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ANGIOTENSIN II INDUCED HYPERTENSION AND THE KIDNEY

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ADDENDUM

Errata

Page v, line 13: "or" rather than "of".

Page 2, line 16: reference to Pickering (1995). This reference was originally published in 1973 in *"Hypertension Manual: Mechanisms, Methods Management"*. The author was in fact G.W. Pickering, who was deceased at the time of the second publication of the chapter.

Page 12, final line: "appears to vasodilation and inhibition angiogenesis" to "appears to mediate vasodilation and inhibition of angiogenesis".

Page 30, line 4: "increased RVR results due to narrowing" to "increased RVR results from narrowing".

Page 44, paragraph 4, line 5: "where as" to "whereas".

Legend to Figure 3.3.1, line 2: "data presented is" to "data presented are"

Legend to Figure 3.3.2, line 7: "data corresponding to the animals light (day) cycle is presented" to "data corresponding to the animals light (day) cycle are presented".

Legend to Figure 3.3.4, line 5: "data corresponding to the animals light (day time) cycle is presented" to "data corresponding to the animals light (day time) cycle are presented".

Page 104, final paragraph: "All data was expressed" to "All data were expressed".

Page 105, line 13: "mRNA could been" to "mRNA could have been".

Addendum

Methodological point: In all studies, human Angiotensin II from Sigma Australia (Chapters 3,4 &5), or Auspep Australia (Chapter 2) was used (peptide sequence: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). All solutions of Ang II were adjusted for actual peptide content.

Chapter 1, page 7, paragraph 2, lines 6-11: Sentence should be omitted.

Chapter 1, page 7, paragraph 2, line 6: Should be added. "The effect of long term infusions of angiotensin II on systemic haemodynamics was first investigated in the conscious rabbit in 1963 *(Dickinson and Lawrence, 1963)*, and later in dogs and rats *(Koletsky et al., 1965; McCubbin et al., 1965)*. The results of these early studies revealed two distinct models of angiotensin II – induced hypertension that differed depending on the dose of angiotensin II used. Long-term infusions of angiotensin II, at doses which produce an immediate pressor response, results in hypertension associated with marked water retention *(Simon et al., 1995)*. The second form of angiotensin II-induced hypertension occurs when doses that initially have no apparent pressor effect, are infused chronically, resulting in a gradual increase in arterial pressure, that is termed the "slow pressor" effect *(Dickinson and Lawrence, 1963)*. It has been suggested that long term infusion of angiotensin II at doses that are initially sub-pressor may be a more accurate model of the development of human hypertension *(Simon et al., 1995)*.

Chapter 1, page 8, line 9: Should be added. "Work by Goormaghtigh established a connection between the observations of Tigerstedt and Bergman and Goldblatt, and a newly described granulated cell type located in the of the afferent arteriole (later known as the juxtaglomerular apparatus) of the glomerulus *(Goormaghtigh, 1945; Goormaghtigh, 1939)*. Goormaghtigh found that in the ischaemic kidney, the granulated cells of the juxtaglomerular apparatus hypertrophied and spread up the afferent arteriole into the interlobular artery *(Goormaghtigh, 1945; Goormaghtigh, 1939)*. These observations led Goormaghtigh to suggest that the granulated cells were responsible for the endocrine secretion of renin, which was important for blood pressure regulation and hypertension, and possibly acted locally to affect vascular function *(Goormaghtigh, 1945)*."

Chapter 1. page 10, paragraph 2, line 6: Should be added. "Within the kidney therefore, there is two very distinct and separate renin angiotensin systems operating. The first one is the classical endocrine system that releases renin and angiotensin II from the juxtaglomerular apparatus into the blood stream where they act on distant sites such as the adrenal cortex and vasculature. The paracrine system releases renin from the juxtaglomerular cells into the extravascular space, where it could act on glomerular arterioles, mesangium, or endothelium to alter glomerular function (for review see *(Navar and Rosivall, 1984; Osborne et al., 1975)*. The concentration of renin in renal lymph (the extravascular

space of the juxtaglomerular apparatus drains into the lymphatic system) is more than 10 times that in renal venous blood collected at the same time *(Navar and Rosivall, 1984; Lever and Peart, 1962)*. The close proximity of the lymph to the periarterial space of the afferent and interlobular arteries *(Kriz, 1987)* suggests a functional connection, perhaps providing a suitable space for the distribution of renin, angiotensin II, or other vasoactive substances to modulate local vascular function.

Chapter 6, page 162, end of paragraph 1: Should be inserted. "There are a number of studies that demonstrate angiotensin II mediated inhibition of renin release, renin gene expression *(Johns et al., 1990; Vander and Geelhoed, 1965)*, and a reduction in renin activity (as seen in Chapter 4), and concentration *(Bean et al., 1979)*. It is possible that infusion of angiotensin II in the experiments described in this thesis may have directly inhibited renin production and/or release which in turn may have counteracted, or made the assessment of the proliferative effect of Ang II *in vivo* difficult. Numerous studies have ruled out the rise in blood pressure mediating the inhibitory effect of Ang II on renin release *(Johns et al., 1990; Kurtz et al., 1986; Van Dongen and Peart, 1974; Blair-West et al., 1971; Vander and Geelhoed, 1965)*. Thus if local increases renal Ang II directly inhibited renin release in my experiments, leading to a reduction in endogenous Ang II levels, it is possible that this cancelled out the effective dose of Ang II delivered. However, there numerous studies that show augmentation of renal Ang II levels to much greater levels than that in plasma, in models of Ang II-induced hypertension. Interestingly, intrarenal Ang II levels are raised even in renin depleted kidneys for review see *(Navar and Harrison-Bernard, 2000)*, suggesting that intrarenal Ang II may be regulated independently from renin. Perhaps in future experiments, higher non-physiological doses of Ang II may be used to over come any possible inhibitory effects on renin and assess the proliferative effect of Ang II *in vivo*.

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DECLARATION

I hereby declare that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, nor material which has been accepted for the award of any other degree or diploma at Monash or another university, except where due acknowledgement is made in the text of the thesis. I also declare this thesis is less than 100,000 words in length exclusive of tables, bibliographies, appendices and footnotes.

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PUBLICATIONS

PAPERS

Stevenson K.M., Edgley A.J., Bergstrom G., Worthy K., Kett M.M. and Anderson W.P. (2000). Angiotensin II Infused Intrarenally Causes Preglomerular Vascular Changes and Hypertension. *Hypertension*, 36: 839-844.

Anderson W.P., Kett M.M., Stevenson K.M., Edgley A.J., Denton K.M. and Fitzgerald S.M. (2000). Renovascular hypertension : structural changes in the renal vasculature [In Process Citation]. *Hypertension*, 36: 648-52.

ABSTRACTS

Amanda J. Edgley, Michelle M. Kett, Nancy R. Nichols and Warwick P. Anderson.
Intrarenal infusion of Angiotensin II and early growth events in the kidney.
18th Scientific Meeting of the International Society of Hypertension
Chicago, 2000

Amanda J. Edgley, Michelle M. Kett, Nancy R. Nichols and Warwick P. Anderson.
Angiotensin II induced hypertension and growth in the kidney.
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Hypertension from intravenous angiotensin II infusion: evidence for structural changes in the kidney
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Amanda J. Edgley, Nancy R. Nichols and Warwick P. Anderson.
Acute intrarenal infusion of Angiotensin II – expression of early growth response genes c-fos and egr-1.
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Angiotensin II induced hypertension and growth in the kidney.
High Blood Pressure Research Council of Australia – 21st Annual Scientific Meeting, Abstract 50, 1999

Kathleen M. Stevenson, Amanda J. Edgley, Goran Bergstrom, Michelle M. Kett, Katrina Worthy & Warwick P. Anderson
Angiotensin II infused intrarenally causes pre-glomerular vascular changes and hypertension
High Blood Pressure Research Council of Australia – 21st Annual Scientific Meeting, Abstract 29, 1999

Amanda J. Edgley and Warwick P. Anderson.
Long term infusion of Angiotensin II into the rat kidney – measurement of blood pressure using telemetry
High Blood Pressure Research Council of Australia – 20th Annual Scientific Meeting, Abstract 61, 1998

SUMMARY

This thesis has examined the hypothesis that increased angiotensin II (Ang II) levels within the kidney may have a pathogenic role in hypertension. One possible pro-hypertensive mechanism by which Ang II may act in the kidney is to promote cellular proliferation and hypertrophy *in vivo*. Cellular proliferation and hypertrophy within the kidney could have important consequences if they were to translate to structural alterations in the renal vasculature and glomerulus, thereby altering renal haemodynamics and acting as a stimulus for hypertension. Whilst a trophic action of Ang II is well described in cultured cells, a similar action of Ang II *in vivo* is not well understood, and the role such an action plays in hypertension has not been investigated. Thus, the major focus of this thesis was to begin to investigate a potential trophic action of Ang II in the kidney *in vivo*, in models of angiotensin II dependent hypertension. To investigate cellular growth, I have used some well accepted markers of cellular proliferation, such as the activation of early growth response genes *c-fos* and *egr-1*, and growth factors genes TGF-β1 and bFGF. These markers have been shown to be upregulated in a variety of cultured cells after exposure to Ang II.

In Chapter 2, acute infusion of a low dose of Ang II (2.5 ng/kg/min) directly into the kidney resulted in a physiological significant renal vasoconstriction yet did not alter mean arterial pressure suggesting that Ang II was contained within the kidney. Despite having significant haemodynamic effects within the kidney, there was no detectable upregulation in the renal expression of early growth response genes *c-fos* or *egr-1* following intrarenal infusion of Ang II for 30 or 240 minutes.

In Chapter 3, infusion of low doses of angiotensin II directly into the kidney for 28 days increased systolic and diastolic pressure in a dose dependent manner. The elevation in arterial pressure was present in both the wake and sleep period of the animals and was not associated with an increase in heart rate or activity. Chronic intrarenal infusion, whilst providing an elegant technique with which to infuse Ang II directly into the kidney, is technically very difficult, and yields a very low success rate. Additionally, recent evidence suggests that Ang II levels within the kidney are increased in models of hypertension induced when Ang II is infused systemically. Thus further studies examining the potential trophic actions of Ang II within the kidney

utilised a second model of hypertension, that of chronic intravenous infusion of low dose Ang II.

In Chapter 4, hypertension induced by infusing initially sub-pressor doses of Ang II intravenously was found to be associated with a reduced slope of the pressure-GFR relationship in maximally dilated kidneys perfused with artificial plasma. These alterations to the pressure-GFR relationship were consistent with structural changes to the renal vascular lumen dimensions, or a reduction in glomerular filtration surface area. Thus to further examine whether these apparent structural changes were indeed due to the trophic effects of Ang II in the kidney, the final study conducted in this thesis investigated some markers of cellular proliferation and hypertrophy in this model of hypertension. In short, infusion of low doses of Ang II intravenously for 5 or 10 days induced a slowly developing hypertension, yet there was no indication that expression of growth factor genes TGF-β1 of bFGF had increased in the kidney in response to the Ang II infusion.

In summary, despite the large body of literature describing a trophic effect of Ang II in cultured cells, it is evident that the trophic actions of Ang II *in vivo* are much more complex and difficult to measure. The primary goal of this thesis was to begin to examine whether slowly developing hypertension, induced with low doses of Ang II, was associated with renal, primarily vascular growth *in vivo*. A unique feature of the studies presented in this thesis was the use of physiologically relevant doses of Ang II, which did not have immediate pressor actions. Hypertension induced with initially subpressor doses of Ang II has been associated with hypertrophy of the systemic vasculature. Whilst the results of this thesis suggest that there are apparent functional and structural changes within the renal vasculature and glomerulus in models of Ang II-dependent hypertension, the findings presented here provide no evidence that these changes are associated with the trophic actions of Ang II in the kidney. Ang II induced proliferation and hypertrophy of individual cells, or distinct populations of cells within the kidney cannot be ruled out in these experiments. However, the functional significance of such small changes *in vivo*, needs also to be examined in relation to the contribution (if any) these would make to the development of hypertension in this model.

SYMBOLS AND ABBREVIATIONS

SYMBOLS

α	alpha
~	approximately
β	beta
Δ	change in
°	degree(s)
>	greater than
<	less than
/	per
%	percentage
±	plus or minus

ABBREVIATIONS

aa	amino acid
ACE	angiotensin converting enzyme
ACEi	ACE inhibition
Ang II	angiotensin II
ANOVA	analysis of variance
AP-1	activation protein 1
AT_1	angiotensin II type I receptor
AT_2	angiotensin II type 2 receptor
ATP	adenosine triphosphate
AVP	arginine vasopressin
βME	β-mercaptoethanol
bFGF	basic fibroblastic growth factor
bp	base pair(s)
bpm	beat(s) per minute
BSA	bovine serum albumin
BW	body weight
°C	degrees Celcius
Ca_2^+	calcium ion
$CaCl_2$	calcium chloride
cDNA	complementary deoxy-ribonucleic acid
Ci	curie(s)
Cl^-	chloride ion
cm	centimetre(s)
Co.	Company
CO_2	carbon dioxide
cpm	count(s) per minute
cRNA	complementary ribonucleic acid
CTP	cytidine triphosphate
DAG	1,2-diacylglycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanodine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpm	dispersion(s) per minute
DTT	dithioltriotol
dUTP	deoxyuridine triphosphate
E.Coli	Escherichia Coli
ECL	enhanced chemiluminesence
EDTA	ethylenediamnietetra acetic acid
egr-1	early growth response gene
ERK	extracellular signal related kinase
et al.	*et alii* (and others)
FCS	fetal calf serum
FF	filtration fraction
FGF	fibroblast growth factor
g	gram(s)
GFR	glomerular filtration rate
GTP	guanidine triphosphate
[^{3}H]	tritiated
H_2O	water
$H_2PO_4^+$	dihydrogen phosphate
HCl	hydrochloric acid
HCO_3^-	bicarbonate ion
HR	heart rate
hr	hour(s)
i.p.	intraperitoneal
i.v.	intravenous
ID	internal diameter
ie	*id est.*
Inc.	Incorporated
IP_3	1,4,5-triphosphate
IRA	intrarenal artery
IU	international units

K^+	potassium ion
kb	kilobase(s)
kDa	kiloDalton(s)
Kf	ultrafiltration coefficient
kg	kilogram(s)
L	litre(s)
LB	luria Bertani
LV	left ventricular
M	molar
MAP	mean arterial pressure
µCi	microcurie(s)
µg	microgram(s)
mg	milligram(s)
Mg^{2+}	magnesium ion
$MgSO_4$	magnesium sulphate
min	minute(s)
µl	microlitre(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mmHg	millimetre(s) of mercury
µmol	micromole(s)
mmol	millimole(s)
MOPS	[3-(N-morpholino)-propanesulfonic acid]
mOsm	milliosmolar
MPT	murine proximal tubule
mQH_20	deionised water
Mr	relative molecular mass
mRNA	messenger ribonucleic acid
n	number
Na^+	sodium ion
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
NaOH	sodium hydroxide
ng	nanogram(s)
NGF	nerve growth factor
nm	nanometre(s)
NS	not significant
O_2	oxygen
OD	optical density
OH^-	hydroxide
OVLT	vascular organ of the lamina terminalis
P	calculated arterial distending pressure
P	degree of significance
[α^{32}P]	radioactive isotope of phosphorus
[α^{33}P]	radiactive isotope of phosphorus
Pa	inflow perfusion pressure
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
pH	power of hydrogen ion
Pi	renal interstitial tissue pressure
PKC	protein kinase C
PO_2	partial pressure of oxygen
PRA	plasma renin activity
Pty. Ltd.	Proprietary Limited
®	registered
R^2	Pearson correlation coefficient
RAS	renin angiotensin system
RBF	renal blood flow
RNA	ribonucleic acid
RNases	ribonucleases
rpm	revolution(s) per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
RVC	renal vascular conductance
RVR	renal vascular resistance
s	sense
[^{35}S]-UTP	radioactive sulphate labelled uridine triphosphate
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
sec	second(s)
SEM	standard error of the mean
SFO	subformical organ
SHR	spontaneous hypotensive rats
SNP	sodium nitroprusside
SP	stroke prone
SSC	standard saline citrate
TE	Tris.EDTA
TGF	tubuloglomerular feedback
TGF-β1	transforming growth factor β1
™	trademark
UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	volt(s)
v	volume
v/v	volume per volume
vs.	versus
VSMC	vascular smooth muscle cell
w/v	weight per volume
WH	weight of heart
WKY	Wistar-Kyoto
wt	weight
x	times

CHAPTER 1

REVIEW OF LITERATURE

1.1 INTRODUCTION

It is well established that common cardiovascular diseases such as stroke, coronary heart disease and heart failure have multiple determinants and risk factors *(Chalmers et al., 1999)*. Amongst these risk factors, high blood pressure has been identified as playing a central role in the pathogenesis of coronary heart disease and stroke. Hypertension increases the risk of cardiovascular disease by 2 to 4 times. In 1995, 17% of the adult population in Australia had high blood pressure (~ 2.2 million people) and/or were receiving treatment for the condition *(Heart Foundation of Australia, 1999; Chalmers et al., 1999)*.

Over the last 30 years much progress has been made in reducing cardiovascular mortality in North America, Western Europe, Japan, and Australasia. In accord with these reductions, the control of hypertension has also improved in these regions, with up to 27% of hypertensive subjects in the USA achieving a blood pressure below 140/90 mmHg by 1991, an increase of 17% over previous figures *(Chalmers et al., 1999)*. Despite major advances in treatment and care worldwide, a reduction in death rates and improved risk factors, it is estimated that blood pressure is still poorly controlled in over 70% of hypertensive people *(Chalmers et al., 1999)*.

In Australia, like most western societies, the incidence of cardiovascular disease related deaths still rates among the highest in the country (~ 38% of all deaths in 1997) *(Heart Foundation of Australia, 1999)*. Furthermore, indigenous Australians die from cardiovascular disease at twice the rate of other Australians *(Heart Foundation of Australia, 1999)*.

1.2 HYPERTENSION

The line dividing normotension and hypertension is an arbitrary one, however for the purposes of diagnosis and treatment, the World Health Organisation has suggested that hypertension be defined by a systolic pressure greater than 140 mmHg and a diastolic pressure greater than 90 mmHg *(Chalmers et al., 1999)*.

There are two main classifications of hypertension;

i) Secondary hypertension which occurs as a manifestation of a known disease such as renal artery stenosis or Cushing's syndrome and makes up a very small percentage of all hypertensive cases (< 5%)

ii) Primary or essential hypertension that comprises nearly 95% of hypertensive cases, yet the cause of the hypertension is unknown.

Speculation exists over whether essential hypertension is a result of several unrelated diseases, or a single process that has been altered in an individual by genetic modification, age, gender, race, or environmental factors *(Hollenberg, 1987)*. In the future it is more likely that an increasing number of distinct phenotypes and genotypes will be identified so decreasing the proportion of essential hypertension diagnoses *(Pickering, 1995)*.

1.2.1 The kidney and hypertension

Over sixty years ago, Goldblatt and co-workers (1934) provided the first convincing evidence for the dominant role of the kidney in the initiation of hypertension when they induced hypertension in normal animals by restricting blood flow to the kidneys *(Goldblatt et al., 1934)*. These studies led to the hypothesis that, a disturbance of intrarenal haemodynamics is critical to the initiation and maintenance of essential hypertension. Goldblatt hypothesised that arterial and arteriolar sclerosis of the intrarenal vessels was the primary stimulus for human essential hypertension, causing a haemodynamic disturbance in the kidney *(Goldblatt, 1947)*.

The prominent role the kidney plays in maintaining the increase in blood pressure, via its role as the long term determinant of sodium balance, is widely accepted *(Cowley, 1992; Guyton et al., 1972; Guyton and Coleman, 1969)*. Faced with a rise in systemic arterial pressure, the kidneys increase salt and water excretion in order to reduce blood volume, and thereby decrease cardiac output, returning arterial pressure to normal *(Guyton et al., 1972)*. This phenomenon, called pressure natriuresis, is a key component in the long-term regulation of arterial pressure and volume homeostasis *(Hall et al., 1986)*. Conversely, when systemic arterial pressure falls, the kidneys retain salt and water, restoring arterial pressure by increasing body

fluid volume and cardiac output. The renal-body fluid feedback mechanism provides an "infinite gain" control of long term arterial pressure *(Guyton, 1990)*. It follows thus, that a change in the kidneys ability to excrete sodium at normal pressures will lead to an elevation in arterial pressure *(Laragh and Brenner, 1995)*.

As set out later in this chapter, the renin-angiotensin system (RAS) is intimately linked to the body-fluid feedback mechanism as it is a potent regulator of both sodium and water balance as well as arterial pressure *(Hall et al., 1990)*. Given the importance of the renin-angiotensin system in this regulation, is it not surprising that blockade of this system via inhibition of angiotensin converting enzyme is one of the most successful regimens for the treatment of essential hypertension *(Hollenberg and Williams, 1988; Hollenberg, 1987)*.

The experiments described in this thesis are concerned with the interaction of the renin-angiotensin system and the kidney in hypertension. Specifically I have investigated the potential trophic actions of angiotensin II in the kidney *in vivo*, in models of angiotensin II-induced hypertension.

1.2.1.1 Previous experimental models of secondary hypertension involving the kidney – a brief history

As mentioned previously, the important role that the kidney plays in controlling long term arterial pressure through its ability to regulate the steady-state relationship between sodium excretion and arterial pressure is well documented *(Guyton, 1990)*. Alterations in the kidneys ability to excrete salt and water will subsequently affect the level of arterial pressure. Indeed, the balance between pressure and fluid homeostasis appears to be altered in essential hypertension, resulting in a rightward shift of the renal function curve (Figure 1.1), so that a higher pressure is required to maintain the balance between sodium and water intake and output *(Guyton et al., 1972)*. This physiology forms the basis of many models of experimentally induced hypertension, developed to investigate the mechanisms involved in essential hypertension. Indeed, most models of experimentally induced hypertension involve some type of renal disturbance, resulting in impairment of normal renal function *(Hall et al., 1996)*.

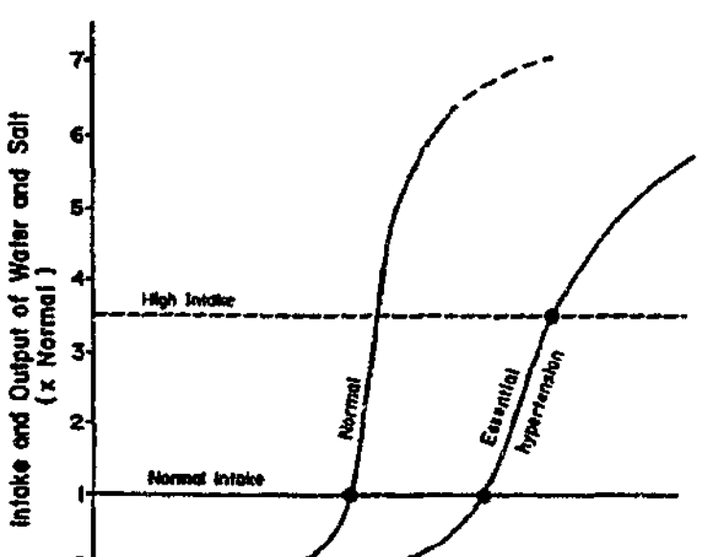


Figure 1.1 Renal function curves in the normal and essential hypertensive state. The renal function curve in essential hypertension is shifted to the right of normal such that higher pressures are required to achieve normal sodium balance (modified from Guyton *et al.* 1972).

1.2.1.1.1 Renal Artery Stenosis

Perhaps the most famous example of renally based experimental hypertension is renal artery stenosis or Goldblatt hypertension *(Goldblatt et al., 1934)*, first described by Goldblatt and colleagues in 1934. In developing this model of hypertension Goldblatt attempted to "produce experimentally, in animals, the probable haemodynamic disturbance in the kidney produced by intrarenal arteriolar sclerosis so frequently found in persons with essential hypertension" *(Goldblatt, 1947)*. By the early 1950's it was evident that arteriosclerosis did not occur in the renal vessels apart from the main renal artery, yet similar types of changes have been documented in peripheral vascular beds in hypertension, which could have similar haemodynamic consequences *(Korner and Angus, 1992; Lever, 1986; Folkow, 1978)*. These changes include hypertrophy of the vessel wall and remodelling of the vessel, both of which act to increase the wall to lumen ratio and can ultimately increase renal vascular resistance (RVR) *(Heagerty and Izzard, 1995; Folkow et al., 1958)*.

In the Goldblatt model of hypertension, surgical constriction of one or both renal arteries with a silver clamp results in graded increases in renal vascular resistance (RVR) and subsequent sustained rises in arterial pressure up to 110 mmHg *(Goldblatt et al., 1934)*. Part of the rise in pressure in this model of hypertension results from the mechanical resistance of the stenosis, increasing the resistance to blood flow in the kidney *(Peach, 1977)*. The increase in renal resistance causes an initial drop in renal blood flow (RBF) and glomerular filtration rate (GFR), which in turn stimulates the rise in arterial pressure via mechanisms that are not yet fully understood but could involve a

combination of effects on; peripheral resistance, the renin-angiotensin system (renin release from the kidney), and an alteration in the relationship between renal sodium excretion and arterial pressure *(Anderson et al., 1995)*. The rise in arterial pressure restores GFR and RBF to normal and renal vascular resistance remains increased *(Anderson et al., 1991; Anderson et al., 1990; Anderson et al., 1987)*. In severe stenosis, angiotensin II maintains GFR in both the initial stage (when angiotensin levels are elevated), and also later when the stenosis has stabilised and angiotensin II levels have returned to normal *(Anderson et al., 1990; Anderson et al., 1987)*.

1.2.1.1.2 Renal Wrap Hypertension

Renal wrap or Page hypertension *(Page, 1939)*, an alternative model of sustained renally based experimental hypertension, was first described in 1939 by Page and colleagues. In this model of hypertension the kidneys are wrapped in cellophane inducing a thickening of the renal capsule, eventually encasing the kidneys in a firm fibrous capsule 3-4mm thick *(Page, 1939)*. The thickened capsule leads to compression of the kidney tissue and thus increases the resistance to blood flow in manner analogous to that seen in renal artery stenosis *(Brace et al., 1974)*. Brace *et al.* (1974) reported tissue pressures of up to 30 mmHg under the thickened capsule. Bilateral renal wrap of rabbits can result in approximately a 50% reduction in RBF and GFR over the first month *(Denton and Anderson, 1985)*, together with a 49 mmHg rise in blood pressure *(Denton and Anderson, 1985)*. In contrast to renal artery stenosis, plasma renin levels in renal wrap hypertension remain elevated for several weeks following wrapping, and angiotensin II is responsible for a substantial proportion of the development of hypertension, and reduction in RBF during this period *(Denton and Anderson, 1985)*.

1.2.1.2 Other models of renally based experimental hypertension

1.2.1.2.1 Renal basis in genetic hypertension

Rat model of genetic hypertension

The spontaneously hypertensive rat (SHR) is probably the most widely used animal model of genetic hypertension. Hypertension in the SHR is similar to human essential hypertension in many aspects including the development of the hypertension and progression to secondary diseases *(Folkow, 1982)*. The central role of the kidney in genetic models of hypertension is highlighted by the finding that transplanting a kidney from a genetically hypertensive animal such as the stroke prone spontaneously

hypertensive rat (SHRSP) into a bilaterally nephrectomised F_1 hybrid (WKY-SHRSP) results in the development of post-transplantation hypertension in the F_1 hybrid *(Rettig et al., 1994)*. Conversely, transplanting a kidney from a normotensive Wistar-Kyoto rat (WKY) into a bilaterally nephrectomised F_1 hybrid animal lowered arterial pressure *(Kawabe et al., 1978)*, for review see *(Rettig et al., 1994)*. Hypertension that 'follows' the kidney has also been demonstrated in other models of genetic hypertension such as the Dahl salt-sensitive rats and their salt-resistant normotensive control strain (for review see *(Rettig et al., 1994)*.

Renal basis for hypertension in humans

Human clinical studies of renal post-transplantation hypertension are more difficult to interpret as there are numerous factors that could contribute to the hypertension such as;

i) graft kidney pathology (immunological rejection, renal artery stenosis, recurrence of original disease)
ii) a genetic alteration of the graft kidney
iii) The use of widespread immunosupressive drugs such as cyclosporine to prevent organ rejection. Cyclosporine also causes renal vasoconstriction which eventually leads to nephrosclerosis

In one study, six African-American patients, that had undergone bilateral nephrectomies, were selected on the basis that an independent nephropathologist had diagnosed them with essential hypertension based on their medical history and careful study of the removed kidneys *(Luke, 1993)*. All six patients received a kidney transplant, which resulted in normalisation of arterial pressure for at least 5 years *(Luke, 1993)*. Additionally, a marked regression of left ventricular hypertrophy and hypertensive retinal changes were also observed *(Luke, 1993)*. As the study was careful to select patients that were diagnosed with hypertension, the results suggest that hypertension can remit after transplantation of the nephrosclerotic kidney, with one free from any damage.

1.2.1.2.2 Other models of hypertension with a renal basis

Other models of hypertension can be induced by interference with normal renal function, including obstruction of the ureter, which produces reductions in renal blood flow, glomerular filtration rate and increases in renal vascular resistance, with a subsequent rise in arterial pressure *(Harris and Yarger, 1974)*. Administration of a single dose of 2-bromoethylamine hydrobromide (BEA) to rats selectively destroys the renal medulla resulting in a rise in arterial pressure *(Taverner et al., 1984; Heptinstall et*

al., 1975). The mechanisms responsible for the increase in pressure upon removal of the renal medulla remain to be firmly established, but might include removal of the source of a putative anti-hypertensive hormone which counteracts the renin-angiotensin system, and is located within the renal medulla *(Heptinstall et al., 1975; Muirhead et al., 1960).*

Infusion of vasoconstrictor agents into the kidney also produces marked changes in renal function and hypertension. Chronic infusion of noradrenaline (1μg/kg/min for 7 days) directly into the kidney of dogs causes an initial reduction in GFR, urinary sodium excretion and renal plasma flow, and an increase in arterial pressure (by 9 ± 4 mmHg over the first 60 minutes). As the infusion continues however, the pressure remains elevated while renal function is restored *(Reinhart et al., 1995).* There is also a large body of literature that shows acute and chronic infusion of angiotensin II (both systemically and intrarenally) can produce a sustained hypertension via mechanisms that are initially pressor or sub-pressor *(Stevenson et al., 2000; Fitzgerald et al., 1997b; Simon and Altman, 1992; Griffin et al., 1991; Brown et al., 1981; Urquhart et al., 1963).* As this thesis is primarily concerned with the role of angiotensin II in hypertension, this literature will be reviewed more thoroughly in subsequent sections.

1.3 ANGIOTENSIN II AND THE KIDNEY

It is now apparent that the kidney and its ability to regulate body fluid volume plays a central role in controlling arterial pressure under normal conditions and in the pathogenesis of hypertension. Control of renal function is intimately linked to the renin-angiotensin system and it follows thus, that a defect in the regulation of this system could be important in the development of some forms of hypertension. The peptide angiotensin II is the major biologically active component of this system.

In addition to the actions of circulating angiotensin II, there is also evidence for a local renin-angiotensin system as all of the components needed for local generation of angiotensin II have been identified within different tissue systems including the brain, heart, adrenal glands, and vasculature. It has been suggested that tissue renin-angiotensin systems responsible for local production of angiotensin II may be involved in the tonic regulatory functions, whilst circulating angiotensin II may be more concerned with acute alterations in vascular resistance *(Dzau, 1988).* In the following section a brief historical review of the renin-angiotensin system will be summarised.

1.3.1 The renin-angiotensin system – an historical perspective

Renin, a proteolytic enzyme secreted by the kidneys was first discovered by Tigerstedt and Bergman in 1898 (cited in *Robertson and Nicholls, 1995)* when they noted that crude extract of rabbit renal cortex produced a rise in arterial pressure when injected intravenously. The significance of these early findings was not fully appreciated until almost 40 years later when Goldblatt and colleagues produced sustained hypertension by constricting the renal arteries *(Goldblatt et al., 1934).* Goldblatts' study refreshed the search for the substance present in the kidney, which could be responsible for the rise in pressure seen during the stenosis. Today, the biochemistry of the renin-angiotensin system is well established and will be overviewed briefly in the following section.

Renin is predominantly produced and stored in specialised smooth muscle cells in the afferent arteriole adjacent to the glomerulus *(Gomez et al., 1989; Ingelfinger et al., 1988; Levens et al., 1981).* Factors controlling the secretion of renin into the circulation include;

i) Macula densa cells; a specialised group of epithelial cells located in the juxtaglomerular apparatus, which detect changes in the sodium and possibly chloride concentrations in the distal tubule *(Johns et al., 1990).*

ii) Stretch of the afferent arteriole that results in changes to wall tension, reducing the release of renin *(Reid et al., 1978).*

iii) The central nervous system, which controls renin secretion via the renal nerves and the adrenal medulla.

iv) A negative feedback loop by which angiotensin II, acts to inhibit the secretion and/or production of renin *(Peach, 1977).*

The renin-angiotensin pathway is represented in Figure 1.2. Circulating renin catalyses the splitting of the glycoprotein angiotensinogen into an inactive decapeptide angiotensin I. Angiotensinogen is produced in the liver but is present in high concentrations in plasma and its production is regulated by various factors including adrenocortical steroids, estrogens, angiotensin II and renin *(Ballermann and Marsden, 1991; Reid et al., 1978).* Expression of the angiotensinogen gene has been demonstrated in the kidney, blood vessel walls, heart, central nervous system and adrenal glands *(Dzau et al., 1987; Campbell and Habener, 1986; Ohkubo et al., 1986).* In the kidney, localisation of angiotensinogen mRNA (messenger ribonucleic acid) has been documented in the proximal tubule, and to a lesser extent in the glomerular tuft and renal vessels *(Terada et al., 1993; Ingelfinger et al., 1990).*

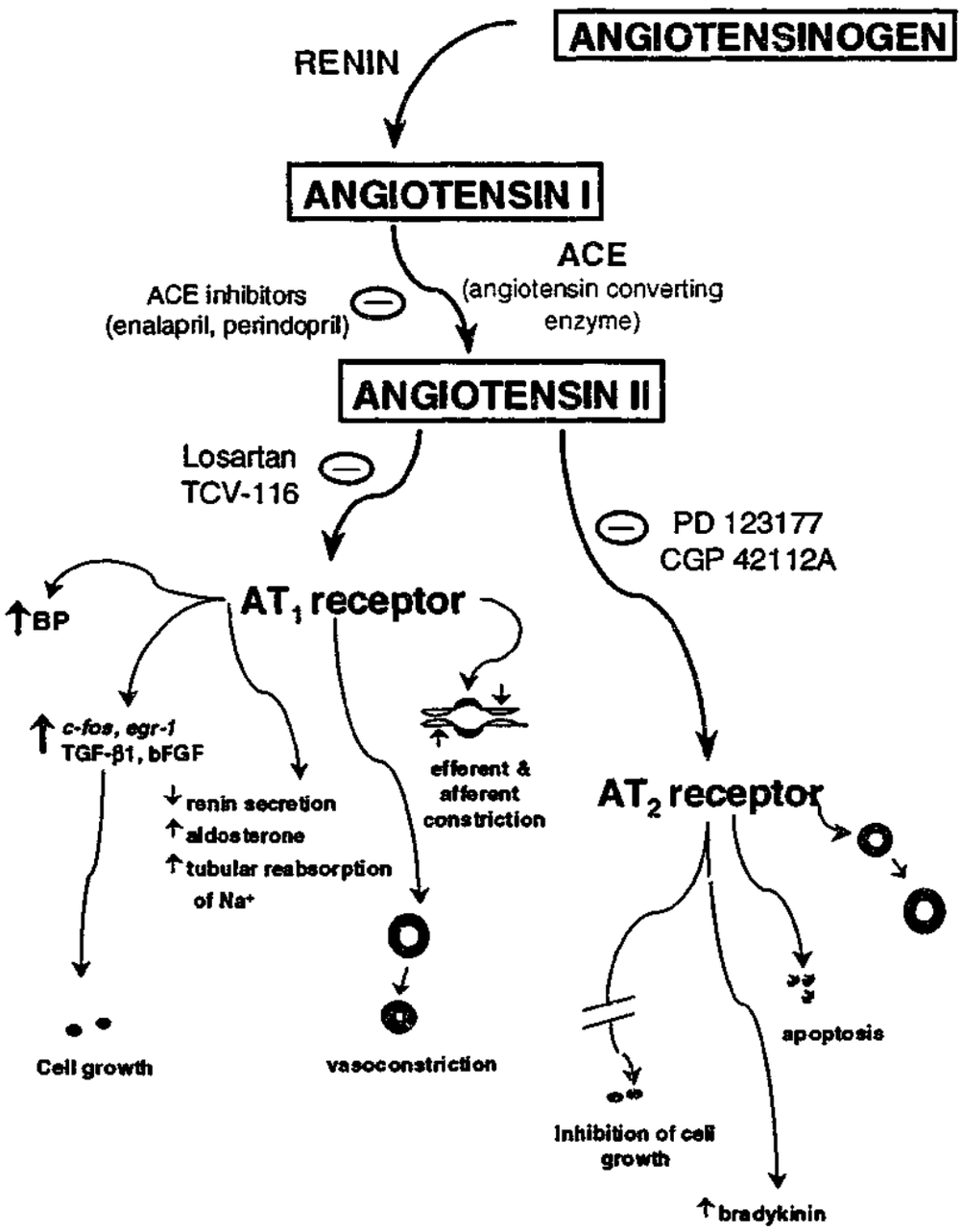


Figure 1.2 The renin-angiotensin system cascade.

The octapeptide angiotensin II, is the major biologically active component of the renin-angiotensin system. Angiotensin II has several actions within the kidney and whole body that function primarily to increase blood pressure and retain sodium.

Angiotensin II-converting enzyme (ACE), cleaves the dipeptide His^{9}-Leu^{10} from angiotensin I to produce the active angiotensin II. Angiotensin-converting enzyme was first isolated by Skeggs and colleagues in 1956 *(Skeggs Jr et al., 1956)*. Found abundantly in human lungs, kidney, ileum, duodenum and the uterus, the enzyme is predominantly located on the plasma membrane of endothelial cells, with small quantities also present in plasma and epithelial cells. *(Lieberman and Sastre, 1983)*. Within the kidney, ACE is found in abundance on endothelial cells of afferent arterioles, glomerular capillaries, and brush border of the proximal tubules *(Danilov et al., 1987; Caldwell et al., 1976)*.

In addition to the circulating renin-angiotensin system, the identification of components of the system in many tissues of the body provides evidence for local autocrine or paracrine actions of angiotensin II. Importantly, all components of the renin-angiotensin system have been identified in the kidney, and elevated intrarenal levels of angiotensin II have been demonstrated in many forms of experimental hypertension *(Levens et al., 1981)*. In experimentally induced, two-kidney, one-clip hypertensive rats, intrarenal angiotensin II levels were increased in the clipped kidney compared unclipped or control kidneys *(Navar and Harrison-Bernard, 2000; Zou et al., 1996a; Navar et al., 1995; Mendelsohn, 1980)*. Accumulation of intrarenal angiotensin II has also been demonstrated in rats given systemic infusions of angiotensin II, and in rats subjected to five weeks of unilateral ureteral obstruction *(Navar and Harrison-Bernard, 2000; Zou et al., 1996b; Zou et al., 1996a; Von Thun et al., 1994; el-Dahr et al., 1993)*. In the context of my work, it is of particular interest that by increasing circulating angiotensin II levels and inducing hypertension, the circulating angiotensin II accumulates in kidney, and also stimulates intrarenal production of angiotensin II, resulting in intrarenal concentrations of the peptide exceeding that in the circulation *(Navar and Harrison-Bernard, 2000; Zou et al., 1996b; Zou et al., 1996a)*. There is also evidence for intrarenal angiotensin II production in humans with hypertension *(Danser et al., 1998; van Kats et al., 1997; Fisher et al., 1995; Admiraal et al., 1993; Cordero et al., 1991)*. The increase in renal angiotensin II in these models of hypertension occurs despite a suppressed renal and circulating renin content, suggesting that intrarenal angiotensin II can be regulated independently of renin *(Navar and Harrison-Bernard, 2000)*. Further accumulation of renal angiotensin II appears to be dependent upon the angiotensin type 1 (AT_1) receptor, as blockade of this receptor prevents the accumulation of intrarenal angiotensin II in these models of hypertension *(Zou et al., 1996b)*. This evidence illustrates a crucial link between intrarenal angiotensin II levels and the development of hypertension. *In vivo*, the physiological relevance of the intrarenal and other local renin-angiotensin systems could include contributions to vascular tone and vascular growth and maintenance of renal haemodynamics and GFR. However, a definitive role for the intrarenal renin-

angiotensin system in the control of arterial pressure, and pathophysiological states such as hypertension is yet to be resolved.

1.3.2 Angiotensin II receptors.

Angiotensin II exerts its actions on target cells and organs in the body via an interaction between the peptide and specific, high affinity receptors in the plasma membrane of the target cells. Specific binding sites for angiotensin II have been identified in all its target tissues such as the adrenal gland, vascular smooth muscle, uterus, brain and kidney *(Mendelsohn, 1985)*. In the kidney, angiotensin II receptor binding sites and receptor mRNA expression have been demonstrated on afferent and efferent arterioles, as well as arcuate arteries, vasa recta bundles, mesangial cells, collecting ducts and proximal tubules *(Miyata et al., 1999; Chatziantoniou et al., 1994; Terada et al., 1993; Paxton et al., 1993; Kakinuma et al., 1993; Sechi et al., 1992)*.

The sequence of the angiotensin II type 1A receptor (AT_{1A}) was first published in 1991 *(Murphy et al., 1991; Sasaki et al., 1991)*. The amino acid sequence analysis of the molecule demonstrates that it shares structural similarities with the G protein coupled superfamily. Humans, rabbits and cows have a single gene encoding the AT_1 receptor. In contrast, rodents have two highly homologous AT_1 receptor genes that encode the receptors isozymes termed AT_{1A}, and AT_{1B} *(Sayeski et al., 1998)*. There are differences in tissue pattern expression between the two isozymes, however no differences have so far been detected in the way each isozyme binds ligand and signal *(Sayeski et al., 1998)*.

When the AT_{1A} receptor gene is inactivated in mice there is a reduction in blood pressure *(Ito et al., 1995)*. In contrast, arterial pressure in mice missing the AT_{1B} receptor gene is indistinguishable from wild type animals suggesting that, in rodents the AT_{1B} receptor has little functional significance *(Chen et al., 1997)*. Tissue responses to angiotensin II are mediated by the activation of second messenger systems, predominantly through the activation of the AT_{1A} receptor. These second messenger pathways include G-protein activation and also tyrosine phosphorylation cascades *(Sayeski et al., 1998)*.

The angiotensin II receptor subtype 2 (AT_2) was identified over ten years ago by its differential pharmacological and biochemical properties compared to the AT_1 receptor *(Whitebread et al., 1989)*. Almost 5 years later the AT_2 receptor was cloned simultaneously by two groups in 1993 *(Mukoyama et al., 1993; Kambayashi et al., 1993)*, and found to share only 34% amino acid sequence homology with the AT_1 receptor. The physiological role played by the receptor is not well understood and actions are hard to elicit as in adult mammals there is a low degree of expression of the AT_2 receptors compared to the AT_1 *(de Gasparo and Siragy, 1999)*. Signal

transduction pathways activated following stimulation of AT_2 receptor are also not well understood, but are thought to include indirect negative coupling to guanylate cyclase, activation of potassium channels and phosphotyrosine phosphatase *(Bottari et al., 1992)*. More recent evidence suggests that the AT_2 receptor also activates mitogen-activated protein kinase phosphatase-1 (MKP-1) and SHP-1 tyrosine phosphatase (for review see *de Gasparo and Siragy, 1999)*.

Regulation of expression of the AT_2 receptor appears to be controlled by numerous factors. Noradrenaline and angiotensin II increase intracellular Ca^{2+} levels and activate protein kinase-C which in turn down-regulates expression of AT_2 receptor mRNA. Likewise, growth factors such as platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) downregulate AT_2 receptor expression in various cell lines including VSMCs (for review see *Matsubara and Inada, 1998)*.

Stimulation of the AT_2 receptor by angiotensin II results in inhibition of DNA synthesis and cell growth stimulated via AT_1 receptor, in endothelial, smooth muscle and fibroblast cells, and also reduces extracellular matrix proteins and induces apoptosis *(de Gasparo and Siragy, 1999)*.

Mutant mice lacking the gene coding for the AT_2 receptor develop normally but have an impaired drinking response to thirst and a reduction in spontaneous movement *(Hein et al., 1995)*. In parallel, another group also developed a mouse with a disrupted AT_2 receptor gene and demonstrated that the disruption was associated with lowered body temperature and attenuated exploratory behaviour *(Ichiki et al., 1995)*. Both mutant variations demonstrated an increased sensitivity to angiotensin II injection, yet the Hein et al *(Hein et al., 1995)* study reported that the mutants had normal blood pressure, whilst the Ichiki et al *(Ichiki et al., 1995)* demonstrated a significantly increased blood pressure.

Intravenous infusion of angiotensin II (5 ng/kg/min) for 3 days resulted in a 12.6% increase in the density of the micro-vessels in the rat cremaster muscle compared to vehicle infused rats *(Munzenmaier and Greene, 1996)*. The action of angiotensin II appeared to be mediated at least in part, by the AT_1 receptor as when the receptor antagonist losartan was co-infused with angiotensin II, blood pressure was less than control and the cremaster vessel density was reduced when compared to the angiotensin II treated group but still greater than control (7.8%). In contrast when the AT_2 receptor antagonist PD123319 was co-infused with angiotensin II, blood pressure and vessel density were increased above that seen with angiotensin II infusion alone (23%) *(Munzenmaier and Greene, 1996)*. This study suggests, at least in the microvasculature, that the AT_1 receptor mediates angiogenesis and vasoconstriction, whilst the AT_2 receptor appears to vasodilatation and inhibition angiogensis.

Despite the growing body of literature detailing a functional role for the AT_2 receptor, most of the known physiological and biochemical actions of angiotensin II in the adult are mediated via the AT_1 receptor *(Robertson and Nicholls, 1995)*. This may be because the AT_2 receptor subtype has very low expression in the adult and makes up only 5-10% of the total angiotensin II receptors in the adult rat kidney *(Zhuo et al., 1993)*. In human fetal kidneys however, the AT_2 receptor subtype is the most abundant, suggesting a role for the AT_2 receptor in development of the kidney *(Grone et al., 1992)*. Furthermore, in embryonic rat kidneys the AT_2 receptor subtype accounts for approximately 80% of the total angiotensin II receptors, yet a week after birth the AT_1 receptor subtype is pre-dominantly expressed *(Zhuo et al., 1993)*.

1.3.3 Actions of angiotensin II

The renin-angiotensin system is widely recognised as an important regulator of vascular vasomotor activity, sodium and water balance, and arterial pressure. It exerts this regulation through the several intra- and extra-renal actions of angiotensin II. Specifically I am interested in the potential trophic actions of angiotensin II on cellular proliferation and hypertrophy within the kidney and its involvement in the initiation and pathogenesis of hypertension. However, the pro-hypertensive actions of angiotensin II are mediated via a number of pathways. In the following section the systemic and intrarenal actions of angiotensin II will be reviewed briefly before the concept of angiotensin II-induced cellular growth and hypertrophy in hypertension is explored more thoroughly.

1.3.3.1 Systemic actions of angiotensin II

1.3.3.1.1 Vasoconstriction

The potent vasoconstrictor effect of angiotensin II on the systemic vasculature was the first property of the renin-angiotensin system to be described and contributes to the acute maintenance of blood pressure by directly affecting total peripheral resistance (TPR). In vascular smooth muscle cell cultures, angiotensin II mediated contraction (cell shortening) results from the interaction with a single class of high affinity receptor (the AT_1) on the vascular smooth muscle cells *(Gunther et al., 1982)*. *In vivo*, the direct vasoconstrictor action of angiotensin II has been demonstrated by numerous investigators, and is mediated through the AT_1 receptor *(Bottari et al., 1993; Brown et al., 1981)*. Activation of the receptor increases the entry of calcium into the

smooth muscle cells via voltage-sensitive channels, thereby prolonging the action potential and increasing the force of contraction of the cells *(Bottari et al., 1993)*.

1.3.3.1.2 Sympathetic nervous system

Activation of the peripheral sympathetic nervous system, and to a lesser extent, withdrawal of parasympathetic discharge facilitated by angiotensin II results in an increase in arterial pressure *(Ferrario et al., 1972)*. The increase in sympathetic activity is thought to be facilitated via:

i) Increasing norepinephrine release and inhibiting re-uptake at central and peripheral adrenergic nerve terminals *(Campbell and Jackson, 1979; Malik and Nasjletti, 1976)*,

ii) Enhancing the sensitivity of the post-synaptic membrane to noradrenaline *(Palaic and Khairallah, 1967)*

iii) Stimulating the synthesis of noradrenaline *(Davila and Khairallah, 1971)*.

Activation of the sympathetic nervous system increases vasoconstriction of the peripheral vasculature and increases cardiac output *(Ferrario et al., 1972)*. The physiological relevance of the actions of angiotensin II to enhance sympathetic transmission are arguable, as the majority of the effects occur in response to administration of angiotensin II at very large doses. Such actions may therefore play a more important role in pathophysiological states where endogenous angiotensin II levels are increased.

1.3.3.1.3 Central Nervous System

Injection of angiotensin II into the area postrema of the caudal medulla results in a systemic pressor response *(Fink et al., 1987; Wright et al., 1985; Peach, 1977)*. Angiotensin II also acts on the central nervous system to increase aldosterone secretion and stimulate thirst centres in the brain, thus influencing volume homeostasis by increasing both water intake and tubular sodium reabsorption *(Peach, 1977)*. When applied directly to the brain via intracerebroventricular injections, angiotensin II causes rats in normal fluid balance to drink water. This response is rapid and repeatable *(Wright et al., 1985; Epstein et al., 1970)*. Direct stimulation of thirst neurones by angiotensin II is achieved when circulating angiotensin II reaches the subfornical organ (SFO) and the vascular organ of the lamina terminalis (OVLT) via fenestrated capillaries *(Robertson and Nicholls, 1995)*.

1.3.3.1.4 Adrenal Gland

Multiple factors regulate the secretion of aldosterone. However, in states of sodium depletion, the renin-angiotensin system appears to be the principal driving force behind aldosterone secretion *(Robertson and Nicholls, 1995)*. In the glomerulosa cells of the adrenal cortex, angiotensin II stimulates the conversion of stored cholesterol to pregnanolone, and then conversion of corticosterone to aldosterone. *(Robertson and Nicholls, 1995)*. The activation of aldosterone secretion appears to be mediated through angiotensin II receptors found on the membrane of adrenal glomerulosa cells, and secretion promotes reabsorption of Na^+ in the collecting ducts of the kidney *(Bottari et al., 1993; Peach, 1977)*. Angiotensin II also directly stimulates the adrenal medulla to release catecholamines *in vivo* and *in vitro (Peach, 1977)*.

1.3.3.1.5 Vascular Growth

The functional consequences of angiotensin II-induced vascular growth, and the role this plays in hypertension is the major focus of this thesis and will be dealt with in detail in the following sections of this review. For completeness, the pro-hypertensive action of angiotensin II via induced vascular growth will also be described briefly below.

Direct evidence implicating angiotensin II in the process of vascular growth comes almost entirely from studies performed on vascular smooth muscle cells in culture. *In vitro*, addition of angiotensin II to vascular smooth muscle cells in culture can elicit both hypertrophic or hyperplastic growth *(Huckle and Earp, 1994; Holycross et al., 1993; Stouffer et al., 1993; Geisterfer et al., 1988; Campbell-Boswell and Robertson, 1981)*. Additionally, angiotensin II has been shown to activate signalling pathways associated with cell growth such as the induction of proto-oncogenes including *c-fos*, and *egr-1 (Huckle and Earp, 1994; Sachinidis et al., 1992; Naftilan, 1992; Naftilan et al., 1989b; Taubman et al., 1989)*. However, It also appears that angiotensin II-induced proliferation in these cells is potentiated by the presence of other growth factors such as platelet derived growth factor –AA and –BB chain (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor-beta1 (TGF-β1) *(Dzau et al., 1991)*.

In summary, the actions of angiotensin II, both direct and indirect, in the systemic circulation provide the body with a mechanism to compensate for sudden reductions in blood pressure. Initially the release of renin and the production of angiotensin II produces a rapid vasoconstriction and activation of the sympathetic nervous system which act in consert to increase total peripheral resistance and cardiac output. The increase in circulating angiotensin II in response to a fall in blood pressure also activates longer acting regulatory systems such as stimulation of aldosterone

secretion and retention of salt and water. The overall result of activation of the renin-angiotensin system is to increase systemic arterial pressure.

1.3.3.2 Intrarenal actions of angiotensin II

As the kidney is an essential component to the development of hypertension, and intimately linked to the renin-angiotensin II system, the potential trophic action of angiotensin II in the kidney and its link with hypertension is the primary focus of this thesis. However, angiotensin II has many renal actions that can act to increase blood pressure, and these will be summarised below.

The major intrarenal actions of angiotensin II are involved in the regulation of renal haemodynamics, electrolyte and fluid balance and thus arterial pressure. There are several sites of action of angiotensin II in the kidney whereby it exerts its haemodynamic influences.

1.3.3.2.1 Renal vasculature

Glomerular capillary pressure is dependent on the relative levels of pre- and post-glomerular resistance and thus changes in diameter of the afferent and efferent arterioles. Therefore, if the afferent arteriole constricts, downstream glomerular capillary pressure decreases and the plasma flow to the glomerulus also decreases resulting in a net decrease in GFR *(Ichikawa and Brenner, 1984)*. Conversely, if the efferent arteriole is constricted glomerular capillary pressure is increased despite a reduction in plasma flow to the glomerulus, with a net conservation of GFR *(Ichikawa and Brenner, 1984)*. An increase in filtration fraction and subsequent maintenance of GFR occurs in the presence of both efferent and afferent arteriolar constriction and this is thought to be due to the greater relative resistance of the efferent arteriole in comparison to the afferent arteriole.

