Endocrine Control of DNA Synthesis in Renal Adrenal and Vascular Tissues of the Rat *in vivo*

By

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Summary

The aims of this study were firstly to investigate the influence of the renin-angiotensin system on growth of various target tissues (kidney, adrenal gland and blood vessels). Secondly, to determine whether changes in growth were a direct consequence of trophic actions of angiotensin II (AII) or secondary to changes in blood pressure. Thirdly to investigate the interactions of the renin-angiotensin system with other factors (nitric oxide synthesis, glucocorticoid hormone) in the control of growth of target tissues.

In each group of rats bromodeoxyuridine (BrdUrd), a thymidine analogue, was given by subcutaneous infusion continuously for two weeks during various treatments. The animals were killed and DNA synthesis was assessed histologically by calculating a BrdUrd index for each cell type. Kidney sections were immunocytochemically stained for renin content. Various morphometric measurements were made in adrenal and vascular tissues.

The renin-angiotensin system was manipulated in three ways: i) by infusing AII subcutaneously, ii) by feeding a low or supplemented sodium diet, iii) by administering captopril in the drinking water. The role of nitric oxide in the regulation of DNA synthesis was investigated by treating rats for up to four weeks with an inhibitor of arginine synthase, L-NAME. The effect of glucocorticoid hormone was tested by implanting a dexamethasone pellet subcutaneously.

Blood Vessels:

In mesenteric blood vessels, AII infusion increased blood pressure and stimulated DNA synthesis in the endothelium, media and adventitia of arteries. Compared with rats fed a sodium supplemented diet, rats given low sodium food had higher BrdUrd indices in all parts of the blood vessels. Captopril lowered the BrdUrd index in the media and adventitia but had no effect on the endothelium. Neither L-NAME nor dexamethasone significantly affected DNA synthesis in vascular smooth muscle cells. DNA synthesis in mesenteric veins was not affected by any treatment. Medial area in transverse sections of arteries was greater after AII treatment. None of the other treatments caused vascular hypertrophy.

Adrenal Gland:

The BrdUrd indices of epithelial and non-epithelial cells were assessed separately in six areas of the adrenal gland: the zona glomerulosa, the zona intermedia, the outer and inner fasciculata, the zona reticularis and the adrenal medulla. AII and low dietary sodium increased the BrdUrd index significantly in the zona glomerulosa and in the zona reticularis but had no effect on the zona fasciculata. Treatment with captopril caused zona glomerulosa atrophy but had no effect on the BrdUrd index. Captopril reduced DNA synthesis in the reticularis. There was hypertrophy of the zona glomerulosa after treatment with either AII or low sodium whereas, high sodium, L-NAME, dexamethasone and captopril caused atrophy. Changes in the BrdUrd index in the medulla appeared to be controlled in a compensatory manner by blood pressure. Treatments which tend to increase blood pressure (AII, dexamethasone, L-NAME) reduced the BrdUrd index whereas captopril caused an increase.

Kidney:

The number of renin secreting cells in the kidney was increased by low sodium and also by captopril. Despite the increase in the number of cells, there was no evidence of DNA synthesis in any renin-secreting cell. BrdUrd indices were calculated for glomerular, tubular and interstitial cells. AII increased the BrdUrd index in glomerular cells, captopril caused an decrease but low and high dietary sodium, L-NAME and dexamethasone had no effect. Dexamethasone increased the BrdUrd index in tubule cells and L-NAME increased DNA synthesis in interstitial cells. AII, captopril or dietary sodium manipulation had no effect on DNA synthesis in tubules or interstitial cells.

Conclusions:

The control of growth of the cardiovascular system is complex, involving direct actions of the renin-angiotensin system. Angiotensin II increases DNA synthesis of many cell types *in vivo*. Angiotensin II directly regulates DNA synthesis in the adrenal cortex and in blood vessels. Some of the effects of AII are compensatory. Many of the effects of AII on growth are influenced by blood pressure and by interactions with other factors such as nitric oxide and glucocorticoid hormones.

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Figure 1.7 on page 10 is a modification of figure 3 by Navar & Rosivall. *In* Clinical Endocrinology and Metabolism. Edited by Bailliere's Tindall, 1989.

List of Abbreviations

Angiotensin II	AII
5-Bromo-2'-Deoxyuridine	BrdUrd
NG-nitro-L-arginine methyl ester	L-NAME
Dexamethasone	Dex
Arginine vasopressin	AVP
Aldosterone	Aldo
Sodium	Na+
Potassium	K+
Endothelial derived relaxing factor	EDRF
Nitric oxide	NO
Antidiuretic hormone	ADH
Adrenocorticotrophic hormone	ACTH
Atrial natriuretic peptide	ANP
Deoxycorticosterone	DOC
Juxtaglomerular apparatus	JGA
Juxtaglomerular	JG
Distal convoluted tubule	DCT
Proximal convoulted tubule	PCT
Afferent arteriole	AA
Glomerulus	G
Bowman's space	BS
Macula densa	MD
Extracellular fluid	ECF
Intracellular calcium concentration	[Ca2+]
Cyclic adenosine monophosphate	Cyclic-AMP
Cyclic guanosine monophosphate	Cyclic-GMP
Inositol 1,4,5-triphosphate	IP3
Phosphatidylinositol	IP2
Phosphate buffered saline	PBS
Normal swine serum	NSS
Normal rabbit serum	NRS
Diaminobenzidine	DAB
Hydrochloric acid	HCl
Plasma renin activity	PRA
Glomerular filtration rate	GFR
Proliferating cell nuclear antigen	PCNA

Spontaneously hypertensive rat	SHR
Wistar-Kyoto rat	WKY
Vascular smooth muscle cell	VSMC
Angiotensin 1 receptor	AT1
Angiotensin 2 receptor	AT2

Chapter 1 General Introduction

1.1. *Endocrine Control of Cell Growth:*

Functional changes in tissues influence their structure. Alterations within cells or in the extracellular matrix may be associated with the differentiation and development, maintenance, physiological adaptation, tumorigenesis or death of tissues. A complex series of signals leads to either a net increase in tissue mass by cell growth (with or without cell proliferation) or a reduction by atrophy and programmed cell death (apoptosis). This thesis will be concerned with changes in cell growth during adaptation to various physiological states in which the activity of the renin-angiotensin system and the cardiovascular system are altered.

The three main tissues responsible for the control of fluid and electrolyte homeostasis and blood pressure are the adrenal glands, the kidneys and the blood vessels. These tissues are under the control of endocrine and nervous systems which regulate their function: the secretory activity of the adrenal gland, the excretory role of the kidney and the vasoconstriction and vasodilation of blood vessels. In addition, these systems have trophic actions which regulate cell growth. Most tissues consist of a heterogeneous population of cells which are differentially regulated according to the imposed homeostatic demands. This means that the mass of a tissue can increase in response to an increased physiological demand or, conversely, if the demand is reduced and the stimulus for growth is removed, the tissue mass decreases.

Although tissues respond to longterm physiological stimulation, cell proliferation is also required to sustain a normal population of cells within each tissue. The rate of cell turnover varies: epithelial cells divide at a slower rate than parenchymal cells which, in turn, proliferate slower than smooth muscle cells. Cells which proliferate rapidly are often those which are susceptible to damage, such as the epithelial lining of the gut (1), or are also subject to increased apoptosis (1, 2) so as to maintain tissue mass. The mechanisms by which the mass of a tissue may be increased are cell proliferation in which mitosis and cell division takes place, and cell hypertrophy. Both may occur synchronously.

1.2. *Endocrine Interactions:*

The adrenal gland, kidney and blood vessels interact to regulate electrolyte and fluid balance and to control blood pressure and flow (Fig.1.1).

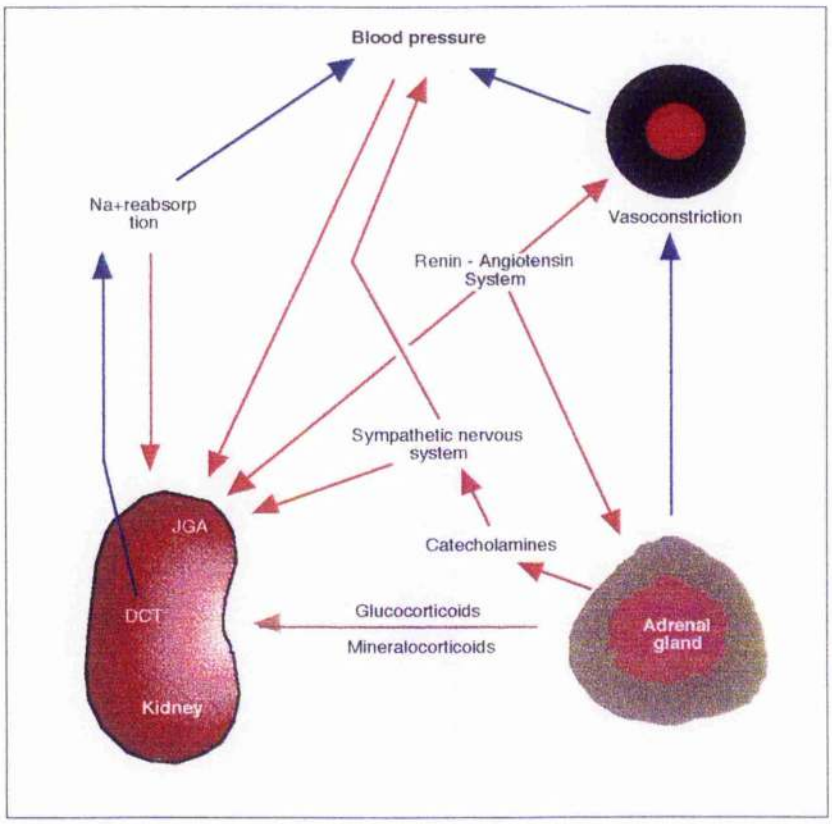


Fig 1.1. The interactions of the adrenal gland, kidney and blood vessels in regulating blood pressure and electrolyte balance.

These interactions involve the control of hormones secreted by the adrenal cortex (mineralocorticoids and glucocorticoids).These target the kidney and the cardiovascular system which respond appropriately to influence this balance. Independent of the adrenal, the kidney is sensitive to changes in blood pressure and sodium concentration which, in turn, have a reciprocal effect on the reactivity of blood vessels. The renin-angiotensin-aldosterone system is central to the control of the interactions between these tissues. Recently, it has been shown that humoral components of this system also have growth-promoting actions (3).

1.3. Physiology of the Renin-Angiotensin-Aldosterone System:

The principal active component of the renin-angiotensin system is the octapeptide angiotensin II (AII). This is derived by a cascade of events (Fig.1.2) involving firstly the cleavage of angiotensinogen (renin substrate) by the aspartyl proteinase renin to produce the decapeptide angiotensin I. Angiotensin I is subsequently converted to AII by the dipeptidase angiotensin converting enzyme (ACE).

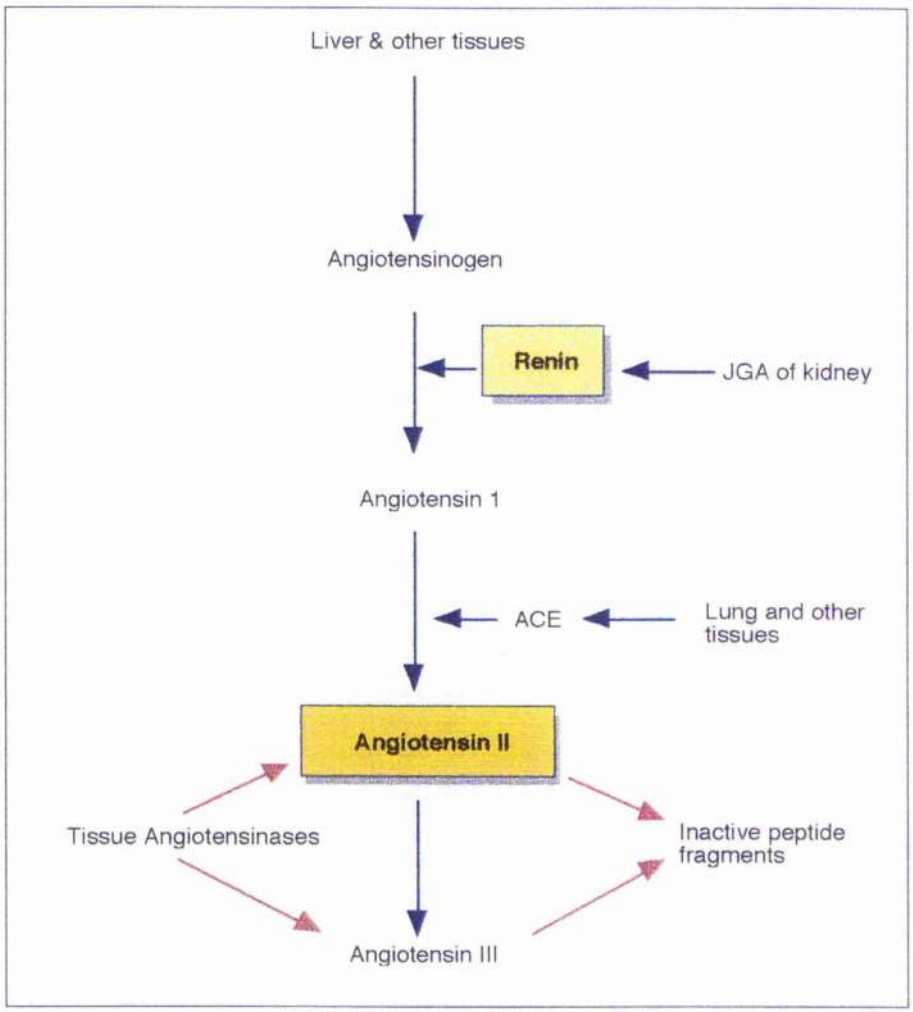


Fig 1.2. The renin - angiotensin cascade.

Angiotensin II acts in several ways to increase sodium retention and blood pressure by i) stimulating the synthesis of the potent antinatriureic hormone aldosterone in the adrenal cortex ii) through direct renal actions iii) as a direct vasoconstrictor of arterial resistance vessels.

The mechanism of the renin-angiotensin system in the control of the volume and composition of the extracellular fluid is complex and is

closely related to blood pressure regulation. Changes in activity of the renin-angiotensin system allow significant variations in sodium intake with only minimal changes in arterial pressure and extracellular fluid volume (4). Elevated blood pressure causes natriuresis and diuresis. As long as excretion exceeds intake, extracellular fluid volume continues to decrease, lowering venous return and cardiac output until pressure returns to normal and fluid intake and output is balanced (Fig.1.3).

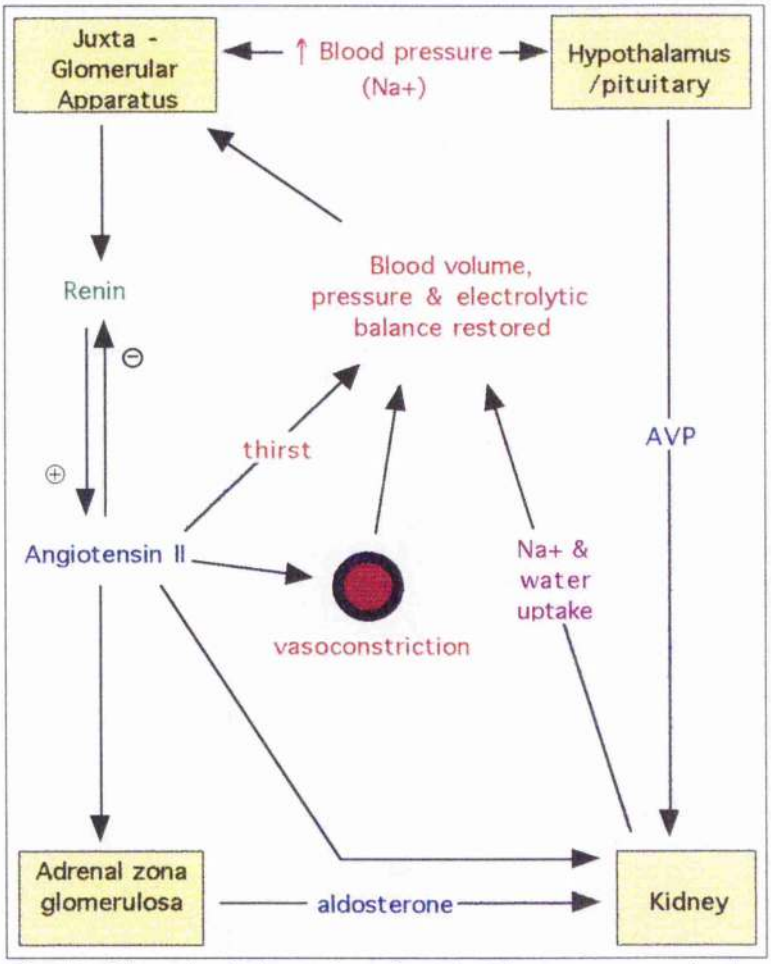


Fig 1.3. The role of the renin-angiotensin-aldosterone system in the regulation of blood pressure and electrolyte balance.

In the kidney, the macula densa of the juxtaglomerular apparatus (JGA) responds to changes in distal tubular sodium/chloride concentration and is, in part, responsible for this feedback loop (Fig 1.3). Under conditions of low sodium or low blood volume, renin secretion is stimulated within juxtaglomerular (JG) cells of the kidney. Angiotensin II generated from the rise in renin increases aldosterone secretion in the adrenal cortex. Blood-borne aldosterone acts on the

distal convoluted and collecting tubules of the kidney to promote reabsorption of sodium from the glomerular filtrate. The net influx of sodium leads to an osmotic influx of water from the filtrate and restores electrolyte composition and blood volume. Arterial baroreceptors respond to changes in pressure and flow. Low blood pressure activates the sympathetic nervous system which, in turn, directly increases renin secretion rate and catecholamine secretion from the adrenal medulla. These substances in turn, directly or indirectly, promote vasoconstriction.

AII stimulates the release of catecholamines from the adrenal medulla and peripheral sympathetic nerve endings and acts on the central nervous system to increase sympathetic nervous system activity (5, 6, 7). Other actions on the central nervous system include (Fig.1.4) the stimulation of thirst, salt appetite and vasopressin secretion (7).

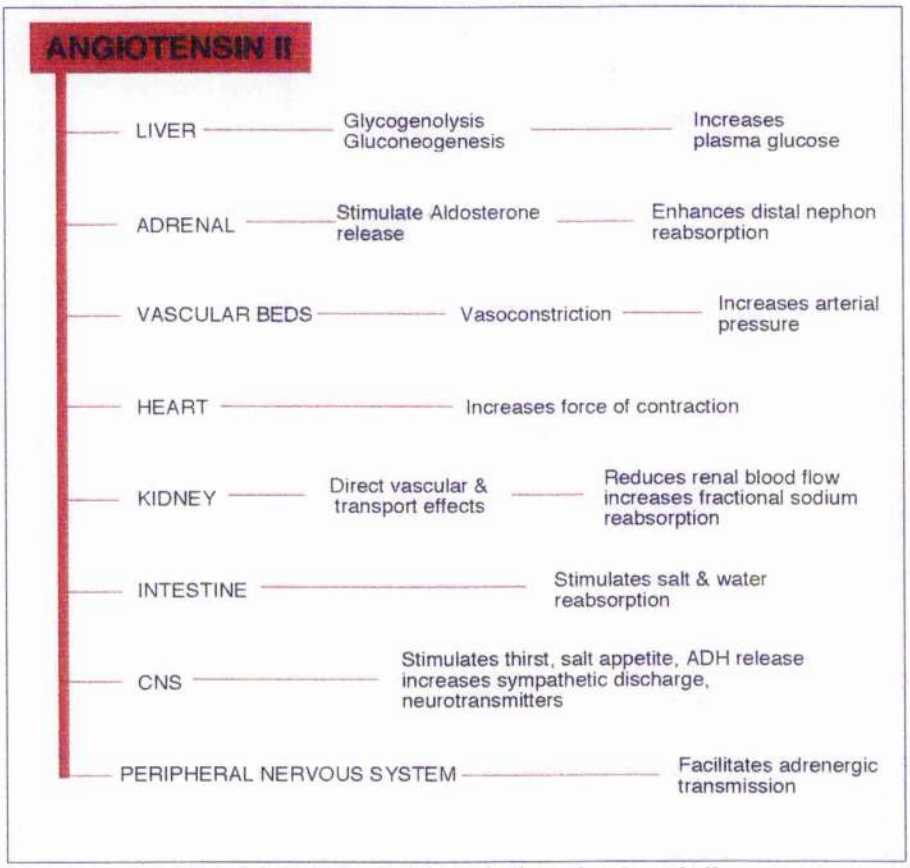


Fig 1.4. Multiple physiological effects of angiotensin II.

AII also increases hepatic gluconeogenesis and glycogenolysis (8, 9) and stimulates salt and water absorption by all segments of the intestine (10). In general, the physiological effects of AII act together to

promote the conservation of salt and water and the maintenance of extracellular fluid volume and arterial blood pressure.

1.3.1. *Renin and the Juxtaglomerular Apparatus:*

Renin is synthesised as a large inactive precursor, preprorenin (Mwt 45 Kd), that is converted first to a smaller inactive form, prorenin (Mwt 42 Kd) and then to active renin (Mwt 35-40 Kd) (11). In plasma, renin circulates in both active and inactive forms (12, 13). Both forms are synthesised and stored in granules within the JG cells of the renal arterioles. JG cells constitute part of the JGA which is sensitive to plasma sodium concentration and is involved in the control of systemic blood pressure (refer to Fig.1.5).

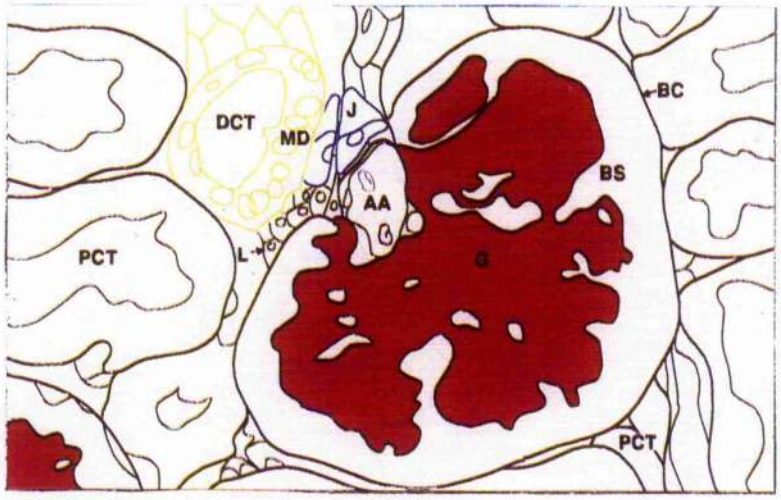


Fig.1.5. The Juxtaglomerular Apparatus

The JGA has three components: JG cells (14), macula densa cells (15) and lacis or Goormaghtigh cells (16) which comprise the extraglomerular mesangium. JG cells, derived from the smooth muscle of the wall of the glomerular arteriole, form a cuff around the vessels just before they enter or leave the glomerulus. They have prominent nuclei and cytoplasm with renin-containing granules (17, 18). The macula densa consists of modified distal convoluted tubular (DCT) cells and is found where the JG cells and DCT are closely apposed. These cells are columnar and have larger more prominent nuclei than other cells of the DCT. The extraglomerular mesangium is a small group of cells which lies between the macula densa and Bowman's capsule at the point of entry of the afferent arteriole. A dense assembly of adrenergic terminal nerve axons is also found in the JGA where smooth muscle

cells and granular cells are target sites for catecholamines; a sympathetic stimulus causes renin release from granular cells (19).

1.3.2. *Regulation of Renin Secretion:*

Changes in renal perfusion pressure directly alter renin secretion (19) and it is postulated that the JG cells, or associated endothelial cells, act as baroreceptors such that changes in the stretch or tension of the afferent arteriolar wall due to alterations in renal perfusion pressure elicit reciprocal changes in renin secretion rate (refer to Fig.1.6).

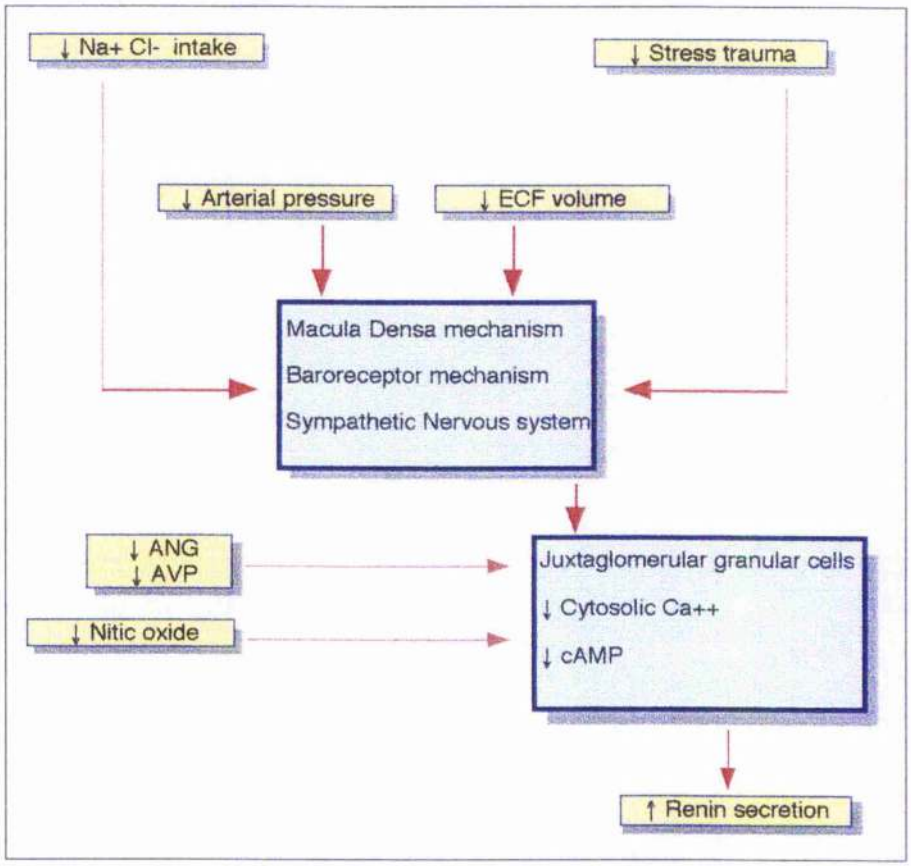


Fig 1.6. Physiological mechanisms regulating renin secretion.

The cytosolic concentration of ionized calcium is an important intracellular signal which regulates the release of enzymes and hormones; the secretion of renin is stimulated by reductions rather than increases in cytosolic calcium levels (19). Increased stretch of the afferent arteriole depolarizes the JG cells resulting in increased calcium entry and elevated cytosolic calcium levels. Subsequently, this leads to a reduction in renin secretion rate (19). Decreased stretch results in

hyperpolarization of the JG cells which leads to decreased calcium influx and stimulation of renin secretion.

Reduced sodium chloride at the macula densa is associated with enhanced renin secretion (20) indicating that renin secretion rate is inversely proportional to the sodium chloride load to the macula densa. Skott and Briggs (21) demonstrated that a decrease in the sodium chloride concentration of the tubular fluid at the macula densa increases renin secretion rate. The mechanism by which this interaction occurs is uncertain but may be due to a suppressive influence of signals from the macula densa cells. Itoh *et al* (22) demonstrated that isolated afferent arterioles with a macula densa segment attached exhibited lower renin secretory rates than arterioles without the macula densa. Although the signal remains unknown, adenosine released from the macula densa cells may act directly on the adjacent JG cells to inhibit renin secretion (22).

The renal nerves and circulating catecholamines also influence renin secretion. JG cells are innervated by adrenergic nerve terminals (23). Low-level renal nerve simulation increases renin secretion through activation of ß-adrenoreceptors on the juxtaglomerular cells (19, 20, 23, 24) whereas direct activation of α-adrenoreceptors by norarenaline inhibits renin secretion. However, activation of α-adrenoreceptors and renal nerve stimulation at higher intensities indirectly stimulate renin secretion via activation of the macula densa and baroreceptor mechanisms. With the exception of noradrenaline, circulating catecholamines released from the adrenal medulla stimulate renin secretion through activation of ß-adrenoreceptors.

AII and vasopressin act directly on the JG cells to inhibit renin secretion (19, 20, 25). The inhibitory effects of these hormones are dependent on the presence of extracellular calcium indicating that both AII and vasopressin (AVP) inhibit renin secretion by enhancing calcium influx into the juxtaglomerular cells (19). *In vivo*, AII inhibits renin secretion by negative feedback mechanisms. *In vitro*, inhibition of renin secretion by AII is well documented (3).

Recent studies have demonstrated direct inhibition of renin secretion by the vasodilator nitric oxide (26). The mechanism of control is not entirely understood but it is thought that renin gene expression is inhibited via activation of cyclic-GMP. Adenosine also inhibits renin release by a direct effect on the secretory cell (27) and by attenuation of ß-adrenoreceptor stimulation (28).

1.3.3. *Angiotensinogen:*

Angiotensinogen, otherwise known as renin substrate, is synthesised and secreted predominantly in the liver (29) but has also been detected in renal lymph (30) and in granules of the proximal tubule cells (31). Angiotensinogen mRNA is present in the cortex and medulla of rat and mouse kidneys (11, 32, 33) although much of this has been localised to proximal tubule cells of the cortex (34). The availability of renin substrate is a prerequisite though not normally a rate-limiting step for AII production.

1.3.4. *Angiotensin Converting Enzyme:*

The main site of conversion of circulating angiotensin I to angiotensin II is the surface of vascular endothelial cells in the lung which are rich in converting enzyme (ACE) (35). ACE has also been found in endothelial cells of the testes, brain, liver adrenal cortex, pancreas, spleen and kidney (36) and it is present on vascular endothelial cells of all kidney arteries, the afferent and efferent arterioles, some glomerular and peritubular capillaries and on the brush border and basolateral membranes of the proximal tubule cells (30, 37). The presence of ACE within such a diversity of tissues is compatible with the existence of local renin-angiotensin systems.

1.3.5. *Angiotensin II:*

AII circulates in plasma at low concentrations and has a very short half-life (38). Within the kidney, AII levels are controlled in several ways (Fig.1.7). Firstly, local renin will convert blood borne renin substrate to angiotensin I which in turn will be converted to AII by ACE located on the luminal surface of the vascular endothelial cells. AII can also be generated intrarenally as a result of renin secretion into the interstitium. Renin in the interstitium (39) finds its way into renal lymph (30, 40). Angiotensin I formed in the interstitium is probably converted to AII by ACE in the interstitial fluid or bound to basolateral membranes of the proximal tubule cells. AII has also been identified in JG cells and although uptake from the extracellular compartment is possible, AII may also be formed intracellularly since both renin and angiotensins have been demonstrated immunocytochemically within secretory granules (17, 18, 40, 41). It follows that AII, whether or not it is generated within juxtaglomerular cells, is probably released together with renin.

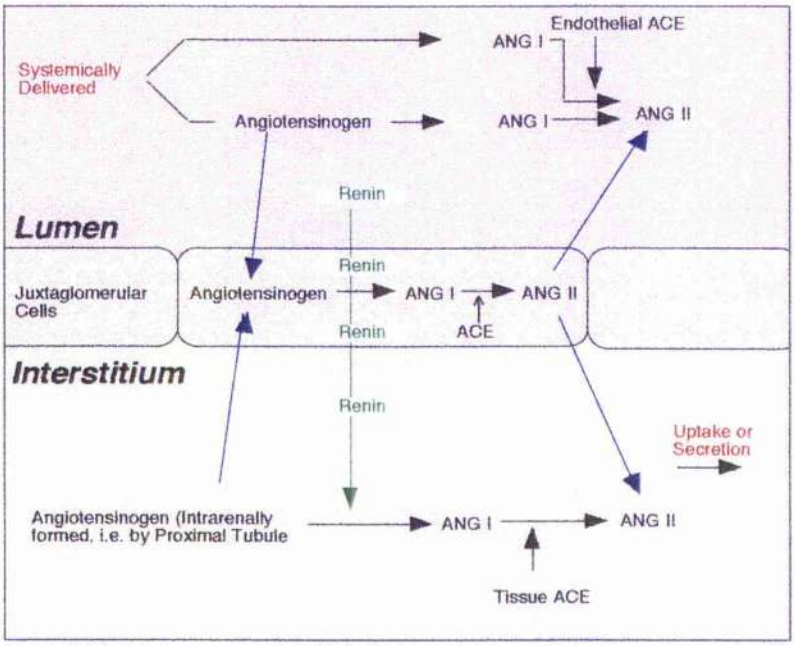


Fig 1.7. The various means by which angiotensin II may be generated intrarenally.

1.3.6. *Physiological Effects of Angiotensin II on the Kidney:*

Intrarenal AII directly regulates renal excretion of electrolytes and water at several levels (42). There is a high density of AII receptors on renal glomeruli (43, 44) and contractile effects of AII on isolated glomeruli and cultured smooth mucle cells are well established (45). AII influences glomerular filtration rate and renal blood flow by direct vasoconstriction of pre- and post-glomerular resistance vessels (46). Mesangial cells contain smooth muscle actin and contract in response to stimulation with AII (47, 48). Mesangial cell contraction reduces glomerular capillary volume and filtration surface area thereby reducing ultrafiltration. In the proximal tubules, AII directly stimulates sodium and fluid resorption (49). Indirectly, AII promotes antinatriuresis in the distal nephron by stimulation of biosynthesis and secretion of aldosterone in the adrenal zona glomerulosa (see later). There is also evidence that AII modulates countercurrent and concentrating mechanisms within the nephron by controlling renal medullary blood flow (50).

Many haemodynamic actions of AII are effected by blood borne AII. However, all the components necessary for the intrarenal generation of AII are present within the kidney indicating that a substantial amount of AII may be generated locally which, in turn, may have paracrine or autocrine functions (49). This is supported by higher renal tissue AII than intrarenal plasma concentrations (51, 52, 53).

Intrarenal levels of AII are increased by sodium deprivation or converting enzyme blockade (52).

Renal Growth Effects:

In addition to its many other physiological actions, the autocrine or paracrine effects of AII may also mediate changes in cell growth (54). Many growth factors are multifunctional regulators, their biological effects being dependent on the target cell type and on the presence of other factors in the local milieu. AII has many of the characteristics of the 'classical' growth factors: it binds to specific cell surface receptors (55); activates a number of intracellular signalling pathways associated with cell growth (56) and induces proliferation of a variety of cells (57, 58). Furthermore, the fact that many renal cell types respond to AII *in vitro*, and possess AII receptors (43, 44, 59) imply a role for AII in the regulation of renal cell growth.

1.3.7. Extrarenal Renin Angiotensin Systems:

There is much evidence for extrarenal renin-angiotensin systems. Large amounts of tissue renin and renin isoenzymes have been found in many tissues including brain, adrenal gland, lung, large arteries and veins, uterus, placenta and the submandibular gland of the mouse (5). Renin has been detected in the adrenal gland following nephrectomy (60) and has been localised to specific intracellular sites within the adrenal cortex. Renin messenger RNA and other components of the system have been detected in brain, heart, adrenal gland and testis in both the mouse and the rat and also in the submandibular gland of the mouse (11, 61). Angiotensinogen (renin substrate) has been detected in the arterial wall (62) suggesting that a local renin-angiotensin system within the vasculature may have paracrine or autocrine effects.

