INHIBITION OF KININ METABOLISM AND THE ROLE OF THE VASCULAR B_1 KININ RECEPTOR IN PATIENTS WITH CONGESTIVE HEART FAILURE

BY

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ABSTRACT

Background Angiotensin-converting enzyme and neutral endopeptidase are endothelial metallopeptidases that metabolise bradykinin. Inhibitors of angiotensin-converting enzyme improve symptoms and survival in patients with heart failure and vascular disease and potentiate bradykinin-mediated vasodilatation and endothelial tissue plasminogen activator release. The vascular actions of kinins are mediated by an inducible B_1 receptor and a constitutively expressed B_2 receptor. Vascular B_1 kinin receptor expression is markedly upregulated with left ventricular dysfunction and angiotensin-converting enzyme inhibition, but its role in man remains unclear.

Objectives The aims of this thesis were first, to confirm biological activity of kinin receptor agonists and antagonists in human vascular tissue *in vitro*: second, to determine the contribution of bradykinin to the systemic haemodynamic effects of angiotensin-converting enzyme inhibition in patients with heart failure: third, to determine the effects of neutral endopeptidase inhibition on the vascular actions of bradykinin in patients treated with angiotensin-converting enzyme inhibition: fourth and finally, to determine the contribution of the B_1 kinin receptor to the vascular actions of kinins in patients with heart failure.

Methods *Myography* The vasomotor effects of kinin peptides were determined using myography of human umbilical vein rings. *Heart failure: Systemic circulation* After 6 weeks of enalapril or losartan therapy, patients underwent right heart catheterisation and received an intravenous infusion of the bradykinin receptor antagonist, B9340. Systemic haemodynamic variables were recorded. *Peripheral circulation* Blood flow and plasma fibrinolytic parameters were determined in both forearms using venous occlusion plethysmography and venous blood sampling. Drugs were administered via the brachial artery of the non-dominant forearm. The effect of the neutral endopeptidase inhibitor, thiorphan, on the vascular actions of bradykinin was examined in patients maintained on angiotensin-converting enzyme inhibition. Vascular B_1 receptor function was examined using selective peptidic kinin receptor agonists and antagonists.

Results Biological activity of kinin receptor agonists and antagonists was confirmed in human umbilical vein. Systemic bradykinin antagonism caused an increase in mean arterial pressure and systemic vascular resistance and attenuated the fall in pulmonary arterial and pulmonary arterial wedge pressures in patients treated with enalapril compared to losartan. Compared to placebo, thiorphan augmented the vasomotor and fibrinolytic actions of bradykinin in patients treated with chronic angiotensin-converting enzyme inhibition. B_1 receptor agonism and antagonism had no effect on vascular tone or enothelial tissue plasminogen activator release in the presence or absence of angiotensin-converting enzyme inhibition. The B_2 receptor agonist, bradykinin, caused vasodilatation and tissue plasminogen activator release and these effects were markedly augmented by angiotensin-converting enzyme inhibition.

Conclusions Bradykinin contributes to the systemic haemodynamic effects of long-term angiotensin-converting enzyme inhibition in patients with heart failure. Neutral endopeptidase contributes to the metabolism of bradykinin in patients with heart failure maintained on angiotensin-converting enzyme inhibitor therapy. Our findings may explain some of the apparent clinical differences between angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, as well as the greater vasodepressor effect observed with combined angiotensin-converting enzyme and neutral endopeptidase inhibition when compared to angiotensin-converting enzyme inhibition alone. Finally, the B_1 kinin receptor does not appear to have a major vasomotor or fibrinolytic role in patients with heart failure. Augmentation of kinin-mediated vasodilatation and tissue plasminogen activator release by angiotensin-converting enzyme inhibition is restricted to the B_2 receptor.

CONTENTS

DECLARATION

The work contained in this thesis was undertaken in the Department of Cardiology, the Cardiovascular Research Unit and within the Clinical Research Facility at the Royal Infirmary, Edinburgh. The substantial part of the work described has been my own and was carried out whilst a Clinical Research Fellow in the Cardiovascular Research Unit during the period between 2001 and 2004. Whilst undertaking this work, I have been fortunate to receive advice and assistance from a number of colleagues and their valuable contribution has been formally acknowledged. All the work presented in this thesis has been published in peer-reviewed journals. The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged.

28.2.08

Nicholas Le May Cruden

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CHAPTER 1

INTRODUCTION:

INHIBITION OF KININ METABOLISM AND

THE ROLE OF THE VASCULAR B_1 KININ RECEPTOR

IN PATIENTS WITH CONGESTIVE HEART FAILURE

1.1 THE KALLIKREIN-KININ SYSTEM

A role for the kallikrein-kinin system in vascular homeostasis was first recognised in 1909 following the demonstration that intravenous injection of fractions extracted from human urine resulted in a transient reduction in blood pressure [Abelous and Bardier, 1909]. Since then, a substantial body of evidence has implicated kinins in a wide range of physiological and pathological processes, including inflammation [Couture et al, 2001], nocioception [Millan, 1999], smooth muscle contraction [Khairallah and Page, 1963], vascular homeostasis [Benjamin et al, 1989; Labinjoh et al, 2000], blood coagulation [Kaplan and Silverberg, 1987] and fibrinolysis [Brown et al, 2000; Labinjoh et al, 2000].

The kallikrein-kinin system can be broadly divided into a plasma-based pathway that results in the formation of the nonapeptide, bradykinin, and a tissue-based pathway producing the decapeptide, Lys-bradykinin (kallidin; Figure 1). Removal of the N-terminal lysine residue from Lys-bradykinin by a plasma aminopeptidase will also result in the generation of bradykinin. Under pathophysiological conditions, such as tissue injury or inflammation, plasma and tissue kallikreins are activated and release bradykinin and Lys-bradykinin through cleavage of high and low molecular weight kininogens respectively.

1.1.1 THE PLASMA KALLIKREIN-KININ SYSTEM

Bradykinin is released into plasma from high molecular weight kininogen following cleavage by the serine protease, plasma kallikrein [Regoli and Barabe, 1980;

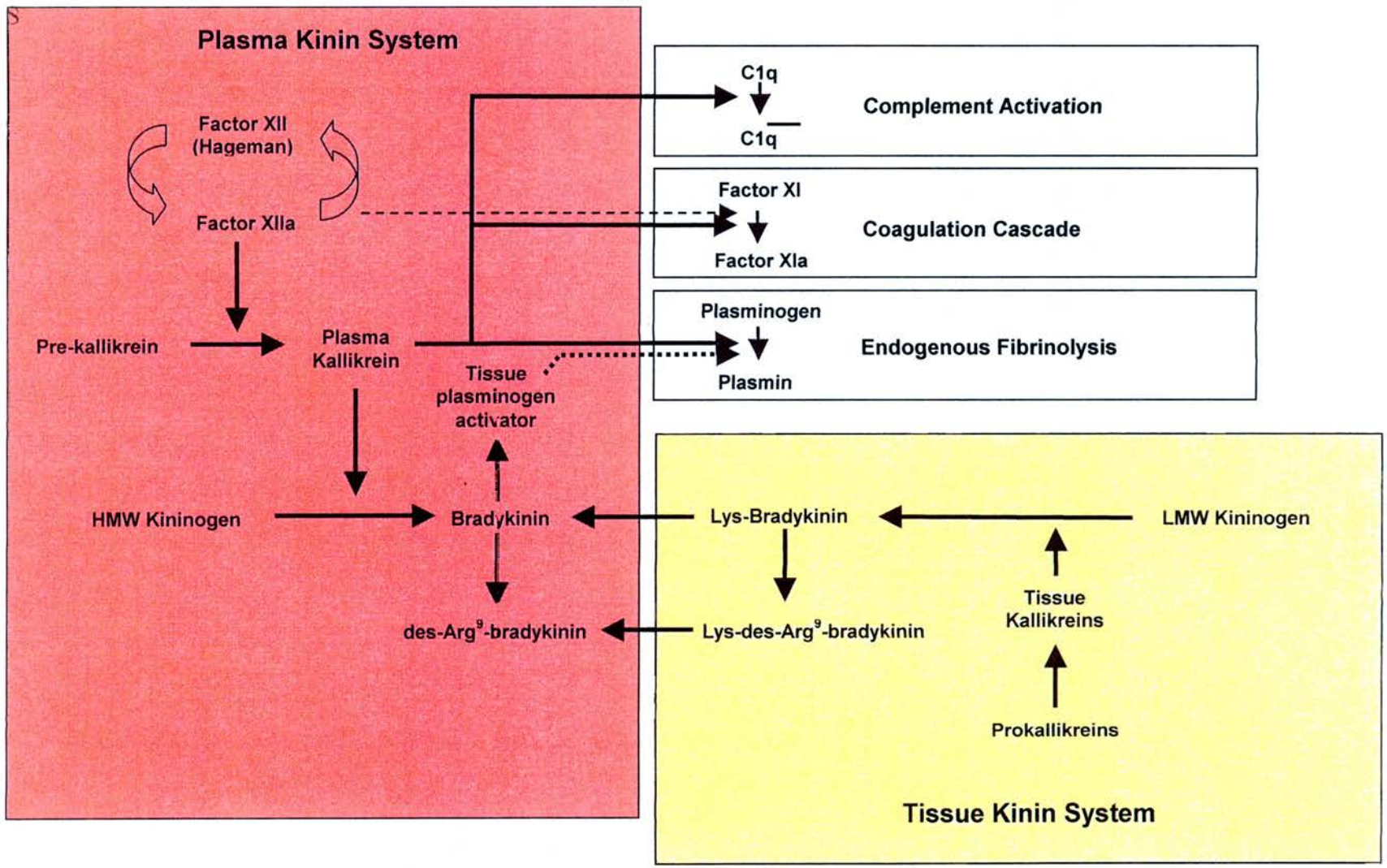

Figure 1. Schematic diagram of plasma and tissue kinin-kallikrein systems. HMW, high molecular weight; LMW, low molecular weight.

Bhoola et al, 1992]. Plasma kallikrein and high molecular weight kininogen are both synthesised in the liver. Kallikrein is synthesised and secreted as the precursor, prekallikrein, whereas high molecular weight kininogen is stored in hepatocytes and undergoes glycolyslation prior to secretion [Bhoola et al, 1992]. Plasma prekallikrein circulates in plasma as a heterodimer bound to high molecular weight kininogen [Mandle et al, 1976] and is activated by coagulation factor XIIa (Hageman factor) and endothelium-derived prekallikrein activators, such as serine protease prolylcarboxypeptidase [Mandle and Kaplan, 1977; Shariat-Madar et al, 2002]. Initially, the liberation of bradykinin was assumed to occur only as a result of "contact activation" of factor XII by negatively charged surfaces. Recently, however, it has been recognised that the components of the plasma kallikrein system may also be assembled and activated on the intravascular surface of endothelial cells through binding to a cell surface receptor complex containing cytokeratin 1, urokinase plasminogen activator (u-PA) receptor and gC1qR (Figure 2) [Joseph et al, 1996; Colman et al, 1997].

1.1.2 The Tissue Kallikrein-Kinin System

The tissue kallikreins are a multigene family of enzymes present in a number of organs including the heart, kidney, pancreas and salivary glands [Bhoola et al, 1992; Marceau et al, 1998]. Three tissue kallikrein enzymes have been identified in man [Marceau et al, 1998]. These enzymes are synthesised in cells as a precursor and converted to their active form by cleavage of an amino-terminal peptide [Takada et al, 1985]. Once activated, the tissue kallikreins preferentially release Lys-bradykinin from low molecular weight kininogen. Like high molecular weight kininogen, low

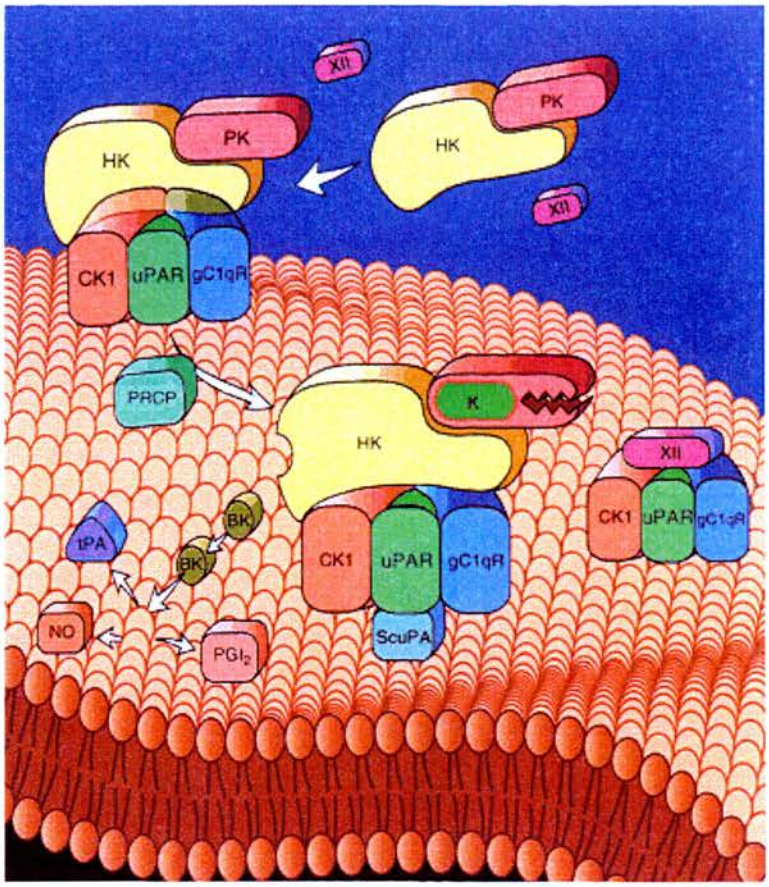

Figure 2. Assembly and activation of the plasma kallikrein-kinin system (KKS) on endothelial cells. Plasma prekallikrein (PK) circulates in complex with high molecular weight kininogen (HK). The HK•PK complex binds to a multiprotein receptor complex that consists of cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR) and gC1qR. The proteins of the HK•PK receptor complex co-localise on endothelial cell membranes. When HK•PK binds to endothelial cells, PK is rapidly converted to kallikrein (K) by the enzyme prolylcarboxypeptidase (PRCP), which is constitutively active on endothelial cell membranes. The resulting kallikrein autodigests its receptor, HK, to liberate bradykinin (BK), which can liberate tissue plasminogen activator (tPA), nitric oxide (NO), and prostacyclin (PGI_2) from endothelial cells. Kallikrein also activates FXII, which binds to the same multiprotein receptor complex as HK in its absence. In this hypothesis for assembly and activation of the proteins of the plasma KKS, FXII is activated by kallikrein after PK activation. ScuPA, single chain urokinase plasminogen activator.[Schmaier, 2002]

molecular weight kininogen is also synthesised in the liver and is present in plasma and other body fluids.

1.2 KININ METABOLISM

The rapid metabolism of bradykinin by a number of peptidases (Figure 3) ensures a half-life in blood of <15 seconds [McCarthy et al, 1965; Ferreira and Vane, 1967; Bonner et al, 1990]. Kininase II (angiotensin-converting enzyme (ACE) or dipeptidyl carboxypeptidase, EC 3.4.15.1) and neutral endopeptidase (enkephalinase, neprilysin, EC.3.4.24.11; NEP) both hydrolyse the Pro^7-Phe^8 bond at the carboxy-terminus of the kinin molecule (Figure 3). Kininase II also splits the Phe^5-Ser^6 bond. The plasma carboxypeptidases, collectively known as kininase I, include carboxypeptidase N (EC 3.4.17.3) and carboxypeptidase B (thrombin activatable fibrinolysis inhibitor (TAFI), carboxypeptidase R or U). Enzymes with kininase I activity cleave the carboxyl-terminal from bradykinin resulting in the formation of des-Arg^9-bradykinin. The half-life of des-Arg^9-bradykinin appears to be 4- to 12-fold greater than bradykinin [Decarie et al, 1996; Blais et al, 1997] and may explain why plasma concentrations of des-Arg^9-bradykinin are greater than bradykinin *in vivo*.

1.3 KININ RECEPTORS

The biological effects of kinins in man are mediated by two principal G-protein coupled receptors, B_1 and B_2, each with high affinity and specificity for their respective ligands, des-Arg^9-bradykinin and bradykinin [Leeb-Lundberg et al, 2005].

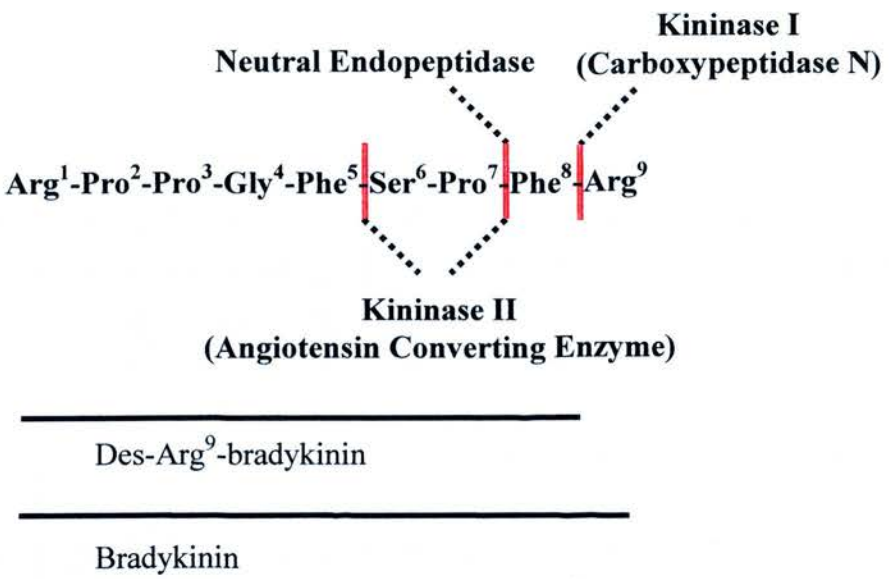

Figure 3. Amino acid structure and C-terminal sites of enzymatic degradation of bradykinin and des-Arg^9-bradykinin.

The B_1 and B_2 kinin receptors share only 36% homology with each other and are distinguishable on the basis of their pharmacological and molecular characteristics [Hess et al, 1992; Menke et al, 1994; Marceau et al, 1998; Leeb-Lundberg et al, 2005]. Both receptors consist of seven helical transmembrane domains (Figure 4), the specificity of receptor binding being determined largely by the sixth transmembrane domain [Leeb et al, 1997].

1.3.1 Peptide Agonists

Binding affinity estimates for the two human kinin receptor subtypes are shown in Table 1. Removal of the C-terminal arginine favours affinity for the B_1 receptor and reduces affinity at the B_2 receptor. The only naturally occurring kinin peptide with subnanomolar affinity at the human B_1 receptor is Lys-des-Arg^9-bradykinin.

1.3.2 Peptide Antagonists

The development of selective peptidic kinin receptor antagonists has greatly facilitated investigation of kinin receptor subtypes (Table 2). In keeping with the kinin receptor agonists, the N-terminal lysine residue confers a greater affinity for the B_1 receptor in man. Lys-[Leu^8]-des-Arg^9-bradykinin, therefore, is the optimal B_1 receptor antagonist based on naturally occurring amino acids. B9340 is a synthetic peptidic antagonist that has similar affinity for both the B_1 and B_2 kinin receptors. The rapid elimination and short duration of action, coupled with the competitive, reversible nature of receptor blockade make these peptides ideal pharmacological tools with which to investigate kinin receptor function *in vivo* in the human vasculature.

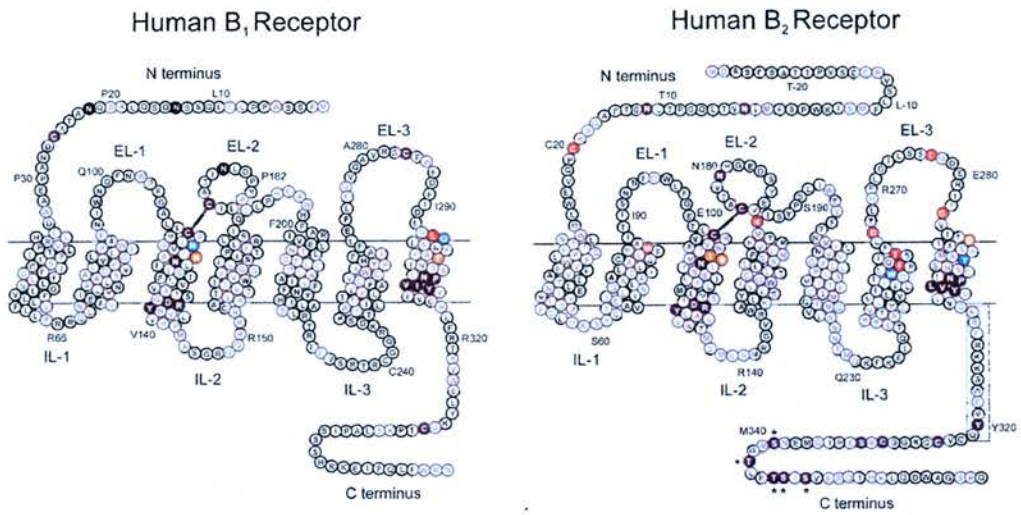

Figure 4. Transmembrane structure of the human B_1 and B_2 kinin receptors.[Leeb-Lundberg et al, 2005]

Table 1. Nanomolar affinity estimates and structure-activity relationships for kinin receptor agonists.

Compound	Amino Acid Structure										Human Kinin Receptors	
	0	1	2	3	4	5	6	7	8	9	B_1R^*	$B_2R^\dagger$
Bradykinin		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg	2000	0.54
Lys-bradykinin	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg	42	0.63
Des-Arg^9-bradykinin		Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe		720	8100
Lys-des-Arg^9-bradykinin	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe		0.2	>30000

*Nanomolar affinity estimates are EC_{50} values from binding competition with 1 nM [H^3]Lys-des-Arg^9-bradykinin to transiently expressed human B_1 kinin receptors in COS-7 cells. † Nanomolar affinity estimates are EC_{50} values from binding competition with 100 pM [H^3]bradykinin to stably expressed human B_2 kinin receptors in chinese hamster ovary cells [Marceau et al, 1998; Menke et al, 1994].

Table 2. Nanomolar affinity estimates and structure-activity relationships for kinin receptor antagonists.

Compound	Amino Acid Structure										Human Kinin Receptors	
	0	1	2	3	4	5	6	7	8	9	B_1R^*	$B_2R^\dagger$
HOE-140	D-Arg	Arg	Pro	Hyp	Gly	Thi	Ser	D-Tic	Oic	Arg	>10000	0.41
Lys-[Leu^8]-des-Arg^9-bradykinin	Lys	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Leu		1.3	>30000
B9340	D-Arg	Arg	Pro	Hyp	Gly	Thi	Ser	D-Igl	Oic	Arg	6.4	0.15

*Nanomolar affinity estimates are IC_{50} values from binding competition with 1 nM [H^3]Lys-des-Arg^9-bradykinin to transiently expressed human B_1 kinin receptors in COS-7 cells or IMR-90 human pulmonary fibroblasts. †Nanomolar affinity estimates are IC_{50} values from binding competition with 100 pM [H^3]bradykinin to stably expressed human B_2 kinin receptors in chinese hamster ovary cells cells [Marceau et al, 1998; Menke et al, 1994; Stewart et al, 1997].

1.3.3 Intracellular Signalling Mechanisms

The B_1 and B_2 kinin receptor subtypes belong to the G-protein coupled receptor superfamily. Although the identity and importance of specific G-protein subunits associated with each receptor subtype are similar, the intracellular transduction mechanisms associated with kinin receptor signalling varies between cell types. Second messenger systems include phospholipase C and phosholipase A_2 that once activated cause a rise in intracellular calcium, inositol phosphate and diacylglycerol. Additional downstream signalling effects include an increase in cyclic AMP and cyclic GMP, activation of protein kinase C and mitogen activated protein kinases, and release of prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor [Levesque et al, 1995; Marceau et al, 1998; Christopher et al, 2001].

Interestingly, B_1 receptor activation appears to be associated with a more persistent intracellular signal than the B_2 receptor suggesting, perhaps, that B_1 receptor activation may lead to more chronic and persistent changes [Mathis et al, 1996; Austin et al, 1997].

1.3.4 Receptor Desensitisation

Kinin receptors differ significantly in their susceptibility to undergo desensitisation. Following agonist mediated activation, the B_2 receptor agonist complex is rapidly internalised and sequestered in calveolae along with associated G-proteins, greatly reducing the availability of unbound B_2 receptors on the cell surface [Sabourin et al, 2002a]. In contrast, the B_1 receptor does not undergo internalisation or significant desensitisation. These differences have been attributed to amino acid sequences in

the cytoplasmic carboxyl terminal of the B_2 receptor, absent from the cytoplasmic domain of the B_1 receptor [Faussner et al, 1998].

1.4 KININ RECEPTOR EXPRESSION AND FUNCTION

1.4.1 B_2 KININ RECEPTOR

The B_2 receptor is the most prevalent receptor subtype expressed under physiological conditions. It is found on the surface of many cell types, including the vascular endothelium and smooth muscle, where it mediates the actions of bradykinin. In animal models, intravascular administration of bradykinin causes arterial vasodilatation and a fall in blood pressure [Regoli and Barabe, 1980]. In man, activation of the endothelial B_2 receptor stimulates endothelium-dependent vasodilatation and release of the pro-lytic factor, tissue plasminogen activator (t-PA), and these effects can be blocked by B_2 receptor antagonists [Benjamin et al, 1989; Groves et al, 1995; Brown et al, 2000; Labinjoh et al, 2000].

1.4.2 B_1 KININ RECEPTOR

In contrast to the B_2 receptor, the vascular B_1 receptor is normally expressed very weakly but is rapidly upregulated in the presence of inflammation [Sardi et al, 1998; McLean et al, 1999; Sardi et al, 1999; McLean et al, 2000b], ischaemic left ventricular dysfunction [Tschope et al, 2000], cardiovascular disease [Raidoo et al, 1997; McLean et al, 2000b] and ACE inhibition [Nwator and Whalley, 1989; Marin-Castano et al, 2002]. In animal models, stimulation of the B_1 receptor produces vasodilatation and a reduction in blood pressure [Drapeau et al, 1991; Nakhostine et

al, 1993; Deblois and Horlick, 2001]. Intense endothelial B_1 receptor expression has been demonstrated in atheromatous human blood vessels [Raidoo et al, 1997] and B_1 receptor stimulation induces dose-dependent vasodilatation in human coronary arteries *in vitro* [Drummond and Cocks, 1995b].

1.4.3 Upregulation of B_1 Kinin Receptor Expression

Functional expression of the B_1 kinin receptor has traditionally been characterised by smooth muscle responses to selective kinin receptor agonists in organ bath preparations. Tissues from a variety of vascular sites and species, including human umbilical vein and coronary artery, examined in this way exhibit B_1 receptor mediated responses which are absent initially but develop in magnitude as a function of the *in vitro* incubation time [Bouthillier et al, 1987; Drummond and Cocks, 1995b; Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999]. These responses can also be invoked by pre-incubation of tissue with bacterial lipopolysaccharide or inflammatory cytokines, such as interleukin-1β [Sardi et al, 1998] and tumour necrosis factor-α [Sardi et al, 1999].

These *in vitro* findings have been confirmed *ex vivo* and *in vivo* in a number of animal models [Marceau, 1995; McLean et al, 1999]. As an example, endotoxaemia induces endothelial expression of B_1 receptors and des-Arg^9-bradykinin causes endothelium-dependent vasodilatation of the coronary arteries [McLean et al, 1999]. In addition, it has been reported that des-Arg^9-bradykinin can cause coronary resistance and conduit vessel dilatation even in the absence of inflammation [Su et al, 2000]. In man, upregulation of B_1 receptor expression in the presence of

inflammation has been demonstrated *in vivo* in the nasal mucosa of patients with allergic airways disease [Christiansen et al, 2002].

1.4.4 Intracellular Mechanisms of B_1 Receptor Upregulation

The induction of B_1 receptor responses is attenuated in tissues treated with the protein synthesis inhibitors, cycloheximide and anisomycin, and the protein trafficking inhibitor, Brefeldin A [Deblois et al, 1991; Audet et al, 1994; Sabourin et al, 2002b]. Brefeldin A selectively inhibits translocation of proteins from the endoplasmic reticulum to the Golgi apparatus, the expected maturation pathway for a G-protein coupled receptor. In addition, the nuclear transcription factor, NF-κβ, has been implicated in the induction of B_1 receptor expression [Ni et al, 1998; Schanstra et al, 1998]. NF-κβ binding domains have been identified in the promoter region of the human B_1 receptor gene [Ni et al, 1998; Schanstra et al, 1998] and cytokine-induced B_1 kinin receptor expression is blocked by pharmacological inhibitors of NF-κβ [Sabourin et al, 2002b; Passos et al, 2004]. Similarly, the protein glycosylation inhibitor, tunicamycin, partially and selectively inhibits B_1 responses suggesting post-translational modification prior to cell surface expression of B_1 receptors [Audet et al, 1994]. Cytokine mediated stabilisation of B_1 receptor mRNA has also been implicated in the upregulation of B_1 receptor expression on the cell surface [Haddad et al, 2000]. Thus, increased B_1 receptor expression appears to result from *de novo* receptor synthesis and occurs over a time period of 2-3 hours.

