Vascular Endothelin-1 Production and Receptor Subtypes In Chronic Heart Failure

A thesis submitted to the University of Edinburgh for the degree of Doctor of Philosophy

By

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Declaration

I declare that this thesis was written entirely by me and represents all my own work, except for the procedures acknowledged in the text.

Acknowledgments

I would first like to thank Professor David Webb and Dr Gillian Gray for having the courage for taking me on in the first place - an extremely brave decision.

As I have discovered, teaching people how to use the perfusion myograph is a tricky business and so 'Cheers' to Paula (Smith) for your patience with me when I first arrived in Edinburgh. To 'Touchie-Feelie Neilie' Neil (Johnston) and Shonna (MacCall), thanks for all the pipetting, extracting etc, I know you enjoyed it really. Ian Megson, well what can I say, we've shared a lab for the past 2+ years and its been an experience - I'm sorry if I've driven you to drink (yeah right!). All the other members of the CPU, past and present, thanks for putting up with me. Moral support has been in the forms of my two other 'Partners in Crime' Amanda (Robson) and Heather (Johnstone). Finally, on the colleague front, the human part of this thesis would not have been possible without Charlie 'SuperBowels' Ferro who has escaped the cold and moved to drier and warmer climes but in particular, Fiona Strachan, all your time, effort, blood, sweat and tears are *so* appreciated, what would have I done without you? However, thanks to the people who quite literally sacrificed the most for me, the CHF patients and control subjects who were brave enough to donate not only an armful of blood, but a huge lump of backside as well! The only thing left to say is "Bottoms Up"!

Abstract

Endothelin-1 (ET-1) is a potent and longlasting vasoconstrictor peptide synthesised and secreted by the vascular endothelium. Plasma ET-1 levels are raised 2 to 3 fold in chronic heart failure (CHF), correlating with disease severity and outcome. Thus, increased ET-1 production may be an important factor in the maintenance of the peripheral vasoconstriction in CHF. Resistance arteries, with internal diameters of 50-350 μm, are the most important blood vessels of the circulation in setting vascular resistance. Therefore, it is important to characterise the ET receptors responsible for ET-1 vasoconstriction in these arteries. The aims of this thesis were i). to characterise the ET receptors on the smooth muscle of normotensive rats, ii). investigate any changes of the ET receptor subtypes in a CHF rat model and in CHF patients and iii). investigate whether there is increased ET-1 synthesis in CHF by measuring plasma ET-1 and big ET-1 levels and if there is an altered localisation of ET-1 and ECE in the wall of the arteries.

Firstly, the ET-1 receptor subtypes responsible for ET-1-induced vasoconstriction in endothelium-denuded mesenteric arteries from normotensive Wistar rats were investigated. Arteries were mounted in a perfusion myograph and ET-1 or sarafotoxin S6c (SRTX S6c) concentration-response curves (CRC) were performed. The relative roles of the ET_A and ET_B receptors in the ET-1-induced vasoconstrictions were evaluated by using either the ET_A receptor antagonist, BQ-123; the ET_B receptor antagonist, BQ-788; the ET_B receptor agonist, SRTX S6c or the non-selective ET_A/ET_B antagonist, TAK-044. It was found that both ET_A and ET_B receptors mediate ET-1 vasoconstriction and that ET_A receptors could compensate for the inhibition of ET_B receptors. The results suggested a potential crosstalk mechanism between the two receptor subtypes.

Any changes in vascular smooth muscle ET receptor responses and subtypes mediating ET-1 vasoconstriction in resistance arteries in CHF were then

investigated. Two sources of arteries were used; i). mesenteric arteries from rats at two different time points after the induction of heart failure by left coronary artery ligation or sham-operation and ii). gluteal arteries dissected from buttock biopsies obtained from Grade II & III CHF patients and age-matched controls.

Endothelium denuded mesenteric arteries from rats 5 or 12 wks after the induction of CHF and the respective sham-operated controls were mounted in a perfusion myograph and ET-1 CRCs were performed. Sensitivity to ET-1 was reduced in arteries from 12 wk but not 5 wk post-ligation rats relative to sham-operated animals. In the arteries from the 12 wk CHF animals sensitivity to ET-1 was restored by prior desensitisation of ET_B receptors with SRTX S6c. SRTX S6c used as an agonist induced a small constrictor response in sham and 5 wk CHF arteries, which was lost in 12 wk CHF arteries. Antagonist studies suggest that ET_A receptors mediate the ET-1-induced vasoconstriction in all arteries.

Functional studies in the perfusion myograph on endothelium denuded human gluteal arteries also demonstrated a reduced sensitivity to ET-1 in the arteries from the CHF patients. ET_B receptor desensitisation had no effect on the ET-1 CRCs in both control and CHF arteries. Again, ET_A receptors appeared to mediate the ET-1-induced vasoconstriction in all arteries. RIA demonstrated increased big ET-1, but not ET-1 levels in the plasma from the CHF patients. Immunohistochemistry demonstrated that ET-1 and ECE was localised to the endothelium, but not smooth muscle, of arteries from both rats and humans. There was no difference seen in the arteries from the CHF rats and humans.

Overall, responses to ET-1 are attenuated in arteries from CHF patients and animals. In the arteries from the 12 wk CHF rats there may be up-regulation of relaxant ET_B receptors on the vascular smooth muscle responsible for the reduced ET-1 sensitivity. However, relaxant ET_B receptors were not evident in

the arteries from the human CHF patients suggesting a down-regulation of ET-1 receptors, most probably ET_A receptors. The down-regulation of the ET-1 receptors is most likely as a result of increased ET-1 synthesis, although not local ET-1 production in these vessels.

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Table of Abbreviations

AA	Arachidonic acid
Ab	Antibody
Ag	Antigen
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ANG I	Angiotensin I
ANG II	Angiotensin II
ANP	Atrial natriuretic peptide
Asp	Aspirin
β-Bl	β-blocker
BNP	Brain natriuretic peptide
BK	Bradykinin
BP	Blood pressure
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CCA	Canine coronary artery
CHF	Chronic heart failure
COX	Cyclo oxygenase
CRC	Concentration response curve
DAB	Diaminobenzidine
Di	Diuretic
Dia	Diameter
Dig	Digoxin
DNS	Did not state
ECE	Endothelin converting enzyme
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
ET	Endothelin
GFR	Glomerular filtration rate
GSA-I	*Griffonia simplicifolia* agglutinin-I
HSA	Human small artery
HR	Heart rate
HUA	Human uterine artery
IP_3	Inositol triphosphate
KCl	Potassium chloride solution
L-Arg	L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester
LCAL	Left coronary artery ligation
L-NMMA	N^G-nitro-monomethyl-L-arginine
Los	Losartan
LV	Left ventricular
LVEDP	Left ventricular end diastolic pressure
MAP	Mean arterial pressure
MI	Myocardial infarction
Min	Minutes
NA	Noradrenaline
NHS	Normal horse serum
Ni	Nitrate
NO	Nitric oxide
NOS	Nitric oxide synthase
NYHA	New York Heart Association
PBS	Phosphate buffer solution
PE	Phenylephrine

PG	Prostaglandin
PGI_2	Prostacyclin
PKC	Protein kinase C
PVR	Peripheral vascular resistance
RAA	Renin-Angiotensin-Aldosterone system
RVP	Rapid ventricular pacing
RVR	Renal vascular resistance
SNP	Sodium nitroprusside
SNS	Sympathetic nervous system
SRTX S6c	Sarafotoxin S6c
SVR	Systemic vascular resistance
TBS	Tris-phosphate buffer solution
TFA	Trifluoroacetic acid
TIVCC	Thoracic inferior vena caval constriction
TxA_2	Thromboxane A_2
UEA-1	*Ulex Europeaus* agglutinin-1
VSMC	Vascular smooth muscle cells

List of Abstracts and Publications Arising from this Thesis

Paper

MICKLEY, E.J., GRAY, G.A. and WEBB, D.J. (1997). Activation of endothelin ET_A receptors masks the constrictor role of endothelin ET_B receptors in rat isolated small mesenteric arteries. *Br. J. Pharmacol.*, **120**, 1376-1382.

Abstracts

MICKLEY, E.J., WEBB, D.J. and GRAY, G.A. (1998). Evidence for an inhibitory ET_B receptor present on the smooth muscle of resistance arteries from a rat model of heart failure. To be presented at the XVI World Congress of the International Society for Heart Research, Rhodes, May 1998.

MICKLEY, E.J., STRACHAN, F.E., FERRO, C.J., GRAY, G.A. and WEBB, D.J. (1997). Endothelin receptors in small arteries from heart failure patients and age-matched controls. Presented as an oral communication at the 5th International Conference on Endothelin, Japan, Sept 1997.

MICKLEY, E.J., WEBB, D.J. and GRAY, G.A. (1997). Vascular endothelin-1 receptors in a rat model of chronic heart failure. *Br. J. Pharmacol.*, **122**, 233P. Presented as an oral communication at BPS, University of Edinburgh, Sept 1997.

MICKLEY, E.J., GRAY, G.A. and WEBB, D.J. (1996). Evidence of crosstalk between constrictor ET_A and ET_B receptors in rat small mesenteric arteries. *Eur. J. Clin. Invest.*, **26**, A49. Presented as a special interest communication at the European Society for Clinical Investigation, Interlaken, Switzerland.

MICKLEY, E.J., SWAN, P.J.H., WEBB, D.J. and GRAY, G.A. (1995). Comparison of two methods of myography for detection of constrictor endothelin ET_B receptors in rat small mesenteric arteries. *Br. J. Pharmacol.*, **116**, 424P.

Introduction

A. 1.1. The Arterial Circulation

The principal function of the circulatory system is the transport of oxygen and nutrients to, and removal of carbon dioxide and other metabolic waste products from, all tissues of the body. However, the circulation is also involved in temperature regulation and the distribution of other substances, such as hormones, and cells such as those of the immune system.

The vascular system is a circuit of blood vessels, comprised of the arterial system, which leads to the capillaries (the main sites of interchange between the tissues and blood) and the venous system. Apart from the capillaries, the whole circulatory system has a common basic structure, being made up of three separate layers: the inner lining *tunica intima,* which consists of a single layer of endothelial cells; the *tunica media*, the intermediate layer of smooth muscle cells; and the outer *tunica adventitia* which consists of connective tissue (collagen and elastin) and fibroblasts (Figure 1.1.). The intima is separated from the media by the internal elastic lamina and acts as a storage function for all blood vessels, in particular the large conduit arteries and veins. Blood vessels are innervated by the sympathetic and non-adrenergic, non-cholinergic neuronal systems, which synapse in the advential and smooth muscle layers.

There are three main types of blood vessel in the arterial system: *elastic arteries*, which are the larger, main distributing vessels such as the aorta; *muscular arteries*, the main distributing branches of the arterial tree such as the radial arteries; and *arterioles* or *resistance arteries*, the terminal branches of the arterial tree leading to the capillary beds. There is a gradual change in structure and function between the three arterial types, as vessel size decreases, the relative amount of elastic tissue decreases and the smooth muscle layer increases. The flow of blood to various organs and tissues is regulated by varying the diameter of the arterial system, a function performed by the smooth muscle layer. It is at the level of the resistance arteries where total peripheral resistance and blood pressure is mainly controlled. It was believed, until recently, that the diameter of these arterioles was under the control of the sympathetic nervous system and circulating hormones alone.

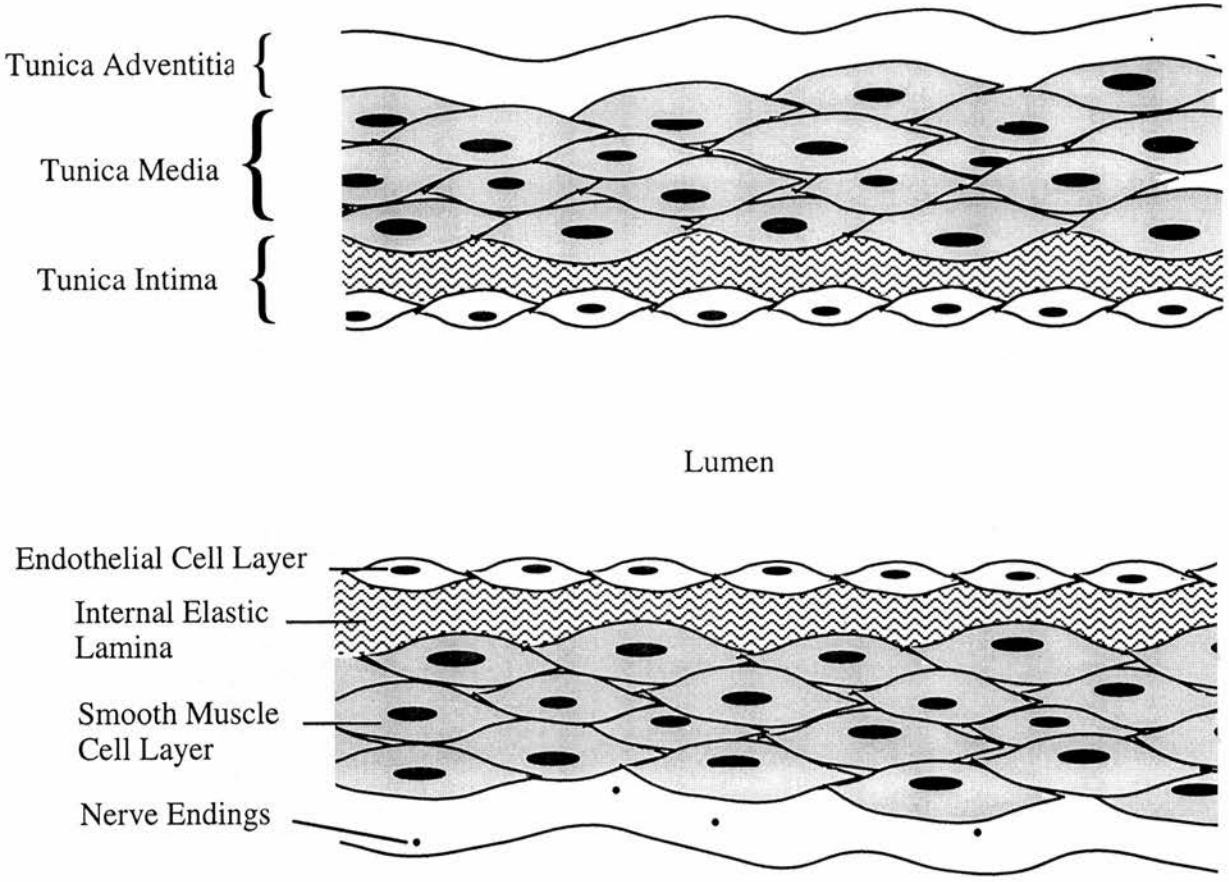


Figure 1.1. A schematic diagram demonstrating the basic structure of a blood vessel.

A. 1.2. The Vascular Endothelium

Over the past two decades it has become obvious that the vascular endothelium has a very important role in regulating vascular tone. The endothelium is a monolayer of cells lining blood vessels of all sizes and it is in a unique position to detect any changes in blood flow, pressure and circulating hormones. As a result of the location, the endothelium helps control the size of blood vessel lumen (along with sympathetic innervation), therefore blood flow and pressure, by producing a number of important vasodilator and constrictor substances. In the 1970s, '80s and '90s several potent vasodilators were discovered to be synthesised and released by endothelial cells, namely prostacyclin (PGI_2; Moncada *et al.*, 1976), endothelial-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) and endothelial-derived hyperpolarising factor (EDHF; Taylor & Weston, 1988). EDRF has since been identified as nitric oxide (NO; Palmer *et al.*, 1987), and a putative candidate for the role of EDHF is the cannabinoid, anandamide (Randall *et al.*, 1996). Due to the discovery of these endothelial produced vasorelaxants, it was hypothesised that counterbalancing constrictor factors would also be released from the endothelium.

A. 1.2.1. Endothelin

Experiments on isolated arteries and veins had already demonstrated that endothelium-dependent contractions induced by hypoxia (Rubanyi & Vanhoutte, 1985) or anoxia (Detar & Bohr, 1972; DeMay & Vanhoutte, 1983) could be elicited. One of the substances released, produced a prolonged vasoconstriction lasting more than 60 minutes, and indirect evidence suggested that it was a peptide (Hickey *et al.*, 1985; Gillespie *et al.*, 1986).

It was in 1988 when Yanagisawa and colleagues isolated and sequenced endothelin (ET) from the culture supernatant of porcine endothelial cells. It is a 21 amino acid peptide and is the most potent vasoconstrictor substance ever characterised. However, it does have some vasodilator properties. In chemically denervated rats, Yanagisawa and co-workers (1988) showed that intravenously administered ET caused an initial short-lived decrease, followed by a marked and sustained (> 60 minutes) increase, in

blood pressure. This slow onset, long lasting pressor effect made it unique amongst all other known vasoconstrictors. For example, in contrast to ET, a bolus of angiotensin II (ANG II) has an immediate vasoconstrictor action which almost immediately returns back to resting baseline (Clarke *et al.*, 1989). However, similar to ET, ANG II can also mediate vasodilatation (Gardiner *et al.*, 1988).

The ETs were found to be a family of three isopeptides, simply called ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989a). The isoform originally characterised was ET-1, with ET-2 and ET-3 being structurally and pharmacologically distinct (Figure 1.2). ET-2 differs by 2 amino acids from ET-1 and ET-3 by 6 amino acids. All three isoforms are encoded by different genes on chromosomes 6, 1 and 20 respectively (Gray & Webb, 1996). However, they are all 21 amino acid peptide chains with two intrachain disulphide bridges between cysteine residues 1 and 15, and 3 and 11. The disulphide bridges and C-terminal domain present in the endothelins are necessary for their actions, as their removal leads to substantial loss of biological activity (Kimura *et al.*, 1988).

The sarafotoxins (SRTX's) are a group of isopeptides isolated from the venom of the Israeli burrowing asp, *Atractaspis engaddensis*. These peptides have been found to be very similar to the ET's both structurally and functionally (Kloog & Sokolovsky, 1989). Indeed, two of these SRTX's, SRTX S6b and SRTX S6c (Figure 1.2) have proved to be useful tools in the studies of the ETs and their receptors.

A. 1.2.2. Generation of endothelin

ET-1 is the major isoform produced by vascular endothelial cells in humans and is the most important of the three ETs in the human body (Gray & Webb. 1996). It is generated in response to physical and chemical forces including vascular shear stress, hypoxia and to other vasoactive mediators such as adrenaline and angiotensin II (Yanagisawa *et al.*, 1988). The synthesis of ET-1, and the other two isoforms, is analogous to other peptides (Figure 1.3). A 212 amino acid polypeptide is the primary translation product from the human ET-1 gene. It contains a 17 amino acid signal

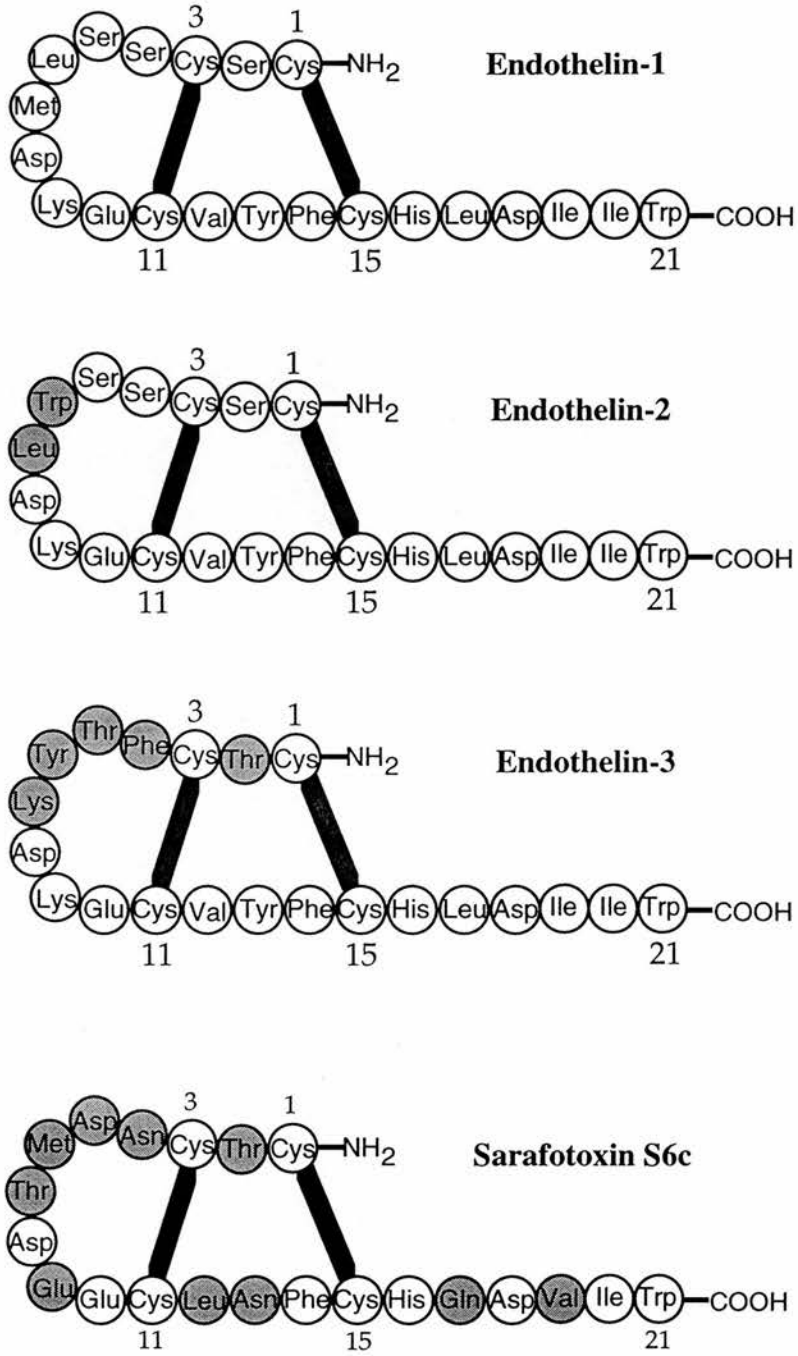


Figure 1.2. Amino acid sequences of the three human endothelin isoforms, and sarafotoxin S6c, one of the sarafotoxin family isolated from snake venom. Amino acids differing from endothelin-1 are shaded, and the lines between residues 1 & 15 and 3 & 11 represent the disulphide bridges linking the cysteine residues.

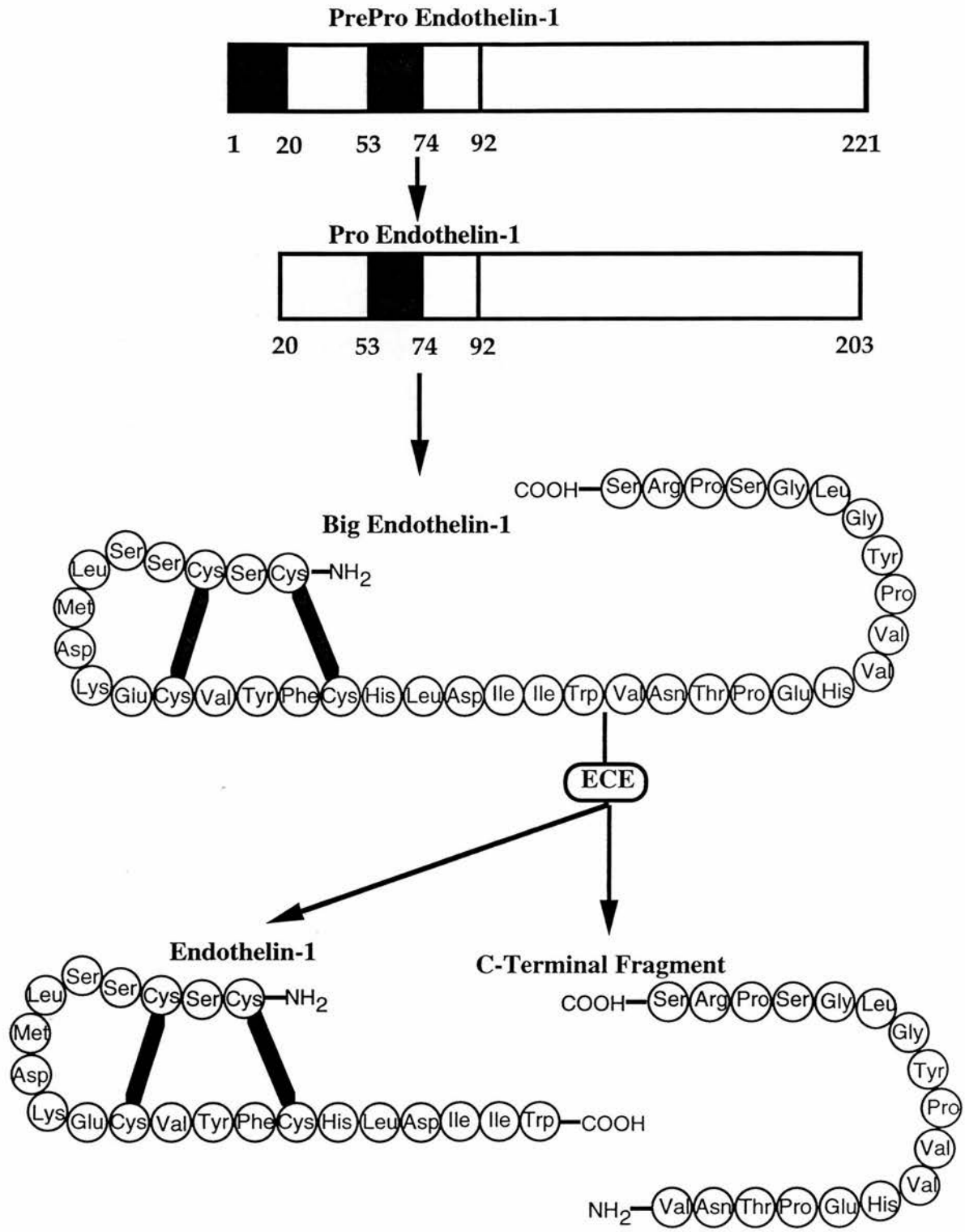


Figure 1.3. A schematic diagram showing the synthesis pathway of the endothelin peptides

sequence which is cleaved once the peptide is secreted from the nucleus (Inoue *et al.*, 1989a; 1989b). This proET-1 is cleaved by dibasic amino acid endopeptidases at two recognition sequences (Lys^{52}-Arg^{53} and Arg^{90}-Arg^{91}), resulting in the 38 amino acid peptide big ET-1. The mature ET-1 is then generated through an unusual proteolytic cleavage between Trp^{21}-Val^{22} of big ET-1 by endothelin-converting enzyme (ECE; Yanagisawa *et al.*, 1988).

There is a great difference in the biological activities of big ET-1 and the mature ET-1 (ET-1 being at least 100 fold more active). Thus, inhibitors of ECE would be useful tools in the investigation of the endothelin system, and as potential therapeutic agents. However, ECE has been difficult to identify. Initially, because of the sensitivity of ECE to metal ion chelators and its pH optima, two enzymatic families, the aspartyl and/or metalloprotease families, were suggested to be responsible for ECE activity (Ikegawa *et al.*, 1990; Lees *et al.*, 1990; Sawamura *et al.*, 1990). Subsequent studies demonstrated that the aspartyl proteases could not account for ECE activity, since aspartyl protease inhibitors did not attenuate ET-1 secretion from cultured endothelial cells (Ikegawa *et al.*, 1990; Shields *et al.*, 1991) or inhibit big ET-1 conversion *in vivo* (Bird *et al.*, 1992). Neutral endopeptidase 24.11 (NEP) was also suggested as a potential candidate for the activity of ECE. However, the NEP specific inhibitor, thiorphan, was shown to be unable to inhibit big ET-1 conversion (Bird *et al.*, 1992; Turner & Murphy, 1996). Furthermore, NEP was found to degrade ET-1 more efficiently than generating ET-1 from big ET-1 (Sokolovsky *et al.*, 1990; Abassi *et al.*, 1993) and is now recognised as a potential mechanism in the removal of ET-1 from the circulation (see A.1.2.3.). In contrast, Gardiner and colleagues (1992a), using SQ 28,603, an NEP and ECE inhibitor, found that in conscious rats, that although the pressor effects of big ET-1 was inhibited, there was no prolongation of the cardiovascular effects of ET-1, but there was of ANP.

However, it was found that the metalloprotease enzyme inhibitor phosphoramidon could consistently inhibit the generation of ET-1 from big ET-1. Phosphoramidon has been shown to inhibit ET-1 production from endothelial cells in culture (Ikegawa *et al.*,

1990), and antagonise the vasoconstrictor effects of big ET-1 *in vitro* (Hisaki *et al.*, 1991; Telemaque & D'Orleans-Juste, 1991) and *in vivo* (Fukuroda *et al.*, 1990; Matsumura *et al.*, 1990; McMahon *et al.*, 1991). Phosphoramidon, however, does not affect vasoconstriction and pressor responses induced by the mature peptide. Furthermore, this enzyme has the ability, albeit less efficiently, to convert the other precursor endothelin peptides, big ET-2 and big ET-3, to the active peptides (Gardiner *et al.*, 1992b; Pollock *et al.*, 1993). This implies that there may be different isoenzymes specifically for each endothelin isoform. Indeed, two forms of ECE have been cloned, ECE-1 and ECE-2 (Xu *et al.*, 1994; Emoto & Yanagisawa, 1995), both of which convert big ET-1 more efficiently than big ET-2 and big ET-3. The differences between the two isoforms are location, pH optimum and affinity to phosphoramidon. ECE-1 is widely distributed throughout the body, although it is not found in the neurones or glia of the brain (Xu *et al.*, 1994) and has an optimum activity of pH 6.8. In contrast, ECE-2 is mainly expressed in neural tissues and has an acidic pH optimum of 5.5 (Emoto & Yanagisawa, 1995). Both isoforms are Type II integral membrane proteins with a short N-terminal cytoplasmic tail, a single transmembranal domain, and a large C-terminal containing a zinc-binding motif in the catalytic domain (Emoto & Yanagisawa, 1995; Turner &Murphy, 1996).

Recently, it has been found that ECE-1 is a family of at least three isoforms. These have been called ECE-1a, ECE-1b and ECE-1c, and vary at the C-terminal (Valdenaire *et al.*, 1995). The relative importance of each isoform is unknown, although experiments have suggested that ECE-1a and ECE-1c are probably the most important physiologically. For the rest of this section, the isoforms will be discussed collectively as ECE-1.

Both ECE-1 and ECE-2 are believed to be membrane proteins either on the plasma membrane or on an intracellular membrane site. Experiments *in vivo* administering exogenous big ET-1 (McMahon *et al.*, 1991; Haynes & Webb, 1994) suggest that this conversion to ET-1 is most likely via an ECE present on the plasma membrane of endothelial and smooth muscle cells. When COS cells were transfected with the ECE-

1 gene, only 5-10% of the exogenous big ET-1 added was converted to the active peptide (Xu *et al.*, 1994) and conversion in ECE-2 transfected COS cells was negligible (Emoto & Yanagisawa, 1995). In contrast, COS cells or endothelial cells co-transfected with preproET-1 and ECE-1 or ECE-2 secreted 50-90% of the "endogenous" precursor as the mature peptide (Sawamura *et al.*, 1990; Xu *et al.*, 1994; Emoto & Yanagisawa, 1995). As a result of these observations, it has been suggested that once secreted from the nucleus, the endogenous preproET-1 is processed to the final mature peptide during transit through the intracellular constitutive secretory pathways, particularly in the Golgi apparatus. Immunohistochemical staining against ET-1 demonstrating its presence in the cytoplasm of endothelial cells (Gui *et al.*, 1993) and conversion of big ET-1 to ET-1 by low density intracellular fractions (Harrison *et al.*, 1993) provide further evidence for this theory. The pH in the vesicles of the Golgi apparatus is between 5.5-5.7, which is optimal acidity for conversion by ECE-2 (Emoto & Yanagisawa, 1995).

In these acidic conditions ECE-1 would not efficiently convert bigET-1. However, using antibodies directed against ECE-1, immunohistochemistry has demonstrated clusters of ECE-1 on the surface of endothelial cells (Barnes *et al.*, 1995). It is suggested that these clusters are localised in caveoli, invaginations of the plasma membrane into which big ET-1 can be secreted for ET-1 generation (Turner & Murphy, 1996).

Therefore, production of the mature peptide could be via two different pathways. During upright tilt there is a rapid release of ET-1 (Stewart *et al.*, 1992), this could be due to a store of ET-1 which is ready for immediate release, perhaps via the prior conversion of big ET-1 by ECE-2 in some Golgi vesicles. However, ECE-1 may be responsible for continuous, longer-term conversion of ET-1 at the cell membrane, whereby both ET-1 and big ET-1 is secreted. Furthermore, ET-1 is secreted in a polar fashion, preferentially towards the smooth muscle cell layer (Wagner *et al*, 1992a).

A. 1.2.3. Clearance and metabolism of endothelin

The metabolism of ET occurs by three different routes: enzymatic degradation, receptor-mediated clearance and urinary excretion. As alluded to above, membrane bound NEP 24.11 is more efficient at degrading ET-1 than generating it (Sokolovsky *et al.*, 1990; Abassi *et al.*, 1993). It is an enzyme which is responsible for the inactivation of many peptides, including bradykinin, atrial natriuretic peptide (ANP) and substance P (Turner & Murphy, 1996). The lungs are important in the removal of ET-1 from the circulation, and porcine lung membrane fractions were found to hydrolyse ET-1. The enzyme responsible for this was shown to be NEP 24.11 (Murphy *et al.*, 1993). NEP inhibition by SQ-29,072 in humans resulted in increased urinary excretion and plasma levels of ET-1 (Abassi *et al.*, 1992) and brachial artery infusion of thiorphan resulted in vasoconstriction of the forearm vascular bed, whereas phosphoramidon infusion produced vasodilatation (Haynes & Webb, 1994). Other enzymes have also been isolated which have the ability to breakdown ET-1, including a protease, probably cathepsin G, which is released from activated polymorphonuclear lymphocytes (Patrignani *et al.*, 1992), deaminase from bovine aortic endothelial cells (Jackman *et. al.*, 1993) and a carboxypeptidase-like enzyme isolated from kidney (Jeng & Deng., 1993). Interestingly a metalloprotease has been found to be released from the isolated perfused mesenteric arterial bed of the rat (Perez-Vizcaino *et al.*, 1995). This enzyme is very active at pH 7.4, implying that it may be physiologically important in the metabolism of ET-1 throughout the vasculature. It was not inhibited by phosphoramidon or captopril, so it is not NEP 24.11 or angiotensin-converting enzyme (ACE). However, the authors suggested that it is similar to a membrane-bound metalloprotease found in the rat kidney which cleaves ET-1 predominantly between Leu^{17} and Asp^{18} (Yamaguchi *et al.*, 1992).

In anaesthetised rats, injection of [^{125}I]-ET-1 results in over 60% being removed during the first minute (Anggard *et al.*, 1989), the majority binding to the lung, followed by the kidney and liver. In humans, however, the lungs do not appear to be as important at removing ET-1 from the circulation, although pulmonary clearance does occur (Stewart *et al.*, 1991). The plasma half-life of ET-1 in humans is less than

90 seconds, mainly due to extraction from the circulation by the splanchnic and renal vascular beds (Weitzberg *et al.*, 1991; Gasic *et al.*, 1992). Despite the species differences in organ extraction, it is accepted that actual removal from the circulation occurs via the same pathway. The circulating ET-1 binds to cell surface receptors, which are internalised. The peptide is then degraded within the cell (Anggard *et al.*, 1989; Shiba *et al.*, 1989; Gandi *et al.*, 1993) by proteases such as deamidase which has been found in human platelets, vascular smooth muscle cells and endothelial cells (Jackman *et al.*, 1992; 1993) and rat kidney (Deng *et al.*, 1994; Janas *et al.*, 1994). It was first suggested that circulating ET-1 was cleared by binding to a receptor, the ET_B receptor, when plasma ET-1 concentrations were increased after rats were treated with the non-selective ET_A/ET_B receptor antagonist, Ro-47-2005, but not after selective ET_A inhibition with BQ-123 or FR 139317 (Loffler *et al.*, 1993). Fukuroda and colleagues (1994c) then demonstrated that the selective ET_B receptor antagonist, BQ-788, inhibited the uptake of intravenously administered [^{125}I]ET-1 in the lungs and kidneys in rats. However, the removal of circulating [^{125}I]ET-1 by the liver was significantly enhanced by ET_B receptor inhibition, implying that hepatic uptake occurs via a receptor-independent mechanism that can compensate for the inhibition of ET-1 removal by the lungs and kidneys (Fukuroda *et al.*, 1994c).

Urinary excretion of ET-1 also occurs, since ET-1 levels can be detected in the urine of humans (Matsumoto *et al.*, 1994) and bilateral nephrectomy in rats results in a reduction of the disappearance of circulating [^{125}I]ET-1 (Kohno *et al.*, 1989).

A. 1.2.4. Endothelin receptors

Receptors are generally classified on the basis of their differing affinities for agonists and antagonists. However, the development of endothelin antagonists was slow, so endothelin receptor subtypes were initially identified using the different properties of the ETs/SRTXs alone. At present two classes of ET receptor subtype in mammalian tissues have been established, the ET_A and ET_B receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990). The ET_A receptor shows a higher affinity to ET-1 or ET-2, than ET-3 whereas the ET_B receptor is non-selective. SRTX S6b has the ability to activate both

Ligand	Selectivity	ET_A	ET_B
ET-1	ET_A/ET_B	160 pM	110 pM
ET-3	ET_B	4.5 nM	70 pM
SRTX S6c	ET_B	4.5 μM	20 pM
IRL 1620	ET_B	16 pM	200 pM
BQ-3020	ET_B	940 nM	200 pM

Table 1.1. Agonists at the ET receptors. No specific ET_A receptor agonists have been identified, however, there are several ET_B receptors currently available. Potency of the ligands at each receptor subtype are shown.

receptors, but SRTX S6c shows 300 000 fold selectivity for the rat ET_B receptor (Williams *et al.*, 1991; Table 1.1).

The genes encoding both receptors were cloned and characterised within two years of the initial description of ET (Arai *et al.*, 1990; Sakurai *et al.*, 1990). The cDNA encoding the human ET_A receptor predicts a structure of 427 amino acids and the cDNA for the ET_B receptor predicts 442 amino acids (Adachi *et al.*, 1991; Hayzer *et al.*, 1992; Arai *et al.*, 1993; Elshourbagy *et al.*, 1993). The sequence homology between the two receptor subtype mature proteins is estimated to be 55 - 64% depending on the tissue origin of the cDNA (Adachi *et al.*, 1991; Hayzer *et al.*, 1992; Arai *et al.*, 1993; Elshourbagy *et al.*, 1993). The ET_A and ET_B receptor genes are located on chromosomes 4 (Hosada *et al.*, 1992) and 13 (Arai *et al.*, 1993) respectively, with similar structural organisation implying that they originated from the same ancestral gene. Functional studies have suggested further ET receptor subtypes (Bax & Saxena, 1994). For instance, the presence of a third receptor subtype was suggested by Emori *et al.* (1990), who demonstrated an ET-3-preferring receptor in cultured bovine endothelial cells. In 1993 an ET-3 selective receptor, called ET_C, was cloned from the amphibian *Xenopus laevis* (Karne *et al.*, 1993). However, if present in the mammalian genome, it has yet to be cloned. Analysis of the human DNA genome with specific probes for the human ET_A and ET_B receptors revealed only two hybridising fragments (Sakamoto *et al.*, 1991). Therefore, if genes encoding other ET receptors exist in the mammalian genome, they must have a low sequence homology with the two established ET receptor genes.

Prior to the cloning of the ET receptors, radioligand-binding studies inferred the existence of two receptor subtypes (Bax & Saxena, 1994). As mentioned above, the receptors were then classified based on the actions of agonists. Maggi and colleagues (1989), used the C-terminal hexapeptide, ET-(16-21), the sequence common to all of the endothelins, and found that it was inactive when tested on rat isolated aorta, but a

full agonist in the guinea-pig isolated bronchus. Thus, the receptors were called ET_A for aorta, and ET_B for bronchus.

The mammalian ET receptors are of the seven transmembrane-spanning, rhodopsin-like, G-protein-coupled receptor superfamily (Hosoda *et al.*, 1992; Arai *et al.*, 1993). Activation of the receptors results in the mobilisation of several intracellular signal transduction pathways. However, the most important pathway is the G-protein-mediated activation of phospholipase C (PLC), resulting in formation of inositol triphosphate (IP_3) and diacylglycerol (DAG; Pang *et al.* , 1989; Griendling *et al.* 1989). IP_3 binds to the IP_3 receptor present on the endoplasmic reticulum, opening the IP_3 receptor Ca^{2+} channel, causing the release of Ca^{2+} (Berridge, 1993) into the cytosol. DAG activates protein kinase C (PKC) which may sensitise the contractile proteins to Ca^{2+} via phosphorylation (Sunako *et al.*, 1989; Abe *et al.*, 1991). PKC can also stimulate the sodium-hydrogen antiporter, resulting in extrusion of H^+ and alkalinisation of vascular smooth muscle cells (VSMC; Lonchampt *et al.*, 1991). The increase in intracellular pH enhances the contractility of the VSMCs, again via sensitisation of the contractile proteins to Ca^{2+}. During the sustained vasoconstrictor response to ET, voltage-operated Ca^{2+} channels are opened (Inoue *et al.*, 1990).

The opposing actions of the two receptor subtypes has been demonstrated at the second messenger level as well. Transfection of ET_A receptors into Chinese hamster ovary cells, when stimulated by ET-1, induced an increase of cAMP levels by stimulation of phospholipase A_2. In contrast, when ET_B receptors were transfected, there was inhibition of forskolin-induced cAMP turnover (Aramori & Nakanishi, 1992).

Following the cloning of the two receptors, antagonists were developed. Antagonists against the ET_A receptor were the first to be described, most notably BQ-123 (Ihara *et al.*, 1992) and FR 139317 (Sogabe *et al.*, 1993). These two antagonists, are the compounds that the majority of research into ET receptor subtypes have been based

around. Disclosure of non-selective ET_A/ET_B receptor antagonists were next, including compounds such as PD142893 and PD 145065 (Warner *et al.*, 1994a,b,c). The development of specific ET_B receptor antagonists took the longest, and indeed the first one to be described, IRL 1038, was found to have highly variable affinity to the receptor between batches (Urade *et al.*, 1994). Thus, all data and studies utilising this compound should be considered with great caution (Bax & Saxena, 1994). It was not until early 1994 that the potent and novel ET_B selective antagonist, BQ-788, provided the field with a tool to investigate the actions of this receptor subtype (Ishikawa *et al.*, 1994). It is worth noting that all of these original antagonists described are peptides, and are therefore of limited use when needed for chronic, oral dosing. Thus, the search for a non-peptide antagonist, be it selective or non-selective, was intense. The first orally active, non-peptide compound synthesised and described in the literature was Ro 46-2005 (Clozel *et al.*, 1993). This structure was optimised to produce Ro 47-0203, otherwise known as bosentan (Clozel *et al.*, 1994). Bosentan is an orally active, non-selective ET_A/ET_B receptor antagonist with reasonable potency against both receptor subtypes. See Table 1.2 for a review of the major antagonists used in the literature.

The development of these antagonists helped in the characterisation and localisation of the ET receptors in various tissues, confirming, in some cases, the agonist results but contradicting the previous conclusions in other studies.

A. 1.2.4.1. Vascular endothelial ET_B receptors

A bolus injection of ET-1 into an anaesthetised rat produces an initial, transient depressor effect, followed by the well-recognised longlasting pressor response (Yanagisawa *et al.*, 1988). This initial vasodilation is also evident if ET-3 or SRTX S6c is administered (Warner *et al.*, 1989; Clozel *et al.*, 1992). *In vitro* tissue experiments on pre-constricted arterial beds showed that the relaxation by the ET/SRTX peptides is abolished on endothelial denudation (Douglas & Hiley, 1991), implying that ET_B receptors present on the endothelium are responsible for mediating the dilatory actions by the ET peptides. Indeed, pre-treatment of anaethetised animals

Ligand	Selectivity	ET_A	ET_B	Structure
BQ-123	ET_A	7.3 nM	18 μM	Cyclic pentapeptide
FR 139317	ET_A	1 nM	7 μM	Pseudo-tripeptide
BMS-182874*	ET_A	55 nM**	>20 μM**	Benzensulfonamide
BQ-788	ET_B	1300 nM	1.2 nM	Tripeptide
Ro 46-8443*	ET_B	7 μM	40 nM	Benzensulfonamide
PD 145065	ET_A/ET_B	3.5 nM	15 nM	Linear hexapeptide
TAK-044	ET_A/ET_B	0.1 nM	1.8 nM	Cyclic hexapeptide
Ro 47-0203 (bosentan)*	ET_A/ET_B	4.7 nM	95 nM	Sulphonamide
SB 209670*	ET_A/ET_B	0.2 nM	18 nM	Carboxylic acid derivative

Table 1.2. Table of the most commonly used selective and non-selective endothelin receptor antagonists in the literature. All antagonists are peptide in nature, except for those indicated by the asterisk (*). Potency of the antagonists at each receptor subtype is shown as IC_{50} or $**K_i$

with BQ-123 was found to potentiate the depressor effect of bolus ET-1, whilst inhibiting the majority of the pressor response (Douglas *et al.*, 1992). However, BQ-788 abolished the vasodilatory response completely (Karaki *et al.*, 1994), as did desensitisation of the ET_B receptors by repeated ET-3 exposure (Le Monnier de Gouville *et al.*, 1990). Furthermore, reverse transcription-polymerase chain reaction experiments in cultured human endothelial cells have demonstrated the mRNA encoding the ET_B receptor only (Hosada *et al.*, 1991; Ogawa *et at.*, 1991; Molenaar *et al.*, 1993).

The mechanism underlying the initial depressor response was found to be by the release of endogenous vasodilator factors. Inhibition of nitric oxide synthase by L-NMMA (L-N^G-monomethyl-arginine) or L-NAME (L-N^G-nitro-arginine methyl ester) inhibited the depressor activities of ET-1 (Whittle *et al.*, 1989). Thus, in vascular endothelial cells, ET_B receptor-mediated liberation of Ca^{2+} results in the activation of constitutive nitric oxide synthase (cNOS), and the subsequent diffusion of NO to the vascular smooth muscle cells. However, NO is not the only vasodilator to be released via stimulation of endothelial ET_B receptors (Gardiner *et al.*, 1989; 1990b,c). Phospholipase A_2 (PLA_2) can also be activated, thus metabolising arachidonic acid to prostacyclin (DeNucci *et al.*, 1988). In resistance arteries, it has been suggested that the release of endothelial-derived hyperpolarising factor (EDHF), an unknown substance different to NO, is the major endothelial factor liberated by ET_B receptor stimulation (Nakashima & Vanhoutte, 1993).

The release of the vasodilator substances, via endothelial ET_B receptor stimulation is believed to be a counteractive mechanism against the powerful vasoconstrictor actions of the ETs at the smooth muscle level. However, the physiological relevance is under question, since ET-1, synthesised by the vascular endothelium is secreted preferentially abluminally, away from the ET_B receptors present on the luminal side of the endothelium. Thus, under normal physiological conditions these vasodilatory actions may not be utilised. Although in pathophysiological conditions, where increased circulating levels of the ET peptides are to be found, e,g, in heart failure (see

section B.1.6.1.), they may have a role in counterbalancing the vasoconstrictions induced.

A. 1.2.4.2. Vascular smooth muscle constrictor ET_A receptors

It was originally believed that only ET_A receptors were responsible for the vasoconstrictor actions of the ET peptides (Masaki *et al.*, 1991). Due to the relative selectivity of the ET_A receptor for ET-1, but not ET-3 or SRTX S6c, the classification of ET receptors mediating vascular constrictions were originally based on the rank order of potency; ET-1>ET-3>SRTX S6c. As a result, the majority of the early, functional studies on isolated tissues confirmed the mRNA localisation studies showing that the ET_A receptor was only found to be expressed in vascular smooth muscle (Arai *et al.*, 1990; Hori *et al.*, 1992; Yang *et al.*, 1993), and mRNA for the ET_B receptors in the endothelium (Hosada *et al.*, 1991; Ogawa *et al.*, 1991; Molenaar *et al.*, 1993). Thus, the consensus of opinion was that ET_A receptors mediate the vasoconstrictor action of the ETs, which are modified by the release of endothelial ET_B receptor stimulated relaxing factors.

Typical vascular preparations with constrictor ET_A receptors include the rat thoracic aorta (Maggi *et al.*, 1989; Sumner *et al.*, 1992; Warner *et al.*, 1993a,b; Moreland *et al.*, 1994), guinea pig aorta and pulmonary artery (Hay *et al.*, 1993), rabbit carotid artery (Moreland *et al.*, 1992 & 1994; White *et al.*, 1993), goat cerebral artery (Salom *et al.*, 1993), porcine carotid artery and monkey saphenous vein (Moreland *et al.*, 1994). *In vivo* experiments also confirmed the major role of the ET_A receptor in ET-1-induced vasoconstriction, the pressor effect of ET-1 infusion being significantly attenuated by ET_A receptor antagonism (Douglas *et al.*, 1992; Bigaud & Pelton, 1992).

Vasoconstrictor ET_A receptors also contribute to basal tone in the whole systemic vasculature. Infusion of BQ-123 alone into anaesthetised rats induced a decrease in femoral artery mean arterial pressure, accompanied by a systemic vasodilatation (Bigaud & Pelton, 1992). This has also been demonstrated in humans. Infusion of

BQ-123 into the forearm of healthy volunteers produced a vasodilatation, as shown by an increase in forearm blood flow, of up to at least 40% (Haynes & Webb, 1994).

A. 1.2.4.3. Vascular smooth muscle constrictor ET_B receptors

The first data suggesting that ET_A receptors are not solely responsible for the vasoconstrictor activity of the ET peptides, was when Williams and colleagues (1991) identified SRTX S6c as a specific ET_B receptor agonist. Using binding assays, they demonstrated that SRTX S6c only inhibited [^{125}I]-ET-1 binding in the rat hippocampus and cerebellum, but not in rat atria and aorta. In fact the K_i values for SRTX S6c were 300 000 fold weaker in the aorta/atria preparations than in the hippocampus/cerebellum. However, when they injected increasing doses of SRTX S6c into pithed rats, a pressor response was seen. It was comparable in potency to equivalent doses of SRTX S6b (the non-selective agonist), up to 0.3 nmol/kg. At doses greater than 0.3 nmol/kg the pressor response to SRTX S6b was significantly higher. These results were confirmed in other studies using either the specific ET_B agonist [Ala ^{1,3,11,15}]ET (Douglas & Hiley, 1991; Clozel *et al.*, 1992; Bigaud & Pelton, 1992) or SRTX S6c (Clozel *et al.*, 1992). Furthermore, there were two vascular beds particularly sensitive to the constrictor properties of these specific ET_B agonists, namely the mesenteric and renal beds (Clozel *et al.*, 1992; Bigaud & Pelton, 1992). Douglas and colleagues (1992) showed regional differences in ET-1-induced vasoconstriction which were sensitive to BQ-123. Flow probes placed around the right carotid artery and the superior mesenteric artery in anaesthetised rats, demonstrated that both vasculatures were susceptible to ET-1-induced vasoconstriction. However, BQ-123 inhibited the increase in vascular resistance in the carotid artery, but had no effect on the mesenteric vasoconstriction. Cristol *et al.*, (1993) saw in the anaethetised rat, that renal blood flow was reduced by ET-1, ET-3, SRTX S6b and STRX S6c equipotently. BQ-123 pre-treatment was ineffective against all the agonists. Similar results have been shown with the other popular ET_A receptor antagonist, FR 139317 in both the mesenteric and renal beds (Gardiner *et al*, 1994a). Furthermore, the ET_A/ET_B receptor antagonist bosentan was shown to significantly inhibit, although not completely abolished, the renal and mesenteric

vasoconstrictions to ET-1 in conscious rats (Gardiner *et al*, 1994b). Thus, implying the presence of vasoconstrictor ET_B receptors in these vascular beds.

It was in veins that vasoconstriction mediated by ET_B receptors became evident in functional, organ bath experiments (Moreland *et al.*, 1992; Sumner *et al.*, 1992). It was in the rabbit saphenous vein (Moreland *et al.*, 1992) and jugular vein (Sumner *et al.*, 1992), that constrictions were seen to ET-1 and ET-3, which could not be inhibited by ET_A receptor antagonists (Moreland *et al.*, 1992; Sumner *et al.*, 1992), and that contractions could be elicted by SRTX S6c (Moreland *et al.*, 1992). The first artery seen to constrict to ET-1, ET-3 and BQ3020 (a selective ET_B agonist) was the rabbit pulmonary artery (Ihara *et al.*, 1992; Warner *et al.*, 1993a). However, the majority of constrictor ET_B receptors appear to be present in venous smooth muscle (Moreland *et al.*, 1994).

A. 1.2.4.4. Mixed constrictor ET_A/ ET_B receptor populations

Vasoconstriction in some blood vessels have been found to be mediated via a mixed population of ET_A and ET_B receptors. For complete inhibition of ET-1 constriction in the rabbit pulmonary artery, both ET receptor subtypes had to be inhibited by BQ-123 treatment in combination with either BQ-788 (Fukuroda *et al.*, 1994a) or ET_B receptor desensitisation by prolonged exposure with SRTX S6c (LaDouceur *et al.*, 1993). The presence of the ET_A receptors was confirmed by radioligand binding studies (LaDouceur *et al.*, 1993). This is despite the fact that this tissue had previously been shown to contract exclusively by ET_B receptors (Ihara *et al.*, 1992; Warner *et al.*, 1993a). This has also been shown to be the case in rabbit saphenous vein (LaDouceur *et al.*, 1993; Sudjarwo *et al.*, 1994) and in the rat renal vasculature (Wellings *et al.*, 1993). Other tissues containing mixed ET_A/ ET_B receptor populations described include porcine coronary artery (Shetty *et al.*, 1993), canine saphenous vein and monkey iliac artery and jugular vein (Moreland *et al.*, 1994).

To summarise the above review on which receptors mediate vasoconstriction, a thorough study by Moreland and colleagues (1994) on six different species, both

arterial and venous preparations demonstrated that, generally, ET_A receptors were the subtype mediating constriction in the high pressured, arterial side of the circulation, whereas ET_B receptors have a role in the low pressured, venous side. They also showed an *in vivo* pressor response to SRTX S6c in cynomolgus monkeys, which they concluded could not occur via constriction of the venous side of the circulation alone. The authors admit that they were only studying large, pre-resistance arteries *in vitro*, and that the pressor response to SRTX S6c could be partially due to constrictor ET_B receptors in the smaller, resistance arteries, as was demonstrated in the canine coronary bed, where intracoronary infusion of SRTX S6c resulted in a pronounced decrease in coronary vascular resistance (Teerlink *et al.*, 1994a).

A. 1.2.4.5. Subtypes of ET_A and ET_B receptors

Some studies have shown an apparent subclassification of both ET_A and ET_B receptors based on antagonist studies. Warner and colleagues (1993a,b) were the first to show that vasodilation to ET-1 in the rat mesenteric bed could be abolished on pre-treatment with the non-selective antagonist PD 142893. However, ET_B-mediated contractions in the rabbit pulmonary artery and rat stomach strip were unaffected. Due to the equipotence of the ET/SRTX peptides in mediating these contractions and dilatations, it was concluded that all these responses were via ET_B receptors, which should be subclassified into ET_{B1} (PD 142893-sensitive) and ET_{B2} (PD 142893-insensitive).

A complementary study by Sudjarwo *et al.*, (1994) using vascular tissue, not only suggested ET_{B1} and ET_{B2} receptors, but also ET_{A1} and ET_{A2} receptors. They suggested these subclassifications based on the relative vasoconstrictions to ET-1, ET-3, SRTX S6c and IRL 1620 in the rabbit saphenous vein, and the subsequent sensitivities of these agonists to inhibition by an assortment of selective ET receptor antagonists. They, again, proposed ET_{B1} receptors as those sensitive to ET_B antagonism (IRL 1038 & RES-701-1-sensitive) and ET_{B2} as those insensitive to antagonism. However, both of these ET_B receptor subtypes could undergo tachyphylaxis, after prolonged activation by SRTX S6c. Furthermore, they suggested ET_{A1} receptors as those which are BQ-123-sensitive, and ET_{A2} receptors being BQ-123-insensitive. The overall conclusions

as to which receptor subtypes are activated by the individual ET/STRX peptides are as follows; ET-1 induced constrictions via all subtypes, whereas ET-3 activates both ET_B receptors, and also the ET_{A1}, BQ-123-sensitive receptor. These results may explain why in some studies ET-3 responses can be inhibited by BQ-123, but the ET-1 responses appear to be insensitive (Sumner *et al.*, 1992). Both SRTX S6c and IRL 1620 activated both subtypes of the ET_B receptor only.

Using the newly described ET_B antagonist, BQ-788, the same group showed, in the rabbit saphenous vein, that BQ-788 inhibited ET-3 contractions more than desensitisation by SRTX S6c. They, therefore, concluded that BQ-788 inhibited both ET_{B1} and ET_{B2}, as well as possessing some weak antagonist properties at the ET_{A1} receptor (Karaki *et al.*, 1994).

In many functional studies, ET-3 vasoconstriction has been found to be inhibited by BQ-123 (Sumner *et al.*, 1992), therefore it would suggest that many of the studies using ET-3 as an agonist delineating between the ET_A and ET_B receptor should be viewed with caution. Other suggestions have been that the agonist-binding kinetics are different. It has been hypothesised that ET-3 has weaker binding characteristics than ET-1, thus it appears that ET-3 responses are antagonised, whereas ET-1 responses are not (Bax & Saxena, 1994).

With respect to the ET_B receptor subclassification, Sokolovsky and colleagues (1992) have proposed different affinity sites, relating to the binding properties and second messenger systems. They suggest that the vasodilatory actions of ET_B receptors is mediated by a "super-high" affinity receptor, which has an affinity for ET-1 in the picomolar range and does not induce phosphoinositide hydrolysis. They called the "super-high" affinity receptor the ET_{B1} receptor, correlating with the vasodilatory, PD 142893-sensitive ET_{B1} receptor of Warner *et al.*, (1993a,b). The vasoconstrictor actions are mediated by the conventional "high" affinity ET_B receptors, with an affinity for ET-1 in the nanomolar range, which does induce phosphoinositide turnover. This

receptor was termed ET_{B2}, again agreeing with the vasoconstrictor, PD142893-insensitive receptor (Warner *et al.*, 1993a,b).

However, further evidence of these apparent subtypes of the established ET_A and ET_B receptors are needed before this nomenclature is accepted. Although it is tempting, in the case of the ET_B receptors present on the endothelium and smooth muscle cell layer, to call them ET_{B1} and ET_{B2} respectively.

A. 1.2.4.6. Atypical ET receptors

Studies on isolated rings of arteries and veins have shown discrepancies in receptors mediating constriction to the ET peptides. Vasoconstrictor receptors not conforming to the rank order of potency at the respective ET_A and ET_B receptors have implied further subtypes (Bax & Saxena, 1994). In the original paper describing the selective ET_A antagonist, BQ-123, it was seen that in the porcine coronary artery, part of the ET-1-induced constriction was resistant to inhibition. This data implies that another ET receptor was partly mediating the constriction to ET-1 (Ihara *et al.*, 1992). This BQ-123 (or FR 139317) resistant portion of the ET-1 curve was found to be mediated by ET_B receptors, since it was sensitive to prolonged SRTX S6c exposure (Seo *et al.*, 1994). In addition, the non-selective ET_A/ ET_B receptor antagonist, bosentan, shifted the whole of the ET-1 curve to the right (Seo *et al.*, 1994).

Harrison and colleagues (1992) also showed that more than one receptor was involved in ET constriction. However, they described, as well as a typical ET_A receptor, an atypical non- ET_A, non- ET_B receptor which recognised ET-3 and SRTX S6c, but not ET-1 or SRTX S6b. Monophasic concentration-response curves (CRCs) to ET-1 and STRX S6b were observed, but biphasic CRC to ET-3. The authors divided the ET-3 CRC into higher and lower sensitivity components. The higher sensitivity component was abolished after prolonged exposure to SRTX S6c, converting it to a curve similar to the ET-1/SRTX S6b CRCs. This is a phenomenon well described for the ET_B receptor (Le Monnier de Gouville *et al.*, 1990). However, it appears that ET-1 could

not stimulate this ET-3/SRTX S6c receptor, which is unlike the non-selective ET_B receptor. Thus, the authors concluded that there was another subtype of ET receptor.

Whether Harrison and colleagues were describing the putative ET-3 preferring ET_C receptor is unknown. In another study, a non- ET_A, non- ET_B receptor was described that contributed to the ET-1 contractile response (Schoeffter & Randriantsoa, 1993). What is clear, is that ET-1-induced constriction in the porcine coronary artery is both ET_A and ET_B receptor mediated. The relevance of ET-3-induced constriction has generally been ignored, as it is believed that ET-1 is the major isoform synthesised and released by the vasculature. However, in pathophysiological states, these putative ET-3 receptors may become important since raised circulating levels of ET-3 have been measured in conditions such as acute myocardial infarction (Teerlink *et al.*, 1994b).

Other atypical receptors have been described in tissues such as pig pulmonary artery and veins, goat cerebral artery and rat aorta (Bax & Saxena, 1994).

A. 1.2.4.7. ET receptors in human blood vessels

It appears that the majority of the constrictor actions of the ET peptides in the human vasculature act via the ET_A receptor. ET-1 infused into the forearm produces an decrease in forearm blood flow (Clarke *et al.*, 1989), which is antagonised completely by co-infusion of BQ-123 (Haynes & Webb, 1994). Furthermore, infusion of BQ-123 alone causes direct vasodilation, demonstrating that ET production is involved in maintaining basal vascular tone (Haynes & Webb, 1994).

Experiments on isolated blood vessels have also demonstrated that the contractile responses of ET-1 are also mainly mediated by ET_A receptors (Davenport & Maguire, 1994). In agreement with the animal studies, larger conduit arteries tend to be populated almost entirely with ET_A receptors mediating constriction. Many studies have been performed on the coronary arteries taken from explanted hearts. All functional constrictor responses to ET-1 have been attributed to ET_A receptors (Bax *et al.*, 1993; Davenport *et al.*, 1993; Godfraind, 1993; Bax *et al.*, 1994). Although ET_B

receptor mRNA has been detected in the smooth muscle layer of human coronary arteries, aorta and pulmonary artery using both reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridisation techniques (Davenport *et al.*, 1993). Biphasic competition binding curves using BQ-123 and BQ-3020 against ^{125}I-ET-1 also confirmed the presence of both receptor subtypes, in the ratio of approximately 85% : 15%, ET_A : ET_B respectively (Davenport *et al.*, 1993). It was concluded that the ET_B receptors detected had no functional constrictor role, since BQ-123 shifted the ET-1 CRCs to the right in a parallel manner, and BQ-3020 had no constrictor activity (Davenport *et al.*, 1993). Other human vascular tissue in which ET_A receptors mediated constriction to ET-1, include aorta (Davenport *et al.*, 1993), small omental arteries (Riezebos *et al.*, 1994), pulmonary arteries (Hay *et al.*, 1993) and umbilical arteries (Bogoni *et al.*, 1996). Subtypes of ET_A receptors have been suggested in human blood vessels, due to the ability of BQ-123 to reverse SRTX S6b constrictions, but not ET-1 (Bax *et al.*, 1994).