1.4.1. Structure and Function of the Rat Adrenal Gland:

The mammalian adrenal glands are encapsulated paired organs which lie superior to the kidneys. The cortex and medulla are histologically and embryologically distinct. The adrenal cortex is divided into four concentric zones: (refer to Fig.1.8).

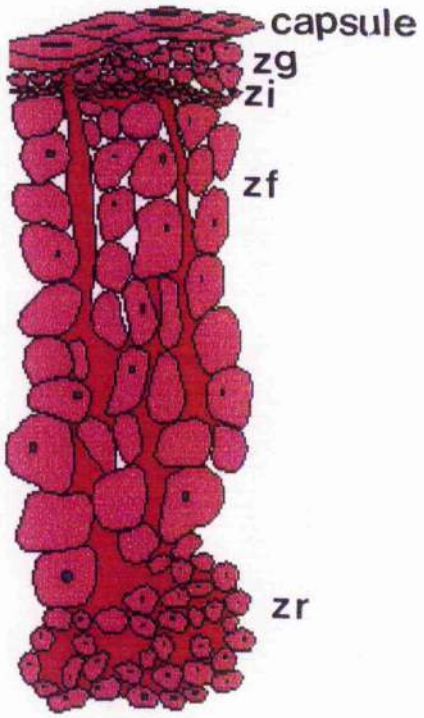


Fig.1.8. The Zones of the Adult Rat Adrenal Cortex. ZG (Zona Glomerulosa), ZI (Zona Intermedia), ZF (Zona Fasciculata), ZR (Zona Reticularis)

i) *Zona Glomerulosa:*

The zona glomerulosa lies just below the connective tissue capsule and consists of layers of subcapsular aggregates of irregularly arranged parenchymal cells which occupy approximately 10-15% of the gland volume. The cells are ovoid or round with cytoplasm containing abundant lipid (63). These cells produce the major mineralocorticoid, aldosterone. (Fig.1.9). The synthetic pathway for aldosterone starts from cholesterol and involves a series of dehydrogenation and hydroxylation reactions. The uptake of cholesterol into mitochondria and subsequent conversion to pregnenolone by the enzyme cytochrome-P450 is the first and one of the rate-limiting steps for aldosterone synthesis; the subsequent conversion of deoxycorticosterone to aldosterone is also closely regulated.

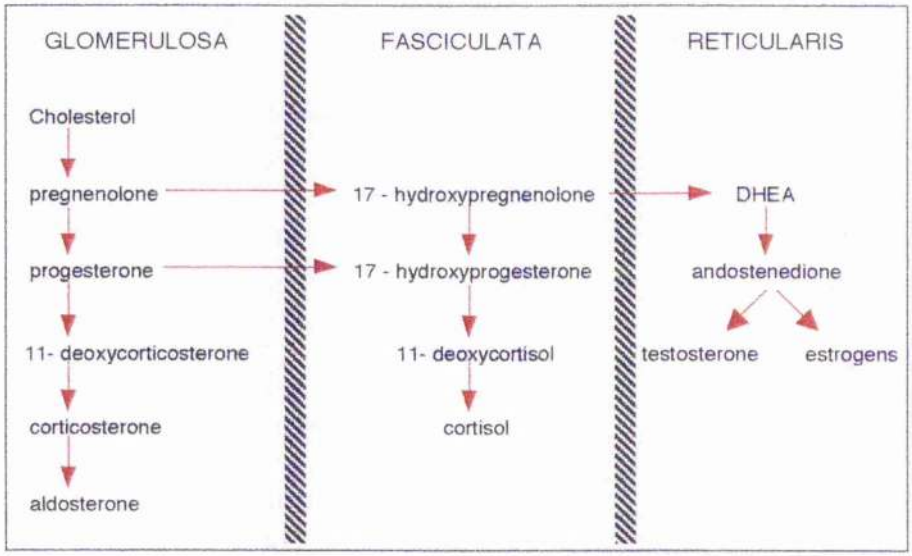


Fig 1.9. The synthetic pathway for the biosynthesis of aldosterone, cortisol / corticosterone and sex steroids.

Aldosterone acts to conserve sodium at several sites (sweat glands, colon, salivary glands, kidney). In the kidney, the hormone stimulates sodium reabsorption from the distal tubule and collecting duct segments of the nephron (64) by two mechanisms: firstly by activation of either luminal membrane sodium channels (64) which increases intracellular sodium concentration, or secondly by activation of sodium channels via a reduction in the intracellular calcium concentration (65). Increased amounts of sodium ions are exchanged for potassium or hydrogen ions leading to decreased sodium excretion (antinatriuresis) and increased potassium (kaliuresis) and hydrogen ion excretion.

In the rat, aldosterone secretion is controlled largely by AII (66) although other factors such as plasma potassium (K^+) concentration (67) and adrenocorticotrophic hormone (ACTH) (68) are also involved. Signal-response coupling mechanisms for AII, ACTH and potassium are compared in Fig.1.10. All three agonists depend on extracellular Ca^{2+}. AII and K^+ both raise intacellular Ca^{2+}; ACTH acts mainly through cyclic-AMP (69). The zona glomerulosa cell is particularly sensitive to the depolarising effects of raised extracellular K^+ which causes calcium uptake through voltage sensitive channels. AII binds to receptors which activate phospholipase C, triggering stored calcium release via inositol 1-4-5 trisphosphate as well as opening plasma membrane calcium channels. Arginine vasopressin (70), serotonin (71) and vasoactive intestinal polypeptide (72) also stimulate aldosterone secretion but are physiologically less important. Atrial natriuretic peptide (73), dopamine (74), and somatostatin (75, 76) inhibit release.

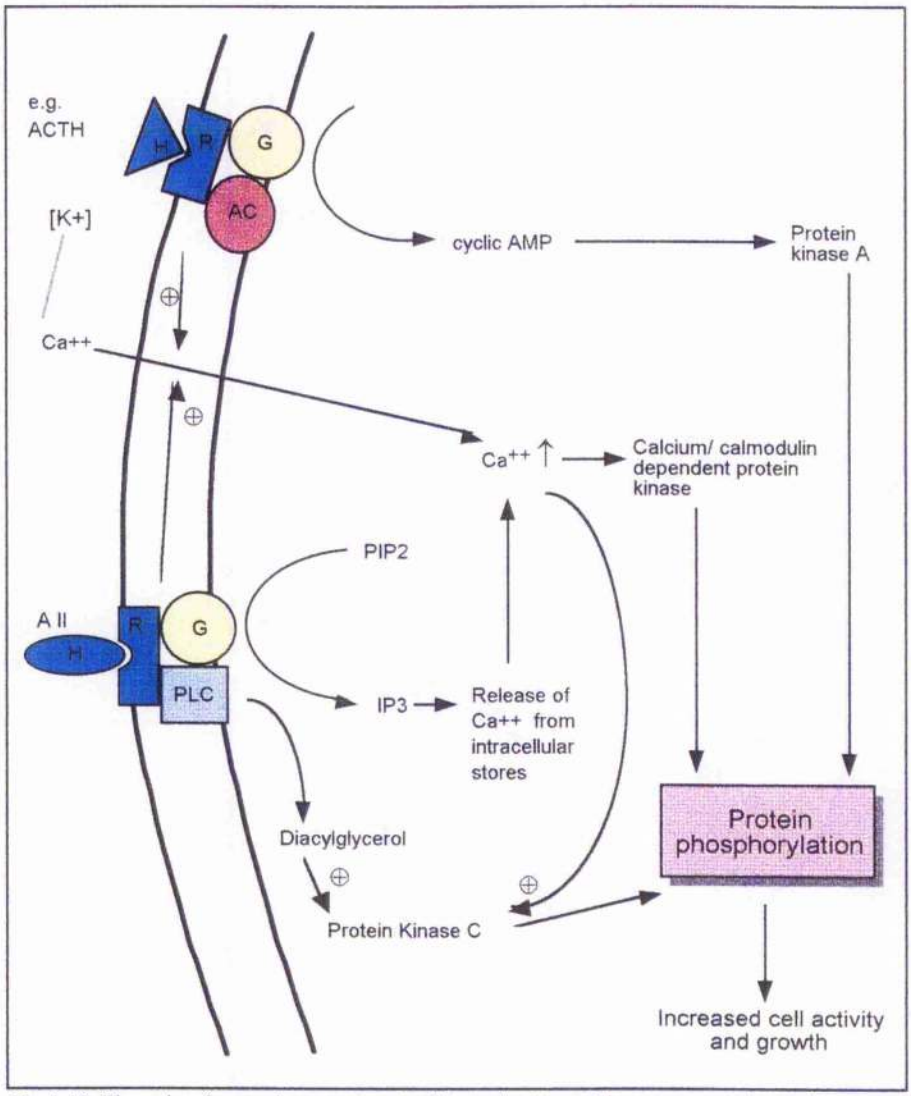


Fig 1.10. The role of second messengers in the mechanism of the response of an endocrine cell to stimulation with potassium, ACTH or AII. (IP 3 = Inositol 1,4,5- triphosphate, PIP 2 = phosphatidylinositol.

In vivo, dietary sodium intake is the major determinant of aldosterone secretion. Dietary sodium restriction enhances aldosterone secretion through raised plasma AII levels (77), up-regulation of AII receptors on glomerulosa cells (78) and removal of the tonic inhibitory influence of dopamine (74). In addition, there is evidence of a local renin-angiotensin system which is more active after adaptation to a low sodium diet (60). However, the contribution that this paracrine system makes to the net output of aldosterone is uncertain.

ii) *Zona Intermedia:*

The zona intermedia is not identifiable in all species. In the rat, it lies immediately below the zona glomerulosa and consists of two or three layers of small cells containing regularly ovoid nuclei and scanty cytoplasm. These cells contain little or no lipid droplets (63) and it has been postulated that they are transitional cells between the zona glomerulosa and zona fasciculata (63). The zona intermedia has also been described as the amitotic zone of the adrenal gland (79, 80). Others claim that zona glomerulosa and zona fasciculata cells originate here (81).

iii) *Zona Fasciculata/Reticularis:*

The major part of the cortex (50-60%) comprises the zona fasciculata. It contains large, clear, lipid-containing cells centripetally arranged in narrow columns or cords between the zona intermedia and the zona reticularis. These secretory cords are normally one cell thick and are separated and supported by connective tissue strands containing capillary sinusoids. The plasma membrane of the secretory cells lies subjacent to the endothelium of the capillary sinusoids. The cytoplasm of the fasciculata cell is packed with mitochondria containing tubulovesicular cristae. In the zona reticularis the secretory cords become less clearly organised and appear as an irregular network of branching cords and clumps of eosinophilic glandular cells separated by numerous wide capillary sinusoids. The glandular cells are much smaller than those of the other zones, the cytoplasm contains few lipid droplets, abundant lipofuscin and the mitochondria are more sparse than in the fasciculata.

The zona fasciculata and zona reticularis are thought to act as a functional unit in the production of glucocorticoids, such as cortisol (human) and corticosterone (rat), and androgens (refer to Fig.1.9).

Glucocorticoid secretion by the zona fasciculata is controlled almost exclusively by the activity of the hypothalamo-pituitary axis and the release of ACTH. The actions of ACTH are mediated by binding to cell surface receptors. The receptor is linked to a plasma membrane adenylate cyclase, and causes an increase in intracellular cyclic-AMP by stimulating adenylate cyclase activity. This, in turn, promotes steroidogenesis. As with aldosterone, the major control point in the pathway is the cleavage of cholesterol to pregnenolone.

Glucocorticoids promote glycogen and protein synthesis in the liver but are catabolic in most other tissues, increasing lipolysis and protein breakdown and reducing glucose uptake from the circulation. Glucocorticoid receptors are also expressed by most cell types in the kidney. They promote natriuresis by raising glomerular filtration rate, and modulating Na^+-H^+ exchange in the proximal tubule (82). Glucocorticoids increase renal plasma flow as a result of increased afferent and efferent arteriolar resistance possibly due to increased contractility of vascular smooth muscle cells (83).

1.4.2. *Cytogenesis of the Adrenal Cortex:*

During normal adult life, following postnatal growth and development, a constant supply of differentiated new cells is required to sustain a normal functional cortical parenchyma (84). Two theories have been proposed to explain adrenocortical cytogenesis. The 'migration theory', originally proposed by Gottschau (85), is based on the interdependence of the cortical zones with respect to their cellular origins and their fate after differentiation. It proposes that cell proliferation occurs mainly at the periphery of the cortex to displace existing cells centripetally. New cells differentiate into zona glomerulosa cells and, as they are displaced inwards they change into fasciculata cells and, finally, as they reach the inner cortex, they become reticularis cells before being eliminated. This theory is backed by experimental evidence: most of the cell proliferative activity appears to occur at the periphery of the cortex in both the immature (86) and the adult rat (87) and there are few mitoses in the inner zones (88, 89). In the reticularis, there are abundant pyknotic nuclei, lipofuscin inclusions and mitochondrial degeneration which are all indicative of cell senescence and death. Apoptosis is common in the reticularis (2, 90). Furthermore, the migration of cells from the outer to the inner zones has been demonstrated by nuclear pulse labelling experiments. DNA labelling with tritiated thymidine in foetal

and neonatal (91), prepubertal (92, 93) and adult animals (93, 94) has shown that cells originally labelled at the glomerulosa or outer fasciculata boundary are sequentially displaced to the inner zones of the cortex, before disappearing.

The alternative 'zonal theory' of adrenocortical cytogenesis was proposed first by Swann (95) and supported later by Race & Green (96). It states that the zones of the adrenal cortex independently increase or reduce mass in response to physiological demand. Each is therefore self-sustaining. The argument for a zonal theory is based on the histological and functional heterogeneity of the cell types.

The three zones of the cortex produce different steroid hormones and are, in part, differentially regulated. The zonal theory is supported by evidence that external stimuli selectively promote changes in cell growth within the different zones of the cortex. The zona glomerulosa is relatively independent of ACTH (97); hypophysectomy leads to profound atrophy of the cortex which is more marked in the inner zones (98) than in the zona glomerulosa although the secretory rate of the zona glomerulosa is affected by removal of ACTH (99). Indeed, ACTH promotes cell hypertrophy of the fasciculata and to some extent the reticularis in response to increased demand for glucocorticoids or androgens (98). The hypertrophic actions of ACTH on the reticularis are enhanced in the presence of prolactin during gonadectomy (100) and there is evidence of mitotic activity in the zona reticularis (96, 101). Although pituitary control of adrenal growth has focussed largely on the effects of ACTH, Estivarez *et al* (1982) (102) have demonstrated that pro-opiomelanocortin (POMC), a peptide from which ACTH is derived, is a potent independent mitogen in the adrenal cortex following adrenalectomy or adrenal damage. Whether POMC influences the differentiation of the cortex during regeneration is not clear.

Chronic treatment with corticosteroids leads to atrophy of the fasciculata and reticularis (103) mediated either directly (103) or indirectly via suppression of ACTH (104). The steroidogenic demands on the zona glomerulosa are reflected by changes in growth; AII raised by dietary sodium restriction leads to an increase in width of the zone (105). Moreover, the zona glomerulosa may be reduced by inhibitors such as atrial natriuretic peptide (106), dopamine (74) or captopril, an angiotensin converting enzyme inhibitor (107).

1.4.3. *The Adrenal Medulla:*

The adrenal medulla is composed of closely packed clumps of secretory chromaffin cells or pheochromocytes supported by a fine reticular network containing numerous wide capillaries. Sustentacular cells are interspersed between the nests of pheochromocytes. Scattered groups of ganglion cells are also present .

The chromaffin cells of the adrenal medulla secrete adrenaline, dopamine and noradrenaline under the control of preganglionic neurones of the sympathetic nervous system. Catecholamines produce a variety of haemodynamic and metabolic effects by binding to either α or ß receptors. The haemodynamic effects include an increase in the rate and force of myocardial contraction (ß1), vasoconstriction (α1), and vasodilation (ß2). Noradrenaline produces generalised vasoconstriction and an increase in both systolic and diastolic blood pressures. Adrenaline produces vasoconstriction in many vascular beds and increases cardiac output and heart rate but also causes vasodilation in tissues populated with ß2 adrenoreceptors including skeletal muscle. Dopamine increases cardiac output, reduces mesenteric and renal vascular resistance while producing vasoconstriction elsewhere. Other effects of catecholamines include stimulating renin secretion (24) and overriding vasorelaxatory effects of nitric oxide (108).

Chromaffin cell growth is not well documented but is thought to be mediated largely through the action of nerves (109). Low blood pressure results in increased DNA synthesis in rats (110). The circulating catecholamines produced by the adrenal medulla are growth factors for some tissues (111, 112) but whether they act in a paracrine or autocrine fashion is not known.

1.5.1. Structure and Function of Blood Vessels.

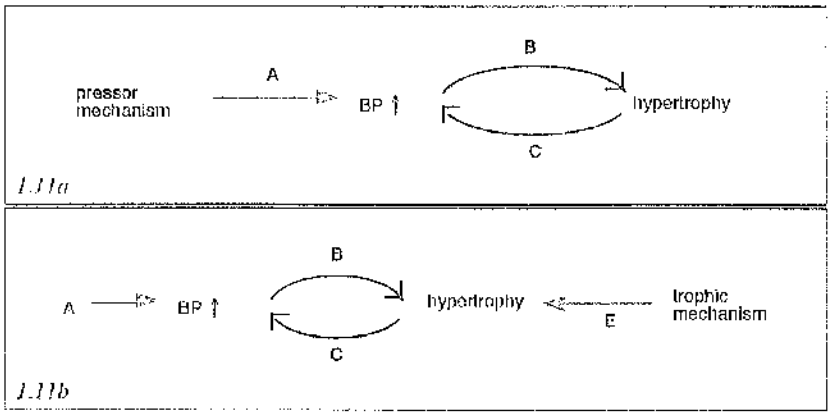
In vertebrates the blood vessels and the heart constitute a closed vascular system. A blood vessel consists of three cell types: endothelial cells, smooth muscle cells and connective tissue cells embedded in nerves. They are organised in three distinct structures, the intima, media and adventitia respectively. The vascular tree is a circuit that conducts blood from the heart through large diameter, low resistance vessels to small arteries and arterioles. The capillaries are thin-walled and allow the exchange of nutrients and waste products between tissue and blood, a process that requires a large surface area. The circuit back to the heart is completed by the veins which are distensible and provide a volume

buffer that acts as a capacitance for the vascular circuit. The physical factors which determine intravascular pressure are cardiac output (a product of stroke volume and heart rate) and total peripheral resistance (narrowness of small arteries and arterioles). The relative contribution that cardiac output and total peripheral resistance make to blood pressure varies. For example, in the Goldblatt two-kidney one clip model of hypertension, cardiac output is initially increased in the presence of normal total peripheral resistance. Later, output falls but resistance increases to maintain high blood pressure. With time, cardiac output falls further and total peripheral resistance increases even more, leading to a sustained form of hypertension. The mechanism for progressive increased peripheral vascular resistance is attributed to thickening of the media of blood vessels with narrowing of the lumen (113).

1.5.2. *Definition of Vascular Hypertrophy:*

Vascular hypertrophy is defined as an increase in mass of any of the histological layers of blood vessels. In hypertension, the earliest changes have been observed in the media of small arteries and arterioles, whereas in other disease states such as arteriosclerosis there is proliferation of the intima (114). Unlike man, the intima of the rat is less well developed and consists only of a single layer of endothelial cells. Therefore, for the purpose of this study, hypertrophy of blood vessels will refer only to thickening of the media.

The process of arterial thickening may be regarded as a necessary adaptation to prevent damage to the microcirculation and to prevent over-perfusion of tissues. This lead Folkow (115) to propose his theory of vascular amplification in which small increases in blood pressure cause an increase in vascular smooth muscle cell mass in resistance vessels which in turn increase blood pressure (Fig.1.11a). This results in hypertrophied blood vessels which are characterised by an increase in the lumen/wall ratio. More recently, others have suggested modifications of this theory involving the direct actions of smooth muscle mitogens (refer to Fig.1.11b) (111).



Figs 1.11a & 1.11b. The amplification of blood pressure by hypertrophy and the possible role of growth factors.

Three mechanisms have been proposed to explain vascular hypertrophy: hyperplasia, cellular hypertrophy and remodelling (refer to Fig.1.12). Hyperplasia is an increased number of smooth muscle cells, whereas cellular hypertrophy is an increase in size of the existing smooth muscle cells as a result of increased protein synthesis (116) or polyploidy (117). Remodelling refers to an increased lumen/wall ratio due to a rearrangement of the existing cells around a smaller lumen (118, 119). These phenomena vary in different parts of the circulation. For example, hypertrophy of smooth muscle cells of large conductance arteries such as the aorta is associated with polyploidy (120), whereas mesenteric resistance vessels are either remodelled (121, 122) or thickened by hyperplasia (123, 58).

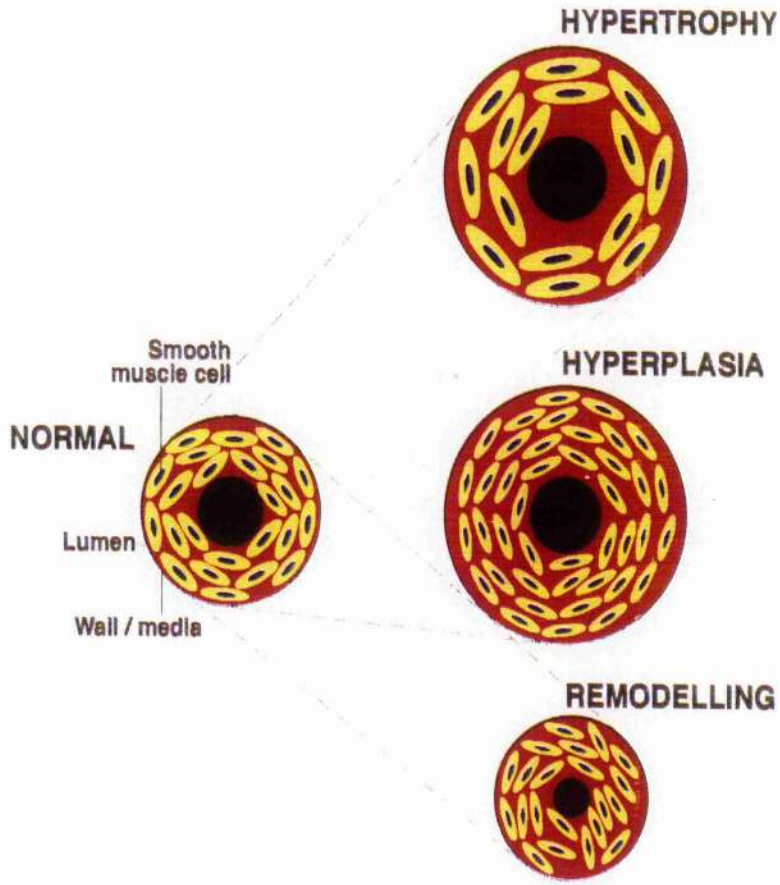


Fig.1.12. The Mechanisms of Vascular Hypertrophy

1.5.3. *Effects of Angiotensin II on Blood Pressure and Vascular Growth:*

AII directly stimulates vascular smooth muscle cell contraction increasing blood pressure acutely by vasoconstriction (55). The excitation-contraction coupling mechanism involves a depolarisation of the cell membrane after binding by AII to specific smooth muscle cell receptors. Depolarisation activates voltage-dependent Ca^{2+} channels leading to an influx of Ca^{2+}. The intracellular free Ca^{2+} concentration regulates vascular contraction by binding to calmodulin which then associates with myosin light chain kinase, initiating contraction.

When infused in animals at a dose below the threshold of its acute vasoconstrictor effect, AII raises blood pressure slowly and progressively (124, 125). Although the mechanism for this slow action is uncertain, it has been postulated that vascular hypertrophy develops during the slow response, and that AII may be an important growth factor *in vivo* (126). Furthermore, in young normotensive Wistar-Kyoto rats, hypertrophy of the arterial media appears to be related specifically to growth effects of AII rather than indirect effects of pressure or growth effects of catecholamines (127). The hypothesis that AII acts as a growth factor has also been strengthened by studies using ACE inhibitors and AII receptor antagonists (128, 129) which demonstrate either prevention or regression of hypertrophy. Tested *in vitro*, AII either stimulates cell proliferation (130, 131) or increases cell size without cell division (132) depending on the type of blood vessel studied.

1.5.4. *Other Factors which Control Blood Vessel Growth:*

Other vascular derived growth factors also contribute to the normal and pathological growth of blood vessels (133). Platelet-derived growth factor, transforming growth factor beta, insulin-like growth factor and fibroblast growth factor regulate smooth muscle cell growth *in vitro* and *in vivo* and are thought to elicit paracrine or autocrine effects on growth (133, 134). They are known to act both independently and synergistically with AII (135) and *in vitro* are dependent on the presence of insulin in the growth medium (3).

Cellular and Molecular Mechanisms:

The cellular and molecular mechanisms of growth of smooth muscle cells are poorly understood and are difficult to study because cell growth requires a multitude of factors which bind to or activate cells in a

finite or temporal sequence (136). The progression of cells from the interphase of the cell cycle to the mitotic phase is not an all or none event but involves programmed activation of a series of genes. If the conditions fail to meet the demands of the cell, growth can be arrested following DNA synthesis (polyploidy) without proceeding to the mitotic phase of the cell cycle. Alternatively, the cell may be eliminated by apoptosis.

Calcium plays an important role in these processes (136); in many cells there is a positive relationship between the cytosolic free calcium concentration ($[Ca^{2+}]$) and mitogenesis. Contractile effects of AII occur together with the mobilization of Ca^{2+} either by uptake via the cell membrane or by release from intracellular stores (55); this relationship may contribute to the trophic action of AII in vascular smooth muscle cells (116). Vascular smooth muscle cell membranes defective in Ca^{2+} handling may increase intracellular free calcium levels and increase peripheral vascular resistance, thereby enhancing the vascular amplifier. Mechanisms are either decreased membrane-bound $[Ca^{2+}]$ leading to a decrease in membrane potential, depolarisation and hyperexcitability (137) or reduced Ca^{2+} efflux by failure of the Ca^{2+} ATPase pump (138)

AII and many other mitogens increase Na^+/H^+ exchange in vascular smooth muscle cells (139); leading to intracellular alkalinisation which is widely considered to be a trigger for growth (140). Enhanced Na^+ permeability also increases intracellular calcium concentrations and promotes vascular tone.

Mechanical Factors:

Blood pressure or wall stress mediates smooth muscle cell hypertrophy in hypertensive animal models (120). Stretching cultured aortic smooth muscle cells increased protein, collagen and elastin synthesis (141). In addition, stretching vascular smooth muscle cells promotes the expression of the proto-oncogene c-fos (142). Expression of c-fos, c-myc and c-jun are also inducible by AII (142, 143, 144, 265) raising the possibility that the growth effects of AII and stretch are mediated by the protein products of these genes.

Endothelial Factors:

The endothelium influences growth of arterial smooth muscle (114). Removal of the endothelium *in vivo* induces proliferation and migration of smooth muscle cells (114, 145). *In vitro*, isolated endothelial cells produce factors that either inhibit or promote the

proliferation of arterial smooth muscle cells. Endothelium derived relaxing factor and prostaglandin E2 are two of many vasoactive agents derived from the endothelium which inhibit proliferation of vascular smooth muscle cells (146).

Extracellular Matrix:

In hypertension, the extracellular matrix expands by an increase in collagen, elastin and glycosaminoglycans (147). Additional matrix contributes to resistance by stiffening the vessel, reducing compliance and increasing resistance (148).

1.6. Methodology:

Hypertrophy of tissues may be determined by various techniques. Changes in weight reflect an increase in number of the components of the tissue and/or the size of its cells. Gross histological changes may be observed by light microscopy aided by the use of special stains or measured with the use of an eyepiece graticule with a Vernier scale or grid (149). More rapid measurements may require an automated computerised image analysis system for calculating area fractions. Pressure or wire myographs which determine force-internal circumference relationships (150) have been used to characterise hypertrophy of blood vessels. However, the size of blood vessel selected is restricted to those with a lumen large enough to accommodate pieces of wire. Subtle changes in the size of cells or their organelles may be determined by electron microscopy.

Growth is often assessed by changes in DNA synthesis. Various techniques may be used to determine this. The simplest is the mitotic index which counts the number of mitoses in a tissue. However, since mitosis is a rapid transient event, large number of cells have to be counted to assess the degree of mitosis accurately. Colchicine has been used in cell replacement studies, however this technique involves arresting cells in mitosis and frequently yields conflicting results (151). The DNA content of cells may be measured by the use of special stains such as Feulgen which binds stoichoimetrically to DNA. These stains lend themselves to quantitative analysis by either microspectrophotometry, static image analysis or flow microfluorimetry and are based on the transmission and/or absorption of light. The light transmitted is related to the staining intensity which is directly proportional to the DNA content. These techniques are particularly useful for measuring ploidy and may be modified for the measurement

of cellular protein content. Nuclear labelling experiments using analogues of pyrimidine such as tritiated thymidine ([^{3}H]TdR, bromodeoxyuridine (BrdUrd) or iododeoxyuridine (IdU) may be used to determine the degree of DNA synthesis of a tissue by being incorporated into the DNA during the synthetic phase of the cell cycle (refer to Fig.1.13).

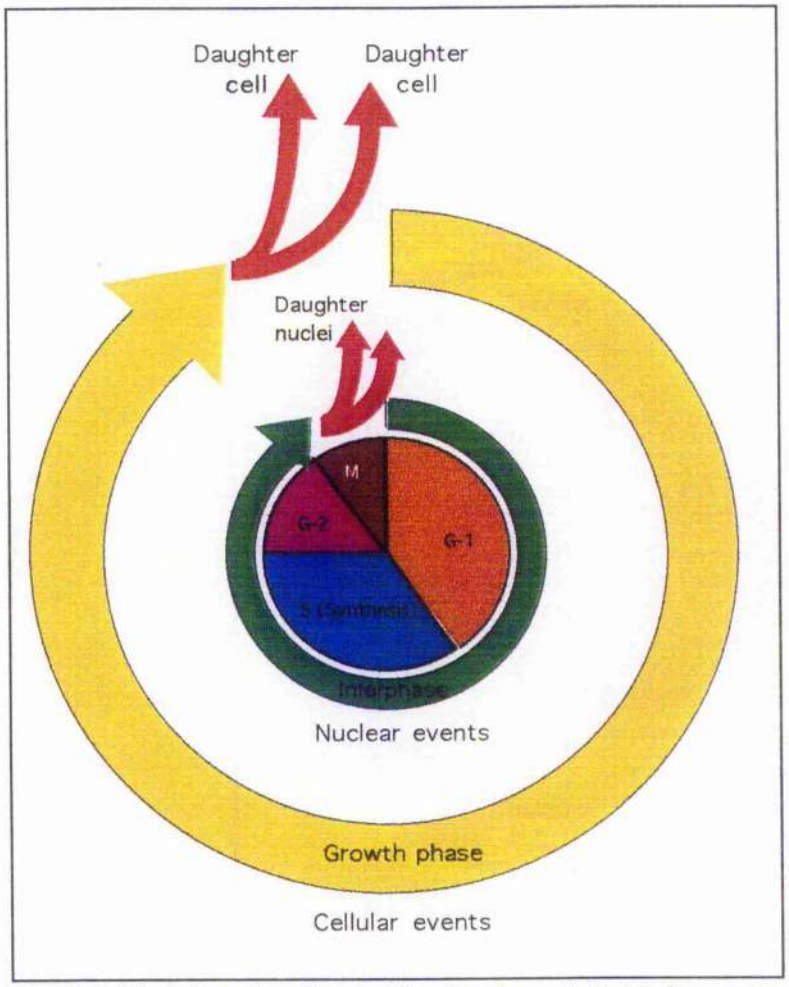


Fig 1.13. The eukaryotic cell cycle. Note that the synthetic (s) phase occurs during the interphase.

The labelled nuclei are detectable in single-stranded DNA by immunocytochemistry or fluorescence microscopy (152, 153). The BrdUrd technique is particularly useful in longterm studies as there is no requirement for expensive radioactive isotopes, the cells which incorporate BrdUrd are quickly and easily detected by immunocytochemistry (152), the coloured end-product is chemically stable and an index of DNA synthesis may be achieved by cell counting either manually or automatically using an image analysis system. This

technique is superior to [^{3}H]TdR where localisation is limited by the distance of travel of the emitted ß particles, the grain size of the photographic emulsion, by the exposure time and by the limited resolution of autoradiographs. The application of this technique may also be extended to include the analysis of wax-embedded tissues after *in vivo* labelling of intact animals (154).

1.7. Image Analysis:

Most image analysis systems consist of a conventional microscope coupled to a video camera which acts as the image sensor. The camera produces a voltage signal proportional to the light intensity. The principal stages thereafter require five main steps: (i) *image capture* which involves the conversion of the image into an electronic signal suitable for digital processing; (ii) *segmentation* describes the act of separating the regions of interest within an image from the background by thresholding which means that all tones below a selected level are treated as black and all above as white. The end product becomes a binary image of the object expressed in pixels; (iii) *object detection* is used to signify each of the individual regions of interest (i.e. radius of the media of a whole blood vessel) and is the component of the image which is to be measured; (iv) *measuring* provides a quantitative value which is transferred from pixels into real 'units'; (v) *analysis*, once the measurements have been made the results can be analysed by computer and decisions can be made on how the measurements can be presented graphically.

1.7.1. *Analysis of Ploidy Using Computerised Image Analysis:*

Static image analysis cytometry has been developed for the assessment of ploidy providing the tissue architecture is maintained. This allows operator interaction in the process of cell selection (155). Image analysis is possible from tissue sections obtained from paraffin-embedded material and facilitates staining of DNA with Feulgen's stain. The Feulgen stain reacts stoichiometrically with DNA to allow the amount of staining to correlate directly with the DNA content of the nucleus. The reaction is based on the hydrolysis of deoxyribose-purine bonds in the DNA to produce reactive sugar aldehydes that can be demonstrated with Schiff's reagent. As light is passed through a feulgen-stained nucleus, a proportion of the light is absorbed and the remaining light is transmitted. The stoichiometric binding of the Feulgen

stain allows for the transmitted light to be interpreted and related directly to the DNA content of the cell. With careful calibration the pixel information can be related to nuclear DNA content.

1.8. Aim of the Study:

The renin-angiotensin-aldosterone system plays an important role in the aetiology and pathogenesis of hypertension. One of the main, but less considered features in this development, is the role of DNA synthesis in the target tissues. Most experiments have been performed *in vitro*. *In vivo* experiments have involved short-term studies applied to a slow adaptive process (156). Few have attempted to study progressive changes in growth over a realistic period of time for tissues to adapt to manipulations of the renin-angiotensin system. The aim of this study is to investigate the effects of longterm modulation of the renin-angiotensin system on cell growth of the target tissues. The renin-angiotensin system will be manipulated in rats by dietary sodium depletion (raising renin and plasma AII), infusions of a slow pressor dose of AII (increase plasma AII and lower plasma renin activity), and treatment with captopril to block the conversion of AI to AII (raising plasma renin activity but lowering AII). Bromodeoxyuridine will be infused continuously throughout and the effects of treatment on DNA synthesis will be assessed by immunocytochemistry in adrenal glands, kidneys and blood vessels. In addition, growth effects on blood vessels from all orders of the vascular tree will be measured morphometrically.

