and renin-angiotensin-aldosterone systems [401]. ANG II increases ET-1 transcription and secretion in vitro in a variety of cell types, including endothelial and vascular smooth muscle cells [21, 402], and ET-1 can increase ACE activity [73, 403]. As discussed in Chapter 4, ET receptor antagonists attenuate the acute haemodynamic effects of ANG II in rats in vivo [377, 379], although this is by no means a uniform finding [375], and has not been replicated in acute studies in dogs [376] or in local [404] or systemic studies in man (Chapter 4). Animal data have, however, demonstrated that ACE inhibition can attenuate ET-1 induced hypertension [405, 406] and suggested that concomitant blockade of the renin-angiotensin and ET systems produces changes greater than those seen with blockade of either system alone [393, 407-409]. In man, synergism between ETA receptor antagonists and angiotensin receptor type 1 antagonists has been demonstrated [410]. Also, many clinical studies with ET receptor antagonists demonstrating clear haemodynamic effects [293, 299] have been performed in patients already receiving ACE inhibitors.

In contrast to forearm studies that have suggested that ETA receptor blockade would be less effective in patient groups compared to healthy controls [195, 222, 388], study 3 (Chapter 5) demonstrated that, despite a greater reduction in blood pressure in CRF patients, reductions in SVRR were similar in CRF patients and healthy volunteers. In light of the above evidence, a possible explanation for the augmented response in CRF, as six of the eight patients were or ACE inhibitors, might be due to an interaction between ET receptor antagonism and ACE inhibition.

The aim of this study was, therefore, to explore the possible systemic and renal interaction between ET receptor antagonism and ACE inhibition, hypothesising that pre-treatment with ACE inhibition would augment the systemic and renal haemodynamic response to ETA receptor antagonism.
6.2 Study design

This was a randomised, double-blind, placebo-controlled study in 6 healthy subjects. For demographic data, see Table 6.1. Subjects attended for 6 visits, each separated by ≥7 days. On visits 1-3, they received either placebo, BQ-123, or the combination of BQ-123 & BQ-788 in a randomised order. They then followed and identical study protocol on visits 4-6, after pre-treatment with enalapril (E). The response to BQ-123 and BQ-123/788 in the presence or absence of E was pre-identified as the comparison of interest.

For 5 days before each study, subjects took enalapril at a dose of 20 mg twice daily (see methods for justification of dosing schedule) taking the last dose at 0830 on the study day. Subjects then underwent a standard clearance study. After baseline measurements, the low dose of antagonist was then administered followed by three 30 min collection periods. The higher dose of antagonist was then administered followed by 5 further 30 min collection periods.

Table 6.1 Subject demographic data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong> (yr)</td>
<td>47 ± 5 (23 - 64)</td>
</tr>
<tr>
<td><strong>Body mass index</strong> (kg/m²)</td>
<td>25 ± 2 (18 - 31)</td>
</tr>
<tr>
<td><strong>MAP</strong> (mmHg)</td>
<td>88.5 ± 2.7 (71.4 - 99.4)</td>
</tr>
<tr>
<td><strong>Creatinine</strong> (µmol/L)</td>
<td>89 ± 7 (62 - 119)</td>
</tr>
<tr>
<td><strong>Urinary Na excretion</strong> (mmol/24 hr)</td>
<td>118 ± 15 (64 - 185)</td>
</tr>
<tr>
<td><strong>Cholesterol</strong> (mmol/L)</td>
<td>5.2 ± 0.3 (3.9 - 6.1)</td>
</tr>
</tbody>
</table>

At 0, 60 & 90 min after the start of low and high dose antagonist, and at the end of the study, blood samples were taken for the measurement of plasma ET-1, aldosterone, ANG II concentrations and PRA. BQ-123 was measured before and at 15, 45 & 90 minutes after the start of each dose of antagonist, and at the end of the
study and serum ACE activity was determined at 0830 am and at 1430 (60 min after the start of the higher dose antagonist).

6.3 Results

All subjects completed all phases of the study without adverse events. Baseline study data (Table 6.2) were not different for any of the 6 individual phases of the study except for plasma aldosterone concentrations which were reduced during the phases when BQ-788 was administered by pre-treatment with enalapril.

Table 6.2: Baseline data

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>BQ-123</th>
<th>BQ-123/788</th>
<th>Enalapril+ E+BQ-123</th>
<th>E+BQ-123</th>
<th>No E vs. E†-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>96.2±2.5</td>
<td>92.6±4.1</td>
<td>94.0±3.2</td>
<td>87.2±1.7</td>
<td>89.2±3.8</td>
<td>90.6±5.0</td>
</tr>
<tr>
<td>SVRI (dyne.s m⁻²/cm⁵)</td>
<td>2868±318</td>
<td>3157±435</td>
<td>3157±410</td>
<td>2726±305</td>
<td>2831±358</td>
<td>2616±353</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>2.94±0.47</td>
<td>2.64±0.43</td>
<td>2.60±0.38</td>
<td>2.77±0.39</td>
<td>2.73±0.36</td>
<td>2.99±0.36</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>61.2±1.5</td>
<td>60.8±1.5</td>
<td>61.3±2.2</td>
<td>60.3±2.5</td>
<td>59.1±3.6</td>
<td>61.8±2.0</td>
</tr>
<tr>
<td>ERBF (ml/min)</td>
<td>587±53</td>
<td>705±66</td>
<td>703±84</td>
<td>795±89</td>
<td>723±138</td>
<td>945±101</td>
</tr>
<tr>
<td>ERVR (mmHg.min/L)</td>
<td>173±19</td>
<td>139±15</td>
<td>142±21</td>
<td>119±16</td>
<td>142±22</td>
<td>108±18</td>
</tr>
<tr>
<td>EFF (%)</td>
<td>28±4</td>
<td>23±2</td>
<td>23±3</td>
<td>21±2</td>
<td>24±3</td>
<td>19±2</td>
</tr>
<tr>
<td>GFR (ml/min/1.73m²)</td>
<td>106±9</td>
<td>110±14</td>
<td>103±13</td>
<td>106±7</td>
<td>103±6</td>
<td>107±9</td>
</tr>
<tr>
<td>UNaV (µmol/min)</td>
<td>157±19</td>
<td>150±32</td>
<td>134±29</td>
<td>154±13</td>
<td>142±22</td>
<td>157±28</td>
</tr>
<tr>
<td>Plasma ET-1 (pg/ml)</td>
<td>4.7±0.5</td>
<td>4.7±0.3</td>
<td>3.5±0.2</td>
<td>3.8±0.3</td>
<td>3.6±0.3</td>
<td>3.9±0.1</td>
</tr>
<tr>
<td>Plasma Aldosterone (pg/ml)</td>
<td>61±14</td>
<td>53±8</td>
<td>76±12</td>
<td>42±4</td>
<td>43±4</td>
<td>29±3*</td>
</tr>
</tbody>
</table>

p<0.01

p<0.01

NS

p<0.05

p<0.05

NS

NS

NS

NS

NS
However comparing the 18 visits where subjects received pre-treatment with enalapril to those where they did not, MAP, SVRI, ERVR and aldosterone were significantly reduced and ERBF was significantly increased.

### 6.3.1 Systemic haemodynamics

Administration of placebo, either alone or in the presence of pre-treatment with enalapril, did not alter systemic haemodynamics. Both selective ETA receptor antagonism with BQ-123, and combined ETA/B receptor antagonism with BQ-123 & BQ-788, reduced MAP (maximum placebo corrected change from baseline: BQ-123: −4.2 ± 1.6 mmHg, ANOVA p<0.05 vs. placebo; BQ-123/788: -4.4 ± 2.0 mmHg). After pre-treatment with E, the effect of BQ-123 on blood pressure was almost doubled (−8.3 ± 3.0 mmHg, p<0.01 vs. BQ-123 alone). However, this synergy was not seen when BQ-788 was co-administered with BQ-123 (−4.4 ± 2.9 mmHg) (Fig 6.1). SVRI followed a similar pattern (Fig 6.2). Pre-treatment with E was associated with a trend towards a smaller increase in HR after BQ-123 or BQ-123/788. No significant changes in CI were noted.

### 6.3.2 Renal haemodynamics

Placebo, E alone, BQ-123 and BQ-123/788 were all neutral in respect of ERBF, ERVR, EFF and GFR. After pre-treatment with E, BQ-123 increased ERBF (+146 ± 41 ml/min, p<0.01 vs. placebo and vs. BQ-123 alone), reduced ERVR (-32 ± 15 mmHg.min/L, p<0.01 vs. placebo and vs. BQ-123 alone) and reduced EFF (-3.4 ± 2.5%, p<0.05 vs. placebo, p<0.01 vs. BQ-123 alone). By contrast, after pre-treatment with E, BQ-123/788 increased ERBF (-224 ± 31 ml/min, p<0.01 vs. placebo and vs. BQ-123/788 alone) and reduced ERVR (26 ± 6 mmHg.min/L, p<0.05 vs. BQ-123/788 alone) (Fig 6.3-6.).
Figure 6.1 MAP

Placebo ± ACEI

ETA antagonism ± ACEI

ETA/B antagonism ± ACEI

Area Under Curve (%)

\[ \text{Placebo} \]
\[ \text{ACEI} \]

\[ \text{BQ-123} \]
\[ \text{ACEI+BQ-123} \]

\[ \text{BQ-123/788} \]
\[ \text{ACEI+BQ-123/788} \]

\( * p < 0.05 \) vs. placebo
\( + p < 0.05 \) vs. ACEI

\( \text{Drug administration} \)
Figure 6.2 SVRI

Placebo ± ACEI

ETA antagonism ± ACEI

ETA/B antagonism ± ACEI

Area Under Curve (%)

- Placebo
- ACEI

- BQ-123
- ACEI+BQ-123

- BQ-123/788
- ACEI+BQ-123/788

* p<0.05 vs. placebo
+ p<0.05 vs. ACEI

Drug administration
Figure 6.3 ERBF

Placebo ± ACEI

Area Under Curve (%)

ET-A antagonism ± ACEI

ET/1 antagonist ± ACEI

* p<0.05 vs. placebo
+ p<0.05 vs. ACEI
** p<0.05 vs. BQ-123

Drug administration

Time (min)
Figure 6.4 ERVR

**Area Under Curve (%)**

- Placebo
- ACEI

![Graph showing Area Under Curve](image)

**ET-A antagonism ± ACEI**

- Placebo ± ACEI
- ACEI

![Graph showing ET-A antagonism](image)

**ETA/B antagonism ± ACEI**

- Placebo ± ACEI
- ACEI

![Graph showing ETA/B antagonism](image)

**Drug administration**

- BQ-123
- ACEI + BQ-123
- BQ-123/788
- ACEI + BQ-123/788

* p<0.05 vs. placebo
+ p<0.05 vs. ACEI
** p<0.05 vs. BQ-123

Time (min): 0 30 60 90 120 150 180 210 240
Figure 6.5 GFR

Placebo ± ACEI

Area under curve (%)

ET-A antagonism ± ACEI

ET-\(A\) antagonism ± ACEI

ETA/B antagonism ± ACEI

Drug administration

* \(p<0.05\) vs. placebo
+ \(p<0.05\) vs. ACEI
Figure 6.6 EFF

Placebo ± ACEI

ET-A antagonism ± ACEI

ET/A/B antagonism ± ACEI

Area Under Curve (%)

-2.5 -2.5 -5
-2.5 -2.5 -5
-5

0 30 60 90 120 150 180 210 240

Time (min)

Panel 1: Placebo and ACEI treatments. Placebo bars are shown in blue, and ACEI in black. Significant differences are indicated with p-values.

Panel 2: ET-A antagonism with and without ACEI. Red bars represent BQ-123, pink bars ACEI + BQ-123. Significant differences are indicated.

Panel 3: ETA/B antagonism with and without ACEI. Green bars are BQ-123/788, blue bars ACEI + BQ-123/788. Significant differences are noted at different levels of significance.
6.3.3 Urinary sodium excretion

No significant changes in natriuresis were observed after placebo, E alone and both BQ-123 or BQ-123/788 alone. However, after pre-treatment with E, BQ-123 produced a striking increase in urinary sodium excretion (UNaV: +58 ± 27 μmol/min, p<0.01 vs. placebo and BQ-123 alone). Fractional excretion of sodium followed a similar pattern. As with renal haemodynamics this increase was not observed with BQ-123/788. (Fig 6.7)

6.3.4 Serum ACE activity

Pre-treatment with E reduced serum ACE activity by 75% at baseline compared to no pre-treatment (9.1 ± 2.3 vs. 36.4 ± 4.3 U, p<0.01), and by 79% at 150 min (5.4 ± 1.7 vs. 26.5 ± 2.1 U, p<0.01). ET-receptor antagonist administration did not alter ACE activity (Table 6.3)

Table 6.3 Serum ACE activity after ET receptor antagonist administration

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>BQ-123</th>
<th>BQ-123/788</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=0 min</td>
<td>36.1 ± 3.2</td>
<td>39.0 ± 11.5</td>
<td>34.1 ± 6.5</td>
</tr>
<tr>
<td>t=210 min</td>
<td>27.4 ± 3.5</td>
<td>27.5 ± 2.7</td>
<td>24.6 ± 5.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Enalapril</th>
<th>Enalapril +BQ-123</th>
<th>Enalapril +BQ-123/788</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=0 min</td>
<td>10.6 ± 3.0</td>
<td>8.6 ± 3.0</td>
<td>11.7 ± 6.6</td>
</tr>
<tr>
<td>t=210 min</td>
<td>8.0 ± 3.2</td>
<td>3.7 ± 2.0</td>
<td>7.0 ± 3.9</td>
</tr>
</tbody>
</table>
Figure 6.7 UNa V

Area Under Curve (%)

- Placebo
- ACEI

% Change from baseline

- ETA antagonism ± ACEI
- ETA/B antagonism ± ACEI

Drug administration

* p<0.05 vs. placebo
+ p<0.05 vs. ACEI
6.3.5 Plasma BQ-123

BQ-123 was detectable in plasma at 15 min post low dose and at 15 & 45 min post high dose administration. Peak BQ-123 concentrations were higher after pre-treatment with E, though the difference was not statistically significant (BQ-123: 1.26 ± 0.18 pg/ml, BQ-123/788:1.32 ± 0.23, BQ-123 + E: 2.06 ± 0.65, BQ-123/788 + E: 2.32 ± 0.53) (Fig 6.8).

**Figure 6.8 Plasma BQ-123 concentrations**

![Graph showing plasma BQ-123 concentrations over time](image)

6.3.6 Plasma ET-1 concentrations

Baseline plasma ET-1 concentrations were not altered by pre-treatment with enalapril (Table 6.2). They were increased following administration of BQ-788 whether administered in the presence of enalapril (+1.7 ± 0.6 pg/ml, 43 ± 16% p<0.05 vs. baseline) or not (4.4 ± 1.3 pg/ml, 132 ± 33%, p<0.05 vs. baseline), but unaffected by placebo or BQ-123 alone.

6.3.7 Plasma aldosterone concentrations

Baseline plasma aldosterone concentrations were lowered by pre-treatment with enalapril (pre treatment with enalapril 37.8±2.8 pg/ml, no pre-treatment 63.6±7.7 pg/ml, p<0.05) (Table 6.2). After administration of BQ-788, plasma aldosterone concentrations fell whether administered in the presence of enalapril (-10.6 ± 3.5 pg/ml, -38 ± 14% p<0.05 vs. baseline) or not (-33.3 ± 9.3 pg/ml, -40 ± 6%, p<0.05 vs. baseline), but unaffected by placebo or BQ-123 alone.
6.4 Discussion

This study demonstrates a synergy between ACE inhibition and ETA receptor antagonism, both on systemic and renal haemodynamics, and on renal tubular function that is lost when the ETB receptor is concomitantly blocked.

The renin-angiotensin and ET systems are known to interact [401] and an *in vivo* synergistic effect between ACE inhibition and ETA receptor antagonism has been demonstrated in animals [393], and in man between ERAs and angiotensin receptor antagonists [410]. This study has shown that, in healthy volunteers subjected to a high degree of ACE inhibition, the effects of ETA receptor blockade on systemic haemodynamics are enhanced. In respect of blood pressure, ACE inhibition almost doubles the effect of ETA receptor blockade. Additionally, in contrast to the absence of an effect of ETA receptor antagonism alone on the renal circulation, the combination with ACE inhibition increased renal perfusion, with an associated fall in EFF, and thus, by inference, a preferential vasodilatation of the efferent arteriole and a fall in glomerular capillary pressure. These alterations in glomerular haemodynamics have the potential to be renoprotective in a manner analogous to the effect of ACE inhibition alone. Additionally, a striking natriuresis was observed that was still developing at the end of the study, 4 hours after initial administration of BQ-123. If sustained over 24 hrs, this would be equivalent to 80 mmol of sodium excreted, a clinically important degree of sodium loss.

These results also demonstrated that, while maximal haemodynamic changes occurred after the higher dose of BQ-123, useful systemic and renal haemodynamic changes were achieved after the lower dose of BQ-123 in the presence of ACE inhibition. As adverse effects in clinical trials with ET receptor antagonists appear to be largely dose related [295], this combination might allow the use of a lower dose of ETA receptor antagonist without losing efficacy.

Montanari and colleagues [410] have demonstrated that ETA receptor antagonism can produce renal haemodynamic changes under conditions of ANG II receptor-1 blockade. This study demonstrates a similar synergy between ETA receptor
antagonism and ACE inhibition. Recently, enalapril has been shown to attenuate ET-1 induced hypertension (but not the reduction in GFR) in rats via increased bradykinin [406]. However, the loss of this synergistic effect in this study on all indices when BQ-788 is also given suggests that the ETB receptor is crucial to the mechanism of this BQ-123-enalapril interaction. In an experimental rat model of interstitial renal fibrosis, enalapril treatment has been shown to increases ETB mRNA expression [411], providing a possible mechanism for this ET-1/ACE interaction. It is possible that ETA receptor antagonism then results in displacement of endogenous ET-1 from the ETA receptor onto the unblocked, upregulated ETB receptor.

As BQ-788 is a vasoconstrictor, it is possible that its ability to abolish the vasodilator effect of enalapril and BQ-123 is non-specific. However, the previous study in healthy volunteers and CRF patients (Chapter 6) has demonstrated that, while this dose of BQ-788 alone produces systemic and renal vasoconstriction, it does not abolish the vasodilator effects of BQ-123 suggesting the effect seen here is specific to the interaction between ACE inhibition and ETA receptor antagonism.

These studies were performed in subjects in a salt replete state (mean 24 hr urine excretion 119 ± 6mmol). A recent study has suggested that ET-1 plays a role in angiotensin-dependent hypertension in humans [412] particularly in respect of blood pressure and proteinuria. Salt depletion, with enhancement of renin-angiotensin activity, might therefore further enhance the synergy seen between ETA receptor antagonism and ACE inhibition.

In this study, in the presence of ACE inhibition, combined ETA/B receptor antagonism actually ERVR and ERBF, underlining the importance of this ETB mediated vasodilatation. This finding has important implications for the therapeutic use of ET receptor antagonists, suggesting that, in conjunction with ACE inhibitors,
ETA will be superior to ETA/B receptor antagonists in respect of haemodynamic benefits. This demonstration of an ETB receptor dependent, synergistic interaction in healthy subjects between ETA receptor antagonism and ACE inhibition suggests these differences observed in renal patients in chapter 5 may be, at least in part, due to concomitant chronic treatment with ACE inhibitors.

It is important to note that BQ-123 concentrations were higher when subjects were pre-treated with ACE inhibition. The number of subjects studied was small and this difference did not reach significance, but it is interesting to note that in Chapter 5, CRF patients also had higher BQ-123 plasma concentrations that healthy volunteers (again, however, not significant). In light of this, a pharmacokinetic interaction between enalapril and BQ-123 cannot be fully excluded without larger studies.

In summary, this mechanistic study demonstrates, in healthy men, that pre-treatment with ACE inhibition increases the systemic haemodynamic effects of ETA receptor antagonism and unmasks a renal haemodynamic and renal tubular effect, and that this synergy is requires an unblocked ETB receptor. These findings would support the use of ETA but not combined ETA/B receptor antagonists as useful adjunctive treatments to ACE inhibitors in the treatment of the systemic and renal vascular consequences of diseases characterised by vasoconstriction particularly in circumstances where sodium loss would also be beneficial such as heart failure or CRF. This interaction should now be explored by longer term studies in patients with such conditions.
Chapter 7

Mechanism of synergism between endothelin-A receptor antagonism and ACE inhibition in healthy volunteers
7.1 Introduction
ACE inhibitors, by preventing the breakdown of bradykinin [392], and ET-1, acting on endothelial ETB receptors [413], both increase endothelium-dependent vasodilatation. Having demonstrated a synergism between ETA receptor antagonism and ACE inhibitors that is dependent upon an unblocked ETB receptor (Chapter 6), the aim of this study was to explore the possible mechanisms by which this interaction might occur. During co-administration of ACE inhibition and ETA receptor antagonism, subjects underwent selective blockade of NO production with L-NMMA and prostaglandin production with indomethacin.

7.2 Study design
This was a randomised, double-blind, placebo-controlled study of 6 healthy subjects. For demographic data, see Table 7.1. Subjects attended for 4 visits, each separated by ≥7 days. For each visit, they received pre-treatment with enalapril. During the study day they then received placebo, BQ-123, BQ-123 + indomethacin or BQ-123 + L-NMMA, in a randomised order (Table 7.1). Comparisons of interest were pre-identified as placebo vs. BQ-123 and vs. BQ-123 + indomethacin/L-NMMA, and BQ-123 vs. BQ-123 + indomethacin/L-NMMA. This study ran concurrently with the study described in Chapter 8 (ie. 5 randomised visits in total). Placebo and BQ-123 phases are the same in the two studies.

Table 7.1 Subject demographic data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>47 ± 2 (30-59)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23 ± 2 (18-29)</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>84.9 ± 3.1 (70.8 - 98.7)</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>86 ± 8 (62 - 119)</td>
</tr>
<tr>
<td>Urinary Na excretion (mmol/24 hr)</td>
<td>103 ± 15 (61 - 162)</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.1 ± 0.3 (3.9 - 6.1)</td>
</tr>
</tbody>
</table>
For 5 days before each study, subjects took enalapril at a dose of 20 mg twice daily (see methods for justification of dosing schedule) taking the last dose at 0830 on the study day, along with indomethacin or a placebo capsule. Subjects then underwent a standard clearance study. After baseline measurements, the low dose of antagonist and L-NMMA or placebo was then administered followed by three 30 min collection periods. The higher dose of antagonist and L-NMMA or placebo was then administered followed by 5 further 30 min collection periods. To ensure blinding with ET-1 (Chapter 8 study), L-NMMA infusion was followed by saline for 55 min, and placebo was administered in 2 stages, over 5 min and 55 min after each dose of antagonist (Fig 8.1).

At 0, 60 & 90 min after the start of low and high dose antagonist, and at the end of the study, blood samples were taken for the measurement of plasma ET-1 concentrations. Serum ACE activity was determined at 0830 am and at 1430 (60 min after the start of the higher dose antagonist).

7.3 Results

One subject experienced an increase in mean arterial blood pressure of 50 mmHg following indomethacin administration and before receiving BQ-123, and was withdrawn from the study and replaced. Baseline measurements were not different in any phase of the study (Table 7.2).

7.3.1 Systemic haemodynamics (Fig 7.1)

The reduction in blood pressure observed after BQ-123 in the presence of enalapril (maximum placebo-corrected change from baseline $-6.0 \pm 1.7$ mmHg, ANOVA $p<0.01$ vs. placebo) was abolished by L-NMMA ($-2.0 \pm 2.7$ mmHg, $p<0.01$ vs. BQ-123+E), and moderately augmented by indomethacin, an effect most evident towards the end of the study ($-11.0 \pm 2.3$ mmHg, $p<0.01$ vs. placebo and vs. BQ-123+E). SVRI was reduced by E+BQ-123 ($-541 \pm 119$ dyne.s $\text{m}^2/\text{cm}^5$, $p<0.01$ vs. placebo), and HR and CI increased. These changes were unaffected by indomethacin
administration (SVRI \(706 \pm 148\) dyne.s \(m^2/cm^5\), \(p<0.01\) vs. placebo), and abolished by L-NMMA (SVRI \(-194 \pm 116\) dyne.s \(m^2/cm^5\), \(p<0.01\) vs. BQ-123+E).

### Table 7.2: Baseline data

<table>
<thead>
<tr>
<th></th>
<th>Enalapril + Placebo</th>
<th>Enalapril + BQ-123</th>
<th>Enalapril + BQ-123 + Indomethacin</th>
<th>Enalapril + BQ-123 + L-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>82.4±2.6</td>
<td>84.2±3.5</td>
<td>90.7±1.8</td>
<td>80.5±2.4</td>
</tr>
<tr>
<td>SVRI (dyne.s (m^2/cm^5))</td>
<td>2291±275</td>
<td>2340±279</td>
<td>2679±348</td>
<td>2108±281</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>3.1±0.4</td>
<td>3.0±0.3</td>
<td>2.9±0.4</td>
<td>3.3±0.3</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>59.6±2.8</td>
<td>60.1±3.2</td>
<td>54.7±2.3</td>
<td>60.1±2.3</td>
</tr>
<tr>
<td>ERBF (ml/min)</td>
<td>760±108</td>
<td>759±134</td>
<td>574±87</td>
<td>862±98</td>
</tr>
<tr>
<td>ERVR (mmHg.min/L)</td>
<td>124±22</td>
<td>126±18</td>
<td>177±25</td>
<td>105±18</td>
</tr>
<tr>
<td>EFF (%)</td>
<td>24±2</td>
<td>25±3</td>
<td>26±1</td>
<td>22±3</td>
</tr>
<tr>
<td>GFR (ml/min/1.73m²)</td>
<td>106±7</td>
<td>106±12</td>
<td>93±13</td>
<td>110±12</td>
</tr>
<tr>
<td>UNaV (µmol/min)</td>
<td>132±16</td>
<td>121±21</td>
<td>60±16</td>
<td>124±24</td>
</tr>
<tr>
<td>Plasma ET-1 (pg/ml)</td>
<td>3.9±0.5</td>
<td>3.4±0.6</td>
<td>5.2±0.6</td>
<td>5.0±0.6</td>
</tr>
</tbody>
</table>

#### 7.3.2 Renal haemodynamics (Fig 7.2)

The effects of BQ-123+E were abolished by NOS inhibition (ERBF: BQ-123+E 179 ± 63 ml/min; BQ-123+E+LNMMA -217 ± 98 ml/min, \(p<0.01\) vs. BQ-123+E. ERVR: BQ-123+E -61 ± 23 mmHg.min/L; BQ-123+E+LNMMA -14 ± 26 mmHg.min/L, \(p<0.01\) vs. BQ-123+E), but unaffected by indomethacin (ERBF: 121 ± 37 ml/min; ERVR: -42 ± 16 mmHg.min/L).
Figure 7.1 Systemic haemodynamics

- MAP
- SVRI
- CI
- HR

Time (min)

Area Under Curve (%)

- ACEI+Placebo
- ACEI+BQ-123
- ACEI+BQ-123+Indomethacin
- ACEI+BQ-123+L-NMMA

* p<0.05 vs ACEI
+ P<0.05 vs ACEI + BQ-123

Drug administration
Figure 7.2 Renal indices

- **ERBF**
- **ERVR**
- **GFR**
- **UNaV**
- **EFF**

* p<0.05 vs ACEI
+ P<0.05 vs ACEI + BQ-123

- **ACEI**
- **ACEI+BQ123**
- **ACEI+BQ-123+Indomethacin**
- **ACEI+BQ-123+L-NMMA**

Drug administration
7.3.3 Urinary sodium excretion (Fig 7.2)
The natriuresis produced by the combination of BQ-123+E (UNaV: 29.3 ± 7.4 μmol/min) was abolished by L-NMMA (−27.4± 20.3 μmol/min, p<0.01 vs. BQ-123+E) and attenuated by indomethacin (−13.6 ± 11.7 μmol/min, p<0.01 vs. BQ-123+E).

7.3.4 Serum ACE activity
Serum ACE activity at t = 0 and 180 min confirmed inhibition of ACE at the start of and throughout each study day. There was no difference in ACE activity during any phase of the study (Table 7.3).