Constrictor ET_B receptors have been seen in some human blood vessels when challenged with SRTX S6c. However, these ET_B receptor constrictions are generally extremely small, usually less than 20% of ET-1 constriction, and highly variable. For instance, Maguire & Davenport saw SRTX S6c constriction in only 50% of saphenous veins (Maguire & Davenport, 1993) and coronary arteries (Davenport & Maguire, 1994) experimented upon. Mixed populations of ET_A/ ET_B receptors have clearly been demonstrated in the internal mammary pre-resistance artery and vein (Seo *et al.*, 1994) and internal mammary resistance artery (Tschudi & Luscher, 1994) and saphenous vein (Bax *et al.*, 1993).

The ET peptides are potent constrictors of human arteries and veins *in vivo* (Haynes & Webb, 1994) and *in vitro* (Bax & Saxena, 1994). In isolated omental resistance arteries mounted in a wire myograph, it was shown that ET-1 was 1000-fold more potent than noradrenaline in inducing constriction (Watt *et al.*, 1989). Furthermore, as with the animal data, it appears that the majority of constrictions in the conduit arteries are mediated by ET_A receptors, that there may be some ET_B receptors present in the

resistance arteries, and that the venous side of the circulation has a mixed population of both receptor subtypes.

A. 1.2.5. Other properties of ET

A. 1.2.5.1. Cardiac actions

As described above, ET-1 is a potent constrictor of the coronary bed, producing marked reductions in coronary blood flow. Due to the long lasting vasoconstrictor action, myocardial ischaemia is often associated with ET-1-induced reduction in blood flow (Gray & Webb, 1995). Coronary angiograms reveal that these effects are primarily due to actions on small coronary arteries (Hirata *et al.*, 1990). Systemic infusion of ET-1 produces an initial hypotension, resulting in an increase in heart rate and cardiac output secondary to systemic vasodilation. In contrast, the subsequent pressor response is associated with bradycardia and a reduction in stroke volume (Miller *et al.*, 1989). The chronotropic response *in vivo* appears to be reflex in origin, because blockade of cardiac efferent neural mechanisms inhibits the increase in heart rate (Gardiner *et al.*, 1990a). The decrease in stroke volume is as a result of both systemic vasoconstriction, increasing afterload, and coronary vasoconstriction, causing myocardial ischaemia (Miller *et al.*, 1989).

In addition to the constrictor effects on the coronary vessels, direct cardiac actions of ET-1 have been described, including positive inotropic and chronotropic responses (Hu *et al.*, 1988a), release of ANP (Hu *et al.*, 1988b) and initiation of hypertrophy (Suzuki *et al.*, 1991). The direct positive inotropic response has been demonstrated *in vitro* on cardiac tissue from many species including the rat (Moravec *et al.*, 1989; Kramer *et al.*, 1991), rabbit (Takanashi & Endoh, 1991) and human (Moravec *et al.*, 1989), an effect generally mediated by ET_B receptors (Kasai *et al.*, 1994; Beyer *et al.*, 1995).

Furthermore, isolated perfused hearts, and myocardial preparations *in vitro* show that ET-1 has both anti- and pro-arrhythmic properties. In ventricular myocytes, electrophysiological techniques have shown that ET-1, via ET_A receptors, inhibits a

protein kinase A-dependent chloride current (James *et al.*, 1994). This is an action which should be anti-arrhythmic. However, pro-arrhythmic tendences have been seen when infused into pigs, whereby fatal ventricular arrhythmias occur (Ezra *et al.*, 1989). In the rat model of acute ischaemia, ET-1 reduces the threshold for fatal arrhythmias (Zhao *et al.*, 1994) and increases the severity and incidence of ischaemic arrhythmias (Garjani *et al.*, 1995). Paradoxically, low dose BQ-123 reduces the incidence of arrhythmias in the rat model of ischaemia, however high dose BQ-123 is pro-arrhythmic (Garjani *et al.*, 1995). At these higher doses, BQ-123 may no longer be ET_A receptor selective.

In humans, intracoronary infusion of big ET-1 and ET-1 both induce coronary vasoconstriction (Pernow *et al.*, 1997). However, systemic ET-1 infusion reduces cardiac output, possibly via a baroreceptor-mediated decrease in heart rate, and an increase in afterload as a consequence of peripheral vasoconstriction (Wagner *et al.*, 1992b).

A. 1.2.5.2. Kidney effects

ET-1 has two main direct actions on the kidney, producing profound renal vasoconstriction, and affecting tubular sodium and water excretion (Miller *et al.*, 1989; Lerman *et al.*, 1991). Of all the vascular resistance beds, the renal vascular bed is the most sensitive to the constrictor actions of ET-1. It constricts both afferent and efferent glomerular arterioles *in vivo*, therefore reducing renal plasma flow and glomerular filtration rate (GFR). This leads to reduced urine flow and Na^+ excretion (anti-diuretic and anti-natriuretic; King *et al.*, 1989; Lerman *et al.*, 1991). Systemic administration of ET-1 into humans consistently causes renal vasoconstriction (Weitzburg *et al.*, 1991; Rabelink *et al.*, 1994) which induces Na^+ retention. However, Na^+ retention occurs at very low doses of ET-1, even when renal vasoconstriction is not apparent (Rabelink *et al.*, 1994).

The ET receptor subtype mediating the renal vasoconstriction is dependent on species under study. For instance, in the rat, renal vasoconstriction to ET-1 is only completely

inhibited by non-selective ET_A/ ET_B receptor antagonism, but not by selective ET_A receptor blockers (Cristol *et al.*, 1993; Wellings *et al.*, 1993). Furthermore, SRTX S6c mimics the renal vasoconstrictor actions of ET-1 (Gellai *et al.*, 1994), thus implying that the predominant renal constrictor receptor is the ET_B receptor. In contrast, in dogs and rabbits, ET-1-induced renal vasoconstriction is entirely inhibited by ET_A receptor antagonism (Brooks *et al.*, 1994; Telemaque *et al.*, 1993). In humans, localisation of ET receptor mRNA, shows ET_A receptors only in the vasculature, whereas ET_B receptor mRNA was present on the tubules (Karet & Davenport, 1995).

In animals, it appears that ET-1 can have diuretic and natriuretic effects (King *et al.*, 1989; Perico *et al.*, 1991). These actions were suggested to be via ET_B receptors present in the tubules and can occur, in rats, despite a fall in GFR and renal blood flow (Perico *et al.*, 1991). This may be due to stimulation of atrial natriuretic peptide (ANP; see section B. 1.3.2.; Munger *et al.*, 1991). Furthermore, ET-1 can inhibit Na^+/K^+ ATPase activity (Zeidel *et al.*, 1989) and vasopressin (see section B. 1.2.3.) effects in rat inner medullary collecting duct cells (Oishi *et al.*, 1991).

All this data suggests that the net effect of ET-1 on Na^+ excretion depends on a balance between Na^+ retaining and natriuretic factors. However, in humans it appears that the anti-natriuretic effects of ET-1 predominate (Rabelink *et al.*, 1994).

A. 1.2.5.3. Endocrine interactions

ET-1 has a close interaction with the renin-angiotensin-aldosterone system (RAAS; see section B. 1.2.1). Apart from inhibiting renin release from isolated rat glomeruli (Rakugi *et al.*, 1988), ET-1 appears to stimulate the RAAS. For instance, ET-1, in the rat mesenteric bed increases generation of renin and ANG II (Rakugi *et al.*, 1990), and stimulates conversion of ANG I to ANG II in pulmonary endothelial cells (Kawaguchi *et al.*, 1990). Furthermore, in the adrenal gland, ET-1 stimulates isolated cortical zona glomerulosa cells to release aldosterone (Cozza *et al.*, 1989), and adrenaline from medullary chromaffin cells (Boarder & Marriot, 1989). *In vivo* administration of ET-1

into animals augments plasma renin, aldosterone, vasopressin and adrenaline concentrations (Miller *et al.*, 1989; Nakamoto *et al.*, 1989; Cao & Banks, 1990). The rise in renin levels could be as a result of renal vasoconstriction. Conversely, ET-1 secretion is potentiated by AII (Emori *et al.*, 1991) and vasopressin (Bakris *et al.*, 1991).

ET-1 also increases circulating ANP (Garcia *et al.*, 1990) and brain natriuretic peptide (BNP; Horio *et al.*, 1992) levels. Furthermore, ANP inhibition, by pretreatment of rats with ANP antibodies, potentiates the ET-1 pressor response (Valentin *et al.*, 1991). Therefore, endogenous natriuretic peptide generation could modulate ET-1 vasoconstriction, although activation of other endocrine systems, such as the RAAS, may enhance ET-1 constrictor actions.

A. 1.2.5.4. Mitogenic properties

ET-1 is a co-mitogen, promoting cell division, hypertrophy and DNA synthesis in vascular and non-vascular smooth muscle cells, fibroblasts, mesangial cells and myocytes (Battisini *et al.*, 1993) by the induction of proto-oncogenes *c-fos, c-jun* and *c-myc* (Simonson *et al.*, 1992). ET-1 has few mitogenic activities upon cells in culture when incubated alone. However, in combination with other mitogens, e.g. ANGII, has powerful synergistic properties (Mattana & Singal, 1995). Nevertheless, ET-1 is a powerful mitogen on melanocytes (Imokawa *et al.*, 1992).

A role for the ET peptides in the hypertrophic process in the formation of a neointima in the rat carotid artery balloon angioplasty model has been suggested. Following balloon angioplasty, an infusion of exogenous ET-1 potentiates the size of lesion formed (Trachtenberg *et al.*, 1993). Furthermore, antagonist treatment, with either BQ-123 (ET_A receptor selective; Douglas *et al.*, 1995) or SB 209670 (ET_A/ ET_B receptor; Douglas *et al.*, 1994) reduces the size of lesion. In addition, the expression of preproET-1, preproET-3, ET_A and ET_B receptor mRNA is increased after angioplasty (Wang *et al.*, 1996). Expression of ET-1 is also enhanced in the endothelium of blood vessels of rats with severe forms of hypertension (Lariviere *et*

al., 1995). This increased expression is in association with the vascular hypertrophy in these animals (Schiffrin *et al.*, 1996).

A. 1.2.5.5. Non-vascular smooth muscle actions

The smooth muscle of the gastrointestinal, respiratory and urogenital tracts are extremely susceptible to contraction by ET-1. However, in these preparations, the constrictor actions are mediated by ET_A and ET_B receptors.

The original agonist classification of the two receptor subtypes was by comparing the abilities of the ET and SRTX peptides to induce constriction in the isolated aorta and bronchus. ET-1 was the most potent at constricting the aorta, thus, the ET_A receptor (Maggi *et al.*, 1989). In the bronchus, all ET/SRTX peptides caused contraction with equal affinity, hence, the ET_B receptor (Maggi *et al.*, 1989). It has subsequently been demonstrated that bronchial tissue from humans also contracts via ET_B receptors (Hay *et al.*, 1993). However, as is comparable to vascular smooth muscle, the larger airways in the respiratory tract, for example trachea, do contain some ET_A receptors, the relative contribution of either receptor subtype being species dependent (Hay *et al.*, 1996).

In the gastrointestinal and urogenital tracts, tissues such as the bladder (Maggi *et al.*, 1990), stomach fundus (Gray and Clozel, 1995), ileum (Warner *et al.*, 1993b,c), and gall bladder (Battistini *et al.*, 1994) all contract to ET-1. In tissues such as the human bladder (Maggi *et al.*, 1990), guinea pig ileum (Warner *et al.*, 1993b,c) and rat uterus (Rae *et al.*, 1993), the contractions are mediated by ET_A receptors, whereas rat stomach fundal strips are ET_B receptor-mediated (Gray & Clozel, 1995). Furthermore, these organs are highly innervated and the ET peptides are able to modulate many neuronal responses (see Section A. 1.2.5.6), as well as induce profound constriction. Thus, the ET peptides have been implicated in the pathophysiology of many non-vascular smooth muscle diseases, such as asthma and Hirschsprung's disease.

A. 1.2.5.6. Neuronal properties

Subthreshold concentrations of ET-1 potentiate contractile responses to catacholamines and 5-HT in isolated rat human arteries (Wong-Dusting *et al*, 1990; Yang *et al.*, 1990). Furthermore, ET-1 markedly elevates venous tone *in vivo* in rats, by a reflex increase in sympathetic nerve activity and activation of α-adrenoceptors (Waite & Pang, 1992). This has also been demonstrated in hypertensive patients, where sympathetically-mediated venoconstriction induced by deep breath was potentiated by infusion of ET-1 (Haynes *et al.*, 1994). However, in healthy subjects no augmentation of this reflex was observed. Perhaps confirming a role for ET-1 in modulating the baroreflex, binding sites for the peptide have been shown in the carotid bifurcation, where upon topical application inhibited the baroreceptor, and stimulated chemoreceptors (Spyer *et al.*, 1991).

ET-1 also has central actions that may contribute to its pressor properties. Intracerebroventricular administration of ET-1, at doses too low to raise blood pressure when administered intravenously, increases blood pressure by stimulating central sympathetic outflow (Ouchi *et al.*, 1989; Matsumara *et al.*, 1991). Furthermore, the inhibitory, parasympathetic pathway appears to be dampened by centrally administered ET-1 (Itoh & De Busse, 1991). Injection of NMDA into the periaquaductal grey area raises blood pressure, an effect that is partially mediated by ET-1. This is through activation of ET_A receptors, since preinjection with FR 139317 inhibited the pressor response to NMDA (D'Amico & Warner, 1995).

ET-1 also modulates non-vascular smooth muscle neuronal responses. It potentiates electrically-induced twitches in the rat vas deferens, but dampens twitches in guinea pig ileum (Warner *et al.*, 1993c). In the rat vas deferens, low concentrations of ET-1 potentiate neurotransmission postsynaptically (Wiklund *et al.*, 1991) by actions on ET_A receptors (Warner *et al.*, 1993c). This response is modified as ET-1 concentrations become higher, since presynaptic ET receptors inhibit noradrenaline release (Wiklund *et al.*, 1991). The identity of the presynaptic ET receptor in the rat vas deferens is

unknown. However, the inhibitory action of ET-1 on the guinea pig ileum neurotransmission is via a presynaptic ET_B receptor (Warner *et al.*, 1993c).

Due to the many actions of the ET peptides described above, they have been suggested to potentially play a role in many pathophysiological states, in particular in diseases of the cardiovascular system. Many conditions have been linked with the ET peptides, including hypertension, chronic renal failure, Raynauld's disease and Hirschsprung's disease (Webb, 1997). Congestive heart failure (CHF) is a condition which is characterised by profound peripheral vasoconstriction, fluid retention and cardiac hypertrophy, all in association with activation of neuroendocrine systems. The ET-1 system may be involved in the pathophysiology of CHF, especially in relation to the chronic vasoconstriction. This thesis is investigating whether there are any alterations in vascular reactivity to ET-1 in small resistance arteries from humans and animals with CHF when compared to controls (see Aims B. 1.7).

B. 1.1. Congestive Heart Failure

Congestive heart failure (CHF) is a common condition affecting ~ 1-2% of the general population (Dargie & McMurray, 1994). It is associated with high morbidity and mortality, and is a major cause of hospitalisation (McMurray & Hart, 1993). As the disease progresses it becomes more uncomfortable and debilitating, and the life quality of the patients becomes extremely poor. Current drug therapy helps relieve some of the symptoms of CHF. However, there is still scope for potential new drugs to increase the life quality of CHF patients .

B. 1.1.1. Pathophysiology

CHF can be defined as a "cardiac disorder which prohibits the delivery of sufficient output to meet the perfusion requirements of metabolising tissues" (Timms & Davis, 1992). In Western countries, CHF is usually the result of damaged myocardium by ischaemia following a myocardial infarct (Dargie & McMurray, 1994). Other causes include coronary blood flow insufficiency, volume overload by valvular incompetence or pressure overload such as hypertension or valvular narrowing. It is dysfunction of the left ventricle which is the main cause of CHF and its symptoms, although right ventricular dysfunction also occurs. The symptoms of CHF manifest themselves as fluid retention, fatigue, exercise intolerance and dyspnoea (difficulty in breathing).

The heart, with its damaged myocardium, responds as if combating blood loss and trauma, rather than myocardial infarction. This is due to evolutionary purposes, to defend against dehydration and restore perfusion pressure to vital organs (Francis *et al.*, 1984). The reduced cardiac output, as a result of poor ventricular performance, activates a series of compensatory neuroendocrine and systemic reflexes in order to maintain cardiovascular homeostasis. These secondary events begin with the stimulation of systems to increase blood volume and maintain perfusion pressure to vital organs. Increased central venous pressure causes augmentation of pre-load and promotes fluid retention in order to increase cardiac output. However, this results in increased pulmonary pressure and oedema. The reduced renal blood flow leads to the

activation of the RAA and arginine vasopressin systems, again promoting sodium and water retention (Francis *et al.*, 1984).

ANG II and vasopressin are also powerful vasoconstrictor agents and these, along with activation of the sympathetic nervous system, increase peripheral vascular resistance in order to maintain perfusion pressure to vital organs and to ensure adequate venous return. As a result of the raised vascular resistance, afterload, or outflow resistance, is also increased. The elevated afterload further exacerbates the ventricular dysfunction. Cerebral and coronary blood flows are usually preserved in CHF whilst the perfusion of skeletal muscle, renal and pulmonary beds is reduced by vasoconstriction. It is the skeletal muscle vasoconstriction which impairs vasodilation during exercise, hence the symptoms of fatigue and exercise intolerance occur (Katz, 1995). As mentioned earlier, the renal vasoconstriction activates the RAA system and pulmonary vasoconstriction contributes further to pulmonary hypertension and oedema (Francis *et al.*, 1994).

The pressure and volume overload caused by the increased preload and afterload stimulates the heart to expand, therefore increasing ventricular volume. However, because of the decreased contractility of the heart muscle in CHF, the ventricle must be stretched to a greater degree for a given stroke volume. This increased ventricle size, in turn, means that a greater tension is needed in the myocardium to expel a certain volume of blood, as explained by Laplace's Law (Julian & Cowan, 1992). The law of Laplace says the tension in the myocardium (T) is proportional to the intraventricular pressure (P) multiplied by the radius of the ventricular chamber ($T \propto PR$). Starling's law also states that the more myocardial fibres are stretched (the end-diastolic fibre length), the greater the energy of the ensuing contraction. However, this is within physiological limits and beyond these limits the energy of contraction falls off. In heart failure there is reduced contractility due to the loss of contractile tissue, and a given amount of stroke work is only achieved with a greater end-diastolic fibre length (see Figure 1.4). In response to the increased tension and volume in CHF, the myocardium hypertrophies, i.e. it increases in weight as a result of an enlargement of

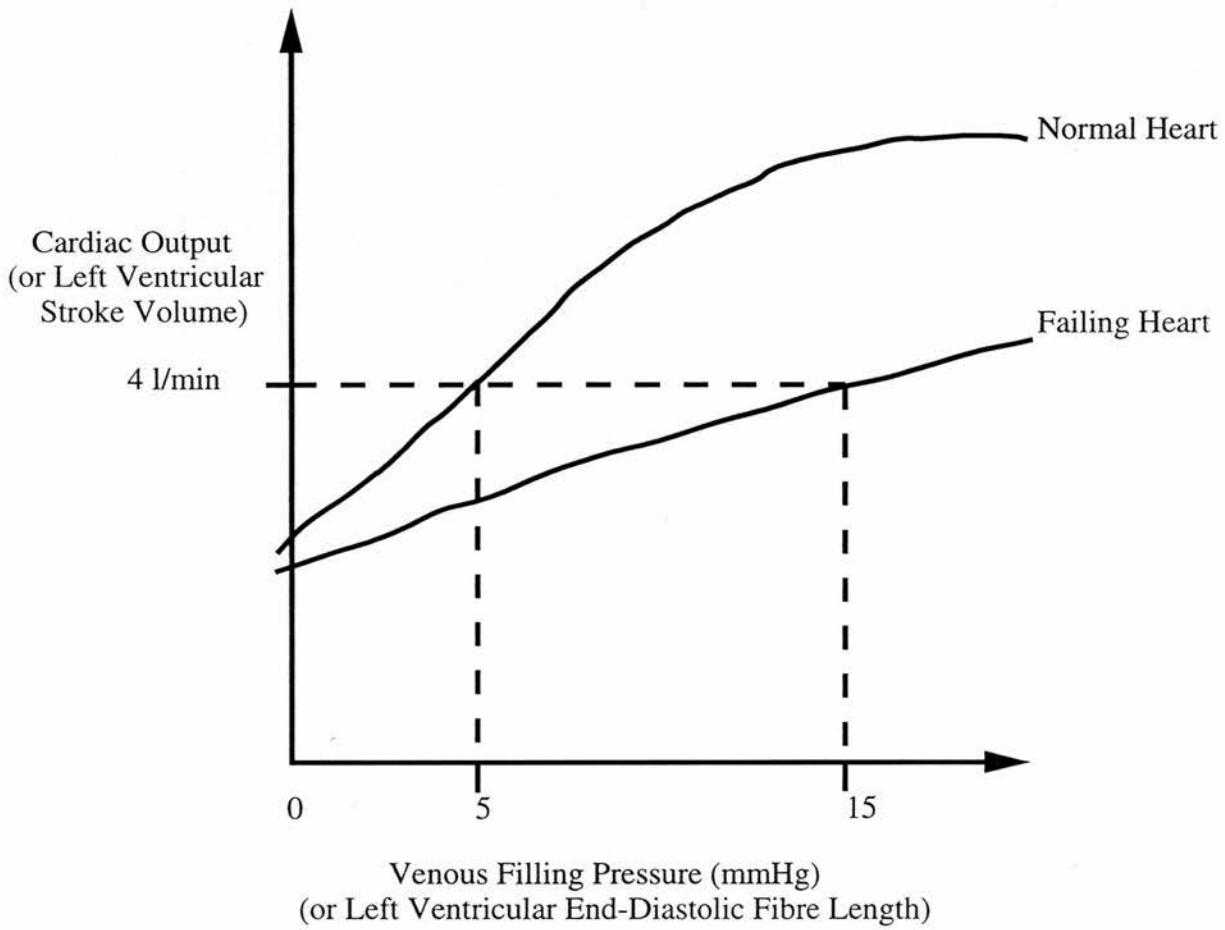


Figure 1.4. Starlings Diagram for left ventricular heart failure. In CHF, the curve is depressed due to reduced contractility. At increased venous filling pressure, the cardiac output is greatly reduced in the failing heart as compared to a healthy heart. This can be expressed as end-diastolic fibre length, therefore in CHF, a given amount of stroke work can only be achieved with a greater end-diastolic fibre length.

individual muscle fibres (Julian & Cowan, 1992). ANG II and aldosterone are known to be mitogenic and are possibly involved in the process of hypertrophy in the heart (Katz, 1995).

Fibrosis frequently develops in hypertrophied muscle. The cause of fibrosis has been suggested to be a consequence of the thickened muscle fibres, increasing the distance oxygen has to diffuse from the capillaries. Impaired oxygenation, hypoxia, occurs at the centre of the fibre and fibrosis develops. Therefore, the heart muscle becomes even less contractile and less able to pump efficiently.

Vascular remodelling also occurs in CHF, where blood vessel structure alters in response to chronic alterations of hemodynamic stress. There is an increase in the ratio of the thickness of tunica media to lumen diameter, in order to maintain raised perfusion pressure more effectively (Weber *et al.*, 1992). The remodelling process involves both systemic and local factors, stimulated by the increase in shear stress. Again, ANG II has been implicated in the vascular remodelling process (Schiffrin, 1995).

Thus, the compensatory reflexes which are initially activated to maintain cardiac output eventually result in the symptoms of CHF. It is known as the "vicious cycle" of CHF (Figure 1.5; Francis *et al.*, 1984).

B. 1.2. Neurohumoral Reflexes in CHF

B. 1.2.1. Renin-Angiotensin-Aldosterone System

B. 1.2.1.1. Renin-Angiotensin

The kidneys are the most important source of renin. In heart failure decreased renal perfusion, due to reduced cardiac output and vasoconstriction, plus direct sympathetic stimulation of the juxtaglomerular cells increases renin secretion. Renin is a proteolytic enzyme which converts angiotensinogen, a circulating plasma globulin, to angiotensin I (ANG I). ANG I is then cleaved by angiotensin-converting enzyme (ACE) to the active octapeptide ANG II (Cockcroft *et al.*, 1995). ACE is widely distributed throughout the body, although its concentration is highest in the lung, where most of

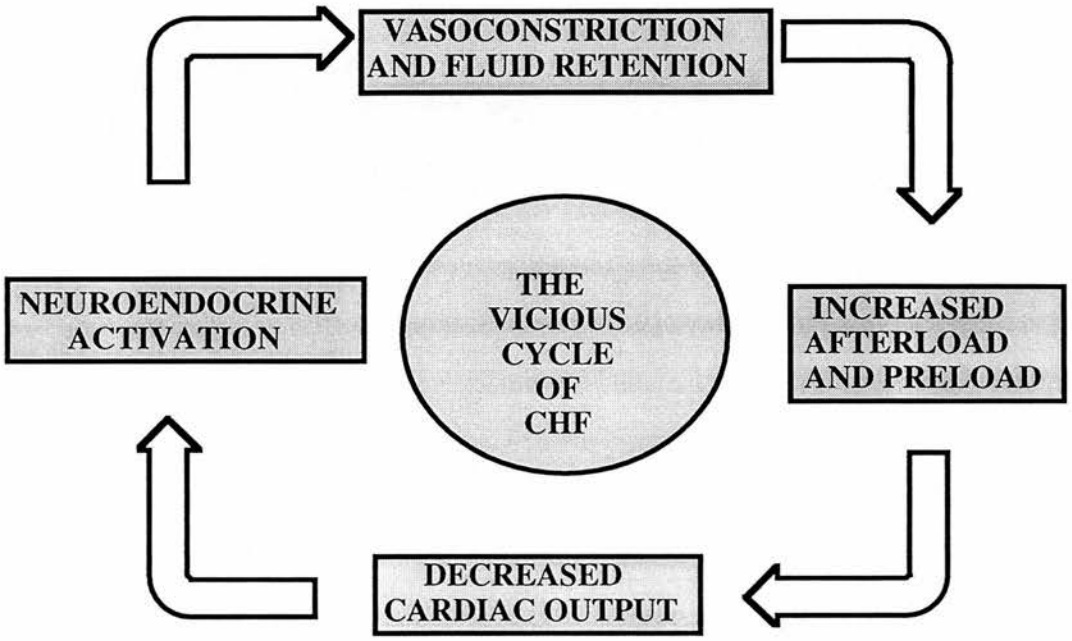


Figure 1.5. The vicious cycle of heart failure. A reduced cardiac output, usually as a result of a myocardial infarction, causes reflex neuroendocrine activation. Vasoconstriction and fluid retention occurs in order to maintain blood flow and oxygen delivery to the vital organs. The increased afterload, due to the peripheral vasoconstriction, and preload, courtesy of the fluid retention and venoconstriction, further reduces the cardiac output, increasing the amount of work the damaged myocardium has to perform.

the conversion of ANG I to ANG II takes place. ANG II is a potent vasoconstrictor which has preferential actions on cutaneous, splanchnic and renal blood flow, all of which are the vascular beds which are most affected by vasoconstriction in CHF. Furthermore, ANG II vasoconstriction is more pronounced in arterioles than veins (Kostis *et al.,* 1987).

Angiotensinogen is predominantly extracellular and is synthesised primarily in the hepatocytes of the liver, but is also found in a variety of cells, including adipocytes, astrocytes and vascular smooth muscle cells (Naftilan *et al.*, 1991; Cockcroft *et al.*, 1995). The presence of mRNA for both renin and angiotensinogen in the walls of blood vessels suggests that there is also a local vascular RAA system, therefore being in an ideal location to interact with the sympathetic nervous system (Cockcroft *et al.*, 1995).

The actions of ANG II are mediated by angiotensin receptors, of which two have been pharmacologically characterised, cloned and defined as AT_1 and AT_2. The majority of the effects of ANG II are via the AT_1 receptor, although the specific functions of the AT_2 receptor are unclear (Matsusaka & Ichikawa, 1997). It has been suggested that the AT_2 receptor has an antiproliferative role in some tissues, including vascular endothelial cells and some neuronal cells (Helin *et al.*, 1997). Both receptors are G-protein-linked.

ANG II infusion causes an increase in arterial pressure within seconds, an effect that is sustained as long as the peptide is infused (Clarke *et al.*, 1989). Upon termination of the infusion, the vasoconstriction disappears due to the rapid degradation of ANG II to ANG III by angiotensinases. The increased blood pressure induced by ANG II is due to an increase in systemic vascular resistance by direct contraction of vascular smooth muscle and potentiation of the sympathetic nervous system (Cockcroft *et al.*, 1995). ANG II enhances sympathetic transmission by augmenting release of noradrenaline out of, and blocking reuptake (uptake 1) into, the nerve terminal. Thus, the direct and indirect vasoconstriction induced by elevated ANG II levels may be part of the

increased systemic vascular resistance indicative of CHF, further exacerbating poor cardiac output by increasing afterload.

The anti-diuretic effects of ANG II are also by direct and indirect mechanisms. It exerts indirect effects on renal tubular function by stimulating aldosterone synthesis and secretion from the zona granulosa. Good and colleagues (1994) demonstrated that forearm infusion of ANG II caused dose-dependent rises in plasma concentrations of both ANG II and aldosterone. Aldosterone induces reabsorption of Na^+ and water and an increase in excretion of K^+ (see Section B. 1.2.1.2.). The direct action of ANG II on the kidney is partly due to the renal vasoconstriction, in particular the efferent glomerular arterioles, increasing renal perfusion pressure, decreasing renal blood flow and enhancing reabsorption by reducing pericapillary hydrostatic pressure and colloid pressure (Cockcroft *et al.*, 1995).

As briefly mentioned, ANG II may potentially have a role in the myocardial and vascular hypertrophy which occurs in CHF. In hypertension it has been shown that ACE inhibitors can alter small vessel structure in previously untreated hypertensives. Schiffrin and colleagues (1994; 1995), used gluteal, subcutaneous small arteries from hypertensive patients before and two years after either ACE inhibition (cilazapril) or β-blockade (atenolol). They showed a correction of media-to-lumen diameter by cilazapril treatment, but not atenolol. This work was confirmed in a separate study, although this study looked specifically at changes in lumen diameter (Thybo *et al.*, 1995). Similar alterations were found in coronary small vessels from spontaneously hypertensive rats (SHR), with perindopril treatment inducing a regression of hypertrophy and remodelling (Thybo *et al.*, 1994).

In the rat model of coronary artery CHF, where a myocardial infarct is induced by a ligature around the coronary artery, increased ACE binding and ANG II receptors (by autoradiography) in the myocardial tissue in and around the infarcted area have been demonstrated (Sun & Weber, 1994; 1996). The increase in AT receptor density was also seen in the smooth muscle cells of blood vessels at the site of infarct (Sun &

Weber, 1994; 1996). *In vivo*, captopril treatment prevents myocardial remodelling in the renovascular hypertensive rats (Jalil *et al.*, 1991).

Many studies have demonstrated a substantial activation of RAA system in CHF by measuring plasma renin activity (PRA; Dzau *et al.*, 1981; Riegger *et al.*, 1982) . Furthermore, a positive correlation between mortality and increased renin activity and ANG II levels has also been shown consistently (Francis *et al.*, 1990; Swedberg *et al.*, 1990). Inhibition of ACE has shown that the RAA system has a major role in the pathophysiology of CHF in humans. The ACE inhibitor captopril was the first drug of its class to be granted a licence for use in CHF, and was found to increase cardiac output, reduce vascular resistance and left ventricular filling pressure, increase exercise tolerance and decrease mortality (Francis *et al.*, 1984). As a consequence of the beneficial effects of captopril and other ACE inhibtors in CHF (see section B. 1.5.1.), they have now become frontline therapy in this condition.

B. 1.2.1.2. Aldosterone

Aldosterone is a steroid hormone derived, via several enzymatic steps, from cholesterol. It is, therefore, pharmacologically possible to inhibit its synthesis, although this potential inhibitory mechanism has not been widely explored (Zannad, 1995). ANG II is the most powerful stimulant of its synthesis and secretion, and consequently, in CHF, plasma levels of both aldosterone and ANGII (or PRA) are found to be increased (Rouleau *et al.*, 1988; Sigurdsson *et al.*, 1993) concomitantly. ACE inhibition in patients with CHF produces decreases in plasma levels of aldosterone as well as ANGII (Brilla *et al.*, 1989). However, it has been shown that with longterm ACE inhibition aldosterone levels, after an initial decrease, can return back to pretherapeutic levels. This is a phenomenon called 'escape' (Struthers, 1995). Aldosterone clearance is closely related to hepatic blood flow and extraction by parenchymal cells, both of which may be impaired in CHF. As a consequence, reduced elimination of aldosterone contributes to elevations in plasma aldosterone levels in CHF patients (Zannad, 1995). Current investigations are underway in order

to ascertain whether spironolactone, an aldosterone receptor antagonist, should be administered as adjunct therapy with ACE inhibitors.

The principal and most well characterised action of aldosterone is the Na^+ and water reabsorption. It binds to a specific intracellular steroid receptor and initiates DNA transcription of specific proteins to activate previously quiescent Na^+ channels in the apical membrane of cells in the cortical collecting tubule. There is also an increase in the number of Na^+/K^+-ATPase molecules in the basement membrane (Rang *et al.*, 1995). This leads to increased K^+ excretion.

As well as its involvement in fluid and sodium retention, aldosterone has other deleterious properties which may contribute to the pathophysiology of CHF. In two experimental models of hypertension, it has been demonstrated that aldosterone stimulates collagen synthesis by myocardial interstitial fibroblasts (Weber & Brilla, 1992). Furthermore, direct vasoconstriction induced by aldosterone has also been hypothesised. In renal failure patients, infusion of AGII resulted in vasoconstriction which was reversed by spironolactone (Schohn *et al.*, 1993).

Aldosterone also potentiates the action of NA in the heart. By inhibiting NA uptake in the myocardium, aldosterone may be partly responsible for the arrhythmias and ischaemia which often cause sudden death in ACE inhibitor treated CHF patients (Remme, 1995). In addition, aldosterone depresses the baroreflex, the inhibitory, parasympathetic pathway, (see Section B. 1.3.1.), at concentrations below those which induce hypertension (Wang *et al*, 1994).

As with ANG II levels, aldosterone concentrations in plasma have also been demonstrated to be elevated in CHF patients and animal models (Swedberg *et al.*, 1990; Weber *et al.*, 1995). In the CONSENSUS trial, investigating the effectiveness of enalapril in CHF, aldosterone levels were reduced alongside ANGII concentrations (Swedberg *et al.*, 1990).

B. 1.2.2. Sympathetic Nervous System

A systemic injection of NA raises blood pressure by increasing heart rate and causing vasoconstriction (Rang *et al.*, 1995). The vascular beds most affected by NA are the splanchnic and skin beds whereas the cerebral, coronary and pulmonary vascular beds are relatively unaffected. Large arteries, arterioles and veins are constricted by actions on $\alpha_1$and α_2 adrenoceptors present on the vascular smooth muscle (VSM). α_1 receptors are mainly responsible for mediating the vasoconstriction via phosphatidylinositol (PI) metabolism, increasing IP_3 and DAG resulting in Ca^{2+} liberation and sensitisation of contractile proteins. However, stimulation of β_2 adrenoreceptors, also present on VSM, causes relaxation through increases in cAMP levels. α_2 adrenoceptors are also located presynaptically, on the nerve terminal where they have negative feedback control on the release on NA, by reducing intracellular cAMP levels, ultimately decreasing Ca^{2+} influx and increasing Ca^{2+} extrusion (Rang *et al.*, 1995).

Stimulation of β_1 adrenoceptors located on the heart raises intracellular cAMP levels. This results in both the heart rate (chronotropic effect) and the force of contraction (inotropic effect) being increased, thus raising cardiac output and cardiac oxygen consumption. It has been shown that raised circulating levels of NA can lead to cardiomyopathy in animal models and humans (Francis *et al.*, 1984). Furthermore, it is hypothesised that augmented NA release is possibly responsible for the fatal arrhythmias which are the major cause of death in CHF patients (Francis *et al.*, 1984).

In heart failure sympathetic activation has been shown to predict survival. Plasma levels of NA increase as the severity of heart failure worsens (Cohn *et al.*, 1984; Francis *et al.*, 1984; Francis *et al.*, 1990; Swedberg *et al.*, 1990). However, plasma NA concentrations do not reflect neurotransmitter release, but the balance between NA spillover into plasma and its clearance (Floras, 1993). In fact, total body spillover was found to be two-fold higher in CHF patients as compared to controls, and clearance reduced by a third (Hasking *et al.*, 1986) The kidney and heart contributed to approximately 60% of the increase in plasma NA (Hasking *et al.*, 1986).

Peripheral modulation of the sympathetic system by antagonising postsynaptic α_1 adrenoceptors demonstrated improvements in CHF patients haemodynamics (Miller *et al.*, 1977; Colucci *et al.*, 1980). However, these improvements are short-lived, thus have made no clinical impact (Manolis *et al.*, 1995). Paradoxically β_1 adrenoceptor antagonists are currently the sympathetic modulatory treatment which is under the most intense scrutiny. Clinical studies have shown a beneficial effect by reducing heart rate, improving relaxation and reducing ischaemia (Remme, 1995; See section B. 1.5.4.)

B. 1.2.3. Vasopressin

Vasopressin is another hormone whose circulating concentration are raised in CHF (Francis *et al.*, 1984; 1990). It has powerful effects in the kidney, explaining its alternative name of antidiuretic hormone (ADH), but is also a powerful vasoconstrictor. In the kidney vasopressin increases water reabsorption in the distal tubules and collecting ducts by increasing water permeability. Action on V_2 receptors raises internal cAMP levels which increase the number water channels in the cell membrane, promoting water re-absorption. Its vascular actions are mediated by stimulation of V_1 receptors, which are coupled to IP_3 turnover. Vasopressin produces vasoconstriction in all vascular beds including the coronary and mesenteric circulations (Rang *et al.*, 1995).

Vasopressin is a nanopeptide synthesised in the posterior pituitary. Its release is principally under the control of the hypothalamus which monitors blood osmolality by osmoreceptors. However, both stretch receptors present in the walls of large veins and ANG II also stimulate the release of vasopressin (Lamb *et al.*, 1991).

Experiments into the role of vasopressin in CHF have been hampered by lack of non-peptide antagonists at the receptor sites and results have been controversial. In animal models, antagonism of the V_2 receptor has proved more beneficial in restoring haemodynamics by increasing fluid excretion (Wang *et al.*, 1991; Nishikimi *et al.*, 1995) , than inhibition of the V_1 receptor (Nishikimi *et al.*, 1995). In patients, a

specific V_1 antagonist did reduce systemic vascular resistance (SVR), but only in 3 out of 11 CHF patients treated. The decrease in SVR occurred in the patients with extremely high plasma levels of vasopressin (Creager *et al.*, 1986). Therefore, it appears that inhibition of the vasoconstrictor properties of vasopressin has a less important role in CHF, than the kidney effects.

B. 1.3. Inhibitory Pathways in CHF

B. 1.3.1. The Baroreflex

As well as an increase in sympathetic drive in CHF, there is a depression of the physiological antagonist mechanism, the baroreflex parasympathetic nervous system. The baroreflex consists of arterial mechanoreceptors present in the carotid sinus and aortic and pulmonary arches and some main arteries near the heart which detect high pressure, and low pressure receptors in the atria and large veins which detect changes in volume. As blood pressure rises, the arterial baroreceptors increase rate of firing. The impulses travel to the area of the brain, in the upper medulla, which is involved in the nervous control of the heart and blood vessels. This parasympathetic mediated afferent pathway causes a lowering of blood pressure by reducing efferent sympathetic vasoconstrictor discharge to the heart and vascular system, and increasing efferent parasympathetic discharge to the heart. Thus, as occurs in CHF, if there is impaired parasympathetic nervous control, either by changes in the inhibitory afferent pathway, efferent innervation to the heart or both, the restraining influence on sympathetic activation is lost. Hence, there is raised sympathetic pathway activity (Lamb *et al.*, 1991; Floras, 1993) .

The loss of baroreflex control in CHF has been demonstrated consistently (Floras, 1993). Reduced baroreceptor firing initially occurs due to the fall in cardiac output, resulting in a decreased stimulus on the arterial baroreceptors which, in turn, increases heart rate and total peripheral resistance (Zucker *et al.*, 1993). Direct measurement of nerve firing, by a technique called microneurography, has demonstrated that the loss of parasympathetic baroreflex control occurs relatively early on in the pathogenesis of the

disease (Ferguson *et al.*, 1992; Grassi *et al.*, 1995). Abnormalities in the central regulation of parasympathetic outflow has also been suggested (Porter *et al.*, 1990).

ANG II has been implicated in decreased baroreflex control. ANG II infusion in humans was demonstrated to inhibit the forearm vascular response to increased arterial pressure (Goldsmith & Haskings, 1995). Furthermore, ANG II inhibition in animals, by either ACE inhibition (Noshiro *et al.*, 1993) or AT receptor blockade (Murakami *et al.*, 1996), enhanced baroreflex control of sympathetic outflow .

Overall, the removal of the restraining influence of the parasympathetic baroreflex in CHF allows increased sympathetic activation. The depressed baroreflex occurs early in heart failure, and, as with many of the pathways known to be activated in this disease, has been correlated with a poor prognosis (Osterzial *et al.*, 1994).

B. 1.3.2. Natriuretic peptides

The natriuretic peptides are a family of three peptides, which circulate in plasma. The first of these peptides to be described and sequenced was atrial natriuretic peptide (ANP; Kangawa & Matsuo, 1984). It is secreted from the cardiac atria, possessing profound natriuretic and diuretic properties. It has also been shown to have vasodilatory actions, in particular being a potent coronary vasodilator (Davidson & Struthers, 1996). Following ANPs discovery, brain natriuretic peptide (BNP) was isolated from porcine brain (Sudoh *et al.*, 1988). In contrast to its name, in humans BNP is secreted almost exclusively from the heart, and in particular the ventricles (Mukoyama *et al.*, 1991). It has similar properties to ANP, and has had its name modified to B-type natriuretic peptide. The final peptide of the family, C-type natriuretic peptide (CNP), is located mainly in the central nervous system, although it is found in the vascular endothelium. However, CNP appears to have extremely limited natriuretic and vasodilatory effects (Hunt *et al.*, 1994). Thus, CNP will not be discussed further.

They are stored as relatively high molecular weight peptides, which are cleaved to the active form on release into the circulation. ANP and BNP are released from storage granules in the myocardium, the main stimulus for secretion being stretching of the atrial and ventricular walls (Kinnunen *et al.*, 1993). Their actions are mediated by three different natriuretic peptide receptors, designated A, B and C. The A- and B-receptors are linked to guanylate cyclase, raising cGMP levels, the second messenger system responsible for the majority of the biological effects of these peptides (Davidson & Struthers, 1996). The C-receptor was originally believed to be responsible for the clearance of the natriuretic peptides, however it is now thought that it may have some other biological functions, although these have not been defined yet (Levin, 1993). All three receptors are widely expressed throughout the body, in particular the kidneys, heart, vascular endothelium and the adrenals (Levin,1993).

The plasma half-lives of ANP and BNP are 3 mins and 22 mins respectively (Yandle *et al.*, 1986). There are two mechanisms by which the natriuretic peptides are removed by the circulation; firstly, as mentioned above, by C-receptor-mediated endocytosis, and secondly by degradation by NEP 24.11 (Kenny *et al.*, 1993). In the plasma of normal, healthy subjects both peptides are detectable at picomolar concentrations, however these levels are markedly increased in the plasma of CHF patients (Grantham & Burnett, 1997). As with the previously described markers of CHF, ANP and BNP concentrations show a close negative correlation with LVEF and CO (Richards *et al.*, 1993; Benedict *et el.*, 1994). Furthermore, the release of ANP and BNP is regulated by the tension of the wall of the left ventricle (Yasue *et al.*, 1994).

Apart from the obvious physiological antagonistic properties of the natriuretic peptides to the activation of the RAA system, sympathetic nervous system and vasopressin, of vasodilatation and fluid excretion, they have other important mechanistic interactions. ANP and BNP produce natriuresis and diuresis in the kidney by a direct action in the collecting duct, however, they also act on the glomerular and tubular cells inhibiting vasopressin (Raine *et al.*, 1989). Furthermore, the natriuretic peptides can interrupt the RAA system at three different sites. ANP can inhibit renin secretion from the

juxtaglomerular cells (Kurtz *et al.*, 1986) *in vitro*, attenuate ACE activity *in vitro* (Kawaguchi *et al.*, 1992) and inhibit ANG II-mediated release of aldosterone from the adrenal cortex *in vivo* (Delkers *et al.*, 1988). ANP has also been shown to modulate the activity of the sympathetic nervous system by inhibiting sympathetically mediated tachycardia (Ebert & Cowley, 1988), and reducing circulating catecholamines (Racz *et al.*, 1989).

The stimulation of the release of natriuretic peptides by the stretching of the heart walls, therefore, is a counterbalancing mechanism in order to modulate the excitatory pathways activiated in CHF. Potentiation of this pathway has been suggested as a possible new avenue of therapy in CHF, but there are currently no non-peptide A- or B-receptor agonists available for experimentation. Decreasing the breakdown of these peptides has been investigated using an NEP inhibitor, candoxatril. However, due to the non-specific nature of NEP to degrade other peptides such as ANG II and ET-1, the actions of these aggrevating peptides is also potentiated, partially counteracting the beneficial effects of increased natriuretic peptide levels.

B. 1.3.3. Nitric oxide

NO and its co-product L-citrulline, are synthesised by the conversion of the amino acid, L-arginine by the enzyme nitric oxide synthase (NOS; Palmer *et al.*, 1988). Three isoforms of NOS have been characterised, of which constitutive NOS (cNOS) is present in normal vascular endothelial cells, producing basal release of NO (Bredt & Snyder, 1990). The other forms of NOS are neuronal NOS (nNOS) present in the CNS and PNS, which synthesises NO to act as an inhibitory neurotransmitter (Bredt *et al.*, 1990), and inducible NOS (iNOS), which is induced in many cells, including endothelial cells, smooth muscle cells and activated macrophages in response to cytokines (Gross *et al.*, 1991). iNOS is a defence mechanism, synthesising large amounts of NO to act as a cytotoxic agent. Both cNOS and nNOS require calcium, calmodulin, NADPH and tetrahydrobioptrin (BH_4) as co-factors in order to produce NO, however, iNOS only requires NADPH and BH_4 (Moncada *et al.*, 1991).

NO, once released from endothelial cells, diffuses to the underlying smooth muscle, and activates soluble guanylate cyclase. Vasodilatation occurs by raised cGMP levels, which has actions on myosin light chain kinase, protein kinases, phosphodiesterases and ion channels resulting in reduced intracellular Ca^{2+} (Moncada *et al.*, 1991).

Endothelial synthesis of NO is induced by hormonal, receptor-mediated stimuli, including ACh, bradykinin, 5-HT, histamine, ATP and NA (Moncada *et al.*, 1991), and by mechanical forces, such as shear stress caused by pulsatile blood flow. Continuous basal release of NO by shear stress is an important phenomenon in the regulation of blood pressure, since inhibition of NOS raises mean arterial blood pressure and peripheral vascular resistance in animals (Rees *et al.*, 1989) and humans (Vallance *et al.*, 1989). The mechanism by which increased flow activates cNOS appears to involve the activation of voltage-dependent K^+ channels (Hutcheson & Griffith, 1994).

NO is extremely labile, courtesy of it being a free radical. It is oxidised by superoxide anions to peroxynitrite ($ONOO^-$), then to nitrate (NO_3^-) and excreted. Superoxide anions are produced endogenously in many physiological processes, including cellular respiration and as a produce of arachidonic acid metabolism (cycloxygenase and lipoxygenase). However these superoxide anions are scavenged by superoxide dismutases present in mitochondria and the cytoplasm of cells (Freeman & Crapo, 1982).

In experimental animal models of CHF, agonist-induced NO production has generally been shown to be impaired as compared to controls. In several studies using either the canine rapid ventricular pacing model (RVP; Kaiser *et al.*, 1989; Elsner *et al.*, 1991; Kiuchi *et al.*, 1993; Wang *et al.*, 1994) or the coronary artery ligation rat model of CHF (Ontkean *et al.*, 1991; Teerlink *et al.*, 1993; 1994c), ACh-induced vasodilation is attenuated in coronary (conduit and resistance), femoral and pulmonary arteries and thoracic aorta. In all but one of these studies (Wang *et al.*, 1994), the vasorelaxations

to the endothelium-independent vasodilator nitroglycerin were similar to those of control dogs. Thus implying that agonist-induced NO release is impaired in CHF.

In contrast to the above studies, a small number of investigations have indicated that agonist-mediated synthesis of NO was unaffected in CHF. There was no change in ACh-induced relaxation in isolated coronary, renal and femoral arteries from dogs with mild CHF induced by 11 days of pacing (O'Murchu *et al.*, 1994). In agreement with the lack of impairment of endothelial NO dysfunction in mild CHF, it was seen that in rats with less than 40% infarcted tissue of the left ventricle (LV), ACh-induced vasodilation to was the same as control. However, in more severe CHF (>40% infarcted LV tissue), there were blunted relaxations to muscarinic receptor stimulation (Drexler & Lu, 1992).

Results of experiments investigating basal release of NO have been even more disparate. Vasoconstriction to systemic infusion of the NOS inhibitor, L-NMMA, was significantly attenuated in conscious CHF dogs when compared to control dogs in two independent studies after 11 days (Elsner *et al.*, 1991) or 4 to 7 weeks (Kiuchi *et al.*, 1993) of RVP. Furthermore, the reactive hyperaemic response, where a reflex vasodilation is induced by hypoxia, has also been seen to be inhibited in the coronary circulation in RVP dogs (Wang *et al.*, 1994). In this study, aortic endothelial cells harvested from CHF dogs had reduced mRNA for cNOS. In contrast, an elegant study using microspheres indicated that L-NMMA infusion reduced blood flow to cerebral, splachnic and renal circulations to similar proportions in both 8 weeks post-MI rats and sham-operated controls (Drexler *et al.*, 1992a). However, vasoconstriction to L-NMMA in the coronary bed was reduced. In another study involving the infusion of L-NMMA into the hind-limb preparation of CHF rats, there was no significant difference in the amplitude of vasoconstriction. This study compared rats with a myocardial infarct of >40%, <40% and sham-operated controls, showing that in this vascular bed, basal release of NO was unaffected by severity of CHF.

A few studies have demonstrated enhanced NO production in CHF. In cardiomyopathic hamsters, vasoconstriction to NA is enhanced when in the presence of L-NMMA, whereas in the control animals it remained the same (Noll *et al.*, 1994). However, this potentiation was only seen in the aorta, but not in the mesenteric arteries from the same animals. In accordance with these findings, NA-mediated NO release via α_2 adrenoceptors in coronary arteries from a canine model of CHF was also enhanced (Main *et al.*, 1991).

Therefore, overall in animal studies there have been mixed results. However generally, reduced NO release, either agonist-mediated or basally produced, is associated with animals with larger myocardial infarcts and therefore severity of CHF. In the human setting, results have been slightly more consistent. Many reports demonstrate reduced receptor-mediated NO release from isolated arteries, such as the epicardial coronary artery (Forstermann *et al.*, 1988), and from intact circulations including the coronary bed (Treasure *et al.*, 1990), skeletal muscle, lower limb (Katz *et al.*, 1992) and forearm circulations (Kubo *et al.*, 1991). However, basally released NO is most probably preserved or even enhanced. Drexler and colleagues (1992b) infused L-NMMA into CHF patients and saw a greater decrease in forearm blood flow, as compared to healthy controls. In agreement, Habib *et al.* (1994) infused systemic doses of L-NMMA in CHF patients. They showed that the increase in systemic vascular resistance in response to L-NMMA was greatest in the most severe heart failure patients. Although they did not have data in healthy controls to properly validate the study. Furthermore, increased plasma nitrate levels were shown in patients with heart failure, as compared to controls (Winlaw *et al.*, 1994). Although this latter observation may be due to activation of the iNOS pathway, since it is known that there are increased circulating levels of cytokines, such as tumour necrosis factor, in heart failure. However, it appears that there could be regional vascular differences. For instance, in the coronary circulation, the basal release of NO was shown to be decreased in CHF patients, as compared to control patients (Mohri *et al.*, 1997).

Thus, although findings have been controversial, overall it appears that receptor-mediated liberation of NO is impaired in both animals and humans with CHF. However, basal production is preserved, and may even be enhanced.

B. 1.3.4. Prostaglandins

Prostaglandins (PGs) are a family of vasoactive substances derived from arachidonic acid (AA). The biosynthesis of PGs is initiated, generally, by the activation of phospholipase A_2, by receptor-G-protein stimulation, which liberates AA from the endothelial cell membrane (Moncada & Vane, 1979). This is the rate limiting step for any PG synthesis. The AA is converted, via cycloxygenase (COX), to the intermediate prostaglandin endoperoxidases, and to the subsequent PGs by their specific synthase enzyme.

The most well recognised PGs are prostacyclin (PGI_2), prostaglandin E_2 (PGE_2) and thromboxane A_2 (TxA_2). The first two metabolites are principally vasodilators, by activating adenylate cyclase and increasing cAMP levels. In contrast, the latter, TxA_2, is a vasoconstrictor acting on its receptors inducing phosphatidylinositol (IP_3) turnover and increasing intracellular Ca^{2+} levels. They are often considered to be physiological antagonists to each others actions. However, in most venous preparations, and in the lung, PGI_2 has little or no dilator activity, whereas TxA_2 is always a potent constrictor (Coleman, 1994). This may have some importance in the setting of CHF. Furthermore, the PG formed varies from cell to cell. In the vascular endothelium PGI_2 is the isoform predominantly synthesised, but in platelets TxA_2 is the major metabolite product. Their biological half-lives are less than 1 min, being rapidly hydrolysed to 6-keto $PGF_{1\alpha}$ (PGI_2) and TxB_2 (TxA_2). As a consequence of their instablity, their levels in plasma and urine are often measured by the stable metabolites (Coleman, 1994).

In CHF, it has been demonstrated that PGE_2 has a major role in maintaining renal blood flow. Inhibition of COX with indomethacin in CHF dogs had no effect on cardiac output or systemic vascular resistance, however decreased renal blood flow by more than 25% (Oliver *et al.*, 1981). This has also been demonstrated in humans,

where urinary PGE_2 concentrations are raised 3-4 times in CHF patients with hyponatremia, but not in those with normal serum sodium concentrations (Dzau *et al.*, 1984).

The presence of a constrictor PG has been detected in CHF in both animal and human studies. The vasodilatory responses to ACh are significantly potentiated in the femoral arteries of CHF dogs (Kaiser *et al.*, 1989), and in the forearm vasculature in CHF patients (Katz *et* al., 1993) when COX is inhibited by indomethacin. The identity of this vasoconstricting COX product in currently unknown. However, it has been seen in canine basilar arteries, that superoxide anion is generated by the hydroperoxidase activity of COX (Katusic & Vanhoutte, 1989). The authors suggested that the superoxide anion could produce vasoconstriction in three ways. Firstly any NO produced could be scavenged, secondly, PGI_2 synthesis can be inhibited and thirdly, that there could be a direct vasoconstrictor action on the smooth muscle. Furthermore, it is known that there is enhanced free radical production in CHF patients (McMurray *et al.*, 1990).

In summary, the vasodilator PGE_2 has an important role in renal blood flow in severe CHF, in the presence of renal dysfunction. However, in stable, less severe CHF patients with normal kidney function, agonist-induced vasodilation is counteracted by the release of a vasoconstricting COX product, the identity of which remains elusive (Katz, 1995).

B. 1.4. Diagnosis of CHF

CHF in the Western world is generally a consequence of ischaemic heart disease. When presented with the full blown symptoms of fluid retention, fatigue, dyspnoea clinical diagnosis is relatively simple. Other symptoms, less common, include tachycardia, a third heart sound and cardiomegaly (Dargie & McMurray, 1994).

There is no single test for CHF; the diagnosis depending on a combination of the symptoms and signs, with confirmatory evidence of impaired cardiac function from a chest *x*-ray or echocardiogram (Timms & Davies, 1992). A chest *x*-ray is used for evidence of pulmonary oedema or venous hypertension, and may show an enlarged heart, although little other information can be gleaned from the radiograph. Echocardiography is possibly the most useful non-invasive technique for helping in a diagnosis of CHF. An echocardiogram permits direct measurement of the dimensions of all the cardiac chambers and allows a dilated poorly contracting left (or right) ventricle to be identified (Dargie & McMurray, 1994).

There are invasive methods of investigating the causes of CHF, including angiography where a contrast medium is introduced by a catheter into the heart, via the femoral artery and cine films taken at 25-50 frames per second (Julian & Cowan, 1992). The pressures within the heart and great vessels and cardiac output can also be measured using a cardiac catheter. However, radionuclide ventriculograms are the most accurate measure of left ventricular function, including contractility of the ventricle, end-systolic and end-diastolic volumes. Ventriculography involves technetium-labelled red cells, so the pool of blood and its movements in the left ventricle can be detected (Julian & Cowan, 1992).

Blood samples measuring the neuroendocrine factors of noradrenaline and renin can be used since their levels are directly related to prognosis. However, these are not widely used in clinical practice, although interest in the cardiac natriuretic peptides as a reliable marker of left ventricular dysfunction has been growing (Barnett, 1993). Many studies have shown a strong correlation between both atrial and brain natriuretic peptides and the severity of heart failure and that during exercise, levels rise in CHF patients but not in control subjects (Chati *et al.*, 1996; Steele *et al.*, 1997). Indeed, exercise can also be used as a test for the severity of CHF, measuring peak oxygen uptake and the ability of the patient to perform exercise. However, exercise testing is not generally used, instead the patient is asked the amount of exercise he/she can endure before becoming uncomfortable.

B. 1.4.1. Severity of Heart Failure and NYHA Class

The standard method for categorising the severity of heart failure once diagnosed is grading the patient by their symptoms. The classification used is the New York Heart Association grading, which was first proposed in 1964, and updated in 1973. Patients are categorised as NYHA Class I, II, III or IV (see table). Classes II, III and IV are regarded as mild, moderate and severe heart failure respectively, patients in class I are effectively normal (Timms & Davis, 1992).

NYHA Grading of Symptoms

Class	Symptoms
I	Cardiac disease but without resulting limitation of physical activity.
II	Cardiac disease with slight limitation of physical activity, comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnoea or anginal pain.
III	Cardiac disease resulting in marked limitation of physical activity but comfortable at rest. Less than ordinary physical activity causes fatigue, palpitation, dyspnoea or anginal pain.
IV	Cardiac disease resulting in the inability to perform any physical activity without discomfort, often discomfort at rest. If any physical activity is undertaken, discomfort is increased.

B. 1.5. Current Drug Therapies

The most effective drugs used in the treatment of CHF has been those which decrease systemic and pulmonary vascular resistance, by direct action on resistance arteries interrupting the neuroendocrine vasoconstrictor reflexes. Reduction of blood volume also helps alleviate symptoms, and improves general well-being.

B. 1.5.1. Angiotensin Converting Enzyme Inhibition

The most effective treatments in helping alleviate the symptoms of CHF and slow down the progression of the disease have been those drug therapies which decrease systemic and pulmonary vascular resistance. The class of drug which has had the greatest impact are the angiotensin converting enzyme (ACE) inhibitors, blocking the conversion of ANG I to ANG II and therefore stopping the vasoconstrictor and anti-naturetic actions of ANG II (Cockcroft *et al.*, 1995).

In healthy volunteers, brachial artery infusion of the ACE inhibitors, enalaprilat (Webb *et al.*, 1988) and ramiprilat (Webb & Collier, 1987) had no effect on forearm blood flow. This suggests that the RAA system has no role in maintaining resting, basal vascular tone in healthy subjects. Unfortunately, these simple experiments have not been repeated in CHF patients. However, trials with ACE inhibitors have consistently shown the beneficial effects of the inhibition of the RAA system in heart failure. The Co-operative North Scandinavian Enalapril Survival Study (CONSENSUS, 1987) was the first large trial to show a reduction in mortality in patients with severe (NYHA class IV) heart failure when treated with the ACE inhibitor, enalapril. It was then demonstrated that enalapril improved survival in patients with less severe heart failure, where 57% and 31% of patients studied were NYHA class II and III respectively (SOLVD, 1991).

The enzyme ACE does not exclusively convert ANG I to ANG II, it is also known as kininase II, having a major role in the breakdown of bradykinin (BK; Cockcroft *et al.*, 1995). BK is an endothelium-dependent vasodilator. It has been shown to produce vasodilation by the production of NO (O'Kane *et al.*, 1994) and PGI_2 (Pitt *et al.*, 1997). ACE inhibition has been shown to improve endothelial dysfunction in animals (Clozel *et al.*, 1990) and in humans (Hirooka *et al.*, 1992). Therefore, decreased breakdown of BK may contribute to the beneficial effects of ACE inhibition. BK, however, may be responsible for some of the side effects of ACE inhibition, including cough and hypotension. The AT_1 receptor antagonist losartan, in clinical trials has proved to better tolerated than captopril, with no incidences of cough and fewer

incidences of initial dosing hypotension (ELITE study; Pitt *et al.*, 1997). In this study, losartan treatment reduced mortality rates, as compared to those on captopril (9.4% vs 13.2%). The authors suggested that the lowering of sudden deaths was potentially due to an increased inhibition of ANG II and its effects. It is known that ANG II can be formed by non-ACE-dependent pathways, therefore captopril may not be suppressing ANG II production completely. Thus blockade at the AT_1 receptor inhibits ANG II actions in a more complete manner than captopril. Furthermore this study implies that bradykinin may have no involvement in the beneficial effects of ACE inhibitors, but is probably responsible for at least one of the side-effects, cough.

B. 1.5.2. Diuretics

Diuretics are extremely effective as a symptomatic treatment for CHF. By increasing the excretion of Na^+ and water, thus reducing the circulatory volume, preload and oedema are decreased (Dargie & McMurray,1994). Until recently, they were used as initial therapy following diagnosis of CHF, however they are now generally used as adjunct treatment with ACE inhibitors. Loop diuretics, such as frusemide, are the main class of diuretics prescribed and are the most powerful of all diuretics, capable of causing 15-25% of Na^+ in the filtrate to be excreted. However, reduced diuretic efficacy to loop diuretics can occur, upon which a second diuretic, either a thiazide (eg. bendrofluazide) or thiazide-related metolazone is used in combination (Dargie & McMurray, 1994).

The loop diuretics inhibit Na^+ reabsorption in the ascending loop of Henle, by inhibiting the $Na^+/K^+/2Cl^-$ carrier in the luminal membrane. This effectively removes the osmotic gradient in the renal medulla, (since the filtrate is now not hypertonic) and therefore water reabsorption does not occur. An additional effect of the loop diuretics is that because more solute is delivered to the distal portion of the nephron, the osmotic pressure further reduces water reabsorption (Rang *et al.*, 1995).