1.4.5 B_1 Kinin Receptor Expression, Atherosclerosis and Cardiac Failure

Systemic inflammation plays an important role in the pathogenesis of atherosclerosis [Ross, 1999] and cardiovascular disease [Levine et al, 1990; Ridker et al, 2000]. In keeping with this, an inducible B_1 receptor response has been reported *in vivo* in animal models of myocardial ischaemia [Mazenot et al, 2001] and infarction [Tschope et al, 2000; Tschope et al, 2004], and balloon angioplasty induced vascular injury [Agata et al, 2000]. In man, autopsy studies have indicated that B_1 receptors are markedly upregulated in the presence of atherosclerosis [Raidoo et al, 1997].

Patients with chronic heart failure have significant elevations in plasma cytokine concentrations including tumour necrosis factor-α [Levine et al, 1990; McMurray et al, 1991; Kelly and Smith, 1997]. Such patients not only demonstrate marked endothelium-dependent vasomotor dysfunction [Kubo et al, 1991; Katz et al, 1992], but this dysfunction is highly correlated with plasma tumour necrosis factor-α concentrations [Katz et al, 1994]. In the face of such systemic inflammation, therefore, it is likely that the expression of kinin receptors will favour the B_1 receptor. However, the effects of vascular B_1 receptor agonism and antagonism and the influence of ACE inhibition on these responses remain unknown.

1.5 ANGIOTENSIN-CONVERTING ENZYME

1.5.1 BACKGROUND

Angiotensin-converting enzyme (kininase II) is a membrane-bound metallopeptidase present on the vascular endothelium. First described for its ability to convert angiotensin I to angiotensin II, ACE also metabolises bradykinin [Erdös and Yang, 1967]. Indeed, ACE has greater affinity for bradykinin than for angiotensin I [Jaspard et al, 1993] and is the principal enzyme responsible for the rapid turnover of bradykinin and its metabolites [Decarie et al, 1996; Blais et al, 1997; Cyr et al, 2001]. Therefore, inhibitors of ACE will not only block the formation of angiotensin II, but will also inhibit the breakdown of bradykinin. As a result, plasma angiotensin I concentrations and renin activity increase, whereas circulating concentrations of angiotensin II and aldosterone fall [Gavras et al, 1978]. Although the short half-life of bradykinin makes accurate measurement of plasma concentrations difficult, elevated plasma bradykinin concentrations have been reported in the presence of ACE inhibition [Pellacani et al, 1994].

1.5.2 PHYSIOLOGICAL EFFECTS OF ANGIOTENSIN-CONVERTING ENZYME INHIBITION

Inhibitors of ACE reduce blood pressure and systemic vascular resistance in a dose-dependent manner with little change in heart rate [Powers et al, 1982; Beermann et al, 1993; Flammang et al, 1993; Mitrovic et al, 1996]. In patients with left ventricular systolic impairment, ACE inhibitors reduce pulmonary artery wedge pressure and increase cardiac output and these changes persist following 3 months of ACE

inhibitor therapy [Beermann et al, 1993; Flammang et al, 1993; Mitrovic et al, 1996]. In the kidney, ACE inhibitors increase renal blood flow without affecting glomerular filtration rate and promote a natriuresis [Hollenberg, 1988].

1.5.3 Angiotensin-Converting Enzyme Inhibition and Endothelial Function

Angiotensin-converting enzyme inhibitors reverse endothelial dysfunction in a number of conditions associated with the development of congestive heart failure including hypertension, coronary artery disease and non-insulin-dependent diabetes mellitus [Antony et al, 1996; O'Driscoll et al, 1999; Anderson et al, 2000; Hornig et al, 2001]. In patients with heart failure, treatment with the ACE inhibitor, quinapril, increases nitric oxide availability and augments endothelial-dependent vasodilatation [Hornig et al, 1998]. Similarly, ACE inhibitors may improve endogenous fibrinolytic parameters in patients with heart failure [Goodfield et al, 1999] through inhibition of angiotensin II-mediated synthesis of plasminogen activator inhibitor type-1 (PAI-1) [Ridker et al, 1993] and potentiation of bradykinin-stimulated endothelial t-PA release [Witherow et al, 2002].

1.5.4 Clinical Effects of Angiotensin-Converting Enzyme Inhibitor Therapy

A number of landmark trials over the last two decades have demonstrated that ACE inhibitors reduce morbidity and mortality in patients with heart failure due to left ventricular systolic impairment [CONSENSUS, 1987; SOLVD, 1991]. These impressive benefits appear to be dose-dependent [Packer et al, 1999] and extend to

patients with asymptomatic left ventricular dysfunction [SOLVD, 1992]. In a recent meta-analysis, data pooled from five major trials evaluating ACE inhibitors in patients with heart failure and myocardial infarction demonstrated a significant reduction in rates of mortality, reinfarction and hospitalisation with ACE inhibitor therapy when compared to placebo [Flather et al, 2000].

Although the reduction in mortality may be primarily due to the prevention of progression of heart failure, there is increasing evidence that ACE inhibitors have anti-ischaemic properties [Pfeffer et al, 1992; Yusuf et al, 1992]. In patients with left ventricular dysfunction - with or without symptoms of heart failure - treatment with an ACE inhibitor reduced the risk of myocardial infarction or sudden cardiac death by 20% [Pfeffer et al, 1992; Yusuf et al, 1992]. More recently, these benefits have been shown to extend to high-risk patients with normal left ventricular function. The HOPE study demonstrated that in patients with normal left ventricular function and known vascular disease or diabetes mellitus plus one other cardiovascular risk factor, ACE inhibition significantly reduced mortality and recurrent ischaemic events by 22% [Yusuf et al, 2000]. These findings are supported by the recent EUROPA trial which demonstrated a significant reduction in the combined endpoint of cardiovascular death, myocardial infarction or cardiac arrest with perindopril when compared to placebo in patients with stable coronary artery disease in the absence of symptomatic heart failure [Fox, 2003]. Although a number of mechanisms including blood pressure reduction, reversal of endothelial dysfunction and alterations in endogenous fibrinolysis have been implicated, the exact mechanisms by which ACE

inhibitors prevent ischaemic vascular events remain unclear [Brown and Vaughan, 1998].

1.5.5 Angiotensin-Converting Enzyme Inhibition and Bradykinin

There is now substantial evidence that the haemodynamic and clinical effects of ACE inhibitor therapy may be due, at least in part, to the inhibition of bradykinin metabolism [Gainer et al, 1998; Witherow et al, 2001]. At a functional level, ACE inhibition potentiates the vascular actions of bradykinin in the human forearm [Benjamin et al, 1989; Labinjoh et al, 2001; Witherow et al, 2002] and coronary [Kuga et al, 1997] circulations and in healthy volunteers and patients with hypertension, bradykinin antagonism attenuates the vasodepressor response to acute ACE inhibition [Gainer et al, 1998; Squire et al, 2000]. We have recently demonstrated that intra-arterial administration of the combined B_1 and B_2 kinin receptor antagonist, B9340, causes vasoconstriction in the forearm circulation of patients with heart failure maintained on chronic ACE inhibitor therapy [Witherow et al, 2001]. In contrast, intra-arterial HOE-140, a selective B_2 receptor antagonist has no effect on forearm blood flow in patients treated with ACE inhibition [Davie et al, 1999; Witherow et al, 2001]. This disparity may reflect the differing pharmacological profiles of the bradykinin antagonists used and provides the first, albeit indirect, evidence of a vasomotor role for the B_1 kinin receptor *in vivo* in man.

1.5.6 Angiotensin-Converting Enzyme Inhibition and B_1 Receptor Responses

Inhibitors of ACE not only increase bradykinin concentrations but also augment production of des-Arg^9-bradykinin. Under physiological conditions, the percentage of bradykinin metabolised to des-Arg^9-bradykinin by plasma carboxypeptidases is limited (~1% – 11%) [Decarie et al, 1996; Blais et al, 1997; Cyr et al, 2001]. In the presence of ACE inhibition, however, this figure increases significantly to ~50% [Cyr et al, 2001]. Thus ACE inhibition will potentially favour the augmentation of both B_1 and B_2 kinin receptor mediated effects [Lamontagne et al, 1995].

There is evidence that ACE inhibition can lead to upregulation of B_1 receptor expression *in vivo* [Nwator and Whalley, 1989; Marin-Castano et al, 2002], although this finding remains controversial [Marceau et al, 1999]. Recently, Marin-Castano and colleagues demonstrated increased expression of functional B_1 receptors in both renal and vascular tissues in rats exposed to chronic ACE inhibitor therapy. This induction was independent of the B_2 kinin receptor and contributed to the hypotensive effect of ACE inhibitor therapy [Marin-Castano et al, 2002]. Previous work *in vitro* has suggested that B_1 agonism can induce upregulation of its own receptor [Schanstra et al, 1998; Phagoo et al, 1999]. Given the anticipated augmentation of des-Arg^9-bradykinin concentrations associated with ACE inhibition, this mechanism may have contributed to the observed increase in B_1 receptor expression [Marin-Castano et al, 2002]. Whether ACE inhibition induces functional B_1 receptor expression in human vascular tissue *in vivo* is not known.

1.6 NEUTRAL ENDOPEPTIDASE

Neutral endopeptidase is a glycosylated metallopeptidase that colocalises with ACE on the cell membrane and is found in a wide variety of tissues including the vascular endothelium [Roques et al, 1993]. Neutral endopeptidase has a broad substrate specificity, metabolising a number of biologically active peptides including endothelin-1, atrial natriuretic peptide, substance P and bradykinin [Roques et al, 1993]. This lack of substrate selectivity may account for the heterogeneity observed with the vasomotor and haemodynamic effects of isolated NEP inhibition in man [Northridge et al, 1989; Richards et al, 1990 Bevan et al, 1992; Favrat et al, 1995; Northridge et al, 1999].

Expression of NEP is upregulated in patients with heart failure [Fielitz et al, 2002; Knecht et al, 2002] and, in the presence of ACE inhibition, the contribution of NEP to bradykinin metabolism is increased [Dumoulin et al, 2001]. Inhibition of NEP potentiates the half-life of bradykinin [Graf et al, 1993] and augments bradykinin-mediated vasodilatation *in vitro* [Krassoi et al, 2003]. It is not known whether NEP inhibition augments the half-life of Lys-des-Arg^9-bradykinin. Indeed, it has been suggested that the Phe^8 residue may protect B_1 ligands from degradation by NEP [Marceau et al, 1998]. Although the effects of NEP inhibition on systemic haemodynamics are variable [Northridge et al, 1989; Richards et al, 1990 Bevan et al, 1992; Favrat et al, 1995; Northridge et al, 1999], clinical improvements have been reported during NEP inhibition in patients with heart failure [Northridge et al, 1999].

Co-administration of ACE and NEP inhibitors may confer additional therapeutic efficacy. Combined ACE and NEP inhibition attenuates bradykinin degradation more effectively than either enzyme alone [Dumoulin et al, 2001] and in animal models, improves cardiac remodelling and survival to a greater extent than isolated ACE inhibition [Trippodo et al, 1999]. These cardioprotective effects are lost in transgenic mice lacking the B_2 kinin receptor [Xu et al, 2004]. In man, combined ACE and NEP inhibition reduces blood pressure to a greater extent than inhibition of either enzyme alone [Favrat et al, 1995; Campese et al, 2001] and is associated with symptomatic and haemodynamic improvements in patients with heart failure [McClean et al, 2000]. The hypothesis that combined ACE and NEP inhibition may improve symptoms and survival in patients with heart failure to a greater extent than ACE inhibition alone has recently been evaluated in a large-scale clinical trial (OVERTURE) [Packer et al, 2002]. Although OVERTURE failed to demonstrate a significant reduction in all-cause mortality with the combined ACE and NEP inhibitor, omapatrilat, when compared to enalapril, omapatrilat did reduce the combined secondary endpoint of cardiovascular death and hospitalisation [Packer et al, 2002]. It remains to be established whether combined ACE and NEP inhibition augments the vascular actions of bradykinin to a greater extent than ACE inhibition alone in patients with heart failure.

1.7 KININS, ENDOGENOUS FIBRINOLYSIS AND ATHEROTHROMBOSIS

1.7.1 THE ENDOGENOUS FIBRINOLYTIC SYSTEM

The endogenous fibrinolytic pathway describes a complex process involving the hydrolytic cleavage of fibrin, by plasmin, to cause clot dissolution and generate fibrin degradation products (Figure 5). Plasmin, a serine protease, is generated by the action of an array of enzymes and inhibitors which have a coordinated action on the zymogen, plasminogen. The major role of this enzymatic system is to protect the circulation from intravascular fibrin formation and thrombosis that would otherwise result in vessel occlusion and tissue ischaemia.

There are two endogenous plasminogen activators responsible for the initiation of fibrinolysis: t-PA and u-PA. The main physiological plasminogen activator involved in the degradation of intravascular fibrin is t-PA (Figure 6) [Astedt, 1979; Kok, 1979]. Tissue plasminogen activator is released from the endothelium through the translocation of a dynamic intracellular storage pool and the efficacy of plasminogen activation and fibrin degradation is determined by the relative balance between the acute local release of t-PA and its subsequent inhibition through formation of complexes with the serpin, PAI-1.

1.7.2 ENDOGENOUS FIBRINOLYSIS AND ATHEROTHROMBOSIS

There is substantial evidence that the endogenous fibrinolytic system plays a crucial role both in the acute thrombotic consequences of plaque disruption and the chronic processes of atherogenesis. Reperfusion of the infarct-related artery occurs

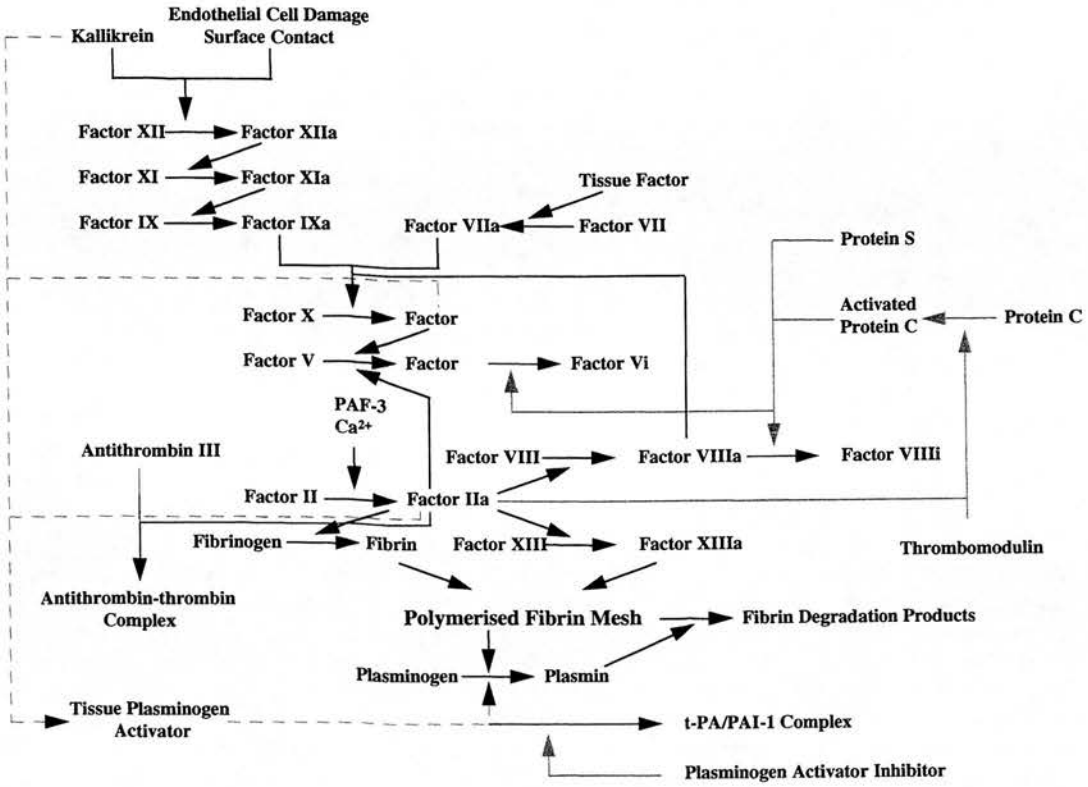

Figure 5. The Coagulation and Fibrinolytic Systems

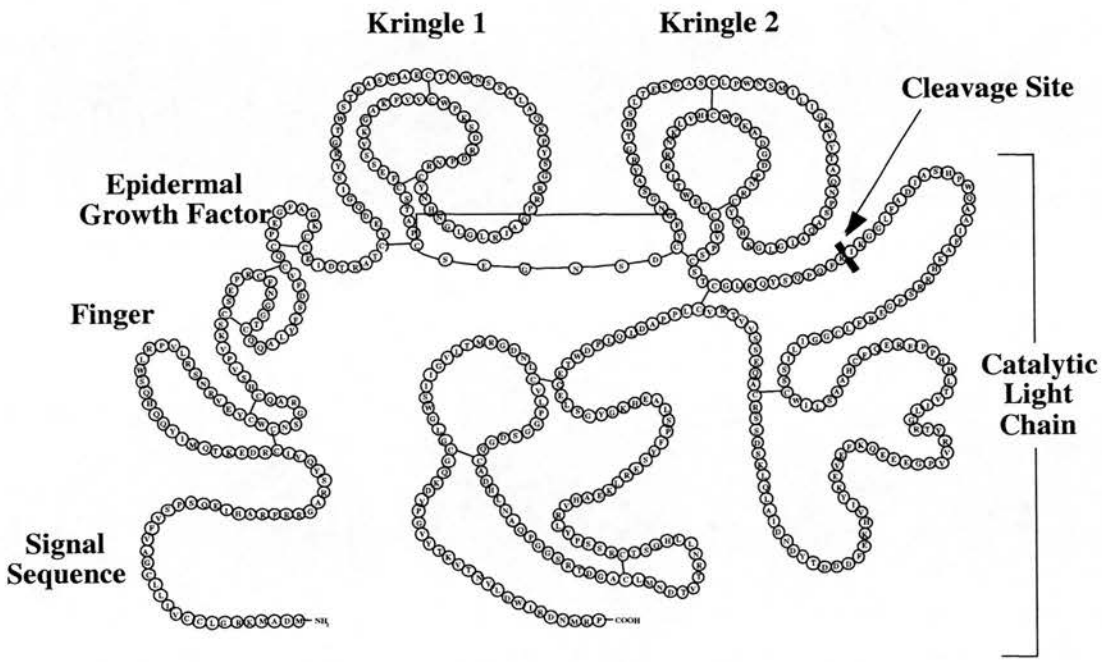

Figure 6. Protein structure of tissue plasminogen activator

spontaneously in a third of patients with acute myocardial infarction within 12 hours of the onset of symptoms [DeWood et al, 1980; Armstrong et al, 1989] and in both healthy individuals [Meade et al, 1993] and patients with cardiovascular disease [Jansson et al, 1993], reduced plasma fibrinolytic activity is associated with an increased risk of myocardial infarction. Detailed postmortem studies have shown that plaque growth is induced by episodic subclinical plaque disruption and thrombus formation [Mann and Davies, 1999]. The prolonged presence of residual thrombus over a disrupted or eroded plaque will provoke smooth muscle migration and the production of new connective tissue, leading to plaque expansion [Mann and Davies, 1999]. Consistent with this, enhanced macrovascular fibrin deposition and atherogenesis are seen in genetic murine models of t-PA deficiency [Christie et al, 1999].

If endogenous fibrinolysis is to be effective then rapid mobilisation of t-PA from the endothelium is essential, because thrombus dissolution is much more effective if t-PA is incorporated during rather than after, thrombus formation [Fox et al, 1985]. The capacity of endothelial cells to release t-PA from intracellular storage pools, and the rapidity with which this can be mobilised, may not necessarily be reflected in the basal plasma concentrations of t-PA antigen or activity. Using a novel model to assess the acute endogenous fibrinolytic potential within the forearm and coronary circulations *in vivo* in man [Newby et al, 1997b], we have demonstrated that acute t-PA release is impaired in smokers [Newby et al, 1999] and in patients with atherosclerosis and the degree of fibrinolytic impairment correlates inversely with atherosclerotic plaque load [Newby et al, 2001].

1.7.3 KININS, ENDOGENOUS FIBRINOLYSIS AND ATHEROTHROMBOSIS

Bradykinin is released during the contact phase of coagulation when high molecular weight kininogen is cleaved by kallikrein to produce a disulphide-linked light and heavy chain [Schiffman et al, 1980; Reddigari and Kaplan, 1988]. Although an inflammatory mediator, bradykinin is also a potent endothelial cell stimulant that can induce the acute release of t-PA from the endothelium [Brown et al, 1999; Labinjoh et al, 2000] through a B_2 receptor mechanism [Brown et al, 2000]. Thus, following activation of the intrinsic coagulation pathway, the liberation of bradykinin may represent an important negative feedback loop in which bradykinin-induced t-PA release inhibits thrombus formation within the vascular lumen when localised endothelial denudation occurs. Moreover, this process is further amplified by the action of plasmin itself which, in combination with kallikrein, augments bradykinin release from high molecular weight kininogen [Kleniewski et al, 1992].

Inhibition of ACE enhances bradykinin-induced t-PA release in healthy volunteers ~2-fold [Labinjoh et al, 2001]. Moreover, in an unblinded, non-randomised study in patients with heart failure, ACE inhibition markedly augmented bradykinin-induced t-PA release (~6- to 10-fold) such that local concentrations of t-PA approach those seen during systemic thrombolytic therapy for acute myocardial infarction [Witherow et al, 2002]. Given the enhanced activation of the kallikrein system and increased bradykinin release seen in clinical studies of patients with unstable angina [Hoffmeister et al, 1995], ACE inhibition may have major beneficial effects on the acute local fibrinolytic balance by markedly enhancing bradykinin-induced t-PA

release in areas of intravascular thrombus formation. Consistent with this, intra-arterial enalprilat administration increases basal t-PA release in the forearm circulation of healthy volunteers and this effect was blocked by the co-administration of the B_2 receptor antagonist, HOE-140 [Pretorius et al, 2003]. Whether combined ACE and NEP inhibition augments bradykinin-induced t-PA release to a greater extent than ACE inhibition alone has not been investigated. Moreover, it has yet to be established whether B_1 kinin receptor agonism causes t-PA release in man.

1.8 HYPOTHESES

In human umbilical vein *in vitro*:

1. The B_1 receptor agonist, Lys-des-Arg^9-bradykinin, causes dose-dependent vasoconstriction that is selectively inhibited by the B_1 antagonist, Lys-des-[Leu^8]-Arg^9-bradykinin, and by the combined kinin receptor antagonist, B9340.
2. The B_2 receptor agonist, bradykinin, causes dose-dependent vasoconstriction that is selectively inhibited by the B_2 antagonist, HOE-140, and by the combined kinin receptor antagonist, B9340.

In patients with heart failure *in vivo*:

1. Bradykinin contributes to the systemic haemodynamic effects of long-term ACE inhibition in patients with heart failure.
2. Combined ACE and NEP inhibition augments the vascular action of bradykinin to a greater extent than ACE inhibition alone.

3. The B_1 kinin receptor mediates vasodilatation and endothelial t-PA release in the peripheral circulation of patients with heart failure treated with ACE inhibition.

1.9 AIMS

The aims of the thesis were:

In human umbilical vein rings (Chapter 3):

- To establish the biological activity of custom-made, selective peptidic kinin receptor agonists and antagonists.

In patients with heart failure treated with long-term ACE inhibition (Chapter 4):

- To determine whether the combined kinin receptor antagonist, B9340, alters systemic haemodynamics.

In patients with heart failure treated with long-term ACE inhibition (Chapter 5):

- To determine whether local NEP inhibition augments bradykinin-mediated vasodilatation and endothelial t-PA release in the forearm vascular bed.
- To determine whether the B_1 kinin receptor agonist, Lys-des-Arg^9-bradykinin, mediates vasodilatation or endothelial t-PA release in the forearm

vascular bed, and if so, to establish whether these effects are augmented by local NEP inhibition.

- To determine whether atrial natriuretic peptide stimulates endothelial t-PA release in the forearm vascular bed.
- To determine whether local NEP inhibition augments atrial natriuretic peptide-mediated vasodilatation in the forearm vascular bed.

In patients with heart failure (Chapter 6):

- To determine the effects of selective B_1 kinin receptor agonism and antagonism on vasodilatation and endothelial t-PA release in the forearm vascular bed.
- To establish the effects of ACE inhibition on B_1 kinin receptor mediated responses in the forearm vascular bed.
- To confirm the effects of ACE inhibition on B_2 kinin receptor mediated responses in the forearm vascular bed.

CHAPTER 2

METHODOLOGY:

UMBILICAL VEIN MYOGRAPHY, IMMUNOHISTOCHEMISTRY AND MEASUREMENT OF FOREARM BLOOD FLOW, SYSTEMIC HAEMODYNAMICS AND PLASMA FIBRINOLYTIC AND NEUROHORMONAL PARAMETERS

2.1 INTRODUCTION

A wide variety of techniques exist for assessing vascular function both *in vitro* and *in vivo*. A general overview of the techniques employed in this thesis is detailed below. Details specific to each study can be found in the methods sections of subsequent chapters.

Myography of isolated vascular tissue *in vitro* is an accurate and reproducible method by which to determine vessel specific vasomotor responses to peptide agonists and antagonists. The major advantage of this *in vitro* technique is that it allows precise and controlled alterations in the local physiological conditions to be made. Extrapolating the results of myography studies to a vascular bed *in vivo* can be difficult, however, as vascular responses may vary significantly depending on the size and type of vessel studied.

These limitations can be largely overcome by combining regional intra-arterial forearm infusion with bilateral venous occlusion plethysmography. This technique allows a direct assessment of peripheral vascular responses to be made *in vivo* using doses 10- to 1,000-fold lower than those required to produce a systemic effect [Benjamin et al, 1995; Webb, 1995]. This dose reduction ensures a reduction in the risk of unwanted or potentially harmful side effects whilst minimising the risk of the confounding cardiac, renal and nervous system reflexes which can occur with systemic dosing. Using these techniques to increase our understanding of tissue and vascular bed specific responses to drug administration will improve our ability to

predict systemic vascular responses and target and design invasive systemic studies appropriately.

Systemic haemodynamic responses to drug administration *in vivo* in man are determined not only by the concomitant effects in different vascular beds, such as the cerebral, pulmonary and coronary circulations, but also by changes in heart rate, myocardial contractility and neurohumoral reflexes [Webb, 1995]. Although a number of invasive and non-invasive techniques exist for assessing systemic haemodynamic changes following drug administration, pulmonary artery catheterisation remains the gold standard [Swan et al, 1970].

2.1.1 Human Umbilical Vein Myography

Myography of isolated vessels from animals has been used traditionally as a sensitive and reliable method with which to examine the vascular effects of the B_1 and B_2 kinin receptors *in vitro* (Figure 7). Recently, however, B_1 kinin receptor vasomotor responses have been identified in two human tissues, human umbilical vein [Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999] and coronary artery [Drummond and Cocks, 1995b]. Of these, the pharmacological responses of human umbilical vein have been characterised most extensively. For this reason, combined with the obvious practical and ethical constraints associated with obtaining viable human coronary artery, we chose to confirm the biological activity of our custom-made kinin peptides *in vitro* using myography of human umbilical vein.

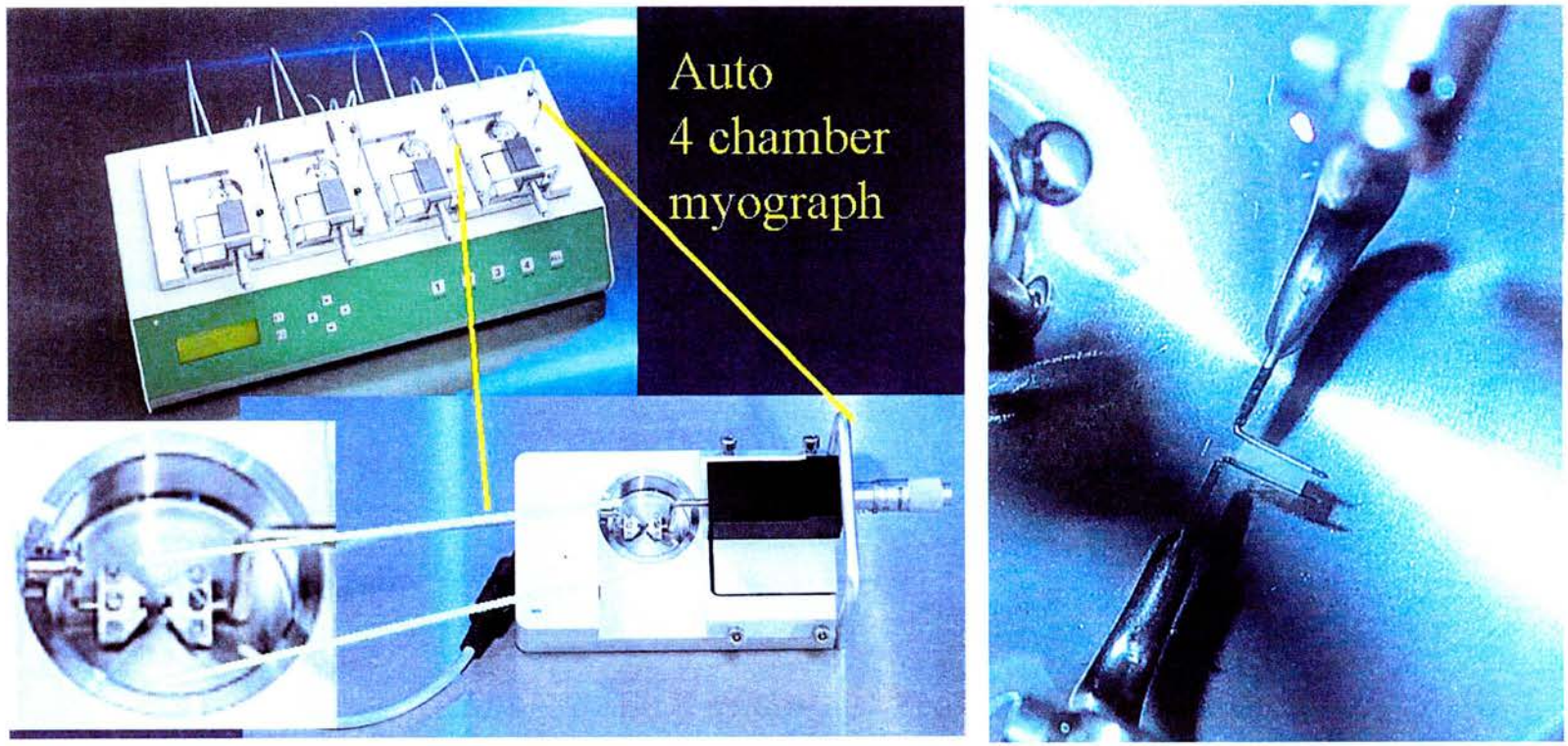

Figure 7. Four chamber myograph (above). Vessel ring under tension (right).