Table 7.3 Serum ACE activity (AU)

<table>
<thead>
<tr>
<th>Serum ACE activity (AU)</th>
<th>E + Placebo</th>
<th>E + BQ-123</th>
<th>E + BQ123 + Indomethacin</th>
<th>E + BQ-123 + L-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>t = 0 min</td>
<td>6.1±3.3</td>
<td>12.6±4.0</td>
<td>11.2±4.1</td>
<td>12.6±4.4</td>
</tr>
<tr>
<td>t = 180 min</td>
<td>12.4±4.9</td>
<td>4.8±2.0</td>
<td>5.7±3.0</td>
<td>2.1±2.1</td>
</tr>
<tr>
<td>0 vs. 180 min</td>
<td>p=0.36</td>
<td>p=0.15</td>
<td>p=0.34</td>
<td>p=0.09</td>
</tr>
</tbody>
</table>

7.3.5 Plasma ET-1 concentrations
Plasma ET-1 concentrations were not affected by pre-treatment with enalapril alone, or by subsequent treatment with BQ-123, indomethacin or L-NMMA (Table 7.4).

Table 7.4 Plasma ET-1 concentrations (pg/ml)

<table>
<thead>
<tr>
<th>Plasma ET-1 (pg/ml)</th>
<th>E + Placebo</th>
<th>E + BQ-123</th>
<th>E + BQ123 + Indomethacin</th>
<th>E + BQ-123 + L-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>t = 0 min</td>
<td>3.9±0.5</td>
<td>3.4±0.6</td>
<td>5.2±0.6</td>
<td>5.0±0.6</td>
</tr>
<tr>
<td>t = 120 min</td>
<td>3.8±0.5</td>
<td>4.7±0.3</td>
<td>4.5±0.7</td>
<td>5.1±0.5</td>
</tr>
<tr>
<td>ANOVA*</td>
<td>p=0.91</td>
<td>p=0.55</td>
<td>p=0.53</td>
<td>p=0.46</td>
</tr>
</tbody>
</table>

* for 0, 30, 90, 120, 180 & 240 min measurements
7.4 Discussion

This study has demonstrated that synergy between ACE inhibition and ETA receptor antagonism is abolished by NOS inhibition but largely unaffected by prostaglandin inhibition, leading us to conclude that ETA receptor antagonism and ACE inhibition act synergistically through an ETB receptor-mediated, NO-dependent and COX-independent mechanism. This systemic data is in keeping with previous forearm work demonstrating that the dilatory effects of BQ-123 are mediated through NO but not prostaglandins [194].

As L-NMMA is a vasoconstrictor, it is possible that its ability to abolish the vasodilator effect of enalapril and BQ-123 is non-specific. However, indomethacin has also been shown to produce renal vasoconstriction in healthy subjects [414] but it had no effect on the renal vasodilatation seen in this study after enalapril and BQ-123, suggesting the effect of L-NMMA seen here is specific to the interaction between ACE inhibition and ETA receptor antagonism.

While prostacyclin is the major COX product of macrovascular endothelium in vitro, COX activity can produce both vasoconstrictor and vasodilator arachadonic acid derivatives. Prostaglandins stimulate renin release [415]. Thus indomethacin may produce both vasodilatation, by inhibition of renin-mediated ANG II generation and COX-1-mediated thromboxane A2 synthesis, and vasoconstriction, by blocking synthesis of vasodilator prostanoids. Studies in animals also suggest that vasoconstrictor COX products, such as thromboxane A2 and prostaglandin H2, might be implicated in ET-1 induced vasoconstriction, particularly in disease models [416-420]. The greater fall in blood pressure when indomethacin was co-administered with enalapril and BQ-123 may represent blockade of the action of these constrictor COX products. Additionally, there is animal evidence that vasodilator prostaglandins may also be involved in the actions of ET in the renal
circulation, particularly the renal medulla [418, 421]. This study did not, however, demonstrate any inhibitory effect of indomethacin on the systemic and renal haemodynamic effects of the combination of ACE inhibition and ETA receptor antagonism. In respect of RBF, however, clearance studies only measure total RBF. It is possible, therefore, that opposite changes are occurring in the renal cortex and medulla.

In respect of natriuresis, knockout and antagonist studies in animals have implicated ETB receptors, in association with NO production, in ET mediated sodium excretion [135]. COX inhibition however, augments big ET mediated natriuresis, suggesting the involvement of prostaglandins, in an anti-natriuretic role [422]. This study demonstrates, in the presence of ACE inhibition, an ETB dependent natriuresis that is mediated by both NO and, to a lesser extent, prostanoids.

In summary, this mechanistic study demonstrates, in healthy men, that the systemic and renal haemodynamic synergy between ACE inhibition and ETA receptor antagonism operates largely via a NO dependent mechanism.
Chapter 8

Effects of exogenous ET-1 administration in the presence of ET-A receptor antagonism and ACE inhibition in healthy volunteers
8.1 Introduction
The ETB receptor appears to have a natriuretic role at least in animals. ETB knockout mice, and ETB antagonist treated rats, develop a sodium dependent hypertension [134, 135]. Dogs subjected to high-grade ETA blockade demonstrate a vasodilatation and natriuresis in response to administration of exogenous low dose ET-1[123]. However, ET-1/ETB induced natriuresis has not been demonstrated in man. In this study exogenous ET-1 was administered in the presence of ACE inhibition and ETA blockade to examine the effects of ETB receptor activation on systemic and renal haemodynamics

8.2 Study design
This was a randomised, double-blind, placebo-controlled study 6 healthy subjects. Subjects attended for 3 visits, each separated by ≥7 days. For each visit, they received pre-treatment with enalapril. During the study day they then received placebo, BQ-123, or BQ-123 + ET-1 in a randomised order (Fig 8.1). Comparisons of interest were pre-identified as placebo vs. BQ-123 and vs. BQ-123 + ET-1, and BQ-123 vs. BQ-123 + ET-1. This study ran concurrently with the study described in Chapter 7 on the same subjects (ie. 5 randomised visits in total). Placebo and BQ-123 phases are the same in the two studies.

For 5 days before each study, subjects took enalapril at a dose of 20 mg twice daily (see methods for justification of dosing schedule) taking the last dose at 0830 on the study day. At 0830 they also received a placebo capsule to allow indomethacin administration (Chapter 7 study) to be blinded. Subjects then underwent a standard clearance study. After baseline measurements, the low dose of antagonist was administered over 15 min and ET-1 or placebo over 60 min followed by three 30 min collection periods. The higher dose of antagonist and ET-1 or placebo was then administered followed by 5 further 30 min collection periods. To ensure blinding for L-NMMA administration (Chapter 7 study), ET-1 and saline placebo were
administered in 2 stages, over 5 min and 55 min after each dose of antagonist (Fig 8.1).

**Fig 8.1 Study protocol (Chapter 7&8)**

<table>
<thead>
<tr>
<th>Time</th>
<th>Placebo or BQ-123 100 nmol/min</th>
<th>Placebo or BQ-123 1000 nmol/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=0</td>
<td>Enalapril 20 mg 0830</td>
<td>1100</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>1200</td>
</tr>
<tr>
<td>t=90 min</td>
<td></td>
<td>1330</td>
</tr>
</tbody>
</table>

- **PAH/Inulin**
  - Saline
  - L-NMMA
  - ET-1

- **Indomethacin 100 mg/Placebo**
  - Saline
  - L-NMMA
  - ET-1

BP & bioimpedance measurements every 15 min
Urinary collection periods every 30 min

At 0, 60 & 90 min after the start of low and high dose antagonist, and at the end of the study, blood samples were taken for the measurement of plasma ET-1 concentrations. BQ-123 plasma concentrations were measured before and at 15, 45 & 90 minutes after the start of each dose of antagonist, and at the end of the study. Serum ACE activity was determined at 0830 am and at 1430 (60 min after the start of the higher dose antagonist).
8.3 Results

All subjects completed the study without adverse effects. Baseline measurements were not different in any phase of the study (Table 8.1).

Table 8.1 Baseline data

<table>
<thead>
<tr>
<th></th>
<th>E+Placebo</th>
<th>E+BQ-123</th>
<th>E+BQ-123+ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>82.4±2.6</td>
<td>84.2±3.5</td>
<td>82.1±3.9</td>
</tr>
<tr>
<td>SVRI (dyne.s m²/cm²)</td>
<td>2291±275</td>
<td>2340±279</td>
<td>2238±297</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>3.1±0.4</td>
<td>3.0±0.3</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>59.6±2.8</td>
<td>60.1±3.2</td>
<td>61.9±2.9</td>
</tr>
<tr>
<td>ERBF (ml/min)</td>
<td>760±108</td>
<td>759±134</td>
<td>675±69</td>
</tr>
<tr>
<td>ERVR (mmHg.min/L)</td>
<td>124±22</td>
<td>126±18</td>
<td>132±14</td>
</tr>
<tr>
<td>EFF (%)</td>
<td>24±2</td>
<td>25±3</td>
<td>24±2</td>
</tr>
<tr>
<td>GFR (ml/min/1.73m²)</td>
<td>106±7</td>
<td>106±12</td>
<td>102±8</td>
</tr>
<tr>
<td>UNaV (μmol/min)</td>
<td>132±16</td>
<td>121±21</td>
<td>137±36</td>
</tr>
<tr>
<td>Plasma ET-1 (pg/ml)</td>
<td>3.9±0.5</td>
<td>3.4±0.6</td>
<td>5.5±0.7</td>
</tr>
</tbody>
</table>

8.3.1 Systemic haemodynamics (Fig 8.2)

The reduction in blood pressure observed after BQ-123 in the presence of enalapril (maximum placebo-corrected change from baseline −6.0 ± 1.7 mmHg) was abolished by ET-1 (−6.0 ± 1.7 mmHg). SVRI was reduced by E+BQ-123, and HR and CI increased. Administration of exogenous ET-1 abolished all systemic haemodynamic changes produced by BQ-123 except for the increase in HR.
Figure 8.2 Systemic haemodynamics

% Change from baseline

MAP

Area Under Curve (%)

SVRI

CI

HR

Time (min)

0 30 60 90 120 150 180 210 240

0 30 60 90 120 150 180 210 240

0 30 60 90 120 150 180 210 240

0 30 60 90 120 150 180 210 240

ACEI+Placebo

ACEI+BQ-123

ACEI+BQ-123+ET-1

Drug administration

ACEI+Placebo

ACEI+BQ-123

ACEI+BQ-123+ET-1

* p<0.05 vs ACEI

+ p<0.05 vs ACEI + BQ-123

Drug administration
Figure 8.3 Renal indices

- **ERBF**
- **ERVR**
- **GFR**
- **UNaV**
- **EFF**

**Drug administration**
- ACEI
- ACEI+BQ123
- ACEI+BQ-123+ET-1

* p<0.05 vs ACEI
+ P<0.05 vs ACEI + BQ-123

Time (min)

Area Under Curve (%)

* * p<0.05 vs ACEI
+ P<0.05 vs ACEI + BQ-123

Drug administration

* * p<0.05 vs ACEI
+ P<0.05 vs ACEI + BQ-123

Drug administration
8.3.2 Renal haemodynamics (Fig 8.3)

ET-1 administration abolished all renal haemodynamic changes produced by BQ-123. (ERBF: BQ-123+E 179 ± 63 ml/min; BQ-123+E+ET-1 -213 ± 56 ml/min, p<0.01 vs. BQ-123+E. ERVR: BQ-123+E -61 ± 23 mmHg.min/L; BQ-123+E+ET-1 +54 ± 1.5 mmHg.min/L, p<0.01 vs. BQ-123+E).

8.3.3 Urinary sodium excretion (Fig 8.3)

The natriuresis produced by the combination of ACE inhibition and ETA receptor antagonism (UNaV: 29.3 ± 7.4 µmol/min) was abolished by exogenous ET-1 infusion (UNaV: -38.2±23.6, p<0.01 vs. BQ-123+E).

8.3.4 Serum ACE activity

Serum ACE activity at t = 0 and 180 min confirmed inhibition of ACE at the start of and throughout each study day. There was no difference in ACE activity during any phase of the study (Table 8.2).

<table>
<thead>
<tr>
<th>Serum ACE activity (AU)</th>
<th>E + Placebo</th>
<th>E + BQ-123</th>
<th>E + BQ-123 + ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>t = 0 min</td>
<td>6.1±3.3</td>
<td>12.6±4.0</td>
<td>6.2±3.1</td>
</tr>
<tr>
<td>t = 180 min</td>
<td>12.4±4.9</td>
<td>4.8±2.0</td>
<td>5.6±2.5</td>
</tr>
<tr>
<td>0 vs. 180 min</td>
<td>p=0.36</td>
<td>p=0.15</td>
<td>p=0.76</td>
</tr>
</tbody>
</table>

8.3.5 Plasma ET-1 concentrations

Administration of exogenous ET-1 resulted in a threefold rise in plasma ET-1 concentrations 30 min after commencing each ET-1 infusion (t=0 min, 5.5±0.7 pg/ml; t=30 min, 12.5±2.8 pg/ml; t=120 min, 15.1±3.6 pg/ml, p<0.05 vs. placebo).
Plasma concentrations had reduced back to baseline 90 min after commencing ET-1 infusion (t=90 min, 5.4±0.5 pg/ml; t=180 min, 6.5±0.8 pg/ml)

8.3.6 Plasma BQ-123 (Fig 8.4)

Plasma BQ-123 concentrations were not altered by infusion of exogenous ET-1

Figure 8.4 Plasma BQ-123 levels ± exogenous ET-1 infusion

8.4 Discussion

This study demonstrates that ET-1 abolishes the systemic and renal vasodilatation and natriuresis produced by combined ACE inhibition and ETA receptor antagonism.
Studies in dogs have used ETA receptor blockade to unmask a renal vasodilatory and natriuretic effect of ET-1, presumed to be due to ETB receptor activation [123]. In administering exogenous ET-1 during combined ACE inhibition and ETA receptor blockade, the aim had been to demonstrate an effect of direct ETB receptor stimulation in man. However, administration of exogenous ET-1 in this study served only to abolish the systemic and renal effects of combined ACE inhibition and ETA receptor blockade. BQ-123 is a competitive antagonist at the ETA receptor [93] whereas ET-1 is a non-competitive agonist [413]. It would be important therefore to ensure that plasma ET-1 concentrations do not achieve levels sufficient to displace BQ-123 from the ETA receptor. A previous study has demonstrated that an equivalent dose of BQ-123 to our low dose is sufficient to abolish the systemic and renal vasoconstriction produced by this dose of ET-1 [139]. Therefore, in designing the study, it was hoped that the high dose of BQ-123, an order of magnitude greater, would be sufficient to sustain ETA blockade in the presence of this dose of ET-1. The abolition of vasodilatation and natriuresis does suggest that a degree of ETA receptor activation is occurring by displacement of BQ-123 by exogenous ET-1. However, no increases in plasma BQ-123 concentrations were observed in the presence of ET-1 to suggest any displacement from its receptor. It is possible, therefore, that the ETB receptor does not play a role in renal vasodilatory tone and sodium regulation in healthy volunteers. However, all studies so far presented (Chapter 5 & 6) and previous work from the department [194, 210] suggests that the net result of ETB receptor activation is to produce vasodilatation in man. This needs to be clarified using lower doses of ET-1 in the presence of high grade ETA receptor blockade.
Figure 8.1: ET-1 administration under conditions of ACE & ETA blockade

Key: Study drugs  Inhibited  Activated
Chapter 9

The systemic haemodynamic and renal response to acute ETA antagonism after a high salt intake in healthy volunteers
9.1 Introduction

Animal models of salt-sensitive hypertension have suggested an important pathogenic role for ET-1 [423] and the therapeutic potential for ETA receptor antagonism in this condition [424-426]. In man, plasma and urinary ET concentrations have been shown to be elevated in salt-sensitive individuals [427, 428], though this is not a uniform finding [215].

A high salt intake has also been shown to increase vascular NO activity in man [429] and the response to NOS inhibition increases on a high salt diet suggesting a greater dependence on the dilatory actions of NO when salt loaded [429, 430]. This upregulation of NO is likely to represent an important adaptation to increased salt intake and studies suggest that a relative deficiency of endothelial NO activity may contribute to the development of salt-sensitive hypertension [426, 431, 432].

ETA antagonism with BQ-123 decreases vascular tone in healthy volunteers and patient groups (Chapters 3 & 5). Forearm studies suggest these dilator effects are largely mediated through nitric oxide (NO) [194]. The hypothesis for this study was, therefore, that in individuals whose blood pressure exhibited a high degree of salt sensitivity, the relative reduction in endothelial NO activity would result in a reduced systemic haemodynamic response to BQ-123 compared to salt-resistant individuals.

9.2 Study design

This was a randomised, double-blind, placebo-controlled study 6 healthy subjects. For demographic data, see Table 9.1. Subjects attended for 4 visits, each separated by $\geq7$ days receiving placebo or BQ-123 in a randomised order in the presence or absence of sodium supplementation. For 3 days before each study subjects adhered to a standardised diet restricting salty foods (restricted salt: RS). On two of the four visits, subjects also took 15 slow sodium tablets a day (10 mmol each) to achieve a high salt (HS) intake. Comparisons of interest were pre-identified as placebo vs. BQ-123 on a RS diet, placebo vs. BQ-123 on a HS diet and BQ-123 on a RS vs. a HS diet. Salt sensitivity
index (SSI) was calculated as the percentage difference between baseline MAP on a high salt compared to a routine salt diet.

Table 9.1 Subject demographic data

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>51±3 (41 - 64)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index (kg/m^2)</td>
<td>24 ± 2 (19 - 33)</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>98.2 ± 8.8 (88.6 – 108.5)</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>93 ± 8 (62 - 119)</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.2±0.2</td>
</tr>
</tbody>
</table>

On each study day, subjects underwent a standard clearance study. After baseline measurements, the low dose of BQ-123 was administered followed by three 30 min collection periods. The higher dose of BQ-123 was then administered followed by 5 further 30 min collection periods.

At 0, 60 & 90 min after the start of low and high dose of BQ-123, and at the end of the study, blood samples were taken for the measurement of plasma ET-1, and urine samples for the measurement of urinary ET-1 excretion rates.

9.3 Results

One subject was unable to tolerate salt tablets, withdrew from the study and was replaced. Six subjects completed the study without adverse events. 24 hr urinary sodium excretion on the third day of a RS diet was 130±19 mmol/24 hrs. After the HS diet, this increased to 279±36 mmol/24 hrs (p<0.01 vs. RS). Baseline data are shown in Table 9.2.

Blood pressure at baseline was higher after the HS diet (RS both visits: 93.2±2.2, HS both visits: 101.0±3.2 p<0.01) but ERBF and GFR were unchanged. Plasma ET-1 concentrations were not increased (RS both visits: 4.8±0.3 pg/ml, HS both visits: 5.1±0.2 pg/ml) but urinary ET-1 excretion rates were higher after the HS diet (RS both visits: 10.6±1.2 pg/min, HS both visits: 13.7±1.4 pg/min p<0.05).
Table 9.2 Baseline data on routine salt (RS) vs. high salt (HS) diet

<table>
<thead>
<tr>
<th></th>
<th>Placebo RS diet</th>
<th>BQ-123 RS diet</th>
<th>Placebo HS diet</th>
<th>BQ-123 HS diet</th>
<th>RS vs. HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>94.5±2.8</td>
<td>93.4±3.7</td>
<td>100.7±4.9</td>
<td>101.4±4.6</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>SVRI (dyne.s m⁻²/cm²)</td>
<td>2527±297</td>
<td>2842±453</td>
<td>3009±512</td>
<td>2787±385</td>
<td>NS</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>3.2±0.4</td>
<td>2.9±0.4</td>
<td>3.0±0.4</td>
<td>3.1±0.4</td>
<td>NS</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>60.8±0.9</td>
<td>58.4±1.1</td>
<td>55.3±1.4*</td>
<td>55.6±1.3*</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>ERBF (ml/min)</td>
<td>695±78</td>
<td>703±75</td>
<td>732±118</td>
<td>737±96</td>
<td>NS</td>
</tr>
<tr>
<td>ERVR (mmHg.min/L)</td>
<td>150±13</td>
<td>144±17</td>
<td>159±32</td>
<td>153±25</td>
<td>NS</td>
</tr>
<tr>
<td>EFF (%)</td>
<td>24±2</td>
<td>22±2</td>
<td>23±2</td>
<td>22±2</td>
<td>NS</td>
</tr>
<tr>
<td>GFR (ml/min/1.73m²)</td>
<td>99±11</td>
<td>102±13</td>
<td>105±10</td>
<td>104±10</td>
<td>NS</td>
</tr>
<tr>
<td>UNaV (µmol/min)</td>
<td>165±11</td>
<td>166±25</td>
<td>253±17</td>
<td>221±24</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Plasma ET-1 (pg/ml)</td>
<td>4.7±0.5</td>
<td>4.9±0.4</td>
<td>5.2±0.2</td>
<td>5.0±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary ET-1 excretion (pg/min)</td>
<td>11.3±1.5</td>
<td>10.0±1.8</td>
<td>12.3±2.2</td>
<td>15.1±1.5</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

*p<0.05 vs. Placebo RS diet

9.3.1 Systemic haemodynamics (Figs 9.1 & 9.2)

BQ-123 significantly reduced MAP and SVRI, and increased HR and CI on both a RS and a HS diet to an equal degree (p<0.01 vs. placebo) (Fig 9.1). However, the increase in baseline MAP on a high salt diet for each individual (SSI) did correlate with the extent to which he responded to ETA antagonism (R²=0.76 p=0.02) (Fig 9.2). There was no correlation observed between the reduction in MAP after BQ-123 and baseline MAP (R²<0.001 p=0.98), or 24 hour urinary sodium excretion (R²<0.001 p=0.99) for that study day.
Figure 9.1 Systemic haemodynamics

**MAP**

**SVRI**

**CI**

**HR**

Area Under Curve (%)

* p<0.05 vs Placebo RS
+ P<0.05 vs Placebo HS

Placebo RS
BQ-123 RS
Placebo HS
BQ-123 HS
9.3.2 Renal haemodynamics (Figs 9.3 & 9.4)

Consistent with previous studies in healthy volunteers (Chapter 5) BQ-123 did not significantly alter ERBF, ERVR or GFR and no correlations were noted between SSI and ERVR ($R^2=0.59 \ p=0.27$), or ERBF ($R^2=0.28 \ p=0.08$).

9.3.3 Urinary sodium excretion

While BQ-123 modestly increased urinary sodium excretion on both diets (RS: $+7.9\pm11.1\%$; HS: $+10.7\pm12.0\%$, $p<0.05$ vs. HS placebo), there was no correlation between SSI and sodium excretion ($R^2<0.001 \ p=1.00$), or fractional excretion ($R^2<0.001 \ p=0.96$).
9.3.4 ET-1 concentrations

Plasma ET-1 concentrations were not altered by any treatment (Table 9.3). Urinary ET-1 excretion rates were higher at baseline on a HS diet (Table 9.2) but did not
correlate with 24 hr urinary sodium excretion or SSI. Urinary ET-1 excretion was not altered by administration of placebo or BQ-123 on either diet (Table 9.3).

**Table 9.3 Plasma ET-1 concentrations (pg/ml) and urinary excretion rates (pg/min)**

<table>
<thead>
<tr>
<th></th>
<th>Plasma ET-1 concentrations (pg/ml)</th>
<th></th>
<th>Urinary ET-1 excretion (pg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>BQ-123</td>
<td>Placebo</td>
</tr>
<tr>
<td>RS diet</td>
<td>t = 0 min</td>
<td>4.7±0.5</td>
<td>4.9±0.4</td>
</tr>
<tr>
<td></td>
<td>t = 120 min</td>
<td>4.8±0.5</td>
<td>4.1±0.3</td>
</tr>
<tr>
<td>ANOVA*</td>
<td>p=0.97</td>
<td>p=0.41</td>
<td>p=0.53</td>
</tr>
<tr>
<td>RS diet</td>
<td>t = 0 min</td>
<td>11.3±1.5</td>
<td>10.0±1.8</td>
</tr>
<tr>
<td></td>
<td>t = 120 min</td>
<td>10.2±1.2</td>
<td>11.7±2.5</td>
</tr>
<tr>
<td>ANOVA*</td>
<td>p=0.85</td>
<td>p=0.91</td>
<td>p=0.72</td>
</tr>
</tbody>
</table>

* for 0, 30, 90, 120, 180 & 240 min measurements

**9.4 Discussion**

While this study did not demonstrate a difference, in the group as a whole, in the effect of BQ-123 on systemic and renal haemodynamics on a HS diet compared to the RS diet, individuals with higher degrees of salt sensitivity of their blood pressure appear to have a blunted blood pressure response to ETA receptor antagonism on a high salt intake. This suggests that ETA antagonists would be more effective as treatments for hypertension in salt-sensitive individuals when such patients are salt restricted.

The role of ET in the response to salt and salt-sensitivity has been supported by a series of studies. Salt loading causes rats to develop greater hypertension on a high salt diet under conditions of chronic ETB receptor blockade [433], and the DOCA-salt hypertensive rat, a salt-sensitive strain demonstrates increased ET-1 production,
upregulation of ETB receptors and hypertension that is blocked by ETA receptor antagonism [434, 435]. Renal ET-1 in particular is important in the response to salt. Urinary ET-1 is increased by a high salt diet [433], and a high salt intake also augments the ETB receptor mediated medullary vasodilatation induced by big-ET-1 [133]. A high salt intake is known to increase renal NO activity [430]. However, salt-sensitive rats fail, unlike salt-resistant strains, to increase renal NOS activity in response to salt loading [426]. Taken together, these studies suggest that the response to an increased salt ingestion is to increase in ETB and NO mediated vasodilatation particularly in the renal medulla where it promotes natriuresis (that may be in part related to this increase in blood flow or direct tubular effect) as an adaptive response to limit hypertension, and that in salt-sensitive individuals, there is a failure to increase NO sufficiently and a consequent reduced sodium excretion and increased systemic vasoconstriction.

It has previously been demonstrated in forearm studies that ETA receptor antagonists achieve their haemodynamic effects through NO [194], presumed due to binding of ET-1 under conditions of ETA receptor blockade to the ETB receptor. It is possible that the lesser response to BQ-123 in salt-sensitive subjects seen in this study is a consequence of reduced endothelial NO activity in such individuals compared to salt-resistant individuals. However, if the lesser response to BQ-123 in salt-sensitive subjects is a consequence of reduced endothelial NO activity, concomitant ETB blockade will be less important in salt-sensitive individuals on a high-salt diet with loss of endothelial NO. This must be borne in mind in comparing the clinical effects of selective ETA and combined ETA/B receptors in unscreened subjects, as a high salt diet in salt-sensitive individuals may limit any differences between these two therapeutic approaches.

In this study, a high salt diet increased baseline blood pressure without an alteration in plasma ET-1 concentrations to suggest an upregulation of the ET system. However, due to the largely paracrine action of vascular endothelial ET-1, plasma ET-1 is not a good marker of the activity of the vascular ET system. Urinary ET-1, a marker of increased renal production [436], was, however, increased on a high salt diet consistent with increased renal ET-1 in response to salt loading. Despite this, no clear changes in renal blood flow or vascular tone could be demonstrated in this study either in the group as a
whole, or relating the changes observed in individuals to their salt-sensitivity, though the subject with the highest degree of salt-sensitivity did exhibit the greatest increase in ERVR and fall in ERBF on a high salt diet. PAH clearances can only measure total renal blood flow so changes in medullary or cortical blood flow may be missed. Nevertheless, SSI, however, had no influence on sodium excretion either in this study.

However, this study is limited by the small number of subjects studied and the fact that 4 out of the 6 subjects had very similar and small increases in blood pressure in response to increased salt intake. While a trend was observed in respect of a correlation between SSI and ERBF and ERVR, this did not reach significance. Further studies are needed in healthy volunteers and patients with a range of SSIs, particularly subjects with a high degree of salt sensitivity to explore this further.

In conclusion, the effectiveness of ETA receptor blockade may be limited in salt-sensitive individuals who continue to take a high salt diet. Identification of such individuals is therefore important, as pharmacological intervention for hypertension must be instituted in conjunction with dietary advice.
Chapter 10

Urinary excretion of endothelin-1
10.1 Introduction

ET-1 is largely removed from the circulation by receptor (ETB) mediated mechanisms, primarily in the pulmonary circulation [86-88]. Renal clearance, however, does account for ~10% of plasma ET-1 clearance in man [86]. ET-1 is a small polypeptide and plasma ET-1 will thus be filtered at the glomerulus, and, because it is dependent upon filtration, this renal clearance does not require receptor-mediated mechanisms. After filtration, in common with most polypeptides, ET-1 is likely to be hydrolysed at the brush border and the constituent peptides and amino acids reabsorbed in the tubules and metabolised. In rats, though up to 15% of plasma ET-1 is extracted across the renal bed [88] less than 1% of injected radiolabelled ET-1 is recovered in the urine [92]. The failure of plasma ET-1 in these isotope studies to appear in the urine implies that neither glomerular filtration nor tubular secretion of plasma ET-1 accounts for urinary ET-1, which is therefore assumed to be primarily of renal origin, and, hence, independent of plasma ET-1 concentrations.

