THE ROLE OF ENDOTHELIN IN THE MULTIHORMONAL REGULATION OF FLUID RETENTION IN CONGESTIVE HEART FAILURE

by

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Abstract

Abnormal fluid retention is characteristic of congestive heart failure (CHF). The mechanisms responsible for this phenomenon are unclear. Recently, the role of endothelin (ET) in the pathophysiology of CHF has been given much attention. Studies were conducted to elucidate how ET may contribute to salt and water retention. Cardiomyopathic (CM) hamsters with a moderate degree of heart failure were employed for in vivo and in vitro trials. Clearance methods were used to determine the level of renal function in CM hamsters in comparison with age- and sex-matched control animals. Plasma and urine samples were collected for determination of haematocrit, plasma protein concentration, glomerular filtration rate (GFR), and sodium and water excretion while mean arterial blood pressure (MAP) was monitored intermittently. Radioligand binding studies were carried out to determine ET receptor distribution in the inner medullary collecting duct (IMCD) of CM and control hamsters. The effects of chronic enalapril therapy on renal function and ET receptors were investigated to deduce any interaction between the renin-angiotensin-aldosterone system (RAAS) and the ET hormonal system. Studies were also performed to measure the stimulation response of IMCD cells to ET-1 or to angiotensin II administration.

The results demonstrated that renal excretion of sodium and water was diminished in the CHF hamsters in response to atrial natriuretic peptide (ANP) infusion and the fractional excretion of these variables decrementally decreased with increasing severity of heart failure. Binding studies revealed an equal distribution of $ET_A$ and $ET_B$ receptor subtypes in the IMCD of the hamsters and these receptors, particularly the B variety, were downregulated in the diseased animals. A similar negative correlation was apparent between the receptor density and the degree of cardiac dysfunction — the number of ET receptors declined as heart failure became more pronounced. Following chronic treatment
with the angiotensin converting enzyme (ACE) inhibitor, enalapril, an improved renal excretory response to ANP was observed with simultaneous restoration of ET receptor density in the IMCD cells. Furthermore, the correlations of the severity of heart failure, fractional excretion of electrolytes, and receptor density, were abolished with enalapril.

Cells of the IMCD in CM hamsters exhibited lower basal levels of cGMP accumulation than those of the normal controls. Stimulation of the IMCD cells with ET-1 showed a dose-dependent increase in cGMP production in both CM and healthy animals, but the responses were attenuated in the former. This blunted response may represent a reduction in ET receptor number in the CM hamster kidneys.

Incubation of rat IMCD cells with low-concentration angiotensin II displayed a downregulation of ET receptors with an increase in receptor affinity. Co-incubation with the angiotensin receptor antagonist, saralasin, prevented this downregulation and the ET-1 binding affinity remained constant. These results suggest that angiotensin II may participate in the regulation of ET receptor expression.

When the data are taken together, there is a strong implication that ET, in combination with angiotensin II, contributes to the regulation of salt and water homeostasis. That renal excretory function is improved with concomitant restoration of ET density after enalapril therapy is convincing evidence that these two hormones interact to promote abnormal fluid retention in the setting of CHF. In conclusion, in the CM hamster model, it is the insufficient ET receptor density, secondary to angiotensin II activation, that results in the unchecked reabsorption of electrolytes by the kidneys in CHF.
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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine vasopressin</td>
</tr>
<tr>
<td>Bmax</td>
<td>Maximum binding capacity</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>([Ca^{2+}]_i)</td>
<td>Intracellular calcium concentration</td>
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<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-cyclic monophosphate</td>
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<tr>
<td>CCD</td>
<td>Cortical collecting duct</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>cGMP</td>
<td>Guanosine 3',5'-cyclic monophosphate</td>
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<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
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<tr>
<td>CM</td>
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<tr>
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<td>C-type natriuretic peptide</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>ECE</td>
<td>Endothelin converting enzyme</td>
</tr>
<tr>
<td>ECFV</td>
<td>Extracellular fluid volume</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
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<td>Endothelin</td>
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<td>ET&lt;sub&gt;A&lt;/sub&gt;</td>
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<td>ET&lt;sub&gt;C&lt;/sub&gt;</td>
<td>Endothelin receptor subtype C</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FE&lt;sub&gt;Na&lt;/sub&gt;</td>
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<tr>
<td>FE&lt;sub&gt;H2O&lt;/sub&gt;</td>
<td>Fractional excretion of water</td>
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<tr>
<td>G</td>
<td>Heterotrimeric G protein</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
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<tr>
<td>IMCD</td>
<td>Inner medullary collecting duct</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>(k_f)</td>
<td>Ultrafiltration coefficient</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
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<td>Phosphatidylcholine</td>
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<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
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<td>PGI₂</td>
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</tr>
<tr>
<td>PI</td>
<td>Phosphoinositide</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
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<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>ROC</td>
<td>Receptor operated calcium channel</td>
</tr>
<tr>
<td>RPF</td>
<td>Renal plasma flow</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RVR</td>
<td>Renal vascular resistance</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
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<tr>
<td>STX</td>
<td>Sarafotoxin</td>
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<tr>
<td>TXA₂</td>
<td>Thromboxane A₂</td>
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<tr>
<td>U₉GMP</td>
<td>Urinary cGMP</td>
</tr>
<tr>
<td>UV</td>
<td>Urine flow</td>
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<tr>
<td>UₙNaV</td>
<td>Urinary sodium excretion</td>
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<tr>
<td>VDCC</td>
<td>Voltage-dependent calcium channel</td>
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# LIST OF LETTER SYMBOLS

Three-letter and One-letter Symbols for Amino Acids

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<th>Amino Acid</th>
<th>Three-letter</th>
<th>One-letter</th>
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</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
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<tr>
<td>Glutamic acid</td>
<td>Glu</td>
<td>E</td>
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<td>Phenylalanine</td>
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<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
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<td>Methionine</td>
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<td>Tyrosine</td>
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Chapter One  Introduction

The syndrome of congestive heart failure (CHF) is defined as the inability of the heart to sufficiently pump blood to meet the metabolic requirements of the body. Typically, one side of the heart fails first, followed by the other. The term "congestive" refers to the backing up of blood in the pulmonary veins leading to congestion of the lungs and viscera. Symptoms and signs of CHF include cardiac hypertrophy, hepatomegaly, oedema, dyspnea, weakness, prolonged circulation time, neck vein distension, and reduced exercise capacity.

A wide array of disturbances can lead to CHF. Myocardial damage due to ischaemic heart disease, idiopathic dilated cardiomyopathy, and myocarditis are examples of intrinsic heart disease that result in heart failure. Toxin-induced injury such as that from drug overdose or alcoholism or viral infection can lead to cardiac dysfunction. Other insults to the heart may be of pressure or volume overload in nature. For example, chronic systemic hypertension and mitral valve regurgitation also stress the heart to the degree of failure.

Despite continued and recent advances in the understanding and treatment of cardiovascular disease, CHF remains a major health concern. The prevalence of CHF is estimated to be 4 million persons in the United States alone (1), with an incidence of 400 000 persons annually (2). Morbidity is substantial, accounting for 6% of all hospital discharges while the mortality rate is greater than 1 quarter million individuals per year. The prognosis of CHF is grim, with the likelihood of 5-year survival less than 50% (3) from first diagnosis. Many factors have been associated with survival time (4-7).
1.1 Structural and Physiological Changes in CHF

As heart failure develops, adaptive processes occur within the heart to compensate for reduced pumping efficiency (8,9). Initially, there is a gradual increase in the chamber size of the heart which is mainly due to 'slippage' of the myocardial fibres. As the heart continues to dilate, destruction and scarring of the myocytes curb this slippage; with the consequence that greater forces of contraction are required to achieve shortening (Frank-Starling Law). Second, cardiac hypertrophy ensues to minimize wall stress. This increase in cell mass maintains adequate cardiac output for some time but at the expense of increased oxygen consumption and depressed contractility.

Compensatory mechanisms in CHF are complex (Figure 1.1). Not only is there remodelling of the heart, but neural, hormonal, haemodynamic, and renal adaptations are manifest as well (3, 10-13). Sympathetic activity is increased upon unloading of arterial baroreceptors by diminished blood pressure or stroke volume (14-16). This is accompanied by a rise in the activity of the renin-angiotensin-aldosterone system (RAAS) (17-19) and in the release of arginine vasopressin (AVP) (20-22). These neuroendocrine changes are appropriate to maintain proper organ function through vasoconstriction and volume retention. Yet these responses tend to become magnified, resulting in increased afterload and volume load to the already compromised heart. These circumstances create such clinical features as oedema, ascites, and hepatomegaly. Endothelin (ET), a more recently discovered vasoconstrictor, has also been implicated to contribute in the exaggerated responses in CHF since the levels of this peptide are elevated in CHF patients (23-29).

Natriuretic and vasodilating factors are concomitantly produced to counter-regulate the vasoconstrictive and fluid-retaining actions of the above systems in CHF. The principal one is the natriuretic peptide family (30) which thus far includes four members. These are
Figure 1.1 Flow chart delineating the adaptive mechanisms which occur during the course of congestive heart failure. See text for a detailed discussion.
atrial natriuretic peptide (ANP) (31), brain natriuretic peptide (BNP) (32), C-type natriuretic peptide (CNP) (33) and urodilatin (34). That the levels of ANP and BNP are elevated in CHF are well documented (35-41). ANP and BNP share similar structure and biological actions (42). Unlike ANP and BNP, CNP does not exhibit natriuretic properties (43) but may be important in paracrine regulation of central systems related to pressure control and vascular tone. Urodilatin appears to be a more potent natriuretic agent than ANP (44). Prostaglandins E₂ (PGE₂) and I₂ (PGI₂) (45,46) and dopamine (47,48) are thought to play a more minor role in minimizing the degree of vasoconstriction and extracellular fluid volume (ECFV) expansion.

Cardiac output and blood pressure are relatively preserved at rest during the early stages of CHF. As the degree of CHF progresses to moderate or severe, cardiac output is reduced and it fails to rise normally upon exercise with the consequence that proportionally less flow is available to the exercising muscle (10). In addition, there is marked vasoconstriction of the cutaneous, renal, and splanchnic beds such that redistribution of the blood flow occurs to provide adequate nourishment to the brain and coronary vessels. However, compensation can only be sustained for a limited period of time. The heart tends to decompensate, with either left or right ventricular failure occurring. Left heart failure will result in increased pulmonary venous pressure and thus pulmonary congestion and oedema. Right heart failure leads to elevated venous and capillary hydrostatic pressure and thus peripheral oedema, hepatomegaly, and ascites. Eventually, both sides of the heart may fail.

The vasculature in heart failure adapts by increasing tone in the venous capacitance and arteriolar resistance systems (8). This increase involves components of the sympathoadrenal systems, the RAAS, and the impaired function of the endothelium in producing the vasodilator nitric oxide (NO). Hypertrophy of the vascular smooth muscle
and increased salt and water content in the vessel wall may also contribute to the reduced compliance of the vessels.

1.2 Renal Adaptation and Excessive Salt and Water Retention
The kidney plays a central role in the pathogenesis of CHF. When the heart is impaired, a diminished effective circulating blood volume is perceived by pressure and volume sensors located in the atria, ventricles, great veins and arteries, lungs, and the juxtaglomerular apparatus of the kidney. The abnormally gross retention of salt and water retention may be attributed to the adaptation of the kidney to the weakened cardiac function, leading to the hallmark features of dependent oedema, ascites, hepatomegaly, etc. Both intrarenal and neurohumoral responses occur to enhance sodium and water reabsorption by the kidneys which results in detrimental extracellular fluid volume expansion.

One consequence of CHF is that there is a reduction in renal plasma flow (RPF) which parallels the decrease in cardiac output (49-51). An increase in the glomerular transcapillary hydrostatic pressure is required to maintain an adequate glomerular filtration rate (GFR) and this is effected by a disproportionate increase in efferent arteriolar resistance that is mediated by angiotensin II constriction (52). The resulting increased filtration fraction raises the oncotic pressure in the post-glomerular circulation, thereby abnormally favouring the reabsorption of tubular fluid from the proximal tubule.

Increased sympathetic tone has been commonly observed in CHF (53-56). It is beneficial in maintaining blood pressure but excessive venoconstriction may add to the already diminished RPF, further enhancing electrolyte conservation. Nord et al (57) demonstrated that the sympathetic nervous system directly influences tubular reabsorption via stimulation of the Na\(^{+}\)-H\(^{+}\) antiport.
The RAAS also has a major role in directing the kidney to retain salt and water in CHF. Renin secretion is elevated early in the course of heart failure (58-62) and facilitates the conversion of angiotensinogen to angiotensin I which is followed by angiotensin II formation. Angiotensin II, as discussed above, is a potent vasoconstrictor as well as a potentiator of catecholamine action (49). Studies have shown that angiotensin II directly stimulates sodium reabsorption in the proximal tubule (63,64). Aldosterone levels are increased which promote sodium reabsorption at distal Na⁺/K⁺ or Na⁺/H⁺ exchange sites of the nephron (64).

Arginine vasopressin levels are generally elevated in patients with CHF (20-22,65). This small peptide hormone facilitates water reabsorption in the collecting ducts of the kidney by signalling the insertion of water channel proteins into the apical membrane (66).

1.3 Endothelin in CHF

Plasma endothelin levels are raised 2 to 3-fold in CHF (67-73). These levels correlate with the severity of heart failure or pulmonary hypertension (29,74-77) which implicates this peptide in the pathophysiological processes of CHF and perhaps other cardiovascular diseases (78). Its diverse biologic actions (79-81), including profound vasoconstriction, make ET a prime candidate in contributing to the reduced systemic and renal perfusion characteristic of CHF.

1.3.1 The endothelin family

The discovery of a novel endothelium-derived constricting factor was by Hickey and colleagues (82) in 1985. In the subsequent two years, this peptidergic vasoconstrictor was isolated, cloned, and sequenced by Yanagisawa et al (83) and given the name, endothelin, since it was derived from culture supernatant of porcine aortic endothelial cells. Further analysis revealed that three separate genes for ET existed and that each encoded a distinct
isopeptide—ET-1, ET-2, and ET-3 (84). Most research has been focussed on ET-1 as the expression of the other isoforms is more limited (85). A fourth peptide, vasoactive intestinal contractor or ET-ß, discovered through genomic cloning of mice (86), is considered to be the murine and rat form of human ET-2 since its precursor mRNA is similar to that of prepro-ET-2 and not of prepro- ET-1 or -ET-3 (87). Sarafotoxins, from the venom of the burrowing asp, *Attractaspis engaddensis*, are four more peptides that share high homology and bioactivity with the ETs (88). Such conservation of the peptides amongst species underscores the physiologic importance of ET.

1.3.2 Structure of the ET family
Considerable sequence homology exists between all constituents of the ET family with between 52% to 81% sequence similarity (89). Each member is composed of a 21-amino acid single chain containing, at identical positions, four cysteine residues that form two disulfide bridges to hold the peptide in a hairpin loop configuration. Polar, charged side chains in the hairpin loop and a hydrophobic COOH-terminus are also characteristic of ET peptides (83,90,91). The flexible hexapeptide C-terminus is greatly conserved and must be intact to confer bioactivity. Figure 1.2 depicts the proposed structure and sequence homology of the ET and sarafotoxin families.

