Studies on the regulation of gene expression of tissue renin-angiotensin systems

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Abstract.

The classic view of the renin-angiotensin system is that of a circulating, systemic hormonal cascade regulating blood pressure and extracellular fluid volume. However, increasing evidence has led to the suggestion that functional renin-angiotensin systems comprising some or all of the components of the enzymatic pathway may exist within several individual organs or tissues. The purpose of the work presented in this thesis was (i). to confirm renin-angiotensin system gene expression in various tissues (ii). to examine the effects of inhibition at different levels on expression (iii). to investigate the effects of experimental post-infarction heart failure on tissue renin-angiotensin systems and to see whether ACE inhibitor treatment, which improves this condition, influences gene expression. In the work presented, first a systematic study of rat tissues by Northern blotting and RT-PCR demonstrated the expression of mRNA for components of the renin-angiotensin system in several tissues, supporting the concept of their existence and resolving controversy over the potential for expression in certain tissues. Subsequently, both acute and chronic pharmacological blockade of the cascade by an ACE inhibitor demonstrated differential regulation of the renin-angiotensin system in different tissues. In further studies, blockade of the cascade at three different levels in the marmoset confirmed differential regulation of specific components in different tissues. In contrast to previous reports, induction of experimental heart failure in rats followed by administration of an ACE inhibitor resulted in a 1.6 fold increase in renal angiotensinogen mRNA and a change in cardiac ACE mRNA was not seen with heart failure. A reduction in mortality was also associated with ACE inhibitor treatment. The results obtained raise interesting questions both of the role of tissue renin-angiotensin systems in contributing to the pathophysiology of disease states and mediating the effects of pharmacological blockade of the renin-angiotensin system, and more generally regarding their organisation.

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Dedication.

Dedicated to Sumita, who supported and encouraged me throughout the project and to my father James: A premature victim of cardiovascular disease.

Abbreviations.

AI	angiotensin I.
AII	angiotensin II.
ACE	angiotensin converting enzyme.
Aogen	angiotensinogen.
Arg	arginine.
Asp	asparagine.
AT_1	type 1 angiotensin II receptor.
AT_2	type 2 angiotensin II receptor.
bp	base pair.
bpm	beats per minute.
cAMP	cyclic adenosine monophosphate.
cDNA	complementary Deoxyribonucleic acid.
cpm	counts per minute.
cps	counts per second.
DEPC	diethyl pyrocarbonate.
EDTA	ethylenediaminetetra-acetic acid . $2H_2O$.
g	acceleration due to gravity.
GAPD	glyceraldehyde-3-phosphate dehydrogenase.
Gln	glutamine.
Glu	glutamic acid.
gm	gram(s).

h/hr	hour(s).
HGPRT/HGPT	hypoxanthine guanine phosphoribosyl transferase.
His	Histidine.
Ile	isoleucine.
kb	kilobasepair.
K_M	Michaelis constant.
Leu	leucine.
min	minute(s).
MOPS	morpholinopropanesulphonic acid.
mRNA	messenger Ribonucleic acid.
O.D.	optical density.
PCR	polymerase chain reaction.
Phe	phenylalanine.
PRA	plasma renin activity.
Pro	proline.
RA	renin-angiotensin.
rpm	revolutions per minute.
SDS	sodium dodecyl sulphate.
sec	second(s).
SEM	standard error of the mean.
SHR	spontaneously hypertensive rat.
SNS	sympathetic nervous system.
SSC	saline sodium citrate.

TAE	Tris-acetate/EDTA.
T.E .	Tris-EDTA.
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol.
Tyr	tyrosine.
U.V.	ultra violet.
V	volts.
VSM	vascular smooth muscle.
Val	valine.
WKY	wistar-kyoto.

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CHAPTER ONE

Historical review

PREFACE

The history of the study of the renin-angiotensin (RA) system has its foundation in the study of blood pressure and its regulation. The most important clinical theme to arise from the study of blood pressure has been the concept of hypertension. Hypertension may be defined as an elevation of arterial blood pressure above the normal range expected in a particular age group. Hypertension may occur without a discoverable organic cause and this is known as *essential* or *primary hypertension*. Essential hypertension was first formally defined by Frank (1911) and the first description in the West of this condition was probably by Schaarschmidt and Nicolai in the mid 1700's who reported agitation of the circulation and constriction of the vascular bed in patients (in Backer, 1953). *Secondary* or *symptomatic hypertension* is defined as hypertension due to, or associated with, a variety of primary diseases such as stenosis of the renal artery (renal hypertension), endocrine disorders (such as Cushings syndrome or phaeochromocytoma) or vascular disease. It is necessary, therefore, to look at the elucidation, function and pathophysiology of the RA system in the general context of the study of blood pressure and hypertension.

1.1 THE EARLY DAYS

Stephen Hales (1733), in his studies on the horse, proved by direct cannulation of a large artery that blood flow exerted a specific pressure on blood vessel walls. He also defined much of the haemodynamics of the circulation and the possible effects on the circulation of pathological lesions. This expanded the famous work of William Harvey, whose demonstration of the circulation of the blood in 1633 (in Swales, 1987) was the beginning of modern cardiovascular research. Hale's work was developed throughout the nineteenth century, most notably by Vierordt, Marey and Mahomed, to give a method of indirect measurement (in Swales, Sever, and Peart, 1991). However, it took until the early part of the twentieth century before the sophistication of indirect measurement was systematised to include diastolic as well as systolic pressure. This is illustrated most famously by the work of Korotkoff (1905), which forms the basis of indirect clinical measurement today.

In the intervening period, the work of Richard Bright (1836) stands out as a landmark in that it established a link between renal disease and hypertension. Bright studied patients who had died with diseases characterised by dropsy and albuminuria. Upon post-mortem examination, he found that the kidneys were shrunken and diseased (nephritis) and the heart enlarged (most apparent in the left ventricle). Such a presentation became known as Bright's disease. Bright recognised that the degree of cardiac hypertrophy was related to the progression of renal disease, but incorrectly postulated that cardiac hypertrophy was a compensatory mechanism to restore adequate renal perfusion in the diseased kidney.

Bright's observations encouraged a plethora of researchers to attempt to elucidate the cause of this new disease. Traube (1856) came to the same incorrect conclusions as Bright but nevertheless was the first person to link raised arterial pressure with the disease. Johnson (1868) recognised arteriolar medial hypertrophy in the pathogenesis of the disease, confirming Bright's observations concerning hypertrophy. Ewald (1877; in Swales, 1987) supported these findings. In contrast, Mahomed (1874) and Allbutt (1896) described raised blood pressure without renal disease, giving increased arteriolar resistance as the cause. This ties in with Leyden (1881), who postulated that thickening of arteries (and consequential narrowing of the lumen) in the renovascular bed could cause elevation of arterial pressure in the absence of renal disease.

1.2 THE ROLE OF THE KIDNEY IN HYPERTENSION

In 1892, Brown-Séquard found that nephrectomised animals lived slightly longer and had a temporary improvement in condition after being given renal extracts. This supported his earlier postulate that body organs could release substances into the circulation that had physiological effects, but were not catabolic products (Brown-Séquard, 1854).

Tigerstedt and Bergman (1898) proposed to test the ideas of Brown-Séquard with respect to the kidney and its relationship with the manifestations of uraemia in Bright's disease; in doing so they discovered renin. They took an extract of fresh rabbit kidney and administered it intravenously into other healthy rabbits. This caused an elevation of blood pressure within a short time; peaking after one to two minutes and gradually returning to

normal within twenty minutes. They termed the pressor substance evidently present in the kidney extract, as renin. They also found that repeated administration of renin had a lesser response and that its action might be mediated indirectly via an effect on vascular smooth muscle. Renin was demonstrated to be present in the renal cortex and renal venous blood. Renin was not found in arterial blood or urine. It was postulated that under certain conditions, the kidney would produce an excess of renin which would be released into the circulation. Blood vessels would thus have increased resistance due to the pressor action of renin, eventually giving rise to cardiac hypertrophy. In addition, Tigerstedt and Bergman investigated the basic physical properties of renin, finding it to be non-dialysable, water soluble but insoluble in ethanol and heat-inactivated above 56° Centigrade.

1.2.1 Experimental models of renal hypertension

The studies of Tigerstedt and Bergman (1898), were essentially not expanded for the next thirty years and the existence of renin was still not widely accepted at the time of Tigerstedt's death in 1925. From a scientific perspective, other investigators had failed to reproduce Tigerstedt and Bergman's work and the biochemical demonstration of renin was technically complex. Perhaps the greatest obstacle to the further study of renin was the lack of a suitable model of renal hypertension. Various attempts at producing models had resulted in renal failure in the experimental animal (Goldblatt, 1937).

In the early 1930's Harry Goldblatt began studies into experimental renal hypertension to test his hypothesis that lumen narrowing in the renal arteries of human 'essential' hypertensives was the primary cause of the observed elevated blood pressure. Together with his co-workers (Goldblatt *et al.*, 1933; 1934), Goldblatt measured the arterial blood pressure of conscious dogs that had hypertension induced by constricting their renal arteries with adjustable silver clamps. Partial constriction of only one artery (two kidney-one clamp model) gave an initial elevation of blood pressure that recovered to normal in a couple of days. Partial constriction of both renal arteries (two kidney-two clamp model) gave a persistent rise in blood pressure. This rise could be reversed by removing the clamps (Goldblatt, 1937). A rise in blood pressure also developed if one of the renal arteries was

constricted and the unclamped kidney removed (one kidney model). The severity of induced hypertension was therefore related to the degree of constriction of the renal arteries. Goldblatt postulated that the elevation of blood pressure was induced by the release of a pressor substance by the kidney. He also showed that constricting blood vessels other than the renal arteries had no effect on blood pressure.

Goldblatt and his group further characterised their model over the next ten years or so. They demonstrated that an intact ischaemic kidney had to be present for hypertension to develop (Goldblatt, 1937). This supported other groups' work, including Prinzmetal and Friedman (1936) and Harrison, Blalock, and Mason (1936), who found that in two kidney-one clamp hypertension in dogs, an extract from the ischaemic kidney had a greater pressor effect than an extract from the unclamped kidney, in recipient dogs. Goldblatt (1937) also showed in the dog, that ligation of the renal veins in both kidneys did not induce hypertension. This implied that a pressor substance was released from the kidneys via the renal veins. Later work (Goldblatt, 1947) that demonstrated the development of hypertension in Goldblatt's models following removal of the sympathetic nervous system, supported Goldblatt's idea that neural factors were not involved in hypertension.

Some investigators in the field failed to reproduce Goldblatt's work or found that the data from different experiments contradicted each other (Page, 1937; Prinzmetal, Friedman, and Oppenheimer, 1938; Pickering, Prinzmetal, and Kelsall, 1939). Failure to demonstrate a pressor action was probably due to technical effects such as the loss of the pressor substance with alcohol extraction techniques and the vasodepressor effects of certain classes of anaesthesia (Pickering, 1968).

Confirmation of a pressor substance being present in renal venous blood came from Houssay and Fasciolo (1937), who studied the effect on blood pressure of transplanting kidneys into the neck of bilaterally nephrectomised dogs. An ischaemic donor kidney from a hypertensive dog caused a rapid increase in blood pressure in the recipient animal. Transplanting a normal kidney from a normotensive dog did not affect blood pressure. Furthermore, renal venous blood from an ischaemic kidney caused vasoconstriction in the

vascular bed of the hind limb of the toad, *Bufo parenarum.* Venous blood from a normal kidney, or from extra-renal tissues in hypertensive models, did not have this level of pressor effect (Houssay and Taquini, 1938).

1.3 ISOLATION OF THE PRESSOR SUBSTANCE

By the late 1930's, it was generally accepted that the ischaemic kidney produced a pressor substance that was released into the venous circulation. Due to the revival of interest in the work of Tigerstedt and Bergman (1898), renin seemed the obvious candidate for the pressor and this was proposed by Prinzmetal and Friedman (1936), amongst others, after they repeated the early work. However, the pressor substance had not yet been isolated from blood.

Braun-Menéndez and co-workers (1939) managed to isolate the pressor substance from renal blood. It must have been a surprise to many that the pressor substance was clearly demonstrated not to be renin. In stark contrast, the pressor substance was dialysable, soluble in acetone but not organic solvents, heat stable and inactivated by acids and proteolytic enzymes. This pointed to it being a polypeptide. It could also be produced by incubating renin with blood globulins. The pressor was termed 'hypertensin'. It was postulated that renin was a proteolytic enzyme (this was later confirmed by Croxatto and Croxatto [1942] who demonstrated that incubating pepsin with blood proteins gave a product with similar properties to 'hypertensin') secreted by the kidney, which acted on blood pseudo-globulins to give the active peptide 'hypertensin'. Page and co-workers (Kohlstaedt, Helmer, and Page, 1938; Page, 1939; Page and Helmer, 1940) simultaneously isolated and characterised a pressor substance. In experiments on an isolated rabbit ear preparation; they showed that vasoconstriction took place if renin was perfused through the vasculature using fresh blood, but not if renin was perfused using physiological saline. This showed that renin did not act directly on blood vessels, but required a 'renin-activator' present in blood. They went on to show, in further work, that incubating renin with 'renin-activator' (isolated by fractional precipitation of bovine serum) gave a pressor substance with similar properties to 'hypertensin'. Page and Helmer (1940) called their pressor substance 'angiotonin'. It

eventually became apparent that 'hypertensin' and 'angiotonin' were the same thing and the common name angiotensin was agreed upon for the pressor, along with angiotensinogen for the renin substrate or 'activator' (Braun-Menéndez and Page, 1958).

1.3.1 Characterisation of angiotensin

In the early 1950's a small group consisting of Leonard Skeggs, Walter Marsh (a recent Ph.D. like Skeggs) and the physiologist Joseph Kahn were involved in the race to purify and determine the structure of angiotensin. They prepared crude extracts of renin from pig kidneys, angiotensinogen from horse blood, incubated the two to form angiotensin and purified the product by counter-current distribution. By a quirk of fate during one experimental run, Skeggs used saline instead of distilled water to dialyse his angiotensinogen. This resulted in purified angiotensin appearing in an earlier fraction of the counter-current distribution to normal. Subsequent experiments showed that this new fraction appeared when renin and angiotensinogen were incubated in the presence of chloride ions. Skeggs called the 'normal' fraction Angiotensin I (AI) and the fraction produced in the presence of chloride ions, Angiotensin II (AII). It was found that renin was not involved in determining whether AII was produced. Skeggs proposed that there was an enzyme present in the crude angiotensinogen extract that was activated by chloride ions and converted AI to AII. Therefore, as well as showing two forms of Angiotensin, this was the first demonstration of angiotensin converting enzyme [ACE] (Skeggs *et al.*, 1954a). AI was purified (Skeggs *et al.*, 1954b) and the amino acid composition determined (Skeggs *et al.*, 1955) by 1955. AII was purified (Skeggs, Kahn and Shumway, 1956a) and had its relationship to AI and amino acid sequence determined by 1956 (Lentz *et al.*, 1956; Skeggs *et al.*, 1956). Skeggs and his colleagues showed that converting enzyme cleaved the C-terminal dipeptide (His^{9}-Leu^{10}) of AI, a decapeptide (Asp^{1}-Arg^{2}-Val^{3}-Tyr^{4}-Ile^{5}-His^{6}-Pro^{7}-Phe^{8}-His^{9}-Leu^{10}) to yield AII, an octapeptide. A couple of months earlier the amino acid sequence of a bovine angiotensin had been published (Elliot and Peart, 1956). This sequence was identical to that of horse AI (Skeggs *et al.*, 1956) with the exception of a valine for isoleucine substitution at position 5 of the decapeptide. AII was also subsequently found to

have up to three times the pressor effect of noradrenaline in the anaesthetised rat (Peart, 1956).

Angiotensin Converting Enzyme was also isolated at this time (Skeggs, Kahn, and Shumway, 1956b) and partially characterised. The part of the angiotensinogen molecule from which AI is generated via enzymatic cleavage by renin was also characterised (Skeggs *et al.*, 1957), AI being demonstrated to be the first ten amino acids at the amino terminal end of the angiotensinogen molecule.

AII was soon synthesised in the laboratory and was categorically identified as the active pressor substance, AI being the precursor inactive form (Bumpus, Schwartz, and Page, 1957). The pathway that was to be termed the renin-angiotensin system had therefore been elucidated before the end of the 1950's.

Since the 1950's roles for AII apart from that of a simple pressor have been postulated. It was suggested that AII might affect gene transcription via receptors on chromatin (Re and Parab, 1984). If this is true, then AII may have comprehensive functions beyond cardiovascular and fluid homeostasis. AII has been shown to influence the growth of vascular smooth muscle cells in culture (Dzau and Gibbons, 1988). This implies that local production of AII may be involved in cardiac and vascular hypertrophy. Such a growth mechanism might arise via activation of the phosphoinositide second-messenger system (Heagerty and Ollerenshaw, 1986; Lever, 1986; Mizuno *et al.*, 1988). Local production of AII has also been postulated as having a role in the maintenance of vascular tone (Nakamaru, Jackson and Inagami, 1986; Mizuno *et al.*, 1988). A full description of the putative roles of AII is given in chapter two.

The most recent developments in the characterisation of AII have come with the discovery of its receptors in the late 1980's. The receptors were placed into two distinct subtypes, type 1 (AT_1) and type 2 (AT_2), based upon their differential pharmacological and biochemical properties (Chiu *et al.*, 1989). A bovine adrenal AT_1 receptor complementary DNA (cDNA) was cloned, sequenced and had the protein expressed, in the early 1990's

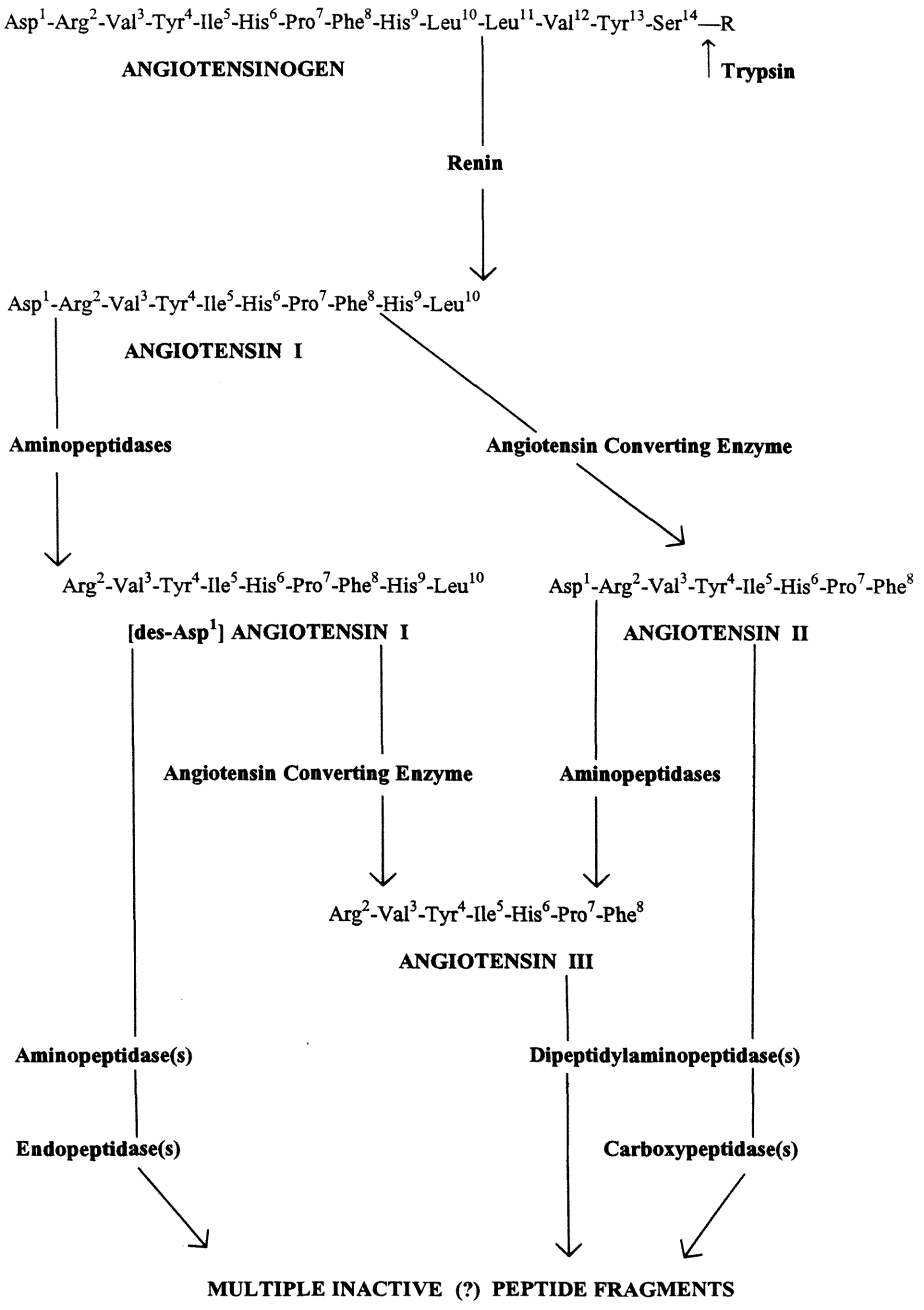


Figure 1A: The renin-angiotensin cascade (after Peach, 1977).

(Sasaki *et al.*, 1991). Simultaneously, similar data for a rat vascular AT_1 receptor cDNA was published (Murphy *et al.*, 1991). From the predicted amino acid sequence, the AT_1 receptor was placed into a seven-transmembrane receptor, G protein-coupled receptor superfamily. Subsequent molecular analyses showed that the AT_1 receptor was comprised of two isoforms AT_{1a} and AT_{1b} (Iwai and Inagami, 1992; Sasamura *et al.*, 1992). Pharmacological data from the cloning and expression studies showed that the AT_1 receptor mediates most known effects of AII via activation of the phosphoinositide/calcium pathway; or inhibition of adenylate cyclase activity. In recent years AT_1 receptors have been cloned from a variety of tissue sites in several species (Timmermans *et al.*, 1993). AT_2 receptors from foetal rat cDNA (Mukoyama *et al.*, 1993), a rat phaeochromocytoma cell line (Kambayashi *et al.*, 1993) and foetal mouse cDNA (Nakajima *et al.*, 1993; Ichiki *et al.*, 1994) have since been cloned, expressed and sequenced. In the kidney, mRNA for the AT_{1a} isoform has been shown to be far more abundant than AT_{1b} mRNA (Du *et al.*, 1995), thus suggesting that AT_{1a} is the predominant renal form of the AT_1 receptor. The AT_2 receptor has been demonstrated to belong to a seven-transmembrane receptor superfamily like the AT_1 receptor. However, it has been postulated to be in a unique part of that family that does not show G protein-coupled signalling or even follow a classic intracellular signalling pathway (Tsutsumi *et al.*, 1991; Dudley, Hubbell, and Summerfelt, 1991). The role of the AT_2 receptor is largely unclear, but as it is predominantly expressed in foetal tissue (Grady *et al.*, 1991; Tsutsumi *et al.*, 1991) it is likely to play an important role in development.

1.4 RENIN

1.4.1 Localisation of renin production

Despite the clarification of the role of renin elucidated in the 1930's, the exact site of renin production in the kidney was unknown. Tigerstedt and Bergman (1898) had shown renin to be present in the renal cortex rather than the renal medulla. This was verified by Prinzmetal and Friedman (1936).

Goormaghtigh (1939) proposed the site of renin production to be acidophil and basophil granules (implying in themselves an endocrine function) within the juxtaglomerular

(JG) cells (these are specialised smooth muscle cells found in the media of the afferent arteriole, close to the glomerulus). He came to this conclusion after observing that constriction of the renal artery in dogs or rabbits was associated with an increase in the size and number of JG cells, thus forming a link with the elevated blood pressure seen by Goldblatt when constricting the renal arteries of the dog (see section 1.2.1). Goormaghtigh's studies were a rewarding epitaph to the work of Ruyter (1925) who had described granules in the terminal part of the afferent arterioles of the glomerulus. However, Goormaghtigh's work was largely ignored until the mid 1950's. Further characterisation by Cook, Gorden, and Peart (1957) showed that renin was located mainly in the outer cortex (where most of the glomeruli are found) whereas no renin was found in the subcapsular region (where there are no glomeruli). Tobian, Janecek, and Tomboulian (1959) found a definite correlation between the amount of juxtaglomerular granulation (JG index) and the amount of extractable renin in the kidneys of rats with experimental hypertension. Finally, categorical evidence of the localisation of renin to granules within the JG cells, came from Edelman and Hartcroft (1961) by the use of fluorescence labelled antibodies to renin.

1.4.2 Further characterisation of renin

Although the basic properties of renin were known, the actual nature of renin itself was largely unknown throughout the 1950's and 1960's. Attempts to isolate and purify the enzyme [e.g. Hass, Lamform, and Goldblatt (1953)] were only partly successful due to the relatively small amount of renin in kidney homogenate and the instability of extracts. This instability was manifest as the techniques used to purify and concentrate renin also purified and concentrated other proteases present in the kidney (Inagami *et al*, 1988).

The discovery that in certain strains of mice the submandibular gland produced relatively large amounts of renin, was exploited to produce a satisfactory working yield of pure, stable renin (Cohen *et al.*, 1972). The high yields of intact renin obtained were instrumental in later biochemical and molecular genetic analysis of renin. Confirmation that renin had been isolated was demonstrated immunologically, using antibodies generated from a mouse renal extract, by radio immunoassay (Michelakis *et al.*, 1974a). The renin from the

submandibular gland was also shown to react specifically with angiotensinogen to generate AI (Michelakis *et al.*, 1974b). New methods were developed to purify kidney renin, including chemical inactivation of proteases by pepstatin A (Aoyagi *et al.*, 1971) and affinity chromatography. This resulted in pure stable renal renin first being isolated in the pig (Murakami and Inagami, 1975; Inagami and Murakami, 1977). Rat renal renin (Matoba, Murakami, and Inagami, 1978) was also isolated by Inagami's group as was human renal renin (Yokosawa, Inagami, and Hass, 1978; Yokosawa *et al.*, 1980). Pro-renin, the inactive precursor of renin first described by Lumbers (1971), was soon isolated (Yokosawa *et al.*, 1979) and purified (Takii and Inagami, 1982).

Renin was demonstrated to be an aspartyl (or acid) protease as it was inactivated by diazoacetylamino acid esters (Inagami, Misono, and Michelakis, 1974) and had a similar active site arrangement to other acid proteases (Misono and Inagami, 1980). However, renin was found to be quite unique amongst acid proteases. It was shown to have a neutral pH optimum (not acid), only one known substrate (angiotensinogen) and weak inhibition by pepstatin A (a phenomenon that proved useful in the isolation of renin: Other proteases were inactivated by pepstatin A allowing the renin to remain intact, whilst inhibition of the biological action of renin did not occur to any great degree).

In the early 1980's, the amino acid sequence of mouse submandibular renin was deduced from DNA sequencing of the cDNA complementary to the renin mRNA (Panthier *et al.*, 1982; Misono, Chang, and Inagami, 1982; Corvol *et al.*, 1983). Homology was shown between the active sites of renin and other aspartyl proteases. In the process, evidence for renin precursor (pro-renin) was substantiated. The cDNA's for human renin (Imai *et al.*, 1983) and rat renin (Burnham *et al.*, 1987) were soon cloned and sequenced.

Perhaps the most interesting development in the story of renin has been the detection of renin activity in extra-renal tissues. Gould, Skeggs, and Kahn (1964) demonstrated renin activity in blood vessel walls. Ganten *et al.* (1971) reported the presence of renin in dog brain. Early reports of such activity were postulated to be due to uptake from the circulation (Loudon *et al.*, 1983) and to the non specific action of cathepsin D on angiotensinogen when

using acidic assay conditions. (Day and Reid, 1976; Hackenthal, Hackenthal, and Hilgenfield, 1978). The use of more stringent purification and detection techniques gave categorical evidence for renin activity in the brain (Hirose, Yokosawa, and Inagami, 1978; Ganten and Speck, 1978). Within a few years renin was shown to be present in a vast range of tissues including the adrenals (Naruse *et al.*, 1984).

The use of molecular biology techniques, within the last ten years or so, has strengthened the concept of extra-renal renin production and lead to the thesis that discrete local renin-angiotensin systems exist. Field *et al.* (1984), using Northern blot analysis, detected renin mRNA in the adrenal gland and testis of the mouse in addition to the expected sites of the kidney and submandibular gland. Renin mRNA went on to be described in a fully comprehensive range of tissues using the more sensitive Ribonuclease Protection Assay (Samani *et al.*, 1987; Samani, Swales, and Brammar, 1988). Since then mRNA for all major components of the renin-angiotensin cascade including renin, angiotensinogen, ACE and the AII receptor have been detected in many tissues using several techniques (Samani, 1991). Methods based on Reverse Trancription Polymerase Chain Reaction (RT-PCR) have allowed the detection of RA system mRNA from very small amounts of starting material, thus giving unrivalled sensitivity (Paul, Wagner, and Dzau, 1993). The existence of extra-renal renin-angiotensin systems is discussed in detail in chapter three.

1.5 ANGIOTENSINOGEN

1.5.1 Localisation of angiotensinogen production

Nasjletti and Masson (1972) demonstrated that perfused rat liver produced angiotensinogen and production could be inhibited by puromycin. Liver homogenates showed only small traces of angiotensinogen. It was therefore proposed that the liver freshly synthesises angiotensinogen and releases it into the circulation. Angiotensinogen was also found to be synthesised in liver slices (Freeman and Rostorfer, 1972) and hepatocytes (Weigand, Warnze, and Falge, 1977). Using antibodies to dog angiotensinogen, Morris, Iwamoto, and Reid (1979) detected angiotensinogen in rat liver, showing a heterogeneous distribution with the greatest localisation in the hepatocytes. Molecular genetic techniques

have also shown that the liver has the greatest amount of angiotensinogen mRNA (Campbell and Habener, 1986; Ohkubo *et al.*, 1986; Dzau *et al.*, 1987b). It is now generally accepted that the liver is the major site for generation of plasma angiotensinogen.

1.5.2 Further characterisation of angiotensinogen

In the early 1960's Skeggs and co-workers (1963) reported the purification of porcine angiotensinogen from plasma. As in 1957, with horse angiotensinogen, they found that AI constituted the amino terminal amino acid sequence of angiotensinogen. They also found that angiotensinogen was a glycoprotein and existed in several forms with slightly differing molecular weights (due to differing amounts of associated carbohydrate). Since then angiotensinogen has been purified from a number of species including man (Dorer *et al.*, 1978). Interestingly, a high molecular weight variant of angiotensinogen has been discovered that exists in high levels in the plasma of pregnant women (Tewksbury and Dart, 1982; Tewksbury and Tyron, 1989).

The cDNA for rat liver angiotensinogen was cloned and sequenced in the early 1980's (Ohkubo *et al.*, 1983), followed later by the mouse cDNA (Clouston *et al.*, 1988) and human (Gaillard, Clauser, and Corvol, 1989). Nucleotide sequence analysis placed angiotensinogen into the serpin gene superfamily. These are serine protease inhibitors. Angiotensinogen has no known protease activity (Tewksbury, 1990) but this interesting classification questions if being renin substrate is the only function of angiotensinogen. Angiotensinogen mRNA has been found in a variety of tissues (Ohkubo *et al.*, 1986) and has been demonstrated to be differentially expressed in different tissues (Campbell and Habener, 1986). This type of evidence has supported the concept of independent local RA systems (see chapter 3).

1.6 ANGIOTENSIN CONVERTING ENZYME

1.6.1 Localisation of angiotensin converting enzyme production

Although Skeggs and his colleagues had shown ACE to be present in plasma, the rate of conversion of AI to AII in plasma was too slow to account for the rapid accumulation of AII *in vivo* (Ng and Vane, 1967). Further study of the conversion of AI to AII demonstrated

that ACE was found predominantly in the vascular endothelium of the lungs. The lungs were therefore postulated to be the most important site for the generation of circulating AII (Ng and Vane, 1967; 1968). More recent thinking has suggested that production of AII in the endothelial lining of peripheral tissues may be as least as important (Campbell, 1987). Since the 1960's, ACE has been shown to be present in a variety of tissues including the brain, heart, kidney and liver (Lieberman and Sastre, 1983; Patchett and Cordes, 1985) and body fluids. This ubiquitous distribution of ACE may have important functional aspects.

1.6.2 Further characterisation of angiotensin converting enzyme

After the initial characterisation of ACE by Skeggs and colleagues, further progress was hindered by the lack of a simple chemical assay. The development of various assays using tripeptide substrates (Piquilloud, Reinharz, and Roth, 1970) and spectrophotometry (Cushman and Cheung, 1971) lead to the identification of ACE as a dipeptidal-carboxypeptidase (Das and Soffer, 1975); carboxypeptidases being a class of zinc-containing metalloproteins that cleave the terminal amino acid from the carboxylic acid end of peptide substrates. ACE was also purified in the early 1970's from porcine lung, using gel filtration (Dorer *et al.*, 1972; Nakajima *et al.*, 1973).

The possibility of inhibitors of the RA system (discussed further in chapter 2) had proved clinically attractive in the 1950's and 1960's. Renin and AII antagonists were developed but they invariably had to be administered parenterally and often had low specificity. A major progression in this field came from a peculiar source. The venom of the South American viper *Bothrops jararaca* was found to lower blood pressure and potentiate the effects of bradykinin, a vasodepressor peptide (in Swales, 1987). The mechanism of bradykinin potentiation appeared to be inhibition of kininase II, the main enzyme responsible for inactivation of bradykinin (Yang, Erdös, and Levin, 1970). It was soon recognised that kininase II and ACE were the same thing. The snake venom therefore also inhibited the conversion of AI to AII. The possibility of a converting enzyme inhibitor that could be used for the management of blood pressure became feasible. Further work culminated in the

design of a class of low molecular weight, orally active, non-peptide ACE inhibitors (Ondetti, Rubin, and Cushman, 1977), the first being Captopril.

As with renin and angiotensinogen, molecular genetic techniques have facilitated the further study of ACE. Since the late 1980's the ACE gene has been cloned and sequenced in a number of species including man (Roy *et al.*, 1988; Soubrier *et al.*, 1988; Ehlers *et al.*, 1989). Remarkably, this analysis has shown that not only is the ACE gene a result of an evolutionary gene duplication (having two homologous domains in the active site), but it also codes for two distinct mRNA species. The larger species corresponds to the duplicated gene and is expressed in endothelial and epithelial cells in many tissues, but is found predominantly in the lung. The smaller species corresponds to the 3' end of the gene and is only expressed in the testis. It has been postulated that the differential transcription of ACE is controlled by alternative promoter sequences (Hubert, Houot, Corvol, and Soubrier, 1991).

CHAPTER TWO

Physiology, pathophysiology and inhibition of the endocrine renin-angiotensin system

2.1 PHYSIOLOGY OF THE ENDOCRINE RENIN-ANGIOTENSIN SYSTEM

Humans and other animals require a constant electrolyte composition of intra- and extracellular fluids to maintain homeostasis. Potassium ions form the major intracellular osmotic component and sodium ions the major extracellular osmotic component, across cell membranes. Changes in these ions concentrations determine not only intra- and extracellular fluid volumes, but also blood volume and arterial blood pressure. Under normal conditions, the kidney regulates these ions via the renin-angiotensin (RA) system. The RA system can therefore essentially considered to be a long term regulator of (i). sodium balance and extra-cellular fluid volume, (ii). arterial blood pressure, and (iii). potassium balance. The RA system is activated when physiological or environmental phenomena act to influence any of these three parameters.

The kidneys secrete renin (a glycoprotein) in response to such influences as changes in posture, sodium depletion, alimentary fluid loss, shock (hypotension), haemorrhage and heart failure. When renin enters the circulation it cleaves angiotensinogen (a plasma protein produced by the liver), to liberate the decapeptide angiotensin I (AI). In a single passage through the pulmonary vasculature, AI is converted to angiotensin II (AII) by the action of angiotensin converting enzyme (ACE). The AII thus produced is delivered to the peripheral tissues where it affects physiological responses via interaction with specific receptors (Campbell, 1987).

2.1.1 Synthesis and physiology of renin

In humans renin is synthesised as a 23-43-340 amino acid pre-pro-renin molecule (Morris, 1986). The arrangement in other species is similar. Translation of renin mRNA yields pre-pro-renin. The pre-segment directs insertion of the protein into the lumen of the rough endoplasmic reticulum where it undergoes co-translational removal of the pre-segment and glycosylation, to become enzymatically inactive pro-renin[§]. Pro-renin is then sorted from the Golgi apparatus to one of two compartments:- A regulated secretory pathway involving lysosymes or a constitutive secretory pathway.

Active renin is the primary product of the regulated secretory pathway (Fritz *et al.*, 1987). This pathway stores secreted proteins, acutely releases them after stimulation by a secretagogue, and is mediated by dense secretory granules which also serve as the site of pro-renin to renin conversion (Kelly, 1986). In humans, cleavage of the pro-segment has been demonstrated to take place after two dibasic amino acid residues (Lys^{-2} Arg^{-1}) to yield a Leucine residue as the first amino acid of active renin. As there are ten pairs of dibasic amino acids in pro-renin and cleavage is at one specific site, it is thought that the pro-segment is removed by the action of an enzyme with high specificity. A wide range of proteases can activate pro-renin, at least *in vitro*. Thiol proteases, such as Cathepsin B which is found in lysosomes of the kidney and liver, have been demonstrated to activate pro-renin and decrease its molecular weight (Slater, Gounaris, and Haber, 1979; Luetscher, Bialek, and Grislis, 1982). Trypsin-like enzymes that also activate pro-renin have been found in the kidney, endothelial cells and neutrophils (Hsueh and Baxter, 1991). Trypsin-like enzymes have been demonstrated to act at the 'authentic' cleavage site but other enzymes e.g. pepsin act at alternative sites (Ishizuka *et al.*, 1991).

Pro-renin is the secretory product of the constitutive pathway (Fritz *et al.*, 1987). This pathway is not regulated acutely and delivers pro-renin to the cell surface at a constant rate. The fate of pro-renin appears to be differentially regulated across species. In humans up to 90% of secreted renin is pro-renin whereas in some mouse strains up to 90% of secreted renin is mature active renin (Fritz *et al.*, 1986; Ladenheim *et al.*, 1989). There is little evidence for pro-renin to renin conversion after secretion (Kim *et al.*, 1990; Anderson *et al.*, 1990).

[§ Recent *in vitro* evidence suggests that pro-renin may be activated directly, without removal of the 43 amino acid pro- segment, by acidification or cold storage (Atlas, Sealy, and Laragh, 1978; Leckie and McGhee, 1980). This partial and reversible activation of pro-renin in the absence of proteolysis is presumably due to an unfolding of the pro-segment that exposes the active site. It is unlikely that pro-renin is activated by non-proteolytic mechanisms once in the circulation, as injection of monkeys with large doses of human

recombinant pro-renin does not result in detectable increases in blood pressure or AII (Lenz *et al.*, 1990).

Four main mechanisms regulate renin release:

(i). Sodium chloride load to the kidney distal tubules is detected by a sodium chloride receptor in the macula densa and is largely responsible for a reduction in renin secretion in response to increased dietary sodium intake (Vander, 1967).

(ii). A drop in blood pressure is interpreted by a baroreceptor in the afferent arteriole of the juxtaglomerular (JG) apparatus and results in an increase in renin secretion in order to defend central blood volume. This change in renin secretion may be mediated by voltage dependent alterations in calcium influx (Fray and Lush, 1984).

(iii). The heart, arterial tree, and other extra-renal loci sense blood volume changes and modulate renin secretion accordingly (a decrease in volume leads to an increase in renin secretion and vice versa) via reflex activation of ß-1-sympathetic pathways to the kidney (Vandongen and Peart, 1974; Davis and Freeman, 1976). This system appears to be important for maintaining the renin response to upright posture in humans (the venous pooling caused by gravitational pull reduces central blood volume, disinhibiting the cardiopulmonary baroreflex, and simultaneously causes a decrease in carotid sinus pressure. These effects result in increased ß-1-sympathetic activity and increased renin secretion).

(iv). AII has a direct negative feedback effect on renin release (perhaps compensating for its sodium retaining effect in the proximal tubule) [Katz and Malvin, 1993].

Other factors may also affect renin release to a lesser degree. Vasopressin and atrial natriuretic factor (ANP) suppress renin release via activation of the ß-1 receptor (Sealy and Laragh, 1990). Kallikrein and kinins can stimulate renin release perhaps via an effect on pro-renin to renin conversion (Atlas, Sealy, and Laragh, 1978) or by a direct effect on renin

release (Carretero and Beierwaltes, 1984). Prostaglandins may also stimulate renin secretion (Weber and Siess, 1980).

All factors affecting renin release seem to do so by changing the concentration of intracellular calcium or cyclic adenosine monophosphate (cAMP). An increase in cytosolic calcium ions in the kidney JG cells suppresses renin release. A decrease in cytosolic calcium in these cells increases renin secretion. A rise in cAMP stimulates renin secretion whereas a fall in cAMP has the opposite effect (Churchill, 1985).

2.1.2 Synthesis and physiology of angiotensinogen

The majority of circulating angiotensinogen is thought to originate in hepatocytes in the pericentral zone of liver lobules (Morris, Iwamoto, and Reid, 1979), although adipocytes and astrocytes are also prominent synthesisers of the protein. Translation of angiotensinogen mRNA yields pre-angiotensinogen. In humans, angiotensinogen is synthesised as a 33-452 amino acid pre-angiotensinogen molecule (Kageyama, Ohkubo, and Nakanishi, 1984), whereas in the rat and mouse the arrangement is 24-453 amino acids (Ohkubo *et al.*, 1983; Clouston *et al.*, 1988). The pre-segment is co-translationally removed to give the mature protein. This is then secreted by the cell into the circulation. Most angiotensinogen is extra-cellular (it is constitutively secreted), therefore there is no apparent mechanism for rapid changes in angiotensinogen concentration by an organism. However, the concentration of angiotensinogen in blood can be rate limiting for angiotensin formation (Newton *et al.*, 1968). The normal circulating level of angiotensinogen is approximately the K_M of renin which means that a fall in plasma angiotensinogen can cause a near to linear decrease in the rate of angiotensin formation. On the other hand, the maximum increase in the rate of angiotensin formation that can occur with an increase in the level of plasma angiotensinogen is two-fold (K_M being the substrate concentration at which half maximum velocity of a reaction occurs). Under normal circumstances, an increase in plasma angiotensinogen will not increase the rate of angiotensin production due to negative feedback on the rate of renin secretion by AII.

The major role of angiotensinogen can be considered to be that of an extra-cellular (plasma) reservoir of angiotensin peptides. However, as angiotensinogen is an unusually large precursor of AI (452 amino acids compared to the 10 amino acids of AI), it has been suggested that angiotensinogen or its product (after AI cleavage) des(angiotensin I)angiotensinogen may have additional functions: Physiological studies have shown that plasma angiotensinogen is up-regulated during pregnancy and oestrogen therapy, and down-regulated in Addisons disease or after thyroidectomy (Clauser *et al.*, 1989). Regulation was shown to be due to changes in hepatic synthesis. This was confirmed by demonstrating that oestrogen, thyroid hormones and glucocorticoids increase angiotensinogen mRNA expression in rat hepatocytes (Chang and Pearlman, 1987). Also, alignment of the 5' sequences of the rat and human angiotensinogen genes, showed consensus sequences for an oestrogen responsive element, a thyroid hormone responsive element and three glucocorticoid responsive elements (Fukamizu *et al.*, 1990). The role that angiotensinogen may play in these conditions is not really understood, but the potential involvement in adrenocorticoid based disorders and regulation of adrenal sex hormones is not altogether surprising as the RA system (AII) is a well known regulator of adrenal function; there is also evidence to suggest that angiotensinogen may be an inflammatory regulator. During the acute phase of inflammation or injury, angiotensinogen levels are greatly increased (Hoj-Nielsen and Knudsen, 1987). This appears to be due to stimulation of liver angiotensinogen synthesis as injection of *Escherichia coli* lipopolysaccharide induces liver angiotensinogen mRNA expression (Kageyama, Ohkubo, and Nakanishi, 1985). Analysis of the 5' terminal sequences of the angiotensinogen gene has also demonstrated a putative acute-phase response element that binds proteins that may be inflammatory mediators.

2.1.3 Synthesis and physiology of ACE

There has been relatively little characterisation of ACE secretion, the thrust of work on ACE being concerned with the complex enzymology and catalytic mechanism. However, as ACE shows a broad specificity, hydrolysing other biologically active peptides besides AI such as bradykinin and substance P, it has been implicated in a number of physiological and

pathophysiological processes such as neuropeptide metabolism, immunity, reproduction and digestion (Unger, Gohlke, and Gruber, 1990).

It has been demonstrated that ACE can be induced by corticosteroids in cultured endothelial cells, alveolar macrophages and the lung (Ehlers and Riordan, 1990). Activation of protein kinase C (PKC) and cAMP seem to increase ACE activity in cultured endothelial cells, but only activators of PKC increase the release of ACE into the culture medium (Iwai *et al.*, 1987). The best evidence for hormonal control of ACE *in vivo* has been for the testicular form of ACE. Follicle stimulating hormone (FSH), leutenising hormone (LH), human chorionic gonadotropin (HCG) and testosterone reverse the depletion of testicular ACE induced by hypophysectomy (Velletri *et al.*, 1985). Also, serum ACE activity has been demonstrated to vary with thyroid function. Activity is elevated in hyperthyroidism and reduced in cases of hypothyroidism (Yotsumoto *et al.*, 1982; Smallridge, Rogers, and Verma, 1983). Recent gene targeting experiments, in which exon 14 of the ACE gene was disrupted (Krege *et al.*, 1995), have also shown male mice homozygous for a disrupted ACE gene have impaired fertility and lower blood pressure than heterozygous males or males with no disruption of the ACE gene. In addition, although females heterozygous for a disrupted ACE gene have a reduced serum ACE activity similar to heterozygous males, they do not share the decrease in blood pressure shown by heterozygous males. As an isoenzyme of ACE is found in post-meiotic spermatogenic cells and sperm in the testis (see section 1.6.2), these findings suggest that testis ACE might be important for overall sperm function and gender related control of blood pressure. In addition, in humans there is evidence to suggest that a deletion polymorphism in the ACE gene is a risk factor for myocardial infarction (Cambien *et al.*, 1992).

2.1.4 Mechanisms of AII action

AII is the principal effector hormone of the renin-angiotensin cascade and is regarded to be one of the most powerful naturally occurring vasopressors (second only perhaps to the recently discovered Endothelins). AII has a short *in vivo* half-life estimated at between 15-150 seconds and is cleared from the circulation in the peripheral vascular bed by tissue

amino-peptidases and endopeptidases (Peach, 1977). AII mediates the physiological functions of the RA system via interaction with its receptors (see section 1.3.1). The major acute actions of AII are on (i). vascular smooth muscle, (ii). the kidney and (iii). the adrenals.

2.1.4.1 Vascular smooth muscle tone

AII is an important modulator of vascular smooth muscle (VSM) tone. AII acts in an acute manner causing VSM to contract, thereby raising peripheral vascular resistance; this pressor response is by far the most rapid physiological action of AII. The response to AII is triggered by its interaction with cell surface receptors. These receptors stimulate phospholipase C (PLC) via intermediary guanine nucleotide regulatory proteins. PLC activation causes the membrane-bound second messenger inositol tri-phosphate (IP_3) to be released and also produces membrane bound diacylglycerol (DAG). IP_3 interacts with receptors on intracellular calcium stores to release calcium. This calcium then binds to calmodulin which activates myosin light-chain kinase to phosphorylate myosin light chains thereby initiating smooth muscle cell contraction. The smooth muscle tone is then maintained by a (DAG and calcium stimulated) PKC dependent mechanism (Marsden, Brenner, and Ballermann, 1990).

2.1.4.2 Renal action

(i). Renal tubular sodium reabsorption: AII has a direct stimulatory effect on renal tubular sodium reabsorption (Johnson and Malvin, 1977). This enhances the reabsorption of sodium ions (down their electrochemical gradient) through sodium channels located on the luminal-apical membrane of the proximal tubule. Increased body sodium content helps to maintain blood pressure by increasing extra-cellular fluid osmolarity which in turn enhances water retention (and hence filling of the vascular tree and hydraulic control of blood pressure). The kidneys are known to contribute to the pathophysiology of heart failure via enhanced sodium and fluid retention (Cannon, 1977).