Some forms of hypertension are renin and therefore AII independent. Administration of deoxycorticosterone (DOC) salt by promoting sodium retention, increases blood pressure and has a negative feedback effect on renin secretion. Increased DNA synthesis in vascular smooth muscle cells in this instance is increased (157) without elevated systemic levels of AII. It is postulated that blood pressure alone is a stimulus for DNA synthesis (117, 157, 158). The second aim of this study, therefore, is to assess whether increased activity of the renin-angiotensin system is a pre-requisite for growth. Hypertension induced by glucocorticoid excess does not appear to involve changes in systemic activity of the renin-angiotensin system. Therefore, the effects of the synthetic glucocorticoid, dexamethasone, on DNA synthesis will be assessed. Furthermore, corticosteroids also cause hypertension by a slow mechanism and it is suggested that amplification by hypertrophy occurs during the slow response (111) however, the effects of

dexamethasone as a trophic factor *in vivo* in smooth muscle cells have not been studied.

Inhibition of nitric oxide synthase promotes local vasoconstriction and, in the longterm, hypertension (159). The mechanism of increased blood pressure may be direct or secondary to changes in the renin-angiotensin system. A direct mechanism is indicated by evidence that renin is suppressed acutely by nitric oxide (26). The longterm effects of nitric oxide suggest a secondary, renin-dependent form of hypertension. However, there is little information regarding alterations in DNA synthesis in blood vessels and whether or not vascular hypertrophy is a main feature in the early stages of hypertension. The secondary effects of stimulating the renin-angiotensin system may also be reflected by changes in growth of the adrenal gland. Furthermore, prolonged inhibition of nitric oxide synthase with L-arginine inhibitors results in malignant hypertension and renal necrosis (160), the early stages may reflect changes in renal cell growth.

Chapter 2 Materials and Methods

2.1. Rats:

Male Sprague Dawley and Wistar rats were purchased from Harlan Olac, Bicester, U.K. and maintained under Home Office Regulations in the animal unit of the MRC Blood Pressure Unit, Western Infirmary, Glasgow. Animals were housed in a temperature-light-controlled room (21^{O}C; 12 hours light, 12 hours dark). All animal handling, surgery, dietary treatments, blood sampling and decapitation was done by Home Office Project licence holder, Dr. C.J. Kenyon except for those described in Chapter 3 which were performed by Dr. J.J. Morton, MRC Blood Pressure Unit, Glasgow. Normal and special low sodium rat chow was obtained from Special Diets Services, Witham, Essex. All animals were given food and water *ad libitum*. Before surgery animals were anaesthetised with sodium pentobarbitone. Mini osmotic pumps containing BrdUrd were inserted subcutanously into the back of the neck of all rats prior to treatment.

2.2. General Materials:

2.2.1. *Chemicals and Reagents:*

The companies cited below are UK based unless otherwise stated.

Bovine Serum Albumin	- Sigma Chemical Company
5'-Bromo-2'-deoxyuridine	- " "
Buffered Formaldehyde	- Diagnostic Developments
Captopril	- E.R. Squibb & Sons, Princeton, N.J., USA.
Cellasolve	- Genta Medical
Chloroform	- Genta Medical
Coat-A-Count Kit	- Diag. Product Co. C.A., USA.
Dexamethasone Acetate	- Sigma Chemical Company.
Diamino Benzidene	- Aldrich Chemical Company Ltd.
Dimethyl Formamide	- Sigma Chemical Company
Dimethyl Sulphoxide	- " "
DPX Mountant	- Merks Ltd.
Ethanol	- Customs and Excise Controlled
Fast Red Violet LB Salt	- Sigma Chemical Company
Fast Blue BB Salt	- " "

Feulgen Kit	- Cell Analysis Systems, Becton Dickinson Ltd.
Glacial Acetic Acid	- BDH Laboratories
Gurrs Eosin	- Diagnostic Development
Histomount	- Hughes & Hughes Ltd.
Hydrogen Peroxide Concentrate	- Merks Ltd.
Hypertensin	- Ciba Geigy, Basle, Switzerland
Isopropanol	- Genta Medical
Levamisole	- Sigma Chemica Company
L-Nitro Arginine Methyl Ester	- " "
Mayers Haematoxylin	- Diagnostic Developments
Methanol	- Genta Medical
Napthol AS-TR-Phosphate	- Sigma Chemical Company
Paraffin Wax	- Bayer Diagnostics Ltd.
Phosphate Buffered Saline Tablets	- Mercia Dignostics
Poly-L-Lysine	- Sigma Chemical Company
Porcine Serum	- Gibco BRL
Rabbit Serum	- Scottish Antibody Production Unit (SAPU)
Silastic Curing Agent	- Dow Corning, Midland, USA.
Swine Serum	- Gibco BRL
Trizma Base	- Sigma Chemical Company
Trizma HCl	- " "
Trypsin	- ICN Biomedicals
Xylene	- Genta Medical
Xylene Substitute	- Life Sciences International

2.2.2. *Antibodies:*

Anti-Bromodeoxyuridine IgG Monoclonal Antibody - Europath Ltd.

Rabbit-Anti Mouse Immunoglobulin/ Alkaline Phosphatase - Dako Ltd.

Swine-Anti Rabbit Immunoglobulins - Dako Ltd.

Peroxidase-Anti-Peroxidase - Dako Ltd.

Rabbit Anti-Mouse Renin FAb fragments - Gift from Professor K.Poulsen, Denmark.

Ulex *Europeus* Lectin (Endothelial Cell Marker) - Vector Laboratories

2.2.3. *Materials and Equipment:*

Glass slides	- Life Sciences International
Tissue cassettes	- Bayer Diagnostics Ltd.

Miniosmotic Pumps (model 2002) - Alzet, Palo Alto, CA, USA.
Coverslips - Life Sciences International
Microtome (rocking rotary) - Lab-Tek
Tissue Processor (Tissue-Tek) - Bayer Diagnostics Ltd.
Optomax V, Image Analysis System - Cambridge Instruments
Cell Analysis Systems 200 Image Analysis System - Becton Dickinson
CCD Camera (JVC TK 870E) - JVC Ltd.

2.3. Preparation of Solutions:

2.3.1. *Bromodeoxyuridine:*

0.5g of BrdUrd was dissolved in 2mls of dimethyl sulphoxide (DMSO) to give a concentration of 0.25mg/ml. 2mls of saline was added to the mixture to give a final concentration of 0.125mg/ml. 226µl of the BrdUrd solution was added to each pump and an estimated release of 10µl/d gave each animal approximately 1.25mg BrdUrd per day.

2.3.2. *Fixatives:*

Methacarn fixative was the preferred fixative for preserving nuclear DNA for all tissues. Methacarn solution contained 60% methanol; 30% chloroform;10% glacial acetic acid.

2.3.3. *Buffers:*

Tris Buffered Saline (TBS): 10mM Tris HCl, 2mM Tris Base, 150mM NaCl, pH 7.2.

Phosphate Buffered Saline (PBS): 1 tablet dissolved in distilled water, pH 7.4.

Veronyl Acetate Buffer: Stock Solution 1.94g sodium acetate trihydrate, 2.94g sodium barbitone/litre double distilled water. Working solution 1ml stock/4ml water, pH to 9.2 with normal HCl.

2.3.4. *Enzyme Substrates:*

Peroxidase Substrate. 1 vial of DAB powder reconstituted in 10ml dimethyl formamide. Working Solution: Reconstituted DAB

Tris Buffer (0.05M), 250ml
Imadizole, 25ml
Hydrogen Peroxide, 125µl

Refer to appendix IV for substrate protocol.

Alkaline Phosphatase Substrate: Veronyl Acetate Buffer, pH 9.2 (10ml)

5mg Levamisole
5mg Fast Red Violet LB or Fast Blue BB
4mg Napthol AS TR Phosphate

Refer to appendix I for substrate protocol.

2.3.5. *Antibody Diluents:*

All antibodies, with the exception of renin, were diluted in 1% bovine serum albumin (BSA) dissolved in TBS. Rabbit anti-mouse renin was diluted in 5% normal swine serum (NSS)/PBS.

2.3.6. *Blocking Agents:*

Endogenous enzyme activity was removed from tissues using either
20% Normal Swine Serum diluted in PBS or
15% Normal Rabbit Serum diluted in TBS

2.3.7. *Angiotensin II:*

Mini osmotic pumps were filled with AII in 0.9% sodium chloride solution at a concentration between 5-7mg/ml depending on the weight of the rat. Pump capacity was 226 ± 6µl (mean ± standard deviation). The rate of infusion was 0.5 ± 0.02µl/h as tested previously *in vitro* by Griffin *et al* (1991) (126). Thus, a pump containing 6mg of AII in a rat of 250g delivered peptide at 200ng.Kg^{-1}.min^{-1}.

2.4. Tissue Processing and Section Cutting:

Following 24 hour fixation in methacarn solution, tissues were immersed in cellasolve and automatically processed through a series of chloroforms followed by infiltration with paraffin wax. Tissues were orientated and embedded in chucks containing molten wax. Section thickness was determined by the experimental requirements. Generally, 3µm thick sections were used except for sections of blood vessels analysed for ploidy (see Chapter 3c). All sections were cut, floated onto a waterbath at 58°C, mounted onto poly-L-Lysine coated microscope slides to prevent detachment of the section from the slide during trypsinisation (see appendix for protocol) and oven-dried at 60°C to enhance section-slide adherence.

2.5. Staining and Immunostaining:

The immunocytochemical procedures used for all tissues were indirect methods which involve visualisation of the antigenic sites using two or more antibodies. The advantage of the 'indirect method' over the 'direct method' is that a greater proportion of antigen can be detected using a minimum concentration of primary antibody.

2.5.1. *Renin:*

Sections of kidney were dewaxed in xylene, immersed in absolute alcohol and submerged in 0.5% H_2O_2/methanol (see Appendix III) for 30 minutes to remove endogenous peroxidase. Sections were washed in PBS and ringed using a wax pencil to prevent removal of the antibody from the section. 100µl of blocker (NSS/PBS) was added to each section to prevent background positivity, drained and blotted after 30 minutes and replaced with rabbit anti-mouse renin (diluted 1/800, 5% NSS/PBS). Sections were incubated overnight at 4°C. Tissue sections were washed in PBS and incubated with swine anti-rabbit antibody diluted in 5% NSS/PBS for 30 minutes. After several washes in PBS the sections were conjugated with peroxidase anti-peroxidase antibody (PAP) diluted 1/1150 in 5% NSS/PBS for 30 minutes followed by washing in distilled water. Renin was visualised by immersing the sections for 8 minutes in diaminobenzidene (see Appendix iv) and appeared dark brown. For double-labelling of renin and BrdUrd in the kidney, sections were treated according to the method described in 2.5.2.

2.5.2. *BrdUrd Immunostaining*:

The alkaline phosphatase technique used for the detection of BrdUrd is a modification of that described previously by Ponder & Wilkinson, (161) and Mason & Sammons (162).

Tissue sections were cleared in xylene and hydrated through decreasing concentrations of alcohol into water. The sections were immersed in 0.7 normal hydrochloric acid (NHCl) for 30-60 minutes at 37°C to hydrolyse the double-stranded DNA molecules and facilitate binding of the BrDUrd antibody to antigenic sites. Following several washes in distilled water, the sections were added to a solution of trypsin (ICN Biomedicals) at 37°C (see Section 2.5.3 for optimising trypsin). Following trypsinisation, the sections were washed vigorously in distilled water and submerged in TBS. The working solution for the primary antibody was optimum at a dilution of 1:20 (anti-BrDUrd in 1%

BSA/TBS) overnight at 4°C. The sections were carefully ringed with a wax pencil and 100µl of diluted antibody was added to each section. The sections were washed in 1% BSA/Tris and rabbit anti-mouse alkaline phosphatase antibody was applied at 1/25 diluted in 1% BSA/Tris for 60 minutes. BrdUrd was visualised by incubating each section with 100µl of alkaline phosphatase substrate (see Appendix I), in the refrigerator in the dark for 10 minutes. To visualise BrdUrd-negative nuclei, sections were counterstained in haematoxylin (see Appendix v). Due to the solubility of alkaline phosphatase reaction product in non-aqueous solutions, the sections were taken immediately from water and mounted onto coverslips containing compatible aqueous mounting medium. BrdUrd-positive nuclei appeared either dark blue or red depending on the chromogen used. In negative control sections, the primary antibody was treated with either an antibody of the same immunoglobulin class not directed against BrdUrd or buffer.

2.5.3. *Optimising trypsinisation*

Trypsin digestion is normally used for revealing antigenic sites; although the mechanism is not entirely understood, it is thought that protein cross-links caused during fixation are released by the protease making more antigenic sites available to the antibody. The concentration of enzyme and duration of trypsinisation was dependent on the tissue type and had to be optimised separately for each tissue. In order to do this, a series of trypsin concentrations ranging from 0.01%-2% diluted in TBS/Calcium Chloride were set up. Trypsinisation times ranged from 30 seconds to 10 minutes. The detection of BrDurd in the adrenal gland was optimised at 0.03% trypsin for 2 minutes. The kidney required a lower concentration of trypsin for a longer time, 0.01% for 3.5 minutes and blood vessels were optimised at 0.1% for 2 minutes.

2.5.4. *Ulex europeus lectin:*

Sections were dewaxed and hydrated. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 minutes. Sections were washed briefly in tap water and trypsinised for 20 minutes at 37°C. Following several washes in tap water a wax ring was circled around the sections and peroxidase-conjugated Ulex *Europeus* lectin diluted 1/200 BSA/Tris was applied and incubated on the sections for 30 minutes at room temperature. The sections were rinsed in TBS and washed for 10 minutes in a TBS bath. The sections were then immersed in DAB

solution for 8 minutes, rinsed in tap water and the DAB chromogen was enhanced in weak 0.1% Copper Sulphate solution. Sections were counterstained with haematoxylin, dehydrated and mounted in DPX. Endothelial cells appeared dark brown.

2.5.5. *Feulgen Staining of DNA:*

10µm sections of blood vessels were dewaxed and hydrated. A Cell Analysis Systems (CAS) calibration slide consisting of an imprint of cultured rat hepatocytes, post fixed in 10% neutral buffered formalin, was included with each staining batch to ensure the quality of Feulgen DNA staining. The tissue sections were hydrolysed for 60 minutes in 5 normal hydrochloric acid (5N HCl). The Feulgen stain containing Schiff's reagent and the blue chromogen thionine was supplied in kit form. The contents of one staining vial were dissolved in 100ml of 0.1N HCl in 125ml Erlenmeyer flask. The flask was sealed with parafilm to prevent the emission of sulphur dioxide gas, and stirred for 1 hour. The Feulgen stain reagent was filtered through grade #1 filter paper into Coplin jars. The slides were transferred from the 5N HCl into the stain reagent and left for 1 hour. The rinse solution was prepared by adding the contents of one vial of CAS rinse reagent containing 10% potassium bisulphate, to 300 ml of 0.05N HCl. The rinse solution was divided into three Coplin jars. Following staining, the slides were transferred into the first rinse for 30 seconds, the second rinse for 5 minutes and the third rinse for 10 minutes. After washing in running tap water for 5 minutes, the slides were differentiated in 1% acid alcohol for a further 5 minutes to remove excess stain. Sections were dehydrated through graded alcohols, cleared in xylene and mounted in histomount.

2.5.6. *Special Stains:*

The special stains which were used to identify deposition of proteoglycans, collagen and reticulin deposition within blood vessels were as follows:

Proteoglycans- Alcian Blue Periodic Acid Schiff's (PAS).

Young and mature collagen and reticulin- Methyl blue van Gieson

Muscle and collagen- Masson's Technique

The protocols for each special stain are summarised in Appendix VI-VIII.

2.6. Plasma Enzyme/Steroid Measurements:

2.6.1. *Plasma Renin Activity (PRA)*: Heparinised trunk blood was collected for measurement of PRA. This was kindly done in the MRC Blood Pressure Unit, Glasgow by Dr. J.J. Morton. In brief, the technique is based on the measurement of trapped angiotensin I generated by plasma when incubated with a known concentration of angiotensinogen (163).

2.6.2. *Plasma Corticosterone Activity:*

Heparinised trunk blood was collected, centrifuged and stored at -20°C until used. Plasma corticosterone was measured by radioimmunoassay as described by Morton *et al.* (164). This was kindly performed in the MRC Blood Pressure Unit by Miss Mary Ingram .

2.7. Blood Pressure:

Blood pressure was measured by a tail cuff method as described by Evans *et al* (165).

2.8. Image Analysis and Cell Counting:

2.8.1. *The Optomax V- Morphometry Image Analysis System:*

The Optomax V-Morphometry Image Analysis System was used to measure the radius and medial area of all blood vessels. The system was initially calibrated using a stage micrometer and magnification objective X40. The stored calibration was used to automatically express the processed image from pixels (256 X 256) into micrometers. Only transverse sections of blood vessels were selected for measurement. The live image from the microscope was digitised using the CCD camera, captured and processed by the image analysis software (Vids-V Software) and transferred into a binary image which facilitated image segmentation. The portion of the blood vessel to be measured (e.g. media) was selected and thresholded by circling the appropriate area with a light pen applied to the television screen. This area was calculated and remaining areas (e.g. lumen) were excluded to give the radius and area of the media automatically.

2.8.2. *CAS Image Analyser:*

Sections of blood vessels were stained with the CAS Feulgen dye as described in section 2.5.5. Control slides containing intact cultured liver cells of known DNA content were stained simultaneously. Prior to

calibration the light intensity was standardised to a reference light level stored in the computer memory. Calibration involved the assessment of the Feulgen stained calibration slide (see section 2.5.5). Computer automated selection of 40 tetraploid nuclei with a single peak and a coefficient of variation of less than 5% indicated acceptable staining quality as well as ensuring camera alignment and correct light setting. A threshold was set to achieve image segmentation, distinguishing smooth muscle cell nuclei from the background. Sections of blood vessel were selected at random and a minimum of 100 smooth muscle cell nuclei were analysed for ploidy status in vessels of all sizes in each mesenteric vascular bed. The data were presented as a histogram of nuclear DNA mass as described previously (166).

2.8.3. *Cell Counting and the BrdUrd Index:*

In random sections of adrenal gland, two thousand cells were counted in the zona glomerulosa, zona intermedia, outer and inner zona fasciculata, reticularis and medulla. Epithelial and endothelial cells which stained positively for BrdUrd were expressed as a percentage of the total number of cells counted in each zone. The BrdUrd index was calculated as follows:

$$\textbf{BrdUrd Index} = \frac{\text{Number of Stained Cells}}{\text{Total Number of Cells}} \times 100\%$$

The cells were counted using a Zeiss Axiophot microscope and camera lucida frame which delineated a fixed area of $64\mu^2$ at x40 magnification. Within each field of view, approximately two hundred and fifty cells were counted. Eight fields for each zone were selected at random to give a total count of two thousand cells. In the kidney, two thousand cortical cells were counted and the number of BrdUrd-positive cells was expressed either as a BrdUrd index or as the number of BrdUrd positive cells per 100 glomeruli.

In blood vessels, a BrdUrd index was obtained separately for endothelial, vascular smooth muscle cells and adventitial cells by counting all of the cells in each blood vessel layer. Blood vessels which had any damage to the endothelium were excluded from measurement.

2.9. Statistical Analysis:

2.9.1. *Student's T-Test.*

Morphometric variables, cell counts and BrdUrd Indices were compared between different groups of rats by calculating a mean for each rat and

then applying a two-sample t-test. As judged by Kolmogorov-Smirnoff and F tests, the distributions of these means were compatible with the assumptions of normality and equality of variance on which the t-test is based.

2.9.2. *Cumulative Distribution Function:*

The medial areas of blood vessels were ranked for each experimental group in ascending order and grouped into centiles according to the method described by Short (167). To assess whether treatment had effects on a sub-population of arteries which could lead to an altered shape of distribution, the pooled measurements of individual arteries were used to construct a cumulative distribution curve for each group of rats. The difference between control and test curves was measured in the direction of the medial area and was expressed as a percentage change in area compared to the control values. The estimated change in area could then be plotted as a function of the control area.

THE RENIN ANGIOTENSIN SYSTEM AND DNA SYNTHESIS IN THE KIDNEY, ADRENAL GLAND AND MESENTERIC BLOOD VESSELS.

The first chapters of this thesis refer to three experiments designed to investigate the effects of manipulations of the renin-angiotensin system on DNA synthesis in some of its target tissues. The effects of treatment with AII, captopril and low and high dietary sodium on the kidney, adrenal gland and mesenteric blood vessels are described separately in chapters 3 a-c but refer to the same groups of animals. Chapters 4 and 5 describe separately the effects of nitric oxide synthase inhibition and administration of glucocorticoids on DNA synthesis of various tissues.

Chapter 3a: DNA SYNTHESIS AND THE SYSTEMIC AND INTRARENAL RENIN-ANGIOTENSIN SYSTEMS.

Introduction:

AII is the major effector peptide of the renin-angiotensin system and, although it is generally regarded as a circulating hormone, there is also strong evidence of an intrarenal renin-angiotensin system (41, 168). All of the components required for generation of AII have been localised within the kidney including angiotensinogen (31) renin (40) angiotensin I, ACE and angiotensinases (41). Intrarenal synthesis of some of them has been established by finding intrarenal mRNAs (169, 170). In addition, local concentrations of AII have been found in renal tissue (51) and in specific intrarenal fluid compartments of rats (171) which are higher than systemic concentrations. These high levels of AII are increased by sodium intake and blockade with ACE (52). In addition, AII has been identified in JGA cells of rat kidney although it is not known whether it is synthesised *in situ* or absorbed from elsewhere (40, 172).

Goldblatt has suggested that the kidney evolved an intrinsic renin-angiotensin system which only later became adapted as a circulating hormone system (173). The role of this intrinsic system as a major modulator of renal function is generally recognised (49, 174). AII influences intrarenal haemodynamics in several ways: by eliciting dose-dependent decreases in renal blood flow and by smaller more variable effects on glomerular filtration rate (GFR), such that filtration fraction increases (49). Locally generated AII may reduce inner cortical blood flow to a greater extent than circulating AII (175).

Although these physiological effects of AII indicate a functional intrarenal renin-angiotensin system, there is little information on the role of AII in the regulation of renal cell growth *in vivo*. Many of the renal cell types possess AII receptors (43, 44. 176) and, *in vitro*, AII promotes hypertrophy of mesangial cells (177) and proximal tubular cells (59). *In vivo*, analysis of the role of specific growth factors is complicated by heterogeneity of the cell types in the kidney each with different rates of turnover. In addition, growth factors may also have different effects on epithelial, endothelial, interstitial or contractile cells (54). *In vivo* it is difficult to distinguish between direct growth effects

of AII from secondary haemodynamic effects induced, for example, by vasoconstriction.

In the normal adult mammalian kidney, renin is synthesised in afferent and efferent arterioles and interlobular arteries (174). The intrarenal distribution of renin changes markedly during maturation (178), adrenalectomy (30) and renal artery constriction (30) and it has been proposed that changes in the distribution of renin may be important in the stabilisation of renal perfusion pressure (179). Furthermore, it has been shown that chronic stimulation of the renin-angiotensin system increases the fraction of renin-secreting cells along the afferent arteriole (30, 33). Dietary sodium restriction and converting enzyme blockade increase the granulation and number of renin-secreting cells (30, 180). The mechanism by which renin secreting cell number increases is controversial. Histological studies have described mitotic figures in cells of the JGA (181, 182) suggesting that cell proliferation may contribute to their increased number. Others have failed to show an increase in DNA synthesis (183) and have argued that existing smooth muscle cells in the afferent arteriole are recruited and transformed into granular epithelial cells in response to stimulation (30, 33, 41, 183).

In part, the controversy has arisen because of the difficulties in detecting small increases in proliferation in cells which normally turnover slowly, or, as with the adaptation of rats to conditions of altered intake of dietary sodium, where the problem is complicated by the slow time course of events.

In order to investigate the effects of AII as a renal growth factor *in vivo*, the renin-angiotensin system was manipulated over two weeks in several ways. Firstly, AII was infused into rats. The main objective of this was to promote the systemic renin-angiotensin system but inhibit the local production of renin/AII. This would help to discriminate between systemic and paracrine growth effects of AII. Also, since the mechanical effects blood pressure are also important in cell growth, a dose of AII which was known to raise blood pressure by a slow mechanism was infused into rats.

Secondly, the renin-angiotensin system was stimulated by adaptation of rats to low dietary sodium. Low dietary sodium is known to raise AII levels but decrease the pressor activity of AII and other vasoactive hormones such as catecholamines or vasopressin (184, 185). Thus, dietary sodium restriction would raise both the intrarenal and systemic AII concentration and would determine whether any growth

effects mediated by AII on the kidney are direct or secondary to blood pressure changes.

Thirdly, rats were given the converting enzyme inhibitor, captopril to inhibit the systemic conversion of AI to AII. This would drive the intrarenal renin-angiotensin system and promote increased renal renin/AII levels. It was hoped that this would distinguish between the systemic and paracrine or autocrine growth effects of AII.

The length of time given to the rats to adapt to treatment was considered appropriate to demonstrate changes in growth of all renal cell types including those which turnover slowly such as mesangial cells and smooth muscle cells of the afferent arteriole. The end result included all DNA synthesis which took place over two weeks.

Materials and Methods:

a) Rats:

Animal treatments described below are applicable to chapters 3a-c. Groups of six, adult, male Sprague-Dawley rats (250g) were anaesthetised using sodium pentobarbitone and were implanted with miniosmotic infusion pumps subcutaneously at the back of the neck. BrdUrd was infused continuously for 14 days.

Angiotensin II:

In one group AII was infused via a second minipump at a rate of 200ng.Kg^{-1}.min^{-1} for 14 days whilst pumps in the control group contained vehicle. Systolic blood pressure was measured after treatment in concious warm rats using a tail-cuff method (165).

Dietary Sodium:

One group of rats received a special low sodium diet for 14 days A second group also ate the low sodium diet and received sodium supplements in the form of 1.5% NaCl in drinking water.

Captopril:

Captopril was administered in drinking water (0.5mg/ml; approx 10mg.kg^{-1}.d^{-1}). Dose was calculated on an average fluid intake of 20ml.kg.d. The control group drank tap water alone. All rats had free access to food and water.

b) Renin: After 14d, heparinised trunk blood was collected for measurement of plasma renin activity (163).

c) Tissue Processing:

Treatment of tissues are described in detail in Chapter 2. In brief, rats were killed by a blow to the back of the head followed by decapitation. Tissues were removed and fixed in Methacarn solution and processed to paraffin wax. Sections of kidney were double immunostained for renin and BrdUrd, whilst adrenal gland and blood vessels were stained for BrdUrd only. All sections were counterstained with haematoxylin.

d) Quantification:

i) Renin-Secreting Cells: All nucleated renin-secreting cells were counted in three transverse kidney sections from each rat and divided by the total number of glomeruli.

ii) BrdUrd-containing nuclei: Using a x40 objective the renal cortex was scanned. A minimum of 2,000 cells per kidney were counted in glomeruli, tubules and interstitium. The renal arterial tree was also examined for the presence of BrdUrd-positive nuclei.

e) Statistics: Data were analysed by unpaired Students' t test.

Results:

Distribution of BrdUrd-positve nuclei:

The highest concentration of BrdUrd-positive nuclei was in the tubular epithelial cells of the deep renal cortex (refer to Fig.3.1); there were few labelled nuclei in the medulla. Labelled nuclei in the distal parts of proximal tubules and in the thick loops of Henle in the deep cortex were distributed as focal aggregates. In the glomeruli the majority of labelled cells were in the mesangium (Fig.3.2) although occasional endothelial and epithelial cells were also positively stained. BrdUrd-positive nuclei were regularly identified in vascular smooth muscle cells and in endothelial cells in each section, however, no renin-containing cell stained positively for BrdUrd (Fig.3.3).

Angiotensin II Infusion:

After two weeks, blood pressure increased significantly (AII 177± 11.1 S.E.M; cf control 131.5± 5.3 mmHg, $p<0.05$); plasma renin activity was reduced to almost undetectable levels and the number of renin-secreting cells decreased (Fig.3.4a). The distribution of immunostainable renin was restricted to JG cells at the glomerular vascular pole. In some JGA's of rats treated with AII, renin was undetectable. In AII-treated rats DNA synthesis increased in glomeruli but there was no significant effect on tubules or interstitial cells (Fig.3.4b).

Dietary Sodium Manipulation:

Compared with rats fed a sodium supplemented diet, plasma renin activity was higher and the number of renin-secreting cells was twofold greater in rats fed a low sodium diet (Fig.3.5a). In the low sodium group, renin-secreting cells were located predominantly at the vascular poles but also extended along the arterioles where, in some sections, they were distant from the JGA. This contrasted with the high sodium group where renin-positive cells were detected only at the vascular pole (Fig.3.5b). Despite high plasma renin activity, low dietary sodium had no effect on DNA synthesis in glomeruli, tubules or interstitial cells (Fig.3.5a).

Angiotensin Converting Enzyme Inhibition:

Treatment with captopril increased plasma renin activity and the number of renin secreting cells (Fig.3.6a) compared with the controls. The distribution of renin in captopril-treated rats was similar to that of the low sodium group (Fig.3. 6b). Opposite to the effects of AII infusion, captopril reduced the number of glomerular BrdUrd-positive nuclei (Fig.3.6b). As with the other experimental groups, captopril had no significant effect on tubule or interstitial cell DNA synthesis (Fig.3.6a).

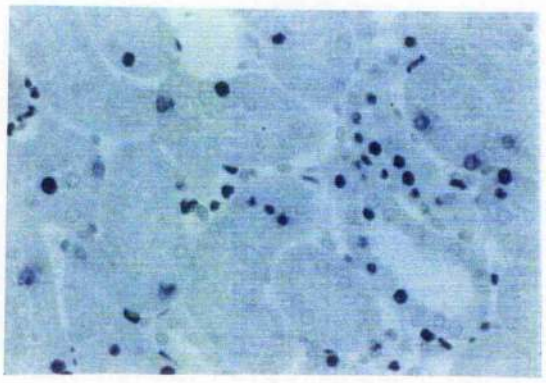


Fig.3.1. The inner renal cortex of the rat. BrdUrd-positive nuclei are detected predominantly in tubule cells.

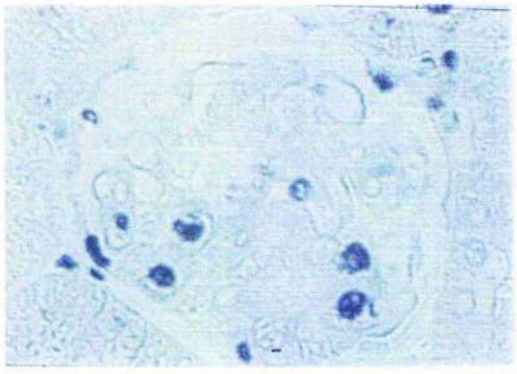


Fig.3.2. Rat Glomerulus. Note that the majority of BrdUrd-positive nuclei are those of mesangial cells.

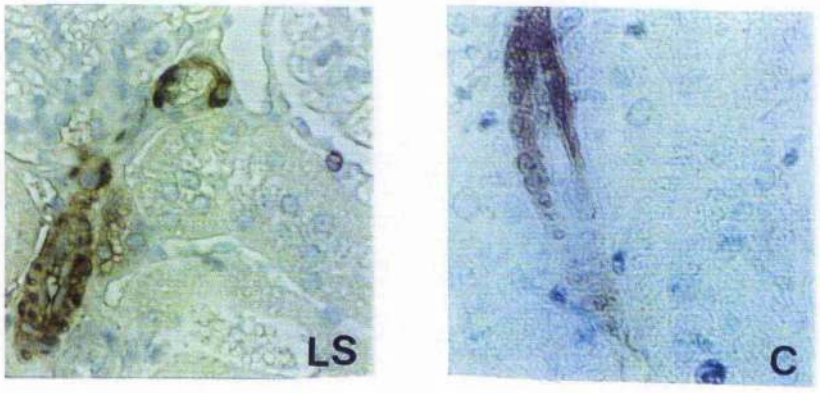


Fig.3.3. Renin detected in the renal arterioles with either low dietary sodium (LS) or captopril (C). Despite increased renin secretion, there is no BrdUrd in any renin cell stained with fast red violet or fast blue BB.

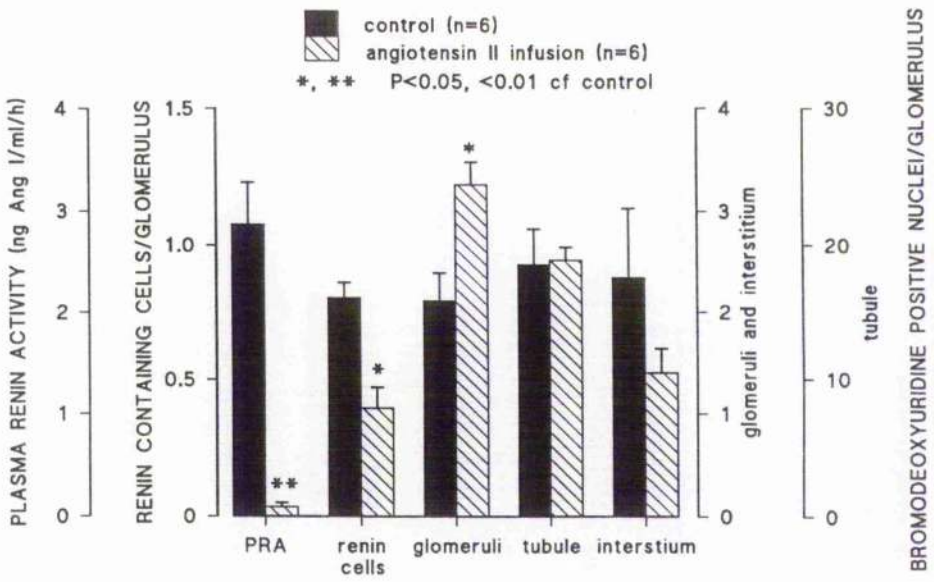


Fig.3.4a The effects of AII infusion on plasma renin activity (PRA), renin-secreting cell number and DNA synthesis of the renal cell types. AII caused a significant increase in mesangial cell DNA synthesis.