In CRF, the consequence of a reduced filtration rate is a reduced filtered load of ET-1 delivered to the tubules. Thus renal ET-1 clearance will fall as renal function is lost, leading to increases in plasma concentrations of ET-1 [216]. Renal ET-1 production, and hence urinary ET-1 excretion, is, however, also increased in CRF [92, 188]. Therefore, while plasma ET-1 concentrations and urinary ET-1 excretion should be independent of each other [436], both will correlate inversely with GFR, and may, thus, appear to correlate with each other.

There is also recent animal evidence to suggest that ETB receptors may participate in the movement of proteins across the renal tubular epithelium [437]. Peri-tubular ET-B receptors are abundant in the human kidney [52]. However, their possible role in urinary ET-1 excretion has not been studied.
Using paired plasma and urine samples from the studies described in studies 3-7 (Chapter 5-9), this chapter compares plasma ET-1 concentrations and ET-1 excretion rates to establish if any relationship exists between these two measurements. Additionally, using measurements from study 3 (Chapter 5), the effect of ET receptor blockade on urinary excretion of ET-1 has been examined.

10.2 Protocol
During all of the clearance studies (studies 3-7), paired plasma and urine samples were taken at 0, 60 & 90 min after the start of both low and high dose antagonist administration, and at the end of the study, for the measurement of plasma and urinary ET-1 concentrations. Urinary excretion rate was calculated by multiplying urinary ET-1 concentration by urine flow rate.

10.3 Results
10.3.1 Correlation of plasma and urinary ET-1
Analysing the results from study 3 (Chapter 5) in isolation, at baseline (t=0), before administration of ET receptor antagonist, a limited correlation between plasma and urinary ET-1 excretion was observed (R²=0.09, p=0.02). However, both plasma ET-1 concentration and urinary ET-1 excretion correlated inversely with baseline GFR (Fig 10.1). Fractional excretion of ET-1 also correlated inversely with baseline GFR (R²=0.44, p<0.01) and only exceeded unity in patients with CRF (Fig 10.2).

Analysis of paired plasma and urine samples from all the phases of study 3 (384 paired samples) did not demonstrate a relationship between plasma ET-1 concentration and urinary ET-1 excretion (R²=0.001, p=0.50) (Fig 10.3).
Figure 10.1 Plasma & urinary ET-1 in baseline samples

Plasma ET-1 concentration vs. GFR

Urine ET-1 excretion vs. GFR

Plasma ET-1 concentration vs. urine ET-1 excretion

Healthy Volunteers

Renal Patients
Figure 10.2 Fe ET-1 vs. GFR

Fig 10.3 Plasma ET-1 concentrations vs. Urinary ET-1 excretion rate - all samples
Plasma ET-1 concentrations were significantly increased acutely in two studies. In study 6 (Chapter 8), administration of exogenous ET-1 achieved a ~ threefold increase in plasma ET-1 concentrations but did not alter urinary ET-1 excretion rates ($R^2=0.009$, $p=0.58$) (Fig 10.4).

**Fig 10.4 Plasma ET-1 concentrations vs. Urinary ET-1 excretion rate during exogenous ET-1 administration**

**R² = 0.009  
**p=0.58
In study 3, plasma ET-1 concentrations increased at 30 min after administration of the high dose of BQ-788 alone (+2.6±0.5, p<0.01), or in combination with BQ-123 (+3.5±0.6 pg/ml, p<0.01). Again, these alterations in plasma ET-1 after ET receptor antagonism were not reflected by changes in urinary ET-1 excretion (R²=0.009, p=0.11) (Fig 10.5).

**Fig 10.5 Plasma ET-1 concentrations vs. Urinary ET-1 excretion rate during ETB receptor blockade**

![Graph showing plasma ET-1 concentrations vs. urinary ET-1 excretion rate during ETB receptor blockade.](image)
10.3.2 Effect of ET receptor antagonism on ET-1 excretion

In study 3, following administration of BQ-788 alone urinary ET-1 excretion decreased (-4.7±1.5 pg/min, p<0.05) (Fig 10.6) but was not altered by placebo, BQ-123 or BQ-123/788. Because GFR was also reduced by ETB receptor antagonism (-11.4±3.4%, p<0.01 vs. placebo), fractional urinary excretion of ET-1 was calculated. This was reduced after both BQ-788 alone (-41±6%, p<0.01) and in combination with BQ-123 (-40±7%, p<0.01), but not by placebo (+6±12%) or BQ-123 alone (-12±8%) (Fig 10.4). No difference was observed in the pattern of responses to ET receptor antagonism between healthy volunteers and CRF patients. In study 4 (Chapter 6), when healthy volunteers were pre-treated with ACE inhibitor, again a reduction in fractional excretion of ET-1 was observed when BQ-123/788 (-23±13%) but not BQ-123 (-6±18%) was given.
Figure 10.6 Plasma ET-1 concentrations and urinary ET-1 excretion after ET receptor antagonism

- Plasma ET-1 concentration (pg/ml)
- Urine ET-1 excretion (pg/min)
- Fractional urinary ET-1 excretion (% change from baseline)

Legend:
- Placebo
- BQ-123
- BQ-123/788
- BQ-788

*p<0.05 vs. BL
10.4 Discussion

The lack of correlation between plasma ET-1 concentrations and urinary ET-1 excretion rates, particularly when drug manoeuvres acutely increase plasma ET-1 concentrations, demonstrates the independence of plasma and urinary ET-1 concentrations, consistent with two separate ET systems in the circulation and the kidney. Additionally, because urinary ET-1 is dramatically and acutely reduced by ETB blockade, these data also suggest a role for ETB receptors in the excretion of ET-1 of renal origin.

Baseline data from study 3 (Chapter 5), before pharmacological manoeuvres to change plasma ET-1, confirm an inverse correlation between plasma ET-1 concentration and GFR, consistent with reduced renal clearance, and between urine ET-1 excretion and GFR, consistent with increased renal production of ET-1 in CRF. Because both plasma ET-1 concentrations and urine ET-1 excretion are related to GFR, a weak correlation does exist between these two measurements at baseline. Additionally, baseline data from study 3 demonstrate that fractional excretion of ET-1 only exceeds unity in patients with CRF. For this to occur more ET-1 must be excreted than filtered. If, as suggested by isotope studies [92], plasma ET-1 does not account for urinary ET-1, a fractional excretion exceeding unity demands renal ET-1 synthesis. Hence our finding supports urinary ET-1 excretion as being a marker of the increased renal ET-1 production in CRF.

There are few data, however, to suggest how renal ET-1 reaches the tubules. A recent study in fish has localised ETB receptors to the basolateral aspect of proximal tubular cells and has demonstrated that low concentrations of ET-1 are able to reduce cell to tubular lumen transport of methotrexate and CyA derivatives. This effect was inhibited by ETB receptor antagonists [437], suggesting a role for ETB receptors in tubular transport mechanisms.

In study 3 and 4, after correcting for changes in GFR by calculating FeET-1, it was evident that urinary ET-1 excretion is reduced specifically by blockade of the ETB receptor either alone or when combined with ETA receptor blockade. Because the acute
nature of our studies make it unlikely that ET receptor blockade had significant effects on renal ET-1 production, these data suggest that the renal tubular excretion of ET-1 appears also to be at least partly mediated through the ETB receptor.

In conclusion, these data provide confirmatory evidence in man for two independent ET systems in the circulation and in the kidney, both related to GFR, and suggest, for the first time, that ETB receptors may be involved in the urinary excretion of renal ET-1. This has implications for the use of ET antagonists that block both the ETA and the ETB receptor. ET-1 is associated with renal fibrosis [438], and therefore failure to excrete renal ET-1 may be detrimental to the kidney, limiting the use of combined ETA/B receptor antagonists. As ET receptor antagonists are in Phase III clinical trials, this important observation warrants further attention.
Chapter 11

Renal Clearance of BQ-123
11.1 Introduction

BQ-123 is a cyclic pentapeptide that is cleared by hepatic anion transport mechanisms [97]. However, though non-significant, renal patients had higher plasma concentrations after BQ-123 infusion than healthy volunteers for the same dosing schedule of BQ-123 (study 3). Concentrations of BQ-123 were therefore measured in the urine in a subgroup of subjects to ascertain if any BQ-123 was excreted renally, and, if so, whether this renal clearance was significantly reduced in CRF to account for the higher plasma concentrations observed in CRF patients.

Urinary concentrations of BQ-123 were therefore measured after BQ-123 administration (alone or with BQ-788) during 10 healthy volunteer visits and 5 patient visits from the study described in Chapter 5.

11.2 Results

BQ-123 was recoverable in the urine after intravenous administration. The profile of BQ-123 appearance in the urine mirrored exactly the profile in the plasma (Fig 11.1).

Fig 11.1 BQ-123 plasma concentrations and urinary excretion rates
It fell to zero and remains there by 2 hours after the high dose infusion. In the group as a whole, the total excreted was 3.7 ± 0.5% (0.3 - 6.2%) of the total given.

An inverse correlation between peak plasma concentrations of BQ-123 at 105 min (at end of high dose BQ-123 infusion) and urinary BQ-123 excretion rate between 90 and 120 min was noted (R²=0.35, p=0.04). Additionally, a correlation between the total BQ-123 excreted by an individual and their GFR was noted (R²=0.31, p=0.03) (Fig 11.2).

**Fig 11.2 % BQ-123 excreted vs. GFR**

11.3 Discussion

BQ-123 is subject to renal clearance and this clearance reduces as GFR falls. However, this renal excretion is of minor importance in respect of overall clearance of BQ-123 from the circulation. A reduced urinary excretion rate of BQ-123 is, nevertheless, associated with higher plasma concentrations, and must be taken into consideration in interpreting the effects of BQ-123 in renal patients. Looking at individual plasma concentrations, this is probably only relevant in patients with a very low GFR (<20 ml/min).
Chapter 12

The effects of single high dose cyclosporin in healthy volunteers
12.1 Introduction

CyA is a fungal derived peptide that is a widely used immunosuppressive agent that inhibits the immune system by suppressing T-helper cell activation via inhibition of the intracellular enzyme calcineurin. The introduction of CyA in the transplant field in the 1980s has significantly improved graft survival [439-441]. Unfortunately, though it is an effective immunosuppressive drug, it has a wide range of side effects including hypertension and nephrotoxicity. It is estimated that 60 - 90% of patients on chronic CyA therapy requiring anti-hypertensive medication [441-443].

The precise mechanisms of CyA induced hypertension and nephrotoxicity however, remain unclear. Impaired vasodilator responsiveness [444] or enhanced vasoconstrictor tone, either as a direct CyA effect [445] or as a consequence of increased sympathetic nervous system (SNS) activity [445-447] and renal vasoconstriction, with salt and water retention and consequent extravascular volume expansion [448], have all been proposed, and it is probable that more than one mechanism is acting to increase blood pressure.

Animal models and patient studies have both demonstrated increases in systemic vascular resistance and reductions in CO in response to CyA [449-452]. CyA is also well documented as causing acute renal vasoconstriction in patients on chronic CyA treatment [453-456]. However, while acute studies in healthy volunteers have demonstrated renal hypoperfusion after a single high dose of CyA [347, 457-459], this is not a uniformly reproducible effect [460, 461], and experimental data suggests that, in both healthy subjects and patients, systemic and renal vasoconstriction may only develop after days to weeks of CyA treatment [462-465]. Additionally, forearm studies have suggested that acute CyA administration might, paradoxically, increase the activity of endothelial vasodilators in healthy subjects naïve to the drug [466].

The purpose of this study was, therefore, to examine the systemic and renal haemodynamic effects of a single, oral, high dose of CyA in healthy volunteers to ascertain if the pattern of systemic and renal haemodynamic changes produced differed
from those documented in patients on chronic CyA therapy, and thus ascertain if this is a relevant model for the investigation of chronic CyA induced systemic vasoconstriction and renal hypoperfusion.

12.2 Study design

The study was a randomised, double-blind, placebo-controlled, crossover study. Subjects attended for 2 visits, separated by ≥5 days. Twelve healthy volunteers were recruited to the study. For subject demographic data see Table 12.1

On each day, subjects then underwent an adapted clearance study. After baseline measurements over three 20 min collection periods, subjects then received placebo (olive oil) or CyA (Neoral) liquid (Novartis), 10 mg/kg. Measurements then continued at 20 min intervals for 4 hours. At 0, 60, 120, 180 and 240 min, blood was collected for the measurement of plasma CyA and ET-1.

Table 12.1 Subject demographic data

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>33±2 (22 - 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24 ± 1 (20 - 26)</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>90.3 ± 1.8 (80.0 - 99.3)</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>84 ± 3 (71 - 104)</td>
</tr>
</tbody>
</table>

12.3 Results

6 subjects completed the CyA arm of the study and 9 completed the placebo arm. The most common reason (in 5 of 24 studies) for non-completion was emesis. Other reasons were not study related. Data was only analysed from those subjects completing a study day. Baseline data did not differ between CyA and placebo phases (Table 12.2).
12.3.1 Systemic haemodynamics

Mean arterial pressure rose in the CyA group by 6.7 ± 1.9% from 92.9 to 99.6 mmHg (p<0.001 compared to placebo). This was accompanied by an increase in HR of 26.2 ± 3.5% (p<0.001), an increase in CI of 20.6 ± 3.1% (p<0.001) and a fall in SVRI of 13.5 ± 1.4% (p<0.001). Changes were maximal at 80 min but persisted throughout the 4 hours of the study (Fig 12.1).

Table 12.2: Baseline data

<table>
<thead>
<tr>
<th></th>
<th>CyA</th>
<th>Placebo</th>
<th>CyA vs. Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mmHg)</td>
<td>(mmHg)</td>
<td>t-test</td>
</tr>
<tr>
<td>MAP</td>
<td>92.9±2.1</td>
<td>89.9±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>SVRI (dyne.s m²/cm⁵)</td>
<td>2432±152</td>
<td>2448±232</td>
<td>NS</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>3.1±0.2</td>
<td>2.9±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>57.3±3.4</td>
<td>54.3±2.6</td>
<td>NS</td>
</tr>
<tr>
<td>ERBF (ml/min)</td>
<td>914±90</td>
<td>862±103</td>
<td>NS</td>
</tr>
<tr>
<td>ERVR (mmHg.min/L)</td>
<td>116±16</td>
<td>122±17</td>
<td>NS</td>
</tr>
<tr>
<td>EFF (%)</td>
<td>27±2</td>
<td>24±3</td>
<td>NS</td>
</tr>
<tr>
<td>GFR (ml/min/1.73m²)</td>
<td>120±8</td>
<td>103±7</td>
<td>NS</td>
</tr>
<tr>
<td>UNaV (µmol/min)</td>
<td>130±41</td>
<td>106±17</td>
<td>NS</td>
</tr>
</tbody>
</table>

12.3.2 Renal haemodynamics

A moderate fall in GFR was seen (-9.0 ± 4.8%, p<0.01), maximal at 180 min. ERBF after CyA was not different from placebo. However, ERBF as a proportion of CO did fall from 14.8 to 11.6% (p<0.01) (Fig 12.2).

12.3.3 Urinary sodium excretion

There were no differences in urinary sodium excretion between the two groups.
Figure 12.1 Systemic haemodynamics

![Graphs showing changes in MAP, SVRI, CI, and HR over time for Cyclosporin and Placebo, with statistical significance noted.](image)

- MAP: *p<0.001
- SVRI: *p<0.001
- CI: *p<0.05 vs. placebo, +p<0.01 vs. placebo
- HR: *p<0.001
Figure 12.2 Renal haemodynamics

- EEBF
- ERVR
- GFR
- ERBF/CO

Change from baseline (ml/min)

Placebo
Cyclosporin

*p<0.05 vs. placebo
p<0.01
12.3.4 Plasma ET-1 concentrations

Plasma ET-1 concentrations were not altered by CyA administration (Placebo baseline: $3.5 \pm 0.9$ pg/ml, t=4 hrs $2.7 \pm 0.5$; CyA baseline $4.8 \pm 2.8$ t=4 hrs: $3.9 \pm 0.6$)

12.3.5 Plasma CyA concentrations

CyA concentrations were maximal at 60 minutes post administration and remained at this level for a further 60 minutes reaching peak values of $1630 \pm 169$ nmol/L (Fig 12.3).

Fig 12.3 Plasma cyclosporin concentrations after administration of 12 mg/Kg oral neoral

![Graph of plasma cyclosporin concentrations](image)

12.4 Discussion

Single, high dose CyA in healthy volunteers caused the expected increase in blood pressure, together with a striking increase in HR. However it was also associated with a decrease in SVRI and an increase in CI. This contrasts with the reported haemodynamics in patients on chronic CyA therapy where an increase in SVRI and a
reduction in CI are commonly seen [451, 452]. Additionally, the renal vasoconstriction reported in patients on treatment [347, 453, 454, 456] was not seen though there was a fall in RBF relative to CO.

Recent forearm studies in healthy volunteers have shown that acute administration of CyA results in increased basal and stimulated NO activity and NOS gene expression [466]. Conversely, renal transplant patients on chronic CyA show reduced basal and stimulated NO activity compared to healthy volunteers [467]. CyA is also known to increase gene transcription and secretion of ET-1 from endothelial and smooth muscle cells [171]. Increased activity of the ET system is also indicated by the ability of ET receptor antagonism to attenuate CyA induced smooth muscle cell contraction [171] and CyA induced renal vasoconstriction [145]. Changes in gene transcription will not be manifest immediately, but will take some days to develop fully. Hence, while CyA acutely upregulates endothelial NO activity, chronic exposure to CyA will cause endothelial dysfunction due to both reduced NO activity and increased ET-1 activity, altering the balance of vasoactive compounds in the endothelium in favour of vasoconstriction. Rat models suggests that this switch in endothelial balance to favour vasoconstriction happens early, with 1-3 weeks being sufficient for reduction in urinary NO metabolites and alterations in vessel responses suggestive of reduced NO activity to occur [468, 469]. Such data suggest the mechanisms behind the hypertensive and renal haemodynamic changes of a single high dose of CyA will be different from those found in chronic CyA administration. The results from this study are consistent with this hypothesis.

The most striking change in this study was the increase in HR after CyA. This study was not designed to look the sympathetic nervous system (SNS), however, it is known that CyA can increase SNS activity [446, 447]. The increase observed in this study was observed in subjects who did not experience nausea after CyA, suggesting this was a direct CyA effect. Such large increases have also been observed after 10mg/kg CyA (Hansen). In the study presented in Chapter 13, subjects given 1-2 mg/kg exhibited small
increases in HR, more consistent with the published literature. This would suggest that the marked increase seen in this study is probably related to the large size of the dose given. Such SNS stimulation will have haemodynamic consequences. As the systemic vasodilatation observed in this study would normally reduce blood pressure, and given that the time course of the study is too short for significant salt and water retention to occur, it is possible that the increase in MAP after CyA in this study is due to this increase in SNS activity. This is provides a second reason why an acute single high dose model of CyA administration might be inappropriate for the investigation of chronic CyA induced changes.

Finally, subjects experienced nausea and vomiting in 5/24 studies and had to discontinue the protocol. Water loading undoubtedly contributed to the nausea as one subject vomited in the placebo phase and before drug dosing. However, 4/5 subjects vomited during the CyA arm of the study with the time course of the vomiting correlating with peak plasma CyA concentrations. This high incidence of emesis renders this model both ethically and physiologically unsound.

In summary therefore, the systemic vasodilatation and lack of changes in RBF in healthy volunteers given single high dose CyA concurs with forearm studies suggesting an initial upregulation of NO in the endothelium in response to CyA. It is at variance with the reported haemodynamic changes in subjects receiving chronic CyA treatment, namely systemic and renal vasoconstriction. This makes an acute single high dose model is an inappropriate one for the assessment potential therapeutic treatments for CyA induced hypertension and nephrotoxicity. The time course of the switch from dilatation to constriction, and its correlation with the induction of endothelial dysfunction by CyA needs more detailed investigation in man.
Chapter 13

The haemodynamic response to cyclosporin in renal transplant patients
13.1 Introduction

Study 8 (chapter 12) demonstrated that in healthy subjects receiving a single large dose of CyA, the acute increase in MAP seen is associated with an increase in CO and a decrease in SVR. In animal models, short-term administration of CyA leads to an increase in SVR and MAP and a decrease in CO [449, 450]. Similar haemodynamic changes have been reported in heart transplant patients when compared with essential hypertensives matched for blood pressure [451]. These different responses to CyA in heart transplant patients compared to healthy subjects might be explained by the altered cardiac responses of a denervated transplanted heart, or the long-term exposure to CyA in these patients. However, vasoconstriction to a single dose of intravenous CyA has also been reported in renal patients on dialysis, but not on chronic CyA treatment [452], suggesting other factors, such as renal impairment, might be involved in CyA induced vasoconstriction.

The purpose of this study was, therefore, to explore the effects of a single standard oral dose of CyA on systemic haemodynamics renal transplant patients to observe if chronicity of CyA treatment was important in the generation of systemic haemodynamic responses and hypertension. To assess this, the systemic haemodynamic responses of renal transplant patients on chronic immunosuppressive therapy including CyA was compared with matched renal transplant patients on long-term immunosuppressive regimes consisting of prednisolone and/or azathioprine (Pred/Aza) but not including CyA. The study hypothesis was that a single dose of CyA would induce vasoconstriction in patients on long-term CyA but not in patients previously naive to CyA.

13.2 Study design

The study was a randomised, single-blind, placebo-controlled, crossover study in 22 renal transplant patients. Subjects attended for 2 visits, separated by ≥5 days. Subjects continued to take all their normal medications except for CyA, where relevant, which was omitted on the morning of the study only. Subjects received CyA 100 mg on one visit and placebo
(icing sugar) on the other. Both CyA and placebo were placed within gelatin capsules to ensure that the subjects remained blinded to the treatment received on each study day. On each study day, an 18 SWG cannula was sited in the left antecubital vein. Subjects then rested supine for 40 min before study drug administration. Subjects remained recumbent throughout the study. Haemodynamic measurements were made at 10 min intervals from 30 min pre-dose until CyA administration, then at 20 min intervals until 3 hours after dosing. At 0 and 3 hours, blood samples were taken for the measurement of plasma ET-1

13.2.1 Subjects
Eleven renal transplant patients on chronic immunosuppressive therapy including CyA, Pred and Aza (Group I) and eleven patients maintained on immunosuppressive therapy with Pred/Aza but not including CyA (Group II), all under long term follow-up by Edinburgh Renal Unit, were recruited to the study. Patients on immunosuppressive agents other than Pred, Aza or CyA were not recruited to the study. Additionally, patients with known ischaemic heart disease, clinical evidence of heart failure, heart transplantation, diabetes mellitus, or other significant cardiac or vascular history were excluded from recruitment.

<table>
<thead>
<tr>
<th>Table 13.1 Subject demographic data</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Number</td>
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<tr>
<td>Age (yr)</td>
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<td>Body mass index (kg/m(^2))</td>
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<tr>
<td>MAP (mmHg)</td>
</tr>
<tr>
<td>Creatinine ((\mu)mol/L)</td>
</tr>
<tr>
<td>Duration of transplant (months)</td>
</tr>
<tr>
<td>CyA dose - chronic (mg/dose)</td>
</tr>
<tr>
<td>CyA given (mg/kg)</td>
</tr>
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</table>
Groups were matched for creatinine, age and weight. However, mean arterial pressure was slightly higher in group I, 9 of the 11 patients in group I, and 8 of the 11 patients in group II were also on anti-hypertensive medication, and the mean duration of immunosuppressive therapy (= duration of transplant) was significantly shorter for this group compared to the patients on long-term Pred/Aza only (Table 13.1). For subject demographics and patient characteristics, see Tables 13.1 & 2

**Table 13.2a Subject characteristics**

<table>
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<tr>
<th></th>
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<th>HT?</th>
<th>Time since Tx (months)</th>
<th>Cre (μmol/L)</th>
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Table 13.2b Subject characteristics

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<th>Renal diagnosis</th>
<th>am CyA dose (mg/kg)</th>
<th>HT?</th>
<th>Anti-HT medication</th>
<th>Time since Tx (months)</th>
<th>Cre (µmol/L)</th>
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<td>Doxazosin Enalapril</td>
<td>115</td>
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</table>

M - male, F - female, CPN - chronic pyelonephritis, GN - glomerulonephritis, HT - hypertension, NK - not known, PKD - polycystic kidney disease, COAD - chronic obstructive airways disease, OA - osteoarthritis, OP - osteoporosis, ROD - renal osteodystrophy, Tx - transplant

13.3 Results

All 22 subjects completed the study. No adverse events were reported. Baseline haemodynamic data did not differ on each of the study phases (Table 13.3).
### Table 13.3 Baseline data

<table>
<thead>
<tr>
<th></th>
<th>Group I Placebo</th>
<th>Group I CyA</th>
<th>Group II Placebo</th>
<th>Group II CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAP (mmHg)</strong></td>
<td>98.1±3.3</td>
<td>101.2±3.8</td>
<td>93.1±3.7</td>
<td>92.2±2.1</td>
</tr>
<tr>
<td><strong>SVRI (dyne.s m²/cm⁵)</strong></td>
<td>2846±347</td>
<td>2996±298</td>
<td>2726±136</td>
<td>2755±167</td>
</tr>
<tr>
<td><strong>CI (L/min/m²)</strong></td>
<td>3.07±0.24</td>
<td>3.05±0.28</td>
<td>2.81±0.21</td>
<td>2.81±0.24</td>
</tr>
<tr>
<td><strong>HR (bpm)</strong></td>
<td>61.1±2.8</td>
<td>61.7±2.5</td>
<td>66.6±2.8</td>
<td>66.8±2.8</td>
</tr>
</tbody>
</table>

#### 13.3.1 Systemic haemodynamics

There was a small increase in MAP in both groups after acute CyA administration (maximum placebo-corrected change from baseline, group I: + 7.4 ± 3.3 mmHg, ANOVA p<0.01 vs. placebo, group II: +6.7 ± 2.5 mmHg, p<0.01). HR was unchanged after CyA administration in both groups relative to baseline values, though increased in group II relative to placebo (group I: +2.3 ± 1.2 bpm, p>0.5 vs. placebo, group II: +3.9 ± 2.1 bpm, p<0.01) (Fig 13.1).

A significant decrease in CI was observed in group I (-0.2 ± 0.11 L/min/m², p<0.05). This contrasted with an increase in CI in group II (+0.27 ± 0.10 L/min/m², P<0.05). SVRI increased in group I both in absolute terms and relative to placebo (+460 ± 128 dyne.sec/cm⁵/m², p<0.01) (Fig 13.1). By contrast, in group II SVRI was unchanged relative to baseline and placebo (-257 ± 137 dyne.sec/cm⁵/m², p>0.1) (Fig 13.1). Both CI and SVRI responses were significantly different (p<0.01) when group I was compared with group II (Fig 13.2).
Figure 13.1 Effect of 100 mg CyA or placebo on systemic haemodynamics

Group I

SVRI (dyne·sec·cm⁻²·m²)

-300

0

300

600

900

P < 0.01

Grp I: CyA

Grp I: Placebo

Group II

SVRI (dyne·sec·cm⁻²·m²)

-300

0

300

600

900

P > 0.1

MAP (mmHg)

-5

0

5

10

15

20

P < 0.01

Grp II: CyA

Grp II: Placebo

HR (bpm)

-8

0

2

4

6

8

P > 0.5

Grp II: CyA

Grp II: Placebo

Cl (L/min/m²)

-0.6

0

0.3

P < 0.01

Grp II: CyA

Grp II: Placebo

P < 0.05

0 20 40 60 80 100 120 140 160 180

Time (min)

0 20 40 60 80 100 120 140 160 180

Time (min)
Figure 13.2 Effect of 100 mg CyA or placebo on systemic haemodynamics. Placebo corrected % change from baseline.
13.3.2 Endothelin-1 concentrations

Plasma ET-1 concentrations were not significantly altered in either group compared to placebo after CyA administration (Table 13.4).

**Table 13.4 Plasma ET-1 concentrations (pg/ml) after acute CyA administration**

<table>
<thead>
<tr>
<th>Study drug</th>
<th>Group I: Patients on long-term CyA</th>
<th>Group II: Patients naïve to CyA</th>
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<tbody>
<tr>
<td></td>
<td>CyA</td>
<td>Placebo</td>
</tr>
<tr>
<td>Baseline</td>
<td>2.78 ± 0.59</td>
<td>3.85 ± 0.29</td>
</tr>
<tr>
<td>3 hr post administration</td>
<td>4.47 ± 0.41</td>
<td>4.43 ± 0.74</td>
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13.4 Discussion

This study demonstrated that the pattern of the haemodynamic response to acute CyA administration differs between renal transplant patients on chronic CyA therapy and those naïve to it. Patients on chronic immunosuppression with CyA demonstrate an increase in blood pressure associated with an increase in vascular resistance and a reduction in CI after acute CyA administration. By contrast, matched patients maintained on drug regimes without CyA increase both blood pressure and CI and do not vasoconstrict after acute CyA administration. These findings in transplant patients on long-term CyA are consistent with published literature in heart transplant patients and chronic dialysis patients [451, 452]. The patients naïve to CyA, however, followed the pattern previously observed in healthy subjects given a large single dose of CyA (Chapter 12) with a small vasodilatation and an increase in CI.