1.3.3 Biosynthesis and expression of ETs
Endothelin is synthesized in a manner analogous to other hormonal peptides. From a single open reading frame, ET-1 is translated into a prepropeptide of 203 residues that undergoes sequential proteolytic cleavages to yield the final mature molecule (79,91). Processing begins with cleavage at, by a dibasic pair-specific endopeptidase, residues Lys\(^{52}\)-Arg\(^{53}\) and at Arg\(^{92}\)-Arg\(^{93}\) which removes the N-terminus containing the secretory signal sequence and the C-terminus to produce the prohormone intermediate, big ET-1, of 38 (human) or 39 (porcine) amino acids. A putative ET-converting enzyme (ECE) then
Figure 1.2 The structure and homology of ET isoforms and the sarafotoxins. Upper panel: a free hydrophobic C-terminus and the hairpin loop configuration formed by the two disulfide bonds is common to all members of the ET and sarafotoxin families. Lower panel: the highly conserved sequence of the ET isopeptides and a representative member (S6b) of the sarafotoxins is depicted. Non-conserved residues are enclosed within the boxes. (Adapted from Simonson (ref. 81)).
cuts big ET between the unique Trp$^{21}$-Val$^{22}$ couplet to give the potent 21-residue peptide (Figure 1.3). The nature of this ECE remains ill-defined although inhibition by phosphorimadon and characterization studies suggest it to be a neutral metalloprotease (92-96).

Expression and secretion of ET was originally believed to be localized exclusively to the vascular endothelium. However, it is now apparent that ET is produced in numerous tissue and cell types, including, but not limited to, endothelium of umbilical vein, glomerulus, corpus cavernosum, and aorta, keratinocytes, macrophages, bone marrow mast cells, astrocytes, mesangial cells, cardiomyocytes, epithelium of the breast, lungs, brain, uterus, stomach, kidney, testis, skeletal muscle, and intestine (reviewed in 80,81,85). Secretion of ET is largely constitutive (83, 97-100) although some evidence indicates there may be a regulated pathway via secretory granules (101). Whether the secretion is constant or regulated may be a function of cell type. Wagner et al (102) reported that ET secretion is polar, given that approximately 80% of ET-1 is secreted basolaterally in cultured endothelial cells.

Many stimuli have been demonstrated to upregulate ET secretion and expression. Growth factors and cytokines such as thrombin (100,103-105), transforming growth factor β (100,106-108), interleukin-1 (107,108), tumour necrosis factor alpha (109), and insulin (110,111) have been shown to increase the mRNA levels of ET. Raised message levels and secretion of ET were also observed in response to endotoxin (112,114), phorbol esters (105,114), high glucose (115), fluid mechanical shear stress (116), angiotensin II (117,118), arginine vasopressin (118,119), bradykinin (105), oxidized low-density protein (120), hypoxia (121), cyclosporine (122), and on occasion, ET itself (123).
Figure 1.3 Schematic diagram of post-translational processing of ET peptides. Large preprohormones are cleaved by dibasic pair-specific endopeptidases to give distinct prohormones that are subsequently cleaved by a novel ET converting enzyme at a specific trp-val recognition site to form the mature 21-residue ET isopeptide. (Adapted from Simonson (ref. 81)).
There are also inhibitory factors to regulate the production of ET. Indeed, ANP and other nitrovasodilators prevent ET secretion from cultured endothelial cells (122,124). In contrast to these in vitro data, one recent investigation found that under circumstances of vasodilator-induced hypotension, there was enhanced systemic release of ET-1 (125), suggesting that the source was of non-endothelial origin. Sandok et al (126) reported that the rise in circulating levels of ET in a model of acute renal ischaemia occurred only in the presence of prostaglandin inhibition or the absence of ANP. Furthermore, nitric oxide (NO) and prostacyclin (PGI₂) released after thrombin stimulation provide negative feedback on ET production via cGMP and cAMP formation, respectively (127,128). Kohan and Padilla (129) confirmed that NO and cGMP reduce ET-1 production and noted that interferon gamma is also a potent inhibitor of ET-1 release. The groups of Stewart et al (130) and Cade et al (131) have reported that vascular smooth muscle cells release an inhibitory substance that reduces ET production by endothelial cells. The chemical properties of this substance await determination.

The levels of local and circulating ET are adjusted through elimination by at least three mechanisms. First, there is hydrolytic degradation by a neutral metalloendopeptidase, EC 24.11, that is present on the cell surface of various kidney, pulmonary, intestinal, and central nervous tissues (132). This enzyme, which inactivates several regulatory peptides, cleaves ET and hydrolyzes its N-terminus, rendering inoperative products (133). Sarafotoxins are four-fold less susceptible to this endopeptidase than ET which may partially explain why the former are so much more toxic than the latter (134). Lysosomal degradation appears to be another mode in which ET is catabolized as lysosomal vesicles in bovine pulmonary endothelial cells have been shown to be rich in ET that has probably been endocytosed (135). The pulmonary circulation was predominantly responsible for the removal of circulating ET isopeptides after ventricular injection in the rat although
there was uptake by the kidneys and liver as well (136,137). Within minutes of injection, more than 60% of the ET-1 was eliminated. Fukuroda et al (138) demonstrated that clearance of exogenous ET by the rat lung and kidney was mediated through ET<sub>B</sub> receptors (see below). While ET is cleared in the lungs of rats and guinea pigs, it is the kidneys, spleen, and hindlimb that clear ET in the pig (139), suggesting a species-specific pathway for degradation.

1.3.4 Endothelin receptors

Prior to molecular evidence of different ET receptor subtypes, pharmacological studies revealed that different agonist affinities and ET isopeptide selectivities for binding sites existed, indicating there was a population of multiple receptor subtypes (140). Differences in physiological responses to the ET isopeptides were also observed. The potency of ET-1 and ET-2 as pressor agents was much greater than that of ET-3 (114) while ET-1 and ET-3 were equipotent in causing the transient vasodilatation (141,142). Two receptor subtypes, termed ET<sub>A</sub> and ET<sub>B</sub>, were proposed to distinguish between vasoconstriction and vasodilation mediation, respectively. In 1990, Arai and colleagues (143), and Sakurai and colleagues (144), independently cloned the ET<sub>A</sub> receptor from bovine lung and ET<sub>B</sub> receptor from rat lung, respectively. The human A-type receptor was subsequently cloned and characterized (145). Numerous studies have confirmed that ET<sub>A</sub> is selective for ET-1 over ET-3 and sarafotoxin S6c (STXS6c) while ET<sub>B</sub> shows similar affinities for all three ET isopeptides (143,144,146). The homology between human and rat ET<sub>A</sub> and ET<sub>B</sub> is about 91% and 88%, respectively while that between human ET<sub>A</sub> and ET<sub>B</sub> is roughly 55% (140).

The development of a wide repertoire of pharmacological tools has enabled further division of the ET<sub>B</sub> receptor classification. It was shown that this receptor exhibited both vasodilating and vasoconstricting abilities (147) and thus given the designations ET<sub>B1</sub> and
ETB2, respectively. Others have distinguished between the ETB subtypes by characterizing their respective pharmacological profiles (148,149). However, no definitive biochemical or molecular evidence for the subtypes is available. Binding studies have revealed the preferential binding of ET-3 over ET-1 by an ET receptor in a number of tissues (150-152). This receptor has been classified as the putative ETc receptor and has been cloned from the dermal melanophores of Xenopus laevis (153). A mammalian version of this receptor has not yet been isolated.

Sequencing of the cDNA clones has demonstrated that ET receptors are peptides of approximately 426-444 residues containing secretory signal sequences of 20-26 amino acids (143,144,153,154). They belong to the superfamily of G-protein-coupled, seven-domain-membrane-spanning receptors. G-proteins are guanine-nucleotide-binding regulatory proteins.

1.3.5 Localization of ET receptors

The distribution of ET receptor subtypes varies between and among species and tissue type (140). For example, in rat heart, mRNAs for both the A-type and the B-type were widespread in myocardial muscle, but only the A variety was present on coronary blood vessels (155). In human kidney, the predominant form is the ETB subtype which is concentrated in the collecting system (156). Similar results have been obtained in the rat and dog in some studies (157,158). However, the receptor profiles in rat kidney cortex and papilla were reversed when compared to that of dog (158,159). Vasculature in the kidney has been found to contain primarily A-type receptors (155,160,161). In general, ETA receptors are expressed more abundantly on vascular smooth muscle cells while ETB ones are expressed primarily on endothelial and other cell types.
1.3.6 Signal transduction from ET receptors

The effects of ETs are transduced via cell surface receptors coupled to various heterotrimeric G-proteins (81,85). Both ET$_A$ and ET$_B$ subtypes are able to couple to at least four types of G-proteins: G$_s$, G$_q$, G$_{11}$, and G$_i$ (pertussis toxin-sensitive) (162,163). Binding of ETs to their receptors stimulates the GTP turnover of these proteins which then regulate (activate or inhibit) one or more effectors. Such effectors include phospholipase C (PLC) (164-167), phospholipase A$_2$ (137), phospholipase D (PLD) (168), and adenylyl cyclase (AC) (163) (Figure 1.4).

Activation of PLC results in the hydrolysis of phosphatidylinositol to the second messengers inositol trisphosphate (IP$_3$) and diacylglycerol (DAG) (165,169) although hydrolysis of phosphatidylcholine by PLC or phosphatidic acid degradation by PLD provide another source for the latter (170). IP$_3$ mobilizes Ca$^{2+}$ from the sarcoplasmic reticulum or calciosome, thereby raising intracellular concentrations of free calcium [Ca$^{2+}$]. The increase, along with influx of extracellular Ca$^{2+}$ through voltage-dependent and non-voltage-dependent (receptor-operated) ion channels, appears to mediate ET-induced smooth muscle cell (SMC) contractions (85,140). DAG, with cofactors Ca$^{2+}$ and phosphatidylserine, activates protein kinase C (PKC) which may sensitize the contractile apparatus to Ca$^{2+}$ (171,172). PKC also mediates the ET-1 stimulation of Na$^+$-H$^+$ exchange that leads to intracellular alkalinization in vascular SMCs (173).

In contrast to the vasoconstricting mechanisms discussed above, a signalling pathway for vasodilation has been observed (174,175). Via ET$_B$, ET stimulates a pertussis toxin-sensitive G protein that results in the release of nitric oxide (NO), which in turn facilitates the relaxation of vascular SMCs through its second messenger, cGMP (176).
Figure 1.4 Schematic representation of the putative G protein-linked ET receptor subtypes and the multiple transmembrane signal transduction cascades evoked by ET ligand binding. Isopeptide selectivity of the ET receptor subtypes is shown. Stimulation of the phosphoinositide (PI) pathway leading to protein kinase C (PKC) activation and release of calcium from intracellular stores is well characterized. Extracellular calcium entry through receptor operated (ROC) and voltage dependent (VDCC) calcium channels also increases intracellular calcium following ET receptor activation. Other effector systems affected by ET include phospholipase A₂ (PLA₂), phospholipase D (PLD), adenylate cyclase (AC), and the electroneutral Na⁺-H⁺ antiporter. ET-1, ET-2, ET-3: endothelin-1, endothelin-2, endothelin-3, respectively; ET₄, ET₅, ET₆: endothelin receptor subtypes A, B, C, respectively; G, heterotrimeric G protein; PC, phosphatidylcholine; PA, phosphatidic acid; DAG, diacylglycerol, IP₃, inositol 1,4,5-trisphosphate; [Ca²⁺]ᵢ, intracellular calcium concentration; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate; PGE₂, PGI₂, prostaglandins E₂ and I₂; TXA₂, thromboxane A₂; MAPK, mitogen activated protein kinase; c-fos/c-jun, immediate early genes.
The signalling pathways mediating long-term effects of ETs, such as mitogenesis, have not been fully elucidated. However, elevated \([Ca^{2+}]_i\), mitogen-activated protein kinase (MAPK) stimulation, and enhanced PKC and Na\(^+\)-H\(^+\) antiporter activity have been suggested to play a role (177). Consistent with this notion is the finding that ETs stimulate the MAPK cascade and 90kD S6 kinase (RSK) (178,179). In addition, increased \([Ca^{2+}]_i\) was directly involved in the ET-1-induced rise in the mRNA levels of several *fos*/*jun* genes (180).

1.4 Potential Roles for ET in the Electrolyte Derangement in CHF

1.4.1 Effects of ET on haemodynamics and renal function

As vasoconstriction is a prominent feature of CHF, it is logical to surmise that this increased tone may be the result of the potent actions of ET, the quantities of which are raised in this pathophysiological state (67-77). Indeed, such observations have been noted in a number of studies (181-184). Systemic infusion of ET-1 has repeatedly caused increases in systemic and renal vascular resistance (RVR) and decreases in RPF and GFR (181,183,185,186).

It is controversial whether ET actions are mediated in an autocrine/paracrine or circulating mode. Due to the involvement of several baroreceptor reflexes in the regulation of kidney function, intrarenal infusion of ET-1 has been completed to separate systemic from intrarenal influences of the peptide. Prolonged and dose-dependent decreases in RPF and GFR and increases in RVR were evident with this route of administration as well (187,188). ET-induced vasoconstriction in isolated perfused rat kidney was a direct effect of ET on the renal vasculature and was mediated through both the A and B subtypes of the ET receptor (189).
Weak pressor doses of ET-1 did not alter GFR in a study by King et al (181) but higher doses caused extreme contraction of both efferent and afferent arterioles in addition to a depressed glomerular flow rate and ultrafiltration coefficient (Kf), thus reducing GFR (181,190). Utilizing micropuncture studies, these authors found that ET-1 diminishes Kf although they could not deduce whether it was due to a change in glomerular capillary surface area or hydraulic conductivity. In this regard, Simonson and Dunn (191) reported ET-1 contracts glomerular mesangial cells, so that it may reduce filtering surface area.

In view of the decline in RPF and in GFR produced by ET-1, it would be expected that the filtered load of sodium would be smaller and thereby reduce excretion, provided tubular transport were unaffected. Furthermore, peritubular Starling forces would favour sodium reabsorption. Both natriuretic (181, 192) and antinatriuretic (186, 193,194) responses have been elicited with systemic infusion of ET-1. The increase in sodium excretion occurred despite reduced RPF and, in another study, GFR (195). Pretreatment with nifedipine, the calcium channel blocker, prevented ET-1 induced renal vasoconstriction and sodium retention (194), implying that ET-1 plays a role in volume equilibrium. Clavell et al (196) found systemic infusion of ET-1 resulted in renal vasoconstriction, diuresis, and antinatriuresis but a natriuretic effect was unmasked with intrarenal BQ123 treatment. Furthermore, the same study found that the A-subtype ET receptor contributed to the vasoconstriction whereas the B-subtype was mainly responsible for the diuresis independent of sodium excretion. Similar results with regard to the roles of the ET receptor subtypes in vasoconstrictor and diuretic responses were reported by Kamphuis et al (197) and Pollock and Opgenorth (198). High-dose intrarenal infusion and isolated perfused kidney studies with lowered GFR also resulted in natriuresis (187,188,199,200). These data suggest that ET induces natriuresis in the kidney, but this may be modulated by other factors that could be intra- or extrarenal in nature.
One mechanism by which ET-1 may decrease sodium absorption is through direct tubular action. Zeidel et al (201) demonstrated that ET-1 evoked natriuresis in vivo by inhibiting the Na\(^+\)/K\(^+\)-ATPase in epithelial cells of the inner medullary collecting duct (IMCD), likely via a PGE\(_2\) pathway. An earlier report by Jabs et al (202) stated that this prostaglandin diminished ATPase activity in rabbit IMCD. Garvin and Sanders (203) showed that suppression of the Na\(^+\)/K\(^+\)-ATPase by ET concomitantly inhibited fluid and bicarbonate transport in the proximal straight tubule. Thus ET affects transport in both the proximal nephron and the distal concentrating segments. Other routes of ET action involve interplay with various neurohumoral systems.