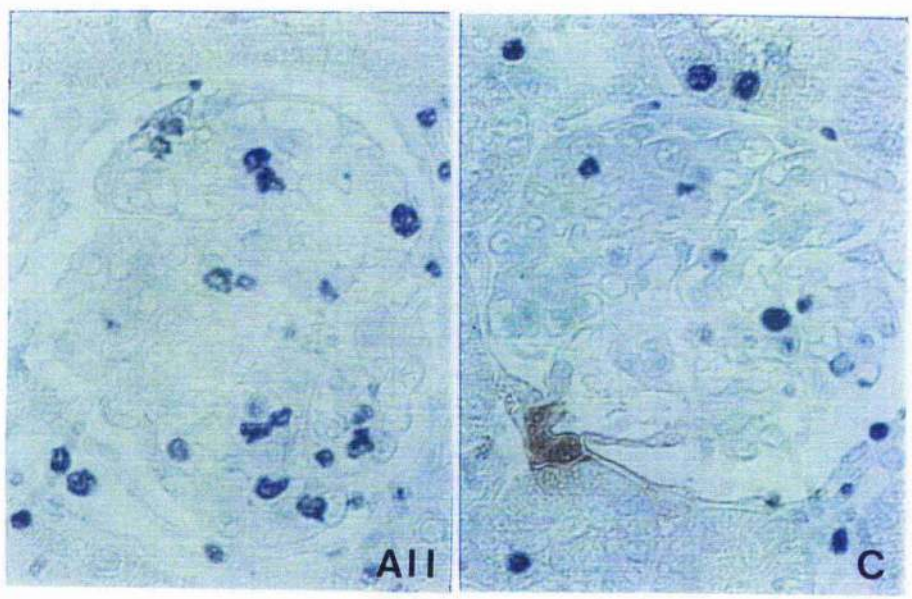


Fig.3.4b. Glomeruli from control (C) and treated (AII) showing renin at the vascular pole and absent in AII glomerulus. BrdUrd uptake is increased in mesangial cells following AII infusion.

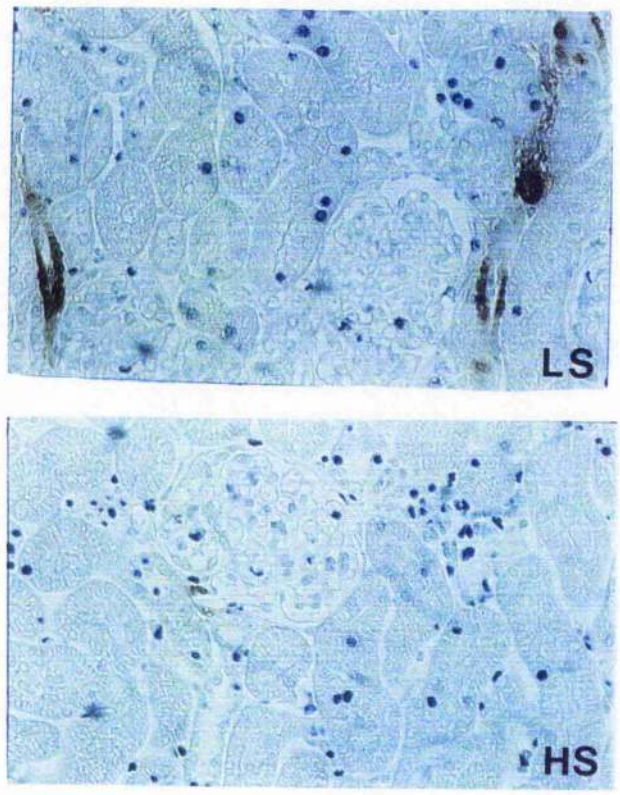


Fig.3.5a. The effects of low (LS) and high sodium (HS) on renin distribution. Note that renin extends distant from the JGA in response to low dietary sodium.

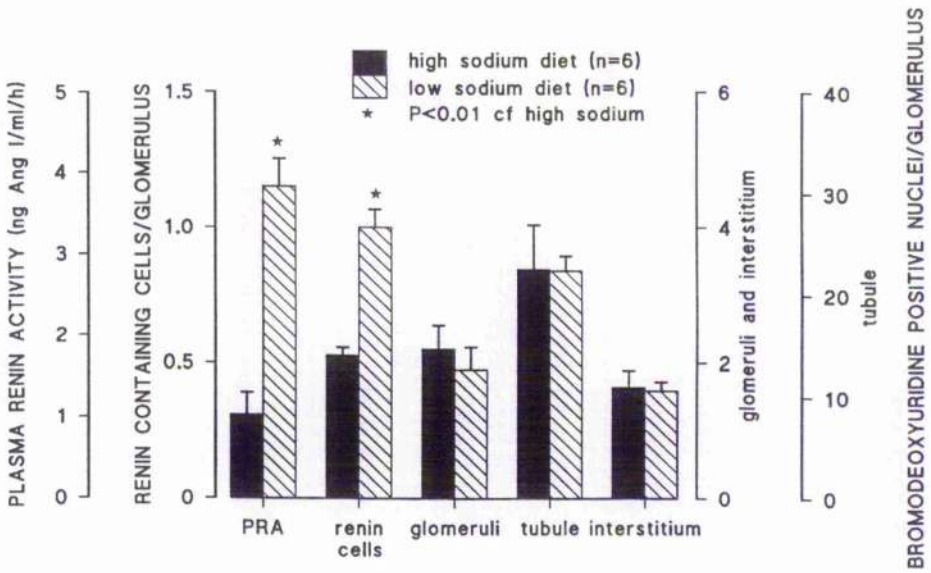


Fig.3.5b. The effects of low and high dietary sodium on plasma renin activity (PRA), renin-secreting cell number and DNA synthesis of the renal cell types. Despite increased renal and plasma renin there were no changes in cell growth.

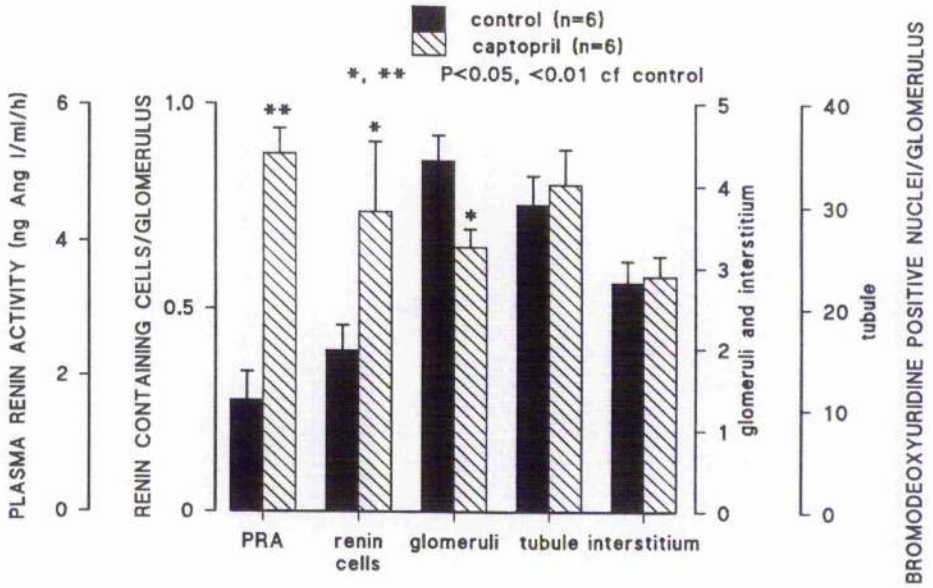


Fig.3.6a. The effects of captopril on plasma renin activity (PRA), renin-secreting cell number and DNA synthesis of the renal cell types.

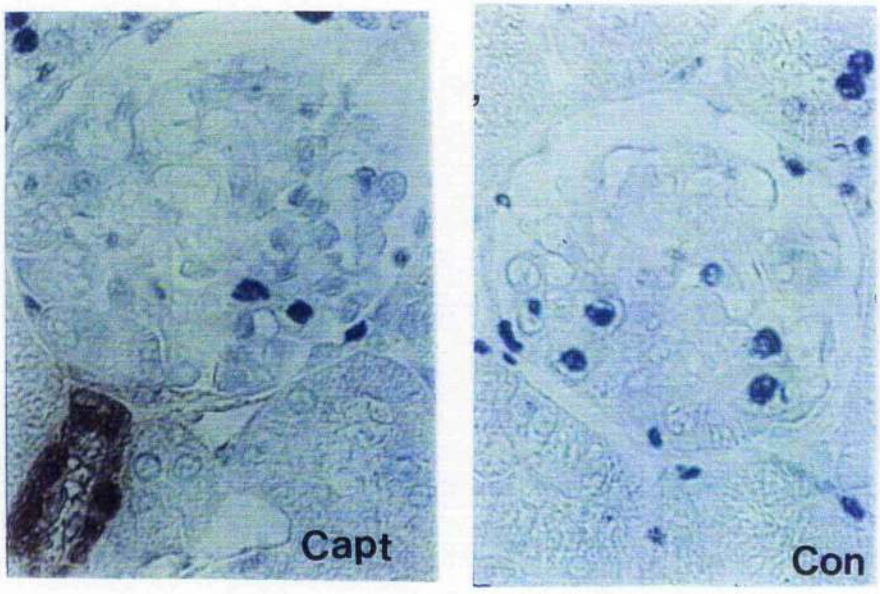


Fig.3.6b. The effects of captopril on renin and DNA synthesis in the kidney. Despite increased intrarenal renin caused by captopril (Capt), DNA synthesis was reduced in glomerular cells. Control (Con).

Discussion

1) *Proliferation of Renin-Secreting Cells:*

Treatment with ACE inhibitors or dietary sodium restriction promotes activity of intrarenal renin-angiotensin systems (33, 34, 52, 170) whose functions, amongst many, may be to promote renal cell growth (54). Treatment with low dietary sodium or captopril are two means by which renin secretion and AII within the kidney may be raised. Low dietary sodium, by increasing renin secretion promotes AII which is required for the subsequent synthesis and secretion of aldosterone to retain sodium. Captopril, by inhibiting converting enzyme, lowers plasma AII concentration and blood pressure which both stimulate renal renin secretion. (19).

Following either treatment, the greater demand for renin results in an increased number of renin-secreting cells within the kidney. However, the mechnism for this response is controversial and it is not clear whether renin-secreting cell numbers increase because of hyperplasia or metaplasia. The use of a double-labelling technique with antibodies to renin and BrdUrd and experiments with captopril and low sodium diet were intended to show whether renin-secreting cells within the renal arterioles proliferate in response to stimulation.

Two enzyme substrates, fast red violet and fast blue were used independently to detect BrdUrd in conjunction with the horseradish peroxidase reaction for renin. Fast red violet and Fast blue b) have different absorption spectra from each other and are both readily distinguishable from diaminobenzidene, the chromogen used in the peroxidase reaction. Despite an increase in the number of immunoreactive renin secreting cells within the kidney with low sodium or captopril, there was no DNA synthesis in any renin-secreting cell. The result was the same whether Fast red or Fast blue was used to detect BrdUrd These results agree, in part, with Cantin *et al* (1977) (183) who failed to show an increase in tritiated thymidine ([^{3}H]TdR) uptake following development of the ischaemic (endocrine) kidney. This experimental model involves the partial ligation of the aorta between the renal arteries with a consequent reduction in renal blood pressure to one kidney. Since low blood pressure is a stimulus for renin secretion, ligation results in an increase in renin-secreting cell number within the JGA and arterioles. However, the lowering of blood pressure may alone have removed a stimulus for vascular smooth muscle cell growth in the

arterial walls. In the present study, blood flow was not restricted, yet neither treatment with low sodium or captopril had any effect on DNA synthesis in the renal arteriole. It would appear, therefore that cell proliferation is not a component of the adaptation of the renal arteriole to stimulation.

Stimulation increases the number of renin secreting cells and renin gene expression within the afferent arterioles (33). This has lead to speculation that existing cells within the arteriole are recruited and phenotypically transformed into myoepithelioid cells by metaplasia (30, 33,41,183). Others believe that migration of smooth muscle cells is possible (264). However, the mechanisms responsible for the changes in distribution of renin gene expression remain largely unknown and require further study.

2) *AII as a Growth Factor for Tubular and Interstitial Cells:*

It has been demonstrated that all renal tissue AII and up to 50% of renal venous AII are of intrarenal and not systemic origin (186). The physiological significance of intrarenal AII is unclear. Mendelson *et al* have demonstrated high density AII receptors in the superficial and deep cortex of the kidney (187). Glomerular AII receptors are distributed predominantly in mesangial cells (43, 187) whilst the highest density of AII receptor binding sites are in proximal convoluted tubules (176) and to a lesser extent in renomedullary interstitial cells (188). The functional role of these receptors with regard to the regulation of salt and water metabolism is only partially known (49) and it is possible that they also mediate the growth-promoting effects of AII formed either intrarenally or systemically (54).

Treatment with low dietary sodium, which raises both intrarenal renin/AII and systemic renin/AII, had no significant effect on DNA synthesis in any renal cell type, indicating that AII does not appear to have direct intrarenal or systemic effects on renal cell growth *in vivo*. Furthermore, neither AII infusion which potentially increases systemic and decreases intrarenal AII simultaneously, nor captopril with opposite effects on AII control, had any significant effect on interstitial or tubular cell growth. Surprisingly, when systemic AII was raised by infusion, a negative trend on growth of interstitial cells was observed. It is possible that a significant result might have been achieved if the response of particular tubules and renal cell types were noted separately. Also, knowledge of plasma and local tissue AII values and plasma renin

activity would have allowed a precise distinction between systemic and paracrine responses.

The present observation that tubular and interstitial cells do not proliferate in response to manipulations of the renin-angiotensin system contrast with those of Johnson *et al* (189), who using PCNA expression as a marker of DNA synthesis, claimed that AII increased proliferation in tubule and interstitial cells. In this instance, however, the dose of AII was almost fourfold greater than that used in the present experiments resulting in severe hypertension with consequent renal injury. There was also evidence of leukocyte infiltration, tubular damage and interstitial fibrosis. Since cytokines produced by neutrophils are growth factors (190), it is difficult to assess whether the growth effects were mediated directly by AII or by haemodynamic effects of AII or as a result of the immune response to injury. Furthermore, this study is subject to error. It is well established that PCNA is functionally active during periods of unscheduled DNA replication, including DNA repair, with the result that PCNA antigen overestimates cells which are in cycle (191). Renal damage alone, therefore, could increase PCNA expression by stimulating regeneration.

3) *AII as a Growth Factor for Mesangial Cells:*

The role of AII as a growth factor for vascular smooth muscle cells has been studied extensively, particularly as a mediator of blood vessel hypertrophy in hypertension (192). The similarity between vascular smooth muscle and mesangial cells suggest that the mechanisms proposed for the AII-induced growth of vascular smooth muscle cells may also apply to the mesangium.

The lack of an effect of low or high dietary sodium on mesangial cell DNA synthesis contrasts with increased DNA synthesis following AII infusion. The main differences between AII raised by infusion and by low sodium are haemodynamic. AII infusion raised blood pressure significantly resulting in mild hypertension whereas low sodium blunts vascular pressor responsiveness both directly, by downregulating receptors to pressor agents such as AII and norepinephrine (78, 185), and, indirectly, by extracellular fluid volume depletion. For these reasons, low dietary sodium has been used clinically as a treatment for hypertension. High blood pressure increases smooth muscle tension and hence may induce expression of growth proto-oncogenes (142). The angiotensin receptor subtype AT1 mediates most of the vascular and

renal proximal tubular actions of AII (193, 265). Down regulating these receptors by feeding a low sodium diet may result in decreased pressor responsiveness, lower blood pressure and reduced smooth muscle cell stretch, which could remove a stimulus for growth. Conversely, AII infusion promotes high blood pressure with increased stretch which could explain increased mesangial cell DNA synthesis in response to AII infusion. Likewise, reduced mesangial cell DNA synthesis in response to captopril may be associated with reduced blood pressure. The insensitivity of rat mesangial cells to the proliferative effects of AII have been confirmed *in vitro*, although AII did induce an increase in protein synthesis (177).

4) *Does Blood Pressure Influence the Response to AII:*

Changes in vascular smooth muscle cell growth are normally associated with high blood pressure. It is not fully known whether AII acts as a smooth muscle cell growth factor *in vivo*, whether blood pressure can, alone, stimulate growth or whether there is a requirement for both factors in order for cells to synthesise DNA. In the Goldblatt two-kidney, one-clip model, rats develop a renin-dependent form of hypertension (113). This model has been used extensively to study independently the role of blood pressure in the control of renin secretion. It is known that renal artery clipping results in a sustained increase in plasma renin activity and a subsequent rise in blood pressure to the unprotected, hypertensive kidney. This results in renovascular hypertension with the rise in blood pressure also sustained by vascular hypertrophy. The clipped kidney receives raised AII but is protected from the increase in blood pressure. Using this experimental model, Eng *et al* (194) demonstrated an increase in DNA synthesis in all renal cell types of the hypertensive kidney, but little or no change in growth in the protected kidney, and concluded that both raised pressure and AII are a pre-requisite for growth. The Goldblatt model is similar to the present study where low dietary sodium represents the clipped kidney being exposed to raised levels of AII in the absence of a rise in pressure. The clipped kidney, on the other hand, parallels the effects of AII infusion being exposed to both high AII and blood pressure. The lack of an effect of low dietary sodium on renal cell growth suggests that AII may not be involved in renal cell growth *in vivo*, but increased blood pressure is probably a pre-requisite for growth. The conclusions that Eng *et al* derived from their experiments are flawed in that they had no

evidence in support of growth effects of AII. An independent study based on raising blood pressure without altering the activity of the renin-angiotensin system is required to clarify the role of AII in renal cell growth. Ideally, blood pressure measurements for all animals would have been appropriate for this study, however, while the tail-cuff method is useful for detecting relatively large positive increments in blood pressure with AII, it is not suitable for detecting smaller negative changes in otherwise normotensive rats. A more sensitive method for studying subtle effects of captopril and dietary sodium on blood pressure would have involved cannulation of a major artery which would have compromised growth aspects of this study.

In conclusion, increased numbers of renin-secreting cells caused by dietary sodium restriction and captopril treatments are due to metaplasia and not changes in cell proliferation. Increased and decreased DNA synthesis in renal glomeruli in response to angiotensin II and captopril are accounted for by known changes in intrarenal hamodynamics and not exclusively by effects on systemic concentration of angiotensin II. Intrarenal changes in angiotensin II, which may have been expected in response to the various treatments, had no significant paracrine or autocrine effects on DNA synthesis in tubule or interstitial cells.

Chapter 3b: THE EFFECTS OF MANIPULATIONS OF THE RENIN-ANGIOTENSIN SYSTEM ON DNA SYNTHESIS IN THE RAT ADRENAL GLAND

Introduction:

Aldosterone secretion by the adrenal zona glomerulosa is controlled predominantly by the renin-angiotensin system. *In vivo*, angiotensin II specifically stimulates the zona glomerulosa to secrete mineralocorticoids with no apparent direct effects on glucocorticoid secretion. Evidence for a zone specific role for AII is derived firstly from studies with low dietary sodium which increases both plasma renin activity and plasma AII levels (77, 195), upregulates zona glomerulosa AII receptors (77) and increases the biosynthetic capacity of the zona glomerulosa (196). Secondly, whilst the zona fasciculata and reticularis atrophy following hypophysectomy, the glomerulosa remains intact, indicating only partial regulation by ACTH (2). Thirdly, AII *in vitro* stimulates steroidogeneis in isolated rat zona glomerulosa cells and not in fasciculata/reticularis cells (197).

There are several mechanisms whereby AII stimulates aldosterone secretion. The acute response to AII is mediated through high affinity zona glomerulosa cell receptors, an effect which can be totally blocked by the ACE inhibitor captopril (77). In the longer term AII also controls, in part, the expression of some of the enzymes involved in the synthesis of aldosterone (81, 198) and increases the size of the mitochondrial and lipid compartments of the cell (63) where the steroid enzymes are secreted and lipid is stored (199). In addition, Hartcroft & Hartcroft (180) described an increase in zona glomerulosa width in response to dietary sodium restriction. However, whether this increase in glomerulosa width is associated with cell proliferation as well as cell hypertrophy remains to be investigated. Other actions of AII in the adrenal gland include its role in intra-adrenal renin-angiotensin systems. Renin and AII concentrations in the zona glomerulosa vary with the plasma electrolyte status (60).

According to Race and Green (96), individual zones of the cortex are responsible for maintaining their own cell populations in response to increased functional demand. The evidence for this is controversial. Some studies have shown that although cells proliferate in the outer zones, the new cells are subsequently displaced inwards to

replace cells which die (85, 86, 93, 200). The evidence for the zonal theory is based on several lines of evidence: Ogishama *et al* (201) demonstrated zone-specific expression of aldosterone synthase cytochrome p-450aldo (zona glomerulosa) and cytochrome p-45011ß (zonae fasciculata/reticularis) indicating relative functional independence of the adrenocortical zones; administration of renin increased zona glomerulosa width in rats (202); the zona reticularis, which is commonly regarded as the apoptotic zone of the cortex, has AII receptors present on these cells albeit to a lesser extent than the zona glomerulosa (203), indicating probable functional significance of AII within this zone.

Stimulation of the adrenal medulla is mediated largely through the action of nerves which synapse on chromaffin catecholamine-secreting cells to promote secretion. Secretion is, in part, also controlled by AII which binds either directly to specific receptors on chromaffin cells (204) or indirectly by increasing sympathetic nervous system activtiy (5). Low blood pressure is a known stimulus for catecholamine secretion (205, 206), however, it is not known whether changes in growth of the cells are a component of this response and whether growth effects of AII are involved. Changes in chromaffin cell proliferation have been described in response to physiological stimulation (207).

The first aim of this study, therefore, was to assess whether the adaptation of zona glomerulosa to continuous stimulation with AII involved changes in DNA synthesis. AII was raised systemically by infusion or by dietary sodium depletion and was inhibited by treatment with captopril. The second aim was to examine whether changes in DNA synthesis are zone specific or whether growth effects of AII are more diverse within the adrenal gland. DNA synthesis was assessed over a two week period by the continuous infusion of BrdUrd.

Materials & Methods:

As described for Chapter 3a.

Results:

Histology

Except for rats treated with captopril, the zona fasciculata was clearly delineated from the zona glomerulosa by the zona intermedia comprising small cells 3-5 cell layers thick, with relatively little lipid content and few BrdUrd-positive nuclei. In all zones of the cortex and the medulla, epithelial cells were distinguished from non-epithelial cells by being bigger and having rounder and larger nuclei. Staining with Ulex *europeus* lectin and immunohistochemistry indicated that most of the non-epithelial cells were endothelial (refer to Fig.3.7).

With the exception of the zona glomerulosa, the numbers of epithelial and non-epithelial cells were similar. BrdUrd indices tended to be greatest in the zona glomerulosa and outer zona fasciculata and least in the inner zona fasciculta and reticularis. Within the zona glomerulosa BrdUrd-positive nuclei were uniformly distributed.

Angiotensin II Infusion:

AII infusion increased blood pressure (AII 177± 11.1 S.E.M; cf control 131.5± 5.3 mmHg, $p<0.05$) and increased the width of the zona glomerulosa (Fig 3.8a). Epithelial zona glomerulosa cells, grouped together in aggregates, were enlarged and contained abundant cytoplasmic lipid (Fig 3.8a). Half of the epithelial cells had BrdUrd-positive nuclei, threefold more than controls ($p<0.001$) (Fig.3.8c). In non-epithelial zona glomerulosa cells and in epithelial and non-epithelial zona intermedia and zona fasciculata cells, the BrdUrd-index was unaffected by treatment.

In the zona reticularis, at the interface with the medulla, groups of BrdUrd-positive cells were abundant in adrenals from AII-treated rats (Fig.3.8b). The BrdUrd indices of non-epithelial cells in the zona reticularis and of both cell types in the medulla were lower in AII-treated glands.

Dietary Sodium Manipulation:

Compared with rats fed a high sodium (supplemented) diet, the pattern of response of the adrenal gland to sodium restriction was similar to the effects of AII (3.9c). The zona glomerulosa was wider

with larger epithelial cells many more of which contained BrdUrd-positive nuclei (3.9a). As with AII treatment, the BrDurd-index of zona reticularis epithelial cells was increased with dietary sodium restriction (Fig.3.9b). However, unlike AII treatment, dietary sodium restriction did not affect DNA synthesis in the medulla.

Captopril Treatment:

Captopril treatment markedly reduced zona glomerulosa width but had little effect on BrdUrd labelling. (Fig.3.10c). However, the morphology of epithelial zona glomerulosa cells was very different from controls (Fig.3.10a). Small cells with lipid-deplete cytoplasm and pyknotic nuclei were arranged in irregular clumps. The BrdUrd index in non-epithelial cells of inner zona fasciculata and reticularis was lower after captopril, whereas twice as many of the epithelial cells of the medulla were positive (Fig.3.10b).

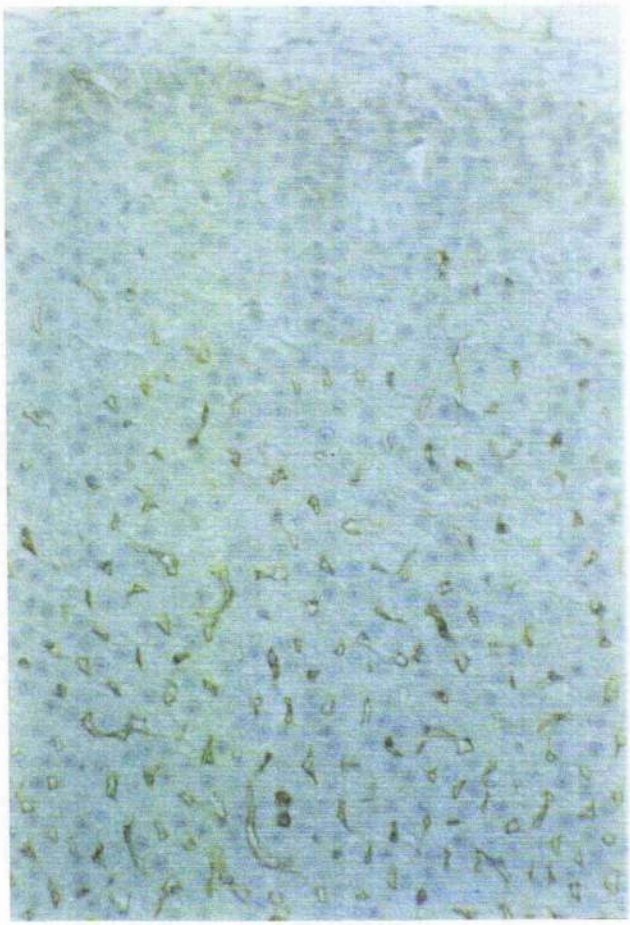


Fig.3.7. The rat adrenal cortex immunostained with Ulex europeus lectin. Endothelial cells are brown.

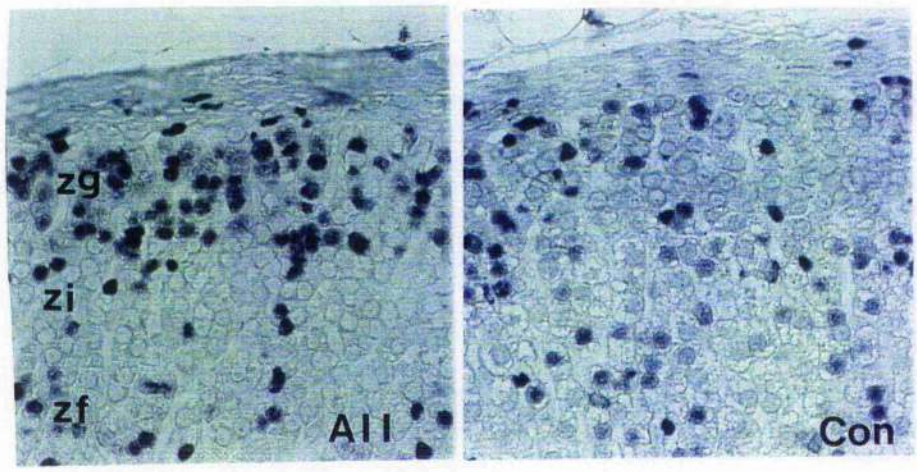


Fig.3.8a. The effects of AII infusion on DNA synthesis in the zona glomerulosa (ZG). Angiotensin II (AII), Control (Con). The zona intermedia (ZI) clearly separates the ZG from the zona fasciculata (ZF).

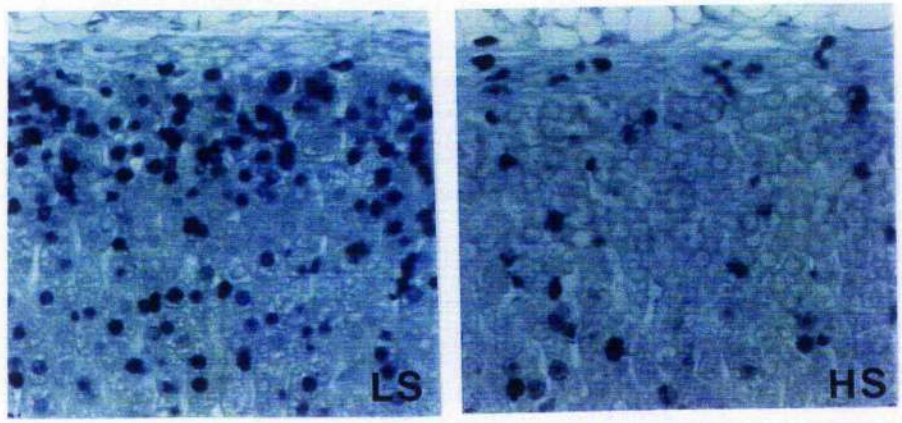


Fig.3.9a. Effects of low and high dietary sodium on DNA synthesis in the zona glomerulosa. Note increased zonal width with low sodium.

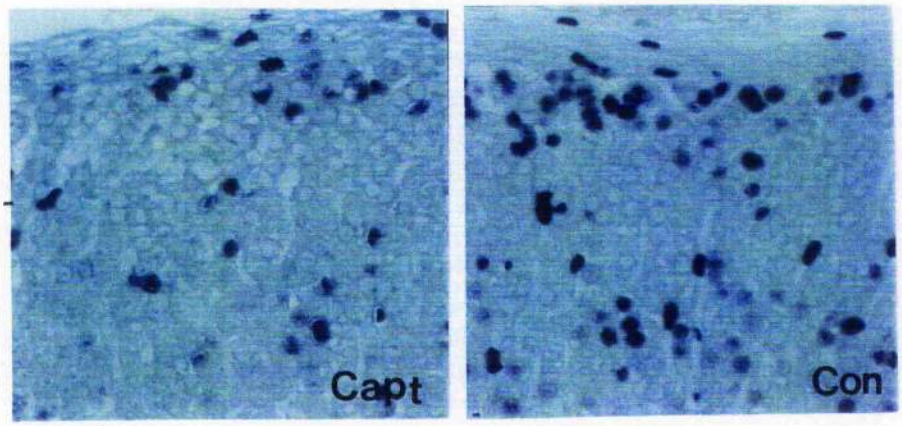


Fig.3.10a. The effects of captopril treatment on the zona glomerulosa. The cells appear smaller and the zone is reduced in width.

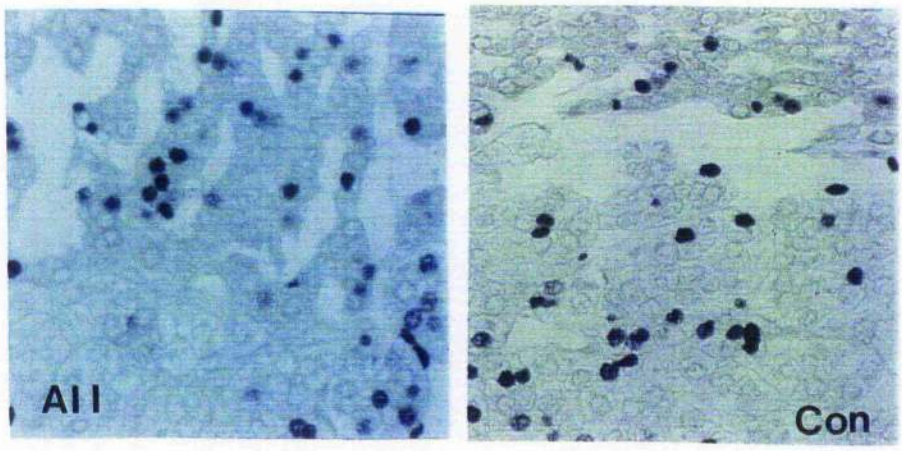


Fig.3.8b. The interface between the cortex and medulla showing increased DNA synthesis in the reticularis following AII infusion.

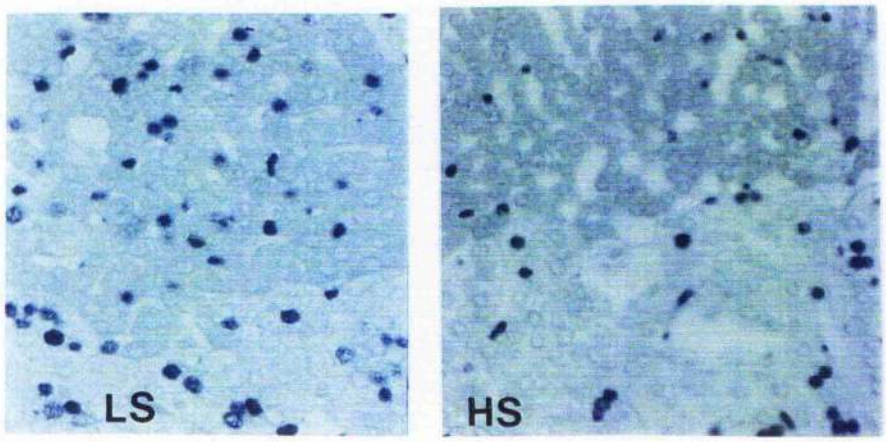


Fig.3.9b. The effects of low and high dietary sodium on the zona reticularis and medulla.

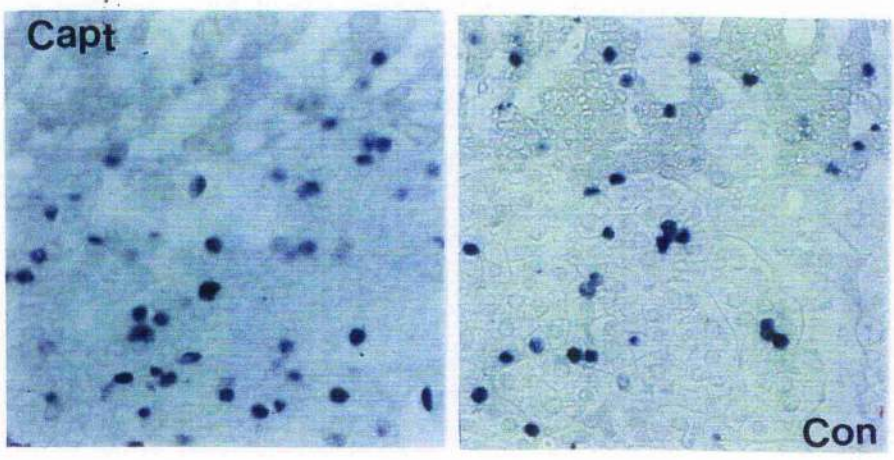


Fig.3.10b. The effects of captopril on DNA synthesis in the reticularis/medulla.