Vascular casting studies have demonstrated that angiotensin II infusion produces marked vasoconstriction of both afferent and efferent arterioles but the physiological importance of relative changes in efferent and afferent diameter are often disputed in the literature *(Denton et al., 1992; Steinhausen et al., 1990; Wilson, 1986)*. A study by Denton and colleagues in 1992 showed that infusion of angiotensin II in rabbits, caused a greater reduction in afferent arteriolar diameter than in efferent. However, the calculated increase in vascular resistance per micron was greater in efferent arterioles due to their smaller resting diameter *(Denton et al., 1992)*. Thus although angiotensin II constricted the afferent arteriole to a greater extent, functionally the effect was greater on the efferent arteriole *(Denton et al., 1992)*.

A more recent study by Denton and colleagues found that the extent of vasoconstriction of afferent and efferent arterioles by angiotensin II changed in different regions of the kidney *(Denton et al., 2000)*. In this study, angiotensin II was infused directly into the renal artery at doses which did not affect systemic blood pressure. This resulted in a progressive decrease in RBF, a simultaneous increase in filtration fraction, and a preservation of GFR. The increase in filtration fraction was the result of a greater increase in relative efferent arteriolar resistance, compared to the resistance in the afferent arterioles of the mid- and outer-cortical glomeruli. These changes occurred despite an apparent preferential effect of angiotensin II on afferent arteriolar diameters of the outer- and mid-cortex *(Denton et al., 2000)*. In contrast, the afferent and efferent arterioles of the juxtamedullary nephrons responded to angiotensin II with a decrease in diameter of similar extent (5.4um and 6.7um respectively) *(Denton et al., 2000)*.

In summary, exogenous application of angiotensin II results in constriction of both efferent and afferent arterioles, with the net effect being to prevent large reductions in GFR in response reductions in RBF *(Blantz, 1987; Wilson, 1986; Rosivall and Navar, 1983)*.

1.3.3.2.2 GFR and mesangial cell contraction

Angiotensin II binds the angiotensin type-1 receptor on isolated glomeruli from rats *(Mendelsohn et al., 1986)*. Infusion of angiotensin II has also been shown to result in changes in GFR and glomerular structure, specifically angiotensin II slightly lowers single-nephron glomerular filtration rate (SNGFR) and whole kidney GFR in part due to reducing ultrafiltration coefficient (Kf) *(Pagtalunan et al., 1995; Blantz, 1987)*. Kf consists of two components; hydraulic conductivity and capillary surface area available for filtration, and is a measure of the permeability of the glomerular filtration barrier. The mechanism responsible for angiotensin II-induced reductions in (Kf) is yet to be determined, however much research has centred around the glomerular mesangium as a potential regulator. Angiotensin II receptors (AT_1) have been demonstrated on glomerular mesangial cells and addition of angiotensin II to mesangial cultures causes contraction *(Mendelsohn et al., 1986; Ausiello et al., 1980)*. The Kf reducing effect of angiotensin II to reduce Kf has been hypothesised to be mediated by contraction of the glomerular mesangial cells, which in turn reduces the glomerular capillary surface area available for filtration. However, while angiotensin II has been shown to contract mesangial cells in culture and reduce glomerular tuft volume *in vivo*, most studies do not detect a decrease in capillary surface area *(Denton et al., 2000; Pagtalunan et al., 1995; Denton et al., 1992; Haley et al., 1987; Ichikawa and Brenner, 1984)*. A more

recent hypothesis has suggested that alterations in the geometry of the glomerular pole due to contraction of the mesangium may have consequences for glomerular haemodynamics *(Denton et al., 2000)*. It has been proposed that contraction and drawing down of the mesangium into the extraglomerular space may lead to distention of the area where the afferent and efferent arterioles enter the glomerulus, possibly leading to changes in distribution of glomerular flow *(Elger et al., 1998; Inkyo-Hayasaka et al., 1996)*. Indeed, Denton and colleagues (2000) have shown that the origins of the efferent and afferent arterioles are further apart in angiotensin II treated kidneys and this effect is dose-dependent. The increase in space between the arterioles may indicate the downward contraction of the mesangium impinging on the extra-glomerular space.

The combined effects of angiotensin II on efferent and afferent arteriolar resistance, Kf act to subsequently increase pre-:post-glomerular resistance, thereby increasing glomerular capillary hydrostatic pressure (Pg), capillary pressure gradient (ΔP) and ultimately conserving GFR in the face of a reduced renal blood flow *(Blantz, 1987; Myers et al., 1975)*. It is possible that the increased levels of intrarenal angiotensin II observed in some forms of hypertension may play a role in the maintainence of the hypertension by a continual angiotensin II dependent suppression of renal function and sodium excretion via its effects on the tubules, vasculature and long term proliferative effects. As long as these renal effects of elevated angiotensin II are sustained, the hypertension could be maintained even when circulating angiotensin II levels return to normal levels.

1.3.3.2.3 Tubular

Reabsorption of water and some solutes that have been filtered through the glomerulus takes place in the renal tubules. Angiotensin II elicits a biphasic effect on sodium reabsorption in the proximal tubule of the kidney, directly stimulating sodium transport at low (physiological) doses ($10^{-12} - 10^{-10}$ M), and reducing it at high doses (3×10^{-7} to 3×10^{-6} M) *(Harris and Young, 1977)*.

The direct actions of angiotensin II on proximal tubular sodium transport are thought to be mediated via high affinity angiotensin II receptors located on the brush border and basolateral membrane of the proximal tubular endothelial cells (for review see *Harris and Navar, 1985)*. *Schuster et al.* (1984) demonstrated that physiological levels of angiotensin II stimulated fluid reabsorption in isolated proximal tubules. *In vivo*, dose response relationships between angiotensin II and whole kidney proximal tubule sodium reabsorption have also been demonstrated *(Olsen et al., 1985)*. In these experiments, low physiological levels of angiotensin II infusion into dogs produced a marked increase in proximal tubular fractional sodium reabsorption,

however at very high rates of angiotensin II infusion sodium reabsorption was inhibited and there was an accompanying natriuresis *(Olsen et al., 1985)*. This inhibition was reversed by artificially controlling renal artery perfusion pressure, thus indicating that the natriuresis was caused by the increase in renal artery pressure and not an inhibition of proximal tubular sodium reabsorption *(Olsen et al., 1985)*. Indirectly, angiotensin II acts to enhance sodium reabsorption by stimulating the secretion of aldosterone from the glomerulosa cells of the adrenal cortex *(Harris and Navar, 1985)*.

In anaesthetised rats treated with the angiotensin receptor antagonist DuP 753, fractional proximal tubule fluid reabsorption decreased nearly 10% and fractional distal sodium reabsorption decreased approximately 4%, indicating that angiotensin II is also involved in fluid reabsorption at distal sites in the tubules via the AT_1 receptor *(Zhuo et al., 1992)*. Similar observations have also been made in animals treated with ACE inhibitors *(Zhuo et al., 1989; Harris et al., 1987)*. The mechanisms by which angiotensin II directly stimulates tubular sodium reabsorption appear to involve activation of the luminal membrane Na^+-H^+ exchanger and the basolateral (brush-border) membrane Na^+ - HCO^3- co-transporter *(Laragh and Brenner, 1995)*.

The intrarenal tubuloglomerular feedback (TGF) mechanism maintains the balance between reabsorption and the amount of solute and fluid filtered at the glomerulus. The macula densa cells of the thick ascending loop of Henle detect increases in sodium chloride and total solute concentration in the tubule fluid, which then trigger increases in glomerular vascular resistance and decreases in GFR at a single nephron level *(Laragh and Brenner, 1995)*. Angiotensin II has been found to enhance the responsiveness of the TGF mechanism *(Mitchell and Navar, 1988)* and also has a role in regulating this responsiveness in models of angiotensin II dependent hypertension such as two-kidney, one-clip hypertension *(Braam et al., 1995)*.

1.3.3.2.4 Renin secretion

Renin synthesis is controlled by a number of factors, including a negative feedback of circulating angiotensin II. Johns and colleagues (1990) correlated alterations in renal renin mRNA and immunohistochemical localisation of renin protein in conditions of altered angiotensin . Specifically they found that treatment of rats with ACE inhibitor enalapril increased renal renin mRNA via an increase in renin gene expression or a decrease in mRNA turnover. A parallel increase in renin protein staining suggested that the production of renin was also stimulated when formation of angiotensin II is inhibited. Addition of exogenous angiotensin II partially reversed these affects by inhibiting renin gene expression, yet did not reduce renal renin protein

staining suggesting that the negative feedback loop of angiotensin II on renal renin distribution reflects a direct inhibition of renin synthesis *(Johns et al., 1990)*.

1.4 TROPHIC ACTIONS OF ANGIOTENSIN II

Having reviewed the overall actions of angiotensin II that regulate blood pressure, the remainder of this literature review will focus on evidence of a trophic action of angiotensin II in models of hypertension.

Chronic hypertension in humans and experimental animals is characterised by an increase in peripheral resistance, a major component of which cannot be abolished by administering drugs that cause smooth muscle cell relaxation *(Folkow et al., 1973)*. From this the "structural" component of vascular resistance was proposed and later shown to be due to an increase in smooth muscle mass of the vasculature *(Folkow, 1978)*. The increase in smooth muscle mass increases the wall to lumen ratio, decreasing the lumen of the vessel and thus increasing total peripheral resistance. The observation of vascular growth, vessel hypertrophy and subsequent lumen reduction in hypertension led to the coining of the term `vascular amplifier' *(Korner, 1982)*. The vascular amplifier theory proposes that the increased wall thickness and reduced lumen diameters seen in hypertensive vessels leads to an amplification of the vessel resistance response to given stimuli when compared to normotensive vessels *(Korner, 1982)*.

At a cellular level, vascular growth relates to the changes in the vascular smooth muscle cells of the artery wall. These cellular changes can be threefold, encompassing vascular smooth muscle cell (VSMC) hypertrophy (increase in size of cells but no cell division), cellular hyperplasia (an increase in cell number but not size) (Figure 1.3). Additionally a process of vascular remodelling can take place, which involves remodelling of the vessel wall around a smaller lumen, with no net change in vessel wall mass

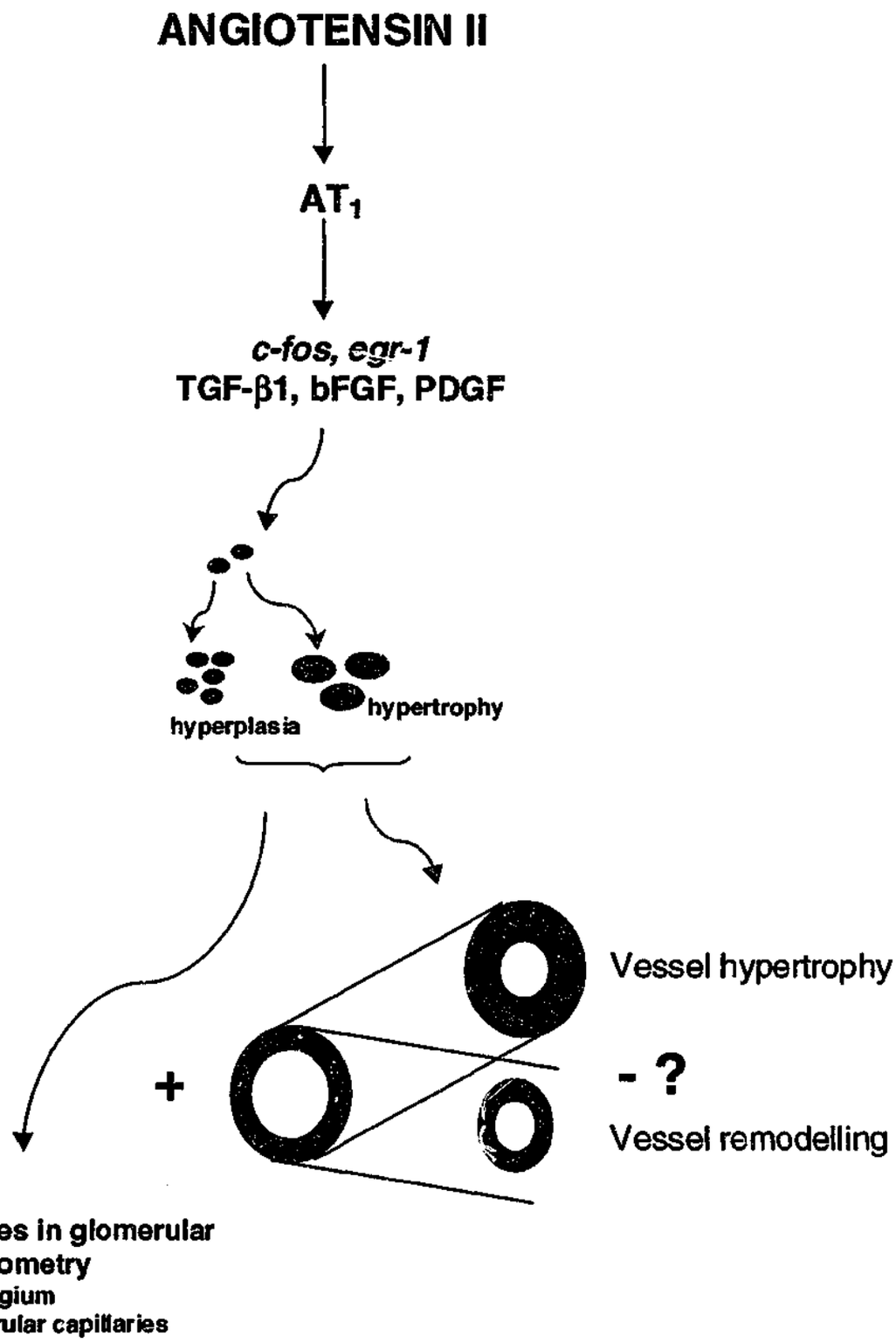


Figure 1.3 Putative cascade of angiotensin II mediated cellular growth and hypertrophy and the consequences in the vasculature.

1.4.1 Cellular hypertrophy and hyperplasia induced by angiotensin II

There is an expanding body of literature demonstrating angiotensin II-induced growth and hypertrophy in VSMCs from the systemic and renal vasculature. In the following section the growth promoting actions of angiotensin II and the molecular and cellular pathways involved in the growth response will be explored.

Although most evidence of growth induced by angiotensin II has been investigated in the systemic vasculature, I am particularly interested in potential angiotensin II related structural changes in the renal vasculature, as the kidney apparently plays an essential role in the development of hypertension. The hypothesis that angiotensin II-induced renal vascular changes could lead to hypertension was stimulated by studies demonstrating that infusion of angiotensin II directly into the kidney produces hypertension and renal changes similar to those described in other forms of renally based models of hypertension *(Fitzgerald et al., 1997a; Lohmeier and Cowley, 1979).*

1.4.1.1 Angiotensin II-induced hypertrophy and hyperplasia in cultured cells

1.4.1.1.1 Systemic (non-renal)

In vitro, angiotensin II has been shown to induce both hypertrophy and hyperplasia of vascular smooth muscle cell cultures. In an early study, addition of angiotensin II to cultures of human aortic smooth muscle cells for 7 days significantly enhanced cell proliferation and induced an increase in the average size of the cells *(Campbell-Boswell and Robertson, 1981).* In contrast, angiotensin II-induced hypertrophy but not hyperplasia of cultured rat aortic smooth muscle cells in defined serum-free media *(Geisterfer et al., 1988).* After four days of angiotensin II treatment the vascular smooth muscle cell protein content had increased by 20% compared to vehicle-treated controls. The hypertrophy was due to an increase in protein synthesis that was evident from 6-9 hours after treatment *(Geisterfer et al., 1988).* Angiotensin II-induced hypertrophic growth of vascular smooth muscle was further found to be selective, with increases in certain proteins within the cell exceeding overall cellular protein increases. Proteins that were found to increase most in the cells included α-actin, vimentin, tropomyosin, and myosin heavy chain, indicating that angiotensin II may also promote expression of smooth muscle-specific contractile proteins *(Turla et al., 1991).*

Hypertrophic responses to angiotensin II in cultured rat aortic smooth muscle cells are blocked by the AT_1 receptor antagonist DuP 753, but not the AT_2 receptor antagonist PD123177, suggesting that the AT_1 receptor is functional in cultured VSMCs, is chiefly responsible for the hypertrophic response to angiotensin II *(Chiu et al., 1991)*.

The different (hypertrophic or hyperplastic) responses of VSMCs to angiotensin II documented in the literature appears to be dependent on the species, the degree of differentiation of the smooth muscle cultures, and the serum used. Indeed Paquet and colleagues demonstrated that angiotensin II exerted a mitogenic effect on cultured aortic smooth muscle cells from SHR, but had very little effect on aortic cells from WKY rats *(Paquet et al., 1990)*.

1.4.1.1.2 Renal Cultures

Vascular smooth muscle cells

Angiotensin II has been shown to induce a dose-dependent increase (with a maximum response at 10^{-5}M angiotensin II) in proliferation and DNA synthesis in subcultures of renal arteriolar smooth muscle cells *(Dubey et al., 1992)*. Dubey and colleagues (1992) found that concentrations of angiotensin II (10^{-9}M) increased cell number by up to 164% by day 12 when compared to control cells grown in 0.4% fetal calf serum (FCS). Furthermore this cellular proliferation was associated with the expression of the early growth response gene *c-fos* following just 30 minutes of exposure to angiotensin II *(Dubey et al., 1992)*. The study was the first to demonstrate the mitogenic action of angiotensin II on renal arteriolar cells and thus suggests that the peptide could potentially be involved in the hypertrophy of renal arterioles in hypertension. However, there is very little known about the mechanisms of action of angiotensin II regulating growth in renal arteriolar cells (ie; receptor activation, second messenger pathways, interaction with growth factors).

Other renal cells

Cultured human adult and fetal mesangial cells undergo both hypertrophy and hyperplasia in response to angiotensin II, suggesting a possible role for angiotensin II in the pathogenesis of glomerulosclerosis in humans *(Orth et al., 1995; Ray et al., 1991)*. Mitogenesis is thought to occur in this case through the AT_1 receptor and involve the induction of growth factors such as PDGF and TGF-β1 *(Wolf, 1995; Rupprecht et al., 1992)*. PDGF mediated proliferation of cultured mesangial cells has also been shown to be associated with *egr-1* mRNA expression *(Rupprecht et al.,*

1992). In fact, both angiotensin II and PDGF stimulated proliferation of mesangial cultures induce the *egr-1* gene *(Rupprecht et al., 1992).* Furthermore, activation of early immediate genes including *egr-1* and *c-fos* is dependent upon the presence of polyamines in the cell, and plays a significant role in the induction of cultured mesangial cell proliferation *(Schulze-Lohoff et al., 1993).*

Primary cultures of glomerular endothelial cells incubated with 10^{-9} and 10^{-10} M of angiotensin II for 24 hours showed a similar proliferative response compared with cultures that were stimulated with 10% FCS *(Wolf et al., 1996).* Furthermore, blockade of the AT_1 receptor with losartan attenuated the proliferative response to angiotensin II. Blockade of the AT_2 receptor did not alter the angiotensin II-induced proliferation of the cultured endothelial cells *(Wolf et al., 1996).* In this paper, proliferation of the cultured glomerular endothelial cells was measured by incorporation of [^{3}H]-thymidine, expression of the early gene *egr-1* and detection of MAP kinase 2 (activated during angiotensin II-mediated proliferation of other cell types). Glomerular endothelial cells form a barrier between the blood and adjacent cell populations such as mesangial cells, and therefore, may play an important role in the interactions between circulating factors and growth of the surrounding cells.

Proximal tubular epithelial cells have the highest density of the angiotensin II receptors of all cell types in the nephron *(Mujais et al., 1986).* Epidermal growth factor induced mitogenesis in rabbit proximal tubular cells is potentiated by angiotensin II, although angiotensin II alone did not stimulate growth in tubular cells *(Norman et al., 1987).* In a permanent cultured murine proximal tubule cell line (MCT), angiotensin II slightly inhibited proliferation but induced cellular hypertrophy in a dose-dependent manner *(Wolf and Neilson, 1990).* Addition of angiotensin II was associated with hypertrophy of the cultured MCT cells and expression of TGF-β1 mRNA and protein. The hypertrophic response to angiotensin II in the MCT cells was mediated by the AT_1 receptor as the response was blocked with losartan *(Wolf and Ziyadeh, 1997).*

1.4.1.2 In vivo effects of angiotensin II (systemic)

Analysis of the role that angiotensin II plays in the regulation of vascular growth, particularly in the kidney *in vivo,* is complicated by an array of factors that influence cellular homeostasis and function. The relative roles played by mechanical forces, vasoactive substances, growth factors and hormones in the development of cardiovascular hypertrophy and hypertension *in vivo* are difficult to assess. Most evidence indicating a role of angiotensin II in vascular growth has been indirectly gathered from studies utilising blockade of the renin-angiotensin system, or infusion of exogenous angiotensin II.

1.4.1.2.1 Angiotensin II blockade (in rats)

Angiotensin II was first implicated in the process of smooth cell muscle growth in hypertension when Owens (1987) examined the effects of antihypertensive therapy on the smooth muscle cell changes in the aorta of SHR. This study demonstrated that the efficacy of the drugs propanolol, hydralazine and captopril in preventing smooth muscle cell hypertrophy was related to the degree of blood pressure reduction *(Owens, 1987)*. However, treatment with captopril, an ACE inhibitor, resulted in a greater reduction of smooth muscle hypertrophy than did hydralazine, a non-specific vasodilator, despite an equivalent reduction in blood pressure *(Owens, 1987)*. A similar result was also demonstrated in the normotensive WKY rat, suggesting that angiotensin II may also play a role in vascular smooth muscle growth under normal physiological conditions *(Owens, 1987)*.

These results have been confirmed by a number of groups. In particular, a study by Wang and Prewitt (1990), demonstrated that captopril treatment resulted in a reduction in the cross-sectional wall area of aortas and arterioles in normotensive, and one kidney-one clip hypertensive rats, suggesting involvement of angiotensin II in smooth muscle cell growth, independent of blood pressure *(Wang and Prewitt, 1990)*. Levy *et al.* (1988) also demonstrated that treatment with captopril (ACE inhibitor), reversed aortic hypertrophy and normalised blood pressure in two-kidney, one-clip hypertensive rats. Treatment with an angiotensin converting enzyme inhibitor also decreased neointimal thickening in rat carotid arteries by approximately 80%, 14 days after balloon catheter injury *(Powell et al., 1989)*. A summary of the literature, which examines the effect of inhibition of the renin-angiotensin system on vascular structure in hypertension, can be found in Lundie *et al.* (1997).

It should be noted that the anti-hypertensive action elicited when the renin-angiotensin system is blocked may be due to a number of mechanisms, including potential stimulation of the vasodilatory bradykinin pathway. However, when ACE inhibitors are administered to rats with angiotensin II-induced hypertension, with a constant and continued presence of the exogenously delivered angiotensin II, there is little or no effect on blood pressure, heart rate, and plasma renin activity *(Textor et al., 1981)*. Thus, in the presence of exogenous angiotensin II, ACE inhibition has no haemodynamic effect suggesting bradykinin plays little or no role in the anti-hypertensive action of chronic ACE inhibition in this model *(Textor et al., 1981)*.

A role for angiotensin II in normal vascular function is also evident in normotensive rats. Chronic ACE inhibition of normotensive Sprague-Dawley rats with captopril led to a significant reduction in mean arterial pressure, yet resting diameter of

arterioles from the cremaster muscle were not different between treated and control animals *(Frisbee et al., 1999)*. Arteriolar responses to endothelium dependent and independent vasodilators, however, were reduced in the captopril treated animals, and maximal vasodilatation was reduced, supporting a role for angiotensin II in maintaining normal vascular tone and vasodilator reactivity *(Frisbee et al., 1999)*.

1.4.1.2.2 Angiotensin II blockade (in humans)

The literature investigating the effect of ACE inhibition on blood pressure in human hypertension is extensive. However, few studies have examined the vascular effects of ACE blockade in humans. In a random, double blind human trial, the effects of treatment with the ACE inhibitor perindopril and a beta-blocker (atenolol) on gluteal small artery structure were compared in previously untreated essential hypertensive patients *(Thybo et al., 1995)*. Despite achieving a greater reduction in blood pressure with atenolol, perindopril treatment resulted in an increase in gluteal small artery diameter and a reduction in the ratio of media thickness to lumen diameter, where as atenolol had no effect *(Thybo et al., 1995)*. Furthermore, 1 or 2 years after commencing treatment of hypertensive patients with the ACE inhibitor cilazapril, and endothelium-dependent vasorelaxation to acetylcholine is slightly improved, an effect which is not observed in gluteal small arteries of patients treated with atenolol *(Schiffrin, 1995)*. Thus, the results of these studies suggest that ACE inhibitors induce regression of abnormal vessel structure and function in hypertension.

1.4.1.2.3 Systemic angiotensin II infusion in rats

The effects of ACE inhibition on vascular growth may be due, at least in part, to inhibition of angiotensin II or a reduction in blood pressure, making it difficult to directly attribute effects on vascular growth specifically to angiotensin II. An alternative method of investigating angiotensin II-induced vascular growth is by infusing exogenous angiotensin II systemically into experimental animals and inducing hypertension. Although angiotensin II-induced hypertension is one of the most common experimental models of hypertension, few studies have directly investigated the effects of angiotensin II on vascular growth.

Chronic, low dose infusion of angiotensin II results in a slowly developing increase in arterial pressure *(Brown et al., 1981)*. The direct or fast pressor effect of angiotensin II infusion occurs at high supra-physiological doses, and is thought to be mediated via the systemic effects discussed in section 1.3.3.1. A component of the slow pressor effect of low, doses of angiotensin II is thought to involve growth of the

vasculature *(Griffin et al., 1991).* Brown *et al.* (1981) infused angiotensin II intravenously at 270 or 810 ng/kg/min for one hour, or at 20ng/kg/min for 7days. By day seven of infusion, the mean arterial pressure of the rats infused with low dose angiotensin II was 153 mmHg, which was similar to the rise seen with the brief high dose, where mean arterial pressure rose to 146 mmHg after one hour of 270ng/kg/min, and 156 mmHg after 810ng/kg/min infusion. The difference between the two infusion methods was that when angiotensin II was infused for 7 days at 20 ng/kg/min the arterial pressure did not change in the first hour of infusion, but had risen by 15 mmHg after 24 hours. The mean arterial pressure then rose progressively in each animal reaching a peak towards the seventh day of infusion. Additionally, there was no detectable change in sodium balance and water intake during the seven days of low dose angiotensin II infusion *(Brown et al., 1981).* The results of this study indicated that the slow pressor effect of angiotensin II develops at near physiological plasma concentrations of the peptide and that the effects did not appear to be due to alterations in sodium and water balance *(Brown et al., 1981).*

A more recent experiment by Griffin *et al.* (1991) demonstrated that low dose infusion of angiotensin II raises blood pressure slowly and also causes vascular hypertrophy that is in part due to a non-pressor mechanism. In this study angiotensin II (200 ng/kg/min subcutaneously; s.c.) was infused for 10-12 days, producing a slowly progressive pressor response that raised systolic pressure by approximately 70 mmHg, inhibited plasma renin activity and increased plasma concentrations of angiotensin II three-fold. Compared with saline infused control rats, angiotensin II treated rats had increased heart weights, and increased media thickness, cross-sectional area, and media:lumen ratio of mesenteric vessels. The mesenteric lumen diameter was not affected. Additional groups of angiotensin II and saline infused rats were simultaneously treated with hydralazine (220 mg/l) in their drinking water. Hydralazine given with angiotensin II prevented the slow pressor response but failed to prevent any of the mesenteric vascular changes associated with the infusion when compared to hydralazine treated controls *(Griffin et al., 1991).* This suggests that angiotensin II can alter vascular structure via a mechanism that is independent of its blood pressure effects. These observations have subsequently been confirmed in a number of studies where hydralazine treatment has been shown to attenuate angiotensin II-induced hypertension in rats, yet did not reduce the augmented smooth muscle cell proliferation in the carotid artery and mesenteric microvessels *(Su et al., 1998)*, or the angiotensin II–induced increase in aortic fibronectin mRNA *(Himeno et al., 1994).*

Simon and Altman (1992) infused angiotensin II (200ng/kg/min, intraperitoneally; i.p.) into normotensive Sprague-Dawley rats for 7-10 days. The systolic blood pressure of the rats was unchanged 24 hours after the infusion

commenced but was increased after 7-10 days. Protein synthesis, measured by incorporation of [^{35}S] –methionine, was increased after 24 hrs of treatment; in the aortic media, portal vein and bladder wall, but not in the diaphragm. In contrast [^{3}H]-thymidine incorporation into the aortic media was inhibited after 24 hrs of angiotensin II treatment. By 7-10 days of angiotensin II treatment protein synthesis had returned to baseline while DNA synthesis in the aorta was altered in a bi-modal fashion; in 53% of the rats it was still inhibited while in 26% there was a two- threefold increase in synthesis *(Simon and Altman, 1992)*.

In a similar study, angiotensin II (200 ng/kg/min, s.c.) was infused for 2 weeks after induction of a balloon-catheter injury in rats *(Daemen et al., 1991)*. Systolic pressure rose by more than 40 mmHg and was associated with significant increases in DNA synthesis in the injured artery, as well as a near doubling of the neointimal cross-sectional area *(Daemen et al., 1991)*.

Vascular growth and hypertrophy observed in angiotensin II–infused, hypertensive rats appears to be mediated via the AT_1 receptor, in accord with data obtained from VSMC cultures. In angiotensin II-infused hypertensive rats (120 ng/kg/min, s.c., 21 days), administration of the AT_1 receptor antagonist losartan normalised the arterial pressure and, among other indices of growth, reduced the media-lumen ratio of coronary, renal arcuate, mesenteric, and femoral arteries *(Li et al., 1998)*. In comparison, the AT_2 receptor antagonist PD123319 did not reduce the cardiovascular growth in this experimental model of hypertension.

In addition to hypertrophy and hyperlasia, angiotensin II has been shown to be involved in the formation of new vessels. Addition of angiotensin II into the avascular rabbit cornea facilitated the activation of pre-existing collateral vascular pathways as well as new vessel formation in 85% of the total corneas treated *(Fernandez et al., 1985)*. Further, treatment of normotensive and hypertensive animals with captopril reduced the number of small arterioles in the cremaster muscle *(Wang and Prewitt, 1990)*.

In summary, systemic infusion of angiotensin II results in hypertension that is accompanied by both hypertrophy and hyperplasia of VSMCs obtained from systemic resistance arteries. Additional evidence suggests that slow-pressor angiotensin II-induced hypertension may be a consequence of the hypertrophy/hyperplasia of the VSMCs and subsequent increases in the wall:lumen ratio of vessel walls. Conversely, blockade of the renin-angiotensin system in hypertension results in a reversal of associated vascular growth that is not seen with other antihypertensive therapies despite similar reductions in blood pressure.

1.4.1.3 Trophic effects of angiotensin II in vivo (in the kidney)

The functional consequences of vascular changes in the kidney and the role these changes play in hypertension have been well described. Likewise, a link between angiotensin II-induced hypertrophy/hyperplasia of VSMCs, and resistance vessel hypertrophy in hypertension has also been discussed. However, to date no studies have directly investigated the trophic actions of angiotensin II within the kidney *in vivo*. An assessment of the role that angiotensin II plays in the regulation of renal or systemic cellular growth is difficult to make *in vivo*, due to the heterogeneity of cell types and complex physiological actions and interactions of angiotensin II in the kidney. There is a great deal of indirect evidence implicating important roles for angiotensin II in regulating cellular growth, and in particular vascular growth, within the kidney and consequently in disease states such as hypertension. One important observation is that the renin angiotensin system is activated in disease states involving compensatory renal growth *(Ibrahim et al., 1997; Johnston et al., 1993)*. However, there is evidence in some experimental models of hypertension that the actions of angiotensin II on renal vascular growth *in vivo* vary from the effects observed in the systemic vasculature.

1.4.1.3.1 Angiotensin II blockade – effects in the kidney

Most research into the effects of angiotensin II blockade in the kidney has been performed using the SHR as a model of hypertension. However, whilst blockade of the renin-angiotensin system in the SHR prevents the development of vascular hypertrophy in non-renal beds, the effect on the renal vasculature in these animals is more controversial. In contrast to other vascular beds it appears that blockade of the renin-angiotensin system in the SHR does not prevent the renal vascular hypertrophy, but does increase the lumen of the afferent arteriole and attenuates hypertension. Thus it appears that the renal vasculature of the SHR is unique in its response to ACE inhibition. The main findings of the experiments investigating the renal effects of angiotensin II blockade in the SHR will be summarised below.

SHR have low to normal plasma renin activity and are generally not considered to be a renin-dependent model of hypertension, yet despite this, inhibitors of the renin-angiotensin effectively lower blood pressure, normalise RVR, GFR and RBF *(Nakamura and Johns, 1995; Henrich and Levi, 1991; Kitami et al., 1989)*. This evidence suggests that angiotensin II plays a role in the renal abnormalities and the development of the hypertension in this strain of rat. This has been illustrated by the effects of infusing an AT_1 receptor antagonist intrarenally into uninephrectomised SHR rats for 48 hours, which resulted in a significant reduction in arterial pressure *(Wood et al., 1993)*. However, infusing the same dose intravenously had no effect on arterial

pressure, thus suggesting that endogenous angiotensin II, acting within the kidney plays an important role in this model of genetic hypertension *(Wood et al., 1993)*.

1.4.1.3.2 Angiotensin II blockade - renal vessels in the SHR

In the SHR, increased RVR results due to narrowing of the afferent arteriole, and this in turn contributes to the development of hypertension *(Norrelund et al., 1994)*. If ACE inhibitors are administered from an early age, elevation of RVR and the development of hypertension can be prevented *(Lee et al., 1991; Li and Jackson, 1989)*.

In the systemic arteries of the SHR vessel wall (medial) hypertrophy or remodelling occurs in vessels of all sizes from the aorta and resistance arteries down to the arterioles, when compared to the normotensive control WKY rat strain *(Rizzoni et al., 1995; Clozel et al., 1989; Owens et al., 1988; Owens, 1987; Mulvany et al., 1985)*. Further, inhibition of the renin-angiotensin system with ACE inhibitors or angiotensin receptor antagonists reverses the vessel hypertrophy in these systemic beds *(Rizzoni et al., 1995; Lee et al., 1991; Owens, 1987)*.

In accord with systemic vessels, medial hypertrophy occurs in the main renal, arcuate and interlobular arteries SHR kidneys *(Kett et al., 1996; Kett et al., 1995; Smeda et al., 1988a; Smeda et al., 1988b; Folkow et al., 1971)*. However, treatment with an ACE inhibitor (enalapril), or AT_1 blockade does not attenuate the renal vessel hypertrophy despite a reduction in arterial pressure *(Kett et al., 1996; Kett et al., 1995)*. These results suggest that neither arterial pressure nor angiotensin II play major roles in the renal vessel hypertrophy seen in these animals.

The afferent arteriole of the SHR, arguably the major site of resistance in the kidney, shows a reduction in lumen diameter compared to the normotensive WKY *(Skov et al., 1992; Gattone et al., 1983)*. The reduction in lumen diameter in the afferent arterioles of the SHR is interestingly, not associated with vessel wall hypertrophy, rather wall mass is often reduced when compared to WKY control rats *(Skov et al., 1992; Gattone et al., 1983)*. In contrast to the other renal vessels, chronic ACE inhibition from 4 weeks of age results in an increase in afferent arteriole lumen diameter in both SHR and WKY rats *(Notoya et al., 1996; Kimura et al., 1991)*. Interestingly though Notoya *et al.* (1996), showed that lisinopril treatment led to an increase in external diameter and media cross-sectional area in both strains, which contradicts findings on systemic vascular hypertrophy *(Notoya et al., 1996)*. Thus, vascular wall remodelling and not hypertrophy appears to be responsible for the reduction in lumen diameter in the afferent arteriole of the SHR. Further, it may be that in the renal afferent arteriole of the SHR and WKY, angiotensin II exerts a growth

inhibitory effect which is reversed upon ACE inhibition, leading to the increase in afferent wall cross-sectional area demonstrated with lisinopril treatment in both SHR and WKY rat strains *(Notoya et al., 1996)*. In contrast to the afferent arteriolar changes, efferent arteriole diameters are not different between SHR and WKY rats, and ACE inhibition does not affect efferent arteriole diameter *(Notoya et al., 1996; Kimura et al., 1991)*.

The structural effects of ACE inhibition on the renal vasculature of the SHR have been confirmed *in vitro* in a perfused kidney preparation following ACE inhibition with perindopril from 4-10 weeks of age *(Bergstrom et al., 1998)*. In this study, renal vessel lumen characteristics in maximally dilated kidneys, perfused with artificial plasma, were assessed as an indicator of the functionally significant, structural changes to renal vascular lumen dimensions. The study concluded that chronic ACE inhibition increased the average renal vessel lumen diameter in SHR, predominantly in the preglomerular vessels, and additionally decreased *in vivo* renal vasoconstrictor responsiveness, further suggesting that in SHR, renal vascular remodelling rather than hypertrophy or hyperplasia, may play a role in the antihypertensive effects of chronic ACE inhibition *(Bergstrom et al., 1998)*.

1.4.1.3.3 Trophic effects of angiotensin II in other renal cells

A large proportion of the evidence for a role of angiotensin II in renal vascular growth and remodelling comes from studies looking at the effect of ACE inhibitors on the progression of renal disease. Anderson and colleagues *(Anderson, 1989a; Anderson, 1989b; Anderson, 1988)* showed that treatment of systemic hypertension with ACE inhibitors reduced renal injury (measured by degree of histological glomerular damage and proteinuria), independent of the effects of blood pressure. In rats with subtotal nephrectomy, high dose enalapril (200 mg/L drinking water) halted compensatory renal hypertrophy and glomerular sclerosis to a greater extent than low dose enalapril (50 mg/L drinking water) *(Ikoma et al., 1991)*. This occurred despite equivalent reductions in glomerular capillary hydraulic pressure. In a similar study, treatment with enalapril eight weeks after subtotal nephrectomy, reversed the substantial glomerular injury, proteinuria and hypertension that was present. (for review see *(Taal and Brenner, 2000)*.

Following unilateral nephrectomy in rats, a reduction in renal mass leads to adaptive hypertrophy of the remaining glomeruli, as well as an increase in single nephron GFR and capillary hydrostatic pressure, all brought about by a relative afferent arteriolar vasodilatation and efferent vasoconstriction *(Hostetter et al., 1981)*. Pre-treatment of normotensive rats with an ACE inhibitor (enalapril) for 4 weeks prior to

unilateral nephrectomy resulted in significant inhibition of compensatory renal growth *(Wight et al., 1990)*. In untreated nephrectomised animals, both protein and DNA content of the remaining kidney increased suggesting both a hyperplastic and hypertrophic growth response. However, treatment with enalapril inhibited only the hyperplastic growth response, providing further evidence for involvement of angiotensin II in renal growth *(Wight et al., 1990)*.

1.4.1.3.4 Systemic infusion of angiotensin II – trophic effects in the kidney

There is considerable evidence to suggest that angiotensin II induces vascular hypertrophy that is partly due a non-pressor mechanism. As mentioned in section 1.4.1.2 earlier, Griffin and colleagues (1991) demonstrated that while angiotensin II-induced hypertension was reversed by treatment with hydralazine, it failed to prevent the hypertrophy of the mesenteric vessels that was associated with the hypertension. In another study by Johnson and colleagues (1992), chronic, low dose infusion of angiotensin II produced a slowly developing, moderate hypertension in rats. The hypertension developed after 3 days infusion of angiotensin II (200 ng/kg/min; s.c.), persisted for the remaining 11 days of infusion and produced a mean systolic pressure of 156-172 mmHg *(Johnson et al., 1992)*. The renal vasculature of 4 out of 6 rats infused with angiotensin II demonstrated focal fibrinoid necrosis of the afferent arteriole and other small arteries by day 14. Additionally, by day 14 of infusion 4.74% of VSMCs in the small arteries of angiotensin II infused rats stained positive for PCNA (proliferating cell nuclear antigen), compared with only 1.45% of cells in vehicle infused rats *(Johnson et al., 1992)*. The moderate hypertension produced in the study *(Johnson et al., 1992)* was also associated with decreased renal renin protein, a mild increase in glomerular cell proliferation, a marked increase in smooth muscle cell-like proteins in the glomerular mesangial regions, and increased positive staining for aminopeptidase A (an enzyme that inactivates angiotensin II) in glomeruli *(Johnson et al., 1992)*. Tubular cell proliferation and injury were also documented.

1.4.1.3.5 Intrarenal infusion of angiotensin II – trophic effects in the kidney

A previous study from our laboratory infused angiotensin II at 0.5 ng/kg/min directly into the renal artery of uninephrectomised dogs for 28 days *(Fitzgerald et al., 1997b*; Figure 1.4). This dose of angiotensin II did not cause a rise in MAP initially, yet by day 7, MAP was significantly increased and remained elevated for a further 21 days with an average increase of 11 mmHg. Renal blood flow was significantly decreased

24 hours after the commencement of the infusion but recovered to pre-infusion levels during days 7-28 of infusion.

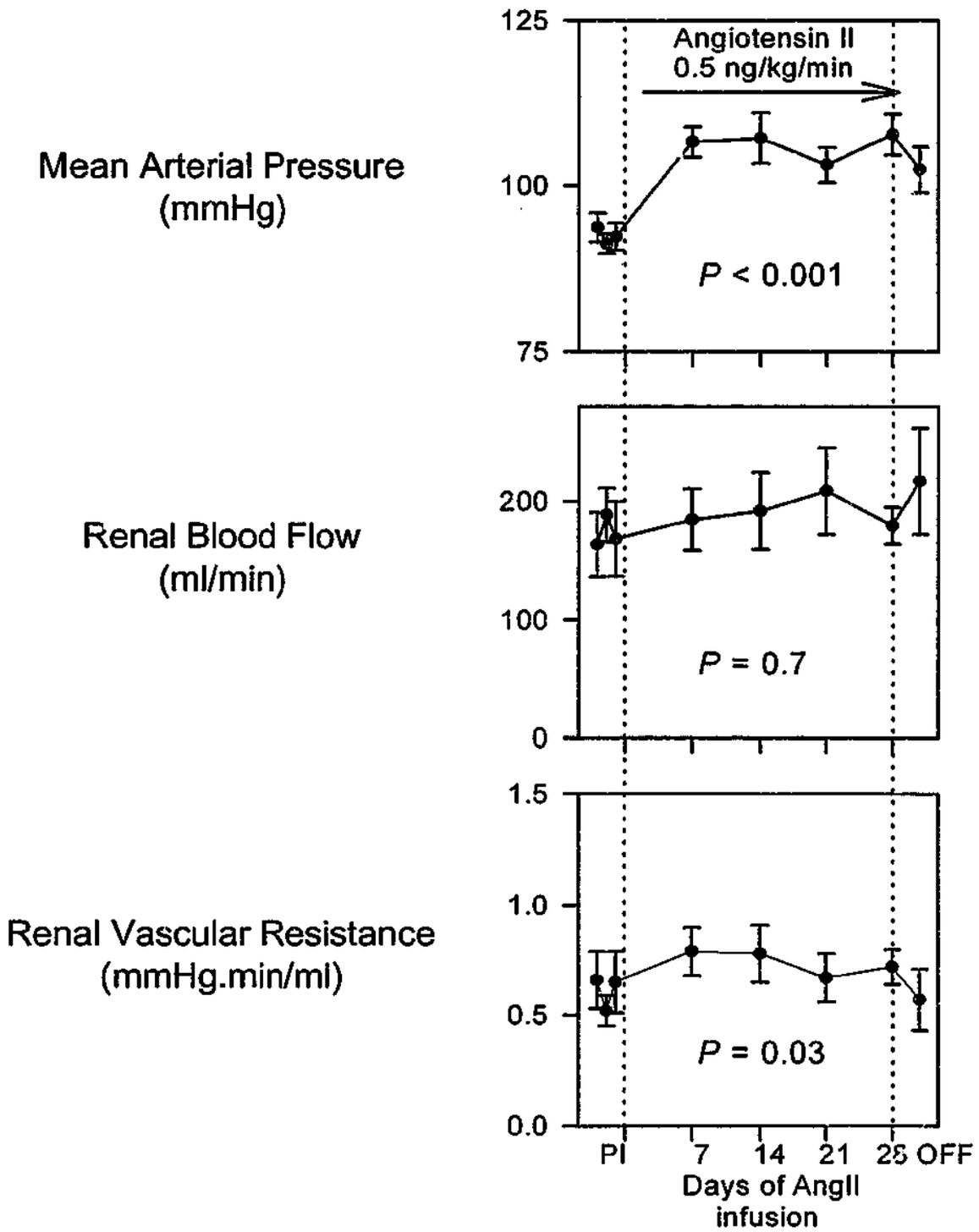


Figure 1.4: The effect of angiotensin II (0.5 ng/kg/min) infused continuously into the renal artery of dogs for 4 weeks on mean arterial pressure, renal blood flow, and renal vascular resistance. Values represented as mean ± SEM, PI = pre-infusion levels of variables, OFF = levels of haemodynamic variables, 24 hours after cessation of the infusion. (Figure modified from Fitzgerald *et. al.* (1997).

Infusion of angiotensin II into the renal artery also resulted in an increase in central venous pressure and haematocrit, but no change in bodyweight, thus providing equivocal evidence at best, for sodium and water retention. Other renal actions that could have contributed to the hypertension include renal vasoconstriction, however there was only a slight increase in RVR during the infusion. The study concluded that the rise in arterial pressure was primarily due to effects of angiotensin II within the kidney, which demonstrated that increased intrarenal angiotensin II within the renal vasculature could result in hypertension. Furthermore, it was speculated that the hypertension might have been, in part, due to the vascular growth promoting effects of angiotensin II within the kidney, although this hypothesis was not directly measured in the study.

A functional test, developed by Gothberg and Folkow (1983) has been used to detect structural remodelling of the renal vascular bed in angiotensin II-dependent renal clip hypertension. In brief, the technique involves constructing pressure-flow and pressure-GFR relationships in maximally dilated kidneys perfused with colliod solution (iso-osmotic with plasma). They demonstrated that the pressure-GFR relationship was shifted to the right in the renal hypertensive rats, suggesting a rise in the pre- to post-glomerular resistance ratio which would result in a lower GFR for any given pressure in the hypertensive rats *(Gothberg and Folkow, 1983)*. Whilst the isolated perfused kidney model is a good functional test for vascular lumen changes in the kidney, the specific cellular and morphometric changes, if any, occurring in the renal vasculature in this model of hypertension still need to be investigated.

In an acute study by Rosenberg and Hostetter (1993), angiotensin II was infused directly into the kidney of rats at a dose of 50 ng/kg/min for one hour. The infusion increased expression of early growth response genes *egr-1* and *c-fos* but not *c-myc*. Mean arterial pressure was unaffected by the infusion, and RBF decreased only in the kidney that received angiotensin II. Rosenberg and Hostetter (1993) concluded, that the stimulation of the growth response genes could not be attributed to the trophic actions of angiotensin II alone, as infusion of another vasoconstrictor, noradrenaline, produced a similar increase in early gene expression, indicating vasoconstriction of the renal vessels could have stimulated early gene expression *(Rosenberg and Hostetter, 1993)*.

In summary, direct *in vitro* and *in vivo* evidence points to an important role of angiotensin II in renal growth, particularly in disease states such as hypertension. Possibly the most important, functional role that angiotensin II-induced renal proliferation plays is via its effects on VSMC mass and replication. Potentially, angiotensin II stimulated growth or remodelling of the vasculature in the kidney could lead to an increase in contractile mass and reduction in renal vessel lumen diameter. If

this were the case then hypertrophy or remodelling of the intra renal vessels around a smaller lumen could lead to an increase in RVR much like main renal artery stenosis does, and thus be an initiating factor in the development of the hypertension (Figure 1.3).

1.4.2 Molecular and cellular pathways of angiotensin II-induced growth

Acutely, angiotensin regulates vasomotor tone and fluid homeostasis. However, the previous section of this literature review suggests the possibility of a longer, proliferative action of angiotensin II on many different cells types in the kidney, and especially in VSMCs. *In vitro*, the trophic action of angiotensin II on cell cultures is intimately linked with a cascade of intracellular signals and interaction with numerous growth factors in an autocrine and paracrine fashion. So how might angiotensin II act to induce cellular proliferation and hypertrophy within the kidney? There are three possible modes of angiotensin II-induced growth:

i) Direct mechanical stimulation of vasoactive cells such as (ie; vasoconstriction of smooth muscle or mesangial cells
ii) Stimulation of growth factors (indirect stimulation of growth)
iii) Interaction with growth factors (direct stimulation of growth)

In the following section, the diverse signal transduction pathways of angiotensin II-induced cellular proliferation and hypertrophy, and its interaction with growth factors will be reviewed.

1.4.2.1 Signal transduction

The renin-angiotensin system impacts on nearly every organ system in the body through the angiotensin II molecule. Virtually all haemodynamic and proliferative effects of angiotensin II are mediated through the AT_1 receptor, yet in different tissues angiotensin II exerts different actions such as vasoconstriction, sodium absorption, increased thirst and aldosterone release. The responses elicited by angiotensin II on different tissues in the body are likely to be mediated by activation of multiple second messenger systems and signal transduction pathways in a tissue specific manner. In the next section the pathways of angiotensin II-induced growth within the kidney will be reviewed with particular emphasis on the signal transduction and interaction with growth factors.

Activation of the AT_1 receptor by angiotensin II results in a rapid increase in intracellular inositol phosphates, in particular, inositol 1,4,5-triphosphate (IP_3), and 1,2-

diacylglycerol (DAG) in VSMCs. The increase in the second messenger molecules result in mobilisation of intracellular calcium stores and activation of protein kinase C (PKC) isoforms leading to a cascade of protein phosphorylations that dictate the response of the cell *(Griendling et al., 1997)*. More recently, the AT_1 receptor has been shown to share similar properties with cytokine receptors such as interleukin-2, and interferon-α and -γ *(Berk and Corson, 1997)*. Similar to the cytokine receptors the AT_1 receptor activates phospho lipase C (PLC), mobilises calcium stores, activates PKC, stimulates phosphorylation of proteins on tyrosine residues, activates mitogen-activated protein (MAP) kinases (including extracellular signal-related kinase; ERK1 and ERK2) and induces proto-oncogene expression (such as *c-fos*) *(Griendling et al., 1997; Berk and Corson, 1997; Du et al., 1996; Huckle et al., 1992; Huckle et al., 1990; Taubman et al., 1989)*.

Generally, the cytokine receptors mediate tyrosine phosphorylation via protein kinases such as Src-related kinases, and the Janus family of kinases (JAK and TYK) *(Darnell et al., 1994; Pleiman et al., 1993)*. The Janus family of kinases are involved in the activation of mRNA expression of early growth response genes such as *c-fos* and c-jun. In cultured VSMCs angiotensin II has been shown to rapidly activate JAK2 and TYK2, and stimulate tyrosine phosphorylation of another important transcription factor; signal transducers and activators of transcription (STAT113) *(Marrero et al., 1995)*. Touyz RM (1997) found that angiotensin II regulates smooth muscle cell growth and contraction via tyrosine kinase-dependent signalling pathways *(Touyz and Schiffrin, 1997)*. Additionally, angiotensin II activated STAT19 in cultured neonatal cardiac fibroblasts *(Bhat et al., 1994)*, suggesting that activation of these tyrosine kinases could play a role in angiotensin II mediated cellular growth.

MAP kinases make up a superfamily of threonine and serine protein kinases that are involved in cell growth and differentiation *(Griendling et al., 1997)*. Activation of MAP kinases by angiotensin II in VSMC cultures is transient, peaking at 2-5 minutes. On the basis of studies using tyrosine kinase inhibitors it appears that angiotensin II regulated tyrosine kinases may be involved in responses to angiotensin II such as vasoconstriction, proto-oncogene expression, and protein synthesis *(Griendling et al., 1997; Duff et al., 1995)*.

Other signal transduction pathways that angiotensin II stimulates include the Src family of kinases that participate in growth factor signal transduction, in particular c-src which is activated by angiotensin II in vascular smooth muscle cells (for review see *(Berk and Corson, 1997)*. Phosphorylation of another tyrosine kinase focal adhesion kinase (FAK), also occurs in VSMC exposed to angiotensin II. The FAK protein has been localised to focal adhesion complexes which act as specialised sites of cell adhesion and cause phosphorylation of talin and paxillin which may be involved in

regulation of cell morphology and movement (for review see *Griendling et al., 1997).* Longer term signalling events include activation of the nicotinamide adenine dinucleotide (NADH/NADPH) oxidase enzyme *(Griendling et al., 1997).* With respect to cell growth events, when the NADH/NADPH pathway is inhibited then angiotensin II stimulated protein synthesis is also inhibited, indicating a role for this oxidase in the growth response to angiotensin II *(Griendling et al., 1997).*

In cultured glomerular mesangial cells, angiotensin II as well as endothelin and arginine vasopressin (AVP) enhance protein tyrosine phosphorylation via PKC-dependent and PKC-independent pathways *(Force et al., 1991).* Stimulation of the same cells with unrelated agonists such as epidermal growth factor (EGF) resulted in tyrosine phosphorylation of similar proteins, suggesting common intermediates in the signalling pathways for growth factors and mitogenic peptides such as angiotensin II *(Force et al., 1991).* The activation of multiple signal transduction pathways via coupling of angiotensin II to the AT_1 receptor demonstrates the ability of angiotensin II to act more like a cytokine and regulate multiple cellular functions.

1.4.2.2 Interaction with early growth response genes

The activation of a variety of signal transduction pathways by angiotensin II and the AT_1 receptor is ultimately linked to a cascade of cellular events that includes stimulation of early growth response genes and growth factors. The number of growth related genes that have been found to interact with angiotensin II are expanding. Some immediate early genes that are activated by angiotensin II include; *c-fos, c-jun, c-myc, JunB, egr-1, and cMGI.*

The mitogenic actions of angiotensin II have, arguably, the most significant effects in the VSMCs of the blood vessels. In this respect, angiotensin II-induced growth in these vessels may play an important role in the normal physiological function of the vessel, and also play a pathophysiological role in hypertension. As mentioned previously, in situations such as essential hypertension, alterations in the renal vasculature due to growth, hypertrophy and remodelling could contribute to the development of the hypertension. It is for this reason that we are most interested in the growth effects of angiotensin II on VSMCs, particularly within the kidney. In the following section, evidence of immediate early gene stimulation by angiotensin II will be presented.