2.1.2 FOREARM RESISTANCE VESSELS

Venous occlusion plethysmography, combined with unilateral brachial artery infusion of pharmacological agonists and antagonists, is a powerful and reproducible method for directly assessing vascular receptor physiology in the forearm circulation *in vivo* (Figure 8) [Wilkinson et al, 2001]. This technique has been utilised very successfully to demonstrate that bradykinin stimulates vasodilatation [Cockcroft et al, 1994] and endothelial t-PA release [Brown et al, 2000] via the B_2 kinin receptor in the peripheral circulation of healthy man. Downstream signalling mechanisms, however, differ between vasodilatation and t-PA release, as well as the vascular bed being studied.

In the human forearm circulation, high K^+ concentrations [Halcox et al, 2001] and inhibitors of K^+ transport across the vascular smooth muscle cell membrane [Honing et al, 2000; Halcox et al, 2001; Inokuchi et al, 2003; Dwivedi et al, 2005], but not inhibitors of prostaglandin synthesis [Honing et al, 2000] attenuate bradykinin-mediated vasodilatation, The effects of inhibition of nitric oxide synthase on bradykinin-mediated endothelium-dependent vasodilatation in the human forearm circulation are less clear, previous work reporting either no effect [Brown et al, 2000] or a modest attenuation of bradykinin-mediated vasodilatation [Cockcroft et al, 1994]. Taken together, these findings suggest a role for endothelium-derived hyperpolarising factor, and potentially, nitric oxide, in mediating the vasodilator effects of bradykinin in the human forearm circulation. In the coronary circulation *in vivo* in man, inhibition of nitric oxide synthase attenuates the bradykinin-mediated increase in coronary artery diameter and blood flow [Kuga et al, 1995]. The mechanism by which bradykinin induces t-PA release downstream of the B_2 kinin

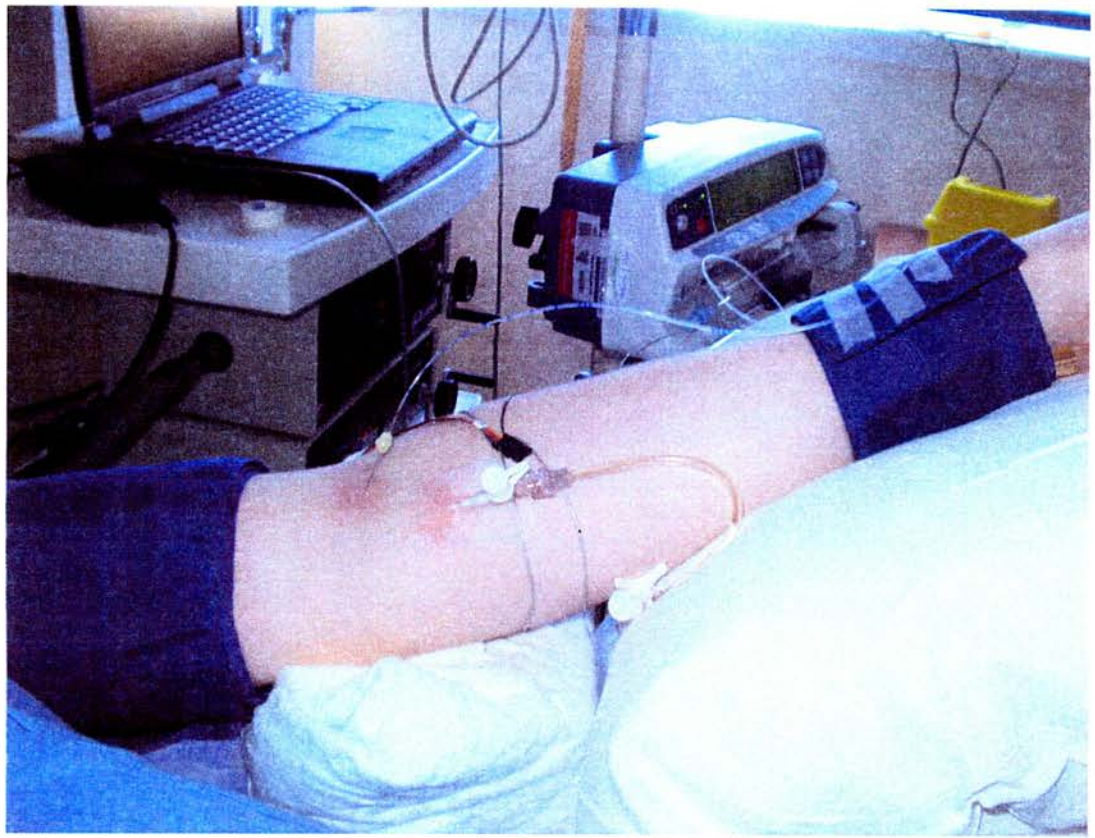

Figure 8. Forearm plethysmography set-up.

receptor is less clear. As with bradykinin-mediated vasodilatation, inhibition of nitric oxide synthase and prostaglandin synthesis does not attenuate the bradykinin-induced increase in t-PA release in the human forearm circulation *in vivo* [Brown et al, 2000]. The effects of inhibitors of endothelium-derived hyperpolarising factor on bradykinin-mediated t-PA release are not known.

Using forearm plethysmography, we have recently demonstrated that combined B_1 and B_2 receptor antagonism, but not B_2 receptor antagonism alone, causes vasoconstriction in the forearm circulation of patients with heart failure treated with ACE inhibition [Witherow et al, 2001]. Besides providing the first evidence of a potential vasomotor role for the B_1 receptor *in vivo* in man, these results would suggest that systemic inhibition of vascular kinin receptors would increase blood pressure and alter systemic haemodyamics in the patients with heart failure treated with ACE inhibition.

2.1.3 Systemic Haemodynamics

Thermodilution-based continuous cardiac output monitoring, in combination with pulmonary artery catheterisation has been established as an accurate and reproducible method for repeatedly assessing systemic haemodynamics *in vivo* in man [Ditmyer et al, 1995]. This technique employs a thermal filament which transfers a safe level of heat to the surrounding blood in a pulsed, on-off manner every 30 seconds (Figure 9). Changes in the temperature of the blood are measured by a distal rapid-response thermistor in the pulmonary artery. The cardiac output is then calculated automatically using a modified Stewart-Hamilton equation [Yelderman, 1993]. This technique, combined with intravenous drug administration, has been used to investigate the haemodynamic effects of systemic drug

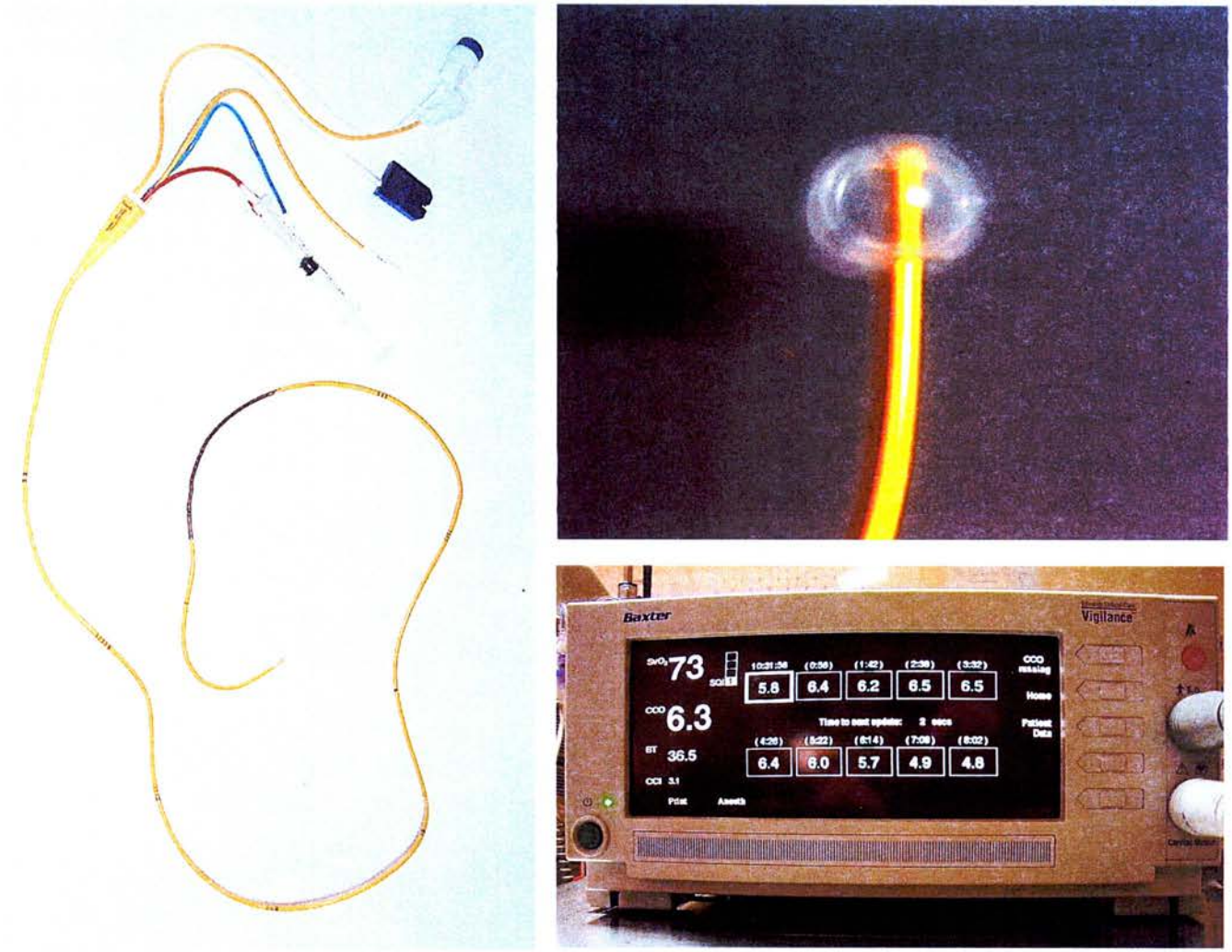

Figure 9. Swan-Ganz catheter (left), with balloon inflated (right upper) and Vigilance monitor (right lower).

administration in a variety of patient populations including patients with heart failure [Leslie et al, 2005].

2.2 GENERAL

2.2.1 ETHICAL CONSIDERATIONS

All studies were performed with the approval of the Lothian Research Ethics Committee in accordance with the Declaration of Helsinki and with the written informed consent of each subject.

2.2.2 UMBILICAL CORD

Human umbilical cord was obtained from women aged 16-40 years undergoing routine caesarean section following uncomplicated pregnancy.

2.2.3 PATIENT PREPARATION

All patients with heart failure abstained from alcohol for 24 hours and from food, tobacco and caffeine-containing drinks for at least 4 hours before each study. Studies were performed in a quiet, temperature controlled room maintained at 23-25°C and diuretics were withheld on days for patient comfort.

2.2.4 BLOOD PRESSURE MEASUREMENT

Blood pressure was recorded at intervals throughout patient studies using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda

Medical Inc, Japan). During forearm venous occlusion plethysmography, blood pressure was recorded in the non-infused arm.

2.3 UMBILICAL VEIN MYOGRAPHY

2.3.1 TISSUE PREPARATION

Immediately after delivery, 10 cm umbilical cord was excised midway between placenta and child, and placed in Krebs buffer solution (NaCl 6.954 nmol/L, KCl 4.7 nmol/L, $CaCl_2$ 2.5 nmol/L, $MgSO_4$ 1.17 nmol/L, $NaHCO_3$ 2 nmol/L, KH_2PO_4 1.18 nmol/L, EDTA 0.027 nmol/L, glucose 5.5 nmol/L; Fisher Scientific UK Ltd, Loughborough UK). Human umbilical vein was carefully dissected from the cord and cut into rings of approximately 3 mm width [Sardi et al, 1998; Sardi et al, 1999].

2.3.2 CHANGES IN VESSEL TENSION

Three hours after delivery, umbilical vein rings were mounted on wire myographs, suspended in organ baths containing 10 mL Krebs solution and stretched with an initial tension of 2 g (Multimyograph System 700MO, JP Trading, Denmark). Kreb's solution was maintained at 37°C and continually bubbled with 95% O_2 / 5% CO_2. Changes in tension were measured using an isometric transducer (Mac Lab 8, Analog Digital Instruments Pty Ltd, Australia). Following 60 minutes equilibration, during which the tension was readjusted at 15 minute intervals, maximal contraction to KCl (60 mmol/L) was determined on three occasions, interspersed by 15 minute washout periods to assess tissue viability. Maximal contraction to 60 mmol/L KCl was determined on a final occasion at the end of each experiment.

2.3.3 Data Analysis

Myography data were extracted from Chart™ data files and changes in tension expressed as a percentage of the maximal contraction to 60 mmol/L KCl determined at the end of each experiment.

2.4 IMMUNOHISTOCHEMISTRY

2.4.1 Tissue Preparation

Following contractile studies, human umbilical vein rings were fixed in formalin and embedded in paraffin wax. Tissue was boiled in 0.01 mol/L sodium citrate (pH 6.0) for 8 minutes to facilitate antigen retrieval. A haemotoxylin and eosin stain was performed for each tissue specimen.

2.4.2 Immunostaining

B_1 and B_2 receptors were immunodetected using diaminobenzidine (DAB) immunoprecipitation. Non-specific binding sites were blocked with 1:100 donkey serum followed by 3% H_2O_2. Tissue sections were then incubated with the primary affinity purified goat polyclonal antibody specific for human the B_1 or B_2 kinin receptor (Santa Cruz Biotechnology, Inc., USA) followed by the secondary antibody (donkey anti-goat IgG; Santa Cruz Biotechnology Inc, USA) or with the secondary antibody alone (control). Finally, tissues were treated with a peroxidase-antiperoxidase strepavidin-biotin conjugating system (Dako K0690) and visualised by conventional light microscopy using liquid DAB precipitant (Dako K3465).

2.5 FOREARM VENOUS OCCLUSION PLETHYSMOGRAPHY

2.5.1 BRACHIAL ARTERY CANNULATION

The brachial artery of the non-dominant arm was cannulated under local anaesthesia using a 27-gauge needle (Cooper's Needle Works Ltd, Birmingham, UK). The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) and patency maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusion remained constant throughout all studies at 1 mL/min.

2.5.2 BLOOD FLOW MEASUREMENT

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges that were applied to the widest part of the forearm [Webb, 1995]. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg pressure for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson) was processed by a MacLab® analogue-to-digital converter and Chart™ *v*3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers Inc,

Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

2.5.3 Data Analysis

Plethysmographic data were extracted from the Chart™ data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel *v*5.0; Microsoft). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this incurs [Webb, 1995]. Usually, the last five flow recordings in each 3 minute measurement period were calculated and averaged for each arm. Blood flow data was expressed as the absolute change in blood flow in the infused arm, or where appropriate, to reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm [Benjamin et al, 1995; Webb, 1995]. Percentage changes in the infused forearm blood flow were calculated [Benjamin et al, 1995; Webb, 1995] as follows:

$$\% \text{ Change in blood flow} = 100 \times \{I_t/NI_t - I_b/NI_b\} / I_b/NI_b$$

where I_b and NI_b are the infused and non-infused forearm blood flows at baseline (time 0) respectively, and I_t and NI_t are the infused and non-infused forearm blood flows at a given time point respectively.

2.6 SWAN-GANZ CATHETERISATION

2.6.1 Right Heart Catheterisation

A 9F venous sheath was inserted aseptically under local anaesthesia via the right femoral vein. Under fluoroscopic screening, a continuous cardiac output thermodilution Swan-Ganz catheter (Edwards Lifesciences, Irvine, CA, USA) was positioned in the pulmonary arterial tree.

2.6.2 Systemic Haemodynamic Measurement

Pulmonary arterial pressure, pulmonary arterial wedge pressure and central venous pressure were recorded using a Hewlett Packard monitor (U78339A, Hewlett Packard, Andover, MA, USA). Continuous cardiac output was recorded using a Vigilance monitor (Edwards Lifesciences, Irvine, CA, USA).

2.6.3 Data Analysis

Mean instantaneous cardiac output was determined as the average of three cardiac output measurements.

2.7 FIBRINOLYTIC PARAMETERS, HAEMATOCRIT AND NEUROHORMONES

2.7.1 Forearm Venous Sampling

Ten millilitres of blood were withdrawn simultaneously from bilateral 17-gauge venous cannulae positioned in the antecubital fossa and collected into acidified

buffered citrate (Biopool Stabilyte, Umeå) for t-PA assays and citrate (Monovette, Sarstedt, Numbrecht) for PAI-1 assays. Samples were kept on ice before being centrifuged at 2,000 *g* for 30 minutes at +4°C. Platelet-free supernatant was decanted and stored at -70°C before assay.

2.7.2 Systemic Venous Sampling

Thirty millilitres of blood was collected from the side arm of the venous sheath into lithium heparin for determination of plasma ACE activity and 0.45% o-phenanthroline/4.65% disodium ethylene diamine tetraacetic acid for determination of plasma angiotensin II concentrations. Blood samples were collected on ice, centrifuged immediately and the resulting supernatant stored at -70°C until assayed.

2.7.3 Plasma Fibrinolytic Parameter Assays

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay; Coaliza® PAI-1 [Declerck and Collen, 1990] and Coaliza® t-PA [Booth et al, 1987] (Chromogenix AB, Mölndal, Sweden) respectively and t-PA activity, by a photometric method, Coaset® t-PA [Gram et al, 1987] (Chromogenix AB). The intra-assay coefficients of variation were 5.5 and 2.4% for t-PA antigen and activity respectively and 7.0% for PAI-1 antigen. The inter-assay coefficients of variation were 4.0, 4.0 and 7.3% respectively. The sensitivities of the assays were 0.5 ng/mL, 0.1 IU/mL and 2.5 ng/mL respectively.

2.7.4 Plasma Haematocrit

Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid.

2.7.5 Plasma Neurohormone Assays

Plasma ACE activity was determined using colourimetric spectrophotometry (Sigma Diagnostics, St Louis, MO, USA) [Holmquist et al, 1979]. Following extraction using Bond Elut® columns (Varian, Harbor City, CA, USA), plasma angiotensin II (Diasorin, Stillwater, MN, USA) concentrations were determined by radioimmunoassay [Morton et al, 1985].

2.7.6 Data Analysis

Estimated net release of t-PA antigen and activity were defined as the product of the infused forearm plasma flow (based on the mean haematocrit and forearm blood flow) and the concentration difference between the infused ($[t\text{-}PA]_{Inf}$) and non-infused arms ($[t\text{-}PA]_{Non\text{-}inf}$) [Newby et al, 1997b; Newby et al, 1999].

Estimated net forearm t-PA release=$FBF \times \{1\text{-}Hct\} \times \{[t\text{-}PA]_{Inf} - [t\text{-}PA]_{Non\text{-}inf}\}$.

CHAPTER 3

VASOMOTOR EFFECTS OF KININ RECEPTOR AGONISTS AND ANTAGONISTS IN ISOLATED HUMAN UMBILICAL VEIN

3.1 SUMMARY

The current study was performed to confirm the biological activity of custom-made selective peptidic kinin receptor agonists and antagonists in human tissue *in vitro* prior to their use in clinical studies *in vivo*. Following pre-incubation with captopril, contraction of human umbilical vein rings to increasing concentrations of Lys-des-Arg^9-bradykinin (B_1 receptor agonist) or bradykinin (B_2 receptor agonist) in the presence or absence of Lys-[Leu^8]-des-Arg^9-bradykinin (B_1 antagonist), HOE-140 (B_2 antagonist) or B9340 (combined B_1/B_2 antagonist) was measured using myography. Lys-des-Arg^9-bradykinin and bradykinin caused dose-dependent constriction of human umbilical vein rings ($p<0.001$ for both). Lys-[Leu^8]-des-Arg^9-bradykinin and B9340 caused an approximate 10-fold rightwards shift, whilst HOE-140 caused a modest leftwards shift in the dose response curve for Lys-des-Arg^9-bradykinin ($p<0.001$, $p<0.001$ and $p<0.05$ respectively). In contrast, HOE-140 and B9340, but not Lys-[Leu^8]-des-Arg^9-bradykinin, caused a rightwards shift in the dose response curve for bradykinin ($p<0.001$, $p<0.01$ and p=not significant respectively). These findings confirm the biological activity of the custom manufactured preparations of peptidic kinin receptor agonists and antagonists in human tissue *in vitro*. In addition, augmentation of B_1 mediated responses in the presence of a B_2 receptor antagonist supports the hypothesis that cross-talk may exist between the kinin receptor subtypes.

3.2 INTRODUCTION

First proposed in 1977 [Regoli et al, 1977], subsequent work has confirmed the existence of two human kinin receptor subtypes, B_1 and B_2. With the advent of molecular cloning, the primary structure of both kinin receptors has been established, identifying them as members of the G-protein superfamily characterised by seven transmembrane α helices [McEachern et al, 1991; Menke et al, 1994].

Functional expression of vascular kinin receptors has traditionally been characterised by smooth muscle responses to selective kinin receptor agonists in organ bath preparations [Regoli et al, 1977; Drummond and Cocks, 1995a; Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999]. Using this technique, B_1 and B_2 receptor mediated responses have been identified in tissues from a wide variety of vascular sites and species, including human umbilical vein [Regoli et al, 1977; Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999] and coronary artery [Drummond and Cocks, 1995b]. Human umbilical vein examined in this way exhibits constitutive B_2 receptor mediated responses, as well as B_1 receptor mediated responses, which are absent initially but develop in magnitude as a function of the *in vitro* incubation time [Bouthillier et al, 1987; Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999]. These responses can also be invoked by pre-incubation of tissue with bacterial lipopolysaccharide or inflammatory cytokines, such as interleukin-1β [Sardi et al, 1998] and tumour necrosis factor-α [Sardi et al, 1999].

Bradykinin has high affinity and selectivity for the human B_2 receptor and is the principal ligand in plasma. Removal of the C-terminal arginine from bradykinin and related peptides is a key determinant of affinity at the human B_1 receptor. Although des-Arg^9-bradykinin is the most abundant B_1 agonist present in plasma, it has only modest affinity for the human B_1 receptor and retains some activity at the human B_2 receptor, being only 100-fold more selective for the human B_1 receptor [Marceau et al, 1998; Leeb-Lundberg et al, 2005]. In contrast, Lys-des-Arg^9-bradykinin, a tissue-based metabolite of kallidin, is the only naturally occurring kinin sequence with subnanomolar affinity at the human B_1 receptor. Indeed, Lys-des-Arg^9-bradykinin has ~1000-fold greater affinity for the B_1 receptor than des-Arg^9-bradykinin, and is inactive at the B_2 receptor in man [Gobeil et al, 1996; Marceau et al, 1998; Christiansen et al, 2002; Leeb-Lundberg et al, 2005].

Development of peptidic B_1 receptor antagonists began in the 1970s with modification of the bradykinin sequence to produce [Leu^8]-des-Arg^9-bradykinin [Regoli et al, 1977; Stewart et al, 1997]. Although highly selective for the human B_1 receptor, [Leu^8]-des-Arg^9-bradykinin retains some partial agonist activity [Marceau et al, 1998]. Further modification led to the development of Lys-[Leu^8]-des-Arg^9-bradykinin, a synthetic peptide with potent selective inhibitory activity at the human B_1 receptor [Marceau et al, 1998; Deblois et al, 2001]. Peptide antagonists for the B_2 receptor were developed later. Of these, icatibant, or HOE-140, is a synthetic peptide antagonist with high affinity and selectivity at the human B_2 receptor that has been used most extensively to investigate B_2 kinin receptor physiology in animal models and in man *in vivo*. In contrast, B9340 is a synthetic peptide sequence with

significant antagonist activity at both the B_1 and B_2 kinin receptor [Stewart et al, 1997].

We obtained bradykinin, Lys-des-Arg^9-bradykinin, Lys-[Leu^8]-des-Arg^9-bradykinin, HOE-140 and B9340 custom manufactured for use in clinical studies. Using organ bath myography, the aims of this study were to confirm the biological activity of these custom-made peptides in human umbilical vein *in vitro* prior to use in clinical studies.

3.3 METHODS

3.3.1 TISSUE

Human umbilical cord was obtained from women aged 16-40 years undergoing routine caesarean section following uncomplicated pregnancy with the approval of the Local Research Ethics Committee, in accordance with the Declaration of Helsinki and with the written informed consent of each subject.

3.3.2 DRUGS

Captopril (Sigma Pharmaceuticals, St Louis, MO) and pharmaceutical grade Lys-des-Arg^9-bradykinin (Clinalfa AG, Läufelfingen, Switzerland), bradykinin (Clinalfa), Lys-[Leu^8]-des-Arg^9-bradykinin (Clinalfa), HOE-140 (Clinalfa) and B9340 (Clinalfa) were dissolved in physiological saline on the day of study. The doses of peptides were chosen based on the results of affinity binding studies [Regoli and Barabe, 1980 Marceau et al, 1998; Leeb-Lundberg et al, 2005] and data from

previous human umbilical vein myography studies [Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999].

3.3.3 Study Design

Tissue rings were mounted on the myograph, allowed to equilibrate for 60 minutes and maximum contraction to KCl (60 mmol/L) determined. Tissue rings were then incubated with captopril (1 μmol/L) 30 minutes prior to performing cumulative dose responses to bradykinin (10^{-11} - 10^{-6} mol/L) and Lys-des-Arg^9-bradykinin (10^{-11} - 10^{-6} mol/L) in the presence or absence of Lys-[Leu^8]-des-Arg^9-bradykinin (1 μmol/L), HOE-140 (1 μmol/L) or B9340 (1 μmol/L). Peptide antagonists were applied 10 minutes before agonists to ensure that equilibrium was obtained. Maximal contraction to 60 mmol/L KCl was determined on a final occasion at the end of each experiment. A single concentration curve for bradykinin and Lys-des-Arg^9-bradykinin was obtained for each ring, and experiments were performed in parallel with rings from the same tissue.

3.3.4 Immunohistochemistry

Following contractile studies, human umbilical vein rings were fixed in formalin and embedded in paraffin wax. A haemotoxylin and eosin stain was performed for each tissue section and B_1 and B_2 receptors were immunodetected using DAB immunoprecipitation.

3.3.5 DATA ANALYSIS AND STATISTICS

Human umbilical vein responses are expressed as a percentage of the maximal contraction to 60 mmol/L KCl obtained at the end of each experiment. Data were examined by two-way analysis of variance (ANOVA) with repeated measures using GraphPad PRISM (GraphPad). All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level.

3.4 RESULTS

Immunohistochemistry confirmed intense immunolabelling of both B_1 and B_2 receptors on human umbilical vein (Figure 10). Consistent with previous work [Gobeil et al 1996; Sardi et al, 1998], bradykinin and Lys-des-Arg^9-bradykinin caused dose-dependent constriction of human umbilical vein rings (Figure 11; $p<0.001$ for both). Lys-[Leu^8]-des-Arg^9-bradykinin and B9340 caused an approximate 10-fold rightwards shift and HOE-140 caused a modest leftwards shift in the dose response curve for Lys-des-Arg^9-bradykinin (Figure 11a; $p<0.001$, $p<0.001$ and $p<0.05$ respectively). In contrast, HOE-140 and B9340, but not Lys-[Leu^8]-des-Arg^9-bradykinin, caused a rightwards shift in the dose response curve for bradykinin (Figure 11b; $p<0.001$, $p<0.001$ and p=not significant respectively).

