



**GENE EXPRESSION OF THE RENIN-ANGIOTENSIN SYSTEM IN THE
SPONTANEOUSLY HYPERTENSIVE RAT**

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DECLARATION

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PUBLICATIONS IN SUPPORT OF THIS THESIS

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ABSTRACT

The principal aim of the studies described in this thesis was to examine the gene expression of the renin-angiotensin system (RAS) in the spontaneously hypertensive rat (SHR) and the normotensive Wistar-Kyoto rat (WKY).

(1) The technique of *in situ* hybridisation was used in order to determine the cellular localisation of the gene expression of the RAS in kidney and liver in the pre-hypertensive and established phases of hypertension and to determine whether the sites of expression of the RAS in vascular tissue were similar in hypertensive and normotensive rats. The developmental regulation of kidney renin gene expression in the SHR was similar to that seen in the WKY. No differences in the cellular localisation of angiotensinogen mRNA in liver was noted at any age in both SHR and WKY. ACE mRNA expression in liver and kidney was also similar in the two rat strains. In the mesenteric vascular bed of both SHR and WKY, angiotensin converting enzyme (ACE) was expressed in the endothelium and smooth muscle layer of the blood vessels as well as in the surrounding adipose tissue. Angiotensinogen expression was noted in the smooth muscle and surrounding adipose tissue, while renin mRNA was inconsistently demonstrated in the smooth muscle layer only. ACE mRNA was also demonstrated in the adipocytes of human subcutaneous and extra-peritoneal adipose tissue.

(2) The mRNA expression for renin, angiotensinogen and ACE was determined in the kidneys and livers from SHR and WKY during chronic treatment with captopril and following its withdrawal. Chronic captopril treatment was associated with a dramatic rise in renin mRNA in the kidney and an elevation in mRNA for ACE in the liver. The release from captopril treatment was associated with a reversal of the increase in kidney renin mRNA but no reversal of the sustained elevation of ACE mRNA in the liver. *In situ*

hybridisation revealed a localisation of renin to the area of the juxtaglomerular apparatus in the kidneys from untreated animals, but recruitment of vascular sites of renin expression in kidneys from captopril-treated animals. In kidneys from released animals, renin mRNA expression was once again confined to the juxtaglomerular apparatus. ACE mRNA was expressed in hepatocytes throughout the livers from animals in all treatment groups. The results highlight a differential effect of captopril withdrawal upon the gene expression of the components of the renin-angiotensin system in kidney and liver.

(3) Neonatal sympathectomy did not influence the gene expression of the RAS components in the kidneys, livers and the mesenteric vascular bed from adult SHR and WKY. ACE activity in plasma, kidney, liver, aorta, lung, skeletal muscle and brain from adult animals was also not affected by neonatal sympathectomy. The accumulation of AII into aorta, mesenteric artery and its branches, kidney and skeletal muscle was not influenced by innervation, hyperinnervation or sympathectomy which indicates that, unlike adrenaline, the facilitation of sympathetic transmission by AII does not involve a process of uptake of the peptide into sympathetic nerves.

(4) The ACE gene was expressed at a similar level in livers and kidneys from both SHR and WKY, despite the fact that the corresponding ACE activity was at least an order of magnitude lower in livers when compared with kidneys. The possibility that endogenous ACE inhibitory activity was present in the liver was investigated by examining the effect of liver homogenates on plasma ACE activity. The effect of hepatotoxicity on plasma ACE activity was also studied, in order to determine whether the liver was a source of circulating ACE. Liver homogenates from both WKY and SHR, when added to plasma, significantly decreased the measurable ACE activity in it by 41% and 50% respectively. When liver homogenates were pre-incubated with the sulphhydryl-blocking drug 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB, 5 mM), the inhibitory effect of the homogenate

on plasma ACE was significantly reduced. Plasma ACE activity was not influenced by carbon tetrachloride-induced hepatotoxicity in either rat strain. In contrast, there was a four-fold increase in liver ACE activity which was not associated with a significant change in the ACE mRNA levels or a significant change in the inhibitory activity of liver homogenates. The results indicate that hepatic ACE activity is subject to endogenous inhibition and suggest that there is an uncoupling of ACE gene expression and activity in the liver.

ABBREVIATIONS

The following abbreviations have been used throughout this thesis.

| | |
|------------------|--|
| βME | β-mercaptoethanol |
| A ₂₆₀ | absorbance at 260 nm |
| A ₂₈₀ | absorbance at 280 nm |
| A ₇₅₀ | absorbance at 750 nm |
| ACE | angiotensin converting enzyme |
| AD | adrenaline |
| AI | angiotensin I |
| AII | angiotensin II |
| AIII | angiotensin III |
| ALaAT | alanine aminotransferase |
| APES | 3-aminopropyltriethoxysilane |
| AT ₁ | angiotensin II receptor, type 1 |
| AT ₂ | angiotensin II receptor, type 2 |
| b | bases |
| C-terminal | carboxyl-terminal |
| cAMP | adenosine 3',5'-monophosphate |
| cDNA | complementary deoxyribonucleic acid |
| cGMP | guanosine 3',5' monophosphate |
| COMT | catechol-O-methyl transferase |
| cRNA | complementary ribonucleic acid |
| CSIRO | Commonwealth Scientific and Industrial Research Organisation |
| DG | diacylglycerol |
| DNA | deoxyribonucleic acid |
| DOCA | deoxycorticosterone acetate |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylenebis(oxyethylenitrilo)tetraacetic acid |
| GRE | glucocorticoid responsive element |
| HHL | hippuryl-histidine-leucine |
| His | histidine |
| IP ₃ | 1,4,5-inositol triphosphate |
| k | rate constant for hydrolysis |
| K _{cat} | maximal velocity |
| kd | kilodaltons |
| K _m | Michaelis constant |

| | |
|----------------|---------------------------------------|
| Leu | leucine |
| L _f | final fragment length in kilobases |
| L ₀ | initial fragment length in kilobases |
| M | moles/litre |
| mM | millimoles/litre |
| mm Hg | millimetres of mercury |
| MOPS | 3-[N-Morpholino]propanesulphonic acid |
| mRNA | messenger ribonucleic acid |
| N-terminal | amino-terminal |
| NA | noradrenaline |
| NADH | nicotine adenine dinucleotide |
| NGF | nerve growth factor |
| NMN | normetanephrine |
| OHDA | 6-hydroxydopamine |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PRA | plasma renin activity |
| ptn | protein |
| R.V. | reaction volume |
| RAS | renin-angiotensin system |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| S.A. | specific activity |
| S.V. | sample volume |
| SAMe | s-adenosyl-methionine |
| SDS | dodecyl sulphate, sodium salt |
| SEM | standard error of the mean |
| SHR | spontaneously hypertensive rat |
| SNS | sympathetic nervous system |
| SSC | standard sodium/citrate buffer |
| SSPE | standard saline/phosphate/EDTA buffer |
| TAE | tris/acetate/EDTA buffer |
| TLC | thin layer chromatography |
| Tris | tris(hydroxymethyl)aminomethane |
| tRNA | transfer ribonucleic acid |
| UTP | uridine 5'-triphosphate |
| UV | ultra-violet |
| WKY | Wistar-Kyoto rat |
| Xaa | unspecified amino acid |

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1.1 Hypertension - definition and aetiology

Hypertension, the pathological elevation of blood pressure, is one of Western society's most common disorders. In Australia, 18% of males and 14% of females are affected [National Heart Foundation, Risk Factor Prevalence Study No. 3, 1989]. Adults with a systolic pressure greater than 140 mm Hg and/or a diastolic pressure greater than 90 mm Hg (measured at least twice on two different occasions) are regarded as suffering from hypertension [Guidelines Sub-Committee of WHO/ISH Mild Hypertension Liaison Committee, 1993]. The consequences of high blood pressure result from the fact that the heart and blood vessels in most species are not designed to withstand the long term pressure elevation. Morbidity and premature death from stroke, coronary artery disease, heart failure or renal failure is a common outcome.

Hypertension can be classified as either

(a) primary, where the aetiology is unknown

or (b) secondary, where the cause is due to a known organic condition, e.g. phaeochromocytoma, renovascular disease or primary aldosteronism.

Although the aetiology of primary hypertension is unknown, it is generally accepted that a multi factorial explanation must be considered. It has been proposed [Folkow, 1978] that primary hypertension results from the interactions of at least three major types of influences.

(1) A polygenetically inherited predisposition constitutes the primary requirement. In humans, this is likely to be individually variable due to different arrays of predisposing genes which may differ in power.

(2) Environmental factors, such as salt intake and psychoemotional elements, may influence or precipitate hypertension depending on the balance of predisposing elements.

(3) Secondary adjustments, including structural adaptation of the heart, arteries and arterioles, may also be genetically reinforced.

Thus, the pro-hypertensive influences of (1) and (2) may interact with (3) to produce a positive feedback situation which gradually shifts blood pressure upwards and resets the barostats.

1.2 The Renin-Angiotensin System

1.2.1 Production and actions of angiotensin II

There are a variety of mechanisms which are involved in the control of blood pressure. The Renin-Angiotensin System (RAS), through its production of Angiotensin II (AII) plays a major role in cardiovascular homeostasis. The enzymatic reactions involved in the RAS cascade are shown in *Figure 1.1.* (page 4). Originally, the RAS was considered to be a circulating endocrine system. The enzyme renin, synthesised in the kidney, acts on angiotensinogen, which is secreted by the liver, to form angiotensin I (AI). AI is then converted to angiotensin II (AII) by angiotensin converting enzyme (ACE). The major site of ACE in the circulatory system appears to be the endothelial lining of the pulmonary blood vessels [Erdös, 1979].

AII interacts with specific receptors and has varied and widespread actions in a number of tissues (which will be outlined only briefly here). The decapeptide AI has limited intrinsic biological activity [Khosla et al, 1974]. The heptapeptide AIII has similar actions to AII, but its potency is much lower and it is present in much lower concentrations than AII [Khosla et al, 1974; Semple et al, 1976].

AII is a potent pressor agent. It causes an immediate vasoconstrictor response by directly acting on vascular smooth muscle [Brown et al, 1964; Peach, 1977] and indirectly causes contraction by enhancing the activity of the sympathetic nervous system [Nicholas, 1970; Van Zweiten and De Jonge, 1986]. At sub-constrictor doses, administered chronically, AII also has a less well understood slowly developing pressor action which results in a gradual, progressive rise in blood pressure [Dickinson and Lawrence, 1963; Bean et al, 1979; Lever, 1993]. AII acts directly on vascular endothelium to influence the release of endothelium-derived constricting and relaxing factors [Lüscher and Vanhoutte, 1990].

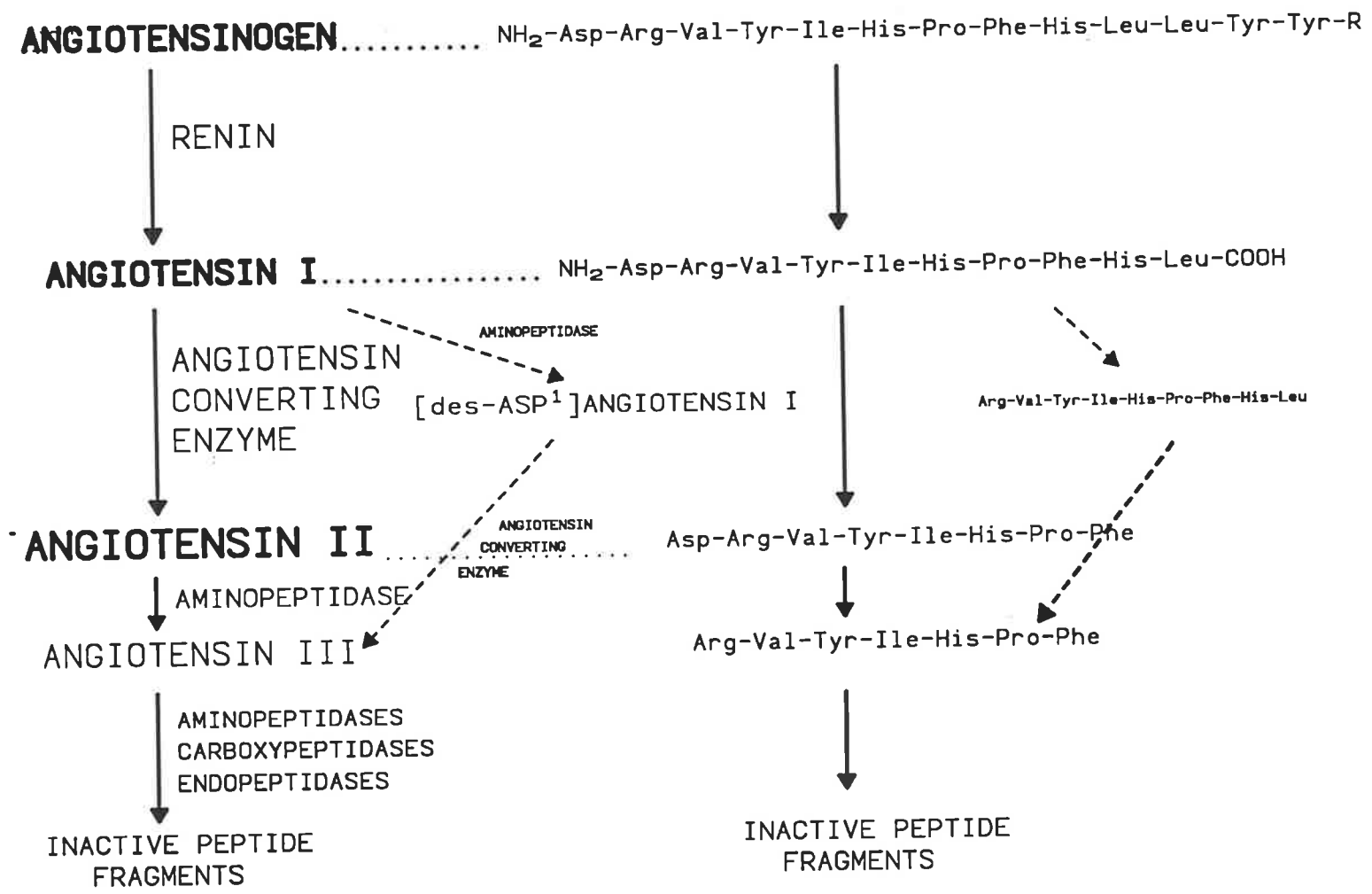


Figure 1.1. Enzymatic reactions of the Renin-Angiotensin System. The solid arrows show the main pathways, while the dashed arrows indicate a minor pathway. The sequence of rat angiotensinogen is shown. (Adapted from [Douglas, 1985])

The volume and composition of extracellular fluid is controlled by AII through multiple central and peripheral actions. These include the stimulation of thirst [Fitzsimmons, 1969; Fitzsimmons, 1993] vasopressin secretion [Bonjour and Malvin, 1979; Brooks and Malvin, 1993] and aldosterone secretion [Carpenter et al, 1961; Robertson, 1984]. In the kidney, AII influences glomerular filtration rate and renal blood flow as a consequence of renal artery and arteriolar vasoconstriction. It also has direct effects on proximal tubule epithelium sodium and water reabsorption [Schalekamp and Derkx, 1993].

The role of AII as a growth factor has been demonstrated in a number of vascular and non-vascular cell types. AII induces DNA and protein synthesis which is associated with the expression of the mitogen platelet-derived growth factor and the proto-oncogenes *c-fos* and *c-jun* [Heagerty, 1991].

While the components of the RAS can be found in plasma, they have also been detected at many other sites where they constitute what is known as local tissue RASs which have the potential to act in an autocrine or paracrine fashion. Although the precise functions of these local RASs are still unknown, it has been suggested that the circulating RAS is important for the acute regulation of blood pressure and that local tissue RASs are involved in the long term determination of cardiovascular homeostasis [Dzau, 1987].

1.1.2 Renin

Renin (EC 3.4.23.15) is a glycoproteolytic enzyme that is responsible for the enzymatic initiation of the RAS cascade - the conversion of angiotensinogen to AI. In humans and rats, transcription from a single renin gene, ~12.5 kilobases (kb) in length, results in a mRNA of ~1450 bases (b) [Imai et al, 1983; Soubrier et al, 1983; Burnham et al, 1987]. In mice there are two renin genes, designated *ren1* and *ren2* [Mullins et al,

1982; Panthier et al, 1982]. The enzyme is a monomeric aspartyl protease of molecular weight 36-40 kilodaltons (kd) and pI 4.8-5.6 (depending on the species) [Inagami, 1993]. The main source of renin is the juxtaglomerular cells of the afferent arterioles of the kidney. Translation of renin mRNA yields preprorenin, which, following post-translational glycosylation and removal of a signal peptide, becomes prorenin. The prorenin can be secreted constitutively into the plasma, or packed into granules and secreted in a regulated fashion. Processing of prorenin to active renin can be species- and tissue-specific. A number of enzymes including trypsin, plasmin and cathepsin B are capable of activating prorenin *in vitro*, but the primary site of conversion of plasma prorenin to renin remains unclear [Griendling et al, 1993; Reudelhuber et al, 1993]

Renin shows extremely stringent substrate specificity [Misono et al, 1974]. Angiotensinogen is the only known substrate for renin. The rate at which renin generates AI from angiotensinogen is species selective due to differences in amino acid sequences of both renin and angiotensinogen in different species. Renin has a pH optimum in the vicinity of 6, although the optimum is broad so the reaction proceeds readily at the physiological pH of plasma (pH 7.3-7.4) [Favre et al, 1973]. Plasma renin activity (PRA) is generally regarded as an index of the activity of the RAS and is a valuable tool in the diagnosis of several disorders including Conn's syndrome, Liddle's syndrome and Gordon's syndrome [Nicholls and Robertson, 1993].

1.2.3 Angiotensinogen

Angiotensinogen is a globular protein of molecular weight 55-65 kd and pI 4.3-4.9, depending on the degree of glycosylation [Clauser et al, 1989]. Transcription from a single gene, ~13 kb in length, results in a mRNA species of ~1600 b. AI is generated by the cleavage of a leucine-valine bond in human angiotensinogen or a leucine-leucine bond in other species. While angiotensinogen is the only known substrate for renin, there are a number of other enzymes which can cleave angiotensinogen to form AI

(Cathepsin D, pepsin and other aspartyl proteases) and AII (tonin, cathepsin G, trypsin and kallikrein) [Campbell, 1987].

The majority of circulating angiotensinogen is derived from the liver, mainly from the pericentral zone of the liver lobules [Morris et al, 1979]. The plasma serves as a major reserve of angiotensinogen, and the plasma level is a major determinant of RAS activity [Clauser et al, 1989].

1.2.4 Angiotensin converting enzyme

Angiotensin converting enzyme (ACE, EC 3.4.15.1) is dipeptidyl carboxypeptidase that converts AI to AII. ACE is a monomeric zinc metallopeptidase which has a diverse substrate specificity. It facilitates the removal of dipeptides and tripeptides from the carboxy-terminal of a number of other substrates, including bradykinin [Erdös, 1990], neurotensin [Skidgel et al, 1984] and opioid peptides. [Skidgel and Erdös, 1967]. ACE also displays some endopeptidase activity towards substance P [Yokosawa et al, 1983] and luteinizing hormone-releasing hormone [Skidgel and Erdös, 1985]. The favoured ACE substrate is bradykinin, for which the Michaelis constant (K_m) is approximately 80 times lower than for AI (0.2 versus 16 $\mu\text{mol/l}$ respectively) [Soubrier et al, 1993a].

Two forms of ACE have been isolated - a pulmonary or somatic form and a testicular form. Somatic ACE is associated with the plasma membrane of vascular endothelial cells and thus has a ubiquitous tissue distribution. High levels of ACE are also found in epithelial cells (e.g. kidney proximal tubules, intestine) and neuroepithelial cells (e.g. striatum, cerebellum, pituitary) [Skidgel and Erdös, 1993]. Although the majority of ACE is a membrane-bound enzyme, a soluble form (which is presumed to be derived from the sloughing off from membranes) is also found in body fluids such as blood, urine, seminal plasma and cerebrospinal fluid [Cushman and Cheung, 1971b; Erdös and

Skidgel, 1987]. The mechanism of solubilisation of ACE from membranes *in vivo* is not clear.

Somatic ACE is a glycoprotein (8-32% carbohydrate) which is heavily sialated (tissue and species dependent) and has a molecular weight of 140-170 kd [Das and Soffer, 1975; Berecek et al, 1993]. In the testis, a distinct smaller isoform (M.W. ~100 kd), representing the C-terminal half of somatic ACE, is present in germinal cells [Brentjens et al, 1986]. Membrane-bound ACE is anchored by its C-terminal and is orientated such that the catalytic sites are exposed at the extracellular surface of the cell.

Monovalent anions, especially chloride, enhance the activity of ACE towards its substrates, although the extent of activation varies with different substrates. While chloride is essential for the cleavage of AI, this is not the case for all substrates. For example, the hydrolysis of bradykinin proceeds at ~35% of the maximal rate in the absence of chloride [Erdös, 1979; Bunning and Riordan, 1983]. This activation of ACE is thought to be the consequence of a change in the conformation of the protein induced by the binding of chloride to a lysine residue located in the region of the active site [Weare, 1982].

Studies which analysed purified ACE showed that the enzyme contains one gram atom of zinc per mole of enzyme, which is indicative of a single active site [Bunning and Riordan, 1985]. Further evidence for a single active site was provided by binding studies with radiolabelled inhibitors [Cumin et al, 1989] and the fact that truncated testicular ACE appeared to be fully active [Lattion et al, 1989]. However, molecular cloning studies have revealed that ACE consists of two large highly homologous domains, designated as the amino or N- and carboxyl or C- domains [Soubrier et al, 1988]. Both domains possess putative active sites, based on sequence homology with the consensus sequence His-Glu-Xaa-Xaa-His for the active site of zinc metallopeptidases [Vallee and Auld, 1990]. *In vitro* mutagenesis studies, in which either one of the two domains was absent or critical residues in one of the active sites

were point-mutated, have demonstrated conclusively that ACE contains two functionally active sites [Wei et al, 1991a; Wei et al, 1991b]. Further, since each domain exhibits differing orders of potency for various ACE inhibitors, the two active sites appear to be structurally distinct [Wei et al, 1991c]. Subsequent reinvestigation of purified somatic ACE has shown that the enzyme contains two atoms of zinc per molecule [Ehlers and Riordan, 1991; Williams et al, 1992] and displays differential binding parameters for radioligands and ACE inhibitors [Brée et al, 1992; Perich et al, 1992].

As illustrated in Table I.A., (page 10) the kinetic parameters and chloride dependence for hydrolysis differ between the two domains. While both domains show similar K_m values for the substrates examined, the rate of hydrolysis is much higher for the C-domain, which is also more dependent on the presence of chloride for hydrolysis. The optimum chloride concentrations for each of the domains (10 mM for the N-terminal and 800 mM for the C-terminal) are markedly different from that which was determined for purified rabbit lung ACE (300 mM) [Bunning and Riordan, 1985]. However, the chloride activation profiles of human kidney ACE and recombinant wild type ACE are indistinguishable [Wei et al, 1991b]. The disparity regarding optimum chloride concentrations for rabbit and human ACE was not addressed by Wei et al, (1991b) but it may be related to the methods of purification of the ACE preparations from the two species.

The functional significance of the two domains of ACE is not clear. Wei et al (1991b) have suggested that the chloride concentration may determine the relative contribution of each domain to the overall activity of the enzyme. Thus, the C-domain might be responsible for the majority of the activity at vascular and extracellular sites where the chloride concentration is high. However, at intracellular locations where the chloride concentration is low, the N-domain may play a greater role.

| Hip-His-Leu | | | | |
|-------------|----------------------|-------------------------------------|-------------------------------|----------------------------------|
| Enzyme | K _m μM | K _{cat} s ⁻¹ | No chloride % max activity | Optimal [Cl ⁻] mM |
| Wild type | 1540 | 408 | 0.8 | 800 |
| N-domain | 2000 | 40 | 13.1 | 10 |
| C-domain | 2000 | 360 | 0.1 | 800 |

| Angiotensin I | | | | |
|---------------|----------------------|-------------------------------------|-------------------------------|----------------------------------|
| Enzyme | K _m μM | K _{cat} s ⁻¹ | No chloride % max activity | Optimal [Cl ⁻] mM |
| Wild type | 16 | 40 | 2.3 | 30 |
| N-domain | 16 | 12 | 5.5 | 10 |
| C-domain | 16 | 35 | 1.8 | 30 |

Table I.A. Kinetic parameters and chloride dependence for hydrolysis of the artificial substrate His-His-Leu and angiotensin I by recombinant wild type and mutant ACE (adapted from Wei et al, 1991b; Soubrier et al, 1993b).

ACE has a wide tissue, cellular and intracellular distribution but at many of the extra-vascular locations, the precise function ACE is not known. The application of the techniques of molecular biology to the study of ACE has resulted in a reappraisal of the structure and function of the enzyme. The discovery of two structurally and functionally distinct domains has raised the possibility that there may be another endogenous substrate, which has more favourable kinetics for the N-domain. The discovery of such a substrate may have important clinical implications.

1.2.5 Angiotensin receptors

AII exerts its effects by interacting with specific, high affinity receptors in the plasma membranes of its target cells. The development of selective AII antagonists has led to the identification of two major receptor types, which have been designated AT₁ and AT₂. The characteristics of these receptor types are outlined in Table 1.2

| | AT ₁ | AT ₂ |
|----------------------------|-----------------------------|---------------------|
| Potency order | AII > AIII | AII = AIII |
| Selective antagonist | losartan | PD123177, CGP42112A |
| Dithiothreitol sensitivity | inhibited | potentiated |
| Effector pathways | ↑IP ₃ /DG; ↓cAMP | not known (↓cGMP?) |

Table I.B. Properties of angiotensin receptors. (Adapted from Catt, 1993; Timmermans et al, 1993) (IP₃, 1,4,5-inositol triphosphate; DG, diacylglycerol; cAMP, adenosine 3',5'- monophosphate; cGMP guanosine 3',5'-monophosphate)

Cloning and sequencing of the AT₁ receptor from rat aortic smooth muscle [Murphy et al, 1991] and subsequent genomic analysis has suggested that further sub-classification (AT_{1A} and AT_{1B}) is warranted [Iwai and Inagami, 1992; Kakar et al, 1992]. Based on sequence analysis, both AT₁ subtypes share structural similarities with other receptors which are coupled to guanyl nucleotide regulatory proteins. Thus, the receptor contains seven hydrophobic regions representing putative membrane-spanning domains and three potential N-glycosylation sites.

To date almost all of the physiological actions of AII have been inhibited by the AT₁-selective antagonist losartan and thus the precise physiological function of the AT₂

receptor remains obscure [Timmermans et al, 1993]. Therefore the designation of the AT₂ receptor subtype remains tentative until there is a definitive demonstration of a physiological role for this AII binding site [Timmermans et al 1993]

1.2.6 The RAS in essential hypertension-therapeutic RAS inhibitors

The role of the RAS in cardiovascular homeostasis has led to the suggestion that abnormalities in this system may be associated with the pathogenesis of essential hypertension. Angiotensinogen concentrations have been reported to be higher in hypertensive subjects [Fasola et al, 1966]. However, in the majority of patients suffering from essential hypertension, PRA and plasma concentrations of AII are distributed throughout the broad range of values found in normotensive individuals [Doyle, 1977]. There have been no reported differences in ACE concentrations in plasma from hypertensive and normotensive humans. [Corvol et al, 1993]. Nevertheless, in many hypertensive patients, interruption of the RAS results in a depressor response which is not related to the pretreatment PRA level [Brunner et al, 1979; Frohlich et al, 1984].

There are now a number of antihypertensive agents which interfere with the RAS. There are three sites at which these agents act:

(1) The renin inhibitors, which prevent the conversion of angiotensinogen to AI, are the least well studied of the three classes of agents. This was initially because the early renin inhibitors were peptides with poor oral availability but more recently because these drugs are species selective. The renin-inhibitors developed for human use have blood pressure-lowering effects in primates only and are ineffective in rodents and so basic research involving these agents has been limited [Hui and Haber, 1993]

(2) The angiotensin converting enzyme inhibitors are the most widely used of the drugs which interfere with the RAS. They have been in clinical use for many years and have been studied extensively. They are extremely effective antihypertensive agents and have the added benefit of preventing or attenuating the deleterious structural changes in the blood vessels and heart which accompany the development of hypertension [Saxena and Man In't Veld, 1991; Johnston, 1993]. The precise mechanism of action of the ACE inhibitors has been a matter of some debate due to two reasons: (a) the antihypertensive effect of the ACE inhibitors does not correlate with the inhibition of plasma ACE [Waeber et al, 1989] and (b) as discussed above, AI is not the only substrate of ACE. ACE also acts on a number of other peptides including neurotensin, substance P and importantly bradykinin. Bradykinin, which has blood pressure-lowering effects, is degraded to an inactive molecule by ACE. Therefore, under conditions of ACE inhibition, the levels of active bradykinin are elevated and some researchers believe that this action of the ACE inhibitors contributes to their blood pressure-lowering effect [Antonaccio et al, 1981]. Other mechanisms which have been proposed to explain the antihypertensive effects of ACE inhibition include a reduction in sympathetic nervous activity [Antonaccio and Kerwin, 1981], alterations in baroreflex activity [Antonaccio et al, 1981] and stimulation of prostaglandin synthesis [Moore et al, 1981].

(3) The third site at which interference with the RAS has been most recently exploited is at the level of the AII receptor. The AT_1 receptor is responsible for the cardiovascular and natriuretic actions of AII. The AT_1 -selective receptor antagonists, e.g. losartan, are effective antihypertensive agents and they mirror many of the effects seen with the ACE inhibitors [Brunner et al, 1993; Timmermans et al, 1993]. Thus, hypotheses which conclude that the antihypertensive effects of the ACE inhibitors are due to "non-AII-mediators" no longer warrant support.

1.3 Animal models of hypertension

The study of hypertensive mechanisms has been greatly facilitated by the development of animal models of the disorder. Several models have been described [De Jonge, 1984]. In 1934, Goldblatt induced secondary hypertension in dogs by clamping the renal artery. Other forms of secondary hypertension have been induced by encasing the kidney in a bag, or kidney removal in conjunction with the administration of deoxycorticosterone acetate (DOCA) and saline.

Experimental study of primary hypertension was greatly expanded with the development of rat strains with inherited hypertension. The first such strain was the New Zealand genetically hypertensive rat developed by Smirk and Hall in 1958, followed a few years later by Okamoto and Aoki with the Kyoto spontaneously hypertensive rat. There are now a number of animal models of primary hypertension including the Dahl, Lyon, Milan and Sabra rat strains. Each of these strains have their own characteristics and it is possible that each corresponds to a subset of human primary hypertension.

The studies described in this thesis used the SHR and its normotensive control the Wistar-Kyoto rat (WKY).

1.3.1 The spontaneously hypertensive rat (SHR)

The SHR was developed by selective brother to sister inbreeding that resulted in 100% of the progeny developing hypertension without a requirement for pharmacological or surgical intervention [Okamoto and Aoki, 1963].

When compared with its genetic control, the WKY, arterial pressure is elevated in the SHR from around four weeks of age. Arterial pressure increases with age in both the SHR and WKY. However, in the SHR it does so at a faster rate and reaches a

maximum level at a later age (16-20 weeks in the SHR compared with 6-10 weeks in the WKY). Three phases have been designated in the life of the SHR. A pre-hypertensive phase spans the period from birth to 4 weeks of age. A period of developing hypertension occurs from 4 to 16-20 weeks of age, followed by a phase of established hypertension. In the conscious unrestrained adult male SHR the systolic arterial pressure averages 190-210 mm Hg compared with 115-130 mm Hg for the WKY.

The SHR exhibits a number of characteristics which are also seen in many human primary hypertensive individuals [Trippodo and Frohlich, 1981]. The elevated arterial pressure is associated with increased total peripheral resistance, a normal cardiac output and normal or slightly reduced blood volume. There is high sympathetic nerve activity (at least in some stages) as well as left ventricular and vascular medial hypertrophy. Hypertension may be aggravated by excess dietary sodium, stress and other environmental influences. The SHR responds to many pharmacological agents that are effective antihypertensive agents in humans. In both humans and the SHR the sequelae of hypertension are cardiac failure, stroke or renal lesions which result in premature mortality. Thus the SHR is considered an excellent laboratory model of clinical primary hypertension.

In both humans and the SHR, the precise genetic lesions responsible for the development of hypertension have not been elucidated.

1.3.2 The RAS in the SHR

Early studies which searched for the cause of hypertension in the SHR discounted defects in the RAS because, despite some conflicting results [De Jonge et al, 1972; Forman and Mulrow, 1974], it was generally agreed that plasma renin activity was not elevated [Koletsky et al, 1970; Sen et al, 1972; Shiono and Sokabe, 1976] However,

kidney renin levels and plasma AII levels are reported to be significantly elevated in young SHR, prior to the development of hypertension [Siniako and Mirkin, 1974; Gomez et al, 1988a]. In adult SHR, when hypertension is established, kidney renin is lower than that seen in normotensive rats and it has been suggested that this decrease is a compensatory reaction to the elevated blood pressure [Shiono and Sokabe, 1976]. A similar pattern of abnormality has been reported in the renin content of mesenteric blood vessels, where renin content is significantly greater at 3 weeks of age but not at 17 weeks of age [Naruse and Inagami, 1982]. Thus, it appears that there are irregularities in renin activity or content that precede the development of hypertension in the SHR and so the RAS remains a candidate in the search for the lesion which causes hypertension. The findings from biochemical analyses have been supported by more recent evidence from molecular studies which indicate that there is a widespread abnormality in renin gene expression in the SHR that is modulated, in some tissues, with the development of hypertension [Samani et al, 1989].

ACE activity is suppressed in a number of tissues in the SHR [Rosenthal et al, 1987; Welsch et al, 1987]. However, the suppression of ACE activity cannot be ascribed simply to a compensatory reaction to elevated blood pressure, as was proposed for renin, since ACE levels are lower in SHR even at three weeks of age, before the development of hypertension [Grima et al, 1990]. Despite the fact that ACE activity is low in the SHR, administration of ACE inhibitors to young animals prevents the development of hypertension and has a potent blood-pressure lowering effect in adults [Bengis et al, 1978; Muirhead et al, 1978; Antonaccio et al, 1979; Ferrone and Antonaccio, 1979]. The profound effect of ACE inhibition on the SHR is discussed in more detail below (1.5)

The slow pressor response to AII is reported to be enhanced in the SHR [Li and Jackson, 1989]. The enhancement is specific to the slow pressor effect of AII since the fast response to intravenous infusion of AII and the slow response to noradrenaline are not different from that seen in WKY. It was proposed that the enhanced slow pressor

response to AII was mediated by a greater direct action of AII on the kidneys of SHR. These findings are particularly significant when considered in conjunction with the evidence that the hypotensive effects of ACE inhibition correlate with tissue inhibition and not plasma inhibition of the enzyme [Waeber et al, 1989].

1.4 Gene expression of the RAS

As mentioned earlier (1.2.1), as well as the classical circulating endocrine RAS, there are local tissue RASs which have the potential to exert autocrine and paracrine influences on local tissue function [Dzau, 1988]. Biochemical and immunohistochemical demonstrations of renin enzyme activity, renin substrate, ACE, angiotensin and angiotensin receptors in multiple tissues provided the initial evidence for local RASs. However, the presence of the RAS components in tissues could have been attributed to uptake from the circulation rather than local synthesis. The demonstration of mRNA for renin and angiotensinogen at multiple extrarenal and extrahepatic sites has provided convincing evidence for their local synthesis [Dzau, 1986; Campbell, 1987].

1.4.1 Regulation of gene expression

Following the discovery of local tissue renin-angiotensin systems, evidence has accumulated that they are regulated independently of the circulating endocrine system. The expression of a gene in a particular cell type is dependent on the interaction of specific DNA elements with a transcription complex which consists of general or cell specific transcription factors, nuclear proteins and RNA polymerase II. Sequence analysis of the promoters of the RAS genes have revealed a number of potential hormone and second-messenger-responsive elements which could contribute to tissue specific gene expression [Lynch and Peach, 1991; Morris, 1992; Soubrier et al, 1993a].

While the regulation of renin and angiotensinogen gene expression has been studied extensively, at the commencement of the studies described in this thesis, there were no reports regarding the control of ACE gene expression.

The expression of the renin gene is developmentally regulated. Kidney renin mRNA levels in newborn rats are 8-fold higher than the levels in kidneys from adults [Gomez et al, 1988a]. A number of studies have investigated the effect of dietary sodium on renin gene expression. Sodium depletion results in a significant rise in renin mRNA levels in kidney, heart and adrenal but not brain or testicular tissue [Nakamura et al, 1985; Ingelfinger et al, 1986; Dzau et al, 1986; Tada et al, 1989]. Kidney renin gene expression is also influenced by β -adrenergic agonist stimulation [Dzau et al, 1987b]. Extra-renal but not renal renin expression is increased by androgens and oestrogen [Dzau et al, 1987a].

The gene expression of angiotensinogen appears to be subject to developmental regulation. Angiotensinogen mRNA is undetectable in foetal rat livers, levels reach a peak 24 hours after birth and gradually decrease into adulthood [Gomez et al, 1988b]. In the kidneys of prepubertal male rats very little angiotensinogen mRNA can be detected, but by puberty and into adulthood it increases significantly [Ellison et al, 1989]. During phenotypic modulation of cultured 3T3 fibroblasts to an adipocyte cell-line, angiotensinogen accumulation increases dramatically, which also suggests that expression is controlled by a developmental regulatory mechanism [Saye et al, 1990].

A number of hormones are known to influence angiotensinogen expression, including androgens [Ellison et al, 1989], thyroid hormones [Kimura et al, 1990] and cytokines [Okamoto et al, 1987]. The most studied modulators regarding angiotensinogen expression are the glucocorticoids. Multiple "Glucocorticoid Responsive Elements" (GRE), which bind to the activated glucocorticoid receptor to enhance transcription, occur in the promoter region of the angiotensinogen gene [Lynch and Peach, 1991]. There is increased liver angiotensinogen mRNA accumulation in response to

glucocorticoids *in vivo*, in dispersed hepatocytes and in cultured cell lines, [Kalinyak and Perlman, 1987; Ben-Ari and Garrison, 1988; Chang and Perlman, 1988].

Dietary sodium restriction results in an increase in kidney and aorta but not liver or adipose tissue angiotensinogen mRNA levels [Ingelfinger et al, 1986]. Adipose tissue but not liver angiotensinogen gene expression is reported to be subject to nutritional regulation [Frederich et al, 1992].

Another important regulator of RAS gene expression is the vasoactive product of the RAS cascade, AII. Both renin and angiotensinogen are subject to feedback regulation by AII. In the kidney, AII suppresses renin gene expression [Johns et al, 1990], while in the kidney and liver AII increases accumulation of angiotensinogen mRNA [Klett et al, 1988b]. The effect of AII on angiotensinogen expression is due to the ability of AII to stabilise the angiotensinogen mRNA [Klett et al, 1988a].

In the SHR, sodium regulation of RAS gene expression is reported to be altered. While sodium depletion causes significant increases in kidney renin mRNA in both SHR and WKY, the response is enhanced in SHR [Kitami et al, 1989]. In WKY, mild sodium restriction is reported to stimulate kidney angiotensinogen mRNA expression. This was not seen in kidneys from SHR maintained on a similar sodium load [Pratt et al, 1989]. However, the finding from Pratt et al, (1989) must be viewed with caution since the expected increases in kidney renin gene expression were not observed in either rat strain, despite the fact that plasma renin activity and kidney renin content were significantly elevated by the sodium restriction.

From the above discussion, it is apparent that the RAS is widespread and subject to influence by a variety of physiological and non-physiological stimuli. The reasons why such a wide variety of tissues synthesise AII is not known and the significance of developmental and tissue-specific regulation of gene expression of the RAS is not yet clear.

1.4.2 Localisation of RAS gene expression

The determination of mRNA levels in tissue homogenates by Northern or slot analyses can determine whether or not a gene of interest is expressed in that tissue, and whether the level of expression is normal. However, these techniques cannot determine which regions or cells within a tissue are responsible for the synthesis of the mRNA. The techniques of differential dissection and *in situ* hybridisation allow localisation of the sites of gene expression within tissues and organs.

The regional distribution of angiotensinogen mRNA was determined in rat kidney by dissection of mid-sagittal sections into three regions [Campbell and Habener, 1986]. Angiotensinogen mRNA levels were found to be highest in the cortex, intermediate in the outer medulla and lowest in the inner medulla and papilla, suggesting a role for the local production of angiotensinogen in glomerular function in the kidney. Differential dissection following collagenase digestion was used to localise angiotensinogen mRNA to the adventitia and surrounding adipose tissue of rat aorta [Cassis et al, 1988a]. Dissection of rat brain into eight anatomically distinct sections demonstrated that angiotensinogen mRNA accumulated in all sections of the brain with the possible exception of the pituitary [Lynch et al, 1986].

The use of ³⁵S-labelled DNA or RNA probes in conjunction with *in situ* hybridisation provides a much more powerful tool to better define the spatial localisation of gene expression. This technique involves the hybridisation of a nucleic acid probe to mRNA in tissues sections which have been adhered to microscope slides. Subsequent coating of the tissue sections with a photographic emulsion, and the deposition of silver grains in the emulsion adjacent to the location of bound radioactive probe (caused by the energetic decay products of the radioisotope), permits precise identification of the cell type responsible for gene expression.

The expression of angiotensinogen in adipose tissue surrounding aorta from Sprague-Dawley rats was confirmed and extended to include the adipose tissue of the mesentery using *in situ* hybridisation. Angiotensinogen mRNA was not found in the smooth muscle cells of these blood vessels [Campbell and Habener, 1987]. In contrast, in aorta from Wistar and WKY rats, angiotensinogen expression was demonstrated in the medial smooth muscle layer, as well as in the periaortic fat [Naftilan et al, 1991]. The disagreement about the sites of angiotensinogen expression is probably not due to differences between rat strains, but due instead to methodological differences (for example, differences in the preparation of the tissue sections for hybridisation and the type and size of the probe used).

The localisation of the developmental regulation of WKY kidney renin gene expression has also been examined using *in situ* hybridisation. In foetal kidneys, renin is expressed in the vascular pole of the juxtamedullary glomeruli, as well as along afferent, intralobular and arcuate arteries. In kidneys from newborn rats, renin mRNA is found throughout the length of afferent arterioles, but not in arcuate or intralobular arteries. Thus, with the maturation of the kidney, the expression of renin is progressively restricted until, in adults, renin gene expression is confined to the juxtaglomerular apparatus [Gomez et al, 1989]. Immunohistochemical studies have reported similar changes in renin distribution for mouse, pig and human [Minuth et al, 1981; Egerer et al, 1984; Celio et al, 1985].

The cellular localisation of the developmental regulation of kidney renin gene expression or the spatial expression of RAS components in tissues such as aorta have not been examined in the SHR. Since angiotensinogen gene expression in response to sodium depletion may be blunted in the SHR (1.4.1) and investigations of renin gene expression in the SHR have reported widespread abnormalities (1.3.2), the cellular localisation of such aberrant expression should be viewed as an important step in the elucidation of the functions of the tissue RAS.

1.5 The effect of ACE inhibition on the SHR

1.5.1 Blood pressure

As discussed previously (1.3.2) the inhibition of ACE has a profound effect on the development of hypertension in the SHR, despite the fact that ACE activity is generally suppressed at all stages of development. Furthermore, following withdrawal from treatment, the blood pressure of ACE inhibitor-treated animals rises very slowly and, some considerable time later, reaches a plateau at a level significantly lower than controls that had not received an ACE inhibitor [Ferrone and Antonaccio, 1979; Cahihac and Guidicelli, 1986; Harrap et al, 1986; Christensen et al, 1988]. This feature is not seen with other antihypertensive agents, including hydralazine and atenolol [Guidicelli et al, 1980; Freslon and Guidicelli, 1983]

Suppression of blood pressure occurs after a relatively brief (four week) period of treatment with an ACE inhibitor [Harrap et al, 1990]. However, the timing of the treatment is critical. Persistent effects on blood pressure are seen only if administration of the ACE inhibitor is commenced in the "pre-hypertensive" phase. If treatment is begun after the development of hypertension, then, following withdrawal of the agent, blood pressure slowly returns to that of untreated controls [Muirhead et al, 1978; Unger et al, 1985]. Replacement of AII during the treatment period prevents the post-treatment suppression of blood pressure [Harrap et al, 1990]. The mechanism responsible for the continued suppression of blood pressure following cessation of treatment is not known, although several hypotheses have been investigated.