1.4.2.2.1 Angiotensin II-induced stimulation of immediate early gene expression in VSMCs

Exposure of rat aortic VSMC cultures to 10^{-7} M angiotensin II resulted in increases in both *egr-1* mRNA, peaking at 30 minutes, and protein, peaking at 60 minutes *(Sachinidis et al., 1992)*. The upregulation of *egr-1* gene and protein expression was abolished by the non-peptide AT_1 receptor antagonist EXP3174, by preventing the intracellular increase of inositolphosphates. These findings further suggest that the response of *egr-1* to angiotensin II is mediated by the phosphoinositide signaling system *(Sachinidis et al., 1993; Lyall et al., 1992)*.

Expression of the *c-fos* gene is also stimulated by angiotensin II, and mediated by activation of PKC and Ca^{2+} mobilisation. Angiotensin II stimulation of VSMC cultures results in rapid induction of *c-fos* and *c-jun* mRNA *(Naftilan et al., 1990)*, and an increase in cell size and protein content *(Millet et al., 1992)*. The ACE inhibitor, perindopril reduced neointimal hyperplasia in injured rabbit arteries and this was also associated with a 50% reduction in *c-jun* expression and a 45% reduction in *c-fos* expression *(Van Belle et al., 1995)*. Angiotensin II exerted a mitogenic effect on cultured VSMCs from SHR, but its effect was weak on cultured cells from the normotensive WKY rats *(Paquet et al., 1990)*. Further, PLC activation and *c-fos*, *c-myc* proto-oncogene expression induced by angiotensin II in these cells was considerably greater in the SHR cells, but may have been linked to a greater number of angiotensin II receptors in the SHR cells *(Paquet et al., 1990)*. In accord with cultures derived from arteries, cultured smooth muscle cells from human saphenous vein illicit a dose-dependent increase in *c-fos* expression and DNA synthesis in response to angiotensin II which is associated with an increase in intracellular calcium, and is blocked by the AT_1 receptor antagonist losartan *(Patel et al., 1996)*. In rat VSMC cultures the angiotensin II stimulated increase in DNA synthesis, cell proliferation and *c-fos* mRNA expression was blunted by pre-incubation of the cells in the dihydropyridine calcium antagonist isradipine *(Ko et al., 1993)*. Conversely, expression of *egr-1* mRNA was not affected by pre-incubation of cells in isradipine *(Ko et al., 1993)*.

Thus *in vitro*, angiotensin II-induced smooth muscle cell proliferation is closely associated with induction of a variety of immediate early genes particularly *c-fos* and *egr-1*. Regulation of expression of these genes by angiotensin II *in vivo* may provide an early indication of vascular growth.

1.4.2.2.2 Angiotensin II-induced expression of early genes in other cells

In a different population of contractile cells, isolated adult rat cardiomyocytes, angiotensin II stimulates expression of both *egr-1* and *c-fos* genes *(Iwami et al., 1996;*

Neyses et al., 1993). Furthermore the increase in gene expression is not seen when cardiomyocytes are incubated with noradrenaline or endothelin, suggesting that angiotensin II induces early gene expression in cardiomyocytes via mechanisms that do not include mechanical stimulation *(Iwami et al., 1996)*. However, conflicting evidence shows that repetitive mechanical stimulation of cardiomyocytes also increased *egr-1* and *c-fos* gene expression *(Kubisch et al., 1993)*. The increase in expression of *egr-1* and *c-fos* in rat cardiomyocytes by angiotensin II can also be blocked by treatment with nisoldipine, affecting the signal transduction pathway of angiotensin II activation (ie; by interfering with PKC) *(Grohe et al., 1994)*.

In neonatal cardiac fibroblasts, addition of 100 mM angiotensin II resulted in an upregulation of *c-fos* and *egr-1* mRNA after 15 minutes, reaching maximal expression at 45 minutes of incubation with angiotensin II *(Sharma et al., 1994)*. Expression of *c-jun* was induced after 45 minutes of angiotensin II treatment and remained elevated for up to 2 hours. Despite the immediate early gene stimulation in the fibroblast by angiotensin II, there was no increase in cell proliferation and growth measured by DNA and protein content *(Sharma et al., 1994)*.

Angiotensin II-stimulated expression of early growth response genes is not restricted to vascular smooth muscle cultures. In rat brain tissue, intracerebroventricular injections of angiotensin II (up to 100 ng) induced a dose-dependent expression of c-Fos and Egr-1 protein in the subfornical organ, the median pre-optic area, the paraventricular nucleus and the supraoptic nucleus of the hypothalamus *(Lebrun et al., 1995)*. These areas are involved in the central osmoregulatory and neuroendocrine actions of angiotensin II *(Lebrun et al., 1995)*. Further to this, administration of losartan, the AT_1 receptor antagonist, 5 minutes prior to angiotensin II injection prevented the angiotensin II-induced immediate early gene and protein expression *(Lebrun et al., 1995)*. Peripheral administration of angiotensin II also induces Fos protein in similar regions of the brain (for review see *Rowland et al., 1996)*. Other cells that undergo angiotensin II-induced immediate early gene expression include adrenal glomerulosa cells *(Clark et al., 1992)*, adrenal cortical and medullary cells, hepatocytes, and intestinal epithelial cells (for review see *(Huckle and Earp, 1994)*.

1.4.2.2.3 Angiotensin II-induced early gene expression in renal cells

Cellular growth stimulated by angiotensin II appears to be consistently associated with upregulation of early gene expression in a variety of cell culture systems, providing insight into the chain of events that interact to initiate cell growth. Investigation into angiotensin II-induced stimulation of early gene expression in renal

cell cultures is possibly of greatest interest because of the potential role for angiotensin II stimulated renal growth *in vivo* as a contributing factor in the onset and maintenance of hypertension.

Angiotensin II activation of early immediate genes in the kidney has been reported in a number of renal cell cultures. In the only study of cultured renal arteriolar smooth muscle cells, Dubey *et al.* (1992) found that incubation of renal arteriolar cells with angiotensin II (10^{-5} M) for 30 minutes resulted in an increase in *c-fos* mRNA expression that lasted for one hour. Likewise, angiotensin II induces only slight increases in cellular proliferation and *egr-1* expression in cultured glomerular mesangial cells *(Rupprecht et al., 1992)*. In contrast however, a study by Schulze-Lohoff *et al.* (1993) found that angiotensin II did not stimulate *egr-1*, *c-fos* or *c-myc* expression in these cells, yet AVP induced maximal expression of these genes after 30 minutes *(Rupprecht et al., 1994; Schulze-Lohoff et al., 1993)*.

In non-contractile renal cell cultures such as murine proximal tubular cells, angiotensin II stimulated an increase in both *c-fos* and *c-myc* expression *(Wolf and Neilson, 1990)*. Also glomerular endothelial cell cultures respond to angiotensin II with a moderate but significant increase in proliferation, and upregulation of *erg-1* gene expression via the AT_1 receptor, protein kinase 2 pathway *(Wolf et al., 1996)*.

To date, demonstration of direct angiotensin II-induced early gene expression in the kidney, *in vivo*, has been reported by only one group, Rosenberg and Hostetter (1993). As described earlier in section 1.4.1, they study infused angiotensin II directly into one kidney of anaesthetised rats for one hour. RBF was measured by para-aminohippuric acid (PAH) clearance and MAP recorded. After one hour of angiotensin II infusion renal blood flow and GFR were decreased only in the infused kidney, and mean arterial pressure was unchanged. *(Rosenberg and Hostetter, 1993)*. Yet RNA isolated from the whole kidney exhibited an increase in expression of *c-fos* and *egr-1* genes, but not *c-myc* when compared to the contralateral control kidney from the same animal *(Rosenberg and Hostetter, 1993)*. Infusion of noradrenaline in the same manner also produced an increase in early gene expression in the whole kidney, raising the possibility that angiotensin II may stimulate early gene expression via a vasoconstrictive action on the kidney and not act directly as a growth factor *(Rosenberg and Hostetter, 1993)*.

In summary, the upregulation of expression of immediate early genes by angiotensin II could provide a useful marker of early growth events *in vivo*. Additionally, clarification of these early growth events mediated by angiotensin II *in vivo*, provides further evidence for a direct role of angiotensin II-induced cellular growth in the latter development of hypertension.

1.4.2.3 Trophic effects of angiotensin II – interaction with growth factors

In addition to promoting the transcription of early growth response genes, which primarily function as transcriptional regulators, angiotensin II has also been shown to increase levels of a number of growth factors and their receptors. In general, it appears that angiotensin II has a synergistic relationship with some of the classical growth factors such as TGF-β1, bFGF, and PDGF, by augmenting growth factor stimulated cellular hyperplasia and hypertrophy in cultured VSMCs, as well as stimulating release of some growth factors from cultured cells. Again, most of the evidence in support of an interaction between angiotensin II and these growth factors comes exclusively from *in vitro* studies of VSMCs. *In vivo*, very few studies have investigated the interaction between angiotensin II-induced vascular hypertrophy and stimulation of these growth factors.

1.4.2.3.1 Angiotension II interaction with growth factors in VSMC

The trophic effects of angiotensin II is thought to be mediated in part by production of growth factors such as TGF-β1, PDGF-A and bFGF, which act in an autocrine or paracrine fashion to enhance cell growth. Indeed, the endogenous synthesis of all three growth factors (TGF-β1, PDGF-A and bFGF) have been demonstrated in VSMC cultures and further, production is stimulated by addition of angiotensin II *(Itoh et al., 1993; Gibbons et al., 1992; Stouffer and Owens, 1992; Naftilan et al., 1989a)*.

The action of angiotensin II to induce hypertrophy or hyperplasia of cultured VSMCs is believed to be the result of the specific induction of either proliferative (enhanced cell division) or anti-proliferative (enhanced cell hypertrophy but not division) growth factors, particularly bFGF and TGF-β1 respectively *(Koibuchi et al., 1993)*. Application of TGF-β1 to VSMC cultures decreases both basal and induced DNA synthesis but increases protein and RNA synthesis, indicating a hypertrophic, non-proliferative role for this growth factor *(Itoh et al., 1993; Koibuchi et al., 1993; Gibbons et al., 1992; Naftilan et al., 1989a)*. In contrast, exogenous bFGF has been demonstrated to increase DNA synthesis VSMCs, dose-dependently *(Itoh et al., 1993)*, suggesting that TGF-β1 has an anti-proliferative, hypertrophic effect in VSMC cultures whilst bFGF is mitogenic. This is further confirmed by blockade of these factors with anti-sense oligonucleotides. Blockade of TGF-β1 with anti-sense oligonucleotides results in a significant increase in angiotensin II-induced DNA synthesis *(Itoh et al., 1993)*. This increase was then blocked by addition of antisense oligonucleotides to bFGF, providing further evidence for a proliferative role of autocrine bFGF *(Itoh et al., 1993)*.

More recent studies have confirmed these earlier findings. For example, angiotensin II stimulated-collagen expression in cultured human VSMCs was shown to be associated with a 6-fold increase in TGF-β1 protein *(Ford et al., 1999)*. The increase in collagen mRNA and TGF-β1 protein was blocked by an AT_1-receptor antagonist (losartan), suggesting that angiotensin II stimulates collagen production in human VSMC via the AT_1 receptor and an autocrine loop inducing TGF-β1 *(Ford et al., 1999)*. This study also demonstrated that stimulation of the autocrine loop of TGF-β1 required tyrosine phosphorylation *(Ford et al., 1999)*. Likewise, in rat aortic vascular smooth muscle cell cultures, a study by Hamaguchi *et al.* (1999) confirmed that angiotensin II mediated increases in TGF-β1 mRNA expression, and accompanying cellular hypertrophy involves activation of (ERK) and activator protein –1 (AP-1).

1.4.2.3.2 Angiotensin II interaction with growth factors in renal cells

In accord with the interaction between angiotensin II and TGF-β1 on vascular smooth muscle cells in culture, there is evidence of a similar relationship within the kidney. Expression of the TGF-β1 gene has been demonstrated in normal glomeruli, in regenerating tubules, and in the developing rat kidney *(Choi et al., 1997; Basile et al., 1996; Rogers et al., 1993; MacKay et al., 1990)*. Additionally, exogenous TGF-β1 induces hypertrophy of rat mesangial cells in culture and inhibits proliferation *(Choi et al., 1993)*. Co-ordinate expression of TGF-β type II (but not type I) receptor immunoreactivity, and renin has been demonstrated in developing renal VSMC and mature juxtaglomerular cells *(Liu and Ballermann, 1998)*. TGF-β1 production and action is enhanced in response to renal injury. In neo-natal rats subjected to three days of unilateral ureteral obstruction, increased TGF-β1 expression was attenuated by treatment with AT_1 receptor antagonist *(Yoo et al., 2000)*.

An interaction between TGF-β1 and the renin-angiotensin system has also been implicated in renal fibrosis. In the human kidney, overproduction of TGF-β1, together with activation of the RAS has been demonstrated in glomerulonephritis, diabetic nephropathy, and hypertensive glomerular injury *(Border and Noble, 1998)*. Inhibition of the renin-angiotensin system reduces over-expression of TGF-β1 and reverses renal fibrosis (for review see *Border and Noble, 1998)*.

Basic fibroblast growth factor is a potent angiogenic and mitogenic peptide growth factor, and a prime candidate to fill the role as a proliferative growth factor in vascular growth *(Goldfarb, 1996; Itoh et al., 1993)*. Reported to be important in cellular differentiation during development, aFGF and bFGF mRNA and protein expression is found abundantly in embryonic rat and human tissues, including in the kidney and

smooth muscle associated with arteries *(Gonzalez et al., 1995; Gonzalez et al., 1990).* In the mid-trimester human fetus bFGF protein was present in arterial smooth muscle cells and bFGF message was widely expressed within the fetal kidney, specifically within the renal tubular epithelia, and mesangial cells of the glomerulus. *(Gonzalez et al., 1995).*

Increased bFGF mRNA levels are first detectable at 30 minutes after incubation of rat aortic smooth muscle cells with 10^{-7}M angiotensin II, and maximum expression occurs at 8 hours *(Peifley and Winkles, 1998).* As demonstrated for TGF-β1, the angiotensin II-induced increase in bFGF levels appears to be mediated via the AT_1 receptor and tyrosine kinase pathway as blockade of the receptor with losartan, or genistein, a tyrosine kinase inhibitor, abolishes the increase in bFGF expression *(Samain et al., 2000; Peifley and Winkles, 1998).* Also, angiotensin II does not stimulate bFGF levels maximally if RNA synthesis or protein synthesis is inhibited, indicating that there is a transcriptional control mechanism in angiotensin II-mediated bFGF mRNA induction, involving synthesis of new proteins (probably transcription factors) *(Peifley and Winkles, 1998).* Angiotensin II stimulation of TGF-β1 is also dependent on synthesis of new proteins *(Gibbons et al., 1992).*

In cultured proximal tubule epithelial cells from the rat kidney, exogenous bFGF stimulates DNA synthesis in a dose-dependent manner *(Zhang et al., 1991).* Likewise, in cultured human fetal mesangial cells, angiotensin II and bFGF increase DNA synthesis via mitogen-activated kinase-dependent and independent signalling pathways *(Izevbigie et al., 2000).*

The study of bFGF gene expression *in vivo*, in normal adult rat tissues is difficult due to the low abundance of FGF mRNAs, which are undetectable by northern blot analysis *(Zhang et al., 1991).* Using reverse transcriptase-polymerase chain reaction (rt-PCR) bFGF mRNA has been identified in RNA from young rat kidneys *(el-Husseini et al., 1992).* In adult Sprague-Dawley rats, expression of aFGF and bFGF has been demonstrated in kidney glomeruli and whole cortex using rt-PCR *(Ford et al., 1997).* aFGF and bFGF protein has been localised to blood vessels, collecting ducts, and Bowmans capsule in adult rat kidneys *(Floege et al., 1999; Cauchi et al., 1996; Takeuchi et al., 1992).* However, immunolocalisation of aFGF or bFGF protein to the smooth muscle layer of arteries varies greatly depending on the preparation and fixation of the kidney *(Cauchi et al., 1996).*

In a rare *in vivo* study, the role of bFGF in angiotensin II stimulated proliferation of balloon-injured arteries was examined in rats *(Su et al., 1998).* Angiotensin II was infused into rats for one week to induce hypertension and smooth muscle cell replication, then on day four of infusion the left carotid artery was subjected to balloon injury *(Su et al., 1998).* Angiotensin II infusion significantly stimulated smooth muscle

cell replication in the balloon injured carotid artery, the intact carotid artery, and three branch levels of the mesenteric vascular tree *(Su et al., 1998)*. Consecutive treatment of the animals with anti-bFGF antibody blocked the mitogenic effect of angiotensin II in the larger vessels, but not in the smallest (type I) vessels in the mesenteric vasculature *(Su et al., 1998)*. The study concluded that the presence of bFGF is necessary for angiotensin II-induced DNA synthesis in large vessels *(Su et al., 1998)*.

Mice that are deficient in the bFGF gene display morphologically normal vessels, yet have significantly lower blood pressure than wildtype mice, and have decreased vascular smooth muscle contractility *(Dono et al., 1998; Zhou et al., 1998)*. This provides evidence of a role for bFGF in the control of vascular tone and blood pressure homeostasis.

It appears thus, that the respective levels of growth factors present in both basal and angiotensin II-induced growth states will determine whether cultured VSMCs will undergo hypertrophic or hyperplastic growth. In pathological states such as hypertension, increased levels of angiotensin II *in vivo* may facilitate an imbalance between proliferative and anti-proliferative growth factors. An abnormal imbalance of these growth factors may augment cardiovascular hypertrophy and perpetuate the progression of the hypertension. But what triggers the up-regulation of one growth factor more than another? There may be a defect in the trophic stimulus or signal transduction pathways that pre-dispose a cell to respond abnormally.

Interestingly, in VSMC cultures from SHR, there is some evidence of abnormal regulation of TGF-β1 gene and receptors by angiotensin II, which could be associated with the exaggerated growth of VSMCs from SHR *(Fukuda et al., 1998; Agrotis et al., 1995)*. In aortic smooth muscle cells from WKY rats, TGF-β1 stimulated DNA synthesis was abolished by angiotensin II, where as TGF-β1-induced DNA synthesis was enhanced by angiotensin II in SHR cells *(Fukuda et al., 1998)*. Additionally, angiotensin II increased the expression of TGF-β type 1 receptor mRNA in cells from WKY rats, but did not effect receptor expression in SHR cells. The authors of this paper concluded that the angiotensin II mediated increase in TGF-β type 1 receptor expression may facilitate endogenous TGF-β1 to counteract the stimulatory effect of angiotensin II in WKY cells, whereas endogenous angiotensin II-induced TGF-β1 cannot counteract angiotensin II growth in SHR cells, resulting in exaggerated vascular growth in these animals *(Fukuda et al., 1998)*.

The evidence summarised above suggests a role for angiotensin II-induced early gene expression and a subsequent interaction with growth factors such as bFGF and TGF-β1 in mediating smooth muscle cell hypertrophy and hyperplasia. *In vivo*, abnormally high levels of angiotensin II in the kidney may facilitate increases in growth factors like bFGF, TGF-β1, and PDGF, which in turn, may contribute to cellular

hypertrophy and hyperplasia leading to vessel hypertrophy or remodelling. Vessel wall hypertrophy or remodelling which results in structural reductions of the vessel lumen, will subsequently increase vascular resistance. Increases in vascular resistance are present in, and potentiate the development and severity of hypertension *(Korner et al., 1991)*

1.5 SUMMARY

The kidney, via the pressure-naturesis mechanism, regulates salt and water homeostasis and therefore plays a crucial role in controlling levels of arterial pressure *(Guyton, 1989)*. Angiotensin II, through its intrarenal and systemic actions, is also intimately linked to the regulation of the pressure-naturesis mechanism and arterial pressure. It follows therefore, that an abnormality in the kidneys' ability to excrete salt and water will lead to alterations in arterial pressure. Abnormal renal function has been associated with the development of hypertension in a variety of experimental animals, and indeed humans.

There are several actions of angiotensin II within the kidney that are pro-hypertensive. These include direct vasoconstriction of the renal vasculature and stimulation of sodium and water reabsorption in the proximal tubule. This thesis has focussed on another potential renal action of angiotensin II that could contribute to the rise in arterial pressure, and looked for evidence of a trophic role for angiotensin II within the kidney *in vivo*.

Evidence demonstrating a trophic action of angiotensin II on VSMC growth *in vitro* is extensive and widely accepted, and it has been reported that angiotensin II induces both hypertrophy and hyperplasia in these cells via the AT_1 receptor. On the basis of the *in vitro* literature, and indirect studies involving blockade or infusion of components of the renin-angiotensin system (see section 1.4.1.2), angiotensin II-induced cellular hypertrophy or growth, particularly in the vasculature, has been implicated to play a role *in vivo* in the development or persistence of some hypertensive states.

The trophic actions of angiotensin II on VSMCs *in vitro*, together with evidence of elevated intrarenal angiotensin II, and a critical role of the kidney in hypertension, suggest that angiotensin II-induced renal vascular growth, and subsequent renal vascular hypertrophy may participate in the initiation and maintenance of some hypertensive states.

There exists, however, a large gap in the current literature. *In vivo* evidence for a direct trophic action of angiotensin II on cellular growth in hypertension is lacking. Indeed, there is little direct *in vivo* evidence of angiotensin II-induced renal cellular growth, irrespective of the potential role this may play in hypertension. The overall aim of this thesis, therefore was to begin to investigate the physiological relevance of angiotensin II mediated cellular growth or hypertrophy within the kidney *in vivo*, and further, to examine its role in the development of hypertension.

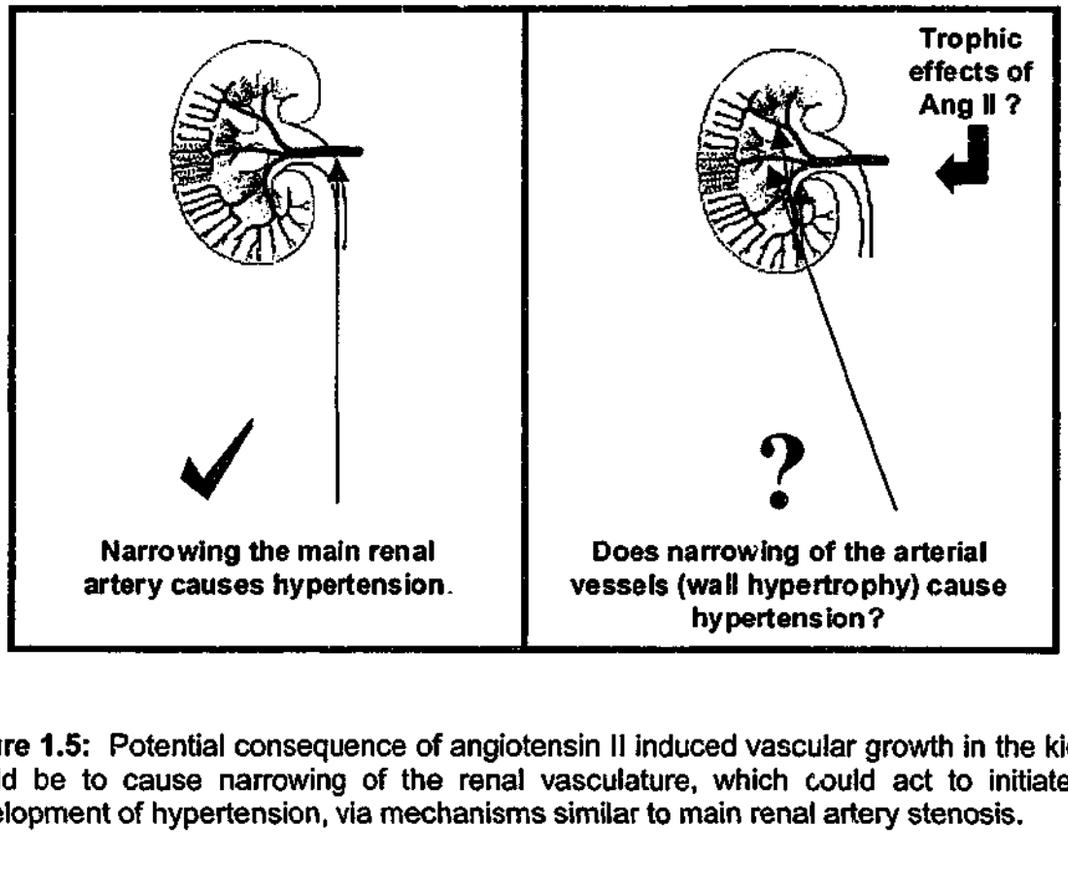


Figure 1.5: Potential consequence of angiotensin II induced vascular growth in the kidney would be to cause narrowing of the renal vasculature, which could act to initiate the development of hypertension, via mechanisms similar to main renal artery stenosis.

1.5.1 Aims:

1: To begin to investigate potential cellular growth induced by angiotensin II *in vivo*, I tested whether physiological doses of angiotensin ii infused directly into the kidney, induced early growth events in the kidney, as indicated by the expression of some well accepted markers of early cellular growth, *c-fos* and *egr*-1 genes.

2: To determine whether angiotensin II acting within the kidney could raise arterial pressure, the effect of infusing small doses of angiotensin II intrarenally (not systemically) on the development of hypertension was investigated in conscious rats.

In order to obtain a precise measure of the effects of angiotensin II on arterial pressure; 24-hour blood pressure, heart rate, and activity was measured in conscious rats housed in their home cages via telemetry.

3: Increased levels of angiotensin II are found in the kidney in several models of hypertension, including hypertension induced with systemic infusion of angiotensin II. The aim of the 3rd experiment was to investigate whether hypertension induced with systemic infusion of angiotensin II was associated with structural changes to the renal vasculature. Structural changes to the renal vasculature were assessed using a functional test in a maximally dilated, isolated perfused kidney preparation developed by Gothberg, Folkow and colleagues (1979).

4: To examine whether angiotensin II-induced hypertension was also associated with cellular growth and hypertrophy in the kidney, renal expression of growth factor genes TGF-β1 and bFGF, shown to be involved in angiotensin II-induced cellular growth *in vitro*, were investigated in the kidney of rats with hypertension induced with systemic angiotensin II infusion. Additionally whole kidney DNA and protein content were also determined as markers of renal growth.

CHAPTER 2
IMMEDIATE EARLY GENE EXPRESSION IN THE KIDNEY AFTER ACUTE INTRARENAL INFUSION OF ANGIOTENSIN II

2.1 INTRODUCTION

As reviewed in Chapter 1, angiotensin II (Ang II) is known to stimulate both hypertrophy and proliferation of vascular smooth muscle cells in culture *(Gibbons et al., 1992; Sachinidis et al., 1992a; Chiu et al., 1991; Geisterfer et al., 1988)* and has also been reported to stimulate expression of early growth response genes such as *egr-1* and *c-fos (Sharma et al., 1994; Ko et al., 1993; Sachinidis et al., 1992b; Millet et al., 1992)*. Likewise, Ang II has trophic actions on many renally derived cells in culture, including renal arteriolar smooth muscle cells *(Wolf and Ziyadeh, 1997; Wolf et al., 1996; Wolf, 1995; Dubey et al., 1992)*.

In contrast to the extensive evidence gathered from cultured cells, there is less direct evidence that exists concerning the trophic actions of Ang II *in vivo*, and even less is known about the effect in the kidney. Up-regulation of these genes in cultured cells that are exposed to Ang II provides some evidence for a role for Ang II in renal growth *in vivo*, perhaps as part of a cascade of growth factors that interact to promote cell growth. It is possible that intrarenal Ang II may stimulate renal cell growth *in vivo*, and subsequently induce structural changes within the kidney, that could potentially alter renal function.

The central focus of this thesis was to investigate whether Ang II induces growth *in vivo*, particularly within the kidney, and also to investigate the relationship of Ang II-induced growth *in vivo* to the development of hypertension. Despite a large body of literature describing an interaction between Ang II and induction of early growth response genes in cultured cells, such a role for Ang II *in vivo* is yet to be directly investigated.

Therefore, to begin investigating the potential trophic actions of Ang II, we have looked at whether small, physiological increases of Ang II within the kidney, *in vivo*, would stimulate early gene expression that has been shown to be associated with Ang II induced growth *in vitro*.

2.1.1 Aims:

Specifically, the aims of the present study were to investigate whether infusion of Ang II (2.5 ng/kg/min) directly into the kidney, at a dose that does not alter systemic haemodynamics, stimulates expression of early growth response genes *egr-1* and *c-fos*, and further, to investigate the time course of expression of these genes.

2.2 MATERIAL AND METHODS

Systemic and renal haemodynamic responses to an acute infusion of Ang II (2.5 ng/kg/min; Auspep, Australia) directly into the left renal artery were investigated. The kidneys were collected and expression of early growth response genes *c-fos* and *egr-1* were investigated via northern blot analysis.

All experiments were conducted under the approval of the Department of Physiology Animal Ethics Committee and were in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Ten week old male outbred Sprague–Dawley rats (240–360 g) were obtained from Monash University Animals Services and maintained on a standard rat chow (GR2; Barastoc Stockfeeds, Pakenham, VIC, Australia) and allowed free access to water.

A stock solution of Ang II (1 mg/ml; Auspep, Australia) was made with 0.9% w/v NaCl (saline) and frozen in aliquots. On the day of experiment, a fresh aliquot was thawed and Ang II made up in 0.9% NaCl to a dose 2.5 ng/kg/min and infused directly into the left renal artery in a volume of 10 µl/min. Once thawed, the concentrations of Ang II used in this thesis were adjusted to take into account the actual peptide amount, as determined by the amino acid analyses provided by the manufacturer. For example, if the actual peptide content was 90%, the amount of angiotensin II stock solution added to the sterile saline was adjusted to account for the less than 100% purity of the substance

2.2.1 Surgical preparation

Refer to Figure 2.2.1a for summary of surgical preparation. The animals were anaethetised with Inactin (thiobutabarbital, 175 mg/kg I.P, Research Biochaemicals International MA, USA) and underwent a tracheostomy to assist with ventilation. The

animals were then instrumented with a jugular vein catheter (PE-50) for infusion of maintenance fluids (2% Bovine Albumin Serum (BSA); fraction V, Sigma Chemical Co., USA) at 6 ml/hr during surgery, and 2 ml/hr for the remainder of the protocol, and a urinary bladder catheter. The right femoral artery was catheterised (PE-50) for measurement of arterial pressure. In order to infuse Ang II directly into the left kidney a tapered PE-10 catheter was inserted into the left femoral artery and advanced through the abdominal aorta until its tip was positioned approximately 1 mm into the left renal artery. The gastrointestinal tract was removed, the left and right kidneys denervated and a transit–time ultrasound flow probe (0.7 V; Transonic Systems, Ithaca, NY, USA) was placed around the left renal artery.

2.2.2 Measurement of haemodynamic variables

Arterial pressure was recorded by connecting the femoral artery catheter to a disposable pressure transducer (Cobe, Avarda, CO, USA) calibrated at the level of the surgical table. Heart rate was measured by a cardiotachometer activated by the arterial pressure pulse. Blood flow (ml/min) to the left kidney was recorded by connecting a transit-time ultrasound flow probe (type 0.7V; Transonic Systems, USA) to an ultrasonic flowmeter (model T206; Transonic systems, USA). All signals were amplified and recorded on a Grass polygraph (model 7D; Quincy, MA, USA) and the values read at 5 minute intervals directly off the chart.

2.2.3 Acute infusion of angiotensin II

At the end of the preparative surgery, the animals were allowed at least a 30 minute period to stabilise before the commencement of the experimental protocol. Animals were randomly allocated into one of four groups consisting of an intrarenal infusion of:

i) 30 min vehicle (heparinised saline 10 IU/ml; n=6)
ii) 30 min Ang II (2.5 ng/kg/min; n=6)
iii) 240 minute vehicle (heparinised saline 10 IU/ml; n=6)
iv) 240 minute Ang II (2.5 ng/kg/min; n=6).

See Figure 2.2.1b for experimental design. Mean arterial pressure, renal blood flow and heart rate were recorded continuously throughout the experimental protocol. During the 15 minute pre-infusion control period, haemodynamic variables were measured at three minute intervals and averaged to generate a mean for the period. Following the control period, infusion of vehicle or Ang II commenced with variables measured every five minutes for the 30 minute infusion, and every ten minutes for the 240 minute infusion.

2.2.3.1 Pilot study: acute infusion of angiotensin II – dose response

Before the commencement of the main study, a pilot study was conducted to determine a dose of Ang II that, when infused into the kidney, reduced renal blood flow by approximately 25%. In two animals, Ang II was infused directly into the kidney in increasing doses for 5 minute intervals. The doses of Ang II infused at consecutive intervals were 1, 2, 5, 10, 20 ng/kg/min. The peak renal blood flow response was taken for each dose and an average generated from the two animals.

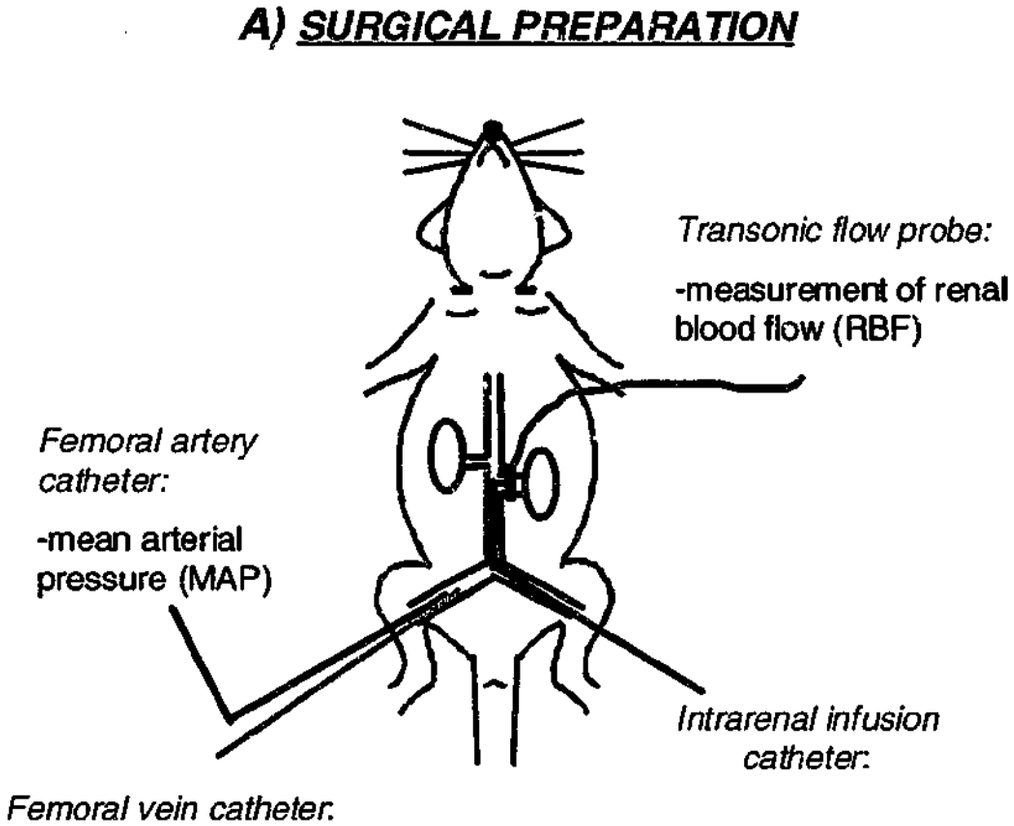


Figure 2.2.1: A) Schematic diagram of surgical preparation of anaesthetised rat. Mean arterial pressure (MAP), renal blood flow (RBF) and heart rate (HR) were recorded continuously throughout the experiment. Infusion of vehicle (heparinised saline 10 IU/ml) or Ang II (2.5 ng/kg/min) into the kidney was facilitated via a catheter positioned in the renal artery.

B) Timeline of the experimental protocol. Ang II (2.5 ng/kg/min) or vehicle (heparinised saline) was infused directly into the renal artery for 30 or 240 minutes.

2.2.4 Determination of c-fos and egr-1 mRNA levels using Northern blot analysis

Northern blot analysis provides a quantitative method to examine gene expression in isolated tissues. The technique can be used to look for the presence of expression of a specific gene, or quantitate changes in gene expression in response to experimental interventions. The technique involves extraction of total RNA (ribonucleic acid) from the tissue of interest, separation of the RNA according to molecular size via electrophoresis, transfer of the RNA to a nylon membrane, and finally, hybridisation of the membrane to radioactive-labelled probe with a complimentary sequence to the gene of interest. Northern blotting provides gross information about gene expression in a tissue but does not provide information on localisation of gene expression to specific cell types.

2.2.4.1 Summary of Northern blot analyses

In a positive control experiment, *c-fos* mRNA levels were determined in left kidney samples from animals that had undergone infusion of Ang II intrarenally (2.5 ng/kg/min) or intravenously (200 ng/kg/min, for 1 hour), and also in samples of rat brain, to determine if detection of the *c-fos* mRNA levels was possible in the kidney.

In the first series of Northern blots, *c-fos* and *egr-1* mRNA levels were determined in left kidney tissue from animals that had received an intrarenal infusion of vehicle (heparinised saline 10 IU/ml) or Ang II (2.5 ng/kg/min) for 30 minutes. In a separate comparison, *c-fos* and *egr-1* mRNA levels were determined in left kidneys from animals that underwent a 240 minute infusion of either vehicle or Ang II.

In the second series of Northern blots, *c-fos* and *egr-1* mRNA levels were determined in left infused and right control kidneys from animals receiving Ang II for 30 minutes, to enable within animal comparisons of gene expression to be made. Similarly, *c-fos* and *egr-1* mRNA levels from left and right kidney samples of the group that underwent a 240 minute infusion of Ang II was compared on the same blot.

2.2.5 Tissue collection and extraction of RNA

Ribonucleases (RNases), which degrade RNA, are found naturally and abundantly in all tissues and the environment. Thus, a number of standard pre-cautionary procedures must be followed whenever RNA is handled in the laboratory. Gloves are used at all times when handling RNA samples and performing experiments

using RNA. To destroy RNases all reagents are made up with molecular grade chemicals and de-ionised filtered water (mQH_2O), and then autoclaved or alternatively, prepared with autoclaved H_2O treated with diethylpyrocarbonate (DEPC). All glassware was cleaned and rinsed with distilled water and then baked at 180 °C for 8 hours before use. RNA samples were thawed for minimal amounts of time to avoid degradation as RNA is labile.

At the end of the infusion left and right kidneys were harvested from the animals, and snap frozen on dry ice, and stored at –70 °C prior to analysis for expression of early immediate genes *c-fos* and *egr-1*. Total RNA was extracted from left and right kidneys using a standard Tri-reagent ™ (Sigma Chemical Co., Australia) protocol which is a modification of the Chomczynski (1987) *(Chomczynski and Sacchi, 1987)* method. Briefly, a mixture of guanidine thiocyanate and phenol is used to extract DNA, RNA and protein in a single step liquid phase separation. Left and right kidneys were pulverised on dry ice and approximately 500 mg of kidney tissue was homogenised with 8 ml of Tri-reagent ™ then incubated at room temperature for 5 minutes. Chloroform (Sigma Chemical Co., Australia; 0.2 ml/ml Tri-reagent ™) was added to remove proteins. The solution was then centrifuged at 12,000 g for 15 minutes at 4 °C. The top aqueous layer containing the RNA was transferred to a fresh tube and precipitated with isopropanol at room temperature for ten minutes. After centrifuging at 12,000 g for 15 minutes at 4 °C the supernatant was discarded and the RNA pellet washed with 75% ethanol. Each pellet was resuspended in approximately 400 µl of DEPC treated water. Purity and yield of RNA were determined by measuring the absorbance of the sample at 260 and 280 nm wavelength, using the GBC UV/VIS 918 Spectrophotometer (UV/VIS 918, GBC Scientific Equipment Pty.Ltd., Australia). Since the optical density of 40 µg/ml of RNA at 260 nm is 1, then the concentration of the RNA sample can be determined using the following formula;

$$\text{RNA } (\mu g/ml) = 40 \times \text{dilution factor} \times OD_{260nm}$$

The integrity of RNA samples was also confirmed by running the samples on a formaldehyde agarose gel and staining with ethidium bromide to visualise the 18s and 28s rRNA bands. The 28s rRNA band should appear twice as intense as the 18s rRNA band, thus if degradation is present then the intensity of the 28s rRNA compared to the 18s rRNA will not be in the correct ratio. Therefore, integrity of the RNA was determined by the presence of sharp clear bands of ribosomal RNA on the fromaldehyde gel, and a ratio of absorbance at 260 nm/280 nm that was greater than 1.8.

2.2.6 DNase treatment and precipitation of samples

The RNA extracted from the kidney tissue was further purified by incubating with DNase to remove DNA and then performing a subsequent phenol-chloroform extraction and ethanol precipitation. In short, 100 μg of total RNA was incubated with RQ1 RNase-free DNase (Promega, Madison, USA; 1 unit/μg RNA), and RQDNase reaction buffer (40mM Tris-HCl, 10mM $MgSO_4$, 1mM $CaCl_2$) at 37°C for 30 minutes. DNase degrades both double and single-strandard DNA endonucleolytically, producing 3'-OH oligonucleotides.

After incubation to remove DNA contamination, the kidney RNA samples were purified by extracting with phenol-chloroform-isoamyl alcohol and precipitating with ethanol. The protocol used for this process has been previously described in *(Ausubel, 1987)*. Briefly, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was added to the enzyme reaction, vortexed and then spun at 12,000 g for 1 minute at room temperature. The top aqueous phase was then transferred to a fresh high recovery tube. The sample was then extracted twice with hydrated ether (Diethyl ether:H_2O, 1:1, v/v) to remove residual phenol. To precipitate RNA, a 1/10 volume of 3 M Sodium Acetate (pH 5.2) was added to the sample followed by 2.5 volumes of ice cold 100% ethanol (Sigma Chemical Co., Australia). The sample was then mixed and placed in the –70 °C freezer for at least one hour, but in most cases overnight. The sample was then spun at 4 °C for 15 minutes to pellet the RNA, then was washed twice in 70% ethanol, the supernatant removed and the pellet resuspended in approximately 25 μl of sterile millipore filtered water. After treatment of RNA with DNase and extraction with phenol: chloroform: isoamyl alcohol the ratio of the absorbances at 260 and 280 nm were all above 1.8 indicating that the purity of the samples had increased considerably.

2.2.7 Electrophoresis and Northern blotting

The extracted RNA was separated according to size using agarose gel electrophoresis. Total RNA from left kidneys infused with Ang II or vehicle were combined on the following Northern blots:

i) Control experiment: Left kidneys from animals infused with Ang II (2.5 ng/kg/min) intrarenally, or vehicle for 240 minutes, or animals infused with Ang II (200 ng/kg/min) intravenously for one hour. Also RNA from samples of rat brain tissue.

ii) RNA from left kidneys of animals infused Ang II or vehicle for 30 minutes.

iii) RNA from left kidneys of animals infused for Ang II or vehicle for 240 minutes.

iv) RNA from left (infused) and right (control) kidneys animals infused with Ang II for 30 minutes.

v) Left and right kidney RNA from animals that received intrarenal Ang II infusion for 240 minutes.

An RNA ladder was run on each gel to establish the size of the transcripts after hybridisation with the probes of interest. Ten micrograms of total RNA in volumes up to 5 µl were added to 14 µl formamide, 3.3 µl formaldehyde (12.3 M) and 1µl 20 x Northern buffer (0.8 M MOPS [3-(*N*-morpholino)-propanesulfonic acid]; pH 7.0, 0.2 M sodium acetate, 0.02 M EDTA [ethylenediaminetetre acetic acid]). The samples were then denatured at 90°C for 5 minutes and put back on ice. Two microliters of northern loading buffer (95% formamide v/v, 0.09% bromophenol blue w/v, 0.09% xylene cyanol FF w/v) was added and the samples loaded into a 1.2% w/v agarose gel containing 2.2 M formaldehyde, which was submerged in a gel cell (HE99X, Hoefer Scientific Instruments, CA, USA) containing electrophoresis buffer (1 x Northern buffer). When a current is applied across the gel the RNA will travel to the positive electrode as it is negatively charged. Larger RNA molecules travel slowly through the gel and thus the RNA separates according to size along the gel (ie; the rate of migration is directly proportional to the size of the RNA). Kidney RNA was electrophoresed at 80 volts for 3-4 hours or until the dye front was 2 cm from the bottom of the gel.

Following complete separation of total RNA, the formaldehyde gel was blotted overnight onto nylon membrane (Duralon-UV™ membrane, Stratagene, USA). The RNA was transferred to the nylon membrane by simple capillary action *(Ausubel, 1987)*. The following morning the transferred RNA was crosslinked to the membrane by exposing the membrane to UV light (UVC50 Hoefer Scientific Instruments, CA, USA). The membrane was then stained with methylene blue (5% v/v acetic acid, 0.04% w/v methylene blue) to visualise the RNA, and a photograph of the membrane was taken before it was stored in a sealed bag until hybridisation.

2.2.8 Northern blot analysis of c-fos mRNA levels

2.2.8.1 c-fos probe preparation and labelling

A 540 bp fragment of rat *c-fos* gene was kindly donated by Dr. Nancy Nichols. Previously, the fragment has been amplified and purified by Nicole Bye, in the Department of Physiology, Monash University. A [α^{32}P]-UTP labelled *c-fos* RNA probe was synthesised by *in vitro* transcription of the linearised anti sense cDNA template

using RNA polymerase T7. Briefly, *c-fos* cDNA template (100 ng), reaction buffer (containing CTP, GTP, ATP nucleotides), RNAsin (to prevent degradation by RNases), and T7 RNA polymerase were added to 250 μCi of dried [α^{32}P]-UTP (NEN Life Sciences, Amersham, Australia). The mixture was then incubated for one hour at 37°C before adding RQDNase enzyme (Promega, USA) to digest the DNA template. The reaction mixture was then passed through a Nuc Trap® push column (Stratagene®, CA, USA) to remove unincorporated nucleotides. Aliquots of the labelling reaction before and after elution from the column were precipitated with acid and counted in a beta liquid scintillation counter (Liquid scintillation systems, LS 5801, Beckman, USA). Incorporation greater than 80% was deemed successful and used for hybridisation on the same day

2.2.8.2 Hybridisation of Northern blots with c-fos cRNA probe

Membranes were pre-washed in 1% w/v sodium dodecyl sulphate (SDS) and 0.1 x standard saline citrate buffer v/v (SSC, [20 X stock; 3 M NaCl, 0.3 M sodium acetate, pH 7.0]) for 30 minutes at 77°C. The pre-wash was discarded and 15 ml of pre-hybridisation solution containing 5 x SSC v/v, 0.5% w/v 'blotto' (instant skim milk powder; Carnation, USA), 1% w/v SDS, 10% w/v dextran sulphate, 25 μg/ml poly A, poly C and 100 μg/ml salmon sperm DNA (Sigma Chemical Co., Australia). The poly A, poly C and salmon sperm DNA were boiled and kept on ice for five minutes before adding to the hybridisation solution. Blots were pre-hybridised for at least 3 hours at 77 °C. Pre-hybridisation solution was discarded after 3 hours and 10 ml of fresh solution added. Radioactive RNA probe was added to achieve 10^6 cpm/ml of hybridisation solution. Blots were hybridised for 18 hours at 77 °C.

The radioactive hybridisation solution was discarded and blots were washed once at 77°C with 5 x SSC v/v, 0.5% w/v blotto, 1% w/v SDS for one hour. The blots were then washed twice (30 minutes each) at 77 °C in 2 x SSC v/v and 0.1% w/v SDS, and twice in 0.5 x SSC v/v. The hybridised membrane was sealed in a plastic bag and exposed to a blanked phosphorimage screen (Molecular Dynamics, CA, USA) for two weeks at room temperature. The exposed phosphor screen was then scanned using a STORM phosphorimager (Molecular Dynamics, CA, USA) and the bands visualised as pixels using ImageQuaNT™ software (Molecular Dynamics, CA, USA).

2.2.9 Nothern blot analysis of egr-1 mRNA levels

2.2.9.1 egr-1 cDNA probe preparation

A 2.1 kb cDNA *egr-1* fragment (OC 68 insert) was kindly supplied by Professor Agapios Sachinidis (Medizinische Universitaets-Poliklinik Bonn Germany). The 2.1 kb cDNA was inserted into the plasmid pUC 13 (2.6 kb in size). A 1 μg aliquot of the plasmid and insert was digested with *Eco* R1 enzyme overnight to liberate the *egr-1* cDNA. The sample was then run together with a molecular weight standard DNA ladder (DNA ladder, Sigma Chemical Co., Australia) on a 1% w/v agarose gel at 80 volts to determine if the 2.1 kb fragment had been released. When the correct size fragment was confirmed, a 30 μg aliquot of the plasmid containing the *egr-1* cDNA insert was digested with *Eco* R1 at 37 °C overnight.

The enzyme digest was run at 40 volts for 8 hours to separate the 2.1 kb cDNA insert, and the 2.6 kb plasmid. After 8 hours, the gel was stained with ethidium bromide and illuminated with an ultraviolet light source. Under ultra-violet illumination a small incision was made in the gel directly right of the 2.1 kb cDNA fragment. A small piece of NA-45 membrane (Schleicher & Schuell, Germany) was inserted into the incision and then the gel was turned 90° in the gel cell and the cDNA fragment was electrophoresed onto the NA-45 paper. The excised *egr-1* cDNA was eluted from the NA-45 paper by heating in the presence of high concentrations of salt. The cDNA was then purified by standard phenol-chloroform purification and ethanol precipitation as described earlier. An aliquot of the 2.1 kb cDNA was run on a 1% w/v agarose gel to confirm the size of the insert and rule out any contamination of the sample with the pUC 13 plasmid.

2.2.9.2 Labelling and hybridisation of egr-1 cDNA probe to Northern blots

The *egr-1* cDNA was labelled with the radioactive nucleotide [α^{32}P]-dCTP (Easytide, NEN Life Sciences, Amersham, Australia) using a random priming labelling kit (Oliglabelling Kit, Pharmacia, Amrad, Australia). Briefly, 50 ng of cDNA template was resuspended in 34 μl of TE buffer (10 mM TRIS-HCl containing 1 mM EDTA, pH 7.5), then denatured for 2-3 minutes in boiling water and immediately put on ice. To the denatured DNA 10 μl of reagent mix (containing dATP, dGTP, dUTP) was added along with 5 μl of [α^{32}P]-dCTP and 1 μl of DNA polymerase I (Klenow fragment in 50 mM potassium phosphate, 0.1 mM dithiotriotol (DTT), and 50% glycerol, Pharmacia, Amrad, Australia). The mixture was then incubated for 60 minutes at 37 °C. The labelled cDNA probe was separated from unincorporated radioactive [α^{32}P]-dCTP by

passing the reaction mixture through a NICK™ column (Sephadex® G-50 Grade, Pharmacia, Australia) and eluting with TE buffer. One microliter of reaction mix was added to 5ml of scintillant fluid before and after the column purification, and counted in a beta liquid scintillation counter (Liquid Scintillation Systems, LS 5801, Beckman, USA). Recovery of labelled probe above 50% was considered successful and used the same day for hybridisation.

Membranes with left kidney RNA from control and Ang II infused kidneys in the 30 minute, and 240 minute group were prehybridised at 42 °C for at least 3 hours with gentle agitation. The hybridisation solution contained 50% v/v formamide (Sigma Chemical Co., Australia), 7% w/v SDS, 0.1 mg/ml salmon sperm DNA (Sigma Chemical Co., Australia), 0.75 M NaCl, 0.05 M $NaH_2PO_4.H_2O$, 5 mM EDTA, pH 7.4. After at least 3 hours, the prehybridisation solution (same as the hybridisation solution) was discarded and fresh hybridisation solution was added to the membranes. The labelled cDNA probe was denatured by boiling for five minutes and then put on ice for five minutes. Radioactive probe was added to the hybridisation solution (2×10^6 cpm/ml of hybridisation solution) and the membranes incubated at 42 °C for 18 hours.

At the end of the hybridisation period, the radioactive hybridisation solution was discarded and the membranes washed three times (20 minutes each) in; 2 x SSC v/v, 1% w/v SDS at room temperature, and then twice (45 minutes each) in 0.2 x SSC v/v, 1% w/v SDS at 65 °C to remove any unbound or non-specific binding of the probe. The hybridised membrane was sealed in a plastic bag and exposed to a blanked phosphorimage screen (Molecular Dynamics, USA) for two weeks at room temperature. The exposed phosphor screen was then scanned using a STORM phosphoimager as mentioned in section 2.2.7.2.

2.2.10 Hybridisation of Northern blots with cDNA probe for 18s RNA

Blots that had been previously hybridised with *egr-1* and *c-fos* underwent a second hybridisation with a [α^{32}P]-labelled cDNA probe directed against 18s rRNA, and were exposed to blanked phophorimage screen overnight. The expression of the ribosomal gene 18s rRNA was used as a marker for gel loading.

2.2.11 Data analysis

Each lane in the Northern blot analyses contained RNA from an individual animal. Using the ImageQuaNT™ software package, total integrated density (the integrated density if all the pixels in a designated area) was determined for each transcript; and a corresponding background area of the lane was subtracted from the

density of the transcript. The total integrated density of the transcript was then divided by the total integrated, background adjusted density of the 18s rRNA transcript. The mRNA levels are expressed as a ratio of 18s rRNA to standardise for minor loading differences in total RNA between lanes, and as such are presented as arbitrary units.

2.2.12 Statistical analysis

All data were analysed using SYSTAT™ 5.0 statistical software package (SPSS Inc., USA). Resting haemodynamic variables before infusion of vehicle or Ang II were compared using an unpaired *t*-test. The haemodynamic effects of acute infusion of Ang II compared to its vehicle were tested using repeated measures analysis of variance *(Ludbrook, 1994)*.

Expression of *c-fos* mRNA and *egr-1* mRNA was adjusted for background and expressed as a ratio of 18s rRNA expression. Statistical comparisons of relative levels of *c-fos* mRNA and *egr-1* mRNA in kidneys from animals treated with Ang II or vehicle were analysed using the rank sign test for non-parametric data *(Snedecor and Cochran, 1967)*. For all data, $P < 0.05$ was considered statistically significant.

