TISSUE RENIN-ANGIOTENSIN SYSTEMS AND CARDIOVASCULAR DISEASE

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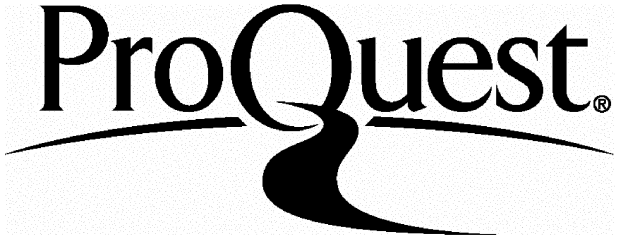

DEDICATION

This work is dedicated to all the friends who became colleagues, and colleagues who became friends, without whose support this thesis would never have been completed. Many are mentioned specifically overleaf.

It is not the critic who counts;
Not the man who points out where the strong man stumbled
or where the doer of deeds could have done better.
The credit belongs to the man who is actually in the arena:
Whose face is marred by dust and sweat and blood;
Who strives valiantly;
Who errs and comes short again and again;
Who knows the great enthusiasms;
Who, at the best, knows the triumph of high achievement;
And who, at the worst, if he fails,
At least fails while daring greatly,
So that his place shall never be with those cold and timid souls
who know neither victory nor defeat.

ANON

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ABSTRACT

Circulating/endocrine renin-angiotensin systems (RAS) play an important role in mammalian blood pressure homeostasis. Renin acts on angiotensinogen to yield angiotensin I, itself cleaved by circulating/endothelially-bound angiotensin converting enzyme (ACE) to yield the vasoconstrictor angiotensin II (AII). AII also stimulates adrenal aldosterone release. However, tissue RAS may also exist. AII generated by such local systems may control tissue growth and injury responses. This thesis examines the role of RAS in mammalian cardiovascular physiology and pathophysiology in an animal model and in humans .

The transgenic TGR(mRen-2d)27 rat expresses the mouse renin-gene in diffuse tissues and is spontaneously hypertensive. Using RNase protection assay and reverse-transcription PCR, transgene expression was identified in the right ventricle, kidney, lung and aorta. The blood-pressure profile of heterozygote male TGRs was characterised by serial measurement of systolic blood pressure (SBP) under light halothane anaesthesia using tail-cuff photoplethysmography. Blood pressure rose steeply after 3 days of age, reaching a maximum at day 69 (mean 256±11 mmHg). Thereafter, blood pressure slowly fell and continued to do so to 120 days (mean 216±30mmHg). The ACE-inhibitor Ramipril (10μg/kg/day) reduced both vascular ACE activity and SBP, whilst 1mg/kg/day reduced blood pressure to that of normotensive control animals. Calcium channel blockade (Amlodipine), even at doses of 20mg/kg/day, could not reduce SBP to normotensive levels, and hydrallazine therapy had no consistent hypotensive action. These data suggest a role for local vascular AII in the pathogenesis of the TGR hypertensive phenotype.

A high incidence of malignant hypertension (MH) was shown to occur amongst TGRs. Tissue ACE inhibition with non-hypotensive doses of ramipril which inhibited tissue (but not circulating) ACE activity (5μg/kg/day) were protective, reducing MH-related death rates from 63% to 4.3% and reducing scores for renovascular damage. Left ventricular hypertrophy (LVH) and collagen deposition amongst untreated TGRs was also reduced by ramipril treatment in a dose-dependent fashion, and partly through a non-hypotensive mechanism: 5μg/kg/day reduced both LV mass and collagen content at 70 and 120 days.

A polymorphism of the human ACE gene has been described which consists of the presence (Insertion, or I allele) or absence (deletion, D allele) of a 287 base pair fragment. The D allele is associated with higher circulating and tissue ACE activity.

It has been postulated that increased cardiac ACE activity may play a role in the development and progression of idiopathic dilated cardiomyopathy (IDC) in humans. However, no excess frequency of the D allele or DD genotype was found amongst 99 individuals with IDC when compared to 364 controls and within patients the D allele did not consistently correlate with markers of disease severity or progression.

Physiological left ventricular growth was studied in UK male military recruits exposed to 10 weeks of physical training. The D allele correlated in a dose-response fashion with increases in left ventricular wall thickness and mass. Plasma beta natriuretic factor (BNP) levels correlate with LV mass, and rose with training to the greatest extent in those of DD genotype. Finally, prevalence of electrocardiographic measures of LVH increased more in those individuals of DD genotype than those of II genotype. These data support a role for tissue RAS in the control of human physiological LV growth.

DECLARATION OF AUTHORSHIP

All the studies presented in this thesis were conceived, designed and initiated by the author. All data analysis and interpretation were also performed by him, and any errors in this thesis are therefore entirely his own.

Dr. Peter Gohlke performed the assays of tissue and plasma ACE activity for me in Frankfurt. Dr. Linda Kiernan performed all the assays of collagen concentration, and assisted in blood pressure measurement and tissue retrieval. David Holt at St. George's Hospital assayed BNP levels in blood.

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LOCAL AND SYSTEMIC RENIN-ANGIOTENSIN SYSTEMS

CHAPTER SUMMARY

Cells of the renal juxta-glomerular apparatus produce the aspartyl protease renin which acts on the alpha-2 globulin angiotensinogen (synthesised in the liver) to generate angiotensin I (AI). This non-pressor decapeptide is converted to angiotensin II (ATII) by contact with the peptidyldipeptidase angiotensin converting enzyme (ACE) (reviewed in Kem and Brown 1990). ATII stimulates the release of aldosterone, and is also a potent vasoconstrictor. The renin angiotensin system (RAS) is therefore important in the maintenance and control of blood pressure as well as the regulation of salt and water metabolism. However, renin, angiotensinogen and ACE have also been identified in cardiovascular tissues including the heart (Yamada, Fabris et al. 1991) and blood vessels as has mRNA for components of this system such as angiotensinogen (Campbell and Habener 1986; Ohkubo et al. 1986; Naftilan et al. 1991). Receptors for angiotensin II have been found on vascular smooth muscle cells(Gunther et al. 1982). Within tissues the RAS may therefore have a local paracrine function (reviewed in (Dzau 1988a; Dzau 1988b) and the expression of the different components can be altered by pathophysiological stimuli such as sodium restriction(Naftilan et al. 1991). In fact, kinetic studies suggest that much of the circulating angiotensin I and II is derived from the both renal and non-renal tissues(Campbell 1987a; Campbell 1987b; Admiraal et al. 1990).The pharmacological actions of ACE-inhibitors may depend upon inhibition of local as well as systemic RAS (Dzau 1988b) and may reset ATII/ ACE feedback on local RAS component synthesis(Schunkert et al. 1993).

This chapter describes our knowledge of both the systemic and circulating renin-angiotensin systems at the time when this work was initiated. It also provides the background evidence which supports a role for tissue RAS in the control of cellular growth. Particular attention will be paid to the existence and role of a cardiac renin-angiotensin system.

1.1: THE CIRCULATING ENDOCRINE RENIN-ANGIOTENSIN SYSTEM

1.11: OUTLINE

The systemic circulating endocrine renin-angiotensin system (endocrine RAS) regulates human salt and water balance and blood pressure. Cells of the renal juxta-glomerular apparatus produce the aspartyl proteinase renin which acts on the alpha-2 globulin angiotensinogen (synthesised in the liver) to generate angiotensin I (ATI). This non-pressor decapeptide is converted to angiotensin II (ATII) by contact with the peptidyldipeptidase angiotensin converting enzyme (ACE) (reviewed in Kem and Brown 1990) (Figure 1.1).

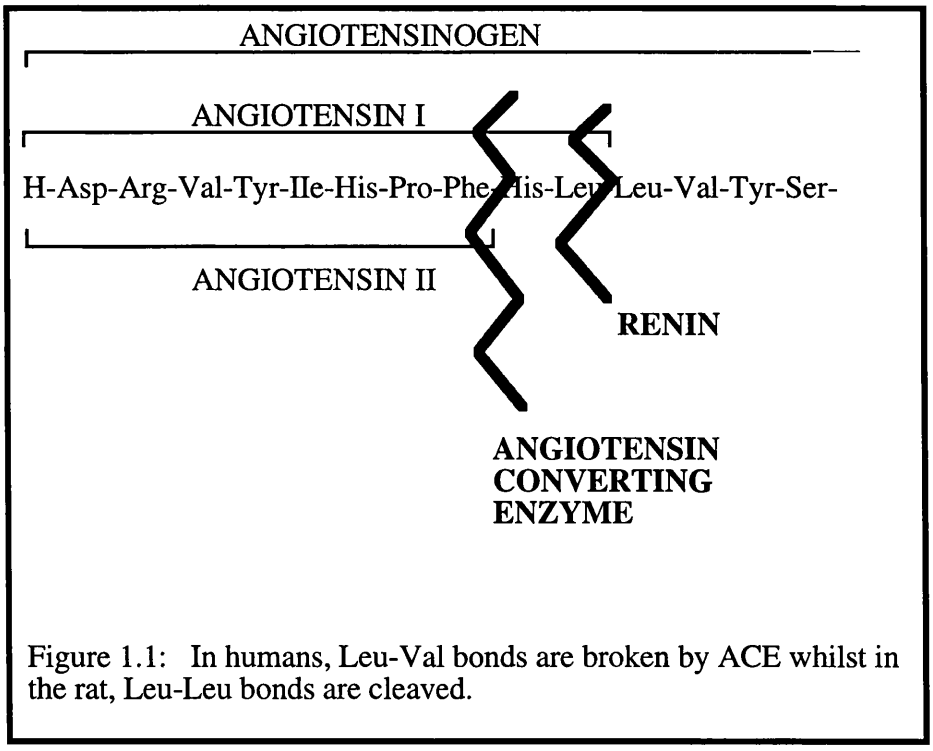


Figure 1.1: In humans, Leu-Val bonds are broken by ACE whilst in the rat, Leu-Leu bonds are cleaved.

ATII is a potent vasoconstrictor which stimulates adrenal aldosterone release. It is degraded by the carboxypeptidase angiotensinase C (cleaving the carboxy-terminal to yield a heptapeptide), by the aminopeptidase angiotensinase A (yielding ATIII), and by the endopeptidase angiotensinase B.

1.12: COMPONENTS OF THE ENDOCRINE RAS

1.121: Renin

In 1889 hypertension was induced in the rabbit by injection of renal extract(Tigerstedt and Bergmann 1889). The active agent is now known to be the aspartyl protease renin -a glycosylated carboxypeptidase (RMM approx. 40-55kD, depending on species) whose sole identified substrate is the hepatically-synthesised alpha-2 globulin angiotensinogen(see review (Skott and Jensen 1993)). Inactive pre-pro renin produced by initial translation of the renin gene (chromosome 1) undergoes signal-peptide cleavage, glycosylation in the endoplasmic reticulum, and packaging in the golgi apparatus to produce prorenin protogranules. Some prorenin is directly released. Other protogranules coalesce forming secretory granules in which lysosomal enzymes act to yield active renin(Hsueh et al. 1991).

Renin activity is the rate-limiting step in ATII generation in the human and rat circulating RAS. The main renin source is a complex of specialised renal cells known as the renal juxtaglomerular apparatus, which consists of

(i) the macula densa, formed from tubular epithelial cells of the thick ascending Loop of Henle and distal tubule and modified mesangial cells. The macula dense is closely associated with ..

(iii) up to 20 specialised modified epithelioid smooth muscle cells in the media of the afferent (>efferent) arteriole of the JGA.

Renin granules in these secretory JGA cells are released by exocytosis when reduced circulating blood volume, sodium load, or systemic blood pressure are perceived(Gibbons et al. 1984).

Release is therefore <u>stimulated by</u>:

(i) A fall in blood pressure leading to a rise in renal nerve sympathetic tone and JGA ß-adrenergic stimulation (Nakamura and Johns 1994).

(ii) Reduced renal sodium ion concentration acting indirectly through the macula densa (Gibbons et al. 1984) or directly on JGA cells (Fray 1976)

and <u>inhibited by:</u>

(i) A rise in renal perfusion pressure causes afferent arteriolar stretch and granular cell depolarisation(Fray 1976).

(ii) ATII (inhibits JGA renin release via a calcium/calmodulin-dependent pathway) (Antonipillai and Horton 1985).

(iii) Both atrial natiuretic peptide (ANP) and anti-diuretic hormone (ADH, or vasopressin) (Chou et al. 1990).

Many stimuli (e.g. hyponatraemia, reduced circulating volume due to diuretic use, and reduced perfusion pressure due to renal artery stenosis) stimulate both release of stored renin, and renin gene expression(Moffett et al. 1986; Gardiner and Lindop 1992; Skott and Jensen 1993).

Cooling, acidification or trypsin addition increases plasma renin activity (as measured by angiotensinogen to ATI conversion) by a factor of ≤ 10 (reviewed in Nielsen and Poulsen 1988). The presence of such 'inactive renin' in plasma persists at lower level (unlike active renin) after bilateral nephrectomy in rats, suggesting either sequestration of renally-derived 'inactive renin', or its non-renal synthesis. However, prorenin is undetectable by immunoassay in rat plasma after nephrectomy (Kim et al. 1991a). Further, in trypsin-activated plasma from rats 24 hours after nephrectomy a specific renin-inhibitor only blocks 20% of the plasma ATI-generating capacity, whilst up to 90% of this capacity was blocked by a broad inhibitor of sulphydryl enzymes(Hagemann et al. 1992). There would thus seem to be another circulating inactive renin-like substance other than prorenin. The same is true in tissues (see below).

1.122: Angiotensinogen

The circulating alpha-2 globulin angiotensinogen is cleaved by renin at the first Leu-Leu bond, yielding the inactive decapeptide Angiotensin I (ATI) (figure 1).

The human angiotensinogen gene is found on chromosome 1q42-43. Promoter sequences are activated by glucocorticoids and cytokines (Ohtani et al. 1992). ATII exerts negative feedback on angiotensinogen synthesis in the endocrine RAS (Herrman and Dzau 1983), in part through inactivation of an unidentified factor stimulating angiotensinogen synthesis (Hasewgawa et al. 1976). Polymorphisms of the angiotensinogen gene have been identified, one of which (the misense mutation with Methionine->Threonine substitution at codon 235: T235) has been associated with human hypertension and coronary artery disease (Katsuya et al. 1995). Gene expression is high in hepatocytes, and the liver is thus the main source of circulating angiotensinogen.

1.123: Angiotensin-1 Converting Enzyme (ACE)

ACE is a zinc metallo-protease which catalyses:

(i) conversion of the inactive decapeptide ATI to the active octapeptide ATII thorough the hydrolytic cleavage of dipeptides from the carboxyl terminus his-leu dipeptide.

(ii) inactivation of bradykinin by two sequential dipeptide hydrolytic steps. In this context, ACE is also known as kininase II (Figure 1.2).

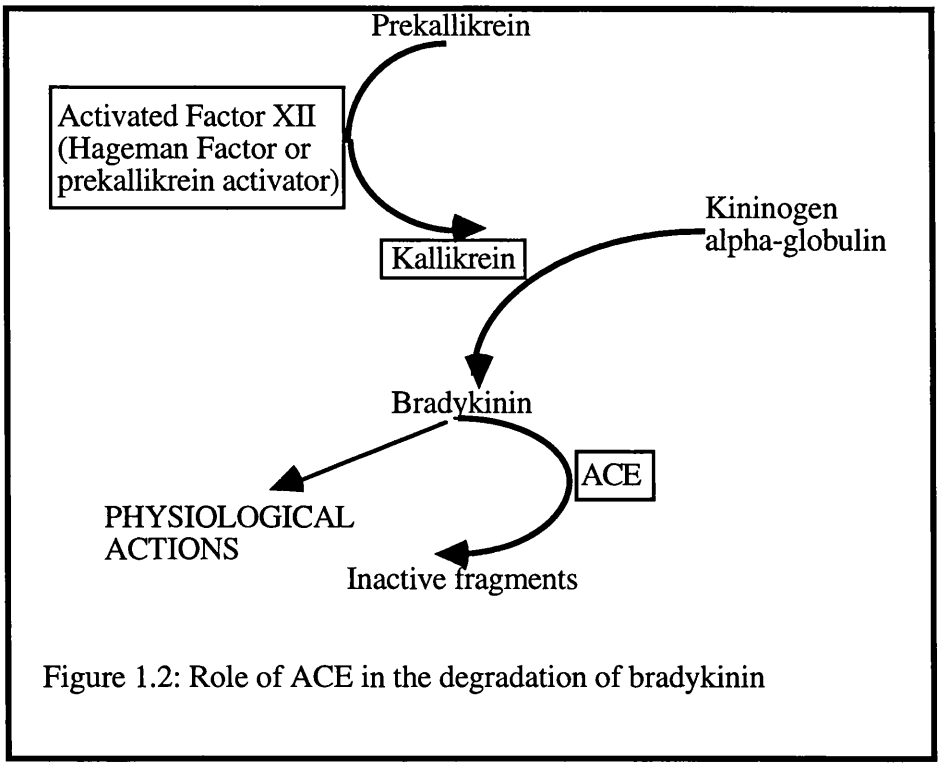


Figure 1.2: Role of ACE in the degradation of bradykinin

Thus, ACE is simultaneously generates a potent vasoconstrictor (ATII) and inactivates a potent vasodilator (bradykinin).

The main ACE isoform is a 170kD glycoprotein found in two compartments (Beldent et al. 1993):

(i) Membrane bound: ACE is an integral membrane protein with the C-terminus protruding intracellularly, and the molecule being anchored by a transmembrane hydrophobic component.

(ii) Soluble circulating ACE specifically synthesised in some animal cell lines (Beldent et al. 1993), and also derived partly from pulmonary capillaries.

The ACE gene is highly expressed in vascular endothelium (Sibony et al. 1993), and >80% of circulating ATI to ATII conversion thus occurs in the extensive pulmonary capillary bed.

Glucocorticoids induce ACE expression (Fishel, et al. 1995a), and negative feedback of ATII on ACE synthesis may occur. ATII infusion (although at concentrations- 100-1000ng/kg/min- associated with haemodynamic and possible neuroendocrine effects) suppresses pulmonary ACE activity (and mRNA levels by >50% at all doses) (Schunkert

et al. 1993). ACE-inhibition increases pulmonary ACE mRNA, and total plasma ACE concentration may rise in other animal models after chronic ACE-inhibition (Unger et al. 1981; Ulm and Vassil 1982).

1.124: Angiotensin II

ACE acts on ATI to produce the octapeptide ATII, the primary identified biological effector generated by the circulating RAS. Its pressor actions are mediated directly through vasoconstriction, and indirectly through renal salt and water retention (reviewed in Dzau 1988a):

a) ATII causes preferential efferent glomerular arteriole vasoconstriction, raising glomerular pressure. Increasing filtration fraction is offset by rising renal vascular resistance and fall in renal blood flow with a net modest reduction in glomerular filtration rate and a reduction in water clearance.

b) Renal sodium retention occurs through action on proximal convoluted tubule AT_1 receptors (Cogan 1990).

c) AT_1 receptors activation stimulates synthesis and release of the mineralocorticoid hormone aldosterone- itself a vasoconstrictor causing renal sodium and water retention.

d) Paracrine or circulatory ATII acting on the central nervous system increases thirst and salt appetite, and releases anti-diuretic hormone (Bunneman et al. 1992).

1.125: Products of ATII Breakdown

Some of the breakdown products of ATII (such as Ang3-8 -so called Angiotensin IV- and Angiotensin 1-7) may have biological activity mediated by AT_1 receptors, and possibly by novel receptor subtypes(Timmermans and Smith 1994). AT1-7 causes neuronal excitation and stimulates vasopressin release with a potency similar to that of ATII (Ferrario et al. 1991). ATIII is a weak vasoconstrictor.

1.126: Receptors for ATII

Two main classes of ATII receptor have been identified -AT_1 and AT_2- defined by the use of non-peptide subtype-selective antagonists Losartan (AT_1) and PD123177 (AT_2) (Timmermans and Smith 1994). Other receptor subtypes are postulated, including specific receptors binding ATII breakdown products(Timmermans and Smith 1994). The role of the different receptor subtypes has been reviewed by Timmermans and Smith (1994). The

AT_2 receptor is present in adult tissues, but expressed maximally in embryonic tissues (Dzau et al. 1993) suggesting a role in growth and development(Mukoyama et al. 1993). The receptor has been characterised using a rat foetal cDNA library, and shares 34% homology and a seven-transmembrane domain with the AT_1 receptor (Mukoyama et al. 1993). Both inhibition and activation of tyrosine phosphatase have been observed with receptor stimulation (Timmermans and Smith 1994).

The AT_1 receptor transduces the majority of the systemic effects of ATII, and is highly expressed in the adult vessel wall. It is further subclassified in rodents into AT_{1A} and AT_{1B} (Dzau et al. 1993). The AT_{1A} gene has been characterised (Takeuchi et al. 1993). Like the AT_2 receptor, the AT_1 receptor has a seven-domain transmembrane structure but, unlike the AT_2 receptor, is coupled to G-proteins. Second messenger activity is achieved through

(i) inhibition of adenylate cyclase causing reduced cAMP levels (as in the proximal convoluted tubule)

(ii) phospholipase C activation (e.g. hepatocytes and vascular smooth muscle cells) causing hydrolysis of phosphoinositide and thus generation of inositol 1,4,5-triphosphate (which mobilises intracellular calcium (Feolde et al. 1993)) and diacylglycerol (which causes to protein kinase C activation) (Dzau et al. 1993).

(iii) Mitogen activated kinases (MAP kinases) which are also activated by diverse cellular growth factors (e.g. platelet-derived growth factor) (Ishida et al. 1992). This, and the demonstration of a glucocorticoid-responsive element in the rat cardiac AT_{1A} promoter sequence (Guo et al. 1995), suggests that the AT_1 receptor might partly mediate the growth responses to ATII.

5 polymorphism sites have been identified at the 5' end of the human AT_1 gene, 2 of which are associated with amino acid switches in the gene product (6 ser<->Pro, 45 Gly<->Arg). 2 have been identified at the 3' end (Rolfs et al. 1994).

Competitive antagonists for AT_1 receptors increase free circulating ATII concentrations through competition at non-specific ATII-binding sites. Some of their biological effects may thus be mediated through increased ATII-activity at the AT_2 receptor (Timmermans and Smith 1994).

1.2: LOCAL PARACRINE OR AUTOCRINE RENIN-ANGIOTENSIN SYSTEMS

1.21: OUTLINE

The possible existence of local tissue renin-angiotensin systems was suggested by 2 observations:

(i) Optimum ATII/receptor binding occurs at ATII concentrations higher (nM) than those seen in plasma(pM) (Assad and Antonaccio 1982; Swales and Samani 1993).

(ii) ACE inhibitors reduce blood pressure in those with essential hypertension and normal/low plasma renin activity, leading some to suggest that they might inhibit a tissue RAS unaffected by plasma RAS activity (Dzau 1988a).

Increasing evidence now supports their existence(reviewed in (Dzau 1988a; Dzau 1988b; Dzau 1993a; Swales and Samani 1993)). In general, a local tissue RAS is suggested by evidence of:

a) Gene expression of RAS components within the tissue
b) Physiological responsiveness of gene expression
c) Local generation of ATII
d) The presence of ATII receptors
e) The demonstration that these receptors are physiologically active (i.e. demonstration of the effects of both ATII and ATII receptor antagonists on the tissue).

In many tissues, these criteria have now been met.

Angiotensinogen mRNA is identified in renal, neural and vascular tissues (see below), and local synthesis may strongly influence its concentration in interstitial fluid (Campbell 1987a).

Renin mRNA (Lilly et al. 1985) and product (Swales et al. 1983) is found in cultured mammalian vascular smooth muscle cells and throughout the vessel wall (Swales et al. 1983). In the SHR, systolic blood pressure correlates better with aortic than plasma renin concentration(Swales et al. 1983). Using competitive reverse transcription PCR (Iwai and Inagami 1992) and conventional PCR (Ekker et al. 1989; Fabian et al. 1989), renin mRNA is demonstrated in rat ileum, brain, adrenal, spleen, lung, thymus and ovaries. Liver renin gene expression is physiologically responsive, being increased 3-fold by sodium deprivation or captopril administration (Iwai and Inagami 1992). However, expression may be at low level or confined to specific cell subtypes as although RNAse protection assay

can still demonstrate renin gene expression in mouse testes, liver, and submandibular gland (Miller et al. 1989), Northern blot hybridisation showed no renin mRNA in mouse or rat liver (Dzau et al. 1987).

Non-renin angiotensinogenases also exist. A neutral aspartyl protease with renin-like activity has been demonstrated in canine brain (reviewed in (Dzau 1989; Ferrario et al. 1991)). Some (e.g. tonin, elastase, cathepsin G and tissue plasminogen activator) can cleave ATII directly from angiotensinogen(reviewed in (Dzau 1989)). The activation of the clotting cascade with vascular injury, and the finding of many of these enzymes within neutrophils, suggests that they may act specifically at sites of vascular injury (Dzau 1989).

ACE expression occur at high level in vascular endothelium, but also in the small intestinal endothelium, the epididymis(Sibony, Gasc et al. 1993) and brain (Ferrario et al. 1991). Tissue-specific /age-related ACE gene transcription occurs in renal tissue (very high proximal tubular epithelial expression), and cardiovascular, hepatic and pulmonary tissues (Yosipov et al. 1994).

Such local systems may be paracrine in nature: receptors for ATII are classically described as existing on cell surfaces, allowing transduction of the effects of endocrine and paracrine ATII. However, true autocrine systems (intracellular production and actions) may also exist. ATII receptors may also exist on the cell nuclei. Specific binding sites for ATII exist on cellular chromatin which may regulate gene transcription(Re, Vizard et al. 1984). Stimulation of ATII receptors on rat hepatocyte nuclei leads to a modest rise in total mRNA levels (approx. 1.5 fold), and a more significant rise in mRNA for renin and angiotensinogen (7.8 fold and 2.5 fold respectively). Interestingly, saturation of these nuclear binding sites results in a reduction in gene transcription, thus suggesting a plausible mechanism for the negative feedback of ATII on its own synthesis (Eggena et al. 1993). Angiotensin II binds to cardiac and renal nuclei. Of this total binding, 21% and 38% respectively is specific and displaceable by receptor antagonists, suggesting the presence of selective ATII receptors on these membranes. Such binding increases gene transcription by 24% and 38% respectively. An *intracellular* renin-angiotensin signalling system may exist, through which the effects of ACE-inhibitors mat be partly mediated (Eggena et al. 1993).

1.22: SPECIFIC TISSUE RENIN-ANGIOTENSIN SYSTEMS

1.221: Adrenal

Both mouse and rat adrenal express the renin gene (Bader et al. 1992; Peters et al. 1993; Zhao et al. 1993), as shown by competitive reverse transcription PCR and conventional PCR amplification (Ekker et al. 1989; Iwai and Inagami 1992), and Northern blot hybridisation (Dzau et al. 1987). Levels of adrenal immunoreactive renin persist after bilateral nephrectomy (Dzau 1988b). Gene expression is largely confined to the zona glomerulosa (Bachmann et al. 1992; Tokita et al. 1992), is responsive to ACTH (Sander et al. 1992), is differently regulated when compared to other tissues (Nishimura et al. 1992), and rises as hypertension develops in the salt-loaded spontaneously hypertensive rat (SHRSP) (Kim et al. 1991b; Kim et al. 1992).

1.222: Brain RAS

A local brain RAS exists (reviewed by Ferrario et al. 1991; Bunneman et al. 1992).)

Angiotensinogen is the principle CSF protein, and its mRNA is found at high concentration in areas of the brain which participate in blood pressure control (e.g. hypothalamus and medulla). Glial cells are probably responsible for its production, although the lack of identifiable glial renin or ACE mRNA might suggest uptake and processing by adjacent cells.

Renin mRNA is detected in rat brain by PCR (Ekker et al. 1989), RNAse protection (Miller et al. 1989) and Northern blot hybridisation (Dzau et al. 1987). Expression is upregulated by the administration of captopril (Iwai and Inagami 1992), is greater in SHRs than in controls (Assad and Antonaccio 1982) and is tissue-specific in terms of response to ACE-inhibition and nephrectomy (Campbell et al. 1993). Brain non-renin aspartyl protease may play a part in processing along with cathepsins.

ACE is synthesised in neurones of the central autonomic and endocrine pathways, as well as in the choroid plexus. Angiotensin-(1-7) is also produced directly from ATI in the brain (possibly by prolyl-endopeptidase), is found at very high concentrations in the hypothalamus and neurohypophysis, and has marked biological activity there and elsewhere in the body (Table 1.1). This system is insensitive to ACE-inhibition.

Coronary Constriction	+++
CNS AVP Release	+++
Baroreceptor transmission	++
Autonomic CNS neuronal excitation	+++
Prostaglandin release	+++
human astrocytes	+++
renal epithelia	+++
vascular smooth muscle	++

Table 1.1: Actions of Angiotensin-(1-7) (Adapted from Ferrario et al. 1991). All actions are of similar potency to those of ATII, except that AT-(1-7) is a much more potent coronary vasoconstrictor

1.223: Renal RAS

As described above, renal renin synthesis occurs predominantly in the juxtaglomerular apparatus(Bruna et al. 1993), and this is the primary source of circulating renin. However, tissue RAS may regulate intrarenal haemodynamics and sodium balance(Mendelsohn 1976; Mendelsohn 1979; Dzau 1988a). All RAS components are synthesised at a local level in renal tissue:

Angiotensinogen expression is high in the proximal convoluted tubules(Ingelfinger, Zui et al. 1990), and responsive to changing salt load (Ingelfinger et al. 1986), renal nerve stimulation(Nakamura and Johns 1994), and hypertension induced by contralateral renal arterial clipping(Von Thun et al. 1994).

Renin is synthesised by cells other than those of the JGA, which also produce renin-like peptides such as cathepsin D. Pulse-labelling experiments support glomerular mesangial cell renin synthesis which, in culture, is responsive to physiological stimuli (e.g. calcium and beta-adrenergic stimulation) (Dzau and Kriesberg 1986).

Angiotensin-I-converting enzyme activity is also present. Intra-arterial ATI injected into the dog renal artery produces vasoconstriction in the renal arterial bed and this effect can be blocked by the administration of ACE inhibitors (Aiken and Vane 1972). Juxtaglomerular cells are capable of the local generation of both ATI and ATII (Naruse et al. 1982). Using a radiolabelled analogue of the ACE-inhibitor lisinopril, ACE has been identified throughout the renal vascular endothelium (including glomeruli) (Chai et al. 1986). Much of the deep proximal convoluted tubule ACE activity (Chai et al. 1986) may be associated with the brush border (Ikemoto et al. 1986b). Non-ACE enzymes may also contribute to the catalysis of ATI conversion to ATII (Schmidt et al. 1986).

Renal ATII-receptors have been identified in the cortical glomeruli and the vasa recta (Chai et al. 1986).

1.224: Vascular RAS

The evidence which suggests the existence of a local vascular RAS has been reviewed by Swales and Samani (1993). The contribution of systemic RAS activity to vascular form and function are hotly debated. However a local RAS certainly seems to exist, with all components of the RAS demonstrated in the vascular wall (Campbell and Habener 1986; Campbell 1987b; Gohlke et al. 1992):

Renin is identified throughout the aortic wall by immunocytochemistry (Swales et al. 1983; Dzau 1988a,b), with dense staining in the inner intima and outer media and adventitia(Dzau 1988a,b). Adventitial staining is heavy around adrenergic nerve terminals.

Rat aortic ***angiotensinogen*** mRNA expression may be as high as 30% of that in the liver (Campbell and Habener 1986). Angiotensinogen synthesis is differentially regulated in the aorta in the presence of renovascular hypertension (Nishimura et al. 1992). Cassis showed that angiotensinogen mRNA expression was highest in para-aortic fat (40% of hepatic levels), low in the adventitia and, (using a full-length rat angiotensinogen DNA probe), undetectable in the media (Cassis et al. 1988). Naftilan, however, demonstrated both medial and para-aortic fat mRNA by Northern blotting and in-situ hybridisation. A low sodium diet (5 days of a 0.2% sodium diet) increased medial (but not para-aortic fat) mRNA by >3-fold when compared to a 1.6% sodium diet (Naftilan et al. 1991). Medial and neointimal expression (determined by in-situ hybridisation) is also induced in SD rat carotid arteries 1 week after endothelial denudation. Media:adventitia ratio of

angiotensinogen mRNA increased one week after injury, with no changes in total adventitial expression (Rakugi et al. 1993b).

ACE: Intra-arterial administration of ATI into the perfused dog leg (Aiken and Vane 1972) and human brachial artery causes ACE-dependent vasoconstriction (Webb and Collier 1986). In both cases, the authors suggest that very little ATI to ATII conversion occurs in the plasma, supporting the existence of local vascular ACE. Administration of ramiprilat alone into the human brachial artery causes a minimal (7%) increase in flow suggesting that ACE-related vascular ATII generation plays little role in control of normal resting male forearm vascular tone (Webb and Collier 1986). Non-hypotensive ACE-inhibition increases human arterial compliance when beta-blockade or hydrallazine fail to do so (Simon et al. 1984; Simon et al. 1985), and brachial artery diameter is increased by administration of ACE inhibitors only in hypertensive subjects and not in normotensive individuals (Dzau and Safar 1988), despite identical inhibition of serum ACE activity. Human studies using ACE inhibitors provide further evidence of excess vascular RAS activity (or sensitivity) in hypertensive subjects (Dzau and Safar 1988).

Gohlke investigated the site of aortic ATI-converting enzyme activity (Gohlke et al. 1992). ATI (+/- ramiprilat) or ATII was instilled into bladders made of isolated rabbit aortas, incubated, and ATI and ATII assayed in the luminal fluid. Tritiated ATI was shown to enter the aortic media and (to a lesser extent) the adventitia. ATI conversion to ATII (14% by 1 hour) was totally blocked by ramiprilat or endothelial denudation, suggesting that the majority of conversion of ATI to ATII is via ACE and occurs in the vascular endothelium. However, the method of denudation of the endothelium is not stated, the luminal pressure not measured, and ATII-generation within the aortic media not quantified. If conversion were slow here or diffusion of ATII out into the lumen restricted (e.g. by binding of ATII to VSMC receptors), then lumenal assays for just 60 minutes may have been insufficient to detect paracrine medial conversion. Further, the assay may have been insufficiently sensitive to detect such conversion. Although the importance of the aortic vascular endothelium to ATI conversion is thus emphasised, paracrine converting enzyme activity within the aortic wall is not excluded.

It has been suggested that part of the dilating effect of ACE-inhibitors might be mediated through the action of kinins in veins but not arteries (Yang et al. 1993; Caputo et al. 1995). However, Losartan produces significant relaxation in both human saphenous vein and internal mammary artery suggesting that ACE-inhibitors might cause dilatation at least partly through the prevention of ATII generation. Caputo showed that AT_1 receptor-

dependent rat carotid artery constriction occurs in the presence or absence of endothelial denudation (Caputo et al. 1995).

Data from all of the above studies do not prove local paracrine synthesis of RAS components (including ACE) in the media. It remains possible that active ATII had reached the VSMC compartment through the prior action of endothelial ACE on blood-derived ATI. However, there is evidence that local ATII synthesis occurs, as endothelially-denuded rat aortic rings contract in response to ATI (Andre et al. 1990) and aortic rat ACE expression has been identified in all layers (Arnal et al. 1994) which is induceable by injury (Rakugi et al. 1993a). In the chronic stages of hypertension, aortic ACE activity is increased in the 2K1C rat (discussed above (Swales et al. 1983)).

ACE inhibitor effects on vascular function and growth may be separated from their hypotensive effects (Wang and Prewitt 1990). In hypertensive animals aortic VSMC size (not number), collagen content, and medial thickness are reduced by treatment with ACE inhibitors (Levy et al. 1993), even at doses that do not inhibit plasma ACE (Albaladejo et al. 1994). Aortic compliance in dogs with high-renin hypertension is improved with ACE-inhibitor treatment (Fischer et al. 1993), and this effect in the hypertensive rat aorta differs with ACE inhibitor used despite their similar haemodynamic profiles (Frohlich and Horinaka 1991). Beta-antagonists and vasodilators lower blood pressure without effect on aortic compliance (Simon et al. 1985; Dzau and Safar 1988). Such effects are probably mediated through inhibition of ATII synthesis. Saralasin causes relaxation of isolated rat carotid arteries and improved compliance (Stefas and Levy 1991) and this effect persists after endothelial denudation and cannot be increased by potassium cyanide poisoning. These data suggest that, in the rat carotid, ATII activity may play a major role in the regulation of vessel tone. Indeed, the vascular effects of ACE-inhibition may be related to short-term relaxation of arterial wall VSMCs (Safar et al. 1986) as well as longer term effects on arterial wall structure. Angiotensin might influence vessel wall structure and function indirectly (by its neuromodulatory effect on noradrenergic release from sympathetic nerve terminals (Dzau and Safar 1988)) or by direct effects on vascular cell growth (Geisterfer et al. 1988; Naftilan et al. 1989; Naftilan et al. 1990b) and collagen synthesis (Hiroshi et al. 1991) (see below).

Aortic ATII receptors (Dzau and Safar 1988) mediate aortic contraction(Dzau 1986). The specific effects of ATII and of ATII receptor antagonists described above also support the presence of specific aortic ATII receptors.

1.23: LOCAL CARDIAC RAS

The isolated perfused rat heart is capable of ATII generation (Lindpaintner et al. 1988). Both ATI and ATII are detected in rhesus monkey cardiac tissue, with levels (by chromatographic assay) being higher in the atria than the ventricles (Lindpaintner et al. 1987). In rabbits pretreated with ramipril, the hearts (after death) showed reduced responses in heart rate and contractility to sympathetic nerve stimulation, suggesting that local ATII synthesis is occurring through an ACE-dependent mechanism (Lindpaintner et al. 1987). Such data have suggested the existence of local cardiac renin-angiotensin systems in rodents and primates.

The concept of a local human myocardial RAS is also gaining increasing acceptance (Kurtz 1992), and its role in cardiac growth and pathophysiology is the subject for much of the research presented in this thesis. Evidence exists for the local synthesis of all necessary components of a cardiac RAS, and suggests that synthesis may be modulated by physiological and pathophysiological stimuli. There is, however, some debate as to whether the heart relies more on uptake of some circulating RAS components than their local generation. This debate focuses particularly on cardiac renin synthesis.

1.231: Cardiac Renin Synthesis

Renin mRNA was identified by Dostal in cell-lysed neonatal rat heart homogenates cultured for eight days (Dostal et al. 1992a). The tissue, rapid loss of cardiocyte phenotype in culture, dependence on reverse-transcription PCR, and lack of internal control mRNA (external renal and skeletal muscle controls were used) prevents extrapolation to the adult human heart *in vivo*. However, in situ hybridisation with autoradiography also suggested the presence of renin mRNA. Renin signal was mainly localised to the LV. Both myocytes and fibroblasts synthesised renin mRNA and protein. *In vivo* cardiac renin synthesis is debated. Murine cardiac Ren-1d (>ren-2d) mRNA is demonstrated by RNAse protection near the limits of detection (Miller et al. 1989). High level cardiac rat renin mRNA is demonstrated by Northern blot hybridisation (approx. 2% of those seen in the rat kidney) (Dzau et al. 1987) and by PCR (similar to adrenal levels) (Okura et al. 1991), and may be physiologically responsive to salt depletion (reviewed by Urata et al. 1994a). By contrast, Iwai was quite unable to demonstrate renin mRNA in rat cardiac tissue despite the use of (highly sensitive) reverse transcription competitive PCR (Iwai and Inagami 1992). Using conventional PCR amplification techniques, Ekker was also unable to detect renin mRNA in rat cardiac tissue (Ekker et al. 1989). Non-specific hybridisation in

Northern blot, and PCR artefact may account for false identification or renin mRNA (Von Lutterotti et al. 1994).

ATI is synthesised by isolated perfused rat hearts in response to hog renin (Lindpaintner et al. 1990). However, the use of hog renin in the rat may have led to altered kinetics of ATI generation (in a similar way to the altered kinetics of transgenic murine renin in the rat (chapter 2), and much lower rates of production may actually occur in the rat exposed to rat renin. This idea is supported by the fact that ATI and ATII generation were undetectable at baseline, suggesting either low tissue renin levels endogenously, or a combination of this with lower activity of native renin.

The inability to reproducibly demonstrate cardiac renin synthesis in healthy cardiac tissue is a major flaw in the demonstration of a complete cardiac RAS (Ekker et al. 1989). The origin of cardiac renin protein detected (for instance) in isolated ventricular myocytes using specific antibodies (Samani et al. 1987) is thus debated. In theory, it might represent locally synthesised product or that taken up from the circulation. Tissue renin activity disappears after binephrectomy in the rat but persistence of cardiac AT II suggests the presence of a non-renin-dependent RAS in the rat (Dzau et al. 1993). The 30-hour anephric pig has no detectable cardiac renin, ATI or ATII in the LV tissue, leading Jan Danser to discount local porcine renin synthesis (Jan Danser et al. 1993)

1.232: Cardiac Angiotensinogen Synthesis

Angiotensinogen gene expression

Cardiac angiotensinogen gene expression is identified by Northern blot hybridisation in adult rats (Dzau et al. 1987) but is strongly associated with ventricular growth. Both myocytes and fibroblasts from neonatal rat heart homogenates (i.e. in a rapid phase of growth) (Dostal et al. 1992a) are capable of angiotensinogen expression. Expression is predominantly ventricular, and greater than that of renin. Young adult rat hearts express angiotensinogen mRNA in all chambers as assessed by Northern blot (Baker et al. 1990), in situ hybridisation and RNAse protection (Lindpaintner et al. 1990), whilst older animals seem to lack expression in the ventricles (Campbell and Habener 1986). Both dexamethasone administration (Lindpaintner et al. 1990) and rat aortic banding increase angiotensinogen expression (Baker et al. 1990). Cardiac angiotensinogen gene expression (message and immunoreactivity) has also been reported in humans, with prominent endocardial ventricular and atrial expression (Urata et al. 1994a).

Substrate angiotensinogen

The *in vitro* generation of ATI in neonatal cultured rat myocytes and fibroblasts also suggests the local synthesis of angiotensinogen substrate (Dostal et al. 1992b). In isolated perfused hearts from young growing rats (rat weight 150-200g), a clear early 'washout' of angiotensinogen occurred which was increased by prior perfusion with angiotensinogen-rich plasma, suggesting that a proportion of the angiotensinogen detectable was of circulatory origin. "Stable" low-level release followed, although at 2 hours perfusion there still seems to be a slow decline in release, raising the possibility of continued steady elution of adsorbed angiotensinogen. However this release and the magnitude of the washout phase was greater in animals pretreated with dexamethasone, arguing in favour of local synthesis, rather than elution, being the major factor in continued release(Lindpaintner et al. 1990).

1.233: Cardiac ACE Synthesis

Physiologically-responsive local cardiac ACE synthesis has been demonstrated. Conversion of ATI to ATII in isolated perfused rat hearts, with (Lindpaintner et al. 1990) or without (Linz et al. 1986) the addition of exogenous renin, is largely prevented by ACE-inhibition (Lindpaintner et al. 1987). Ramipril pretreatment blocks the vasoconstrictor and inotropic responses of isolated perfused rat, guineapig, and rabbit hearts to the addition of ATI but not ATII (Lindpaintner et al. 1987).

Radioligand binding and immunofluorescent studies confirm the presence of ACE, and molecular techniques demonstrate its local synthesis. Cultured neonatal rat myocytes and fibroblasts can synthesise ATII peptide, suggesting the presence of an ATI-converting enzyme (Dostal et al. 1992b). Immunoreactive ACE is demonstrable in the perinuclear region of these cells (Dostal et al. 1992b). Whole heart studies also demonstrate cardiac ACE synthesis although the anatomical, histological, cellular and cell-structural distribution varies with species, animal age, and the presence of different cardiac growth stimuli. Rat cardiac valvular and vascular ACE is detected in the 19 day embryo and myocardial ACE on the first day after birth after which levels increase (Hunt et al. 1995). Expression (both of mRNA and protein) is high in adult rat cardiac valves (Yamada et al. 1991) (particularly in the aortic valve endothelium (Sun et al. 1993)) and the coronary arteries (Yamada et al. 1991), moderate in the atria (Sun et al. 1993), although usually <4-fold higher in the atria than ventricles (Yamada, et al. 1991; Sun et al. 1993; Hunt et al. 1995). Left-ventricular binding-site count (of radioligand in a membrane-rich fraction) was 2.5x less in the LV than the RV (Yamada et al. 1991). Within the myocardium, ACE (as

detected by autoradiography) is found at highest concentration in association with the intramyocardial coronary arteries (Sun et al. 1993). In adult rat heart, Yamada was unable to detect ACE (by radioligand binding) in the SA or AV nodes (Yamada et al. 1991). In the hearts of a healthy male organ donors ACE-activity was confirmed at 16 points throughout the heart (Urata et al. 1990a). Activity fell 3-fold from the RA, through the base of the heart, to the LV apex and were 2-fold higher in the RV than the LV (Urata and Ganten 1993)), possibly through downregulation of myocardial ACE by the higher plasma ATII in pulmonary venous drainage (Urata et al. 1990b).

1.234: Non-ACE-dependent ATI-conversion

Most of the basal conversion of ATI to ATII in the rat heart is carried out by ACE (Lindpaintner et al. 1990; Schunkert et al. 1993). However, ATII generation in the ischaemic canine LV is not ACE-dependent, but is blocked by aprotinin (a non-specific serine protease inhibitor) (Gondo et al. 1989; Noda et al. 1993), suggesting the presence of a non-ACE ATI-converting enzyme. ATI may be positively inotropic in the Chinese hamster despite almost complete ACE-inhibition with captopril, suggesting that an alternative pathway of ATI->II conversion exists (quoted by Bumpus (1991). Rusicka supported this concept in his study of minoxidil-induced LVH (Rusicka and Leenen 1993). There were 2 treatment limbs: a) Animals were untreated, treated with Enalapril alone, Minoxidil alone, or minoxidil plus enalapril b) Untreated, losartan alone, minoxidil, or minoxidil plus losartan. ATII-receptor antagonism reduced resting LV mass, and completely prevented LVH development in response to minoxidil. ACE-inhibition reduced basal LV mass to a greater degree than losartan. LV mass was similar in untreated and minoxidil + enalapril-treated animals, but remained higher than in the enalapril-alone group. These data suggests that the minoxidil treatment had increased cardiac ATII expression as the sole mediator of the hypertrophic response, but that part of this ATII-generation was not mediated by ACE, but by another protease. This non-ACE enzyme may be induced during the hypertrophic process. Hypertrophied rat hearts have an increased capacity to convert ATI to ATII (Schunkert et al. 1990; Schunkert et al. 1993). Enalaprilat reduced this conversion rate by 70%, but to levels a little higher than those seen in non-hypertrophied hearts (Schunkert et al. 1993). Thus, 30% of rat cardiac ATI-conversion is mediated by an enzyme other than ACE, and this enzyme might have been induced by cardiac hypertrophy.

Urata was the first to suggest that most of the capacity for the conversion of ATI to ATII in the *human* heart did not lie with ACE (Urata and Ganten 1993). Using membrane

preparations of normal cardiac tissue, he showed that ACE-inhibition blocked only 5% of ATI-conversion capacity compare to an 80% inhibition by a non-specific serine protease inhibitor (soybean trypsin inhibitor). The responsible membrane-associated neutral serine protease was first isolated from human ventricular tissue by Urata in 1990 (Urata et al. 1990a) as a 30kD glycoprotein chymase active at optimum pH 7.5-9.0. Chymases are chymotrypsin-like serine proteases (Urata et al. 1994b) found in the secretory granules of mast cells. Unlike rodent chymase, human chymase is a potent and highly specific generator of ATII. It cleaves the Phe^8-His^9 bond to yield ATII and a His-Leu fragment, and seems to have maximal substrate specificity for ATI and neurotensin (Kinoshita et al. 1991). The gene structure and putative gene control sequence for the chymase gene have been reviewed (Urata and Ganten 1993). EM-histochemistry shows that most human heart chymase is identified in the myocardial interstitium, and is particularly associated with the extracellular matrix structure (Urata and Ganten 1993). No immuno-gold deposits were observed in association with myocytes. In situ hybridisation studies using human cardiac tissue identify chymase expression in cardiac mast cells, endothelial cells and mesenchymal cells (Urata et al. 1994a).The specific localisation of chymase may suggest a role in the modification of matrix.

Non-chymase cardiac ATI-converting enzymes may also exist. Chymase is not inhibitable by aprotinin- an important fact, as the papers by Gondo and Noda (Gondo et al. 1989; Noda et al. 1993) are often quoted as evidence in support of chymase being the major dog pathway of cardiac ATII generation. In fact, a non-ACE and non-chymase pathway of ATII generation may be in operation during the induction of ATII generation by ischaemia in the dog.

1.235: Cardiac ATII Receptors

Cardiac ATII receptors are identifiable in the 14-day rat embryo, and in cardiac vasculature by day 19. In adulthood, receptors are evenly distributed between atria and ventricles (Hunt et al. 1995). Most are of the classical AT_1 and AT_2 subtypes although receptors for the depleted hexapeptide fragment of ATII (ATIV) have also been identified (Hanesworth et al. 1993), which may mediate growth-inhibitory effects (Baker and Aceto 1990). In humans, high affinity low capacity ATII receptors have been demonstrated in membrane preparations (Urata et al. 1994a).

The AT_2 receptor seems to disappear rapidly with cell culture (de Gasparo et al. 1994) which might explain the finding of predominantly AT_2 receptors in adult rat atria by autoradiography, but AT_1 receptors in 3-day cultured neonatal rat atrial cells (Feolde et al.

1993). The distribution of receptors in human heart has not been extensively studied, but AT_2 receptors seem more common in human atrial tissue (2:1 ratio) (Timmermans and Smith 1994), especially around the sino-atrial node (Urata et al. 1994a). The ventricular inotropic effect of ATII seems to be AT_1-mediated (Timmermans and Smith 1994).

The poor resolution of radioligand and immunobinding studies has made the localisation of receptors within cardiac tissue hard to define (Timmermans and Smith 1994). In general, myocardial binding of ATII is low, moderate in the conduction system, and high around autonomic nerve terminals (de Gasparo et al. 1994). Cardiac fibroblasts may account for much of the total cardiac AT_1 receptor expression (Sadoshima and Izumo 1993a; Villarreal et al. 1993; Timmermans and Smith 1994). As well as cell surface receptors, cardiac nuclear receptors may also exist (Eggena et al. 1993). Radiolabelled ATII injected into rat LV localises around cardiomyocyte nuclei (Robertson and Khairallah 1971)of which 20% is receptor bound, causing an increase of 24% in gene transcription. An *intracellular* renin-angiotensin system may thus exist.

Functional responses to ATII have been characterised in a variety of cardiac tissues (Table 1.2). Through such mechanisms, a number of physiological responses are possible. ATII has also been implicated in the control of cardiac hypertrophy, fibrosis, and electrophysiology as well as playing a role in the development of/compensation for cardiac systolic failure.

Cell Type	Source	Response to ATII
MYOCYTES	Neonatal rat	*Increase in:* Leu incorporation Protein content c-fos/c-jun gene expression free cytosolic calcium Endothelin receptor mRNA
	Rabbit ventricular	*Increase in*: Chloride channels
	Dog ventricular	Increased calcium currents
FIBROBLASTS	Neonatal rat	*Increase in*: Phe and Thy uptake MAP kinase Protein and DNA content
	Adult rat	*Increase in*: Collagen/ protein synthesis
	Human	*Increase in*: Collagen synthesis
ENDOTHELIAL CELLS	Rat	*Increase in*: Endothelin mRNA *Reduction in*: TGF-beta1
	SHR	*Reduction in*: Proliferation with serum
	Human	*Increase in*:: E-Selectin

TABLE 1.2: (Adapted from Timmermans and Smith (1994))
SHR = spontaneously hypertensive rat
TGF= transforming growth factor
Most of these effects are mediated through the AT receptor. However, adult rat fibroblast collagen synthesis, and that in the human, may be AT mediated as may the reduced endothelial proliferation in SHR vessels stimulated with serum.

1.24: SUMMARY OF HUMAN CARDIAC RAS

RAS components are synthesised in myocardial tissues. The evidence for local renin synthesis remains tenuous at the present time, although other candidate angiotensinogenases have been identified (see also chapter 10). As well as paracrine systems, cardiac cells may also possess an intracellular autocrine RAS.

The chapters which follow explore the possible role of such paracrine RAS in the regulation of cardiac growth and function. Experimentation in the TGR has been extended to studies of human cardiac physiological growth and pathophysiology.

1.3: INTEGRATION OF CIRCULATING AND LOCAL RENIN-ANGIOTENSIN SYSTEMS

Systemic endocrine as well as local paracrine and autocrine RAS may thus exist. Their relative roles in control of cell and tissue response are much debated. In theory, one of three situations might be envisaged:

(i) There are two completely separate systems- one endocrine and one paracrine- which operate independently.

(ii) The circulating endocrine system supplies all of the RAS components. All tissue responses are due to uptake of these components from the circulation.

(iii) Local renin-angiotensin systems are 'depleted systems'. They lack some components which are drawn from the circulating RAS, while regulation of expression of other components controls tissue response.

Situations (ii) and (iii) may both pertain to different degrees in different tissues (Swales and Samani 1993). In the heart, for instance, a RAS comprising locally-synthesised components may require the activity of renin derived from the circulation in order to function. The evidence concerning the contributions of local synthesis and uptake of RAS components will now be discussed.

1.31: PRORENIN

As detailed above, most (>90%) of the circulating renin in rat and human is in the form of 'inactive' renin including prorenin, which can be activated by acid and proteases such as trypsin. Kim questioned whether prorenin might be extracted from the circulation and converted in tissues to active renin by such a mechanism (Kim et al. 1990). ^{125}I-radiolabelled human recombinant prorenin (and labelled albumin in order to assess plasma volume) was injected into marmosets, and plasma clearance followed. In three, sequential plasma sampling was followed by sacrifice of the animal at 60 minutes when organs were removed, total labelling assessed, and prorenin and renin concentrations assayed. Prorenin was rapidly taken up by the liver and kidneys and converted to active renin. However, it must be noted that very little (<1%) of the injected prorenin was taken up by the sum of all the other tissues assayed, including the heart and aorta (with ovary, testis, adrenal, spleen and uterus), suggesting that such uptake may be of little biological significance in these tissues (Kim et al. 1990). However, Mullins (personal communication) has shown that the production of prorenin by the liver alone in a transgenic rodent model is associated with the development of massive cardiac hypertrophy, raising the intriguing possibility of local

uptake and activation in the myocardium. Various possible mechanisms of conversion of prorenin to renin in tissues have been postulated (reviewed in(Dzau 1989; Kim et al. 1990)), including the activity of tissue plasminogen activator, plasmin, kallikrein, elastase, tonin and cathepsin G. However, the biological role for these candidates remains to be proven.

1.32: RENIN

Part of the renin pool identified in the vascular wall (see above) may be derived through uptake from the circulating pool. Labelled pure rat renal active renin is rapidly cleared by renal and hepatic tissue (Kim et al. 1987). Louden has demonstrated that exogenous renin may be sequestered and very tightly bound in the vascular wall, where it exerts its vasoconstrictor action (Louden et al. 1984). Swales has demonstrated similar findings in the nephrectomised rat (Swales and Samani 1993), showing that intravenously-administered renin is taken up by the aorta (Swales and Samani 1993) and that the subsequent rise in BP correlates better with aortic tissue renin activity than plasma renin activity. Consequently, the persistence of renin activity in vascular tissues for 24-48 hours after nephrectomy (reviewed in (Dzau 1989)) may merely represent the persistence of this adsorbed renin, and not local production.

However, part of the vascular renin may be locally synthesised (Swales et al. 1983). Vascular renin activity is raised in the SHR in the absence of elevated plasma renin (Assad and Antonaccio 1982), and in these animals systolic BP correlates better with aortic than plasma renin activity (Swales et al. 1983). Aortic renin-like activity persists long after that of plasma following nephrectomy (Basso and Taquin 1971), and ACE-inhibitors exert a persistent hypotensive effect long after nephrectomy (Man-in't-Veld et al. 1979). Re has identified renin mRNA in mammalian vascular smooth muscle in culture (Re et al. 1992), although the presence of message does not necessarily imply generation of the active product, and normally quiescent genes may become activated during the phenotypic changes which follow culture. Dzau was able to show expression of renin in human umbilical vein using in situ hybridisation (Dzau 1988a), and high-level expression in the rat aorta using Northern blotting (Dzau et al. 1987), whilst Okura made the same findings using PCR (Okura et al. 1991). It is strange, therefore that, using highly sensitive reverse-transcription competitive PCR, Iwai and Inagami were unable to reproduce these findings (Iwai and Inagami 1992).

The possible dual roles of local RAS component synthesis and uptake from the circulation have also been studied in other tissues. Campbell studied 5 groups of male rats: control animals, those treated with ramipril 10mg/kg/day for 24 hours, animals who had undergone bilateral nephrectomy either 24 or 48 hours previously, and animals 24 hours after nephrectomy given ramipril for a further 24 hours (Campbell et al. 1993). Nephrectomy reduced plasma levels of renin by 98%, and generally reduced tissue levels of ATII by 59-78%. ACE-inhibition further reduced ATII levels in aorta, lung and adrenal, but had no effect on levels in brown fat or heart suggesting that cardiac ATII generation might involve a non-renin and non-ACE dependent series of pathways, or (alternatively) the action of a single enzyme capable of cleaving angiotensinogen to yield ATII. The fall in tissue ATII levels after nephrectomy , yet the persistence of ATII, was taken to suggest an involvement of both renally-derived and locally synthesised renin in driving tissue RAS. It must be noted that levels of ATII in tissues were still falling between 24 and 48 hours after nephrectomy, and in the absence of a stable baseline, continued local synthesis is not proven.

Jan Danser has shown that the local generation of ATI and ATII in the rat heart seems to be dependent on a supply of renal renin (Jan Danser et al. 1995). Cardiac renin uptake was also studied in eleven pigs (Jan Danser et al. 1993). Prior to death, blood samples were drawn for assay of RAS components. Immediately after death, the four cardiac chambers were dissected free and frozen at -70^{0}C. Cardiac angiotensinogen levels were 75-85% lower than plasma levels. Cardiac renin, ATI and ATII levels were of a similar scale to, and correlated well with, plasma levels. However, only 5% of cardiac weight is due to trapped plasma and thus the concentration of these components is too high to be due to mere plasma contamination. This data suggested either that local and systemic synthesis of renin is similarly regulated, or that cardiac renin is derived from the circulation. To address this question, two further pigs were nephrectomised and killed 30 hours later when cardiac renin, ATI and ATII were below the limits of assay detection. The author's conclusion that all of the ATI-generating enzyme activity in the heart is derived from renal renin may be true in gross terms. However, local cellular systems may contribute little to the overall total cardiac renin content, whilst exerting powerful physiological effects. One might speculate that restriction of supply, selective uptake, or selective degradation of RAS components derived from the circulation might allow tissues to control local concentrations of such components and use them in a paracrine fashion. In this respect, the close association between circulating and cardiac renin, ATI and ATII levels and the lack of association between levels of angiotensinogen in this study is interesting.

Finally, it must not be forgotten that physiologically-responsive renin gene transcription has been demonstrated in the hearts of animal models (see above), although Von Lutterotti argues vehemently that there is no conclusive proof of local tissue renin synthesis, and that all tissue renin is derived from the circulation (Von Lutterotti et al. 1994). Nonetheless, it remains true that many studies supposedly demonstrating renin gene expression in cardiovascular tissues are flawed, and that the relative roles in such tissues of local renin synthesis and uptake by them of circulating renin, is still debated.

1.33: ACE

In Jan Danser's study, the chamber distribution of concentrations of ATI and ATII differed (right atrium, RA> left atrium, LA> right ventricle, RV> left ventricle, LV: and LA>RA>LV>RV respectively) (Jan Danser et al. 1993), suggesting that mere local passive sequestration of circulating ACE is not occurring. Furthermore, the cardiac ATI to ATII ratio was 3 fold higher than that in the plasma, again supporting local synthesis.

1.4: SUMMARY OF LOCAL AND SYSTEMIC RAS

Circulating endocrine and local autocrine or paracrine renin-angiotensin systems exist and interact, with the circulating endocrine RAS possibly supplying components for local paracrine systems. Such interaction might account for the finding that renin-inhibitory peptide infused into dogs causes a hypotensive response, although blood pressure continues to fall long after plasma renin activity is maximally suppressed (Dzau 1988a). Dzau (Dzau 1988a) postulates that the two systems, at least in the cardiovascular system, play two independent roles. The short-term regulation of cardiovascular homeostasis is carried out by the endocrine system. Tonic control is due to local systems which regulate thirst and salt appetite (brain), sodium handling (kidney), vascular tone (vascular walls), and cardiac contractility and function (heart). This hypothesis is supported by evidence from the study of acute and chronic systolic cardiac failure. In chronic compensated human cardiac failure (as in renovascular hypertension), plasma renin activity returns to a normal level after an initial rise. Tissue RAS activity remains elevated, however (Okamura et al. 1986; Finckh et al. 1991; Dzau 1993b; Paul et al. 1994). The acute hypotensive response to ACE-inhibition correlates well with the initial plasma renin activity, but this does not remain the case with chronic treatment. ACE-inhibition may also lower blood pressure in patients with low circulating renin levels (reviewed by Dzau (1988a)).