Hypertension is associated with deleterious structural modifications of the cardiovascular system that are proposed to exert a reinforcing influence on the maintenance of hypertension [Folkow, 1982]. Thus, in the SHR there is left ventricular hypertrophy, which is revealed in the increased heart weight to body weight ratio, as

well as vascular medial hypertrophy, which is manifested as an increased ratio of media to lumen diameter of the blood vessels [Mulvany et al, 1978; Limas et al, 1980; Lee et al, 1983a; Mulvany et al, 1985; Lee et al, 1987; Owens et al, 1988]. There is also evidence that the blood vessels of humans show similar structural alterations [Aalkjaer et al, 1987; Korsgaard et al, 1990]. As well as preventing hypertension, the chronic administration of ACE inhibitors to SHR, is associated with a beneficial effect on the structure of the heart and the blood vessel walls. This has been demonstrated histologically [Mulvany et al, 1978; Freslon and Guidicelli, 1983; Harrap et al, 1990] and biochemically [Jonsson et al, 1991]. Accordingly it has been proposed that the continued suppression of blood pressure following withdrawal from ACE inhibitor treatment is a consequence of the sustained regression of cardiovascular hypertrophy [Freslon and Guidicelli, 1983; Unger et al, 1985; Harrap et al, 1990]. However, this hypothesis has been questioned because there appears to be a discrepancy between the effect of ACE inhibition on structure and the sustained effect on blood pressure [Christensen et al, 1988; Christensen et al, 1989; Mulvany, 1991]. Three chronic treatment regimens (captopril, isradipine and hydralazine) all lowered blood pressure and had similar effects on the media to lumen ratio of biopsied mesenteric vessels from SHR. However, only captopril had a sustained effect on blood pressure following cessation of treatment. The blood pressures of animals treated with isradipine or hydralazine rose rapidly when treatment was stopped [Christensen et al, 1989]. Further, although the effect perindopril on blood pressure and regression of vascular structure is dose dependent, the sustained effect on blood pressure following withdrawal is not [Christensen et al, 1988].

The gene expression of elastin and actin in mesenteric vessels from animals treated with enalapril or hydralazine has also been examined [King et al, 1992]. There was no significant difference between treatment groups in the expression of these structural genes following withdrawal from treatment, despite the fact that only enalapril was associated with a persistent suppression of blood pressure. Similarly, although chronic

captopril administration is associated with a decrease in the enhanced vascular levels of 3-methylhistidine, a biochemical marker for contractile proteins, following withdrawal from treatment, the levels rise to those seen in untreated controls at a time when blood pressure is still significantly reduced [Smid et al, 1993].

ACE inhibition also has effects on certain abnormalities of vascular function in the SHR and it has been proposed that this mechanism might be responsible for the sustained lowering of blood pressure. Captopril reverses the enhanced constriction in response to both sympathetic nerve stimulation and intraluminal noradrenaline in the SHR, but after release from treatment the decreased vasoconstriction of the mesenteric bed returns to its abnormally high levels [Head et al, 1993]. The relaxation of constricted vessels in response to acetylcholine is impaired in the SHR, but treatment with an ACE inhibitor normalises this response [Clozel et al, 1990]. However, this beneficial effect of ACE inhibition is also not sustained following release from treatment, at a time when blood pressure is still significantly lower than untreated controls [Dyer et al, 1993].

From the preceding discussion, it can be recognised that while many studies have explored a number of possible explanations for the persistent low blood pressure in SHR following cessation of ACE inhibitor treatment, the precise mechanism of the response is still unclear. The phenomenon represents a situation where environmental influences interact with and disrupt the genetic factors which are responsible for the development of high blood pressure. These observations should therefore be regarded as more than just a vagary of academic interest, for if a corollary exists in humans, it may be possible to prevent hypertension, as well as its associated morbidity and premature mortality, by brief therapeutic intervention early in life.

1.5.2 Gene expression of the RAS

As discussed previously (1.4.1) AII exerts feedback control on renin and angiotensinogen gene expression. Therefore, under conditions of ACE inhibition, it would be predicted that the mRNA levels for renin and angiotensinogen might be altered.

The expression of the renin and angiotensinogen genes in the kidney has been examined following short-term (5-7 days) administration of ACE inhibitors. Angiotensinogen mRNA levels were not altered, but there was a significant increase in the levels of kidney renin mRNA in both SHR and normotensive rats [Gomez et al, 1988c; Kitami et al, 1989; Tada et al, 1989]. The increased renin expression was associated with a demonstration of immunoreactive renin at vascular sites upstream from the glomerulus [Gomez et al, 1988c]. Furthermore, the effect of ACE inhibition on renin gene expression appears to be tissue specific since brain renin mRNA levels were not affected [Tada et al, 1989].

At the time of the commencement of the studies described in this thesis, the effect of long-term ACE inhibition on RAS gene expression had not been reported. This would be considered to be important since the minimum treatment period required to prevent hypertension in the SHR is at least one week [Harrap et al, 1990]. Furthermore, since, as discussed previously (1.4.1), there are numerous physiological and non-physiological stimuli which influence RAS gene expression, it might be expected that other factors would come into play during extended treatment with ACE inhibitors. In particular, the gene expression following release from ACE inhibition has not been examined.

1.6 The sympathetic nervous system (SNS)

Through the cardiovascular control centre in the medulla and its innervation of the heart and blood vessels in the periphery, the sympathetic nervous system (SNS) is

intimately involved in the control of blood pressure. The SNS emits a tonic, low frequency discharge which responds to pressure, volume and chemoreceptor information and therefore reflexly integrates circulatory functions. SNS activity is also affected by environmental influences [Folkow, 1982].

1.6.1 The SNS in human primary hypertension

Overactivity of the SNS has also been proposed as a causative factor in human essential hypertension. The activity of the SNS can be assessed by measuring changes in cardiovascular reflex activity, electrophysiological recording of nerve impulses or quantitation of the levels of catecholamines (in particular noradrenaline (NA) and adrenaline (AD)) in urine or plasma.

Many hypertensive patients demonstrate a reduced baroreceptor sensitivity [Takeshita et al, 1975; Simon et al, 1977; Eckberg, 1979; Goldstein, 1983a]. However, studies in young subjects from hypertension-prone families show normal baroreceptor function and therefore impairment of baroreflex function may be a secondary effect and not involved in the genesis of hypertension [Kotchen et al, 1982]. Direct recordings of tonic sympathetic discharge to skeletal muscle blood vessels have found no clear difference between controls and patients with early primary hypertension [Wallin et al, 1973; Wallin and Sundlöf, 1979].

Measurement of plasma NA has been employed as a convenient measure of monitoring sympathetic tone. The concentration of NA in the circulation is dependent on spillover from the synaptic cleft following binding to postsynaptic receptors, re-uptake by the nerve endings, uptake into extra-neuronal tissues as well as metabolic degradation and therefore the validity of circulating catecholamines as an index of SNS activity has been questioned [Tuck, 1986]. An analysis of 64 studies which measured plasma NA in hypertensive and normotensive subjects found that 80% of the studies reported a higher mean plasma NA in the hypertensive groups [Goldstein, 1983b]. However, a statistically significant difference between the groups was seen only in 40 % of the

studies. As plasma NA increases with age in normotensive subjects, the differences in plasma NA between hypertensive and normotensive subjects are most evident among young persons [Goldstein, 1983b].

The contribution of sympathetic overactivity to human primary hypertension has been difficult to document, due to the complex rapid responses of the SNS to environmental influences, including those imposed on it by experimenters, as well as restrictions on the sites of access in humans for measurement of indices of SNS activity. Neurogenic trigger elements in hypertension may be intermittent and since SNS activity is widely affected by numerous factors including psychological and physical stress, age, diet, body weight, caffeine and smoking [Tuck, 1986], it is not surprising that the question of whether enhanced activity of the SNS contributes to primary hypertension has remained unanswered. Further, heterogeneity in the pathophysiology of primary hypertension may mean that only subsets of hypertensives have SNS overactivity [Messerli et al, 1986].

Many agents which are (or have been) used in the treatment of hypertension interfere with SNS activity. These include agents which act centrally, e.g. clonidine, to produce a diffuse inhibition of sympathetic outflow, agents such as guanethidine which influence transmitter release from sympathetic neurones as well as peripheral α - and β -antagonists such as prazosin and propranolol [Weiner, 1985]. That such a wide range of therapeutic agents which disrupt SNS activity are effective in the treatment of hypertension indirectly implicates a role for the SNS in the maintenance of hypertension.

1.6.2 Interactions between the RAS and the SNS

There are many sites of interaction between the SNS and the RAS under both physiological conditions and in pathological states when both systems are activated.

Renin release is partly under the influence of SNS control. The juxtaglomerular cells of the kidney are innervated by sympathetic nerves. With low levels of sympathetic activation, which are below the threshold for changes in renal blood flow or urinary sodium excretion, the renal nerves stimulate renin release via the action of β_1 -adrenoceptors [Thames, 1984]. At higher levels of renal nerve stimulation, antinatriuretic and α_1 -adrenoceptor vasoconstrictor activity also influence renin release [Thames, 1984].

Stimulation by AII of sympathetic neurotransmission has been demonstrated in a number of tissues including heart [Starke et al, 1970], rat caudal artery [Nicholas, 1970] and the rat mesenteric vascular bed [Cline, 1985]. The enhanced response to sympathetic nerve stimulation can be blocked by the AII antagonist saralasin [Cline, 1985]. A number of mechanisms have been proposed, including increased synthesis of NA [Roth, 1972], enhanced release of NA [Schumann and Guthrie, 1967], inhibition of NA uptake [Panisset and Bourdois, 1968; Peach et al, 1969; Palaic and Khairallah, 1970], facilitation of NA release via pre-synaptic AII-receptors [Hughes and Roth, 1971; Zimmerman et al, 1972; Zimmerman and Kraft, 1979] and sensitisation of target tissues and α -receptors [Pals et al, 1968; Johnson et al, 1974]. It is generally agreed that the most physiologically relevant of these proposed mechanisms is a primary effect on transmitter release via prejunctional activation of AII receptors, which occurs at low AII levels, so that the vasoconstrictor activity of AII is significantly increased by the facilitation of sympathetic neurotransmission [Van Zweiten and De Jonge, 1986].

1.6.3 The SNS in the SHR

While sympathetic overactivity in human primary hypertensives has been difficult to document, in part because of restricted access to relevant sites of measurement, it is without doubt that an enhanced SNS activity contributes to both the initiation and maintenance of hypertension in the SHR [Head, 1989].

The existence of hypernoradrenergic innervation of blood vessels from SHRs is supported by numerous studies which have used a variety of techniques to demonstrate an overdeveloped peripheral SNS compared with the WKY. These techniques include fluorescence histochemistry [Haebera et al, 1968; Ichijima, 1969; Scott and Pang, 1983], morphometric analysis of nerve bundles [Lee et al, 1983b; Lee, 1985], chemical assay of NA in tissues [Berkowitz and Spector, 1976], radioenzymatic analysis of NA in tissues [Berkowitz et al, 1980; Head and Berkowitz, 1982] and HPLC-ECD assay of NA in tissues [Head et al, 1984; Head et al, 1985; Cassis et al, 1985; Donohue et al, 1988]. The increased innervation occurs immediately after birth and prior to the development of hypertension in the SHR [Donohue et al, 1988].

The activity of renal and splanchnic nerves in the SHR is significantly higher than that seen in WKY [Iriuchijima, 1973; Judy et al, 1976]. Further support for an overactive SNS in the SHR has come from studies which have shown that the response of blood vessels to nerve stimulation is enhanced in the SHR [Nilsson and Folkow, 1982; Yamamoto and Cline 1988; Stephens et al, 1991]. When neuronal uptake of NA is blocked with cocaine, the shift in the frequency response curve - the so-called "cocaine shift" - is much greater in the SHR [Cassis et al, 1985; Stephens et al, 1991].

Sympathetically innervated tissues influence the level of their innervation by the production of the peptide nerve growth factor (NGF) [Levi-Montalcini and Angeletti, 1968]. In the rat, development of the peripheral sympathetic nerves occurs in the first few weeks after birth. NGF levels in vascular tissues from SHR are significantly elevated in young, but not adult SHR [Donohue et al, 1989] and moreover, the increased levels of the peptide are associated with an increased expression of the NGF gene [Falckh et al, 1992].

Neonatal sympathectomy with 6-hydroxydopamine (OHDA) or antibodies to NGF in combination with guanethidine can markedly attenuate the development of hypertension in the SHR [Yamori et al, 1972, Provoost and de Jonge, 1978; Bevan and

Tsuru, 1981; Lee et al, 1987]. It has also been suggested that sympathetic nerves exert a trophic influence on vascular smooth muscle [Head, 1989], since, in addition to preventing hypertension, neonatal sympathectomy can also prevent vascular medial hypertrophy [Bevan and Tsuru, 1981; Lee et al, 1987]. The precise trophic interactions between sympathetic nerves and vascular smooth muscle are yet to be elucidated.

As discussed previously (1.6.2), there are many potential sites of interaction between the RAS and the SNS. Therefore it could be predicted that, as well as disrupting SNS function, neonatal sympathectomy would also have profound effects on the RAS. Neonatal sympathectomy with OHDA has been shown to cause a significant reduction in the basal activity of the RAS, as measured by PRA, although the release of renin in response to hypotensive stress was not affected [Sinaiko et al, 1980]. The mechanism responsible for the decrease in basal PRA has not been investigated, although it would follow that since renal nerve activity influences the release of renin from the kidney, ablation of sympathetic nerves might have some effect on kidney renin gene expression. However, the effect of neonatal sympathectomy on the gene expression of renin or other RAS components has not been reported. Furthermore, the effects of sympathectomy on local tissue RAS at sites other than the kidney have also not been investigated. The mesenteric vascular bed is a highly innervated tissue and contributes greatly to the total peripheral resistance in the rat. In the SHR the mesenteric vascular bed is hyperinnervated when compared with the WKY [Head, 1989] and therefore it is of interest to examine the influence of sympathetic denervation on the RAS gene expression in this tissue.

1.7 Introduction summary

The RAS comprises both a classical circulating endocrine system as well as local tissue systems which have the potential to act in a paracrine or autocrine fashion. The precise functions of the local tissue RASs are not known but it has been suggested that they play a role in the long-term regulation of blood pressure.

Both renin and angiotensinogen gene expression are subject to developmental and tissue-specific regulation. In the kidneys from normotensive rats, renin gene expression is progressively restricted with age so that while in foetal and newborn kidneys renin is expressed at vascular sites as well in the glomerulus, adult kidney renin gene expression is confined to the juxtaglomerular apparatus. Kidney renin gene expression is reported to be elevated in young, but not adult SHR. However, the cellular localisation of the developmental regulation of kidney renin gene expression has not been examined in this strain. In addition, the spatial expression of the RAS components in vascular tissue from the SHR has not been investigated. The cellular localisation of RAS gene expression in this rat model is viewed as an important step in the elucidation of the functions of the tissue RASs and the genesis of hypertension.

Although there appears to be no abnormality in the circulating RAS, ACE inhibitors have a profound effect on the development of hypertension and the development of vascular medial hypertrophy in the SHR. Moreover, following discontinuation of treatment with ACE inhibitors, blood pressure remains suppressed. Although it has been suggested that the beneficial effect of ACE inhibitors on vascular structure or function may contribute to the sustained low blood pressure, the precise mechanism is not known. Short-term administration of ACE inhibitors is associated with dramatic effects on renin but not angiotensinogen gene expression in the kidney. However, the effects of long-term treatment with ACE inhibitors on gene expression of the RAS has not been reported. In particular, the effect of withdrawal from ACE inhibition on RAS

gene expression has not been examined. The effect of long-term ACE inhibition on the expression of ACE is unknown.

There is convincing evidence that overactivity of the SNS plays a role in the genesis of hypertension in the SHR. There are many potential sites of interaction between the RAS and the SNS. In particular, renin release from the kidney is partly under SNS control. Neonatal sympathectomy prevents the development of hypertension in the SHR and also prevents vascular medial hypertrophy as well as significantly decreasing the basal activity of the RAS. The mechanism for this is not known but it is likely that denervation has an effect on renal renin gene expression. The effect of denervation on the gene expression of the RAS components in vascular tissue has also not been examined.

1.8 Aims

The principal aims of this study follow from the preceding discussion, i.e.:

To examine the gene expression of the renin-angiotensin system in the SHR.

In particular:

(1) To establish the technique of *in situ* hybridisation in order to determine the cellular localisation of the gene expression of the RAS in kidney and liver in the pre-hypertensive and established phases of hypertension and to determine whether the sites of expression of the RAS in vascular tissue are similar in hypertensive and normotensive rats.

(2) To determine whether long-term administration of ACE inhibitors to SHR and WKY results in sustained effects on the gene expression of the RAS in kidney and liver, and in particular, to determine whether the continued suppression of blood

pressure following withdrawal from ACE inhibitor treatment is associated with continued effects on gene expression.

(3) To determine whether neonatal sympathectomy in SHR and WKY influences the gene expression of the RAS in kidney, liver and vascular tissue.

CHAPTER 2**METHODS**

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2.1 Animals

2.1.1 Source of animals and housing conditions

Male SHR and WKY were obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) breeding colony, located at O'Halloran Hill, South Australia. In studies in which the blood pressure changes during the developmental phase of hypertension were examined, 4 week old animals were transferred to the animal holding facility at the CSIRO Division of Human Nutrition, Kintore Avenue, Adelaide. For studies in which age related changes were not monitored, the rats were held at the O'Halloran Hill facility until immediately prior to the commencement of the experiments. For selected studies, adult male Sprague-Dawley rats and 6 week old balb/c mice were obtained from the University of Adelaide Central Animal House.

The rats were housed in wire-bottomed cages, with 4-6 rats per cage. Mice were housed 10-12 to each cage, in plastic boxes with wire lids. The animals were fed standard chow (Purina). The animal holding facilities were illuminated by artificial light sources which were maintained on strict 12 hour light/dark cycles. Water was allowed *ad libitum*, and in selected studies, pharmacological agents were added to the drinking water.

During the course of the conduct of the studies, the animals at the O'Halloran Hill breeding facility were randomly subjected to genetic analysis in order to verify the purity of the strains used for breeding (i.e. WKY, SHR and SHR-stroke prone). There was no evidence of genetic contamination in any strain. All animals representing a particular strain were identical at the 13 genetic markers tested (See Appendix I). The strain profiles which were obtained were consistent with those found in previous screens and those published in the literature.

Prior to the commencement of the studies described in this thesis, approval for the experiments was obtained from the University of Adelaide Animal Ethics Committee and the CSIRO Animal Care and Ethics Committee.

2.1.2 Blood pressure measurements

Blood pressure was monitored using a tail-cuff procedure (IITC-Life Sciences, California). Rats were placed in a perspex restraining tube which was, in turn, placed in a warming cabinet maintained at 30-32°C. After 20-30 minutes in this environment, the tail cuff blood pressure was measured 5-6 times for each rat. The final blood pressure value was the average of the individual measurements, after obvious outliers had been excluded.

2.1.3 Tissue collection

Animals were sacrificed either by decapitation or by lethal dose of pentobarbitone sodium (70 mg/kg i.p.). Tissues were excised immediately, cleared of adhering tissue and thoroughly rinsed in ice-cold saline. The tissues were weighed, wrapped in aluminium foil, snap frozen in liquid nitrogen and then stored at -80°C until analysis. For some experiments, selected tissue segments were placed in at least 30 volumes of fixative solution (4% paraformaldehyde in phosphate buffered saline (PBS, 20 mM sodium phosphate, 130 mM sodium chloride, pH 7.4)) and stored at 4°C overnight.

2.2 Biochemical analyses

2.2.1 ACE activity

The ACE activity of tissue homogenates and plasma was determined using a radiochemical procedure [Rohrbach, 1978]. This procedure used the artificial substrate [Glycine-1-¹⁴C]Hippuryl-L-Histidyl-L-Leucine (HHL, Dupont) and the rate of hydrolysis was quantified by measuring the liberated ¹⁴C-Hippuric Acid using liquid scintillation counting.

Blood was collected from decapitated rats and from mice by cardiac puncture into heparinised tubes. Plasma was removed following centrifugation at 3000g for 15 minutes at room temperature and stored at -20°C until analysis. Tissues which had been thoroughly rinsed in ice-cold saline, snap frozen in liquid nitrogen then stored at -80°C were homogenised in 50 mM potassium phosphate (pH 8.3) using an Ultra-Turrax homogeniser or glass/glass homogeniser with a motor driven pestle (aorta only). Lungs were homogenised in 30 volumes, aorta was homogenised in 20 volumes and all other tissues were homogenised in 10 volumes of buffer.

The incubation mixture comprised;

- 10µl homogenate or plasma
- 10µl 400 mM sodium borate, pH 8.3
- 10µl 1.5 M sodium chloride
- 10µl water (or other solution as required)
- 9µl 27.52 mM HHL
- 1µl ¹⁴C-HHL (3.0 mCi/mmol)

The reaction was carried out at 37°C and stopped by the addition of 50µl 1M hydrochloric acid and cooling on ice. Blanks, in which the acid was added to the sample prior to the addition of other reaction components, were run concurrently in each assay. The [glycine-1-¹⁴C]hippuric acid which was formed was extracted from the aqueous layer with 300µl ethyl acetate. The solution was vortexed vigorously for 10 seconds and the organic and aqueous phases were separated by centrifugation at 3000g for 3 minutes at room temperature. 200 µl of the upper organic layer were placed into 5 ml Beckman Ready-Value scintillation fluid and the amount of radioactivity in each sample was determined by liquid scintillation counting.

The enzyme activity of each sample was given by the formula:

$$\text{nmol HHL hydrolysed /min/ml} = (\text{reaction dpm} - \text{blank dpm}) / (\text{S.A.} \times 0.91 \times t \times V)$$

where S.A is the specific activity of the ^{14}C -HHL (87 dpm / nmol)
0.91 is the hippuric acid extraction coefficient
0.67 is the fraction of ethyl acetate counted
 t is the time in minutes of the reaction
 V is the volume of the enzyme in millilitres

The reaction time was determined for each tissue individually. The time chosen lay on the linear portion of the time versus hydrolysis curve for each tissue and ranged from 15 minutes for plasma and lung to 120 minutes for liver and skeletal muscle. ACE activity was expressed as nmol HHL hydrolysed per minute per milligram of protein (assay described below) for each tissue sample or as nmol HHL hydrolysed per minute per ml for plasma.

All samples were assayed in duplicate. The intra- and inter-assay coefficients of variation were determined to be 6% and 8% respectively.

2.2.2 Protein

The protein content of tissue homogenates was analysed using a folin phenol reagent [Lowry et al, 1951]. Tissue homogenates or standards were diluted in 0.1 M sodium hydroxide (typically 10-50 μl homogenate to 0.5 ml sodium hydroxide). Blank samples, which were included in each assay, consisted of sodium hydroxide alone. To 50 μl of standard, sample or blank, 2.5 ml of 2% sodium carbonate in 0.1 M sodium hydroxide / 1% sodium tartrate / 1% copper sulphate (100: 1: 1) was added and the mixture was allowed to stand at room temperature for 10 minutes. 50% Folin-Ciocalteu Reagent (0.25 ml,) was then added and after the colour had been allowed to develop for 30 minutes, the absorbance at 750 nm (A_{750}) was determined. The protein concentration of each sample was determined by comparison with standard solutions which were prepared from bovine serum albumin. A_{750} was proportional to a protein content in the concentration range 0.125 - 1 mg/ml.

The intra- and inter-assay coefficients of variation were determined to be 3% and 4% respectively.

2.2.3 Noradrenaline

Noradrenaline (NA) was measured in tissue samples using a radioenzymatic procedure based on that described by [Da Prada and Zürcher, 1976].

Tissues, which had been rinsed in ice-cold saline, were weighed and placed in 0.3 M perchloric acid containing 0.5 mM magnesium chloride and 5 mM ethylenebis(oxyethylenitrilo)-tetraacetic acid (EGTA). Noradrenaline was extracted from the tissues at 4°C overnight and the extracts were then stored at -80°C until analysis.

NA was converted to its radioactively-labelled methyl derivative, normetanephrine (NMN) by the liver enzyme catechol-O-methyl transferase (COMT) (See Appendix II) using ³H-S-adenosyl-methionine (SAmE) as the methyl donor.

The incubation mixture comprised;

- 100ul sample or standard or blank (as appropriate)
- 50ul 2M tris(hydroxymethyl)aminomethane (Tris), pH 9.6
- 25ul COMT
- 20ul 50 mM MgCl₂
- 5ul ³H-SAmE

After 60 minutes, the reaction was stopped with the addition of 1 M sodium borate, pH 8 (150 µl), 1.5% tetraphenylboron (50 µl) and 0.5 mg/ml NMN (50 µl, prepared in 0.01 M hydrochloric acid).

The radioactively-labelled methyl derivative and cold carrier were separated from unreacted ³H-SAmE by extraction into ether (10 ml), which was followed by back

extraction into 1 M acetic acid (0.5 ml). The organic and aqueous phases were separated by centrifugation, followed by snap-freezing of the aqueous phase in an ethanol/dry ice bath. The samples were then evaporated to dryness using a Buchler Vortex Evaporator and redissolved in methanol containing acetic acid (0.01 M, 4:1, 50 μ l). ^3H -NMN was separated from other radioactively-labelled compounds (e.g. metanephrine and 3-methoxytyramine) by thin layer chromatography (TLC) on silica-coated plastic sheets containing a fluorescence indicator (Merck). The developing solvent was chloroform / methanol /ethylamine (16:3:2). and the location of the NMN on the TLC sheet was determined by visualisation under ultraviolet (UV) light. The region corresponding to NMN was cut from the sheet and placed directly into a 20 ml scintillation vial. After extraction with 0.5 M hydrochloric acid for 30 minutes at room temperature, 15 ml Beckman Ready Value Scintillation fluid was added.

The concentration of radioactivity in each sample was measured by liquid scintillation spectrometry and the concentration of NA in each sample was determined by comparison with standard solutions (prepared from authentic NA) and corrected for blank values which were generated concurrently with the tissue samples. Blank samples comprised 0.3 M perchloric acid containing 0.5 mM magnesium chloride and 5 mM EGTA. The radioactivity measured in standard solutions was proportional to NA content in the concentration range 0.05-100 ng/ml.

All samples were assayed in duplicate. The intra-assay coefficient of variation was 6%. Inter-assay comparisons were not made.

2.2.4 Angiotensin II uptake

The method used to examine the uptake of AII into rat tissues was a modification of a previously reported procedure which had been used to examine the uptake of noradrenaline in the rat intestine [Suvannapura and Levens, 1988].

Immediately after excision, tissues were rinsed in ice-cold saline and then pre-incubated in Krebs solution (gassed with a mixture of 95% O₂ and 5% CO₂) for 30 minutes. The Krebs solution comprised the following; 118 mM sodium chloride, 3.9 mM potassium chloride, 0.92 mM potassium phosphate, 25 mM sodium bicarbonate, 5.6 mM glucose, 2.5 mM calcium chloride, 1.0 mM magnesium chloride.

Following an equilibration period, the tissues were incubated in gassed Krebs (2 ml) containing angiotensin II (5-L-isoleucine)[tyrosol-3,-5³H(N)] (³H-AII, 14 nM) at 37°C for 30 min.

The tissues were rinsed in ice-cold saline (5 ml, 3 times) and solubilised (at 55°C for 2 hours) in 0.5 M ammonium hydroxide in toluene. The tritium content of each sample was assayed by liquid scintillation spectrometry. The AII uptake into each tissue was calculated from the specific activity of the peptide and expressed as picomoles of angiotensin II per gram of tissue.

2.2.5 Alanine aminotransferase (AlaAT)

The AlaAT concentration in plasma was measured using a spectrophotometric procedure [Horder and Rej, 1985] in which the decrease in absorbance due to the oxidation of the reduced form of nicotine adenine dinucleotide (NADH) was monitored spectrophotometrically.

Plasma was collected and stored as described above (2.2.1). A working reagent mixture consisting of 1.23 M dl-Alanine (prepared in 0.11 M Tris, pH 7.3) / lactate dehydrogenase 3000 U/L / 3.3 mg/ml NADH (20: 0.1: 1) was prepared on the day of the assay. Working reagent (1 ml) was added to plasma (0.1 ml) in a spectrophotometric cuvette and after thorough mixing, allowed to incubate at 30°C until a stable absorbance reading was obtained (usually 10 minutes). 180 mM α-

ketoglutarate (0.1 ml, prepared in 0.11 M Tris, pH 7.3)) was added and the change in absorbance at 340 nm was monitored for 3 minutes (following an initial lag time of 1 minute).

The AlaAT activity (U/l)

where 1 U = 1 micromole of NADH oxidised per minute

was given by the formula

$$\Delta A \times R.V. \times 1000 / 6.22 \times S.V.$$

where ΔA is the change in absorbance per minute

R.V. is the reaction volume (1.2 ml)

1000 converts U/ml to U/l

6.22 is the micromolar absorptivity of NADH at 340 nm

S.V. is the sample volume (0.1 ml)

If the change in absorbance was not linear for the observation period, the plasma sample was diluted (1/5 or 1/10) with water and re-assayed.

The intra-assay coefficient of variation was 4%. No inter-assay comparisons were made.

2.3 RNA analyses

Sterile equipment was used in all procedures that involved the analysis of RNA. Gloves were worn at all times. Solutions and plasticware were autoclaved (120°C) and glassware was baked (200°C for 5 hours). Molecular Biology Grade chemicals were used where possible.

2.3.1 Quantification of nucleic acids

RNA was quantified by spectrophotometric analysis [Sambrook et al, 1989]. An aliquot of the RNA solution (usually 3-5 μ l) was diluted in 2 ml H₂O. The concentration of RNA was calculated from the absorbance of the solution at 260 nm (given that an optical density of 1 corresponds to a RNA concentration of 40 μ g/ml). The purity of the RNA sample was estimated from the ratio of absorbances at 280 nm (A_{280}) and 260 nm (A_{260}) (given that pure RNA has an A_{280}/A_{260} ratio of 2). Only RNA samples with an A_{280}/A_{260} of greater than 1.65 were used for analysis.

The DNA concentration of the cDNA templates which were used to make RNA probes was quantified using a technique in which the fluorescence of bisbenzimidazole is enhanced when bound to DNA [Labarca and Paigen, 1980]. Samples or standards were diluted to a volume of 1 ml with DNA Assay Buffer (50 mM sodium phosphate, 2 M sodium chloride, pH 7.4). The diluted sample was then mixed with DNA Assay Buffer (1 ml) containing bisbenzimidazole (0.2 μ g/ml). The fluorescence of each sample was determined using a Perkin-Elmer LS-5 Luminescence Spectrometer with the excitation and emission wavelengths set at 360 nm and 460 nm respectively. The DNA content of each sample was determined by comparison with standard solutions which were prepared from λ DNA. Blank samples consisted of DNA Assay Buffer alone. The fluorescence of each standard solution was proportional to the DNA concentration in the range 25-500 ng/ml. It should be noted that, except for the pipette tips that were used to remove aliquots from the DNA samples, sterile equipment was not used for this assay.

2.3.2 Mini Gel Electrophoresis

Various preparative procedures (e.g. plasmid linearisation, RNA transcription) were verified by mini gel electrophoresis. Each sample (9.5 μ l) was mixed with Gel Loading

Buffer (2.5 μ l; 50% glycerol, 0.2% bromophenolblue, 0.2% xylene cyanol) and then subjected to electrophoresis in an agarose gel (0.8% agarose in 1x TAE) (1x TAE is 40 mM Tris, 20 mM acetic acid, 1 mM ethylenediamine tetraacetic acid (EDTA)). Ethidium bromide (0.001%) was added to the agarose solution immediately prior to pouring the gel. Typically, electrophoresis was carried out at 60-80V for 1-2 hours.

2.3.3 Preparation of hybridisation probes

Hybridisation reactions were performed using cRNA probes which were synthesised from pGEM4 templates containing cDNA to the rat renin (#879A, pREN.44 ceb) or angiotensinogen (#693, pRANG6) genes and a Bluescript template containing cDNA for the human ACE (pB3519) gene. The plasmids were generous gifts from Dr. Kevin Lynch, University of Virginia Health Sciences Center (renin, angiotensinogen) and Dr Pierre Corvol, Institute National de la Sante et de la Recherche Medicals, Paris (ACE).

Prior to synthesis of RNA transcripts the cDNA templates were linearised (see Table II.A below).

The incubation reaction comprised:

- 30 μ g template DNA
- 5 μ l restriction enzyme
- 5 μ l 10x Buffer (as supplied by the manufacturer)
- H₂O to 50 μ l

Linearisation was allowed to proceed for 3 hours at 37°C. The reaction mixture was then diluted with H₂O to a total volume of 100 μ l and extracted with an equal volume of Tris-saturated phenol/chloroform/isoamyl alcohol (50:49:1) (See Appendix II). The aqueous supernatant which was recovered following centrifugation at 8,000 g for 3 minutes was then extracted with an equal volume of chloroform. Following further centrifugation at 8,000 g for 3 minutes linearised DNA was precipitated from the

supernatant by adding 2 volumes of ice-cold ethanol followed by cooling on ice for 60 minutes. After centrifugation (12,000 g, 30 minutes, 4°C), 0.5 ml 70% ice-cold ethanol was added to the precipitate and which was centrifuged again. The precipitate was dried under vacuum at 37°C for 5 minutes, resuspended in 30 µl H₂O and stored at -80°C.

The ³⁵S-cRNA probes were synthesised using a Message Maker *in vitro* Transcription Kit (Bresatec) and ³⁵S-UTP (Dupont) according to a protocol supplied by Bresatec Limited.

The incubation reaction comprised of:

1µg linearised template DNA (see Table II.A below)
 2µl 10x Nucleotide/Buffer cocktail (composition not specified by the manufacturer)
 2µl 100 mM Dithiothreitol (DTT)
 1µl RNase Inhibitor (human placental, 20 U/µl)
 5-10µl ³⁵S-UTP (>1000 Ci/mmol, aqueous)
 or 2µl 5 mM UTP (for preparation of unlabelled RNA)
 H₂O to 18µl
 2µl RNA polymerase (see Table II.A below)

After gentle mixing, the reaction was allowed to proceed at 37°C for 60 minutes. The DNA template was then removed by adding 1 µl RNase-free DNase I (10 U/µl) to the reaction and incubating the solution at 37°C for a further 10 minutes. The reaction mixture was then diluted with H₂O to a total volume of 100 µl and extracted with an equal volume of Tris-saturated phenol/chloroform/isoamyl alcohol (50:49:1). The aqueous supernatant was recovered following centrifugation at 8,000 g for 3 minutes. Newly synthesised RNA was precipitated by adding 10 µl 3 M sodium acetate, pH 6, and 2.5 volumes of ice-cold ethanol, followed by cooling at -70°C for 30 minutes. After centrifugation (12,000 g, 30 minutes, 4°C), 0.5 ml 70% ice-cold ethanol was added to the precipitate which was centrifuged again. The precipitate was dried under vacuum at 37°C for 5 minutes and resuspended in 100 µl 20 mM β-mercaptoethanol (

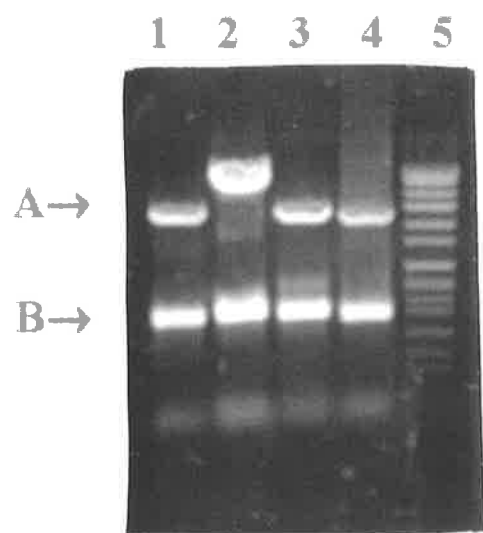
β ME). The probes were either stored in aliquots at -80°C , or, subjected to hydrolysis if they were to be used for *in situ* hybridisation studies (see below).

| | Restriction enzyme | RNA polymerase | Transcript size |
|----------------------|---------------------------------|---------------------------|-----------------|
| Renin cRNA | Bam H1 (10 U/ μl) | T7 (2 U/ μl) | ~1400 b |
| Angiotensinogen cRNA | EcoR1 (10 U/ μl) | T7 (2 U/ μl) | ~1600 b |
| ACE cRNA | Bgl 1 (8 U/ μl) | T3 (20 U/ μl) | ~1600 b |
| Renin mRNA | EcoR1 (10 U/ μl) | SP6 (5U/ μl) | ~1400 b |
| Angiotensinogen mRNA | Hind 111 (12 U/ μl) | SP6 (5U/ μl) | ~1600 b |

Table II.A The restriction enzymes used to linearise the cDNA templates, the RNA polymerase enzymes used to synthesise RNA transcripts and the size of the transcripts generated (given as approximate number of bases (b)).

Unlabelled RNA was prepared to allow visualisation of the transcription reaction in order to verify that RNA of the correct molecular weight was synthesised. At the end of the 60 minute incubation period, prior to the addition of DNase I, 9.5 μl of the reaction solution was analysed by mini-gel electrophoresis and UV illumination of the ethidium bromide-stained nucleotides. Molecular weight size markers (SPP-1 bacteriophage digested with *EcoRI*) were run concurrently on each gel to allow the estimation of the molecular weight of the RNA transcripts. For each of the transcripts, a single band of the expected molecular weight was observed (*Figure 2.1*, page 47). Occasionally, a faint band of larger than expected molecular weight was observed (*Figure 2.1*, Lane 3) which was probably a consequence of transcription from non-linearised templates.

Figure 2.1. Verification of transcription reactions by electrophoresis and staining with ethidium bromide. Lane 1, angiotensinogen mRNA. (negative control); Lane 2, ACE cRNA; Lane 3, angiotensinogen cRNA; Lane 4, renin cRNA; lane 5, molecular weight ladder A: linearised cDNA template. B: RNA transcripts.



The fragment length of RNA probes which were used for *in situ* hybridisation studies was adjusted to a mass average of approximately 150-250 bases by limited alkaline hydrolysis [Cox et al, 1984]. Samples were hydrolysed in 40 mM sodium bicarbonate / 60 mM sodium carbonate at 60°C. The hydrolysis time was calculated from the formula:

$$t = L_o - L_f / kL_oL_f$$

where t is the time in minutes

L_o and L_f are the initial and final fragment lengths in kilobases

k is the rate constant for hydrolysis (approximately $0.11 \text{ kb}^{-1}\text{min}^{-1}$)

Samples were neutralised by the addition of sodium acetate, pH 6, and glacial acetic acid to 0.1 M and 0.5% respectively. The hydrolysed RNA was recovered by ethanol precipitation, resuspended in 20 mM β ME and stored in aliquots at -80°C .

The radioactivity of ^{35}S -labelled RNA probes, determined by liquid scintillation counting, was generally $1.8-2 \times 10^6$ dpm/ μl . The incorporation of ^{35}S -UTP into transcripts was $>90\%$, and the specific activity of the probes was calculated to be $0.5-1 \times 10^8$ dpm/ μg .

2.3.4 RNA isolation

Total RNA was isolated from tissues using a guanidinium thiocyanate-phenol-chloroform extraction procedure [Chomczynski and Sacchi, 1987].

Frozen tissue samples were placed in 9.5 volumes of Solution A (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 100 mM β ME) and immediately homogenised (without thawing) using an Ultra-Turrax for 60 seconds at setting 4. Following the addition of 0.5 volumes of 10% sarcosyl the sample was rehomogenised for a further 30 seconds. After the sequential addition, with mixing after each addition,

of 0.1 ml 2 M sodium acetate (pH 4), 1 ml Tris-saturated phenol and 0.2 ml chloroform/isoamylalcohol (49:1) to 0.9 ml homogenate, the mixture was cooled on ice for 15 minutes. Following centrifugation at 12,000 g at 4°C for 20 minutes, 1 volume of ice-cold isopropanol was added to the supernatant, which was then stored at -20°C for at least 2 hours. The RNA was pelleted by centrifugation at 12,000g at 4°C for 30 minutes. The pellet was redissolved in 0.3 ml Solution A containing 0.5% sarcosyl and an equal volume of ice-cold isopropanol was added. Following at least 2 hours at -20°C, the solution was centrifuged at 12,000 g for 30 minutes at 4°C. The pelleted RNA was washed twice with 75% ice-cold ethanol followed by centrifugation at 12,000 g for 20 minutes at 4°C. The precipitate was dried under vacuum at 37°C for 5 minutes and resuspended in H₂O by heating at 37°C for 30 minutes. The concentration of RNA in each sample was determined by spectrophotometric analysis, as described above (2.3.1), before storage at -80°C.

2.3.5 Slot analysis

Slot blots of RNA samples were prepared as described by Sambrook et al (1989). Serial dilutions (10, 2 and 0.5 µg) of each RNA sample were made in water. The RNA concentration was confirmed in an aliquot of each 10 µg sample by spectrophotometric analysis. Each dilution was mixed with formamide, formaldehyde and SSC (final concentrations 50%, 7% and 1x respectively). (1x SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.) The samples were heated at 65°C for 15 minutes followed by rapid cooling in an ice bath for 5 minutes. Ice-cold 20x SSC was then added to each sample to give a final SSC concentration of 10x. Sufficient RNA sample was prepared in this way to subsequently allow 3 duplicate blots to be made for each series of samples. The samples were transferred to Hybond N filters (Amersham) under vacuum using a slot blot apparatus (Millipore or Biorad). The RNA was covalently bound to the membrane by crosslinking under UV light. Where the number of samples being

compared exceeded the number that could be applied to a single membrane, then at least two samples were common to each of the membranes required. The filters were stored individually in envelopes at room temperature until hybridisation.

2.3.6 Northern analysis

Selected RNA samples were examined by Northern blot analysis. The extracts (usually 10 µg) were heated at 65°C for 15 minutes in the presence of 50% formamide, 0.66 mM formaldehyde and 1x MOPS buffer (20 mM 3-[N-Morpholino]propanesulphonic acid, 3 mM sodium acetate, 1 mM EDTA, pH 7) and ethidium bromide (0.0005%) before placing on ice for 15 minutes. Gel Loading Buffer was added to each sample to give a final concentration of 20%. The treated extracts were fractionated on 1% agarose gel, prepared in 1x MOPS and 0.66 mM formaldehyde, for 6 hours at 40 V in 1x MOPS buffer. After electrophoresis, the gels were soaked, with gentle shaking, for 15 minutes in 0.05 M sodium hydroxide made up in 1x SSC, followed by two washes in 10x SSC, also with gentle shaking, for 10 minutes each. The RNA was transferred by capillary action to Hybond N nylon membranes in 10x SSC [Sambrook et al, 1989] and bound to the membrane by UV cross-linking. Visualisation under UV light allowed the positions of the 18s and 28s bands of RNA to be marked on each membrane. The membranes were stored individually in envelopes at room temperature until subjected to hybridisation.

2.3.7 Hybridisation

The Hybond membranes were incubated for 6 hours at 60°C in plastic bags containing a pre-hybridisation solution (1 ml/cm²) which consisted of 50% formamide, 50 mM Tris, pH 7.5), 1 mM sodium chloride, 10% dextran sulphate, 2x Denhardt's solution (1x Denhardt's is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum



albumin), 1% sodium dodecylsulphate (SDS) and 1 mg/ml salmon sperm DNA. Following pre-hybridisation, sufficient ^{35}S -labelled cRNA probe was added to each bag to achieve an ^{35}S radioactive concentration of 1.5×10^6 dpm/ml. The membranes were incubated for an additional 16 hours at 65°C , washed three times with $1\times$ SSC/0.1% SDS for 15 minutes at room temperature and three times with $0.1\times$ SSC/0.1% SDS for 20 minutes at 65°C . The membranes were air dried and subjected to autoradiography.

2.3.8 Autoradiography

The membranes which had been hybridised were placed against X-ray film (β max, Amersham) contained in film cassettes at -80°C . When comparisons were to be made of samples on different membranes, those membranes were laid adjacent to one another, against the same piece of film. The exposure times varied from 3 days to 5 weeks, depending on the gene and tissue being studied.

The films were developed in Phenisol X-ray developer (diluted 1:5 in water) for 5 minutes. Development was stopped with 3% acetic acid (1 minute), followed by fixation with Kodak Rapid Fixer (diluted 1:3 with water) for 5 minutes. After washing in running tap water for 15 minutes, the films were dried at 37°C .

2.3.9 Quantification of mRNA levels

The autoradiographic signals from the slot blot analyses were scanned (three times each slot) using a laser densitometer (LKB Ultrascan XL, Pharmacia LKB Biotechnology) and the area under each peak analysed using an integration system that was incorporated in the operations program for the densitometer. The absorbance of hybrid images was plotted against the concentrations of total RNA taken from

individual animals. Linear regression lines were established for each tissue sample and comparisons between treatment groups made on the basis of the slopes of the regression lines.

The intra- and inter-blot coefficients of variation were determined to be 12% and 15% respectively.