The thiazides are moderately powerful diuretics and increase Na^+ excretion (and Cl^-) by inhibiting the Na^+/Cl^- co-transporter in the distal convoluted tubule. They do not have any action on the thick ascending loop of Henle (Rang *et al.*, 1995).

Both types of diuretics have some actions on blood vessels. Loop diuretics have venodilator actions, although the mode of action is unknown. When intravenously infused into patients with acute CHF, a venodilation occurs before the onset of the diuretic effect, thus reducing preload on the heart. Thiazides, however, have direct vasodilator actions after the diuretic effects by opening K^+ channels (Rang *et al.*, 1995).

B. 1.5.3. Nitrates

The nitrates act by releasing NO. Some nitrates, such as nitroprusside, produce arteriolar dilatation, reducing peripheral vascular resistance, thus improving cardiac output. Other nitrates, such as nitroglycerin, cause venous dilatation, increasing venous pooling, reducing venous return to the heart, therefore decreasing left ventricular end diastolic pressure (LVEDP). Overall, all nitrates improve cardiac output and, more importantly, do not produce a reflex increase in heart rate (Elkayam, 1996).

There are two mechanisms by which nitrates donate NO. The organic nitrates, a group of compounds which include nitroglycerin and isosorbide dinitrate, are converted by a thiol-containing enzyme present at, or near, the plasma membrane of VSMCs (Seth & Fung, 1993). The final bioactive product of this enzymatic process is probably not NO itself, but a closely related molecule, nitrosothiol, which is also able to activate soluble guanylate cyclase. Nitroprusside and molsidomine are compounds which donate NO in a non-enzymatic manner, they simply breakdown to NO directly (Abrams, 1996).

The different mechanisms by which the two classes of NO-donating drugs produce vasodilation possibly explains why the organic nitrates act preferentially on the venous circulation, whereas nitroprusside is both an arterial and venous dilator. The organic nitrates are taken up into the VSMC layer more avidly by veins than arteries, therefore at lower concentrations the benefical effects are due to reduced preload, by increased venous capacity. At higher concentrations, the organic nitrates do produce arterial dilation. However they have little effect in the microcirculation possibly because of the lack of the thiol-containing enzyme in the VSMCs of these arterioles (Harrison & Bates, 1993).

Unfortunately, long term treatment with the nitrates lead to tolerance, where the dilatory actions become less over a period of time. The mechanisms behind this phenomenon are currently unknown (Abrams, 1996). Haemodynamic benefits of nitrates in CHF during exercise include significant increases in stroke volume and work indexes, as well as decreases in pulmonary wedge and arterial pressures, systemic vascular resistance and heart rate (Hecht *et al.*, 1982). Furthermore, the Veterans Administration Heart Failure Trials (V-HeFT) showed a small, but significant improvement in maximum oxygen consumption in patients when treated with isosorbide dinitrate in combination with hydralazine (Cohn *et al.*, 1987).

The effects of nitrate therapy alone on survival in CHF patients has never been studied. However, the V-HeFT studied the effects when in combination with hydralazine, showing significant reduction in mortality in comparison with the placebo and prazosin groups. In comparison to ACE inhibition with enalapril, lower mortality rates due to sudden death was found in the enalapril group versus direct vasodilation therapy (Cohn *et al.*, 1991). Thus, nitrate therapy is a useful treatment in CHF, but is secondary to ACE inhibition. However, some patients cannot tolerate ACE inhibitors, so nitrate therapy, in combination with hydralazine, is a useful alternative, although care should be taken to avoid nitrate tolerance (Elkayam, 1996).

B. 1.5.4. Beta-blockers

In recent years, the use of β-adrenoceptor blockers in the treatment of CHF has received increasing attention. Inhibition of the positive inotropic β_1-adrenoceptors may be surprising in CHF, however the results of several clinical trials have demonstrated a significant reduction in mortality in those groups of patients assigned a β-blocker (BBPP, 1988; CIBIS, 1994).

As described earlier, raised NA plasma levels from CHF patients have consistently been described, often correlating with many of the parameters of the disease (Krum, 1997). There is generalised sympathetic activation, however NA spillover is specifically increased in the heart. There is depletion of catecholamines from storage vesicles in the cardiac nerve terminals (Chidsey *et al.*, 1965) and downregulation of β-adrenoceptors, in particular the β_1-adrenoceptors on myocardial cells, as well as adenylate cyclase uncoupling (Bristow *et al.*, 1990).

In acute heart failure, sympathetic activation is important to increase force of contraction and maintain cardiac output. As the disease progresses this sympathetic activation contributes to the disease by several actions including, myocardial toxicity, increased myocardial oxygen consumption, and activation and potentiation of other aggrevating systems. However, the reduction in the threshold for the induction of arrhythmias is probably the most important detrimental mechanism, since it is believed that sudden death, caused by fatal arrhythmias, in CHF patients is most likely mediated by NA. This is implied by the clinical trials of xamoterol, a partial β-agonist (Xamoterol study group, 1990), and milrinone, a phosphodiesterase inhibitor (Packer *et al.*, 1991), which were both associated with adverse mortality outcomes. Furthermore, as mentioned above, of the few published studies into β-blockers in CHF, all of them demonstrate reductions in mortality (CIBIS, MDC, US Multi-centre carvedilol study program). Hospitalisation rates of these groups of patients were also reduced (US Multi-centre carvedilol study program).

The clinical benefits of β-blockade occur after long-term therapy, that is treatment of more than 2 months duration. To avoid sudden interference of the inotropic support to the failing myocardium commencement of β-blocker therapy has to be of extremely low doses (Krum, 1997). In the long-term, improvements described include increased ejection fractions and patients overall well-being. However, effects on exercise tolerance have been variable. This is most likely due to the heart-rate limiting effects of this class of drugs. Furthermore, β-blockade inhibits other neurohormonal systems activated in CHF, such as the RAA system (Krum *et al.*, 1995), and the ET system (Krum *et al.*, 1996) as well as improving parasympathetic activity (Goldsmith *et al.*, 1993).

Some of the β-blockers, for instance carvedilol, have other pharmacological properties. Carvedilol possesses a vasodilator component, albeit a modest one which possibly helps initial toleration by the patient of the drug (Krum, 1997). It also has anti-proliferative and anti-oxidant properties, which could potentially help slow the progression of the disease. However, these ancillary properties, in combination with β-blockade appear to produce clinical and mortality benefits in CHF. Thus the use of a β-blocker in the treatment of CHF is now becoming standard therapy, despite the exact mechanism of benefit being unknown.

B. 1.5.5. Cardiac glycosides

Digoxin and digitalis have been used in the treatment of CHF for over 200 years (Anand, 1995). They are members of a group of drugs called the cardiac glycosides, which are the active principles from the leaves of the foxglove. The mechanism of action of the cardiac glycosides is by direct action on the myocardium. They increase the force of contraction by raising the size of the intracellular Ca^{2+} gradient by binding to the K^+ binding site in the Na^+/K^+ ATPase pump. Inhibition of the pump results in a rise in $[Na^+]_i$, which, in turn, slows down the Na^+/Ca^{2+} exchanger. Under normal conditions the Na^+/Ca^{2+} exchanger pumps Na^+ into the cell, whilst extruding intracellular Ca^{2+}. Therefore, because there is increased $[Na^+]_i$ during cardiac

glycoside blockade, the gradient for Na^+/Ca^{2+} exchange is reduced. Thus the $[Ca^{2+}]_i$ is retained for longer within the cell (Rang *et al.*, 1995).

In CHF the oedema is partly relieved by the cardiac glycosides due to the extra-cardiac property of venous dilation, reducing preload. There is also an overall reflex vasodilation in response to the increased cardiac output and arterial pressure. The diuresis seen to cardiac glycoside treatment is also partly due to the increased renal blood flow, as well as inhibition of the Na^+/K^+ ATPase in the renal tubules, similar to the diuretics (see Section B. 1.5.2.). Cardiac glyocosides increase the efficiency of the failing myocardium with regard to oxygen consumption. This is possibly as a result of a reduction of excessive diastolic stretching of the myocardium. They also depress AV conduction, which has beneficial effects in CHF due to reducing the likelihood of arrhythmias and sudden death.

Several small and large trials have demonstrated the beneficial effects of digoxin in CHF. Withdrawal of digoxin therapy increases the chances of deterioration of the condition (Captopril-Digoxin Trial, 1988; Guyatt *et al.*, 1988; Pugh *et al.*, 1989; Packer *et al.*, 1993). However, the worsening heart failure in some patients could be controlled by increasing diuretic therapy (Pugh *et al.*, 1989). In some studies digoxin treatment also significantly improves exercise capacity, breathlessness and NYHA class (Guyatt *et al.*, 1988; DiBianco *et al.*, 1989; van Veldhuisen *et al.*, 1993), but in others digoxin appears to have no effect on these symptoms at all (Captopril-Digoxin Trial, 1988; German & Austrian Xamoterol Study Group, 1988; Just *et al.*, 1993). In all of these studies, however, improved contractility due the inotropic properties of digoxin was clearly demonstrated.

Overall, the most effective drug therapies in the treatment of CHF are those which produce peripheral vasodilation causing decreased after and preload, therefore reducing the amount of work the heart has to perform. The ACE inhibitors are currently the most successful group of drugs in CHF at present. However abolition of the actions

of the RAA system does not halt the symptoms and progression of the disease completely. Thus, there is still room for improved drug therapy.

B. 1.6. Endothelin and CHF

As described earlier, ET-1 has several different actions which implies that it could have a potential role in CHF. The most obvious, direct actions implicating ET-1 are i). its long-lasting vasoconstrictor actions on both the arterial and venous beds (Yanagisawa *et al.*, 1988), and ii). its powerful effects on the kidney, reducing both renal plasma flow and glomerular filtration rate and causing sodium retention (Miller *et al.*, 1989; Lerman *et al.*, 1991).

ET-1 also has indirect effects suggesting its involvement in CHF including interactions with the other neuroendocrine mediators released in CHF. Subthreshold concentrations of ET-1 potentiated contractile responses to catecholamines and 5-HT (Yang *et al.*, 1990) in isolated human arteries, and the pressor response to AGII infusion in rats (Yoshida *et al.*, 1992). Thus basal, physiological levels of ET-1 could also enhance sympathetic tone and other vasoconstrictor reflexes in CHF. Indeed, ET-1 potentiation of neuronal contractile responses to catacholamines has already been demonstrated in hypertensive patients (Haynes *et al.*, 1994) whereby the sympathetically mediated venoconstriction was elicited by a deep breath, infusion of ET-1 enhancing this reflex in the hypertensive but not normotensive subjects.

The circulating levels of ANG II, vasopressin and catecholamines can also be modulated by ET-1. In pulmonary artery endothelial cells, ET-1 stimulated the conversion of ANG I to ANG II (Kawaguchi *et al.*, 1990) and furthermore, intravenous infusion of ET-1 *in vivo* augmented plasma renin activity (Miller *et al.*, 1989). Increased adrenal synthesis of both adrenaline (Boarder & Marriot, 1989) and aldosterone (Cao & Banks, 1990) by ET-1 has also been demonstrated. Conversely, ET-1 secretion from cultured endothelial cells is augmented by ANG II (Emori *et al.*, 1991) and arginine vasopressin (Bakris *et al.*, 1991). Thus, RAA system activation,

catacholamine and ET-1 secretion may potentiate each other, synergistically augmenting vasoconstriction and sodium retention in CHF.

As described earlier, the renal vasculture is extremely sensitive to ET-1, constricting both afferent and efferent renal arterioles *in vitro* (Edwards *et al.*, 1990). In contrast to animal data, a low dose infusion of ET-1 in healthy volunteers is anti-natriuretic in the absence of significant reductions in renal plasma flow and GFR (Rabelink *et al.*, 1994). Infusion of higher concentrations of ET-1 sufficient to increase plasma levels threefold, such as are seen in experimental and human CHF, causes more profound sodium retention and reductions in both renal plasma flow and GFR (Rabelink *et al.*, 1994).

ET-1 is also a co-mitogen, promoting cell division, hypertrophy and DNA synthesis in vascular smooth muscle cells and myocytes via the induction of proto-oncogenes *c-fos* and *c-myc* (Battistini *et al.*, 1993). Thus, similar to AGII, ET-1 may have a role in myocardial and peripheral vascular remodelling in CHF. ET-1 also has direct cardiac actions. Infusion of low concentrations into animals results in heart rate and cardiac output to rise. However higher doses cause cardiac output to fall as a result of direct coronary vasoconstriction and increased systemic vascular resistance (Lerman *et al.*, 1991; Miller *et al.*, 1989).

The properties of ET-1 described implies that it could have a potential role in all aspects of the pathophysiology of CHF, from the obvious vasoconstriction and renal dysfunction, to potentiation of neuroendocrine reflexes, ventricular and vascular re-modelling whilst also depressing myocardial contractility.

B. 1.6.1. Plasma endothelin concentrations in CHF

The first paper describing ET-1 concentrations in the plasma from CHF patients demonstrated that there was not a significant difference in ET-1 levels as compared to healthy controls (Cernacek & Stewart, 1989). However, this study only assayed the plasma from 5 CHF patients, who were in mild to moderate heart failure (NYHA Class

II). Furthermore in the same study, it was found that there were elevated plasma levels of ET-1 in patients with cardiogenic shock (fourteen fold) and pulmonary hypertension (six fold). The latter finding is important, since pulmonary hypertension manifests itself as heart failure becomes more severe. Indeed, plasma ET-1 correlates with the extent of pulmonary hypertension in CHF patients (Cody *et al.*, 1992; Cacoub *et al.*, 1993).

Following the findings of Cernacek & Stewart, most reports on plasma ET-1 levels in CHF patients have been shown to be increased between 2-3 fold (Table 1.3). These studies have tended to concentrate on the more severe CHF patients, those in NYHA Class III - IV. Rodeheffer and colleagues (1992), were the first study to report a correlation between plasma ET-1 concentrations and severity of CHF. They split their patients into 2 groups; mild CHF, which constituted NYHA I and II, and severe CHF, NYHA III and IV, with a third group of age- and sex-matched controls. The plasma ET-1 from the mild CHF group was significantly raised from controls (11.1 vs 7.1 pg/ml, $P<0.001$), and the levels in the severe CHF group were then significantly higher than those from the mild group (13.8 vs 11.1 pg/ml, $p=0.03$). In addition, they saw a negative correlation between left ventricular ejection fraction and plasma ET-1. This study was then followed by a report showing that patients with end-stage heart failure, who had undergone cardiac transplantation, had significantly higher ET-1 levels than patients with severe, but stable CHF (Lerman *et al.*, 1992). The plasma ET-1 remained chronically elevated for at least 12 months. ET-1 plasma levels are also predictors of mortality. Tomoda (1993) showed that in the group of severe CHF patients studied, those who died had greater than 2 fold higher circulating ET-1, than the patients who were stable.

Animal studies have demonstrated similar findings (Table 1.4). In dogs with heart failure induced by pacing (RVP; Cavero *et al.*, 1990; Marguiles *et al.*, 1990; Calderone *et al.*, 1993) or by thoracic inferior vena caval constriction (TIVCC; Underwood *et al.*, 1992) ET-1 levels are raised 2-3 fold. In rats with CHF following coronary artery ligation ET-1 levels are raised significantly at 1 week, 4 weeks and 16 weeks post-

STUDY	CONTROL SUBJECTS	CHF SUBJECTS	NYHA CLASS	DIFFERENCE	ANTIBODY X-REACTIVITY
Cernacek & Stewart, 1989	0.29 ± 0.24 pg/ml (n=14)	0.46 ± 0.36 pg/ml	II, n=5	ND	DNS
Cody *et al.*, 1992	3.7 ± 0.6 pg/ml (n=12)	9.07 ± 4.13 pg/ml	II (3), III (14), IV (3), n=20	2.5 fold ↑	bigET-1 = 17%
Stewart *et al.*, 1992	0.74 ± 0.11 pg/ml (n=9)	3.7 ± 0.47 pg/ml	III, n=6	5 fold ↑	bigET-1 - 10%
McMurray *et al.*, 1992	6.4 ± 0.3 pmol/l (n=16)	12.4 ± 0.6 pmol/l	II (9), III (32), IV (6), n=47	2 fold ↑	DNS
Lerman *et al.*, 1992	6.8 ± 0.3 pg/ml (n=24)	11.7 ± 1.1 pg/ml	III-IV, n=24	1.7 fold ↑	bigET-1 < 37%
Tomoda, 1993	1.51 ± 0.39 pg/ml (n=12)	2.74 ± 1.02 pg/ml	DNS	1.8 fold ↑	bigET-1 = 17%
Cacoub *et al.*, 1993	4.4 ± 0.3 pg/ml (n=20)	7.7 ± 0.3 pg/ml	II (6), III (27), IV (9), n=39	1.8 fold ↑	bigET-1 < 5%
Wei *et al.*, 1994	~7 pg/ml (n=6)	~8 - 9 pg/ml	I (14), II (5), III (7), IV (4), n=30	ND	bigET-1 = 37%
Krum *et al.*, 1995	~3 pg/ml (n=10)	~10 pg/ml	II (4), III (8), n=12	~3 fold ↑	DNS

Table 1.3. Plasma endothelin-1 levels, as measured by radioimmunoassay, in chronic heart failure patients as compared to normotensive, control subjects. Abbreviations include ND, no difference; DNS, did not specify

STUDY	MODEL	DIFFERENCE IN ET-1 LEVELS	ANTAGONIST TREATMENT	ET-1 LEVELS AFTER TREATMENT
Cavero *et al.*, 1990	RVP in dogs	3 fold ↑	-	-
Marguiles *et al.*, 1990	RVP in dogs	2 fold ↑	-	-
Underwood *et al.*, 1992	TIVCC in dogs	2.5 fold ↑	-	-
Calderone *et al.*, 1993	RVP in dogs	3 fold ↑	-	-
Fu *et al.*, 1993	LCAL in rats	DNS	-	-
Loffler *et al.*, 1993	AVIAS in rabbits	29 fold ↑	-	-
Teerlink *et al.*, 1994b	LCAL in rats	1.5 fold ↑	Bosentan	DNS
Clavell *et al.*, 1996	TIVCC in dogs	1.5 fold ↑	FR 139317	DNS
Sakai *et al.*, 1996b	LCAL in rats	3 fold ↑	BQ-123	DNS
Cannan *et al.*, 1996	TIVCC in dogs	2 fold ↑	-	-
Shimoyama *et al.*, 1996	ICE in dogs	DNS	Bosentan	8-10 fold ↑
Mulder *et al.*, 1997	LCAL in rats	↔	Bosentan	2.5 fold ↑
Spinale *et al.*, 1997	RVP in rabbits	2 fold ↑	PD 156707	3 fold ↑

Table 1.4. Change in plasma endothelin-1 levels in various animal models of CHF as compared to sham-operated animals. The effects on plasma ET-1 levels after endothelin receptor antagonism are also shown. Abbreviations include, AVIAS, aortic valvular insufficiency and stenosis; ICE, intracoronary microembolism; LCAL, left coronary artery ligation; RVP, rapid ventricular pacing; TIVCC, thoracic inferior vena caval constriction; DNS, did not state.

infarct (Teerlink *et al.*, 1994). Furthermore, the plasma ET-1 levels were found to correlate with the size of infarct and right ventricular hypertrophy. Interestingly in this study, plasma ET-3 levels were also assayed, and it was found that there was a transient rise in ET-3 levels at 1 week. This latter response was suggested to be as a result of the acute myocardial infarction. However, rises in plasma ET-3 levels were not seen in humans with acute myocardial infarcts (Miyauchi *et al.*, 1991), although both ET-1 and bigET-1 concentrations were increased. In rabbits, where CHF was induced by aortic valvular insufficiency and stenosis, again, plasma ET levels were significantly augmented (Loffler *et al.*, 1993).

The physical levels of ET-1-immunoreactivity detected in plasma described vary from paper to paper, this may be due to several reasons. The levels of ET present in plasma are detected using the radioimmunoassay (RIA) technique (see methods B3.1). RIA is where an antibody (Ab) specific for the substance, in this case ET-1, to be assayed, is added to the sample. A known amount of radiolabelled antigen (Ag), usually ^{125}I-ET-1, is then added. Thus, the Ag present in the sample competes with the labelled Ag for binding to the specific Ab. This means that the higher the amount of endogenous Ag present in the sample, the lower the binding of labelled Ag. After separation of the free and bound labelled Ag, the amount of bound radioactivity is measured. The amount of ET-1 present in the sample is deduced by comparing against a standard curve which is run at the same time as the assay is being performed. Standards contain known concentrations of ET-1, the radioactivity in each tube is measured and the standard curve constructed.

The reasons for the differences between the levels measured in plasma, are probably due to differences in either extraction procedure or the specificity of primary Ab used. The latter explanation is the most likely, since the crossreactivity with other members of the ET family and the precursor big ET-1 varies from Ab to Ab. The most common ET-1 Ab used in RIAs described in the literature, has been the Amersham International, polyclonal anti-ET-1 Ab. This Ab has a cross-reactivity to ET-2 = 100%, ET-3 < 1% and big ET-1 = 37%. It is known that ET-2 is not present in

plasma, therefore the immunoreactive ET detected is likely to be a combination of ET-1 and big ET-1.

As a result of this cross-reactivity with big ET-1, it is recognised that in CHF, the rise in ET-1-immunoreactivity is probably an increase in big ET-1 as well (Table 1.5; Pacher *et al.*, 1993; Wei *et al.*, 1994). Pacher and colleagues (1993) were the first to delineate this. They used an Ab specifically raised against the C-terminal of big ET-1, which has a crossreactivity with the mature isoforms of ET of less than 1%. They saw that hypertensive non-CHF patients had similar levels of circulating big ET-1 to age-matched controls. However, in CHF patients there was a significant augmentation of plasma levels. Again, the big ET-1 levels correlated with the severity of CHF. Wei and colleagues (1994), also demonstrated that the rise in immunoreactive ET was due to increased big ET-1. They used a gel permeation chromatography (GPC) technique, and saw equivalent peaks for ET-1 in samples from both CHF patients and healthy, age-matched controls. However, a peak correlating to bigET-1 was seen only in the plasma from the CHF patients.

In all the studies investigating plasma ET-1/big ET-1 levels, there was no difference seen between patients with different aetiologies i.e. generally, ischaemic cardiomyopathy or idiopathic dilated cardiomyopathy.

B. 1.6.2. Increased production of ET-1 in CHF

As described above, it appears that there is elevated circulating big ET-1 in CHF, implying that there is the potential for increased production of ET-1. It has been shown in the RVP CHF dogs that there is a correlation between immunoreactive plasma ET levels and elevated right and left atrial pressures. Furthermore, in humans there is a correlation with pulmonary hypertension (Cody *et al.*, 1992). Therefore, this elevated production could be stimulated by raised cardiac pulmonary pressures. In accordance with this, mRNA for ET-1 in the lungs and hearts of dogs with CHF, have been shown to be increased (Wei *et al.*, 1994). In contrast, higher levels of plasma ET have been observed in the TIVCC dog model of CHF, than in the RVP dog model.

STUDY	CONTROL SUBJECTS	CHF SUBJECTS	NYHA CLASS	DIFFERENCE	ANTIBODY CROSSREACTIVITY
Pacher *et al.*, 1993	1.3 ± 0.3 fmol/ml (n=22) *~5.6 pg/ml*	3.1 ± 0.9 fmol/ml * *~13.3 pg/ml*	II (13)	2.4 fold ↑	ET-1 < 1%
		5.2 ± 2.3 fmol/ml * *~22.4 pg/ml*	III-IV (14)	4 fold ↑	
		2.7 ± 0.9 fmol/ml ** *~11.6 pg/ml*	II (19)	2.1 fold ↑	
		5.7 ± 3.3 fmol/ml ** *~24.5 pg/ml*	III-IV (7)	4.4 fold ↑	
Wei *et al.*, 1994	ND (n=4)	14.6 ± 2.4 pg/ml	IV (4)		ET-1 - 100% BigET-1 = 37%

Table 1.5. Studies investigating big ET-1 plasma levels. Pacher and colleagues studied both mild (II) and severe (III-IV) CHF patient bigET-1 levels without (*)or with (**) concomitant hypertension. The study by Wei *et al.*, 1994, used gel filtration chromatography to distinguish the relative amounts of ET-1 and bigET-1 contributing to the final levels of immunoreactive ET in severe CHF (NYHA IV only) and normal subjects plasma samples. In healthy controls, there was only one peak seen, correlating to the ET-1 fraction, however in the plasma from the patients with CHF, two peaks were detected, the largest representing bigET-1. It was calculated that of the total immunoreactive ET, 62±7% was bigET-1 and 38±5% was ET-1.

The TIVCC is a model without atrial distension, as demonstrated by the lack of raised ANP levels, suggesting that increased cardiac filling pressures are not essential for increased plasma ET-1-immunoreactivity in CHF (Underwood *et al.*, 1992).

In heart failure, there is low cardiac output, and often hypotension, which results in reduced vascular shear stress. Sharefkin and colleagues (1991) demonstrated an inverse relationship between shear stress and ET synthesis and release in cultured human endothelial cells. This observation was corroborated by a study *in vivo*, where chronic increases in blood flow in canine femoral arteries, secondary to a fistula, resulted in a decrease in ET-1 content in the arteries exposed to increased shear stress (Miller & Burnett, 1992).

There is widespread expression of ET-1 mRNA throughout many tissues of the body, which could be the potential sources of excessive ET-1 production (Nunez *et al.*, 1990). Of particular focus, especially in the setting of CHF and its reduced blood flow, the kidney has come under scrutiny. Suprarenal aortic constriction, reducing renal perfusion pressure, markedly increased circulating ET-1 (Sandok *et al.*, 1992). The rise in ET-1 may be due to decreased shear stress in the renal vascular bed, but could also be as a result of activation of the RAA system. It has been shown that ANG II stimulates ET-1 production *in vitro* (Dohi *et al.*, 1992). Moreover, in the two dog models of CHF, the TIVCC dog model (Underwood *et al.*, 1992), which is a high renin model, there is greater increases in plasma ET, than in the low renin model, produced by RVP (Cavero *et al.*, 1990; Marguiles *et al.*, 1990). However, this phenomenon may be as a result of the lack of the restraining hormone ANP being produced in the TIVCC model. Although, some studies in animals (Clavell *et al.*, 1994) and humans (Galattins Jensen *et al.*, 1996) have shown an inhibition of the activation of the ET system on ACE inhibition.

Reduced clearance of ET-1 has been suggested to be involved in CHF. In renal failure, massive increases in circulating ET-1, but not big ET-1, has demonstrated that impaired renal performance affects the bodys ability to remove circulating ET (Webb,

1997). This could become an important mechanism in the later stages of CHF. Exogenous bolus ET-1 produced greater increments in plasma ET-1 in experimental CHF, than in sham-operated controls (Cavero *et al.*, 1990). Although that may be due to clearance and metabolism mechanisms already performing to maximum capacity in the CHF animals. However, using a rabbit model of CHF, Loffler and colleagues (1993) measured tissue ET-immunoreactivity and density of ET receptors in the kidney and cardiac ventricles, and saw a decrease in both tissue ET-1 content and density of ET receptors. Since the ET_B receptor is known to be important in clearance of the ETs (Fukuroda *et al.*, 1994c), downregulation in the kidney could be an important mechanism contributing to the increases in circulating ET. However, in the human condition, McMurray *et al.*, 1992, measured plasma ET-1 levels from two sites, aorta and renal vein, and found that there was significant renal extraction in CHF patients.

B. 1.6.3. Functional effects of ET-1 in CHF

The significance of elevated, pathophysiological levels of circulating ET-1 was addressed by Lerman and colleagues (1991), who infused ET-1 into anaesthetised dogs producing a twofold increase in circulating ET-1, as is comparable with the levels seen in CHF. This ET-1 resulted in significant systemic and renal vasoconstriction, in association with a decrease in heart rate and cardiac output. There was no increase in mean arterial pressure. However, this model does not truely reflect the pathophysiological condition since there will probably be increased levels of ET-1 at the VSMC interface due to the polar secretion of ET-1 from endothelial cells in CHF (Wagner *et al.*, 1992a)

In RVP, CHF dogs, with 2 fold raised plasma ET-1 levels, systemic and renal vasoconstrictor responses to low dose exogenous ET-1 were attenuated in comparison to control dogs (Cavero *et al.*, 1990). However, the decrease in glomerular filtration rate was preserved in CHF animals. This suggests that there is downregulation of ET-1 receptors when chronically exposed to high levels of ET-1, or that there is up-regulation of an effective counterbalancing factor, such as ANP. This last point was addressed, again comparing the TIVCC and the RVP models of CHF. In the TIVCC

model, there is no atrial stretch, thus there is no rise in ANP levels. Hence, Underwood and colleagues (1992) administered low doses ET-1 and saw no attenuation of the vasoconstrictor actions in these animals when compared to controls. They then co-infused ANP and ET-1, and the vascular responses were markedly reduced, similar to those seen in the RVP model. In contrast to the systemic circulation, there was little modifying effect of ANP on ET-1 action in the kidney, in accordance with the response seen in RVP dogs.

Other studies have suggested down-regulation of ET receptors (Calderone *et al.*, 1993; Loffler *et al.*, 1993; Fu *et al.*, 1993) in the face of chronic exposure to high circulating levels of ET-1. As mentioned previously, in rabbits with CHF, the density of ET-1 receptors (they did not discriminate between the ET_A or ET_B receptor) was reduced in the ventricles of the heart and in the kidney (Loffler *et al.*, 1993). However, the receptor affinity remained unaltered in the cardiac tissue, but was increased in the kidney. They also demonstrated that in the left ventricle of the heart and the kidney, that there was reduced tissue immunoreactive ET, when compared to the non-CHF animals. In the right ventricle tissue ET-1 levels were increased 60% in comparison to control rabbits. Unfortunately, the functional importance and consequence of the alteration in the balance of plasma, tissue ET-1 levels and the receptor number and affinity were not addressed (Loffler *et al.*, 1993).

In an interesting study by Calderone and colleagues (1993), it was demonstrated that in the the circumflex coronary artery (CCA) in the RVP dog model of CHF, there is reduced basal accumulation of inositol phosphates (IPs) and ET-1-induced activation of phosphatidylinositol turnover. Furthermore, in CCA taken from normal, healthy dogs, when exposed to ET-1 for 60 minutes, there was also a decrease in ET-1-induced IP production. The authors went on to evaluate the role of protein kinase C in this phenomenon, using the phorbol ester, phorbol 12-myristate 13-acetate (PMA), which actives PKC. They incubated the CCA for 60 minutes with PMA and saw a similar reduction of ET-1-induced IP accumulation. Thus it implies that chronic exposure to ET-1 desensitises the ET receptors present in the CCA, by reducing PI

turnover. The authors suggest that PKC could be acting as a negative feedback mechanism regulating the responsiveness of the CCA, when under chronic exposure to an agonist (Calderone *et al.*, 1993). A similar effect has been demonstrated to chronic exposure of other agonists to their respective receptors, including α_1 adrenoceptors (Fredrik Leeb-Lundberg *et al.*, 1985) and ANG II receptors (Pfeilschifter *et al.*, 1989).

In the rat coronary artery ligation model of CHF, a reduced pressor response to bolus injection of ET-1 was shown (Fu *et al.*, 1993), whilst the vasodilatory action was preserved. It was found that there was a reduced ET-1 receptor density in the mesenteric arteries of these animals, but no alteration in the myocardium. Furthermore, the dissociation constant was increased approximately 3-fold in the mesenteric arteries from the CHF rats. Thus there appears to be a preferential loss of vascular smooth muscle ET-1 receptors, but not of endothelial (ET_B) or myocardial ET-1 receptors. However, in the cardiomyopathic hamster no alterations in cardiac tissue ET-1 density or dissociation constants were shown (Bolger *et al.*, 1992).Furthermore, although not significant, there was a trend towards an increased vasoconstrictor response to exogenous ET-1 in mesenteric arteries taken from this model (Noll *et al.*, 1994).

B. 1.6.4. ET-1 Antagonists in CHF

Despite the suggestion of down-regulation of ET receptors, antagonism of the ET system has shown improvement in CHF animals. In the TIVCC dog model, ET_A receptor antagonism resulted in a marked decrease in arterial pressure, affecting both renal and systemic vasoconstriction (Clavell *et al.*, 1994). The newly developed orally active non-selective ET_A/ET_B receptor antagonist, bosentan, proved extremely successful in reducing mean arterial blood pressure in coronary artery ligated rats (Teerlink *et al.*, 1994b). This study demonstrated the importance of the ET system in maintaining blood pressure and systemic vasoconstriction as heart failure progresses, because bosentan had a greater effect at reducing blood pressure and systemic vasoconstriction at 16 weeks post-MI, than at the earlier time points of 1 and 4 weeks.

More importantly, bosentan produced improvements in haemodynamics when administered in the presence of ACE inhibition.

Therefore, on the evidence described above it appears that the ET system is an attractive target for drug intervention in the management of CHF. Reduction in peripheral vaso- and venoconstriction will help to reduce afterload and preload, therefore reducing the amount of work the already damaged myocardium has to perform. Antagonism of the system in the kidney, improving renal perfusion and GFR, could help in natriuresis and inhibit the stimulation of the RAA system. ET-1 may also be involved in hypertrophy and re-modelling, so that ET inhibition could slow any structural changes. Hence, overall, ET system antagonism could be of potential therapeutic value in slowing the progression of CHF.

The ET system can be inhibited at two levels, either at the production of the mature peptide, by inhibiting endothelin-converting enzyme, or the actions of ET-1 by antagonism at the receptor level. Due to the recognition that it is raised production of ET-1, as demonstrated by increased circulating big ET-1 levels, it is an attractive idea to inhibit conversion, a situation comparable to ACE inhibition. However, firstly, selective, potent inhibitors against ECE have not been forthcoming and secondly, the knowledge on the subtypes of ECE, and which form is the most physiologically important is confused. Thus, this possibility as potential therapy, although attractive, has tended to be upstaged by the alternative mechanism, receptor antagonism. There are problems with receptor antagonism as well. Initially it was believed that ET_A receptors solely mediated vasoconstriction. However, since the discovery that some vascular beds also have constrictor ET_B receptors, the question arises whether both receptor subtypes should be antagonised. Unless an antagonist can be synthesised which discriminates between the endothelial and smooth muscle ET_B receptors, the vasodilatory actions of ET-1 will be lost. Because it is believed that ET-1 is released abluminally, the loss of the vasodilatory ET_B receptor by inhibition may not be as important. However, due to the increased circulating levels of big ET-1/ET-1, one would expect the endothelial ET_B receptors to play some modulatory role. One of the

clearance mechanisms of ET-1 is the ET_B receptor, and as has been demonstrated *in vivo*, inhibition of this receptor subtype results in massive increases in circulating ET-1. Furthermore, the relative contribution of smooth muscle ET_B receptors to peripheral vasoconstriction is also unknown.

B. 1.7. AIMS OF THESIS

It was suggested in 1990 (Cavero *et al.* & Marguiles *et al.*), that the ET system could also be activated in heart failure, being a major mediator of the peripheral vasoconstriction . It is the aim of this thesis to investigate whether ET-1 has a role in heart failure, and if there is an alteration of ET receptor responses and subtypes mediating the responses in small resistance arteries. The small resistance arteries have an internal lumen diameter of between 100 - 400 μm (Schiffrin, 1995) and are the most important blood vessels of the circulatory system in controlling the vascular resistance. Raised peripheral vascular resistance is an important aggravating phenomenon in heart failure. Therefore, if ET-1 and its receptors are involved in the overall vasoconstriction responsible for the increased vascular resistance, antagonists of the ET pathway could be potential novel therapies in the treatment of heart failure. Thus, it is important to identify which ET receptors mediate ET-1 vasoconstriction in CHF. Two sources of arteries have been used in this thesis, i). mesenteric arteries from the coronary artery ligation rat model of CHF at two different time points after the induction of heart failure, and ii). gluteal arteries dissected from buttock biopsies obtained from Grade II and III CHF patients and age-matched controls.

The aims of this thesis were;

1. To characterise which ET receptor subtype(s) mediate ET-1 vasoconstriction on the smooth muscle of mesenteric resistance arteries from normotensive rats.
2. To investigate whether there are any changes in the ET receptor subtypes mediating ET-1 vasoconstriction on the smooth muscle of mesenteric resistance arteries from a rat model of CHF at two different time points (5 and 12 weeks) after the induction of CHF.

3. To investigate the vasoconstrictor ET receptor subtype(s) on the smooth muscle of resistance arteries removed from Grade II and III CHF patients.

4. To ascertain whether there are increased circulating levels of big ET-1 and/or ET-1 in the plasma of CHF patients.

5. To assess if there is an altered localisation of ET-1 and ECE in the walls of the arteries from both the rat model and human CHF patients when compared to the respective control arteries.

Methods

C. 1.0. Functional studies of the endothelin system

It has long been known that small arterial vessels - the resistance arteries - are chiefly responsible for regulating blood flow and capillary pressure, thereby controlling total peripheral resistance (Furness & Marshall, 1974). However, most *in vitro* studies using isolated vessels have concentrated on responses of large conduit arteries, such as the aorta. In 1976 a new technique called wire myography was published which allowed the study of resistance arteries in isolation (Mulvany & Halpern, 1976). Wire myography involves mounting the resistance artery as a ring preparation between two fine wires which are passed through the lumen. The response to agonists are then recorded as a change in wall tension as measured by isometric force exerted on the wires. It is, therefore, similar to organ bath experiments on larger vessels, but on a smaller scale. This method is an extremely useful way of studying resistance arteries.

However, recently a more physiological technique has been introduced, the small vessel arteriograph, which is also known as the perfusion myograph (Halpern & Kelley, 1991). It is this technique, the perfusion myograph, which has been used throughout this thesis in order to study any changes in the endothelin receptor population in resistance arteries before, during and after the onset of congestive heart failure in rats, and in patients with established heart failure as compared to controls.

C. 1.1. Source of tissue

C. 1.1.1. *Rat mesenteric vessels*

Male Wistar rats were used from an in-house stock bred at the Biomedical Research Facility (Western General Hospital, Edinburgh) and maintained on standard chow and tap water *ad libitum*. The animals (10-16 weeks old) were killed by exsanguination and a ventral midline incision was made. The mesenteric bed was immediately excised and pinned out in a silicone-coated (Sylgard, Dow-Corning, U.K.) dissecting dish containing Krebs-Henseleit solution (mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 5.5 glucose; BDH-Merck, Dorset, U.K.) at room temperature (22-24°C).

For resistance arteries of internal diameter 200 - 350 μm, third order branches of the mesenteric artery, i.e. those appearing after the 3rd branch of the superior mesenteric artery (Figure 2.1), were excised under a dissection microscope (Zeiss, U.K.) using No.5 watchmaker forceps and fine ocular scissors (Altomed Ltd, Tyne & Wear, U.K.). In order to avoid touching the section of vessel to be studied, the surrounding fat was gently pulled away exposing the thin membrane running between the fat and the artery. This membrane was cut, therefore clearing the fat from the artery. The artery was removed, but a small area of fat was left intact in order to mark the proximal end of the vessel. This was so the ends of the vessel could be differentiated in order to mount the artery in the correct orientation (proximal to distal) in the myograph, thus any flow through the lumen would be in the same direction as that of blood *in vivo*.

Mesenteric arteries were studied for several reasons. The mesenteric bed receives a relatively high proportion of cardiac output (~10%) and so any change in resistance in this vascular bed can affect the total peripheral resistance and blood pressure profoundly. Due to the structure of the mesenteric tree (Figure 2.1.), 3rd order arteries, which are usually between 200-350 μm, are easy to identify and dissect. Also there are many branches (which consistute the 1st order arteries) off the superior mesenteric artery, hence many preparations can be obtained from one bed, thus reducing the number of animals sacrificed.

C. 1.1.2. *Mesenteric vessels from rat congestive heart failure model*

Male Wistar rats were used from an in-house stock bred at the Department of Pharmacology Animal Unit (George Square, Edinburgh) and maintained on standard chow and tap water *ad libitum*. The animals had surgery performed on them at 5 weeks of age. The animal was anaethetised with sodium pentobarbitol (60 mg/kg Sagital), and when fully unconscious placed on a small animal respirator to aid breathing (60 breaths/min). An incision down the chest of the animal exposed the ribs, where upon the ribs were opened and the heart exteriorised. A ligature was tied round the proximal left coronary artery, in rats selected for heart failure, or pulled out through the heart muscle in the sham-operated control animals. The chest was closed, and animals allowed to recover (Selye *et al.*, 1960; Pfeffer *et al.*, 1979). Surviving rats

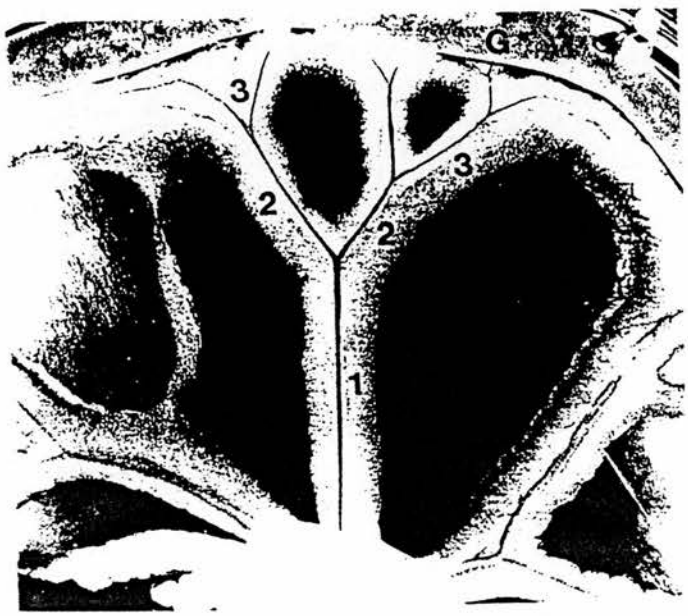


Figure 2.1. Photograph of an excised rat mesenteric bed pinned out on a silicone-coated dissecting dish, showing the location of 1st (1), 2nd (2) and 3rd (3) order mesenteric arteries. G = gut wall. (x3 magnification).

were divided into groups of 5 and 12 weeks post-ligation, whereupon the animals were sacrificied and studies performed on the vessels. Heart failure was verified by placement of a pressure transducer tipped catheter (Millar, U.K.) in the left ventricle for the measurement of left ventricular end diastolic pressure (LVEDP). Rats with LVEDP > 15 mmHg were considered to have CHF. After sacrifice, the mesenteric bed was removed and treated as descibed above. In addition to the vessels removed for functional myography studies, several other 3rd order branches were dissected and fixed in 10% formalin for 36 hours for immunohistochemical staining. However, for this dissection the fat was left intact around the artery (and vein) to protect it as much as possible from handling damage.

All surgical and *in vivo* procedures were performed by Dr Gillian Gray, Department of Pharmacology, George Square.

C. 1.1.3. *Human vessels from gluteal buttock biopsies*

The protocol of this study was approved by the Lothian Research Ethics Committe. Written, witnessed, informed consent was obtained from each subject. All subjects were asked to abstain from their usual course of therapy 24 hours before the procedure and from caffeine-containing drinks or alcohol 12 hours before the biopsy was taken. A 20 ml venous blood sample was taken from each subject for measurements of ET-1 and big ET-1. The blood samples were immediately stored on ice until centrifugation at 4°C, 3 000 g for 20 minutes, the plasma separated into tubes and stored at -80°C until assay. A further 10 ml blood sample was taken for basic haematology and clinical chemistry tests (glucose, creatinine).

Skin biopsies, approximately 2cm long, 0.75 cm wide and 0.75 cm deep, were removed from the gluteal region of the left buttock under local anaesthetic (1% lignocaine, Astra Pharmaceuticals Ltd., U.K.) by Mrs Fiona Strachan or Dr Charlie Ferro at the Clinical Pharmacology Unit and Research Centre, Western General Hospital. Upon excision, the biopsy was immediately placed in cold Krebs-Henseleit solution. Small arteries were carefully dissected from the biopsy, in a manner similar to that described above for rat mesenteric blood vessels. However, it was not possible

to know which direction the blood passed through the lumen. The biopsy usually yielded more than one vessel. If there were two or more arteries, one was mounted immediately in the myograph and the second stored in the refrigerator overnight for experimentation the next day. To avoid any influence of overnight storage on the results, the order of experiments was randomised. Any surplus vessels were snap frozen in isopentane (BDH-Merck) prechilled in solid CO_2 and stored at -80°C until immunohistochemical staining was performed (see Section C. 2.).

C. 1.2. Perfusion myography

As mentioned earlier, two different techniques have been described which allow the study of isolated resistance arteries, i.e. the wire myograph and the perfusion myograph. When a vessel is mounted in the wire myograph the wires pull at the vessel walls pulling it into two flat planes. The perfusion myograph however, involves the cannulation of both ends of a length of vessel, pressure is applied intraluminally (by infusing a physiological solution) allowing the blood vessel to assume its natural cylindrical shape (Figure 2.2).

There are several advantages of the perfusion myograph over the wire myograph (Halpern & Kelley, 1991; Schiffrin, 1995), allowing the vessels to be studied under physiological pressurised conditions, these include:

a). Allowing the diameter of the vessel to change when contracting and relaxing

b). An equal transmural pressure across the vessel wall, whereas in the wire myograph the wall is subject to distension from two ends resulting in unequal stretch and pressure

c). The pressurised vessel is able to assume a more physiological shape

d). The endothelium is untouched and the rest of the vessel wall is less likely to be damaged, whereas wires cause local damage to the endothelium

e). The axial length can be set to compensate for the extension or retraction of arteries after dissection and mounting

f). Agonists and antagonists can be perfused luminally or superfused

g). Allows myogenic and spontaneous tone to develop, which is rarely seen in vessels mounted in the wire myograph

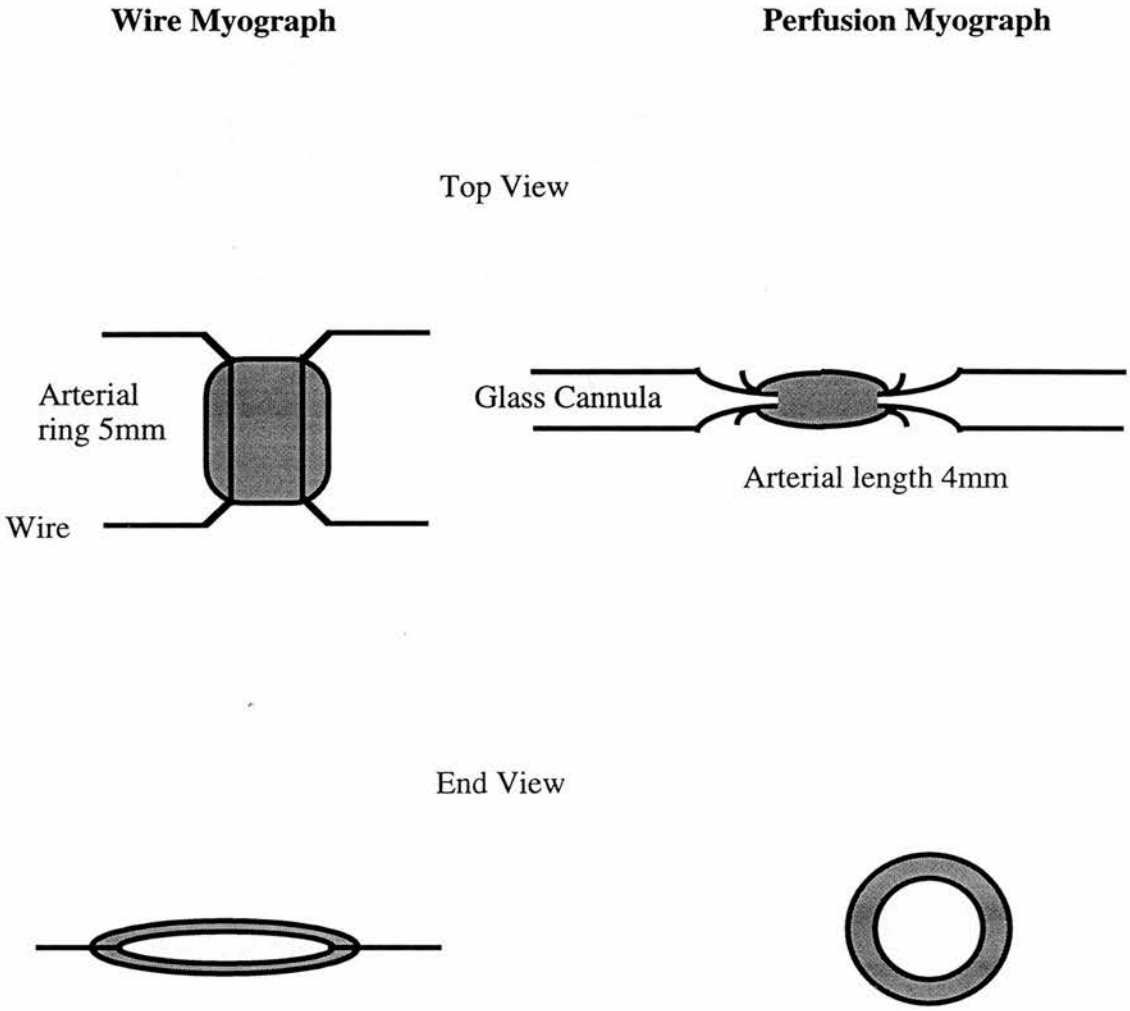


Figure 2.2. A schematic diagram comparing wire and perfusion myography. The views from above and side demonstrate the shapes of the blood vessel segments once mounted. As shown, the wire myograph pulls the vessel flat, whereas the perfusion myograph cannulation forms a cylindrical shape.

The sensitivity of the vessels to agonists has also been demonstrated to vary between the two methods. For example, it has been shown that vessels mounted in the pressure myograph are significantly more responsive to α-adrenoceptor agonists than those mounted in the wire myograph (Buus *et al.*, 1994; Falloon *et al.*, 1995). This implies that responses found using the pressure myograph may be of more relevance to an *in vivo* situation. Some results described in this thesis also demonstrate a similar finding, see Section 2.4.

The perfusion myograph does have its disadvantages. In the pressurised system a length of artery (usually 3 - 4 mm) is needed which is intact, without holes or branches in order to maintain the pressure. However many small vessels, such as coronary and renal arteries, have small branches which can be difficult to see, even under the microscope, thus introducing holes, when isolated, into the vessel wall. In these situations it may be advantageous to study these vessels using the wire myograph (Schiffrin, 1995). However, because the perfusion myograph allows a closer approximation to *in vivo* conditions, the small vessel arteriograph is the technique employed throughout this thesis for the functional studies performed on the resistance arteries from rat and humans.

C. 1.2.1. *Mounting and pressurising of the vessels in the myograph*

After dissection, the arteries were transferred to the myograph vessel chamber (Living Systems Instrumentation, Burlington, Vermont, U.S.A) containing 10ml of Krebs-Henseleit solution. The resistance artery was then mounted onto the two fine glass cannulae (~ 100-150 μm tip diameter), and secured by single-fibre silk threads. The procedure of mounting the vessel was as follows. The proximal end of the vessel was gently pulled onto the cannula tip until approximately 200 μm of the tip was inserted into the lumen of the vessel and secured by two silk threads, which had already been looped onto the cannula. Any blood present in the lumen of the artery was removed by opening the stopcock to the proximal cannula and infusing a slow flow of Krebs-Henseleit solution through the lumen by means of a miniature peristaltic pump (PS/200, Living Systems Instrumentation Inc., Burlington, Vermont, USA). Care

was taken not to allow the intra-luminal pressure to rise above 10 mmHg , in order to avoid damage to the endothelium and vessel wall. After the blood had been removed, the stopcock was closed and the distal end of the artery was tied onto the distal cannula in the same way as described for the proximal end.

An intraluminal pressure of 60 mmHg was reached by slowly introducing Krebs-Henseleit solution into the vessel lumen using the miniature peristaltic pump, connected to a pressure servo unit (Figure 2.3). This pressure was chosen because it has been estimated that vessels of this size would experience pressures approximately 50% of mean arterial pressure *in vivo* (Halpern & Kelley, 1991). As the pressure increased, the vessels usually developed a bend as a consequence of axial lengthening. These buckles were removed by gently retracting the proximal cannula, using the length transducer, to the original axial length prior to dissection, being careful not to introduce any axial stretch. The vessels were checked for leaks by changing the pressure servo unit from automatic to manual mode, this means that any change in pressure will not be compensated for by the pump infusing Krebs solution. If a drop in pressure occurred, either the ties were re-secured or the artery was discarded and another one mounted. Throughout the experiments, the pressure servo unit was kept on automatic and maintained at the set intraluminal pressure, with checks at random to make sure no leaks had appeared during the course of the experimental procedure.

The myograph was placed on an inverted stage microscope (Nikon TMS-F, Japan) which was connected to a monochrome television camera (Burle, USA), and the vessel visualised on a television monitor (Figure 2.3). The lumen diameter and wall thickness were measured using a video diamension analyser (Living Systems Instrumentation, Burlington, Vermont, USA) which had been calibrated against a stage micrometer (resolution = 1μm). The video dimension analyser senses changes in optical density of the vessel at a chosen scan line. The walls have a higher optical density than the rest of the vessel, appearing on the television screen as two thick bands, so continuous measurements of both wall thicknesses and lumen diameter can be made. However, smaller lumen diameters were measured by hand using a calibrated micrometer since the differences in the optical density at diameters of > 150

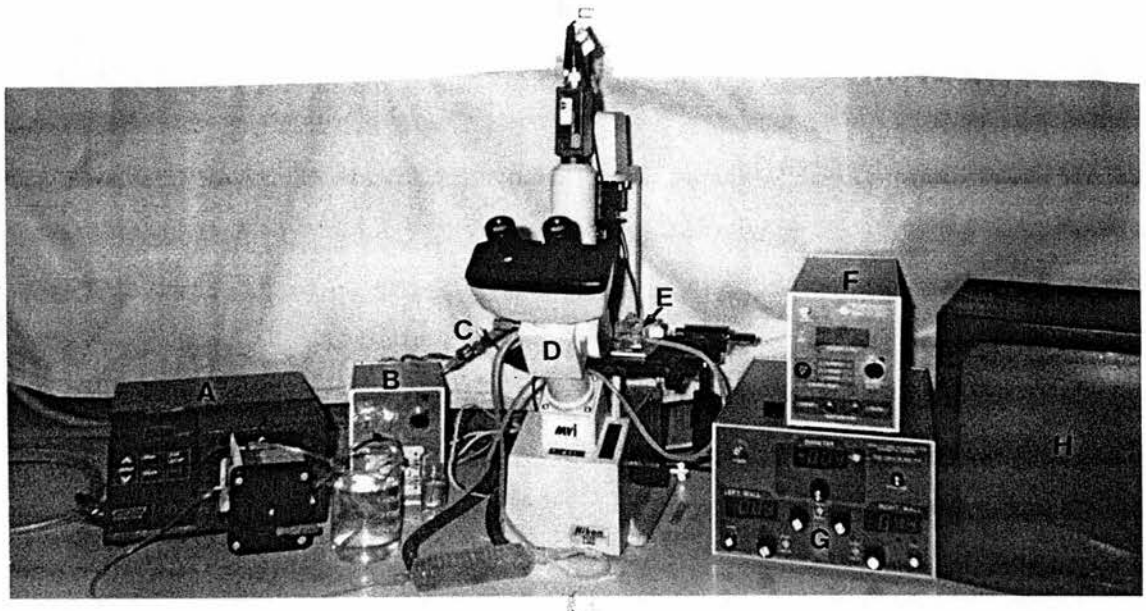


Figure 2.3. Photograph showing the components of the small vessel arteriograph (perfusion myograph): Watson-Marlowe pump (A); minature peristaltic pump (B); pressure transducer (C); inverted stage microscope with camera unit (D); vessel chamber (E); pressure servo unit (F); video dimension analyser (G); and television screen (H).

μm were not distinct enough for the optical dimension analyser to detect and was, therefore, unable to measure the distances between the vessel walls.

After mounting and pressurisation, the arteries were continuously superfused with Krebs-Henseleit solution which was gassed with 95% O_2 and 5% CO_2. The temperature of the Krebs-Henseleit solution was raised by passing through a glass-jacketed heating coil which was warmed with circulating water from a water bath (Grant Systems, UK) so the temperature in the myograph chamber was 37°C, as monitored by a digital thermometer (TM-903, Lutron Equiment, UK).

C. 1.2.2. *De-endothelialisation*

In larger arteries, the most common method for removing the endothelium is by mechanical disruption, usually by rubbing the luminal surface with a cotton bud. However, this technique is not applicable to small resistance arteries because of their size and fragility. Methods which have been employed to remove the endothelium have included perfusion with detergents such as 3-[(3-cholamidopropyl)dimethyl ammonio]-1-propane sulphonate (CHAPS) (Hiley *et al.*, 1987; Takase *et al.*, 1995) or sodium deoxycholate (Byfield *et al.*, 1986), dissolving the intracellular matrix with enzymes such as collagenase (Carvalho & Furchgott, 1981), rupturing endothelial cells osmotically with distilled water (Criscione *et al.*, 1984) or perfusion with 40 mM potassium chloride (Griffith *el al.*, 1985). These chemical and enzymatic techniques are difficult to control i.e. exposure time, shear rate and carry the risk of damaging the adjacent smooth muscle cells, and so any changes in vessel response cannot clearly be attributed to endothelial cell removal.

However, mechanical techniques have also been devised which denude resistance arteries of their endothelium. Osol *et al.*, (1989) demonstrated that the introduction of a single human hair into the vessel lumen was an effective method of denudation. An alternative technique of passing an air bubble through the lumen of vessel is possibly the most common method used in perfusion myography (Ralevic *et al.*, 1989; Bjorling *et al.*, 1992; Falloon *et al.*, 1993; Touyz *et al.*, 1995) and it is this procedure which was employed throughout this thesis.

C. 1.2.2.1. *Air bubble denudation technique*

The endothelium was removed by perfusion of an air bubble through the lumen of the mounted vessel, in a method similar to that described by Falloon *et al.*, (1993).

Firstly, the axial length of the vessel was noted by recording the setting on the length transducer, so the vessel could later be reset to its original length. The intraluminal pressure was slowly reduced manually, whilst the axial length was simultaneously re-adjusted to prevent any axial stretch on the artery. The distal stopcock of the chamber was opened and an air bubble approximately 2 centimetres in length was introduced into the tubing (Tygon: I.D. 1/32") feeding the proximal cannula at the proximal end. This was done by disconnecting the proximal end luer fitting and absorbing some of the Krebs-Henseleit solution in the tubing using a tissue, and re-inserting the luer fitting. The air bubble was infused slowly through the tubing, proximal cannula, artery, distal cannula, tubing and stopcock by switching the pressure-servo box to flow mode, at a flow rate which produced a pressure of 20-25 mmHg. This procedure resulted in a series of small air bubbles, usually between 10 and 15, being passed through the lumen of the vessel. Once the air bubbles had passed through, flow was maintained for a further 5 minutes to ensure all endothelial debris was washed away. The pressure-servo box was then switched back to pressure mode, the distal stopcock closed and the vessel restored to its original axial length and pressure.

In all experiments the removal of the endothelium was assessed by utilising the endothelium-dependent vasodilator, acetylcholine (ACh 10^{-6}M) after pre-contraction to the α_1-adrenoceptor agonist phenylephrine (PE, 10^{-5}M). This air bubble method of endothelial denudation has previously been demonstrated to completely disrupt and remove the endothelium using confocal, scanning and transmission electron microscopy (Smith, 1995) without causing damage to the underlying smooth muscle.

C. 1.2.3. *Reperfusion circuit*

In organ bath studies, cumulative concentration-response curves are performed by direct addition of increasing concentrations of drug to the bathing solution. The

temperature of the bathing solution is maintained at 37°C by the jacketed organ bath. However in the perfusion myograph, the chamber in which the vessel is mounted is not heated; the temperature of the Krebs-Henseleit solution is maintained by being circulated through a heating coil. This means that the concentration-response curve cannot be performed by direct addition to the chamber, since the superfusate would have to be switched off (the time required to complete an ET-1 response curve is approximately 40 minutes) resulting in a steady decline in chamber temperature. Thus a 'reperfusion circuit' technique was used for generation of a cumulative-response curve.

In all experiments, after the preliminary viability checks (see C. 1.2.1.), a reperfusion circuit was set up. This was a closed system with a total volume of 30 mls of Krebs-Henseleit solution being constantly superfused at a flow rate of 5 mls/min. It was to this reservoir of Krebs-Henseleit solution that the agonists and antagonists were applied, keeping the volume at 30 mls by removing one ml of Krebs and adding one ml of the drug in a stepwise fashion. Responses were recorded 5 minutes after addition of each agonist concentration.

C. 1.2.4. *General experimental protocol*

After mounting, the vessels were allowed to equilbrate for 60 minutes and then exposed to the general 'wake up' procedure described as follows. All vessels were then exposed to 60 mM potassium chloride (KCl, equimolar replacement of NaCl with KCl), by changing the superfusate from Krebs-Henseleit solution to 60 mM KCl solution. After the maximum constriction was reached (usually 3 minutes after the commencement of superfusion with 60 mM KCl solution), the superfusate was changed to Krebs-Henseleit solution for washout. Following a 10 minute washout, the superfusion circuit was stopped and phenylephrine (PE; 10^{-5}M; Sigma, Dorset, U.K.) added directly to the vessel chamber, to produce a contraction <35% of resting lumen diameter. After washout, the vessels were exposed to 60 mM KCl for a second time, to ensure maximum constriction had been reached, all constrictions were compared to this KCl response. The presence of endothelium was confirmed by pre-

constricting with PE (10^{-5}M) and subsequent addition of acetylcholine (ACh; 10^{-6}M) directly to the vessel chamber.

In all experiments the endothelium was removed by the passing of an air bubble (see above), and confirmed functionally by the loss of ACh-induced relaxation during PE-induced constriction. Following denudation, the vessels were exposed to 60 mM KCl for a third time to check that the smooth muscle had not been damaged.

This initial viability check and endothelial removal protocol was used in all functional experiments throughout the thesis. In all the subsequent studies undertaken, the experimental procedure is described in each individual chapter.

C. 1.3. Validation study:

A comparison of techniques; wire myography vs perfusion myography for demonstration of constrictor ET_B receptors

The wire myograph differs from the perfusion myograph by the vessel being mounted as a ring preparation, as opposed to an even cylindrical shaped preparation. The response of the vessel to agonists in the wire myograph is recorded by isometric force exerted onto the wires, whereas the responses in the perfusion myograph are measured as differences in lumen diameter. This study was designed to compare the two methodologies in the detection of constrictor ET_B receptors in the mesenteric artery from the normotensive rat.

C 1.3.1. *Wire myograph*

Third order rat mesenteric small arteries were dissected as described above (section C.1.1.1.) and mounted in a wire myograph (Mulvany & Halpern, 1976). Segments ~2 mm in length were mounted, using a light microscope, in the wire myograph by carefully feeding the two fine wires through the lumen of the vessel. Once mounted, the vessels were incubated in Krebs-Henseleit solution, warmed to 37°C and allowed to equilibrate for 30 minutes. During this period, to ensure endothelial cell removal, a third wire was passed through the lumen and gently agitated to carefully rub the endothelium off without damaging the smooth muscle layer. After this initial

equilibration period, a set tension of 200mg had been applied and which was maintained throughout the whole experiment, being re-adjusted if the tension dropped below 200mg.

