Vascular Remodelling In Malignant Hypertension.

by Tara Collidge

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Department Of Molecular Physiology
University Of Edinburgh
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I declare that all the work presented in this thesis is my own except where stated otherwise, and that it has been entirely composed by myself.

Tara Collidge

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DEDICATION

For

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ABBREVIATIONS

1C2K	one-clip-two-kidney model (Goldblatt)
ACE	angiotensin converting enzyme
AP-1	activator protein one
AT1/2	angiotensin II receptor type one/two
bFGF	basic fibroblast growth factor
bp	base pairs
BrDU	5'bromo-2'deoxyuridine
CCR2	chemokine (C-C motif) receptor two
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
DOCA	deoxycorticosterone acetate
DT	diphtheria toxin
DTR	diphtheria toxin receptor
ET	endothelin
FACS	fluorescence activated cell sorting
g	gram
G	gravity
I3C	indole-3-carbinol
ICAM-1	intercellular adhesion molecule one
IHR	Inducible Hypertensive Rat
IκB	inhibitory factor kappa B
IL-2	interleukin two
L	litre
LDL	low density lipoprotein
LFA-1	lymphocyte function associated antigen one
L-NAME	N(omega)-nitro-L-arginine methyl ester
M	molar
MAPK	mitogen associated protein kinase

MCP-1	macrophage chemoattractant protein one
MH	malignant hypertension
min	minute
MMF	Mycophenolate Mofetil
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa B
NO	nitric oxide
i/e/nNOS	inducible/endothelial/neuronal nitric oxide synthase
OCP	oral contraceptive pill
PCR	polymerase chain reaction
RAS	renin angiotensin system
RNA	ribonucleic acid
RNase	ribonuclease
rtPCR	real time polymerase chain reaction
sFlt-1	soluble fms-like tyrosine kinase
SHR	spontaneously hypertensive rat
SHRSP	spontaneously hypertensive rat, stoke prone
TGFβ	transforming growth factor beta
TNFα/β	tumour necrosis factor alpha/beta
VCAM-1	vascular cell adhesion molecule one
VEGF	vascular endothelial growth factor

Standard prefixes used were

c	centi (10^{-2})
m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})

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ABSTRACT

The incidence of malignant hypertension (MH) fails to decline. Improved anti-hypertensive agents have reduced mortality, but the economic burden remains high due to complications such as end stage renal failure. Since the condition is initially asymptomatic, little is known about early pathogenesis. However the principal pathology is vascular remodelling of resistance vessels, termed fibrinoid necrosis, which causes ischaemic end-organ injury. Cardiovascular disease is the leading cause of mortality in western society and typically the consequence of atheromatous and/or hypertensive remodelling. As such, attention has focused on understanding the processes that contribute to various forms of vascular remodelling.

It is apparent that inflammation plays an important role in modulating, and possibly initiating, some types of large vessel disease including atheroma. Less is known regarding hypertensive remodelling of resistance vessels.

Recently a highly controllable and reproducible animal model of MH was developed in the rat. The Inducible Hypertensive Rat (IHR) exploits conditional transgenic technology allowing renin expression to be switched on, and hypertension to develop, following exposure to a dietary inducing agent. The resulting phenotype resembles human MH, where inappropriate activation of the renin angiotensin system is also seen.

This study used the IHR to characterise the development of MH with specific reference to the renal vasculature. Histological injury and hypertension were pre-dated

by adventitial fibroblast proliferation and inflammatory cell infiltration. In order to determine the role of inflammatory cells the immunosuppressant FK506 was administered pre-emptively, resulting in the total abolition of hypertension and end-organ injury.

To allow further investigation of inflammation, the MH phenotype was developed in mice using subcutaneous angiotensin II infusion. When MH was superimposed on a transgenic line susceptible to conditional macrophage depletion, vascular remodelling failed to occur in the mesenteric circulation where depletion was greatest.

The effect of volume expansion on the IHR was assessed. Transgenic animals craved saline and the resulting fluid overload overcame cerebral autoregulation resulting in ischaemic stroke without alteration in systemic hypertension or pathology. The onset of stroke was tightly predictable and reproducible. Accordingly, the saline-loaded IHR represents a novel and inducible model of ischaemic stroke.

In conclusion, this study has identified inflammation as an early and important event in the pathogenesis of MH in two rodent models. Additionally, cerebral autoregulation in the IHR could be overcome by fluid overload resulting in the dissociation of central and systemic pathology.

CHAPTER ONE.

INTRODUCTION.

1.1 MALIGNANT HYPERTENSION.

Pulm.

Accelerated or malignant phase hypertension (MH) describes the combination of severe hypertension accompanied by necrotising vascular injury. Clinically it is recognised by elevated blood pressure together with bilateral retinal haemorrhages or exudates, with or without papilledema (grades III or IV hypertensive retinopathy) [Ahmed et al., 1986]. A microangiopathic haemolytic anaemia may also be present. A list of clinical features is given in table 1.1.

PRESENTING SIGNS	PRESENTING SYMPTOMS
Elevated blood pressure	Tiredness
Grade III or IV hypertensive retinopathy	Increased urine output
Visual field defects - scotoma	Loss of appetite
Left ventricular hypertrophy	Weight loss
Renal impairment and proteinuria	Headache
Confusion	Nose bleeds
Seizures	Visual disturbance
Pulmonary oedema	

Table 1.1. Clinical Features Of Malignant Hypertension.

The vascular injury observed in MH is characteristic; fibrinoid necrosis (vascular smooth muscle cell death with intimal plasma protein, including fibrin, deposition) and proliferative endarteritis (myointimal proliferation with lumenal narrowing) affects resistance vessels of around 200 μm. (figure 1.1).

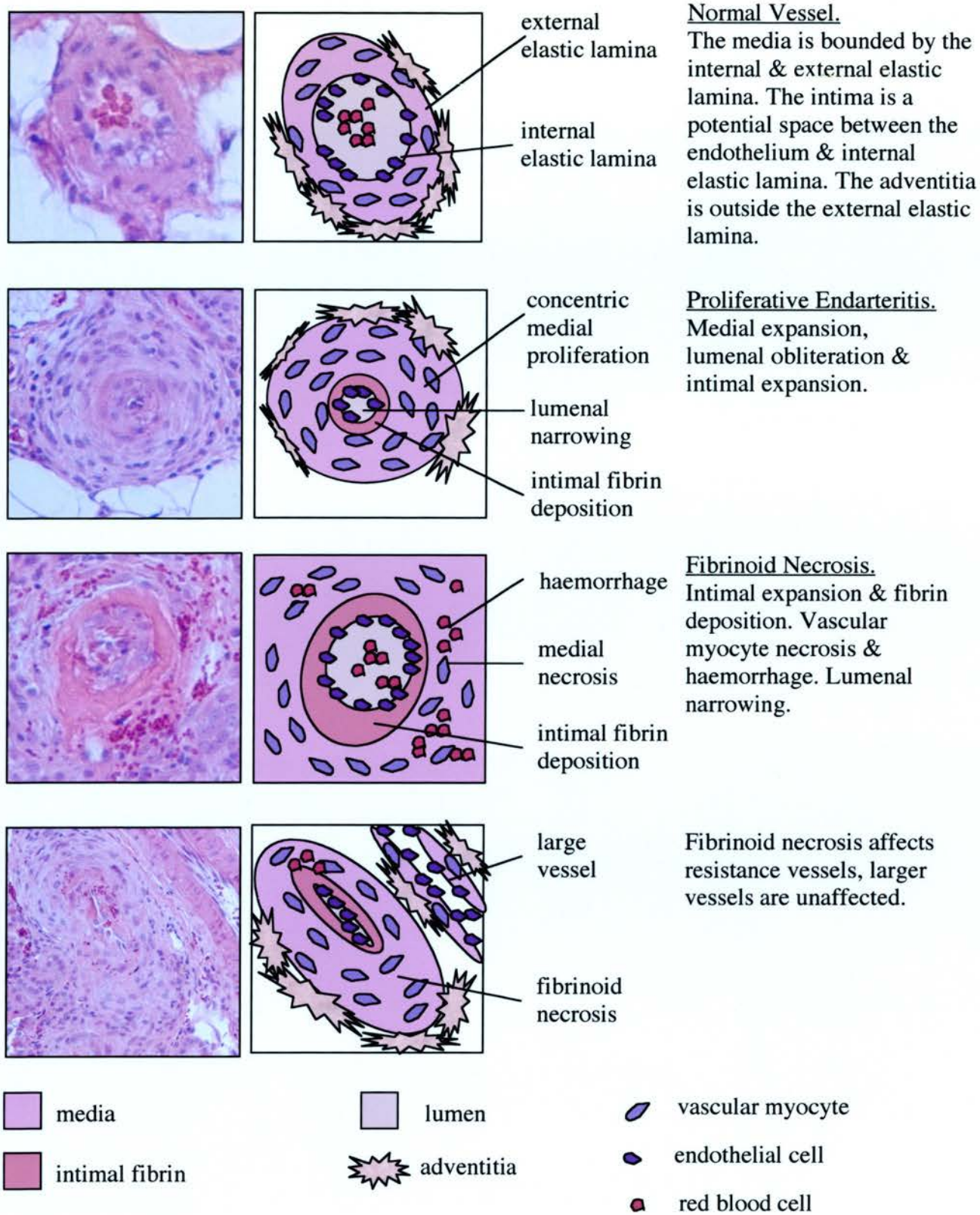

Fig 1.1. Vascular Structure.
H&E histology is shown with schematic diagrams adjacent. Vessels were not perfusion fixed.

Fibrinoid necrosis is not specific to MH and this appearance can be seen within the kidney in other conditions such as haemolytic uraemic syndrome and scleroderma renal crisis. The kidneys, heart and brain are principally affected, however despite an awareness of MH for many decades [Keith, 1939], the triggers and mechanisms involved in vascular injury remain unclear.

Direct pressure effects ("forced dilatation"), excessive vasoconstriction, volume depletion, the endocrine and paracrine renin-angiotensin system (RAS), nitric oxide, natriuretic peptides, endothelins and oxidative stress have all been implicated in the pathogenesis of this condition [Adam and Raij, 2000; Fleming, 2000; Ruiz-Ortega et al., 2001a; Ruiz-Ortega et al., 2003]. And recently, it has become apparent that inflammatory mediators also play a role in some experimental models [Mervaala et al., 2000; Muller et al., 2000a].

Prior to effective anti-hypertensive treatment, mortality was 99% at five-years [Keith, 1939]. With the development of improved anti-hypertensive agents, five-year survival has improved to 63-75% [Gudbrandsson et al., 1979; Isles et al., 1985; Lip et al., 1995], but remains poorer than a population of patients with essential hypertension, matched for blood pressure [Isles et al., 1985]. Despite this fall in mortality, the incidence fails to decline, remaining approximately 1-2 per 100,000 per year [Edmunds et al., 2000; Lip et al., 1994]. As such, it remains a significant cause of end stage renal failure, cardiovascular and cerebrovascular morbidity and mortality in developed countries. The impact is higher still in developing countries with less health care provision and greater socio-economic disadvantage [Kadiri et al., 2000].

1.1.1 Risk Factors And Outcome Predictors.

A number of patient series have been published looking at associated risk factors and patient outcome [Gudbrandsson et al., 1979; Isles et al., 1985; Lip et al., 1995; Webster et al., 1993]. The largest published series to date comes from the group in Birmingham, U.K [Lip et al., 1995]. They and others have identified Negro race, especially males and evidence of end-organ injury at presentation as conveying increased mortality (see table 1.2).

Data from the Glasgow Blood Pressure Clinic suggested that if serum creatinine was less than 300μmol/L at presentation, then five and ten-year mortality approached that of matched essential hypertensive controls [Isles et al., 1985]. There is only limited evidence that aggressive treatment of blood pressure slows renal deterioration in MH, but it has been repeatedly linked to reduced mortality and as such this high risk group require tight blood pressure control.

AT PRESENTATION:	DURING FOLLOW UP:
Elevated serum urea	Poor control of hypertension
Elevated serum creatinine	Cigarette smoking*
Proteinuria	
Left ventricular hypertrophy on echocardiogram	
Race – Negro	
Previous hypertension	
Advanced age*	

Table 1.2. Predictors Of Poor Outcome In Malignant Hypertension.

*Have not consistently been found to increase mortality in all series.

Smoking carries an increased risk of developing MH in some series [Bloxham et al., 1979] but it is less clear from the literature if continued smoking will translate into a higher mortality or susceptibility to renal dysfunction in those people who already have the condition. Given the low prevalence of MH in the general population, patient series are often small and collected over long time periods so that trends may be masked by changes in population characteristics or disease management over time. It seems likely however that the increased rate of cardiovascular events among patients that continue to smoke will have an adverse impact.

The oral contraceptive pill (OCP) can predispose to MH although OCP associated hypertension is usually mild and reversible on discontinuing therapy [Harris, 1969]. The majority of literature relates to the older OCP preparations. More recently the Glasgow group found that 32.4% of women with MH of childbearing age (n=34) were taking the OCP compared to 20.2% at that time in the general population [Lim et al., 1987]. Conversely, the Birmingham group found a very low frequency of OCP use in their series of 39 women, which may reflect their relatively high (44%) non-Caucasian, population [Lip et al., 1997]. Male sex was a risk factor for developing MH but did not convey an adverse prognosis [Bloxham et al., 1979; Isles et al., 1985; Lip et al., 1995].

Mortality occurs as a consequence of ischaemic end-organ injury in the form of renal failure, stroke, myocardial infarction or heart failure. The degree to which these contribute varies with the population studied. In predominantly white populations cardiac disease is the commonest cause of death while renal failure predominates where there is a greater proportion of Negro or Asian patients [Lip et al., 1995; Webster et al., 1993].

Given the increased risk of death from cardiovascular disease over the normal population, standard risk factor modification should be aggressively implemented with patients considered for lipid lowering therapy and aspirin where appropriate.

Although mortality has improved steadily over the last three decades, there is still room for improved blood pressure control. Despite this we may reach a plateau that will only be overcome with additional understanding of the condition.

1.1.2 Diagnosis And Treatment.

Fundoscopy is critical in the detection of MH as it provides a non-invasive window onto the vasculature. There is no difference in outcome between patients with or without papilledema and this distinction is no longer diagnostically relevant [Ahmed et al., 1986].

In the majority of cases where life threatening complications such as encephalopathy, aortic dissection or pulmonary oedema are absent, oral anti-hypertensives are recommended with the aim of reducing diastolic blood pressure to 110mmHg over twenty-four hours and normalising it over a period of weeks. MH presenting as a medical emergency requires intravenous anti-hypertensive therapy and intensive observation. There is no evidence that one group of agents are better than any other for initial treatment, however it is important to avoid a rapid fall in blood pressure that could result in cerebral ischaemia [Graham, 1975; Ledingham and Rajagopalan, 1979]. Unless phaeochromocytoma is suspected, longer acting beta-blocking agents have an effect within a few hours, are unlikely to cause sudden hypotension and convey an

additional cardio-protective effect. Long acting calcium antagonists are also of benefit and are frequently combined with beta-blockers to offset reflex tachycardia. Diuretics have been used with good effect in patients without volume depletion. However there are theoretical reasons for not using diuretics as they could, by increasing natriuresis, increase activation of the renin-angiotensin system. Volume depletion is common in patients with MH and diuretics should generally be avoided. Multiple agents are frequently required particularly in the Negro population where drug resistance is more common [Saunders et al., 1990].

Initially it has been advised to avoid therapy with agents that block the RAS due to the increased prevalence of renovascular disease in this population [Webster et al., 1993]. However once renovascular disease has been excluded, these agents should be included as inappropriate activation of the RAS is invariably seen in MH. A wealth of information now exists from animal models indicating that ACE inhibitors and angiotensin II receptor antagonists are highly efficacious and for conditions such as scleroderma renal crisis, ACE inhibitors are established as the treatment of choice [Steen et al., 1990; Steen and Medsger, 2000].

Most importantly, the major determinant of patient survival has repeatedly been shown to be good, long-term blood pressure control [Isles et al., 1985; Lip et al., 1995; Webster et al., 1993].

1.2 RODENT MODELS OF HYPERTENSION.

Traditionally the majority of hypertensive animal models have been developed in the rat in preference to the mouse and primarily this is due to their tendency to develop hypertension together with larger size, allowing a wider range of physiological and surgical interventions. The first animal model of hypertension was created by surgical constriction of the renal artery, resulting in subsequent renin hyper-secretion. Developed in dogs by Goldblatt in 1934, this original model was adapted for the rat by Wilson and Byrom in 1939 [Goldblatt et al., 1934; Wilson, 1939]. The experimental use of larger animals has fallen from favour over the years due to a combination of ethical objections and long breeding time.

In the rat, hypertension can be selected by breeding and this has resulted in a variety of strains including the spontaneously hypertensive rat strains, Dahl salt sensitive rats and Lyon hypertensive rats now available. A detailed list is given in table 1.3. While these genetic forms of hypertension mimic the polygenetic background observed in human hypertension and represent a sophisticated model, they are subject to limitations. Namely their genetic background is complex and largely unknown, resulting in difficulty in selecting appropriate control animals and in isolating relevant candidate genes. The former is relevant in light of the importance of strain i.e. genetic background, on complex traits such as hypertension, where the interaction of multiple gene effects produces the phenotype making subtle strain differences important [Rapp, 2000; Rapp et al., 1990]. The latter necessitates large animal numbers and breeding strategies to decipher individual gene effects on a complex background. Despite these

problems, the majority of quantitative trait loci identified, chromosomal regions predicted to underlie multi-factorial traits, have relied heavily on such inbred strains.

It is now possible to insert exogenous genetic material encoding for a gene (or genes) of interest from the same or different species, into a fertilised embryo by pronuclear microinjection and produce live, transgenic offspring harbouring that gene. The advantage that these transgenic animals have over traditional phenotype-selected strains is that they have a clearly defined (mono)genetic cause for their hypertension, permitting the study of a specific candidate gene as well as more conventional pathophysiological investigation. Their disadvantage is that gross and often ectopic gene expression is not observed in most disease states, including essential hypertension but due to their relative genetic simplicity, transgenic animals form a powerful tool with which to investigate the role of candidate genes in hypertension (and other conditions) and to answer specific questions posed by the experimenter.

For descriptive purposes, rat models of hypertension can be classified into groups based on the type of end-organ injury they exhibit, biochemical markers such as "low-renin", "high-renin", "endothelin-dependant" or "endothelin-independent" models and their response to classes of anti-hypertensive agent. Such classifications are arbitrary and below they are summarized according to the presence of a transgene or not.

STRAIN	LINE	ORIGINAL STOCK	YEAR REPORTED
New Zealand (Dunedin):genetically hypertensive (GH) rats	H, C	Wistar derived	[Smirk and Hall, 1958]
USA (Brookhaven):Dahl salt-sensitive and resistant rats	H, L	Sprague-Dawley	[Dahl et al., 1962a; Dahl et al., 1962b]
Japan (Kyoto):spontaneously hypertensive rats (SHR)	H	Wistar derived	[Okamoto and Aoki, 1963]
Japan (Kyoto):spontaneously hypertensive rats-stroke prone (SHRSP)	H	Wistar derived	[Okamoto et al., 1974]
Israel (Jerusalem):DOCA salt-sensitive and resistant rats	H, L	unknown	[Ben-Ishay et al., 1972]
France: Lyon hypertensive, normotensive and hypotensive rats	H, C, L	Sprague-Dawley	[Dupont et al., 1973]
Italy: Milan hypertensive and normotensive rats	H, C	Wistar	[Bianchi et al., 1974]
Netherlands (Utrecht):fawn-hooded hypertensive and hypotensive rats	H, L	German brown X white Lashley	[Kuijpers and Gruys, 1984]
Russia (Novosibirsk):inherited stress induced arterial hypertension (ISIAH) rats	H	Wistar derived	[Markel A, 1985]
Czech Republic:Prague hypertensive (PHR) and normotensive (PNR) rats	H, L	Wistar derived	[Heller et al., 1993]

Table 1.3 Rat Strains Selectively Bred For Blood Pressure.