2.3.10 *In situ* hybridisation

The method used for *in situ* hybridisation studies was a compilation of previously published techniques [Krishnan and Cleary, 1990; Naftilan et al, 1991]. Tissues which had been stored in fixative solution at 4°C overnight were embedded in paraffin wax using standard histological techniques. Sections (5-7 μ) were mounted on 3-aminopropyltriethoxysilane (APES) treated slides (See Appendix II). Typically, sections from 3 or 4 different tissues were arranged on each slide. The sections were melted at 55°C for 45 minutes, then deparaffinised (2 x 4 minutes in Safsolvent), hydrated (4 minutes each in 100%, 95% and 70% ethanol) and subjected to a number of pre-hybridisation washes (at room temperature unless otherwise stated): 4% paraformaldehyde in PBS for 20 minutes; 2x SSPE (1x SSPE is 150 mM sodium chloride, 10 mM sodium phosphate, 0.8 mM EDTA, pH 7.4) for 5 minutes; 0.2 M hydrochloric acid for 20 minutes; 2x SSPE for 5 minutes; Proteinase K (3 μ g/ml) in 50 mM Tris (pH 7.5), 5 mM EDTA for 30 minutes at 37°C; 4% paraformaldehyde in PBS for 5 minutes; 2x SSPE for 5 minutes; glycine (200 μ g/ml) in 2x SSPE for 5 minutes; 4% paraformaldehyde in PBS for 20 minutes; 2x SSPE for 5 minutes; 0.25% acetic anhydride in 0.1 M triethanolamine HCL (pH 8) for 10 minutes; 2x SSPE for 5 minutes. The sections were then dehydrated, air dried and warmed on a metal heating block at 52°C for 15 minutes.

Hybridisation solution was applied to the warmed sections, which were then overlaid with siliconised coverslips (See Appendix II). The hybridisation solution was 50% formamide, 2x SSPE, 10% polyethyleneglycol (MW 8000), 0.1% yeast tRNA, 0.05% salmon sperm DNA, 1% sodium dodecyl sulphate (SDS), 0.5% Blotto, 50 mM DTT. RNA probes were used at a final concentration of 20,000 dpm/ μ l. 35 S-labelled mRNA (either renin or angiotensinogen) was used to monitor non-specific binding. Hybridisation was performed at 52°C for 20 hours in an humidified chamber.

Following hybridisation, the sections were subjected to a number of washes to remove non-specifically bound probe: 50% formamide /2x SSPE containing 20 mM β ME at 52°C for 20 minutes (twice); 2x SSPE at room temperature for 5 minutes (twice); 5x SSC containing RNase A (5 ug/ml) and RNase T1 (10 U/ml) at room temperature for 15 minutes; 2x SSPE /20 mM β ME at 52°C for 5 minutes; 2x SSPE at 52°C for 20 minutes; 0.1x SSPE at 52°C for 20 minutes. The sections were dehydrated (2 minutes each in 50%, 70% and 95% ethanol) and air dried. The sections were then coated with Ilford K5 nuclear track emulsion (diluted 1:1 with water) or Amersham LM-1 emulsion (undiluted), dried and exposed at 4°C in light proof boxes. When Amersham emulsion was used, desiccant was added to the slide boxes. The time of exposure varied from 1-5 weeks, depending on the gene and the tissue under investigation.

The emulsion was developed with Kodak D19 developer (diluted 1:1 with water) for 5 minutes. Development was stopped with 1% acetic acid (1 minute), followed by fixation with Kodak Rapid Fixer (diluted 1:3 with water) for 3 minutes. After washing the slides in running tap water for 15 minutes, the slides were counterstained with haematoxylin and eosin and coverslips were affixed with Pix mounting medium. The deposition of black silver grains indicated the binding of RNA probe to the tissue section.

2.4 Statistical analyses

Values are expressed as the mean \pm SEM for the number of measurements shown for each experiment. Statistical significance was determined using the GraphPad InStat computer program, which allowed a choice of a number of statistical tests. In general, appropriate parametric tests were used (e.g. Student's *t*-test for paired data, Student's *t*-test for unpaired data or analysis of variance) unless the variances of sample groups were significantly different, in which case equivalent non-parametric tests were used (Wilcoxon signed matched pairs test, Mann-Whitney U-test or Kruskal-Wallis analysis of variance). In all studies $P < 0.05$ was considered statistically significant.

The data was tabulated using the Lotus 123 spreadsheet computer program and the results were represented graphically using the GraphPad computer program. All computer software used in the analysis of the data was licensed to the Department of Clinical and Experimental Pharmacology, University of Adelaide, South Australia.

CHAPTER 3**LOCALISATION OF THE GENE EXPRESSION OF THE RAS IN SHR AND
WKY USING *in situ* HYBRIDISATION**

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3.1 Introduction

While the analysis of RNA in tissue homogenates may provide evidence for abnormal levels of expression of a particular gene in a certain tissue, the cell types responsible for aberrant expression are not identified by this technique. *In situ* hybridisation involves hybridisation of a nucleic acid probe to tissue sections which have been adhered to a microscope slide and thus the cell types responsible for gene expression can be identified.

In the kidneys from several species, immunocytochemistry studies have demonstrated that the sites at which renin immunoreactive protein may be found depends on the age of the animal. There is a progressive decrease in the number of renin-containing cells in arcuate and intralobular arteries as the animal develops to maturity, so that in adults immunoreactive renin is found almost exclusively in the juxtaglomerular apparatus [Minuth et al, 1981; Egerer et al, 1984; Celio et al, 1985; Gomez et al, 1988a]. In the rat, *in situ* hybridisation has demonstrated that the shift in renin distribution is a consequence of changes in renin gene expression and not due to decreased renin uptake from the circulation [Gomez et al, 1989]. The distribution of renin protein in kidneys from SHR has been examined in newborn (2 days) and adult (90 days) animals [Gomez et al, 1988a]. No significant difference in renin immunodistribution was noted in newborn SHR when compared with WKY, despite the finding that in the SHR renal renin concentration was twice that seen in WKY. In adults there was a small but significant decrease in the percentage of blood vessels with longer trajectory of immunostaining in the SHR, which was in agreement with the small but significant decrease in renin content in kidneys from adult SHR [Gomez et al, 1988a]. The developmental regulation of kidney renin gene expression in the SHR has not been examined using *in situ* hybridisation. Since, in 5 week old SHR the level of renin mRNA in kidney is significantly elevated [Samani et al, 1989], a closer examination of the ontogeny of renin expression in kidneys from SHR at time points between the ages of 2 days and 90 days seems warranted. In addition, since the expression of

angiotensinogen in kidney and liver is also reported to be subject to developmental regulation [Gomez et al, 1988b; Ellison et al, 1989], it is of interest to determine whether the spatial localisation of such regulation is similar in hypertensive and normotensive rats.

It has been suggested that the vascular RAS may influence local vascular function and be involved in the long-term regulation of blood pressure [Dzau, 1987]. There is still much controversy regarding whether renin is expressed in vascular tissue [Samani et al, 1988; Ekker et al, 1989] and some dispute about the precise sites of expression of angiotensinogen [Campbell and Habener, 1987; Naftilan et al, 1991]. There has been no comparison between hypertensive and normotensive rats of the vascular sites of expression of the RAS components.

In the studies described in this chapter, *in situ* hybridisation was used to examine the developmental regulation of gene expression of the RAS components in kidneys and livers from hypertensive and normotensive rats and to determine whether the vascular sites of expression of RAS components were similar in the two rat strains.

3.2 Methods

3.2.1 Animals

Male SHR and WKY were sourced and housed as described previously (2.1.1). Kidneys and livers were removed from 1 week (4 SHR, 4 WKY), 4 week (4 SHR, 4 WKY) and 21 week old animals (5 SHR, 5 WKY) following sacrifice by decapitation (2.1.3). Mesenteric artery segments, including adherent fat, were removed from 21 week old SHR and WKY.

The effect of perfusion fixation of the animals on the subsequent detection of mRNA sequences in tissue sections was investigated in preliminary experiments.

Pentobarbitone sodium anaesthetised adult rats were perfused through the left ventricle with phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in PBS (2.1.3) to 1 ml/g body weight. Tissues were then removed and processed as described below.

3.2.2 *Verification of probes*

³⁵S-labelled RNA probes were prepared as described previously (2.3.3). The specificity of each of the cRNA probes was verified by Northern analysis (2.3.6) of total RNA extracted from kidney and liver homogenates (2.3.4) followed by hybridisation (2.3.7).

In initial experiments, RNA probes labelled with Digoxigenin (DIG, Boehringer Mannheim) were prepared for use in the hybridisation according to the manufacturer's instructions. When DIG-labelled probes were used, the hybridisation reaction and immunological detection of bound probe was also performed in accordance with the manufacturer's instructions.

3.2.3 *In situ hybridisation*

Tissue segments were placed in 4% paraformaldehyde fixative overnight (2.1.3) before embedding in paraffin wax. Sections (5-7 μ , 3 or 4 sections per slide) were mounted on APES-treated microscope slides (*Appendix II*), and subjected to *in situ* hybridisation (2.3.10).

In preliminary experiments, a much less complicated pre-hybridisation protocol than the one described was investigated. In this simplified protocol, sections were melted and hydrated as described (2.3.10) and then subjected to a number of pre-hybridisation washes (at room temperature unless otherwise stated): 2x SSPE for 5 minutes;

Proteinase K (3 µg/ml) in 50 mM Tris (pH 7.5), 5 mM EDTA for 30 minutes at 37°C; 2x SSPE for 5 minutes; 0.25% acetic anhydride in 0.1 M Triethanolamine HCl (pH 8) for 10 minutes; 2x SSPE for 1 minute. All subsequent procedures were as described earlier (2.3.10).

The use of RNase digestion to monitor non-specific binding was also investigated in preliminary experiments. Duplicate tissue sections were subjected to digestion by RNase A (5 µg/ml) and RNase T1 (10 U/ml) in 5x SSC at room temperature for 15 minutes, followed by 2x SSPE for 5 minutes at room temperature, prior to acetylation with 0.25% acetic anhydride in 0.1 M Triethanolamine HCl (pH 8) for 10 minutes. After washing in 2x SSPE for 1 minute, these sections were subjected to hybridisation with cRNA probes and post-hybridisation washes as described earlier (2.3.10).

3.3 Results

3.3.1 Preliminary experiments

Initially, DIG-labelled probes were used in the hybridisation reaction. DIG is a non-radioactive label and therefore likely to be less hazardous in the laboratory than the traditional radioactively-labelled compounds. Detection of DIG-labelled probes was achieved by an antibody linked to the enzyme alkaline phosphatase, followed by the generation of a coloured precipitate. Despite strict adherence to the manufacturer's instructions, DIG-labelled probes were found to be unsuitable for *in situ* hybridisation due to the high level and widespread non-specific generation of colour precipitate in tissue sections. The technical problems encountered when using DIG-labelled probes were almost certainly due to deficiencies in the DIG system because as soon as this system was abandoned in favour of ³⁵S-labelled probes followed by autoradiographic detection, specifically-bound probe was readily detected.

Tissue morphology may be improved when tissue segments are removed from animals which have been perfusion fixed. When tissue sections from perfusion-fixed animals were compared with sections from animals which had been sacrificed by decapitation, no apparent differences were noted either in the morphology of the tissue sections or in the detection of specific mRNA sequences following hybridisation. Animals which were one week old were considered to be too small to successfully perfuse. In some experiments, different tissue segments from the same animal were analysed for enzyme activity as well as RNA concentration. However, perfusion with fixative inactivates enzymes and thus prevents their analysis. Collectively, the procedure of perfusion-fixation offered no apparent benefit and was therefore not routinely performed.

The non-specific binding of RNA probes may be monitored by one or more of three methods: i.e. (1) assessment of the binding of radioactively labelled mRNA probes, (2) digestion of mRNA in the tissue section prior to hybridisation with radioactively labelled cRNA probes and (3) competition for binding to mRNA in the tissue section by the presence of excess unlabelled cRNA in the hybridisation reaction. The first two procedures were assessed for use as a "negative control" in the present studies. Duplicate tissue sections which had been hybridised with either radioactively labelled mRNA or radioactively labelled cRNA following RNase digestion showed similar low levels of silver grains in the photographic emulsion which indicated that either method was suitable for monitoring non-specific binding. For all subsequent experiments, non-specific binding of the cRNA probe was assessed only by comparison with adjacent tissue sections which had been hybridised with the mRNA probe.

During the preliminary experiments which were performed to establish the optimum conditions for *in situ* hybridisation, a simple pre-hybridisation protocol (3.2.3) was used [Krishnan and Cleary, 1990]. This protocol readily detected mRNA that was expressed at a high level, such as renin in kidney and angiotensinogen in liver, but did not appear to be suitable for detecting the low level of expression of these genes in vascular tissue. Therefore a more complex prehybridisation protocol was developed

(2.3.10), based on a method that had been reported to detect the expression of angiotensinogen in aorta [Naftilan et al, 1991]. This second protocol, which was used for all subsequent experiments, included an additional deproteinisation step (using hydrochloric acid) which was designed to remove ribosomal proteins from the RNA and several post-fixation washes with paraformaldehyde which were designed to minimise the loss of RNA following deproteinisation.

3.3.2 Verification of probes

Northern analysis of total RNA from kidney and liver homogenates (*Figure 3.3.1*) revealed a single hybridisation band for each of the cRNA probes that corresponded with the expected molecular weight: renin ~1450 bases; angiotensinogen ~1600 bases; ACE ~4300 bases [Lynch et al, 1986; Burnham et al, 1987; Soubrier et al, 1988].

3.3.3 Renin expression in kidney and liver

In kidneys from both WKY (*Figures 3.3.2a, 3.3.2c, and 3.3.2e*) and SHR (*Figures 3.3.2b, 3.3.2d, and 3.3.2f*) at each of the ages studied, renin expression was restricted to the cortex. In one week old animals from both WKY (*Figure 3.3.3a*) and SHR (*Figure 3.3.3b*) renin expression was very often detected in intralobular arteries and extended for some distance along the afferent arteriole. By 4 weeks of age, in both WKY (*Figure 3.3.3c*) and SHR (*Figure 3.3.3d*), renin was expressed almost exclusively in the juxtaglomerular apparatus, although occasionally mRNA expression extended a short distance along the afferent arteriole. In kidneys from adult WKY (*Figure 3.3.3e*) and SHR (*Figure 3.3.3f*) renin expression was restricted to the juxtaglomerular apparatus. Specific binding of renin cRNA was assessed by comparison with sections that had been hybridised with mRNA (*Figures 3.3.2g and 3.3.3g*).

Renin mRNA was not detectable in liver sections from either SHR or WKY at any of the ages studied (not shown).

3.3.4 Angiotensinogen expression in kidney and liver

Angiotensinogen mRNA was not detected in kidneys from either SHR or WKY at one and 4 weeks of age (data not shown). In adult animals, angiotensinogen mRNA was demonstrated in the proximal tubule cells of kidneys from both WKY (*Figure 3.3.4a*) and SHR (*Figure 3.3.4b*). Specific binding of angiotensinogen cRNA was assessed by comparison with sections that had been hybridised with mRNA (*Figure 3.3.4c*). Extended autoradiographic exposure, which was performed to amplify the signal and to better demonstrate the distribution of cells which expressed angiotensinogen, showed that the gene was expressed primarily in the inner cortex and the medullary rays (*Figure 3.3.4d*).

In liver sections from both WKY (*Figures 3.3.5a, 3.3.5c and 3.3.5e*) and SHR (*Figures 3.3.5b, 3.3.5d and 3.3.5f*) at all ages, angiotensinogen mRNA was detected in hepatocytes throughout the tissue. At all ages and in both rat strains, expression appeared to be more marked in the hepatocytes surrounding the portal triangles.

3.3.5 ACE expression in kidney and liver

The developmental regulation of ACE gene expression was not studied, although the expression of this gene in kidneys and livers from adult animals was examined. Demonstration of ACE mRNA in both tissues required much longer exposure times than for renin or angiotensinogen (3-10 times longer) and consequently the level of non-specific deposition of silver grains in the tissue sections was much higher. In both WKY (*Figure 3.3.6a*) and SHR (*Figure 3.3.6b*) ACE mRNA was detected in the

proximal tubule cells, particularly in the inner cortex, as well as in the endothelium of the intralobular arteries. Specific binding of the ACE cRNA probe was assessed by comparison with sections which had been probed with mRNA (*Figure 3.3.6c*).

In the liver, ACE was expressed in hepatocytes throughout the tissue sections (*Figure 3.3.7a*) as well as in the endothelial lining of blood vessels (*Figure 3.3.7b*). Although not as clearly defined as for liver angiotensinogen expression, in some liver sections from both rat strains, it appeared that the level of expression was not uniform throughout the lobules but increased in the hepatocytes surrounding the portal areas (*Figure 3.3.7a*).

3.3.6 RAS expression in vascular tissue

Extended autoradiographic exposure was required to demonstrate RAS expression in mesenteric vascular tissue and consequently the level of non-specific deposition of silver grains in these tissue sections was very high.

In both SHR (*Figure 3.3.8a*) and WKY (*Figure 3.3.9a*), renin mRNA was detected in the smooth muscle cells of the blood vessel wall. Demonstration of renin mRNA in vascular tissue was inconsistent and quite often the number of silver grains in tissue sections probed with renin cRNA was not different from that seen in the negative control sections (*Figure 3.3.8d*, *Figure 3.3.9d*). Angiotensinogen mRNA was evident in the smooth muscle cells of the vessel wall as well as overlying adipocytes (or pre-adipocytes) in the adipose tissue surrounding the blood vessels (*Figure 3.3.8b*, *Figure 3.3.9b*). ACE was expressed throughout the vascular preparation including the endothelium, medial smooth muscle layer, adventitia and surrounding adipose tissue (*Figure 3.3.8c*, *Figure 3.3.9c*).

Figure 3.3.1. Representative examples of Northern blot analysis. Kidney (lanes 1 and 2) and liver (lanes 3 and 4). Total RNA (10 ug) from WKY (lanes 1 and 3) and SHR (lanes 2 and 4) was hybridised with cRNA probes for (a) renin, (b) angiotensinogen and (c) angiotensin converting enzyme.

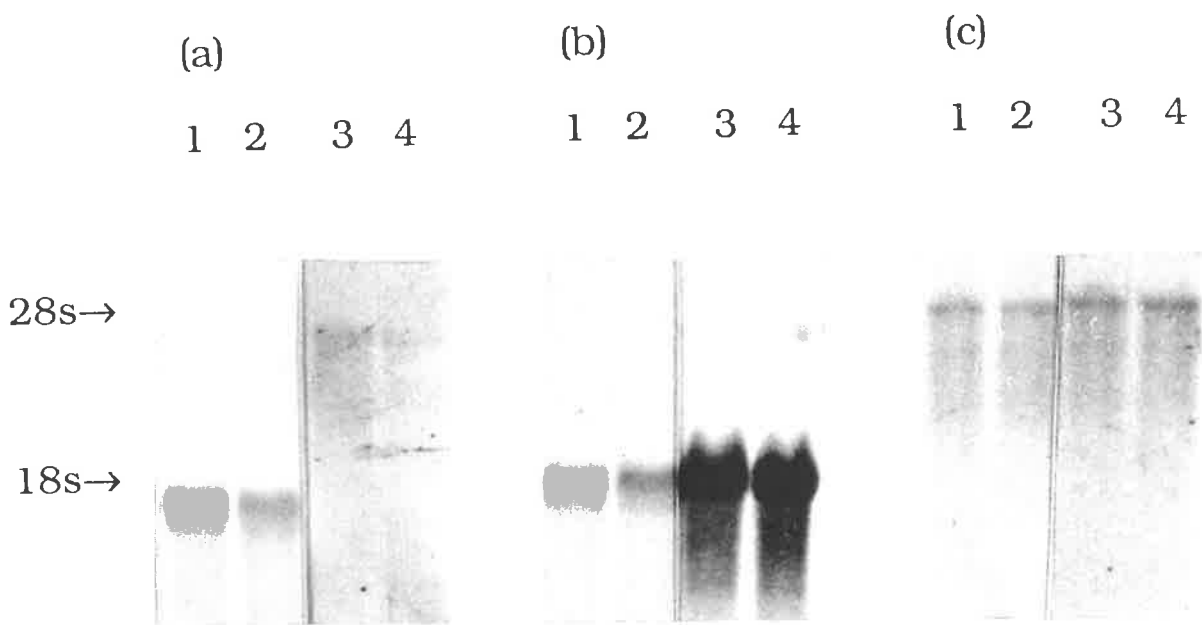
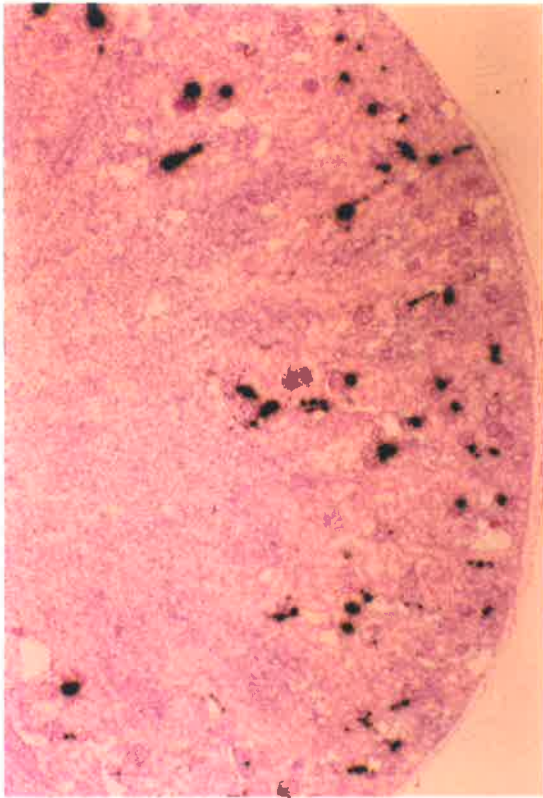
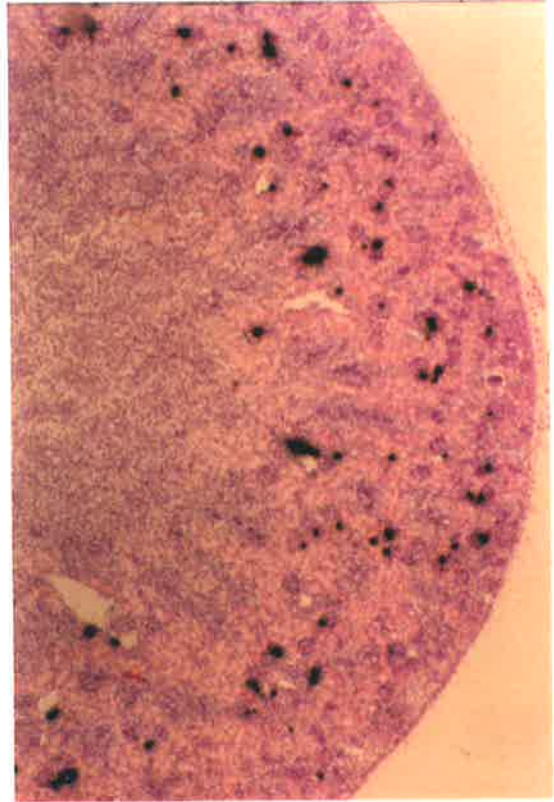


Figure 3.3.2. In situ hybridisation pattern for renin mRNA seen in kidney from (a) 1 week old WKY, (b) 1 week old SHR, (c) 4 week old WKY, (d) 4 week old SHR, (e) 21 week old WKY and (f) 21 week old SHR. The deposition of black silver grains represents the binding of the ³⁵S-labelled RNA probe. Renin mRNA was found in the cortex at all ages studied in both rat strains. (g) Specific binding of RNA probe was assessed by comparison with sections which were probed with ³⁵S-labelled mRNA. (Magnification X40)

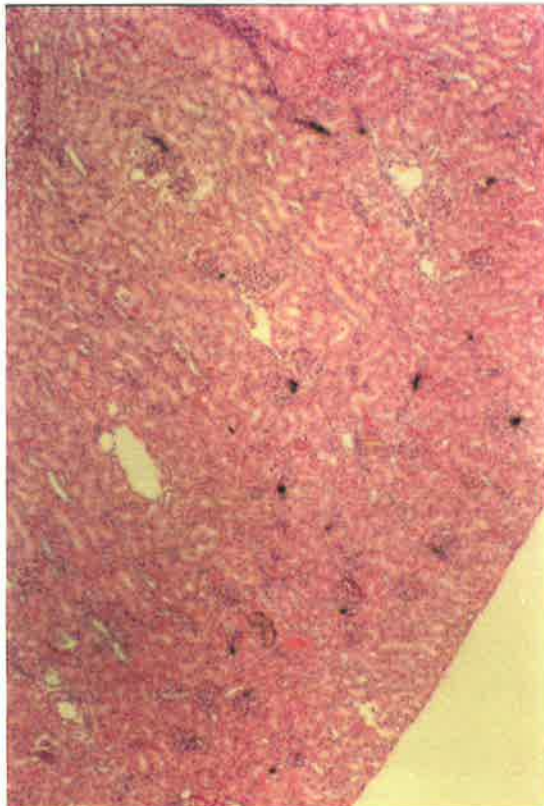
(a)



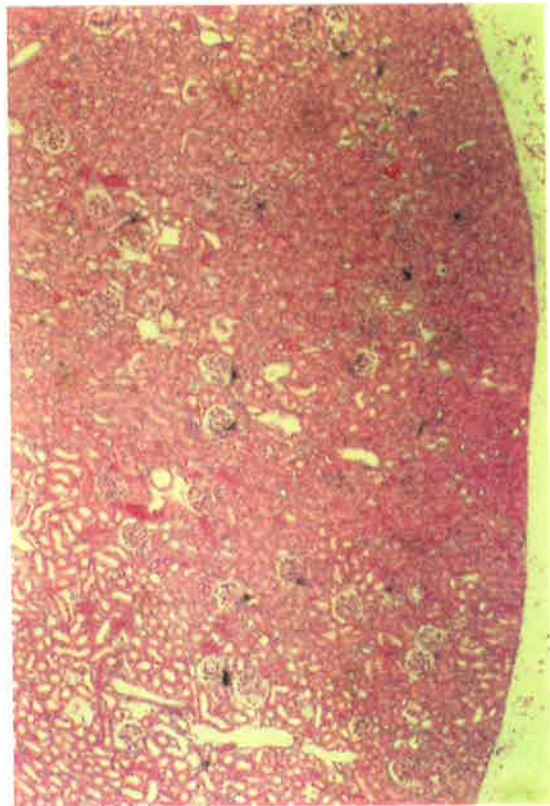
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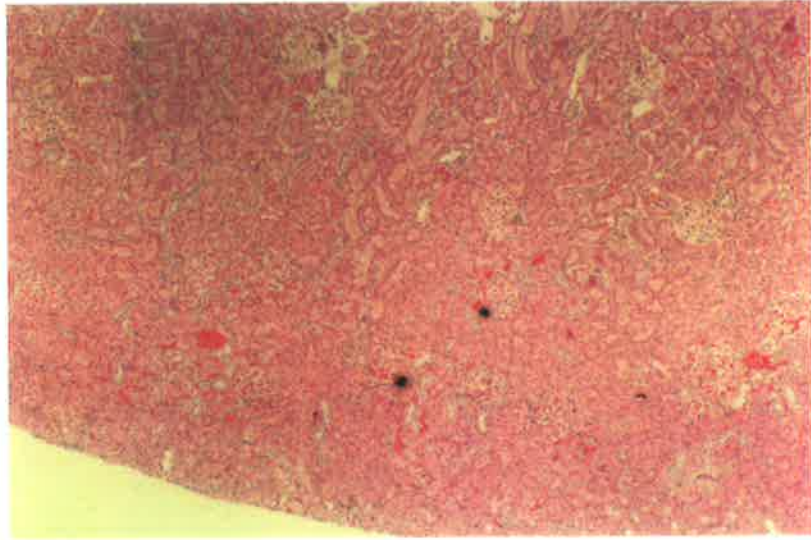
(c)



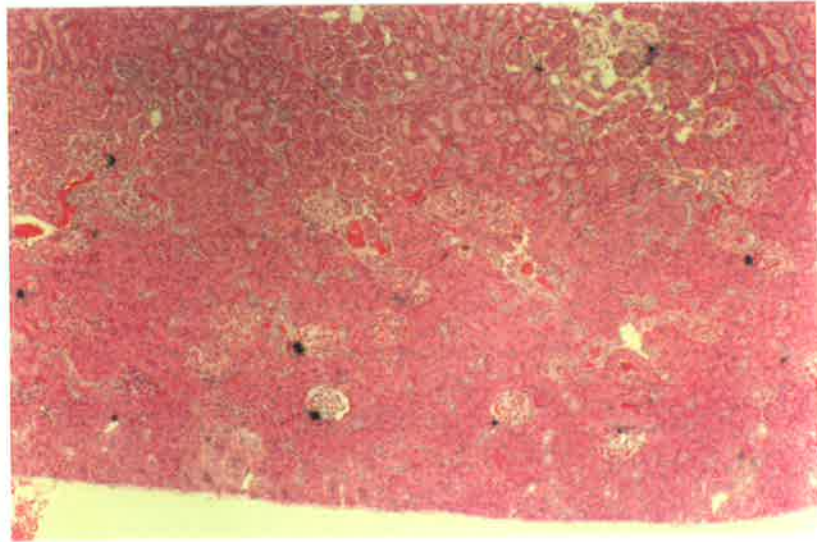
(d)



(e)



(f)



(g)

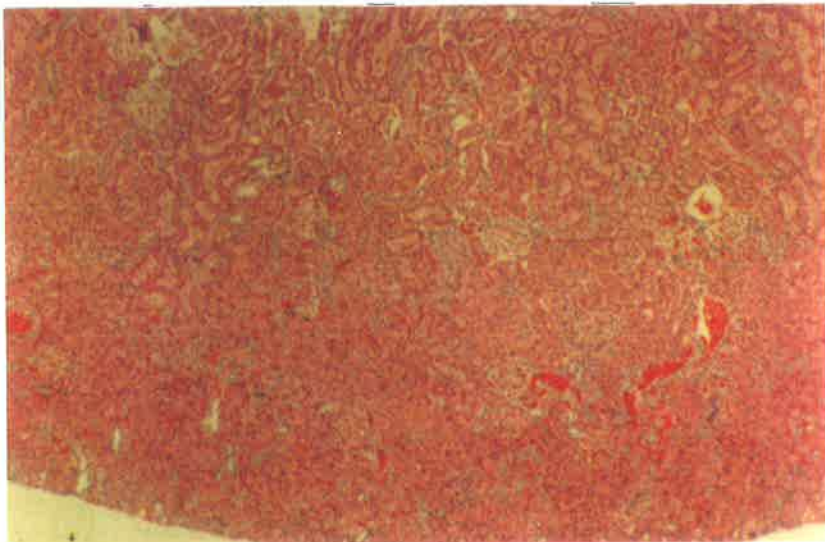
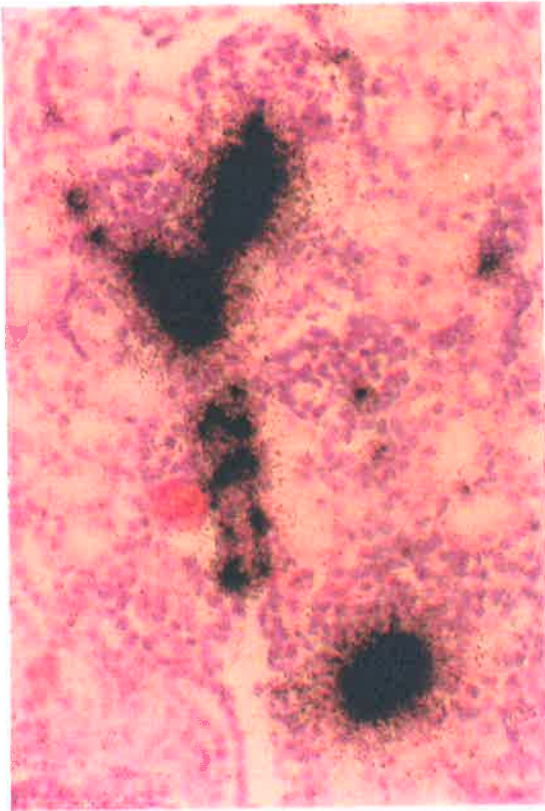
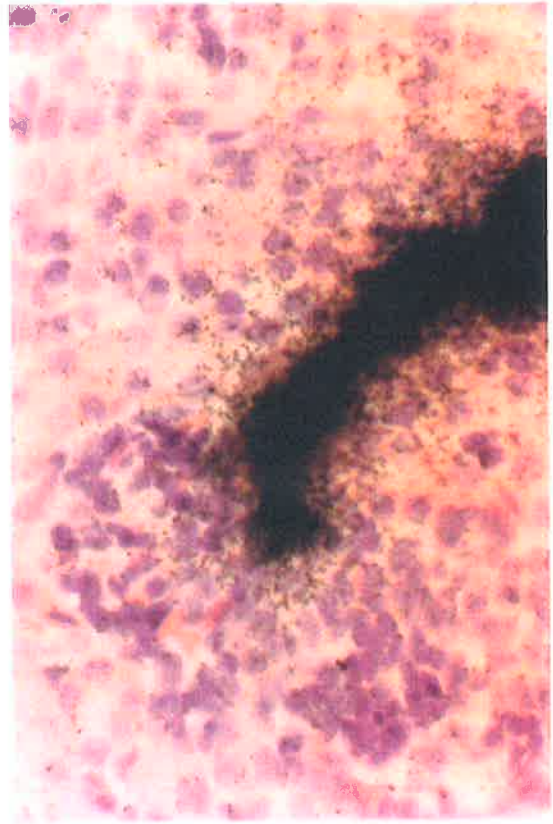


Figure 3.3.3. In situ hybridisation pattern for renin mRNA seen in kidney from (a) 1 week old WKY, (b) 1 week old SHR, (c) 4 week old WKY, (d) 4 week old SHR, (e) 21 week old WKY and (f) 21 week old SHR. The deposition of black silver grains represents the binding of the ³⁵S-labelled RNA probe. In one week old animals from both WKY renin mRNA is evident in intralobular arteries and extending for some distance along the afferent arteriole. In four week old animals renin is expressed almost exclusively in the juxtaglomerular apparatus, although occasionally renin mRNA expression extends a short distance along the afferent arteriole. In kidneys from adult animals WKY renin expression is restricted to the juxtaglomerular apparatus. Specific binding of renin cRNA was assessed by comparison with sections that had been hybridised with mRNA (g). (Magnification X200)

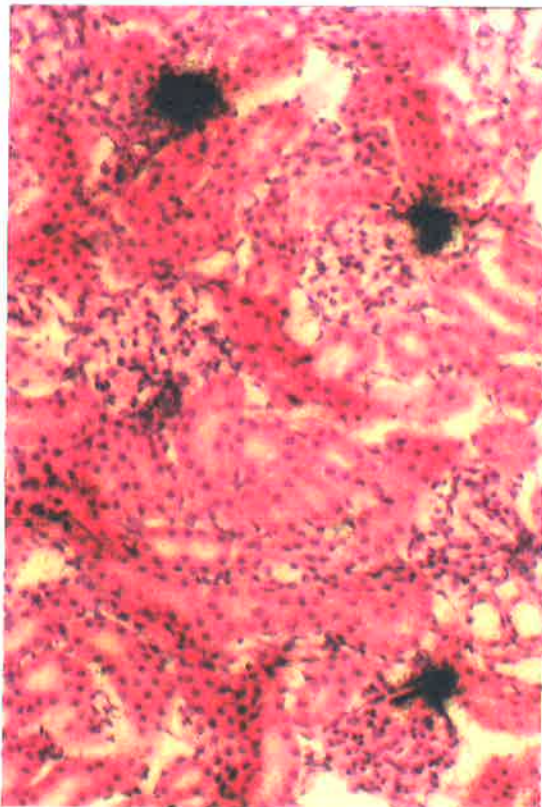
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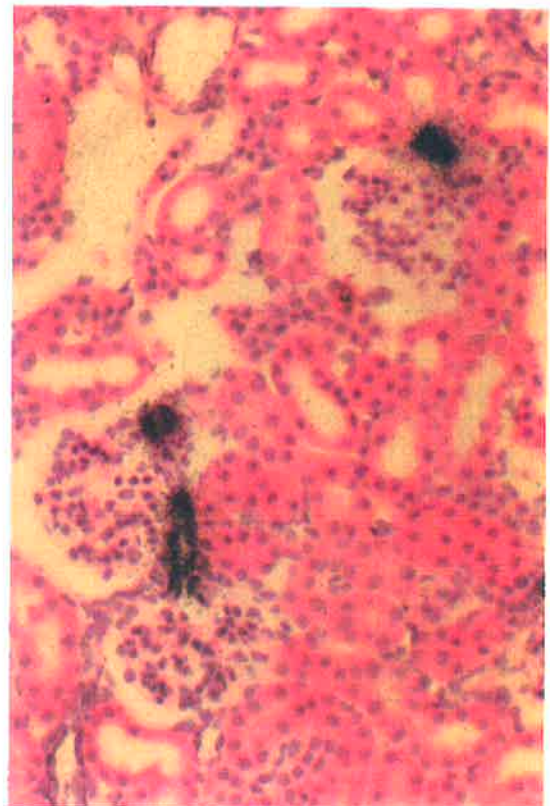
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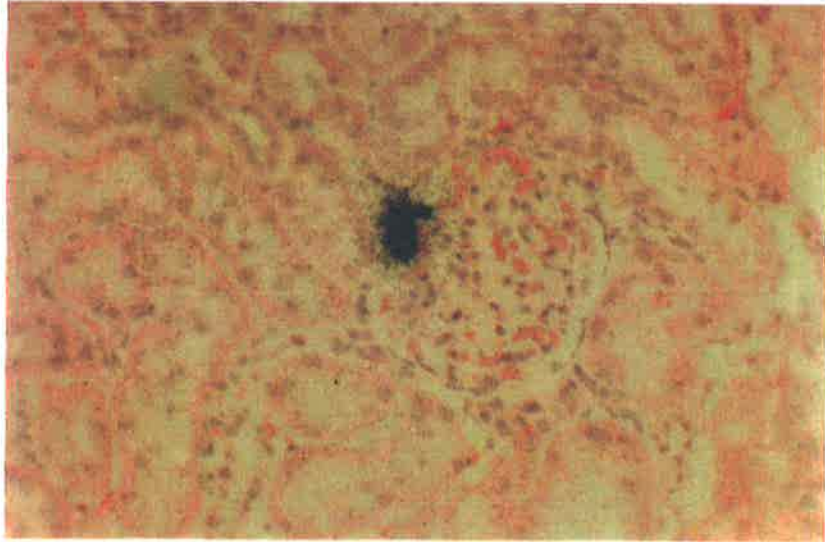
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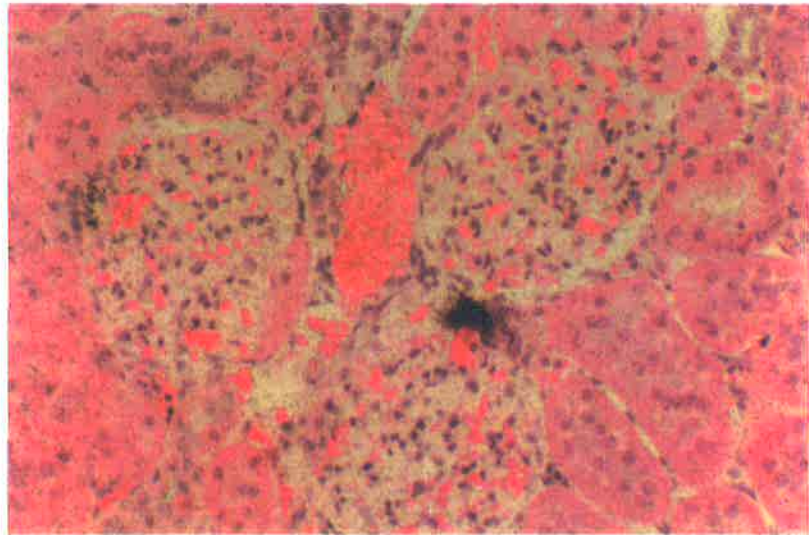
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(e)



(f)



(g)

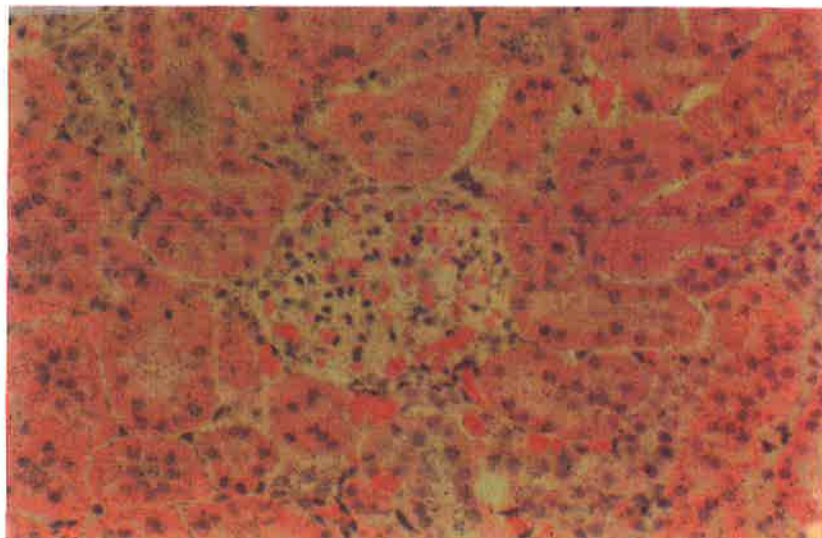
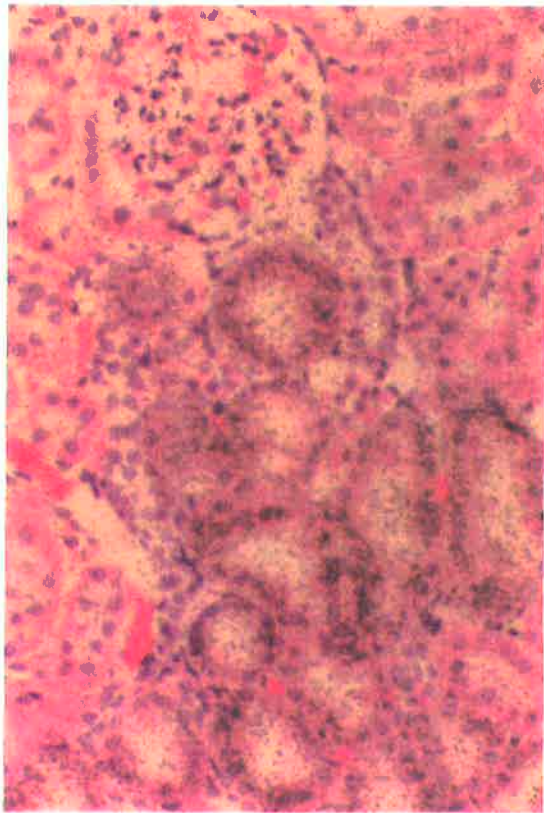
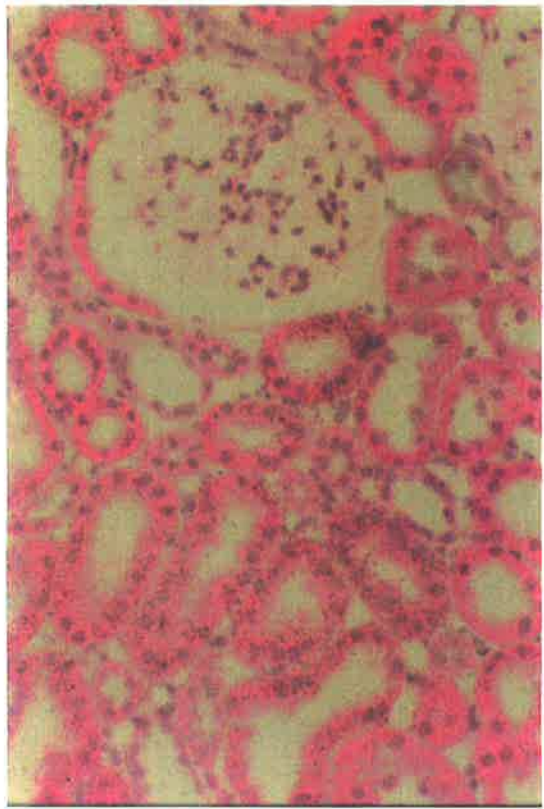


Figure 3.3.4. In situ hybridisation pattern for kidney sections from adult animals. (a) WKY kidney section probed with angiotensinogen cRNA (b) SHR kidney section probed with angiotensinogen cRNA (c) WKY kidney section probed with mRNA and (d) SHR kidney section probed with angiotensinogen cRNA. Angiotensinogen mRNA is demonstrated in the proximal tubule cells. (Magnification (a) X200, (b) X200 (c) X200 (d) X40)