CyA can induce smooth muscle cell contraction [171], and cause increased contractile responsiveness of blood vessels in vitro [445]. However, in healthy subjects with
presumed normal endothelial function, acute administration of CyA into the brachial artery only causes vasoconstriction in the presence of a nitric oxide clamp, and, in the presence of stimulated endothelial NO release, in fact, increased forearm blood flow, suggesting that nitric oxide is an important regulating mechanism protecting against CyA-associated vasoconstriction in vivo [466]. Morris et al. have demonstrated that renal transplant patients on chronic CyA have reduced basal and stimulated NO activity compared to those on Pred/Aza and to healthy volunteers [467]. Hence, chronic exposure to CyA is associated with reduced NO activity, and thus a loss of protection against further CyA associated vasoconstriction. Rat models suggests that this switch in the balance of endothelial vasomotor tone to favour vasoconstriction during CyA administration happens early, with 1-3 weeks being sufficient to see a reduction in urinary NO metabolites and alterations in vessel responses, consistent with reduced NO activity [468, 469].

The results from this study would be consistent with this loss of NO activity in long-term CyA administration. The observed vasoconstriction after acute CyA suggests that chronic exposure to CyA has resulted in endothelial dysfunction with reduced NO activity, and loss of an NO related protective mechanism. The lack of vasoconstriction to acute CyA in the Pred/Aza patients suggests that endothelial function in this group is comparatively well preserved and thus NO activity is protecting against CyA induced increases in vascular tone.

It is possible that, in addition to reduced NO activity during chronic CyA treatment, increased activity of vasoconstrictor systems also occurs. In respect of ET-1, CyA is known to stimulate ET release from endothelial and smooth muscle cells and activates ET-1 gene expression [171]. ET receptor antagonism attenuates CyA induced renal vasoconstriction [145] and CyA induced smooth muscle cell contraction [171]. If ET-1 is upregulated in patients on chronic CyA treatment, and further augmented cyclically by twice daily CyA ingestion, then ET receptor antagonists, which improve endothelial function, might provide a therapeutic option for CyA induced hypertension and renal
dysfunction. Indeed, Binet et al. have recently demonstrated that the ET receptor antagonist bosentan can attenuate CyA associated renal vasoconstriction, though not hypertension in healthy subjects treated with 7 days of CyA [177]. This study does not show any convincing alterations either in baseline plasma ET-1 concentrations between the two groups studied, or after single dose CyA within each group. However the secretion of ET-1 from endothelial cells is mainly abluminal and plasma concentrations may not be truly reflective of alterations in the activity of the ET system. Further studies in patients with ET receptor antagonists are needed to clarify this issue.

It must be noted, however, that, despite differences in SVRI and CI, similar rises in MAP occurred in both groups, thus blood pressure changes cannot be wholly attributed to alterations in vessel tone. Increased SNS activity [446, 447], and salt and water retention [448] have also been proposed as mediators of CyA associated hypertension. This study was not designed to look at either of these possibilities. However, in respect of salt and water retention, it is highly unlikely that, given the short time course, this can account for the acute blood pressure response seen here. Previous studies have suggested that hypertension precedes the onset of antinatriuresis by some hours [347] to days [461, 464, 465] supporting the hypothesis that hypertension on CyA is unlikely to be initiated by renal vasoconstriction and consequent sodium retention.

The groups studied were well matched except for the length of time that they had been transplanted. It is important to note, given the association of renal impairment with endothelial dysfunction [470], and the finding that acute administration of CyA to dialysis patients naïve to CyA causes vasoconstriction [452], that they have similar creatinines. The different responses between the groups to CyA observed in this study are therefore not associated with differences in renal function. The two groups do differ, however, in the duration of immunosuppressive treatment. Since the introduction of CyA in the early 1980s, dual therapy with Pred/Aza has become rare, with most immunosuppressive regimens containing a calcineurin inhibitor. As such, the Pred/Aza treated patients represent an historical group. Hence it is not possible to match for
duration of transplant with CyA treated patients. However, one would anticipate greater not less endothelial dysfunction with increasing time as a renal patient.

In summary, the observed haemodynamic response of renal transplant patients to CyA is altered by previous long-term exposure to CyA from mild vasodilatation to vasoconstriction, consistent with CyA associated endothelial dysfunction. Identification of such a chronic CyA induced endothelial dysfunction is important, as restoration of endothelial balance in favour of vasodilatation might help protect against both hypertension and the renal vasoconstriction common in CyA treated patients. Further work is needed to clarify the time course over which this endothelial dysfunction develops in man, and its relative contribution to the overall elevations in blood pressure seen in CyA treated patients.
Chapter 14

Conclusions
The acute studies presented in this thesis explore the acute actions and interactions of ET-1 and its receptors in the systemic and renal circulations in health and disease. These studies have helped to establish a clearer picture of ET physiology and pathophysiology. Such observations suggest there is clinical potential for ET receptor antagonists in the treatment of renal dysfunction. As indicated in Chapter 1, in vitro and in vivo observations suggest a pathogenic role for ET-1 and a possible therapeutic role for ET receptor blockade in a number of cardiovascular and renal diseases. In this respect, the observations in this thesis in respect of ETA receptor antagonism, particularly in conjunction with ACE inhibition are promising. However, the observations of these acute studies need to be borne out by long-term clinical intervention studies.

14.1 Role of ET-1 and ETA in the maintenance of normal vascular tone

These studies suggest a role for ET-1 acting via the ETA receptor in the systemic but not renal vascular tone in health.

Though initial studies with ET-1 and ECE inhibitors or ET receptor antagonists suggested a role for ET-1 in the maintenance of normal vascular tone [191, 192, 194, 195, 201], further local [197, 471] and systemic studies [139, 142, 199, 202] with ET receptor antagonists have been conflicting in respect of this function of ET-1.

The studies in this thesis in healthy subjects repeatedly demonstrate systemic vasodilatation with ETA receptor antagonism when this is used at doses sufficient to produce significant ETA receptor blockade, consistent with a role for ET-1 acting via the ETA receptor in the maintenance of normal vascular tone. However, consistent with previous studies, these data suggests that ET-1 does not play a role in the maintenance of renovascular tone in the "unchallenged" renal circulation in health [120, 139-141, 199].

It is important to remember that ETA receptor antagonism can cause vasodilatation not only by blocking ETA mediated vasoconstriction but also by allowing endogenous ET-1 to bind preferentially to the unblocked endothelial ETB receptor promoting ETB
receptor mediated vasodilatation. However, concomitant ETB blockade in healthy volunteers does not alter the systemic haemodynamic effects of ETA receptor antagonism suggesting that it is blockade of ETA mediated vasoconstriction rather than promotion of ETB mediated vasodilatation that is responsible for the major part of the decrease in vascular tone observed after ETA receptor antagonism.

14.2 Role of endothelial ETB receptor mediated vasodilatation and vascular smooth muscle ETB receptor mediated vasoconstriction in the maintenance of normal vascular tone

These studies suggest that the net effect of ETB receptor activation in health is to produce vasodilatation, evident in both systemic and renal circulations.

Local studies with ET receptor agonists and antagonists [207, 208] have suggested that ETB receptors also play a vasoconstrictor role in the maintenance of normal vascular tone. However, there may be a difference depending upon vessel type in respect of the presence and clinical significance of vasoconstrictor ETB receptors.

Local forearm [194] and systemic [210] administration of ETB receptor antagonist in this department has been shown to produce vasoconstriction. The studies in this thesis have confirmed this finding in healthy subjects, suggesting that endothelial vasodilator ETB receptors have a role in maintaining a basal vasodilatation in the systemic circulation, and that the balance of the constrictor and dilator functions of ETB receptors is, in these circulations at least, in favour of vasodilatation in health.

As with ETA receptor antagonism however, ETB receptor blockade may produce vasoconstriction not only by loss of ETB mediated vasodilatation but also by disproportionate binding of endogenous ET-1 to the unblocked ETA receptor producing ETA mediated vasoconstriction. Additionally, loss of the clearance function of the ETB
receptor will increase circulating levels of ET-1 that may further augment ETA receptor activation.

In this respect it is important to note that, contrary to expectations if the clinical effect of ETB receptor activation is vasodilatation, concomitant administration of ETB to ETA receptor antagonism does not affect the systemic vasodilatation produced by ETA receptor antagonism alone. This is in contrast to previous forearm studies when ETB receptor antagonism attenuated the vasodilatation produced by ETA receptor blockade [194]. There are a number of possible reasons for this discordance. Firstly, the forearm circulation is not necessarily indicative of total peripheral vascular resistance. The measurement of SVR may include local circulations where ETB mediated vasoconstriction is important. Secondly, the vasodilatation produced by ETA receptor antagonism may produce reflex neurohormonal mechanisms that act to limit any changes in the circulation. In forearm studies, this is avoided by using concentrations of drugs that are only locally active and so should not activate systemic compensatory mechanisms [365]. Thirdly, the role of ETB mediated vasodilatation in health may be minor by comparison to ETA mediated vasoconstriction and so loss of this vasodilatation in healthy blood vessels in an "unchallenged" state may be clinically insignificant in the presence of blockade of the constrictor ETA receptor. Therefore, the vasoconstriction produced by ETB receptor antagonism alone, and the failure of ETB receptor blockade to augment ETA receptor mediated vasodilatation does not support a role for constrictor ETB receptors in health, but is consistent with a role for ETB in the maintenance of systemic vasodilator tone.

Within the renal circulation, the lack of effect of ETA receptor blockade suggests that ETA mediated tone in not important in the kidney in health. The absence of dilatation after ETA receptor blockade would also suggest that ETB mediated vasodilatation (via binding of "displaced" ET-1) is not important. However, in health, the "unchallenged" renal circulation is near-maximally dilated hence further reductions in renal vascular resistance are unlikely. The renal vasoconstriction seen after ETB receptor blockade
suggests that the endothelial ETB receptor is an important mediator of this dilatation. The fact that concomitant ETA blockade abolishes this dilatation suggests that the ETA receptor does have a constrictor function in the renal circulation that may become more important in circumstances where the renal circulation is "challenged". As with the systemic circulation, the data from this thesis do not support clinically relevant ETB mediated vasoconstriction in the renal circulation in health.

14.3 Role of ET-1 and ETA in the maintenance of vascular tone in renal failure

These studies suggest a role for ET-1 acting via the ETA receptor in the systemic and, in contrast to health, in renal vascular tone in patients with CRF.

In respect of systemic haemodynamics, in forearm studies in patients with renal failure, ETA receptor antagonism produced vasodilatation, but to a lesser degree than that seen in healthy controls [195]. Similarly other studies in hypertensives [222] and patients with heart failure [285] did not show enhanced vasodilatation to ETA receptor blockade. Studies where patient groups do show enhanced vasodilatation after ETA receptor blockade compared to controls [221] have used doses of ET antagonists (BQ-123: 100 nmol/min) that are, on the basis of these studies, systemically active, and therefore will activate systemic homeostatic mechanisms that will make interpretation of results difficult. Additionally, it is possible that, locally, this dose reaches sufficiently high concentrations to lose ETA selectivity and begin to have effects at the ETB receptor [198].

However, these systemic studies show an equal effect of ETA receptor antagonism on SVR, and a greater effect on blood pressure in CRF compared to healthy controls. Additionally, renal haemodynamic changes occur only in subjects with renal impairment. In attributing these findings to ETA receptor blockade alone, the preliminary dose ranging study and measurement of BQ-123 concentrations allows a
degree of certainty in stating that maximal ETA receptor blockade has been achieved without losing ETA selectivity. BQ-123 is not therefore directly activating the ETB receptor in these studies.

Therefore, the presence of renal failure and (treated) hypertension may alter the vascular sensitivity to ET receptor antagonists and reflect an enhanced activity of the ET system in renal disease, particularly in the renal circulation. The observed increased urinary ET-1 excretion rates in renal failure would support this argument at least in respect of the kidney. The findings may not be consistent with local studies for the aforementioned reasons pertaining to the extrapolation of local to systemic data. However, there are a number of other possible explanations for these findings. Firstly, ET receptor antagonists may be having direct effects on cardiac function [212] and venous capacitance [472] that may alter blood pressure. An increase in ETA mediated venodilation, or an ETA mediated decrease in CO for example, may result in a greater reduction in blood pressure. These studies were not designed to look at these possibilities beyond a basic measure of CI and HR. Secondly, there is the possibility of altered drug pharmacokinetics in renal failure. BQ-123 is primarily metabolised in the liver [97]. Though these studies have demonstrated some renal excretion of BQ-123 that is GFR dependent, this does not impact significantly on systemic pharmacokinetics and is unlikely to be responsible for the observed differences between patients and healthy subjects. Thirdly, there is the possibility of interactions with other drugs taken by the patients. The studies presented in this thesis investigating the interaction of ET receptor antagonists and ACE inhibition do raise the possibility that the different responses to ET receptor antagonists seen between CRF patients and healthy controls, both in the degree of systemic haemodynamic change and the presence of renal haemodynamic changes, are all attributable to concurrent ACE inhibition in the renal patients, though it is worth noting, that 2 patients that were not on ACE inhibitors had similar responses after ETA receptor antagonism to the other patients.
14.4 Role of endothelial ETB receptor mediated vasodilatation and vascular smooth muscle ETB receptor mediated vasoconstriction in the maintenance of vascular tone in renal failure

These studies suggest that the net effect of ETB receptor activation in renal failure is to produce vasodilatation, evident in both systemic and renal circulations.

Forearm studies in subjects with hypertension [196] or atherosclerosis [473] have demonstrated that ETB receptor antagonism produces vasodilatation, and augments ETA mediated vasodilatation, suggesting an increase in the importance of ETB mediated vasoconstriction in hypertension. It is likely, however, these findings are due to a dose of ETB receptor antagonism (BQ-788: 50 nmol/min) that has been shown to be systemically active in these studies.

The studies in patients with renal impairment do not suggest any increase in the importance of vasoconstrictor ETB receptors in the systemic or renal circulations. By contrast, the ETB receptor appears to be of greater importance in the maintenance of systemic and renal vasodilator tone in renal failure. Not only does ETB receptor blockade alone produce vasoconstriction, but concomitant administration of ETB receptor blockade, does, in renal patients attenuate the haemodynamic effects of ETA receptor blockade, suggesting that ETB mediated vasodilatation is clinically significant in renal disease, particularly in the renal circulation. It is worth noting that absolute renal vascular resistance at baseline in the renal patients is three times that of the healthy volunteers indicating a basal state of renal vasoconstriction, and that the absolute increase in renal vascular resistance, though equal in percentage terms, is also three times that of healthy controls. This suggests that the balance of dilators and constrictors in the blood vessels in CRF is shifted towards constrictors. Thus blockade of one of these constrictors, ET-1 via the ETA receptor, or loss of one of the dilator functions, ET-1 via the ETB receptor, becomes clinically more significant.
Of course, given the demonstrated synergism between ETA receptor antagonists and ACE inhibitors that is abolished by concomitant ETB blockade in healthy volunteers, it is possible that the differential effect of ETA and ETA/B observed in the renal patients but not the healthy subjects is attributable to pre-treatment with ACE inhibition. Again, however, the 2 patients that were not on ACE inhibitors had similar responses to combined ETA/B receptor blockade to the other patients.

### 14.5 Renoprotective profile of ETA receptor antagonism in renal failure

These studies suggest that ETA receptor antagonism offers a potentially renoprotective profile.

These studies have demonstrated that, acutely ETA receptor antagonism produces systemic vasodilatation and a clinically relevant reduction in blood pressure in patients with renal failure, without sodium retention. This effect, though slightly greater with ETA receptor antagonism, can be seen with combined ETA/B receptor blockade. ET receptor antagonists have been shown in large scale studies to be effective in lowering blood pressure [224], but, given the large numbers of anti-hypertensive agents already available, this alone is insufficient to focus attention on ET receptor antagonists.

However, these studies have also demonstrated that ETA receptor antagonism can reduce RVR and increase RBF in CRF patients. This renal vasodilatation is associated with a reduction in EFF. In the absence of changes in mesangial contraction, this is suggestive of a preferential efferent arteriolar dilatation that will therefore act to reduce glomerular capillary perfusion pressure. Consistent with this proposed reduction in glomerular capillary pressure, there is a reduction in proteinuria.

In support of these observations, a previous study in type I diabetics with proteinuria but essentially normal or only mildly impaired renal function has demonstrated that 6 weeks of ETA receptor blockade does reduce blood pressure and, though not significantly affecting overall renal haemodynamics, does reduce proteinuria [190].
In respect of clinical potential, it is important to note that these renal effects are not produced by combined ETA/B receptor antagonism.

14.6 Natriuresis

These studies have not clearly demonstrated an ET mediated natriuresis.

Animal evidence to date points to a paracrine action of renal ET-1 via the ETB receptor in salt and water homeostasis [135, 394]. The peritubular distribution of ETB receptors [11, 52] suggests that the ETB receptor also mediates these actions in man. These studies have demonstrated that selective ETB receptor blockade does reduce sodium excretion in both health and disease. However, correcting for GFR suggests that his antinatriuresis is accounted for by renal vasoconstriction and not a blockade of ETB mediated natriuresis. Also, ETA blockade alone, though not producing sodium retention despite a fall in blood pressure, did not increase sodium excretion despite leaving the ETB receptor available to endogenous ET-1. These results are consistent with previous studies in man where no clear effect can be demonstrated.

In administering exogenous ET-1 in the presence of ETA blockade, it was hoped to stimulate an unblocked ETB receptor to observe any tubular effects, an approach used effectively in dogs [123]. However, as discussed, the dose of ET-1 was probably too high for the dose of BQ-123 used, resulting in drug displacement from the ETA receptor, as evidenced by loss of ETA mediated vasodilatation. Thus the failure to demonstrate natriuresis is not indicative of a lack of ETB tubular effects in man.

Only when ETA receptor antagonism was given in the presence of ACE inhibition was a significant, and indeed striking, increase in sodium excretion observed. It is possible that this is entirely a consequence of the renal vasodilatation observed, and therefore again a haemodynamic effect. However, the renal haemodynamic effects are not as great as those seen in renal patients, where no net changes in sodium excretion were observed.
Animal studies suggest that the effects of exogenous ET-1 are regional within the kidney with cortical vasoconstriction and medullary vasodilatation [474]. It is possible therefore that these regional variations are further enhanced with concomitant ETA-ACEI treatment increasing blood flow to the medullary tubular segments promoting sodium excretion. Because concomitant ETB receptor blockade equally abolished the renal haemodynamic and natriuretic changes of this combination treatment, it is impossible for us to attribute this natriuresis directly to the unblocked tubular ETB receptor.

14.7 Angiotensin - endothelin interaction

These studies have demonstrated that the acute effects of ANG II are not mediated through ET-1 via the ETA receptor.

Clinically, this is consistent with studies demonstrating an effect of ET receptor antagonism in patients already treated with drugs blocking angiotensin activity [292, 293, 299]. The distinct nature of ANG II and ET-1, at least acutely, therefore allows for ET receptor antagonists to be seen as an adjunctive treatment to RAAS inhibition. However, animal studies suggest a more chronic interaction, particularly in respect of vascular remodelling [371], that needs to be explored in man.

However, a synergistic interaction is demonstrable between ETA receptor blockade and ACE inhibition. As shown, this is largely mediated, through an unblocked ETB receptor, by nitric oxide but not prostaglandin. This synergism may, in part, be responsible for the greater effect of ETA receptor antagonism in renal patients compared to healthy controls. Another possibility, however, is a pharmacokinetic interaction between BQ-123 and enalapril. Though BQ-123 plasma concentrations were not significantly different in the presence or absence of enalapril, they were higher during combination treatment. Given the small numbers of subjects studied, it is possible that a pharmacokinetic interaction has been missed.
These current studies cannot differentiate if the role of ACE inhibition in this interaction is to reduce ANG II formation or reduce the breakdown of bradykinin. However, a synergistic interaction has been demonstrated between angiotensin receptor antagonists and ETA receptor antagonists [410] suggesting the former is more likely.

14.8 Evidence for the deleterious effect of ETB receptor blockade

These studies suggest that all the potentially beneficial haemodynamic effects of ET receptor antagonism are mediated through the ETA receptor. All the data in this thesis points to the net balance between endothelial and vascular smooth muscle ETB receptors being in favour of vasodilatation. Concomitant ETB blockade, at best, does not alter the effects of ETA receptor blockade. In these studies, it reduces the hypotensive and renal vasodilatory effects of ETA blockade in patients with renal failure. Possibly connected to these responses, it also abolishes the synergism that operates between ETA receptor blockade and ACE inhibition.

Additionally ETB is known to act as a clearance receptor for ET-1 [87]. On every occasion where the ETB receptor was blocked in these studies, plasma ET-1 concentrations increased consistent with this clearance role. Though the clinical significance of increased plasma concentrations of this paracrine hormone is unclear, there is some evidence that circulating ET-1 may play an important role in renal regulation in cardiorenal states of ET activation [109].

Finally, ETB receptor blockade reduced the urinary excretion of ET-1. Given the mitogenic potential of ET-1 [64, 65] and the association of increased intra-renal production of ET-1 in low renal mass models correlating with glomerulosclerosis [148, 180-182], the retention of ET-1 within the kidney thus has the potential to be deleterious.
14.9 Studies with cyclosporin
The initial aim of high dose CyA administration to healthy subjects was to provide a model of systemic and renal vasoconstriction for interventional studies with ET receptor antagonists. Instead these studies demonstrated that the initial effect of CyA is to produce vasodilatation, both in healthy subjects, and in transplant patients naïve to the drug. This is consistent with forearm studies in transplant patients where patients on CyA demonstrate a greater degree of endothelial dysfunction than matched controls [467], and in healthy subjects demonstrating that CyA acutely increases NOS activity on first exposure [466].

14.10 Future Studies
The observations in this thesis raise further questions to be answered and areas to explore. Some of these are discussed below

1. Clarification of the effect of ETA receptor antagonism on proteinuria
The reduction in proteinuria in renal patients after ETA receptor antagonists was clearest in subjects with higher baseline degrees of proteinuria. The renal subjects recruited had low grades of proteinuria (all < 1.5 g/24hr). Nephrotic range proteinuria was a specific exclusion criteria to avoid issues with altered protein binding of drugs in the circulation. Given that evidence with ACE inhibitors is that the greater renoprotective effect is afforded in patients with higher degrees of urinary protein leak [475], clarification of this effect by recruiting subjects with >3g/24hrs of proteinuria (with maintained serum albumin concentrations) would be important.

2. Natriuretic effect of ETB receptor
To demonstrate the natriuretic effect of ETB receptor activation, a further study of low dose exogenous ET-1 administration in the presence of high grade ETA blockade is required.
3. ETA-ACEI interaction - bradykinin

Having demonstrated that this interaction is ETB dependent and NO mediated, because ACE inhibitors also block the breakdown of bradykinin, bradykinin receptor antagonism would be useful to delineate whether the interaction with ACE inhibition is acting via a reduction in angiotensin or an increase in bradykinin.

4. ETA-ACEI interaction - renal patients

To clarify if the greater effect of ETA antagonism in renal patients is due to an interaction between ETA receptor blockade and ACE inhibition, or because of an altered activity of the ET systemic in renal disease, a study needs to be performed with ETA receptor antagonists in patients on and off ACE inhibition. Recruitment will need to take into account the requirement for equal control of blood pressure in study phases.

5. Salt sensitivity

Study 7 demonstrated a link between salt sensitivity and the response to ETA receptor antagonism on a high salt diet. However, this was a small scale study, and only one subject exhibited a high degree of salt-sensitivity. Examining the effects of ET receptor antagonism in two hypertensive groups, one clearly identified as salt-sensitive, one not, on both high and low salt diets would clarify the role of ET, particularly of renal origin in salt sensitivity. Because salt sensitivity may be linked to a blunted increase in endothelial NO in response to a high salt diet [426], the comparison of ETA with ETA/B blockade may be interesting in this respect.

6. Chronic ETA receptor blockade in patients with renal impairment

Given the renoprotective profile demonstrated in our studies in renal patients, this needs to be confirmed in chronic studies, with particular attention to any slowing of the rate of progression of renal impairment, or reduction in proteinuria as a surrogate marker [476].
7. Renal effects of ET receptor antagonism in patients with chronic heart failure

Renal dysfunction occurs in CHF partly to reduced renal perfusion pressure. Its presence is a poor prognostic marker [477]. Animal studies have suggested that ET receptor antagonism may improve renal function in CHF. However, despite many studies in man into the role of ET receptor antagonism in heart failure, many with non-selective antagonists, none have specifically examined the effects on renal function. Given that these studies suggest that ETB blockade will abolish any beneficial effects of ETA-ACE interaction both systemically and in respect of renal haemodynamic and tubular function, a clearer picture of the renal effects of ET receptor antagonism should be sought in both acute and chronic studies in this condition characterised by vasoconstriction and sodium retention.

8. Acute intervention in scleroderma hypertensive crisis

Scleroderma associated pulmonary hypertension responds to ET receptor blockade [268, 269]. Though not directly studied in this thesis, given the vasoactive nature of these drugs, and the pro-fibrotic effects of ET-1 experimentally, this condition would represent a potential target for ETA receptor antagonists, particularly given that the current treatment for this condition is ACE inhibition.

9. Studies with cyclosporin

The ability of ET receptor antagonism to influence chronic CyA induced endothelial dysfunction should be explored. However, a model of acute administration to healthy subjects is not applicable for these purposes.

Additionally, though CyA induced hypertension is likely to be multi-factorial, the endothelial dysfunction and sympathetic activation might be amenable to treatment with nebivolol, a beta-adrenoreceptor blocker with nitric oxide donor capabilities.


282. Iwanaga Y, Kihara Y, Hasegawa K, et al. Cardiac endothelin-1 plays a critical role in the functional deterioration of left ventricles during the transition from compensatory


237


Publications arising from work presented in this thesis


Endothelin Receptor Antagonists
Promising New Agents in the Management of Cardiovascular Disorders

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Abstract

Since its discovery in 1988, endothelin (ET) has been widely implicated in the pathophysiology of cardiovascular disease. ET antagonists have favourable effects in experimental models of these conditions and have proved useful in elucidating the role of the ET system. Orally acting ET antagonists appear very promising in clinical trials, particularly in patients with chronic heart failure and hypertension, but more information on the roles of the ET receptor subtypes in health and disease is required so that an informed choice can be made between the use of endothelin-A (ET-A) receptor-selective and nonselective receptor antagonists.

The endothelins (ET) are a family of 21 amino acid peptides with powerful vasoconstrictor and pressor properties that were first described by Yanagisawa et al. in 1988. Three different isopeptides, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), have so far been identified, each with distinct genes and tissue distributions. Of the 3 peptides, ET-1 is the major endothelial isoform and is therefore likely to be the most important in the regulation of cardiovascular function. Therefore, this article, focuses primarily on ET-1. Its main site of production is the vascular endothelial cell but it is also produced by other cell types, including renal tubular and mesangial cells, vascular smooth muscle cells and epicardial cells. In addition to its vasoconstric- tor properties, ET-1 functions as a mitogen, modulates other hormone systems and influences ion and fluid transport in the gut and kidney.

Regulation of the production of ET-1 is thought to be at the level of gene transcription. Enhanced gene transcription occurs in response to a wide range of stimuli including other vasoactive hormones [such as angiotensin II, adrenaline (epinephrine) and vasopressin], cytokines (such as interleukin-1 and endotoxin), and hypoxia, glucose and oxidised low density lipoprotein (LDL). In contrast, prostacyc- lin, nitric oxide (NO) and the natriuretic peptides all inhibit transcription of endothelin genes. The gene product is prepro-ET-1, a protein with 212 amino acids which is cleaved in stages to yield the largely inactive big ET-1, a 38-amino-acid precursor peptide. Endothelin converting enzyme (ECE), a metalloproteinase, then splits big ET-1 into the active mediator, ET-1, and its C terminal frag- ment (fig. 1).