1.4.2 ETs in the integrated handling of fluid homeostasis in CHF

As discussed earlier, there is a balance between vasoconstriction and vasodilation, and between fluid retention and fluid excretion to regulate ECFV and blood pressure. Not only do ET levels reach pathophysiological concentrations in CHF, but enhanced release of other hormones and peptides is either stimulated by ET-1 or vice versa in an attempt to maintain functional homeostasis. Furthermore, the physiological effects of ET and the various neurohumoral substances may potentiate or interfere with one another, thereby modulating their respective actions.

High plasma levels of ANP are found in CHF. Although the primary stimulus for ANP release is atrial stretch (204), it is also produced (205) and secreted (205-207) by isolated atria and cultured myocytes in response to ET-1. Two recent studies determined that this secretion of ANP was mediated via ET\(_A\) rather than ET\(_B\) receptors since BQ123, an A-subtype-selective antagonist, inhibited ET-1-stimulated ANP secretion while ET\(_B\)-specific agonists did not activate ANP secretion (208,209). By enhancing circulating levels of ANP, ET theoretically would indirectly cause natriuresis since ANP blocks sodium reabsorption at the collecting ducts and increases GFR (210). Despite higher quantities of
circulating ANP, a blunted renal response to this peptide is consistently seen in those with CHF (211,212). The cause(s) of this attenuation has not been determined, but one may speculate that it is due to poor renal blood flow which would result in poor delivery of this hormone to the distal sites of the nephron. In addition, intrinsic end-organ resistance or antagonistic fluid-retaining systems may predominate in this setting. Therefore, some investigators argue that the purpose of ET-stimulated ANP secretion is, instead, to oppose the contractile effects of ET (81). Some studies have suggested that ANP modulates the actions of ET (213,214). Jaiswal (215) found ET inhibits ANP-stimulated cGMP production while Cernacek et al (216) found no interaction between ET and ANP-induced cGMP expression. It is possible that ET and ANP inhibit distal tubular sodium reabsorption via separate mechanisms.

ANP also indirectly minimizes sodium reabsorption by antagonizing the release of renin and aldosterone. In addition, ANP inhibits angiotensin II-stimulated fluid absorption in the proximal nephron through a mechanism involving cGMP-dependent protein kinase (217). Other studies have focussed on the interactions between ANP and AVP; the levels of which are also abnormally high in CHF (20,22,65). ANP has been shown to suppress AVP release (218) while AVP has the reverse effect on ANP (219). Moreover, AVP attenuated the renal effects of ANP (220) while ANP counteracted the antidiuretic effect of AVP in patients with moderate CHF (221), albeit through unknown mechanisms.

There appear to be two components comprising the RAAS. The circulating RAAS seems to be activated in acute situations of homeostasis disturbance and then returns to basal levels of activity after cardiovascular compensation (222). On the other hand, the autocrine/paracrine tissue component appears to mediate longterm effects that influence cardiovascular function and structure.
Activation of the RAAS, in patients with CHF is most likely in response to perceived low renal plasma flow and pressure which may be due, in part, to the exquisite sensitivity of the kidney (223) to the potent vasoconstricting properties of ET-1. Yet the effects of ET-1 on the RAAS have varied, depending on its source. Infusion of ET-1 has been shown to elevate circulating aldosterone (186,193) which may be through direct stimulation of the adrenal glomerulosa cells (224). However, plasma renin activity is raised after systemic infusions of ET-1 and would therefore activate the angiotensinogen-angiotensin-aldosterone cascade. Whatever the stimulus for aldosterone secretion, enhanced sodium reabsorption by the distal tubules and collecting ducts should ensue. Moreover, angiotensin II formation is also increased, and this peptide directly stimulates sodium reabsorption from the proximal tubule. In contrast, local intrarenal ET appears to inhibit the RAAS. ET-1 production in the glomeruli and IMCD has been documented (225). A number of studies has established that ET-1 inhibits renin secretion in vitro by a calcium-dependent pathway (226-229). One group recently found that ETs selectively inhibit cAMP-stimulated renin secretion in murine juxtaglomerular cells through a calcium-sensitive process mediated by ET\textsubscript{B} receptor activation (230). Presumably, aldosterone secretion and angiotensin II formation would decrease following depressed renin activity, leading to increased sodium output. Thus Simonson (81) has suggested ET is natriuretic when locally released but antinatriuretic when circulating levels of ET are elevated, as in CHF. This rise in circulating ET may reflect spillover from autocrine/paracrine secretion.

While ET may activate the RAAS, one component of the RAAS, angiotensin II, also exerts effects on ET. Angiotensin II has been shown to increase ET-1 synthesis in endothelial cells (118) as well as upregulate ET receptor and mRNA levels for the A subtype in vascular smooth muscle cells (231). These data further support the notion of a
link between the RAAS and ET. In contrast, Wilkins et al (232) found that the pressor
and renal actions of ET in vivo apparently were not mediated by the RAAS since results
with captopril treatment were similar to those without it.

Water metabolism is most notably regulated through the actions of AVP, but current
evidence indicates ET also participates in fluid homeostasis. The finding that volume
deployment in rats and increased osmolality reduced ET-1 production in rat IMCD cells in a
dose-dependent fashion supports the idea that ET may provide a communication link
between volume status and sodium and water transport (233). That immunoreactive ET
peptides were present in secretory vesicles in the rat posterior pituitary and that the
vesicles were depleted during water deprivation also implies an active role for ET in body
fluid balance (234). Although AVP and ET stimulate the production of one another
(186,235), they are also mutually antagonistic in the renal collecting system. AVP
downregulated ET-1 binding to ET\textsubscript{B} receptors in the rat cortical collecting duct via a
protein kinase A-dependent mechanism (236) while ET-1 inhibited the antidiuretic effect
of AVP (185). More specifically, endothelin inhibited the AVP-induced accumulation of
cAMP in the cortical collecting duct, outer and inner medullary collecting ducts, and the
papillary tubules of the microdissected rat nephron via a pathway involving PKC
(237,238). Subsequent studies demonstrated that endothelin inhibits AVP-stimulated
water permeability in rat IMCD by blocking cAMP generation (239,240). Edwards and
colleagues (241) have since revealed that the inhibition by ET of AVP-induced increases in
water permeability and cAMP accumulation is achieved through activation of the ET\textsubscript{B}
receptor rather than the A subtype. A current publication by Ozaki et al (242)
demonstrates the coupling of ET\textsubscript{B} receptors in porcine kidney tubular epithelial LLC-PK\textsubscript{1}
cells to two distinct signal transduction cascades. The increase in cGMP in response to
ET-1 was pertussis toxin-insensitive while the decrease of AVP-induced cAMP was
pertussis toxin-sensitive, suggesting that the latter response was mediated by a pertussis toxin-sensitive \( G_i \) protein. As cGMP has been shown to inhibit amiloride-sensitive sodium transport in LLC-PK\(_1\) cells (243), these authors have suggested that ET-stimulated cGMP increases may also inhibit sodium channels in epithelial cells to evoke natriuresis. Thus ET may act as a natriuretic factor by inhibiting AVP-induced cAMP and by stimulating cGMP production.

Collectively, the above data indicate that many complex interactions may be occurring simultaneously during the course of heart failure with the increased activity of several neurohormonal systems. With the observation that salt and water retention is characteristic of CHF, it is possible that the effects of one or more fluid-retaining systems are overriding those of the excretion-promoting systems. This may be achieved through increased sensitivity of the vasculature and kidneys to the vasoconstricting factors or decreased responsiveness to the vasodilating substances, but the explicit mechanism(s) of how fluid retention persists remain a mystery. To elucidate the retentive mechanisms, the relative importance of each hormonal system in contributing to this pathophysiology must be determined. Utilization of inhibitors of the various systems, such as an angiotensin converting enzyme (ACE) inhibitor, may help to delineate the impact of each hormone on ECF volume expansion in CHF.

1.5 Angiotensin Converting Enzyme (ACE) Inhibitors

Angiotensin converting enzyme (ACE) inhibitor is a peptidyl dipeptidase whose primary action is to prevent the conversion of angiotensin I to angiotensin II, both in the circulation and in the autocrine/paracrine tissue system. The discovery of the nonapeptide inhibitor, teprotide, from the snake venom of Bothrops jararaca (244) provided the first observation of inhibition of ACE. Since then, synthetic ACE inhibitors have been produced that are employed in animal and clinical studies.
1.5.1 Chemical structure and classification

Angiotensin converting enzyme inhibitors interact with a zinc ion that is present in the active site of ACE (245). To ensure efficient catalytic conversion, ACE inhibitors are designed with specific chemical groups that will bind effectively to the zinc ion. Thus, ACE inhibitors are classified on the basis of their zinc ligand. Three classes of ACE inhibitors have been produced with the following moieties: sulphydryl-containing, carboxyl-containing, and phosphoryl-containing. Captopril, enalapril, and fosinopril are examples of ACE inhibitors in each class, respectively. The extent of inhibitory action, or its potency, is determined by the strength of binding to the zinc ion and the presence of additional binding sites. Carboxyl groups bind less tightly to the zinc ion than sulphydryl groups. However, binding by enalapril is strengthened by the addition of two more binding sites, thereby increasing duration of action. Several ACE inhibitors are administered as prodrugs to enhance absorption of the drug. Prodrugs are esterified forms of the molecules which are activated by hydrolysis to the diacids in the liver, following intestinal absorption. Enalapril is one compound that is orally delivered as a prodrug.

1.5.1 Pharmacokinetics

Following oral ingestion, ACE inhibitors may be 25% to 80% bioavailable. The presence of food decreases absorption with some compounds. Generally, ACE inhibitors are bound to plasma proteins and the amount of binding varies between 10% to 95%. The majority of ACE inhibitors are eliminated predominantly via the kidneys whether they are metabolized or not. Enalapril is approximately 60% to 70% bioavailable and food does not affect its absorption (244). Maximum levels of enalapril are attained within one-half to one and one-half hours after ingestion while those of the active enalaprilat form are achieved within 2 to 4 hours after ingestion. Both forms circulate virtually protein-free.
and peak activity occurs 4 to 6 hours after administration. The effective half-life of enalaprilat is about 11 hours.

Numerous studies have shown that ACE inhibitors exhibit a steep dose-response curve, in contrast to previously-held notions (246). Only low dosages are required to block angiotensin conversion, and the effect of increasing the dose is to augment the duration but not the intensity of inhibition (245). High dosages have been used to maximize efficacy and duration (246). Nussberger et al (247,248) observed a dissociation between the quantities of plasma angiotensin II and the antipressor effect with some ACE inhibitors. This dissociation could be related to other consequences of ACE inhibitor action such as attenuation of sympathetic activity, increased vasodilating hormones and parasympathetic activity, and inhibition of the tissue component of angiotensin (245,249).

1.5.3 ACE inhibitors in CHF
Several intensive studies have been conducted that indicate ACE inhibitors benefit patients with varying degrees of heart failure (250). The CONSENSUS (251) demonstrated that mortality from CHF decreased in patients with severe heart failure while the SOLVD trial (252) gave similar results in patients with mild-to-moderate symptomatic heart failure. Furthermore, the SOLVD prevention study (253) showed that ACE inhibitors could circumvent any progression to heart failure in asymptomatic patients with left ventricular dysfunction.

1.6 ET Receptor Antagonist
BQ123, an ET$_A$-selective antagonist, was designed based on the structure of a cyclic pentapeptide, found in the fermentation products of Streptomyces misakiensis, which behaved as an ET-receptor antagonist. Structure-function studies led to the development
of BQ123 cyclo[D-Asp-Pro-D-Val-Leu-D-Trp] which is recognized by the first extracellular loop of the ET\textsubscript{A} receptor (85). BQ123 binds to ET\textsubscript{A} with an IC\textsubscript{50} of 22 nM.

1.7 Rationale

In light of the fact that ET infusion has been repeatedly demonstrated to affect haemodynamic properties as well as renal function, the elevated levels of this peptide in CHF make it a prime candidate as a major contributor to the pathophysiological process. Furthermore, interplay between ET and other neurohormonal systems, including the RAAS, natriuretic peptides, and AVP, has been documented in numerous studies. Each component is likely able to modulate the effects of one or more of the other systems in an antagonistic or synergistic manner, depending on the extent of activation and on signals that are received by various sensors in the body. In the environment of heart failure, ET, the RAAS, and AVP (and also the sympathetic nervous system) act jointly to increase vascular tone while ANP opposes it. The RAAS and AVP also act to conserve fluid by the kidney while ANP tries to promote its loss. Normally, ET is believed to be natriuretic and diuretic, but in the course of CHF, when the circulating levels are anomalously high, it appears to work in reverse. In addition, most of the hormones are able to regulate the expression of one another, adding another dimension of interaction. Therefore, which physiological responses will prevail in CHF is determined by the balance of forces created by the different regulatory elements (Figure 1.5).

When the available data is taken together, a logical approach to take would be to determine how the neuroendocrine systems interact with one another in CHF. By administering an ACE inhibitor, one can dissect out the importance of the RAAS to some extent. The effect of angiotensin II blockade on ET can be examined through characterizing the ET receptor distribution in the IMCD of CM hamsters. Because angiotensin II potently stimulates ET production and release, the circulating and tissue
Figure 1.5 Neurohumoral systems involved in body fluid homeostasis. An imbalance between the vasodilating/natriuretic and vasoconstricting/anti-natriuretic forces, favouring fluid retention as observed in CHF, is shown above.
levels of ET will rise. If ET and its receptors have a typical hormone-receptor relationship, the high circulating levels of ET would be expected to downregulate the ET receptors. Indeed, certain populations of ET receptors have been found to be downregulated in heart failure (73,254). We hypothesize that the increased levels of ET in CHF downregulate the ET$_B$ receptors, which have been shown to antagonize the actions of AVP, thereby removing the tonic inhibition of AVP action. Downregulation of ET receptors would also decrease sodium excretion which is probably mediated by these receptors as well. By decreasing the ET receptor density in the IMCD, AVP will stimulate cAMP accumulation which will in turn direct insertion of water-channel proteins into the apical membrane for increased water reabsorption. Fewer ET receptors would result in a diminished capacity to mediate salt output. Such consequences could lead to the hallmark fluid retention of CHF. This hypothesis was tested. Figure 1.6 summarizes the current hypothesis.

1.8 Objectives and Approach

The overall objective of this thesis is to gain further insight into the pathophysiology of salt and water retention. Specifically, one objective was to determine whether ET participates in this fluid retention, and if so, by what mechanism(s). Second, the study also aimed at answering whether angiotensin II plays a role in mediating the actions of ET. The approach of the study is outlined below.

Both in vivo and in vitro experiments were conducted to correlate physiological responses with physical differences in the kidneys of normal and CM hamsters. Standard clearance measurements were obtained from CM and normal control hamsters for basal values and to demonstrate a blunted response to ANP in CM hamsters as compared to normal. The IMCD were then carefully isolated from the kidneys of both groups to characterize ET
Figure 1.6  Schematic summary of the hypothetical role of ET in salt and water handling by the kidney in CHF. The rise in ET levels, secondary to angiotensin II and vasopressin stimulation, results in downregulation of ET receptors in the kidneys of CHF individuals. Reduction in ET receptor density leads to enhanced electrolyte reabsorption. See text for discussion.
receptor subtype distribution and density through radioligand binding methods. Binding experiments were performed using $^{125}$I-labelled ET-1 and the specific receptor antagonist, BQ123. The clearance and radioligand binding studies were then repeated on additional CM and normal animals that had been treated with the ACE inhibitor, enalapril, for one week prior to the experiments. Chronic enalapril therapy was administered to determine whether ACE inhibition would restore the natriuretic response by cardiomyopathic (CM) hamsters to exogenous ANP. Urine samples were collected for basal values pre- and post- enalapril, and to ascertain the response to ANP before and after enalapril treatment via measurement of its second messenger, cGMP (Figure 1.7).