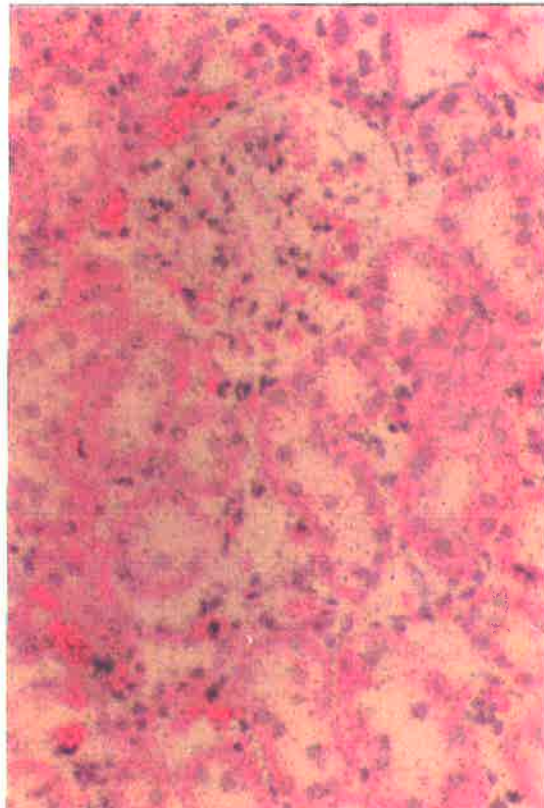
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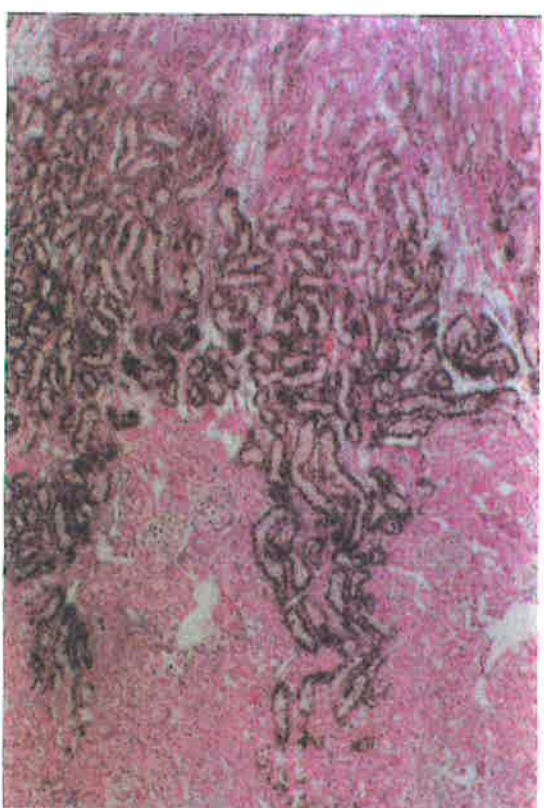
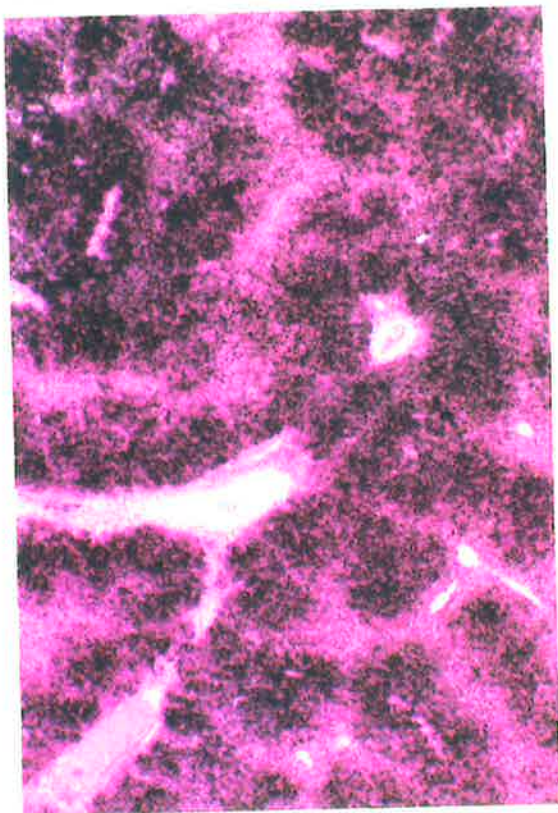
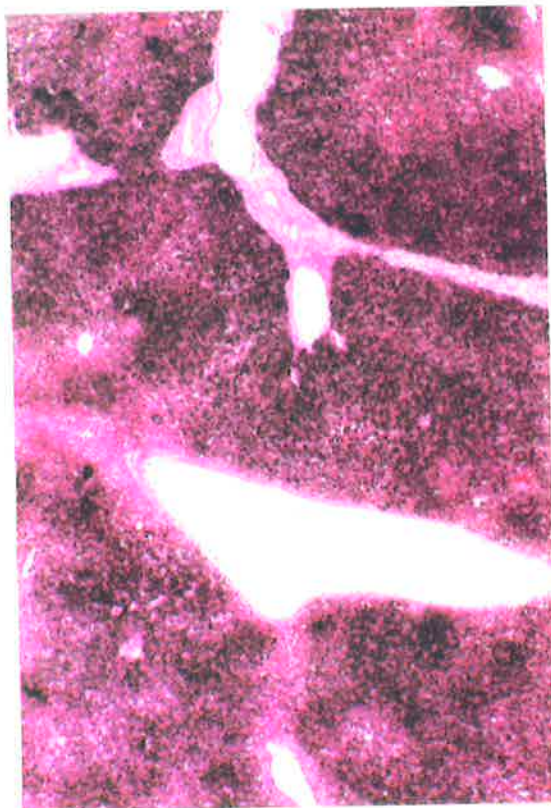


Figure 3.3.5. In situ hybridisation pattern for angiotensinogen mRNA seen in liver from (a) 1 week old WKY, (b) 1 week old SHR, (c) 4 week old WKY, (d) 4 week old SHR, (e) 21 week old WKY and (f) 21 week old SHR. Angiotensinogen is expressed in hepatocytes throughout the liver sections, with increased expression evident in the hepatocytes surrounding the portal areas. (Magnification X40)

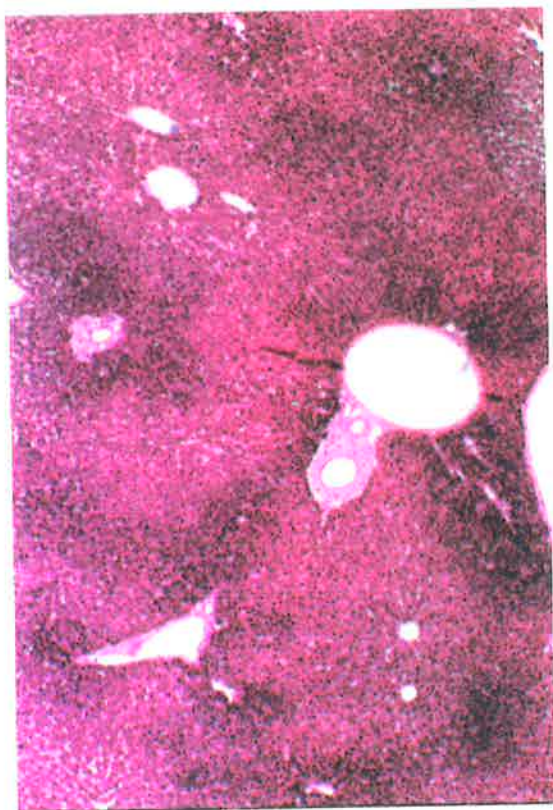
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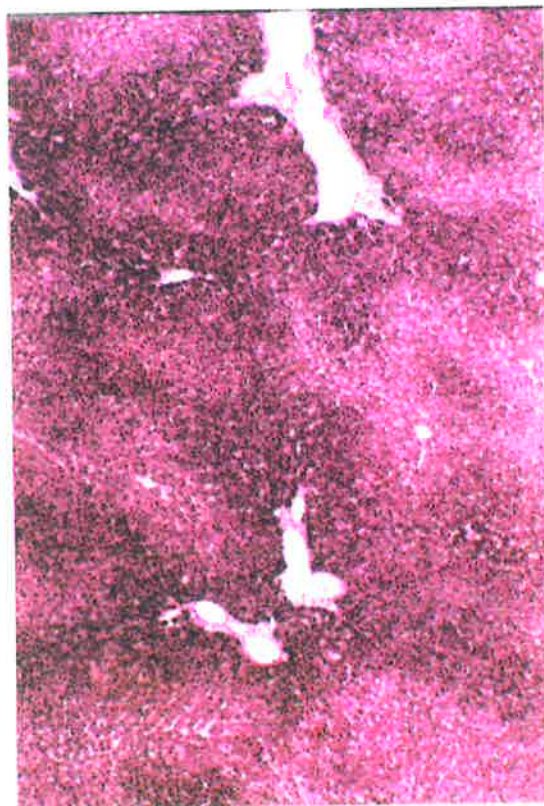
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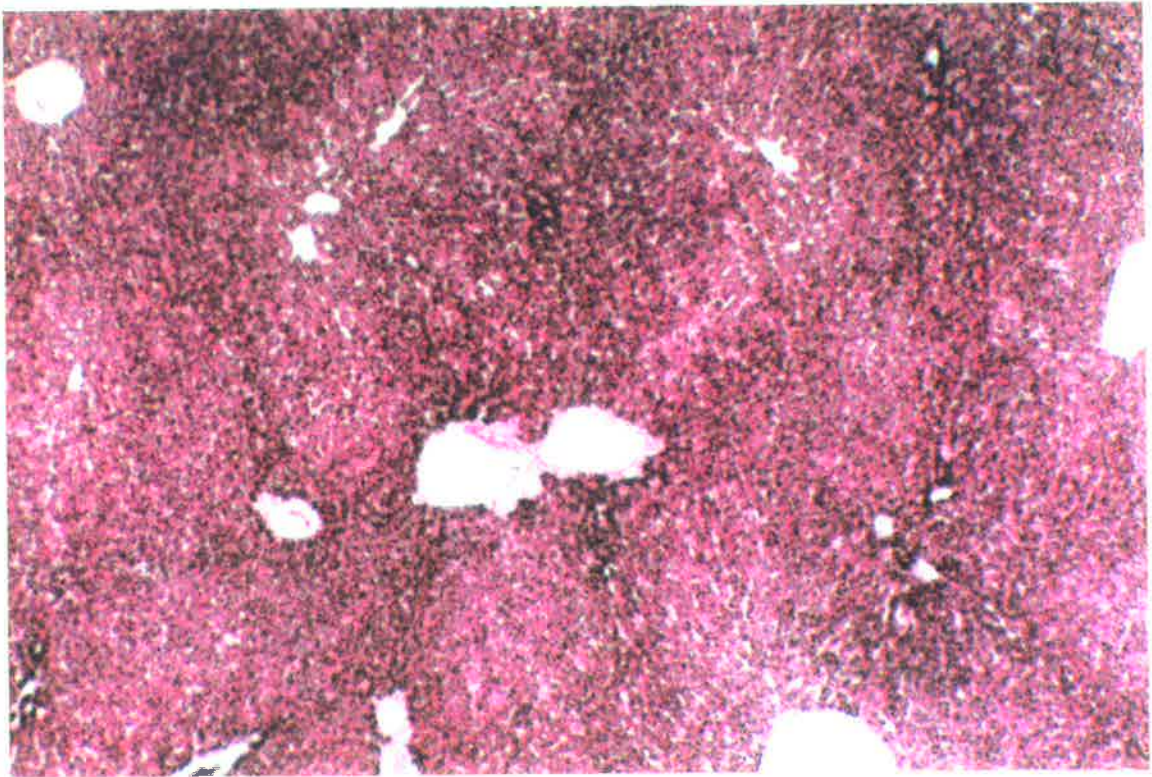
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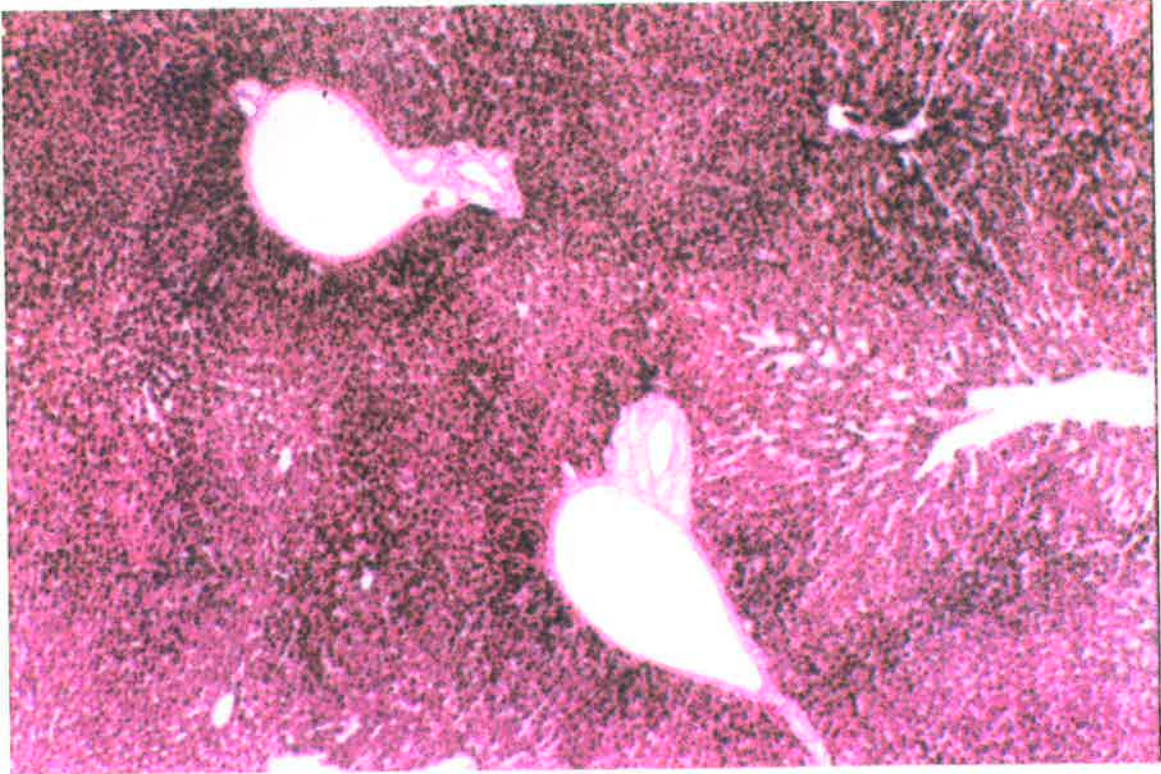
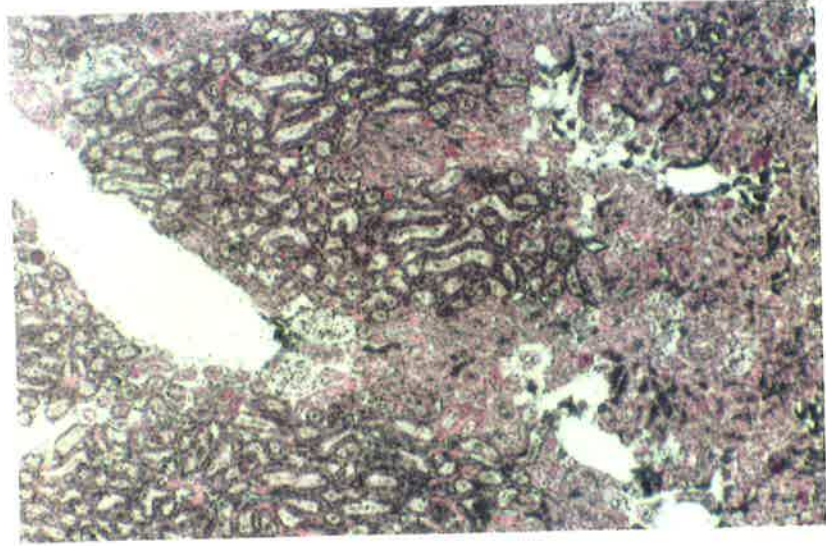
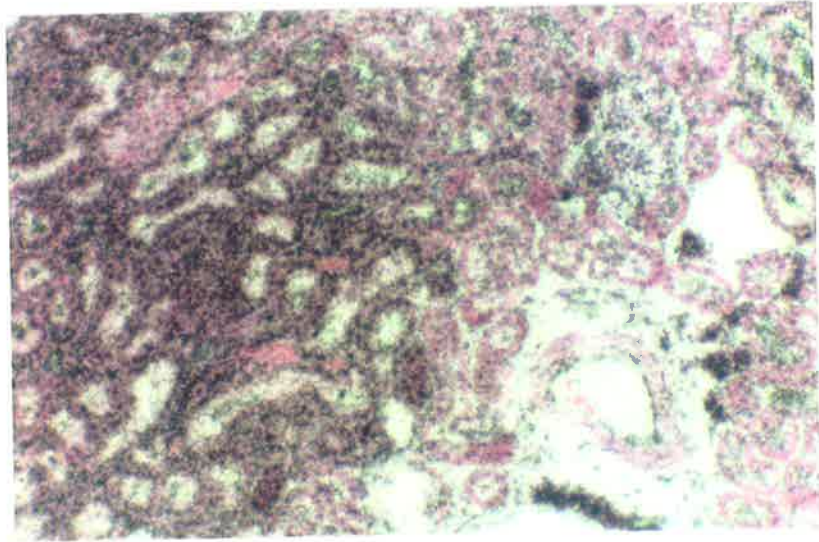


Figure 3.3.6. In situ hybridisation pattern for kidney sections from adult animals (a) WKY kidney section probed with ACE cRNA, (b) SHR kidney section probed with ACE cRNA and (c) SHR kidney section probed with mRNA. ACE is expressed in proximal tubule cells in the cortex. (Magnification (a) X40 (b) X200 (c) X40)

(a)



(b)



(c)

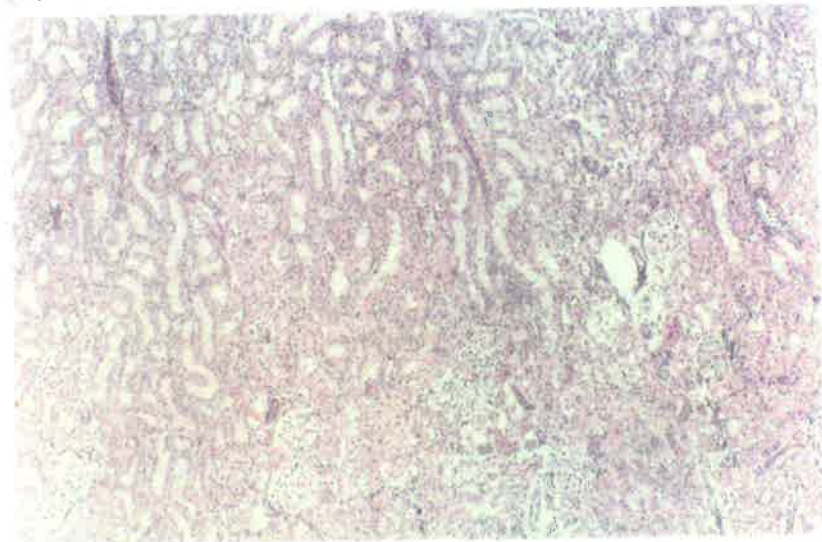
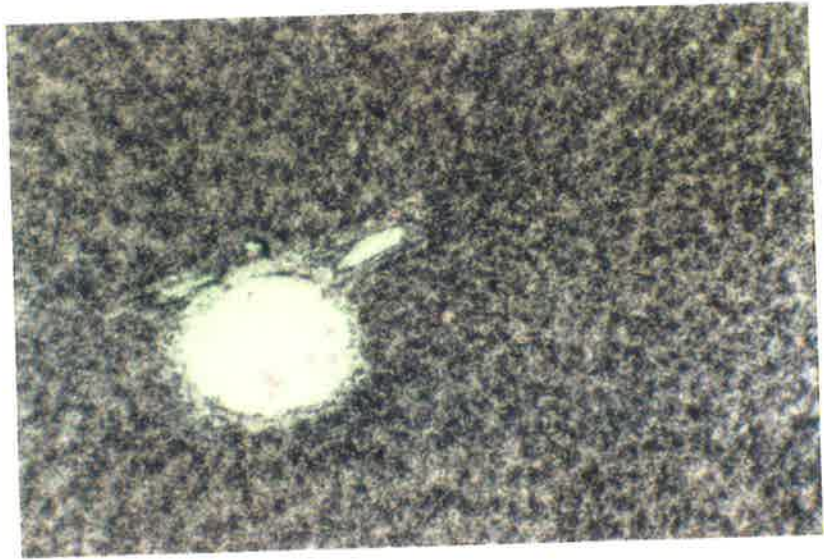


Figure 3.3.7. In situ hybridisation pattern for liver sections from adult animals. (a) WKY liver section probed with ACE cRNA, (b) SHR liver section probed with ACE mRNA and (c) SHR liver section probed with mRNA. ACE is expressed in hepatocytes throughout the sections as well as in the endothelial lining of blood vessels. (Magnification (a) X40 (b) X200 (c) X200)

(a)



(b)



(c)

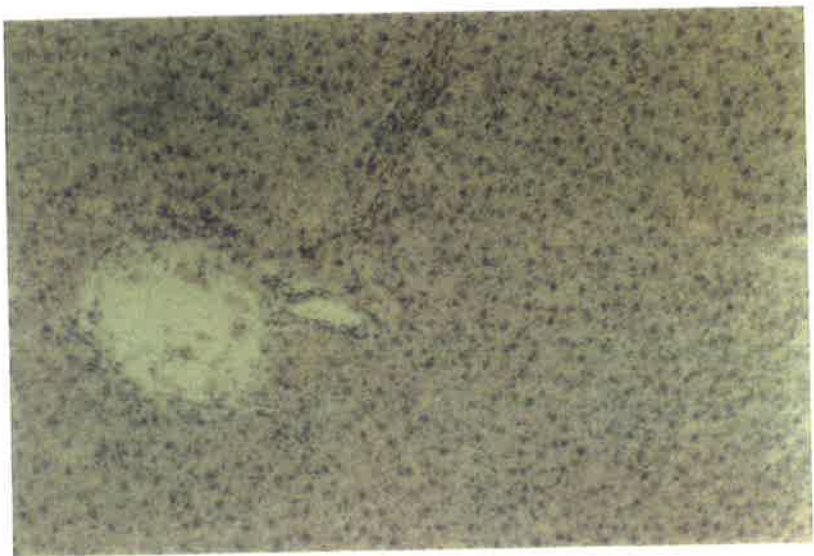
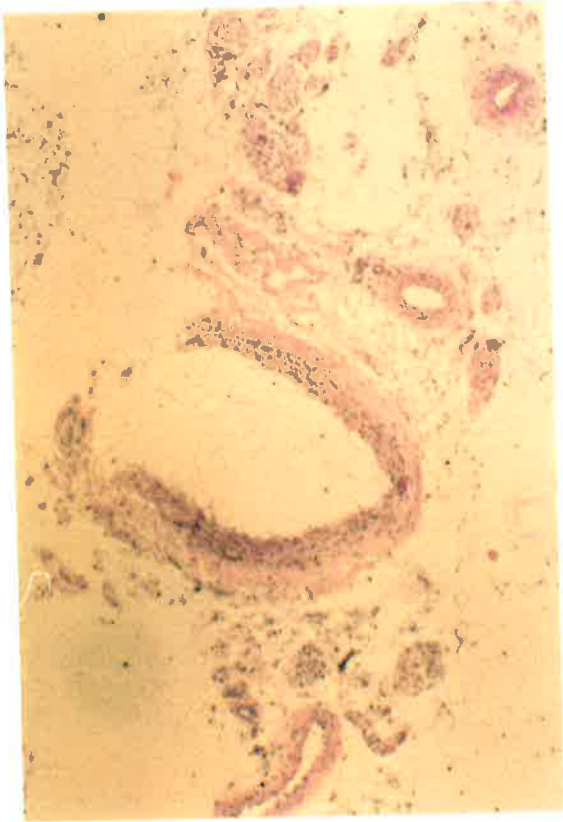
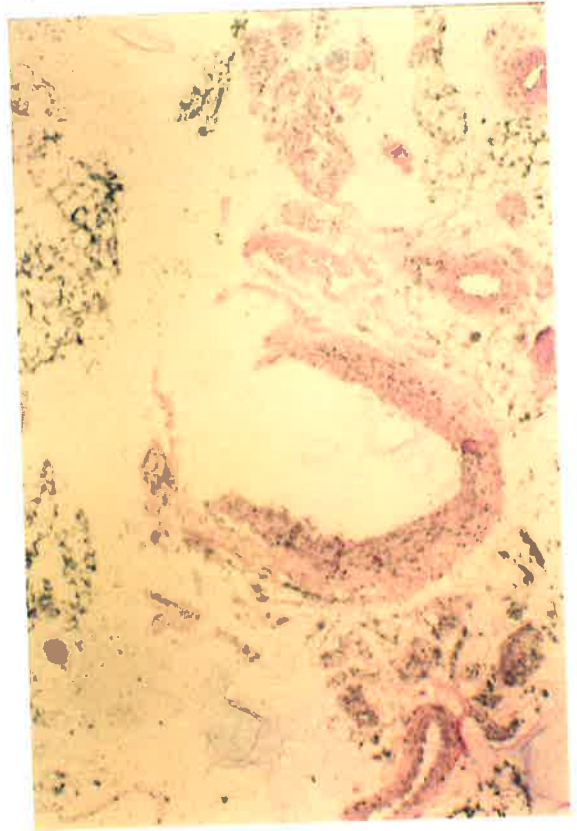


Figure 3.3.8. In situ hybridisation pattern for (a) renin (b) angiotensinogen (c) ACE and (d) negative control in the mesenteric vascular bed from adult SHR. Renin mRNA is demonstrated in the smooth muscle cells of the blood vessels. Angiotensinogen mRNA can be seen in the vascular smooth muscle as well as the surrounding adipose tissue. ACE mRNA is expressed throughout the vascular preparation i.e. in the endothelium, the vascular smooth muscle and also in the surrounding adipose tissue. (Magnification X40)

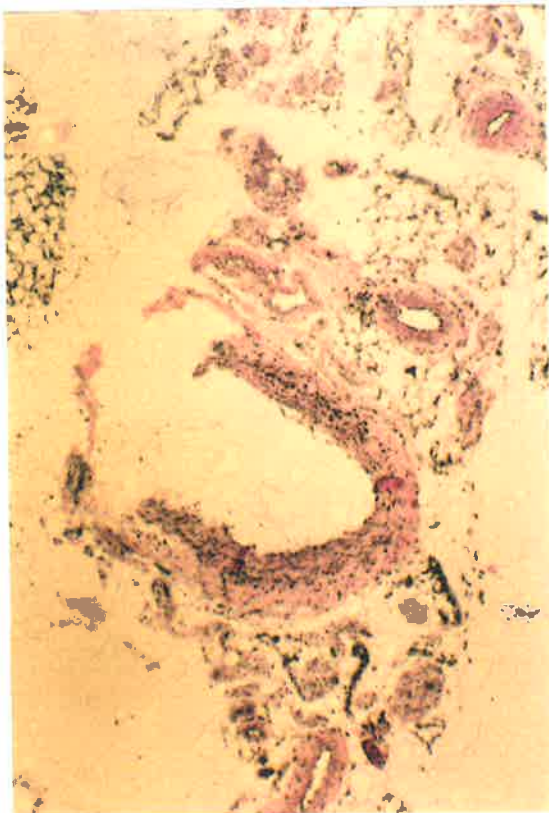
(a)



(b)



(c)



(d)

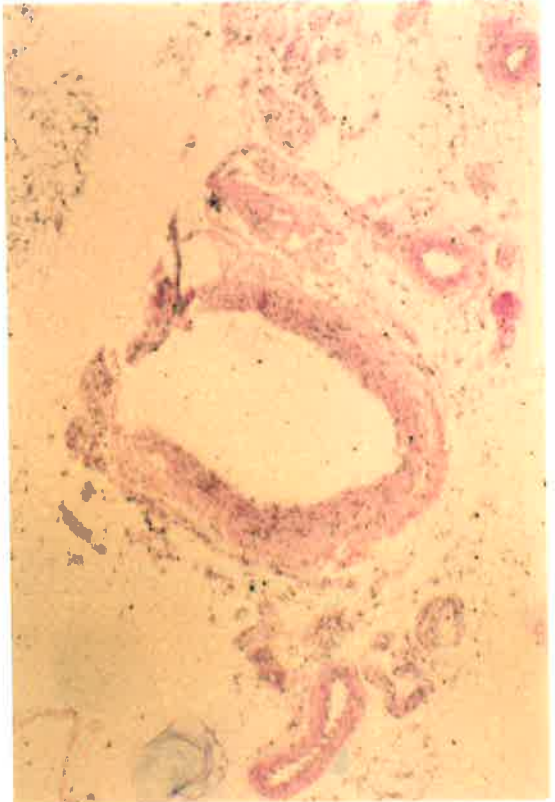
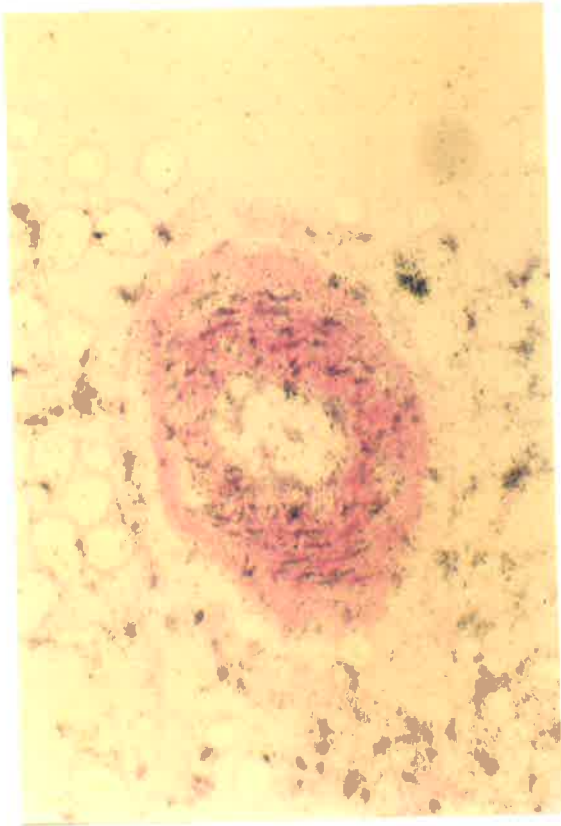
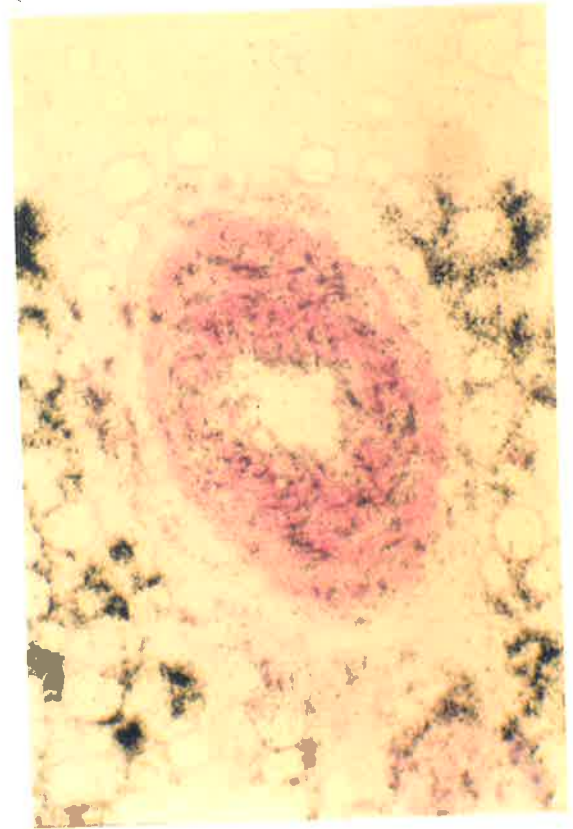


Figure 3.3.9. In situ hybridisation pattern for (a) renin (b) angiotensinogen (c) ACE and (d) negative control in the mesenteric vascular bed from adult WKY. Renin mRNA is demonstrated in the smooth muscle cells of the blood vessels. Angiotensinogen mRNA can be seen in the vascular smooth muscle as well as the surrounding adipose tissue. ACE mRNA is expressed throughout the vascular preparation i.e. in the endothelium, the vascular smooth muscle and also in the surrounding adipose tissue. (Magnification X200)

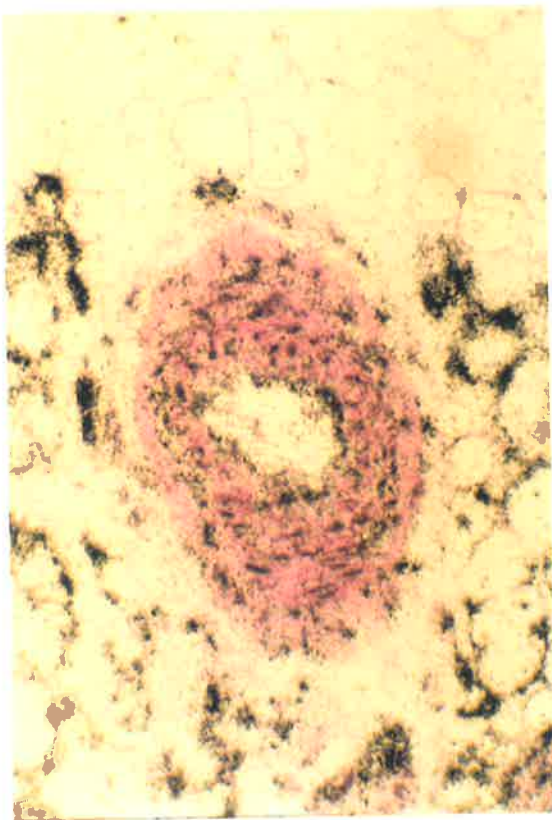
(a)



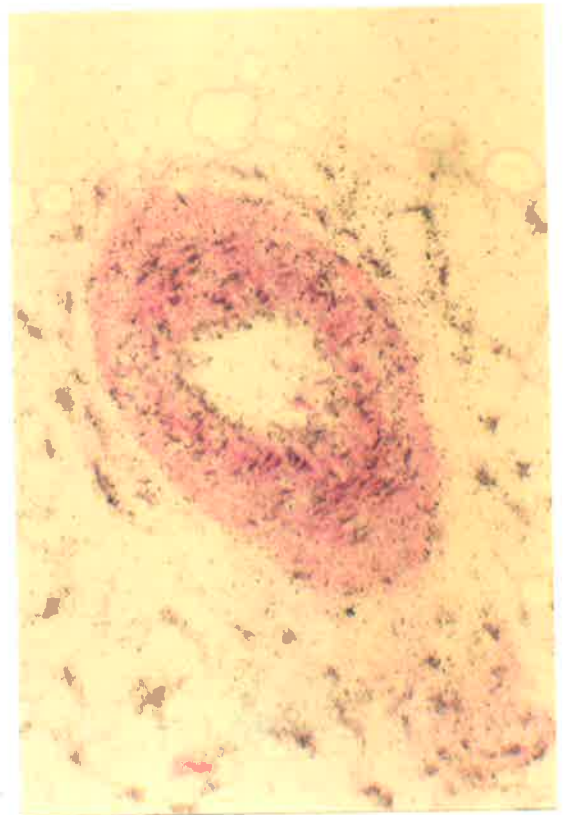
(b)



(c)



(d)



3.4 Discussion

The present study used the technique of *in situ* hybridisation to localise the sites of expression of RAS components in tissues from SHR and WKY. The specificity of the probes used for hybridisation was confirmed by Northern analysis. For each of the genes studied, hybridisation bands of the expected molecular weight were observed following hybridisation of total RNA samples.

Numerous methods for the detection of mRNA in tissue sections using *in situ* hybridisation have been reported. As with all assay techniques, the goal of protocol optimisation is to achieve the maximum signal to noise ratio. In *in situ* hybridisation the pre-hybridisation washes serve to remove ribosomal and other bound proteins from the mRNA and to acetylate macromolecular sites in the tissue which might bind non-specifically to the RNA probe. Loss of mRNA following deproteinisation can be minimised by including several post-fixation washes, but the preservation of mRNA is at the cost of increased non-specific binding of RNA probes. In the present study, a relatively simple pre-hybridisation protocol, without post-fixation washes was adequate to detect mRNA which was expressed in relative abundance, such as renin in kidney and angiotensinogen in liver. However, the prehybridisation protocol required the inclusion of more severe deproteinisation as well as post-fixation for the demonstration of lower levels of mRNA. The probability of detecting low levels of mRNA can also be increased by extending the autoradiographic exposure time, but this may also result in an increase in the apparent non-specific binding.

The level of non-specific deposition of silver grains was markedly different between the two brands of photographic emulsion which were used in the study. Ilford K5 emulsion was a far superior product and therefore was used in preference to the Amersham emulsion where possible. However, the manufacturers often could not supply the Ilford emulsion, so that on some occasions it was necessary to use the Amersham product. The difference in quality between the two products was probably due to the fact that

Iford supplied only freshly-prepared emulsion and recommended it be used within two-three months, whereas Amersham often stored their product "on the shelf" for quite some time (months) before sale.

It should be noted that no attempt was made to quantify the levels of mRNA detected in tissue sections by *in situ* hybridisation, other than a subjective "greater than" or "less than" assessment of expression. The reliability of quantification is critically dependent of the reproducibility of the hybridisation technique. Since it is usually possible to examine only three or four tissue sections per microscope slide and each microscope slide represents an individual hybridisation reaction, concurrent analysis of appropriate standards in each hybridisation reaction is technically difficult and demanding. While there have been reports of methods which propose quantitation of *in situ* hybridisation reactions, these techniques have incorporated computer-assisted image analysis to minimise variation in silver grain counting [Nunez et al, 1989; Escot et al, 1991]. This technology was not available for the present study and thus quantification of the *in situ* results was not considered appropriate.

The developmental regulation of renin expression in kidney from WKY was similar to that reported previously for normotensive rats [Gomez et al, 1989] and follows closely the reported pattern of distribution of immunoreactive renin [Gomez et al, 1988a]. In newborn animals, renin mRNA was evident in the intralobular arteries, but in older animals there was a progressive restriction of the sites of renin expression. In the present study it was demonstrated that the developmental regulation of kidney renin expression in hypertensive rats is qualitatively similar to that seen in normotensive rats. Thus the elevated renin mRNA levels that have been reported in 5 week old SHR [Samani et al, 1988] are not likely to be as a result of recruitment of renin-expressing cell types different to that of WKY, but rather due to increased levels of expression in renin-expressing cell types common to both hypertensive and normotensive rats.

The localisation of angiotensinogen and ACE mRNAs in the proximal tubule cells of kidneys from adult WKY and SHR confirms previous studies in normotensive rats which have demonstrated angiotensinogen mRNA [Ingelfinger et al, 1990b] and ACE immunoreactivity [Ikemoto et al, 1990] at this site. AII has been shown to stimulate sodium transport in the proximal tubule epithelium as well as enhance the reabsorption of bicarbonate and acidification of tubule fluid [Harris and Young, 1977; Liu and Cogan, 1987; Liu and Cogan, 1989]. Thus, the demonstration of angiotensinogen and ACE mRNA at this site correlates with some of the intrarenal actions of AII.

It has previously been reported that there is very little angiotensinogen mRNA expressed in the kidneys of prepubertal male rats and that the level of expression of this gene increases dramatically following puberty [Ellison et al, 1989]. The developmental regulation of renal angiotensinogen was confirmed in the present study since angiotensinogen mRNA was not detectable in kidney sections from one and four week old rats (pre-puberty) but was readily demonstrated in adult kidneys. The cellular localisation angiotensinogen expression in kidneys from animals between four weeks of age and adulthood is required to further characterise the developmental regulation of kidney angiotensinogen gene expression.

While the mRNAs for each of the components of the RAS were demonstrated in kidneys from both hypertensive and normotensive adult rats, providing further evidence for an intrarenal RAS, the same cell types were not responsible for the expression of all components. There appeared to be co-localisation of angiotensinogen and ACE expression in the proximal tubule cells, but renin expression was confined to the juxtaglomerular apparatus. It follows that if the RAS is acting in an autocrine or paracrine fashion in the kidney, then, either renin is taken up from the tubular lumen or the interstitial fluid into the tubule cells or there is another enzyme which is responsible for the conversion of angiotensinogen to AI at this site.

Hepatic expression of renin was either absent, or beyond the limit of detection using Northern analysis and *in situ* hybridisation techniques. The absence of renin expression in liver is consistent with the results of some studies using similar, and in some cases, more sensitive techniques [Dzau et al, 1987c; Samani et al, 1987; Suzuki et al, 1988] but is contrary to findings in other studies which clearly demonstrate renin expression in this tissue [Samani et al, 1988; Ekker et al, 1989]. Thus, the expression of hepatic renin remains open to further investigation.

Hepatocytes throughout the liver sections from both WKY and SHR expressed both angiotensinogen and ACE mRNA. Although the level of angiotensinogen gene expression in the liver is reported to decrease with age there was no apparent change in the cellular pattern of expression of this gene in normotensive or hypertensive rats. Hepatocytes surrounding the portal areas appeared to have an increased expression of angiotensinogen mRNA which is a feature consistent with the finding that the majority of circulating angiotensinogen is secreted by the liver [Clauser et al, 1989]. In contrast, the pattern of ACE expression in hepatocytes was more homogenous with only marginally increased expression in the periportal areas.

The major focus of the study described in this chapter was the investigation of the expression of renin, angiotensinogen and ACE in vascular tissue. However, the results of these experiments were often inconsistent due to several reasons. The vascular tissue sections were extremely small and consequently it was very difficult to maintain adherence of the tissue sections to the microscope slides throughout the extensive pre- and post-hybridisation washing procedures. Even though tissue sections were examined in duplicate, it was very rare that sections from the same tissue block were successfully recovered from hybridisation reactions with all of the four probes used. This was particularly frustrating in situations where the "negative control" was lost. Another factor which contributed to the inconsistent results with renin was the low level of gene expression in the blood vessels which necessitated extended exposure in order to detect specifically bound probe. This resulted in an increase in the non-specific

deposition of silver grains in the photographic emulsion so that the negative control vascular sections had a "dirtier" appearance than other tissue sections. It was also unfortunate that for many of the assay runs which examined vascular tissue, the Ilford emulsion was unavailable and the inferior Amersham product was used. Despite these technical difficulties, it was possible to draw some conclusions regarding the expression of the RAS components in vascular tissue.

There has been much debate about the expression of renin mRNA in vascular smooth muscle cells. Renin-like activity has been demonstrated in the medial smooth muscle layer of the aorta, but is dramatically reduced following nephrectomy [Thurston et al, 1979]. Thus it was concluded that the renin is taken up into the smooth muscle cells from the circulation and not synthesised locally. The demonstration of renin mRNA in homogenates of aorta as well as in vascular smooth muscle cells in culture has provided evidence for the local synthesis of this protein [Re et al, 1982; Dzau et al, 1987c; Samani et al, 1987; Metzger et al, 1988; Suzuki et al, 1988]. However, the controversy is still open to debate following the report that renin mRNA was not detected in vascular tissue using the extremely sensitive polymerase chain reaction (PCR) [Ekker et al, 1989]. Even though PCR is an extremely sensitive technique for the detection of mRNA, the sensitivity is entirely dependent on the recovery of undegraded RNA from tissue homogenates. Thus failure to detect a mRNA species using this technique, as for other hybridisation techniques, must be viewed with caution unless supporting evidence is presented that good quality RNA was used for reverse transcription. Extraction of RNA from vascular tissue is technically much more difficult than for other tissues and is more likely to result in the recovery of degraded RNA [Falckh, 1992]. The results from the present study provide little further evidence to settle the debate about the local synthesis of renin in vascular tissue. In both WKY and SHR renin mRNA could be demonstrated in vascular smooth muscle cells, but the results were inconsistent and usually specific binding of the renin cRNA probe was barely above the level of non-specific binding. Thus, the level of renin expression in

vascular smooth muscle cells, if present at all, appears to be extremely low. The combination of *in situ* hybridisation with PCR may provide a suitable technique for the further investigation of renin expression in blood vessels.