2.3 RESULTS

2.3.1 Haemodynamic variables

2.3.1.1 Acute infusion of angiotensin II – Pilot study

The dose-dependent effects of infusing increasing doses of Ang II, into the kidney, on renal blood flow (RBF) are illustrated in Figure 2.3.1. Intrarenal infusion of 1, 2 and 5 ng/kg/min Ang II resulted in a mean reductions of approximately 13, 25 and 40% respectively in RBF, when compared to pre-infusion levels. In order to achieve a reduction in RBF of approximately 25% for the main study, a dose of 2.5 ng/kg/min of Ang II was chosen on the basis of the findings in this pilot study.

2.3.1.2 Acute infusion of angiotensin II

Pre-infusion control measurements for mean arterial pressure (MAP), heart rate (HR), renal blood flow (RBF), renal vascular resistance (RVR), and renal vascular conductance (RVC), of animals receiving vehicle or Ang II for 30 or 240 minutes are presented in Table 2.1. There was no significant difference in resting haemodynamic variables between animals prior to commencement of the 30 minute or 240 minute infusion of vehicle (heparinised saline 10 IU/ml), or Ang II (2.5 ng/kg/min). Animals receiving a 240 minute Ang II infusion had significantly greater body weight (body wt) than the vehicle animals (332 ± 6 g, 298 ± 9 g respectively $P < 0.05$, unpaired *t*-test).

	30 minute infusion			*240 minute infusion*		
	VEHICLE	**ANG II**	***t*-test**	**VEHICLE**	**ANG II**	***t*-test**
MAP (mmHg)	89 ± 3	82 ± 2	NS	87 ± 6	88 ± 4	NS
HR (bpm)	407 ± 13	387 ± 19	NS	381 ± 14	358 ± 6	NS
RBF (ml/min)	5.0 ± 0.2	5.2 ± 0.5	NS	5.3 ± 0.6	6.5 ± 0.5	NS
RVR (ml/mmHg)	18.2 ± 1.4	16.8 ± 1.9	NS	18.0 ± 2.9	13.8 ± 0.8	NS
RVC (ml/min/ mmHg)	0.057 ± 0.004	0.063 ± 0.008	NS	0.062 ± 0.009	0.073 ± 0.004	NS
Body wt *(g)*	300 ± 20	322 ± 17	NS	332 ± 6	298 ± 9	*P<0.05*

Table 2.1: Control measurements in anaesthetised rats prior to commencement of infusion of either vehicle (heparinised saline 10 IU/ml) or Ang II (2.5 ng/kg/min) into the renal artery. Values represented as mean ± SEM. Control measurements were recorded for 15 minutes and analysed at 3 minutes intervals. An unpaired t-test was used to test for significant differences in control measurements between treatment groups.

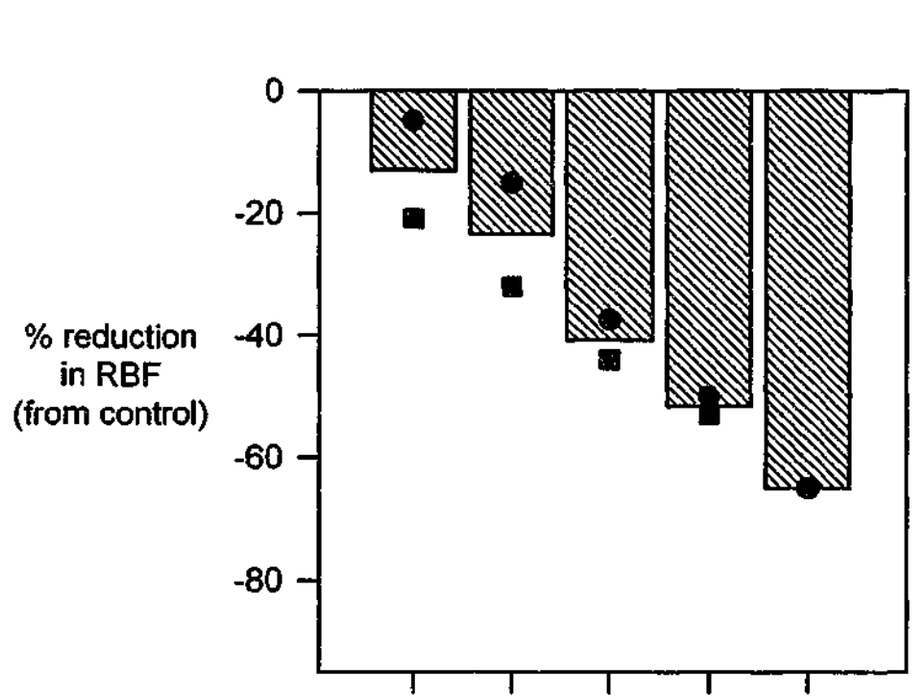


Figure 2.3.1: Percentage reduction (from control) in renal blood flow in response to 5 minute infusions of increasing doses of Ang II into the renal artery of anaesthetised rats. Mean renal blood flow response represented by hatched bars (n=2). Individual animal responses represented by soild circle and squares.

30 minute infusion period

During the final 15 minutes of Ang II infusion, mean arterial pressure was not different from the pre-infusion control period in both the vehicle (88.8 ± 1.3 mmHg vs 87.5 ± 1.2 mmHg respectively; $P = 0.4$), and Ang II group (82.2 ± 0.8 mmHg vs 81.8 ± 0.9 mmHg; $P = 0.4$; Figure 2.3.2a).

Intrarenal infusion of Ang II for 30 minutes significantly reduced renal blood flow compared to the pre-infusion control levels (4.7 ± 0.2 ml/min vs 5.2 ± 0.2 ml/min respectively; $P = 0.02$; Figure 2.3.2b). In contrast, renal blood flow during the last 15 minutes of the infusion period was unchanged compared to the pre-infusion control period, in animals that received a vehicle infusion (5.0 ± 0.1 ml/min vs. 5.0 ± 0.1 ml/min respectively $P = 0.96$; Figure 2.3.2b).

Concomitant with the fall in renal blood flow, renal vascular resistance significantly increased during 30 minute Ang II infusion when compared to pre-infusion control levels (18.9 ± 0.9 ml/min/mmHg vs 16.8 ± 0.7 ml/min/mmHg respectively; $P = 0.03$; Figure 2.3.2c). In animals receiving vehicle infusion, renal vascular resistance was not significantly changed from control levels (Figure 2.3.2c).

30 and 240 minute angiotensin II infusion – change from control period

MAP, RBF, and RVC expressed as change from pre-infusion control levels in animals receiving either vehicle or Ang II infusion for 30 or 240 minutes are presented in Figure 2.3.3. Intrarenal infusion of Ang II for 30 or 240 minutes did not increased mean arterial pressure when compared animals treated with vehicle (Figure 2.3.3a).

Renal blood flow was significantly reduced by 240 minutes of Ang II infusion when compared to the vehicle infusion ($P = 0.026$, Figure 2.3.3b), however the fall in renal blood flow in animals that received a 30 minute infusion of Ang II did not reach significance when compared the vehicle animals ($P = 0.28$, Figure 2.3.3b). In accord with the reduction in renal blood flow, renal vascular conductance was significantly decreased in animals infused with Ang II for 30 and 240 minutes compared with animals that received a vehicle infusion ($P = 0.004$, $P = 0.016$ respectively, Figure 2.3.3c).

In summary, intrarenal infusion of Ang II at 2.5 ng/kg/min for 30 or 240 minutes, had no significant effects on MAP. In contrast, RBF was significantly reduced in animals receiving Ang II for 240 minutes when compared to the vehicle group, and was reduced in animals receiving Ang for 30 minutes when compared to the pre-infusion control period. Renal vascular conductance was significantly reduced in both groups receiving Ang II for 30 or 240 minutes when compared to the vehicle infused control animals.

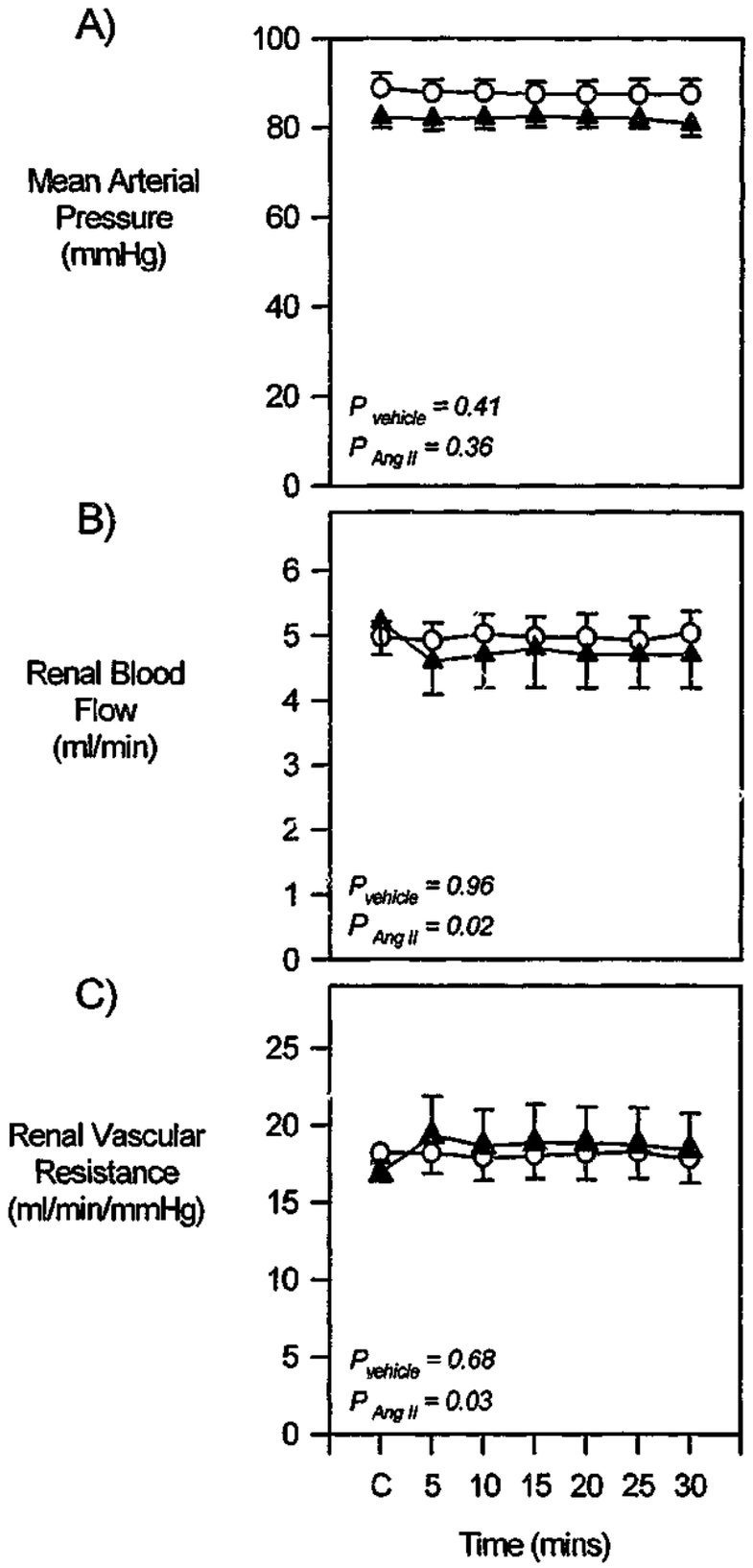


Figure 2.3.2: Mean arterial pressure (A), renal blood flow (B), and renal vascular resistance (C) in animals receiving an intrarenal infusion of vehicle (heparinised saline 10 IU/ml; n=6; open circles) or Ang II (2.5 ng/kg/min; n=6; solid triangles) for 30 minutes. Data presented as group mean ± SEM. A paired *t*-test was used to determine significant differences between the 15 minute control period and the last 15 minutes of infusion within each experimental group.

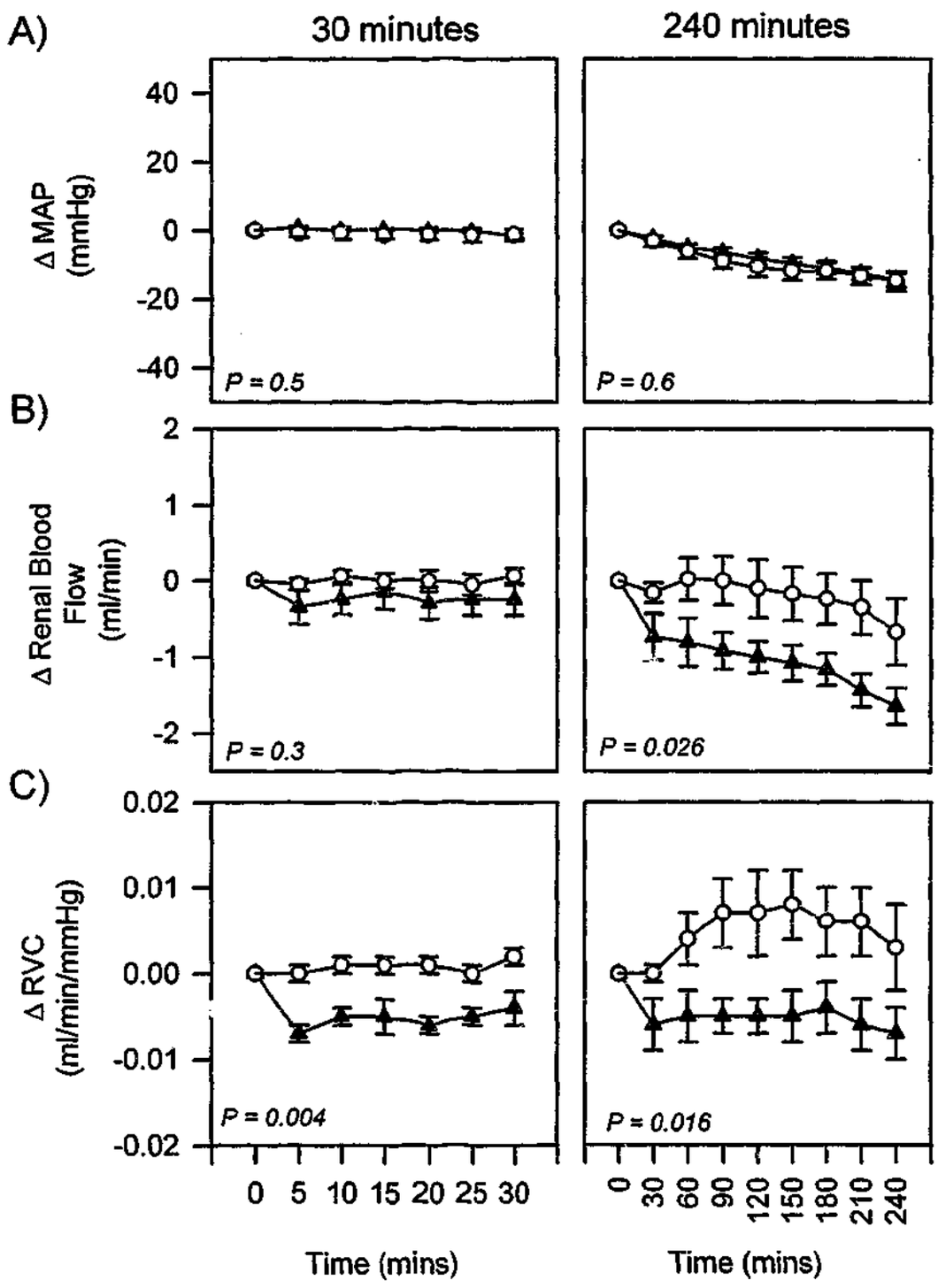


Figure 2.3.3: (a) Mean arterial pressure (MAP), (b) renal blood flow (RBF), and (c) renal vascular conductance (RVC) in rats that received an intrarenal infusion of vehicle (Heparinised saline; open circles) or Ang II (2.5 ng/kg/min; solid triangles) for 30 or 240 minutes. Data presented as mean ± SEM of the difference between the averaged control period and levels at the indicated time after commencement of infusion of Ang II or vehicle. Repeated measures ANOVA was used to test for significant differences ($p < 0.05$) in measured variables, between vehicle and Ang II groups in each infusion period (refer to test statistics on graphs).

2.3.2 Northern blot analysis

2.3.2.1 Detection of c-fos mRNA level in rat kidney and brain

In order to determine if *c-fos* mRNA levels were detectable in rat kidney tissue by Northern blot analyses, a positive control Northern blot analyses was performed. Detectable levels of *c-fos* mRNA were confirmed in rat kidney tissue from animals that had undergone an infusion of; Ang II (2.5 ng/kg/min, intrarenally), vehicle (heparinised saline 10 IU/ml, intrarenally), or an intravenous infusion of Ang II (200 ng/kg/min for one hour). Expression of *c-fos* mRNA is highest in the kidneys of animals that received Ang II intravenously (Figure 2.3.4). Expression in kidneys treated with intrarenal Ang II is lower but still detectable. In contrast, *c-fos* mRNA levels were barely detectable in rat brain tissue (cortex and hippocampus) from animals that had under gone adrenalectomy, or been subjected to environmental stressors (Figure 2.3.4).

2.3.2.2 Effect of intrarenal angiotensin II infusion on c-fos mRNA levels in the kidney

Figures 2.3.5 and 2.3.6 are duplicate experiments representing Northern blot analyses of *c-fos* mRNA levels in left kidney tissue from rats that received an intrarenal infusion of Ang II (2.5 ng/kg/min) or vehicle (heparinised saline 10 IU/ml) for 30 or 240 minutes. The radioactive signal, representative of *c-fos* mRNA levels in rat kidney tissue, was very weak and thus a long exposure (2 weeks) to the phosphorimage screen was required to visualise the signal. This indicates that the levels of *c-fos* mRNA in rat kidney tissue are very low and close to the limit of sensitivity of the phosphor technology. Despite this however, duplicate Northern blot analyses of *c-fos* mRNA levels suggest that expression of *c-fos* mRNA is reproducible between blots and that the levels of expression detected are similar on different blots (Figure 2.3.5; Ang II (30 min infusion) 52 ± 13 vs. vehicle 34 ± 8 arbitrary units, and Ang II (240 min infusion) 51 ± 4 vs. vehicle 51 ± 10 arbitrary units; Figure 2.3.6; Ang II (30 min infusion) 42 ± 13 vs. vehicle 27 ± 9 arbitrary units, and Ang II (240 min infusion) 53 ± 15 vs. vehicle 50 ± 10 arbitrary units.).

The individual data (Figure 2.3.5 and 2.3.6, upper panels) indicate that *c-fos* mRNA levels in rat kidney tissue from animals that received a vehicle and Ang II infusion were variable within the same treatment group. Furthermore, the spread of *c-fos* mRNA levels overlapped greatly between treatment groups. Rank sign analysis of the non-parametric data demonstrate that *c-fos* mRNA levels were not significantly different in kidney tissue from animals that received an intrarenal infusion of Ang II for 30 or 240 minutes when compared to the respective vehicle group (Figure 2.3.5 and 2.3.6).

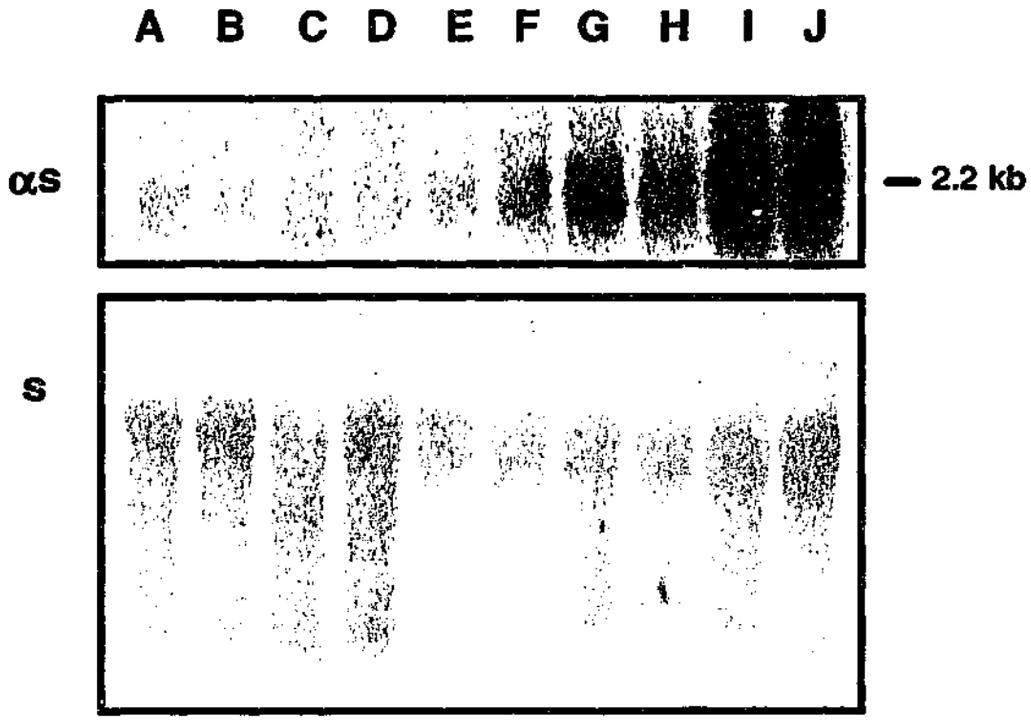


Figure 2.3.4: *c-fos* mRNA levels in rat brain and kidney tissue

Northern blot analysis of total RNA (10 μg) from rat brain and kidney tissue. Membranes were hybridised with ^{32}P-labelled *c-fos* cRNA probes (anti-sense, αs; top panel), and (sense, s; lower panel). Lane **A)** rat brain cortex (adrenalectomised), **B)** rat brain cortex (control), **C)** rat brain hippocampus (stress), **D)** rat brain hippocampus (control), **E)** and **F)** rat kidney (intrarenal infusion of Ang II (2.5ng/kg/min; 4hr), **G)** and **H)** rat kidney (intrarenal infusion of vehicle; 4hr), **I)** and **J)** rat kidney (intravenous Ang II (200ng/kg/min; 1hr).

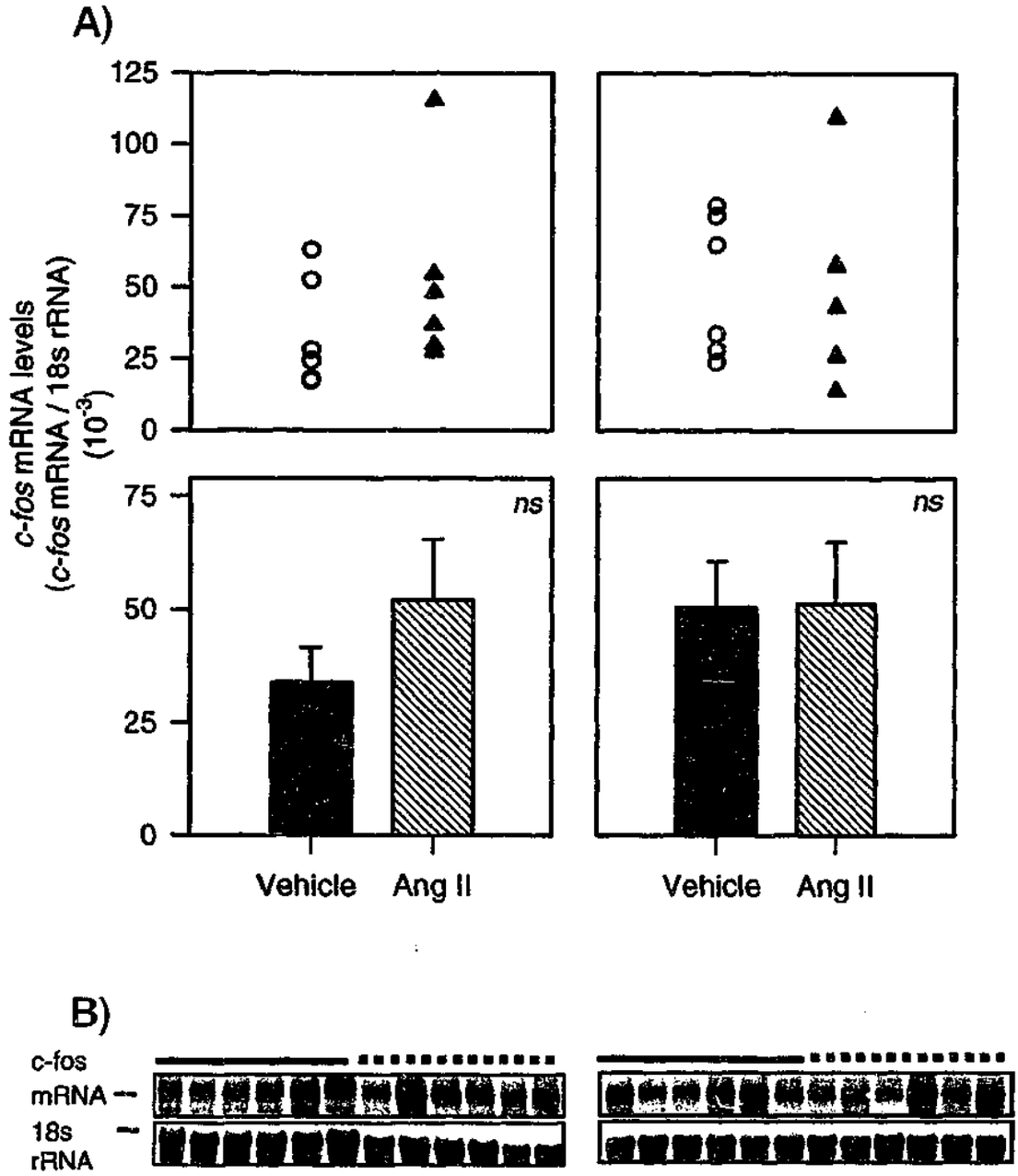


Figure 2.3.5: Effect of intrarenal Ang II infusion on *c-fos* mRNA levels in the kidney
A) Individual (upper panels), and group mean ± SEM (lower panels) *c-fos* mRNA levels in rat kidney tissue from animals that received an intrarenal infusion of vehicle (heparinised saline 10 IU/ml; open circles and black bars) or Ang II (2.5 ng/kg/min; solid triangles and hatched bars). The mRNA levels have been corrected for background and expressed as a ratio of density of the 18s rRNA transcript. Rank sign analysis was used to test for significant differences in *c-fos* mRNA levels between rats treated with Ang II and vehicle for 30 or 240 minutes, '*ns*' indicates values that are not significantly different from vehicle.

B) Northern blot analyses of total RNA (10 µg) from the kidneys of animals that received an intrarenal infusion of Ang II (dashed line) or vehicle (solid line), as indicated above. Membranes were hybridised with a ^{32}P-labelled *c-fos* cRNA probe (upper panel), followed by rehybridisation with a ^{32}P-labelled cDNA probe coding for 18s rRNA. The relative densities of the *c-fos* mRNA transcripts were expressed as a proportion of the 18s rRNA transcript to adjust for minor loading differences in total RNA between each of the samples.

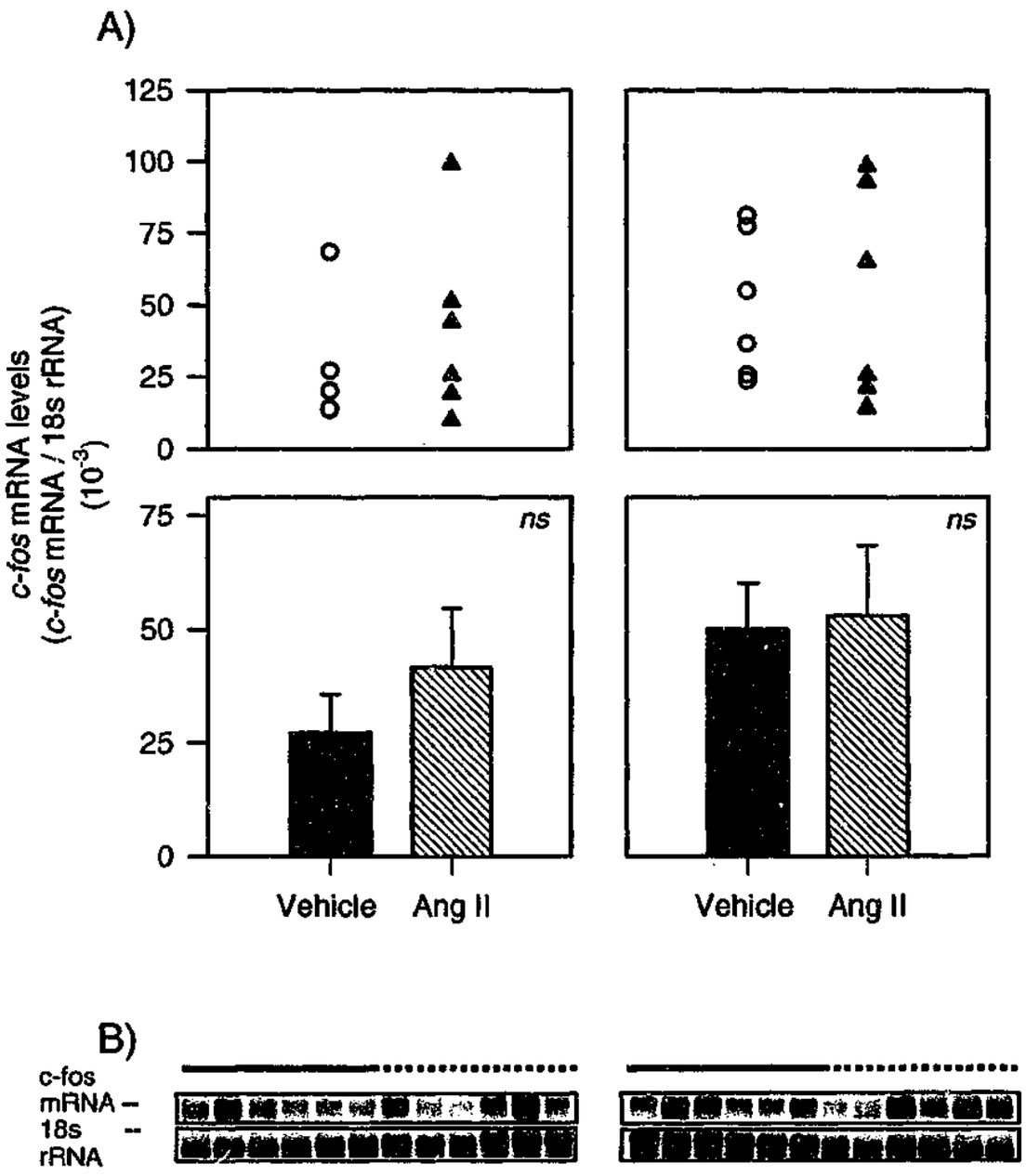


Figure 2.3.6: Effect of intrarenal Ang II infusion on c-fos mRNA levels in the kidney
A) Individual (upper panels), and group mean ± SEM (lower panels) c-fos mRNA levels in rat kidney tissue from animals that received an intrarenal infusion of vehicle (heparinised saline 10 IU/ml; open circles and black bars) or Ang II (2.5 ng/kg/min; solid triangles and hatched bars). The mRNA levels have been corrected for background and expressed as a ratio of density of the 18s rRNA transcript. Rank sign analysis was used to test for significant differences in *c-fos* mRNA levels between rats treated with Ang II and vehicle for 30 or 240 minutes, 'ns' indicates values that are not significantly different from vehicle.

B) Northern blot analyses of total RNA (10 µg) from the kidneys of animals that received an intrarenal infusion of Ang II (dashed line) or vehicle (solid line), as indicated above. Membranes were hybridised with a ^{32}P-labelled *c-fos* cRNA probe (upper panel), followed by rehybridisation with a ^{32}P-labelled cDNA probe coding for 18s rRNA. The relative densities of the *c-fos* mRNA transcripts were expressed as a proportion of the 18s rRNA transcript to adjust for minor loading differences in total RNA between each of the samples.

2.3.2.3 Effect of intrarenal angiotensin II infusion on egr-1 mRNA levels in rat kidney tissue.

Rank sign analysis of data indicates no significant difference in *egr-1* mRNA levels in rat kidney tissue from animals that received an intrarenal infusion of Ang II or vehicle, for 30 or 240 minutes, when compared to the *egr-1* mRNA levels in rat kidney tissue from the respective vehicle groups (Figure 2.3.7). Levels of *egr-1* mRNA in rat kidney were very low and close to the limit of the phosphor technology. Individual data (Figure 2.3.7a; top panels) indicate that *egr-1* mRNA levels are variable within the same treatment group. Further, *egr-1* mRNA levels also overlap between treatment groups (Figure 2.3.7; Ang II (30 min) 59 ± 32 vs. vehicle 27 ± 5 arbitrary units, and Ang II (240 min) 21 ± 5 vs. vehicle 37 ± 6 arbitrary units).

2.3.3 Within animal comparisons of gene expression

2.3.3.1 Within animals comparison of c-fos and egr-1 mRNA levels in left (infused) and right (control) kidney.

Levels of *c-fos* and *egr-1* mRNA in rat kidney tissue showed considerable variation between animals in the same treatment groups. In an attempt to reduce this variation between animals in the same treatment group, *c-fos* and *egr-1* mRNA levels were compared between left kidneys from animals that received an intrarenal Ang II infusion for 30 or 240 minutes, and in the right control kidneys from the same animals (Figure 2.3.8 and 2.3.9). Although comparable amounts of total RNA were loaded onto the Northern blots (apparent from the methylene blue stain, Figure 2.3.8 and 2.3.9, lower panels), there was no apparent consistent pattern of upregulation of *c-fos* or *egr-1* mRNA levels in left kidney tissue, exposed to Ang II compared to the right control kidneys from the same animal (Figure 2.3.8 and 2.3.9, upper panels).

Qualitatively, levels of *c-fos* and *egr-1* mRNA in left kidney tissue appear upregulated in some animals, but suppressed in others, when compared to the right control kidneys from the same animal, further confirming that levels of both *c-fos* and *egr-1* mRNA are not upregulated to detectable levels in response intrarenal infusion of Ang II.

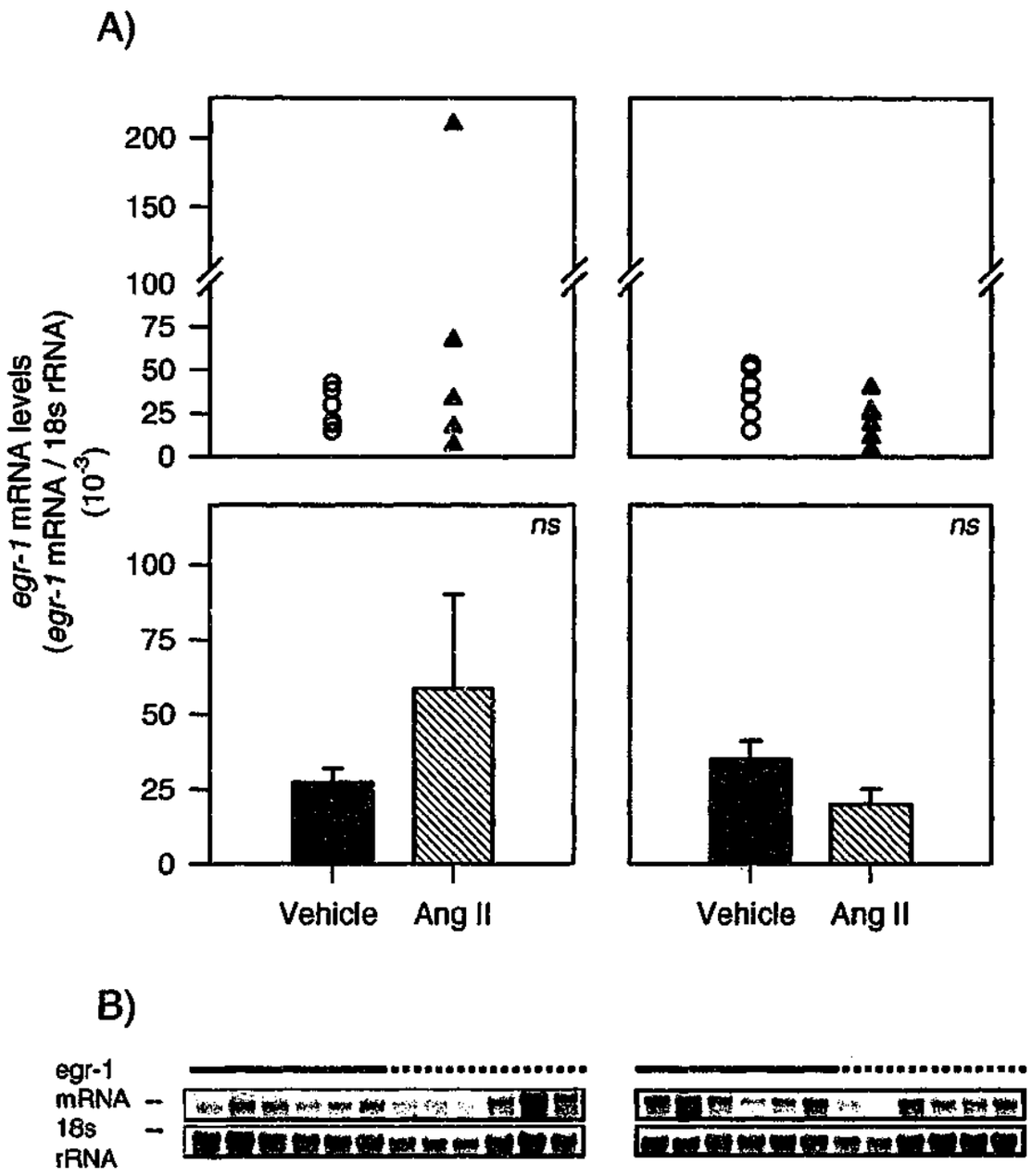


Figure 2.3.7: Effect of intrarenal Ang II infusion on *egr-1* mRNA levels in the kidney
A) Individual (upper panels), and group mean ± SEM (lower panels) *egr-1* mRNA levels in rat kidney tissue from animals that received an intrarenal infusion of vehicle (heparinised saline 10 IU/ml; open circles and black bars) or Ang II (2.5 ng/kg/min; solid triangles and hatched bars). The mRNA levels have been corrected for background and expressed as a ratio of density of the 18s rRNA transcript. Rank sign analysis was used to test for significant differences in *egr-1* mRNA levels between rats treated with Ang II and vehicle for 30 or 240 minutes, '*ns*' indicates values that are not significantly different from vehicle.

B) Northern blot analyses of total RNA (10 μg) from the kidneys of animals that received an intrarenal infusion of Ang II (dashed line) or vehicle (solid line), as indicated above. Membranes were hybridised with a ^{32}P-labelled *egr-1* cRNA probe (upper panel), followed by rehybridisation with a ^{32}P-labelled cDNA probe coding for 18s rRNA. The relative densities of the *egr-1* mRNA transcripts were expressed as a proportion of the 18s rRNA transcript to adjust for minor loading differences in total RNA between each of the samples.

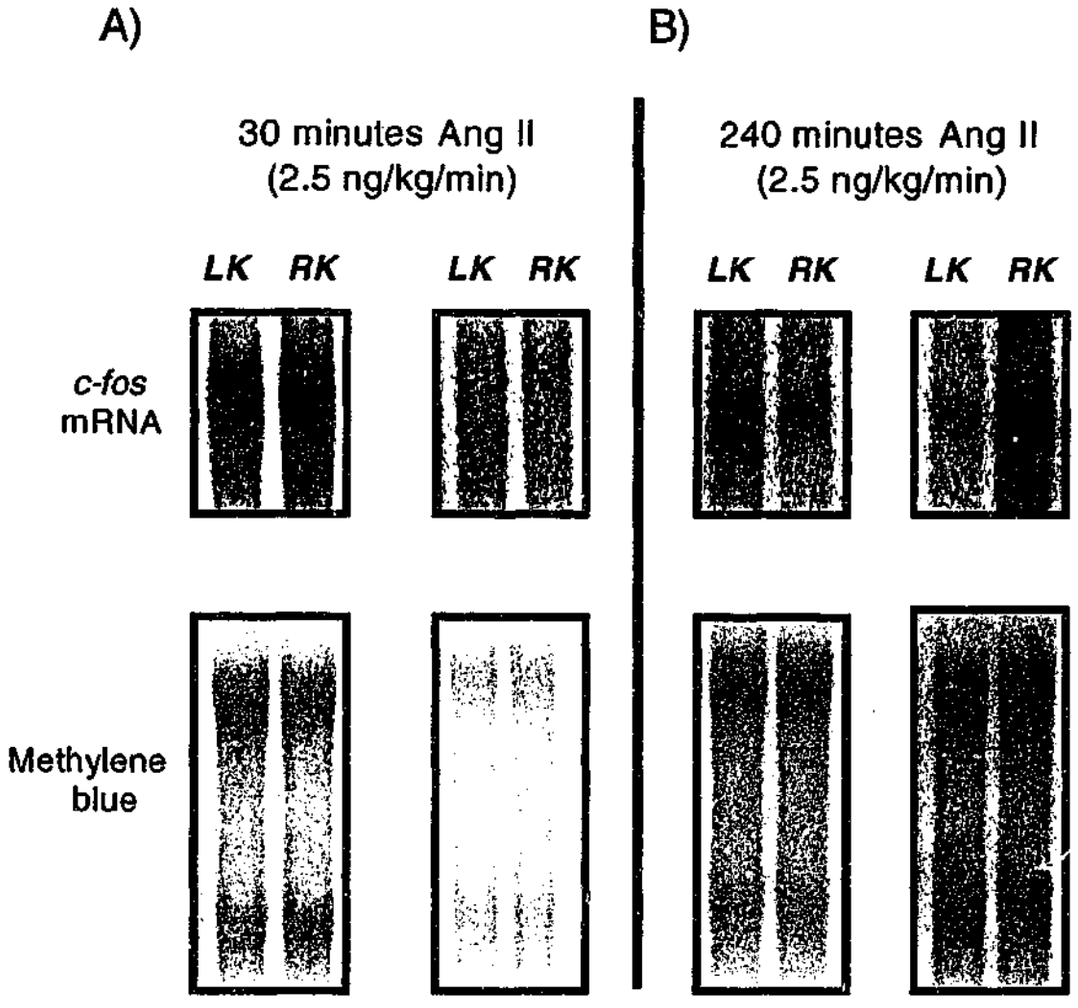


Figure 2.3.8: *c-fos* mRNA levels in left (treated) and right (control) kidneys from rats treated with intrarenal Ang II

Upper panels: Northern blot analyses of *c-fos* mRNA in left (LK; infused) and right (RK; control) kidneys from animals that received an intrarenal infusion of Ang II (2.5 ng/kg/min) for 30 (A) or 240 (B) minutes.
Lower panels: Northern blot membrane stained with methylene blue to visualise total RNA from left and right kidneys prior to hybridisation with ^{32}P-labelled *c-fos* probe. Methylene blue RNA stain indicates that there was no apparent loading differences in total RNA between lanes, that could account for the variability of *c-fos* mRNA levels.

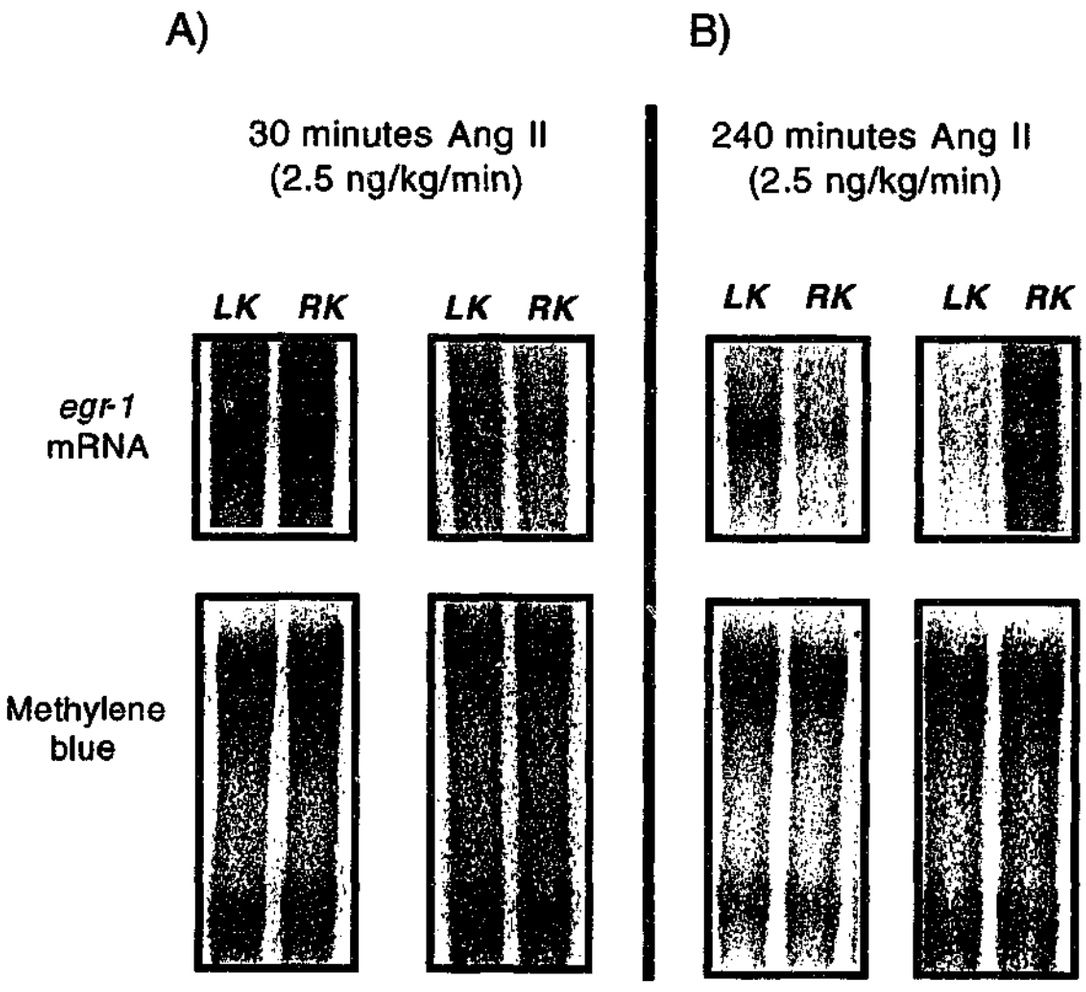


Figure 2.3.9: *egr-1* mRNA levels in left (treated) and right (control) kidneys from rats treated with intrarenal Ang II

Upper panels: Northern blot analyses of *egr-1* mRNA in left (LK; infused) and right (RK; control) kidneys from animals that received an intrarenal infusion of Ang II (2.5 ng/kg/min) for 30 (A) or 240 (B) minutes.
Lower panels: Northern blot membrane stained with methylene blue to visualise total RNA from left and right kidney tissue prior to hybridisation with ^{32}P-labelled *egr-1* probe. Methylene blue RNA stain indicates that there was no apparent loading differences in total RNA between lanes, that could account for the variability of *egr-1* mRNA levels.

2.4 DISCUSSION

2.4.1 Evidence for trophic effects of angiotensin II in cultured cells

The present study was based on evidence from *in vitro* studies that demonstrate Ang II induces hypertrophy and hyperplasia of cultured vascular smooth muscle cells (VSMC), including renal arterial cells *(Dubey et al., 1992)*, as well as many other renally derived cultured cells *(Wolf, 1995)*. The trophic action of Ang II in cultured cells is also accompanied by stimulation of immediate early genes such as *c-fos* and *egr-1 (Sachinidis et al., 1992b; Millet et al., 1992; Taubman et al., 1989)*, and it may be that Ang II could promote cellular hypertrophy and hyperplasia via an interaction with a cascade of growth factors.

2.4.2 Acute infusion of angiotensin II – effect on renal and systemic haemodynamics

Infusion of Ang II at 2.5 ng/kg/min, directly into the left kidney for 30 or 240 minutes, produced a significant decrease in renal vascular conductance yet did not affect mean arterial pressure. Intravenous infusion of Ang II for 30 minutes significantly reduced renal blood flow compared to pre-infusion control measurements, however this change in renal blood flow was not significant when compared to the animals that received an intrarenal vehicle infusion for 30 minutes. Renal blood flow was significantly reduced in animals that received an intrarenal infusion of Ang II for 240 minutes when compared to the vehicle controls.

In summary, infusion of low physiological doses of Ang II directly into the kidney for 30 or 240 minutes, produced a sustained renal vasoconstriction but did not affect mean arterial pressure, indicating that the Ang II was contained within the kidney, and did not spill over to affect systemic haemodynamics.

2.4.3 Angiotensin II-induced early gene expression in the kidney in vivo

Despite the physiologically significant renal vasoconstriction, infusion of Ang II for 30 or 240 minutes, directly into the left kidney, did not produce a significant increase in expression of immediate early genes *c-fos* or *egr-1* when compared to vehicle controls. These results are in direct contrast to evidence gathered from cells in culture (see Chapter 1 Section 1.4.2.2.1). Upregulation of *egr-1* mRNA and protein after incubation with Ang II has been demonstrated in a number of cell culture systems

including vascular smooth muscle cells *(Sachinidis et al., 1992b; Dubey et al., 1992; Rupprecht et al., 1992)*. Likewise, expression of *c-fos* mRNA has been shown to be upregulated by Ang II in cultured rat cardiomyocytes and smooth muscle cells *(Neyses et al., 1993; Millet et al., 1992; Kawahara et al., 1988)*.

More importantly, the results of the present study are also in direct contrast to a previous study by Rosenberg and Hostetter (1993) in which Ang II (50 ng/kg/min) was infused intrarenally into anaesthetised rats for one hour before investigating early gene expression in the kidney. After one hour of Ang II infusion at 50 ng/kg/min, the study reported a significant increase in renal expression of early genes *c-fos* and *egr-1 (Rosenberg and Hostetter, 1993)*. In contrast to the present study where 2.5 ng/kg/min Ang II was infused intrarenally, Rosenberg and Hostetter (1993) infused an approximately 20 times higher, non-physiological dose of Ang II (50 ng/kg/min). They reported significant decreases in renal plasma flow and GFR (measured by PAH, and ^{3}H-inulin clearance) only in the left kidney infused with Ang II, and no effect on mean arterial pressure. Renal plasma flow and GFR were decreased by 26 and 22% respectively with Ang II infusion and this was associated with a significant increase in *c-fos* and *egr-1* gene expression in the infused kidneys when compared to the right control kidney *(Rosenberg and Hostetter, 1993)*. Measurement of renal blood flow by clearance of PAH provides an average renal plasma flow reading for the entire experimental period and thus does not provide information about transient fluctuations in renal blood flow. The pilot study conducted before the beginning of this experiment indicated that renal blood flow was reduced by over 60% when Ang II is infused at 20 ng/kg/min for five minutes. Additionally, other studies have reported significant reductions in renal blood flow with intrarenal doses of Ang II as low as 0.5 ng/kg/min in dogs *(Fitzgerald et al., 1997)*, and 0.3 pmol/min in rats *(Fitzgerald et al., 1999)*. It is possible that the differences in gene expression seen between the Rosenberg and Hostetter (1993) study, and the series of experiments presented here, may arise from a different stimulus produced by the two doses of Ang II. Over one hour, the Rosenberg and Hostetter (1993) study reported an average decrease in renal plasma flow of approximately 22%, but the evidence presented here suggests that initially renal blood flow may have been reduced by as much as 60% or more. It is possible therefore, that the transient severe renal ischaemia produced by Ang II infusion, could have stimulated expression of early genes in this case, and that it was not associated with a growth response stimulated directly by Ang II.

2.4.3.1 Time-course of induction of gene expression in the kidney

In the present experiments, two time points were chosen to investigate the time course of induction of early gene expression in the kidney, in response to intrarenal infusion Ang II. The 30 minute infusion period was chosen as it is consistent with the time taken for expression of immediate early genes to be induced following administration of Ang II in culture systems. The 240 minute infusion period was chosen to investigate whether the stimulation of immediate early genes by Ang II was maintained with a prolonged stimulus. Indeed, both *egr-1* and *c-fos* gene expression is induced between 15 - 30 minutes in rat mesangial cell cultures, rat and human vascular smooth muscle cells cultures and rat cardiomyocytes *(Patel et al., 1996; Iwami et al., 1996; Sharma et al., 1994; Schulze-Lohoff et al., 1993; Neyses et al., 1993; Sachinidis et al., 1992b; Rupprecht et al., 1992; Lyall et al., 1992; Millet et al., 1992; Paquet et al., 1990; Naftilan et al., 1989; Kawahara et al., 1988)*. Expression of both genes has been shown to reach maximum levels anywhere between 30 minutes and 2 hours, and seems partly dependent on the length of the exposure to Ang II *(Patel et al., 1996; Iwami et al., 1996; Sharma et al., 1994; Schulze-Lohoff et al., 1993; Neyses et al., 1993; Sachinidis et al., 1992b; Rupprecht et al., 1992; Lyall et al., 1992; Millet et al., 1992; Paquet et al., 1990; Naftilan et al., 1989; Kawahara et al., 1988)*.

2.4.3.2 Technical considerations

Hybridisation of the northern blots with the *c-fos* riboprobe elicited a transcript approximately 2.2 kb in length which corresponded to the published size of the full length *c-fos* gene *(Schreiber et al., 1991)*. The size of the transcript was determined using a methylene blue stained RNA ladder that was run and blotted with the hybridised samples. Similarly, hybridisation with the *egr-1* cDNA probe elicited a 2.1 kb transcript that corresponded to the published size of the *egr-1* gene *(Sachinidis et al., 1992b)*. Both *egr-1* and *c-fos* mRNA levels were extremely low, requiring a two-week exposure to the phosphor screen to visualise the expression. The levels of both *c-fos* and *egr-1* mRNA overlapped greatly between animals in the same treatment group, and between treatments, leading to the conclusion that there was no detectable effect of Ang II treatment on mRNA levels of these genes in the kidney. Initially, *c-fos* mRNA levels were determined in a variety of tissues, indicating that levels were highest in kidney tissue from animals that received large doses of Ang II (200 ng/kg/min) intravenously, and lower yet detectable in kidneys that received intrarenal Ang II or vehicle, suggesting that levels of *c-fos* mRNA in the kidney are upregulated to a greater extent by large, supra-physiological doses of Ang II.

To overcome some of the variation between animals within the same treatment group, *c-fos* and *egr-1* expression were compared between the left infused and right control kidney from the same animal. Even within animal comparisons indicated that there was no consistent pattern of expression of *c-fos* or *egr-1* in the kidneys infused with Ang II.