Two ET receptors have been identified and cloned: endothelin-A (ET-A) receptors have a higher affinity for ET-1 than either ET-2 or ET-3, whereas endothelin-B (ET-B) receptors have equal affinity for the 3 ET peptides. Within the circulation, ET-A receptors are found on vascular smooth muscle cells and their activation results in vasoconstriction. ET-B receptors are also found on vascular smooth muscle cells, where they mediate vasoconstriction. However, ET-B receptors are more abundant on the vascular endothelium where their
Fig. 1. The endothelin (ET) pathway: stimuli for ET production, pathway for generation and major effects mediated by ET-A and ET-B receptors in blood vessels. ET-1 is generated by endothelial cells and secreted abluminally to act on ET receptors on vascular smooth muscle cells promoting contraction. ET-1 can initiate relaxation via stimulation of ET-B receptors on the vascular endothelium promoting the generation of NO from L-arginine and PGI₂ from arachidonic acid. cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; ECE = endothelin converting enzyme; ET-A = endothelin-A receptor; ET-B = endothelin-B receptor; ET-1 = endothelin-1; PGI₂ = prostacyclin; mRNA = messenger RNA; NO = nitric oxide.

activation results in vasodilatation mediated by NO and prostacyclin. In the human kidney, ET-A receptors are localised to vascular smooth muscle, notably in the glomeruli, vasa recta and arcuate arteries. ET-B receptors are more numerous (ET-B to ET-A ratio 2 : 1) and more widespread with a high concentration in the collecting system. The distribution of these receptors within the kidney suggests a vasoactive role for ET-A receptors and a role in sodium and water handling for ET-B receptors.

Signal transduction is complex and reviewed elsewhere. ET-1 is removed in the renal, splanchnic and pulmonary circulations, probably by ET-B receptor binding and internalisation and also enzymatic degradation by neutral endopeptidases.

1. Endothelin Receptor Antagonists

Compounds that block the ET system have been used to define the role of the endogenous ET system and confirm its importance in cardiovascular function and dysfunction. Attention is now focusing increasingly on drugs that may have a role in clinical medicine. Though ECE inhibitors and monoclonal antibodies have been used experimentally,
and the former may be a therapeutic option in the future, most clinical work to date has been done with ET receptor antagonists.

ET receptor antagonists can be divided into orally active compounds and parenteral preparations. In certain situations an intravenous preparation will be preferred, notably to counter acute, isolated insults (e.g. cerebral ischaemia following subarachnoid haemorrhage or stroke, unstable angina, acute myocardial infarction, acute renal failure or postoperative pulmonary hypertension). However, many of the anticipated indications for ET receptor antagonists, including hypertension, chronic heart failure and coronary restenosis, will require chronic treatment for which oral agents are best suited (table I).

ET receptor antagonists can be further subdivided into nonselective ET receptor antagonists and ET-A–selective receptor antagonists. ET-A selectivity generally refers to compounds with >1000-fold affinity for the ET-A over the ET-B receptor. It is important to appreciate that so-called 'nonselective' antagonists are still generally selective for the ET-A over the ET-B receptor (table I). More work is needed to better delineate the respective roles of the ET-A and ET-B receptors in normal physiology and, more importantly, in pathophysiology before conclusions can be drawn regarding the relative benefit of nonselective versus selective ET-A receptor blockade in a given clinical situation.

A series of peptide antagonists that bind to, but do not activate ET receptors have been developed through the modification of ET peptides. Cyclic peptides were designed to mimic the hairpin loop of ET. For example, BQ 123 is a cyclic pentapeptide and ET-A–selective receptor antagonist that was developed from BE 18257B (a natural product of *Streptomyces misakiensis*). TAK 044 is a cyclic hexapeptide with nonselective ET receptor antagonist properties. A series of linear peptides with ET-B–selective receptor antagonist properties have also been developed (e.g. BQ 788). These peptides are broken down in the gut and, therefore, must be given parenterally after which they have relatively short half-lives. Experimentally, the half-life of BQ 123 can be increased by side-chain modifications that reduce hepatic extraction,[16] but the clinical utility of these compounds is limited by the lack of oral analogues.

A series of nonpeptide ET receptor antagonists with greater bioavailability than the peptides have been developed through the use of screening libraries and molecular modelling (table I). Bosentan (RO 470203), is a sulfonamide with nonselective receptor blocking activity that has the benefit of being available as both an oral and intravenous preparation. This will allow treatment to be rapidly initiated with the intravenous preparation in acute situations (e.g. exacerbations of heart failure) and continued thereafter with the oral formulation. SB 209670, a carboxylic acid derivative, is a more potent nonselective receptor antagonist than bosentan, but it is only available as an intravenous preparation. Enrasentan (SB 217242) is an analogue of SB 209670 with improved bioavailability that is currently in development as an oral drug.[17]

Orally active and selective ET-A receptor antagonists are being developed. ABT 627 is a carboxylic acid derivative with 2000-fold greater selectivity for the ET-A versus the ET-B receptor and an elimination half-life (t1/2) of 24 hours in humans[18] making it an attractive choice for situations requiring long term treatment (e.g. prevention of coronary restenosis). Minor structural modifications to ABT 627 yield highly selective ET-B receptor antagonists.[18] TBC 11251 and TA 0201 are sulfonamide derivatives with high bioavailability and ET-A receptor selectivity.[19] TA 0201 has a t1/2 of only 0.9 hours, but it is converted to an active metabolite with demonstrable inhibitory effect 8 hours after oral administration in rats.[20]

An interesting agent in preclinical development is L 747072, an angiotensin receptor antagonist discovered to have nonselective ET receptor antagonist activity, providing the potential for a dual therapeutic approach to the treatment of hypertension and other cardiovascular conditions.[17]
<table>
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<th>Name</th>
<th>Route</th>
<th>ET-A selectivity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Phase</th>
<th>Anticipated indication</th>
<th>Structure</th>
<th>Manufacturer (co-developer)</th>
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<td><strong>ET-A–selective receptor antagonists</strong></td>
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<td>Cyclic pentapeptide</td>
<td>Banyu</td>
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<tr>
<td>PD 147953 (FR 139317)</td>
<td>IV</td>
<td>×7000</td>
<td>Preclinical</td>
<td>Heart failure, diabetic complications, hypertension, ischaemic heart disease, renal failure</td>
<td>Linear tripeptide</td>
<td>Parke-Davis</td>
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<td>(Fujisawa)</td>
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</table>

<sup>a</sup> Drugs are listed (oral then IV) in order of the highest reported phase of development.

<sup>b</sup> Based on binding and functional assays."14"151

ET-A = endothelin-A; IV = intravenous; NK = not known; PO = oral.
2. Endothelin and the Cardiovascular System

The importance of ET-1 in cardiovascular function is suggested by the actions of exogenous ET-1, the administration of which is associated with an increase in blood pressure and peripheral vascular resistance.\textsuperscript{[21,22]} The direct effects of ET-1 on the heart are probably species specific but a positive inotropic response to ET-1 is seen in isolated cardiac tissue from rats and humans,\textsuperscript{[23,24]} and in in vivo models where coronary vasoconstriction is countered with a vasodilator.\textsuperscript{[25]} The integrated haemodynamic response is, however, much more complicated. Increased peripheral vascular resistance and blood pressure produce reflex reductions in heart rate and cardiac output, and coronary vasoconstriction can cause myocardial ischaemia and cardiodepression. The effectiveness of receptor antagonists should clearly be judged on their integrated effects, as well as local effects on target organs or tissues.

In addition to its haemodynamic actions, endogenous ET-1 is mitogenic \textit{in vitro}, thus causing cell proliferation and vascular remodelling, and caused renal sodium retention,\textsuperscript{[26]} features of congestive heart failure (CHF) and hypertension.

3. Coronary Artery Disease

3.1 Ischaemia and Myocardial Infarction

Inducing coronary ischaemia in patients with coronary artery disease (CAD) results in a significant release of ET-1 of cardiac origin.\textsuperscript{[27]} Plasma ET-1 concentrations are high in CAD, particularly in patients with myocardial infarction or coronary vasospasm,\textsuperscript{[28-31]} and elevations of plasma ET-1 and big ET-1 in unstable angina and after myocardial infarction are correlated with a worse prognosis,\textsuperscript{[32,33]} suggesting that an activated ET system may contribute to a poor outcome.

Activation of ET-A and -B receptors in the coronary circulation contribute to vasoconstriction\textsuperscript{[34,35]} and in isolated porcine and canine coronary arteries, TAK 044 inhibits ET-1–induced coronary vasoconstriction to a greater extent than BQ 123.\textsuperscript{[35]} These data suggest that nonselective receptor blockade may be more effective than selective. In patients with CAD, acute intravenous administration of the nonselective antagonist bosentan increased the diameter of coronary epithelial arteries; an effect that was not augmented by administration of intravenous nitrates. This effect was, however, only clear in normal or mildly diseased arteries and was correlated inversely with plasma LDL-cholesterol levels.\textsuperscript{[36]}

Both ET-A-selective (BQ 123) and nonselective (TAK 044) ET receptor antagonists have been shown to reduce infarct size in coronary ligation models.\textsuperscript{[37,38]} Though these animal studies are promising, studies in humans with myocardial infarction have not been published.

3.2 Atherosclerosis

Plasma ET-1 concentrations are elevated in patients with atherosclerosis\textsuperscript{[39]} and increased ET-1 levels have been demonstrated in atherosclerotic lesions\textsuperscript{[40]} and throughout the vessel walls of patients with CAD\textsuperscript{[6]} suggesting up-regulation of the ET system in atherosclerosis.

In hypercholesterolaemic hamsters, ET-A receptor inhibition decreases the progression of atherosclerosis mainly by reducing the number and size of macrophage–foam cells.\textsuperscript{[41]} In addition, apolipoprotein E-deficient mice, which are genetically predisposed to severe atheroma and endothelial dysfunction, are protected from atherosclerosis when treated with ET-A receptor antagonists and show an increase in NO-mediated endothelium-dependent relaxation.\textsuperscript{[42]} Similarly, hypercholesterolaemic pigs treated for a prolonged period with both selective and nonselective ET receptor antagonists have improved coronary vascular function.\textsuperscript{[43]} In view of these results, it is possible that the failure of bosentan to significantly dilate severely diseased coronary arteries in humans\textsuperscript{[36]} may relate to the greater degree of endothelial dysfunction in these arteries, and that this may respond to longer term administration of the drug.
3.3 Postangioplasty Restenosis

ET-1 has a mitogenic effect on vascular smooth muscle cells in vitro. In animal studies, expression of messenger RNA (mRNA) for all of the components of the ET system is increased at balloon angioplasty sites, and intra-arterial administration of ET-1 after angioplasty increases neointima formation. In humans, an increase in plasma ET-1 concentration proportional to the duration and pressure of the balloon inflation is seen distal to the angioplasty site. Nonselective ET receptor antagonism with SB 209670 or its analogue enrasentan has reduced neointima formation after endothelial injury by balloon angioplasty, whereas selective ET-A receptor antagonism with BQ 123 has been ineffective. However, highly selective ET-A receptor blockade with the potent oral agent ABT 627 has been shown to reduce restenosis, indicating that combined receptor antagonism may not be required to achieve this effect. Thus, prevention of restenosis with ET antagonists seems possible, although many other potentially useful agents have failed to fulfil their promise in this condition.

3.4 Arrhythmias

In animals, both combined ET receptor antagonism with SB 209670 and selective ET-A receptor antagonism with LU 135252 reduce the incidence of arrhythmias induced by ischaemia or exogenous ET-1. Of note, however, while low doses (10 µg/kg/min) of BQ 123 reduced the frequency of ischaemic arrhythmias in rats, higher doses (100 µg/kg/min) increased mortality rates because of an increase in refractory ventricular fibrillation.

4. Congestive Heart Failure

There is considerable evidence that ET-1 plays a role in CHF. ET-1 causes hypertrophy of cardiac myocytes and has a direct toxic effect on these cells. In animal models of CHF, increased cardiac and pulmonary ET-1 synthesis has been documented. In a Dahl salt-sensitive, hypertensive rat model, the initial compensated left ventricular hypertrophy (LVH) was not associated with an increase in cardiac ET-1 levels, but a 5-fold increase was noted after the development of left ventricular (LV) dilatation and global hypokinesis. ET-A receptors are more numerous than ET-B receptors in both the normal and failing heart, but ET-A receptors may be up-regulated in the failing heart suggesting that they may be the major therapeutic target. As with CAD, however, some evidence points to the enhancement of vascular smooth muscle ET-B mediated vasoconstriction in CHF. Also, although the contractility of myocytes obtained from animals and humans with CHF is attenuated in response to exogenous ET-1, there is some evidence that endogenous ET-1 is involved in the maintenance of cardiac function in CHF. Thus, caution is necessary in contemplating blockade of the ET system in patients with heart failure.

Animal models of CHF have shown substantially improved survival with both selective ET-A and nonselective ET-A/B receptor antagonists. In rats with CHF induced by coronary artery ligation, though acute ET-A receptor blockade with BQ 123 reduced myocardial contractility, long term treatment resulted in a 90% survival rate, compared with 40% in nontreated animals. This improved survival was associated with an amelioration of LV dysfunction and prevention of ventricular remodelling. Treatment with the nonselective receptor antagonist bosentan for 9 months in a coronary artery ligation model of CHF resulted in beneficial alterations in haemodynamics, cardiac geometry and function and an 18% increase in survival compared with controls. In the Dahl salt-sensitive, hypertensive model of CHF, long term treatment with bosentan, started at the stage of compensated LVH, did not alter LV mass but improved survival by 36%. Of note, in a pacing CHF model, long term (6 weeks) nonselective receptor antagonism (L 753037) increased coronary sinus NO concentrations and improved endothelium-dependent relaxation in coronary arteries.

In humans, the increase in circulating big ET-1 or ET-1 seen in patients with CHF is correlated with the severity of symptoms and with prognosis.
A series of clinical trials point towards potential usefulness of ET receptor antagonists in this condition (table II), though it is unclear whether selective or nonselective receptor antagonism will provide the greatest benefit.

Studies with bosentan have consistently reported marked haemodynamic benefits both acutely and in the longer term. In patients on chronic triple therapy of ACE inhibitors, diuretic and digoxin, acute administration of intravenous bosentan (300mg in total) 2 days after withdrawal of the ACE inhibitor, resulted in a 33% decrease in pulmonary vascular resistance (PVR). Oral bosentan (1g twice daily for 2 weeks) improved systemic vascular resistance index (SVR; -25%), PVR (-20%) and cardiac index (CI; +15%) on day 1 of treatment. After 2 weeks of continuous oral therapy, additional haemodynamic improvements (PVR; -10%, SVR; -10%, CI; +15%) were documented in patients receiving ongoing ACE inhibitor therapy. The REACH study, in which 370 patients with CHF received bosentan (500mg twice daily) or placebo for 6 months, was terminated early because of asymptomatic, reversible increases in hepatic transaminases in the treatment group, but improvements in symptoms, and fewer hospitalisations were reported in bosentan-treated patients who completed the 6 month protocol. Further clinical studies of bosentan in CHF are being planned.

The theoretical benefits of selective ET-A receptor antagonism in patients with CHF have, so far, only been explored in small acute studies. Intravenous BQ 123 (100 nmol/min for 60 min for 2 patients; 200 nmol/min for 60 min for 8 patients) and TBC 11251 (1.5 or 3 mg/kg) have produced significant systemic and pulmonary haemodynamic benefits, respectively (table II). Acute ET-B receptor antagonism with BQ 788, caused systemic vasoconstriction in CHF patients, an effect which was reversed by the addition of BQ 123, suggesting that ET-B receptors contribute to vasodilator tone in CHF. Longer term studies with selective ET-A receptor antagonists have yet to be performed. Similarly, the long term effects of ET antagonism on vascular and ventricular remodelling remain to be explored in patients with CHF.

### 5. Hypertension

Exogenous administration of ET-1 increases peripheral vascular resistance and elevates blood pressure, and studies with ET receptor antagonists have revealed that endogenous ET-1 has an important role in the maintenance of normal vascular tone. It is also possible that ET-1 may modulate blood pressure by renal mechanisms, by altering the balance between its potentially favourable natriuretic action (ET-B receptors) and sodium retention as a consequence of renal vasoconstriction (ET-A

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**Table II. Studies with endothelin receptor antagonists in patients with congestive heart failure (CHF)**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Study design</th>
<th>n</th>
<th>Drug</th>
<th>Route and duration of administration</th>
<th>NYHA</th>
<th>MAP</th>
<th>PAP</th>
<th>SVR</th>
<th>PVR</th>
<th>HR</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kiczewski et al.</td>
<td>nb, nc</td>
<td>24</td>
<td>Bosentan</td>
<td>IV bolus IV bolus</td>
<td>III</td>
<td>↓</td>
<td></td>
<td>↓</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sütlich et al.</td>
<td>r, db, pc</td>
<td>36</td>
<td>Bosentan</td>
<td>Oral 14 days Oral 14 days</td>
<td>III</td>
<td>↓</td>
<td></td>
<td>↓</td>
<td></td>
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</tr>
<tr>
<td>Packer et al.</td>
<td>r, db, pc</td>
<td>370</td>
<td>Bosentan</td>
<td>Oral 6 months Oral 6 months</td>
<td>IIB-IV</td>
<td>Clinical improvement = reduced hospital admissions</td>
<td>↓</td>
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<tr>
<td>Cowburn et al.</td>
<td>nb, nc</td>
<td>10</td>
<td>BQ 123</td>
<td>IV bolus IV bolus</td>
<td>Stable CHF</td>
<td>↑</td>
<td></td>
<td>(↑)</td>
<td></td>
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<tr>
<td>Cowburn et al.</td>
<td>8</td>
<td>BQ 788</td>
<td>IV bolus</td>
<td>IV bolus</td>
<td>Stable CHF</td>
<td>↑</td>
<td></td>
<td>(↑)</td>
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<td></td>
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<tr>
<td></td>
<td>7</td>
<td>BQ 788 + BQ 123</td>
<td>IV bolus</td>
<td>IV bolus</td>
<td>Stable CHF</td>
<td>↑</td>
<td></td>
<td>(↑)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Givertz et al.</td>
<td>r, db, pc</td>
<td>24</td>
<td>TBC 11251</td>
<td>IV bolus IV bolus</td>
<td>III-IV</td>
<td>↓</td>
<td></td>
<td>↓</td>
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</table>

* a  Seven patients of the original 8 received BQ 123 + BQ 188.

**CI** = cardiac index; **db** = double-blind; **HR** = heart rate; **IV** = intravenous; **MAP** = mean arterial pressure; **n** = number of participants; **nb** = nonblind; **nc** = noncomparative; **ns** = not specified; **NYHA** = New York Heart Association grade of CHF; **PAP** = pulmonary artery pressure; **pe** = placebo-controlled; **PVR** = pulmonary vascular resistance; **r** = randomised; **SVR** = systemic vascular resistance; **↑** = significant increase; **↓** = significant decrease; **↔** = no change; **(↑)** = nonsignificant increase; **(↓)** = nonsignificant decrease.
receptors), by interactions with other hormone systems, such as the renin-angiotensin system, by effects on the central and peripheral nervous system, and, in the longer term, by its mitogenic properties, which will influence the calibre and responsiveness of blood vessels. Knockout rodent models suggest that ET-B receptors participate in blood pressure regulation by causing vasodilatation and natriuresis. Forearm models in healthy human volunteers have shown that selective ET-A receptor antagonism (BQ 123) causes local vasodilatation, combined ET-A and ET-B (BQ 123 plus BQ 788) or nonselective ET-A/B receptor antagonism (TAK 044) causes vasodilatation, but to a lesser extent than ET-A antagonism alone, whereas selective ET-B blockade (BQ 788) causes vasoconstriction. Systemic studies with nonselective and selective ET-A or -B receptor antagonists have confirmed these local findings; selective ET-A (BQ 123) and nonselective receptor antagonists (TAK 044) produce similar reductions in mean arterial pressure and SVR while ET-B-selective receptor antagonists (BQ 788) produce an increase in peripheral vascular resistance.

With respect to the pathophysiology of hypertension, production of ET-1 is increased in some (e.g. Dahl salt-sensitive and stroke-prone spontaneously hypertensive rats), but not all rat models. Those models in which ET-1 production is increased (mostly, but not exclusively salt-dependent types) are associated with increased vascular growth and are responsive to both selective and nonselective ET receptor antagonists. This response comprises not only a modest reduction in blood pressure but also a marked regression of vascular growth.

In humans, elevated plasma ET concentrations are found in hypertension, though this is not consistent. These high concentrations would appear, mostly, to be a feature of severe hypertension or indicative of the presence of complications or coexisting disease. Levels of prepro-ET-1 and ECE mRNA are increased in the vascular smooth muscle cells of patients with hypertension, and there is evidence that endothelial function, specifically endothelium-dependent vascular relaxation, is impaired both in patients with hypertension and in groups at risk of developing hypertension. Forearm studies with selective ET-A or -B receptor antagonism (BQ 123 and BQ 788, respectively) suggest increased vascular ET activity in patients with essential hypertension compared with normotensive controls, and demonstrate a greater response to nonselective antagonism compared with selective ET-A antagonism. These data need to be confirmed, but suggest a mechanism for ET in the pathogenesis of hypertension.

To date, one major study has examined the antihypertensive effects of an ET receptor antagonist in humans. In 293 patients with hypertension, oral bosentan 500 to 2000 mg/day for 4 weeks achieved a reduction in blood pressure equivalent to that achieved with 20mg of enalapril. This reduction was achieved without a reflex increase in the activity of the sympathetic nervous system (as measured by plasma noradrenaline levels and heart rate) or renin-angiotensin system. Further confirmatory work is needed, but given the range of available antihypertensive drugs, a new class of agents would have to demonstrate greater efficacy, a better adverse effect profile, or other benefits in addition to blood pressure lowering properties in order to obtain a place in the therapy for hypertension. In addition to antihypertensive effects, the potential benefits of ET antagonists in patients with hypertension might include reducing long term structural changes in blood vessels (vascular hypertrophy or atherogenesis) or the myocardium, ameliorating endothelial dysfunction, and/or beneficial effects on salt and water balance. Subgroups such as salt-sensitive individuals or black patients with hypertension (with higher plasma ET levels and/or impaired endothelium-dependent relaxation) might benefit the most from these agents.

6. Vasospastic Conditions

A role for ET has been suggested in vasospastic conditions, such as migraine and Raynaud’s disease, because higher plasma ET concentrations are seen during active episodes in patients with these conditions. It must be remembered, though, that ET
plays an important role in normal developmental biology. ET antagonists are teratogenic in the first trimester of pregnancy and therefore use of these drugs in women of child-bearing age is precluded, limiting their usefulness in these and other conditions. However, this limitation is probably less important in the delayed cerebral vasospasm seen after subarachnoid haemorrhage or in ischaemic stroke. Selective and nonselective ET receptor antagonists (RO 611790 and SB 217242, respectively) have both been shown to reduce ischaemic brain injury in animals. Phase II trials with ET antagonists for these indications are currently in progress.

7. Pulmonary Hypertension

ET-1 is elevated in animal models of pulmonary hypertension and in humans, possibly because of increased pulmonary production, as the pulmonary venous concentration of ET-1 exceeds the arterial concentration. In addition, tissue levels of mRNA for ET-1 appear to be correlated with the PVR. Thus, pulmonary hypertension, a disease characterised by endothelial injury, vascular smooth muscle cell proliferation and pulmonary vasoconstriction, is another potential therapeutic target for ET receptor antagonists.

Nonselective and selective ET-A receptor antagonism both prevent NG-monomethyl-L-arginine-induced pulmonary vasoconstriction in a rat monocrotaline model of pulmonary hypertension. The lack of effect of ET-B antagonism suggests this is an ET-A-mediated effect. In a pig hypoxic model of pulmonary hypertension, selective ET-A and nonselective receptor antagonists were equally effective in countering hypoxia-induced increases in pulmonary artery pressure; ET-B-selective drugs had no effect. Both ET-A-selective and nonselective drugs (table I) are currently in development for this indication.

8. Conclusion

ET receptor antagonists have been, and continue to be, crucial experimental tools in elucidating the role of the ET system in cardiovascular physiology and pathophysiology. With the increasing availability of orally administered selective and nonselective antagonists, it should be possible to more fully establish the clinical role of these drugs in patients with cardiovascular disease. If they live up to their promise, ET receptor antagonists could offer a wide range of benefits including the reduction of pre- and after-load, reduction of pulmonary and systemic blood pressure, improvement in coronary blood flow, and, in the longer term, a reduction in abnormal ventricular and vascular remodelling and atherosclerosis. Through these effects, they might be useful in the management of patients with CAD, hypertension and CHF. Though the cardiovascular system appears to be a promising target for these new drugs, further work is needed to clarify when selective as opposed to nonselective antagonism is required.

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References


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Summary: The endothelins comprise a family of potent vasoconstricting peptides. Endothelin-1 appears to be the predominant isoform produced by the vascular endothelium, acting mainly in a paracrine fashion on vascular smooth muscle cells to cause vasoconstriction. It also has a range of other local actions – in the kidney, in the nervous system and on other hormone systems – that could, potentially, play a part in the genesis of hypertension. The association of raised plasma endothelin concentrations in human hypertension has caused much interest, but the literature is not consistent. Given the generally low plasma concentration of the endothelins, and their mainly paracrine actions, it remains unclear whether plasma endothelin has a functional role in hypertension. Additionally, problems remain with the measurement of plasma endothelin that raise doubts about the validity of conclusions drawn from these measurements. Key Words: Plasma—Endothelin—Hypertension—Radioimmunoassay—Enzyme-linked immunoassay.

INTRODUCTION

The endothelins (ETs) are a family of 21-amino-acid peptides with vasoactive, inotropic and mitogenic properties and the ability to modulate other hormone systems, as well as gut, kidney and lung function. Three different peptides, endothelin (ET)-1, ET-2 and ET-3, each with distinct genes and tissue distributions, have so far been identified. ET-2 differs from ET-1 by two amino acids and ET-3 from ET-1 by six amino acids. Each has a common structure of a hydrophobic C terminal end and two intra-chain disulphide bonds that form a hairpin structure (1–4).

ET-1 is primarily produced by endothelial cells but is also found in a variety of other cell types in heart (epicardium), lung (bronchial epithelial cells), kidney (tubular cells), central and peripheral nervous system tissue (astrocytes/nerve plexi/pituitary tissue), macrophages and vascular smooth muscle cells. ET-2 mRNA is found to a lesser extent in endothelial cells and also in other cells in heart and kidney. ET-3 is found in gut, kidney, adrenal secretory tissue, macrophages and CNS tissue but not endothelium (2,5–7).

ET-1 is synthesized from preproendothelin-1, the 212-amino-acid gene product. Endothelin genes are thought to be regulated at the transcription level with stimuli to gene transcription including hormones (such as adrenalin, angiotensin II, insulin and arginine vasopressin), cytokines (such as platelet-derived growth factor and transforming growth factor beta), hypoxia, oxidized low density lipoprotein and cyclosporin. Inhibition has been documented in response to prostacyclin, nitric oxide, atrial and other natriuretic peptides and heparin (4,7,8).

Preproendothelin-1 is processed to form proendothelin-1 which is then cleaved by intracellular furin-like proteases to generate big ET-1 (38 amino acids) which is biologically inactive. Endothelin-converting enzyme (ECE), a metalloprotease, then cleaves big ET-1 to the biologically active ET-1 and a 17-amino-acid C-terminal fragment-1 (CTF-1). A group of ECEs for ET-1 have been identified, each with a different tissue distribution and different pH at which they act. ECEs have not yet been identified that are selective for ET-2 and ET-3 (9).

Two receptors for ET have been identified. ET\textsubscript{A} receptors are found in the aorta, heart, kidney and vascular smooth muscle cells where their activation causes cell contraction. They exhibit a marked selectivity for ET-1. ET\textsubscript{B} receptors are present on endothelial cells where their activation results in the production of nitric oxide and prostacyclin, resulting in vasodilatation. They are also present on vascular smooth muscle cells where their acti-
ution causes vasoconstriction. They have equal affinity for ET-1, ET-2 and ET-3. Signal transduction is via G protein coupling and activation of intracellular phospholipase-C with subsequent hydrolysis of phosphatidyl inositol and changes in intracellular calcium. Removal of ET-1 from the circulation is by receptor binding and internalization (ETB effect). renal clearance and enzymatic degradation by neutral endopeptidases (8-10).

MEASUREMENT

Plasma endothelin-like immunoreactivity is said to comprise around 60% big ET-1 and 30% ET-1. ET-3 but not ET-2 also circulates in the blood at lower concentrations (<10%) (4,11,12). Most of the focus has been on plasma ET-1 because it is the major active isoform in blood, although it is possible that big ET-1 could act as a circulating hormone which is processed to ET-1 at its site of action.

There are many problems associated with the measurement and interpretation of plasma ET-1 concentrations. Most ET-1 (<90%) is released from endothelial cells abuminally (13) where it acts on endothelial and smooth muscle cells as a predominantly autocrine and paracrine mediator. Plasma ET-1 may not, therefore, be an accurate reflection of endothelial synthesis of ET-1. In addition, the ET-1 concentration in plasma is likely to reflect a balance between production/overspill and its clearance. Under normal circumstances, ET-1 is rapidly cleared from the circulation by ETB receptors, the kidney and neutral endopeptidases and so plasma concentrations may be largely determined by production. Consistent with this, although ET-1 exerts effects for up to 60 min, the plasma half-life in healthy subjects is only ~1 min (14). Reduction in ETB receptor numbers, enzymatic degradation, or renal function, or administration of an ETB receptor antagonist, may reduce this clearance and increase plasma ET-1 half-life.