Following a further equilibration period of 30 minutes after tension had been placed on the vessels, the vessels were exposed to the same preliminary checks as the vessels mounted in the perfusion myograph as described above. As for the perfusion mounted vessels, denudation was confirmed by the lack of relaxation to ACh following PE pre-constriction.

All experiments in the wire myograph were performed by Mr Philip Swan, Vascular Laboratory, Department of Medicine, University of Edinburgh, Western General Hospital, Edinburgh.

C. 1.3.2. *Perfusion myograph*

The vessels were dissected, mounted and pressurised as descibed in sections C. 1.1.1. and C. 1.2.1., and the prelimary checks and de-endothelisation procedures carried out (sections C. 1.2.1. & C. 1.2.2.).

C. 1.3.3. *Experimental protocol for comparison of detection of constrictor ET_B receptors in both wire and perfusion myography*

Following confirmation of endothelial denudation and smooth muscle integrity, concentration-response curves to SRTX S6c (10^{-12}-10^{-7}M) were constructed on vessels in both types of myograph. The effects of partial pre-constriction to the thromboxane A_2 mimetic, U46619 ($3x10^{-8}$M) on SRTX S6c concentration-response curves was investigated to demonstrate whether partial tone could unmask a SRTX S6c response.

In the perfusion myograph, a reperfusion circuit was set up 30 minutes before starting the SRTX S6c curve in the absence or presence of U46619. The SRTX S6c concentration-response curve was then performed in the manner described in section C. 1.2.3.

In the wire myograph the cumulative concentration-response curve was performed by draining the chamber and adding pre-heated Krebs-Henseleit solution (in a water bath to 37°C), with or without U46619, to the vessel. The concentration of SRTX S6c was then added directly to the chamber. In the experiments with U46619, following the first exposure to U46619 the constriction was extremely stable, and between changes of Krebs-Henseleit solution remained constricted. Once the constriction to U46619 was established the SRTX S6c CRC was constructed. All the responses on the wire myograph were recorded on a MacLab system (MacLab, Australia).

C. 1.3.4. *Results of validation experiments*

All responses were calculated as a percentage of the maximum constriction obtained with the second exposure to 60mM KCl solution (see section C.4.0.).

SRTX S6c did not induce any constrictions in the rat mesenteric arteries when mounted in the wire myograph (n=7). However, a pre-constriction with U46619 resulted in a concentration-dependent response to SRTX S6c (n=7; Figure 2.4.A.).

When mounted in the perfusion myograph, in the absence of pre-constriction, concentration-dependent responses to SRTX S6c were seen in 9 out of the 17 vessels (Figure 2.5) studied. A vessel was classified as being a responding artery if SRTX S6c produced a constriction of > 4% of KCl, this was because electical interference, such as the water bath switching on/off, could affect the picture of the vessel on the TV screen, slightly altering the optical density. This meant that the walls of the artery could be alittle darker than when originally measured, therefore appearing to reduce lumen size, usually by between 5-10 μm which generally equated to approximately 4% of a KCl constriction. In the presence of U46619, there was no potentiation of the constrictions elicted by SRTX S6c, although all vessels when pre-contracted, did constrict to SRTX S6c (n=7; Figure 2.4.B.).

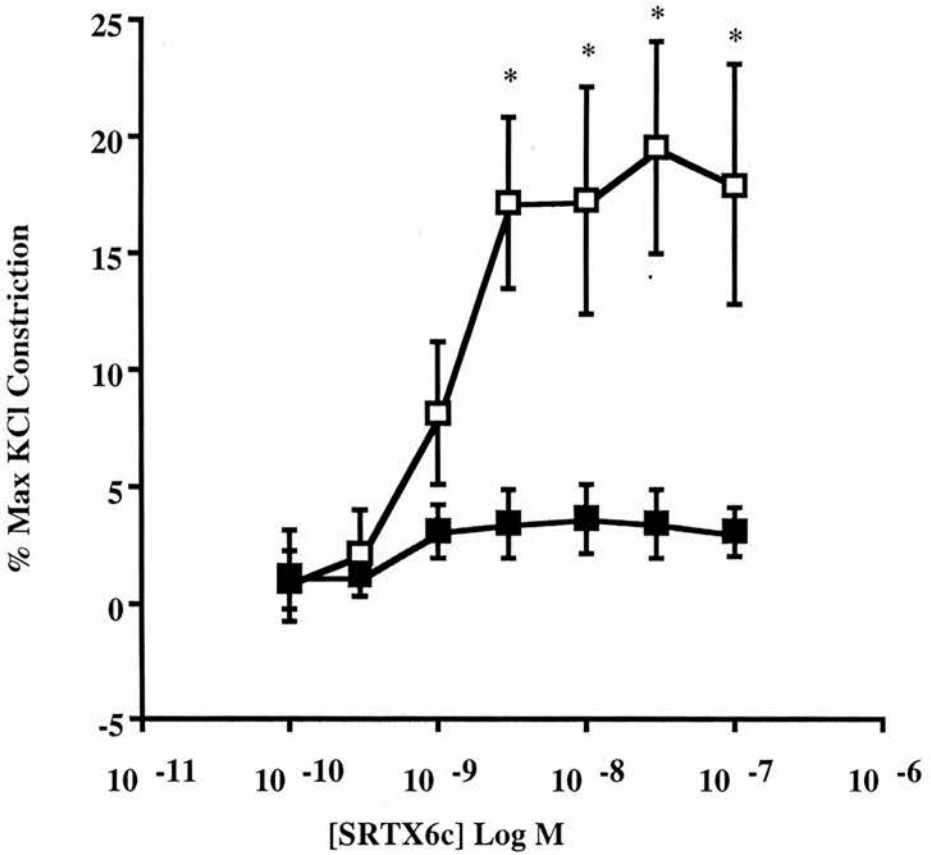


Figure 2.5.A. Wire Myograph

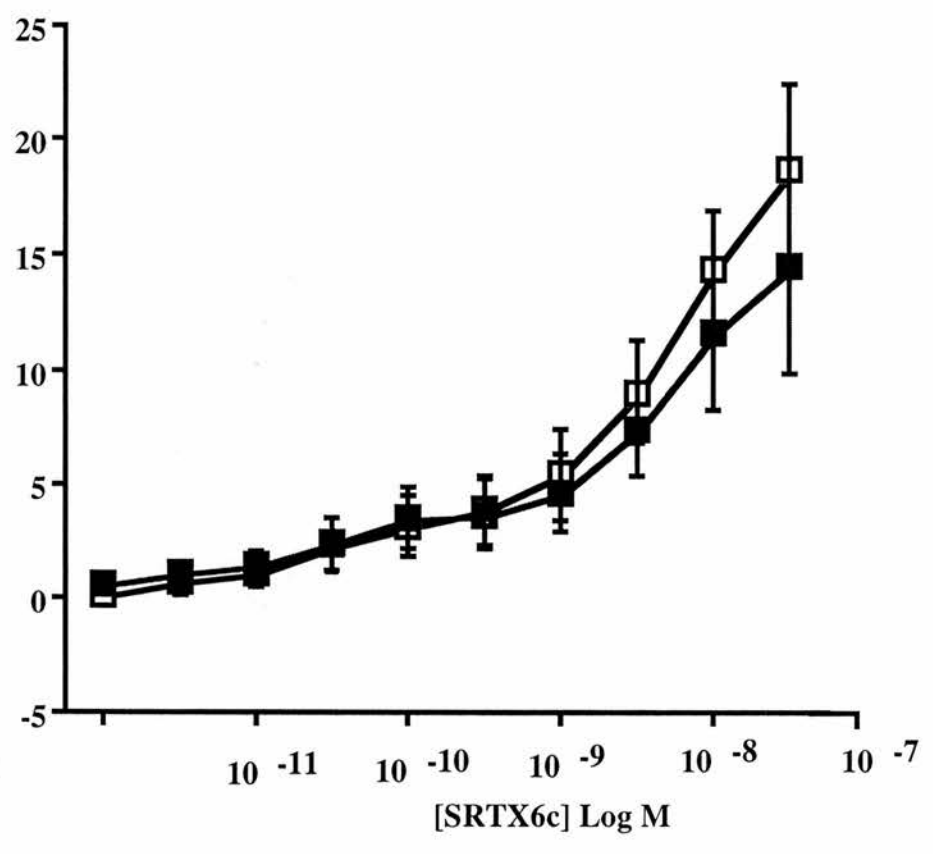


Figure 2.5.B. Perfusion Myograph

Figures 2.5.A. & 2.5.B. The effects of pre-constriction with U46619 on the constrictor actions of SRTX S6c on rat small mesenteric arteries when mounted in either a wire myograph (2.5.A.) or perfusion myograph (2.5.B.). In the wire myograph, constrictions to SRTX S6c (■) were only seen after the artery had been partially pre-constricted with U46619 (□ ,* P>0.05, Students t-test). However, U46619 had no potentiating effect on the constrictions induced by SRTX S6c when mounted in the perfusion myograph.

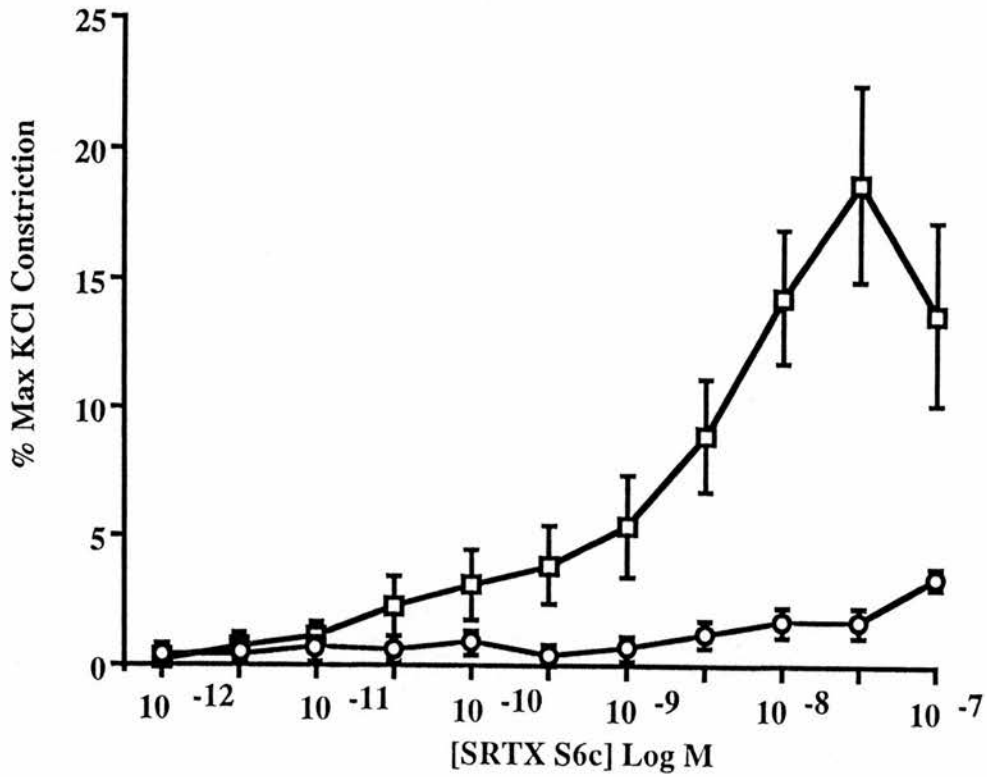


Figure 2.6. The effects of SRTX S6c on rat small mesenteric arteries when mounted in a perfusion myograph. SRTX S6c induced constrictions in 9 out of the 17 rat arterial segments mounted. The graph shows the amount of constriction in the 9 responding arteries (□), EC_{50} = 3± 1x10^{-9}M, E_{max}= 19±4%) compared to the non-responding arteries (○), which were classified on the basis that the artery did not constrict above 4% of KCl-induced constriction.

C. 1.3.5. *Discussion of comparison of wire vs perfusion myography*

These results suggest that the rat mesenteric small arteries contain a population of constrictor ET_B receptors. These receptors could not be detected in the wire myograph without an initial pre-constriction by the thromboxane A_2 analogue, U46619. This pre-constriction showed that SRTX S6c could induce up to 20% of the maximum constriction seen to 60 mM KCl solution. In the perfusion myograph, SRTX S6c caused constrictions in 53% of all arteries studied, when no prior tone had been induced by U46619. Pre-constriction resulted in all vessels responding to SRTX S6c. However, the constrictions were not potentiated. Similar maximum responses to SRTX S6c were seen in vessels mounted in the perfusion myograph, whether they had been pre-constricted or not, as compared with the arteries mounted and pre-constricted in the wire myograph. It is known that arteries mounted in the perfusion myograph can develop intrinsic tone (Falloon *et* al., 1993). Thus, the variability of responses to SRTX S6c when mounted in the perfusion myograph could be explained by the development of tone in those arteries which did respond. However, intrinsic tone does not appear to develop in the wire myograph technique, hence the ineffectiveness of SRTX S6c to produce a response without the aid of exogenously administered tone via U46619. This would also explain why all the pre-constricted (by U46619), perfusion myograph mounted arteries responded to the ET_B receptor agonist.

Thus, because the perfusion myograph demonstrated the presence of constrictor ET_B receptors more reliably than the alternative wire technique, this was the methodology chosen for all functional studies in this thesis .

C. 2.0. Immunohistochemistal localisation of the endothelin system

Immunohistochemical staining techniques have been described as "another special stain" (Boenisch, 1989) like that of the classic histochemical stains such as eosin and haematoxylin. However, what is special about immunohistochemistry is that the antibody (Ab) is the pivotal reagent used in the methodology. This means that this technique can be used to detect anything which is antigenic in nature, producing highly specific results. Because ET-1 is a peptide, both monoclonal and polyclonal Abs can be raised against it. Polyclonal Abs to ET-1 have been successfully used for measuring plasma ET-1 levels in radioimmunoassays (RIA; See C. 3.0., Cernacek & Stewart, 1989; Cody *et al.*, 1992). However, for immunohistochemistry the monoclonal Ab to ET-1 is favoured, because it is more specific than the polyclonal antiET-1.

ET-1 is secreted from cells and can then act in a autocrine or paracrine fashion. Thus, visualising the mature peptide via antiET-1 does not show whether there is an induction or upregulation of the production of ET-1 in cells that would not synthesise ET-1 under normal conditions. However, it was hypothesised that there may be an alteration in the localisation of ECE, perhaps an upregulation of ECE in the vascular smooth muscle cells. In order to see if there is altered production in CHF, two monoclonal Abs against ECE have been used. These Abs were a gift from Dr K. Tanzawa and colleagues (Sankyo Co. Ltd, Tokyo, Japan) who raised them against purified rat lung ECE. AEC 27-121 (ECE 27) is highly specific to ECE particularly rat ECE, whereas AEC 32-236 (ECE 32) recognises ECE from various species including rat and human and is not as specific as ECE 27 (Takahashi *et al.*, 1995).

Furthermore, to identify the particular area and cells in which the ET-1 and ECE immunoreactivity is found, other monoclonal Abs have been used. These Abs are directed against specific targets characteristic of different cell types and are then used on adjacent tissue sections. Due to the simple structure of blood vessels, only the endothelial and smooth muscle layers need to be visualised. An Ab raised against smooth muscle α-actin has been used to detect the smooth muscle cell layer. For the endothelial cells, *Ulex europaeus* agglutinin 1 (UEA 1), which is a glyocoprotein

isolated from *Ulex europaeus* (Furze gorse) seeds was used. UEA 1 has been established as excellent marker for endothelial cells, which cross-reacts with a specific lectin present on the endothelial cell surface (Roussel & Dalion, 1988). The UEA-1 used was previously biotinylated, hence, a secondary biotinylated Ab directed against the UEA-1 molecule was not needed. Although UEA-1 is not an Ab *per se*, and is being used as an alternative to a primary Ab, for the simplicity of the text it is referred to as a primary Ab.

C. 2.1. *ABC Peroxidase Method*

The method of immunohistochemistry chosen for this thesis was the three-step indirect method. In this technique, the unconjugated primary Ab binds to the antigen (e.g. ET-1). A biotinylated-labelled secondary Ab directed against the primary Ab (which is now the antigen) is then applied, followed by the avidin biotinylated enzyme complex (ABC) conjugated to peroxidase. Finally the chromogen, 3,3'-diaminobenzidine tetrahydrochloride (DAB) is added as the substrate to the peroxidase, producing a brown, highly insoluble precipitate (Figure 2.6; Hsu *et al.*, 1981a, b). It is a highly sensitive technique, being more sensitive than a one Ab, direct method, because the secondary Ab usually binds to several different epitopes on the primary Ab, consequently attaching more enzyme molecules at the site of antigen.

Use of the ABC peroxidase method also increases sensitivity because of the unique, high affinity ($10^{15}M^{-1}$) of avidin, a 68 000 molecular weight protein, for the vitamin biotin. In fact, the affinity of avidin for biotin is over a million times greater than the affinity of antibodies for most antigens, and is essentially irreversible. As well as this extraordinary affinity, the ABC method has two other properties to enhance sensitivity. Firstly, avidin has four binding sites for biotin, and secondly the enzyme, peroxidase (and all other enzymes) can be conjugated to several molecules of biotin (Hsu *et al.*, 1981a, b). Thus, due to the multiple linkages, macromolecular complexes can be formed between avidin and biotinylated enzymes. The exact structures of these complexes are not fully known, but it is believed that there are three dimensional crosslinkages between many enzyme and avidin molecules. These complexes have

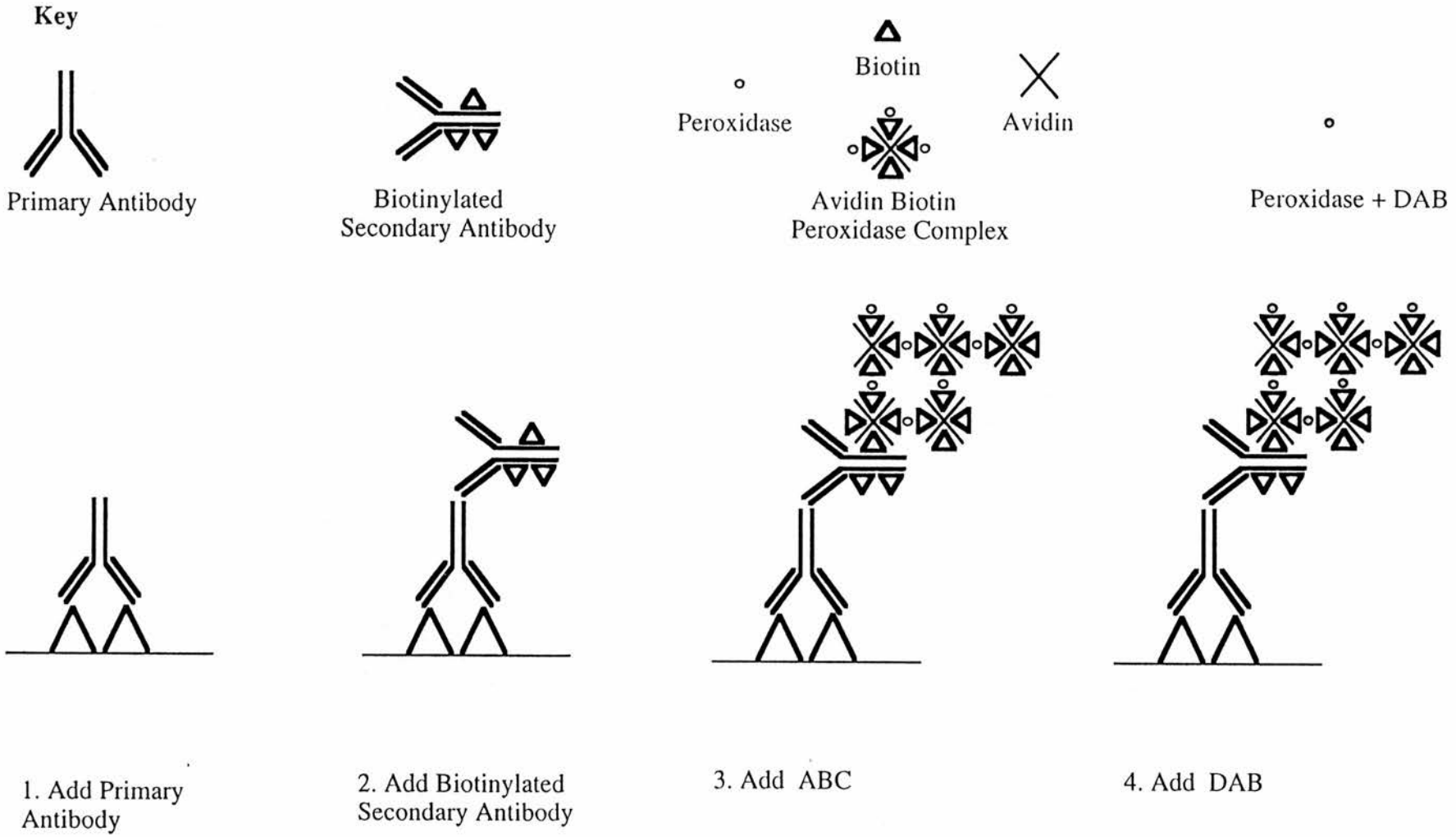


Figure 2.7. A simplified diagram showing the three-step indirect Avidin/Biotinylated Enzyme Complex (ABC) immunohistochemical technique. Once the tissue has been prepared and the endogenous peroxidase blocked (see text for details), the primary antibody is incubated for 60 minutes.A secondary biotinylated antibody is added, followed by the preformed ABC . To visualise the sites of binding, the chromagen,Diaminobenzidine Tetrahydrochlorine (DAB) is added, which produces an insoluble, brown precipitate.

few exposed biotin residues, but have one or more available biotin binding sites for potential crosslinking with the biotinylated secondary Ab (Beltz & Burd, 1989).

C. 2.2. *Tissue Fixation and Embedding*

The majority of published studies using immunohistochemical techniques localising ET-1 have utilised the ABC peroxidase method (Giaid *et al.*, 1989; Giaid *et al.*, 1991; Li *et al.*, 1994; Giaid *et al.*, 1995; Timm *et al.*, 1995). However, in many of these studies (Giaid *et al.*, 1989; Giaid *et al.*, 1991; Timm *et al.*, 1995), the immunohistochemical procedure was performed on fixed, frozen tissues, cryostated to between 5 - 10mm and thaw-mounted onto poly(L-lysine)-coated glass slides. For this thesis it was decided to perform the technique on 4% paraformaldehyde fixed, paraffin-embedded tissue (Li *et al.*, 1994; Giaid *et al.*, 1995), since the tissues can be cut into 3mm sections, resulting in easier handling, finer focussing and better histology.

The tissues were initially isolated, and snap frozen in isopentane (BDH-Merck) which had been chilled by solid CO_2 and stored at -80°C until required for fixation. The vessels were fixed for 24 hours in 4% paraformaldehyde at room temperature. Following fixation, the tissues were infiltrated with paraffin-wax using the paraffin-wax processing machine (Pathology Department, Western General Hospital) on an overnight cycle and mounted into wax blocks the next day.

Formaldehyde fixation preserves the tissues by forming 'methylene bridges' with basic amino acids in the tissue. This results in conservation of the intracytoplasmic proteins and low permeability to macromolecules. However, formaldehyde fixation can result in low exposure of antigens, due to decreased penetrating ability of the Ab to the antigen. However, this can be overcome by proteolytic digestion. Trypsin has been used in this thesis to counteract any potential masking of antigens to the Abs, which cleaves adjacent to lysine and arginine, the two amino acids most likely to react with formaldehyde (Beltz & Burd, 1989).

Following the embedding procedure, tissues were cut (3μm thick) using a microtome (Pharmacology Department, George Square) and a ribbon of sections floated on a water bath (R.A. Lamb, London, U.K.) at 35°C, and left to flatten for 20 minutes before being mounted onto glass slides (BDH-Merck).

C. 2.2.1. *Immunohistochemical Experimental Procedure*

Sections were dewaxed by immersing in xylene (BDH-Merck) for 10 minutes twice, and rehydrated by rinsing in absolute alcohol for 5 minutes twice and 90 % methanol for 5 minutes and washing in distilled water. Endogenous peroxide production was suppressed by incubating the tissues in methanolic H_2O_2 (1 part 3 % H_2O_2 in 4 parts absolute methanol; BDH-Merck) for 10 minutes and washing in distilled water.

Tissues were then trypsinised. Sections were brought up to 37°C by immersing in a prewarmed coplin jar filled with distilled water. The sections were transferred to a coplin jar containing 0.1 % trypsin (Sigma) in 0.05 M Tris buffer solution (TBS; see section 2.2.4.1; pH 7.8) at 37°C and incubated for 2 minutes. All tissues were washed for 30 minutes in TBS + 0.1 % $CaCl_2$, pH 7.8.

After washing, sections were ringed and isolated on the slide by drawing round the tissues with a paraffin-wax pen (Dako Ltd, Buckinghamshire). This is to reduce the volume of antibody needed to be added to the tissues. A solution of blocker was applied to sections for 30 minutes. The blocker contained 1% normal horse serum (Vector Laboratories, Peterborough) in phosphate buffer solution (PBS; see section 2.2.4.1. for solutions recipes). This step is included in order to reduce background staining due to hydrophobic interactions of the primary antibody with irrelevant proteins.

Following blocking, the primary antibodies were applied and the tissues incubated overnight at 4°C. The primary antibodies used were;

a). mouse Anti ET-1, IgG isotype (ams Biotechnology, Witney, Oxon), working dilution 1/500.

b). mouse Anti Smooth Muscle Actin, IgG isotype (Novocastra Labs Ltd, Newcastle upon Tyne), working dilution 1/50.

c). mouse Anti ECE, AEC 27-121, IgG isotype (donated by Dr M Takahashi, Sankyo Co. Ltd, Tokyo, Japan), working dilution 1/10.

d). mouse Anti ECE, AEC 32-236, IgG isotype (donated by Dr M Takahashi, Sankyo Co. Ltd, Tokyo, Japan), working dilution 1/20.

e). Biotinylated Ulex europaeus agglutinin 1 (Vector Labs, Peterborough), working dilution 1/100. This Ab was previously biotinylated, and did not require addition of the secondary biotinylated Ab.

All the primary Abs were diluted in 1% normal horse serum in PBS. The negative control used the 1% normal horse serum in PBS alone.

Following primary antibody incubation, all sections, except the sections being stained for endothelial cells, were washed in PBS for 10 minutes and the secondary anti mouse biotinylated IgG, diluted 1/200 in 1% normal horse serum PBS, was applied, and incubated for 60 minutes at room temperature. The sections for endothelial cell staining were washed and retained in PBS, until addition of the ABC complex. Tissues were rinsed of excess secondary antibody by washing in PBS for 10 minutes and incubated with ABC complex (see section 2.2.4.1.; Vector Stain ABC kit, Vector Labs, Peterborough) at room temperature for 30 mins. After washing in PBS for 10 minutes, sections were exposed to DAB solution (Sigma) for 5 minutes. After a PBS wash, the tissues were counterstained using haematoxylin (BDH-Merck) for 3 minutes and rinsed in tap water. All sections were dehydrated through alcohols (70%, 80%, 90%, 100%), cleared in xylene (to remove paraffin pen markings) and coverslipped using DPx mountant (BDH-Merck).

The results were viewed under a light microscope (Nikon) and photographs taken.

C. 2.2.2.1. *Solutions*

Phosphate Buffer Solution (PBS) used for washes

1). 63.5g of disodium hydrogen orthophosphate (BDH-Merck) was dissolved in 400 mls of deionised water by microwaving on full power for 5 minutes.

2). 8.5g of sodium dihydrogen orthophosphate (BDH-Merck) was dissolved in 400 mls of deionised water.

3). The two solutions were mixed and volume made up to 5 litres with deionised water.

4). The pH was adjusted to 7.6 using 4N HCl (BDH-Merck).

0.05M Tris buffer for trypsinisation

1). 6.04g of Tris (hydroxymethyl)-methylamine (BDH-Merck) was dissolved in 80 mls of deionised water.

2).2.77 ml of 4N HCl was added to Tris solution.

3). 8.1g of NaCl (BDH-Merck) was dissolved in 900 ml of deionised water.

4). The two solutions were mixed, pH adjusted to 7.8 using 4N HCl and the volume made up to 1 litre.

Vector ABC complex kit

1). To 5 mls PBS 2 drops of solution A was added.

2). To the PBS + solution A mix 2 drops of solution B was added.

3). The solution was left to stand for at least 30 minutes before use.

C. 2.3. Alkaline phosphatase staining

To validate the specificity of the ABC peroxidase immunohistochemical methodology, an alternative enzyme/substrate protocol was used in some tissues. Instead of biotinylated IgG, the secondary Ab used was IgG conjugated to alkaline phosphatase (AP-IgG; Dako Labs). The revealing substrate was New Fuschin (Dako Labs), which is converted to a pink precipitate. The protocol followed was exactly as for the ABC peroxidase technique, except that the AP-IgG, instead of biotinylated IgG, was incubated on the tissues for 60 minutes at room temperature. After washing, the tissues were exposed to the New Fuschin substrate for 15 minutes. The sections were

then washed in PBS and counterstained with Mayers haematoxylin for 3 minutes. The new fuschin substrate is soluble in alcohol, unlike the sections stained with DAB, so the tissues were not dehydrated through alcohols, but immediately coverslipped using an aqueous based mountant, Faramount (Dako Labs).

C. 3.0. Radioimmunoassay of ET-1 and bigET-1 from human plasma

Introduction

Radioimmunoassay (RIA), is a well validated technique used to measure any antigenic component present in a liquid sample. Similar to immunohistochemistry, this technique utilises specific Abs raised against the antigenic substance in question, followed by the addition of radiolabelled antigen (Ag). The radiolabelled, precipitated Ag/Ab complexes are then measured in a gamma counter. Therefore, the higher the concentrations of endogenous antigen, the lower the amount of bound radiolabelled antigen and the subsequent radiation counts. For this thesis, both ET-1 and big ET-1 levels were measured in the plasma samples from the human CHF patients and control subjects to ascertain whether there was increased circulating production of the peptide. Increased circulating levels of ET-1 have been demonstrated in many studies (see Section B. 1.6.1; Table 1.3). However, it is controversial whether these raised levels are ET-1 or the precursor big ET-1 (Pacher *et al.*; Wei *et al.*, 1994). It is important to delineate between the two forms of the peptide, since it may help in demonstrating whether there is an up-regulation of the production of ET, or reduced clearance and elimination of the mature peptide in CHF. In order to answer this question, primary, polyclonal antibodies raised in rabbits against human ET-1 and big ET-1 (Peninsula Labs, USA) were used. The ET-1 Ab had a crossreactivity with big ET-1 of 10%, and the big ET-1 Ab had a crossreactivity with ET-1 of 8%.

C. 3.1. Sample collection

As described previously (Section A. 1.1.3.), two 10ml blood samples were collected in a tube containing Na^{+}EDTA (20mg; Sigma) and aprotinin (1000 KIH units; Sigma) and immediately placed on ice. The plasma was separated from the cellular components of blood by centrifugation at 4°C, 3000 rpm for 20 minutes. The cell free plasma was pipetted into a storage tube, and stored at -80°C until assay.

C.3.2. RIA technique

There are two main components to the measurement of ET-1 or big ET-1 from the plasma samples in the RIA technique. These are, the extraction of either ET-1 or big ET-1 from the sample, and subsequent RIA of the extracts.

C. 3.2.1. *Extraction*

The ET-1 and big ET-1 had to be extracted from the plasma samples due to the extremely low circulating levels of these peptides. The peptides were extracted using Bond Elut Extraction Cartridges (Varian Sample Preparation Products, Switzerland). These cartridges, similar to the shaft of a syringe, are internally coated with a silica sorbent. The sorbent is a covalently bonded silica gel with functional groups which have polar characteristics to interact with any compounds also containing polar groups, such as ET-1 and big ET-1.

Prior to extraction of the sample, the extraction columns had to be activated to obtain the correct conditions for the extraction of either ET-1 or big ET-1. For ET-1 extraction, 3ml of 100% methanol (BDH -Merck) was added to the column and allowed to flow through. The columns were washed with deionised water, and then brought to pH 5.5, by the addition of 2ml of 10% acetic acid (BDH -Merck). After thawing, the plasma samples were acidified with 20% acetic acid (1:1 v/v; 2.5ml:2.5ml), added to the column for extraction and left to flow through by gravity. The column was washed with 2ml of 10% acetic acid, followed by 3ml of ethyl acetetate (BDH -Merck) to remove any unwanted proteins or lipids from the column. To elute the ET-1 fraction from the silica gel, collection tubes were placed under the extraction columns, and 1.5ml of elution buffer (80% methanol/20% 0.05M ammonium bicarbonate solution; BDH-Merck) was added to the columns (Rolinski *et al.*, 1994).

For the big ET-1 extraction, a slightly different protocol was followed due to the larger nature and slightly different properties of the 38 amino acid peptide. Instead of using acetic acid, the column was acidified with trifluoroacetic acid (TFA; BDH -Merck) in order to obtain pH 2. Furthermore, the elution buffer used was 80% methanol/20% TFA (Rolinski *et al.*, 1994).

Following the collection of the eluted ET-1 or bigET-1 extracts, the eluates were dried down under nitrogen (BOC) at 37°C. The dried elutes were reconstituted with 0.4ml

assay buffer (see Section C. 3.2.2.4.), and stored at -40°C until assay (generally the next day).

Previous validation studies in the laboratory, have demonstrated that the extraction/recovery rate is 89% for the ET-1 protocol and 63% for the big ET-1 methodology.

C. 3.2.2. *Radioimmunoassay protocol*

As described in Section B.1.6.1., the samples are incubated with a primary Ab, and the appropriate radiolabelled antigen. The resulting complexes are separated from the unbound label, the radioactivity measured and compared against standards of known concentrations.

C.3.2.2.1 *Standard curves*

The standard concentrations used in the ET-1 and big ET-1 RIAs were as follows;

The stock concentration of ET-1 (Peninsula Labs, USA) was 64 pg/ml, this was the highest standard (S_{64}) used. Serial 1:1 dilutions using assay buffer were performed, giving concentrations of 32 pg/ml (S_{32}), 16 pg/ml (S_{16}), 8 pg/ml (S_8), 4 pg/ml (S_4), 2 pg/ml (S_2) and 1 pg/ml (S_1).

The stock concentration of big ET-1 (Peninsula Labs) was 128 pg/ml (S_{128}), as for the ET-1 standard curve, serial 1:1 dilutions using assay buffer gave standards of S_{64}, S_{32}, S_{16}, S_8, S_4, S_2 and S_1.

Duplicates of 100µl of each sample and standard were assayed. Furthermore, 3 other control tubes were made up and assayed. These were the 'Blank', which contained only assay buffer solution, ^{125}I-ET-1 or bigET-1 and Amerlex (see Section C. 3.2.2.2.), a 'Reference' tube which contained assay buffer solution, primary Ab, ^{125}I-ET-1 or bigET-1 and Amerlex and the 'Total Counts' tube which consisted of assay buffer and ^{125}I-ET-1 or bigET-1.

C. 3.2.2.2. *ET-1 RIA protocol*

After thawing of the ET-1 extracts, 100µl of the primary anti-human ET-1 Ab (1: 20 000; Peninsula Labs) was added to 100µl of sample, standard and reference tube (but not the blank or total tubes), vortexed and left to incubate at room temperature for 4 hours. The ET-1 Ab had previously been diluted 1:20 in 0.1% Triton X (BDH-Merck) in assay buffer solution. Following incubation, 100µl of ^{125}I-ET-1, which is diluted to contain 6000 cpm, was added to the samples, standards, reference, blank and total counts tubes, vortexed and allowed to incubate overnight at 4°C.

In order to separate out the Ag/Ab complexes from the unbound ^{125}I-ET-1, 200µl of Amerlex (Amersham International, Buckinghamshire) was added to all tubes, except the total tube, vortexed and incubated for 30 minutes at room temperature. Amerlex is a separation reagent, which consists of magnetizable polymer beads coated with donkey, anti-rabbit IgG, which binds to the bound Ag/Ab complex. Ideally, the separation of the free and bound fractions are carried out using a magnetic separator, where the beads migrate to the base of the tube, taking with them any bound, labelled complex. However, a magnetic separator was not available at the time of assay, thus the samples were separated by centrifugation at 2500rpm for 20 minutes at 4°C. The unwanted supernatant containing any unbound ^{125}I-ET-1, as well as any other substances present in the sample, was aspirated off and the resulting pellets were counted for radioactivity in the Gamma counter (Wallac, Finland). The standard curves were plotted using the programme within the counter, and the levels of ET-1 present in each sample calculated from the standard curve.

C. 3.2.2.3. *Big ET-1 RIA protocol*

A similar methodology to the ET-1 RIA was performed for measurement of bigET-1 levels. However, 100µl of rabbit anti-human bigET-1 Ab (Peninsula Labs) was added to the relevant tubes and samples and left to incubate overnight at room temperature. 100µl of ^{125}I-bigET-1 was added on the second day of the assay. After overnight incubation at 4°C, 200µl of Amerlex was added to each tube (except the Total), and the rest of the protocol performed as for the ET-1 RIA.

C. 3.2.2.4. *Solutions*

RIA buffer

To 1 litre of deionised water the following compounds were added;

3.853g of sodium dihydrogen phosphate (BDH -Merck)

18.07g of disodiumhydrogen phosphate (BDH -Merck)

2.927g of sodium chloride (BDH -Merck)

1g bovine serum albumen (Sigma)

100mg sodium azide (BDH -Merck)

1ml Triton X-100 (BDH-Merck)

All RIAs performed were with the assistance and under the supervision of Mr Neil Johnston, Clinical Pharmacology Unit, Western General Hospital.

C. 4.0. Data analysis

For all experiments described in section C. 1.

a). the results are calculated as a percentage of maximum constriction obtained with the second exposure to 60 mM KCl Krebs-Henseleit solution and are expressed as mean $\pm$ s.e.mean

b). where a maximum response to the agonist was obtained, the negative log of the concentration causing half-maximal contraction (pD_2) was calculated by linear regression analysis, and compared using a Student unpaired two-tailed t-test

c). concentration-response curves were compared by one-way ANOVA followed by Fisher's least significant difference test.

d). Significance was taken at $P \leq 0.05$.

In section C. 3. all plasma levels of ET-1 and big ET-1 were compared by unpaired Students t-test, and significance taken at $P \leq 0.05$.

Results Chapter 1

Activation of endothelin ET_A receptors masks the constrictor role of endothelin ET_B receptors in rat isolated small mesenteric arteries

D. 1.1. Introduction

It is now well established that the vasoactive effects of the peptide endothelin-1 (ET-1) are mediated via both ET_A (Arai *et al.*, 1990) and ET_B receptors (Sakurai *et al.*, 1990) as discussed in section A. 1. 4. Administration of ET-1 to anaesthetised or conscious rats leads to a brief decrease, followed by a long lasting increase, in blood pressure (Yanagisawa *et al.*, 1988) that is accompanied by increased resistance in virtually all vascular beds studied (Gardiner *et al.*, 1994; Allcock *et al.*, 1995). Prior administration of an ET_A receptor antagonist, e.g. BQ-123 or FR 139317, enhances the initial depressor effect of ET-1 (an ET_B receptor mediated effect) and reduces the pressor effect (McMurdo *et al.*, 1993; Gardiner *et al.*, 1994). However, the pressor and regional constrictor effect of ET-1 is not fully inhibited by ET_A receptor antagonists, even with high doses, implying that ET_B receptors may also have a vasoconstrictor role (McMurdo *et al.*, 1993). Consistent with this possibility, the ET_B receptor selective agonist, sarafotoxin S6c (SRTX S6c) was found to produce vasoconstriction in pithed rats (Williams *et al.*, 1991; Clozel *et al.*, 1992).

In vitro experiments have also demonstrated ET_A receptor antagonist resistant responses to ET-1 (Ihara *et al.*, 1992; Sumner *et al.*, 1992; Fukuroda *et al.*, 1994b) and constrictions to SRTX S6c (Moreland *et al.*, 1992; Sumner *et al.*, 1992; La Douceur *et al.*, 1993; Gray *et al.*, 1994). As a consequence of these *in vitro* data, it has been suggested that constrictor ET_B receptors have a role only in large calibre vessels and in the venous circulation (Moreland *et al.*, 1992; Davenport & Maguire, 1995). However, in the conscious rat (Gardiner *et al.*, 1994) and the anaesthetised ganglion-blocked rat (Allcock *et al.*, 1995), ET-1 induced reduction of blood flow to the mesenteric resistance bed is partly resistant to ET_A receptor inhibition. Reduction of regional blood flow in response to SRTX S6c is also most marked in the mesenteric bed of the pithed rat (Clozel *et al.*, 1992). In humans, ET-1 constrictions in forearm blood vessels are also partly resistant to BQ-123 and constrictions to SRTX S6c can be seen (Haynes *et al.*, 1995; Strachan *et al.*, 1995). Thus, there may be an important

role for constrictor ET_B receptors in mediating vascular resistance and blood pressure. Indeed, the recently described non-peptide ET_B receptor antagonist, Ro 46-8443, produced a reduction in blood pressure in anaesthetised, normotensive rats (Clozel & Breu, 1996).

In contrast to the evidence for ET_B receptor mediated constriction of the rat mesenteric bed *in vivo*, *in vitro* studies of perfused mesenteric beds or isolated human and rat mesenteric arteries mounted in wire or perfusion myographs have led to the conclusion that constrictor ET_B receptors have little (Tschudi & Luscher, 1994; Tasake *et al.*, 1995; Deng *et al.*, 1995; Touyz *et al.*, 1995) or no role (D'Orleans-Juste *et al.*, 1993) in this vascular bed. All of these studies have based their conclusions on inhibition of ET-1-induced contraction by ET_A receptor antagonists, or responses to ET_B selective agonists. The aim of the present study was to further investigate the role of ET_B receptors in mediating constriction in pressurised rat mesenteric arteries using ET-1, the ET_A receptor antagonist, BQ-123 (Ihara *et al.*, 1992), the ET_B selective agonist SRTX S6c (Williams *et al.*, 1991), the ET_B receptor selective antagonist, BQ-788 (Ishikawa *et al.*, 1994) and the ET_A/ET_B antagonist, TAK-044 (Kikuchi *et al.*, 1994).

D. 1.2. Methods

Male Wistar rats (10-16 weeks old) were killed by exsanguination and the mesenteric bed immediately excised and placed into cold, oxygenated Krebs-Henseleit solution. Third order branches of the mesenteric artery (internal diameter 150 - 350 μm) were dissected (~3 mm length; section C.1.1.1.) and mounted between two glass microcannulae in the perfusion myograph chamber, as described in section C. 1.2.1. The vessel was constantly superfused with warmed (37°C), oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution. The intraluminal pressure of the vessel was raised to 60 mmHg and maintained at this pressure without further intraluminal perfusion. Luminal diameter was measured using a video dimension analyser and by hand, using a calibrated micrometer, when the optical dimension analyser was unable to detect

differences in optical density at smaller lumen diameters (i.e. constriction; C. 1.2.2.). After an equilibration period of 60 min, the initial "wake up" procedure was followed as described in section C. 1.2.4. Briefly, the vessels were exposed twice to modified Krebs-Henseleit solution containing 60mM KCl. The endothelium was removed by passing an air bubble through the lumen of the vessel (Falloon *et al.*, 1993; Smith, 1996; C.1.2.2.) and complete denudation was confirmed by addition of acetylcholine (ACh 10^{-6}M) to vessels pre-constricted with phenylephrine (PE 10^{-5}M). In all vessels, the relaxation induced by ACh prior to the passage of an air bubble (usually back to resting diameter), was completely abolished after endothelial denudation. A maximum constriction to 60mM KCl was also obtained after denudation to confirm that the smooth muscle had not been damaged, and for the standardisation of all constrictor responses. After washing, the reperfusion circuit of Krebs-Henseleit solution was set up and constantly superfused at a constant flow rate of 5 ml/min (C. 1.2.3.). It was this reservoir of Krebs-Henseleit solution to which the agonists and antagonists were applied, keeping the volume at 30 mls by removing one ml of Krebs and adding one ml of the drug in a stepwise fashion (C. 1.2.3., Smith *et al.*, 1995). Responses were recorded 5 min after addition of each agonist concentration, which was sufficient time for an equilibrium response. All of the following studies were carried out in random order and only one concentration response curve to ET-1 or SRTX S6c was performed per tissue.

D. 1.2.1. *ET-1 and SRTX S6c Study*

In the first set of experiments cumulative concentration-response curves to ET-1 (10^{-13}-$3x10^{-8}$M, n=8) or SRTX S6c (10^{-12}-10^{-7}M, n=17) were obtained as described above.

D. 1.2.2. *Receptor Antagonism Study*

In the second set of experiments, vessels were exposed to either BQ-123 (10^{-6} M, n=8), BQ-788 ($3x10^{-8}$M, n=8), TAK-044 (10^{-8} & $3x10^{-7}$ M, n=4 & 8 respectively), BQ-123 + BQ-788 (concentrations as before, n=8) or vehicle (n=8) for 30 min, before obtaining concentration-response curves to ET-1 (10^{-13}-$3x10^{-8}$M). For these experiments, agonists were prepared in a solution of antagonist so that addition to the

perfusion circuit did not dilute the antagonist solution superfusing the tissue. In some experiments, the vessels were exposed for 30 min to SRTX S6c ($3x10^{-8}$M) twice (a wash out period of 10 min between each exposure), in order to desensitise the ET_B receptor prior to commencement of the ET-1 concentration-response curve. This was carried out both in the absence and in the presence of BQ-123 (n=8 each). In all experiments, the time-course of the protocol was the same; 2 hours after verification of the removal of the endothelium, the concentration-response curve to ET-1 was begun.

D. 1.2.3. *Materials*

ET-1 and SRTX S6c were purchased from Novabiochem (Nottingham, U.K.) and BQ-788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-g-MeLeu-D-Trp($COOCH_3$)-D-Nle, sodium salt) from Neosystems (Strasbourg, France) and were reconstituted in 50:50 methanol:distilled water. BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Vel-L-Leu]) from Neosystems (France) and TAK-044 (cyclo[D-a-Asp-3-[(4-phenylpiperazin-1-yl)carbonyl]-L-Ala-L-a-Asp-D-2-(2-thienyl)-Gly-L-Leu-D-Trp] disodium salt) synthesised by Takeda Chemical Industries (Osaka, Japan) and were reconstituted in 0.9% saline, aliquoted and stored frozen at -20°C until use. All peptide agonists and antagonists were diluted in Krebs-Henseleit solution containing 0.1% bovine serum albumin (BSA: Sigma, Poole, U.K.). In all antagonist experiments the ET-1 concentrations were diluted in 0.1% BSA Krebs-Henseleit solution with the appropriate antagonist. ACh (Chloride salt) and PE (Hydrochloride salt;BDH-Merck, UK) were prepared in saline at stock concentration of 10^{-2}M, aliquoted, and stored at -20°C until use when diluted in Krebs-Henseleit solution. These were the sources for all materials used throughout the thesis.

D. 1.3. Results

D. 1.3.1. *Effects of 60mM KCl*

In all experiments 60mM KCl superfusion constricted the arteries, an effect which was reversible back to initial resting diameter on washout (Table 3.1.). The initial diameter remained constant until agonist-induced constriction was generated.

D. 1.3.2. *Effects of ET-1 and SRTX S6c*

ET-1 constricted the pressurised arteries in a concentration-dependent manner (Figure 3.1, pD_2 9.8 , E_{max} 101.9± 2.6 % KCl induced contraction at 10^{-8}M ET-1, n=10). SRTX S6c also produced concentration dependent contraction (Figure 3.1.), but the response was extremely variable, the maximum response obtained with 3 x 10^{-8} M SRTX S6c ranging from 0 to 39% of KCl contraction (mean response = 10.7 ± 2.9%, n=17). In fact, only 9 of the 17 vessels (53%) constricted to the SRTX S6c concentration-response curves.

D. 1.3.3. *Effect of ET_A receptor blockade*

Incubation with BQ-123 (10^{-6}M) before and during exposure to ET-1 (Figure 3.2.) had no effect on contractile responses to low concentrations of ET-1 (10^{-13} to 10^{-11}M) but resulted in inhibition of responses to concentrations of ET-1 between 10^{-11} and 3 x 10^{-8}M. Incubation with BQ-123 significantly inhibited the constrictions to 10^{-9} and 3x10^{-9}M ET-1 (*P*=0.006 & 0.01 respectively) when compared using ANOVA. However, the effect of BQ-123 on the overall pD_2 of the ET-1 concentration response curve did not reach statistical significance (pD_2 9.2, n=8 vs 9.8, n=10, n.s., *P*=0.094).

D. 1.3.4. *Effect of ET_B receptor desensitisation or blockade*

ET_B receptor desensitisation, by exposure to supra-maximal concentration of SRTX S6c (3x10^{-8}M), produced an initial constriction in 4 out of the 8 vessels studied (mean response = 8.1 ± 3.5 % KCl constriction). During the first 30 min exposure to SRTX S6c, the vessel diameter returned to the initial resting value. No constriction was seen, in any of the vessels studied, during the second exposure to SRTX S6c confirming that tachyphylaxis had occurred. The ET-1 concentration response curve was not significantly altered by either ET_B receptor desensitisation (Figure 3.3.A, pD_2 = 9.9, n=8) or following incubation with the selective ET_B receptor antagonist, BQ-788 (3x10^{-8}M, Figure 3.3.B, pD_2 = 10.0, n=8), although both treatments tended to shift the ET-1 concentration response curve to the left (*P*=0.5 & 0.34 respectively).

D. 1.3.5. *Effects of combined ET_A and ET_B receptor blockade*

Co-incubation of vessels with BQ-123 (10^{-6}M) and BQ-788 (3 x 10^{-8}M) resulted in a parallel shift of the ET-1 concentration response curve to the right (Figure 3.4, n=8). Incubation with BQ-123 (10^{-6}M) following desensitisation of ET_B receptors with 10^{-7} M SRTX S6c caused a similar rightward shift (Figure 3.4, n=8). Incubation of vessels with the ET_A/ET_B receptor antagonist, TAK-044 (Figure 3.5., 10^{-8}M, n = 4 and 3 x 10^{-7}M, n = 8) also caused a parallel concentration-dependent shift to the right of the ET-1 concentration-response curve. As the maximum response to ET-1 was not reached within the concentration range studied it was not possible to calculate pD_2 values for ET-1 in experiments with BQ-123 plus either BQ-788 or SRTX S6c desensitisation or with TAK-044 (both concentrations).

D. 1.4. Discussion

Previous *in vivo* studies have clearly indicated a role for ET_B receptors in mediating vasoconstriction in resistance beds, but their role has been difficult to demonstrate in isolated resistance vessels. In the present study it was seen that a role for ET_B receptors in isolated rat mesenteric arteries emerges when both ET_A and ET_B receptors are blocked, whereas blockade of ET_A receptors alone only partially inhibited ET-1 induced contraction and inhibition of ET_B receptors alone had no effect. This phenomenon is similar to previous observations in rabbit pulmonary artery (Fukuroda *et al.,* 1994a), rat trachea (Clozel and Gray, 1995) and human bronchus (Fukuroda *et al.*, 1996), and may be explained by the existence of a 'crosstalk' mechanism between the ET_A and ET_B receptors.

In initial experiments the highly selective ET_B receptor agonist SRTX S6c (Williams *et al.*, 1991) was used to investigate the presence of ET_B receptors in pressurised mesenteric arteries. SRTX S6c produced concentration-dependent constriction but the maximum constriction reached only ~10% of that routinely seen with ET-1, much less than would have been predicted from previous *in vivo* experiments (Clozel *et al.,* 1992). However, the magnitude of responses to SRTX S6c is in agreement with

responses obtained by Takase *et al.* (1995) and Deng *et al.* (1995), in rat mesenteric arteries studied in the perfusion and wire myograph respectively. Interestingly, in all three studies, the contractions to SRTX S6c occurred at relatively high concentrations (10 nM). The ET_B receptor agonists BQ-3020 and IRL 1620 were equally ineffective in the perfused rat mesenteric bed at concentrations up to 1 nM (D'Orleans-Juste *et al.*, 1993). This is quite different to the ET_B agonist responses induced in large blood vessels, which are generally larger and occur at lower concentrations (Moreland *et al.*, 1992, Sumner *et al.*, 1992, La Douceur *et al.*, 1993, Gray *et al.*, 1994). Another interesting feature of the results, not mentioned by previous investigators, is the variability in responsiveness to SRTX S6c. While some vessels failed to respond, others gave up to ~40% of the maximum contraction obtained with ET-1. This might be explained by differential distribution of ET_B receptors in the mesenteric bed, although 3rd generation branches of the main mesenteric artery were routinely used for these studies. Another possibility is variation in intrinsic myogenic tone that these vessels can develop when pressurised. As seen in the validation study (C. 1.3.), using vessels mounted in the wire myograph, no responses were obtained to SRTX S6c until some tone was introduced by a low concentration of the stable thromboxane analogue, U46619 (Mickley *et al.*, 1995).

It has been suggested that SRTX S6c produces a pressor effect in rats via a receptor which is not of the ET_A or ET_B receptor subtype (Flynn *et al.*, 1995). In pithed rats SRTX S6c induced a depressor, followed by pressor response. The depressor response was blocked by BQ-788 administration, as was part of the pressor response. However, a significant part of the pressor response remained. Co-administration of BQ-123 with BQ-788 produced no further antagonism of the SRTX S6c-mediated pressor response, although the pressor response to ET-1 was completely abolished (Flynn *et al.*, 1995). The authors concluded that SRTX S6c was either acting on a novel ET-1 receptor, or was producing a non-specific effect. However, Giller and co-workers (1997), using Piebald-lethal (s^l) mice, a naturally occurring mutant with an inheritant deletion of the ET_B receptor gene, showed that the pressor response to an infusion of SRTX S6c was absent. Thus, they demonstrated that SRTX S6c induces

constrictions by ET_B receptors only (Giller *et al.*, 1997). Furthermore, the heterogenous responses to ET_B receptor agonists, of vasodilatation and constriction, are mediated by receptors derived from the same ET_B receptor gene (Giller *et al.*, 1997; Mizuguchi *et al.*, 1997) because the transient vasodilatation was also abolished.

An alternative approach to investigation of the role of ET_B receptors is to remove the influence of ET_B receptors, either by desensitisation (LaDouceur *et al.*, 1993) or by use of a selective ET_B receptor antagonist like BQ-788 (Ishikawa *et al.*, 1994). In the present study, neither of these interventions inhibited ET-1 induced contraction, a result which would support the view that ET_B receptors have little or no role in rat mesenteric arteries. Interestingly, both desensitisation and BQ-788 treatment seemed to slightly potentiate responses to ET-1, although this effect was not significant. Seo (1996) recently reported similar potentiation of ET-1 induced constriction by the ET_B receptor antagonist, Res 701-1 in human gastroepiploic arteries. There are several possible explanations for these observations. Potentiation of contractions by ET_B receptor antagonists would be expected in the presence of the vascular endothelium due to blockade of endothelial ET_B receptor mediated release of relaxing factors by ET-1. However, this is an unlikely explanation for the present results as the endothelium was effectively removed by passing of an air bubble through the lumen of the vessels, as evidenced by the loss of relaxant responses to acetylcholine.. Previous histological studies in the laboratory have also shown complete removal of the endothelium by this method (Smith, 1996) and also immunohistochemical evidence as demonstrated in section E. 1.4.1., Figure 4.1. The experiments of Seo (1996) were also conducted in endothelium-denuded vessels. Alternatively, potentiation might have been caused by displacement of ET-1 from low affinity ET_B clearance receptors (Fukuroda *et al.*, 1994c) by BQ-788, but this would not account for the similar effect of receptor desensitisation. Another alternative, suggested by Seo (1996), is the presence of sensitive ET_B receptors on smooth muscle which inhibit or negatively modulate ET_A receptor-mediated constrictions to ET-1.

From the results obtained with SRTX S6c, BQ-788, and desensitisation alone, one would predict that blockade of ET_A receptors, using a selective competitive antagonist, like BQ-123 (Ihara *et al.*, 1992) would cause a parallel rightward shift of the ET-1 concentration response curve. However, in the presence of BQ-123 the ET-1 concentration-response curve in pressurised mesenteric arteries was biphasic, only responses to high concentrations of ET-1 being shifted to the right in a parallel manner by BQ-123, consistent with competitive antagonism at the ET_A receptor. Interestingly, the BQ-123 resistant, possibly ET_B mediated, responses to ET-1 were at the lower end of the dose-response curve, consistent with the presence of a high affinity ET_B receptor. Takase *et al.* (1995) reported similar results with the ET_A receptor antagonist, FR139317 in rat mesenteric arteries, although in that case the ET_A resistant component was smaller than seen here. Takase *et al.* perfused the vessels at a pressure of 30 mmHg, half of that used in the present study. Given the observation that increased tone may reveal constrictor ET_B receptors, as implied by the responses to SRTX S6c (Mickley *et al.*, 1995), the lower pressure used by Takase *et al.* (1995) may account for the smaller ET_A receptor antagonist resistant element of the ET-1 curve. The results of the present study are consistent with the reported ET_A receptor antagonist resistant reduction in mesenteric blood flow induced by ET-1 *in vivo* (Gardiner *et al.*, 1994, Allcock *et al.*, 1995).

In order to investigate whether the residual ET_A antagonist resistant portion of the ET-1 response was mediated by ET_B receptors combined treatment with BQ-123 and either desensitisation or BQ-788 was used. Both of these combination treatments resulted in a parallel shift of the ET-1 concentration response curve. In fact, the BQ-123 sensitive portion was moved further to the right than with BQ-123 alone, in agreement with Fukuroda *et al.* (1996) who described a similar phenomenon in human bronchi. Responses to ET-1 were also inhibited, in a concentration dependent manner, by TAK-044, a peptide antagonist with similar potency at both ET_A and ET_B receptors (Kikuchi *et al.*, 1994). Ironically, in a study by the group who synthesised and described TAK-044 (Awane-Igata *et al.*, 1997), ET-1-induced constrictions in canine mesenteric arteries were not inhibited by TAK-044.

These results demonstrate a clear role for ET_B receptors in mediation of constrictor responses to ET-1 in small mesenteric arteries that is only revealed when ET_A receptors, in addition to ET_B receptors, are blocked. The lack of effect of ET_B receptor blockade or desensitisation alone seems to indicate that ET_A receptors can somehow compensate for the inactivation of ET_B receptors. Similar observations have been reported in vascular (Fukuroda *et al.*, 1994a) and non-vascular (Clozel & Gray, 1995; Fukuroda *et al.*, 1996) tissues. The concept of receptor 'crosstalk' has been proposed to explain these observations. The mechanism is not fully understood, although interactions at the second messenger level have been suggested, such that blockade of the ET_B receptor releases an inhibitory mechanism acting at the ET_A receptor (Fukoroda *et al.*, 1996). Allosteric interactions between ET receptors have been suggested to account for the results of radioligand binding studies in rat heart (Sokolovsky, 1993). Further biochemical studies are required to elucidate the interactions between ET receptors co-existing in the same tissue and the mechanism of the apparent crosstalk phenomenon. Interestingly, similar interactions have been reported between α_1 and α_2 adrenoceptors activated by noradrenaline (Daly *et al.*, 1988).

In the rat, the mesenteric bed receives up to 10% of cardiac output and thus resistance in this bed is an important determinant of total peripheral resistance and of blood pressure. The present results show that simultaneous blockade of both ET_A and ET_B receptors is required for complete inhibition of constrictor responses to ET-1 in the rat mesentery *in vitro*. This agrees with observations that blockade of both receptors is required to inhibit ET-1 induced increases in blood pressure *in vivo* (McMurdo *et al.*, 1993). The role of ET_B receptors in regulating constrictor responses to ET-1 might be even greater in human resistance vessels, where ET_B agonists have a greater direct effect than in other species *in vitro* (Takase *et al.*, 1995) and *in vivo* (Haynes *et al.*, 1995).

In some pathophysiological states associated with increased peripheral resistance and increased plasma concentrations of ET-1, there is evidence for an upregulation of

smooth muscle ET_B receptor; most notably in heart failure in dogs (Cannan *et al.*, 1996) and humans (Love *et al.*, 1996); in atherosclerosis (Winkles *et al.*, 1993; Dagassan *et al.*, 1996) and in hypertension (Kanno *et al.*, 1993, Batra *et al.*, 1993). The results of the present study suggest that blockade of both ET_A and ET_B receptors may be required for effective inhibition of ET-1 induced constriction in these diseases. This study was conducted in vessels without endothelium. However, in the presence of endothelium, ET_B receptor blockade can actually enhance responses to ET-1 by blocking the release of nitric oxide and prostacyclin through endothelial ET_B receptor stimulation (De Nucci *et al.*, 1988). Thus, the effectiveness of endothelin receptor blockade therapeutically will depend on the level of endothelial ET_B receptor stimulation and on the relative selectivity of the antagonist for endothelial and smooth muscle ET_B receptors, the ideal antagonist allowing ET-1 to act at the endothelial ET_B receptor while blocking its effects at smooth muscle ET_A and ET_B receptors.

	ET-1 Control	*SRTX S6c*	*+ BQ-123*	*+ BQ-788*	*+ SRTX S6c desens*	*+ BQ-123 + BQ-788*	*+ BQ-123 + SRTX S6c desens*	*+ TAK-044 (10^{-8}M)*	*+ TAK-044 ($3x10^{-7}$M)*
Resting Diameter	277 ± 15	300 ± 9	261 ± 13	287 ± 15	281 ± 7	273 ± 21	304 ± 19	300 ± 12	301 ± 12
+ 60 mM KCl Diameter	51 ± 3	48 ± 2	53 ± 3	51 ± 1	48 ± 3	55 ± 2	50 ± 3	45 ± 3	50 ± 2
Max ET-1/SRTX S6c Diameter	47 ± 3	273 ± 12	56 ± 7	50 ± 2	48 ± 3	63.8 ± 8.4	118 ± 27	118 ± 39	233 ± 31

Table 3.1. Mean resting lumen diameters and lumen diameters (μm) after exposure to 60 mM KCl solution or after the maximum concentration of endothelin-1 (ET-1) or sarafotoxin S6c (SRTX S6c) in each experimental group.