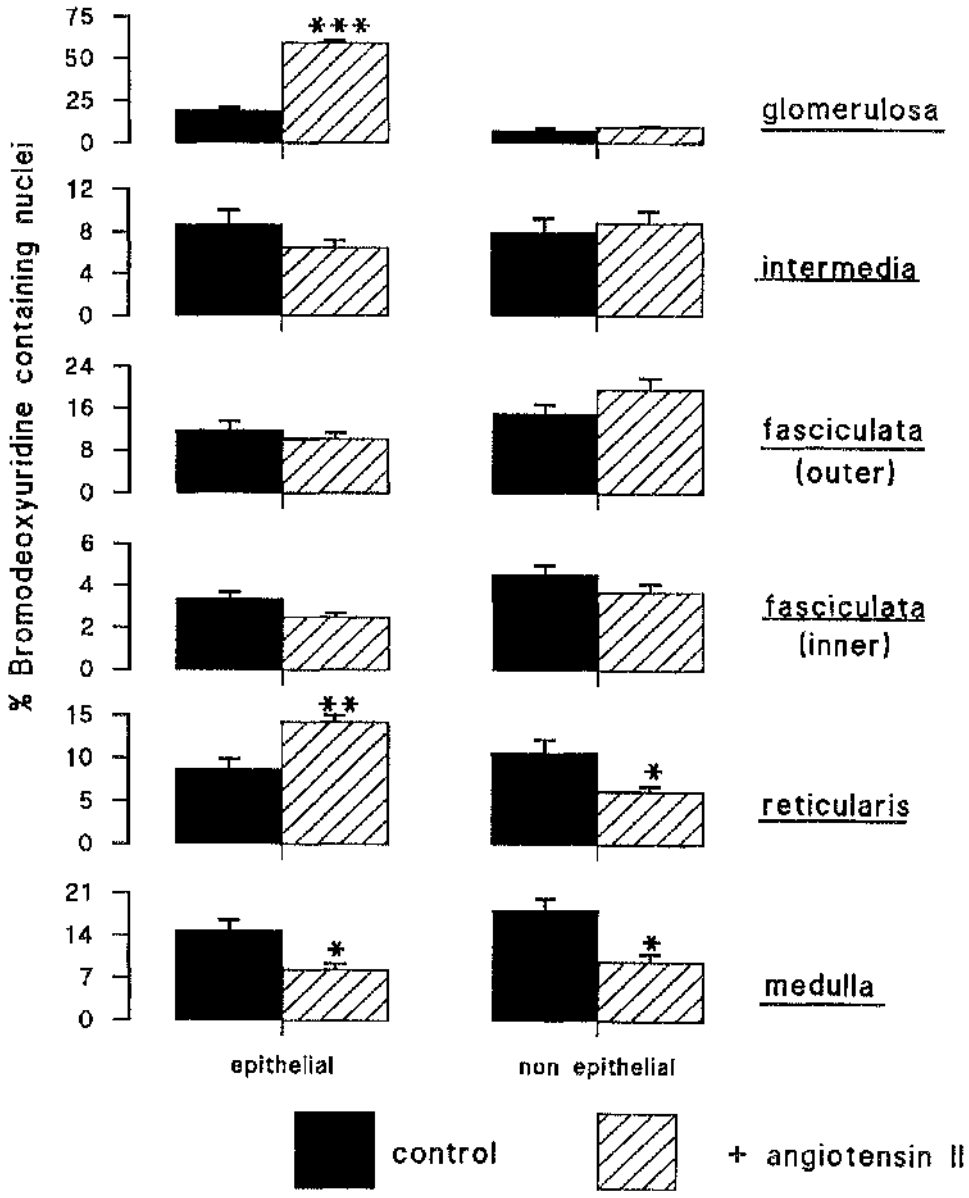


Fig.3.8c. DNA synthesis in the rat adrenal cortex and medulla following infusion of AII. The most significant increases are in the epithelial cells of the zona glomerulosa and zona reticularis.

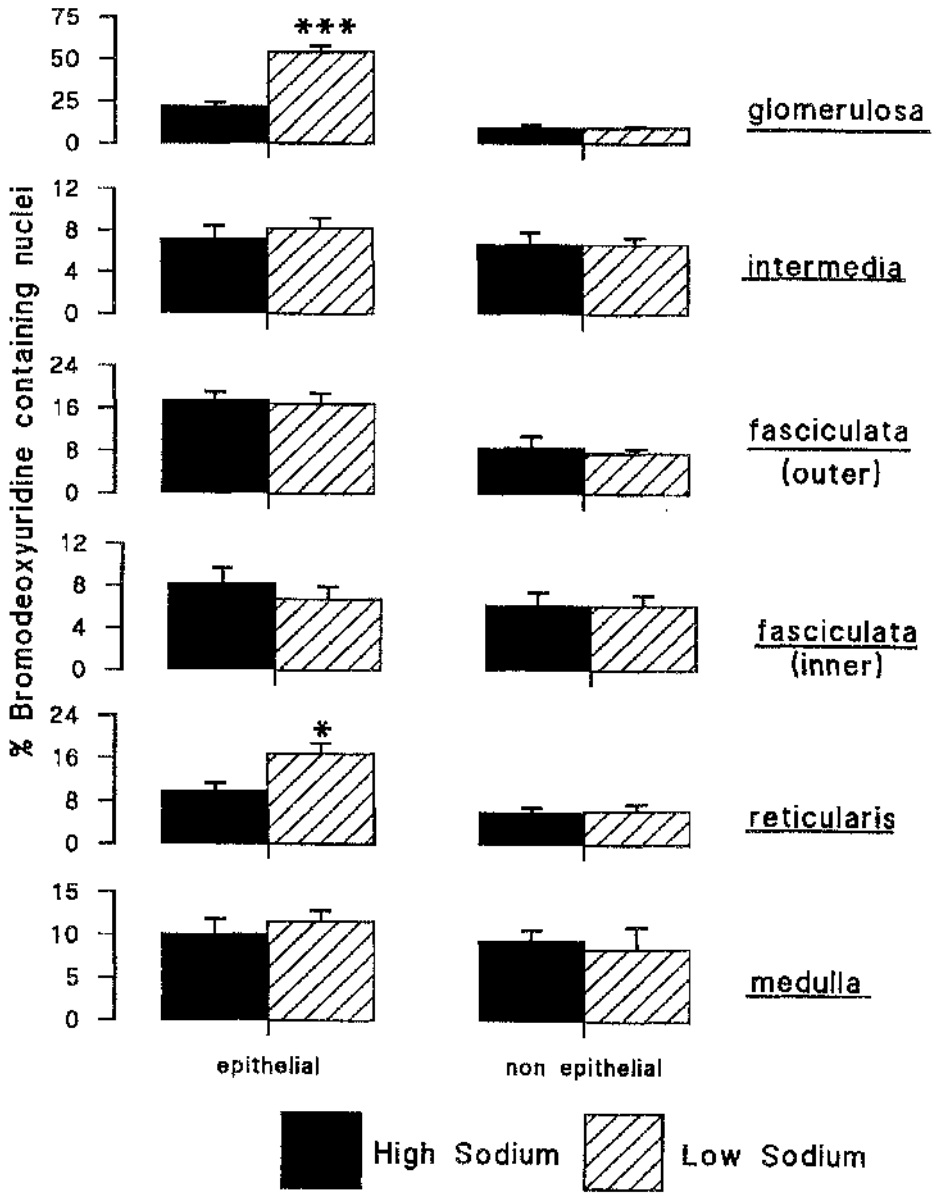


Fig.3.9c. DNA synthesis in the adrenal cortex and medulla following low and high dietary sodium. Low sodium increased DNA synthesis in the zona glomerulosa and reticularis.

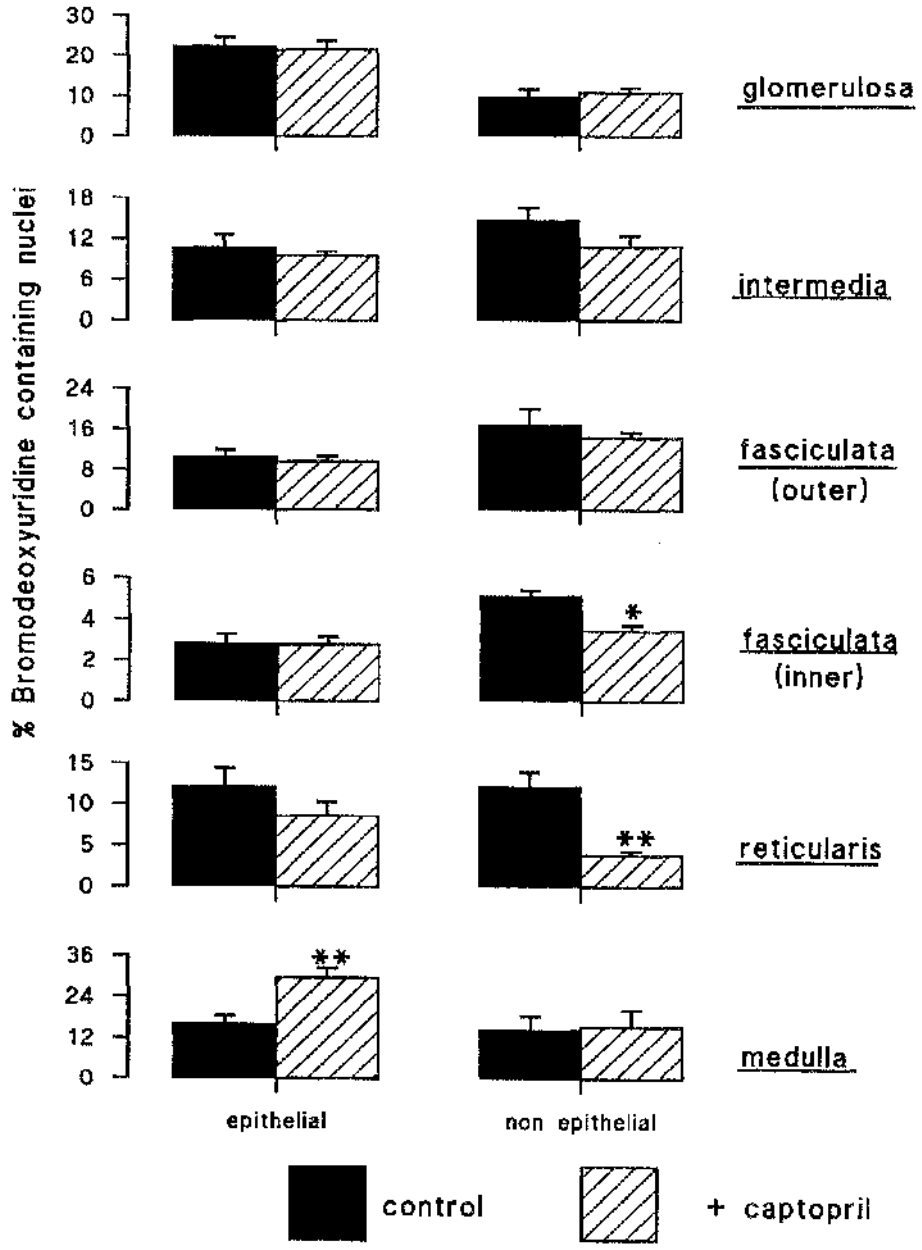


Fig.3.10c. The BrdUrd indices in the adrenal cortex and medulla following treatment with captopril.

Discussion:

Adult male rats were selected for this study to avoid any prepubertal (208) or oestrus-related (209) changes in proliferative activity and the timecourse of the study avoided any changes in growth associated with circadian rhythm (210).

In the control groups, maximal DNA synthesis occurred in the zona glomerulosa and outer zona fasciculata. This is consistent with previous reports of mitotic activity in the adult male rat (87, 200). Interestingly, those cells which had incorporated BrdUrd, were situated on either side of an almost unlabelled, clearly delineated, zona intermedia which appeared not to change even when the cortex was stimulated with AII. The lack of DNA synthesis in the zona intermedia is in accord with Engstrom (211) and van Dorp & Deane (80) but contrasts with Mitani *et al* (81) who, also using BrdUrd, described the zona intermedia as being a novel stem cell zone with apparent proliferative activity. The main disadvantage of the latter study, however, is the timecourse used to demonstrate changes in DNA synthesis. BrdUrd administered by single injection following low dietary sodium for a period of hours rather than days, whilst showing some labelling, is an insufficient time for the adrenal gland to adapt properly to alterations in diet.

AII raised by infusion and by low dietary sodium both increased DNA synthesis in epithelial cells of the zona glomerulosa and caused a twofold increase in zona glomerulosa width. It would appear, therefore, that cell growth is an intrinsic component of the zona glomerulosa in response to increased activity of the renin-angiotensin system. In addition, many zona glomerulosa cells were enlarged and contained abundant lipid. These cells also have enlarged mitochondria and a more extensive smooth endoplasmic reticulum (98, 212), the organelles which are responsible for steroid synthesis (199). The growth effects are specific to the rise in AII since zonal atrophy occurred following treatment with captopril. Although, in this instance, the BrdUrd index was unchanged, there was nuclear pyknosis and atrophy of cells of the zona glomerulosa which had not incorporated BrdUrd indicating that cell death also probably occurred. Since the BrdUrd index was based on the number of BrdUrd-positive cells as a proportion of all cells within the zone, increased cell death would probably be reflected by a higher BrDurd index. Although DNA synthesis was probably reduced in the zona glomerulosa, programmed cell death may also have occurred

following withdrawal of the trophic stimulus, this may have obscured an increase in the BrdUrd index. Despite evidence that the zona reticularis is the zone where cells die by apoptosis (2), the effect of captopril this study raises the possibility that cell death also occurs zonally. Although Wyllie et al (2) demonstrated increased apoptosis only in the inner zones of the cortex following dexamethasone treatment or hypoxia, there are few other experiements to show that cell death also occurs in the outer zones.

Low sodium and AII infusion increased epithelial cell DNA synthesis in the zona reticularis, independent of any changes in the zona intermedia or zona fasciculata. Furthermore, treatment with captopril caused a reduction, albeit small, in epithelial cell DNA synthesis in the reticularis. These results imply that AII could also act as a specific growth factor for cells of the zona reticularis. The lack of a growth effect of AII on the epithelial cells of the zona fasciculata despite changes in the zona reticularis argues against the migration theory.

AII infusion caused a decrease in DNA synthesis in the adrenal medulla, in contrast to low sodium which had no effect. Opposite to the effects of AII, captopril caused an increase in medullary cell DNA synthesis. It would appear, therefore, that AII does not have a direct growth-promoting effect on the adrenal medulla but may have influences mediated by haemodynamic processes. Although, under normal circumstances, raised systemic AII stimulates sympathetic activity and promotes catecholamine secretion (5), it is possible that prolonged infusion of AII which increases blood pressure also results in reduced demand for catecholamines to compensate for the rise in blood pressure. Similarly, the hypotensive effects of captopril are perhaps compensated by increased catecholamine secretion which is enhanced in response to hypotension (110).

Immunostaining with the lectin, Ulex *europeus,* demonstrated that the majority of non-epithelial cells within the adrenal gland were endothelial. Although the effects of manipulating the renin-angiotensin system on DNA sythesis of these cells are difficult to interpret and appear not to be specific, it is known that adrenocortical secretory activity can be modulated by changes in blood flow (110). Since vasoreactivity is modulated by vasoactive substances secreted by endothelial cells, it is possible that changes in DNA synthesis of adrenocortical endothelial cells reflects changes in adrenal haemodynamics.

In conclusion, increased DNA synthesis contributes to the hypertrophy of the zona glomerulosa which occurs in longterm increased activity of the circulating renin-angiotensin system. AII appears to be a specific growth factor for zona glomerulosa and zona reticularis cells *in vivo*. The lack of growth effects in other zones supports the zonation theory of cytogenesis and suggests that cell proliferation is an important component of the response of the adrenal cortex to stimulation. The changes in DNA synthesis in the adrenal medulla are probably due secondary to variations in the demand for catecholamines caused by changes in systemic blood pressure.

Chapter 3C: VASCULAR HYPERTROPHY AND THE RENIN-ANGIOTENSIN SYSTEM

Introduction:

Structural alterations of the vascular wall play an important role in the pathogenesis and perhaps the aetiology of hypertension (114, 213). Three important changes have been identified in blood vessels: hyperplasia, which is an increase in the number of smooth muscle cells by cell division; cellular hypertrophy which is an increase in the size of existing smooth muscle cells and remodelling which is an a rearrangement of existing cells around a smaller lumen (118).

The factors which contribute to cardiovascular growth are complex and although there is a strong genetic component in essential hypertensive patients and in genetically hypertensive rats (214, 215), other physiological factors are also involved. The renin-angiotensin system is implicated both in the control of blood pressure and in the control of hypertrophy. Treatment of SHR strains with ACE inhibitors prevents the development of hypertension and reverses cardiovascular hypertrophy (215) whereas replacement of AII during ACE inhibitor treatment restores hypertrophy (215). *In vitro*, AII promotes mitogenesis of vascular smooth muscle cells (130, 131) protein synthesis (116) and activates some of the proto-oncogenes responsible for growth (265). Taken together, these data suggest that AII plays an important role as a cardiovascular mitogen.

Investigations of the effect of AII on blood vessels are limited by the techniques employed. For example, myography has demonstrated hypertrophy in blood vessels following AII infusions, however the range and size of blood vessels selected for measurement are restricted to those which can be manipulated without damage in either wire or perfusion myographs. Small resistance vessels which are the main determinants of peripheral vascular resistance are therefore excluded. In myography, it is not possible to assess whether thickening of the media is due to increased cell size or whether cells become polyploid or have divided. In the present study no vessels are excluded and the techniques have focussed on changes in DNA synthesis, cell number and ploidy.

Vascular smooth muscle cells grow slowly *in vivo* (216), and some previously applied techniques are suited to measuring changes over periods of hours and are inadequate to detect changes over a period

of days (3, 158). *In vitro* techniques have drawbacks because tissues are separated from intrinsic growth factors and interactions with other cells. The present study has investigated whether AII is a growth factor *in vivo* using the continuous infusion of bromodeoxyuridine over two weeks, a timecourse that allows for the slow progressive growth of blood vessels.

A further complication is that vasoactive hormones like AII may have dual effects on vascular morphology causing hypertrophy either directly by acting as a mitogen, or indirectly by increasing blood pressure. To overcome this problem, previous studies have examined the vascular effects of AII in the presence or absence of other antihypertensive agents (126, 217, 218). This too is unsatisfactory since blood pressure lowering drugs might also directly affect smooth muscle cell growth rates (158) or have other secondary effects on humoral factors controlling growth. In the present study we have tried to dissociate the mitogenic effects of AII from its effects on blood pressure by adapting rats to low and high dietary sodium.

Whilst low dietary sodium endogenously raises AII levels, there is also the tendency for prolonged sodium restriction to reduce blood pressure (267). The dose of captopril given in the present study has also been shown experimentally to reduce blood pressure and simultaneously lower plasma angiotensin levels in rats (129).

Materials & Methods:

The animal experiments described in Chapter 3a are the same for this chapter. Changes in deposition of proteoglycans, collagen and matrix proteins were assessed using special stains; Masson's trichrome, Alcian blue PAS and Methyl Blue Van Gieson. Refer to general materials and methods chapter..

Quantification: BrdUrd indices were obtained for a range of blood vessels in cross section. Medial area was calculated for the same blood vessels.

Results:

1) *Histology:*

Histological sections of the entire mesenteric bed contained transverse sections of arteries, arterioles and veins. Only endothelial cells were identified in the intima. The media consisted entirely of smooth muscle cells in all vessels examined. By contrast, the adventitia contained fibrocytes, nerves, capillary and lymphatic blood vessels and a few mononuclear cells. Histological examination of sections treated with histochemical stains (see "special stains" Chapter 2) showed no alteration in the collagen, matrix protein or proteoglycan content of the vessel wall in any of the experiments.

2) *DNA Synthesis:*

AII infusion increased DNA synthesis in the arterial tree by twofold in the endothelium of arterioles, and by threefold in the media and adventitia (Fig.3.11). Fig.3.12. shows that the number of BrdUrd-positive nuclei in the media of vessels of all sizes increased, indicating that DNA synthesis was not confined to resistance vessels. The effect of AII infusion on BrdUrd uptake is shown in Fig 3.17.

Low dietary sodium increased BrdUrd indices by 2-3 fold in the endothelium, media and adventitia when compared with vessels from rats with a sodium supplement (Fig. 3.13). The effect of dietary sodium intake on the media of all vessel sizes is shown in Fig.3.18. The number of BrdUrd-positive nuclei was consistently higher in vessels irrespective of size in the low sodium group (Fig.3.14).

Treatment with captopril halved the BrdUrd index in the media and adventitia, but had no effect on the endothelial BrdUrd index (Fig.3.15). This effect was observed in arteries and arterioles of all sizes (Fig.3.16). The effect of captopril on BrdUrd uptake in all vessels

is shown in Fig.3.19. None of the treatments had any significant effect on DNA synthesis in venules or veins.

3) *Analysis of Ploidy:*

In all experimental groups, analysis of the histograms plotting the frequency distribution of nuclear DNA content indicated that the majority of smooth muscle cells were diploid in all sizes of vessels; there was an occasional tetraploid nucleus typical of cells undergoing proliferation, but the number of these was not significantly affected by any of the treatments. Representative histograms from mesenteric vessels from an AII-treated rat and a control are presented in (Fig.3.20).

4) *Vascular Morphometry and Cell Number*:

Table 3.1. Summarises the effects of all treatments on medial vascular DNA synthesis, medial area and cell number as compared by t-test; *, **, ***, P <0.05, 0.01, 0.001. Data show mean ± S.E.M (n= 5, 6).

	Angiotensin II	**Dietary Sodium**	**Captopril**
BrDurd Index (% Staining)	AII 37.4±1.7 *** Con 10.9±1.4	Low Na 19.2±1.7 ** High Na 6.9±0.8	Cap 3.1±0.7 ** Con 13.7±0.9
Medial Area (μm^2)	AII 14864±980 * Con 9414±1212	Low Na 9120±946 High Na 8199±882	Cap 7285±704 Con 10501±934
No. Nuclei/Section	AII 53.8±3.2 * Con 37±3.1	Low Na 41.9±3.7 High Na 34.6±2.9	Cap 34.5±2.8 Con 54.7±5.7
No.Nuclei /UnitArea (mm^2)	AII 4350±230 Con 5550±270	Low Na 5450±300 High Na 4940±240	Cap 5550±280 Con 5850±270

Angiotensin II Infusion:

All transeversely sectioned vessels were included in the analysis so that representative sampling of all order resistance vessels was assured. The size of blood vessels ranged from first to fifth order in AII and control groups. Medial area was normally distributed and was compared by t-test. Medial areas in the AII group, however, were greater for all sizes of vessel. To depict this effect graphically, the medial area of each group were ranked separately in ascending order and arranged into centiles. The first centile from the control group was compared directly with the first centile from the treated group. The standardised rank order of AII

AII treated vessels compared with those of controls is shown in Fig.3.21. At no point do the lines overlap suggesting that AII affects vessels of all sizes. The extent to which medial area is affected by treatment is estimated in Fig.3.24. Although AII has a pronounced effect on the media of all order resistance vessels, with increases in some resistance vessels up to 250%, AII also caused a significant increase in the number of medial nuclei per cross-section of media ($P<0.05$; Fig.3.25). There was a tendency for fewer nuclei per unit area of media but this was not statistically significant (Fig.3.26).

Low Sodium:

The standardised rank order of vessels in both groups are shown in Fig.3.22 Although the medial area of vessels from low sodium rats tended to be slightly higher than those of the high sodium group, this effect was not statistically significant. The estimated increase in medial area is depicted in Fig.3.24 Dietary sodium did not significantly affect the number of nuclei per cross-section of media (Fig.3.25) nor the number of nuclei per μm^2 of media (Fig.3.26).

Captopril:

The vessels ranked in order of medial area showed no significant effect of treatment (Fig.3.23). Treatment with captopril tended to reduce medial area (Fig.3.24), however this effect was not statistically significant. Similarly, the number of nuclei per cross section of media tended to be reduced with captopril but the effect was not significant (3.25) and there was no change in the number of nuclei per μm^2 of media (3.26). The structure of veins was unaffected by any treatment.

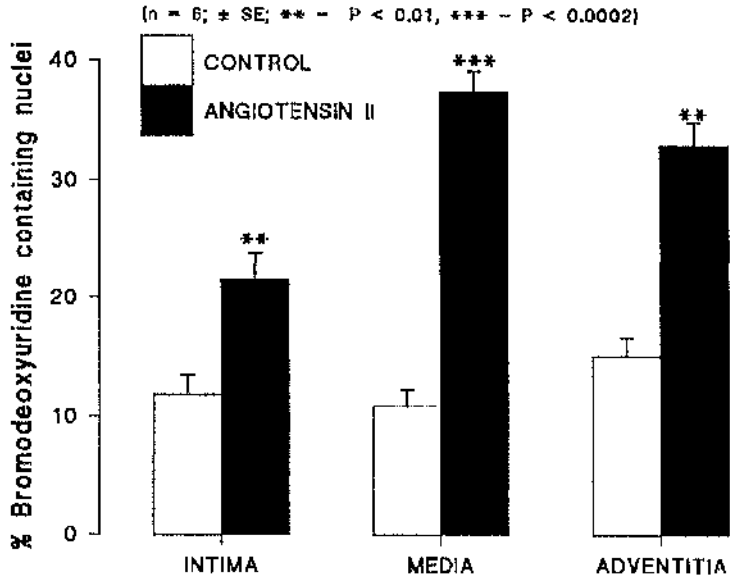


Fig.3.11. The effects of Angiotensin II infusion on DNA synthesis of intima, media and adventitia of mesenteric blood vessels.

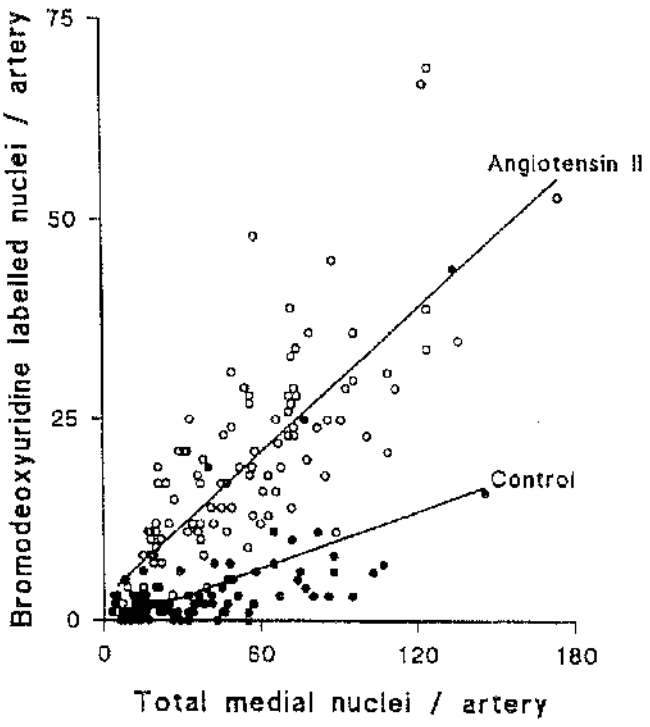


Fig.3.12. The effects of AII infusion on the relationship between BrdUrd index and vessel size. The number of BrdUrd-positive nuclei is increased in arterioles/arteries of all sizes. by AII treatment

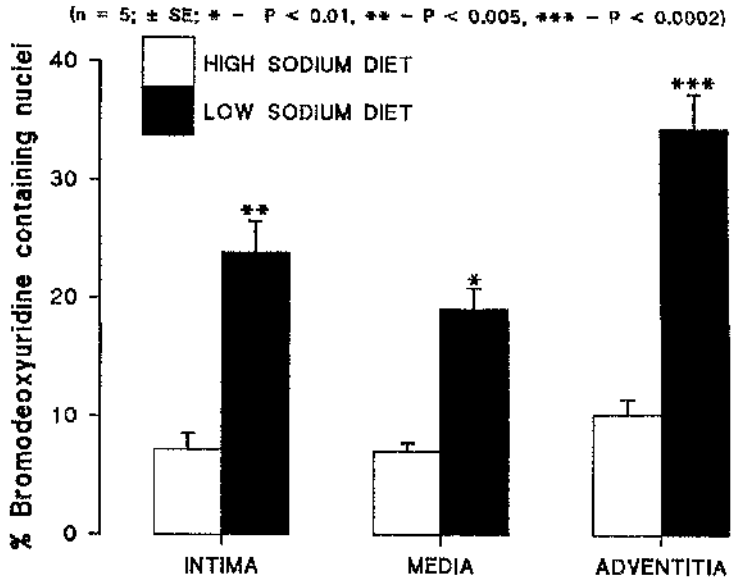


Fig.3.13. The effects of dietary sodium on DNA synthesis in mesenteric arteries. Note that the effects of low sodium are similar to AII infusion.

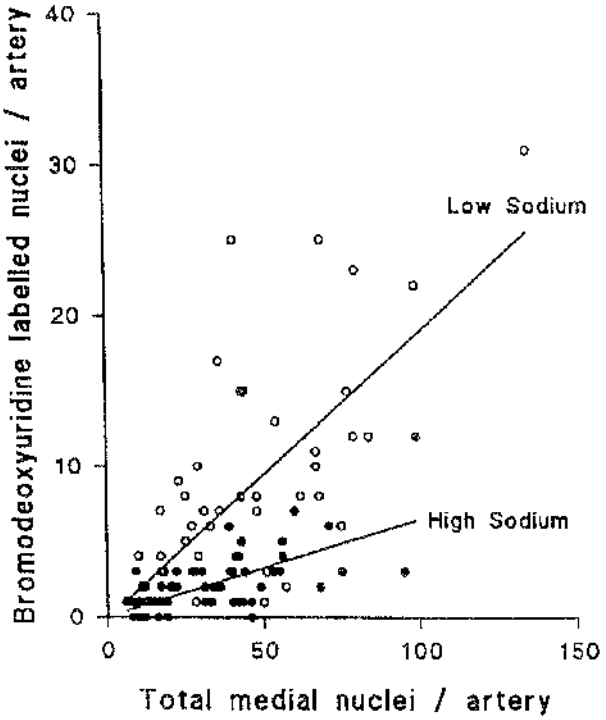


Fig.3.14. The relationship between BrdUrd index and vessel size. Low sodium increases BrdUrd uptake in vessels of all sizes.

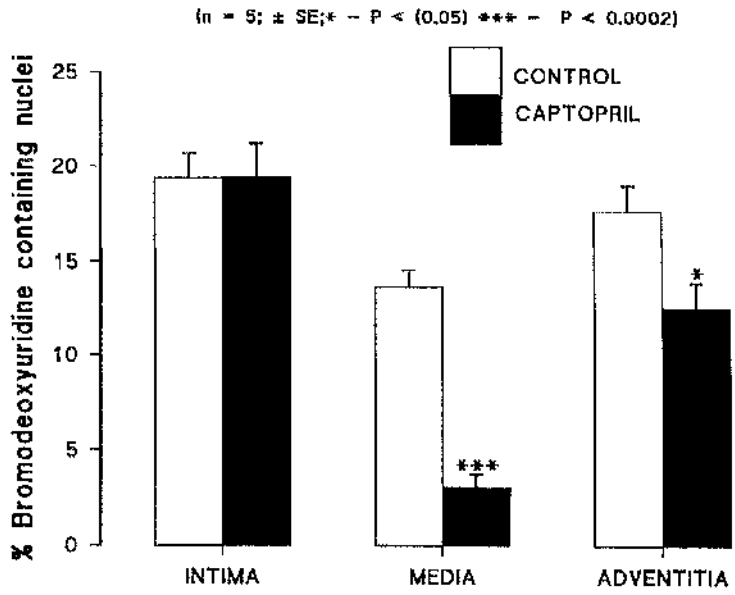


Fig.3.15. The effect of captopril on the BrdUrd index of mesenteric arteries. Note the lack of effect on the intima.

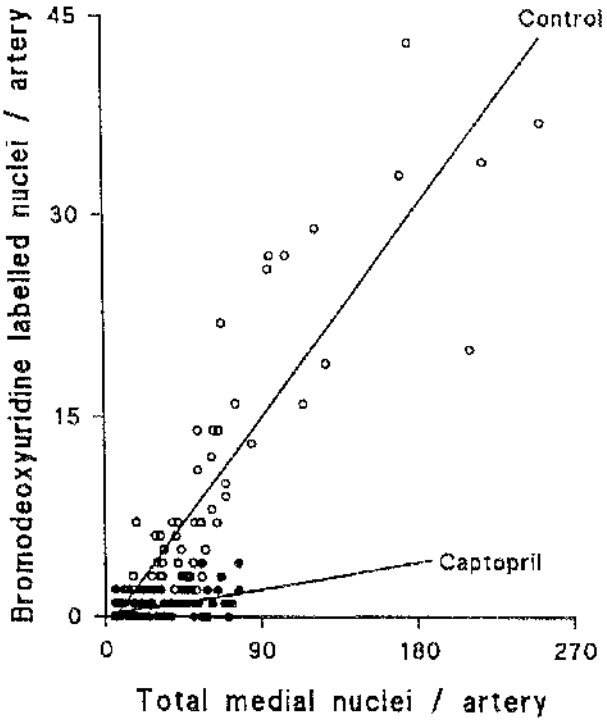


Fig.3.16. The effect of captopril on the relationship between vessel size and BrdUrd index.

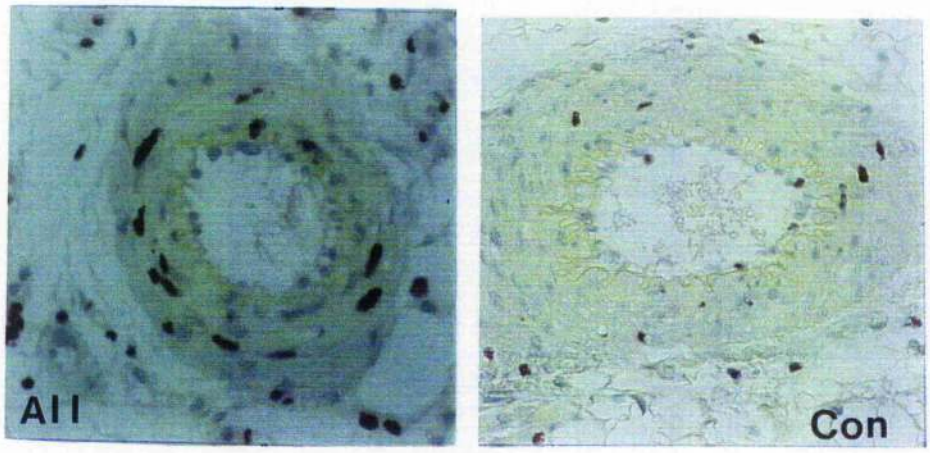


Fig.3.17. A mesenteric arteriole showing BrdUrd immunostaining following AII infusion. Angiotensin II (AII), Control (Con).