Angiotensinogen and ACE mRNA were consistently demonstrated in both the vascular smooth muscle and in the adipose tissue surrounding the mesenteric blood vessels. In addition, as expected, ACE mRNA was demonstrated in the vascular endothelium. The pattern of distribution of mRNA for these two genes was similar in hypertensive and normotensive rats. Angiotensinogen expression is reported to be widespread in many tissues of the rat [Campbell and Habener, 1986] and has been demonstrated in the smooth muscle cells of the aorta from normotensive rats [Naftilan et al, 1991]. In rats treated with oestrogen, thyroxine and dexamethasone, angiotensinogen expression was localised by *in situ* hybridisation to the periaortic fat and not the medial smooth muscle [Campbell and Habener, 1987]. However, it has been suggested [Naftilan et al, 1991] that this drug regimen influences angiotensinogen expression in a region-specific manner such that adipose tissue angiotensinogen expression is increased 12-fold with minimal changes in smooth muscle cell expression. Thus the marked increase in periaortic angiotensinogen expression might favour its detection by *in situ* hybridisation and result in failure to detect the lower levels of expression in the smooth muscle cells of aorta from steroid-treated animals [Naftilan et al, 1991].

While several studies have demonstrated the expression of angiotensinogen in adipose tissue or adipocytes in culture [Campbell and Habener, 1987; Cassis et al, 1988a; Cassis et al, 1988b; Saye et al, 1989; Frederich et al, 1992], the present study is the first to demonstrate ACE mRNA in adipose tissue. The precise function of AII which is generated locally in adipocytes is not known, but the expression of angiotensinogen in adipocytes is reported to be subject to tissue-specific nutritional regulation [Frederich et al, 1992]. The expression of ACE mRNA in adipose tissue will be considered in more detail in a subsequent chapter (*Chapter 8*).

In conclusion, the results reported in this chapter suggest that the developmental regulation of kidney renin and angiotensinogen gene expression is similar in hypertensive and normotensive rats. Furthermore, there appear to be no significant differences in the cellular localisation of RAS components in the kidney, liver or mesenteric vascular bed from hypertensive and normotensive rats. Thus the development of hypertension in the SHR is unlikely to be a consequence of the abnormal recruitment of RAS-expressing cells in any of the tissues studied, although the results do not preclude the possibility that the level of expression of RAS components in these tissues may be a causative factor in the pathogenesis of hypertension.

CHAPTER 4**THE EFFECT OF CHRONIC CAPTOPRIL TREATMENT AND ITS
WITHDRAWAL ON THE GENE EXPRESSION OF THE RAS IN THE SHR**

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4.1 Introduction

Administration of ACE inhibitors to SHRs during the developmental phase of hypertension effectively prevents its occurrence [Bengis et al, 1978; Antonaccio et al, 1979; Ferrone and Antonaccio, 1979]. Furthermore, the withdrawal of ACE inhibitors following chronic treatment in the SHR is associated with a long term suppression of the expected rise in blood pressure [Ferrone and Antonaccio, 1979; Harrap et al, 1986]. This feature is not seen for other antihypertensive drugs including hydralazine and atenolol [Guidicelli et al, 1980; Freslon and Guidicelli, 1983].

It has been suggested that persistent effects on the vasculature are responsible for the sustained lowering of blood pressure following release from ACE inhibitor treatment [Freslon and Guidicelli, 1983; Unger et al, 1985; Harrap et al 1990]. However, as discussed in detail previously (1.5.1), following the withdrawal of ACE inhibitors in the SHR, key indices of vascular change, including those relating to vascular structure, vascular relaxation and vascular contraction, all rapidly return to abnormal levels shortly after the withdrawal of the drugs, despite the fact that blood pressure persists at a lower level [Mulvany, 1991; King et al, 1992; Dyer et al, 1993; Head et al, 1993; Smid et al, 1993].

Short-term (5-7 days) administration of ACE inhibitors is characterised by the presence of an increase in the levels of kidney renin mRNA in both SHR and normotensive rats [Gomez et al, 1988c; Kitami et al, 1989; Tada et al, 1989]. The increased renin expression is associated with a demonstration of immunoreactive renin at vascular sites upstream from the glomerulus [Gomez et al, 1988c]. Short-term ACE inhibition is not associated with changes in the mRNA levels of angiotensinogen in kidney or liver [Gomez et al, 1988c; Campbell et al, 1991]. The effect of chronic administration of an ACE inhibitor and its subsequent withdrawal, on gene expression of the RAS has not been reported. A number of stimuli influence the gene expression of the RAS (1.4.1) and consequently it could be expected that, during chronic ACE inhibition, other

factors might attenuate or alter the effects on RAS gene expression that are observed with short-term ACE inhibition.

The study described in this chapter was designed to explore the hypothesis that chronic ACE inhibition might have a prolonged influence on the gene expression of the components of the RAS in the SHR, distinct from that seen with short-term ACE inhibition, with the effect persisting after the withdrawal of the agent. Accordingly, the levels of mRNA for renin, angiotensinogen and ACE were measured in kidney and liver homogenates from SHR and WKY which had been treated with the ACE inhibitor captopril during the development of hypertension. The expression of the mRNAs for the RAS components was viewed against the blood pressure lowering influence of captopril and particularly the suppression of blood pressure after its withdrawal.

4.2 Methods

4.2.1 Treatment of animals and removal of tissues

Male SHR and WKY were obtained from the CSIRO breeding colony at 4 weeks of age (2.1.1). At 4¹/₂ weeks of age, selected groups of animals including both SHR and WKY, were treated with captopril in their drinking water (100 mg/kg per day) and maintained on the drug therapy until they were 17 weeks of age. Sub-groups of these animals were withdrawn from the drug treatment at 13 weeks of age and subsequently allowed water without captopril until they were 17 weeks of age. Control animals received water without captopril from 4¹/₂ until 17 weeks of age. The number of animals in each treatment group was either 7 or 8. During the treatment period, blood pressure was monitored every two weeks from 5 to 13 weeks of age and weekly thereafter using a tail-cuff procedure as described previously (2.1.2).

At 17 weeks of age, the animals were stunned and decapitated and their kidneys and livers removed (2.1.3). For some animals, kidney and liver segments were placed in

fixative (2.1.3). The remaining kidneys and livers were stored at -80°C until processed for the isolation of RNA, or the measurement of ACE activity.

4.2.2 Biochemical and RNA analyses

Kidneys and livers were homogenised in 50 mmol/l potassium phosphate buffer (pH 8.3) for the determination of ACE activity (2.2.1). ACE activity is expressed as nmol ^{14}C -Hippuryl-L-Histidyl-L-Leucine [Glycine-1- ^{14}C] (HHL) hydrolysed per minute per milligram of protein (2.2.2) for each sample.

Preparation of full-length and hydrolysed probes (2.3.3), isolation of RNA from tissue homogenates (2.3.4), slot analysis (2.3.5), Northern analysis (2.3.6), hybridisation (2.3.7), autoradiography (2.3.8), mRNA quantification (2.3.9) and *in situ* hybridisation (2.3.10) were performed as previously described.

4.2.3 Statistical analyses

Values are expressed as the mean \pm SEM for the number of measurements shown in the results. Statistical significance was determined using analysis of variance. $P < 0.05$ was considered statistically significant.

For graphical representation of the mRNA levels, the relative slopes of the regression lines fitted for each sample were compared with the mean slope of the regression lines calculated for untreated WKY.

4.3 Results

4.3.1 Blood pressure development

The tail cuff blood pressure profiles for each treatment group are shown in *Figure 4.3.1*. Blood pressure increased markedly in the SHR from 5-17 weeks of age. In

contrast, there was only a modest rise in blood pressure in WKY. There was a dramatic suppression of the rise in blood pressure in SHR which were given captopril throughout the experiment. SHR maintained on captopril from 4¹/₂-13 weeks of age and then withdrawn from the drug displayed a modest rise in blood pressure after drug withdrawal, but had a significantly lower blood pressure than their untreated controls throughout the release period. A similar influence of captopril was seen in WKY.

4.3.2 ACE activity

The ACE activity measured in the kidneys from untreated SHR was significantly lower when compared to that from WKY (*Figure 4.3.2a*). In the kidney, captopril treatment from 4¹/₂ to 17 weeks of age led to a dramatic decline in the ACE activity in both WKY and SHR. Four weeks following release from captopril treatment, the ACE activity measured in kidneys from both SHR and WKY was not significantly different from the level seen in kidneys from untreated animals.

The liver ACE activity levels were an order of magnitude or more smaller than those measured in the kidney and were not significantly different between tissues from the WKY and SHR (*Figure 4.3.2b*). In contrast to the kidney, captopril treatment had little effect on the low levels of ACE activity in livers from SHR and WKY.

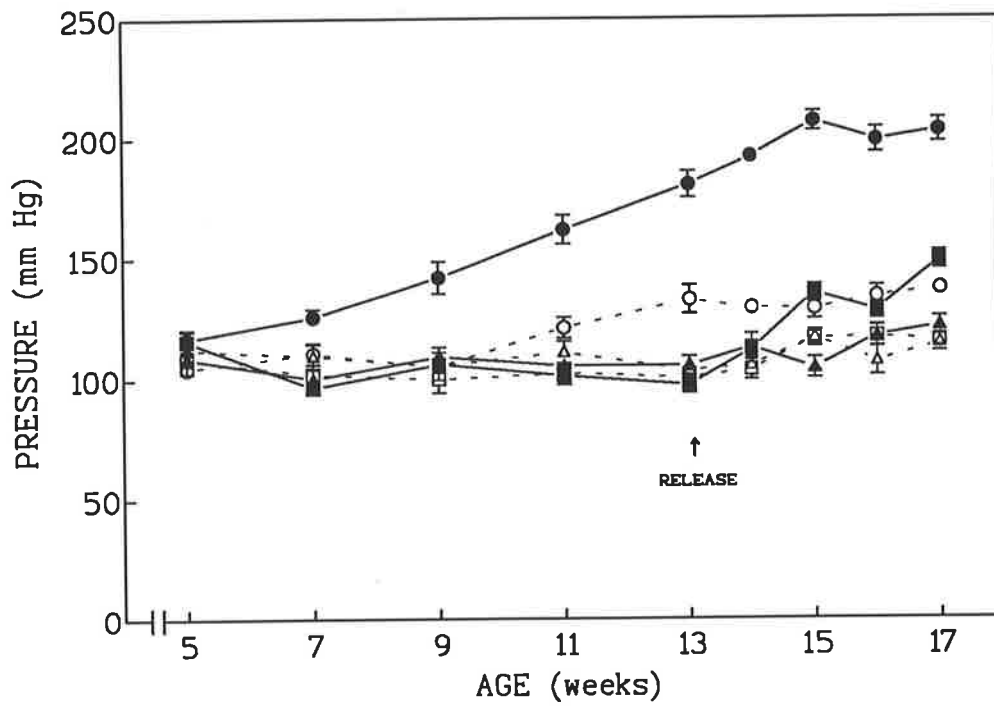


Figure 4.3.1 Tail-cuff blood pressure readings (mean \pm SEM, $n = 7-8$) for animals which had received no drugs (SHR ●; WKY ○), captopril treatment from 4½ to 17 weeks of age (SHR ▲; WKY △), or captopril treatment from 4½ to 13 weeks and then released from the drug for a period of 4 weeks (SHR ■; WKY □)

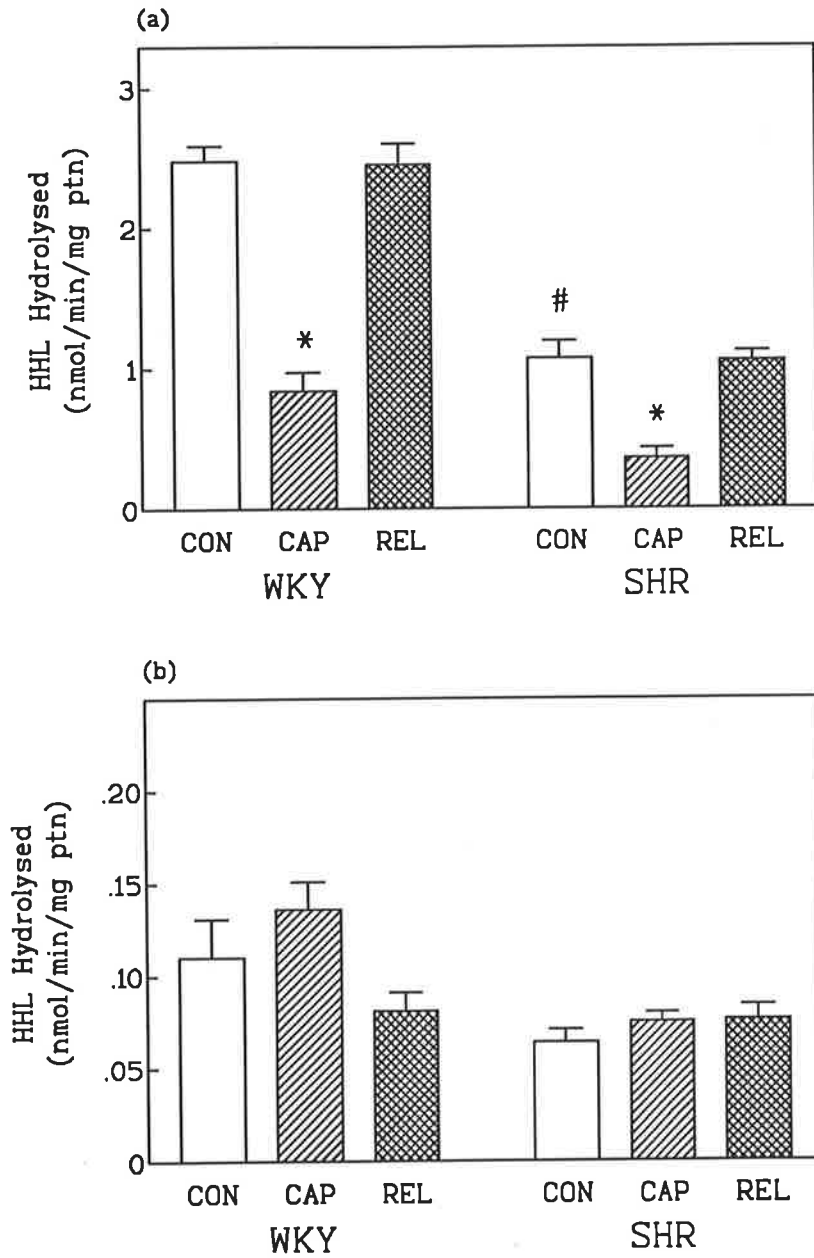


Figure 4.3.2. ACE activity measured in (a) kidneys and (b) livers from control (□), captopril-treated (▨), and captopril-released (▩) WKY and SHR. Shown are the means \pm SEM for $n = 7-8$ observations. # $P < 0.05$ versus WKY control * $P < 0.05$ versus control for each strain.

4.3.3 Slot analyses

The level of kidney renin mRNA was similar in tissues from the SHR when compared with the WKY. Both strains of rat showed a dramatic increase in kidney renin mRNA when the animals were treated chronically with captopril (Figure 4.3.3). The withdrawal of captopril from 13 weeks to 17 weeks of age was associated with a restoration of the lower levels of renin mRNA which were seen in kidneys from untreated animals. Renin mRNA in the livers from both rat strains was below the level of detection .

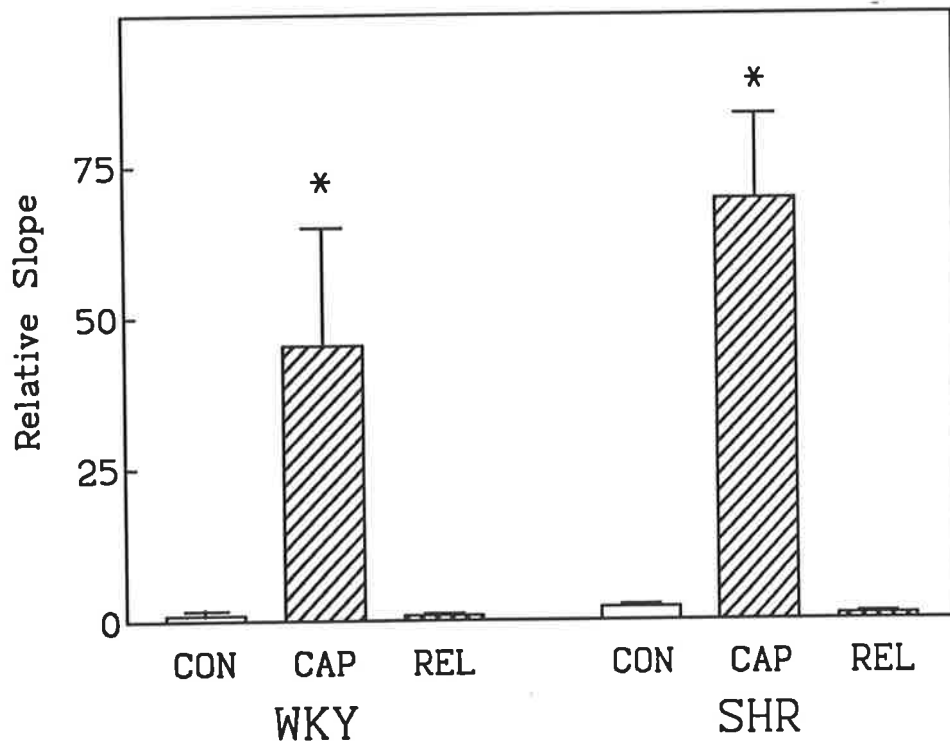


Figure 4.3.3. The kidney renin mRNA levels from control (□), captopril-treated (▨), and captopril-released (▩) WKY and SHR. Shown are the relative slopes of the regression lines fitted for each sample, compared with the mean slope of the regression lines calculated for control WKY. (Means ± SEM for n = 7-8 observations.) * P<0.01 versus control and release for each strain.

Neither captopril treatment nor its release influenced the levels of angiotensinogen mRNA expression in kidney or liver from both rat strains (*Figure 4.3.4*). There was however a tendency for the levels of angiotensinogen mRNA in kidneys from the SHR to be somewhat lower than the levels present in the kidney from the WKY but this difference did not achieve statistical significance.

The levels of ACE mRNA were similar in untreated rats of both strains for both kidney and liver (*Figure 4.3.5*). Kidney ACE mRNA expression was not influenced either by captopril treatment or its withdrawal (*Figure 4.3.5a*). However, captopril treatment caused a significant rise in liver ACE gene expression which was not reversed four weeks following release from the drug (*Figure 4.3.5b*).

4.3.4 In situ hybridisation

In situ hybridisation was used to identify the cells which produced renin mRNA in the kidney in this study. No strain differences were observed in the localisation of expression of either gene and the effect of captopril on the pattern of expression was similar for both SHR and WKY. In kidneys from animals which had received no drug treatment, renin expression was localised to the area of the juxtaglomerular apparatus (*Figure 4.3.6a*). However, there was recruitment of vascular sites of renin expression in kidneys from captopril-treated animals (*Figure 4.3.6b*). In the kidneys from 17 week old animals which had been released from captopril treatment at 13 weeks of age the localisation of renin expression was similar to that seen in untreated animals (*Figure 4.3.6c*). In particular, there was no evidence of continued expression in extra-glomerular vascular sites.

Captopril treatment, or its release, did not influence the localisation of gene expression for angiotensinogen in kidney and liver or ACE in kidney and liver (not shown).

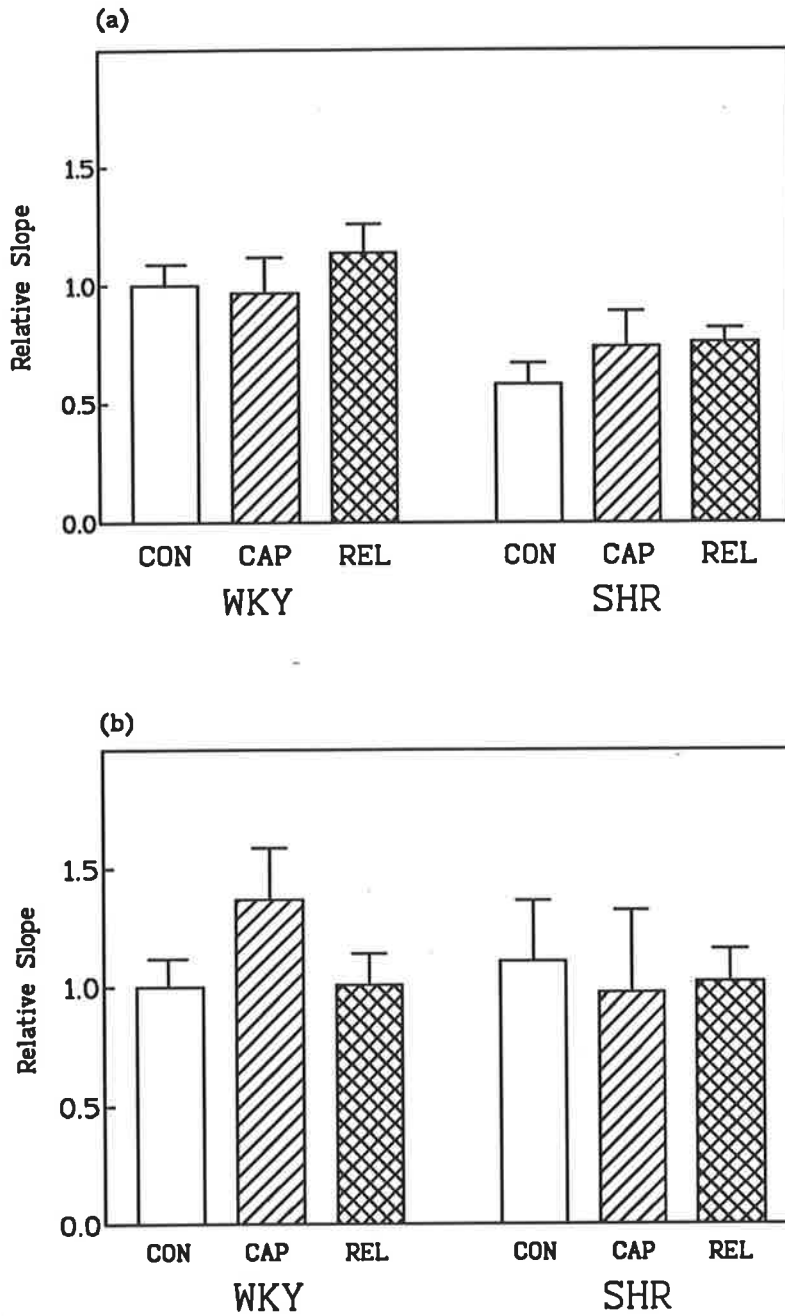


Figure 4.3.4 Angiotensinogen mRNA levels measured in (a) kidneys and (b) livers from control (□), captopril-treated (▨), and captopril-released (▩) WKY and SHR. The data are presented as described in the legend to *Figure 4.3.4*. Shown are the means \pm SEM for $n = 5-8$ observations.

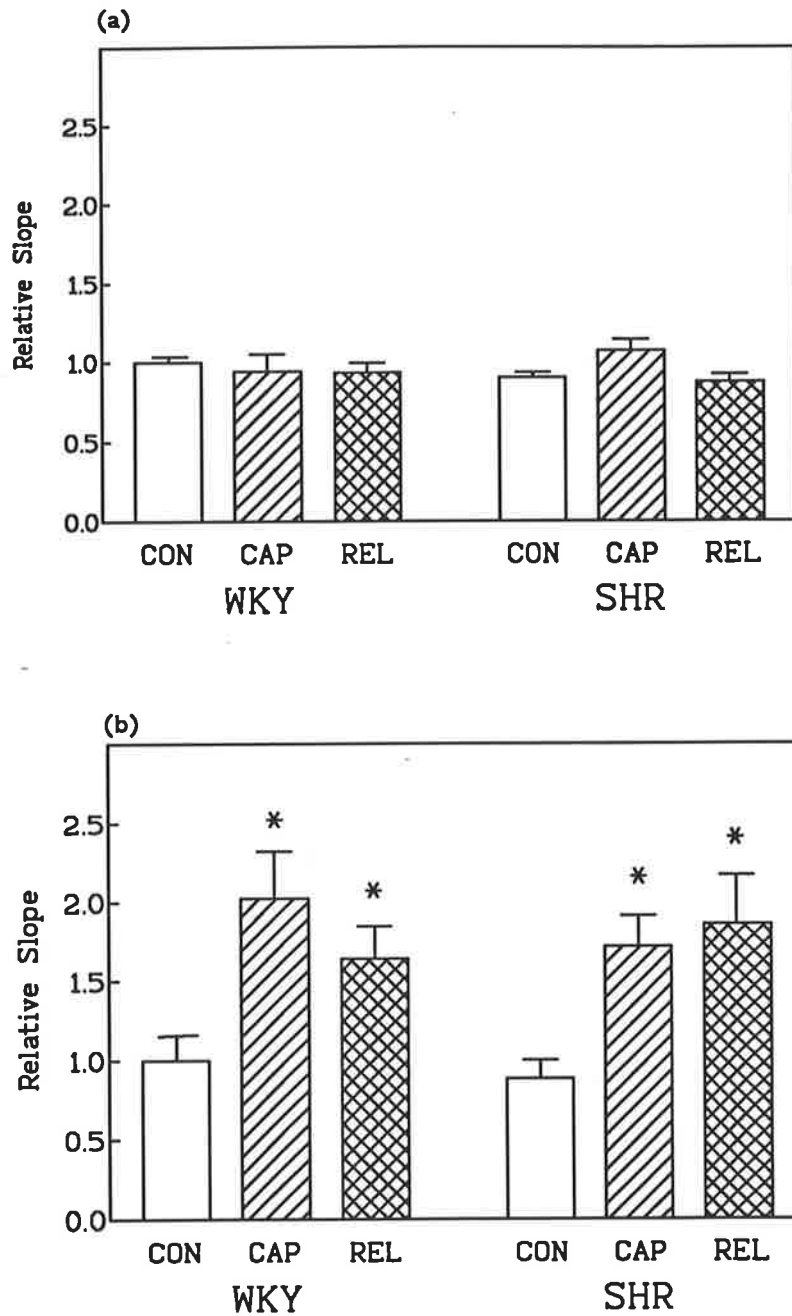
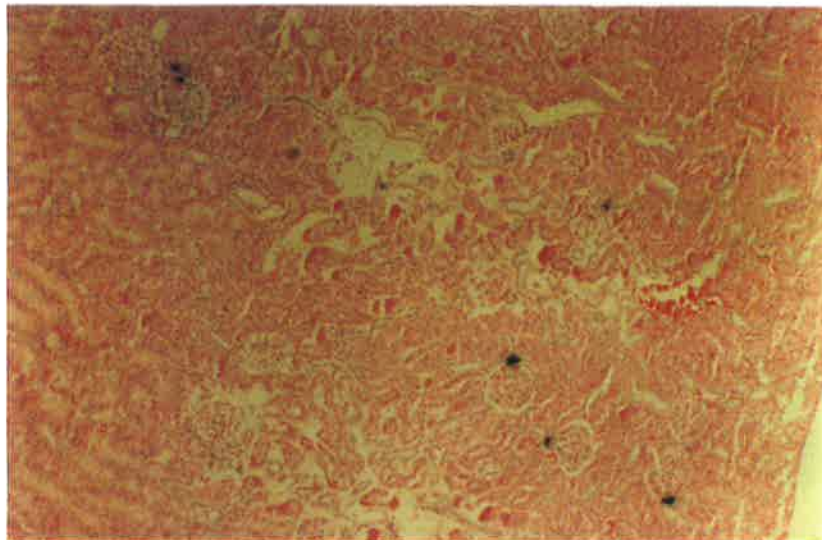


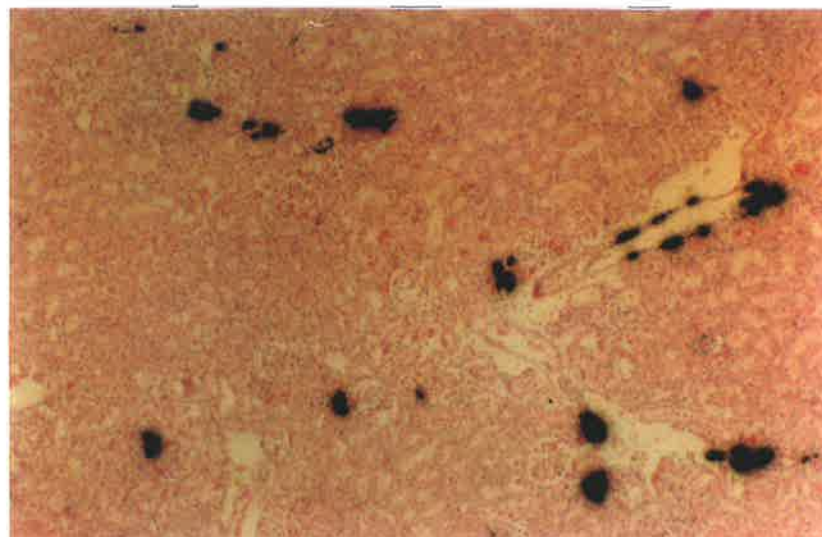
Figure 4.3.5 ACE mRNA levels measured in (a) kidneys and (b) livers from control (□), captopril-treated (▨), and captopril-released (▩) WKY and SHR. The data is presented as described in the legend to Figure 4.3.5. Shown are the means \pm SEM for $n = 5-8$ observations. * $P < 0.05$ versus control for each strain.

Figure 4.3.6 *In situ* hybridisation pattern for renin mRNA seen in kidney from (a) control, (b) captopril-treated and (c) captopril-released SHR. Note the localisation of renin mRNA in the area of the juxtaglomerular apparatus in kidneys from control and captopril-released animals. In kidneys from captopril-treated animals, the signal for renin mRNA can be seen along the length of the afferent arteriole and in the intralobular artery. (Magnification X40)

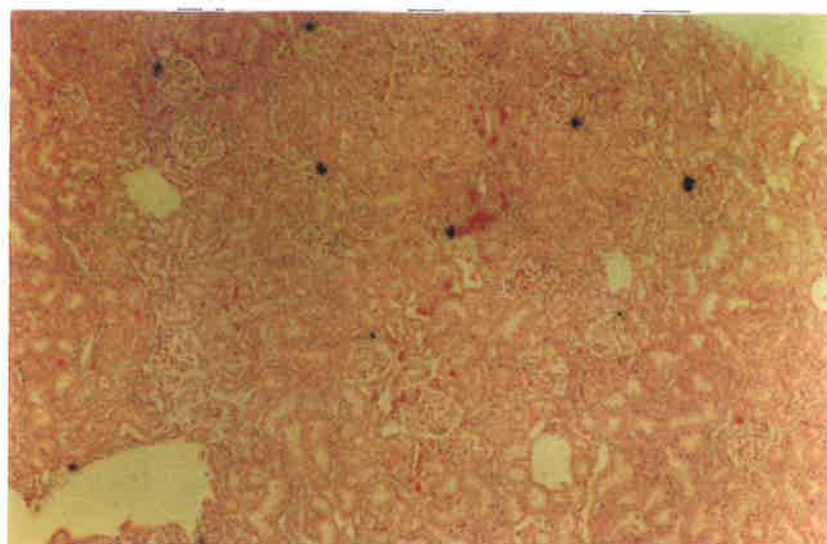
(a)



(b)



(c)



4.4. Discussion

The results presented in this chapter confirm the findings of previous studies which have shown that captopril and other ACE inhibitors exert a profound effect in preventing the development of elevated blood pressure in the SHR and that blood pressure remains low even when the agent is withdrawn [Bengis et al, 1978; Antonaccio et al, 1979; Guidicelli et al, 1980; Freslon and Guidicelli, 1983; Harrap et al, 1990; King et al, 1992].

The antihypertensive effect of ACE inhibitors correlates with tissue levels of inhibition but poorly with plasma inhibition. Following a single dose of ACE inhibitor, aortic and kidney but not plasma ACE activity remains suppressed [Cohen and Kurz, 1982; Unger et al, 1984]. Therefore it might be suggested that the prolonged suppression of blood pressure following withdrawal from ACE inhibitor treatment may be associated with an accumulation of the agent in tissues and a continued suppression of ACE activity in them [Freslon and Guidicelli, 1983; Unger et al, 1985; Dzau, 1987]. In the present study, the ACE activity in kidneys from SHR and WKY treated with captopril was significantly reduced. However, four weeks following release from treatment, ACE activity had returned to levels that were not different from untreated controls. Thus, a continued suppression of kidney ACE activity is not likely to be the mechanism responsible for the continued suppression of blood pressure following release from captopril. It is of interest to note that the kidney ACE activity in the hypertensive animals was lower than that seen in kidneys of the normotensive WKY rats. This has been reported previously [Welsch et al, 1987; Grima et al, 1990].

In contrast to the kidney, there was no significant inhibition of ACE activity in livers from captopril-treated animals. However, it should be noted that the levels of hepatic ACE activity were extremely low and only marginally above the limit of sensitivity of the assay. Therefore it was not surprising that inhibition of activity could not be demonstrated in this tissue.

The effect of short term treatment with ACE inhibitors on renin gene expression has been well documented. In kidneys from normotensive rats, renin mRNA levels increase 5-fold, 10-fold and 20-fold following 5, 7 and 10 days of ACE inhibitor treatment respectively [Gomez et al, 1988c; Kitami et al, 1989; Tada et al, 1989]. The effect of ACE inhibition on kidney renin gene expression is reportedly enhanced in the SHR following 10 days of treatment [Kitami et al, 1989]. While there was a trend for the kidney renin mRNA response to be greater in the SHR following prolonged treatment in the present study, this did not reach statistical significance. Since renin gene expression is subject to a number of different regulatory influences, it could be expected that during prolonged ACE inhibition the increase in renin gene expression might be attenuated. In the present study, animals were administered captopril for a period of 12 weeks. In both SHR and WKY there was no diminution of the increase in kidney renin gene expression at the conclusion of this extended treatment period. Instead, the relative levels of renin mRNA, when compared with untreated controls, appeared to be even greater than seen under short-term ACE inhibitor administration. In the previously published studies which were described above the kidney renin mRNA response to ACE inhibition appeared to increase with the length of the treatment period and thus the results of the present study confirm and extend the observed trend. This enormous increase in renin expression must represent a significant commitment of energy and resources by the kidney to the maintenance of AII levels and therefore highlights the importance of this peptide in renal physiology.

The ACE inhibitor-induced increase in renin mRNA levels in kidney is associated with the demonstration of immunoreactive renin at vascular sites upstream from the glomerulus [Gomez et al, 1988c]. In untreated animals, immunoreactive renin is not seen at vascular sites other than the juxtaglomerular apparatus. In the present study, the cells responsible for renin mRNA expression were localised using the technique of *in situ* hybridisation. As previously demonstrated (3.4) the sites of renin expression in kidneys from adult untreated SHR and WKY are similar and restricted to the

juxtaglomerular apparatus. The pattern of expression in response to captopril was also similar in the two rat strains. Renin mRNA was demonstrated in the intralobular arteries as well as in extended lengths of the afferent arterioles in both SHR and WKY. Thus, the demonstration of immunoreactive renin at vascular sites upstream from the juxtaglomerular apparatus following short-term ACE inhibition [Gomez et al, 1988c] was likely to be as a result of local synthesis at those sites and not due to uptake of renin from the circulation. Importantly, the pattern of kidney renin expression four weeks following release from captopril treatment was once again similar to that seen in untreated controls.

The levels of angiotensinogen mRNA in kidney and liver were not changed by prolonged captopril treatment or its release. This finding is consistent with previous studies which have demonstrated that short-term ACE inhibitor administration also does not influence angiotensinogen gene expression [Gomez et al, 1988c; Campbell et al, 1991]. Since AII is reported to increase angiotensinogen mRNA levels in kidney and liver [Klett et al, 1988a], it would be expected that under conditions of converting enzyme inhibition angiotensinogen mRNA levels might be decreased. Two hypotheses can be proposed to explain this apparently contradictory finding. While the plasma levels of AII decrease significantly following acute ACE inhibitor administration [Mento and Wilkes, 1987], chronic administration is associated with normal (or even elevated) plasma AII levels [Mento and Wilkes, 1987; Mento et al, 1989; Campbell et al, 1991]. However, tissue levels of AII appear to be differentially regulated by ACE inhibition since the kidney levels of AII are significantly reduced [Campbell et al, 1991]. Therefore, it might be proposed that the influence of AII on angiotensinogen gene expression may depend on circulating and not local levels of AII. In contrast, since kidney renin gene expression is profoundly influenced by ACE inhibition, this hypothesis would argue for an influence of local rather than circulating AII levels in the regulation of renin gene expression. A second more likely explanation for the lack of effect of ACE inhibition on angiotensinogen gene expression relates to the mechanism

by which AII stimulates angiotensinogen mRNA levels. The effect of AII on angiotensinogen expression is due to the ability of AII to stabilise the angiotensinogen message and thus extend its lifetime [Klett et al, 1988b]. Under basal conditions, the message would retain an inherent stability, so that even when local AII levels are decreased, the stability and lifespan of the message would not be decreased and therefore changes in the level of angiotensinogen mRNA would not be detected.

In the present study the levels of kidney ACE mRNA were also not influenced by captopril treatment or its release. ACE gene expression in lung but not testis is reported to be subject to negative feedback control by AII and ACE inhibition is associated with an increase in ACE mRNA levels [Schunkert et al, 1993]. Further evidence for the tissue specific regulation of ACE expression comes from studies which examined the level of ACE protein following ACE inhibition. Total ACE protein is increased in lung but not kidney or aorta following chronic short-term administration of ACE inhibitors [Fyhrquist et al, 1980; Chai et al, 1992]. Thus the results of the present study provide further evidence that ACE is not induced in kidney following ACE inhibition.

The most significant influence of captopril treatment and release on the liver occurred with respect to ACE mRNA levels. Captopril treatment in both the WKY and SHR was associated with a marked increase in liver ACE mRNA levels. Furthermore, following release from chronic captopril treatment, these elevated levels did not return to the levels seen in the control animals even though during the release period, the blood pressure in the SHRs was still very much lower than that seen in untreated SHRs. This finding may be viewed in two ways. It is tempting to speculate that the lowered blood pressure achieved by chronic captopril treatment and sustained after the withdrawal of captopril may well be a consequence of the increase in ACE mRNA in the liver. This hypothesis would require that the protein product of the elevated hepatic ACE mRNA be involved in the metabolism of a substrate which subsequently exerts a hypotensive effect in captopril-treated and released animals. Such a substrate of ACE

has not yet been identified. Alternatively it could be argued that the increase in hepatic ACE mRNA is a consequence of the lowered blood pressure and represents an attempt by the liver to restore blood pressure to "normal" levels. While such a role for the liver in cardiovascular homeostasis may be considered unlikely, it should be remembered that the liver is responsible for the synthesis and secretion of the precursor for one of the most potent known vasoconstrictor agents - i.e. angiotensinogen.

A possible criticism of the present study might be that during quantification of the levels of the mRNA for the RAS components, the autoradiographic signals were not standardised to the signal of a "housekeeping" gene to control for amounts of RNA loaded to each well or slot. A commonly used "house keeping" gene is β -actin. Under most circumstances the expression of β -actin does not vary significantly and it is commonly used as an unregulated RNA. However, the concept of "housekeeping" genes must be reconsidered. In circumstances where the levels of AII are variable, the levels of β -actin can be altered significantly [Turla et al, 1991; Nishimura et al, 1992; King, 1993]. Another commonly used "housekeeping" gene is β_2 -microglobulin, although in our hands and others [Robertson, 1993] the mRNA levels for this gene also change under pathological and physiological conditions. In the light of these findings, it was decided that standardisation of autoradiographic signals to the signal of a "housekeeping" gene would not be appropriate.

An additional important feature became apparent with respect to the expression of ACE and its activity in liver. When comparing the levels of ACE mRNA with the activity of ACE in the kidney and the liver, it was noted that in the liver there is a relatively large expression of ACE mRNA, with a poor ACE activity in the tissue. The expression and activity of hepatic ACE will be considered in more detail in a subsequent chapter (*Chapter 7*).

In conclusion, the results of the present study highlight a role for angiotensin converting enzyme inhibition in elevating kidney renin mRNA levels as well as

elevating liver levels of ACE mRNA. During the period of drug withdrawal when blood pressure is still significantly lower than in untreated SHR, the kidney renin gene expression returns rapidly to normal, whereas the liver ACE gene expression remains elevated. Collectively the results suggest that the long-term blood pressure suppressing effects of the ACE inhibitors may not be related to the activities of kidney renin, but may be influenced in some way by the activities and levels of liver ACE mRNA. It may also be possible that the mechanisms responsible for the lowering of blood pressure during the administration of ACE inhibitors are distinct from the mechanisms responsible for the continued suppression of blood pressure following withdrawal of these agents.

CHAPTER 5**THE EFFECT OF NEONATAL SYMPATHECTOMY ON THE RAS IN
ADULT SHR**

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5.1 Introduction

The enhanced activity of the sympathetic nervous system (SNS) contributes to both the initiation and maintenance of hypertension in the SHR [Provoost and De Jonge, 1978; Head, 1989]. In the rat, development of the peripheral nervous system occurs in the first 2-3 weeks following birth. In the SHR, some blood vessels are hyperinnervated when compared with the WKY and this increased innervation occurs immediately after birth [Donohue et al, 1988].

The level of sympathetic innervation in blood vessels (and other tissues) is influenced by the trophic peptide nerve growth factor (NGF), which is elevated in young SHR as is its gene expression [Donohue et al, 1989; Falckh et al, 1992]. Administration of antibodies to NGF in combination with the adrenergic neurone blocking agent guanethidine to neonatal SHR disrupts the development of the peripheral SNS and also prevents the development of hypertension [Lee et al, 1987]. Chemical denervation of neonate SHR with 6-hydroxydopamine (OHDA) also attenuates the development of hypertension in these animals [Yamori et al, 1972; Provoost and De Jonge, 1978; Bevan and Tsuru, 1981].

Treatment of neonatal SHR with OHDA has been shown to cause a significant reduction in the basal PRA levels, although the release of renin in response to the hypotensive stress caused by hydralazine was not affected [Sinaiko et al, 1980]. The mechanism responsible for the decrease in basal PRA has not been investigated but it is likely that the removal of sympathetic nerves may have some effect on the expression of the renin gene in the kidney.

There are many possible sites of interaction between the SNS and the RAS (1.6.2) and thus the expression of the RAS in tissues other than kidney may also be influenced by neonatal sympathectomy. The mesenteric vascular bed, which contributes greatly to the total peripheral resistance is hyperinnervated in the SHR [Head, 1989]. The response of the rat mesenteric vascular bed to sympathetic nerve stimulation is

potentiated by the presence of renin substrate, which suggests that the sympathetic nerves may stimulate the local production of AII [Weishaar et al, 1991]. This hypothesis is supported by evidence that nerve stimulation augments the local overflow of AII from canine gracilis muscle [Schweiler et al, 1991]. Therefore, disruption of the vascular RAS may contribute to the attenuation of the development of hypertension following neonatal sympathectomy.

The study described in this chapter was designed to investigate the hypothesis that neonatal sympathectomy might have a prolonged effect on the RAS in SHR and WKY. Accordingly, rat pups were treated with OHDA and the development of blood pressure was subsequently monitored until the animals were 16 weeks old, at which time, the gene expression of the RAS components in kidney, liver and mesenteric artery (and its branches) as well as the ACE activity in several tissues was examined

5.2 Methods

5.2.1 Treatment of animals and removal of tissues

Neonate SHR and WKY (1 or 2 days old) were injected intraperitoneally with 6-hydroxydopamine (OHDA, 100 mg/kg). The injection was repeated on six occasions every 2-3 days until the rats were 13 or 14 days of age. In each litter, half the males were injected with OHDA, while the remaining males were injected with ascorbic saline. Ascorbic saline-treated rats had their right front toe clipped for identification. OHDA (20 mg/ml) was prepared in ascorbic saline (ascorbic acid 50 mg/L in sterile 9% sodium chloride) immediately prior to injection. The injection volume was 5 μ l per gram body weight and insulin "Lo-dose" syringes (0.5 ml) were used for injection. The number of animals in each treatment group was 13 (SHR control and SHR OHDA) or 15 (WKY control and WKY OHDA). Following the treatment period, blood pressure



was monitored every two weeks from 4 to 16 weeks of age using a tail-cuff procedure as described previously (2.1.2).

At 16 weeks of age, the animals were stunned and decapitated and kidney, liver, lung, caudal artery, aorta, whole brain, mesenteric artery and its branches (from which adhering fat was removed) and skeletal muscle (from the posterior aspect of the hind limb) were removed and rinsed in ice-cold saline (2.1.3). Blood was collected from the decapitated rats into heparinised tubes. Plasma was removed following centrifugation of the blood at 3000g for 15 minutes at room temperature and the plasma stored at -20 °C until analysis. The tissues included kidney, liver, lung, caudal artery, aorta, whole brain, skeletal muscle (from the posterior aspect of the hind limb) and mesenteric artery and branches (from which adhering fat was removed). The tissues were stored at -80°C until processed for the isolation of RNA (kidney, liver and mesenteric artery), or the measurement of ACE activity (kidney, liver, aorta, lung, skeletal muscle and brain). Some tissues (kidney, liver, lung, caudal artery, skeletal muscle and brain) were weighed prior to being placed in ice-cold 0.3M perchloric acid containing 0.5 mM magnesium chloride and 5 mM EGTA. Following overnight extraction at 4°C, the perchloric acid tissue extracts were stored at -80°C until processed for the determination of NA.