The diffuse presence of the RAS in a variety of tissues might infer a common tissue role. RAS gene expression is high in situations of vascular injury or growth (such as occur in atherosclerosis, hypertension, or endothelial denudation) suggesting that local RAS may have a growth-promoting function (Katz 1990a; Katz 1992).

It will be seen from the above review that renin is the rate limiting step in the generation of circulating ATII. In cardiac tissue, this has long also been assumed to be the case. However, the data reviewed above might suggest that, in fact, cardiac ACE is the rate limiting step within this tissue system.

1.5: THE ACE GENE INSERTION/DELETION POLYMORPHISM

1.51: BACKGROUND

Large interindividual differences in plasma ACE levels exist, but levels are similar within families (Cambien et al. 1988) suggesting a strong genetic influence in the control of ACE levels. The human ACE gene (DCP1) is found on chromosome 17q23 (Mattu et al. 1995) and contains a restriction fragment length polymorphism (Rigat et al. 1990) consisting of the presence (Insertion, I) or absence (Deletion, D) of a 287 base pair alu repeat sequence (Rigat et al. 1992) in intron 16 (Tiret et al. 1992). D allele frequency is approximately 0.57-0.59 (Rigat et al. 1992; Mattu et al. 1995).

1.52: INFLUENCE OF THE I/D POLYMORPHISM ON PLASMA AND TISSUE ACE LEVELS

Amongst 80 healthy caucasians (age unknown) the polymorphism accounted for 47% of the variance in plasma ACE, although considerable overlap existed between groups(Rigat et al. 1990). Tissue ACE levels might be similarly influenced. T-cells express ACE. The fact that most of the ACE activity is microsomal, and that B-cells lack ACE mRNA expression while monocyte ACE levels are 28-fold lower, all support the conclusion that T-cell ACE activity is due to cellular synthesis not passive adsorption from the circulation. ACE activity in those of DD genotype is 75% and 39% higher in plasma and T-lymphocytes respectively than in those of II genotype (Costerousse et al. 1993). Local ATII generation in human internal mammary artery may also be increased in those of DD genotype (Pinto et al. 1994). However, there is no evidence of an association of ACE genotype with circulating ATII levels (Harrap et al. 1993). These data suggest an influence of the I/D polymorphism on tissue and plasma ACE activity. Increasing D-allele burden might thus be associated with increased 'net RAS activity' in tissue systems. Any phenotype critically-regulated by tissue RAS may be more prominent within a population amongst those of DD genotype if tissue ACE levels are the rate limiting step in the tissue RAS. We have utilised this concept in seeking a role for cardiac RAS in the development of idiopathic dilated cardiomyopathy (chapter 7) and left ventricular hypertrophy (chapters 6 and 8).

The mechanism by which the polymorphism influences ACE levels is unknown. If truly an intron polymorphism, it might mark another polymorphism elsewhere in the gene, such as in the promoter region where it might influence transcription kinetics. However, part of the polymorphism might be translated and incorporated into mRNA, altering mRNA stability or splicing, or ACE protein stability (Rigat et al. 1990).

Many physiological stimuli cause induction of RAS (including ACE) gene expression (see above, and subsequent chapters). Prospective studies of polymorphism influence on the phenotypic response to a physiological challenge therefore allow not only elucidation of a role for tissue RAS in the control of that phenotype, but also examination of the molecular control of tissue ACE expression. For instance, if the D allele is associated with more responsive gene transcription, any given physiological challenge will cause a disproportionate change in RAS-dependent phenotype in association with the D allele. This concept has been utilised in the studies described later in this thesis.

1.6: RAS AND CELLULAR GROWTH

Evidence which intimately connects RAS activity and cellular growth comes from a number of lines of investigation:

(i) The RAS genes are upregulated in response to stimuli which stimulate cellular growth e.g. hypertension and vascular injury

(ii) ATII directly stimulates cellular growth

(iii) Inhibitors of ATII generation or action may prevent growth responses

These lines of evidence are now discussed in turn.

1.61 RAS GENE UPREGULATION IN RESPONSE TO GROWTH STIMULI

RAS activity is increased in response to growth stimuli such as hypertension, glucocorticoids, and vascular injury.

Increased vascular RAS activity may play a primary pathogenic role in the development of hypertension in the 2-kidney 1-clip rat (Shiota et al. 1992) and SHR through local ATII generation. Unilateral rat renal artery clipping (2K1C hypertension) causes a rapid rise in plasma, pulmonary, aortic and mesenteric artery renin activity, with a subsequent fall during the chronic phase of hypertension (Okamura et al. 1986). Vascular ACE activity (and thus the constrictive effect of AT1 in isolated aorta and mesenteric artery) remains elevated during the chronic stage of hypertension, and is reduced (along with BP) by enalapril and ATII receptor antagonists. Vascular renin levels are raised in hypertensive models (Dzau 1986) including the SHR, where levels correlate with the degree of hypertension (Assad and Antonaccio 1982). Vascular ACE (Okunishi et al. 1991) and angiotensinogen levels (Naftilan, J. et al. 1988; Okunishi et al. 1991) are also raised in the SHR.

Growth factors such as glucocorticoids and fibroblast growth factor (FGF) can stimulate ACE expression, as is seen by assay of ACE activity, and measurement of ACE mRNA by Northern blot densitometry in cultured vascular smooth muscle cells. Transfection of porcine iliac artery with recombinant FGF-1 also induces ACE expression (Fishel et al. 1995b).

Balloon vascular injury increases ACE expression in vascular smooth muscle cells (Rakugi et al. 1993a). This is identified by immunohistochemistry by 1 week, and carotid ACE activity increases by 3.6 fold within 2 weeks (Fishel et al. 1995b). The angiotensinogen gene is similarly induced in both the media and neointima (Rakugi et al.

1993b). Finally, a polymorphism of renin gene associated with greater renin gene expression has recently been identified in the spontaneously hypertensive rat (Samani et al. 1989), and the smooth muscle cell proliferation (fibrocellular intimal hyperplasia or FCIH) seen in response to balloon catheter damage is greater in such rats than in normal control animals (Clowes and Clowes 1980).

1.62 ANGIOTENSIN II ACTS AS A DIRECT STIMULATOR OF CELLULAR GROWTH

ATII may exert both hypertrophic an hyperplastic effects on vascular cells. Using a transfection of a chloramphenicol acetyl transferase reporter construct linked to the VSMC alpha-actin promoter into cultured VSMCs, ATII has been shown to stimulate alpha-actin gene expression (Andrawis et al. 1993). Quiescent well-differentiated rat aortic smooth muscle cells cultured in serum-free medium hypertrophy when exposed to ATII (Geisterfer et al. 1988), with both cell mass and protein content increasing. In a similar experiment, Berk demonstrated ATII receptor-dependent hypertrophy (but not hyperplasia) with an increase in protein synthesis (80%), protein content (30%), and cell volume (45%) (Berk et al. 1989).

By contrast, ATII-induced hyperplasia was shown in human aortic VSMCs (Campbell-Boswell and Robertson 1989) cultured in serum-containing media. ATII might thus have been potentiating the hyperplastic response to serum growth factors- a situation which may be closer to that seen *in vivo*. DNA synthesis (measured by ^{3}H-thymidine incorporation) is increased by balloon-injury and by ATII infusion in the rat thoracic aorta and carotid, with the two stimuli exerting synergistic effects (Daemen et al. 1991). The mitogenic effects of ATII in cell culture seem delayed (onset at 48 hours), but are profound at doses ranging between 3-100nM (Weber et al. 1994).

Finally, an ACE gene polymorphism associated with higher serum and possibly tissue ACE activity increases the risk of restenosis at the site of emergency coronary angioplasty (Ohishi et al. 1993).

1.63 EFFECTS OF ACE INHIBITORS AND ATII RECEPTOR ANTAGONISTS ON TISSUE GROWTH

1.631: Evidence from models of atherosclerosis (reviewed in (Sharpe 1993)).
In models of accelerated atherosclerosis, vascular growth (and development of the neointima) is reduced by ACE inhibition, endothelial migration is increased, plasminogen activator levels raised, and VSMC migration reduced. In high cholesterol models, endothelial function (such as flow-dependent relaxation) is relatively preserved by treatment with ACE inhibitors. Further, in the Watanabe heritable hyperlipidaemic rabbit and in cholesterol-fed cyomolgus monkeys, ACE inhibitors (but not beta-blocking agents or calcium channel blockers) demonstrate a potent antiatherosclerotic action. Although free-radical scavenging by the sulphydryl group of captopril may have played a protective role in these studies, it seems likely that much of the benefit of these drugs is derived from inhibition of vascular ACE.

1.632: Evidence from hypertensive models
Captopril produces a greater reduction in vascular VSMC hypertrophy than an equihypotensive dose of hydrallazine (Owens 1987). In the one-kidney one-clip rat, captopril reduces vascular growth (Wang and Prewitt 1990).

1.633: Evidence from models of vascular injury
FCIH in response to rat carotid balloon injury is reduced by up to 80% by ACE-inhibitors such as cilazapril (Powell et al. 1989; Powell et al. 1991). A non-hypotensive mechanism is suggested by the inhibitory effect of non-hypotensive doses of the ACE inhibitor ramipril (McEwan et al. 1992) and the negligible effects of verapamil and minoxidil (Powell et al. 1991). ACE-inhibition seems ineffective in low-circulating-renin models of hypertension when compared to high circulating renin models, suggesting that RAS activity influences the degree of vascular response to injury. However, these data are flawed to some degree, as the total postoperative blood pressure burden was reduced far less by ACE-inhibition in the low-circulating renin group than in the high-circulating renin group (Clozel et al. 1993), and tissue RAS activity was not considered in this study.

The effect of ACE-inhibition seems to depend on the inhibition of ATII generation as ATII antagonists are effective in preventing neointimal proliferation (Laporte and Escher 1992). TCV-116 (an antagonist of the AT_1 receptor fifty times as potent as losartan)

administered to rats after balloon catheter carotid injury reduces carotid DNA content at 3 days by <85% and intimal thickening at 14 days by 58% (Kawamura et al. 1993). These effects are independent of those on blood pressure, as local application of losartan (formerly DuP753) is also effective (Laporte and Escher 1992; Farhy et al. 1993).

A similar reduction in neointimal generation is seen when rats are treated with ACE inhibitors or losartan (Osterrieder et al. 1991), and the effect of ACE inhibition is lost if ATII is co-administered systemically or locally (Osterrieder et al. 1991; Laporte and Escher 1992). Further, kinin antagonism does not block the effects of ACE inhibition on VSMC growth in culture (Uehara et al. 1993). Nonetheless, a component of the effect of ACE-inhibitors may be through increased kinin levels. Administration of a kinin antagonist (Hoe 140) may reduce the inhibitory effects of ACE inhibition on neointimal formation by 42-72% (Farhy et al. 1992; Farhy et al. 1993). The degree of remaining inhibition was similar to that seen with AT_1 antagonism due to losartan, suggesting that ACE inhibition acted by both ATII antagonism and kinin agonist effects (Farhy et al. 1993). It may be that kinins themselves suppress ATII synthesis or that ATII suppresses kinin generation, and that the two mechanisms are therefore intimately linked rather than being truly independent (Peter Gohlke, personal communication December 1995).

In humans, trials of ACE inhibition have shown no benefit in reducing the incidence of coronary restenosis after angioplasty. This lack of effect may be due to low local levels of the drug (doses in general are many hundreds of times lower than those administered to rats due to the more marked hypotensive effect seen in humans), mixed case anatomy and disease severity, and to the timing of drug administration. Thus, the MERCATOR trial initiated treatment with 2.5mg cilazapril on the evening after angioplasty (MERCATOR study group 1992). Even the initiation of fosinopril therapy in Desmet's study 18 hours prior to angioplasty may have been insufficient (Desmet et al. 1994).

1.634: Evidence from cell culture

ACE inhibition with trandolaprilat increases cell doubling time, reduces proliferation (^{3}H-thymidine uptake) and cellular protein synthesis (^{3}H-Leucine uptake) in serum-induced growth of rat cultured vascular smooth muscle cells (Uehara et al. 1993). These responses were not mediated by kinins, as Hoe 140 (a kinin antagonist) had no effect.

1.64 CELLULAR MECHANISMS BY WHICH ATII MAY LEAD TO CELLULAR GROWTH

The growth-related effects of ATII, like those of many other growth factors (e.g. PDGF and FGF-1), may be partly mediated through induction of proto-oncogenes. ATII induces the rapid expression of the growth-associated nuclear proto-oncogenes c-myc (Naftilan et al. 1989a)(within 30 minutes), c-fos (Kawahara et al. 1989; Naftilan et al. 1989b; Weber et al. 1994), and c-jun (Naftilan et al. 1990) in cultured VSMCs, through increased gene transcription rate in the case of c-fos (Naftilan et al. 1989b). Proto-oncogene products may form complexes which bind with AP-1 promoter sequences to induce expression of TGFß1 which, in its active form, may mediate cellular hypertrophy. ATII induces the expression of PDGF and TGFß (Weber et al. 1994), although antibodies to these products do not seem to block the mitogenic activity of ATII. This does not prove lack of their involvement in the mitotic response, as internalisation of the antibodies was not demonstrated and intracellular antagonism was therefore unproved. Expression of platelet-derived growth factor A-chain is induced in cultured VSMCs by ATII (Naftilan et al. 1989a), the homodimer of which is already known to be mitogenic (Walker et al. 1986:) and the gene for which is also expressed in rat vascular and skeletal myocytes (Sejersen et al. 1986). Gene expression is followed by an increase in the detectable PDGF protein product in the culture medium (Naftilan et al. 1989a).

1.7: RAS AND CARDIAC HYPERTROPHY

1.71 RISKS OF HUMAN LEFT VENTRICULAR HYPERTROPHY

Electrocardiographically-defined human left ventricular hypertrophy (LVH) occurs in 20% of unselected hypertensives and in the majority of those with severe/malignant hypertension (Baxter and Yellon 1993). Although serving to increase myocardial work capacity and normalise wall stress (Grossman et al. 1990), LVH is associated with poor outcome (Katz 1990b), an excess total mortality independent of systolic blood pressure (SBP), and increased risk of cardiovascular death and disease (table 1.3).

VARIABLE	INCREASE IN RELATIVE RISK	
	Male	Female
Cardiovascular disease	1.49	1.57
Death from Cardiovascular disease	1.73	2.12
Death from all causes	1.49	2.01

Table 1.3: Risk-factor adjusted relative risks associated with a 50g/m increase in height adjusted LV mass (:4-year follow-up of Framingham Heart Study cohort(Levy et al. 1990a))

Overall, LVH is associated with a 10-fold increase in mortality rate (Massie et al. 1989) and risk of developing congestive cardiac failure(CCF) (Vogt et al. 1993). Some of this risk can be attributed to the hypertrophy itself (Kannel et al. 1969; Katz 1990b; Levy et al. 1990a) through early diastolic dysfunction and later fibrosis, myocyte necrosis, failed myocardial capillary bed expansion and flow autoregulation, and subsequent overt heart failure (Massie et al. 1989) and sudden cardiac death. Treatment of hypertension thus targets the prevention or regression of LVH(Baxter and Yellon 1993). Although its impact on outcome is unknown(Massie, Tubau et al. 1989), experimental data suggest benefit (Baxter and Yellon 1992b; Bruckschlegel et al. 1995).

1.72: THE HISTOPATHOLOGY OF LVH

The changes in cardiac form and function of human LVH have been reviewed by Katz (Katz 1990b). Ventricular hypertrophy is associated with both individual myocyte hypertrophy (through the addition of contractile protein units) and increases in cardiac matrix deposition.

Although 75% of heart volume is occupied by muscle cells, at least 2/3 of its cells are non-myocytes. The majority are fibroblasts producing collagen (reviewed in (Villarreal et al. 1993; Brilla et al. 1995)). At least six types of cardiac collagen exist (Kuzuo et al. 1993). Types I, III and V are fibroblast-derived (although rat cardiac myocytes may synthesise type III collagen (Eghali et al. 1988)). They comprise >90% of total cardiac collagen (Eghali et al. 1988; Bishop and Laurent 1995) and (with fibronectin) most of the cardiac matrix (Eghali et al. 1988; Weber and Brilla 1991). Type I collagen forms thick rods of high tensile strength, whilst the fine fibrillar network of type III collagen is more elastic. Basal cardiac collagen deposition rate is low, due to low synthesis (rates 30% lower than other proteins in rabbit LV), and high degradation rates (30% of synthesised collagen is rapidly degraded and not deposited).

Since 1976 (Sen et al. 1976), LVH has been known to be associated with interstitial collagen accumulation in animals and humans (Caspari et al. 1977; Oldershaw et al. 1980; Weber et al. 1989; Linz et al. 1992) through increased synthesis and reduced degradation. Collagen accumulation early after aortic banding is due to decreased degradation, and later (16 weeks) to increased synthesis (Eleftheriades et al. 1993). Early increased synthesis may also occur in other models (reviewed in (Bishop and Laurent 1995)). Paradoxically, increased synthesis and degradation may go hand in hand (Brilla et al. 1995). This causes a net reduction in RV collagen content early (<6 days) after bleomycin-induced rabbit lung injury but later (14 days), collagen synthesis outstrips degradation and net RV collagen content rises (Turner et al. 1986). Collagen subtype ratios may change during the hypertrophic process (Muckerjee and Sen 1993) and may influence the physical properties of the tissue (reviewed in (Bishop and Laurent 1995)). In general, there is an early increase in type III and V collagen with a later increase in type I collagen.

Such changes in matrix content and composition have been associated with diastolic dysfunction and reduced ventricular compliance, and later with impaired systolic function (Weber et al. 1988; Weber et al. 1993). Pharmacological reversal of LVH might also detrimentally alter cardiac function if rapid reduction in myocyte mass occurred leaving the excess matrix largely intact. Indeed, LV collagen concentration increases during spontaneous regression of LVH in the DOCA-salt model of hypertension (Baxter and Yellon 1992b). Furthermore, the relative contributions of collagen deposition and

myocyte hypertrophy to the ventricular hypertrophic response vary with time and aetiology of the LVH in humans (Caspari et al. 1977) and animals (Bishop and Laurent 1995).

1.73 POSSIBLE ROLE FOR RAS IN THE DEVELOPMENT OF LVH

The development of LVH in hypertension and its regression with therapy correlate poorly with SBP (Lee and Lindpainter 1993). BP reduction alone is insufficient to induce LVH regression in animal models (Sen and Tarazi 1983) and some hypotensive agents are associated with either failure of regression of LVH (e.g. hydrallazine or dihydropyridine calcium-channel blockers(Massie et al. 1989)), or with progression of LVH in humans (e.g. minoxidil and tiapamil)[reviewed in (Massie et al. 1989; Baxter and Yellon 1993)]. These findings focus attention on the genetic and transduction mechanisms regulating LV mass.

Early studies suggested that ACE-inhibitor treatment were more effective at inducing regression of LVH than other drug classes (Massie et al. 1989; Baxter and Yellon 1993), suggesting that cardiac RAS may be involved. Such a concept is supported by evidence from animal models. Cardiac ACE and angiotensinogen gene expression increase during LV hypertrophy (Baker et al. 1990; Schunkert et al. 1990; Bruckschlegel et al. 1995; Hunt et al. 1995), and ATII receptor numbers also rise (Suzuki, et al. 1993; de Gasparo et al. 1994).

Exposure of the porcine heart to ATII infusion increases physiological cardiac hypertrophy (Beinlich et al. 1991), while RAS antagonists impair such growth (Beinlich et al. 1991). Hypertrophy due to pressure-loading can be similarly reduced by ACE-inhibition (Baker et al. 1990; Linz et al. 1992). Finally, regression of established LVH is more readily accomplished with ACE-inhibition than with equihypotensive doses of other agents (Veniant et al. 1993).

Such data strongly support a role for RAS in the control of both physiological an pathophysiological growth.

1.8: THIS THESIS

The above background suggests that local tissue renin-angiotensin systems may control tissue growth and injury responses. The corpus of work presented here explores this possibility further, and also examines the possibility that cardiac tissue ACE might be the rate limiting step in the generation of local ATII in this tissue.

The investigations can be divided into 2 methodological groups:

(i) This utilising a transgenic rat model- the TGR(mREN2)27 rat. The nature of this model is the subject of the following chapter (chapter 2).

(ii) Investigations into the role of renin-angiotensin systems in human cardiac physiology and pathophysiology, based upon the use of the ACE gene I/D polymorphism.

These studies address the role of RAS in the development of cardiac growth and injury responses (both human and animal studies), and their role in the genesis of the tissue growth and injury responses which accompany hypertension (animal studies).

THE TGR(mREN2)27 RAT

2.1: TRANSGENIC TECHNOLOGY

2.11 DEFINITION OF A TRANSGENE

A transgene is a gene or gene construct introduced into a species which integrates into the native chromosomes and is transmitted through the germ line to subsequent generations. Transgenesis allows the effects of manipulation of individual gene expression to be studied *in vivo*.

2.12: THE SCIENCE OF TRANSGENESIS

The phenotypic and genotypic diversity of the animal kingdom has hampered investigation of the genetic mechanisms and origins of cardiovascular disease. Transgenic science partly overcomes such problems by allowing integration of added homologous (from the same species) or heterologous (from a different species) lengths of DNA into host chromosomes. Several methods are commonly used :

(i) DNA injection into the pronucleus of a single-celled fertilised egg.

(ii) Injection into a pluripotential embryonic stem cell with subsequent implantation into a blastocyst to yield a chimeric animal.

(iii) Infection of an embryo with a retrovirus carrying the transgene

The embryo is implanted in a surrogate or 'pseudo-pregnant' (by hormonal manipulation) mother, reared, and used for breeding to establish a transgenic line. The DNA used varies:

(i) It may be a gene e.g. the murine renin gene Ren-2^d inserted into the rat genome (Mullins et al. 1990).

(ii) It may be a gene promoter sequence inserted in order to study its behaviour, with its activity being 'reported' by a linked gene whose activity is easily measured (e.g. chloramphenicol acetyltransferase gene expression is reflected by generation of acetylchloramphenicol from chloramphenicol). The promoter sequence can be modified to allow recognition of sequences important to regulation of gene expression. The promoter sequence for renin (Mullins et al. 1988)has been so studied.

(iii) It may be a promoter/target gene construct. The use of specific promoters allows modification of quantitative and anatomical transgene expression (e.g. transthyretin promoter and hepatic expression (Steinhelper et al. 1990)). Metallothionein promoters (which respond to dietary heavy metals through sensitive response elements in the promoter sequence) allow activation of gene expression at different timepoints.

(iv) It may prevent expression of a target native gene by disrupting a portion of the host genome at its point of insertion(see review (Sharp and Mullins 1993)). Targeted gene knockout is achieved by introducing an artificial transgene generating either antisense RNA (which binds to the target native mRNA or pre-mRNA and interferes with processing or translation) or enzymes which cleave target mRNA. Outcome in one model is not predictable. The genotype of a given transgenic animal may be affected by (a) the (often) random site of integration of the transgene into the native genome (b) possible scrambling of both the transgene and the chromosomal DNA at the insertion points (c) the variable extent of integration causing differences in cell/animal gene copy number (d) Accidental insertion of unwanted vector sequences (e) Patchy gene expression even within one tissue in one animal. The phenotypic response to expression may also depend on (a) Species differences. Thus, the mouse may carry either the renin Ren-1^c alone or with the Ren-2^d gene. Transferring the Ren-2^d gene into a Ren-1^c mouse has no effect (Tronik et al. 1987; Mullins et al. 1989)but causes hypertension in the rat (Mullins et al. 1990). (b) The native strain into which the transgene is inserted, with different lines of Sprague-Dawley rat responding differently to murine renin gene insertion (chapter 5).

2.13: PRODUCTION OF TRANSGENIC ANIMALS BY MICROINJECTION

Superovulation is induced in female rats (porcine FSH for 2 days by osmotic minipump followed by intraperitoneal injection of human chorionic gonadotrophin (hCG) (Paul et al. 1994), the animals mated and the fertilised eggs removed, isolated (hyaluronidase digestion), cultured, the pronucleii microinjected with the transgene construct, and the eggs reimplanted into the oviducts of pseudopregnant females (mated with vasectomized males). The offspring are screened for transgene integration by Southern blot hybridisation of tail-snip DNA using a transgene-specific sequence as the probe, or polymerase chain reaction (PCR) amplification using transgene-specific primers. Any phenotypic change in transgenic progeny is assumed to be due to transgene expression (rather than 'knock-out effects') if the same effect occurs in at least two offspring (Jaenisch 1988). In some, the transgene will have been incorporated into the germ cells of the animal, and thus breeding with normal control animals will produce a new generation of animals which are heterozygotes for the transgene. These are screened in a similar way, and can be used to derive a transgenic breeding stock.

2.2: GENERATION OF THE TGR(mREN-2d)27 RAT

2.21: THE *Ren-2* TRANSGENE

Some mouse strains have a single renin gene (known as the Ren-1c gene), but others (e.g. the DBA/2J strain) have two such genes (Ren-1d and Ren-2d respectively (Mullins et al. 1982; Panthier and Rougeon 1983). The genes and products are 97-99% homologous (Sigmund and Gross 1991). In 2-gene strains (e.g. DBA/2J), the Ren-2d gene is expressed at a similar low level in the SMG (Field and Gross 1985; Sigmund and Gross 1991) and kidney. In the kidney, Ren-2d and Ren-1d gene transcript levels are similar, with the Ren-1d gene being principally expressed in the JGA cells,. In diverse other tissues, both genes are expressed to a lesser degree (Sigmund and Gross 1991), influenced by animal sex (especially high in male mouse SMG, and female rat and mouse liver (Ekker et al. 1989)), and physiological stimuli (e.g. altered sodium intake (Miller et al. 1989)). Transcription of the *Ren-1c* gene is highly responsive to intracellular cAMP (Bruna et al. 1993).

The solitary rat renin gene (on Chromosome 1) shares 90% homology with mouse Ren-1 (mRen-1) cDNA (Sigmund and Gross 1991). Distribution of expression is similar, although the rat lacks expression in the SMG (Ekker et al. 1989).

2.22: THE TGR(mRen-2d) LINE 27 RAT

In generating the TGR(mRen-2d)27 rat, the entire Ren-2d gene and flanking sequence (Mullins et al. 1988) was microinjected into fertilised oocyte pronucleii of a Sprague-Dawley/WKY cross. Thirty-seven eggs yielded eight progeny. Five were transgene positive (TG+), and three transmitted the transgene to the next generation. Four of the five TG+ founders had transgene-dependent hypertension (systolic BP>200 mmHg by ten weeks)(Mullins, Peters et al. 1990) and one was a normotensive mosaic, although transgene transmission to her offspring was associated with similar hypertension. Significant hypertension persisted in subsequent generations in two lines(Lee, Zhao et al. 1991). Male infertility prevented breeding from one of these lines. The line TGR(mREN2)27 was derived from the remaining breeding founder (number 27), and offspring used in the experiments which will be described in this thesis. Transgenic rats derived from this original line 27 [TGR(mREN2)27] are also referred to as *Ren2* rats.

2.3: DISTRIBUTION OF TRANSGENE EXPRESSION IN THE TGR(mREN2)27 RAT

2.31: GENERAL

Zhao quantified and compared murine and rat renin gene expression (by RNase protection assay using a *Ren*-2 rat renin specific riboprobe respectively) in the DBA/2 mouse and the transgenic rat (Zhao et al. 1993). Results are shown below (Table 2.1). Mixed sex 'adult rats' (ages and numbers used for assay not described) were used. The pattern for males of defined age alone is not known. The DBA/2 mouse Ren-2 expression tabulated is drawn from Zhao's review of the published literature (see ref.(Zhao et al. 1993) for further details).

The distribution of the *Ren*-2 gene was similar in the mouse and in the TGR(mREN-2)27 rat, except in the submandibular gland. The transgene was detected in 20 of 31 tissues and endogenous rat renin was detected "at rather low level" at all sites aside from the great vessels. The co-expression of native rat renin genes with the Ren-2 transgene allows for the stimulation of local tissue renin-angiotensin systems (see above) and co-induction of native and transgenes by physiological stimuli. Low levels of renin might also be produced in tissues in which mRNA levels were beyond the limit of detection in this study. The absence of detectable native rat renin therefore does not prove the absence of a local RAS which may be driven in the transgenic rat. Such a supposition would agree with the hypothesis (chapters 1 and 10) that tissue RAS may require both locally-synthesised components as well as some taken up from the circulation with a key regulatory factor in ATII generation being tissue ACE expression.

TABLE 2.1 (After (Bader et al. 1992; Zhao et al. 1993).)

A: Tissues in which native rat renin gene expression parallels Ren-2 expression in transgenic rat

TISSUE	RAT	TGR	MOUSE
Testis	+	+	++
Brain	+	+	+
SMG	-	-	+++++

B: Tissues in which Ren-2 expression in TGR exceeds native rat renin gene expression

TISSUE	RAT	TGR	MOUSE
Ovary	+	++	++
Small Intestine	+	++	++
Jejunum/ileum	+	+++	+
Adrenal	+	+++++	+++

C: In liver and kidney:

TISSUE	RAT	TGR	MOUSE
Kidney	++++	++	++++
Liver	+	-	+

D: Assay results uncertain in the following tissues:

TISSUE	RAT	TGR	MOUSE
Lung	?+	?+	+
Aorta	?+	+	No assay
Heart	?+	?+	?+

2.32: SPECIFIC TISSUE DISTRIBUTION

2.321 Adrenal

Mullins originally described high adrenal renin activity and mRNA(Mullins et al. 1990), of almost exclusively of murine origin. The lack of evidence of high renin stores implied active secretion. These data have been confirmed(Bader et al. 1992; Tokita et al. 1992; Tokita et al. 1994a), and the observations extended. By RNase protection assay, adrenal transgene expression is >10 times greater than in any of the other tissue (Zhao et al. 1993). Expression begins before birth, is sustained throughout adult life, and rises both before and during the established hypertensive phase. Cultured transgenic glomerulosa cells have high renin and prorenin levels, and secretion of both is increased by ACTH exposure (Tokita et al. 1992).

In situ hybridisation using a non-specific renin riboprobe and immunohistochemistry showed that enhanced renin expression was largely confined to the cortex (inner zona glomerulosa and outer zona fasciculata), where renin gene transcripts were 10-100 times as common as in controls (Bachmann et al. 1992). Tokita confirmed heavy expression in the glomerulosa, but also showed greater renin and prorenin concentrations in non-glomerulosa tissues in TGRs than in control rats (Tokita et al. 1992). Plasma prorenin levels were 12-fold higher in TGRs than in controls. Bilateral adrenalectomy (with oral NaCl maintenance) reduces circulating prorenin levels by 80%, although levels remain 2.5 fold elevated above control levels (Bachmann et al. 1992). Bilateral nephrectomy leads to elevation in plasma and adrenal renin activity in the TGR, demonstrating that most of the circulating renin in the TGR is of non-renal (and possibly largely of adrenal) origin (Tokita et al. 1992; Tokita et al. 1994a).

2.322 Kidney

Renal renin secretion is suppressed and tissue renin activity reduced by 75-80% in the TGR(mREN2)27 rat compared to controls (Mullins et al. 1990; Hackenthal et al. 1992). In adults, native renin gene (Hackenthal et al. 1992) and transgene expression(Veniant et al. 1995) are suppressed to a similar degree(RNase protection assay) (Zhao et al. 1993). Both fall with age and the development of hypertension, with a dramatic fall even at 2-4 weeks of age (Zhao et al. 1993). Co-regulation of expression of both genes (Bader et al. 1992) suggests that similar gene control systems may operate. Bachmann has confirmed these observations using heterozygote animals of both sexes (Bachmann et al. 1992). In situ hybridisation (animal weight 200-400g) showed low juxtaglomerular apparatus expression of renin, and immunohistochemistry

confirmed low afferent arteriolar ATII and renin expression at the JGA. Staining with an anti-rat renin antibody yielded similar results. Granular cells are lost and appear to have transformed into smooth muscle cells.

ATII antagonists or ACE-inhibition reverse this suppression. This may be partly through a loss of ATII negative feedback (Bader et al. 1992), and partly through a reduction in BP as in later life some animals have both near-normotensive blood pressures and near-normal renal renin expression (Bachmann et al. 1992).

2.323 Heart and blood vessels

Aortic and cardiac transgene expression is disputed (see chapter 4). ATI and ATII release from isolated perfused 12-18 week old female TGR hindlimbs is three-fold higher than that in controls, suggesting vascular transgene expression (Hilgers et al. 1992). ACE-inhibition (and not inhibition of any other Angiotensin I-converting protease) increased ATI release and reduced ATII release in both controls and in TGRs, suggesting feedback regulation of the native and ren-2 genes. This issue is discussed further in chapter 4.

2.4: THE HYPERTENSIVE PHENOTYPE

2.41: BACKGROUND

In founders, blood pressure rose from 4 weeks of age to a maximum level of 265 mmHg by 10 weeks(Mullins, Peters et al. 1990). The cause of this hypertension is still not known. The nature of the transgene and the sensitivity of the TGR to the hypotensive effects of ACE-inhibitors and ATII receptor antagonists (Mullins et al. 1990; Bader et al. 1992; Barrett and Mullins 1992; Moriguchi et al. 1994) when compared to calcium-channel blockers (Hirth-Dietrich et al. 1994) or aldosterone antagonists (Sander et al. 1992) suggests an ATII-dependent mechanism. Indeed, just 0.5mg/kg/day of the ATII receptor antagonist DuP753 reduces blood pressure by approx. 25% (i.e. 50 mmHg) (Bader et al. 1992).

2.42: ROLE OF RAISED CIRCULATING RAS ACTIVITY

High circulating RAS activity might in theory be responsible through one of three means:

(i) High circulating levels of (inactive) prorenin are taken up by vascular tissues, converted to renin, and drives a local RAS (Kim et al. 1990). High local concentrations of ATII would cause vasoconstrictive and inotropic effects.

(ii) High concentrations of circulating renin or ATI are taken up by vascular tissues, with the same effect

(iii) High circulating concentrations of ATII have a direct vasoconstrictor effect.

None of the above possibilities seem to be the case: each is discussed in turn.

2.421 Prorenin levels

Plasma (inactive) prorenin levels (renin activity subtracted from total plasma renin activity after trypsin activation) are raised <12-fold (Mullins et al. 1990; Bachmann et al. 1992) and rise in parallel with plasma ATI levels and blood pressure in the heterozygote TGR, without alteration in plasma active renin levels (Sander et al. 1992). However, bilateral adrenalectomy (with oral NaCl maintenance) reduces circulating prorenin levels by 80% with a fall in blood pressure of only 22% (Bachmann et al. 1992), suggesting that the high prorenin levels are not the sole cause of the hypertension. Bilateral nephrectomy increased adrenal and circulating plasma renin and prorenin concentration without effect (20 hours later) on blood pressure (198 mmHg vs. 203 mmHg) (Tokita et al. 1994a) and bilateral adrenalectomy (with

dexamethasone replacement therapy) had no effect on BP at 20 hours, despite a fall in total plasma renin concentration. Circulating prorenin levels do not correlate with blood pressure (Veniant et al. 1995). These data suggest that high circulating prorenin concentrations are not the cause of the hypertension. It remains possible, however, that tissue ACE levels are rate-limiting in an ATII-dependent hypertension. In this circumstance, it is still possible that circulating prorenin could drive the hypertensive phenotype and for falls in circulating prorenin not to correlate well with falls in blood pressure.

2.422 Circulating renin *levels*

Original studies suggested that plasma renin was up to 6-fold reduced in transgenic rats (Mullins, et al. 1990; Sander et al. 1992). If true, the hypertensive phenotype is only partly responsible for this suppression: although PRA in spontaneously hypertensive rats is also suppressed it is still four-fold higher than in the TGR (Sander et al. 1992), and treatment with spironolactone increases renin levels without any hypotensive action(Bader et al. 1992; Sander et al. 1992).

However, the true activity of plasma renin in the TGR (and the acceptance that it is indeed low) is disputed. Angiotensin-I generating capacity in TGR plasma is due to renin, as shown by studies using CP 71362 (a renin inhibitor) (Tokita et al. 1992), and most of the plasma renin activity is of transgene origin (Yamaguchi, et al. 1992; Veniant et al. 1995). Further, Tokita reported (in contrast to the findings of Mullins and Sander (Mullins 1990; Sander et al. 1992)) that plasma renin activity was higher in TGRs than in control animals (Tokita et al. 1994b). They ascribed this difference to an induction of renin release from the kidney by the ether anaesthetic used by Mullins (although the effect of ether would have had to have been selectively greater on controls than TGRs). In addition, despite consistent previous reports of plasma ATII levels of 32-35 fmol/ml in TGRs vs. 50-98 fmol/ml in control animals (Mullins et al. 1990; Hilgers et al. 1992; Sander et al. 1992; Moriguchi et al. 1994), Tokita alone reports ATII levels 3-4 fold higher in TGRs than controls. This suggests perhaps that there was a true difference in RAS profile between the animals used in each study. Alternatively, different anaesthetic profiles, stress, methods of plasma sample collection, sample handling or assay method may have been responsible.

2.423 Circulating renin *activity*

Tokita went on to study the kinetics of reaction of mouse Ren-2 renin with rat angiotensinogen at rat pH (the situation in the TGR) (Tokita et al. 1994b). Based on these calculations, they suggest in fact that the concentration of Ren-2 renin in the TGR

is lower than that of native rat renin in the normal (non-TGR) Sprague Dawley and tens of thousands of fold lower than in the DBA/2 mouse from which the renin is derived. However, rat angiotensinogen concentrations are much higher than those of the mouse (approximately 7-fold), and the kinetics of activity of mouse renin on this rat substrate at rat pH is 10-fold faster than with mouse angiotensinogen substrate. Thus, although TGR plasma renin concentration is probably three-fold lower (by their estimations) than that in control animals, *in vivo activity* is probably a great deal higher. Veniant supports this concept, suggesting that active plasma renin levels assayed at physiological pH are raised after 35 days in TGRs when compared to controls(Veniant, et al. 1995).

However, plasma ATI levels and ATII levels are not elevated in the TGR in any other studies (Mullins et al. 1990; Hilgers et al. 1992; Sander et al. 1992; Moriguchi, et al. 1994) despite normal (Mullins et al. 1990) or low (Veniant et al. 1995) angiotensinogen levels, suggesting that true renin activity in the rat is 'functionally' low, either through substrate limitation or truly low activity.

If Veniant and Tokita are correct (Tokita et al. 1994b; Veniant et al. 1995), even modest tissue transgene expression (or exposure to adsorbed circulating renin) could generate high local ATII production in tissues capable of abundant angiotensinogen synthesis. In vascular tissue, this would cause vasoconstriction. Indeed, circulating renin levels (in contrast to those of prorenin) correlate with systolic blood pressure (Veniant et al. 1995).

In summary, there remains great debate as to whether the TGR(mREN-2)27 rat represents a low or high-circulating renin model of hypertension. The recent work of Veniant suggests that the latter may be the case (Veniant et al. 1995).

2.43: HIGH TISSUE RAS ACTIVITY

2.431 General Comments

High circulating RAS activity does not therefore seem to be the cause of the hypertension in the TGR(mREN-2)27 rat. High local tissue RAS activity might be responsible: co-expression of native and transgenic renin genes allows a role for transgenic stimulation of local tissue renin-angiotensin systems in the development of hypertension in this model. Systolic blood pressure in mature female TGRs falls in 2 stages with 13 day of treatment with Lisinopril 20 mg/kg (by 30-40%) or losartan 10mg/kg/day. Rapid inhibition of plasma ACE might account for the initial rapid fall in BP over 2-3 days, whilst the slower fall over the next 4-5 days might be due to tissue

ACE inhibition (Moriguchi et al. 1994). The role of expression in many different tissues has been studied.

2.432 Role of brain RAS in the aetiology of the hypertensive phenotype

In the TGR, tissue-specific down-regulation of Ren-2 gene transcription occurs at 2-4 weeks of life in areas of the brain concerned with sympathetic outflow (Zhao et al. 1993). However, sympathetic outflow in TGRs is similar to that seen in genetically hypertensive Lyon rat control (Lo et al. 1993) and ganglion blockade produces a similar percentage fall in BP in both, suggesting a similar contribution of sympathetic activity to the hypertension in both. These data suggest that the hypertensive phenotype is not centrally or sympathetically-driven.

2.433 Role of renal RAS in the aetiology of the hypertensive phenotype

Renal levels of native and transgene renin mRNA are low, and fall as hypertension develops (Zhao et al. 1993) making it unlikely that the kidney is the driving force behind the hypertensive phenotype (Bachmann et al. 1992; Bader et al. 1992). Nonetheless, transgenic rats have a degree of RAS-mediated salt and water retention (Lo et al. 1993). Tubular sodium resorption in isolated kidneys is increased, and the natiuretic and diuretic response to frusemide *in vivo* is greater. Although this might be thought to account for the TGR sensitivity to the hypotensive effect of ACE inhibitors, captopril produces an *anti*-natiuretic effect in these animals not seen in controls, while hypotension induced by a calcium channel blocker has the opposite effect (Hirth-Dietrich et al. 1994).

2.434 Role of adrenal RAS in the aetiology of the hypertensive phenotype

The high levels of Ren-2 transcript found in the adrenal gland precede the development of hypertension in the TGR (Zhao et al. 1993) and might in theory generate hypertension by one of five mechanisms:

(i) Increasing aldosterone generation, producing salt and water retention. It is possible that local ATII generation could have a paracrine effect within the adrenal in stimulating aldosterone synthesis or release. Basal steroid synthesis (assessed by urinary steroid hormone and metabolite excretion) may be increased during the development of hypertension but not in older rats (i.e. 30 weeks). although (inexplicably) deoxycorticosterone plasma levels are depressed in the presence of raised

urinary levels. Plasma corticosterone levels are similar in Sprague Dawley controls and transgenic homozygotes but higher in heterozygotes than in controls(Sander, Bader et al. 1992). The adrenal tissue maximal response (assessed by urinary aldosterone and deoxycorticosterone levels) to ACTH stimulation is greater in the TGR *in vivo* (Sander et al. 1992) but strangely not *in vitro* using dispersed zona glomerulosa cells from female TGR(mREN2)27 animals (Rocco et al. 1994). In any event, basal urinary corticosteroid and mineralocorticoid/aldosterone levels are only 2-fold raised (Mullins et al. 1990; Sander et al. 1992), and treatment with the aldosterone antagonist spironolactone does not significantly lower blood pressure making hypertension due to pure mineralocorticoid excess unlikely (Sander et al. 1992). This finding has been confirmed in both young developing rats and in mature animals (Bader et al. 1992).

(ii) Direct generation of ATII, acting as a circulating vasoconstrictor. Plasma ATII is not significantly elevated in the TGR (see above)

(iii) The generation of excessive catecholamines. Plasma catecholamines are not significantly elevated in the TGR (Bachmann et al. 1992).

(iv) Circulating adrenal prorenin is taken up by tissues (e.g. vascular wall) and converted to active renin (Kim et al. 1990), driving a local RAS to produce local ATII. Plasma prorenin levels rise in parallel with plasma ATI levels and blood pressure in the heterozygote TGR (Sander et al. 1992). Male TGR adrenal prorenin release in response to ACTH is greater (maximal 10-fold rise in plasma prorenin) than in controls (four-fold rise). However, prorenin levels do not correlate individually with BP (Veniant et al. 1995), and bilateral adrenalectomy (with oral NaCl or dexamethasone maintenance), markedly reduces circulating prorenin and total renin levels with little effect on blood pressure (Bachmann et al. 1992; Tokita et al. 1994a).

(v) Generation of high circulating renin activity (see above). The possible correlation between plasma renin and BP is debated (Sander et al. 1992; Veniant et al. 1995).

2.435 Role of ovarian RAS in the aetiology of the hypertensive phenotype

Kidney native and transgene renin transcription and plasma ATII (57% reduction) fall after ovariectomy in TGR(mREN2)27 females. In SHRs, total renin levels did not change, but plasma active renin fell by 54% and prorenin rose by 134%. Blood pressure fell by approximately 10%. In TGRs, blood pressure also fell by 10%.

Plasma prorenin levels fell non-significantly, but total plasma renin fell, and plasma active renin fell by 48%. This might suggest either a) ovarian hormone-dependent regulation of gene transcription or b) a role for the ovary in conversion of prorenin to renin in TGR (Bachmann et al. 1993). Although steroid control of renin gene expression is different in the female TGR than in controls the overall involvement of this factor in the genesis of the hypertension seems negligible. Blood pressure is also higher in male heterozygote TGRs than females.

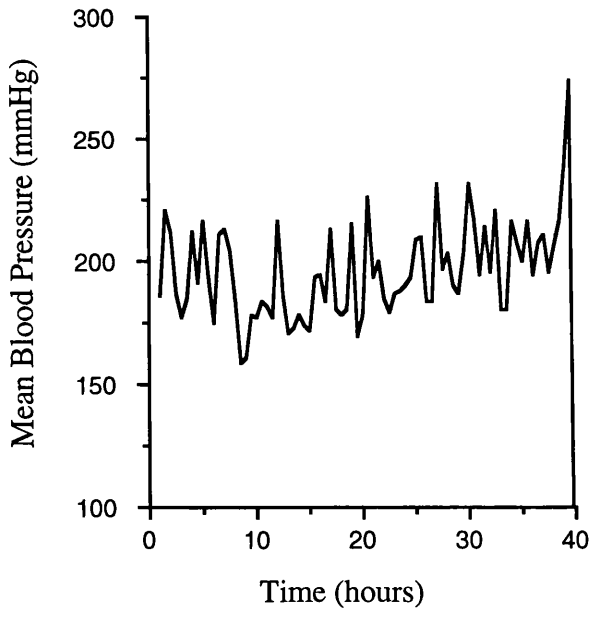
2.436 Role of vascular RAS in the aetiology of the hypertensive phenotype

Thus, high circulating RAS activity, as well as adrenal, renal, cerebral, hepatic and ovarian RAS do not seem to be driving the hypertension although this is clearly due to RAS activity somewhere. Vascular RAS activity may be responsible. Angiotensin peptide release from isolated perfused hindlimbs of adult (12-18 week old) female TGRs was significantly raised when compared to controls (Hilgers et al. 1992), and is not significantly reduced by bilateral nephrectomy. The site of transgene expression in the hind-limb was not determined, but it was suggested that expression might be vascular in order to explain the very high renin release. Basal plasma ATII was significantly depressed in this study, as has been confirmed by others (see above). The sensitivity of blood pressure to RAS antagonism (above), the high vascular ATII generation and low plasma ATII levels all suggest that vascular Ren-2 expression may be driving the hypertension, which in turn suppresses native ATII generation elsewhere in the body.

2.5: THE TGR(mREN2)27 RAT AS A MODEL OF MALIGNANT HYPERTENSION

Between 66 and 81% (95% confidence limits) of heterozygote male TGR(mREN2)27 rats derived from crossing homozygous TGR(mREN2)27 rats with the Edinburgh Sprague-Dawley strain develop malignant hypertension (MH) by 100 days. Typical features include an accelerated rise in BP, fibrinoid necrosis principally of renal afferent arterioles and interlobular arteries, significant increases in circulating renin, ATII and aldosterone together with increased immunostaining for renin in afferent arterioles (Whitworth et al. 1995a). Changes may thus be partly due to activation of the (normally suppressed) renal RAS. Native rat renin is the primary renal transcript in the Ren-2 rat (data from pH profiling: C. Whitworth, personal communication), suggesting that the transition from benign hypertension (BH) to MH involves activation of the native renal RAS rather than transgenic RAS. Gene-specific renal RNAse protection assays during BH and MH are required to clarify this issue.

The development of MH is rapid process, as may be seen from figure 2.1 (C. Whitworth: reproduced with kind permission):



Terminal accelerated phase from the HanRen2/Han-- male aged 81 days that developed MH. Mean BP (by continuous telemetry recording) is shown over the last 40 hours to illustrate the steep rise occurring in the terminal six hours. Such a rise was also observed in 5 HanRen2/Edin-- rats on telemetry where MH was allowed to progress without intervention.

Figure 2.1

Development of MH is independent of environmental factors, absolute preceding BP and its rate of rise (Whitworth et al. 1994). The fact that a cross of original Hanover stock with the Edinburgh SD colony was associated with MH, that specific SD fathers consistently yield litters of high MH prevalence, and recent studies in Edinburgh, suggest that an additional genetic factor may be involved. A monogenic mode of inheritance seems to operate (J. J. Mullins, personal communication. February 1996) .

2.6: IN SUMMARY

The TGR(mREN2)27 rat is a monogenic model of hypertension whose phenotype is probably dependent upon high vascular RAS ATII generation. Such a model therefore provides a unique opportunity for the investigation of the role of local renin-angiotensin systems in cardiovascular disease, and was chosen for further study for this reason. However, the model also has the drawback of having two independent co-existing RAS (native and transgenic), and two sites of RAS activity (circulating and tissue). The relative importance of each of these is unclear (Veniant et al. 1995).

By complementing studies of TGR(mREN2)27 rat with the utilisation of ACE I/D polymorphism genotyping, we were able to extend our investigations of this rodent model to cover human physiology and pathophysiology.

GENERAL METHODS

The studies described in this thesis were extended from an animal model through to human beings. Core methods applying to each group of experiments are described below. Methods peculiar to an individual experiment are described in the appropriate chapter describing that experiment.

3.1: HUMAN STUDIES: GENOTYPING ACCORDING TO THE ACE I/D POLYMORPHISM

3.11: DNA EXTRACTION

5ml EDTA blood samples stored at -20^0 C were used.

To lyse blood cells, one litre of sucrose lysis buffer (SLB) was made (and stored at 4^{0}C) using the following constituents in distilled water:

CONSTITUENT	QUANTITY
0.32M sucrose solution	109.54g
10mM tris pH 7.5	10ml of 1M stock solution
5mM $MgCl_2$	5ml of 1M stock solution
1% Triton-X100	10ml

Table 3.1: Constituents of sucrose lysis buffer

100ml Nuclei Lysis Buffer (NLB) was made with distilled water and the following constituents, autoclaved and stored in a sealed bottle:

CONSTITUENT	QUANTITY
10mM tris-HCl pH 8.2	1ml of 1M stock solution
400mM NaCl	2.34g
2mM Na_2EDTA	0.2ml of 1M stock solution

Table 3.2: Constituents of nuclei lysis buffer

Twenty-four samples were processed on ice at any one time. Samples were defrosted, emptied into 30ml polypropylene tubes with 20 mls cold SLB, inverted repeatedly to mix, and left for 10 mins. Samples were centrifuged (10000 rpm: 15 minutes:4^0 C), supernatant decanted, 3ml of SLB added to the nuclear pellet, and the pellet resuspended in 22ml SLB by vortex. Washing was repeated twice. 3ml of NLB was then added, the pellet vortexed and 1ml 5M sodium perchlorate added. The samples were agitated (room temperature: 15 min) before incubation in a waterbath (60^0 C: 90 min). 1ml cold (-20^0 C) chloroform was now added, and the samples then agitated for 15 minutes and centrifuged (3000rpm: 3 min). The supernatant discarded. The DNA was finally precipitated by gentle agitation with an excess of iced (-4^0 C) 100% ethanol, wrapped around a drawn glass pipette tip then deposited with the broken tip in a 3ml Eppendorf tube containing 0.5ml of TE buffer (pH 7.2) . The DNA was allowed to dissolve, and the samples stored in a refrigerator at 4^0 C.

3.12: POLYMERASE CHAIN REACTION AMPLIFICATION

The I/D polymorphism was identified by polymerase chain reaction amplification (PCR) and subsequent electrophoretic separation of fragments. Two PCR methods were used. The first-reported method of PCR amplification used two primers (Cambien et al. 1988; Rigat et al. 1992), has since been used in the majority of published studies (Rigat et al. 1990; Cambien et al. 1992; Tiret et al. 1992; Marian et al. 1993; Raynolds et al. 1993; Tiret et al. 1993). It has since become clear that this system is prone to systematic bias in that the shorter (deletion) fragment is preferentially amplified at the expense of the larger insertion (I) allele. This causes misclassification of a small proportion (5-15%) of heterozygotes as being D homozygotes (Shanmugam et al. 1993; Fogarty et al. 1994). Such misclassification may be prevented by specific alterations in the PCR conditions (such as the addition of a denaturing agent such as desmethylsulphoxide, which increases the stringency of the reaction), or by the use of an insertion allele-specific third primer as described by Evans (Evans et al. 1994).

The first human study described in this thesis (chapter 7) was performed before the potential misclassification problem had been identified, and thus utilised the original 2-primer system, as originally described (Cambien et al. 1988; Rigat et al. 1992). The latter studies utilised a 3-primer PCR system, with primers as described by Evans (Evans et al. 1994) but with a modified protocol as subsequently described herewith. Two priming oligonucleotides flank the insertion sequence in intron 16 and a third oligonucleotide is specifically within the insertion sequence. This method yields shorter allele fragments. This, together with I-allele-specific amplification, eliminates the mistyping of heterozygotes as DD homozygotes. We used primer ratios corresponding

to the 50pmol ACE1 (5' or left hand oligo) and 3 (3' or right hand oligo) and 15pmol ACE2 (insertion specific oligo) used by Evans et al in a 50µl reaction, giving amplification products of 84bp for allele ACE D and 65bp for allele ACE I. Our amplification conditions were as follows: 1 cycle 95^0C 5 min.; 40 cycles 95^0C 1 min, 50^0C 1 min., 72^0C 5 min. 20µl PCR reactions contained 50mM KCl, 10mM Tris HCl pH8.3, 1.5mM MgCl2 , 0.01mg/ml gelatin, 200µM each dNTP, 0.2 units Taq polymerase (Gibco BRL, Paisley, U.K.) and 8pmol of primers ACE1 and ACE3, outside the insertion (Alu) sequence, and 2.4pmol of primer ACE2, inside the insertion sequence. Reactions were overlaid with 20µl mineral oil. All 96 wells were always filled with reagents (mix or dummy reagents) to ensure constant thermal mass on the block. Amplification products were visualised using electrophoresis on 7.5% polyacrylamide gels. The accuracy of our genotyping was confirmed under conditions previously reported(O'Dell, Humphries et al. 1995), such that replica PCRs set up using only the primer pair ACE1 and ACE3, both at 8pmol per 20µl PCR reaction, always confirmed the presence of the D allele.

DNA fragments were separated using agarose gel electrophoresis (in the case of the 2-primer system), and electrophoresis on an 8.4% polyacrylamide gel (in the case of the 3-primer system). Fragments were identified by the incorporation of ethidium bromide into the gels, and viewing under ultraviolet light. A representative gel is shown below (figure 3.1).

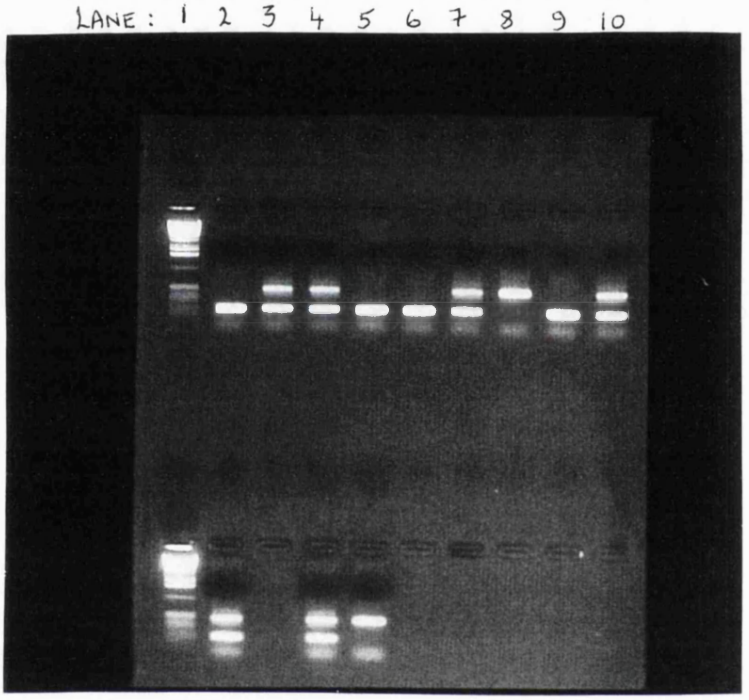


Figure 3.1: Reproduction of polaroid photograph (taken under ultraviolet light) of an ethidium bromide-stained agarose gel. Electrophoresis has separated the insertion and deletion-specific fragments of DNA from the region of the ACE gene The gel shown used DNA amplified using the 2-primer system. Note that the insertion bands are a little weaker than the deletion bands in heterozygotes (eg lanes 3 and 4), suggesting preferential amplification of the smaller deletion fragment. Lane 1 shows a kilobase ladder, lane 5 and 6 patients of DD genotype, and lane 8 a patient of II genotype.

3.2: ANIMAL STUDIES

3.21: ANIMAL STOCK

All studies were performed in accordance with The Home Office Animal (Scientific Procedures) Act 1986 (Home-Office 1986), and in accordance with the regulations of the University of London Biological Services Unit. Hanover stock TGR(mREN2)27 rats homozygote for the *Ren*-2 transgene were obtained from John Mullins at the Centre for Genome Research, West Mains Road, Edinburgh UK. Homozygote status had been confirmed by Southern Blot analysis of tail-snip DNA taken from at least three litters as described by Mullins (Mullins et al. 1990). Animals of their Sprague Dawley control strain were also supplied. Homozygote TGRs were maintained with Lisinopril 25 mg/litre added to their drinking water.

Heterozygote TGRs were derived by mating male homozygote TGR(mREN2)27 rats with control Sprague Dawley females. Pups were weaned at 24-26 days and females killed. Male heterozygotes were housed 3-4 per cage on wood shavings and maintained in a 12 hour light-dark cycle with controlled temperature (18-20^0 C), and humidity (50-60%). There was free access to food (normal sodium dry fish-meal diet) and tap water.

Three breeding pairs of both Sprague Dawley and TGR(mREN2)27 rats were used to maintained the colony.

3.22: MEASUREMENT OF BLOOD PRESSURE

Intermittent BP measurement may induce stress responses associated with an artifactual rise in BP- an effect which is influenced by the strain of animal used and the training effect of repeated handling and frequent BP measurement(Bunag 1983). Training alone is insufficient to negate this effect(Bidani, Griffin et al. 1993). For these reasons, animals were handled twice weekly when cages were cleaned out and systolic blood pressures (SBP) were recorded frequently (1-3 times/wk) in a room at half-light at 07.00hrs to 0900hrs each day (i.e. before normal working hours in order to minimise noise). A regime was devised which produced light anaesthesia (animals blinking and with good body tone, but immobile) and a stable SBP over a 2 minute period (data not shown). Animals were exposed to 4 parts per million (ppm) of halothane (Fluothane-Halothane Ph. Eur: ICI, Macclesfield, Cheshire, England; Fluovac delivery system and 1400g scrubber cartridge: International Medical Supplies, Brough, Bradwell, Sheffield, UK) in 100% oxygen in a closed perspex box for 90 seconds with transfer to a heated mat (35^0C) and exposed to halothane 1.5 ppm by nose cone. The mean of five systolic

blood pressure (SBP) measurements was recorded by tail-cuff (3/8" and 7/16") photoplethysmography (MacLab Bioamplifier ML-130, ADInstruments Pty Ltd Australia and Apple Macintosh LC475 computer, IITC Mod 29 Pulse Amplifier and tail cuff system IITC Life Science, Woodland Hills, California, USA). The tail cuff was placed perpendicular to the tail axis at the base of the tail, which was cleaned with alcohol swabs if dirty. The recording system was 2-point calibrated with an aneroid sphygmomanometer on every occasion of use. SBP was defined as the cuff pressure at which arterial pulsation was first detected (Figure 3.2). Only systolic blood pressure was documented. In a validation study using TGR(mREN2)27 rats, SBP (but not diastolic BP) recorded by plethysmography correlated well with that recorded by direct carotid cannulation, with a mean difference of 8.5mmHg (direct cannulation higher) between the two methods (C. Whitworth, personal communication).