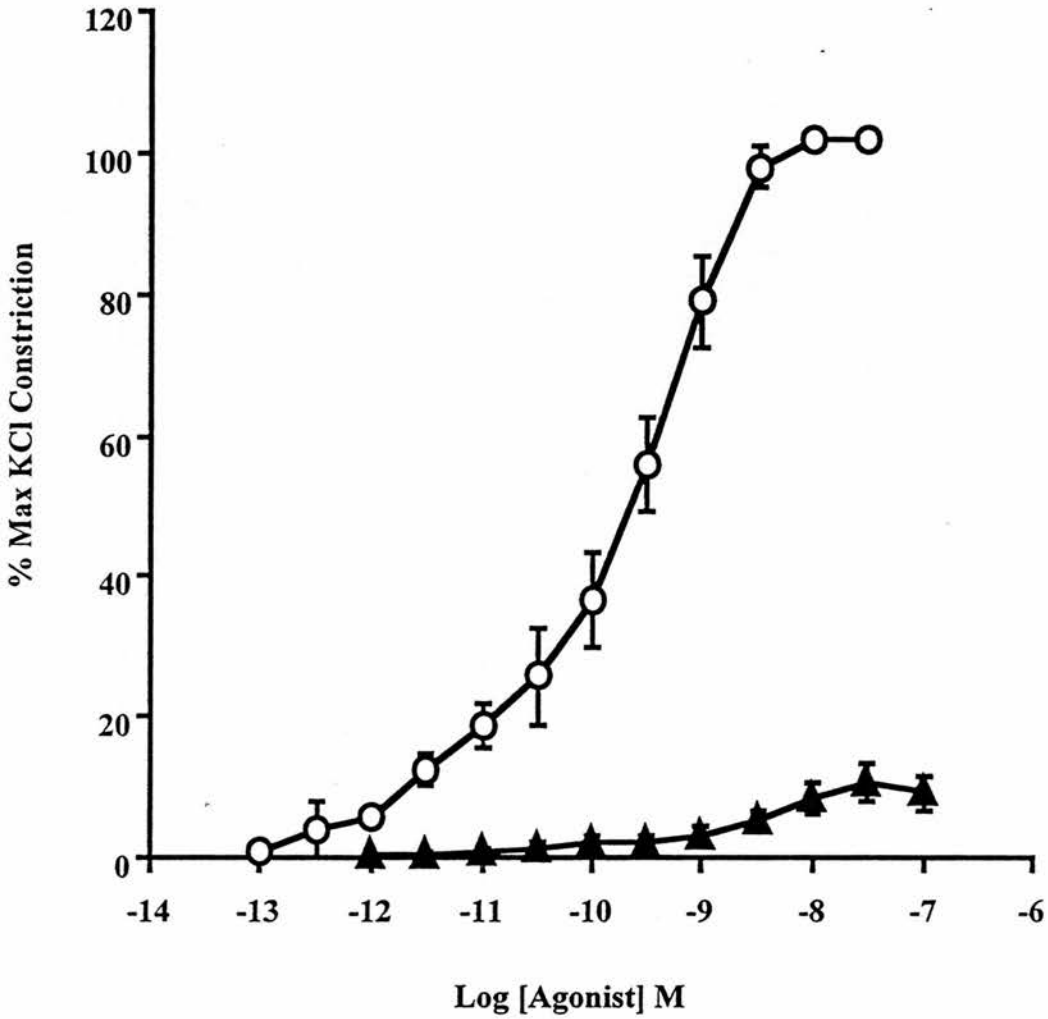


Figure 3.1 Comparison of the contractile responses to endothelin-1 (ET-1,○) and sarafotoxin S6c (SRTX S6c,▲) in rat small mesenteric arteries. ET-1 (n=10) produced a maximal constrictions of similar proportions to 60mM KCl at $3x10^{-9}$M. SRTX S6c (n=17) induced small constrictions at the highest concentrations, suggesting a small population of ET_B receptors present on the smooth muscle of the resistance arteries. All values shown are mean ± s.e.mean.

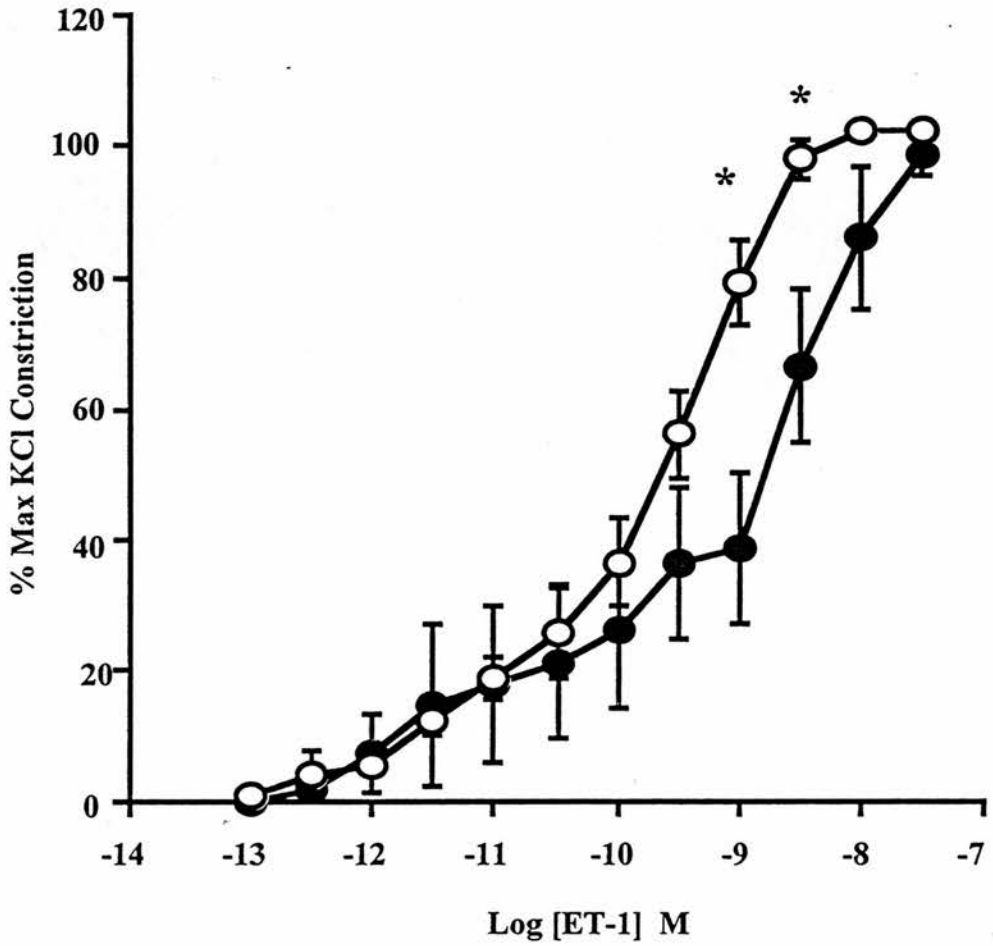


Figure 3.2. Effect of the ET_A receptor antagonist BQ-123 on the ET-1 concentration-response curve in rat small mesenteric arteries. Pre-incubation with BQ-123 (10^{-6}M) for 30 minutes (● , n=8) shifted the responses to the higher concentrations of ET-1 in a parallel fashion to the right. All values are mean ± s.e.mean. *P<0.05 compared to control (O) ET-1 responses.

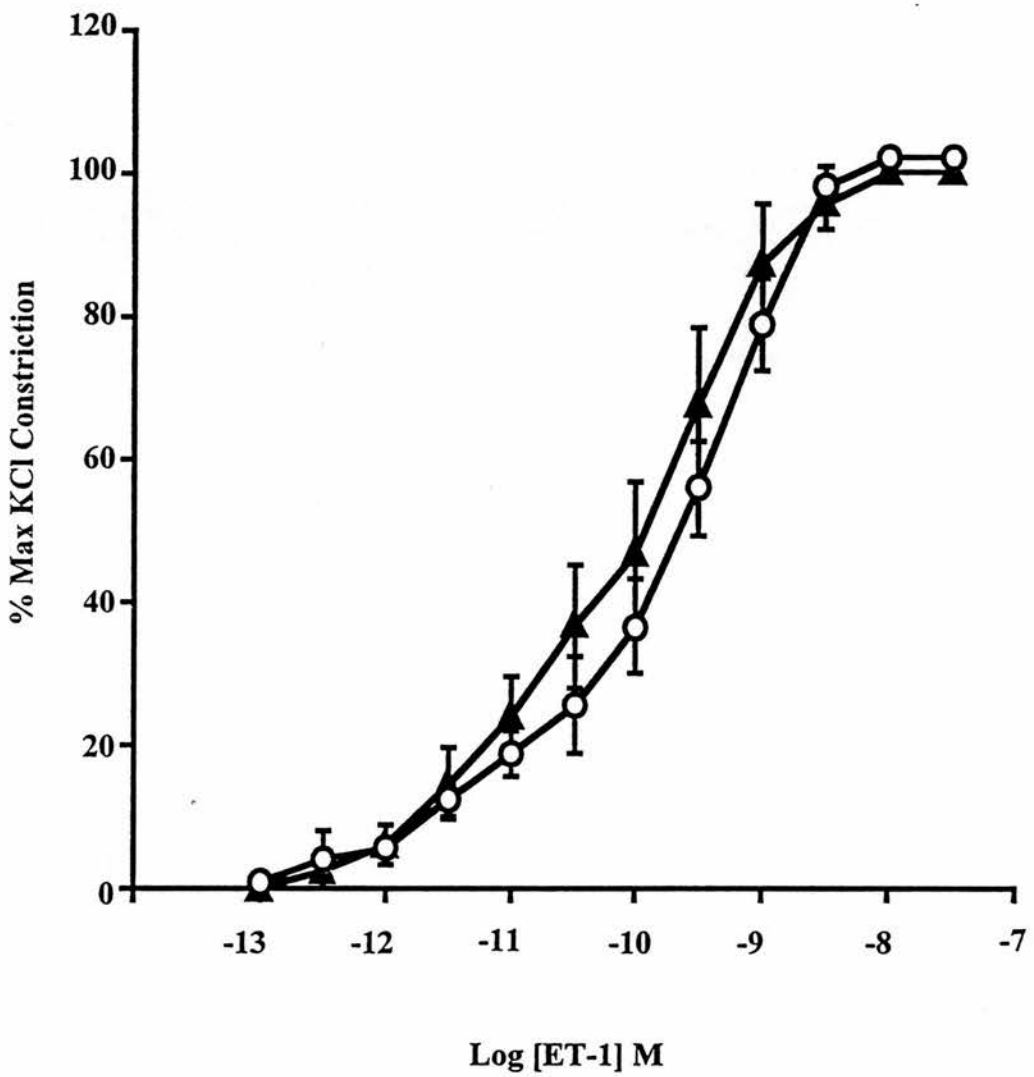


Figure 3.3.A. The effects of selective ET_B receptor blockade on ET-1-induced constrictions in rat small mesenteric arteries. The vessels were exposed to SRTX S6c (10^{-7}M,▲, n=8) twice for 30 minutes (each exposure) prior to the start of ET-1 concentration-response curve. The ET-1 concentration response curves tended to be shifted slightly to the left as compared to control (O), though not significant, P=0.54 as compared using ANOVA. All values are mean ± s.e.mean.

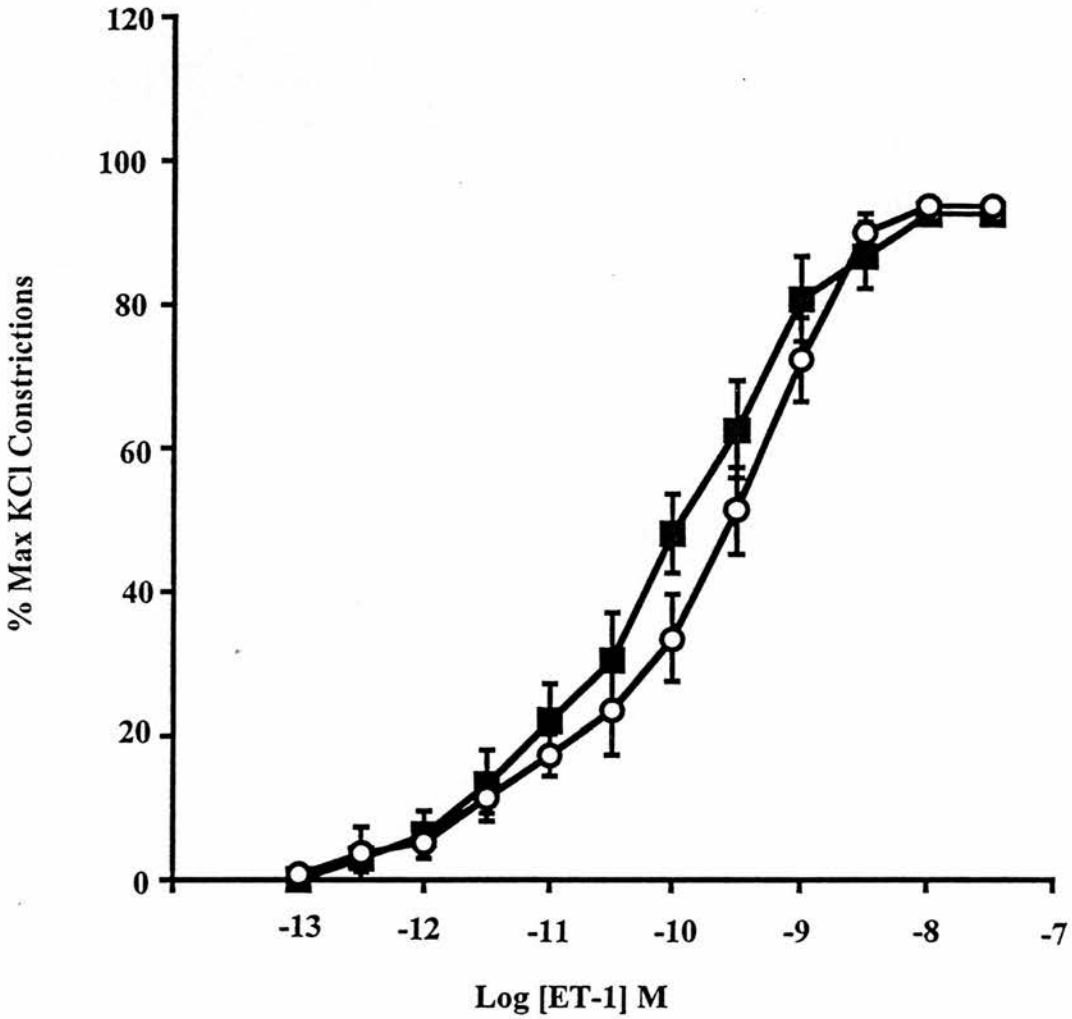


Figure 3.3.B. The effects of selective ET_B receptor blockade on ET-1-induced constrictions in rat small mesenteric arteries. The vessels were exposed to BQ-788 (Figure 3b, 3x10-8M, ■ , n=8) pre-incubated for 30 minutes prior to the start of ET-1 concentration-response curve. The ET-1 concentration response curve tended to be shifted slightly to the left as compared to control (O), though not significant, P= 0.42 as compared using ANOVA. All values are mean ± s.e.mean.

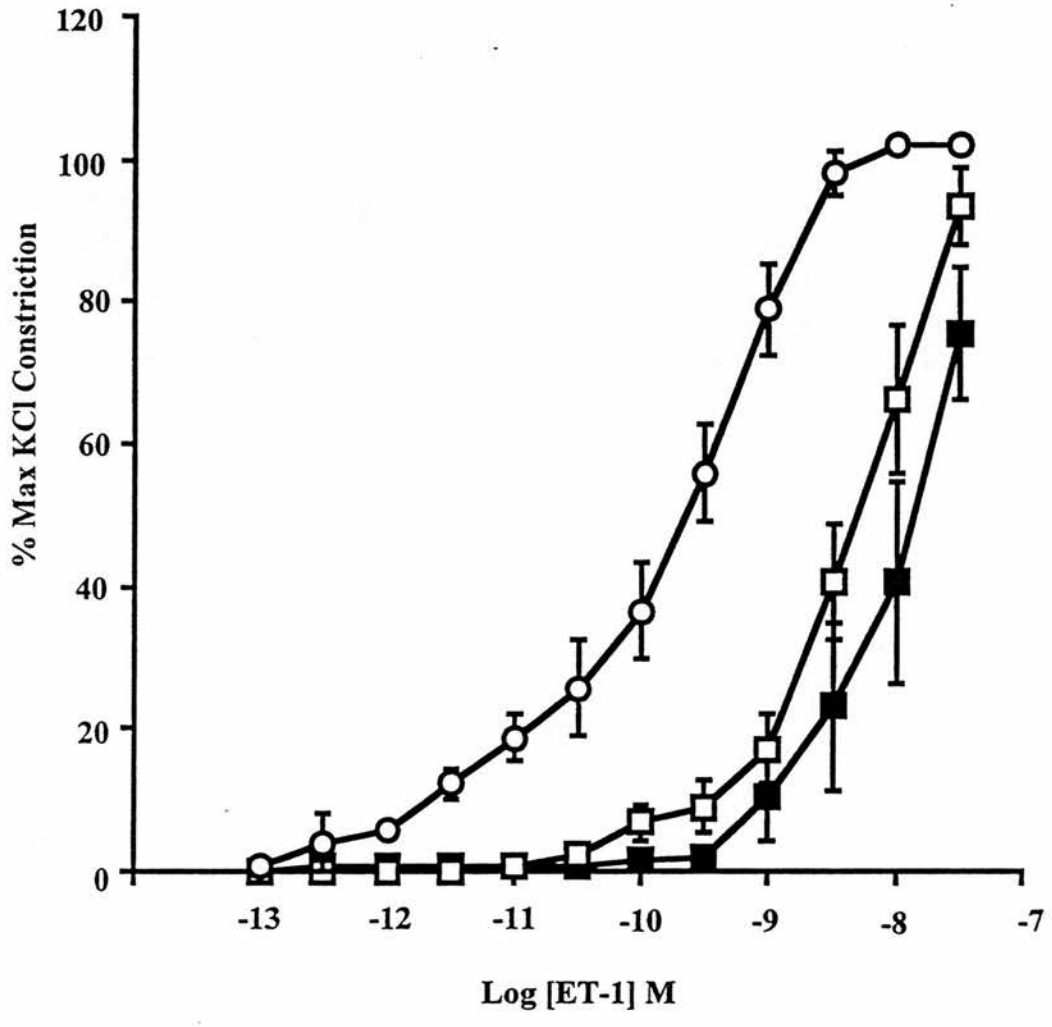


Figure 3.4. The effects of non-selective ET_A/ET_B combination treatment on ET-1-induced constrictions in rat small mesenteric arteries. The vessels were exposed to either vehicle (O), BQ-123 plus BQ-788 (10^{-6}M & $3x10^{-8}$,□, n=8) or pre-incubated with SRTX S6c twice (each 10^{-7}M) plus BQ-123 (10^{-6}M, ■ , n=8). Both treatments significantly shifted the ET-1 concentration-response curve to the right in a parallel fashion (P=0.0001 for both). All values are mean ± s.e.mean.

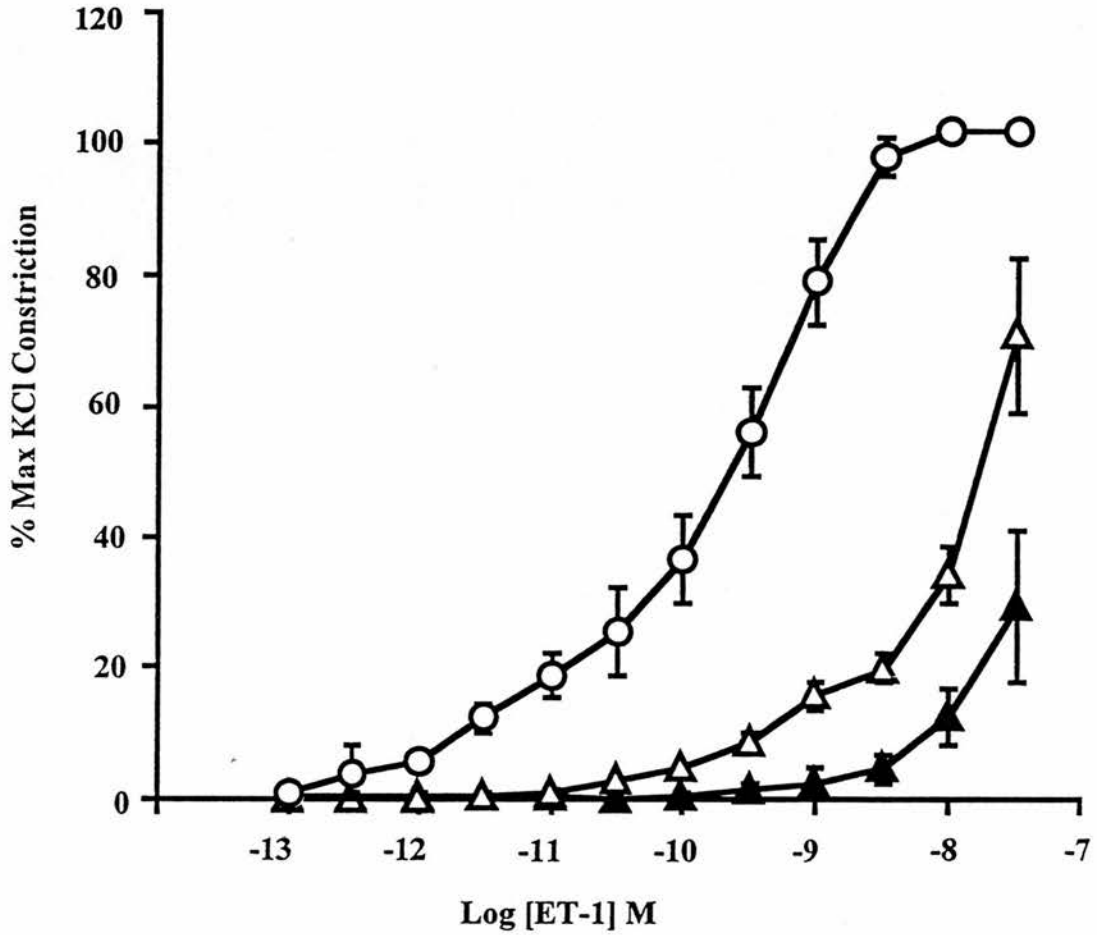


Figure 3.5. The effects of the non-selective ET_A/ET_B receptor antagonist TAK-044 on ET-1-induced constrictions in rat small mesenteric arteries. The vessels were pre-incubated for 30 minutes with either 10^{-8} M (△, n=4) or 3 x 10^{-7}M (▲, n=8) TAK-044. Both treatments significantly inhibiting the ET-1 concentration-response curve (P=0.0002 & 0.0001 respectively) as compared to control (○). All points are mean ± s.e.mean.

Results Chapter 2

Evidence for an inhibitory endothelin ET_B receptor on the smooth muscle of small mesenteric arteries from a rat model of CHF

E. 1.1. Introduction

The ET system has been investigated in several different models of CHF, including dogs, rabbits, hamsters and rats. In all of the animals models used, circulating plasma ET-1 levels are consistently elevated. As discussed in Section B. 1.6.3., several studies have demonstrated decreased ET receptor density in cardiac, renal and arterial tissues from various animal models of CHF in association with the raised circulating ET-1 levels (Loffler *et al.*, 1993; Fu *et al.*, 1993). Furthermore, in anaesthetised CHF dogs, cardiorenal vasoconstrictor responses to exogenously administered ET-1 were significantly attenuated in comparison to control dogs (Cavero *et al.*, 1990). In agreement, in pithed CHF rats there was a reduced pressor response to a bolus injection of ET-1 (Fu *et al.*, 1993). Interestingly, the vasodilator response was preserved (Fu *et al.*, 1993). However, despite the apparent down-regulation/desensitisation of ET receptors in CHF, ET antagonists, both selective ET_A and non-selective ET_A/ET_B receptor antagonists, have improved survival in CHF animals (Shimoyama *et al.*, 1996; Sakai *et al.*, 1996b) perhaps in part by reducing the raised vascular resistance (Clavell *et al.*, 1996).

In the previous chapter it was shown that both ET_A and ET_B receptors are involved in the vasoconstriction to ET-1 in endothelium denuded mesenteric resistance arteries from normotensive, Wistar rats (Mickley *et al.*, 1997). Furthermore, there could potentially be a crosstalk mechanism between the two receptor subtypes communicating (Mickley *et al.*, 1997), most probably, at the G-protein second messenger level (Ozaki *et al.*, 1997). The ET_A receptor had the ability to compensate for the "loss" of smooth muscle constrictor ET_B receptors when either inhibited with BQ-788 or desensitised by prolonged exposure to STRX S6c. It has been suggested that selective ET_A receptor blockade would be desirable in reducing peripheral vascular resistance in CHF, since concomitant inhibition of ET_B receptors would lead to the loss of the counterbalancing vasodilatory actions of the endothelial ET_B receptors and

would further increase circulating ET-1 by inhibiting ET_B receptor clearance (Davenport & Maguire, 1994). However, BQ-123 in the small mesenteric arteries of normotensive rats antagonised responses only at the higher concentrations of ET-1-induced vasoconstriction (Mickley *et al.*, 1997). Only when both ET_A and ET_B receptors were inhibited were the vasoconstrictions to all concentrations of ET-1 antagonised, suggesting that for effective removal of ET-1-induced vasoconstriction both ET_A and ET_B receptors should be inhibited. Thus, it is important to identify the receptors involved in the constrictor responses to ET-1 in arteries in heart failure.

In this chapter, any changes in responses to ET-1, and the receptor subtypes mediating ET-1-induced vasoconstriction, have been investigated using the rat coronary artery ligation (LCAL) model of CHF (Pfeffer *et al.*, 1964). Furthermore, since heart failure is a progressive disease and the rise in circulating ET-1/big ET-1 levels occur during the later stages of the condition (Rodeheffer *et al.*, 1992; McMurray *et al.*, 1992; Cacoub *et al.*, 1993; Wei *et al.*, 1994), any alteration in arterial responsiveness was followed using arteries taken from animals with heart failure at two different time points of 5 weeks and 12 weeks post-ligation. This model has proved useful in studying the mechanisms involved in CHF (Witchel, 1997) as it mimics the human pathophysiology including cardiac dilatation, hypertrophy, infarct thinning and collagen deposition and the animals respond to drug therapies, such as ACE inhibitors and β-blockers (Witchel, 1997).

It has been reported that ET-1 produces its characteristically long-lasting vasoconstriction by binding to its receptors and the receptor-ligand complex being internalised (Resink *et al.*, 1990). Autoradiographic studies have indicated the existence of ET-1 receptors in both the plasma membrane and intracellular compartments of rat aortic smooth muscle cells (Resink *et al.*, 1990). After the receptor-ligand complex has been internalised and the intracellular second messenger

systems triggered, the ET-1 is degraded and the receptor is recycled back to the cell surface (Marsault *et al.*, 1993). It has been estimated that ~ 40% of receptors are recycled after 1 hour once ET-1 has been dissociated or degraded from its receptors following internalisation (Marsault *et al.*, 1993). Thus, it has been suggested that ET-1 receptor antagonists cannot reverse established ET-1 vasoconstriction until the ET-1 receptors have been recycled back to the cell surface. In rat aortic rings, BQ-123 took over 40 min to reverse an established ET-1 constriction (Marsault *et al.*, 1993; Warner *et al.*, 1994). This was investigated using the small arteries which were exposed to the ET-1 CRC alone. Once the vessel had been exposed to the highest concentration of ET-1 and maximum constriction established, either BQ-123 or the non-selective ET_A/ET_B receptor antagonist, TAK 044 was added to the reperfusion circuit in order to ascertain whether the ET-1 tone could be reversed.

E. 1.2. Methods

E. 1.2.1. *Left Coronary Artery Ligation Rat Model of Heart Failure*

Heart failure was induced by ligation of the left anterior descending coronary artery in 6 week old male Wistar rats as described in Section C. 1.1.2. Either 5 weeks or 12 weeks after ligation, the right carotid artery was was cannulated and a transducer-tipped catheter fed into the left ventricle to measure the left ventricular end diastolic pressure (LVEDP) to confirm CHF. Animals were classified as having CHF if the LVEDP was > 15 mmHg. The mesenteric bed was excised and placed immediately into cold Krebs solution. Sham-operated rats were used as controls, where the ligature was pulled out of the heart.

All operational procedures and haemodynamic measurements were performed by Dr Gillian Gray, Department of Pharmacology, George Square, Edinburgh.

E. 1.2.2. *Perfusion myograph studies*

Arteries were dissected from the mesenteric bed whilst immersed in cold Krebs-Henseleit solution. All of the following studies were carried out in random order and only one ET-1 CRC was performed per tissue.

E. 1.2.2.1. *ET-1 and SRTX S6c study*

After dissection, arteries were mounted in a perfusion myograph and the initial 'wake up' and denudation procedures performed. After the reperfusion circuit had been started, arteries were superfused for 1 hour with Krebs-Henseleit solution and a ET-1 CRC (10^{-13}-$3x10^{-8}$M) was obtained. Cumulative CRCs to SRTX S6c (10^{-12}-$3x10^{-8}$M) were also performed in different arteries. However, in these arteries the SRTX S6c CRC was constructed 5 min after the reperfusion circuit had been set up because these arteries were used as the SRTX S6c desensitisation group (See below F. 1.2.2.2.).

E. 1.2.2.2. *Receptor antagonism study*

Some arteries, after mounting and denudation procedures, were exposed to 30 min reperfusion of Krebs-Henseleit solution followed by a 30 min incubation with BQ-123 (10^{-6}M) and an ET-1 CRC (10^{-13}-$3x10^{-8}$M) obtained. In the arteries which had been exposed to the SRTX S6c CRC, the arteries were washed with Krebs-Henseleit solution, a new reperfusion circuit started and challenged with SRTX S6c ($3x10^{-8}$M) for 30 min and an ET-1 CRC constructed. This constituted the ET_B receptor desensitisation group. For the non-selective ET_A/ET_B receptor antagonist group, arteries were challenged with SRTX S6c ($3x10^{-8}$M) for 30 min twice (with a wash out period of 10 min between each exposure) plus BQ-123 (10^{-6}M) in the second 30 min period prior to a ET-1 CRC.

E. 1.2.2.3. *Reversal study*

In the vehicle control vessels which only had an ET-1 CRC performed, once the maximum constriction to the highest concentration of ET-1 ($3x10^{-8}$M) had been obtained BQ-123 (10^{-5}M) or TAK-044 (10^{-5}M) or vehicle control (Krebs-Henseleit solution) was added to the reperfusion circuit and reperfused for 20 min, with lumen diameter measurements taken at 0, 5, 10, 15 and 20 min after the addition of antagonist.

E. 1.2.2.4. *Intrinsic tone*

As described in the previous chapter, SRTX S6c induced constrictions in 53% of arteries challenged with a SRTX S6c CRC. It was suggested that the arteries which constricted to SRTX S6c may have some intrinsic tone, thus revealing the presence of constrictor ET_B receptors as demonstrated in the wire myograph studies (Section C. 1.3.5.). The presence of intrinsic tone was investigated in the arteries studied by exposing all arteries to Ca^{2+}-free Krebs-Henseleit solution (by addition of 10^{-4} M EDTA) and 10^{-5}M sodium nitroprusside (SNP) at the end of each experiment. The lumen size after the subsequent relaxation was compared to the lumen size at the beginning of the experiment, before the SRTX S6c CRC and ET-1 CRC.

All experiments were performed in the presence of indomethacin (10^{-4}M) added directly to the superfusing Krebs-Henseleit solution.

E. 1.3. Results

E. 1.3.1. *Effects of coronary artery ligation*

During the study period none of the sham-operated animals died. However, in the coronary artery ligation group mortality usually occurred during the 24 hour period after the ligation procedure had been performed. The average survival 24 hour post-ligation was 70%. There was no difference in weight between the sham-operated rats and the 5 week CHF rats (454.3±12.2g vs 466.5±16.5g) or 12 week CHF rats (521.7±36g vs 553.9±7.2g). The mean LVEDP for the sham-operated animals was4.4±0.9 mmHg. Consequently the rats with heart failure had significantly raised LVEDP of 20.8±1.9 mmHg. The area of infarcts, between 30-40% of the left ventricle, in the ligated rats were clearly thinner than the ventricles of the sham-operated rats, with collagen and fibrous scar tissue replacing healthy myocardium.

E. 1.3.2. *Effects of 60mM KCl and PE*

There was no difference in the maximum constrictions to KCl or PE in the arteries from the 5 and 12 week CHF rats and the respective controls (Table 4.1.).

E. 1.3.3. *Effects of ET-1 and SRTX S6c*

ET-1 constricted all arteries in a concentration-dependent manner (Figures 4.1.A. and B.).

In the arteries from the 5 week sham-operated rats (n=8; mean lumen dia = 307±17μm) there was a biphasic response, with a shallow, gradual vasoconstriction with the lower concentrations of ET-1 < 10^{-10}M, and a sharper vasoconstriction to maximum at the higher concentrations > 10^{-10}M. There was no difference in the sensitivity to the ET-1 CRC in the 5 week post-ligation rats (n=8; mean lumen dia =

301±10.5 μm) in comparison to the 5 week sham-operated controls (Figure 4.1.A.) when compared by ANOVA (P=0.5). Furthermore, the pD_2 values were similar (Table 4.2.) when compared using a Students two-tailed t-test. However, the constrictions to the lower concentrations of ET-1 appeared to be attenuated in the arteries from the 5 week post-ligation rats (Figure 4.1.A).

The response to ET-1 in the 12 week sham-operated rats (n=8; mean lumen dia = 280±18.8 μm) was also biphasic in nature (Figure 4.1.B.). However, the arteries from the 12 week post-ligation rats (n=8; mean lumen dia = 305±17 μm) were significantly less sensitive to the ET-1 CRC than the arteries from the 12 week sham-operated controls (ANOVA P=0.04) and the response was monophasic in nature. The pD_2 values were also significantly different (Table 4.2.).

SRTX S6c induced small constrictions in some of the arteries studied, similar to responses described in Chapter 3.

In the 5 week sham-operated rats, SRTX S6c induced small constrictions in 3 out of the 6 arteries exposed to the SRTX S6c CRC (Figure 4.2.A.; mean lumen dia = 286.7±26.8 μm), with constrictions to the highest concentration of SRTX S6c (10^{-7}M) ranging from 0% to 20% of maximum KCl constriction, E_{max}=7±4.8%. In the 5 week CHF rats, SRTX S6c induced small constrictions in 3 out of the 4 arteries exposed to an SRTX S6c CRC (Figure 4.2.A.; mean lumen dia = 274±21.1 μm), E_{max}=7.3±1.8% max KCl constriction. In the 5 week CHF arteries tachyphylaxis of the ET_B receptors to the two highest SRTX S6c concentrations could be seen to be occurring.

SRTX S6c-induced constrictions in 4 out of 6 arteries dissected from 12 week sham-operated rats with tachyphylaxis occurring at the highest concentration (Figure 4.2. B.; mean lumen dia = 281±23.2 μm). Responses to SRTX S6c ranged from 0% to 17% KCl constriction, E_{max}=10±2.2% KCl constriction. In contrast, only 1 out of 6 arteries removed from 12 week CHF rats constricted to SRTX S6c, this response was only 7% KCl constriction (Figure 4.2.B; mean lumen dia = 286±28.9 μm).

E. 1.3.4. *Effects of ET_A receptor antagonism*

ET_A receptor antagonism with BQ-123 inhibited the ET-1-induced constrictions in the 5 week sham-operated rat arteries (n=6, Figure 4.3.A.; mean lumen dia = 311±13.5 μm). The whole ET-1 CRC was significantly shifted to the right in a parallel manner (ANOVA, P=0.0008) and threshold concentration for constriction was shifted from 10^{-12}M to $3x10^{-11}$M. The ET-1 CRC was also significantly inhibited in the arteries from the 5 week CHF rats (n=6, P=0.0001; mean lumen dia = 305±15.2 μm). However, the constrictions to the lower concentrations of ET-1, up to 10^{-10}M remained in the presence of BQ-123, with the threshold constrictions occurring at 10^{-12}M ET-1 in both the vehicle control ET-1 CRC and the BQ-123 treated ET-1 CRC (Figure 4.3.B.). The pD_2 values could not be calculated as maximum ET-1 constrictions were not reached in either group.

BQ-123 inhibited the ET-1-induced constrictions in the vessels from the 12 week sham-operated rats (n=6; ANOVA P=0.0008; mean lumen dia = 315±10.6 μm). Similar to the results in 5 week sham-operated rat arteries, the threshold constrictions were shifted from 10^{-12}M to $3x10^{-11}$M in the 12 week sham-operated arteries (Figure 4.3.C.). The ET-1-induced vasoconstrictions were also significantly inhibited in the arteries from the 12 week CHF rats (n=6; ANOVA P=0.003; mean lumen dia = 315±23.8 μm). Interestingly, the ET-1 CRC in the 12 week CHF vessels was

inhibited by BQ-123 to a similar point on the graph as the ET-1 CRC in the 12 week sham-operated vessels. For instance the constrictions at 10^{-8}M ET-1were 28±5.8% vs 20±6.1%, and at $3x10^{-7}$M ET-1 83±12% vs 88.5±8.1% (sham vs CHF). Again, the pD_2 values could not be calculated as maximum ET-1 constrictions were not reached in either group.

E. 1.3.5. *Effects of ET_B receptor antagonism*

ET_B receptor down-regulation using SRTX S6c had no effect on ET-1 vasoconstrictions in the arteries from 5 week sham-operated rats (n=6; Table 4.2.; Figure 4.4.A; ANOVA P=0.4; mean lumen dia = 286.7±26.8 μm) or 5 week CHF rats.(n=5; ANOVA P=0.6; mean lumen dia = 274±21.1 μm).

In the 12 week sham-operated rat arteries ET_B receptor down-regulation had no effect on the ET-1 response (n=6; Table 4.2; Figure 4.4.B; ANOVA P=0.57; mean lumen dia = 281.7±23.2 μm). However, ET_B receptor desensitisation restored the biphasic ET-1 response in the 12 week CHF rat arteries, similar to the vasoconstrictions in the 12 week sham-operated arteries. The ET-1 CRC was significantly shifted to the left (ANOVA P=0.037; mean lumen dia = 286±28.9 μm) and the pD_2 similar to the sham-operated arteries (Table 4.2.). In particular, the vasoconstrictions to the lower concentrations of ET-1 (<10^{-10}M) were enhanced (Figure 4.4.B.).

E. 1.3.6. *Effects of ET_A and ET_B receptor antagonism*

Non-selective ET_A and ET_B receptor antagonism significantly inhibited the entire ET-1 CRC in the 5 week sham-operated arteries (n=6; Figure 4.5.A; ANOVA P=0.0006; mean lumen dia = 271.7±18.2 μm). Threshold constrictions were moved from 10^{-12}M to 10^{-10}M. ET-1 vasoconstrictions in the 5 week CHF rat arteries were also inhibited

to a similar extent (n=4; ANOVA P=0.0001; mean lumen dia = 292.5±14.9 μm). Since maximal constrictions to ET-1 were not reached the pD_2 values could not be calculated.

ET-1-induced vasoconstrictions were significantly shifted to the right by non-selective ET_A and ET_B receptor antagonism in the 12 week sham-operated rat arteries (n=6; Figure 4.5.B.; ANOVA P=0.0006; mean lumen dia = 276.7±13.3 μm). Threshold constrictions were moved from 10^{-12}M to 10^{-10}M. Non-selective ET_A and ET_B receptor antagonism also significantly inhibited the entire ET-1 CRC in the arteries from the 12 week CHF rats (n=6; ANOVA, P=0.003; mean lumen dia = 285±29.7 μm), again to a similar point on graph as the responses of the 12 week sham-operated rat arteries (Figure 4.5.B.).

E. 1.3.7. *Reversal study*

Unfortunately, due to the small numbers (8 per group) exposed to an ET-1 CRC only, there were not enough arteries per animal group to divide into the 3 reversal treatment groups of BQ-123, TAK-044 or vehicle to perform any analyses on the results. As a consequence the reversal data has been combined for the 5 week animals (sham & CHF; Figure 4.6.A.) and 12 week animals (Figure 4.6.B.).

As can be seen in Figures 4.6. A and B established ET-1-induced vasoconstrictions were reversed in arteries from all experimental groups within 20 minutes of application of the appropriate antagonist to the reperfusion circuit. However, if no antagonist was applied, the vasoconstriction to the maximal concentration of ET-1 remained constant for at least the duration of the reperfusion time. BQ-123 effectively reversed the ET-1 vasoconstriction within 15 minutes, however, a small constriction remained of 28.3 ±

8.4 μm and 28 ± 4.2 μm (5 and 12 week rats respectively). At 20 min, TAK-044 appeared to reverse the ET-1 tone slightly more effectively than BQ-123 (although not to a significant extent) with a small constriction remaining of 9.4 ± 7.1 μm and 15.3 ± 9 μm (5 and 12 week rats respectively).

E. 1.3.8. *Intrinsic tone investigation*

None of the arteries from any experimental group had intrinsic tone present. The combination of Ca^{2+} -free Krebs and chelation using EDTA and direct smooth muscle relaxation with SNP immediately relaxed the arteries to the initial resting tone measured at the beginning of the experiments and prior to the SRTX S6c or ET-1 CRCs (data not shown).

E. 1.4. Discussion

Ligation of the left descending coronary artery in a rat causes ischaemia of the myocardium of the left ventricle, resulting in an infarcted area and compromised cardiac output, thus triggering the neurohumoral reflexes which begin the 'vicious cycle' of CHF. In this way the effects of left coronary artery ligation are similar to the main cause of CHF in humans. A myocardial infarction produced by the rupture of atheroma in one of the coronary arteries perfusing the left ventricular myocardium and occlusion by the subsequent formation of a blood clot also results in myocardial ischaemia.

The LCAL rat model of heart failure has been demonstrated previously to mimic the pathophysiology of the disease in man. For instance, both plasma ANG II and NA levels are increased up to 9 months post-ligation (Mulder *et al.*, 1996; 1997) and the animals respond favourably to ACE inhibition (Mulder *et al.*, 1996). Due to the

similarities between the ANG II and ET-1 systems, it is reasonable to assume that this rat model is useful for the study of the ET-1 pathway in CHF. Indeed, in the rat LCAL model of CHF it has been shown that ET-1 production is up-regulated (Teerlink *et al.,* 1994b; Sakai *et al.,* 1996a & b). Sakai and colleagues (1996a) saw that 3 weeks post-ligation, plasma ET-1 concentrations were >3 fold higher than those of the sham-operated rats, and was significantly correlated with LVEDP. Raised plasma ET-1 levels persist as the condition progresses in this model since Teerlink and co-workers (1994b) found that from 1 to 16 weeks post-ligation circulating ET-1 levels were 1.5 times greater than the sham rats. In contrast, one study saw no increase in plasma ET-1 levels (Mulder *et al.,* 1997) at 2 or 9 months post-ligation. It was suggested that this may be as a result of the rats having only moderate cardiac dysfunction due to the induction of smaller infarcts, whereas in the previous studies described the infarcts were larger leading to more severe cardiac dysfunction (Mulder *et al.,* 1997). Unfortunately the plasma ET-1 concentrations were not measured for this thesis because the amount of plasma needed in the RIA protocol was too large to be obtained per animal. A commercial ELISA kit is available which uses a smaller volume of plasma, but during validation studies of the kit in the department it was found that the reliability of the results was questionable. Therefore, activation of the ET-1 system could not be quantified, although immunohistochemical studies (see Sections G. 1.3.1.) have been performed in order to visualise whether there is an alteration in ET-1 production in the vascular smooth muscle layer. Nevertheless it has been assumed that the ET-1 system is activated during the progression of CHF in these rats based on the results of Teerlink *et al.*, (1994b) and Sakai *et al.*, (1996a,b). Thus, the main aim of this chapter was to assess whether there was any alteration in ET-1 receptor characteristics at two time points after the induction of heart failure, since down-regulation of receptors commonly occurs during prolonged agonist exposure.

In all mesenteric arteries studied, ET-1 was a potent constrictor. The constrictions to ET-1 in the sham-operated rats from both groups were biphasic in nature, with a

higher affinity first shallow component to the lower ET-1 concentrations, followed by a lower affinity steeper second component to higher concentrations. The shapes of these ET-1 CRCs would suggest a heterogenous population of ET-1 receptors with constrictor ET_B receptors responsible for the high affinity phase and ET_A receptors producing the lower affinity phase (Mickley *et al.*, 1997). Although not overall significantly different from the sham-operated constrictions in sensitivity (as determined by pD_2) and maximum response, the shape of the ET-1 CRC in the 5 week CHF rat mesenteric arteries appears to be different. The first shallow component is attenuated in the 5 week post-ligation rat arteries, suggesting a down-regulation of the high affinity constrictor receptors. As mentioned above, protracted exposure to a specific agonist can lead to receptor down-regulation and this appears to be borne out as the constrictions to ET-1 in the arteries removed from the 12 week CHF rats were significantly different, the whole CRC being shifted to the right. Furthermore, not only is the sensitivity to ET-1 reduced, but the curve is clearly monophasic in nature, with the shallow component completely lost. Thus, the responses to ET-1 alone tend to suggest that as the disease progresses, the receptors responsible for the constrictions to the lower concentrations of ET-1 are down-regulated.

Down-regulation of ET receptor binding sites has been observed following incubation of ET-1 with cultured vascular smooth muscle cells (Hirata *et al.*, 1988) and cardiocytes (Hirata *et al.*, 1989). More specifically, it has been demonstrated that ET_B receptor mRNA is down-regulated by prolonged exposure to ET-1 by a decrease in stability of the mRNA in rat osteosarcoma cells (Sakurai *et al.*, 1992). ET_B receptors undergo tachyphylaxis readily, and it is the possible down-regulation of these receptors which results in the loss of the high affinity phase of the curve. At 12 weeks post-ligation the lower affinity part of the curve is also shifted, therefore suggesting that ET_A receptors are also down-regulated as the disease state progresses. In the previous chapter, it was demonstrated that the ET_A receptor could accommodate the antagonism/desensitisation of the ET_B receptors by receptor crosstalk. The overall ET-

1 CRC in the 5 week CHF arteries was found not to be significantly different from that of the sham-operated controls. Therefore at this time point of heart failure, the ET_A receptors could be partially compensating for the down-regulated ET_B receptors. If the ET_A receptors were beginning to downregulate at 5 weeks post-ligation, this could explain why the constrictions to the lower concentrations of ET-1 were not completely restored. Therefore, it would be interesting to investigate the responses to exogenous ET-1 in arteries removed from CHF rats at a slightly earlier time point after artery ligation such as 2 or 3 weeks post-ligation.

One study has investigated the ET-1 receptor density and affinity in mesenteric arteries and ventricles from 4 weeks post-LCAL rats, using binding assays and Scatchard analysis (Fu *et al.*, 1993). The Scatchard plots revealed a single population of ET-1 binding sites in both vascular and ventricular membranes. However, in the mesenteric artery membranes from rats with CHF the density of ET-1 receptors was significantly decreased by 59% as compared to sham animals (Fu *et al.*, 1993). Furthermore, the dissociation constant was significantly increased 2.8 times in the mesenteric arteries from the CHF rats. In contrast, neither the density or dissociation constant of ET-1 receptors in the ventricular membranes from the CHF rats were different from the controls (Fu *et al.*, 1993). Unfortunately, this study did not investigate specifically the vascular responsiveness of the mesenteric arteries from these animals to exogenous ET-1. However, a bolus injection of ET-1 into pithed animals showed that there was a decreased pressor response but a preserved depressor response in the CHF animals when compared to control animals (Fu *et al.*, 1993). It was therefore concluded that there is down-regulation of ET_A receptors responsible for vascular constriction, but not relaxant ET_B receptors (Fu *et al.*, 1993).

Other studies have investigated regulation of ET-1 receptors in CHF, although these studies have primarily focused on the receptors present in cardiac and renal tissue.

Raised circulating and tissue ET-1 levels in CHF rabbits were found to be associated with reduced density of ET-1 receptor binding sites in both heart and kidney tissues (Loffler *et al.*, 1993). In another rabbit model of CHF (3 weeks of RVP), Spinale and co-workers (1997) studied the isolated myocyte inotropic response to exogenous ET-1. They found that the ET-1 response was significantly reduced in the myocytes from the CHF rabbits, and this was restored to control levels in CHF rabbits treated with the ET_A receptor antagonist PD 156707 (Spinale *et al.*, 1997). However, in a previous study from the same laboratory no change in myocyte ET_A receptor density of affinity was found in pacing-induced CHF (Thomas *et al.*, 1996). The reduced responsiveness to ET-1, if not by receptor down-regulation, has been suggested to be via the desensitisation of the ET-1 transmembrane signalling pathway (Calderone *et al.*, 1993). A reduced generation of ET-1 stimulated IP_3 accumulation via decreased activiation of PI turnover in the circumflex coronary artery (CCA) of RVP dogs (4-7 weeks of RVP) has been demonstrated (Calderone *et al.*, 1993). Receptor phosphorylation by protein kinase C (PKC) activation resulting in receptor uncoupling was suggested to be acting as a negative feedback mechanism regulating the functional responsiveness of the CCA to ET-1 (Calderone *et al.*, 1993).

In contrast, responsiveness to exogenous ET-1 has been shown to be enhanced in the papillary muscle from RVP dogs (Li & Rouleau, 1996). The sensitivity to ET-1 was augmented in the tissues from the dogs 4-7 weeks after pacing began (Li & Rouleau, 1996). Furthermore, in LCAL rats an increase in myocardial ET receptors was found in association with enhanced ventricular ET-1 mRNA and mature peptide (Sakai *et al.*, 1996a & b). It has been suggested that ET-1 is involved in the maintenance of cardiac function in early heart failure, but all the studies described have been performed between 3 and 7 weeks after the induction of heart failure. Therefore, it appears that ET receptor regulation and responsiveness varies from tissue to tissue, study to study, and model to model.

In order to investigate the receptor subtypes involved in the altered constrictions seen to ET-1, either SRTX S6c, BQ-123, or a combination of both drugs were used to antagonise the ET-1-induced constrictions. Similar to the previous chapter, SRTX S6c was used both as an agonist and antagonist of the ET_B receptor in order to evaluate the relative contribution of the ET_B receptor to the vasoconstrictor response. As an agonist, SRTX S6c evoked small constrictions in 50% and 66% of the 5 and 12 week sham-operated rats respectively of approximately 10% of maximum KCl constriction comparable to those seen in the previous chapter. Small constrictions to SRTX S6c were also seen in 75% of the arteries from the 5 week LCAL rats. However, only one artery from the 12 week LCAL rats constricted (and only 7% of max KCl) to SRTX S6c. It was suggested previously that the arteries which did respond to SRTX S6c had some intrinsic tone. This was investigated by exposing the arteries, at the end of the experiment, to Ca^{2+}-free Krebs-Henseleit solution plus SNP. All arteries, whether exposed to ET-1 alone, SRTX S6c as an agonist or antagonist or any of the other treatments relaxed back to the resting tone measured at the beginning of each individual experiment. Thus, it appears that these arteries had no intrinsic tone present. It has been suggested that constrictor ET_B receptors are present only in the low pressure venous side of the circulation though some may be present in the pre-capillary arterioles. Thus, the arteries in which SRTX S6c induced a constriction could have been of a smaller lumen diameter than those arteries which did not respond. However, all the arteries experimented upon were of a similar size, there was no difference in the lumen diameter of those vessels which responded and those that did not.

Another explanation for the presence of constrictor ET_B receptors in some of the arteries could be the 'plasticity' phenomenon first described for contractile ET_B receptors by Adner and colleagues (1996). They demonstrated that constrictor ET_B receptors become apparent in human omental arteries after being left for up to 5 days in

serum-free medium, which they called cultured arteries. In organ baths they showed a significantly weaker and less potent constriction to ET-3 than ET-1 in fresh omental arteries, of which FR 139317 induced a parallel rightward shift. However, in the cultured arteries contractions to ET-3 were significantly greater in magnitude (100 fold) and sensitivity than the fresh arteries, and there was now no significant difference between the potency of ET-1 and ET-3 in the cultured arteries (Adner *et al.*, 1996). Furthermore, FR 139317 shifted the responses to the high concentrations of ET-1, not the whole ET-1 CRC as seen in the fresh arterial segments. RT-PCR showed mRNA encoding both receptor subtypes of equal intensity in the fresh segments, but in the cultured arteries the band representing the ET_B receptor was a more intense signal than that for the ET_A receptor. The authors concluded that in fresh arteries, despite the presence of mRNA for both receptor subtypes the ET_A receptor only is responsible for contraction. However, after organ culture an increase in mRNA for the ET_B receptor occurs, and there is a corresponding appearance of a functional response (Adner *et al.*, 1996). It was hypothesised that during the organ culture period, the arteries are in an inactive environment where there is no blood flow or nervous or humoral stimulation, and this may result in an up-regulation of functional ET_B receptors (Adner *et al.*, 1996). The loss of pressure on the arterial wall, it was theorised, may reflect the low pressure venous system where ET_B receptors are known to have an important functional constrictor role (Moreland *et al.*, 1994; Haynes *et al.*, 1995).

This 'plasticity' phenomenon may be occurring in the rat mesenteric arteries during this study, since varying times elapsed between removal of the mesenteric bed and the mounting of the arteries in the perfusion myograph. Furthermore, these experiments per mesenteric bed were conducted over a period of two days since there were 4 experimental conditions (ET-1 control, SRTX S6c desensitisation, BQ-123 and combination antagonism), but only two perfusion myographs. Thus, 2 experiments were performed on day 1 with the intact mesenteric bed stored in Krebs solution overnight at 4°C for the second 2 experiments to be performed on Day 2.

Experimental protocols were randomised and studying the data there were reponses to SRTX S6c on both Days 1 and 2 (6 & 4 out of 22 responded on Days 1 & 2 respectively, and 6 & 6 out of 22 did not respond on Days 1 & 2 respectively). However, this explanation cannot be ignored due to the transportation of the mesenteric bed after removal from the Pharmacology department to the laboratory at the Western General Hospital varied from between 1 hour to 3 hours, thus, plasticity of the ET_B receptor could be occurring over this period.

Interestingly, the results of the SRTX S6c used as an agonist do suggest that the arteries from the 12 week CHF rats have no functional contractile ET_B receptors, which could partially explain the reduced ET-1 vasoconstriction. However, when STRX S6c was used as an antagonist, whereby the ET_B receptors were desensitised, the lack of vasoconstriction to STRX S6c as an agonist became more understandable. Comparable to the previous chapters results, STRX S6c desensitisation in the arteries from the sham-operated rats and the 5 weeks post-ligation animals had no effect on the ET-1 CRCs, but the ET-1 CRC in the 12 week CHF arteries was shifted to the left. In fact, the ET-1 response, after SRTX S6c desensitisation, was now biphasic and similar to the vasoconstrictions in the sham-operated control arteries. These results suggest that an inhibitory, ET_B receptor on the VSMC layer has become active by either up-regulation or an alteration of the second messenger systems associated with the ET_B receptors already present on the VSMC layer. Indeed, inhibitory ET_B receptors have been demonstrated previously (Seo, 1996; Mickley *et al.*, 1997). Inhibition of ET_B receptors with Res 701-1 in human denuded gastroepiploic arteries potentiated ET-1 constrictions suggesting the presence of inhibitory ET_B receptors negatively modulating ET_A receptor stimulation (Seo, 1996). Similarly in the previous chapter, SRTX S6c desensitisation or BQ-788 pretreatment slightly, but not significantly, enhanced ET-1 vasoconstrictions (Mickley *et al.*, 1997).

The endothelial ET_B receptor produces vasodilatation by stimulating the release of NO, PGI_2 and possibly EDHF. Whether this putative inhibitory VSMC ET_B receptor acts via similar mechanisms should be investigated by the repeating experiments when in the presence of a NOS or EDHF inhibitor. More specifically, the experiments should be repeated using a cNOS inhibitor since it is believed that the inducible form of NOS is up-regulated in heart failure (or comparison of a non-specific NOS inhibitor such as L-NAME and a specific iNOS inhibitor, e.g. aminoguanidine). However, it is unlikely that these ET_B receptors activate cNOS, since it is usually located in the endothelium, but not in VSMC layer of blood vessels. A COX product like PGI_2 is unlikely to be relevant, as all experiments were performed in the presence of indomethacin.

Intracellular crosstalk might also be involved where the ET_B receptor may lower the affinity of ET-1 for ET_A receptors. Ozaki and colleagues (1997) performed an interesting experiment using transfected human Girardi heart cells. Girardi cells are derived from human atria and are a unique cell line which express ET_B, but not ET_A receptors. Transfection of ET_A receptor cDNA into Girardi cells resulted in co-expression of ET_A/ET_B in the ratio of 4:6. Using binding experiments they showed in the transfected cells that ET_B receptor ligands (BQ-3020 and BQ-788) had low binding affinities, especially when compared to the binding affinities in the non-transfected, ET_B receptor Girardi cells. BQ-123 displaced ^{125}I-ET-1 in the transfected cells in a biphasic manner. However, BQ-3020 and BQ-788 had high affinities for the ET_B receptors in binding experiments where the ET_A receptors had been masked using BQ-123 (Ozaki *et al.*, 1997). The functional abilities of the ET receptors in these cells were assessed by measuring intracellular Ca^{2+} concentrations. It was demonstrated that BQ-123 inhibited 80% of the ET-1-induced increase in intracellular Ca^{2+} concentrations in the transfected cells, whereas BQ-788 had no effect at all. However when BQ-123 and BQ-788 were combined, ET-1-mediated increases in intracellular Ca^{2+} concentrations were completely abolished. In a further experiment, investigating

the inhibitory effects of ET-1 on forskolin-stimulated cAMP accumulation, in the non-transfected cells BQ-788 abolished the ET-1-induced decrease in cAMP levels. However, there was no significant effect of BQ-788 in the transfected cells. Interestingly, there was also no inhibition by BQ-123 of the ET-1 inhibition of adenylate cyclase activity in the transfected cells, but once again, when in combination with BQ-788, BQ-123 completely blocked the ET-1 inhibition of forskolin-induced cAMP accumulation (Ozaki *et al.*, 1997). The authors concluded from these experimental results that intracellular crosstalk mechanisms were occurring, and that stimulation of ET_A receptors induces a characteristic change in ET_B receptors through intracellular signalling (since similar experiments performed using only the cell membranes resulted in no lowering of affinity of the ET_B receptor ligands). Thus, this change alters the affinity of the ET_B receptor ligands for the ET_B receptor possibly by a partial desensitisation of the ET_B receptor (Ozaki *et al.*, 1997). Although these results show an action of the ET_A receptor on the ET_B receptor, it is quite plausible that the ET_B receptor could have an action, via the intracellular G-proteins, on the ET_A receptor. Therefore, the inhibitory ET_B receptor present on the 12 week CHF rat mesenteric arteries could be acting by either: i) inducing a slight conformational change in the ET_A receptor such that it lowers the binding affinity of ET-1; or ii) acting as a break on the stimulatory G-proteins of the ET_A receptor, by part of the ET_B receptor inhibitory G-protein (α or $\beta\gamma$-subunits) binding to that of the ET_A receptor apparatus. Deglycosylation of ET_B receptors can result in a decreased ability of the receptor to bind ligands (Sokolovsky *et al.*, 1992). Furthermore, it has been demonstrated that ET-1 can induce ADP-ribosylation of the inhibitory subunit of G-protein complexes (Kelly *et al.*, 1990; Sokolovsky, 1993). Hence, stimulated ET-1 receptors have the ability to influence G-protein complexes. The paper from Ozaki and colleagues (1997) may also provide a partial explanation for the results in the previous chapter. This will be discussed in the General discussion as this paper was not published until after the completion of the work for the last chapter.

BQ-123 was used to investigate the role of the ET_A receptor. In the sham-operated rat mesenteric arteries BQ-123 inhibited the whole ET-1 vasoconstriction, shifting the whole ET-1 CRC to the right. In contrast to the results of the previous chapters, there was not a biphasic inhibition. The reasons for this are not clear. Although it may be due to the different environments where the animals were bred and housed, as well as seasonal changes and the animals being of different ages. Furthermore, the sham-operated rats did have a surgical procedure performed on them, whereas the rats in the previous chapter did not. The only difference in the experimental conditions, was the presence of indomethacin in the Krebs-Henseleit solution in this study, suggesting that the ET_B receptors in the previous study were releasing a constrictor COX product. However, in this study, in these arteries it appears that ET_A receptors mediate the majority of ET-1-induced vasoconstriction despite the apparent biphasic shape of the ET-1 CRC, although ET_B receptors may play a minor role in the constrictions, as suggested by the small constrictions seen to SRTX S6c. The ET-1 CRC was also shifted to the right by BQ-123 pretreatment in the 5 week post-ligation rat mesenteric arteries. On close examination of the graph the biphasic nature of the CRC appears to be retained, with similar vasoconstrictions to the lower concentrations as those of the control ET-1 CRC,.but with the higher concentrations shifted to the right. This BQ-123-insensitive portion of the ET-1 CRC is probably ET_B receptor mediated. However, as with the sham-operated rat arteries it is the ET_A receptor which mediates the majority of the ET-1 vasoconstriction. The whole monophasic ET-1 CRC in the 12 week CHF rat arteries was shifted to the right in a parallel manner, implying that there were no functional constrictor ET_B receptors present. Curiously, the CRC in the 12 week CHF arteries was shifted less than the CRC in the 12 week sham-operated vessels, but to the same point on the graph such that they were superimposed. This is surprising if there are inhibitory ET_B receptors present. It would be expected that once the ET_A receptors are antagonised that the ET-1 CRC would be shifted further to the right since the inhibitory ET_B receptors would be stimulated and ET-1 will be competing with the antagonist to stimulate the ET_A receptors. A simple explanation for this could be because there are more molecules of ET-1 to bind to the ET_B receptors

when the ET_A receptor binding sites are occupied by BQ-123, the ET_B receptors undergo tachyphylaxis and the restraining influence on the ET_A receptors is removed. Thus, at the higher concentrations of ET-1 and the BQ-123 blockade is being overcome, the ET_A receptors are now free to induce constriction.

Combination non-selective blockade of the ET receptors by BQ-123 and SRTX S6c desensitisation inhibited the entire ET-1 CRC in the sham-operated rat arteries in a parallel manner. In the arteries removed from the 5 week CHF rats the ET-1 CRC was also shifted in a parallel manner, with the vasoconstrictions to the lower concentrations of ET-1 inhibited. This suggests that, similar to the observations in the previous chapter, ET_B receptor inhibition alone (by desensitisation) was compensated for by the ET_A receptor (Mickley *et al.*, 1997), although the ET_B receptor constrictor contribution in these vessels was less than that reported before. Finally, non-selective blockade in the 12 week CHF rat arteries also shifted the ET-1 CRC to the right in a parallel manner.

As has been demonstrated by the acute infusion studies, ET-1 receptor antagonists reverse established ET-1-induced tone (Warner *et al.*, 1994). However it is not known how quickly ET-1 vasoconstriction is reversed by ET-1 receptor antagonism, especially as it believed that ET-1 binding, once it occurs, is essentially irreversible (Marsault *et al.*, 1993). Furthermore, it is believed that the ET-1-receptor complex is internalised where ET-1 is degraded slowly off the receptor which is then externalised for further stimulation (Marsault *et al.*, 1993). Thus, an additional aim of this chapter was to investigate how quickly the antagonists BQ-123 or TAK-044 reversed the established constriction to the highest concentration of ET-1 ($3x10^{-8}M$).