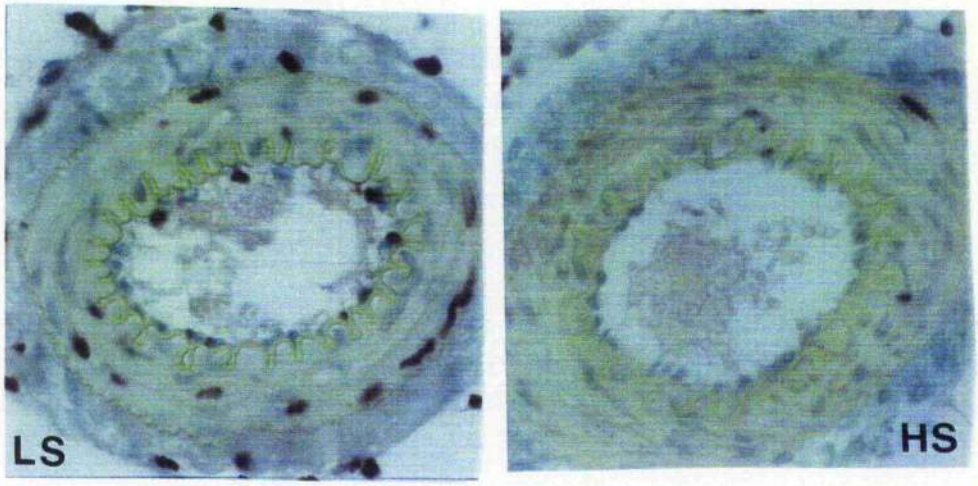


Fig.3.18. The effect of dietary sodium manipulation on DNA synthesis of representative arterioles. Low sodium (LS), High sodium (HS).

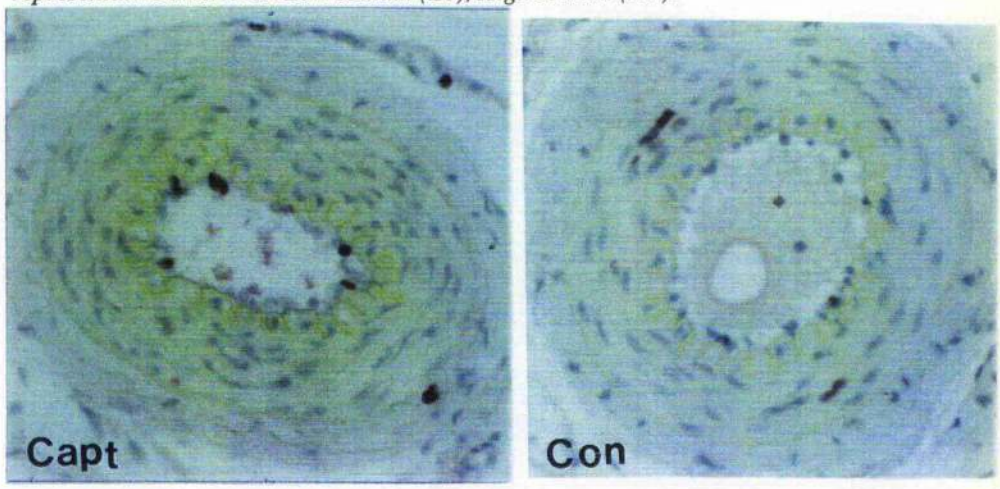


Fig.3.19. DNA synthesis in the media following treatment with captopril. Note the lack of effect of treatment on the intima.

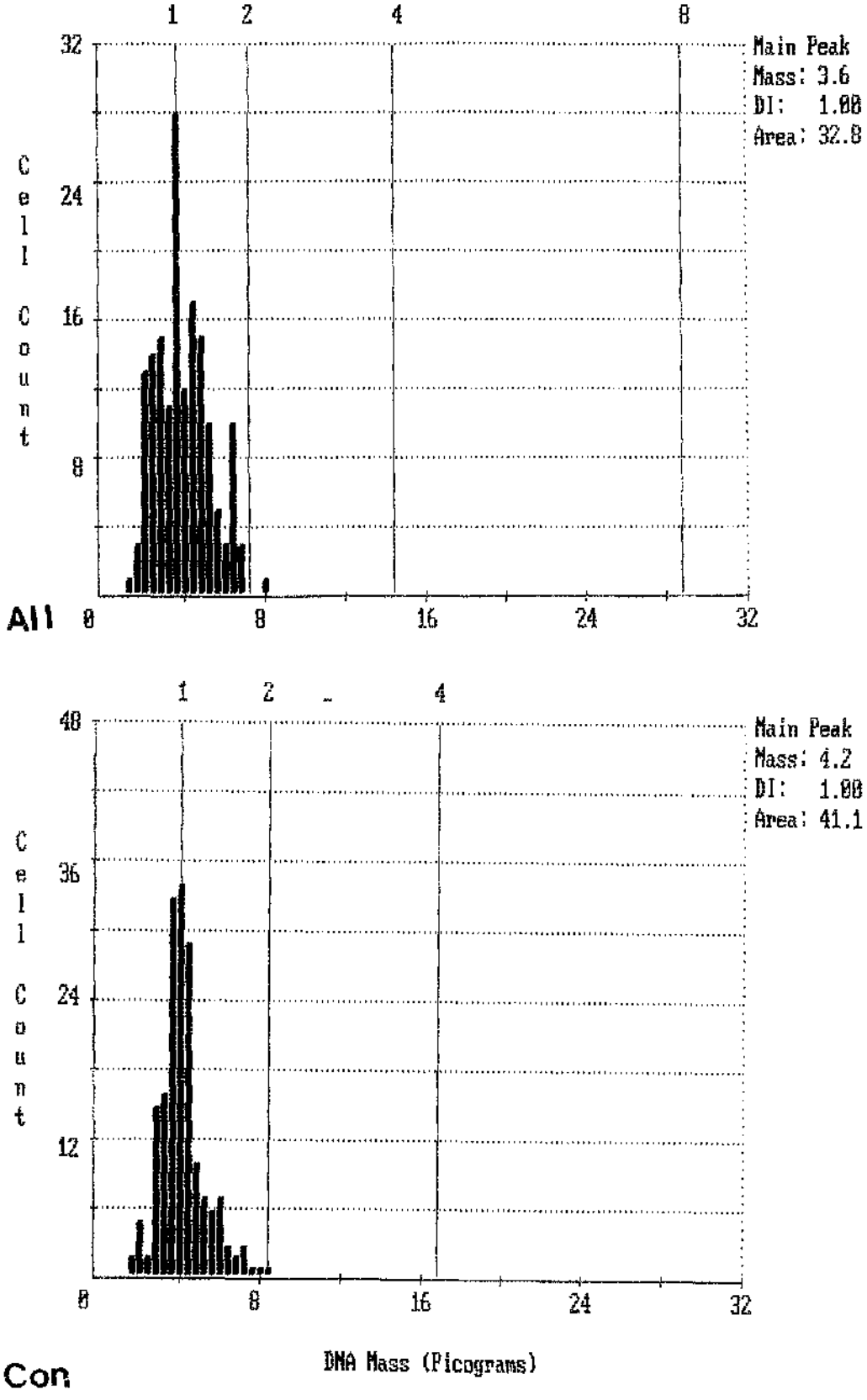


Fig.3.20. Representative histograms of ploidy analysis. Note that the nuclei from both All and control groups are predominantly diploid.

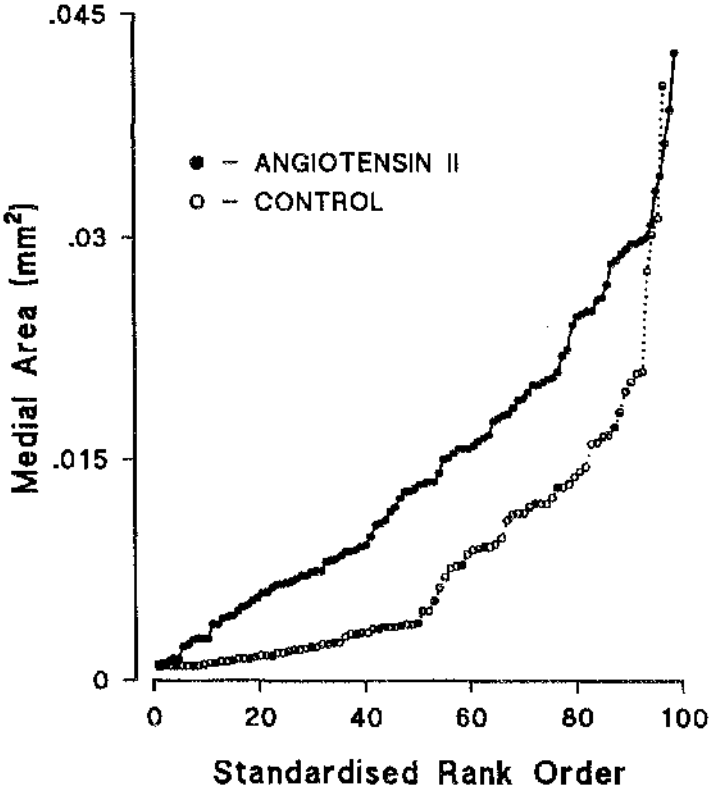


Fig.3.21. The standardised rank order of the medial area of mesenteric blood vessels following infusion with Angiotensin II. Note that at no point do the lines overlap.

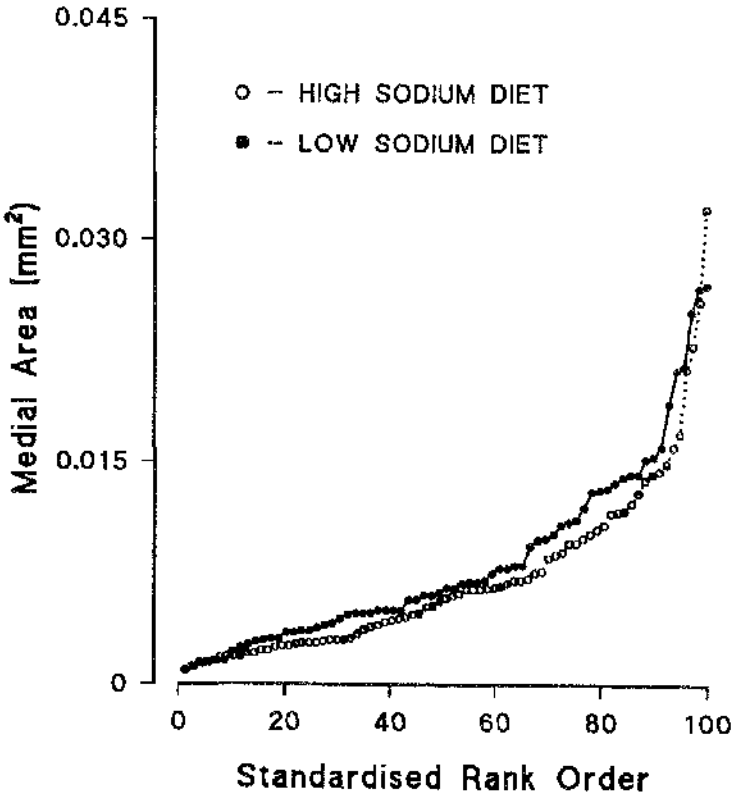


Fig.3.22. The standardised rank order of mesenteric blood vessels from low and high sodium groups. Note the lack of effect of treatment. on medial area.

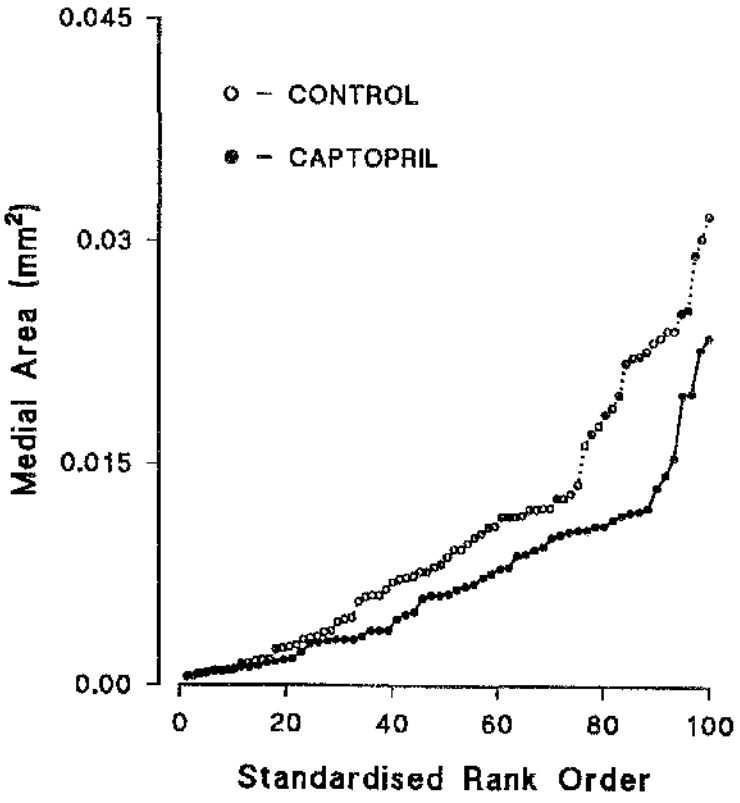


Fig.3.23. Standardised rank order of medial area following treatment with captopril. Note the tendency for captopril to reduce medial area.

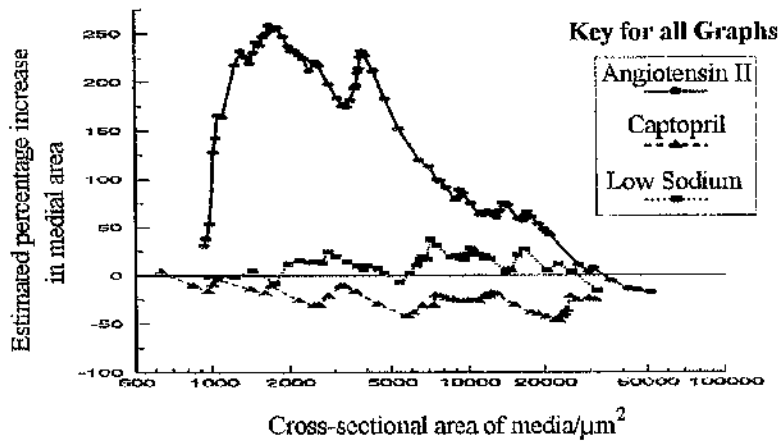


Fig.3.24. The estimated change in medial area after all treatments. Note the pronounced effect of AII on a range of small resistance vessels.

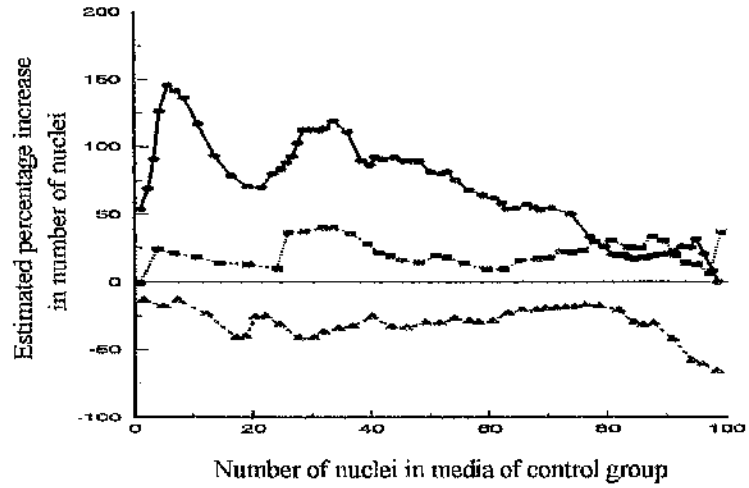


Fig.3.25. The effects of AII, low sodium and captopril on the number of medial nuclei per cross section of media.

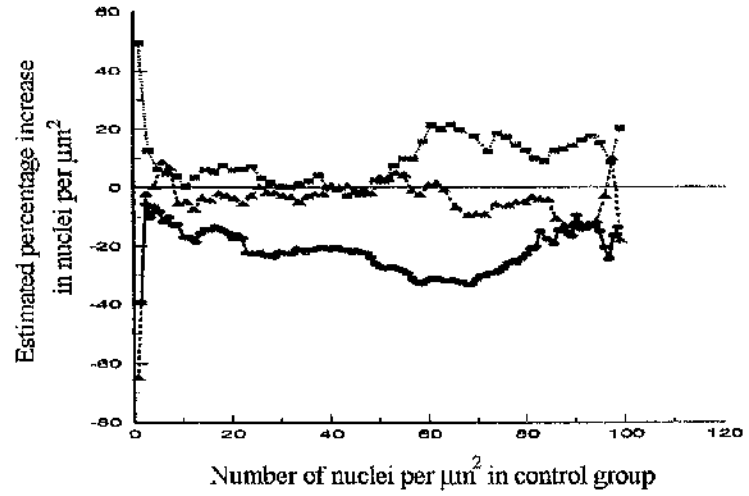


Fig.3.26. The effects of AII, low sodium and captopril on the number of nuclei per unit area of media.

Discussion:

Tunica Media:

Two-threefold differences in DNA synthesis of vascular smooth muscle cells (VSMC) of resistance vessels of the mesenteric bed were observed in response to AII infusion, captopril treatment or variations in dietary sodium intake. These effects are surprisingly large given firstly that changes in circulating AII concentrations caused by these treatments are predicted to be modest (125) and also that increased plasma AII levels are known to downregulate angiotensin receptors in vascular tissues (219) thereby, potentially, limiting responsiveness. However, the magnitiude of these differences have to be put in perspective. Firstly, by infusing BrdUrd continuously over the entire period of treatment, DNA synthesis has been monitored for a longer time period than others have used routinely (156, 158). Secondly, apparent increases in BrdUrd labelling could be affected by changes in cell death. Apoptosis is a gene-regulated programmed event which is controlled separately from cell division. Unlike cell division, apoptosis leaves no trace after dead cells have been phagocytosed. The BrdUrd index would appear greater if cell division was unaffected but mature cells were dying at a faster rate. Thirdly, increased DNA synthesis does not necessarily result in cell division. VSMC's may become polyploid particularly in conductance vessels in hypertension (156). It should be noted that that the present BrdUrd index does not necessarily reflect cell division.

Assessing changes in structure of blood vessels depends on how the tissues are treated following experimentation. Perfusion fixation of blood vessels at pressure (e.g.100mmHg) is used to enable standardisation of lumen/wall ratio measurements. This standardisation is, however, arbitrary since perfusion may over or underestimate the intravascular pressure of the animal thus leading to collapse or distension of the lumen (167). The method that Short used to compare vessels from normotensive and hypertensive patients is better because although lumen diameter was a consideration, representative vessels of all sizes were included in the analysis. In this latter respect, the present studies are similar in that no vessel was excluded. This has two advantages; it obviates the need to standardise by lumen diameter and it includes smaller resistance vessels which myography studies tend to ignore. Using this approach, it was possible to identify a crucial difference between the effects of AII caused by exogenous infusion and

those caused by raising endogenous AII concentration by dietary sodium restriction. Whereas AII infusions increased medial area by up to two hundred and fifty percent in very small vessels, low sodium had no effect despite a twofold increase in DNA synthesis. AII infusion caused a significant increase in the number of VSMC's per cross section of media and a decrease, albeit non-significant, in the number of VSMC's per unit area of media indicating that cell proliferation was possibly accompanied by increased cell size. On the other hand, given that there was no increase in extracellular matrix, low sodium caused an increase in the number of nuclei per unit area with no overall effect on medial area indicating cell division, but suggesting smaller individual VSMC's. The possibility that differences between the effects of exogenous and endogenous AII are caused by polyploidy, has been excluded. VSMC's of mesenteric vessels of all sizes and all experimental conditions were predominantly diploid.

Morphologically, increased cell division caused by exogenous and endogenous AII has different consequences. Exogenous AII caused vascular hypertrophy whereas endogenous AII had no effect on medial area. The increase could be explained by the mechanical effects of blood pressure on growth. Evidence for this is derived from studies in which smooth muscle cell stretching promotes the expression of some of the proto-oncogenes which induce growth (142) and blood pressure lowering drugs which either prevent or reverse vascular hypertrophy (126, 218). However, this evidence is controversial. Firstly, proto-oncogenes are activated during stretch (142) and also in response to AII (144, 265), yet there was no evidence of hypertrophy following treatment with low dietary sodium. Assuming that AII induced endogenously, in response to low dietary sodium, also stimulates proto-oncogene expression, the lack of effect on hypertrophy makes a single role for proto-oncogenes difficult to reconcile. Secondly, the effects of blood pressure lowering drugs are difficult to interpret since they may interact directly with growth processes (158).

Neverthelesss, given the wealth of evidence from various sources that hypertension can itself cause hypertrophy (115, 220, 221), a direct effect of blood pressure cannot be excluded. The obvious explanation for the effects of endogenous and exogenous AII is that AII infusion increased blood pressure whereas low sodium diets tend to have the opposite effect. It is possible that other blood pressure-induced changes in blood vessel structure may be responsible for the increase in

medial area such as cell hypertrophy or remodelling of existing smooth muscle cells. The former is more likely given that remodelling does not result in an increase in media mass but an alteration in lumen size due to rearrangement of the existing cells (118, 119).

It is also possible, however, that the vascular effects of low sodium are unrelated to changes in blood pressure since it has been postulated that growth effects of AII are mediated via its effects on modulating renal sodium metabolism (222).

The effects of captopril are interesting in two respects. Firstly, the decrease in BrdUrd uptake would suggest that even under normal dietary conditions AII tonically maintains rates of VSMC DNA synthesis. Secondly, the tendency for captopril to reduce medial area is more likely to be due to the negative effects of captopril on VSMC DNA synthesis (less cell renewal) rather than increased cell death since the number of cells per unit of media remained constant. This is in contrast to the effects of captopril in the adrenal gland where the zona glomerulosa was atrophied and the rate of DNA synthesis appeared unchanged as a result of increased cell death within the zone (see Chapter 3b). Although it is possible that captopril by lowering blood pressure reduces cell proliferation, the tendency for medial area to be reduced is more likely to be due to reduced cell proliferation caused by the absence of AII. Apoptosis induced by ACE inhibitiors remains to be investigated.

Endothelium:

Both AII infusion and low dietary sodium increased DNA synthesis in endothelial cells of resistance arteries. This finding is novel and is surprising based firstly on the actions of AII in promoting the release of vasoactive anti-mitogenic factors which cause endothelium-dependent relaxation such as endothelin-1(223) and prostaglandin (266). It is possible, however, that increased DNA synthesis occurs as a consequence of increased functional demand. Secondly, AII inhibits coronary endothelial cell DNA synthesis in culture via the angiotensin II receptor AT2 (224). The mechanisms of AII-induced growth of endothelial cells remains to be investigated. Captopril, on the other hand caused no change in DNA synthesis. This indicates first that endothelial cell DNA synthesis is not controlled exclusively by AII, or secondly that the tendency for reduced growth is compensated by other stimulatory factors such as the direct actions of ACE on bradykinin degradation

(225). *In vitro* the presence of the endothelium reduces DNA synthesis in VSMC of isolated arteries (226). It is possible, therefore, that ACE inhibitors promote the release of endothelial cell anti-mitogenic factors *in vivo*.

Adventitia:

The adventitia showed an increase in DNA synthesis when AII was raised by low sodium and by infusion. This is in accord with increased DNA synthesis in the adventitia of isolated arterial segments demonstrated by Daemen *et al* (1991) (227). Fibroblasts possess AII receptors and also proliferate in response to AII-stimulation *in vitro* (3).

In conclusion, AII promotes DNA synthesis in all cell types of the mesenteric bed and is a potent mitogen for vascular smooth muscle cells *in vivo*. AII alone may not be able to cause vascular hypertrophy *in vivo*. A likely additional stimulus is blood pressure. Treatment with a low sodium diet also increases vascular smooth muscle cell DNA synthesis without vascular hypertrophy indicating that other humoral factors may be modulated by sodium intake. DNA synthesis in veins is unaffected by the renin-angiotensin system.

Chapter 4: THE EFFECTS OF NITRIC OXIDE SYNTHESIS INHIBITION ON DNA SYNTHESIS OF TISSUES: INTERACTIONS WITH THE RENIN-ANGIOTENSIN SYSTEM

Introduction:

The regulation of blood pressure by the renin-angiotensin system is influenced by many other factors. The actions of AII, for example, are enhanced in the presence of other vasoconstrictors such as noradrenaline and PDGF and conversely, are attenuated by a variety of vasodilators such as atrial natriuretic peptide, prostaglandins and endothelium-derived relaxing factor (EDRF) (See Vanhoutte for review, 228). It has been shown that many endogenous vasoactive substances such as acetylcholine, bradykinin, histamine, adenine nucleotides, thrombin, substance P and serotonin (229) influence the release of EDRF to elicit endothelium-dependent vasodilation. Thus, EDRF has become established as an important mediator of cardiovascular and renal haemodynamics, neurotransmission and immunological responses (229).

Furchgott and Ignarro *et al* (230, 231) independently showed that EDRF had biological properties similar to nitric oxide (NO). Palmer *et al* (1987) (232) subsequently established that NO accounted for the biological activity of EDRF. NO is derived from L-arginine (233) under the control of two synthase enzymes; one which is expressed constitutively and depends on calcium ions, the second, which is inducible, is calcium independent (229).

The use of NO synthase inhibitors such as the arginine analogues NG-monomethyl L-arginine and NG-nitro-L-arginine methyl ester (L-NAME) have increased our understanding of the role of NO in controlling haemodynamics and other neuroendocrine systems. L-NAME inhibits both the constitutive and the inducible NO synthases and has been used extensively to study the influence of NO on changes in blood pressure and the renin-angiotensin system (234, 235). Acute inhibition of the formation of NO caused vasoconstriction and marked increases in blood presssure in the normotensive Brattleboro and Long-Evans rats (236, 237). Chronic inhibition of NO synthase in adult rats causes hypertension and a rise in plasma renin activity (234, 236, 238). This led to the hypothesis that NO synthase inhibition, by removing the

vasodilatory effects of NO, potentiates the action of vasoconstrictors such as AII which, in turn, cause hypertension (235). Treatment with the AII receptor antagonist losartan, (234, 235) or the ACE inhibitor, enalapril, (235) either prevented or reduced hypertension caused by NO synthase inhibition. Longterm studies, however, have also shown that NO synthase inhibition results in a persistent form of hypertension involving the development of vascular hypertrophy (160). The mechanism by which vascular hypertrophy occurs in this experimental model is unclear but may involve the mitogenic actions of AII. In this chapter, time-dependent changes in vascular DNA synthesis following L-NAME treatment have been assessed using protocols established in previous chapters.

As well as the dose of NO synthase inhibitor, previous studies have shown that the duration of the experiment is important in determining when activation of the renin-angiotensin system is the cause of increased blood pressure (160). In the present study blood pressure, plasma renin activity and vascular DNA synthesis was measured in young normotensive Wistar-Kyoto rats after treatment for two or four weeks with L-NAME. The possible effects of L-NAME on renin secretory activity in the kidney and on a second angiotensin target tissue, the adrenal gland, are also considered.

Materials and Methods:

NO synthase was inhibited in two experiments lasting two and four weeks. Two groups of six and two groups of eight, three-week old, male Wistar rats (40-50g), maintained on a regular diet were given either water containing L-NAME or tap water alone for two weeks and four weeks respectively. The calculated dose of 40mg/kg/day (0.2mg) was based on an average fluid intake of 20ml/kg/day. At the beginning of the two-week experiment, mini osmotic pumps conatining BrdUrd were implanted (see General Materials and Methods). In the four-week experiment, the mini osmotic pumps were implanted at the beginning of the third experimental week so that in both experiments, all animals received a two-week infusion of BrdUrd.

Cells were counted in the adrenal glands as described in Chapter 2. In kidneys, however, four thousand cells were counted in the renal cortex to distinguish effects of treatment from normal growth of young animals. Adrenal zona glomerulosa widths were measured using an automated image analysis system.

Results:

Table 4.1: The effects of L-NAME treatment on various physiological variables. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Treatment	Exp. Group	Blood Pressure (mm Hg)	PRA (ng/AngI/ml/h)	Body Weight (g)
L-NAME (2wk)	L-NAME	128±7	$0.12\pm0.1^{***}$	157.5±16
	Control	116±10	1.63±0.7	162±14
L-NAME (4wk)	L-NAME	$161\pm5^{**}$	0.78±0.2	190±10
	Control	134±5	1.36±0.6	199±23

i) *Arteries:*

The medial area of arteries ranged from 14 - $435x10^{-4}mm^2$ and 1.43 - $211x10^{-4}mm^2$ in the two control groups and 18 - $357x10^{-4}mm^2$ and $1.6x10^{-4}$ - $172x10^{-4}mm^2$ in the groups treated for two and four weeks respectively with L-NAME. Between 158 and 238 arteries were measured in each group. As described previously, measurements from each group were ranked and compared (Fig. 4.1, 4.2). L-NAME did not significantly affect medial area in either experiment. There were no differences in the BrdUrd indices of the intima, media and adventitia between control and L-NAME groups after either two or four weeks (Figs.4.3, 4.4). At two weeks there was a tendency for DNA synthesis to be greater than at four weeks. However, this rate was not affected significantly by treatment with L-NAME. When the total number of medial nuclei/artery was plotted against the number of BrdUrd-positive nuclei/artery the slopes of the lines of experimental and control data at both two and four weeks were almost superimposable (Figs.4.5, 4.6). Together, these results show that, in spite of hypertension, L-NAME treatment has no significant effect on DNA synthesis in mesenteric arteries. Similarly, L-NAME has no effect on medial area or DNA synthesis in veins.

ii) *Adrenal Glands:*

At two weeks the width of the zona glomerulosa decreased in the L-NAME group (Control: 0.036± 0.002mm; L-NAME: 0.029±0.002mm, $p<0.02$), while at four weeks the zona glomerulosa did not differ from the controls (Control: 0.048±0.002mm; L-NAME: 0.053±0.002mm).

Adrenal gland weight corrected for body weight was unchanged after treatment with L-NAME. The BrdUrd index of epithelial cells in the zona glomerulosa was significantly lower in the L-NAME group at two weeks compared with controls (Fig. 4.7a). This effect is presented pictorially in figure 4.7b. At four weeks, however, there was a tendency for there to be more DNA synthesis in the glomerulosa following treatment with L-NAME although this did not reach statistical significance.(Fig 4.8a, b.).The zona intermedia showed little evidence of DNA synthesis and no significant differences in BrdUrd indices in treated and control groups in either experiment. The BrdUrd index of non-epithelial cells in the outer fasciculata was lower in the L-NAME-treated group after two weeks ($P<0.01$) but not at four weeks. These non-epithelial cells stained positively with an endothelial cell marker. There were no significant differences in epithelial cell BrdUrd uptake at either two or four weeks. DNA synthesis in the inner zona fasciculata was consistently low in all groups and of all cell types. L-NAME treatment for two or four weeks did not affect the BrdUrd index in either the inner zona fasciculata or the zona reticularis. In the medulla, the BrdUrd index of epithelial cells was lower in the L-NAME group at two weeks ($P<0.01$,). After four weeks however, the index was again similar to the controls.

iii) *Kidney:*

Kidney weights were unaffected by either period of treatment with L-NAME. After two weeks L-NAME, the number of renin-positive glomeruli/kidney decreased significantly (Fig 4.9). After four weeks, renin-secreting cells were not counted due to the lack of available anti-renin antibody. BrdUrd uptake was predominantly in the inner cortex and was generally greater in the first two weeks than the second two. In glomeruli, the majority of BrdUrd uptake was in mesangial cells, although uptake in tubular epithelial cells, particularly in proximal tubules, was also high. There were no significant differences between control and treated groups in the glomeruli or tubules. After the first two weeks of L-NAME there was a significant increase in non-epithelial (interstitial) cell DNA synthesis ($p<0.001$; Fig 4.9) while at four weeks, the BrdUrd index was still higher than controls but not significantly (Fig. 4.10) .

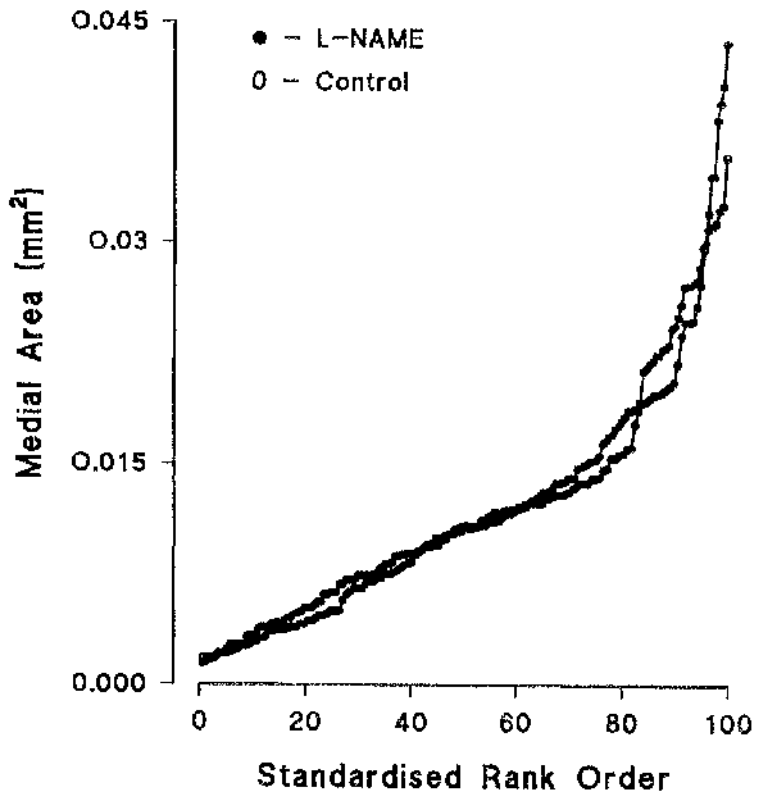


Fig.4.1. The standardised rank order of mesenteric arteries following treatment with L-NAME for two weeks.

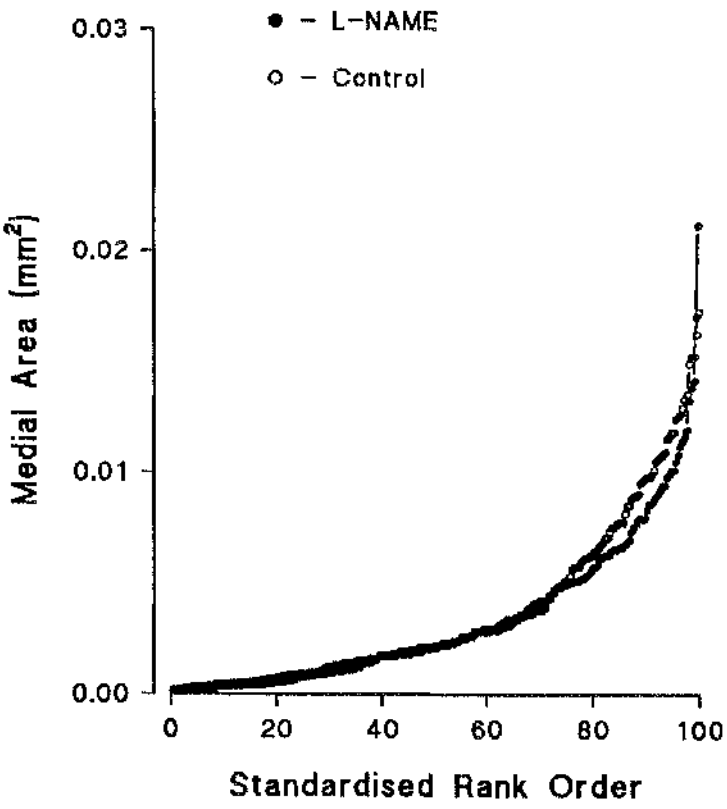


Fig.4.2. The standardised rank order of mesenteric arteries following treatment with L-NAME for four weeks.