Reproduced from [Rapp, 2000]. Selected for high blood pressure (H), low pressure (L), control strain or unselected (C).

1.2.1 Non-Transgenic Models Resulting In Hypertension.

1.2.1.1 One Clip Two Kidney Model.

The earliest model of experimental hypertension is the one clip, two kidney or Goldblatt hypertensive rat. It was developed originally in dogs during the early 1930's [Goldblatt et al., 1934; Wilson, 1939]. Adult rats undergo surgery to constrict one renal artery resulting in an iatrogenic renal artery stenosis, mimicking a recognised cause of M.H. in man. The ischaemic kidney secretes renin with subsequent hypertension and pathological changes typical of hypertension are seen in vascular beds of the kidney, heart and mesentery. Similar results are found in mice although with less marked hypertension and end-organ injury [Wiesel et al., 1997]. The development of hypertension is dependent upon the degree of stenosis delivered to the renal artery and will vary depending upon operative technique and individual animal susceptibility. In one series of 157 rats, 41% developed chronic, stable hypertension with the remainder split between no hypertension (36%) and severe hypertension (23%) with malignant transformation by two to three weeks [Wilson, 1939]. Commercially available standardised clips now allow greater control over the final level of hypertension. This basic model suggests that pressure is important for malignant vascular injury since the clipped kidney, protected from systemic hypertension, escapes insult despite high circulating renin levels. As a consequence of hypertension the animals develop a pressure natriuresis and hence salt and water depletion. This in turn promotes RAS activation and renin production from the unclipped kidney exacerbating the process

further. It was possible to suppress the development of pathology in animals with evolving MH, despite the development of high blood pressure, by offering 0.9% saline. Animals converted to MH when access to saline was denied [Mohring et al., 1976]. The mechanism behind saline protection is not fully understood, but it may be that replacement of salt and water loss incurred during pressure natriuresis can prevent the transition to MH. Pressure natriuresis induced salt-water depletion detected by the unclipped kidney would result in increased renin production, exacerbating hypertension. The authors suggest that this local renin production may be a factor in triggering the transition to MH. Protection from vascular injury by administration of saline has not been found in other "high renin" models of MH including the aortic ligation model [Rojo-Ortega et al., 1979]. Clearly the timing of saline administration to patients with MH, advocated in some settings, who are at risk of developing cerebral or pulmonary oedema, is critical and potentially not recommended. The role of the paracrine RAS is likely to be important in the development of MH [Fleming, 2000]. This is further supported by more sophisticated micropuncture studies demonstrating normal levels (i.e. inappropriately high) of angiotensin II within the proximal tubule of the unclipped kidney despite suppressed renin at that time point [Cervenka et al., 1999b]. Chronic administration of caffeine (0.1% in drinking water) to this model greatly exacerbated hypertension and pathology consistent with MH was observed six weeks post surgery [Ohnishi et al., 1986]. The ability of caffeine to exacerbate hypertension was not found in the SHR and caffeine ameliorated hypertension in DOCA-salt rats possibly by increasing salt and water excretion [Choi et al., 1993; Ohnishi et al., 1986]. Circulating renin was increased in the Goldblatt plus caffeine

model and hypertcnsion could still be suppressed with ACE-inhibition [Ohnishi et al., 1986]. Caffeine-induced renin secretion is mediated by peripheral, renal adenosine receptor blockade hence its' powerful effect on this renin driven model [Tofovic et al., 1991]. Similar increases in circulating renin were observed in rats fed on a low salt diet with no associated renal injury suggesting that caffeine may have effects in a more physiological setting [Tseng et al., 1993]. Caffeine also augmented the slow-pressor, but not rapid-pressor response to angiotensin II infusion via a sympathetic nervous system dependant mechanism [Ohnishi et al., 1987].

1.2.1.2 Angiotensin II Infusion.

Angiotensin II infusion has been used alone or as an additional stressor in a variety of animal models, in order to study hypertension and the mechanisms underlying vascular injury. Infusion of angiotensin II into rats at a dose of 400ng/kg/min or above will result in a rapid elevation of blood pressure and development of fibrinoid necrosis in the vascular beds of the kidney, heart and mesentery over a one to two week period. There is often an associated perivascular, small vessel, inflammatory cell infiltrate [Ishizaka et al., 2000; Su et al., 1998a; Wiener et al., 1996]. Smaller doses result in chronic hypertension without malignant transformation. The effects of angiotensin II infusion on vascular remodelling and responses to pharmacological manipulation are discussed in section 1.3.1.

1.2.1.3 Inhibition Of Nitric Oxide.

Nitric oxide (NO), originally called endothelial derived relaxing factor, is a labile substance generated by the action of nitric oxide synthase (NOS) on its substrate L-arginine. There are three isoforms of NOS, two constitutive forms regulated by calcium, endothelial (eNOS) and neuronal (nNOS) and one inducible form (iNOS). NO acts as a vasodilating agent to maintain normal vascular tone via eNOS and regulates glomerular haemodynamics via nNOS.

Administration of the general NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) to normal rats results in hypertension in part by removing opposition to the vasoconstricting agents angiotensin II, endothelin and thromboxane A2. Vascular injury is accompanied by an inflammatory infiltrate. Typical hypertensive end-organ damage, namely fibrosis develops and associates with increased expression of TGFβ [Kashiwagi et al., 2000]. These changes are associated with a local increase in ACE activity and angiotensin II and hypertension and pathology can be abolished by co-administration of an ACE inhibitor or AT1 antagonist [Kashiwagi et al., 2000; Ribeiro et al., 1992; Takemoto et al., 1997; Tojo et al., 1996] but not by endothelin blockade [Li et al., 1996a; Sventek et al., 1997]. However hypertension and to a variable extent, end-organ injury, could be ameliorated by other non-RAS agents [Tojo et al., 1996].

Interestingly, only mice lacking the eNOS gene display a hypertensive phenotype not seen in nNOS or iNOS knockouts, but NOS inhibition using L-NAME on an eNOS knockout background fails to elicit a pressor response, confirming the important role of nitric oxide production via eNOS in maintaining vascular tone [Huang et al., 1995; Van

Vlict et al., 2003]. It is likely that the loss of eNOS is partially compensated for by other vasodilators and that subsequent hypertension is augmented by the lack of eNOS derived nitric oxide from the kidney and heart [Ortiz and Garvin, 2003].

NO also has a role in preventing platelet aggregation and inflammatory cell adherence to the endothelium. NOS inhibition results in MCP-1 upregulation in vitro [Tsao et al., 1997] and in vivo in association with monocyte adherence and vascular injury [Usui et al., 2000]. Inhibition of MCP-1 by monoclonal antibody therapy was able to attenuate monocyte recruitment and medial proliferation, but unable to influence perivascular fibrosis, cardiac fibrosis, TGF-β_1 and collagen type 1 expression or hypertension. Local ACE activity remained high despite antibody treatment. It has also been shown that TGF-β_1 upregulation and fibrosis were prevented by AT_1 receptor blockade, suggesting that fibrotic end-organ damage is angiotensin II dependant [Koyanagi et al., 2000].

One candidate system linking the RAS and inflammation is oxidative stress. Oxidative stress upregulates MCP-1 via activation of NFκB and anti-oxidants have been demonstrated to abolish increased ACE activity in this model [Usui et al., 1999; Usui et al., 2000].

In short, NO inhibition appears to have its' hypertensive and pathological effects predominantly via the RAS and RAS inhibition remains the most sensitive way of blocking vascular and end-organ damage.

1.2.1.4 The Deoxycorticosterone Acetate Salt Sensitive Rat.

Administration of deoxycorticosterone acetate (DOCA) in combination with uni-nephrectomy and high (1% saline) salt diet (DOCA-salt model) results in "low renin" hypertension, renal injury in the form of proteinuria and glomerulosclerosis and cardiac hypertrophy [Besse et al., 1994; Lafferty et al., 1991]. The term "low renin" is based on the theory proposed by Laragh and Resnick suggesting that hypertension could be classified into a "high renin" form, also termed "dry", with an activated RAS and responsive to treatment with agents inhibiting this system, and a "low-renin" form also termed "wet" where salt and water retention is the principal abnormality [Laragh and Resnick, 1988]. This distinction is clinically relevant as each type is responsive to different treatment modalities.

The DOCA-salt model is unusual in that antagonism of the RAS does not lower blood pressure or prevent end-organ injury [Aono et al., 1988; Li et al., 1996b]. However, treatment with calcium antagonists, endothelin antagonists, neutral endopeptidase inhibitors (possibly via endothelin metabolism) and diuretics are all able to lower blood pressure and prevent end-organ injury [Cabral et al., 1994; Fujita et al., 1996; Li et al., 1996b; Pu et al., 2002]. Although in some reports, inhibition of the RAS, while not lowering blood pressure, did improve cardiac fibrosis [Brown et al., 1999; Hara et al., 2001].

As with other forms of salt-dependant hypertension, the vascular endothelin system is activated with elevated levels of protein and mRNA detectable [Lariviere et al., 1993a; Lariviere et al., 1993b; Schiffrin, 2000]. It is not surprising in this context that

endothelin blockade is efficacious in lowering blood pressure and ameliorating end-organ injury.

In addition to the renal and vascular effects of DOCA, a component of the DOCA-salt model is centrally mediated and intracerebroventricular infusion of low dose aldosterone (at doses too low to have systemic effect) resulted in hypertension which could be blocked by mineralocorticoid receptor antagonists [Gomez-Sanchez, 1986]. Additionally, intracerebroventricular administered mineralocorticoid receptor antagonists attenuated the blood pressure response to chronic systemic aldosterone and DOCA administration [Gomez-Sanchez et al., 1990; Janiak et al., 1990].

1.2.2 Transgenic Models Of Malignant Hypertension Over-Expressing prorenin.

A summary of transgenic, hypertensive rodent models is given in table 1.4.

1.2.2.1 TGR(mRen2)27.

The TGR(mRen2)27 was the first transgenic hypertensive rat described [Mullins et al., 1990]. The mouse *Ren2* gene encoding renin, together with large 5' and 3' flanking sequences including the endogenous promoter and other control elements, was inserted into the Hanover Sprague-Dawley rat. This model was originally developed to investigate mouse *Ren2* gene expression and function under the control of its own promoter in the rat, which unlike the mouse and in common with man, possesses a single renin gene. Although not designed to emulate human hypertension, it has

provided an excellent model of hypertension exhibiting many phenotypic similarities with the human disease. It remains one of the widest used experimental models of hypertension and has been extensively reviewed [Engler et al., 1998; Lee et al., 1996].

Animals have severe, chronic hypertension with systolic blood pressures reaching 240mmHg in heterozygous males and 290mmHg in homozygotes by 8-10 weeks of age. Death from hypertensive crisis due to malignant transformation occurs in some homozygotes but is unpredictable. Indefinite treatment with ACE inhibitors is required to prevent death and to maintain a breeding line.

Mouse prorenin is highly expressed in the adrenal gland which is also the major source of circulating renin activity [Tokita et al., 1994], but expression occurs at many sites including the kidney, heart, resistance vasculature and brain [Hilgers et al., 1992; Mullins et al., 1990]. Expression is absent from the rat submandibular gland where *Ren2* is expressed at high levels in the original two-gene DBA/2 mouse strain, possibly suggesting that other unidentified factors capable of activating *Ren2* exist within the mouse submandibular gland that are absent in the rat [Mullins et al., 1990; Zhao et al., 1993].

Females exhibit less hypertension and lower mortality than males, with female homozygotes equivalent to heterozygote males in terms of blood pressure. Such sexual dimorphism has been observed in other hypertensive rat strains including spontaneously hypertensive rats and the DOCA-salt model [Crofton et al., 1989; Ganten et al., 1989] also large human epidemiological studies suggest that being female is cardio-protective [Kannel et al., 1980]. Androgens are capable of modulating *Ren2* expression in the host DBA/2 mouse strain and administration of androgens to female

mice results in higher levels of submandibular *Ren2* transcription [Wagner et al., 1990]. Additionally levels of circulating RAS components appear to be higher in males compared to females [Chen et al., 1992]. Hence, in this model, enhanced expression of the transgene mediated by androgens could account for the increased severity of the male phenotype rather than a pre-existing, sexual predisposition. This idea is countered by the observation of sexual dimorphism in a different transgenic rat strain: The TGR(hAT-rpR) rat line expresses rat prorenin under the control of the liver specific human α1-anti-trypsin promoter. Males have a sixty-fold greater plasma prorenin level compared to female animals (the α1-AT promoter typically associates with a seven-fold higher expression in males) and only male animals display target-organ pathology [Veniant et al., 1996].

Vascular remodelling in TGR(mRen2)27 occurs in resistance as well as in large blood vessels and nephrosclerosis with proteinuria is seen as early as eight weeks [Bachmann et al., 1992; Bohm et al., 1994; Springate et al., 1994; Tawfik-Schlieper et al., 1995; Thybo et al., 1992]. In common with other hypertensive models, vascular remodelling of resistance vessels is associated with medial hyperplasia while medial hypertrophy and polyploidy are associated with larger vessels such as the aorta [Brosnan et al., 1999]. In MH this progresses to the two features of malignant vascular injury namely fibrinoid necrosis and endarteritis proliferans. Young animals show changes of left ventricular hypertrophy progressing to failure by 30 weeks. The hypertrophic response has molecular features, such as reduced response to the inotropic effects of isoprenaline and phenylephrine, in common with human left ventricular hypertrophy and other animal models [Bohm et al., 1994; Tawfik-Schlieper et al., 1995]. The similarity

between this model and human hypertensive pathology is striking and the underlying mechanisms in TGR(mRen2)27 rats are likely to have relevance in the human setting. Reaction kinetics for angiotensin II generation in the rat and man, suggest that renin is rate limiting. Hence, insertion of extra copies of the renin gene is a powerful way of driving the RAS in this species.

The development of end-organ pathology appears to be angiotensin II-dependent. Anti-hypertensive agents will lower blood pressure but are not as effective in attenuating end-organ damage as drugs specifically targeting the RAS [Bohm et al., 1995; Lee et al., 1995]. In TGR(mRen2)27 transgenic rats, transgene-derived prorenin is elevated while endogenous rat renin is appropriately suppressed. Angiotensin I and II are both decreased in the plasma. The levels of transgene derived prorenin and angiotensin II at the tissue level however are likely to be more relevant than circulating levels in predicting end-organ injury in this and other animal models. Administration of low dose lisinopril (0.5mg/kg/day) to TGR(mRen2)27 rats resulted in partial attenuation of blood pressure, lower cardiac to body mass ratio and reduced renal injury in association with decreased renal angiotensin II levels. Dihydralazine treated animals, despite correction of blood pressure to control levels, displayed no improvement in target-organ injury or alteration in renal angiotensin II levels [Lee et al., 1995]. Low doses of the angiotensin II receptor blocker telmisartan reduced renal and cardiac injury in TGR(mRen2)27 animals with only modest effect on hypertension [Bohm et al., 1995]. Low dose ramipril treatment (5mg/kg/day) of the TGR(mRen2)27 heterozygote (HanRen2/Edin) rat strain, resulted in increased survival and failure to transform from benign to MH, without lowering blood pressure. Tissue, but not plasma, ACE activity

was decreased suggesting that the paracrine RAS is important for transformation to MH and lethality in this model, independent of blood pressure [Montgomery et al., 1998]. Although tail-cuff plethysmography used by Montgomery *et al.* would fail to detect subtle or intermittent changes in blood pressure, their treatment group had comparable medial hypertrophy to control animals, suggesting a similar level of hypertension. However, identical subdepressor ramipril treatment of TGR(mRen2)27 rats could not prevent left ventricular hypertrophy and fibrosis developing or peri-vascular fibrosis within the right ventricle, implicating pressure, rather than the RAS, as the dominant effector for hypertrophic cardiac remodelling in this model [Bishop et al., 2000]. Imidapril, at subdepressor doses, prevented the progression of left ventricular hypertrophy to cardiac failure in Dahl salt-sensitive hypertensive rats despite not lowering blood pressure [Kobayashi et al., 2000a]. Heart failure in this model was associated with an increase in left ventricular ACE expression, which was attenuated by imidapril. A similar increase in left ventricular ACE expression has been found in human heart failure [Studer et al., 1994]. Cardiac ACE activity, but not circulating activity, was found to be elevated in another heart failure model induced by coronary artery ligation in rats [Hirsch et al., 1991]. Chronic administration of L-NAME to rats results in accelerated hypertension and end-organ injury as discussed earlier. A subdepressor dose of ACE inhibitor was able to reduce vascular wall-to-lumen ratio and peri-vascular fibrosis in this model [Kobayashi et al., 2000b]. Additionally, angiotensin II receptor blockade using candesartan, reduced stroke and proteinuria in a stroke-prone spontaneously hypertensive rat model, without affecting blood pressure, implicating angiotensin II directly rather than factors also affected by ACE inhibition

such as bradykinin potentiation [Inada et al., 1997]. Minute falls or fluctuations in blood pressure with so called "subdepressor doses" of agents antagonizing the RAS are difficult to exclude as no studies of this type have used continuous arterial monitoring via telemetry. As such, pressure effects may partially contribute to the outcome of tissue RAS inhibition discussed above.

Increasingly, there is evidence that individual tissues can regulate their own RAS and all components of the RAS are present in some vessels [Gibbons, 1998]. Cardiomyocytes release angiotensin II on mechanical stretch [Sadoshima et al., 1993] and the brain has an isolated renin-angiotensin system with important roles in systemic blood pressure regulation in addition to non-haemodynamic effects [Culman et al., 2002; Veerasingham and Raizada, 2003]. The demonstration that mouse *Ren2* expression in the TGR(mRen2)27 rat occurs in all tissues exhibiting injury, suggests strongly, but does not prove, that local angiotensin II formation is enhanced and could mediate organ injury. Evidence suggesting upregulation of the paracrine RAS and the powerful effect of agents that block this system (even administered at doses that have little or no effect on blood pressure), provide strong evidence to link angiotensin II with target-organ injury in a variety of hypertensive states. Direct and indirect pressure effects are also important and may be the dominant effector in certain types of target-organ response.

1.2.2.2 Constitutive Tissue Specific Renin Expressing Transgenics.

To address the role of (pro)renin in the development of cardiovascular pathology when not subject to control by regulatory elements within the *Ren2* promoter and to bypass target organ expression, a constitutive prorenin expressing model has been developed TGR(hAT-mRen2) using the human α1 anti-trypsin (hAT) promoter to drive ectopic mouse *Ren2* cDNA expression from the liver. Elevated plasma mouse prorenin levels and hypertension (190mmHg systolic by seven weeks of age) were observed in transgenic animals. Circulating prorenin levels were elevated $1X10^3$ fold in this model compared to only 10 to 20-fold elevated in TGR(mRen2)27 and in both models angiotensin II and aldosterone were not elevated. Initial studies suggested that left ventricular hypertrophy occurred prior to the onset of significant hypertension, eluding to transgene expression rather than hypertension *per se* being important in the pathogenesis of end-organ injury in this model [Ogg, 1997]. It is likely that this model will provide additional information on the role of prorenin in the development of hypertensive pathology.