Hence, in a number of situations, plasma ET-1 concentrations can be altered without a change in ET-1 production. It may not, therefore, be valid to draw conclusions about ET-1 production from plasma concentrations. Plasma big ET-1 has been proposed as a more reliable measure of endothelial ET-1 synthesis because its removal from the circulation is slower and less variable (15,16).

Turning to measurement techniques, plasma ETs are currently analysed by either radioimmunoassay (RIA) or enzyme-linked immunoassay (ELISA). Because normal plasma concentrations are in the low picomolar range (12,17-25), direct measurement in the plasma is difficult and both RIA and ELISA require ET to be extracted from the plasma and concentrated prior to measurement. Recovery rates during extraction are variable depending on, for example, the size and nature of the extraction columns and the fluid used to elute the ET; acetic acid extraction (26) achieves the highest recovery rate of 90% but recovery can be as low as around 50% (see Table 1). ET-1 is also partly degraded during the extraction process (26), further reducing the amount of plasma ET-1 that will then be assayed.

Antibodies commonly used in current assays exhibit cross-reactivity between the subtypes of ET, depending on which part of the molecule the antibody is raised against and depending on the antibody used; for example, using ET-1 detection of 100% as the standard, cross-reactivity of the antibody can range between 0 and 200% with big ET-1, between 10 and 1300% for ET-2, and between 0% and 120% for ET-3 (see Table 1). Many papers quote the concentration of ‘plasma ET-1’, when a more realistic term, given the cross-reactivity of the antibodies used, is ‘plasma ET-like immunoreactivity’. As a separate issue, it is not clear whether the ET assayed in blood comprises mature peptides or degraded fragments of these. Therefore, it is possible that the high plasma ET-1 concentrations, for instance in renal failure, may bind ET antibodies but not be biologically active.

The sensitivity of a good immunoassay may be sufficient to detect levels down to 0.5 pg/ml. However, some kits have a sensitivity no lower than 8 pg/ml, higher than the range in which blood concentrations in healthy subjects are found. Also, intra- and inter-assay variability tends to be relatively high for ET assays, with inter-assay variations of >10% reported, thus reducing the precision of the measurement (see Table 1). In addition, sample handling is important because haemolysis can increase measured ET concentrations tenfold. Lipaemic or icteric samples also give falsely high readings. Furthermore, dilution curves of synthetic pure ET-1 from different suppliers also show considerable variation (26). Hence, the reference point for biological samples varies depending on the supplier of the standard. Finally, the conditions under which blood is sampled must be borne in mind given that changes in position (45), temperature (24) and mental stress (46) have been reported to affect plasma ET-1 concentrations, and given the known interaction between ET-1 and other neurohumoral mechanisms (41,47,48). Taking these many factors into account, comparisons between studies performed in different laboratories are currently probably not warranted, although the ratios of concentrations in patients relative to controls may be a useful measure (Table 2).

PLASMA ENDOTHELIN AND ESSENTIAL HYPERTENSION

ET-1 is a profound vasoconstrictor, causes cell proliferation, and is involved in the maintenance of basal vascular tone in a balance between constricting and dilating substances. It also activates the sympathetic nervous system and the renin–angiotensin–aldosterone axis. Hence, a role has been sought for the peptide in the pathophysiology of essential hypertension, a condition characterized by endothelial dysfunction and increased vascular tone (1). In this review, we have considered measurements of plasma ET concentration in patients with essential hypertension in all papers published in English or abstracted in English that appear on Medline. Although plasma ET-1 levels have been reported as elevated in patients with essential hypertension in some
### TABLE 1. Plasma endothelin concentrations in essential hypertension: assay employed

<table>
<thead>
<tr>
<th>Study (ref.)</th>
<th>Difference between essential hypertension and control detected?</th>
<th>Number of patients/controls</th>
<th>Recovery (during extraction process)</th>
<th>Assay</th>
<th>Inter-assay coefficient of variation</th>
<th>Intra-assay coefficient of variation</th>
<th>Cross-reactivity – as stated in papers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kohno 1990 (11)</td>
<td>Yes (not borderline group) +ve correlation with BP</td>
<td>54/25</td>
<td>63%</td>
<td>Peninsula RIA</td>
<td>Not stated</td>
<td>Not stated</td>
<td>84%</td>
</tr>
<tr>
<td>Saito 1990 (27,28)</td>
<td>Yes (especially with target organ damage)</td>
<td>20/12</td>
<td>52–55%</td>
<td>Monoclonals RIA</td>
<td>6.8%</td>
<td>7.3%</td>
<td>80–100%</td>
</tr>
<tr>
<td>Shichiri 1990 (20)</td>
<td>Yes</td>
<td>11/21</td>
<td>61%</td>
<td>RIA</td>
<td>13%</td>
<td>10%</td>
<td>100%</td>
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<td>Naruse 1991 (29)</td>
<td>Yes (in patients with target organ damage)</td>
<td>82/91</td>
<td>90%</td>
<td>Dalnippo RIA</td>
<td>Not stated</td>
<td>Not stated</td>
<td>277%</td>
</tr>
<tr>
<td>Widimsky 1991 (30)</td>
<td>Yes (in severe hypertension; +ve correlation with BP)</td>
<td>16/8</td>
<td>Not stated</td>
<td>Amersham RIA</td>
<td>Not stated</td>
<td>Not stated</td>
<td>200%</td>
</tr>
<tr>
<td>Fernandez-Cruz 1993 (31)</td>
<td>Yes (in young but not old)</td>
<td>62/119</td>
<td>78%</td>
<td>Peninsula RIA</td>
<td>15%</td>
<td>12%</td>
<td>7%</td>
</tr>
<tr>
<td>Fogari 1994 (32)</td>
<td>Yes (and +ve correlation with ABPM (no control group))</td>
<td>15</td>
<td>Not stated</td>
<td>Amersham RIA</td>
<td>9%</td>
<td>6%</td>
<td>204%</td>
</tr>
<tr>
<td>Lemme 1994 (33)</td>
<td>Yes (mild to moderate hypertensives)</td>
<td>75/75</td>
<td>Not stated</td>
<td>RIA</td>
<td>8%</td>
<td>5%</td>
<td>32%</td>
</tr>
<tr>
<td>Januszewicz 1994 (34)</td>
<td>Yes (mild to moderate hypertensives)</td>
<td>37/21</td>
<td>100%</td>
<td>Amersham RIA</td>
<td>13.8%</td>
<td>4.8%</td>
<td>Not stated</td>
</tr>
<tr>
<td>Zoccoli 1995 (25)</td>
<td>Yes (particularly black hypertensives)</td>
<td>20/8</td>
<td>75–85%</td>
<td>Peninsula RIA</td>
<td>15%</td>
<td>8%</td>
<td>Not stated</td>
</tr>
<tr>
<td>Ergul 1996 (35)</td>
<td>Yes (with +ve correlation with DBP)</td>
<td>50/50</td>
<td>Direct measurement Not stated</td>
<td>Amersham RIA</td>
<td>Not stated</td>
<td>Not stated</td>
<td>1305%</td>
</tr>
<tr>
<td>Zapolowska-Stachowiak 1997 (36)</td>
<td>Yes (partially black hypertensives)</td>
<td>64/44</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>7%</td>
</tr>
<tr>
<td>Davenport 1990 (37)</td>
<td>Yes (other correlation with BP)</td>
<td>25/25</td>
<td>73%</td>
<td>Amersham RIA</td>
<td>13%</td>
<td>9%</td>
<td>Not stated</td>
</tr>
<tr>
<td>Predel 1990 (19)</td>
<td>No (mild to moderate hypertensives)</td>
<td>12/20</td>
<td>Not stated</td>
<td>Amersham RIA</td>
<td>Not stated</td>
<td>Not stated</td>
<td>100%</td>
</tr>
<tr>
<td>Schifferin 1991 (38)</td>
<td>No (plus to more hypertensives; +ve correlation with BP)</td>
<td>22/17</td>
<td>Not stated</td>
<td>Peninsula RIA</td>
<td>Not stated</td>
<td>Not stated</td>
<td>7%</td>
</tr>
<tr>
<td>Miyachi 1992 (39)</td>
<td>No</td>
<td>51/75</td>
<td>Not stated</td>
<td>ELISA (Banyu)</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
</tr>
<tr>
<td>Baldys-Waligorska 1993 (40)</td>
<td>No (mild hypertensives; +ve correlation with BP)</td>
<td>10/36</td>
<td>88%</td>
<td>Biomedica RIA</td>
<td>8%</td>
<td>5%</td>
<td>100%</td>
</tr>
<tr>
<td>Veglio 1993 (42)</td>
<td>No (mild to moderate hypertensives)</td>
<td>25/30</td>
<td>90%</td>
<td>Peninsula RIA</td>
<td>9%</td>
<td>6%</td>
<td>7%</td>
</tr>
<tr>
<td>Hoffman 1994 (23)</td>
<td>No</td>
<td>17/19</td>
<td>85–92%</td>
<td>Peninsula RIA</td>
<td>18%</td>
<td>7%</td>
<td>7%</td>
</tr>
<tr>
<td>Haynes 1994 (41)</td>
<td>No</td>
<td>12/12</td>
<td>84%</td>
<td>RIA</td>
<td>4.2%</td>
<td>2.4%</td>
<td>2.4%</td>
</tr>
<tr>
<td>Sorensen 1994 (42)</td>
<td>No</td>
<td>12/12</td>
<td>85%</td>
<td>RIA</td>
<td>11%</td>
<td>8%</td>
<td>54%</td>
</tr>
<tr>
<td>Ferri 1995 (43)</td>
<td>No (obese)</td>
<td>15/10</td>
<td>85%</td>
<td>Peninsula RIA</td>
<td>10%</td>
<td>7%</td>
<td>7%</td>
</tr>
<tr>
<td>Letizia 1995 (24)</td>
<td>No (non-obese)</td>
<td>12/11</td>
<td>Not stated</td>
<td>Peninsula RIA</td>
<td>Not stated</td>
<td>Not stated</td>
<td>7%</td>
</tr>
<tr>
<td>Schifferin 1997 (44)</td>
<td>No (tend to higher in severe hypertensives)</td>
<td>8/5</td>
<td>Not stated</td>
<td>Peninsula RIA</td>
<td>Not stated</td>
<td>Not stated</td>
<td>7%</td>
</tr>
</tbody>
</table>

NS, no significant cross-reactivity; BP, blood pressure; DBP, diastolic blood pressure, ABPM, ambulatory blood pressure monitoring; RIA, radioimmunoassay; ELISA, enzyme-linked immunoassay.
studies (11,20,25,27-36,58), this is by no means universal (19,22-24,37-44).

Plasma ET concentrations, even when elevated, are generally not as high as those associated with haemodynamic effects when ET-1 is given exogenously in man (59-61). However, when ET-1 is given exogenously there is likely to be a gradient of concentration of ET-1 between blood and the endothelium/smooth muscle interface, such that the vascular smooth muscle is exposed to less ET-1 than is indicated by assays of plasma concentration. In contrast, the gradient is in the opposite direction for endogenously generated ET-1, so that the concentration in plasma is considerably less than at the endothelium/smooth muscle interface. Therefore, for the same plasma concentration, vascular smooth muscle 'sees' considerably more ET-1 when it is generated endogenously than given exogenously. Indeed, in prostatic carcinoma, where plasma ET-1 concentrations are as high as 50 pg/ml, but are probably not generated at the endothelium/smooth muscle interface, blood pressure is not raised (49). Hence, comparisons based on plasma concentrations are unlikely to be valid and locally increased ET-1 generation may occur in the blood vessels of hypertensive subjects without it being clearly reflected in the blood, and may have important effects on vascular structure and function, either directly or indirectly, such as through potentiation of the sympathetic nervous system (41).

Why do only some studies show an elevated plasma ET concentration in hypertension? It may relate to the use of different assays with different cross-reactivities. First, if the main plasma ET species are ET-1 and big ET-1, with a smaller contribution from ET-3, then those assays purporting to measure ET-1 but with a high cross-reactivity for these other species might give artificially high values. However, this does not appear to explain the disagreement because even those groups using antibodies with a low (<10%) cross-reactivity for these species are divided in their findings with respect to plasma ET-1 concentrations in hypertension (see Table 1). Second, two groups have reported that the plasma ET immunoreactivity in patients with essential hypertension has the same high performance liquid chromatography (HPLC) profile as in normotensives i.e. the same relative concentrations of the ET subtypes (11,62). Hence, inaccuracy in ET-1 measurement resulting from cross-reactivity should be the same for hypertensive and normotensive groups and a true difference, therefore, still exist whatever the cross-reactivity of the antibody used in the assay.

Does the size of the populations studied account for the difference of opinion? Davenport et al. (37) studied 25 patients and 25 controls and showed no differences in plasma ET-1 concentrations. They calculated that for the power of the test to be able to detect a 25% difference in concentrations, at a 5% significance level, 100 patients would be required. No study meets this size requirement. From Table 1 it can be seen that, although there is one larger study failing to detect an elevation in plasma ET-1 in hypertensives, the majority of larger studies are in the group that do detect a difference. Whereas the published studies mostly account for other important confounding factors, including age, creatinine and antihypertensive drugs, a meta-analysis is likely to be unhelpful because the assays and the populations are not comparable, or sufficiently defined, and a strong possibility of publication bias for the positive studies exists.

It does appear, though, fairly consistently, that patients with severe hypertension are much more likely to have elevated plasma ET-1 concentrations. Several groups who report a difference in plasma (ET-1) note this is limited to patients with more advanced disease, in terms of target organ damage or higher blood pressures (11, 28-30,58). Additionally, one group who observed no significant difference did see a trend towards higher plasma ET-1 concentrations in severe disease (44). This could be explained by the observation that patients with atherosclerosis (a common condition in hypertensive patients) have higher circulating ET-1 concentrations irrespective of blood pressure (51) but groups specifically excluding patients with vascular disease have still detected this effect (25). If plasma ET-1 levels are higher in more severe hypertension, one might expect to find a correlation between plasma ET-1 and blood pressure. Some groups do observe such a correlation (11,25,30,31,33), even when no absolute difference has been detected (38,40) whereas some do not (19,23,42,43) even when a difference between groups is observed (28,29,34,35).
Indeed, one study observed a significant negative correlation between blood pressure and plasma ET-1 in the hypertensive group (37). Fogari et al. (32) did not find a correlation between plasma ET concentration and blood pressure measured in the clinic (three readings, supine) but did find a significant correlation when blood pressure was measured using 24-h ambulatory blood pressure monitoring. Interestingly, in relation to the plasma findings in severe hypertension, Schiffrin et al. (44) have recently demonstrated an increase in ET-1 gene expression in resistance arteries that is confined to patients with severe hypertension, and only associated with a trend for plasma ET-1 to be elevated, suggesting that ET-1 production may be increased in hypertension but this is poorly reflected by changes in plasma ET-1.

Ergul et al. (35) report a striking threefold higher plasma ET-1 concentration in black patients with hypertension compared to white patients with hypertension. Hoffman et al. (23) did not detect this racial difference but the numbers in each group in both studies were small and the issue needs clarification with a larger study. In salt-sensitive compared to salt-resistant patients with essential hypertension findings are similarly equivocal (23, 63). What is clear is that a substantial overlap in plasma ET-1 concentrations exists between the hypertensive group, however it is described, and the control group in all of the studies. Thus, with respect to plasma ET concentrations in essential hypertension, they are neither a sensitive nor a specific index of this condition.

Two groups have found abnormalities in ET-1 clearance in hypertensive patients with normal renal function, suggesting an abnormality in renal ET handling consistent with reduced ET-1 clearance or renal production (23,25). This is in marked contrast to the situation in renal failure where ET-1 clearance/glomerular filtration rate (GFR) ratio is substantially raised (25). Within the kidney, ET has the potential to generate hypertension by several mechanisms. The effect of exogenous ET-1 on the renal vasculature is to cause vasoconstriction (which will activate the renin–angiotensin system) and profound salt and water retention, both having the potential to raise blood pressure (64). It is not clear yet whether the elevated plasma ET-1 concentrations seen in various disease states are able to cause such renal vasoconstriction but it is known that ET-1 can cause a reduction in renal blood flow and GFR at concentrations that do not alter systemic haemodynamics (65). However, ET-1 is also produced by inner medullary collecting duct cells (IMCD) where it inhibits the arginine vasopressin (AVP) stimulated reten¬tion of water (66). There is evidence that extracellular sodium concentrations may regulate IMCD ET-1 production (67) and hence ET-1 may have a role in the renal regulation of volume homeostasis. If, as the urinary data suggest (23,25), nephron ET-1 production or handling is altered in essential hypertension, there may be inappropriate sodium and water retention aiding the development or maintenance of hypertension. Interestingly, spontaneously hypertensive rats have been shown to have reduced medullary ET-1 levels (68), whereas in the renanat kidney model of renal failure ET-1 production is increased (69).

CONCLUSIONS

Studies using receptor antagonists show that the ETs are important mediators in physiological blood pressure regulation in humans (70,71). Endothelin antagonists have also been shown to reduce blood pressure in hypertensive patients (72) and may have additional benefits in terms of potential effects on the heart, blood vessels and kidney (73). Measurement of plasma ET concentrations in hypertension is likely to be of limited value, and a poor reflection of ET-1 generation, although it may indicate hypertensive subgroups in whom ET antagonists might be particularly effective.

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J Cardiovasc Pharmacol 33 (Suppl. 2), 2000


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Endothelin Antagonists and Hypertension: A Question of Dose?

To the Editor:
We read with interest the recent report in Hypertension from Martin et al. on the effects of intra-arterial administration of the endothelin ET<sub>B</sub> receptor antagonist, SB 209670, on forearm blood flow in hypertensive subjects and matched controls. Their finding of forearm vasodilation to intra-arterial SB 209670 in healthy controls suggests a role for endothelin (ET)-1 in regulation of basal vascular tone and is consistent with our own work<sup>2</sup>-<sup>4</sup> and that of some<sup>4</sup>-<sup>6</sup> but not other groups<sup>6</sup>-<sup>7</sup> with both ET<sub>A</sub> receptor selective and ET<sub>B</sub> receptor antagonists. Also, contrary to some earlier work<sup>6</sup>-<sup>7</sup> they find no difference from controls in the in vivo response of the resistance vessels of hypertensive subjects to ET receptor antagonists.

Our early intra-arterial studies were undertaken with the ET<sub>A</sub> selective antagonist, BQ-123, at a dose of 100 nmol/min. We have since undertaken pharmacodynamic and kinetic dose-ranging systemic studies with BQ-123 and find that this dose of BQ-123 has modest systemic effects, more on vascular resistance than blood pressure.<sup>9</sup> Maximum plasma concentrations of BQ-123 at 100 nmol/min were 58±158 nmol/L and IC<sub>50</sub> values for BQ-123 at the ET<sub>A</sub> and ET<sub>B</sub> receptors in vitro are 9 to 24 nmol/L and 10 to 18 000 nmol/L, respectively, depending on cell type.<sup>10</sup> Hence, when given locally into the forearm (blood flow ~50 mL/min) rather than the systemic circulation (~5 000 mL/min), this dose of BQ-123 will achieve concentrations (~60 000 nmol/L) that may have functionally important inhibitory effects at the ET<sub>B</sub> receptor.

On this basis, our laboratory has more recently delivered BQ-123 in forearm studies at a dose of 10 nmol/min, with which, if anything, greater effects on local blood flow have been seen.<sup>4</sup> This may be explained by the major role of the vascular ET<sub>B</sub> receptor being to mediate vasodilation.<sup>4</sup> It is such that combined ET<sub>A</sub> inhibition may, by blocking ET<sub>B</sub> mediated effects, attenuate the vasodilation associated with selective ET<sub>A</sub> receptor antagonism.<sup>8</sup> We have also used BQ-788 intra-arterially (at 1 nmol/min) as an ET<sub>B</sub> selective antagonist, here based on systemic studies showing that 30 nmol/min, but not 3 nmol/min, increases systemic vascular resistance.<sup>11</sup>

However, a key issue arises for the published body of work using intra-arterial administration of ET receptor antagonists. These investigations have generally used high doses that are likely to be both nonselective and systemically active, conditions that interfere with a clear interpretation of these studies. Cardillo and colleagues gave BQ-123 (at 100 nmol/min) and BQ-788 (at 50 nmol/min) by intra-arterial coadministration to hypertensives and controls to achieve dual ET receptor blockade.<sup>4</sup> However, it would now appear that both of these agents were given at systemically active doses. A similar problem of using a systemically active dose of TAK-044 may account for a rather modest effect on vascular tone in healthy subjects in one study<sup>7</sup> and the lesser effect of a greater dose of TAK-044 in another.<sup>3</sup> By giving systemic doses of the pharmacological probes, responses in the infused forearm may have been influenced directly by changes in systemic vascular resistance or indirectly by the activation of reflex neurohormonal mechanisms.<sup>12</sup> In this regard, there must remain some uncertainty about interpretation of work examining the role of endothelin in hypertension and other vascular diseases using drug administration via the brachial artery until these studies are repeated with doses of drugs that are demonstrably confined to a local action.

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Response
We thank Doctors Goddard and Webb for their comments regarding intra-arterial infusion of endothelin (ET) antagonists. Goddard and Webb raise a number of relevant issues. They reiterate the importance of using systemic doses of vasoactive substances to test hypotheses regarding direct effects on the vasculature. The key issue is how to establish that doses are indeed systemic. The usual approach includes careful assessment of systemic blood...
pressure and heart rate responses and/or measurement of forearm blood flow and vascular resistance in the contralateral limb. This is really a general issue of the technique itself and not limited to endothelin blockade as the vasoactive substance.

Specific to endothelin blockade, however, is the issue of selectivity of the receptor antagonist. Goddard and Webb suggest that doses of the ETA, "selective" antagonist BQ-123, assumed to be subsystemic in a number of studies, may indeed be systemic even after active generation of endogenous endothelin-1 to basal vascular tone. 

However, this still does not explain differences in vascular responses to this agent given at the same dose and at the same infusion rate over similar periods of time. We used SB209670 in our study specifically because it is nonselective, and we wished to test the impact of blockade of all major ET receptor subtypes on the vasculature, between normal subjects and patients with cardiovascular disease.

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Systemic ETA receptor antagonism with BQ-123 blocks ET-1 induced forearm vasoconstriction and decreases peripheral vascular resistance in healthy men


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1 The effect on systemic haemodynamics of BQ-123, a selective endothelin A (ETA) receptor antagonist, was investigated in healthy men by giving, on separate occasions, ascending intravenous doses of 100, 300, 1000 and 3000 nmol min⁻¹ BQ-123, each for 15 min, in a randomized, placebo-controlled, double-blind study. The response of forearm blood flow to brachial artery infusion of endothelin-1 (ET-1; 5 pmol min⁻¹ for 90 min) was also studied using bilateral forearm plethysmography, after systemic pre-treatment, on separate occasions, with one of two doses of BQ-123 (300 and 1000 nmol min⁻¹ for 15 min) or placebo.

2 Systemic BQ-123 dose-dependently decreased systemic vascular resistance (P<0.01 for all doses vs placebo) and mean arterial pressure (P<0.05 for 300 nmol min⁻¹ and P<0.01 for 1000 and 3000 nmol min⁻¹) during the 60 min following infusion. There were concurrent increases in heart rate and cardiac index. BQ-123, when infused systemically for 15 min, appeared to reach a maximum effect at 1000 nmol min⁻¹.

3 Intra-brachial ET-1 infusion, after pre-treatment with placebo, caused a slow onset progressive forearm vasoconstriction without systemic effects. This vasoconstriction was attenuated by pre-treatment with BQ-123 at 300 nmol min⁻¹ and abolished by BQ-123 at 1000 nmol min⁻¹ (P<0.01 vs placebo).

4 These effects occurred at concentrations of BQ-123 in the plasma (510±64 nmol l⁻¹) that were ETA receptor selective, and were not accompanied by an increase in plasma ET-1 that would have indicated ETB receptor blockade.

5 We conclude that ETA-mediated vascular tone contributes to the maintenance of basal systemic vascular resistance and blood pressure in healthy men.

Keywords: Endothelin; vasodilatation; BQ-123; human; systemic

Introduction

Endothelin-1, which was first identified by Yanagisawa et al. (1988), is a well characterized, 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 characterized pharmacologically in blood vessels. ETA receptors (Arat et al., 1990) have higher affinity for ET-1 than ET-3, are found on vascular smooth muscle cells, and mediate vasoconstriction. ETB 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). ETB receptors are also present on vascular smooth muscle cells, where they may contribute to vasoconstriction (Clozel et al., 1992; Reizebos et al., 1994; Sco 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 ETA receptor antagonist, first demonstrated the importance of ET-1 in maintaining basal resistance vessel tone, in large part through an action on the ETA receptor (Haynes & Webb, 1994). These observations have since been confirmed by others (Berrua 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 ETA receptor antagonism would produce systemic vasodilatation. Recently, however, acute systemic administration of the selective ETA antagonist, BQ-123, was reported to have no effect on systemic haemodynamics (Schmetterer et al., 1998; Montanari et al., 2000).

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We hypothesized that a systemic haemodynamic effect of ETA receptor blockade was not seen in these studies because the doses of BQ-123 used provided insufficient ETA receptor blockade to affect blood pressure. In addition, because healthy subjects have a number of reflex mechanisms that serve to defend blood pressure, we hypothesized that an important effect on systemic vascular resistance might have been missed by measurement of blood pressure alone. We also hypothesized that systematically effective ETA 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 forearm vascular resistance. We, therefore, undertook two studies. First, we assessed the haemodynamic effects of increasing doses of BQ-123, using bioimpedance cardiology, with the aim of achieving a high degree of ETA selective receptor blockade. Second, we examined whether haemodynamically active doses of BQ-123 would antagonize 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.

Methods

Subjects

Five healthy men (age range 18–30 years) were recruited to each of the two 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 08.30 h in a quiet room kept at a controlled temperature (22–24°C).

Drugs

BQ-123 (Clinalfa AG, Laufelfingen, Switzerland, molecular weight 632.7) at doses ranging from 100–300 nmol min⁻¹ was used as a selective ETA antagonist, with 2500 lower affinity for the ETB receptor (IC₅₀ ETA of 7.3 nm against IC₅₀ ETB 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, U.K.). 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.

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.

Measurements

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. Blood pressure was recorded in duplicate at each time-point using a well-validated semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA-751 sphygmomanometer, Takeda Medical Inc) (Winberg et al., 1988), in the right arm. Cardiac output (CO) and heart rate (HR) were recorded using a well validated non-invasive bioimpedance technique as previously described (NCCOM 3; BoMed Medical Manufacturer Ltd) (Goldstein et al., 1986).

Forearm blood flow Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic gauges (Benjamin et al., 1995; Webb, 1995) that were securely applied to the widest part of each forearm. The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mmHg. Upper arm cuffs were intermittently inflated to 40 mmHg for 10 in every 15 s to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Recordings of forearm blood flow were made repeatedly at 10 min intervals over 3-min periods. Voltage output from a dual-channel Vasculab SPG 16 strain gauge plethysmograph (Medasonics Inc) was transferred to a Macintosh personal computer (Perforama 475, Apple Computer Inc, Cupertino, CA, U.S.A.) using a MacLab analogue digital converter and Chart software (v. 3.2.8; both from AD Instruments, Castle Hill, NSW, Australia). Calibration was achieved using the internal standard of the Vasculab plethysmography units.

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. Samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Beckton Dickinson) centrifuged 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 determined by standard radioimmunoassay (Peninsula Laboratories), as previously described (Newby et al., 1998a).

BQ-123 assay BQ-123 concentrations in plasma were measured by high performance liquid chromatography (HPLC) with fluorescence detection. One volume of plasma was precipitated with 4 volumes of ethanol, ultracentrifuged at 4 C for 15 min at 10,000 x g, and the resulting supernatant injected into the HPLC column. The HPLC system consisted
of a Waters 510 HPLC pump, WISP (Waters Intelligent Sample Processor) and Spherisorb S5 ODS column (Waters Ltd, Watford, Herts, U.K.) with detection by an LS-5 fluorometric detector (Perkin-Elmer Ltd, Beaconsfield, Bucks, U.K.), with excitation and emission wavelengths of 284 and 348 nm respectively. The mobile phase consisted of 60:40 acetonitrile: de-ionized water with tri-fluoroacetic acid at a concentration of 0.1%. The peptide TAK-044 was found to fluoresce at identical wavelengths to BQ-123 and was eluted from the column with a retention time similar to but not identical with BQ-123, allowing its separate measurement. Hence, TAK-044 was used as a standard in this assay. 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.