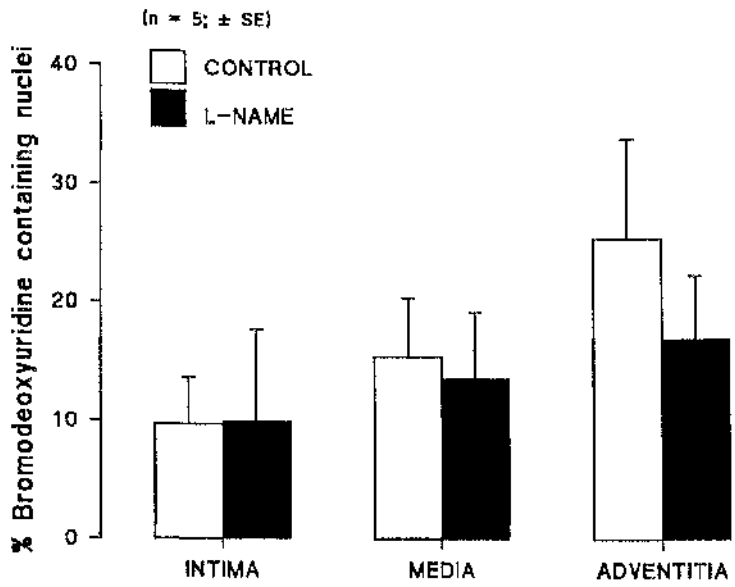


Fig.4.3. The effect of L-NAME (2wk) on DNA synthesis of blood vessels.

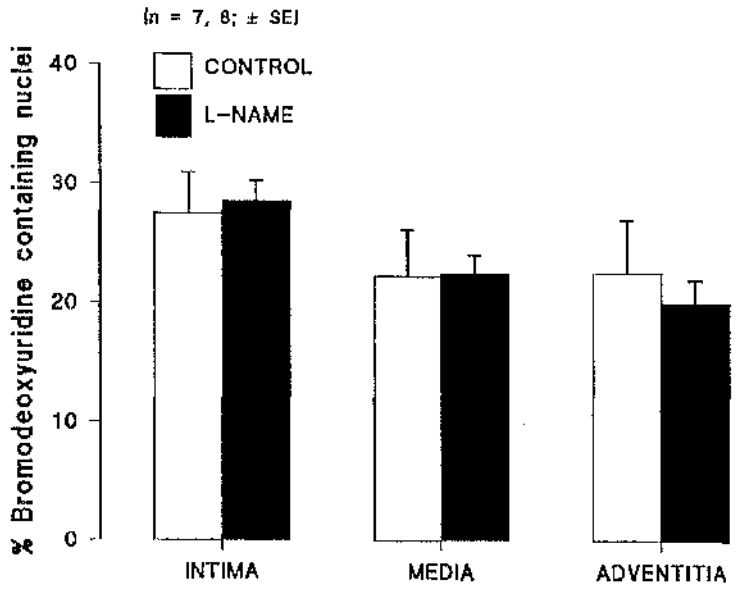


Fig.4.4. The effect of L-NAME (4wk) on DNA synthesis in the intima, media and adventitia of blood vessels.

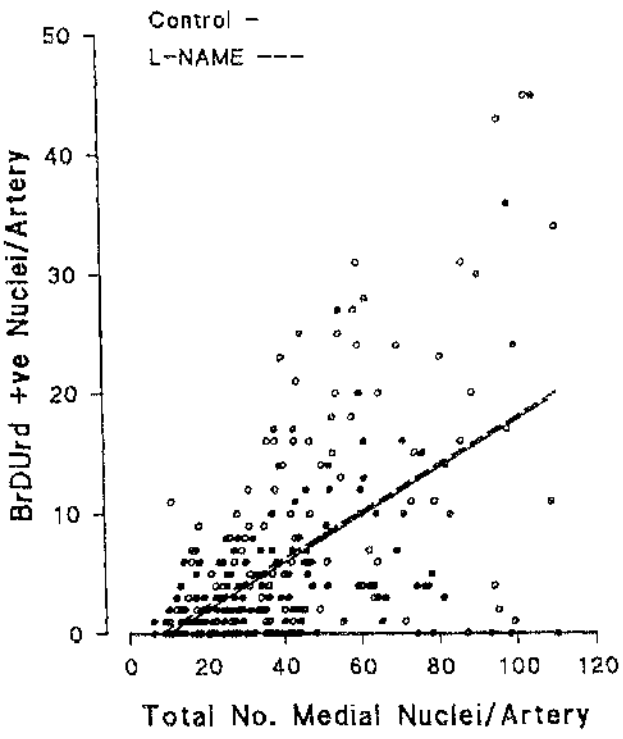


Fig.4.5. The relationship between BrdUrd index and vessel size. Shows that the lack of effect of L-NAME (2wk) on DNA synthesis applies to all sizes of blood vessels.

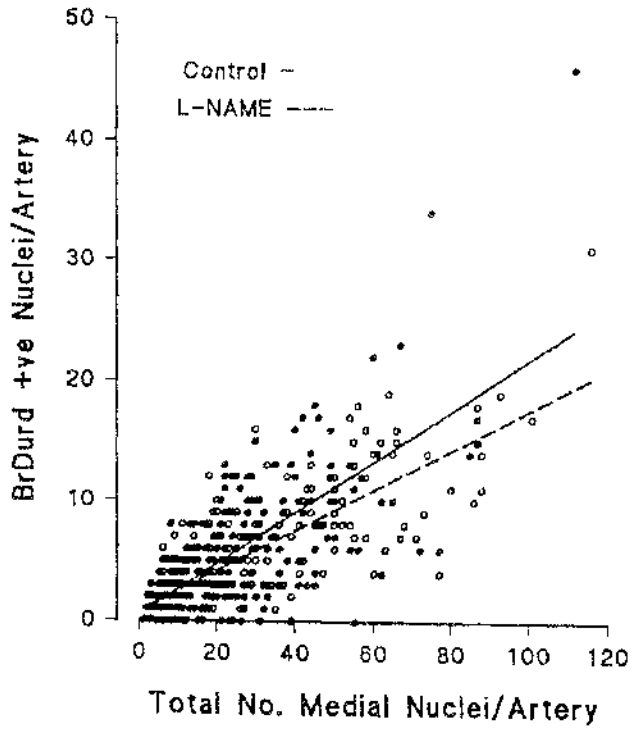


Fig.4.6. The relationship between BrdUrd index and vessel size.Demonstrates that L-NAME (4wk) has no effect on DNA synthesis in all sizes of blood vessel.

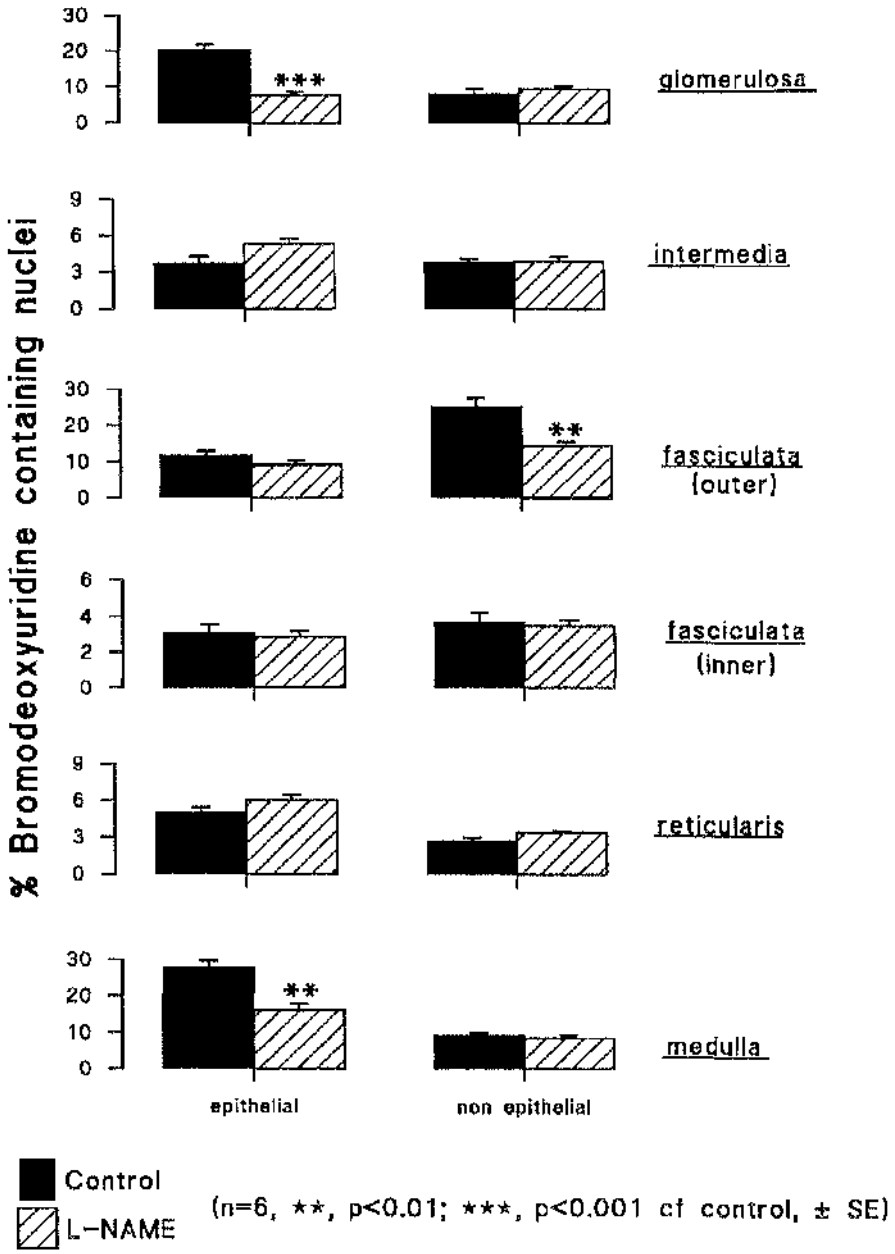


Fig.4.7a. Adrenal Gland: BrdUrd indices for the cortex and medulla. Note the effect of L-NAME (2wk) on the zona glomerulosa.

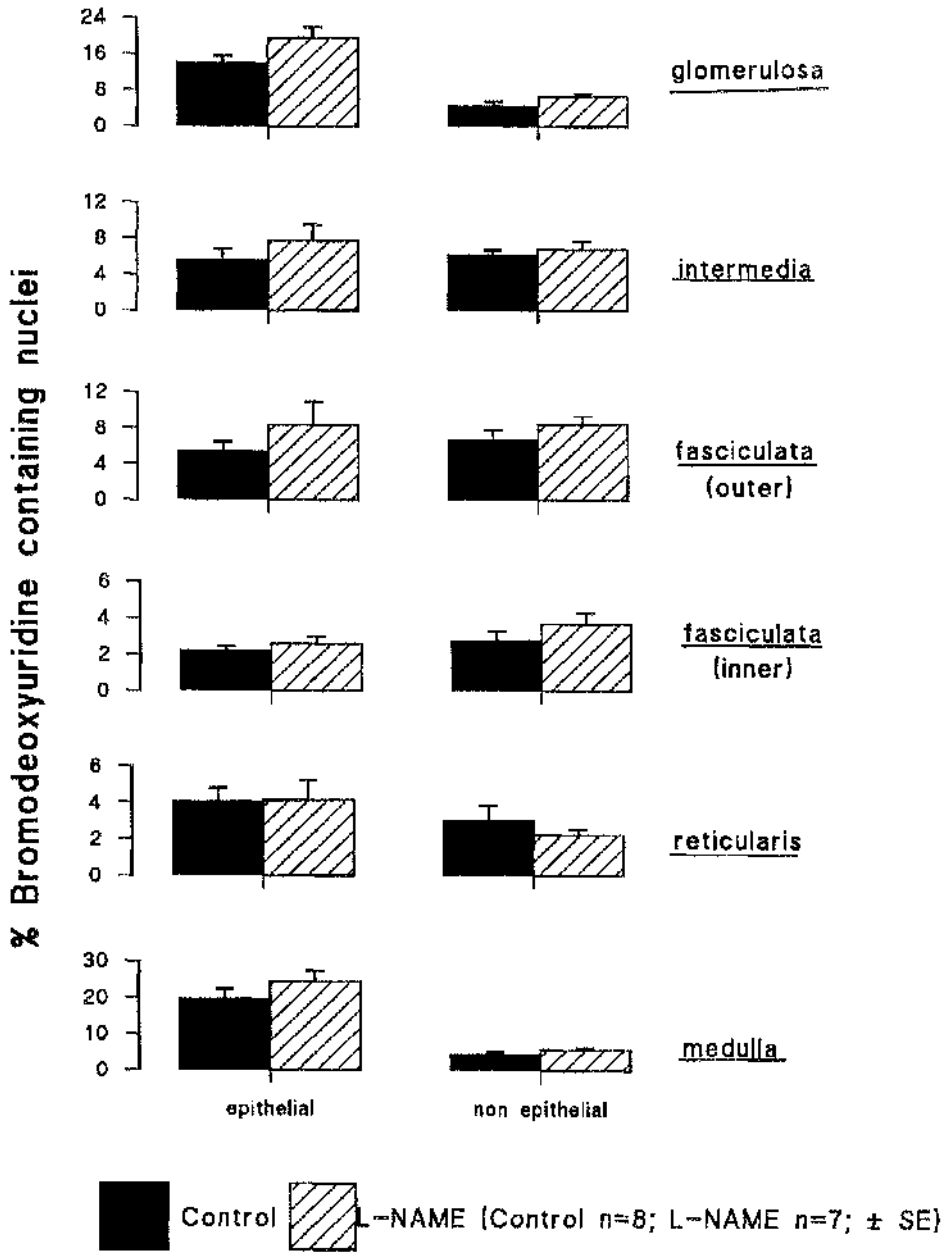


Fig.4.8a. The effect of L-NAME (4wk) on the BrdUrd index on the adrenal cortex and medulla.

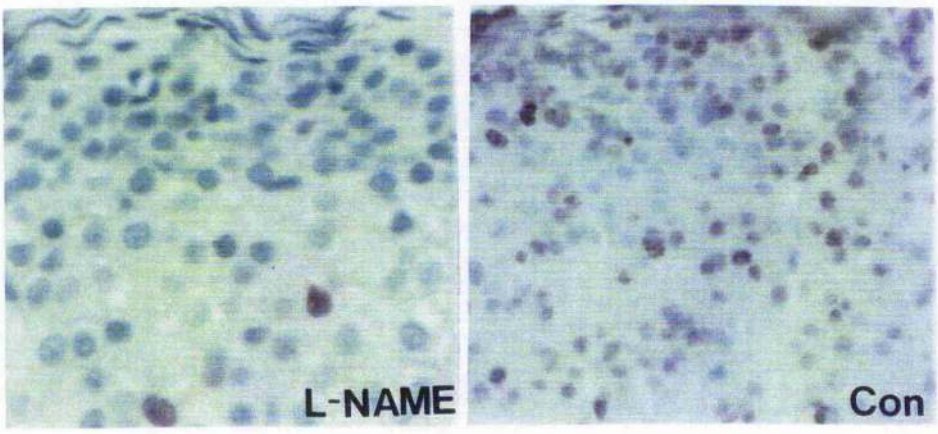


Fig.4.7b. The effect of L-NAME (2wk) on the zona glomerulosa. The zone is reduced in width and there are few BrdUrd-positive nuclei.

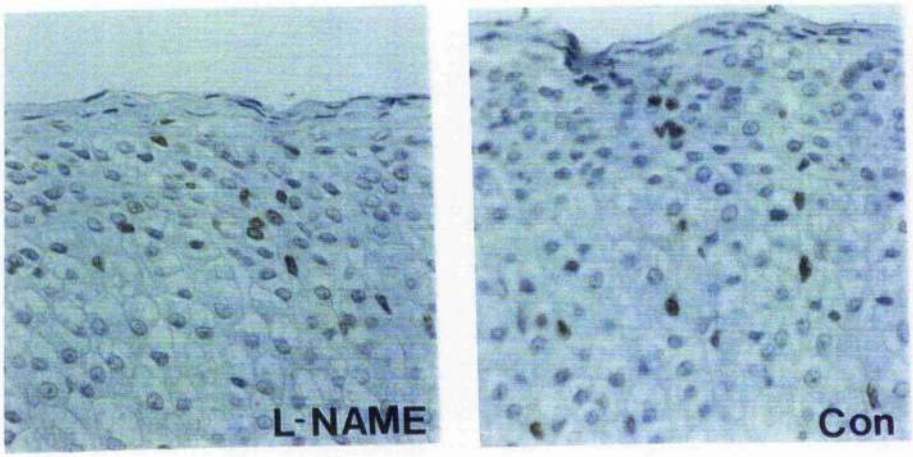


Fig.4.8b. The effect of L-NAME (4wk) on the zona glomerulosa. Note the recovery of the zone.

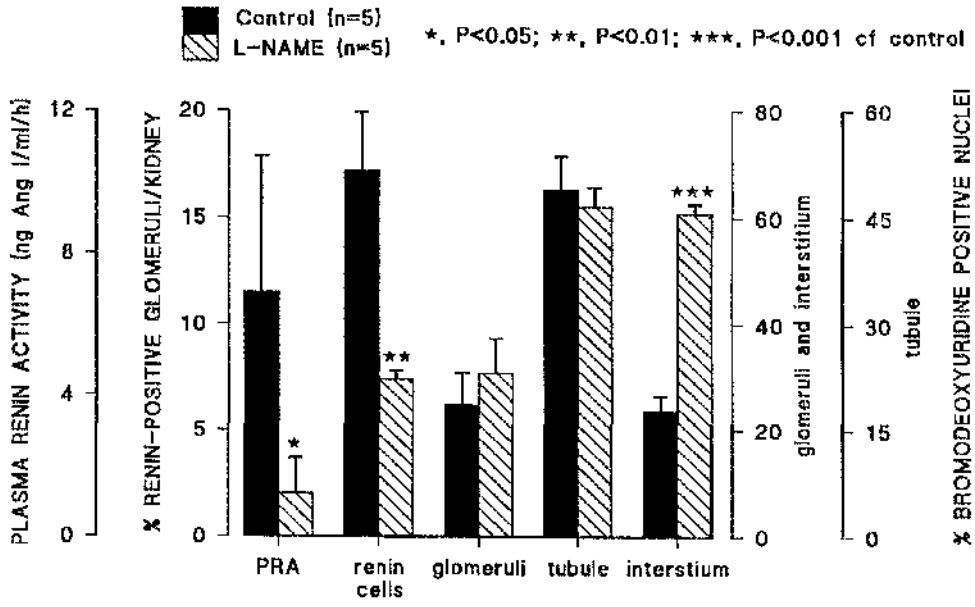


Fig.4.9. The effect of L-NAME (2wk) on plasma renin activity (PRA), renin-secreting cell number, and DNA synthesis of the renal cell types. Note the effect of L-NAME on renin secretion.

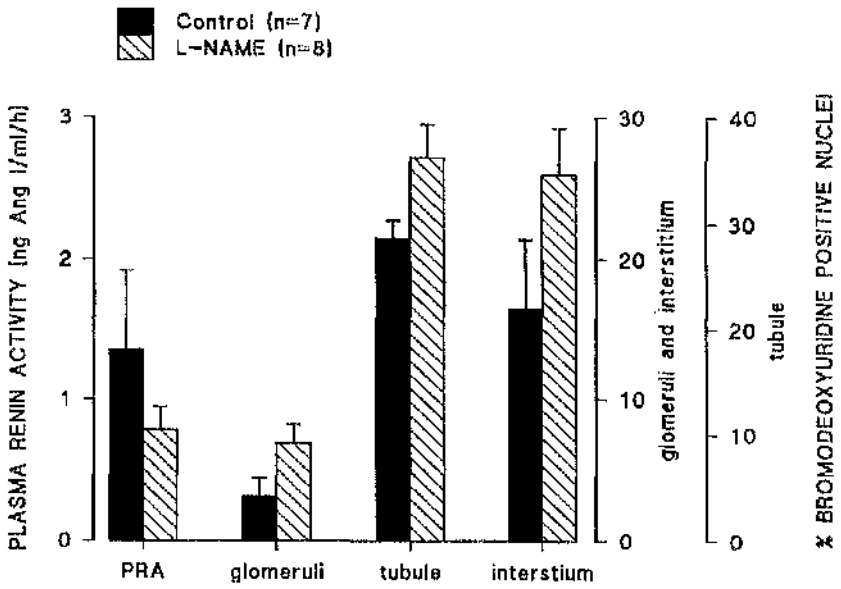


Fig.4.10. The effect of L-NAME (4wk) on plasma renin activity (PRA), renin cell number and DNA synthesis of the renal cell types.

Discussion:

Theoretically, inhibition of NO synthesis could influence the structure and growth of the adrenal gland, mesenteric bed and kidney in several ways: i) directly, by antagonising antiproliferative effects of NO, ii) indirectly, in mesenteric arteries, by increasing vascular tone, iii) indirectly, by influencing the synthesis of other neuroendocrine factors. Since it is known that the development of hypertension following NO inhibition is a gradual process (160), one might also expect temporal differences in growth and development. These possibilities are discussed for each tissue in turn.

Kidney:

DNA synthesis increased in all cell types at two and four weeks but the increase was only significant in interstitial cells after two weeks of L-NAME. The general increase in DNA synthesis may be explained by the actions of L-arginine analogues on renal function. Chronic administration of L-NAME is known to elicit dose-dependent increases in glomerular capillary pressure and renal vasoconstriction resulting in increased diuresis (234, 235, 239). Therefore, DNA synthesis of the renal cell types may reflect increased functional activity of the kidney.

The results demonstrate temporal changes in the activity of the RAS. After two weeks, PRA and the number of renin secreting cells in the kidney were reduced. Suppression of the renin-angiotensin system may be a consequence of increased blood pressure which was observed during L-NAME treatment. This effect is surprising since NO is known to inhibit renin secretion directly *in vivo* (26) and one would perhaps expect an increase in renin secretion following inhibition of NO synthesis. The effects of NO inhibition after four weeks of L-NAME are different and cannot be compared directly with any other experiment. PRA was within the normal range although hypertension was established. The increase in PRA from two to four weeks, despite the sustained negative influence of high blood pressure, may be explained by overriding changes in other stimuli, for example, sodium metabolism or blood volume. Renal damage caused by persistent high blood pressure (160) is also associated with activation of the RAS. However, in the present experiment, this explanation is unlikely since there was no evidence of hypertensive damage to the kidney.

Throughout L-NAME treatment, PRA was either suppressed or normal indicating that general increases in renal cell DNA synthesis following treatment with L-NAME are not mediated by systemic AII.

Even when AII is raised by infusion, (see chapter 3a) DNA synthesis in most of the renal cell types is not raised significantly. The effects of L-NAME on renal cell growth might indicate a hitherto unrealised direct antiproliferative effect of NO on these cell types.

Changes in DNA synthesis of mesangial cells were not observed at either two or four weeks even although blood pressure was high in all rats at the end of treatment. As discussed in chapter 3a, increased blood pressure appears to be an important component for mesangial cell growth. However, the results from this experiment show that in the absence of AII, pressure alone is not a sufficient stimulus. Mesangial cell growth possibly requires a combination of raised AII and raised blood pressure.

Adrenal Gland:

Following treatment with L-NAME, DNA synthesis in the zona glomerulosa varied. After two weeks, the BrDUrd index was less than half of the controls whereas during the subsequent two weeks DNA synthesis appeared the same in control and experimental glands. If the effects observed during the first two weeks were due directly to the removal of NO, it is not clear why these effects were not sustained for the second two weeks. The acute stimulation of aldosterone synthesis is not influenced greatly by NO (240) and since reduced zona glomerulosa DNA synthesis is normally associated with reduced functional activity, it is unlikely that the changes in DNA synthesis are due to direct effects of NO. It is possible that effects of L-NAME at two weeks are the indirect consequence of an attenuation of NO-mediated blood flow through the adrenal gland (241). However, again this is unlikely since no effects were observed at four weeks. Furthermore, *in vitro* studies with perfused glands have shown that the minor influence that NO has on trophin-stimulated steroidogenesis (240; 241) are not due to the effects on flow (241). The fluctuations in PRA correlate closely with the effects observed in the zona glomerulosa. This argues strongly that the changes in growth observed in the zona glomerulosa are secondary to changes in the plasma levels of AII, the major stimulus for aldosterone secretion.

Inhibition of NO production by L-NAME resulted in reduced DNA synthesis in non-epithelial cells of the outer zona fasciculata. There were significant effects only during the first two weeks of treatment and no effect at any time in epithelial cells. These results differ

from previous experiments (see chapter 3b) in which AII raised by infusion and low dietary sodium or blocked by captopril had no effect on the non-epithelial cells of the outer zona fasciculata. It is unlikely, therefore, that AII is a stimulus for growth of these cells. Most of the non-epithelial cells in the adrenal gland identified positively with an endothelial marker. Since NO is produced predominantly from endothelial cells, it is possible that by blocking the synthesis of both inducible and constitutive NO synthase in these cells with L-NAME, DNA synthesis decreased. This effect was not observed at four weeks and it is possible that effects of treatment produce transient changes in cell growth with the subsequent loss of effect after four weeks. Another possible explanation for reduced DNA synthesis may relate to the established inhibitory effects of glucocorticoids on the NO synthase enzyme (242), however this is unlikely since neither L-arginine nor L-NAME had any significant effect on corticosterone secretion (241). The most likely explanation is the reduction in blood flow through the adrenal gland following treatment with L-NAME (241) which may alone remove the stimulus for growth. However, why this effect is specific to the outer zona fasciculata is uncertain. It is perhaps significant that in studies with radioactive microspheres to measure blood flow in the adrenal, most of the radioactivity accumulates in the region of the outer zona fasciculata.

The reduction in DNA synthesis of the adrenal medulla at two weeks may be due (i) directly to the removal of NO, (ii) indirectly to decreased medullary blood flow or (iii) indirectly by increased blood pressure. In previous experiments (see chapter 3b), the reduction in medullary cell DNA synthesis appeared to be due either directly to negative effects of AII on cell growth or indirectly as a result of increased blood pressure. These experiments clarify that reduced DNA synthesis can occur even when AII levels are reduced, indicating that the reduction in DNA synthesis may occur indirectly to compensate for the rise in blood pressure. However, the effect was only apparent after two weeks and not after four weeks treatment.

Mesenteric Arteries:

NO inhibits vascular smooth muscle cell proliferation *in vitro* (146). One might expect, therefore, that L-NAME would promote DNA synthesis following removal of a growth inhibitor. However, treatment with L-NAME *in vivo* for two or four weeks had no significant effect

on medial DNA synthesis or medial area. In a previous experiment (see chapter 3c), treatment with captopril and high dietary sodium decreased vascular smooth muscle cell DNA synthesis by twofold providing strong evidence that removal of AII has negative effects on vascular smooth muscle cell growth. In this experiment, PRA levels were transiently suppressed during four weeks of treatment. One could anticipate, especially at two weeks, a corresponding reduction in DNA synthesis. Although the results fail to show any statistical significance, when PRA was reduced, there was a tendency for DNA synthesis to decrease in both the media and the adventitia. Similarly, when PRA was normal, medial DNA synthesis was very similar to the control group. However, the lack of any real effect on blood vessels at two weeks may be explained by the combined effects of growth promotion and growth inhibition. The negative effects of low PRA counterbalanced by effects of growth promoters following NO inhibition. Alternatively, the lack of effect may be explained by the nature and timescale of the experiment. In previous experiments, treatment with L-NAME resulted in an immediate and sustained increase in PRA (160, 240). In the present experiments PRA was measured at the end of two and four weeks. However, if PRA rises initially as shown by others (160, 240) and then falls to low levels at two weeks as shown in the present experiments, the fluctuations in PRA may explain why DNA synthesis was not reduced significantly. This experiment is unlike the others described in that PRA following infusion with AII or captopril would have been maintained constantly high or low.

Interestingly, blood pressure which was increased at two and four weeks did not, alone, appear to be a stimulus for growth of vascular smooth muscle cells, nor did it result in hypertrophy of any of the blood vessels measured. Similarly, in a previous experiment (see chapter 3c) AII raised by low sodium increased VSMC DNA synthesis but did not cause vascular hypertrophy. This reinforces any previous conclusion that both raised AII and blood pressure are required for the development of vascular hypertrophy.

In summary, two weeks of inhibition of NO synthesis by L-NAME, increased blood pressure with compensatory suppression of the RAS. Secondary effects of reduced PRA and AII probably result in negative effects on DNA synthesis in the adrenal zona glomerulosa. Reduced DNA synthesis in zona fasciculata endothelial cells is possibly due to a combination of the negative effects of L-NAME on blood flow

and the inhibition of glucocorticoids on NO synthase. Whereas previous studies have shown vascular smooth muscle to be sensitive to alterations in the RAS, DNA synthesis was not affected by suppression of the RAS with L-NAME. This lack of effect on vascular tissues may reflect the combined influence of the removal of a tonic anti-proliferative factor, NO, and the suppression of a potent mitogen, angiotensin II. Vascular hypertrophy over the timescales and with the dose of L-NAME indicated does not appear to be a feature of hypertension following inhibition of NO synthase.

Chapter 5: THE EFFECTS OF GLUCOCORTICOIDS ON GROWTH OF VARIOUS TISSUES

Introduction:

The effects of glucocorticoid hormones on growth of tissues are complex. *In vivo*, stimulatory effects of low replacement doses of corticosterone (2μg/d) are observed in underweight, adrenalectomised rats (243), whereas equivalent (supraphysiological) doses of the synthetic glucocorticoid, dexamethasone in intact animals reduce body weight gain (244). Furthermore, contrasting effects are seen depending on the tissue type and on the glucocorticoid concentration. For example, glucocorticoids induce apoptosis of thymic lymphocytes *in vitro* (245), promote adrenocortical atrophy (103) and inhibit growth hormone secretion by most secretagogues (246). Conversely, glucocorticoids repress apoptosis of the prostate (245), facilitate feeding and weight gain by acting directly on central nervous system tissues (243) promote growth hormone secretion (263) and proto-oncogene expression (247).

In addition to changes in growth, doses of glucocorticoids as low as 2μg/d can alter neuromuscular function, salt and water metabolism and cause hypertension (82). It has been shown that the mechanisms of glucocorticoid-induced hypertension are not fluid-volume dependent (248) but blood pressure may be elevated by increased peripheral vascular resistance (249) caused by the potentiation of vasoconstrictors (250; 251) or inhibition of vasodilators (252). Lever, (111) has suggested that glucocorticoids can cause vascular hypertrophy.

Glucocorticoids interact with the renin-angiotensin system (RAS) in several ways. High concentrations of the naturally-occurring glucocorticoids, cortisol (man) and corticosterone (rat) have mineralocorticoid-type actions with marked suppression of the RAS (253). Synthetic glucocorticoids, such as dexamethasone, have little mineralocorticoid activity. There are glucocorticoid receptor elements on many of the genes controlling the RAS and although angiotensinogen (254), ACE (255) prorenin (256) and the AT-type1 receptor (257) are dexamethasone-inducible, only exceptionally does plasma renin activity alter.

In chapter 3C, vascular hypertrophy and hyperplasia resulted in response to infusion of AII and high blood pressure. In chapter 4, nitric oxide synthase inhibition suppressed the RAS yet blood pressure was raised but there was no evidence of vascular hypertrophy. In this chapter, low dose dexamethasone has been given since it offers the opportunity to investigate the effects of blood pressure on blood vessel structure without significantly affecting plasma renin activity. Furthermore, it provides an *in vivo* model to investigate the effects of glucocorticoids as potential growth factors/inhibitors for a variety of tissues.

Materials and Methods:

Twelve adult male Sprague-Dawley rats (300g) had free access to rat chow and tap water *ad libitum* in a temperature-light-controlled room (21°C; 12 hours light, 12 hours dark). At day 0 the rats were intraperitonaelly anaesthetised (fentanyl citrate 67.5 ng/g, fluanisone 5 μg/g, midazolam 2.5 μg/g) and silastic pellets releasing dexamethasone or vehicle were implanted subcutaneously at the back of the neck. Pellets were prepared by mixing dexamethasone with Silastic medical grade elastometer in a ratio of 1:40. After curing, pellet were cut into pieces which were calculated to release 4μg/d of dexamethasone. At the same time, osmotic minipumps releasing BrdUrd were also implanted as described previously (See Chapter 2). After two weeks treatment rats were killed and various tissues fixed for the immunocytochemical detection of BrdUrd-positive nuclei Two thousand cells were counted in each adrenocortical zone and in the adrenal medulla. In the kidney, one hundred glomeruli per rat were examined for the presence of renin immunostaining. A BrdUrd index for lipocytes was obtained by counting one hundred lipocytes surrounding mesenteric arteries.

Results:

Table 5.1. Shows the effects of dexamethasone in rats: N.B. Thymus, adrenal gland and kidney weight are corrected for body weight. Data shown are means±SEM; *p<0.05, **p<0.01, ***p<0.001.

	Dexamethasone	Control
Blood Pressure (mmHg)	176±7.5**	151.5±8.5
Plasma Renin Activity (ng/AngI/ml/hr)	3.7±2.8	5.4±2.06
Plasma Corticosterone (nmol/ml)	164±117	204±117
Body Weight (g)	293±17*	314±8
Thymus Weight (mg/100g)	92±12***	177±21
Adrenal Weight (mg/100g)	14.5±0.8	14.8±0.6
Kidney Weight (g/Kg)	3.37±0.01	3.21±0.001

1) *Morphometry:*

i) Arteries:

Despite significantly increasing blood pressure, treatment with dexamethasone had no significant effect on the medial area of mesenteric arterioles (Fig.5.1), nor was there any evidence of vascular hypertrophy.

ii) Kidney:

Dexamethasone tended to reduce the percentage of renin-positive glomeruli in the kidney, however this effect was not significant. (Fig. 5.2).

2) *DNA Synthesis:*

i) Arteries:

The number of BrdUrd-positive nuclei in the media of mesenteric arterioles was low in both the controls and treated animals,

approximately 2-4% compared with other experiments where control, adult Sprague-Dawley rats show approximately 10% of BrdUrd positive nuclei after two weeks. Examination of all blood vessels showed that the number of BrdUrd-positive nuclei was consistently low indicating a lack of effect of treatment.

ii) Adrenal Glands:

Treatment with dexamethasone caused a significant reduction in DNA synthesis within the epithelial cell population in the zona glomerulosa, outer zona fasciculata, reticularis and also reduced DNA synthesis in the catecholamine-secreting cells of the medulla. These results are represented in Fig (5.4). Photomicrographs of the effects of dexamethasone on the adrenal gland are shown in figures 5.5-5.6.

iii) Kidneys:

DNA synthesis in the kidneys was mostly in cells of the inner cortex, aggregating as small foci. Treatment with dexamethasone increased the BrdUrd index in tubular cells (Fig. 5.3) but had no significant effect on either glomerular or interstitial cells, Fig (5.2).

iv) Thymus:

Most cells in the thymus of control rats stained positively for BrdUrd indicating rapid cell proliferation. The negative effects of dexamethasone on growth of thymic lymphocytes were so profound that measurements were not made. The effects are shown in Fig.5.7.

v) Adipose Tissue:

The BrdUrd index of fat cells surrounding the mesenteric blood vessels was reduced following treatment with dexamethasone (4.7±1; cf control 1.3±0.7 % BrdUrd-positive cells; $p<0.01$).