In this study *c-fos* and *egr-1* mRNA levels were not detectably upregulated by intrarenal infusion of Ang II at a dose that produced significant renal vasoconstriction. Further to this, the basal mRNA levels of these genes in the kidney were extremely low and close to the limit of detection of the phosphorimaging technology. The kidney is a very dense and heterogeneous tissue consisting of many different types of cells. It is possible that the infusion of Ang II may be upregulating the expression of these genes in some tissue types but not others, and thus the increase in expression was diluted in the total kidney RNA. Indeed Ang II has been shown to increase *egr-1* expression by only 1.5 times in cultured rat mesangial cells *(Rupprecht et al., 1992)*. Likewise, if the Ang II stimulated increase in expression of these genes was occurring only in the vasculature of the kidney then the signal would be diluted by the rest of the total kidney RNA as the renal arterial vasculature comprises only approximately 1% of the total kidney volume *(Kett et al. 1995)*. It is possible that *in vivo* detection of Ang II induced changes in gene expression could be better estimated by further dissecting out the kidney tissue where these changes are thought to be important, such as the renal vasculature or the renal glomeruli. Alternatively, *in situ* hybridisation could be used to localise expression of these genes to structures within the kidney, and then compare differences in expression between animals. It is also possible that *in vivo* regulation of cellular growth cycles may be different to that *in vitro*, and require only small intermittent increases in early growth response genes to begin the cascade of cell growth.

2.4.4 Conclusion

In conclusion, our results indicate that intrarenal infusion of Ang II, at a dose that causes physiologically significant renal vasoconstriction was not associated with a detectable upregulation of *c-fos* and *egr-1* mRNA levels in the kidney *in vivo*. Nevertheless, evidence for a trophic action of Ang II in cultured cells including vascular smooth muscle cells is expansive and cannot be discounted. Additionally, evidence demonstrates that long term, low dose intra-renal and systemic infusion of Ang II causes hypertension which may, in part, be due to growth of the vasculature *(Stevenson et al., 2000; Fitzgerald et al., 1997)*. Thus the cellular changes occurring *in vivo* in situations of elevated Ang II, particularly within the kidney, and the relationship to the initiation or maintenance of hypertension still remain to be determined.

CHAPTER 3

HYPERTENSION FROM CHRONIC INTRARENAL INFUSION OF ANGIOTENSIN II

3.1 INTRODUCTION

One possible way in which Ang II may contribute to hypertension is to induce renal cellular growth or hypertrophy *in vivo*, and subsequently cause structural changes to the renal vasculature, which could alter renal function, and increase arterial pressure. To begin to investigate whether Ang II does indeed induce renal structural changes, we examined the effect of chronic, local infusion of Ang II directly into the kidney, on arterial pressure of conscious rats.

A recent publication from our laboratory demonstrated that chronic, intrarenal infusion of Ang II produces moderate hypertension in uni-nephrectomised dogs *(Fitzgerald et al., 1997)*. The rise in pressure in this study appeared to be due to the effects of Ang II primarily within the kidney.

Additional evidence also exists, supporting a role for Ang II in the kidney in hypertension, as Ang II levels are increased within the kidney in several forms of experimental hypertension, including during systemic infusion of Ang II, main renal artery stenosis and renal wrap hypertension *(Navar and Harrison-Bernard, 2000; Anderson et al., 1987; Anderson et al., 1978)*. There is also some indirect evidence for increased Ang II activity within the kidney in human hypertension including renal artery stenosis and essential hypertension *(Bender et al., 1984; Hricik et al., 1983)*.

Potentially, elevated intrarenal Ang II levels may contribute to the development of the hypertension in these models, by inducing growth related structural changes in the renal pre-glomerular vessels, subsequently increasing renal vascular resistance and stimulating the development of hypertension.

3.1.1 Aims

The aim of the present study therefore, was to determine whether chronic intrarenal infusion of low doses of Ang II, elicits hypertension in a dose-dependent manner in conscious unrestrained rats. Accurate recordings of arterial pressure are difficult to obtain in rats and thus, in this study, arterial pressure was measured at regular intervals over 24-hour periods using radio-telemetry in conscious rats, housed in their home cages and free from environmental stressors.

3.2 MATERIALS AND METHODS

In this study three groups of animals underwent a six week protocol that involved a 28 day infusion directly into the renal artery (IRA) of:

i) Vehicle (heparinised saline, 10 IU/ml)

ii) Ang II (1.5 ng/kg/min)

iii) Ang II (4.5 ng/kg/min)

An additional group of animals underwent the same six week protocol, and received a 28 day infusion of:

iv) Ang II (4.5 ng/kg/min) delivered intravenously (IV).

Cardiovascular parameters, including systolic and diastolic pressure, heart rate and activity, were recorded for a 24-hour period, twice weekly by radio-telemetry monitoring. These recordings continued throughout the twenty eight-day infusion and then for ten days after the infusion had ceased.

3.2.1 Preparation

All experimental and surgical procedures were approved by the Department of Physiology Animal Experimentation Ethics Committee as being in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

A stock solution of Ang II (500 µg/ml; Sigma Chemical Co., Australia) was made with 0.9% w/v sodium chloride (NaCl) and frozen in aliquots. On the day of experiment, a fresh aliquot was thawed and Ang II made up in 0.9% (NaCl) to the appropriate dose. Once thawed, the concentration of Ang II was adjusted to take into account the actual peptide amount, as determined by the amino acid analyses provided by the manufacturer.

At 10 weeks of age, male outbred Sprague-Dawley rats were anaesthetised with an intraperitoneal injection of a mixture of sodium pentobarbitone (30 mg/kg; Nembutal Rhone Merieux, Australia), methohexitone sodium (40 mg/kg; Brietal Sodium, Eli Lilly, Australia), sterile 0.9% NaCl and underwent a left nephrectomy.

Four weeks later the animals underwent a second operation involving the implantation of an osmotic minipump (Alzet, model 2ML4, California, USA) and telemetry device (Data Sciences International, MN, USA).

3.2.1.1 Implantation of minipump

The technique of infusing directly into the kidney via the suprarenal artery in rats was adapted from a method first described by Smits *et al.* (1983). Rats were anaesthetised as described above and placed on a heating table. The renal artery of the right kidney was exposed via a midline incision and the right suprarenal artery was isolated and cleared. A soft vinyl catheter, filled with heparinised saline (10 IU/ml) was inserted into the suprarenal artery and advanced to the junction of the renal artery, then secured in place with silk and tissue glue (Vet bond, 3M animal care products, Germany). The free end of the catheter was attached to a 28 day osmotic minipump (primed overnight at 37°C in 0.9% NaCl), that contained either Ang II (Sigma Chemical Co., Australia) dissolved in heparinised saline (10 IU/ml in 0.9% w/v NaCl), or vehicle (heparinised saline, 10 IU/ml in 0.9% w/v NaCl). The catheter was secured to the minipump using silk thread and superglue. The minipump was allowed to float freely within the intraperitoneal cavity. Rats were divided into four groups and received a 28 day infusion of either vehicle (n=3), Ang II (1.5 ng/kg/min; n=4), Ang II (4.5 ng/kg/min; n=4) delivered intrarenally, or Ang II (4.5 ng/kg/min; n=6), delivered intravenously via a catheter inserted into a lumbar branch of the vena cava.

3.2.1.2 Implantation of telemetry device

Following attachment of the minipump, a radio telemetry blood pressure device was implanted into the lower abdominal aorta (see Figure 3.2.1). In brief, the lower abdominal aorta was isolated and the aorta was clamped below the level of the renal arteries. A small hole was made in the aorta and the catheter of the telemetry device was quickly inserted then secured with a small piece of gauze and tissue glue. The clamp on the aorta was then released to restore blood flow to the hindlimb region. The telemetry implant was secured into the muscle layer with silk thread to prevent tension being applied to the catheter.

3.2.1.3 Recovery

During the post-operative period the rats were given a subcutaneous injection of buprenorphine hydrochloride (Temgesic, Reckitt & Coleman Pharmaceuticals, Australia) at a dose of 0.1 mg/kg. The animals were allowed 3-7 days to recover from surgery before any measurements were made.

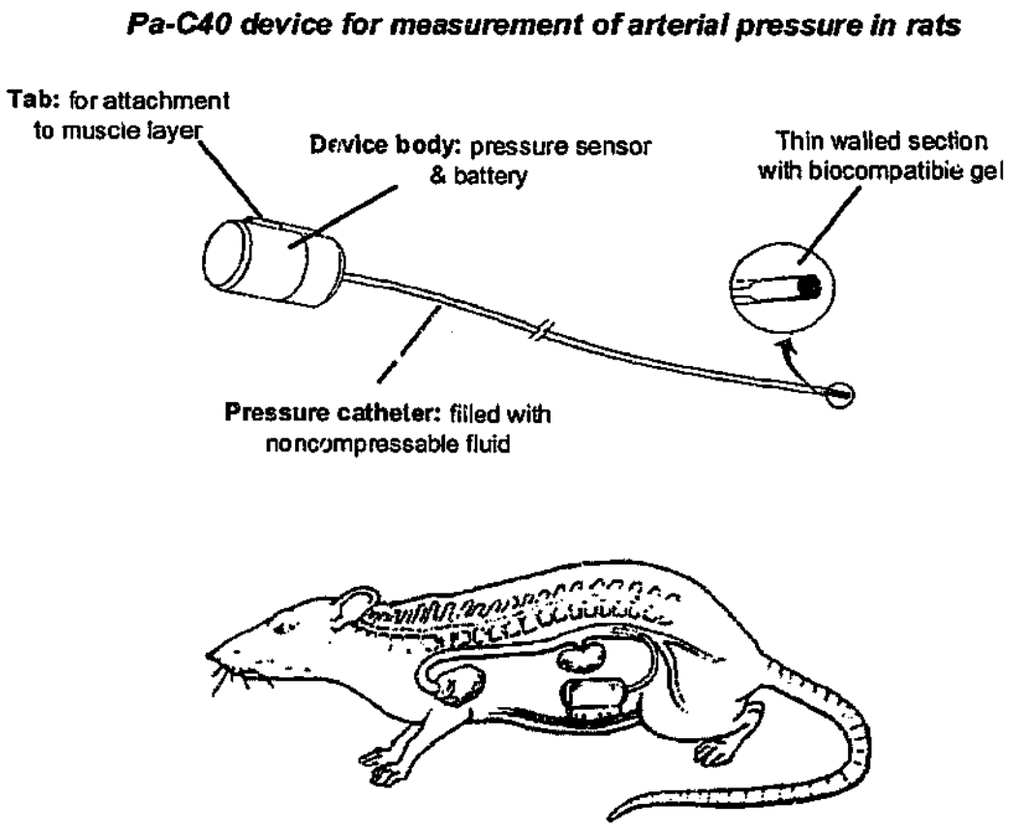


Figure 3.2.1: Pa-C40 telemetry implant for the measurement of arterial pressure in conscious, freely moving rats. The telemetry implant is glued into the lower abdominal aorta and the pressure sensor device secured to the muscle layer of the abdominal wall. Figure modified from 'PA device surgical manual' (DSI, Data Sciences International, 1995, USA).

3.2.2 Experimental protocol/data collection

Unrestrained rats, housed in their home cages, were placed onto the telemetry receiver plates for the measurement of cardiovascular parameters. Receiver plates were connected to a computer with the Data Sciences International software (Dataquest® LabPRO™, Data Sciences International, MN, USA) to enable collection of data. Recordings of systolic and diastolic pressure, heart rate and activity of the animals were made twice a week for a 24-hour period. The collection of data occurred in this manner for the entire infusion period and for ten days post-infusion. Within the 24-hour period, the data was collected for a 60-second interval every 15 minutes. The data for each animal was averaged into twelve-hour night or day blocks according to the light/dark cycle of the animal house. Both day and night data were then grouped into four-day intervals and this was used for analysis. Grouping the data into four-day intervals was necessary because the animals had surgery on different days and there were also only six receiver plates available at one time. The four-day interval ensured that measurements were made in every animal during that period.

Throughout the experimental period rats were weighed three times a week. At *post-mortem* the heart and right kidney were collected and wet and dry weights recorded for whole heart, left ventricle and right kidney. Also, at *post-mortem* the position of the catheter in the suprarenal artery and its attachment to the osmotic minipump was confirmed, and the telemetry implant was carefully removed and the pressure offset measured on the receiver plates. All arterial pressure measurements were corrected for the pressure offset value before data was manipulated.

3.2.3 Criteria for successful intrarenal infusion

The osmotic minipump and catheter were removed at *post-mortem*, ten days after the infusion ceased. At this time, not one of the suprarenal catheters was patent (ie could not pass fluid through into the kidney). Therefore a criteria was established to best determine if the infusion had been successful.

The following criteria were established in order for the infusion to be deemed successful:

i) The catheter tubing had to be clear of blood or blood clots.

ii) The diameter of catheter tubing was not increased in size.

iii) The catheter had to be in the correct position in the animal (ie still in place in the suprarenal artery)

iv) No evidence of blood in the pump.

Only those animals deemed to have received a successful infusion by the criteria were included in the study.

	Vehicle *Heparinised saline 10 IU/ml; IRA*	*Ang II* *1.5 ng/kg/min; IRA*	*Ang II* *4.5 ng/kg/min; IRA*	*Ang II* *4.5 ng/kg/min; IV*
TOTAL	9	9	11	9
"Successful" infusion	3	4	4	6
"Unsuccessful" infusion	6	5	6	3

Table 3.1: Total number of 'successful' and 'unsuccessful' animals used in telemetry study.
All data presented in this chapter is from animals deemed to have received a successful infusion based on the above criteria.

3.2.4 Statistical analysis

Data was pooled into 4 day intervals and a group mean ± SEM generated for all parameters during both the light and dark cycle of the animals. Statistical analysis was performed using SYSTAT™ 5.0 statistical software package (SPSS Inc., USA).

In all groups that received an intrarenal infusion of Ang II (1.5 and 4.5 ng/kg/min), there appeared to be a peak in arterial pressure around day 8 to 15 of the infusion (refer to Section 3.3). After day fifteen the response to intrarenal infusion of Ang II became inconsistent, and we concluded that this may have been due to the catheters becoming blocked. For this reason, statistical analysis was performed on the data only over day four to fifteen of the infusion.

Two way analysis of variance (ANOVA), partitioned for dose, was used to test for a significant dose-dependent and time dependent effect of intrarenal Ang II infusion on systolic and diastolic pressure in conscious rats over day 4-15 of the infusion period. Heart rate over day 4-15 was analysed for statistical differences between intrarenal infusion treatments using a two-way ANOVA. A one way ANOVA, partitioned for dose, was used to test for a significant dose-dependent effect of intrarenal infusion of Ang II on systolic and diastolic pressure in rats during day 8-11 of the infusion period. Activity over day 8-11 was analysed for differences in intrarenal treatments using a one way ANOVA. Likewise, a one way ANOVA was used to test if there was a significant difference in systolic and diastolic pressure of animals that received Ang II (4.5 ng/kg/min) administered intrarenally, or intravenously.

3.3 RESULTS

3.3.1 Chronic intrarenal infusion of angiotensin II – individual rat data

Twenty-four hour telemetry recordings of systolic pressure, diastolic pressure, and heart rate in individual rats that received an intrarenal infusion of Ang II (1.5 or 4.5 ng/kg/min) or vehicle (heparinised saline) were performed twice weekly, over the over the entire six week experimental period. In animals that received intrarenal Ang II there is evidence of an early peak in arterial pressure around day 8-15 of the infusion (Figure 3.3.1). Additionally, intrarenal infusion of Ang II appears to markedly increase the variability of the arterial pressure between animals in the same treatment group, and also within individual animals (Figure 3.3.1, middle and right hand panels). In contrast, animals that received an intrarenal infusion of vehicle displayed little variation in arterial pressure both between animals in the same treatment group, and within the same animal on consecutive measurements (Figure 3.3.1, left hand panels). Moreover, intrarenal vehicle infusion was not associated with any apparent peak in arterial pressure around day 8-15 of the infusion period. Similar trends were observed during both the night and day time recording periods in animals that received either vehicle or Ang II (1.5 or 4.5 ng/kg/min) intrarenally.

3.3.2 Chronic intrarenal infusion of angiotensin II – group data

Over day four to fifteen of the infusion period, the night time systolic pressure was, on average, approximately 17 mmHg greater in the group that received an intrarenal infusion of Ang II (4.5 ng/kg/min; 143 ± 5 mmHg) when compared to the vehicle infused group (126 ± 1 mmHg; Figure 3.3.2, upper right hand panel). Likewise, during the same period, night time systolic pressure was on average 6 mmHg greater in rats that received intrarenal Ang II (1.5 ng/kg/min; 132 ± 5 mmHg) when compared to the vehicle treated group. Diastolic pressure of the animals followed similar trends (Figure 3.3.2, lower left and right hand panels). The mean night time diastolic pressure was also approximately 17 mmHg greater in the animals infused with intrarenal Ang II (4.5 ng/kg/min; 110 ± 4 mmHg) when compared to the vehicle infused group (93 ± 2 mmHg). Also, the diastolic pressure in the Ang II (1.5 ng/kg/min) group was approximately 5 mmHg greater on average when compared to the control group.

A two way analysis of variance demonstrated a significant effect of intrarenal Ang II infusion on night time systolic and diastolic pressures over day 4-15 of the infusion period (P_{dose} = 0.02 and P_{dose} = 0.008 respectively; Figure 3.3.2 upper and lower right hand panels). However, the increase in night time arterial pressure in

response to intrarenal Ang II infusion did not change with time (P_{time} = 0.62, and P_{time} = 0.53, systolic and diastolic pressure respectively). Similar results were demonstrated during the day time period (P_{dose} = 0.01, 0.002, P_{time} = 0.63, 0.66 for day time systolic and diastolic pressures respectively; Figure 3.3.2 upper and lower left hand panels).

At the end of the experimental period on days 36-39, the arterial pressure in animals treated with Ang II (1.5 ng/kg/min and 4.5 ng/kg/min) appeared not different from the vehicle treated group, across both night and day periods (Figure 3.3.2). Heart rate (Figure 3.3.3) over day four to fifteen of the infusion, was not significantly different between animals that received intrarenal infusions of vehicle, Ang II (1.5 ng/kg/min), or Ang II (4.5 ng/kg/min; P_{dose} = 0.5).

3.3.3 Intrarenal infusion of angiotensin II – day 8 –11 of the infusion period

When the period in which arterial pressure appeared to peak in animals treated with intrarenal Ang II was analysed separately, night time systolic pressure over day 8-11 of the intrarenal infusion was 124 ± 1 mmHg in the vehicle treated group compared with 137 ± 8 and 150 ± 7 mmHg respectively in animals that received an intrarenal infusion of Ang II (1.5 and 4.5 ng/kg/min, respectively; P_{sys} = 0.03, Figure 3.3.4 lower panel). Likewise, intrarenal Ang II infusion had a dose-dependent effect on night time diastolic pressure during this period (Figure 3.3.4, lower panel). Night time diastolic pressure was 92 ± 4 mmHg in the vehicle treated animals, 105 ± 5 mmHg and 115 ± 6 mmHg in the 1.5 and 4.5 ng/kg/min Ang II treated animals, respectively (P_{sys} = 0.02; Figure 3.3.4 upper panel). The dose-dependent effect of intrarenal infusion of Ang II was also evident in the day time diastolic and systolic pressures over day 8 – 11 of the infusion (P_{sys} = 0.03 and P_{dia} = 0.03 respectively, Figure 3.3.4 upper panel. Whilst activity was, as expected, higher during night time than during the day, there was no significant difference in activity between treatment groups, for either period over day 8-11 of the infusion (P_{dose} = 0.65 and P_{dose} = 0.59; activity during day and night respectively; Figure 3.3.5).

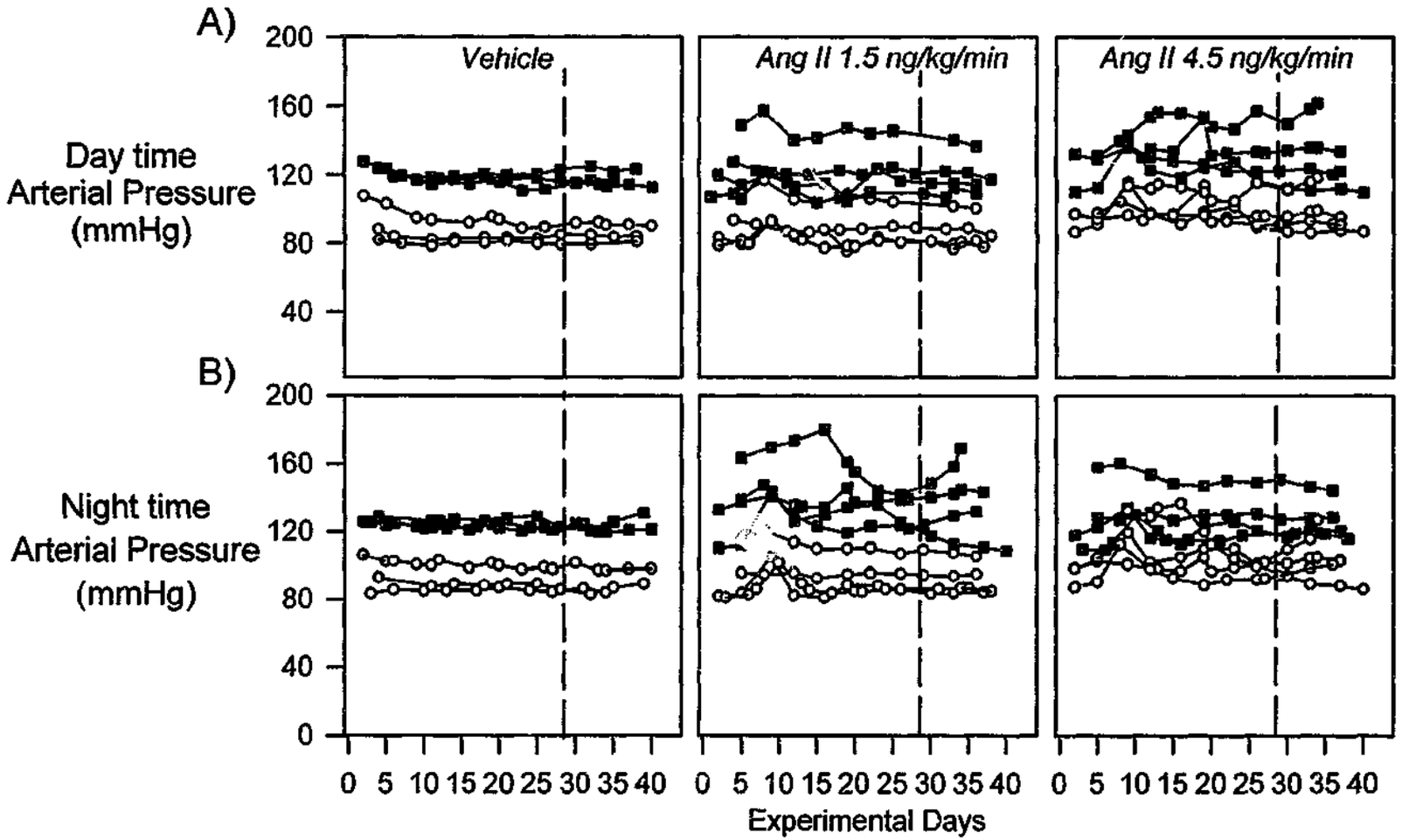


Figure 3.3.1: Diastolic (open circles) and systolic (closed squares) pressure in individual animals that received an intrarenal infusion of vehicle (heparinised saline 10 IU/ml; left hand panels), Ang II (1.5 ng/kg/min; middle panels) or Ang Ii (4.5 ng/kg/min; right hand panels) for 28 days. Data presented is only from those animals deemed to have received a successful infusion as indicated in the text. Each data point was obtained by averaging measurements over a 12-hour interval which corresponded to the animals light (daytime; A), or dark (night time; B) cycle. The dashed line represents the end of the infusion period. Arterial pressure measurements were made twice weekly for the entire 28 day infusion period and for 10 days after the infusion had ceased.

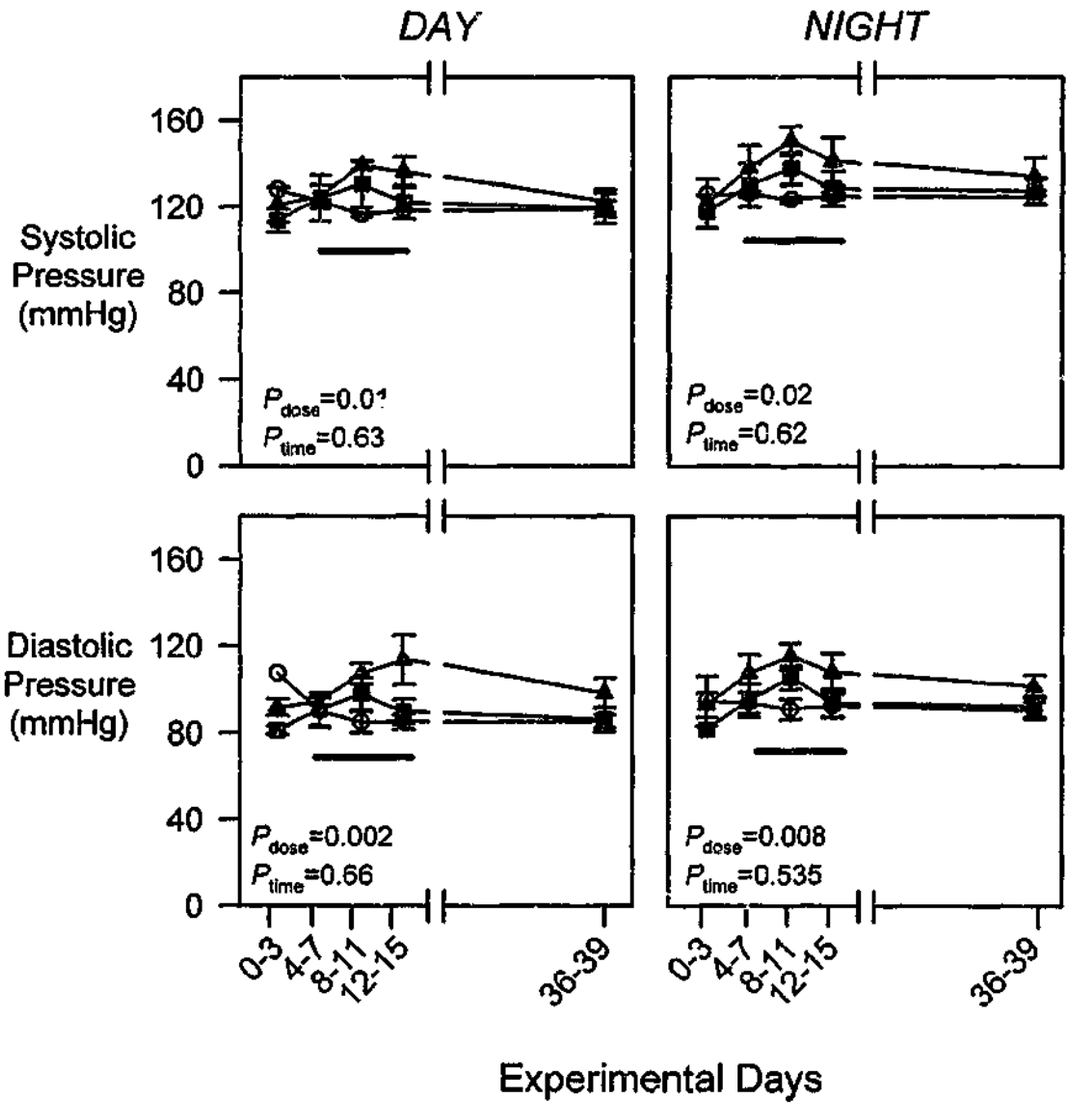


Figure 3.3.2: The effect of intrarenal infusion of vehicle (heparinised saline 10 IU/ml; open circles, n = 3), Ang II (1.5 ng/kg/min; solid squares, n = 4), or Ang II (4.5 ng/kg/min; solid triangles, n = 4) on systolic (upper panels) and diastolic (lower panels) arterial pressure in conscious rats. Data was pooled into 4 day intervals and presented as group mean ± SEM. Experimental days 36-39 represent the 'off' period, after the intrarenal infusion had ceased. Data corresponding to the animals light (day) cycle is presented in the left hand panels, and dark (night) cycle in the right hand panels. A two-way ANOVA (partitioned for dose) was used to test for a significant dose-dependent (P_{dose}) or time-dependent (P_{time}) effect of intrarenal Ang II infusion on arterial pressure over day 4–15 of the infusion period.

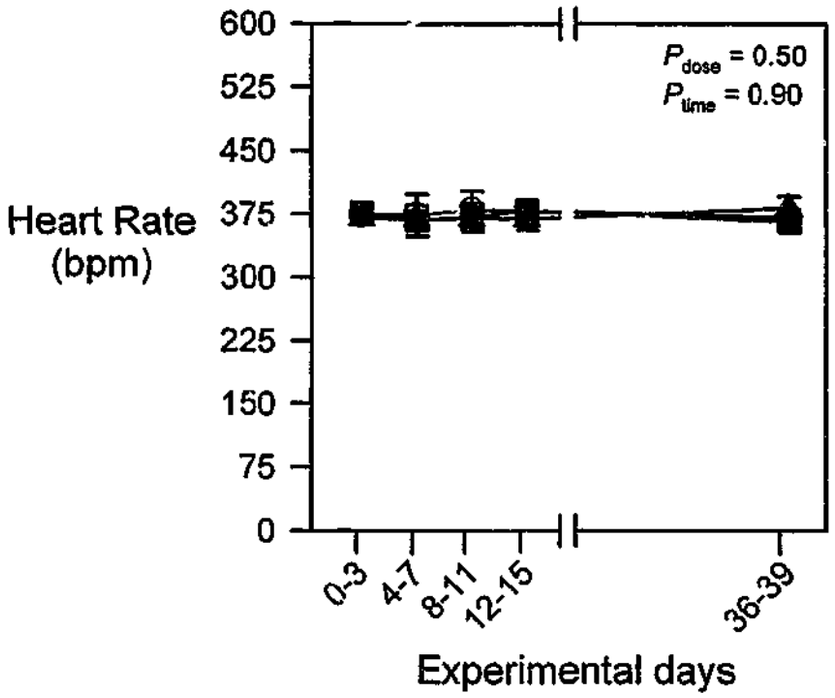


Figure 3.3.3: Effect of a 28 day intrarenal infusion of vehicle (heparinised saline 10 IU/ml; open circles), Ang II (1.5 ng/kg/min; solid squares), or Ang II (4.5 ng/kg/min; solid triangles) on night time heart rate in conscious rats. The data was pooled into 4 day intervals and represented as group mean ± SEM. Experimental days 36-39 represent the 'off' period, after the intrarenal infusion had ceased. A two way ANOVA (partitioned for dose) demonstrated that there was no significant dose-dependent, or time-dependent effect of intrarenal Ang II infusion on conscious night time heart rate, analysed over day 4-15 of the infusion period.

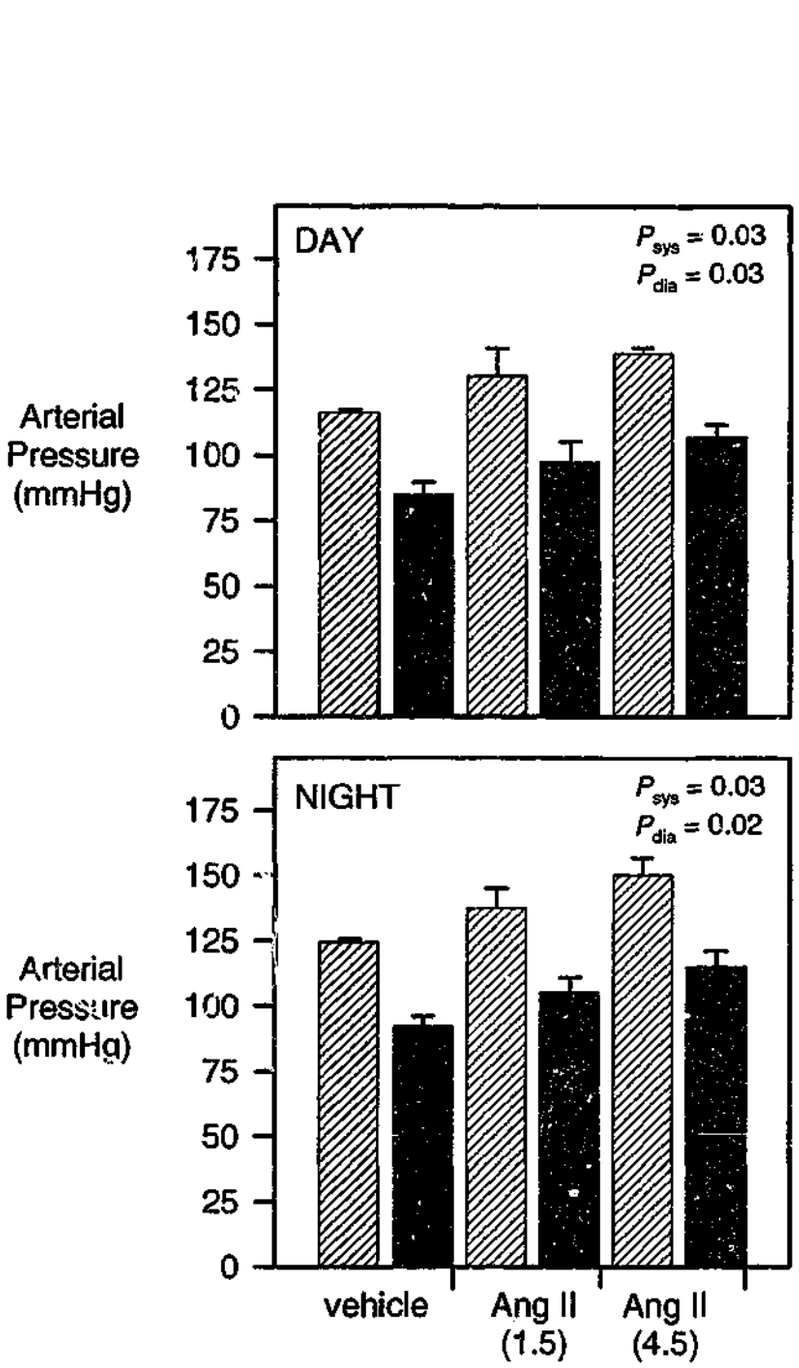


Figure 3.3.4: Systolic (hatched bars) and diastolic (solid bars) pressure in conscious rats that received an intrarenal infusion of either vehicle (heparinised saline 10 IU/ml; blue bars), Ang II (1.5 ng/kg/min; red bars, middle) or Ang II (4.5 ng/kg/min; red bars, right). The data was pooled over day 8 - 11 of the infusion period and presented as group mean ± SEM. Data corresponding to the animals light (day time) cycle is presented in the upper panel, and dark (night time) cycle in the lower panel. A one way ANOVA was used to test for a significant dose-dependent effect of intrarenal Ang II infusion on systolic (P_{sys}) and diastolic pressure (P_{dia}) over day 8 – 11 of the infusion, during both day and night time periods.

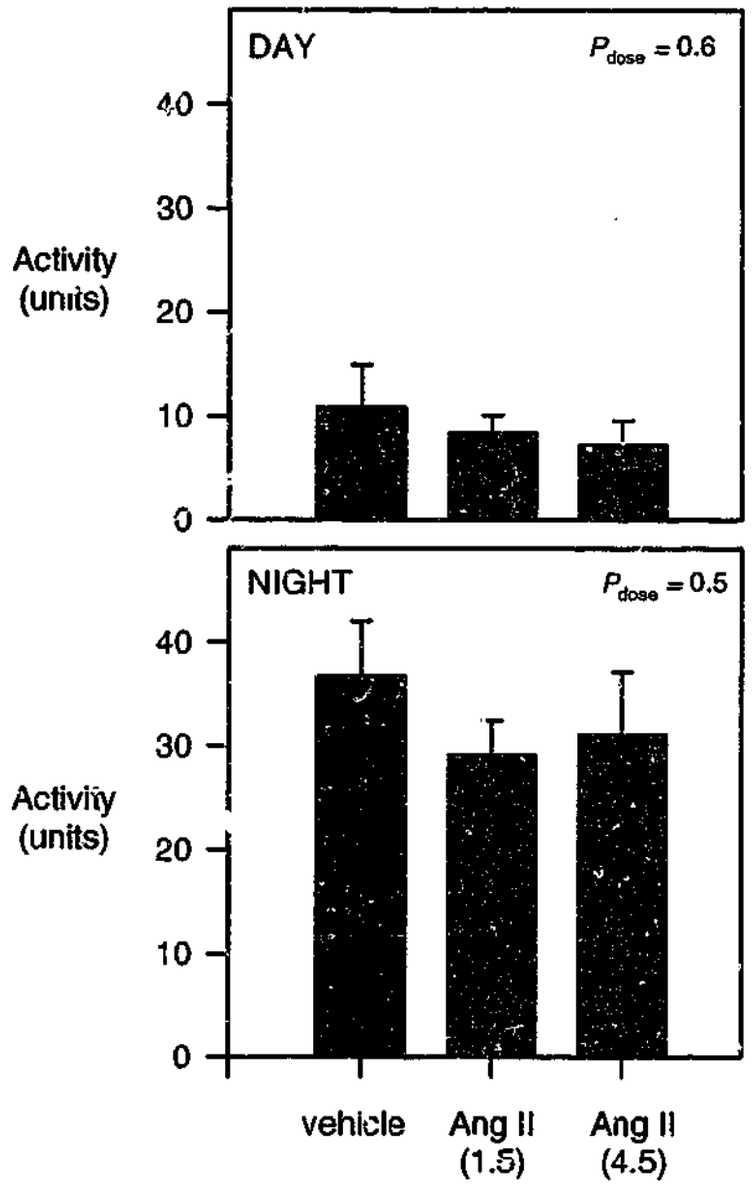


Figure 3.3.5: The effect of intrarenal infusion of either vehicle (heparinised saline 10 IU/ml; blue bars), Ang II (1.5 ng/kg/min; pink bars), or Ang II (4.5 ng/kg/min; red bars) on activity (arbitrary units) in conscious rats during both day (upper panel) and night (lower panel). Data was pooled over day 8 – 11 of the infusion period and presented as mean ± SEM. A one way ANOVA demonstrated that there was no significant dose-dependent effect of intrarenal Ang II infusion on activity in conscious rats.

3.3.4 Intravenous versus intrarenal infusion of angiotensin II (4.5 ng/kg/min)

An additional group of animals received an intravenous infusion of Ang II (4.5 ng/kg/min, n=6). As an intravenous vehicle group was not performed in these experiments, night time systolic pressure in the intrarenal vehicle group was therefore compared with animals that received an intravenous infusion of Ang II (4.5 ng/kg/min), across the entire experimental period (Figure 3.3.6). Over the twenty-eight day infusion, the night time systolic blood pressure of the intravenous Ang II group (4.5 ng/kg/min) averaged 154 ± 5 mmHg. This was approximately 20 mmHg above the systolic pressure recorded in these animals on day 36-39, beyond the life of the pump, and therefore when the infusion had ceased (Figure 3.3.6).

Over day 8-11 of the infusion period, night time systolic (156 ± 7 mmHg), and diastolic pressure (124 ± 6 mmHg) of animals that received an intravenous infusion of Ang II (4.5 ng/kg/min) were not significantly different from the systolic (150 ± 6.6 mmHg) and diastolic (115 ± 6 mmHg) pressure of animals treated with intrarenal Ang II (4.5 ng/kg/min; P_{sys} = 0.5, P_{dia} = 0.4, respectively; Figure 3.3.7). Similar results were also observed during the day time recording period (P_{sys} = 0.2, P_{dia} = 0.3).

3.3.5 Post-mortem measurements

There was no significant dose-dependent effect of intrarenal Ang II infusion on any of the parameters measured at *post-mortem* (Table 3.2).

	Vehicle	Ang II 1.5ng/kg/min	Ang II 4.5ng/kg/min	P<0.05
Body weight (BW) (g)	505 ± 38	509 ± 22	525 ± 6	NS
Heart wet weight (WH) (g)	1.2 ± 0.2	1.2 ± 0.1	1.27 ± 0.03	NS
WH:BW (10^{-3})	2.3 ± 0.2	2.3 ± 0.1	2.4 ± 0.1	NS
Left ventricle wet weight (LV) (g)	0.87 ± 0.13	0.85 ± 0.08	0.95 ± 0.04	NS
LV:BW (10^{-3})	1.70 ± 0.14	1.66 ± 0.08	1.80 ± 0.08	NS
Right kidney dry weight (g)	0.54 ± 0.06	0.50 ± 0.04	0.54 ± 0.03	NS
Right kidney: BW (10^{-3})	1.06 ± 0.03	0.97 ± 0.04	1.02 ± 0.05	NS

Table 3.2: Organ and body weight data on the day of *post-mortem* for all animals receiving an intrarenal (IRA) infusion. All data represented as mean ± SEM. A one way ANOVA was used to test for a significant dose-dependent effect of intrarenal Ang II infusion.

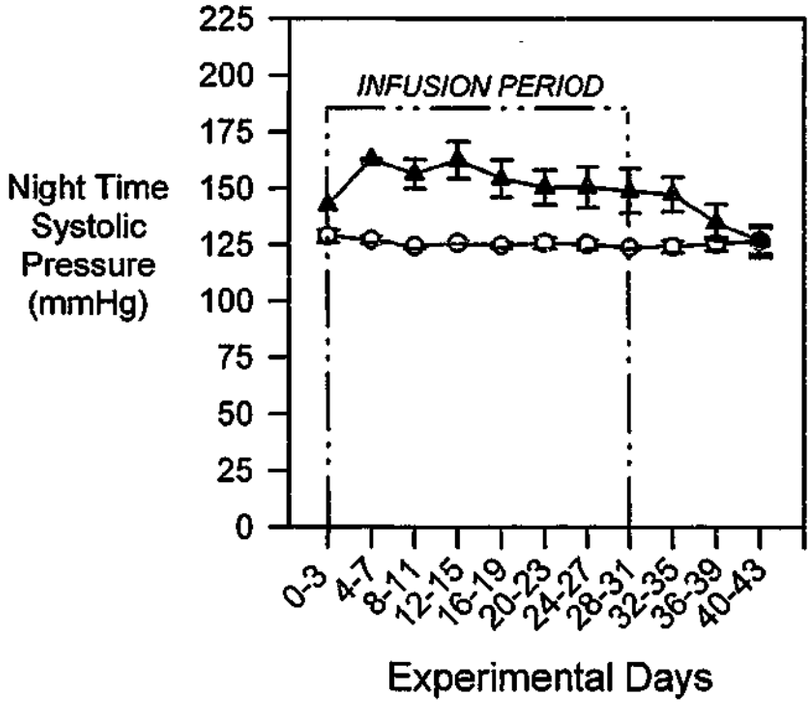


Figure 3.3.6: Effect of a 28 day intravenous infusion of Ang II (4.5 ng/kg/min; solid triangles), or intrarenal infusion of vehicle (heparinised saline 10 IU/ml; open circles) on night time systolic pressure in conscious rats. Data was pooled into 4 day intervals and is presented as group mean ± SEM. Where error bars cannot be seen, they fall within the symbol.

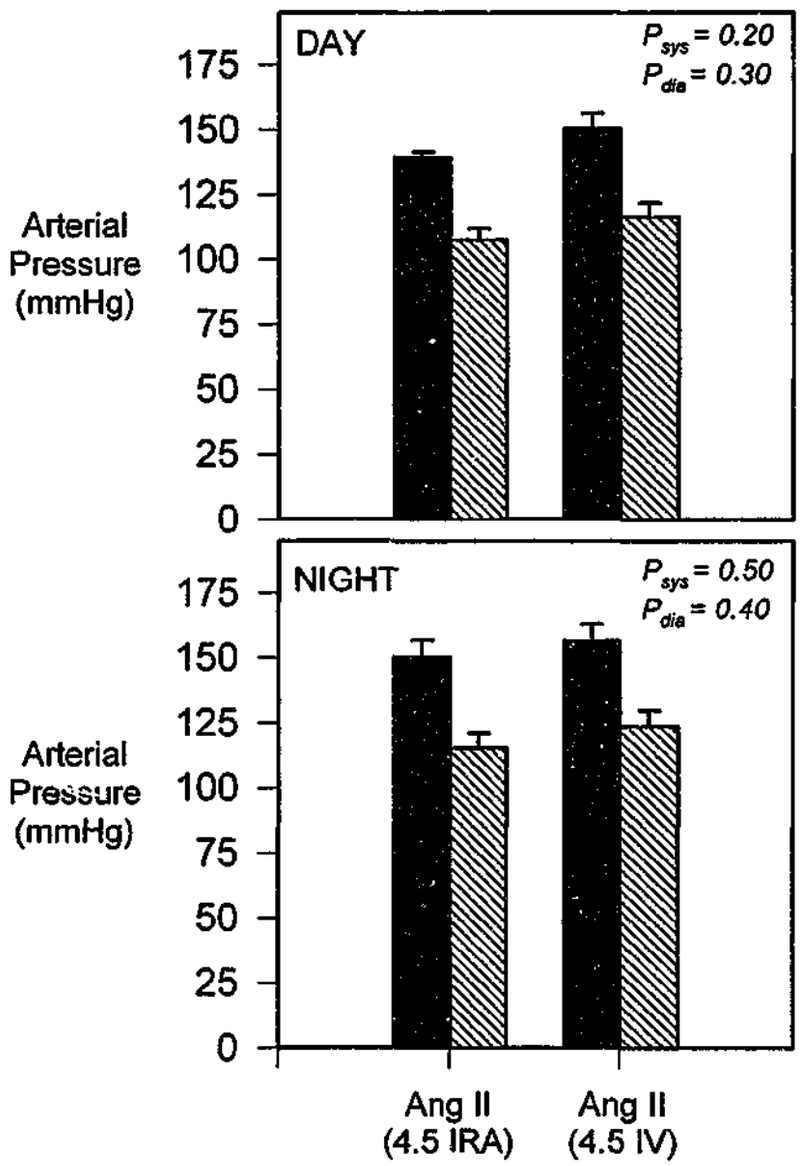


Figure 3.3.7: Effect of intrarenal infusion of Ang II (4.5 ng/kg/min; left hand bars), or intravenous infusion of Ang II (4.5 ng/kg/min, right hand bars) on systolic (black bars) and diastolic (hatched bars) pressure in conscious rats. Data was pooled over day 8 – 11 of the infusion period and is presented as group mean ± SEM during the corresponding light (day; upper panel) and dark (night; lower panel) cycle of the animals. Analysis of variance demonstrated that there was no significant effect of the route of administration of Ang II (4.5 ng/kg/min) on diastolic (P_{dia}) or systolic pressure (P_{sys}) in conscious rats during both the day and night time period.

3.4 DISCUSSION

The purpose of the present study was to determine if chronic intrarenal infusion of low doses of Ang II, induced hypertension in a dose-dependent manner in rats. To further dissect out the effect of Ang II on arterial pressure in these animals, we used telemetry to monitor 24-hour blood pressure in freely moving conscious rats housed in their home cages. As the rats are not subjected to human handling, or invasive procedures to measure arterial pressure, they are consequently free of any environmental stressors, and thus, telemetry recordings provide an accurate and reliable method of arterial pressure measurement.

3.4.1 Successful infusion into the renal artery – a difficult technique

I was initially interested in using telemetry to monitor the animals' blood pressure throughout the infusion period and then for 10 days following cessation of infusion. However the protocol was complicated by the fact that the technique of renal arterial infusion in rats was difficult and had a very low success rate. Additionally, at *post-mortem*, 10 days after the intrarenal infusion had ceased, not one of the catheters placed in the suprarenal artery were patent and it was therefore difficult to assess if the infusion into the kidney had been successful. To overcome these problems, criteria were established to determine at *post-mortem*, whether the infusion into the kidney had been successful. It is evident from the animals that met the criteria, that infusion into the suprarenal artery in rats was successful in only approximately 30% of cases. This is in contrast to a report by Smits *et al.* (1983), who originally developed the technique and reported a success ratio over 90% for infusion periods up to 14 days. I can only suggest that the length of the current protocol might have contributed to the considerably lower success rate of infusion in this study.

The arterial pressure recorded in individual animals treated with intrarenal Ang II appeared to peak around day 8-15 of the infusion period, when compared to the vehicle group. After day 15 of the infusion the arterial pressure response in these animals became more ambiguous and tended to trail off. As the criteria that were used to determine whether the intrarenal infusion was successful did not require that the infusion catheters were patent, it is possible that the decline in arterial pressure after day 15 in the Ang II treated animals, might be due to the infusion catheters becoming blocked or endothelialised. It is unlikely that the inconsistent arterial pressure response after day 15 of Ang II infusion was due to a technical problem with the 2ML4 osmotic minipumps as they have been shown to accurately pump at the described rate (2.25

µl/hr) for 28 days (Alzet, model 2ML4, California, USA). In an attempt to minimise the factors that complicated the present protocol, the data presented in this chapter are only from animals in which the renal arterial infusion was deemed successful by the criteria mentioned in Section 3.2.3. As the blood pressure response to intrarenal Ang II in individual animals became ambiguous after day 15, and assessment of catheter patency was impossible at this time, statistical analysis of cardiovascular variables was therefore performed only over days 4-15 of the infusion.

3.4.2 Individual blood pressure responses to intrarenal infusion of angiotensin II

One particularly noticeable feature of the results is that individual rats that received an intrarenal infusion of Ang II displayed greatly increased variability of arterial pressure both within the same animal on consecutive measurements, and between animals in the same treatment group when compared to the vehicle group. Increased variability of blood pressure over time has also been demonstrated in human hypertensive patients and has been related to end-organ hypertensive damage *(Parati et al., 1986; Mancia et al., 1985)*. In contrast, the arterial pressure of animals receiving an intrarenal infusion of vehicle displayed very little variability between animals and within the same animal on consecutive measurements. This observation suggests that arterial pressure measurements recorded using radio telemetry provide reliable and highly reproducible information about arterial pressure in conscious, unrestrained animals. Previously studies have also demonstrated that telemetry measurement of arterial pressure correlates closely with conventional methods of recording, and additionally, provides an accurate and reliable method for detailed investigation of blood pressure data in conscious and non-stressed animals *(Guiol et al., 1992; Brockway et al., 1991; Armentano et al., 1990)*.

3.4.3 Intrarenal angiotensin II infusion – effect on blood pressure

Intrarenal infusion of Ang II resulted in a dose-dependent increase in both systolic and diastolic pressures. Further, the effect of intrarenal Ang II on arterial pressure was consistent throughout the 12-hour day time and night time, or sleep and wake periods, of the animals. Recent studies arising from our laboratory have also demonstrated hypertension induced by intrarenal infusion of low doses of Ang II in dogs *(Fitzgerald et al., 1997)* and more recently in rats *(Stevenson et al., 2000)*. However, mean arterial pressure was measured only once a week in the dogs receiving intrarenal Ang II *(Fitzgerald et al., 1997)*, and once only on the final day of

infusion in the rats, that had undergone brief anaesthesia to implant a catheter to measure arterial pressure *(Stevenson et al., 2000)*. In the present study, the use of radio telemetry to monitor arterial pressure in conscious, unrestrained rats housed in their home cages, allowed for a more thorough investigation into the arterial pressure response to intrarenal Ang II infusion. Using telemetry, arterial pressure, heart rate and activity can be recorded at regular intervals throughout the day and night, while the animals were free to move, housed in their home cages, and not exposed to any environmental stressors. Additionally, measurements made at regular intervals over long periods of time are beneficial, as a 24-hour profile can be constructed for individual animals to more closely examine the effect of infusing Ang II intrarenally on arterial pressure.

Consistent with the previous studies *(Fitzgerald et al., 1997, Stevenson, 2000 #367)* of intrarenal Ang II infusion in our laboratory, the dose-dependent increase in arterial pressure recorded in this study was not due to an increase in the heart rate as there was no significant difference in heart rate between any treatment groups.

3.4.3.1 Day 8-11 of the infusion period

The dose-dependent rise in arterial pressure of the animals treated with Ang II appeared to peak around day 8-11 of the infusion. Moreover, both systolic and diastolic pressure was increased to a greater extent during the night time period, which corresponded to the rats most active period. Rats are nocturnal animals and this is reflected in the higher activity recorded during the night time period, and is also consistent with increase in arterial pressure seen in every group throughout the same period. Despite this apparent increase in activity during the night time period, intrarenal Ang II infusion had no effect on activity during both the day and night periods, when compared to the vehicle treated animals, suggesting that the increase in arterial pressure in animals treated with Ang II was not due to increase in activity.

3.4.3.2 Organ and body weights at post-mortem

There was no significant difference in any of the organ or body weights measured at *post-mortem*, on day 36-39 of the protocol, including left ventricular:body weight ratio, an indicator of left ventricular hypertrophy. Hypertension is commonly associated with left ventricular hypertrophy *(Frohlich, 1986)*, and thus it might be expected that there would be some degree of hypertrophy in the animals with hypertension induced with intrarenal Ang II infusion. However, as the animals were sacrificed ten days after the infusion had ceased, it is possible that any left ventricular hypertrophy may have been resolved in this time.

3.4.4 Intravenous vs intrarenal infusion of angiotensin II

An additional group of animals were infused with Ang II at 4.5 ng/kg/min intravenously. On average, over the entire experimental period intravenous infusion of Ang II increased systolic blood pressure approximately 20 mmHg above the level recorded once the infusion had ceased. Systolic and diastolic pressure increased to a similar extent when Ang II (4.5 ng/kg/min) was administered systemically or directly into the kidney. There are multiple systemic and intrarenal actions of Ang II that could be acting in both models to increase arterial pressure, but the exact mechanisms responsible for the similar increase in arterial pressure seen when Ang II is infused intrarenally, or intravenously have not been investigated in this study, and indeed remain somewhat unresolved in the literature.