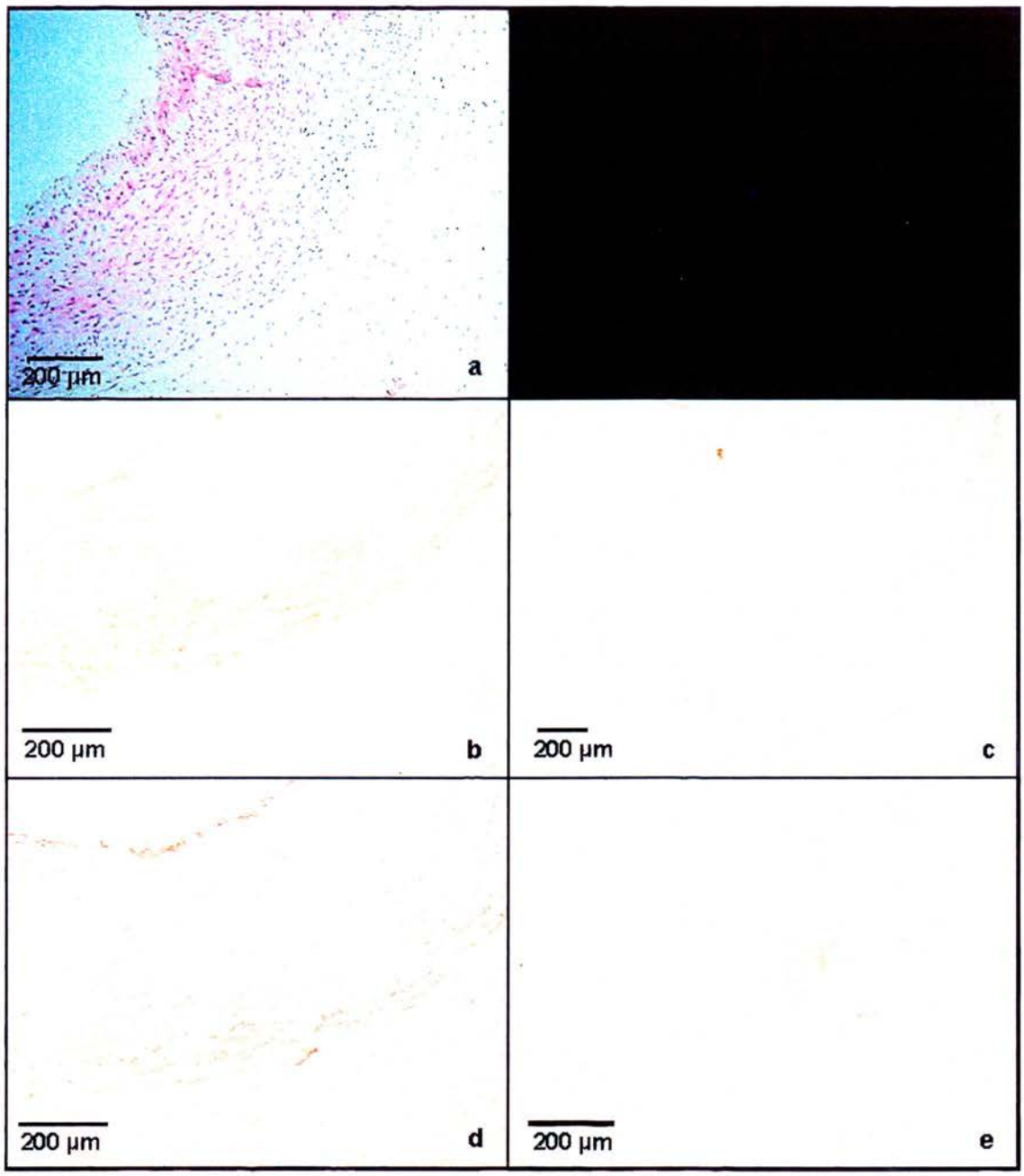

Figure 10. Human umbilical vein ring stained with haemotoxylin and eosin (a). Immunolabelling (DAB) of B_1 (b) and B_2 (d) kinin receptors on smooth muscle cells of human umbilical vein rings and their respective controls (c and e).

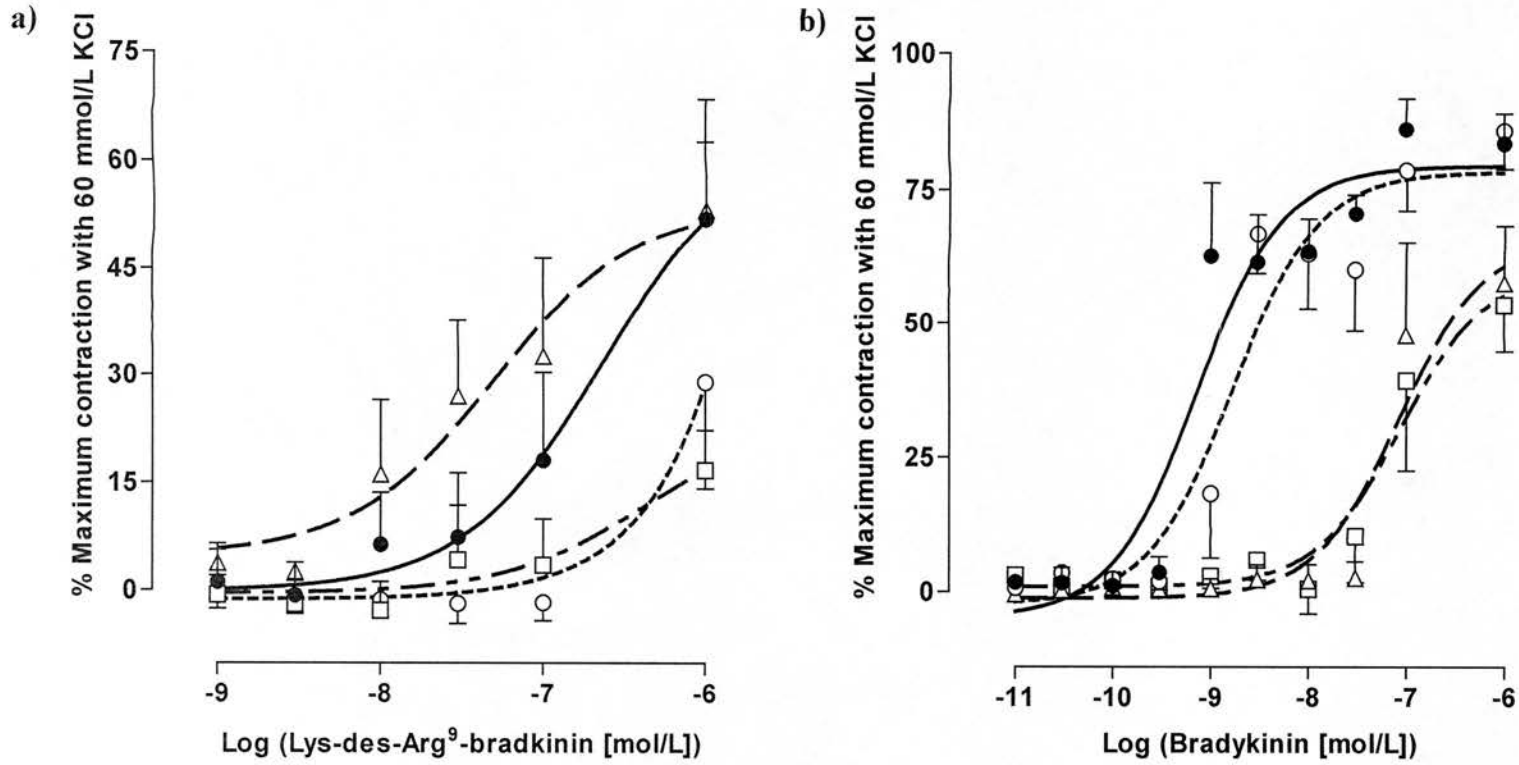

Figure 11. Contraction of umbilical vein rings to (a) Lys-des-Arg^9-bradykinin (solid circles, solid line) and (b) bradykinin alone (solid circles, solid line) or in the presence of Lys-[Leu^8]-des-Arg^9-bradykinin (1 μmol/L; open circles, fine dash), HOE-140 (1 μmol/L; open triangles, large dash) or B9340 (1 μmol/L; open square, intermittent dash). (a) Lys-[Leu^8]-des-Arg^9-bradykinin and B9340 caused a rightwards shift and HOE-140 caused a modest leftward shift in the dose response curve for Lys-des-Arg^9-bradykinin ($p<0.001$, $p<0.001$ and $p<0.05$ respectively; ANOVA). (b) HOE-140 and B9340, but not Lys-[Leu^8]-des-Arg^9-bradykinin, caused a rightwards shift in the dose response curve for bradykinin ($p<0.001$, $p<0.001$ and p=not significant respectively; ANOVA).

3.5 DISCUSSION

We have demonstrated both histologically and functionally that B_1 and B_2 kinin receptors are present in human umbilical vein. Using myography of human umbilical vein rings, we have confirmed that the vasomotor effects of the B_1 agonist, Lys-des-Arg^9-bradykinin, and the B_2 agonist, bradykinin, are selectively attenuated by the B_1 kinin receptor antagonist, Lys-[Leu^8]-des-Arg^9-bradykinin, and the B_2 receptor antagonist, HOE-140, respectively. In addition, the combined B_1/B_2 receptor antagonist, B9340, significantly attenuated the vasomotor activity of both Lys-des-Arg^9-bradykinin and bradykinin confirming blockade at both the B_1 and B_2 receptor. These findings confirm that clinical grade preparations of kinin and kinin-related peptides are biologically active in human tissue.

Human umbilical vein cut into rings has greater mechanical activity than when cut longitudinally due to the circular arrangement of the outer layer of smooth muscle [Altura et al, 1972]. Previous studies have demonstrated that myography of human umbilical vein is an appropriate model with which to assess vascular kinin receptor function [Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999]. In the present studies, as with previous work, the biological activity of peptides was assessed in the presence of captopril to prevent degradation by ACE [Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999]. The concentration-response curves obtained for bradykinin and Lys-des-Arg^9-bradykinin were comparable with data from these previous studies [Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999] and confirm efficacy at concentrations predicted to be achieved in the infused human forearm circulation.

The vasomotor effects of bradykinin have been studied in arteries and veins from a variety of different human tissues. In man, bradykinin is relaxant in all vessels except umbilical vein and artery although the mechanisms responsible for this remain unclear [Regoli and Barabe, 1980]. It has been suggested that stimulation of kinin receptors expressed on vascular smooth muscle leads to vasoconstriction, whilst stimulation of endothelial kinin receptors results in vasodilatation. Differences in downstream signalling pathways may also account for the contrasting vasomotor responses.

The B_1 and B_2 kinin receptors are coupled to similar G-protein subtypes and share the same intracellular signalling pathways [Leeb-Lundberg et al, 2005]. It has been suggested that cross-talk may exist between the two receptor subtypes [Duka et al, 2001; Barki-Harrington et al, 2003]. In transgenic mice lacking the B_2 kinin receptor, for example, the B_1 receptor is upregulated and assumes vascular functions normally associated with the B_2 receptor [Duka et al, 2001]. Our findings that the B_2 receptor antagonist, HOE-140, augments the vasomotor responses to the B_1 agonist, Lys-des-Arg^9-bradykinin, in human umbilical vein *in vitro* are in keeping with this hypothesis.

In summary, we have confirmed that Lys-des-Arg^9-bradykinin and bradykinin are potent vasoconstrictors in human umbilical vein *in vitro* and these effects are blocked by the selective kinin receptor antagonists, Lys-[Leu^8]-des-Arg^9-bradykinin and HOE-140 respectively, and in both cases by the combined receptor antagonist,

B9340. Our findings confirm the biological activity of clinical grade peptidic kinin receptor agonists and antagonists in human vascular tissue.

CHAPTER 4

BRADYKININ CONTRIBUTES TO THE SYSTEMIC HAEMODYNAMIC EFFECTS OF CHRONIC ANGIOTENSIN-CONVERTING ENZYME INHIBITION IN PATIENTS WITH HEART FAILURE

4.1 SUMMARY

Bradykinin is an endogenous vasodilator that may contribute to the systemic effects of ACE inhibitor therapy. Using B9340, a bradykinin receptor antagonist, we determined the contribution of bradykinin to the systemic haemodynamic effects of long-term ACE inhibition in patients with chronic heart failure. Fourteen patients with heart failure received enalapril (10 mg twice daily) or losartan (50 mg twice daily) in a randomised double-blind cross-over trial. After 6 weeks treatment, patients underwent right heart catheterisation and were randomised to an intravenous infusion of B9340 (2-20 μg/kg/min) or saline placebo. Following B9340 infusion in patients treated with enalapril, mean arterial pressure (+5.2 mmHg), systemic vascular resistance (+315 dynes.s/cm^5), pulmonary arterial wedge pressure (-1.4 mmHg) and mean pulmonary arterial pressure (-1.3 mmHg) were greater compared to losartan ($p<0.005$, $p=0.07$, $p<0.0001$ and $p<0.05$ respectively) or placebo infusion ($p\leq0.005$ for all). There was a reduction in cardiac output after B9340 with enalapril compared to placebo ($p<0.001$) but not losartan. We have demonstrated that bradykinin contributes to the systemic haemodynamic effects of long-term ACE inhibition in patients with heart failure. Our findings may explain the apparent clinical differences between ACE inhibitors and angiotensin receptor blockers in the treatment of heart failure.

4.2 INTRODUCTION

Activation of the renin-angiotensin-aldosterone system plays a key role in the pathogenesis of chronic heart failure, leading to salt and water retention and peripheral vasoconstriction. Inhibitors of ACE reduce peripheral vascular tone and systemic arterial pressure [Powers et al, 1982] and improve symptoms and survival in patients with chronic heart failure [CONSENSUS, 1987]. Although previously attributed to a reduction in angiotensin-mediated vasoconstriction, recent data have indicated that the haemodynamic changes associated with ACE inhibitor therapy may be due, at least in part, to the inhibition of bradykinin metabolism.

The bradykinin metabolising properties of ACE (kininase II) were first described in 1967 [Erdös and Yang, 1967]. Indeed, ACE has greater affinity for bradykinin than for angiotensin I [Jaspard et al, 1993] and is the principal enzyme responsible for the rapid turnover of bradykinin (plasma half-life ~15 seconds) [Bonner et al, 1990]. This rapid metabolism makes accurate measurement of bradykinin difficult, although elevated plasma bradykinin concentrations have been reported in the presence of ACE inhibition [Pellacani et al, 1994]. Bradykinin is a powerful endothelium-dependent vasodilator and, at a functional level, inhibition of ACE potentiates the action of exogenous bradykinin in the human forearm and coronary circulations [Benjamin et al, 1989; Kuga et al, 1997; Witherow et al, 2002].

There are two principal kinin receptor subtypes in man, B_1 and B_2 [Leeb-Lundberg et al, 2005]. The endothelial B_2 receptor is constitutively expressed and mediates the

vasodilator and pro-fibrinolytic effects of bradykinin [Cockcroft et al, 1994; Brown et al, 2000]. The vascular B_1 receptor is normally expressed very weakly but is markedly upregulated in the presence of inflammation, cardiovascular disease states [McLean et al, 2000b] and ACE inhibition [Nwator and Whalley, 1989; Marin-Castano et al, 2002], where it also mediates vasodilatation [Drummond and Cocks, 1995b].

The selective kinin receptor antagonists, HOE-140 (icatibant) and B9340, are synthetic peptide analogues of bradykinin with different inhibitory activities at the specific kinin receptor subtypes. HOE-140 acts solely at the B_2 receptor, whereas B9340 blocks kinin activity mediated via both B_1 and B_2 receptors [Stewart et al, 1997]. Several investigators have reported that intravenous HOE-140 attenuates the vasodepressor response associated with acute ACE inhibition, both in healthy volunteers and in hypertensive patients [Gainer et al, 1998; Squire et al, 2000]. In contrast, B9340, but not HOE-140, induced vasoconstriction in the forearm circulation of patients with heart failure treated with chronic ACE inhibitor therapy [Davie et al, 1999; Witherow et al, 2001]. Besides implicating bradykinin in the haemodynamic changes associated with ACE inhibition, these data raise the possibility of a role for the B_1 receptor in patients with chronic heart failure [Witherow et al, 2001].

The aims of the present study were to demonstrate that the kinin receptor antagonist, B9340, inhibits bradykinin activity in the systemic circulation and to determine

whether endogenous bradykinin contributes to the haemodynamic effects of long-term ACE inhibition in patients with chronic symptomatic heart failure.

4.3 METHODS

4.3.1 PATIENTS

Fourteen patients with stable New York Heart Association (NYHA) class II-III chronic heart failure and objective evidence of left ventricular impairment (left ventricular ejection fraction <40%, shortening fraction <20%, or left ventricular end-diastolic diameter >5.5 cm) were enrolled into the study. Patients were included only if they had been established on maximally tolerated ACE inhibitor therapy for at least 6 months. Patients were excluded if they had significant valvular heart disease, renal or hepatic failure, or had suffered previous malignant ventricular arrhythmias.

4.3.2 DRUGS

B9340 (molecular weight 1318.6) is a synthetic peptide antagonist of bradykinin with potent inhibitory activity at both the B_1 and B_2 receptors (pIC50 *in vitro* of 8.1 and 9.8, respectively) [Stewart et al, 1997]. The doses of B9340 and bradykinin were chosen based on the results of dose ranging studies performed in the forearm and systemic circulations of healthy volunteers [Witherow et al, 2003; Cruden et al, 2004] (Figure 12). Pharmaceutical grade B9340 was supplied by Clinalfa AG (Läufelfingen, Switzerland) and dissolved in saline on the day of study.

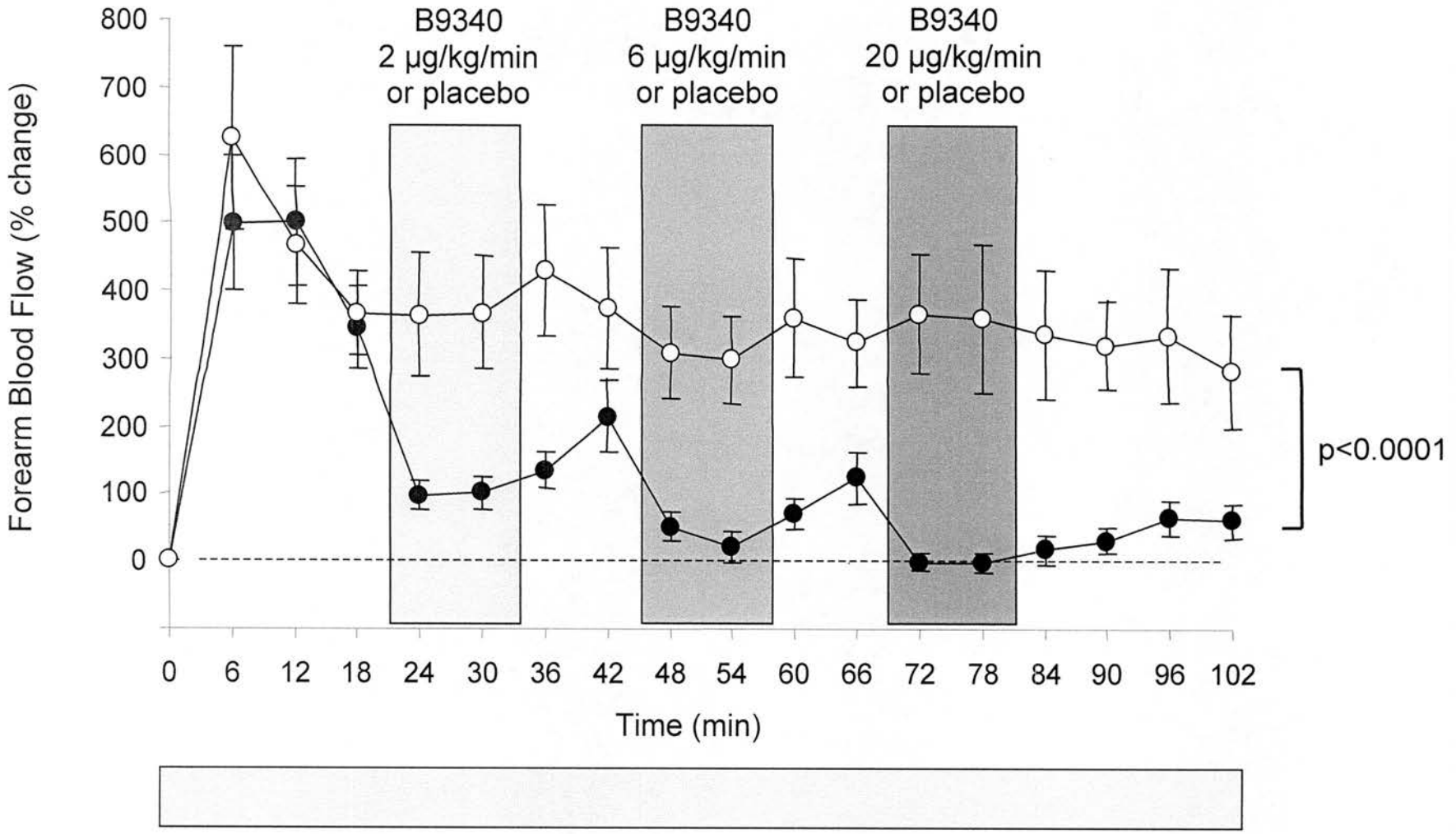

Figure 12. Effect of systemic intravenous B9340 (2-20 µg/kg/min; closed circles) or placebo infusion (open circles) on forearm blood flow measured using venous occlusion plethysmography during intra-brachial infusion of bradykinin in healthy volunteers (n=6) [Cruden et al, 2004].

4.3.3 Study Design

Following initial screening, patients received enalapril 10 mg [CONSENSUS, 1987] or losartan 50 mg [Pitt et al, 2000], both twice daily, in place of their usual ACE inhibitor in a randomised double-blind cross-over trial. After 6 weeks of each treatment, subjects attended fasted at 8.00 a.m. and underwent right heart catheterisation. On the morning of each visit, oral study medication was administered at 7.00 a.m. to achieve peak plasma concentrations of the active metabolites, enalaprilat or E-3174 respectively, during B9340 or placebo infusion (10-11.00 a.m.). Diuretics were withheld on the morning of each study for patient comfort.

4.3.4 Haemodynamic Study Protocol

Following a 30 minute infusion of 50 mL saline, patients received an intravenous infusion of B9340 at 2, 6 and 20 μg/kg/min for 15 minutes at each dose or saline placebo (75 mL) in a randomised, double-blind manner. The randomisation was weighted such that ten patients on enalapril received B9340, ten patients on losartan received B9340 and six patients on either enalapril or losartan received placebo (Figure 13). Haemodynamic measurements were recorded at -40, -30, -10, 0 (baseline), +7, +15, +22, +30, +37, +45, +60, +75, +90 and +105 minutes during each study. Venous blood was collected at 0 (baseline), +45 and +105 minutes for determination of plasma ACE activity and plasma angiotensin II concentrations.

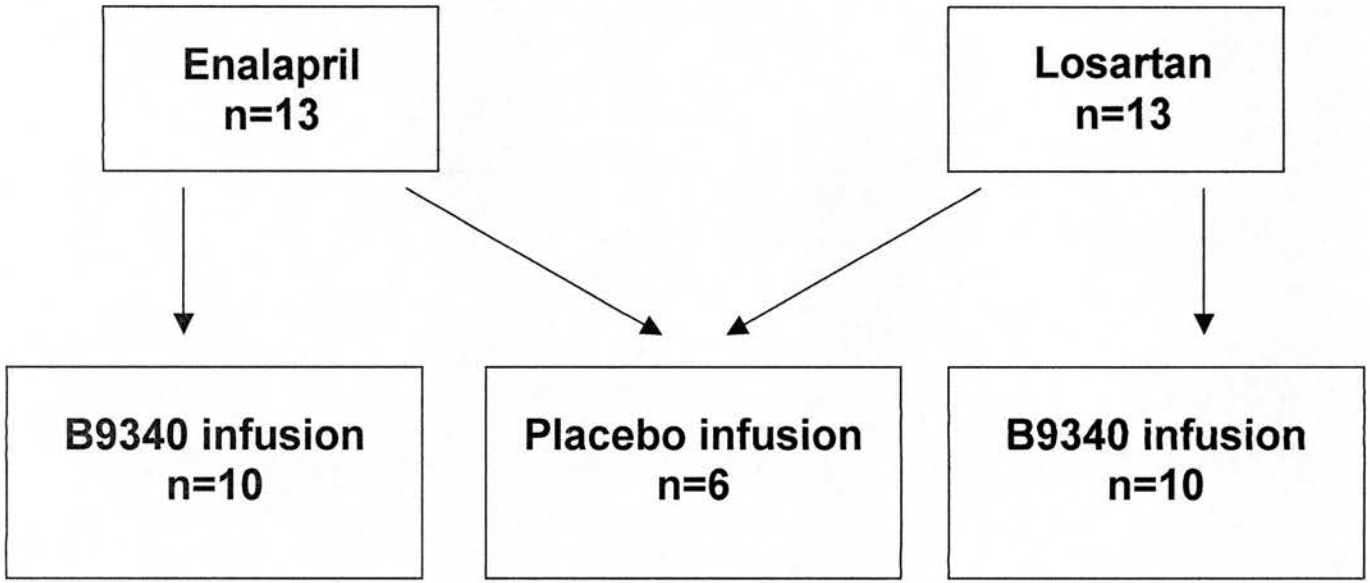

Figure 13. Heart failure study design. After 6 weeks of treatment with either enalapril or losartan in a double-blind cross-over trial, patients were randomised to receive a systemic intravenous infusion of B9340 or saline placebo in a double-blind manner.

4.3.5 Laboratory Analysis

Blood samples were collected on ice, centrifuged immediately, and the resulting supernatant stored at -70°C until assayed. Plasma ACE activity was determined using colourimetric spectrophotometry (SIGMA Diagnostics, St Louis, Mo) [Holmquist et al, 1979]. After extraction using Bond Elut columns (Varian; Harbor City, Calif), plasma angiotensin II (Diasorin, Stillwater, Minn) concentrations were determined by radioimmunoassay [Morton and Webb, 1985].

4.3.6 Data Analysis and Statistics

Data are expressed as mean ± standard error of the mean. Haemodynamic parameters were assessed over time as absolute change from baseline (0 minutes) using one-way ANOVA with repeated measures. Comparisons between treatment groups were made using two-way ANOVA for which the within-subject variables were drug and time. Plasma neurohormone concentrations were compared at baseline using an unpaired *t*-test. Statistical significance was taken at the 5% level.

4.4 RESULTS

Following the first visit, one patient withdrew due to worsening heart failure unrelated to treatment and was replaced. There were no significant differences in patient characteristics or baseline haemodynamic parameters between treatment groups (Table 3).

Table 3. Patient characteristics and baseline haemodynamics.

Variable	Enalapril + B9340 Infusion (n=10)	Losartan + B9340 Infusion (n=10)	Enalapril/Losartan + Placebo Infusion (n=6)
Age yrs (range)	62 (45 - 75)	63 (45 - 78)	66 (60 - 73)
Gender (male/female)	8 / 2	9 / 1	5 / 1
Diagnosis (IHD/DCM)	9 / 1	8 / 2	5 / 1
NYHA class (II/III)	8 / 2	6 / 4	3 / 3
Ejection fraction (%)	28 ± 3	28 ± 3	31 ± 2
Body mass index	26 ± 1	26 ± 1	25 ± 2
Concomitant medications			
Aspirin	8	8	6
Diuretic	9	8	5
Beta-blocker	7	6	3
Statin	7	6	5
Nitrate	3	3	3
Digoxin	2	1	3
Calcium antagonist	1	2	1
Spironolactone	0	1	1
Baseline haemodynamics			
Heart rate (beats/min)	62 (3)	62 (4)	61 (3)
MAP (mmHg)	81 (4)	83 (4)	83 (3)
SVR (dynes.s/cm^5)	1228 (116)	1194 (110)	1443 (80)
Cardiac output (L/min)	5.4 (0.4)	5.6 (0.5)	4.7 (0.2)
CVP (mmHg)	1 (1)	1 (1)	1 (1)
MPAP (mmHg)	15 (2)	18 (2)	15 (2)
PAWP (mmHg)	5 (1)	9 (2)	7 (1)

Data are expressed as number of patients or mean ± SEM. IHD, ischaemic heart disease; DCM, idiopathic dilated cardiomyopathy; NYHA, New York Heart Association; MAP, mean arterial pressure; SVR, systemic vascular resistance; CVP central venous pressure; MPAP, mean pulmonary arterial pressure; PAWP, pulmonary arterial wedge pressure.

4.4.1 PLASMA NEUROHORMONES

Plasma ACE activity (13.2 ± 2.4 *versus* 38.8 ± 4.8 units/L; $p<0.0001$) and plasma angiotensin II concentrations (3.5 ± 0.5 *versus* 12.3 ± 2.3 pg/mL; $p<0.005$) at baseline on the morning of haemodynamic studies were significantly lower in patients treated with enalapril compared to losartan respectively (Figure 14). There were no significant changes in plasma ACE activity or angiotensin II concentrations during or following B9340 or placebo infusion (Table 4).

4.4.2 HAEMODYNAMIC PARAMETERS

4.4.2.1 *Mean Arterial Pressure and Heart Rate*

There were no significant changes in heart rate or mean arterial pressure during placebo infusion or following losartan therapy. Mean arterial pressure increased after administration of B9340 in patients treated with enalapril ($p<0.005$; Figure 15) although there was no significant change in heart rate. This pressor effect was greater in patients treated with enalapril than those given losartan therapy or placebo infusion ($p<0.005$ and $p<0.0001$ respectively; Figure 15).