Another line, the TGR(hAT-rpR) similar to the TGR(hAT-mRen2) line except that rat renin was used in place of mouse, was not hypertensive (determined by tail plethysmography), but did develop end-organ damage in the form of cardiac fibrosis, hypertrophy, glomerulosclerosis and large vessel remodelling. Rat prorenin levels were 400-fold elevated in the plasma of male animals but other components of the RAS were similar to non-transgenic controls [Veniant et al., 1996]. Again, in the absence of

hypertension, it is tempting to speculate that pathology in this model is mediated by elevated, circulating prorenin.

1.2.2.3 The Double "Human" Transgenic Rat.

Double transgenic rats carrying the human renin and angiotensinogen genes have been established. Species specificity within the RAS results in rats expressing either human renin or angiotensinogen being normotensive. Inter-crossing rats from these strains generated a double transgenic on a Sprague-Dawley background heterozygous for each transgenic site and capable of angiotensin II formation. Both transgenes used genomic sequences driven by their endogenous promoters and as such expression was observed from many sites including target organs [Bohlender et al., 1997]. These double transgenic animals exhibited severe hypertension (200mmHg by seven weeks) and developed MH with typical end-organ injury [Bohlender et al., 1997].

The resulting double transgenic strain allows for the development of targeted treatment in humans. In addition to the human renin antagonist Ro42-5892, which corrected hypertension and pathology, blood pressure could also be reduced and end organ damage ameliorated by the NFκB inhibitor pyrrolidine dithiocarbamate [Muller et al., 2000a]. Cyclosporin, an immunosuppressant acting via calcineurin inhibition to block IL-2 dependant T-cell responses, was also able to significantly attenuate vascular proliferation and monocyte infiltration in the presence of only a small reduction in systolic blood pressure [Mervaala et al., 2000]. NFκB is a pro-inflammatory transcription factor upregulated in this model along with AP-1. As with other forms of

vascular injury, especially angiotensin II mediated, perivascular monocyte accumulation and cell adhesion molecule upregulation is described in association with vascular injury [Grafe et al., 1997; Mervaala et al., 1999; Muller et al., 2000b]. It is significant that in this setting, anti-inflammatory treatment has attenuated both inflammatory cell accumulation and target organ injury. However, to date more specific pro-inflammatory mediators have not been mechanistically implicated in the pathogenesis of MH in this model.

Similarly in mice due to species-specific interaction between renin and angiotensinogen, strains transgenic for either the human renin or angiotensinogen (*hAGT*) genes are normotensive. However, hypertension and end-organ injury developed following inter-crossing these stains [Fukamizu et al., 1993; Merrill et al., 1996]. Peripheral ACE inhibition lowered but failed to normalise blood pressure in these animals suggesting an additional factor was contributing to hypertension. Due to expression of both transgenes (under the control of their endogenous promoters) in the brain, a central contribution to hypertension was suspected and confirmed by attenuating blood pressure with intracerebroventricular administration of the AT1 antagonist losartan [Davisson et al., 1998]. The development of a proximal tubule targeted human angiotensinogen expressing mouse where *hAGT* was placed under the control of the kidney androgen-inducible promoter (KAP) has highlighted the importance of the renal paracrine RAS in this model. Crossed with the original single renin strain the combined *hRen*/KAP*hAGT* strain was hypertensive with normal plasma and raised urinary angiotensin II levels and hypertension failed to correct with losartan [Davisson et al., 1999].

1.2.2.4 Brain Specific Angiotensinogen Depletion.

Systemic blood pressure can be regulated via central mechanisms and agents such as methyldopa owe their mode of action to, as yet poorly understood central mechanisms. The extent to which neurological mechanisms contribute to human hypertension, or experimental animal models, is unclear. In health the blood brain barrier excludes circulating angiotensin II from the brain, which has an independent RAS capable of angiotensin II generation with roles in regulating salt-water balance and vascular tone via arginine-vasopressin (AVP), adrenocorticotropic hormone, mineralocorticoids and sympathetic outflow. Additionally, intracerebroventricular injections of renin or angiotensin II into the brains of rats will result in marked salt-water thirst and increased drinking behaviour [Fitzsimons, 1998]. Some strains of spontaneously hypertensive rats and the TGR(mRen2)27 line have elevated protein levels of angiotensinogen or angiotensins within the brain and central administration of RAS antagonists can attenuate hypertension [DiNicolantonio et al., 1982].

With the purpose of dissecting out the relative importance of neurological influences, a transgenic rat was developed to express reverse orientation sequence encoding the 5'end, exon 1 and partial exon 2 of the rat angiotensinogen gene. The construct was driven by a glial fibrillary acid protein (GFAP) promoter localising expression to the central nervous system and put onto a Hanover Sprague-Dawley background. This new strain exhibited a 90% reduction in brain angiotensinogen protein while plasma levels remained unchanged. The animals were mildly hypotensive (systolic pressure 5mmHg

lower than controls) and passed larger quantities of dilute urine and this corresponded to reduced plasma AVP. In response to intracerebroventricular renin injection they exhibited an attenuated drinking response and the degree of hypertension and left ventricular hypertrophy induced during systemic subcutaneous angiotensin II infusion was reduced [Baltatu et al., 2000].

An additional cross was performed onto the TGR(mRen2)27 background [Schinke et al., 1999]. This cross, exhibited less hypertension (systolic pressures of 200mmmHg compared to 220mmHg) suggesting that in the TGR(mRen2)27, as with other hypertensive models, the central nervous system contributes to the aetiology of hypertension and target-organ injury.

1.2.2.5 The Inducible Hypertensive Rat.

Transgenic approaches can result in high and ectopic levels of gene expression not typical of human hypertension. However, it is now possible to insert genes in a highly regulated way and control both the level and site of transgene expression. Both forms of control have been used in the Inducible Hypertensive Rat (IHR) developed in this laboratory [Kantachuvesiri et al., 2001]. In the IHR, mouse *Ren2* cDNA is regulated by the *Cyp1a1* promoter and expressed only in the presence of specific inducing agents (figure 1.2).

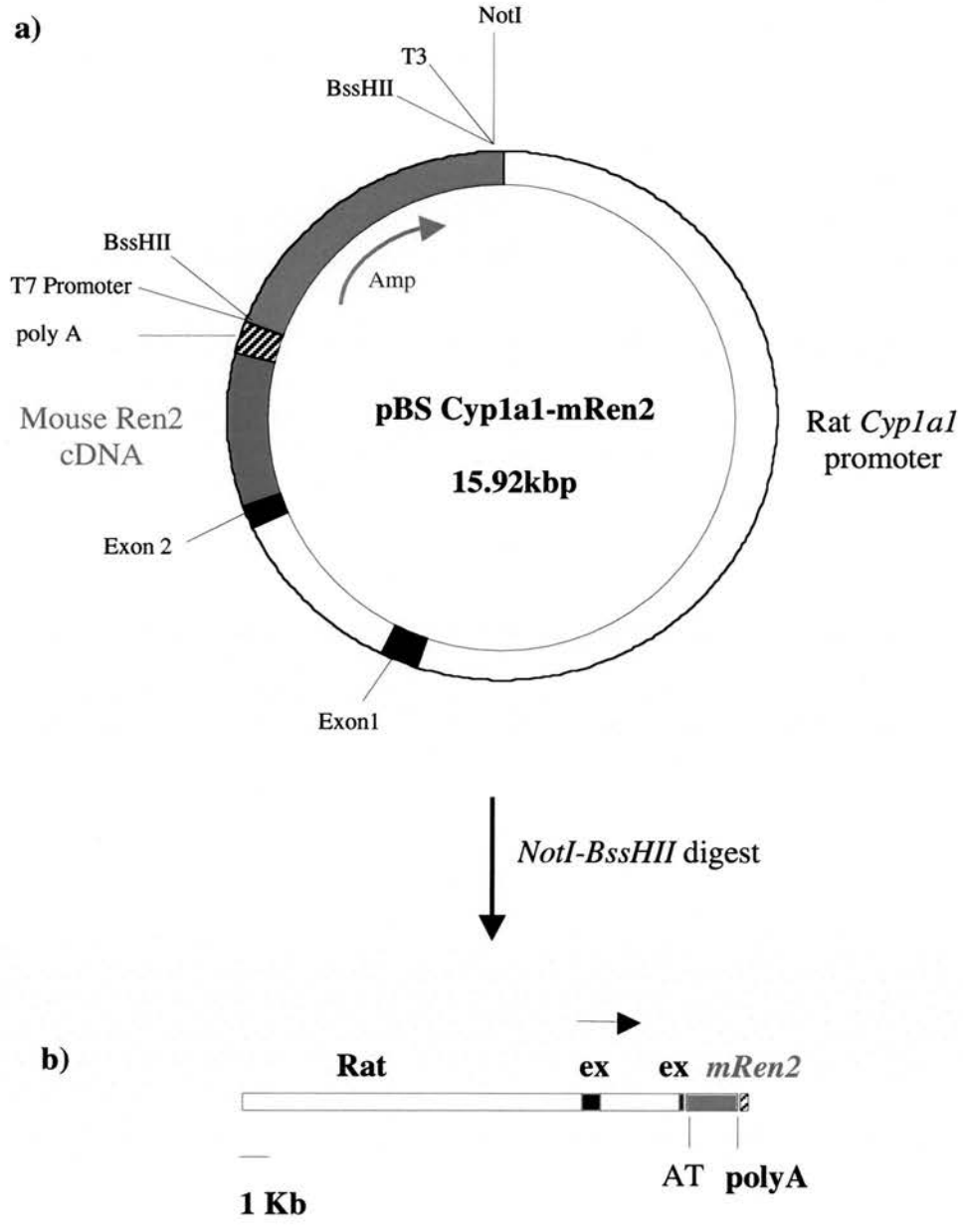

Fig. 1.2 Transgene Structure.

pBluescript Cyp1a1-mRen2 structure (1.2a). The Cyp1a1-mRen2 transgene comprised the rat *Cyp1a1* promoter, including exon1 and partial exon2, fused to 1.2 kb mRen2 cDNA with SV40 polyadenylation site (polyA). The transgene was excised by *NotI-BssHII* digest (1.2b).

The IHR is the major model of hypertension used in the experiments described in this thesis. There are three important differences from the earlier TGR(mRen2)27 strain. Firstly, mouse prorenin is expressed from an extra-renal site and not from organs affected by hypertensive injury. Second, expression is inducible and reversible so that hypertension can be generated reproducibly and at will. Third, since constitutive transcription is absent, hypertension is generated de novo and does not influence cardiovascular development. It is appreciated that manipulation of the RAS early in life can alter vascular development, renal morphology and blood pressure in adult animals, including humans, and this phenomenon may complicate the adult phenotype of constitutive transgenics: Half of the foetuses from women taking angiotensin II receptor antagonists during early pregnancy developed renal abnormalities and oligohydramnios [Serreau et al., 2005]. Components of the RAS are expressed in characteristic patterns throughout nephrogenesis and inhibition of this system during late (neonatal) nephrogenesis in the rat, results in vascular, tubular and papillary abnormalities. Additionally, gene deletion studies of ACE, angiotensinogen and $AT1_{A/B}$ show similar renal malformations [Sequeira Lopez and Gomez, 2004; Wu et al., 1994]. Such abnormalities result in reduced nephron number, which is postulated to contribute to adult hypertension [Woods and Rasch, 1998].

As part of the hepatic cytochrome p450 system *Cyp1a1* expression is induced by xenobiotics such as indole-3 carbinol (I3C) and 3-methylcholanthrene acting via the aryl hydrocarbon receptor. Inclusion of dietary I3C results in rapid expression and secretion of mouse prorenin from the liver. Very low levels of expression are also

found in the skin and small intestine but these tissues do not suffer hypertensive injury in this model [Kantachuvesiri et al., 2001].

The transgene was introduced onto an inbred Fisher F334 background with good breeding characteristics, to yield a pure inbred strain with a highly reproducible phenotype. Only male animals carry the transgene due to integration onto the Y chromosome. Within 24 hours of addition of I3C to standard feed, blood pressure rises and peaks at seven days, remaining stable at a level of 220mmHg systolic. Due to the potentially beneficial effects of I3C on human breast cancer, there is a history of human exposure to this compound without adverse effects [Bradlow et al., 1994; Wong et al., 1997]. Animals develop MH over 14 days and display clinical signs consistent with accelerated hypertension such as polyuria, weight loss, haemolysis and reduced appetite. Fibrinoid necrosis and proliferative endarteritis are seen in the mesenteric and cardiac circulations by day seven and renal bed by day 14 (figure 1.3).

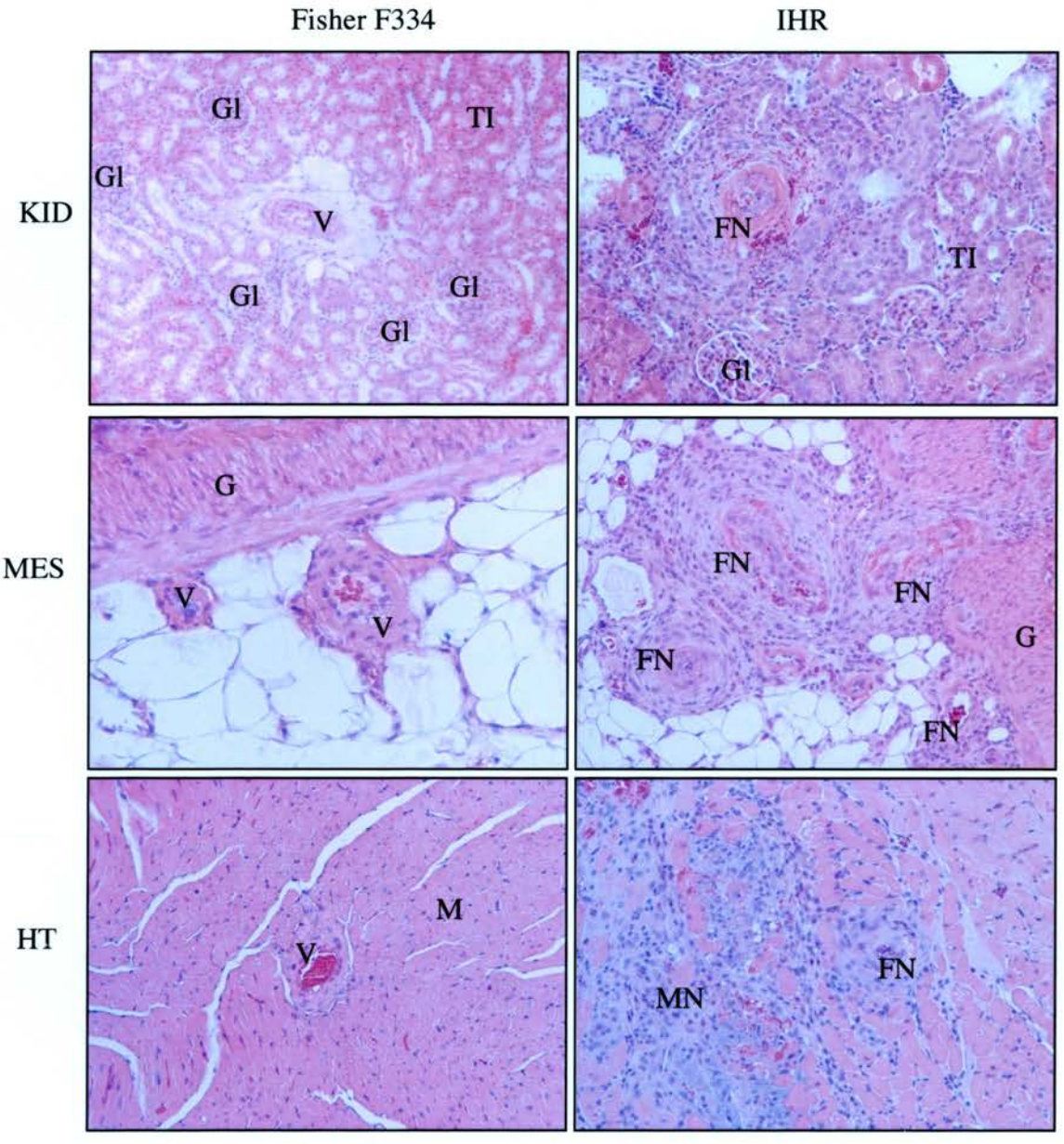

Figure 1.3 Histology Following Fourteen Days Of Induction In The IHR.

Fisher F334 and IHRs were induced for 14 days with 0.3% w/w I3C. Fibrinoid necrosis (FN) is seen in the kidney (KID), mesentery (MES) and heart (HT). MN, myocardial necrosis; G, gut wall; Gl, glomerulus; TI, tubulointerstitium; V, normal vessel; M, myocardium.

The cerebral circulation is spared. The reason for this hierarchy of vascular bed susceptibility is unknown, but may relate to the differential autoregulatory capacity within these organs. All components of the RAS are significantly elevated in the plasma. Transgene derived prorenin is $5X10^4$ times greater, active renin $1X10^2$ times greater and circulating angiotensin II double compared to Fisher F334 controls by day seven. Prorenin elevation occurs rapidly together with an elevation in blood pressure, which precedes the rise in circulating active renin and angiotensin II. This suggests that processing of the transgene at a local, tissue level is responsible in part for the phenotype. For example, it has been suggested that one percent of prorenin can exist in an active form without cleavage via conformational shift alone [Yamauchi et al., 1990]. If this occurs in the IHR model it would result in significant levels of circulating "active" renin.

Endogenous rat renin within the kidney is undetectable by immunostaining during the early development of MH suggesting appropriate suppression.

This novel model is extremely versatile and the experimenter is able to switch transgene expression on and off at will. Notably, blood pressure can return to normal after fourteen days of induction [Howard et al., 2005], therefore offering the opportunity to study repair mechanisms without excessive mechanical or pharmacological manipulation. Patients with MH also have activation of the RAS and frequently present with established renal and cardiac impairment. As such, this model provides an excellent opportunity to observe the development of this condition, without surgical or pharmacological manipulation, for the first time.

The abrupt onset of transgene expression in the IHR may account for severity of the hypertensive phenotype compared to the TGR(mRen2)27 strain. Adaptive changes within the vasculature of TGR(mRen2)27 animals due to constitutive expression and continuous prorenin exposure may favour the development of a chronic, rather than malignant, hypertension in this model. Another factor influencing the severity of the phenotype seen in the IHR model appears to be the genetic background, which differs from TGR(mRen2)27. Indeed when the transgene in the TGR(mRen2)27 strain was moved onto Fisher F334 and Lewis backgrounds, differences in lethality were observed between the two transgenic strains [Whitworth et al., 1995a]. Linkage analysis suggested that this trait was influenced by two loci spanning the *ACE* and *AT1* genes [Kantachuvesiri et al., 1999]. Plasma ACE activity is significantly higher in Fisher F334 rats compared to Lewis rats suggesting involvement of the RAS in modulating this phenotype.

Since there is no transgene expression in target organs and in the kidney, endogenous renin is down regulated, this model provides us with an opportunity to dissect the role of the paracrine RAS. Initial studies looking at cardiomyocytes in vitro and in vivo using this model suggested that transgene-derived prorenin was elevated. This raises the possibility of uptake and activation of prorenin within cardiac and perhaps other tissues [Peters et al., 2002]. This has been alluded to in other studies where prorenin binding and uptake by vessels and cardiomyocytes via a renin receptor has been suggested [Danser et al., 1994; Prescott et al., 2000; Saris et al., 2001; van Kesteren et al., 1997].