5.2.2 Biochemical and RNA analyses

The NA content of perchloric acid tissue extracts was determined as described previously (2.2.3). The NA concentration is expressed as ng or µg per gram of tissue.

Tissues were homogenised in 50 mM potassium phosphate buffer (pH 8.3) for the determination of ACE activity (2.2.1). ACE activity is expressed as nmol ¹⁴C-Hippuryl-L-Histidyl-L-Leucine [Glycine-1-¹⁴C] (HHL) hydrolysed per minute per millilitre of plasma or per milligram of protein (2.2.2) for each tissue sample.

Preparation of full-length probes (2.3.3), isolation of RNA from tissue homogenates (2.3.4), slot analysis (2.3.5), hybridisation (2.3.7), autoradiography (2.3.8), and mRNA quantification (2.3.9) were performed as previously described.

Although the number of animals in each treatment group was either 13 or 15, biochemical and RNA analyses were not performed on all tissues from each animal. For NA determination, the number of tissues analysed from each treatment group was 9 (except for brain where $n=6$). For ACE activity assessment, the number of tissues from each treatment group which were analysed was either 6 or 7. The assignment of the brain of each animal to either the determination of NA concentration or the assessment of ACE activity was decided randomly. RNA from 6 or 7 kidneys and livers from each treatment group was subjected to slot analysis. Due to the small tissue size of the mesenteric artery, samples from 2-3 animals (randomly selected within each treatment group) were pooled prior to the extraction of RNA. This provided an n of 5 for each treatment group.

4.2.3 Statistical analyses

Values are expressed as the mean \pm SEM for the number of measurements shown in the results. Statistical significance was determined using analysis of variance. $P < 0.05$ was considered statistically significant.

For graphical representation of the mRNA levels, the relative slopes of the regression lines fitted for each sample were compared with the mean slope of the regression lines calculated for untreated WKY.

6.3 Results

6.3.1 Blood pressure development

The tail cuff blood pressure profiles for each treatment group are shown in *Figure 5.3.1*. The mean blood pressures of SHRs which had been treated with OHDA at birth

were significantly lower, at 8 weeks of age and older, than control SHR treated with ascorbic saline. In WKY, while there was a trend for the tail cuff blood pressures of rats treated with OHDA to be lower than the ascorbic saline-treated controls; however, the differences failed to reach statistical significance at all time points except 14 weeks of age.

6.3.2 Tissue NA concentration

The determination of the NA concentrations in tissues from control SHR and WKY provided evidence for the presence of hypernoradrenergic innervation in the SHR (*Figure 5.3.2*). The NA concentration in caudal artery and skeletal muscle was significantly higher in control SHR when compared with control WKY (*Figure 5.3.2 (d) & (e)*). In kidney and lung the NA concentration tended to be higher in SHR, but the differences failed to reach statistical significance (*Figure 5.3.2 (a) & (c)*).

Neonatal administration of OHDA resulted in a profound and widespread peripheral sympathectomy in adult SHR and WKY. In all peripheral tissues examined, including kidney, liver, lung, caudal artery and skeletal muscle, the NA concentration in perchloric acid tissue extracts was significantly lower in OHDA-treated animals when compared with the NA concentration in tissues from control animals (*Figure 5.3.2 (a)-(e)*). In contrast, there was no significant alteration in brain NA concentration in rats from both strains which had been treated with OHDA at birth when compared with ascorbic saline treated-controls (*Figure 5.3.2 (f)*).

5.3.3 ACE activity

In the SHR, ACE activity was significantly lower in plasma and kidney (*Figure 5.3.3 (a) and (b)*) and tended to be lower in liver and lung (*Figure 5.3.3 (c) and (e)*).

However, this lower level of ACE activity was not universal since in aorta (*Figure 5.3.3 (d)*) skeletal muscle (*Figure 5.3.3 (f)*) and brain (*Figure 5.3.3 (g)*) there was either a similar level or a trend for increased ACE activity in the SHR. Neonatal sympathectomy did not influence the ACE activity in either the peripheral tissues or the brain.

5.3.4 Slot analysis

The gene expression of the RAS components was examined in kidney, liver and mesenteric artery from control and OHDA-treated animals. In the kidney (*Figure 5.3.4*) and the liver (*Figure 5.3.5*) the levels of mRNA for each of the RAS components was not significantly different when control SHR was compared with control WKY. In both rat strains, the RAS mRNA levels in adult animals were not influenced by neonatal treatment with OHDA (*Figure 5.3.4, Figure 5.3.5*).

The expression of renin and angiotensinogen in mesenteric artery was below the limit of detection using the slot technique. When ACE gene expression in the mesenteric artery and branches was examined by slot analysis, it was found that the level of ACE mRNA was significantly higher in mesenteric arteries and its from the SHR (*Figure 5.3.6*). ACE gene expression in the mesenteric artery from adult SHR and WKY was not significantly influenced by neonatal treatment with OHDA (*Figure 5.3.6*).

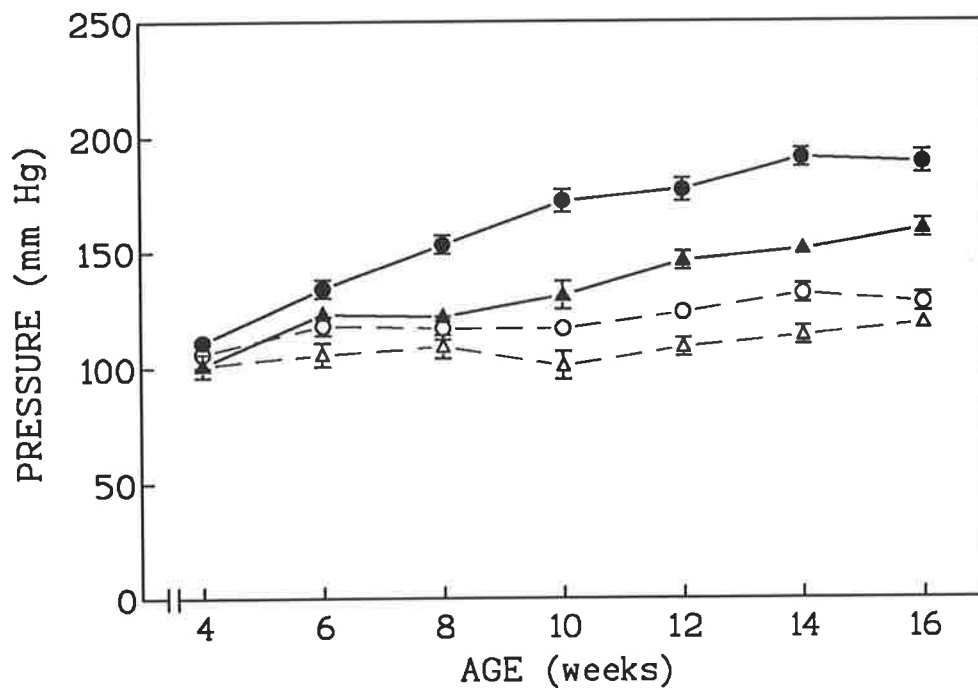


Figure 5.3.1 Tail-cuff blood pressure readings (mean \pm SEM, $n = 13-15$) for animals which had received ascorbic saline (SHR ●; WKY ○) or OHDA (100 mg/kg, SHR ▲; WKY △) on 6 occasions between 1 and 14 days of age.

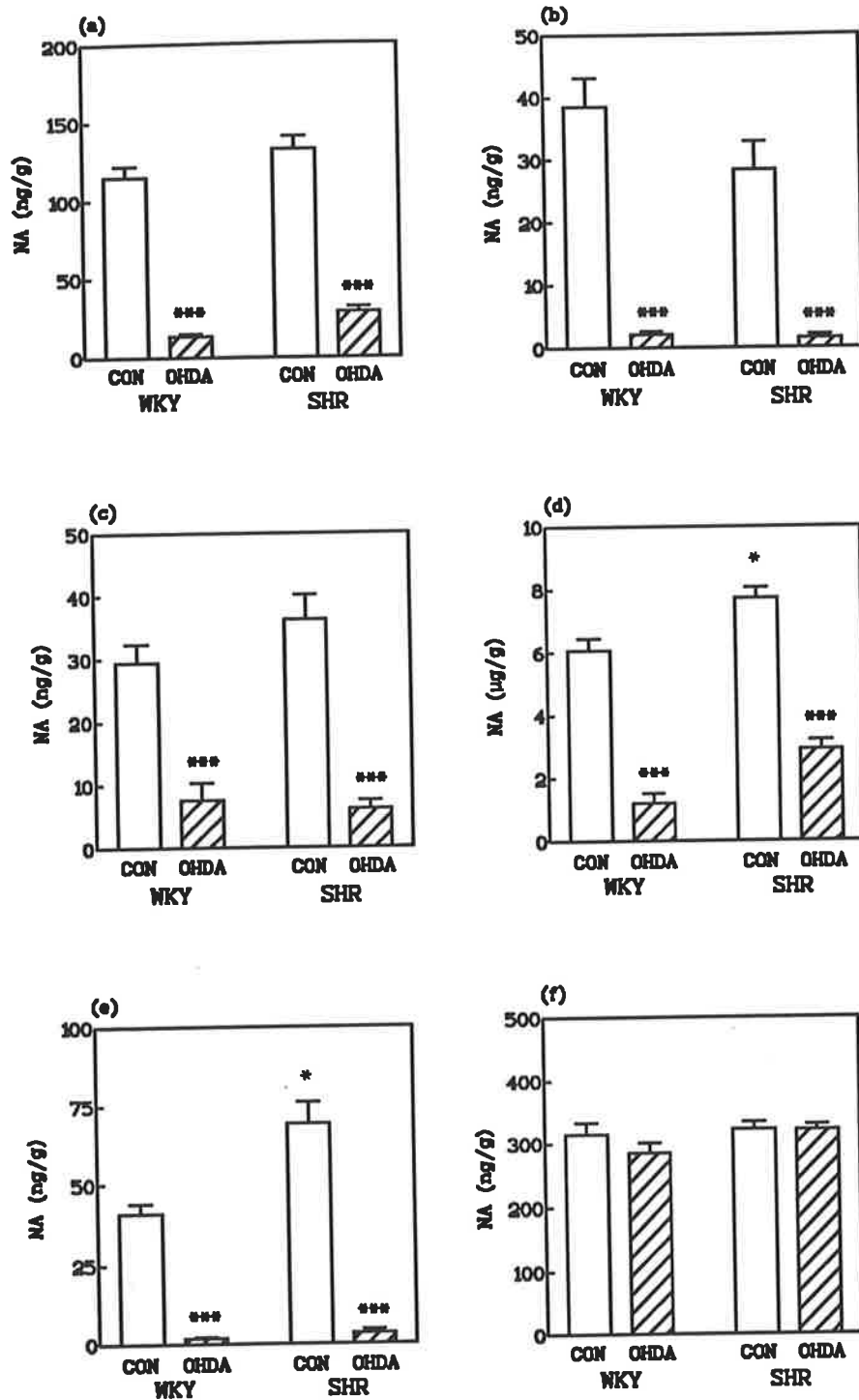


Figure 5.3.2 Tissue NA concentration (ng or µg/g) measured in (a) kidney, (b) liver, (c) lung, (d) caudal artery, (e) skeletal muscle and (f) brain from control (□) and OHDA-treated (▨) WKY and SHR. Shown are the means ± SEM for n = 6 (brain) or 9 observations. * SHR control significantly different from WKY control (P<0.05). *** OHDA significantly different from control for each rat strain (P<0.001).

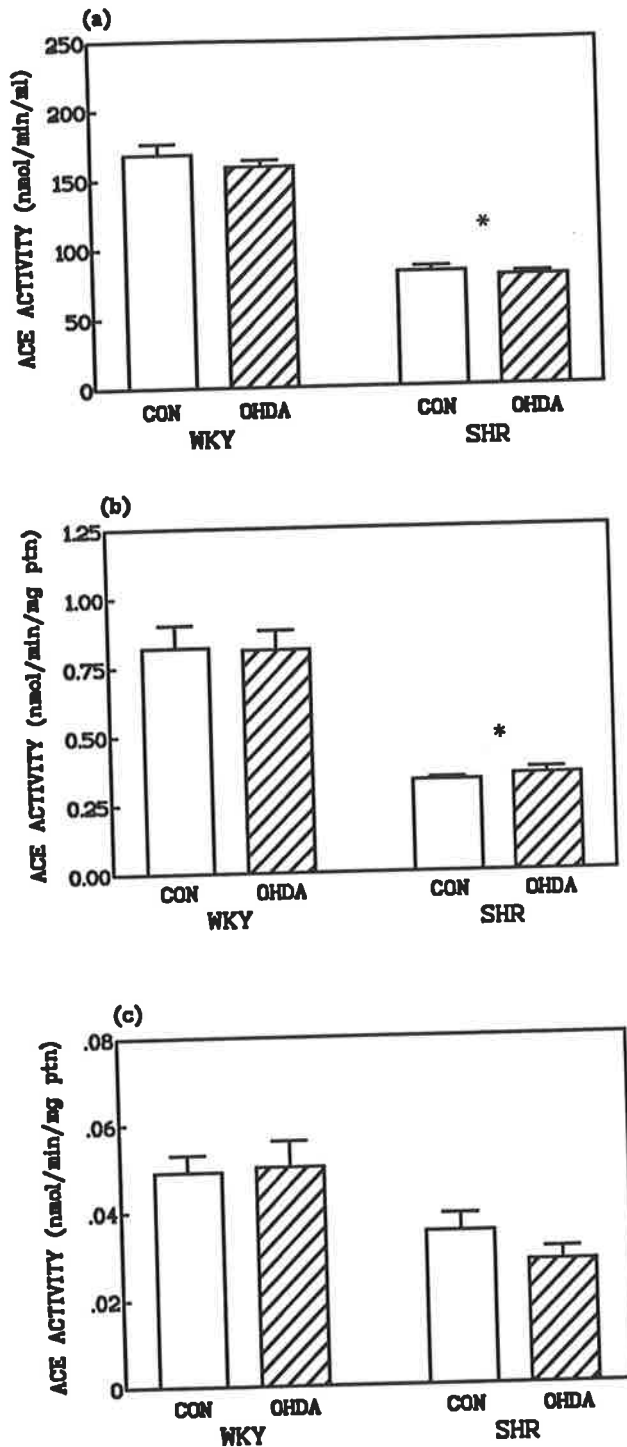
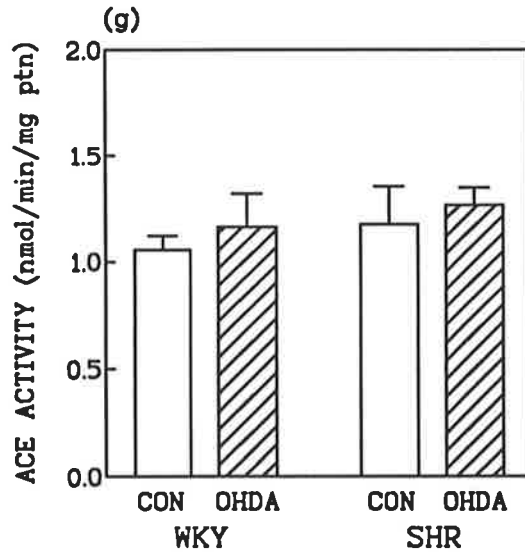
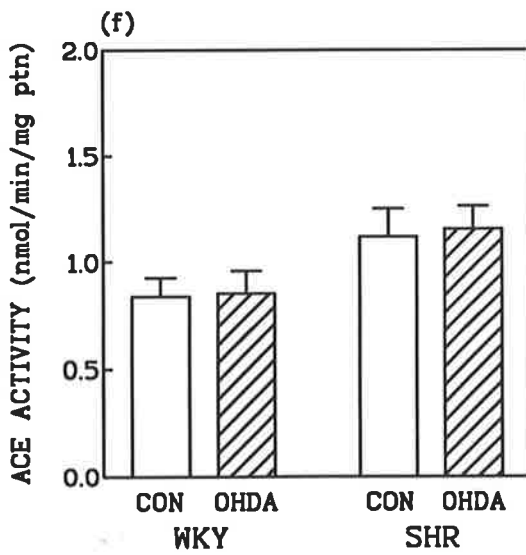
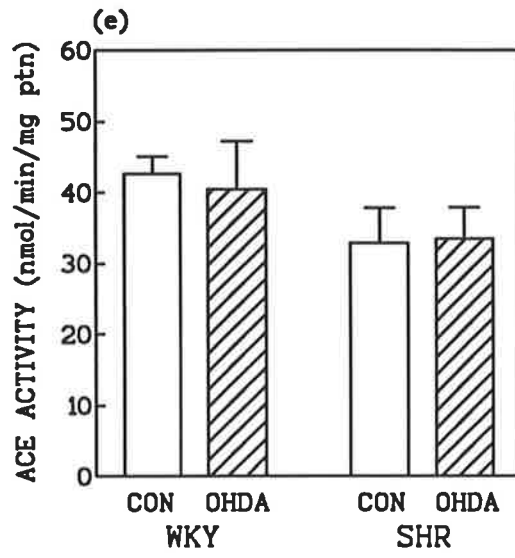
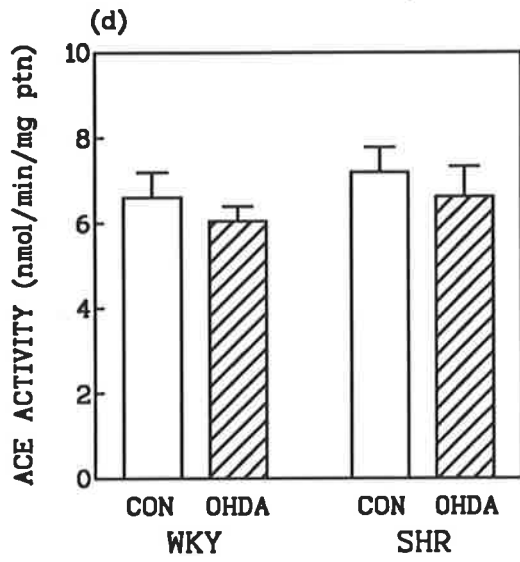


Figure 5.3.3 ACE activity (nmol/min/ml or nmol/min/mg protein) measured in (a) plasma, (b) kidney, (c) liver, (d) aorta, (e) lung, (f) skeletal muscle and (g) brain from control (□) and OHDA-treated (▨) WKY and SHR. Shown are the means \pm SEM for $n = 6$ or 7 observations. * SHR (both control and OHDA) significantly different from WKY (both control and OHDA) ($P < 0.05$).



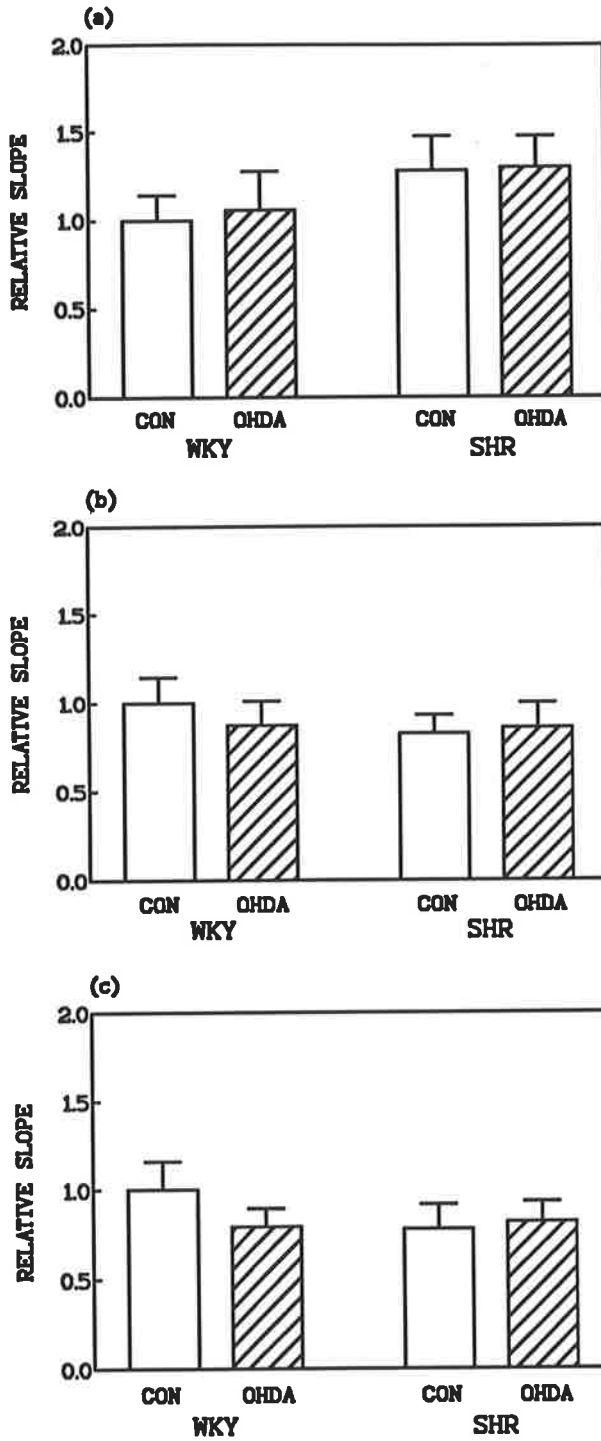


Figure 5.3.4. The mRNA levels for (a) renin, (b) angiotensinogen and (c) ACE in kidneys from control (□) and OHDA-treated (▨) WKY and SHR. Shown are the relative slopes of the regression lines fitted for each sample, compared with the mean slope of the regression lines calculated for control WKY. (Means \pm SEM for $n = 6$ or 7 observations in each case.)

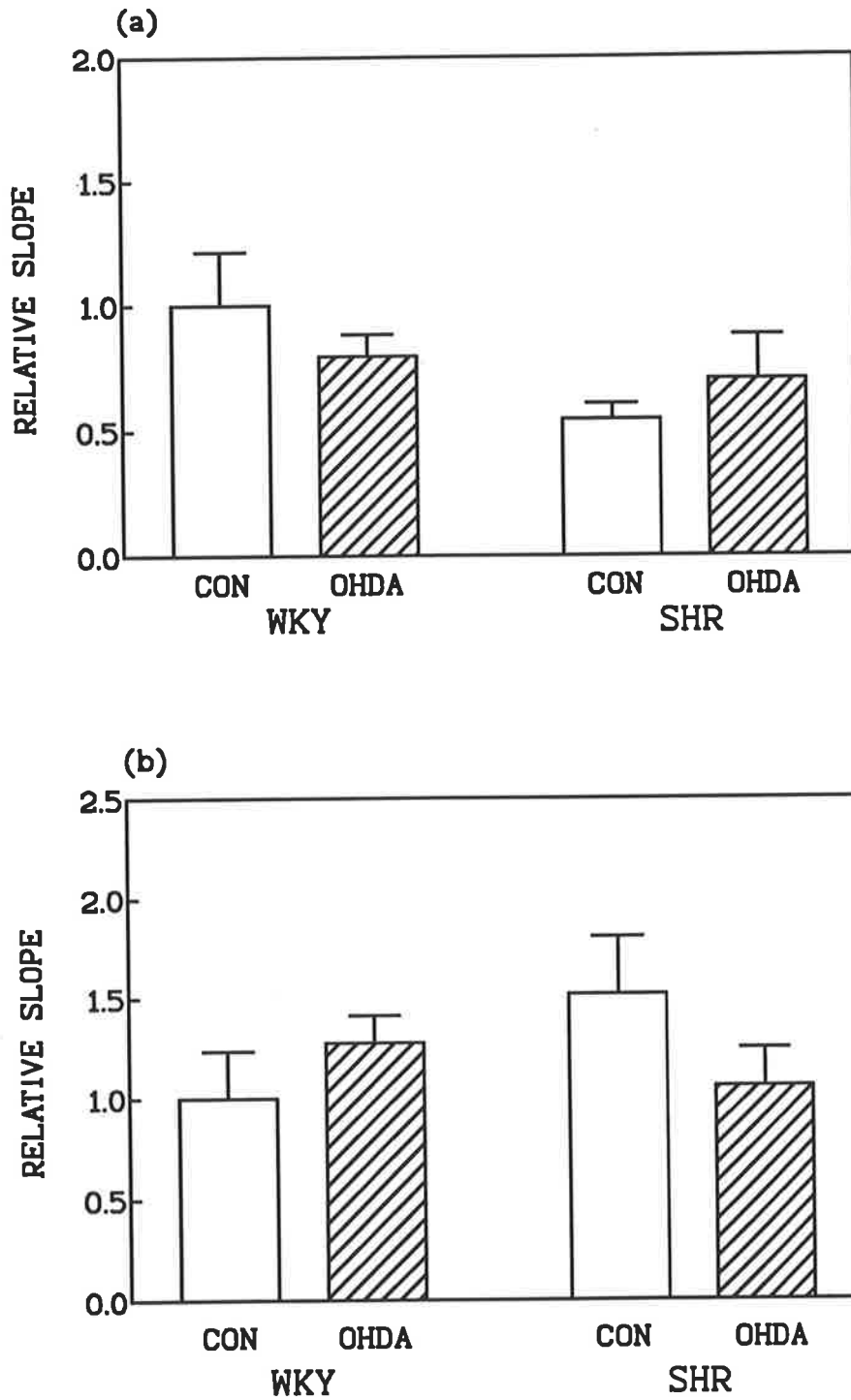


Figure 5.3.5 The mRNA levels for (a) angiotensinogen and (b) ACE measured in livers from control (\square), OHDA-treated (▨) WKY and SHR. The data are presented as described in the legend to *Figure 5.3.4*. Shown are the means \pm SEM for $n = 6$ or 7 observations.

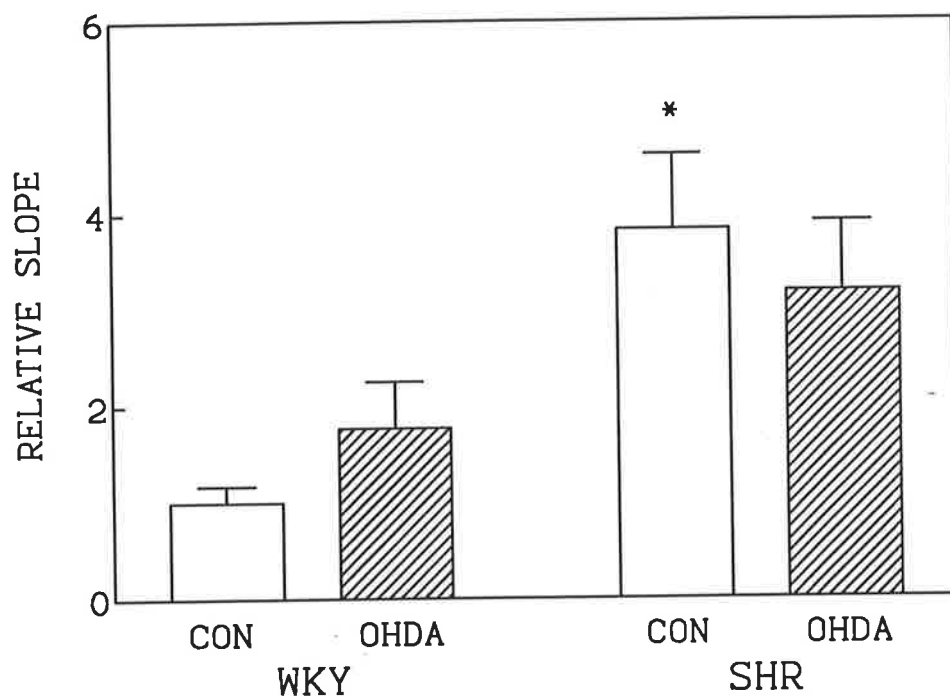


Figure 5.3.6 The mRNA levels for ACE measured in mesenteric artery from control (□) and OHDA-treated (▨) WKY and SHR. The data are presented as described in the legend to *Figure 5.3.4*. Shown are the means \pm SEM for $n = 5$ observations (pooled from $n = 13$ or 15 individual animals). * Significantly different from WKY control ($P < 0.05$).

5.4 Discussion

The results presented in this chapter confirm the findings of previous studies which have shown that neonatal sympathectomy with OHDA attenuates the development of hypertension in the SHR [Yamori et al, 1972; Provoost and De Jonge, 1978; Bevan and Tsuru, 1981]. Although normotension was not achieved, the blood pressure in SHR that had been treated with OHDA was substantially lower than that in the ascorbic saline-treated controls, a finding which highlights the importance of the SNS in the development of blood pressure in this rat strain.

The treatment schedule which was used in the present study produced a widespread sympathectomy in peripheral tissues but not in the central nervous system (CNS). Although OHDA does not penetrate the CNS from the blood stream [Weiner, 1985], it has been reported previously that a similar treatment regimen resulted in a significantly lower NA concentration in several areas of brain [Clark et al, 1972]. In the present study there were no apparent central effects of OHDA. When OHDA is administered intraventricularly, central neurones are damaged and there is little regeneration [Weiner, 1985] thus in the present study, it is likely that the peripherally administered OHDA did not penetrate the blood brain barrier. It has previously been reported that blood vessels are resistant to the depletion of noradrenaline by OHDA [Berkowitz et al, 1972] and in the present study, the caudal artery was the peripheral tissue which was the least sensitive to the actions of OHDA. It is most likely that this resistance is due to poor penetration across the vessel wall to the adventitia by the OHDA. The ability of the vascular sympathetic nerves to regenerate may prevent the complete attenuation of the development of hypertension in the SHR.

ACE activity is reported to be suppressed in some tissues of the SHR [Grima et al, 1990]. A similar finding was noted previously (*Chapter 4*) and has been confirmed in the study described in the present chapter. However, not all tissues from the SHR have a lower ACE activity than comparable tissues from the WKY. ACE activity in aorta,

brain and skeletal muscle from the SHR is at least as high as the activity seen in these tissues from WKY. Thus, it is likely that regulatory mechanisms in each tissue are independently controlled. Neonatal sympathectomy did not influence the ACE activity in plasma, peripheral and central tissues in adult animals. This finding parallels the situation in humans where plasma or serum ACE concentration is remarkably stable in a given individual and with the exception of some pathological conditions (e.g. granulomatous disease, hyperthyroidism and diabetes) it is not affected by hormonal or environmental factors [Lieberman, 1975; Alhenc-Gelas et al, 1983].

Basal PRA is reported to be lower in SHR which have been chemically sympathectomised with OHDA at birth [Sinaiko et al, 1980]. The kidney is the major source of plasma renin and therefore it was expected that kidney renin gene expression would be decreased in OHDA-treated animals. However, the levels of kidney renin mRNA were not influenced by neonatal sympathectomy in either SHR or WKY. The contradictory findings may be related to differences in the treatment schedules resulting in differing degrees of denervation. In the previously reported study [Sinaiko et al, 1980], OHDA was administered on 3 occasions during the first week of life and weekly thereafter until 6 weeks of age whereas in the present study, OHDA was administered on 6 occasions during the first 2 weeks of life. The extended administration of OHDA may have resulted in a greater level of denervation. In the previously reported study, the mean level of NA in kidneys from OHDA-treated animals was 6% of the mean level determined for saline treated controls, while in the present study, the mean level of NA in kidneys from OHDA-treated animals was 12% and 20 % of the mean NA concentration in kidneys from control WKY and SHR respectively. In the previously reported study the NA concentration was significantly reduced in brains from OHDA-treated animals. Thus the reduction in PRA noted in Sinaiko et al's study may have been the result of central as well as peripheral mechanisms. It is also possible that a significant decrease in PRA can be accommodated by a relatively small decrease in basal renin gene expression and that

the technique of slot analysis lacks the sensitivity to detect such a change. In light of the lack of influence of OHDA treatment on kidney renin expression, it was therefore not surprising that angiotensinogen and ACE gene expression in kidney and liver were also not influenced by neonatal sympathectomy.

An interesting finding to emerge was the significantly higher level of ACE mRNA in the mesenteric artery (and its branches) from ascorbic saline-treated SHR. However, this finding must be viewed with caution for several reasons. Due to the small tissue weight of the mesenteric artery, it was necessary to pool tissues from 2 or 3 animals in order to get sufficient RNA for analysis. This resulted in an n of five in each group for statistical analysis. The variation in the results for ACE mRNA levels from SHR and OHDA-treated WKY was very large and as a result, the only statistical difference between the groups was between saline-treated WKY and saline-treated SHR. Even though there appeared to be a large difference in the ACE mRNA level from OHDA-treated SHR and both WKY treatment groups, this failed to reach statistical significance. In addition to the problems with the statistical analysis with this data, there may also have been technical artefacts which contributed to the apparently large differences in ACE mRNA levels in the mesenteric arteries from WKY and SHR. In particular, it was much more difficult to remove the adhering fat from the mesenteric artery and branches in SHR and thus tissues which were harvested from these animals contained a much higher amount of adipose tissue. As discussed previously (*Chapter 3*) it is apparent that adipose tissue expresses a high level of ACE mRNA and thus the data presented in this study may represent the ACE mRNA levels in adipose tissue as well as vascular tissue from SHR, but only ACE mRNA expression from vascular tissue from WKY. Future investigations of the expression of ACE in the mesenteric artery must therefore ensure the complete removal of all adhering adipose tissue before RNA extraction and assessment of the ACE mRNA levels.

In conclusion, the results presented in this chapter do not support the hypothesis that the administration of OHDA at birth has a prolonged effect on the gene expression and

activity of the RAS and therefore the disruption of the RAS is not likely to be a contributing factor to the antihypertensive effects of neonatal sympathectomy.

CHAPTER 6**IS ANGIOTENSIN II TAKEN UP INTO SYMPATHETIC NERVES?**

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6.1 Introduction

AII can modulate neuroeffector responses in a variety of tissues from both hypertensive and normotensive animals (1.6.2). Included in this modulation is an action of AII in facilitating the release of neurotransmitter from sympathetic nerves by a mechanism involving prejunctional angiotensin receptors [Hughes and Roth, 1971; Zimmerman et al, 1972; Jackson and Campbell, 1979; Zimmerman and Kraft, 1979]. For example, AII causes a marked potentiation of the responses of the perfused mesenteric vascular bed to sympathetic nerve stimulation and this phenomenon is enhanced in preparations from the SHR [Kawasaki et al, 1982; Kawasaki et al, 1984]. It has also been reported that AII can block neuronal reuptake of NA [Palaic and Khairallah, 1967; Panisset and Bourdois, 1968; Peach et al, 1969; Jackson and Campbell, 1979]. A similar prejunctional facilitatory modulation process has also been described for the catecholamine adrenaline [Majewski and Rand, 1981]. The prejunctional facilitatory action of adrenaline is dependent upon the uptake of adrenaline into the sympathetic nerves and its subsequent release from those nerves by stimulation prior to activation of prejunctional receptors [Majewski and Rand, 1981; Majewski et al, 1982].

While there is evidence for a prejunctional facilitatory role for angiotensin II, it is not known whether there is a requirement for angiotensin II to be accumulated within the sympathetic nerves or whether such an uptake process is altered in the hypertensive state. The study described in this chapter determined the extent to which angiotensin II accumulates in vascular tissue of SHR and WKY, at the same time isolating that component which occurred within the sympathetic nerves by the use of chemical sympathetic denervation.

6.2 Methods

6.2.1 Animals, treatments and tissue collection

Adult (6-12 months of age) male SHR and normotensive WKY were obtained from the CSIRO animal facility at Glenthorne, Adelaide (2.1.1). Randomly selected rats from each strain were pretreated with 6-hydroxydopamine (OHDA, 50 mg/kg i.p. on 2 occasions, 3 days prior to sacrifice and 100 mg/kg i.p. on 2 occasions, 1 day prior to sacrifice). This regimen is exactly as that described by Suvannapura and Levens (1988). OHDA was freshly prepared in ascorbic saline immediately prior to injection. Control animals received intraperitoneal injections of ascorbic saline.

Immediately following sacrifice of the animals (2.1.3), tissues were removed, rinsed in ice-cold saline and placed in gassed Krebs solution (2.2.4).

6.2.2 NA determination

In a preliminary experiment, the NA concentration in several tissues from one animal in each treatment group was determined to ensure that a sympathectomy was produced by the proposed treatment regimen. Ventricle, vas deferens, mesenteric artery and branches, aorta, kidney, and skeletal muscle (from the posterior aspect of the hind limb) were removed from each animal and weighed prior to being placed in ice-cold 0.3 M perchloric acid containing 0.5 mM magnesium chloride and 5 mM EGTA. Following overnight extraction at 4°C, the NA content of each extract was determined as described previously (2.2.3)

6.2.3 AII uptake

Mesenteric artery and branches, aorta, skeletal muscle and kidney from control and OHDA-treated SHR and WKY were pre-incubated in gassed Krebs solution for 30 minutes (2.2.4). Following the equilibration period, the tissues were incubated in gassed Krebs (2 ml) containing angiotensin II (5-L-isoleucine)[tyrosol-3,-5³H(N)] (³H-AII, 14 nM) at 37°C for 30 min. (In preliminary experiments this concentration of AII and this incubation time were shown to lie on the linear portion of the accumulation curve). In some experiments, the selective AT₁-antagonist losartan (10⁻³M or 10⁻⁶M) or the metabolic inhibitor 2,4-dinitrophenol (DNP, 10⁻³M) were present throughout the equilibration and incubation periods. Upon removal, the tissues were rinsed in ice-cold saline (5 ml, 3 times), weighed and solubilised (at 55°C for 2 hours) in 0.5M ammonium hydroxide in toluene. The conditions for incubation and washing were based on those described by Suvannapura and Levens (1988) for the uptake of noradrenaline into rat intestine. The tritium content of each sample was assayed by liquid scintillation counting. The AII uptake into each tissue was calculated from the specific activity of the peptide and expressed as picomoles of angiotensin II per gram of tissue.

6.2.4 Statistical analyses

The results were expressed as mean ± SEM; values were compared using ANOVA and statistical significance was established when P<0.05.

6.3 Results

6.3.1 Tissue NA concentration

A preliminary examination of several tissues from control and OHDA-treated animals (one animal per treatment group) demonstrated that the treatment regimen produced a

profound and widespread sympathectomy in both WKY and SHR (*Figure 6.3.1*). The NA concentrations in all tissues from OHDA-treated animals were reduced by 75-99 % when compared with the concentrations in tissues from control animals (with the exception of aorta from OHDA-treated WKY in which there was a 35 % reduction (*Figure 6.3.1d*)). The mesenteric artery sample from the WKY control animal was lost (*Figure 6.3.1c #*).

6.3.2 Angiotensin II uptake

Incubation of the mesenteric artery, aorta, kidney and skeletal muscle in Krebs containing ^3H -AII for 30 min resulted in an accumulation of the peptide to a similar magnitude in all tissues within the range 5-15 pmol/g (*Figure 6.3.2*). There were no differences between the accumulation of angiotensin II in tissues from SHR and WKY (*Figure 6.3.2*). In addition, pretreatment of animals with OHDA failed to reduce the accumulation of angiotensin II in the tissues examined (*Figure 6.3.2*). Subsequent studies using kidney and skeletal muscle segments demonstrated that the accumulation of angiotensin II was temperature sensitive and attenuated by the metabolic inhibitor DNP (*Figure 6.3.3*). The angiotensin II receptor antagonist losartan, when present at high a concentration (10^{-3}M), inhibited the accumulation of angiotensin II into skeletal muscle (*Figure 6.3.3b*).

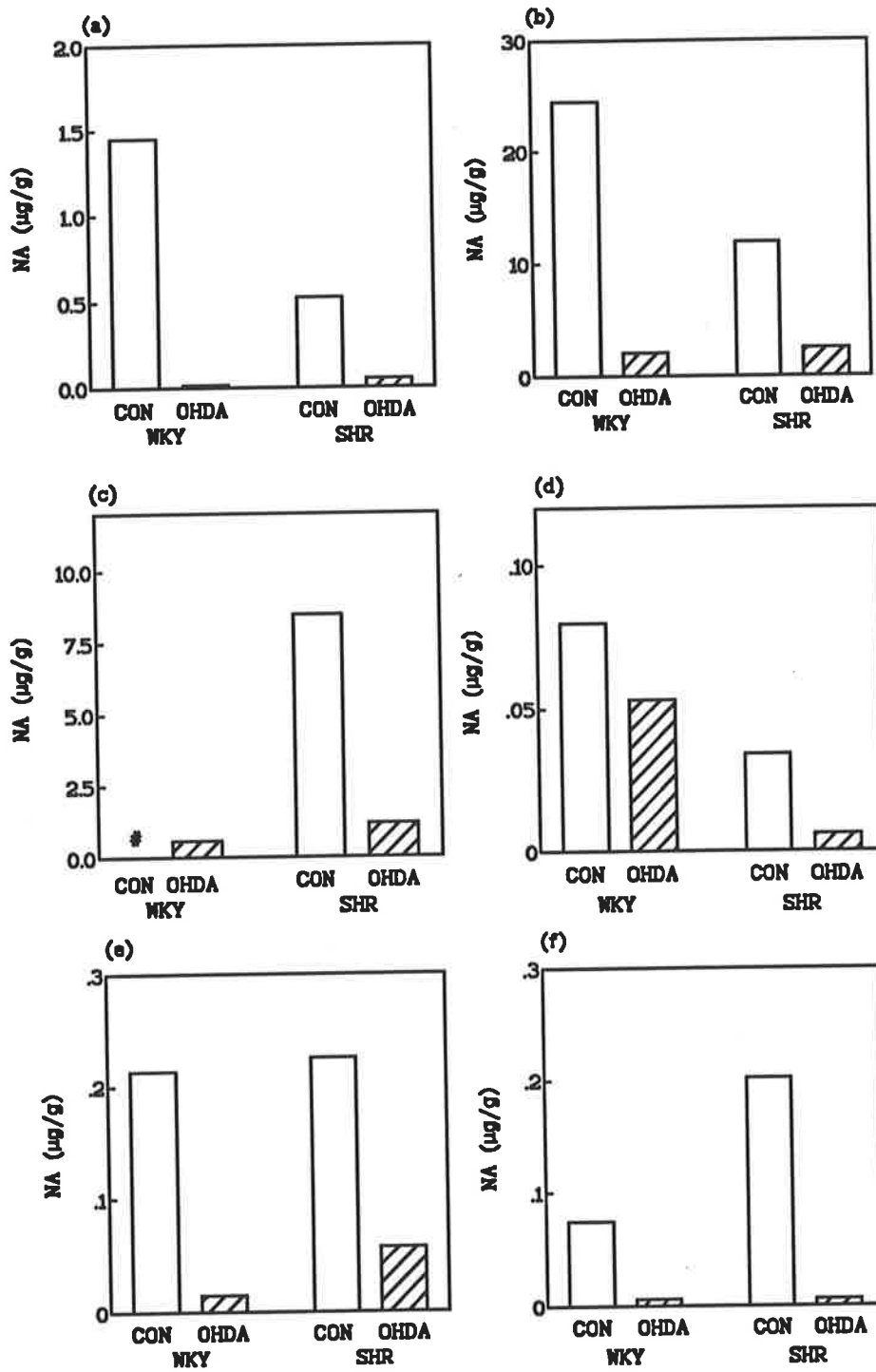


Figure 6.3.1. NA concentration ($\mu\text{g/g}$ tissue) in (a) ventricle, (b) vas deferens, (c) mesenteric artery, (d) aorta, (e) kidney and (f) muscle from control SHR and WKY (\square) as well as from OHDA-treated animals (▨). Columns represent a single determination. (# in (c) refers to the fact that this sample was lost.)