4.4.2.2 *Cardiac Output and Systemic Vascular Resistance*

There were no significant changes in cardiac output or systemic vascular resistance during placebo infusion or following losartan therapy. In patients treated with enalapril receiving B9340, there was a trend towards an increase in systemic vascular resistance ($p=0.08$) but no change in cardiac output (Figure 15). Compared to placebo infusion, there was an increase in systemic vascular resistance and a fall in cardiac output following B9340 infusion in enalapril treated patients ($p<0.0005$ and

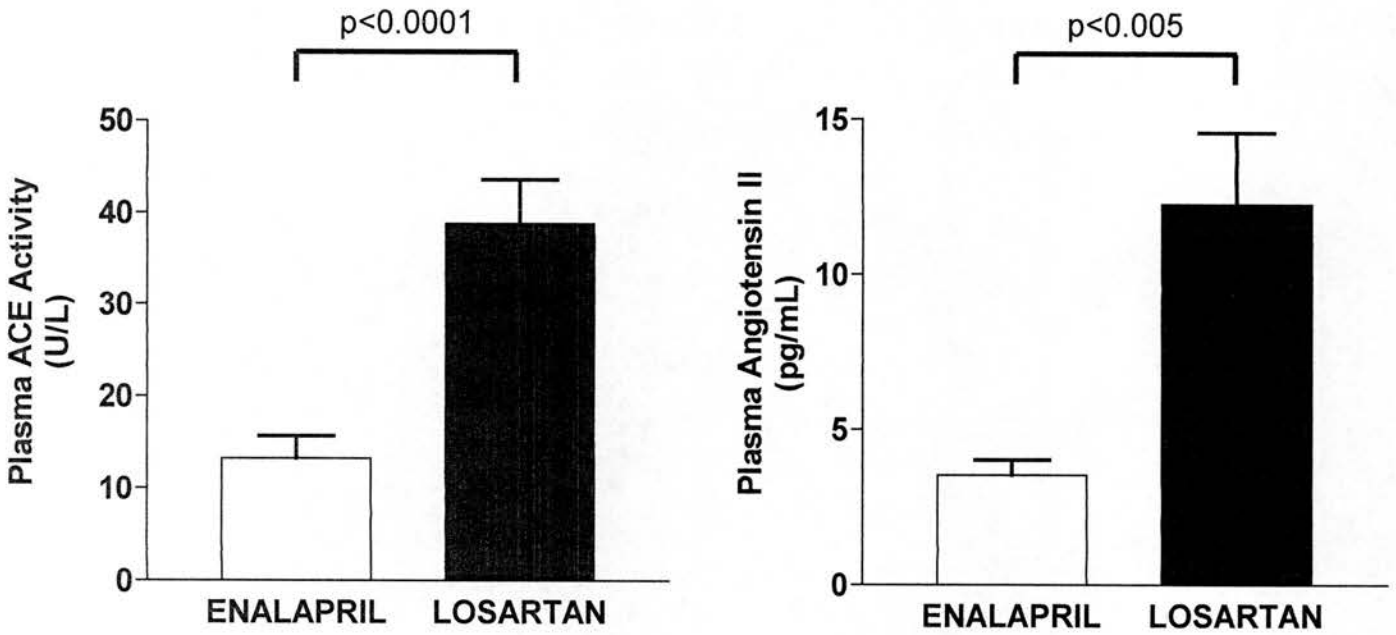

Figure 14. Baseline plasma ACE activity (n=13) and angiotensin II (n=12) concentrations in patients with heart failure after 6 weeks of therapy with enalapril (open bar) or losartan (solid bar).

Table 4. Plasma neurohormone concentrations at baseline, during B9340 infusion at 20μg/kg/min (peak; +45 mins) and following a 60 minute washout period (washout; +105 minutes).

	Plasma ACE Activity (U/L)			Plasma Angiotensin II Concentrations (pg/mL)		
	Baseline	Peak (+45 min)	Washout (+ 105 min)	Baseline	Peak (+45 min)	Washout (+ 105 min)
Enalapril + B9340 (n=10)	14.8 ± 2.9	12.2 ± 2.2	12.4 ± 1.9	3.5 ± 0.7	3.3 ± 0.6	3.4 ± 0.6
Losartan + B9340 (n=10)	40.3 ± 5.2	41.2 ± 4.2	43.8 ± 4.9	8.7 ± 1.5	7.0 ± 1.3	9.9 ± 2.8
Enalapril/Losartan + Placebo Infusion (n=6)	23.0 ± 8.3	24.5 ± 6.3	33.9 ± 7.4	13.5 ± 4.5	14.2 ± 5.9	9.9 ± 4.5

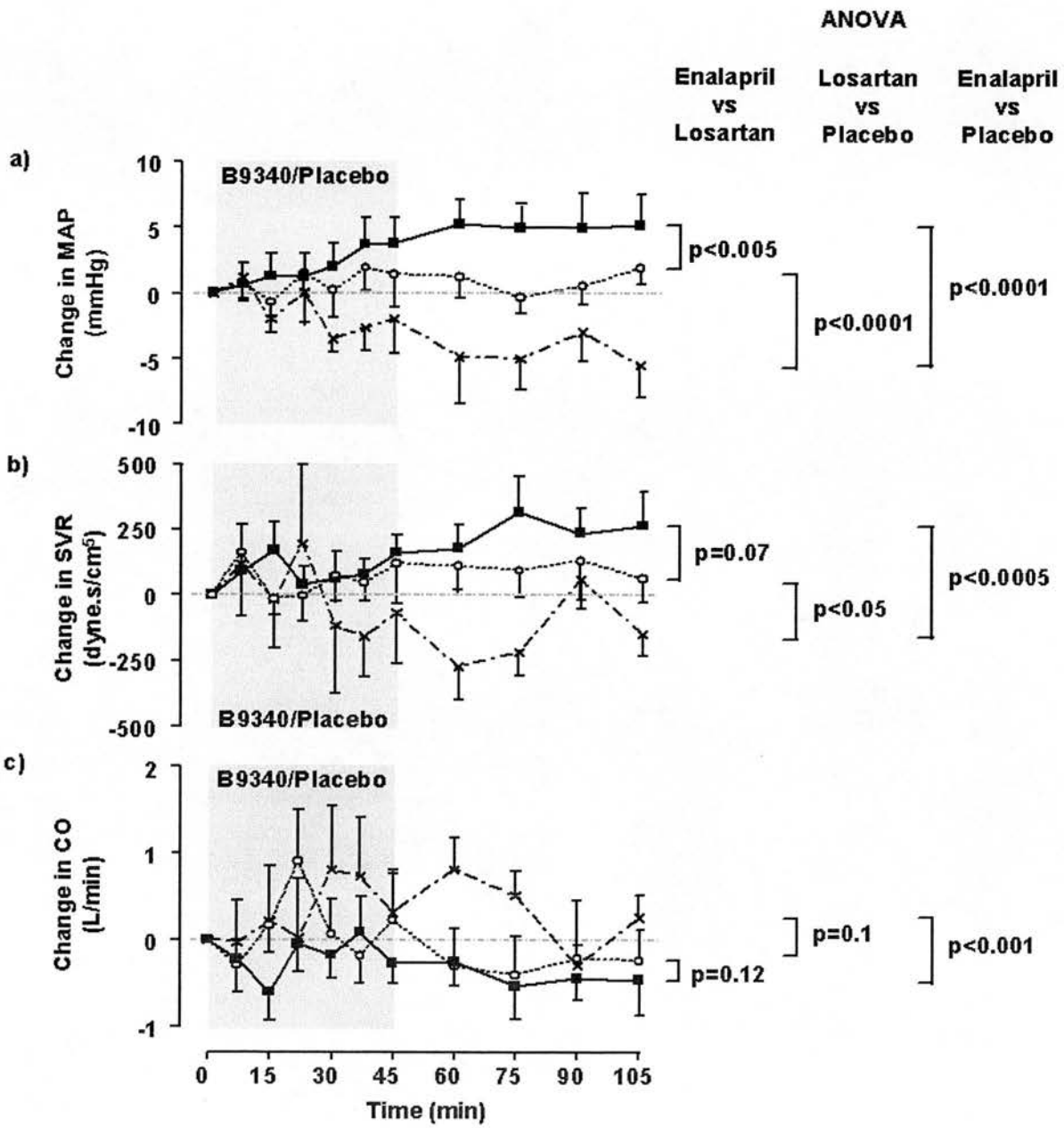

Figure 15. Changes in (a) mean arterial pressure (MAP), (b) systemic vascular resistance (SVR) and (c) cardiac output (CO) following intravenous infusion of saline placebo (crosses) or B9340 (2-20 μg/kg/min) in patients with chronic heart failure treated with enalapril (closed squares) or losartan (open circles).

$p<0.001$ respectively; Figure 15). There was a trend towards an increase in systemic vascular resistance in the enalapril treated group compared to those receiving losartan ($p=0.07$; Figure 15).

4.4.2.3 *Central Pressures*

Central venous pressure, pulmonary arterial wedge pressure and mean pulmonary arterial pressure all fell significantly over time during placebo infusion ($p<0.05$ for all; Figure 16). Compared to losartan therapy or placebo infusion, B9340 attenuated the fall in pulmonary arterial wedge pressure ($p<0.0001$ for both) and mean pulmonary arterial pressure ($p<0.05$ and $p=0.005$ respectively) in patients treated with enalapril (Figure 16). There were no significant differences in the reductions of central venous pressure between treatment groups (Figure 16).

4.4.3 ADVERSE EVENTS

There were no major drug-related adverse events reported. One patient reported mild self-limiting diarrhoea following B9340 infusion.

4.5 DISCUSSION

We have demonstrated that intravenous administration of B9340, a selective peptidic antagonist of bradykinin receptors, has a significant pressor effect in patients with chronic heart failure maintained on long-term ACE inhibitor but not angiotensin receptor blocker therapy. These findings provide convincing evidence that

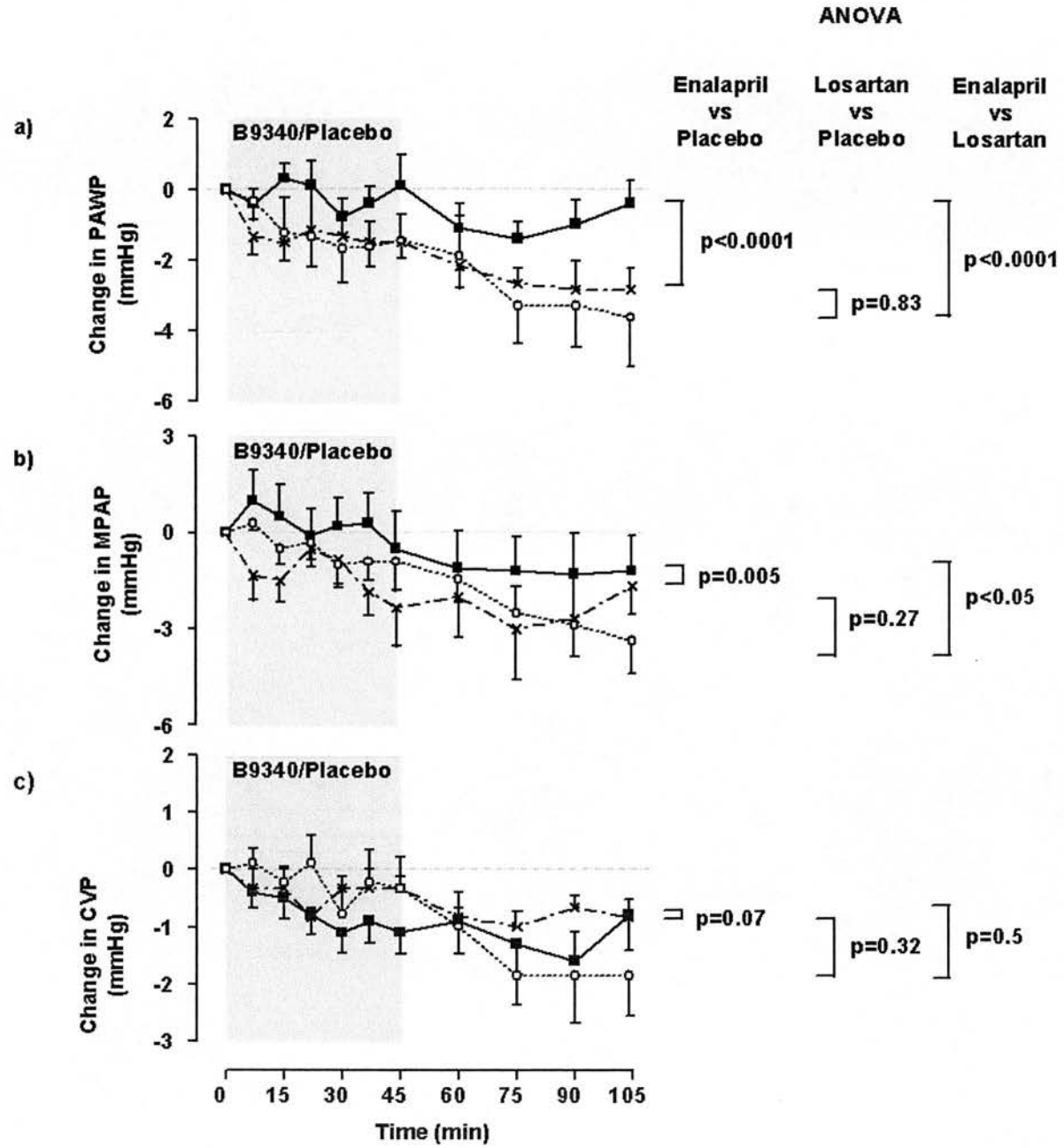

Figure 16. Changes in (a) pulmonary arterial wedge pressure (PAWP), (b) mean pulmonary arterial pressure (MPAP) and (c) central venous pressure (CVP) following intravenous infusion of saline placebo (crosses) or B9340 (2-20 μg/kg/min) in patients with chronic heart failure treated with enalapril (closed squares) or losartan (open circles).

bradykinin contributes to the hypotensive effects of chronic ACE inhibitor therapy in patients with heart failure.

We have previously shown that B9340 causes vasoconstriction in the forearm circulation in patients with heart failure treated with chronic ACE inhibitor therapy [Witherow et al, 2001]. The results of the present study confirm that these effects are important in the systemic circulation. This is consistent with published data demonstrating that the bradykinin receptor antagonist, HOE-140, attenuates the acute vasodepressor response to ACE inhibition. Gainer and colleagues demonstrated that in salt deplete healthy volunteers as well as hypertensive subjects, a systemic infusion of HOE-140 attenuated the hypotensive response to a single dose of captopril [Gainer et al, 1998]. The reduction in blood pressure observed following co-administration of HOE-140 and captopril was similar to that seen with the angiotensin receptor blocker, losartan [Gainer et al, 1998]. As the authors point out, previous studies have demonstrated that the peak hypotensive responses to captopril and losartan occur 1 and 4 hours respectively after drug administration [Gainer et al, 1998].

It has been suggested that these differences could account for the observed differences in the blood pressure response [Gainer et al, 1998]. To overcome this potential methodological difficulty, we chose to compare the ACE inhibitor, enalapril, at a dose previously shown to reduce mortality in patients with heart failure [CONSENSUS, 1987] with the angiotensin receptor antagonist, losartan, administered at the maximum daily dose. Both these agents have long-acting active

metabolites, enalaprilat and E-3174 respectively, achieving peak plasma concentrations 3-4 hours post ingestion that coincided with systemic intravenous administration of B9340 or placebo [Abrams et al, 1984; Ohtawa et al, 1993]. It should also be recognised that we have assessed the effects of kinin receptor blockade in patients maintained on long-term ACE inhibitor therapy rather than the acute effects of a single oral dose. Moreover, patients with heart failure maintained on chronic ACE inhibitor therapy may upregulate vascular B_1 kinin receptor expression [Nwator and Whalley, 1989; Witherow et al, 2001; Marin-Castano et al, 2002] and we therefore used a combined B_1 and B_2 receptor antagonist to characterise more precisely the role of bradykinin.

It has been argued [Schiffrin, 2002] that angiotensin receptor blockade might potentially be more effective at blocking the detrimental effects of angiotensin II than ACE inhibition because in a substantial proportion of patients treated with chronic ACE inhibition, plasma angiotensin II concentrations rise over time returning towards pre-treatment values [MacFadyen et al, 1999]. This "ACE escape" is thought to be due to angiotensin II formation by non-ACE dependent pathways and may be associated with a poorer prognosis [Roig et al, 2000; Petrie et al, 2001]. However, clinical evidence that angiotensin receptor blockers have superior efficacy to ACE inhibitor therapy has proved elusive. In contrast, recent trials have confirmed that the therapeutic benefits of ACE inhibitors and angiotensin receptor blockers are additive: combination therapy improving symptoms and cardiovascular mortality in patients with heart failure when compared to ACE inhibition alone [Cohn and Tognoni, 2001;

McMurray et al, 2003]. Our findings may help to explain the additive benefits of combined ACE inhibition and angiotensin receptor blockade.

Large-scale clinical trials have demonstrated that ACE inhibitors not only improve survival but reduce the incidence of myocardial ischaemia [Flather et al, 2000]. This may be explained, in part, by the observation that ACE inhibition improves the fibrinolytic balance in patients with heart failure and ischaemic heart disease [Wright et al, 1994]. Bradykinin is not only a potent vasodilator but is intimately involved with the coagulation and fibrinolytic cascades. Indeed, it is a powerful mediator of endogenous fibrinolysis through the release of endothelium-derived t-PA [Brown et al, 2000; Witherow et al, 2002; Pretorius et al, 2003]. In patients with heart failure, chronic ACE inhibition markedly augments local bradykinin-mediated release of t-PA [Witherow et al, 2002]. Thus, potentiation of the other vascular actions of bradykinin may also contribute to the clinical benefits of ACE inhibitor therapy.

In patients treated with losartan, there was a small but significant increase in mean arterial pressure and systemic vascular resistance following B9340 infusion compared to placebo. This may have reflected the significant protein load associated with B9340 infusion and a protein-based placebo, such as albumin, may have been a more appropriate control. There are data indicating that bradykinin may contribute to the vascular effects of angiotensin receptor blockers. In transgenic mice overexpressing the AT2 receptor, angiotensin II causes vasodilatation that is attenuated by HOE-140 [Tsutsumi et al, 1999] and in man, HOE-140 inhibits the improvement in flow-mediated vasodilatation associated with the angiotensin

receptor blocker, candesartan [Hornig et al, 2003]. Finally, the findings are consistent with the possibility that bradykinin also contributes to the maintenance of blood pressure and vascular tone in patients with heart failure independently of ACE.

In summary, we have shown that in patients with chronic heart failure, systemic infusion of the combined kinin receptor antagonist, B9340, attenuates the vasodepressor effects associated with long-term enalapril therapy when compared to treatment with the angiotensin receptor blocker, losartan. We conclude that, in patients with chronic symptomatic heart failure, bradykinin contributes to the systemic haemodynamic effects associated with long-term ACE inhibitor therapy.

CHAPTER 5

NEUTRAL ENDOPEPTIDASE INHIBITION AUGMENTS THE VASCULAR ACTIONS OF BRADYKININ IN PATIENTS TREATED WITH ANGIOTENSIN-CONVERTING ENZYME INHIBITOR THERAPY

5.1 SUMMARY

Angiotensin-converting enzyme and neutral endopeptidase (EC 3.4.24.11; neprilysin) are metallopeptidases present on the endothelium that metabolise bradykinin. Inhibitors of ACE potentiate bradykinin-mediated vasodilatation and endothelial t-PA release. Combined ACE and NEP inhibition may have additional beneficial cardiovascular effects mediated through potentiation of bradykinin. We investigated the effects of local NEP inhibition on the vascular actions of bradykinin in patients with heart failure maintained on chronic ACE inhibition. Ten patients received an intrabrachial infusion of thiorphan (30 nmol/min), a NEP inhibitor, in a randomised double-blind placebo-controlled cross-over trial. Thiorphan was co-infused with Lys-des-Arg^9-bradykinin (1-10 nmol/min), bradykinin (30-300 pmol/min), atrial natriuretic peptide (10-100 pmol/min) and sodium nitroprusside (2-8 μg/min). Bradykinin, atrial natriuretic peptide and sodium nitroprusside caused dose-dependent vasodilatation (peak blood flow 14.4 ± 2.2, 3.6 ± 0.6 and 8.6 ± 1.3 mL/100mL/min respectively; $p<0.0001$). Bradykinin caused dose-dependent increases in t-PA antigen and activity (peak concentration 31.8 ± 3.4 ng/mL and 21.9 ± 7.6 IU/mL respectively; $p<0.001$) and estimated antigen and activity release (peak release 152 ± 46 ng/100mL/min and 154 ± 22 IU/100mL/min respectively; $p<0.005$). Compared to placebo, thiorphan augmented bradykinin-mediated vasodilatation (1.4-fold; $p<0.0001$) and net t-PA release (1.5-fold; $p<0.005$). We have demonstrated that NEP contributes to the metabolism of bradykinin in patients with heart failure maintained on ACE inhibitor therapy. Our findings may explain some of the clinical effects of combined ACE and

NEP inhibition, including the greater vasodepressor effect observed with combined therapy when compared to ACE inhibition alone.

5.2 INTRODUCTION

Bradykinin is a potent endothelium-dependent vasodilator peptide released at sites of inflammation and coagulation. Apart from vasodilatation, it also stimulates endothelial release of the pro-lytic factor, t-PA, and these effects are mediated by the constitutively expressed B_2 receptor [Brown et al, 2000; Witherow et al, 2002]. Removal of the C-terminal arginine from bradykinin results in the formation of des-Arg^9-bradykinin, the principal ligand for the B_1 kinin receptor in plasma [Marceau et al, 1998]. However, the most potent endogenous ligand for the B_1 kinin receptor is Lys-des-Arg^9-bradykinin [Marceau et al, 1998]. The vascular B_1 receptor is normally expressed very weakly but is markedly upregulated in the presence of inflammation [McLean et al, 1999; Deblois et al, 2001], cardiovascular disease [McLean et al, 2000b] and ACE inhibition [Nwator and Whalley, 1989; Marin-Castano et al, 2002], where it also mediates vasodilatation [Marceau et al, 1998].

ACE is the principal enzyme responsible for the rapid turnover of bradykinin (plasma half-life ~15 seconds) and its metabolites [Marceau et al, 1998]. It has been widely established that inhibitors of ACE improve morbidity and mortality in patients with heart failure [CONSENSUS, 1987] and these benefits may be due, at least in part, to the inhibition of bradykinin metabolism [Gainer et al, 1998; Witherow et al, 2001]. Inhibition of ACE increases the plasma half-lives of

bradykinin and des-Arg^9-bradykinin approximately 9- and 2-fold respectively [Cyr et al, 2001] and, at a functional level, potentiates the vascular actions of bradykinin in the human forearm [Benjamin et al, 1989; Witherow et al, 2002] and coronary [Kuga et al, 1997] circulations. Moreover, bradykinin antagonism causes vasoconstriction [Witherow et al, 2001] and attenuates the fall in blood pressure [Gainer et al, 1998] in patients treated with ACE inhibitor therapy.

Neutral endopeptidase (EC 3.4.24.11; neprilysin) is a membrane-bound metallopeptidase that co-localises with ACE and metabolises a number of vasodilator and vasoconstrictor peptides, including atrial natriuretic peptide, substance P, endothelin-1 and bradykinin [Roques et al, 1993]. Expression of NEP is upregulated in patients with heart failure [Fielitz et al, 2002; Knecht et al, 2002] and, in the presence of ACE inhibition, the contribution of NEP to bradykinin metabolism is increased [Dumoulin et al, 1998]. Inhibition of NEP potentiates the half-life of bradykinin [Graf et al, 1993] and augments bradykinin-mediated vasodilatation *in vitro* [Krassoi et al, 2003]. It is not known whether NEP inhibition augments the half-life of Lys-des-Arg^9-bradykinin. Indeed, it has been suggested that the Phe^8 residue may protect B_1 ligands from degradation by NEP [Marceau et al, 1998]. Although the effects of NEP inhibition on systemic haemodynamics are variable [Northridge et al, 1989; Richards et al, 1990 Bevan et al, 1992; Favrat et al, 1995], clinical improvements have been reported during NEP inhibition in patients with heart failure [Northridge et al, 1999].

Co-administration of ACE and NEP inhibitors may confer additional therapeutic efficacy. Combined ACE and NEP inhibition attenuates bradykinin degradation more effectively than either enzyme alone [Dumoulin et al, 2001] and in animal models, improves cardiac remodelling and survival to a greater extent than isolated ACE inhibition [Trippodo et al, 1999]. These cardioprotective effects are lost in transgenic mice lacking the B_2 kinin receptor [Xu et al, 2004]. In man, combined ACE and NEP inhibition reduces blood pressure to a greater extent than inhibition of either enzyme alone [Favrat et al, 1995; Campese et al, 2001] and is associated with symptomatic and haemodynamic improvements in patients with heart failure [McClean et al, 2000]. The hypothesis that combined ACE and NEP inhibition may improve symptoms and survival in patients with heart failure to a greater extent than ACE inhibition alone has recently been evaluated in a large-scale clinical trial (OVERTURE) [Packer et al, 2002].

We have previously demonstrated that chronic ACE inhibition potentiates bradykinin-mediated vasodilatation and endothelial release of t-PA in the forearm circulation of patients with heart failure [Witherow et al, 2002]. The aims of this study were to investigate whether local NEP inhibition augments the vascular actions of bradykinin, and to examine the effects of B_1 receptor agonism, in patients with heart failure maintained on long-term ACE inhibitor therapy.

5.3 METHODS

5.3.1 PATIENTS

Ten patients with symptomatic heart failure and echocardiographic evidence of left ventricular systolic dysfunction attended fasted at 9 a.m. on two occasions at least 2 weeks apart. The protocol was performed with the approval of the Local Research Ethics Committee, in accordance with the Declaration of Helsinki and with the written informed consent of each patient. Patients were maintained on maximally tolerated ACE inhibitor therapy (ramipril - 10 mg daily (n=4), 5 mg daily (n=3); enalapril - 20 mg daily (n=1), 10mg daily (n=1); lisinopril - 40 mg daily (n=1)) for at least 6 months prior to recruitment. On the morning of each visit, ACE inhibitor therapy was administered at 8 a.m. and diuretics were withheld.

5.3.2 DRUGS

Pharmaceutical grade thiorphan (Clinalfa AG, Läufelfingen, Switzerland), bradykinin (Clinalfa AG), Lys-des-Arg^9-bradykinin (Clinalfa AG), atrial natriuretic peptide (Clinalfa AG) and sodium nitroprusside (David Bull Laboratories, Warwick, United Kingdom) were dissolved in 0.9% saline on the day of study.

5.3.3 STUDY DESIGN

Following 15 minutes equilibration with 0.9% saline, patients were randomised to receive an intrabrachial infusion of thiorphan (30 nmol/min) or saline placebo for 3 hours. Thiorphan or placebo was co-infused with bradykinin (30, 100 and 300 pmol/min) [Witherow et al, 2001], Lys-des-Arg^9-bradykinin (1, 3 and 10 nmol/min),

atrial natriuretic peptide (10, 30 and 100 pmol/min; Clinalfa AG) [van der Zander et al, 1999] and sodium nitroprusside (2, 4 and 8 μg/min) [Newby et al, 1997a] for 10 minutes at each dose. There was a 20 minute washout infusion of 0.9% saline between compounds. The order of infusion was randomised between patients but was maintained for both visits. The dose of thiorphan was chosen based on previous forearm studies [Ferro et al, 1998] to achieve a local plasma concentration >10-fold the IC_{50} of thiorphan for NEP *in vitro* [Barclay et al, 1990]. The doses of Lys-des-Arg^9-bradykinin were chosen based on binding affinity data [Gobeil et al, 1996] and the hypotensive dose response in rodents and non-human primates [Drapeau et al, 1991; Deblois et al, 2001]. The combined rate of infusion remained constant throughout each study at 1 mL/min.

5.3.4 Measurements

Bilateral forearm blood flow was measured using venous occlusion plethysmography [Webb, 1995; Newby et al, 1997b; Wilkinson and Webb, 2001]. Heart rate and blood pressure were recorded in the non-infused arm using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751; Takeda) at baseline and in the final minute of each drug infusion period, after forearm blood flow measurements and venous sampling. Venous cannulae (17-gauge) were inserted bilaterally into a large antecubital vein. Throughout each study, 10 mL of blood was collected from each arm into acidified buffered citrate (Biopool Stabilyte; Umeå; for t-PA assays) and citrate (Monovette, Sarstedt, Numbrecht; for PAI-1 assays). Platelet-free plasma was prepared as described previously [Newby et al, 1997b] and stored at -70°C before assay. Plasma concentrations of t-PA and PAI-1 antigen were determined

using an ELISA, t-PA activity using a photometric method [Newby et al, 1997b], and ACE activity using colourimetric spectrophotometry (reference range 8 to 55 U/L; Sigma) [Holmquist et al, 1979].

5.3.5 Data Analysis and Statistics

Data are expressed as mean ± standard error of the mean. Statistical analyses were performed using ANOVA or, where appropriate, paired *t*-tests.

5.4 RESULTS

There were no significant differences in heart rate, blood pressure or baseline forearm blood flow during or between study days (Table 5). Consistent with previous studies [Brown et al, 2000], one subject developed transient upper limb oedema with 300 pmol/min of bradykinin that rapidly resolved on cessation of the infusion. There were no other reported side effects.

5.4.1 Plasma ACE Activity

Baseline plasma ACE activity was similar between thiorphan and placebo study visits (12.3 ± 2.6 *versus* 9.7 ± 1.5 U/mL respectively; p=0.7). Compared to baseline, there were no significant differences in plasma ACE activity measured after 90 minutes of thiorphan (10.7 ± 2.5 U/mL; p=0.3) or placebo (10.2 ± 1.7 U/mL; p=0.3) co-infusion.

Table 5. Patient characteristics and baseline haemodynamics.