Stimulation studies were completed on the IMCD of CM and normal hamsters to assess whether there was a difference in the response of the respective cells to ET-1 administration. Rat IMCD were also stimulated with low-concentrations of angiotensin II to clarify whether this peptide has regulatory functions for ET receptor expression.

It is expected that this research will aid in a better understanding of the physiological and molecular mechanisms underlying the predominant expanded extracellular fluid volume in CHF. A greater understanding of these mechanisms would be important in designing therapeutic regimes.
Figure 1.7 Schematic summary of the approach employed in the present study. (A) Flow chart of the in vivo scheme. (B) Flow chart of the in vitro scheme.
Chapter Two  Materials and Methods

2.1  Animals
Male UM-X7.1 strain cardiomyopathic (CM) Syrian hamsters and age/genetically-matched inbred albino normal controls were purchased at 290 days of age (Canadian Hybrid Farms, King's County, Nova Scotia). All animals, weighing between 110 and 160 grams, were fed regular laboratory chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. They were housed in standard plastic cages in a climate-controlled room (22°C) and allowed to adapt to the new environment before use for studies.

2.1.1  Determination of severity of CHF
Careful assessment of the degree of heart failure was performed on each animal based on a previously established grading scale (255,256). Grade 0 designates no lesions. Grade 1 is considered to be mild heart failure with minimal subcutaneous oedema, no measurable exudates in the body cavities, and liver enlargement. Moderate heart failure may be Grade 2 when there is diffuse subcutaneous oedema and mild pleural effusions and ascites, or Grade 3 when ascites and pleural effusions are at measurable volumes of 2.5 ml or less. Grade 4 represents severe heart failure and symptoms include profound generalized oedema over the entire body and face in addition to conspicuous ascites and pleural effusions that measure more than 2.5 ml.

2.2  Reagents
Inactin was obtained from Byk Gulden Konstanz (Germany). Enalapril was generously donated by Merck Frosst Canada Inc. Endothelin (ET)-1 and the ET\textsubscript{A}-specific antagonist, BQ123, were purchased from Peninsula Laboratories Inc., Belmont, California. The following compounds were obtained from Sigma Chemical, St. Louis, MO.: atrial natriuretic peptide (ANP), saralasin (Sarlle), an angiotensin II receptor antagonist,
polyethylene glycol, and tris-HCl buffer. [3H]-inulin-methoxy was from NEN Dupont (Boston, MA) and Iodine-125 (125I) was from Dupont Canada Inc. The other chemicals and reagents were obtained from Fisher.

2.3 In vivo Studies

2.3.1 Clearance protocol

Each hamster, whether untreated or enalapril-treated, was anaesthetized with Inactin (25 mg/kg) by intraperitoneal injection and carefully weighed. Surgical anaesthesia typically ensued within 10-20 minutes. The animal was then placed on a temperature-regulated table and body temperature was monitored with a rectal probe. A tracheostomy using polyethylene tubing (PE 160) was performed to maintain a clear airway. The external jugular vein and carotid artery were cannulated with polyethylene catheters (PE 50) for infusion and blood sampling, respectively. The carotid cannula was connected to a Gilson recorder (Middleton, WI) connected to a pressure transducer to record blood pressure. Through a suprapubic incision, a polyethylene catheter was inserted into the bladder for urine collection. An intravenous infusion (compact infusion pump, Harvard Apparatus Co., Inc., Millis MA) of 0.9% saline containing [3H]inulin was administered at a rate of 0.0201 ml/min for the duration of the experiment. Following a 60-minute equilibration period, two 30-minute baseline clearance collections were made. In the next phase, a bolus dose (1 µg/kg) of ANP was given followed by its addition to the saline infusion at a concentration of 1 µg/kg/min for 90 minutes. Two 30-minute clearance periods were obtained 30 minutes after the initiation of ANP infusion. Blood samples were drawn into heparinized tubes for haematocrit and plasma protein concentration determination at the midpoint of each clearance phase. Urine was collected into preweighed tubes for gravimetric volume calculation. At the end of each experiment, animals were sacrificed by
removing their hearts for weight measurement. Kidneys were harvested for receptor studies.

Time control experiments were conducted using analogous clearance methods. Cannulated male golden Syrian hamsters were continuously infused with [\( ^3\)H]inulin-saline. Blood pressure was monitored as above. Following 45 minutes of equilibration, three 30-minute clearance collections at 60-minute intervals were performed. Blood samples were obtained during each collection phase for calculation of glomerular filtration rate (GFR).

2.3.2 Chronic enalapril treatment
Enalapril (2.5 mg/kg) was administered subcutaneously once per day, for six days, prior to study. On the seventh day of treatment, clearance studies were performed as described above.

2.3.3 Blood and urine analysis
2.3.3.1 Haematocrit and plasma protein concentration
Haematocrit was determined using a micro haematocrit reader (Phillips-Drucker, Astoria Oregon, USA) while plasma protein was ascertained with a hand refractometer (Erma, Tokyo).

2.3.3.2 Ion concentrations
Plasma and urinary sodium and potassium were measured with the IL943 flame photometer (Instrumentation Laboratory, Lexington Mass.).

2.3.3.3 Glomerular filtration rate
10 µl of plasma or urine were aliquoted to plastic scintillation vials and 5 ml of Scinti Verse (Fisher Scientific, USA) were added for quenching. Radioactivity was measured by a liquid scintillation counter (Beckman Scientific, Irvine CA).
2.3.3.4 Urinary cGMP measurement

cGMP was measured with a commercially available radioimmunoassay kit (NEN, Du Pont) after acetylation.

2.4 In vitro Studies

2.4.1 Tissue preparation

2.4.1.1 Inner medullary collecting duct (IMCD) cell extraction

Kidneys were excised and thoroughly rinsed in ice-cold PBS buffer after removal of perirenal fat and capsules. After bisection with a sterile scalpel, the renal medulla and papilla were carefully dissected out and minced in 1 ml of collagenase (1.5 mg/ml) (United States Biochemical, Ohio) dissolved in 37°C 1640 medium. The minced tissues were transferred to a tube containing 5 ml of 1640 medium and collagenase and then incubated at 37°C for 30 minutes. To prevent clumping, the digested tissues were pipetted in and out with a pasteur pipet. Digestion was stopped with the addition of an equal volume of 37°C 1640 medium with 10% fetal calf serum (FCS) and the mixture was centrifuged at 1000 rpm for 3 minutes. Once the supernatant was discarded, the digested tissues were rinsed with 5 ml of the same medium and centrifuged at 1000 rpm for a further 3 minutes. The supernatant was aspirated and 10 ml of 37°C 1640 medium with 10% FCS was added and mixed. The mixture was further incubated for one hour and then centrifuged again at 1000 rpm for 3 minutes. The supernatant was discarded and for a) stimulation studies, the pellet was resuspended with 37°C 1640 medium (about 1 ml/100 μg protein) containing isobutylmethylxanthine (50 μl/ml) and for b) receptor binding studies, the pellet was resuspended with 2 ml of 1% Tris-Tyrode buffer and frozen at -80°C until use for studies.

A sample of the IMCD cell preparation was sent to the Division of Anatomic Pathology at the University of British Columbia for histological identification. Histological examination of tissue sections confirmed that the cells were low columnar with distinct cell borders and
formed tubular structures, and that brush borders were absent. Sections stained with mouse monoclonal antibodies specific for epithelial membrane antigen (Dako, Santa Barbara, CA), low molecular weight cytokeratin (Enzo, New York), and high molecular weight cytokeratin (Enzo, New York) were positive for the first two but not the latter. These results were consistent with the cells being of collecting duct origin (257).

2.4.1.2 Preparation of IMCD for binding studies

The IMCD cells obtained above were homogenized with a Caframo Stirrer (Wiatron, Ontario) for 2 minutes at 4°C. Low speed (1000 rpm) centrifugation for 5 minutes at 4°C sedimented the cellular debris, unbroken cells, and nuclei which were discarded. The remaining supernatant was kept in Tris-Tyrode buffer (1%) and subjected to high speed centrifugation of 15 000 rpm for 20 minutes at 4°C. The resulting supernatant was discarded and the pellet resuspended with 8.5 ml Tris-Tyrode (1%). This suspension was sonified at low speed for 5 seconds while on ice. A 20 µl aliquot of this suspension was taken for protein assay by the Lowry method.

2.4.2 Receptor binding studies

Homogenized IMCD cells were used for binding studies to determine the ET receptor profile in normal and cardiomyopathic hamsters. 125I-ET-1 was prepared by the chloramine T method (258) and served as the hot ligand while ET-1 served as the cold ligand. BQ123 blocked the ET<sub>A</sub> receptor. The binding buffer used consisted of 1% Tris-Tyrode with 1 mM PMSF (pH 7.4). A standard curve for ET-1 was constructed with concentrations ranging from 0 to 100 ng/20 µl buffer. The experiments were carried out in duplicate in plastic culture tubes with a final volume of 240 µl (20 µl BQ123 (100 ng), 100 µl homogenate, 100 µl (120 000 cpm) hot label, 20 µl cold ET-1) incubated at room temperature for 30 minutes. BQ123 was allowed to completely bind all ET<sub>A</sub> subtype receptors in the homogenate before cold and hot ET-1 were added to the mixture. At the
end of the reaction, bound and unbound labelled peptides were separated by double antibody precipitation; 50 μl of 10% normal rabbit serum, 100 μl of goat anti-rabbit IgG, and 1 ml of 5% polyethylene glycol 8000 were added sequentially. The mixture was left at room temperature for 15 minutes before centrifugation at 3000 rpm for 30 minutes at 4°C. The supernatant was aspirated by vacuum suction and the pellet radioactivity was counted by an LKB minigamma counter (Wallec, Finland). These counts were entered into the computer Ligand Program (305) to calculate the dissociation constant (Kd) and maximum binding capacity (Bmax) of ET receptors for ET-1.

2.4.2.1 Acid-washed receptor studies
To show that the ET receptors were not previously occupied by elevated levels of endogenous ET-1, the IMCD samples were first washed with acid to ensure any occupied cell-surface receptors were free. IMCD cells were isolated as described above and homogenized for 2 minutes on ice. The homogenate was then centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was retained and centrifuged at 15 000 rpm for 16 minutes at 4°C. Then the resulting pellet was resuspended with 2 ml of sodium acetate buffer, pH 5.0, and allowed to stand for 10 minutes at room temperature. This suspension was again centrifuged as in the preceding step. A gentle rinse with 2 ml of 1% Tris-Tyrode was performed to remove any residual acid without disturbing the pellet. Finally, the pellet was resuspended with an appropriate amount of 1% Tris-Tyrode solution. The cells were now ready for binding studies as described above.

2.4.3 Stimulation Studies
2.4.3.1 ET-induced cGMP Accumulation
Fresh, digested IMCD cells were pre-conditioned in IMDM culture medium with 10% FCS at 37°C for 1 hour prior to stimulation with ET-1. They were then spun down at 1000 rpm for 3 minutes at 4°C. The supernatant was discarded and the cells resuspended
with an appropriate volume of IMDM medium. A 100 µl aliquot of the cell suspension was transferred to each of sixteen labelled tubes in a 37°C water bath. Various concentrations (0 M, 10⁻⁹ M, 10⁻⁸ M, 10⁻⁷ M) of ET-1 in stimulating medium (IMDM + 1 mM IBMX, 5 mM Na acetate, 3 mM lactic acid) were added to the labelled tubes and the mixture was incubated for 5 minutes. The tubes were then placed into an ice bath and the reaction stopped by the addition of 10 µl of 100 mM EDTA solution. Samples were frozen at -80°C for 15 minutes and thawed to rupture cell membranes, allowing the release of soluble cGMP. After a vigorous mixing, the freezing, thawing, and mixing procedure was repeated. Addition of 300 µl of Tris-EDTA (50 mM Tris, 4 mM EDTA) buffer brought the final volume to 0.5 ml and the sample was centrifuged at 3000 rpm for 30 minutes followed by acetylation. The supernatant was retained for cGMP measurement by radioimmunoassay and the pellet for protein content by the Lowry method.

2.4.3.2 Angiotensin II Stimulation of IMCD Cells

IMCD cells of Long-Evans rats were extracted as above. The digested cells were grown to confluence in 5 ml of 37°C 1640 medium with 10% FCS in 6-well culture dishes. Incubation of IMCD cells with different concentrations (10⁻¹⁰ M, 10⁻⁹ M, 10⁻⁸ M, 10⁻⁷ M, 10⁻⁶ M) of angiotensin II indicated it had little effect on ET receptor number in the IMCD cells. However, IMCD cells that were incubated with 10⁻¹¹ M angiotensin II showed a distinct change in ET receptor density (Figure 2.1). This concentration of angiotensin II was chosen for further studies as it represents a more physiological concentration. Angiotensin II (10⁻¹¹ M) was added to the cell cultures for 18 hours and grown at 37°C in 1640 medium plus 10% FCS. Cells were then harvested and centrifuged at low speed for 3 minutes. The supernatant was discarded and the pellet resuspended with 2 ml of the same medium. The mixture was centrifuged again and the supernatant aspirated. Two ml of Tris-Tyrode was then added and the suspension was homogenized for 2 minutes at
Figure 2.1 Effects of varying concentrations of angiotensin II on ET receptor density. Ratios of ET receptor density in IMCD cells treated with different concentrations of angiotensin II compared to control (no angiotensin II) are illustrated. At higher concentrations, little effect was seen on receptor number. Experiments were performed in duplicate or triplicate.
Fractional Change in ET Receptor Density

Fractional Change in ET Receptor Density

.control 10-11 10-10 10-9 10-8 10-7 10-6

Angiotensin II (M)
4°C. The supernatant was collected and centrifuged at high speed (15,000 rpm) for 20 minutes at 4°C. The resulting supernatant was discarded and the pellet resuspended with an appropriate volume of 1% Tris-Tyrode. Receptor studies were performed as described above.

Another set of experiments was conducted as outlined above except with one additional modification. The same experiment was repeated but in the presence of the angiotensin receptor antagonist, saralasin.