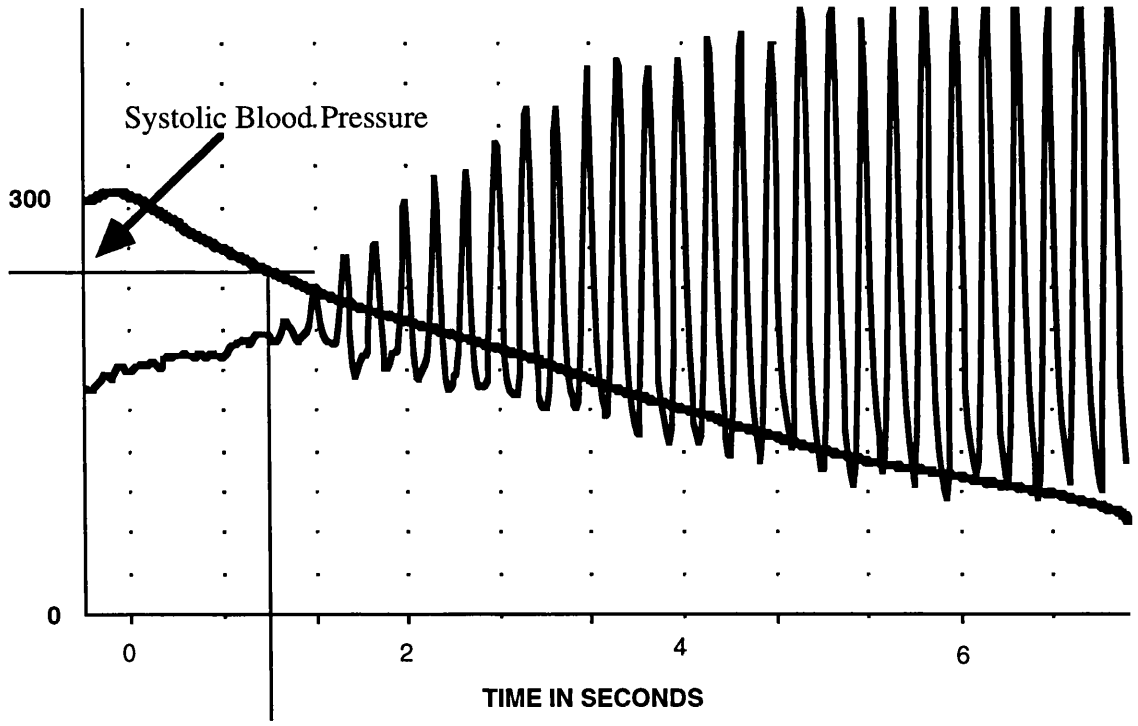


Figure 3.2: Maclab recording of photoplethysmographic trace, superimposed upon the cuff pressure trace. Cuff pressure has been allowed to fall from 300 mmHg to 40 mmHg over a ten second period. Systolic pressure is taken as that cuff pressure at which systolic photocell pulsations are recorded. By positioning a cursor at the precise origin of the first pulsation, a cursor automatically exhibits the actual blood pressure at this timepoint in a separate analogue window. Variation is clearly introduced in deciding the precise origin of the first pulsation. Each recording was repeated five times, and the mean of these systolic blood pressures recorded.

3.23: ASSAY OF PLASMA AND TISSUE ACE ACTIVITY

Animals were killed by aortic exanguination through an 18 gauge cannula inserted above the aortic bifurcation. Blood was drawn into iced lithium heparin tubes, centrifuged (2000G: 12 min: 4^0C), and the plasma stored at -70^0C. The animals were perfused with 0.9% sodium chloride solution (4^0C: 120 mmHg for control animals: 160 mmHg for transgenic animals) for 3 minutes or until the tissues became pallid. Tissues were removed, dissected free of surrounding tissue, rinsed three times in iced saline, blotted dry, weighted, and flash frozen in liquid nitrogen. They were then ground in liquid nitrogen in a mortar and pestle to a fine powder, suspended by sonication in triton X-100 (0.3%v/v in distilled water: 5ml per gram weight, or 0.5mls for all samples <0.1g), and stored at -70^0C.

The samples were thawed, centrifuged, and supernatant ACE activity kindly assayed by Dr. Peter Gohlke. 50 µl supernatant was diluted with 400ml of phosphate buffered saline (pH 8), and 50ml of carbobenzoxy-phenyl-analyl-histidyl-leucine substrate (Z-Phe-His-Leu) added. During incubation at 37°C for 60 minutes, ACE catalyses hydrolysis of this substrate to yield *o*-phthalaldehyde (*o*-phthal) and dipeptide-histidyl-leucine (His-Leu) which fluoresces at wavelength 365nm-495nm. The reaction was stopped by the addition of 1mL of sodium hydroxide to a 100ml aliquot of the incubation mixture. Now, in darkness, 25ml of orthophthaldialdehyde solution [2% solution in dimethysulfide (DMSO)] was added. After 30 minutes, 1mL 1M HCl was added, and the samples centrifuged at 3,000g. Supernatant fluorescence was measured within 60 minutes, and ACE activity calculated using the following formula, where molecular weight of the substrate (RMM) was 263.3 and protein content was calculated by Lowry's method (Lowry, et al. 1951):

$$\frac{\text{Sample absorbance}}{\text{Substrate absorbance}} \times \frac{\text{Factor for Each Tissue}}{(\text{Substrate RMM}) \times \text{time in minutes} \times (\text{mg Protein})}$$

ACE activity was expressed in nmole His/Leu/ml/min.

TISSUE TRANSGENE EXPRESSION IN THE TGR(mREN2)27 RAT

4.1: INTRODUCTION

The TGR(mREN2)27 rat might make a useful model in which to study cardiac and vascular responses to hypertension, and the role of renin-angiotensin systems in such responses. However, such studies are currently hampered by debate concerning the level of *Ren-2* transgene and native renin expression in both the heart and in vascular tissue (Ekker et al. 1989; Hilgers et al. 1992; Iwai and Inagami 1992; Zhao et al. 1993). Methodological differences, the use of ill-defined numbers of animals, and the use of animals of mixed sex and age might account for much of the current confusion. I therefore set out to re-examination transgene expression in the aorta, carotid and in specific cardiac chambers of adult male TGR(mREN2)27 heterozygotes of defined number, weight and sex.

4.2: METHODS

The RNase protection assay is a sensitive method of identifying transgene expression in animal models, and was therefore used in this study. Confirmation of low-level expression was sought using the much more sensitive technique of reverse transcription- polymerase chain reaction amplification of transgene RNA. In order to prevent contamination and consequent 'false positive' results, disposable equipment was used throughout, with all non-disposable equipment being sterilised (using hydrochloric acid where appropriate) between handling each tissue and each animal type.

4.21: RNA EXTRACTION USING THE 'RNAzol B' METHOD

4.211 Tissues

Fifteen male heterozygote TGR(mREN2)27 rats and 15 SD controls (all 250-300g) were anaesthetised with an intraperitoneal injection of 0.5ml Hypnorm (fentanyl 0.025mg per 100g/fluanisone 0.8mg per 100g:Jansson), and 0.5ml midazolam (0.42mg/100g: Hypnovel, Roche). The abdomen was opened, the abdominal aorta tied with 3/0 braided silk, and the animal perfused with iced saline for 1 minute (180mmHg for transgenics, 120mmHg for controls) through a proximally placed cannula. The left lung, both carotids, and proximal 1-2cm of aorta were rapidly removed, cleaned of surrounding tissue on ice, washed in iced saline, blotted dry, weighed, and snap frozen

in liquid nitrogen. The heart was removed, cleaned and rinsed, the right ventricle dissected free from the left ventricle and septum, and the tissues similarly treated.

4.212 RNA extraction

RNA extraction was performed at the Centre for Genome Research, Edinburgh in a laboratory previously unexposed to *Ren-2* mRNA or cDNA. RNAzol contains phenol and guanidium thiocyanate (Chomczynski and Sacchi 1987) promotes the formation of RNA-Guanidium-water complexes, abolishes the hydrophilic interactions of DNA and proteins, and thus selectively leaves RNA in aqueous solution. Yields are usually approximately 5-6μg RNA per 1mg tissue, but can be as low as 1μg per 1mg tissue.

The weights of each tissue sample varied, and therefore the number of animals used as a source for RNA varied with the tissue being studied (12 lungs, 6 right ventricles, 3 left ventricles, 15 carotids and 15 aortic samples). Like tissues were pooled and processed together, although the lung samples were processed in two batches of 6 ($lung^1$ and $lung^2$).

The samples were homogenised in 50ml Corning Tubes using an Ultra-Turrax Drive T25 350 watt homogeniser (8000-24000 revs/min: IKA Labortechnik, Germany), with the addition of approx. 2ml RNAzol per 100mg/tissue (15 aortas in 15 mls, 15 carotids in 15 mls, 12 lungs in 50 mls, 6RV in 25 mls,3LV in 25 mls) for 0.5-1 minute. Each homogeniser head was stripped and cleaned in 2M HCl (5 minutes soak) before use and between samples, before being rinsed in dH20 (RNA free). 0.1 volumes of chloroform were added and the samples covered, shaken for 15 seconds, left on ice (4^0C) for 5 minutes, and centrifuged (2100g: 4^0C:15 mins). The aqueous phase (excluding interface) was transferred to a 30ml glass Corex tube (PYREX), an equal volume of isopropanol added, the samples left on ice for 15 minutes and then centrifuged (15 mins: 4^0C: 16500g). A yellow-white pellet of RNA was identified at the bottom of the tube. After removal of the supernatant, the RNA pellet was washed with 1ml 70% ethanol added to the RNA (>0.8ml ethanol per 50-100μg RNA), and the sample vortexed and recentrifuged (16500g:4^0C:8 mins). This process was repeated. The RNA pellet was now incompletely air-dried (10-15 minutes) and redissolved by vortexing in pure formamide (Chomczynski, P; 1992). The use of pure chemicals, and the strong denaturing effect of RNAzol meant that DEPC-treated solutions were not required. Large grey pellets were derived from the ventricular samples suggesting glycogen contamination. The process of RNAzol extraction was therefore repeated on these samples.

4.22: RNA QUANTITY AND QUALITY

Final RNA concentrations are usually in the range of 4mg/ml.

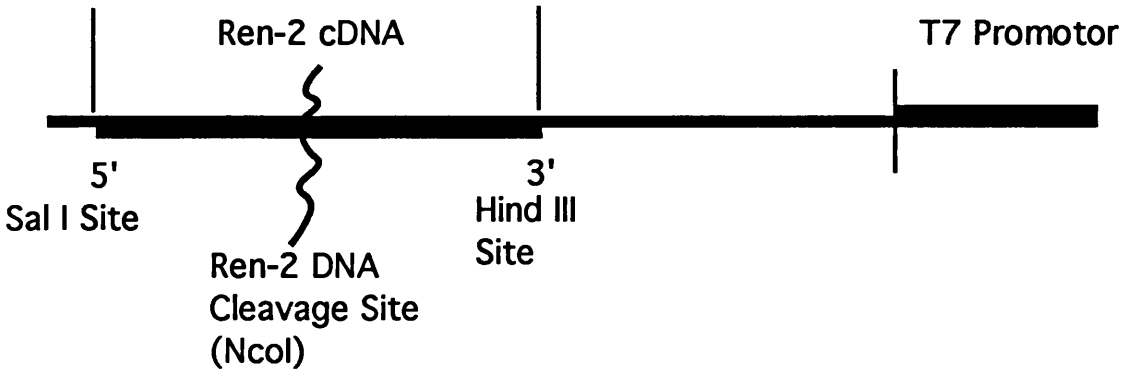
The RNA samples were diluted 1/200 for quantification by UV absorption spectrophotometry at 260nm. Absorption at 280nm was also quantified, as higher absorption at this wavelength suggests protein contamination. Concentration of RNA was calculated from the following equation: Absorbance at 260nm x dilution x 40=µg RNA /ml). RNA was shown to be intact by agarose gel electrophoresis.

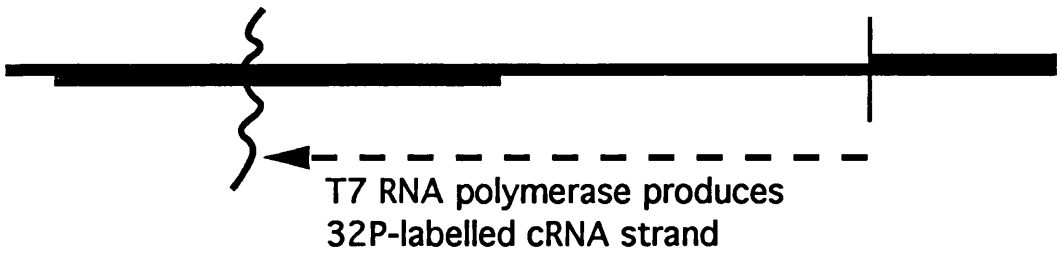
4.23: RNAse protection assay for Ren-2 mRNA

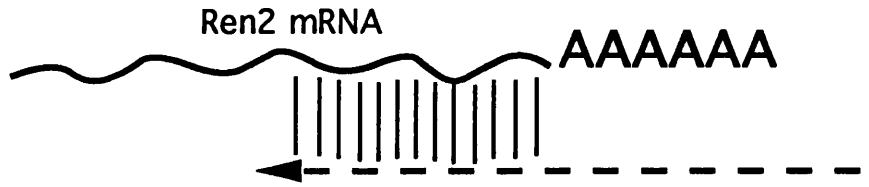
4.231 Making the riboprobe (Figure 4.1)

cDNA for the Ren-2 gene has been cloned into the Bluescript II KS plasmid vector (Strategene Ltd, Cambridge, UK). The full-length cDNA is cloned into the Sal I and Hind III sites of the vector (M. Sharp, personal communication). This plasmid was linearised using Nco I, which cuts once in the *Ren2* cDNA, and transcribed using bacteriophage T7 DNA-dependent RNA polymerase, which reads from the T7 promoter site through the Hind III site, and onwards through the Ren2 cDNA, to the NcoI cleavage point. This produces a partial complementary RNA (cRNA) 216nt in length.

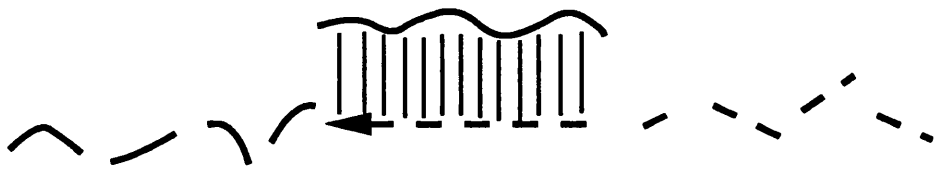
In the order listed, the following reagents were mixed: 1.0µl Ren2 cDNA (20-100ng/µl), 1.5µl DTT (100mM), 0.5µl bovine serum albumin (BSA: 2mg/ml), 1.0µl base mix (ATP/UTP/GTP: 33mM each), 1.0µl RNAGuard (Pharmacia), 1.0µl alpha-^{32}P]CTP (4000Ci/mmol), 3.0µl Stratagene transcription buffer (5x) (see appendix at end of chapter), and 1.0µl T7 RNA-polymerase. Total reagent mixture volume was therefore 15.25µl. These were then incubated at room temperature for 90 minutes. One µl of RNAse-free DNAse I (Promega) was now added, and incubation continued for 20 minutes at 37 degrees. Water was added to a total of 100µls, and extraction performed with phenol: chloroform: isoamylalcohol. Precipitation was performed with 100µls 4M NH4Ac, 200µls isopropanol followed by immediate centrifugation and resuspension in 100µls of DEPC H2O. Radioactivity incorporation was monitored by scintillation counting. The probe was diluted to a final count of 450 000cpm /1µl .







Probe RNA binds to native mRNA
(hybridise at 55 degrees C overnight)



RNAse digests single-stranded DNA, leaving
native target mRNA undigested

5

Protected RNA now separated by electrophoresis on denaturing gel (which separates RNA into single strands). Nul sample and undigested probe RNA also run in separate lanes.

Figure 4.1 RNAse protection assay for mouse Ren-2d gene mRNA (see text for details)

4.232 RNA coprecipitation

120µg RNA from each tissue was co-precipitate with 4.5 x 10^5 cpm of riboprobe in Sodium acetate and Ethanol in dry ice, centrifuged (12000g) for 20 minutes and the pellets washed in 70% EtOH (DEPC) before recentrifugation. The damp pellets were resuspended in 30µls of RNAse protection hybridisation buffer (RPH buffer: see appendix at end of chapter), heated to 85^{0}C for 15 minutes, then incubated overnight (55^{0}C) to allow hybridisation between renin mRNA and cRNA riboprobe.

4.233 RNA Digestion

Ribonucleases A and T1 were diluted in digestion buffer (see appendix at end of chapter) such that single-stranded RNA, but not double-stranded RNA is cleaved. 350µls was added to each tube, and incubated at 30^{0}C for 30 minutes. 20µl SDS (10%) and 2.5µl Proteinase K (20mg/ml) were added, and the samples incubated at 30^{0}C for 10 minutes to inactivate the ribonucleases. Hybridisation allows the *Ren2* mRNA to protect a region of 153nt of the cRNA riboprobe from being digested in these conditions.

4.234 Polyacrylamide Gel Electrophoresis

One extraction with phenol:chloroform:isoamylalcohol was performed, with subsequent precipitation with 5µl of tRNA (Promega: as a carrier to bring down the small amount of mRNA/riboprobe left) and 1ml of ethanol. The samples were left on dry ice for 20 minutes, centrifuged for 20 minutes at 12000g, and rinsed in 70% EtOH. The pellets were aspirated with a drawn-out Pasteur pipette, air dried, resuspended in 4µls of sequencing gel loading buffer (95% formamide, 20mM EDTA, 0.05% M Bromophenol Blue), denatured at 95^{0}C for 5 minutes, and electrophoreses on a 6% denaturing polyacrylamide gel containing 7.3M urea. The gel was fixed in 10% methanol/10% acetic acid for 10 minutes, dried, and exposed on a PhosphorImage plate for 2 weeks (Molecular Dynamics, Sevenoaks, Kent, UK).

4.24 REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION AMPLIFICATION DETECTION OF TRANSGENE EXPRESSION

Complementary DNA was made from 1µg of total RNA from each rat tissue using Expand-RT (Boehringer Mannheim, Lewes, Sussex), according to instructions. Synthesis of cDNA was primed with random hexanucleotides, and then a portion of

this used as a template for the amplification of renin DNA, using primers specific for exons 6 and 7 of the renin genes. The amplified product is 125bp from both the rat and mouse Ren2 genes. Amplification was performed using Expand (Boehringer Mannheim) according to manufacturers instructions. This reaction was then electrophoresed through a 1.5% agarose slab gel, capillary blotted onto nylon membrane and hybridized with the mouse Ren2 cDNA probe ($3x10^9$ cpm/μg) for 65 hours at 68^0C in 20mls of 0.25M NaH_2PO_4, pH 7.2; 7% SDS; 1mM EDTA and 250μg/ml denatured salmon testis DNA. The membrane was then washed to a final stringency of 0.2xSSC, 0.1% SDS at 68^0C, and subjected to autoradiography at -70^0C with intensifying screens for 8 days.

4.3: RESULTS

4.31: QUANTITY OF RNA EXTRACTED

The concentration of RNA extracted from each tissue was measured by ultraviolet absorption spectrophotometry as described above. Absorption at each wavelength (260nm and 280nm), together with the calculated RNA concentration of each sample, is shown in table 4.1 below:

Control	260 nm	280 nm	Ratio	mg RNA/ ml	TGR	260 nm	280 nm	Ratio	mg RNA /ml
Lung[1]	2.20	1.01	2.19	*17.56*	**Lung[1]**	2.18	0.99	2.20	*17.4*
Lung[2]	1.80	0.86	2.08	*14.32*	**Lung[2]**	1.98	0.92	2.15	*15.8*
RV	0.94	0.47	1.99	*7.54*	**RV**	1.23	0.61	2.01	*9.84*
LV	0.98	0.49	1.99	*7.86*	**LV**	1.30	0.64	2.02	*10.41*
Carotid	0.06	0.05	1.27	*0.456*	**Carotid**	0.06	0.04	1.30	*0.448*
Aorta	0.19	0.11	1.74	*1.5*	**Aorta**	0.25	0.15	1.71	*2.000*

Table 4.1: Concentration of total RNA extracted from tissue samples as determined by ultraviolet absorption spectrophotometry at 260 and 280nm wavelengths.
RV= right ventricle LV= left ventricle

4.32: DEMONSTRATION OF INTEGRITY OF RNA

The RNA was shown to be intact by agarose gel electrophoresis (figure 4.2)

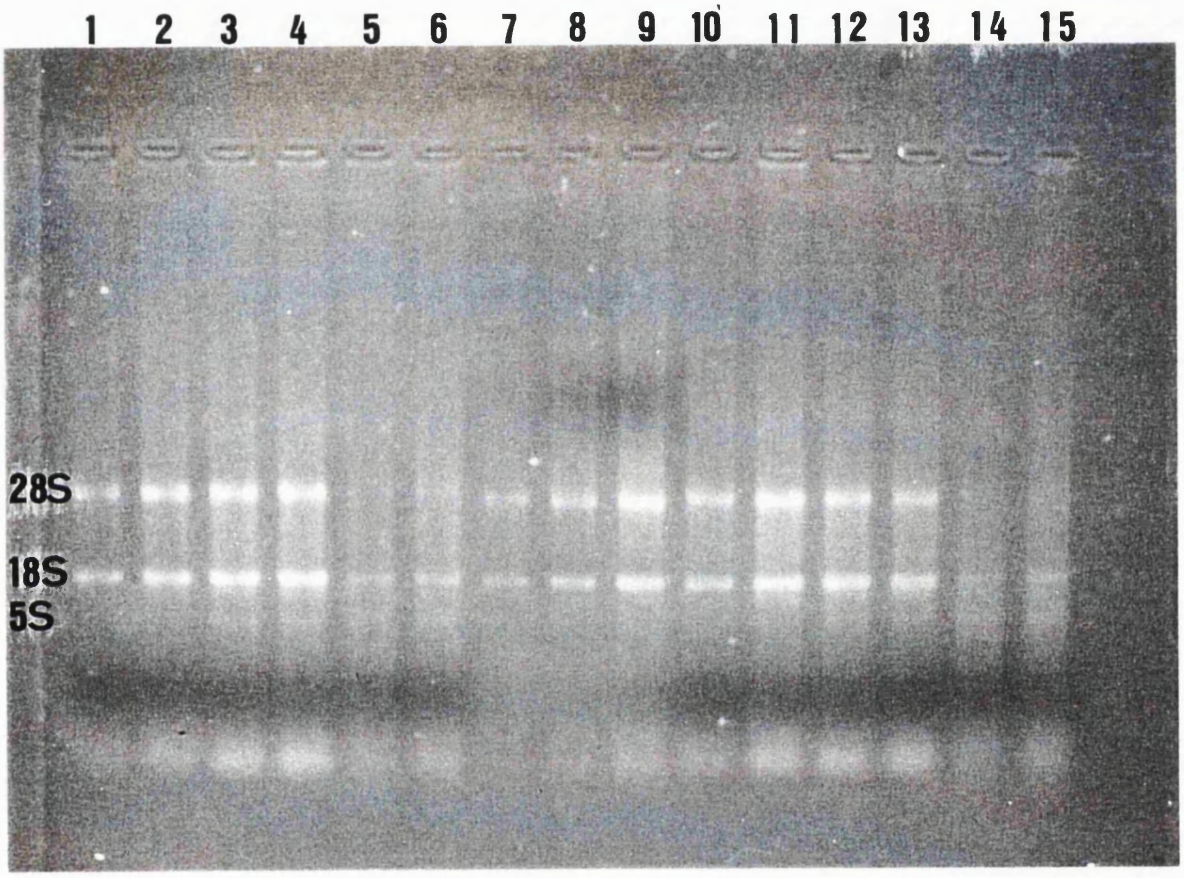


Figure 4.2 1µg per sample was loaded onto a 1% agarose gel in 0.5x TrisAcetateEDTA buffer.
The lane order is: Control Lung1, Lung2, RV, LV, Carotid, Aorta.
Track 7-9 are 0.5, 1, and 2 µg of ES cell RNA as a quality control
then transgenic Lung1, lung2, RV, LV, Carotid, Aorta.
Three ribosomal bands are visible: 28S, 18S, and 5S. The minor RNA species of intermediate size is a short-lived precursor which is processed further to produce the 5S ribosomal RNA band, seen as a faint line below the 2 larger species (big bars). This a mark of high quality RNA. The carotid and aorta look lower in quality and quantity. The reason for this is unclear.

4.33: TISSUE TRANSGENE EXPRESSION

4.331 RNase Protection Assay

The Phosphorimage derived from this experiment is shown in figures 4.3a-c.

The PhosphImages demonstrate convincing evidence of transgene expression in the lung and right ventricle of transgenic rats, with absence of expression (as expected) in the tissues of control animals. Pseudo-colour images (figure 4.3c) suggest possible expression in the aorta, although this is by no means certain.

The renin signal was only present in TGR samples, and not in the tissues of control animals. By inference, therefore, this renin signal is of transgene (mouse) origin, and not of native (rat) origin. The primers used, as mentioned, do not prevent amplification of endogenous rat renin, and yield products of the same length. Proof of renin origin would require cloning of the PCR bands and sequencing.

Figures 4.3a-c. Phosphorimage of gel separating fragments resulting from RNAse protection assay as described in the text. Each track contains 120µg of total RNA, except 'mouse' which contains 20µg of xsubmaxillary gland RNA from C57/B16 x CBA F_1 males (single renin gene mice which carry the Ren 1^c gene). The markers have burned out, but are M13 DNA sequenced with ddTTP terminator only (i.e. a 'T' track). The image of the markers can be seen more clearly by adjusting the range of the greyscale used by the computer, and by the use of pseudocolour computer enhancement (4.3c). These show that the full-length riboprobe and mRen2 bands are of the expected sizes.

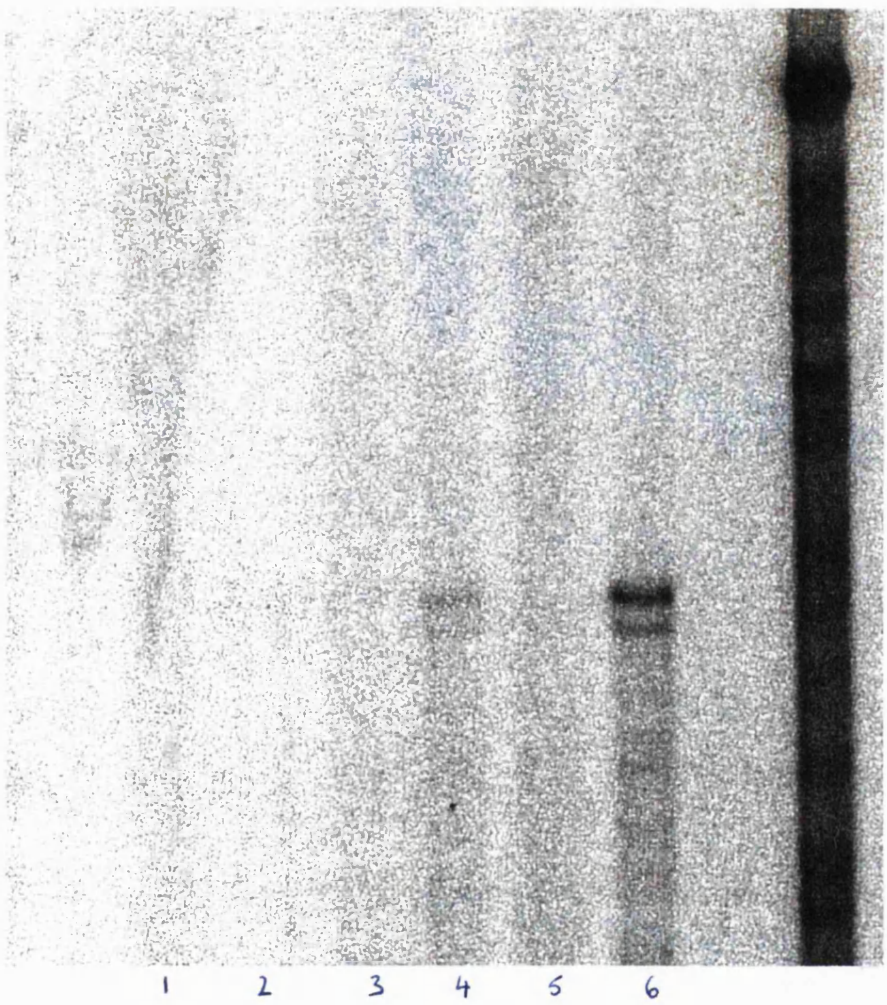


Figure 4.3a (see legend above).

Lane 1	LV
Lane 2	TGR LV
Lane 3	RV
Lane 4	TGR RV
Lane 5	tRNA
Lane 6	mouse
Lane 7	probe

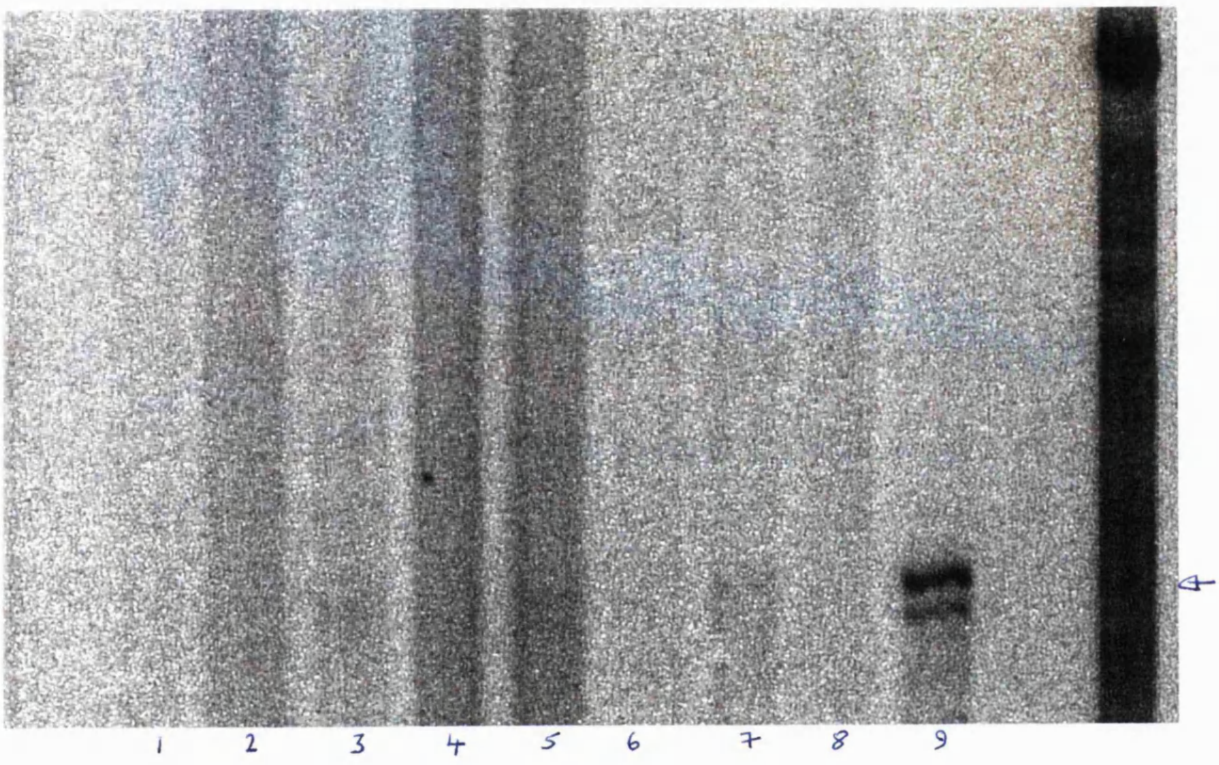


Figure 4.3b (see legend above)

Lane 1	tRNA
Lane 2	Carotid
Lane 3	TGR Carotid
Lane 4	Aorta
Lane 5	TGR Aorta
Lane 6	Lung
Lane 7	TGR Lung
Lane 8	tRNA
Lane 9	mouse
Lane 10	probe

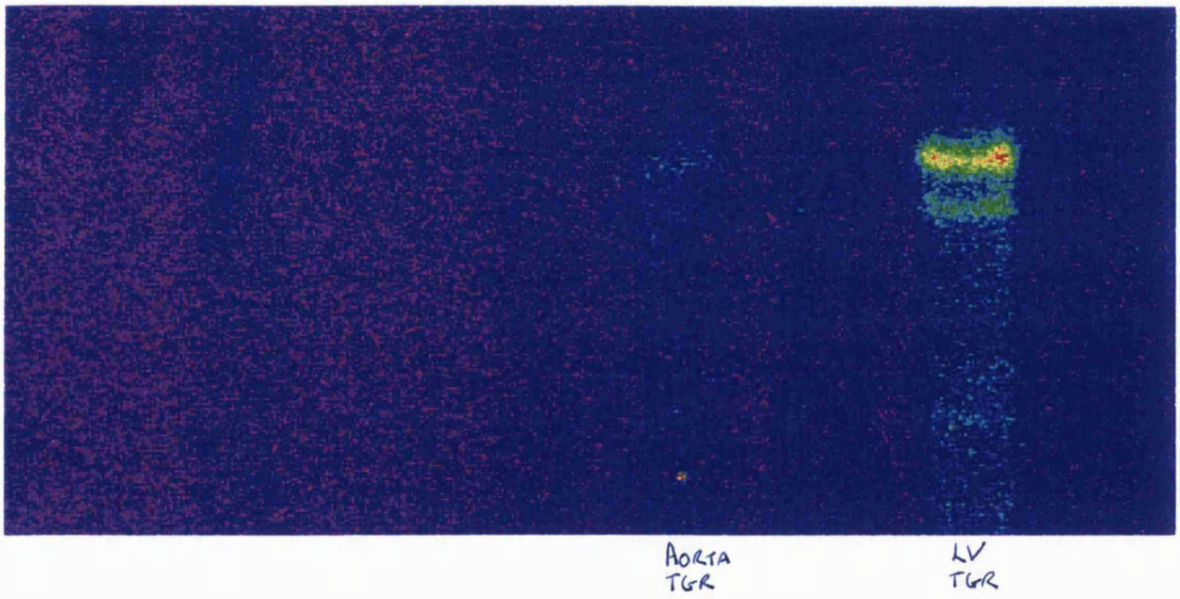


Figure 4.3c (pseudocolour image: see legend above)

4.332: RT-PCR

In view of the lack of evidence of transgene expression in the LV, and of possible expression in the carotid on RNase protection, reverse transcription PCR was performed using the RNA samples extracted above. The resulting autoradiograph is presented (Figure 4.4). Expression of the renin gene is confirmed in the line 27 right ventricle and lung, as was shown by RNAse protection assay. The possible gene expression in the aorta suggested by RNAse protection is confirmed by RT-PCR. In addition, a weak band suggests expression in the carotid. Difficulty in demonstrating expression in the carotid might be partly due to the poor quality of derived RNA from this tissue (see above).

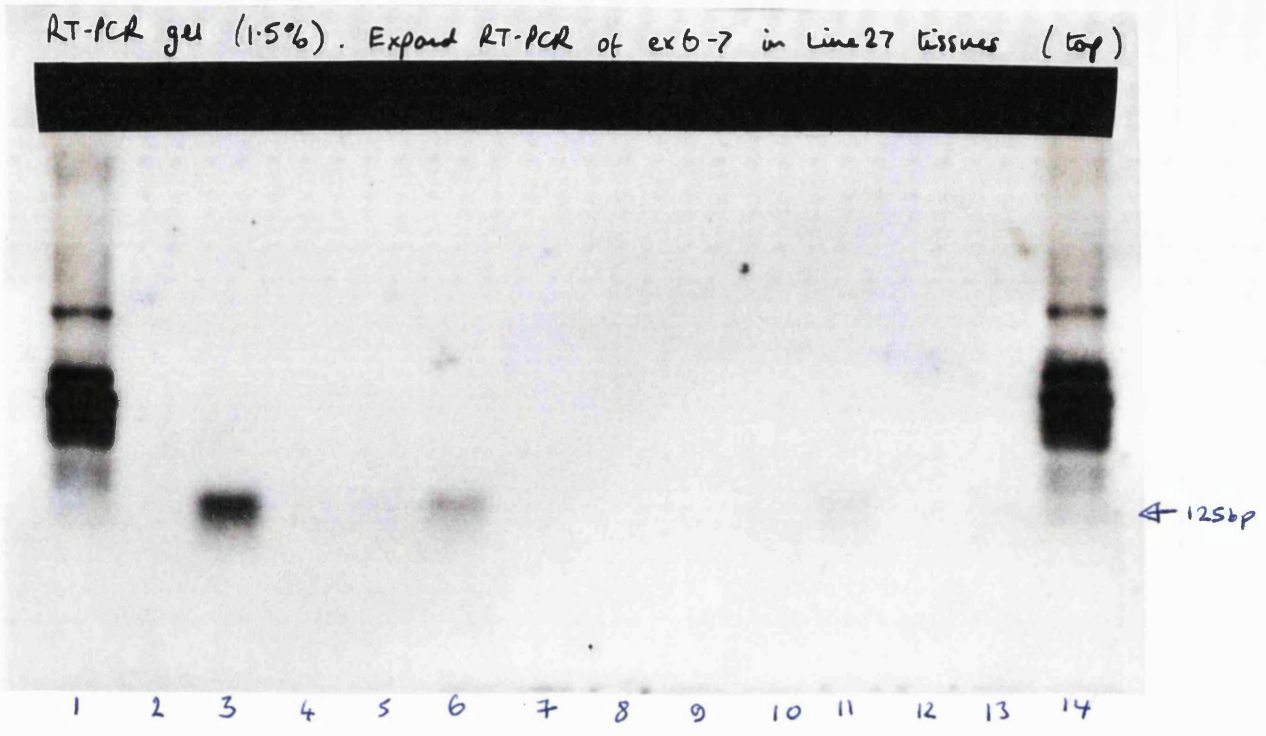


Figure 4.4 Autoradiograph of RT-PCR analysis of renin gene expression. Renin DNA was amplified from 1µg of reverse-transcribed RNA from the indicated tissues, using the following primers:
Forward:5'-CCTGGCAGATCACAATGAAGG-3' (exon 6)
Reverse. 5'-GCATGATCAACTACAGGGAGC-3' (exon 7)
Cycling conditions were 94^{0}C for 2 mins (1 cycle); 94^{0}C for 10 seconds, 58^{0}C for 30 seconds, 68^{0}C for 45 seconds (10 cycles); 68^{0}C for 7 minutes (1 cycle). The products of these reactions were subjected to gel electrophoresis, transfer and filter hybridisation as described above. The size of the hybridising band is 125bp, which does not distinguish between rat and mouse renin mRNAs. Positive bands are shown in bold below:

Lanes 1 and 14:	Plasmid size markers
Lane 2:	Control rat aorta
Lane 3:	**Line 27 aorta**
Lane 4:	Control rat carotid
Lane 5:	**Line 27 carotid**
Lane 6:	**Line 27 right ventricle**
Lane 7:	Control rat right ventricle
Lane 8:	Control rat left ventricle
Lane 9:	Line 27 left ventricle
Lane 10:	Control rat lung
Lane 11:	**Line 27 lung**
Lane 12:	Control rat right ventricle (minus reverse transcriptase)
Lane 13:	Line 27 right ventricle (minus reverse transcriptase)

4.4: DISCUSSION

Three methods are commonly used to identify transgene expression in animal models. Northern blotting is relatively insensitive to the potentially low level expression expected in these tissues, and it was for this reason that other techniques were utilised. RNase protection assay is more sensitive. Reverse-transcription polymerase chain reaction amplification (RT-PCR) is extremely sensitive to very low level expression. However, due to such very great sensitivity, the significance of a positive result in the absence of demonstration of expression by RNase protection might be debated.

Using RNase protection assay, ren2 transgene expression in the right ventricle and lung of the TGR(mREN2)27 rat is confirmed, with supportive positive results from RT-PCR analysis. Weaker aortic transgene expression suggested by RNase protection is confirmed by RT-PCR. In addition, a weak signal band on RT-PCR suggests transgene expression in the carotid.

These data go some way to clarifying the results of others. Zhao used RNase protection assay to suggest possible transgene expression at the lowest limits of detection in the heart (Zhao et al. 1993). However, the result is annotated as 'controversial', the cardiac chambers were not distinguished and mixed sex animals of unspecified age ranges were used. We can confirm transgene expression in the right ventricle, but interestingly are unable to confirm expression in the left ventricle. If this pattern of expression were to be confirmed, then it might account for the possible negative results of Zhao, when extracts of whole heart were studied. Under such circumstances, the greater bulk (and hence RNA content) of the left ventricle might have diluted the low level expression of RNA from the right ventricle.

We also confirm the low level expression of the Ren-2 renin transgene found by Zhao in the aorta (Zhao et al. 1993). Northern blot analysis and RNase protection assays have both suggested low level expression of renin in the heart and aorta in TGR(mREN2)27 and control animals (Dzau et al. 1987; Miller et al. 1989). Hilgers, using RNase protection and Northern blotting, was only able to demonstrate transgene (and not endogenous renin gene) expression in the TGR aorta (Hilgers et al. 1992). Strangely, highly sensitive PCR amplification has failed to confirm these positive results (Ekker et al. 1989; Iwai and Inagami 1992). Methodological differences and a sex-specific effect (female rats have less severe hypertension that male TGRs, and live longer (Hilgers et al. 1992)) may account for such differences in results.

This transgene expression may also occur in other great vessels. Until now, no other group has examined transgene expression in these vessels (such as the carotid). RT-PCR suggests possible low-level transgene expression in the carotid in our study. Difficulty in demonstrating expression in the carotid might be partly due to the poor quality of derived RNA from this tissue: the 260/280 absorption ratio is expected to be higher than 1.8 for good quality RNA, and this is evidently not the case for our carotid samples. It is unclear why this is so. However, it was the case that the carotid samples were the last to be removed from the animals, and took considerable time to strip of surrounding tissue before freezing. RNA might have degraded during this time.

The seemingly low expression in the carotid might be due to a low yield of PCR product secondary to poor RNA quality, or the presence of trace contaminating factors which might reduce the efficiency of the reverse transcription or PCR steps. This is suggested by the low 260/280nm absorption ratio of ther RNA discussed earlier.

The RT-PCR assay is in no way quantitative. However, the RNAse protection assay is quantitative, and demonstrates that carotid m*Ren-2* expression is at least lower than that seen in the aorta. Whether this low level expression is sufficient to affect aortic morphology or physiology is unknown, as is the ultrastructural site of expression: although global expression might be low, expression in one cell type might actually be high.

4.5: IN CONCLUSION

The TGR(mREN2)27 rat expressed the Ren-2 transgene in the right ventricle, aorta and lung. Expression is also shown in the carotid artery, where expression is probably lower. However, the seemingly low expression might be due to a low yield of PCR product secondary to poor RNA quality, or the presence of trace contaminating factors which might reduce the efficiency of the reverse transcription or PCR steps. This is suggested by the low 260/280nm absorption ratio of ther RNA discussed earlier.

4.6: APPENDIX TO CHAPTER 4 SOLUTIONS USED IN RNase PROTECTION ASSAY

RIBOPROBE TRANSCRIPTION BUFFER (x10)

Tris-HCl pH 7.5	400mM
MgCl	60mM
Spermidine-HCl	20mM

Store at room Temperature

RNAse PROTECTION HYBRIDISATION (RPH) BUFFER (RNAseA/RNAse T)

Formamide (deionised with 3x BioRad AG 501 x8 resin)	8mls
PIPES pH 6.4 (1M)	400µls
NaCl (5M, DEPC)	800µls
EDTA (0.5M, DEPC)	20µls
H20	780µls

DIGESTION BUFFER

Tris-HCL pH 7.5 (1M)	60µls
EDTA pH 8.0 (0.5M)	60µls
NaCl (5M)	360µls
H20	5.52mls
RNAse T1 (1700-2000 u/µl)	8.0µl
RNAse A (10 mg/ml)	80µl

THE ROLE OF RENIN-ANGIOTENSIN SYSTEMS IN THE DEVELOPMENT OF MALIGNANT HYPERTENSION IN THE TGR(mREN2)27 RAT

5.1 INTRODUCTION

5.11: BENIGN AND MALIGNANT HYPERTENSION

5.111: Pathophysiology

Systemic arterial hypertension is associated with an increased risk of renal, neurological and cardiovascular disease (Veterans Administration Cooperative Study Group on Antihypertensive Agents 1967; Veterans Administration Cooperative Study Group on Antihypertensive Agents 1970) and may be complicated by a severe and rapidly accelerating phase. Such 'malignant hypertension' (MH) (Houston 1989) is associated with renal disease in one half of cases (Gudbrandsson et al. 1979), and 1-2% of the essential hypertensive population are affected (Houston 1989). Untreated MH was almost universally fatal (Keith, et al. 1928; Kincaid-Smith et al. 1958). Even a decade ago, 25% of sufferers had died within 5 years of diagnosis (Yu et al. 1986). Its clinical and histopathological features are well defined (Kincaid-Smith, et al. 1958; Houston 1989). Hypertension accelerates rapidly (Laragh et al. 1972) due to a sudden severe rise in peripheral vascular resistance (Giese 1976). A diffuse arteriolar vasculopathy occurs, usually accompanied by rapidly declining renal function (Laragh et al. 1972). Neurological symptoms (e.g. headache, ataxia, nausea, vomiting, reduced level of consciousness, seizure activity), renal dysfunction (polyuria with weight loss preceding classical renal failure) and cardiac dysfunction (due to diastolic failure, LVH, and microvascular disease causing breathlessness and chest pain) are common (Keith et al. 1928). Retinal changes are routinely seen, with grades IV (with papilloedema) and III retinopathy (with exudates and haemorrhages) (Keith et al. 1928; Kincaid-Smith et al. 1958) defining 'malignant hypertension' (Ahmed et al. 1986).

Renal glomerulosclerosis, and glomerular tuft collapse and thrombosis occur (Klemperer and Otani 1931) as are cortical renal haemorrhages and intimal hyperplasia ('onion skinning') due to smooth muscle cell hypertrophy and layering causing luminal encroachment in small-vessels (Keith et al. 1928; Kincaid-Smith et al. 1958). Alternate dilations and constrictions occur in single vessels (Giese 1976). Areas of 'fibrinoid necrosis' (patchy smooth muscle cell necrosis with fibrin deposition and possible thrombosis) are identified (Kincaid-Smith et al. 1958). The renal vasculature (especially the afferent glomerular and interlobular arteries) are especially affected although similar vascular changes are identified in other organs such as the heart and brain. Secondary ischaemia (Kincaid-Smith et al. 1958; Sanerkin 1971) leads to target organ damage (Laragh 1972).

5.12 THE TGR(mREN2)27 RAT AS A MODEL OF HYPERTENSION

The TGR(mREN2)27 rat has been shown to suffer severe spontaneous hypertension (chapters 2 and 5), and as such is a useful genetic model of hypertension. However, it has also become clear that animals from some colonies may, in addition, develop malignant hypertension (chapters 2 and 5). Our colony was derived from that kept in Edinburgh, and as such used Edinburgh Sprague Dawley rats as normotensive control strains to breed with the transgenic rats, thus deriving heterozygote offspring. As such, our colony suffered a similar incidence of malignant hypertension amongst heterozygote male offspring (see below). Use of these animals thus allowed us to investigate the role of RAS in the genesis of both the hypertensive state itself, and the transition to the malignant hypertensive state.

5.13 RAS INVOLVEMENT IN 'BENIGN' HYPERTENSIVE STATES

Tissue renin-angiotensin systems may have an important role in the pathogenesis of a variety of hypertensive states in animal models. This is true of both secondary hypertension (e.g. renovascular hypertension) (Miyazaki et al. 1986; Moffett et al. 1986; Mai et al. 1995), as well as spontaneous or genetic hypertension (e.g. the SHRSP rat (Ikemoto et al. 1986a) or SHR (Unger et al. 1985; Ikemoto et al. 1986a; Ikemoto et al. 1986b). Tissue RAS activity might also play a part in the pathogenesis of some of the histopathological changes associated with hypertension (Masson et al. 1964; Gavras et al. 1971; Johnson et al. 1992).

Similarly, amongst humans, secondary (renovascular) hypertension is well known to be associated with an increase in renal renin release, and RAS activity my again play a role in the genesis of hypertensive states in some cases of primary or 'essential' hypertension (Laragh et al. 1972; Laragh 1973; Laragh et al. 1982; Laragh 1992). As in animals, many of the histopathological sequelae of sustained hypertension in humans may be mediated through RAS activity (Brunner et al. 1972; Alderman et al. 1991; Laragh 1992).

5.14: TISSUE RAS AND MALIGNANT HYPERTENSION

Local (especially renal) (Kim et al. 1992) or circulating RAS activity may play an important role in the pathogenesis of the malignant hypertensive phenotype in both humans (Helmer 1964; Hollenberg et al. 1969; Brunner et al. 1972; McLaren and MacDonald 1983; Alderman et al. 1991; Laragh 1992; MacGregor 1992) and in animal models (Matsunaga et al. 1975; Shibota et al. 1979). An initial pressure diuresis/natiuresis may stimulate renal renin production (Gardiner and Lindop 1992; Orth and Ritz 1975), which may be directly pathogenic (Volpe et al 1990) or may cause ATII-induced vasoconstriction and a further rise in BP (Saragoca et al. 1983; Houston 1989), leading indirectly to histopathological damage.

5.2: AIMS

5.21: STUDY A

To date, the hypertensive phenotype of the TGR(mREN2)27 rat has not been characterised over time. Such characterisation is important if the effects of hypotensive agents are to be studied in animals of different age. A descriptive temporal study of the blood pressures of such animals is thus required. Furthermore, studies of their response to hypotensive agents is required for two reasons:

(i) The cause of the hypertensive phenotype in the TGR(mREN2)27 rat is unclear (chapter 2). Demonstration of sensitivity to ACE-inhibition, and lack of sensitivity to direct vasodilator agents, would support a role for tissue ATII generation in the genesis of this hypertension.

(ii) The relative roles of tissue renin-angiotensin systems themselves and of the direct effects of hypertension in the aetiology of the cardiovascular changes associated with hypertension are unclear. Further studies were planned (chapter 6) to dissociate the effects of RAS activity and systolic pressure burden on LV growth, and these would require a knowledge of the dose-response curves of TGR blood pressure to hypotensive agents. Dose response curves for such agents have not been established and their effects with age remain unknown.

We addressed these issues using the calcium channel blocker Amlodipine (Pfizer UK, Sandwich, Kent), the vasodilator hydrallazine, and the ACE-inhibitor Ramipril (Hoechst-Roussel, Frankfurt, Germany). For use in future experiments, we also sought to identify a maximal non-hypotensive dose of the ACE-inhibitor Ramipril, and a dose of both agents which would reduce blood pressure to that of control (non-TGR) rats.

5.22: STUDY B

This study was designed to investigate the role of tissue RAS in the development of malignant hypertension in the TGR(mREN2)27 rat model. In order to differentiate the effects of high blood pressure from the effects of renal RAS activation in MH, we examined the effects of non-hypotensive doses of Ramipril (5mg/kg/day) on plasma and tissue ACE activity, mortality from MH, and resulting renal pathology in a rat model of MH. Results suggest that tissue ACE inhibition offers dramatic protection against the development of MH.

STUDY A

5.3: METHODS

5.31: STUDY DESIGN

Male heterozygote TGR(mREN2)27 rats (TGRs) were weaned by day 26, drugs administered, where appropriate, in drinking water from day 28, and animals followed for 70 days. Animals and water bottles were weighed three times per week, and drug administration per cage adjusted accordingly. At these times, blood pressure was also recorded using tail-cuff photoplethysmography as previously described (chapter 3). At the end of the study period, animals were anaesthetised (intraperitoneal injection of fentanyl 0.025mg/100g and fluanisone 0.8mg/100g [Hypnorm, Jansson, USA], 0.5ml: and midazolam 0.42mg/100g [Hypnovel, Roche, Switzerland], 0.5ml), and killed by aortic exanguination. In addition, and on the basis of the blood pressure data accruing, some animals were selected for studies of LV hypertrophy. After exanguination, these animals were perfused with a fixative solution, and cardiac tissue harvested (see chapter 6 for details).

On the advice of UCL statistical department, mean SBPs were compared between treatment groups at each timepoint by t-test. Mean group SBP over the entire 70 days was also compared between groups. $p<0.05$ was accepted as suggesting statistical significance.

Four studies were undertaken:

5.311 Study 1: Characterisation of the hypertensive phenotype of the TGR(mREN2)27 rat over time

The systolic blood pressures (SBP) of 12 control Sprague Dawley rats were compared with those of 14 heterozygote male TGRs which survived to 70 days (from an original cohort of 25 weaners).

5.312 Study 2: Response to the ACE inhibitor Ramipril

The effects of four doses of Ramipril were studied:

10mg/kg/day (n=13 survivors of 13 weaners)
5mg/kg/day (n=10 survivors of 10 weaners)
2mg/kg/day (n=10 survivors of 10 weaners)
1mg/kg/day (n=14 survivors of 14 weaners)

5.313 Study 3: Establishing a non-hypotensive dose of Ramipril

Ramipril at a dose of 10μg/kg/day has been shown to be non-hypotensive in normotensive Sprague Dawley rats (J. McEwan, personal communication), and therefore this dose was initially studied (n=20).

5.314 Study 4: Response to Vasodilator Agents

Amlodipine (Pfizer Central Research, Sandwich, Kent, UK.) is a calcium channel blocking agent. Of this class of drug, it was chosen due to its long biological half-life, solubility (up to 5mg/ml in tap water), stability in tap water, lack of photosensitivity and its neutral flavour with proven acceptability to rats. It had also been shown to be highly effective in reducing systolic blood pressure in spontaneously hypertensive rats: a dose of 10mg/kg/day reduced SBP from 190mmHg to control levels (130mmHg) (Dr. Howard Why, Dept. of Cardiology, Kings College Hospital: personal communication). An initial dose of 10mg/kg/day was therefore used (n= 18 of 18 weaners).

5.4: RESULTS

5.41 CHARACTERISATION OF THE HYPERTENSIVE PHENOTYPE OF THE TGR(mREN2)27 RAT

The blood pressures of the 12 control and 14 heterozygote male transgenic animals are shown in figure 5.1. Overall, SBP was similar at 30 days (144±6 and 149±9 mmHg for control and transgenic animals respectively). Thereafter blood pressure rose in all animals, but very steeply amongst the transgenic animals, reaching a maximum of 256 ± 11 mmHg on day 69. At all timepoints after day 30, transgenic blood pressure was significantly higher in the untreated TGRs than in the control Sprague Dawley, although on days 44 and 46, blood pressures were again similar (p= 0.53 and 0.98 respectively). This was due to a short-lived rise in blood pressures in the control animals. No explanation can be offered for this finding, although the abrupt and transient rise suggests a possible environmental factor leading to arousal . After 60 days amongst transgenic animals (and 50 days in control animals), blood pressure did not change significantly over time ($p > 0.3$ by ANOVA).

5.42: RESPONSE TO THE ACE INHIBITOR RAMIPRIL

The systolic blood pressure of heterozygote male TGRs proved highly sensitive to the hypotensive effects of Ramipril (figure 5.2). A significant effect was seen at all doses and at all time-points, except at the lowest dose of 1mg/kg/day at 44 days, where BP was not significantly different between the untreated and Ramipril 1mg/kg/day groups. Throughout, the lowest dose of Ramipril has the least effect. However, a clear dose-response relationship was only evident in mid-life (38-55 days: p for heterogeneity <0.05 at all points).

Comparison with the data for control animals (derived from figure 5.1) suggested that Ramipril 1mg/kg day produced a blood pressure profile similar to that in control SD animals (figure 5.3). Although significant differences in blood pressure early in treatment (121±11mmHg vs. 144±6 mmHg at day 30, p<0.04: and 158±8mmHg vs. 134±7mmHg at day 36, p=0.03: for treated TGRs and control Sprague Dawley animals respectively), and with the sudden unexpected rise in SBP amongst controls on day 46 (as discussed above: p<0.05), later in life mean SBPs were similar.

5.43 ESTABLISHING A NON HYPOTENSIVE DOSE OF RAMIPRIL

Ramipril 10μg/kg/day was found to be associated with a significant hypotensive effect (p for heterogeneity <0.001 for whole period: figure 5.4, table 5.1).

On this basis, we proceeded to investigate the effect of an even lower dose (5μg/kg/day: n=12), using the same conditions and treatment protocols as outlined above. The effects of such treatment are shown in figure 5.5. Mean blood pressure over this period was not significantly different in the treated and untreated groups (225±7 vs. 212±3 mmHg: p=0.091), being lower on day 36 alone (173±7 vs. 154±5 p=0.031) and non-significantly lower on day 41 (197±10 vs. 177±5 p=0.056).

Age (days)	Mean±SEM SBP for Untreated TGRs (mmHg)	Mean±SEM SBP Ramipril 10μg/kg/day (mmHg)	Significantly different BP? ($p<0.05$)
36	173±7	137±4	*
41	197±10	174±5	*
44	190±14	182±9	
46	210±16	176±8	*
50	212±5	190±8	*
55	236±13	217±6	
58	251±13	235±8	
62	233±7	223±8	
69	256±11	211±6	*
71	250±10	232±10	*

Table 5.1 Effect of treatment with Ramipril 10μg/kg/day on the mean systolic blood pressure of heterozygote male TGR(mREN2)27 rats.

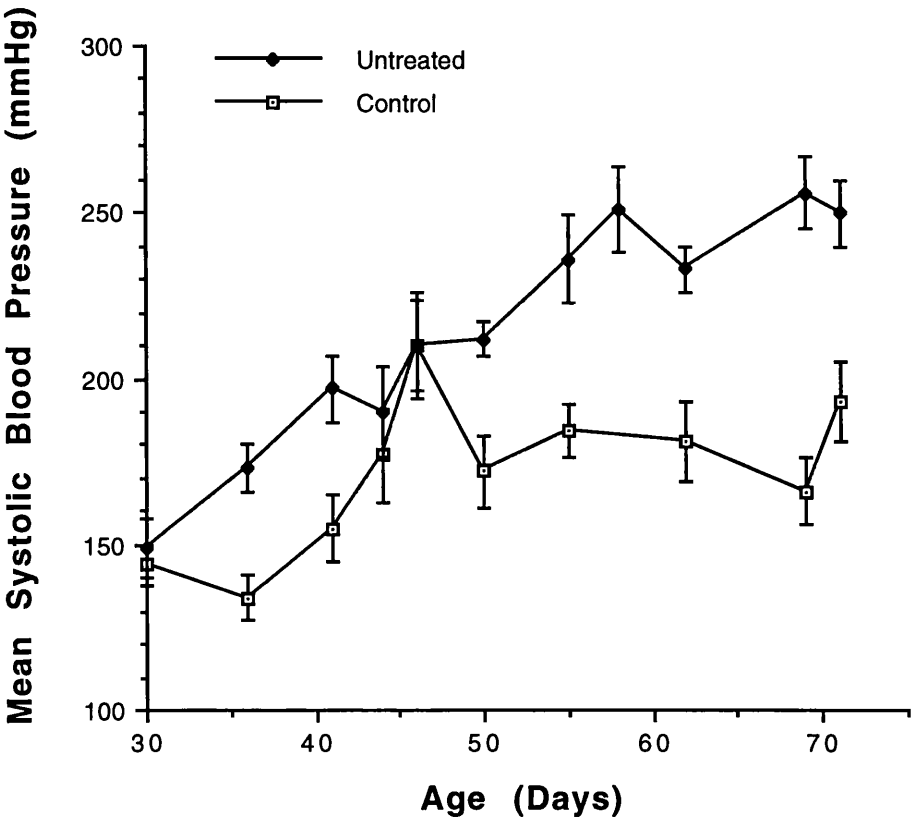


Figure 5.1: Blood mean (±SEM) blood pressures of 12 control Sprague Dawley rats (controls) and 14 untreated heterozygote male TGR(mRen-2d) 27 rats (untreated) from 30-72 days of age.