AII is known to inhibit adenylate cyclase in proximal tubule cells via binding to a G-protein. This has been demonstrated to increase activity of Sodium/Hydrogen exchanger (NHE) transport proteins on the apical membrane, thus enhancing sodium reabsorption. Activation of NHEs has been implicated in the pathogenesis of hypertension in the SHR and human essential hypertension (Berk *et al.*, 1989; Ng, Fennell, and Dudley, 1990). Other tubular transport mechanisms may also be affected by AII.

(ii). Renal vascular effect: The contraction of VSM in response to AII reduces intra-renal blood flow, and to a lesser extent, Glomerular Filtration Rate (Earley and Friedler, 1966). When AII causes renal constriction, proximal sodium reabsorption may be enhanced via induced changes in capillary physical forces, and by a vascular action to reduce renal medullary blood flow (Hall, 1986). These responses may enhance medullary osmolality and promote passive sodium reabsorption in the loop of Henle. In addition, increased sodium may heighten vascular sensitivity to AII.

2.1.4.3 Adrenal action

In response to stimulation by AII, the zona glomerulosa (ZG) cells of the adrenal cortex augment release of the mineralocorticoid steroid hormone aldosterone. As aldosterone is not stored by the adrenal, AII stimulates biosynthesis and secretion of aldosterone rather than the release of pre-formed stores (Boyd and Peart, 1971). The primary target of aldosterone is the kidney where it stimulates sodium retention and hence regulates blood pressure.

Stimulation of the ZG cells by AII leads to activation of PKC and an increase in DAG, which mobilises intracellular calcium stores. These cellular events result in increased aldosterone synthesis and secretion. When aldosterone reaches the kidney it binds to specific cytoplasmic receptors in cells of the distal tubule of nephrons. This has the effect of actively pumping sodium ions (against their electrochemical gradient) across the serosal-basolateral membrane by the sodium-potassium-ATPase enzyme. This enzyme hydrolyses adenosine 5' tri-phosphate (ATP) to produce energy to facilitate the exchange of three sodium ions for

two potassium ions. The potassium ions then move down their electrochemical gradient into the tubular lumen (through a calcium activated potassium channel) and are excreted.

Increases or decreases in plasma potassium directly stimulate the increase or decrease of aldosterone secretion. Increases or decreases in sodium have the opposite effect via modulation of renin secretion. The action of aldosterone in the distal tubule determines the final ratio of potassium to sodium in the urine. Simultaneously, dietary sodium and potassium-related changes in Glomerular Filtration Rate (GFR) and sodium reabsorption in the proximal tubule, adjust the amount of sodium reaching the distal tubule thereby modulating the absolute levels of these ions available to the action of aldosterone. A mechanism of electrolyte balance is therefore achieved that does not interfere with the maintenance of blood pressure homeostasis (Sealy and Laragh, 1990).

AII has also been found to have a trophic effect on the adrenal glomerulosa. During chronic administration of AII, the width of the glomerulosa has been found to increase (Blair-West *et al.*, 1962) influencing haemodynamics. In addition AII augments the production of corticosteroids (in addition to aldosterone), which include important anti-inflammatory glucocorticoids such as hydrocortisone, by the adrenal fasiculata, and stimulates catecholamine release from the adrenal medulla (Vallotton *et al.*, 1981).

As aldosterone promotes sodium reabsorption, and catecholamines increase mean arterial blood pressure, it is evident that the combined actions of AII on the adrenal gland play a major role in the regulation of extracellular fluid volume and blood pressure by the RA system; and are involved in the pathophysiology of hypertension and hormone imbalance.

2.1.4.4 Other actions of AII

(i). Growth of vascular smooth muscle cells: It has been demonstrated in cultured VSM cells that AII stimulates protein synthesis and hence growth (Geisterfer, Peach, and Owens, 1988). Hypertrophy in these cells can be prevented by addition of a transcriptional

inhibitor to the media (Berk *et al.*, 1989). This suggests that the increase in protein is dependent on an increase in gene transcription.

AII induces the expression of early proto-oncogenes such as *c-myc*, *c-jun* and *c-fos* in VSM cells (Geisterfer, Peach, and Owens, 1988; Naftilan, Pratt, and Dzau, 1989). The products of these genes are important regulators of the cell cycle and hence mitogenesis. AII also regulates the expression of several other genes including those coding for growth factors. The late expression of platelet-derived growth factor (PDGF) A-chain mRNA and fibroblast growth factor (FGF) mRNA is induced by AII (Naftilan, Pratt, and Dzau, 1989). PDGF and FGF play an important role in promoting VSM cell proliferation. AII may also stimulate mRNA expression of inhibitors of growth and proliferation such as transforming growth factor beta (TGF-β) [Powell *et al.*, 1991].

(ii). AII and the nervous system: Stimulation of the sympathetic nerves by AII has been found to augment the vaso-constricter action of sympathetic nervous system (SNS) activity by encouraging enhanced release of noradrenaline into the synaptic cleft or modulating the re-uptake of noradrenaline at nerve endings (Malik and Nasjletti, 1976). AII may also have an action on the central nervous system leading to an increase in SNS activity as direct injection of AII into the brain stem or AII infusion into the vertebral arteries increases peripheral resistance. This pressor effect is greater than the effect of the same intra-venous dose of AII (Scroop and Lowe, 1969). The interaction of AII and the nervous system has been especially implicated in the pathogenesis of renovascular hypertension (Mistry, 1984).

Fitzsimons (1972) showed that a direct injection of AII into the central nervous system stimulated drinking. This effect can be inhibited by administration of an AII receptor antagonist. Subsequent studies have demonstrated that AII regulates stimulation of thirst, water drinking and the release of vasopressin, and that these actions of AII appear to be mediated by a direct action in the brain. All of the components of the RA system have been demonstrated in the brain (see chapter three), so the exciting scenario of local production of AII serving neurotransmission functions is a possibility.

(iii). Vascular Permeability: The pathogenesis of vascular injury including endothelial dysfunction and atherosclerosis is associated with hypertension and diabetes mellitus. Increased vascular permeability is an early manifestation of these phenomena. AII has been generally thought to increase vascular permeability by its pressor action (Goldby and Beilin, 1972). However, increasing evidence supports a role for the modulation of vascular permeability by AII independent of its pressor activity (Naftilan, Pratt, and Dzau, 1989). A recent elegant study by Williams and co-workers (1995) demonstrated an up to seven-fold increase in mRNA for the glycoprotein Vascular Permeability Factor (VPF) in human VSM cells, when exposed to AII *in vitro*. This response was also shown to be inhibited by the non-peptide AII receptor antagonist Losartan, thus demonstrating this to be an AT_1 receptor sub-type mediated event. This work has therefore proposed a mechanism whereby AII might act locally influencing permeability, growth and function of the vascular endothelium, independent of its pressor effect. Further demonstration of the regulation of cytokines such as VPF by AII may strengthen the link between activation of the RA system and vascular disease.

(iv). Prostaglandins and AII: Prostaglandin levels have been shown to be controlled by hormones that stimulate their biosynthesis (Nasjletti and Malik, 1982). AII enhances arachidonic acid release therefore resulting in increased prostaglandin production. AII has been demonstrated to stimulate the release of Prostaglandin E_2 (PGE_2) from most tissues (including kidney) and the release of Prostaglandin I_2 (PGI_2) from kidney, heart, lung and arterial blood vessels. In turn, these vasodepressor prostaglandins may antagonise the pressor effect of AII (Sealy and Laragh, 1990), thus playing an anti-hypertensive role in homeostasis. AII may also stimulate (to a lesser degree) the release of the vasopressor prostaglandins Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and Thromboxane A_2 (TxA_2). These prostaglandins stimulate calcium mobilisation and therefore modulate the contraction of VSM (Fukuo *et al.*, 1986).

Enhanced release of PGE_2 and PGI_2 has been postulated to reduce vascular responsiveness to AII during normal pregnancy. Conversely, the pathogenesis of pre-

eclampsia has been attributed in part to reduced release of these vasodepressor prostaglandins, thus giving an imbalance of vasopressor $PGF_{2\alpha}$ and TxA_2 (Walsh, 1985).

It has to be considered, however, that the interaction of AII and prostaglandins is complex. Prostaglandins have been shown to give species and vascular bed specific responses that both antagonise and augment the response to AII (Gerber and Niles, 1979). AII may therefore differentially regulate regional haemodynamics.

(v). Endothelium-derived relaxation factor and AII: In addition to prostaglandins, endothelial cells release transient mediators termed endothelium-derived relaxation factor (EDRF) [Griffith *et al.*, 1984]. Most (if not all) of this activity has been attributed to nitric oxide which stimulates soluble guanylate cyclase activity (Palmer, Ferrige, and Moncada, 1987). It has been demonstrated that EDRF may modulate the response of VSM cells to AII, as removal of endothelium potentiates the contractile response of VSM cells to AII (Oshiro, Paiva, and Paiva, 1985). Studies on cultured endothelial and VSM cells have also shown that AII-induced mobilisation of calcium and phospholipids are attenuated by EDRF (Ganz *et al.*, 1986).

(vi). Slow pressor effect of AII: Dickinson and Lawrence (1963), first demonstrated in the rabbit that infusion of small sub-pressor doses of AII over several days produced a gradual rise in blood pressure. This 'slow pressor' effect of AII cannot be prevented by adrenalectomy (Dickinson and Yu, 1967) but can be prevented by restricting dietary salt intake (Cowley and McCaa, 1976). Lever (1986) proposed that hypertension is induced (in models such as renovascular hypertension) by the acute 'fast pressor' action of AII and is maintained by the trophic action of the hormone to induce vascular hypertrophy. There are three main arguments to support this hypothesis (i). the direct, immediate pressor actions of AII are less than the degree of chronic hypertension that occurs with equal concentrations of the hormone, suggesting an additional contribution of the 'slow pressor' effect, (ii). lower concentrations of AII are required to maintain hypertension than to initiate it, and (iii). AII is a known growth stimulant for VSM. As plasma renin and angiotensin levels are near normal in established experimental and human renovascular hypertension then

the RA system may maintain the long term hypertensive state by non-acute actions of AII such as the 'slow pressor' effect.

(vii). Vasodepressor action of AII: Paradoxically, AII appears to exert an opposing effect to its powerful vasoconstricting property depending upon its concentration. Continuous infusion of small amounts of AII sustains (AII induced) increases in blood pressure and renal sodium retention (Ames *et al.*, 1965). However, high doses of AII cannot maintain an (AII induced) increase in blood pressure and actually encourage natriuresis (Louis and Doyle, 1965): *in vitro*, isolated strips of vascular tissue cannot maintain a vasoconstricting response to AII (Khairallah *et al.*, 1966). This tachyphylaxis is specific to AII as the tissue continues to respond to norepinephrine. Certain vascular tissues may even dilate when exposed to AII (Webb, 1982). A biphasic vasoconstrictor/vasodilator response of blood pressure to AII has also been demonstrated (Rowe and Nasjletti, 1983). The release of vasodilator substances such as EDRF in response to AII stimulation has been heavily implicated in this mechanism (Ito, Johnson, and Carretero, 1991).

2.2 PATHOPHYSIOLOGY OF THE ENDOCRINE RENIN-ANGIOTENSIN SYSTEM

2.2.1 Hypertension

In normotensive people, plasma renin activity (PRA) is very low when salt intake is high. Conversely, when salt is in short supply PRA rises acutely. These changes in renin, in normal individuals, offset any effects of accompanying volume expansion or contraction so that blood pressure does not change significantly during wide variations in salt intake. Therefore, at high levels of sodium intake renin is not required for maintenance of blood pressure. However, as sodium becomes less available, plasma renin plays an important role in sustaining normal levels of blood pressure. For a long time it was generally thought that the RA system played no part in the pathophysiology of elevated blood pressure or essential hypertension (Laragh and Sealy, 1990). This view was assumed because as in normotensives, a wide range of plasma renin values were seen in patients with essential hypertension. However, since it was discovered that AII is the major regulator of aldosterone secretion

(Laragh, 1960) and that wide fluctuations in PRA can be meaningfully related to salt balance (Laragh, Sealy, and Sommers, 1966), evidence has accumulated to support the involvement of the RA system in the pathogenesis of hypertension. As hypertension is associated with an increased risk of stroke, acute myocardial infarction, coronary heart disease, heart failure, and renal disease (Laragh and Sealy, 1990), the RA system may be considered to play a direct role in the pathophysiology of these conditions.

Hypertensives may be profiled according to whether they have a relatively low, or normal to high level of circulating renin, if their PRA is indexed against their concurrent 24 hour urinary sodium excretion. The indexed value can be compared to a normotensive profile to give the relative 'renin status' of the hypertensive individual. High-renin hypertensives are typically more vasoconstricted and sodium/fluid depleted than low-renin hypertensives with similar levels of blood pressure.

(i). Low-renin hypertension: Around 30% of hypertensive patients have low or suppressed levels of PRA. Increased mineralocorticoid (modulated by the action of AII) is known to cause sodium retention / volume expansion and hence an elevated blood pressure. This in turn suppresses renin secretion by a negative feedback mechanism (see section 2.1.1). Interest has therefore centered on whether low-renin hypertensive patients have an excess of mineralocorticoid hormones. Low-renin hypertensives often respond to dietary sodium depletion or to natriuretic drugs with a complete normalisation of their blood pressure, thus supporting the thesis that mineralocorticoid imbalance and (consequent) sodium retention is at the root of the problem (Laragh, 1978). Although elevated levels of deoxycorticosterone and 19-nor deoxycorticosterone have been reported in a few cases, no such elevations were found in a systematic study of 46 low-renin hypertensives (Gomez-Sanchez, Holland, and Upcavage, 1985). The absence of significantly large changes in volume expansion in most low-renin hypertensives also argues against a central role played by mineralocorticoid excess. Individuals with low-renin hypertension may in fact have a more normal renin mechanism than high-renin hypertensives, as one would expect an elevation in blood pressure to diminish renin release via interaction with renal baroreceptors. However, it must

be borne in mind that there is plenty of evidence to suggest that a normal kidney exposed to high arterial pressure and normal salt intake will reduce its renin secretion to near zero (Collins *et al.*, 1970; Case *et al.*, 1976; Sealy *et al.*, 1977; Laragh, 1984). Therefore any renin secretion, even at a low level, in a hypertensive setting may be considered to be abnormal and the RA system may play a direct pathophysiological role in the maintenance of hypertension.

A more direct explanation of 'low-renin' hypertension has been postulated by Brenner and colleagues (Brenner, Garcia, and Anderson, 1988). They proposed that a renal abnormality, due to a reduced number of nephrons and / or a decrease in filtration surface area (FSA) per nephron, is responsible for systemic hypertension. A decrease in renal function due to decreased FSA results in increased renal sodium retention. This in turn leads to increased mean arterial blood pressure, glomerular capillary hypertension and eventual glomerular sclerosis. Glomerular sclerosis further decreases FSA, perpetuating a vicious circle. This scenario may also provide an explanation for the low renin levels and increasing loss of renal function seen in conditions such as diabetic nephropathy.

(ii). Normal and high-renin hypertension: As noted above, renin levels should be suppressed in the presence of elevated blood pressure. Therefore, the presence of 'normal' renin levels seen in about 50% of hypertensive patients and the higher than 'normal' renin levels seen in about 20% of hypertensives, may be grossly inappropriate and play a role in sustaining hypertension. In this setting, the long term mechanism of hypertension is renin-mediated and one would expect pharmacological blockade by a renin or ACE inhibitor to reduce the elevated blood pressure. This is in fact the case (Case *et al.*, 1978), in contrast to low-renin hypertension which does not respond significantly to RA system inhibitors.

Several hypotheses have been proposed to explain 'inappropriately high or normal' renin levels. Perhaps the most attractive of these is the concept of 'nephron heterogeneity' proposed by Sealy and colleagues (Sealy *et al.*, 1988). Under this scenario, a mixed population of normal and ischemic (caused by afferent arteriolar narrowing) nephrons is assumed to exist in essential hypertensives (ischemic nephrons constituting the minority of

the nephron population, as most essential hypertensives do not exhibit evidence of renal insufficiency). Hypersecretion of renin from the ischemic nephrons could work to impair sodium excretion in the larger group of normal nephrons, which are in adaptive natriuresis and are secreting little or no renin of their own. This inter-nephron discordance may create a hypertensive renin-volume interaction that results in a 'normal' or 'high' circulating renin level.

The classification of 'normal- renin' hypertensives into modulators or non-modulators according to their renal haemodynamic responses to saline and AII infusion (Williams and Hollenberg, 1991) lends some support to the idea of nephron heterogeneity. Non-modulators have impaired plasma renin suppression, renal blood flow and natriuresis during saline infusion. These impaired functions can be corrected by administration of an ACE inhibitor, suggesting that 'inappropriate' renin secretion (perhaps from an ischemic subpopulation of ischemic nephrons) is responsible. In contrast, modulators respond to saline infusion with an exaggerated natriuresis, and ACE inhibition does not alter their renal haemodynamic response (Sealy *et al.*, 1988).

2.2.2 Vascular hypertrophy

One of the primary features of hypertension is hypertrophy and polyploidy of the smooth muscle cells of large arteries. The involvement of AII has been implicated in these processes, as pharmacological agents (especially ACE inhibitors) that prevent the formation of AII and lower blood pressure also prevent smooth muscle cell hypertrophy (Owens, 1987). ACE inhibitors have also been shown to prevent VSM growth in one kidney-one clamp Goldblatt hypertensive rats without any effect on blood pressure (Wang and Prewitt, 1990). This demonstrates that the effect is not simply due to a reduction in blood pressure as a consequence of reduced AII. Indeed, there is evidence that sub-pressor doses of AII as low as 100 pg/ml have a trophic effect on VSM cells in culture (Schelling, Fischer, and Ganten, 1991).

AII has been demonstrated to cause increased production of extracellular matrix proteins (which are in themselves mitogenic) at a greater rate in hypertensive rat strains than

in normotensive strains (Scott-Burden *et al.*, 1991). In addition, VSM from hypertensive animals is more responsive to AII giving increased tone and vascular resistance. AII may therefore be an important factor in the development of the increased peripheral resistance seen in hypertension via growth mechanisms.

Current evidence suggests that the signalling pathways used by AII in smooth muscle in hypertensives may be altered to favour a growth related response. In the SHR, PLC activation by AII is enhanced. This results in increased production of IP_3 and a subsequent elevation of intracellular calcium release in VSM cells (Osanai and Dunn, 1992). Increased AII-induced activity of the Sodium/Hydrogen exchanger (Davies *et al.*, 1991) and activation of S6 kinase (Resink *et al.*, 1989) are also present in the SHR. The consequent increases in intracellular calcium, alkalinisation and entry into the cell cycle, support the increased growth and contractility of vascular smooth muscle cells that may lead to hypertension.

2.2.3 The Heart

AII has a number of inotropic and chronotropic effects on myocardial cells. AII can influence cardiac fuction by actions on myocytes (contraction, growth, metabolism), fibroblasts (matrix deposition), conduction tissue, coronary artery endothelium (release of vasoactive peptides, altered permeability) and sympathetic norepinephrine release (may indirectly affect contractile state, conduction, growth, metabolic state and coronary resistance)[Lindpaintner and Ganten, 1991]. Reflex bradycardia and reduced cardiac output follow the rise in blood pressure induced by injection with AII (Mistry, 1984). AII may also increase heart rate via sympatho-adrenal mechanisms (Peach, 1977).

2.2.4 Diabetes Mellitus

Hypertension is more prevalent in diabetics than non-diabetics (Christlieb, 1978), and hypertensives who are also diabetic have almost double the rate of cardiovascular mortality seen in non-diabetic hypertensives (Aromaa, Reunanen, and Pyörälä, 1984). Hypertensive diabetics may have few functional JG cells and a reduced ability to stimulate the release of renin. Consequently, very low renin levels are often seen in these individuals, and their

ability to stimulate aldosterone production and excrete potassium is impaired. In severe cases, this may result in the development of hyporeninemic hypoaldosteronism syndrome (Christlieb, 1978).

Nephropathy and retinopathy are major microvascular complications of hypertension that are especially seen in patients with diabetes mellitus. Studies have shown that elevated plasma pro-renin levels can identify (diabetic) individuals at risk of microvascular complications (Wilson and Luetscher, 1990). In addition, pro-renin levels are increased in the ocular vitreous fluid of individuals with proliferative retinopathy (Danser *et al.*, 1989). ACE inhibitors have been shown to be successful in reducing microvascular complications and this effect is greater than one would expect by reducing the pressor effect of AII alone. (Kasiske *et al.*, 1993; Lewis *et al.*, 1993).

2.2.5 Renin secreting tumours

Renin secreting tumours are made up of JG cells or haemangiopericytomas. They are generally found in young patients with severe hypertension. Very high renin levels are seen in the peripheral blood and (tumour) kidney resulting in the observed increase in blood pressure via increased pressor action of AII and increased salt and water retention (Duprez *et al.*, 1990).

2.3 INHIBITION OF THE RENIN-ANGIOTENSIN SYSTEM

In the past 30 years, inhibitors of the RA system have played a fundamental role in elucidating the various roles of the system. Clinically, inhibitors of the RA system, especially ACE inhibitors, are today central to the routine management of pathophysiological states such as hypertension and heart failure.

2.3.1 Angiotensin converting enzyme inhibitors

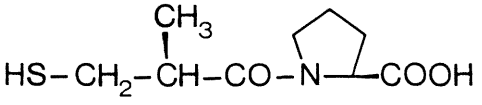
In 1965, Ferreira discovered that the venom of the South American Viper *Bothrops jararaca* contained a substance that potentiated the effects of bradykinin. It was soon realised that this substance might be an inhibitor of ACE (see section 1.6.2). Further work found that

the active substance was a series of peptides in the venom and these were termed bradykinin potentiating peptides (BPP) [Ferreira, Bartelt and Greene, 1970; Ondetti *et al.*, 1971].

Further work on the peptides demonstrated that a nonapeptide with several proline substitutions (Glu^1-Tyr^2-Pro^3-Arg^4-Pro^5-Gln^6-Ile^7-Pro^8-Pro^9) would be the most pharmacologically potent. This peptide was synthesised and termed Teprotide (SQ 20,881). Initially, Teprotide seemed clinically attractive because as well as being a competitive inhibitor of ACE, it was a poorer substrate than BPP and did not seem to increase plasma bradykinin levels at therapeutic doses. However, as Teprotide is a straight chain peptide it had to be administered intravenously. It also only has a half-life of less than an hour. Teprotide's clinical usefulness was therefore limited.

In the late 1970's, Ondetti and co-workers synthesised a non-peptide inhibitor of ACE working on the principle that ACE was similar to pancreatic carboxypeptidase A, a peptidase whose pharmacokinetics had already been well characterised. The new inhibitor was termed Captopril (SQ 14,225) [Ondetti, Rubin and Cushman, 1977]. Captopril was found to have a number of advantages over Teprotide. It could be administered orally, had a half-life of up to 4½ hours and had ten times the potency of Teprotide (Gavras *et al.,* 1978). Orally active ACE inhibitors are classified according to their zinc ligand. Captopril is therefore a sulphydryl ACE inhibitor. The other two main classes that have since been developed are carboxyl (e.g. Enalapril) and phosphorous (e.g. Fosinopril).

Figure 2A: Captopril (SQ 14,225; D-3-mercapto-2-methyl-propanoyl-L-proline).



2.3.1.1 Therapeutic effects of ACE inhibitors

(i). Ace inhibition and AII: The classic functional role of ACE inhibitors is to block ACE in the vascular endothelium of the lung and other tissues, resulting in the lowering of the level of circulating AII. This decreases the effect of AII on things such as vascular tone, structural alterations of vessels and cardiac tissue contributing to hypertension, aldosterone release, SNS transmission and renal sodium handling (see section 2.1.4). The net effect of lowering circulating AII is a reduction in blood pressure. However, as ACE inhibitors have been shown to lower blood pressure without the plasma RA system being activated, the effect of ACE inhibitors cannot be explained solely by their effect on circulating AII levels: As ACE and kininase II are identical (see section 1.6.2), the breakdown of bradykinin by ACE may also be inhibited, thus augmenting the vasodepressor effect of bradykinin; ACE inhibitors have also been demonstrated to interfere with SNS transmission (Zimmerman, 1981), alter permeability of VSM to sodium (Ito *et al.*, 1981) and affect cell surface peptidases other than ACE (Hooper *et al.*, 1992). Perhaps the most important way in which ACE inhibition may modulate cardiovascular function, is by inhibition of putative local RA systems (see chapter three). This is implied because renin inhibitors and AII-receptor antagonists can also elicit depressor responses to some extent without the plasma RA system being activated (Dzau, 1988b) and this mechanism is not via bradykinin potentiation or any of the other 'non-renin dependent' actions of ACE inhibitors.

(ii). Coronary heart disease: Ace inhibitors have been found to be useful in the treatment of atherosclerosis and ischaemic heart disease. They reduce myocardial oxygen demand, counter elevations in systemic vascular resistance, improve coronary blood flow, reduce blood pressure and ventricular wall tension without reflex tachycardia and reduction of myocardial contractility, and prevent myointimal proliferation (Daly *et al.*, 1985; Chobanian, 1990). 'Sulphydryl' ACE inhibitors may have a role as scavengers of cytotoxic free radicals which gives them an advantage in the treatment of myocardial infarction or reperfusion injuries (Westlin and Mullane, 1988).

ACE inhibitors have been found to have especially beneficial effects in the treatment of myocardial infarction and heart failure. There is evidence of ventricular remodelling following myocardial infarction (Pfeffer and Braunwald, 1990). Non-infarcted areas of the ventricle are thought to undergo compensatory hypertrophy probably due to similar mechanisms of wall stretching seen in volume-overload hypertrophy. This enlargement of the ventricle can be diminished by administration of an ACE inhibitor. In addition, ACE inhibition has been demonstrated to attenuate cardiac dilation and improve the survival of rats with experimental heart failure induced by coronary ligation (Pfeffer, Pfeffer, and Braunwald, 1985; Pfeffer, Pfeffer, Steinberg, and Finn, 1985). In humans, administration of an ACE inhibitor to patients with left ventricular dysfunction within a couple of weeks following myocardial infarction prevents cardiac dilation and improves ventricular function (Pfeffer *et al.*, 1988; Sharpe *et al.*, 1988). As in rats, not only does administration of an ACE inhibitor improve symptoms in humans, but interestingly mortality following myocardial infarction and left ventricular dysfunction is also reduced (The CONSENSUS trial study group, 1987; Pfeffer *et al.* [The SAVE-study trial group], 1992).

Following heart failure the circulating RA system is not usually activated in the stable chronic phase (Dzau *et al.*, 1981; Hodsman *et al.*, 1988) therefore it is possible that local tissue RA systems may be activated in early and compensated heart failure and may contribute to the pathophysiology of the disease state.

(iii). Renal protection: In experimental models of renal failure such as induction of diabetes mellitus (Zatz *et al.*, 1986), ACE inhibitors reverse glomerulosclerosis, improve haemodynamics and lower glomerular pressure (Meyer *et al.*, 1985). ACE inhibitors also improve insulin resistance and consequently lower the degree of associated hypertension and cardiovascular risk. Renal blood flow in diabetic nephropathy (Björck *et al.*, 1986) is also improved.

2.3.2 Renin inhibitors

Inhibition of renin, as a therapeutic tool, has a number of virtues. Renin inhibition interrupts the RA cascade at the first and rate-limiting step preventing the accumulation of bio-active products and precluding subsequent interaction of AII with its receptors (Chiu *et al.*, 1989). The high degree of specificity of renin to its only naturally occurring substrate, angiotensinogen (this is especially evident in humans as AI is liberated from angiotensinogen by renin cleavage of a leucine-valine bond as opposed to a leucine-leucine bond in most other species studied [Clauser *et al.*, 1989]), gives renin inhibitors a theoretical advantage over ACE inhibitors that are promiscuous in their recognition of functionally active peptides such as bradykinin, substance P and neuropeptide Y (Erdos, 1990); this relatively low specificity of ACE inhibitors is probably responsible for observed side effects such as cough and angio-oedema.

Early attempts at inhibiting the RA system were made with antisera to kidney extracts (Johnson and Wakerlin, 1940; Goldblatt, Hass, and Lamfrom, 1951) and practically all attempts since, to inhibit the RA system with antibodies, have been directed at renin. These studies demonstrated the successful inhibition of renin, and consequent prevention of hypertension, in response to experimentally induced renal ischemia. Unfortunately, subsequent studies using antibodies to components of the RA system gave conflicting results (Peart, 1975; Haber and Burton, 1979) and have resulted in the low profile of anti-RA antibodies in both the experimental and clinical setting. This variability in data was probably related to the fact that impure antigen was used to raise the antibodies, resulting in problems of antibody specificity. Feedback control of the RA system (especially renin) may also have been instrumental in the variability observed (Ganten and Gross, 1979). The complete purification of renin, largely by Inagami and co-workers (Inagami and Murakami, 1977; Matoba, Murakami, and Inagami, 1978; Yokosawa, Inagami, and Haas, 1978), allowed more specific antibodies to renin to be produced. Goat antibody to dog renin was found to decrease the blood pressure of conscious normotensive sodium depleted dogs and Goldblatt one kidney-one clamp hypertensive dogs (Dzau *et al.*, 1980).

The first non-antibody renin inhibitors developed were derivatives of the tetrapeptide sequence Leu^{10}-Leu^{11}-Val^{12}-Tyr^{13} from the amino end of horse angiotensinogen (Kokubu, Ueda and Fujimoto, 1968). These had the disadvantage of being hydrolysed by renin thereby reducing the time-scale of their activity considerably. Further peptide inhibitors were designed that were not cleaved by renin (Burton *et al.*, 1980); these were found to be good regulators of blood pressure in animal models but were not well tolerated in humans (Zusman *et al.*, 1983). Subsequent substrate-analogue inhibitors have largely been based on pepstatin. In these inhibitors, non-hydrolysable bonds have been replaced by the scissile peptide bond hydrolysed by renin in order to increase specificity; at the same time modifications have been made to the scissile bond to prevent it being hydrolysed by renin. These compounds therefore have a relatively long half-life as well as being highly specific.

The most recent renin inhibitors developed are small non-peptides (molecular weight between 500 and 1000) and are currently starting to make an appearance in the clinical management of hypertension. Some of these compounds (Kleinert *et al.*, 1992; Fischli *et al.*, 1994) are suitable for administering orally (an absolute pre-requisite if they are to be used on a widespread clinical basis), have a half-life of several hours (prototypes of these sort of compound had a low bio-availability due to high liver first pass and biliary excretion [Greenlee, 1990]), reduce blood pressure to a similar extent as ACE inhibitors and have other beneficial effects such as improving haemodynamic status during congestive heart failure (Neuberg *et al.*, 1991).

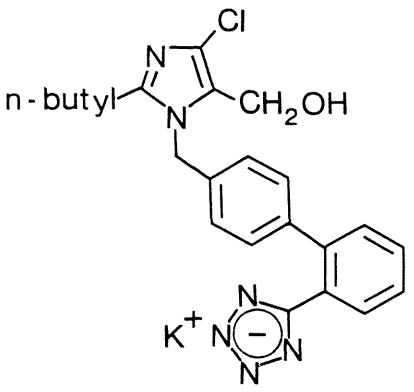
2.3.3 Angiotensin II Antagonists

After the use of antibodies to renin, competitive antagonism of AII receptors was the approach used to block the RA system. After Bumpus, Schwarz, and Page (1957) synthesised AII in the laboratory, AII analogues were used to study the functioning of the RA system. The most effective of these was obtained by making amino acid substitutions at positions 1 and 8 of bovine AII and was named 'Saralasin' (Sar^1-Arg^2-Val^3-Tyr^4-Val^5-His^6-Pro^7-Ala^8) [Marshall, Vine, and Needleman, 1970; Pals *et al.*, 1971]. Unfortunately, Saralasin was found to lower blood pressure only in high renin situations (such as sodium depletion in the

experimental subject). Saralasin also showed agonist properties when initially perfused (Dunn *et al.*, 1976) and this was amplified in low renin situations e.g. deoxycorticosterone (DOC) - salt hypertensive rats, and salt loaded normotensive and 'low renin' hypertensive patients (Ganten and Gross, 1979). Saralasin also had to be administered intravenously (due to its peptide nature) and had a half-life of only four minutes. The experimental and clinical potential of saralasin was therefore severely limited, and the development of orally active ACE inhibitors became the first choice as a therapeutic approach to blockade of the RA system.

Interest in AII antagonists was resurrected with the development of non-peptide, orally active compounds based upon a bi-phenyl tetrazole moiety. The best known of these compounds is 'Losartan (DuP753)' developed by the Du Pont company (Chiu *et al.*, 1990). Losartan was found to inhibit only about 80% of AII receptors in microsomes from adrenal cortical cells, whereas Saralasin inhibited 100%. A compound, PD123177, developed by the Parke Davis company, was also found to inhibit the 20% of receptors that Losartan had no effect on. On this basis two main groups of AII receptors were defined (see section 1.3.1). The receptors blocked by Losartan were termed AT_1 and those resistant to Losartan inhibition but sensitive to PD123177 were termed AT_2. All defined actions of AII in mammals are blocked by Losartan and are therefore AT_1 mediated.

Figure 2B: Losartan (Dup 753; 2-n-butyl-4-chloro-5-hydroxymethyl-1-[2'-{1H-tetrazol-5-yl}-{biphenyl-4-yl}methyl]imidazole, Potassium salt).



2.3.3.1 Therapeutic effects of AII receptor antagonists

The *in vivo* pharmacology of modern AII receptor antagonists has not been studied to anywhere approaching the same level as ACE inhibitors but the basic properties are known. A small number of clinical trials have been carried out with Losartan and it has been found to be well tolerated and as effective as ACE inhibitors (Weber, 1992).

AII receptor antagonists such as Losartan have been found to have a very similar action to ACE inhibitors on blood pressure and other AII induced effects such as aldosterone release and water intake (Wong *et al.*, 1990). The improvement in haemodynamics and survival seen in rat models of heart failure treated with ACE inhibitors is also seen with Losartan (Raya *et al.*, 1991). Losartan has also been shown to prolong survival in the stroke-prone SHR (Camargo *et al.*, 1991) and Dahl salt-sensitive rat (von Lutterotti *et al.*, 1991), when they are salt loaded.

There are differences between the effects of Losartan and ACE inhibitors: Losartan has not been found to lower blood pressure to any great degree when the RA system is not activated (Wong *et al.*, 1990) which implies that it mainly functions by blocking circulating

AII. Renal effects of Losartan are also different in some respects; an improvement in haemodynamics is seen in renal failure, but a reduction in albuminuria is not seen in nephritis (Hutchinson and Webster, 1992). The most impressive unique renal effect of Losartan is an up to 300% increase in uric acid excretion (Burnier *et al.*, 1993). This appears to be independent of blocking AII as the effect is similar in both salt-depleted and salt-repleted subjects.

CHAPTER THREE

Tissue renin-angiotensin systems

3.1 EVIDENCE FOR INVOLVEMENT OF THE CIRCULATING RA SYSTEM OUTSIDE THE VASCULAR COMPARTMENT

The classic view of the (circulating) RA system as a systemic hormonal system was moulded from a history of research that focused its attention on blood, a readily accessible material. However, if one considers the high turnover of conversion of AI to AII and clearance of both peptides across peripheral tissues, the levels of AI and AII found in venous blood are far too great to be explained by generation in the circulation alone. Daum, Uehsleke, and Klaus (1966) studied the affect of aminopeptidase infusion on the regulation of blood pressure by renin and AII in pithed (hence autonomic regulation removed) rats. They found that an infusion of aminopeptidase reduced the pressor effect of an infusion of AII but was much less effective on an infusion of renin. They concluded that some AII production (resulting from the renin infusion) must take place away from the circulation where the aminopeptidase could not degrade the AII as efficiently.

The development of inhibitors of the RA system stimulated further study. Amongst these were AII antisera and AII antagonists such as Saralasin. In the rat, AII antiserum was demonstrated to inhibit AII induced hypertension but did not affect the high renin, Goldblatt two kidney-one clamp induced hypertension. The blood pressure of nephrectomised rats infused with renin was also unaffected by antiserum. In contrast, Saralasin was found to lower blood pressure under all these regimes (Thurston and Swales, 1974a; 1974b). Nearly ten years later, evidence for the uptake of renal renin into the arterial wall was obtained by Loudon and co-workers (Loudon *et al.*, 1983). They measured aortic renin concentrations in nephrectomised rats injected with renin. The level of plasma renin decreased rapidly with time whilst the level of aortic renin persisted for a number of hours. The blood pressure response to the injected renin was found to correlate more closely to aortic renin than plasma renin. This implied that local angiotensin generation was of physiological importance.

Further evidence for generation of angiotensins outside of the vascular compartment came from the study of vascular beds isolated from the circulation and perfused with synthetic media. These studies demonstrated the release of AI and AII by the perfused

vascular bed. Angiotensin levels could be modulated by the addition of renin and ACE inhibitors thus supporting the concept of *de novo* synthesis of angiotensins rather than an experimental 'washout' effect of angiotensins accumulated in vivo. Mizuno and co-workers (1987a) showed direct release of AI and AII from isolated rat kidney perfused with angiotensin-free medium. In addition, Hilgers and co-workers (1991) showed that de novo synthesis of angiotensins could be prevented by bilateral nephrectomy of the animal before isolation of the vascular bed. This demonstrated that probably all *de novo* synthesis was due to renally derived renin. They also found that adding renin or angiotensinogen to the perfusate increased angiotensin II release, thus endorsing the postulate that uptake mechanisms have a crucial role.

The most impressive evidence that generation of angiotensins by the endocrine RA system takes place outside the vascular compartment, comes from a recent series of experiments by Admiraal and co-workers (Admiraal *et al.*, 1990; Danser *et al.*, 1990; Danser *et al.* 1992) on various vascular beds *in vivo*. These studies have shown that the rate at which vascular beds take up AI is in excess of the rate at which it is generated within the plasma. Local generation therefore replaces AI taken up from the circulation. Perhaps the most elegant of these studies (Admiraal *et al.*, 1990) measured the uptake of infused ^{125}Iodine labelled AI by tissue beds and looked at AI levels in the aorta, and renal, hepatic, femoral and antecubital veins. Extraction ratios of AI by the different vascular beds were between 47% (in the forearm) and 96% (in the liver). Despite the high extraction ratios, venous levels of AI in forearm, leg and kidney were essentially the same as arterial levels (liver venous levels of AI being about 51% of the arterial levels). About 50-80% of AI in the veins appeared to result from local de novo production. The metabolism of AI was also studied. From estimates of blood transit times in the vascular beds and PRA, they calculated that only 20-30% of AI activity in the kidney and limbs and about 60% of activity in the hepatomesenteric circulation could be accounted for (in terms of AI generation) by circulating renin. Further evidence suggests that most of this extra-plasmatic generation of AI is by renally derived renin as changes in local angiotensin production correlate well with

PRA (Danser *et al.*, 1992). Also, in anephric humans AI levels are practically zero (Wilkes *et al.*, 1991).

3.2 EVIDENCE FOR LOCAL PRODUCTION OF RA SYSTEM COMPONENTS IN TISSUES

Recent molecular and biochemical approaches to angiotensin physiology have raised the possibility that there are distinct, local RA systems that may be differentially regulated and that may target the actions of AII to specific organs or physiological events. Anti-renin antibodies were developed and used extensively throughout the 1980's to demonstrate the existence of renin in a comprehensive range of extra-renal sites (Slater, Defendini, and Zimmerman, 1980; Naruse, Takil, and Inagami, 1981; Naruse *et al.*, 1984). The 1980's also saw an exponential increase in the use of molecular biology techniques in research. Field and co-workers (Field *et al.*, 1984) were the first to look at renin gene expression in a range of tissues. They presented convincing Northern blot data for the presence of renin messenger RNA (mRNA) in the adrenals and testes of the mouse as well as the kidney and sub-mandibular gland. This laid the foundation for the mass of molecular analyses that have taken place over the last ten years, supporting the concept of tissue RA systems. Renin has by far been the best characterised tissue component of putative RA systems.

3.2.1 Renin

In the human female reproductive tract (Skinner, Lumbers, and Symonds, 1968) and the sub-mandibular gland (SMG) of the mouse (Michelakis *et al.*, 1974b; Field *et al.*, 1984), renin levels are exceptionally high. This cannot be explained by uptake of renin from the circulation. High SMG levels in the mouse have been explained on a molecular genetic basis. In some mouse strains (that have a low level of sub-mandibular gland renin) e.g. C57-BL10, there is a single renin gene locus (*ren-1*^{c}). In other strains e.g. DBA/2 (that have a high level of sub-mandibular gland renin) there is also a duplication of the renin structural gene present, (*ren-1*^{d}, *ren-2*^{d}). The three genes have different tissue specificities but all have been shown to be expressed in the kidney (Fabian *et al.*, 1989; Miller *et al.*, 1989; Mullins *et al.*, 1989). The high level of expression of the *ren-2*^{d} gene in the SMG of two-gene mice accounts for

the increased renin activity seen in the SMG of these animals (Miller *et al.*, 1989) and provides categorical evidence for extra-renal renin production.

Sealey and Rubattu (1989) demonstrated an increase in prorenin in the venous blood of baboon ovary, human testis and human adrenal. However, a change in veno-arterial levels of active renin was not found.

Renin has also been detected in a variety of cultured cells including vascular smooth muscle (Iwai *et al.*, 1989) and endothelial cells (Tang, Stevenson, and Dzau, 1990). However, local synthesis in culture does not necessarily mean that the cells would produce renin *in vivo*. The presence of angiotensins in culture has also been described (Naruse, Shizume, and Inagami, 1985; Hariba, Nomura, and Shizume, 1990) thus supporting the concept of complete local tissue systems.

In some sites, tissue renin has been demonstrated to rise after nephrectomy. This is best documented in the adrenal gland where bilateral nephrectomy results in an increase in detectable renin with a simultaneous drop of PRA to practically zero (Doi *et al.*, 1984; Baba *et al.*, 1986; Kim *et al.*, 1992). Historically, renin activity in the vascular wall has been demonstrated after nephrectomy (Basso and Taquini, 1971) but work by Thurston *et al.* (1979) demonstrated that renin-like activity in rat aorta was due to other acid proteases. More recent work (Saito *et al.*, 1988), however, in different isolated vascular beds, has shown persistence and even elevation of renin activity. Regulation mechanisms may therefore be different according to the site examined.

Renin is found in locations outside the blood-tissue barrier such as the brain (Hirose, Yokosawa, and Inagami, 1978; Dzau *et al.*, 1980a; Genain, van Loon, and Kotchen, 1985), the testes (Parmentier *et al.*, 1983; Inagami, 1993b) and the vitreous fluid of the eye (Deinum *et al.*, 1990). It is unlikely that renin is taken up from the circulation from these sites. In addition, Immunohistochemical techniques have localised renin to specific cells within certain tissues such as the brain (Slater, Defendini, and Zimmerman, 1980), placental membranes (Poisner *et al.*, 1981), testes, adrenal and pituitary (Deschepper *et al.*, 1986). In

the adrenal cortex, sub-cellular sites of production have been identified (Mizuno *et al.*, 1987b). Intracellular localisation of renin is more likely to be a consequence of local synthesis than uptake from the circulation.

Perhaps the strongest evidence for local extra-renal production of renin is the detection of renin mRNA in RNA extracted from various extra-renal tissues using complementary nucleic acid probes (Mesterovic, Catanzaro, and Morris, 1983; Field *et al.*, 1984; Samani *et al.*, 1987; Samani, Swales, and Brammar, 1988). This work not only confirmed existing biochemical data, but also found expression in unexpected sites such as the liver (Samani, Swales, and Brammar, 1988).

Whilst mRNA work has presented good evidence for local synthesis of renin, it has not escaped criticism. Although mRNA is an absolute pre-requisite for protein synthesis, its presence does not necessarily result in the translation of protein. There is evidence to suggest that perhaps all genes are minimally transcribed in all cell types (illegitimate transcription), without any subsequent translation of protein (Chelly *et al.*, 1989). However, the level of expression is so small that it can only be detected by multiple rounds of polymerase chain reaction (PCR) amplification after reverse transcription of the mRNA. Renin mRNA described at most sites is expressed at a far greater level than this and detection techniques such as Northern blotting are unaffected by this background level of expression. The use of *in situ* hybridisation has localised renin gene mRNA to specific cells types (Deschepper *et al.*, 1986; Racz *et al.*, 1992) thus disputing illegitimate transcription as the cause of renin message. These studies have also shown that renin mRNA is detected in different cell types at different extra-renal sites. Levels of renin mRNA in different tissues are therefore not simply a reflection of the differential frequency of one particular cell type in the different tissues: Another criticism of the gene expression studies is that renin mRNA detected in tissues codes for pro-renin and not mature renin. As the evidence that pro-renin can be processed to active renin outside of the kidney is debatable, the physiological relevance of extra-renal renin is controversial: Perhaps the most worrying argument against tissue RA systems having an essential role at some sites, is the variation in the reported level of renin

mRNA in certain tissues (Dzau *et al.*, 1987b; Samani, Swales, and Brammar, 1988; Seo *et al.*, 1991; Holycross *et al.*, 1992). This phenomenon is most acute in the liver, brain, heart and aorta where some investigators have easily detected message and others have found nothing despite using PCR analysis. The variation may be accounted for by the range of species and strains examined, but this explanation makes a unified theory on the role of tissue RA systems difficult to formulate.

Transgenic technology has further strengthened the case for extra-renal renin production. Mullins and co-workers introduced the *Ren-2* gene into rat embryos and found that the resulting animals exhibited fulminant hypertension (Mullins *et al.*, 1989; Mullins, Peters, and Ganten, 1990). The offspring of these rats that inherited the *Ren-2* gene also showed fulminant hypertension. Mullins therefore had produced the first model of inheritable hypertension with a known genetic basis (the presence of an extra copy of the renin gene). The animals have low kidney (cannot be detected by immunohistochemistry) and plasma active renin levels but high plasma pro-renin levels. Vascular angiotensin generation is also increased. These animals are therefore a good model of low-renin human hypertension (Thurston *et al.*, 1978). As the kidney is excluded as a source of pro-renin in these animals then it has to be assumed that extra-renal production is taking place. Several tissues have been demonstrated to express the transgene at very high levels especially the adrenal gland (*Ren-2* mRNA expression is 1000-fold higher than endogenous rat renin mRNA levels and renin enzyme activity is four times greater than controls). Interestingly, renin mRNA was not detected in the SMG of these animals in contrast to the high level of *Ren-2*^{d} expressed in the SMG of native mice (Ganten *et al.*, 1991).

3.2.2 Angiotensinogen

The presence of angiotensinogen has been demonstrated directly both biochemically and immunohistochemically in a variety of tissues (in addition to liver) including kidney (Richoux *et al.*,1983), brain (Imboden *et al.*, 1987) and heart (Sawa *et al.*, 1992). As for renin, this has been reinforced by the detection of angiotensinogen mRNA in these sites (Dzau *et al.*, 1987b). There is no direct correlation between the relative amounts of

angiotensinogen and renin found in a particular tissue. This may be due to different cell types within certain tissues producing different components of the RA system (Racz *et al.*, 1992). As angiotensinogen is the only known precursor of the angiotensin peptides, the presence of angiotensinogen in tissues can also be inferred from the detection of AI and AII in tissues.

3.2.3 Angiotensin converting enzyme

ACE has been described in practically every tissue and body fluid (Ehlers and Riordan, 1990). As for renin and angiotensinogen, molecular genetic analysis has detected ACE mRNA in a number of tissue sites (Griendling, Murphy, and Alexander, 1993). In addition to the well characterised endothelial sites of production, there is plenty of evidence for non-endothelial production in sites such as the epithelial brush borders of the kidney, the testes and adrenal (Ehlers and Riordan, 1990). ACE is generally located on the external surface of the cell membrane, but may also be located intracellularly (Okamura, Clemens, and Inagami, 1981).