Table 1.4 Summary of transgenic rat models with altered blood pressure.

NAME	TGR(mREN2)27	INDUCIBLE HYPERTENSIVE RAT	TGR(α1AT-mRen2)	TGR(α1AR-rpR)	TGR(hAOGEN/hRen)
TRANSGENE	Mouse Ren2 complete gene plus 3' and 5' flanking sequences	Cyp1a1 promoter fused to mouse Ren2 cDNA (integrated onto Y chromosome)	Human α1AT promoter fused to mouse Ren2 cDNA	Human α1AT promoter fused to rat Renin cDNA	Human angiotensinogen and renin genes
BACKGROUND	Hanover Sprague-Dawley	Fisher F334	Fisher F334	Fisher F334	Sprague-Dawley
PHENOTYPE	SBP 200mmHg at 9 weeks (heterozygote) >250mmHg (homozygote).	Normal prior to induction. SBP 220mmHg after 7 days	SBP 190mmHg at 7 weeks	Not hypertensive at 10 weeks	Hypertensive 200mmHg at 7weeks
SEX INFLUENCE	Male > female	All Male	Not determined	Males>>females	Not reported
DRUG CORRECTION	ACE inhibitors AT antagonism Not spironolactone	Salt sensitivity attenuated by superoxide dismutase mimetic.	ACE inhibitors		ACE inhibition. Human renin inhibitor. Bosentan. Cyclosporin. NFκB inhibitor
EXPRESSION SITE	Widespread. Mostly adrenal cortex	Mostly liver	Liver	Liver	Widespread for both transgenes but hRenin greatest in kidney and hAngiotensinogen in the liver
END-ORGAN INJURY	Vascular remodelling, cardiac hypertrophy & fibrosis, nephrosclerosis and stroke. Variable MH.	Fibrinoid necrosis of mesentery, coronary and renal circulations	Vascular remodelling Cardiac hypertrophy & fibrosis Nephrosclerosis. Transformation to M.H.	Cardiac fibrosis and nephrosclerosis	Cardiac hypertrophy, nephrosclerosis and M.H
PLASMA LEVELS	10-20 X mouse prorenin levels. Dispute over other RAS components but not greatly elevated	10^5 X mouse prorenin levels. Elevated angiotensin II and aldosterone.	10^3 X mouse prorenin levels. Other RAS components normal.	400 X rat prorenin levels. Renin, angiotensin II normal	Human prorenin and angiotensinogen elevated. Plasma angiotensin II elevated

Table 1.4 Summary of transgenic rat models with altered blood pressure (continued).

NAME	DAHL SS TGR(α1NK-ATPase)	TGR(ASRAOGEN) TGR(ASHAOGEN - mREN2)	NPY-TGR	TGR(DbH-ETB:ETBsl:sl)	TGR(hKLK1)
TRANSGENE	Rat wild-type α1Na-K-ATP-ase cDNA and promoter	200bp reverse orientation construct producing anti-sense oligonucleotide to rat angiotensinogen. Driven by GFAP.	rat neuropeptide Y gene and promoter	DbHETB rescue of spotting lethal.	Human tissue kallikrein under the mouse metallothionein promoter.
BACKGROUND	Dahl Salt sensitive	Sprague-Dawley. TGR(mRen2)27.	Sprague-Dawley	Spotting lethal	Sprague-Dawley
PHENOTYPE	improves hypertension & glomerulosclerosis	Hypotension or reduced hypertension in TGR(mRen2)27. Polyuria	Stress-induced hypertension. Increased vascular resistance & pressor response. Reduced hypotension in response to haemorrhage.	salt sensitive hypertension	Hypotension. Reduced hypertrophy and fibrosis to pressor agents. Altered circadian rhythm.
DRUG CORRECTION				amiloride	B2 antagonist
EXPRESSION SITE	Heart, brain, kidney, aorta	Brain	Heart, vessels, spleen	Adrenergic tissue.	Heart
PLASMA LEVELS		Normal plasma angiotensinogen. 90% reduction in brain level Reduced AVP	Normal at rest NPY elevated post haemorrhage	ET-1 increased	

1.3 THE PATHOPHYSIOLOGY OF MALIGNANT HYPERTENSION.

Multiple factors may interact to generate and sustain vascular injury and hypertension in M.H. (table 1.5). Although hypertension must be present to diagnose MH clinically, the absolute level appears relatively unimportant since it is possible to have either benign or MH with identical blood pressure values [Isles et al., 1985]. Typically blood pressure will be high and in most published series the mean diastolic blood pressure at presentation is approximately 140mmHg. Acute onset of hypertension, not permitting adaptive responses, may predispose to vascular injury and people who develop MH tend to be young (mean age 50 years), often without a previous history of hypertension or evidence of left ventricular hypertrophy at presentation [Lip et al., 1995].

1. Pressure	Rate of rise
2. Shear Stress	
3. Neurohumoral factors	Renin-angiotensin system
	Kallikrein-kinin system
	Endothelins
	Neuropeptides
	Natriuretic peptides
	Nitric oxide
4. Oxidative stress	
5. Inflammation	
6. Genetic background	
7. Renal salt handling	
8. Renal Mass	

Table 1.5. Factors Contributing To The Pathogenesis Of Malignant Hypertension.

In the Goldblatt model fibrinoid necrosis develops only in the contralateral, unclipped kidney exposed to high pressures, suggesting that hypertension is a requirement for end-organ injury. However, with a lesser degree of stenosis, where the same level of systemic hypertension is attained over a longer time course, MH does not develop suggesting that rate of blood pressure rise may be critical [Wilson, 1939]. Similarly, in the Inducible Hypertensive Rat model [Kantachuvesiri et al., 2001] inclusion of less I3C in the diet resulted in a slower rise, but similar final level of blood pressure and was accompanied by changes of chronic, but not malignant, hypertension.

1. Renal	Renal artery stenosis
	Cholesterol emboli
	Glomerulonephritis
	Parenchymal disease
	Tumours – renal cell carcinoma, reninoma
	Scleroderma renal crisis
2. Endocrine	Conn's syndrome
	Pheochromocytoma
	Cushing's
	Thyroid disorders
	Hyperparathyroidism
3. Vascular	Aortic coarctation
4. Drugs	Cocaine
	Amphetamines
	Eryrthropoietin
	Oral contraceptive pill
5. Other	Sleep apnoea syndrome
	Pre-eclampsia

Table 1.6. Secondary Causes Of Malignant Hypertension.

Secondary causes of hypertension account for 30-40% of MH [Isles et al., 1985; Webster et al., 1993] and provide an explanation for sudden onset hypertension (table 1.6). The remainder mostly transform from benign hypertension and it is estimated that 1% of benign hypertensives will do so [Bechgaard, 1956].

In human MH, the RAS is inappropriately active and patients, with the exception of those with primary aldosteronism and phaeochromocytoma, have inappropriately high levels of circulating renin [Zarifis et al., 1996]. Bartter's syndrome and familial chloride loosing diarrhoea are states of RAS activation with normal or low blood pressure. Little is known about vascular remodelling in these conditions, but some authors have observed vascular lesions similar to those seen in hypertensive disease [Brackett et al., 1968; Pasternack and Perheentupa, 1966; Pasternack et al., 1967; Sutherland et al., 1970]. This inappropriate activation may be primary or a consequence of salt-water loss due to pressure natriuresis or afferent arteriolar injury, stimulating renin production. The homozygous deletion polymorphism (DD) affecting the *ACE* gene is associated with an increase in plasma ACE activity and occurs twice as often in patients with MH than the general population [Stefansson et al., 2000]. The impact of this polymorphism on human survival is not yet known, but mortality from MH in the TGR(mRen2)27 line appears linked to two genetic regions containing the *ACE* and *AT1* receptor gene loci [Kantachuvesiri et al., 1999].

Given the presence of high circulating renin in these patients and the suggestion from animal studies that the RAS may be contributing to, or even generating, pathology at a tissue level, this is currently an area of great interest.

1.3.1 The Role Of Angiotensin II In Hypertensive Vascular Remodelling.

Angiotensin II is an octapeptide and major effector molecule of the RAS via its actions on two classes of receptor, termed AT1 and AT2 respectively. Uniquely, rodents have a duplication of AT1 ($AT1_a$ and $AT1_b$) (figure 1.4).

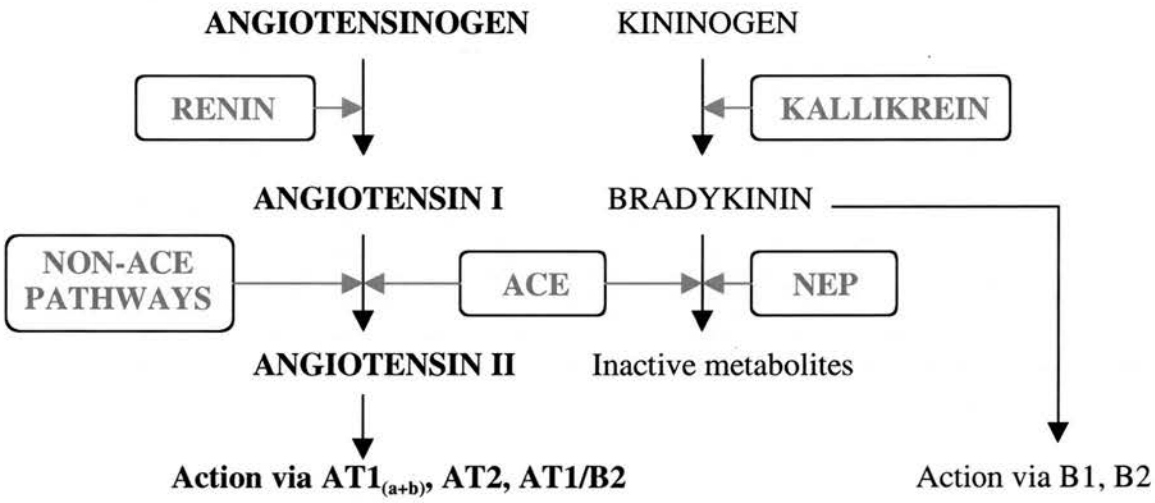

Fig. 1.4 The Renin Angiotensin System And Kallikrein Kinin System.

ACE, angiotensin converting enzyme, identical to kininase II and active principally within the pulmonary circulation; "non-ACE pathways" include cathepsin G, chymostatin-sensitive angiotensin II generating enzyme (CAGE), chymase, tonin and tissue plasminogen activator (tPA) are important in non-pulmonary tissues and may account for 40% of angiotensin II generation in humans. AT, angiotensin II receptor. AT1 subdivisions $AT1_{a+b}$, only exist in rodents. NEP, neural endopeptidase. B1/2, bradykinin receptor type 1/2.

Angiotensin II infusions have been used to explore the pathophysiology of vascular injury and infusion into rats at a dose of 400ng/kg/min, or above, will result in a rapid elevation of blood pressure and development of fibrinoid necrosis in the vascular beds of the kidney, heart and mesentery over a one to two week period. Mice are also susceptible to hypertensive vascular injury in this way with an associated perivascular, small vessel, inflammatory cell infiltrate [Su et al., 1998a; Wiener et al., 1996]. Lower doses result in chronic hypertension without malignant transformation.

Given that direct infusion of angiotensin II results in vascular remodelling and hypertensive end-organ injury and that agents blocking the RAS are highly efficacious in treating animal models of hypertension, much attention has focused on the role of angiotensin II and the molecular mechanisms involved. In addition to regulating vascular tone and salt-water balance, angiotensin II behaves as a cytokine influencing a variety of cellular processes including oxidative stress via NAD(P)H oxidase [Griendling et al., 1994; Ushio-Fukai et al., 1996], growth and inflammation [Brasier et al., 2002; Ruiz-Ortega et al., 2001b]. Within vessels, angiotensin II has been shown to effect tone, inflammation, matrix remodelling and thrombosis via actions on endothelial cells, vascular smooth muscle cells and fibroblasts. The resulting angiotensin II-induced breakdown in vascular homeostasis, specifically endothelial dysfunction, has been suggested as critical in mediating vascular remodelling in hypertensive states as well as other conditions where vascular remodelling is central, such as atherosclerosis [Dzau, 2001]. Although the list of molecular effectors of angiotensin II is still incomplete, evidence suggests that alterations in the redox state of cells is an important and early event in the molecular cascade leading to aberrant vascular function.

Angiotensin II can stimulate the formation of reactive oxygen species (ROS) in vitro via a membrane bound NAD(P)H oxidase [Griendling et al., 1994]. ROS in turn, avidly inactivates nitric oxide (NO) necessary for normal endothelial function [Harrison, 1997]. The consequence of NO inactivation, in addition to vasoconstriction, is NFκB activation through loss of IκB stabilization triggering expression of pro-inflammatory cytokines, leukocyte adhesion and vascular smooth muscle cell proliferation and migration [De Caterina et al., 1995; Dubey et al., 1995; Peng et al., 1995]. Evidence also exists for increased levels of ROS in certain hypertensive conditions. Infusion of angiotensin II into rats increased the levels of NAD(P)H derived ROS. Both hypertension and NAD(P)H expression were decreased by pre-treatment with superoxide dismutase (SOD) [Laursen et al., 1997]. Heparin-binding SOD, targeted to the endothelium, was capable of reducing blood pressure in a spontaneously hypertensive rat model known to have high levels of vascular ROS [Nakazono et al., 1991].

Angiotensin II is also capable of inducing leukocyte recruitment and activation by ROS-dependant pathways. Vascular VCAM-1 binds circulating leukocytes initiating adhesion of inflammatory cells. Endothelial VCAM-1 expression was induced by angiotensin II via NFκB and inhibited by an anti-oxidant [Pueyo et al., 2000]. Angiotensin II was also found to increase VCAM-1 expression in rat vascular smooth muscle cells [Tummala et al., 1999].

The role of macrophages and other inflammatory cells is increasingly a target for investigation, not only in malignant vascular injury, but also in vasculopathy related to atheroma, diabetes, post angioplasty re-stenosis and benign hypertension. Whilst the

association of inflammatory cells with such lesions is not in dispute, their significance is still unclear, although it would seem that they are not necessarily a response to tissue injury. Factors that initiate the recruitment of these cells are also receiving attention. MCP-1, a member of the C-C chemokine family, is a macrophage chemoattractant that acts via the CCR2 receptor and is upregulated in the arteries of animals rendered hypertensive by angiotensin II infusion. Bush *et al* infused angiotensin II on a CCR2 knockout background and observed greatly reduced monocyte infiltration and vascular hypertrophy, indicating that in this model, macrophage recruitment, via CCR2, was essential for vascular hypertrophy [Bush et al., 2000].

In vivo immunomodulatory treatments have been used to target inflammatory pathways induced by angiotensin II (discussed in chapter four). Additionally, mycophenolate mofetil (MMF) prevented the development of salt-sensitive hypertension following angiotensin II infusion. Although angiotensin II induced hypertension was not prevented by MMF, T cell infiltration and tubulointerstitial injury believed to cause salt sensitivity, were significantly reduced [Rodriguez-Iturbe et al., 2001]. Angiotensin II is known to induce the adhesion molecule and MCP-1 expression and inflammatory cell infiltration into the kidney. Osteopontin, a chemoattractant with a role in macrophage recruitment into vascular lesions, is also upregulated by angiotensin II [Giachelli et al., 1991; Liaw et al., 1994]. Treatment of double transgenic rats described above, where circulating levels of angiotensin II are high, with dexamethasone, resulted in amelioration of renal injury and inflammatory cell infiltration independent of blood pressure. Similar results were seen with the more specific immunosuppressant agents MMF and soluble TNF receptor, entanercept [Muller et al., 2002].

It is certain that the bulk of angiotensin II response is mediated via AT1 in the adult and infusion of angiotensin II onto an $AT1_a$ /$AT1_b$ double knockout background had no pressor effect. [Tsuchida et al., 1998]. However AT2 is not entirely passive and despite low expression in adult vasculature, there is evidence to suggest a modulatory role on angiotensin II response as illustrated by the attenuation of presser responses to angiotensin II in the face of AT2 over-expression specifically targeted to vascular smooth muscle [Tsutsumi et al., 1999]. The author suggests that this may be mediated via NO. Interplay between the NO system and the RAS has been demonstrated in other settings including pressure diuresis and natriuresis [Madrid et al., 1997]. Of interest, AT2 expression is relatively high in normal resistance vessels affected during the development of MH [Nora et al., 1998], but the extent to which AT1 antagonists exert their effect via increasing angiotensin II levels and subsequent binding to AT2 as has been suggested, is unclear. The finding that chronic AT2 antagonism modulates the pressor response to angiotensin II [Munzenmaier and Greene, 1996; Scheuer and Perrone, 1993; Wesseling et al., 2005] together with similar findings in AT2 knockout mice [Hein et al., 1995; Ichiki et al., 1995] further support a regulatory role for AT2. It has been suggested that AT2 can interact directly, and without agonist binding, with AT1 resulting in direct antagonism via heterodimer formation [AbdAlla et al., 2001a]. This would explain why AT2 associated AT1 antagonism can exist despite AT2 specific antagonism.

As stated above, angiotensin II receptors can exist as heterodimer combinations and form heterodimers with other vasoactive peptide receptors including bradykinin. Termed AT1/B2 heterodimers, these receptors demonstrated enhanced signal

transduction and production of inositol phosphates in response to angiotensin II, as well as altered pathways of endocytosis in vitro [AbdAlla et al., 2000]. AT1/B2 heterodimers have been suggested to contribute to angiotensin II hyper-responsiveness in pre-eclamptic women [AbdAlla et al., 2001b]. AT1/B2 receptors were more abundant on the mesangial cells of spontaneously hypertensive rats than normotensive controls, suggesting that they may contribute to mesangial cell hyper-responsiveness to angiotensin II in this model [AbdAlla et al., 2005]. Since other G-protein-coupled receptors can exist as dimers and share cellular location, it is possible that additional combinations may exist which affect signal transduction.

1.3.2 The Role Of The Endothelin System In Malignant Vascular Remodelling.

Endothelins 1-3 (ETs) with their potent vasoconstrictor and mitogenic actions have been implicated in the development of M.H. [Weber et al., 1994; Yanagisawa et al., 1988]. The effects of ETs are mediated in mammals through two functionally distinct classes of receptor, ETA and ETB. ETB displays a dual role, able to induce vasoconstriction when situated on vascular smooth muscle cells, as well as NO mediated vasodilatation on endothelial cells. Angiotensin II can induce ET expression suggesting the possibility of cross-talk between the RAS and ET systems. In animals, ET(s) infusion results in increases in blood pressure and vascular resistance [Mortensen et al., 1990; Yokokawa et al., 1989]. Induction of ET expression within vascular endothelial cells occurs in response to low levels of shear stress, and in response to mediators such as thrombin, and angiotensin II [Yanagisawa et al., 1988] which could

be involved in the pathogenesis of MH. Pressure alone, without inducing shear stress, caused an increase in ET-1 release in vitro via a mechanism involving protein kinase C and phospholipase C [Hishikawa et al., 1995]. As mentioned above, elevated ET-1 levels have been reported in "low-renin" as well as some "high-renin" models of MH, including the DOCA-salt model and chronic administration of caffeine to Goldblatt rats [Fujita et al., 1996; Kohno et al., 1991]. Renal ET-1 mRNA levels were also increased in the TGR(mRen2)27 Edinburgh Sprague-Dawley cross HanRen2/Edin- with MH, but administration of bosentan, a combined ETA/B antagonist, failed to attenuate hypertension or affect outcome [Whitworth et al., 1995b]. Similarly bosentan had no effect on the vascular or renal lesions of MH in SHRs treated with L-NAME [Li et al., 1996a] and had only a small effect on blood pressure in DOCA-salt-treated spontaneously hypertensive rats [Schiffrin et al., 1995]. Additionally, ETA antagonists failed to influence blood pressure or vascular pathology in wild-type rats treated with NO inhibition or only had a partial effect [Sventek et al., 1997; Verhagen et al., 1998]. However, glomerular ischaemic injury and proteinuria were prevented in this model by ETA blockade [Verhagen et al., 1998].