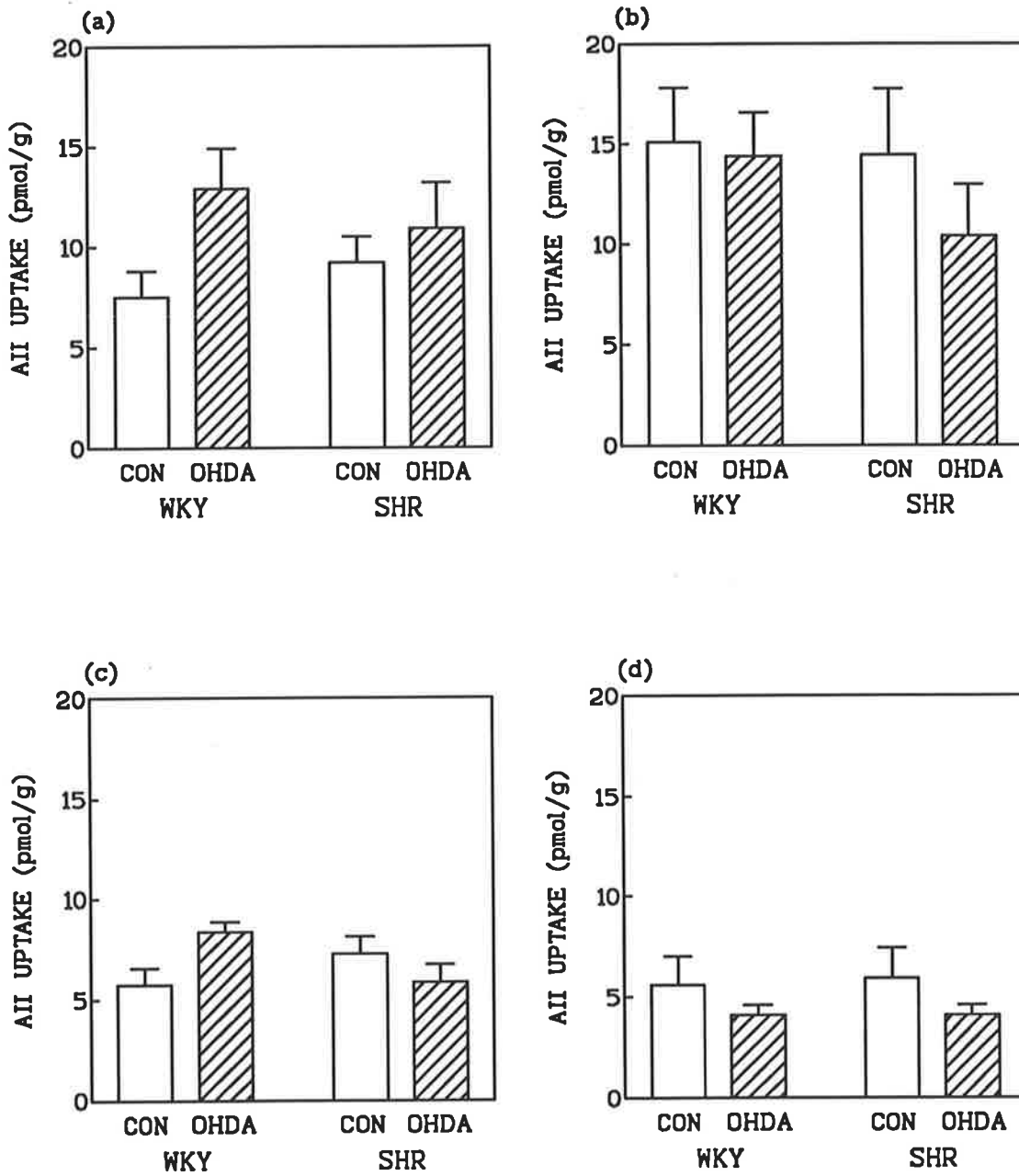


Figure 6.3.2 The accumulation of ^3H -AII into (a) mesenteric artery, (b) aorta, (c) kidney and (d) muscle from control SHR and WKY (□) as well as from OHDA-treated animals (▨). Columns are the mean \pm SEM of $n = 5$ determinations.

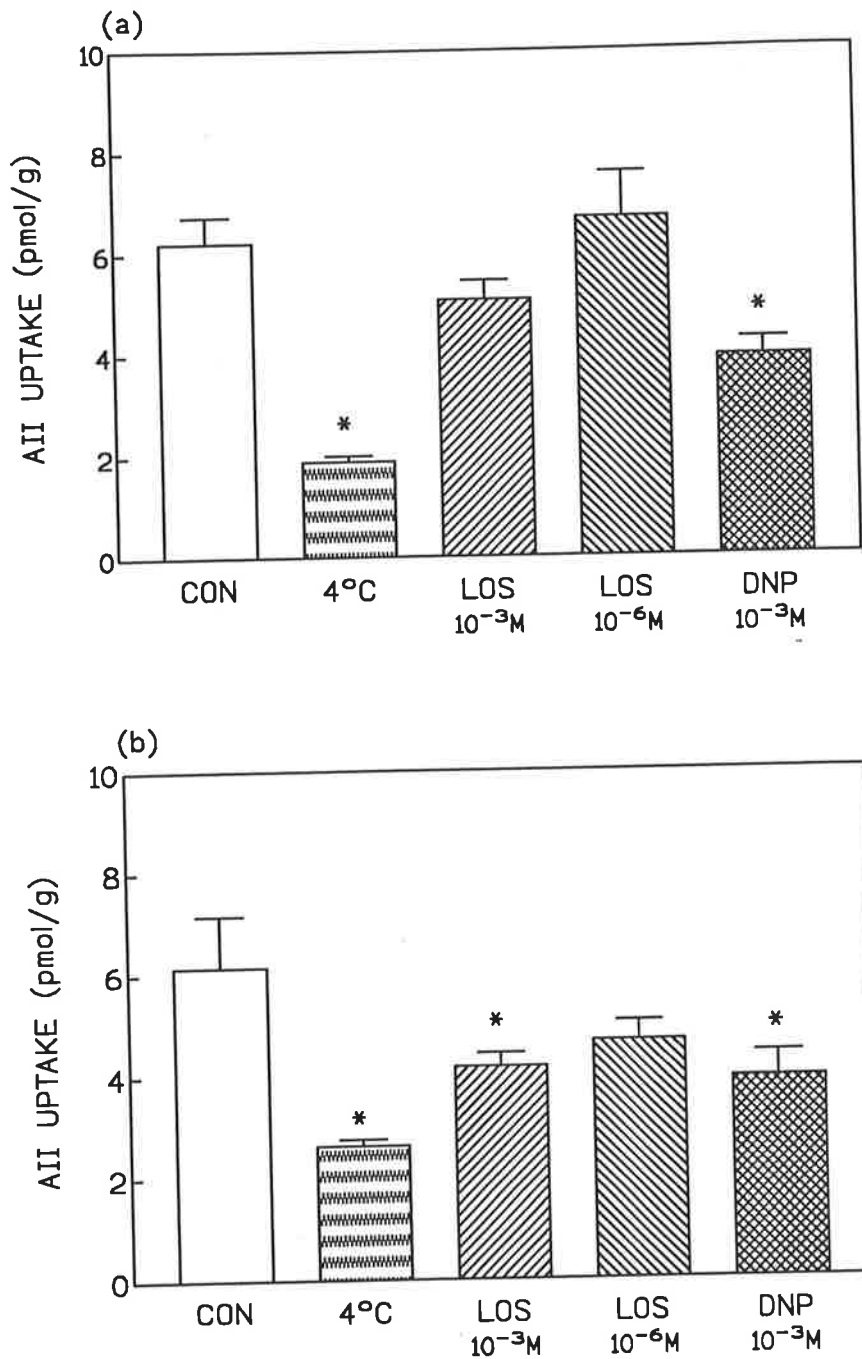


Figure 6.3.3 The accumulation of ^3H -AII in the (a) kidney and (b) skeletal muscle. Shown is the accumulation of ^3H -AII at 4°C (▨), in the presence of losartan (10^{-3}M , ▩, 10^{-6}M , ▧) and in the presence of DNP (10^{-6}M , ▣). Columns are the mean \pm SEM for $n = 6 - 11$ observations. * Significantly different from control ($P < 0.05$).

6.4 Discussion

Adrenaline potentiates the responses to sympathetic nerve stimulation in a variety of tissues and this process has been implicated in the development of hypertension [Majewski and Rand, 1981]. The adrenaline-mediated hypertension hypothesis suggests that adrenaline is taken into sympathetic nerves, then subsequently released to act upon presynaptic receptors on the neuronal surface which facilitate the release of transmitter and hence, potentiate responses to sympathetic nerve stimulation [Majewski and Rand, 1981; Majewski et al, 1982]. It could be suggested that the inhibition by AII of NA uptake into neuronal tissue [Palaic and Khairallah, 1967; Panisset and Bourdois, 1968; Peach et al, 1969; Jackson and Campbell, 1979] is a consequence of a competitive process in which the peptide utilises the same uptake carrier to enter the sympathetic nerve terminal. Furthermore, it could be argued that AII may exert its action on neuroeffector processes in a similar way to adrenaline by being incorporated into the nerve ending, subsequently released and then interacting with prejunctional receptors. If this process was operating, it would be characterised by an accumulation of angiotensin II into sympathetically innervated tissues and would demonstrate a sensitivity towards sympathetic denervation.

The sympathetic innervation of many tissues from the SHR, including the mesenteric artery and kidney, is significantly greater when compared with tissues from the WKY [Head, 1989]. In both SHR and WKY the sympathetic innervation of the mesenteric artery is at least 10-fold greater than that seen in the aorta [Head, 1989]. If AII is taken up into sympathetic nerves it would be predicted that the accumulation of the peptide would be greater in tissues with a relatively greater sympathetic innervation. In the present study, there was no apparent influence of innervation, hyperinnervation or sympathetic denervation on the accumulation of angiotensin II in a variety of sympathetically innervated tissues suggesting that there is either little or no incorporation of the peptide into neuronal structures. Thus, the failure to demonstrate a significant accumulation of angiotensin into neuronal structures suggests that a

process involving incorporation, release and subsequent activation of prejunctional receptors is not responsible for the enhanced facilitation of neurotransmission in the blood vessels of the SHR.

The uptake of AII into skeletal muscle was significantly reduced by the specific AT₁-receptor antagonist losartan, although this was evident only at a high concentration of the agent. In both kidney and skeletal muscle, AII accumulation was significantly reduced by the metabolic inhibitor 2,4-dinitrophenol and incubation at 4°C. Therefore, it may be concluded that the uptake of AII into these tissues involves a specific energy-dependent process, although there is a large non-specific component of this accumulation.

The use of autoradiography may provide information regarding the precise localisation of the sites of AII accumulation into these tissues. In the present study autoradiographical analyses of tissues which had been incubated in ³H-AII and subsequently exposed to photographic emulsion for one year were not successful. This was probably due to the extremely long half-life (12 years) of the radiolabel (³H) used in these experiments, coupled with the relatively low level of accumulation.

In conclusion, the results of the study described in this chapter indicate that while AII is accumulated in tissues from both SHR and WKY and a proportion of this accumulation appears to be by a specific energy-dependent process, AII is not taken up into sympathetic nerves.

CHAPTER 7**EXPRESSION AND ACTIVITY OF HEPATIC ACE**

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7.1 Introduction

The majority of studies which have examined the regulation of gene expression of the components of the RAS have focused on renin and angiotensinogen. This has been due, in part, to the relatively wide availability of cDNA for these genes. However, the rate limiting step in the renin-angiotensin cascade is considered to be the conversion of angiotensinogen to AI by renin, and understandably, many studies have examined changes in the expression and levels of this enzyme and its substrate. Less attention has been paid to concomitant alterations in ACE expression and activity. Furthermore, those studies which have examined ACE gene regulation have concentrated on tissues, such as lung and testis, which exhibit high levels of activity of the enzyme [King et al, 1989; Schunkert et al, 1993].

It was noted previously (*Chapter 4*) that the mRNA for the ACE gene is readily detected in rat liver using the relatively insensitive techniques of slot and Northern analyses. This was surprising, given that the measurable ACE activity in liver homogenates was extremely low. There are at least two possible explanations for this observation. Firstly, it has been reported that in some tissues there is an endogenous inhibitor of ACE activity [Ondetti et al, 1971; Hazato and Kase, 1986; Ikemoto et al, 1988]. Accordingly, it is possible that in the liver, the considerable levels of ACE mRNA code for significant amounts of the ACE protein but that the enzyme activity may be inhibited by the presence of an endogenous inhibitor. Alternatively, it is conceivable that ACE is packaged and released from the tissue into circulation, leading to a situation of high levels of gene expression and low levels of enzyme activity within the tissue. It has been reported previously that interference with hepatic function leads to a decrease in circulating levels of ACE activity, a feature consistent with this explanation [Loyke, 1970].

The purpose of the study described in this chapter was to further examine hepatic expression and activity of ACE. The possibility that endogenous ACE inhibitory

activity was present in the liver was investigated by examining the effect of liver homogenates on plasma ACE activity. The effect of hepatotoxicity on plasma ACE activity was also studied, in order to determine whether the liver was a source of circulating ACE.

7.2 Methods

7.2.1 Animals and tissue treatments

Adult male SHR and WKY were obtained from the CSIRO animal facility at Glenthorne, Adelaide. Balb/c mice (6 weeks of age) were obtained from the University of Adelaide Central Animal House. Animals were housed under a 12 hour light/dark cycle, fed standard chow and allowed water ad libitum (2.1.1).

Hepatotoxicity was induced in randomly selected WKY and SHR with subcutaneous injections of carbon tetrachloride (2 ml/kg in olive oil, twice a week for 2 weeks). Control animals received injections of vehicle (olive oil). The rats were sacrificed 72 hours after the final injection. In mice, hepatotoxicity was induced with a single intraperitoneal injection of paracetamol (200 mg/kg). Control animals received injections of saline. The mice were sacrificed 24 hours after administration of paracetamol or saline. Some experiments used animals that had received no treatment.

The mice were administered a lethal dose of pentobarbitone sodium prior to cardiac puncture. The rats were stunned and decapitated. Blood was collected into heparinised tubes and plasma removed following centrifugation at 3000g for 15 minutes. Tissues were thoroughly rinsed in ice-cold saline, snap frozen in liquid nitrogen and then stored at -80°C until processed for the isolation of RNA, or the measurement of ACE activity.

7.2.2 Biochemical analyses

Kidneys, livers and lungs were homogenised in 50 mmol/l potassium phosphate buffer (pH 8.3) for the determination of ACE activity (2.2.1). ACE activity is expressed as nmol ^{14}C -Hippuryl-L-Histidyl-L-Leucine [Glycine-1- ^{14}C] (HHL) hydrolysed per minute per milligram of protein (2.2.2) for each tissue sample or per millilitre for plasma.

For experiments in which endogenous ACE inhibitory activity was examined, bovine serum albumin (20 mg/ml) and liver or kidney homogenates were co-incubated with plasma from WKY for 30 minutes at room temperature, prior to assessment of ACE activity. In some experiments, the effect of 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB, 5mM) on the measurable ACE activity and endogenous inhibitory activity was examined in order to determine whether the endogenous inhibitor possessed a sulphhydryl moiety in its structure. DTNB was prepared in 25% ethanol immediately prior to use and pre-incubated with liver homogenates for 30 minutes at room temperature, such that the final ethanol concentration was 0.5%. This concentration of ethanol was found to be without effect on the measurement of ACE activity.

The effect of tissue homogenates on the extraction of the cleavage product (^{14}C -hippuric acid) from the incubation mixture was also assessed. ^{14}C -Hippuric acid was prepared from ^{14}C -HHL using control plasma. Following extraction into ethyl acetate, the cleavage product ^{14}C -hippuric acid was evaporated to dryness under vacuum and then redissolved in water at a concentration of 1000 dpm/ μl . This ^{14}C -hippuric acid solution was then used in place of ^{14}C -HHL in a standard ACE activity incubation reaction. The effect of tissue homogenates on the recovery of radioactivity from the incubation mixture was compared with that seen for BSA and plasma. The results were expressed as a percentage of the expected recovery of radioactivity based on the known amount of radioactivity added to each reaction and the extraction coefficient of hippuric acid into ethyl acetate (0.91).

AlaAT was measured in plasma according to the procedure described previously (2.2.5).

7.2.3 RNA analyses

Preparation of full-length probe (2.3.3), isolation of RNA from tissue homogenates (2.3.4), slot analysis (2.3.5), Northern analysis (2.3.6), hybridisation (2.3.7), autoradiography (2.3.8) and mRNA quantification (2.3.9) were performed as previously described.

7.2.4 Statistical analysis

Values are expressed as the mean \pm SEM for the number of measurements shown in the results. Homogeneity of variances was determined using Bartlett's Test. Statistical significance was determined using a paired *t*-test, an unpaired *t*-test, a Mann-Whitney Test or analysis of variance (ANOVA) followed by the Tukey-Kramer Multiple Comparisons Test for individual comparison of means. Non-parametric tests were used only when the variances of the samples were shown to be significantly different. $P < 0.05$ was considered statistically significant.

7.3 Results

7.3.1 Comparison of kidney and liver ACE

When the relative amounts of ACE mRNA were determined in kidney and liver by slot blot analysis, it was found that the ACE gene was expressed at a similar level in livers and kidneys from both SHR (*Figure 7.3.1*) and WKY (not shown). However, the measurable level of ACE activity in liver, expressed as nmoles HHL hydrolysed per

min per mg protein, was at least an order of magnitude lower in livers when compared with kidneys (*Figure 7.3.1*).

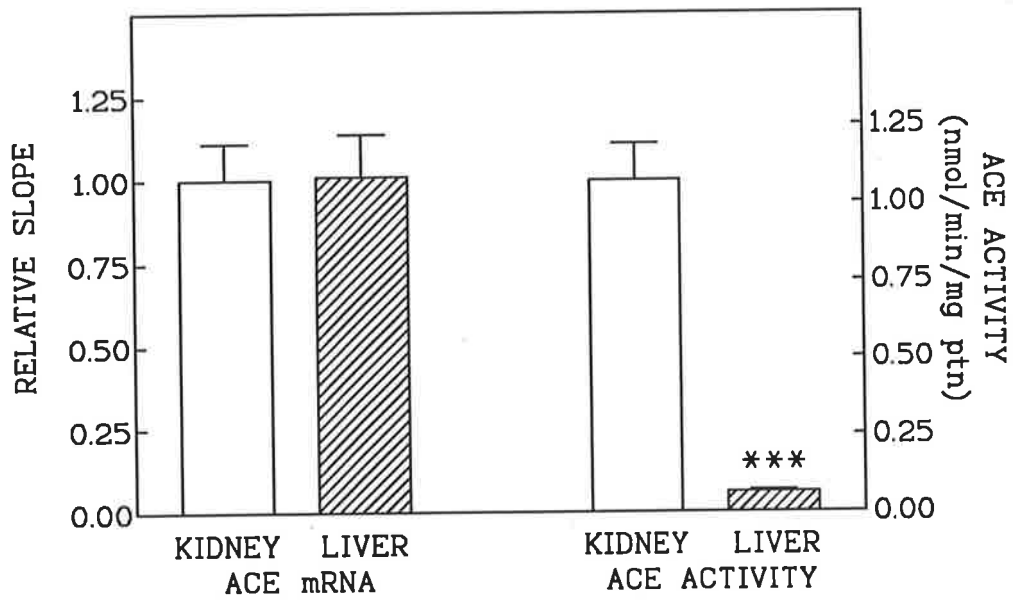


Figure 7.3.1. ACE mRNA and ACE activity measured in kidney and liver from SHR. The columns represent the means \pm SEM for $n = 5$ determinations. *** $P < 0.001$.

7.3.2 Endogenous inhibitory activity

The presence of endogenous ACE inhibitory activity in tissues was investigated by pre-incubating plasma with kidney and liver homogenates and subsequently assessing the measurable ACE activity in the plasma sample. For this experiment, ACE activity is expressed as a percentage of the activity which was measured in plasma when it was pre-incubated with buffer rather than homogenate. Liver homogenates from both WKY and SHR significantly decreased the measurable ACE activity in plasma by $41 \pm 7\%$ and $50 \pm 4\%$ respectively (*Figure 7.3.2*). Although there was a tendency for kidney homogenate to also inhibit plasma ACE activity, this failed to reach statistical significance (*Figure 7.3.2*). When plasma was pre-incubated with BSA (20 mg/ml), to provide an equivalent protein concentration comparable with test samples, there was no effect on plasma ACE activity (*Figure 7.3.2*), indicating that the presence of increased protein in the incubation mixture was not responsible for the inhibitory effect observed with tissue homogenates. The presence of tissue homogenates did not affect the extraction of the substrate cleavage product (^{14}C -Hippuric Acid) from the reaction mixture indicating that the inhibitory activity seen in liver homogenates was not a result of impaired partitioning of hippuric acid (*Figure 7.3.3*).

The effect of the sulfhydryl-blocking drug 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB, 5mM) on ACE activity and endogenous inhibitory activity was also examined. In this experiment, the inhibitory activity is expressed as the percentage difference of the plasma ACE activity measured in the presence of tissue homogenate. When liver homogenates were pre-incubated with DTNB, the inhibitory effect on plasma ACE was significantly reduced (*Figure 7.3.4a*). Pre-incubation with DTNB significantly increased the measurable ACE activity in liver homogenates (*Figure 7.3.4b*).

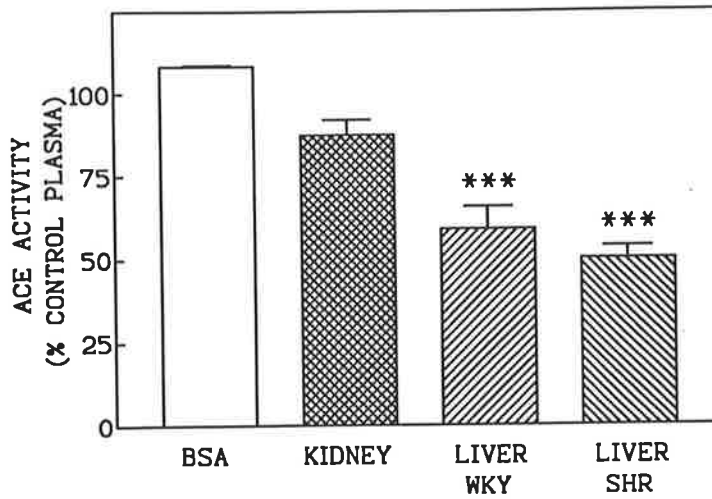


Figure 7.3.2. Effect of tissue homogenates on plasma ACE activity. Bovine serum albumin \square (n=3); kidney \blacksquare (n=7); WKY liver ▨ (n=6); SHR liver ▩ (n=6). The columns represent the means \pm SEM for the number of determinations indicated..
***P<0.001.

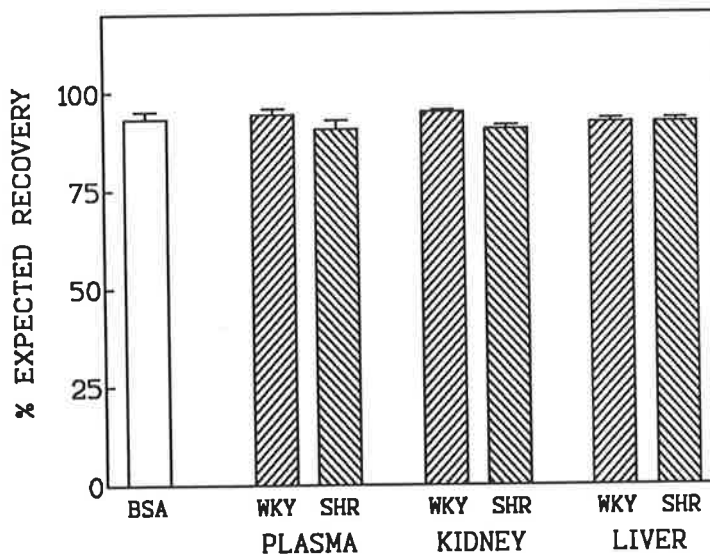


Figure 7.3.3. Effect of tissue homogenates on the extraction of the cleavage product ¹⁴C-Hippuric acid. WKY ▨ ; SHR ▩ . The columns represent the means \pm SEM for n = 5 determinations.

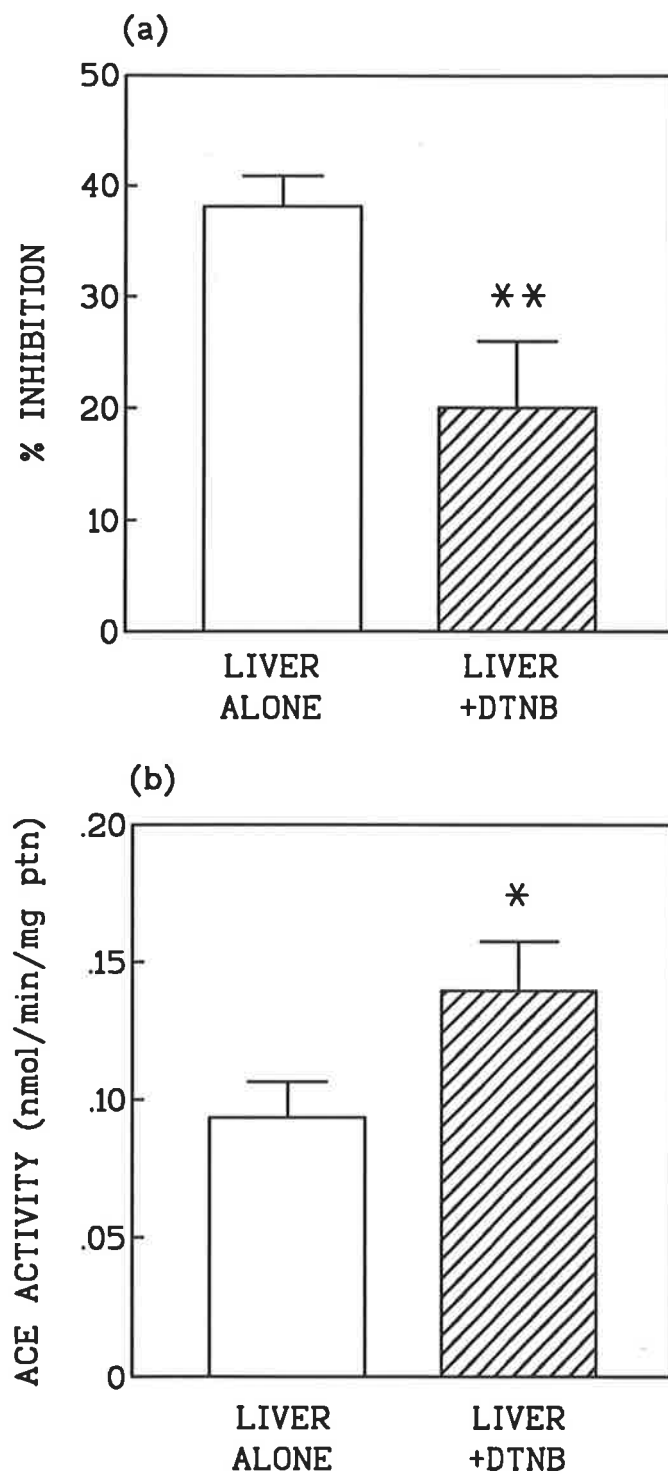


Figure 7.3.4. Effect of DTNB (5 mM) on (a) the endogenous ACE inhibitory activity of liver homogenates from SHR and (b) the ACE activity in liver homogenates from SHR. Control \square ; DTNB ▨ . The columns represent means \pm SEM for $n = 6$ determinations. * $P < 0.05$, ** $P < 0.01$.

7.3.3 Hepatotoxicity and ACE activity

Northern analysis revealed that ACE mRNA of the expected size (~4.3 kb) was observed in liver from both WKY and SHR and that carbon tetrachloride treatment did not influence the transcript size for ACE in either rat strain (*Figure 7.3.5*).

Hepatotoxicity was induced in rats by the subcutaneous injection of carbon tetrachloride. The plasma level of the hepatic enzyme AlaAT was used as an indicator of hepatotoxicity. In both WKY and SHR, AlaAT was significantly increased in plasma from carbon tetrachloride-treated animals when compared with olive oil-treated controls (*Figure 7.3.6a*). It is noteworthy that the increase in circulating levels of AlaAT were greater in SHR than in WKY (*Figure 7.3.6a*). The yellow appearance of livers from rats treated with carbon tetrachloride also confirmed that liver damage had occurred. Although hepatotoxicity was induced, plasma ACE activity was not decreased by carbon tetrachloride treatment in either rat strain (*Figure 7.3.6b*). Hepatotoxicity also did not affect ACE activity in lung (*Figure 7.3.7a*) or kidney (*Figure 7.3.7b*). However, when the effect of carbon tetrachloride on liver ACE activity was examined, it was found that there was a four-fold increase when compared with controls (*Figure 7.3.8a*). This increase in liver ACE activity which was seen in damaged livers was not associated with a significant change in the mRNA levels for ACE (*Figure 7.3.8b*).

When the effect of liver homogenates from carbon tetrachloride-treated rats on the inhibition of plasma ACE was examined, it was found that while the inhibitory activity tended to be lower in damaged livers, this did not reach statistical significance (*Figure 7.3.9a*). Similarly, the relative increase in liver ACE activity induced by DTNB tended to be lower in livers from carbon tetrachloride-treated animals, but this also did not reach statistical significance (*Figure 7.3.9b*).

The effect of hepatotoxicity on ACE activity in mice was also examined in order to exclude the possibility that the effect of carbon tetrachloride on rat ACE activity was a

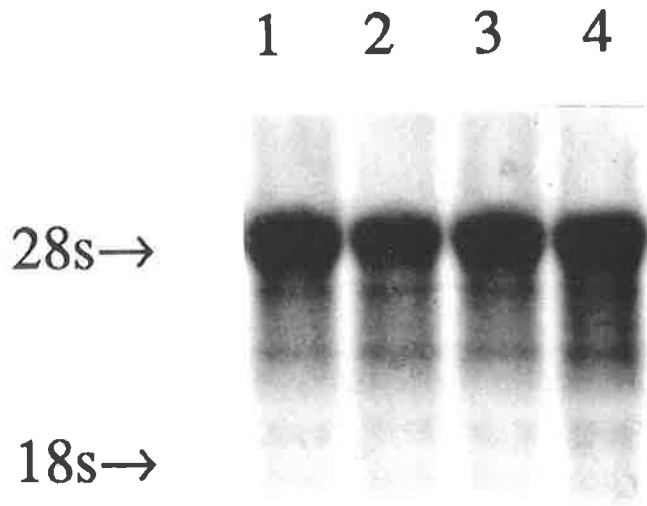
species or drug-specific effect. Paracetamol treatment caused a significant increase in plasma ALaAT (*Figure 7.3.10a*) but had no influence on plasma ACE activity (*Figure 7.3.10b*). When the effect of paracetamol on tissue ACE activity was examined, the pattern of effects was similar to that seen for carbon tetrachloride in rats. Paracetamol caused a significant increase in liver ACE activity but had no influence on the levels of activity of the enzyme in kidney or lung (*Figure 7.3.11*).

The chloride sensitivity profile of ACE was examined in randomly selected liver samples from control and carbon tetrachloride-treated SHR. In both treatment groups, the sensitivity of ACE to chloride ion concentration appeared to be similar with an optimum chloride ion concentration of at least 305 mM (Table VII.A.)

| | 5 mM | 10 mM | 15 mM | 55 mM | 105 mM | 305 mM |
|--------------------|-------|-------|-------|-------|--------|--------|
| CON 1 | 0.00 | 0.01 | 0.01 | 0.03 | 0.04 | 0.06 |
| CON 2 | 0.02 | 0.01 | 0.00 | 0.02 | 0.04 | 0.07 |
| CON 3 | 0.01 | 0.01 | 0.00 | 0.02 | 0.04 | 0.03 |
| mean | 0.01 | 0.009 | 0.002 | 0.024 | 0.039 | 0.055 |
| SEM | 0.005 | 0.001 | 0.003 | 0.004 | 0.0001 | 0.012 |
| CCl ₄ 1 | 0.04 | 0.03 | 0.05 | 0.09 | 0.16 | 0.50 |
| CCl ₄ 2 | 0.01 | 0.01 | 0.01 | 0.03 | 0.07 | 0.14 |
| CCl ₄ 3 | 0.01 | 0.02 | 0.00 | 0.04 | 0.08 | 0.13 |
| mean | 0.019 | 0.021 | 0.02 | 0.052 | 0.103 | 0.258 |
| SEM | 0.009 | 0.005 | 0.016 | 0.02 | 0.028 | 0.122 |

Table VII.A Effect of chloride ion concentration on ACE activity (nmol/min/mg protein) in livers from control and carbon tetrachloride treated rats.

Figure 7.3.5. Representative Northern analysis showing the hybridisation band for ACE mRNA in liver. Lane 1, WKY control; Lane 2, carbon tetrachloride-treated WKY; Lane 3 SHR control; Lane 4, carbon tetrachloride-treated SHR.



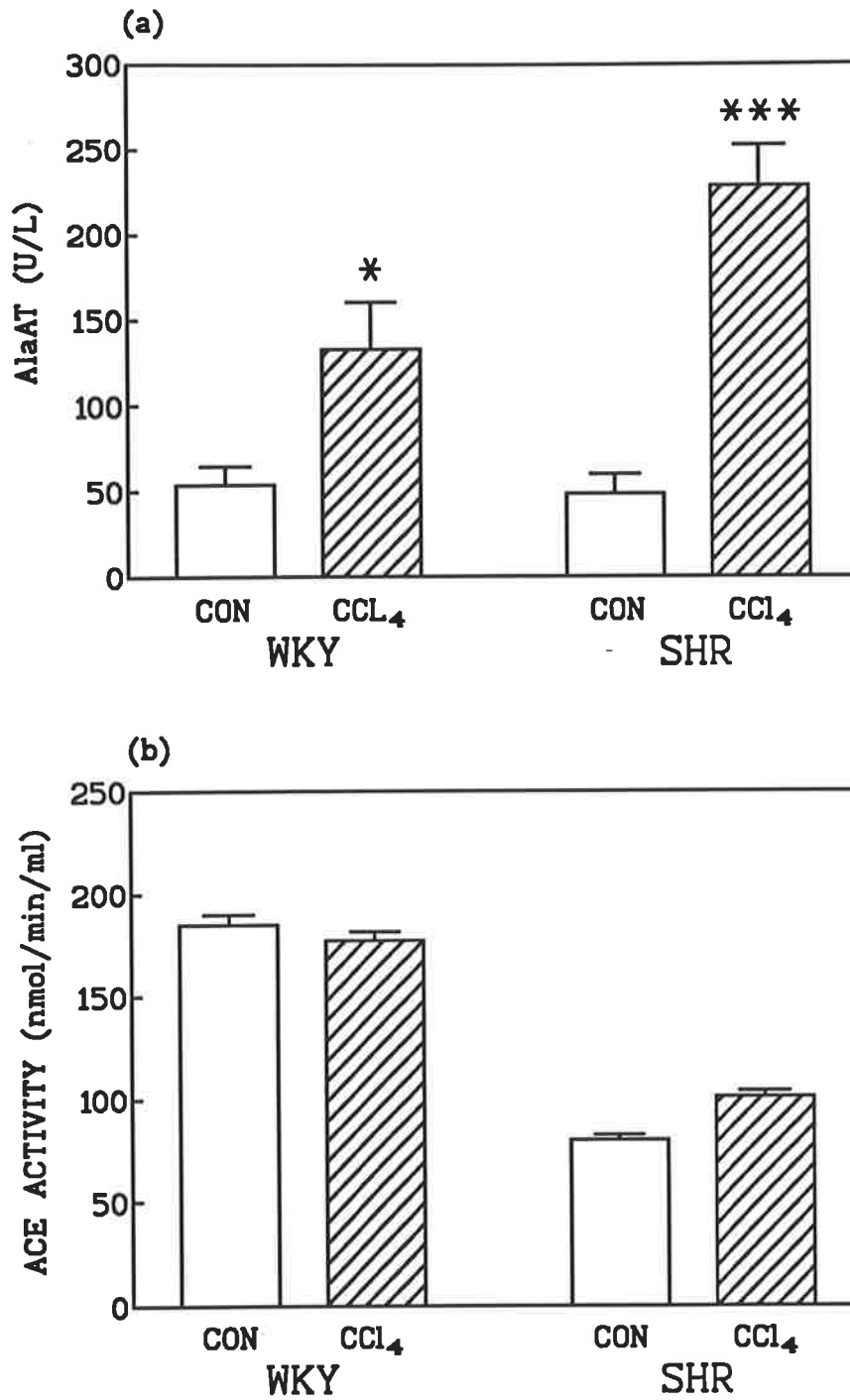


Figure 7.3.6. The effect of carbon tetrachloride on (a) plasma AlaAT activity and (b) plasma ACE activity. Control \square ; Carbon tetrachloride ▨ . The columns represent the means \pm SEM for $n = 6$ determinations. * $P < 0.05$, *** $P < 0.001$.

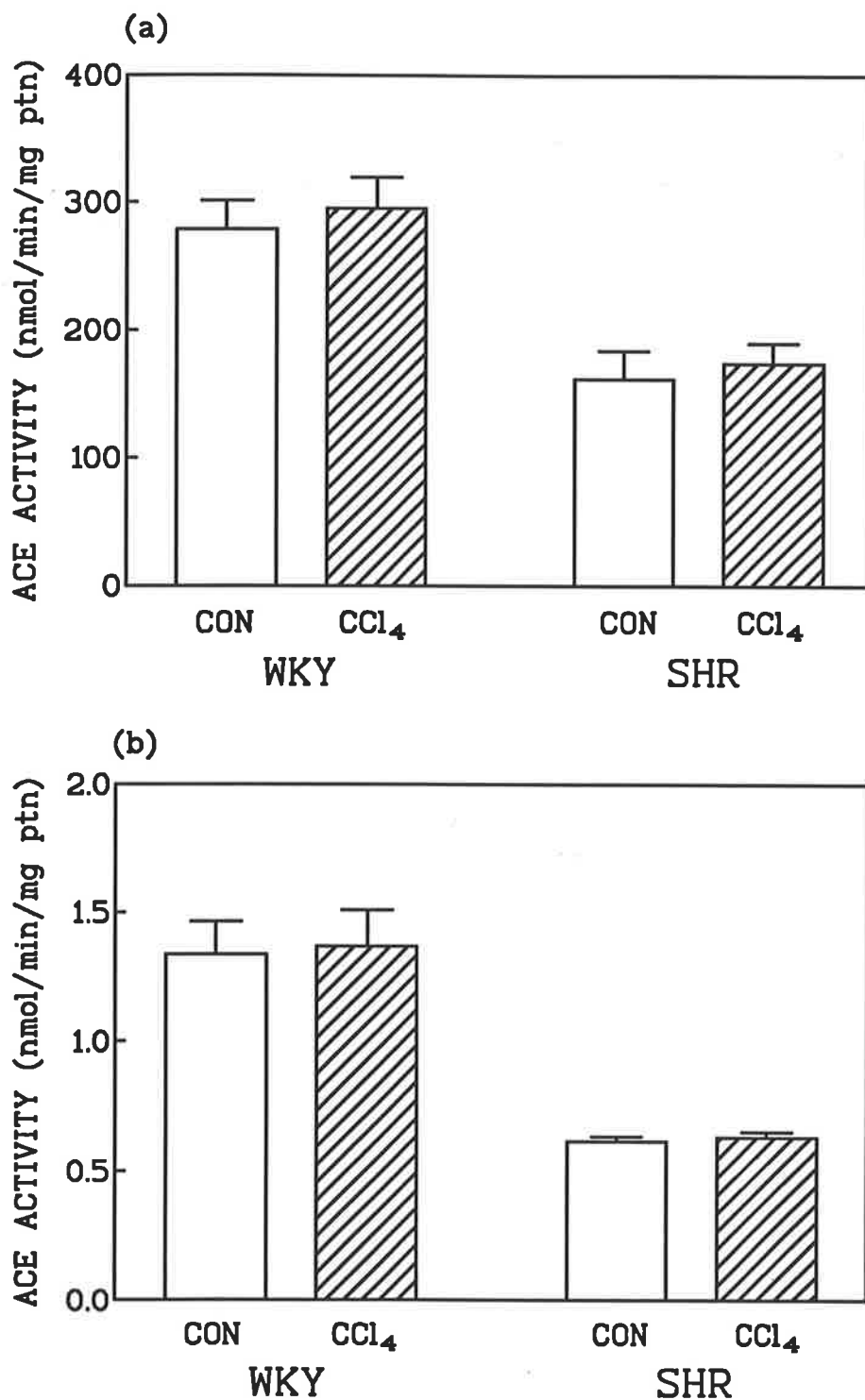




Figure 7.3.7. The effect of carbon tetrachloride on (a) lung and (b) kidney ACE activity. Control  ; Carbon tetrachloride  . The columns represent the means \pm SEM for n = 6 determinations.

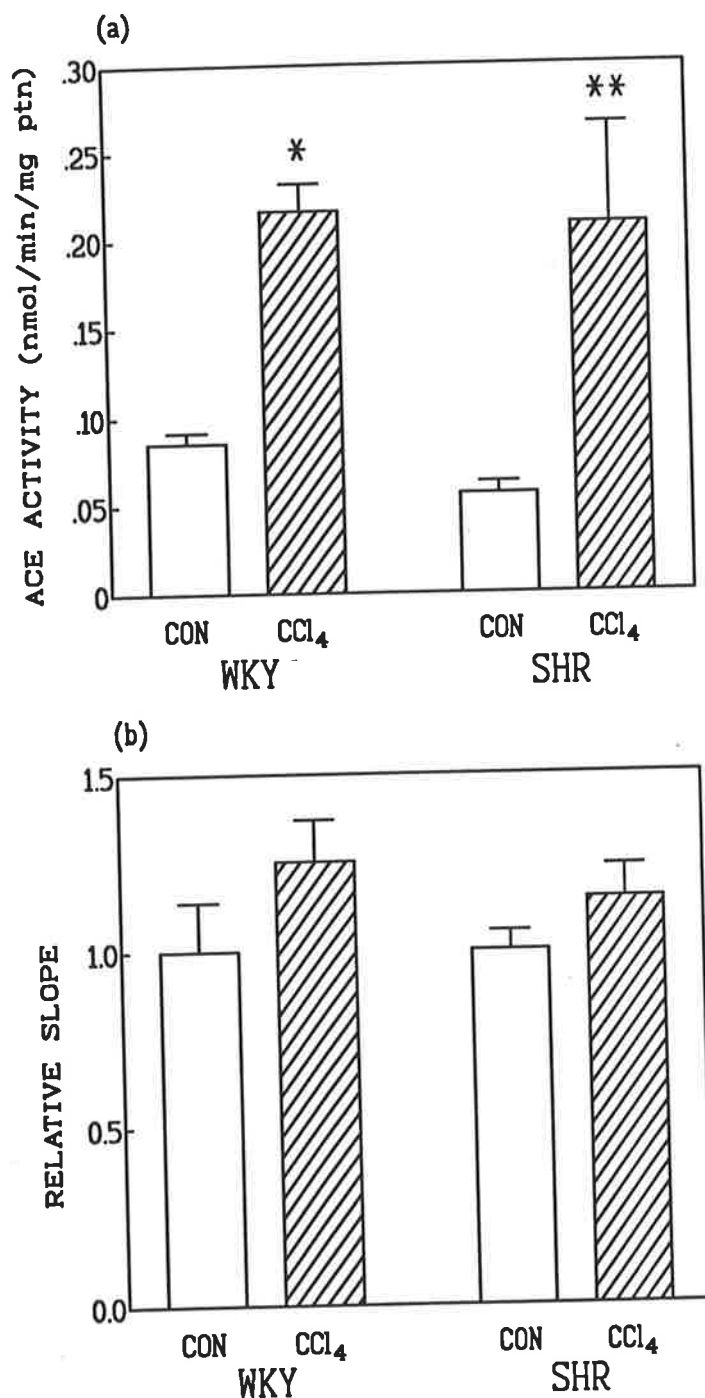


Figure 7.3.8. The effect of carbon tetrachloride on (a) liver ACE activity and (b) liver ACE mRNA. Control \square ; Carbon tetrachloride ▨ . The columns represent the means \pm SEM for $n = 6$ determinations. * $P < 0.05$, ** $P < 0.01$.

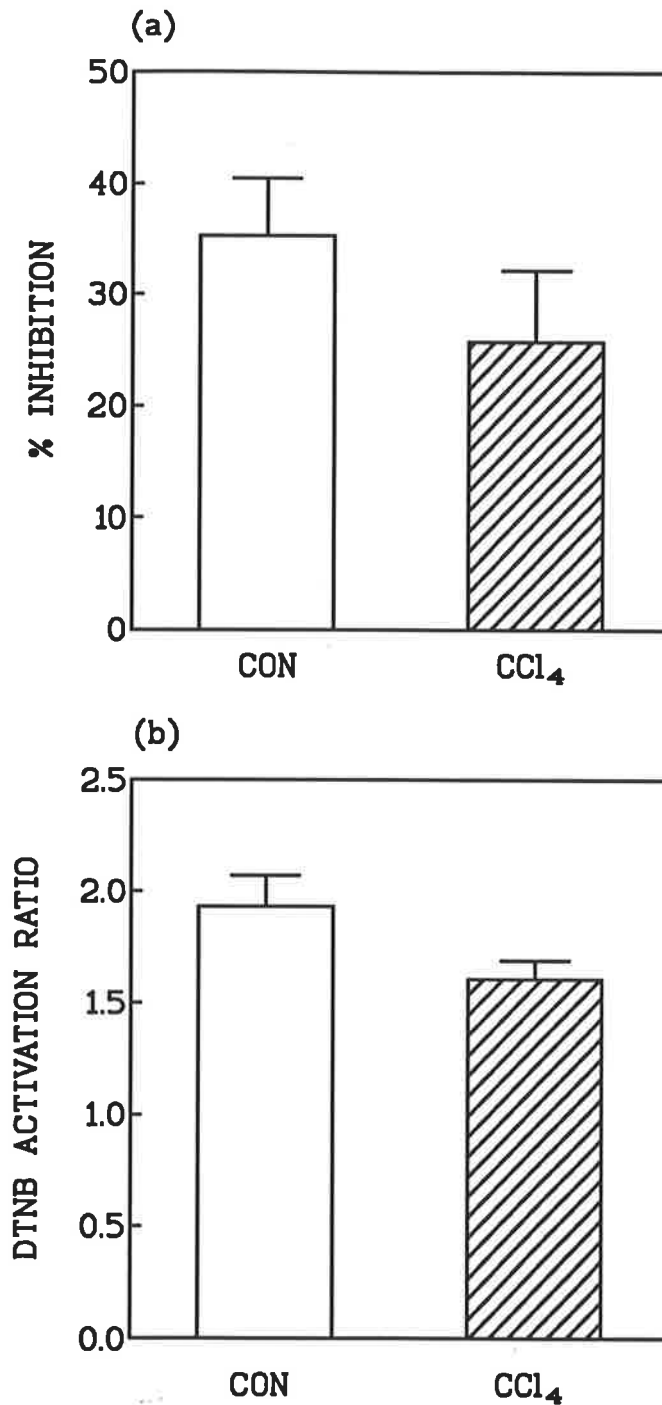


Figure 7.3.9. The effect of carbon tetrachloride on (a) endogenous ACE inhibition in liver homogenates and (b) the increase in ACE activity in liver homogenates produced by DTNB (5 mM) from control and carbon tetrachloride-treated SHR. The columns represent the means \pm SEM for n = 6 determinations.