2.5 Statistics
All data were expressed as the mean ± standard error of the mean (SEM). Paired student's t-test was used to determine differences between control versus experimental phases within the same animals. Unpaired student's t-test was used to compare the means between two populations. Linear regression analysis (method of least squares) was used to estimate correlation coefficients. Differences were considered significant at the level of p<0.05.
Chapter Three  Results

3.1  Categorization of CM Hamsters

Based on the grading system described previously (255,256), all CM hamsters included in this study presented with a moderate degree of heart failure (Grades 2 and 3) with the exception of one manifesting severe heart failure. The former had subcutaneous oedema accompanied by pleural effusions, minor body cavity exudates, and liver congestion while the latter exhibited more gross oedema and organ pathology. Dilatation of the centrolobular veins and central cyanosis with bluish tongue were evident. Despite fluid retention, the CM animals were smaller in body size than the normal ones, with a mean body weight of 133 ± 4 g compared to 150 ± 1 g (p<0.0001) (Figure 3.1). In contrast, the total heart weight of the CM group was 735 ± 42 mg compared to 577 ± 6 mg in the normal controls (p<0.001) (Figure 3.2). There was overt hypertrophy of the atria and ventricles of the CM group. A comparison of the total heart/body weight ratios between the two groups showed a significant difference (CHF: 5.5 ± 0.3 mg/g, control: 3.8 ± 0.1 mg/g, p<0.0001) (Figure 3.3). No difference was observed between the body weights of untreated (150 ± 1 g) and enalapril-treated (153 ± 2 g) control hamsters or between that of the untreated (133 ± 4 g) and enalapril-treated (132 ± 2 g) CM animals (Figure 3.1). Thus, enalapril had no effect on body mass. On the other hand, the total heart weights of the enalapril-treated CM hamsters (604 ± 13 mg) were significantly lower (p<0.01) than that of their untreated counterparts (735 ± 42 mg), and both these values were higher than those of either of the controls (Figure 3.2). Furthermore, there was a parallel decrease (p<0.01) in the total heart/body weight ratio of the enalapril-treated CM hamsters (4.5 ± 0.1 mg/g) compared to that of those without treatment (5.5 ± 0.3 mg/g) (Figure 3.3).
Figure 3.1 Body weights of normal and cardiomyopathic (CHF) hamsters prior to and after enalapril therapy (2.5 µg/kg/day s.c. for one week). Values are the mean ± SEM of 6 (untreated CHF) or 8 animals. *p<0.0001 vs. normal.
Body Weight (g)

- Normal: Untreated
- Normal: Enalapril-treated
- CHF: Untreated
- CHF: Enalapril-treated

* indicates a significant difference.
Figure 3.2 Heart weights of normal and cardiomyopathic hamsters prior to and following enalapril therapy (2.5 μg/kg/day s.c. for one week). Values are the mean ± SEM of 6 (untreated CHF) or 8 animals (untreated or treated normal, treated CHF). *p<0.05 vs. normal. **p<0.001 vs. normal. *p<0.01 vs. untreated CHF.
Total Heart Weight (mg)

- Normal
- Normal
- CHF
- CHF

- Untreated
- Enalapril-treated

* * +
Figure 3.3 Heart weight/body weight ratios of normal and cardiomyopathic hamsters prior to and after enalapril therapy (2.5 μg/kg/day s.c. for one week). Values are the mean ± SEM of 6 (untreated CHF) or 8 animals (untreated or treated normal, treated CHF). *p<0.0001 vs. normal. †p<0.01 vs. untreated CHF.
3.2. *In vivo* Studies

3.2.1 Golden Syrian hamsters as a time control

Table 3.1 contains haemodynamic and renal excretion indices as a function of time. Values represent three collection phases as described in the methods section. There was a slight tendency for mean arterial pressure (MAP) to decline (from 126 ± 6 to 120 ± 6 to 111 ± 4 mmHg, p<0.05) from phase I to phase III while glomerular filtration rate (GFR) remained quite stable (from 0.53 ± 0.07 to 0.53 ± 0.09 to 0.52 ± 0.07 ml/min). With the exception of an increase in the urine flow rate during the last phase (23.2 ± 3.4 μl/min, p<0.05), there was no significant difference in the patterns of all other excretory parameters.

3.2.2 Untreated CM hamster studies

3.2.2.1 Baseline and ANP-phase haemodynamic characteristics

The haemodynamic characteristics of CM and normal hamsters are summarized in Table 3.2. The baseline MAP of CHF hamsters (92 ± 3 mm Hg) was significantly lower (p<0.01) than those of controls (119 ± 8 mm Hg) as were the haematocrit (47 ± 1 % versus 52 ± 1 %, p<0.01) and plasma protein concentration (4.3 ± 0.2 g/dl versus 4.9 ± 0.1 g/dl, p<0.04). The GFR, however, did not differ significantly between the two groups (CHF: 0.55 ± 0.12 ml/min; control: 0.40 ± 0.07 ml/min). Administration of exogenous ANP resulted in a 25% drop in MAP of both groups (CHF, 70 ± 4 mm Hg; control, 88 ± 6 mm Hg), but no significant change in GFR (CHF: 0.52 ± 0.08 ml/min; control: 0.55 ± 0.10 ml/min).

3.2.2.2 Renal effects of ANP administration

Baseline urine flow (4.2 ± 1.0 versus 6.0 ± 1.6 μl/min) as well as absolute and fractional excretion rates of sodium (1.42 ± 0.41 versus 1.88 ± 0.50 %; 2.3 ± 0.5 versus 1.9 ± 0.3 %) and water (1.1 ± 0.2 versus 0.8 ± 0.1 %) did not differ significantly between the
Table 3.1 Haemodynamic and renal excretory function in golden Syrian hamsters

<table>
<thead>
<tr>
<th></th>
<th>Phase I</th>
<th>Phase II</th>
<th>Phase III</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>126 ± 6</td>
<td>120 ± 6</td>
<td>111 ± 4*</td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>0.53 ± 0.07</td>
<td>0.53 ± 0.09</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td>P&lt;sub&gt;Na&lt;/sub&gt;, mmol/l</td>
<td>142.2 ± 1.3</td>
<td>140.5 ± 1.8</td>
<td>139.8 ± 1.6</td>
</tr>
<tr>
<td>UV, μl/min</td>
<td>12.7 ± 2.5</td>
<td>16.3 ± 3.9</td>
<td>23.2 ± 3.4*</td>
</tr>
<tr>
<td>U&lt;sub&gt;Na&lt;/sub&gt;V, mmol/min</td>
<td>4.43 ± 0.68</td>
<td>4.36 ± 0.42</td>
<td>4.81 ± 0.54</td>
</tr>
<tr>
<td>FE&lt;sub&gt;Na&lt;/sub&gt;, %</td>
<td>6.6 ± 1.2</td>
<td>6.4 ± 0.5</td>
<td>6.9 ± 0.7</td>
</tr>
<tr>
<td>FE&lt;sub&gt;H2O&lt;/sub&gt;, %</td>
<td>2.7 ± 0.5</td>
<td>3.5 ± 1.0</td>
<td>3.9 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SEM. MAP, mean arterial pressure; GFR, glomerular filtration rate; P<sub>Na</sub>, plasma sodium concentration; UV, urine flow; U<sub>Na</sub>V, urinary sodium excretion; FE<sub>Na</sub>, fractional excretion of sodium; FE<sub>H2O</sub>, fractional excretion of water. *p<0.05 vs. phase I.
## Table 3.2 Haemodynamic and renal excretory function in response to ANP

<table>
<thead>
<tr>
<th></th>
<th>Control Group (n= 8)</th>
<th>CHF Group (n= 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>ANP</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>119 ± 8</td>
<td>88 ± 6*</td>
</tr>
<tr>
<td>Haematocrit, %</td>
<td>52 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td>Plasma protein, g/dl</td>
<td>4.9 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>0.40 ± 0.07</td>
<td>0.55 ± 0.10</td>
</tr>
<tr>
<td>GFR(^a), ml/min/kg</td>
<td>2.7 ± 0.5</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>UV, μl/min</td>
<td>4.2 ± 1.0</td>
<td>21.4 ± 3.9*</td>
</tr>
<tr>
<td>U(_{Na})V, mmol/min</td>
<td>1.42 ± 0.41</td>
<td>4.36 ± 0.74*</td>
</tr>
<tr>
<td>FE(_{Na}), %</td>
<td>2.3 ± 0.5</td>
<td>5.9 ± 0.8*</td>
</tr>
<tr>
<td>FE(_{H2O}), %</td>
<td>1.1 ± 0.2</td>
<td>3.2 ± 0.4*</td>
</tr>
<tr>
<td>U(_{cGMP}), pmol/ml</td>
<td>7.6 ± 1.3</td>
<td>33.4 ± 4.3*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. ANP, atrial natriuretic peptide; MAP, mean arterial pressure; GFR, glomerular filtration rate; GFR\(^a\), glomerular filtration rate normalized to body weight; UV, urine flow; U\(_{Na}\)V, urinary sodium excretion; FE\(_{Na}\), fractional excretion of sodium; FE\(_{H2O}\), fractional excretion of water; U\(_{cGMP}\), urinary cyclic guanosine monophosphate; ND, not determined. *p<0.002 vs. baseline. +p<0.02 vs. baseline. †p<0.01 vs. control. ‡p<0.03 vs. baseline.
normal and CHF hamsters (Table 3.2 and figures 3.4 and 3.5). Urinary cGMP levels (7.6 ± 1.3 versus 11.7 ± 1.9 pmol/ml) were also not significantly different (Figure 3.6). However, there was a marked difference in the response to ANP infusion by the two groups. In the control group, there were significant rises in all of the parameters (urine flow: 21.4 ± 3.9 µl/min, p<0.02, FE_{Na}: 5.9 ± 0.8%, p<0.02, FE_{H2O}: 3.2 ± 0.4%, p<0.02, cGMP: 33.4 ± 4.3 pmol/ml; p<0.001) but only marginal increases in the CM animals (urine flow: 8.9 ± 2.5 µl/min, FE_{Na}: 2.7 ± 0.5%, FE_{H2O}: 1.7 ± 0.3%, cGMP: 23.8 ± 3.5 pmol/ml, p<0.03) (Figures 3.4, 3.5, 3.6 and Table 3.2). The natriuretic response to ANP by the normal animals occurred despite a decrease in MAP and no increase in GFR.

3.2.2.3 Heart failure severity and urinary excretion rates
Retention of salt and water as well as a diminished response to ANP were evident in the CM hamsters. There was a significant negative correlation between the degree of heart failure and ANP-induced fractional sodium excretion (Figure 3.7A) and of ANP-induced cGMP excretion (Figure 3.7B). A more severe degree of heart failure was expressed as a larger heart weight/body weight ratio. A negative correlation was also observed between the stage of heart failure and ANP-evoked fractional water excretion although this did not achieve statistical significance.

3.2.3 Enalapril-treated CM hamster studies
3.2.3.1 Baseline and ANP-phase haemodynamic characteristics
The baseline MAP and haematocrit of CHF animals improved to 114 ± 5 mmHg and 52 ± 1 % (Table 3.3) after chronic enalapril treatment although these values were slightly lower than that of the control group (123 ± 8 mmHg; 54 ± 1 %). The plasma protein concentration (4.8 ± 0.2 g/dl) of CHF hamsters was no longer diluted when compared to
Figure 3.4 Fractional excretion of sodium in normal and cardiomyopathic hamsters. Baseline values and responses to acute ANP infusion (1 μg/kg/min) are shown. Data are expressed as the mean ± SEM of 6 (CHF) or 8 (control) animals. *p<0.002 vs. respective baseline. **p<0.01 vs. respective normal phase.
Figure 3.5 Fractional excretion of water in normal and cardiomyopathic hamsters. Baseline values and responses to acute ANP infusion (1 µg/kg/min) are shown. Data are expressed as the mean ± SEM of 6 (CHF) or 8 (control) animals. *p<0.002 vs. respective baseline. +p<0.02 vs. respective baseline. **p<0.01 vs. respective normal phase.
Figure 3.6 Urinary cGMP excretion in normal and cardiomyopathic hamsters. Baseline values and the responses to acute ANP infusion (1 μg/kg/min) are shown. Data are expressed as the mean ± SEM of 6 (CHF) or 8 (control) animals. *p<0.001 vs. respective baseline. **p<0.03 vs. respective baseline.
Figure 3.7 Relationships between heart weight/body weight ratios and excretory indices in hamsters. (A) Negative correlation between heart/body weight ratios and the fractional excretion of sodium. (B) Negative correlation between heart/body weight ratios and the urinary excretion of cGMP. Open symbols = control animals; closed symbols = CM animals. (n=14).
A. 

FE Na (%) 

\[ r = -0.55 \quad p < 0.05 \]

Heart Wt/ Body Wt (mg/g)
Table 3.3 Haemodynamic and renal excretory function of enalapril-treated hamsters in response to ANP

<table>
<thead>
<tr>
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<th>Control Group (n= 8)</th>
<th>CHF Group (n= 8)</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>ANP</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>123 ± 8</td>
<td>88 ± 5*</td>
</tr>
<tr>
<td>Haematocrit, %</td>
<td>54 ± 1</td>
<td>58 ± 1*</td>
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<tr>
<td>Plasma protein, g/dl</td>
<td>5.1 ± 0.1</td>
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<tr>
<td>GFR, ml/min</td>
<td>0.52 ± 0.08</td>
<td>0.61 ± 0.04</td>
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<tr>
<td>GFR&lt;sub&gt;a&lt;/sub&gt;, ml/min/kg</td>
<td>3.4 ± 0.6</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>UV, µl/min</td>
<td>8.5 ± 1.5</td>
<td>21.4 ± 1.1*</td>
</tr>
<tr>
<td>U&lt;sub&gt;Na&lt;/sub&gt;V, mmol/min</td>
<td>2.53 ± 0.58</td>
<td>5.30 ± 0.39*</td>
</tr>
<tr>
<td>FE&lt;sub&gt;Na&lt;/sub&gt;, %</td>
<td>3.4 ± 0.5</td>
<td>6.7 ± 0.5*</td>
</tr>
<tr>
<td>FE&lt;sub&gt;H2O&lt;/sub&gt;, %</td>
<td>1.7 ± 0.2</td>
<td>3.8 ± 0.3*</td>
</tr>
<tr>
<td>U&lt;sub&gt;GMP&lt;/sub&gt;, pmol/ml</td>
<td>6.2 ± 0.6</td>
<td>33.8 ± 2.3*</td>
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Values are mean ± SEM. Abbreviations are as in Table 3.2. *p<0.01 vs. baseline. †p<0.01 vs. control. ‡p<0.02 vs. control.
the control value (5.1 ± 0.1 g/dl). No difference was displayed in the GFR of the CHF and controls (0.65 ± 0.04 ml/min vs. 0.52 ± 0.08 ml/min, respectively). A larger than 25% decrease in MAP occurred in both groups following ANP infusion (control: 88 ± 5.4; CHF: 81 ± 4 mmHg) while GFR remained unaffected. The haematocrit was raised to a greater extent in the control than the CHF group whereas the plasma protein concentration fell slightly in both cases after exogenous ANP (Table 3.3).

3.2.3.2 Renal effects of ANP administration

In comparison with the control group (8.5 ± 1.5 µl/min), enalapril therapy resulted in a higher basal urine flow in the CHF group (21.6 ± 2.3 µl/min, p<0.01) (Table 3.3). This was also accompanied by higher fractional and absolute rates of sodium excretion (5.5 ± 0.5 versus 3.4 ± 0.5 %; 6.06 ± 0.55 versus 2.53 ± 0.58 mmol/min, p<0.01) as well as of fractional water excretion (2.7 ± 0.3 versus 1.7 ± 0.2 %, p<0.01) (Figures 3.8 and 3.9). Baseline cGMP levels of the CHF animals (10.9 ± 1.1 pmol/ml) were significantly higher (p<0.01) than those of normal ones (6.2 ± 0.6 pmol/ml) (Figure 3.10). The effects of ANF infusion were no longer blunted in the CHF group with enalapril. The ANP-induced levels of the above variables, with respect to the CHF group (urine flow: 23.3 ± 2.2 µl/min, FE<sub>Na</sub>: 7.5 ± 0.6 %, FE<sub>H2O</sub>: 3.9 ± 0.1%, cGMP: 39.1 ± 1.6 pmol/ml), were at least comparable, if not marginally greater, than those of the normal controls (21.4 ± 1.1 µl/min, 6.7 ± 0.5%, 3.8 ±0.3%, cGMP: 33.8 ± 2.3 pmol/ml) (Table 3.3 and figures 3.8, 3.9, 3.10).
Figure 3.8 Fractional excretion of sodium in normal and cardiomyopathic hamsters following enalapril therapy (2.5 μg/kg/day s.c. for one week). Baseline values and responses to acute ANP infusion (1 μg/kg/min) are shown. Data are expressed as the mean ± SEM of 8 animals. *p<0.01 vs. respective baseline. **p<0.01 vs. normal baseline.
Figure 3.9 Fractional excretion of water in normal and cardiomyopathic hamsters chronically treated with enalapril (2.5 µg/kg/day s.c. for one week). Baseline values and responses to acute ANP infusion (1 µg/kg/min) are shown. Data are expressed as the mean ± SEM of 8 animals. *p<0.01 vs. respective baseline. **p<0.01 vs. normal baseline.
Figure 3.10 Urinary excretion of cGMP in normal and cardiomyopathic hamsters after chronic enalapril therapy (2.5 μg/kg/day s.c. for one week). Baseline values and responses to acute ANP infusion (1 μg/kg/min) are shown. Data are expressed as the mean ± SEM of 8 animals. *p<0.01 vs. respective baseline. **p<0.01 vs. normal baseline.
3.2.3.3 Heart failure severity and urinary excretion rates

Animals treated with the ACE inhibitor, enalapril, did not exhibit the same relationship between heart failure severity and ANP-induced fractional sodium and water excretion (Figures 3.11A and 3.11B) as those untreated. The negative correlation between the degree of heart failure and ANP-induced cGMP excretion was also abolished upon enalapril therapy (Figure 3.11C).