In contrast, treatment of the combined ETA/B receptor antagonist, TAK-044 effectively prevented DOCA-salt-induced renal injury and functional deterioration in malignant DOCA-salt SHR [Kohno et al., 1997]. Treatment of the salt-loaded SHRSP with the ETA antagonist A127722, increased survival and prevented the development of cerebral oedema and proteinuria if given early [Blezer et al., 1999]. This protective effect was lost if treatment was delayed suggesting that ETA is implicated early in the pathogenesis of hypertension and end-organ injury in this model. The discrepancy in

results between these studies could be due to the different animal models of MH studied and pharmacological properties of drugs tested, particularly the specificity of receptor blockage.

Data from human disease is conflicting with plasma levels of ETs being both increased and reduced in separate studies [Lemne et al., 1994; Schiffrin and Thibault, 1991]. As a consequence, transgenic strategies have been employed to further investigate the role of endothelins in the development of hypertension.

A natural deletion of the ETB gene exists called spotting-lethal. Animals with this condition die neonatally from aganglionic megacolon, the rodent equivalent of Hirshsprungs disease (where ETB mutations compose 5%). Genetic rescue of these animals by targeting the rat ETB cDNA under the direction of the human dopamine hydroxylase promoter replaces ETB at adrenergic sites including the enteric nervous system (termed TGR(DBH-ETB:ETBsl/sl)) [Gariepy et al., 2000]. As mentioned above ETB can induce vasoconstriction or vasodilatation depending on location and when situated on renal tubular epithelium promotes natriuresis. The rescued phenotype lacked ETB receptor expression within the kidney and appeared healthy and normotensive on a low salt diet. However, they exhibited a hypertensive response to high (8%) dietary sodium chloride that corrected with amiloride implicating abnormal endothelial sodium channel (ENaC) activity, known to be important in sodium handling within the distal nephron. In these animals under salt stress, the renin-angiotensin system is appropriately suppressed. These results are consistent with ETB providing tonic inhibition of ENaC during the normal maintenance of blood pressure. A gain of

function mutation of this gene results in one of the few monogenetic causes of human hypertension, Liddle syndrome [Hansson et al., 1995; Shimkets et al., 1994].

Although ETA antagonists are beneficial in DOCA-salt treated rats, ETB antagonism can sometimes be detrimental. To address this TGR(DBH-ETB:ETBsl/sl) rats were treated with unilateral nephrectomy and DOCA-salt. The result was worsening of hypertension and end-organ injury in the form of left ventricular hypertrophy and renal dysfunction again suggesting a protective role for ETs acting via ETB [Matsumura et al., 2000]. The authors mention that an increase of ETA mediated activity rather than less ETB activation cannot be excluded.

Endothelins have also been implicated in renal injury through their actions on mesangial cell proliferation [Haneda et al., 1995] and are associated with human renal injury such as cyclosporin nephropathy and lupus nephritis [Busauschina et al., 2004; Nassar and Badr, 1994]. TGR(hET-2)37 is a transgenic rat line generated by insertion of the human ET-2 gene under the control of its' own promoter. ET-2 is highly expressed within the glomerulus of transgenic rats but absent from normal rat kidney. TGR(hET-2)37 rats were normotensive but developed glomerulosclerosis with proteinuria by 12 months of age suggesting a role for ET-2 in the development of fibrotic injury within the kidney independent of blood pressure. The reason for these animals normotension, despite a two-fold elevation in circulating ET-2, is unclear and may be partially accounted for by activation of the NO system, since inhibition of this system resulted in greater than expected hypertension. However this line also exhibited developmental abnormalities within the kidneys and heart which may also contribute to overall blood pressure [Hocher et al., 1996; Liefeldt et al., 1999].

1.3.3 Neuropeptide Y.

Neuropeptide Y (NPY) is a ubiquitous neuropeptide, which co-localises with norepinephrine in the secretory vesicles of central and peripheral neurones. Exogenous administration results in persisting pressor and vasoconstrictor responses and in some instances may potentiate the effects of other vasoactive substances such as angiotensin II or norepinephrine [Dahlof et al., 1985; Wahlestedt et al., 1990]. Spontaneously hypertensive rats have higher NPY levels within the myocardium and an enhanced pressor response to NPY administration and the NPY locus has been shown to segregate with blood pressure [Katsuya et al., 1993; Westfall et al., 1990].

Even less is known regarding human hypertension but links to exercise induced blood pressure elevation have been made [Solt et al., 1990].

In order to examine the role of over expression of this peptide during rest and stress, transgenic animals were created expressing the rat NPY gene under control of its' own promoter on a Sprague-Dawley background [Michalkiewicz et al., 2001]. Animals had increased levels of NPY protein in many organs mirroring the distribution of the endogenous peptide. In a resting state, transgenic animals displayed a greater total peripheral resistance than non-transgenic controls. Interestingly, blood pressure determined by direct arterial measurement was normal but found to be elevated when estimated using tail-cuff plethesmography. The authors went on to confirm an enhanced stress response by illustrating greater pressor responses to noradrenaline infusion and a reduced fall in blood pressure and heart rate induced by haemorrhage. They suggest that in this setting NPY acts by modulating α-adrenoreceptor sensitivity shedding light

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on an important aspect of blood pressure regulation. Rats harbouring the NPY transgene demonstrated a reduced pressor response to NO inhibition with L-NAME. This affect was centrally mediated and could be mimicked by intracerebroventricular infusion of NPY and inhibited by an NPY antagonist [Michalkiewicz et al., 2005]. Since both NPY and NO act as inhibitory neurotransmitters sometimes within the same neural circuits, it is plausible that one can compensate for deficiency of the other.

Chromogranin A (CHGA) is a peptide stored and co-released from autonomic neurones together with catecholamines and NPY and is over expressed in human, essential hypertension. One biologically active product of CHGA, termed catestatin, functions to inhibit neurotransmitter release. Creation of *Chga* gene-ablated mice resulted in hypertension with additional loss of diurnal blood pressure variation. Secretory granules were depleted of catecholamines and NPY while circulating levels were elevated in keeping with a lack of catestatin. These elevated levels may be responsible for the hypertension observed via altered autonomic response. Hypertension was abolished by addition of the human *Chga* gene [Mahapatra et al., 2005].

1.3.4 Renal Salt Handling.

Renal salt handling has been proposed as central to the aetiology of hypertension in human disease and animal models. Clear examples exist where altered sodium handling result directly in hypertension such as Liddle syndrome. Here dominantly inherited mutations of the sodium channel on the collecting tubule lead to sustained activation with sodium retention and associated hypertension. A transgenic strategy was employed

to investigate the role of the sodium potassium ATP-ase (Na-K-ATPase) in hypertension and salt handling in the kidney. Dahl salt-sensitive (D S-S) rats have a mutation in the α1 Na-K-ATPase that segregates with hypertension [Herrera et al., 1998]. The authors went on to express the wild-type α1 Na-K-ATPase under the control of its' own promoter on a D S-S background to obtain a reduction in blood pressure and glomerulosclerosis, suggesting strongly that α1 Na-K-ATPase acts as a susceptibility factor in salt sensitive hypertension in this model.

Salt sensitive hypertension exists when sodium balance within the body is related to systemic blood pressure; blood pressure rising with increased sodium intake. In this state, the pressure natriuresis curve is shifted to the right i.e. it requires a higher pressure to excrete a given sodium load. Salt sensitivity is common in human essential hypertension, especially elderly and black populations, and can be induced experimentally in rodents by infusing angiotensin II, cyclosporin and phenylephrine and also by nitric oxide inhibition [Andoh et al., 2001; Franco et al., 2001; Johnson et al., 1999; Lombardi et al., 1999; Quiroz et al., 2001]. Tubulointerstitial injury consisting of peritubular capillary loss and an inflammatory infiltrate (predominantly macrophage) is common to both human and rodent pathology. These various methods of inducing renal injury alter renal blood flow among other things. In describing the "vicious cycle" of Bright's disease, Wilson and Byrom observed that after release of the clip in the one clip two kidney model, hypertension persisted. Renal injury was histologically minor and predominantly microvascular and interstitial, similar to that described above, suggesting that ischaemia is one possible mechanism for inducing altered salt handling [Wilson, 1941].

Vasoconstriction resulting in reduced nutrient delivery and hypoxia are thought to contribute to tubulointerstitial injury and, as a consequence, altered sodium handling and hypertension. Hypoxia can induce expression of leukocyte adhesion molecules, cytokines and chemokines resulting in inflammatory cell infiltration as well as extracellular matrix deposition, interstitial and tubular proliferation [Fine et al., 1998; Lu et al., 1999; Norman et al., 1999]. In the rodent models described, tubulointerstitial injury is greatest in the outer medullar, most susceptible to hypoxia, suggesting that this mechanism is important in the development of salt sensitivity. Afferent arteriolar injury or increased resistance has been proposed as important for the development of renal hypoxia, reduced single nephron glomerular filtration rate with decreased sodium filtration and increased sodium reabsorption via tubuloglomerular feedback [Johnson et al., 2002]. This cycle leads ultimately to sodium retention, increased blood pressure causing increased renal perfusion, sodium excretion and reducing hypoxia.

VEGF, an angiogenic factor induced by hypoxia, is reduced in cyclosporin-induced salt sensitive hypertension in rats. Hypertension and pathology were attenuated by treatment with VEGF, suggesting that pro-angiogenic factors such as this may have relevance for the treatment of cyclosporin-induced nephropathy in humans [Kang et al., 2001]. However, the role of VEGF is complex since it can also have pro-inflammatory actions and facilitate monocyte recruitment.

The immunosuppressant agent mycophenolate mofetil can prevent the development of salt sensitivity and reduce T cell infiltration caused by angiotensin II infusion and nitric oxide blockade in rats suggesting that tubulointerstitial inflammation is important for the development of this condition [Quiroz et al., 2001; Rodriguez-Iturbe et al., 2001].

Recently the IHR model has been shown to display salt sensitivity if transient induction and hypertension are allowed to develop. When induction was stopped blood pressure levels returned to normal but increased if the animals were fed a diet containing 8% sodium chloride. If a superoxide dismutase mimetic was co-administered, salt sensitivity was attenuated suggesting that in the IHR, superoxide production plays a role in this mechanism [Howard et al., 2005].

1.3.5 Kallikrein Kinin System.

The kallikrein kinin system is widespread and can exert powerful vasodilating, antioxidant, antiproliferative and antithrombotic effects. This system is also interconnected with the RAS and the contribution of kinins to the efficacious effects of ACE inhibitors has long been debated (figure 1.4). The effects of kinins are mediated mostly via its receptor B2, which is constitutively and widely expressed [Bhoola et al., 1992]. B2 expression on the renal collecting duct may mediate natriuresis via prostaglandin E_2 generation [Garcia-Perez and Smith, 1984; Stokes and Kokko, 1977]. Kinins can also inhibit the actions of vasopressin (ADH) contributing to their diuretic effect [Schuster et al., 1984].

Human essential hypertension is associated with low urinary kininogen excretion [Balsano, 1991]. This defect is more common within the salt sensitive sub-population and it has been suggested that kinins mediate excretion of excess salt and counter the effects of sympathetic system and RAS activation [Katori and Majima, 1997; Katori et al., 2001]. Reduced kallikrein expression was found in three rodent models displaying

salt sensitivity; the nitric oxide inhibition model, four-fifths nephrectomy model and protein overload model [Ardiles et al., 2003]. Administration of kallikrein to humans and rats was able to lower blood pressure but only in salt sensitive individuals [Bellini et al., 1993; Chao and Chao, 1997] and B2 null mice develop salt sensitive hypertension at a young age [Cervenka et al., 1999a].

A transgenic rat which over expresses human kallikrein under the mouse metallothionein promoter was generated and the cardiovascular effects assessed [Silva et al., 2000]. High levels of expression were observed in the hearts of these animals and expression was also seen in the kidney, lung and brain. Transgenic animals were hypotensive (5mmHg lower) and had a reduced response in terms of left ventricular hypertrophy, atrial natriuretic peptide (ANP) expression and fibrosis to isoproterenol infusion. Hypotension was improved, but not corrected by a B2 antagonist suggesting that increased kinin generation is at least partially responsible. An additional finding was that the circadian rhythm in these animals was altered such that the amplitude of the dominant twenty-four hour period was reduced suggesting that the kallikrein kinin system may have a role in regulating blood pressure at the level of circadian rhythm.

1.3.6 Renal Mass.

The "foetal origins hypothesis" introduced by Barker observed that low infant birth weight associated with the development of hypertension and heart disease in adult life [Barker, 1994; Barker, 1995a; Barker, 1995b; Vijayakumar et al., 1995]. This idea is now well established and additionally non-insulin dependant diabetes, osteoporosis,

pulmonary infections, obstructive airways disease and increased mortality are now linked to low birth weight [Barker et al., 1991; Hales et al., 1991; Syddall et al., 2005]. Similar data from animal models suggests foetal programming is not restricted to humans.

In 1988 Brenner *et al* suggested that the nephron number at birth was inversely linked to the development of hypertension and renal injury in later life. Similarly, if a critical number of nephrons were lost later in life hypertension and renal injury could develop [Brenner et al., 1988; Mackenzie et al., 1996]. Simply, if nephrons are lost, glomerular filtration rate is increased through those that remain and sodium balanced maintained. This increase in glomerular pressure causes injury and proteinuria (seen histologically as focal and segmental glomerulosclerosis), which in turn damages more nephrons resulting ultimately in renal dysfunction, sodium and water retention and hypertension. The critical nephron number required to be lost is about to two-thirds of previously normal renal mass in humans [Hayes et al., 1991; Novick et al., 1991].

African Americans and Aboriginals are at greater risk of developing hypertension, proteinuria and renal injury and have small kidneys containing less nephrons. The Milan hypertensive and spontaneously hypertensive rats selected for hypertension, also have less nephrons than normotensive controls [Mackenzie et al., 1996; Singh and Hoy, 2004].

Poor nutritional status may be in part, responsible for these changes since maternal protein restriction in rats between E0 and E16 (the human equivalent of five to six weeks gestation), prior to nephron formation, results in twenty percent fewer nephrons [Welham et al., 2005]. It is possible that historically, this conveyed a survival

advantage now lost. The molecular mechanisms underlying nephron reduction are not clear and factors other than reduced nephron number, such as islet cell number and vascular resistance determined during foetal development, may also contribute to the predisposition to disease in adult life. But crudely, a reduction in renal mass will predispose an animal to renal injury and hypertension and this is exploited in the generation of some animal models of hypertension such as the DOCA-salt and remnant kidney models. If five-sixth nephrectomy is performed in spontaneously hypertensive rats, malignant nephrosclerosis develops in the remnant kidney [Bidani et al., 1994].

Angiogenic factors such as VEGF are important in restoring blood supply and maintaining tissue in the injured kidney. In models of tubulointerstitial injury known to result in salt sensitivity and hypertension, VEGF is not produced by injured tubules, and the peri-tubular, microvascular injury is not repaired [Grone et al., 1995].

1.4 CEREBROVASCULAR INJURY.

Stroke, both ischaemic and haemorrhagic, associates strongly with hypertension in man. The relationship between increasing blood pressure and stroke is linear, doubling for every 7.5mmHg rise in diastolic pressure [1996]. Once cerebral autoregulation is overcome by the rapid onset of severe hypertension which characterises MH, the brain is susceptible to injury and a picture of cerebral oedema, small infarcts and haemorrhages with associated small vessel fibrinoid necrosis is seen [Fisher, 1968; Fisher, 1982; Lassen and Agnoli, 1972].

How hypertension causes stroke is poorly understood, but it likely mediates brain damage in a variety of ways, for example by increasing the extent and severity of atheroma, via "malignant" hypertensive encephalopathy and eclampsia, causing expansion and rupture of berry aneurysms and by causing diffuse white matter changes or leukoaraiosis. By convention however, the term "hypertensive small vessel stroke" is commonly used to refer to small, deep ("lacunar") cerebral infarcts and spontaneous intracerebral haemorrhages. These stroke sub-types are also to some extent heterogeneous, but are linked by the probable importance of small vessel fibrinoid necrosis in their pathogenesis [Lammie, 2002]. Due perhaps to efficient autoregulation, cerebral vessels in chronic hypertensives do not consistently display the striking medial hypertrophy observed systemically [Dickinson and Thomson, 1960] and even blood vessels not previously exposed to sustained hypertension are capable of withstanding high pressures without leak [Dickinson, 2001; Giese, 1973; Margolis and Sadowsky, 1976]. Both "forced dilatation" and excessive autoregulatory vasospasm of the microvasculature have been suggested as early events in hypertensive small vessel

stroke, both perhaps capable of mediating fibrinoid necrosis [Byrom, 1954; Byrom, 1975].

Much research has focused on animal models where invasive investigation is permissible. The rat is a useful model for the investigation of human stroke as they share many features of vessel anatomy, histology and ultrastructure [Lee, 1995]. Its size and longevity also make this species an appropriate model of cardiovascular disease.

To date the SHRSP is the best documented non-surgical model of stroke, exhibiting fibrinoid small vessel necrosis, cortical infarction and haemorrhage in the context of MH [Okamoto et al., 1974]. Cerebral infarction is the principal pathology, but small foci of haemorrhage can be found within some infarcts (a common finding in human stroke) as well as apparently unrelated to areas of infarction. Unfortunately, the method of phenotype selection used to generate this model precludes an ideal control matched for genetic background and hypertension. Similarly, SHR strains without the stroke prone phenotype differ in their genetic background. Although this offers the advantage of identifying susceptibility genes, it makes direct comparison difficult. Differences in the microstructure of cerebral vessels and reduced cerebral blood flow in the SHR compared to Wistar-Kyoto rats have been described [Johansson, 1984; Yamori and Horie, 1977]. Infarct size in response to cerebral artery ligation is also larger in the SHR [Coyle and Feng, 1993]. TGR(mRen2)27 homozygote rats with MH develop stroke similar to those seen in the SHRSP but much less frequently. The difficulty with both these models is the inability to accurately predict the onset of stoke and hence to more easily investigate its causal vessel lesions and early brain changes. Vessel

pathology identified some time after a stroke may be as much a response to brain injury as causal of it.

Models that surgically induce stroke by means of mechanical or chemical arterial occlusion overcome the problem of unpredictability, but require surgical consistency and impose additional procedural stress on the animal. They also place limitations on the vascular distributions that can be investigated and shed no light on stroke cause, being most useful in studies of the brain's response to hypoxic-ischaemic damage.