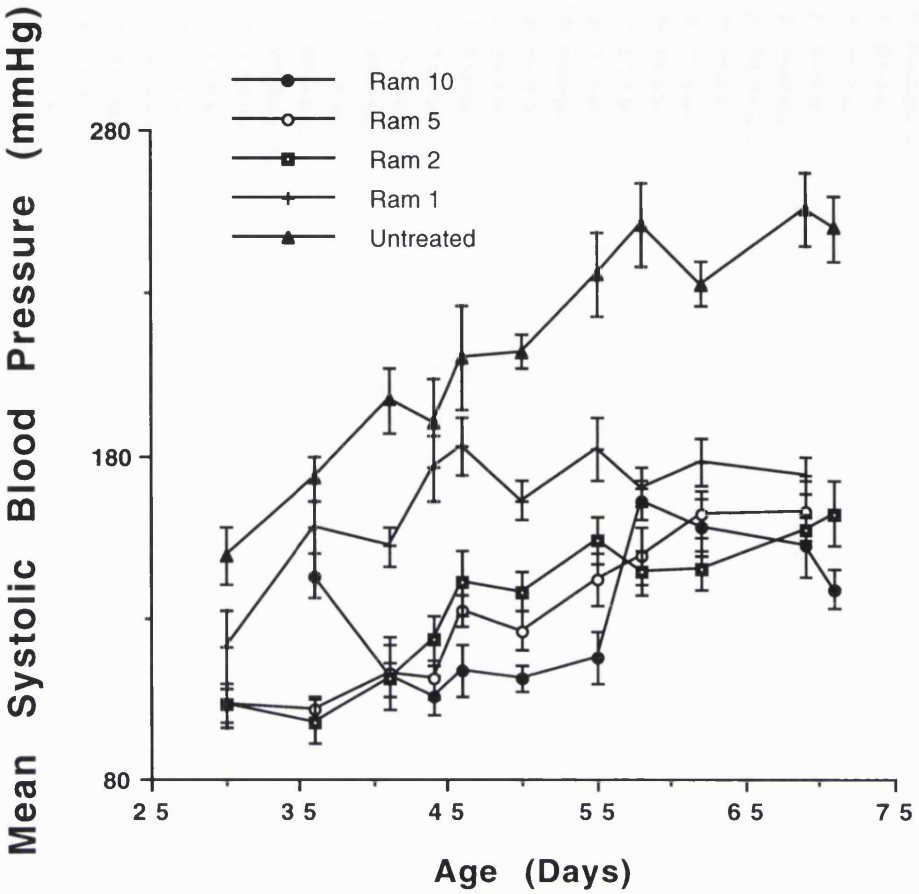


Figure 5.2: Dose-response curves of systolic blood pressure against animal age, for heterozygote male TGR(mREN2)27 animals treated with 4 different doses of the ACE-inhibitor Ramipril. The blood pressure curve for untreated transgenic TGR(mREN2)27 rats is also shown for comparison

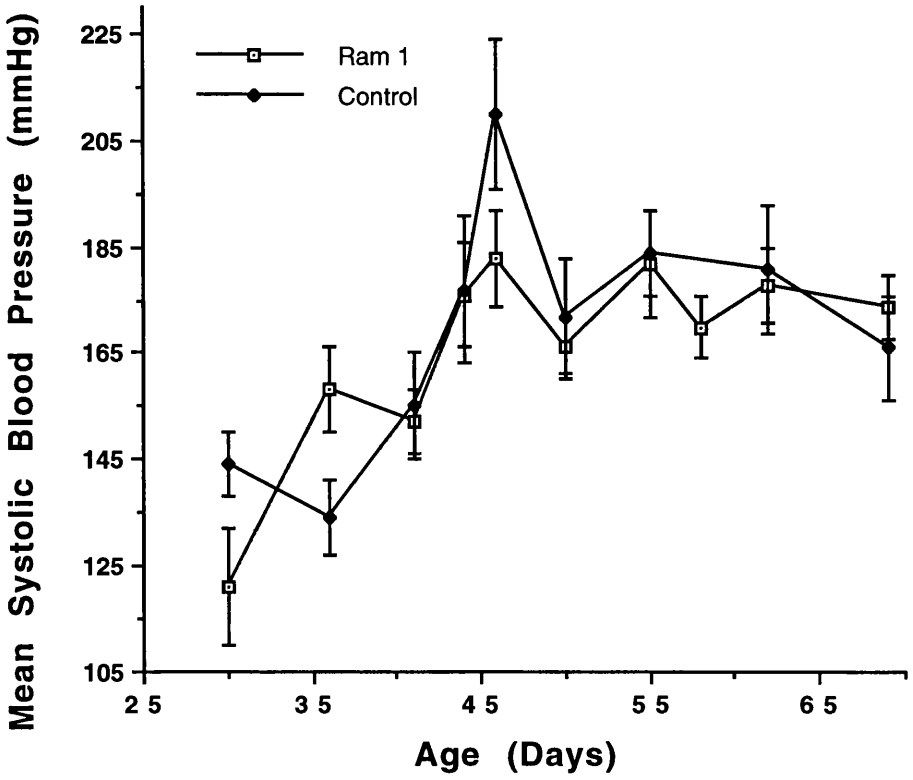


Figure 5.3: Comparison of mean (±SEM) systolic blood pressures of untreated control Sprague Dawley rats with those of TGR(mREN2)27 heterozygote male rats treated with the ACE-inhibitor Ramipril at a dose of 1mg/kg/day.

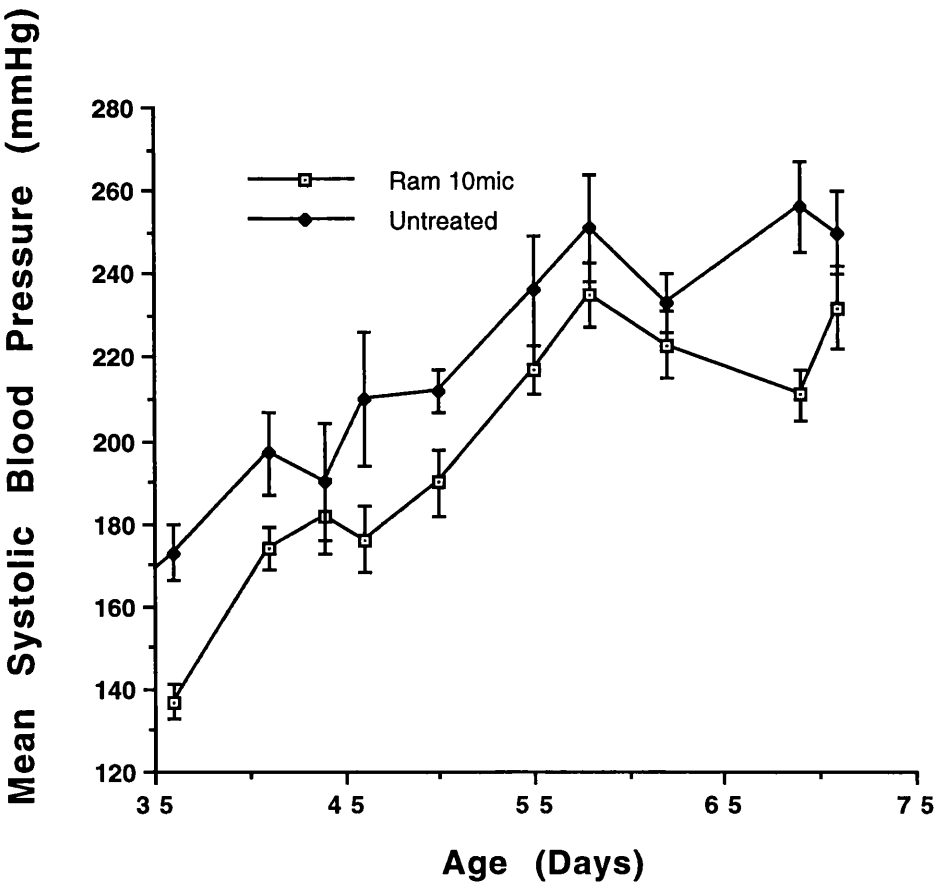


Figure 5.4: Effect of treatment with 10μg/kg/day of Ramipril (ram 10 mic) on the mean (±SEM) systolic blood pressure of heterozygote male TGR(mRen-2d)27 rats. Data for untreated animals are shown for comparison.

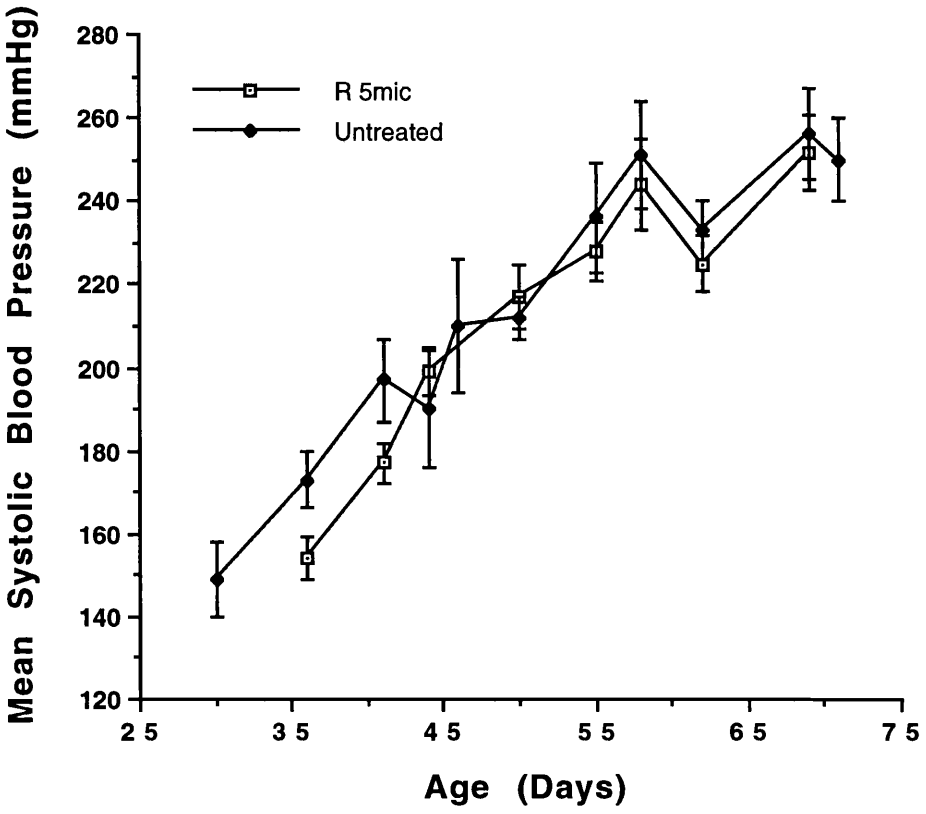


Figure 5.5: Effect of treatment with Ramipril 5µg/kg/day (R 5 mic) on the mean systolic blood pressure of heterozygote male TGR(mREN2)27 rats

5.44 RESPONSE TO VASODILATOR AGENTS

Amlodipine 10mg/kg/day had only modest effects upon the SBP of heterozygote male TGR(mREN2)27 rats (table 5.2: figure 5.6). Consequently, a higher dose was studied (20mg/kg/day: n=17 of 17 weaners), with little additional hypotensive effect being observed. Neither dose of Amlodipine was capable of reducing mean systolic blood pressure to that of the control SD group.

Age	Untreated TGR(mREN2) 27 rats	Amlodipine 10mg/kg/day mean systolic BP (mmHg)	Amlodipine 20mg/kg/day mean systolic BP (mmHg)	Sprague Dawley Rats mean systolic BP (mmHg)
36	173±7	141±13	140±6	134±2
41	197±10	160±7	184±13	155±2
44	190±14	175±7	186±8	177±2
46	210±16	203±9	185±4	210±2
50	212±5	205±8	215±6	172±2
55	236±13	224±10	218±6	184±2
58	251±13	190±10	214±6	
62	233±7	201±8	201±7	181±2
69	256±11	187±5	205±7	166±2
71	250±10	220±16	197±9	193±2

Table 5.2: Mean (±SEM) systolic blood pressure of Sprague Dawley Control rats, untreated heterozygote male TGR(mREN2)27 rats, and similar transgenic animals treated with Amlodipine.

In an effort to establish whether this blunting of effect might apply to other vasodilator agents, the effect of the potent vasodilator hydrallazine were also studied in a further small pilot study. Doses previously shown to be hypotensive in the SHR were chosen, with doses rising above those shown to reduce BP in SHRs to control levels (Clowes and Clowes 1908; Albaladajo et al 1994; Smeda et al 1988): (beginning at of 80mg/litre [n=9 survivors of 12 weaners]), 160mg/litre [n=9 survivors of 12 weaners] and 200mg/litre [n=4 survivors of 7 weaners] (figure 5.7: error bars omitted for clarity). Hydrallazine failed to produce a significant reduction in blood pressure at any dose.

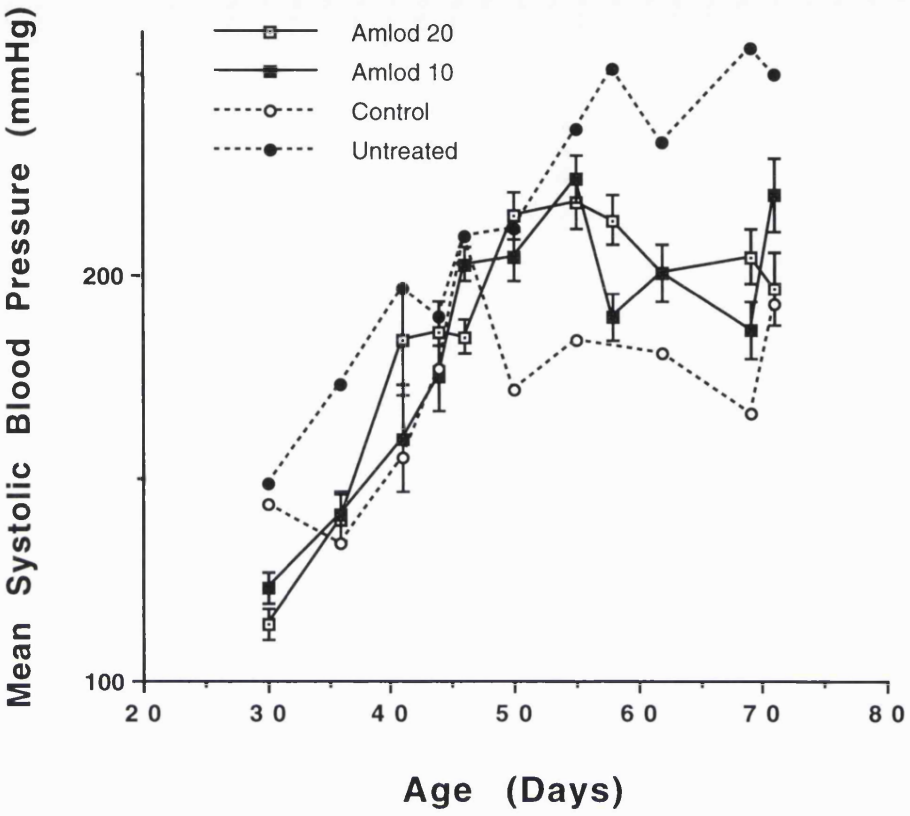


Figure 5.6: Mean (±SEM) systolic blood pressure against age, of Sprague Dawley Control rats (control), untreated heterozygote male TGR(mREN2)27 rats (untreated), and similar transgenic animals treated with Amlodipine 10mg/kg/day (Amlod 10) or 20mg/kg/day (Amlod 20).

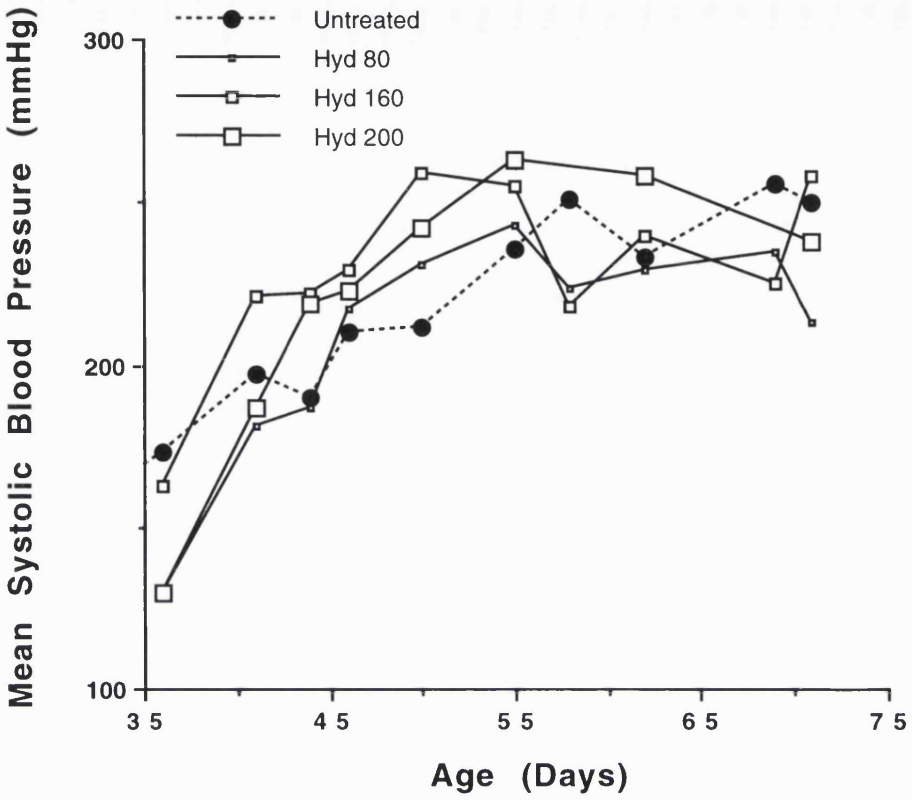


Figure 5.7: Effect of treatment with hydrallazine 80mg/day (hyd 80), 160 mg/day (hyd160) and 200mg/day (hyd 200) on the mean systolic blood pressure of untreated male heterozygote TGR(mREN2)27 rats.

STUDY B

5.5: METHODS

5.51: STUDY STRUCTURE

Male heterozygotes were derived from homozygous TGR(mREN2)27 males crossed with female Edinburgh Sprague Dawleys. Offspring were weaned at 28 days and randomly allocated into one of two groups:

Group 1: Untreated controls (n=40).

Group 2 Treatment group receiving a non-hypotensive dose ramipril (5μg/kg/day: Hoescht-Roussel U.K) in drinking water from age 28-120 days(n=24).

Systolic blood pressure was recorded weekly for each animal. Method of blood pressure recording, and animal husbandry, were as previously described (chapter 3). Mortality from MH was defined using previously-described clinical features (Whitworth et al 1994; Whitworth et al 1995a,b). After 120 days all animals were anaesthetised (as above) and killed by exanguination through an 18 gauge aorta cannula inserted just proximal to the aortic bifurcation. Any animal showing the characteristic features of MH and still alive were immediately killed and tissue harvested in the same way.

5.52: ANGIOTENSIN CONVERTING ENZYME ACTIVITY

Plasma ACE activity were assessed in all animals as described previously (Chapter 3). Tissue ACE activity was quantified in 6 animals from each group (method described in chapter 3). After exanguination, each animal was pressure-perfused with cold saline (4^0C) for 60-90 seconds (160mmHg) until the kidneys and heart were pallid and free of blood. The hearts were rapidly removed, cleaned of connective tissue on ice, the atria removed, the right ventricle dissected free from the left ventricle and septum, and the samples snap frozen in liquid nitrogen. The left carotid artery, left kidney, and a 1cm length of aorta just distal to the left subclavian artery were similarly removed, cleaned and snap-frozen. All samples were stored at -70°C for ≤4 weeks, ground in liquid nitrogen in a cooled mortar and pestle (-70°C) and resuspended in Triton X100 as described. ACE activity was assayed (Unger et al 1982) and expressed as nmol His/Leu/ml per minute.

5.53: HISTOLOGICAL EXAMINATION

Kidneys were removed after exanguination and placed in 4% paraformaldehyde/0.1% glutaraldehyde in phosphate buffer (pH 7.3). Further processing was performed by Stuart Flemming, an experienced renal histopathologist. Briefly, tissues were processed using a Citadel tissue processor (Shandon Southern Products Ltd., Cheshire, UK) on an 18 hour programme of serial dehydration in graded ethanol concentrations (70%, 80%, 90%, 100% x3), followed by three de-alcoholisation phases using the clearing agent 'Histoclear™' (National Diagnostics, Atlanta, USA), and two paraffin wax immersions at 60^0C. Tissues were embedded in wax blocks (Raymond Lamb Blockmaster III embedding centre, London, UK) prior to sectioning and staining.

Three mm sections were stained with haematoxylin & eosin (Stevens 1990) . Briefly, sections were de-waxed in xylol, rehydrated through graded ethanols to water, stained with an alum haematoxylin, washed in running water, immersed in 1% HCl in 70% ethanol for 10 seconds, washed again for 5 minutes, stained with 1% eosin for 10 minutes and washed again. Slides were then serially dehydrated through graded ethanols (25%, 50%, 75%, 90%, 100%), cleared with xylol and mounted with DPX paraffin (BDH Laboratory Supplies, Poole, UK).

Stained sections were reviewed by Stuart Flemming (an experienced renal histopathologist) blinded to animal group. Tissues were taken from randomly selected untreated survivors (n=8), treated survivors (n=9) and untreated animals who were showing signs of MH and were about to die (n=7). Three elements, namely arterial intimal proliferation, arteriolar necrosis and nephron injury were scored on a scale of 0-5 as follows:

1:	0-5%	scored 1 for X^2_{trend} analysis
2:	6-25%	scored 2 for X^2_{trend} analysis
3:	26-50%	scored 3 for X^2_{trend} analysis
4:	51-75%	scored 4 for X^2_{trend} analysis
5:	>75%	scored 5 for X^2_{trend} analysis

In addition, vascular medial thickening was scored on a scale of 1-5 depending on the degree of medial hypertrophy and hyperplasia.

5.54: STATISTICAL ANALYSIS

Blood pressures and tissue and plasma ACE activity were compared in untreated and treated groups by t-test. The renal histopathology scores were compared by X^2_{trend} analysis, as (upon statistical advice) too many scores were <5 for simple X^2 analysis, and this would not accurately compare a non-linear trend such as changes in histological pattern. Values of $p<0.05$ were considered statistically significant.

5.6: RESULTS

5.61: SYSTOLIC BLOOD PRESSURES

5.611 Untreated and treated transgenic animals

Throughout the study period, SBP was similar in the treated and untreated groups. Ramipril caused no sustained hypotensive action : SBP was actually slightly higher in the treated group than the untreated at three timepoints (days 32, 35, 38, 116 and 119: p=0.012, <0.001, 0.005, <0.001, and 0.03 respectively). At only three timepoints (days 53, 83 and 110) was treated SBP< untreated (p=0.046, 0.03, and 0.03 respectively: figure 5.8)

5.612 Untreated transgenic animals: survivors vs. non-survivors

SBP in animals with sustained benign hypertension are similar to those who develop MH up to 55 days of age, at which point animals begin to die of MH (figures 5.9 and 5.10). The diversity of blood pressures in the MH group, and the radical changes seen over time after 60 days, may be explained in one of two ways:

(i) Those animals going on to develop MH suffer increasing vascular instability

(ii) Blood pressure recordings occasionally were taken in healthy-looking animals which were in the early stages of the MH process.

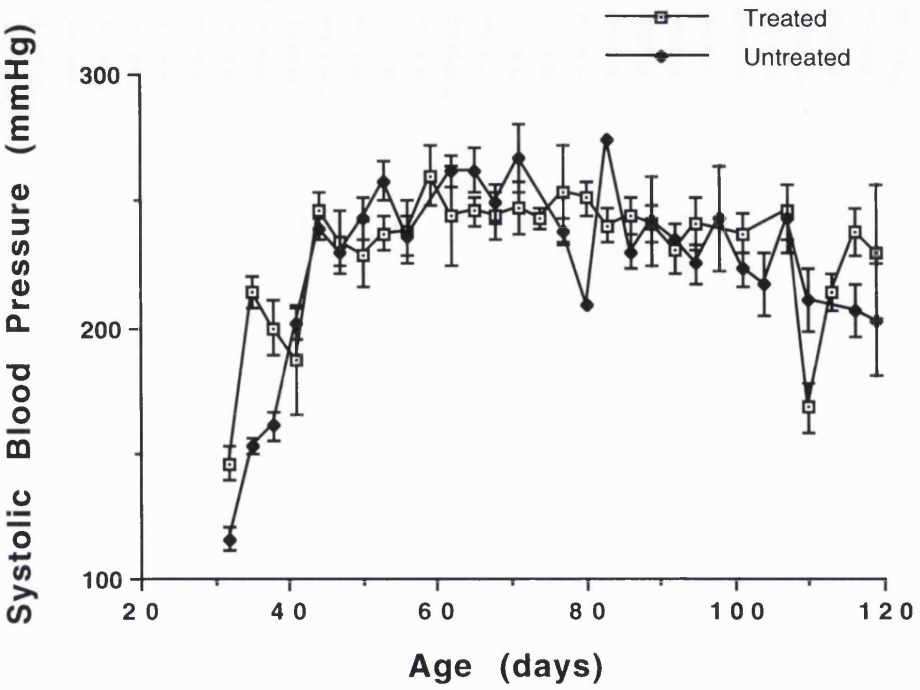


Figure 5.8: Mean (±SEM) systolic blood pressures in male heterozygote TGR(mREN2)27 rats treated with ramipril 5µg/kg/day ('treated'), and similar untreated animals. Blood pressure recorded by tail-cuff plethysmography.

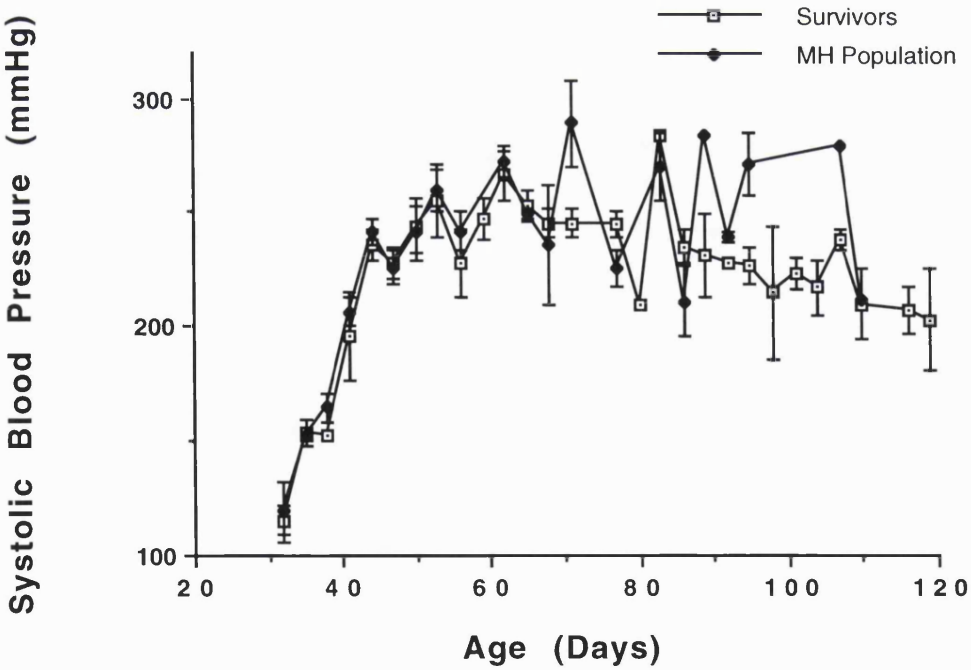


Figure 5.9: Mean systolic blood pressures (±SEM) of surviving untreated TGR(mREN2)27 heterozygote male rats, when compared to those ultimately dying of malignant hypertension.

5.62: SURVIVAL

Untreated MH mortality was 25/40 (63%) compared to 1/24 (4.2%) in the treated group (Fig 6.4). Death was confirmed as being due to malignant hypertension on the basis characteristic features:

1. Lethargy, hunched posture, shivering and piloerection. Occasional neurological features (such as tremor, fitting or limb paresis) were noted.
2. Dramatic weight loss , redundant skin folds and low tissue turgor.

Exanguination confirmed the low circulating blood volume (4ml in MH animals compared to >10ml in animals with BH).

3. At sacrifice, serosal and pulmonary haemorrhages were common.
4. The gut was thin and translucent, and green liquid stool was occasionally observed within.
5. The kidneys were small, shrunken, and 'flea-bitten', with surface pitting and punctate surface haemorrhages.

In general, animals of our colony suffered much more rapidly-progressive MH than the Edinburgh colony, with severe symptoms often appearing within hours.

Survival benefit is confirmed by the complete absence of deaths from malignant hypertension in those animals treated with ramipril 5µg/kg/day who were part of a later study of left ventricular mass and collagen content (chapter 6): i.e. these findings are reproducible.

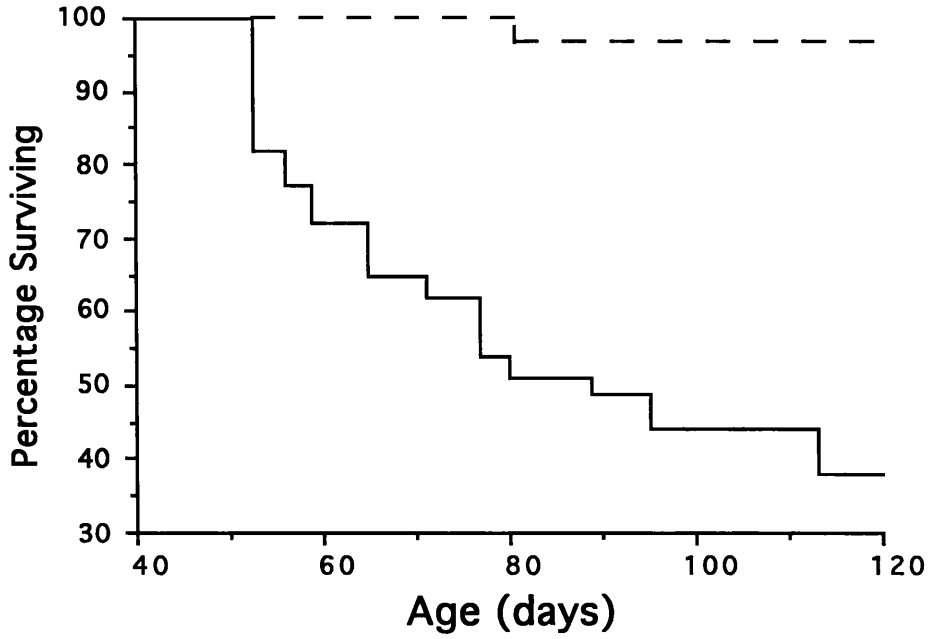


Figure 5.10 :Percentage survival of each cohort with time.

— — — — Treated TGR(mRen-2d)27 rats (5µg/kg/day Ramipril)
———— Untreated TGR(mRen-2d)27 rats

*The single death in the treated group occurred in a cage accidentally treated with plain tap water for 2 days, 3 days prior to death.

5.63: ACE ACTIVITY

Ramipril 5μg/kg/day significantly inhibited ACE activity (nmol HisLeu/ml/min) in all tissues: by >60% in resistance vessels (119.62±23.6 vs. 45.83±14.78 for carotid: 152.38±35.4 vs. 55.9±18.8 for aorta: mean±SEM, untreated vs. treated respectively), >55% in myocardium (2.94±0.55 vs. 1.28± 0.24 for LV: 4.11±0.92 vs. 1.68±0.94 for RV) and >40% in the kidney (0.75±0.08 vs. 0.44±0.04 nmol HisLeu/ml/min). Paradoxically, plasma ACE activity rose by 52% with treatment (45.83±2.11 vs. 69.53± 3.06 nmol HisLeu/ml/min, untreated vs. treated). (Figure 5.11)

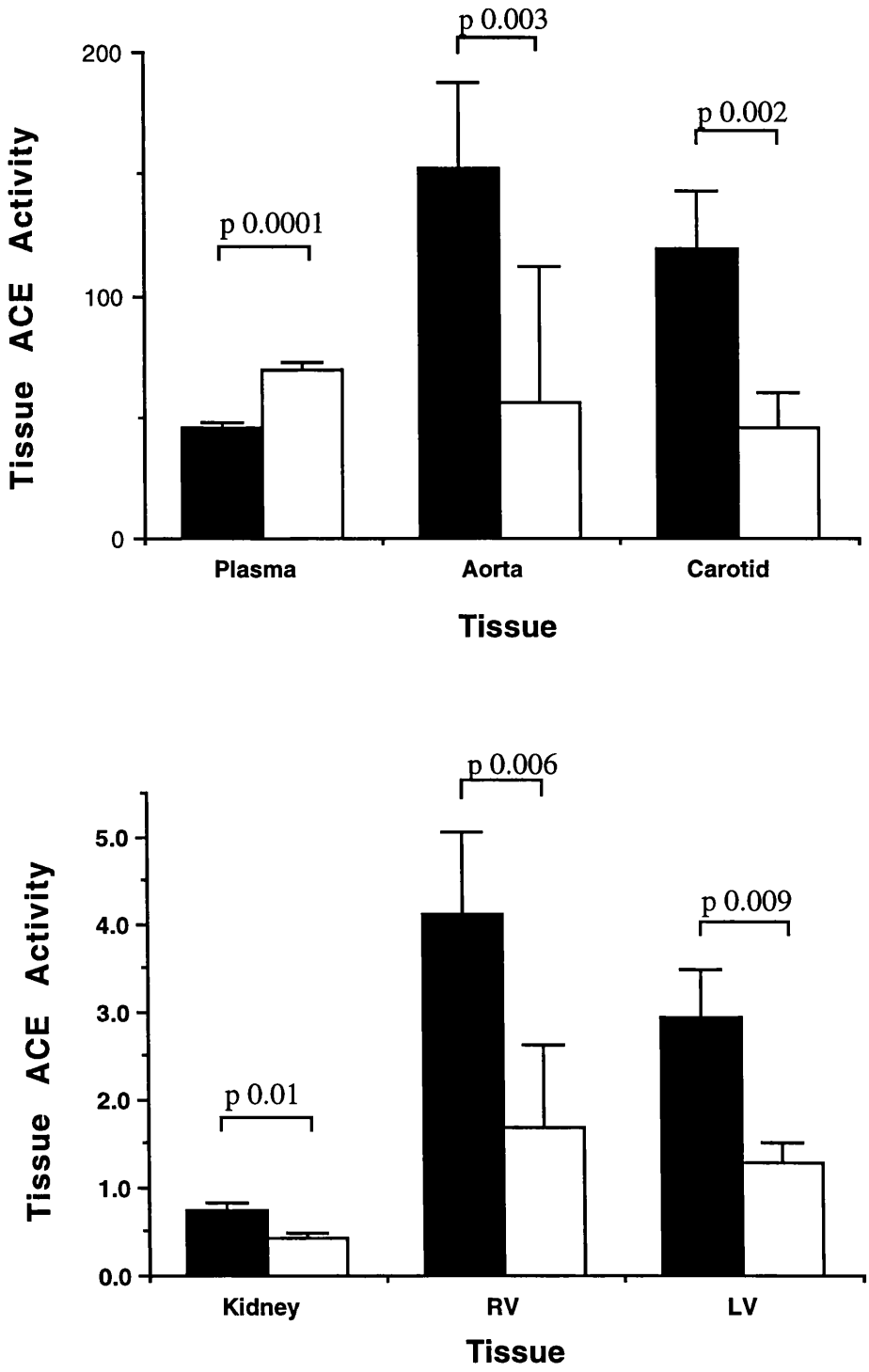


Figure 5.11: Tissue and plasma Angiotensin-converting enzyme activity in male heterozygote TGR(mRen-2d)27 rats (n=6 per group).
RV=right ventricle LV= left ventricle
Units of ACE activity =nmol HisLeu/ml/hour
Filled bars represent untreated animals. Open bars represent animals treated with ramipril 5 microgrammes/kg/day.

5.64: RENAL HISTOLOGY

Representative examples of renal histology (under light microscopy) are shown in Figures 5.12 a-f. Review of histology revealed two morphological patterns distinguished by the presence or absence of nephron injury. One pattern was characterised by vascular changes. There was hypertrophy and hyperplasia of the smooth muscle cells of the media of arcuate and interlobular arteries and of afferent arterioles (a). These changes were of moderate severity, with vessel walls being approximately twice as thick as that of control non-transgenic animals. There was no intimal injury, endothelial swelling or subendothelial protein exudation. The second pattern of histopathology consisted of changes superimposed upon these vascular smooth muscle cell responses. There was endothelial swelling and intimal proliferation affecting the interlobular arteries. Afferent arterioles showed fibrinoid necrosis and endothelial swelling (b). Glomerular collapse (c), fibrosis (d), and occasional crescent formation were evident. Associated with damaged glomeruli there was tubular epithelial cell injury and tubular atrophy (e) with interstitial oedema, inflammation and fibrosis. Tubular injury associated with glomerular damage strongly suggests a single nephron pattern of tissue injury (f).

Figures 5.12 a-f (following pages)
Light photomicrographs (x40) of renal tissue from untreated animals, showing the patterns of injury seen and scored.

a Medial hyperplasia and hypertrophy affecting interlobular and arcuate arteries in cross-section but with normal glomerular and tubular structure.
b Fibrinoid necrosis and endothelial swelling affecting afferent arterioles
c Glomerular tuft collapse
d Glomerular tuft sclerosis
e Tubular epithelial cell injury and tubular atrophy with interstitial oedema, inflammation and fibrosis
f Histology suggestive of a single nephron pattern of injury

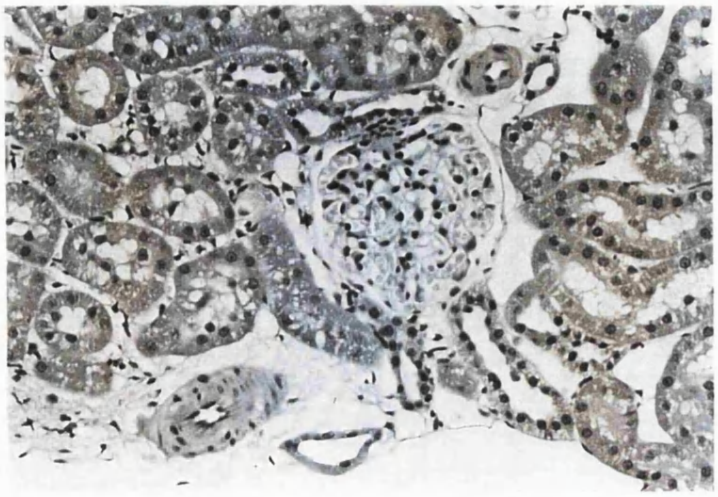


Fig 5.12a

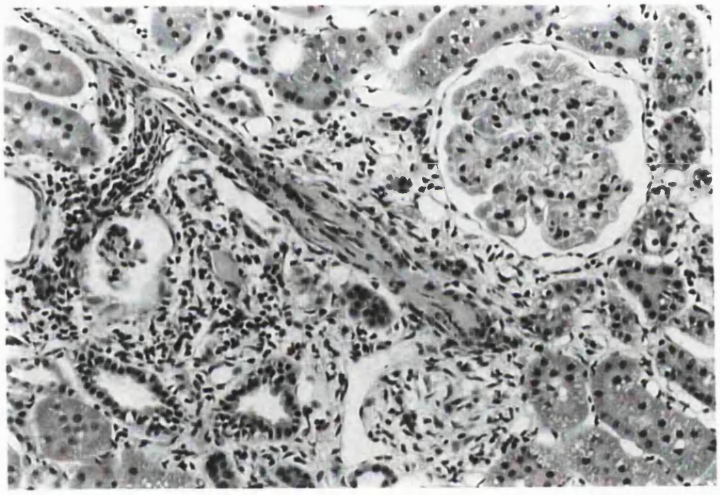


Fig 5.12b

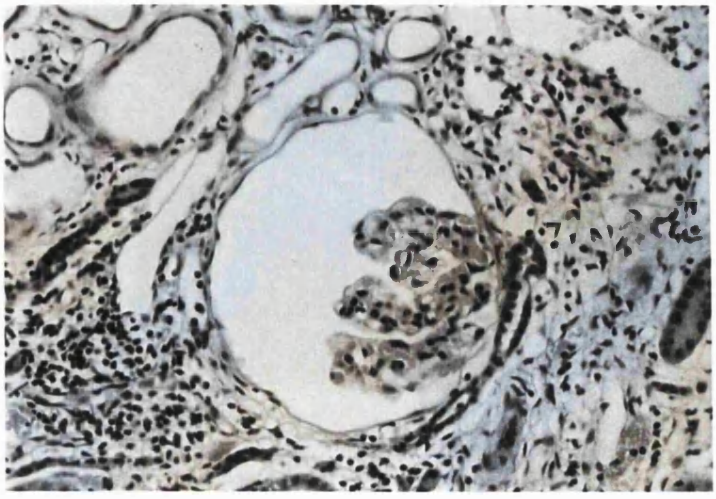


Fig 5.12c

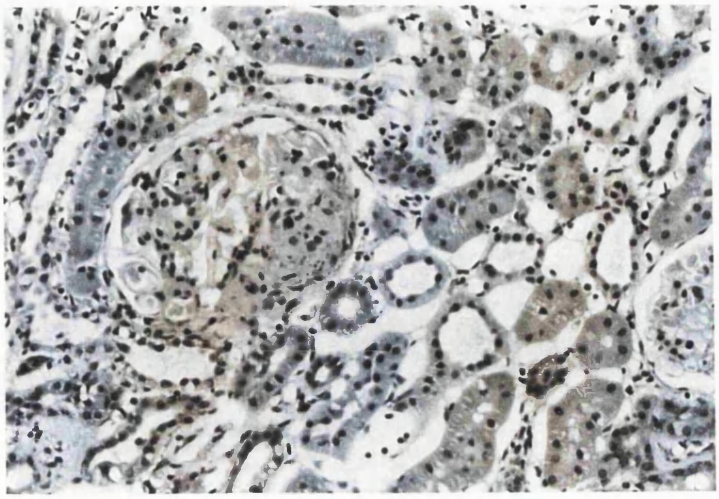


Fig 5.12d

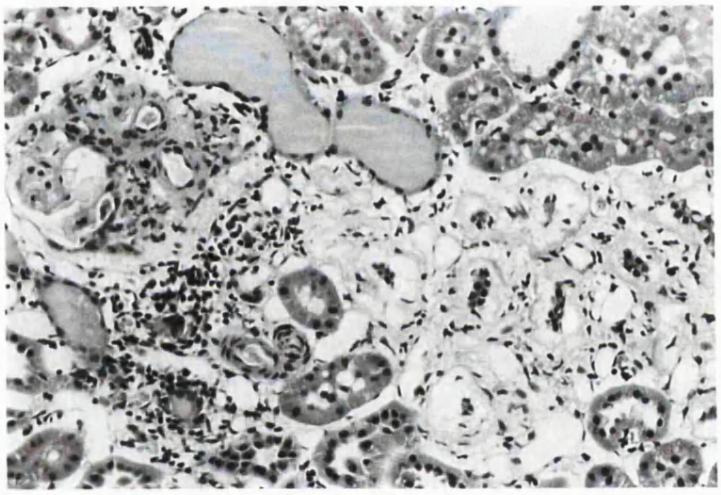


Fig 5.12e

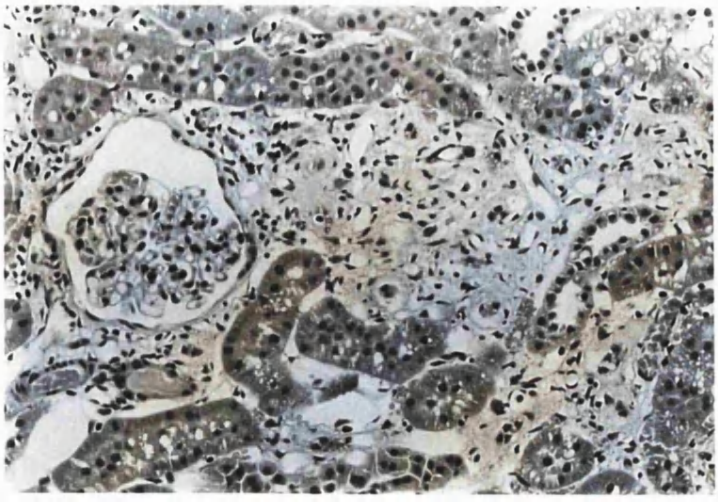


Fig 5.12f.

These separate elements, namely vascular muscle response, arterial intimal proliferation and nephron injury were scored as described above (table 6.1). When histological scores for the untreated group was compared with those of the treated group, there was no difference in the degree of medial hyperplasia and hypertrophy (X^2_{trend}=0.01: p>0.02). The untreated group showed a greater degree of intimal fibrosis and hyperplasia (X^2_{trend}=9.16: p<0.01), fibrinoid arteriolar necrosis (X^2_{trend}=10.11: p<0.01) and evidence of nephron injury (X^2_{trend}=10.2: p<0.01).

On comparing scores for the renal tissue of untreated animals who survived with those in the terminal phase of MH, there was no significant difference in the degree of arteriolar medial hypertrophy and hyperplasia (X^2_{trend}=0.27: p>0.05) or intimal damage (X^2_{trend}=0.03: p>0.05). Those in the terminal phase of MH actually had less evidence of arteriolar damage (X^2_{trend}=7.16: p<0.01) and nephron injury (X^2_{trend}=4.06: p>0.05), possibly as they had a shorter life and reduced net blood pressure burden.

Group	arterial score	intimal score	arteriolar score	nephron score
untreated survivors	2	3	4	4
untreated survivors	3	2	3	3
untreated survivors	2	**0**	2	2
untreated survivors	2	3	3	3
untreated survivors	2	2	3	3
untreated survivors	2	3	4	4
untreated survivors	4	3	3	3
untreated survivors	2	3	3	4
low dose ram	2	**0**	**0**	**0**
low dose ram	3	**0**	**0**	**0**
low dose ram	2	**0**	**0**	**0**
low dose ram	2	**0**	**0**	**0**
low dose ram	2	**0**	**0**	**0**
low dose ram	3	**0**	3	3
low dose ram	3	3	3	3
low dose ram	2	**0**	2	2
low dose ram	2	2	2	2
MH sufferers	2	2	2	2
MH sufferers	2	3	3	3
MH sufferers	2	2	2	3
MH sufferers	3	**0**	**0**	**0**
MH sufferers	2	3	3	3
MH sufferers	3	3	3	3
MH sufferers	2	2	2	2

Table 5.3: Renal histopathological scores for individual animals randomly drawn from 3 groups of heterozygote male TGR(mREN2)27 rats: untreated survivors, rats in the terminal stages of malignant hypertension, and rats treated with ramipril 5µg/kg/day from weaning. Arterial intimal proliferation, arteriolar necrosis, nephron injury and vascular medial thickening were scored on a scale of 1-5 (see above). MH sufferers were untreated animals dying of MH. 'Low dose ram' refers to animals treated with Ramipril 5µg/kg/day.

5.7: GENERAL DISCUSSION

We have described in detail, for the first time, the timecourse of the rise in blood pressure of the heterozygote male TGR(mREN2)27 rat. Systolic blood pressure was found to rise early in the life of these animals, as has been noted before (Mullins et al. 1990), and to be sustained throughout the 70 day period. The hypertensive phenotype was associated with marked renal histological changes. Our observations support those previously reported in this model by Bachmann, although our data are more detailed. He showed such changes to be progressive over the 4-6 month period and to be more prominent in males. Fibrinoid necrosis was not described, but no comment is made either of the presence of an MH phenotype in the animal population used for this study. Arterial wall thickness was notably increased in the arcuate and interlobular arteries and in the proximal afferent arteriole. At 4 month, there were few changes in the nephron unit (including the glomeruli), although prominent changes appeared at 6 months when 10% of units were damaged. The frequency of such damage rose by eight months. In some glomeruli, sclerosis or tuft collapse were seen, and in others enlarged Bowman's capsular volume also occurred. Focal tubular atrophy was noted (Bachmann et al. 1992).

Our studies also confirm that the blood pressure of the TGR(mREN2)27 rat is highly sensitive to inhibition of the renin-angiotensin system by ACE inhibition. Such sensitivity to ACE inhibitor treatment has been previously demonstrated, as has a marked sensitivity to ATII receptor antagonists (Mullins et al. 1990; Bader et al. 1992; Barrett and Mullins 1992; Moriguchi et al. 1994). Thus, even a dose of captopril of 10mg/kg/day in drinking water begun at 51 days reproducibly reducing mean BP by 40-60mmHg (Mullins et al. 1990). Our data also support the resistance of these animals to vasodilator treatment, as has been suggested for the calcium-channel blocker Nitrendipine (Hirth-Dietrich et al. 1994). Doses of Amlodipine which would normally render the Spontaneously Hypertensive Rat (SHR) normotensive had only limited effect on the TGR model. This resistance is not exclusive to the calcium channel blockers as a group, as we also found hydrallazine to be ineffective. Unlike ACE-inhibitors, hydrallazine may increase brain and cardiac sympathetic activity, and may enhance the synaptic effects of noradrenaline (Kohlmann et al. 1984). This might partly explain the lack of response. However, such effects have not been shown for Amlodipine, and are unlikely to account for its limited effects.

The striking difference in response to these different hypotensive agents may have parallels in human hypertension, where different forms of hypertension associated with different renin-angiotensin system activity have been shown to have

quite different profiles of response to a spectrum of different pharmacological agents (Helmer 1964; Hollenberg et al. 1969; Buhler et al. 1972; Laragh 1973; Laragh 1992).

Such data support a primary role for the renin-angiotensin system in the genesis of the hypertension in the TGR(mREN2)27 rat. The potent effect of AT_2 receptor antagonists referred to above suggest that the hypotensive effects of the ACE inhibitor ramipril are unlikely to be mediated through the action of kinins. Concurrent treatment with Hoe 140 (a kinin receptor antagonist) might confirm this interpretation.

Our data also suggests (study 2) that, in the TGR(mREN2)27 rat, tissue ACE inhibition protects against the development of
(i) the malignant hypertensive phenotype (MH)
(ii) death associated with this condition
(iii) the appearance of some of the renal histopathological features of MH
and that these protective effects are independent of any reduction in SBP.
Thus, these data support a role for tissue RAS in the development of MH. Low-dose ramipril treatment reduces tissue ACE activity without a detectable effect on systolic blood pressure. Despite persistent severe hypertension, ramipril treatment almost completely protected against the development of the malignant hypertensive phenotype. Furthermore, treatment with a non-hypotensive dose of the ACE-inhibitor ramipril significantly modify the pattern of hypertensive vascular and nephron damage described above. The degree of medial wall thickening did not differ between treated and untreated groups (either survivors or MH sufferers), suggesting that such effects are related merely to the hypertensive phenotype and are not directly due to increased RAS activity. However, the degrees of arteriolar myointimal proliferation, fibrinoid change and nephron injury were significantly reduced or abolished in the treated group despite similar blood pressures, suggesting that an intact RAS is important in tissue injury in the kidney in this model. It is interesting to note the very slight reduction in severity of vascular injury in those animals dying with MH, when compared to survivors. This suggests that the vascular injury may be mediated through RAS activity (cf. the protective effects of ACE inhibition) but are related to the longevity of exposure to this stimulus. These data suggest that the vascular injury pattern is a necessary associated precursor to the development of MH, but is not pathognomic of its presence, nor directly solely pathogenic in its cause.

These studies are open to criticism. The use of light halothane anaesthesia may have influenced the blood pressure of these animals. However, as has been reported before (chapter 2), a validation study performed by Dr. Whitworth in Edinburgh shows that

such an effect is minimal in her hands. It remains possible, however, that halothane had different effects on the blood pressures of the control Sprague Dawley strain and the transgenic animals. This might partly explain the seemingly unusually high systolic blood pressures seen in this control strain. Neither can we discount an interaction between treatment with a hypotensive agent and the halothane administration. A further study of a large cohort of animals trained to have blood pressures recorded without sedation is warranted, although data during the early training period would remain unreliable. Perhaps more reliable would be to use a continuous BP recording system, with BP telemetered from implanted transducers. Such systems do exist, but have 3 major draw-backs: significant expense, inability to implant the devices into young animals (e.g. <130g), and short transmission life (<4 weeks).

The lack of recording of diastolic blood pressure is a major limitation to any study using photoplethysmographic data recording. We therefore cannot discount an excess effect of treatment with hypotensive agents on diastolic blood pressure (and hence mean blood pressure). This might be particularly true of treatment with vasodilating agents. Furthermore, the finding of similar blood pressure at a single time of each day do not exclude an effect of drug therapy on blood pressure variability over 24 hours. Once again, telemetry would seem to provide a partial answer. However, in a small pilot study of 4 animals treated with Amlodipine 10mg/kg/day and monitored by telemetry (kindly carried out by Dr. Whitworth, Edinburgh), the effect on diastolic blood pressure was found to be similar to that on systolic blood pressure, and the data for reduction in SBP closely matched our own.

Administration of ramipril at the low dose of 5μg/kg/day was associated with significant inhibition of tissue ACE. This is probably related to its very high tissue binding in comparison to other ACE inhibitors. Conversely, plasma ACE activity actually seemed raised in the treatment group. The possible cause for this finding needs to be addressed. Ramipril at antihypertensive doses (at least 60 times higher than those used in this study: 0.2-1mg/kg) will almost completely block ACE activity in rats if administered by gavage. Administration in drinking water (i.e. steady overnight intake) causes only a 50-80% of circulating ACE activity. Ramipril at lower doses (e.g. 0.01-0.3mg/kg) does not affect plasma ACE activity. The apparent increase in plasma ACE activity with low-dose ramipril treatment may be due to induction of ACE expression, possibly through loss of negative feedback by ATII on ACE synthesis (Schunkert et al. 1993a). After captopril usage, this effect is detected in assay due to rapid dissociation of the drug-ACE complex, and to the instability of the drug/enzyme complex in storage (Unger et al. 1981) (Peter Gohlke, Institut Fur Pharmakologie, Kiel, Germany: personal communication October 1995). Similar

effects can be seen with enalapril treatment (Ulm and Vassil 1982). Although we might assume that the increase in converting-enzyme activity is an assay artefact due to dissociation of the drug from the enzyme during storage it is possible that drug complex stability is particularly low *in vivo*. For some part of the day, the treated animals might thus have been exposed to a higher circulating ACE activity than untreated animals- perhaps accounting for the initially (<day 10) raised SBP while tissue loading with ramipril was perhaps continuing. If this were the case, then the argument that tissue ACE inhibition is the crucial factor in MH development becomes even clearer, as despite this higher circulating RAS activity, the animals remain protected.

It is clear that treatment with hydrallazine (without BP reduction) is not protective, suggesting that the protective effect of ramipril is exclusive to that drug. Hypotensive treatment with Amlodipine 10-20mg/kg/day prevented deaths from MH. Taken with the protective effect of low-dose ramipril, these data suggest that sustained hypertension alone may be necessary, but is not in itself sufficient, to lead to MH. In this process, tissue RAS activity seems to play a crucial role.

5.8: RAS AND THE PATHOGENESIS OF MALIGNANT HYPERTENSION

5.81 MECHANISTIC THEORIES OF MALIGNANT HYPERTENSION

The mechanism of transformation of benign to malignant hypertension is not understood. Although conversion may seem spontaneous, it may be triggered by direct vascular injury (e.g. due to smoking, which increases the risk of developing MH and is associated with more severe disease and a poorer outcome (Bloxham et al. 1979; Tuomilehto et al. 1982)) or vascular damage through a sudden rise in blood pressure (e.g. phaeochromocytoma (Houston 1989), withdrawal of hypotensive medication (Houston 1989), and treatment with steroid hormones (Petitti and Klatsky 1983)). Vascular injury might thus act on a substrate of chronic hypertensive damage. This 'vascular injury initiation' hypothesis is supported by the finding of very similar vascular histopathological lesions in both MH and in a variety of systemic vasculitides such as autoimmune vasculitis and haemolytic uraemic syndrome (Kincaid-Smith 1975; Bohle et al. 1976).

Whatever the nature of the trigger, the appearance of the classical vascular changes of MH requires exposure to high vascular pressure. Renal artery stenosis may protect the affected kidney from the damage afflicting the contralateral kidney (Derow and Altschule 1935) and hypotensive treatment attenuates the vascular changes of MH (Kincaid-Smith 1982). In the SHRSP, a slightly lower BP in earlier life might protect from MH (Stier et al. 1989; Volpe et al. 1990; Whitworth et al. 1994). However, we found no consistent difference in SBP between those animals who later developed MH, and those who did not, although such measurements (using tail cuff plethysmography, halothane anaesthesia, only SBP measurement at one time of day) may allow some changes in BP or its circadian rhythm to escape detection. Finally a small decline in diastolic pressure with treatment, although unlikely, cannot be excluded. Cohort studies using 24 hour BP telemetry are ideally needed.

Finally, a specific genetic substrate may be required. A gene 'trigger' may be monogenetically inherited in the TGR(mREN2)27 rat line (see chapter 3). There is little evidence of such a factor in humans, although associations of MH with specific HLA types has been suggested (Gudbrandsson et al. 1980; Forsberg and Low 1983).

We thus postulate that sustained hypertension may lead to a spiral of increasing vasoconstriction and escalating blood pressure, if 'triggered' by vascular injury. If this were so, then a positive feedback within the circuit may then operate (figure 5.13):

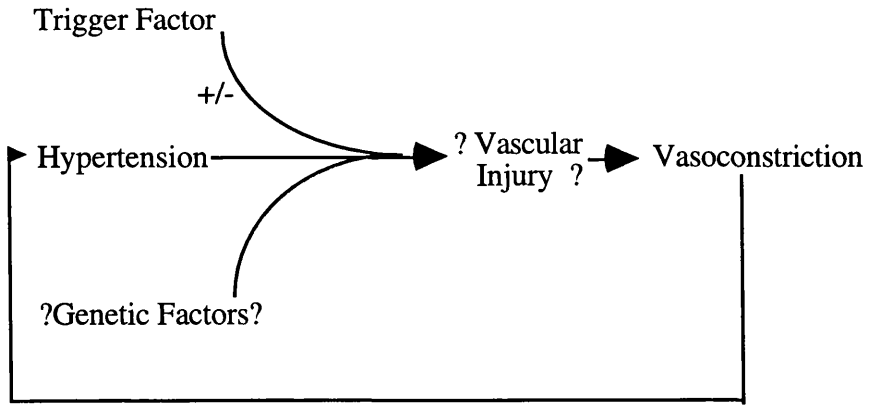


Figure 5.13: Possible scheme for the initiation and maintenance of malignant hypertension

The escalating hypertension of the malignant phase is due to profound vasoconstriction (Giese 1976). This is likely to hold true in our MH model, given that similar volume depletion is seen to that in other MH models (Orth and Ritz 1975). This vasoconstriction may be RAS-dependent:

(i) In MH patients, vasoconstriction is highly responsive to ACE-inhibition (Saragoca et al. 1983), but often less to vasodilators such as nitroprusside (Rouby et al. 1982).

(ii) Circulating RAS activation has been recognised as a key feature of MH since 1976 (Giese 1976). Transition to MH in the Ren-2 rat is similarly associated with significant increases in circulating renin and ATII (Whitworth et al. 1995a).

5.82 THE ROLE OF RENIN-ANGIOTENSIN SYSTEMS IN THE PATHOPHYSIOLOGY OF MALIGNANT HYPERTENSION

5.821 RAS involvement in 'benign' hypertensive states

Expression of local tissue RAS components may play an important role in the initiation or maintenance of hypertensive states. Clipping of a single renal artery in the 2K1C hypertensive rat model increases renin gene expression (Mai et al. 1995) and post-translational processing, activation, half-life and activity (Moffett et al. 1986). Renal ATII levels (Von Thun et al. 1994) and renal vascular and brush-border ACE expression also increase (Mai et al. 1995). In the hypertensive SHRSP rat, the rise in BP with age correlates closely with the rise in renal ACE activity (Ikemoto et al. 1986b). Aortic ACE activity rises after renal artery clipping in the rat (Miyazaki et al. 1986), whilst the sustained hypotensive effect of a single dose of enalapril in the SHR (Ikemoto et al. 1986b) and of longer-term treatment (Unger et al. 1985; Ikemoto et al. 1986a) is paralleled by a sustained reduction in renal and aortic ACE activity which persists long after the recovery of plasma ACE activity.

RAS activation might also cause some of the vascular complications and histopathological changes associated with hypertension. ATII infusion at high concentration causes extensive renal damage in the rabbit (Gavras et al. 1971) and at doses producing only modest hypertension causes marked histopathological changes: focal arteriolar injury and fibrinoid necrosis, vascular smooth muscle cell proliferation, expression of contractile proteins by glomerular mesangial cells, and tubulointerstitial injury (Johnson et al. 1992). Similarly, renin administration to uninephrectomised rats causes hypertension and histopathological changes including the arteriolar necrosis (Masson et al. 1964). Such effects may be partly independent of BP itself as non-hypotensive RAS inhibition protects against extrarenal vascular injury in rats with hypertension and chronic renal failure (reviewed in (Kim et al. 1994)). RAS activity has also been implicated in the genesis of many of the

cardiovascular sequelae of sustained hypertension in humans (Brunner et al. 1972; Alderman et al. 1991; Laragh 1992).

5.822 Malignant hypertension is associated with RAS activation in animal models

RAS activation is an integral component of MH. The SHRSP rat (Okamoto et al. 1974) is a useful model of human MH (Matsunaga et al. 1975; Shibota et al. 1979), in which the frequency of malignant crises is increased by dietary salt-loading (Kim et al. 1991b; Camargo et al. 1993). In their MH phase, both ATII levels and plasma renin activity (PRA) rise (Camargo et al. 1990; Volpe et al. 1990; Kim et al. 1992), the latter correlating with the presence of renovascular and cerebrovascular lesions (Matsunaga et al. 1975; Shibota et al. 1979). Similar observations are made in other animal models. Some two kidney one clip (2K1C) rats go on to develop MH, and in these PRA and renal renin content are raised (Mohring et al. 1975). In the Goldblatt dog, tightening of the renal arterial clamps leads to a sudden rise in plasma renin coinciding with the development of MH (Carpenter et al. 1961). Similarly, chronic renal arterial noradrenaline infusion into unilaterally nephrectomised dogs leads to a steady rise in systemic BP with initial RAS suppression, followed by a period of natiuresis, a sudden 10-fold rise in PRA and a classical hypertensive crisis (Lohmeier et al. 1984). Interestingly, in humans, the efficacy of ß-adrenergic blockade with propranolol may be related to the initial PRA, being less effective in those with low-PRA essential hypertension, but effective in those with MH and with high-PRA hypertension (Buhler et al. 1972). Such data might support the concept that noradrenergic stimulation in the dog model is active in promoting hypertension through the activation of the renal RAS.