Part of the mechanism of ACE inhibitors (see chapter two) may be attributed to inhibition of locally produced ACE. This in itself provides evidence of the importance of local ACE production for the functioning of a putative tissue RA system. The importance of tissue ACE, rather than serum ACE, in the long-term response to ACE inhibition has been examined in a number of studies (Dzau, 1988c). These studies showed that the magnitude and duration of blood pressure reduction correlated better with inhibition of ACE activity in certain tissues, rather than inhibition of serum ACE.

3.2.4 Angiotensin II receptor

The AT_1 receptor has been detected in various tissues by using pharmacological binding assays and specific cDNA probes. AT_1 receptor mRNA has been shown to be easily detectable in the kidney, liver, lung, spleen and adrenals using Northern blotting, and to show a much smaller level of expression in the heart, aorta, thymus, ovary and uterus (Murphy *et al.*, 1991). Studies that have used probes capable of distinguishing between the

AT_{1a} and AT_{1b} isoforms have shown that the AT_{1a} isoform is also found in the brain, whereas the AT_{1b} isoform is absent from the brain, heart and spleen (Kakar *et al.*, 1992).

3.3 THE FATE OF LOCALLY DERIVED COMPONENTS OF TISSUE RENIN-ANGIOTENSIN SYSTEMS

At present there is no consensus of opinion on the destiny of locally produced RA system components. However, if one assumes that the tissue gene expression of RA system components leads to the synthesis of their respective proteins, three possible scenarios regarding the distribution and functions of these locally synthesised components can be identified (Samani and Kelly, 1991).

(i). Locally synthesised renin and angiotensinogen supplement the extracellular AII generating capacity of a tissue:

Much evidence has accumulated (largely in relation to vascular tissues) to support the concept that apart from generating AI within the circulation, renin and angiotensinogen may also be taken up into tissues and cause local AI production (Campbell, 1985; Admiraal *et al.*, 1990; Danser *et al.*, 1990, 1992). That this local angiotensin generation makes a physiological contribution, at least in the regulation of blood pressure by the RA system, has been suggested by direct measurement of vascular uptake of renin in relation to blood pressure change and by examining the effects of inhibitors of the system (Swales, 1979). There is also evidence to suggest that, at least in some tissues, locally derived renin is transported to the extracellular space (Deinum *et al.*, 1990; Osmond, Sealy, and McKenzie, 1991).

Under this scenario, locally synthesised renin and angiotensinogen would not have a role separate from the endocrine system but simply add to the capacity to generate angiotensins within the extracellular space of the tissue. While the contribution made by the local synthesis of RA system components could vary not only from tissue to tissue but also in a single tissue under different circumstances, the main implication of this scenario is that in the end the effects of locally synthesised components are mediated through the same AII receptors that are responsive to AII produced either locally from components taken up from

the circulation, or indeed to plasma derived AII. This in turn implies that agents capable of blocking the interstitial generation of AII or its actions at the extracellular receptor would as much influence the contribution of the locally synthesised components as they would those derived by uptake from the circulation.

Although this scenario provides the simplest way of looking at the role of locally synthesised renin and angiotensinogen, is there any hard evidence for it? Overflow of locally synthesised components into the circulation has been demonstrated, for instance, for the adrenal gland and gonads (Sealy and Rubattu, 1989) and the persistence of circulating angiotensins following nephrectomy (Deheneffe, 1976) are in keeping with this scenario. In some sites, in response to specific stimuli, similar changes have been observed in locally derived components as in the circulating RA system. For example, renin mRNA in the adrenal gland has been reported as showing upregulation in response to a low salt diet just as it does in the kidney (Dzau *et al.*, 1987a). Angiotensinogen mRNA has also been shown to increase in the kidney as well as the liver in response to a low salt diet (Iwao *et al.*, 1988). This similar response of the circulating and locally derived components to a stimulus might suggest that they subserve a common function and therefore support this scenario.

If AII generated from locally derived components acts at the same site as AII derived from the circulating system, a situation could be envisaged whereby enhanced local AII production as a consequence of increased local synthesis of RA system components could modulate the effect of systemically administered AII. In most people, a low salt diet increases the sensitivity of the adrenal gland to AII (Hollenberg *et al.*, 1974). This response has been reported to be blunted in a group of patients with essential hypertension labelled non-modulators (Taylor *et al.*, 1984) and in the spontaneously hypertensive rat (SHR) [Williams *et al.*, 1982]. This blunting is specifically reversed by the administration of ACE inhibitors (Taylor *et al.*, 1984). One possible mechanism of this so called non-modulation may be that the blunted response is due to increased local AII production in the adrenal gland. There is evidence for this, at least in the SHR model, as both increased adrenal renin

mRNA (Samani, Swales, and Brammar, 1989) and activity (Naruse and Inagami, 1982) have been observed.

(ii). The result of locally produced RA system components is distinct independent local tissue renin-angiotensin systems:

The most exciting scenario for the function of tissue RA systems is the formation of independent local systems. By their very nature, these systems would have to be isolated from components derived from the circulating RA system and hence located in extracellular spaces that have a blood-tissue barrier or in intracellular compartments. Given these criteria, the most obvious place where a local RA system might exist is the brain, which has a well characterised blood-tissue barrier. Indeed, there is much evidence to suggest that a unique brain RA system exists (Hirose, Yokosawa, and Inagami, 1971; Dzau *et al.*, 1987b; Bunnemann, Fuxe, and Ganten, 1993). The case for intracellular localisation of tissue RA systems has been strengthened over the last ten years by the demonstration of generation of AII in tumour and other cultured cell lines (Inagami *et al.*, 1986) and the discovery of intranuclear AII receptors that have been demonstrated to regulate RNA synthesis when exposed to AII (Re and Parab, 1984; Tang *et al.*, 1992).

Despite cellular localisation of RA system components by biochemical means, evidence for expression of mRNAs for all of the components of the RA system within the same cell types is lacking. There is also a lack of evidence for processing of pre-pro-renin outside of the kidney allowing it to be sequestered intracellularly at extra-renal sites, or indeed conversion of pro-renin to active renin, outside of the kidney (Hackenthal *et al.*, 1990). It has been suggested that some tissues may use alternative start-sites for the transcription of the renin gene (which has been shown in some strains of mice) which might result in pro-renin being directly produced (Morris, 1986); however, experimental proof of this is lacking. Both renin and angiotensinogen produced in their primary sites of synthesis have strong signals on their molecules directing them into the secretory pathway of the cell, therefore if intracellular systems exist then such a fate for locally synthesised components must be avoided. To date, there is still no convincing proof for extrarenal intracellular pro-

renin to renin conversion, but several interesting postulates have been made by Sealy and co-workers. Sealy and Rubattu (1989) suggested that pro-renin may be reversibly activated by binding to a receptor that stearically modifies it, allowing the active sites to have access to angiotensinogen. This seemingly attractive hypothesis gained support when cDNAs for a renin binding protein that inhibits renin activity were subsequently cloned (Inoue *et al.*, 1990; Takahashi, Inoue, and Miyake, 1992) and expression of the mRNA detected in several tissues. Despite this development, a related pro-renin binding protein that would constitute the postulated pro-renin receptor has not been found, and Sealy and Rubattu have subsequently changed their minds with regard to their original hypothesis. More recent thinking by Sealy and co-workers (Sealy *et al.*, 1995) has suggested that pro-renin may undergo non-proteolytic site specific activation *in vivo* in a similar fashion to the reversible intrinsic renin catalytic activity seen when pro-renin is exposed to acid or the cold *in vitro* (see section 2.1.1). However, there is no hard experimental evidence to support this line of thought.

Perhaps the most interesting aspect of this scenario relates to the action of inhibitors of the RA system. In scenario one, the action of inhibitors on locally produced components of the RA system is easy to predict as it is simply a reflection of the general effects on the circulating RA system. However, in this scenario, the action of RA system inhibitors on local RA systems cannot be predicted by reference to the circulating RA system. The relative effectiveness of inhibitors will be dependent upon them gaining access to the sites of AII production and local AII receptors. Factors such as relative lipophilicity and size of the inhibitor molecule become crucial to its effectiveness. Experimental evidence has demonstrated that lipophilic ACE inhibitors have a much enhanced activity than less lipophilic inhibitors, in the brain and cerebro-spinal fluid (Unger, Gohlke, and Gruber, 1990). Lipophilicity also appears to be involved in the relative intracellular effectiveness of ACE inhibitors (Mizuno *et al.*, 1988).

(iii). Locally produced RA system components have functions unrelated to AII generation:

This is perhaps the least considered scenario and has been used as a vehicle to explain away anomalies such as the high level of pro-renin found at extra-renal sites (such as the ovary); and the abundance of (pro)renin in the testis with no local production or access to circulating angiotensinogen (Deschepper *et al.*, 1986; Campbell and Habener, 1986). Theoretically, as renin and angiotensinogen are related to other physiologically active proteins, they may have other functions. There is little evidence for this in the case of renin, but the arguments for angiotensinogen are perhaps more convincing (see section 2.1.2).

The major implication of this scenario is the total lack of a relationship between locally derived components and the endocrine RA system. Under this scenario, most of the currently available inhibitors and antagonists of the system, perhaps with the exception of renin inhibitors, should not influence the actions of the locally derived components. Although evidence for alternate roles (from angiotensin generation) for locally derived RA system components is lacking, this scenario remains a possibility until the fate of locally derived RA system components is unequivocally determined.

3.4 DIFFERENTIAL REGULATION OF GENE EXPRESSION OF RA SYSTEM COMPONENTS IN TISSUES

Activation of the circulating RA system in early studies made it impossible to differentiate between local production of RA system components and uptake from the circulation, using classic biochemical techniques. The recent application of molecular genetic techniques for the detection of mRNA for RA system components has made it possible to look at RA system regulation in tissues independently of the circulating system.

The expression of renin mRNA has been found to be differentially regulated in different tissues by phenomena such as the level of salt intake, β-adrenergic receptor activation, androgens, corticosteroids, pharmacological blockade of the RA cascade and AII (Dzau, Ingelfinger, and Pratt, 1986; Sigmund *et al.*, 1992). For example, sodium depletion or ß-adrenergic receptor activation increases expression in the kidney, heart and adrenal gland

but not in the genitals or submandibular gland. In addition, renin gene expression in the genitals and submandibular gland was found to be regulated by androgens. Perhaps most interestingly, extra-renal renin gene expression does not seem to be modulated by feedback inhibition of AII as is the case in the kidney. This has been shown in a number of studies where administration of an ACE inhibitor (and consequent drop in circulating AII) resulted in an increase in kidney renin gene expression, but no change in other tissues looked at (Samani, Kelly, Read, and Swales, 1991; Sigmund *et al.*, 1992).

Regulation of renin gene expression (and other components of the RA system) appears to be largely controlled by cis-acting elements in the 5' region of the respective genes. In the renin gene, consensus sequences have been found for cAMP, glucocorticoid, and sex hormone responsive elements (Dzau, Burt, and Pratt, 1988). Transient expression studies (Morris, 1992), and transgenic studies (Miller *et al.*, 1989) have also demonstrated that the presence of these elements is required for tissue related expression of the renin gene. Although two promoter sequences are present in the renin gene, transcription in most species generally occurs using only the promoter nearest to the transcription start site (Imai *et al.*, 1983). In certain mouse strains the choice of promoter used is tissue specific (Morris, 1992).

Like renin, angiotensinogen gene expression has also been shown to be differentially expressed. For example, following bilateral nephrectomy liver angiotensinogen expression is upregulated; whereas expression in the brain and heart remains the same, and expression in the adrenal lung and vascular tissue decreases (Campbell and Habener, 1986). Angotensinogen also appears to be regulated during various pathological states (see chapter two). In the SHR, brain angiotensinogen mRNA is increased compared with Wistar-Kyoto (WKY) normotensive controls; renal mRNA is decreased and liver mRNA is unchanged (Yongue *et al.*, 1991). A recent analysis of the role of angiotensinogen in spontaneous hypertension by Samani and co-workers (Lodwick *et al.*, 1995) has also suggested that the effect of the angiotensinogen locus on pulse pressure (a significant cardiovascular risk factor) may be mediated through a tissue-specific abnormality of angiotensinogen gene expression, at least in the SHR. Angiotensinogen gene expression has been shown to be regulated by cis-

acting elements in the angiotensinogen gene (see chapter two) and a choice of at least four polyadenylation sites (Ohkubo *et al.*, 1986) and two additional promoters upstream of the main transcription start site (Ben-Ari, Lynch, and Garrison, 1989). Transcription from these extra start sites has been found to be regulated by glucocorticoid action.

The regulation of tissue ACE gene expression is not as well characterised as that of renin and angiotensinogen, partially because detection of ACE mRNA in tissues (apart from the lung) was difficult until the relatively recent adoption of PCR technology (personal observation). Nevertheless interesting observations have been made especially in relation to the heart: ACE mRNA has been shown to be increased in cardiac tissue in response to experimental heart failure with no corresponding increase in other tissue or plasma ACE activity (Hirsch *et al.*, 1991). This type of study has profound implications for the site of action of ACE inhibitors and their possible role in the management of pathological cardiovascular events.

There is evidence to suggest that AT_1 receptor gene expression is modulated by AII itself. AII has been shown to reduce AT_{1a} expression in cultured rat glomerular mesangial cells (Makita *et al.*, 1992). In addition, an infusion of AII increased AT_1 receptor expression in the adrenal gland, but had no effect on expression in the kidney or aorta (Iwai and Inagami, 1992). In culture, the addition of compounds that increase intracellular cAMP has a similar effect on AT_1 mRNA expression to that of AII (Makita *et al.*, 1992). Recent studies have suggested that the AT_{1a} and AT_{1b} AII receptor subtypes are differentially regulated by dietary sodium intake. In the brain (Sandberg, Ji, and Catt, 1994) sodium deprivation increases the expression of AT_{1b} mRNA whereas high sodium intake increases the expression of AT_{1a} mRNA. The effect of dietary sodium on AII receptor gene expression in the kidney has recently been shown to be opposite to the effect on the brain (Du *et al.*, 1995). In response to low sodium intake an increase in AT_{1a} subtype mRNA levels in the kidney was demonstrated. This increase was also shown to have an inverse relationship with the level of AT_{1b} mRNA expression. As there are differences in the 5' -regulatory regions of the AT_{1a} and AT_{1b} genes (Takeuchi *et al.*, 1993; Guo and Inagami, 1994), differential regulation of

AT_1 receptor subtypes in different tissues in response to sodium may indicate distinct functional specificities.

The AT_2 receptor has been shown to be expressed in foetal and neonatal tissue in amounts far greater than in adult tissue. In the neonate rat brain, AT_2 receptors are found in areas concerned with learning and acquisition of motor skills. This expression falls off significantly as the animal matures. Expression of the AT_2 receptor in adult animals is highly tissue- and species- specific (Timmermans *et al.*, 1993).

3.5 PHYSIOLOGY AND PATHOPHYSIOLOGY OF TISSUE RENIN-ANGIOTENSIN SYSTEMS

Most of the physiological roles proposed for tissue RA systems are based on functions modulated by AII (see chapter two). A kidney RA system would therefore modulate renal haemodynamics, a cardiovascular system vascular tone and hypertrophy, an adrenal system aldosterone secretion and so on.

Interesting observations have been made in the context of reproduction and development. The ovary has been shown to have renin and angiotensinogen mRNA expression and ovulation can be prevented by inhibitors to the RA system (Lumbers, 1993). The demonstration of mRNA expression for components of the RA system in foetal tissue may have major implications in development e.g. expression of RA system mRNAs was seen in several rat foetal tissues, and also an apparent difference in expression in certain tissues was observed between 18 and 20 days of development (Kelly, Bradley, and Samani, unpublished data).

The main pathophysiological interest in tissue RA systems revolves around their role in hypertension. This interest is mainly a consequence of three major observations:

(i). Hypertensive rat models such as the SHR have elevated blood pressure but little or no activation of the circulating RA system (Ruiz *et al.*, 1990). Tissue RA systems may therefore play a role in the generation of the increased blood pressure seen in hypertension.

(ii). ACE inhibitors and some AII receptor antagonists (see section 2.3) have been demonstrated to reduce blood pressure independently of an effect on plasma renin or AII in hypertensive animal models and human hypertensives. These pharmacological agents may therefore function, at least in part, by inhibiting the hypertensive effect of tissue RA systems.

(iii). The high level of renin mRNA expression in the adrenal gland, but low kidney renin expression and low PRA seen in the original hypertensive transgenic rat created by Mullins and co-workers (see section 3.2.1) also provides strong corroborative evidence for the existence of tissue RA systems and their role in hypertension.

Most studies into the pathophysiology of tissue RA systems have centered around six main tissues: kidney, adrenal, brain, heart, gonad and vascular tissue.

(i). The kidney renin-angiotensin system:

It is very fitting a local RA system has been described in the tissue that initiated the vast amount of hypertension-related research over the last ninety or so years. All components of the RA system have been localised in the kidney by immunocytochemistry (Taugner *et al.*, 1982). Northern blot analysis has demonstrated that renin and angiotensinogen mRNA are expressed in the kidney (Ingelfinger *et al.*, 1986; Dzau *et al.*, 1987b) and ACE mRNA has been detected by RT-PCR (Kelly and Samani, unpublished data). Whilst *in situ* hybridisation has shown renin mRNA is found primarily at the vascular pole of the glomerulus (Deschepper *et al.*, 1986), angiotensinogen mRNA is expressed mainly in the proximal tubule (Ingelfinger *et al.*, 1990) as is ACE (Ehlers and Riordan, 1990). Possible intrarenal sites of AII production include the proximal tubule, JG cells (where both renin and AT_1 receptors are co-localised), vasculature and the interstitial fluid. Indeed, the studies by Admiraal and co-workers (see section 3.1) have demonstrated that the majority of AI found in renal venous blood is of intrarenal origin. Evidence for the predominant intrarenal production of AII has been suggested by the demonstration that AII concentrations in renal lymph (Bailie, Rector, and Seldin, 1971) and the proximal tubule (Seikaly, Arant, and Seney, 1990) greatly exceed (up to 1000 times) that in plasma. Recent studies by Allan and co-workers have shown that whilst the ratio of AII to AI is approximately 1: 1.5 in plasma, this

ratio is reversed in renal lymph and renal tissue, thus showing that intrarenal AII formation differs from AII formation in plasma (Allan *et al.*, 1994). In addition, recent work by Fisher and co-workers (Fisher *et al.*, 1995) has shown that in some subjects, the time course of the effect of renin inhibition on renal plasma flow is not congruent with the time course of the effect of renin inhibition on the RA system in the vascular compartment. After infusion of a renin inhibitor was discontinued, plasma AII levels were seen to rise steadily in proportion to a drop in plasma drug levels, but renal plasma flow remained at peak levels seen during infusion or even continued to rise. This implies that the renin inhibitor exerts most of its influence outside the vascular compartment, most probably by interruption of local intrarenal AII formation.

The strongest evidence for the involvement of the kidney RA system in hypertension is in the SHR before the rapid increase in blood pressure at 6 weeks onwards. Kidney mRNA and renal renin levels are increased (Samani, Swales, and Brammar, 1989) along with renal AII content and AII receptors (Matsushima *et al.*, 1988), compared to WKY normotensive controls. PRA is not increased in these animals which suggests that the over-activity of the intrarenal RA system serves a local function (perhaps crucial in the development of sustained hypertension). When the SHR matures, all of these phenomena (with the exception of elevated blood pressure) are about the same compared to the WKY strain suggesting that the increased blood pressure in itself then modulates the local RA system back to 'control' levels. A difference between the intrarenal and circulating RA systems is also seen in the chronic stage of Goldblatt two kidney-one clamp hypertension; renin mRNA is still elevated despite the drop in PRA to sham levels (Samani *et al.*, 1989). There is also evidence of differential regulation of renal angiotensinogen mRNA expression in the SHR by sodium, compared to WKY (Pratt *et al.*, 1989).

Interesting observations have been seen with respect to the intrarenal kidney RA system in experimental diabetic studies. In the adult biobreeding (BB) spontaneously diabetic rat and in Streptozocin-induced diabetic rats, the kidney expresses a higher level of renin mRNA (even after onset of diabetes) and has a higher renin content than that seen in control

animals (Everett *et al.*, 1992). This appears to suggest that in the setting of plasma volume expansion seen in diabetic rats, control of the intrarenal RA system is disordered in diabetes with the possibility of enhanced AII production. Further evidence for this possibility comes from studies that show both ACE inhibitors and AII receptor antagonists are effective in delaying the progression of renal injury (Lewis *et al.*, 1993; Remuzzi *et al.*, 1993).

A recent line of investigation has been the study of the renal RA system in experimental heart failure. There is plenty of evidence to suggest that the intrarenal RA system may be involved in the alterations in sodium and fluid balance seen in chronic heart failure, via direct tubular effects or haemodynamic changes (Hall, 1986). In addition, in some models of experimentally induced chronic heart failure, the circulating RA system is not activated (Hodsman *et al.*, 1988). Recently, Schunkert and co-workers (Schunkert *et al.*, 1992b) found that renal angiotensinogen mRNA levels were significantly elevated in rats with chronic heart failure. In addition, kidney renin and liver angiotensinogen mRNAs did not alter, and circulating levels of renin and AII did not change. The increase in angiotensinogen mRNA was found to be proportional to the size of myocardial infarction induced in the animal, suggesting that activation of the intrarenal RA system was influenced by the degree of heart failure. As renin and ACE are in a large excess in the kidney, angiotensinogen, rather than renin (as is the case with the circulating RA system), should influence the rate of intrarenal AII production. This was indeed demonstrated, as kidney AII concentration was also shown to be elevated and this correlated with the increase in angiotensinogen mRNA. This model of experimental heart failure has therefore provided evidence both to support the concept of a local kidney RA system and to explain one of its potential pathological roles.

(ii). The adrenal renin-angiotensin system:

The existence of a local adrenal RA system has been suggested over the years mainly by various biochemical/immunological, and gene expression demonstrations of renin (Ryan, 1967; Deschepper *et al.*, 1986); and the persistence of adrenal renin and AII following nephrectomy (Baba *et al.*, 1986).

In a similar fashion to the kidney RA system, the main evidence for the involvement of a local adrenal RA system in pathological states comes from the observation that adrenal renin activity and mRNA levels in both young and mature SHRs is elevated compared to WKY controls (Naruse and Inagami, 1982). A recent genetic analysis of renin gene expression in the rat adrenal gland (Iwai, Shimoike, and Kinoshita, 1996) using an F_2 cross of SHR/WKY demonstrated that the higher renin mRNA concentration seen in SHR adrenals is due to the SHR allele of the renin gene or renin gene locus. Aldosterone levels were also explained by PRA, adrenal renin mRNA level and the renin genotype. These correlations disappeared when the animals were put onto a high salt diet. In addition, whilst the adrenal renin mRNA concentration was found to exert only a minor influence on plasma aldosterone concentration when the animals were put on a high salt diet, paradoxically, the SHR allele of the renin gene or renin gene locus was found to confer a lower rate of aldosterone synthesis at 25-27 weeks of age in animals with a high dietary salt intake.

A difference between the adrenal and circulating RA systems is also seen in the chronic stage of Goldblatt two kidney-one clamp hypertension, as adrenal renin mRNA is elevated despite the drop in PRA to sham levels (Samani, Swales, and Brammar, 1989). A high level of adrenal renin gene expression is also seen in the Mullins transgenic rat model of low renin hypertension (see section 3.2.1).

(iii). The brain renin-angiotensin system:

All of the components of the renin-angiotensin system have been described in the brain (Fischer-Ferraro *et al.*, 1971; Slater, Defendini, and Zimmerman, 1980) and expression of RA system mRNAs have been demonstrated (Dzau *et al.*, 1986). AII receptors (predominantly AT_1 receptors) have been localised to areas of the brain that participate in cardiovascular regulation such as the anterior hypothalamic region and the circumventricular organs (Rowe *et al.*, 1990). Brain AII has been shown to participate in blood pressure, fluid and electrolyte homeostasis via mechanisms distinct from those in the periphery. Regulatory mechanisms include enhancement of sympathetic outflow, stimulation of vasopressin and corticotrophin release and increased catecholamine production (Bunnemann, Fuxe, and

Ganten, 1993). Brain AII also has behavioural effects such as thirst and salt appetite, essential to fluid and electrolyte homeostasis (Epstein, Fitzsimons, and Rolls, 1970; Fluharty and Epstein, 1983).

There is evidence to suggest that production of AII by the brain RA system may play a role in the pathogenesis of hypertension in the SHR. Compared to normotensive WKY controls, SHRs have increased numbers of brain AII receptors and show increased sympathetic tone (Schömig *et al.*, 1978; Pinto, Nazarali, and Saavedra, 1988). Renin activity and mRNA levels are also increased in the SHR brain (Naruse *et al.*, 1985; Samani, Swales, and Brammar, 1989), ACE levels are higher in SHR cerebrospinal fluid (Israel and Saavedra, 1987) and transgenic mice carrying the rat angiotensinogen gene have a high level of transgene expression in the brain and elevated blood pressure (Kimura *et al.*, 1992). Central administration of an ACE inhibitor or AII receptor antagonist reduces blood pressure in mature SHRs and attenuates the development of hypertension in young SHRs (Ruiz *et al.*, 1990). Intracerebroventricular Captopril administration has also been shown to attenuate the development of hypertension in the young SHR without altering PRA, aldosterone and vasopressin concentrations, fluid balance, sodium excretion, or resting sympathetic nervous activity (Okuno *et al.*, 1983).

The brain RA system has also been shown to be responsive to dietary sodium chloride supplementation in the salt sensitive / stroke-prone strain (SHR-SP) of the SHR. The depressor response to centrally administered ACE inhibitors has been demonstrated to be exaggerated in the SHR-SP with addition of extra sodium chloride to the diet (Takata *et al.*, 1986). This suggests that salt sensitive hypertension in this model may be mediated, at least in part, by brain AII. In contrast, the depressor effect of centrally administered Captopril in the WKY or the pressor effect of intracerebroventricular AII in the SHR are not enhanced by dietary sodium supplementation (Ashida *et al.*, 1982). In general, the central and peripheral RA systems have been found to be differentially regulated by sodium chloride intake in salt sensitive animals: The brain RA system appears to be upregulated and the peripheral RA system downregulated in response to sodium chloride supplementation in the SHR-SP. This

upregulation of the brain RA system may be modulated by the anterior hypothalamic area (AHA), as the pressor response to locally injected AII in SHR-SP animals is proportional to the amount of sodium chloride in their diet. This implies that dietary sodium supplementation increases the number of AT_1 receptors in the AHA of the SHR-SP (Yang *et al.*, 1992).

The brain RA system has recently been implicated in other clinical conditions such as hypothyroidism. Angiotensinogen mRNA expression has been shown to be decreased in the diencephalon and the brain stem of hypothyroidic rats (Hong-Brown and Deschepper, 1992). Further evidence that thyroid hormones modulate the brain RA system is also presented in this study; addition of thyroid hormone to primary cultures of astrocytes derived from diencephalons was shown to increase angiotensinogen secretion. This is a surprising observation, as classic thought suggests that the brain is not sensitive to thyroid hormones (Schwartz and Oppenheimer, 1978). However, the pathophysiological effects that may be mediated by the brain RA system, in conditions such hypothyroidism, are largely unclear.

(iv). The cardiac renin-angiotensin system:

Renin enzymatic activity in myocytes was first demonstrated nearly ten years ago (Dzau and Re, 1987) and both renin and angiotensinogen mRNAs have been shown to be present in atrial and ventricular myocytes using *in situ* hybridisation (Dzau 1988a; 1988b). Local AII production in the beating heart was also demonstrated by Linz and colleagues (1986) who measured coronary sinus AII levels in isolated rat heart perfused with AI. Addition of ACE inhibitors prevented this conversion, thus supporting the existence of functionally active cardiac ACE. Recently, Hirsch and colleagues (1991) demonstrated increased right ventricular ACE mRNA levels and ACE enzyme activity in experimental heart failure using RT-PCR analysis. Plasma and extra-cardiac tissue ACE activity was not activated. This therefore provides good evidence for independent ACE production in the heart.

The involvement of a local cardiac RA system (see chapter three) in cardiac disorders has been postulated (Lindpaintner and Ganten, 1991). In the SHR, cardiac hypertrophy is

seen to develop and this is accompanied by an increase in renin and angiotensinogen mRNA in the myocardium (compared to normotensive rat strains) which may result in local production of AII. In vitro studies suggest that AII may exert a pathophysiological effect intracellularly on cardiac myocytes (Robertson and Khairallah, 1971; Re *et al.*, 1984) Angiotensinogen mRNA levels are also increased in the hypertrophic left ventricle that results from coronary occlusion (Drexler *et al.*, 1989). Other possible pathophysiological roles of a local cardiac RA system include coronary vasoconstriction (Ertl, Alexander, and Kloner, 1983), increased cardiac contractility (Ahmed *et al.*, 1975; Sadoshima *et al.*, 1993), involvement in ischaemia and reperfusion injury (Westlin and Mullane, 1988) and hypertrophy (Kim *et al.*, 1995).

The possible involvement of a local cardiac RA system in cardiac hypertrophy is of particular interest as cardiac hypertrophy is a major risk factor for heart failure in humans (Kannel, 1987). In vivo studies in the rat have shown that RA system inhibitors regress cardiac hypertrophy induced by hypertension (Childs, Adams, and Mak, 1990), aortic coarctation (Everett *et al.*, 1994) and myocardial infarction (Yamagishi *et al.*, 1993), thereby suggesting that AII may play a fundamental role in the pathophysiology of cardiac hypertrophy. This effect is seen even at low doses that do not alter systemic blood pressure. Both ACE inhibition and AT_1 receptor antagonism have also been shown to blunt molecular adaptations in left ventricular hypertrophy, such as upregulation of left ventricular ANP and downregulation of sarcoplasmic reticulum Ca^{2+}- ATPase mRNA levels (Bruckschlegel *et al.*, 1995). Hypertrophic regression due to blockade of the RA system has also been shown to associate with reduced mortality, in rats, despite persistent pressure overload (Bruckschlegel *et al.*, 1995).

(v). The gonadal renin-angiotensin system:

Studies have shown that AII can inhibit testosterone biosynthesis in cultured rat Leydig cells by inhibition of cAMP production (Khanum and Dufau, 1988). The AII receptor antagonist, Saralasin, has also been shown to inhibit ovulation in rats, an effect that can be reversed by AII (Pellicer *et al.*, 1988). The expression of components of the RA system in

the reproductive organs may therefore lead to local mediation of reproductive function by AII.

(vi). The vascular renin-angiotensin system:

Despite indirect evidence, the existence of a vascular RA system has remained probably the most controversial of all proposed local RA systems. Studies on vascular production of renin in nephrectomised animals in the 1970's gave conflicting results. Basso and Taquini (1971) found that in nephrectomised rats, renin-like activity in the aorta persisted for several hours after PRA was no longer detectable. In contrast, Thurston and co-workers (Thurston *et al.*, 1979) found that bilateral nephrectomy caused a dramatic decrease in arterial renin-like activity, suggesting that activity associated with the vascular wall resulted from uptake of renally-derived circulating renin. Recent studies on the Mullins transgenic rat (see section 3.2.1) have strengthened the argument for locally derived vascular renin. In these animals, bilateral nephrectomy 24 hours prior to hindquarter perfusion did not affect angiotensin release (angiotensin release in control hindquarters being dramatically reduced). This suggests that renin in these animals post-nephrectomy is locally derived. In addition, mRNA from the *ren-2* transgene was detected in the aorta and mesentery of these animals (Hilgers *et al.*, 1992).

The possible existence of a vascular RA system has major implications for the pathogenesis of cardiovascular disease and hypertension. A number of studies have reported higher levels of renin in arterial tissues of the SHR compared with WKY controls (Asaad and Antonaccio, 1982; Rosenthal *et al.*, 1984), but this has not been found to be reflected in a change in renin mRNA levels in vascular tissue (Samani, Swales, and Brammar, 1989): In two kidney-one clamp experimentally hypertensive rats, aortic angiotensinogen mRNA levels have been found to be five times higher four weeks after clamping, compared to sham operated animals (Shioto, Miyazaki, and Okunishi, 1992). Within twelve weeks, mRNA levels in clamped animals are about the same as those in sham animals. The actual vascular origin of angiotensinogen mRNA in this study is debatable, however, as it is widely suggested that detectable angiotensinogen mRNA at vascular sites is associated with

periadventitial adipose tissue (Frederich *et al.*, 1992): The best documented involvement of a vascular RA system in hypertension comes from work on ACE. In two kidney-one clamp hypertension, an increase in aortic ACE activity was found to correlate with ACE mRNA levels in the aorta (Shioto, Miyazaki, and Okunishi, 1992). Increased vascular ACE may therefore be important in maintaining the hypertensive state by increasing local production of AII.

The most interesting role that a local vascular RA system may serve is in defining vascular structure. Changes in resistance vessel structure have long been considered to play an important part in hypertension (Folkow, 1982). There is recent evidence to suggest that resistance vessel structure is affected by the vascular RA system in immature individuals and that this effect is independent of that induced by increased blood pressure: Young SHRs that are treated with an ACE inhibitor over a short period have a permanent reduction in blood pressure (Harrap *et al.*, 1990). No such long term effect is seen in adult SHRs. Overactivity of the vascular RA system at a specific stage in development or growth may therefore lead to permanent vascular changes that initiate or maintain the pathological state. The form that such changes may take is the subject of much debate. Classic actions of AII on vascular cells such as hypertrophy and hyperplasia may not uniquely be responsible for the structural changes seen in hypertension, as there is increasing evidence to suggest that vascular remodelling (rearrangement of existing components of the vessel wall to give a smaller lumen without any change in the amount of material) may be just as important (Baumbach and Heistad, 1989). Overactivity of a vascular RA system may also be important in the contribution that large blood vessels make to hypertension. ACE inhibitors have been shown to reduce the stiffening of large conduit arteries (seen in hypertension) that may contribute to elevated blood pressure and pathological lesions (Dzau and Safar, 1988).

The vascular RA system especially raises a number of questions that have to be asked about local RA systems in any tissue. Although vascular and other tissues can be shown to have all components of the RA system present and even express mRNA for them (although evidence for significant levels of renin mRNA in vascular and certain other tissues *in vivo* is

still not conclusive), are all of these components actively synthesised and secreted? Are there local mechanisms in place for the control of production of AII and subsequent intracellular signalling? Do the components of local systems have the necessary spatial organisation to actually produce AII and play a physiological role that can be related to that of the circulating RA system? These and numerous other questions are continually being addressed but have not yet been unequivocally resolved.

CHAPTER FOUR

OUTLINE OF EXPERIMENTS

When the studies presented in this thesis were being formulated, a systematic study of gene expression of renin-angiotensin (RA) system components in a range of tissues and regulation of this expression by inhibitors of the system had not been previously reported, although it had been suggested that renin gene expression in the kidney and extrarenal sites might be differentially regulated. It was therefore decided first of all to examine tissue renin and angiotensinogen gene expression (and plasma levels of renin and AII) in rats treated with an angiotensin converting enzyme inhibitor versus a control group (the rat being the standard animal model used in cardiovascular research). One effect of ACE inhibition is to cause a large drop in plasma AII. Since AII is already known to be an important regulator of kidney renin production, it was hoped that this experiment would highlight any potential differences in the regulation of gene expression in renal and extrarenal sites. The results from the ACE Inhibitor study form the basis of the first experimental chapter (chapter 6) presented in this thesis.

The next stage of the experimental work was to apply the approach taken in the previous study to a system as close as possible to the human situation. The actual use of human subjects was obviously unfeasible, but it was felt that an alternative primate model would be a good approximation. I was fortunate to have an ongoing collaboration at the time with Dr. Frederic Cumin and Dr. Jeanette Wood of the cardiovascular research department at Ciba Geigy Ltd., Basal, Switzerland. Most importantly, Dr. Cumin and Dr. Wood had several years experience of studies using the Marmoset monkey, *Callithrix jacchus*. As inhibition of the RA system at different levels i.e. renin inhibition, ACE inhibition or AII antagonism may have different effects, a question of obvious pharmaceutical interest - the effects of inhibition at these three levels - was examined. Blood pressure was to be measured by a novel radiotelemetry system giving unparalleled accuracy and biochemical measurements were to be extensive. In light of the findings of Kitami and co-workers (1991) in a primate study and the design of the previous rat study, it was decided to focus the study of the regulation of gene expression on renin and angiotensinogen in the kidney and liver. The results of the marmoset study are presented in chapter seven.

After the first two studies were completed, it was decided that a logical application of this experience would be to examine the relative activities of the circulating and tissue RA systems in a pathophysiological situation. During the 1980's, a model of post-infarction heart failure in the rat had implicated activation of the RA system in the pathophysiology of heart failure and provided the experimental basis for the therapeutic utility of ACE inhibitors in heart failure. Whether tissue RA systems were activated in heart failure or if ACE inhibition affects tissue RA systems in this setting was not known. An extensive heart failure study, looking in particular at the regulation of gene expression of components of tissue RA systems, was therefore planned. Initially, a pilot study comprising of a sham operated group of rats versus an infarcted (induced by coronary artery ligation) group was undertaken. The results from this pilot study were encouraging so rather than present the data as it stood, it was decided to undertake an independent expanded study based upon the pilot study. The revised study consisted of four groups of rats: A, Sham operated; B, Infarcted; C, Sham operated + ACE inhibitor treated; D, Infarcted + ACE inhibitor treated. An important facet of the revised protocol was that it allowed examination of the potential mechanisms by which ACE inhibition may reverse the pathophysiology of myocardial infarction and heart failure (see section 2.3.1.1), with respect to modulation of tissue RA systems. The heart failure study also allowed an informal comparison with the results of the ACE inhibitor study (chapter 6), as two of the groups (Sham operated, and Sham operated + ACE inhibitor treated) were similar to the two experimental groups in the ACE inhibitor study. However, there is an important contrast between the design of these two studies. Whilst the ACE inhibition in the ACE inhibitor study was only over 5 days and could therefore be considered to be an acute treatment, ACE inhibition in the heart failure study was over 30 days and could therefore be considered to be a chronic treatment. This enables acute versus chronic ACE inhibition and any consequent differences in the regulation of tissue RA systems to be examined. The heart failure study is presented in chapter 8 of this thesis.

Chapter nine, the final experimental chapter presented, consists of a short study undertaken to examine a deficiency of previous studies - namely the failure to detect ACE mRNA by Northern blotting in extra-pulmonary tissues. The development of a Reverse-

Transcription Polymerase Chain Reaction (RT-PCR) protocol is described and preliminary data presented.

CHAPTER FIVE

MATERIALS AND METHODS

5.1 GENERAL COMMENTS

In this chapter the main techniques used in the experimental studies are described. Practically all of the techniques are well established in Molecular Biology, therefore original references have not been given in all cases. The main guide to designing protocols has been 'Molecular Cloning, a laboratory manual 2nd edition' (Sambrook, Maniatis, and Fritsch, 1989) in tandem with personal modification of existing techniques. The composition of buffers and other information related to the preparation of specific solutions is given in Appendix 5A.

5.2 SOLUTIONS

All aqueous solutions were made with water purified by activated carbon, reverse osmosis and finally ion-exchange resins. Solutions were sterilised and made Deoxyribonuclease (DNAase) free by autoclaving for 20 minutes at 15 atmospheres pressure (121 °C). To destroy Ribonucleases (RNAase) that may be present in solutions used for Ribonucleic Acid (RNA) work, solutions were mixed well with 0.1% diethyl pyrocarbonate (DEPC; Sigma, UK), and incubated for at least 12 hours at 37 °C before autoclaving. The DEPC degrades into volatile ethanol and carbon dioxide upon autoclaving. It is important to ensure that all of the DEPC is removed by thorough autoclaving after treatment, as it may modify RNA by carboxymethylation. Certain solutions that cannot be autoclaved e.g. solutions containing Sodium Dodecyl Sulphate (SDS) or Urea were made up with DEPC treated autoclaved water, as were solutions containing Tris base (Tris chemically reacts with DEPC).

5.3 GLASSWARE AND PLASTICWARE

All glassware used for RNA work was sterilised and made RNAase free by wrapping in aluminium foil and heat baking at 160 °C for 12 hours, before use. Glassware that needed to be siliconised e.g. Corex® tubes (Fisons, UK) for RNA preparations were treated by immersing for 10 minutes in dimethyl silane (2% solution in 1.1.1. trichloroethane, Fisons, UK). This was followed by drying at room temperature for 10 minutes and rinsing in

distilled water to remove any HCl produced by the degradation of the siliconising solution. Siliconised glassware was then baked as described above.

Plasticware including micro-pipettor tips was either bought pre-sterilised (by γ-irradiation) or sterilised by dry baking at 120 °C for 12 hours.

5.4 PURIFICATION OF NUCLEIC ACIDS

The purification of nucleic acids is one of the most fundamental techniques in molecular biology. The major purification step, the removal of proteins, can be simply and efficiently carried out by extracting an aqueous solution of the nucleic acid with organic solvents.

Phenol:Chloroform extraction:

The standard method for removal of proteins from nucleic acid solutions is by extraction with a phenol:chloroform mixture. Although phenol alone is good at denaturing proteins, it does not completely inhibit RNAase and is a solvent for RNA molecules that have long poly(A) tails. Combining phenol with chloroform solves these shortcomings. Chloroform also helps to denature proteins, facilitates the separation of the aqueous and organic phases and removes polysaccharides that may be present in the preparation. Deproteinisation is also more efficient when two different solvents are used rather than one.

(i). Liquefied Phenol (Fisons, U.K) was supplied pre-buffered with Tris (pH 7.4) and stabilised with 0.1% hydroxyquinoline (as well as behaving as an antioxidant, hydroxyquinoline is a weak chelator of metal ions, partial inhibitor of RNAase and a useful indicator of the organic phase of an extraction due to its yellow colour). Phenol was stored at -20 °C and thawed immediately prior to use. Chloroform (AR grade; Fisons, UK) was supplied as a 100% solution. Iso Amyl Alcohol (AR grade; Fisons, UK) was added to the chloroform solution to a final concentration of 4 % (v/v) as an anti-foaming agent.

(ii). Phenol:chloroform extraction was carried out by adding an equal volume of 1:1 phenol:chloroform to the crude nucleic acid solution and vortexing the contents vigorously to

form an emulsion. Extractions were then shaken for between 3 and 20 minutes depending on the volume and nature of the nucleic acid preparation. Centrifugation was then carried out either in a minifuge for 10 minutes (for samples in eppendorf tubes) or for 20 minutes at 10,000 rpm in a Beckman RC5B/C centrifuge fitted with a Sorvall SS34 rotor, for larger volumes in Corex® tubes. The upper aqueous phase was then removed carefully, avoiding the denatured protein interface between the aqueous and organic phases, and transferred to a new tube using a pipette. To achieve greater recovery, the organic phase and interface were usually back-extracted by adding up to ½ volume of aqueous buffer, vortexing and centrifuging as before and combining the resultant aqueous phase with the original aqueous phase.

(iii). Finally, chloroform extraction, using an equal volume of chloroform mixture added to the pooled aqueous phase, was carried out to remove any residual phenol from the aqueous phase.

5.5 CONCENTRATING NUCLEIC ACIDS

The standard method of concentrating nucleic acids is to precipitate them with ethanol in the presence of moderate concentrations of monovalent cations. The precipitation mixture is generally left at a low temperature (-20 °C or -70 °C) for several hours. The precipitate is then recovered by high speed centrifugation. The salt used to provide the cations is largely a matter of personal choice although the office of the nucleic acid being prepared may influence this choice. For most routine precipitations, sodium acetate p.H. 5.2 at a final concentration of 0.3M is the salt of choice.

Ethanol precipitation of nucleic acids:

(i). After estimating the volume of nucleic acid solution, 1/10 volume of 3M sodium acetate (Sigma, UK) pH 5.2 was added.

(ii). Two volumes (2½-3 volumes if RNA rather than DNA was being precipitated) of 100% ethanol were then added and the mixture placed at -20 °C (DNA) or -70 °C (RNA) for several hours to allow precipitation.

(iii). The nucleic acid was then pelleted by centrifugation at 4 °C in either a Microcentaur minifuge (high speed ~ 12,000g +, 15 minutes) for eppendorf tubes, or in a Beckman RC5B/C centrifuge fitted with a Sorvall SS34 rotor (10,000 rpm, 30 minutes) for Corex® tubes.

(iv). The supernatant was carefully removed and discarded leaving the pelleted nucleic acid in the centrifuge tube. The pellet was then washed with 70% ethanol to remove any co-precipitated salt, followed by further centrifugation to re-pellet the nucleic acid. After discarding the 70% wash, the pellet was dried under a standard table lamp. The pellet was then resuspended in an appropriate aqueous buffer. Care was taken not to over-dry the pellet as this makes resuspension difficult. However, if ethanol remains in the sample it may be difficult to pipette or load the sample onto a gel, so care was taken to ensure that no ethanol remained in the sample tube.

5.6 PREPARATION OF TOTAL CELLULAR RNA

Several procedures have been described for extracting total cellular RNA. They all incorporate measures to prevent the degradation of RNA by endogenous RNAase released from disrupted cells at the same time as the RNA. Such measures include the use of protein denaturants (e.g. guanidinium isothiocyanate, urea and SDS), deproteinisation procedures (e.g. with guanadinium hydrochloride or phenol), and using RNAase inhibitors such as heparin or iodoacetate.

In these studies a modification of the method described by Auffray and Rougeon (1980) was used. The major advantage of this method is that ultra-centrifugation is not required.

(i). Tissues for RNA extraction were removed from the animal immediately after sacrifice, frozen in liquid nitrogen and stored at -70 °C to reduce any enzymatic degradation of cellular RNA.

(ii). After weighing, the tissue was homogenised in RNA lysis buffer containing 6M urea, 3M LiCl, 20 μg/ml heparin sulphate (Pharmacy, Leicester Royal Infirmary), 0.1% SDS and 10mM sodium acetate pH 5.2. At least 10ml buffer / gm tissue was used and homogenisation was carried out in 30ml capacity Corex® tubes (Fisons, UK) for 3 minutes at room temperature using a polytron homogeniser (Brinkmann Instruments) at 80-100% power.

(iii). The samples were placed on ice and stored overnight (12-16 hours) at 4 °C to allow the RNA to selectively precipitate.

(iv). The RNA was pelleted by centrifugation in a Sorvall SS34 rotor in a Beckman RC5B centrifuge at 10,000 rpm for 30 minutes at 4 °C.

(v). The pellet was washed once with 5ml of 6M urea, 3M LiCl and recentrifuged as above.

(vi). The washed pellet was resuspended in 5ml aqueous buffer (10mM Tris-HCl pH 7.6, 0.5% SDS), and the suspension transferred to a fresh Corex® tube.

(vii). The crude RNA suspension was then phenol:chloroform extracted as described in section 5.4.

(viii). The aqueous phase was then ethanol precipitated overnight (12-24 hours) as described in section 5.5 and the RNA recovered by centrifugation in a Beckman RC5B/C centrifuge fitted with a Sorvall SS34 rotor (10,000 rpm, 30 minutes).

(ix). The pellet was washed in 70% ethanol, dried and resuspended at high concentration (>10 μg/μl) in DEPC treated water and stored at -70 °C.

(x). RNA concentration was determined by absorbance measurements on a spectrophotometer (Philips Pu 8620) at 260nm, and checked by comparing the relative ethidium bromide fluorescence of a 5 μg heat denatured (65 °C, 10 min) sample aliquot run on an agarose gel. Integrity of the sample was verified by clearly defined 28S and 18S

ribosomal and 7.8S / 5S / tRNA bands, a relatively high 28S : 18S fluorescence, and the presence of a light 'smear' of high molecular weight mRNA species. Any significant contamination with chromosomal DNA could be seen visible near the wells. A similar intensity of fluorescence from different 5 μg sample aliquots run on the same gel verified accuracy of the spectrophotometric determination of the concentrations. Yields of RNA varied according to tissue and ranged from around 500 μg RNA / g tissue for aorta to over 5000 μg RNA / g tissue for liver.

5.7 AGAROSE GEL ELECTROPHORESIS

(i). The appropriate amount of agarose (BRL, ultrapure) for the concentration of gel required was weighed out and added to gel running buffer (1×TAE) in a conical flask and dissolved by bringing to the boil in a microwave oven (after weighing complete to assess for water loss resulting from boiling).