Variable	All Patients (n=10)	Units
Age (range)	64 (52 - 73)	years
Gender (male/female)	5/5	n
Diagnosis (IHD/DCM)	8/2	n
NYHA class (II/III)	6/4	n
Risk factors for IHD		n
Smokers/non-smokers	4/6	
Hypertension	4	
Diabetes mellitus	0	
Hypercholesterolaemia	6	
Body mass index	30 ± 1	kg/m^2
Concomitant medications		n
ACE inhibitor	10	
Aspirin	7	
Diuretic	10	
Beta-blocker	8	
Statin	6	
Nitrate	1	
Digoxin	4	
Calcium Antagonist	0	
Spironolactone	2	
Warfarin	3	
Amiodarone	2	
Echocardiography		
Ejection fraction	31 ± 2	%
LVEDD	61 ± 3	mm
Baseline heart rate		beats/min
Thiorphan visit	58 ± 3	
Placebo visit	56 ± 2	
Baseline mean arterial pressure		mmHg
Thiorphan visit	92 ± 5	
Placebo visit	86 ± 4	
Baseline forearm blood flow		mL/100mL/min
Thiorphan visit	2.0 ± 0.4	
Placebo visit	1.9 ± 0.2	

Data are expressed as number of patients or mean ± SEM. Mean arterial pressure was calculated as diastolic pressure plus one-third pulse pressure. IHD, ischaemic heart disease; DCM, idiopathic dilated cardiomyopathy; NYHA, New York Heart Association; ACE, angiotensin-converting enzyme; LVEDD, left ventricular end diastolic diameter.

5.4.2 Forearm Blood Flow

Bradykinin, atrial natriuretic peptide and sodium nitroprusside caused dose-dependent increases in forearm blood flow in all studies ($p<0.0001$ for all; Figure 17). Forearm blood flow did not change during Lys-des-Arg^9-bradykinin infusion (Figure 17). Compared to saline placebo, co-infusion of thiorphan augmented forearm vasodilatation to bradykinin 1.4-fold ($p<0.0001$; Figure 17) but not atrial natriuretic peptide or sodium nitroprusside.

5.4.3 Plasma Fibrinolytic Factors

5.4.3.1 *Release of t-PA*

There were no significant differences in baseline plasma t-PA antigen (10.8 ± 0.9 *versus* 9.8 ± 0.9 ng/mL), t-PA activity (0.1 ± 0.1 *versus* 0.4 ± 0.1) or PAI-1 antigen concentrations (37.3 ± 3.7 *versus* 38.4 ± 6.2 ng/mL) between thiorphan and placebo respectively. Bradykinin caused a dose-dependent increase in plasma t-PA antigen and activity concentrations in the infused arm ($p<0.001$ for all; Table 6), and the net release of t-PA antigen and activity in all studies ($p<0.005$ for all; Figure 18). Compared to placebo, thiorphan augmented the increase in plasma t-PA activity concentration (21.9 ± 2.4 *versus* 24.8 ± 2.6 IU/mL respectively at bradykinin 300 pmol/min; $p<0.05$; Table 6) in the infused arm and the net release of t-PA antigen (157 ± 46 *versus* 233 ± 46 ng/100mL/min respectively at bradykinin 300 pmol/min; $p<0.005$, Figure 18) and activity (155 ± 22 *versus* 244 ± 51 ng/100mL/min respectively at bradykinin 300 pmol/min; $p<0.005$; Figure 18). There was a trend towards an increase in t-PA antigen in the infused arm during thiorphan infusion compared to placebo (32.5 ± 3.4 *versus* 36.5 ± 4.2 ng/mL respectively at

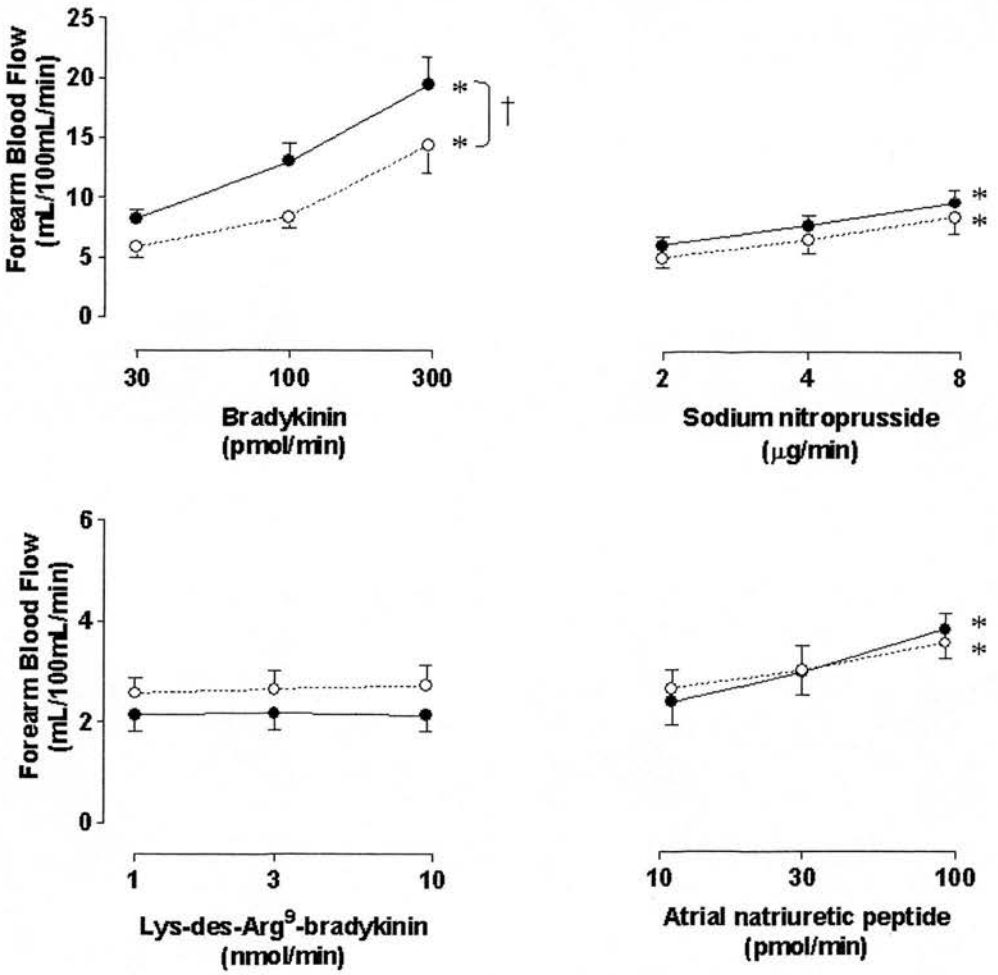

Figure 17. Effect of thiorphan (solid circles) and placebo (open circles) on infused forearm blood flow during intra-arterial infusion of bradykinin, sodium nitroprusside, atrial natriuretic peptide and Lys-des-Arg^9-bradykinin (*$p<0.0001$, ANOVA dose response; †$p<0.0001$, ANOVA thiorphan versus placebo).

Table 6. Effect of thiorphan and placebo on forearm blood flow (FBF) and plasma tissue plasminogen activator (t-PA) antigen and activity concentrations during co-infusion of bradykinin, atrial natriuretic peptide and Lys-des-Arg^9-bradykinin.

Parameter		Bradykinin pmol / min				Atrial Natriuretic Peptide pmol / min				Lys-des-Arg^9-bradykinin nmol / min			
	Baseline	30	100	300	Baseline	10	30	100	Baseline	1	3	10
Thiorphan Infusion												
FBF, mL/100mL/min												
Infused	2.1±0.2	8.2±0.7	13±1.5	19±2.2*‡	2±0.3	2.4±0.4	3±0.4	3.8±0.6*	2.2±0.3	2.1±0.3	2.2±0.3	2.1±0.3
Non-infused	1.5±0.3	2±0.4	1.9±0.3	1.7±0.3	1.4±0.2	1.5±0.3	1.6±0.3	1.7±0.3	1.6±0.3	1.5±0.3	1.5±0.3	1.6±0.3
t-PA antigen, ng/mL												
Infused	9.3±0.8	18.0±1.8	26.2±2.9	36.5±4.2*	10.8±0.9	10.7±0.8	10.2±0.7	9.5±0.8	10.1±0.9	10.2±0.9	10.1±0.8	9.8±0.8
Non-infused	9.6±0.7	10.8±1	12.9±1	17.6±2.5*	9.9±0.8	10.4±0.9	10.3±0.9	9.8±0.8	10.2±0.9	10.2±0.9	10±0.8	9.6±0.8
t-PA activity, IU/mL												
Infused	0.1±0.1	8.7±1.4	16.2±2.4	24.8±2.6*§	1.3±0.5	1.1±0.4	0.8±0.4	0.5±0.2	0.8±0.4	0.8±0.5	0.5±0.3	0.4±0.2
Non-infused	0.1±0.1	1.0±0.2	3.1±1	6±1.2†§	0.6±0.2	0.7±0.3	0.5±0.3	0.4±0.3	0.7±0.3	0.2±0.2	0.4±0.3	0.3±0.2
Placebo Infusion												
FBF, mL/100mL/min												
Infused	2±0.3	5.8±0.8	8.3±0.9	14.4±2.2*‡	2.2±0.4	2.6±0.4	3±0.5	3.6±0.6*	2.5±0.3	2.6±0.3	2.7±0.4	2.7±0.4
Non-infused	1.9±0.3	2.1±0.4	2±0.3	2.1±0.4	2±0.4	2±0.3	2±0.3	2±0.3	1.8±0.2	1.9±0.3	1.9±0.3	1.9±0.3
t-PA antigen, ng/mL												
Infused	9.1±0.7	14.2±1	22.8±2.2	32.5±3.4*	9.8±0.8	9.4±0.7	9.2±0.7	9.5±0.8	9.6±0.8	9.7±0.8	9.5±0.8	9.4±0.8
Non-infused	9.3±0.8	9.8±0.7	11.2±0.8	14.6±1.2*	10±0.7	9.8±0.6	10±0.7	9.6±0.7	9.4±0.8	9.3±0.8	9.2±0.7	9±9.7
t-PA activity, IU/mL												
Infused	0.3±0.1	4.6±1.3	12.6±2.4	21.9±2.4*§	0.6±0.2	0.5±0.1	0.4±0.2	0.3±0.1	0.8±0.2	0.6±0.2	0.6±0.2	0.6±0.2
Non-infused	0.3±0.1	0.8±0.3	1.4±0.4	2.8±0.7†§	0.2±0.1	0.3±0.1	0.2±0.1	0.1±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.2

*p<0.001, †p<0.05 ANOVA dose response; ‡p<0.001, §p<0.05 ANOVA thiorphan vs placebo.

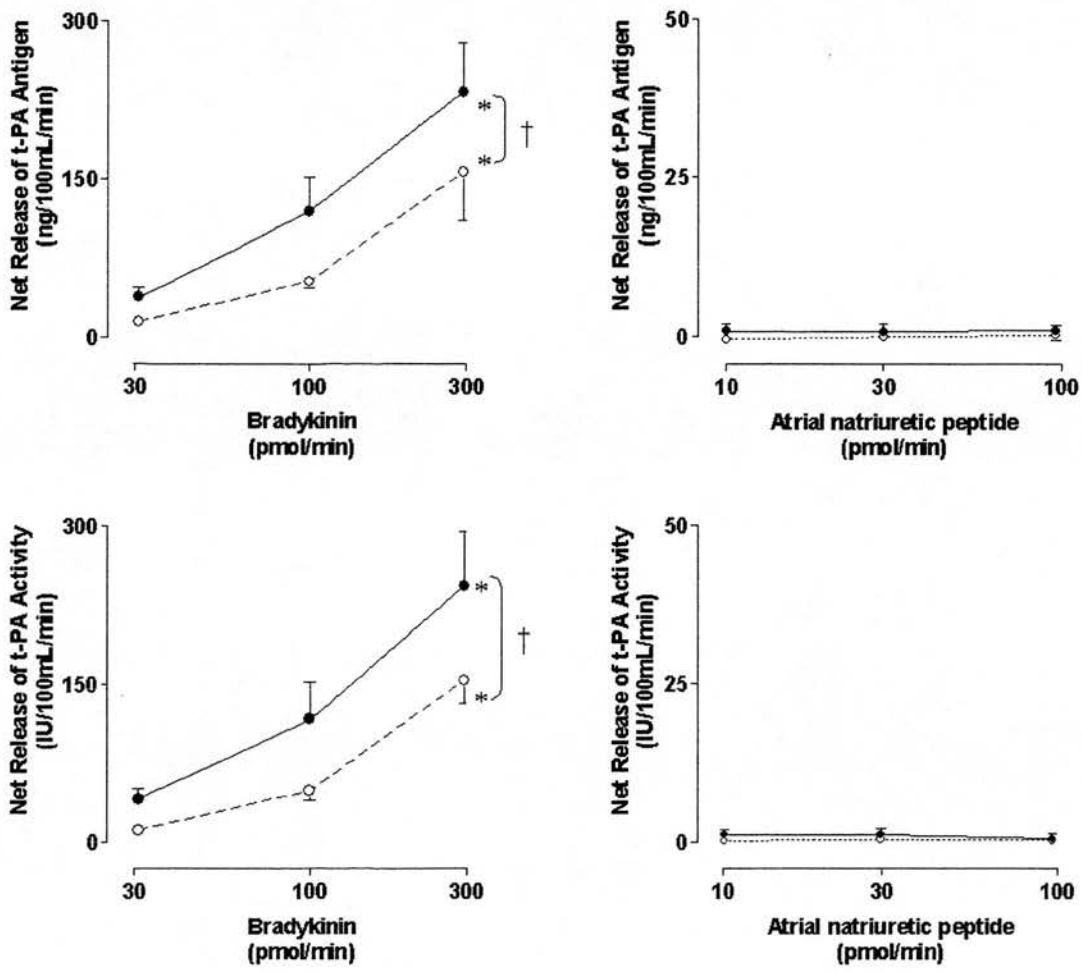

Figure 18. Effect of thiorphan (solid circles) and placebo (open circles) on net tissue plasminogen activator (t-PA) antigen (top) and activity (bottom) release during intra-arterial infusion of bradykinin, and atrial natriuretic peptide (*$p<0.005$, ANOVA dose response; †$p<0.005$, ANOVA thiorphan versus placebo).

bradykinin 300 pmol/min; p=0.058; Table 6). Due to systemic overspill, bradykinin increased plasma t-PA antigen and activity concentrations in the non-infused arm ($p<0.01$ and $p<0.05$ respectively; Table 6) that, for t-PA activity, was greater during thiorphan infusion ($p<0.05$; Table 6). There were no significant changes in t-PA antigen, activity or net t-PA release during infusion of atrial natriuretic peptide or Lys-des-Arg^9-bradykinin.

5.4.3.2 *Plasma PAI-1 Antigen*

Consistent with the augmentation of t-PA release and subsequent PAI-1 mediated clearance, there was a significant reduction in plasma PAI-1 antigen concentrations in the infused arm during bradykinin co-infusion with thiorphan (baseline 33.3 ± 3.5 *versus* 29.4 ± 3.4 ng/mL at bradykinin 300 pmol/min; $p<0.05$) but not placebo (Table 7). There were no significant changes in PAI-1 antigen concentration during infusion of Lys-des-Arg^9-bradykinin or atrial natriuretic peptide (Table 7).

5.5 DISCUSSION

We have, for the first time, demonstrated that acute local NEP inhibition augments bradykinin-mediated endothelium-dependent vasodilatation and endogenous t-PA release in patients with heart failure maintained on chronic ACE inhibitor therapy. In addition, this is the first clinical study to show that the B_1 receptor does not mediate vasodilatation or endothelial t-PA release in these patients. Our findings support the hypothesis that bradykinin may contribute to the systemic haemodynamic differences observed between combined ACE and NEP inhibition, and ACE inhibition alone.

Table 7. Effect of thiorphan and placebo on plasminogen activator inhibitor type-1 (PAI-1) antigen concentrations in the infused and non-infused forearms during co-infusion of bradykinin, atrial natriuretic peptide and Lys-des-Arg^9-bradykinin.

Parameter	Bradykinin pmol / min		Atrial Natriuretic Peptide pmol / min		Lys-des-Arg^9-bradykinin nmol / min	
	Baseline	300	Baseline	100	Baseline	10
Thiorphan Infusion						
PAI-1, ng/mL						
Infused	33.3 ± 3.5*	29.4 ± 3.4*	33.0 ± 4.7	31.7 ± 4.4	32.6 ± 4.4	31.0 ± 3.5
Non-infused	37.3 ± 4.0	32.5 ± 3.1	34.4 ± 5.0	33.0 ± 4.3	35.7 ± 4.3	31.1 ± 3.3
Placebo Infusion						
PAI-1, ng/mL						
Infused	33.2 ± 4.3	32.8 ± 3.2	33.0 ± 4.6	33.9 ± 4.8	30.6 ± 3.0	30.7 ± 3.7
Non-infused	34.7 ± 4.3	30 ± 2.5	36.8 ± 4.5	35.3 ± 4.5	29.9 ± 3.1	30.2 ± 3.2

*$p<0.05$ Students *t*-test, baseline vs 300 pmol/min bradykinin.

5.5.1 Clinical Implications

There is now substantial evidence that bradykinin contributes to the systemic haemodynamic and anti-ischaemic effects of ACE inhibitor therapy [Gainer et al, 1998; Witherow et al, 2001; Witherow et al, 2002; Pretorius et al, 2003]. Our findings suggest that bradykinin-mediated vasodilatation may contribute to the greater vasodepressor actions demonstrated with combined ACE and NEP inhibition compared to isolated ACE inhibition [Favrat et al, 1995; Campese et al, 2001]. Moreover, despite the marked increase in bradykinin-induced t-PA release by ACE inhibition alone [Witherow et al, 2002], additional NEP inhibition causes further substantial augmentation of acute t-PA release. Taken together, these haemodynamic and pro-fibrinolytic effects would be expected to have important therapeutic consequences. However, in the recent OVERTURE trial [Packer et al, 2002] of patients with heart failure, treatment with omapatrilat, a combined ACE and NEP inhibitor, failed to reduce all-cause mortality when compared to enalapril, although it did reduce the combined secondary endpoint of cardiovascular death and hospitalisation. Post-hoc analysis redefining endpoints according to the Studies of Left Ventricular Dysfunction (SOLVD) criteria suggested that omapatrilat may be more effective at preventing cardiovascular events than enalapril, but that the additional benefit was substantially smaller than anticipated [Packer et al, 2002]. This may, in part, reflect the shorter duration of NEP inhibition compared to ACE inhibition with omapatrilat [Azizi et al, 2002]. Given our findings, pharmacological strategies offering a more balanced and prolonged duration of combined ACE and NEP inhibition may confer greater cardiovascular benefits.

Augmentation of bradykinin-mediated vasodilatation within the kidney may also contribute to the greater increase in renal blood flow observed with combined ACE and NEP inhibition than ACE inhibition alone [Regamey et al, 2002]. As a result, it has been suggested that combined ACE and NEP inhibition may afford greater renal protection than ACE inhibition alone [Regamey et al, 2002]. It should be noted, however, that potentiating the vascular actions of kinins may have detrimental effects. Bradykinin has been implicated in the pathogenesis of ACE inhibitor-mediated angio-oedema [Nussberger et al, 1998]. An even greater incidence of angio-oedema has been reported following treatment with combined ACE and NEP inhibition [Armstrong et al, 2002]. Our findings are consistent with the suggestion that bradykinin may contribute to this rare, but potentially life-threatening side effect.

5.5.2 Role of the B_1 Receptor

We have demonstrated that intra-arterial Lys-des-Arg^9-bradykinin, a potent and highly selective agonist at the human B_1 kinin receptor, has no effect on blood flow or endothelial t-PA release in the forearm circulation of patients with heart failure maintained on long-term ACE inhibitor therapy. This is in contrast to our previous findings that combined B_1 and B_2 receptor blockade but not isolated selective blockade of the B_2 receptor causes vasoconstriction in patients with heart failure treated with ACE inhibition [Witherow et al, 2001]. We infused Lys-des-Arg^9-bradykinin at a dose that would achieve a local plasma concentration twenty times greater than those previously shown to produce 50% of the maximal vasomotor response in both human [Gobeil et al, 1996] and animal studies [Drapeau et al, 1991; Deblois et al, 2001] and, therefore, our findings are unlikely to reflect an inadequate

dose. Additional clinical studies using a selective B_1 kinin receptor antagonist are required to more fully investigate the role of the vascular B_1 receptor in man.

5.5.3 Atrial Natriuretic Peptide and Neutral Endopeptidase

Consistent with previous work demonstrating an impaired forearm vasodilator response to atrial natriuretic peptide in patients with heart failure [Cockcroft et al, 1989; Hirooka et al, 1990; van der Zander et al, 1999], atrial natriuretic peptide caused a modest dose-dependent increase in forearm blood flow that was not augmented by local NEP inhibition. Although suppression of PAI-1 expression in endothelial cells has been reported *in vitro* [Pawlowska et al, 2002], we report for the first time that intra-arterial atrial natriuretic peptide does not directly alter either plasma PAI-1 or t-PA antigen concentrations *in vivo* in man.

Local NEP inhibition did not potentiate atrial natriuretic peptide-mediated forearm vasodilatation in keeping with previous data demonstrating that intrabrachial thiorphan (30 nmol/min) does not increase endogenous plasma atrial natriuretic peptide concentrations in the human forearm circulation [Ferro et al, 1998]. This may, at first, appear surprising, given that systemic NEP inhibition augments plasma atrial natriuretic peptide concentrations in patients with heart failure [Northridge et al, 1999]. However, it is likely to reflect differences in the rate of clearance of atrial natriuretic peptide and bradykinin from the forearm circulation. The half-life of atrial natriuretic peptide (~5 minutes) is greater than that of bradykinin (~15 seconds). Assuming a transit time of the forearm vascular bed of ~30 seconds, NEP inhibition with intrabrachial thiorphan is unlikely to result in sufficient local accumulation of

atrial natriuretic peptide to augment forearm vasomotor responses. Moreover, the natriuretic peptide C receptor contributes equally to the clearance of plasma atrial natriuretic peptide and this pathway is unaffected by NEP inhibition.

5.5.4 Study Limitation

Although selective for NEP, thiorphan may cause some inhibition of ACE activity and theoretically our findings could represent further inhibition of ACE activity. Thiorphan exists as two enantiomers that, whilst equipotent for NEP inhibition, have differing potencies against ACE: selectivity of NEP compared to ACE inhibition of ~50-fold for (S)-thiorphan and 200-fold for (R)-thiorphan [Roques et al, 1993]. The preparation of thiorphan used in this study contains equal proportions of both isomers [Roques et al, 1993]. We do not believe that the effects of thiorphan were mediated through additional inhibition of ACE activity because local thiorphan infusion did not alter plasma ACE activity. Moreover, in a previous study [Ferro et al, 1998] using the same dose of thiorphan, there were no effects on plasma angiotensin II concentrations in the forearm circulation of healthy volunteers treated acutely with enalapril. However, we acknowledge that ACE inhibition by maximally tolerated doses of an ACE inhibitor may be incomplete [Jorde et al, 2000], and we cannot completely exclude a contribution of additional ACE inhibition to our study findings.

In conclusion, we have demonstrated that local NEP inhibition augments bradykinin-mediated vasodilatation and endothelial t-PA release in patients with heart failure maintained on long-term ACE inhibitor therapy. Using a potent B_1 receptor agonist,

we have shown that isolated stimulation of the B_1 kinin receptor does not cause vasodilatation or endothelial t-PA release in man. These findings confirm that NEP contributes to the metabolism of bradykinin in patients with heart failure and suggest that potentiation of the vascular and pro-fibrinolytic actions of bradykinin may explain some of the observed effects in recent clinical trials of combined ACE and NEP inhibitor therapy.

CHAPTER 6

THE B_1 KININ RECEPTOR DOES NOT CONTRIBUTE TO VASCULAR TONE OR TISSUE PLASMINOGEN ACTIVATOR RELEASE IN PATIENTS WITH HEART FAILURE TREATED WITH ANGIOTENSIN-CONVERTING ENZYME INHIBITION

6.1 SUMMARY

Vascular B_1 kinin receptor expression is markedly upregulated with left ventricular dysfunction and ACE inhibition but its function in man remains unknown. We investigated the contribution of the B_1 receptor to the maintenance of vascular tone and t-PA release in patients with heart failure. Eleven patients with heart failure were withdrawn from ACE inhibitor therapy for 2 weeks before receiving 6 weeks of enalapril (10 mg bd) or losartan (50 mg bd) in a randomised double-blind cross-over study. Patients received an intrabrachial infusion of Lys-des-Arg^9-bradykinin (B_1 agonist; 1-10 nmol/min), bradykinin (B_2 agonist; 30-300 pmol/min), sodium nitroprusside (2-8 μg/min), Lys-[Leu^8]-des-Arg^9-bradykinin (B_1 antagonist; 1-10 nmol/min), HOE-140 (1.5-13nmol/min) and norepinephrine (60-540 pmol/min). Blood flow and t-PA release were measured using venous occlusion plethysmography and blood sampling. Bradykinin and sodium nitroprusside caused dose-dependent vasodilatation ($p<0.0001$ for all). Norepinephrine caused vasoconstriction ($p<0.0005$ for all). Lys-des-Arg^9-bradykinin, Lys-[Leu^8]-des-Arg^9-bradykinin and HOE-140 had no effect on blood flow. Bradykinin ($p<0.001$ for all), but not Lys-des-Arg^9-bradykinin, caused dose-dependent t-PA antigen and activity release. Compared to ACE inhibitor withdrawal or losartan, enalapril augmented bradykinin-mediated vasodilatation ($p<0.05$ for both) and t-PA release ($p<0.01$ for all) but had no effect on responses to sodium nitroprusside, Lys-des-Arg^9-bradykinin, Lys-[Leu^8]-des-Arg^9-bradykinin, HOE-140 or noradrenaline. The B_1 kinin receptor does not have a major vasomotor or fibrinolytic role in patients with heart failure.

Augmentation of kinin-mediated vasodilatation and t-PA release by ACE inhibitor therapy is restricted to the B_2 receptor.

6.2 INTRODUCTION

Bradykinin is the major effector for the kinin family of peptides in man. It is released at sites of inflammation and coagulation and contributes to the systemic haemodynamic (Chapter 4) [Gainer et al, 1998] and anti-ischaemic [Witherow et al, 2002; Pretorius et al, 2003] effects of ACE inhibitor therapy. Besides vasodilatation, bradykinin stimulates endothelial release of the pro-lytic factor, t-PA, and these effects are mediated by the constitutively expressed B_2 kinin receptor [Brown et al, 2000].

Des-Arg^9-bradykinin is the principal ligand for the B_1 kinin receptor in human plasma and is generated by carboxypeptidases following removal of the C-terminal arginine from bradykinin. The vascular B_1 receptor is normally expressed very weakly but is markedly upregulated in the presence of inflammation [Deblois and Horlick, 2001], ischaemic left ventricular dysfunction [Tschope et al, 2000], cardiovascular disease [McLean et al, 2000b] and ACE inhibition [Whalley et al, 1989; Marin-Castano et al, 2002]. In animal studies, stimulation of the B_1 receptor produces vasodilatation and a reduction in blood pressure [Drapeau et al, 1991; Nakhostine et al, 1993; Su et al, 2000; Deblois and Horlick, 2001]. Intense endothelial B_1 receptor expression has been demonstrated in atheromatous human blood vessels [Raidoo et al, 1997] and B_1 receptor stimulation induces dose-

dependent vasodilatation in human coronary arteries *in vitro* [Drummond and Cocks, 1995b]. Whether the B_1 receptor contributes to the vascular effects of kinins *in vivo* in man remains unknown.

Angiotensin-converting enzyme is the principal enzyme responsible for the rapid breakdown of bradykinin (plasma half-life ~15 seconds) to its inactive metabolites [Marceau et al, 1998]. In addition to increasing plasma bradykinin concentrations, ACE inhibition will favour bradykinin breakdown by alternative metabolic pathways including plasma carboxypeptidases, augmenting the generation of des-Arg^9-bradykinin and thereby potentiating both B_1 and B_2 receptor mediated effects [Marceau et al, 1998]. B_2 receptor antagonism attenuates the vasodepressor effect of a single oral dose of captopril in healthy volunteers and subjects with hypertension [Gainer et al, 1998]. In patients with heart failure, the combined B_1 and B_2 kinin receptor antagonist, B9340, causes vasoconstriction in the forearm circulation in the presence, but not absence of ACE inhibition [Witherow et al, 2001], and when administered systemically, attenuates the haemodynamic effects of chronic ACE inhibition (Chapter 4). However, the role of the vascular B_1 kinin receptor in patients with heart failure and those treated with ACE inhibitor therapy remains to be established.

Although des-Arg^9-bradykinin is the principal B_1 agonist present in plasma, it has only modest affinity for the human B_1 receptor and retains some activity at the human B_2 receptor [Marceau et al, 1998]. Indeed, des-Arg^9-bradykinin is only 100-fold more selective for the human B_1 receptor [Marceau et al, 1998]. In contrast, Lys-

des-Arg^9-bradykinin, an endogenous tissue-based metabolite of kallidin, has ~1000-fold greater affinity for the B_1 receptor than des-Arg^9-bradykinin, and is inactive at the B_2 receptor in man [Gobeil et al, 1996; Marceau et al, 1998; Christiansen et al, 2002]. Substitution of the Phe^8 residue in Lys-des-Arg^9-bradykinin with leucine results in the formation of Lys-[Leu^8]-des-Arg^9-bradykinin, a synthetic peptide with potent selective inhibitory activity at the human B_1 receptor [Marceau et al, 1998; Deblois and Horlick, 2001].

The aims of this study were to investigate whether the B_1 receptor contributes to the vascular actions of kinins in the patients with heart failure treated with ACE inhibitor therapy. Using intrabrachial infusion of custom manufactured, clinical grade peptides, we examined the effects of selective kinin receptor agonism and antagonism on vascular tone and endothelial t-PA release in patients with heart failure maintained on long-term ACE inhibition.