3.4.4.1 Systemic vs intrarenal angiotensin II – different mechanisms to increase pressure?

Possible intrarenal mechanisms by which Ang II may cause hypertension are summarised in Chapter 1, Section 1.3.3.2 but include Ang II dependent vasoconstriction of the renal vasculature, subsequently increasing renal vascular resistance, and stimulation of proximal tubule sodium reabsorption among other effects. Alternatively, Ang II may cause growth of the renal vasculature, as previously shown in cultured renal arteriolar cells *(Dubey et al., 1992)*. Vascular hypertrophy is common in many hypertensive states, and infusion of Ang II in low doses systemically is associated with hypertrophy of systemic vascular beds, that is partly attributable to Ang II-induced growth *(Simon and Altman, 1992; Griffin et al., 1991)*. However Guyton and others have suggested that hypertension cannot be maintained without renal defect *(Guyton et al., 1972)*. Increased Ang II levels within the kidney *in vivo*, therefore, might induce cellular growth, leading to hypertrophy of the renal vasculature and a reduction in lumen diameter, increasing pre-glomerular vascular resistance, and subsequently acting to increase blood pressure via a mechanism similar to main renal artery stenosis *(Anderson et al., 1995; Ruilope, 1994)*.

There is evidence suggesting that some of the mechanisms responsible for the increase in arterial pressure seen when low doses of Ang II are infused intrarenally, may also play role in increasing arterial pressure when low doses of Ang II are infused systemically. Angiotensin II levels are elevated in the kidney in many forms of experimental hypertension including when Ang II is infused systemically *(Zou et al., 1996a; Von Thun et al., 1994)*. In these models of hypertension, intrarenal Ang II levels are elevated above the level found in plasma and appear to result from renal accumulation of the systemically delivered Ang II, as well as stimulation of intrarenal

production of Ang II *(Navar and Harrison-Bernard, 2000; Zou et al., 1996b)*. Therefore, increases in Ang II within the kidney, through intrarenal or systemic infusion, could play a critical role in the development of hypertension in this model, potentially via multiple intrarenal actions, including stimulation of renal vascular cellular growth and hypertrophy by Ang II. Although there is little evidence for salt and water retention, or direct vasoconstriction when Ang II is infused at low doses both intrarenally *(Fitzgerald et al., 1997)*, or systemically *(Griffin et al., 1991; Brown et al., 1981)*, very small changes in salt and water balance can lead to alterations in arterial pressure *(Guyton, 1992)*. Hence alterations in salt and water balance induced when Ang II is infused systemically or intrarenally may be difficult to detect, and thus the contributions these mechanisms make to the increase in arterial pressure would be very hard to assess.

It is also important to note however, that there are also many actions of Ang II outside the kidney that could equally contribute to the increase in arterial pressure observed when Ang II was infused systemically, and these are summarised in Chapter 1, Section 1.3.3.1, but include actions on the sympathetic nervous system, adrenal gland, and a direct vasoconstrictive action. In addition, while evidence suggests that intrarenal Ang II levels are increased in a number of Ang II-dependent models of hypertension, including when Ang II is infused systemically, intrarenal or systemic levels of Ang II were not measured in this study, and as such need to be confirmed in future studies where hypertension is induced with both intrarenal and intravenous infusion of Ang II. Measurement of both intrarenal and plasma levels of Ang II in these models of hypertension, may help to dissect the mechanism by which hypertension is induced with intrarenal and intravenous Ang II infusion, and may also confirm that the renal levels of Ang II are similar in both models.

3.4.5 Conclusions

In summary, the present study demonstrates that infusing low dose Ang II intrarenally into uni-nephrectomised rats produces a significant and dose-dependent increase in arterial pressure. This finding is in agreement with previous reports from our laboratory of intrarenal infusion of Ang II, in dogs and rats studies *(Fitzgerald et al., 1997, Stevenson, 2000 #367)*. In addition to these earlier studies, the effect of intrarenal Ang II infusion on arterial pressure has been examined using radio-telemetry recording in conscious, unrestrained animals, housed in their home cages and free of any environmental stressors. The dose-dependent effects of intrarenal Ang II infusion were observed in both systolic and diastolic pressure, and were also consistent across the sleep and wake periods of the animals.

There was some technical difficulty associated with the infusion into the renal artery, resulting in a very low success rate of the protocol. Therefore, the data

presented in this Chapter were only from those animals deemed to have received successful infusion, according to the criteria established in Section 3.2.3. Further, the addition of a fifth group to this study, in which Ang II (4.5 ng/kg/min) was infused intravenously, resulted in a similar increase in arterial pressure over day 8-11 compared to those animals that received that same dose of Ang II, delivered intrarenally. There are many actions of Ang II that could have contributed to the rise in arterial pressure in both models of hypertension and these mechanisms were not investigated in this study. However, in addition to the other pro-hypertensive actions of Ang II, the renal mechanisms contributing to the hypertension may be similar in both models of hypertension, induced by infusion of Ang II intrarenally, or intravenously. Thus in order to increase the productivity of the technique, all future studies reported in this thesis will use the intravenous route of administration of Ang II to induce hypertension, and study the effects of such in the kidney.

CHAPTER 4

HYPERTENSION FROM INTRAVENOUS ANGIOTENSIN II INFUSION: EVIDENCE FOR STRUCTURAL CHANGES IN THE KIDNEY

4.1 INTRODUCTION

In the previous Chapter, long term infusion of low doses of Ang II directly into the kidney induced dose–dependent rises in arterial pressure of conscious rats housed in their home cages. The increase in arterial pressure was observed during both day and night time, and was not associated with an increase in heart rate. In other forms of Ang II-dependent hypertension, including hypertension induced with systemic infusion of Ang II, intrarenal levels of Ang II increase above plasma levels, suggesting there is an activation of intrarenal Ang II production, as well as an accumulation of circulating Ang II in the kidney *(Navar and Harrison-Bernard, 2000; Zou et al., 1996; Von Thun et al., 1994)*. Increased Ang II levels in the kidney might play a role in the development of the hypertension in these experimental models (as outlined in Chapter 1, Section 1.3.3.2). In addition there is also indirect evidence for increased Ang II activity in the kidney in some forms of human hypertension *(Fommei et al., 1987; Bender et al., 1984; Hricik et al., 1983)*.

Systemic infusion of low doses of Ang II results in a slowly developing hypertension *(Griffin et al., 1991; Brown et al., 1981; Bean et al., 1978)*. There is evidence to suggest that the trophic actions of Ang II acting on the systemic vasculature could induce vascular hypertrophy, and subsequently contribute to the rise in arterial pressure via this mechanism, which is separate from the better known vasoconstrictive action of the hormone *(Lever et al., 1992; Griffin et al., 1991)*.

One possible consequence of renal accumulation of Ang II in these models of hypertension may be to induce renal vascular growth and hypertrophy. Vascular

hypertrophy or vessel remodelling may lead to narrowing of vessel lumen, increasing renal vascular resistance, and ultimately leading to the development of hypertension.

4.1.1 Aims

Thus the aim of the present study was to determine if hypertension induced by an intravenous infusion of low dose Ang II resulted in structural changes of the renal vascular bed *in vivo*, in particular reduced resistance vessel lumen dimensions. This was tested using an established functional test of vessel lumen dimensions, used previously to detect vessel lumen changes in a number of experimental models of hypertension *(Bergstrom et al., 1998; Tomoda et al., 1997; Gothberg and Folkow, 1983)*.

4.2 MATERIALS AND METHODS

Animals received an intravenous infusion of Ang II (4.5-10 ng/kg/min) or vehicle (heparinised saline 10 IU/ml) for 10 days. On day 10, awake mean arterial pressure was measured prior to investigation of renal vascular structural changes in maximally dilated kidneys, using an isolated-perfused kidney preparation *(Bergstrom et al., 1998; Gothberg et al., 1979)*

Experiments were performed on male Sprague-Dawley rats (10-12 weeks old) bred at Monash University, Victoria, Australia. All experiments were approved in advance by the Monash University Standing Committee on Ethics in Animal Experimentation as being in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

A stock solution of angiotensin II (500 μg/ml, Sigma Chemical Co., USA) was dissolved in 0.9% w/v sterile saline and frozen in one ml aliquots. Only one aliquot was thawed and used at a time, and partially used aliquots were discarded. Once thawed, the concentration of angiotensin II was adjusted to take into account the actual peptide content, as determined by the amino acid analyses provided by the manufacturer.

4.2.1 Surgical preparation & commencement of angiotensin II infusion (day 0)

Rats were anaesthetised with an intraperitoneal injection of anaesthetic mixture containing sodium pentobarbitone (30 mg/kg; Nembutal Rhone Merieux, Australia) and methohexitone sodium (40 mg/kg; Brietal Sodium, Lilly Australia), and 0.9% w/v NaCl. When pedal and corneal reflexes were abolished, a midline abdominal incision was made, and the right branch to the lumber vein isolated. A tapered soft vinyl catheter

(SV45) attached to a osmotic minipump (Alzet, model 2ML4, California, USA), primed overnight in 0.9% NaCl at 37°C, was inserted into the branch of the lumbar vein and then advanced 2 cm into the *vena cava*. The catheter was tied into place with silk and glued with tissue glue (Vet bond, 3M animal care products, Germany). Absorbable sutures were used to sew the abdominal muscle layer and skin layer. Recovery from the anaesthetic was aided with the administration of a subcutaneous injection of buprenorphine hydrochloride (Temgesic, Reckitt & Coleman Pharmaceuticals, Australia) at 0.1 mg/kg.

4.2.2 Measurement of awake pressure (day 10)

On day 10 of infusion, rats were brought up to the laboratory and anaesthetised with the short acting anaesthetic methohexitone sodium (30 mg/kg i.p.; Brietal Sodium, Eli Lilly, Australia). A teflon catheter was inserted into the tail artery, secured with tape and then protected with the barrel of a 10 ml syringe. Rats were allowed to recover for at least one hour, after which, awake mean arterial pressure and heart rate were recorded for 20 minutes. The first 5 minutes of recorded data was discarded and the remaining 15 minutes averaged to generate a mean blood pressure for each animal. At the end of the recording period, an arterial blood sample was taken for analysis of renin activity and haematocrit.

4.2.2.1 Analysis of plasma renin activity

One ml samples of arterial blood were collected into chilled tubes, containing 50 µl of an inhibitor cocktail (0.21% w/v $NaH_2PO_4.2H_2O$, 0.74% w/v Na_2HPO_4, 1.0% neomyocin sulphate, 3.72% w/v ethylenediaminetetraacetate, and 1.0% v/v 2,3-dimercaptopropanol: a renin inhibitor, pH 7.4), for the determination of plasma renin activity (PRA). All samples were centrifuged at for 10 min at 4°C, and the plasma transferred to a fresh tube and frozen for subsequent analysis of plasma renin activity via established radioimmunoassay methods *(Oliver et al., 1990)*.

4.2.3 In vitro investigation of renal vascular structure (day 10)

The isolated-perfused kidney preparation was first developed by Gothberg, Folkow and colleagues (1979) and involves maximally dilating the renal vascular bed *in situ* before construction of pressure-flow and pressure-GFR relationships.

4.2.3.1 Preparative surgery and confirmation of intravenous infusion

Once awake arterial pressure had been recorded and a blood sample collected for renin activity and haematocrit, rats were anaesthetised with pentobarbitone sodium (40 mg/kg i.p., with supplementation to effect) and placed on a warm operating table. When all reflexes were abolished, the jugular vein was cannulated (PE-50) and a continuous infusion of 2% w/v bovine serum albumin (BSA; dissolved in 0.9% w/v NaCl) (Sigma Chemical Co. St Louis, USA) was begun at 6 ml/hr. A midline incision was performed and the skin and abdominal muscles cauterised to prevent blood loss.

At this point, the position of the intravenous catheter was confirmed in all animals, and patency of the catheter was verified by detaching the catheter from the pump and drawing back venous blood. The activity of the Ang II in the pump was also confirmed by aspirating the fluid left in the pump and injecting it into the *vena cava* and noting a rise in arterial pressure.

The intestines were removed by first ligating the mesenteric artery and then ligating the small intestine just distal to the duodenum and also the distal section of the large intestine. The abdominal aorta was then isolated 1 cm proximal and 1 cm distal to the left renal artery. The left ureter was cannulated (PE-10, 7 cm) for the collection of urine and the left testicular and adrenal arteries ligated. After the rat was heparinised (500 IU i.v.; Fisons, Sydney, Australia), a PE-160 catheter that was attached to the perfusion circuit was inserted retrogradely into the distal aorta and advanced to the junction of the left renal artery. A catheter branching from the perfusion circuit just prior to its insertion point into the aorta was used to measure kidney arterial inflow pressure.

4.2.3.2 Perfusion of the left kidney

Immediately after the perfusion to the left kidney was started, the aorta was tied off just above the left renal artery and the renal vein cut to allow the perfusate to exit the kidney (Figure 4.1; experimental design). The animal was then killed via an overdose of pentobarbitone. The renal capsule was removed to minimise increments in renal tissue pressure, and a 25 gauge needle (ID 0.3 mm) inserted 4 mm into the renal mid-cortex to measure renal tissue interstitial pressure (Pi; Figure 4.1). The resting perfusate flow was maintained at an average of 5.5 ml/min using a peristaltic pump (Ismatec, Switzerland). In the isolated kidney, perfusion flow, arterial distending pressure and GFR were used to construct pressure-flow and pressure-GFR relationships. In this situation arterial distending pressure (P) was calculated as the inflow pressure (Pa) minus the renal interstitial pressure (Pi) *(Bergstrom et al., 1998).*

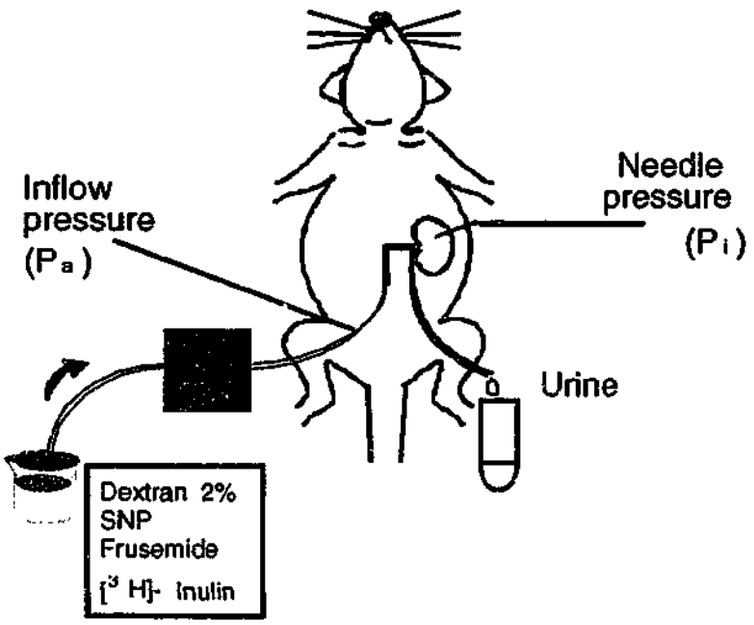


Figure 4.1: Schematic diagram of *in situ* isolated-perfused kidney preparation.

4.2.3.3 Composition of the perfusate solution

The perfusate consisted of a modified Tyrode solution containing Na^+ 148, K^+ 4.3, Cl^- 133, Ca^{2+}2.5, Mg^{2+} 0.8, HCO_3^- 25, and $H_2PO_4^+$ 0.5 mmol/L as well as D-glucose at 5.6 mmol/L, and Dextran 70 at 20 g/L, with a pH of 7.4 and a PO_2 of 903 mmHg when bubbled with 95% O_2 and 5% CO_2. The perfusate was iso-osmolar at 300 mOsm/L, and also contained 0.04 µCi/mL of [^{3}H]-inulin (NEN life sciences, Amersham, Australia) for measurement of GFR, 0.9 mmol/L sodium nitroprusside (Sigma Chemical Co. St Louis, USA) that, when combined with the low perfusion temperature (20°C-23°C), assured that the vasculature of the kidney was maximally dilated. Frusemide 20 mg/L was also added to the perfusate to inhibit tubulo-glomerular feedback. The maximal dilation of the kidney, due to the low perfusate temperature and the sodium nitroprusside, has been previously confirmed in experiments from our laboratory where infusion of acetylcholine (0.07 mg/min) did not further dilate the kidney *(Bergstrom et. al., 1998)*. Throughout the experiment the perfusate was protected from light.

4.2.3.4 Step-wise increases in perfusion flow

Thirty minutes after the beginning of the perfusion, a baseline recording of perfusion inflow pressure (Pa) and tissue pressure (Pi) were taken. Then, collection of urine and measurement of perfusion pressure and tissue interstitial pressure were made over eight step-wise increments in perfusion flow (range: 5 ml/min to 46 ml/min).

Before each step increase in perfusion flow, the pump was returned to baseline levels. Volume of urine collected during each perfusion flow period was measured gravimetrically and GFR calculated from [^{3}H]-inulin clearance.

4.2.3.5 Maximum vasoconstrictor response

In order to functionally assess the presence of vascular hypertrophy (increased vessel wall mass), the maximum renal vascular resistance (measured as peak arterial distending pressure; P) in response to supramaximal doses of constrictor cocktail were measured *(Bergstrom et al., 1998)*. After the final collection period the perfusion flow was returned to baseline and the perfusate changed to a solution containing the identical ingredients but without the sodium nitroprusside. After a twenty minute run in period, a vasoconstrictor cocktail containing phenylephrine (100 μmol/L, Sigma Chemical Co, St Louis, USA), Ang II (1.0 μmol/L, Auspep, Australia), and vasopressin (1.25 μmol/L, Peninsula laboratories, USA) was added, via infusion, to the perfusate for 5 minutes to assess the maximal renal vasoconstrictor response, measured as maximum pressure reached. At the end of the experiment the left kidney was removed from the animal, weighed and then dried at 70°C to determine dry kidney weight.

4.2.4 Data collection and statistical analysis

Pressure was measured using a disposable strain gauge (Cobe, Arvarda, USA) and signals were amplified and recorded on a neotrace pen recorder (Neomedix Systems, Sydney, Australia) and collected on an IBM compatible computer equipped with an analogue to digital converter and data acquisition software.

All data was expressed as mean ± SEM. Unpaired student's *t* test was used to compare mean arterial pressure, heart rate, weight data and maximal vasoconstrictor response at the end of the experiment. Pressure-flow and pressure-GFR relationships were analysed for differences in slope and elevation using family regression co-variant analysis *(Feldman, 1988)* and a line of symmetry fitted to each group using Model II regression *(Brace, 1977)*. In each rat individual pressure-flow, pressure-GFR, GFR-flow and urine flow-flow relationships were tested for a linear relationship using the Pearson correlation coefficient.

4.3 RESULTS

4.3.1 Cardiovascular parameters

Awake mean arterial pressure was approximately 27 mmHg greater in animals that received an Ang II infusion for 10 days when compared to vehicle controls (P=0.002; Table 4.1, Fig. 4.3.1). Left ventricle to body weight ratio was significantly increased in parallel with arterial pressure in the Ang II group compared to vehicle controls (P=0.04; Fig. 4.3.1, Table 4.1). There was no significant difference in heart rate, body weight on day of perfusion, or left kidney dry weight between animals treated with Ang II or vehicle (Table 4.1). Plasma renin activity (Figure 4.3.2) was significantly inhibited in animals that were infused with Angiotensin II for 10 days (P<0.001, unpaired t-test).

	Vehicle (n=9)	*Ang II (n=12)*	(p-value) t-test
Mean Arterial Pressure (mmHg)	113 ± 2	140.± 7	0.002
Heart Rate (BPM)	386 ± 6	408 ± 9	0.07
Body Weight (BW) (g)	354 ± 11	348 ± 10	0.72
Left Ventricle : Body Weight (10^{-3})	1.88± 0.04	2.00 ± 0.1	0.04
Dry Kidney Weight (g)	0.24 ± 0.01	0.23 ± 0.01	0.38

Table 4.1: Haemodynamic variables and organ weights from animals receiving a 10 day intravenous infusion of Ang II (4.5-10 ng/kg/min) or vehicle (Hep saline 10 IU/ml). Data collected on day of isolated perfusion experiment (day 10). Data presented as mean ± SEM, statistical significance was obtained using an unpaired t-test.

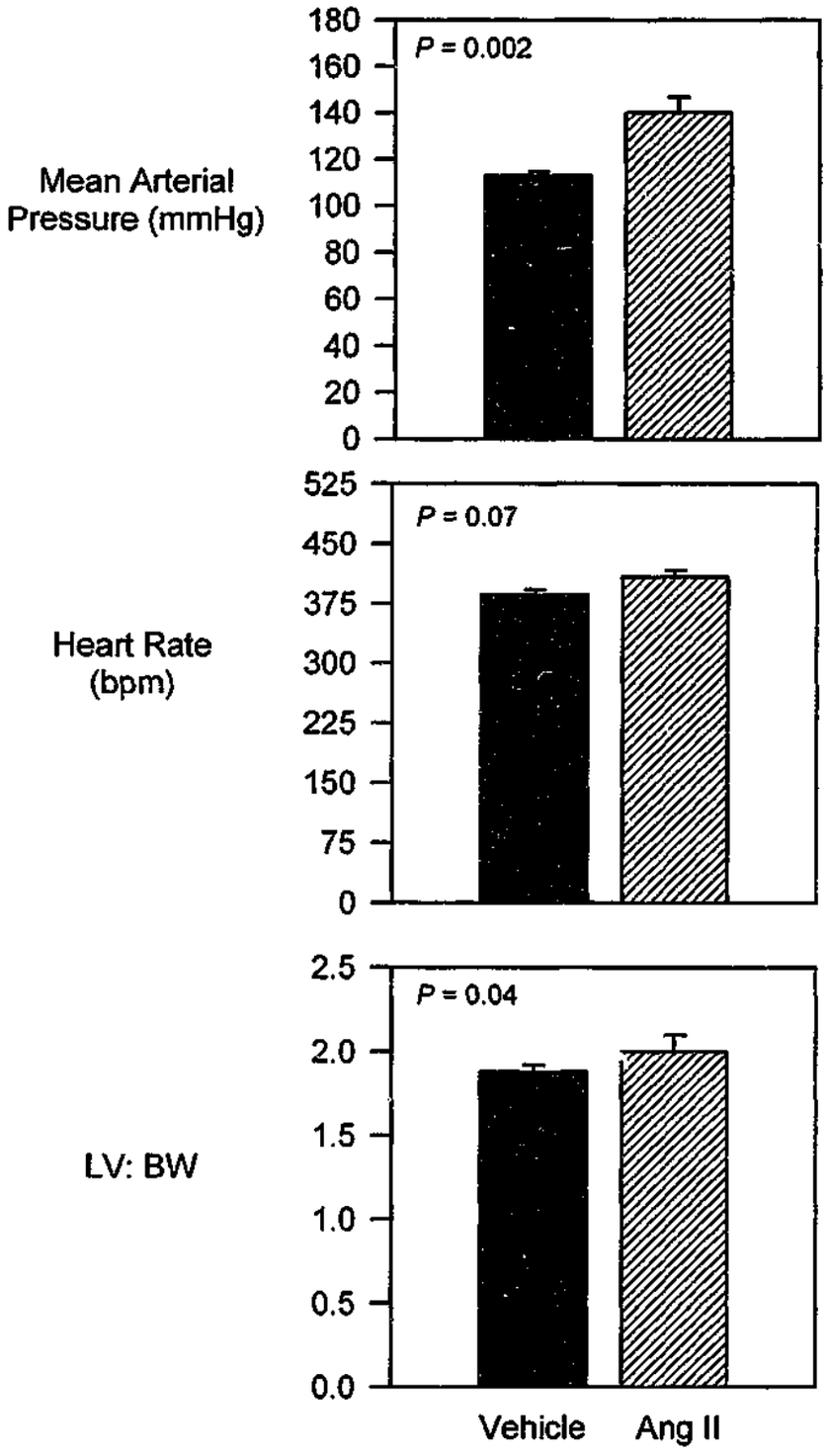


Figure 4.3.1: Mean arterial pressure, heart rate and left ventricle to body weight ratio (LV:BW) in animals that received a 10 day intravenous infusion of Ang II (4.5-10 ng/kg/min; hatched bars) or vehicle (Heparinised saline 10 IU/ml; black bars). Data presented as mean ± SEM, recorded on day 10 on the infusion. An unpaired *t*-test was used to detect statistical differences in cardiovascular parameters between animals treated with Ang II or vehicle.

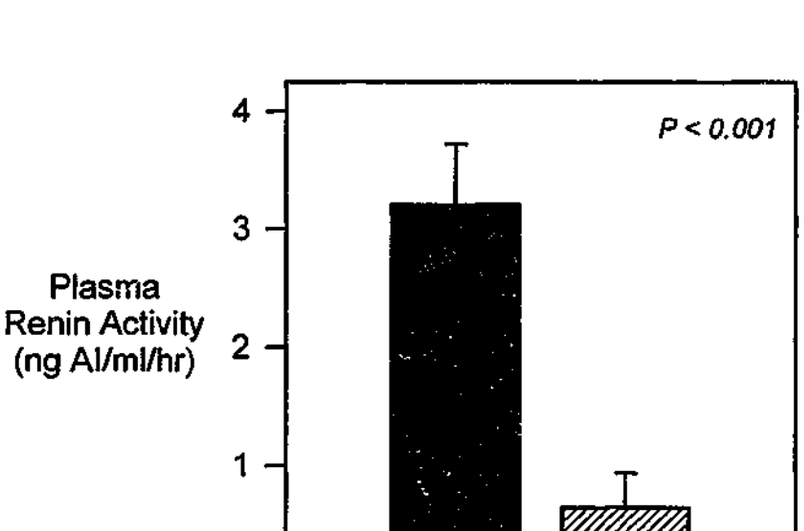


Figure 4.3.2: Plasma Renin Activity (PRA) in animals following intravenous infusion of vehicle (heparinised saline 10 IU/ml; black bars) or Ang II (4.5-10 ng/kg/min; hatched bars). Data presented as mean ± SEM, and statisitcal differences in PRA between treatment groups tested using an unpaired *t*-test ($P<0.001$).

4.3.2 Functional characteristics of renal vasculature in maximally dilated, perfused kidneys

Perfusion inflow pressure (Pa), renal interstitial tissue pressure (Pi), and calculated arterial distending pressure (P) versus the actual perfusion pump rate in animals treated with Ang II (n = 6) or vehicle (n = 7) are represented in Fig. 4.3.3. Data presented is from animals that completed the entire perfusion experiment. It can be seen that inflow pressure, interstitial pressure and therefore arterial distending pressure do not appear to be different in animals treated with Ang II or vehicle, at all pump rates. Additionally, pressure increases to similar levels in both groups with step-wise increases in flow (pump rate). For simplicity, arterial distending pressure will be referred to as pressure for remainder of this chapter.

4.3.2.1 Perfusate flow – GFR, and perfusate flow – urine flow relationships in maximally dilated kidneys

Regression analysis of individual perfusate flow - GFR and perfusate flow - urine flow relationships from Ang II and vehicle treated animals, determined that there was a significant linear relationship between GFR and perfusate flow within each experiment, with Pearson correlation coefficients (R^2) ranging from 0.937 to 0.994 (Figure 4.3.4., upper left hand panels). Family regression analysis demonstrated that the slope of the perfusate flow-GFR relationship was significantly reduced in Ang II treated group (P=0.005), suggesting that for a given increase in perfusion flow, animals treated with Ang II generally had a lower GFR when compared to the vehicle treated animals (Figure 4.3.4 lower left hand panels). Elevation of the flow-GFR relationship was not different between the Ang II or Vehicle animals (P=0.27, family regression analysis).

A significant linear relationship was also confirmed for perfusate flow - urine flow relationship in each individual animal with Pearson correlation coefficients (R^2) ranging from 0.968 to 0.999 (Figure 4.3.4 upper right hand panels). In accord with the flow - GFR relationship, the slope of the perfusate flow - urine flow relationship was also significantly reduced in the Ang II group (P=0.002; see Figure 4.5 lower right hand panels), suggesting that in rats treated with Ang II, there was a smaller increase in urine flow on average, with a given increase in perfusate flow. Likewise Ang II infusion was not associated with a shift in the perfusate flow - urine flow relationship (P=0.35, family regression analysis).

4.3.2.2 Pressure-GFR and pressure-perfusate flow relationships in maximally dilated kidneys

Individual pressure-GFR and pressure-flow relationships in both Ang II and vehicle treated animals were linear with Pearson correlation coefficients ranging from 0.918 – 0.993 for pressure-GFR relationships, and 0.988 – 0.997 for pressure-flow relationships (Figure 4.3.5 upper panels). The slope of the pressure-GFR relationship was significantly reduced in rats that received Ang II infusion for 10 days when compared to the vehicle control group (P=0.002; Figure 4.3.5 lower right hand panel), indicating that the increase in GFR with increasing pressure was smaller in rats that received Ang II. In contrast, there was no significant change in the elevation of the pressure-GFR relationship between treatment groups (P=0.26; Figure 4.3.5 lower right panels).

There was no significant change in slope or elevation of the pressure-flow relationship (P=0.64, 0.96 respectively; Figure 4.3.5 lower left hand panels), indicating that overall resistance in the kidney was not different in rats treated with Ang II when compared to the vehicle treated group.

The y-intercept and gradient of the lines of symmetry averaged from the pressure-GFR relationships, and pressure-flow relationships in individual rats that received Ang II or vehicle infusion are presented in Table 4.2. This data confirms the family regression analysis, and demonstrates that there was no significant change in the slope (b) of the pressure-flow relationship yet the slope of the pressure-GFR relationship was reduced in those animals that received Ang II when compared to the vehicle treated animals.

	Pressure-Flow relationship (n=6)		Pressure-GFR relationship (n=7)	
	Flow = a+b (pressure)		GFR = a+b (pressure)	
GROUP	**a**	**b**	**a**	**b**
Vehicle	-0.069 ± 2.3	0.90±0.06	770 ± 118	24 ± 4
Ang II	-4.99 ± 3.3	0.95 ± 0.09	867 ± 93	21 ± 2
P	0.96	0.64	0.26	0.002

Table 4.2: Intercept (a) and slope of the relationship between arterial distending pressure and perfusate flow or GFR in animals that received a 10 day intravenous infusion of vehicle (heparinised saline 10 IU/ml) or Ang II (4.5-10 ng/kg/min). Data presented as mean ± SEM, averaged from the pressure-GFR, pressure-flow relationships of individual animals. An unpaired t-test was used to detect significant differences in the slope and y-intercept between treatment groups.

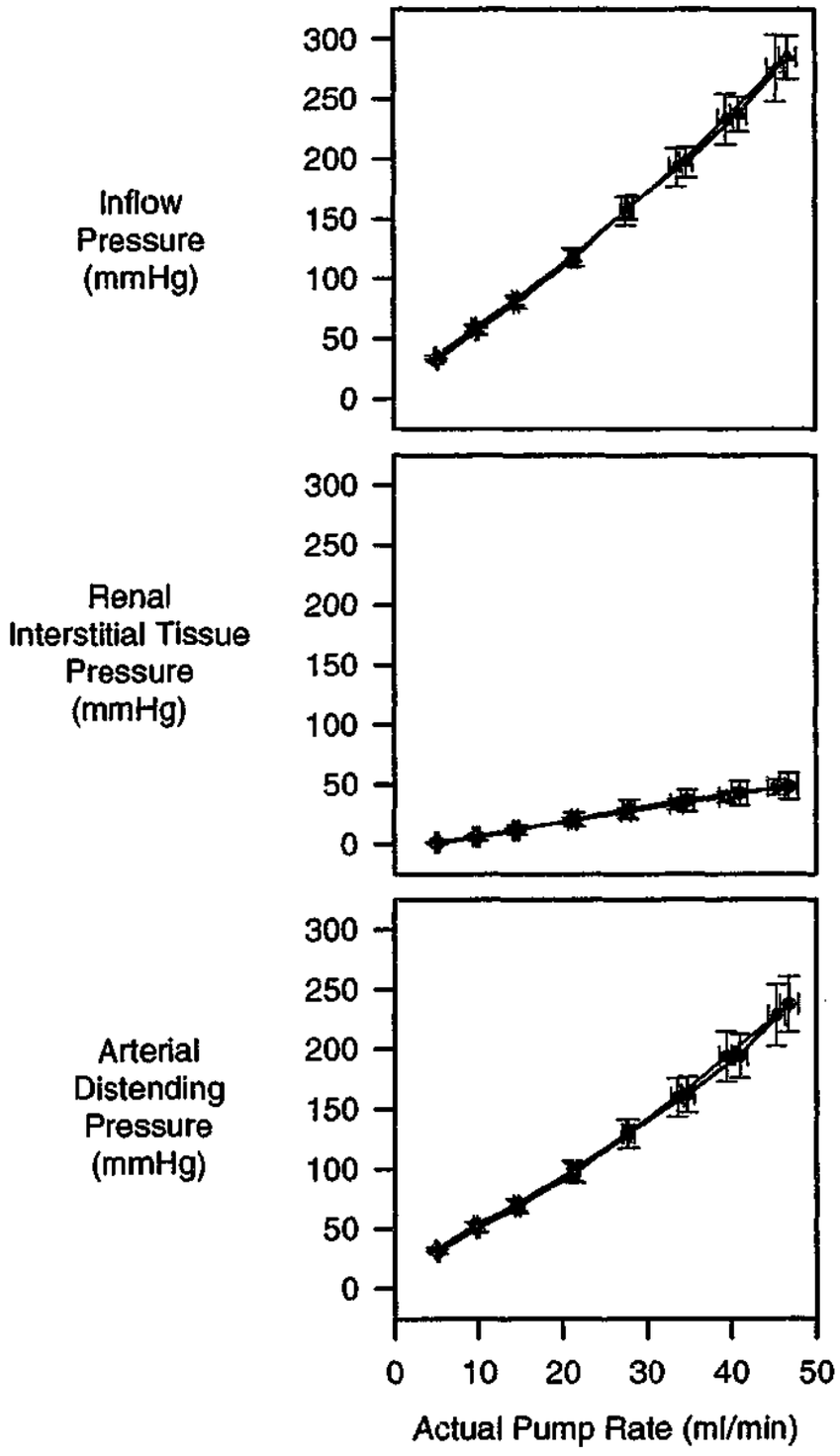


Figure 4.3.3: Inflow pressure (Pa; upper panel), renal tissue interstitial pressure (Pi; middle panel) and calculated arterial distending pressure (P = Pa - Pi; lower panel) plotted against actual pump rate in animals treated with Ang II (4.5 –10 ng/kg/min,I.V.; n=7; red symbols and lines) or vehicle (Heparinised saline, I.V.; n=6; blue symbols and lines) for ten days. Data is presented as group mean ± SEM for both actual pump rate (abscissa), and pressure measurements (ordinate).

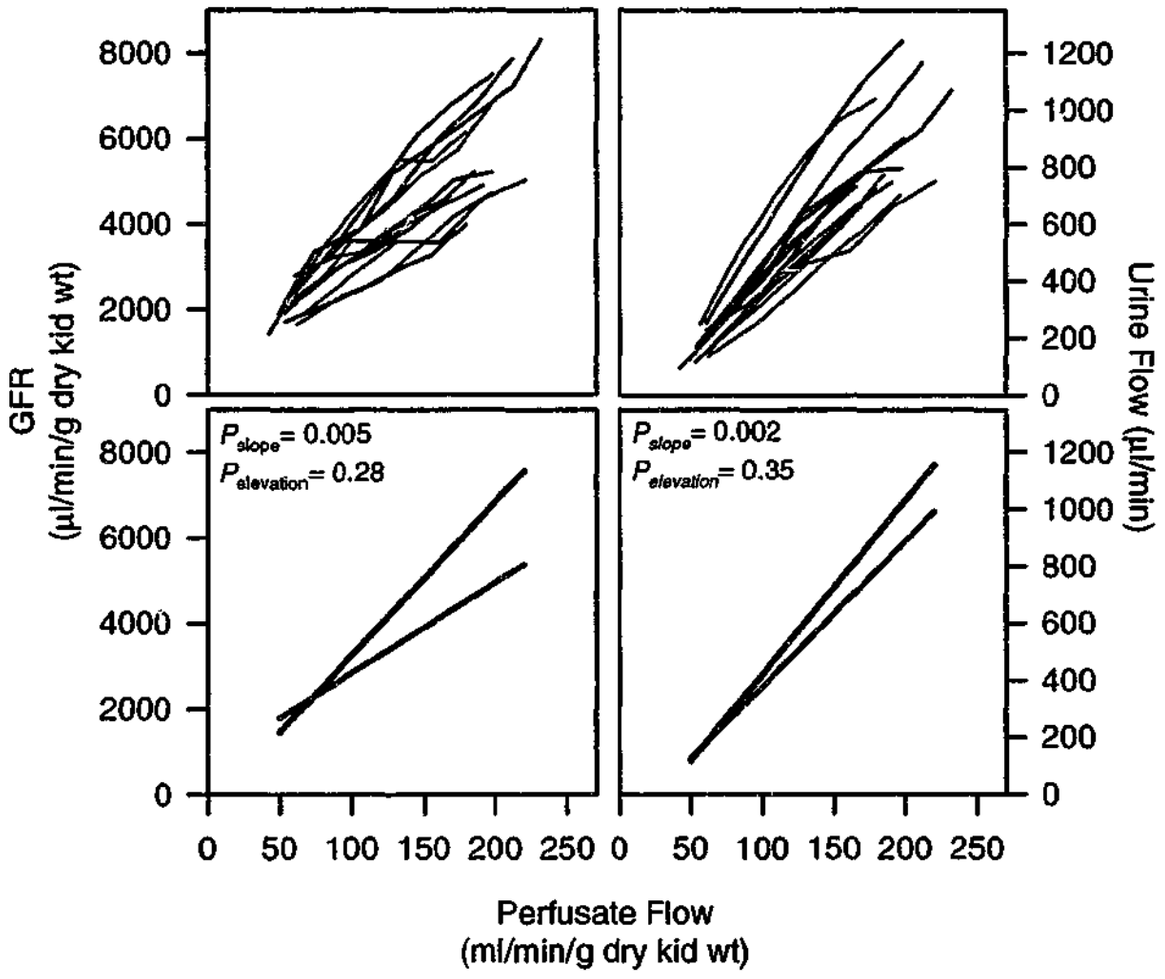


Figure 4.3.4: Relationship between perfusate flow - GFR (left panel) and urine flow (right panel) in kidneys from animals that received Ang II (red lines), or vehicle (blue lines) infusion for 10 days. Upper panels represent each individual experiment result (Ang II n=7, vehicle n=6). Lower panels represent the regression line fitted to the data by model II regression analysis. Equation for the perfusate flow - GFR regression line in Ang II treated rats is GFR= 464 + 26.6(perfusate flow), and in vehicle treated animals is GFR= -334 + 35.9(perfusate flow). Equation for the perfusate flow - urine flow line of symmetry in Ang II treated rats is urine= -131 + 5.1(perfusate flow), and vehicle line is urine= -186 + 6.1(perfusate flow). Family regression analysis was used to test for differences in the slope (P_{slope}) and elevation ($P_{elevation}$) between treated with Ang II or vehicle.

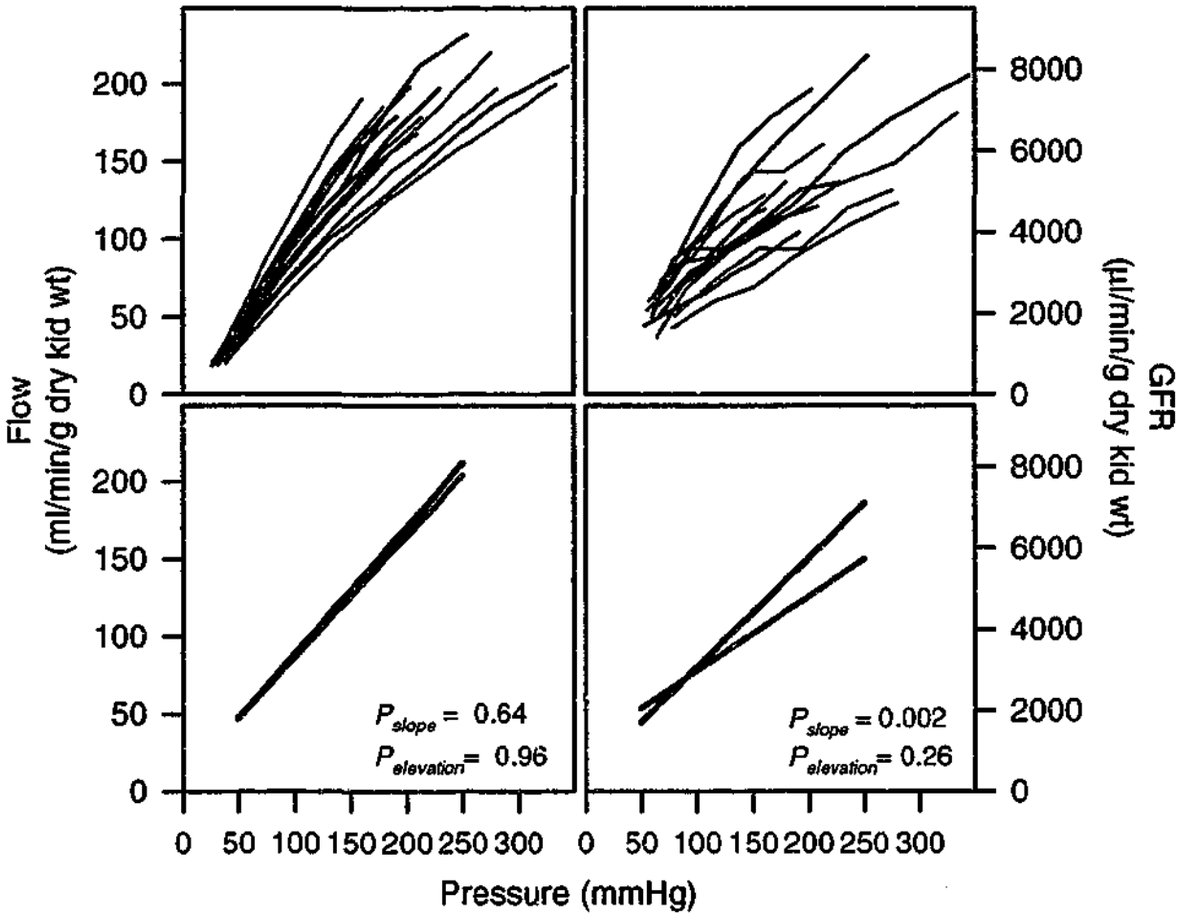


Figure 4.3.5. Relationship between arterial distending pressure and perfusate flow (left hand panels), or glomerular filtration rate (GFR; right hand panels) in maximally dilated, isolated perfused kidneys from animals receiving intravenous Ang II (4.5-10 ng/kg/min; red lines) or vehicle (Heparinised saline; blue lines) for 10 days. Upper panels represent each individual experiment (vehicle n=6, Ang II n=7). The lower panels represent the line of symmetry fitted to the data using model II regression analysis. Equation for the pressure - GFR line of symmetry in Ang II treated rats is GFR= 1124 + 18(pressure), and in vehicle treated animals is GFR= 362 + 27(pressure). Equation for the pressure - flow line of symmetry in Ang II treated rats is Flow= 8.4 + 0.78(pressure), and vehicle line is Flow= 6.7 + 0.82(pressure). Family regression analysis was used to test for differences in the slope (P_{slope}) and elevation ($P_{elevation}$) between treated with Ang II or vehicle.

The maximal arterial distending pressure response to supramaximal doses of the vasoconstrictor cocktail were not different between the Vehicle and Ang II treated animals ($P = 0.3$, unpaired t-test; Figure 4.3.6.), indicating that there was no evidence for vessel hypertrophy in rats that received an intravenous Ang II infusion.

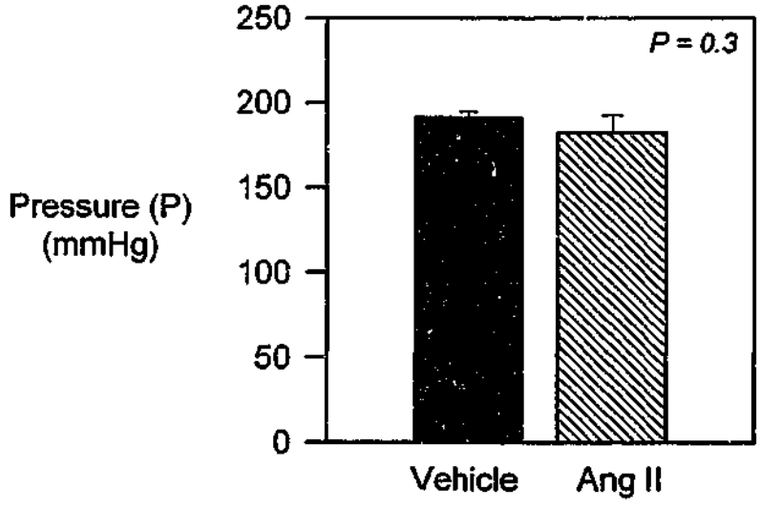


Figure 4.3.6: Maximum arterial distending pressure response to supramaximal doses of a vasoconstrictor cocktail in perfused kidneys of animals that underwent an intravenous infusion of vehicle (open bars) or Ang II (4.5-10 ng/kg/min; hatched bars). Data presented as mean ± SEM, and statistical differences between vehicle and Ang II treated rats were tested using an unpaired t-test.

4.4 DISCUSSION

4.4.1 Hypertension from intravenous infusion of angiotensin II

Infusion of Ang II for ten days resulted in an increase in mean arterial pressure of about 30 mmHg. Arterial pressure was measured directly via the tail artery, in conscious, freely moving rats. The time-course of the arterial pressure rise was not examined in these experiments but is described in experiments presented in the following Chapter. The increase arterial pressure in rats that received Ang II was also, as expected, associated with significant left ventricular hypertrophy *(Frohlich, 1986)*.

Plasma renin activity was significantly reduced in the animals infused with Ang II indicating that endogenous production and/or release of renin was inhibited *(Johns et al., 1990)*. Heart rate, organ and body weights were not different compared to control animals.

4.4.1.1 Evidence for slow pressor effect of angiotensin II on blood pressure

Previous studies have indicated that intravenous infusion of Ang II at doses ranging from 10 ng/kg/min-20 ng/kg/min, are initially sub-pressor, but over 7-14 days can increase arterial pressure by up to 50 mmHg *(Hu et al., 1999; Melaragno and Fink, 1995; Gorbea-Oppliger and Fink, 1994; Brown et al., 1981; Koletsky et al., 1965)*. Thus, the increase in arterial pressure observed in response to Ang II in this study is consistent with previous reports of slow onset hypertension, however a definitive mechanism responsible for the development of the hypertension has not been described. It has been suggested that trophic effects of Ang II may induce vascular hypertrophy and thus contribute to the hypertension by increasing systemic resistance *(Griffin et al., 1991)*. Comparative studies of slow pressor hypertension induced with systemic Ang II infusions have also demonstrated increases in protein and DNA synthesis in systemic arteries 24 hours and one week after commencement of Ang II infusion *(Su et al., 1998; Simon and Altman, 1992)*. Likewise systemic vascular hypertrophy is evident after 10-12 days of low dose Ang II infusion *(Griffin et al., 1991)*. Thus the time period over which Ang II-induced slow pressor hypertension develops appears consistent with the time taken for cellular growth and subsequent vascular hypertrophy to occur. Therefore, after 10 days of sub-pressor Ang II infusion, such as used in the current study, any proliferative actions of Ang II, and subsequent vasculature hypertrophy should be detectable.

The aim of the present study was to look for evidence of renal vascular changes in slow pressor Ang II induced hypertension, particularly evidence of structural narrowing of the renal vasculature. While hypertrophy and structural changes in response to Ang II have been documented for several systemic vascular beds, and for large vessels such as the aorta, the effect of intravenous infusion of small doses of Ang II on the renal vasculature has not been investigated. In other models of hypertension induced by administering initially sub-pressor doses of Ang II systemically, intrarenal Ang II levels increase above that in the circulation, suggesting that the kidney maybe an important site of action for Ang II *(Navar and Harrison-Bernard, 2000)*.

In order to determine whether intravenous Ang II infusion was associated with structural changes in the renal vascular bed, I used a well established, functional test of vessel lumen dimensions in maximally dilated isolated-perfused kidneys. The technique established by Gothberg and colleagues (1979), is a simple and indirect test that provides information about gross structural luminal changes in the renal vasculature. Whilst the technique can accurately predict average structural changes across the entire vascular bed, these need to be confirmed using techniques such as stereology.

4.4.2 Determining renal vessel lumen changes: functional test of structural changes

4.4.2.1 The Gothberg kidney – what is it?

When a vascular bed is maximally dilated, pressure-flow relationships provide information about average vessel lumen dimensions in the bed *(Folkow et al., 1977)*. Gothberg *et al.* (1979) first investigated pressure-flow relationships at maximal dilatation in isolated-perfused kidneys and proposed there were two functional indices of vessel lumen changes; i) the minimal total renovascular resistance which is an indicator of average vascular diameter, and ii) the pre-:post-glomerular resistance ratio which is derived from the relationship between GFR and perfusion pressure and can provide information about structural changes in the pre-glomerular vasculature. Important features of the technique include perfusing the kidney with an iso-oncotic solution containing sodium nitroprusside to ensure that the renal vascular bed is maximally dilated *(Gothberg et al., 1979)*. Thus, the apparent 'true' levels of total vascular resistance can be compared as there is no contribution of vascular tone from constriction of the smooth muscle of the vessel, and any resistance differences must be therefore, due to the structural properties of the vessels *(Folkow et al., 1977)*. Frusemide is also added to the perfusate solution to inhibit tubulo-glomerular feedback.

The maximally dilated isolated, perfused kidney technique has proven to be a reliable and sensitive method of assessing average renal structural alterations across the entire kidney *(Bergstrom et al., 1998; Folkow et al., 1977)*.

i) Firstly, exploration of renal vascular lumen changes using this method are advantageous as the average vessel lumen characteristics are assessed in the whole – organ.

ii) Secondly, the changes are measured in the most relevant way; by their contribution to renal flow resistance, glomerular filtration pressure, and glomerular filtering capacity (although, these measurements are made in maximally dilated kidneys).

iii) Thirdly, estimations of lumen diameter are measured by resistance and thus are a far more sensitive way to detect vessel lumen changes. According to Poiseuilles law (Resistance $\propto$ 1/radius^4, *Korner et al., 1989*), lumen radius changes are multiplied by the fourth power to calculate resistance, thus by measuring resistance, very small lumen changes can be detected *(Gothberg and Folkow, 1983)*

4.4.2.2 Isolated-perfused kidney – who has used it?

The results of renal haemodynamic studies in SHR rats, using the perfused kidney technique *(Gothberg and Folkow, 1983; Gothberg et al., 1979; Folkow et al., 1977)* have been found to be in good agreement with other techniques investigating renal vascular lumen dimensions *(Skov et al., 1992; Kimura et al., 1991; Gattone et al., 1983)*. More recently, in our laboratory, the isolated-perfused kidney preparation has been modified and improved by replacing the original kerosene perfusate with a modified iso-oncotic Tyrode's solution *(Tomoda et al., 1997)*. The improved technique has been used by a number of investigators in our laboratory to assess renal vascular changes in spontaneously hypertensive rats that underwent renal denervation or ACE inhibition *(Bergstrom et al., 1998; Tomoda et al., 1997)*, and in rats made hypertensive with an intrarenal Ang II infusion *(Stevenson et al., 2000)*. These studies have been published in leading hypertension journals.

The isolated-perfused kidney preparation

In the maximally dilated kidney, perfusion pressure is increased step-wise to construct pressure-flow and pressure-GFR curves. The relationship between the perfusion flow and arterial distending pressure reflects the total resistance of the renal vascular bed at maximal dilation. On the other hand the relationship between GFR and perfusion pressure is a little more complex. As there are no hormonal, neural or autoregulatory mechanisms acting in the maximally dilated, isolated-perfused kidney

there are three possible factors that determine the slope and the position of the pressure-GFR relationship:

i) Intra-glomerular hydrostatic pressure: Determined by the perfusion pressure and the pre-:post-glomerular resistance ratio, the lower the resistance ratio, the higher glomerular hydrostatic pressure, and thus GFR will be. The position of the pressure-GFR curve along the pressure (x) axis, at a constant overall resistance is determined solely by the pre-:post-glomerular resistance ratio. For example, if there was an increase in the pre-:post-glomerular resistance ratio then it would be predicted that GFR would be reduced by a uniform factor across a spread of perfusion pressures (ie: the position of the slope would move to the right yet the slope would remain the same).

ii) Whole-kidney ultrafiltration coefficient: Defined as the total glomerular surface area multiplied by the mean hydraulic conductance. The slope of the pressure-GFR curve is determined by both the pre-:post-glomerular resistance ratio and the whole kidney filtration capacity (the sum of all single glomerular filtration coefficients) *(Gothberg et al., 1979; Folkow et al., 1977)*. Thus, changes in the slope of the pressure-GFR relationship may be due to alterations in pre:post-glomerular resistance ratio, or changes in glomerular filtration coefficient, and the relative contributions each of these factors make to the change in slope cannot be determined in the perfused kidney model *(Folkow et al., 1977)*.

iii) Intratubular pressure: Intra-tubular pressure is important in determining the pressure that drives glomerular filtration, as the pressure in the glomerulus must overcome that in the tubules in order to filter. As the intravascular pressure increases (ie; step-wise increases in perfusion flow), glomerular filtration and intratubular pressure also increase, leading to an increase in tissue pressure in the renal parenchyma. Increases in renal tissue pressure will act to reduce the bore of the renal vessels, particularly in low-pressure veins, and hence affect GFR. In this study renal tissue interstitial pressure (an estimate of tubular hydrostatic pressure) was identical in both Ang II and vehicle treated animals, suggesting that renal interstitial pressure was counteracting GFR to a similar extent in both groups *(Gothberg et al., 1979; Folkow et al., 1977)*.

Renal vascular structural changes in Ang II hypertension

In the present study, Ang II induced hypertension did not significantly affect the slope or the elevation of the pressure-flow relationship in the maximally dilated kidney, suggesting that, total renal vascular resistance was not different between animals treated with Ang II or vehicle.

If Ang II-induced hypertension had induced structural changes in the renal vasculature (in particular the renal pre-glomerular vessels as originally hypothesised); in the maximally dilated, perfused kidney model, a right-shift in the position of the pressure-GFR relationship along the x-axis would have been expected. Indeed, such an effect has been recently described in rats made hypertensive with an intrarenal infusion of Ang II *(Stevenson et al., 2000)*. However, in the present study a change in the position (ie the elevation) of the pressure-GFR relationship along the abscissa was not observed in animals treated with intravenous Ang II when compared to the vehicle group. Rather, intravenous infusion of small doses of Ang II, resulted in a significant reduction in the slope of the pressure-GFR relationship such that, increases in pressure resulted in smaller increases in GFR in rats that received Ang II compared to vehicle rats. As discussed earlier, the slope of the pressure-GFR relationship in maximally dilated kidneys is determined by increases in the pre-:post-glomerular resistance ratio, or alterations in glomerular ultrafiltration coefficient *(Gothberg et al., 1979; Folkow et al., 1977)*.