In the IHR model the cerebral circulation is spared during the first fourteen days of induction, during which time systemic vascular remodelling is advanced and is associated with end-organ damage. Extending the time of transgene induction beyond fourteen days to determine the point at which the cerebral circulation succumbed to systemic hypertension was deemed unsuitable due to the general condition of the animals at this stage. This apparent "sparing" of the cerebral circulation appears paradoxical in that, at least in humans, the brain vasculature is traditionally considered to be uniquely vulnerable to raised blood pressure and to suffer pathologies such as fibrinoid necrosis at "benign" levels of hypertension.

In conclusion, most animal models of hypertensive vascular injury to date either require surgical or drug intervention to initiate pathology or rely on constitutive expression of a transgene, which can complicate vascular injury by altered foetal development and chronic adaptation. Additionally using outbred backgrounds enhances phenotypic variation. In this respect, the IHR offers a unique opportunity to investigate hypertensive vascular remodelling without these constraints. The IHR is characterised

in detail in this thesis to provide an insight into the early stages of vascular remodelling that pre-date hypertensive end-organ injury. Immunomodulatory therapy was then employed to target these processes in the IHR. Subcutaneous infusion of angiotensin II was adapted to create a malignant hypertensive phenotype in two mice strains and was used to investigate the role of inflammation on vascular remodelling. Additionally, it is hoped that experimental techniques currently only available in the mouse will be used to investigate angiotensin II-induced malignant vascular remodelling in the future.

CHAPTER TWO.

MATERIALS AND METHODS.

2.1 Animals.

Inducible Hypertensive Rats (TGRCyp1a1mRen2) were bred at The Biological Resource Facility, Western General Hospital, Edinburgh. CD11bDTR mice were bred at the Hugh Robson Building, University of Edinburgh. Fisher F334 rats, FVB/NJ and C57Bl/6 mice were purchased from Harlan. All animals were ear-tagged and clipped or marked with permanent ink on the tail base for identification. Only male mice and rats were used in the studies described. Animals were housed with free access to food and water throughout the experimental period and were fed standard commercial rat chow containing 0.32% sodium chloride (Special Diet Services Witham, Essex, UK) in pellet or powdered form. Regular twelve-hour diurnal light dark cycles were continuously maintained and the breeding, maintenance and study of animals were performed according to Home Office regulations.

2.2 Animal Techniques.

2.2.1 Induction Of Transgene Expression In Cyp1a1Ren2 Rats.

As previously described, indole-3-carbinol (I3C) was used to induce transgene expression in IHRs [Kantachuvesiri et al., 2001]. Transgenic IHR and Fisher F334 rats aged 8-12 weeks (around 250g) were studied. Induction was preceded by transferring animals from standard pellet feed to powdered feed for two days. I3C 0.3% w/w was mixed with standard powdered feed using a food processor to ensure even distribution and stored at 4°C. Powdered diet, approximately 25g per day mixed with a little water, was fed to the animals and free access to water was allowed throughout the experimental period.

2.2.2 Blood Pressure Measurement.

Indirect estimation of systolic blood pressure was performed using tail cuff plethesmography (Harvard Apparatus, Linton Electronics, UK) on non-sedated, conscious rats and mice. Animals were trained prior to the period of experimentation on five to seven occasions to minimize stress during the experimental period.

Animals were removed from the experimental room and placed in the procedure room. Ten minutes prior to the procedure they were placed in a heating chamber at 38°C with adequate ventilation to maximise vasodilatation. Mice were then encouraged into a

perspex restraining tube (Vet Tech Solutions) and rats were swaddled (figure 2.1a). Animals were placed on a heated pad at 38°C. The tail was fed through the cuff so that it was positioned approximately 25% along the length of the tail from the base. Inflation pressures were not allowed to exceed 250mmHg for rats and 200mmHg for mice to avoid tail necrosis and unnecessary discomfort. Systolic blood pressure was defined as the pressure when arterial blood flow was first detected. An average of three reading were taken from each animal on each occasion. After the procedure, animals were returned to their cage and experimental room. Tail cuff plethesmography was performed between 9am and 12 am on each day. If the experimental protocol required injection of the animals, blood pressure recordings were done prior to this (figure 2.1).

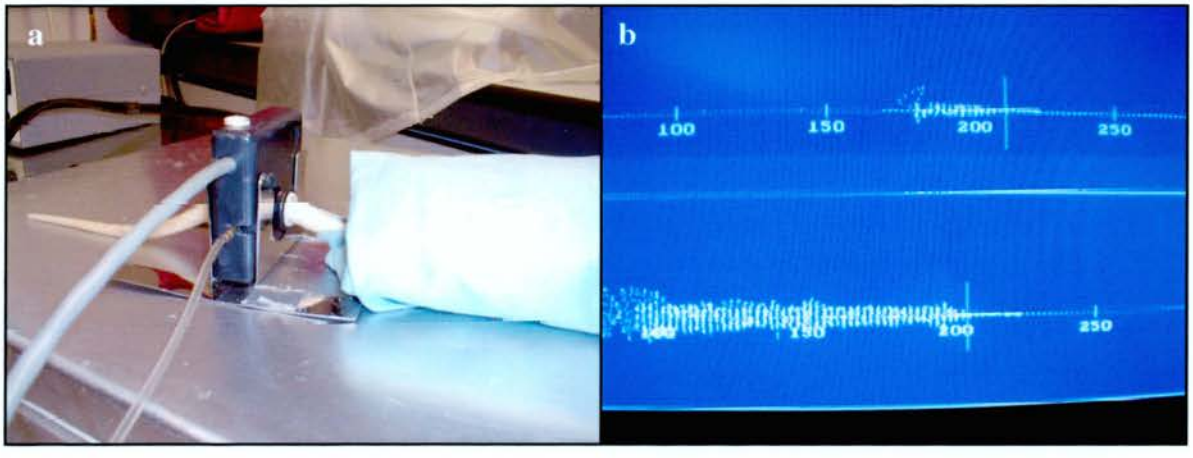

Figure 2.1 Tail Cuff Plethysmography Using Harvard Apparatus.
Conscious restrained rat is shown with tail cuff in place (a). A tracing from a hypertensive mouse showing 210mmHg systolic blood pressure (b).

Direct arterial pressure measurements were achieved via implantation of radiotelemetry devices (Dataquest IV 2.2, from Data Sciences International, St. Paul, Minnesota,

USA), under halothane anaesthesia. Animals were allowed to recover and observed for a one-week period prior to the start of study to ensure any post-operative complications were apparent and reasonable recovery had taken place.

2.2.2.1 Telemetry Equipment.

The implanted transmitter (TA11PA-C40) consisted of a module containing a battery-powered sensor (a stable ion implant semiconductor strain gauge sensor). Arterial pressure was conducted to the sensor via 0.7 mm diameter catheter (figure 2.2). To maintain patentcy, the distal 1 cm of the catheter was coated with an anti-thrombotic film and the tip was filled with a biocompatible gel plug to prevent blood refluxing into the tip. Receivers (RA 1010) were placed under standard plastic rat cages. Telemetric animals were housed singly and aluminium sheets were placed between cages to reduce radio-interference between animals. A dual ambient pressure monitoring (C11PR) measured barometric pressure to correct for the implant measuring pressure relative to a vacuum and all input signals were transmitted to a BCM 100 consolidation matrix. Systolic, diastolic and mean arterial pressure data was transmitted together with HR and activity data to a computer for processing, storage and analysis using the data acquisition system (Dataquest IV2.2) with Microsoft OS/2.

Figure 2.2 DataScience International TA11PA-C40 Device.
Device implanted within the abdominal cavity under general anaesthetic.

2.2.2.2 Implantation technique.

Implantation of a transmitter (TA11PA-C40) was performed by aseptic technique. Anaesthesia was induced using 4-6% halothane (M&B, RMB Animal Health Ltd., Dagenham, UK) in oxygen using an Acoma vaporizer F (International Market Supply, Congleton, UK) via a chamber. The anterior abdominal wall was shaved and the rat placed supine on a heating mat (38^{o}C) while anaesthesia was maintained with 1-3% halothane in oxygen via a mask. 70% ethanol was used to clean the skin prior to midline to open the peritoneal cavity. Saline (0.9%) swabs were used to cover small and large bowel and the retroperitonium was exposed. Using an operating microscope (Wild 650) the descending aorta distal to the origin of the renal arteries was dissected free from surrounding tissue by blunt dissection using a cotton bud. The aorta was tied

loosely (2/0 Ethilon) distal to the origin of the renal arteries. About 2-5 mm above the iliac bifurcation the aorta was punctured with a 19-gauge needle tip and the distal portion of the sensing catheter was inserted into the aorta while aortic blood flow was transiently restricted by lifting the tie. The catheter was advanced up to the level of the tie then fixed *in situ* using tissue adhesive (VetBond, Braun-Melsungen, Germany) with a 2 mm square piece of gauze placed beneath the catheter at the point of entry. The tie was released and removed. Patentcy of the sensing catheter was verified at this stage by eliciting an audible fluctuating note from a radio tuned to a low frequency AM band. Saline swabs were then removed and the transmitter module was placed in the abdominal cavity and sutured to the anterior abdominal wall. The muscle layer was sutured and the skin was closed with surgical clips. Surgery was performed by Mrs Gillian Brooker. During the post-operative period rats were kept on heating pads until recovery and were usually eating and drinking within four hours. Analgesia and prophylactic antibiotic were administered. If haemorrhagic or ischaemic complications occurred, animals were humanely culled.

2.2.3 Insertion Of ALZET Miniosmotic Pumps.

Alzet mini-osmotic pumps model 2002 (Charles River UK Ltd), were inserted into C57Bl/6 mice weighing at least 20g to infuse angiotensin II (Sigma). Halothane anaesthesia was administered at induction dose via a perspex chamber. When fully unconscious mice were placed prone on a clean table and an area between the scapulae was moistened, shaved and the skin cleaned with an alcohol wipe. A 1cm skin incision

was made and subcutaneous pocket created. The prepared pump was inserted and the skin joined with one surgical clip. Mice were allowed to recover and analgesia was administered orally (figure 2.3).

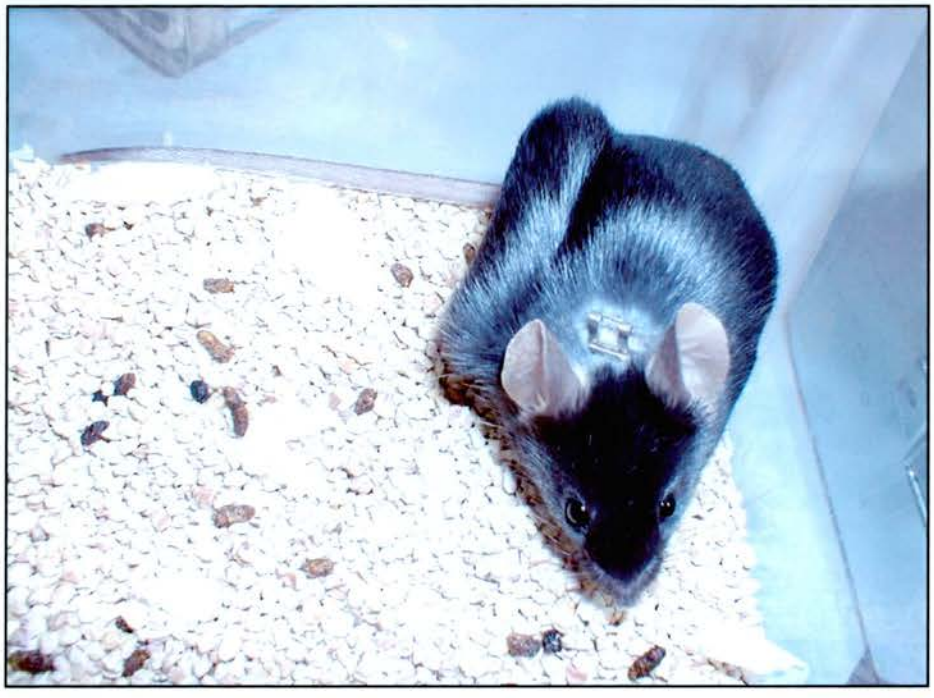

Figure 2.3. Alzet 2002 Miniosmotic Pump In Situ.
Mice behaved normally post insertion and were able to move freely.

Pumps were prepared as follows using sterile technique: The correct concentration of angiotensin II used was sufficient to fill each pump (200µl) allowing for a delivery rate of 0.5µl/hr +/- 0.2µl/hr (variation per pack) at 37^{0}C. Angiotensin II was diluted in sterile 0.9% saline. 0.9% saline was used in control pumps. Pumps were overfilled and the moderator inserted to remove trapped air and prevent leakage from the insertion

site. To allow immediate delivery of drug from the time of insertion, pumps were incubated in sterile 0.9% saline at 37^{0}C overnight.

2.2.4 Urine Collection.

Timed urine collections for estimation of renal function were achieved by placing the animals in metabolic cages (Techniplast 3700M071). Urine was collected at least 48 hours after exposure to these new conditions to allow acclimatisation. The total volume of the resulting urine was measured and 2ml retained and stored at 4 or –20°C prior to analysis.

2.2.5 Intra-Peritoneal Administration of Drugs.

Rats were given intra-peritoneal injections of BrDU (Sigma) 40mg/kg, twenty-four hours prior to cull if required. BrDU was prepared under sterile conditions to allow the final injection volume not to exceed 0.5ml. Injections were administered into the lower outer quarter of the abdomen using a 1ml syringe and 23-guage needle.

FK506 (a kind donation from Fugisawa Corporation) at a dose of 1mg/kg/day was prepared under sterile conditions and injected as above. The compound is insoluble in water and was suspended in sterile distilled water prior to injection.

2.2.6 Euthanasia.

At the end of a study period, the animals were culled individually by a rising partial pressure of carbon dioxide within a chamber. The animal was then removed and death ensured by cervical dislocation. With the animal positioned supine the coat was smoothed with water and a midline incision made from lower abdomen to high thorax, exposing the abdominal and thoracic cavities. Blood was removed via cardiac puncture using a 21-guage needle and 5ml (rats) or 1ml (mice) syringe. This was divided into two 1.5ml microfuge tubes one containing (0.1 volume) 20µl 0.5M EDTA, pH 8 per ml of whole blood, mixed and kept on ice for full blood count analysis and the other allowed to clot on ice, spun at $13X10^3G$ for five minutes at 4^0C and the serum removed and stored at -80^oC for biochemical analysis. If required a sample was collected into 0.5M EDTA and inhibitor mix on ice, spun at $13X10^3G$ for five minutes at 4^0C and the supernatant removed for RAS assay (see below). The kidneys were removed, decapsulated, halved longitudinally and each half placed in either 10 ml 10% formalin buffered saline (Sigma) or methacarn (six volumes of methanol to three volumes of chloroform to one volume of acetic acid, freshly made) for a maximum of twenty hours, depending on size, at room temperature. Kidneys were then dehydrated and paraffin embedded for sectioning later. Another half was placed cut face down in a tissue mould containing OCT (frozen tissue matrix, Sigma), rapidly frozen on dry ice and stored at -80^oC for cryo-sectioning. Sections of small bowel with associated mesentery was removed, cleaned with normal saline to avoid sectioning artefact and fixed in formalin or methacarn prior to dehydration. Additionally pieces of mesentery,

without associated bowel, were frozen in OCT and stored at -80^{0}C for cryosection. The heart was removed, drained of blood, cut longitudinally and formalin-fixed as above. Hearts were either weighed whole or following dissection of the left ventricle. Two 5mm cubes of liver were snap frozen and stored at –80^{0}C for RNA extraction. The brain was removed last with the animal placed on its front, by incision into the base of skull. Strong scissors were used to open the skull via two lateral incisions towards the orbits. With the brain exposed, careful dissection was performed to free the brainstem and optic nerves and the brain removed whole and fixed in formalin for 48 hours or snap frozen for nucleic acid extraction later. Brain tissue was processed and examination by Prof. A. Lammie, Cardiff University.

The left tibia was removed and measured in mm.

2.3 Histology.

2.3.1 Preparation Of Sections.

Paraffin sections were prepared as follows: Fixed tissues were individually placed in labelled tissue cassettes and dehydrated sequentially in industrial methylated spirits (70%, 90% and two 100% baths), 100% ethanol, three xylene baths and then immersed in paraffin wax (two baths at 58^{0}C) using an automated tissue processor overnight (Shandon Citadel 2000). Each tissue was mounted using heated forceps at a paraffin embedding centre (Raymond Lamb Blockmaster) dispensing wax at 58^{0}C and then cooled to solidify. Paraffin blocks were stored at 4^{O}C prior to cutting.

4μm sections were cut using a Bright 5040 microtome and floated onto a water bath at 45^{0}C. Sections were picked up onto labelled plain or poly-L-lysine coated slides for immunostaining (Sigma-Aldrich, UK), allowed to drain then dried fully overnight at 37^{0}C. Slides were stored at 4^{0}C until use.

For frozen sections the insulating block was removed from the cryostat (Bright, containing 5040 microtome) and the temperature set to –20^{0}C. Tissue was removed form –80^{0}C store, the case was discarded and tissue adhered to a pre-chilled cryostat chuck using OCT and rapid freeze spray (Cryospray, Bright). The tissue and chuck were allowed to chill for at least fifteen minutes to allow good adherence of the tissue. After trimming, 6μm sections of tissue were cut and picked up onto plain labelled slides. Slides were stored within the cryostat prior to fixation to reduced drying artefact. Sections were fixed for one minute in 95% ethanol rinsed in tap water, dried and stained or stored at –20^{0}C until use.

2.3.2. Haematoxylin And Eosin Staining.

Paraffin sections were dewaxed by immersing in xylene for ten minutes within a fume hood. This was repeated once. Sections were then hydrated by successive immersions in ethanol (100% twice, then 74% followed by 64%) for two-minute each then finally into tap water. Slides were then immersed in haematoxylin (1:10 dilution Shandon Gill No.2) for between two and five minutes depending upon the intensity of stain required. Slides were then rinsed twice in tap water and immersed in eosin 1% in tap water filtered solution for four seconds then placed into tap water to wash before rapid

dehydration and emersion in xylene prior to pertex mounting. Unlike haematoxylin, eosin is water-soluble and colour continues to leach out of the slides until they are fully dehydrated.

2.3.3. Immunostaining Procedures.

An ABC peroxidase method was used effectively on formalin fixed, paraffin embedded tissues as well as less stringent forms of fixation, for the following primary antibodies (table 2.1).

Sections were placed in a vertical rack and de-waxed (section 2.3.2). Antigen retrieval was carried out by placing the slides, still within a vertical rack, into a microwaveable sealed bowl with lid holes as vents containing 500ml 1mM EDTA, pH 8. Slides were then microwaved from cold at 80% power for three times five minutes with one-minute rests and then cooled for five minutes. The bowl was then placed into a sink and tap water run in until all the antigen retrieval solution was replaced and the slides cold. Care was taken no to let the hot slides dry out. EDTA retrieval alone was used successfully for all of the above antibodies except frozen sections when no antigen retrieval was performed.

The slides and rack were immersed in 3% hydrogen peroxide solution for 15mins to quench endogenous peroxidases. Hydrogen peroxide 3% was re-used for one week and stored at 4^0C. Slides were then washed twice in phosphate buffered saline pH 7.4 for two minutes each time.

ANTIBODY	EPITOPE	D	T	SP	SO
ED-1	macrophage lysosomal glycoprotein	1:50	60	R	S
αSMA	alpha smooth muscle actin	1:50	60	R	SI
CD3	T cell membrane receptor	1:100	30	R	S
CD8	Cytotoxic T cell membrane receptor	1:100	30	R	S
BrDU	5'bromo-3'deoxy-uridine	1:200	40	Any	D
Renin	Rodent prorenin/renin protein	1:500	O/N	R/M	I
CD4*	T helper cell membrane protein	1:100	O/N	R	S
$F4/80^{M}$	Macrophage	1:500	60	M	C
B cell*	B cell	1:40	O/N	R	S
Desmin	desmin	1:50	O/N	R/M	D

Table 2.1 List Of Primary Antibodies Used For Immunostaining.