6.3 METHODS

6.3.1 PATIENTS

Eleven patients with symptomatic heart failure (NYHA class II or III) and evidence of left ventricular systolic dysfunction (ejection fraction <40%, shortening fraction <20% or left ventricular end-diastolic dimension >55 mm) were recruited. Patients were maintained on maximally tolerated ACE inhibitor therapy for at least 6 months prior to enrolment.

6.3.2 DRUGS

Pharmaceutical grade bradykinin (Clinalfa AG, Läufelfingen, Switzerland), Lys-des-Arg^9-bradykinin (Clinalfa), Lys-[Leu^8]-des-Arg^9-bradykinin (Clinalfa), HOE-140 (Clinalfa), sodium nitroprusside (Mayne Pharma plc, Warwickshire, UK) and norepinephrine (Abbott Laboratories Ltd, Maidenhead, UK) were dissolved in physiological saline on the day of study. The doses of bradykinin, HOE-140, sodium nitroprusside and norepinephrine were chosen based on the results of previous studies [Newby et al, 1997a]. The doses of Lys-des-Arg^9-bradykinin and Lys-[Leu^8]-des-Arg^9-bradykinin were chosen based on the EC_{50} (B_1 receptor, 0.2 nmol/L; B_2 receptor, >30000 nmol/L) and IC_{50} (B_1, 1.3 nmol/L; B_2, >30000 nmol/L) respectively for human kinin receptors *in vitro* [Marceau et al, 1998], data from human umbilical vein myography studies [Gobeil et al, 1996; Sardi et al, 1998] and the hypotensive dose response in rodents and non-human primates [Drapeau et al, 1991; Deblois and Horlick, 2001].

6.3.3 STUDY DESIGN

Where symptoms would allow, ACE inhibitor therapy was withdrawn for a period of 2 weeks (n=7). Following this and in place of their usual ACE inhibitor, patients were randomised to receive 6 weeks of treatment with enalapril 10 mg [CONSENSUS, 1987] twice daily or losartan 50 mg [Pitt et al, 2000] twice daily in a double-blind, cross-over trial. One patient withdrew due to worsening symptoms and was replaced. During week 2 of ACE inhibitor withdrawal and weeks 6 and 12 of the cross-over trial, patients attended on two occasions at least 3 days apart and

underwent an agonist infusion protocol on one occasion and an antagonist infusion protocol on the other. The study order remained constant for each patient but was randomised between patients.

6.3.4 Drug Infusion Protocol

Study drugs were infused in random order for 10 minutes at each dose and separated by a 20 minute infusion of 0.9% saline.

6.3.4.1 *Agonist Protocol*

After 30 minutes equilibration with 0.9% saline, patients received an intrabrachial infusion of Lys-des-Arg^9-bradykinin (1, 3 and 10 nmol/min), bradykinin (30, 100 and 300 pmol/min) and sodium nitroprusside (2, 4 and 8 μg/min).

6.3.4.2 *Antagonist Protocol*

After 30 minutes equilibration with 0.9% saline, patients received an intrabrachial infusion of Lys-[Leu^8]-des-Arg^9-bradykinin (1, 3 and 10 nmol/min), HOE-140 (1.5, 4 and 13 nmol/min) and norepinephrine (60, 180 and 540 pmol/min).

6.3.5 Data Analysis

Data were examined by two-way ANOVA with repeated measures and two-tailed Student's *t*-test using GraphPad PRISM (GraphPad). All results are expressed as mean ± standard error of the mean. Statistical significance was taken at the 5% level.

6.4 RESULTS

Patients were predominantly male with mild to moderate congestive heart failure due to ischaemic heart disease (Table 8). There were no significant differences in heart rate, blood pressure or baseline forearm blood flow during or between study days (Table 9).

6.4.1 PLASMA NEUROHORMONE CONCENTRATIONS

Compared to losartan, plasma ACE activity (42.2 ± 11 *versus* 10.5 ± 6.1 Units/L respectively; $p<0.05$) and angiotensin II concentrations (24.4 ± 6.3 *versus* 7.8 ± 1.6 pg/mL respectively; $p<0.05$) were lower during treatment with enalapril (Figure 19).

6.4.2 FOREARM BLOOD FLOW

6.4.2.1 *Agonist Protocol*

Bradykinin ($p<0.0001$ for all) and sodium nitroprusside ($p<0.0001$ for all), but not Lys-des-Arg^9-bradykinin (p=not significant for all), caused dose-dependent vasodilatation in all studies (Figure 20). Bradykinin-mediated vasodilatation was augmented in patients treated with enalapril compared to losartan ($p<0.005$; Figure 20) or ACE inhibitor withdrawal ($p<0.05$; Figure 20).

6.4.2.2 *Antagonist Protocol*

Norepinephrine ($p<0.0005$ for all), but not Lys-[Leu^8]-des-Arg^9-bradykinin or HOE-140 (p=not significant for all), caused dose-dependent vasoconstriction in all studies (Figure 21).

Table 8. Patient characteristics.

Variable	All Patients (n=10)	Units
Age	68 (53 - 79)	years (range)
Gender	10/1	male/female
Diagnosis	9/2	IHD/DCM
NYHA class	7/4	II/III
Risk factors for IHD		n
Smokers/non-smokers	2/9	
Hypertension	3	
Diabetes mellitus	0	
Hypercholesterolaemia	8	
Body mass index	28 ± 1	kg/m^2
Concomitant medications		n
ACE inhibitor	11	
Aspirin	10	
Diuretic	9	
Beta-blocker	3	
Statin	8	
Nitrate	5	
Digoxin	1	
Calcium Antagonist	4	
Echocardiography		
Ejection fraction	36 ± 3	%
LVEDD	63 ± 2	mm

Data are expressed as number of patients or mean ± SEM unless indicated. IHD, ischaemic heart disease; DCM, idiopathic dilated cardiomyopathy; NYHA, New York Heart Association; ACE, angiotensin-converting enzyme; LVEDD, left ventricular end diastolic diameter.

Table 9. Baseline haemodynamics.

Parameter	Agonist Study	Antagonist Study
Baseline heart rate	beats/min	beats/min
Withdrawal	68 ± 5	72 ± 4
Enalapril	61 ± 4	61 ± 5
Losartan	64 ± 4	67 ± 3
Baseline mean arterial pressure	mmHg	mmHg
Withdrawal	97 ± 6	99 ± 5
Enalapril	96 ± 6	94 ± 5
Losartan	98 ± 6	97 ± 5
Forearm blood flow	mL/100mL/min	mL/100mL/min
Withdrawal	3.2 ± 0.3	2.8 ± 0.3
Enalapril	2.4 ± 0.3	2.7 ± 0.2
Losartan	2.6 ± 0.3	2.9 ± 0.5

Data are expressed as mean ± SEM. Mean arterial pressure was calculated as diastolic pressure plus one-third pulse pressure.

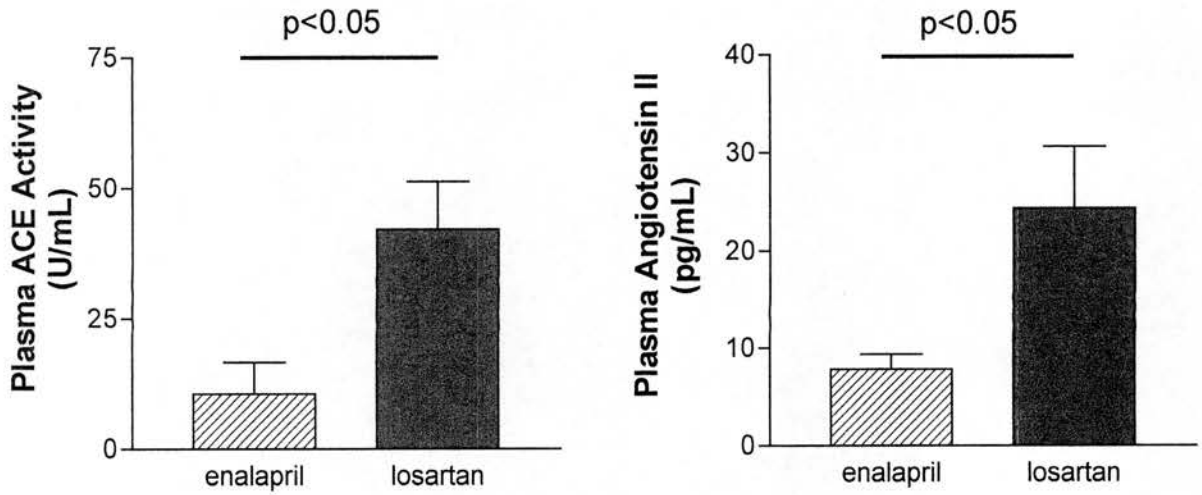

Figure 19. Plasma ACE activity and angiotensin II concentrations in patients with heart failure after 5 weeks of treatment with enalapril (open bar) or losartan (solid bar).

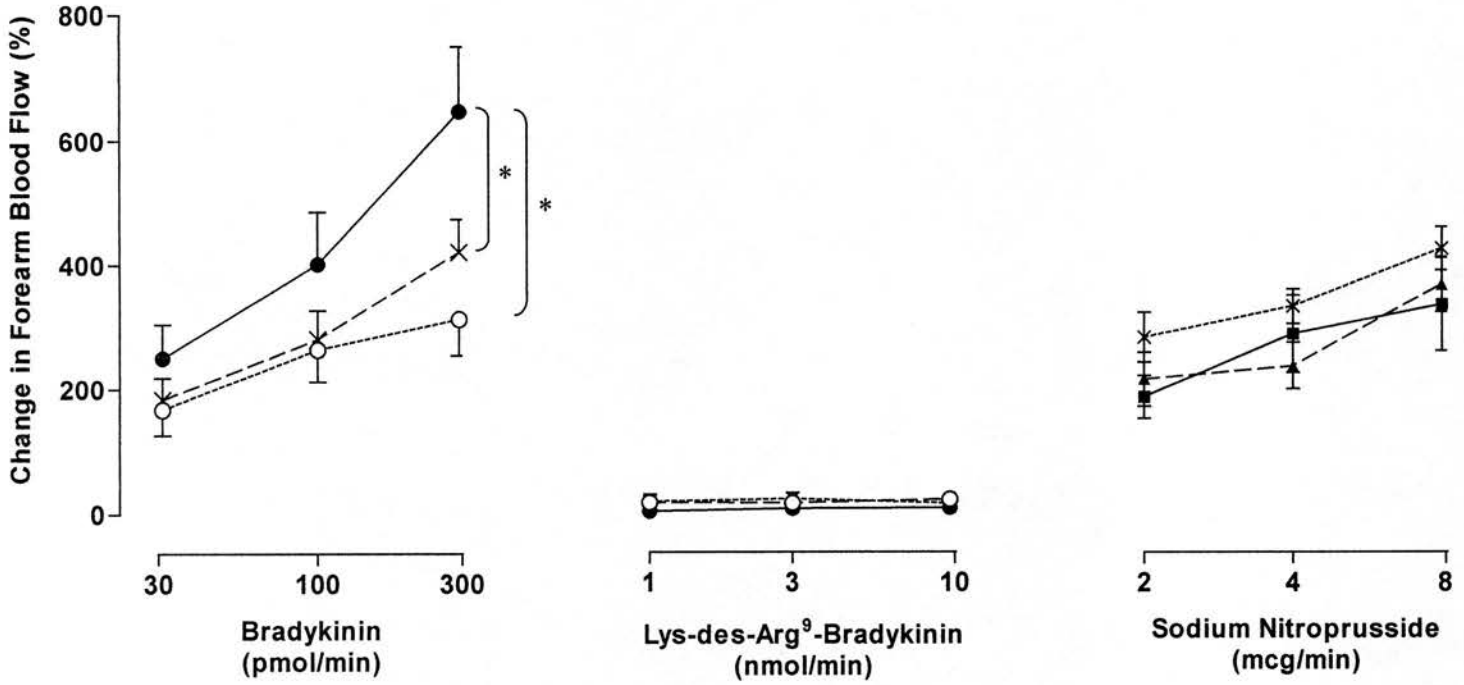

Figure 20. Effect of bradykinin, Lys-des-Arg^9-bradykinin and sodium nitroprusside on forearm blood flow in patients treated with enalapril (solid circles), losartan (open circles) or following ACE inhibitor withdrawal (crosses).*$p<0.05$, †$p<0.005$ by analysis of variance.

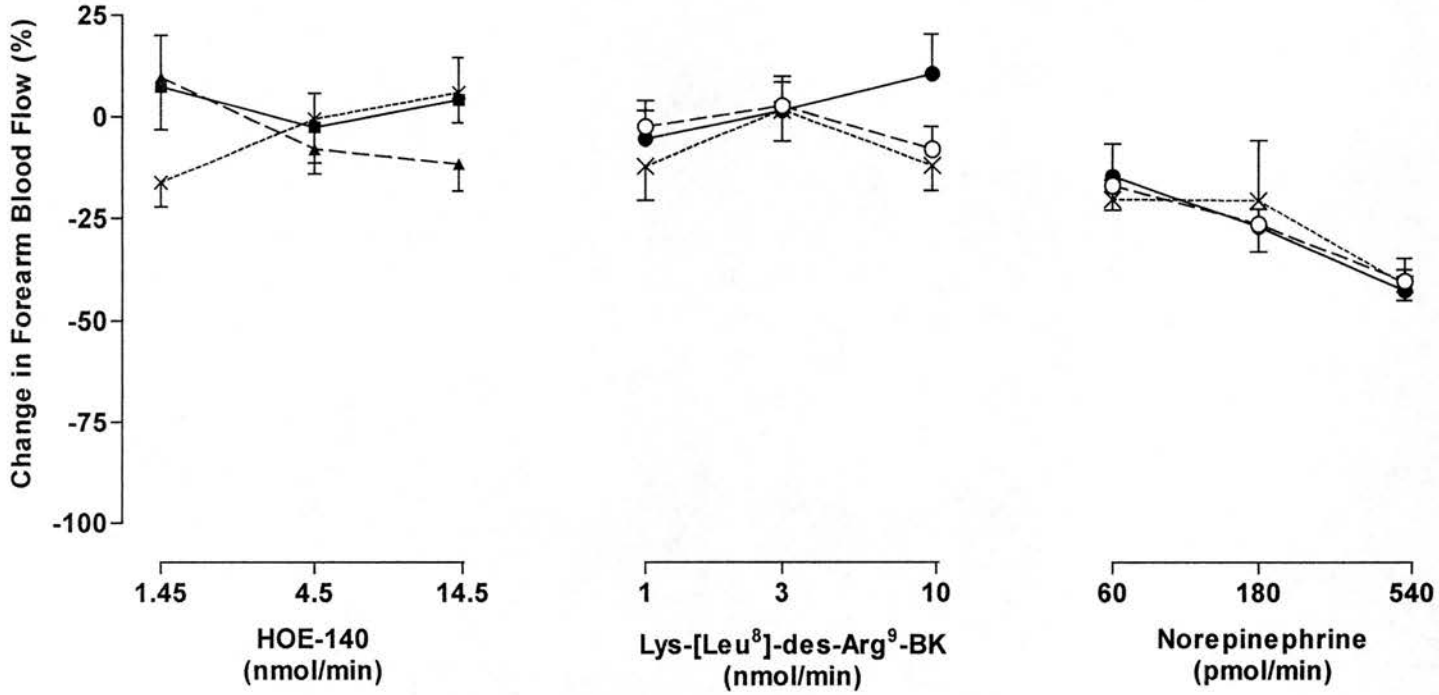

Figure 21. Effect of HOE-140, Lys-[Leu^8]-des-Arg^9-bradykinin (Lys-[Leu^8]-des-Arg^9-BK) and norepinephrine on forearm blood flow in patients treated with enalapril (solid circles), losartan (open circles) or following ACE inhibitor withdrawal (crosses).

6.4.3 FIBRINOLYTIC FACTORS

6.4.3.1 *Release of t-PA*

Bradykinin ($p<0.001$ for all), but not Lys-des-Arg^9-bradykinin (p=not significant for all), caused dose-dependent increases in plasma concentrations of t-PA antigen and activity in the infused arm and net release of t-PA antigen and activity in all studies (Figure 22). The bradykinin-mediated increase in plasma t-PA antigen and activity in the infused arm, and net release of t-PA antigen and activity was augmented in patients treated with enalapril compared to losartan ($p\leq0.0005$ for all) or ACE inhibitor withdrawal ($p<0.005$ for all; Figure 22).

Consistent with systemic overspill [Witherow et al, 2002], bradykinin caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the non-infused arm with enalapril therapy, and plasma t-PA antigen alone following ACE inhibitor withdrawal ($p\leq0.0001$ for all; Figure 22). Enalapril augmented the increase in t-PA compared to losartan therapy or ACE inhibitor withdrawal ($p<0.01$ for both; Figures 22 and 23).

6.4.3.2 *Plasma PAI-1 Antigen*

There were no significant differences in basal PAI-1 antigen concentrations between study days. Consistent with an increase in PAI-1 mediated clearance following marked t-PA release [Chandler et al, 1997], and a potential time effect [Brown et al, 2002], plasma PAI-1 antigen concentrations fell during infusion of bradykinin in both the infused (47.6 ± 7.9 ng/mL at baseline *versus* 43.8 ± 7 ng/mL during bradykinin 300 pmol/min; $p<0.05$) and non-infused arms (51 ± 8.2 ng/mL at baseline

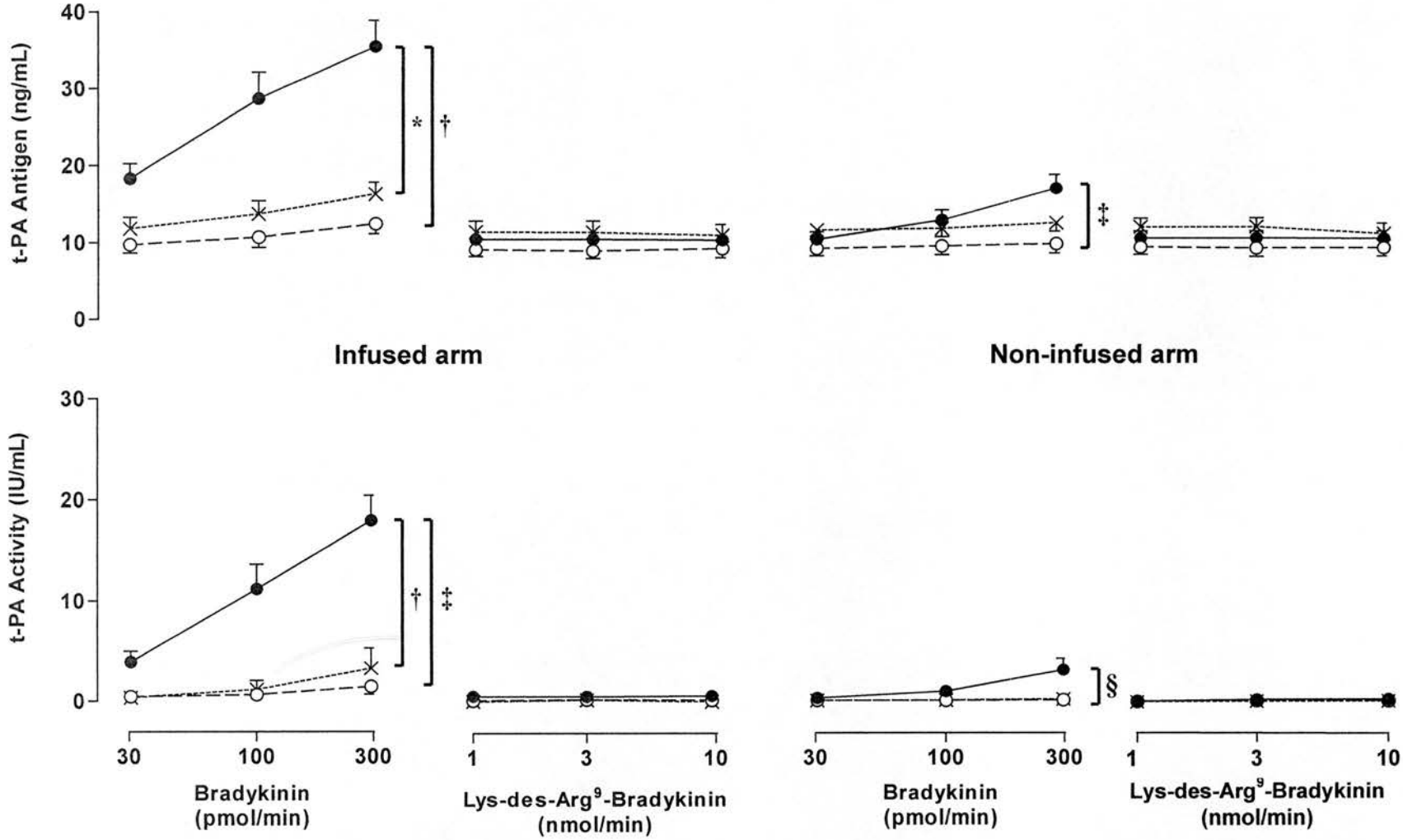

Figure 22. Effect of bradykinin and Lys-des-Arg^9-bradykinin on plasma t-PA antigen and activity concentrations in the infused and non-infused arm in patients treated with enalapril (solid circles), losartan (open circles) or following ACE inhibitor withdrawal (crosses).*$p<0.005$, †$p\leq0.0005$, ‡$p<0.0001$, §$p<0.01$ by analysis of variance.

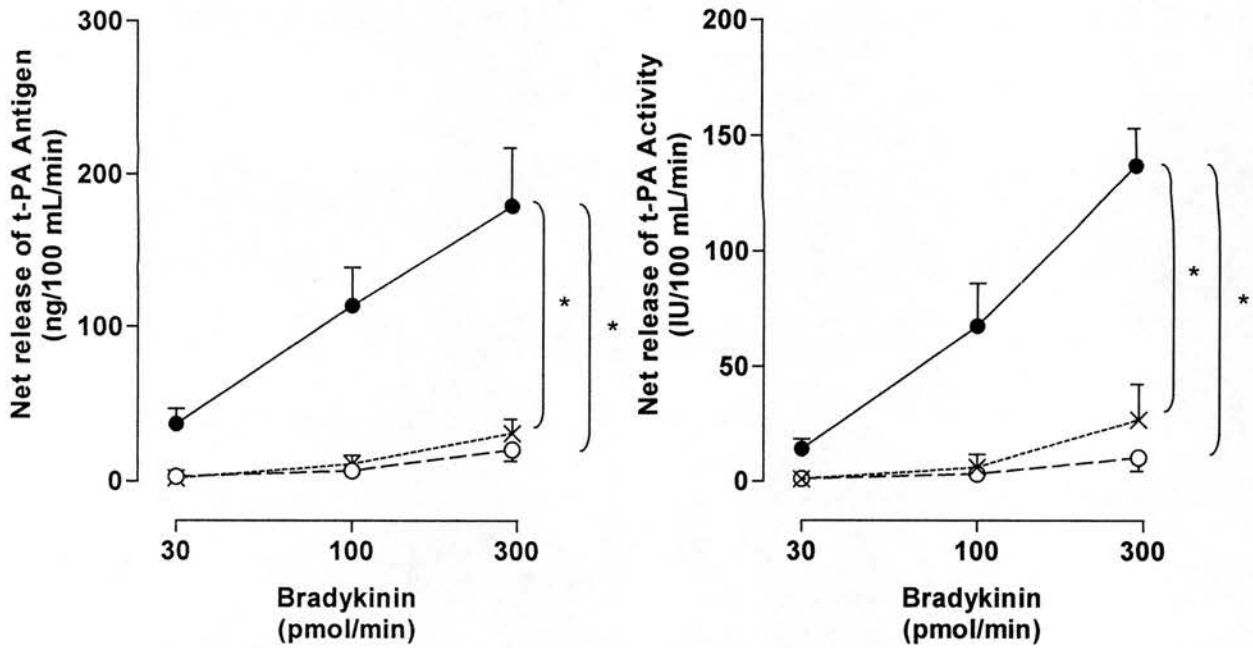

Figure 23. Effect of bradykinin on net release of t-PA antigen and activity in patients treated with enalapril (solid circles), losartan (open circles) or following ACE inhibitor withdrawal (crosses).*$p<0.0001$ by analysis of variance.

versus 44.5 ± 7 ng/mL during bradykinin 300 pmol/min; $p<0.05$) in patients treated with enalapril but not losartan or during ACE inhibitor withdrawal.

6.5 DISCUSSION

This is the first study to characterise the potential vasomotor and fibrinolytic role of the vascular B_1 kinin receptor *in vivo*. We have demonstrated that the peptidic B_1 receptor agonist, Lys-des-Arg^9-bradykinin, and antagonist, Lys-[Leu^8]-des-Arg^9-bradykinin have no effect on vascular tone or endothelial t-PA release in the presence or absence of ACE inhibition. In contrast, and consistent with our previous unblinded and non-randomised data [Witherow et al, 2002], ACE inhibition markedly augmented the vascular actions of bradykinin mediated via the B_2 receptor. We conclude that the B_1 receptor does not appear to have a major vasomotor or fibrinolytic role in the forearm circulation of patients with heart failure treated with chronic ACE inhibition.

Our findings are in contrast to previous *in vitro* and animal work demonstrating vasodilatation following B_1 receptor stimulation [Drapeau et al, 1991; Nakhostine et al, 1993; Su et al, 2000; Deblois and Horlick, 2001; Marin-Castano et al, 2002]. Before concluding that the B_1 kinin receptor does not mediate vasodilatation or endothelial t-PA release in the forearm circulation of patients with heart failure, we must first consider the following possibilities: the doses of the Lys-des-Arg^9-bradykinin and Lys-[Leu^8]-des-Arg^9-bradykinin used in this study were inadequate;

the custom-made peptides lacked biological activity; or the extent of ACE inhibition was insufficient to upregulate B_1 receptor expression.

We infused Lys-des-Arg^9-bradykinin at a dose which was at least 20-fold greater than that previously shown to produce 50% of the maximal hypotensive response in both primate ($EC_{50} \sim 0.1$ pmol/kg)[6] and rodent studies ($EC_{50} \sim 0.3$ pmol/kg) [Drapeau et al, 1991]. Similarly, Lys-[Leu^8]-des-Arg^9-bradykinin was infused at a dose 20-fold greater than that previously shown to abolish B_1 receptor mediated vasomotor responses in animal models *in vivo* [Drapeau et al, 1991; Deblois and Horlick, 2001]. To address the issue of biological activity, we have examined vasomotor responses to the custom manufactured B_1 agonist and antagonist in isolated human umbilical vein (Chapter 3) [Sardi et al, 1998]. The concentration-response curves obtained for Lys-des-Arg^9-bradykinin and Lys-[Leu^8]-des-Arg^9-bradykinin were comparable with data from previous studies (Chapter 3) [Gobeil et al, 1996; Sardi et al, 1998] and confirm efficacy at concentrations predicted to be achieved in the infused human forearm circulation.

Previous rodent studies have demonstrated that, besides cardiovascular inflammation, chronic ACE inhibition upregulates functional vascular B_1 receptor expression [Marin-Castano et al, 2002]. We have examined B_1 receptor function in patients with heart failure treated with an effective evidence based dose of enalapril [CONSENSUS, 1987]. Moreover, plasma concentrations of angiotensin II and ACE activity confirmed significant inhibition of the renin-angiotensin system with enalapril at this dose. From our findings, therefore, we can conclude that the B_1 kinin

receptor does not mediate vasodilatation or endothelial t-PA release in patients with mild to moderate heart failure treated with long-term ACE inhibitor therapy.

We have previously demonstrated that combined B_1 and B_2 receptor blockade, but not B_2 receptor blockade, causes peripheral vasoconstriction in patients with heart failure treated with ACE inhibition. In our current study, however, selective B_1 receptor antagonism had no effect on peripheral vascular tone. One potential explanation for this discrepancy is that the B_1 receptor may only mediate the vasomotor effects of kinins in the absence of B_2 receptor mediated signalling. In support of this hypothesis, the B_1 and B_2 kinin receptors are coupled to similar G-protein subtypes and share the same intracellular signalling pathways [Leeb et al, 1997; Marceau et al, 1998]. In transgenic mice lacking the B_2 kinin receptor, the B_1 receptor is upregulated and assumes vascular functions normally associated with the B_2 receptor [Duka et al, 2001]. Consistent with these data, we have demonstrated that the B_2 receptor antagonist, HOE-140, augments the vasomotor responses to the B_1 agonist, Lys-des-Arg^9-bradykinin, in human umbilical vein *in vitro* (Chapter 3). Future studies examining the effects of B_1 receptor agonism and antagonism during concomitant administration of HOE-140 may help clarify the issue of kinin receptor cross-talk in the peripheral circulation of patients with heart failure.

6.5.1 Study Limitations

It has been suggested that the extent of the inflammatory response in patients with congestive cardiac failure correlates with the severity of underlying heart failure [Levine et al, 1990]. We have examined B_1 receptor function in patients with mild to

moderate (NYHA class II-III) heart failure. We cannot exclude the possibility that vascular B_1 receptor expression may be restricted to patients with severe end-stage heart failure. In addition, the B_1 kinin receptor has been implicated in a number of alternative biological processes, including leukocyte trafficking [McLean et al, 2000a] and ischaemia-induced angiogenesis [Emanueli et al, 2002]. It remains possible that B_1 receptors, mediating processes other than vasodilatation or endogenous fibrinolysis, may be present in the human forearm vasculature. Finally, we have examined B_1 mediated responses in the forearm circulation of patients with heart failure. Specific vascular beds may differ in their response to B_1 agonists and the current findings cannot be extrapolated to the entire vasculature.