RAS activation in MH is not just a secondary phenomenon: MH crises can be triggered by RAS stimulation and agonists. In Sprague Dawley rats rendered hypertensive by aldosterone administration, the additional administration of renin triggers MH (Masson et al. 1961). Renin administration to single-kidney rats causes arteriolar necrosis (Masson et al. 1964), and arterial ATII infusion into rats causes hypertension, renal fibrinoid necrosis and intimal hyperplasia of afferent arterioles (Johnson et al. 1992). Meanwhile, RAS antagonists at non-hypotensive doses are protective. In SHRSP animals, a high potassium diet suppresses or delays the rise in PRA seen with salt loading, and reduces 12 week mortality three-fold without affecting BP for most of the study period (Volpe et al. 1990). Enalapril (15mg/kg/day) led to 90% survival at 24 weeks (with normal renal histology) compared to a 100% mortality in untreated animals (Stier et al. 1989). Although BP was lower up to 12 weeks of age in the treated group (Stier et al. 1989), BP reduction

alone may not be protective against stroke in this model (Stier et al. 1988) and sustained hypertension alone is not sufficient in animal models to cause MH (Bidani et al. 1994).

The protective effect of ramipril seen in our study is likely to be at least partly mediated by a reduction in ATII synthesis rather than reduced kinin degradation. In the salt-loaded SHRSP model of MH, ATII antagonism reduces both the blood pressure and plasma renin activity of MH animals, reduces renal dysfunction and renovascular damage, and may abolish stroke(Shibota, et al. 1979; Camargo et al. 1993). Notably, ATII antagonism (using 1-sarcosine, 8-alanine ATII) caused hypotension only in those animals with MH, again suggesting that the malignant (and not benign) hypertension of SHRSP is ATII-mediated (Shibota, et al. 1979). Losartan at the non-hypotensive dose of 1mg/kg/day completely prevents mortality from MH and limits the development of its histological changes in the SHRSP (Fornes et al. 1993).

5.823 Malignant hypertension is associated with RAS activation in humans

Together with hypertension and the rapid development of necrotising arteriolar disease, RAS activation is now also accepted as one of the three cardinal features of **human** MH (Giese 1976). Juxta-glomerular apparatus hyperplasia (McLaren and MacDonald 1983; Gardiner and Lindop 1992), and raised renal renin secretion (Hollenberg et al. 1969) and PRA (Helmer 1964; Hollenberg et al. 1969) may all be seen, and ATII generation across the pulmonary vascular bed is increased (Giese 1976). Such findings may not be universal (McAllister et al. 1971), being subject to the confounding influences of varying durations and aetiologies of antecedent hypertension and the effects of treatment (which, due to the nature of this medical emergency, are often initiated prior to the drawing of research samples). Thus only 36% of 22 MH patients had an elevated PRA in one study although in only 8 patients was the sample drawn prior to the initiation of therapy. PRA was raised in 5 of these eight (McAllister et al. 1971). Finally, there are difficulties in defining what constitutes an 'elevated PRA'. In all 7 patients with elevated PRA available for follow-up, PRA fell to 'normal values' at the end of one year. It would perhaps be more interesting to know what happened to the absolute values of PRA in all patients over this time, to identify those who had had a 'relative' rise in PRA (inappropriate for the hypertension).

Raised aldosterone levels also suggest raised RAS activity in MH. Aldosterone levels were raised amongst 14 MH sufferers after variable periods of treatment (Laragh et al. 1960) suggesting that, once initiated, RAS activation was persistent. Similarly, aldosterone levels were elevated in 19 (86%) of 22 MH

sufferers, and remained elevated despite falling PRA. Six exhibited transient hyperaldosteronism in the face of normal or low PRA after treatment, and one developed an adrenal adenoma (McAllister et al. 1971). Eleven patients at presentation exhibited high aldosterone levels in the absence of raised PRA (McAllister et al. 1971). Chronic RAS activation might therefore be associated with disrupted adrenal autonomy and feedback regulation in MH, which might be sustained (Figure 5.14, after McAllister 1971).

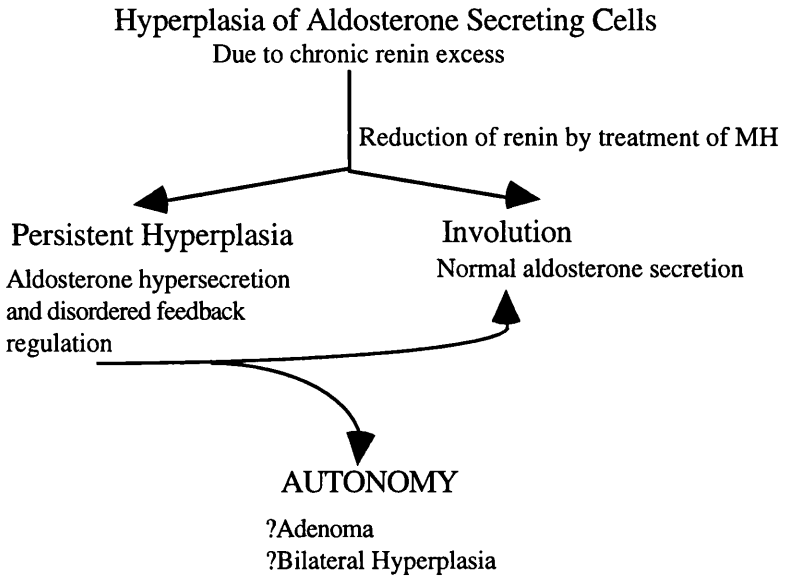


Figure 5.14: Possible role of the adrenal gland in the initiation and maintenance of malignant hypertension.

Finally, the high peripheral vascular resistance of the MH patient is highly responsive to ACE-inhibition (Saragoca et al. 1983) whilst often proving resistant to vasodilators such as nitroprusside (Rouby et al. 1982), suggesting that ATII may be driving the vasoconstriction of MH. In addition, the response of vascular resistance to captopril correlates closely with PRA prior to treatment (Saragoca et al. 1983). Treatment targeted against RAS in humans "may reverse the entire clinical syndrome of malignant hypertension" (Laragh 1992).

5.824 Local vs. Systemic RAS in the pathogenesis of MH

The above evidence argues strongly that RAS activation may be a key factor in the development of MH. This might involve:

(i) *Activation of the conventional systemic endocrine RAS, led by renin released from the JGA*

(ii) *Activation of local renal RAS, leading to*:

a) production of ATII which is directly pathogenic or which drives synthesis/release of other biologically active substances

b) production of RAS components (e.g. ATII or renin) of sufficient activity or concentration to exert an endocrine role

(iii) *Local vascular RAS activation in distant (i.e. non-renal) organs.* This in turn may be true intrinsic activation, or may be driven/modulated by endocrine RAS components (such a renin or ATII) of renal origin.

Increased circulating RAS activity in models of MH seems at least partly renally driven, given the findings of raised renal renin secretion and JGA hyperplasia and hypergranularity described above. In the dog with unilateral nephrectomy, chronic intrarenal arterial infusion of noradrenaline elevates PRA, and triggers an MH crisis which can be terminated by the cessation of infusion (Lohmeier et al. 1984). This may suggest that the RAS-dependent systemic increase in TPR, and the associated systemic MH changes, are due to a renally-derived substance. This agent, or its production, is sensitive to ACE-inhibition in humans, as TPR in MH is highly responsive to ACE-inhibition and may, under some circumstances, respond to nephrectomy (Saragoca et al. 1983). The SHR-SP becomes increasingly sensitive to the hypotensive actions of an ATII antagonist (1-sarcosine, 8-alanine), but increasingly resistant to the pressor effects of exogenous ATII infusion. Such data suggests that the hypertension of MH is driven by RAS activation, leading to an increase in circulating ATII (Shibota et al. 1979). PRA may rise without an increase in renal renin synthesis in DOCA-salt hypertensive animals, possibly through alterations in prorenin metabolism and renin glycosylation (Kim et al. 1994).

However, increased local tissue RAS activity may play a part in the development of some features of MH. In renovascular hypertension, Mai (Mai et al. 1995) suggests that 'gene expression ....(of RAS components)... is regulated by local rather than systemic factors'. In the salt-loaded SHRSP, bilateral nephrectomy reduces plasma renin and ATII, whilst leaving both the blood pressure and adrenal ATII still markedly elevated (Kim et al. 1991b), suggesting that (at least in the later stages) endocrine RAS activity is no longer a key component. After treatment of MH by blood pressure reduction, aldosterone levels may remain elevated despite a fall in PRA (McAllister et al. 1971). This dissociation of adrenal and circulating RAS activity together with the fact that, in the SHRSP, MH does not seem to be due to increased activation of circulating prorenin, may suggest that extrarenal RAS activity is involved in the pathogenesis of malignant hypertension (Kim et al. 1992).

That tissue RAS is crucial to the development of MH is strongly suggested by our study using the TGR(mREN2)27 rat. I have already described (chapter 2) the low circulating RAS activity during the BH phase, and how (during the malignant phase)

there is a sudden increase in plasma RAS activity. This is unlikely to be derived from the classical JGA route, as JGA hypoplasia is observed (chapter 2). The TGR itself has hypertension which is highly likely to be due to high local tissue (and probably vascular) RAS activity, and the sudden elevation in RAS components in the circulation during BH/MH transition may well be derived from the same source. Although renal tissue renin is suppressed, local ATII generation may in fact be very high due to the kinetics of action of mouse Ren-2 with rat angiotensinogen (Tokita et al. 1994b). In our study, the fact that tissue ACE inhibition prevented the development of both clinical and histopathological changes of MH confirms the importance of tissue RAS activity in this model, and its importance in initiating the MH phenotype. That this protective effect was due to inhibition of tissue ATII synthesis, and not inhibition of kinin metabolism, has not been shown. However, the studies in SHR-SP rats showing a similar protective effect of low-dose losartan support the former hypothesis.

But at what step might such inhibition of RAS (by ATII antagonism or ACE inhibition) be active?

5.825 Pathogenic mechanisms by which RAS activation may contribute to the malignant hypertensive process.

If RAS activation may mediate the clinical and histopathological changes of MH, through a number of mechanisms including a reduction in glomerular capillary ultrafiltration coefficient, glomerular cell hypertrophy and reduced mesangial cell function (MacGregor 1992).

Vascular Damage due to directly to ATII ATII may induce renal ischaemic damage by inducing renal afferent arteriolar vasoconstriction (Steinhausen et al. 1986). Through both an elevation in arteriolar lumenal pressure and wall permeability, ATII may contribute to the development of fibrinoid necrosis (Fornes, et al. 1993). Focal arteriolar dilatations are seen in the MH patient in which vascular pressure might be raised (Giese 1976). ATII might raise this pressure further by exacerbating distal vasoconstriction and increasing distal occlusion by promoting vascular smooth muscle cell hypertrophy (Geisterfer et al. 1988; Heagerty 1991). It might also increase transendothelial permeability by inducing endothelial cell constriction (Fornes, et al. 1993). ATII increases cardiac myocyte permeability and causes myocyte death (Tan et al. 1991). Similar actions on vascular smooth muscle cells might further weaken vessel wall integrity: indeed, the myocardiotoxic effects of ATII may be secondary to such a vasculotoxic effect (Tan et al. 1991). Our histopathological data suggest that such vascular damage may be prevented with low dose ramipril. However, a similar degree of such damage was seen in animals suffering MH as those who survived

untreated without an MH crisis, suggesting that such vascular damage is not the causal precipitant alone of an MH phase. The potent vasoconstrictor endothelin has been postulated to be a mediator of RAS-related tissue damage and hypertension in MH: RAS components may interact with both the expression of endothelin (which is increased in some models of hypertension during conversion to a malignant phase) (Kohno et al. 1991) and the renal medullipin system (which may be depressed by ATII) (Muirhead 1993). However, although renal endothelin-1 expression is increased in the TGR model of MH, endothelin-1 antagonists offer no protection against MH phenotype (Whitworth et al. 1995b).

Effects on local gene induction Renal levels of mRNA for collagen, fibronectin, and TGF-ß1 are increased in MH- a rise which can be significantly blunted by the use of ATII receptor antagonists and ACE-inhibitors. ATII may thus contribute to fibrinoid necrosis in MH through the local induction of specific gene pathways (Kim et al. 1994).

Effects of ATII on sodium handling Renal ATII might in theory cause sodium retention contributing to MH development. Through the action of local ACE, ATII is produced at the brush border (Ikemoto et al. 1986b) which may bind to local receptors (Brown and Douglas 1982; Cox et al. 1983) to promote sodium resorption(Harris and Navar 1985). Against this hypothesis, ACE inhibition and ATII receptor antagonism both reduce renal sodium loss in the TGR (Hirth-Dietrich et al. 1994). However, a herald natiuresis and diuresis marks the transition from BH to MH (Kincaid-Smith et al. 1958). Although the potential sodium conservation in the TGR during BH makes it unlikely, a role for a sudden local ATII activation causing sodium loss cannot be discounted.

Glomerular hyperfiltration injury Renal ATII may cause glomerular hyperfiltration injury through effects on glomerular mesangial cells (which are sites of renin synthesis, contractile, ATII-responsive, and which may regulate glomerular pressure and filtration) (Dzau and Kriesberg 1986) and through selective vasoconstriction of the efferent arteriole (Steinhausen et al. 1986). However, RAS inhibition protects against many of the histopathological effects of hypertension in rat chronic renal failure without any effect on glomerular pressure (reviewed in (Kim et al. 1994)). The sudden natiuresis and diuresis of the transition to MH, thought to be due to hyperfiltration injury, may itself be pathogenic by increasing activation of the RAS cascade. Thus, in the SHR, 5/6 nephrectomy leads to loss of renal vascular autoregulation and the appearance of the classical renal histological features of MH (despite no further rise in BP) (Bidani et al. 1994). Renal histological changes and

increasing proteinuria in 400g (approx. four month old) TGR rats are likely consequences of early glomerular damage (Chung et al. 1993) of the type identified ultrastructurally in older rats (Bachmann et al. 1992). The pattern of nephron injury associated with MH was prevented by low dose ramipril in our study. These data support the concept that RAS-dependent hyperperfusion is the final mechanism of injury (although this may of course be triggered by initial vascular ischaemic damage) (Bidani et al. 1994). However, once again this is not the sole cause of the MH crisis, as similar nephron damage was seen in those animals developing MH as those untreated animals who survived without an MH crisis. The latter, however, were much older, suggesting perhaps that the nephron injury had been accelerated in the MH group. Whether ACE-inhibition enhances renal autoregulatory capacity is unclear although it has been reported that the cerebral autoregulatory range is increased in hypertensive patients treated with ACE-inhibition(Paulson, Vorstrup et al. 1985).

5.9: IN CONCLUSION

The TGR(mREN2)27 male heterozygote is highly sensitive to ACE-inhibition, with doses as low as 10μg/kg/day lowering SBP. Such data support a role for RAS activity in the generation of the hypertensive phenotype. A dose of 1mg/kg/day was shown to reduce blood pressure to that of control SD rats, whilst 5μg/kg/day left blood pressure unaffected. By contrast, blood pressure is highly resistant to the actions of vasodilating agents. Hydrallazine- even at very high dose- has little effect at all on SBP. Amlodipine does reduce systolic blood pressure, but is unable to reduce blood pressure to control levels.

Sustained elevated blood pressure is necessary for the development of MH in this model. in combination with specific genetic background or a variety of trigger factors, may cause malignant hypertension. The likely initiator of the MH cycle is the development of vascular damage within the kidney, although other factors are evidently also important.

Activation of circulating, renal, and non-renal (e.g. adrenal) RAS accompanies MH. MH syndrome can be caused by administration of RAS components and can be prevented by ACE-inhibitors or ATII antagonists. ATII may cause the profound vasoconstriction and accelerating hypertension of MH. It may also increase intraluminal pressure and arteriolar permeability, yielding a vascular filtration injury. A profound natiuresis and diuresis heralding the onset of MH will further drive RAS activation(Giese 1976), as will afferent arteriolar fibrinoid necrosis (as suggested by the hyperplasia of the renin containing cells of the JGA)(Gardiner and Lindop 1992). In the TGR the JGA is essentially obliterated by smooth muscle cells, and local vascular ATII generation is thus postulated as the cause of this effect in the TGR. It would be of importance, in further studies, to demonstrate whether RAS activation precedes the natiuresis seen in some models. This would be difficult however, given the unpredictable timing of onset of MH, the difficulty in quantifying renal flow and water loss, and the very rapid sudden and severe pattern followed by MH (in our model, profound hypertension leading to severe dehydration and polyuric renal failure occurs within 6 hours).

Further studies are required in order to elucidate the point at which pharmacotherapy directed against RAS is effective in the MH cycle. Does it offer a vasculoprotective effect, thus preventing the initiation of MH by blocking vascular injury in response to hypertension? Does it prevent triggering of RAS activation through inhibition of renal ATII/ACE/renin feedback? Does it allow the original RAS activation, but prevent the pathological actions of ATII generation? Our data

suggest that the actions of ramipril are targeted early in the MH cascade, given that no evidence of diuresis/weight loss/dehydration was observed at any time-point in our animals. ACE-inhibition might therefore be the treatment of choice for hypertension if MH is to be prevented. Low-dose ACE-inhibition might also have advantages in the treatment of established MH. An intervention study of treatment of the TGR in its malignant phase is thus planned to help clarify this point.

CARDIAC HYPERTROPHY IN THE TGR(mREN2)27 RAT

6.1: INTRODUCTION

6.11: BACKGROUND

Considerable evidence suggests that renin-angiotensin system activity my regulate left ventricular growth in both physiological and pathophysiological situations (chapters 1 and 10). Such cardiac growth comprises both an increase in left ventricular myocyte mass, and an increase in collagen matrix accumulation (chapters, 6, 8, 10), and RAS activity may thus influence either or both of these components.

6.12 CARDIAC HYPERTROPHY IN THE TGR (mRen-2d)27 RAT

The severe hypertensive phenotype of the Ren-2 rat may make this animal a potentially useful model of hypertensive cardiac hypertrophy. Villarreal has demonstrated an increase in LV mass of 30% at 4 months (Villarreal et al. 1995), and Bachmann has also noted cardiac hypertrophy in animals of up to 8 months of age (Bachmann et al. 1992). However, no studies have accurately assessed the contribution of myocyte hypertrophy or collagen deposition to this hypertrophy, and none has utilised the model to address the role of RAS in the generation of this hypertrophic response.

6.2: STUDY AIMS

This study was performed to address 2 issues:

1. To confirm the presence of left ventricular hypertrophy in the TGR(mREN2)27 rat
2. To characterise the nature of this hypertrophy in terms of both total LV mass changes, and changes in LV collagen content
3. To examine the role of tissue renin-angiotensin systems in the control of LV hypertrophy in this model.

6.3: METHODS

Animals were studied at two timepoints: 70 and 120 days.

6.31: TREATMENT GROUPS

These comprised heterozygote male TGR(mREN2)27 rats (TGR) and normotensive Sprague Dawley control strain animal (SD).

6.311 70 day timepoint

Group 1: Untreated TGRs (n= 6)
Group 2: TGRs treated with ramipril 5 µg/kg/day (n= 6)
Group 3: SDs (n= 6)
Group 4: TGRs treated with ramipril 1mg/kg/day (n= 6)

On the basis of preliminary studies (chapter 5), doses were selected which yielded similar blood pressures in a treated and untreated TGR group (groups 1 and 2), and in a treated TGR group and SDs (groups 3 and 4).
The animals for this study were drawn from those used in the studies of the responses to hypotensive agents described in chapter 5.

6.312 120 day timepoint

As for the 70-day timepoint, four groups were studied:

Group 1: Untreated TGRs (n=19 survivors of 43 weaners)
Group 2: TGRs treated with ramipril 5 µg/kg/day (n=12 survivors of 14 weaners)
Group 3: SDs (n=12 survivors of 12 weaners)
Group 4: TGRs treated with ramipril 1mg/kg/day (n=13 survivors of 13 weaners)

Drug doses were adjusted thrice weekly, blood pressures recorded weekly, and animals housed as previously described (chapter 3). At the end of each study period, the animals were anaesthetised and killed by aortic exanguination as previously described (chapter 5).
Comparison of groups 1 and 3 would allow the demonstration and quantification of the degree of cardiac hypertrophy in TGRs when compared to their normotensive control strain. Meanwhile, comparison of group 1 with group 2, and of group 3 with group 4 would allow the direct effects of the hypertensive phenotype to be distinguished from the effects of RAS activity. Each pair has an identical blood pressure, but one of each pair (groups 2 and 4 respectively) underwent ACE inhibition. Differences within each pair are therefore not due to alterations in SBP, but to ACE-inhibition.

6.32: ASSESSMENT OF LV HYPERTROPHY

6.321 Tissue Harvesting

All animals were weighed immediately prior to death. After exanguination, six animals were randomly selected from each study group. These were perfused for 5 minutes with 4% paraformaldehyde/0.1% glutaraldehyde solution (160mmHg for groups 1 and 2, and 120mmHg for groups 3 and 4). The heart was removed, freed from surrounding tissues and the atria removed. The right ventricle (RV), and the left ventricle and septum (LV) were dissected free, blotted dry, weighed (wet weight) and then stored in fixative for assay of collagen.

6.322 Assay of ventricular collagen content

Interstitial cardiac collagen has 3 polypeptide a-chains which contain a high proportion of hydroxyproline. This imino acid is present in low concentration in only a few other proteins (such as elastin), and measurement of hydroxyproline content may therefore be used to assay cardiac collagen content (Bishop and Laurent 1995). Ventricular collagen content and concentration was determined using reverse phase HPLC assay of hydroxyproline as described by Campa et al (Campa et al. 1990). The LV and RV were blotted dry and weighed. A portion of ventricle of approximately 50mg weight was dried in an oven at 50^0C overnight to a constant weight, and hydrolysed in 1ml of 6M hydrochloric acid at 110^0C for 16 hours. Hydrolysates were decolourised with 70mg activated charcoal and fixative removed by mixing with anion exchange resin (70mg Dowex-AG2-XGS mesh 100-200 chloride form, Bio-Rad, UK). To remove the charcoal and resin, the hydrolysate was filtered (Millipore type GA pore size 0.65μm). A 10μl aliquot of supernatant was evaporated to dryness under a vacuum on a sample concentrator (Savant Speedvac Plus SC110 AR; Life Science International, Basingstoke, Hampshire, UK), the residue redissolved in 100μl HPLC grade water, buffered with potassium tetraborate (100μl: 0.4M: pH9.5: Sigma) and reacted with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl, Sigma) in methanol (100μl, 36mM). Samples were protected from the light and incubated at 37^0C for 20 minutes, and the reaction stopped by addition of hydrochloric acid (50μl, 1.5M). Sodium acetate (150μl: 167mM: pH6.4: Fisons, Loughborough, UK) in acetonitrile (26% vol/vol) was added, and samples filtered (Millipore type GV, 0.22μm pores). A 100μl aliquot (of a total derived mixture volume of 500μl) was loaded onto the column (LiChroCART LiChrospher 250mm length x 4mm diameter: 5μm particle size, 100RP-18) and eluted with an acetonitrile gradient as described (Campa et al. 1990) using the Beckman System Gold, High Wycombe, UK). The hydroxyproline content of each sample was

determined by comparing peak areas of samples from the chromatogram (achieved by monitoring the absorbance at 495nm) to those generated from standard solutions handled under identical conditions (Concentration OHPro standard solution =25nm: 10µl of this is handled as above to yield 500µl mixture as before, and 100µl loaded onto the column. Hence, 50pmol OHPro is loaded onto the column, and this equated to 50 absorbance units (HPLC units), and 1HPLC unit = 1 pmol OHPro). Collagen concentration and content were calculated using the following formulae, assuming a molecular weight for hydroxyproline (OHPro) of 131.1 and the proportion of collagen that is OHPro (by weight) to be 12.2%.

nmols OHPro=
HPLC units (pmol) x [500µl (total volume derived mixture)/100µl (injectate volume)]
x [1000µl (hydrolysate volume)/10µl (volume dried down)] x 0.001
and thus
=> nmols OHPro = HPLC units/2

Now collagen content (mg in hydrolysate) = [nmol OHPro/10^{6}] x 131.1 x [100/12.2]
Collagen concentration (mg/g/dry tissue) = collagen content (mg)/dry weight (g) and ventricular collagen content = collagen content of sample (mg) x [wet weight of ventricle/wet weight of sample].

6.33 MEASUREMENT OF PLASMA AND TISSUE ACE ACTIVITY

After exanguination, 6 animals from each group at the 120 day timepoint were used for assay of plasma and tissue ACE activities, as previously described (chapter 3).

6.34: STATISTICAL ANALYSIS

The effects of different drug doses (comparison across treatment groups) was examined by ANOVA and Fischer's test. Individual comparison of LV mass and collagen data between groups 1 and 2, and between groups 3 and 4 were made by student's t-test. A value of $p<0.05$ was taken as statistically significant.

6.4: RESULTS

6.41: SURVIVAL DATA

As predicted from our previous study (chapter 5), there was a significant (56%) mortality amongst the untreated animals. The majority of these deaths were attributable to a classical malignant hypertensive phase. However, amongst the survivors of the MH risk period, some went on to die suddenly later in life (day 76, 106 and 111: figure 6.1). There was no mortality from malignant hypertension in animals treated with ramipril 5μg/kg/day, although again two later sudden deaths (14% mortality) occurred (at 86 and 123 days). There was no mortality amongst the control or normotensive TGR groups (i.e. treated with ramipril 1mg/kg/day).

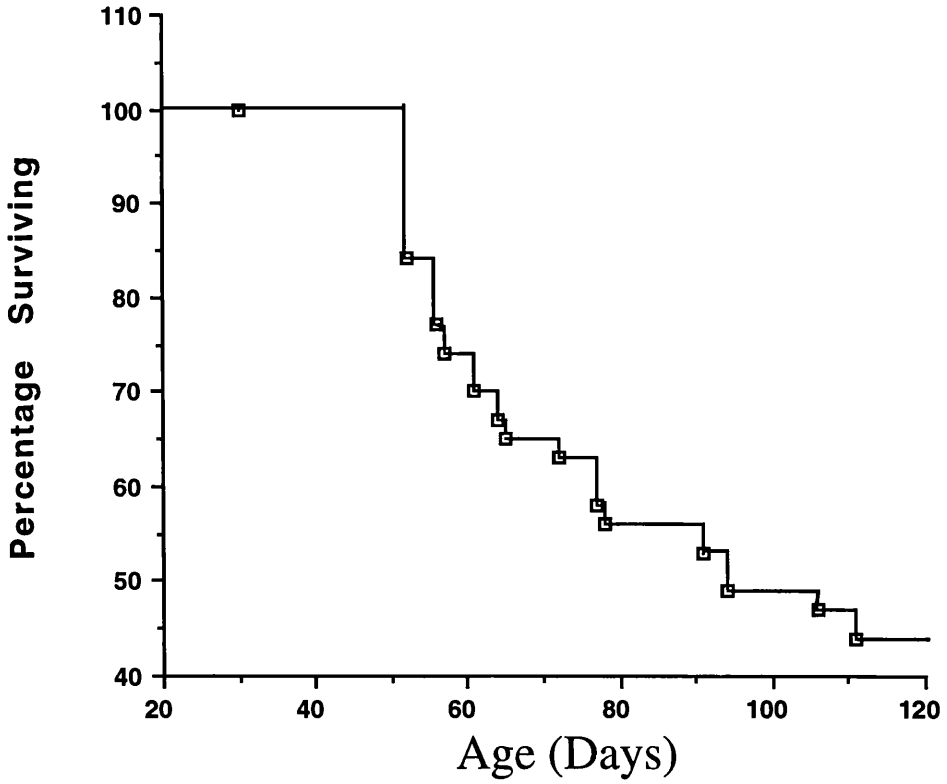


Figure 6.1: Progressive mortality amongst untreated heterozygote male TGR(mRen-2d)27 rats over a 120 day period.

6.42: BLOOD PRESSURE DATA

Blood pressure had been shown to be similar between groups 1 and 2, and between groups 3 and 4 (chapter 5). This matching of mean systolic blood pressures continued in those animals studied up to 120 days (figure 6.2). Mean SBP was significantly greater in the groups 1 and 2 than in groups 3 and 4 ($p<0.0001$ for lifetime mean BP: $p<0.05$ for comparisons at each timepoint). Mean SBPs were similar when groups 1 and 2 were compared, and when groups 3 and 4 were compared ($p>0.5$ for mean lifetime SBP). Blood pressure in group 2 was significantly lower than that in group one only on day 35 ($p=0.004$) although non-significantly lower on days 49 and 56 ($p=0.069$ and 0.131 respectively). Ramipril 1mg/kg/day (group 4) was only associated with a statistically significantly lower blood pressure than that in controls on days 35 ($p=0.001$) and 112 ($0=0.035$), and bordered on significance on day 119 ($p=0.051$). Mean SBP was actually slightly higher in group 4 than group 3 on three occasions (table 6.1).

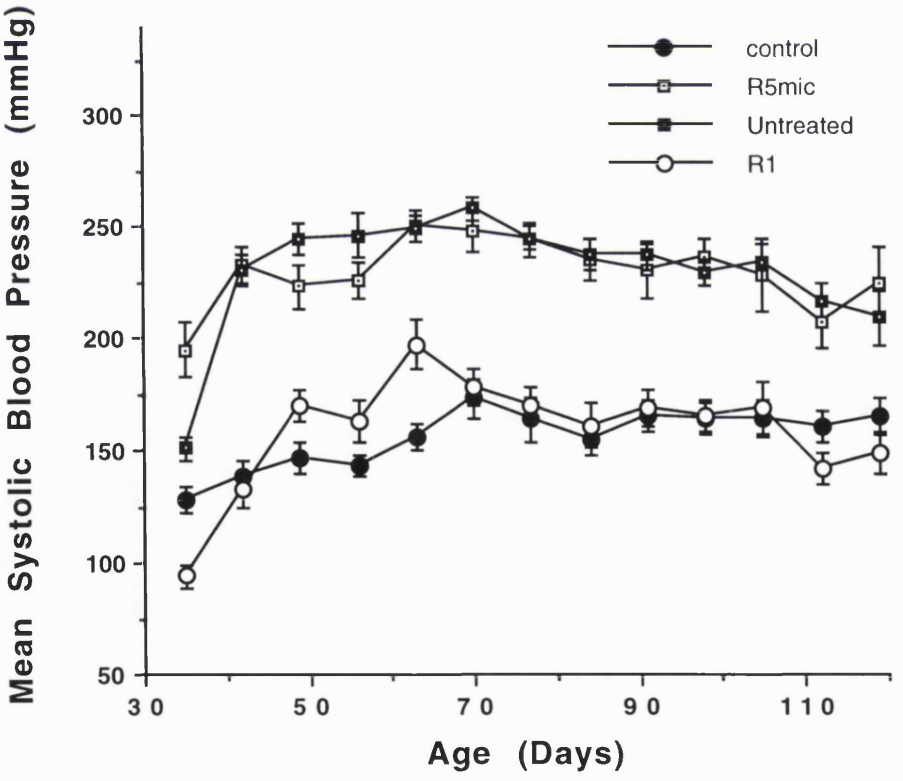


Figure 6.2: Mean systolic blood pressures (±SEM) for untreated heterozygote male TGR(mREN2)27 rats (untreated), similar animals treated with ramipril 1mg/kg/day and 5µg/kg/day (R1 and R5mic respectively), and control Sprague Dawley rats (controls).

Animal age	Group 1	Group 2	Group 3	Group 4
35	151±5	195±12	128±6	94±5
42	230±7	233±8	138±7	133±8
49	245±7	223±10	147±7	170±7
56	246±10	226±8	143±5	163±9
63	249±6	250±7	156±6	197±11
70	258±5	248±5	173±9	178±8
77	245±5	244±8	164±10	170±8
84	237±7	235±9	155±7	161±10
91	237±6	230±12	165±7	169±8
98	229±6	236±9	164±7	165±7
105	234±8	228±16	164±8	169±12
112	217±8	207±11	161±7	142±7
119	209±12	225±16	165±8	149±9

Table 6.1: Mean (±SEM) systolic blood pressures (mmHg) at different ages for each treatment group (see text for details).

6.43: ACE ACTIVITY

Due to technical difficulties, tissues ACE activity was available in tissues from only 3 of the control animals. Data are presented graphically below (figures 6.3 a-f), and numerically in table 6.2.

Treatment with ramipril inhibited plasma and tissue ACE significantly, with greater inhibition seen at greater dose (p for heterogeneity for groups 1, 2 and 4 being 0.014, 0.0004. 0.0015, 0.001 and 0.0001 for renal, RV, LV, aortic and carotid ACE activity respectively). However, plasma ACE activity was actually higher in the low dose group (group 2: p 0.001 for both comparisons). Tissue ACE activity was lower in group 4 than group 2 (i.e. 1mg/kg than 0.5μg/kg), but statistically higher in group 4 than group 2 in plasma ($p<0.001$).

Tissue	Group 1	Group 2	Group 3	Group 4
Plasma	34.1±3.9	56.9±1.8	92.3±4.5	23.6±2.8
Renal	0.76±0.08	0.44±0.02	0.931±0.23	0.41±0.11
RV	4.11±0.41	1.68±0.47	2.13±0.79	0.90±0.23
LV	2.94±0.32	1.28±0.14	2.20±0.58	0.95±0.16
Aorta	152.38±17.7	55.9±9.4	108±35.88	23.95±3.75
Carotid	119.63±11.8	45.83±7.39	39±6.41	8.43±1.53

Table 6.2: Plasma and tissue ACE activity amongst different treatment groups at 120 days

We had succeeded in producing similar blood pressures in groups 1 and 2, and groups 3 and 4, and planned for the second of each pair of groups to have lower tissue ACE activity. As planned, ACE activity was lower in group 2 than group 1 (p 0.011, 0.006, 0.009, 0.003 and 0.002 for renal, RV, LV, aortic and carotid ACE activity respectively), although higher in plasma (p=0.0001). ACE activity was lower in group 4 than group 3 (i.e. animals treated with ramipril 1mg/kg than untreated controls), but due to the small numbers of control tissues ACE levels available (n=3), these comparisons only reached statistical significance for the carotid samples (p 0.1, 0.11, 0.15, 0.13, 0.16, and 0.015 for plasma, renal, RV, LV, aortic and carotid ACE activity respectively).

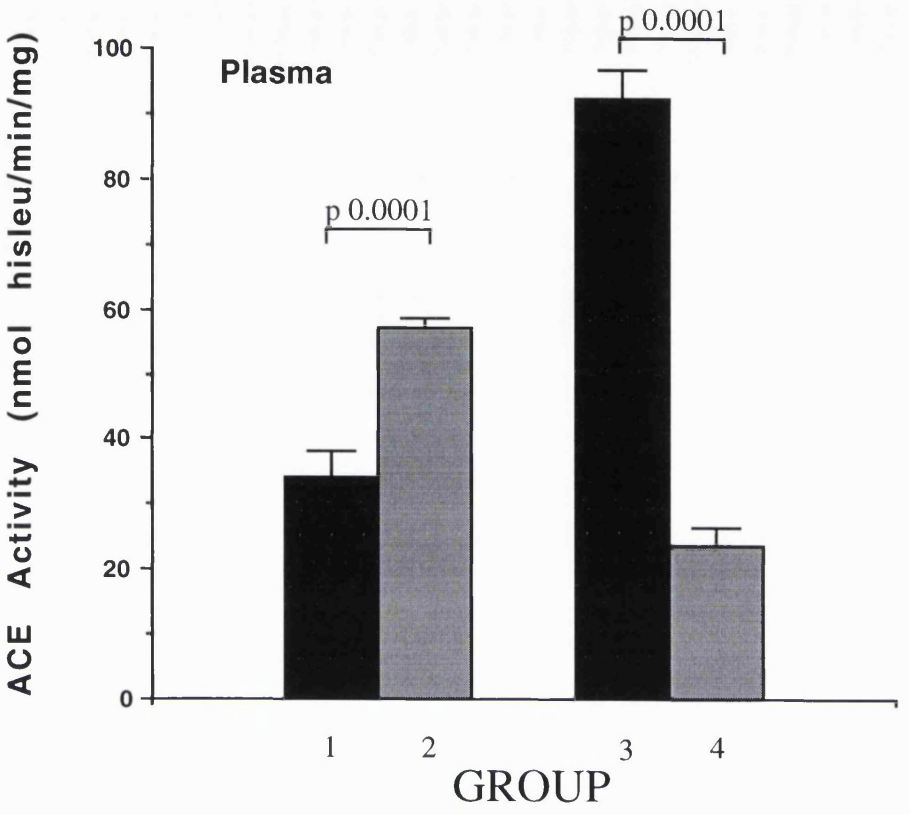


Figure 6.3a: Mean plasma ACE activity (±SEM) by treatment group.

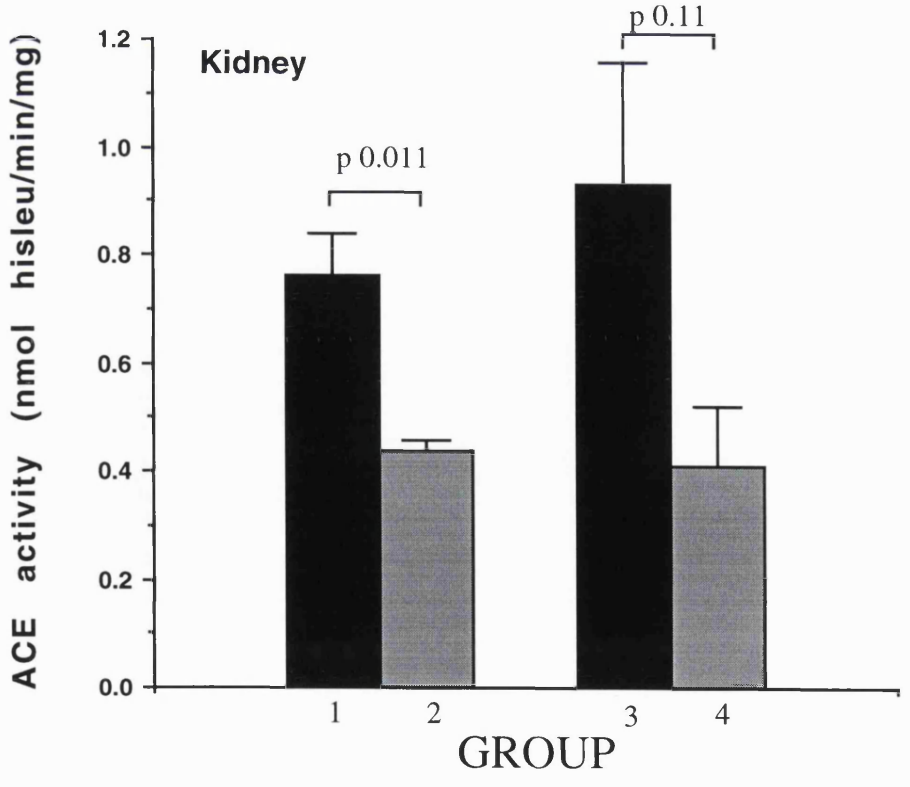


Figure 6.3b: Mean renal ACE activity (±SEM) by treatment group.

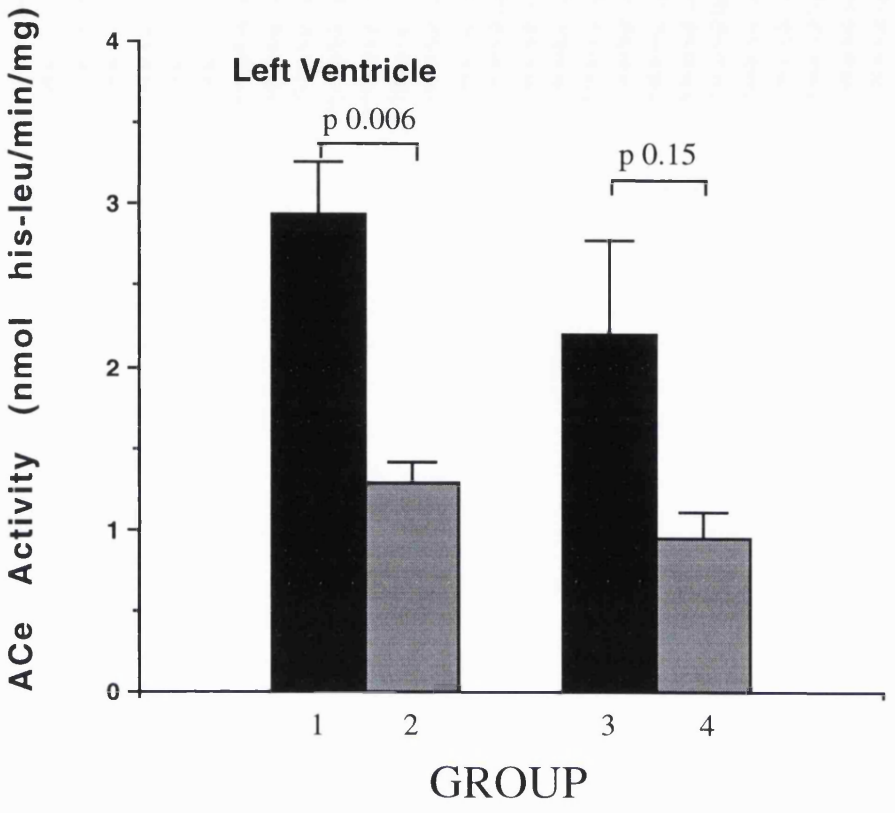


Figure 6.3c: Mean left ventricular ACE activity (±SEM) by treatment group.

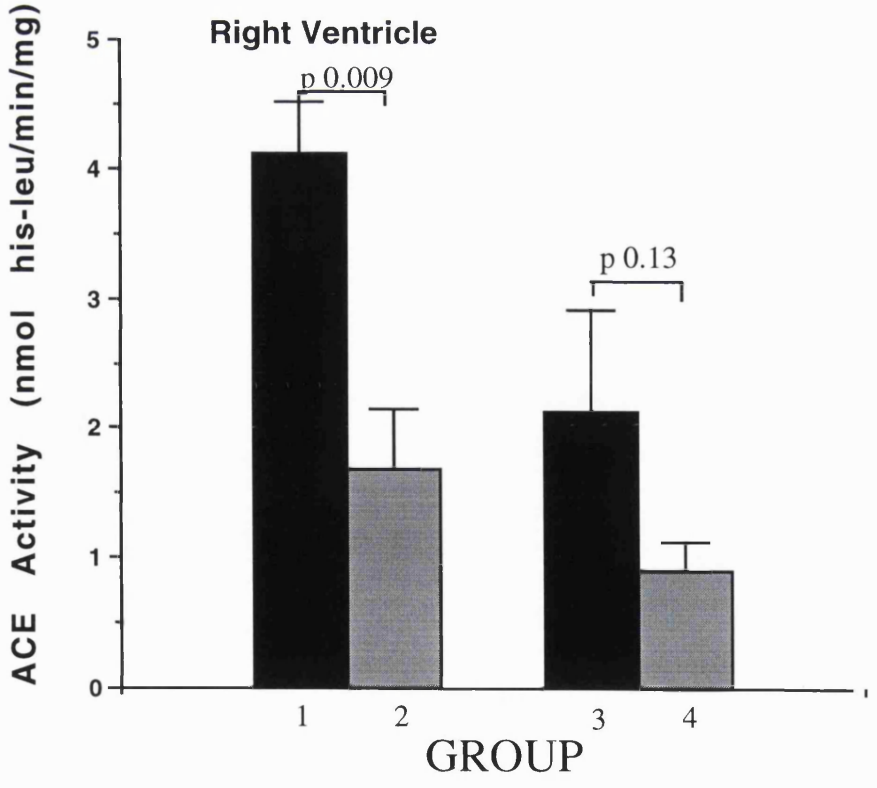


Figure 6.3d: Mean right ventricular ACE activity (±SEM) by treatment group.

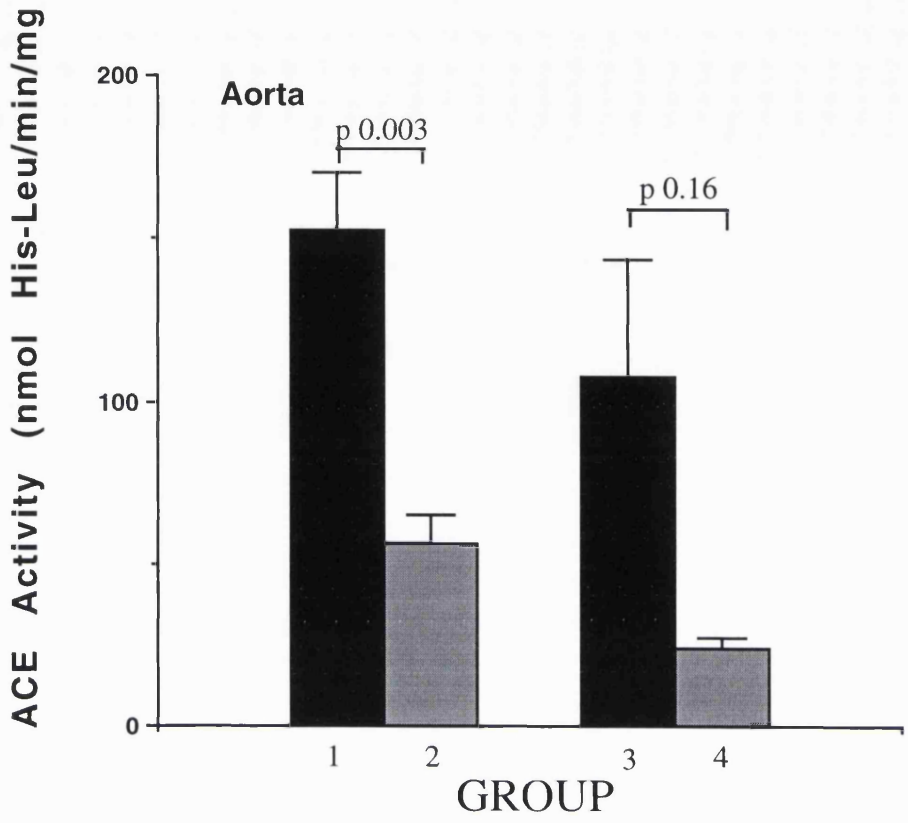


Figure 6.3e: Mean aortic ACE activity (±SEM) by treatment group.

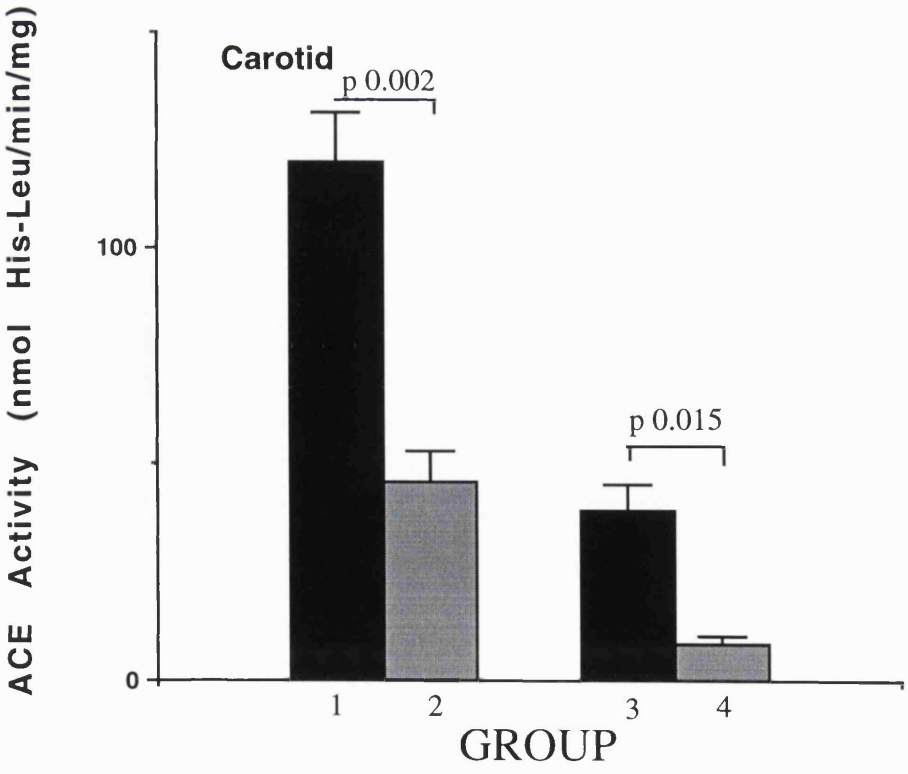


Figure 6.3f: Mean carotid ACE activity (±SEM) by treatment group.

6.44: BODY WEIGHTS

Body weights were similar at 70 days. However, at 120 days weights differed significantly but seemed unrelated to blood pressure burden or to ACE-inhibition (533±11.2, 477±15.5, 427±23.3, 533±3.1g for groups 1, 2, 3 and 4 respectively: p for heterogeneity <0.0001: p<0.05 for comparisons of group 1> 2 and 3:, and group 3< 1, 2 and 4)

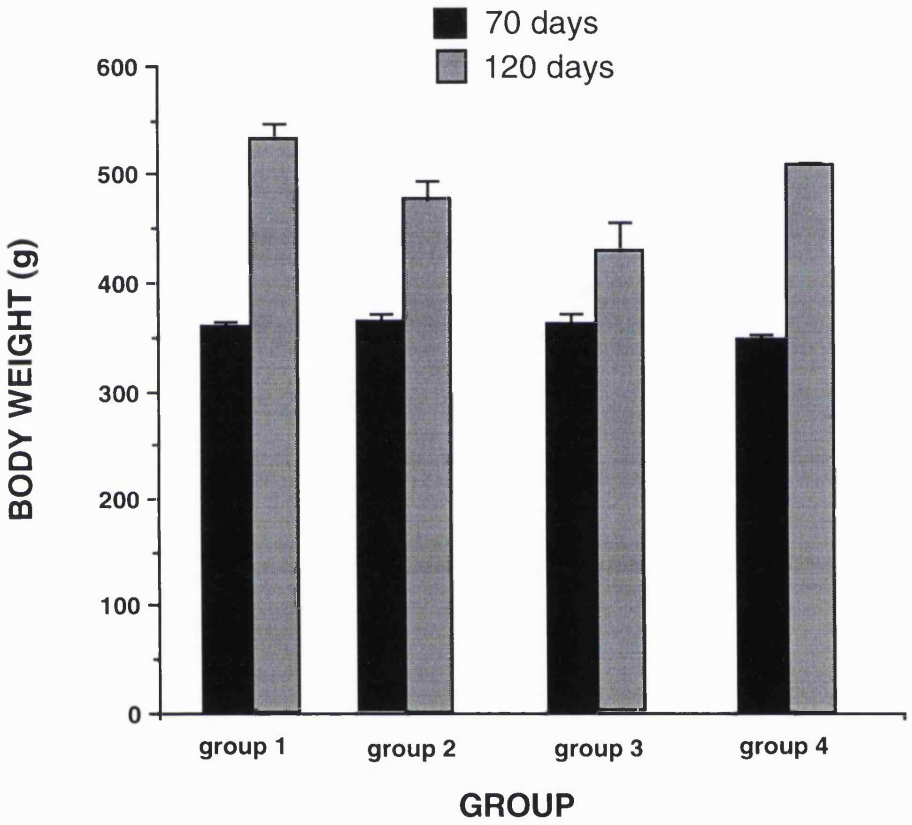


Figure 6.4: Mean body weights (±SEM) of animals in each treatment group at age 70 and 120 days.

6.45 CARDIAC HYPERTROPHY IN THE TGR(mREN2)27 RAT

6.451 Left ventricle

Significant left ventricular hypertrophy is seen in untreated TGR(mREN2)27 rats when compared to controls (groups 1 and 3 respectively) at both 70 and 120 days (figure 6.5). This difference remains after adjustment for body mass (3.66±0.05 vs. 2.69±0.05 g/kg at 70 days, p<0.0001: and 2.87±0.20 vs. 2.57± 0.10g/kg, p=0.04 for groups 1 vs. 3 respectively). When compared to group 3, LV collagen content in group 1 is raised by 9% at 70 days and 90% at day 120 (4.51±0.55 vs. 4.14±0.19mg, [NS]: 8.74±0.44 vs. 5.16±0.67mg respectively, p<0.002), and this difference also persists after adjustment for body weight (12.83±1.5 vs. 12.14±0.6mg/kg body wt at 70 days: 18.08±1.7 vs. 12±1.1 mg/kg at 120 days, p=0.01).

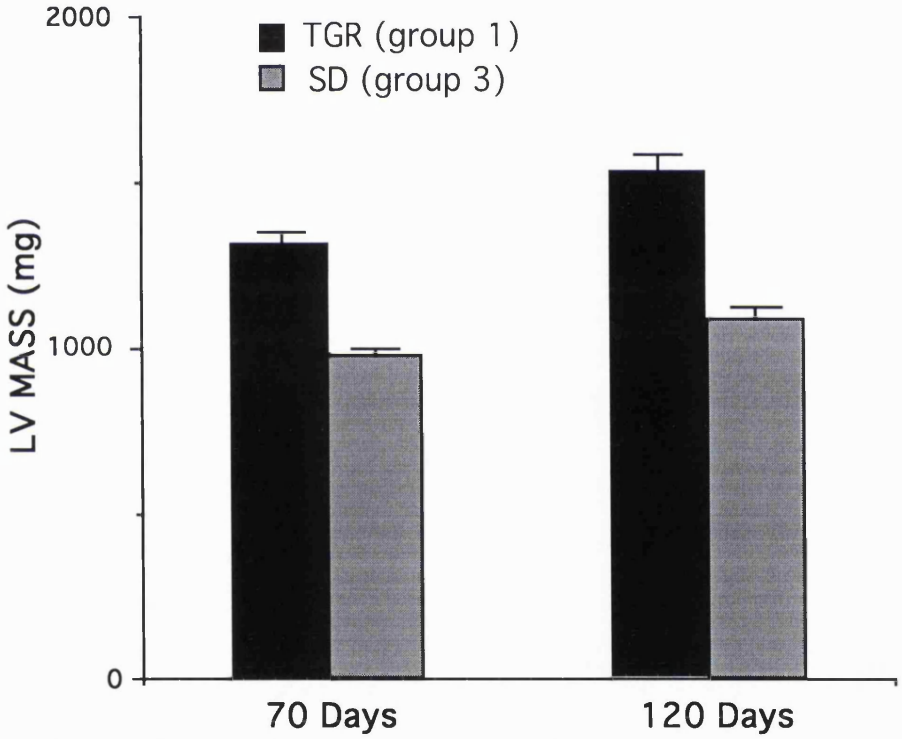


Figure 6.5: Mean (±SEM) LV mass (mg) of untreated heterozygote male TGR(mREN2)27 rats (TGR, group 1) compared to normotensive Sprague Dawley control animals (SD, group 3).

6.452 Right Ventricle

RV masses are similar in groups 1 and 3 , with differences depending largely on differences in animal weight. This holds true at both 70 days (214±7 vs. 236±11g unadjusted, p=0.1: 0.6±0.02 vs. 0.65±0.03g/kg body weight adjusted, p=0.15) and 120 days (301±56 vs. 267±9g, and 0.56±0.1 vs. 0.63±0.03g/kg body weight

adjusted) for group 1 vs. 3. RV collagen content is initially higher in group 3 than group 1 (at 70 days: 1.39±0.17 vs. 1.8±0.12 mg, p=0.04), even after adjustment for body weight (3.91±0.49 vs. 5.36±0.35mg LV collagen/kg body weight, p=0.04). At 120 days, however, the situation is reversed with unadjusted and adjusted RV collagen content being non-significantly greater in the group 1 than 3 (unadjusted: 2.49±0.37 vs. 1.79±0.15mg, p=0.07; adjusted: 4.66±0.59 vs. 4.19±0.17mg/kg body weight, p=0.42).

6.46: THE EFFECTS OF ACE-INHIBITION

6.461 Left Ventricle

ACE inhibition is associated with a reduction in LVH at both 70 and 120 days (p for heterogeneity <0.0001 at both timepoints: p <0.05 for group 1 vs. group 4 and group 1 vs. 2). (figure 6.6).

This effect was independent of body mass (data adjusted for body mass: 3.66±0.05 vs. 3.45±0.03 vs. 2.36±0.04g/kg at 70 days, and 2.87±0.1 vs. 3.03±0.03 vs. 1.82±0.03g/kg at 120 days: p for heterogeneity <0.0001 in both cases).

LV collagen content was also reduced by ACE-inhibitor treatment, with the effect becoming more noticeable over time. At 70 days, LV collagen contents were 4.57±0.55, 3.45±0.09 and 3.81±0.21mg for groups 1, 2 and 4 respectively (p for heterogeneity 0.065: group 1 vs. group 4 p<0.05), whilst at 120 days the respective contents were 9.65±0.44, 7.10±0.31 and 4.28±0.24mg (p<0.0001: p<0.05 for all comparisons). This effect was also independent of body mass, with p<0.05 for all comparisons at 120 days, and p for heterogeneity <0.0001 (figure 6.7)

6.462 Right Ventricle

At 70 days, RV mass is similar in groups 1, 2 and 4 respectively (214±7.0 vs. 226±4.0 vs. 217±7.0mg: p=0.32). However, by 120 days, ACE inhibition is associated with a relative reduction in RV mass (301±56 vs. 247± 11 vs. 234±6 mg: p 0.2) and collagen content (2.49±0.33 vs. 1.83±0.11 vs. 1.53±0.08 mg: p0.01). This effect is independent of body mass for both RV mass (at 120 days : 0.56±0.1 vs. 0.52±0.01 vs. 0.46±0.01 :p<0.05) and collagen content (4.66±0.59 vs. 3.83±0.57 vs. 3.02±0.40mg/kg: p=0.01)

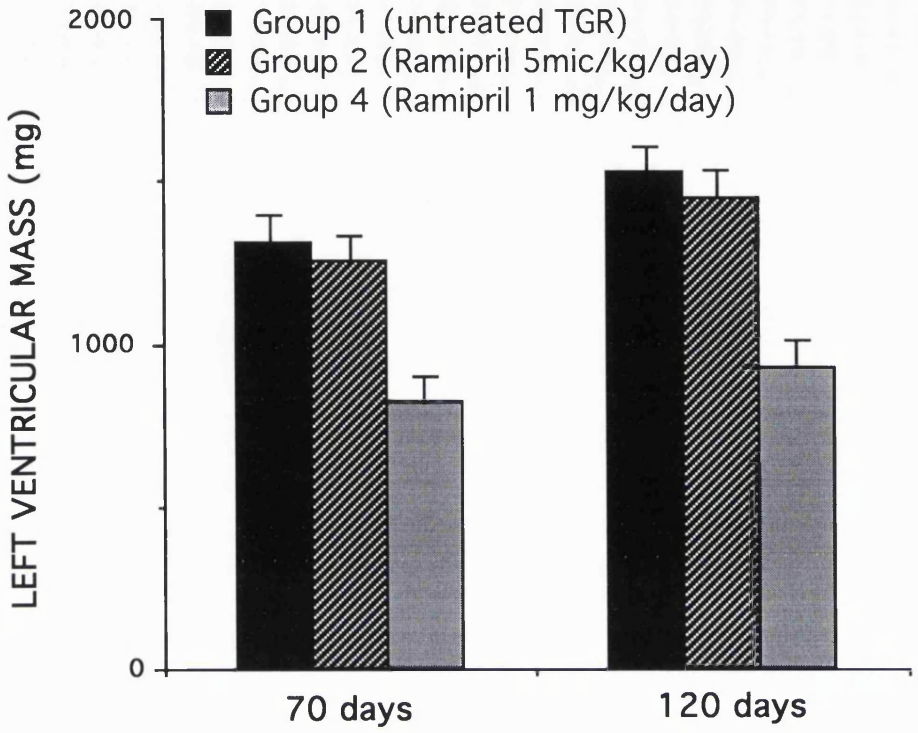


Figure 6.6 Effects of increasing dose of the ACE-inhibitor Ramipril on the left ventricular masses of heterozygote male TGR(mREN2)27 rats.