(ii). Once the agarose had completely dissolved, the flask was re-weighed and returned to the original weight with sterile distilled water. Ethidium bromide (10 mg/ml stock) was added to a final concentration of 0.5 μg/ml and the agarose solution allowed to cool to < 50 °C before pouring onto the gel tray. The gel tray for supporting the gel consisted of an ultra-violet - transparent perspex base (Biological Workshop, Leicester University) with raised side walls having notches at one end to accept a variety of gel combs. For most analyses gel combs with teeth 5mm long and 1.5mm wide with 1.5mm between teeth were used. The tray was prepared by setting the gap between the teeth of the comb and the base of the gel tray to 0.5 - 1mm and sealing the ends of the tray with autoclave tape to form a liquid-tight seal.

(iii). After leaving for at least 30 minutes to set, the gel comb and autoclave tape were carefully removed and the gel carefully placed into an appropriately sized gel tank filled with 1×TAE containing ethidium bromide. The gel was covered in buffer to a depth of 5mm.

(iv). After loading nucleic acid samples in the wells left by the removal of the gel comb (teeth), a power pack (Biorad, UK) was connected with the cathode at the well end of the tank and the anode, to which the nucleic acid migrates, at the bottom. Gels were run at a maximum of 5 V/cm length of gel.

(v). The rate and distance of migration of nucleic acids was judged, by using the fact that the dye(s) that forms part of the sample loading buffer runs concurrently with nucleic acid fragments of specific size according to the concentration of the gel. When sufficiently run, the nucleic acids were visualised under ultra violet light from an ultra violet transilluminator. This visualisation is possible due to the planar groups of the ethidium bromide molecule intercalating between the stacked bases of nucleic acid, absorbing non-visible 260nm wavelengths transmitted by the transilluminator, and reemitting light of 590nm to give an orange fluorescence. The amount of dye bound and the resulting fluorescence is directly proportional to the amount of nucleic acid present.

(vi). For permanent records the gels were photographed on the transilluminator using Polaroid 667 Land film and a Polaroid Land camera (both Eastman-Kodak Ltd, UK) .

5.8 PREPARATION OF COMPLEMENTARY DNA (cDNA) PROBES

The cDNA probes used in the studies in this thesis were donated as kind gifts from a variety of sources. The probes were usually supplied in the form of 1-2 μg of recombinant plasmid (probe ligated to vector DNA). It was therefore necessary to introduce the plasmid into a suitable bacterial host that could then be grown up on a large scale to produce a working stock of plasmid.

5.8.1 Transformation of competent bacterial cells

(i). 100 μl of an overnight culture of an appropriate *E. coli* strain were inoculated into a universal containing 10ml of Luria-Bertani (L-) broth [L-broth consists of 10 gm peptone (Oxoid), 5 gm yeast extract (Oxoid), 5 gm NaCl per litre (pH 7.0)]. The culture was grown to mid-log phase, with an O.D. 600nm of 0.4- 0.6. The cells were harvested by centrifugation at 3,000g for 10 min at 4 °C. The cell pellet was washed by resuspension in

4ml of ice-cold 10mM NaCl and centrifugation performed as before. The cells were resuspended in 4ml of ice-cold 100mM $CaCl_2$ and left on ice for 30 min. Following centrifugation at 2,500g, the cells were resuspended in 1ml of ice-cold 100mM $CaCl_2$. The cells were placed on ice for 1 hour before use in transformation.

(ii). A total volume of 25 μl of plasmid DNA in T.E. buffer were added to 100 μl of competent cells, mixed well and placed on ice for 1 hour. The cells were heat-shocked at 42 °C for 3 min. 500 μl of L-broth were added and the cells incubated at 37 °C for 1 hour. After mixing, 100 μl aliquots were plated onto Luria-Bertani (L-) agar plates [L-agar consists of L-Broth + 15 gm/litre of agar (Oxoid)] containing the appropriate antibiotics where necessary. The plates were incubated at 37 °C overnight.

5.8.2 Large scale extraction of plasmid DNA

(i). The following solutions were used in the extraction procedure:

Solution I.

50mM glucose.

25mM Tris-HCl pH 8.0.

10mM EDTA.

Solution II.

0.2M NaOH.

1% SDS.

Solution III.

5M acetate (11.5ml glacial acetic acid).

3M potassium ions (60ml 5M potassium acetate).

(ii). 400ml of bacterial culture (resulting from the inoculation of a single transformed clone) was grown up to stationary phase (overnight) with appropriate antibiotics where necessary. The cells were collected by centrifugation, resuspended in 10ml of solution I containing 5mg/ml lysozyme (Sigma, UK) and placed on ice for 30 min. 10ml of freshly

made solution II were added, the contents mixed, and left on ice for a further 10 min. 15ml of ice-cold solution III were added, the contents mixed well, and the tube placed on ice for another 10 min. Centrifugation was performed at 20,000 rpm in a Sorvall SS34 rotor for 20 min at 4 °C to remove the cellular DNA and bacterial debris.

(iii). Equal volumes of the resulting supernatant were transferred into 30ml Corex® tubes, 0.6 volume of isopropanol was added, the contents mixed, and the tubes left to stand at room temperature for 15 min. DNA was recovered by centrifugation in a Sorvall SS34 rotor at 12,000g for 30 min at 20 °C. The supernatant was discarded and the DNA pellet dried briefly in a vacuum desiccator. The DNA was then resupended in sterile distilled water to a final volume of 17ml.

(iv). Caesium chloride was then added to the DNA solution to a final concentration of 1mg/ml and ethidium bromide to 50 μg/ml. The solution was then transferred to an ultracentrifuge tube. Chromosomal and plasmid DNA were then separated by density gradient ultracentrifugation in a Sorvall TV850 rotor at 40,000 rpm for 20 hours at 20 °C.

(v). After centrifugation, the DNA separated on the density gradient was visualised under ultraviolet light and the lower band of plasmid DNA removed from the tube by inserting an 18 gauge hypodermic needle into the side of the ultracentrifuge tube beneath the plasmid band and collecting the fraction. Ethidium bromide was removed from the fraction by equilibration with caesium chloride-saturated isopropanol, while caesium chloride was removed by dialysis against distilled water at room temperature. Plasmid DNA was recovered from the 'cleaned up' fraction by ethanol precipitation, resupended in an appropriate volume of sterile distilled water or T.E. buffer, and stored at -20 °C.

5.8.3 Isolation of cDNA inserts by restriction endonuclease digestion of recombinant plasmid DNA and electrophoresis through low-melting-point agarose

(i). Restriction enzymes were purchased from Life Technologies/BRL. Digests were performed according to the manufacturer's recommendations using the specific buffer supplied with the enzyme. 'Double' digests that had two enzymes requiring different

buffering conditions were performed using one buffer that gave an acceptable level of activity for both enzymes (compatibility information is available in the form of a chart from the manufacturer). Generally, one unit of enzyme was added per μg of DNA. Total reaction volume was generally set between 30- 60 μl and reactions incubated for 4-16 hours at the appropriate temperature (usually 37 °C). One unit of enzyme digests 1 μg DNA in 1 hour under optimal conditions. An excess of enzyme was used and incubation performed for several hours to ensure that there were no partial digest artefacts. Digestions were stopped and prepared for electrophoresis by the addition of 1/10 volume of sample loading buffer.

(ii). After incubation with restriction enzyme(s) the digest was electrophoresed in an appropriate percentage (usually 1%) low-melting-point (LMP) agarose gel to separate the cDNA insert and plasmid vector. As the proportion of the recombinant plasmid that constitutes the cDNA probe is known, then the amount of cDNA separated on the gel is that same proportion of the total amount of recombinant plasmid digested.

(iii). After the gel had been run long enough to adequately separate the vector and insert, the bands were visualised on a U.V. transilluminator and the portion of agarose containing the probe cut out of the gel using a sterile industrial razor blade (Richardsons, Leicester, UK).

(iv). The agarose block was trimmed of any agarose not containing probe, the trimmed block weighed, and transferred to a sterile screw-capped Eppendorf tube. It was assumed that the agarose block roughly had the same density as water (1g/ml), therefore the concentration of probe in the agarose block was easy to calculate.

(v). Sterile 18 MΩ water (Sigma, UK) was added to the eppendorf tube to give a final concentration of 20 ng of probe per 21 μl of agarose/water. It is important to ensure that no more than ¼ of the volume is occupied by the agarose to prevent interference with any subsequent radiolabelling reactions.

(vi). The agarose/water mix was then boiled for 5 minutes to melt the agarose, mixed carefully, allowed to cool to 40 °C, dispensed into 21 μl aliquots in screw-capped Eppendorf tubes, and stored at -20 °C until use.

5.8.4 Radiolabelling of cDNA probe in low-melting-point agarose

Double stranded cDNA probes were labelled by denaturing them to form single stranded templates, priming the template with random hexamer/octamer oligonucleotides, and extending the primers with radioactively labelled α-^{32}P dCTP and unlabelled dATP, dTTP, dGTP using the Klenow fragment of *E. coli* DNA polymerase I. A commercial kit (BRL/Life technologies, UK) was used for this purpose.

(i). 20 ng of probe in 21μl of 18 MΩ water was denatured at 100 °C for 12 minutes in a 1ml screw-capped Eppendorf tube.

(ii). The tube was given a 3 second low speed spin (minifuge) and components from a RadPrime kit (BRL/Life technologies, UK) added in the following order:

2.5× RadPrime buffer mix	20 μl
AGT mix	3 μl
α^{32}P dCTP (3000μCi/mmol, Amersham, UK)	5 μl (50 μCi)
Klenow fragment of DNA polymerase1	1 μl
TOTAL	**50 μl**

(iii). The contents of the tube were gently mixed, given a 3 second spin and incubated in a water bath for 45 minutes at 37 °C.

(iv). The labelling reaction was passed down a Sephadex G-50 (Pharmacia, UK) column, to remove unincorporated nucleotides, before use in a hybridisation.

5.8.5 Purification of radiolabelled cDNA probe using gel filtration column chromatography

(i). A Gel filtration resin (suitable for separating radiolabelled probes > 80 bases long from unincorporated nucleotides) was prepared by adding 10g of Sephadex G-50 (Pharmacia, UK) to 160ml of water, washing the swollen resin with sterile distilled water several times and equilibrating in T.E. buffer (pH 7.6). The resin was then autoclaved and stored at room temperature.

(ii). A glass Pasteur pipette was loosely plugged at the end with a small amount of sterile glass wool and clamped upright in a retort stand. The Pasteur pipette was then filled with gel filtration resin to form a column using a Gilson p1000 micropipette, taking care not to introduce any air bubbles. Resin was added until it packed at a level 1cm below the top of the Pasteur pipette.

(iii). The labelled probe was made up to a volume of 150 μl with T.E. buffer and applied to the top of the column. The resulting fraction was collected at the bottom of the column in a microcentrifuge tube. 150 μl of T.E. buffer was then applied to the top of the column and the resulting fraction was collected. This was repeated until ~ 15 fractions had been collected. The fractions were then held next to a hand-held mini-monitor (with a plastic cap fitted on the end of the Geiger tube) to assess their radioactive content. Typically, the first 5-7 fractions contained no counts; the next 4-5 fractions had counts near to or in excess of the limit of the scale of the mini-monitor (2000 cps); the next 2-3 fractions had counts between 200-800 cps; the following fractions had increased counts typically near to or in excess of the limit of the scale of the mini-monitor.

As smaller molecules are retained by the pores of the resin beads and larger molecules pass straight through the column, the first radioactive peak represents labelled probe, the radioactive 'dip' represents the tail end of labelled probe and the beginning of unincorporated radioactive nucleotides being eluted from the column, and the second radioactive peak represents unincorporated radioactive nucleotides.

(iv). Fractions from the first radioactive peak were pooled and 1/50 of the volume applied to a GFC filter (Whatman) and liquid scintillation counted. Probes typically labelled up to a specific activity of 1-3 $\times 10^9$ cpm/μg. The remainder of the pooled fractions was used immediately in the hybridisation.

5.9 SOUTHERN BLOTTING AND HYBRIDISATION

The term Southern blotting (Southern, 1975) is used to describe the transfer of DNA fragments, separated on an agarose gel, onto a filter membrane, which can then be hybridised to ^{32}P-labelled DNA or RNA probes. The relative positions of the DNA fragments in the gel are preserved during the transfer to the filter. Any bands complementary to the radioactive probe can be located by autoradiography.

5.9.1 Southern gel electrophoresis

(i). 1.5% agarose gels were prepared as described in section 5.7. Gels measuring 19.5cm x 20.5cm with 24 wells were routinely used.

(ii). Samples were loaded using a p100 Gilson micropipette and run at 35 V until the DNA size markers (3 μl Gibco 1Kb ladder) had migrated a sufficient distance.

(iii). The gel was photographed with a fluorescent ruler aligned with its length. A standard curve was then generated according to the distance migrated by the DNA size markers. From this curve, the size of probed fragments that 'lit up' on the final autoradiograph could be determined.

5.9.2 Denaturation of DNA fragments prior to blotting

Before transferring to a supporting membrane for hybridisation, the double stranded fragments had to be made single stranded in order for the single stranded complementary DNA probe to bind. Prior to denaturation, if one wishes to transfer large fragments (> 20 kb) a 15 minute soak in 0.25M HCl depurinates the DNA to allow easy transfer of the DNA. As fragments smaller than 20 kb were to be analysed, depurination was not carried out, the

cleavage that occurs by U.V. nicking during photography of the gel being sufficient for good transfer.

(i). After photography, the gel was submerged in a tray containing denaturing solution and gently agitated for one hour with a change of solution after 30 minutes.

(ii). The denaturing solution was then removed from the tray and the gel submerged in neutralising solution for one hour with a change of solution after 30 minutes, whilst being gently agitated.

5.9.3 Gel blotting

(i). The blotting apparatus was set up as follows: A glass plate several centimetres longer and wider than the gel was placed over a tray filled with transfer buffer (20×SSC). A 'wick' consisting of a piece of Whatman 3MM paper cut slightly wider than the gel was wetted in 3×SSC and wrapped around the plate so that the ends dipped in the transfer buffer. A 1ml plastic pipette was used to roll out any air bubbles trapped underneath the paper.

(ii). Using a glass plate as support, the gel was lifted from the (neutralising) buffer and lowered onto the wet 3MM paper, making sure there were no air bubbles between the gel and paper. To ensure that transfer buffer did not by-pass the gel via the edges of the wick, the gel was surrounded with a water-tight border of Saran Wrap (Dow).

(iii). A piece of Hybond-N (Amersham, UK) nylon membrane the same size as the gel was cut using a clean blade and ruler, wet in 3×SSC and placed carefully on top of the gel. After rolling out any air bubbles from the Hybond-N, two prewetted pieces of 3MM paper were placed on top of the Hybond-N and newly introduced air bubbles were rolled out. Twelve blotting towels (Sigma, UK) were placed on top of the 3MM paper and a 5cm stack of paper towels (Kleenex, UK) were placed on top of the blotting towels. Finally a glass plate and 200gm weight were placed on top of the stack to compress the components of the blot.

(iv). Capillary transfer of nucleic acids from the gel to the nylon membrane was allowed to proceed overnight (16-24 hours).

(v). The next morning the towels and Whatman papers were removed to reveal the membrane and the position of the gel slots marked with an indelible ball-point pen (Fisher, UK).

(vi). The membrane was carefully removed, placed in 3×SSC (DNA side up) to remove excess salt and agarose, air dried on 3MM paper with the DNA side up for 30 minutes and wrapped in Saran Wrap.

(vii). The membrane was then exposed to U.V. light (312nm) for 60 seconds on a U.V. transilluminator (Vilber Lourmat, France) to covalently cross-link the DNA with the filter (Optimum fixation time was determined previously by fixing blots of an identical sample for varying lengths of time, hybridising with a radioactively labelled cDNA probe under identical conditions and observing which fixation time gave the strongest signal when exposed to X-ray film.

(viii). If not hybridised straight away, the membrane was stored at 4 °C.

5.9.4 Hybridisation of Southern membranes

DNA hybridisation was carried out at 65 °C in a rotating hybridisation system (Hybaid, UK). With this system, membranes to be hybridised were soaked in 3× SSC and placed in between coarse Nylon support meshes cut slightly larger than the membrane (if several membranes were to be hybridised simultaneously, then meshes and membranes were simply stacked in an alternate fashion). A 'protective' piece of Hybond-N (prevents aggregation of non-specific probe signal on the uppermost membrane) was placed on the top mesh and the mesh/membrane stack rolled up to form a tube. The tube was then inserted into a cylindrical hybridisation bottle, some 3× SSC added, and the bottle was rotated to partially unroll the 'tube' so that the mesh/membrane stack adhered to the wall of the hybridisation bottle. Prior to addition of the hybridisation solution the 3× SSC was poured off.

(i). Pre-hybridisation: Membranes were pre-hybridised in 30ml of hybridisation buffer containing 6× SSC, 5× Denhardt's solution, 0.5% SDS, 6% polyethylene glycol 6000 and 200 μg/ml sonicated salmon sperm DNA. The salmon sperm DNA was heat-denatured at 100 °C for 10 minutes and added to the buffer just before use. Hybridisation bottles were rotated at approximately 12 rpm on the 'rotisserie' device inside the oven for at least 4 hours.

(ii). Hybridisation: Following pre-hybridisation, the buffer was poured off and fresh hybridisation buffer of the same composition but containing the labelled cDNA probe was added. The probe was denatured by boiling it together with the salmon sperm DNA before addition to the buffer. Hybridisation was allowed to proceed for at least 16 hours (maximum 24 hours).

5.9.5 Membrane washing and autoradiography

Washing the membranes after hybridisation removes a large proportion of any non-specifically bound probe. The degree of probe removal is determined by the stringency of the washes. The lower the salt (SSC) concentration of the washing buffer and the higher the washing temperature, the higher the stringency.

(i). Membranes were first washed twice in 3× SSC, 0.1% SDS for 20 minutes. The membranes were then washed for 40 minutes in two changes of 1×SSC, 0.1% SDS. The membranes were finally washed twice in 0.1× SSC, 0.1% SDS for 20 minutes each time. All washes were at 65 °C.

(ii). Following washing, membranes were air dried briefly so that they were still damp, and wrapped in Saran Wrap. The filters were then placed DNA side down on pre-flashed Kodak X-omat AR X-ray film in cassettes with intensifying screens (Highspeed extra, Eastman Kodak, UK). After overnight exposure at -70 °C, the films were developed using an automatic film processor to assess the strength of signal. The appropriate exposure time for an optimum result could then be estimated. The size of any fragments detected by autoradiography could be determined from a standard curve obtained from the relative migration distances of the DNA size markers.

5.9.6 Reprobing of membranes

To remove the probe from the filters before hybridisation with a different probe, membranes were placed in 1 litre of freshly boiled 0.5% SDS solution, gently agitated and allowed to cool to < 40 °C. After briefly drying on Whatman 3MM paper, the filters were wrapped in Saran Wrap and autoradiographed overnight to check efficient probe removal. Filters were stored at 4 °C until needed. Reprobing was carried out as for fresh membranes.

5.10 NORTHERN BLOTTING AND HYBRIDISATION.

Once Southern's technique was used successfully for detecting sequences within DNA, it was soon extended to blotting RNA (Northerns) separated by electrophoresis. RNA being single-stranded, must be kept fully denatured during electrophoresis, to prevent self folding and allow identical RNA molecules to run at the same rate within the gel. In these studies this was achieved by running RNA samples in agarose gels containing formaldehyde.

5.10.1 Northern gel electrophoresis

(i). A 1.2% agarose gel containing 2.2M formaldehyde and 1×MOPS (morpholinopropanesulphonic acid) buffer p.H. 7.0 was prepared by dissolving the appropriate amount of agarose in 7/10 volume of water, allowing the solution to cool to 50 °C, adding to 1/10 volume 10×MOPS and 1.74/10 volumes formaldehyde (38% v/v, Fisons, U.K.), topping up to final volume with water, and pouring. Ethidium bromide was included in the gel for visualisation, but only ¼ of the standard concentration was used as efficiency of Northern hybridisation may be reduced by ethidium bromide.

(ii). RNA samples for electrophoresis were prepared as follows:

RNA + DEPC water	6.75 μl
10× MOPS	3.00 μl
Formamide	15.00 μl
Formaldehyde	5.25 μl
TOTAL	**30.00 μl**

(iii). The samples were heated at 65 °C for 10 minutes to remove any RNA secondary structure, chilled on ice and 3 μl of 10× RNA loading buffer added before loading onto the formaldehyde agarose gel.

(iv). Electrophoresis was carried out in 1× MOPS buffer at a maximum of 3.5 volts/cm length of gel; current limited to 70mA until the bromophenol blue in the loading buffer had run ~ 10-12 cm (6-7 hours for a 20.5 cm length gel). 4 μg of 0.25-9.4 kb RNA ladder (BRL/Life technologies, UK) was used as a size marker.

5.10.2 Northern gel blotting

After electrophoresis, the gel was then soaked in 20× SSC for 1 hour before blotting to remove the formaldehyde. Blotting was carried out as for Southern gels (see section 5.9.3) and the RNA was fixed to the membrane by U.V.-irradiation for 60 seconds as for DNA. After the membrane had been fixed, the portion of the membrane containing the RNA size markers was cut off and visualised using a staining solution of 0.04 % methylene blue in 0.5 M sodium acetate pH 5.2.

5.10.3 Hybridisation of Northern membranes

Hybridisation of Northern membranes was essentially performed in the same manner as for Southern membranes (see section 5.9.4) except that the composition of the hybridisation buffer, and the hybridisation temperature were different. Hybridisations were performed in 50ml of a buffer containing 50% Formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, 6% polyethylene glycol 6000 and 200 μg/ml sonicated salmon sperm DNA. The use of a 50% formamide buffer changes the hybridisation kinetics in such a way that a lower temperature (42 °C) is required than for an aqueous buffer. This combination of buffer and temperature favours RNA:DNA hybridisation. The more sensitive Northern membranes are also exposed to less harsh conditions for the duration of the hybridisation, thus maximising their potential for successful reprobes. SSPE was used in preference to SSC as it has a much superior buffering capacity against formamide.

5.10.4 Northern membrane washing and autoradiography

Northern membranes were washed and autoradiographed in essentially the same manner as described in section 5.9.5 except that SSPE was used in place of SSC. The initial 3× SSPE washes were also performed at 42 °C rather than 65 °C, as it was assumed that as the membrane was still saturated with formamide based solution immediately post-hybridisation, local kinetics on the membrane would dictate the lower temperature. The following washes were generally performed at 60 °C.

5.11 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Within the last few years, the polymerase chain reaction has been utilised to indirectly demonstrate levels of mRNA expression of a gene in tissues where the level of expression is below the detection limits of Northern Blotting and Ribonuclease Protection Assay, or where the amount of RNA extracted from a tissue is insufficient for use in these standard techniques. Total RNA is first reverse transcribed to first strand cDNA using a commercially available high activity reverse transcriptase. The cDNA is then amplified by PCR using suitable primers (designed from published nucleotide sequence of the gene of interest). The resulting product (the size of which can be predicted) can be separated on an agarose gel and subjected to Southern analysis (see section 5.9). Whilst the method is excellent at detecting tiny amounts of starting material and verifying the results of other methods, it is very difficult to obtain truly quantitative results. The efficiency of the RT reaction may not be the same from sample to sample. More importantly, by its very nature, PCR is an exponential reaction and therefore small changes in amplification efficiency can dramatically affect the amount of product (e.g. a change in amplification efficiency from 85% to 80% at 30 cycles would reduce the yield of product more than two-fold). The various constituents of the PCR reaction may become rate-limiting with successive cycles, and factors such as different levels of impurities may give a large inter-sample variability. The PCR analyses undertaken in this thesis were therefore interpreted in this light.

5.11.1 Reverse transcription of RNA template

Total RNA was reverse transcribed to first strand cDNA using oligo $(dT)_{12\text{-}18}$ (Pharmacia, UK) priming and Maloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT, BRL/Life Technologies, UK).

(i). 1- 5 μg of total RNA was made up to a volume of 8 μl with 18 MΩ water (Sigma, UK) in a 0.5ml DNA Thermal Cycler-compatible microfuge tube. This was heated at 94 °C for 5 minutes, given a brief spin in a microcentrifuge and put on ice for one minute.

(ii). 2 μl of oligo $(dT)_{12\text{-}18}$ (50 ng/μl; Pharmacia, UK) was then added to the tube, at room temperature. This was left for two minutes.

(iii). 10 μl of a reverse transcription master mix (kept on ice until use) was then added to the tube. The master mix consisted of 4 μl of 5x First Strand Buffer (supplied with the enzyme), 2 μl of 100mM Dithiothreitol (supplied with the enzyme), 2 μl of 10mM deoxy-nucleotide tri-phosphates (dGTP, dATP, dTTP, dCTP; Pharmacia, UK), 1 μl of Human Placental Ribonuclease Inhibitor (20-40 U/μl; Pharmacia, UK), and 1 μl of M-MLV RT (200 U/μl).

(iv). The reaction was then given a brief mix and spin and incubated at 37 °C for one hour.

(v). After incubation, the reaction was heated at 94 °C for 10 minutes, spun briefly and put on ice. If the reaction was not to be subjected to PCR amplification straight away it was stored at -20 °C.

5.11.2 Amplification of cDNA product by Polymerase Chain Reaction

(i). A 10 × stock of PCR buffer consisting of 500mM KCl, 200mM Tris-HCl pH 8.4, and 2.5mM $MgCl_2$ made up with 18 MΩ water was kept frozen in aliquots. Prior to use, an aliquot was defrosted and Bovine Serum Albumin (BSA) solution (10mg/ml stock, Pharmacia, UK) was added to a final concentration of 1mg/ml.

(ii). 80 μl of a 1 in 10 dilution of 10 × PCR buffer/BSA solution, containing 0.4 μl of DNA *Taq* polymerase (5U/μl; Bioline, UK) and an appropriate amount of the specific amplification primers, was mixed gently and briefly spun. This was then added to the 20 μl of reverse transcribed RNA to give a total PCR reaction volume of 100 μl. This was mixed gently, briefly spun and ~ 50 μl of mineral oil (Sigma, UK) layered on top. The PCR reaction was kept on ice until it was put into the DNA thermal cycler (Perkin Elmer/Cetus, USA).

(iii). Mineral oil (~ 50 μl) was put into the tube wells of the DNA thermal cycler heating block and the block pre-heated to 94 °C. The reaction tube was then inserted into one of the tube wells on the heating block and subjected to the following regime for an appropriate number of cycles (usually 25-30):

Denaturation: 1 minute @ 94 °C

Annealing: 1 minute @ the appropriate temperature for the primers being used

Extension: 30 seconds per 500 base-pairs of extension @ 72 °C

(iv). After the reaction was complete, an aliquot of the PCR product (usually 30 μl) was taken for Southern analysis and the remainder stored at -20 °C.

5.12 BIOCHEMICAL ASSAYS FOR COMPONENTS OF THE RA SYSTEM IN PLASMA AND TISSUE

Assays for plasma renin concentration (PRC), plasma renin activity (PRA), and plasma renin substrate (PRS) [plasma angiotensinogen] were all essentially variations of the in vitro generation of AI and subsequent radioimmunoassay (RIA) as described by Menard and Catt (1972). This method gives an estimate of the level of the component being assayed in terms of the ability of a fixed volume of plasma to generate AI over a fixed time period. The first step of the method is the incubation of plasma at 37 °C at a controlled pH (7.0 - 7.4), AI formed is protected from degradation and enzymatic conversion by the addition of inhibitors known to block angiotensinases and ACE, but that do not interfere with the action of renin. In order to quantitate the AI formed, the second step is the addition of AI antiserum and a known amount of ^{125} Iodine - labelled AI in a relatively large volume at 0 - 4 °C. The

cooling and dilution practically stop enzymatic reactions, and the AI produced in the first step competes with the radioactively labelled AI for the antibody. The sample is then kept at 4 °C for 18 hours, unbound labelled AI removed by adsorption onto plasma or dextran coated charcoal, and the resulting supernatant liquid scintillation counted. AI generated by the plasma sample is quantified by reference to a standard curve constructed from standard amounts of AI that have been subjected to RIA in a similar fashion to the sample being assayed.

Detailed protocols are given for the assays of rat plasma. The equivalent assays in the marmoset study (chapter 7) were performed in a similar fashion.

5.12.1 Measurement of plasma renin concentration (PRC)

(i). Duplicate plasma samples (20 μl) were incubated at 37 °C for 5, 30, 60, or 120 minutes with 60 μl of a mixture of plasma renin substrate[§] in 0.1M Tris-acetate buffer pH 7.4 containing 2,3-dimercaptopropanol (16mM) and phenylmethylsulfonyl fluoride [PMSF] (0.5 mg/ml).

(ii). The enzyme incubation was stopped by rapidly cooling the samples in ice water.

(iii). The AI generated was estimated in the same tube as follows by a modification of the RIA described by Menard and Catt (1972) : 400 μl of cold 0.1M Tris-acetate buffer, pH 7.4 containing human serum albumin (1 mg/ml) and AI antiserum was added to each sample, followed by 10 pg of ^{125} Iodine - labelled AI (in 50 μl of 0.1M Tris-acetate, pH 7.4). The samples were incubated at 4 °C for 18 hours.

(iv). 200 μl of dextran coated charcoal mix (Sigma, UK) was added to each sample and thoroughly mixed for 30 seconds. The samples were then immediately centrifuged at 3000 × g for 15 minutes at 4 °C. The supernatant was immediately transferred to a scintillation tube and counted.

[§] The plasma renin substrate was plasma that was obtained from 24 hour bi-nephrectomised rats which had been given a subcutaneous injection of long acting polyestradiol phosphate

(Estradurin, U.K.) ten days before nephrectomy. This substrate preparation was shown to be completely renin-free and sufficient to cause zero-order kinetics when added to the sample incubation.

5.12.2 Measurement of plasma renin activity (PRA)

Assays for PRA were carried using a modification of the antibody trapping method of Poulson and Jørgensen (1974) and was technically similar to the assay for PRC except that plasma renin substrate was not added to the reaction. The first step of the method is addition of AI antiserum to the plasma sample (the antibody dilution was worked out previously and shown to be in excess of that required over the range of the standard curve used). During incubation at 37 °C at a controlled pH (7.0 - 7.4), AI formed is captured by its antibody protecting it from degradation and enzymatic conversion. The quantitation of AI formed is then identical to that of the PRC assay except that AI antiserum is not added to the reaction (The cooling and dilution after the incubation cause the 'trapped' AI to dissociate from the antibody so therefore the AI formed during the incubation competes with the radioactively labelled AI for the 'trapping' antibody which now serves as the RIA antibody). AI generated by the plasma sample is quantified by reference to a standard curve constructed from standard amounts of AI that have been subjected to antibody trapping and competition with radioactively - labelled AI in a similar fashion to the sample being assayed.

5.12.3 Measurement of plasma angiotensinogen (PRS)

(i). Duplicate Plasma samples (10 μl) were mixed with mouse submaxillary gland renin (20 μl, 112ng) and 30 μl of 1M TES buffer (T.E. with 100mM NaCl) pH 7.2 containing EDTA (50mM), 2,3-dimercaptopropanol (8mM) and PMSF (0.5 mg/ml) for 0, 60 and 90 minutes at 37 °C (the last time point ensured that the reaction had reached a plateau).

(ii). Reactions were stopped by chilling the sample and adding 300 μl of ice-cold 0.1M Tris-acetate pH 7.4, containing human serum albumin (1 mg/ml). After dilution, the angiotensin I generated was measured by a modification of the RIA described by Menard and

Catt (1972). PRS was calculated from the 90 minute result assuming that one mole of angiotensinogen generates one mole of angiotensin I.

5.12.4 Measurement of plasma angiotensin II

Blood was collected into an enzyme inhibitor solution consisting of *ortho*-phenanthroline (final concentration 30 mM), K-EDTA (final concentration 1%) and the rat renin inhibitor GCP44099A (final concentration 5 μM = ~ 1300 x IC_{50}). Plasma AII concentrations were measured by direct RIA after extraction of AII from the plasma with phenylsilyl silica gel cartridges. The sensitivity of the RIA was 1 fmol angiotensin II per fraction and cross-reactivity with angiotensin I was < 0.1%.

5.12.5 Measurement of plasma angiotensin converting enzyme (ACE) activity

(i). Plasma samples (100 μl) were collected on heparin and incubated in 50mM Hepes buffer pH 8.15, 300mM NaCl, 10 μM $ZnCl_2$, containing 5mM Hippuryl-Histidyl-Leucine (HHL)and a trace amount ($2x10^5$ cpm/tube) of [glycine-1-^{14}C]-hippuryl-L-histidyl-L-leucine (3 mCi/mmol, Amersham, UK) at 37 °C for 2.5 hours (total incubation volume 500 μl). Each plasma sample was assayed in duplicate in the absence or presence of 12.5 μM of enalaprilat.

(ii). The incubation was stopped by adding 500 μl of 1M HCl to each sample.

(iii). Cleaved [^{14}C]-Hippuric acid was extracted from the acidified samples by the addition of 1 ml of ethyl acetate, vigorous shaking and a one hour incubation at 4 °C, followed by separation of the two phases by centrifugation at 5,000 g for 20 minutes at 4 °C.

(iv). 200 μl aliquots of the organic phase were then added to 5 ml of scintillation cocktail (75% toluene / 25% ethyl acetate) and counted in a Tri-Carb scintillation counter (Packard Instrument Company, USA) for 5 minutes.

(v). Specific ACE activity was determined from the rate of formation of [^{14}C]-Hippuric acid under conditions of initial velocity (< 10 % of substrate hydrolysed). Results were expressed as nmol of substrate hydrolysed per ml of plasma per hour of incubation.

5.12.6 Measurement of tissue angiotensin converting enzyme (ACE) activity

Tissue ACE activity was measured in a similar manner to plasma ACE, using freshly prepared homogenates prepared in ice-cooled assay buffer containing 0.5% Triton X-100. The protein content of the homogenates was determined by the method of Bradford (1976). Results were expressed in nmol substrate hydrolysed per mg protein per min incubation.

APPENDIX 5A: SOLUTIONS AND BUFFERS

1× T.E. BUFFER

10mM Tris-HCl pH 8·0 (Fisons, UK).

1mM EDTA (Sigma, UK).

10× SAMPLE LOADING BUFFER:

50% Glycerol v/v (Sigma, UK).

49.5% DEPC treated water.

0.25% Bromophenol Blue (Sigma, UK).

0.25% Xylene Cyanol (Sigma, UK).

1× TAE GEL RUNNING BUFFER:

40mM Tris-acetate (Fisons, UK).

1mM EDTA (Fisons, UK).

0.5 μg/ml Ethidium Bromide (Sigma, UK).

SOUTHERN BLOTTING DENATURING SOLUTION:

1.5M NaCl (Fisons, UK).

0.5M NaOH (Fisons, UK).

SOUTHERN BLOTTING NEUTRALISING SOLUTION:

1M Tris-HCl pH 8.0 (Fisons, UK).

1.5M NaCl (Fisons, UK).

20× SALINE SODIUM CITRATE (SSC):

3.3M NaCl (Fisons, UK).

0.3M Na_3 Citrate (Fisons, UK).

pH 7.0

20× SALINE SODIUM PHOSPHATE EDTA (SSPE)

3.6M NaCl (Fisons, UK).

0.2M NaH_2PO_4 (Fisons, UK).

0.02M Na_2 EDTA (Fisons, UK).

pH 7.4

10 × MOPS FORMALDEHYDE GEL RUNNING BUFFER

0.2M morpholinopropanesulphonic acid (Sigma, UK).

50mM sodium acetate (Sigma, UK).

5mM EDTA (Fisons, UK).

pH 7.0

2.5 × RADPRIME BUFFER

125mM Tris- HCl pH 6.8

12.5mM $MgCl_2$

25mM 2-mercaptoethanol

150 μg/ml oligodeoxyribonucleotide primers (random octamers)

AGT mix (deoxyribonucleotide tri-phosphate mix)

10mM each of dATP, dGTP, dTTP in 1mM Tris-HCl (pH 7.2)

5× FIRST STRAND BUFFER:

250mM Tris-HCl (pH 8.3)

375mM KCl

15mM $MgCl_2$

10× PCR REACTION BUFFER:

500mM KCl (Sigma, UK).

200mM Tris-HCl pH 8.4 (Fisons, UK).

25mM $MgCl_2$ (Sigma, UK).

1 mg/ml Bovine Serum Albumin (Pharmacia, UK).

CHAPTER SIX

Effects of ACE inhibition on tissue renin and angiotensinogen gene expression in the rat

6.1 INTRODUCTION

Since their introduction into clinical practice in 1977, inhibitors of angiotensin converting enzyme (ACE) have found an increasing role in the management of several cardiovascular disorders. They have been found to be useful not only in the treatment of hypertension (Brunner, Gavras, and Waeber, 1979) and left ventricular dysfunction (The CONSENSUS Trial Study Group, 1987; The SOLVD Investigators, 1991), but also to reduce the risk of recurrent myocardial infarction (Pfeffer *et al.*, 1992), to cause regression of cardiac hypertrophy (Dahlof, Pennert, and Hansson, 1992), to improve arterial compliance and to prevent the progression of diabetic nephropathy (Parving, Hommel, and Smidt, 1988). Given such multiple effects, it has been proposed that tissue RA systems may be important targets for the actions of ACE inhibitors (Dzau, 1987). In the kidney, ACE inhibitors increase renin production (Nakamura *et al.*, 1985; Ludwig *et al.*, 1987), primarily by disrupting the negative feedback exerted by AII. Their reported effect on renin production in extra-renal tissues is still poorly understood and controversial. In this study, it was therefore decided to examine the effects of ACE inhibition on renin as well as angiotensinogen mRNA levels in several rat tissues where these genes are expressed.

6.2 METHODS

(i). Animals and ACE inhibitor treatment

Male Wistar rats (300-400 gm, 12 weeks old), n=12 were purchased from Charles River laboratories (UK). The animals were housed under controlled conditions (temperature 21 ± 1 °C, humidity 60 ± 10%, 12 hour day/night cycle) and fed standard rat chow (Rat & Mouse no. 3 Breeding Diet, Special Diet Services Ltd., Witham, Essex, UK) containing 0.25% Sodium and 0.66% Potassium. The animals were allowed free access to drinking water throughout the study. The animals were divided into two groups (n=6 per group). The treated group animals (Group A) had the ACE inhibitor perindopril (kindly provided by Servier Laboratories Ltd.) administered by gavage at a dose of 1.5 mg/kg/day dissolved in 1ml of water, for a period of 5 days. The control group animals (Group C) were given the vehicle (1ml water) daily for the same time period. Animals had daily weights and water

intakes measured. Four hours after the dose on day 5 of the study, the animals were killed by cervical stunning and decapitation. 1ml of blood was collected in pre-chilled syringes containing 100 μl of 10% K-EDTA and *ortho*-phenanthroline (final concentration 30 mM). The blood was immediately centrifuged at 4 °C and the plasma stored at a constant temperature of -20 °C until assayed for plasma renin concentration (PRC) and plasma AII concentration. Organs for RNA analysis were removed within a few minutes of death, snap-frozen in liquid nitrogen and stored at -70 °C prior to RNA extraction.

(ii). Measurement of PRC and plasma AII

PRC was measured by RIA of generated angiotensinogen I in the presence of excess plasma renin substrate. Plasma AII was measured by direct Radio Immunoassay (see section 5.12).

(iii). RNA extraction

The following tissues were collected for Northern blot analysis of RNA: brain, heart, liver, kidney, testes, adrenal, and aorta. Total tissue RNA was extracted by a modification of the lithium chloride / urea selective precipitation technique (Auffray and Rougeon, 1980) as described in section 5.6. For all tissues, apart from adrenal and aorta, RNA was prepared and analysed for individual animals. Due to the limited amount of material obtained per animal for adrenal and aorta, samples were pooled (n=6) for each group before RNA extraction.

(iv). Northern blot analysis

Analysis for renin and angiotensinogen mRNA was carried out by standard Northern blotting procedures as described in section 5.10, using 60 μg per sample. The probes used were a mouse submandibular gland renin cDNA (pSMG 5913) for renin kindly supplied by Professor W. Brammar, University of Leicester, and a rat liver angiotensinogen cDNA (pRang6) for angiotensinogen (Lynch *et al.*, 1986) kindly provided by Dr. K. Lynch, University of Virginia, Charlottesville, USA. Probes were labelled with α-^{32}P-dCTP by the random oligo-primer method as described in section 5.8.4. In both cases, blots were routinely washed post-hybridisation to a stringency of 0.5 - 0.1× SSPE at 60 °C and exposed to X-ray

film at -70 °C with intensifying screens. For internal control of even RNA loading and transfer, blots were stripped (see section 5.9.6) and reprobed with a cDNA for hypoxanthine guanine phosphoribosyl transferase (HGPRT) an enzyme of the purine salvage pathway constitutively expressed in most cells (Brennand, Konecki, and Caskey, 1983), kindly supplied by Dr. C. Thomas Caskey, Baylor College of Medicine, Houston, Texas, USA. Molecular weights of the bands† seen on the membranes were estimated from a concurrently run RNA molecular weight ladder (BRL/Life Technologies UK). Intensities of autoradiographic signals, reflecting the amount of specific mRNA, were quantitated by 2D densitometry scanning using an LKB Ultrascan 2222-010 Scanner (LKB-Produkter AB, Bromma, Sweden), and values for renin and angiotensinogen mRNA normalised by the corresponding HGPRT level before making comparisons. All samples were analysed at least three times.

† The apparent molecular weights of the mRNAs were: renin, 1.6 kb; angiotensinogen, 1.9 kb; HGPRT, 1.6 kb.

(v). RT-PCR analysis

The presence of renin mRNA in the various tissues was also evaluated by reverse transcription of total RNA followed by PCR amplification (RT-PCR) [Sambrook, Fritsch, and Maniatis, 1989]. Two pairs of renin specific oligonucleotide primers were used in the analysis: Pair 1 from exons 3 and 4 respectively of the gene; upstream primer (ACCCAG TACTATGGTGAGATCGGC) from nucleotides +276 to +299 and downstream primer (TCAATGGTGTAGAACCGACTCCTT) from nucleotides +518 to +495 in the rat renin cDNA sequence (Burnham *et al.*, 1987), and Pair 2 from exons 7 and 8 respectively; upstream primer (CACCTTGTTGTGTGAGGAGG) from nucleotides +854 to +873 and downstream primer (ATCTTCCTCTACAGCCCCTC) from nucleotides +1030 to +1011. In both cases, reverse transcription was carried out using oligo-dT (see section 5.11.1). For all tissues 5 μg of total RNA was used except for the kidney where 1 μg was used. For primer Pair 1, PCR was carried out for 30 cycles as follows: denaturation, 1 minute at 94 °C; annealing, 1 minute at 60 °C; extension 30 seconds at 72 °C. Fifty picomoles were used in

each reaction (total volume of reaction 100 μl) and the final magnesium concentration per reaction was 2.5mM. For primer Pair 2, PCR conditions were identical except for an annealing temperature of 65 °C. Following PCR, amplified products ($^1/_3$ of each reaction) were run out on 1.5% agarose gels containing ethidium bromide. Sizes of amplified products were determined from concurrently run DNA markers (1kb ladder, BRL/Life Technologies, UK). To confirm that the amplified product seen with both pairs of primers came from renin mRNA, the amplified products were transferred to nylon membranes (Hybond N, Amersham, UK) by Southern blotting (see section 5.9) and hybridised to pSMG 5913.

(vi). Statistical analysis

Where appropriate, results were compared using Student's unpaired t-test. $P < 0.05$ was considered statistically significant.

6.3 RESULTS

(i). Both control (Group C) and perindopril treated (Group A) animals showed a similar weight gain over the 5 days of the experiment (Table 6.1). Average daily water intake was also not different between the groups. Nevertheless, at 5 days PRC was markedly increased in the treated group while the circulating AII was reduced to a low level.

(ii). Figures 6A and 6B show the findings of the effects of perindopril treatment on renin mRNA levels. In the kidney, treatment caused a greater than four-fold average increase in renin mRNA level. In contrast, no significant change was observed in renin mRNA levels in any of the extra-renal tissues.

(iii). Figures 6C and 6D shows the corresponding findings for angiotensinogen mRNA. Even taking into account the fact that the very weak signals encountered in tissues such as the heart made accurate quantitation difficult, treatment was again not associated with a significant change in angiotensinogen mRNA levels in any of the tissues including liver and kidney.

(iv). The presence of renin mRNA in the tissues studied was independently confirmed by two different RT-PCR studies (Figure 6E). With each pair of primers, a product of precisely the size expected from renin mRNA (Pair 1, 243 base pairs; Pair 2, 177 base pairs) was amplified from all tissues. The derivation of the amplified product from renin mRNA was further confirmed in each case by hybridisation to pSMG 5913. By choosing primer pairs that spanned an intron for both amplifications, the possibility of getting an identical sized product from any contaminating DNA in the RNA sample was avoided. Amplification from genomic DNA would have yielded products of 901 base pairs (Primer Pair 1) and 441 base pairs (Primer Pair 2), respectively.

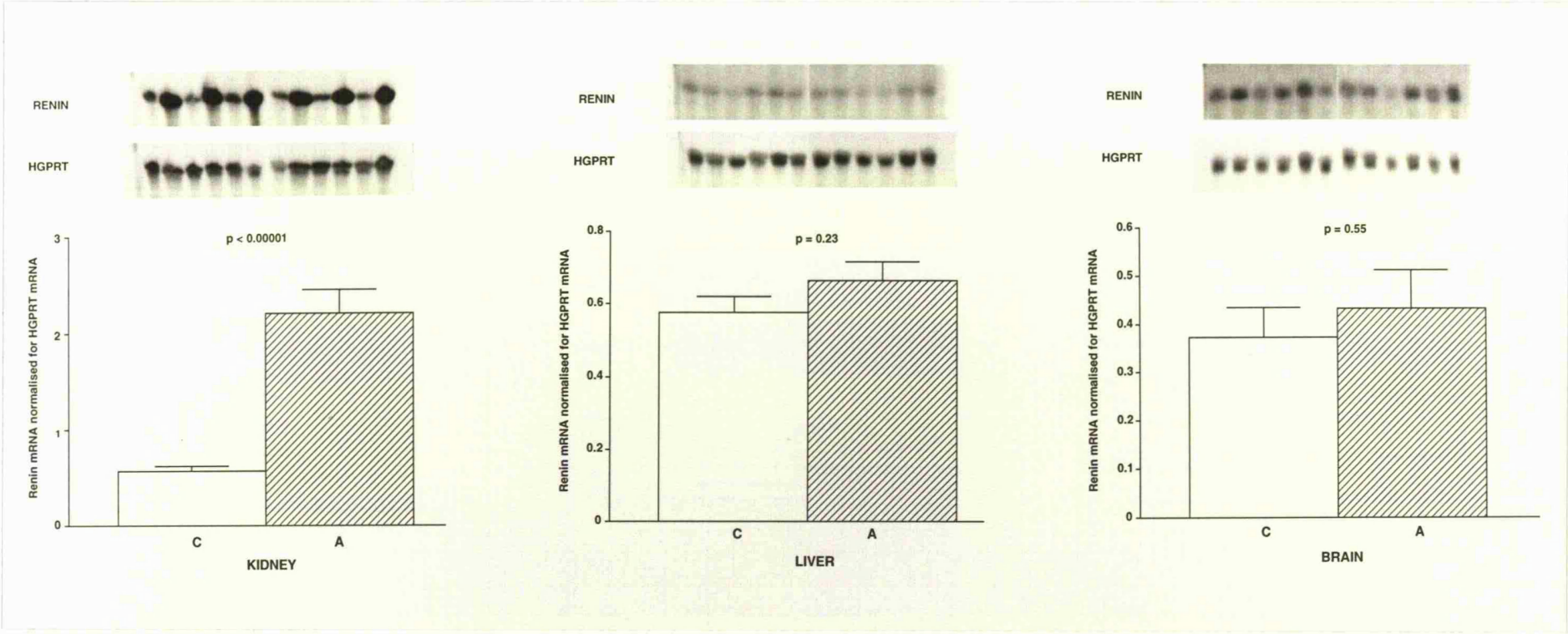
It is important to note that the RT-PCR analyses were designed to confirm the presence of renin mRNA and were not designed to be quantitative. e.g. After 30 cycles of PCR, the difference in kidney renin mRNA between control and perindopril treated rats demonstrated by Northern blotting (Figure 6A) was not apparent in the levels of amplified PCR product (Lanes 1-2, Figure 6E).