Formalin fixed paraffin sections were used except ^{M}, methacarn fixed; * frozen section. All primary antibodies were monoclonal, raised in mouse except F4/80 which was a rat anti-mouse monoclonal. D, dilution; T, incubation time in minutes at 37^{0}C; O/N, overnight incubation at 4^{0}C; R, rat; M, mouse; SP, species directed against; SO, source; S, Serotec; SI, sigma; D, Dako; C, Caltag; I, kindly provided by Dr. Tadeshi Inagami, Vanderbilt University, USA.

Normal rabbit serum (Scottish Transfusion Services) diluted 1:5 with phosphate buffered saline pH 7.4 was used as a blocking agent. This solution was also used for dilutions of all primary antibodies. Slides were individually removed from the phosphate buffered saline wash bath and wiped back and around the specimen with a

paper towel. 65µl of rabbit serum 1:5 and one drop of Avidin D (Vector Laboratories) was applied to the specimen, covered with a plastic coverslip and placed in a humidified chamber for twenty minutes. High endogenous biotin was particularly found in kidney sections. The chamber was saturated with 5ml phosphate buffered saline. Slides were then transferred back into a vertical rack for two phosphate buffered saline washes as above.

65µl of primary antibody was applied to individual slides (except control) together with one drop of Biotin (Vector Laboratories) using the coverslip method as above. Incubation times, dilutions and temperatures are given in table 2.1. Slides were then transferred back into a vertical rack for two phosphate buffered saline washes as above.

A secondary biotinylated rabbit anti-mouse IgG (DAKO, UK) at 1:400 dilution in normal rabbit serum 1:5 was applied using the coverslip method (65µl) and incubated for thirty minutes at 37^0C (except control). For F4/80, a biotinylated rabbit anti-rat IgG was used (1:1000, Vector Laboratories). This was followed by two phosphate buffered saline washes.

One drop of Vectastain Elite ABC-peroxidase Reagent (Vector Laboratories) was applied and slides incubated at 37^0C for thirty minutes. This was followed by two phosphate buffered saline washes.

For colour development, DAB (dark brown/black, Boeringher Mannheim) was made up as directed and applied using the coverslip method, approximately 200µl per slide for five to ten minutes. Alternatively, AEC (red, Vector Labs) was used. The incubation time was sometimes longer and was assisted by development in the dark. Colour

development was observed under a microscope via a clear plastic coverslip during development to avoid over staining.

Slides were then rinsed in phosphate buffered saline and immersed in a 1:10 dilution of Shannon Gill No2 Haematoxylin for five to ten minutes depending on freshness. Haematoxylin was made weekly and any metallic scum was removed with a tissue prior to immersing the slides.

For DAB stained slides a permanent dehydrated mount was used. Slides were washes in tap water after counterstaining then sequentially dehydrated through alcohols (75%, 95%, 100%) for two minutes each, and then placed into a clearing xylene and mounting xylene (separate from the initial de-waxing xylenes). Slides were then mounted pertex and allowed to air dry. For AEC, aqueous Glycergel (DAKO,UK) was used to mount directly after staining.

Control slides lacking either primary or secondary antibody were run simultaneously and washed individually.

For double straining, the method from 2.3.3 was followed exactly through to the staining step with DAB/AEC. Then the process was repeated using a second primary antibody. Secondary and tertiary steps remain the same and the stain used is the alternative to the first stain. Counterstaining and mounting are as already described.

2.3.4 Quantification Of Proliferating Cells.

BrDU positive cells were counted on non-perfusion fixed kidney tissue immunostained for BrDU and counterstained with haematoxylin (as above). The vascular compartments quantified were defined as follows:

Endothelial compartment; a single layer of cells internal to the internal elastic lamina and adjacent to the lumen. Medial compartment; cells contained within the external and internal elastic laminae. Adventitial compartment, cells contained within the external elastic lamina and a one media depth circumference external to it (figure 2.4).

The total number of BrDU positive cells was counted using a hand held counter for each compartment and expressed as positive number of cells per section of kidney when an intact longitudinal section was contained on the slide. If the section was incomplete (for example due to poor positioning of the tissue within the wax or sectioning deeper within the block) then positive cells were expressed per vessel. All vessels per section were examined. The labelled ends of slides were masked and coded during counting in order to blind the observer. Counts were repeated using the same method by the same observer on a separate day.

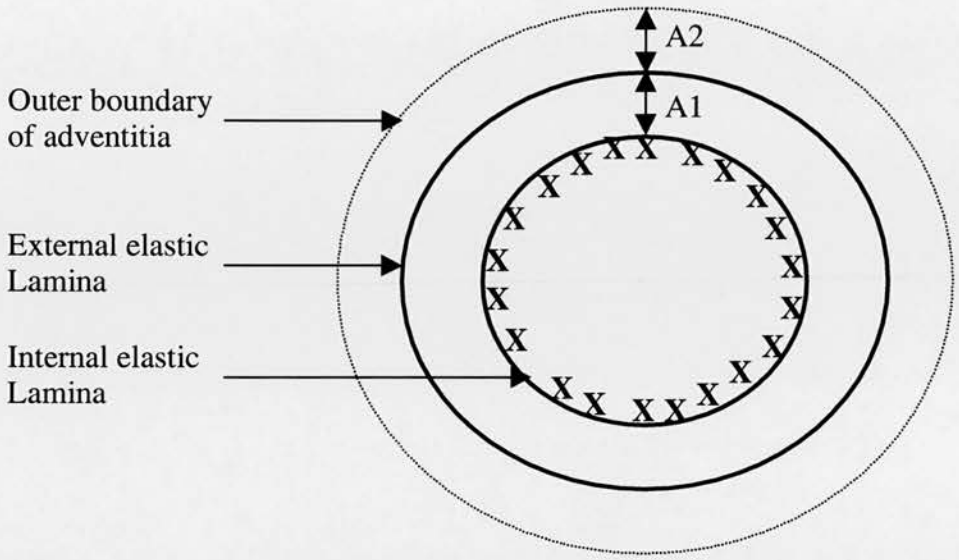

Figure 2.4. Schematic Diagram Of Vascular Compartments.

X, endothelium; A1, medial thickness; A2, adventitial thickness. Distance A1=A2.

2.4 Assays.

2.4.1 Urea And Electrolytes.

Serum was collected as described in section 2.2.6 and stored at -20^{0}C prior to analysis. Samples were relabelled with consecutive numbers for ease and transported on dry ice. Analysis was performed by the biochemistry department at The Sick Children's Hospital in Edinburgh using a Hitachi 911 analyser (Roche). Sodium and potassium were analysed using an indirect ion-selective method. The samples were diluted and the potential across the cell used to calculate the concentration of salt. The sodium electrode consists of a sodium sensitive glass membrane which has silicate ion-

exchange sites. This forms one half of the electrochemical cell, with the reference electrode assembly constituting the complementary half of the cell. The sensing surface of the potassium electrode is a polyvinyl chloride (PVC) membrane containing a potassium sensitive neutral carrier, vanilomycin. This forms one half of the electrochemical cell, with the reference electrode assembly constituting the complementary half of the cell.

The concentration of urea in the sample is directly proportional to rate of NAD^+ formation in the following reaction:

Urea + H_2O reacts with urease to form $2NH_4^+ + CO_2$.

2α-ketoglutarate + $2NH_4^+$ + 2NADH reacts with glutamate dehydrogenase to form 2L-glutamate + $2NAD^+ + 2H_2O$.

NAD^+ is measured by a decrease in absorption at 340nm.

Creatinine was assyed by colourmetric assay utilizing the Jaffe reaction. Briefly, creatinine reacts with picric acid in alkaline conditions to form a red pigment. Bilirubin is oxidised prior to the reaction.

2.4.2 Glucose.

Analysis was performed by the biochemistry department at The Sick Children's Hospital in Edinburgh using a Hitachi 911 analyser on serum samples as above. Glucose is analysed using the following reaction:

Glucose + ATP reacts with hexokinase to form Glucose-6-phosphate + ADP

Glucose-6-phosphate + NAD^+ reacts with glucose-6-phosphate dehydrogenase to form 6 phosphogluconate + NADH + H^+

Although the hexokinase catalyses the phosphorylation of hexoses other than glucose, the glucose-6-phosphate dehydrogenase reaction is specific for glucose-6-phosphate and therefore glucose. The rate of absorbance increases due to the conversion of NAD^+ to NADH and is directly proportional to the glucose in the sample.

2.4.3 Osmolality.

Osmolality was calculated using the following equation:

Osmolality = 2 X (Plasma sodium + potassium) + urea + glucose.

2.4.4 Full Blood Count.

EDTA blood samples stored and transported at 4^0C were analysed at the Department of Haematology, Western General Hospital, Edinburgh. Analysis was based on flow cytometry using a Beckman Coulter GEN.S analyser.

2.4.5 Renin Angiotensin System.

Samples were transported on dry ice to the Department of Pharmacology, University Of Heidelberg, Germany for analysis by Dr J. Peters [Oster et al., 1973; Peters et al., 1996].

Blood for angiotensin II determination was placed onto ice-cold inhibitor mix containing 4mM PMSF, 2.5μg/μl O-phenantrolin, 250mM EDTA pH 8.0, 4% β-mercaptoethanol, 40μg/ml pepstatin and 0.16mg/ml captopril, spun at $13X10^3G$ 4^0C for five minutes and the supernatant removed and stored at -80^0C prior to analysis. Angiotensin II levels were quantified by radioimmunoassay. An EDTA plasma sample was used for active and inactive renin levels. To determine inactive renin (prorenin) the sample was first activated by incubation with trypsin. Substrate was added and the reaction allowed to precede for one hour at 37^0C. The reaction was then terminated and angiotensin I generation determined by radioimmunoassay. Active renin was determined in parallel (without the addition of trypsin) and the value subtracted from the total "inactive" value to deduce the amount of prorenin.

The relative amounts of transgene derived (mouse) and endogenous rat renin were determined by renin assay before and after immunoprecipitation using a mouse specific renin antibody and formalin fixed *Staphlococcus aureus* protein A.

2.4.6 Urinary Creatinine.

Analysis was performed by the biochemistry department at The Sick Children's Hospital in Edinburgh using a Hitachi 911 analyser. The technique was identical to that described for serum analysis except bilirubin was not oxidised prior to the reaction.

2.4.7 Urinary Protein.

Urinary total protein concentration was estimated by protein dye-binding assay using the Microprotein-PR kit (Sigma). The increase in absorbance at 600nm when pyrogallol red-molybdate combines with basic amino acids was measured and is directly proportional to the concentration of protein within the sample. Samples were compared with a standard as per the manufacturers instructions. 4µl of urine was mixed with 200µl of reagent in a 96-well plate and measured after fifteen minutes. Proteinuria was calculated as follows:

Proteinuria (mg/dl) = ((test – reagent blank) / (standard – reagent blank)) X concentration of the standard.

2.4.8 Creatinine Clearance.

Creatinine clearance was estimated using the following calculation:

((urine creatinine mmol/l X urine volume in mls) / plasma creatinine µmol/l) X 1000 / duration of collection in minutes and expressed in ml/min. This was further adjusted for weight and expressed as ml/min/100g.

2.5 Determining Transgene Expression In The Liver And Brain.

RNA was extracted from approximately 200mg of liver or whole brain tissue using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Following

precipitation, RNA was resuspended and incubated with two units of DNase I (DNA-free, Ambion) for 30 minutes at 37^{0}C. 4mg of total RNA was reverse-transcribed using 200 units of SuperScriptII RNaseH$^-$ reverse transcriptase (Invitrogen) and then diluted to 10ng/ml ready for polymerase chain reaction (PCR). Two separate reverse transcription reactions were carried out for each sample, and each was assayed in triplicate wells for the gene of interest, and 18S ribosomal RNA. No RNA and no reverse transcriptase reactions were included as controls. Primers were designed using PrimerExpress 1.5 software (Applied BioScience). 18S ribosomal RNA expression was measured using commercial Taqman 18S Ribosomal Vic probe and primers (Applied Biosystems). Hepatic renin expression was quantitated relative to 18S RNA using Taqman probes and primers that detected both mouse and rat renin: (forward: 5' GGTGCCCTCCACCAAGTG, reverse: 5' AGTGTCGGAGATACTCAGGAGACT, probe (Fam-Tamra): 5' AGCCGCCTCTACCT TGCTTGTGGG).

Real time PCR was carried out using the following standard conditions: 50^{0}C for 2 minutes, 95^{0}C for 10 minutes and forty cycles of 95^{0}C for 15 seconds and 60^{0}C for 1 minute. The reactions were performed using the ABI Prism 7000 Sequence Detection System and analysed using Sequence Detector System software version 1.0 software. 18S and renin assays were performed in separate reaction wells with standard curves from zero to 75ng of RNA template. Renin was expressed as a ratio of 18S from the same sample.

2.6 Statistical Analysis.

Within the text numerical data is expressed as the mean value +/- the standard deviation of the mean. For data represented graphically, error bars represent the standard error of the mean. Animal studies contained six per group unless stated. Results were analysed using InStat software by conventional nonparametric methods. Blood pressure was analysed assuming a normal distribution. All tests were two-sided and a p value of 0.05 or less was taken to indicate significance.

CHAPTER THREE.

THE DEVELOPMENT OF MALIGNANT HYPERTENSION IN THE INDUCIBLE HYPERTENSIVE RAT: CHARACTERISATION AND VASCULAR REMODELLING RESPONSE.

3.1 INTRODUCTION.

Patients with MH often present late with established end-organ injury and as a result, morbidity and mortality remain high [Lip et al., 1995]. Consequently, MH is difficult to study in humans and relies mostly on post mortem data [Akikusa et al., 1983]. The histological injury observed in the resistance vessels of the IHR closely resembles that of the human condition developing de novo and accordingly, is a good model with which to study this form of vascular remodelling [Fujimoto, 1978; Kadiri and Thomas, 1993; Kantachuvesiri et al., 2001; Sinclair et al., 1976]. In man, renal failure, cardiac infarction and stroke, result from end-organ ischaemia as a direct consequence of occlusive vascular injury affecting resistance vessels throughout the body and the vascular remodelling events that occur early in the development of MH, predating fibrinoid necrosis, are of particular interest, but remain poorly defined.

Good MH models exist including the stroke prone spontaneously hypertensive rat and Dahl salt sensitive rat strains [Hampton et al., 1989; Ogata et al., 1982] and renovascular models such as the Goldblatt 1C2K model and aortic ligation model. However, the former are often on out-bred backgrounds and lack the controllability and

tight reproducibility of pathology required to investigate events occurring early in vascular remodelling and in the latter, the onset of hypertension is controlled, but the rate of rise of blood pressure varies depending on the surgical technique and rat stain used. Consequently, despite studies describing vascular pathology in detail, there is little detail of the period before vascular injury becomes established [Chatelain et al., 1983].

The controllable properties of the IHR have been used here to investigate the early stages of vascular remodelling. Transgene induction using dietary 0.3% indole-3-carbinol (I3C) for two-weeks is known to generate a severe hypertensive phenotype [Kantachuvesiri et al., 2001]. Fibrinoid necrosis develops in the mesenteric, renal and cardiac circulations and animals loose weight and display other features typical of MH such as polyuria and lethargy.

I3C has been established as a rapidly metabolised, relatively non-toxic inducer of *Cyp1a1* expression known to exist naturally as a component of cruciferous vegetables [Kantachuvesiri, 1999; Stresser et al., 1995]. I3C and other lipophilic compounds such as β-naphthoflavone and tetrachlorodibenzo-ρ-dioxin are capable of binding transcription factors containing the constitutively expressed arylhydrocarbon receptor (AhR), resulting in *Cyp1a1* transcription [Elferink and Whitlock, 1990]. In addition to *Cyp1a1*, *Cyp1a2* and *Cyp1b1* are also regulated by the AhR system, however, only *Cyp1a1* is not constitutively expressed in the liver, making it a suitable inducible system [Shen et al., 1994; Sutter et al., 1994]. A number of unrelated studies have explored the potential anti-carcinogenic effects of I3C, possibly mediated by inactivation of endogenous oestrogens in the setting of oestrogen dependant tumours,

although these anti-carcinogenic effects remain controversial [Bradlow et al., 1991; Kim et al., 1994; Nixon et al., 1984]. However, these studies required long-term administration of I3C and did not result in significant toxicity. In addition to its' rapid metabolism, I3C is converted in the acid environment of the stomach into a series of breakdown products which are potent AhR agonists, hence it is highly effective as an oral agent [Bjeldanes et al., 1991; Bradfield and Bjeldanes, 1991].

A series of timed inductions were carried out using adult IHR and Fisher F334 rats as a control population, for between two and 14 days, in order to study the development of hypertension and pathology in the IHR model and extend our knowledge of end-organ responses in this model.

3.2 EXPERIMENTAL DESIGN.

Fisher F334 and transgenic IHR adult male rats approximately 12 weeks of age, were induced with 0.3% w/w dietary I3C for two, four, seven, nine, 11 and 14 days to span the period up to and including the onset of well defined vascular injury. The number of animals per group was between five and six.

Twenty-four hours prior to cull, animals were injected with intra-peritoneal 5'bromo-2'deoxyuridine (BrDU) at standard dose to mark S-phase events during the last day of life. Urine volumes for the final 24 hours were recorded and aliquots assayed for creatinine clearance and proteinuria.

At cull, blood was taken for creatinine and RAS assays and body weight, left kidney and heart weights were recorded. Tissues were fixed and processed for histology and since the vascular remodelling observed in MH is principally proliferative, vascular proliferation events were counted.

Blood pressure was recorded telemetrically in a separate two-week experiment with a group size of four.

3.3 RESULTS.

3.3.1 Clinical Outcome Following Two Weeks Of Induction.

There were no deaths in any group during the experimental period and Fisher F334 rats consuming I3C, used as controls, remained well throughout the maximum two-week induction period. The IHR group visibly lost weight and coat condition from day nine onwards. Transgenic rats consumed less food from day three onwards and displayed reduced movement beyond day twelve. From day ten, transgenic rats developed obvious polyuria indicated by requiring more frequent bedding changes. The sleep pattern of both groups was preserved with periods of sleep during the day observed in transgenic and non-transgenic groups.

All non-transgenic animals were normal at post-mortem examination. At sacrifice there was loss of the mesenteric and peri-nephric fat pads in the IHR group visible on opening the abdomen. In three transgenic rats, patechei were seen on the sub-capsular surface of the kidney suggestive of micro-infarction. In two transgenics, regions of cardiac pallor overlying the right ventricle could be seen in keeping with infarction. Longitudinal transection of the hearts of transgenic rats revealed concentric cardiac enlargement affecting the left ventricle and intra-ventricular septum. The gut, brain and other organs were macroscopically normal. These observations are in keeping established MH and similar to those reported in other animals including humans.

3.3.2 Severe Hypertension Develops Predictably In The IHR.

Fisher F334 rats remained normotensive throughout the experimental period with a mean systolic pressure of 121 +/- 12.6mmHg (figure 3.1a). There was little intra-group variation between blood pressure or diurnal variability in blood pressure, heart rate and activity (figures 3.1a-c).