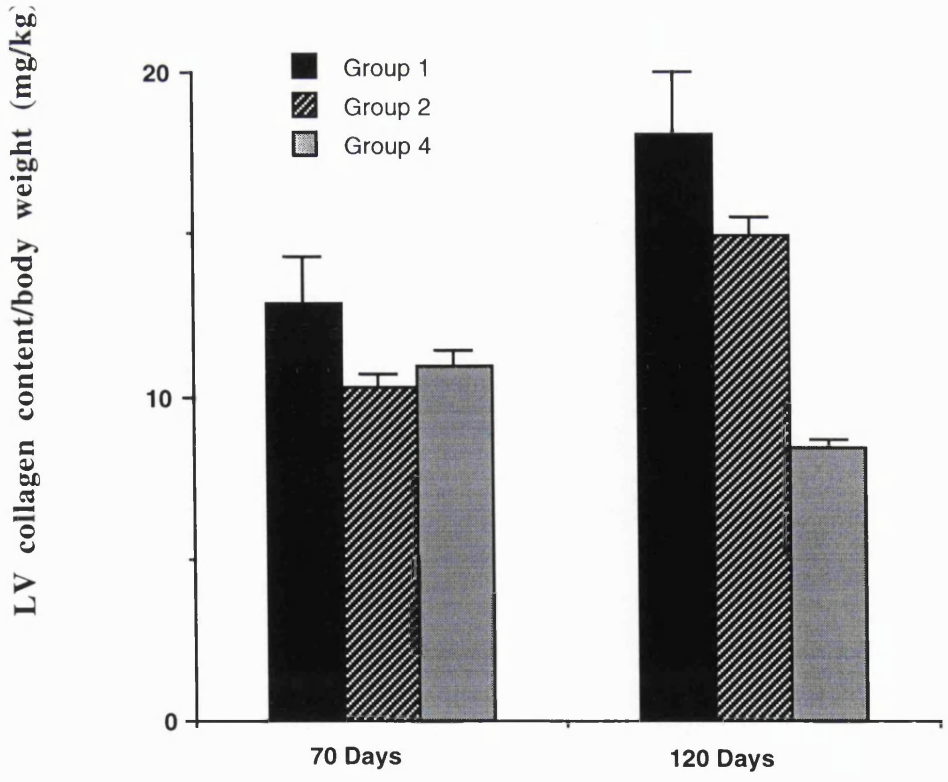


Figure 6.7: Effect of Ramipril treatment on left ventricular collagen content, when adjusted for body mass.

6.47 THE NON-HYPOTENSIVE MECHANISM OF ACTION OF ACE-INHIBITION

Systolic pressure burden was identical in paired groups 1 and 2, and groups 3 and 4. In each pair, one group had lower ACE activity than the other (groups 2 and 4) through treatment with ramipril (see above). Comparison of these paired groups thus allows us to distinguish non-hypotensive actions of ACE inhibition.

Hypertensive animals (groups 1 and 2) were initially compared (table 6.3). In every case, parameters of hypertrophy were lower in the ramipril-treated animals than in control SD animals, although these differences did not quite reach statistical significance in most cases. p-values for each comparison are given in table 6.4

Ventricle	Parameter	Time	Unadjusted		Adjusted	
			Group 1	Group 2	Group 1	Group 2
LEFT	Mass	70	1312	1250	3.66	3.45
		120	1529	1444	2.87	3.03
	Collagen	70	4.57	3.45	12.83	10.26
		120	9.65	7.10	18.08	14.92
RIGHT	Mass	70	214	226	0.6	0.62
		120	301	247	0.56	0.52
	Collagen	70	1.39	1.17	3.91	3.25
		120	2.49	1.83	4.66	3.83

Table 6.3: Effect of low dose ramipril (group 2) on mean left ventricular mass (mg) and collagen content (mg) in heterozygote male TGR(mREN2)27 rats. Group 1 are untreated animals. Data presented in unadjusted form, and after adjustment for animal body mass.

Parameter	Timepoint	Unadjusted	Adjusted
LV Mass	70	0.20	**0.02**
	120	0.2	0.10
LV Collagen	70	**0.05**	0.13
	120	**0.02**	0.09
RV Mass	70	0.15	0.19
	120	0.27	0.59
RV Collagen	70	0.27	0.27
	120	0.07	0.18

Table 6.4: p-values for comparisons between groups 1 and 2, of left ventricular mass and collagen content. Comparisons made for both unadjusted data, and data adjusted for animal body mass.

Normotensive animals were now compared (groups 3 and 4: control SD and TGR treated with ramipril 1 mg/kg/day respectively: table 6.5).

Ventricle	Parameter	Time	Unadjusted		Adjusted	
			Group 3	Group 4	Group 3	Group 4
LEFT	Mass	70	975	821*	2.69	2.36*
		120	1087	926+	2.57	1.82*
	Collagen	70	4.1	3.8	11.42	10.95
		120	5.16	4.28+	12	8.42+
RIGHT	Mass	70	236	217*	0.6	0.63
		120	267	234+	0.63	0.46*
	Collagen	70	1.83	1.69	5.04	4.87
		120	1.79	1.53	4.19	3.02*

Table 6.5: Mean LV mass and collagen content amongst normotensive animals (group 3= Sprague Dawley controls: group 4 = heterozygote male TGR(mREN2)27 rats treated with Ramipril 1mg/kg/day). Statistical significance of comparisons between groups at each timepoint is shown by the following symbols:

* $p<0.0001$ * $p< 0.001$

+ $p< 0.01$ + $p <0.05$

6.5: DISCUSSION

These data support a role for tissue RAS in the control of both physiological cardiac growth and pathophysiological cardiac hypertrophy. Both tissue aspects of growth (increase in myocyte and collagen mass) seem affected by ACE inhibition.

6.51: MALIGNANT HYPERTENSION

As expected (chapter 5), there was a significant (56%) mortality amongst the untreated animals. The majority of these deaths were attributable to a classical malignant hypertensive phase. However, amongst the survivors of the MH risk period, some went on to die suddenly later in life (day 76, 106 and 111: figure 6.1). We might assume that such deaths were related to the presence of sustained long-term severe hypertension. The lack of mortality from malignant hypertension in animals treated with ramipril 5μg/kg/day and amongst control or normotensive TGR groups (i.e. treated with ramipril 1mg/kg/day) confirm the protective effect of low dose ramipril therapy, but suggest that it does not wholly protect against the long-term effects of hypertension.

6.52: THE HYPERTENSIVE PHENOTYPE

This is the first study to have characterised the blood pressure profile of the TGR into adulthood, and confirms the continuation of hypertension for at least 120 days. We also note a steady reduction in SBP in all TGRs (treated or untreated) from 60 to 120 days. Thus when data for days 63 and 112 are compared, mean SBP fell from 249±19 to 216±30mmHg, from 250±22 to 207±38, and from 197±38mmHg for groups 1, 2 and 4 respectively ($p<0.0001$ in each case). No such fall was seen in control SD animals. Such a fall (over the age range of 2-8 months) has been remarked upon before although never quantified (Bachmann et al. 1992). The cause of this effect is unknown.

6.53 THE DEVELOPMENT OF LEFT VENTRICULAR HYPERTROPHY

6.531 Increase in LV mass

There have been few previous studies of the cardiac hypertrophic response of the TGR(mREN2)27 rat, and the hypertrophic response has consequently been

inadequately characterised. Villareal examined five male (?heterozygote) TGR(mRen2)27 rats at 16 weeks of age, and 6 Sprague Dawley controls (Villarreal et al. 1995). However, blood pressure was not recorded over the lifespan of these animals. The nature of training, sedation, timing and frequency of BP measurement was not stated. Hypertensive phenotypic varies in different outbred lines (Mullins and Mullins 1994), yet we do not know the pedigree of the animals used. Heart weight was 30% higher in TGRs (1.9g vs. 1.43g in controls), and heart weight:body mass ratio increased (4.1 vs. 3.1g/kg) but no differentiation was made between the different cardiac chambers. Bachmann noted a consistent increase in heart size at ages 2-8 months (200-500g) with differences in myocardial vasculature (increased medial thickness in arteries and arterioles with vascular smooth muscle cell hypertrophy) only prominent in male rats. Precise data (including cardiac or chamber masses, ages, strain and blood pressure profile) are not provided (Bachmann et al. 1992). Systematic analysis of these changes "was not possible... due to the small number of animals available for this study".

Our studies show that LV mass was consistently greater in TGRs than in SD control animals even when adjusted for body mass, and that the TGR would thus seem to be a useful model of LVH. The hypertrophic process is due to both an accumulation in LV collagen, and to an increase in non-collagen component (of which we might expect the bulk to be due to myocyte hypertrophy: see below).

6.532 Increase in LV collagen content

Cardiac perivascular fibrosis has been identified in the TGR (Bachmann et al. 1992; Villarreal et al. 1995). Villareal (Villarreal, et al. 1995) attempted to explore the changes in collagen deposition, but (along with the flaws listed above) did not separate the different chambers, and measured not collagen *content* but *concentration* . This was reduced (3.4 vs. 4.7mg/g dry weight), as was interstitial collagen area fraction. They suggest that the finding of 'reduced interstitial collagen' conflicts with higher collagen type III mRNA. However, LV collagen content may well have been increasing, in the presence of a greater degree of myocyte hypertrophy, thus causing a fall in concentration. Such a lack of distinction between collagen contents and concentrations has marred previous work in this field. The intensity and relative contributions of collagen deposition and myocyte hypertrophy to the hypertrophic response are known to vary over time and with the cause of the LVH. This is true of humans (Caspari et al. 1977) as well as animal models where the timing of assessment, the animal model, the animal species and sex may also have effects (Bishop and Laurent 1995). In the SHR, an early increase in collagen concentration (animal weight 100g) is then lost (falling at weight 200-250g) before returning to control levels (300g). Collagen content,

however, continues to climb steadily to this point (Sen et al. 1976). On the other hand, DOCA-salt treatment for 12 weeks was associated with an increase in both collagen content and concentration(Baxter and Yellon 1992a). The importance of timing in the assessment of matrix parameters, and of the direct measurement of collagen content, is also shown in Baxter's study (Baxter and Yellon 1992a). As LV collagen concentration increased with LVH regression after withdrawal of DOCA-salt treatment, continued collagen deposition was proposed. However, if percentage LV water content changes minimally in each animal group, their data shows a reduction in LV collagen content as LVH regresses (5.02mg at 2 weeks vs. 4.75mg 14 weeks after cessation of DOCA-salt treatment). LV mass fell overall by approx. 17%, and LV collagen content by only 5.4%. Similarly, Kuzuo examined regression of LVH in the SHR with treatment (Kuzuo et al. 1993). Collagen concentration in 32 week old SHRs (with LVH), normotensive controls, and age-matched animals who had received 12 weeks of treatment with captopril were similar, although total LV collagen content was reduced in SHRs by ACE-inhibitor treatment (Kuzuo et al. 1993). Collagen *concentration is* therefore an unreliable guide to collagen *deposition*, and wherever possible collagen content should be measured, as was the case in our study.

LVH in the TGR comprises an increase in overall LV mass, and mass of the collagen component. It would seem that in the initial phases (up to 70 days) , the contribution of myocyte hypertrophy may be equivalent to (or perhaps greater than) that of collagen deposition, as collagen concentration at 70 days is slightly lower in the TGR than in the SD (16.6$\pm$1.04mg/g cf. 18.54$\pm$0.48 mg/g dry weight: p=0.1). Later, however, collagen deposition proportionately exceeds myocyte hypertrophy, and collagen concentration rises significantly by 120 days in the TGR (to 21.21$\pm$1.35mg/g: p=0.02) but not in the SD control (18.48$\pm$1.71mg/g: p>0.5). This conclusion is supported by the finding of similar collagen contents at 70 days in both groups (4.57$\pm$ 0.55 vs. 4.14$\pm$0.19mg unadjusted: 12.83$\pm$1.5 vs. 12.14$\pm$0.6mg/kg body weight adjusted) but markedly elevated LV collagen contents in TGRs at 120 days (unadjusted 8.74$\pm$0.44 vs. 5.16$\pm$0.67, p<0.002: adjusted for body mass 18.08$\pm$1.69 vs. 12$\pm$1.07mg/kg, p=0.01).

6.54: ACTION OF ACE-INHIBITORS

Administration of ramipril limits the progression of hypertrophy. LV mass is consistently lower in groups 2 and 4 (ramipril 5µg/kg/day and 1mg/kg/day) than in untreated animals whether or not data are unadjusted for body mass. Once again, this is through both the inhibition of both the deposition of collagen and of the increase in non-collagen components. The effect is most prominent on collagen deposition,

however. Thus, at 120 days, total body-weight-adjusted LV mass is similar in groups 1 and 2 (2.87±0.1 vs. 3.03±0.03g) whilst collagen content is substantially reduced (18.08±1.69 vs. 14.92±0.57mg: p=0.09). This inhibitory effect is manifest even at the 70 day time-point, prior to which excess collagen deposition in untreated animals seems to have been minimal (10%: see above). That ACE-inhibition reduces physiological (growth related) and pathophysiological (stimulated) collagen deposition is also demonstrated when groups 3 and 4 are compared. Despite equal blood pressure, LV collagen content is lower in ramipril-treated TGRs than control SDs at both 70 and 120 days ($p<0.0001$ in both cases: see above). Cardiac collagen content has been previously shown to be reduced in the SHR by 12 weeks of captopril treatment (Kuzuo et al. 1993).

Ramipril does not merely act by inducing a reduction in SBP. LV mass adjusted for body weight is reduced at 70 days in TGRs treated with a non-hypotensive dose of Ramipril ($p<0.02$). At both 70 and 120 days, LV collagen content is also reduced ($p=0.09$ and 0.01 respectively). This non-hypotensive effect is even more prominent when groups 3 and 4 are compared (control SD and Ramipril 1mg/kg/day). LV mass at 70 and 120 days ($p<0.001$ and $p=0.002$ respectively), and LV collagen content at 120 days ($p=0.01$) were both lower in ramipril treated animals than controls. The more limited effect of ACE inhibition at low dose may be partly due to a lower percentage inhibition of ACE activity, and to a greater overall tissue RAS activity in the face of hypertension. The TGR itself would also seem to have higher myocardial RAS activity than equivalently hypertensive animals: Paul et al, commenting on 'work in progress', claim to have compared TGRs with SHRs matched for plasma renin activity, age, weight and sex and blood pressure. LV:body weight ratios were quoted as being higher for the TGR than the SHR (Paul et al. 1994).

It must be noted that ramipril is seen to have effects at doses of just 5µg/kg/day, which inhibits tissue ACE activity but not plasma ACE activity. At least part of the effect of ACE inhibition with ramipril on ventricular growth is thus dependent upon inhibition of a tissue RAS.

6.55 THE DEVELOPMENT OF RIGHT VENTRICULAR HYPERTROPHY

We have been unable to demonstrate RVH in the TGR when compared to control animals. At no timepoint was RV mass greater in group 1 than group 3. RV collagen content is actually lower in the TGR than in controls at 70 days ($p<0.04$ whether adjusted for body mass or not). In theory, ventricular hypertrophy could be due to mere pressure overload, pressure overload being transduced by a myocardial RAS, to

transgene expression in myocardial tissue, or to uptake of the raised levels of circulating prorenin. The latter two options seem unlikely, as we might have expected RV mass or collagen content to be higher in the TGRs by 70 days (compared to SD controls), not lower. At 120 days, RV collagen content has risen to a level greater than that in controls (4.66±0.59 vs. 4.19 ±0.17mg/kg body weight). This amounts to an *increase* of body-mass adjusted RV collagen content of 19% in the TGR (3.91 to 4.66mg/kg: p=0.3) , with a *reduction* in RV collagen content in controls of 22% (5.36mg/kg to 4.19mg/kg: p=0.03). Paul claims to have demonstrated increased RV:body weight ratios in the TGRs, but not SHRs of equal blood pressure when compared to control (7.338 vs. 5.606 vs. 5.241 respectively) (Paul et al. 1994), a finding which would be consistent with our demonstration of transgene expression in the right ventricle. The age of these animals was not given. Taken together, such data suggest either that increased RAS activity in the absence of pressure overload only takes effect as the animal ages, or that a progressive increase in pulmonary vascular resistance occurs in the TGR. This might be possible, given the vascular and pulmonary transgene expression previously discussed.

Ramipril treatment is associated with a significant reduction in RV mass at both 70 and 120 days (p for heterogeneity<0.0001 in both cases). As in the LV, this effect is especially marked on collagen deposition and at later timepoints (120 days: figure 6.8). This preferential inhibition of collagen deposition leads to reduced RV collagen concentration by 120 days in animals of group 4 when compared to those of group 1 (25.16±0.9 vs. 30.63±1/73mg/g: p<0.05)

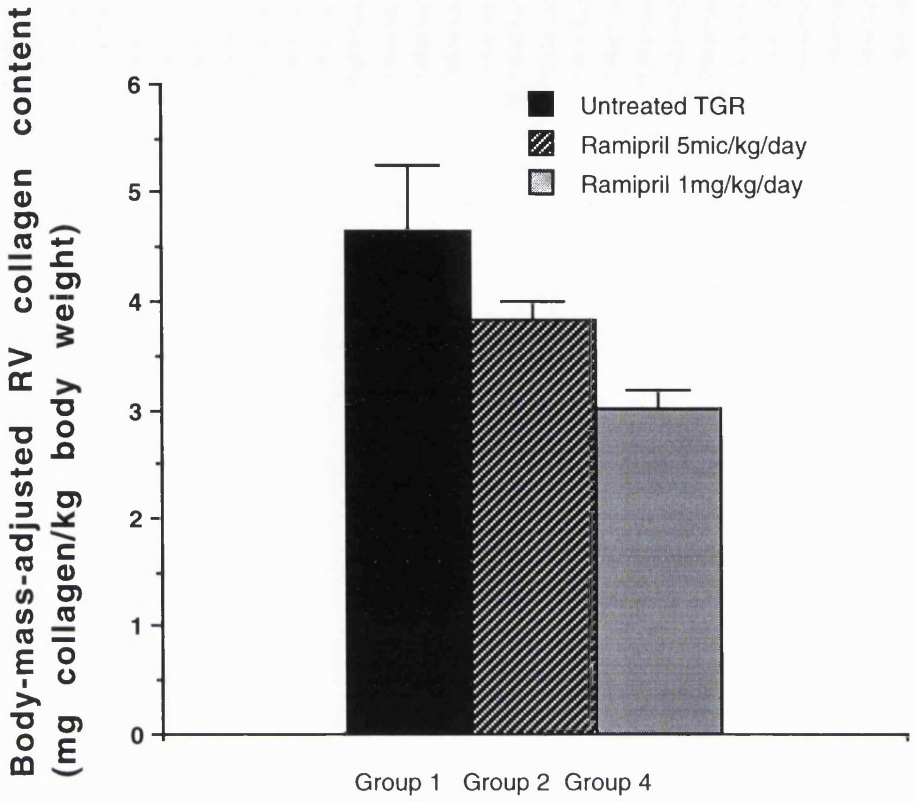


Figure 6.8: Effects of ACE inhibition on right ventricular collagen content (mean ± SEM) after adjustment for animal body mass.

This action on the RV might be mediated either through a reduction in pulmonary vascular resistance, or through a direct effect on the RV itself suggesting once again an important role for tissue RAS in the regulation of physiological ventricular collagen content and growth.

In Baxter's study (Baxter and Yellon 1992a) (above) regression of LVH in rats 14 weeks after withdrawal of DOCA-salt treatment was associated with an increase in LV collagen concentration, due to a fall in overall mass of 17%, and of LV collagen content of only 5.4%. Similar disparate responses of myocyte hypertrophy and collagen deposition might explain the delayed increase in ventricular collagen content (and hence early reduction in collagen concentration) seen in the TGR. Such data might suggest that myocyte hypertrophy is more rapidly responsive to changes in hypertrophic stimuli than is collagen matrix. This in itself may be due to differences in the sensitivity and regulation of transduction mechanisms, or merely to the very long half-life (over 100 day) of collagen(Turner, Oliver et al. 1986). It is also possible that, early in life, increased collagen synthesis and degradation had gone hand in hand (which can reduce

collagen half-life by <6-fold (Brilla et al. 1995))leading to an early net reduction in collagen content (as is seen in the hypertrophying RV of the rabbit in the bleomycin model (Turner et al. 1986)).

The differential effect of ACE inhibition on ventricular growth and collagen deposition at different times has been noted in other models. As discussed above, the cause of hypertrophy will also influence the nature of the hypertrophic process. In the suprarenal banded model, fibronectin dominates collagen deposition (Linz et al. 1992). The overall contribution of matrix and myocyte also varies: in the suprarenal banded rat, most of the increase in cardiac mass is due to collagen deposition and not to myocyte hypertrophy (Linz et al. 1992; Nagasawa et al. 1995). Variation even occurs within one model: Baxter identified a previously denied increase in collagen concentration and content in the DOCA-salt model of hypertension. Differences in duration of hypertension (Baxter used animals treated for 12 weeks, whilst many others had used treatment durations of only 6 weeks (Baxter and Yellon 1992a)) may account for such disparity.

6.6: IN CONCLUSION

The heterozygote male TGR(mREN2)27 rat exhibits a sustained hypertensive phenotype up to 120 days, although there is a slow fall in blood pressure towards control levels from 70 days onwards. The profile of response to ACE-inhibition is also consistent over time. The hypertensive phenotype is associated with significant left ventricular hypertrophy and collagen deposition. ACE-inhibition reduces ventricular hypertrophy partly through a hypotensive mechanism, and partly through an effect independent of altered systolic pressure burden. This component of its action is not dependent upon inhibition of plasma ACE, but is associated with an inhibition of myocardial ACE activity. These data support the existence of a myocardial RAS which is involved in the regulation of cardiac growth and hypertrophy. The effects of ramipril might be due either to a reduction in ATII synthesis leading to a reduced trophic drive, or to a reduction in kinin degradation causing an antihypertrophic effect. The possible mechanistic implications are discussed in greater detail in chapter 10.

Caution must be exercised in the interpretation of all of this data, which apply to a self-selected group of surviving animals in group 1 (namely, those resistant to severe hypertension and who were not susceptible to MH). A number of animals prone to the MH phenotype or hypertension-related death may have survived in groups 2 and 4, and their cardiac behaviour may have thus been different from the 'non-MH-prone' animals. We cannot control for the bias that such an effect would incorporate.

IDIOPATHIC DILATED CARDIOMYOPATHY AND THE INSERTION/DELETION POLYMORPHISM OF THE ANGIOTENSIN CONVERTING ENZYME GENE.

This study was published in: J Am Coll Cardiol 1995:25 (7); 1627-1631

7.1: INTRODUCTION

The cardiomyopathies are a group of diseases of the heart muscle from which cases due to hypertension, congenital, coronary or valvular disease have been excluded. There are three main subtypes. Hypertrophic (an inherited condition which is increasingly associated with defects of the beta-myosin heavy chain gene). There is gross asymmetrical cardiac hypertrophy, marked fibre disarray, a predisposition to cardiac dysrhythmia, and an increased incidence of sudden cardiac death. Early diastolic failure may lead to later systolic failure. Restrictive (myocardial infiltration with inelastic tissue such as amyloid or fibrous tissue as in sarcoid). Early diastolic failure may lead to later systolic failure. Dilated cardiomyopathy is characterised by a primary reduction in myocardial systolic function, with reduced ejection fraction and ventricular dilatation. The cause is usually occult, when cases are referred to as 'idiopathic dilated cardiomyopathy, or IDC. Myocarditis and sarcoidosis may be occasional precipitants. Rarely, familial cases are seen (see below). The trabeculae of the left ventricle are flattened, with mural thrombus often seen. Myocyte nuclei are enlarged and 'smudged' as may be seen in cases of hypertrophy. There is little or no cellular infiltration. There is marked smooth muscle infiltration of the subendocardium. Interstitial fibrosis is a very prominent feature throughout the whole myocardium, and especially in the subendocardium, with heavy deposition of collagen between muscle fibres (Oakley 1987).

Given the potential role of the cardiac RAS in the control of interstitial matrix, cardiac remodelling and fibrosis (Linz et al. 1989; Brilla et al. 1990; Linz et al. 1992) (and see chapters 1, 6 and 10) , and the association of the D polymorphism of the ACE gene with raised tissue ACE levels (chapter 1), an association of the D allele with more severe IDC might be postulated. Against this background, Raynolds et al reported that possession of the ACE DD genotype was a risk factor for the development of end-stage heart failure due to IDC (Raynolds et al. 1993). However, this conclusion has been questioned (Woodrow 1994), and the involvement of ACE genotype in the genesis of idiopathic dilated cardiomyopathy (IDC) as opposed to its progression was not directly addressed. We therefore examined the relationship between ACE genotype, the diagnosis of IDC itself, and markers of disease severity and clinical disease progression in a case-control study.

7.2: METHODS

7.21: SUBJECTS

7.211: Patients

Diagnosis of IDC was based on the criteria recommended by the World Health Organisation and the National Heart, Lung and Blood Institute (Brandenberg et al. 1981). All had left ventricular dilatation (end diastolic diameter > 2.7 $cm/m^{2)}$ (Manolio et al. 1992) and impaired systolic contraction (left ventricular ejection fraction <40% or fractional shortening <25%). Cases with ≥50% obstruction of one or more coronary arteries, active myocarditis (Aretz et al. 1987) specific primary or secondary heart muscle disease, sustained systemic arterial hypertension, isolated right ventricular dilatation and valvular or pericardial disease were excluded. Twenty-seven patients with a history of chronic alcohol consumption of >8 units/day for males and > 6 units/day for females were included.

We studied 99 consecutive unrelated Caucasian patients with IDC (mean (± standard deviation) age 41±14 years: range 12-73: 79 male) who presented to St. George's Hospital (January 1989 and March 1994). Forty-two were in New York Heart Association class I, 17 in class II, 25 in class III and 15 in class IV when first seen. They were assessed using 12-lead electrocardiography, chest radiography, two-dimensional transthoracic doppler echocardiography, 24-hour ambulatory electrocardiographic monitoring, radionucleotide ventriculography and maximal symptom-limited exercise testing. LV cavity dimensions were assessed by short axis views at the level of the papillary muscles. Coronary angiography was performed in 81 patients. The remaining 18 patients were all under the age of 40 years with no risk factors for ischaemic heart disease and no evidence of ischaemia on exercise electrocardiographic testing. Endomyocardial biopsy in the 56 patients who consented to the procedure was normal in 23, and showed fibrosis in 33. The clinical and demographic characteristics of those consenting to biopsy and those who declined were similar. Mean (± SD) left ventricular ejection fraction at diagnosis was 25±11%, and left ventricular end-diastolic volume 68 ±11mm^3.

Duration of symptoms was defined empirically as the time from first reported symptoms to time of first confirmation of diagnosis of IDC recorded in the case notes. Patients were followed for a mean of 28±33 months, during which time 35 patients clinically deteriorated (22 requiring cardiac transplantation) and 4 suffered sudden cardiac death (as defined by Greene (Greene et al. 1989)).

7.212: Control Group

The control group consisted of 364 caucasian males (age 54±3 years: range 49-60). drawn from a local General Practice population. Those with symptoms suggestive of coronary artery disease or with a known diagnosis of ischaemic heart disease were excluded. We also compared our patients to previously reported healthy European control samples (Rigat et al. 1990) including one from the ECTIM study(Cambien et al. 1992).

7.22 DNA EXTRACTION AND GENOTYPING THE ACE I/D POLYMORPHISM

DNA was extracted from leucocytes by a process of nuclear lysis, DNA precipitation, chloroform separation, and ethanol precipitation, as described in chapter 3. Detection of the I/D polymorphism in intron 16 of the ACE gene used two-primer polymerase chain reaction amplification as previously described (Tiret et al. 1992) (chapter 3) with subsequent separation of the fragments by agarose gel electrophoresis.

7.23: STATISTICS

Differences in the distribution of the DD, ID and II genotypes were assessed by contingency table/chi squared analysis. Numerical data between different groups were compared using unpaired 2-tailed t-tests, and outcomes compared by a one-tailed ANOVA test. p-values were expressed with continuity corrections. A p-value of <0.05 was considered statistically significant. We assumed that the frequency of the DD genotype in our control subjects would be similar to that previously reported (Cambien et al. 1992). This study thus had a power of 0.9 to detect a 42% difference in prevalence in the patient group.

7.3: RESULTS

ACE genotype and allele frequencies in the IDC and control groups were compared (table 7.1). Allele frequencies in both were similar (p=0.5), and consistent with Hardy Weinberg equilibrium. Control genotype distribution was similar to that previously reported (p=0.1 (Cambien et al. 1992): p>0.1(Rigat et al. 1990)) and to that in the IDC group (p=0.75).

	Genotype			Allele Frequency	
	DD	ID	II	D	I
IDC patients	31 (31)	50 (51)	18 (18)	0.57	0.57
Controls	112 (31)	168 (46)	(84) 23	0.53	0.47
ECTIM controls	200 (27)	390 (53)	143 (20)	0.53	0.47

Table 7.1: Distribution of ACE genotypes [number (%)] and allele frequencies amongst patients with dilated cardiomyopathy, study controls, and controls from the European ECTIM study (Cambien et al. 1992) (see text for details).

The relationship between ACE genotype and clinical markers of disease severity and progression were examined amongst patients (tables 7.2 and 7.3). Those with the DD genotype were compared to individuals homozygous for the I allele, and also to the total 'non-DD' (ID and II) patients. Only two features were associated with DD genotype: duration of symptoms prior to diagnosis was longer and the number of ventricular ectopics/hour was greater. In particular, fibrosis on endomyocardial biopsy was not associated with ACE genotype. The distribution of patients amongst the DD, ID and II groups was similar in those with progressive heart failure or requiring orthoptic heart transplant when compared either to the clinically stable patients (p=0.5),the European controls (p=0.5), or our control group (p> 0.5). The same is true when comparing the allele frequency in these subgroups (p >0.5 in all cases.)

	ACE Genotype		
	DD	ID	II
Progressive Heart Failure or Cardiac Transplant	12	15	8
Sudden Cardiac Death	0	3	1
Clinically Stable	19	32	9

Table 7.2: Lack of association of ACE genotype with disease progression amongst patients with dilated cardiomyopathy (see text for details)

	GENOTYPE				p VALUE	
	DD	ID	II	ID+II	DD/II	DD/other
Age (years)	43±13	36±14	47±17	40±15	0.32	0.56
Sex: male	27	41	11	52		
female	4	9	7	16	0.08	0.34
NYHA I/II	17	33	9	42		
III/IV	14	17	9	26	0.98	0.67
Duration of follow-up (months)	30±29	29±34	26±37	28±35	0.71	0.8
Duration of symptoms before diagnosis (months)	50+52	29+34	26+40	27+51	0.16	0.02
Ejection Fraction (%) n=58	22±8	28±12	22±11	26±12	0.93	0.2
Left ventricular end- diastolic dimension(mm) n=77	72±12	65±9	72±12	67±10	0.96	0.51
Left ventricular fractional shortening (%) n=77	14±6	15±7	14±7	14±7	0.91	>0.9
Pulmonary artery wedge pressure (mmHg) n=45	20±11	18±7	28±7	20±8	0.095	0.78
Ventricular ectopics (/hour) n=48	333 ±334	143 ±294	81 ±134	128 ±264	0.06	0.03
Patients with	11	12	5	17		
without non- sustained ventricular tachycardia	7	18	6	24	0.66	0.27
Fibrosis:	14	13	6	19		
No fibrosis: on endomyocardial biopsy	7	13	3	16	0.67	0.53

Table 7.3: Relationship between ACE genotype and markers of disease progression and severtity amongst patients with dilated cardiomyopathy (see text for details)

7.4: DISCUSSION

RAS activity may be associated with the development of cardiac fibrosis, hypertrophy, remodelling, electrical instability, and systolic and diastolic dysfunction (reviewed in chapter 10). Consequently, an association between ACE genotype and dilated cardiomyopathy would seem feasible. Raynolds et al (Raynolds et al. 1993) compared 112 caucasian patients with end-stage IDC, (93 requiring heart transplant) and 79 younger white control subjects. The frequency of the DD genotype was 36% in the IDC patients, and 24% in the controls ($p<0.01$). However, their IDC patient cohort also had an excess of the II genotype, which would not support the concept that the DD genotype exerts a pathophysiological action mediated by the associated raised levels of ACE activity. Further, they did not examine the association of ACE gene polymorphisms with the diagnosis of IDC per se, or with disease severity or progression within a cohort. Our study has addressed these issues.

Our control group was drawn from a local general practice register, with clinical cases of ischaemic heart disease (IHD) excluded. Invasive investigation to exclude IHD was considered ethically unjustifiable. However, the DD genotype has only been reported to be strongly associated with myocardial infarction in an otherwise low risk (non-hypertensive, normo-cholesterolaemic, non-diabetic) group, in whom only 35% of cases might be ascribed to its possession (Cambien et al. 1992). With so few myocardial infarctions likely to have occurred in this subgroup, potential distortion of ACE allele distribution in our control group due to the presence of, or mortality from, ischaemic heart disease can only therefore be minimal. Similarly, the incidence of IDC is very low, and undiagnosed cases in the community are unlikely to introduce significant bias. Our patients were highly characterised consecutive cases of IDC referred for management of heart failure or dysrrhythmia.

Distribution of ACE genotypes and allele frequencies were similar in patients and controls, with an excess of neither the D allele nor the DD genotype being associated with a diagnosis of IDC. Although the frequency of the D allele is slightly raised amongst patients when compared to controls, the difference is not statistically significant ($p=0.5$). Furthermore, if increased ACE activity is linked to myocardial fibrosis in IDC, then DD genotype might be associated with an excess number of cases of fibrosis found on myocardial biopsy. Although numbers were small, we found no evidence to support such a link. However, as for Raynolds (Raynolds et al. 1993), our study lacked the power to detect a small increase in relative risk associated with the presence of the D allele.

We also examined the association of the ACE polymorphism with progression of the disease by comparing clinical parameters in those with the DD genotype (n=31) with those in the 'II' (n=18) and the 'non-DD' groups (II and ID: n=68) (Table 7.3). The age/sex characteristics of these groups were not significantly different. The DD genotype itself was significantly associated with only two parameters: they had more ventricular ectopic beats per hour (although numbers assessed were limited and confidence intervals wide), and a longer time from first recalled symptoms to first recorded diagnosis of IDC. The later is an empirical timespan, and subject to a number of social, personal, professional and pathological influences (see below). However, it might cautiously be suggested that those with a DD genotype had slower (rather than more rapid) disease progression, as clinical grading of symptoms at presentation and all measures of ventricular contractility were similar irrespective of patient genotype.

The frequency of the DD genotype and D allele was similar in the subgroup of 35 patients with progressive disease, (even with the inclusion of the four who died suddenly) when compared to those with a stable course (n=60). However, the risk of end-stage IDC previously attributed to having two D alleles is small (odds ratio 1.43 when compared to the ECTIM data (Cambien et al. 1992), and these numbers may be insufficient to rule out such a weak association of the ACE genotype with progression to end-stage heart failure.

The results from this study do accord with some features of the study of Raynolds et al. The frequency of the D allele in their IDC and control groups (M Raynolds, personal communication), and our IDC group, was remarkably similar (D frequency 0.56,0.57,0.57 respectively). However, we were unable to demonstrate any association between the ACE polymorphism and the diagnosis of IDC per se. Many factors may account for the divergence between our findings.

(i) There are theoretical difficulties in any population study in determining what makes an association (or lack of it) 'significant', and pitfalls in applying the investigational standards derived for Mendelian traits to studies of more complex phenotypes or diseases (Risch et al. 1993).

(ii) There were differences in patient populations. Theirs comprised patients with end-stage IDC of undefined aetiology and characteristics (most being transplant recipients), whilst ours were unselected well characterised IDC patients of varied disease severity in our study.

(iii) Control groups characteristics differed. Ours was drawn from a local General Practice population and was slightly older than our patient group (mean $\pm$standard deviation 54$\pm$3 compared to 41$\pm$14 years). Theirs largely comprised potential heart donors and was younger (mean $\pm$SEM 33$\pm$1.8 and 44.8$\pm$1.5 years respectively). Neither group had proven normal hearts. None of our controls routinely underwent either echocardiography or coronary angiography, although those with symptomatic or

documented coronary disease were excluded. However, few of their controls underwent angiography (males over 40 and women over 45 years of age in a young population), and ejection fractions as low as 30% were accepted as normal. The allele distribution in their control group did not obey the Hardy Weinberg equilibrium (see appendix to this chapter), and the incidence of II genotype (12.7%) was far lower than that previously reported in control groups (Woodrow 1994) (19.5% (Cambien et al. 1992) and 17.5% (Rigat et al. 1990)). The frequency of the DD genotype (24%) was also lower than expected (Woodrow 1994), and lower than previously reported (36.25%(Rigat et al. 1990), and 27% ECTIM controls (Cambien et al. 1992).) This may have been due to the nature of the control group itself, or due to sampling error due to the small number of controls (n=79) studied. It is this unusual distribution of alleles amongst controls which accounts for much of the statistically significant excess of the DD genotype in their IDC patient group when compared to their controls. In contrast, our control population was much larger (n=364), and the data did obey the Hardy Weinberg equilibrium. The genotype and allele frequencies were similar to that seen in a much larger sample of European (ECTIM) controls(n=733) (Cambien et al. 1992).

Comment must be made with regards to the method of ACE genotyping used in this study. We used the accepted methodology of the time (Rigat et al. 1990)- namely a two-primer system for the amplification reaction of our polymerase chain reaction, as was originally described. This may be prone to misclassification of some ID individuals as DD genotypes (chapter 3). The scale of such error is likely to be small, and in our laboratory, comparison of historical control data derived from the two-primer system shows that the error is of a similar magnitude (Prof. S. Humphries, personal communication). DNA samples extracted for this study have since been exhausted at another institution, and we are unable to exclude small errors by the use of a three-primer (allele-specific) system. It must be noted that Raynold's original paper used an identical system.

There are also other cautions which must be sounded in the interpretation in of this study. Any evidence concerning the influence of the ACE D allele on disease progression and severity can only be viewed as tentative. The 'time from diagnosis' is taken as the time from first recorded diagnosis in the clinical notes of the patient. This is clearly likely to be inaccurate. Many cases may well have been suspected for some years, and referred to us with this diagnosis in mind. Loss of this recorded 'lead time' may have operated to a greater extent against those with more benign disease, who may have presented to their clinician much later, and also been referred much later for assessment. Those with more aggressive disease, on the other hand, may well have been referred much more quickly. There may also have been an age bias (see below) causing a skew in referral timing.

This study is also complicated by the very possibility of a genetic influence on the development or progression of disease, or indeed in its aetiology. Literature review reveals several case reports of dilated cardiomyopathy in at least 2 members of the same family (Evans 1949; Emanuel et al. 1971; Berko and Swift 1987; Goldblatt et al. 1987; Maclennan et al. 1987). Retrospective and prospective studies(Evans 1949; Maclennan et al. 1987; Mestroni et al. 1990; Michels et al. 1992) suggest that true familial cases (i.e. where the aetiological agent is inherited) largely demonstrate autosomal dominant inheritance although autosomal recessive (Emanuel et al. 1971; Goldblatt et al. 1987) and X-linked inheritance (Berko and Swift 1987) are sometimes shown. All subjects entered into this study were unselected unrelated probands. It is possible that some of these patients were therefore sufferers of a true 'monogenic' inherited/sporadic disease whose behaviour was quite different from the other cases, and in whom the presence or absence of a D allele may have had quite different impact.

It is also possible that our case mix was biased in other ways. There may well have been referral bias in the nature and severity of the cases sent to St. George's hospital. St. George's is a centre for heart transplantation, and referrals of more severe case (with this in mind) may well have occurred. Many rapidly progressive or severe cases may not survive to reach referral, whilst the elderly (who may have suffered a much more benign clinical course) may not be referred at all (due to the lack of transplant provision and 'ageist bias').

There are also problems with the design of the study in terms of inclusion or exclusion of known causes. It is possible that the clinical cases of active myocarditic cardiomyopathy are strongly influenced by ACE genotype, as may cases of granulomatous disease such as sarcoid which was excluded from this study. Due to the frequent occurrence of viral infections in the community, it is also difficult to include/exclude cases of cardiomyopathy which may have had a viral origin. Similarly, conventionally moderately heavy alcohol intake is tolerated in the diagnosis of IDC. Individual sensitivity to the cardiotoxic effects of alcohol varies greatly. It is likely that cases of alcoholic and viral cardiomyopathy were included, and that these were of varying severity and duration from 'insult'. Finally, this 'mixed bag' effect of various unknown aetiologies and possible genetic influences means that we may well not be looking at one pathological condition at all, but at a spectrum of diseases whose net result (cardiac dilatation, systolic dysfunction, and arrhythmias) is the same. The influence of a single gene (such as the D polymorphism of the ACE gene) may thus vary considerably.

7.5: IN CONCLUSION

Raynolds et al (1993) question whether the D polymorphism of the ACE gene predisposes individuals to develop cardiomyopathy. We can find no evidence that it does. Neither does our data suggest a major role for the ACE DD genotype in the progression of idiopathic dilated cardiomyopathy. However, due to the numerous problems inherent in studies such as this, the only way forward if such associations of this ACE gene polymorphism are to be investigated further is for large long-term population-surveillance and follow-up studies to be performed.

APPENDIX TO CHAPTER 7

The Hardy Weinberg Equilibrium

The Hardy Weinberg Equilibrium related the frequency of heterozygotes to homozygotes for an allele at a particular locus. If in equilibrium, then the population fulfills the criteria for random mating in a large population meaning that the genetic constitution of the population will remain stable form one generation to the next. Considering the 2 alleles I and D at the ACE gene locus, the proportion of each allele in a population might be described as i and d respectively, where i+d=1. If we selected given individuals (i.e. pairs of ACE alleles) the probability of getting 2 D alleles would be d x d (or d^2), of 2 I alleles i x i (or i^2), and of one of each allele (a D and an I allele, or an I and a D allele) i x d plus d x i (or 2id). In other words, the proportion of homozygotes and heterozygotes can be represented as:

$$i^2 + 2id + d^2 + 1.$$

This was shown to be the case for the early studies of ACE gene polymorphism (e.g. Cambien(Cambien, Poirer et al. 1992)). However, in Reynolds study, the population was not in Hardy Weinberg Equilibrium, suggesting that the sample was not representative of the population from which it was drawn. This makes it difficult to draw conclusions about causation of progression of a given disease.

THE ROLE OF THE ANGIOTENSIN-CONVERTING ENZYME GENE I/D POLYMORPHISM IN THE LEFT VENTRICULAR HYPERTROPHIC RESPONSE TO PHYSICAL TRAINING

8.1: INTRODUCTION

Increased left ventricular mass is associated with excess cardiovascular mortality and morbidity (Levy et al. 1990a), and is more strongly associated with cardiovascular risk than blood pressure itself (Koren et al. 1991) (chapter 10). The mechanisms which regulate myocardial mass and the genetic elements involved in their control are poorly understood (Post et al. 1994). ATII-generation by local myocardial RAS may play a part (Lee and Lindpainter 1993) (and see chapter 1), although the inaccessibility of human normal left ventricular tissue has impaired further investigation of this hypothesis. However, it has been suggested (chapter 1) that cardiac tissue ACE may be the rate-limiting step in the generation of local cardiac ATII, and (chapter 1 and 3) that the deletion allele (D) rather than the insertion allele (I) of the ACE gene may be associated with elevated tissue ACE levels (Costerousse et al. 1993; Pinto et al. 1994). If cardiac renin-angiotensin systems are important regulators of myocardial growth, then the D allele might be associated with a greater response to a given hypertrophic stimulus. The study described in this chapter was designed to test this hypothesis. In order to do this, an appropriate hypertrophic stimulus needed to be identified (section 7.2 below), as did an appropriate population to whom to apply this stimulus (section 7.3).

8.2: IDENTIFICATION OF AN APPROPRIATE CARDIAC HYPERTROPHIC STIMULUS

Any study of a gene-environment interaction will be strengthened by the ability to apply a reproducible standardised environmental stimulus to a phenotypically uniform population. There are few cardiac hypertrophic stimuli which one is able to standardise in this way and apply prospectively, and the application of pharmacological agents for this purpose (such as pressor agents or thyroxine) is ethically unacceptable. However, physical training has ethical advantages as a stimulus, as it associated with improved health. It is known to increase cardiac mass (DeMaria et al. 1978; Kanakis and Hickson 1980) and aside from sustained untreated hypertension is the only non-pharmacological stimulus available for the prospective study of human LV hypertrophic responses.

8.21 EXERCISE AS A CARDIAC HYPERTROPHIC STIMULUS

8.211: Physiological responses to exercise

The trained state is characterised by the capacity to the increased tissue oxygen demands of vigorous exercise. Exercise may be dynamic or isometric and heart rate (HR), cardiac output (CO) and oxygen consumption increase in both. Dynamic exercise is typified by the rhythmic contraction and shortening of large muscle groups, (e.g. running). Isometric exercise accomplishes little external work (e.g. training against high resistances: extremes of weight training, or carrying heavy loads), and is associated with greater increases in cardiac work due to a greater rise in SBP, with a rise in diastolic BP and lack of fall in total peripheral vascular resistance (TPR) not seen in dynamic exercise (Nutter et al. 1972).

Isometric exercise: Lower intensity work is essentially aerobic, but higher resistance increases muscle tension and work until work exceeds blood supply. Contraction of greater than 15% of maximum voluntary force (MVC) thus tend to be anaerobic, and effort of >70% of MVC may halt muscle blood supply altogether (Nutter et al. 1972). Circulatory responses occur within seconds. Stroke volume (SV) changes little at lower workloads but falls with higher resistances and when HR has risen significantly. In young individuals TPR does not change greatly. Both BP and HR increase with the duration and relative intensity of the isometric exercise.

Dynamic (or Isotonic) Exercise: HR and CO increase greatly with little change in BP burden (Astrand and Rodal 1970). Although SBP rises in proportion to workload, DBP remains stable or falls (Schaible and Scheur 1985). However responses do vary with the muscle groups used, with forearm work often producing a more 'isometric' response (Blomqvist and Saltin 1983). Contractile state is enhanced, as SV and rate of ventricular pressure generation rise and end-systolic volume falls. Left ventricular end-diastolic volume (LVEDD) increases variably, but seems greater with increasing exercise intensity. This factor may be important in determining the degree of cardiac hypertrophy seen with training (Schaible and Scheur 1985).

Combined Isometric and Dynamic Exercise: Although much time may be spent in one form at a time (e.g. running, or weight training), combined exercise is typical in military training (e.g. running whilst carrying heavy packs or logs). The circulatory burdens of each exercise form are synergistic: handgrip whilst running leads to an increase in blood pressure, despite the lower TPR seen with the aerobic work (Nutter et al. 1972).

8.212 The Effects of Exercise Training on the Human Heart (Table 8.1)

Unlike pathological hypertrophy, the increase in LVM associated with physical training (Cohen et al. 1980; Shapiro 1984; Pelliccia et al. 1991) first noted in 1910 (Barach 1910) is associated with improved myocardial function (Schaible and Scheur 1985). LVEDD (Maron 1986) and wall thickness may both rise (Rost 1982), although the final pattern will be partly determined by the balance of exertional pressure-loading (i.e. static exercise) which increases septal and free-wall thickness (Morganroth et al. 1975), and volume-loading (i.e. dynamic exercise) which also increases LVEDD (Morganroth et al. 1975; DeMaria et al. 1978; Parker et al. 1978; Longhurst et al. 1980; Keul et al. 1981; Paulsen et al. 1981; Wieling et al. 1981). Since both LVEDD, ventricular volume (Pyorala et al. 1967) and wall thickness increase with dynamic exercise training, then LVM overall rises (DeMaria et al. 1978; Huston et al. 1985). Thus, although LVM increased in both groups, LVM and skeletal muscle mass increased only proportionately in competitive weight trainers, whilst LVEDD was only raised significantly in the long-distance runners (Longhurst et al. 1980; Longhurst et al. 1981). Rost also noted an increase in LVM in endurance athletes, with that in those taking 'static' exercise being merely commensurate with the increase in their total body mass (Rost 1982). Thus, the nature of the training may better determine the pattern of the hypertrophic response (Shapiro 1984) and its intensity the scale (Cohen et al. 1980; Shapiro 1984; Schaible and Scheur 1985), with elite runners having larger LV dimensions than good runners (Underwood and Schwade 1977).

Scale of Hypertrophic response

Most studies of physiological (training-related) LVH have been *cross-sectional* comparisons of non-athletes with professional athletes. The adjudged degree of hypertrophy is dependent upon the nature of the control group used. Endurance athletes are lighter than the general population. Thus, a 29% or 60% increase in LVM will be determined depending on whether a heavy or light control group are used (Longhurst et al. 1980). The hypertrophic response is variable and influenced by the training intensity (perhaps most importantly (Shapiro 1984)) and nature of the sport (Ikaheimo et al. 1979; Bekaert et al. 1981; Huston et al. 1985; Lombardo et al. 1991). Thus in a study of 947 elite athletes, rowers or canoeists were found to have the greatest left ventricular wall thicknesses, being $\geq$13mm in 7% of this cohort (Pelliccia et al. 1991). At least six other cross-sectional echocardiographic studies have shown significant increases in LVM in endurance athletes. LVEDD is <33% greater in competitive runners, cyclists and swimmers (Lengyel and Gyarfus 1979; Bekaert et al. 1981) than in controls, although an 8-14% increase is more usually seen in both runners and cyclists (Bekaert et al. 1981). The increase in LVEDD may be dramatic: 13% of elite athletes have an LVEDD >60mm, with an accepted 'normal range' of <70mm (Pelliccia et al. 1994). Posterior wall and septal thicknesses may increase by

<40% and LVM by 40-80% in professional endurance athletes (Lengyel and Gyarfus 1979; Bekaert et al. 1981; Schaible and Scheur 1985; Maron 1986). Thus, wall thickening seems essentially symmetrical in endurance athletes, and associated with increased chamber dimensions (Grant et al. 1965; Maron 1986). In general, athletes have LVEDDs 10% larger, and wall thicknesses 15-20% larger than matched control subjects. LV mass is usually increased by 45% (Maron 1986).

Prospective studies of physiological LVH have generally involved shorter and less intensive training schedules than those in cross-sectional studies, and the changes in cardiac dimensions are consequently less marked (Schaible and Scheur 1985). Three studies have shown an increase in LVM in response to a short-term training program (DeMaria et al. 1978; Kanakis and Hickson 1980; Shapiro and Smith 1983): LVM rose by 28% (corrected for body surface area) with 6 weeks of a running programme (Shapiro and Smith 1983), and by 12.6% with 10 weeks of resistance lower limb exercise (Kanakis and Hickson 1980). Eleven weeks of modest exercise (walk-jog-run at 70% of predicted maximal heart rate for an hour a day, 4 days a week) increased LV mass by 22% through a rise in both wall thickness and LVEDD (DeMaria et al. 1978). Freshmen in Amsterdam who train in rowing increase septal thickness by 12% in 7 months, and LVEDD by only 4% (Wieling et al. 1981). Others have shown lesser changes with modest exercise (Schaible and Scheur 1985; Maron 1986). Differences in exercise pattern and intensity may account for such differences (Schaible and Scheur 1985). Thus 50 minutes jogging/day for 5 days/week for 12 weeks significantly increased LVEDD but not wall thickness (Adams et al. 1981), and trained young female hockey players at peak season had an LVEDD 40% larger than controls, but little change in septal thickness (Zeldis et al. 1978). Wolfe failed to show any effect of a 6 month training programme on LVM and dimensions in 12 men although a higher mean age and entry level of fitness, and low workload (30 minutes jogging 4x/wk) may account for this lack of effect (Wolfe et al. 1979). UK infantry recruits (used in our study) have a low entry level of fitness, and are subjected to intensive and prolonged training.

Timing of Hypertrophic Response

The timescale of the hypertrophic response to exercise is even less well documented than its regression.

Regression: LVM is seen to regress within four days of training reduction(Huston, Puffer et al. 1985; Maron 1986), and was clear in 6 rowers when training intensity was reduced for 90 days the 1988 Olympic Games (Pelliccia et al. 1991). With 3 weeks of inactivity, LVM fell by 20% in highly-trained athletes within 3 weeks of inactivity (Martin et al. 1986) and by 1/3 in competitive runners in whom posterior wall thickness decreased by 25% and LVEDD by 9% (Ehsani et al. 1978). Ultimately, LV dimensions and mass return to normal (Shapiro 1984) although continued activity

at lower level limits regression. In cyclists in the 'non-racing' season, therefore, LVEDD was unchanged although wall thickness reduced (Fagard et al. 1983).

Progression: LVEDD increases within 1 week of training in cyclists(Nishimura, Yamada et al. 1980). Increases in wall thickness and LVEDD are seen within 6 weeks of running 5x/wk (Shapiro and Smith 1983), 10 weeks of resistance lower limb exercise(Kanakis and Hickson 1980), 11 weeks of walking/running (DeMaria et al. 1978) and 14 weeks of interval bicycle training (Stein et al. 1980) although they may occur within a week amongst swimmers out of competitive training for up to 7 months (7% rise in both LVEDD and wall thickness) (Ehsani et al. 1978). Amongst rowers differences in septal thickness and LVEDD only becoming marked in 16 weeks (Wieling et al. 1981). An increase in cardiac mass in recruits over a similar timescale to our study has been demonstrated (Frick et al. 1963).

Author	Study Nature	Subjects	Training	Result
Bekaert 1981	Cross-sectional	runners cyclists		LVEDD increased by 8-14%
Lengyel 1979	Cross-sectional	swimmers		LVEDD increased by <33%
Pelliccia 1991	Cross-sectional	elite athletes rowers canoeists		7% have wall thickness >13mm
Pelliccia 1994	Cross-sectional	elite athletes rowers canoeists		13% have LVEDD >60mm
Adams 1981	Prospective growth	25 male students vs 11 controls aged 22 years	50 min jog 5 days/wk 12 weeks	No change in wall thickness
DeMaria 1978	Prospective growth	male volunteers	11 wks walk/ jog/run 4x/wk	LVM increase of 22%
Ehsani 1978	Prospective growth	8 competitive swimmers	9 wks swim training after 7 month rest	LVEDD +6% post wall+7% Rise within 1 wk
Frick . 1963	Prospective growth	14 male infantry aged18-26	basic training 2 months	LV volume rose 30ml/m^2
Kanakis 1980	Prospective growth	9 males aged 18-27 aged 18-27	10 wk leg resistance 5x/wk	LVM increase of 12.6%
Nishimura 1980	Prospective growth	professional cyclists	professional training	LVEDD rises in <1 week
Shapiro 1983	Prospective growth	15 male volunteers	6 weeks run 5x/wk for 30 min	LVM increase of 28%. Rise by wk 2
Stein 1980	Prospective growth	12 male 12 female cyclists	14 weeks interval training	LV dimensions increased 6%
Wieling 1981	Prospective growth	23 rowers (14 seniors)	7 months rowing	Septum +12% LVEDD+4%
Wolfe 1979	Prospective growth	12 males age 36.8 yrs 10 controls	30 min jog 4x/wk 6 months	No effect on LV mass although LVEDD rose
Zeldis 1978	Prospective growth	10 female hockey players	routine season	LVM increased 40% over season

Table 8.1 Key studies of effects of physical exercise on LV growth

Other variables

The effects of age on the hypertrophic response are disputed. Consistent with findings in animal models (Bloor et al. 1970), the response of the young may be greater (Wolfe et al. 1979). Others suggest the reverse (Nishimura et al. 1980). Sex, race and the nature and intensity of training programmes may influence physiological LVH (Cohen et al. 1980; Shapiro 1984; Schaible and Scheur 1985; Lombardo et al. 1991; Pelliccia et al. 1991) although female athletes still develop significant LVH (LVM 40% greater than controls (Zeldis et al. 1978)). Finally, prior training may reduce the LVH response (Saltin et al. 1968).

8.3: IDENTIFICATION OF AN APPROPRIATE POPULATION TO WHOM TO APPLY THE CARDIAC HYPERTROPHIC STIMULUS

8.31 MILITARY RECRUITS AS A POTENTIAL STUDY POPULATION

8.311: Military recruits as a homogeneous population

It has been shown above that the extent and pattern of physiological LVH (Cohen et al. 1980; Rost 1982; Shapiro 1984; Maron 1986; Pelliccia et al. 1991) is influenced by :

-the age and sex of the subjects (Bloor et al. 1970; Wolfe et al. 1979; Nishimura, et al. 1980), and their initial level of fitness (Saltin et al. 1968)

-the nature and intensity of their training program (Cohen et al. 1980; Shapiro 1984; Schaible and Scheur 1985; Lombardo et al. 1991; Pelliccia et al. 1991)

-the associated cardiac pressure-load (Morganroth et al. 1975) and volume-load experienced (Morganroth et al. 1975; DeMaria et al. 1978; Parker et al. 1978; Longhurst et al. 1980; Keul et al. 1981; Paulsen et al. 1981; Wieling et al. 1981)

If the association of genotype with phenotypic response to a given stimulus are to be effectively examined, the effects of these confounding factors (and other environmental factors which may have unknown effects) must therefore be negated. The use of UK military recruits offers a unique opportunity to do this. All are of similar age, live under identical environmental conditions, have identical daily patterns (sleep, diet, etc.), and groups of single sex can be examined. They undergo an identical supervised 10-week training pattern comprising 69 periods of physical training (each of 40 minutes). Thirty-five periods emphasise endurance alone. Much of the remaining time also involves identical physical activity (weapons, assault course

and battlefield training, and marching). Such training is likely to be of sufficient intensity and pattern to cause demonstrable LVH of between 10 and 30% (DeMaria et al. 1978; Kanakis and Hickson 1980; Shapiro and Smith 1983) over a 10 week period (Frick et al. 1963; Ehsani et al. 1978; Stein et al. 1980). The standardised nature of the population and their environment makes identification of a gene-stimulus interaction feasible.

8.312 Past studies of cardiac hypertrophy in military recruits

The use of military recruits in the study of physiological cardiac hypertrophy has been partially validated. One early study (Frick et al. 1970) used 29 young male signaller draftees with low entry level of fitness who trained for 2 hours/day for 2 months (lower intensity and duration than in the UK). Technologically primitive cardiac assessment showed no change in heart rate or cardiac dimensions. More intensive training of recruits demonstrate a non-significant fall in HR and BP, but an increase in stroke volume of 14%, CO of 10%, and heart size of 30cc/m^2 (Frick et al. 1963).

8.4: METHODS

8.41: STUDY POPULATION

The study population comprised all 460 consecutive males recruited to the Army Training Regiment Bassingbourn, UK over a 9 month period. All were normotensive and free of cardiovascular disease, and underwent an identical 10 week period of intensive strength and endurance training. This consisted of sixty-nine periods of physical training, each of 40 minutes duration, of which 35 periods emphasised lower limb endurance alone. At entry, the time taken to complete standard 1.5 mile run at maximal exertion was recorded.

Subjects were studied on the first day of training (pre-training data), and again 10 weeks later (post-training data). At entry, height and weight were documented, and venous blood was drawn for ACE genotype analysis. On both occasions, blood pressure (mean of three manual measurements after 5 minutes supine rest, each 1 minute apart) was documented, and transthoracic echocardiography performed. In random subsamples, blood was taken for assay of beta-natriuretic peptide and a 12-lead electrocardiographic recording performed before and after

training. Only subjects who completed training without interruption were included in follow-up.

This study was conducted with Army Medical Services Research Executive (ethical committee) approval and written informed consent was obtained from each participant.

8.42: ASSESSMENT OF LEFT VENTRICULAR MASS AND GROWTH

Left ventricular growth was primarily assessed by echocardiographic examinations, and confirmed by two further independent methods.

8.421: Echocardiographic Methods

Left ventricular mass was determined by echocardiographic examination (Acuson 128/XP10c:Hewlett Packard Sonos 2500: 2.5-3MHz probes) performed to strict protocol by experienced technicians. All subjects lay in a standard left-lateral position so as to negate the influence of body position on calculated LV mass (Martin et al. 1986). Septal thickness, posterior wall thickness, and left ventricular end-diastolic dimension were measured from 2-dimensional left ventricular short-axis views recorded at the level of the mitral valve leaflet tips, using the American Society of Echocardiography protocols (Sahn, et al. 1978). Measurements were made independently by two observers blinded to ACE genotype and training status, and expressed as the mean of 3 readings made by each observer. Left ventricular mass was calculated as suggested by Devereux and co-workers (Devereux 1987) and also adjusted for height and surface area (de Simone et al. 1992). Only echocardiograms considered technically excellent by both observers were analysed.

8.422: Electrocardiographic Methods

With the subjects supine, standard 12-lead electrocardiograms (ECGs) were recorded using the Marquette MAC VU (C)-12SL machine (Marquette Electronics, Wisconsin, USA). Simultaneous recordings were made for each lead at a paper speed of 25mm/sec, 40-150Hz frequency and 10mm/mV gain over a ten second period. The data were stored on computer disc and analysed using MUSE software (Marquette Electronics, Manchester, UK). Left ventricular mass index (LVMI) was calculated from paired electrocardiographic data using the Rautaharju equation(Rautaharju et al. 1988). An increase in calculated LVMI of more than $5g/m^2$ was chosen empirically as a measure of significant LV growth. Sokolow-Lyon criteria (SV_1+ the greater of RV_5

or RV_6 >3.5 mV) were used to define the presence of LVH (Sokolow and Lyon 1949).

8.423: Assay of beta-natriuretic peptide (BNP)

15ml venous blood was drawn into cooled tubes containing 100µl EDTA and 100µl Aprotinin (Bayer, Newbury, Berkshire UK.) The samples were immediately centrifuged (4^0C: 3000 rpm for 10 minutes) and the plasma separated and placed on dry ice for up to four hours before storage at -80^0C. Within 3 months, BNP was recovered (65-88%) and assayed by radioimmunoassay (Peninsula Laboratories, Belmont, CA, USA) as previously described (Lang et al. 1991). The technician was blinded to the timing of the sample and subject genotype. The minimum concentration detected was 1.25pg/ml.