TABLE 6.1: Effects of 5 days perindopril treatment on weight, water intake, plasma renin concentration and plasma angiotensin II levels.

	C	A	*P*
Weight gain (gm)	28.0 ± 2.3	31.8 ± 2.4	> 0.05
Average daily fluid intake (ml)	31.2 ± 1.8	32.6 ± 2.2	> 0.05
PRC (ng AI/ml/hr)	9.4 ± 1.2	317.0 ± 46.8	< 0.001
AII (fmol/ml)	9.5 ± 1.8	< 1.0	

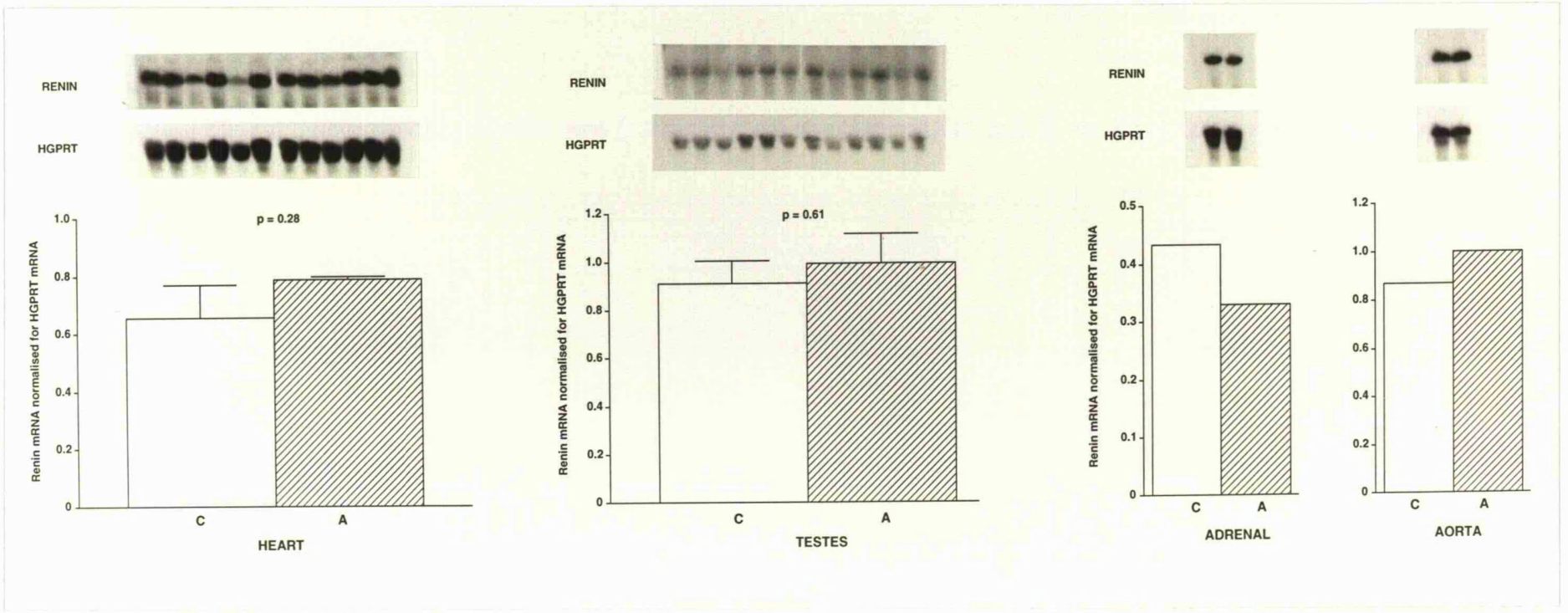
C, control treated animals; A, perindopril treated animals; PRC, plasma renin concentration; AII, plasma angiotensin II; *P*, probability; mean ± SEM are shown. Angiotensin II levels in all perindopril treated animals were below the upper limit of sensitivity of the assay (1.0 fmol/ml).

FIGURE 6A: Effects of 5 days perindopril treatment (1.5 mg/kg/day) on renin mRNA levels in rat kidney, liver and brain.



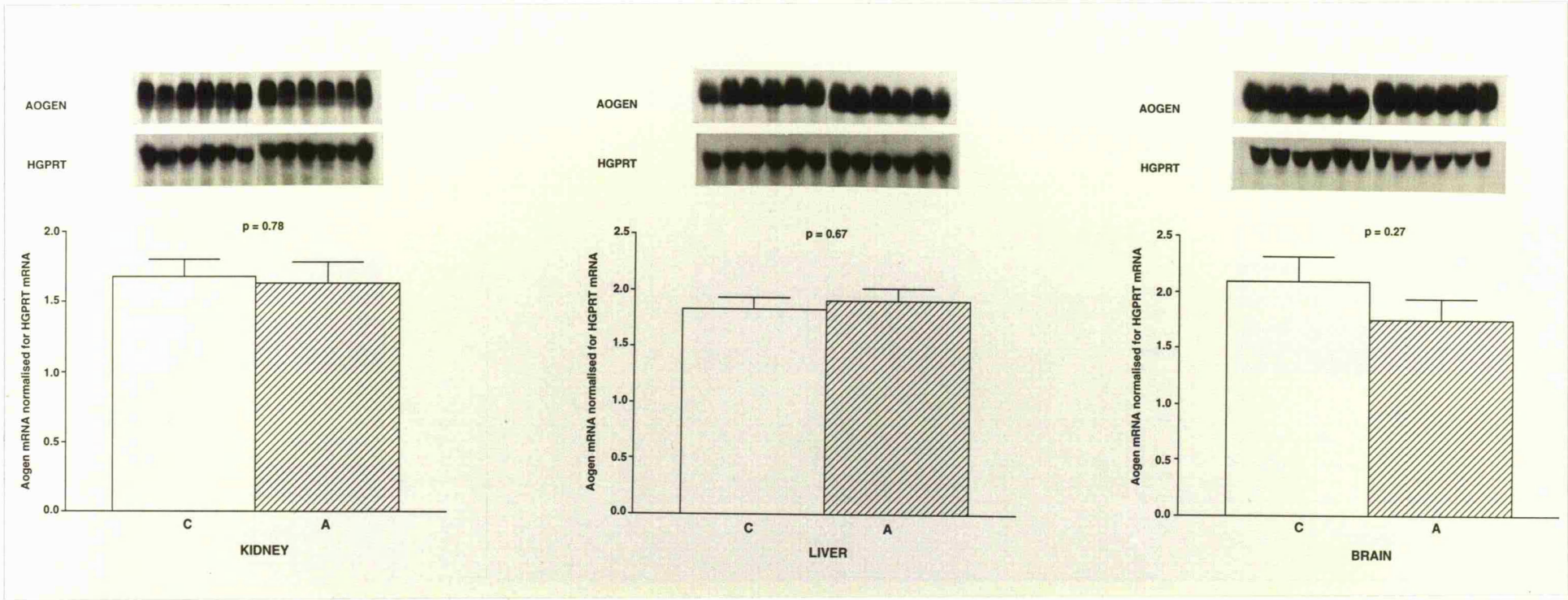
C, control treated animals; A, perindopril treated animals; HGPRT, hypoxanthine guanine phosphoribosyl transferase. For each tissue the upper panel shows the results of the probing with the renin probe and the lower panel the probing with the control HGPRT probe. For kidney, liver and brain, RNAs from control and perindopril treated animals were run in alternate lanes starting with a control sample. 60 μg of total RNA was loaded per sample. Autoradiograph exposure times following probing for renin mRNA were 24 hours for kidney gel and between 7-14 days for liver and brain. Following probing for HGPRT mRNA, the exposure times were between 1-3 days. The bar charts show the relative levels of renin mRNA in control and perindopril treated rats normalised for HGPRT mRNA (mean ± SEM are shown), expressed in arbitrary densitometric units specific to the individual gel. *P* values are from Student's t-test. Please note that levels across tissues run on different gels are **not** directly comparable.

FIGURE 6B: Effects of 5 days perindopril treatment (1.5 mg/kg/day) on renin mRNA levels in rat heart, testis, adrenal and aorta.



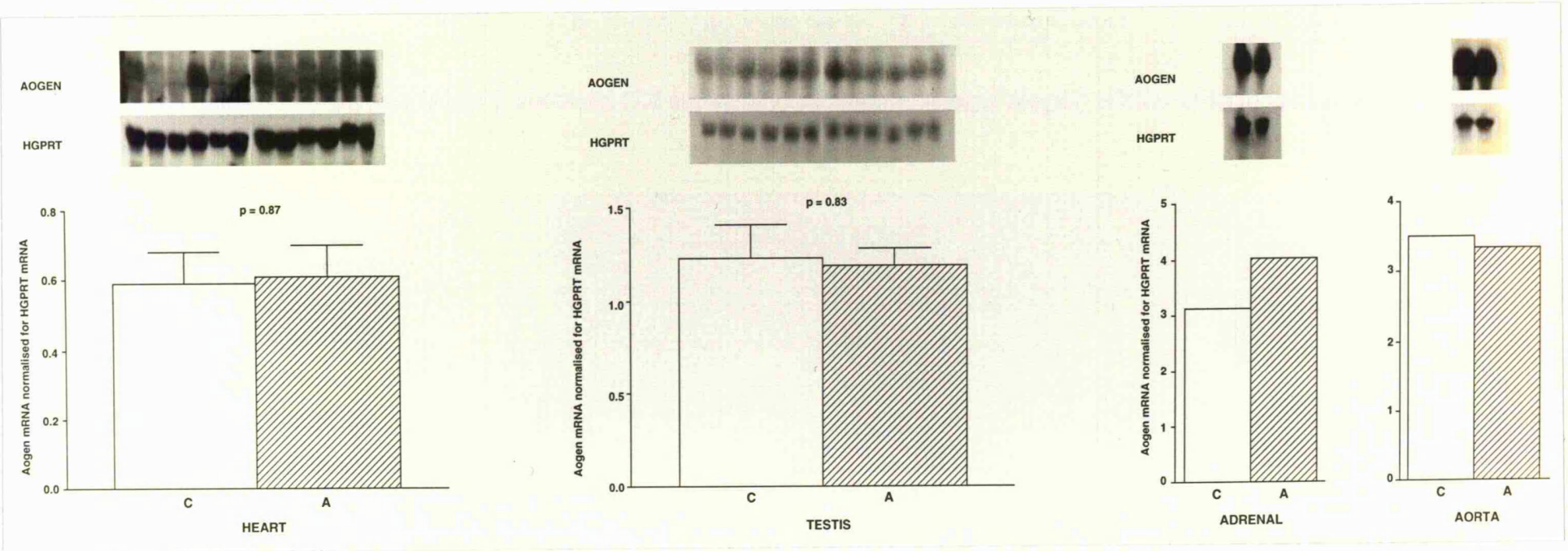
C, control treated animals; A, perindopril treated animals; HGPRT, hypoxanthine guanine phosphoribosyl transferase. For each tissue the upper panel shows the results of the probing with the renin probe and the lower panel the probing with the control HGPRT probe. For heart and testis, RNAs from control and perindopril treated animals were run in alternate lanes starting with a control sample. For adrenal and aorta single results are shown (as samples collected from all animals of each group were pooled prior to RNA extraction). 60 μg of total RNA was loaded per sample. Autoradiograph exposure times following probing for renin mRNA were between 7-14 days for heart, testis, adrenal and aorta. Following probing for HGPRT mRNA, the exposure times were between 1-3 days. The bar charts show the relative levels of renin mRNA in control and perindopril treated rats normalised for HGPRT mRNA (mean ± SEM are shown), expressed in arbitrary densitometric units specific to the individual gel. *P* values are from Student's t-test. Please note that levels across tissues run on different gels are **not** directly comparable.

FIGURE 6C: Effects of 5 days perindopril treatment (1.5 mg/kg/day) on angiotensinogen (aogen) mRNA levels in rat kidney, liver and brain.



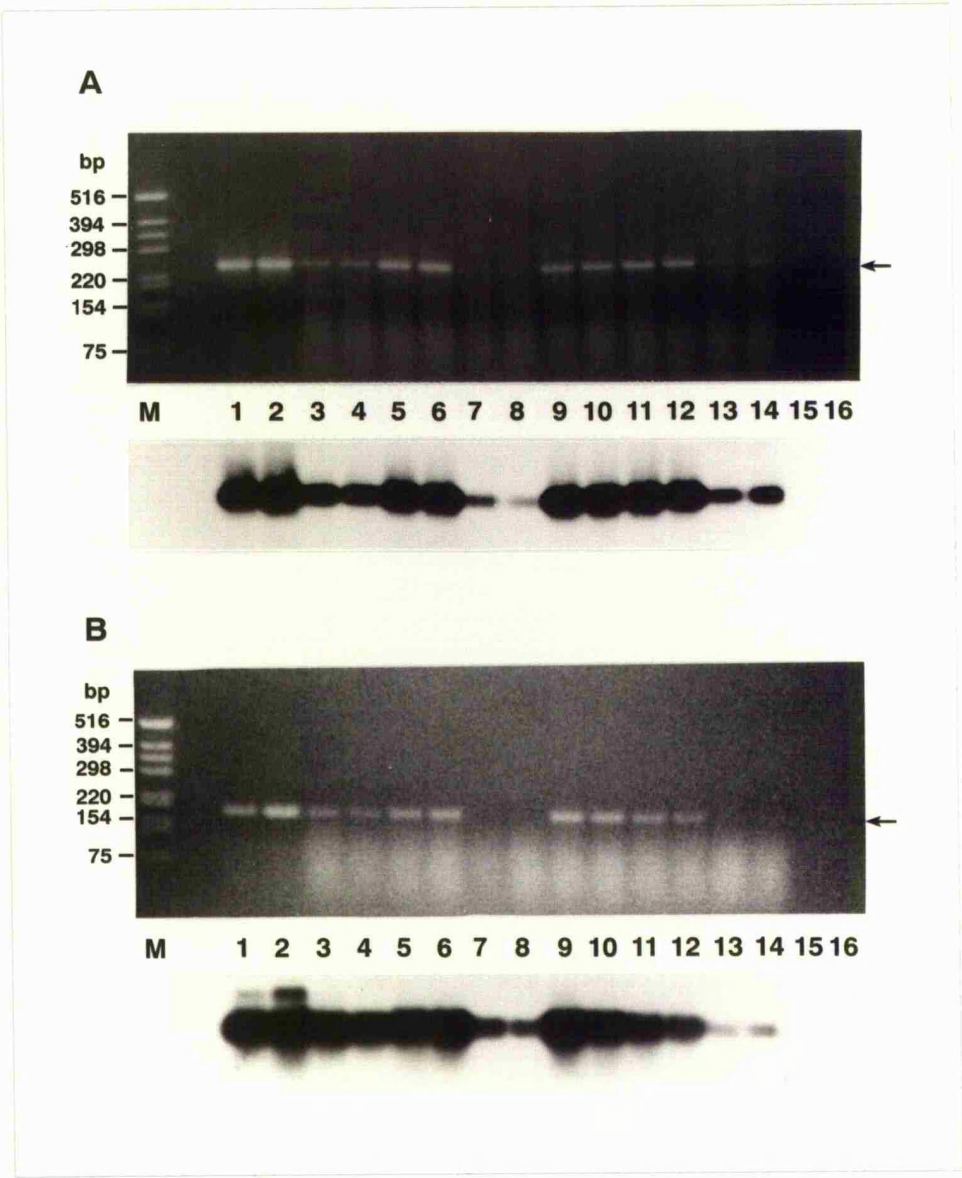
C, control treated animals; A, perindopril treated animals; HGPRT, hypoxanthine guanine phosphoribosyl transferase. For each tissue the upper panel shows the results of the probing with the angiotensinogen probe and the lower panel the probing with the control HGPRT probe. For kidney, liver and brain, RNAs from control and perindopril treated animals were run in alternate lanes starting with a control sample. 60 μg of total RNA was loaded per sample. Autoradiograph exposure times following probing for angiotensinogen mRNA were 8 hours for liver gel and between 1-2 days for kidney and brain. Following probing for HGPRT mRNA, the exposure times were between 1-3 days. The bar charts show the relative levels of angiotensinogen mRNA in control and perindopril treated rats normalised for HGPRT mRNA (mean ± SEM are shown), expressed in arbitrary densitometric units specific to the individual gel. *P* values are from Student's t-test. Please note that levels across tissues run on different gels are **not** directly comparable.

FIGURE 6D: Effects of 5 days perindopril treatment (1.5 mg/kg/day) on angiotensinogen (aogen) mRNA levels in rat heart, testis, adrenal and aorta.



C, control treated animals; A, perindopril treated animals; HGPRT, hypoxanthine guanine phosphoribosyl transferase. For each tissue the upper panel shows the results of the probing with the angiotensinogen probe and the lower panel the probing with the control HGPRT probe. For heart and testis, RNAs from control and perindopril treated animals were run in alternate lanes starting with a control sample. For adrenal and aorta single results are shown (as samples collected from all animals of each group were pooled prior to RNA extraction). 60 μg of total RNA was loaded per sample. Autoradiograph exposure times following probing for angiotensinogen mRNA were between 12-14 days for heart and testis and between 1-2 days for adrenal and aorta. Following probing for HGPRT mRNA, the exposure times were between 1-3 days. The bar charts show the relative levels of angiotensinogen mRNA in control and perindopril treated rats normalised for HGPRT mRNA (mean ± SEM are shown), expressed in arbitrary densitometric units specific to the individual gel. *P* values are from Student's t-test. Please note that levels across tissues run on different gels are **not** directly comparable.

FIGURE 6E: Demonstration of renin mRNA in rat tissues using RT-PCR.



Panel A shows the results obtained using primer pair 1 (from exons 3 and 4) and Panel B the results obtained using primer pair 2 (from exons 7 and 8). In each case the upper part of the panel shows the amplified PCR products on ethidium-bromide stained agarose gels and the lower part, the results of hybridisation with renin probe pSMG 5913 after Southern transfer of the PCR products (autoradiograph exposure times < 2 hours). M, marker track (fragments from 1 kb ladder, BRL/Life Technologies); Samples: 1-2 kidney; 3-4 liver; 5-6 brain; 7-8 heart; 9-10 testis; 11-12 adrenal; 13-14 aorta. For each tissue, the first sample is from a control rat and the second from a perindopril-treated rat. Tracks 15-16 control reactions: 15 RT-PCR in absence of any RNA; 16 PCR of kidney RNA without RT step. Arrows point to the amplified products of 243 bp (Panel A) and 177 bp (Panel B) respectively. Although the amplified product was in some cases not easily visible on the agarose gel, in all tissues the hybridisation with pSMG 5913 confirmed its presence.

6.4 DISCUSSION

Ever since the 1970's there has been controversy regarding the existence and functions of tissue renin-angiotensin systems. Initial concerns centered around specificity of biochemical assays for renin (Reid, 1977). Later concern shifted to the source of the renin detected in tissues - whether it was taken up from the circulation or synthesised locally (Loudon *et al.*, 1983). The application of molecular-genetic techniques and the demonstration of (pro)renin mRNA in various tissues in the mid to late 1980's and early 1990's (Dzau *et al.*, 1987a, 1987b; Paul *et al.*, 1988; Samani, Swales, and Brammar, 1988; Ekker, Tronik, and Rougeon, 1989; Okura *et al.*, 1991; Lou *et al.*, 1991; Holycross *et al.*, 1992; Iwai and Inagami, 1992; Della Bruna *et al.*, 1993; Lou, Robinson, and Morris, 1993) suggested that at least part of the renin detected in tissues was produced locally. However, even this finding has proved controversial because of the variability reported in terms of both the ability to detect renin mRNA in extra-renal sites and the ease with which this could be done. The reasons for the inconsistency are not clear but may reflect methodological as well as important strain/species differences.

The detection of renin mRNA in tissues of the animals of this study is in agreement with most of the studies mentioned above. Several features support the view that the mRNA species detected by Northern blotting in the various tissues in this study was indeed that for renin: (i). Only a single mRNA band was seen in all tissues. It has previously been shown in Southern blot analysis of rat genomic DNA that, under similar conditions to those used here, pSMG 5913 only binds to DNA fragments containing portions of the renin gene and does not detect heterologous genes (Samani, Brammar, and Swales, 1989); (ii). The band was of the anticipated size for renin mRNA (1.6 kb); (iii). The band detected in the extra-renal tissues co-migrated with the band from kidney RNA which was included as a positive control in all blots; (iv). The kidney mRNA species detected showed positive modulation with ACE inhibition which is precisely what would be expected for renin mRNA (Figure 6A). In addition, the presence of renin mRNA in all tissues studied was confirmed using an alternate technique - RT-PCR. Oligonucleotide primer pairs, located in two different regions of the renin transcript, amplified products of the predicted sizes which hybridised to pSMG 5913.

The widespread expression of renin mRNA in rat tissues has also been demonstrated previously using a further technique, RNAase-protection (Samani, Swales, and Brammar, 1988).

The results indicate that there are differences in the control of renin gene expression in renal and extra-renal tissues. Specifically, they suggest that, at least in the short term, a fall in circulating AII does not markedly affect renin gene expression in several extra-renal tissues. These findings are consistent with previous observations on the effects of changes in dietary salt intake on renal and extra-renal renin RNA levels (Samani, Swales, and Brammar, 1988). Taken together with the results of other studies which have shown similar dissociation in the effects of other acute stimuli (Catanzaro, Mesterovic, and Morris, 1985; Dzau *et al.*, 1987a; Suzuki *et al.*, 1988) the findings support the concept that, even if they ultimately serve similar functions, the circulating and tissue RA systems differ: The circulating system acting to maintain acute cardiovascular homeostasis while the tissue system works more chronically (Dzau, 1987).

Apart from interruption of the negative feedback effect of AII on the renin producing juxtaglomerular (JG) cells of the kidney, stimulation of the renal baroreceptor as well as other mechanisms (Skott and Jensen, 1993) may contribute to the increase in kidney renin mRNA by ACE inhibition. At a molecular level, this increase most likely represents increased transcriptional activity of the renin gene, although this study cannot exclude changes in renin mRNA turnover. Gomez *et al.* (1990) have also reported an additional mechanism that can operate - namely recruitment of afferent arteriolar cells upstream of the JG cells to produce renin.

A notable finding of this study was that while kidney renin mRNA increased four-fold in the perindopril-treated group, PRC increased by over 30 fold (Table 6.1). Such disproportional changes in kidney renin mRNA and PRC have been reported both previously (Nakamura *et al.*, 1985; Ludwig *et al.*, 1987) and since this study was undertaken (Morishita *et al.*, 1993) in relation to both ACE inhibition and other stimuli; and indicate important post-transcriptional regulation of renin production and secretion by the kidney. Considerable

evidence now exists that, in particular, the secretion of renin is complexly regulated and can occur through multiple pathways (Dzau, Burt, and Pratt, 1988).

The distribution of angiotensinogen mRNA found in the animals in this study agrees with previous reports in rats (Campbell and Habener, 1986; Ohkubo *et al.*, 1986). Although the size of the band (~ 1.9 kb) detected by Northern blotting in different tissues did not vary, it was quite broad (Figures 6C and 6D). Minor heterogeneity of rat angiotensinogen mRNA transcripts due to the presence of multiple closely spaced 3' polyadenylation sites has been reported (Ohkubo *et al.*, 1986) and the broadness of the band most likely simply reflects this. Any functional significance of such heterogeneity remains to be clarified but with regard to potential tissue specific effects it is notable that the structure of transcripts in different tissues have not been shown to differ (Ohkubo *et al.*, 1986).

Situations in which circulating AII is perturbed have previously been reported to affect both liver (Iwao *et al.*, 1988; Iwao *et al.*, 1990) and kidney angiotensinogen (Ingelfinger *et al.*, 1986; Iwao *et al.*, 1988) mRNA levels. Further, direct infusion of AII has been shown to stimulate liver and renal angiotensinogen synthesis (Nakamura *et al.*, 1990; Schunkert *et al.*, 1992a) and a single dose of the ACE inhibitor captopril or infusion of the AII antagonist saralasin to transiently decrease the level of liver angiotensinogen mRNA (Nakamura *et al.*, 1990). Therefore, the lack of effect of perindopril on angiotensinogen mRNA levels in the liver and kidney in this study, despite the marked suppression of circulating AII, was somewhat surprising. However, with ACE inhibitor treatment for several days, factors other than AII that may affect angiotensinogen synthesis, such as renin itself (Hermann and Dzau, 1983) and aldosterone (Chang and Perlman, 1987), are also altered and the overall absence of an effect may simply reflect a balance of inhibitory and stimulatory factors. In the kidney, Gomez *et al.* (1990) similarly found that 5 days treatment with the ACE inhibitor enalapril had no effect on the angiotensinogen mRNA level and, likewise, Schunkert *et al.* (1992a) reported liver and renal angiotensinogen mRNA levels to be unaffected in male Sprague-Dawley rats after quinapril treatment for three days. However, it needs to be emphasised that under other conditions than those used here, ACE

inhibition may have an effect on renin/angiotensinogen gene expression. Thus ACE inhibition has been reported to normalise the increased renal angiotensinogen gene expression that occurs in rats with experimental heart failure (Schunkert *et al.*, 1992b) or in those receiving a low sodium diet (Iwao *et al.*, 1988), and expression of renin mRNA has been found to be switched on by enalapril in the atrium in female Wistar rats on a low sodium diet (Lou, Robinson, and Morris, 1993).

The lack of effect on renin/angiotensinogen gene expression in some sites could also reflect inadequate interruption of an important local AII feedback loop because of either the relatively short period of ACE inhibitor treatment and/or poor tissue penetration of perindopril in some sites, e.g. across the blood-brain and blood-testicular barriers (Sakaguchi *et al.*, 1988; Johnston *et al.*, 1989). In this context, it is notable that in several extra-renal sites there was a trend towards higher renin mRNA levels in the ACE inhibitor treated group, although none achieved statistical significance (Figures 6A and 6B). The effect of longer term ACE inhibition needs to be studied before one can totally rule out any consequence. This is particularly relevant as many of the clinical benefits of ACE inhibitor treatment (Brunner, Gavras, and Waeber, 1979; Gavras *et al.*, 1981; The CONSENSUS Trial Study Group, 1987; Asmar *et al.*, 1988; Parving, Hommel, and Smidt, 1988; The SOLVD Investigators, 1991; Dahlof, Pennert, and Hansson, 1992; Pfeffer *et al.*, 1992; The AIRE Study Investigators, 1993) are only apparent after prolonged treatment.

As noted previously, there is an additional source for renin and angiotensinogen present in tissues - uptake from the circulation. Indeed, there is now substantial evidence that the vast majority of circulating angiotensins (both AI and AII) are actually generated within tissues from components derived in this way (Campbell, 1985; Admiraal *et al.*, 1990). It is therefore logical to hypothesise that inhibiting such 'tissue' generation of AII is important in at least some of the effects of ACE inhibitors. The results of this study do not in any way impinge on the validity of this proposition. Nevertheless, they do raise important questions regarding the organisation of tissue renin-angiotensin systems and particularly the fate of the locally synthesised components (Samani and Kelly, 1991). Three scenarios are possible:

(i). Although differently regulated, locally derived renin and angiotensinogen add to the extracellular pool of angiotensin generating capacity also contributed to by components derived from the circulation and ultimately subserve the same functions as the endocrine system; (ii). They form independent local renin-angiotensin systems (perhaps intracellularly) subserving functions entirely distinct from the circulating system; or (iii). Given that both (pro)renin and angiotensinogen share homology with proteins involved in a variety of other functions, they subserve functions not mediated via AII generation at all. The actual scenario may indeed vary from tissue to tissue. Whilst this study, and others reported in the vast amount of published literature shed light on the regulation of expression of the locally derived components, a better understanding of the functions of tissue renin-angiotensin systems is only likely to occur when more is learnt about their fate.

In conclusion, this study has for the first time systematically investigated the effects of ACE inhibition on expression of the genes for renin and angiotensinogen in several rat tissues. The results obtained raise interesting questions not only in terms of the role of tissue renin-angiotensin systems in mediating the effects of ACE inhibition, but more generally regarding their organisation.

CHAPTER SEVEN

Expression of components of the renin-angiotensin system during prolonged blockade at different levels in the marmoset

7.1 INTRODUCTION

The RA cascade offers several potential sites for blockade. To date, three different pharmacological modes of blockade of the RA system have been extensively studied: (i). renin inhibition, (ii). ACE inhibition and (iii). AII receptor antagonism (see section 2.3). These different modes of interference have a common goal in that they prevent AII exerting its physiological responses. However, blockade of the RA system at different points should highlight differences in the way that a specific class of inhibitor limits AII. The enzyme renin catalyses the first and rate-limiting step in the reaction cascade leading to the formation of AI. In contrast to the other enzymes in the cascade, renin has a unique substrate specificity; angiotensinogen is its only known naturally occurring substrate. Inhibition of renin therefore theoretically provides a very specific way to block the RA system. Angiotensin-converting enzyme (ACE), on the other hand, is an enzyme with a broad substrate specificity. In addition to AI, it cleaves other peptides such as bradykinin and substance P. ACE inhibitors may therefore influence cascades other than the RA system. AII-receptor antagonists, in contrast to renin inhibitors and ACE inhibitors, block the actions of AII rather than its formation. They therefore have the potential to block the effects of any AII generated via pathways other than the classical RA system (Samani and Kelly, 1991). As there is more than one subtype of the AII receptor, such antagonists, depending on their affinities for the various subtypes, may not block all of the actions of AII.

AII exerts a negative feedback on renin synthesis and release (Katz and Malvin, 1993). Therefore, agents blocking the synthesis or effects of AII might be expected to increase renin synthesis and release. After ACE inhibition, plasma concentrations of AII should decrease, but AI may increase. Renin concentrations should also increase. These predicted changes have been shown to occur after ACE inhibition (Johnston, 1984). After AII subtype 1 (AT_1) receptor antagonism, plasma concentrations of AI and AII should not decrease and may even increase. If the effects of AII on renin synthesis and release are mediated via the AT_1 receptor, renin should also increase. These predicted changes after AT_1 receptor antagonism have also been reported (Bunkenburg *et al.*, 1991). After renin inhibition, plasma concentrations of AI and AII should decrease. Although renin activity

should be inhibited, the amount of renin in the plasma can be expected to increase. Most studies with renin inhibitors performed in monkeys or humans have shown that circulating concentrations of renin are increased after renin inhibition (Wood *et al.*, 1985). Recently, however, renin synthesis and release were found to be actually inhibited in the marmoset with the nonpeptide renin inhibitor ES-8891 (Kitami *et al.*, 1991).

The purpose of this study was to compare the effects of blockade of the RA system at these three different levels not only on the circulating components of the RA system, but also on the expression of the renin and angiotensinogen genes, in a primate. The common marmoset (*Callithrix jacchus*) is a New World monkey that has been extensively used for drug safety testing, the study of developmental abnormalities and in recent times, cardiovascular research (McPhearson, 1980; Hearn, 1983). The marmoset was first used in cardiovascular research as a model for evaluating the effects of primate-specific renin inhibitors. As the first renin inhibitors were antibodies or large peptides (see section 2.3.2) that are difficult to produce, the small size of the marmoset (body weight being similar to that of the rat) was an advantage when evaluating the pharmacokinetics of compounds that were in such a limited supply.

As conflicting results have been reported with renin inhibitors (Wood *et al.*, 1985; Kitami *et al.*, 1991), the following two different types of renin inhibitor were chosen for these studies: CGP-29287, a potent synthetic renin inhibitor (Wood *et al.*, 1985), and R-3-36-16, a potent renin inhibitory monoclonal antibody (Wood *et al.*, 1986). The ACE inhibitor was benazeprilat (Kaiser *et al.*, 1990) and the AT_1-receptor antagonist was valsartan (Criscione *et al.*, 1993). Each inhibitor was given in a dose that had been previously shown to produce a near-maximum fall in blood pressure in conscious sodium-depleted marmosets (Criscione *et al.*, 1993; Wood *et al.*, 1985; Wood *et al.*, 1986). The compounds were given by continuous infusion to eliminate influences of the different pharmacokinetic properties of the various agents. Blood pressure was monitored by a new telemetry system designed to record continuously in conscious marmosets moving freely in their home cage (Schnell and

Wood, 1993). After 8 days the animals were killed, and blood and tissues were taken for humoral and mRNA measurements, respectively.

7.2 METHODS

(i). Animals and administration of drugs

Normotensive adult (4-7 years old) marmoset monkeys (*Callithrix jacchus*: Ciba, Sesseln, Switzerland) of both sexes weighing 300-400g were used. The animals were kept in family pairs in cages under controlled conditions (temperature 24 - 27 °C; relative humidity 50 - 80%; 12 hour day/night cycle) in the Cardiovascular Department, Ciba-Geigy Limited, Basal, Switzerland). They were fed a low-salt pellet diet (9 mmol/kg sodium; NAFAG, Gossau Switzerland) supplemented with fruit and were allowed free access to drinking water throughout the study. The animals were fed this diet for one week before beginning the drug treatments and during treatment to stimulate activity of the RA system and make the blood pressure sensitive to blockade of the RA system.

The various blockers of the RA system were given by continuous intraperitoneal infusion using osmotic minipumps (2001; Alzet, Palo Alto, CA, USA) implanted in the peritoneal cavity. The minipumps were implanted under anaesthesia (10 mg/kg ketamine supplemented with 0.75 mg/kg diazepam and 0.15 mg/kg atropine [intramuscular administration]). The ACE inhibitor benazeprilat was given in a dose of 10 mg/kg/day (n=6), the AT_1-receptor antagonist valsartan in a dose of 10 mg/kg/day (n=9), the renin inhibitor CGP-29287 in a dose of 30 mg/kg/day (n=7), and the renin inhibitory antibody R-3-36-16 in a dose of 10 mg/kg/day (n=6). As a control, a separate group of marmosets received vehicle only (0.9% saline, n=6). It has previously been shown that the doses used lower blood pressure in sodium-depleted marmosets to a similar and maximum extent (Criscione *et al.*, 1993, Wood *et al.*, 1985; 1986).

(ii). Experimental protocol

Blood pressure and heart rate were measured continuously for one week before and during the treatment period. The marmosets were killed on the 8th day of treatment, and

blood samples were taken for the measurement of the various biochemical factors. The kidneys and liver were removed within a few minutes of death, snap frozen in liquid nitrogen and stored at -70 °C prior to RNA extraction.

(iii). Measurement of blood pressure.

Mean arterial blood pressure was measured in the marmosets, while freely moving in their own cages, by a telemetry system (Data Sciences, St Paul, MN, USA) as described previously (Schnell and Wood, 1993). The pressure transmitters (AM model TA11PA-C40) were implanted in the peritoneal cavity with the sensor catheter placed in the aorta (below the renal artery) pointing upstream. The surgery was performed under anaesthesia induced by Saffan (10 mg/kg), supplemented with diazepam (0.75 mg/kg [intramuscular administration]) and atropine (0.15 mg/kg [intramuscular administration]). The marmosets were allowed at least one month for recovery before an experiment was performed. The marmoset cages were fitted with biotelemetry receivers, and blood pressure and heart rate were recorded continuously beginning one week before treatment. Values were averaged over periods. Delta blood pressure was calculated by using each animal as its own control i.e. each post-treatment value was subtracted from the value for the corresponding period of time in the pre-treatment period.

(iv). Measurement of biochemical parameters

Blood was collected on EDTA for the measurement of plasma renin activity (PRA), plasma total immunoreactive renin (TR), plasma active immunoreactive renin (AR), and plasma angiotensinogen (PRS). For the measurement of plasma concentrations of AII, blood was collected into pre-cooled syringes containing an enzyme inhibitor solution (Bunkenburg *et al.*, 1991) to prevent in vitro generation and/or degradation of AII. Heparinized blood was collected for the measurement of plasma concentrations of urea, creatinine, sodium and potassium.

Determinations of PRA were made by the AI antibody-trapping method (Poulson and Jørgensen, 1974). PRS was determined by incubation of plasma with an excess of human

renin. PRS was not determined in the R-3-36-16 group because, even with an excess of renin, it was not possible to overcome the inhibitory action of the antibody. The AI formed in the PRA and PRS assays was determined by RIA (Menard and Catt, 1972).

TR and AR were measured by immunoradiometric assay using antihuman renin monoclonal antibodies (Heusser *et al.*, 1987). The antibodies used to measure TR did not distinguish AR from inactive renin. One of the antibodies used in the assay for AR selectively binds AR. This assay could not be used to measure AR in plasma from animals treated with the renin inhibitor CGP-29287, as it interferes with this assay. Neither of the immunoradiometric assays could be used for plasma from the marmosets receiving R-3-36-16, since this antibody is used in both assays.

Plasma AII concentrations were measured by RIA after extraction of AII from the plasma with phenylsilyl silica and separation from other immunoreactive material by high-performance liquid chromatography (HPLC).

Plasma urea and creatinine were measured by photometric detection of products formed after enzymatic degradation of these substrates. Plasma electrolyte concentrations were measured by flame photometry.

(v). RNA extraction

Total cellular RNA was extracted from kidney and liver samples by a modification of the lithium chloride/urea selective precipitation technique (Auffray and Rougeon, 1980) as described in section 5.6. Tissues of four to five marmosets per group were used for Northern blot analysis.

(vi). Northern blot analysis

Analysis for renin and angiotensinogen mRNA was carried out by standard Northern blotting procedures as described in section 5.10, using 60 μg of total RNA per sample. The probes used were a 750 base pair human kidney renin cDNA encompassing exons 2-9 of the human renin gene (Hardman *et al.*, 1984) kindly supplied by Professor W. Brammar,

University of Leicester, and a rat liver angiotensinogen cDNA (pRang6) for angiotensinogen (Lynch *et al.*, 1986) kindly provided by Dr. K. Lynch, University of Virginia, Charlottesville, USA. Probes were labelled with α-^{32}P-dCTP by the random oligo-primer method as described in section 5.8.4. In both cases, blots were routinely washed post-hybridisation as described in section 5.10.4 and exposed to X-ray film at -70 °C with intensifying screens. For internal control of even RNA loading and transfer, blots were stripped (see section 5.9.6) and reprobed with a cDNA for hypoxanthine guanine phosphoribosyl transferase (HGPT) an enzyme of the purine salvage pathway constitutively expressed in most cells (Brennand, Konecki, and Caskey, 1983), kindly supplied by Dr. C. Thomas Caskey, Baylor College of Medicine, Houston, Texas, USA. Intensities of autoradiographic signals, reflecting the amount of specific mRNA, were quantitated by 2D densitometry scanning using an LKB Ultrascan 2222-010 Scanner (LKB-Produkter AB, Bromma, Sweden), and values for renin and angiotensinogen mRNA normalised by the corresponding HGPT level before making comparisons. All samples were analysed at least three times.

(vii). Statistical analysis.

Results for all treatment groups and all parameters were first compared by analysis of variance (ANOVA). Where this proved significant, pairwise comparisons were carried out using Tukey's test for multiple comparisons. As the variability of the biochemical and RNA results increased with the means of the values, analyses were carried out on log-transformed data. $P < 0.05$ was considered statistically significant.

7.3 RESULTS

(i). All of the compounds induced a significant reduction in blood pressure starting on the first day and persisting throughout the treatment period (Figure 7A). The total area under the change in blood pressure curve was slightly greater for the converting enzyme inhibitor-treated group than for the antibody-treated group ($P < 0.05$) but not significantly different from the other treatment groups.

(ii). Plasma urea tended to increase after CGP-29287, benazeprilat and valsartan but not R3-36-16 treatment (Table 7.1). This increase was only statistically significant for benazeprilat administration. Plasma creatinine also tended to increase after benazeprilat, valsartan and CGP-29287, but this was not statistically significant (Table 7.1). Plasma sodium and potassium concentrations were not changed by any of the treatments (data not shown). The more marked effects of benazeprilat on plasma urea were probably due to its slightly greater effect on blood pressure and therefore renal perfusion pressure.

(iii). Plasma concentrations of TR (AR and inactive renin) significantly increased (14- 20 fold) after all three modes of interference (Figure 7B). After benazeprilat and valsartan, AR also significantly increased ~ 100-fold (Table 7.1). The ratio of AR to TR increased approximately fivefold in these groups (Table 7.1). PRA also significantly increased after these two blockers and to a greater extent with the converting enzyme inhibitor (36- fold vs. 17-fold, Figure 7B). AR could not be measured in the marmosets treated with CGP-29287 or R-3-36-16 because the renin inhibitors interfere with this assay. PRA was not reduced in the CGP-29287 - treated marmosets compared with the control group. However, PRA was much lower in the CGP-29287 - treated animals than in the benazeprilat- or valsartan- treated animals, although the total number of renin molecules was similar after the three different modes of interference. Moreover, the ratio of PRA to TR was significantly lower in the CGP-29287 - treated animals than in the other groups (Table 7.1).

(iv). PRS was significantly reduced in the benazeprilat and valsartan - treated marmosets but not in the CGP-29287- treated animals (Figure 7C). Plasma concentrations of AII were significantly decreased in the CGP-29287, benazeprilat, and R-3-36-16- treated marmosets. The decrease in the R-3-36-16- treated group was significantly less than in the CGP-29287- and benazeprilat- treated groups. In contrast, AII was markedly increased in the valsartan- treated animals compared with all of the other groups (Figure 7C).

(v). The effects of the various treatments on kidney renin and angiotensinogen mRNA levels are shown in Figure 7D, and the densitometric data are summarised in Figure 7F. All four types of treatment had highly significant effects on renin mRNA levels (F =

65.49, $P < 0.001$). Effects ranged from an eightfold increase in the R-3-36-16 - treated group to a 15-fold increase in the valsartan - treated group (Figure 7F, top). On pairwise comparisons, the differences between the valsartan and R-3-36-16 treatment groups were statistically significant. Please note that whilst the renin signal in the control animals is weak, it was easily discernible on the original autoradiograph but has not reproduced well photographically. Kidney angiotensinogen levels showed considerable variations between individual animals (Figure 7D). However, there was no overall significant effect of any of the treatments ($F = 1.18$, $P = 0.35$; Figure 7F, middle).

(vi). The effects of the various treatments on liver angiotensinogen mRNA levels are shown in Figure 7E. Liver angiotensinogen mRNA level was ~ 30% higher compared with controls in the group of animals treated with CGP-29287 and between 30 and 40% lower in the other treatment groups (Figure 7F, bottom). ANOVA was significant ($F = 4.29$, $P = 0.012$). On pairwise comparisons the difference between the control group and the various treatment groups was not significant. However, there was a significant difference between the CGP-29287 - treated group and the other treatment groups.

PARAMETER	VEHICLE	CGP-29287	BENAZEPRILAT	VALSARTAN	R-3-36-16
Change in blood pressure (mmHg) [total area under change in blood pressure curve]	962 ± 678	-2,682 ± 347	-3,838 ± 298	-3,140 ± 243	-2,271 ± 428
Plasma urea (mmol/l)	6.8 ± 0.3	11.7 ± 1.7	15.1 ± 2.2	12.2 ± 1.8	4.2 ± 0.5
Plasma creatinine (mmol/l)	33.0 ± 3.0	41.0 ± 2.0	46.0 ± 7.0	47.0 ± 7.0	34.0 ± 5.0
Active renin (AR) [ng/ml]	0.1 ± 0.04	NA	9.8 ± 1.3	11.0 ± 2.4	NA
Active renin : Total renin (AR/TR)	0.06 ± 0.02	NA	0.28 ± 0.03	0.40 ± 0.06	NA
Plasma renin activity : Total renin (PRA/TR) [ng AI/hr/ng renin]	26.5 ± 6.2	1.7 ± 0.4	44.0 ± 5.0	27.0 ± 5.0	NA

TABLE 7.1: Physiological and biochemical parameters of study groups.
Vehicle (control); CGP-29287 (renin inhibitor); benazeprilat (angiotensin converting enzyme inhibitor); valsartan (Angiotensin II antagonist); R-3-36-16 (renin inhibitory monoclonal antibody). NA, not assayed (see text for details). Mean ± SEM are shown.

FIGURE 7A: Effects of 7 days continuous treatment of sodium-depleted marmosets with vehicle (control), benazeprilat (angiotensin I-converting enzyme inhibitor), CGP-29287 (synthetic renin inhibitor), valsartan (AII subtype I- receptor antagonist) and R-3-36-16 (human renin inhibitory monoclonal antibody) on mean arterial blood pressure (MABP) measured in conscious freely moving animals by telemetry.

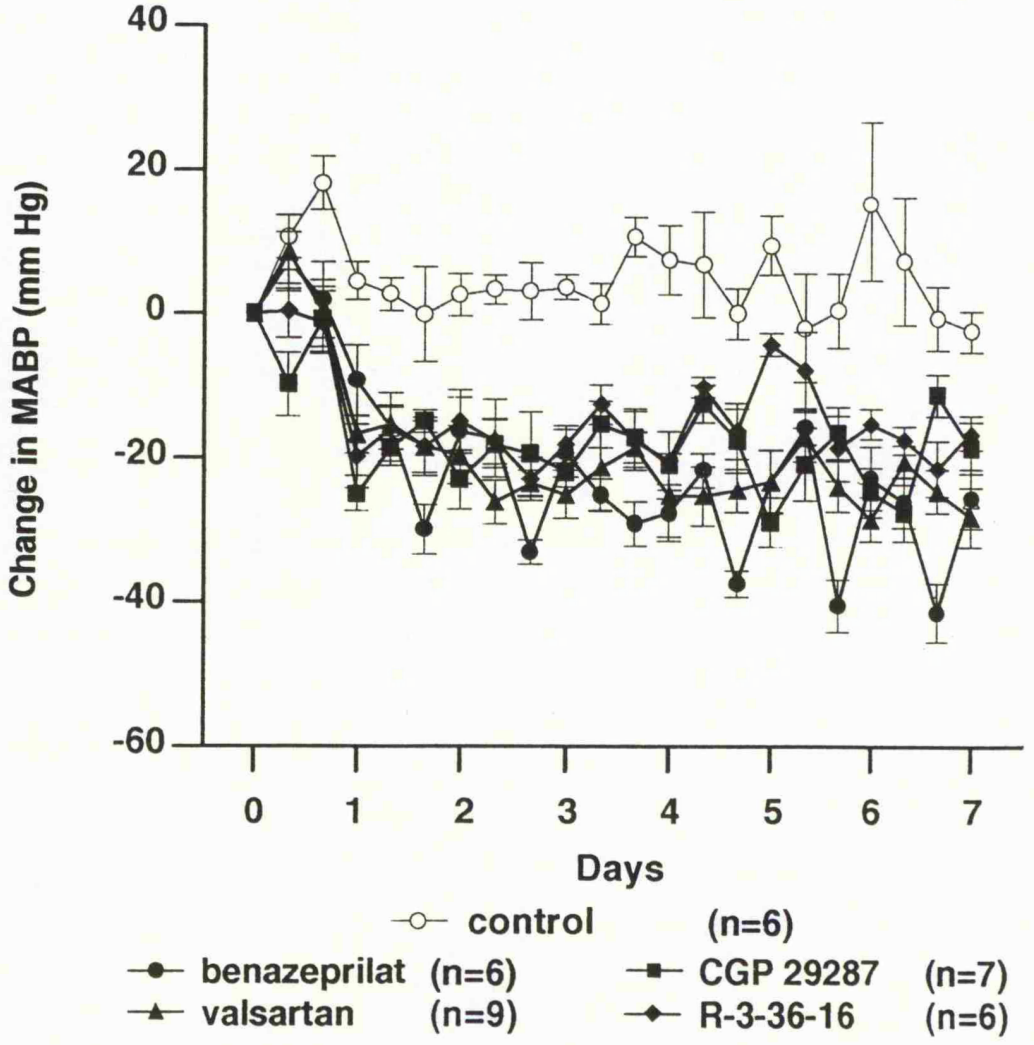
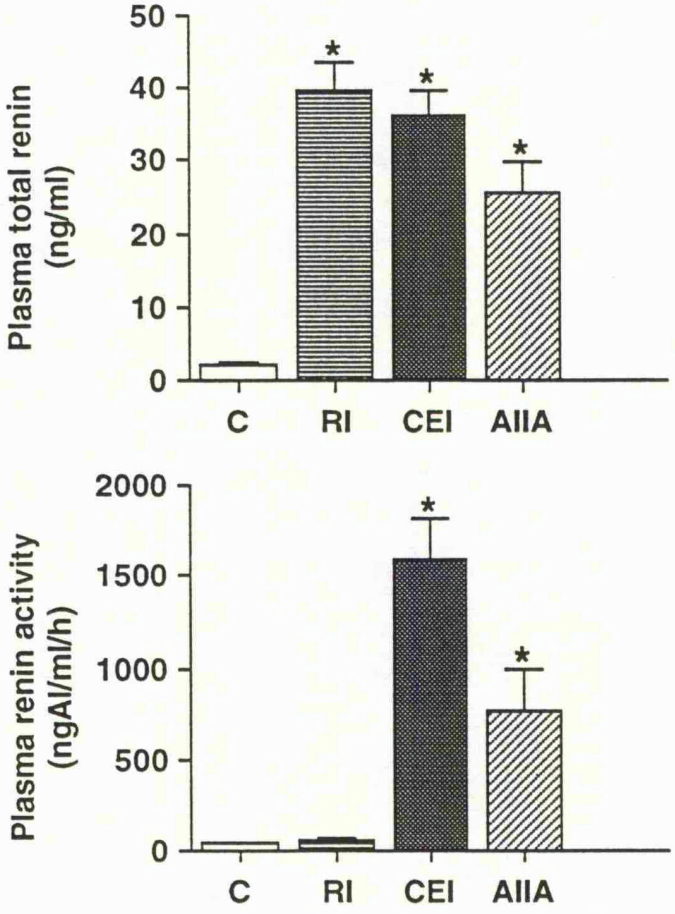
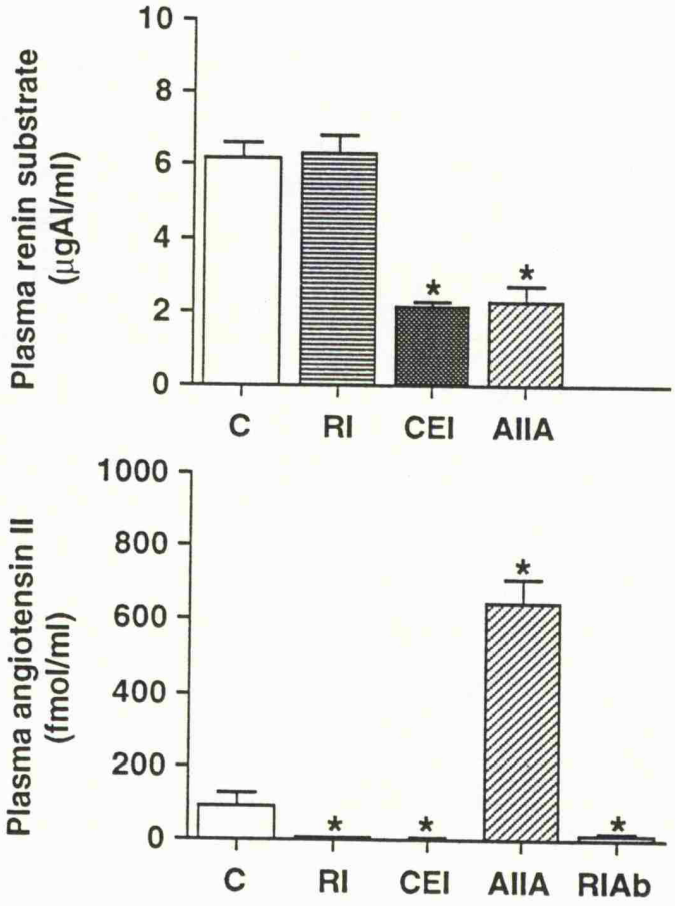


FIGURE 7B: Effects of 7 days continuous treatment of sodium-depleted marmosets with vehicle (control [C]), CGP-29287 (synthetic renin inhibitor [RI]), benazeprilat (angiotensin I-converting enzyme inhibitor [CEI]) and valsartan (AII subtype I-receptor antagonist [AIIA]) on total immunoreactive renin and plasma renin activity.