Both BQ-123 and TAK-044 reversed ET-1-induced tone. With both antagonists there remained a partial constriction, although TAK-044 apparently reversed the tone faster and in a more complete manner than the BQ-123. This might reflect the dual inhibitory properties of TAK-044, blocking the constrictor ET_A and ET_B receptors since combination antagonist pretreatment resulted in greater inhibition of the ET-1 CRC than BQ-123 alone. However, it could also reflect the different potencies of the antagonists at the ET_A receptor. The IC_{50}s for BQ-123 (Ihara *et al.*, 1991) and TAK-044 (Kukuchi *et al.*, 1994) at the ET_A receptor are $7.3x10^{-9}$ & $1x10^{-10}$M respectively. The concentration of both antagonists used was 10^{-5}M which is clearly greater than the estimated IC_{50}s. In rat aortic rings established ET-1 constrictions were reversed slowly by BQ-123 (10^{-5}M) and PD 145065 (10^{-5}M), a non-selective ET_A/ET_B receptor antagonist (Warner *et al.*, 1994). However, in this preparation it took approximately 40 min to reverse the tone to similar levels that were attained in the mesenteric arteries within 20 minutes in this study. Furthermore, a 10x higher concentration of ET-1 was used in this study prior to reversal, than was used in the rat aortic ring study (Warner *et al.*, 1994). These results could imply that the whole ET-1-receptor complex internalisation, degradation and subsequent receptor externalisation might occur at a faster rate in resistance arteries than in the larger conduit arteries.

To summarise the results, it appears that in the earlier stages of CHF there is no difference in sensitivity to ET-1 in the resistance arteries of the mesenteric bed. ET_A receptors on the VSMC layer mediated the majority of the response, if not all to ET-1 in the arteries from the sham-operated animals. In the arteries from the 5 week CHF rats, some constrictor ET_B receptors were evident, although ET_A receptors were responsible for most of the constrictions to ET-1. However, at 12 weeks post-infarction the mesenteric arteries have a reduced sensitivity to ET-1 which is not due to receptor down-regulation but appears to be mediated by the emergence of a inhibitory ET_B receptor on the VSMC layer. As a consequence of these results it would suggest that dual non-selective ET_A/ET_B receptor antagonism would be a less advantageous

method than selective ET_A antagonism to reduce ET-1 vasoconstriction in heart failure. Furthermore, it must be stressed that these studies were performed in denuded arteries and the actions of the dilatory endothelial ET_B receptor in counterbalancing ET-1 vasoconstriction have not been investigated. Thus, if in heart failure the endothelial ET_B receptor is preserved, it would be sensible not to inhibit ET_B receptors. In addition, ET_B receptors are partly responsible for the removal of circulating ET-1 and antagonism would result in the further rise of plasma ET-1 levels (Fukuroda *et al.*, 1994c).

Other studies have demonstrated a blunted vasoconstriction to exogenous ET-1 in CHF *in vivo* usually by infusion of ET-1 into anaesthetised animals. A reduced pressor response to ET-1 was shown in 3 week post-ligation CHF rats (Fu *et al.*, 1993) and attenuated systemic and regional vascular constrictor responses in the RVP dog model were also demonstrated (Cavero *et al.*, 1990). In the TIVCC dog model of CHF an intracoronary infusion of ET-1 produced an attenuated reduction of coronary blood flow and vascular resistance when compared to control dogs (Cannan *et al.*, 1996). Curiously, it was found that intracoronary SRTX S6c induced a significant decrease in coronary blood flow and vascular resistance in the TIVCC dogs, whereas in the control animals there was no effect on the coronary vasculature. Furthermore, SRTX S6c induced a 2 to 3 times greater coronary vasoconstriction in the CHF dogs than ET-1. Therefore, in this model of CHF in the coronary bed, there may have been an hypotensive ET_A receptor modulating the vasoconstrictor actions of up-regulated ET_B receptors. If this occurs in the human setting of CHF, it would obviously be advantageous to inhibit ET_B receptors.

Endothelin receptor antagonists have been used in several animal models of CHF to assess whether blockade of the ET system lowers vascular resistance, reduces cardiac hypertrophy and improves survival. The first study investigating the usefulness of ET

antagonists in heart failure using the LCAL rat model was Teerlink and colleagues (1994b) utilising the orally active non-selective ET_A/ET_B receptor antagonist bosentan. A single high dose of bosentan (100mg/kg) administered to rats 2, 4 and 8 weeks post-ligation significantly decreased mean arterial pressure in conscious CHF rats over a 48 hour period, with the effect on MAP increasing in the later stages of heart failure. However, it was found that bosentan also lowered that MAP in the respective sham-operated rats to a similar degree. Thus, bosentan was acting as a hypotensive agent. However, similar results were seen using a single dose of the ACE inhibitor cilazapril. The most important finding of this study was that a combination therapy of bosentan and cilazapril reduced the MAP to a greater extent than when given alone, having synergistic effects on each others actions (Teerlink *et al.*, 1994b).

Acute administration of BQ-123 for 2 hours in 3 week CHF rats (Sakai *et al.*, 1996a) resulted in a decrease in heart rate and myocardial contractility, but did not affect MAP or any of the haemodynamic parameters in the sham-operated rats. Since the heart rate of the CHF rats was similar to the sham-operated rats prior to, and was significantly reduced after, BQ-123 infusion, and the LVEDP was *increased* by BQ-123, it was suggested that the activation of the ET-1 system at this early time-point in heart failure was to maintain cardiac function.

ET_A blockade by an acute infusion of FR 139317 into TIVCC dogs resulted in significant decreases in MAP and, more importantly in relation to this thesis, systemic vascular resistance (SVR). However, the renal vascular resistance (RVR) was increased (Clavell *et al.*, 1996). In the microembolism dog model of CHF, bolus dose of bosentan also significantly atttenuated SVR, as well as LVEDP, and as a result increased cardiac output (Shimoyama *et al.*, 1996). However, in both studies heart rate in the CHF dogs was unaffected by ET_A receptor inhibition, as were all the haemodynamic parameters in the sham-operated control dogs (Clavell *et al.*, 1996;

Shimoyama *et al.*, 1996). Therefore, in these studies it is clear that ET-1 is involved in the increased SVR, and the antagonists were not acting simply as general hypotensive agents. Thus, the ET-1 system is activated and is involved in the systemic vasoconstriction characteristic of CHF.

Sakai *et al.* (1996b) were the first group to demonstrate the beneficial effects of ET-1 receptor antagonism in heart failure. In LCAL rats, BQ-123 was administered using a subcutaneously implanted mini osmotic pump for 12 weeks. BQ-123 administration almost doubled the number of CHF rats surviving. The 12 week survival of rats treated with BQ-123 was 85% as compared with 43% of the CHF animals treated with saline (Sakai *et al.*,1996b). The LVEDP was significantly reduced and myocardial contractility improved in the treated CHF group. Furthermore, the raised central venous pressure was completely reversed back to that of the sham-operated animals (Sakai *et al.*,1996b). In addition, BQ-123 treatment significantly slowed the progression of left ventricular dysfunction and prevented ventricular remodelling (Sakai *et al.*,1996b).

In an impressive study by Mulder and colleagues (1997), LCAL rats were treated for either 2 or 9 months with bosentan or placebo control. Two doses of bosentan were used, 30 or 100 mg/kg/day taken in the food. Only the highest dose had any effect (Mulder *et al.*, 1997), so all the results discussed are concerned with the animals treated with 100 mg/kg. At 9 months, bosentan significantly improved survival (47% untreated vs 65% bosentan). At 2 months, bosentan treatment had no effect on mortality, because survival was so high (Mulder *et al.*, 1997). Furthermore, bosentan treatment had significant effects on the haemodynamics of CHF rats. MAP, heart rate, central venous pressure, LVEDP, plasma catecholamines, urinary cGMP, left ventricular (LV) collagen, LV dilatation and hypertrophy were all significantly reduced and contractility of the non-infarcted LV wall improved by treatment. Therefore, high

dose bosentan reduced afterload and preload via arterial and venous dilatation, and improved cardiac output through a combination of the arterial and venous effects and the cardiac effects of reduced hypertrophy, fibrosis and dilatation (Mulder *et al.*, 1997).

From the results of Sakai and colleagues (1996b) and Mulder and co-workers (1997) it is obvious that ET-1 receptor antagonism is beneficial in heart failure. However, it is still unclear whether a selective ET_A or non-selective ET_A/ET_B antagonist should be used for the treatment of CHF. It was the aim of this chapter to elucidate the receptors present on the VSMC wall of resistance arteries removed from CHF rats at two different time points. In the earlier stage of CHF there was no alteration in the sensitivty to ET-1, although there was an emergence of an ET_B receptor mediating the constrictions at the lower concentrations of ET-1. However, as in the sham-operated healthy mesenteric arteries, the ET_A receptor mediates the majority of the vasoconstriction. At a later stage of CHF there was a reduced vasoconstrictor response to ET-1 which could be attributed to the emergence of an inhibitory ET_B receptor on the VSMC layer. This result is in agreement with *in vivo* studies showing a reduced pressor response to exogenous ET-1 although no other study has investigated the functional ET-1 receptors present on the vasculature of CHF animals. Therefore, these findings are novel and suggest that an ET_A receptor antagonist, rather than a combined ET_A/ET_B receptor antagonist, should be more effective in reducing the peripheral vascular resistance characteristic of CHF. Furthermore, despite powerful constriction to ET-1 in these vessels the established constrictor response can be reversed quite readily. This latter observation might be important in other clinical conditions, such as acute renal failure or subarachnoid haemorrage, where rapid reversal of ET-1 constriction of renal and cerebral resistance vessels would be required.

	5 week Sham-Operated	*5 week Post-Ligated*	*12 week Sham-Operated*	*12 week Post-Ligated*
Resting Diameter	292 ± 9.8	294.4 ± 7.6	284.8 ± 8.8	304.6 ± 12.1
KCl Diameter	57.6 ± 2.8	55.5 ± 1.8	55.6 ± 2.2	59.6 ± 2.9
PE Diameter	54.4 ± 2.4	52.6 ± 2.8	56 ± 2.8	61.7 ± 2.7

Table 4.1. Lumen diameter of arteries (μm) from all experimental groups when resting, during maximum 60 mM KCl constriction and maximum PE 10^{-5} M constriction. No difference in maximal KCl or PE induced constrictions was observed in the arteries from the 5 week and 12 week CHF rats when compared to the respective sham-operated controls.

	5 week Sham-operated	*5 week Post-ligated*	*12 week Sham-operated*	*12 week Post-ligated*
pD_2 value for ET-1 CRC	9.29	9.2	9.49	8.87 *
pD_2 value for SRTX S6c desensitisation	9.33	9.25	9.42	9.4 **

Table 4.2. Table comparing the pD_2 values of the control ET-1 CRCs and after SRTX S6c desensitisation in 5 week and 12 week CHF rat arteries compared with the respective sham-operated controls. The pD_2 value for the ET-1 CRC in the arteries from the 12 week CHF rats is significantly lower than the pD_2 value for the ET-1 CRC in the respective sham-operated control (* P=0.04, Students t-test). After SRTX S6c desensitisation in these 12 week CHF rat arteries the pD_2 value was restored to similar value of the 12 week sham-operated controls, and is now significantly different from the control ET-1 CRC pD_2 (** P=0.04).

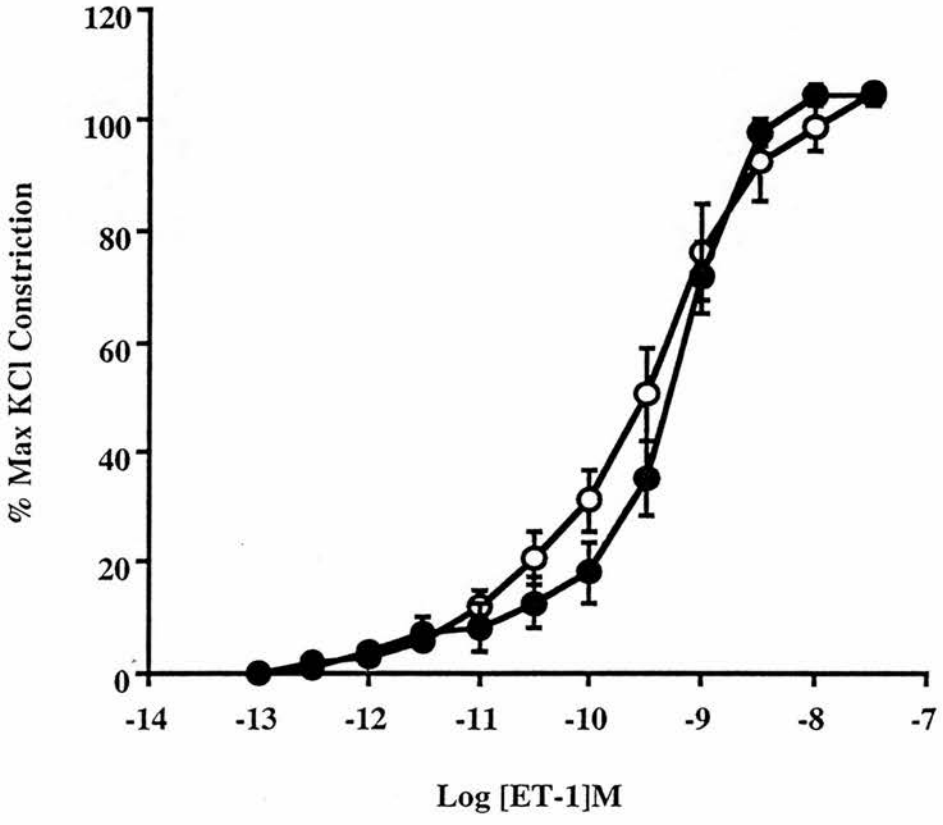


Figure 4.1.A. Comparison of the contractile responses to ET-1 in small mesenteric arteries from 5 week post infarct, CHF rat (● , n=8) and control 5 week sham-operated rats (O , n=8). There was no difference in the sensitivity of the arteries to ET-1 from the CHF rats when compared with the sham-operated animals (P>0.05 ANOVA).

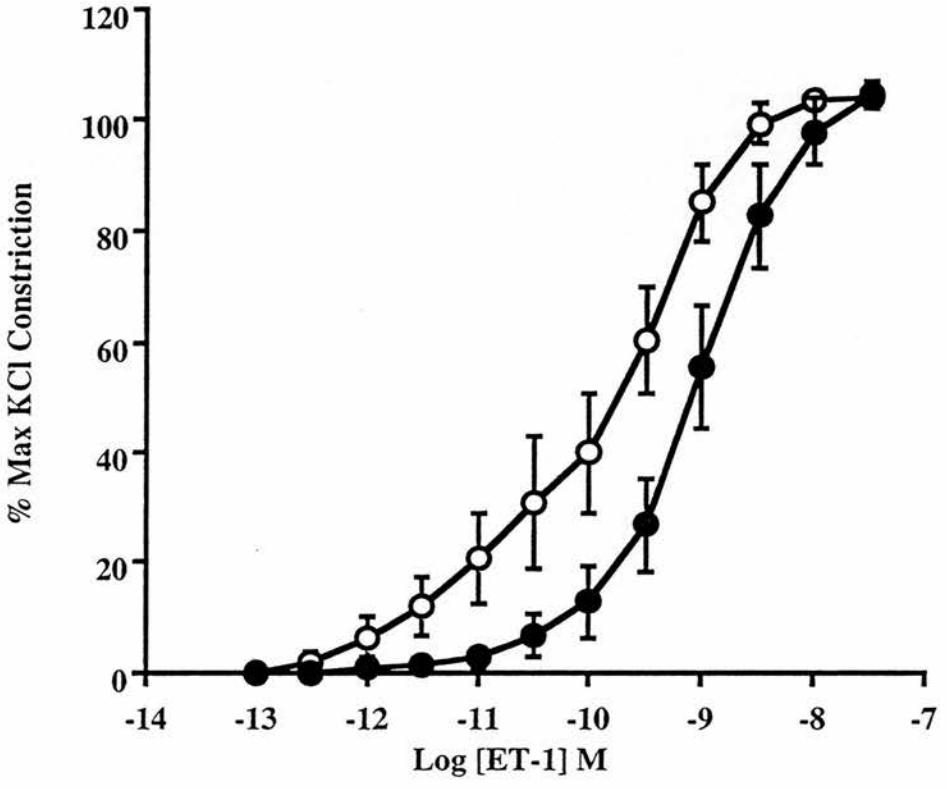


Figure 4.1.B. Comparison of the contractile responses to ET-1 in small mesenteric arteries from 12 week post infarct, CHF rat (●, n=8) and control 12 week sham-operated rats (O , n=8). The sensitivity of the arteries from the CHF rats was significantly reduced when compared with the sham-operated animals ($P<0.05$ ANOVA).

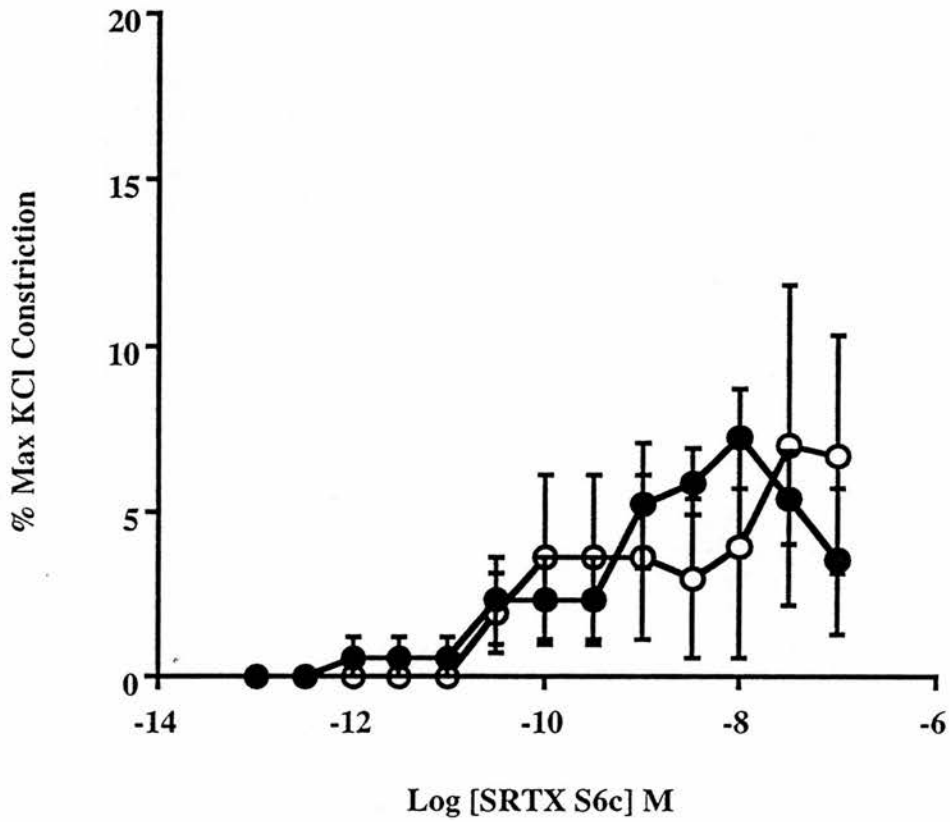


Figure 4.2.A. SRTX S6c-induced constrictions in the mesenteric arteries from the 5 week animal groups. SRTX S6c induced small constrictions in 3 out of 6 of the vessels studied from the 5 week sham-operated rats (O) and 3 out of 4 of the vessels studied from the 5 week post-ligation rats (●). In the latter group, tachyphlaxis of the ET_B receptor can be seen at the highest concentration of SRTX S6c.

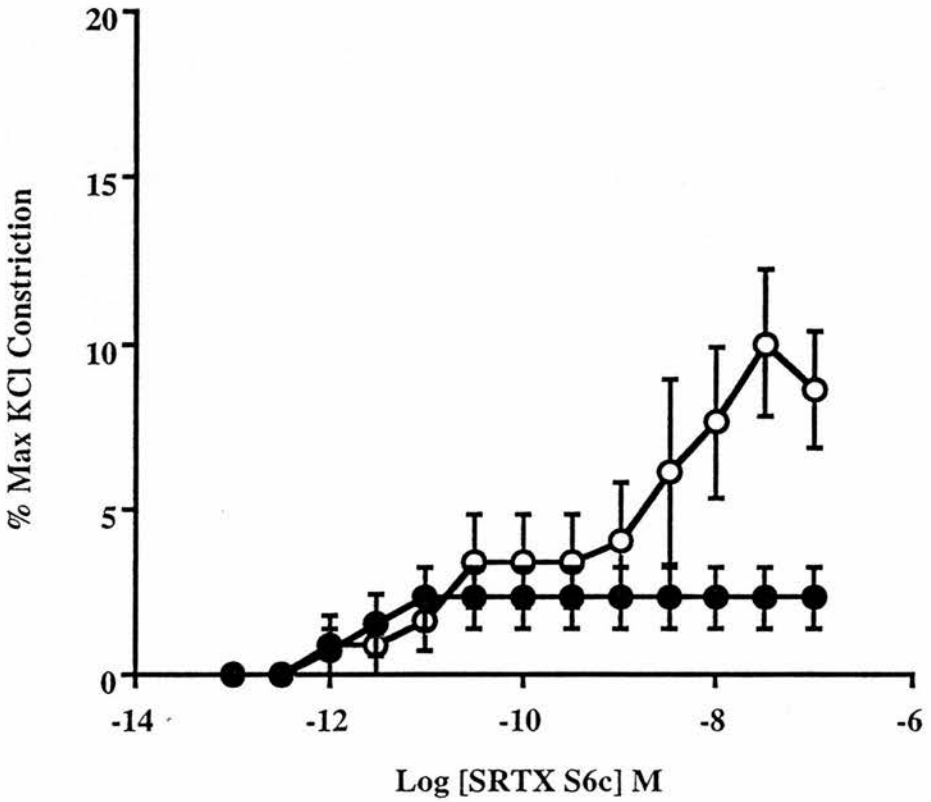


Figure 4.2.B. SRTX S6c-induced constrictions in the mesenteric arteries from the 12 week animal groups. SRTX S6c induced small constrictions in 4 out of 6 of the vessels studied from the 12 week sham-operated rats (O). However, only 1 out of 6 of the vessels studied from the 12 week post-ligation rats (●) constricted to SRTX S6c. In the sham-operated rats, tachyphlaxis of the ET_B receptor can be seen at the highest concentration of SRTX S6c.

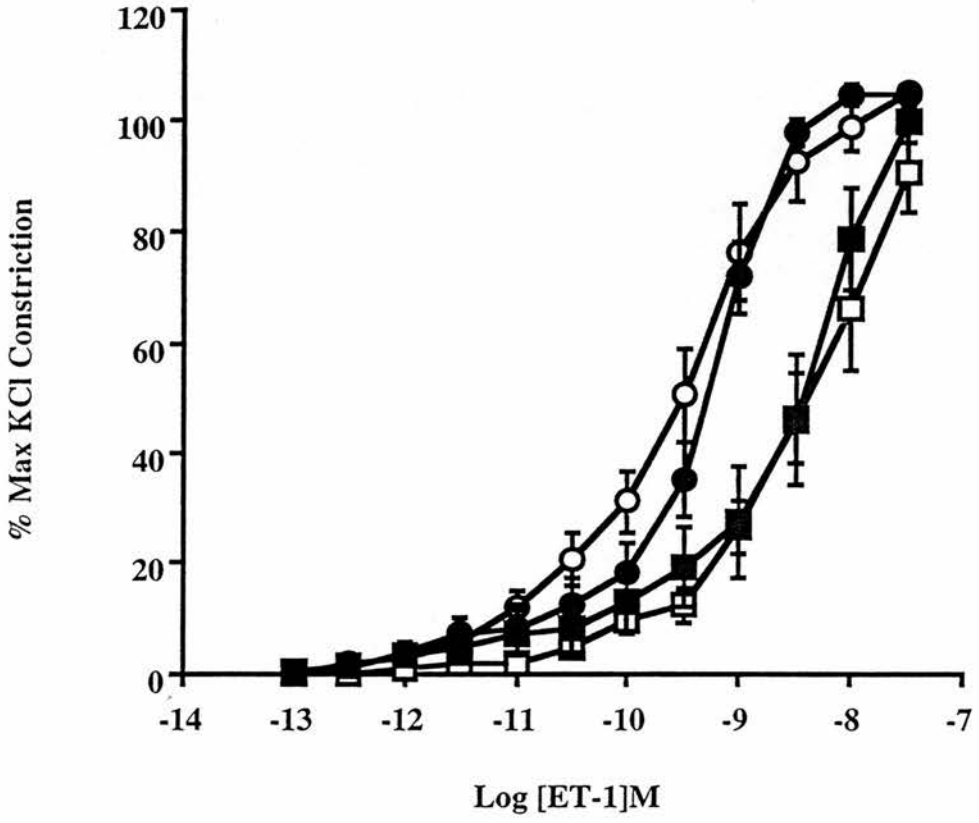


Figure 4.3.A. The effect of the ET_A receptor antagonist BQ-123 on the ET-1 concentration-response curve (CRC) in the small mesenteric arteries from 5 week post- infarct, CHF rats (●, n=8) and 5 week sham-operated control rats (O , n=8). Preincubation with BQ-123 (10^{-6}M) for 30 min shifted the entire ET-1 CRC in the mesenteric arteries from both the CHF rats (■ , n=6) and sham rats (□ , n=6). $P<0.05$ as compared to the respective control ET-1 CRCs.

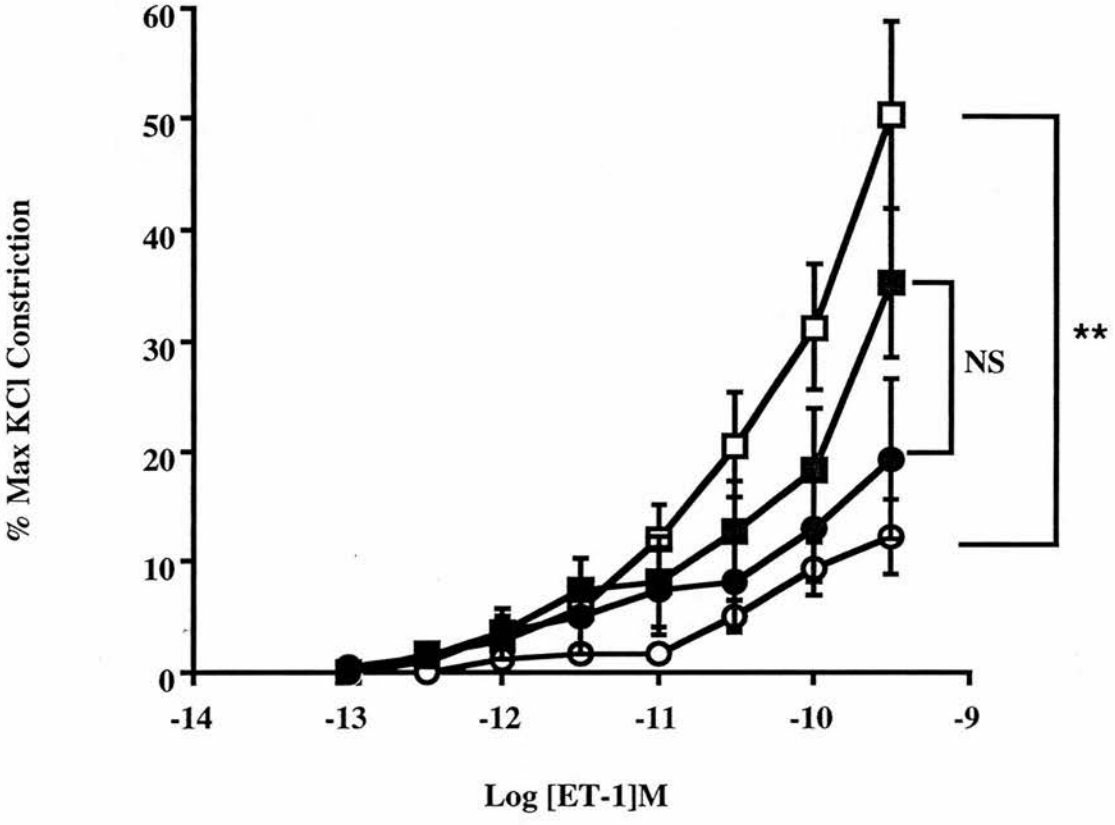


Figure 4.3. B. The effects of BQ-123 on the lower concentrations of ET-1-induced constrictions in arteries from 5 week post-infarct CHF rats and sham-operated controls. In the sham-operated rat arteries the constrictions to ET-1 (□ ,n=8) were significantly inhibited by BQ-123 (O , n=6, **P=0.003, ANOVA). However, the constrictions to the lower concentrations of ET-1 (■ , n=8) in the CHF rat arteries were not significantly (NS) inhibited by BQ-123 (● , n=6) when compared by ANOVA (P=026).

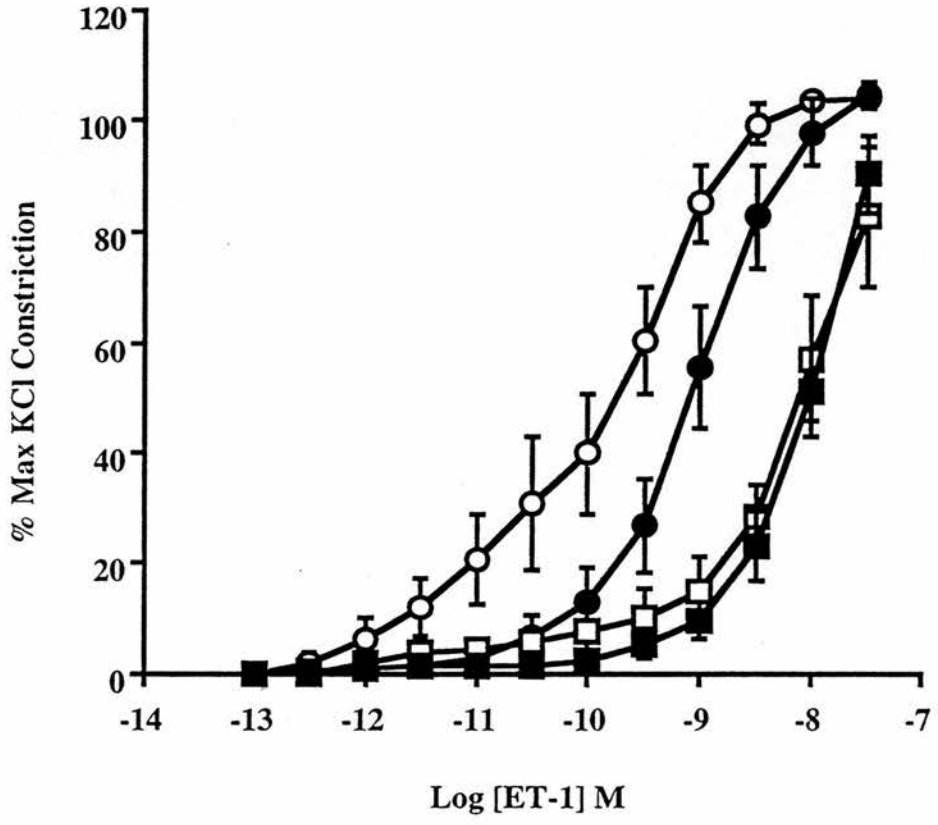


Figure 4.3.C. The effect of the ET_A receptor antagonist BQ-123 on the ET-1 concentration-response curve (CRC) in the small mesenteric arteries from 12 week post- infarct, CHF rats (●, n=8) and 12 week sham-operated control rats (O , n=8). Preincubation with BQ-123 (10^{-6}M) for 30 min shifted the entire ET-1 CRC in the mesenteric arteries from both the CHF rats (■ , n=6) and sham rats (□ , n=6). $P<0.05$ as compared to the respective control ET-1 CRCs.

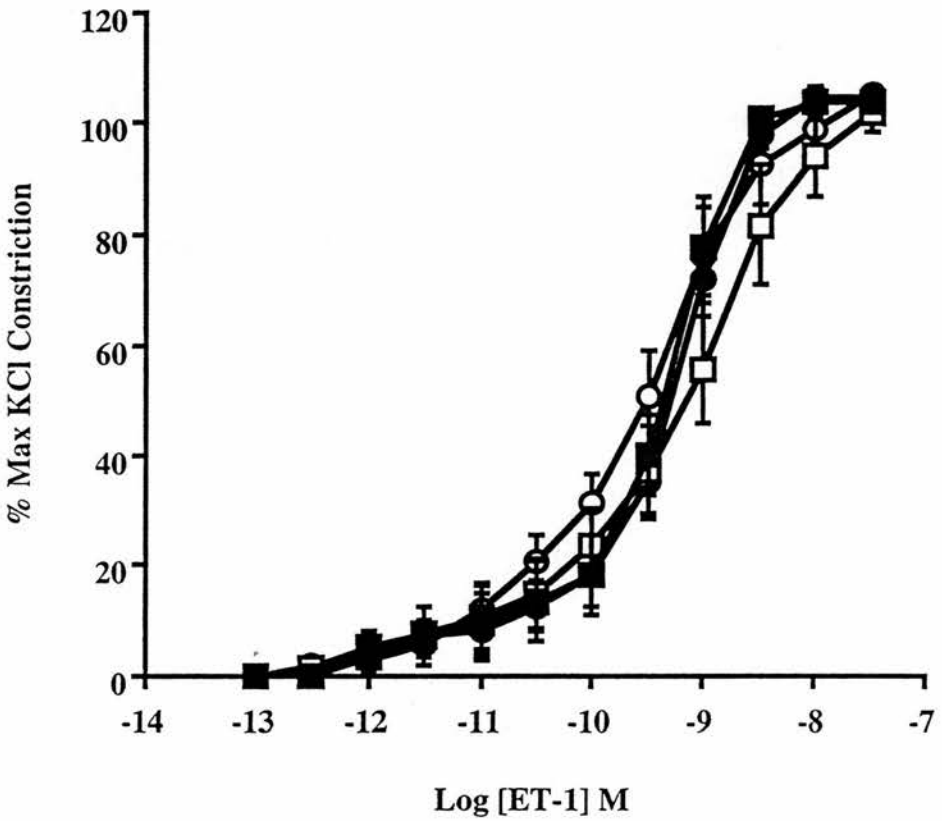


Figure 4.4.A. The effect of ET_B receptor desensitisation on ET-1-induced vasoconstrictions in the small mesenteric arteries from 5 week post-infarct, CHF rats and 5 week sham-operated rats. ET_B receptor desensitisation by prolonged SRTX S6c exposure had no effect on the ET-1 CRC in the sham-operated arteries (□ , n=6) as compared to the arteries challenged with ET-1 CRC alone (○ , n=8) or in the 5 week CHF rat mesenteric arteries (■ , n=5) when compared to the vehicle control ET-1 CRC (●, n=8).

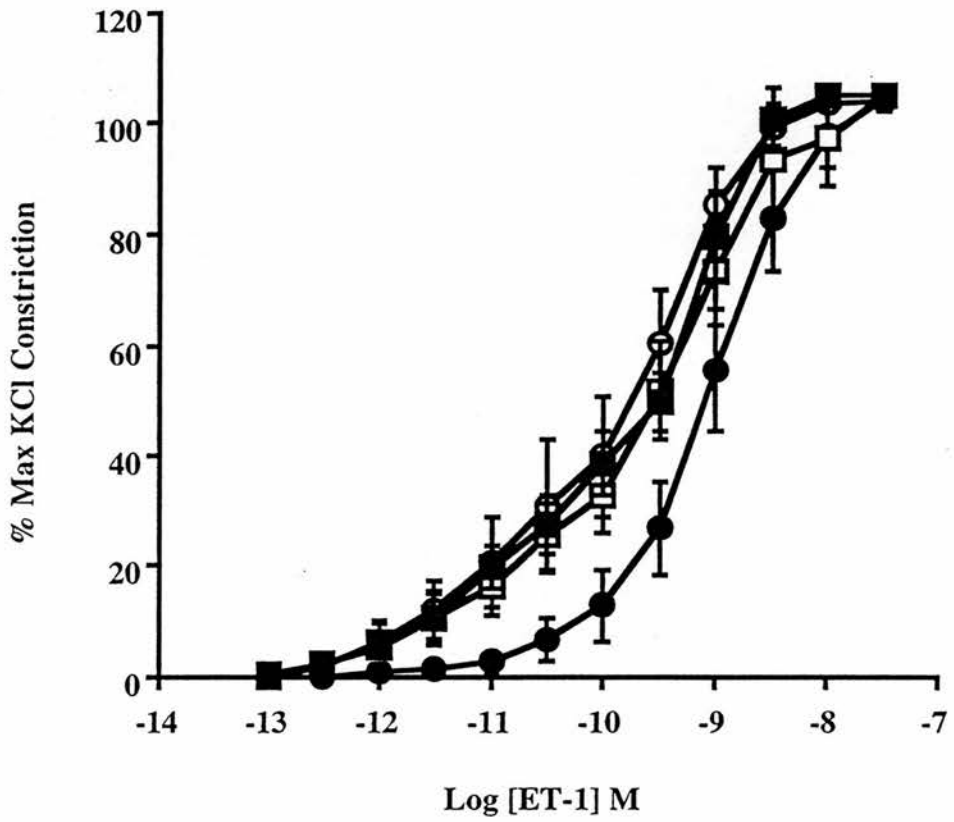


Figure 4.4.B. The effect of ET_B receptor desensitisation on ET-1-induced vasoconstrictions in the small mesenteric arteries from 12 week post-infarct, CHF rats and 12 week sham-operated rats. ET_B receptor desensitisation by prolonged SRTX S6c exposure had no effect on the ET-1 CRC in the sham-operated arteries (□ , n=6) as compared to the arteries challenged with ET-1 CRC alone (○, n=8). However, the sensitivity to ET-1 was restored by ET_B receptor desensitisation in the 12 week CHF rat mesenteric arteries (■ , n=6), being significantly shifted to the left when compared to the vehicle control ET-1 CRC (●, n=8; ANOVA $P<0.05$).

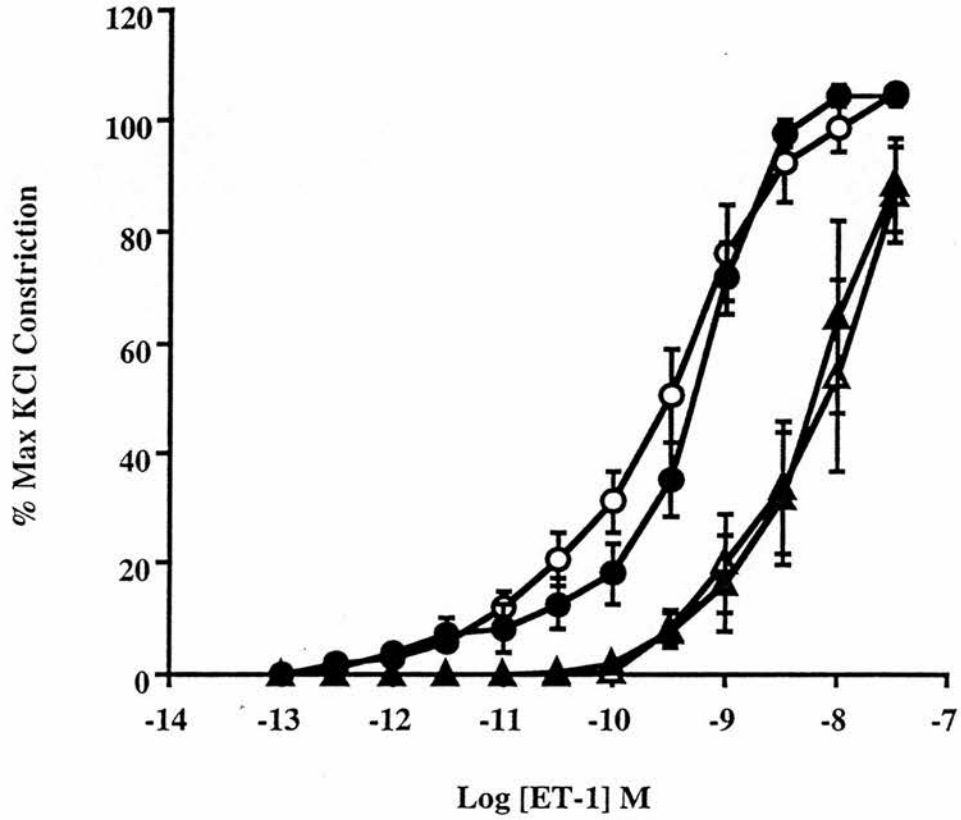


Figure 4.5.A. Non-selective ET_A/ET_B receptor antagonism shifted the ET-1 CRC in arteries from both 5 week animal groups to the right. The constrictions to ET-1 after BQ-123 and SRTX S6c exposure in the 5 week CHF (▲, n=4) and sham-operated controls (Δ, n=6) were significantly difference from the respective ET-1 CRC controls, 5 week CHF (●, n=8) and sham-operated rats (O, n=8), $P<0.05$ ANOVA.

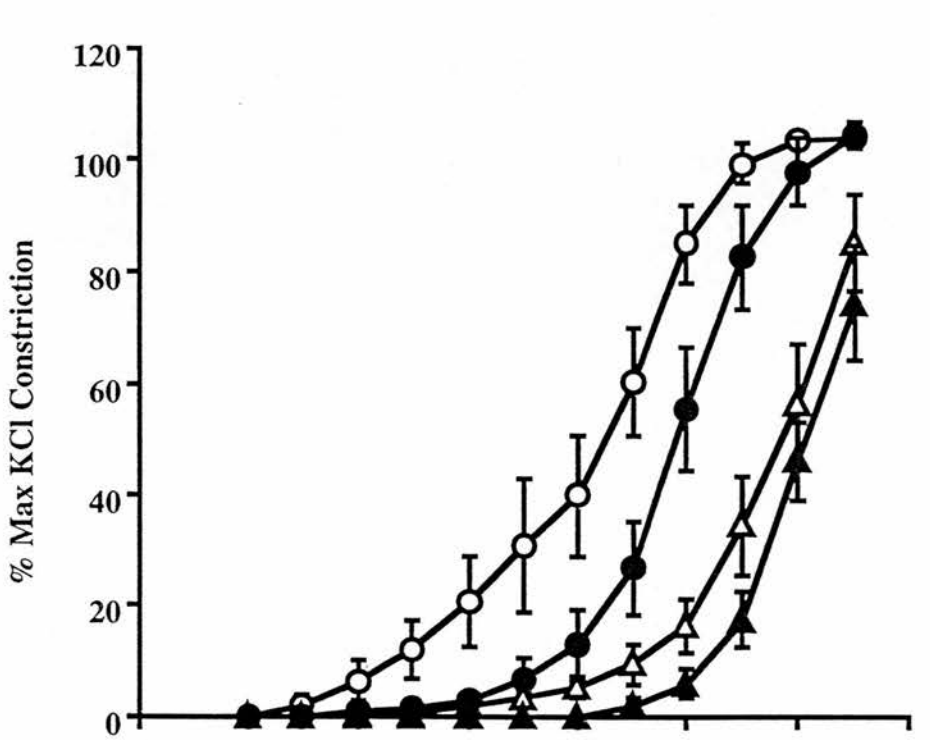


Figure 4.5.B. Non-selective ET_A/ET_B receptor antagonism shifted the ET-1 CRC in arteries from both 12 week animal groups to the right. The constrictions to ET-1 after BQ-123 and SRTX S6c exposure in the 12 week CHF (▲, n=6) and sham-operated controls (Δ, n=6) were significantly difference from the respective ET-1 CRC controls, 12 week CHF (●, n=8) and sham-operated rats (O, n=8), $P<0.05$ ANOVA.

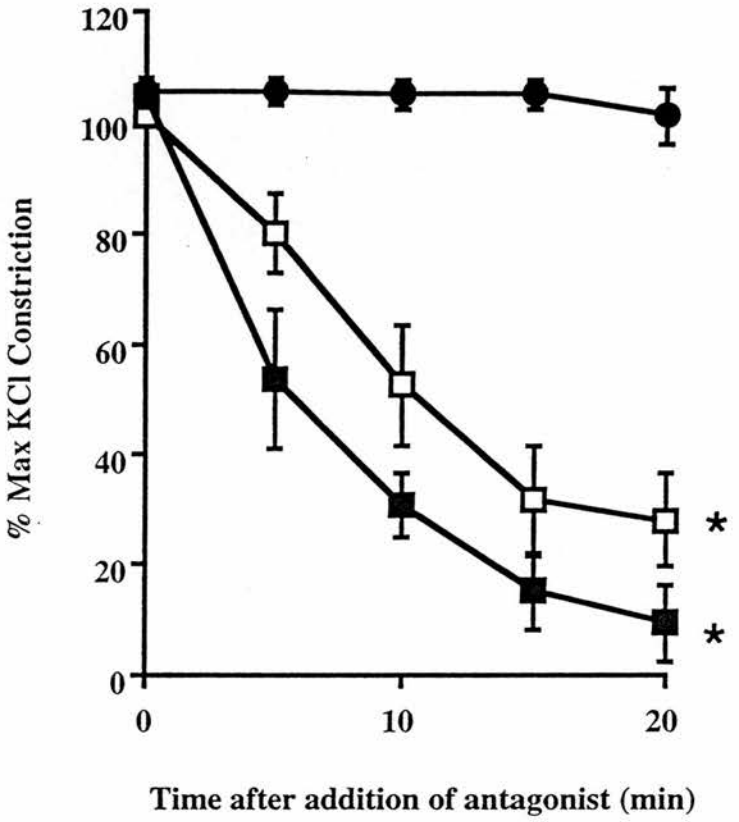


Figure 4.6. A. Reversal of established ET-1-induced tone in arteries from 5 week CHF and sham-operated rats. After maximum constriction was established with the highest concentration of ET-1 either BQ-123 (10^{-5}M, n=5,□), TAK-044 (10^{-5}M, n=6,■) or vehicle (n=5,●) was added to the superfusate and reperfused for 20 minutes with lumen diameters recorded at 0, 5, 10, 15 and 20 minutes. * indicates P<0.01 ANOVA when either BQ-123 or TAK-044 reversal curves were compared to the vehicle control curve.

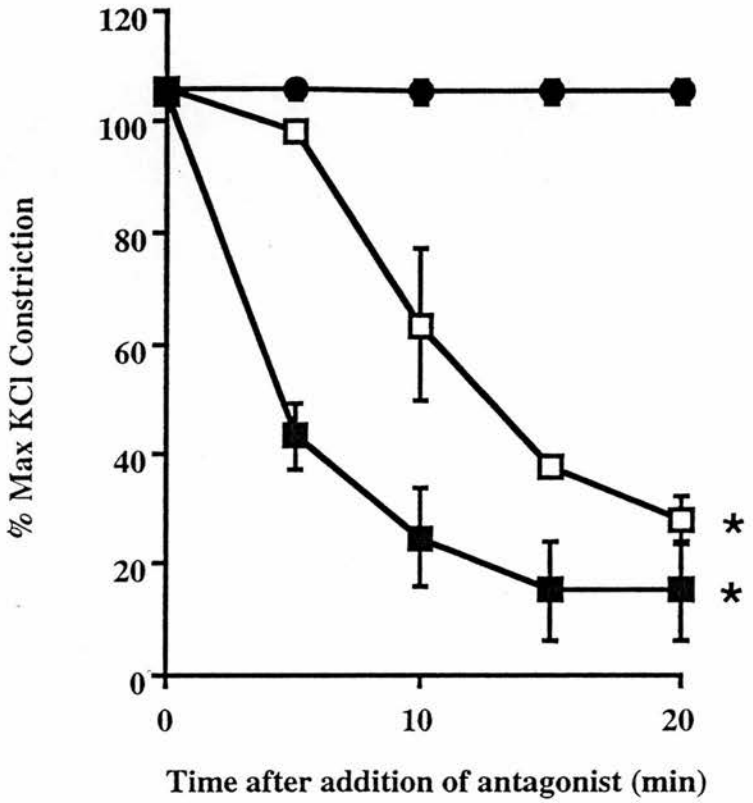


Figure 4.6. B. Reversal of established ET-1-induced tone in arteries from 12 week CHF and sham-operated rats. After maximum constriction was established with the highest concentration of ET-1 either BQ-123 (10^{-5}M, n=5,□), TAK-044 (10^{-5}M, n=6,■) or vehicle (n=5,●) was added to the superfusate and reperfused for 20 minutes with lumen diameters recorded at 0, 5, 10, 15 and 20 minutes. * indicates $P<0.01$ ANOVA when either BQ-123 or TAK-044 reversal curves were compared to the vehicle control curve.

Results Chapter 3

Attenuated constrictor responses to ET-1 in gluteal small arteries from CHF patients: A result of raised plasma big ET-1 levels ?

F. 1.1. Introduction

The majority of studies in human heart failure subjects have focused solely on the circulating levels of ET-1. Activation of the ET-1 system has been demonstrated repeatedly in heart failure where plasma ET-1 (Cody *et al.*, 1992; Lerman *et al.*, 1992; McMurray *et al.*, 1992; Rodeheffer *et al.*, 1992) or big ET-1 (Pacher *et al.*, 1993; Wei *et al.*, 1994; Pacher *et al.*, 1996) levels are raised. However, functional studies, where locally acting doses of ET-1 and SRTX S6c were infused into the forearm resistance bed (Love *et al.*, 1996a), have also been performed. Consistent with increased ET-1 generation in CHF, there was a reduced vasoconstrictor response to ET-1 in the resistance beds of the CHF patients when compared to the normal control subjects. In contrast, the vasoconstriction to SRTX S6c was enhanced in the CHF patients (Love *et al.*, 1996a). This work suggests there is an up-regulation of constrictor ET_B receptors in human CHF. Furthermore, similar observations were reported in the coronary resistance bed in a dog model of CHF (Cannan *et al.*, 1996; See E. 1.4.).

A handful of ET antagonist studies have recently been performed in human heart failure. Forearm infusions of the ECE inhibitor phosphoramidon and the ET_A receptor antagonist BQ-123 both increased forearm blood flow (vasodilatation; Love *et al.*, 1996a) in CHF patients. Intriguingly, the selective ET_B receptor antagonist BQ-788, when infused into the brachial arteries of CHF patients, induced a decrease in forearm blood flow (Love *et al.*, 1996b). This observation implies that the endothelial ET_B receptor dilator response predominates in the vasculature of CHF patients despite possibly up-regulated ET_B constriction. Nevertheless, the first clinical trial of an ET antagonist in CHF showed that a systemic infusion of the non-selective ET_A/ET_B antagonist bosentan increased cardiac output and decreased systemic and pulmonary vascular resistance (Kiowski *et al.*, 1995).

In vitro experiments on resistance arteries from heart failure patients have shown impaired constrictions to various agonists (Angus *et al.*, 1993). Arteries were obtained from CHF patients by means of a skin biopsy from the buttock area and maximum

contractions to KCl, NA, ANG I and ANG II were significantly reduced compared to the responses seen in arteries from healthy volunteers. These observations may reflect the activation of the sympathetic nervous and RAA systems in CHF.

The aim of this chapter was to assess the reactivity and sensitivity to ET-1 of small arteries from gluteal biopsies in patients with heart failure and age-matched controls. Furthermore, up-regulation of constrictor ET_B receptors in the arteries from the CHF patients was investigated using STRX S6c as an agonist. The relative contributions of the ET-1 receptors were also assessed using BQ-123, SRTX S6c desensitisation and a combination of both treatments. The reversal of established ET-1 tone was investigated using BQ-123 and TAK-044. Furthermore, in order to ascertain whether there is an activation of the ET-1 system in the CHF patients, radioimmunoassay for ET-1 and big ET-1 was performed on plasma samples from all subjects.

F. 1.2. Methods

F. 1.2.1. *Biopsy procedure*

All biopsies were undertaken in the morning and the subjects were asked to abstain from eating and drinking caffeine-containing beverages from 12 hours prior to the biopsy being performed. Furthermore, the CHF patients were asked to refrain from taking their medication on the morning of the biopsy. In all subjects an ECG, blood pressure and heart rate were measured and blood samples were taken before starting the biopsy procedure. The gluteal biopsy (~0.75 cm wide x 0.75 cm deep x 2 cm long) was removed from the right buttock under local anaesthesia and immediately transferred to cold Krebs-Henseleit solution (See Section C. 1.1.3.)

F. 1.2.2. *ET-1 & big ET-1 radioimmunoassay*

Plasma ET-1 and big ET-1 levels were measured by radioimmunoassay. ET-1 and big ET-1 were extracted by acidification of the plasma samples using 20% acetic acid (2.5ml:2.5ml) and applied to preactivated extraction columns (using 10% acetic acid or trifluoroacetic acid for ET-1 or big ET-1 extraction respectively) and eluted with the

appropriate elution buffer (See Section C. 3.2.1.). The eluates were dried under nitrogen and reconstituted with assay buffer. The samples were incubated with the appropriate antibody (Ab). The anti-human ET-1 Ab was incubated for 4 hours at room temperature or with the anti-human big ET-1 Ab overnight at room temperature. Following Ab incubation, ^{125}I-ET-1 or ^{125}I-big ET-1 were added to the samples and incubated overnight at 4°C. The Ag/Ab complexes were separated using Amerlex, the supernatant aspirated and the radioactivity of the resulting pellets counted in a Gamma counter.

F. 1.2.3. *Perfusion myograph studies*

Arteries were dissected from the biopsy whilst immersed in cold Krebs-Henseleit solution. All of the following studies were carried out in random order and only one ET-1 CRC was performed per tissue.

F. 1.2.3.1. *ET-1 and SRTX S6c study*

After dissection, arteries were mounted in a perfusion myograph and the initial 'wake up' and denudation procedures performed. After the reperfusion circuit had been started, arteries were superfused for 1 hour with Krebs-Henseleit solution and a ET-1 CRC (10^{-13}-$3x10^{-8}$M) was obtained. Cumulative CRCs to SRTX S6c (10^{-12}-$3x10^{-8}$M) were also performed in different arteries. However, in these arteries the SRTX S6c CRC was constructed 5 min after the reperfusion circuit had been set up because these arteries were used as the SRTX S6c desensitisation group (See below F. 1.2.3.2.).

F. 1.2.3.2. *Receptor antagonism study*

Some arteries were exposed to 30 min reperfusion of Krebs-Henseleit solution followed by a 30 min incubation with BQ-123 (10^{-6}M) and an ET-1 CRC (10^{-13}-$3x10^{-8}$M) obtained. In the arteries which had been exposed to the SRTX S6c CRC, the arteries were washed with Krebs-Henseleit solution, a new reperfusion circuit started and challenged with SRTX S6c ($3x10^{-8}$M) for 30 min and an ET-1 CRC constructed. This constituted the ET_B receptor desensitisation group. For the non-selective ET_A/ET_B

receptor antagonist group, arteries were challenged with SRTX S6c ($3x10^{-8}$M) for 30 min twice (with a wash out period of 10 min between each exposure) plus BQ-123 (10^{-6}M) in the second 30 min period prior to a ET-1 CRC.

F. 1.2.3.3. *Reversal study*

In the vehicle control vessels which were exposed to an ET-1 CRC only, once the maximum constriction to the highest concentration of ET-1 ($3x10^{-8}$M) was established, either BQ-123 (10^{-5}M), TAK-044 (10^{-5}M) or vehicle control (Krebs-Henseleit solution) was added to the reperfusion circuit and reperfused for 20 min. Lumen diameter measurements were taken at 0, 5, 10, 15 and 20 min after the addition of the antagonist.

F. 1.2.3.4. *Intrinsic tone*

At the conclusion of all experiments, arteries were exposed to Ca^{2+}-free Krebs-Henseleit solution (by addition of 10^{-4}M EDTA) and 10^{-5}M SNP. The lumen size of the relaxation was compared to the lumen size at the beginning of the experiment, before the SRTX S6c CRC and ET-1 CRC.

F. 1.3. Results

F. 1.3.1. *Haemodynamic parameters*

BP, HR and ages were similar between the CHF patients and control (Tables 5.1. & 5.2., mean ages were 67.9±3.6 vs 66.7±3.0 years, controls vs CHF; P=0.8, Students t-test). Mean heart rate 67.7±2.3 vs 71.6±4.3 beats per min, controls vs CHF; P=0.5, Students t-test), systolic blood pressure (136.2±5.9 vs 137.5±7.9 mmHg, controls vs CHF; P=0.9, Students t-test) and diastolic blood pressure (80.0±2.7 vs 77.5±3.7 mmHg, controls vs CHF; P=0.6 Students t-test) were all similar. Serum creatinine, urea and glucose were all within normal limits, confirming the lack of renal impairment in the CHF patients. The NYHA Class and drugs prescribed were all obtained from the patients' notes (Table 5.1.). One patient was NYHA Grade I, seven patients were NYHA Grade II and four patients were NYHA Grade III (See Table 5.1). All patients

took aspirin and 11 out of 12 patients took diuretics. All patients were using ACE inhibitors, except for one patient who was administered losartan as an alternative. Other drugs administered were nitrates (5 out of 12), digoxin (3 out of 12) and β-blockers (1 out of 12). Ischaemic heart disease was the cause of heart failure in all patients.

F. 1.3.2. *Plasma ET-1 and big ET-1 levels*

The plasma ET-1 levels were similar between age-matched controls and CHF patients (3.4±0.3 vs 3.3±0.1 pg/ml patients vs controls; P=0.7, Students t-test; Figure 5.1.). However, the levels of big ET-1 were significantly higher in the plasma from the CHF patients as compared to the age-matched controls (8.9±1.3 vs 17.1±1.7 pg/ml controls vs patients, P=0.002, Students t-test).

F. 1.3.3. *Effects of KCl, PE and ACh*

There was no difference in the maximum constrictions to KCl or PE in the arteries from the age-matched controls and CHF patients. Furthermore, the relaxations to ACh in PE preconstricted arteries before endothelial denudation were similar between the two experimental groups (Table 5.3.). After denudation the relaxation to ACh was abolished (Table 5.3.).

F. 1.3.4. *Effects of ET-1 and SRTX S6c*

ET-1 constricted all arteries in a concentration-dependent manner (Figure 5.2.). However, the arteries dissected from the CHF patient biopsies were significantly less sensitive to ET-1 (pD_2s = 9.4 vs 8.7 controls vs CHF; n=8 each group; P=0.025 ANOVA; Table 5.4.). The maximal constrictions to ET-1 were not different between the two groups (Figure 5.2.) nor were the mean lumen diameters (227.1±21.6 vs 218.6±20.2 μm control vs CHF).

SRTX S6c induced constrictions in all arteries from both groups (Figure 5.3.). SRTX S6c constricted the arteries from the control subjects (n=5, mean lumen dia =

178.3±15.9 μm), E_{max}=14±7 % max KCl constriction at $3x10^{-8}$M. The mean constrictions to SRTX S6c in the arteries from the CHF patients (n=6, mean lumen dia = 204.3±6.2 μm) tended to be slightly enhanced when compared to the control arteries, E_{max}=21.1±9.4 % max KCl constriction at $1x10^{-8}$M, however, this effect was not significant (P=0.4, ANOVA).

F. 1.3.5. *Effects of ET_A receptor antagonism*

ET_A receptor antagonism by pre-incubation with BQ-123 (n=6; mean lumen dia =235±30 μm) shifted the ET-1 CRC in the control arteries significantly to the right (Figure 5.4.; P=0.0001 ANOVA). The ET-1 constrictions in the arteries from the CHF patients were also significantly inhibited by BQ-123 (n=6; P=0.015; mean lumen dia = 221.7±11.4 μm), although the shift was less marked than the control arteries. The pD_2 values could not be calculated as maximum constrictions to ET-1 in some arteries were not reached.

F. 1.3.6. *Effects of ET_B receptor desensitisation*

SRTX S6c desensitisation (Figure 5.5.; Table 5.4.) had no effect on the ET-1 constrictions and sensitivities in the arteries from the control subjects (n=6; P=0.52 ANOVA; mean lumen dia = 178.3±15.9 μm) or from the CHF patients (n=6; P=0.49 ANOVA; mean lumen dia = 204.3±6.2 μm).

F. 1.3.7. *Effects of ET_A/ET_B receptor antagonism*

Combined inhibition with BQ-123 and SRTX S6c desensitisation (Figure 5.6.A.) significantly inhibited the vasoconstrictions to ET-1 in the arteries from the control subjects (n=4; P=0.0004 ANOVA; mean lumen dia = 190±26.5 μm). Combined antagonism also significantly inhibited the ET-1 CRC in the CHF arteries (n=6; P=0.0003 ANOVA; mean lumen dia = 203.3±26.5 μm). In control arteries, the non-selective ET_A/ET_B receptor antagonism inhibited the ET-1 constrictions to a similar extent as with BQ-123. However, in the CHF arteries, non-selective ET_A/ET_B receptor

antagonism was more effective in inhibiting the ET-1 CRC than BQ-123 (Figure 5.6.B.), the whole curve being shifted further to the right.

F. 1.3.8. *Reversal study*

Similar to the previous chapter, due to the small numbers exposed to an ET-1 CRC only, there were not enough arteries per group to divide into the 3 reversal treatment groups of BQ-123, TAK-044 or vehicle to perform any analyses on the results. As a consequence the reversal data have been combined (Figure 5.7.).

Established ET-1-induced vasoconstrictions were maintained for the 20 min period when vehicle control (Krebs-Henseleit solution; n=4; Figure 5.7) was added to the reperfusion circuit. BQ-123 reversed the ET-1 vasoconstriction to 11.3±6.6 % max KCl constriction after 20 min of reperfusion (n=6; P=0.004 ANOVA), however, TAK-044 completely reversed the established ET-1 tone by 15 min of reperfusion (-4.0±2.5 % max KCl constriction; P=0.0001 ANOVA). However, statistically (ANOVA) there was no diference between the reversal abilities of BQ-123 and TAK-044 (P=0.13).

F. 1.3.9. *Intrinsic tone investigation*

There was no intrinsic tone in any of the arteries studies. The combination of Ca^{2+}-free Krebs and chelation using EDTA and direct smooth muscle relaxation with SNP immediately relaxed the arteries to the initial resting tone measured at the beginning of the experiment (data not shown).

F. 1.4. Discussion

The gluteal biopsy was an effective method of obtaining resistance arteries from human subjects with relatively little discomfort to the subject. The vessels were obtained from either CHF subjects who had established heart failure as a result of ischaemic heart disease, or healthy age-matched controls. Dissecting small arteries from a gluteal biopsy is a well-established technique, and has been used mainly in studies

investigating vascular hypertrophy in hypertension (Schiffrin *et al.*, 1994; Thybo *et al.*, 1994; 1995). Although it is not known how much these arteries contribute to peripheral vascular resistance, the studies using small arteries from hypertensive subjects showed that there is an increase in the media thickness of the vessels from the hypertensives as compared to the normotensive control arteries (Aalkjaer *et al.*, 1987; Schiffrin *et al.*, 1994; Thybo *et al.*, 1994; 1995). Furthermore, ACE inhibitor therapy corrects these structural changes (Schiffrin *et al.*, 1994; Thybo *et al.*, 1994; 1995). Thus, it is suggested that these arteries are involved in the pathogenesis of hypertension and that the structural alterations in these vessels could account for part of the increased peripheral vascular resistance (Aalkjaer *et al.*, 1987).