8.43: DNA EXTRACTION

5ml blood samples were drawn into tubes containing ethylene diamine tetra-acetic acid (EDTA), and samples stored at -70°C. Leucocyte DNA was extracted as previously described (chapter 3).

8.44 ASSESSMENT OF ACE POLYMORPHISM GENOTYPE

The potential misclassification of subjects of ID genotype as being of DD genotype has been previously discussed (chapter 3). ACE gene insertion/deletion polymorphism genotype was thus determined using a three primer system (Evans et al. 1994) (chapter 3). DNA fragments were subsequently separated by electrophoresis on an 8.4% polyacrylamide gel, and identified as previously described.

8.45: STATISTICAL ANALYSIS

The measurements of left ventricular dimension made by the two observers agreed closely, with 92-98% lying within ±1.96 standard deviations of the mean of the two measures. Bland-Altman plot suggested no systematic differences between the paired measurements made by each observer (Bland and Altman 1986). The mean of the two observers' measurements was therefore used in all analyses.

Change in blood pressure, left ventricular dimensions and mass were assessed by calculating the difference between pre- and post-training measurements for each subject. Data were analysed for the study population as a whole, and differences in response between different genotype groups compared. Pre- and post-training data were compared using two-tailed paired t-tests. Mean changes between groups and their statistical significance were compared by analysis of variance. It has been recognised that changes in biological variables cannot be analysed by studying simple differences alone, as the magnitude of the change may be influenced by baseline values (Glynn et al. 1982; Cain et al. 1992). Therefore, analysis of covariance was used to adjust for the potential confounding effects of pretraining left ventricular mass, as well as for age and systolic blood pressure. Differences in proportions were assessed by the McNemar chi square statistic for paired data. P-values <0.05 were considered to be statistically significant. All analyses were performed in SAS (SAS Institute 1985).

8.5: RESULTS

8.51: ECHOCARDIOGRAPHIC (Table 8.2: Figure 8.1)

All of the 460 eligible subjects agreed to participate in the study and ACE genotype obtained in 458. 150 failed to complete training, and where thus unavailable for post-training analysis. Paired echocardiograms suitable for analysis were obtained in 140 of the remaining subjects. The physical characteristics (mean +/-standard deviation) of those studied (age 18.9$\pm$ 2.1 years: height 176$\pm$ 6.1 cm: weight 67.7$\pm$ 11.1 Kg: systolic BP 120$\pm$ 10.9: diastolic BP 71$\pm$ 9.7 mmHg) did not differ from those excluded, and were the same across genotypes. The genotype distribution (n[%]: II 34[24.3], ID 77[55], DD 29[20.7] did not differ between those studied and those ultimately excluded (through training failure and inadequate imaging) from final analysis (p= 0.67).

Blood pressures pre-training (120$\pm$0.94 systolic, 71$\pm$0.84 mmHg diastolic) and post-training (121$\pm$0.99 systolic, 69$\pm$0.89 mmHg diastolic) were no different (p=0.50 and 0.31 respectively).

Pre-training left ventricular dimensions are shown by genotype in the first column of table 8.2. End-diastolic dimension differed between genotypes (II>DD>ID: p for heterogeneity = 0.009). Presence of the D allele was associated with a trend towards lower initial left ventricular mass (II>ID>DD) although this was not

statistically significant even when adjusted for height$^{2.7}$(table 8.2) (de Simone et al. 1992).

Overall, training was associated with left ventricular growth (table 8.2). Left ventricular dimensions increased significantly ($p<0.0001$ for each measure), with mean left ventricular mass rising from 167g to 197g ($p<0.0001$). The magnitude of these changes was strongly associated with ACE genotype. LV mass altered by +2.0g, +38.5g and +42.3g for II, ID and DD genotypes respectively (p for heterogeneity <0.0001: Figure 8.1). The association of ACE genotype with increase in LV mass persisted after adjustment for subject height$^{2.7}$ (table 8.2) (de Simone et al. 1992), as well as pretraining LV mass, age, and systolic blood pressure (figure 8.1). Septal thickness changed by -0.02 vs. 0.09 vs. 0.15cm, and posterior wall thickness by -0.02 vs. 0.08 vs. 0.14 cm for II, ID and DD respectively (p for heterogeneity <0.0001 in both cases.)

	Pre-training (n=140)	Post-training (n=140)	Change (cm)	p (paired t-test)
Septal thickness (cm)				
II	0.96	0.94	-0.02(0.02)	0.47
ID	0.97	1.05	0.09(0.01)	0.0001
DD	0.92	1.06	0.15(0.03)	0.0001
All	0.96	1.02	0.08(0.01)	0.0001
p (heterogeneity)	(0.08)	(0.0001)	(0.0001)	
Posterior wall (cm)				
II	0.92	0.98	-0.02(0.02)	0.29
ID	0.93	0.99	0.08(0.01)	0.0001
DD	0.89	1.00	0.14(0.03)	0.0001
All	0.92	0.97	0.07(0.01)	0.0001
p (heterogeneity)	(0.35)	(0.0001)	(0.0001)	
End-diastolic dimension (cm)				
II	5.12	5.27	0.15(0.07)	0.04
ID	4.90	5.26	0.35(0.05)	0.0001
DD	5.01	5.21	0.20(0.06)	0.004
All	4.98	5.25	0.27(0.03)	0.0001
p (heterogeneity)	(0.009)	(0.80)	(0.02)	
LV mass (g)				
II	175	177	2(5.02)	0.69
ID	165	204	38.5(3.7)	0.0001
DD	162	204	42.3(6.0)	0.0001
All	167	197	30.4(3.0)	0.0001
p (heterogeneity)	(0.18)	(0.0005)	(0.0001)	
LV mass/height$^{2.7}$ ($g/m^{2.7}$)				
II	37.7	37.9	0.3 (1.1)	0.81
ID	36.2	44.7	8.6(0.8)	0.0001
DD	34.7	43.6	9.0(1.3)	0.0001
All	36.2	42.8	6.6(0.7)	0.0001
p (heterogeneity)	(0.20)	(0.0001)	(0.0001)	

Table 8.2: Pre- and post- training left ventricular dimensions and mass [mean, (standard error)] by ACE genotype

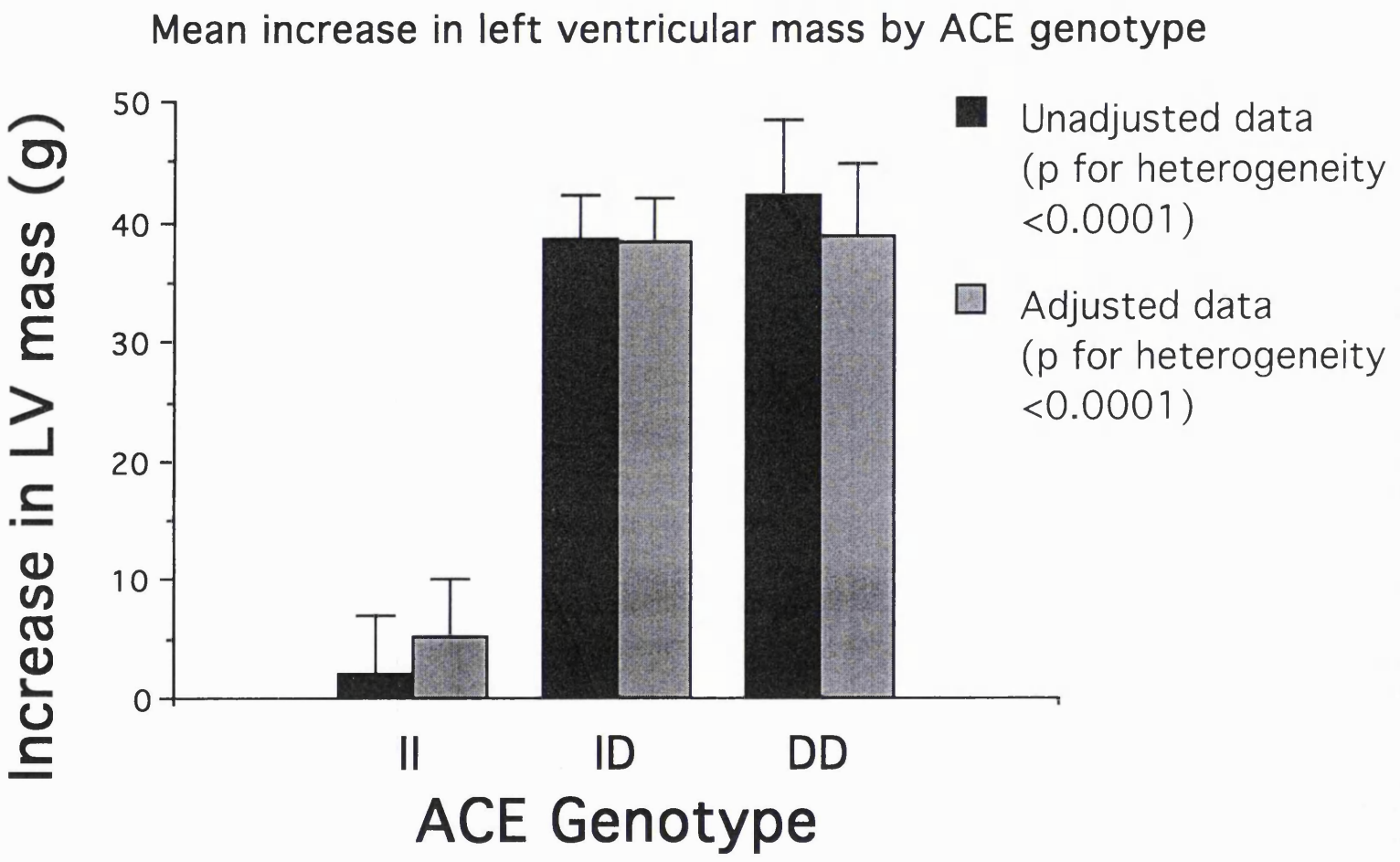


Figure 8.1 Mean (±SEM) increase in left ventricular mass associated with 10 weeks of basic military training. Data is presented in unadjusted form, and when adjusted for height, age, systolic blood pressure and pretraining LV mass

8.52: ELECTROCARDIOGRAPHIC DATA (Table 8.3)

On each visit, study logistics and time constraints prevented complete data acquisition in 14% of those completing training. Thus, of the 308 training survivors, pre- and post-training ECGs were available for 121 individuals. All electrocardiograms were visually reviewed by 2 observers blinded to subject genotype. No subjects exhibited complete bundle branch block, evidence of accessory pathway conduction or evidence of myocardial infarction, which would have prevented assessment of LVH. The prevalence of electrocardiographically-defined LVH was genotype-related, rising from 6/24 before training to 11/24 afterwards in those of DD genotype ($p<0.01$), but from 8/30 to only 9/30 in those of II genotype. The D allele was associated with an increase in calculated LV mass, although this difference did not reach statistical significance (increase of more than $5g.m^2$ in 26.7 vs. 35.8% vs. 37.5% for II, ID and DD respectively)

	ACE Genotype		
	II	ID	DD
ALL	n=30	n=67	n=24
Number (%) with LVH Pre-Training	8(26.7)	35(52.2)	6(25.0)
Number (%) with LVH Post-Training	9(30.0)	36(53.7)	11(45.8)
Number (%) with $>5g/m^2$ increase in LV Mass	8(28.6)	24(35.8)	9(37.5)

Table 8.3: Training-related changes in prevalence of left ventricular hypertrophy by voltage criteria (Sokolow and Lyon 1949) and increase in left ventricular mass (Rautaharju et al. 1988) by genotype in 121 individuals.

8.53: PLASMA BNP (Figure 8.2)

Of the 460 subjects entered into the study, one cohort of 84 was randomly selected for assay of plasma BNP. The paired pre- and post-training BNP data of the 49 who completed training were used for analysis.

Pre-training plasma BNP levels did not differ between genotypes (p for heterogeneity = 0.69: figure 8.2). Training was associated with a significant rise in plasma BNP levels in the group as a whole (n=49: mean$\pm$SE 44.6$\pm$2.5pg/ml vs. 66.4$\pm$4.7pg/ml: $p<0.001$). The magnitude of this rise was related to the presence of the D allele (figure 8.2). BNP levels rose significantly only in those with one or more D alleles (n=35, pre-training 43.7$\pm$2.8pg/ml, post-training 69.6$\pm$6.1pg/ml, $p<0.0001$), and was greatest for those of DD genotype (mean increase in plasma BNP of 11.5$\pm$6.3pg/ml in those of II genotype vs. 56.0$\pm$17.3pg/ml for DD genotype: $p<0.01$). Post-training BNP levels, unlike pre-training levels, were associated with ACE genotype (DD>ID and DD>II: $p<0.001$ for both comparisons).

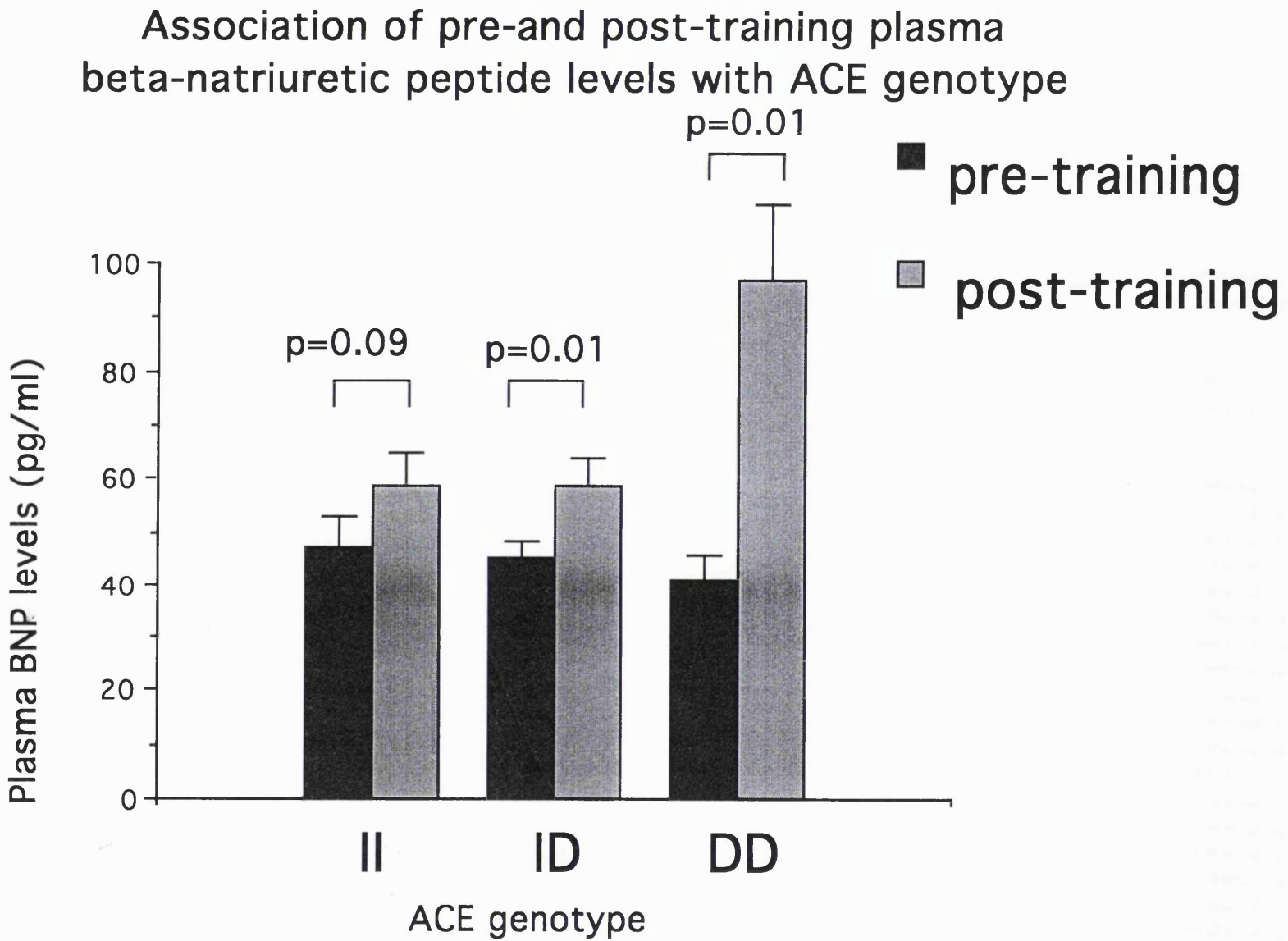


Figure 8.2 Plasma BNP levels before and after 10 weeks of basic military training compared by t-test.

8.6: DISCUSSION

As postulated, the extent of exercise-induced left ventricular growth is strongly influenced by the insertion/deletion polymorphism of the angiotensin converting enzyme gene.

8.61: ECHOCARDIOGRAPHIC FINDINGS

In the group as a whole, left ventricular mass and dimensions increased significantly with exercise training ($p<0.0001$ for all measurements). Hypertrophy was to be expected in this timescale (Ehsani et al. 1978), has been previously demonstrated in military recruits (Frick et al. 1963), and its magnitude was similar to that previously reported(12.5-22%) (DeMaria et al. 1978; Kanakis and Hickson 1980). Increase in LV mass and wall thickness was associated with the D allele. This effect was not due to identified confounding factors. The potentially confounding effects of subject age and sex (Wolfe et al. 1979; Nishimura et al. 1980), initial fitness (Saltin et al. 1968) and nature and intensity of training program (Cohen et al. 1980; Schaible and Scheur 1985; Maron 1986; Pelliccia et al. 1991) were minimised by the uniform nature of the study population and the application of an identical training program to all subjects. Multivariate analysis confirmed a modest influence of systolic blood pressure on LV mass (Koren et al. 1991), although blood pressures were similar between all genotypes pre- and post-training. The influence of genotype remained when data were standardised for height and body morphology (de Simone et al. 1992), or when multivariate adjustment was made for the effects of height, age, blood pressure and initial fitness.

Although resting blood pressure was no different between genotypes either before or after training, it remains possible that the D allele was associated with a greater rise in blood pressure in response to exercise. It was not possible to address this issue in this study. The very nature of military training, where it is carried out (often in the field), the clothing worn , and of the form of exercises it entails (e.g. pull-ups to a bar) make non-invasive 24-hour blood pressure monitoring, or invasive monitoring, impractical. However this issue was addressed in a follow-up study, the data of which are presented in the following chapter.

Training-associated changes in cardiovascular haemodynamics might in theory influence LVEDD, and hence calculated LV mass. However, heart rate changes are modest in recruits, correlate poorly with LVEDD, are unlikely to be ACE-genotype associated, and their influence on calculated LV mass is very small (Frick et al. 1963; DeMaria et al. 1978; Adams et al. 1981; Schaible and Scheur 1985). Furthermore, physical training has little effect on resting cardiac output and peripheral vascular resistance (Frick et al. 1963; DeMaria et al. 1978).

The greater LV growth associated with the D allele is not merely due to genotype-related differences in pretraining LV dimensions i.e. the effect is not due to exercise causing hypertrophy towards a mean 'fixed ceiling': (i)There was a trend for the D allele to be associated with lower pre-training LV mass, yet LV mass increased for those of DD genotype ($p<0.0001$) and not II genotype, and showed no evidence of growing to a common mean (post-training LV mass DD> II genotype, $p<0.001$). (ii) Although pretraining septal thickness and end-diastolic dimension differed between genotypes, such differences were small and were not confined to any one genotype (septal thickness ID>II>DD: end-diastolic dimension II>DD>ID). Septal thickness rose with training to a greater extent as the number of D alleles increased. Those of ID genotype had a pretraining septal thickness slightly (but not significantly) greater than those of II genotype (0.97 vs. 0.96cm), yet grew more (+0.09 vs. -0.02 cm respectively). (iii) The association remained after adjustment for pretraining left ventricular mass (either directly or by adjustment of individual component measurements used to calculate LV mass). (iv) Finally, BNP levels in each genotype were similar prior to training and were significantly different between genotypes afterwards, again refuting the possibility that LV mass increased significantly more in those of DD genotype only because initial LV mass was smaller in this group.

It will be noted that paired perfect echocardiograms suitable for analysis were available in only 140 of those completing training, amounting to an exclusion of 27% of echocardiograms at either the start or end of training. Exclusion rates of 20% or above are not unusual in studies of this nature (Lindpaintner et al. 1996), where perfect image quality and axis orientation is crucial. Our exclusion rates were perhaps somewhat increased by the conditions under which recordings were obtained, with high throughput of large numbers of individuals in confined space being routine. Such exclusions were not a source of bias, however. They were made before genotypes were available. The physical characteristics and ACE genotype distribution of those included and subsequently excluded from analysis did not differ.

8.62: ELECTROCARDIOGRAPHIC DATA

These data, derived by 2 methods, support the association of ACE genotype with LV growth and are consistent with the findings of Schunkert et al. (1994). The presence or absence of significant (dichotomous) left ventricular hypertrophy was defined by voltage criteria. The use of voltage combinations minimises the error of repeated measures in an individual (Farb et al. 1990). The Sokolow-Lyon voltage (SV_1+ the greater of RV_5 or RV_6 >3.5mV) (Sokolow and Lyon 1949), is the most reproducible of these combinations (Farb et al. 1990), and has been previously used in studies of

the ACE gene polymorphism and LVH (Schunkert et al. 1994). The number of individuals with voltage-defined LVH ($LVH^{+}ve$) rose minimally in those of II and ID genotypes (from 8 to 9 of 30, and from 35 to 36 of 67 respectively), but nearly doubled (from 6 to 11) in the 24 of DD genotype. This increase in prevalence was due partly to a genotype-related increase in the number of new cases. Of the 22 individuals of II genotype whose pre-training ECGs did not satisfy the voltage criteria for LVH ($LVH^{-}ve$), 7 (32%) were $LVH^{+}ve$ after training. This compared to 8 of 36 (22%) of the ID group, and 5 of 18 (27%) of the DD group. However, genotype also influenced the number of individuals who were initially $LVH^{+}ve$ and who became $LVH^{-}ve$ after training (6 of 8[75%], 7 of 35[20%] and 0 of 6[0%]) for II, ID and DD respectively). A balance of a genotype-dependent increase in ECG signal amplitude and increased signal attenuation (due to training-related changes in chest wall muscle mass, lung volume, thoracic impedance, body morphology and obesity (Rudy, et al. 1982; Levy et al. 1990b)) may account for these findings.

The high prevalence of 'LVH' as defined by voltage criteria will be noted in this population (table 8.3). Low septum-to-skin distance increases voltage-defined LVH prevalence (Devereux et al. 1983), the frequency of which is therefore inversely related to body mass index (Xie et al. 1994). This is increasingly true amongst younger individuals (Otterstad et al. 1991; Xie et al. 1994). LVH defined by Sokolow-Lyon criteria is therefore not uncommon amongst the young, fit or lean (Lie and Erikssen 1984; Otterstad et al. 1991; Xie et al. 1994). The high prevalence of ECG LVH in this young fit lean population is therefore not unexpected. Indeed, a similar study has shown a frequency of ECG LVH of nearly 20% amongst middle aged males (Schunkert et al. 1994).

Electrocardiography is an insensitive (Schillaci et al. 1994) tool with which to prospectively examine left ventricular hypertrophy. Recordings are dependent on lead and body position (Farb et al. 1990), vary greatly with repeated measurement (Farb, et al. 1990) and may be influenced by training-associated changes in thoracic impedance (above). However, in the context of the echocardiographic and hormonal changes reported, they provide supporting evidence of an association of ACE genotype with exercise-related hypertrophy.

8.63: BNP

These data strongly support the association of ACE genotype with left ventricular growth.

Beta-natriuretic peptide (BNP) is a peptide hormone of predominantly left ventricular origin (Nakao et al. 1991). Human left ventricular hypertrophy is associated

with raised plasma BNP levels (Hasegawa et al. 1993; Cheung and Brown 1994) which correlate with LV mass during both hypertrophic progression and regression (Kohno et al. 1992a; Kohno et al. 1995). BNP synthesis can thus be considered a marker of myocyte growth (Takahashi et al. 1992; Harding et al. 1995), and it is in this capacity which we used BNP assay in this study. Pre-training plasma BNP levels did not differ between genotypes. Levels increased significantly with training in the whole group- an effect strongly associated with ACE genotype (figure 8.2: p for heterogeneity of rise in BNP levels =0.003). Levels did not rise significantly for those of II genotype (47.0±5.6 vs. 58.4±6.3pg/ml), ID genotype (44.9±3.4 vs. 58.8±5.1pg/ml: p=0.02), but rose by 209.3±89.8% (mean of paired differences) amongst those of DD genotype (40.7±4.8 vs. 96.7±14.2pg/ml: p<0.0001). Post-training BNP levels were thus associated with genotype(p for heterogeneity <0.005). We do not believe that known confounding factors influenced these data. Exercise has little influence on plasma BNP concentration (Kohno et al. 1992b; Nicholson et al. 1993; Matsumoto et al. 1995), all blood samples were taken in the absence of recent exercise, and any such effect of exercise is likely to be shortlived due to the very short plasma half-life of BNP (Vanneste et al. 1990). No subjects had identifiable cardiac disease associated with raised plasma BNP levels (Nakao et al. 1991; Matsumoto et al. 1995).

8.64: EXCLUSION OF BIAS

A major strength of this study was the use of a uniform study population, environment and stressor. Nonetheless, if the D allele was associated with improved echocardiographic image, selection bias may then have influenced our echocardiographic data. However, the physical characteristics and genotype distribution of those whose echocardiographic data were analysed were similar to those ultimately excluded from analysis. Even if some unidentified selection bias had influenced echocardiographic data, such bias could not have applied to the BNP and ECG data. Acceptability of echocardiographic images was not a criterion for ECG or BNP analysis. Nonetheless, the genotype association with growth held for ECG data despite the fact that almost half (60 of 121) of the individuals whose paired ECGs were analysed were not members of the group of 140 subjected to echocardiographic analysis. Similarly, one third (11 ID, 5 DD) of the individuals used in BNP analysis were also independent of the echocardiographic data set, and the genotype association holds even for this small number: BNP levels rose with training for the 11 of ID genotype (50.93± 6.16 vs. 59.36± 7.57 pg/ml, p=0.40) but significantly more in the 5 of DD genotype (39.5±5.05 vs. 76.28±12.56, p=0.026: p= 0.028 for change in BNP

for ID vs. DD). Measurement bias and error was reduced by blinding all investigators to recruit genotype and the use of high quality echocardiographic records, and repeated measures by each of two observers. Recruits performed similarly in tests of fitness and physical performance at entry. For instance, run times over 1.5 miles were independent of genotype: (mean$\pm$SD time in minutes 9.55$\pm$0.64 vs. 9.80$\pm$ 0.60 vs. 9.91$\pm$0.64 for II, ID and DD respectively).

8.65: COMPARISON WITH DATA FROM OTHER STUDIES

These data therefore convincingly demonstrate an association of ACE genotype with left ventricular growth. Previous studies relating ACE genotype to LV mass, such as those of Lindpaintner and Schunkert (Schunkert et al. 1994; Lindpaintner et al. 1996) may have provided conflicting conclusions for several reasons:

Firstly, past studies have often been small: Gharavi reported a study of only 38 homozygotes (Gharavi et al. 1995). Kupari studied only 86 individuals in total (aged 36-37), of whom 25 were of II genotype and 26 of DD genotype (Kupari et al. 1994). Secondly, cross-sectional population studies such as these might also be confounded by deaths attributable to ACE genotype-associated disease including LVH itself (Levy et al. 1990a; Cambien et al. 1992; Raynolds et al. 1993; Tiret et al. 1993).

Finally, cardiac renin-angiotensin systems may have little basal effect, but may transduce and amplify hypertrophic stimuli (such as hypertension or exercise) to which individuals are exposed to varying degrees in a general population. Several observations support this concept. The presence of, or a trend towards, an association of LVM with ACE genotype is stronger in groups containing large numbers of hypertensive individuals (Iwai et al. 1994; Prasad et al. 1994) than in disease-free populations (Kupari et al. 1994; Lindpaintner et al. 1996). LV mass may correlate with systolic blood pressure only amongst those of DD genotype (Prasad et al. 1994). DD genotype has been associated with concentric LV remodelling in hypertensives (Gharavi et al. 1995) and increased LV mass and phenotypic expression in hypertrophic cardiomyopathy (Marian et al. 1993; Lechin et al. 1995). The association of ACE genotype with LV mass is thus likely to differ with varying exposure to hypertrophic stimuli. Even prior to training, ACE genotype is associated with differences in LV conformation (septal thickness ID>II>DD p=0.08: LVEDD II>DD>ID p=0.009): (Table 8.2, column 1), with the net balance of such differences producing a 'reversed association' with resting genotype and height-adjusted and unadjusted LV mass (II>ID>DD: p=0.18 for LVM and 0.20 for LVM/$ht^{2.7}$). A different pattern of LV structure was associated with ACE genotype after training (Table 8.2, column 2). Data from individuals under various levels of hypertrophic

stimulation (as might be found in a general population) might therefore no longer show a clear association of LV mass with genotype.

Such factors might account for the recently-reported lack of association of ACE genotype with LV mass in patients from the Framingham database (Lindpaintner et al. 1996). This retrospective cross-sectional population study examined the association of an allelic variant with a phenotypic characteristic (dichotamously-defined 'LVH'.). Of 10333 individuals, 24% (2439) of mixed sex, race, and age (mainly late middle-aged) were studied. A diverse population such as this would have been exposed to a highly variable lifetime hypertrophic burden or even antihypertrophic burden (between 12 and 16% of each genotype were taking antihypertensive medication, some of which might have even included ACE inhibitors).

Our finding of an association of LV geometry with ACE genotype has been suggested before (Gharavi et al. 1995), and is important given the possible association of geometry with outcome (Devereux 1995; Krumholz et al. 1995).

8.66 MECHANISM OF EFFECT OF ACE GENE I/D POLYMORPHISM

The slight decline in baseline LV mass with increasing numbers of D alleles, and a reversal of this association after training, would be consistent with an increased responsiveness of ACE synthesis being associated with the D allele. The association of the ACE gene I/D polymorphism with ACE levels suggests that the effects on LV growth are mediated through the renin-angiotensin system. In theory, this might be through alterations in tissue kinin metabolism or, perhaps more likely, through effects on ATII synthesis. However, the ACE gene lies in close proximity to the human growth hormone gene, and the I/D polymorphism might thus be a marker for differences in tissue growth hormone activity. Current evidence suggests that such an association is unlikely (Jeunemaitre et al. 1992; McKenzie et al. 1995). Secondly, The D allele might be merely associated with a greater increase in exercise-related blood pressure, and hence hypertrophic burden. We cannot discount this possibility. However, non-invasive measurement of blood pressure during training proved impossible in this study. However, resting blood pressures before and after training were not influenced by ACE genotype, the ACE gene is not associated either with a predisposition to raised blood pressure in hypertensive individuals (Harrap et al. 1993), presence of essential hypertension (Jeunemaitre et al. 1992), or to differences in blood pressure amongst normal individuals (Rigat et al. 1990; Berge and Berg 1994). Finally, in a separate study, we have been unable to demonstrate an association between blood pressure response to bicycle ergometric exercise and ACE genotype (data not shown).

8.67: IMPLICATIONS FOR HUMAN PATHOPHYSIOLOGY

Caution is required if these conclusions are to be extended to disease states and to other populations. Myocardial growth response to exercise is influenced by sex and age (Wolfe et al. 1979; Nishimura et al. 1980). Even different exercise patterns may have differing effects, although both experimental pressure or volume loading may increase myocardial ACE (Schunkert et al. 1990; Finckh et al. 1991; Schunkert et al. 1993b). Exercise-related hypertrophy differs from that associated with disease states in that it is associated with improved myocardial function (Schaible and Scheur 1985). An association of ACE-genotype with such physiological hypertrophy does therefore not necessarily imply an association with pathophysiological hypertrophy. However, these findings are consistent with a role for paracrine renin-angiotensin systems in the control of LV growth (Baker et al. 1990; Dzau 1993b; Lee and Lindpainter 1993; Malhotra et al. 1994), whose inhibition may partly account for the effect of ACE inhibitors in reducing myocardial mass (Lievre et al. 1995).

8.7: CONCLUSIONS

The insertion/deletion polymorphism of the angiotensin-converting enzyme gene is associated with the left ventricular growth response to exercise. These data support the presence of a myocardial renin-angiotensin system acting as a transduction system for growth stimuli.

THE I/D POLYMORPHISM OF THE ANGIOTENSIN CONVERTING ENZYME GENE AND EXERCISE-RELATED BLOOD PRESSURE CHANGES

9.1: INTRODUCTION

It has been shown in the preceding study that the insertion/deletion polymorphism of the angiotensin-converting enzyme gene is associated with the left ventricular growth response to exercise. It was concluded that these data supported a role for myocardial renin-angiotensin systems in the control of left ventricular growth. However, it remains possible that the effect of the D allele was not related directly to differences in the myocardial renin-angiotensin system, but that the effect was mediated indirectly through a genotype-dependent difference in exertional pressor response. Superficial analysis would not support this concept. Blood pressures before or after training did not differ significantly across genotypes amongst the individual study populations examined echocardiographically, by ECG, or through BNP assay. However, a possible influence of ACE genotype on systolic blood pressure is seen in the cohort as a whole i.e. amongst the 308 individuals who completed training (figure 9.1). This trend is not statistically significant prior to training (p for heterogeneity =0.35), but approaches significance at the end of training (p for heterogeneity 0.07) when systolic blood pressure for those of II genotype was significantly lower than those of DD genotype (122.7$\pm$1.4 vs. 118.0$\pm$1.5mmHg: $p<0.05$). Diastolic blood pressures did not differ before or after basic training between those of different genotype (pre-training 70.3$\pm$1.37 vs. 70.6$\pm$0.8 vs. 69.4$\pm$1.3 mmHg, p=0.75: post-training 69.7$\pm$1.23 vs. 70.1$\pm$0.81 vs. 69.9$\pm$ 1.23 mmHg, p=0.96: for II, ID and DD respectively).

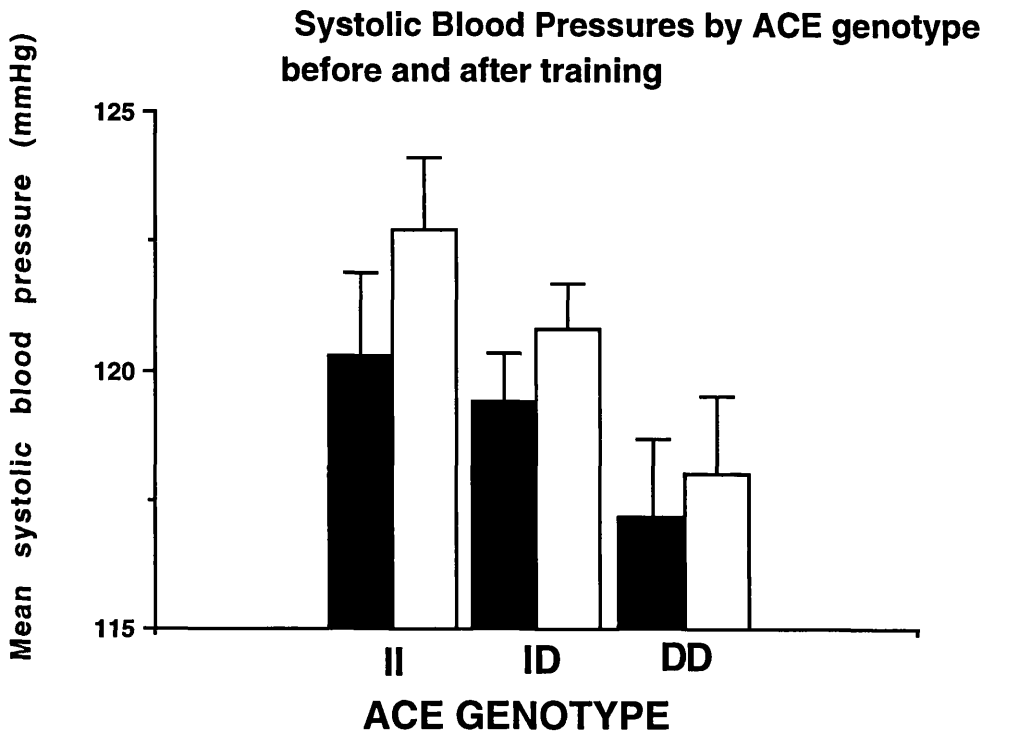


Figure 9.1: Systolic blood pressure (mean of three readings per recruit) by ACE genotype amongst 308 military recruits both before and after 10 weeks of basic military training (see chapter 7 for methods)

The association of the D allele with a slightly lower resting systolic blood pressure tends to argue against D-allele associated LV growth being mediated through changes in BP burden: a greater reduction in BP associated with the D allele would, if anything, have reduced LV growth. However, at the end of the primary study (chapter 8) it remained a possibility that the D allele was associated with a greater rise in systolic blood pressure during exercise. The greater increase in LV mass associated with the D allele (rather than the I allele) might thus not have been due to a greater growth response to an identical cardiac workload, but instead have been due to the D allele being faced with greater cardiac afterload and work during exercise. It had not proven possible in the original study to assess blood pressure response to conventional day-to-day training (see chapter 8). This study was designed, therefore, to determine whether ACE I/D polymorphism is associated with an increased exercise-induced blood pressure.

9.2: METHODS

9.21: STUDY POPULATION

This study was conducted with Army Medical Services Research Executive (ethical committee) approval and written informed consent was obtained from each participant. Once again, military recruits at the Army Training Regiment, Bassingbourn were recruited to the study. All were undergoing an identical training programme, as previously described (chapter 8). One intake of twenty-seven caucasian males (age 18.3$\pm$3.4 years, height 175$\pm$7.3 cm, weight 69.2$\pm$9.3 kg) were assessed during their final week of training. All consented to participate. None had undertaken physical exercise during the two days prior to the study.

9.22: ASSESSMENT OF BLOOD PRESSURE RESPONSE TO EXERCISE

Blood pressure response to exercise was assessed according to previously described methods(Schairer, Stein et al. 1992). Upright bicycle exercise was performed on a bicycle ergometer (electromechanically-braked: work independent of wheel rotation speed: Cycline 4000, NY. USA). Two identical machines were used, both of which had identical gearing ratios. Each subject wore similar light clothing, and all training was done in the same external environment. Each subject wore a Polar Accurex Heart

Rate Monitor (Polar CIC Inc., Port Washington, New York, USA), with continuous wrist-mounted heart rate display. Subjects were asked to pedal to maintain a fixed heart rate, and to maintain that rate for three minutes. Three stages were used: 110 beats per minute (bpm), 130 bpm, and 150 bpm. Blood pressure was manually recorded using a sphigmomanometer cuff at rest and at the end of each stage. External work rate was measured automatically by the bicycle ergometer by calculation of the work required to rotate the ergometer flywheel at the various recorded speeds of revolution with known gearing ratios.

9.23: ACE GENOTYPING

This was performed using 3 primer polymerase chain reaction amplification with subsequent electrophoretic fragment separation on a polyacrylamide gel, as previously described (chapter 3). Results were compared by two individuals blinded to the results of the investigation.

9.24: STATISTICAL ANALYSIS

Heterogeneity in mean blood pressure across genotypes were evaluated using ANOVA, and differences in blood pressure between genotypes compared by t-test. $p<0.05$ was taken to be statistically significant.

9.3: RESULTS

9.31: ACE GENOTYPE

3 (11.1%) individuals were of DD genotype, 15 (55.6%) of ID genotype, and 9 (33.3%) of II genotype. Heights, weights and ages did not differ across genotypes ($p>0.3$ in all cases).

9.32: BLOOD PRESSURE RESPONSE TO EXERCISE

With increasing heart rate and work, diastolic blood pressure did not rise. However, there was a steady increase in systolic blood pressure of similar degree across genotypes. At no time-point were either systolic or diastolic blood pressures significantly different between genotypes ($p>0.05$ for all comparisons: p for heterogeneity 0.84/0.69, 0.30/0.73, 0.27/0.95, and 0.50/0.81 for systolic/diastolic blood pressure at rest, 110 bpm, 130 bpm, and 150 bpm respectively: figure 9.2) Blood pressures do not differ when those with a D allele (i.e. DD and ID grouped data) are compared to those without a D allele (i.e. those homozygote for the I allele: figure 9.3)

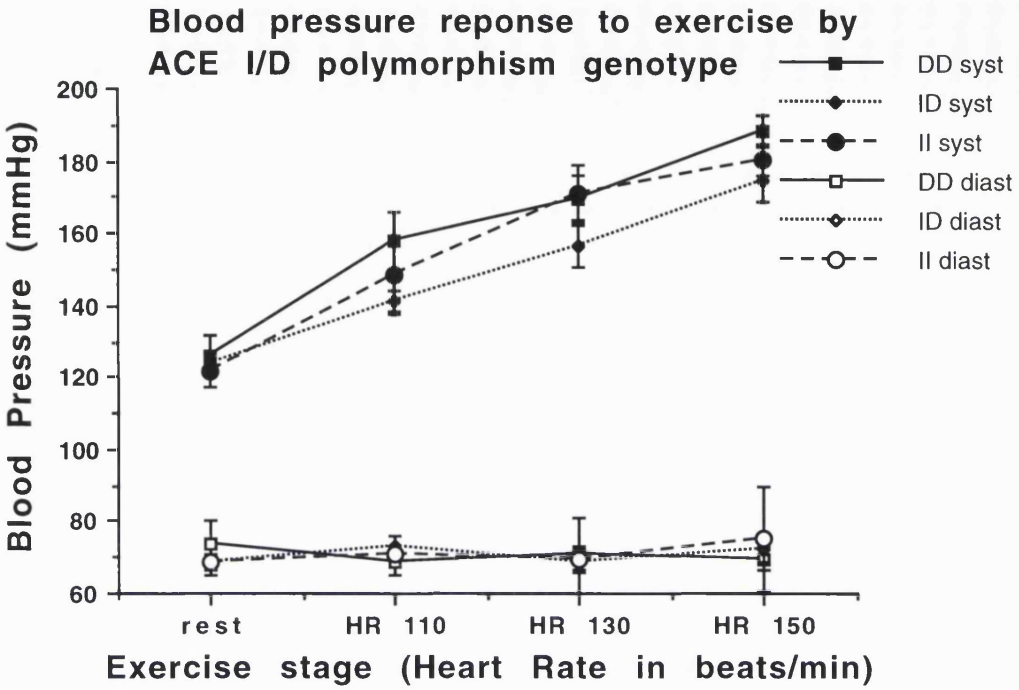


Figure 9.2: Blood pressure (mean +/- SEM) at 3 fixed heart rates during exercise, by ACE I/D genotype (see text for details)

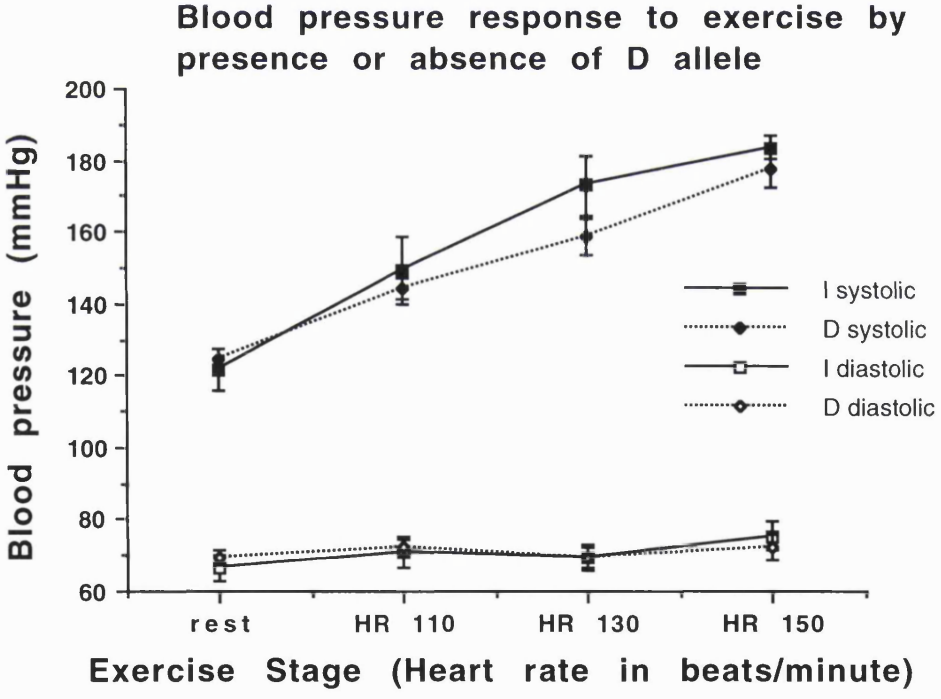


Figure 9.3: Mean=/- SEM blood pressures at fixed heart rates amongst those with one or more D alleles (D) or homoygous for the I allele (I): see text for details.

9.33: INFLUENCE OF GENOTYPE ON WORK RATE

At any given heart rate, mechanical power output (watts) was similar across genotypes (p for heterogeneity 0.85, 0.23 and 0.67 respectively: p>0.05 for all genotype comparisons) (figure 9.4). The same is true when those with a D allele (ID+DD data) are compared to those without (II genotype) (figure 9.5).

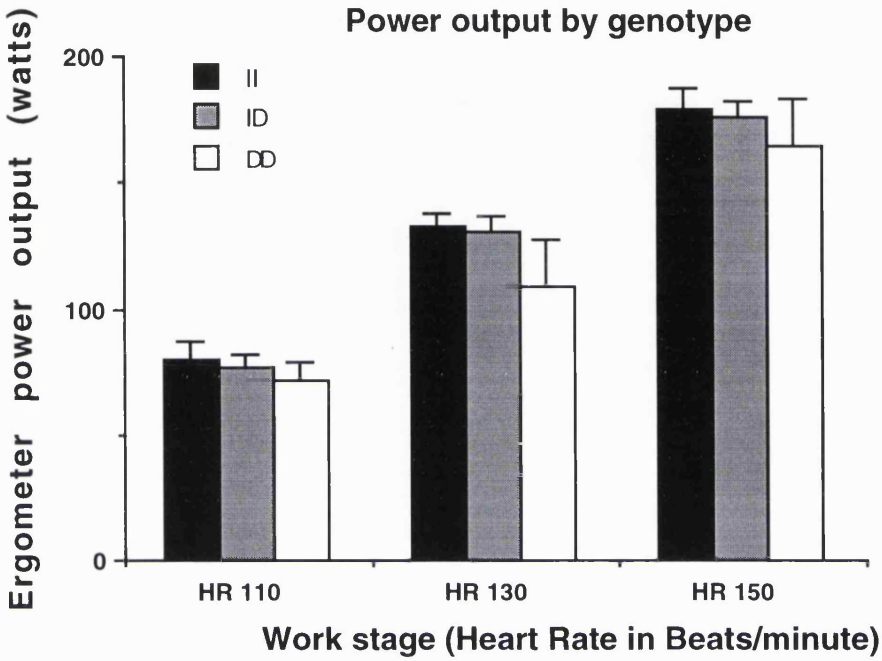


Figure 9.4: Power output (watts) at each fixed heart rate during exercise by ACE I/D genotype (see text for details)

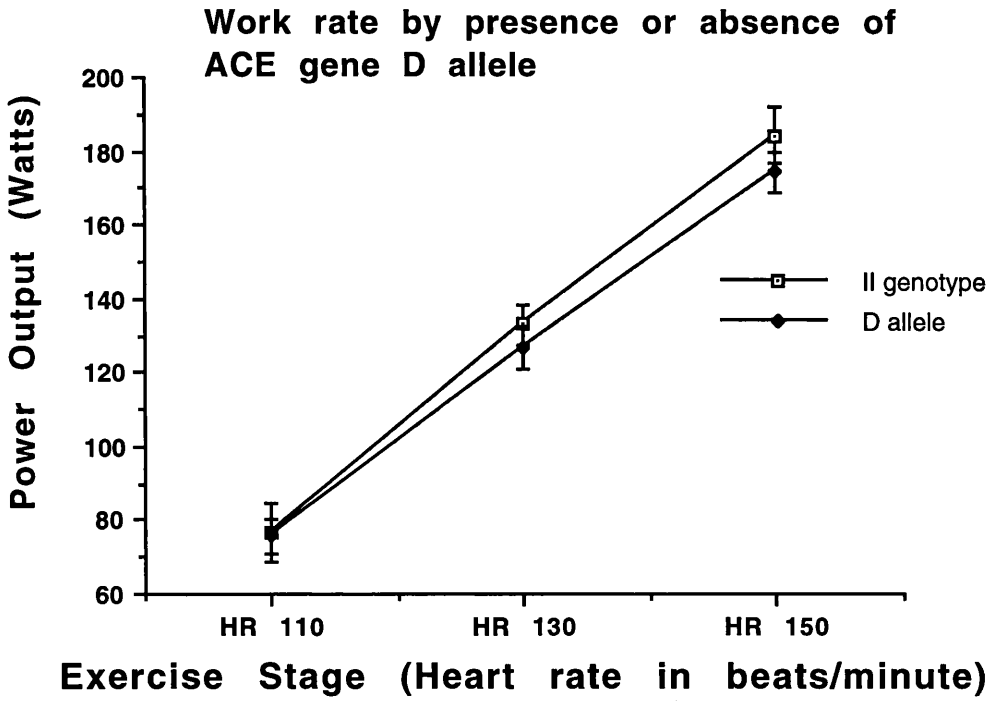


Figure 9.5: Power output at fixed heart rates during exercise amongst those with one or more D alleles (D), or homozygote for the I allele (see text for details)

9.4: DISCUSSION

Our original data showed that the D polymorphism of the ACE gene was associated with a greater exercise-related increase in left ventricular mass than the I polymorphism. This new data does not conclusively dismiss the possibility that at least some of this effect was due to the association of the D allele with greater cardiac work when completing a given task.

Increased cardiac work due to increased pressure or volume loading may induce a left ventricular hypertrophic response (Cohen et al. 1980; Shapiro 1984; Levy et al. 1990a; Pelliccia et al. 1991; Vogt et al. 1993). Our previous data has confirmed the modest influence of resting systolic blood pressure on LV mass noted by Koren and others (Koren et al. 1991). However, there was only a modest influence of resting systolic blood pressure on the LV growth response to exercise, and the association of the D allele with LV growth remained when multivariate adjustment was made for factors including blood pressure. It is possible, however, that the haemodynamic response to exercise differed between genotypes, and that this association was responsible for the excess LV growth associated with the D allele. This study was designed to address this issue.

Initial analysis of the data tends to suggest that there is no significant effect of ACE genotype on the blood pressure response to exercise. In keeping with the findings of others, systolic pressure rises in proportion to workload, whilst diastolic blood pressure does not (Schaible and Scheur 1985; Schairer et al. 1992). At any given heart rate the systolic blood pressures were not significantly different between genotype/allele groups. Total work performed on the bicycle ergometer was also similar between genotypes and allele groups. One might therefore conclude that cardiac work responses to exercise are unrelated to ACE genotype. However, a more detailed examination of the data are warranted.

Despite the small number of subjects in this group, those of DD genotype had higher systolic blood pressures at several time points: namely at rest (126 ± 6.1 vs. 124 ± 2.8 vs. 121.9 ± 4.8mmHg), at heart rate 110 bpm (157.7 ± 8.2 vs. 141.4 ± 2.9 vs. 148.4 ± 11.0 mmHg), and at heart rate 150bpm (188.3 ± 4.1 vs. 174.9 ± 5.8 vs. 180.6 ± 4.2mmHg for DD vs. ID vs. II respectively). Cardiac rate/pressure product (product of mean blood pressure and heart rate, as a measure of cardiac work at any given exercise stage) is thus slightly higher for much of the time for those with a D allele than those without, although this does not reach statistical significance (p for heterogeneity 0.92, 0.33 and 0.41 for heart rate 110bpm, 130 bpm, and 150 bpm respectively: all comparisons between genotypes non-significant) (figure 9.6).

At any given heart rate, work performed was similar between genotypes and allele groups. However, there is a consistent trend (although not statistically significant

for any comparisons) for the D allele to be associated with a lower work output for every given heart rate: heart rate 110 bpm (71.7±6.7 vs. 76.3±5.8 vs. 79.4±7.4 watts), 130 bpm (108.3±19.2 vs. 130.7±5.6 vs. 132.8±4.8 watts) and 150 bpm (165±18 vs. 176.3±5.7 vs. 179.4± 8.4 watts, for DD vs. ID vs. II respectively). This study was performed with troops exercising to a target heart rate. However, under normal training conditions much of the exercise undertaken will be aimed at target task completion, for which a similar amount of physical work output will be required. It is thus instructive to calculate peak cardiac rate-pressure product for each stage of exercise undertaken in this study (mean blood pressure at end of stage x heart rate at end of stage) per unit of external work i.e. A measure of cardiac work necessary to achieve a unit measure of external work. This data is shown in figure 9.7:

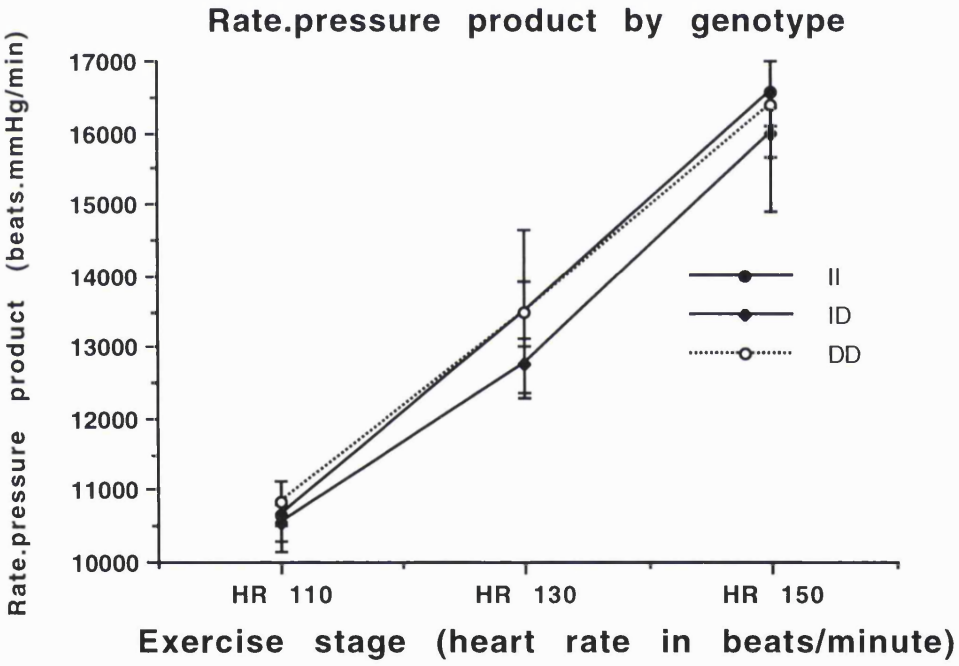


Figure 9.6: Cardiac rate-pressure product (mean blood pressure x heart rate) at each heart rate stage during graded exercise, according to ACE I/D genotype

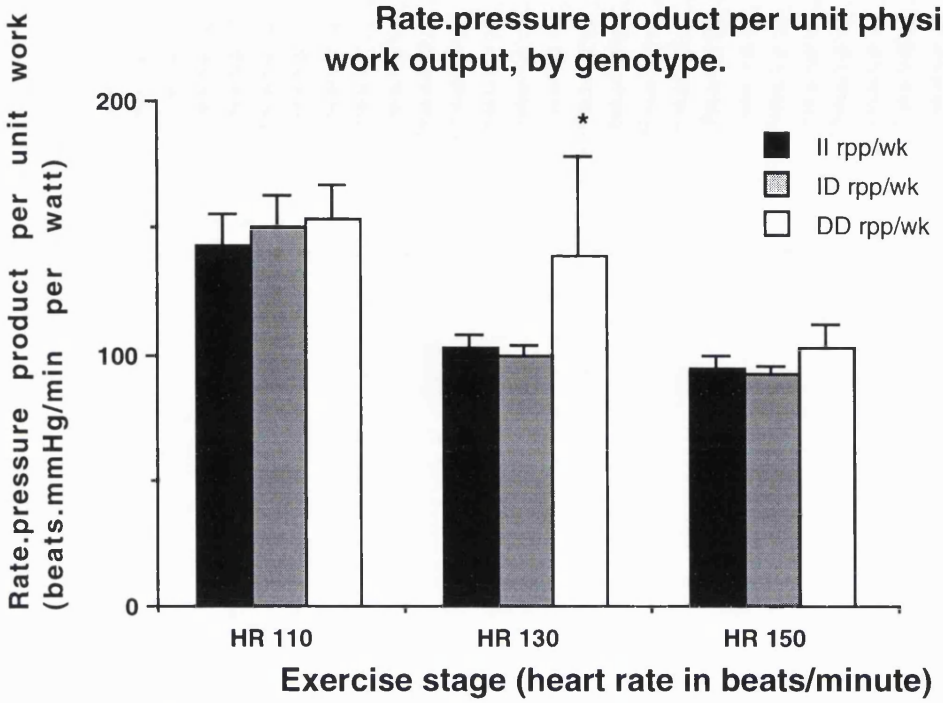


Figure 9.7: Cardiac work (heart rate/mean BP product) per unit of external work at each heart rate during graded exercise, according to ACE I/D genotype (see text for details)

These data suggest that for any given amount of physical work performed on the bicycle ergometer, those of DD genotype may perform slightly more cardiac work than those of other genotypes. This especially marked at moderate loads where rate.pressure product per watt is 102.5±5.0 vs. 99.3±4.3 vs. 138.4±39.6 beats.mmHg.min^{-1}.watt^{-1} for II vs. ID vs. DD respectively: mean±SEM: $p<0.05$ for II vs. DD, and for ID vs. DD).

This finding may suggest that those of DD genotype perform higher cardiac work to produce the same physical work output. It might be that those of DD genotype are actually less physically fit. We have no direct evidence of this being the case, although formal studies using accurate measures of physical performance and aerobic capacity are required. However, it is worth noting that, in the first and major study, minimum run times at entry over 1.5 miles showed a very slight non-significant trend in this direction (mean±SD time in minutes 9.76±0.63 vs. 9.83± 0.62 vs. 9.90±0.69 minutes for II, ID and DD respectively). Such data are sufficient to raise the question as to whether ACE genotype is associated with reduced physical fitness, altered training capacity/'trainability', or is related to cardiovascular control during exercise.

This study has many flaws which limit the interpretation of the derived results.

As discussed, we have no accurate measure of physical fitness in these troops. Further, our access to further troops was limited, with only one cohort being available to us. Consequently, the study is small, with only three individuals homozygote for the D allele being included. Evidently, the study should be repeated using larger numbers of individuals all of whom should have accurate assessments of physical fitness performed (including aerobic capacity and assessment of maximal oxygen delivery [VO2-max]). Power-targeted staging of exercise rather than heart-rate-targeted staging would also be of use. Recruits were also only assessed at one point in their training (namely, at the end of 10 weeks). Loading conditions cannot be proven to have been the same in all recruits, and biomechanical assessment (i.e. of lower limb muscle bulk, thigh length etc) was not undertaken. Further, haemodynamic responses to exercise might vary between genotypes at different levels of fitness. Any further study should therefore examine a large cohort serially throughout training. Responses may also depend on the type of exercise being undertaken, the muscle groups being used, whether the exercise is aerobic or anaerobic, and exercise duration (Nutter t al. 1972; Blomqvist and Saltin 1983; Schaible and Scheur 1985).

9.5: CONCLUSIONS

The ACE gene I/D polymorphism is not associated either with a predisposition to raised blood pressure in hypertensive individuals (Harrap et al. 1993), presence of essential hypertension (Jeunemaitre et al. 1992), or to differences in blood pressure amongst normal individuals (Rigat et al. 1990; Berge and Berg 1994). However, this does not discount an effect on exertional blood pressure. It is not possible to decide from this study to what extent ACE genotype-dependent differences in the haemodynamic response to exercise contributed to the changes in LV mass seen in the first study (chapter 8). It would seem likely that any such confounding effect was not complete. Mean increase in LV mass was +2.0g for those of II genotype, but +42.3g for those of DD genotype. The small differences in rate.pressure product which one might expect for a given power output , and the fact that (in this study) significant differences were only observed in one target heart-rate band, both mitigate against the sole effect of ACE genotype on LV growth being mediated through changes in cardiac work.

THE ROLE OF TISSUE RAS IN THE CONTROL OF LEFT VENTRICULAR GROWTH AND HYPERTROPHY

10.1 THE ROLE OF MYOCARDIAL RAS IN LVH

10.11 LINES OF EVIDENCE SUPPORTING A ROLE FOR MYOCARDIAL RAS IN THE CONTROL OF LV GROWTH

Data suggesting the presence of a myocardial tissue RAS has been discussed at length in chapter 1. The data presented in this thesis support the existence of such a local system, and suggest that it plays an important role in the regulation of both physiological and pathophysiological myocardial growth. Three other main lines of inquiry have suggested such a role for tissue RAS:

1. ***Myocardial RAS activity alters during the hypertrophic process.***
2. ***RAS agonists increase cardiac growth***
3. ***RAS antagonists inhibit cardiac growth***

These observations apply both to global cardiac hypertrophy, and to the specific changes in the cardiac extracellular matrix and in myocyte volume which accompany cardiac hypertrophy. Each of these three lines of evidence will now be discussed in relation to both global cardiac hypertrophy, myocyte growth, and collagen matrix deposition.