In animals treated with R-3-36-16 (human renin inhibitory monoclonal antibody), total immunoreactive renin or plasma renin activity could not be measured. Mean ± SEM are shown. For each parameter, overall differences between groups by analysis of variance were highly significant ($P < 0.0001$). * Significantly different ($P < 0.05$) from control group on pairwise comparisons.

FIGURE 7C: Effects of 7 days continuous treatment of sodium-depleted marmosets with vehicle (control [C]), CGP-29287 (synthetic renin inhibitor [RI]), benazeprilat (angiotensin I-converting enzyme inhibitor [CEI]), valsartan (AII subtype I- receptor antagonist [AIIA]) and R-3-36-16 (human renin inhibitory monoclonal antibody [RIAb]) on plasma angiotensinogen (plasma renin substrate) and plasma AII concentrations.



In animals treated with R-3-36-16 (human renin inhibitory monoclonal antibody [RIAb]), only plasma AII could be measured. Mean ± SEM are shown. For each parameter, overall differences between groups by analysis of variance were highly significant ($P < 0.0001$).
* Significantly different ($P < 0.05$) from control group on pairwise comparisons.

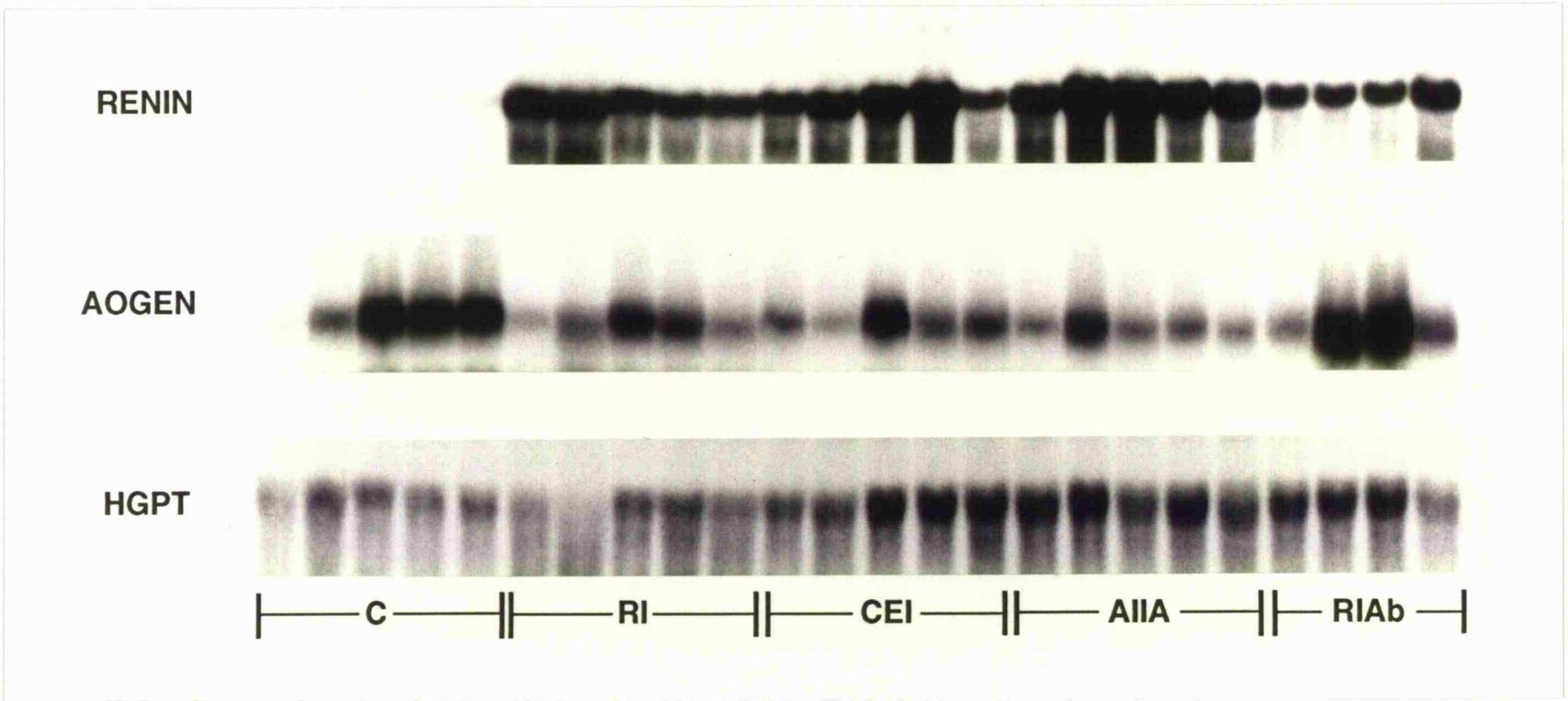


FIGURE 7D: Northern blot showing effects of 7 days continuous treatment of sodium-depleted marmosets with vehicle (control [C]); CGP-29287 (synthetic renin inhibitor [RI]), benazeprilat (angiotensin I-converting enzyme inhibitor [CEI]), valsartan (AII subtype I-receptor antagonist [AIIA]) and R-3-36-16 (human renin inhibitory monoclonal antibody [RIAb]) on kidney renin and angiotensinogen (AOGEN) mRNA levels.

Also shown are results of probing for control mRNA species, hypoxanthine guanine phosphoribosyl transferase (HGPT). Each track contains 60 μg of kidney RNA prepared from an individual animal. Exposure times of autoradiographs were as follows: renin (3 days); angiotensinogen (9 days); HGPT (2 days).

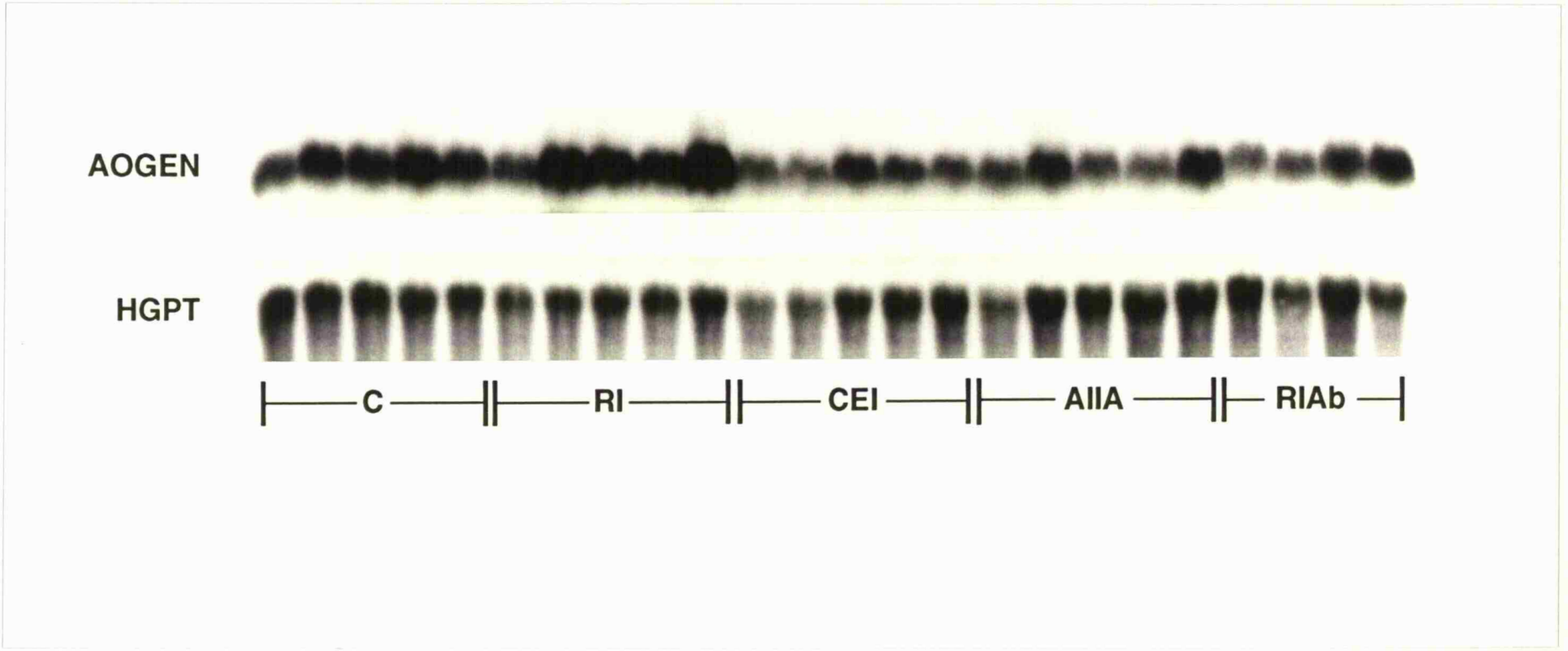


FIGURE 7E: Northern blot showing effects of 7 days continuous treatment of sodium-depleted marmosets with vehicle (control [C]); CGP-29287 (synthetic renin inhibitor [RI]), benazeprilat (angiotensin I-converting enzyme inhibitor [CEI]), valsartan (AII subtype I-receptor antagonist [AIIA]) and R-3-36-16 (human renin inhibitory monoclonal antibody [RIAb]) on hepatic angiotensinogen (AOGEN) mRNA levels.

Also shown are results of probing for control mRNA species, hypoxanthine guanine phosphoribosyl transferase (HGPT). Each track contains 60 μg of liver RNA prepared from an individual animal. Exposure times of autoradiographs were as follows: angiotensinogen (3 days); HGPT (2 days).

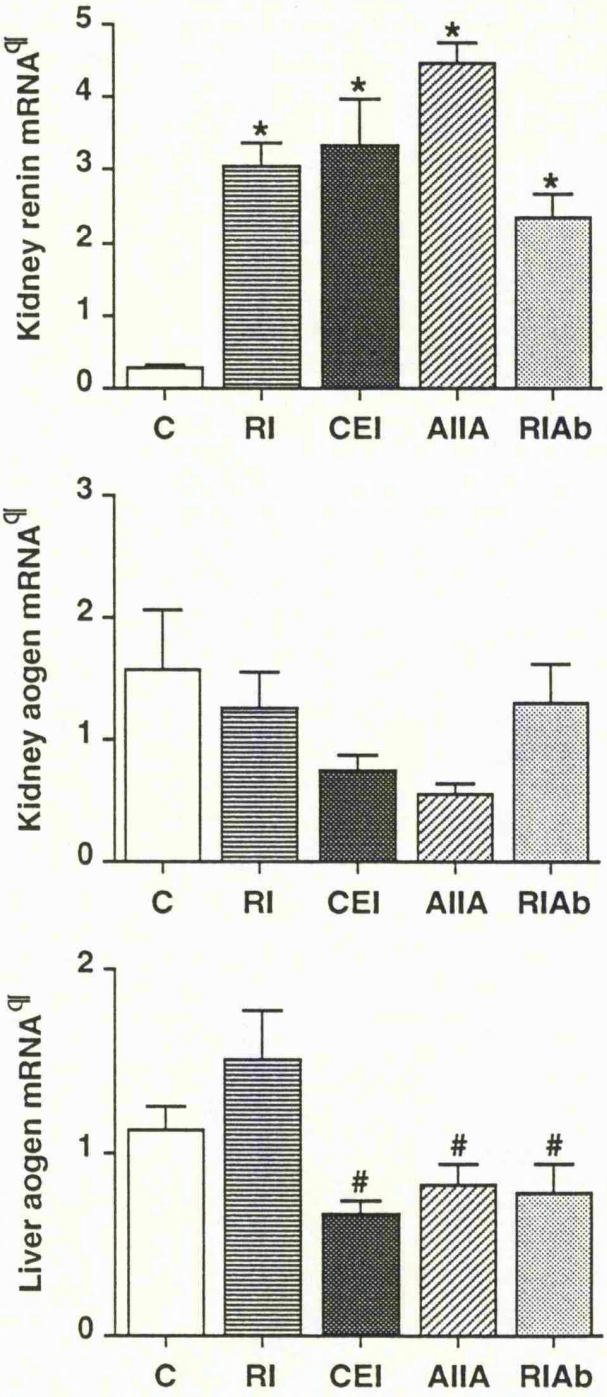


FIGURE 7F: Results of densitometric analysis of Northern blots (Figures 7D and 7E) assessing effects of 7 days continuous treatment of sodium-depleted marmosets with vehicle (control [C]), CGP-29287 (synthetic renin inhibitor [RI]), benazeprilat (angiotensin I-converting enzyme inhibitor [CEI]), valsartan (AII subtype I- receptor antagonist [AIIA]) and R-3-36-16 (human renin inhibitory monoclonal antibody [RIAb]) on kidney renin, angiotensinogen (AOGEN) and hepatic angiotensinogen mRNA levels. Mean ± SEM are shown. ¶ Normalised for hypoxanthine guanine phosphoribosyl transferase (HGPT) mRNA level, expressed in arbitrary densitometric units specific to the individual gel. * Significantly different ($P < 0.05$) from control group on pairwise comparisons. # Significantly different ($P < 0.05$) from RI group on pairwise comparisons.

7.4 DISCUSSION

Inhibition of ACE is now a well-established form of therapy in the treatment of hypertension and other cardiovascular disorders (Johnston, 1984). Renin and the AII receptor offer alternate target sites for inhibiting the RA system, and several compounds that act at these sites are currently being developed for clinical use. From a theoretical point of view, blockade of the RA system individually at the three levels would be predicted to have different effects on plasma levels of components of the system, depending not only on the enzyme inhibited (renin or ACE), but also from varying effects on renin and angiotensinogen synthesis (Hermann and Dzau, 1983). In turn, these differences could be relevant in determining the therapeutic effects of the drugs. The primary purpose of this study was to compare the effect of blockade of the RA system at various levels on expression and plasma level of components of the system. The decision to use a primate in this study was especially pertinent as all currently available renin inhibitors, unlike ACE inhibitors and AII-receptor antagonists, show high species specificity. The renin inhibitor CGP-29287 used in this study was developed for human use and has previously been shown to be highly effective in blocking marmoset renin (Wood, 1985). The results obtained from this study are therefore of special relevance to the human clinical situation. Furthermore, this study is the first in which a comparison of the full range of RA system inhibitors has been directly made under the same conditions in a primate.

In addition, to the biochemical measurements, effects on blood pressure were also determined by telemetry. All of the treatments produced a highly significant fall in blood pressure, indicating the major dependence of the blood pressure on the RA system under the conditions employed. In all cases, the fall in blood pressure was maintained throughout the experimental period. Overall, the effect on blood pressure was greatest in the group receiving benazeprilat and least in the animals receiving R-3-36-16. The former may reflect additional effects of the ACE inhibitor compared with the other agents, and in particular its inhibition of bradykinin metabolism (Johnston, 1984); the latter may reflect the relative lack of access of antibody to relevant sites of tissue angiotensin generation (Campbell, 1985), as indicated

by the lesser effect on plasma AII. However, as only one dose of each compound was tested, the small difference may just be related to different degrees of blockade of the RA system.

A predicted fall in plasma AII levels was seen after converting enzyme and renin inhibition. Treatment with the AT_1 receptor antagonist was conversely associated with a marked rise in plasma AII levels, this effect most probably reflecting the increase in PRA after such treatment.

All modes of inhibition were associated with an increase in plasma TR. The main cause of this is probably the removal of the negative feedback exerted by AII on renin release (Katz and Malvin, 1993). The finding that the rise in TR was similar in the valsartan- treated group as in the groups treated with benazeprilat and CGP-29287, suggests that the negative feedback of AII is mediated predominantly via the AT_1 receptor subtype. The fall in blood pressure seen in all treated groups was probably also a stimulus for renin release (Katz and Malvin, 1993). Other mechanisms including effects of the drugs on central pathways regulating renin release (Katz and Malvin, 1993; Kohara *et al.*, 1992) may also have played a role.

The results show that the increase in plasma renin is accompanied in all cases by an increase in kidney renin mRNA levels, suggesting that both increased synthesis and secretion are involved in causing the rise in plasma renin. The results do not, however, distinguish between whether the increase in kidney renin mRNA levels reflects an increase in renin gene transcription or a decrease in renin mRNA turnover.

The findings with benazeprilat are in agreement with several previous studies in rats that have shown that ACE inhibition is accompanied by an increase in kidney renin mRNA level (Nakamura *et al.* 1985, Gomez *et al.*, 1988; Iwao *et al.*, 1988; Kelly, Read, Swales and Samani, 1992; Morishita *et al.*, 1993). However, the results of blockade by CGP-29287 are in contrast to those obtained by Kitami and co-workers (1991) with another synthetic nonpeptide renin inhibitor, ES-8891. Using a very similar experimental protocol to that used in this study (apart from the drug being administered orally), they found a significant

decrease in plasma immunoreactive renin and a lower kidney renin mRNA content in ES-8891 - treated marmosets compared with control. They suggested that, because it is small (molecular weight, 795) and highly lipophilic, ES-8891 may penetrate the cell membrane to form a renin-ES-8891 complex within the juxtaglomerular cell itself. The complex may then reduce renin secretion from the cell and signal a reduction in the transcription rate of the renin gene, or an increase in the turnover rate of its transcript, resulting in the observed change in the level of renin mRNA. In contrast, CGP-29287 is hydrophilic and larger (molecular weight, 1,496), and its effect on renin production may only reflect its inhibition of extracellular AII formation.

Evidence for the mechanisms proposed by Kitami and co-workers (1991) remains to be obtained. As far as the endocrine RA system is concerned, the ultimate effects of CGP-29287 and ES-8891 are the same:- a decrease in circulating AII. However, if different types of nonpeptide renin inhibitors exert different effects on renin synthesis, this may have great relevance in another context. Recent studies have shown that the renin gene is expressed not only in the kidney but also in several extrarenal tissues (see section 3.5). The function of extrarenal renin remains largely unknown (Samani and Kelly, 1991). It is also unclear whether it is secreted, and there is evidence that in some sites it acts intracellularly to generate angiotensins (Inagami, 1993a). Thus renin inhibitors with varying access to the intracellular compartment may have markedly different effects on the synthesis and activity of renin produced in extrarenal tissues.

In addition to the rise in total renin, the ratio of active renin to total renin was increased after treatment with benazeprilat or valsartan. It is now clear that secretion of renin from the juxtaglomerular cells occurs through at least two pathways, a constitutive pathway, directly from the Golgi apparatus or the protogranules, and a regulated pathway involving exocytosis of mature secretory granules (see section 2.1.1). Prorenin is secreted principally through the constitutive pathway, whereas active renin is mainly released from the secretory granules, at least in humans. The finding that the ratio of active renin to TR was increased after treatment with benazeprilat or valsartan suggests either a relative switch to the regulated

pathway of kidney renin secretion after these treatments, or a more rapid rate of processing of prorenin to active renin, allowing its secretion via the constitutive pathway.

PRS (angiotensinogen) decreased to about one-third of the control level after benazeprilat or valsartan treatment. This was probably largely due to increased consumption of angiotensinogen by the accompanying high levels of renin. However, previous studies have shown that liver angiotensinogen secretion is also decreased by manoeuvres that suppress plasma AII and/or raise plasma renin (Hermann and Dzau, 1983). Liver angiotensinogen mRNA in rats has been shown to be increased by AII infusion (Kohara *et al.*, 1992; Nakamura *et al.*, 1990) and decreased by ACE inhibitor treatment (Iwao *et al.*, 1988, Nakamura *et al.*, 1990). Consistent with these observations, a decrease in liver angiotensinogen mRNA levels was seen in the benazeprilat- and valsartan-treated groups (Figure 7F, bottom) although these did not reach statistical significance. With CGP-29287 treatment, liver angiotensinogen mRNA levels increased despite the suppression of plasma AII. It has been shown that renin tonically inhibits liver angiotensinogen production (Hermann and Dzau, 1983), and the increase in liver angiotensinogen mRNA levels in this group most probably reflects the highly efficient suppression of plasma (and tissue) renin in much the same way as that achieved by nephrectomy, which also raises liver angiotensinogen production (Iwao *et al.*, 1990). In contrast, in the group treated with the renin antibody, the liver angiotensinogen mRNA level decreased. Plasma AII was slightly less suppressed in this group than in the other groups (Figure 7C), perhaps reflecting, as discussed earlier, reduced access of the antibody to tissue sites of angiotensin generation. The overall effect of renin antibody on liver angiotensinogen mRNA levels probably represents the net balance of its effect on inhibitory and stimulatory factors for liver angiotensinogen gene expression (Hermann and Dzau, 1983).

As in the case of renin, molecular studies have shown that the gene for angiotensinogen is also widely expressed, including in the kidney (Campbell and Habener, 1986). Previous studies in rats have shown kidney angiotensinogen mRNA levels to be increased by a low-salt diet (Inglefinger *et al.*, 1986; Iwao *et al.*, 1988). Results with ACE

inhibitor treatment have been conflicting with either a decrease (Iwao *et al.*, 1988) or no change (Gomez *et al.*, 1988). In this study, kidney angiotensinogen mRNA levels were found to be quite variable, but there was no overall effect of any of the treatments. It is possible that a stimulatory effect of the low-salt diet employed masked any effects. Nevertheless, the findings support previous observations suggesting that hepatic and renal expression of angiotensinogen are differently regulated (Inglefinger, Pratt, Ellison, and Dzau, 1986).

In conclusion, the various modes of inhibition of the RA system used in this study (the first such comparison in a primate) all produced a marked increase in renal renin synthesis and secretion, in keeping with the known role of AII in feedback regulation of renin synthesis. The various drugs had different effects on angiotensinogen mRNA levels in the liver, probably reflecting their different effects on renin activity and AII levels within the circulation and tissues. Kidney angiotensinogen mRNA level was not affected by any treatment, indicating different regulation of hepatic and renal expression of angiotensinogen.

CHAPTER EIGHT

Tissue expression of components of the renin-angiotensin system in experimental post-infarction heart failure in the rat: effects of heart failure and ACE inhibitor treatment

8.1 INTRODUCTION

Heart failure is a condition in which the pumping action of the ventricle of the heart is insufficient to supply blood at the rate required by metabolising peripheral tissues. As heart failure progresses, back pressure of blood combined with abnormal salt and water retention lead to congestion of the lungs and liver and accumulation of fluid in peripheral tissues (oedema). At this stage the heart failure is said to be congestive (CHF). The reduced flow of arterial blood from the heart may in extreme cases result in peripheral circulatory failure (cardiogenic shock). Heart failure may result from any situation that overloads, damages or reduces the efficiency of the heart muscle. Common causes are hypertension, coronary thrombosis, chronic disease of the heart valves and arrhythmias, and acute myocardial infarction (MI). In the clinical environment, CHF is a common complication of MI and is an important indicator of an adverse short and medium term prognosis (Pfeffer *et al.*, 1992; The AIRE Study Investigators, 1993). It usually occurs as a result of left ventricular dysfunction due to myocardial damage at the time of the MI and is associated with activation of neurohumoral compensatory mechanisms including the sympathetic nervous system and the renin-angiotensin (RA) system (Francis *et al.*, 1984).

Heart failure is one of the primary cardiovascular conditions in which aberrations of the RA system has been found. Activation of the RA system has been reported to occur as a compensatory mechanism in both clinical and experimental CHF, PRA and renal renin content being increased (Michel *et al.*, 1988). Plasma angiotensinogen has also been shown to be decreased in patients with severe heart failure (Arnal *et al.*, 1991). In addition, activation of the RA system is also thought to have some detrimental cardiovascular effects in heart failure arising from the peripheral vasoconstriction, aldosterone and vasopressin secretion, and sodium and water retention caused by the action of AII (see section 2.1.4).

Several recent large clinical trials have shown that treatment of individuals with post-infarction left ventricular dysfunction with an ACE inhibitor improves prognosis (The CONSENSUS Trial Study Group, 1987; The SOLVD Investigators, 1991; Pfeffer *et al.* [The SAVE-Study Trial Group], 1992; The AIRE Study Investigators, 1993). While the

haemodynamic sequelae of inhibition of the circulating RA system and prevention of degradation of the vasodilator peptide bradykinin may play a role, the mechanisms underlying the beneficial effects of ACE inhibitors in heart failure remain to be fully elucidated (see section 2.3.1.1). Specifically, there is current interest as to whether tissue RA systems are activated in post-infarction heart failure and whether ACE inhibitor treatment modulates tissue expression of the genes for various components of the RA system (Dzau, 1993). Studies to date have reported increased cardiac ACE activity and mRNA level (Hirsch *et al.*, 1991; Pinto *et al.*, 1993) and angiotensinogen mRNA (Lindpaintner *et al.*, 1993) in rodent models of experimental heart failure which may play an important role in the ventricular remodelling that occurs after a myocardial infarction. Less attention has focused on changes that may occur in other sites of RA system expression that may be of relevance to cardiovascular homeostasis in heart failure, and a systematic study of the regulation of gene expression of components of the RA system in heart failure has not been carried out to date. It was therefore decided to examine renin, angiotensinogen, ACE and AT_1 receptor gene expression at various tissue sites in a well-described rodent model of post-infarction heart failure and also to see whether such expression is modified by ACE inhibitor treatment. At the same time changes in circulating levels of components of the RA system were measured to compare and contrast with any changes at tissue sites.

8.2 METHODS

(i). Animals and induction of experimental myocardial infarction

An experimental model of heart failure may be induced in the rat by ligation of the left coronary artery to give consequent infarctions in the left ventricular wall. The resultant haemodynamic changes are comparable to those seen in man following myocardial infarction.

Myocardial infarction was induced in 20 weeks old male Wistar rats (~ 400 gm) following a modification of the surgical procedure of Selye *et al.* (1960). Briefly, the animal was anaesthetised using Halothane in oxygen/nitrous oxide. A thermometer was inserted into the animal's rectum and body temperature regulated using a heat-emitting lamp. The trachea

was dissected free and the animal intubated and placed on a positive pressure ventilator (Rodent ventilator, Hugo Sachs Elektronik). A 4cm incision was made midsternally, the pectoral muscles split and the thorax opened between the fifth and sixth ribs. The pericardium was severed and a curved needle with no. 6 silk was introduced near the insertion of the left auricular appendage and brought to the surface at the extreme left of the pulmonary cone no more than 2-3mm from the aorta to ensure that the left main coronary artery rather than a distal branch was ligated. The ligature was then tied and the success of the ligation indicated by observation of the anterior and lateral left ventricular wall becoming pale and hypodynamic and by dilatation of the left auricular appendage due to an acute rise in intracardiac pressure. The thorax was then closed by tying a thread around the fifth and sixth ribs and slight pressure applied to ensure that air and blood were excluded from the thorax. The pectoral muscles and skin incision were then stitched back together and the animal given pure oxygen via the ventilator. When respiration was sufficient, the animal was extubated and the trachea closed prior to the animal regaining consciousness. Preoperatively, the animal was given 0.1 gm (intraperitoneal administration) of ampicillin to prevent infection and 0.15 ml of Temgesic for two days postoperatively to reduce pain. Sham animals were operated on identically except for placement of the ligature.

Preliminary experiments indicated that this procedure resulted in an essentially 100% success rate of induction of a large myocardial infarction (average ~ 35-37% of left ventricular surface area) as calculated by the method of Chien *et al.* (1988). Briefly, the left ventricle was opened, a piece of acetate sheet pressed flat on its surface and the circumference of the ventricle outlined. The infarcted region was then outlined (both for endo- and epicardial surfaces). Infarct size was then calculated as a percentage of left ventricular surface area (averaged from endo- and epicardial surface infarcted areas).

(ii). ACE inhibitor treatment

The animals were allowed to recover postoperatively for 24 hours and survivors [100% (16/16) for sham operated rats; 96% (23/24) for MI rats] were then randomly assigned to either receive or not receive the ACE inhibitor, perindopril, 1.5 mg/kg/day given

in drinking water with adjustment of concentrations to ensure appropriate dosing on the basis of twice weekly assessment of fluid intake. The animals were housed singly for the duration of the experiments under controlled conditions (temperature 21 ± 1°C, humidity 60 ± 10%, 12 hour day/night cycle), humidity and light periods and had free access to standard rat chow containing 0.25% Na and 0.66% K, and water.

(iii). Blood Pressure measurement

Two days prior to sacrifice at 30 days, animals has their blood pressure measured indirectly using the tail cuff technique. Briefly, an 11 mm tubular inflatable cuff was placed near the proximal portion of the tail, while a pneumatic sensor connected to a pulse transducer (Physiograph; Marco Bio Systems Inc., Houston, Texas) was attached distal to the cuff. The cuff was connected to a calibrated electrosphygmomanometer which had set maxima for cut off inflation pressure and released the pressure automatically at a constant rate of 3mm Hg/second, while displaying the current occluding pressure. The pulse transducer was connected to a small amplifier which was input to an oscilloscope to visually observe the cut off and return of blood flow (pulse). The arterial pulsation was most easily detected when the vessels of the tail were fully dilated. To facilitate dilation of the vessels, the animals were placed in a warming chamber. The rats were first weighed to assess their size and placed in a suitable ventilated restraining tube which held them snugly. The restrained rats were placed five at a time in a rack that prevented them leaving their tubes but allowed the tail to emerge through a small opening. The rack was then kept in a large thermostatically controlled heating cabinet maintained at 32 °C for 20 minutes before readings were taken. Five consecutive consistent readings were taken and averaged per animal.

(iv). Blood and tissue collection

Animals were killed by cervical dislocation. Samples of blood were rapidly collected in pre-chilled tubes containing EDTA (for measurement of renin, angiotensinogen and angiotensin II) and lithium heparin (for measurement of plasma ACE). The tubes were immediately placed on ice and the plasma rapidly separated and stored in aliquots at -70 °C

until assays were carried out. Tissues were then rapidly removed, individually frozen in liquid nitrogen and stored at -70 °C pending RNA extraction. The lungs and right ventricle were weighed prior to freezing.

(v). Biochemical measurements

Plasma renin concentration (PRC), Plasma renin substrate (PRS), Plasma angiotensin II concentration and Plasma (and tissue) ACE activity were assayed as described in section 5.12.

(vi). RNA extraction and mRNA analysis

Total tissue RNA was extracted as by a modification of the lithium chloride/urea selective precipitation technique (Auffray and Rougeon, 1980) as described in section 5.6. For kidney, brain, liver and lung, RNA was prepared individually from 5 animals of each group. For left ventricle, right ventricle and adrenal because of limited amounts of material, samples were pooled from 2-3 animals before RNA extraction. RNA concentrations were determined by spectrophotometry at 260nm and prior to Northern blot analysis substantial degradation of RNA during extraction was excluded by running aliquots on agarose gels stained with ethidium bromide.

Northern blotting (60 μg of RNA per sample) and hybridisation were performed as described in section 5.10. The Northern membranes were probed in random order to determine mRNA levels for renin, angiotensinogen, ACE, angiotensin type 1 receptor (AT1R) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA (internal control) using the following probes: a 1.4 kb rat kidney renin cDNA pREN44.ceb† (Burnham *et al.*, 1987); a 1.6 kb rat liver angiotensinogen cDNA pRang6 (Lynch *et al.*, 1986) both kindly provided by Dr K Lynch, University of Virginia, Charlottesville, USA; a 3.3 kb human ACE cDNA (Soubrier *et al.*, 1988) kindly provided by Dr F. Soubrier, INSERM Unit 36, Paris, France; a 2.2 kb rat vascular angiotensin subtype IA receptor cDNA (Murphy *et al.*, 1991) kindly supplied by Dr K. Bernstein, Emory University, Atlanta, Georgia, USA; and a 1.2 kb human foetal liver glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA¶

(commercially available) kindly supplied by Dr. A. Hahn, University of Basel, Switzerland. All cDNA probes were radioactively labelled as described in section 5.8.4.

† This rat renin probe was unavailable at the beginning of this thesis and therefore pSMG5913, a mouse renin probe was used in the chapter six study. Extensive personal experiments were undertaken to compare these two probes and no difference in the tissue specific or drug modulated pattern of hybridisation in rats was found.

¶ GAPD was chosen as a replacement for HGP(R)T (as used in chapters six and seven) as an internal control as it was found to give a "cleaner" hybridisation (thus facilitating easier densitometric determination of signal) and exposure times were greatly reduced (allowing for a greater rate of reprocessing of filters). GAPD has also recently become the internal standard of choice for other groups in the field.

For all probes, blots were washed post-hybridisation to a stringency of 0.5 - 0.1 x SSPE and 0.1% SDS at 60 °C. Autoradiography was carried out at -70 °C using Kodak X-Omat AR film (Eastman Kodak, UK) and intensifying screens. Before reprobing a previous probe was stripped from the membranes as described in section 5.9.6. Molecular weights of the bands& seen on the membranes were estimated from a concurrently run RNA molecular weight ladder (BRL/Life Technologies UK). Intensities of autoradiographic signals were quantitated by 2D densitometry scanning using an LKB Ultrascan 2222-010 XL Scanner (LKB-Produkter AB, Bromma, Sweden). All samples to be compared were run, hybridised and autoradiographed together. To allow for variability related to loading and transfer, the densitometric value for each test mRNA was normalised by dividing with the corresponding GAPD mRNA value, before comparisons were made. All samples were analysed at least three times.

& The apparent molecular weights of the mRNAs were: renin, 1.6 kb; angiotensinogen, 1.9 kb; ACE, 4.2 kb; AT_1 receptor, 2.3 kb; GAPD, 1.4 kb.

(vii). Statistical analysis

Survival differences were analysed by Fisher's exact test. Results for quantitative variables were first compared by analysis of variance (ANOVA) for all groups. Where this proved significant, pair-wise comparisons were carried out using Tukey's test for multiple comparisons. Interactions between the effects of myocardial infarction and ACE inhibitor treatment were examined using general linear modelling using Minitab version 7.0. Spearman's rank correlation was used to analyse the relationship between lung weight and lung ACE mRNA level. For data, where the variability increased with the means of the values, analysis were carried out on log-transformed data. $P < 0.05$ was considered statistically significant.

8.3 RESULTS

(i). Four groups of rats were studied: A, sham operated rats with no ACE inhibitor (n=8); B, Myocardial Infarction (MI) rats with no ACE inhibitor (n=12); C, sham operated rats with perindopril treatment (n=8); D, MI rats with perindopril treatment (n=11). Table 8.1 shows the survival of the rats in the different groups at 30 days. There was no mortality in either of the sham operated groups, whereas mortality was 42% (5/12) in untreated MI rats ($P = 0.05$ compared with group A) which was reduced to 9% (1/11) in MI rats treated with perindopril ($P = 0.09$ compared with group B).

(ii). Body weight of survivors was not significantly different between the groups ($P = 0.362$) [Table 8.1]. Blood pressure was however significantly different between all groups ($P < 0.01$) with independent effects of both MI ($P < 0.001$) and ACE inhibitor treatment ($P < 0.001$) [Figure 8A]. Pulse rate was not significantly different between the groups ($P = 0.830$) [Table 8.1; Figure 8A]. Lung weights were markedly increased, about 2.5 fold on average, in the MI groups ($P < 0.001$) [Table 8.1; Figure 8B]. Although lung weight was slightly reduced in the groups receiving perindopril, this was not statistically significant. Likewise there was a marked increase in right ventricular (RV) weights in the MI groups ($P < 0.001$), with again no statistically significant effect of perindopril treatment (Table 8.1; Figure 8B).

(iii). The effects on circulating levels of components of the RA system in the different groups are shown in Table 8.2 and Figure 8C. Compared with sham operated rats (Group A), there was a nine-fold increase in plasma renin concentration (PRC) with MI alone (Group B, $P < 0.01$). PRC was massively increased more than hundred-fold by ACE inhibitor treatment in both groups C and D ($P < 0.001$) but there was no significant interaction between MI and ACE inhibitor ($P = 0.18$). Plasma angiotensinogen (PRS) was slightly but not significantly decreased by MI alone (Group B) but more than two-fold decreased in the groups receiving the ACE inhibitor treatment ($P < 0.001$). PRS was lowest in Group D and in pair-wise comparisons this was significantly different from all other groups. Across the groups there was a strong negative correlation ($r = - 0.71$, $P < 0.001$) between PRC and PRS levels. Plasma ACE level was unchanged with MI, but as expected, was markedly decreased (> 15-fold, $P < 0.001$) in both groups receiving perindopril (Table 8.2; Figure 8C). Plasma immunoreactive angiotensin II showed a small but insignificant rise with MI. Despite the marked suppression of plasma ACE activity, the AII level was not reduced in either group of rats treated with perindopril and indeed the highest level, was seen in group D rats (MI rats treated with perindopril). However, because of considerable inter-rat variability, there was no overall difference in plasma immunoreactive angiotensin II levels between the groups by ANOVA ($P = 0.17$).

(iv). Steady-state levels of mRNAs of components of the RA system in the kidney at 30 days in the different groups are shown in Figure 8D (n=5 per group). There was a two-fold ($P < 0.01$) increase in renin mRNA in animals with MI alone compared with sham controls, and an increase of 5-6 fold ($P < 0.001$) with ACE inhibitor treatment (Figure 8E). The highest levels were seen in animals with MI treated with perindopril. The effects of MI and treatment were both independently highly significant ($P < 0.001$) but not the interaction between the two ($P = 0.18$). Kidney angiotensinogen mRNA level was unchanged with MI but there was a relatively small but highly significant ($P = 0.002$) 1.6 fold increase with ACE inhibitor treatment (compare groups C+D v's A+B, Aogen panel, Figure 8D; Figure 8E). Angiotensin I receptor (ATIR) mRNA level was unchanged by either MI or ACE inhibitor treatment. mRNA for ACE was not detectable in the kidney using Northern blotting.

(v). Messenger RNA levels in the liver, brain and adrenal are shown respectively in Figures 8F-8H. Unlike in the kidney, there was no significant change in hepatic angiotensinogen mRNA level with ACE inhibitor treatment (or with MI). Likewise, there was no change in hepatic expression of ATIR or renin in any of the groups (Figure 8F). In the brain only angiotensinogen and renin mRNAs were detectable and there was no change in expression of either after MI or with perindopril treatment (Figure 8G). In the adrenal gland, only renin and ATIR mRNAs were detectable and again there was no alteration in the expression of either of these genes (Figure 8H).

(vi). In both the left and right ventricles only renin mRNA was detectable and there was no difference in the level of expression between the different experimental groups (Figures 8I and 8J).

(vii). In the lung, ACE mRNA level decreased by 50% ($P < 0.001$) after MI (Figures 8K and 8E). ACE inhibitor treatment had no effect either by itself or after MI (Figures 8K and 8E). In the MI group as a whole, there was a significant inverse relationship ($r = - 0.68$, $P = 0.029$) between lung weight and lung ACE mRNA level (Figure 8L). ATIR mRNA was unchanged in any of the groups. Renin‡ and angiotensinogen mRNA levels were not detectable.

‡ Although a faint signal could be seen with some lung RNA samples e.g. the positive control used in Figure 8I, this was not a consistent observation between samples and was most probably due to lung renin mRNA being on the limits of detectability of Northern blotting. Therefore renin mRNA in lung, as detected by Northern blotting, has been reported as a negative result.

(viii). To investigate whether the observed change in lung ACE mRNA level with MI was associated with a change in ACE activity, a further series of sham (group A) and infarct rats (group B) [n=5 per group] were studied. Pulmonary ACE activity was found to be significantly lower in animals with MI than sham-operated rats ($P < 0.01$) [Figure 8M]. In

contrast, renal ACE activity was much lower than pulmonary ACE activity and was unchanged after MI.

TABLE 8.1: Characteristics of studied rats.

	Group			
	A	**B**	**C**	**D**
n	(8)	(12)	(8)	(11)
Mortality	0 (0%)	5 (42%)	0 (0%)	1 (9%)
Body weight (gm)	426 ± 18	424 ± 22	437 ± 11	401 ± 9
Blood pressure (mmHg)	125 ± 5	89 ± 4	105 ± 4	80 ± 3
Pulse rate (bpm)	395 ± 11	392 ± 11	392 ± 7	402 ± 11
Lung weight (mg)	1895 ± 216	4638 ± 572	1714 ± 115	4411 ± 503
RV weight (mg)	196 ± 10	508 ± 57	209 ± 10	458 ± 32

A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment. Mean ± SEM or (number / %) are shown. Body weight, blood pressure, pulse rate, lung weight and right ventricle (RV) weight are given for animals that survived to the end of the study (30 days).

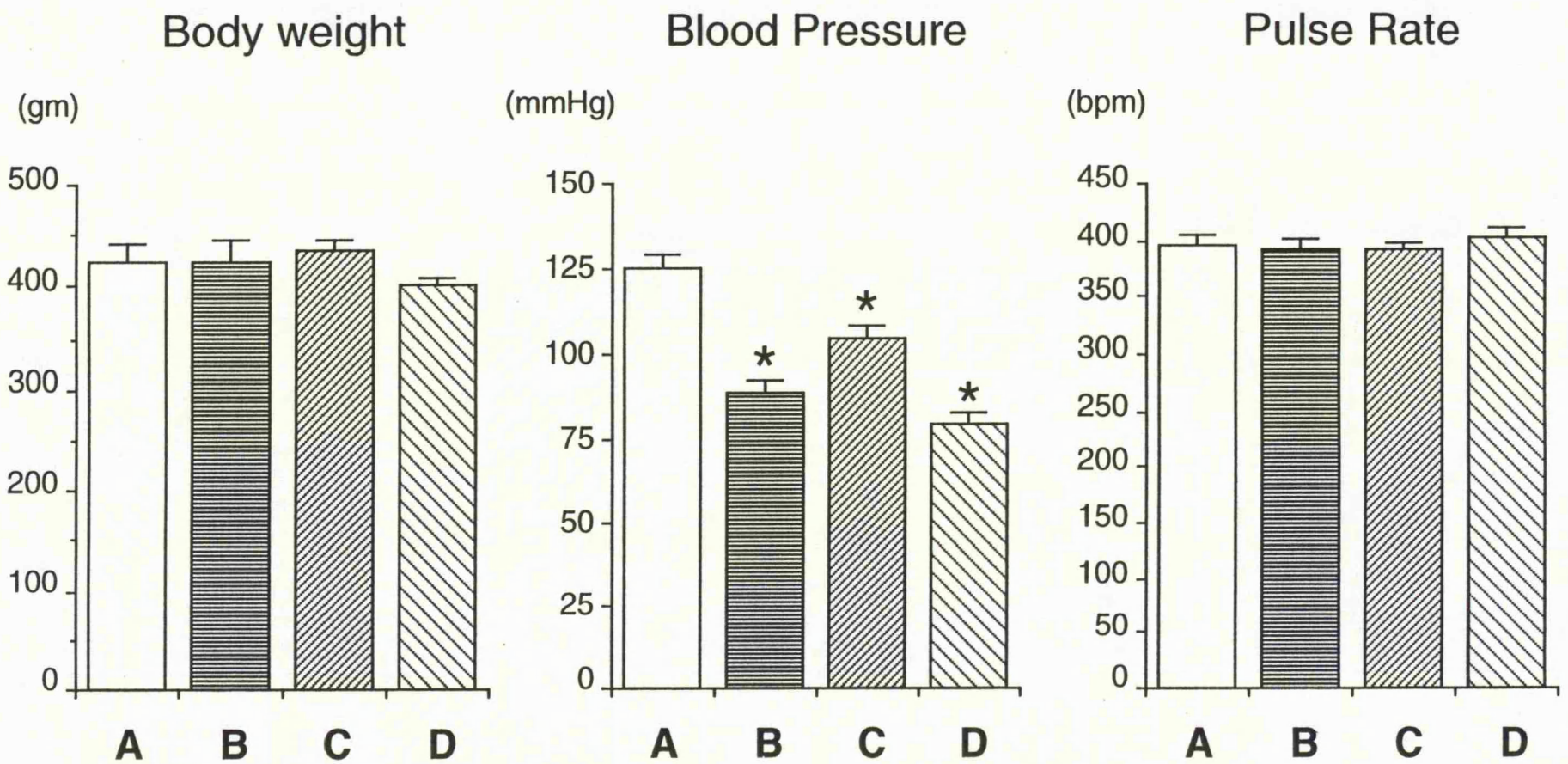


Figure 8A: Body weight, blood pressure and pulse rate in post-infarction heart failure and after ACE inhibitor treatment.
Animals had blood pressure and pulse measured 2 days prior to sacrifice and were weighed immediately prior to sacrifice. A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment. Mean ± SEM are shown. * significantly different ($P < 0.05$) from relevant control group on pairwise comparisons.