Study design

Study 1: Systemic haemodynamic study This was a double-blind, placebo-controlled, balanced, 5-way crossover study in five 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.

Study 2: ET-1 challenge study This was a double-blind, placebo-controlled, 3-way crossover study in five subjects (three of whom participated in the systemic study), investigating the effects of intra-arterial ET-1 on forearm blood flow (FFB), 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.

Data analysis

Study 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 and 0 min recordings. Haemodynamic data are expressed as placebo-corrected percentage change from baseline ± s.e.mean. 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 five subjects. The number of subjects was agreed with the local ethics committee on that basis.

Study 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). As flow only stabilizes after 60 s of wrist cuff inflation, recordings made in the first 60 s were not used for analysis. The last five flow recordings in each measurement period were calculated and averaged for the infused and non-infused arms (Webb, 1995). To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point, in effect using the non-infused arm as a contemporaneous control for the active treatment arm (Benjamin et al., 1995). Forearm blood flow 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 five subjects.

Results

Study 1: Systemic haemodynamics

All five 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 between 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,B).

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 (ETA receptor IC₅₀ 7.3 nM; ETB receptor IC₅₀ 18 μM) (Ihara et al.,...
Table 1 (A) Plasma ET-1 (pg ml⁻¹) levels at baseline, 5, 15 and 240 min following infusion of BQ-123. (B) Plasma big ET-1 (pg ml⁻¹) levels at baseline, 5, 15 and 240 min following infusion of BQ-123

<table>
<thead>
<tr>
<th>BQ-123 (nmol min⁻¹)</th>
<th>Baseline (pg ml⁻¹)</th>
<th>5 min (pg ml⁻¹)</th>
<th>15 min (pg ml⁻¹)</th>
<th>240 min (pg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Placebo</td>
<td>4.91 ± 0.69</td>
<td>5.16 ± 0.74</td>
<td>5.81 ± 0.50</td>
<td>5.77 ± 0.75</td>
</tr>
<tr>
<td>100</td>
<td>4.57 ± 0.48</td>
<td>4.08 ± 0.39</td>
<td>3.95 ± 0.65</td>
<td>4.44 ± 0.29</td>
</tr>
<tr>
<td>300</td>
<td>5.23 ± 0.52</td>
<td>5.29 ± 0.49</td>
<td>5.49 ± 0.95</td>
<td>5.88 ± 0.65</td>
</tr>
<tr>
<td>1000</td>
<td>4.43 ± 0.28</td>
<td>6.07 ± 0.49</td>
<td>5.56 ± 0.98</td>
<td>5.80 ± 0.65</td>
</tr>
<tr>
<td>3000</td>
<td>2.88 ± 0.10</td>
<td>4.08 ± 0.49</td>
<td>4.03 ± 0.63</td>
<td>4.22 ± 0.41</td>
</tr>
<tr>
<td>(B) Placebo</td>
<td>45.7 ± 14.4</td>
<td>49.3 ± 10.7</td>
<td>66.2 ± 14.4</td>
<td>38.8 ± 7.1</td>
</tr>
<tr>
<td>100</td>
<td>37.8 ± 7.4</td>
<td>29.2 ± 1.9</td>
<td>26.0 ± 2.4</td>
<td>35.8 ± 5.2</td>
</tr>
<tr>
<td>300</td>
<td>40.2 ± 6.5</td>
<td>38.5 ± 6.3</td>
<td>49.5 ± 14.1</td>
<td>48.4 ± 6.0</td>
</tr>
<tr>
<td>1000</td>
<td>57.5 ± 9.1</td>
<td>51.9 ± 8.5</td>
<td>51.4 ± 9.4</td>
<td>51.5 ± 9.2</td>
</tr>
<tr>
<td>3000</td>
<td>26.6 ± 6.7</td>
<td>34.7 ± 0.6</td>
<td>30.7 ± 3.1</td>
<td>30.1 ± 4.6</td>
</tr>
</tbody>
</table>

Table 2 Baseline data – absolute values

<table>
<thead>
<tr>
<th>BQ-123 (nmol min⁻¹)</th>
<th>MAP (mmHg)</th>
<th>SVRI (L min⁻¹ m⁻²)</th>
<th>CI (L min⁻¹ m⁻²)</th>
<th>HR (b.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>78.9 ± 1.5</td>
<td>22.7 ± 1.3</td>
<td>3.52 ± 0.21</td>
<td>64.2 ± 6.6</td>
</tr>
<tr>
<td>100</td>
<td>79.2 ± 2.3</td>
<td>21.9 ± 1.7</td>
<td>3.69 ± 0.25</td>
<td>57.1 ± 5.2</td>
</tr>
<tr>
<td>300</td>
<td>80.8 ± 3.5</td>
<td>21.8 ± 1.8</td>
<td>3.80 ± 0.31</td>
<td>63.9 ± 6.6</td>
</tr>
<tr>
<td>1000</td>
<td>78.2 ± 2.1</td>
<td>22.5 ± 2.9</td>
<td>3.63 ± 0.38</td>
<td>54.5 ± 4.8</td>
</tr>
<tr>
<td>3000</td>
<td>78.6 ± 3.3</td>
<td>23.5 ± 2.2</td>
<td>3.45 ± 0.35</td>
<td>57.3 ± 5.9</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P = 0.96</td>
<td>P = 0.98</td>
<td>P = 0.89</td>
<td>P = 0.69</td>
</tr>
</tbody>
</table>

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 two 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 and 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 and Figure 1).

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

Study 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).

Discussion

We have shown, in healthy humans, that BQ-123 causes substantial systemic vasodilatation, associated with a small but significant reduction in arterial blood pressure. 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 ETA receptor in controlling vascular tone and blood pressure (Haynes & Webb, 1998), and is in accord with the results of previous local forearm infusion studies (Haynes & Webb, 1994; Berrazuta et al., 1997; Verhaar et al., 1998).

We have several reasons for concluding that the effects on vascular tone and blood pressure are mediated by the ETA receptor. Measured BQ-123 concentrations in plasma at both 300 and 1000 nmol min⁻¹ were substantially greater than the IC₅₀ for BQ-123 at the ETA receptor. Even so, at 1000 nmol min⁻¹ the plasma concentration of 510 nmol l⁻¹, was more than 35 fold lower than the IC₅₀ for the ETB receptor (18 µM), consistent with effective but selective ETA receptor blockade (Ihara et al., 1992). In addition, there is a substantial body of evidence that the ETB 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 ETB receptor in vivo cause increases in plasma ET-1 concentrations (Haynes et al., 1992; 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 (Table 1A,B). Finally, we have previously shown that the net effect of systemic ETB receptor blockade is to cause systemic vasoconstriction, and therefore, we would anticipate that any ETB blockade would attenuate the vasodilatation associated with ETA 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 ETB receptor at this dose.

Doses sufficient to lower blood pressure (300 and 1000 nmol min⁻¹) also antagonized the forearm vasoconstriction to brachial artery administration of ET-1 and, in keeping with its submaximal effect on SVRI, the lower dose of BQ-123 (300 nmol min⁻¹) only partially antagonized forearm vasoconstriction to ET-1. Of note, however, in the presence of a higher degree of ETA blockade, exogenous ET-1 failed to produce vasodilatation. This possibly reflects the local balance of dilator and constrictor effects mediated by endothelial and vascular smooth muscle ETB receptors. However it is also possible that the locally administered ET-1 was washed out by an increase in forearm blood flow consequent upon the vasodilatation induced by systemic BQ-123. Comparison with a constrictor agent unaffected by ETA antagonism would be needed to clarify this further.

Previous studies in healthy men (Schmetterer et al., 1998; Montanari et al., 2000) failed to demonstrate a significant effect of BQ-123 on basal haemodynamics. However, the doses used were substantially lower, at 23.7 nmol min⁻¹ for 60 min, followed by 94.8 nmol min⁻¹ for 60 min (Schmetterer et al., 1998) and ~9 nmol min⁻¹ for 90 min (Montanari et al., 2000). These should be compared with 100 nmol min⁻¹ BQ-123 for 15 min as the threshold dose for a systemic haemodynamic effect in our studies. In addition, both other studies measured blood pressure but not systemic vascular resistance, 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 blood pressure, with both bosentan (mixed ETA/ETB) (Weber et al., 1996) and ABT-627 (ETA selective) (Verhaar et al., 2000).

In the latter study, although systemic haemodynamics were only recorded at 30 min and 8 h after dosing, systemic

Table 3 Haemodynamic changes after BA-123 administration

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>SVRI (L min⁻¹ m⁻²)</th>
<th>CI (L min⁻¹ m⁻²)</th>
<th>HR (b.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 (nmol min⁻¹)</td>
<td>-4.8±2.6%</td>
<td>-15.8±7.6%</td>
<td>14.3±8.9%</td>
<td>23.8±13.4%</td>
</tr>
<tr>
<td></td>
<td>(-4.0±2.5)</td>
<td>(-3.9±2.1)</td>
<td>(0.49±0.32)</td>
<td>(12.1±6.5)</td>
</tr>
<tr>
<td>300</td>
<td>-6.8±3.6%</td>
<td>-20.6±5.0%*</td>
<td>13.0±2.2%*</td>
<td>11.8±4.4%</td>
</tr>
<tr>
<td></td>
<td>(-5.4±2.8)</td>
<td>(-4.5±0.7)</td>
<td>(0.47±0.07)</td>
<td>(6.8±2.4)</td>
</tr>
<tr>
<td>1000</td>
<td>-8.2±3.1%</td>
<td>-22.7±5.2%*</td>
<td>17.9±5.7%*</td>
<td>20.0±5.9%*</td>
</tr>
<tr>
<td></td>
<td>(-6.4±2.4)</td>
<td>(-5.4±1.9)</td>
<td>(0.59±0.16)</td>
<td>(11.6±3.0)</td>
</tr>
<tr>
<td>3000</td>
<td>-12.4±3.5%</td>
<td>-23.3±4.3%*</td>
<td>12.7±1.6%*</td>
<td>18.7±6.8%*</td>
</tr>
<tr>
<td></td>
<td>(-10.1±3.2)</td>
<td>(-5.6±2.6)</td>
<td>(0.43±0.04)</td>
<td>(10.7±3.1)</td>
</tr>
</tbody>
</table>

Results given are maximum placebo corrected percentage change from baseline ± s.e.mean. *P<0.05 vs placebo, †P<0.01 vs placebo: ANOVA + Bonferroni correction.

Figure 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.
vascular resistance was measured and significant effects on this parameter were found in both acute and chronic dosing.

It should be noted that we have also previously failed to detect significant systemic effects, as measured by blood pressure and heart rate, at our lowest dose of BQ-123 (100 nmol min⁻¹) when administered into the brachial artery (Haynes & Webb, 1994). These current results suggest that 100 nmol min⁻¹ does have a small systemic action, most apparent in its effects on systemic vascular resistance, that was not detected in forearm studies, perhaps because the major vasodilatation is in other vascular beds. In recognition of this potential problem, we have more recently used a 10 fold lower dose of 10 nmol min⁻¹ BQ-123, (Verharr et al., 1998), as a local dose for forearm studies. The current study confirms the rationale for this approach.

It is also interesting to note that a 15 min infusion of BQ-123 produces haemodynamic effects for up to 4 h at the higher doses. Although we have plasma estimations of BQ-123 concentrations only at 0, 15 and 240 min in the current study, subsequent experiments, with identical dosing schedules of BQ-123, demonstrate that the peak concentration is achieved at the end of the infusion, falls to ~10% by 30 min and is undetectable by 75 min post infusion (unpublished data). This suggests that the observed responses are a pharmacodynamic effect rather that a reflection of the plasma half-life of BQ-123. This is similar to our experience with the non-selective ET antagonist TAK 044 where the systemic haemodynamic effects of a 15 min bolus were still observable at 24 h, whereas the peptide had a plasma half-life of 30–60 min (Haynes et al., 1996).

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 cardipulmonary 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 mean arterial pressure, no side effects were observed or reported by the subjects.

In conclusion, this study with BQ-123 demonstrates that systemic ETA receptor antagonism causes substantial peripheral vasodilatation and modest lowering of blood pressure, 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 ETA and mixed ETA/B receptor antagonists, which of the therapeutic approaches will offer the greater haemodynamic benefit in specific clinical indications.

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References


(Received March 23, 2000 Revised July 19, 2001 Accepted July 25, 2001)
Endothelin-A Receptor Antagonism Reduces Blood Pressure and Increases Renal Blood Flow in Hypertensive Patients With Chronic Renal Failure

A Comparison of Selective and Combined Endothelin Receptor Blockade

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Background—Endothelin (ET) is implicated in the pathophysiology of chronic renal failure (CRF). We therefore studied the systemic and renal hemodynamic effects of ET receptor antagonists in CRF and examined differences between selective ETA, selective ETB, and combined ETA/B receptor blockade.

Methods and Results—We conducted a randomized, placebo-controlled, double-blind, 4-way crossover study comparing selective ET receptor antagonists BQ-123 (ETA) and BQ-788 (ETB), given alone and in combination, in acute studies in 8 hypertensive CRF patients and 8 matched healthy controls. BQ-123, alone and in combination with BQ-788, reduced blood pressure in CRF, particularly with BQ-123 alone (mean arterial pressure: controls −4±2%, CRF −13±2%, P<0.01 versus placebo). In CRF, in the face of this fall in blood pressure, BQ-123 substantially increased renal blood flow (38.8±23.9%, P<0.01 versus placebo) and reduced renal vascular resistance (−44.5±11.3%, P<0.01 versus placebo) when given alone but not when combined with BQ-788. These changes were accompanied by a reduction in effective filtration fraction. BQ-123, alone or in combination with BQ-788, had minimal effects on the renal circulation in healthy controls, and BQ-788 alone produced both systemic and renal vasoconstriction in CRF and healthy controls.

Conclusions—ETA receptor antagonism was highly effective in lowering blood pressure in CRF patients currently treated for hypertension. In addition, there were effects consistent with a renoprotective action. However, because the ETB receptor appears to play a key role in the maintenance of tonic renal vasoconstriction, combined ETA/B receptor antagonism, although it lowered blood pressure, did not confer these renal benefits. (Circulation. 2004;109:1186-1193.)

Key Words: blood flow ■ blood pressure ■ endothelin ■ kidney ■ hemodynamics

The endothelins are powerful vasoconstrictor peptides, of which endothelin-1 (ET-1) is the major isofrom. It is produced by vascular endothelium and acts through two receptors in the blood vessels to modulate vascular tone. Endothelin-A (ETA) and endothelin-B (ETB) receptors are found in vascular smooth muscle, where their activation mediate vasoconstriction. ETB receptors are also found on vascular endothelium, where their activation promotes vasoconstriction through the release of nitric oxide and prostaglandins. Additionally, vascular ETB receptors act as a major source of ET-1 clearance from the circulation, and renal tubular ETB receptors may contribute to natriuresis. Studies in humans show the importance of ET-1 in the maintenance of vascular tone and blood pressure and suggest therapeutic potential for endothelin receptor antagonists in pathophysiological states characterized by vasoconstriction, such as essential hypertension and pulmonary arterial hypertension.

Chronic renal failure (CRF) is characterized by systemic and renal vasoconstriction and commonly associated with hypertension. Given that endothelin receptor antagonists cause vasodilation, lower blood pressure, and ameliorate renal dysfunction in experimental models of kidney disease, they may be useful in the treatment of CRF. The aim of the present study was to show whether endothelin receptor antagonists would produce beneficial systemic or renal hemodynamic effects in CRF. Because of the potential opposing effects at ETA and ETB receptors, we directly compared the effects of regimens involving ETA and ETB.
TABLE 1. Subject Demographic Data

<table>
<thead>
<tr>
<th></th>
<th>Healthy Volunteers</th>
<th>Renal Patients</th>
<th>P (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>47±5 (23-64)</td>
<td>46±5 (23-67)</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26±2 (18-31)</td>
<td>27±1 (24-33)</td>
<td>NS</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>92.8±3.1 (83.0-103.6)</td>
<td>98.8±3.5 (78.8-109.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>85±5 (62-111)</td>
<td>255±41 (122-434)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.3±0.3 (3.9-6.1)</td>
<td>5.6±0.2 (4.5-6.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary sodium excretion, mmol/24 h</td>
<td>136±14 (56-199)</td>
<td>150±14 (74-246)</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary protein excretion, mg/24 h</td>
<td>0*</td>
<td>476±110 (n=7; 27-2033)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are mean±SEM (range).
*Values below limit of laboratory assay.
†n=7; 1 value below limit of detection.

receptor blockade separately and in combination in CRF patients and matched healthy controls.

Methods

Subjects
We aimed to recruit 8 men with stable CRF and 8 matched healthy controls to the studies, which were performed in the University of Edinburgh’s Clinical Research Centre 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.

For 3 days before each study, subjects adhered to a standard diet containing 150 mmol of sodium. All subjects abstained from alcohol, nicotine, and caffeine-containing products for 24 hours and had a light breakfast before attending on each study day. All studies were performed in a quiet, temperature-controlled room at 22°C to 24°C, with the subject recumbent throughout, except when voiding urine.

Healthy subjects who had taken any medications in the previous 2 weeks were excluded from the study. Patients continued taking their normal medication up to and including each study day with the exception of diuretics, which they omitted that morning. To enhance homogeneity and avoid other influences on vascular reactivity, patients with vasculitis, other systemic inflammatory disease, poly cyclic kidney disease, nephrotic syndrome, or obstructive uropathy were excluded. Additionally, patients with significant comorbid disease, including diabetes mellitus, heart or lung disease, peripheral vascular disease, or hypercholesterolemia, were excluded. The 2 study groups were matched for age, weight, serum cholesterol, and blood pressure (Table 1).

Drugs
BQ-123 (Cinilafa AG), a selective ETA receptor antagonist, was infused at 100 and 1000 mmol/min for 15 minutes at each dose. These doses were selected from a previous study as having a threshold and maximum hemodynamic effect in healthy controls. BQ-788 (Cinilafa), a selective ETB receptor antagonist, was infused at 30 and 300 mmol/min for 15 minutes, doses shown to be hemodynamically active in a previous systemic dose-ranging study. Drugs were dissolved in physiological saline (0.9%; Baxter Healthcare Ltd) and infused intravenously at a constant rate of 1 mL/min. Saline was administered as placebo.

Para-aminohippurate sodium (PAH; Cinilafa) and inutest (Fresenius Pharma, Austria GmbH) were dissolved in dextrose 5% (Baxter) and administered as a bolus loading dose of 0.4 g of PAH and 3.5 g of inutest in 100 mL of dextrose over 15 minutes and a maintenance infusion of 6.6 g/L PAH and 10 g/L inutest at a rate of 2 mL/min. For subjects with a calculated glomerular filtration rate (GFR) <40 mL/min, doses of PAH and inutest were reduced by one third.

Assays
At prespecified time points, venous blood was collected into EDTA tubes (Sarstedt) for measurement of PAH, inulin, and hematocrit (Hct), plasma ET-1, plasma renin activity, angiotensin II, and aldosterone, and into plain tubes (Sarstedt) for the measurement of serum sodium. Additionally, 20 mL aliquots of urine from each voiding were collected into plain tubes for the measurement of urinary PAH, inulin, sodium, and protein.

Hct was measured on whole blood with a Coulter counter. All other blood samples were centrifuged immediately at 1000g at 4°C for 20 minutes, and plasma and urine were stored in plain tubes at −80°C. Inulin was determined by spectrophotometry after hydrolysis to fructose, and PAH and BQ-123 were determined by high-performance liquid chromatography. BQ-788 assay was not sufficiently sensitive for its detection in plasma. Urinary and plasma sodium concentrations were measured by flame photometry. ET-1, angiotensin II, plasma renin activity, and aldosterone were determined by radiioimmunoassay.

Study Protocol
This was a randomized, double-blind, placebo-controlled study. Subjects attended for 4 visits, receiving placebo, BQ-123, BQ-788, or the combination of BQ-123 and BQ-788. Because previous studies with the same doses of BQ-123 or BQ-788 have demonstrated that hemodynamic changes return to baseline after 4 hours, each visit was separated by ≥7 days to ensure complete washout of the study drugs. An individual otherwise unconnected with the study prepared the drugs to maintain blinding. On each study day, an 18 standard wire gauge cannula was sited in an antecubital vein in each arm. Diuresis was induced by 500 mL of 5% dextrose over 30 minutes through the left arm cannula. After 15 minutes, loading doses of PAH and inutest were administered through the same cannula. Thereafter, maintenance infusions of PAH and inutest and 5% dextrose at 260 mL/h continued throughout the study. Blood pressure, cardiac output and heart rate were recorded by validated noninvasive automated techniques every 15 minutes, and urine was collected every 30 minutes by spontaneous voiding. After a 2-hour equilibration period, baseline measurements were made over 2 30-minute urine-collection periods. The low dose of antagonist was then administered through the right antecubital cannula, followed by 30-minute collection periods. The higher dose of antagonist was then administered, followed by 5 further 30-minute collection periods.

At the midpoint of each collection period, blood was sampled from the right antecubital cannula for PAH, inulin, sodium, and Hct. At 0, 60, and 90 minutes after the start of low- and high-dose antagonist and at the end of the study, additional samples were taken for plasma hormone measurements. BQ-123 was measured before and at 15, 45, and 90 minutes after the start of each dose of antagonist and at the end of the study.
Data Analysis

Data were stored and analyzed with Microsoft Excel (version 5.0, Microsoft Ltd). Demographic data are expressed as mean ± SEM, and comparisons between groups were examined by unpaired Student's t tests. Blood pressure at each time point was calculated as the mean of 2 recordings and represented as mean arterial pressure (MAP = diastolic blood pressure + 1/3 pulse pressure). Bioimpedance data at each time point were calculated as the mean of 4 recordings, each the average of 15 consecutive heart beats. Data were corrected with body surface area to give cardiac index for direct comparison of subjects. Systemic vascular resistance index (SVRI) was calculated by dividing MAP by cardiac index and expressed in dyne · s · m⁻² · cm⁻⁵. GFR and effective renal plasma flow (ERPF) were calculated from inulin and PAH clearances, respectively. The effective renal blood flow (ERBF) was calculated by dividing ERPF by (1 + Hct), effective renal vascular resistance (ERVR) by dividing MAP by ERBF, and effective filtration fraction (EFF) by dividing GFR by ERPF×100%. Urinary sodium excretion was calculated as urinary sodium excretion divided by plasma sodium × plasma inulin × urinary flow rate and fractional excretion as urinary sodium excretion divided by plasma sodium × plasma inulin.

Baseline data were calculated as the mean of the 2 time points that immediately preceded administration of the first study drug. Hemodynamic results are expressed as mean ± SEM placebo-corrected maximum change from baseline. Statistical analysis was performed on untransformed data. Four comparisons of interest were pre-defined as placebo versus BQ-123, versus BQ-788, and versus BQ-123+788, and BQ-123 versus BQ-123+788. Responses were examined by repeated-measures ANOVA, and Bonferroni correction was used to assess significance at specific time points. Statistical significance was taken at the 5% level.

Results

Twelve renal patients were recruited; 1 developed nausea after receiving BQ-123, and 1 was unable to void urine at 30-minute intervals. Two withdrew for reasons unrelated to the study. Eight patients and all healthy controls completed all phases of the study without adverse events. The renal diagnosis and concomitant medication taken by the patients who completed the study are given in Table 2. Baseline parameters are shown in Table 3.

Systemic Hemodynamics

In CRF patients, placebo was associated with increases in SVRI to study end (3478±240 versus 3797±301 dyne · s · m⁻² · cm⁻⁵, P<0.05) and MAP (100.7±3.8 versus 108.0±4.1 mm Hg, P<0.01), consistent with the waning effects of antihypertensive medication (Figures 1 and 2).

<table>
<thead>
<tr>
<th>TABLE 2. Renal Patients: Diagnosis and Medications</th>
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<tbody>
<tr>
<td>Subject</td>
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HSP indicates Henoch-Schönlein purpura; GN, glomerulonephritis.

<table>
<thead>
<tr>
<th>TABLE 3. Baseline Data</th>
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<tbody>
<tr>
<td>Healthy Volunteers</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
</tr>
<tr>
<td>SVRI, dyne · s · m⁻² · cm⁻⁵</td>
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<tr>
<td>Cardiac index, L · min⁻¹ · m⁻²</td>
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<tr>
<td>Heart rate, bpm</td>
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<tr>
<td>ERBF, mL/min</td>
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<tr>
<td>ERVR, mm Hg · min⁻¹ · L⁻¹</td>
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<tr>
<td>EFF, %</td>
</tr>
<tr>
<td>GFR, mL · min⁻¹ · L⁻¹ · 1.73 m⁻²</td>
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<tr>
<td>UNaV, µmol/min</td>
</tr>
<tr>
<td>Urinary protein, mg/L</td>
</tr>
<tr>
<td>Plasma ET-1, pg/mL</td>
</tr>
<tr>
<td>PRA, pg · mL⁻¹ · h⁻¹</td>
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<tr>
<td>Plasma ANG II, pg/mL</td>
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<td>Plasma aldosterone, pg/mL</td>
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</tbody>
</table>

NS indicates not significant; UNaV, urinary sodium excretion; PRA, plasma renin activity; and ANG II, angiotensin II.

Values are given as mean of 2 baseline periods over the 4 study days ± SEM (range).

*Values below limit of laboratory assay.

n=7; † value below limit of detection.
SVRI was reduced to a similar extent by BQ-123 and BQ-123/788 (BQ-123 -630±145 dyne · s · m⁻² · cm⁻⁵, BQ-123/788 -617±158 dyne · s · m⁻² · cm⁻⁵, both P<0.01 versus placebo). MAP was reduced in CRF patients after BQ-123/788 (-7.4±1.6 mm Hg, P<0.01 versus placebo) but to a greater extent after BQ-123 alone (BQ-123 -12.9±1.7 mm Hg, P<0.01 versus placebo and BQ-123/788). Systemic hemodynamic responses to endothelin receptor antagonism showed no correlation with baseline blood pressure.

In healthy controls, placebo did not alter systemic hemodynamics. Both BQ-123 and BQ-123/788 reduced SVRI to a similar extent, equivalent to that seen in CRF (BQ-123 -591±104 dyne · s · m⁻² · cm⁻⁵, BQ-123/788 -498±159 dyne · s · m⁻² · cm⁻⁵, both P<0.01 versus placebo). The reductions in MAP after BQ-123 and BQ-123/788 were equal (BQ-123 -3.6±1.4 mm Hg, BQ-123/788 -3.7±1.6 mm Hg, both P<0.01 versus placebo) and less than those seen in CRF (P<0.05). BQ-788 increased MAP (CRF 7.0±1.6 mm Hg, healthy controls 5.8±1.5 mm Hg, both P<0.01 versus placebo) and SVRI (CRF 454±114 dyne · s · m⁻² · cm⁻⁵, P<0.01 versus placebo; healthy controls 390±76 dyne · s⁻¹ · m⁻² · cm⁻⁵, P<0.05 versus placebo) to a similar extent in CRF patients and controls.

Renal Hemodynamics
In CRF patients, BQ-123 but not BQ-123/788 produced striking increases in ERBF and reductions in ERVR and EFF (ERBF 102±74 mL/min, ERVR -243±91 mm Hg · min · L⁻¹, EFF -4.2±2.9%; all P<0.01 versus placebo and BQ-123/788). GFR did not change. By contrast, in healthy controls, BQ-123 and BQ-123/788 were neutral with respect to ERBF, ERVR, EFF, and GFR. (See Figures 2 and 3.)

In both groups, BQ-788 reduced ERBF (CRF -77±72 mL/min, healthy controls -134±47 mL/min; both P<0.05 versus placebo) and increased ERVR (CRF 112±63 mm Hg · min · L⁻¹, healthy controls 39±12 mm Hg · min · L⁻¹; both P<0.05 versus placebo). These changes were apparent even
at the low dose and were associated with a reduction in GFR and an increase in EFF.

**Urinary Sodium Excretion and Protein Excretion**

No changes in sodium excretion or fractional excretion were observed in either CRF or healthy controls. Urinary protein excretion was undetectable for 1 CRF subject and all healthy controls. In the remaining 7 CRF patients, with measurements uncorrected for changes in GFR, BQ-788 produced a small increase in urinary protein excretion ($P<0.01$ versus placebo), but no changes were observed after BQ-123 or BQ-123/788 (Figure 4). After correction for GFR, BQ-788 and BQ-123/788 did not affect proteinuria, but BQ-123 reduced protein leak by 46% ($-8.1\pm4.9\,\mu g/min$; ANOVA $P<0.01$ versus placebo, $P<0.05$ versus BQ-123/788), an effect most apparent in subjects with higher baseline urinary protein excretion.