10.12 LEFT VENTRICULAR HYPERTROPHY AS A WHOLE

10.121 Myocardial RAS activity alters during the hypertrophic process.

An increase in cardiac ACE and angiotensinogen expression is noted during both physiological and pathophysiological LVH.

During ***physiological cardiac growth***, ACE binding in rat myocardium, cardiac vasculature and valves increases with physiological cardiac growth (Hunt et al. 1995).

In two models of ***pathophysiological hypertrophy*** (involving aortic banding at different site), RAS activity increases. Seven-to-fifteen days after suprarenal aortic banding, rat LV angiotensinogen expression (Northern blot) is increased 4-fold (Baker et al. 1990). LVH due to rat ascending aortic banding is associated with increased cardiac ACE expression (in vitro autoradiography and biochemical assay) (Bruckschlegel et al. 1995), LV ACE mRNA (Northern blot) (Schunkert et al. 1990),

ACE density (ligand-binding studies) and LV tissue ACE activity (Schunkert et al. 1990).

A non-ACE converting enzyme may also be induced in the hypertrophic process. Capacity to convert ATI to ATII is increased in isolated hypertrophied rat hearts (Schunkert et al. 1990; Schunkert et al. 1993b). It is reduced by 70% with ACE-inhibition, but only to levels a little higher than those seen in non-hypertrophied hearts (i.e. from 12% to 3%, cf. 2.6% in control hearts) (Schunkert et al. 1993b) suggesting that at least 30% of ATI-converting activity is due to a non-ACE enzyme induced in LVH.

Finally, LVH is associated with an increase in LV ATII receptor numbers (de Gasparo et al. 1994) and, in 2 models, a three-fold rise in AT_{1A} receptor mRNA levels which reverses with LVH regression (Suzuki et al. 1993). Both AT_1 and AT_2 receptor density were more than doubled in the presence of hypertrophy.

10.122 Effects of RAS agonists and antagonists

Studies of the direct effects of RAS agonists and antagonists on LV growth are hampered by confounding indirect pressor, chronotropic and inotropic effects. Nonetheless, RAS inhibition limits progression of LVH, and may also induce regression.

ATII infusion increases ***physiological cardiac hypertrophy*** in the newborn pig heart (Beinlich et al. 1991), while treatment with either enalapril or DuP753 from birth to day 3 - a period of maximal LV growth rate- reduces LV mass and LV total RNA (Beinlich et al. 1991). Enalapril reduced LV protein content, and LV ribosome formation and protein synthesis. The contribution of a hypotensive effect was not assessed, however. Our studies suggest that ACE inhibition which reduces BP to that of control animals reduces cardiac mass to sub-control levels, suggesting that ACE-inhibition might influence 'basal' physiological cardiac growth as well as pathological hypertrophy. Similarly, our human data suggest a role for myocardial RAS in the control of physiological cardiac growth.

Pathophysiological hypertrophy can be similarly prevented. Our data in the TGR suggest that non-hypotensive ACE-inhibition may reduce pathological LVH. Linz showed that treatment of rats with ramipril 10μg/kg/day (a non-hypotensive dose without effect on plasma ACE activity) for 1 year after suprarenal aortic-banding prevented LVH (Linz et al. 1992). A similar effect is seen with enalapril(Baker, Mitchell et al. 1990). This effect cannot be ascribed with certainty to the reduction in tissue ACE activity (demonstrated by reduced ATII-induced contraction of aortic rings

on exposure to ATI). Low dose ramipril normalised plasma adrenaline levels (19.9nm/l in untreated animals, and 10.2 and 9.1nM/l in treated and sham-operated animals respectively) as well as noradrenaline levels. ATII may facilitate adrenergic neurotransmission, and reserpine (a depletor of peripheral nerve terminal catecholamines) and methyldopa can both inhibit cardiac fibrosis in SHRs. Non-hypotensive beta-receptor blockade with propranolol also leads to an anti-hypertrophic effect in the SHR (Sen et al. 1977). The adrenergic system contribution to the hypertrophic process probably varies in different models, as minoxidil-associated hypertrophy in the SHR is attenuated by methyldopa but not by propranolol. The role of altered aldosterone levels (not measured) and of kinins in the antifibrotic process were also not assessed. Many of these criticisms can be levied at similar experiments.

In the regression of such LVH, Enalapril is more effective than an equihypotensive dose of a calcium channel blocker in inducing regression of established LVH in the 2K1C hypertensive rat (treatment for 6 weeks begun 5 weeks after operation) (Veniant et al. 1993). Bruckschlegel questioned whether such regression was through a reduction in myocardial ATII (Bruckschlegel et al. 1995). Young male rats (3-4 weeks) underwent ascending aortic banding, and 6 weeks later (t=0) were randomised to receive ramipril (10mg/kg/day), hydrallazine (20mg/kg/day), vehicle or losartan. Sham-operated rats were also followed. Blood pressure was recorded at weeks 6 and 8 by tail-cuff plethysmography in conscious restrained rats (no details of rat training given), and echocardiography performed at t -2, +3 and +6 weeks. The effect of intravenous hydrallazine and losartan on trans-stenotic gradient was also studied. LVH regressed significantly over weeks 3-6 of treatment to a similar degree with both ACE inhibition and AT_1 receptor blockade. Hydrallazine treatment was associated with an increase in LVMI over this time. The authors suggest that this was not due to a difference in haemodynamics between treated groups. However, blood pressure was measured by plethysmography distal to the aortic band (i.e. using the tail artery). To try to overcome this problem, direct measurement of LV peak pressure and trans-stenotic gradient was performed in a second experiment after the administration of intravenous hydrallazine or losartan at doses which reduced post-stenotic pressure to a level seen with chronic treatment. These measurements were similar in both groups. One cannot of course be sure that the effects of short term intravenous loading on LV systolic pressure were similar to those seen with chronic treatment, when a variety of adaptive responses may have come into play. It must also be noted that LVH progressed despite a reduction in systolic pressure: in fact, although no direct comparison is made, LVMI was nearly 6g/kg in hydrallazine-treated animals, and <5g/kg in untreated stenotic animals. No statistical comparison is made, but this data suggests that hydrallazine has had a hypertrophic effect of its own, (an effect also seen with the vasodilator minoxidil (Rusicka and Leenen 1993)), possibly through an

increase in cardiac volume load or adrenergic activation. Certainly, the absence of regression of LVH with hydrallazine has been noted before (Sen and Tarazi 1983). Once again, therefore, the conclusion that ramipril causes regression (and prevention of progression) of LVH through a non-hypotensive mechanism that is likely to be ATII mediated cannot be wholly relied upon. Interestingly, LVH regression was associated with improved survival in this study.

Similar effects of ramipril therapy have been observed in the human, and this data is perhaps more convincing. Michel Lievre studied the effect of ramipril therapy on the progression and regression of LV in human hypertensive patients (Lievre et al. 1995). Study design and results are summarised below (figure 10.1):

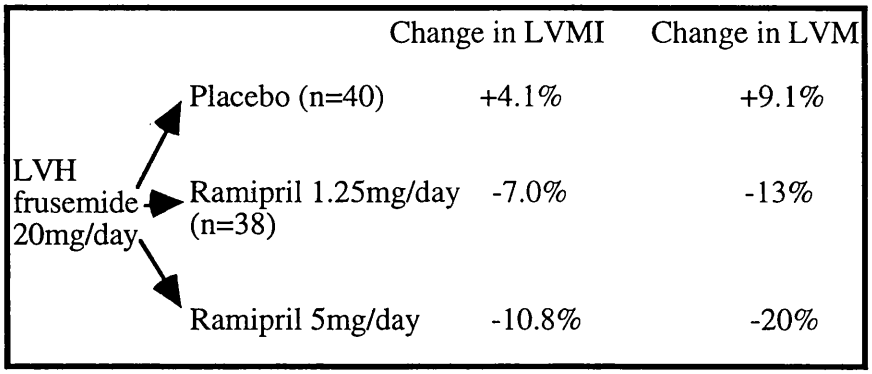


Figure 10.1 Trial design investigating effect of ACE-inhibition on regression of human hypertensive LVH (Lievre et al. 1995) (see text for details)

All patients had moderate hypertension at entry (treated diastolic BP<110mmHg or untreated 95-110mmHg, systolic >160mmHg), and echocardiographically-defined LVH. A run-in period of 4-6 weeks was given for stabilisation of therapy with frusemide 20mg/day. However, duration of hypertension is not described, and previous therapy is not documented. Six months of randomised double-blind placebo-controlled treatment was then initiated for 6 months with ramipril at a dose of 1.25 or 5mg/day. Echocardiography was performed before and after treatment. The effects of therapy on LV mass and LV mass index are shown. These were statistically significant in all cases bar the reduction in LVMI with low dose ramipril (p=0.6). There was a very small non-significant effect of drug treatment on blood pressures recorded at 3 or 6 months, and on ambulatory BP monitoring (performed at the start and end of the treatment phase). As previous therapy and duration of disease are not described, selection bias of only those patients with previously resistant LVH may have occurred. Alternatively, if treatment with ACE inhibitors does lead to regression of LVH, a selected population of non-ACE-inhibitor treated patients may have been chosen. In fact, this makes the effect of ramipril even more remarkable. These data support a non-

hypotensive mode of action for ACE-inhibition in causing the regression of human hypertensive LVH (Lievre et al. 1995).

It seems likely that these actions of ACE inhibitors are due at least in part to inhibition of ATII synthesis. In the ascending aortic banded rat, ATII receptor antagonism is as effective as ACE-inhibition at producing non-hypotensive regression in LVH (Bruckschlegel et al. 1995). ATII-antagonism prevents the increase in tritiated phenylalanine incorporation seen when neonatal rat myocytes are stretched (as a growth stimulus) (Kojima et al. 1994). In vivo, daily intragastric treatment of SHRs with hydrallazine 10mg/kg lowers BP more than TCV-116 1mg/kg, yet has weaker antihypertrophic effects in both a regression (established LVH treated from 13 weeks for 4-12 weeks) and a prevention study (treated from 5 weeks of age) (Kojima et al. 1994). Lack of documented precise technique and timing of BP recording in relation to drug dosing and discussion of the hypotensive profile over time prevents unreserved acceptance of this data.

10.123 Our studies

We carried out no direct studies of gene induction in relation to hypertrophy in either humans or rats. Our only data come from the study of LV growth in the TGR(mREN2)27 rat, where we might compare the tissue ACE activity in untreated hypertensive transgenic animals (group 1) with those in normotensive Sprague Dawley control animals (group 3) (table 11.1). It is of interest that ACE levels were lower amongst control than TGR rats in the kidney, whilst in vascular tissue exposed to the hypertrophic stimulus of a pressure burden, ACE levels were higher (although not significantly so). These data would support an association of increased RAS activity being associated with vascular and cardiac growth. However, against this hypothesis, ACE activity was also raised in the right ventricle of the TGR when compared to control animals. As discussed (chapter 6), it remains possible that vascular resistance was raised in the TGR (as ATII is a potent pulmonary vasoconstrictor, and the renin transgene is expressed in the lung, see chapter 3).

TISSUE	TGR	Control	p value
Renal	0.76±0.08	0.931±0.23	**0.02**
RV	4.11±0.41	2.13±0.79	0.09
LV	2.94±0.32	2.20±0.58	0.36
Aorta	152.38±17.7	108±35.88	0.29
Carotid	119.63±11.8	39±6.41	**0.0004**

Table 10.1: Plasma and tissue ACE activity amongst heterozygote male TGR(mREN2)27 rats at 120 days

Our rat data were shown support a role for tissue RAS in the control of pathophysiological hypertrophy (chapter 6), whilst our human data also support a role for tissue RAS in the control of physiological hypertrophy.

10.13 RAS AND THE EXTRACELLULAR MATRIX IN LVH

RAS components are associated with the cellular components involved in generating interstitial matrix. Angiotensinogen mRNA expression (in-situ hybridisation) (Dostal et al. 1992a), presence of surface AT_1 receptors (ligand binding) (Sadoshima and Izumo 1993a; Villarreal et al. 1993) and presence of ATI and ATII (Dostal et al. 1992b) have all been shown in cardiac fibroblasts, and human cardiac chymase (generating ATII, see below) may act predominantly in the cardiac matrix (Urata et al. 1994 a,b).

10.131 The effects of RAS agonists and antagonists

RAS Agonist Effects At concentrations above those seen in plasma, ATII can increase collagen synthesis and reduce degradation. *In vitro*, ATII increases neonatal fibroblast number and protein and DNA synthesis (Sadoshima and Izumo 1993a) through action on the AT_1 receptor, increases ^{3}H-proline incorporation in cultured adult rat cardiac fibroblasts (Brilla et al. 1994), specifically induces collagen type I and III synthesis (collagen assay and Northern blot) (Villarreal et al. 1993), and causes an AT_2-receptor mediated decrease in collagenase and matrix metalloproteinase I synthesis (Brilla et al. 1994; Brilla et al. 1995).

Meanwhile, *in vivo*, sub-pressor doses of ATII cause ventricular fibrosis in rats within 2 days (Tan et al. 1991), with increased ACE expression seen in the scarred areas (Sun et al. 1993). The greater ACE expression seen with ATII treatment (Sun et al. 1993) and the reduction in cardiac angiotensinogen synthesis with ACE-inhibitor treatment (Urata et al. 1994a) implies a possible ATII/ACE positive feedback loop during cardiac growth- a concept which would be supported by our rat data (lesser effect of ACE-inhibition on collagen deposition in association with the hypertensive state than in normotensives) and human data (possible increased expression of cardiac ACE in the presence of growth, where ATII levels may also be raised (chapters 6 and 8). Crawford, however, could only demonstrate cardiac fibrosis and sustained induction of collagen type I and fibronectin gene expression using pressor doses of ATII (Crawford et al. 1994). Simultaneous ACE-inhibition or treatment with losartan attenuated the induction of the fibronectin gene, although hypotensive doses of prazocin failed to do so. Thus, an autocrine cardiac RAS may be induced by ATII treatment, either through the pressor effect or by direct action (as Sun's data ((Sun et al.

1993)) might suggest (above). Weber suggests that ATII might lead to increased vessel permeability with subsequent accumulation of plasma-derived fibronectin stimulating perivascular fibrosis (Weber et al. 1995). The induction of collagen and fibronectin synthesis by ATII application to cultured fibroblasts, and evidence from co-culture experiments (reviewed in (Weber et al. 1995)) suggest that the effects of ATII on collagen synthesis are more likely to be mediated through paracrine/autocrine effects on fibroblasts.

RAS Antagonist Effects Captopril-treatment of the adult SHR for 12 weeks reduces collagen content to levels lower than those of normotensive controls (despite still having a higher blood pressure) (Kuzuo et al. 1993). Administration of ramipril at non-hypotensive doses (10μg/kg/day) to supra-renally banded rats reduces both fibronectin and collagen deposition. Extracellular space was reduced to normotensive control levels (Linz et al. 1992). Such observations are matched by our finding that ramipril treatment of growing TGRs for a similar period reduces LV collagen content to levels lower than those of controls, despite similar blood pressures. Veniant, however, was unable to demonstrate an effect of enalapril on perivascular fibrosis in the 2K1C rat (Veniant et al. 1993) possibly due to her choice of drug, the different model, and by the shorter treatment period than that in other comparative studies (especially given the long half-life of collagen (Bishop and Laurent 1995)). Biochemical quantification of LV collagen content was not performed, and full transmural collagen area (by specific staining) not assessed. Finally, the increase in perivascular collagen deposition was inhibited to a greater extent by enalapril than by calcium-channel blockade (by 46% and 40% respectively), but wide group error bars in the relatively small groups (n=16) meant that such effects lacked statistical significance. Once again, our data support these findings, with ACE inhibition capable of reducing both physiological and pathophysiological cardiac collagen deposition.

10.14: RAS AND MYOCYTE HYPERTROPHY IN LVH

10.141 RAS and second messenger systems

Komuro showed that mechanical stretch of neonatal rat myocytes increases activity of elements of the protein kinase cascade including mitogen-activated protein kinase (MAP kinase), raf-1 kinase, and protein kinase C (PKC). It also induces an associated increase in protein synthesis of about 1.5 fold, as assessed by phenylalanine incorporation assay. At least 75% of this response is prevented by the addition of an ATII type 1 receptor antagonist, with an associated 60% suppression of the stretch-induced increase MAP kinase activity (Komuro et al. 1995). However, at least 10% of

the cells in this plate were non-myocytes, and so the localisation of the origin of the ATII cannot be defined. We might thus represent Komuro's data as follows (figure 10.2):

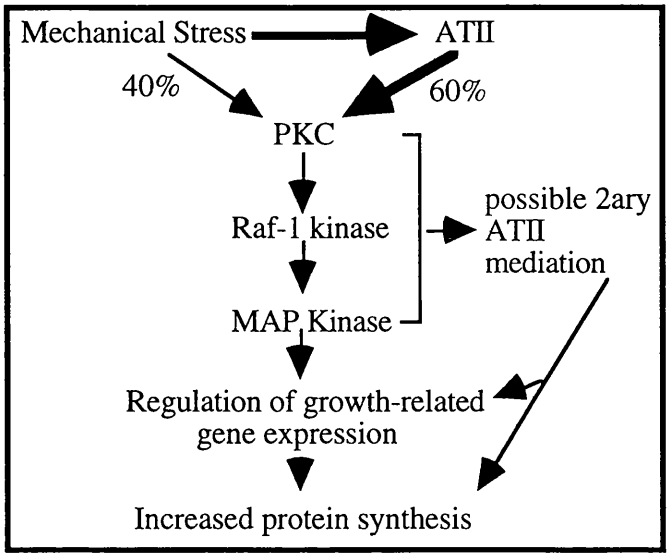


Figure 10.2 Summary of implications of findings by Komuro(Komuro et al. 1995) concerning the role of ATII in stimulating PKC activity as a second messenger in cardiac growth.

In neonatal rat myocytes stretched on silicone trays, the active metabolite of the ATII antagonist TCV-116 prevents an increase in MAP kinase activity, c-fos expression and tritiated phenylalanine incorporation(Kojima et al. 1994).

10.142 Effects of Agonists and Antagonists

Agonist Effects ATII increases protein synthesis in cultured chick myocytes (Baker and Aceto 1990). Applied to neonatal rat cardiomyocytes, it activates multiple phospholipid-derived second messenger systems (e.g. phospholipase C, D and possibly A2) (Sadoshima and Izumo 1993a) as well as the PKC-dependent pathways described above, induces a variety of early response genes (such as c-myc) and many 'late' response genes (such as skeletal actin), increases protein synthesis through an effect on the AT_1 receptor (Sadoshima and Izumo 1993b) and upregulates angiotensinogen gene expression.

Antagonist effects Few studies have addressed the effect of RAS antagonists on myocyte hypertrophy in LVH. Twenty-two days of captopril treatment reduces cardiac myocyte diameter when compared to animals of equal blood pressure (Rossi and Peres

1992) and non-hypotensive doses of ramipril reduce myocyte diameter by 39% through non-hypotensive mechanisms in ascending aortic banded rats (Bruckschlegel et al. 1995). Losartan (6 weeks treatment) had a similar effect, reducing myocyte diameter by nearly 50% through non-hypotensive mechanism (Bruckschlegel et al. 1995). Our rat studies have suggested a differential effect of ACE inhibition on myocyte growth at different timepoints (chapter 6). Furthermore, the bulk of human physiological hypertrophy (unlike pathophysiological hypertrophy) (Oldershaw et al. 1980) is due not to collagen matrix deposition, but to myocyte hypertrophy(Caspari et al. 1977; Grossman et al. 1990). The association of ACE the D allele of the ACE gene polymorphism with physiological hypertrophy therefore suggests a role for tissue RAS in control of human myocyte hypertrophy under such circumstances.

10.15 MECHANISM OF ACTION OF LOCAL RAS IN CONTROLLING LVH

It thus seems clear that local myocardial RAS activity may influence cardiac growth in rats and humans. In theory, this might occur through one or more of a number of different mechanisms:

10.151 ATII acts as a direct local trophic factor

This concept is supported by the *in vitro* effects of ATII and ATII receptor antagonists reported above.

10.152 Cardiac ACE yields a trophic factor other than ATII

Compartmentalisation of ACE with an ATII-degrading system might in theory generate locally active ATII-degradation products. There has, for instance, been no significant work examining AT(1-7) cardiac localisation or action, nor the possible existence of receptors specific to such peptides (see chapter 1).

10.153 Modulation of Myocardial Diastolic and Systolic Function

It is possible that induction of myocardial RAS activity could cause cardiac hypertrophy in part by impairing diastolic function (and hence reducing myocardial efficiency, increasing wall tension, and increasing myocardial work), or by directly acting as an inotrope (thus increasing myocardial work).

Diastolic Function Failure of diastolic function is a prominent feature of the hypertrophied ventricle. *In vitro*, ATII slows relaxation velocity in isolated rat myocytes (Neyes and Vetter 1989a) and potentiates delayed relaxation in hypertrophied myocytes (Neyes and Vetter 1989b). *In vivo*, the increased conversion of ATI to ATII in LVH is associated with depressed myocardial diastolic relaxation (Schunkert et al. 1990). In both animal LVH models (Eberli et al. 1992) and in the hypertensive human heart (Marmor et al. 1989a), ACE inhibition improves diastolic function. In a study of 8 hypertensive patients, a single dose of cilazapril reduced the fractional proportion of diastole taken for peak LV filling to have occurred. This effect, occurring within 3 hours of treatment, suggested an improvement in diastolic function (Marmor et al. 1989). It has also been demonstrated for captopril, and was significantly greater than that seen with calcium channel blockade using nifedipine (Marmor et al. 1989a).

Systolic Function ATII is a positive inotrope in canine atria and ventricles (Koch-Weser 1965). In the rat, it may be both positively inotropic (e.g. in the atria through AT_1 receptor activation) (Feolde et al. 1993) or negatively inotropic (De Mello 1995). Positive inotropy may partly indirect (mediated by modulation of sympathetic transmission), or direct (Timmermans and Smith 1994; De Mello 1995). The positive inotropic effect in the rabbit papillary muscle mediated by the AT_1 receptor (Scott et al. 1992) is independent of catecholamine release as it persists in the presence of alpha and beta blockade (Ishihata and Endoh 1993). ATII-mediated positive inotropy in canine atria and ventricles (Kobayashi et al. 1978) and kitten papillary muscle (Koch-Weser 1965) are similarly unaffected by beta-receptor blockade (but prevented by salarasin, an ATII antagonist) or reserpine respectively. However, these were isolated perfused preparations, with no constant sympathetic discharge as might be seen *in vivo*.. ATII is a positive inotrope in normal human atrial (Holubarsch et al. 1994) and ventricular tissue (reviewed by Timmermans and Smith (1994), although the latter conclusion is disputed. Holubarsch et al were unable to demonstrate any increase in peak developed force in human papillary muscle and free wall strip preparations (Holubarsch et al. 1994) although all of this tissue was derived from diseased hearts (i.e. free wall heart muscle came from patients with dilated cardiomyopathy, whilst papillary muscle was derived from patients with mitral valve disease).

Some of the effects of ACE-inhibitors might be mediated by improving cardiac systolic function. In Gohlke's study of SHRSP rats (Gohlke et al. 1994), ramipril 1mg or 10μg/kg/day significantly improved cardiac function (dP/dt_{max} increase: coronary flow increase: reduced coronary venous sinus lactate dehydrogenase, creatine kinase and lactate concentrations). These benefits were associated with increased myocardial tissue concentrations of glycogen, creatine phosphate and ATP. All of these effects were abolished by the administration of Hoe-140, however, suggesting a significant

role for reduced kinin degradation. Hoe 140 treatment seems, however, to have no effect on the antihypertrophic or antihypertensive actions of the ACE-inhibitor, which would therefore seem to be ATII-mediated (Scicli 1994).

The effects of ACE-inhibition on cardiac function may thus be mediated through changes in cardiac metabolism, change in contractile mechanism or excitation-contraction coupling, or might even reflect increased angiogenesis (Scicli 1994).

10.154 Modulation of aortic compliance

As has been discussed (chapter 1), ACE inhibition increases great vessel compliance in hypertension. This would reduce myocardial systolic work, and hence possibly generate an antihypertrophic effect.

10.155 Modulation of cardiac sympathetic activity

Alpha-receptor activation by noradrenaline induces c-myc expression and hypertrophy in primary neonatal rat cardiomyocyte cultures (Starksen et al. 1986). In the SHR, hydrallazine lowers blood pressure and increases myocardial catecholamine content although regression of LVH only occurs with the co-administration of non-hypotensive doses of propranolol or methyldopa (Sen and Tarazi 1983). However, such doses of propranolol alone had no effect (Sen and Tarazi 1983), and failed to blunt the cardiac hypertrophic response to minoxidil (Sen et al. 1977). Nonetheless, the efficacy of methyldopa therapy contrasts starkly with the lack of effect on LVH of hypotensive agents which reflexly increases adrenergic drive despite excellent blood pressure control (Sen et al. 1977).

Angiotensin II might interact with the sympathetic nervous system at a number of different levels (reviewed by Dominiak (1993):

(i) It facilitates presynaptic noradrenaline (NA) release through a specific action on AT_1 receptors

(ii) It facilitates adrenergic release from chromaffin cells of the adrenal medulla

(iii) It enhances the effects of catecholamines by acting synergistically on the same intracellular second-messenger system (inositoltriphosphate) in vascular smooth muscle cells.

Yasuda showed that ATI and ATII applied to spiral preparations of guinea-pig pulmonary artery increase field-stimulation-induced noradrenaline release through an effect on ATII receptors (Yasuda et al. 1987). Local ATII may therefore augment cardiac sympathetic neurotransmission (or its induced effector responses). *In vitro* heart rate and contractile responsiveness to sympathetic nerve stimulation is reduced in the hearts of rabbits pretreated with ramipril (Lindpaintner et al. 1987). However, in

Yasuda's study, captopril but not an ATII antagonist reduced field-stimulated noradrenaline release, suggesting that ACE inhibition may reduce noradrenergic neurotransmission by a non-ATII-dependent mechanism. ACE inhibition may actually *increase* sympathetic transmission under some circumstances e.g. through reduced noradrenaline reuptake and reduced NA degradation (monoamine oxidase is inhibited by <35% by ramipril) in the rat heart. The net effect of ACE inhibition on sympathetic neurotransmission is thus debated and may be altered by interactions with ATI and bradykinin, levels of which rise with ACE-inhibition (Dominiak 1993). Overall, ACE-inhibition is likely to result in increased noradrenergic transmission and reduced effector responses. (figure 10.3)

ACE-INHIBITOR EFFECTS ON NA TRANSMISSION

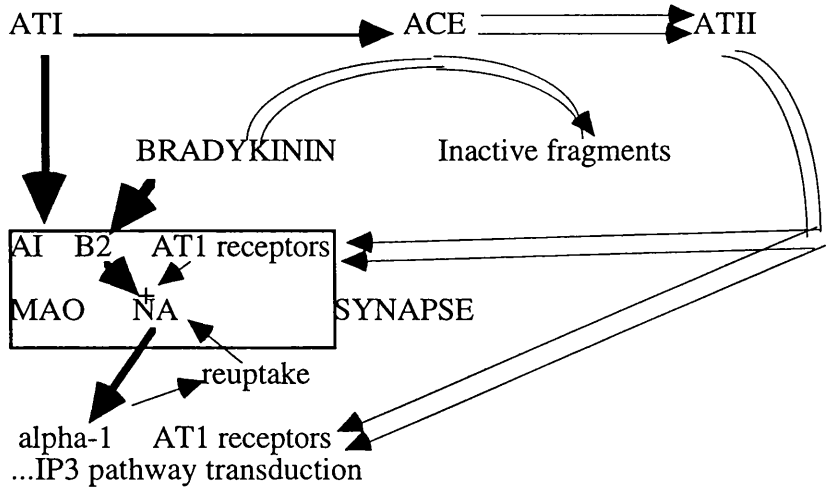


Figure 10.3: Effects of ACE inhibition on noradrenergic transmission. Thick lines represent effects increased by ACE-inhibition, double lines effects reduced.

10.156 Effects on cardiac electrophysiology

The cardiac RAS has direct cardiac electrophysiological effects which may (through alterations in excitation-contraction coupling and cardiac electrical synchrony) affect cardiac growth responses (reviewed by De Mello (1995)). In the rat, ATII shortens action potential (AP) duration. It also reduces gap junction conductance through activation of protein kinase C (PKC) in both the rat and hamster, which may partly explain the associated increases in intracellular resistance and reduction in conduction velocity in myocardial trabeculae. Cardiac refractoriness may be reduced, and myocyte

automaticity increased. Such changes may predispose to cardiac arrhythmogenesis, and seem to be mediated directly through AT_1 receptors. In the hamster, unlike the rat, action potential duration is increased and yet the potential for ATII to predispose to arrhythmias remains, through changes in gap junction conductance, conductance threshold and refractoriness. These effects may be due to the intracellular actions of ATII, generated by ACE, on intracellular AT_1 receptors (Pahor et al. 1994; De Mello 1995). ACE-inhibition rapidly increases cardiac gap junction conductance *in vitro* , an effect associated with increased conductance velocity and decreased intracellular resistance in the rat. Rat cardiac cells become hyperpolarised and action potential amplitude is increased with ACE-inhibition, especially in the presence of hypoxia. Associated increases in electrical synchronisation, and reduction in dispersion of refractoriness, may reduce the predisposition to arrhythmias and may increase ventricular force of contraction (De Mello 1995).

10.157 Effects on kinin degradation

There is evidence (reviewed by Scicli (1994)) of the existence of a tissue kallikrein-kinin system in the rat heart, in which kallikrein levels are much higher than in plasma. It is also possible that the circulating kallikrein/kininogen system may be locally activated in the heart. If myocardial ACE activity controls LV growth, as the data above and our human and rat data suggest, then this might be due either to the generation of ATII by ACE (i.e. increasing a hypertrophic factor), or to the degradation of kinins (i.e. reducing an inhibitory factor). Such effects of kinins could be either direct (as an antihypertrophic agent) or indirect (through effects on cardiac mechanical and electrical function). The increase in basal release of intact bradykinin from the isolated perfused rat heart under ischaemic conditions may be increased >3-fold by ACE-inhibition (Baumgarten et al. 1989). ACE-inhibitors cause kinin-dependent improvements in cardiac function and energy status in the SHR-SP hypertrophied heart (Gohlke et al. 1994) and improved electrical stability of the post-infarct pig heart (Tobe et al. 1991).

Although the potential mechanisms by which kinins might alter LV growth therefore exist, in practice kinins do not seem to play an important role. ATII antagonism and ACE-inhibition have similar effects on LV hypertrophy (Qing and Garcia 1992). Thus, the induction of LVH regression by ramipril in the ascending-aortic banded rat is reproduced with losartan at a dose with similar haemodynamic effects (Bruckschlegel et al. 1995). Gohlke et al studied the hypertrophied hearts of SHR-SP rats (Gohlke et al. 1994). Treatment (initiated prenatally) with ramipril at a hypotensive dose (1mg/kg/day) prevented the appearance of hypertension and LVH. Hoe 140 treatment seemed to have no effect on the antihypertrophic or antihypertensive actions of the ACE-inhibitor, which would therefore seem to be ATII-mediated. The

antihypertrophic effects of ramipril at hypotensive doses do not seem to be kinin dependent. In contrast to these studies, Linz and Scholkens suggested that Hoe 140 treatment could abolish the antihypertensive and antihypertrophic effect of ramipril(Linz and Scholkens 1992), although these results could not be reproduced by others (data reported by Scicli (1994)). Gohlke accounts for the discrepancy in his findings and those of Linz by suggesting that kinins might mediate an antihypertrophic effect in high ATII-models of hypertension (e.g. suprarenal coarctation in Linz's model), compared to spontaneous (genetic) hypertension in Gohlke's study.

10.2 CHYMASE AND ACE IN HUMAN CARDIAC GROWTH

10.21: BACKGROUND

It has recently been suggested that ACE is of little importance to human cardiac ATI-to-ATII conversion and that human cardiac chymase, an enzyme unresponsive to ACE-inhibition and known, is dominant. If true, the benefits currently ascribed to ACE-inhibitor treatment might actually be mediated not by a reduction in cardiac ATII generation but by a paradoxical rise (Bumpus 1991). ATI levels rise by up to 300% in the plasma during chronic ACE inhibitor treatment (Nussberger et al. 1986; Mento and Wilkes 1987). Cardiac chymase, unblocked by the ACE inhibitor, would thus be able to convert this to ATII. Growth-inhibitory effects of ACE-inhibitors would have to be ascribed to actions other than to the inhibition of ATII generation, such as prevention of kinin breakdown (Urata et al. 1990b). The reported relevance of human cardiac chymase and, by inference, the lack of significance of human cardiac ACE, must be closely examined.

10.22 THE DISCOVERY OF NON-ACE DEPENDENT ATI-CONVERSION

Urata was the first to suggest that most of the capacity for the conversion of ATI to ATII in the *human* heart did not lie with ACE (Urata and Ganten 1993). He obtained human cardiac tissue from a cardiac transplant programme. The structure of the study is shown below (figure 10.4):

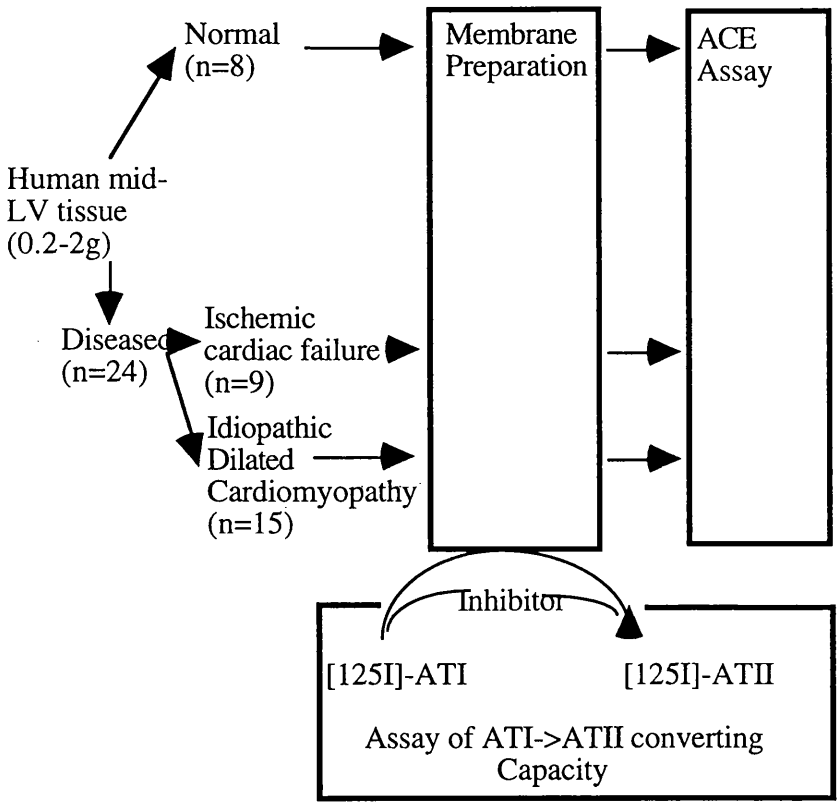


Figure 10.4: Structure of study by Urata(Urata and Ganten 1993) (see text for details)

All ventricular tissue was derived from the mid-LV. Comparisons of ATI-converting activity ([^{125}I]-ATI to [^{125}I]-ATII conversion) and ACE activity (release of tritiated hippuric acid from tritiated hippuryl-gly-gly) was made between different disease states, and with controls. However, these comparisons were, in my opinion, flawed:

(i) Sampling error due to the use of small (as little as 0.2g) biopsies from only the mid-LV may have been introduced due to the patchy distribution of diseased tissue within one heart. Indeed, some sections may have been composed largely of non-viable tissue. Further, only endocardial tissue was studied and epicardial enzyme profile remains unknown.

(ii) We are not told which patients were on ACE-inhibitor treatment, the duration of this treatment, nor when it was stopped. In the preoperative phase, some may have gone without this treatment for some hours (perhaps even up to 24 hours). The tissue and plasma half-lives of these drugs vary, as do their dissociation kinetics.

(iii) There is no record of treatment with beta-agonists, beta-blockers, or vasodilators- all of which influence RAS. It is possible that recipients (IHD and IDC hearts) had received long-term treatment of this sort, whilst those with normal hearts (donors) would have received minimal short -term therapy

These data will not therefore be discussed further, other than to state that they suggested a reduction in ATII-generating capacity in the failing hearts.

Of more importance, ACE-inhibition in membrane preparations from normal heart tissue blocked only 5% of ATI to ATII conversion capacity whilst nearly 80% could be blocked by a non-specific serine protease inhibitor (soybean trypsin inhibitor). It was suggested that most of the biologically-important ATI-converting enzyme activity of the heart is not due to ACE (Urata and Ganten 1993). This conclusion cannot stand.

(i) Using the same membrane preparations from the same sites and from the same hearts, we might expect ACE-activity to be similar if measured by 2 different methods. This is not, however, the case. Thus, the ratio of ACE activities (by hippuric acid generation) were 1: 1.019: 0.685 for normals, IDC and IHD respectively. However, the amount of ATI to ATII converting capacity which was inhibitable by captopril was in the ratio of 1: 0.500: 0.323. This disparity is not explained, but might suggest either a methodological flaw, or the existence of a non-ACE but ACE-inhibitor-sensitive enzyme converting ATI to ATII.

(ii) Small samples of epicardially-stripped tissue from just the mid-LV were used. It is thus not possible to extrapolate these data to the rest of the heart. In particular, regions of the heart such as the septum which are exposed to maximal wall stress may have greater overall induced ACE activity (when compared to chymase activity).

(iii) Membrane preparations do not reflect *in vivo* system activity. Not all ACE is membrane bound, and interstitial matrix-bound and fluid ACE activity may have been ignored. Furthermore, intracellular cytosolic RAS (above) may have far greater biological significance in the control of cell growth, although its total activity per gram of cardiac tissue may be low.

(iii) These assays took no account of compartmental or generalised limitations in substrate availability.

10.23 CHYMASE AND THE MECHANISM OF ACTION OF ACE INHIBITORS IN ALTERING LV HYPERTROPHY

Despite these criticisms, the view that ACE is of little importance in ATII-generation in the human heart is gaining increasing acceptance. If we concur with this conclusion, then the dramatic effects of ACE-inhibitors on human cardiac hypertrophy- even at non-hypotensive doses- must be explained. There might be several plausible alternatives:

10.231 Cardiac ACE generates a biological growth factor other than ATII

This issue is discussed above, and in chapter 1. As yet, no other major mediators have been identified.

10.232 ACE-inhibitor effects are mediated by increased kinin levels

The role for kinins as a tonic inhibitors of growth, or as a mediator of the antihypertrophic effects of ACE-inhibitors, is not strong in animal models (chapter 1, and section 11.157 above). However, the role in humans has not been examined due partly to the lack of biologically stable kinin antagonists of long half-life available for trials, and partly to the marked systemic effects of raised kinin concentrations seen in humans. For these reasons, the role of kinins in the regulation of human cardiac growth is unclear.

10.233 The localisation of cardiac ACE makes it more important than chymase in controlling cardiac growth

Urata (Urata and Ganten 1993) suggests that chymase is predominantly interstitially-distributed in the human heart, and EM-histochemistry shows it to be particularly associated with matrix components (above, and chapters 1 and 6). This might suggest a specific role for chymase in the modification of cardiac matrix, in which RAS participation is clear (above). On the other hand, ACE seems to be particularly associated with the cardiac vascular endothelium, with low LV activity by radioligand studies (Sun et al. 1993; Urata and Ganten 1993). Superficially, therefore, the potential for human ACE to play an important role in cardiac growth seems small. However, autocrine myocardial RAS may exist as well as paracrine systems. Although chymase may play an important paracrine role, intracellular RAS generation of ATII may be far more important in control of growth. Neonatal cardiomyocytes synthesise renin, angiotensinogen, and ACE (Dostal et al. 1992a,b), and rates of synthesis are higher than shown for endothelial cells (Dostal et al. 1992a). Further, perinuclear immunoreactive ACE is demonstrable in cardiac myocytes (Dostal et al. 1992b). Radiolabelled ATII injected into rat LV rapidly localises around the nuclei of cardiac myocytes (Robertson and Khairallah 1971). Nuclear ATII receptors may regulate gene transcription (mRNA levels rise 1.5-fold), and specifically amplify synthesis of intracellular RAS components such as renin (mRNA rises 7.8 fold) and angiotensinogen (mRNA rise x 2.5)(Re, Vizard et al. 1984). Such nuclear ATII receptors exist in the heart (specific- i.e receptor-ATII binding level 21%: stimulation increases gene transcription by 24%). Thus a local intramyocyte RAS exists in which

ACE is a component. This system may be far more important than the chymase-containing paracrine system in the control of cardiac growth.

10.234 The inducability of cardiac ACE makes it more important than chymase in controlling cardiac growth.

Basal levels of angiotensin-I converting enzyme activity (whether ACE or chymase) cannot be expected to reflect activity during the hypertrophic process. ACE expression is induced in the hypertrophied ventricle (above) and it seems that chymase may also be upregulated, but to a lesser extent. Rat LV ACE mRNA and enzymatic activity both increase in the presence of hypertrophy (Schunkert et al. 1990). Enalaprilat reduces conversion rate of ATI to ATII by 70%, but only to levels a little higher than those seen in non-hypertrophied hearts (i.e. from 12% to 3%, cf. 2.6% in control hearts) (Schunkert et al. 1993b). This suggests that in the hypertrophied rat heart, at least 70% of ATI-converting activity is due to ACE, but that at least 30% is due to a none-ACE enzyme such as tissue chymase. This would also seem to have been induced in the hypertrophied heart, as a conversion rate of 3% ATI remains after ACE inhibition, whilst a basal level of 2.6% is seen in control hearts. Both ACE and chymase seem to be induced in the rat with cardiac hypertrophy, although evidently most of the induced capacity to generate ATII from ATI has been due to an increase in ACE activity.

Upregulation of *intracellular* RAS may be even more important, with a positive feedback loop in operation. Stimulation of nuclear ATII receptors may selectively activate transcription of the renin and angiotensinogen genes (see above).

10.24 OUR STUDIES

Our studies of the TGR(mREN2)27 rat suggest that rat cardiac ACE is of great importance in the control of LVH. Ramipril does not inhibit chymase but only ACE, and any biological effects are likely therefore to be ascribed to this action. The study of ACE I/D polymorphism genotype and physiological hypertrophy (chapter 8) suggests that this may also apply to the human, and strongly mitigates against the prevailing view that chymase, and not ACE, is the key angiotensin-II-generating enzyme in the human heart. Whether or not the bulk of ATI-converting enzyme capacity in the human is due to chymase, an inducable human cardiac RAS utilising ACE seems to control human LV physiological growth.

10.3 TISSUE RAS, LVH AND CARDIOVASCULAR DISEASE

10.31: LVH AND CARDIOVASCULAR RISK

Overall, LVH is associated with a 10-fold increase in mortality rate (Massie et al. 1989). However, risk of cerebrovascular accident and death, coronary artery disease, cerebral transient ischaemic attack and intermittent claudication prevalence are all more frequent in those with LVH (Massie et al. 1989; Levy et al. 1990a). This suggests that a common mechanism may mediate both cardiac hypertrophy and predispose to vascular disease. Local RAS activity increases vascular growth, mediates some of the vessel responses to injury, and may thus play a role in the pathogenesis of atherosclerosis. Local RAS may also mediate some of the vessel responses to hypertension, such as increased vessel wall thickness and reduced compliance (chapter 1). Given that local RAS activity also regulates myocardial hypertrophy, it is possible that increased general tissue RAS activity might predispose not only to LVH, but to other forms of vascular disease as well and thus forms the link between LVH and increased cardiovascular morbidity and mortality. If this were true, then we might expect the D allele of the ACE gene polymorphism to be associated not only with LVH (as we have shown) but with other forms of cardiovascular disease as well. Such associations do seem to exist. Thus, in a randomly selected unrelated adult Italian sample (aged 50-64, n=199) DD genotype was associated with a significantly increased vessel wall thickness (12%), but only in the common carotid artery of those not chronically treated for cardiovascular diseases (Castellano et al. 1995).

10.32 ACE GENE POLYMORPHISM AND ISCHAEMIC HEART DISEASE

10.321 Primary ischaemic heart disease

The association of ACE gene polymorphism with ischaemic heart disease is much debated (Teo 1995). In a case-control study, Cambien showed that the DD genotype was more common in male French and Irish survivors seen 3-9 months after myocardial infarction. The association was independent of other risk factors, and exclusive to an otherwise 'low-risk' group in whom the I/D polymorphism accounted for 35% of the infarcts. The D allele is also found more frequently in the offspring of parents who had suffered myocardial infarction (Tiret et al. 1993). In 1226 males (822 with no ischaemic heart disease), a significant association of the D allele was again seen only in lower-risk individuals (as defined by elevation of blood pressure and serum

lipid levels) (Mattu et al. 1995). This independent and non-additive effect of the ACE genotype with other risk factors is most unusual (Swales 1993), although the findings did fit with the proposed association of plasma renin levels and risk of myocardial infarction (Laragh et al. 1972; Laragh 1992). This association was not confirmed in an analysis of 185 male survivors 2.7 years after MI (amongst whom the DD genotype was less common than amongst controls), and 49 women (Bohn et al. 1993). Nor was it noted amongst an Caucasian population of 422 patients in New Zealand when compared to 406 controls (Katsuya et al. 1995). The only published prospective study so far (1250 men with IHD compared to 2340 controls in the USA) showed no association of ACE genotype with IHD or infarction (Lindpaintner et al. 1995). These discrepancies might partly be explained by an apparent synergistic interaction between the D allele and the AGT_1R-C polymorphism of the AT_1 receptor gene.

10.322 ACE gene polymorphism and angioplasty restenosis

Ohishi studied 82 consecutive cases of acute myocardial infarction successfully treated with coronary angioplasty. The DD genotype was associated with presentation with myocardial infarction, and with restenosis (defined as a >50% reduction in the luminal diameter of the stenosis) (Ohishi et al. 1993). However, in a series of 118 cases undergoing angioplasty for single-vessel coronary disease, no association with late luminal loss could be identified (Hamon et al. 1995). The vessel pathology fell into at least 2 groups, as 1/3 of patients had unstable angina. Thus patient populations differed. So too did genotype distribution, which was not in Hardy-Weinberg equilibrium in Ohishi's study (with a deficit in ID cases). Further, Ohsihi used 2-primer amplification whilst the latter study used an allele-specific third primer.

10.33 ROLE OF VASCULAR RAS IN THE PATHOGENESIS OF ESSENTIAL HYPERTENSION

As well as linking the cardiac and vascular effects of sustained hypertension, there is also a suggestion that alterations in vascular RAS may be involved in the pathogenesis of the hypertension itself. Genetic studies implicate the ACE gene locus as a candidate gene for hypertension in the SHRSP rat (reviewed by Soubrier et al. (1994)). Three studies have shown diverse results in associating the polymorphism with hypertension. Using 170 healthy Caucasian adults of varying genetic predisposition to hypertension from the Scottish 'four corners' study, no association of the I/D polymorphism with resting blood pressure was identified (Harrap et al. 1993). However, in a poorly-detailed small Japanese study, the D allele was associated with essential hypertension

(Morise et al. 1993). The difference in results is largely accounted for by a marked difference in allele distribution in control groups. In total contrast, a third study found the insertion allele frequency to be greater (0.56) in 80 hypertensive subjects than in the 93 normotensive controls (0.41) (Zee et al. 1992). The association of the I/D polymorphism with the hypertensive state per se thus remains unproved.

10.34: CONCLUSIONS

There is now convincing evidence of a role for tissue RAS in the genesis of vascular disease, and (from our data and that of others) for a role in the regulation of pathophysiological myocardial hypertrophy. It is thus possible that increased tissue RAS activity provides the mechanistic link between the presence of LVH, and the increased risk of cardiovascular and cerebrovascular events.

10.4: LOCAL vs. SYSTEMIC UPTAKE

The relative roles of uptake of circulating RAS components and of an intrinsic myocardial RAS in the control of cardiac growth have been debated (chapter 1), although the marked chamber differences of ATI and ATII concentration in rhesus monkey heart suggest that the presence of these peptides is due to more than mere adsorption from the circulation (Lindpaintner et al. 1987). 20 hours after bilateral nephrectomy, Lindpainter gave rabbits ramipril or vehicle alone (Lindpaintner et al. 1987). Four hours later, plasma ATII levels were similarly low in both groups, whilst cardiac ATII levels were reduced in the ramipril-treated animals. These data suggest that local synthesis of ATII occurs rather than mere uptake of circulating ATII.

Our studies of the TGR(mREN2)27 rat have certainly shown that inhibition of tissue ACE (without inhibition of plasma ACE) has profound effects on cardiac growth. However, the origin of this tissue ACE (i.e. locally-synthesised or taken up from the circulation) has not been conclusively proven.

High non-renal tissue prorenin levels may play an important role in driving cardiac growth, (Nielsen and Poulsen 1988) either by direct action or indirectly through the action of a prorenin-processing enzyme (Laragh 1992; Dzau et al. 1993)). However, the lack of early right ventricular hypertrophy in our transgenic model suggests either that the right ventricle is unable to take up and activate circulating

prorenin, that uptake of circulating prorenin is not important in driving myocardial RAS, or that uptake only becomes important in later life (note the more rapid RV growth in the 70-120 day period in TGRs than SD controls). In humans, however, the strong association of ACE genotype with cardiac growth speaks of a separate myocardial RAS with local ACE synthesis being under local control and independent of uptake from plasma. This is especially true as no association between circulating ATII levels and ACE polymorphism genotype has ever been demonstrated (chapter 3). Further, these data suggest that in the heart (unlike the plasma) ACE may be a rate-limiting step in ATII generation. It must be remembered, however, that evidence exists of parallel upregulation of other RAS components, and for positive feedback loops within cells whereby increased ATII synthesis may drive increased renin and angiotensinogen supply.

10.5: CLINICAL ROLE OF INHIBITION OF MYOCARDIAL RAS

10.51: ANTIARRHYTHMIC EFFECTS

A hallmark of ACE-inhibition in animal *in vitro* models is a reduction of reperfusion arrhythmia generation (reviewed by Pahor et al. (1994)), although species differences are evident in *in vivo* studies. ACE-inhibitors may exert an antiarrhythmic effect in humans (Webster et al. 1985). Pahor (Pahor et al. 1994) has reviewed the data from 17 trials designed to determine the effects of ACE-inhibition on the incidence of ventricular arrhythmias. Overall, 7 of these studies showed an antiarrhythmic effect, 2 of which specifically examined patients after acute myocardial infarction. These findings may bear a clinical impact. Four of nine large studies have also demonstrated a reduction in sudden cardiac death in patients, usually suffering with impaired cardiac systolic function, who were treated with ACE-inhibition (reviewed by Paulson et al. (1985)). Post-MI sudden death rate in those with clinically impaired LV function is reduced by ramipril treatment (AIRE Study Investigators 1993). In the Hy-C trial, treatment was associated with a reduction in baseline ventricular tachycardia (VT) and a prevention of emergence of new VT- effects which correlated with the clinical benefit seen (Fletcher et al. 1993). The lack of benefit in other trials may have in part been due to frequent open-label ACE-inhibitor treatment in control groups (Pahor et al. 1994). Such data is

supported by our finding of an excess numbers of ventricular premature beats per hour in amongst suffers of dilated cardiomyopathy who are of DD genotype.

Such antidysrhythmic effects might be due to several mechanisms (Pahor et al. 1994; De Mello 1995):

10.511 Changes in cardiac structure

Regression of LVH is associated with a reduced tendency to reperfusion arrhythmias in the Langendorff heart (Baxter and Yellon 1992b). Our studies show a major effect of ACE inhibition on the fibrotic component of LVH in a rat model. By reducing cardiac fibrosis, the potential for myocyte isolation and the appearance of 'circus' arrhythmias is reduced.

10.512 The increase in serum potassium associated with ACE-inhibitor therapy

Hypokalaemia is a risk factor for ventricular arrhythmias in heart failure. It has been suggested that, in studies of ACE-inhibitor treatment, no antiarrhythmic effect is seen if serum potassium is held constant (Pahor et al. 1994).

10.513 Protection of the ischaemic myocardium

ACE-inhibitors may prevent ATP depletion (reviewed by De Mello (1995)) in hypoxic myocardium and protect the ischaemic myocardium from arrhythmia and ischaemic damage if pre-treated (reviewedby Pahor et al. (1994) and Parratt (1994)).

10.514 Modulation of electromechanical feedback

10.515 Reduced diastolic volume resulting in reduced dispersion of refractoriness

10.516 Decreased wall stress

10.517 Reduced collagen deposition and cardiac fibrosis may also reduce myocyte isolation, and reduce the risk of re-entry arrhythmias and automaticity

10.518 Decreased sympathetic tone (see above).

ATII may increase cardiac sympathetic nerve noradrenaline synthesis and release. An increase in sympathetic outflow is a hallmark of cardiac failure, and may be reduced by ACE-inhibition. Cardiac noradrenaline outflow may specifically be reduced by ACE-inhibitor treatment (Pahor et al. 1994).

10.52: BENEFITS AFTER MYOCARDIAL INFARCTION

After myocardial infarction, ventricular remodelling occurs (Smits et al. 1992; Vannan et al. 1993; Yamagishi et al. 1993; Nishikimi et al. 1995), comprising:

(i) Infarct expansion (thinning and 'stretching' of the infarcted area). This occurs within hours of infarction, and is usually complete within 3 weeks.

(ii) Global ventricular dilatation through myocyte lengthening:

(iii) Compensatory myocyte hypertrophy.

Remodelling raises wall tensions and risk of myocardial thrombus, aneurysm, infarct rupture and clinical heart failure, and is associated with a poorer prognosis (Vannan et al. 1993): clinical left ventricular failure after MI is associated with a 6-fold increase in mortality at 30 days (Ball et al. 1994). The process may be partly controlled by cardiac RAS. Cardiac ACE expression increases after MI, and immunostaining suggests that this is mainly due to increased endothelial ACE expression. In rats and humans ACE expression increases early (3-7 days) in macrophages and sprouting capillaries in the marginal zone around the infarct. With the onset of fibrosis, very intense ACE immunoreactivity is seen in the marginal zone of tissue repair, with fibroblasts and vascular tissue contributing heavily (Falkenhahn et al. 1995). Although tissue from 8 human MI patients was examined in this study, the characteristics of these patients and treatment nature and duration is not described. In fact, increased RAS activity may be a universal feature of the dysfunctioning heart. Using reversed-transcription PCR, Paul et al quantified ACE gene expression in heart tissue from 8 patients with dilated cardiomyopathy and 6 patients with ischaemic cardiomyopathy (Paul et al. 1994). ACE expression was increased in those with dilated cardiomyopathy. The nature of drug treatment, which is likely to have included ACE-inhibition, is not described however. Neither is the history of use of inotropic agents. The relative contributions of different cardiac chambers to the tissue was not described, and it is possible that the excess of microscopic scarring and the avoidance of macroscopic scars (localised to true ischaemic territories) may have contributed to their results. Further, RT-PCR is a contentious means by which to assess gene expression, although good controls (use of quantified ACE-cDNA) support their data.

Remodelling is reduced in humans and animal models by RAS inhibition. Smits infarcted 39% of the rat ventricle. Compensatory hypertrophy (suggested by a lack of cardiac weight loss) after a 39% LV infarct in the rat is abolished by losartan treatment, with a reduction in cardiac weight of 34-38% resulting (Smits et al. 1992). This data conflicts with that of Raya who found no such effect in rats with more extensive (<approx. 50%) infarcts (quoted in (Smits et al. 1992)). Stained collagen area was reduced in non-infarcted areas in the MI animals, but not in normal animals. These effects might have been partly mediated by changes in haemodynamic load using their data one can calculate that rate-pressure product was reduced by 15% at 21 days with a fall in mean BP of 20%. These data suggest a negatively inotropic action of losartan, as a slight reduction in peripheral resistance was associated with a slight reduction (not increase) in cardiac output. Nishikimi noted similar reductions in cardiac work in normotensive and hypertensive rats with ATII receptor antagonist treatments after MI (Nishikimi et al. 1995). In this study, compensatory hypertrophy actually led to an increase in LV mass after MI in hypertensive rats. In both rat groups, treatment reduced RV and LV weight and prevented LV dilatation- an effect comparable to that achieved with ACE-inhibition in the same study. Previous studies have shown similar effects of both ACE-inhibition and ATII antagonism, and have suggested that both act through a reduction in cardiac ATII (Yamagishi et al. 1993).

ACE-inhibition benefits humans after MI, possibly through antidysrhythmic effects, the inhibition of all component parts of the remodelling process (Pfeffer et al. 1985; Pfeffer et al. 1988; Sharpe et al. 1991), and vascular-stabilising effects. The SAVE study (Pfeffer et al. 1992) recruited 2231 individuals with MI and ejection fraction of <40% but with no symptoms of heart failure (although treatment was allowed for this condition) or ongoing ischaemia. They were followed for a mean of 41 months. Captopril treatment was initiated at a mean of 11 days, and led to a reduction in all cause and cardiovascular mortality of 19 and 21% respectively. The AIRE study(AIRE Study Investigators 1993) recruited 2006 patients with MI and clinically-diagnosed left ventricular failure who were treated with ramipril and followed for a mean of 15 months. On an intention-to-treat basis, mortality was reduced by 27%. Most of the benefit was through a reduction in the development of severe cardiac failure. By contrast, two studies showed no such benefit of ACE-inhibitor treatment. The SOLVD prevention study (The SOLVD Investigators 1992) examined 4210 asymptomatic patients (80% with a history of MI) with ejection fractions of $\leq$35%. They were followed for a mean of 17.4 months. A non-significant trend to reduced mortality (8% reduction, confidence intervals -8-21%) was seen, although risk of development of heart failure or death combined was reduced by 29% (95% confidence intervals 9-30%). Similarly, the Consensus II study (Swedberg et al. 1992) showed a significant incidence of hypotension with intravenous enalaprilat given early after MI

(mean 15hours) to 6090 patients. Hypotension was associated with increased mortality, and the study was therefore stopped after a follow-up of only 14-180 days. The lack of benefit seen in Consensus II was probably related to the hypotensive effect (10.5%) of early intravenous treatment. The study excluded those who might have benefited most (i.e. those with symptomatic heart failure), and included many who would benefit least (all individuals were included irrespective of ventricular function). In the SOLVD prevention study, 19% of the placebo group received open-label ACE-inhibitor treatment.

It is interesting to note that in the SAVE study, and in a reanalysis of the SOLVD data (Yusuf et al. 1992; Yusuf 1993), myocardial infarction rates seem to have been reduced by ACE inhibitor treatment. The SOLVD reanalysis examined 6797 patients with$\leq$35% (3401 placebo and 3396 enalapril) from both the prevention and treatment limbs of the study (asymptomatic or treated for symptoms of heart failure). None had suffered MI within 30 days of entry or had had a revascularisation procedure within 6 months. Myocardial infarction occurred in 362 of the placebo group and 288 of the treated group (10.6% vs. 8.5%: risk reduction 23%). Unstable angina was also less common in both limbs of the study (risk reduction 20%). These effects became significant after only 6 months of treatment. The SAVE data also showed a 25% reduction in rate of recurrent myocardial infarction. A trend towards reduced infarction rates was noted in the AIRE study, although this did not reach statistical significance. The mechanism of such benefit is unclear, but may involve reduced plaque shear stress (through lower blood pressure), although the reduction in BP in the trials cited seems too small to account for this. However, ACE-inhibitors have profound effects on the development of atherosclerosis and on plaque composition in animal models (Sharpe 1993), direct effects on plaque stability have been postulated (Fuster 1994).

10.6: CONCLUSIONS

A local myocardial RAS seems to exist in association with both myocyte and matrix components. Elements of the cardiac RAS are upregulated during physiological and pathophysiological cardiac growth, leading to increased ATII generation. Angiotensin II is capable of stimulating myocyte growth and matrix generation both *in vivo* and *in vitro* , through both direct and indirect actions. As our data and that discussed above demonstrate, ACE-inhibitors can limit such changes. ATII receptor antagonists have similar effects, suggesting that at least part of the action of ACE-inhibitors is mediated through prevention of ATII generation. The data from studies of the TGR(mREN2)27 rat presented in this thesis support the view that, at least in the rat, cardiac ACE is more important than cardiac chymase in the regulation of cardiac growth. The study of ACE I/D Polymorphism genotype in military recruits suggests that this is also true of human physiological cardiac hypertrophy. We must therefore postulate that an inducable intracellular (and possibly intrafibroblastic) RAS exists in the human heart in which ACE is the main ATI-converting enzyme, and that this system plays a critical role in the regulation of LVH. In the rat, this would seem to be through control of ATII levels. In the human, the relative roles of kinins and ATII have yet to be determined.

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