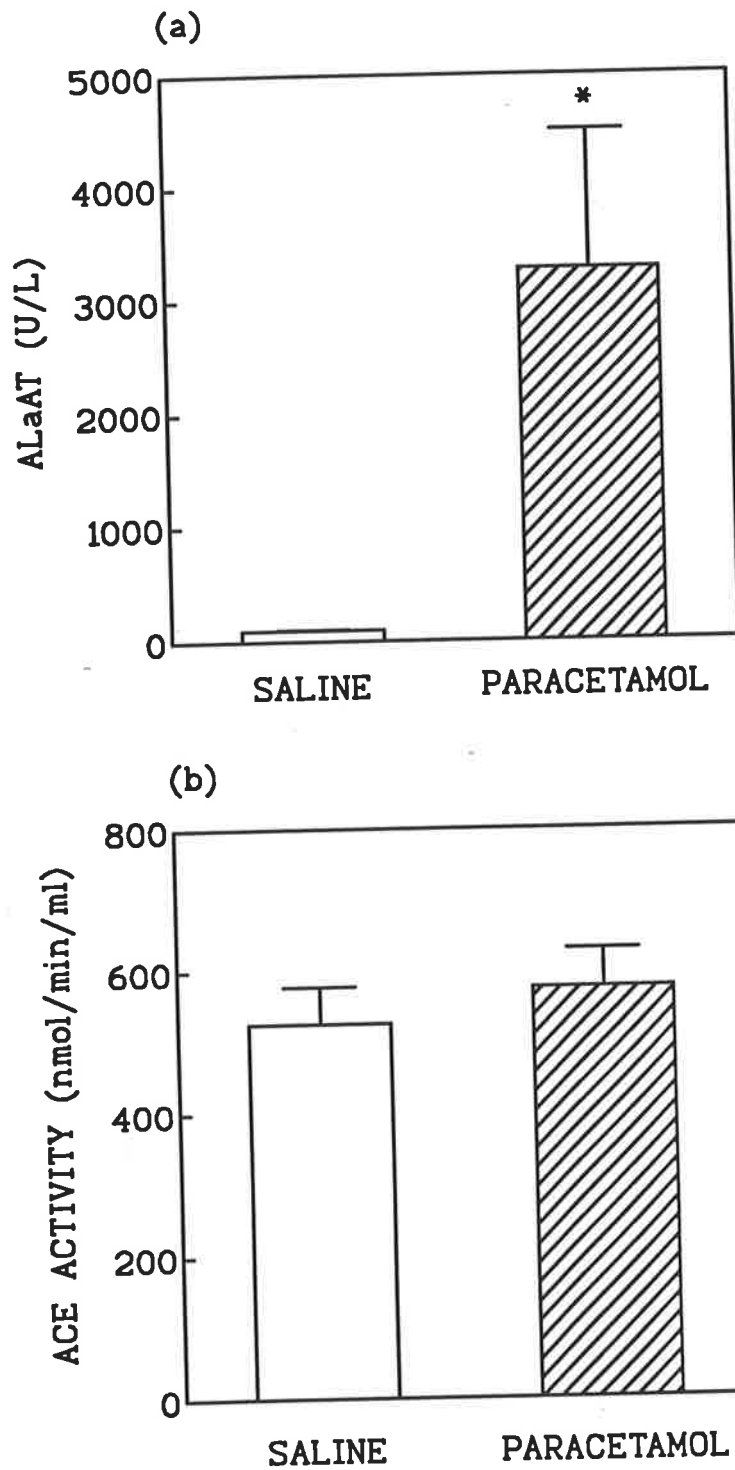


Figure 7.310. The effect of paracetamol on (a) plasma AlaAT activity and (b) plasma ACE activity in mice. Control \square ; paracetamol ▨ . The columns represent the means \pm SEM for $n = 6$ determinations. * $P < 0.05$.

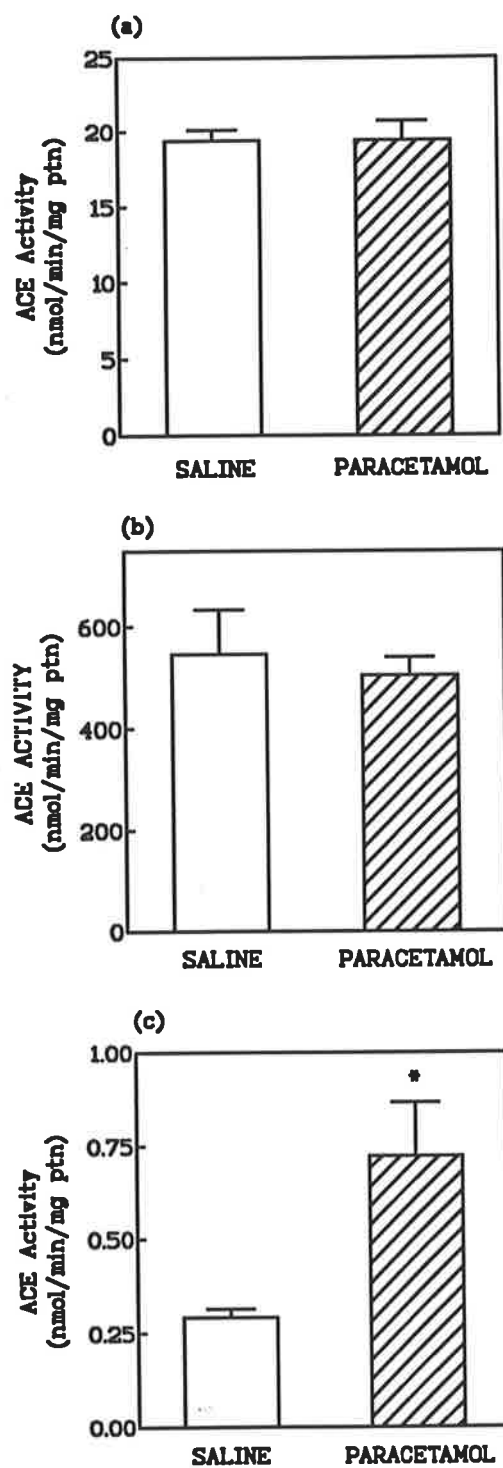


Figure 7.3.11. The effect of paracetamol on (a) kidney (b) lung and (c) liver ACE activity in mice. Control \square ; paracetamol ▨ . The columns represent the means \pm SEM for n = 6 determinations. *P<0.05.

7.4 Discussion

The expression of ACE mRNA in liver is readily detected using the relatively insensitive techniques of slot and Northern analyses. The study described in this chapter established that the ACE gene was expressed in liver at a similar level to that seen in the kidney. This finding was surprising in the light of the extremely low levels of ACE activity which were measured in liver and thus further examination of the expression and activity of hepatic ACE was performed.

The first description of a naturally occurring ACE inhibitor was by Ondetti et al, (1971) who observed ACE inhibitory peptides in snake venom [Ondetti et al, 1971]. An inhibitory tri-peptide of ACE was purified from porcine plasma [Hazato and Kase, 1986] and the presence of an endogenous ACE inhibitor in rat heart has also been reported [Ikemoto et al, 1989]. The rat heart ACE inhibitor is a heat sensitive, sulfhydryl-containing protein of molecular weight greater than 10,000. The present study found that an endogenous inhibitor of ACE was also present in livers from both hypertensive and normotensive rats. The inhibitory activity in liver was reduced while the measurable ACE activity was increased by the sulfhydryl-blocking drug DTNB, which suggested that the inhibitor possessed similar properties to those reported for the inhibitor found in rat heart. The presence of endogenous ACE inhibitors in various tissues is evidence for a further level of control of the activity of the renin angiotensin cascade.

While the results of the present study have provided convincing evidence for the existence of an endogenous ACE inhibitor in the rat liver, the degree of inhibition found did not fully account for the large differences in hepatic ACE mRNA expression and activity. Earlier studies in which ACE activity was measured by indirect methods had found that the liver possessed high levels of the enzyme, and it was suggested that plasma converting enzyme was synthesised in the liver [Page and Bumpus, 1961]. However, support for this hypothesis waned following the results of later studies

which, when using synthetic and specific substrates to determine ACE activity, established that there were low levels of ACE activity in the liver [Oparil et al, 1970; Cushman and Cheung, 1971].

The origins of plasma ACE remain uncertain. It has been assumed that plasma ACE is derived by the sloughing off of membrane-bound enzyme from the endothelial lining of the lung. The evidence for this is based on the similarity between the circulating and pulmonary forms of the enzyme [Das et al, 1977]. Further evidence comes from *in vitro* studies in which ACE activity can be measured in the medium surrounding endothelial cells in culture [Ching et al, 1983; Krulewitz and Fanburg, 1984]. The present study explored the hypothesis that the liver might also be a source of plasma ACE by examining the effect of carbon tetrachloride-induced hepatotoxicity on plasma converting enzyme activity. It would be predicted that, if the liver was a significant source of plasma converting enzyme, then the level of plasma ACE activity would be decreased in rats with hepatotoxicity. The results of the present study do not give clear support to the hypothesis that the liver is a source of plasma ACE, since the level of converting enzyme activity in plasma from rats with damaged livers was not significantly decreased when compared with that seen in rats without liver damage. However, this simplistic interpretation of the results was complicated by the significantly increased levels of converting enzyme activity which were seen in the damaged livers, despite the fact that the mRNA expression for ACE in these livers was not significantly increased. Although there was a trend for the ACE inhibitory activity to be reduced, it appears that there was an initiation of other mechanisms which may have resulted in the maintenance of the level of circulating ACE. The effect of carbon tetrachloride on rat liver ACE activity was not a species- or drug-specific effect since a similar pattern of effects was seen in mice with paracetamol-induced hepatotoxicity. Clearly, the measurement of ACE protein as well as activity will be required to fully elucidate the mechanism of the effects of hepatotoxicity on liver ACE expression and activity.

A dissociation of ACE synthesis and ACE activity has been previously reported. When pulmonary artery endothelial cells were exposed to hypoxia, there was an increase in ACE protein synthesis without a concomitant increase in ACE activity [King et al, 1989]. It was suggested that ACE may be synthesised as an inactive precursor that requires further processing for activation or that ACE may be subject to endogenous inhibition. The study described in this chapter has provided supporting evidence for the latter hypothesis. Further, as summarised in *Figure 7.4.1.*, the results of the present study suggest that mechanisms in addition to transcription and translation are involved in the synthetic pathway that results in the formation of active angiotensin converting enzyme in the liver.

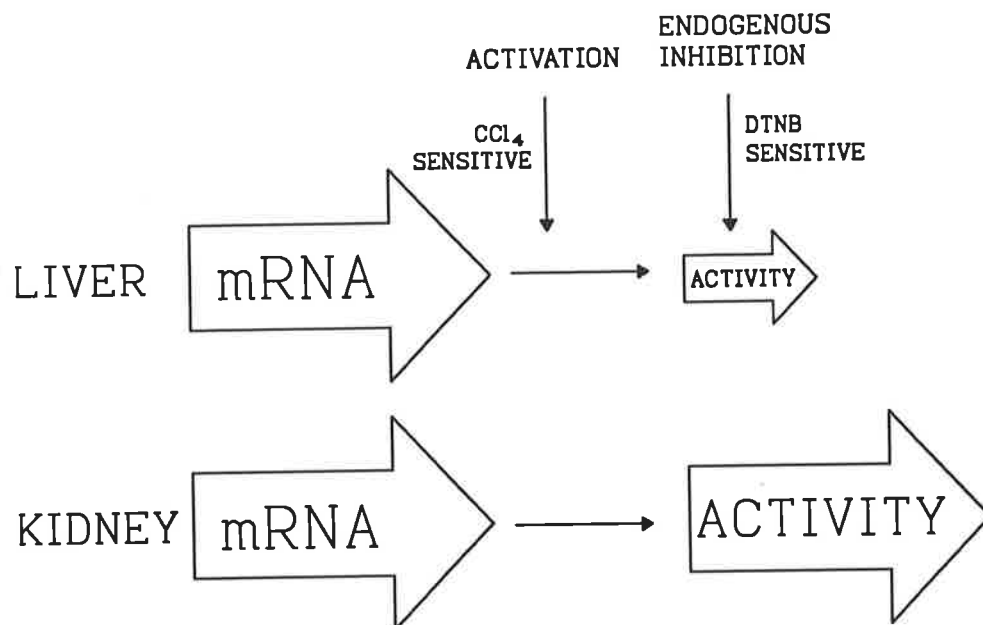


Figure 7.4.1. Summary of factors which influence ACE expression and activity in kidney and liver.

Molecular cloning studies have revealed that somatic ACE consists of two domains (designated the N- and C-domains) which are functionally and structurally distinct (1.2.4) [Wei et al, 1991a; Wei et al, 1991b; Wei et al, 1991c]. While both domains show similar K_m values for HHL, the rate of hydrolysis is 10-fold lower for the N-domain. Furthermore, the optimum chloride concentration for the N-domain is considerably lower (10 mM) than that observed for the C-domain (800 mM). The high level of ACE mRNA but low level of activity that is observed in the liver raises the possibility that in this tissue the N-domain is responsible for the majority of the activity and that following hepatotoxicity the C-domain makes a relatively greater contribution to the total ACE activity in the liver. The large increase in liver ACE activity without a concomitant increase in ACE mRNA levels which was observed in rats with hepatotoxicity is consistent with this hypothesis. However, in liver homogenates from both control and carbon tetrachloride-treated rats the ACE activity which was observed when the chloride concentration was 10 mM (the reported optimum concentration for the N-domain) was negligible and the optimum chloride concentration was clearly at least 305 mM. The large increase which occurred in the measured ACE activity with increasing chloride ion concentration which was observed in the livers from carbon tetrachloride-treated rats paralleled the increase which was also observed in the livers from control animals. Thus it appears that the N-domain of ACE does not exert a major influence in the overall activity of liver ACE.

In conclusion, the present study highlights the complex mechanisms involved in the regulation of hepatic ACE activity and suggest that there is an uncoupling of ACE gene expression and activity in the liver. It is apparent that there is a significant role for non-genomic processes in the regulation of rat hepatic ACE activity. Furthermore, the results emphasise the requirement for studies which are designed to investigate the regulation of ACE to measure the relative changes in mRNA expression, ACE protein, endogenous ACE inhibition and also ACE activity.

CHAPTER 8

**THE EXPRESSION AND LOCALISATION OF THE ANGIOTENSIN
CONVERTING ENZYME mRNA IN HUMAN ADIPOSE TISSUE**

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8.1 Introduction

In the past decade there has been an accumulation of evidence to suggest that as well as the classical endocrine RAS, independently regulated tissue RASs are responsible for the local production of AII which may act in a paracrine or autocrine manner (1.2.1, 1.4) [Dzau, 1986; Campbell, 1987]. In rats, the expression of angiotensinogen mRNA has been demonstrated in several tissues, including adipose tissue surrounding the aorta and mesentery [Campbell and Habener, 1987; Cassis et al, 1988a]. Angiotensinogen secretion has also been demonstrated *in vitro* from fat pads and from isolated adipocyte lines [Cassis et al, 1988a; Cassis et al, 1988b]. Recently, it has been demonstrated that angiotensinogen mRNA expression in adipose tissue, but not liver, is subject to nutritional regulation [Frederich et al, 1992]. It was proposed by those authors that a local renin-angiotensin system may regulate adipose tissue blood supply and the efflux of fatty acids from fat. This hypothesis is critically dependent upon the demonstration of local expression of the ACE gene in adipocytes. While evidence for the expression of ACE mRNA in adipose tissue from rats has been described previously (3.3.6) the study described in this chapter was conducted to further explore this possibility and was based upon an analysis of the gene expression of ACE in adipose tissue obtained from humans.

8.2 Methods

8.2.1 Patients and tissue treatments

Human adipose tissue (both subcutaneous and extraperitoneal in location) was obtained from five subjects (four females, one male) undergoing gastroplasty for morbid obesity. The characteristics (mean \pm SEM) of the group were: age: 27 ± 1.3 years, weight: 122 ± 9.0 kg, Blood pressure: $131 \pm 5.2 / 75 \pm 6.5$ mm Hg. A section of adipose tissue from four patients was frozen at -80°C prior to extraction of RNA and

Northern analysis. For three patients, a segment of the adipose tissue was placed in 4% paraformaldehyde fixative overnight at 4°C (2.1.3) for subsequent *in situ* hybridisation studies.

8.2.2 RNA analyses

Preparation of full-length and hydrolysed probes (2.3.3), isolation of RNA from tissue homogenates (2.3.4), Northern analysis (2.3.6), hybridisation (2.3.7), autoradiography (2.3.8), and *in situ* hybridisation (2.3.10) were performed as previously described.

8.3 Results

8.3.1 RNA recovery

The recovery of RNA from both subcutaneous and extraperitoneal human adipose tissue was extremely low. For the subcutaneous tissue from patient #4 the yield was just 13 µg/g tissue; for the remaining tissues, the recoveries were 62 ± 2.8 µg/g tissue. In the studies described in previous chapters, substantially higher yields were routinely obtained from rat liver (4-5 mg/g tissue) and rat kidney (3-4 mg/g).

8.3.2 Northern analysis

Following electrophoresis, the ethidium bromide-stained RNA was transferred to a nylon membrane (Figure 8.3.1a). The RNA which was extracted from the subcutaneous and extraperitoneal tissues from patient #1 (lanes 1 and 2) appeared degraded, but all other samples provided RNA of good quality. It should be noted that for lanes 1-7, 10µg of total RNA was applied to each well, but for lane 8, only 2.5 µg

was applied due to the very much lower recovery of RNA from this sample of the tissue.

When the RNA was probed with ^{35}S -cRNA for human ACE, a single hybridisation band of the expected size was observed in both subcutaneous (lanes 3, 5 and 7) and extraperitoneal (lanes 4, 6 and 8) adipose tissue (*Figure 8.3.1b*). This band was not observed in degraded RNA (lanes 1 and 2).

8.3.3 Localisation of ACE mRNA

Following *in situ* hybridisation of paraffin-embedded tissue sections with hydrolysed ^{35}S -cRNA, numerous silver grains, representing expression of the ACE gene, were observed overlying adipocytes in both subcutaneous (*Figure 8.3.2a*) and extraperitoneal (not shown) adipose tissue. The density of silver grains was substantially less when adjacent sections were probed with ^{35}S -mRNA (*Figure 8.3.2b*) representing non-specific binding of radiolabelled probe.

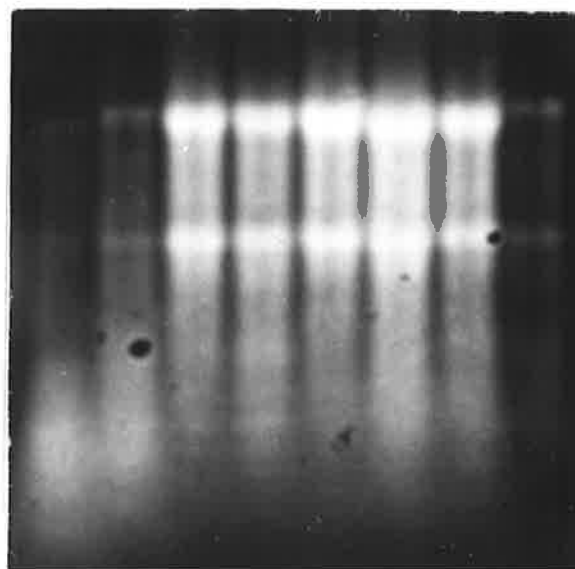
Figure 8.3.1 (a) Ethidium bromide-stained RNA from human adipose tissue following Northern transfer to a nylon membrane. (b) Autoradiograph following hybridisation with ^{35}S -cRNA for ACE. *Lane 1*, patient #1, subcutaneous; *lane 2*, patient #1, extraperitoneal; *lane 3*, patient #2, subcutaneous; *lane 4*, patient #2, extraperitoneal; *lane 5*, patient #3, subcutaneous; *lane 6*, patient #3, extraperitoneal; *lane 7*, patient #4, subcutaneous; *lane 8*, patient #4, extraperitoneal. Note that $10\mu\text{g}$ of total RNA was applied to each well except for *lane 8* to which $2.5\mu\text{g}$ was applied.

(a)

1 2 3 4 5 6 7 8

28s→

18s→



(b)

1 2 3 4 5 6 7 8

28s→

18s→

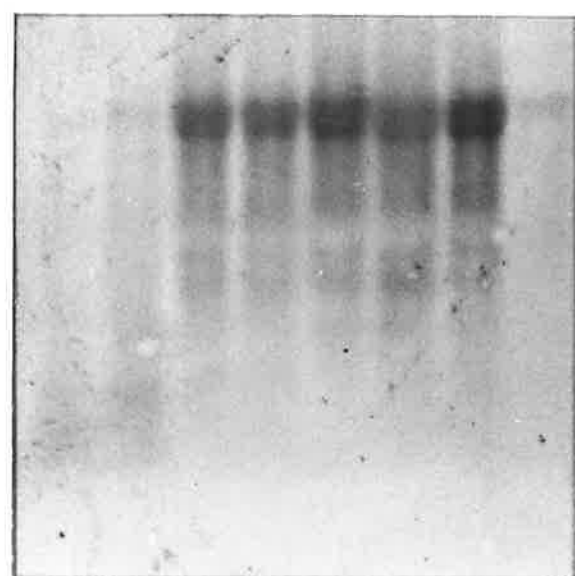
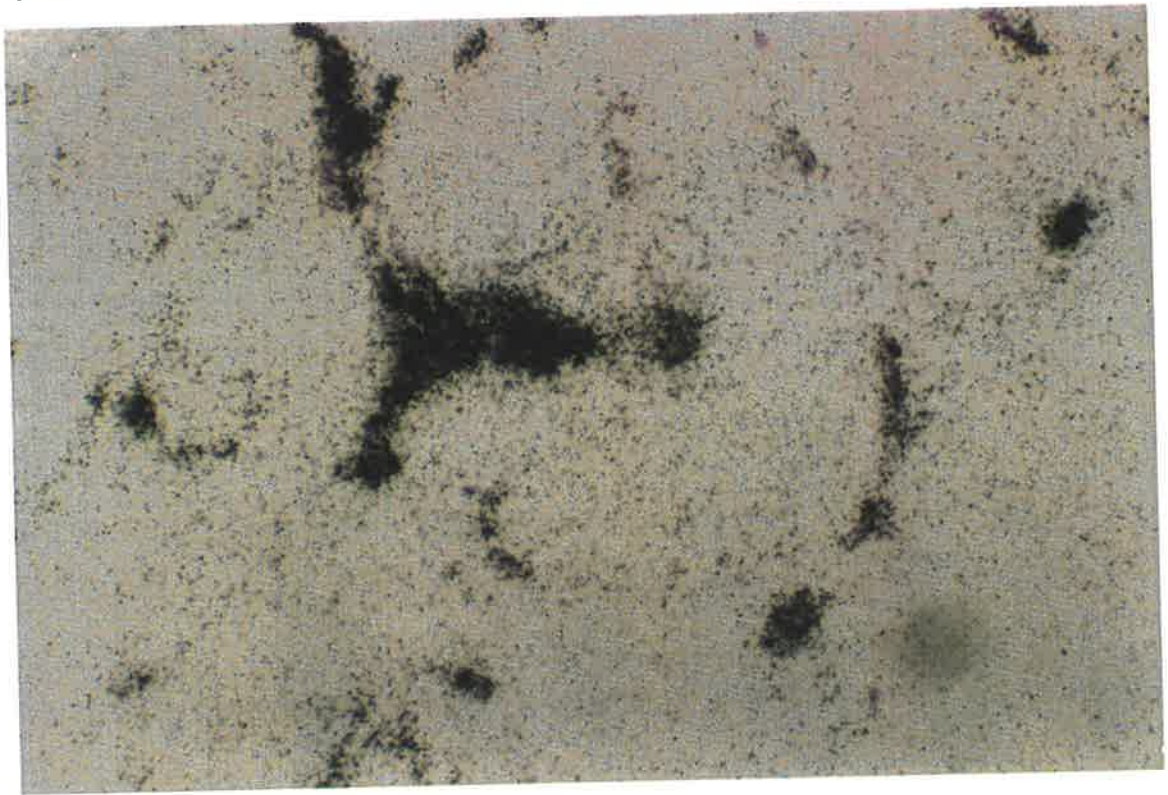


Figure 8.3.2. In situ hybridisation pattern for (a) ACE and (b) negative control, seen in human subcutaneous adipose tissue.

(a)



(b)



8.4 Discussion

The powerful techniques used in studies within the field of molecular biology have provided conclusive evidence for the existence of renin-angiotensin systems at sites distinct from those considered to be part of the classical endocrine RAS. The mRNA expression for the genes of the RAS has been demonstrated in many tissues and these results provide convincing proof of the local synthesis of the RAS components [Dzau, 1986; Campbell, 1987; Dzau, 1987]. Almost all of these studies have examined the gene expression of the RAS in animals rather than humans. This may be due to the relative ease of obtaining tissues samples for RNA extraction from animals. In some tissues the level of RAS mRNA is low and there is the additional difficulty in obtaining large quantities of viable tissue from humans. The first systematic examination of renin, angiotensinogen and ACE gene expression in human tissues used the extremely sensitive polymerase chain reaction (PCR). The expression of all three genes was detected in renal cortex and medulla, adrenal gland, aorta, saphenous and umbilical vein and the heart [Paul et al, 1993].

It has been proposed that adipose tissue also possesses a local RAS. In the rat, angiotensinogen is expressed in fat pads from many sites, including the adipose tissue surrounding blood vessels [Campbell and Habener, 1987; Cassis et al, 1988a] (*Chapter 3*). The study described in this chapter explored the possibility that the ACE gene is expressed in human adipose tissue. Relatively large amounts of adipose tissue are discarded during the surgical procedure of gastroplasty and it was therefore possible to obtain sufficient material to use the relatively insensitive technique of Northern analysis to demonstrate ACE mRNA in this tissue. The results indicate that the ACE gene is expressed in both subcutaneous and extraperitoneal adipose tissue from humans. Although no attempt was made to quantify the ACE mRNA levels, it was apparent from examination of the autoradiograph that the relative level of expression was similar in both types of adipose tissue examined. The sensitive technique of *in situ*

hybridisation was used to identify the sites of expression of ACE within the tissue. In both subcutaneous and extraperitoneal tissue, ACE was expressed in adipocytes.

There is abundant epidemiological evidence which indicates an association between hypertension and obesity. Several studies have shown a correlation between body weight and blood pressure [Dustan, 1983] and weight loss is associated with a corresponding decrease in blood pressure in hypertensive humans [Reisen et al, 1978; Sowers et al, 1982]. It has also been proposed that insulin resistance and consequently hyperinsulinaemia, is the link between hypertension and obesity, although the mechanism responsible is not known [Hall et al, 1992]. It has been reported that angiotensinogen is subject to tissue specific nutritional regulation [Frederich et al, 1992]. Under fasting conditions, angiotensinogen mRNA and angiotensinogen secretion was drastically reduced in epididymal fat pads but not liver. Refeeding was associated with an enhancement of angiotensinogen. Systolic blood pressure was modulated in a fashion that paralleled the adipocyte angiotensinogen expression and secretion [Frederich et al, 1992]. Based on these results, and the results of earlier studies [Belfrage et al, 1979; Bulow and Madsen, 1981], an hypothesis was proposed that may provide an explanation for the association between obesity, hypertension and insulin resistance. It has been suggested that local adipocyte AII production plays an important role in regulating tissue blood supply and fatty acid efflux from fat. This hypothesis is supported by studies in both animals and humans where administration of an ACE inhibitor is associated with significant weight loss as well as the expected decrease in blood pressure [Enalapril in Hypertension Study Group (UK), 1984; McGrath et al, 1990].

In conclusion, the study described in this chapter provides evidence for the expression of the ACE gene in human adipocytes. The results provide support for the hypothesis that local RAS expression in adipocytes may play a role in elevating blood pressure in an obese hypertensive subject.

CHAPTER 9

GENERAL CONCLUSIONS

The study of human primary hypertension has been greatly facilitated by the development of animal models of the disorder, in which high blood pressure is inherited and thus develops without experimental intervention. Although there are several genetic animal models of hypertension, the spontaneously hypertensive rat, which was developed by Okamoto and Aoki in 1963, is the most widely available and has been the most extensively studied. The SHR is generally regarded to be an excellent model for primary hypertension as it shares many of the characteristics and sequelae of the human disorder.

Despite the fact the SHR has been studied intensively by numerous investigators for more than 30 years, the precise pathophysiological mechanisms responsible for the development of hypertension have not been elucidated. A common approach in the investigation of the aetiology of hypertension in the SHR (an approach also adopted in the studies described in this thesis) is to subject the SHR and its normotensive control, the WKY, to pharmacological manipulation and then to examine a candidate parameter and compare the responses of the two rat strains with respect to this parameter. Although this approach has so far proved to be unsuccessful in defining the precise aetiology of hypertension, due mainly to fact that hypertension appears to be caused by the interaction of a number of factors, the information gained from these studies has contributed greatly to the knowledge of basic physiological mechanisms, particularly those involved in cardiovascular homeostasis.

Although initial investigations of the SHR discounted an abnormality of the renin-angiotensin system, the profound blood pressure-lowering effects of the angiotensin-converting enzyme inhibitors in this rat strain have led to a reappraisal of the contribution of the RAS to the development of hypertension. In particular, attention has focussed on the gene expression of the RAS components and on local tissue RASs, which appear to be regulated independently of the circulating system. The studies described in this thesis were undertaken to further explore the gene expression of the RAS in the SHR.

A major focus has been the investigation of the cellular localisation of the gene expression of the components of the RAS in the kidney, liver and the mesenteric vascular bed of SHR and WKY, in order to determine whether hypertension is associated with RAS expression in cell types different to that in the normotensive animals. While the results of this study were negative in that there were no apparent differences in the spatial localisation of RAS expression in the hypertensive rats, even during development, the results did provide new information about the expression of the RAS components, in particular ACE. The co-localisation of ACE and angiotensinogen expression in the proximal tubules of the kidney correlates with evidence from functional studies which have shown that AII has a direct action at this site. Angiotensinogen expression in the proximal tubules of the kidney suggests that it is locally produced there. Circulating AII also plays a major role in kidney function.

The *in situ* hybridisation study of RAS expression in the mesenteric vascular bed from SHR and WKY demonstrated, for the first time, that adipocytes express the ACE gene. Furthermore, this significant finding was extended to include human tissues where it was demonstrated that the ACE gene is expressed in adipocytes from both subcutaneous and extraperitoneal adipose tissue in males and females. This novel demonstration of ACE expression in adipocytes, together with the previous documentation of tissue-specific nutritional regulation of angiotensinogen expression at this site [Frederich et al, 1992], provide an explanation for the abundant

epidemiological evidence which indicates an association between hypertension and obesity. However, since the patients which were examined in this study were not hypertensive, despite presenting with morbid obesity, this emphasises the fact that obesity alone is not a causative factor in hypertension. Indeed, obesity is not necessarily a requirement for hypertension since many hypertensive patients (and the SHR) are not overweight when compared with normotensive controls. However, since hypertension is a heterogenous disorder, with many subsets each having their own characteristics, it is likely that the overproduction of AII in adipocytes in association with obesity may lead to high blood pressure in some hypertensive patients.

The two anti-hypertensive regimens which were used in the studies described in this thesis, i.e. neonatal administration of OHDA and treatment of young SHR with the ACE inhibitor captopril, share the characteristic of continued suppression of blood pressure long after drug treatment is ceased. In the case of OHDA, the mechanism of the continued low blood pressure can be attributed to the permanent destruction of the peripheral sympathetic nerves, but it is not known why blood pressure remains low following withdrawal of ACE inhibitor treatment. Short term treatment with ACE inhibitors has a dramatic effect on the expression of the renin gene in the kidney and it was hypothesised that an effect on the expression of renin might persist or that renin gene expression might rebound to a low level following release from ACE-inhibitor treatment. However, the kidney renin mRNA levels as well as the cellular localisation of kidney renin expression in released animals were not significantly different from untreated controls. Angiotensinogen and ACE expression in the kidney were also not influenced by captopril and thus it can be concluded that abnormal expression of the RAS components in the kidney is not responsible for the lower blood pressure following release.

An unexpected finding in this study was the elevated levels of ACE mRNA in the livers of captopril-treated rats which persisted in released animals. It is possible that the elevation of hepatic ACE mRNA is a reflex increase in response to the lowered blood

pressure in captopril treated and released animals. This is probably not the case for two reasons. Firstly, in the captopril-release study, hepatic ACE mRNA levels were elevated to a similar degree in both SHR and WKY, even though the magnitude of the difference in blood pressure between the control and released animals was far greater in the SHR. Secondly, at the time of sacrifice, the drop in blood pressure mediated by sympathectomy and that present after captopril release were similar, but the sympathectomised animals did not have elevated liver ACE mRNA levels. Therefore, the elevation in hepatic ACE mRNA levels which was observed in response to captopril treatment and which was maintained following release from captopril treatment is likely to be due to a specific effect of the ACE inhibitor and is not a consequence of the lowered blood pressure *per se*. It is likely that in the liver, the expression of the ACE gene is subject to negative feedback control by AII.

There are many sites of interaction between the RAS and the SNS. Thus, it was hypothesised that ablation of the sympathetic nerves at birth might have a prolonged effect on the activity of the RAS. This prolonged effect might have gone either way. That is, since sympathetic nerve stimulation is known to enhance the activity of the RAS, it could be predicted that sympathectomy would result in a decreased activity of the RAS. In contrast, since sympathectomy results in a lowering of blood pressure, then perhaps there might have been a reflex increase in the activity of the RAS. Neither of these two situations occurred. It is possible that acute disruption of the SNS induces changes in the RAS, although in the chronic situation other mechanisms restore the normal level of activity of the RAS.

The possibility that AII was taken up into sympathetic nerves (in a similar fashion to adrenaline) prior to its facilitating effect on sympathetic transmission, and that this process was abnormal in hypertensive rats was also examined. This mechanism would provide an explanation for the apparent requirement for both an intact SNS and a fully functional RAS for the development of hypertension in the SHR. There was no apparent influence of the level of innervation on the uptake of AII into a number of

tissues which indicates that the proposed mechanism is not relevant. However, despite the finding that AII is not taken up into sympathetic nerves, this does not preclude the possibility that it might be synthesised in the nerves and released as a co-transmitter in a manner analogous to neuropeptide Y. This possibility could be investigated by examining the gene expression of the RAS as well as ACE activity in sympathetic ganglia. Due to the extremely small size of the sympathetic ganglia (at least in the rat) the sensitive technique of PCR would be required to examine gene expression in this tissue.

A significant and surprising finding was the relatively high level of expression of the ACE gene in the liver, despite the very low levels of activity in this tissue. ACE mRNA was detected using the relatively insensitive techniques of slot and Northern analysis and, using the technique of *in situ* hybridisation, the expression was localised to hepatocytes as well as the endothelial lining of the blood vessels in the liver. Further investigation of hepatic ACE revealed the presence of an endogenous inhibitor, which apparently contained a sulfhydryl moiety in its structure. The results of this study clearly represent preliminary findings that require significant further examination. For example, it will be necessary to isolate the proposed inhibitor from liver in order to demonstrate that the inhibition of the plasma ACE which was observed in the presence of liver homogenates was not merely a technical artefact of the methodology employed. It is possible that the addition of the liver homogenates to plasma disrupts the association of zinc or other ions with the ACE, which results in a decrease in activity. It is also possible that the inhibition is non-specific in nature and due instead to the presence of NADH or some other similar molecule in the liver homogenate. Finally, evidence of the physiological relevance of the endogenous inhibitor will require demonstration of the co-localisation of the inhibitor and ACE in an *in vivo* situation.

Studies which have investigated the regulation of ACE have been few in number compared with for, for example, renin. The effect of liver damage on hepatic ACE in both rats and mice has provided further evidence to confirm previous findings of a

dissociation between ACE gene expression and activity. The elucidation of the precise relationship between ACE mRNA synthesis and enzyme activity will require the measurement of ACE protein in order to determine whether ACE is synthesised as an inactive precursor that requires further processing for activation. Post-translational activation is commonly required for many proteins (including renin) and so it will not be surprising if this process is also required for ACE.

In conclusion, the studies described in this thesis indicate several areas of research which warrant further investigation, including the expression and activity of the RAS in adipose tissue, the regulation of hepatic ACE and the nature and relevance of endogenous ACE inhibitors. The results presented provide new information, in particular relating to ACE expression and activity, which highlight the complex and fascinating nature of the renin-angiotensin system.

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Appendix I

The results of the electrophoretic screen carried out in 1991 on rats at the CSIRO breeding facility are shown in Table I.

Table I. Strain profiles at thirteen genetic markers for the three strains.

| | SHR n=8 | SHRSP n=8 | WKY n=8 |
|---------------|------------|--------------|------------|
| <i>Acon-1</i> | b | b | b |
| <i>Ahd-2</i> | b | b | b |
| <i>Ahd-c</i> | b | b | b |
| <i>Akp-1</i> | a | b | b |
| <i>Alp-1</i> | a | b | b |
| <i>Br-1</i> | b | b | a |
| <i>Es-2</i> | a | a | d |
| <i>Es-4</i> | a | a | b |
| <i>Es-10</i> | a | a | b |
| <i>Fh</i> | b | b | b |
| <i>Hbb</i> | a | a | a |
| <i>Pep-3</i> | a | a | b |
| <i>Pk</i> | a | a | a |

The electrophoretic screen was carried out by M. Adams, Evolutionary Biology Unit, South Australian Museum, North terrace, Adelaide, SA 500.

Appendix II

This section describes the preparation of solutions and equipment.

Chromic Acid

Chromic acid was used to clean microscope slides and coverslips. Potassium dichromate (18.4 g) was added to water (360 ml), then concentrated sulphuric acid (648 ml) was added at a rate of 20-30 ml every 5 to 10 minutes with a swirling action. If the acid was added too quickly, the extreme heat which was generated mixture cracked the glass flask.

APES (3-aminopropyltriethoxy-silane) slide preparation

Microscope slides were treated with APES to facilitate the adherence of tissue sections during *in situ* hybridisation. The slides were cleaned by soaking in chromic acid overnight. The slides were washed in running water for 2 hours, rinsed several times in deionised water and dried at 37°C. Acid washed slides were immersed in APES (2% in ethanol) for 5 seconds, followed by washing in ethanol (3x 5 seconds) and water (3x 5 seconds). After the slides had been dried at 37°C overnight, they were stored wrapped in aluminium foil at room temperature for up to 4 months.

Due to the toxicity of APES, extreme care was taken when using this substance.

APES-treated slides were activated on the evening prior to day on which they were to be used for the mounting of tissue sections. Dry APES-coated slides were soaked in 10% Gluteraldehyde in PBS (pH 7) for 30 minutes, followed by washing in deionised water (3x 5 minutes). The slides were then dried at 37°C overnight.

Tissue sections were mounted on the activated slides using Elmer's Glue.

Siliconisation of coverslips

Glass coverslips were siliconised in order to minimise the binding of the hybridisation solution during *in situ* hybridisation. Coverslips of various sizes were cleaned by soaking in chromic acid overnight. (The coverslips were posted into the acid solution individually to ensure that each one was coated with acid.) The coverslips were washed in running water for 2 hours, rinsed several times in deionised water and dried

at 37°C. Acid washed coverslips were soaked in dimethyldichlorosilane (5% in chloroform) for 15 minutes, then rinsed in deionised water (3x 5 seconds). (The coverslips were posted into the siliconising solution individually to ensure that each one was coated with silicon.) The coverslips were then dried at 37°C overnight, or at 80°C for 2 hours. The dried siliconised coverslips were wrapped in aluminium foil in packs of 10, with coverslips being separated from each other by aluminium foil inserts. The siliconised coverslips were then sterilised by baking at 200°C for 5 hours. Sterile siliconised coverslips were stored for several weeks at room temperature. Subsequently, sterile coverslips were handled only with sterile forceps.

Due to the toxicity of dimethyldichlorosilane, extreme care was taken when using this substance.

Tris-saturated phenol

Tris-saturated phenol was used in the analyses of RNA. Phenol was melted at 65°C under a fume hood. 8-hydroxyquinoline (1 mg/100 ml) and an equal volume of water was added. The mixture was shaken vigorously and the aqueous and organic phases allowed to separate at 4°C overnight. The upper aqueous phase was aspirated and then water was added again -sufficient to cover the phenol. The bottle was wrapped in aluminium foil and stored at 4°C until use (for up to 4 months).

When Tris-saturated phenol was required, an aliquot of water-saturated phenol was removed from the stock. An equal volume of 0.5 M Tris, pH 8 was added and after vigorous mixing, the solution was centrifuged at 2000g for 5 minutes at room temperature. This process was repeated using 0.5 m Tris, pH 8 and then repeated twice again using 0.1 M Tris, pH 8. If necessary, Tris-saturated phenol was stored, protected from light, for up to one month.

Extreme care was taken when using phenol due to the toxic nature of this compound.

Preparation of COMT (Catechol-O-methyl transferase)

COMT, for use in the measurement of noradrenaline (2.2.3), was prepared from rat liver [Axelrod and Tomchick, 1958].

Livers were removed from 12 stunned and exsanguinated male Sprague-Dawley rats. All subsequent procedures were performed at 4°C

The livers were homogenised in isotonic potassium chloride (1.11%, 4 ml/g) and centrifuged at 78,000 g for 30 minutes. The soluble supernatant was filtered through glass wool and 1M acetic acid was added, dropwise with stirring, until the pH reached 5. After being allowed to stand for 10 minutes the solution was centrifuged at 10,000 g for 10 minutes.

Ammonium sulphate was added to the supernatant to give 0-30% saturation (176 g/l). After being allowed to stand for 10 minutes the solution was centrifuged at 10,000 g for 10 minutes.

Ammonium sulphate was added to the supernatant to give 30-50% saturation (127 g/l). After being allowed to stand for 10 minutes the solution was centrifuged at 10,000 g for 10 minutes. The supernatant was discarded and the precipitate was dissolved in distilled water (50 mls).

The enzyme solution was dialysed for 12 hours against 6l 1 mM sodium phosphate (pH 7), followed by further dialysis for 6 hours against 6l 1 mM Tris (pH 7). The enzyme solution was stored frozen in 1 ml aliquots at -20°C for no longer than 6 months.

APPENDIX III

This section lists the source of all chemicals, reagents and kits used in the studies described in this thesis.

Except for those listed below, all chemicals and reagents were obtained from Sigma Chemical Company. Unless the chemical was available in "Molecular Biology Grade", standard laboratory grade (Univar) was used.

Amersham

Hybond N nylon membranes
LM-1 Nuclear Emulsion

BDH

Ether
Chloroform
Methanol

Beckman

Ready Value Scintillation Fluid

Boehringer-Mannheim

Restriction Enzymes: Bam H1, EcoR1, Bgl 1, Hind 111
Salmon Sperm DNA
RNase A
RNase T1

Bresatec Australia Limited

Message Maker *in vitro* Transcription Kit
SPP-1 DNA molecular weight markers

Dupont

³⁵S-Uridine Triphosphate
5-L-isoleucine[tyrosol-3,-³H(N)]
[Glycine-1-¹⁴C]Hippuryl-L-Histidyl-L-Leucine
³H-S-adenosyl-methionine

Ilford

Phenisol X-ray Developer

K5 Nuclear Track Emulsion

Kodak

Rapid Fixer

D19 developer

Merck

Silica Gel TLC Plates with Fluorescence Indicator

Losartan

Promega

T3 RNA polymerase

Hoescht

Bisbenzimidazole

Squibb

Captopril