In a previous study investigating agonist-induced constrictions in resistance arteries from CHF patient gluteal biopsies, greatly reduced responses to NA, ANG I and ANG II were found (Angus *et al.*, 1993). It was concluded that the attenuated responses were as a result of the marked activation of the sympathetic nervous and RAA systems in CHF (Angus *et al.*, 1993). Thus, the aims of this study were to investigate whether there was activation of the ET-1 system, by measurement of plasma big ET-1 and ET-1 levels in CHF and if there was an associated reduction in vascular responsiveness to exogenous ET-1 in resistance arteries from CHF patients.

Circulating ET-1 levels were not found to be raised in the CHF patients. In contrast, the levels of the precursor peptide, big ET-1, was significantly higher in the plasma of the CHF patients when compared to the healthy controls. This result is in agreement with Wei and colleagues (1994) and Pacher and co-workers (1993; 1997) and shows there is an activation of the ET-1 synthesis pathway. The raised big ET-1, but not ET-1 levels in the plasma of the CHF subjects suggests an accelerated expression of prepro ET-1, but a decreased conversion of big ET-1 to ET-1 by reduced ECE activity. However, there could be an up-regulation of ECE within the VSMCs, such that an increase in big ET-1 secretion may result in increased concentrations of ET-1 at the VSMC layer by being converted locally in the media. This is investigated using

immunohistochemistry in the next experimental chapter (Section G. 1.2. & 1.3.3.). Furthermore, since ET-1 is preferentially secreted towards the VSMC layer (Wagner *et al.*, 1992a), the raised big ET-1 may reflect an overspill of endothelially produced big ET-1 (Wei *et al.*, 1994). Any overspill of the mature form of the peptide may not be evident due to an increase in clearance/degradation mechanisms.

The RIA results of this chapter show raised big ET-1 levels in the plasma of CHF patients, the majority of whom are in moderate heart failure (NYHA Grade II). There is a doubling of big ET-1 levels, which is in agreement with Pacher and colleagues (1993). This group found that there is a further increase in big ET-1 levels as the condition deteriorates, correlating with the severity of CHF (Pacher *et al.*, 1993). Moreover, it was found that plasma big ET-1 levels predict 1 year mortality better than haemodynamic parameters and levels of plasma ANP (Pacher *et al.*, 1997). Thus, it has been suggested that plasma big ET-1 levels may be of prognostic value in determining heart failure severity and outcome.

The viability of the smooth muscle layer in the CHF patients was assessed first, using agonist-dependent and independent constrictors. The maximum constrictions induced by KCl solution or PE were not different between the arteries removed from CHF subjects and age-matched controls. Furthermore, the endothelium-dependent relaxations induced by ACh were also similar, showing that vascular integrity is maintained in the small arteries from CHF patients. This is in contrast to the study by Angus and colleagues (1993) who found that maximal constrictor responses to KCl (124 mM solution) and NA (1μM) were significantly attenuated, as were the relaxations induced by ACh. The main difference between the two studies is that Angus *et al.*, (1993) used the wire myograph in contrast to the perfusion myograph utilised in the present study. However, the CHF patient groups used in both studies are similar (NYHA II-III). Therefore, severity of heart failure is unlikely to be the cause of the discrepancies. Nevertheless, in the present study, agonist-dependent and independent maximal constrictions are similar in the arteries from the CHF patients and

the control subjects demonstrating that there is no overall damage to the VSMCs in the resistance arteries from the CHF patients.

ET-1 was a potent constrictor in arteries from both groups, with the maximal constrictions to ET-1 no different between heart failure and control subjects. In both experiemental groups of arteries, there is a biphasic response to ET-1, similar to that demonstrated in the rat arteries in the previous chapter, with a shallow, high affinity phase and a steeper, low affinity phase. However, the sensitivity to ET-1 was attenuated in the CHF arteries. Despite the reduced sensitivity of the CHF arteries, the biphasic CRC to ET-1 remained. Cowburn and co-workers (1996) also saw a reduced sensitivity to ET-1 in isolated arteries from CHF patients. However, in their study the maximal constriction to ET-1 was not achieved in the vessels from the CHF patients (Cowburn *et al.*, 1996). Furthermore, they also saw no difference in the contractile responses to other constrictor agents (KCl, NA & ANG II). In contrast, gluteal small arteries from hypertensive patients have an attenuated maximal constriction to ET-1(Schiffrin *et al.*, 1992), the maximal responses being normalised to those seen in arteries from normotensive subjects after 1 year of treatment with cilazapril (Schiffrin *et al.*, 1994).

The reduced sensitivity to ET-1 in the CHF arteries could reflect the up-regulation of ET-1 synthesis pathway. Although the plasma levels of ET-1 were not raised, increased big ET-1 secreted towards the VSMC layer could result in higher ET-1 levels in the media by conversion to the mature peptide by VSMC located ECE. Thus, the impairment of ET-1-mediated vasoconstriction could be due to ET receptor down-regulation as a consequence of chronically elevated big ET-1/ET-1 levels (Cowburn *et al.*, 1996). The constrictor response to ET-1 is similar to those described in previous chapter in the arteries from the rat model of CHF, therefore, the reduced sensitivity could be due to the presence of an inhibitory ET_B receptor, as opposed to receptor down-regulation.

However, unlike the rat mesenteric arteries, SRTX S6c, used as an agonist, consistently induced small constrictions in all of the human arteries from both groups. Furthermore, the mean constrictions to SRTX S6c appear to be augmented in the CHF vessels, although this was not significant. If the study had larger numbers, this trend towards enhanced ET_B receptor-mediated constriction could become significant and complement the *in vivo* results of Love *et al.* (1996a; human forearm resistance arteries) and Cannan *et al.* (1996; canine coronary arteries). Both of these studies saw a reduced constriction to ET-1, but increased vasoconstriction to STRX S6c (Love *et al.*, 1996a; Cannan *et al.*, 1996). The RAA system is activated in CHF, and it has shown that although ANG II levels down-regulate total ET-1 binding sites (Roubert *et al.*, 1989), it also up-regulates ET_B receptor mRNA (Kanno *et al.*, 1993). Thus, it has been suggested that ET_B receptors are up-regulated, and that, perhaps, ET_A receptors down-regulated in heart failure resistance arteries.

The involvement of the respective ET_A and ET_B receptor subtypes in the constrictions to the native peptide, ET-1, were investigated using BQ-123, SRTX S6c desensitisation and a combination of these treatments. Antagonism of the ET_A receptor using BQ-123, inhibited the ET-1 vasoconstrictions in vessels from CHF patients and age-matched control subjects. In all the arteries, there was no part of the ET-1 CRC which was resistant to BQ-123 inhibition, suggesting that ET_A receptors alone mediate all of the ET-1 response. This is in contrast to Deng and co-workers (1995), who found that in subcutaneous arteries from healthy subjects, there was a residual response to ET-1 (~15-20% of the maximum ET-1 constriction) resistant to ET_A receptor blockade.

Interestingly, the ET-1 CRC was shifted further to the right in the control arteries than in the CHF arteries, suggesting that BQ-123 was a more potent antagonist in these vessels. This may simply be due to the reduced sensitivity to ET-1 in the CHF arteries. Indeed, Love *et al.* (1996a) observed that vasodilatation to a brachial artery infusion of BQ-123 in CHF patients was blunted in comparison to control subjects. It

was cautiously suggested that ET_A receptors might be downregulated in CHF. As in this study, Love and colleagues (1996a) were reluctant to withold drug treatment from the CHF patients for any longer than 24 hours before the study and it is possible that the persisting vascular effects of the medication may contribute to the differences observed between patients and control subjects (Love *et al.*, 1996a).

ET_B receptor desensitisation did not affect the ET-1 constrictions in the control arteries. Furthermore, SRTX S6c desensitisation did not alter the sensitivity to ET-1 in the arteries removed from the CHF patients. This is in contrast to the results in the 12 week CHF rat vessels where ET_B receptor desensitisation restored the sensitivity to that of the 12 week sham-operated arteries. Thus, in the human condition there is not the appearance of an inhibitory ET_B receptor. The differing vascular beds used in the two studies may account for this discrepancy. The mesenteric bed can receive up to 10% of the total cardiac output and is extremely susceptible to constrictions induced by ET-1 and BQ-3020 (ET_B receptor agonist; Gardiner *et al.*, 1994), whereas the subcutaneous/gluteal vascular bed contributes very little to total peripheral vascular resistance. Moreover, the rat model of heart failure has developed as a consequence of left ventricular dysfunction over a period of 3 months, whereas human heart failure has developed over years. Once again, despite the presence of some constrictor ET_B receptors in the vessels from both groups, as shown by the small constrictions to SRTX S6c, inhibition of the constrictor actions of the ET_B receptors does not affect the ET-1 constriction. Thus, the ET_A receptors could be compensating for the loss of the ET_B receptors, and ET-1 receptor crosstalk may also be a phenomenon in human small arteries. However, BQ-123 pretreatment did inhibit the entire ET-1 CRC, suggesting that ET_A receptors mediate both the high affinity part of the curve, which is usually attributed to constrictor ET_B receptors (Deng *et al.*, 1995), as well as the low affinity portion of the curve. Deng and colleagues (1995), who demonstrated an ET_A receptor antagonist resistant portion of ET-1 constriction in human subcutaneous small arteries, concluded that the constrictions to low concentrations of ET-1 were mediated via constrictor ET_B receptors. Unfortunately, they did not inhibit ET_B receptors, either by

receptor desensitisation or antagonism, but based this conclusion on the constrictions they saw to SRTX S6c (Deng *et al.*, 1995).

Non-selective ET_A/ET_B receptor inhibition antagonised the ET-1 constrictions in vessels from both groups. However, BQ-123 and SRTX S6c pretreatment inhibited the ET-1 constrictions to a larger extent than BQ-123 alone in the arteries from the CHF subjects, but to a similar extent in the arteries of the control subjects. These results imply that both ET_A and ET_B receptors are actively involved in the constrictions to ET-1 in the arteries of the CHF patients. If these small arteries are truly representative of the other vascular beds in the human body, then it would suggest that both ET_A/ET_B receptors should be antagonised for effective anti-endothelin therapy in CHF. Indeed, BQ-123 and TAK-044 both reversed the established ET-1 constrictions in the human arteries. However, TAK-044 restored the arteries back to resting lumen diameters within 15 min of exposure, again, suggesting that both ET_A and ET_B receptors should be antagonised.

Obviously, these experiments should be repeated in resistance arteries with an intact endothelium in order to evaluate the balance of the endothelial, dilator ET_B receptor and VSMC constrictor ET_B receptor in the response to ET-1. In the forearm resistance bed of healthy subjects, infusion of either BQ-123 (Haynes & Webb, 1994) or TAK-044 (Haynes *et al.*, 1996) caused vasodilatation. However, TAK-044, the non-selective ET_A/ET_B receptor antagonist, produced a smaller increase in forearm blood flow than BQ-123 (Haynes *et al.*, 1996). This suggests that the endothelial ET_B receptors prevail in the balance of effects to ET-1. Furthermore, BQ-788 infusion resulted in a small, but significant constriction in this vascular bed, and when co-infused with BQ-123 modulated the vasodilatation, again, producing a lesser degree of vasodilatation compared to BQ-123 alone (Verhaar *et al.*, 1998). As previously described, SRTX S6c constriction is enhanced in CHF (Cannan *et al.*, 1996; Love *et al.*, 1996a). Therefore, the balance of dilator/constrictor ET_B receptors might be altered to favour vasoconstriction. However, BQ-788 infusion into heart failure patients induced a

vasoconstriction similar to that seen in healthy control subjects (Love *et al.*, 1996b). Thus, it appears that dilator ET_B receptors on the endothelium are functionally more important than constrictor ET_B receptors on the arterial smooth muscle in both healthy subjects and CHF patients (Love *et al.*, 1996b). However, the constrictor ET_B receptors may be antagonised, and if crosstalk is occurring between the two ET-1 receptor subtypes present on the VSMC layer, then the ET_A receptor will be compensating for its constrictor loss. ET_B receptor desensitisation in this study had no effect on the ET-1 constrictions in the CHF arteries, but non-selective ET_A/ET_B receptor blockade had a greater inhibitory influence on the ET-1 CRC than ET_A receptor antagonism alone. Therefore, in the forearm studies, even though the VSMC ET_B receptors are antagonised, the ET_A receptors will compensate and it would appear that the endothelial ET_B receptor counteracting vasodilatation is the only mechanism inhibited. Thus, in order to prove that the dilator ET_B receptors are functionally more important than the constrictor ET_B receptors in the resistance arteries of CHF patients, the responses to selective ET_A receptor inhibition and non-selective ET_A/ET_B receptors need to be compared.

The systemic haemodynamic effects of the non-selective ET-1 receptor antagonist, bosentan in CHF patients has been assessed (Kiowski *et al.*, 1995). An infusion of bosentan reduced the mean arterial pressure, pulmonary artery pressure, right atrial pressure, systemic vascular resistance and pulmonary vascular resistance. Bosentan increased cardiac output and stroke volume, but did not alter heart rate (Kiowski *et al.*, 1995). Thus, these changes in haemodynamics suggest that ET-1 antagonism induced both arterial and venous dilatation in these patients, although which effect dominated cannot be delineated. This study was performed in CHF patients whose ACE inhibition had been withdrawn for four plasma half-lives. Whether ET-1 antagonism has additional benefits when in combination with ACE inhibition has to be evaluated in humans. Furthermore, plasma concentrations of ET-1, but not big ET-1, increased at least twofold after bosentan infusion. It was suggested that this could be due to either displacement of ET-1 from the receptor sites or a decreased clearance of ET-1 due to

the inhibition of ET_B receptors (Kiowski *et al.*, 1995). As it appears that dilatory ET_B receptors are preserved in heart failure, and that ET_B receptors are involved in the removal of ET-1 from the circulation, the evidence implies that ET_A receptor inhibition would be more effective as a vasodilator therapy in CHF, despite the evidence for up-regulation of constrictor ET_B receptors.

In summary, the results of the present study demonstrate that resistance arteries from CHF patients are less sensitive to the constrictor actions of ET-1. Down-regulation of ET_A receptors is the most likely explanation, since ET_B-mediated constrictions to SRTX S6c remained in the CHF arteries. Increased circulating levels of big ET-1 demonstrate that the ET-1 synthesis pathway is up-regulated, although plasma ET-1 levels were not raised. Plasma levels of ET-1 are thought to be as an overspill from the endothelium, with the majority of the peptide being released abluminally (Wagner *et al.*, 1992a). Therefore, there might be an up-regulation of ECE on the VSMCs to convert big ET-1 to the mature, vasoactive peptide, which could be responsible for the decreased sensitivity of the arteries to exogenous ET-1. It cannot be concluded from the functional experiments in the myograph that there is receptor down-regulation. Other techniques, such as *in situ* hybridisation and binding assays, are needed to confirm this hypothesis. However, the decreased vascular responsiveness to ET-1 is not due to the presence of a putative inhibitory ET_B receptor as demonstrated in the arteries of the rat model of CHF. Both ET_A and ET_B receptors capable of mediating constriction are present on the VSMCs of resistance arteries from gluteal biopsies from CHF patients and control subjects. Therefore, these results imply that both ET-1 receptor subtypes should be inhibited to effectively remove endogenous ET-1 vasoconstrictor tone in CHF patients. However, endothelial dilatory ET_B receptors appear to be preserved in the forearm vascular bed of CHF patients, and could be functionally more important than constrictor ET_B receptors (Love *et al.*, 1996b). Hence, the ideal receptor antagonist would be one that blocked constrictor ET_A and ET_B receptors, but preserved endothelial ET_B receptors.

PATIENT No.	NYHA Grade	Age	Blood Pressure	Heart Rate	Drugs
1	I	42	112/72	61	ACE I, Di, Ni, Asp
2	II	73	195/95	NA	ACE I, Di, Asp
3	II	62	143/105	56	ACE I, Di, Asp
4	II	77	132/66	68	ACE I, Asp, Dig
5	II	72	175/80	NA	ACE I, Di, Asp
6	III	79	132/74	80	ACE I, Di, Ni, Asp, β-Bl
7	III	77	134/70	NA	ACE I, Di, Ni, Asp, Dig
8	II	71	140/80	100	ACE I, Di, Asp
9	III	78	112/74	78	ACE I, Di, Ni, Asp
10	II	64	121/68	62	ACE I, Di, Asp
11	III	52	116/68	68	ACE I, Di, Ni, Asp
12	II	75	192/96	68	Los, Di, Asp, Dig

Table 5.1. Demographics of the CHF patients who underwent a buttock biopsy. 1 patient was classified as NYHA Grade I, 7 patients were classified as being NYHA Grade II and 4 patients were classified as NYHA Grade III as determined by each individual patients cardiologist. Age, blood pressure and heart rate taken at the time of biopsy are shown. Drugs prescribed are also shown. All patients took aspirin (Asp), diuretics (Di) and angiotensin converting enzyme inhibitors (ACE I), except patient 4 who did not use a diuretic and patient 12 who was administered losartan (Los) as an alternative to ACE I. Other drugs used were nitrates (Ni), digoxin (Dig) and β-blockers (β-Bl). NA represents data not available.

CONTROL No.	*Age*	*Blood Pressure*	*Heart Rate*	*Drugs*
1	63	145/87	71	None
2	73	117/77	55	None
3	69	135/72	70	None
4	69	132/88	75	None
5	75	NA	NA	Asp/Ni
6	61	134/80	62	None
7	76	127/64	60	None
8	73	182/92	60	None
9	44	128/80	72	None
10	63	126/80	68	None

Table 5.2. Demographics of the age-matched control subjects who underwent a buttock biopsy. Age, blood pressure and heart rate are shown. Only one subject, subject 5, was taking any prescribed medication of aspirin (Asp) and a nitrate (Ni). NA represents data not available.

	Control Subject Arteries	*CHF Patient Arteries*
Resting Lumen Diameter	219±14	221±10
+ 60mM KCl Lumen Diameter	70±5	70±4
+ 10^{-5}M PE Lumen Diameter	68±5	72±4
+ 10^{-6}M ACh Lumen Diameter, endothelium intact	199±11	203±9
+ 10^{-6}M ACh Lumen Diameters after de-endothelialisation	65±5	62±4

Table 5.3. Lumen diameter of arteries (μm) dissected from buttock biopsies removed from CHF patients and age-matched controls when resting, during maximum 60 mM KCl constriction and maximum 10^{-5}M PE constriction. Also shown is the lumen diameter after relaxation with ACh before and after endothelial denudation. No difference in maximal KCl or PE induced constrictions or ACh-induced relaxations were observed in the arteries from the CHF patients when compared to the arteries from the age-matched controls (Students t-test).

	Control Subject Arteries	*CHF Patient Arteries*
pD_2 value for ET-1 CRC, n=8 per group	9.4	8.7*
pD_2 value for SRTX S6c desensitisation, n=6 per group	9.03	8.6**

Table 5.4. Table comparing the pD_2 values of the vehicle control ET-1 CRCs and after SRTX S6c desensitisation in arteries from age-matched control subjects and CHF patients. The sensitivity of the arteries from CHF patients to ET-1 is significantly different when compared to the control subjects in the vehicle control group (*P=0.01, Students t-test) and after SRTX S6c desensitisation (**P=0.04, Students t-test).

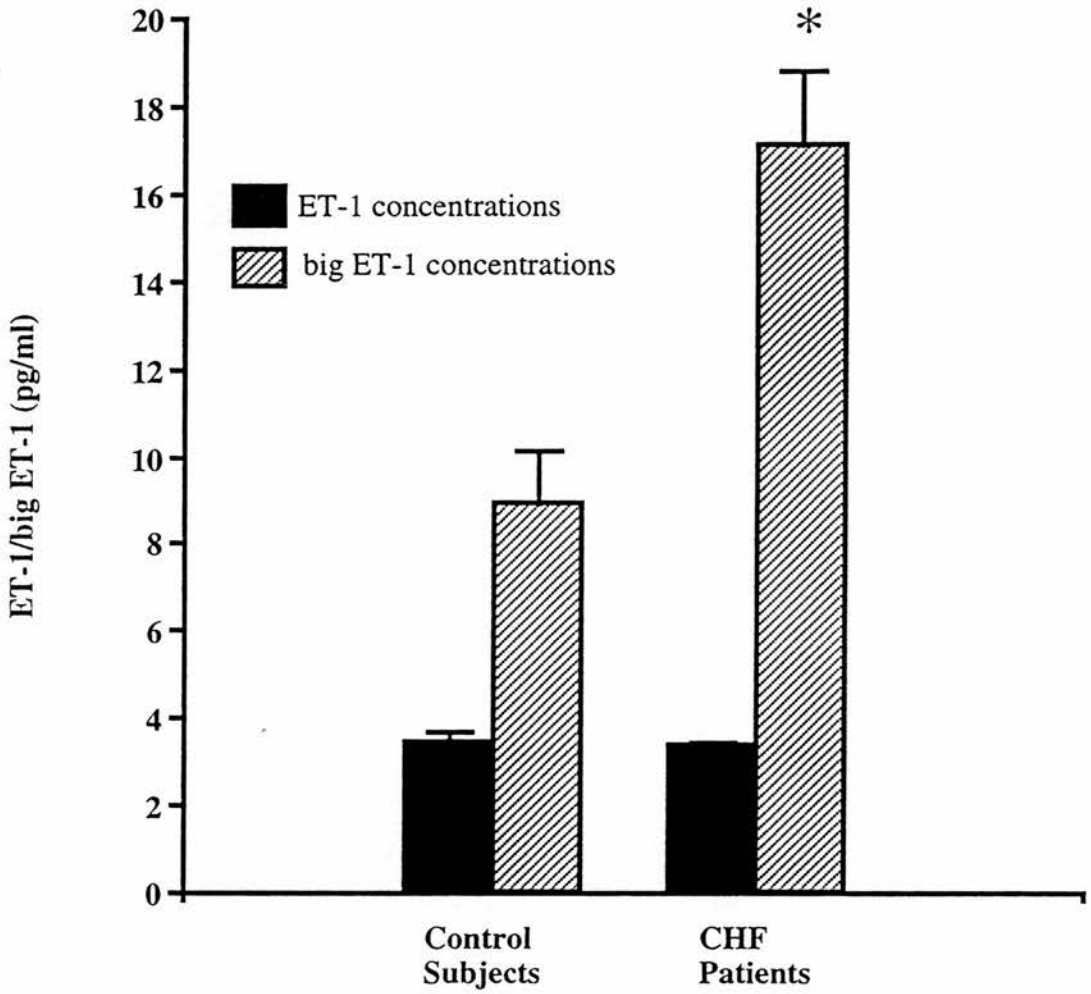


Figure 5.1.
Bar graph of plasma ET-1 (filled columns) and big ET-1 (striped columns) concentrations from healthy control subjects and CHF patients. ET-1 plasma levels were similar in both groups, however the plasma big ET-1 levels from CHF patients of NYHA Class II/III were significantly higher as compared to controls. Values are mean ± sem, *P<0.01, students t-test.

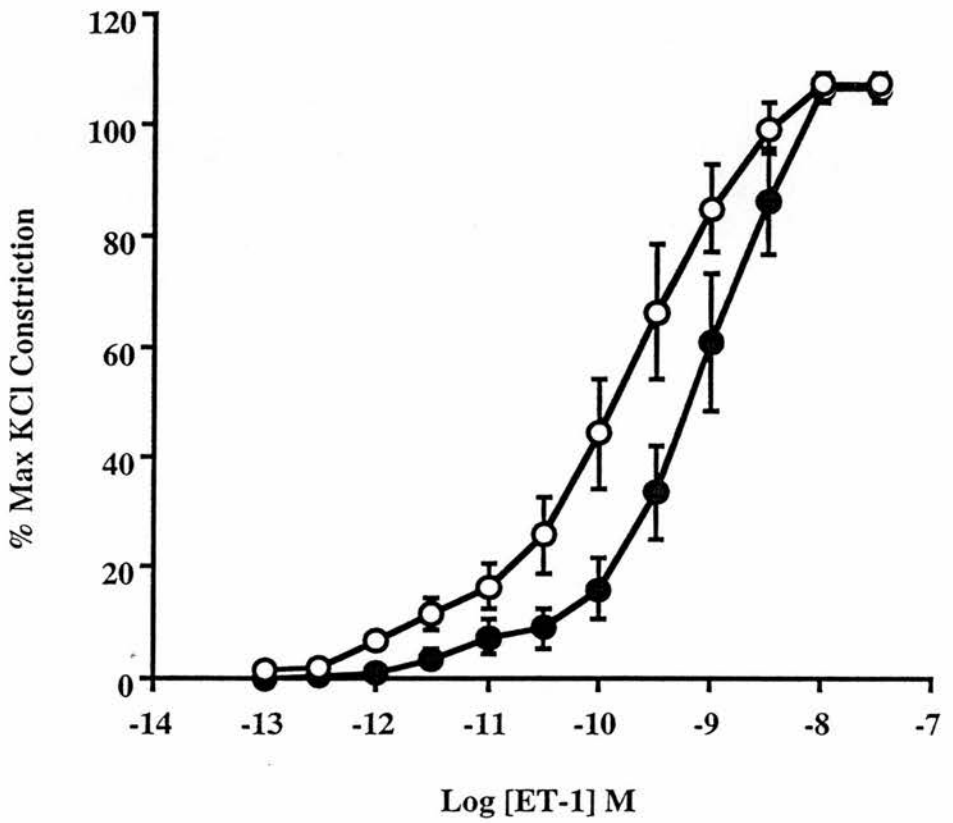


Figure 5.2. Comparison of the contractile responses to ET-1 in small arteries dissected from gluteal buttock biopsies from CHF patients (●, n=8) and age-matched controls (O, n=8). The sensitivity to ET-1 of the arteries from the CHF patients was significantly less than the respective age-matched control arteries (P=0.04, ANOVA).

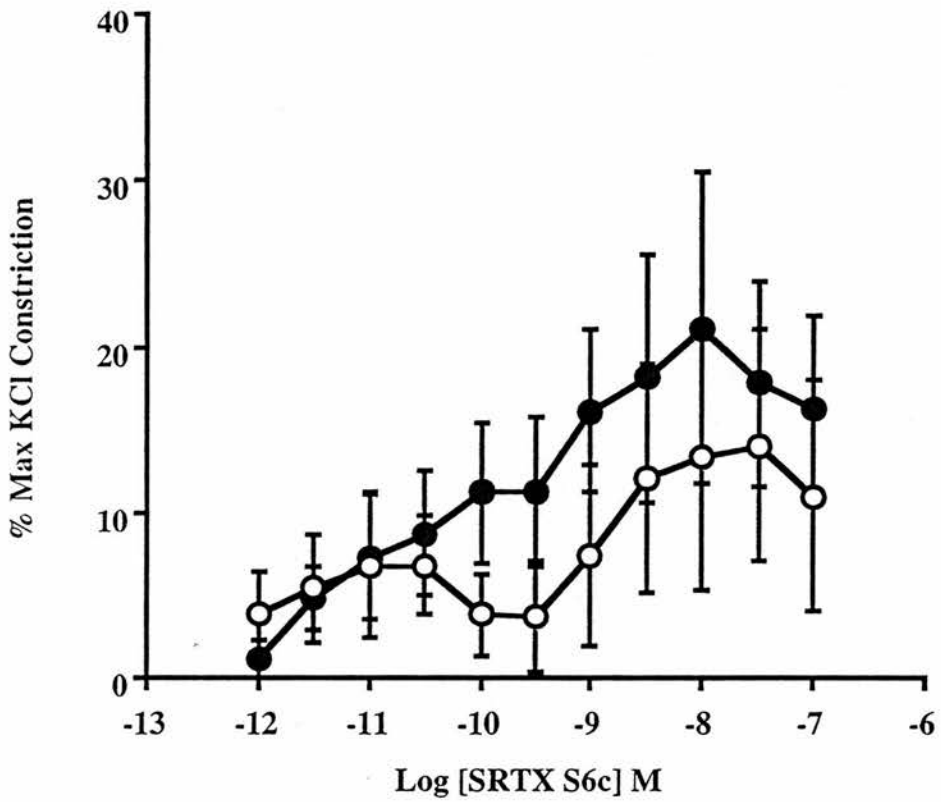


Figure 5.3. SRTX S6c-induced constrictions in the small arteries from CHF patients (●, n=6) and age-matched controls (O , n=5). STRX S6c-induced small constrictions in all vessels. There was no significant difference in the size of constrictions induced SRTX S6c in the CHF arteries when compared to the control arteries (P>0.05, ANOVA).

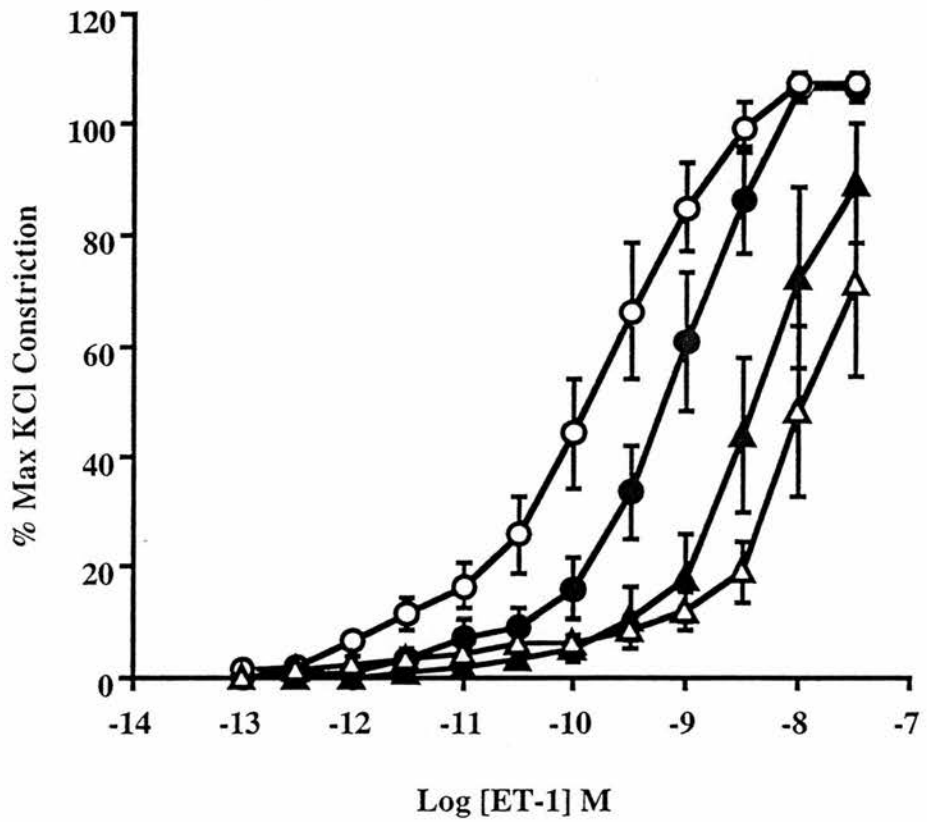


Figure 5.4. The effects of the ET_A receptor antagonist BQ-123 on the ET-1 constrictions in resistance arteries from CHF patients and age-matched controls. Preincubation with BQ-123 for 30 min inhibited the ET-1 CRC in the control arteries (Δ , n=6, P=0.0001 ANOVA) when compared to vehicle control (O , n=8). BQ-123 also inhibited the ET-1 constrictions in the CHF arteries (▲, n=6, P=0.02 ANOVA) when compared to vehicle control (●, n=8).

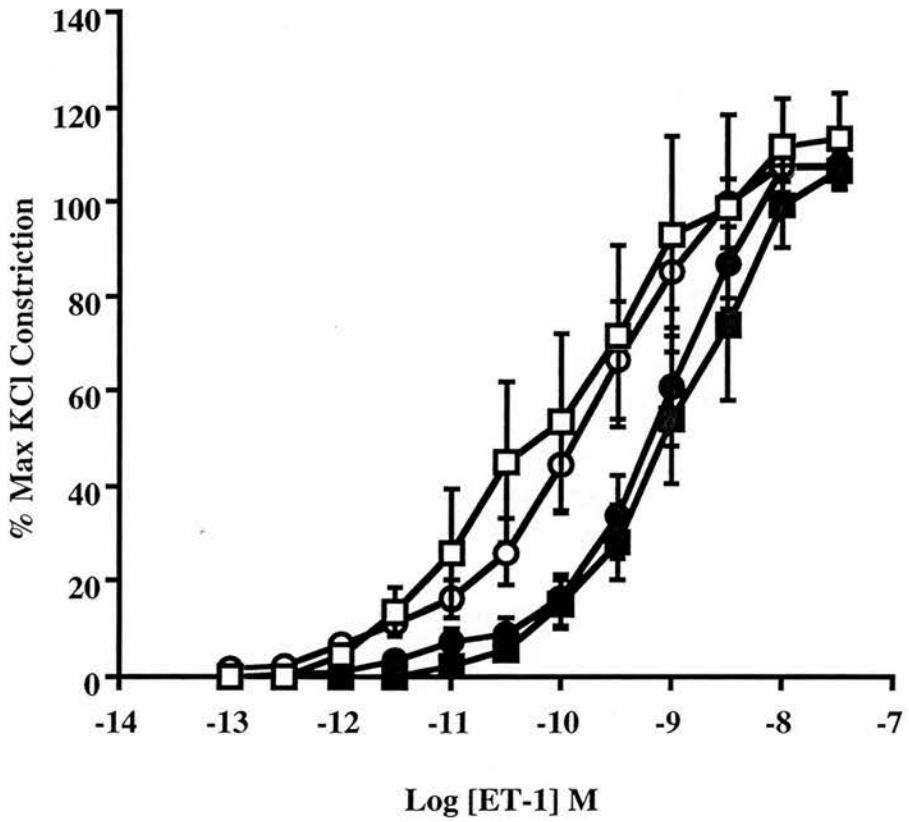


Figure 5.5. The effect of ET_B receptor desensitisation on ET-1-induced vasoconstrictions in the resistance arteries from CHF patients and age-matched controls. ET_B receptor desensitisation by prolonged SRTX S6c exposure had no effect on the ET-1 CRCs in the vessels from control subjects (□ , n=6, P=0.52 ANOVA) or in the arteries from CHF patients (■ , n=6, P=0.49 ANOVA) as compared to the respective vehicle controls (○ , n=8 Age-matched controls & ,● , n=8, CHF).

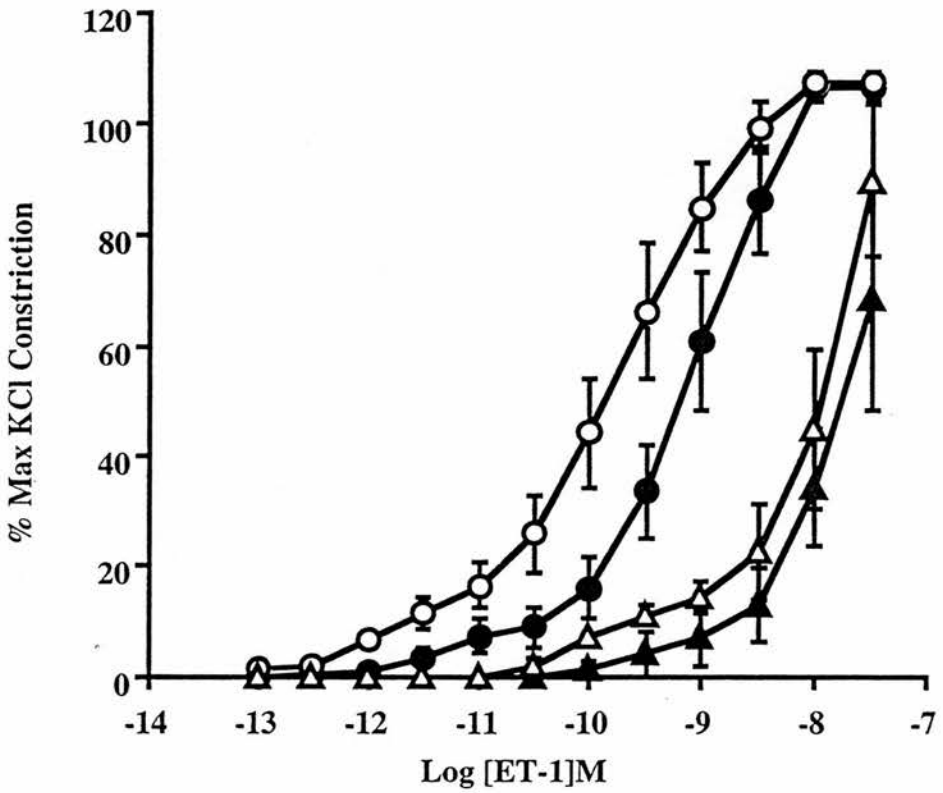


Figure 5.6.A. The effects of combined BQ-123 and SRTX S6c desensitisation on ET-1 constrictions. Non-selective ET_A/ET_B receptor antagonism shifted the ET-1 CRC in arteries from both CHF subjects and controls to the right. The constrictions to ET-1 after BQ-123 and SRTX S6c pretreatment in the control arteries (Δ , n=4) and CHF arteries (▲ , n=6) were significantly different when compared to the respective vehicle control ET-1 CRCs (O, n=8, P=0.0004 ANOVA, control arteries & ● , n=8, P=0.0003, CHF arteries).

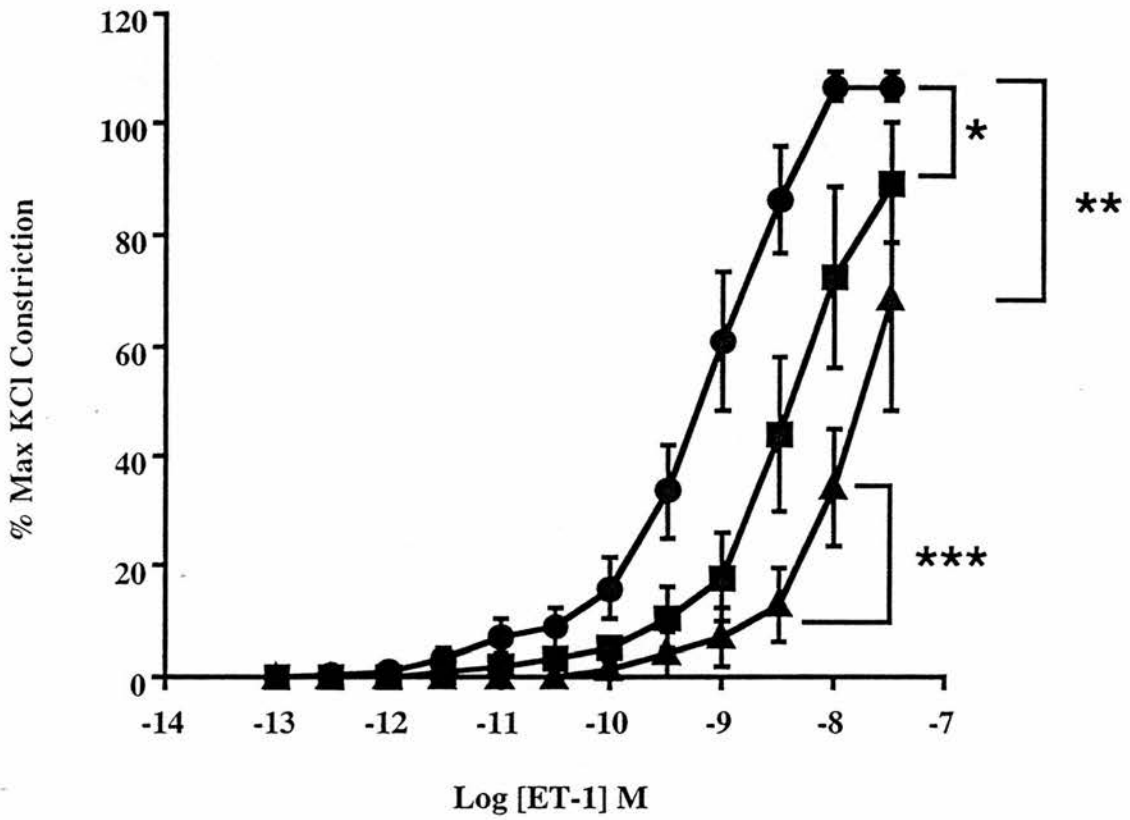


Figure 5.6.B. Comparison of the effects of selective ETA receptor antagonism (■ , n=6) and non-selective ETA/ETB receptor blockade (▲ n=6) on ET-1 constrictions (● , n=8 vehicle control) in arteries from CHF patients. Both treatments were significantly different from the vehicle control constrictions, * represents P=0.02; ** represents P=0.0003 ANOVA respectively. The constrictions at the ET-1 concentrations of $3x10^{-9}$ & $1x10^{-8}$M in the presence of BQ-123 or BQ-123 + SRTX S6c desensitisation were significantly different from each other (***P=0.05, Students t-test).

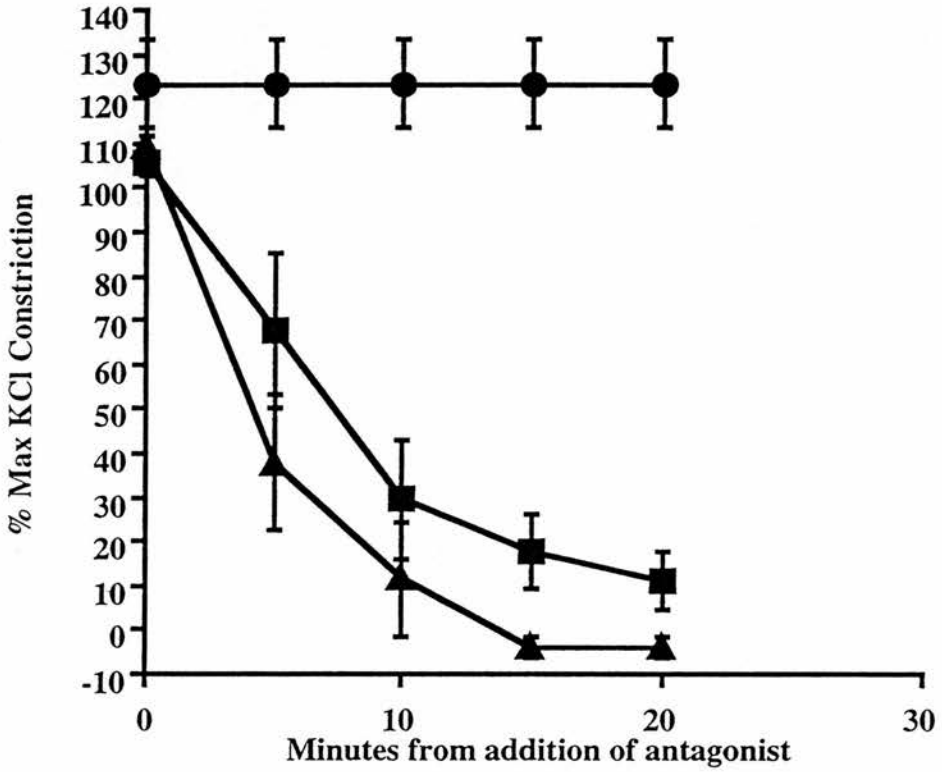


Figure 5.7. Reversal of established ET-1-induced tone by BQ-123 (■ , n=5) and TAK-044 (▲ , n-6). Both antagonists reversed the ET-1 vasoconstriction effectively over the 20 min reperfusion period($P<0.01$, ANOVA), whereas full constriction was maintained in the arteries reperfused with Krebs-Henseleit solution as vehicle control (● , n=5).

Results Chapter 4

Immunohistochemical localisation of ET-1 and ECE-1 in resistance arteries from rats and humans: No difference in CHF

G. 1.1. Introduction

It is now well established that ET-1 is synthesised and secreted from the vascular endothelial cells, but not smooth muscle cells, of healthy blood vessels (Ravalli *et al.*, 1996). Furthermore, the ET-1 released is secreted from the endothelial cells in a polar fashion (Wagner *et al.*, 1992a). As a consequence it is postulated that, under normal physiological circumstances, ET-1 and big ET-1 are secreted from endothelial cells preferentially towards the underlying smooth muscle cell layer, and not into the circulation (Yu & Davenport, 1995). This may be the explanation why circulating levels of ET-1 in healthy humans are so low, below the concentrations needed to produce any effects *in vitro* (Frelin & Guedin, 1994) and ET-1 levels measured in human plasma by RIA probably do not reflect reliably the concentrations of ET-1 present in the media of arteries.

In CHF circulating levels of ET-1 and/or big ET-1 are augmented, reflecting the severity of the disease (see Section B. 1.6.1.). This suggests that, although a crude measure, plasma ET-1/big ET-1 concentrations can demonstrate when the ET synthesis pathway is activated above normal. The increase in ET-1 production could be due to an induction of the ET synthesis pathway in the SMCs of the vasculature as well as an upregulation in the endothelium. The induction of mRNA for ET-1 and the mature peptide has been demonstrated in human and rat VSMCs in culture (Resink *et al* ., 1990). In human diseased, atherosclerotic coronary arteries, Winkles and colleagues (1993) showed that ET-1 mRNA is present in the VSMC-containing atherogenic lesion. Furthermore, diffuse ET-1 immunostaining was seen in transplanted coronary arteries which had undergone vasculopathy (or graft arteriosclerosis; Ravalli *et al.*, 1996). The double-labelling immunohistochemical technique employed showed that the ET-1 staining was predominantly found in the α-actin-positive myointimal cells (Ravalli *et al.*, 1996). However, in intact, undiseased vessels, such as human

saphenous vein and left internal mammary artery, ET-1 and big ET-1 were found to be localised only in the cytoplasm of endothelial cells (Howard *et al.*, 1992).

All these studies suggest that VSMCs can synthesize ET-1 in pathological circumstances. It was the aim of this chapter to demonstrate whether, in CHF, there is an alteration of ET-1 localisation in the small arteries from the human gluteal biopsies between patients and controls, and in the mesenteric arteries from the rat model of CHF at the two time points. Since it was found that there was an increase in circulating big ET-1, but not ET-1 levels, it was hypothesised that there maybe an induction of ECE-1 expression in the VSMCs of the small arteries for local tissue conversion. Furthermore, ET-1 localised in VSMCs of arteries using immunohistochemistry may not necessarily demonstrate synthesis of ET-1 in this layer, but could represent ET-1 bound to its receptors before the complex has been internalised. Thus, it is important to identify whether ECE is present in the VSMC layer for local synthesis. Therefore, to investigate the ET-1 localisation in these small arteries, monoclonal Abs directed against ET-1 and ECE-1 were used.

G. 1.2. Methods

The methods used were as described in Section C. 2.0. Briefly, vessels were isolated and dissected from either the human gluteal fat biopsies or the mesenteric beds from the CHF rat model and sham-operated controls (Sections C. 1.1.1., 1.1.2., 1.1.3.), and snap frozen in isopentane cooled in solid CO_2. The tissues were stored at -80°C until the tissues were fixed in 4% paraformaldehyde for 24 hours. The tissues were infiltrated with paraffin-wax and mounted into blocks. 3μm thick sections were cut and mounted onto Poly-L-Lysine coated slides, three sections per slide.

Sections were dewaxed in xylene, rehydrated through a series of alcohol baths and washed in water followed by phosphate buffer solution (PBS). Endogenous peroxidase was inhibited with 3% H_2O_2 in methanol (1:4) for 10 minutes. The sections were trypsinised to expose the antigens for 2 minutes per slide, and non-specific Ab binding suppressed by incubated with 10% normal horse serum (NHS) in PBS for 30 minutes. Sections were incubated in a humidifier chamber overnight at 4°C with the relevant primary Ab. These primary Abs were; ET-1 (1:500), ECE 27 (1:20), ECE 32 (1:20), α-actin (1:100) or the lectin, biotinylated *Ulex Europeaus* Agglutininen (UEA-1). All primary Abs were diluted in 1% NHS in PBS. For all experiments the negative control used 1% NHS in PBS alone.

All sections, except the UEA-1 treated slides were incubated with the secondary biotinylated horse, anti-mouse IgG (1:200) at room temperature for 60 minutes. All sections were incubated with the Vectorstain ABC complex for 30 minutes and peroxidase activity visualised with diaminobenzidine (DAB). Sections were counterstained with Mayers haematoxylin, dehydrated through alcohols and xylene and mounted with coverslips.

It was found that the lectin marker for endothelial cells, UEA-1, labelled only human endothelial cells and not rat endothelial cells. An alternative lectin label was used, *Griffonia simplicifolia* agglutinin-I (GSA-I), of which the enzyme-substrate was not peroxidase and DAB, but alkaline phosphatase and New Fuchsin (any immunoreactivity present stained pink). However, unlike DAB this stain is soluble in organic solvents so the sections were not dehydrated in alcohols and xylene, but were directly coverslipped using Faramount, an aqueous based mountant (Dako Labs, Bucks.).

In all experiments performed negative controls were included for each tissue. Furthermore, two positive controls were included during each experiment; i). Sections of human uterine artery taken from hysterectomy surgery, which had been previously shown in the laboratory to express ET-1 in the endothelial cell layer (Figure 6.1.D.). and ii). A human biopsy artery which had been experimented upon in the perfusion myograph and had, therefore, been exposed to a full ET-1 concentration-response curve (Figures 6.1. A-C).

G. 1.3. Results

G. 1.3.1. *Control Arteries*

The control sections used were an endothelium denuded human small artery (HSA) which had been exposed to a full ET-1 CRC and a human uterine artery (HUA). The human endothelial cell marker, UEA-1, clearly labelled the endothelium of the HUA (not shown), however, there was no brown UEA-1 immunoreactivity in the denuded HSA (Figure 6.1.A). In both arteries α-actin DAB staining was present in the VSMC layer. In Figure 6.1.B. α-actin staining is shown in the HSA. The surrounding adventitial and connective tissue, which had not been removed during the dissection of the artery from the fat biopsy, has no brown staining, the purple/blue of the haematoxylin staining contrasting against the brown labelled VSMCs. However, in the HSA section exposed to the ET-1 Ab, the whole section is stained brown including the connective tissue (Figure 6.1.C.). ET-1 (Figure 6.1.D) and ECE-1 immunoreactivity were localised to the endothelium of the HUA. However, some discrete areas of ET-1 immunoreactivity are evident in the VSMCs.

G. 1.3.2. *Rat Mesenteric Arteries*

In the arteries from the sham-operated rats at both 5 and 12 weeks post surgery ET-1 and ECE-1 immunoreactivity was clearly visible in the endothelial cell layer (Figures 6.2.F.,G. and 6.3.F.,G.), as confirmed by the GSA-I marker (Figures 6.2.H. and

6.3.H.). However, some discreet areas of ET-1 staining were visualised in the smooth muscle. Furthermore, the adventitia also stained positive for ET-1 and ECE-1, although the brown staining does not appear to be quite as intense as the immunostaining in the endothelium (Figures 6.2.F., G. and 6.3.F.G.). No difference in the localisation of ET-1 or ECE could be seen in any of the mesenteric arteries taken from animals with heart failure for 5 and 12 weeks (Figures 6.2.A-D and 6.3.A-D). Both ET-1 and ECE-1 immunoreactivity was clearly seen in the endothelial cell and adventitial layers, with occasional areas of ET-1 staining in the smooth muscle layer (n=4 for each group).

G. 1.3.3. *Human Gluteal Biopsy Arteries*

ET-1 and ECE-1 staining was localised only to the endothelial cell layer in the arteries from both the CHF patients and age-matched controls (n=5 for both groups; Figures 6.4.A-J). The staining in all the human arteries was not as intense as that seen in the rat arteries and as a consequence did not photograph as well as the rat arterial sections (Figures 6.4.C & G). Therefore, in some arterial sections, instead of using DAB as a chromogen, alkaline phosphatase and New Fuchsin were used as an alternative chromagen with the idea that better photographs could be obtained (Sections 6.4.B & H; n=2). There was no adventitial ET-1 or ECE-1 immunostaining seen in any of the human arteries.

G. 1.4. Discussion

It has been accepted that ET-1 is synthesized in the endothelium, but not the SMCs of healthy blood vessels (Howard *et al.*, 1992). However, it has been shown that animal and human VSMCs, in culture at least, do have the ability to synthesize and secrete ET-1 (Resink *et al.*, 1990; Kanse *et al.*, 1991). For instance, Yu & Davenport (1995), measured secreted ET-1 levels from cultured VSMCs isolated from different vascular

beds. Human epicardial coronary artery VSMCs secreted 2, 3 and 6 times greater amount of ET-1 than human left internal mammary artery, saphenous vein and umbilical vein VSMCs respectively and double the levels of big ET-1 (Yu & Davenport, 1995). They had previously shown by immunohistochemistry that ET-1 staining could only be seen in the endothelium of healthy, intact human saphenous vein and left internal mammary artery (Howard *et al.*, 1992). It was suggested that the culture environment may approximate pathophysiological conditions explaining the induction of the ET-1 synthesis pathway (Yu & Davenport, 1995).

One pathophysiological condition shown consistently to alter ET-1 cellular production is atherosclerosis. Several different studies on human atherosclerotic arteries have demonstrated ET-1 in cells other than the endothelium. In atherogenic plaques ET-1 expression is present (Winkles *et al.*, 1993), specifically being localised to the macrophages and intimal and medial VSMCs of the lesions (Lerman *et al.*, 1991; Zeiher *et al.*, 1995; Ravalli *et al.*, 1997). In contrast Bacon and colleagues (1996) did not visualise ET-1 staining in the VSMCs of atherosclerotic plaques, despite demonstrating an increase in big ET-1 and ET-1 levels in the plaque by RIA. The endothelium lining the atherosclerotic coronary arteries consistently stained for ET-1 immunoreactivity. Thus, they suggested that the endothelium is most probably the source of the raised big ET-1 and ET-1 (Bacon *et al.*, 1996).

Resink and co-workers (1990) induced ET-1 production and secretion in both rat and human VSMCs, as well as endothelial cells by exposing them to various growth factors such as platelet derived growth factor (PDGF), vasopressin and ANG II. All these factors, plus other cytokines known to enhance ET-1 generation, are raised in CHF as well as hypertension and atherosclerosis and could be involved in the induction and up-regulation of the ET-1 synthesis pathway in VSMCs and the endothelium. Thus, it was hypothesised that there might be a change in tissue ET-1

localisation in arteries from CHF patients and rats as compared to the respective controls. It was also hypothesised that there might be an induction of ECE-1 in the VSMCs of the human arteries in particular, since it was seen that circulating levels of big ET-1, but not ET-1, in the plasma of CHF patients were significantly raised (Section F. 1.3.). Furthermore, the functional studies on the small arteries from both CHF patients and rats show that there is decreased responsiveness when exposed to the exogenous ET-1 concentration-response curve. Therefore, enhanced ET-1 production could be responsible for the down-regulation of ET-1 receptors responses, as has been demonstrated in a rat model of hypertension (Lariviere *et al.*, 1993a, b). In the small mesenteric resistance arteries of DOCA-salt hypertensive rats increased ET-1 mRNA and immunoreactive ET-1 was found in association with a reduced density of ET-1 receptors, thereby potentially accounting for the receptor down-regulation (Lariviere *et al.*, 1993a, b).

However, despite the results of the functional and RIA studies, the immunohistochemical studies appear to show no difference in ET-1 and ECE localisation in the arteries from both human CHF patients and the two CHF rat groups when compared to the arteries from their controls. In all arteries studied the endothelium consistently stained for ET-1 and ECE-1 immunoreactivity. Any brown staining in the VSMC layer seen with the ET-1 Ab is most probably ET-1 bound to the ET-1 receptors. However, the monoclonal ET-1 Ab used does not label big ET-1. Thus, these studies do not eliminate the possibility that there is ET-1 synthesis in the VSMCs of the human gluteal arteries or rat mesenteric arteries. The only way it is possible to show whether there is an induction of the ET-1 synthesis pathway in the VSMCs of resistance arteries in heart failure would be via molecular techniques such as *in situ* hybridisation which target ET-1 mRNA expression. However, ECE is required for full synthesis of ET-1, and no ECE-1 staining was observed in the VSMCs. Thus, unless another isoform of ECE is present in the VSMCs of the small arteries of both humans and rats which the ECE-1 Ab does not recognise and react

with, the results imply that there is no ET-1 synthesis pathway in these arteries from either "normal" sham-operated or CHF rats.

The endothelium of all arteries stained positively for ET-1 and ECE-1. Unfortunately, immunohistochemistry is also a non-quantifiable technique, and can not demonstrate whether there is an increase in ET-1 levels, only whether there is an alteration in location. Thus, there could be an up-regulation of the ET-1 pathway in the endothelium alone, as was seen in the DOCA-salt hypertensive rat mesenteric arteries (Lariviere *et al.*, 1993a, b). Some studies do attempt to semi-quantify immunoreactivity in immunohistochemical studies using intensity of staining (Wei *et al.*, 1994; 1997). However, it was not possible to differentiate levels of staining intensity since all staining was not grossly different.

It is believed that in heart failure there could be general endothelial dysfunction, with the loss of counterbalancing vasodilatory factors such as NO (Katz, 1995). However, it is the elusive EDHF, and not NO, which is the major vasodilatory mechanism in resistance arteries. It is not known whether there is attenuated EDHF in CHF, but if the counterbalancing dilatory response is lost, then constriction to ET-1 will be enhanced whether there is an increase in synthesis or not. The immunohistochemical studies performed were unable to show whether there is an increase of ET-1 synthesis in the wall of the resistance arteries from CHF rats and patients. However, high circulating levels of ET-1 and/or big ET-1 have been demonstrated repeatedly, although the source of the elevated ET-1/big ET-1 remains unclear. The heart has been suggested as potential source of ET-1 production. Increased ET-1 mRNA has been reported in the ventricular myocardium of rats with CHF induced by coronary artery ligation (Sakai *et al.*, 1996b) and in the atria of CHF dogs (Clavell *et al.*, 1996; Wei *et al.*, 1997). Contrary to these studies, Wei *et al.*, (1994), using immunohistochemistry to investigate whether there is an alteration of ET-1 production in cardiac atrial and

ventricular tissue from human failing and healthy hearts, found that ET-1 immunoreactivity was present in similar distribution and intensity in both the healthy and failing hearts. Furthermore, similar to the findings reported in this thesis, they also reported a significant augmentation of circulating big ET-1 levels (Wei *et al.*, 1994).

The lungs may also be responsible for increased ET-1 synthesis in CHF, especially when pulmonary hypertension occurs, a phenomenon seen in the latter stages of the disease. In CHF patients ET-1 "spillover" from the lungs has been reported (Tsutamoto *et al.*, 1994), as has a significant positive correlation between circulating ET-1 levels and pulmonary vascular resistance (Cody *et al.*, 1992). Furthermore, an increased expression of ET-1 has been shown in the lungs of patients with pulmonary hypertension (Giaid *et al.*, 1993). In the TIVCC canine low output model of heart failure increased tissue expression and concentrations of ET-1 was found in the lung, which was localised to the pulmonary epithelial cells using immunohistochemistry.(Wei *et al.*, 1997). However, this study did not exclude increased pulmonary endothelial generation (Wei *et al.*, 1997).

Interestingly, in the rat arteries, there was ET-1 and ECE staining in the adventitial layers. It has been suggested that ET-1 might be involved in neuronal responses, either potentiating or inhibiting neural transmission depending on the tissue and nerves being stimulted (Warner *et al.*, 1993a,b). Thus, the nerves present in the adventitial layer of the mesenteric arteries might be synthesising ET-1 in order to potentiate sympathetic nervous activation. Furthermore, fibroblasts are also present in the adventitial layer synthesising collagen and other structural components. However, fibroblasts also have the ability to synthesise ET-1 (Battistini *et al.*, 1993) and they may be a source of the ECE and ET-1 production. Curiously, no ET-1 or ECE-1

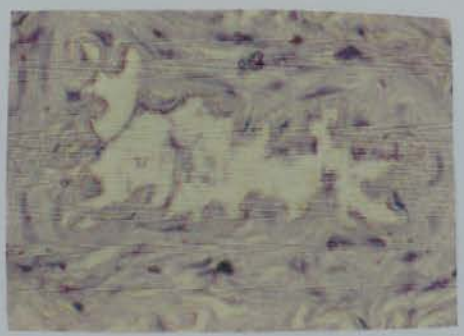
immunostaining was observed in the human arteries suggesting that the cells present in the adventitia of gluteal arteries do not synthesise ET-1.

Searching the literature, no immunohistochemistry studies against ET-1 in peripheral resistance arteries have been reported. Thus, this is the first study specifically investigating the ET-1 synthesis pathway in these small arteries from both rats and humans and furthermore, whether there is an alteration of the pathway in CHF. In conclusion, immunohistochemistry studies performed in this chapter suggest that there is not an induction of ET-1 synthesis in the VSMC layers of the resistance arteries from either human CHF patients and CHF rats. These results show that in the human resistance arteries, the endothelium is the sole manufacturer of ET-1 whereas in the rat mesenteric arteries the adventita also contributes to ET-1 generation. However, due to the limitations of the technique, it does not eliminate the possibility that there could be an up-regulation of ET-1 generation in the endothelium of these arteries in heart failure.

Figures 6.1. A-D.

Positive control sections. Pictures A, B, C & D are all representative immunohistochemical images of the positive control slides included in each immunohistochemical run. Pictures A, B & C are the human resistance artery used as a vehicle control in one of the perfusion myograph studies. As can be seen in Section A, the anti-endothelial cell marker UEA-1 did not stain any of the section showing that the artery had been effecively denuded of its endothelium by the passing of an air bubble through the lumen. Section B shows the DAB brown staining in the VSMC layer when using the anti-α-actin Ab, note no staining was seen in the surrounding adventitia and connective tissue. However, Section C is a section stained with the anti-ET-1 Ab. The artery had been exposed to a full ET-1 CRC, immediately snap frozen and stored at -80°C until fixation with paraformaldehyde. The entire section, including the surrounding adventitial and connective tissue stained brown. Section D is an image of a human uterine artery immunostained with anti-ET-1. The endothelium is stained as demonstrated by the arrows. However, some discrete patches of ET-1 can also be seen in the VSMCs of the artery. All sections are counterstained with haematoxylin.

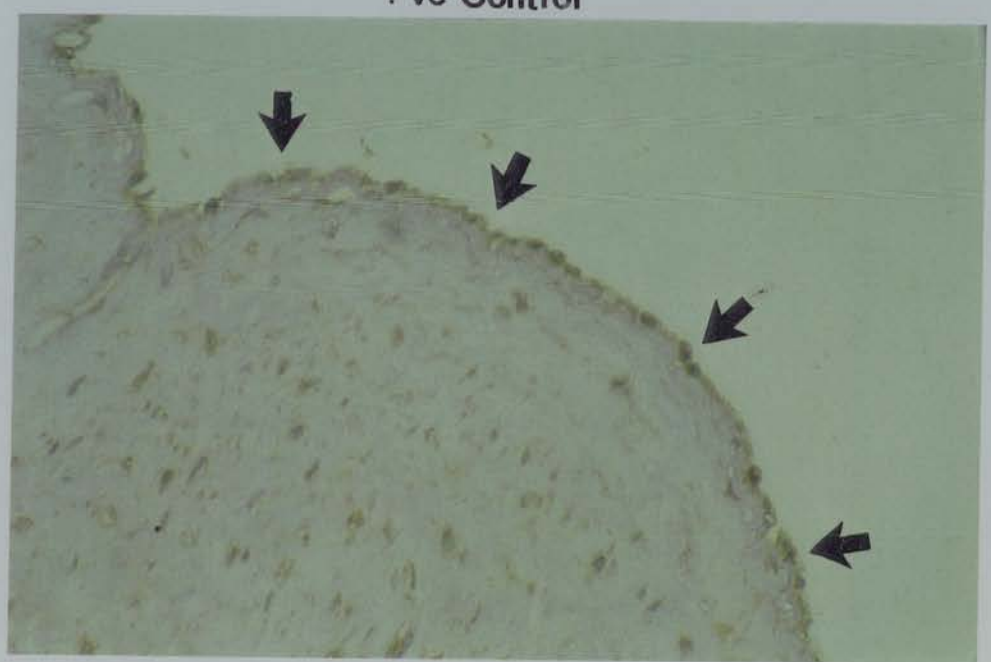
Ulex



α-Actin

ET-I

+ve Control



Scale
50 μm

Figures 6.2. A-H.