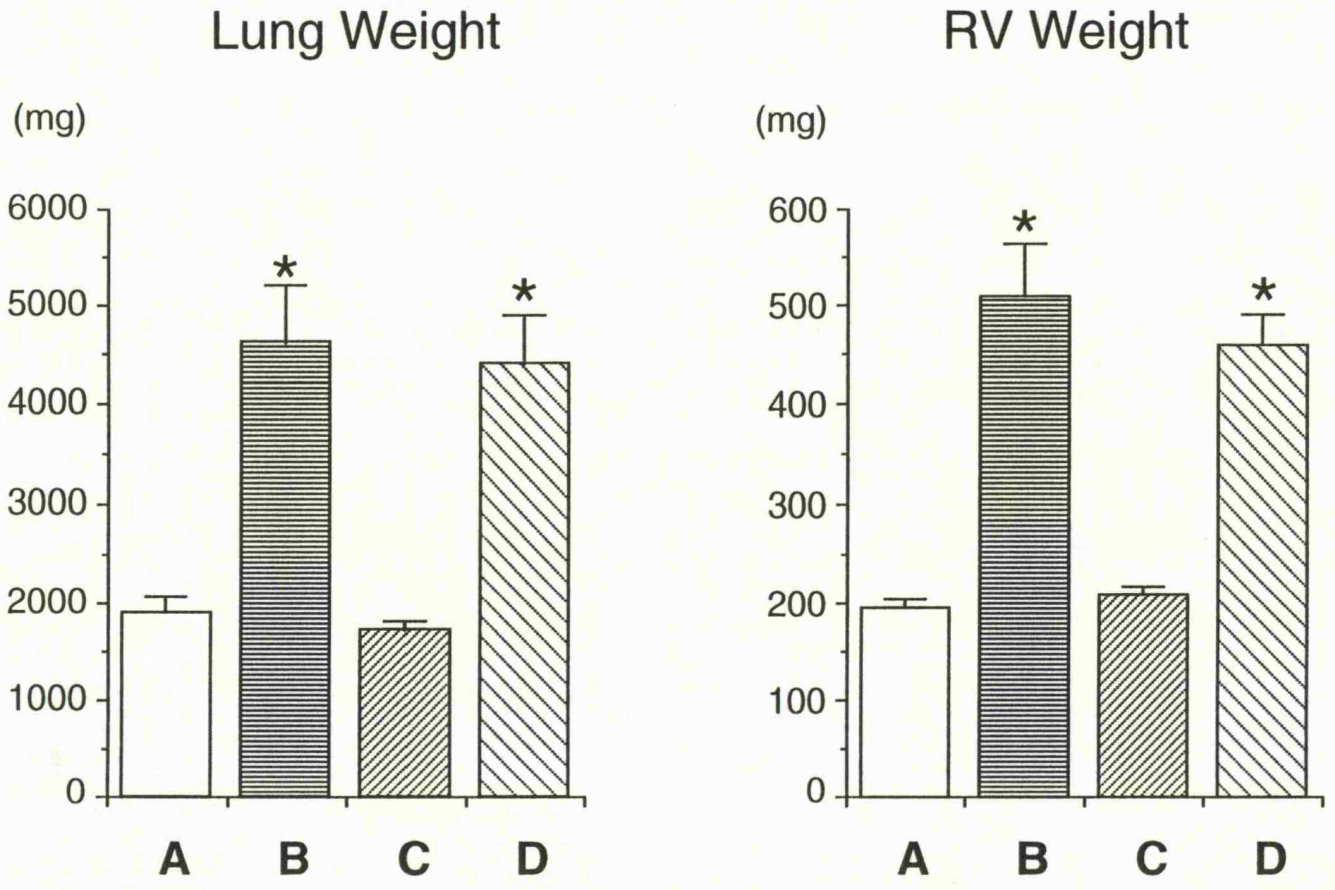


Figure 8B: Lung weight and right ventricular weight in post-infarction heart failure and after ACE inhibitor treatment.
A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment. Means ± SEM are shown. * significantly different ($P < 0.05$) from relevant control group on pairwise comparisons.

TABLE 8.2: Circulating levels of components of the renin-angiotensin system in studied rats.

		Group			
		A	B	C	D
	n	(8)	(7)	(8)	(10)
PRC (ng AI/ml/hr)		2.6 ± 0.6	23.7 ± 9.4	279 ± 66	735 ± 162
PRS (μmol/l)		1.9 ± 0.2	1.6 ± 0.1	0.8 ± 0.1	0.4 ± 0.1
ACE (nmol/ml/hr)		308 ± 10	331 ± 4	19 ± 3	20 ± 4
AII (fmol/ml)		12.3 ± 4.8	21.4 ± 6.3	9.7 ± 1.4	39.9 ± 16.0

A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment. PRC, plasma renin concentration; PRS, plasma renin substrate (angiotensinogen) concentration; ACE, plasma angiotensin-converting enzyme activity; AII, plasma AII concentration. Mean ± SEM are shown. Values are given for animals that survived to the end of the study (30 days).

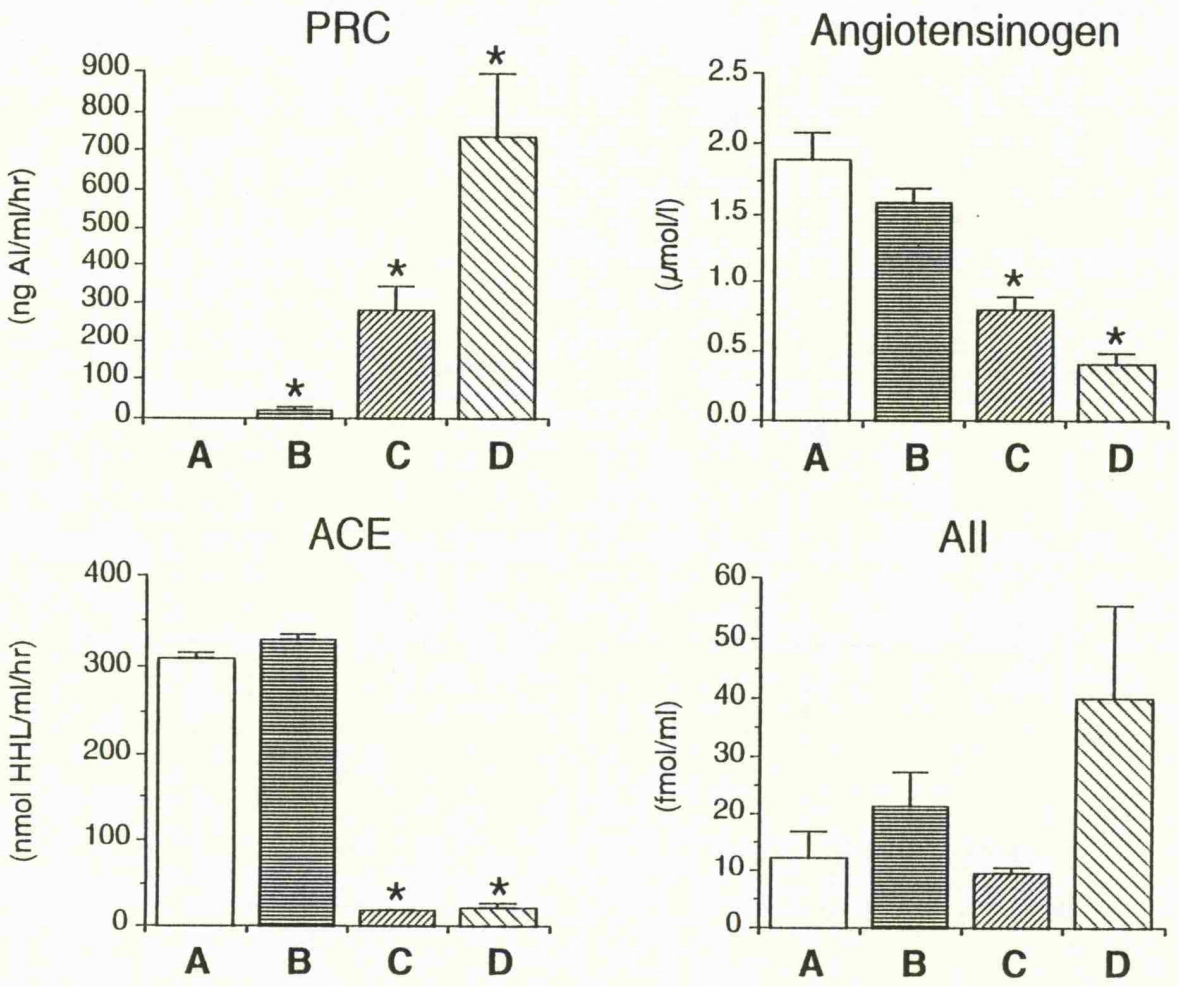


FIGURE 8C: Circulating components of the RA system in post-infarction heart failure and after ACE inhibitor treatment.
A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment. Mean ± SEM are shown. * significantly different ($P < 0.05$) from relevant control group on pairwise comparisons.

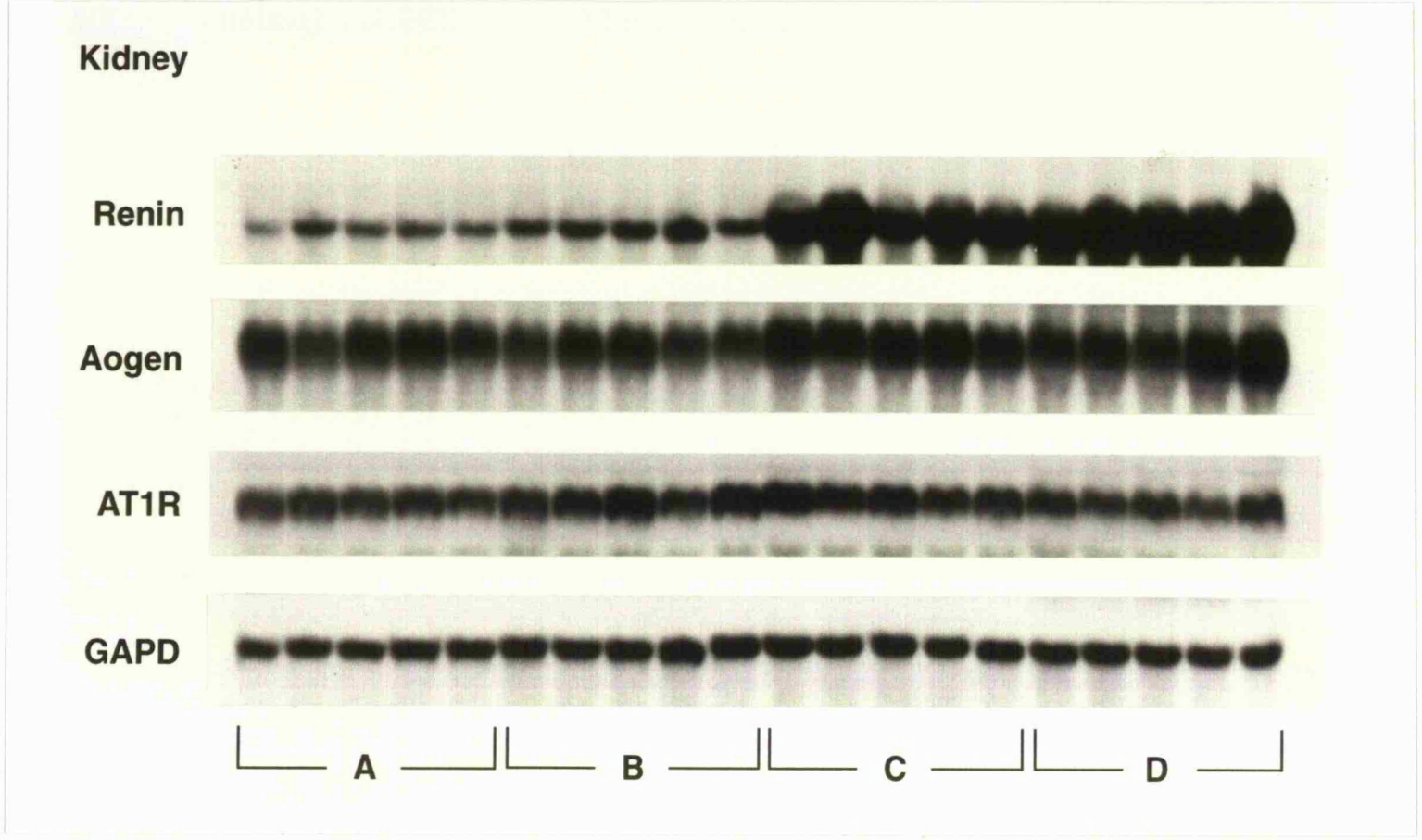


Figure 8D: Expression of RA system components in the kidney in post-infarction heart failure and after ACE inhibitor treatment. Northern blot of RNAs from: A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment; probed serially for renin, angiotensinogen (Aogen), angiotensin I receptor (AT1R) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNAs is shown. Each track contains RNA prepared from an individual animal. Exposure times of autoradiographs were as follows: renin (16 hours); Aogen (3 days); AT1R (5 days); GAPD (12 hours).

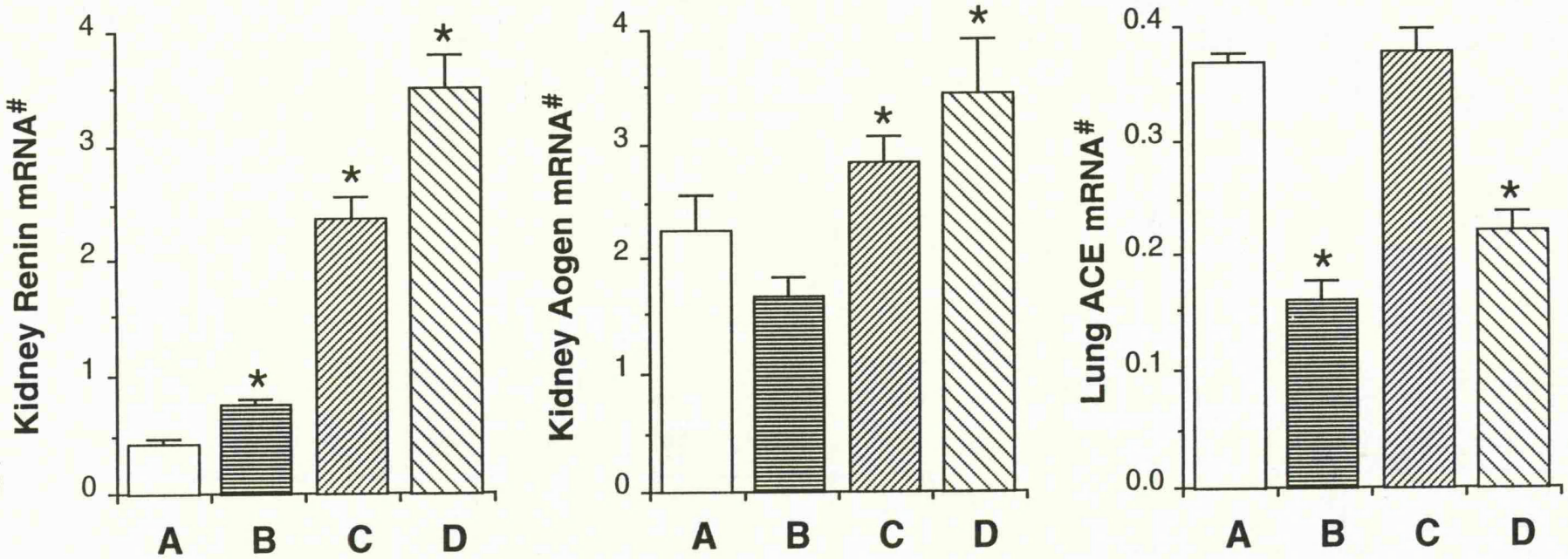


Figure 8E: Changes in kidney renin, kidney angiotensinogen and lung ACE mRNA levels in post-infarction heart failure and after ACE inhibitor treatment.

Results of densitometric analysis of Northern blots shown in Figures 8D (kidney renin and angiotensinogen) and Figure 8K (lung ACE) are shown. A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment. Mean ± SEM are shown. # mRNA levels have been normalised for the respective glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA level and are expressed in arbitrary densitometric units, on the y-axis, specific to the individual gel.

* significantly different ($P < 0.05$) from relevant control group on pairwise comparisons.

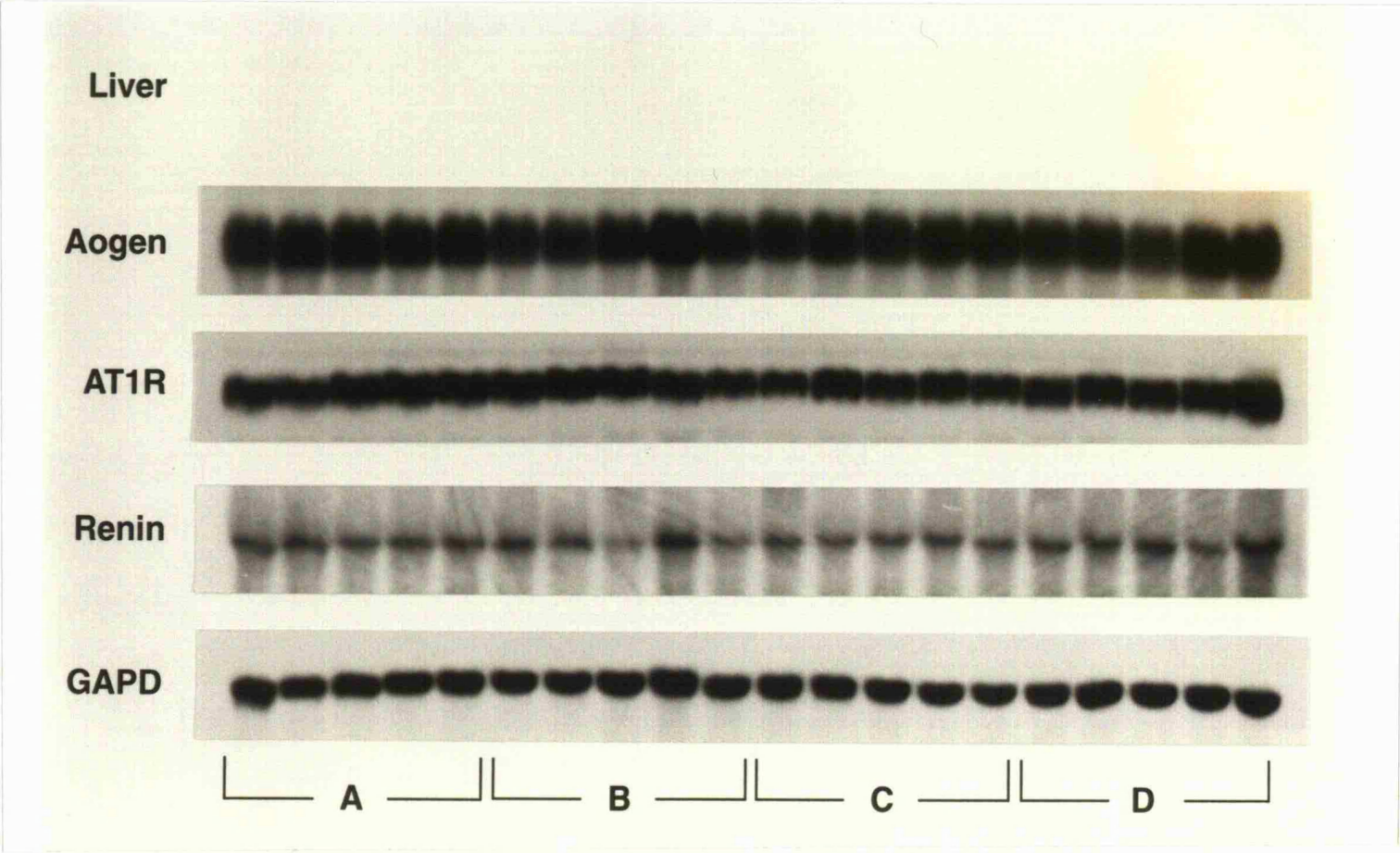


Figure 8F: Expression of RA system components in the liver in post-infarction heart failure and after ACE inhibitor treatment. Northern blot of RNAs from: A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment; probed serially for angiotensinogen (Aogen), angiotensin I receptor (AT1R), renin and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNAs is shown. Each track contains RNA prepared from an individual animal. Exposure times of autoradiographs were as follows: Aogen (4 hours); AT1R (4 days); renin (14 days); GAPD (12 hours).

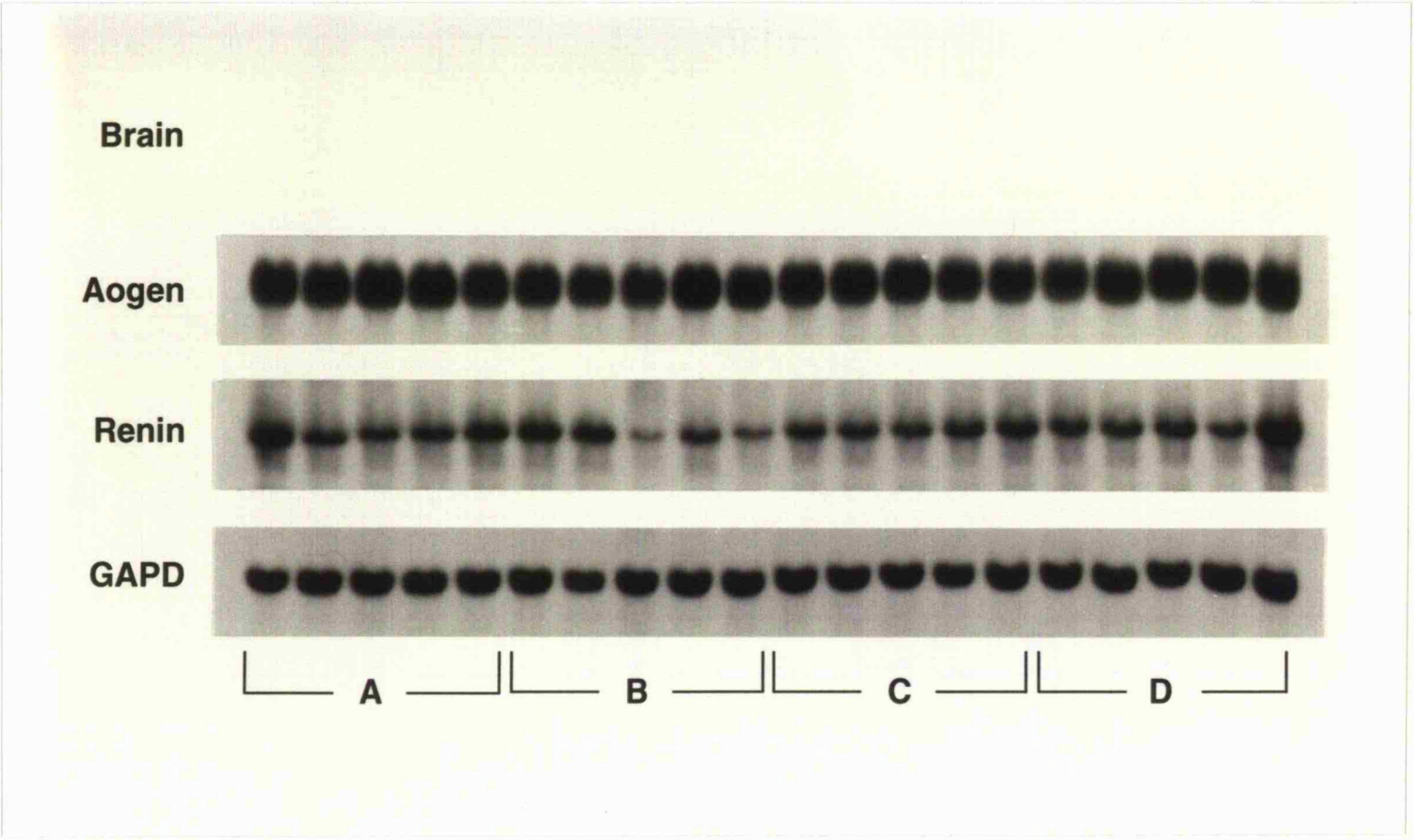


Figure 8G: Expression of RA system components in the brain in post-infarction heart failure and after ACE inhibitor treatment. Northern blot of RNAs from: A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment; probed serially for angiotensinogen (Aogen), renin and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNAs is shown. Each track contains RNA prepared from an individual animal. Exposure times of autoradiographs were as follows: Aogen (16 hours); renin (3 days); GAPD (8 hours).

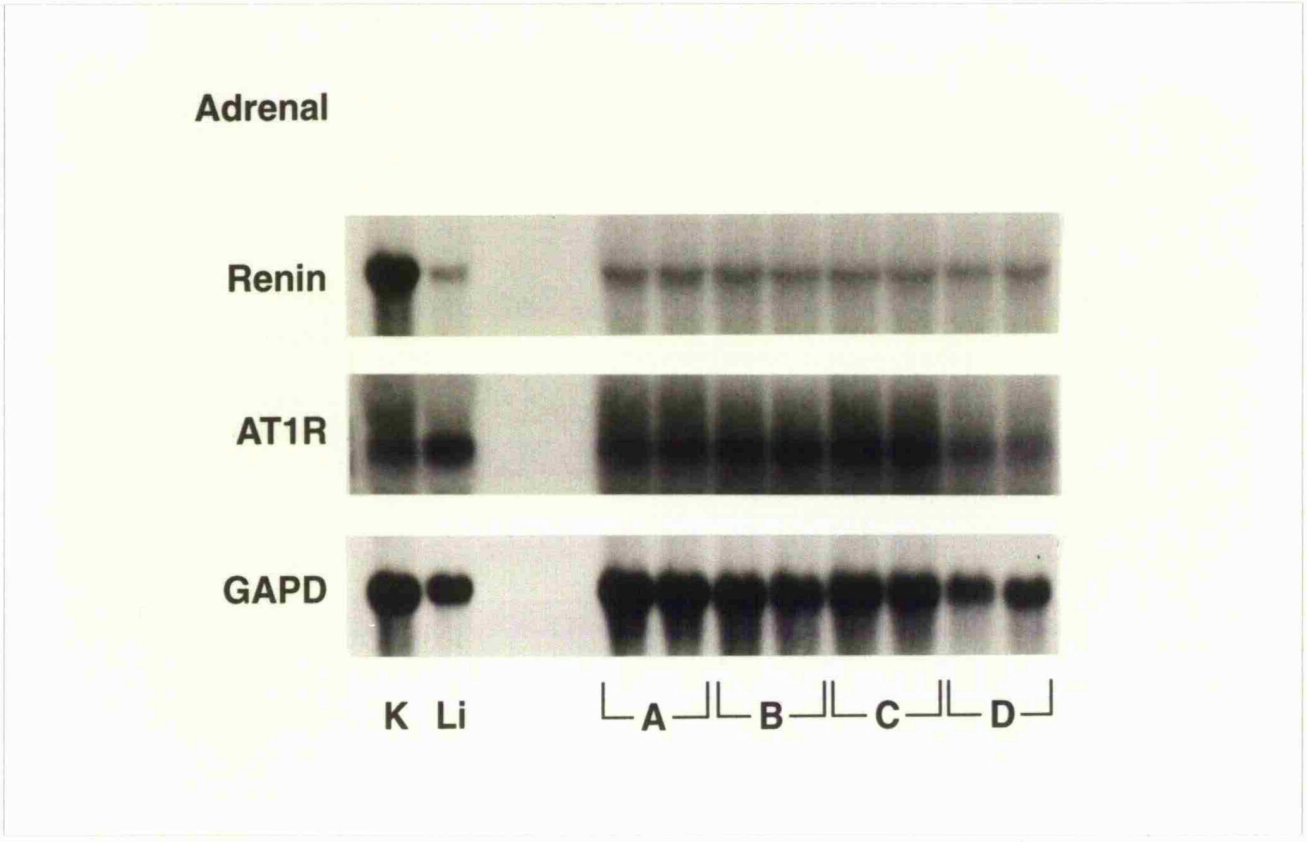


Figure 8H: Expression of RA system components in the adrenal in post-infarction heart failure and after ACE inhibitor treatment. Northern blot of RNAs from: A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment; probed serially for renin, angiotensin I receptor (AT1R) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNAs is shown. Because of limiting amounts of tissue, adrenals from 3 rats in each group were pooled before RNA extraction. The blots also contain kidney (K) and liver (Li) RNA samples as positive controls. Exposure times of autoradiographs were as follows: renin (9 days); AT1R (14 days); GAPD (9 hours).

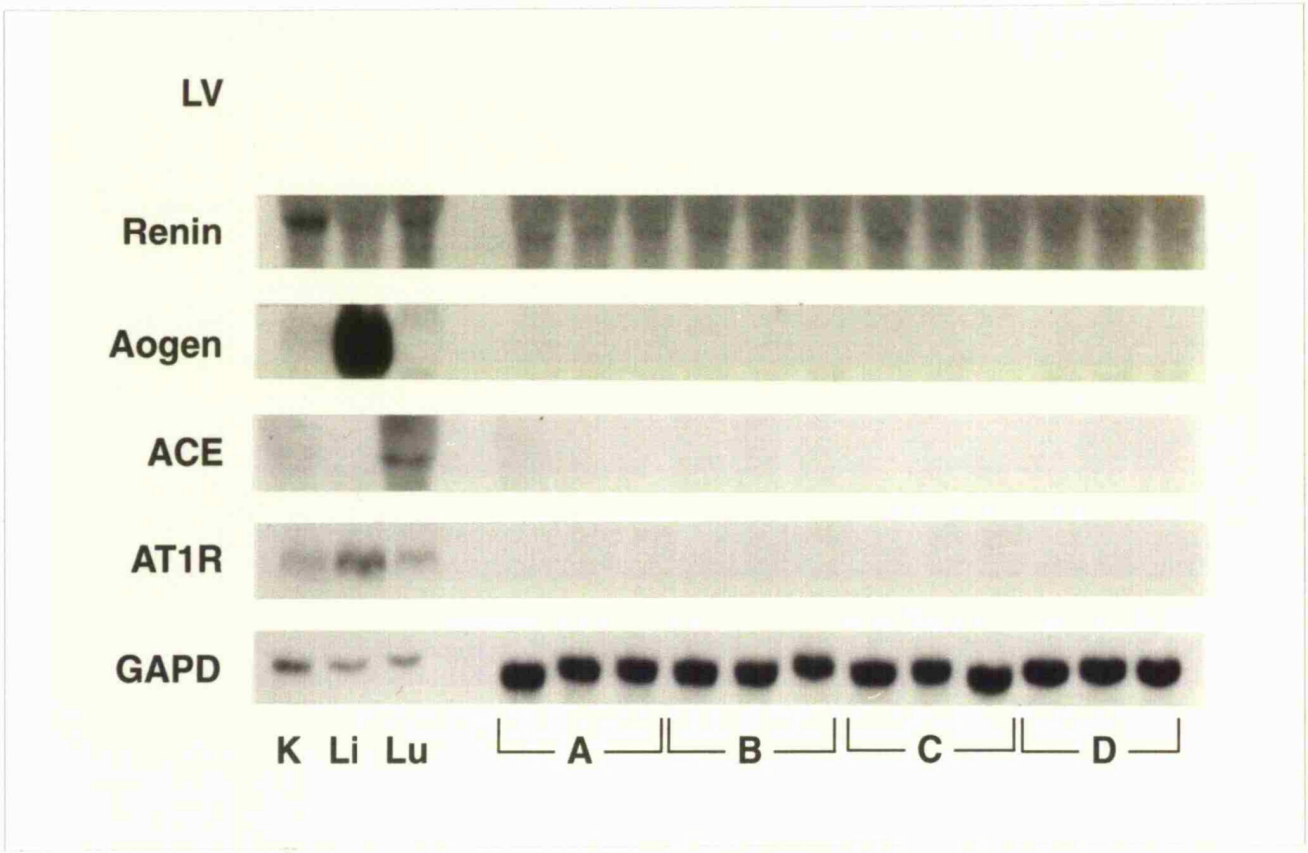


Figure 8I: Expression of RA system components in the left ventricle in post-infarction heart failure and after ACE inhibitor treatment. Northern blot of RNAs from: A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment; probed serially for renin, angiotensinogen (Aogen), angiotensin-converting enzyme (ACE), angiotensin I receptor (AT1R) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNAs is shown. Because of limiting amounts of tissue, left ventricles from 2 rats in each group were pooled before RNA extraction. The blots also contain kidney (K), liver (Li) and lung (Lu) RNA samples as positive controls. Exposure times of autoradiographs were as follows: renin (10 days); GAPD (2 hours).

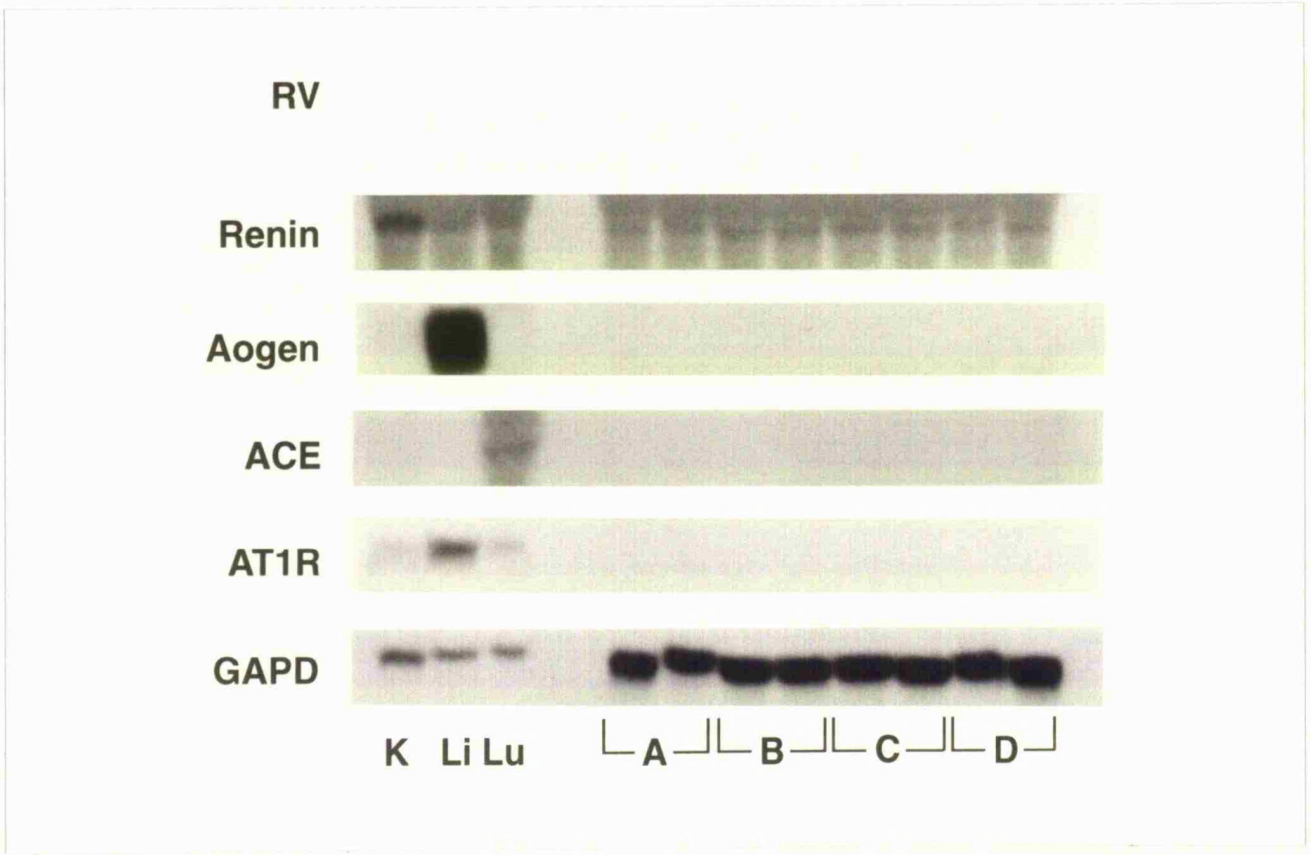


Figure 8J: Expression of RA system components in the right ventricle in post-infarction heart failure and after ACE inhibitor treatment. Northern blot of RNAs from: A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment; probed serially for renin, angiotensinogen (Aogen), angiotensin-converting enzyme (ACE), angiotensin I receptor (AT1R) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNAs is shown. Because of limiting amounts of tissue, right ventricles from 3 rats in each group were pooled before RNA extraction. The blots also contain kidney (K), liver (Li) and lung (Lu) RNA samples as positive controls. Exposure times of autoradiographs were as follows: renin (10 days); GAPD (2 hours).

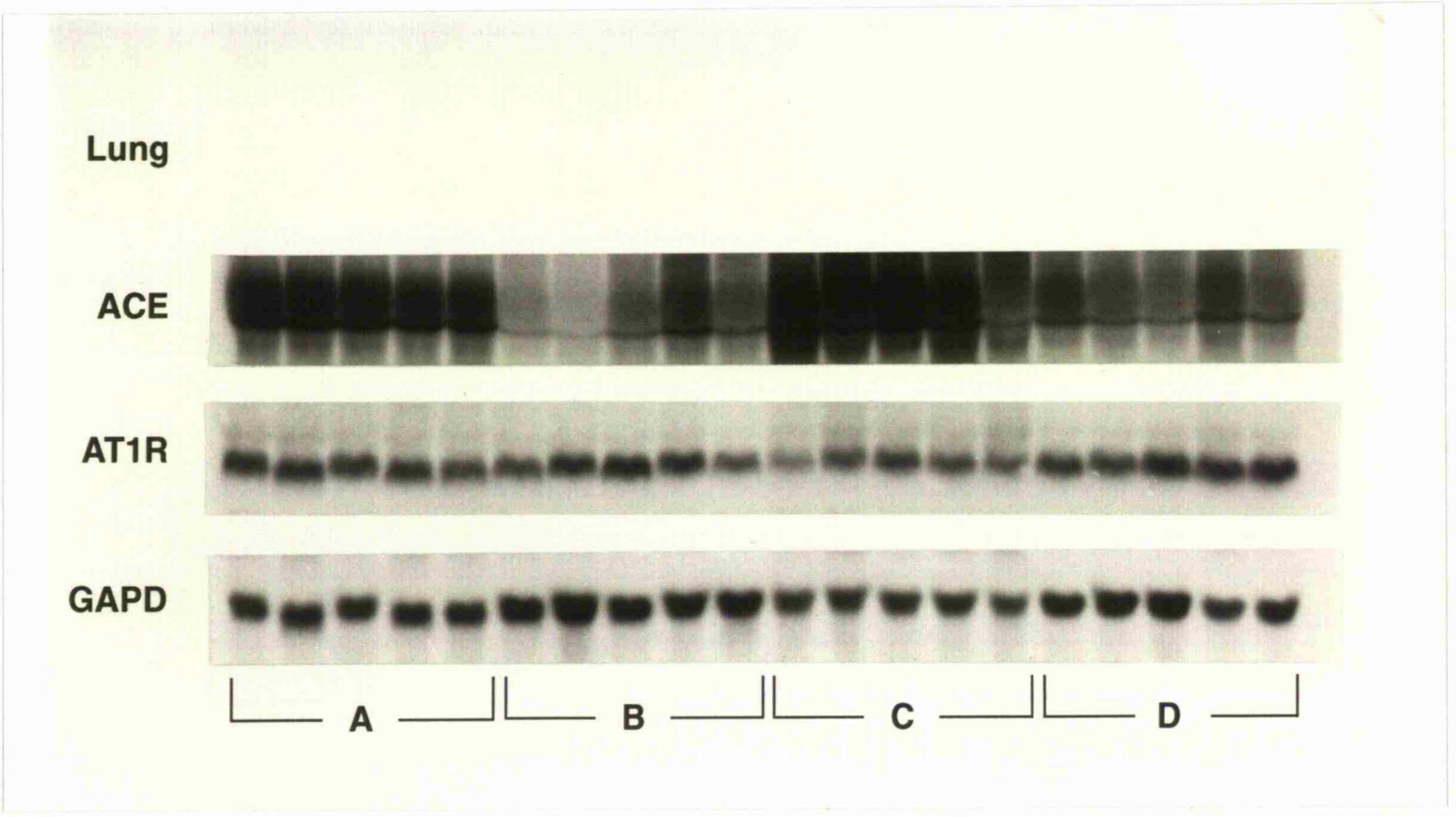
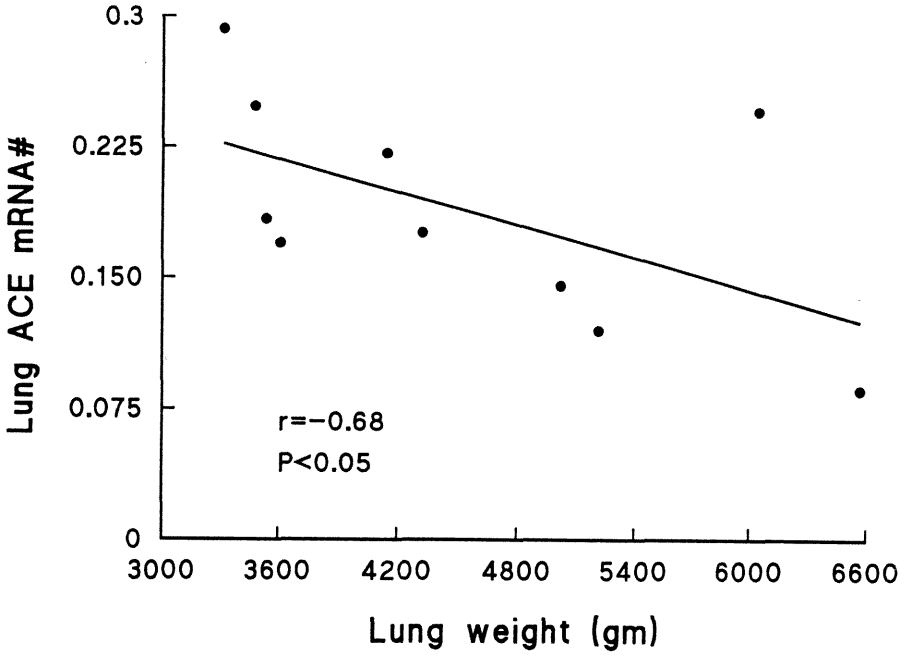


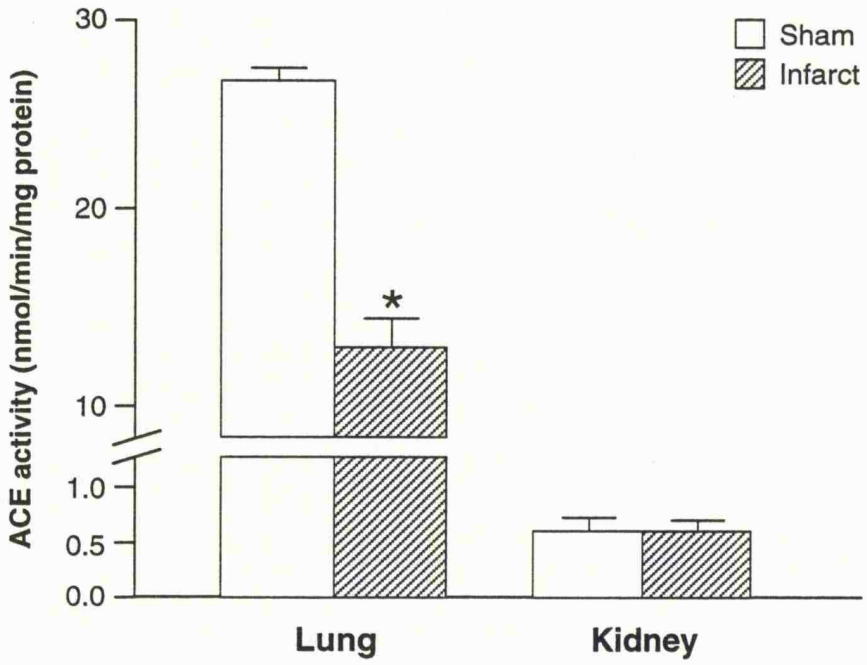
Figure 8K: Expression of RA system components in the lung in post-infarction heart failure and after ACE inhibitor treatment. Northern blot of RNAs from: A, sham operated rats with no ACE inhibitor; B, MI rats with no ACE inhibitor; C, sham operated rats with perindopril treatment; D, MI rats with perindopril treatment; probed serially for angiotensin-converting enzyme (ACE), angiotensin I receptor (ATIR) and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNAs is shown. Each track contains RNA prepared from an individual animal. Exposure times of autoradiographs were as follows: ACE (7 hours); AT1R (8 days); GAPD (12 hours).

Figure 8L: Inverse relationship of lung ACE mRNA to lung weight in animals with myocardial infarction.



ACE mRNA level normalised for GAPD level.

Figure 8M: Effects of post-infarction heart failure on ACE activity in lung and kidney.



Results are expressed as ACE activity per milligram total protein. Mean ± SEM are shown.
* $P < 0.01$ v's control.

8.4 DISCUSSION

In this study tissue- and gene-specific changes in expression of components of the RA system are shown in relation to experimental post-infarction heart failure and ACE inhibitor treatment. The findings are of clinical relevance because of the substantial evidence pointing to a detrimental effect of an activated RA system in this situation (Francis *et al.*, 1984) and the demonstration that ACE inhibitor treatment improves both symptoms and prognosis (The CONSENSUS Trial Study Group, 1987; The SOLVD Investigators, 1991; Pfeffer *et al.* [The SAVE-Study Trial Group], 1992; The AIRE Study Investigators, 1993).

Several previous studies (Pfeffer *et al.*, 1979; Pfeffer, Pfeffer, and Braunwald, 1985; Pfeffer, Pfeffer, Steinburg, and Finn, 1985; Bech *et al.*, 1989) have demonstrated that the rat coronary ligation model is characterised by decreased cardiac output, regional blood flow redistribution, elevated left ventricular end-diastolic pressure and reduced survival. Thus, the haemodynamics of the model resemble the most common features of moderate to severe human congestive heart failure. Consistent with this, a marked increase in lung and right ventricular weights, decreased blood pressure and a high mortality in the group of rats with MI, was observed. To investigate the effects of ACE inhibition, an agent, perindopril, with a prolonged duration of action was chosen. The dose (1.5 mg/kg/day) was chosen on the basis of a previous report showing a substantial anti-hypertensive effect in spontaneously hypertensive rats (Christensen, Jespersen, and Mulvaney, 1989) and on preliminary studies related to those presented in this chapter, showing that it was associated with >70% tissue ACE activity inhibition in a wide variety of tissues of both normal and infarcted rats (Ole Kahr, unpublished data). Although the choice of dose was somewhat empirical, the treatment was nonetheless associated with a substantial reduction in mortality, a finding both previously reported in the model with other ACE inhibitors (Pfeffer, Pfeffer, Steinburg, and Finn, 1985; Sweet *et al.*, 1987; Richer *et al.*, 1992) and consistent with the observations in man (The CONSENSUS Trial Study Group, 1987; The SOLVD Investigators, 1991; Pfeffer *et al.* [The SAVE-Study Trial Group], 1992; The AIRE Study Investigators, 1993). However an interesting observation, within the time-frame of this study, is that the improvement in

mortality was not associated with a significant reduction in either lung or RV weights in the surviving animals.

The increase in PRC in animals with MI was anticipated and consistent with the well-documented activation of the endocrine RA system in moderate to severe heart failure (Francis *et al.*, 1984). Several mechanisms probably contribute to the increased secretion of renin by the kidney in this situation, including reduced renal perfusion pressure (baroreceptor mechanism), reduced sodium delivery to the distal tubule (macula densa mechanism) and increased direct sympathetic stimulation of the renin-producing juxtaglomerular cells (see section 2.1.1). It is notable that the rise in PRC was several-fold greater than the increase seen in kidney mRNA levels. Such disproportional changes in kidney renin mRNA and PRC have been reported previously in relation to ACE inhibition and other stimuli (Nakamura *et al.*, 1985; Ludwig et al., 1987; Morishita *et al.*, 1993) and indicate important post-transcriptional regulation of renin production and secretion by the kidney. With perindopril treatment, a further and marked rise in PRC was seen in both normal and MI animals. This is probably mainly due to a removal of the negative feedback effect of angiotensin II (AII) on kidney renin synthesis and secretion (see below).