3.3 In vitro Studies

3.3.1 Receptor binding assays

3.3.1.1 ET receptor distribution in untreated CM hamsters

Total ET receptor number (Bmax) and ET$_B$ receptor number were much lower (p<0.005, p<0.003, respectively) in CM hamsters (532 ± 77; 214 ± 26 fmol/mg protein, respectively) when compared to normal controls (959 ± 154; 483 ± 88 fmol/mg protein, respectively) (Figure 3.12A). Blocking of ET$_A$ receptors with receptor-specific BQ123 revealed a 50% distribution of ET$_A$ (476 ± 81 fmol/mg protein) and of ET$_B$ (483 ± 88 fmol/mg protein) receptors in the IMCD of normal hamsters (Figure 3.12B). However, the percentage of A and B receptors in the CHF animals was 60% and 40%, respectively; with 318 ± 57 fmol/mg protein being the former and only 214 ± 26 fmol/mg protein comprising the latter. The number of A receptors did not differ significantly (p=0.07) between the two groups but the number of the B subtype did (p<0.003). Thus the downregulation of ET receptors was attributed more to the downregulation of ET$_B$ receptors. Although the maximum binding capacity, Bmax, was depressed in the IMCD cells of CHF hamsters, the affinity of the receptors did not differ between the two groups. The dissociation constant, Kd, of control animals was 0.496 ± 0.087 nM while that of CHF animals was 0.675 ± 0.147 nM, p = 0.16. The affinity of the ET$_B$ receptors was similar between the two groups: normal Kd = 0.203 nM, CHF Kd = 0.302 nM, p = 0.15. These values fall within the range of those reported by others. Please see appendix.
Figure 3.11 Absence of correlative relationships between heart weight/body weight ratios and excretory indices following enalapril therapy (2.5 μg/kg/day s.c. for one week). Negative correlations were abolished between heart/body weight ratios and (A) fractional sodium excretion, (B) fractional water excretion, and (C) urinary cGMP excretion. Open symbols = control animals; closed symbols = CM animals. (n=16).
A.

FE Na (%)

Heart Wt/ Body Wt (mg/g)

$r = -5.8E-02 \quad p = \text{ns}$
B.

FE H2O (%)

Heart Wt/Body Wt (mg/g)

r = 9.4E-02  p = ns
cGMP (pmol/ml)

Heart Wt/Body Wt (mg/g)

r = 0.21  p = ns
Figure 3.12 Effect of CHF on the density of ET receptor distribution in cardiomyopathic hamster IMCD cells. (A) Comparison of total ET receptor and ET$_B$ receptor density and (B) comparison of ET$_A$ and ET$_B$ receptor density in normal and cardiomyopathic hamsters. Values are the mean ± SEM of 8 animals. Experiments were performed in duplicate as described in Materials and Methods. *p<0.005 vs. normal. **p<0.003 vs. normal.
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3.3.1.2 ET receptor distribution in enalapril-treated CM hamsters

After enalapril therapy, the total number of ET receptors in CHF animals was 1016 ± 140 fmol/mg protein which was similar to the 1040 ± 216 fmol/mg protein in normal controls (Figure 3.13A). Addition of BQ123 showed that there was no difference in the density of ET<sub>B</sub> receptors between normal and CM animals (559 ± 72 fmol/mg and 582 ± 110 fmol/mg protein, respectively) (Figure 3.13A), and the percentage of each receptor subtype was roughly 50% (Figure 3.13B). This indicates there was no longer downregulation of ET receptors in the CHF group. Again, the affinity of the receptors was not different between groups. The K<sub>d</sub> for the normal controls was 0.983 ± 0.228 nM and 1.162 ± 0.189 nM for the CHF animals, p = 0.28. Similarly, the K<sub>d</sub> for ET<sub>B</sub> receptors was not significantly higher in the CHF group (0.712 ± 0.169 nM vs. 0.666 ± 0.201 nM, p = 0.43).

3.3.1.3 Effect of CHF on ET receptor density

The number of ET receptors declined as the severity of CHF became more pronounced. The inverse relationship was highly significant in reference to the total ET receptor density (Figure 3.14A) and a parallel interdependence was observed with regard to the ET<sub>A</sub> or ET<sub>B</sub> receptor density (Figures 3.14B and 3.14C). These negative correlations were abolished (Figures 3.15A and 3.15C) or rendered non-significant (Figure 3.15B) in animals treated with enalapril, suggesting enalapril prevents downregulation of ET receptors.

3.3.1.4 Effect of acid-washing on receptor density

Downregulation of ET receptors in CHF tissue was still observed after an acid wash to remove ligands from previously-occupied receptors. The numbers of total ET (725 ± 67 fmol/mg protein) and ET<sub>B</sub> receptors (419 ± 100 fmol/mg protein) were significantly lower than those on normal cells (1029 ± 91 fmol/mg protein and 686 ± 70 fmol/mg protein,
Figure 3.13 Effect of enalapril treatment (2.5 mg/kg/day s.c. for one week) on the density of ET receptor distribution in IMCD cells of cardiomyopathic hamsters. (A) Comparison of total ET receptor and ET\textsubscript{B} receptor density and (B) comparison of ET\textsubscript{A} and ET\textsubscript{B} receptor density in normal and cardiomyopathic hamsters. Values are the mean ± SEM of 8 animals. Experiments were performed in duplicate.
Figure 3.14 Relationship between heart weight/body weight ratios and ET receptor density in IMCD cells. Negative correlations are apparent between the heart/body weight ratios and (A) total ET receptor number, (B) ET$_{A}$ receptor number and, (C) ET$_{B}$ receptor number. Open symbols = control animals; closed symbols = CM animals. (n=16).
c.

Bmax (fmol/mg)

Heart Wt/Body Wt (mg/g)

$r = -0.60 \ p < 0.05$
Figure 3.15 Absence of correlative relationships between heart weight/body weight ratios and ET receptor distribution in IMCD cells following enalapril treatment (2.5 mg/kg/day s.c. for one week). Negative correlations between heart/body weight ratios and (A) total ET receptor density, (B) ET$_A$ receptor density, and (C) ET$_B$ receptor density were abolished after chronic enalapril therapy. Open symbols = control animals; closed symbols = CM animals. (n=16).
A.

fmol/mg protein

![Graph showing correlation between Heart Wt/Body Wt (mg/g) and fmol/mg protein.](image)

- $r = 5.2 \times 10^{-3}$
- $p = ns$

Heart Wt/ Body Wt (mg/g)
B.

fmol/mg protein

\[ r = -0.10 \quad p = \text{ns} \]

Heart Wt/ Body Wt (mg/g)
Heart Wt/ Body Wt (mg/g)

r = 0.12  p = ns.
p<0.01 and p<0.05, respectively) (Figure 3.16A). The number of $ET_A$ receptors was comparable between the two groups (306 ± 62 fmol/mg protein vs. 343 ± 42 fmol/mg protein) (Figure 3.16B).

3.3.2 Stimulation studies

3.3.2.1 ET-stimulated cGMP accumulation in IMCD cells

Basal levels of cGMP production were 6.34 ± 0.37 fmol/μg/5 min in cells derived from CHF IMCD. This was significantly less than the 7.60 ± 0.35 fmol/μg/5 min produced in IMCD cells of normal controls. Cyclic GMP accumulation was dose-dependent in the cells of both groups of animals. At each dose of ET given, radioimmunoassay detected a lower level of cGMP in the CHF cells when compared to the normal control cells. Figure 3.17 illustrates the dose-dependent effect of ET stimulation.

3.3.2.2 Effects of angiotensin stimulation on IMCD cells

Figure 3.18A shows that angiotensin II at a concentration of $10^{-11}$ M mediated downregulation of ET receptors in IMCD tissue (p<0.02 versus control) and that $ET_B$ receptors were substantially fewer by 29% (p<0.003 versus control). Nearly 80% of the ET receptors in these Long-Evans rat IMCD cells were of the B variety. Endothelin A receptors, of which there were about 20%, were downregulated by 39% (Figure 3.18B). Furthermore, the affinity of the ET receptors increased (Kd decreased to 1.16 ± 0.38 nM from 1.98 ± 0.43 nM, (p<0.001)) after angiotensin II treatment. Similarly, the $ET_B$ receptor affinity increased (Kd decreased from 1.43 ± 0.31 nM to 0.932 ± 0.346 nM, (p<0.02)). Cells co-incubated with angiotensin II and saralasin did not exhibit this downregulation (Figures 3.19A and 3.19B) nor the change in affinity. In fact, a comparison of receptor number in control and saralasin/angiotensin II - stimulated cells showed no difference between the two (Figures 3.20A and 3.20B) and the dissociation constants were quite comparable (1.98 ± 0.43 nM vs. 1.64 ± 0.18 nM (p=0.25) and
Figure 3.16 Effect of acid washing on ET receptor density. IMCD cells were washed with sodium acetate (pH 5.0) for 10 minutes and then rinsed with Tris-Tyrode before completion of receptor binding studies as delineated in Materials and Methods. Removal from previously occupied ET receptors of endogenous ET shows a true downregulation of total ET receptor and ET\textsubscript{B} receptor numbers in cardiomyopathic hamsters (A). Downregulation of ET\textsubscript{A} receptors was minimal in cardiomyopathic hamsters (B). Values are expressed as the mean ± SEM of 5 animals. Experiments were performed in duplicate. \*p<0.01 vs. normal. \**p<0.05 vs. normal.
A.

fmol/mg protein

---

Normal  Normal  CHF  CHF

ET  ETB

*  **
B.

fmol/mg protein

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<tr>
<td>ETB</td>
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94
Figure 3.17 ET-stimulated cGMP accumulation in IMCD cells. Cells from either normal or cardiomyopathic hamsters were incubated with different concentrations of ET-1 for 5 minutes and cGMP was measured by radioimmunoassay as described in Materials and Methods. Values are the mean ± SEM of 5 animals with experiments performed in quadruplicate. *p<0.01 vs. normal at respective ET-1 concentrations.
cGMP (fmol/ug/15 min) vs Endothelin (M)

- Normal
- CHF

* indicates statistical significance.
Figure 3.18 Effect of low-concentration angiotensin II on ET receptor density in the IMCD cells of Long-Evans rats. (A) Downregulation of total ET and ET$_{B}$ receptors and (B) total and ET$_{A}$ receptors following incubation of IMCD cells with $10^{-11}$ M angiotensin II. Ang II, angiotensin II. Values are the mean ± SEM of 5 experiments. *p<0.02 vs. control. **p<0.003 vs. control.
A. 3000 fmol/mg protein

Control Control Ang II Ang II

ET ETB

*  

**
B.

![Graph showing fmol/mg protein levels for control and Ang II treatments.](image)

- **Control**: Control
- **Ang II**: ET, ETA

The graph illustrates the fmol/mg protein levels for control and Ang II treatments. It shows a significant increase in fmol/mg protein for ET in the Ang II condition compared to control. Asterisks indicate statistical significance.
Figure 3.19 Prevention by saralasin of angiotensin II-induced ET receptor downregulation. IMCD cells from Long-Evans rats co-incubated with saralasin (10⁻⁹ M) and angiotensin II (10⁻¹¹ M) show no downregulation of (A) total ET and ET₄ receptors or of (B) total ET and ET₃ receptors. Ang II, angiotensin II; Ang + Sarlle, angiotensin II + saralasin. *p<0.02, **p<0.05 vs. angiotensin II alone.
B.

Ang II  |  Ang II  |  Ang + Sartle  |  Ang + Sartle

0  |  500  |  2000  |  2500

ET  |  ETA

*
Figure 3.20 ET receptor distribution in control IMCD cells and in saralasin-treated IMCD cells of Long-Evans rats. (A) Total ET receptor and ET$_B$ receptor densities and (B) total ET and ET$_A$ receptor densities are similar in untreated cells and in cells co-incubated with saralasin (10$^{-9}$ M) and angiotensin II (10$^{-11}$ M). Ang II + Sarlle, angiotensin II + saralasin. Values are the mean ± SEM of 6 experiments.
B.

3000
2500
2000
1500
1000
500
0

Control

Control

Ang + Sarlle

Ang + Sarlle

ET

ETA
1.43 ± 0.31 nM vs. 1.48 ± 0.37 nM (p=0.46), control ET and ETₐ vs. saralasin/angiotensin II ET and ETₐ, respectively).
Chapter Four  Discussion

Through the use of ACE inhibitors, the cycle of CHF can be interrupted via inhibition of angiotensin II-mediated vasoconstriction. In addition, this blockade would impede the sodium and water retention normally induced by angiotensin II and aldosterone. It has been demonstrated that ACE inhibitors can prolong the lives of patients with CHF (250), although the exact mechanism has not been determined. This study was designed to elucidate how enalapril could improve renal function in CHF hamsters by combining in vivo and in vitro methods. By drawing comparisons between the in vivo and in vitro characteristics of untreated with enalapril-treated CM hamsters, the mechanism of ACE inhibition could be derived. The study was based on the premise that the increased levels of angiotensin II would stimulate ET production which, in turn, would downregulate ET receptors and thereby diminish the natriuretic effects of ET. This is the first line of evidence showing that ET and its receptors play a major role in the neurohormonally-integrated excessive fluid retention in CHF.

4.1 Cardiomyopathic Hamster as the CHF Model of Choice

Cardiomyopathic hamsters were initially introduced over thirty years ago by Homburger et al (259). Establishment of various strains (eg. Bio 14.6, Bio F1B, Bio TO-2, UM-X7-1) of CM hamsters was derived through the cross-breeding of diseased animals with unrelated hamsters followed by the inbreeding of affected animals in the F2 generation. Transmission of the hereditary heart and skeletal muscle disorder is through an autosomal recessive gene and animals homozygous for this allele show complete phenotypic penetrance. Focal myocytolytic necrosis develops within 30 days post-partum and maximal degeneration and necrosis is seen 30 to 60 days thereafter (259-261). Scarring, hypertrophy, and dilatation of the heart occur, with heart failure ensuing by about 200 days after birth. The estimated lifespan of CM hamsters is 320 to 360 days although many
die at a younger age (250 days). Classic CHF is the cause of death in most animals, with multiple organ congestion generally manifest.