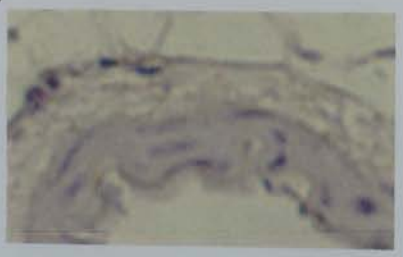
Pictures are representative of the immunostaining seen in mesenteric arteries removed from 5 week post-ligation rats (n=4) and 5 week sham-operated rats (n=4). All tissues shown are serial sections from the same artery removed from a CHF rat (Figures A-D) and a sham-operated rat (Figures E-H). Pictures A & E are the respective negative controls where the sections were not incubated with a primary Ab. Sections B & F are arterial sections incubated with the anti-ET-1 Ab and sections C & G exposed to anti-ECE-27 Ab. In all arteries, both Abs resulted in intense staining in the endothelium and adventitia, with some discreete patches of staining in the VSMC layer. Section D is the serial tissue section of the CHF rat artery which was incubated with the anti-α-actin Ab where only the VSMC layer was stained brown. Section H is the serial arterial section from the sham-operated rat incubated with the endothelial cell marker GSA-1 and stained pink with New Fuchsin. Unfortunately this section has a high degree of background staining. However, the endothelium is clearly stained a darker pink.

5 Week Rat

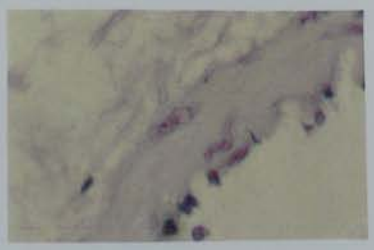
CHF

SHAM

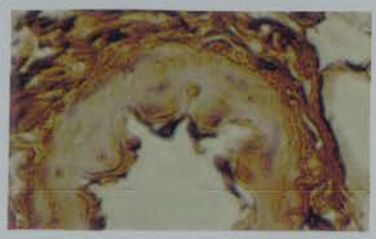
A



E



B



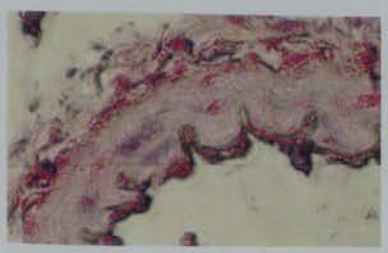
F

C

G

D

H



Scale
50 µm

Figure 6.3. A-H.

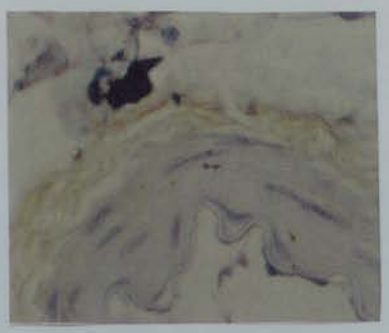
Serial sections of mesenteric arteries from a 12 week post-ligation rat (Sections A-D, n=4) and a 12 week sham-operated rat (Sections E-H, n=4). Sections A & E are the negative control tissues. Dark brown staining can be seen to be localised to the endothelium and adventitia in the sections immunostained with the anti-ET-1 Ab (Sections B & F) and the anti-ECE-27 Ab (Sections C & G). Sections D & H were incubated with anti-α-actin Ab (D) and GSA-I (H).

12 Week Rat

CHF

SHAM

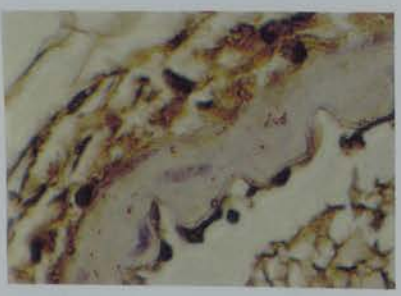
A



E

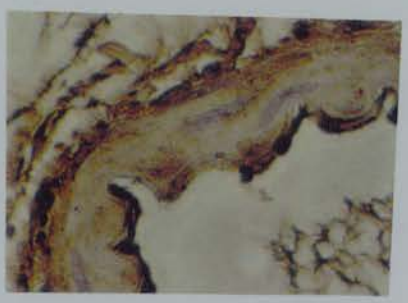
B

F



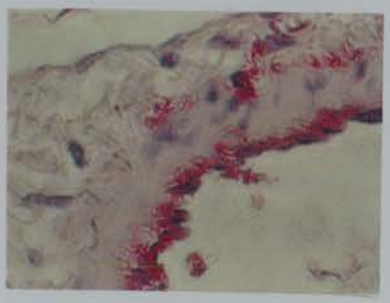
C

G



D

H



Scale
50 µm

Figures 6.4. A-J.

Immunohistochemistry performed on human arteries from a CHF patient (Sections A-E) and control subject (Sections F-J). The staining seen in the human arteries (n=5 for each experiment groups) was consistently lighter than that seen in the rat arteries. Sections A & F are the respective negative controls. Sections B & G are arteries incubated with the anti-ET-1 Ab, although the chromagen used on Section B was New Fuchsin, but on Section G was DAB. Using either visualising chromagen, ET-1 was localised to the endothelium alone. Sections C & H were immunostained using ECE-27 with the chromagen for Section C being DAB and Section H was New Fuchsin. Once again the faint staining using either chromagen was localised to the endothelial cell layer. Sections D & I were immunostained with anti-α-actin visualising the VSMC layer and Sections E & J are stained with the endothelial cell marker UEA-1.

Human Arteries

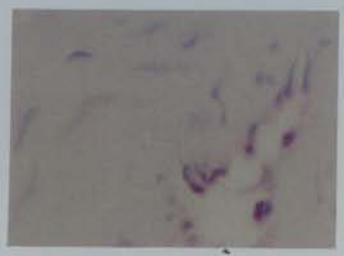
CHF

Control

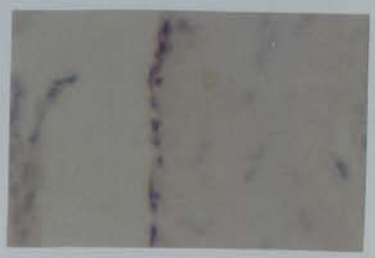
A

F

B

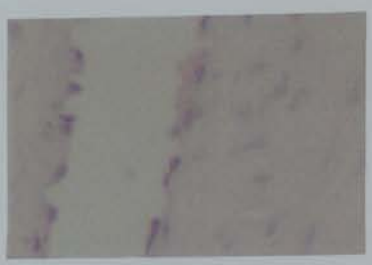


G

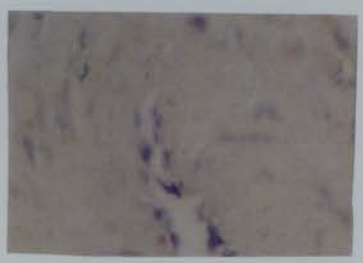


C

H



D



I

E

J

Scale
50 µm

General Discussion

H. 1.1. Summary

Resistance arteries, with internal lumen diameters of between 100-400 μm, are known to be the most important vessels in the body in determining total peripheral vascular resistance (PVR; Schiffrin, 1995). In heart failure, a chronic increase in PVR occurs by the activation of neurohumoral reflexes such as RAA and sympathetic nervous systems, inducing vasoconstriction throughout the body increasing cardiac workload (Francis *et al.*, 1984). Thus, the 'vicious cycle' of heart failure begins. It is now known that the ET-1 system is also activated in heart failure and, because of its powerful constrictor properties, particularly in resistance arteries, it has been identified as a potential target for therapeutic intervention. The most effective drugs in slowing the progression of the condition have been the ACE inhibitors. There are many similarities between the ET-1 and RAA systems, such as their vasoconstrictor, anti-natriuretic and mitogenic properties, all of which are aggravating phenomenon in the progression of CHF.

It was the aim of this thesis to investigate the ET-1 system in resistance arteries from rats and humans with CHF. The initial aim was to investigate the vascular reactivity of denuded small arteries from normotensive rats to ET-1, and the ET-1 receptor subtypes mediating constriction. The second aim was to assess whether the sensitivity to ET-1 and the basic ET-1 receptor population present on the VSMCs of mesenteric arteries are altered in rats with heart failure. The third aim was to ascertain whether the ET-1 synthesis pathway is activated in CHF patients by measuring plasma ET-1 and big ET-1 levels. The fourth aim was to study the responses to ET-1 and the ET-1 receptors responsible for constriction in human resistance arteries from CHF patients and age-matched controls. Finally, the fifth aim was to investigate the local pathway for synthesis of ET-1 in the resistance arteries of rats and humans, and to see if there was an up-regulation of the synthetic pathway within the medial layer in the arteries from the rats and humans with CHF. Ultimately, all this information was designed to examine whether the ET-1 pathway is a viable system against which to target future drugs. Particular attention has been focused upon which constrictor ET-1 receptor subtypes present on VSMCs of resistance arteries to antagonise in order to reduce PVR in CHF.

The results from the first experimental chapter showed that ET-1 is a powerful vasoconstrictor in endothelium-denuded rat mesenteric arteries. Using STRX S6c, BQ-123, BQ-788 and TAK-044 it was demonstrated that both ET_A and ET_B receptors are responsible for mediating ET-1 constrictions. BQ-123 treatment resulted in an inhibition only of constrictions to the highest concentrations of ET-1, implying the constrictions to the lower concentrations of ET-1 are mediated via constrictor ET_B receptors. Circulating ET-1 levels are extremely low, even in disease, and these data suggest that ET_B receptors may be more important under physiological and pathophysiological conditions, as they mediate constrictions to the lower concentrations of ET-1. However, it was shown that ET_B receptor blockade does not inhibit ET-1 constrictions and only when combined with ET_A receptor antagonism was the entire ET-1 constrictor response inhibited. These observations suggest that ET_A receptors can compensate for ET_B receptor "loss" and it was hypothesised that the phenomenon of "crosstalk" between the ET-1 receptor subtypes was occurring. Crosstalk interactions have also been described between α_1- and α_2-adrenoceptors (Daly *et al.*, 1988) and δ-opioid receptor subtypes and μ-receptors (Traynor & Elliott, 1993).

Crosstalk between the ET-1 receptor subtypes had been demonstrated functionally in several different preparations, including the pulmonary artery (Fukuroda *et al.*, 1994a), fundal strips (Clozel & Gray, 1995) and tracheal and bronchial rings (Fukuroda *et al.*, 1996). The mechanism underlying crosstalk was hypothesised to be through interactions at the second messenger level, and it was Ozaki and colleagues (1997), using co-transfection studies who demonstrated the involvement of intracellular signalling in this phenomenon. They showed that when they transfected ET_A receptors into ET_B receptor-expressing Girardi heart cells, the binding characteristics of ET_B receptor agonists were altered. However, when the experiments were repeated using only the cell membranes, the binding characteristics of the agonists were unchanged (Ozaki *et al.*, 1997). Furthermore in the whole co-transfected cells, ET-1-mediated intracellular Ca^{2+} increases could only be completely abolished by a combination of BQ-123 and BQ-788, whereas

BQ-123 alone inhibited 80% of the response, but BQ-788 had no effect at all (Ozaki *et al.*, 1997). Thus, these observations support the functional data that has been reported, including the results of Chapter 3. The precise nature of the crosstalk mechanism warrants further investigation, particularly if ET-1 receptors communicate in a similar manner in human tissues, since this experimental chapter, plus the other reports of similar observations (Fukuroda *et al.*, 1994a; 1996; Clozel & Gray, 1995; Ozaki *et al.,* 1997), show that for effective ET-1 constrictor antagonism, both ET-1 receptor subtypes need to be inhibited.

In Chapter 4, the ET-1 receptor subtypes present on the smooth muscle of mesenteric arteries from a rat model of heart failure was investigated. The rat LV dysfunction model of CHF was used to assess whether there are alterations in the ET-1 receptor subtypes in resistance arteries at different time points after induction of heart failure. In rat arteries 5 weeks after left anterior descending coronary artery ligation, the sensitivity to ET-1 was unchanged. However, there appeared to be a change in the receptor subtypes mediating the differing components of the ET-1 CRC. BQ-123 inhibited entirely the ET-1 CRC in the arteries from the sham-operated rats, but a small BQ-123-insensitive part of the ET-1 CRC in the arteries from the 5 week CHF rats remained. This BQ-123-insensitive ET-1 response was only removed on combined ET_A/ET_B receptor inhibition, but not ET_B receptor desensitisation alone. Thus, in the arteries from the 5 week CHF rats, an ET_B receptor component had been activated, and on specific ET_B receptor blockade was compensated for by constrictor ET_A receptors. Therefore, as in Chapter 3, crosstalk between the ET-1 receptor was occurring, although to a lesser extent.

A further change occurred from 5 weeks post-ligation to 12 weeks post-ligation. The sensitivity of the arteries from the 12 week CHF rats was significantly reduced in comparison to control rats. However, BQ-123 antagonism shifted all ET-1 constrictions in both experimental groups to the right. Thus, the BQ-123-insensitive, ET_B receptor was not present in the 12 week CHF rat arteries. Indeed, when ET_B receptors were desensitised, the ET-1 sensitivity in the 12 week CHF rat arteries were restored to that of

the sham-operated animals, implying the presence of an inhibitory ET_B receptor on the VSMCs of these arteries, as opposed to a down-regulation of ET_A receptors.

In the arteries from the 5 and 12 week sham-operated and the 12 week CHF rats, the studies with BQ-123 and non-selective ET_A and ET_B receptor blockade imply that receptor crosstalk was not occurring. Furthermore, in the 5 week CHF rat arteries, the BQ-123-insensitive part of the ET-1 constriction was less marked than that seen in Chapter 3. In the 12 week CHF arteries the crosstalk mechanism may not have become apparent due to the presence of inhibitory ET_B receptors. However, the difference in results between the two chapters could have been due to the presence of indomethacin in the bathing Krebs-Heneseleit solution, suggesting that a COX product may have a role in the crosstalk mechanism between the two receptor subtypes.

Sokolovsky and co-workers (1992) demonstrated the presence of super-high (SH) and high (H) affinity ET_B receptor sites using binding assays. It was suggested that the SH affinity site represents the vasodilator ET_B receptor, whereas the H affinity site is the vasoconstrictor ET_B receptor (Sokolovsky *et al.*, 1992). Interconversion between the two states of the ET_B receptor might occur, and this may partially explain the results of the CHF rat model. For instance, at 5 weeks CHF, the H affinity, constrictor ET_B receptor may predominate, but at 12 weeks CHF the receptor state converts to the SH affinity, dilatory ET_B receptor. However, manipulation of G-proteins, using Gpp(NH)p did not affect either SH or H affinity states (Sokolosky *et al.*, 1992). Therefore, it appears that cycling between the two states does not occur. However, deglycosylation of the H affinity, vasoconstrictor ET_B receptor resulted in a decreased ability of the receptor to bind ligands, but had no effect on the SH affinity, vasodilatory ET_B receptor binding characteristics (Sokolosky *et al.*, 1992). This is a mechanism by which the crosstalk that Ozaki and colleagues (1997) described may occur.

Up-regulation of the mRNA for ET_B receptors in pathophysiological conditions such as atherosclerosis (Dagassan *et al.*, 1996), hypertension (Kanno *et al.*, 1993) and post

angioplasty (Wang *et al.*, 1995) have been demonstrated. However, in these studies the functional roles of the ET_B receptors have not been investigated, and it is not known whether they possess any constrictor or dilator properties or are expressed in a mitogenic capacity. The results seen in the 12 week CHF rat mesenteric arteries need to be further investigated, initially repeating the experiments with the endothelium intact, but also using molecular techniques to demonstrate whether there is an up-regulation of ET_B receptor, or down-regulation of ET_A receptor, mRNA and protein expression.

In Chapter 5 it was shown that human arteries from CHF patients also have attenuated constrictor responses to ET-1. In contrast to the rat studies, the presence of inhibitory ET_B receptors on the VSMC of the arteries were not responsible for the reduction in sensitivity to ET-1. Experiments using SRTX S6c as an agonist demonstrated that the small constrictor responses remained in the CHF arteries, suggesting that down-regulation of ET_A receptors is the most likely explanation for the decreased ET-1 sensitivity. Again, molecular techniques investigating ET-1 receptor subtype expression need to be performed. The desensitisation/down-regulation of ET-1 receptors is not wholly unsurprising as the activation of the ET-1 synthesis pathway was clearly demonstrated by the doubling of circulating big ET-1 levels. Curiously, there was no concomitant rise in circulating levels of ET-1, suggesting that local tissue conversion by VSMC expressed ECE may occur.

Immunohistochemical methods were employed in Chapter 6 to visualise ET-1 and ECE-1 in the vascular wall of the resistance arteries from rats and humans, and whether there is an alteration in the localisation of ET-1 and ECE-1 in CHF. In the rat arteries intense staining against both ET-1 and ECE-1 was found in the vascular endothelium and the adventitial layer, and there was no apparent difference between the vessels from CHF animals and the sham-operated controls. Interestingly, the staining against both ET-1 and ECE-1 in the human arteries was much less intense than seen in the rat vessels. Furthermore, the staining was only localised to the vascular endothelium, and there appeared to be no difference between the CHF patients arteries and the age-matched

control arteries. The immunohistochemical studies in the human arteries dispute the idea that there is up-regulated ECE-1, and there is a greater concentration of ET-1 on the VSMCs of arteries from the CHF patients. Any staining for ET-1 on the VSMC layer is most likely ET-1 binding to ET-1 receptors, and if there is a down-regulation of ET-1 receptors in the CHF vessels, as demonstrated functionally, then it may be expected that less ET-1 staining is likely in the media.

The reduced intensity of staining between the rat and human arteries might be due to the length of time the arteries from the biopsy or mesenteric bed arteries were snap frozen. The human arteries used in the histological studies were all dissected and frozen on the day after the biopsy had been performed. Dissection of the human arteries was a much more demanding task than the rat mesenteric bed dissection. The physical process of removing the artery from the surrounding fat of the biopsy was more difficult and time-consuming, and there was a greater chance of damaging the artery before mounting into the myograph. Furthermore, there were also fewer arteries within the biopsy of the correct lumen diameter for the studies. Therefore, the myography experiments were prioritised, and all suitable arteries mounted in the myograph first. Myography experiments were performed over two days. Once viable arteries had been mounted in the myograph on the second day, the remaining arteries were frozen to be used in the immunohistochemical studies. However, in the rat mesenteric bed there are many suitable arteries, so once dissected from the bed (a very fast process), the vessels were snap frozen immediately, within a few hours of removal from the animal. Therefore, the ET-1 and ECE-1 peptides could have been degraded in the human arteries over the two day period, such that the intensity of the staining was reduced. Furthermore, this could also explain why no difference in the localisation of ET-1 and ECE-1 in the arteries CHF patients and controls were observed.

Where the source of the raised big ET-1 levels originates is unknown. Although this immunohistochemistry study implies there is no change in ET-1/ECE-1 localisation the resistance arteries of CHF patients, it does not conclusively show that the vessels themselves are not the source of the big ET-1. There could be up-regulated synthesis in

the endothelium which was not shown using immunohistochemistry; a more sensitive technique being needed to definitively resolve this question.

The failing heart has also been suggested as a source of raised big ET-1/ET-1. Increased big ET-1, but not ET-1 levels in severe CHF patients were also observed by Wei and colleagues (1994). Using immunohistochemistry on cardiac atrial and ventricular tissue removed from human hearts, they found no difference in the intensity or localisation of staining in tissue from failing and healthy donor hearts. Furthermore, no big ET-1 was found in any of the heart tissue, and it was suggested that the heart is a target of ET-1, and not the source of raised circulating big ET-1/ET-1 (Wei *et al.*, 1994). Curiously, a study from the same group showed that atrial tissue from CHF dogs had significantly raised prepro ET-1 mRNA and mature peptide as compared to control dog hearts (Wei *et al.*, 1997). Another potential source of increased ET-1 synthesis is the lung. Pulmonary tissue prepro ET-1 mRNA and immunoreactivity is raised in CHF dogs (Wei *et al.*,1997) and in pulmonary hypertensive patients (Giaid *et al.*, 1993). In the lungs from the pulmonary hypertensive patients, the prepro ET-1 mRNA and immunoreactivity was localised to the endothelial cells of pulmonary arteries with medial thickening and intimal fibrosis (Giaid *et al.*, 1993). Furthermore, plasma ET-1 levels have been shown to correlate with pulmonary hypertension in CHF patients (Cody *et al.*, 1992). Reduced clearance of ET-1 may also contribute to the raised circulating ET-1 levels in patients with the most severe heart failure. Indeed, reduced ET_B receptors, but not ET_A receptors, have been shown in the lungs of CHF rats (Kobayashi *et al.*, 1997). Therefore, in the later stages of the condition a combination of increased synthesis and reduced clearance might be responsible for significantly increased big ET-1 and ET-1 levels.

What is clear from the results of this thesis is that peripheral resistance arteries have a reduced sensitivity to ET-1 in heart failure, and this reflects enhanced synthesis in humans. The clearance of the mature peptide does not appear to be compromised in the CHF patients sampled, who are in the moderate stages of heart failure. The findings of this thesis have shown that changes in the vascular responsiveness to ET-1 occur before

raised plasma levels are evident, suggesting that the involvement of the ET-1 system in the pathophysiology of CHF occurs earlier than had previously been predicted. In human CHF the reduced sensitivity seems to be occurring via a down-regulation or desensitisation of the constrictor receptors, whereas in the rat arteries the presence of an inhibitory ET_B receptor is responsible. As discussed previously, these discrepancies in the mechanisms by which the resistance arteries from humans and rats adapt to the constrictor responses to ET-1 could be due to the length of time over which heart failure manifests itself. However, in both species there is a definite decrease in constriction to exogenous ET-1.

H. 1.2. Future studies

To strengthen the results and conclusions of this thesis, other experiments should be performed in these isolated arteries. Complementary to the functional results, molecular techniques such as *in situ* hybridisation, rt-PCR or autoradiography should be performed. *In situ* hybridisation and rt-PCR against mRNA for prepro ET-1, ECE-1, ET_A and ET_B receptors could demonstrate whether there is an alteration in localisation and expression in the genes for the ET-1 pathway Autoradiography would show whether the mRNA signals for the receptor subtypes had been translated to the final protein binding sites.

These studies obviously need to be repeated in endothelium-intact arteries in order to assess any moderating actions of endothelial-derived factors. There is conflicting evidence concerning the production of counter-balancing factors, such as NO, in CHF, be it decreased, increased or no different from healthy arteries (Treasure & Alexandra, 1993). It has often been hypothesised that there is generalised endothelial dysfunction (Treasure & Alexandra, 1993) favouring constriction. In this thesis, there was no impairment of ACh-induced relaxation in the arteries from CHF rats or patients implying there was no agonist-induced endothelial dysfunction. However, this would need to be examined with different, more physiological agonists (endogenous ACh is highly unlikely to be stimulating the endothelium) and over concentration ranges to conclude whether there is agonist-induced endothelial dysfunction or not. Furthermore, the tonic effects of the

endothelium would also need to be investigated to conclude that there is generalised endothelial dysfunction. The advantages of using an *in vitro* technique such as perfusion myography, allows the removal of influencing factors like the endothelium, allowing easier and simpler interpretation of the results. The main aim of this thesis was to investigate ET-1 receptors on the VSMC layers in resistance arteries. Arteries *in vivo* will be influenced by phenomena including innervation, blood-borne factors, shear stress and the endothelium. Therefore, it is important to take the results of this thesis in context, and to combine these findings with *in vivo* studies where all of the regulatory mechanisms are intact.

H. 1.3. Clinical implications of this thesis

Despite the reduced vascular sensitivity in resistance arteries in CHF, the ET-1 system obviously still contributes to the enhanced vascular tone in man (Kiowski *et al.*, 1995; Love *et al.*, 1996a, b) and animals (Clavell *et al.*, 1996; Shimoyama *et al.*, 1996). 'To inhibit or not to inhibit' the ET_B receptor has been the conundrum since it was first suggested that the ET-1 system could be a potential therapeutic target in cardiovascular diseases such as CHF. The results of this thesis using the rat CHF model would imply that selective ET_A receptor inhibition would be the most advantageous method of reducing peripheral vascular resistance in heart failure. This would retain the dilator ET_B receptors on the endothelium as well as the up-regulated ET_B receptors on the VSMC layer. However, in the human arteries constrictor ET_B receptors were present, possibly mediating the constrictions to the lower concentrations of ET-1, whereas the constrictor actions of ET_A receptors were down-regulated. Therefore, non-selective ET_A/ET_B receptor blockade at the smooth muscle layer would be most appropriate. In addition, this thesis has further clinical implications in that it was shown that ET-1 constriction could be reversed relatively quickly in resistance arteries. This is an important finding for use of ET-1 receptor antagonists in acute vasospastic syndromes such as subarachnoid haemorrhage or Raynaud's disease.

As demonstrated by Sakai and colleagues (1996a, b) and Mulder and co-workers (1997), antagonism of the ET-1 pathway would go further than simply reducing systemic vascular resistance, by affecting the cardiac, mitogenic, anti-natriuretic and neuromodulating properties of the peptide. In parallel with the vasoconstriction question, which ET-1 receptor(s) to inhibit for all the detrimental properties of ET-1 has to be elucidated for the maximum beneficial effects of an anti-ET-1 therapy. Furthermore, at what stage of CHF should anti-ET-1 drugs be prescribed, since the ET-1 system may be important in the maintenance of cardiac contractility (Sakai *et al.*, 1996a) in the earlier stages of heart failure. However, in order for ET-1 antagonism, either inhibition of its synthesis or at the receptor level, to be accepted as a novel therapy in CHF, it has to be proved that it has additional vasodilator actions when administered in conjunction with ACE inhibitors.

H. 1.4. Conclusions

The results presented in this thesis demonstrate that CHF causes a disturbance of the ET-1 system under the pathophysiological condition of CHF. Although the physical activation of the ET-1 system was not demonstrated by measurement of plasma big ET-1 and ET-1 levels in the rat model of CHF and could not be seen using immunohistochemical techniques, at 12 weeks of CHF there was clearly an alteration in the vascular responsiveness to ET-1. This was due to a change in the ET-1 receptors on the VSMC layer of the small arteries. In human CHF, a change in the sensitivity to ET-1 was also shown and attributed to ET_A receptor down-regulation. Furthermore, the synthesis pathway of ET-1 was increased in the CHF patients, as demonstrated by plasma big ET-1. Thus, it is likely that the ET-1 system has a role in the raised systemic vascular resistance in CHF.

The prevalence of CHF is rising in the Western world, and once established impairs the quality of life more than most other chronic medical illnesses, killing between 60 to 70% of patients within five years of diagnosis. Although the current vasodilator therapies are fairly successful in the treatment of the condition there is still scope for new treatments. ET-1 inhibition is a promising novel therapy for CHF. Since the search for effective,

selective ECE inhibitors has proved extremely difficult, it is believed that ET-1 receptor antagonism is the most likely way in which the ET-1 system could be counteracted in CHF. The results of this thesis, together with the suggestions of complementary, future work, may help in the understanding of the ET-1 system in CHF, and the development of future ET-1 receptor antagonists for the treatment of CHF.

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Activation of endothelin ET_A receptors masks the constrictor role of endothelin ET_B receptors in rat isolated small mesenteric arteries

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1 Endothelin-1 (ET-1) produces constriction of the rat mesenteric vascular bed *in vivo* via ET_A and ET_B receptor subtypes. The aim of this study was to investigate the relative roles of these receptor subtypes in rat isolated, endothelium-denuded, small mesenteric arteries, under pressure, by use of ET-1; the ET_A receptor antagonist, BQ-123; the ET_B receptor selective agonist, sarafotoxin S6c (SRTX S6c); the ET_B receptor selective antagonist, BQ-788; and the ET_A/ET_B antagonist, TAK-044.

2 In 3rd generation mesenteric arteries, ET-1 (10^{-13}–10^{-7} M) produced concentration-dependent contractions (pD_2 9.86). SRTX S6c (10^{-12}–10^{-7} M) also induced concentration-dependent contractions in 53% of arteries studied, although the E_{max} was much less than that obtained with ET-1 (10.7±2.9% vs 101.9±2.6% of the 60 mM KCl-induced contraction).

3 Neither ET_B receptor desensitization, by a supra-maximal concentration of SRTX S6c (10^{-7} M), nor incubation with BQ-788 (3×10^{-8} M), had any significant effect on the ET-1 concentration-response curve, although both treatments tended to enhance rather than inhibit responses to ET-1.

4 In the presence of BQ-123 (10^{-6} M), responses to low concentrations of ET-1 (up to 10^{-10} M) were unaffected but responses to concentrations of ET-1 above 10^{-10} M were significantly inhibited.

5 SRTX S6c desensitization followed by incubation with BQ-123 (10^{-6} M) or co-incubation with BQ-788 (3×10^{-8} M) and BQ-123 caused inhibition of responses to all concentrations of ET-1, resulting in a rightward shift of the ET-1 concentration-response curve. The same effect was obtained by incubation with TAK-044 (10^{-8} M and 3×10^{-7} M).

6 Thus, responses of rat small mesenteric arteries to ET-1 are mediated by both ET_A and ET_B receptors. The relative role of ET_B receptors is greater than that predicted by the small responses to SRTX S6c or by resistance of ET-1-induced contraction to ET_B receptor desensitization or BQ-788. The effect of ET_B receptor desensitization or blockade is only revealed in the presence of ET_A receptor blockade, suggesting the presence of a 'crosstalk' mechanism between the receptors. These results support the concept that dual receptor antagonists, like TAK-044, may be required to inhibit completely constrictor responses to ET-1.

Keywords: Endothelin-1; sarafotoxin S6c; ET_A receptors; ET_B receptors; BQ-123; BQ-788; TAK-044

Introduction

It is now well established that the vasoactive effects of the peptide endothelin-1 (ET-1) are mediated via both ET_A (Arai *et al.*, 1990) and ET_B receptors (Sakurai *et al.*, 1990). Administration of ET-1 to anaesthetized or conscious rats leads to a brief decrease, followed by a long lasting increase, in blood pressure (Yanagisawa *et al.*, 1988) that is accompanied by increased resistance in virtually all vascular beds studied (Gardiner *et al.*, 1994; Allcock *et al.*, 1995). Prior administration of an ET_A receptor antagonist, e.g. BQ-123 or FR 139317, enhances the initial depressor effect of ET-1 (an ET_B receptor-mediated effect) and reduces the pressor effect (McMurdo *et al.*, 1993; Gardiner *et al.*, 1994). However, the pressor and regional constrictor effect of ET-1 is not fully inhibited by ET_A receptor antagonists, even with high doses, implying that ET_B receptors may also have a vasoconstrictor role (McMurdo *et al.*, 1993). Consistent with this possibility, the ET_B receptor selective agonist, sarafotoxin S6c (SRTX S6c) was found to produce vasoconstriction in pithed rats (Williams *et al.*, 1991; Clozel *et al.*, 1992).

In vitro experiments have also demonstrated ET_A receptor antagonist-resistant responses to ET-1 (Ihara *et al.*, 1992; Sumner *et al.*, 1992; Fukuroda *et al.*, 1994b) and constrictions to SRTX S6c (Moreland *et al.*, 1992; Sumner *et al.*, 1992; La Douceur *et al.*, 1993; Gray *et al.*, 1994). As a consequence of these *in vitro* data, it has been suggested that constrictor ET_B receptors have a role only in large calibre vessels and in the venous circulation (Moreland *et al.*, 1992; Davenport & Maguire, 1995). However, in the conscious rat (Gardiner *et al.*, 1994) and the anaesthetized ganglion-blocked rat (Allcock *et al.*, 1995), ET-1-induced reduction of blood flow to the mesenteric resistance bed is partly resistant to ET_A receptor inhibition. Reduction of regional blood flow in response to SRTX S6c is also most marked in the mesenteric bed of the pithed rat (Clozel *et al.*, 1992). In man, ET-1 constrictions in upper limb blood vessels are also partly resistant to BQ-123 and constrictions to SRTX S6c can be seen (Haynes *et al.*, 1995; Strachan *et al.*, 1995). Thus, there may be an important role for constrictor ET_B receptors in mediating vascular resistance and blood pressure. Indeed, the recently described non-peptide ET_B receptor antagonist, Ro 46-8443, causes a reduction in blood pressure in anaesthetized, normotensive rats (Clozel & Breu, 1996).

In contrast to the evidence for ET_B receptor-mediated constriction of the rat mesenteric bed *in vivo*, *in vitro* studies of perfused mesenteric beds or human and rat isolated mesenteric arteries mounted in wire or perfusion myographs have led to the conclusion that constrictor ET_B receptors have little (Tschudi & Luscher, 1994; Takase *et al.*, 1995; Deng *et al.*, 1995; Touyz *et al.*, 1995) or no role (D'Orleans-Juste *et al.*, 1993) in this vascular bed. All of these studies have based their conclusions on inhibition of ET-1-induced contraction by ET_A

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receptor antagonists, or responses to ET_B selective agonists. The aim of the present study was to investigate further the role of ET_B receptors in mediating constriction in pressurized rat mesenteric arteries by use of ET-1, the ET_A receptor antagonist, BQ-123 (Ihara *et al.*, 1992), the ET_B selective agonist SRTX S6c (Williams *et al.*, 1991), the ET_B receptor selective antagonist, BQ-788 (Ishikawa *et al.*, 1994) and the ET_A/ET_B antagonist, TAK-044 (Kikuchi *et al.*, 1994).

Some of this work has been presented to the British Pharmacological Society (Mickley *et al.*, 1995).

Methods

Male Wistar rats (10–16 weeks old) were killed by exsanguination and the mesenteric bed immediately excised and placed into cold, oxygenated Krebs-Henseleit solution. Third order branches of the mesenteric artery (internal diameter 150–350 μm) were dissected (~3 mm length) and mounted between two glass microcannulae in a small vessel arteriograph (Living Systems Instrumentation Inc., Burlington, U.S.A.). The vessel was constantly superfused with warmed (37°C), oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution (composition, in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.5). The intraluminal pressure of the vessel was raised to 60 mmHg and maintained at this pressure with a pressure servo unit without further intraluminal perfusion. Luminal diameter was measured with a video dimension analyser (Living Systems Instrumentation Inc., U.S.A.) and by hand, with a calibrated micrometer, when the optical dimension analyser was unable to detect differences in optical density at smaller lumen diameters. After an equilibration period of 60 min, the vessels were exposed twice to modified Krebs-Henseleit solution containing 60 mM KCl (equimolar replacement of NaCl by KCl) in order to produce maximum constriction. KCl induced a reduction in lumen diameter but never to the level where the lumen was completely occluded (see Table 1). The endothelium was removed by passing an air bubble through the lumen of the vessel (Falloon *et al.*, 1993; Smith, 1996) and complete denudation was confirmed by addition of acetylcholine (ACh 10^{-6} M) to vessels pre-constricted with phenylephrine (PE 10^{-5} M). In all vessels, the relaxation induced by ACh before the passage of an air bubble (usually back to resting diameter), was completely abolished after endothelial denudation. After washing, a closed system with a total volume of 30 ml of Krebs-Henseleit solution was constantly superfused at a constant flow rate of 5 ml min^{-1}. It was this reservoir of Krebs-Henseleit solution to which the agonists and antagonists were applied, keeping the volume at 30 ml by removing one ml of Krebs and adding one ml of the drug in a stepwise fashion (as previously described, Smith *et al.*, 1995). Responses were recorded 5 min after addition of each agonist concentration, which was sufficient time for an equilibrium response. All of the following studies were carried out in random order and only one concentration response curve to ET-1 or SRTX S6c was performed per tissue. None of the drug treatments resulted in complete occlusion of the vessel lumen within the concentration range studied (see Table 1).

ET-1 and SRTX S6c study

In the first set of experiments cumulative concentration-response curves to ET-1 (10^{-13}–3×10^{-8} M, $n=10$) or SRTX S6c (10^{-12}–10^{-7} M, $n=17$) were obtained as described above.

Receptor antagonism study

In the second set of experiments, vessels were exposed to either BQ-123 (10^{-6} M, $n=8$), BQ-788 (3×10^{-8} M, $n=8$), TAK-044 (10^{-8} and 3×10^{-7} M, $n=4$ and 8 respectively), BQ-123 + BQ-788 (concentrations as before, $n=8$) or vehicle ($n=8$) for 30 min, before concentration-response curves to ET-1 (10^{-13}–3×10^{-8} M) were obtained. For these experiments, agonists were prepared in a solution of antagonist so that addition to the perfusion circuit did not dilute the antagonist solution superfusing the tissue. In some experiments, the vessels were exposed for 30 min to SRTX S6c (10^{-7} M) twice (with a wash out period of 10 min between each exposure), in order to desensitise the ET_B receptor before commencement of the ET-1 concentration-response curve. This was carried out both in the absence and in the presence of BQ-123 ($n=8$ each). In all experiments, the time-course of the protocol was the same; 2 h after verification of the removal of the endothelium, the concentration-response curve to ET-1 was begun.

Data analysis

The results are calculated as a percentage of maximum constriction obtained with the second exposure to 60 mM KCl Krebs solution and are expressed as mean ± s.e.mean. Where a maximum response to the agonist was obtained, the negative log of the concentration causing half-maximal contraction (pD_2) was calculated by linear regression analysis and compared by unpaired one-tailed t test. The concentration-response curves were compared by one-way ANOVA followed by Fisher's least significant difference test. Significance was taken at $P<0.05$.

Materials

ET-1 and SRTX S6c were purchased from Novabiochem (Nottingham, U.K.) and BQ-788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-MeLeu-D-Trp($COOCH_3$)-D-Nle, sodium salt) from Neosystems (Strasbourg, France), all were reconstituted in 50:50 methanol:distilled water. BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Vel-L-Leu]) from Neosystems (France) and TAK-044 (cyclo[D-α-Asp-3-[(phenylpiperazin-1-yl)carbonyl]-L-Ala-α-Asp-D-2-(2-thienyl)-Gly-L-Leu-D-Trp] disodium salt) synthesised by Takeda Chemical Industries (Osaka, Japan) were reconstituted in 0.9% saline, placed in aliquots and stored frozen at −20°C until use. All peptide agonists and antagonists were diluted in Krebs-Henseleit solution containing 0.1% bovine serum albumin (BSA: Sigma, Poole, U.K.). In

Table 1 Mean resting lumen diameters and lumen diameters after exposure to 60 mM KCl solution or after the maximum concentration of endothelin-1 (ET-1) or sarafotoxin S6c (SRTX S6c) in each experimental group

	ET-1 control	*SRTX S6c*	*+ BQ-123*	*+ BQ-788*	*+ SRTX S6c desens*	*+ BQ-123 + BQ-788*	*+ BQ-123 + SRTX S6c desens*	*+ TAK-044* (10^{-8} M)	*+ TAK-044* (3×10^{-7} M)
Resting diameter	277 ± 15	300 ± 9	261 ± 13	287 ± 15	281 ± 7	273 ± 21	304 ± 19	300 ± 12	301 ± 12
+ 60 mM KCl diameter	51 ± 3	48 ± 2	53 ± 3	51 ± 1	48 ± 3	55 ± 2	50 ± 3	45 ± 3	50 ± 2
Max ET-1/SRTX S6c diameter	47 ± 3	273 ± 12	56 ± 7	50 ± 2	48 ± 3	64 ± 8	118 ± 27	118 ± 39	233 ± 31

Data shown are mean ± s.e.mean.

all antagonist experiments the ET-1 concentrations were diluted in 0.1% BSA Krebs-Henseleit solution with the appropriate antagonist. ACh (chloride salt, Sigma, Poole, U.K.) and PE (hydrochloride salt; Fisons, U.K.) were prepared in saline at stock concentration of 10^{-2} M, placed in aliquots, and stored at −20°C until use when diluted in Krebs-Henseleit solution.

Results

Effects of 60 mM KCl

In all experiments 60 mM KCl superfusion constricted the arteries, an effect which was reversible, back to initial resting diameter, on washout (Table 1). The initial diameter remained constant until agonist-induced constriction was generated.

Effects of ET-1 and SRTX S6c

ET-1 constricted the arteries in a concentration-dependent manner (Figure 1, pD_2 9.86, E_{max} 101.9±2.6% KCl induced contraction at 10^{-8} M ET-1, $n=10$). SRTX S6c also produced a concentration-dependent contraction (Figure 1), but the response was extremely variable, the maximum response obtained with 3×10^{-8} M SRTX S6c ranging from 0 to 39% of KCl contraction (mean response = 10.7±2.9%, $n=17$). In fact, only 9 of the 17 vessels (53%) responded to SRTX S6c.

Effect of ET_A receptor blockade

Incubation with BQ-123 (10^{-6} M) before and during exposure to ET-1 (Figure 2) had no effect on contractile responses to low concentrations of ET-1 (10^{-13} to 10^{-10} M) but resulted in inhibition of responses to concentrations of ET-1 between 10^{-10} and 3×10^{-8} M. Incubation with BQ-123 significantly inhibited the constrictions to 10^{-9} and 3×10^{-9} M ET-1 ($P=0.006$ and 0.01, respectively) when compared by ANOVA. However, the effect of BQ-123 on the overall pD_2 of the ET-1 concentration-response curve did not reach statistical significance (pD_2 9.15 ($n=8$) vs 9.86 ($n=10$) NS, $P=0.094$).

Effect of ET_B receptor desensitization or blockade

Exposure to a supra-maximal concentration of SRTX S6c (10^{-7} M), to achieve ET_B receptor desensitization, produced an initial constriction in 4 out of the 8 vessels studied (mean response = 8.1±3.5% KCl constriction). The vessel diameter returned to the initial resting value during the first 30 min exposure to SRTX S6c. No constriction was seen, in any of the vessels studied, during the second exposure to SRTX S6c confirming that tachyphlaxis had occurred. The ET-1 concentration-response curve was not significantly altered by either ET_B receptor desensitisation (Figure 3a, $pD_2=9.88$, $n=8$) or following incubation with the selective ET_B receptor antagonist, BQ-788 (3×10^{-8} M, Figure 3b, $pD_2=10.02$, $n=8$), although both treatments tended to shift the ET-1 concentration-response curve to the left ($P=0.5$ and 0.34, respectively).

Effect of combined ET_A and ET_B receptor blockade

Co-incubation of vessels with BQ-123 (10^{-6} M) and BQ-788 (3×10^{-8} M) resulted in a parallel shift of the ET-1 concentration-response curve to the right (Figure 4, $n=8$). Incubation with BQ-123 (10^{-6} M) following desensitization of ET_B receptors with 10^{-7} M SRTX S6c caused a similar rightward shift (Figure 4, $n=8$). Incubation of vessels with the ET_A/ET_B receptor antagonist, TAK-044 (Figure 5, 10^{-8} M, $n=4$ and 3×10^{-7} M, $n=8$) also caused a parallel concentration-dependent shift to the right of the ET-1 concentration-response curve. As the maximum response to ET-1 was not reached within the concentration range studied it was not possible to calculate pD_2 values for ET-1 in experiments with BQ-123 plus either BQ-788 or SRTX S6c desensitization, or with TAK-044 (both concentrations).

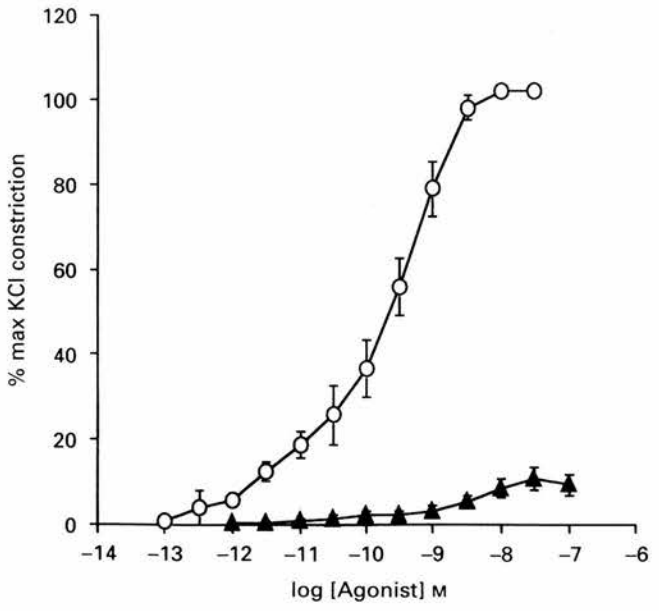

Figure 1 Comparison of the contractile responses to endothelin-1 (ET-1, ○) and sarafotoxin S6c (SRTX S6c, ▲) in rat small mesenteric arteries. ET-1 ($n=10$) produced a maximal constriction of similar proportion to 60 mM KCl at 3×10^{-9} M. SRTX S6c ($n=17$) induced small constrictions at the highest concentrations, suggesting a small population of ET_B receptors present on the smooth muscle of the resistance arteries. All values are mean and vertical lines show s.e.mean.

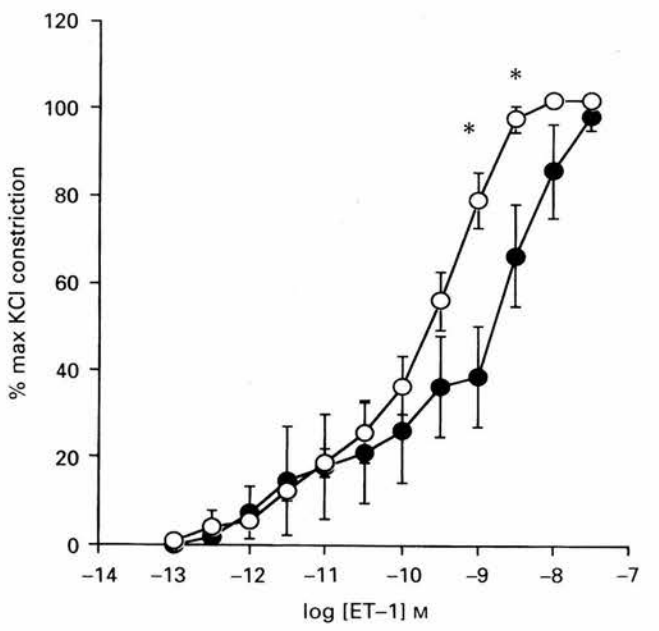

Figure 2 Effect of the ET_A receptor antagonist BQ-123 on the endothelin-1 (ET-1) concentration-response curve in rat small mesenteric arteries. Pre-incubation with BQ-123 (10^{-6} M) for 30 min (●, $n=8$) shifted the responses to the higher concentrations of ET-1 in a parallel fashion to the right. All values are mean and vertical lines show s.e.mean. *$P<0.05$ compared to control (○) ET-1 responses.

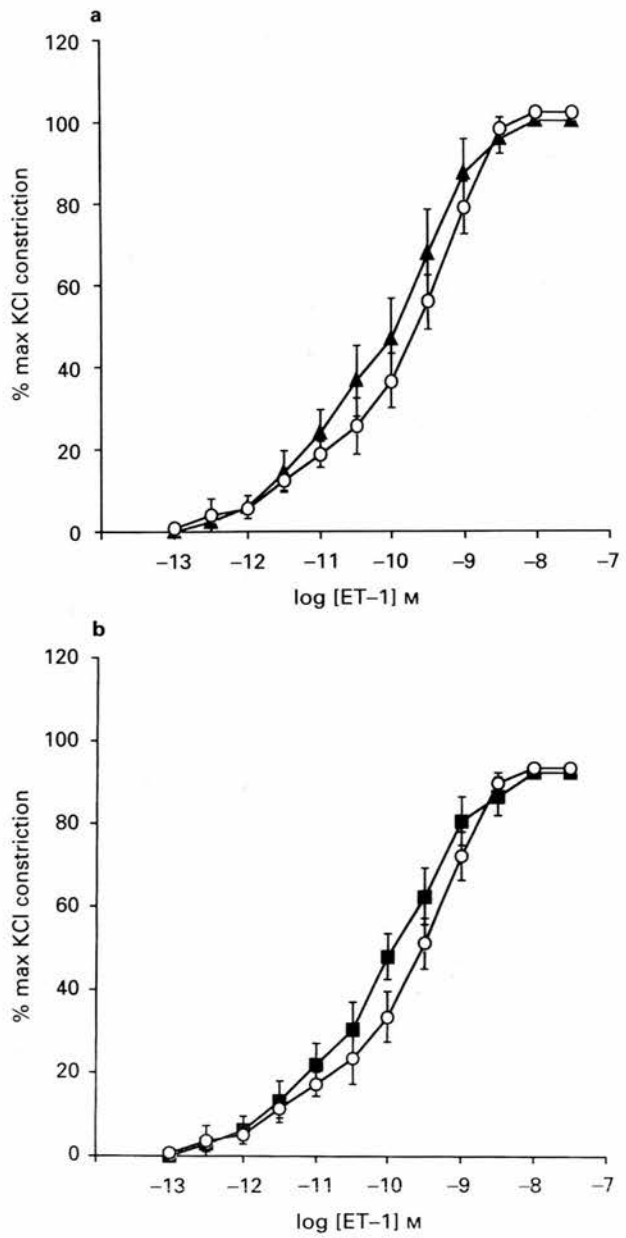

Figure 3 The effects of selective ET_B receptor blockade on endothelin-1 (ET-1)-induced constrictions in rat small mesenteric arteries. The vessels were exposed to either (a) sarafotoxin S6c (SRTX S6c; 10^{-7} M, ▲, $n=8$) twice before addition of ET-1 or (b) BQ-788 (3×10^{-8} M, ■, $n=8$) pre-incubated for 30 min before the start of the ET-1 concentration-response curve. In both treatments the ET-1 concentration-response curves tended to be shifted slightly to the left as compared to control (○), (though not significant, $P=0.54$ and 0.42, respectively, as compared by ANOVA). All values are mean and vertical lines show s.e.mean.

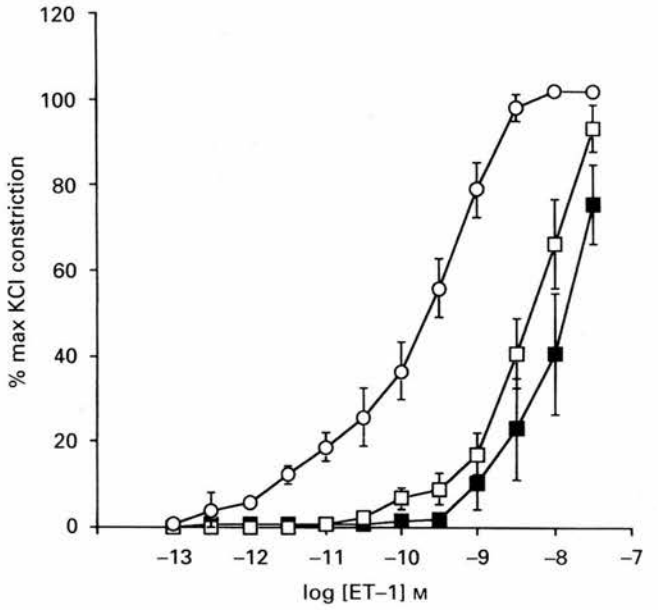

Figure 4 The effects of non-selective ET_A/ET_B combination treatment on endothelin-1 (ET-1)-induced constrictions in rat small mesenteric arteries. The vessels were exposed to either vehicle (○), BQ-123 plus BQ-788 (10^{-6} M and 3×10^{-8} M, □, $n=8$) or pre-incubated with sarafotoxin S6c twice (each 10^{-7} M) plus BQ-123 (10^{-6} M, ■, $n=8$). Both treatments significantly shifted the ET-1 concentration-response curve to the right in a parallel fashion ($P=0.0001$ for both). All values are mean and vertical lines show s.e.mean.

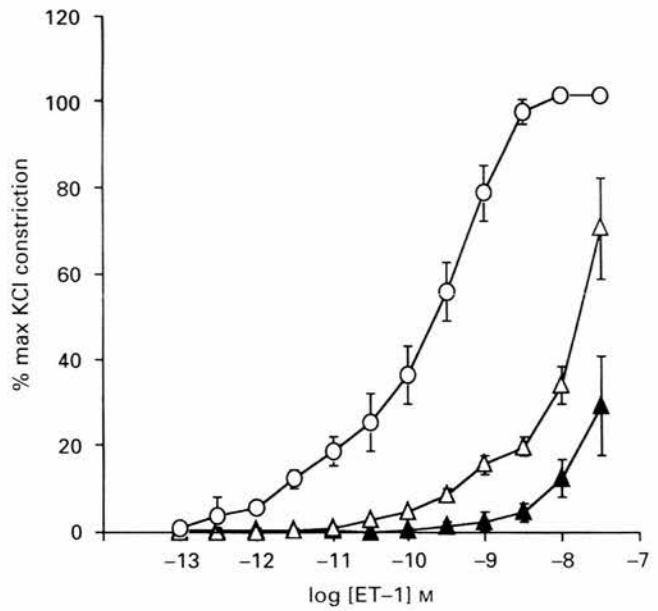

Figure 5 The effects of the non-selective ET_A/ET_B receptor antagonist TAK-044 on endothelin (ET-1)-induced constrictions in rat small mesenteric arteries. The vessels were pre-incubated for 30 min with either 10^{-8} M (△, $n=4$) or 3×10^{-7} M (▲, $n=8$) TAK-044. Both treatments significantly inhibited the ET-1 concentration-response curve ($P=0.0002$ and 0.0001 respectively) as compared to control (○). All values are mean and vertical lines show s.e.mean.

Discussion

Previous *in vivo* studies have clearly indicated a role for ET_B receptors in mediating vasoconstriction in resistance beds, but their role has been difficult to demonstrate in isolated resistance vessels. In the present study, we show that a role for ET_B receptors in rat isolated mesenteric arteries emerges when both ET_A and ET_B receptors are blocked, whereas blockade of ET_A receptors alone only partially inhibited ET-1-induced contraction and inhibition of ET_B receptors alone had no effect. This phenomenon is similar to previous observations in rabbit pulmonary artery (Fukuroda *et al.*, 1994c), rat trachea (Clozel & Gray, 1995) and human bronchus (Fukuroda *et al.*, 1996), and may be explained by the existence of a 'crosstalk' mechanism between the ET_A and ET_B receptors.

In initial experiments we used the highly selective ET_B receptor agonist SRTX S6c (Williams *et al.*, 1991) to investigate the presence of ET_B receptors in pressurised mesenteric arteries. SRTX S6c produced concentration-dependent con-

striction but the maximum constriction reached only ~10% of that routinely seen with ET-1, much less than would have been predicted from previous *in vivo* experiments (Clozel *et al.*, 1992). However, the magnitude of responses to SRTX S6c is in agreement with responses obtained by Takase *et al.* (1995) and Deng *et al.* (1995), in rat mesenteric arteries studied in the perfusion and wire myograph, respectively. Interestingly, in all three studies, the contractions of SRTX S6c occurred at relatively high concentrations (10 nM). The ET_B receptor agonists, BQ-3020 and IRL 1620, were equally ineffective in the rat perfused mesenteric bed at concentrations up to 1 nM (D'Orleans-Juste *et al.*, 1993). This is quite different to the ET_B agonist responses induced in large blood vessels, which are generally larger and occur at lower concentrations (Moreland *et al.*, 1992, Sumner *et al.*, 1992; LaDouceur *et al.*, 1993; Gray *et al.*, 1994). Another interesting feature of our results, not mentioned by previous investigators, is the variability in responsiveness to SRTX S6c. While some vessels failed to respond, others gave up to ~40% of the maximum contraction obtained with ET-1. This might be explained by differential distribution of ET_B receptors in the mesenteric bed, although 3rd generation branches of the main mesenteric artery were routinely used for these studies. Another possibility is variation in intrinsic myogenic tone that these vessels can develop when under pressure. In a separate experiment, in which vessels mounted in the wire myograph were studied, we found that no responses were obtained to STRX S6c until some tone was introduced by a low concentration of the stable thromboxane analogue, U46619 (Mickley *et al.*, 1995).

An alternative approach for the investigation of the role of ET_B receptors is to remove the influence of ET_B receptors, either by desensitization (LaDouceur *et al.*, 1993) or by use of a selective ET_B receptor antagonist, like BQ-788 (Ishikawa *et al.*, 1994). In the present study, neither of these interventions inhibited ET-1 induced contraction, a result which would support the view that ET_B receptors have little or no role in rat mesenteric arteries. Interestingly, both desensitization and BQ-788 treatment seemed to potentiate responses to ET-1 slighty, although this effect was not significant. Seo (1996) recently found a similar potentiation of ET-1-induced constriction by the ET_B receptor antagonist, Res 701-1 in human gastroepiploic arteries. There are several possible explanations for these observations. Potentiation of contractions by ET_B receptor antagonists would be expected in the presence of the vascular endothelium due to blockade of endothelial ET_B receptor-mediated release of relaxing factors by ET-1. However, this is an unlikely explanation for the present results as the endothelium was effectively removed by passing of an air bubble through the lumen of the vessels, as evidenced by the loss of relaxant responses to acetylcholine. Previous histological studies in our laboratory have also shown complete removal of the endothelium by this method (Smith, 1996). The experiments of Seo (1996) were also conducted in endothelium-denuded vessels. Alternatively, potentiation might have been caused by displacement of ET-1 from low affinity ET_B clearance receptors (Fukuroda *et al.*, 1994a) by BQ-788, but this would not account for the similar effect of receptor desensitisation. Another alternative, suggested by Seo (1996), is the presence of sensitive ET_B receptors on smooth muscle which inhibit or negatively modulate ET_A receptor-mediated constrictions to ET-1.

From the results obtained with SRTX S6c, BQ-788, and desensitization alone, one would predict that blockade of ET_A receptors, by use of a selective competitive antagonist, like BQ-123 (Ihara *et al.*, 1992), would cause a parallel rightward shift of the ET-1 concentration-response curve. However, in the presence of BQ-123 the ET-1 concentration-response curve in mesenteric arteries under pressure was biphasic, only responses to high concentrations of ET-1 being shifted to the right in a parallel manner by BQ-123, consistent with competitive antagonism at the ET_A receptor. Interestingly, the BQ-123-resistant, possibly ET_B-mediated, responses to ET-1 were at the lower end of the dose-response curve, consistent with the presence of a high affinity ET_B receptor. Takase *et al.* (1995) obtained similar results with the ET_A receptor antagonist, FR139317 in rat mesenteric arteries, although in that case the ET_A-resistant component was smaller than seen here. Takase *et al.* perfused the vessels at a pressure of 30 mmHg, half of that used in the present study. Given our observation that increased tone may reveal constrictor ET_B receptors, as implied by the responses to SRTX S6c (Mickley *et al.*, 1995), the lower pressure used by Takase *et al.* (1995) may account for the smaller ET_A receptor antagonist-resistant element of the ET-1 curve. The results of the present study are consistent with the ET_A receptor antagonist resistant reduction in mesenteric blood flow induced by ET-1 *in vivo* found by Gardiner *et al.* (1994) and Allcock *et al.* (1995).

In order to investigate whether the residual ET_A antagonist resistant portion of the ET-1 response is mediated by ET_B receptors, we used combined treatment with BQ-123 and either desensitization or BQ-788. Both of these combination treatments resulted in a parallel shift of the ET-1 concentration-response curve. In fact, the BQ-123-sensitive portion was moved further to the right than with BQ-123 alone, in agreement with Fukuroda *et al.* (1996) who described a similar phenomenon in human bronchi. Responses to ET-1 were also inhibited, in a concentration-dependent manner, by TAK-044, a peptide antagonist with similar potency at both ET_A and ET_B receptors (Kikuchi *et al.*, 1994).

These results demonstrate a clear role for ET_B receptors in mediation of constrictor responses to ET-1 in small mesenteric arteries that is only revealed when ET_A receptors, in addition to ET_B receptors, are blocked. The lack of effect of ET_B receptor blockade or desensitization alone seems to indicate that ET_A receptors can somehow compensate for the inactivation of ET_B receptors. Similar observations have been obtained in vascular (Fukuroda *et al.*, 1994c) and non-vascular (Clozel & Gray, 1995; Fukuroda *et al.*, 1996) tissues. The concept of receptor 'crosstalk' has been proposed to explain these observations. The mechanism is not fully understood, although interactions at the second messenger level have been suggested, such that blockade of the ET_B receptor releases an inhibitory mechanism acting at the ET_A receptor (Fukuroda *et al.*, 1996). Allosteric interactions between ET receptors have been suggested to account for the results of radioligand binding studies in rat heart (Sokolovsky, 1993). Further biochemical studies are required to elucidate the interactions between ET receptors co-existing in the same tissue and the mechanism of the apparent crosstalk phenomenon. Interestingly, similar interactions have been described between α_1- and α_2-adrenoceptors activated by noradrenaline (Daly *et al.*, 1988).

In the rat, the mesenteric bed receives a high proportion of cardiac output and thus resistance in this bed is an important determinant of total peripheral resistance and of blood pressure. The present results show that simultaneous blockade of both ET_A and ET_B receptors is required for complete inhibition of constrictor responses to ET-1 in the rat mesentery *in vitro*. This agrees with observations that blockade of both receptors is required to inhibit ET-1-induced increases in blood pressure *in vivo* (McMurdo *et al.*, 1993). The role of ET_B receptors in regulating constrictor responses to ET-1 might be even greater in human resistance vessels, where ET_B agonists have a greater direct effect than in other species *in vitro* (Takase *et al.*, 1995, Mickley, unpublished observations) and *in vivo* (Haynes *et al.*, 1995).

In some pathophysiological states associated with increased peripheral resistance and increased plasma concentrations of ET-1, there is evidence for an upregulation of smooth muscle ET_B receptors; most notably in heart failure in dogs (Cannan *et al.*, 1996) and man (Love *et al.*, 1996); in atherosclerosis (Winkles *et al.*, 1993; Dagassan *et al.*, 1996) and in hypertension (Kanno *et al.*, 1993; Batra *et al.*, 1993). The results of the present study suggest that blockade of both ET_A and ET_B receptors may be required for effective inhibition of ET-1-induced constriction in these diseases. This study was conducted

in vessels without endothelium. However, in the presence of endothelium, ET_B receptor blockade can actually enhance responses to ET-1 by blocking the release of nitric oxide and prostacyclin through endothelial ET_B receptor stimulation (De Nucci *et al.*, 1988). Thus, the effectiveness of endothelin receptor blockade therapeutically will depend on the level of endothelial ET_B receptor stimulation and on the relative selectivity of the antagonist for endothelial and smooth muscle ET_B receptors, the ideal antagonist allowing ET-1 to act at the endothelial ET_B receptor while blocking its effects at smooth muscle ET_A and ET_B receptors.

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