A potential increase in the pre-:post-glomerular resistance ratio could result from an decrease in pre-glomerular vessel lumen dimensions relative to the post-glomerular vessels dimensions. While the contributions of a change in the pre:post-glomerular ratio cannot be ruled out, the lack of change in total renal vascular resistance (ie; no change in the pressure-flow relationship) and indeed the absence of a shift of the position of the pressure-GFR relationship along the abscissa, would suggest that changes in ultrafiltration coefficient are more likely to contribute to the reduced slope in the intravenous Ang II model of hypertension.

4.4.2.3 Possible changes in glomerular filtration coefficient

Direct effects Ang II on the glomerulus

In vitro evidence supporting a role for Ang II induced growth in the glomerulus may indicate possible mechanisms by which Ang II could alter glomerular filtration surface area *in vivo*. Cultured adult and fetal mesangial cells, and glomerular endothelial cells undergo both hypertrophy and hyperplasia in response to angiotensin II *(Orth et al., 1995; Wolf, 1995; Rupprecht et al., 1994; Rupprecht et al., 1992; Ray et al., 1991)*, see section1.4.1.1). Trophic effects of Ang II on the glomerulus *in vivo* could potentially induce structural changes in the glomerular capillaries or mesangium, and induce alterations the surface area available for filtration.

Pressure induced effects on the glomerulus

In vivo, there is some suggestion that Ang II mediated contraction of the glomerular mesangium may subsequently reduce capillary surface area, and therefore act to reduce the glomerular filtration coefficient *(Denton et al., 2000; Denton et al., 1992; Haley et al., 1987; Ichikawa and Brenner, 1984).* However, whilst Ang II has been shown to reduce glomerular tuft volume *in vivo*, there is no evidence to support a decrease in capillary surface area *(Denton et al., 2000; Denton et al., 1992; Haley et al., 1987; Ichikawa and Brenner, 1984).*

Alternatively, infusion of Ang II could have led to a preferential constriction of the efferent arteriole (see Chapter 1, Section 1.3.3.2) and an increase in glomerular capillary pressure *in vivo.* An increase in glomerular capillary pressure would, in general, act to increase GFR, an effect in contrast to the results found in the present study. However, if Ang II preferentially constricted that efferent arteriole for the entire 10 day period, the increase in glomerular capillary pressure could potentially damage the glomerular capillaries, reducing Kf or glomerular surface area available for filtration.

In the present study therefore, Ang II induced changes on renal vascular and glomerular structure could be responsible for the significant reduction in the slope of the pressure-GFR curve in maximally dilated kidneys. However such changes still need to be confirmed using stereological and morphological techniques, and to assess in detail, the relative contribution to the development of the hypertension. The effects of Ang II on the glomerulus *in vivo* during the infusion period, could be investigated more thoroughly using micropuncture techniques to directly measure prospective changes to glomerular capillary pressure, single nephron GFR, and efferent and afferent arteriolar resistances *(Blantz, 1987; Blantz and Gabbai, 1987).*

Indicator of vessel wall hypertrophy

To assess potential vessel wall hypertrophy (and thus vessel wall mass; wall:lumen ratio), a crude functional test, measuring whole kidney response to supra-maximal doses of vasoconstrictor agents was used at the end of each perfusion experiment *(Stevenson et al., 2000; Bergstrom et al., 1998).* The arterial distending pressure and thus total renal vascular resistance response to supra-maximal doses of vasoconstrictors did not differ in kidneys from rats with Ang II-induced hypertension, or rats treated with vehicle. This test reflects the maximal contractile strength of the total kidney vasculature (in relation to the vascular lumen ie; the wall:lumen ratio) and thus suggests that there is no renal vessel wall hypertrophy in the animals with Ang II-induced hypertension. However, the test is only a simple method used to measure total kidney vessel wall hypertrophy and needs to be confirmed using stereological

analysis. Additionally, the test cannot rule out the possibility of renal vessel remodelling (ie remodelling of the same amount of wall mass around a smaller lumen).

Other studies of renal vasculature using this technique (angiotensin II)

Previous studies conducted in our laboratory using the maximally dilated, isolated-perfused kidney preparation, have also supported a role for Ang II in renal vascular lumen changes in hypertension. Perindopril treatment of young SHR rats for 6 weeks resulted in a left-ward shift of both the pressure-flow and pressure-GFR curves, suggesting that chronic ACE inhibition increased average lumen diameter in the SHR, predominantly in the pre-glomerular vessels *(Bergstrom et al., 1998)*. A recent study from our laboratory found that intrarenal infusion of Ang II into Sprague-Dawley rats results in hypertension and a significant right-ward shift in the pressure-GFR relationship, suggesting that intrarenal Ang II had increased the pre:post glomerular resistance ratio, which is consistent with structural reductions in renal pre-glomerular vessel lumens *(Stevenson et al., 2000)*. Similar to the findings of the present study, there was also a significant reduction in the slope of the pressure-GFR relationship in maximally dilated kidneys of rats treated with intrarenal Ang II, suggesting that alterations in Kf may also play a role in this model of hypertension *(Stevenson et al., 2000)*. These studies have yet to confirm the renal vascular lumen changes using stereology.

Other pro-hypertensive actions of angiotensin II in the kidney

There are other actions of Ang II that are pro-hypertensive and could have also contributed to the development of hypertension in this model. These actions are summarised in Chapter 1 Section 1.3.3, but include retention of salt and water, and activation of the renal and systemic sympathetic nerves, and direct vasoconstriction in the kidney and peripheral vasculature acting to reduce GFR. Slow-pressor hypertension, by its very name, suggests there is little role for the direct vasoconstrictor action of Ang II on systemic and renal arteries in this model of hypertension. However, multiple renal and systemic actions of Ang II that could potentially also contribute to the development of hypertension in the present model cannot be ruled out, and were not investigated in this study. Although there may be multiple actions of Ang II that contribute to the hypertension, we have primarily focussed on whether renal structural changes also occur in association with this model of hypertension.

In summary, the results of the present study suggest that long term intravenous infusion of low dose Ang II may have induced structural changes in glomerular or renal pre and post glomerular vessels, resulting in changes to the glomerular filtration surface area and pre-:post-glomerular resistance ratio. These changes translated to a

reduced slope of the pressure-GFR relationship in maximally dilated kidneys from animals that received intravenous Ang II. Whilst the results of this study indicate that the potential renal changes may contribute to the development of hypertension in this model, changes to both the renal vasculature and the glomerulus, associated with Ang II-induced hypertension need to be confirmed using stereological techniques. Further, a link between Ang II-induced renal growth *in vivo*, and structural vascular and glomerular changes, and the role these play in the development of the hypertension need to be investigated. In an attempt to address this issue further, in the following Chapter, cellular growth and hypertrophy in the kidney have been examined *in vivo*, in connection with slowly developing hypertension induced with low doses of Ang II.

CHAPTER 5

ANGIOTENSIN II-INDUCED HYPERTENSION: EXPRESSION OF GROWTH FACTORS TGF-β1 AND bFGF IN THE KIDNEY

5.1 INTRODUCTION

In Chapter 4, Ang II, infused intravenously into rats for ten days, induced hypertension and was associated with renal functional changes, that were consistent with structural alterations to renal vessel lumen dimensions, or a reduction in glomerular filtration surface area. However, these putative renal vascular and glomerular structural alterations need to be confirmed using more direct methods.

Systemic infusion of low doses of Ang II is known to cause a slow, progressive hypertension that may result from several mechanisms including growth and hypertrophy of resistance vessels *(Simon and Altman, 1992; Lever et al., 1992; Griffin et al., 1991; Lever, 1986)*. The vascular hypertrophy that develops in this model of hypertension is thought to be due partly to the trophic, and hence non-pressor actions of Ang II on the systemic vasculature *(Griffin et al., 1991)*.

In vivo, the slowly developing hypertension produced when low dose Ang II is infused systemically may potentially involve Ang II stimulated increases in growth factors such as TGF-β1 and bFGF, that in turn induce cellular growth and hypertrophy. In the kidney, growth factor stimulated cellular hypertrophy and DNA synthesis could contribute to renal vascular or haemodynamic changes, altering renal function, and ultimately contributing to the development of hypertension.

As discussed in Chapter 1, evidence from *in vitro* experiments demonstrate that Ang II is a potent mitogen of cultured renal vascular smooth muscle cells and mesangial cells *(Wolf, 1995; Dubey et al., 1992)*. The cellular mechanisms involved in the Ang II mediated stimulation of cellular growth and hypertrophy still remain to be

elucidated. However a number of growth factors have been implicated in the process. In vascular smooth muscle cell cultures, expression of transforming growth factor-β1 (TBF-β1), platelet derived growth factor A and B chain (PDGF-AA,-BB), and basic fibroblast growth factor (bFGF) genes are upregulated by addition of Ang II *(Itoh et al., 1993; Stouffer et al., 1993; Gibbons et al., 1992; Naftilan et al., 1989).* In addition, Ang II, TGF-β1 and bFGF have been shown to interact to modulate proliferation in a number of renally derived cell cultures including; cultured human fetal, and rat mesangial cells *(Izevbigie et al., 2000)*, proximal tubule epithelial cells *(Zhang et al., 1991)*, and juxtaglomerular cells *(Liu and Ballermann, 1998*; see Section 1.4.2.3).

5.1.1 Aims

Thus, the aims of the present study were to: i) investigate whether intravenous infusion of Ang II (4.5 ng/kg/min) into conscious rats, for 5 or 10 days, induced a slowly developing hypertension, and was associated with ii) increased expression of growth factor genes TGF-β1 and bFGF in the kidney, or iii) increases in total kidney DNA and protein content.

5.2 MATERIALS AND METHODS

Low dose Ang II (4.5 ng/kg/min) or vehicle (heparinised saline 10 IU/ml) was infused intravenously for 5 or 10 days into conscious rats. On day 5 or 10, awake mean arterial pressure was recorded via the tail artery, and the left and right kidneys were collected for subsequent analysis of whole kidney DNA and protein content. Growth factor TGF-β1 and bFGF mRNA expression was measured via *in situ* hybridisation in renal glomeruli, non-glomerular cortical regions, and medullary regions.

All experiments were conducted in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Ten week old outbred male Sprague-Dawley rats were obtained from Monash University Animal Services and maintained on a standard rat chow diet (GR2; Barastoc Stockfeeds, VIC, Australia), and were allowed free access to water.

A stock solution of Ang II (500 μg/ml, Sigma Chemical Co, USA) was dissolved in 0.9% sterile saline and frozen in one ml aliquots. Only one aliquot was thawed and used at a time. Partially used aliquots were not re-frozen.

5.2.1 Surgical Preparation

Animals underwent surgery to implant a venous catheter attached to an osmotic minipump containing either Ang II (4.5 ng/kg/min) or vehicle (heparinised saline 10 IU/ml) as described previously in Chapter 4, Section 4.2.1.

5.2.2 Measurement of awake pressure

On day 5 or 10 of the infusion animals were brought up to the laboratory and briefly anaesthetised to implant a tail artery catheter for the measurement of awake arterial pressure as described previously in Chapter 4, Section 4.2.2. The animals were then re-anaesthetised with 40 mg/kg of sodium pentobarbitone (Nembutal; Rhone Merieux, Australia) and the left and right kidneys harvested, frozen in isopentane (Sigma Chemical Co., Australia) on dry ice and stored at -70°C for subsequent analysis. At *post-mortem*, the position of the catheter in the vena cava was confirmed and left and right ventricle weights recorded.

5.2.3 Tissue analysis

5.2.3.1 Whole kidney DNA content

Right kidney DNA content was determined using a fluorescent marker that specifically binds to the DNA molecular backbone structure (bisBenzimide Hoechst 33258, Calbiochem, USA). A stock solution of the fluorescent dye was made up to 100 µg/ml in distilled H_2O and stored at 4°C in the dark. Salmon Testes DNA (Sigma Chemical Co., Australia) was dissolved at 2 mg/ml in distilled H_2O and then further diluted 1/80 with sodium phopsphate buffer (0.05 M Na_2HPO_4, 0.05 M $NaH_2PO_4.2H_2O$, 2 M NaCl, 0.002 M EDTA, pH 7.4). The absorbance at 260 nm of the standard was measured using a spectrophotometer (UV/VIS 918, GBC Scientific Equipment Pty.Ltd, Australia). An accurate concentration of the standard was determined according to the relation that absorbance at 260 nm of 50 µg/ml DNA is equal to 1. The standard was then diluted further to achieve a concentration of 100 µg/ml and stored as 500 µl aliquots at -20°C.

When each assay was performed a standard curve was made from the stock solution of salmon testes DNA at concentrations of 100, 80, 50, 30, and 10 µg/ml of DNA. Next, frozen right kidneys were pulverised in a mortar and pestle on dry ice and roughly half the kidney mass weighed accurately and placed in a 50 ml plastic tube on ice. Sodium phosphate buffer was added to give a final volume of 10 ml and the tissue then homogenised (Ultra-Turrax T25, Janke & Kunkel, IKA-Labortechnik, Germany) at

high speed for approximately 60 seconds. The homogenate was then centrifuged at 2,000 rpm for 5 min (Model J-6B, Beckman Instruments, USA). A 100 µl aliquot of the supernatant was added to 400 µl of the sodium phosphate buffer. Triplicate 50 µl aliquots of the standards, and duplicate 50 µl aliquots of the samples were added into plastic cuvettes. Next, 850 µl of 0.002 M EDTA/NaCl, and 600 µl of the fluorochrome stock (diluted 1/40 to 2.4 µg/ml with sodium phosphate buffer) was added to the cuvettes, vortexed and then left in the dark for 15 min at room temperature. The standards and samples were read on a fluorimeter (F-2000 Fluorescence Spectrophotometer, Hitachi, Japan) at excitation wavelength of 356 nm and emission wavelength of 480 nm. The average fluorescence value for the DNA standards was used to construct a standard curve (see Figure 5.2.1) and the x- and y-axis intercepts calculated. The concentration of DNA in the experimental samples was determined by interpolation of the DNA standard curve using the mean fluorescence value for each sample. The total DNA content for the whole kidney was then determined by multiplying the DNA concentration (in mg/g) by the kidney wet weight.

5.2.3.2 Whole kidney protein content

The assay used to determine the protein content of the right kidneys was a modification of the Lowry method *(Lowry et al., 1951)*. A stock standard was made using 2.5 g of Bovine Serum Albumin (BSA; Sigma Chemical Co., Australia) dissolved on 25 mls of distilled H_2O and frozen in aliquots at –20°C. On the day the assay was performed a standard curve was constructed from the stock solution of BSA at concentrations 20, 16, 12, 8, 4, and 0 mg/ml of protein. To a 10 ml plastic screwcap tube, 5 ml of reagent A (2% Na_2CO_3 in 0.1 M NaOH, 2% sodium tartrate, 1% cupric sulfate) was added. To this, 10 µl of standard or the homogenate supernatant used in the DNA assay was added to the tube, vortexed and let stand at room temperature. After 7 min 500 µl of reagent B (1 M Folin-Ciocalteu phenol reagent) was added to the sample, the tubes were vortexed again and let stand at room temperature for 1 hour. The absorbance of each sample (in triplicate) was read at 620 nm, and the average absorbances for the protein standards were used to construct a standard curve and the x- and y-axis intercepts were calculated (see Figure 5.2.2 for mean standard curve). The concentration of protein in the experimental samples was determined by the average absorbance and by interpolation of the protein standard curve (to give a concentration in mg/ml). Protein concentration was adjusted to mg/gm of kidney tissue and the total protein content of the kidneys determined by multiplying the protein concentration by the wet weight of the kidneys.

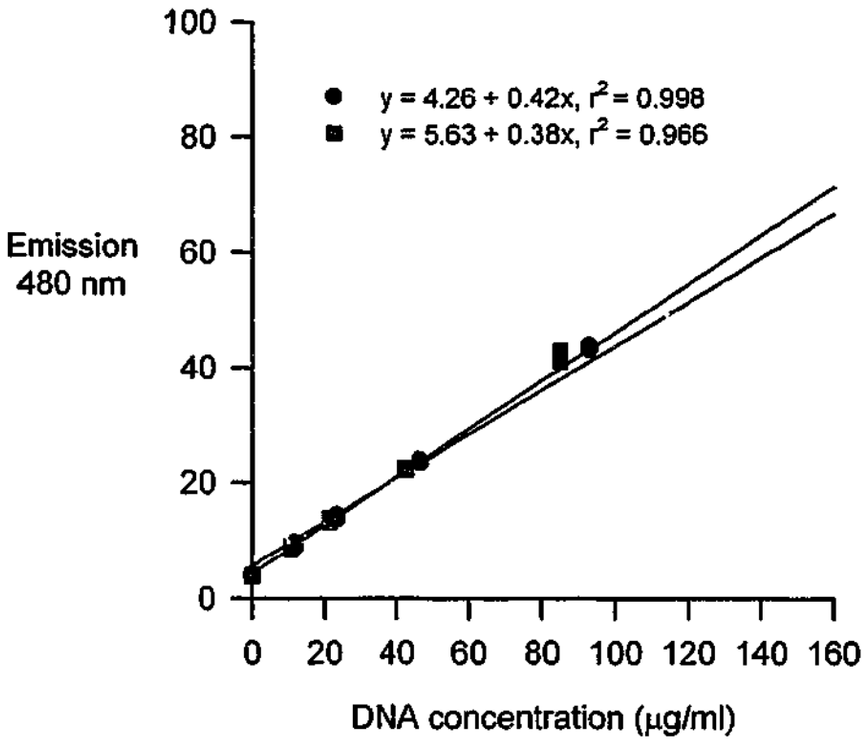


Figure 5.2.1: DNA standard curves constructed using known concentrations of salmon testes DNA. Concentration of DNA in experimental samples was determined by interpolation of standard curve using the mean fluorescence value for each sample.

In a separate qualitative control test, the small amount of phosphate buffer contained in the kidney homogenate was found not to interfere with the assay (assessed by comparing standard curves generated by adding the standard to water, or the phosphate buffer, see Figure 5.2.3).

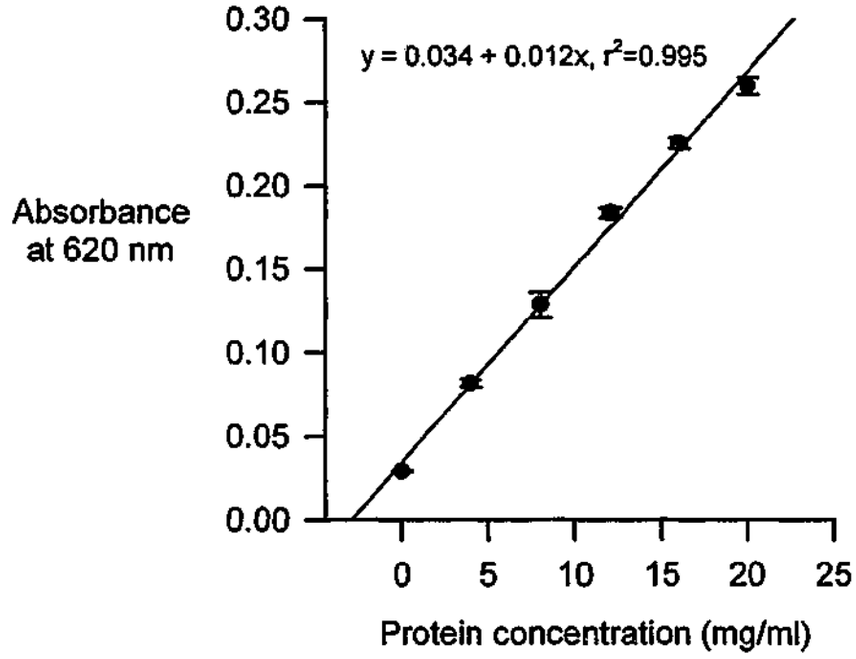


Figure 5.2.2: Depicts the average absorbance (mean ± SEM) for each standard protein concentration; values averaged for 6 protein assays. Protein concentrations in experimental samples were determined by interpolation of the standard curve using average absorbance values.

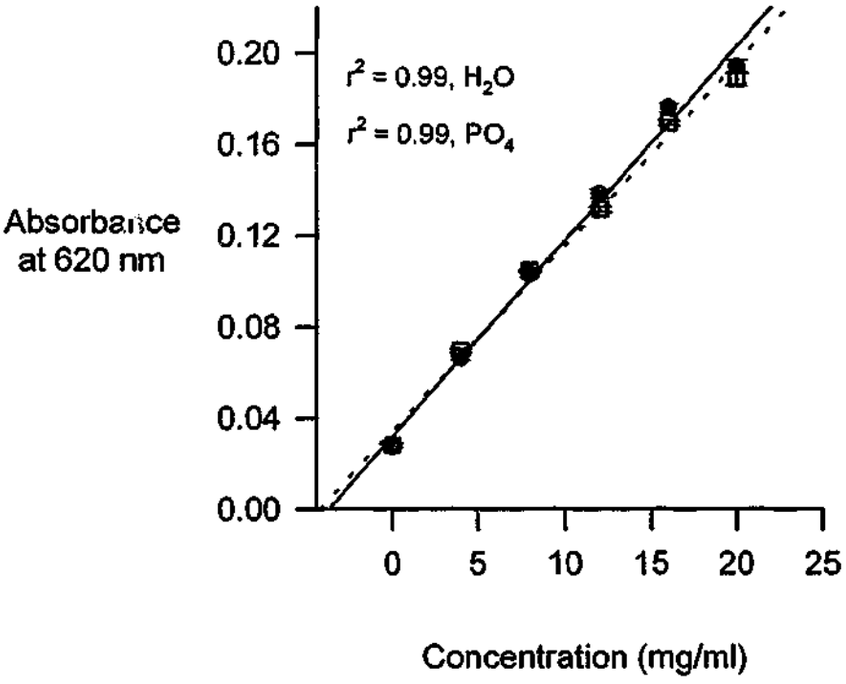


Figure 5.2.3: Comparison of water (H_2O; open square and dashed line) and phosphate (PO_4; closed circle, solid line) buffers used in protein assay to determine total kidney protein content and concentration. The small amount of phosphate buffer present in the kidney homogenate does not affect the absorbance values of the known protein standards.

5.2.4 *In situ* hybridisation: expression of growth factor genes in the kidney

In situ hybridisation provides a technique that can be used to localise gene expression to individual cells or populations of cell. The technique is based on the use of anti-sense single-strand fragments of RNA or DNA that are complementary to nucleic acid sequence of the gene of interest. RNA fragments labelled with radioactivity are hybridised to the complementary cellular nucleic acid sequence under appropriate conditions to form stable hybrids. The radioactive hybrid can then be visualised using autoradiographic techniques and gene expression localised to structures within a tissue or organ *(Wilkinson, 1992; Angerer and Angerer, 1992).*

There are a number of factors that can affect the formation of stable hybrids and thus the sensitivity of the *in situ* hybridisation technique. These include; i) acessibility and integrity of mRNA in the tissue, ii) type of probe, how well it was labelled, and how abundant the message is in the tissue (ie limits of detection), iii) the hybridisation conditions (ie hybridisation temperature, salt conditions), and iv) the degree of non-specific background binding of the probe to the tissue *(Wilkinson, 1992).* The selectivity of the technique depends on the choice of probe, in that it is vital to ensure that the sequence of the probe is unique to the nucleic acid sequence of interest in the tissue and will not cross hybridise to other similar nucleic acid sequences. As the entire genome for the rat has yet to be fully identified, the selectivity of the probe for the target mRNA cannot be guaranteed, so appropriate controls are necessary.

The protocol used in this study was based on previous experiments performed by Morgan *et al.* (1993).

5.2.4.1 Tissue preparation

Ten micrometer coronal sections of left kidneys from animals that had received a 5 or 10 day intravenous infusion of vehicle (heparinised saline) or Ang II (4.5 ng/kg/min) were cryo-sectioned and thaw mounted on gelatin coated slides. Slide mounted sections were stored at –80°C until needed for *in situ* hybridisation.

5.2.4.2 Tissue fixation and dehydration

Frozen, mounted sections were thawed and air dried for approximately 20 min before being placed in glass racks and passed through a series of washes. First, the sections were lightly fixed for 20 min at 4°C in 4% paraformaldehyde/ phosphate buffered saline (PBS) pH 7.4. Mounted sections were then rinsed in PBS (2 x 5 min

each) soaked with 0.1 M triethanolamine.HCl (5 min) and then incubated in 0.1 M triethanolamine.HCl/ 0.25% acetic anhydride (10 min). The triethanolamine/ acetic anhydride incubation is used to facilitate tissue acetylation to improve signal quality. Acetylation blocks positively charged amino groups on tissue proteins and reduces non-specific electrostatic binding of the probe to the tissue *(Angerer and Angerer, 1992)*. The sections were then rinsed in fresh PBS for 5 min and then dehydrated through a series of ethanol solutions (30%, 50%, 70%, 85%, 95%, 95%, 100%, 100% v/v; 3 min each), and air dried ready for pre-hybridisation.

5.2.4.3 Probe amplification and purification

Basic Fibroblast Growth Factor (bFGF)

Plasmids containing the bFGF DNA were prepared and kindly donated by Dr. Miriam Ford, Department of Anatomy and Cell Biology, University of Melbourne. The preparation of the plasmids involved end-filling of RT-PCR products for FGF1 (aFGF) (~190bp) or FGF2 (bFGF) (~180 bp; mouse sequence) with Klenow DNA polymerase *(Sambrook et al., 1989)*. The DNA was then blunt-end inserted into the *SmaI* restriction site of the pGEm-3Zf(+) vectors (Promega, WI, USA) and transformed into DH5α strain *Escherichia coli (E.coli)*. The FGF DNA was inserted into the plasmids orientated such that T7 RNA polymerase would produce sense FGF cRNA and SP6 RNA polymerase would produce antisense FGF cRNA *(Nurcombe et al., 1993)*. Both aFGF and bFGF probes were used previously for *in situ* hybridisation studies in the embryonic rat kidney *(Cancilla, 1998)*. Kidney sections were hybridised to bFGF antisense cRNA probe (complimentary to the bFGF gene sequence), and aFGF sense probe (complementary to the non-coding strand of the aFGF gene). The bFGF gene also encodes an antisense mRNA which is transcribed from the opposite DNA strand of the bFGF gene *(Li et al., 1996b; Li et al., 1996a; Knee et al., 1994; Kimelman and Kirschner, 1989)*. The level of bFGF antisense mRNA in adult rat kidneys is high, much greater than the level of the bFGF sense mRNA, and codes for a protein approximately 25 kDa in size, that has a putative regulatory role in bFGF sense mRNA expression and turnover *(Li et al., 1996b; Li et al., 1996a; Kimelman and Kirschner, 1989)*. For this reason, in the present experiments a aFGF sense probe was used as a negative control (ie: a probe similar in size, and specific activity that should not hybridise to transcribed RNA) for the *in situ* hybridisation.

Nutrient agar plates were innoculated with the *E.coli* bacterial stocks and incubated at 37°C overnight. From this, a single bacterial colony was picked and an overnight culture grown in 5 ml of Luria-Bertani (LB) nutrient broth at 37°C containing 50 μg/ml Ampicillan. One ml of the LB broth was frozen in 100% glycerol and stored at

–80°C, to be used as bacterial stock for future use. Plasmid DNA was purified from the bacterial cells using a Wizard™ minipreps DNA purification kit (Promega, WI, USA) which is a modified alkaline lysis procedure *(Birnboim and Doly, 1979)*, followed by a column purification using Wizard™ minipreps DNA purification resin and minicolumns.

An aliquot of the DNA was incubated with *Kpn*I restriction enzyme (Gibco BRL, Life Technologies, Australia) in order to confirm the presence of the aFGF and bFGF DNA in the appropriate bacterial stocks. The digest reaction was run on a 2% agarose gel (DNA grade agarose, Progen Industries Ltd., Australia) and the bands of plasmid DNA examined under ultraviolet light to determine the molecular size by comparison with a molecular weight DNA ladder (DNA ladder, Sigma Chemical Co., Australia).

When the presence of aFGF and bFGF DNA in the appropriate bacterial stocks was confirmed, a fresh overnight culture of *E.coli*, picked from the new frozen stocks, was grown in 5 ml of LB broth at 37°C. From this a 250 ml overnight culture of *E.coli* was grown and a stock of plasmid DNA containing the aFGF and bFGF DNA was collected by purification using a Wizard™ maxiprep DNA purification kit (Promega, WI, USA) and a yield determined by reading absorption at 260 nm on a spectrophotometer UV/VIS 918, GBC Scientific Equipment Pty Ltd, Australia).

Next, stocks of linearised plasmid DNA were made by incubating at 37°C overnight with restriction enzyme *Sal*I (Gibco BRL, Life Technologies, Australia) to give aFGF sense and bFGF sense DNA templates, and *Eco*RI (Gibco BRL, Life Technologies, Australia) to give aFGF anti-sense and bFGF anti-sense DNA templates. An aliquot of digested plasmid DNA was run on a 1% agarose gel to confirm linearisation. The remaining linearised stocks were purified by extracting with 25:24:1 phenol:chloroform:iso-amyl alcohol. The aqueous phase was then precipitated with 95% ethanol, centrifuged (Eppendorf mini centrifuge, 5415C, Crown Scientific, Australia) at 14,000rpm for 15 min and the pellets air dried before being resuspended in deionised H_2O. A working stock of linearised plasmid was then diluted to approximately 100 ng/µl for probe labelling reactions.

Transforming Growth Factor β1 (TGF-β1)

Transforming growth factor β1 probe was generously supplied by Dr. Nancy Nichols, Department of Physiology, Monash University *(Morgan et al., 1993)*. Plasmids containing DNA complementary to the 5' coding region sequences of TGF-β1 mRNA (0.7kb *kpn*I-*Bam*HI fragment) were previously amplified, purified and linearised by Nicole Bye, Department of Physiology, Monash University *(Bye, 1996)*. The 0.7kb fragment of TGF-β1 DNA was orientated such that transcription with SP6 RNA polymerase produced TGF-β1 anti-sense cRNA (complimentary to the coding strand of

the TGF-β1 gene), and T7 RNA polymerase synthesised TGF-β1 sense cRNA (complimentary to the non-coding strand of the TGF-β1 gene). Kidney sections were hybridised with TGF-β1 anti-sense probe (indicative of TGF-β1 mRNA levels), and TGF-β1 sense probe (complimentary to the non-coding strand of the TGF-β1 gene, and thus a negative control for the hybridisation experiment).

5.2.4.4 *In vitro transcription of radioactive labelled cRNA probes*

Radioactive Isotopes

TGF-β1 cRNA probes were synthesised using 250 μCi [^{35}S]-UTP (specific activity of 1200 Ci/mmol, 30 μM). Recently, an alternate radionucleotide has been increasingly used in *in situ* hybridisation experiments. [α-^{33}P] radionucleotides have a lower tendancy to produce non-specific background binding (they are less 'sticky' than the bulky [^{35}S] radionucleotide), and the β-particles emitted by this isotope have energies intermediate between those emitted from [^{35}S] (0.167 MeV) and [^{32}P] (1.71 MeV) *(McLaughlin and Margolskee, 1993)*. The higher energy and lower background binding achieved with [α-^{33}P] is a benefit when detecting transcripts expressed at very low levels. For this reason [α-^{33}P]-UTP (specfic activity 2000 ci/mmol, 30 μM) was used to label FGF cRNA probes.

Synthesis of cRNA probes

In vitro transcription of [α^{33}P]-UTP and [^{35}S]-UTP cRNA probes was performed in a reaction volume of 10 μl which contained 250μCi [α^{33}P]- or [^{35}S]-UTP (NEN™ Life Science Products, MA, USA; dried down), 1 x transcription buffer (Gibco BRL, Life Technologies, Australia), 10mM DTT, 2 units/μl RNasin (Promega, WI, USA), 500μM nucleotide mix (ATP, CTP, GTP; Pharmacia Biotech, Australia), and 100 ng/μl of DNA template for aFGF, bFGF, or TGF-β1 and 1 unit/μl of SP6 RNA polymerase or T7 RNA polymerase (Gibco BRL, Life Technologies, Australia). The reaction was incubated at 37°C for one hour. Template DNA was then digested in by adding 60 μl of 1 x transcription buffer, 1 unit/μl of RQ1 DNase (Gibco BRL, Life Technologies, Australia) at 37°C for 15 min.

The solution was then passed through a NucTrap® Push Column (Stratagene, CA, USA) to remove unincorporated nucleotides. A 2 μl aliquot of the column purified solution was diluted 1 in 10 with water and used for quantitation of nucleotide incorporation via liquid scintillation counting. A further 2 μl aliquot of the diluted probe was added to 97 μl of deionised H_2O and 5 μl salmon sperm DNA. A 5 μl aliquot was

dried onto filter (total counts in reaction) and the remaining solution precipitated with acid (incorporated counts) and dried under vacuum. The filters were then counted in a β-counter (Liquid Scintillation systems, LS 5801, Beckman, USA). The percentage incorporation of radioactive nucleotide into the cRNA probe was calculated by the ratio of total counts in the reaction to counts from the precipitated probe.

Labelled probes were used in *in situ* hybridisation histochemistry experiments at a concentration of 0.3 µg/ml/kb.

5.2.4.5 In situ hybridisation protocol

Tissue sections that had been fixed in paraformaldehyde, dehydrated in ethanol and air dried (section 4.2.4.2) were incubated with ~100 µl of hybridisation buffer 50% deionised formamide v/v, 4 x standard saline citrate (SSC) buffer v/v [20 x stock; 3 M NaCl, 0.3 M sodium acetate, pH 7.0], and 10% w/v dextran sulphate, 1 x Denhardt's solution [0.2% w/v Bovine Serum Albumin, 0.2% w/v Ficoll, 0.2% w/v polyvinyl-pyrollidone, 0.1 M dithiothreietel DTT]) for 1 hour at 55°C in a sealed humidified chamber. After 1 hour pre-hybridisation the slides were removed from the oven and the hybridisation buffer drained off. Fresh hybridisation buffer containing [^{33}P]- or [^{35}S]-labelled probe (1-3 ng probe/ 10 µl of hybridisation buffer) was added at 10 µl per section. The sections were covered with glass cover slips and incubated again at 55°C in a humidified chamber for 3 hours. The hybridsation temperature of 55°C, did not exceed the melting temperature for double stranded bFGF and TGF-β1 hybrids, and consequently allowed the radioactive probe to bind to the target mRNA in the tissue.

5.2.4.6 Post-hybridisation washes

Post-hybridisation washes were used to remove inbound probe from the tissue sections and RNase A treatment digested partial, non-specific hybrids. Following hybridisation the slides were soaked for 15 min in 4 x SSC, 100 mM Beta-Mercaptoethanol (βME) to soak off coverslips. In hybridisations using [^{33}P]-labelled probe βME was omitted from all wash solutions. The slides were rinsed in 0.5 M NaCl, 0.05 M phosphate buffer (PB) for 10 min before being digested with RNase (0.025 mg/ml in 0.5 M NaCl, 0.05 M PB) for 30 min at 37°C to remove partial hybrids and any single stranded RNA molecules in the tissue (leaving just the radioactive double stranded hybrids). Hybridised sections were then incubated in a high stringency wash containing low salt, formamide and high temperature ensuring that only probe bound to target mRNA remained. The high stringency wash contained 50% formamide, 0.5 M NaCl, 0.05 M PB and 100 mM βME and slides were washed at 63°C for 30 min before

being rinsed in 0.5 x SSC overnight. The next morning the sections were dehydrated in a graded series of ammonium acetate/ ethanol solutions (30, 50, 70% ethanol with 300 mM ammonium acetate, then 95, 99, 99% ethanol, 3 min each) before exposing to autoradiographic film or liquid film emulsion.

5.2.4.7 Localisation of gene expression: film and emulsion autoradiography

Following hybridisation with TGF-β1 sense and anti-sense cRNA probes, slides were exposed to autoradiographic film (Kodak Biomax MR, Australia) for three days to determine the length of time needed for exposure to liquid film emulsion. Because of the very short half life of the [^{33}P]-labelled bFGF probe, kidney sections that were hybridised with bFGF were not exposed to autoradiographic film, but rather exposed directly to liquid film emulsion.

Kodak emulsion (NBT2, Kodak, Australia) that had been previously diluted 1:1 with 600 mM ammonium acetate, pre-warmed to 42°C in a water bath. The liquid photographic emulsion was always handled in total darkness. Slides were coated by dipping them (x3) into emulsion, then when dry, transferring to a light tight container which was stored at 4°C for three weeks. The sections were developed in darkness by incubating in Kodak D19 developer (2.5 min, 15°C), dH_2O (30 seconds), Kodak Fixative (#197-1573, 5 min), dH_2O (15 min), and then running tap H_2O for 30 min. Sections hybridised to TGF-β1 cRNA probes were stained with celestine blue and Mayer's Haemotoxylin.

Kidney sections hybridised with [^{33}P]-labelled FGF probes were exposed to liquid film emulsion for 8 weeks. Tester slides were developed at 4 weeks to ensure there was no over exposure. Based on the complete absence of silver grains at this time the rest of the slides were left for 8 weeks before developing and staining with 0.5% cresyl-violet.

5.2.4.8 Image analysis of in situ hybridisation

Quantitative image analysis was conducted on kidney sections hybridised with TGF-β1 cRNA probe. Kidney sections from four animals in each treatment group were analysed for density of silver grains which represents TGF-β1 gene expression. Two sections from each animal were quantitated. In each section, the number of silver grains were counted over 25 glomeruli, 10 non-glomerular cortical regions, and 10 medullary regions. Grain counting was conducted under bright field conditions at a magnification of x 400, using a Fuji HC-2000 high-resolution digital camera and Analytical Imaging Station 4.0 software (Imaging Research Inc, Ontario, Canada). The

number of silver grains were counted in one image frame, which was placed over glomeruli, cortical regions, or medullary regions. An animal average was calculated for each kidney area quantitated and then group mean ± SEM was calculated for each treatment group and each region.

5.2.5 Statisical analysis

Cardiovascular data, total kidney DNA and protein content were analysed for significant differences between animals that received an intravenous infusion of vehicle or Ang II using an unpaired student *t*-test, on days 5 and 10. Two way analysis of variance was used to test whether there was a significant effect of treatment (vehicle or Ang II infusion) or time (5 or 10 days) on the density of silver grains, representative of TGF-β1 mRNA expression, over glomeruli, non-glomerular cortical regions and medullary regions of the kidney. A probability of 0.05 or less was accepted as statistically significant.

5.3 RESULTS

5.3.1 Cardiovascular measurements

Intravenous infusion of low doses of Ang II for 5 days did not significantly alter mean arterial pressure (P = 0.15, Figure 5.3.1, upper panel). However, intravenous infusion of the same dose of Ang II for 10 days resulted in a significant increase in arterial pressure of approximately 50 mmHg compared to vehicle treated animals (mean arterial pressure 158 ± 7 mmHg and 107 ± 2 mmHg respectively; P <0.001, Figure 5.3.1, upper panel). The increase in mean arterial pressure in the animals treated with Ang II (4.5 ng/kg/min) for 10 days was also associated with an increased left ventricular to bodyweight ratio when compared to vehicle controls (P =<0.001, Figure 5.3.1, lower panel). Likewise the animals that received an intravenous infusion of Ang II for 5 days also demonstrated a slightly larger left ventricular to bodyweight ratio compared to control animals ($2.2 \pm 0.05 \times 10^{-3}$ vs $2.0 \pm 0.05 \times 10^{-3}$ respectively, P =0.03, Figure 5.3.1 lower panel). Intravenous infusion of Ang II for 5 or 10 days did not significantly alter heart rate, haematocrit or body-weight when compared to respective vehicle treated animals (refer to Table 5.1).

5.3.2 Whole kidney DNA and protein content

Whole kidney growth was estimated by measuring total kidney DNA and protein content. Ang II infusion for 5 or 10 days was not associated with an increase in total kidney DNA or protein content when compared to the respective vehicle treated group (Table 5.2). Likewise, DNA:protein ratio was not significantly different in the animals treated with Ang II for 5 or 10 days, when compared to the animals that received a vehicle infusion for 5 or 10 days (P = 0.14, 0.67 respectively, Figure 5.3.2).

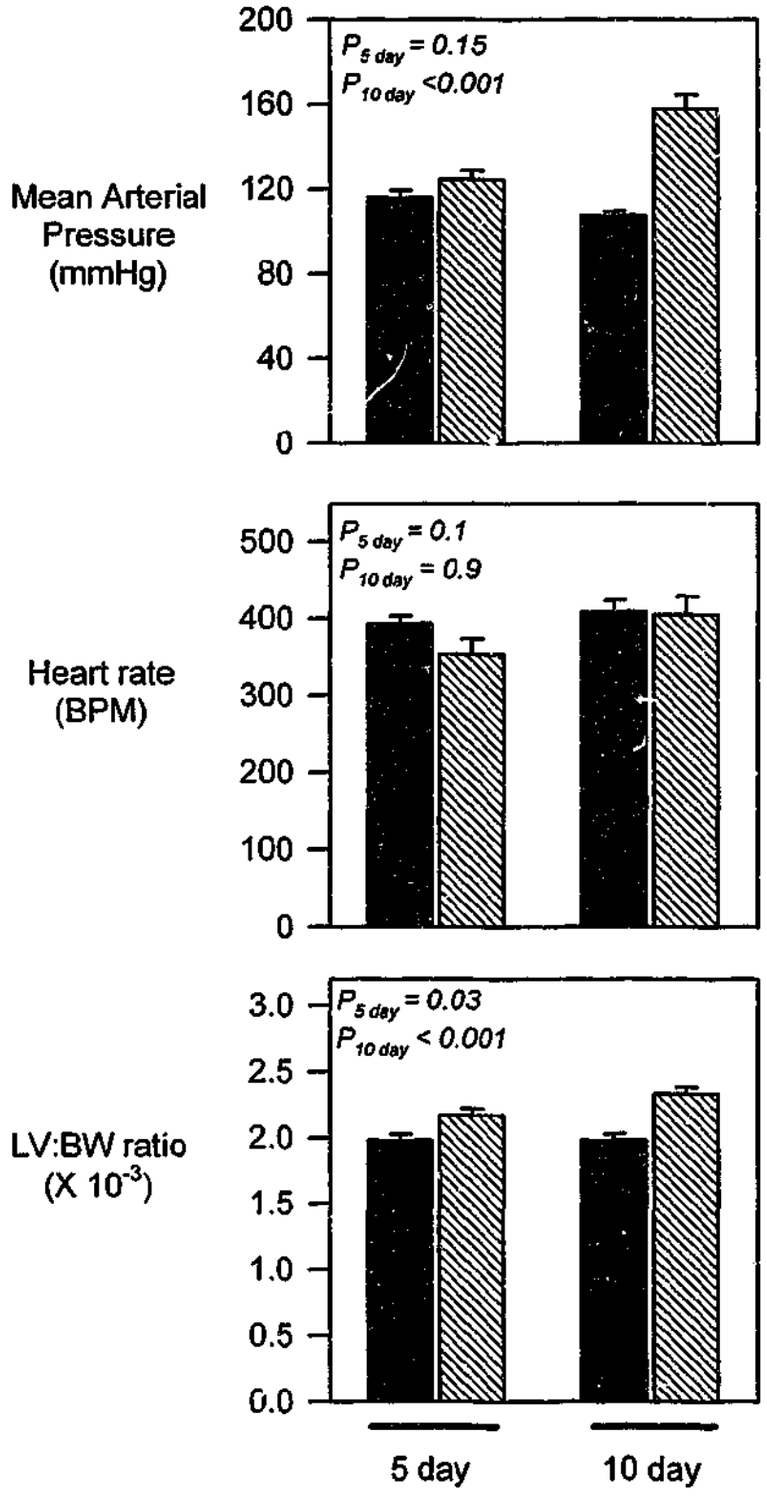


Figure 5.3.1: Conscious mean arterial pressure, heart rate and left ventricle: bodyweight ratio (LV:BW) in rats infused with vehicle (heparinised saline 10 IU/ml; black bars) or Ang II (4.5 ng/kg/min; hatched bars) for 5 or 10 days. Unpaired student *t*-tests were used to test for significant differences in mean arterial pressure, heart rate or LV:BW ratio between animals that received an intravenous infusion of vehicle or Ang II for 5 ($P_{5\ day}$) or 10 ($P_{10\ day}$) days.

	5 day infusion			10 day infusion		
	Vehicle (n=6)	Ang II (n=6)	*t*-test (*P*-value)	Vehicle (n=5)	Ang II (n=6)	*t*-test (*P*-value)
Mean Arterial Pressure (mmHg)	116 ± 3.5	124 ± 4.3	0.15	107 ± 2.0	158 ± 6.7	< 0.001
Heart Rate (bpm)	394 ± 11	354 ± 20	0.11	409 ± 15	405 ± 24	0.89
Body-Weight (g)	295 ± 14	311 ± 13	0.41	356 ± 20	359 ± 8	0.88
Left Ventricle : Body-Weight ($x10^{-3}$)	2.0 ± 0.05	2.2 ± 0.05	0.03	2.0 ± 0.05	2.3 ± 0.05	< 0.001
Haematocrit (%)	40.4 ± 1.4	41.3 ± 0.6	0.55	40.7 ± 0.96	41.7 ± 0.9	0.54
Whole heart : Body-Weight ($x10^{-3}$)	2.5 ± 0.07	2.7 ± 0.08	0.13	2.5 ± 0.05	2.9 ± 0.06	< 0.001
Right Ventricle: Body-Weight ($x10^{-3}$)	0.56 ± 0.03	0.54 ± 0.03	0.77	0.52 ± 0.03	0.58 ± 0.05	0.32

Table 5.1: Cardiovascular variables and organ weights in rats that received an intravenous infusion of vehicle (heparinised saline 10 IU/ml) or Ang II (4.5 ng/kg/min) for 5 or 10 days.

All parameters were analysed using unpaired student *t*-tests to test for significant differences between animals that received an intravenous infusion of vehicle or Ang II, for 5 or 10 days.

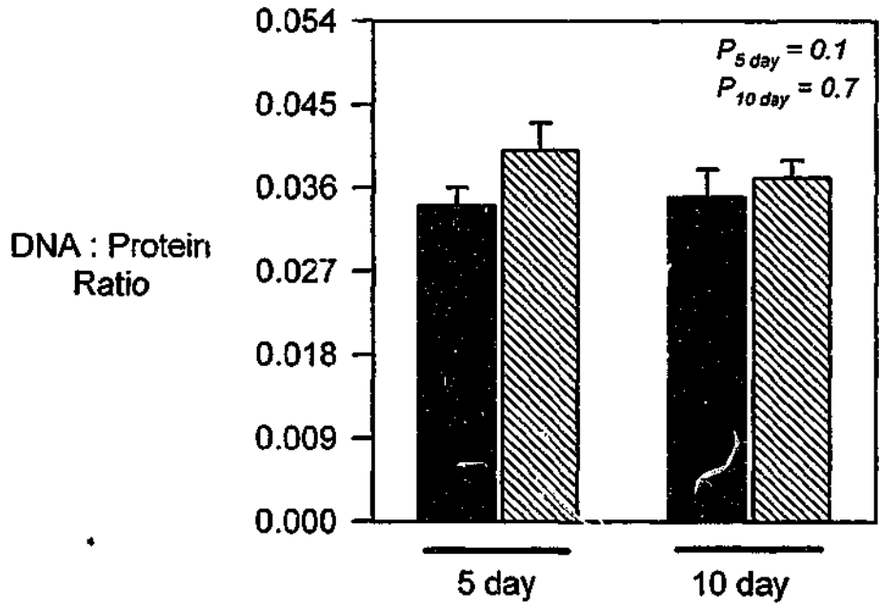


Figure 5.3.2: Right kidney DNA: protein ratio in rats that received an intravenous infusion of vehicle (heparinised saline 10 IU/ml; black bars) or Ang II (4.5 ng/kg/min; hatched bars), for 5 or 10 days. Data was analysed using unpaired students *t*-tests to test for significant differences between vehicle or Ang II treated animals after 5 or 10 days of infusion.

	5 day infusion			10 day infusion		
	Vehicle (n=6)	Ang II (n=6)	*t*-test (*P*-value)	Vehicle (n=5)	Ang II (n=6)	*t*-test (*P*-value)
[DNA] (mg/gm kidney tissue)	5.12 ± 0.30	5.28 ± 0.20	0.64	5.13 ± 0.21	5.19 ± 0.27	0.86
Total DNA content (mg)	6.32 ± 0.5	7.91 ± 1.2	0.23	6.93 ± 0.51	7.07 ± 0.6	0.86
[Protein] (mg/gm kidney tissue)	152 ± 3.5	134 ± 6.9	0.05	147 ± 6.0	142 ± 4.9	0.53
Total protein content (mg)	187 ± 11	194 ± 14	0.71	200 ± 19	192 ± 10	0.72
DNA:Protein ($x10^{-3}$)	34 ± 2.0	40 ± 2.0	0.14	35 ± 3.0	37 ± 2.0	0.67
Right kidney frozen wt (g)	1.23 ± 0.06	1.47 ± 0.17	0.20	1.36 ± 0.11	1.36 ± 0.08	0.99

Table 5.2: DNA and protein concentration, total DNA and protein content, and DNA: protein ratio in the right kidney of rats infused with vehicle (heparinised saline 10 IU/ml) or Ang II (4.5 ng/kg/min) for 5 or 10 days.
All measurements were analysed using unpaired student *t*-tests to test for significant differences between animals that received an intravenous infusion of vehicle or Ang II, for 5 or 10 days.

5.3.3 Expression of growth factor genes TGF-β1 and bFGF in the kidney

5.3.3.1 TGF-β1 mRNA levels in the kidney

Expression of TGF-β1 mRNA in kidney sections was extremely low, requiring a 4 week exposure to liquid emulsion. Blood vessels in the frozen kidney sections were hard to visualise as the total renal vasculature comprises only about 1% of the volume of the kidney (*Kett et al., 1995*). Additionally, as the kidneys were fresh frozen, most vessels had collapsed during the freezing process. For this reason vascular expression of TGF-β1 mRNA was not analysed. In general, as the tissue was not perfusion fixed, identification of specific cell types was difficult. Thus, silver grain density, representative of TGF-β1 mRNA levels was analysed in a region specific manner, in glomeruli, non-glomerular cortical regions, and medullary regions of the kidney. Sliver grain density was measured per scan frame, which was positioned over one of the three regions. Qualitatively, silver grain density was appeared greatest in glomeruli of the kidney.

TGF-β1 mRNA levels in the kidney, represented as proportional area of sliver grains within the scanned area are presented in Figure 5.3.3. In all areas (glomerular, cortical, and medullary), TGF-β1 mRNA levels were significantly increased in animals that received an intravenous infusion of vehicle or Ang II for 5 days, compared to animals that received a 10 day infusion or either vehicle or Ang II (P_{time} = 0.04 (glomeruli), 0.003 (cortex), 0.03 (medulla), Figure 5.3.3). There was no significant effect of intravenous infusion of Ang II for 5 or 10 days on TGF-β1 mRNA levels in glomerular, cortical, or medullary regions, when compared to the respective vehicle groups (Figure 5.3.3).

Silver grain density (representative of TGF-1 mRNA levels) was also expressed as total number of silver grains in scan area (Figure 5.3.4), and total silver grain area (Figure 5.3.5). Statistical analysis produced consistent results, regardless of the way the density was analysed (refer Figure 5.3.4 and 5.3.5).

5.3.3.2 Autoradiographic localisation of TGF-β1 mRNA in the kidney

Silver grain density, representative of TGF-β1 mRNA levels over glomeruli from animals that received an intravenous infusion of Ang II or vehicle for 5 or 10 days is depicted in Figure 5.3.6. Representative glomeruli from animals that received infusions of vehicle for 5 days (Figure 5.3.6, panel A), and Ang II for 5 days (Figure 5.3.6, panel B) were hybridised with ^{35}S-labelled cRNA probe complimentary to the coding

sequence of the TGF-β1 gene. A distinct lack of silver grains is apparent in a representative glomerulus from an animal that received an infusion of Ang II for 5 days, and was hybridised with a ^{35}S-labelled cRNA probe complimentary to the non-coding strand of the TGF-β1 gene kidney (a negative control, for the hyridisation experiment), suggesting that no specific binding occurred with the TGF-β1 sense probe (Figure 5.3.6, panel C).

5.3.3.3 Autoradiographic localisation of bFGF mRNA in the kidney

Kidney sections hybridised with bFGF probes were emulsion dipped and left to expose for 8 weeks. Expression of bFGF mRNA was undetectable in kidneys from animals that received Ang II or vehicle for 5 or 10 days (Figure 5.3.7, panel B). In contrast, bFGF was abundantly expressed in a positive control, the CA2 region of the rat brain hippocampus (Figure 5.3.7, panel A). Expression in the positive control section of rat brain, indicates that hybridisation with the bFGF cRNA probe resulted in a detectable signal using the present protocol. The absence of expression in the kidneys of both Ang II and vehicle rats therefore suggests that bFGF mRNA is not expressed in the kidney, or expression was below the level of the sensitivity of the assay.