In choosing a model to study various aspects of CHF, the obvious choice would be human subjects. However, heterogeneity of the disorder (with respect to ethnic diversity and to cause and development in-patients) and ethical considerations place restrictions on their use. Consequently, researchers have turned to animal models of CHF, of which there are several. Examples include dogs with tricuspid insufficiency (212), inferior vena caval constriction (182), or rapid ventricular pacing (67) and rats with high output CHF by aorto-caval shunting (263) or ventricular infarct by coronary artery ligation (264). However, these models are also heterogeneous and the development of CHF varies in relation to the insult to the heart. Furthermore, these models necessitate the use of complicated and time-consuming procedures to prepare the animals for experimentation, and thereby may produce artificial disturbances in the animals which could lead to unreliable results. The use of CM hamsters can circumvent these impediments to some extent as the disorder is fairly homogeneous with a predictable clinicopathological course in these animals. Another advantage is the natural occurrence of the disorder rather than by surgical induction. Cardiomyopathic hamsters of the UM-X7.1 line were employed in this study.

Hamsters of a specific age (290 days) were used to minimize any differences in developmental stages of CHF. The hamsters use in this study had a moderate level of heart failure. The degree of heart failure was assessed based on the grading scale established previously as discussed in the Materials and Methods section. This grading system can assure homogeneity of the hamsters by excluding those animals identified as having asymptomatic CHF. In addition, in view of previous studies applying this grading system, comparison of current and previous findings is facilitated.
4.2 Physical Characteristics of the Hamsters

The total body weights of the CM hamsters were significantly lower than those of the age- and sex-matched control animals. One explanation for this lower weight may be due to poorer feeding and decreased nutrient delivery to body tissues secondary to inadequate circulatory function.

Total heart weights between the CM and normal hamsters also differed significantly but in this instance, it was the former that had higher heart weights. That the increase in heart weight was in accordance with body size can be ruled out since the diseased animals displayed higher total heart/body weight ratios. This increased heart weight is consistent with previous reports (265-267). Higher heart weights probably reflect congestion of the heart as well as the increase in cell mass (hypertrophy), features characteristic of CHF. That animals treated with enalapril presented with lower heart weights and lower heart/body weight ratios indicates that ACE inhibition can slow or reverse the process of compensatory cardiac hypertrophy, although not completely. This growth suppression probably stems from the reduced proliferative effects of diminished angiotensin II levels.

4.3 The Importance of a Time Control

It is necessary to determine whether the physiological functions of animals undergoing anaesthesia and surgical manipulations remain stable with time. The use of this type of control ensures that any differences in physiological parameters observed during the course of an experiment can be attributed to the treatments applied rather than to changes that occur as a result of systemic perturbations brought on by prolonged anaesthesia. In this study, haemodynamic and renal indices of function were relatively unaltered over a 4-hour period. Although there was a mildly significant decrease in MAP, the GFR was maintained by the kidneys. The increase in urine flow rate and the high percentage of sodium excretion could be ascribed to activation of compensatory mechanisms in response
to volume expansion induced by a high rate of saline infusion. Nevertheless, the data indicate the preparations were stable.

4.4 Haemodynamic and Renal Function in Untreated CM Hamsters

Mean arterial pressures (MAP) were markedly lower in the CM hamsters in comparison with the normal control group. A lower MAP was not unexpected as the effective circulating volume is usually diminished in CHF and this is in agreement with observations made by others (67,182,263,268,269). Glomerular filtration was relatively preserved in the heart failure animals and thus was similar to that in the control group. In heart failure dogs and rats, the GFR was reduced when compared to normal animals (67,182,268,269) which could be due to differences in species, stage of CHF, or methodology. Both haematocrit and plasma protein concentration in the diseased animals were also decreased relative to that in the controls, reflecting extracellular fluid volume expansion.

Infusion of ANP had similar effects on MAP and GFR in CHF and control hamsters; there was a dramatic drop in MAP with no significant change in GFR. The steady GFR indicated the preparation was stable. Similarly, Isnard and colleagues (270) saw MAP fall in both control and CHF rabbits after ANP dosing while Koepke et al (212) also found no differences in the effects of ANP infusion on haemodynamic variables between control and heart failure dogs. The vasodilating effects of ANP were clearly evident in these studies.

Similar to the results of studies in other animals (212,268,269), baseline measurements of urine flow and sodium and water excretion were diminished in CHF hamsters. The lower excretion rates may be explained by the lower MAP which may have resulted in poorer renal blood flow and perfusion pressure. Urinary cGMP levels at baseline were also comparable between normal and CM animals, with a nonsignificant greater value in the former. In agreement with the finding by Isnard et al (270), this higher cGMP production
by the sick animals could reflect stimulation of ANP receptors by higher endogenous levels of ANP. According to Wong et al (271), urinary cGMP is a biological marker of renal activity of ANP. These values imply that renal function in the CHF hamsters was relatively preserved, although there was a tendency toward mild electrolyte retention.

Attenuated responses to exogenous ANP in animals with CHF have been repeatedly reported (212,268,269) and this study further confirmed this observation. The measurements of all the above parameters following ANP administration were significantly lower in CHF versus normal animals. The reasons for the blunted response are unclear. Several explanations have been proposed. It is possible that the attenuated renal effects may be due to the overactivation of the RAAS (270) or other neuroendocrine systems since the antagonism between ANP and angiotensin II or AVP has been documented (217,220). Another hypothesis involves increased ANP degradation or incomplete processing, resulting in loss of biological activity; Isnard et al (270) showed that the ANP structure was intact in CHF rabbits. Others have claimed that renal resistance to ANP is due to downregulation of ANP receptors after chronic plasma ANP elevation since decreased receptor density has been observed in a number of studies (264,272,273). On the other hand, there have also been reports of no ANP receptor downregulation occurrence in CHF and that renal resistance is not due to second messenger defects (270, 274). A limitation imposed by most of the previously reported studies is the lack of investigation into the receptor subtype(s) responsible for regulation. The discrepancies between the published reports may be partially accounted for by the possibility of species or severity-dependent differences. The negative correlation between the degree of heart failure and response to ANP found in this study supports the concept that severity of CHF is an important determinant in responsiveness to ANP activity. Since the available data cannot be reconciled between the various studies, it is a likely possibility that other factors
are contributing to the observed results, depending on the model chosen and the degree of CHF. Perhaps other neuroendocrine systems, activated according to the stage of heart failure, modulate the effects of ANP. Endothelin is a definite contender in this regard as its plasma levels correlate with the extent of CHF (74) and is known to affect ANP release (205-209).

4.5 ET Receptor Distribution in the IMCD of Untreated CM Hamsters

Consistent with previous reports (73,254), downregulation of ET receptors in CHF was observed in this study; the IMCD of the CHF hamsters exhibited a 45% decline in receptor number in comparison to the normal controls. Blockade of ET\textsubscript{A} receptors with the antagonist BQ123 indicated that there was roughly equal distribution of the A and B receptor subtypes in the normal controls. The pattern of distribution in the CHF animals favoured the expression of the A subtype by about 10%. The downregulation in the earlier studies was selective; decreased density of receptors was seen in mesenteric arteries but not in myocardium of CHF rats (254) while downregulation occurred in the ventricles and kidneys of CHF rabbits. However, neither the receptor subtype distribution in these studies nor the nephron segments in the latter were specified and thus offer no point of reference for comparison. Alternatively, collation of the present data with characterization studies in canines (158) and rats (159,275) shows a deviation of ET receptor distribution in the medulla of CM hamsters from these species. Canine and rat species show a preponderance of the ET\textsubscript{B} subtype in medullary preparations. Another study (276) examining the ET binding sites in CM hamsters found no difference between these and matched controls in the amount of ET-1 binding in a number of tissues. However, the experiments do not allow for direct comparison as they were conducted in another strain and at a much younger (90 days) age. Neither the total receptor affinity nor the ET\textsubscript{B} receptor affinity in the CHF animals differed significantly from that in the controls.
in our study. In contrast, the binding affinity of ET-1 receptors was increased after downregulation in a rabbit model of heart failure as compared to the control (73). Although the reason for the change in binding affinity is uncertain, it may represent a mechanism in which the decrease in receptor number is compensated by an enhanced binding affinity.

The revelation that ET receptors are downregulated in the present study lends support to the hypothesis that ET levels are raised in CHF and thus effect downregulation and/or desensitization of its receptors. Albeit that levels of ET were not measured in this investigation, it has been widely documented that ET concentrations are raised in the plasma of CHF patients (67-77) as well as in the plasma and hearts of CM hamsters (277). Moreover, the capability of most renal cell types to produce ETs (278-281) could increase local concentrations in the kidney that serve to downregulate the ET receptors in an autocrine or paracrine mode. Reduced clearance or metabolism of ET may also contribute to the elevated levels of the peptide and thereby effect downregulation. Prior receptor occupancy by endogenous ET could be excluded in this study as a stringent acid wash of the IMCD cells also showed receptor downregulation.

The mechanism(s) by which ET receptor downregulation occurs have not been extensively examined. Receptor-mediated endocytosis is likely to be at least one pathway for decreasing receptor density. In cultured smooth muscle cells incubated with ET, the observed downregulation was postulated to occur as a result of internalization of the ET receptor with its tightly bound ligand (282). However, regulation of receptor number may be at the level of transcription or translation. Indeed, downregulation of the ET\textsubscript{B} receptor mRNA in cultured osteoblasts, after treatment with sufficient ET, was reported by Sakurai et al (283). The reduced quantities of ET\textsubscript{B} mRNA could be ascribed to decreased mRNA stability rather than to inhibition of transcription since the RNA polymerase inhibitor,
actinomycin D, did not affect mRNA levels. Activation of PKC appears to be involved in the downregulation of ET receptors by increasing the susceptibility of the mRNA to degradation. This may be accomplished by preventing the synthesis of an ET receptor mRNA stabilizing factor (283,284). Further studies addressing this aspect of downregulation are required.

Stimulation experiments conducted in this study showed that ET-1-stimulated cGMP production by IMCD cells was lower in CHF animals when compared with that in normal controls, both basally and in response to several concentrations of ET-1. That this cGMP production evoked by ET-1, which occurred in a dose-dependent manner, was diminished in the CM animals suggests that the ET$_B$ receptor was decreased in number since cGMP production is known to be increased by activation of this receptor subtype (176,285). If this is the case, these results provide further confirmation that downregulation of the ET$_B$ receptors occurs in the setting of CHF. However, it cannot be excluded that the hyposensitivity is at the level of the second messenger rather than at the receptor.

Since ET is known to inhibit AVP-stimulated water permeability and cAMP accumulation (237-241) via the ET$_B$ receptor, downregulation of this receptor subtype in particular will abate the normally diuretic effect of ET with the consequence of increased water retention. Reduced density of the A-receptor subtype in IMCD cells may be synergistic in permitting exaggerated water reabsorption. A preliminary report demonstrated that BQ123 abolished the inhibitory effect of 10$^{-10}$ M ET on AVP-stimulated water permeability in rat IMCD (286), suggesting that ET$_A$ normally assumes a role in water homeostasis as well. Furthermore, sodium excretion appears to be mediated by the A-subtype (78). The inhibition by ET of the Na$^+/K^+$-ATPase in the rat proximal nephron and collecting duct (201-203) may well be mediated through the A receptor although the finding that this inhibition may occur via a PGE$_2$ suggests otherwise (202). A decrease in
the density of this receptor subtype would thus leave the pump activity unchecked and allow unimpeaded sodium reabsorption. On the other hand, the recent report by Clavell and colleagues (196) suggests that natriuresis is not regulated by the A-receptor subtype based on the finding that ET-1 infusion alone elicited no change in sodium excretion but blockade of this receptor resulted in a natriuretic action at the level of the proximal tubule. These authors proposed that ET regulation of sodium excretion was independent of both the A and B receptor and that there may be an unidentified subtype responsible. Other investigators have conducted experiments that indicate the B-receptor subtype is responsible for ET-induced natriuresis as well as diuresis. Renal artery infusion of STXS6c, an ET
B
 agonist, resulted in a significant rise in fractional excretion of sodium in the dog and simultaneous infusion of BQ123 with ET-1 produced a similar result, possibly due to unmasking of ET
B
 effects (158). Pollock and Opgenorth (198) found that BQ123 had no effect on the diuretic and natriuretic response to big ET-1, supporting possible ET
B
-mediated inhibition of tubular reabsorption. If the B-subtype regulates natriuresis, then downregulation of this receptor subtype would also lead to decreased sodium output, as seen in CHF. Additional studies are required to resolve what role, if any, the A receptor plays in the renal handling of sodium balance.

4.6 Haemodynamic and Renal Effects of Enalapril in CHF

Contrary to other published studies (287-289), ACE inhibitor treatment resulted in an increase in MAP of the CHF animals in the present study. This is an unexpected finding given the known effects of angiotensin II blockade. One explanation for this increase may be due to an improved cardiac output by ACE inhibition without a significant change in vascular resistance, thereby raising MAP. Enalapril did not change the GFR of the hamsters. Infusion of ANP resulted in a large drop in MAP while it did not affect GFR.
Thus, the effect of ANP on these haemodynamic functions remained the same as in the untreated hamsters.

In agreement with the study of Winaver et al (268), enalapril restored the natriuretic and diuretic responses to ANP in the sick hamsters as evidenced by the increased urine flow, sodium and water excretion, and urinary cGMP levels. The increase in each of the parameters was similar to or greater than that in the normal controls after ANP infusion. Moreover, the baseline measurements of these variables were significantly improved after enalapril treatment. The raised baseline values are likely attributed to the activity of already elevated levels of endogenous ANP in the CHF animals, and infusion of ANP further enhanced fluid excretion. In addition, the baseline plasma protein concentration and haematocrit were restored to nondiluted values comparable to those of the normal animals. The haematocrit increased in response to the diuretic effects of exogenous ANP.

Chronic enalapril therapy abrogated the relationship between the severity of heart failure and ANP-induced responses. Absent were the negative correlations between the degree of cardiac dysfunction and ANP-induced cGMP, and fractional sodium and water excretion.

The improvement of haemodynamic and renal excretory functions following ACE inhibition was likely due, in part, to the mitigated effects of angiotensin II and aldosterone. By curtailing angiotensin II formation, the intense systemic and renal vasoconstriction can be relaxed, allowing better perfusion to the kidneys. Furthermore, the direct reabsorptive actions of angiotensin II are diminished as well as its indirect actions via angiotensin-stimulated aldosterone. Indeed, ACE inhibition with lisinopril improved haemodynamic indices in patients with left ventricular dysfunction such that RBF increased while GFR decreased, thereby lowering filtration fraction and thus fluid reabsorption (290). The same
study reported lisinopril lowered plasma concentrations of angiotensin II, aldosterone, and ANP. In another study by Tepel and colleagues (291), captopril and enalapril inhibited the cytosolic free calcium increase induced by angiotensin II, platelet derived growth factor, ET, and AVP in mesangial cells. These results suggest that ACE inhibition may confer renal protective effects through modulation of calcium-dependent contractile responses to angiotensin II, ET, or other pressor agents in the kidneys.

4.7 ET Receptor Distribution in the IMCD of Enalapril-treated CM Hamsters

Comparison of ET receptor density in heart failure animals and in healthy controls showed no difference between the two groups after chronic enalapril administration. Addition of the ET\textsubscript{A}-specific antagonist, BQ123, revealed that the density of the ET\textsubscript{B} subtype was also similar between the two groups, and there was equal distribution of the two receptor subtypes in the IMCD. Thus, downregulation of ET receptors did not occur and this was probably the result of ACE inhibition. To date, these are the only data showing the distinct downregulation of ET receptors in the IMCD of a CHF model and the restoration to normal density following ACE inhibition therapy.