**Plasma Hormone and BQ-123 Concentrations**

Plasma ET-1 increased to a similar extent after high dose BQ-788 and BQ-123/788 but was unaltered by placebo or BQ-123 (Figure 5). Other hormones were unaffected by endothelin receptor antagonism. BQ-123 was detectable in plasma at 15 minutes after low-dose BQ-123 and at 15 and 45 minutes after high-dose BQ-123 and did not differ between CRF and healthy controls (CRF $1.52\pm0.36\,pg/mL$, healthy controls $1.21\pm0.17\,pg/mL$ at 15 minutes after high dose). BQ-123 concentrations were unaffected by coadministration of BQ-788 (CRF $1.62\pm0.20\,pg/mL$, healthy controls $1.26\pm0.20\,pg/mL$).

**Discussion**

This is the first clinical study to directly compare ETA, ETB, and combined ETA and ETB receptor antagonism at systemic doses in humans. We have shown in CRF patients that selective ETA receptor antagonism produces substantial re-
Figures 3. Renal hemodynamics after endothelin receptor antagonist. Values are given as mean placebo-corrected % change from baseline ±SEM. Black blocks, Administration of endothelin antagonist/placebo; red diamonds, BQ-123; green circles, BQ-123/788; and blue triangles, BQ-788. *P<0.01 vs placebo; **P<0.05 vs BQ-123 (ANOVA + Bonferroni correction for significance at specific time points).

These data confirm the physiological importance of ET-1, through activation of the ETA receptor, in the maintenance of basal systemic but not renal vascular resistance. They also show that ET-1 plays a major role in regulating blood pressure and renal vascular resistance in CRF, consistent with activation of the endothelin system in this condition. The reduction in filtration fraction after ETA receptor antagonism suggests an effect primarily on efferent arteriolar tone, which may serve to reduce glomerular pressure. Consistent with our observations, an ETA receptor-selective antagonist has been shown to reduce proteinuria in type 1 diabetes.

The effects of BQ-788, whether in the presence of BQ-123 or not, suggest that the net effect of ETB receptor activation on the circulation in health and renal disease is to produce vasodilation. Therefore, similar to observations in healthy subjects and patients with heart failure, any enhanced effects of constrictor ETB receptors that potentially exist in CRF are outweighed by ETB receptor-mediated vasodilation. Of particular interest, ETB receptor antagonism increased renal vascular resistance twice as much as systemic vascular resistance, which suggests that tonic ETB receptor-mediated renal vasodilation plays a key role in maintenance of renal vascular tone. This is likely to be of particular importance in CRF, in which baseline renal vascular resistance is high, and suggests ETA receptor antagonism and not dual ETA/B
blockade might be the best approach in renal patients. However, based on earlier work, the present study was designed to achieve effective and selective blockade of the ETA and ETB receptor and not specifically to reproduce the effects of existing endothelin receptor antagonists, all of which block ETA to a greater extent than ETB receptors. Therefore, although the main effects of therapeutic interest in the present study appear to derive from ETA receptor blockade and are countered by ETB blockade, drugs that cause only modest ETB receptor inhibition might produce similar effects.

Perhaps surprisingly, given the evidence for ETB receptor-mediated natriuresis shown in animal studies, no changes in sodium excretion or fractional excretion were observed in the present study. However, during ETA receptor blockade, despite substantial systemic and renal vasodilation, sodium retention did not occur, which is important if these drugs are to be prescribed safely to patients with CRF.

As a limitation, the present studies were acute studies. We also studied a relatively homogeneous CRF population. Therefore, longer-term studies are now needed in a broader population of patients with kidney disease, including those with low renal perfusion pressure. In particular, because ACE inhibitors are most effective as renoprotective agents in patients with proteinuria >3 g per 24 hours, studies are needed in patients with higher degrees of urinary protein leak to address what may be a major benefit of these agents. Importantly, ethical considerations dictated that patients con-
Webb was Global Research & disease. As with CRF additional improve endothelial function, patients with CRF patients and so are unlikely to account for any differences in renal responses to endothelin antagonists between patients and controls.

In conclusion, we have shown that selective ETA receptor antagonists may be valuable antihypertensive drugs in patients with CRF as well as offering additional benefits, including renoprotection. Endothelin receptor antagonists also improve endothelial function, reduce inflammation and fibrosis, and reverse vascular remodeling and so may offer additional benefits to renal patients, who are at high cardiovascular risk. On this basis, longer-term studies in patients with CRF are justified, with particular attention to effects on proteinuria as a surrogate marker for the progression of renal disease.

Acknowledgments

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References

Endothelin A Receptor Antagonism and Angiotensin-Converting Enzyme Inhibition Are Synergistic via an Endothelin B Receptor–Mediated and Nitric Oxide–Dependent Mechanism

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*Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom; and †Pfizer Global Research & Development, Sandwich, United Kingdom

Abstract. Animal studies suggest that endothelin A (ETA) receptor antagonism and angiotensin-converting enzyme (ACE) inhibition may be synergistic. This interaction and the role of ETB receptors and endothelial mediators were investigated in terms of systemic and renal effects in humans in two studies. In one study, six subjects received placebo, the ETA receptor antagonist BQ-123 alone, and BQ-123 in combination with the ETB receptor antagonist BQ-788 after pretreatment with the ACE inhibitor enalapril (E) or placebo. In the other, six subjects who were pretreated with E received placebo, BQ-123, and BQ-123 with concomitant inhibition of nitric oxide (NO) synthase or cyclo-oxygenase (COX). Both were randomized, double-blind, crossover studies. Mean arterial pressure was reduced by BQ-123, an effect that was doubled during ACE inhibition (mean area under curve ± SEM; BQ-123, −2.3 ± 1.8%; BQ-123+E, −5.1 ± 1.1%; P < 0.05 versus placebo). BQ-123 increased effective renal blood flow (BQ-123, −0.1 ± 2.4%; BQ-123+E, 10.9 ± 4.2%; P < 0.01 versus BQ-123), reduced effective renal vascular resistance (BQ-123, −1.2 ± 3.1%; BQ-123+E, −12.8 ± 3.0%; P < 0.01 versus placebo and versus BQ-123), and increased urinary sodium excretion markedly (BQ-123, 2.6 ± 12.8%; BQ-123+E, 25.2 ± 12.6%; P < 0.05 versus BQ-123, P < 0.01 versus placebo and versus E) only during ACE inhibition. These effects were abolished by both ETB receptor blockade and NO synthase inhibition, whereas COX inhibition had no effect. In conclusion, the combination of ETA receptor antagonism and ACE inhibition synergistic via an ETB receptor–mediated, NO-dependent, COX-independent mechanism. The reduction of BP and renal vascular resistance and associated substantial natriuresis makes this a potentially attractive therapeutic combination in renal disease.

Endothelin-1 (ET-1) is a vasoactive peptide that is produced by the vascular endothelium (1) and acts through two receptors to regulate vascular tone. Vascular smooth muscle ETA and ETB receptors (2,3) mediate vasoconstriction, whereas endothelial ETB receptors mediate vasodilation through generation of nitric oxide (NO) and prostanooids (3). Angiotensin II (Ang II) is another powerful vasoconstrictor involved in the regulation of vascular tone, and there is considerable evidence for an interaction between the endothelin and renin-angiotensin systems (4). Ang II increases ET-1 transcription and secretion in vitro in a variety of cell types, including endothelial and vascular smooth muscle cells (5,6), and ET receptor antagonists attenuate the acute hemodynamic effects of Ang II in rats in vivo (7,8). However, this is by no means a uniform finding (9) and has not been replicated in dogs (10) or humans (11). Data in animals suggest that concomitant ET blockade and angiotensin-converting enzyme (ACE) inhibition produce changes greater than those seen with blockade of either system alone (12–15). In addition, clinical studies that demonstrated major hemodynamic effects of ET receptor antagonists have generally been performed in patients who were already receiving ACE inhibitors (16,17).

ACE inhibition, by promoting the effects of bradykinin (18), and ET-1 acting on endothelial ETB receptors (19) both enhance endothelium-dependent vasodilation. Vascular studies in humans suggest that the vasodilator effects of ETA receptor antagonism are dependent on the unblocked ETB receptor and NO generation (20). The aim of this study, therefore, was to explore the possible systemic and renal interaction between ET receptor antagonism and ACE inhibition and the mechanisms by which such an interaction might occur. We hypothesized that previous ACE inhibition would augment the systemic and renal hemodynamic response to ETA receptor antagonism and that this interaction would be dependent on NO production and mediated, at least in part, through the ETB receptor.
Materials and Methods
A total of eight healthy men (six in each protocol) were recruited to the studies, which were performed in the University of Edinburgh’s Clinical Research Centre with the approval of the local research ethics committee and the written informed consent of each participant. The investigations conformed to the principles outlined in the Declaration of Helsinki.

For 3 d before each study, participants adhered to a standard diet that contained 150 mmol of sodium. All participants abstained from over-the-counter medication for 2 wk, alcohol, nicotine, and caffeine-containing products for 24 h and had a light breakfast before attending on each study day. All studies were carried out in a quiet, temperature-controlled room, at 22 to 24°C, with the participant recumbent throughout, except when voiding urine.

Drugs
BQ-123 (Clinalfa AG, Laufelfingen, Switzerland), a selective ETA receptor antagonist (21), was infused at 100 and 1000 nmol/min for 15 min at each dose. These doses were selected from a previous study as having a threshold and maximum hemodynamic effect in healthy control subjects (22). The higher dose produces a peak plasma concentration of BQ-123 (22) 7-fold higher than the Kᵢ for ETA receptor inhibition and 20-fold lower than the Kᵢ for ETB receptor inhibition (21), confirming ETA selectivity, and did not increase plasma ET-1, an index of ETB receptor blockade (23). BQ-788 (Clinalfa AG), a selective ETB receptor antagonist (24), was infused at 30 and 300 nmol/min for 15 min, doses shown to be hemodynamically active in a previous systemic dose-ranging study (25). Drugs were dissolved in physiologic saline (0.9%; Baxter Healthcare Ltd., Thetford, UK) and infused intravenously at a constant rate of 1 ml/min via an 18 standard wire gauge (SWG) cannula sited in the right antecubital fossa. Saline was administered as placebo.

Enalapril (Dexcel Pharma Ltd, Daventry, UK) was the chosen ACE inhibitor and was administered orally for 5 d before each visit at a dose of 20 mg twice daily, taking the final dose at 8:30 a.m. on each study day. This dose was chosen from data suggesting that it would achieve significant inhibition of serum ACE activity (26,27), with steady-state plasma concentrations reached by 3 to 4 d (28). With this dosing regimen, the maximal effect of enalapril occurs 2 to 3 h after administration and persists for 5 to 7 h (27,29). By administering a final dose 2.5 h before baseline measurements, therefore, we aimed to achieve maximal ACE inhibition throughout each study visit.

The nonselective cyclo-oxygenase (COX) inhibitor indomethacin was administered as a single 100-mg oral dose at 8:30 a.m. on the relevant study day to reach peak plasma concentrations by the time of baseline measurements. The dose is known to inhibit prostaglandin production over the time course of the study, as evidenced by decreased urinary excretion rates of prostaglandins (30). N⁰-monomethyl-L-arginine (L-NMMA; Clinalfa AG, Laufelfingen, Switzerland) was administered at a dose of 3 mg/kg over 5 min as an NO synthase inhibitor, with each dose of BQ-123, during the relevant study day. This dose is sufficient to cause a small transient increase in BP (by ~7 to 10%) and decrease renal blood flow (by ~10%) (31). Thereafter, BP returns to baseline by 30 min and renal blood flow by 60 min, with a plasma half-life of 75 min (31).

Para-aminophenpirpure sodium (PAH; Clinalfa AG) and inutest (Freinenski Pharma, Austria) were dissolved in dextrose 5% (Baxter Healthcare) and administered as a bolus loading dose of 0.4 g of PAH and 3.5 g of inutest in 100 ml of dextrose over 15 min and a maintenance infusion of 6.6 g/L PAH and 10 g/L inutest at a rate of 2 ml/min via an 18SWG cannula in the left antecubital fossa. All drugs were prepared from sterile stock solutions on the day of the study.

Assays
At prespecified time points, venous blood was collected into tubes that contained EDTA (Sarstedt, Newton, CA) for the measurement of PAH, inulin, and hematocrit (Hct) and into plain tubes (Sarstedt) for the measurement of plasma sodium. Twenty-milliliter aliquots of urine from each voiding were collected into plain tubes for the measurement of urinary PAH, inulin, and sodium.

Hct was measured on whole blood using a Coulter counter. All other blood samples were centrifuged immediately at 1000 × g at 4°C for 20 min, and plasma and urine were stored in plain tubes at −20°C. Inulin concentrations were determined by spectrophotometry after hydrolysis to fructose, and PAH and BQ-123 (22) were determined by HPLC. BQ-788 assay was not sufficiently sensitive for its detection in plasma. Urinary and plasma sodium concentrations were determined using standard flame photometry. Subaliquots of plasma were used to measure serum ACE activity by generation of Ang II from Ang I (32).

Study Design
Both studies were double blind and placebo controlled. Visits were separated by at least 1 wk. The first study was designed to examine the interaction between ET receptor antagonism and ACE inhibition, and the second was designed to examine the effect of NO synthase inhibition and inhibition of prostaglandin synthesis on this interaction.

Study 1. Participants attended a total of six visits. On two sets of three visits, they received placebo, BQ-123, or the combination of BQ-123 and BQ-788 in a randomized order. With one set, they received pretreatment with enalapril and the other placebo.

Study 2. Participants attended a total of four visits. On each visit, they received pretreatment with enalapril. During the study day, they then received placebo, BQ-123, BQ-123 + indomethacin, or BQ-123 + L-NMMA in a randomized order.

Study Protocol
On each study day, 18-SWG cannulae were sited in an antecubital vein in each arm. At 8:30 a.m., diuresis was induced by the administration of 500 ml of 5% dextrose over 30 min through the cannula in the left arm. The loading dose of PAH and inutest was administered through the same cannula from 8:45 a.m. Thereafter, maintenance infusions of PAH and inutest, and 5% dextrose at 260 ml/h, continued throughout the study. BP, cardiac output, and heart rate were recorded using well-validated noninvasive automated techniques every 15 min (33,34), and urine was collected every 30 min by spontaneous voiding. After a 2-h equilibration period, baseline measurements were made over two 30-min urine collection periods. The lower dose of ET receptor antagonist was then administered at 12:00 p.m. through the right antecubital cannula, followed by three 30-min collection periods. At 1:30 p.m., the higher dose of antagonist was administered followed by five additional 30-min collection periods.

At the midpoint of each urine collection period, blood was sampled from the right antecubital cannula for the measurement of PAH, inulin, sodium, and Hct. BQ-123 was measured before and at 15, 45, and 90 min after the start of each dose of antagonist and at the end of the study, and serum ACE activity was determined at 8:30 a.m. and at 2:30 p.m. (60 min after the start of the higher dose of antagonist).

Statistical Analyses
Data were stored and analyzed using a Microsoft Excel data analysis package (Excel 5.0, Microsoft, Wokingham, UK). Demographic
data are expressed as mean ± SEM. BP at each time point was calculated as the mean of two recordings and represented as mean arterial pressure (MAP, = diastolic BP + 1/3 pulse pressure). Bioimpedance data at each time point were calculated as the mean of four recordings, each the average of 15 consecutive heart beats. Data were corrected using body surface area to give cardiac index for direct comparison of participants. Systemic vascular resistance index (SVRI) was calculated by dividing MAP by cardiac index and expressed in dynes m²/cm⁵. GFR and effective renal plasma flow (ERPF) were calculated from inulin and PAH clearances, respectively (35). Effective renal blood flow (ERBF) was calculated by dividing ERPF by (1 − Hct); effective renal vascular resistance (ERVR) was calculated by dividing MAP by ERBF. Effective filtration fraction (EFF) was calculated as GFR divided by ERPF × 100%. Urinary sodium excretion rate was calculated as urinary sodium × urinary flow rate; and fractional excretion of sodium (FeNa) was calculated as urinary sodium × plasma inulin, divided by plasma sodium × urine inulin.

Study baseline data were calculated as the mean of the two time points immediately preceding the administration of the first study drug. Hemodynamic results are expressed as maximum placebo-corrected change from baseline (mean ± SEM). Statistical analysis was performed on untransformed data, and responses were examined by repeated measures ANOVA. In addition, area under the curve was calculated as a summary statistic of each time curve, and responses were compared by paired t test. Statistical significance was taken at the 5% level. Power calculations from previous studies (22,36) suggested that, with n = 6 participants, the studies had 80% power to achieve statistical significance for systemic hemodynamic indices. In study 1, the response to BQ-123 and BQ-123/788 in the presence or absence of enalapril was preidentified as the comparison of interest. In study 2, comparison of BQ-123 + enalapril with the other three drug combinations individually was preidentified as the comparison of interest.

Results
Six participants were recruited to study 1, and seven were recruited to study 2. Participants completed all phases of study 1 without adverse event. During study 2, one participant experienced an increase in MAP of 50 mmHg after indomethacin administration and before receiving BQ-123 and was withdrawn from the study. Baseline characteristics for the eight participants who completed the studies (four of whom were common to both studies) are shown in Table 1.

Study 1: Interaction between ET Receptor Antagonism and ACE Inhibition
Systemic and Renal Hemodynamics. Administration of placebo, either alone or after pretreatment with enalapril, did not alter systemic hemodynamics. MAP was reduced after both BQ-123 by 4.2 ± 1.6 mmHg (ANOVA P < 0.05 versus placebo) and BQ-123/788 by 4.4 ± 2.0 mmHg (Figure 1A). After pretreatment with enalapril, the BP reduction after BQ-123 was almost doubled, with MAP falling by 8.3 ± 3.0 mmHg (P < 0.01 versus BQ-123 alone). However, this synergy was not seen when BQ-788 was co-administered with BQ-123, when MAP was reduced by 4.3 ± 3.3 mmHg. SVRI followed a similar pattern (Figure 1B).

Placebo, enalapril alone, BQ-123, and BQ-123/788 all were neutral in respect of ERBF, ERVR, EFF, and GFR. After pretreatment with enalapril, BQ-123 increased ERBF by 146 ± 41 ml/min (P < 0.01 versus placebo and versus BQ-123 alone), reduced ERVR by 32 ± 15 mmHg/min per L (P < 0.01 versus placebo and versus BQ-123 alone), and reduced EFF by 3.4 ± 2.5% (P < 0.05 versus placebo, P < 0.01 versus BQ-123 alone; Figure 2). By contrast, after pretreatment with enalapril, BQ-123/788 reduced ERBF by 224 ± 31 ml/min (P < 0.01 versus placebo and versus BQ-123/788 alone) and increased ERVR by 26 ± 6 mmHg/min per L (P < 0.05 versus BQ-123/788 alone).

Sodium Excretion. No significant natriuresis was observed after placebo, enalapril alone, and either BQ-123 or BQ-123/788 alone. However, after pretreatment with enalapril, BQ-123 produced a striking increase in urinary sodium excretion with a maximum excretion rate of 58 ± 27 μmol/min (P < 0.01 versus placebo and BQ-123 alone). FeNa followed a similar pattern. As with renal hemodynamics, this increase was not observed with BQ-123/788 (Figure 3).

Serum ACE Activity. Compared with placebo, pretreatment with enalapril reduced serum ACE activity by 75% at baseline (36.4 ± 4.3 versus 9.1 ± 2.3 U; P < 0.01) and by 79% at 150 min (26.5 ± 2.1 versus 5.4 ± 1.7 U; P < 0.01). ET receptor antagonist administration did not alter ACE activity.

Plasma BQ-123. BQ-123 was detectable in plasma at 15 min after the start of the low-dose and at 15 and 45 min after the start of the high-dose infusion. Peak BQ-123 concentrations tended to be higher after pretreatment with enalapril, although the difference was not statistically significant (BQ-123, 1.26 ± 0.18 pg/ml; BQ-123/788, 1.32 ± 0.23; BQ-123 + enalapril, 2.06 ± 0.65; BQ-123/788 + enalapril, 2.32 ± 0.53).

Study 2: Effect of NO Synthase Inhibition and Inhibition of Prostaglandin Synthesis on Combined ETA Receptor Antagonism and ACE Inhibition
Systemic and Renal Hemodynamics. MAP was reduced by 6.0 ± 1.7 mmHg after BQ-123 in the presence of enalapril. This effect was augmented by indomethacin, with MAP falling by 11.0 ± 2.3 mmHg at the end of the study (P < 0.01 versus BQ-123; Figure 4) and abolished by L-NMMA (P < 0.01)

Table 1. Participant demographic data

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>47 ± 5 (23–64)</th>
</tr>
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<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 2 (18–31)</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>88.5 ± 2.7 (71.4–99.4)</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.01 ± 0.08 (0.70–1.35)</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>96 ± 7 (70–132)</td>
</tr>
<tr>
<td>24-h sodium excretion (mEq/24 h)</td>
<td>118 ± 15 (64–185)</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>201 ± 12 (151–236)</td>
</tr>
</tbody>
</table>

a Values are given as mean ± SEM with the range of values given in parentheses.
b To convert to μmol/L, multiply by 88.4.
c To convert to mmol/L, multiply by 0.0259.
versus BQ-123). Again, SVRI followed a similar pattern (Figure 4). Indomethacin did not affect the renovascular changes induced by BQ-123 + enalapril, but L-NMMA abolished the effects of BQ-123 + enalapril on both ERBF and ERVR (P < 0.01) (Figure 4).

Sodium Excretion. The natriuresis produced by the combination of BQ-123 and enalapril was attenuated by indomethacin (P < 0.01 versus BQ-123 + enalapril) and abolished by L-NMMA (P < 0.01 versus BQ-123 + enalapril) (Figure 4).

Discussion

In these studies, we demonstrated a synergy between the effects of ACE inhibition and ETA receptor antagonism in humans, affecting both systemic and renal hemodynamics, as well as renal tubular function. This synergy is abolished by either ETB receptor blockade or NO synthase inhibition but not COX inhibition. We conclude that ETA receptor antagonism and ACE inhibition act synergistically through an ETB receptor-mediated, NO-dependent, and COX-independent mechanism.

The ET and renin-angiotensin systems are known to interact (4), and an in vivo synergistic effect between ETA receptor antagonism and ACE inhibition has been demonstrated in animals (12) and between ET receptor antagonism and angiotensin AT1 receptor antagonism in humans (37). We have shown, in healthy men who were subjected to systemic ACE inhibition with a clinically relevant dose of enalapril, that the effects of ETA receptor blockade on systemic hemodynamics are enhanced. With respect to BP, ACE inhibition almost doubled the hypotensive effect of ETA receptor blockade. In addition, in contrast to the absence of an effect of ETA receptor antagonism alone on the renal circulation, the combination with ACE inhibition increased renal perfusion, in association with a fall in EFF. Thus, by inference, this combination causes preferential efferent renal arteriolar vasodilatation that should be associated with a fall in glomerular capillary pressure. Such changes in glomerular hemodynamics have the potential to be renoprotective in a manner analogous to the effect of ACE inhibition alone. In addition, this combination produced a striking natriuresis that was still developing to the end of the study, 4 h after initial administration of BQ-123. At its maximum, this natriuresis measured 10 mmol/h, a clinically important degree of sodium loss.

Our results also demonstrate that although maximal hemodynamic changes occurred after the higher dose of BQ-123, potentially useful systemic and renal hemodynamic changes were achieved after a lower dose of BQ-123 in the presence of ACE inhibition. As adverse effects in clinical trials with ET receptor antagonists seem to be largely dose related (38), this
Figure 2. (A) Effects of ET receptor antagonism and ACE inhibition on effective renal blood flow. Legend as for Figure 1A. (B) Effects of ET receptor antagonism and ACE inhibition on effective renal vascular resistance. Legend as for Figure 1A. (C) Effects of ET receptor antagonism and ACE inhibition on effective filtration fraction. Legend as for Figure 1A.
Combination might allow the use of a lower and better tolerated dose of ETA receptor antagonist without compromising efficacy.

Montanari et al. (37) demonstrated that ETA receptor antagonism can produce renal hemodynamic changes under conditions of angiotensin AT1 receptor blockade and that this action is inhibited by NO synthase inhibition. We demonstrated a similar synergy between ETA receptor antagonism and ACE inhibition, again dependent on NO, but extended this work by showing that this synergistic effect is abolished, for all indices studied, when the ETB receptor is blocked, suggesting that the ETB receptor is crucial to the mechanism of this interaction. As both L-NMMA and BQ-788 are vasoconstrictors, it is possible that their ability to abolish the vasodilator effect of enalapril and BQ-123 is nonspecific. However, indo- methacin has also been shown to produce renal vasoconstriction in healthy subjects (39) but had no effect on the renal vasodilation seen in this study after enalapril and BQ-123. Similarly, previous studies in healthy volunteers have demonstrated that although this dose of BQ-788 alone produces systemic and renal vasoconstriction, it does not abolish the vasodilator effects of BQ-123 (36), suggesting that the effect seen here is specific to the interaction between ACE inhibition and ETA receptor antagonism. In an experimental rat model of interstitial renal fibrosis, enalapril treatment was shown to increase ETB mRNA expression (40), providing a possible mechanism for this ET-1/ACE interaction. It is possible that ETA receptor antagonism then results in displacement of endogenous ET-1 from the ETA receptor onto the unblocked, upregulated ETB receptor.
These studies were performed in subjects in a salt-replete state (mean 24-h urine excretion, 119 ± 6 mmol). A recent study suggested that ET-1 plays a role in angiotensin-dependent hypertension in humans (41), particularly with respect to BP and proteinuria. Salt depletion, with enhancement of renin-angiotensin activity, therefore might further enhance the synergy seen between ETA receptor antagonism and ACE inhibition.
It is interesting that in the presence of ACE inhibition in our study, combined ETA/B receptor antagonist tended to increase renal vascular tone and reduce blood flow, underlining the importance of ETB-mediated vasodilation. This finding has important implications for the therapeutic use of ET receptor antagonists, suggesting that, in conjunction with ACE inhibitors, ETA receptor antagonists may be superior to nonselective ETA/B receptor antagonists with respect to some important hemodynamic effects. Indeed, we previously demonstrated in patients who had chronic renal failure and were already being treated with ACE inhibitors that ETA but not combined ETA/B receptor antagonism increases renal blood flow (36). The current demonstration of an ETB receptor-dependent, synergistic interaction between ETA receptor antagonism and ACE inhibition suggests that these differences observed in renal patients may, at least in part, be due to concomitant chronic treatment with ACE inhibitors.

Although prostacyclin is the major COX product of macrovascular endothelium in vitro, COX activity can produce both vasoconstrictor and vasodilator acyclic acid derivatives. Prostaglandins stimulate renin release (42). Thus, indomethacin may produce both vasodilation, by inhibition of renin-mediated Ang II generation and COX-1-mediated thromboxane A2 synthesis, and vasoconstriction, by blocking synthesis of vasodilator prostanooids. Studies in animals also suggest that vasoconstrictor COX products, such as thromboxane A2 and prostaglandin H2, might be implicated in ET-1-induced vasoconstriction, particularly in disease models (43–47). The greater fall in BP when indomethacin was co-administered with enalapril and BQ-123 may represent blockade of the action of these constrictor COX products. There is evidence from studies in animals that vasodilator prostaglandins may additionally be involved in the actions of ET-1 in the renal circulation, particularly the renal medulla (45,48). We could not demonstrate, however, any inhibitory effect of indomethacin on either the systemic or the renal hemodynamic effects of the combination of ACE inhibition and ETA receptor antagonism. With respect to renal blood flow, however, clearance studies only measure total renal blood flow. It is possible, therefore, that opposite changes are occurring in the renal cortex and medulla.

With respect to natriuresis, knockout and antagonist studies in animals have implicated ETB receptors, linked to NO production, in ET-1-mediated sodium excretion (49). COX inhibition, however, augments big ET-1-mediated natriuresis, suggesting an antinatriuretic role for prostaglandins (50). Our studies demonstrate, in the presence of ACE inhibition, an ETB-dependent natriuresis that is mediated by both NO and, to a lesser extent, prostanooids.

In summary, in this mechanistic study, we demonstrated that ACE inhibition increases the systemic hemodynamic effects of ETA receptor antagonism, at both low and high dose, and unmask a renal hemodynamic and tubular effect. This synergy is mediated mainly by NO and requires an unblocked ETB receptor. These findings would preferentially support the further investigation of selective ETA receptor antagonism, over combined ETA/B receptor antagonism, as a useful adjunct to ACE inhibition in the management of the systemic and renal vascular consequences of diseases characterized by vasocostruction. This may be particularly important in circumstances in which sodium loss would also be beneficial, such as hypertension and chronic renal failure. This interaction should now be explored in longer term studies in patients with such conditions.

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