In conclusion, contrary to data from animal studies, we have demonstrated for the first time that the B_1 kinin receptor does not mediate vasodilatation or endothelial t-PA release in the peripheral circulation of patients with heart failure treated with long-term ACE inhibition. Our findings suggest that the beneficial vascular effects of ACE inhibitor therapy attributed to kinins are restricted to those mediated by the B_2 receptor and do not support a major role for the B_1 kinin receptor as a potential therapeutic target in patients with heart failure.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 INTRODUCTION

Chronic heart failure is a common condition in western society that is associated with significant mortality and morbidity. Activation of the renin-angiotensin-aldosterone system plays a key role in the salt and water retention and vasoconstriction central to the pathogenesis of this condition. Recently, it has become clear that the plasma kinin system acts as a counterbalance for the renin-angiotensin-aldosterone system [Schmaier, 2002]. Intimately involved with both these biological systems is the membrane-bound metallopeptidase, ACE, responsible both for the breakdown of bradykinin and the production of angiotensin II. It is now widely accepted that inhibitors of ACE improve symptoms and survival in patients with chronic heart failure and there is increasing evidence that these effects may be mediated, at least in part by potentiation of bradykinin. Confirmation of this hypothesis in patients with heart failure should support the development of novel therapeutic strategies to further augment the vascular actions of bradykinin in the belief that this may afford greater cardiovascular benefit.

The vascular effects of plasma kinins are mediated by two principal receptor subtypes, B_1 and B_2. Until recently, attention has focused on the constitutively expressed endothelial B_2 receptor and the role of the inducible human vascular B_1 receptor *in vivo* remains unclear. A number of metabolic pathways exist in plasma that result in the production or degradation of kinins selective for a specific receptor subtype. Therefore, inhibitors of specific plasma enzymes responsible for kinin metabolism may shift the equilibrium within the plasma kinin system in favour of

either the B_1 or B_2 kinin receptor. Hence, determining the relative contribution of the B_1 kinin receptor to the vasomotor and fibrinolytic effects of plasma kinins in patients with heart failure is crucial both for our understanding of the mechanism of action of ACE inhibitor therapy and for the development of novel therapies targeting specific kinin receptor mediated effects such as vasodilatation and endothelial t-PA release.

Using local and systemic infusions of bradykinin and related peptides combined with robust physiological models for assessing vascular function *in vivo*, we have addressed these important questions through a series of randomised, double-blind, controlled trials in patients with heart failure.

7.2 BIOLOGICAL EFFICACY OF KININ-RELATED PEPTIDES

Prior to use in clinical studies, the biological efficacy of the custom-made peptides, Lys-des-Arg^9-bradykinin, bradykinin, Lys-[Leu^8]-des-Arg^9-bradykinin and HOE-140, obtained from Clinalfa was examined in human umbilical vein *in vitro*. The data obtained confirm that these custom-made peptides have similar biological efficacy and potency at the human B_1 receptor as established commercially available laboratory grade peptides [Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999]. Consistent with previous work [Gobeil et al, 1996; Sardi et al, 1998; Sardi et al, 1999], Lys-des-Arg^9-bradykinin, a B_1 receptor agonist, and bradykinin, a B_2 receptor agonist, caused dose-dependent vasoconstriction that was selectively inhibited by their respective antagonists, Lys-[Leu^8]-des-Arg^9-bradykinin and HOE-140. The

combined B_1 and B_2 receptor antagonist, B9340, attenuated vasomotor responses to both Lys-des-Arg^9-bradykinin and bradykinin. In contrast, however, the B_2 receptor antagonist, HOE-140, shifted the dose response curve for Lys-des-Arg^9-bradykinin to the left, suggesting potentiation of B_1 receptor mediated responses in the presence of B_2 receptor antagonism. These functional data are supported by immunohistochemical evidence of B_1 and B_2 kinin receptor expression on vascular smooth muscle cells in human umbilical vein rings.

7.3 BRADYKININ CONTRIBUTES TO THE SYSTEMIC HAEMODYNAMIC EFFECTS OF ACE INHIBITION

Consistent with previous findings *in vitro* [Stewart et al, 1997] and *in vivo* [Witherow et al, 2003], we have demonstrated using human umbilical myography that B9340 is a selective, reversible peptidic kinin antagonist with similar efficacy at both the B_1 and B_2 kinin receptor. When administered intravenously, B9340 caused systemic inhibition of bradykinin-mediated vasodilatation in a dose-dependent manner in healthy volunteers. Furthermore, we have demonstrated that B9340 has a significant pressor effect (~5 mmHg increase in mean arterial pressure) in patients with chronic heart failure maintained on long-term ACE inhibitor but not angiotensin receptor blocker therapy. These data provide convincing evidence that bradykinin contributes to the systemic haemodynamic effects of long-term ACE inhibition in patients with heart failure.

Given the proven therapeutic benefits of ACE inhibitor therapy in patients with heart failure [CONSENSUS, 1987; Flather et al, 2000] and vascular disease [Yusuf et al, 2000; Fox, 2003], these findings provide additional support for bradykinin and, in particular, inhibitors of bradykinin metabolism as potential therapeutic targets in the treatment of cardiovascular disease. Indeed, additional inhibitors of bradykinin metabolism may further potentiate the vascular effects of bradykinin in patients already maintained on ACE inhibitor therapy. To investigate this hypothesis, we chose to examine the effect of inhibition of NEP on the vascular actions of bradykinin in the peripheral circulation of patients with heart failure maintained on chronic ACE inhibitor therapy.

7.4 EFFECT OF COMBINED ACE AND NEP INHIBITION ON THE VASCULAR ACTIONS OF BRADYKININ

We have demonstrated for the first time in patients with heart failure treated with ACE inhibition, that the haemodynamic and pro-fibrinolytic effects of bradykinin are significantly augmented by additional local NEP inhibition. Our findings suggest that bradykinin-mediated vasodilatation may contribute to the greater vasodepressor action observed with combined ACE and NEP inhibition when compared to isolated ACE inhibition [Favrat et al, 1995; Armstrong et al, 2002]. Moreover, given the undeniable success of ACE inhibitor therapy in the treatment of patients with heart failure, these findings would be expected to have important therapeutic consequences.

In the recent OVERTURE trial [Packer et al, 2002] of patients with heart failure, treatment with omapatrilat, a combined ACE and NEP inhibitor, failed to reduce all-cause mortality when compared to enalapril, although it did reduce the combined secondary endpoint of cardiovascular death and hospitalisation. Post-hoc analysis redefining endpoints according to the SOLVD criteria suggested that omapatrilat may be more effective at preventing cardiovascular events than enalapril, but that the additional benefit was substantially smaller than anticipated [Packer et al, 2002]. These data are in keeping with the marked augmentation of endothelial t-PA release with ACE inhibition previously observed in patients with heart failure [Witherow et al, 2002] compared to the smaller additional benefit associated with combined ACE and NEP inhibition demonstrated in this study. The apparent lack of benefit in OVERTURE (Packer et al, 2002) may also, in part, reflect the shorter duration of NEP inhibition, relative to ACE inhibition, with omapatrilat [Azizi et al, 2002]. Given our findings, pharmacological strategies that offer a more balanced and prolonged duration of combined ACE and NEP inhibition may confer greater cardiovascular benefit.

In contrast to bradykinin, local NEP inhibition did not potentiate atrial natriuretic peptide-mediated forearm vasodilatation. This may, at first, appear surprising, given that systemic NEP inhibition augments plasma atrial natriuretic peptide concentrations in patients with heart failure [Northridge et al, 1999]. However, local NEP inhibition with thiorphan (30 nmol/min) does not increase endogenous plasma atrial natriuretic peptide concentrations in the human forearm circulation [Ferro et al, 1998]. This is likely to reflect differences in the rate of clearance of atrial natriuretic

peptide and bradykinin from the forearm circulation. The half-life of atrial natriuretic peptide (~5 minutes) is greater than that of bradykinin (~15 seconds). Assuming a transit time of the forearm vascular bed of ~30 seconds, acute local NEP inhibition is unlikely to result in sufficient local accumulation of atrial natriuretic peptide to augment forearm vasomotor responses. Moreover, besides NEP, the natriuretic peptide C receptor contributes to a similar extent to the clearance of plasma atrial natriuretic peptide and this pathway is unaffected by NEP inhibition.

7.5 FUNCTIONAL ROLE OF SPECIFIC KININ RECEPTORS

7.5.1 B_1 KININ RECEPTOR

In contrast to data from animal models of inflammation and cardiovascular disease, we have demonstrated that the peptidic B_1 receptor agonist, Lys-des-Arg^9-bradykinin, and antagonist, Lys-[Leu^8]-des-Arg^9-bradykinin, have no effect on vascular tone or endothelial t-PA release. Whilst these data hold true for the forearm circulation of patients with mild to moderate heart failure, we must first consider a number of potential limitations before extrapolating our findings to the human vasculature as a whole.

Historically, the forearm circulation has been considered relatively protected from atheroma for reasons that remain unclear. The only previous study of human vascular B_1 kinin receptor expression has suggested that B_1 kinin receptor expression may be concentrated in atheromatous plaques [Raidoo et al, 1997]. Therefore, functional B_1 kinin receptors may be restricted to vascular beds with high atheroma burden such as

the coronary or femoral circulations. Moreover, specific vascular beds may differ in their response to B_1 agonists, independent of the burden of atheromatous disease.

We have examined B_1 receptor function in patients with mild to moderate (NYHA class II-III) heart failure. It has been suggested that the inflammation associated with heart failure correlates with the severity of disease. Assuming a sufficient inflammatory stimulus is required for B_1 receptor expression, we cannot exclude the possibility that vascular B_1 receptor expression may be restricted to patients with severe end-stage heart failure.

The B_1 kinin receptor has been implicated in a number of alternative biological processes, including leucocyte trafficking [McLean et al, 2000a] and ischaemia-induced angiogenesis [Emanueli et al, 2002]. It remains possible that B_1 receptors, mediating processes other than vasodilatation or endogenous fibrinolysis, may be present in the human forearm vasculature.

7.5.2 B_2 Kinin Receptor

Consistent with previous work [Witherow et al, 2001; Witherow et al, 2002], bradykinin caused dose-dependent vasodilatation and endothelial t-PA release in the peripheral circulation of patients with heart failure. Previous work in healthy volunteers has demonstrated that these effects are mediated through the B_2 kinin receptor by a nitric oxide synthase- and cyclooxygenase-independent pathway [Brown et al, 2000]. Although endothelium-derived hyperpolarising factor has been

implicated, the downstream signalling mechanism resulting in t-PA release following B_2 receptor activation remains unclear [Brown et al, 2000].

7.5.3 ACE Inhibition and Kinin Receptor Function

Inhibition of ACE had no effect on B_1 receptor mediated vasodilatation or t-PA release in patients with heart failure. Bearing in mind the potential limitations discussed above, it can be concluded that the B_1 receptor does not have a major vasomotor or fibrinolytic role in the forearm circulation of patients with heart failure treated with chronic ACE inhibition.

The effect of ACE inhibition on vascular B_1 expression remains controversial. Whilst some investigators have reported upregulation of functional B_1 receptor expression in the systemic circulation [Nwator and Whalley, 1989; Marin-Castano et al, 2002], data from the present study and others [Deblois et al, 1991; Marceau et al, 1999] do not support this hypothesis. This apparent conflict may be due to differences in species- or vascular bed-specific responses, or may reflect a requirement for concomitant signalling processes occurring in specific pathological states associated with increased B_1 receptor expression.

In contrast to B_1 receptor mediated responses, inhibition of ACE significantly augmented bradykinin-mediated vasodilatation (~2-fold) and endothelial t-PA release (~6-fold) in a randomised, double-blind, controlled trial of patients with heart failure, when compared to losartan therapy or withdrawal of agents blocking the renin-angiotensin system. These findings confirm the results of our earlier

non-randomised and unblinded work [Witherow et al, 2002] and provide further support for a role for bradykinin in the anti-ischaemic effects of ACE inhibitor therapy.

The major clinical benefits of ACE inhibition have been incontrovertibly established in patients with coronary artery disease. Although first demonstrated in patients with heart failure, it is now clear from the HOPE [Yusuf et al, 2000], EUROPA [Fox, 2003] and PEACE [Braunwald et al, 2004] trials that the clinical benefits of ACE inhibitor therapy extend to patients with cardiovascular disease and normal left ventricular systolic function. Indeed, when comparing these studies with the meta-analyses of heart failure trials [Flather et al, 2000] there is a clear and remarkably consistent anti-ischaemic effect of ACE inhibitors with a 20-22% relative risk reduction in the rate of myocardial infarction.

In contrast, the beneficial effects on mortality appear to depend upon the overall cardiovascular risk of the patient: greatest in those with severe left ventricular dysfunction and least in those at low risk with preserved left ventricular dysfunction. Thus, both the augmentation of bradykinin-induced vasodilatation and the marked (6- to 10-fold) increase in t-PA release described with ACE inhibition in patients with coronary artery disease may contribute to the primary mechanism of the anti-ischaemic effects associated with chronic ACE inhibitor therapy. Consistent with this hypothesis, ACE inhibitor therapy reduces myocardial troponin release in patients with acute coronary syndromes [Kennon et al, 2001]. The development of novel therapies potentiating the vascular actions of bradykinin mediated by the B_2 receptor

has the potential, therefore, to provide clinical benefits both in the treatment of congestive heart failure and atheromatous vascular disease. These will be discussed below.

7.6 FUTURE DIRECTIONS

7.6.1 B_1 Receptor and Atherosclerosis

There is now a substantial body of evidence that systemic inflammation plays an important role in the pathogenesis of atherosclerosis [Ross, 1999]. Moreover, human postmortem studies have indicated that B_1 receptors are markedly upregulated in the presence of atherosclerosis [Raidoo et al, 1997]. Although we have examined the effects of B_1 receptor agonism and antagonism in the forearm circulation of patients with heart failure predominantly due to ischaemic heart disease, it is widely accepted that the forearm vascular bed is relatively protected from atherosclerotic plaque formation. Further work is required to define the effects of B_1 kinin receptor agonism and antagonism on vascular tone and endogenous fibrinolysis in human atherosclerotic arteries *in vivo*.

We are currently examining the relationship between atherosclerosis and B_1 kinin receptor mediated vasomotor and fibrinolytic effects as part of an on-going British Heart Foundation Research Project (PG/02/092). Using intravascular ultrasound (IVUS), Doppler flow measurements and arteriovenous sampling combined with selective intra-arterial infusions [Newby, 2000], this study will examine the effect of selective kinin receptor agonists and antagonists on blood flow and t-PA release in

the femoral circulation of patients undergoing routine diagnostic coronary angiography. During the study, motorised pullback of the IVUS catheter permits accurate quantification of the atheromatous plaque burden for a given length of the vessel being studied. Given the significant levels of plaque burden in the femoral circulation in patients with suspected coronary artery disease, this study will allow us to directly address the question of whether atherosclerosis is associated with functional B_1 receptor expression.

7.6.2 Kinin Receptor Interactions

It has previously been demonstrated that combined B_1 and B_2 receptor blockade, but not B_2 receptor blockade alone, causes peripheral vasoconstriction in patients with heart failure treated with ACE inhibition [Witherow et al, 2001]. In the data reported here, however, selective B_1 receptor antagonism had no effect on peripheral vascular tone. One potential explanation for this discrepancy is that the B_1 receptor may only mediate the vasomotor effects of kinins in the absence of B_2 receptor mediated signalling. In support of this hypothesis, the B_1 receptor is upregulated in transgenic mice lacking the B_2 kinin receptor where it assumes the vasomotor functions normally associated with the B_2 receptor [Duka et al, 2001]. These findings are perhaps not surprising given that the B_1 and B_2 kinin receptors are coupled to similar membrane associated G-proteins and share common intracellular signalling pathways [Marceau et al, 1998; Leeb-Lundberg et al, 2005]. The hypothesis that significant cross-talk exists between the B_1 and B_2 kinin receptors is supported by the data presented in Chapter 3 demonstrating that B_2 receptor antagonism augments B_1 mediated vasomotor responses in human umbilical vein *in vitro*.

We are currently undertaking further studies to characterise the nature of this receptor interaction *in vivo* in the forearm circulation of patients with heart failure. Using the forearm model employed in Chapters 5 and 6, the effects of B_1 receptor antagonism on vasomotor tone and t-PA release will be examined during co-infusion of the B_2 antagonist in a randomised double-blind placebo controlled trial. A similar study will be performed to examine the effect of B_2 receptor antagonism on B_1 agonist mediated responses.

7.6.3 KININ METABOLISM

Inhibition of ACE augments the vasodilator and fibrinolytic effects of bradykinin in patients with heart failure. We have clearly demonstrated that bradykinin contributes to the systemic haemodynamic effects of ACE inhibition in this population. Consistent with the hypothesis that inhibiting additional enzymes responsible for the metabolism of plasma bradykinin may be beneficial in patients with heart failure, we have demonstrated that combined inhibition of ACE and NEP potentiates the vascular actions of bradykinin to a greater extent than ACE inhibition alone.

The absence of a significant treatment benefit with combined ACE and NEP inhibition in the OVERTURE trial [Packer et al, 2002] may reflect the broad substrate specificity of NEP [Roques et al, 1993]. Inhibition of NEP will result not only in accumulation of plasma bradykinin but alters plasma concentrations of a number of vasoactive peptides including atrial natriuretic peptide, substance P, angiotensin II and endothelin-1 [Roques et al, 1993; Ferro et al, 1998]. Targeting a

bradykinin metabolising enzyme with more restricted substrate specificity may afford greater therapeutic benefit in patients with heart failure. Three such candidate enzymes are aminopeptidase P, carboxypeptidase N and carboxypeptidase B.

7.6.3.1 *Aminopeptidase* P

Aminopeptidase P is a zinc metallopeptidase that inactivates bradykinin by cleaving the N terminal Arg^1-Pro^2 bond (Figure 24) [Hooper et al, 1992; Simmons and Orawski, 1992]. Aminopeptidase P exists both in a membrane-bound form and a cytosolic form [Yoshimoto et al, 1994], is present on the plasma membrane of human vascular endothelial cells [Ryan et al, 1996] and is thought to contribute to the degradation of bradykinin in the pulmonary and coronary circulations [Ryan et al, 1994].

Inhibition of aminopeptidase P blocks bradykinin degradation in a number of vascular beds *in vitro.* In rodents *in vivo*, inhibition of aminopeptidase P in the pulmonary circulation increases plasma bradykinin concentrations [Ryan et al, 1994], whilst systemic aminopeptidase P inhibition augments the vasodepressor response to intravenous, but not intra-arterial, bradykinin [Kitamura et al, 1999]. Furthermore, apastatin, a selective inhibitor of aminopeptidase P, reduces myocardial infarct size by a kinin-dependent pathway in rodent models of myocardial ischaemia and reperfusion, both alone and in combination with ACE inhibition [Wolfrum et al, 2001; Veeravalli et al, 2003].

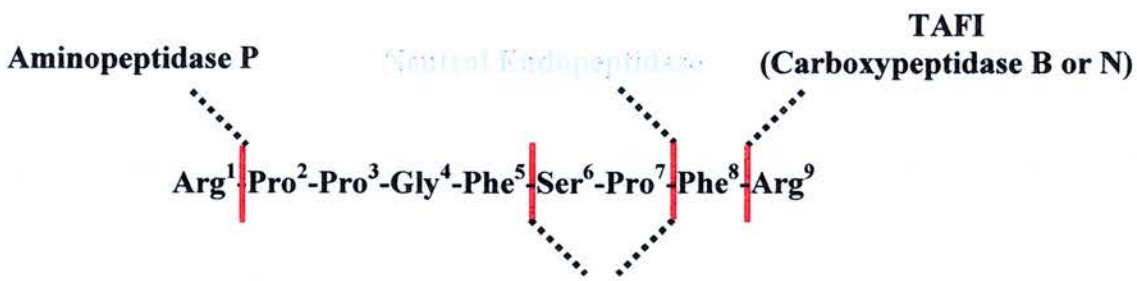

Figure 24. Chemical structure of bradykinin and additional sites of enzymatic cleavage. TAFI, Thrombin activatable fibrinolysis inhibitor.

In man, lower plasma aminopeptidase P activity is associated with a previous history of angio-oedema [Adam et al, 2002] and co-administration of apstatin and ACE inhibition markedly augments the bradykinin wheal response compared to either inhibitor alone [Kim et al, 2000]. Further work is required to determine whether inhibition of aminopeptidase P, in combination with ACE inhibition, augments the vascular actions of bradykinin in the human peripheral and systemic circulations *in vivo* to a greater extent than ACE inhibition alone.

7.6.3.2 *Carboxypeptidase N*

Synthesised in the liver and secreted into the circulation in its active form, carboxypeptidase N is a constitutively active metallocarboxypeptidase that cleaves several biologically active peptides and proteins including bradykinin, anaphylatoxins (C3a, C4a and C5a) and fibrinopeptides A and B, by removing the C-terminal arginine or lysine residues (Figure 24). In human plasma, carboxypeptidase N contributes to <10% of bradykinin metabolism at low concentrations of the nonapeptide [Kuoppala et al, 2000]. However, at higher concentrations of bradykinin, the contribution of carboxypeptidase N to bradykinin degradation is significantly increased [Kuoppala et al, 2000]. As with aminopeptidase P, it remains to be determined whether inhibition of carboxypeptidase N augments the vascular actions of bradykinin.

7.6.3.3 *Carboxypeptidase B (Thrombin Activatable Fibrinolysis Inhibitor)*

Plasma carboxypeptidase B, or thrombin activatable fibrinolysis inhibitor, is another metallocarboxypeptidase present in plasma. Carboxypeptidase B is synthesised in the

liver, but unlike the constitutively active carboxypeptidase N, is secreted into the circulation as an inactive pro-enzyme precursor [Bouma et al, 2001]. In the presence of thrombus formation and in particular, the thrombin-thrombomodulin complex, carboxypeptidase B undergo proteolytic conversion to its active form [Bouma et al, 2001]. Once activated carboxypeptidase B preferentially hydrolyses basic amino acids, removing the C-terminal arginine or lysine residue from a number of substrates including bradykinin (Figure 24), the enkephalins and partially degraded fibrin. In addition to reducing bradykinin-mediated endothelial t-PA release, carboxypeptidase B directly inhibits t-PA mediated fibrinolysis at sites of thrombus formation by removing the C-terminal lysine residues from partially degraded fibrin, thus preventing the binding of t-PA [Bouma et al, 2001]. Interestingly, carboxypeptidase B hydrolyses bradykinin *in vitro* as efficiently as it does plasmin cleaved fibrin peptides, and more effectively than carboxypeptidase N [Myles et al, 2003].

Besides the potentially beneficial effects attributable to the inhibition of bradykinin metabolism, inhibitors of carboxypeptidase B may confer additional therapeutic benefits in patients receiving thrombolytic therapy for acute myocardial infarction. In almost a third of these patients, fibrinolytic therapy fails to restore satisfactory reperfusion of the occluded vessel [DeWood et al, 1980]. The presence of a persistently occluded coronary artery is associated with increased rates of mortality and left ventricular dysfunction [GUSTO, 1993]. Plasma concentrations of activated carboxypeptidase B are elevated during thrombosis and thrombolysis administration [Redlitz et al, 1996] and in animal models, inhibition of carboxypeptidase B with the

selective antagonist, potato carboxypeptidase inhibitor, enhances t-PA mediated clot lysis [Klement et al, 1999; Nagashima et al, 2000; Refino et al, 2000]. We have recently demonstrated that inhibition of carboxypeptidase B augments t-PA mediated fibrinolysis in human whole blood *in vitro* [Cruden et al, 2005]. Thus inhibition of carboxypeptidase B may be a novel therapeutic adjunct to thrombolytic therapy in man.

Inhibitors of carboxypeptidase B may also have a role in the secondary prevention of atherosclerotic vascular disease. Low circulating fibrinolytic activity is associated with an increased risk of myocardial infarction in young men [Meade et al, 1993] and predicts which patients with unstable angina will progress to myocardial infarction [Munkvad et al, 1990]. Acute endothelial t-PA release is impaired in smokers [Newby et al, 1999; Pretorius et al, 2002] and patients with coronary artery disease [Newby et al, 2001], and the degree of impairment correlates with atheromatous plaque load [Newby et al, 2001]. In patients with atheromatous vascular disease, inhibition of carboxypeptidase B may augment endogenous fibrinolytic activity, shifting the balance in favour of clot dissolution rather than propagation, and interrupting the cycle of sub-clinical thrombus formation, organisation and vessel remodelling that underlies chronic atheromatous plaque growth [Mann and Davies, 1999].

To date, however, there has been no assessment of the contribution of carboxypeptidase B to bradykinin metabolism and inhibition of t-PA mediated fibrinolysis *in vivo* in man.

The Badimon chamber provides a powerful and elegant method of assessing *ex vivo* thrombus formation in an extracorporeal flow chamber [Badimon et al, 1999]. The technique utilises a continuous flow of venous effluent blood that is passed into a series of chambers containing a thrombogenic surface usually consisting of denuded porcine aortic strips. The characteristics of the flow chamber can be modified to generate low and high shear stress conditions that generate fibrin or platelet rich thrombi respectively. Using this model, the effects of inhibition of a number of biological pathways including the glycoprotein IIb/IIIa receptor [Lev et al, 2004], HMG-CoA reductase and cholesterol synthesis [Dangas et al, 1999], thrombin [Sarich et al, 2003] and tissue factor [Badimon et al, 1999] on thrombus formation *ex vivo* have been examined.

We propose to expose venous effluent from the bradykinin-infused human forearm model to the Badimon Chamber. The resultant model will permit a direct assessment of the effects of bradykinin-mediated endothelial t-PA release on acute dynamic formation of thrombus formation in man. Using this model, we intend to examine the effect of selective carboxypeptidase B inhibition on thrombus formation in the presence of endogenous bradykinin-induced t-PA. This work is being undertaken as part of a British Heart Foundation research programme (BHF PG/04/131).

7.6.4 Cardiac Effects of Bradykinin and ACE Inhibition

In contrast to endothelial-mediated vasomotor and fibrinolytic function, the effects of bradykinin and ACE inhibition on myocardial contractility in man remain unclear.

Bradykinin exerts a negative inotropic effect on porcine atrial myoctyes *in vitro* [Tom et al, 2001] and *ex vivo* [Baydoun and Woodward, 1991], and its antagonism *in vivo* attenuates left ventricular relaxation and contractile performance both in the presence [Fujii et al, 2002] and absence of ACE inhibition in models of heart failure [Cheng et al, 1998]. In a canine model of pacing induced congestive heart failure, chronic bradykinin infusion preserves left ventricular systolic and diastolic function and delays the progression of heart failure [Tonduangu et al, 2004].

In *ex vivo* animal models, ACE inhibition reduces basal left ventricular contractility and improves left ventricular relaxation via a B_2 receptor and nitric oxide dependent mechanism [Anning et al, 1995; Cargnoni et al, 2001]. Although the effects on basal myocardial contractility in man are not known, acute ACE inhibition attenuates the beta-adrenergic mediated increase in contractility by a nitric oxide dependent pathway in patients with dilated cardiomyopathy, but not control subjects [Wittstein et al, 2001]. It remains to be established whether bradykinin contributes to the cardiac effects of ACE inhibition *in vivo* in man. Using a validated micromanometer tipped left ventricular impedance catheter to enable real time pressure volume measurement within the left ventricular cavity *in vivo*, we intend to examine the effect of acute intra-coronary bradykinin antagonism on myocardial contractility in the normal and failing human heart in the presence or absence of ACE inhibition in patients undergoing elective diagnostic coronary angiography.

7.7 CLINICAL RELEVANCE

The major benefits associated with ACE inhibitor therapy in patients with heart failure and vascular disease are well established [CONSENSUS, 1987; Flather et al, 2000; Yusuf et al, 2000; Fox, 2003]. We have convincingly demonstrated that bradykinin contributes to the systemic haemodynamic effects of ACE inhibition. Moreover, we have shown that systemic ACE inhibition significantly augments bradykinin-mediated vasodilatation and endothelial t-PA release in these patients and these effects appear to be restricted to the vascular B_2 kinin receptor. These data provide further support for the hypothesis that bradykinin is an important mediator of the anti-ischaemic effects of ACE inhibition in patients with cardiovascular disease.

Besides ACE, bradykinin is metabolised by a number of enzyme systems in plasma. We have shown that inhibition of one such enzyme, NEP, in combination with ACE inhibition augments bradykinin-mediated vasodilatation and endothelial t-PA release in the peripheral circulation of patients with heart failure to a greater extent than ACE inhibition alone. Neutral endopeptidase, however, is involved in the metabolism of a large number of vasoactive peptides and this lack of substrate specificity would appear to limit the potential therapeutic benefits of additional bradykinin accumulation in large scale trials of combined ACE and NEP inhibition in patients with heart failure [Packer et al, 2002]. Pharmacological strategies targeting bradykinin metabolism and the B_2 kinin receptor more specifically may afford greater cardiovascular protection.

On a more cautious note, it should be noted that potentiating the vascular actions of bradykinin may have detrimental effects. Bradykinin has been implicated in the pathogenesis of ACE inhibitor-mediated angio-oedema [Nussberger et al, 1998]. Moreover, compared to ACE inhibition an even greater incidence of angio-oedema has been reported following treatment with combined ACE and NEP inhibition [Armstrong et al, 2002]. Further work is required to establish the role of bradykinin in the pathophysiology of this potentially life-threatening condition.

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