In addition to the beneficial effects of ACE inhibition on angiotensin II actions, the fact that ET receptor density was no longer compromised in CHF animals after enalapril suggests that ACE inhibition may have lowered the levels of ET indirectly by blockade of angiotensin II-stimulated ET production. In light of a number of reports demonstrating that angiotensin II stimulates synthesis and release of ET-1 (118,292), as well as upregulation of ET-1 mRNA (293), it is plausible to surmise that angiotensin II blockade would lower ET-1 levels. Supporting this hypothesis are recent reports investigating the possible interaction of angiotensin II with ET and vice versa (294). Captopril was shown to suppress ET-1 release in cultured human endothelial cells perhaps through a bradykinin pathway (295,296). Furthermore, in patients with essential hypertension, captopril
diminished circulating concentrations of ET-1 (297). With the advent of lowered ET concentrations upon enalapril administration, the ET receptors are no longer subjected to the desensitizing effects of pathophysiological elevations of ET. Thus, the observed ET receptor density in the IMCD of the diseased hamsters is similar to that in the IMCD of the healthy controls.

That improved renal function is coincident with restoration of ET receptor number in the CM hamsters following ACE inhibitor therapy has at least two implications. First, this finding suggests that, as discussed above, angiotensin II does exert influence on ET levels and consequently, ET receptor density. Second, re-establishment of sufficient ET receptor density enables the kidney to promote appropriate sodium and water excretion in response to both endogenous and exogenous ANP. Normalization of ET receptor number, especially of the B variety, can allow regulated reabsorption of water by the IMCD in response to AVP stimulation. The ET$_B$ receptor accomplishes dose-dependent inhibition of AVP-induced cAMP accumulation or increased water permeability through the activation of PKC (237,238). However, the inhibition of AVP-stimulated increase in water permeability appears to be mediated through at least one other pathway. The latest publication by Garcia et al (298) shows that NO decreases this water permeability by lowering cAMP levels subsequent to the activation of a cGMP-dependent protein kinase (PKG) by augmented cGMP formation. NO release after ET$_B$ activation by ETs has been documented (176,285,299).

Whether the A or B subtype, both, or neither is involved in sodium balance is controversial. The data accumulated to date favour the association of the B subtype. To lend credence to this theory is the finding that NO appears to contribute to the regulation of renal excretory function (300). Inhibition of NO production decreased absolute and fractional excretion of sodium after renal nerve stimulation in anaesthetized dogs,
suggesting it has a modulatory role in electrolyte homeostasis. New reports assert that nitric oxide blocks basal and AVP-induced sodium reabsorption in cortical collecting tubules (301,302).

Taken together, the data show enalapril therapy restored the ET$_B$ receptor density, and activation of these receptors prompted diuresis through a PKC-dependent and/or PKG-dependent mechanism and natriuresis through a nitric oxide pathway.

The total receptor and ET$_B$ affinities for ET-1 did not differ significantly between the CM and control hamsters after enalapril administration. However, these affinities were decreased relative to those in the untreated hamsters. The reasons for this discrepancy are open to interpretation. One explanation is that the increased affinity of the receptors in the untreated hamsters was a compensatory response to the diminished number of ET receptors and that the affinity decreased once the ET receptor population returned to the usual density. Yet this does not account for the same pattern seen in the healthy group. It is conceivable that enalapril may have had a direct influence on the binding characteristics of ET receptors and that the differences in the affinities observed were not a function of counterbalancing mechanisms. Whether ACE inhibitors exert direct effects on ET receptor binding affinities must be examined. In addition, there is the remote possibility that the inconsistencies may be attributable to variations of binding efficiency amongst the hamsters.

4.8 Angiotensin II Stimulation of IMCD Cells

To conclusively determine whether a link exists between angiotensin II and ET, this assay was carried out. Incubation of IMCD cells with angiotensin II showed a significant downregulation of ET receptors when compared to controls without angiotensin II. Eighty percent of the ET receptors were of the B variety while the A subtype made up the
remainder. There was substantial reduction of both the A and B subtypes after angiotensin II treatment, further corroborating that angiotensin II does cause downregulation of ET receptors. This finding is in agreement with that of Roubert and associates (303) whose work revealed a downregulation of ET-1 binding sites by angiotensin II in the VSMC of rat thoracic aorta. Nevertheless, recent publications have disclosed the upregulating effects of angiotensin II on the ET$_B$ receptor subtype mRNA in rat cardiomyocytes (304) and on the ET$_A$ receptor subtype in human VSMC cultivated from the pulmonary artery (231). Furthermore, the latter study reported no change in receptor affinity for ET-1 after incubation with angiotensin II while the current study found an increase in receptor affinity. The enhanced receptor affinity observed in the present study likely represents a compensatory response to counterbalance the decline in receptor number. The reasons for these opposing results are not clear. Angiotensin II may differentially regulate ET receptor populations depending on the species or cell type involved. In addition, the increased expression of the type A ET receptor in the above study may be related to the concentrations of angiotensin II utilized. Upregulation of the ET$_A$ receptors was most prominent at higher concentrations ($>10^{-10}$ M) of angiotensin II but the present study showed downregulation at a concentration of $10^{-11}$ M. Thus the dosage of angiotensin II may be a determining factor.

Co-incubation of rat IMCD cells with angiotensin II and the angiotensin non-specific receptor antagonist, saralasin (Sarllie), prevented downregulation of ET receptors in the current study. Furthermore, the previous increase in receptor affinity of the saralasin-minus IMCD cells was not observed in IMCD cells incubated with saralasin. These results definitively demonstrate that angiotensin II is an important participant in the regulation of ET receptor expression.
Chapter Five  Summary

Clearance experiments performed in this study have demonstrated that CM hamsters with moderate heart failure exhibit a blunted response to exogenous ANP infusion in comparison to that of age- and sex-matched controls. The urinary excretion of sodium, water, and cGMP were all diminished after ANP dosing in the CHF animals. A significant negative correlation was observed between these measured parameters and the severity of heart failure. Chronic treatment with the ACE inhibitor, enalapril, heightened hydromineral output as well as cGMP production by the CHF hamsters after ANP stimulation. No correlation was present between these variables and the degree of heart failure following enalapril therapy. Therefore, ACE inhibition was beneficial in relieving the symptoms due to volume overload.

*In vitro* studies revealed that ET receptor distribution of the A and B subtypes was nearly equal at about 50% each in the IMCD cells of the normal hamster controls. The ET receptors of CM hamsters were downregulated relative to the binding site density seen in normal control animals and the level of downregulation had an inverse relationship with the degree of heart failure. This decrease in receptor density no longer occurred in the CHF hamsters after they were treated with enalapril for one week. Enalapril was also instrumental in abolishing the negative correlation between ET receptor density and cardiac dysfunction. The dissociation constants for ET-1 binding did not differ significantly between the diseased and healthy animals prior to enalapril treatment and this was also the case in enalapril-treated hamsters. However, the dissociation constants were higher (decreased affinity) in the enalapril-treated animals when compared to those values of the non-treated.
Stimulation with ET-1 of a primary culture of IMCD cells in CHF and normal hamsters showed an attenuated response in the former as determined by the quantification of the second messenger, cGMP. The response to ET-1 stimulation was dose-dependent. Moreover, basal accumulation of cGMP was significantly lower in the CHF animals, suggesting that ET receptor density may have been decreased.

Finally, stimulation studies established there is indeed interaction between the RAAS and ET system. Angiotensin II, at low concentration, was shown to cause downregulation of ET receptors of both the A and B subtype in the IMCD cells of rats. The ET receptor distribution in rat IMCD was composed of approximately 80% B subtype and 20% A subtype. Binding affinity for ET-1 increased following incubation of the IMCD cells with angiotensin II. However, the simultaneous addition of angiotensin II and the angiotensin receptor antagonist, saralasin, precluded any decrease in binding sites for ET-1 in the IMCD cells. Not only was the maximum binding capacity similar between control and cells with receptor inhibition by saralasin, but the dissociation constants remained at comparable values. These experiments illustrate that angiotensin II is involved in the regulation of ET receptor expression.
Chapter Six  Conclusions

The results obtained in this study have elucidated that ET, in conjunction with other hormones like angiotensin II, plays a role in the pathophysiologic salt and water retention characteristic of CHF. The data present compelling evidence for participation by ETs in the regulation of hydromineral balance in that downregulation of ET receptors is strongly associated with abnormal sodium and water reabsorption in the setting of CHF. That enalapril concomitantly restores appropriate renal function and ET receptor density provides a solid argument that angiotensin II and ET synergistically promote increased fluid reabsorption to the detriment of the body.
REFERENCES


169. Lee TS, Chao T, Hu KQ, King GL. Endothelin stimulates a sustained 1,2 diacylglycerol increase and protein kinase C activation in bovine aortic smooth muscle cells. *Biochem Biophys Res Commun* 1989; 162:381-386.


## Summary of reported kinetic properties of ET receptors in the kidney

<table>
<thead>
<tr>
<th>Kidney Tissue</th>
<th>Species/Strain</th>
<th>Methodology</th>
<th>Bmax (fmol/mg protein)</th>
<th>Kd (nM)</th>
<th>% ETB Receptor</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Glomeruli</td>
<td>Wistar rats</td>
<td>Competition binding with $^{125}$I-ET-1 and $^{125}$I-ET-3</td>
<td>~6200 (0.435 ± 0.134 fmol-glomerulus)</td>
<td>2.62 ± 0.52</td>
<td>predominant</td>
<td>Takemoto et al (157)</td>
</tr>
<tr>
<td>CCD</td>
<td>Wistar rats</td>
<td>Competition binding with $^{125}$I-ET-1 and $^{125}$I-ET-3</td>
<td>$0.408 ± 0.058$ fmol/mm</td>
<td>2.05 ± 0.72</td>
<td>predominant</td>
<td>Takemoto et al (157)</td>
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<td>CCD</td>
<td>Wistar rats</td>
<td>Saturation binding with $^{125}$I-ET-1 and $^{125}$I-ET-3</td>
<td>0.598 fmol/mm</td>
<td>2.26</td>
<td>predominant</td>
<td>Takemoto et al (157)</td>
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Summary of reported kinetic properties of ET receptors in the kidney (cont'd)

<table>
<thead>
<tr>
<th>Kidney Tissue</th>
<th>Species/Strain</th>
<th>Methodology</th>
<th>Bmax</th>
<th>Kd</th>
<th>% ET&lt;sub&gt;B&lt;/sub&gt;</th>
<th>Reference</th>
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<tr>
<td>CCD</td>
<td>Wistar rats</td>
<td>Competition binding with &lt;sup&gt;125&lt;/sup&gt;I-ET-1</td>
<td>0.41 ± 0.06 fmol/mm</td>
<td>2.1 ± 0.7</td>
<td>predominant</td>
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<td>Cortext</td>
<td>Sprague-Dawley rats</td>
<td>Saturation binding with &lt;sup&gt;125&lt;/sup&gt;I-ET-1 and BQ123</td>
<td>251 ± 58 (Total)</td>
<td>0.235 ± 0.057</td>
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<td>Gellai et al (159)</td>
</tr>
<tr>
<td>Cortext</td>
<td>Mongrel dogs</td>
<td>Competition binding with &lt;sup&gt;125&lt;/sup&gt;I-ET-1</td>
<td>350</td>
<td>0.9</td>
<td>78</td>
<td>Brooks et al (158)</td>
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<tr>
<td>Cortext</td>
<td>Human</td>
<td>Competition binding with &lt;sup&gt;125&lt;/sup&gt;I-ET-1 and BQ3020&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.6 ± 3.5 (ET&lt;sub&gt;A&lt;/sub&gt;)</td>
<td>2070 ± 800</td>
<td></td>
<td>Karet et al (156)</td>
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<sup>a</sup>ET<sub>B</sub>-selective agonist
Summary of reported kinetic properties of ET receptors in the kidney (cont'd)

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<th>Kidney Tissue</th>
<th>Species/Strain</th>
<th>Methodology</th>
<th>Bmax</th>
<th>Kd</th>
<th>% ET&lt;sub&gt;B&lt;/sub&gt;</th>
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<td></td>
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<td>Competition binding with 125I-ET-1 and</td>
<td>5.3 ± 0.2 (ET&lt;sub&gt;A&lt;/sub&gt;)</td>
<td>26.0 ± 15.0</td>
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<tr>
<td>Cortex</td>
<td>Human</td>
<td>BQ123</td>
<td>14.9 ± 1.6 (ET&lt;sub&gt;B&lt;/sub&gt;)</td>
<td>6500 ± 2200</td>
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<td>Competition binding with 125I-ET-1 and</td>
<td>11.3 ± 2.7 (ET&lt;sub&gt;A&lt;/sub&gt;)</td>
<td>5000 ± 1300</td>
<td>63</td>
<td>Karet et al (156)</td>
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<td>Medulla</td>
<td>Human</td>
<td>BQ3020&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.7 ± 2.2 (ET&lt;sub&gt;B&lt;/sub&gt;)</td>
<td>3.0 ± 1.4</td>
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<td>Competition binding with 125I-ET-1 and</td>
<td>11.1 ± 4.1 (ET&lt;sub&gt;A&lt;/sub&gt;)</td>
<td>11.8 ± 4.0</td>
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<tr>
<td>Medulla</td>
<td>Human</td>
<td>BQ123</td>
<td>36.2 ± 5.6 (ET&lt;sub&gt;B&lt;/sub&gt;)</td>
<td>32800 ± 8000</td>
<td>76</td>
<td>Karet et al (156)</td>
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<sup>a</sup>ET<sub>B</sub>-selective agonist
<table>
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<th>Methodology</th>
<th>B\text{max} (fmol/mg protein)</th>
<th>Kd (nM)</th>
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<td>Medulla</td>
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<td>Saturation binding with $^{125}$I-ET-1</td>
<td>57.7 ± 15.4</td>
<td>0.17 ± 0.04</td>
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<td>Medulla</td>
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<td>Saturation binding with $^{125}$I-ET-1 and BQ123</td>
<td>388 ± 19 (Total)</td>
<td>0.146 ± 0.43</td>
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<td>0.16</td>
<td>61</td>
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<td>Papilla</td>
<td>Mongrel dogs</td>
<td>Competition binding with $^{125}$I-ET-1</td>
<td>183</td>
<td>0.01</td>
<td>50</td>
<td>Brooks et al (158)</td>
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<td>Kidney Tissue</td>
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<td>Kd</td>
<td>% ET&lt;sub&gt;B&lt;/sub&gt;</td>
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<td>Papilla</td>
<td>Sprague-Dawley rats</td>
<td>Saturation binding with 125I-ET-1 and</td>
<td>2020 ± 144</td>
<td>0.075 ± 0.017</td>
<td>97</td>
<td>Gellai et al (159)</td>
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<td></td>
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<td>BQ123</td>
<td>1964 ± 146</td>
<td>0.067 ± 0.004</td>
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<td>IMCD</td>
<td>Sprague-Dawley rats</td>
<td>Saturation binding with 125I-ET-1</td>
<td>3200</td>
<td>0.0401</td>
<td>&gt; 80</td>
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<td>IMCD</td>
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<td>Saturation binding with 125I-ET-1</td>
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<td>Cernacek et al (216)</td>
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<td>Kidney</td>
<td>Burgundy rabbits</td>
<td>Competition binding with 125I-ET-1</td>
<td>129 ± 19 (control)</td>
<td>0.154 ± 0.017</td>
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<td>Löffler et al (73)</td>
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<td></td>
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<td>67 ± 6 (CHF)</td>
<td>0.099 ± 0.009</td>
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<td>Kidney Tissue</td>
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<td>% ET₂B</td>
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<td>Kidney</td>
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<td>Saturation binding with ¹²⁵I-ET-1</td>
<td>756 ± 14</td>
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<td>Bolger et al (276)</td>
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