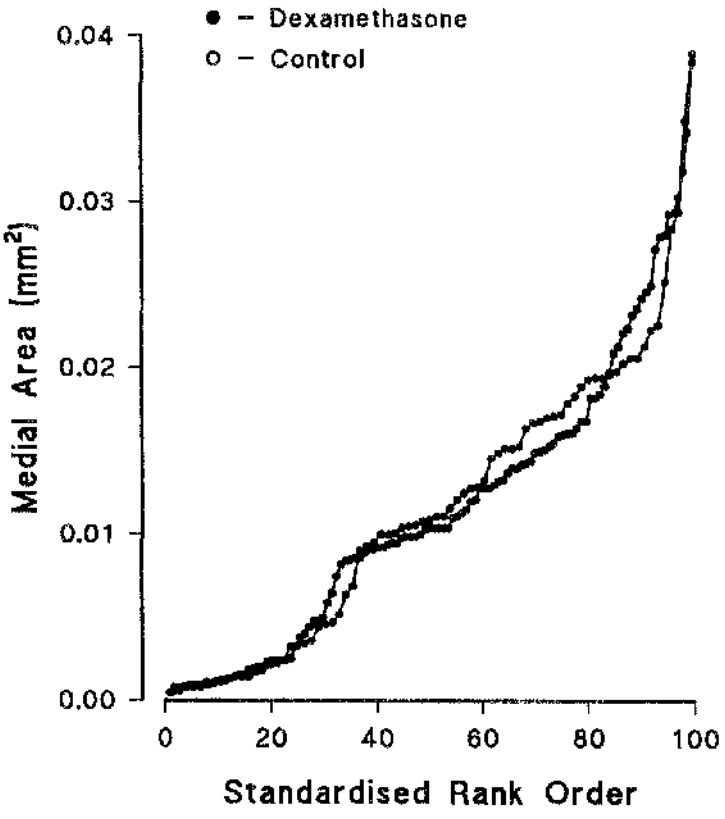


Fig.5.1. The standardised rank order of arterioles/arteries following treatment of rats with dexamethasone (4μg/day). Demonstrates the lack of effect of treatment on medial area.

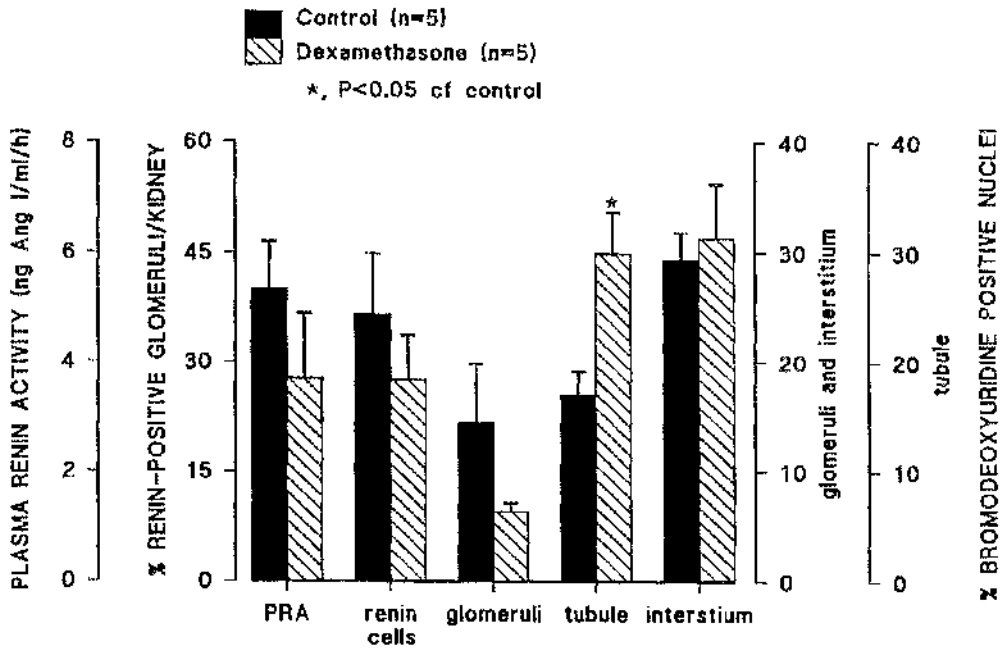


Fig.5.2. The effect of dexamethasone on plasma renin activity (PRA), renin-secreting cell number and DNA synthesis of the renal cell types. Note the increase in DNA synthesis in the renal tubules.

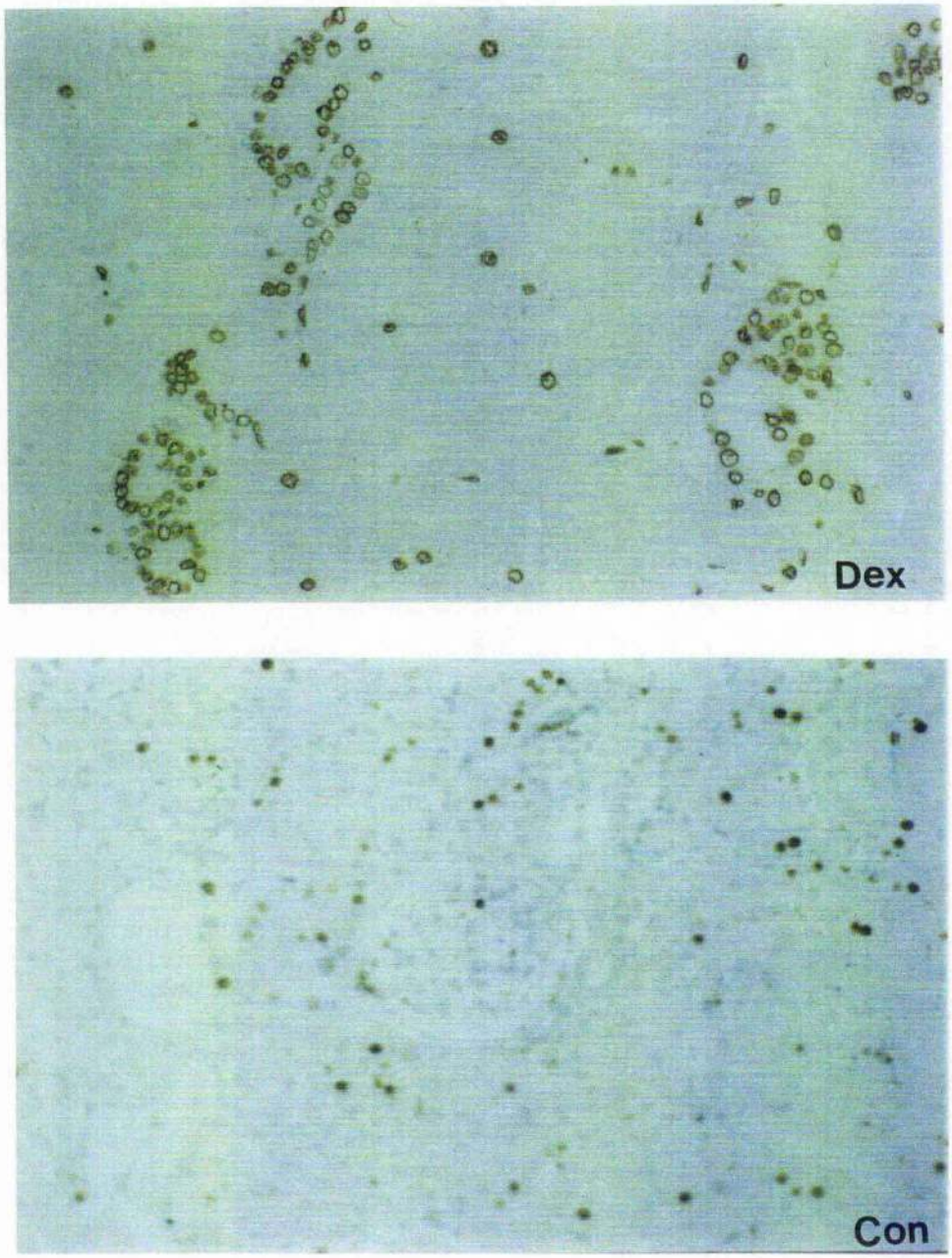


Fig.5.3. The effect of dexamethasone on renal tubule DNA synthesis. [1] *Dexamethasone (Dex), Control (Con).*

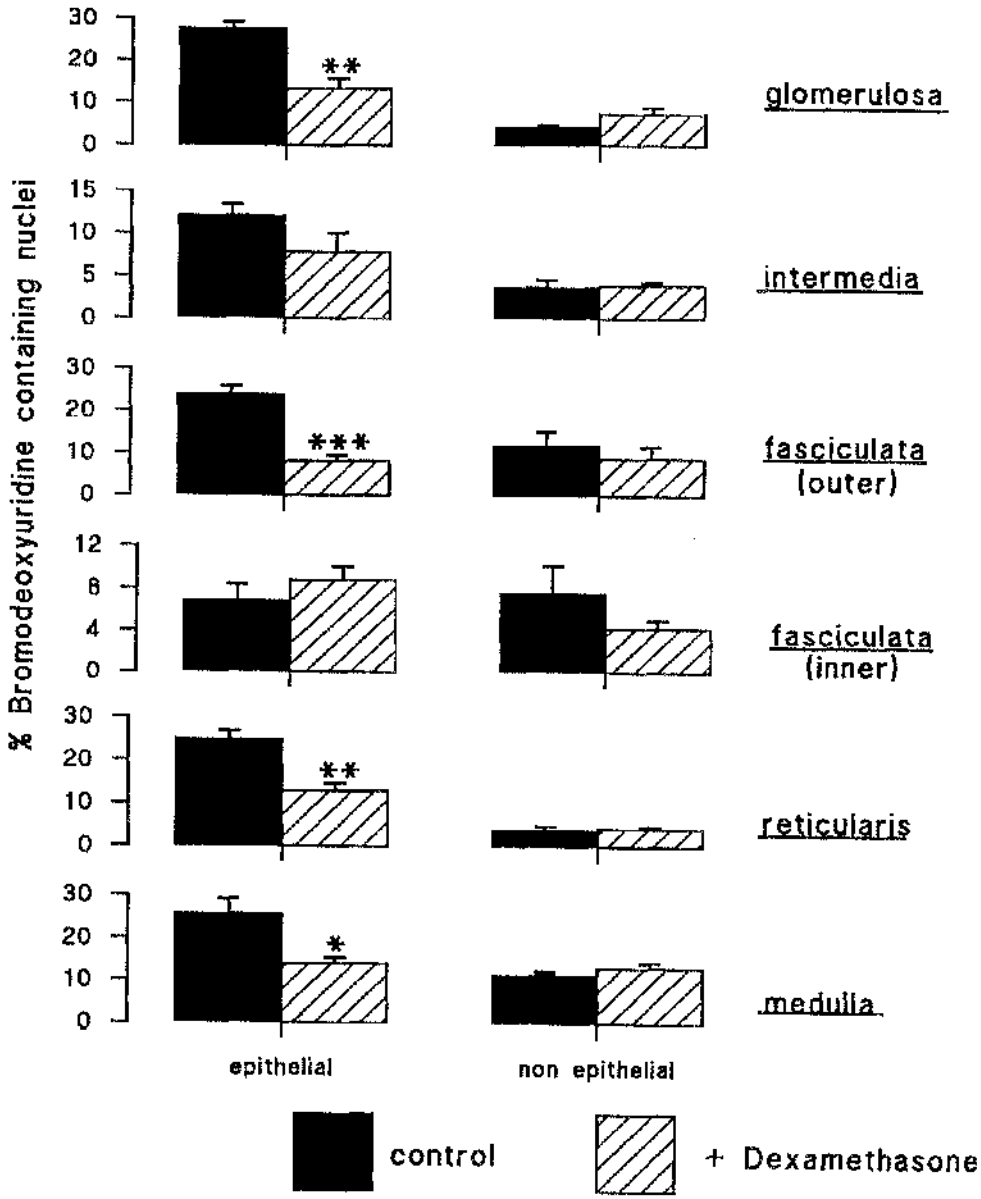


Fig.5.4. The pronounced effect of dexamethasone (4µg/day) on the adrenal cortex and medulla.

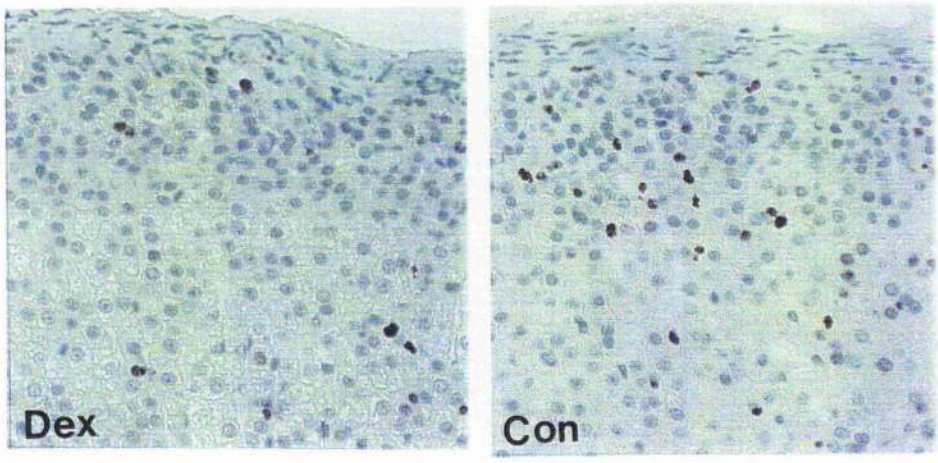


Fig.5.5. The effect of dexamethasone on DNA synthesis in the zona glomerulosa/zona fasciculata.

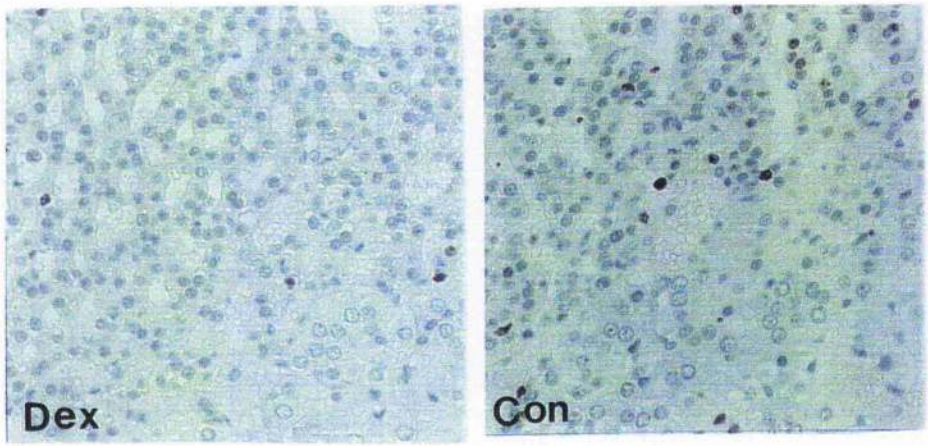


Fig.5.6. The effects of dexamethasone on DNA synthesis in the zona reticularis/medulla.

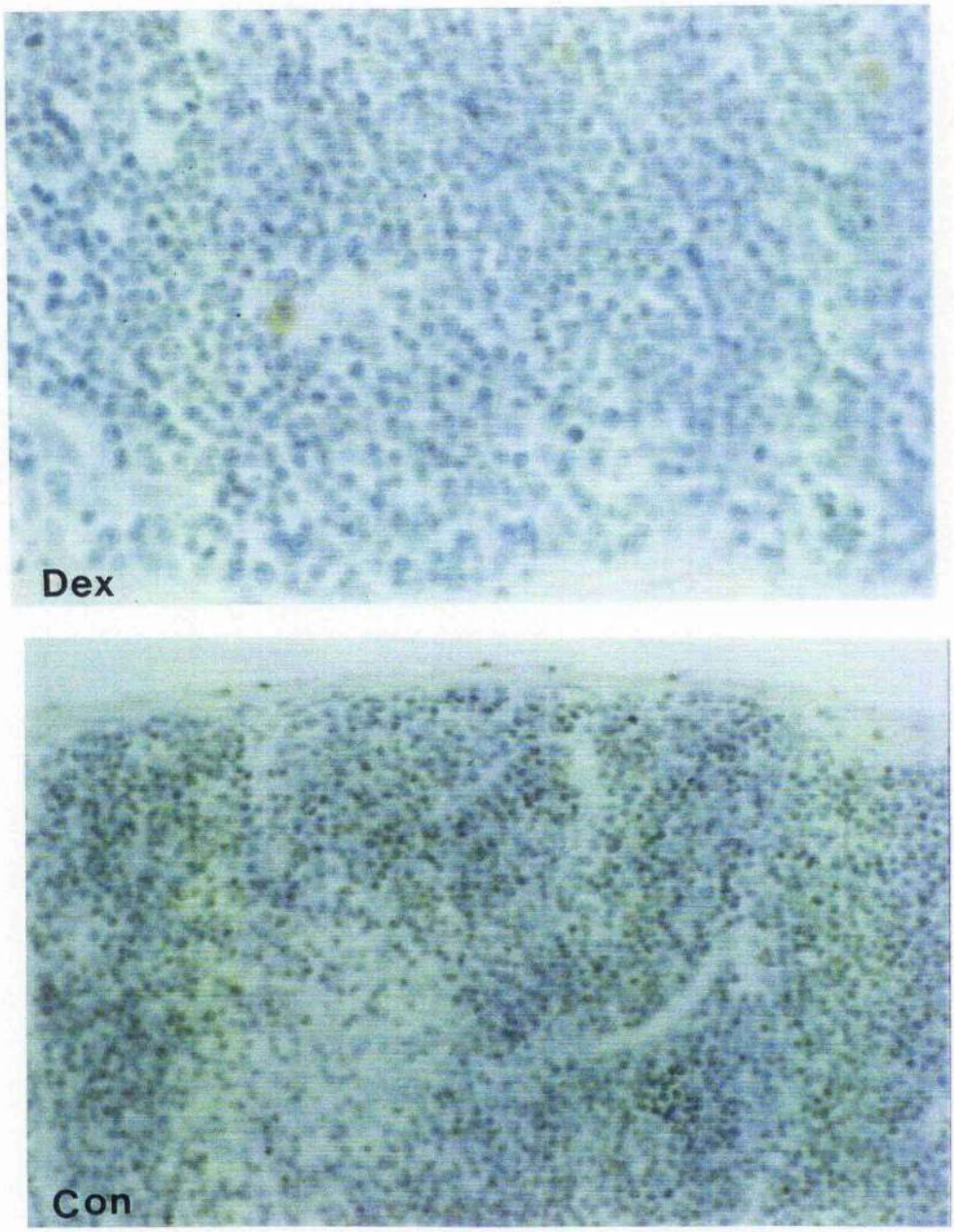


Fig.5.7. The effect of dexamethasone on DNA synthesis in the thymus. Dexamethasone (Dex), Control (Con).

Discussion:

Glucocorticoid treatment caused a significant reduction in body weight gain and profound inhibition of cell proliferation within a variety of tissues *in vivo*. Although it is not clear whether the phenotypic changes in tissues were a direct inhibitory effect of dexamethasone or an indirect consequence of the catabolic effects of glucocorticoids, the extent of the changes which occurred merit comment. The effects of treatment are therefore discussed for each tissue in turn.

Blood Vessels:

As expected, treatment with dexamethasone had no significant effect on the systemic renin-angiotensin system; any slight suppression of renin secretion may be compensated by increased angiotensinogen production. Despite a significant increase in blood pressure, dexamethasone had no effect on BrdUrd uptake into vascular smooth muscle cells nor did it affect medial area. The BrdUrd index of these cells was, in fact, much lower than that described in other studies (see Chapters 3C and 4). This is probably due to age, since the rats used for this study were older by several weeks and therefore gained weight more slowly than younger animals. Experimental and control animals were affected similarly. The lack of effect of dexamethasone on vascular structure suggests that blood pressure alone is not sufficient to cause hypertrophy and supports previous findings that the combined influence of a mitogen and hypertension are necessary.

Bearing in mind the dual catabolic and antiproliferative effects of glucocorticoids, one might expect a reduction in DNA synthesis of VSMCs of blood vessels. Indeed, weight gain was reduced significantly after two weeks. However, the lack of effect perhaps suggests that general growth inhibition is balanced by local factors which promote growth in certain tissues. Further studies on the direct effects of glucocorticoids on VSMC DNA synthesis are required to confirm this.

Adrenal Gland:

Reduced DNA synthesis was observed in the zona glomerulosa after treatment with dexamethasone. Although AII is the major trophic factor for this zone, atrophy has been observed following hypophysectomy (2) indicating partial control of growth by the pituitary gland. Indeed, studies have shown that ACTH increases the size of the intracellular compartment of the glomerulosa cell (258). Doses of

dexamethasone as low as 2μg/d inhibit ACTH secretion (259) and its activity can be assessed indirectly by measuring plasma corticosterone levels. In the present study, corticosterone levels were reduced slightly but the effect was not significant. The values observed are particularly high due to the effects of anaesthesia.

It has been suggested that doses of dexamethasone as low as 3μg/d directly inhibit adrenal cell growth (103). However, due to the local adrenal levels of corticosterone which are 100-1000-fold greater than elsewhere in the body (260) low doses are unlikley to significantly affect net glucocorticoid activity within the adrenal gland. The present study suggests that the negative feedback effect of dexamethasone on ACTH secretion causes a reduction in adrenocortical DNA synthesis and indicates that ACTH helps to maintain the zona glomerulosa *in vivo*.

Dexamethasone had no effect on the zona intermedia, indicating that the effects on growth are selective within zones. In previous studies (See Chapter 3b), DNA synthesis was low in the intermedia and was unaffected by manipulations of the RAS. Taken together, these studies do not support the zona intermedia as a novel stem cell zone as suggested by others (81).

Responses of the zona fasciculata to dexamethasone varied. Decreased DNA synthesis in the outer zona fasciculata is probably due to reduced ACTH since it is the major stimulus for corticosterone secretion and is the major trophic factor for the zona fasciculata. This study suggests that removal of this trophic factor involves reduced outer zona fasciculata cell proliferation *in vivo*.

Previous studies have provided evidence that inner zona fasciculata cells migrate from the outer fasciculata (93). One might therefore expect that the BrdUrd index in the inner part of the zone would reflect that of the outer zone. Since there was no effect of dexamethasone in the inner zona fasciculata, two possibilities are suggested. Firstly, that reduced ACTH has a negative effect on inner cell growth but decreased proliferation is compensated by increased cell death. In this instance, the BrdUrd index would remain constant although zonal atrophy would occur. This explanantion is unlikely since there was no evidence of atrophy within the zone or decreased adrenal gland weight. The second explanation is that the BrdUrd-labelled cells in the inner zona fasciculata may be a different population of cells from those in the outer zona fasciculata. If proliferation of these inner zone cells is not controlled by ACTH, then dexamethasone would have no

effect. The differential effects in the zona fasciculata again demonstrate that the effects are specific to selected populations of cells suggesting that direct effects of dexamethasone are unlikely. These results highlight the independence of zones in maintaining cell populations.

Changes in the reticularis are also interesting. This zone has been identified as the zone in which cell death occurs. However, from our present observations and those in chapter 3b, DNA synthesis also takes place. Cell proliferation and death are not incompatible and both will influence the BrdUrd index. Since dexamethasone has previously been shown to cause apoptosis in the zona reticularis, it would appear that there is a population of cells which are sensitive to the mitogenic effects of ACTH and possibly of AII within the zona reticularis.

Reduced DNA synthesis in the adrenal medulla may be due to the inhibitory effects of dexamethasone, however, similar effects have been observed in the medulla following treatment with factors which raise blood pressure (see Chapters 3b & 4). These inhibitory effects are most likely to compensate for the increase in blood pressure.

Kidney:

Dexamethasone tended to reduce DNA synthesis in most of the renal cell types but increased growth of tubular cells.The overall lack of effect of dexamethasone on kidney weight perhaps reflects this mixed response. The differential effects of treatment again suggest a combination of various factors in the regulation of growth. In mesangial cells, despite increased blood pressure, there was no effect of dexamethasone indicating that blood pressure alone is not a sufficient stimulus for growth of these cells. The tendency for dexamethasone to reduce DNA synthesis in interstitial and glomerular cells points to either direct inhibition or increased catabolism. The increase in tubular cell DNA synthesis may be explained by the direct actions of glucocorticoids on tubular cation exchange (261). The activity of the Na/H antiporter in tubular cells is known to be increased with glucocorticoid hormones. Intracellular alkalinisation is known to be a trigger for cell growth. The overall lack of effect of dexamethasone on the kidney reflects the susceptibility of some tissues to changes in growth relative to others and provides evidence for the multifactorial regulation of growth of many of the cell types.

Lymph Node and Thymus:

The most notable effects of treatment with dexamethasone were observed in the B and T lymphocyte populations where DNA synthesis was reduced by up to ninety percent. In addition, thymus weight was reduced providing evidence firstly that low dose dexamethasone is an antiproliferative factor for these cells, and secondly, that they are controlled predominantly by glucocorticoids since there appeared to be little or no protection from factors which promote growth. Somatic cell and molecular genetic studies on lymphoid cell lines have established that intracellular glucocorticoid receptors are the mediators of glucocorticoid evoked apoptosis (245). Evidence from the present study suggests that reduced cell proliferation is a major component of the thymolytic activity of low doses of glucocorticoid hormones. The extent to which apoptotic processes contribute to reduced thymus weight *in vivo* remains to be determined.

Adipose Tissue:

Within adipocytes, glucocorticoid receptors mediate the breakdown of intracellular lipid by increased lipase activity. There is some controversy, however, as to whether lipocytes proliferate or whether existing lipocytes merely change in size in response to changes in metabolism (262). The effects of glucocorticoids on reducing DNA synthesis suggests firstly that lipocytes synthesise DNA *in vivo* and secondly that DNA synthesis of these cells is controlled, at least in part, by glucocorticoids. Reduced lipocyte proliferation partially explains the catabolic effects of glucocorticoids and lack of weight gain in rats.

In conclusion, treatment with dexamethasone caused a significant reduction in DNA synthesis in most of the cell types studied. This effect was most apparent within the thymus and lymph node indicating that glucocorticoids are potent antiproliferative factors for these cells *in vivo*. The effects on the adrenal cortex are likely to be due to the negative effects of dexamethasone on ACTH secretion. In the kidney, the combination of reduced DNA synthesis and increased DNA synthesis imply that glucocorticoids are both stimulate and inhibit cell growth. Dexamethasone has no obvious effect on DNA synthesis of blood vessels nor on medial area. Blood pressure alone is not a stimulus for vascular hypertrophy *in vivo*.

General Conclusions

Throughout two weeks of infusion of BrdUrd, the patterns of DNA synthesis in blood vessels, adrenal gland and kidney were similar within control groups indicating consistency of the infusion technique. The rates of DNA synthesis were affected by age and weight; prepubertal rats showing a tendency for DNA synthesis to be greater than adult rats. In turn, tissues of older rats (those greater than 250g) tended to show less DNA synthesis. There were between-tissue variations in growth. Uptake of BrdUrd was greatest in the lymphoreticular tissues such as the lymph node and thymus and least in VSMC's of blood vessels. The treatment period was sufficient to detect changes in DNA synthesis of all cell types and was particularly suited to those which have a slower turnover.

This study has shown that the RAS, in addition to its role in the regulation of blood pressure, promotes DNA synthesis and growth of many of its target tissues *in vivo*. The actions of AII appear to be specific for many cell types which possess angiotensin II receptors. *In vivo*, AII promotes proliferation of vascular smooth muscle cells of all sizes of blood vessels independent of blood pressure changes and is also mitogenic for specific adrenocortical cells. Contrary to expectation, raised systemic AII and renin/AII raised locally did not promote growth of any of the cell types in the kidney, except mesangial cells. However, these cells only synthesised DNA when subjected to a combination of raised blood pressure and increased AII. This suggests that growth of some cells may require additional stimulation, possibly blood pressure. Other renal cell types which grow in response to AII in culture do not respond similarly *in vivo*. This may be dose-related or due to variations associated with *in vitro* conditions.

This study highlights the variation in responsiveness of smooth muscle cells which are phenotypically different to growth stimuli. Mesangial cells, renin-secreting cells in the renal arteriole and smooth muscle cells within blood vessels all showed variations in responsiveness to AII. Whilst stimulating DNA synthesis in vascular smooth muscle, AII had no effect on the renal arterioles and stimulated mesangial cells only in the presence of raised blood pressure. In isolation, blood pressure was not a sufficient stimulus for growth in any

of these cells. However, AII and blood pressure combined increased medial thickening in a large proportion of mesenteric resistance vessels. This data suggests that drugs which have the capacity to reduce AII and blood pressure will produce the most potent effects in patients with severe hypertension.

More importantly, this study has also identified that the regulation of cell growth *in vivo* is complicated by the interaction of the RAS with other physiological systems. The interaction of the RAS with the L-arginine derived NO system and with glucocorticoids induces many changes in growth of tissues, many of which are difficult to interpret without having a full understanding of the individual effects. This study suggests largely that mechanisms which promote growth are often counterbalanced by factors which inhibit growth and the interactions between stystems are possibly one way in which tissues are protected from becoming either depleted or overstimulated.

Prospects for Future Work:

This project has opened many avenues for potential research. Consistently in these experiments the growth effects in blood vessels and in the adrenal gland have been attributed to specific effects of AII. The concept that steroid hormones are also involved in the regulation of cell growth, raises the possibility that aldosterone and an as yet unidentified product of the zona reticularis may also be involved in the control of growth of the cardiovascular system. Bearing in mind that AII stimulated growth of the zona glomerulosa and zona reticularis cells, mitogenic actions of the steroids produced by these cells cannot be excluded. Investigating the effects of the androgens and oestrogens *in vivo* would be complicated by the lack of available antagonists, however, the effects of aldosterone could be investigated directly by adrenalectomy or by administration of spironolactone, an aldosterone antagonist. Furthermore, the interactions of AII with low sodium merit further investigation, since the vascular responses to AII are modulated in the presence of sodium. Further study into the AT receptors which control the growth responses is required.

A more diverse study to identify further which growth factors are involved in cardiovascular growth and more importantly, the mechanisms of growth is necessary. This would involve more comprehensive investigation on the actions of local renin-angiotensin systems and analysis of the expression of growth factors *in vivo*.

The concept of genetic variation is an important consideration in further investigating the control of growth by the RAS. Genetic strains of rat with relative insensitivity and hypersensitivity to AII are available. Moreover, many of the 'early genes' or proto-oncogenes have been identified, however not many studies have yet been able to ascribe the expression of these genes with specific physiological functions. Further characterisation of these genes will probably be useful in investigating the mechanisms of cardiovascular disease.

APPENDIX

Appendix I: Alkaline Phosphatase Substrate

Method: Alkaline phosphate substrate is extremely light and heat sensitive.

1. Dissolve Napthol AS TR phosphate in a few drops of dimethyl formamide.
2. Dissolve levamisole in veronyl acetate buffer and add tetrazolium salt (fast blue B or fast red violet).
3. Add Napthol AS TR phosphate- solution becomes cloudy.
4. Filter solution, refrigerated in the dark.
5. Add clear solution to sections for 10 minutes.

BrdUrd positive nuclei appear dark blue or red depending on the dye used.

Appendix II: Slide Coating

Concentrated poly-L-lysine solution was diluted 1:10 with deionised distilled water.

1. Place uncoated, clean slides in a clean rack.
2. Immerse rack in solution for 5 minutes.
3. Drain and dry sections in oven at 60°C for one hour.

Appendix III: O.5 % Hydrogen peroxide (H_2O_2) and methanol.

1part H_2O_2/ 60 parts methanol.

Appendix IV: Diaminobenzidene (DAB)

Stock Solution: The vial containing diaminobenzidene is flushed clean with dimethyl formamide to dissolve the DAB. The contents of the flask are reconstituted with tris buffer pH 7.6 to make to a final volume of 25ml. DAB is dissolved fully overnight in the dark and aliquoted into 200µl quantities and frozen at -70°C.

Working solution: Allow vial to come to room temperature. Add 50ml of 0.1M imadazole, 450ml 0.05M Tris/HCl buffer and 250µl of 30% H_2O_2.

Tissue sections taken from distilled water are submerged in working solution for 8 minutes. Renin appears dark brown.

Appendix V: Counterstaining with haematoxylin

All sections were counterstained by agitating in 0.1% Mayer's Haematoxylin diluted in distilled water for 2 minutes.

Appendix VI: Alcian Blue PAS

Solutions:

1% Alcian Blue in 3% Acetic Acid,

1% Aqueous Periodic Acid

Schiff's Reagent

Technique:

(1) Take sections to water

(2) Treat sections with the alcian blue solution for 5 minutes

(3) Wash well in distilled water

(4) Treat with the periodic acid solution for 2 minutes

(5) Wash well in distilled water. Then wash in Schiff's reagent for 8 minutes

(6) Wash in running water for 10 minutes

(7) Stain the nuclei with Mayer's haematoxylin solution. Differentiate and blue.

(8) Dehydrate, clear and mount.

Results:

acid mucins	-blue
neutral mucins	-red
mixtures	-purple
nuclei	-pale blue

Appendix VII: Methyl Blue van Gieson technique

Solutions:

Modified van Gieson's Stain

0.05% Aqueous Methyl Blue	- 50ml
0.1% Acid Fuchsin in Saturated Aqueous Picric Acid	- 50ml

Mix, then add

Glycerol	- 10ml
Saturated Aqueous Lithium Carbonate	- 0.5ml

Technique:

(1) Take sections to water

(2) Stain nuclei with an iron haematoxylin solution. Differentiate and blue.

(3) Stain with modified van Gieson's solution for 2 minutes.

(4) Wash in 1% acetic acid solution for 2 minutes.

(5) Dehydrate, clear and mount.

Results:

nuclei	- brown-black
reticulin & young collagen	- blue
mature collagen	- red
red blood cells, muscle	- yellow

Appendix VIII: Masson's Technique

Solutions:

Ponceau-acid fuchsin solution

1% aqueous phosphomolybdic acid

1% light green diluted 1:10 with distilled water

Technique:

(1) Take sections to water

(2) Stain nuclei with an iron haematoxylin solution. Differentiate and blue. Wash in water.

(3) Treat with the ponceau acid-fuchsin solution for 2-3 minutes.

(4) Wash in water and differentiate in the phosphomolybdic acid solution (between 5-15 minutes at room temperature).

(5) Wash well in water.

(6) Counterstain with light green solution for 1 minute.

(7) Wash, dehydrate, clear and mount.

Results:

nuclei	-blue-black
muscle	- red
connective tissue	- green.

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