In contrast to PRC, PRS (angiotensinogen) was reduced both with MI and particularly with ACE inhibitor treatment. There was no significant change in liver angiotensinogen mRNA level in any of the groups and the decrease in PRS probably reflects increased consumption by the raised PRC. Indeed across the groups there was a strong negative correlation between PRC and PRS. In keeping with other similarities between this model and the human condition, PRS is also decreased in patients with severe heart failure (Arnal *et al.*, 1991).

In agreement with some (Hirsch *et al.*, 1991; Pinto *et al.*, 1993) but not all (Huang *et al.*,1994) previous reports, no change was found in plasma ACE level in post-infarction heart failure. Plasma ACE is believed to be mainly derived from endothelial ACE released from tissue vascular beds (see section 1.6). Since the pulmonary vascular bed contains the highest concentration of endothelial ACE and it is likely that it makes a major contribution to plasma

ACE, one might have expected plasma ACE to be decreased in rats with heart failure given the changes found in lung ACE expression and activity in these animals. The reasons why this did not happen need to be identified, but it is possible that increased expression and release from other sites e.g. heart (Hirsch *et al.*, 1991; Pinto *et al.*, 1993) compensates for reduced production by the lung, or that turnover of plasma ACE is reduced in heart failure and a change was not detectable by the time the experiments were terminated. However, the findings emphasise the importance of looking at both circulating and tissue components of the RA system in pathophysiological conditions.

The findings on plasma AII in animals treated with perindopril may at first sight appear inconsistent with ACE inhibition, and point to possible measurement problems. Indeed, there are limitations to the technique used. In particular, since HPLC was not employed in this study to separate angiotensin II from angiotensin degradation products such as angiotensin (2-8) and angiotensin (3-8), which are also recognised by the antibody used (although the inhibitors used in the collection of the blood should have kept such degradation to a minimum), a significant contribution from these peptides cannot be excluded. However, several studies using HPLC (Mento and Wilkes, 1987; Bunkenberg *et al.*, 1991; Campbell *et al.*, 1991; Nagano *et al.*, 1991; Yamagishi *et al.*, 1993; Campbell, Kladis, and Duncan, 1994; Tokita *et al.*, 1995) have shown a similar lack of reduction of circulating AII level with chronic ACE inhibitor treatment. Two mechanisms have been advanced to explain the failure of AII level to fall with ACE inhibitor treatment. First, a partial "escape" of ACE inhibition driven by the high level of angiotensin I consequent of the rise in PRC (Mooser *et al.*, 1990). Thus, a combination of renin and ACE inhibitors have been found to cause a reduction in plasma AII (Mento *et al.*, 1989). Alternately, it has been proposed that persistent AII may reflect its generation by alternative pathways not involving ACE (Dzau, 1989). Considerable, especially *in vitro*, evidence exists for the presence of such pathways (Dzau, 1989) but their physiological significance remains unclear (see chapter three). Some of these pathways may also depend on angiotensin I (Dzau, 1989), thus providing a possible explanation for the finding of the highest AII levels in animals with the highest PRC (group D). Whatever the mechanism involved, the findings on plasma AII are important for two reasons. First, they

support the hypothesis that the beneficial actions of ACE inhibitors in heart failure are through mechanisms that extend beyond the simple suppression of circulating AII. Second, the findings provide a potential explanation for some of the tissue-specific changes seen in expression of specific RA system components following ACE inhibition as discussed below.

With regard to post-infarction heart failure, apart from an increase in kidney renin mRNA level, the main change found in tissue expression of components of the RA system was in lung ACE. ACE mRNA level was reduced by 50% and accompanied by a similar reduction in ACE activity. These findings are very similar to those recently reported by Huang *et al.* (1994) at 3 months after infarction in the same model, but contrast with the findings of Hirsch *et al.* (1991) at 85 days. However, since the reduction in pulmonary ACE expression was strongly correlated with the severity of the heart failure as assessed by lung weight (Figure 8L) and Hirsch *et al.* (1991) studied rats with compensated (mild) heart failure, it is possible that the stimulus in their study was not sufficient to significantly affect pulmonary ACE expression.

The mechanism(s) responsible for the change in lung ACE mRNA level and activity remain to be elucidated. The effect seems to be specific both with regard to tissue, as a similar effect was not seen on kidney ACE (Figure 8M), and molecule as in the study by Huang *et al.* (1994) no concomitant changes were found in the activity of two other pulmonary ectoenzymes, neutral endopeptidase and aminopeptidase A. The finding particularly contrasts with the increased cardiac ACE expression that has been reported post MI (Hirsch *et al.*, 1991; Pinto *et al.*, 1993). The effect also seems to be model-specific as no change in lung ACE expression was found in a volume overload model of heart failure induced by creation of an aortocaval fistula (Pieruzzi, Abassi, and Keiser, 1995). The findings presented here clearly suggest that the reduced activity is mainly due to decreased synthesis, although whether the reduction in ACE mRNA level is itself due to reduced transcription or increased transcript turnover remains to be determined. Multiple hormonal and physiological factors are known to influence pulmonary ACE activity. A potential factor relevant here is chronic hypoxia, a likely feature of severe heart failure, which has been

shown to lead to a decrease in pulmonary ACE activity (Oparil *et al.*, 1988). Alternatively, it is being increasingly recognised that haemodynamic factors, and in particular shear-stress, are important regulators of endothelial endocrine activity and the down-regulation of pulmonary ACE in heart failure could therefore be a direct consequence of increased pulmonary pressure. Keane *et al.* (1982) induced pulmonary hypertension in rats using two different (non-MI) methods. In both situations, they found lung ACE activity to be reduced and inversely related to the severity of the pulmonary hypertension. The negative correlation found here between lung weight and pulmonary ACE mRNA level is consistent with this. Finally, there is substantial evidence that lung ACE activity and mRNA level, at least in the short-term, are under feedback regulation by AII (Schunkert *et al.*, 1993), and this could have been a contributing factor as AII levels were higher in both groups of animals with MI (Table 8.2).

The physiological consequences of the reduction of pulmonary ACE activity in post-infarction heart failure remain to be determined. Since pulmonary ACE makes a major contribution to angiotensin I conversion (Erdos, 1990), Huang and co-workers (1994) postulated that its reduction in heart failure may be beneficial by damping the elevation in circulating AII, with consequent detrimental effects (Francis *et al.*, 1984), that would have otherwise resulted from the increase in PRC. The substantial decrease in the ratio of AII/PRC in MI rats (Group B) compared with control rats (Group A) in this study (Table 8.2) is consistent with this hypothesis. At a local pulmonary level, decreased generation of AII may be a protective mechanism to limit the extent of elevation of pulmonary arterial pressure in heart failure.

With regard to ACE inhibitor treatment two main changes were observed in mRNA levels, both in the kidney: a marked rise in renin mRNA and a more modest rise in angiotensinogen mRNA. As with PRC, the main reason for the rise in renin mRNA is probably a reduction in feedback inhibition by AII (Davis and Freeman, 1976). There is strong evidence that despite a lack of effect on plasma AII level, AII concentrations are reduced in several tissues following chronic ACE inhibitor treatment (Campbell *et al.*, 1991;

Nagano *et al.*, 1991; Yamagishi *et al.*, 1993; Campbell, Cladis, and Duncan, 1994; Tokita *et al.*, 1995). Specifically, Campbell and co-workers (1991) demonstrated kidney AII level to be reduced in the rat with perindopril when plasma level was unaffected. Thus, a local renal reduction in negative feedback inhibition by AII is still a plausible explanation for the increase in renin mRNA level despite the unchanged or even increased plasma AII level.

An analogous scenario may explain the differential effect found of ACE inhibition on renal and hepatic angiotensinogen mRNA (Figures 8D and 8F). Angiotensinogen expression is known to be under regulation by angiotensin II (Nakamura *et al.*, 1990; Schunkert *et al.*, 1992a). It is possible that hepatic expression is directly regulated by circulating levels of angiotensin II, and hence did not change significantly in the study presented here, while renal expression is more responsive to local angiotensin II level, and hence, like renin mRNA, increased after ACE inhibition. Renal angiotensinogen expression has been localised to the proximal tubule which is also a key site of action for angiotensin II (Ingelfinger *et al.*, 1990). Thus, increased angiotensinogen synthesis with ACE inhibition could affect proximal tubular function although functional correlates of such an effect remain to be identified.

The findings presented here for renal angiotensinogen expression contrast with those of Schunkert *et al.* (1992b; 1993). They found an increase in renal angiotensinogen mRNA level in rats with either stable compensated or severe chronic heart failure, induced in a similar manner to that in this study. The increase ranged from 30-80% and was related to the severity of the MI. The increase was reversed by enalapril but interestingly not by another ACE inhibitor captopril (Hirsch *et al.*, 1992). They concluded that renal angiotensinogen was under positive regulation by angiotensin II, which was reversed by enalapril. Since their experimental model was identical to the one used in this study, except that perhaps the rats in this study had even more severe heart failure (judged by the increase in PRC), the discrepant findings are surprising and difficult to reconcile easily.

No obvious changes in RA system gene expression in heart failure were found in three important sites where tissue RA systems have been implicated in cardiovascular control - the brain (through effects on the sympathetic nervous system [SNS]), adrenal gland

(through effects on aldosterone production and secretion) and the heart (see section 3.5). The results for brain and the adrenal are particularly important negative findings because both the SNS and aldosterone have been implicated in the pathophysiology and downward progression of heart failure (Packer *et al.*, 1987). The lack of modulation of renin mRNA in the left and right ventricles was interesting as Pieruzzi and co-workers (1995) demonstrated a significant increase in cardiac renin expression with heart failure, albeit in a different model. Renin mRNA has also been found to be upregulated in cardiac tissue with ACE inhibitor treatment (Lou, Robinson, and Morris, 1993) although such reports pertain to atrial expression. Likewise, no systematic or tissue-specific up or down regulation in AT_1 receptor mRNA level in response to either heart failure or ACE inhibitor treatment was found in this study. The AT_1 receptor mediates all the known cardiovascular effects of the RA system (Timmermans *et al.*, 1993). Thus, in so far as the steady state AT_1 receptor mRNA level reflects the AT_1 receptor density in a tissue, this study suggests that changes at this level do not influence the activity of the RA system in either chronic heart failure or after ACE inhibitor treatment. At least two subtypes (AT_{1a} and AT_{1b}) are known to exist for the AT_1 receptor (Iwai and Inagami, 1992). The precise physiological roles of the AT_1 receptor subtypes remain to be established, although their expression is known to vary in different tissues (Kakar *et al.*, 1992). It should be noted that because of strong homology between mRNAs for the AT_{1a} and AT_{1b} subtypes of the AT_1 receptor (Iwai and Inagami, 1992; Kakar *et al.*, 1992) and the manner in which the probe was radio-labelled (random priming cannot produce a homogeneous population of full-length labelled fragments from a template 2.2 kb long), the AT_1 receptor cDNA probe cloned from AT_{1a} receptor subtype mRNA (Murphy *et al.*, 1991) could not distinguish between subtype transcripts by Northern hybridisation. This study therefore cannot exclude subtype specific changes in expression.

A somewhat disappointing finding was that apart from an additive effect of heart failure and ACE inhibitor treatment on kidney renin mRNA levels and PRC, no other effect was found, especially in tissue gene expression, that specifically resulted from a combination of both factors (i.e. in rats of group D). Clearly, only limited parameters of tissue RA systems were studied, but this finding leaves open the question of the importance of the role

of tissue RA systems in the pathophysiology of post-infarction heart failure and specifically their role in mediating the beneficial effects of ACE inhibitors in this condition.

In addition to limitations already alluded to, other short-comings of this study include the fact that only one time-point was examined and therefore it is possible that period-specific changes in tissue RA systems following MI were missed. Some evidence for such changes comes from the finding of Lindpaintner *et al.* (1993) of early activation of cardiac angiotensinogen gene expression following MI at 5 days which had reversed by 25 days. Also where an mRNA species could not be detected by Northern blotting in a particular tissue, a more sensitive technique was not utilised to see if changes, albeit at a much lower level, had occurred.

In conclusion this study has demonstrated: (i). As in humans, ACE inhibitor treatment in this model is associated with improved survival after MI (However, due to relatively small *n* it was not possible to attach statistical significance to this association); (ii). There are selective tissue-specific changes in expression of specific components of the renin-angiotensin system in chronic post-infarction heart failure and following ACE inhibitor treatment in the rat. Namely, an increase in kidney renin expression after both MI and ACE inhibitor treatment, an increase in kidney angiotensinogen expression after ACE inhibitor treatment and a decrease in lung ACE expression and activity after MI. The changes were not reflected in all instances by changes in circulating components, supporting the proposition that tissue and circulating RA systems are regulated differently and subserve different pathophysiological roles. The significance of these tissue-specific changes in gene expression remain to be elucidated. However, an activated renal RA system may play an important role in the regulation of fluid balance which is disturbed in heart failure while the downregulation of lung ACE expression may be a protective mechanism to limit the extent of elevation of pulmonary arterial pressure in heart failure; (iii). There is no down-regulation of the system as determined by expression of the AT_1 receptor and (iv). There is no gross activation (as determined by Northern blotting) of the putative cardiac renin-angiotensin system at this stage of evolution of post-infarction heart failure in this model.

Despite the limitations outlined, this study provides novel information on changes that occur in circulating and tissue RA systems in relation to severe post-infarction heart failure and ACE inhibitor treatment and suggests new aspects of pathophysiology that merit further investigation.

CHAPTER NINE

Analysis of tissue ACE mRNA using RT-PCR

9.1 INTRODUCTION

ACE mRNA has been previously demonstrated in a variety of extra-pulmonary tissue sites (Griendling, Murphy, and Alexander, 1993). However, in the study described in chapter eight, ACE mRNA was not detectable in extra-pulmonary tissues using Northern blotting. It was decided to test the hypothesis that this failure to detect ACE mRNA was due to low levels of message that were below the level of sensitivity of detection offered by Northern blotting. Reverse Transcription Polymerase Chain Reaction (RT-PCR) was chosen to do this as it is the most sensitive technique available. However, under normal experimental conditions, RT-PCR is not quantitative (see section 5.11). Recent modifications of the technique have incorporated the use of co-amplification of various forms of internal standards and claim to be quantitative (Pieruzzi, Zaid, and Keiser, 1995). Unfortunately, these methods are not as robust as Northern Blotting with respect to a truly quantitative nature and it can take a lot of time and effort to develop a quantitative reaction that is specifically tailored to the system to be studied.

Constraints of time and resources prevented the development of a quantitative assay, so a "semi-quantitative" approach was taken in which dose-product curves were produced for each of the samples to determine conditions that were in the linear portion of the PCR with respect to starting material (total RNA reverse transcribed to first strand cDNA). If an amount of starting material that was common to the linear phase of the dose-product curves of all of the samples being compared could be determined, then that amount was chosen for comparison of the samples.

9.2 METHODS

(i). Reverse transcription and PCR

Reverse transcription and PCR were carried out essentially as described in section 5.11. PCR for ACE was based upon that of Hirsch *et al.* (1991) using a primer set obtained from the human ACE cDNA sequence corresponding to bases 492-512 (GCCTCCCCAACAAGACTGCCA; upstream primer) and bases 860-880 (CCACATGTCTCCCAGCAGATG; downstream primer) of the human ACE cDNA

sequence (Soubrier *et al.*, 1988). The optimum PCR conditions were determined empirically on 1 μg aliquots of reverse transcribed control lung RNA which were subjected to PCR and quantified for ACE mRNA by Southern blotting and hybridisation (see section 5.9) to an α-^{32}P-labelled 3.3 kb ACE cDNA obtained from a human umbilical vein endothelial cDNA library (Soubrier *et al.*, 1988) kindly provided by Dr. Florent Soubrier.

The optimum PCR conditions were found to be as follows: denaturation, 1 minute at 94 °C; annealing, 1 minute at 57 °C; extension 30 seconds at 72 °C. The number of cycles was restricted to 25 to keep the PCR reaction in the exponential phase. Magnesium ion concentration in the PCR buffer was 2.5mM and 10 picomoles of each primer was added per reaction.

Control reactions were also amplified concurrently with the samples to be assayed. The positive control consisted of 0.1 ng of recombinant ACE cDNA. Two negative controls were utilised to discount artefacts such as the reamplification of contaminating PCR product from a previous reaction. The first negative control was a reverse transcription reaction in the absence of any RNA and the second an aliquot of lung RNA that had been put through the process of a reverse transcription reaction in which no reverse transcriptase had been added.

(ii). Production of dose-product curves to assess linearity of reaction

Dose-product curves were produced for each of the study samples to control for linearity of the Reverse Transcription reaction and the PCR reaction:

Curve 1: Briefly, 5 μg of total RNA was reverse transcribed to first strand cDNA. This was then serially diluted and identical PCR reactions performed for each dilution. An equal volume of each PCR reaction was then run on an agarose gel, Southern blotted and hybridised with radioactively labelled ACE cDNA. The resulting bands on the autoradiograph were then quantified by 2D densitometry scanning using an LKB Ultrascan 2222-010 Scanner (LKB-Produkter AB, Bromma, Sweden). From this profile, the linearity of the PCR reaction with respect to starting material (first strand cDNA) could be tested and

the amount of starting material whereby components in the reaction became rate-limiting (and the reaction therefore non-linear) determined.

Curve 2: Once an optimum linear range for the PCR reaction had been established, different amounts of total RNA equivalent to points within this range were reverse transcribed to first strand cDNA and identical PCR reactions performed for each amount. This profile tests for linearity of the Reverse Transcription reaction and ideally should be equivalent to the linear portion of curve 1. An equal volume of each PCR reaction was then run on an agarose gel, Southern blotted, hybridised and quantified in the same manner as for curve one.

(iii). Statistical analysis

Where appropriate, results were compared using Student's unpaired t-test.

9.3 RESULTS

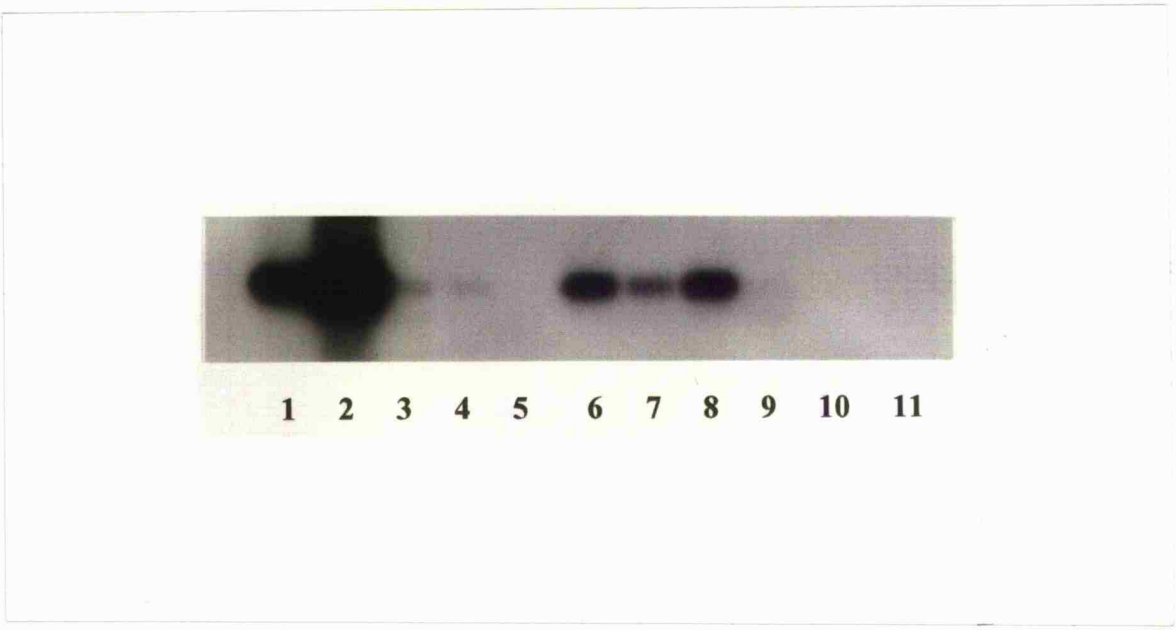
(i). Figure 9A shows the results of RT-PCR followed by Southern blotting of a $^1/_3$ aliquot of the products (run on a 1.5% ethidium bromide stained agarose gel) and hybridisation to ACE cDNA on a range of control (Group A, sham operated rats with no ACE inhibitor) animals from the study presented in chapter eight. Sizes of amplified products were determined from concurrently run DNA markers (1kb ladder, BRL/Life Technologies, UK). A 389 bp band corresponding to the precise size expected for the RT-PCR product from ACE mRNA was detected in all of the tissues examined. By choosing a primer set that spanned three intron/exon boundaries, the possibility of getting an identical sized product from any contaminating DNA in the RNA sample was avoided. Amplification from genomic DNA would have yielded a product of approximately 2.1 kb (intron 3 ≈700 bp; intron 4 ≈450 bp; intron 5 ≈600 bp). The strongest signal was seen in lung followed by brain, kidney, testis, left ventricle, and right ventricle. Very weak signals were apparent for adrenal and liver on the original autoradiograph but these have not reproduced very well photographically. On a longer exposure of this gel (not shown) the presence of ACE mRNA for these tissues was easily discernible.

The ethidium bromide stained gel is not shown as due to the relatively low number of PCR cycles used to ensure linearity of the reaction, only the PCR product for the lung sample was visible prior to Southern blotting and hybridisation.

(ii). Figure 9B shows the results of RT-PCR followed with Southern blotting and hybridisation to ACE cDNA on lung samples from Group A (sham operated rats with no ACE inhibitor) and Group B (Myocardial Infarction [MI] rats with no ACE inhibitor) animals from the study presented in chapter 8. The difference in average signal between the two groups (sham operated 27.02 ± 4.81 v's Infarct 6.37 ± 1.29, arbitrary densitometric units; $P < 0.01$ by Student's unpaired t-test) correlates with that seen between the same groups in the Northern blot (Figure 8K) presented in chapter 8 but the magnitude of the difference between the two groups is greater as assayed by RT-PCR.

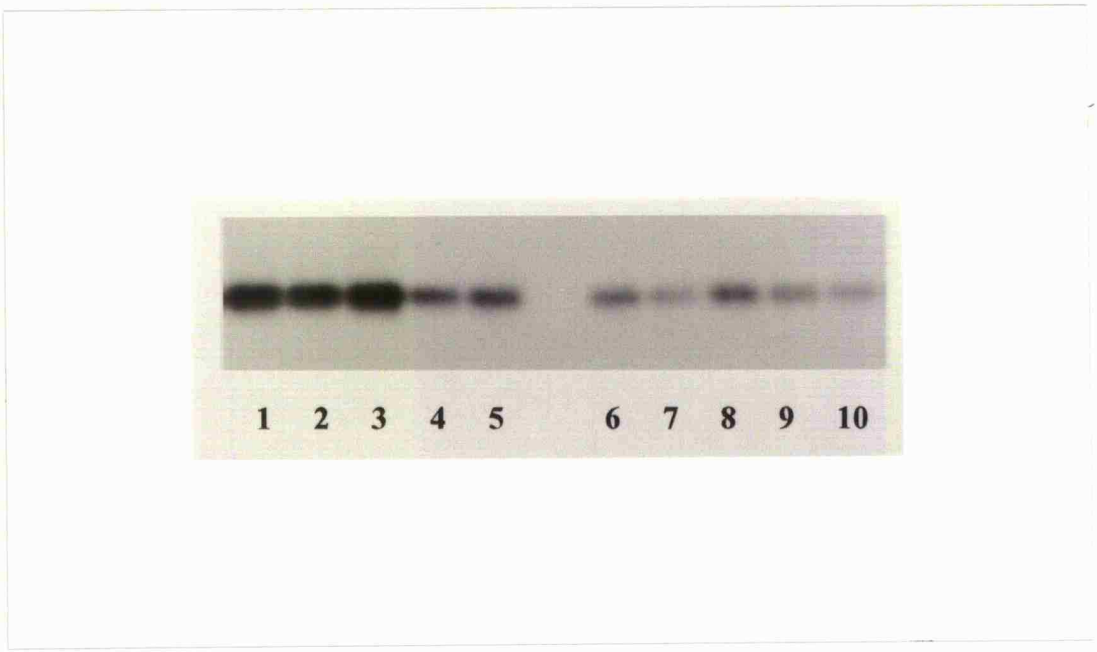
(iii). RT-PCR followed by Southern blotting and hybridisation were also carried out for kidney, liver, brain, adrenal, left ventricle and right ventricle samples from Group A and Group B animals. No difference in expression was seen between the two groups in any of these tissues. Figure 9C shows the results obtained for left (sham operated 47.71 ± 9.59 v's Infarct 66.38 ± 2.95, arbitrary densitometric units; $P = 0.122$ by Student's unpaired t-test) and right ventricle (sham operated 34.61 ± 6.56 v's Infarct 35.11 ± 3.89, arbitrary densitometric units; $P = 0.950$ by Student's unpaired t-test) which were typical of the results seen with the other extra-pulmonary tissues.

Figure 9A: Demonstration of ACE mRNA in rat tissues using RT-PCR followed by Southern blotting and hybridisation.



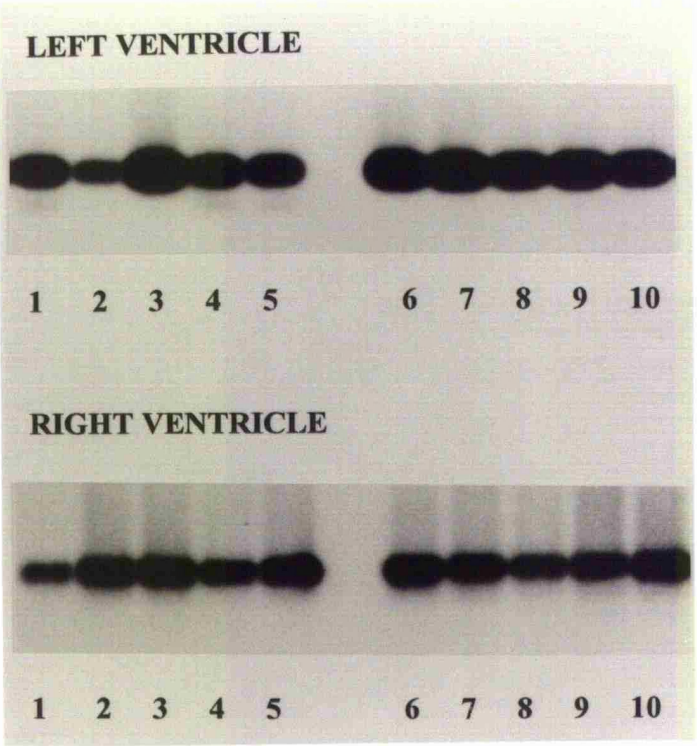
Samples: Track 1, Positive control of 0.1 ng of plasmid containing ACE cDNA subjected to PCR using the ACE primer set; 2, lung; 3, left ventricle; 4, right ventricle; 5, liver; 6, kidney; 7, testis; 8, brain; 9, adrenal. Tracks 10-11 control reactions: 10, RT-PCR in absence of any RNA; 11, PCR of lung RNA without RT step. Autoradiograph exposure time was 5 hours.

Figure 9B: Expression of lung ACE mRNA in sham operated rats and post-infarction heart failure rats.



Southern blot of lung RNAs subjected to reverse transcription and PCR for ACE from: Tracks 1-5, sham operated rats (group A from chapter 8 study); Tracks 6-10, MI rats (group B from chapter 8 study). The amplified product was sized at 389 bp. Each track pertains to an individual animal. Autoradiograph exposure time was 1 hour.

Figure 9C: Expression of left and right ventricle ACE mRNA in sham operated rats and post-infarction heart failure rats.



Southern blots of left and right ventricle RNAs subjected to reverse transcription and PCR for ACE from: Tracks 1-5, sham operated rats (group A from chapter 8 study); Tracks 6-10, MI rats (group B from chapter 8 study). The amplified product was sized at 389 bp. Each track pertains to an individual animal. Autoradiograph exposure time was 24 hours.

9.4 DISCUSSION

This study has demonstrated that ACE mRNA is present in varying amounts in a range of extra-pulmonary tissues. The failure to detect ACE mRNA in extra-pulmonary tissues by Northern Blotting in the study presented in chapter 8 can therefore be attributed to a limitation of the method of detection rather than an intrinsic lack of gene expression. The relative amounts of ACE message detected in each tissue broadly follow that reported by other studies (Griendling, Murphy, and Alexander, 1993), but it has to be borne in mind that this is not a truly quantitative study. Therefore, whilst relative comparisons of the amount of ACE expression between tissues may be valid, one cannot attribute an absolute value to the levels of expression seen.

The result for the relative expression between lung samples from Group A (sham operated rats) and Group B (MI rats) animals mirrors those obtained for the same groups using Northern blotting. This emphasises the potential validity of the RT-PCR assay, but the discrepancy in the magnitude of the difference observed between the groups as assayed by PCR and Northern Blotting emphasises that the assay is not yet truly quantitative. Bearing this in mind, it is possible that the failure to show any difference between these groups in the extra-pulmonary tissues may be due to a limitation of the assay. In particular, using the same primer set, Hirsch and co-workers (1991) found a two-fold increase in ACE mRNA level in the right ventricle following MI that correlated with infarct size. However, their study looked at animals 85 days postoperatively (compared to 30 days in the Group A and B animals) where the heart failure has entered the chronic compensated phase and this may be reflected in the differences observed. Also, Hirsch *et al.* controlled for linearity of their RT-PCR in a similar fashion to that presented here and so both studies may be comparable. As the RT-PCR performed by Hirsch *et al.* was not truly quantitative, and was therefore subject to the same limitations as the study presented here, both sets of data have to be interpreted carefully. Pieruzzi, Zaid, and Keiser (1995) also found an increase in cardiac ACE mRNA in heart failure using a quantitative RT-PCR but their model of heart failure was different to the one employed here.

Apart from the limitations already outlined, only two (sham operated rats and MI rats) of the four (sham operated rats; MI rats; sham operated rats with perindopril treatment; MI rats with perindopril treatment) experimental groups examined by Northern blotting in chapter 8 were studied here. Preliminary attempts to produce dose-product curves for five samples from each of the four groups with a common linear portion were unsuccessful. It was therefore not possible to formulate a single set of reaction conditions that would have given a concurrent linear amplification for the four groups (but this was possible when just assaying two groups). This phenomenon was encountered regardless of the specific tissue RNA being studied and highlights the limitations of semi-quantitative methods.

In conclusion, this study has demonstrated the presence of ACE mRNA in a range of extra-pulmonary tissues where detection by Northern blotting was unsuccessful. The study also confirmed the downregulation in pulmonary ACE gene expression in heart failure as demonstrated by Northern blotting in chapter 8. Bearing in mind the limitations of the assay, this study also suggests that there may not be modulation of extra-pulmonary ACE gene expression in heart failure at key tissue sites in this model during the acute stage (when the circulating RA system is activated). Further development of the assay is required before it can be routinely applied for quantitation of ACE mRNA levels.

CHAPTER TEN

General Discussion

The renin-angiotensin (RA) system has traditionally been thought to consist of a group of proteins and peptides that act in a cascade fashion to maintain extracellular fluid volume, arterial blood pressure and salt balance. In addition to this endocrine-view of the RA system, over the last 10 years or so it has been suggested that locally expressed tissue renin-angiotensin systems exist (see chapter 3). This novel concept has broadened the way in which the RA system is viewed and challenged its traditional endocrine role.

At the onset of this thesis, a number of studies had applied molecular biology techniques to the question of the existence of local tissue RA systems and the study of their regulation (Field *et al.*, 1984; Dzau, Ingelfinger, and Pratt, 1986; Ingelfinger *et al.*, 1986; Dzau *et al.*, 1987; Gomez *et al.*, 1988; Samani, Swales, and Brammar, 1988; Drexler *et al.*, 1989; Ellison *et al.*, 1989). However, systematic studies of the regulation of RA system gene expression in a variety of tissue sites had not been previously reported. The studies presented in this thesis have helped to address this gap in knowledge.

The ACE inhibition study presented in chapter six demonstrated by Northern blotting that renin mRNA is present not only in the rat kidney but also at a range of extra-renal sites. This was (and still is to a certain degree) a highly controversial concept when this study was being undertaken. Two independent RT-PCR assays were therefore designed to verify the Northern blot data result and they succeeded in substantiating the original finding. Renin mRNA in the kidney was found to significantly increase with the removal of feedback inhibition by AII and interestingly this increase was found to be disproportionately small compared to the increase in plasma renin concentration. This finding was subsequently verified in a similar study by Morishita *et al.*, (1993). In the heart failure study presented in chapter eight, a similar increase in kidney renin mRNA was seen with ACE inhibition. However, a much bigger increase in plasma renin was seen with ACE inhibition in this study than in the chapter six study. This may be reflected by the failure of chronic ACE inhibition in this study to suppress plasma AII levels, which were greatly reduced (to levels below the sensitivity of the assay) by the relatively acute administration of perindopril in the chapter six study. A partial "escape" of ACE inhibition may have occurred in the perindopril-treated

groups in the heart failure study due to a high level of angiotensin I resulting from the grossly elevated plasma renin. Alternatively, the differences observed may reflect modulation by alternative pathways of angiotensin generation not involving ACE. Whatever the cause of the differential effect of ACE inhibition on plasma AII between these two studies, this finding highlights the potential for differences in mechanistic effect between acute and chronic administration of an ACE inhibitor. Such mechanistic differences in turn, may explain differential regulation of tissue RA systems independent of changes in the endocrine system. In both the chapter six and the heart failure studies, however, no change was seen with ACE inhibition of renin mRNA levels at extra-renal tissue sites.

The ACE inhibition study demonstrated by Northern blotting that angiotensinogen mRNA is present not only in rat liver but also in a range of extra-hepatic sites. No differences in the level of angiotensinogen mRNA were observed with perindopril administration either in the liver or any of the other tissue sites examined. This was perhaps surprising as one might have expected liver and kidney angiotensinogen mRNA expression to decrease as a consequence of the marked suppression of plasma AII (Ingelfinger *et al.* 1986; Iwao *et al.*, 1988, 1990). However, the lack of modulation of angiotensinogen mRNA observed is consistent with previous studies (Gomez *et al.*, 1990). In contrast, in the heart failure study there was a small but significant increase in kidney angiotensinogen mRNA with ACE inhibition in the sham operated group of rats (as well as the MI group) in spite of no change in plasma AII levels. No such increase was observed in this study as a consequence of MI alone. This again supports the postulate that ACE inhibitors may have mechanisms of action distinct from the simple suppression of plasma AII levels (Ito *et al.*, 1981; Zimmerman, 1981; Dzau, 1988b; Hooper *et al.*, 1992) related, for example, to duration of treatment or other parameters. Therefore, an individual inhibitor may differentially regulate RA system gene expression in the same tissue under different circumstances. The increase in renal angiotensinogen mRNA with ACE inhibition is interesting as it differs from the observations of Hirsch and co-workers (1992). They found a small but significant increase in renal angiotensinogen in heart failure (MI) rats that was lowered to significantly smaller than sham operated levels after enalapril treatment. They

suggested that a positive feedback of AII on angiotensinogen was responsible for this, but limitations in their study (such as the failure to measure plasma AII levels in the enalapril treated animals) means that this argument is speculative.

The existence of a cardiac RA system, and especially the presence or absence of renin mRNA, has been debatable in recent years. Studies in the rat have detected cardiac renin mRNA by RNAase protection (Samani, Swales, and Brammar, 1988) and RT-PCR (Lou, Robinson, and Morris, 1993) and renin message has previously been detected in the mouse heart by Northern blotting (Dzau and Re, 1987). In the ACE inhibition study, RNA was extracted from whole hearts and a relatively strong signal for renin mRNA was observed. In the heart failure study, renin mRNA was detectable in RNA derived from left and right ventricles but at a much lower level than observed for whole hearts. Previous reports have shown that although renin mRNA is found in all four chambers of the heart, the highest levels are found in the right atrium (Baker, Booz, and Dostal, 1992). A disproportionate contribution of atrial renin mRNA expression in the whole heart extractions may therefore be responsible for the observed discrepancy in signal between the two comparative groups of rats in the studies presented here. This possibility could be investigated in future studies. No change in the level of cardiac renin mRNA with administration of perindopril was observed in either study and renin mRNA did not change with MI in the left or right ventricles in the heart failure study. This is in contrast to the study by Pieruzzi and co-workers (1995) who showed that cardiac renin mRNA increased concomitantly with the severity of experimentally induced heart failure in rats. In addition, they presented Western blot data that demonstrated that this increase paralleled an increase in the level of ACE protein. However, heart failure in their model was induced by the creation of an aortocaval fistula and therefore the differences observed may be model specific. Cardiac angiotensinogen mRNA was just about detectable by Northern blotting in the ACE inhibitor study but could not be detected by this method in the left or right ventricles of the heart failure study animals. Angiotensinogen mRNA has a reported cardiac chamber distribution similar to renin mRNA (Baker, Booz, and Dostal, 1992). Therefore, the difference observed in detection between the

two studies may be accounted for by the predominant atrial mRNA expression of angiotensinogen.

The only major discrepancy in the pattern of tissue gene expression between the two rat studies is for adrenal angiotensinogen. Whilst adrenal angiotensinogen mRNA was easily detectable in the ACE inhibitor study (chapter six), it could not be detected in the heart failure study (chapter eight) using the same probe. The reason for this discrepancy is unclear as both studies used adult male Wistar rats (although the ages were different) and so strain-specific expression cannot explain the observations. The major difference between these two sets of animals is that the heart failure study rats underwent surgery (even the controls had a sham operation) whereas the ACE inhibitor study animals did not. It may therefore be possible that surgery induced a physiological response that reduced adrenal angiotensinogen mRNA expression below the detectability of Northern blotting. However, this is a purely speculative explanation as such a response has not previously been described.

The marmoset study, presented in chapter seven, showed an increase in kidney renin mRNA with ACE inhibition in keeping with the rat studies. In addition, the other modes of blockade of the RA system employed in this study increased renal renin mRNA to varying degrees. Active plasma renin increased significantly with ACE inhibition as in the rat studies. Kidney angiotensinogen mRNA did not alter after ACE inhibition (or any of the other treatments) as was found with the chapter six study in rats, but in contrast to the heart failure study (chapter eight). Liver angiotensinogen mRNA was reduced by ~ 40% with ACE inhibition and whilst this was statistically significant as analysed by ANOVA, pairwise comparisons on log-transformed data did not quite achieve statistical significance. This tendency, at least, for a reduction in angiotensinogen mRNA was in contrast to the rat studies where no difference was seen with ACE inhibition. Plasma angiotensinogen was markedly decreased with ACE inhibition in the marmoset study as in the heart failure study and this is likely to be a result of increased consumption of angiotensinogen by elevated plasma renin levels. Hepatic renin mRNA was probed for but could not be detected in contrast to the rat studies. However, this may have been a consequence of not quite enough sensitivity using the

human renin probe. Likewise, ACE mRNA was probed for but could not be detected in either liver or kidney by Northern blotting, in keeping with the rat studies.

An interesting global theme arising from the results presented in this thesis is the tissue specific modulation of RA system gene expression by ACE inhibition, with differences observed between the different studies. In the case of the marmoset study differences were also seen with comparison to renin inhibition and when comparing the various modes of inhibition to each other. ACE inhibitors appear to have multiple mechanisms of action (see Table 10.1). Their principal antihypertensive action is inhibition of ACE in the blood and other tissues such as the kidney. The major antihypertensive effect of ACE inhibitors is the short term rather than long term depressive effect. This is supported by the observation that activity of the endocrine RA system follows the principle of a closed loop hormonal system. With the exception of states of severe cardiovascular decompensation, such as that seen in the heart failure study, activity of the circulating RA system almost always returns to normal when homeostasis is achieved. This can be readily observed in the chronic compensated phase of heart failure where plasma renin is no longer elevated (Dzau *et al.*, 1981). This "non- (circulating) renin dependant" phase may be a stage of heart failure in which the acute action of the circulating RA system is no longer necessary. At this stage, local RA systems may maintain vascular tone and cardiac function. Therefore, during prolonged administration of an ACE inhibitor, activity of the inhibitor may be determined by its relative distribution and half-life in different tissues rather than its serum half-life (Unger, Gohlke, and Gruber, 1990). The results presented in this thesis support such an argument.

TABLE 10.1 Possible mechanisms of the action of converting enzyme inhibitors.

Inhibition of circulating AII formation
Inhibition of local AII in the kidney and other sites
Increasing regional blood flow (especially in the kidney)
Increase in bradykinin levels at tissue sites
Vasodilatory prostaglandin biosynthesis
Blunting of arterial baroreceptor reflex
Attenuation of catecholamine release from noradrenergic nerve endings
Vasodilatory action via activation of endothelial vasodilatory pathways involving nitric oxide or nitrosothiol compounds
Scavenging free oxygen radicals (sulphydryl class of inhibitors)

Whilst inhibitors of ACE, renin and the AT_1 receptor are commonplace in the study of the RA system and ACE inhibitors the major clinical choice for blockade of the RA system, angiotensinogen is a largely unexplored clinical target for blockade of the RA. This is despite the observation that both its plasma level and tissue mRNA expression can be altered indirectly by blockade of other components of the RA system. There are two main reasons for the lack of research in this area:

(i). Although it is that known the concentration of angiotensinogen in blood can be rate limiting for angiotensin formation (See section 2.1.2), there is a distinct lack of pharmacological agents that specifically block angiotensinogen.

(ii). Although angiotensinogen exhibits genetic linkage to and association with essential hypertension and preeclampsia (Jeunemaitre *et al.*, 1992), it is only recently that molecular genetic studies have suggested functional clues as to how angiotensinogen may be a determinant of disease (Lodwick *et al.*, 1995).

In a recent study by Inoue and co-workers (1995), a mutation was identified in a preeclamptic patient leading to the replacement of leucine by phenylalanine at position 10

(the site of renin cleavage) of mature angiotensinogen. Kinetic analyses demonstrated that this substitution leads to a 10-fold decrease in the K_M for renin. New strategies have also been developed to blockade angiotensinogen and thus study its regulation and role in disease. A recent study by Tomita and co-workers (1995) using antisense oligonucleotides against angiotensinogen, transfected directly into the liver of male SHRs, demonstrated a transient decrease in blood pressure. This was accompanied by a significant decrease in liver angiotensinogen protein content and mRNA expression, decreased plasma angiotensinogen levels, and decreased levels of AII. Hepatocytes cultured from the livers of animals treated with antisense oligonucleotides also released significantly less angiotensinogen into the culture media than hepatocytes derived from animals treated with sense oligonucleotides. These results suggest that angiotensin production may be proportional to plasma angiotensinogen concentration and that angiotensinogen is a potential future target for antihypertensive prophylaxis.

Future studies:

The work presented in this thesis has addressed gaps in our knowledge regarding the organisation and regulation of expression of tissue renin-angiotensin systems and raised exciting questions and possibilities. The nature of a Ph.D. thesis is necessarily finite and there is much scope for this work to be extended.

The most obvious way in which to complement the Northern blot data presented here would be to investigate the tissues where an mRNA species could not be detected by Northern blotting, with a more sensitive technique such as RNAase protection or PCR. Work to circumvent this particular limitation was initiated by the development of a RT-PCR assay for ACE mRNA as presented in chapter 9. However, as alluded to previously, the construction of a truly quantitative assay is extremely consuming of time and resources. The basic development of quantitative assays for renin, angiotensinogen, ACE and the AT_1 receptor would be at least a thesis in itself and was therefore outside the scope of this thesis.

Another issue not directly addressed by this thesis is the precise tissue localisation of the RA system mRNAs detected e.g. whole brains and kidneys were used for extraction of

RNA in the rat studies, but a number of studies have localised the expression of components of the RA system to specific tissue areas and cell types (see section 3.5). It also possible that when tissue expression is modulated, e.g. by ACE inhibition, the pattern of distribution of expression within the tissue may also alter and this cannot be discounted by the approach taken here. These limitations could be addressed by undertaking similar studies and using *in situ* hybridisation to assess the tissue distribution of mRNA.

The regulation of RA system gene expression by cis-acting 5' elements and alternative promoter sequences has been well documented (see section 3.4). It is also possible that gene expression may be regulated by the adoption of alternative start sites for initiation of transcription. This could be examined by the application of primer extension analysis to the RNAs isolated for the studies presented in this thesis, in future studies.

Perhaps the biggest limitation of analysis using Northern blotting is that steady-state levels of mRNA are measured. Therefore, although differences detected in gene expression are highly likely to be due to differences in the level of transcription (and an absence of a difference due to the level of transcription being identical), altered stability (with no change in transcription) of the mRNA molecule (or a combination of both phenomena) cannot be ruled out. This question could be resolved by the use of nuclear run-off assays in future studies.

A major expansion of the work presented in this thesis would be to look at expression and regulation of the foetal tissue RA system as this is an area that has largely been ignored in the literature. In a preliminary study of outbred Wistar foetii undertook (but not presented in the experimental chapters of this thesis) to test the feasibility of such a study, expression of mRNA was detectable by Northern blotting for all of the components of the RA system in a wide range of tissues (see Table 10.2 for a summary of results). Interestingly, ACE mRNA was detectable in all tissues except the liver. This is in stark contrast to the studies presented in the experimental chapters of this thesis where attempts to detect ACE mRNA (using the same cDNA probe) by Northern blotting only gave a positive result in the lung. Although, as demonstrated in chapter 9, ACE mRNA could be detected by RT-PCR in extra-pulmonary

TABLE 10.2 Expression of the components of the renin-angiotensin system in rat foetal tissues (After Kelly, Bradley, and Samani, unpublished data).

18 DAYS GESTATION

Tissue	Renin	Angiotensinogen	ACE	AT_1 receptor
Kidney	++	+(+)	(+)	+++++
Liver	+	+++++	---	+++(+)
Brain	+	+(+)	+	+
Heart	+	+	+	++
Lung	+	+	++	+++++
Placenta	+	+++	+++	+++(+)

20 DAYS GESTATION

Tissue	Renin	Angiotensinogen	ACE	AT_1 receptor
Kidney	+++++	---	(+)	++++
Liver	++	+++++	---	++++
Brain	++	+(+)	+(+)	++
Heart	+(+)	---	+	++
Lung	+(+)	(+)	+++++	+++++
Placenta	+(+)	++++	+++	++++
Umbilical cord	(+)	++	(+)	++

\+ mRNA expression detected by Northern blotting (scale + to +++++)

--- mRNA expression not detectable by Northern blotting

tissues in adult rats, the difference in sensitivity between Northern blotting and RT-PCR implies that expression of extra-pulmonary ACE mRNA (at least) is greatly enhanced in foetal tissue. Angiotensinogen mRNA could also be detected in the lung and AT_1 receptor mRNA in the brain and heart of foetal rats but could not be detected in adult rats using Northern blotting (see chapter 8). Large differences in expression of renin and ACE mRNA between 18 and 20 days gestation of rat foetii (full term is 21 days) were also seen in the primary sites of production. Kidney renin expression at 20 days gestation was 2.5 x that at 18 days gestation and lung ACE expression also increased by the same magnitude. Angiotensinogen and AT_1 receptor expression were largely unchanged in all of the tissues examined between 18 and 20 days gestation. These results highlight the importance of the rate-limiting enzymes of the RA cascade (renin and ACE) and suggests a 'switch' to adult levels of expression immediately prior to birth. Such an upregulation in expression is perhaps not too surprising from the point of view of the endocrine RA system, as the newly born animal will be responsible for the maintenance of its own blood pressure and homeostasis. What is interesting, however, is the concept that the foetus may have a functional RA system when homeostatic functions are largely regulated by the mother, via the placenta. The importance of local generation of angiotensins, a fundamental theme throughout this thesis, with functions such as mitogenesis would seem to be especially pertinent to the foetal situation.

There has been an exponential increase in our understanding of the role of the renin-angiotensin system in normal and pathological physiology since the introduction of pharmacological agents that can effectively blockade the system. Prior to the availability of these agents the only way to disrupt the production of angiotensins was the literal ablation of the tissue source of hormone production. This was a crude, invasive methodology and, given that there is now overwhelming evidence for the existence of functional tissue RA systems, somewhat ineffective. Despite the increase in understanding and almost 100 years passing since the discovery of renin (Tigerstedt and Bergman, 1898), there is still much scope, and indeed necessity, for further research into the precise organisation, regulation and multiple functions of tissue renin-angiotensin systems.

CHAPTER ELEVEN

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