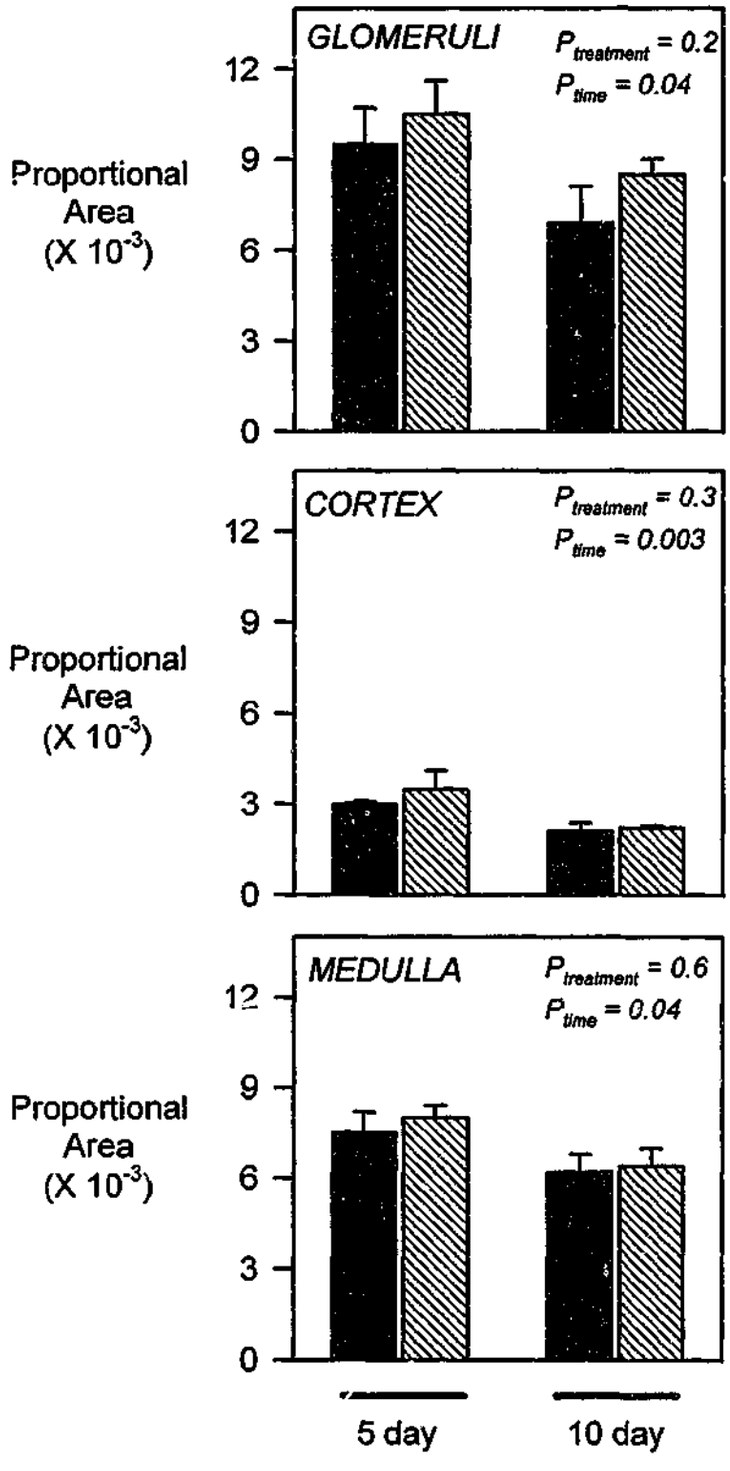


Figure 5.3.3: Proportional area of silver grains over glomeruli (upper panel), non-glomerular cortical (middle panel), and medullary regions (lower panel) in left kidneys from rats that received an intravenous infusion of vehicle (heparinised saline 10 IU/ml; n=4, black bars) or Ang II (4.5 ng/kg/min; n=4, hatched bars), for 5 or 10 days. Silver grain density is respresentative of TGF-β1 mRNA expression. A two-way analysis of variance was used to test for a significant of treatment (vehicle or Ang II, $P_{treatment}$) or time (5 or 10 days, P_{time}).

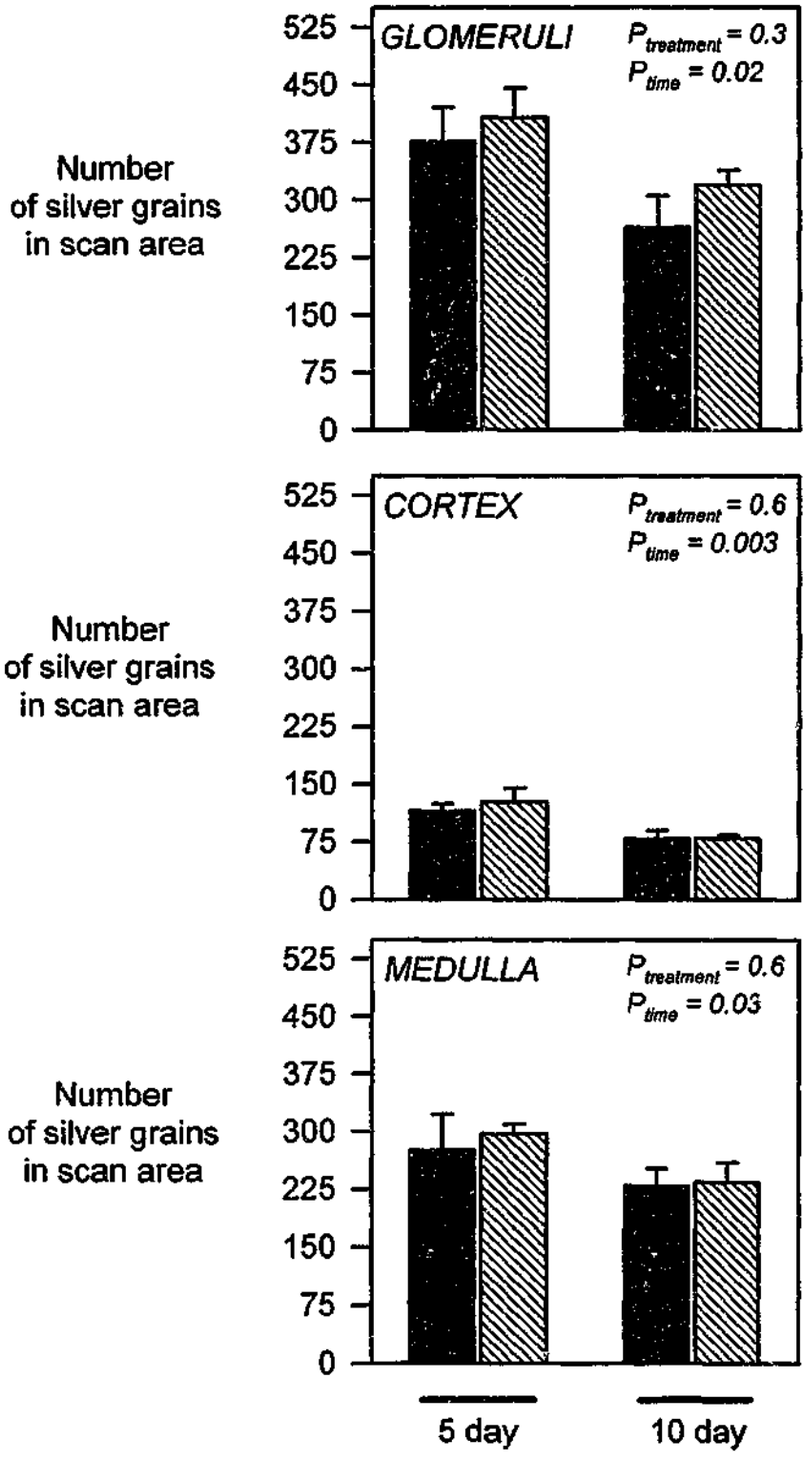


Figure 5.3.4: Number of silver grains in scan area, over glomeruli (upper panel), non-glomerular cortical (middle panel), and medullary regions (lower panel) in left kidneys from rats that received an intravenous infusion of vehicle (heparinised saline 10 IU/ml; n=4, black bars) or Ang II (4.5 ng/kg/min; n=4, hatched bars), for 5 or 10 days. Silver grain density is respresentative of TGF-β1 mRNA expression. A two-way analysis of variance was used to test for a significant of treatment (vehicle or Ang II, $P_{treatment}$) or time (5 or 10 days, P_{time}).

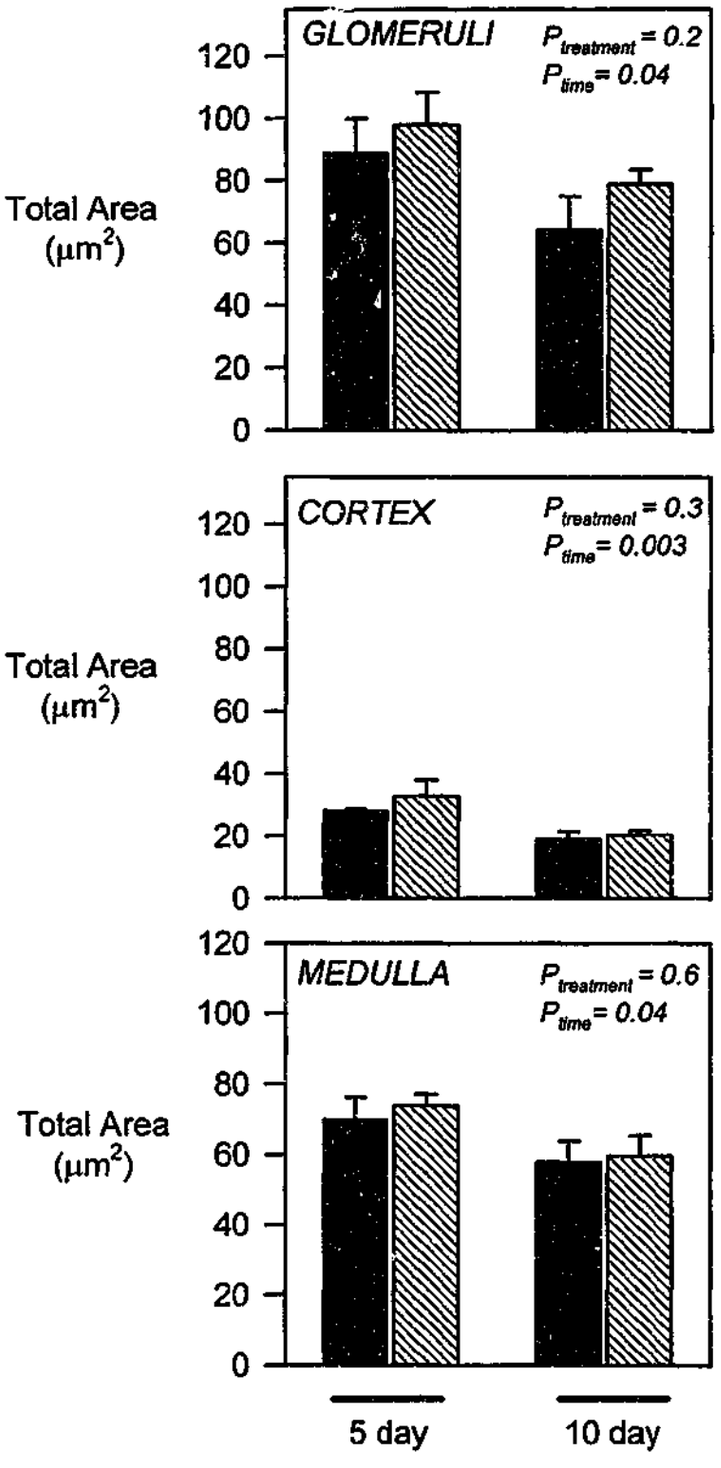


Figure 5.3.5: Total area of silver grains, over glomeruli (upper panel), non-glomerular cortical (middle panel), and medullary regions (lower panel) in left kidneys from rats that received an intravenous infusion of vehicle (heparinised saline 10 IU/ml; n=4, black bars) or Ang II (4.5 ng/kg/min; n=4, hatched bars), for 5 or 10 days. Silver grain density is respresentative of TGF-β1 mRNA expression. A two-way analysis of variance was used to test for a significant of treatment (vehicle or Ang II, $P_{treatment}$) or time (5 or 10 days, P_{time}).

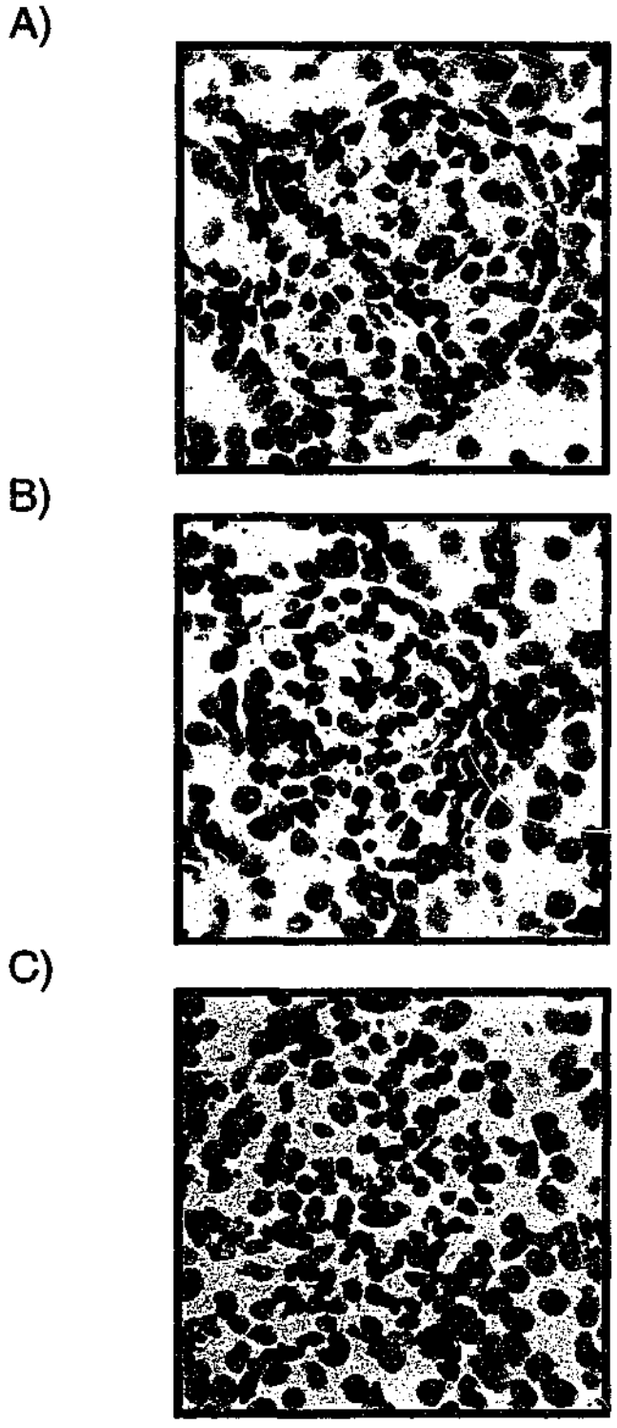
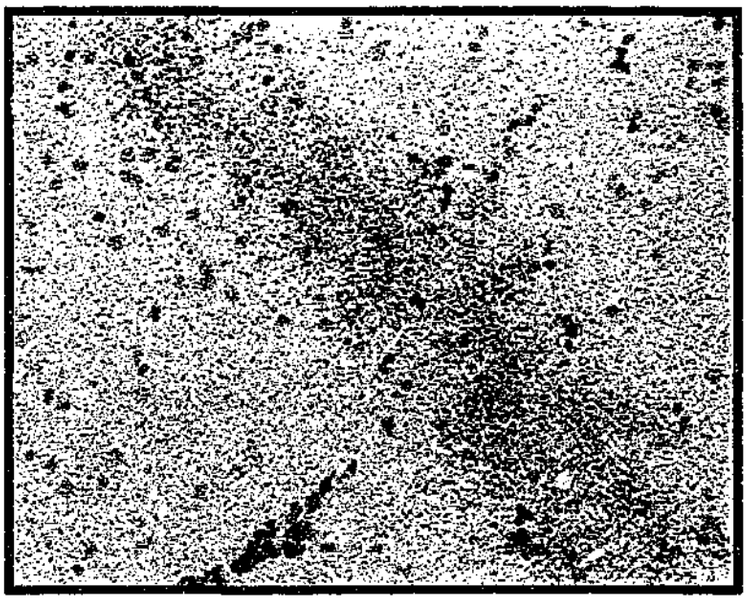


Figure 5.3.6: Representative glomeruli (x 200 magnification) from kidneys of animals that received a 5 day intravenous infusion of vehicle (heparinised saline 10 IU/ml; panel A), or Ang II (4.5 ng/kg/min; panel B and C). Frozen kidney sections, were hybridised with a ^{35}S-labelled cRNA probe complimentary to the TGF-β1 gene sequence. After hybridisation, the slides were exposed to liquid film emulsion for 4 weeks, then developed and stained to visualise the silver grains which are representative of TGF-β1 gene expression (panels A and B). Panel C shows a glomeruli from a kidney that was hybridised with a ^{35}S-labelled cRNA probe complimentary to the non-coding strand of the TGF-β1 gene, and thus acts as a negative control for the in situ hybrisidation experiment.

A)



B)

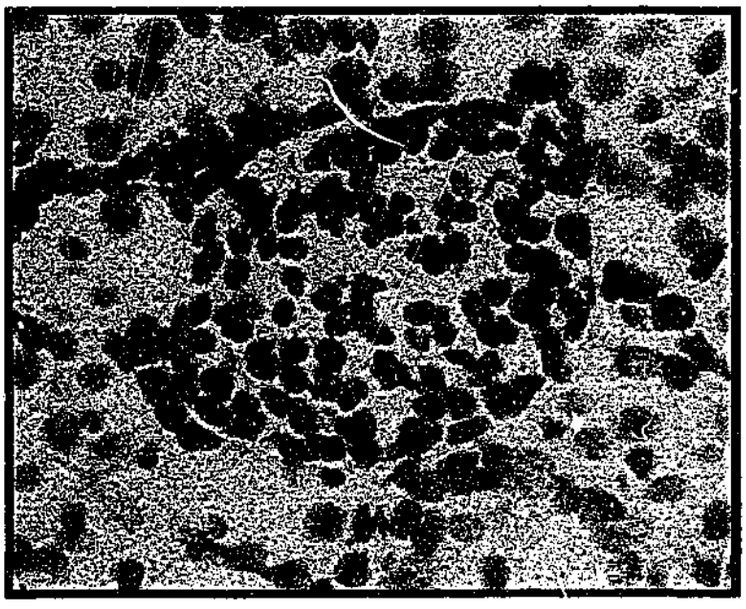


Figure 5.3.7: Panel A depicts abundant silver grain density representative of bFGF mRNA expression in the CA2 region of the rat brain hippocampus (x100 magnification). In contrast, silver grains representative of bFGF mRNA, were undetectable in the glomeruli (x 200 magnification; Panel B) from kidneys of animals that received a 5 or 10 day intravenous infusion of vehicle (heparinised saline 10 IU/ml; panel B), or Ang II (4.5 ng/kg/min). Frozen kidney sections, were hybridised with a ^{33}P-labelled cRNA probe complimentary to the bFGF gene sequence. After hybridisation, the slides were exposed to liquid film emulsion for 8 weeks, then developed and stained to visualise the silver grains which are representative of bFGF gene expression (panels A and B).

5.4 DISCUSSION

In Chapter 4, rats with Ang II-induced hypertension displayed an increased renal pre-:post-glomerular resistance ratio, that was consistent with structural alterations in the renal vasculature, or changes in glomerular filtration surface area. In the present study, the renal vascular, and glomerular structural changes reported in Chapter 4 were examined further, by investigating whether these apparent changes were associated with an increase in the renal expression of growth factor genes TGF-β1 or bFGF, or with increased total kidney DNA and protein content.

5.4.1 Slow pressor effect of angiotensin II on arterial pressure

Infusion of Ang II intravenously increased mean arterial pressure by approximately 50 mmHg over the 10-day infusion period. At the midway point, on day 5 of the infusion however, arterial pressure of the Ang II-infused animals was not statistically different from the animals that received a vehicle infusion, indicating that low-dose, intravenous Ang II infusion resulted in a delayed hypertension which was significant by day 10 of the infusion. A component of the slow onset hypertension achieved by administering small doses of Ang II, is thought to be due to growth or hypertrophy of resistance vessels *(Griffin et al., 1991; Brown et al., 1981*; refer to Chapter 1, Section 1.4.1.2). Thus, the increase in mean arterial pressure reported in this study is consistent with the slowly developing model of Ang II-induced hypertension, and is potentially associated with activation of cellular growth and vascular hypertrophy.

5.4.2 Whole kidney DNA & Protein content

Angiotensin II infusion for 5 or 10 days was not associated with changes in total kidney DNA and protein content. Likewise, DNA:protein ratio was not different in animals that received Ang II compared to the vehicle groups. These results suggest that Ang II-induced, slowly developing hypertension is not associated with changes in DNA or protein measured in whole kidney homogenates.

It is possible that cellular changes induced by Ang II were not detectable by measuring DNA and protein content in whole kidney homogenates *in vivo. In vitro,* Ang II has a wide range of proliferative effects in cultured cells derived from the kidney, and has been reported to induce increases in DNA and protein in cultured renal VSMCs *(Dubey et al., 1992)*, mesangial cells *(Orth et al., 1995; Ray et al., 1991)*, glomerular

endothelial cells *(Wolf et al., 1996)*, and tubule cells *(Norman et al., 1987; Mujais et al., 1986)*. Furthermore, Ang II-induced hypertension had been associated, *in vivo*, with increases in DNA and protein synthesis in systemic arteries *(Su et al., 1998; Simon and Altman, 1992)*. The absence of Ang II-induced increases in whole kidney DNA and protein content does not rule out the possibility of Ang II-induced hypertrophy or hyperplasia in individual cells or populations of cells within the kidney. Potentially, *in vivo*, Ang II stimulated proliferation and hypertrophy in only a small percentage of cells, or in distinct cell populations, and the detection of an increase in DNA or protein in these cells could have been below the sensitivity of the assay. Thus, in order to differentiate the effects of Ang II in certain cell populations within the kidney, in the second part of the study expression of growth factor genes at a tissue level were examined.

5.4.3 Expression of TGF-β1 and bFGF in the kidney

5.4.3.1 TGF-β1 mRNA levels in the kidney

In situ hybridisation was used to localise growth factor TGF-β1 and bFGF gene expression in kidneys from animals that received Ang II or vehicle over 5 or 10 days. As discussed in Chapter 1, Section 1.4.2.3.1, Ang II upregulates expression of growth factors including bFGF, TGF-β1 and PDGF-AA & -BB, *in vitro (Itoh et al., 1993; Stouffer et al., 1993; Koibuchi et al., 1993; Gibbons et al., 1992)*, and also induces growth in a number of cell types. In renal mesangial and tubular cell cultures, Ang II stimulates TGF-β1 production *(Wolf, 1998; Wolf et al., 1995)*. The balance between the proliferative and anti-proliferative actions of these factors may determine whether a cell under goes hypertrophy or hyperplasia.

In the present study, TGF-β1 mRNA expression was detectable in kidneys of rats that received Ang II or vehicle, and analysed in specific tissue regions within the kidney. TGF-β1 mRNA expression was highest over glomeruli in both the vehicle and Ang II groups, but was also detectable in non-glomerular cortical regions, and medullary regions. Expression of TGF-β1 mRNA was not different in kidneys from animals receiving Ang II for 5 or 10 days when compared to their respective vehicle controls, in any of the regions analysed. This result suggests that in the present study, Ang II–induced hypertension was not associated with upregulation of TGF-β1 mRNA expression in glomerular, non-glomerular cortical regions, or medullary regions of the kidney.

Visualisation of renal vessels was difficult as the total renal vasculature comprises of only a very small percentage of the total kidney volume, and as the

kidneys were not perfused before being frozen, most vessels had collapsed, making it impossible to quantitate TGF-β1 mRNA levels using image analysis. Qualitatively, TGF-β1 mRNA in the renal vasculature was extremely low, and the distribution of silver grains could not be differentiated from background. The lack of expression of TGF-β1 in the renal vasculature of animals treated with Ang II or vehicle indicates it is unlikely that *in vivo*, TGF-β1 plays a role in the renal vessel growth, both basally or in Ang II-hypertension. In general, identification of specific cell types within the kidney was difficult in the unfixed tissue. For this reason TGF-β1 mRNA levels were analysed in a region specific manner, to identify potential areas of the kidney where TGF-β1 gene expression may be important.

TGF-β1 mRNA expression in kidneys of Ang II and vehicle treated rats was very low, requiring long exposure to liquid film emulsion. It is possible that changes in TGF-β1 expression were below the level of the sensitivity of the technique used, and therefore not detected. This appears unlikely though, as TGF-β1 expression was significantly lower in animals that received a 10-day infusion when compared to those treated for 5 days. The significant time effect was evident in both vehicle and Ang II-infused animals, and thus indicates that surgery probably induced TGF-β1 expression in the kidneys of both groups, resulting in an up-regulation of TGF-β1 mRNA at 5 days that declined by 10 days. This suggests that, although the level of TGF-β1 expression in all kidneys was low, changes in expression were detectable to a statistically significant level, indicating that TGF-β1 mRNA expression was within the detection limits of the assay.

There are few comparative studies investigating the role of TGF-β1 in the kidney in Ang II-induced hypertension. As reviewed in Chapter 1, Ang II stimulates TGF-β1 production in a number of cell types in culture (see Section 1.4.2.3). *In vivo*, TGF-β1 is up-regulated in the kidney in a number of experimental models of renal disease including unilateral ureteral obstruction *(Yoo et al., 2000)* acute renal ischemia *(Basile et al., 1996)*, rat and human renal transplantation *(Ziai et al., 2000; Campistol et al., 1999)*, and renal fibrosis induced with aldosterone *(Sun et al., 2000)*. There is also evidence of an enhanced proliferative response to TGF-β1 in vascular smooth muscle cells of SHR rats *(Agrotis et al., 1993)*.

In a recent *in vivo* study, performed on rats with two kidney-one clip hypertension, glomerular TGF-β1 mRNA and protein expression was elevated in the clipped kidney 6 days after clipping, and then later in the contralateral kidney *(Wolf et al., 1998)*. However treatment with losartan or triple therapy (hydralazine, reserpine, and hydrochlorothiazide) were equally effective in reducing blood pressure and

glomerular TGF-β1 expression in the contralateral kidney, suggesting that TGF-β1 expression is regulated by the increase in arterial pressure rather than Ang II in this model of hypertension *(Wolf et al., 1998)*. Furthermore, in rats with experimental glomerulosclerosis, ACE inhibition simultaneously decreased intrarenal Ang II, TGF-β1 expression and reduced the glomerular sclerosis index *(Li et al., 1996a)*.

TGF-β1 expression in the present study was not up-regulated in kidneys from animals with Ang II-induced hypertension, suggesting that TGF-β1 does not play a role in producing renal changes observed with Ang II-induced hypertension. An alternative explanation may be that TGF-β1 expression is upregulated in the kidney in some cells, or populations of cells, but not others. In the current study TGF-β1 expression was analysed at the tissue level, not the cellular level due to the poor cellular morphology of the freshly frozen tissue. Therefore, it is possible that increased expression of TGF-β1 mRNA could been induced in individual cells, yet not be detected at the tissue level. A comparable study by Johnson and colleagues (1992), demonstrated that slowly developing Ang II-induced hypertension induced proliferation in only 4.74% of smooth muscle cells in small arteries of the rat kidney, compared to 1.45% of cells in kidneys of rats infused with vehicle. Mild glomerular cell proliferation, increased smooth muscle cell proteins in glomerular mesangial regions, and tubule cell proliferation and injury was also present in animals that received Ang II *(Johnson et al., 1992)*. Analysis of gene expression at a cellular level may be more beneficial and differentiate between cells or populations of cells in the kidney, indicating if, and where in the kidney, TGF-β1 is upregulated in association with Ang II-induced hypertension.

5.4.3.2 bFGF mRNA levels in the kidney

Expression of bFGF mRNA was undetectable in any region of the kidney. In contrast, in a positive control tissue, expression of bFGF was abundant in the CA2 region of the rat brain hippocampus, indicating that the *in situ* hybridisation experiment was successful. The lack of detectable expression of bFGF mRNA in any region of the kidney may indicate that *in vivo*, bFGF is not a primary mitogen within the kidney, in either basal and Ang II-induced hypertensive states. Alternatively, the expression of bFGF mRNA in the kidney may be too low to be detected by *in situ* hybridisation, indeed this notion is consistent with reports that bFGF expression cannot be detected in the kidney by northern blot analysis *(Zhang et al., 1991)*. Likewise, in the young and embryonic rat kidney, bFGF expression has been demonstrated by using RT-PCR to amplify the gene message *(Cancilla et al., 1999; el-Husseini et al., 1992)*, and it is possible that low levels such as those detected using this method could still influence cellular growth.

In the human kidney bFGF mRNA has been localised by *in situ* hybridisation to glomerular, vascular and tubular cells *(Strutz et al., 2000; Floege et al., 1999)*. Additionally, bFGF expression is up-regulated in interstitial and tubular cells from fibrotic human kidneys *(Strutz et al., 2000)*.

Further investigation of the role of bFGF in renal changes induced in Ang II hypertension could utilise *in situ* polymersase chain reaction (PCR) to amplify the expression of bFGF in the kidney. The technique of *in situ* PCR is only semi-quantitative however, and it would therefore make it difficult to compare levels of expression in animals treated with Ang II or vehicle. Furthermore, the lack of detectable expression of bFGF in the present study could indicate two possibilities; that the expression is below the sensitivity of the assay, or that it is not present and therefore does not play a functionally significant role in Ang II- induced renal changes in hypertension.

5.4.4 Technical considerations

The dose of Ang II used in this study to elicit a slowly developing hypertension was 4.5 ng/kg/min. This dose is not consistent with that used in the study presented in Chapter 4 of this thesis. A comparison of previous studies, indicates that intravenous infusion of Ang II produces variable effects on arterial pressure that seem to be dependent on dose, length of infusion, and the time and method of arterial pressure measurement *(Hu et al., 1999; Gorbea-Oppliger and Fink, 1994)*. A summary of studies of intravenous Ang II infusion is provided in Table 5.3. I have no adequate explanation as to why the animals in the present study appeared to be more sensitive to Ang II. However, it is possible that seasonal variations, and the health of the animals contributed to the inconsistent arterial pressure response to Ang II. Regardless of the dose of Ang II used, the aim of the study was to produce a slowly developing hypertension consistent with previous reports that this model of hypertension involves growth or vascular hypertrophy, and to achieve a similar blood pressure response to the study presented in Chapter 4. Thus the dose of Ang II used in the present study increased mean arterial pressure by approximately 50 mmHg by day 10, compared to 30 mmHg in Chapter 4. Direct measurement of arterial pressure in awake animals confirmed that Ang II-induced an increase in arterial pressure that developed slowly and was significant by day 10 of infusion.

TGF-β1 mRNA expression was detected using a 0.7 kb (*Kpn*I - *Bam*HI fragment) probe generated from the 5' coding region of the TGF-β1 gene derived from previously cloned and sequenced cDNAs *(Nichols and Finch, 1994)*. The same probe has been previously used to localise TGF-β1 mRNA in the rat brain *(Bye and Nichols,*

1998), and the specificity of the probe has been confirmed by Northern blot analysis *(Bye, 1996)*. Likewise, bFGF mRNA expression was investigated using a 0.18 kb probe developed by Dr. Miriam Ford *(Nurcombe et al., 1993)*. Previously the probe has been used to detect bFGF expression in the developing rat kidney *(Cancilla, 1998)*. Detection of bFGF mRNA expression in the CA2 region of the rat brain hippocampus, but not in kidneys of rats treated with Ang II or vehicle suggests that the *in situ* hybridisation protocol used in the present study was able to detect bFGF expression. Expression of bFGF mRNA in the CA2 region has since been confirmed in a separate experiment using the same probe *(Young, 1999)*, and has also been demonstrated in a previous study *(Eckenstein et al., 1994)*.

5.4.5 Summary

The results of the present study, are not consistent with the hypothesis that Ang-II induced hypertension is associated *in vivo* with renal growth or the up-regulation of growth factor genes TGF-β1 or bFGF in the kidney. These results are at odds with reports of the actions of Ang II in *in vitro* cell cultures, and suggest that Ang II-induced renal cellular proliferation and hypertrophy *in vivo* may not be as important in the apparent renal structural changes present in this model of hypertension. Alternatively, it is possible that Ang II may stimulate DNA, protein and growth factor expression at a cellular level, in distinct cell populations within the kidney, which cannot be detected by tissue level analysis, as performed in this study. However, the functional significance of such small changes would also need to be evaluated.

Dose (ng/kg/min)	Acute effect (<2 day)	Route	Duration (days)	Arterial Pressure at end of infusion (mmHg)	Heart Rate	Salt and water balance	Urinary Na^+ excretion	Reference
10	-	i.v.	8-12	Increased	-	-	-	*Koletsky et al., 1965]*
5-5.7	Subpressor	i.v.	15	~16mmHg	No change	Not affected	-	*Gorbea-Oppliger et al., 1994*
9.7 – 11	-	i.v.	14	30 by day 6	No change	Not affected	No change	*Melaragno and Fink, 1995*
20	Subpressor	i.v.	7	~50 mmHg	Tended to decrease	-	-	*Brown et al., 1981*
10-28	Pressor	i.v.	15	37-62mmHg	No change	Not affected	No change	*Gorbea-Oppliger et al., 1994 Gorbea-Oppliger and Fink, 1994*
10	Pressor	i.v.	21	Acute ↑ 26 mmHg Additional ↑ 23 mmHG by end of infusion (49 mmHg total)	Decrease in first 3 days then return to baseline	-	-	*Hu et al., 1999*
27-115	-	i.v.	7-10	26-29	-	-	Decreased	*Van der Mark and Kline, 1994*
25	Pressor	i.v.	10	~45 mmHg by day 9	No change	Not affected	-	*Kline et al., 1990*
25-33	Pressor	i.v.	5	24-44mmHg	No change	Not affected	Not affected	*Fink et al., 1986*

Table 5.3: Summary of effects of intravenous infusion of varying doses of angiotensin II on arterial pressure in rats

CHAPTER 6

GENERAL DISCUSSION

Cellular growth induced by angiotensin II – functional consequences

Whilst the literature describing the effects of Ang II on growth factor expression, cellular hypertrophy and hyperplasia *in vitro* is expansive, a large gap exists regarding similar actions of Ang II *in vivo*, particularly within the kidney. Evidence for a direct trophic action of Ang II in the kidney *in vivo* is lacking, and the role this mechanism may play in the development of hypertension has not been investigated. Indeed, demonstrating that Ang II-induced renal growth may contribute to the development of hypertension is dependent on many assumptions. Firstly, Ang II-induced growth within the kidney in hypertension needs to be demonstrated. Secondly, renal cellular growth induced by Ang II would have to result in functionally significant changes, particularly in the renal vasculature, and subsequently alter renal haemodynamics to be considered pro-hypertensive. Thirdly, the mechanism of Ang II-induced renal growth is assumed from *in vitro* evidence to include, stimulation of, and interaction with growth factors such as TGF-β1, leading to cellular growth and renal changes that are thought to contribute to the hypertension. It appears thus, that there is a great deal of indirect evidence suggesting that Ang II-induced renal growth may be important in pathogenesis of some forms of hypertension. However a direct, definitive role for Ang II stimulated renal growth *in vivo* has not been described.

The major focus of this thesis therefore, was to explore the trophic actions of Ang II within the kidney, *in vivo,* in models of hypertension induced by Ang II infusion.

In the first study presented in Chapter 2, the effect of an acute infusion of Ang II directly into the kidney, was investigated on the time-course of early growth response gene expression, *c-fos* and *egr-1*. In this study, a low dose of Ang II (2.5 ng/kg/min) was infused into the kidney resulting in a physiological significant renal vasoconstriction, without affecting mean arterial pressure. Expression of early genes *c-fos* and *egr-1* were not upregulated in the kidney in animals treated with Ang II. The

results of this study were in direct contrast to a previous study by Rosenberg & Hostetter (1993), that reported an increase in the renal expression of *c-fos* and *egr-1* genes in response to a 20 fold dose of Ang II (50 ng/kg/min), infused into the kidney for one hour. However, it is possible that infusion of such a high, non-physiological dose of Ang II into the kidney, as used in the Rosenberg and Hostetter (1993) study, induced a severe reduction in renal blood flow and stimulated compensatory mechanisms within the kidney, therefore stimulating early gene expression via mechanisms not directly attributable to the trophic effects of Ang II.

In the second study undertaken in this thesis, the effect of increasing intrarenal levels of Ang II on arterial pressure, measured in unrestrained rats, was investigated using telemetry. Previous studies in our laboratory have described a similar model of hypertension in dogs. However, in order to more thoroughly examine the effect of intrarenal Ang II infusion, arterial pressure was measured over 24-hour periods, in conscious unrestrained rats, housed in their home cages. Chronic infusion of low doses of Ang II into the kidney, increased both systolic and diastolic pressure in conscious rats, in a dose-dependent manner. Further, the effect of Ang II on arterial pressure was consistent during both the day and night time periods. While chronic infusion of agents into the renal artery of rats is an elegant experimental tool, the technique is difficult, yielding a low success rate (refer Section 3.2.3, Table 3.1). Therefore, to increase productivity, intravenous infusion of Ang II was used to induce hypertension in the following studies in this thesis. Furthermore, during the course of this thesis, evidence has been published indicating that intrarenal levels of Ang II are elevated in models of hypertension where Ang II is infused systemically, above that expected by the infusion alone. That is, Ang II appears to accumulate within the kidney *(Zou et al., 1996b; Zou et al., 1996a)*.

The aim of the experiments described in Chapter 4 therefore, was to investigate if hypertension induced with intravenous infusion of Ang II at a dose that was initially subpressor, was associated with any structural changes in the renal vasculature. Structural changes were assessed using a functional assay to investigate pressure-GFR and pressure-flow relationships in maximally dilated kidneys perfused with artificial plasma. Slowly developing hypertension induced with Ang II was associated with a reduction in the slope of the pressure-GFR relationship, but no change in the slope of the pressure-flow curve suggesting that the pre-: post-glomerular resistance ratio had increased or the ultrafiltration coefficient was reduced within the kidney *(Gothberg et al., 1979; Folkow et al., 1977)*. These changes were consistent with structural alterations to the renal pre-:post-glomerular vessel lumen dimensions, or a reduction in glomerular filtration surface area. Thus, the results from this study demonstrated that there were structural alterations within the kidney associated with

Ang II-induced hypertension. Further, it is possible that these changes could be attributed to cellular proliferation and hypertrophy stimulated by Ang II.

To explore the potential trophic actions of Ang II within the kidney further, the final study undertaken in this thesis investigated some markers of renal growth in this model of hypertension. In this study, infusion of low doses of Ang II intravenously for ten days induced a slowly developing hypertension, but this was not associated with an increase in the renal expression of growth factor genes TGF-β1 or bFGF in the pre-glomerular vessels or renal glomeruli. Indeed, expression of TGF-β1 and bFGF genes in the renal vasculature were undetectable by *in situ* hybridisation. In contrast, whilst TGF-β1 expression was detectable by *in situ* hybridisation in renal glomeruli, non-glomerular cortical regions, and medullary regions, TGF-β1 mRNA levels were not upregulated in animals treated with Ang II. Additionally, there was no evidence of increased cell division or hypertrophy as measured by total kidney DNA and protein content.

In summary therefore, this thesis has provided evidence that hypertension develops when low doses of Ang II are infused intrarenally or intravenously. This slowly developing hypertension is associated with an increased pre-: post-glomerular resistance ratio, consistent with alterations to renal vessel lumen dimensions, or changes in glomerular filtration surface area. Despite these apparent structural changes in the kidney, there was no evidence to indicate that this model of hypertension was associated with detectable upregulation in the expression of growth factor genes TGF-β1 or bFGF in the kidney, *in vivo*, or cellular growth/hypertrophy associated with Ang II-induced hypertension. Additionally, there was no detectable stimulation of renal expression of early genes *c-fos* and *egr-1* following acute infusion of Ang II into the kidney.

The trophic actions of angiotensin II in vivo compared to in vitro

It appears that *in vivo*, potential cellular growth and hypertrophy induced by Ang II is much more difficult to demonstrate, when compared to similar effects documented *in vitro*. There are a number of possible explanations as to why we found no evidence for renal growth induced by Ang II throughout the experiments described in this thesis. Firstly, it is possible that in the experimental models used in this thesis, cellular growth or hypertrophy induced by Ang II is not a principal action of the peptide *in vivo*, and therefore may not be as physiologically relevant or important in the development of hypertension as some of the other pro-hypertensive actions of Ang II. Regulation of cellular growth *in vivo* is complex and there are hundreds of factors that can influence cellular division and hypertrophy. Ang II is just one of these factors, that *in vitro*, causes a variety of cells to undergo hypertrophy and hyperplasia, yet *in vivo* may not

have a significant influence over cellular growth. Alternatively, *in vivo*, other growth factors besides TGF-β1 or bFGF might be involve in Ang II-induced cellular growth and hypertrophy in the kidney. Another possibility is that Ang II could have induced changes in distinct cell populations within the kidney and that these changes were not detected using the methods described in this thesis. Indeed, it is true that the kidney is a very heterogeneous organ comprising of many different cell types.

The main focus of this thesis was to explore potential renal growth induced by Ang II. We chose to investigate markers of renal growth at a whole organ and tissue level. Ang II stimulation of markers of cellular growth in individual cells, or populations of cells within the kidney cannot be ruled out by the studies conducted in this thesis. Indeed a previous study by Johnson and colleagues (1992), demonstrated that slowly developing Ang II-induced hypertension induced proliferation in only 4.74% of smooth muscle cells in small arteries of the rat kidney, compared to 1.45% of cells in kidneys of rats infused with vehicle. These data suggest that growth stimulated by Ang II *in vivo* may indeed be restricted to a very small percentage of cells. Investigation of cellular hyperplasia and hypertrophy stimulated by Ang II in distinct cell populations within the kidney is still necessary. However, more importantly, the functional significance of such small changes, and the potential contribution they make to the development of hypertension *in vivo* also needs to be assessed.

Probably the most significant pro-hypertensive effect of Ang II-induced cellular growth and hypertrophy would be that which occurs in the vasculature of the kidney. Increased levels of Ang II within the kidney could stimulate vascular smooth muscle cell growth and hypertrophy, leading to vascular hypertrophy and remodelling of resistance vessels in the kidney, and could also have effects on the glomerulus. Since vessel wall hypertrophy and remodelling in hypertension have been reported to result in reduction in vessel lumen dimensions *(Korner, 1995; Korner and Angus, 1992)*, hypertrophy of pre-glomerular walls may narrow pre-glomerular resistance vessel lumens, thereby increasing renal pre-glomerular vascular resistance. This would be predicted to be pro-hypertensive, mimicking the haemodynamic effects of main renal artery stenosis (as predicted originally by Goldblatt in 1947 *(Anderson et al., 1995; Ruilope, 1994; Anderson et al., 1984; Goldblatt, 1947)*.

Some alternative approaches that could be used to investigate the functional consequences of renal growth induced by Ang II could include investigating renal vascular responsiveness *in vivo* by constructing vasoconstrictor and dilators curves *(Korner et al., 2000; Wright et al., 1986)*, and investigating the vascular amplifier properties of the renal vascular bed. However controversy exists in the literature as to the interpretation of such experiments, and whether alterations in the vasodilator, constrictor curves relate to changes in the vessel wall structure, wall:lumen ratio,

vessel lumen diameter, or an enhanced responsiveness of the contractile elements (via alterations in second messenger or reaction pathways within the smooth muscle cells) *(Korner et al., 2000; Izzard and Heagerty, 1995)*.

Alternatively, arteriolar resistances, single nephron glomerular filtration rate and renal function could be studied using micropuncture techniques *(Blantz and Gabbai, 1987)*. Structural changes in the renal vasculature or glomerular filtration surface area could also be assessed using standard stereological and morphometric techniques. Such techniques have been used to assess structural changes in the renal vasculature of the SHR *(Kett et al., 1996; Kett et al., 1995)*. However, given the relationship between resistance and vessel radius (Resistance $\propto$ 1/radius^4; Poiseuilles law; *Korner et al., 1989)*, one would expect that methods examining resistance of a vasculature bed (by techniques such as used in Chapter 4) would be more sensitive in detecting small changes in lumen dimensions, than techniques that examine vessel lumen dimensions directly.

Hypertension from intrarenal and intravenous angiotensin II infusion

Throughout this thesis I have used two different models of hypertension induced by Ang II, by infusion of low doses directly into the renal artery, or intravenously. In both models, hypertension develops slowly, and we propose that the mechanisms acting within the kidney to increase arterial pressure may be similar in both models. There is evidence that intrarenal Ang II levels are raised in many forms of Ang II-dependent hypertension including from systemic infusion of Ang II, and in hypertension from renal artery stenosis *(Navar and Harrison-Bernard, 2000)*. Furthermore it is accepted that the kidney, like many other organs, contains all the components of the renin-angiotensin system necessary for the generation of Ang II *(Levens et al., 1981)*. The raised levels of Ang II within the kidney in these forms of hypertension appear to result from accumulation of circulating Ang II, and also activation of intrarenal Ang II production *(Zou et al., 1996a)*. Thus increased levels of Ang II in the kidney are a common feature of many models of hypertension and may be important in the pathogenesis of the hypertension in these experimental models and possibly also in human hypertension. It is important to note however, that in the studies conducted in this thesis, intrarenal levels of Ang II were not measured in either model of Ang II-dependent hypertension. Thus, in future studies, increased levels of Ang II within the kidney need to be confirmed in both hypertension induced with intravenous and intrarenal infusion of Ang II.

There are various systemic actions of Ang II that are pro-hypertensive and could also contribute to the hypertension when Ang II is infused intravenously. These actions have been outlined in Chapter 1 (section 1.3.3.1) but could include stimulation

of the sympathetic nervous system, activation of the central nervous system and subsequent stimulation of thirst and salt appetite and AVP release, stimulation of aldosterone and cortisol synthesis and release, and systemic vasoconstriction. The studies conducted in this thesis focussed on investigating just one potential action of Ang II in the kidney. The additional systemic actions of Ang II were not investigated, and a possible role for these actions in the development of hypertension when Ang II is infused intravenously or intrarenally cannot be ruled out in the studies conducted in this thesis.

Renal actions of angiotensin II

In addition to its systemic actions, Ang II has many renal actions that could also contribute to the increase in arterial pressure when Ang II is infused chronically into the kidney or systemic circulation. Other renal actions of Ang II that are pro-hypertensive are outlined in Chapter 1, section 1.3.3.2, and include a potent vasoconstrictor action on the renal vasculature *(Zhuo et al., 1993; Wilson, 1986)*. Elevated renal vascular resistance induced by Ang II-mediated vasoconstriction could raise arterial pressure by increasing total peripheral resistance. However, in a previous study in dogs, chronic infusion of Ang II into the kidney produced a significant renal vasoconstriction that accounted for only 4% of the rise in total peripheral resistance, suggesting that elevated renal vascular resistance was not a major factor contributing to increase total peripheral resistance *(Fitzgerald et al., 1997)*. A second possible renal action of Ang II that would be pro-hypertensive is to promote salt and water retention in the proximal tubule *(Harris and Navar, 1985)*. Increases in salt and water retention could lead to an increase in cardiac output and thus arterial pressure *(Guyton et al., 1972)*. In the present studies, salt and water retention in hypertension induced by infusing Ang II intrarenally, or systemically was not investigated. The possibility that these mechanisms contributed to the hypertension cannot be ruled out, as very small alterations in whole body sodium balance can cause large changes in arterial pressure *(Guyton et al., 1972)*. Alternatively, the role of salt and water retention in the models of hypertension used in this thesis could be studied further by undertaking continuous 24 hour metabolic studies, measuring intake and output of fluids and therefore subsequent changes in the balance between the two.

Other possible mechanisms by which increased Ang II levels in the kidney could increase arterial pressure would be a reduction in glomerular filtration rate. Reducing the filtered load of sodium and water, would promote salt and water retention. Indeed, in the present studies, when Ang II was infused systemically, there was evidence of an reduced slope of the pressure-GFR relationship in maximally dilated kidneys perfused with artificial plasma, showing that for any given pressure,

GFR in the maximally dilated kidney was less compared to kidneys from vehicle treated animals. Additionally, as there was no overall change in total renal vascular resistance, then the alterations in the pressure-GFR curve were consistent with equal and opposite structural changes in the renal pre- and post-glomerular resistance vessel lumen diameters or a reduction in the glomerular filtration surface area. A recent study from our laboratory has also confirmed a similar reduction in the slope of the pressure-GFR relationship in maximally dilated kidneys from rats with hypertension induced with intrarenal Ang II *(Stevenson et al., 2000)*. In addition to the reduction in slope, Stevenson *et. al.* (2000) also demonstrated a significant right-ward shift of the pressure-GFR curve in the maximally dilated kidneys, suggesting that in hypertension induced with intrarenal Ang II infusion, the renal vascular changes are consistent with a reduction in the pre-glomerular resistance vessel lumens.

Other endogenous substances potentially mediating renal growth in hypertension

There are other endogenous substances, apart from Ang II, that could also act to promote cellular growth and hypertrophy *in vivo*, and are potentially also upregulated in some forms of hypertension. One such mechanism that may have a trophic effect, particularly on vascular smooth muscle cells, is increased renal sympathetic drive. This could result from increased renal sympathetic nerve activity *(Head, 1989)*, renal hyper-innervation *(Tuttle et al., 1995)*, and increased levels of noradrenaline within the renal nerves *(Chen et al., 1995)*. Increased sympathetic nerve activity as well as hyper-innervation has been demonstrated in the SHR *(DiBona and Kopp, 1997)*, and indirect evidence suggests that human hypertensive patients also exhibit increased sympathetic drive *(Esler, 1995; Goldstein, 1983)*. Interestingly, the increase in sympathetic drive in both human and animal models of hypertension appears to have a predominantly renal origin *(DiBona and Kopp, 1997; Esler, 1995)*. *In vitro*, noradrenaline, and its co-neurotransmitters neuropeptide Y (NPY), and adenosine triphosphate (ATP) induce hypertrophy and proliferation in cultured vascular smooth muscle cells *(Chen et al., 1995; Erlinge et al., 1993)*.

Another endogenous substance that could be involved in cellular proliferation *in vivo*, and is potentially upregulated in some forms of hypertension is endothelin. Endothelins are a family of peptide hormones that are powerful vasoconstrictors, and can also induce vascular hypertrophy *(Schiffrin, 1995)*. There is evidence both in favour and against a role for endothelin in hypertension (for review see *Schiffrin, 1995*). Indeed plasma levels of endothelin-1 are either normal or slightly elevated in experimental and human hypertension *(Schiffrin, 1995)*. However, as endothelin is primarily considered to be a paracrine hormone, information about plasma levels of the

hormone can be misleading. Blockade of endogenous endothelins reduces arterial pressure and causes dilation of the vasculature in humans and anaesthetised rabbits *(Evans et al., 1998; Haynes et al., 1996)*. Multiple renal actions of endothelin could play a role in alterations in systemic arterial pressure, including constriction of the renal vascular bed, resulting in increased fluid retention and possibly contributing to glomerular sclerosis. Addtionally, as the kidney is responsible for clearing a large percentage of endothelin from blood, a decreased renal endothelin clearance may also contribute to hypertension *(Markewitz and Kohan, 1995)*.

Cellular growth and hypertrophy in vivo

The studies described in this thesis were stimulated by the overwhelming amount of evidence generated from cell culture studies, suggesting that Ang II can act as a growth-promoting factor (see Chapter 1, Section 1.4). Combined with some indirect evidence of Ang II-induced growth in the systemic vasculature in hypertension, the hypothesis that Ang II, via its trophic effects, may induce growth related structural changes within the kidney and thus contribute to the development of hypertension has been proposed. However, demonstration of vascular growth induced by Ang II *in vivo* is difficult and literature describing such an effect in the kidney is particularly lacking. Thus, as a starting point, this thesis has investigated some markers of cellular growth in the kidney in models of Ang II hypertension.

The results of the studies conducted provide evidence that Ang II induced hypertension was associated with an increase in the pre:post-glomerular resistance ratio, consistent with structural reductions in renal vascular lumen dimensions, or alterations in the glomerular filtration surface area. A recent publication from our laboratory has also confirmed these findings in rats made hypertensive via an intrarenal infusion of Ang II *(Stevenson et al., 2000)*. Despite these apparent renal vascular structural changes, there was no evidence to indicate that Ang II hypertension was associated with a detectable upregulation of early growth response genes in the kidney, or an increase in the expression of growth factor genes TGF-β1 and bFGF. As the experiments were designed as a starting point to investigate growth *in vivo*, all analysis was undertaken at the total kidney or tissue level. However, analysis of early gene expression in total kidney RNA, and growth factor gene expression at the tissue level proved difficult due to the extremely low prevalence of these factors. It is possible that Ang II induced expression of growth factor genes and cellular growth in a small percentage of cells within the kidney, or within distinct cell populations, and that these changes were not detected using the current methods. Another possibility is that *in vivo* Ang II may not upregulate the expression of the genes studied in this thesis, and alternate growth factors may be more important in the cascade of Ang II mediated cell

hypertrophy and proliferation *in vivo*. Future studies could investigate renal growth in models of Ang II dependent hypertension at the cellular level, using such tools as radioactively labelled thymidine or amino acids to investigate cell replication or hypertrophy. Likewise cellular expression of growth factors and early growth genes could be investigated by separating components of the kidney such as glomeruli, tubules or vessels, and amplifying the message using PCR.

Demonstration of cellular proliferation and hypertrophy within the kidney *in vivo*, due to the trophic actions of Ang II, is only a starting point to elucidate a role for such a mechanism in hypertension. Investigation of the functional significance of such changes is critical. It is widely accepted that hypertension itself stimulates cellular growth and hypertrophy, particularly in the vasculature. Thus, cellular growth and hypertrophy would be difficult to directly attribute to Ang II, and might indeed be a secondary consequence of increased arterial pressure. However, the slowly developing model of hypertension used in this thesis increases arterial pressure through a mechanism that is initially sub-pressor and has a time course consistent with the induction of cellular growth and hypertrophy, and subsequent structural vascular changes *(Griffin et al., 1991; Lever, 1986)*. Ultimately, in order to contribute to the increase in arterial pressure, potential cellular changes induced by Ang II would need to translate into structural and functional changes in the kidney, subsequently altering renal haemodynamics.

Thus, there are several avenues of investigation that need to be considered.

- Firstly, cellular growth or hypertrophy, particularly in the vasculature of the kidney needs to be demonstrated in models of Ang II-induced hypertension.
- Secondly, renal vascular growth would need to precede the development of the hypertension in these models, indicating a direct trophic action of Ang II.
- Thirdly, cellular changes induced by Ang II would have to translate to structural vascular changes that are pro-hypertensive.

Elucidation of cellular proliferation and hypertrophy induced by Ang II in the kidney *in vivo* is merely the starting point to investigate this potential role for Ang II in some forms of hypertension, and further research is needed to establish a definitive cascade of events by which cellular growth translates to hypertension.

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