As previously documented, blood pressure rises between two to seven days after the introduction of dietary I3C into the IHR diet. Beyond seven days a plateau was reached of 182 +/- 19.1mmHg systolic (figure 3.1a) [Kantachuvesiri et al., 2001]. Variation of both the rate of development and the plateau phase of hypertension between transgenic group members was low. This is likely to be a consequence of the inbred nature of the IHR line in addition to consistency of the experimental conditions.

Diurnal rhythm of blood pressure, heart rate and activity were maintained in the transgenic group despite the development of severe hypertension although the amplitude of blood pressure recordings was increased (figures 3.1a-c). Heart rate in the IHR group increased in week two when hypertension was established (374 +/- 4.8bpm on day one of induction vs. 392 +/- 4.5bpm on day 14 of induction, $p<0.02$). The IHR group displayed greater nocturnal activity then the control group even prior to induction and this reduced as hypertension developed (figure 3.1b). This late reduction in activity would be expected in ill animals, but the explanation for the enhanced baseline activity of the transgenic group is unclear. As expected, maximal activity, heart rate and blood pressures were recorded nocturnally in both groups.

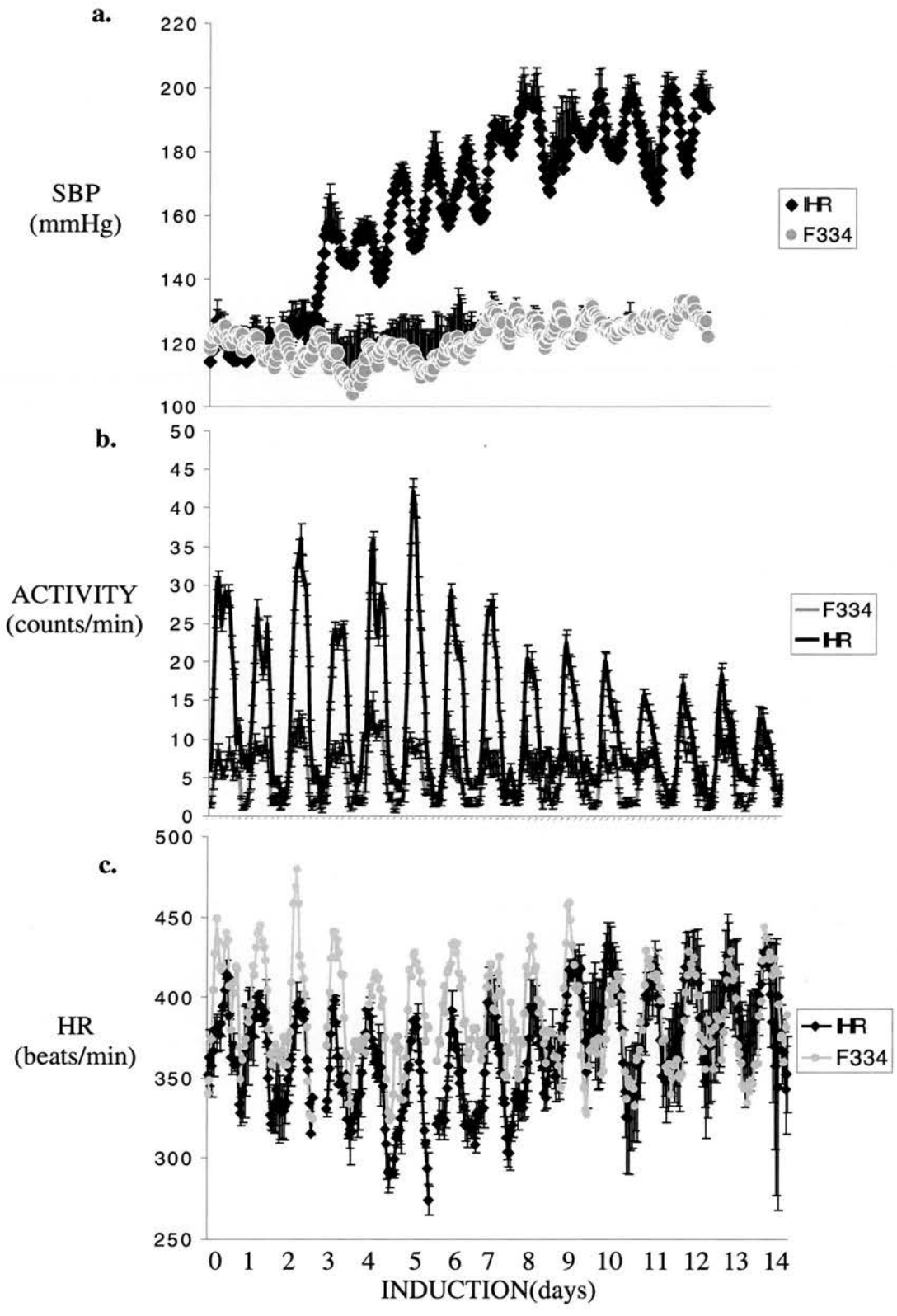

Figure 3.1 Telemetric Data During Dietary 0.3% w/w Indole-3-Carbinol Induction.

Systolic blood pressure (SBP) recordings (figure 1a), activity recordings (figure 1b) and heart rate (HR, figure 1c) during transgene induction. Data is expressed as four hour moving averages +/- standard error of the mean (n=4 except heart rate where only one control animal is shown). IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

3.3.3 End-Organ Response.

3.3.3.1 Cardiac Hypertrophy Accompanies Hypertension.

Transgenic rats progressively lost weight over the two-week induction period compared to Fisher F334 rats who gained weight (-21 +/- 6.1 vs. 5.6 +/- 6.7% of their starting body weight at fourteen days, p=0.0043, figure 3.2). In part this was explained by reduced food intake in the transgenic group but catabolism, associated with an illness state, is also likely to have contributed.

Cardiac hypertrophy, principally involving the left ventricle, developed with sustained hypertension of seven or more days in the IHR (0.376 +/- 0.01 vs. 0.308 +/- 0.013 heart weight : end body weight ratio day seven, p=0.0022, 0.416 +/- 0.019 vs. 0.298 +/- 0.011 heart weight : end body weight ratio day 14, p=0.0043, figure 3.3). When left kidney weight was compared to end body weight no significant difference between the groups was found suggesting that the cardiac weight increase was an organ specific response (figure 3.4).

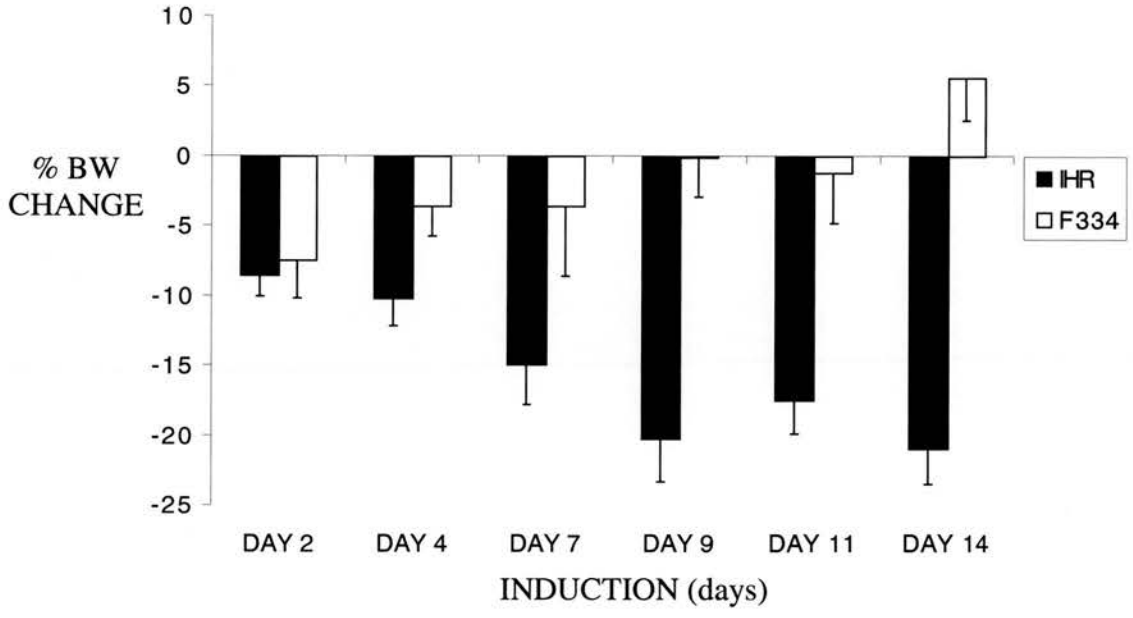

Figure 3.2. Weight Loss During The Development Of Malignant Hypertension.

Percentage body weight (BW) change over two weeks of transgene induction. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

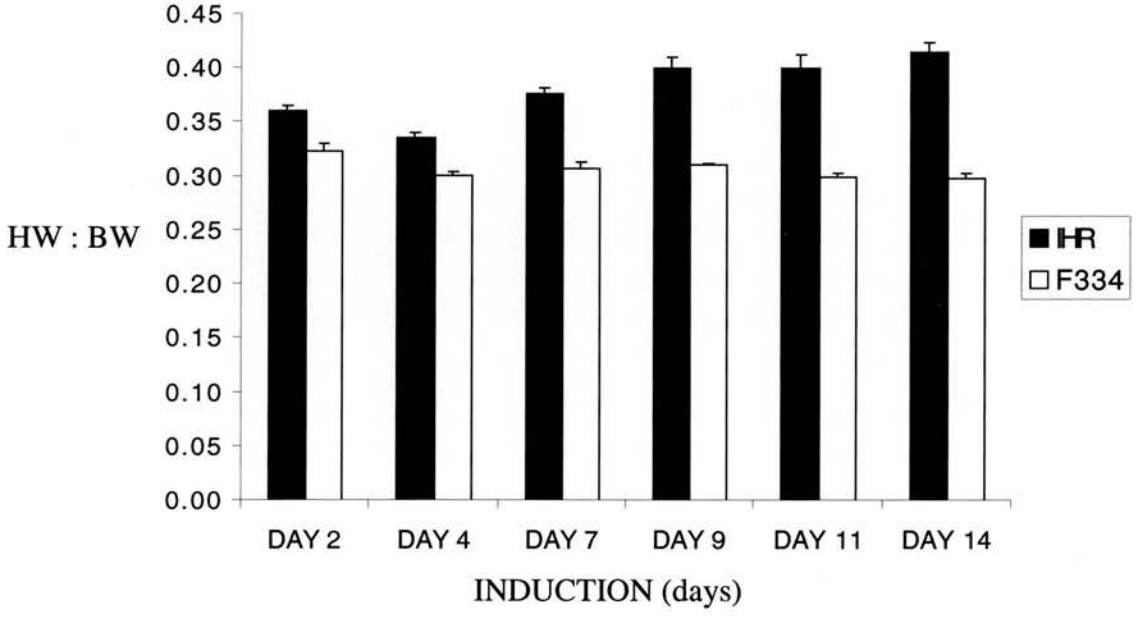

Figure 3.3. Cardiac Hypertrophy Develops In Malignant Hypertension.

Heart weight to end body weight ratio (HW:BW) during transgene induction from two to fourteen days. Cardiac hypertrophy is significant from day seven onwards in the IHR group. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

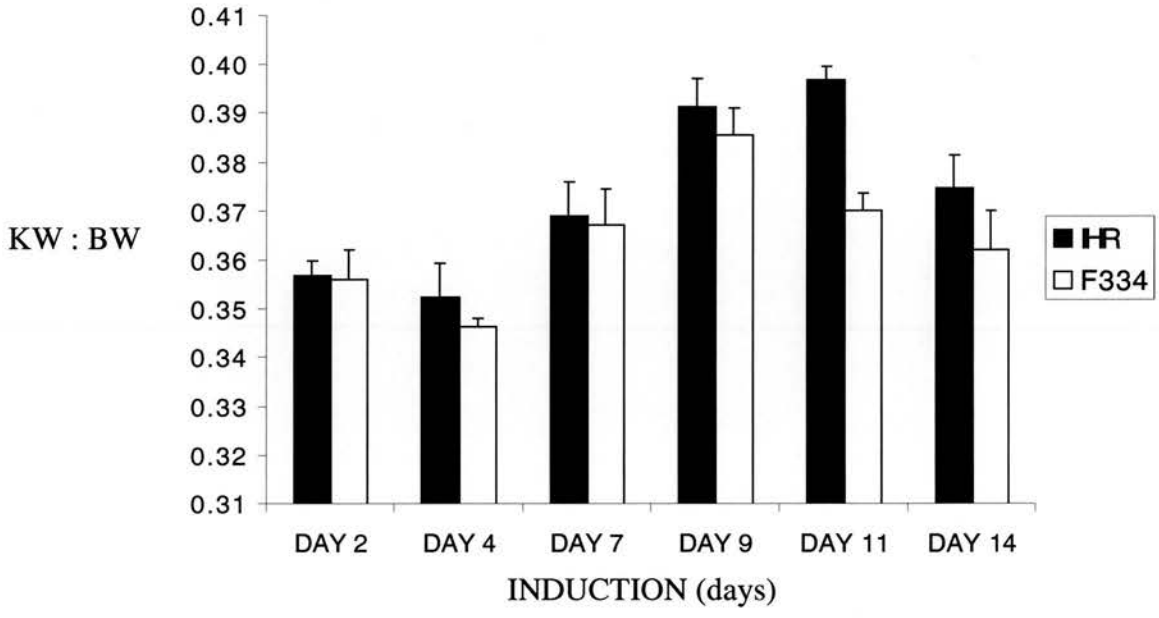

Figure 3.4. Hypertension Has No Effect On Renal Weight.

Left kidney weight to end body weight ratio (KW:BW) during transgene induction from two to fourteen days. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

3.3.3.2 Renal Function Was Well Preserved In The IHR.

With the onset of hypertension, transgenic animals developed polyuria similar to humans with severe hypertension although this did not reach statistical significance by day fourteen (0.58 +/- 0.5 vs. 0.12 +/- 0.04 mls/hour/100g, p=0.056, figure 3.5). In some cases volumes in excess of 40ml per rat were recorded over 24 hours. Water consumption increased accordingly.

Creatinine clearance was not affected over the time period studied (figure 3.6) and despite marked vascular injury within the kidneys of IHRs, the glomeruli and bulk of the tubulointerstitium remained histologically intact. Transgenic animals did develop significant proteinuria (0.036 +/- 0.02 vs. 0.008 +/- 0.01 mg/ml/g, p=0.047) after two

weeks of induction compared to controls consistent with mild renal dysfunction or occurring as a consequence of high glomerular filtration pressure and polyuria (figure 3.7).

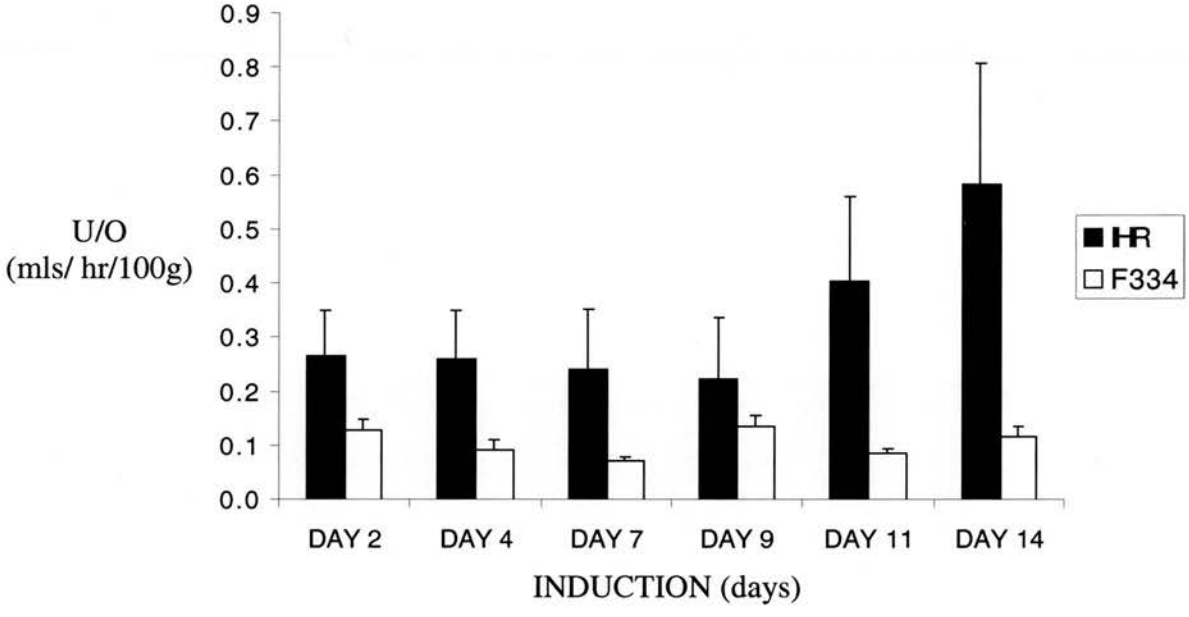

Figure 3.5. Polyuria Develops During Malignant Hypertension.

Weight adjusted urine output (U/O) during transgene induction from two to fourteen days. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

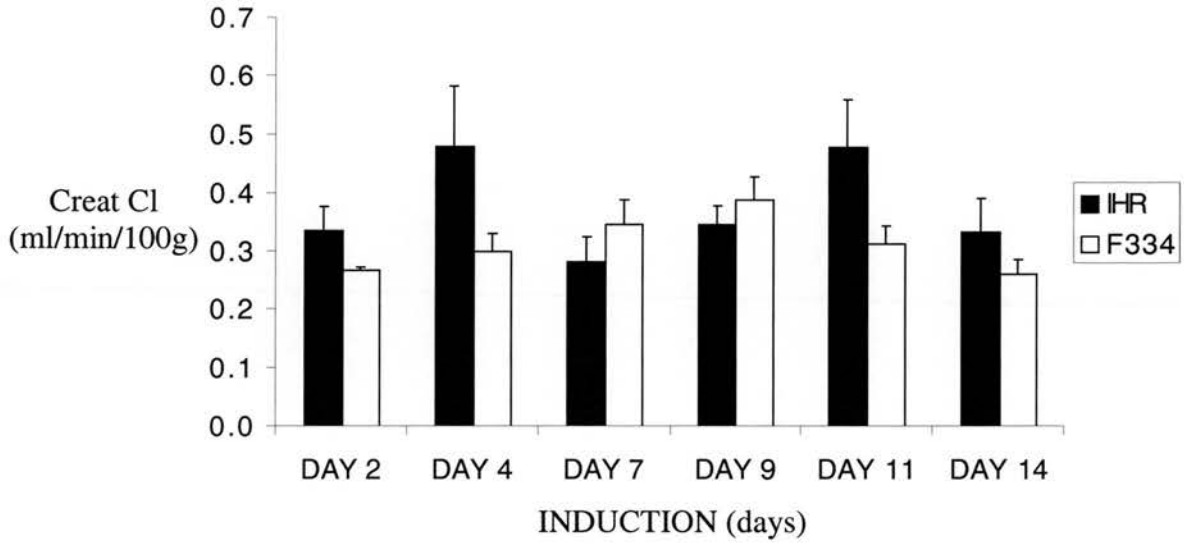

Figure 3.6. Creatinine Clearance Is Not Affected By The Development Of Malignant Hypertension.

Weight adjusted creatinine clearance (Creat Cl) during transgene induction from two to fourteen days. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

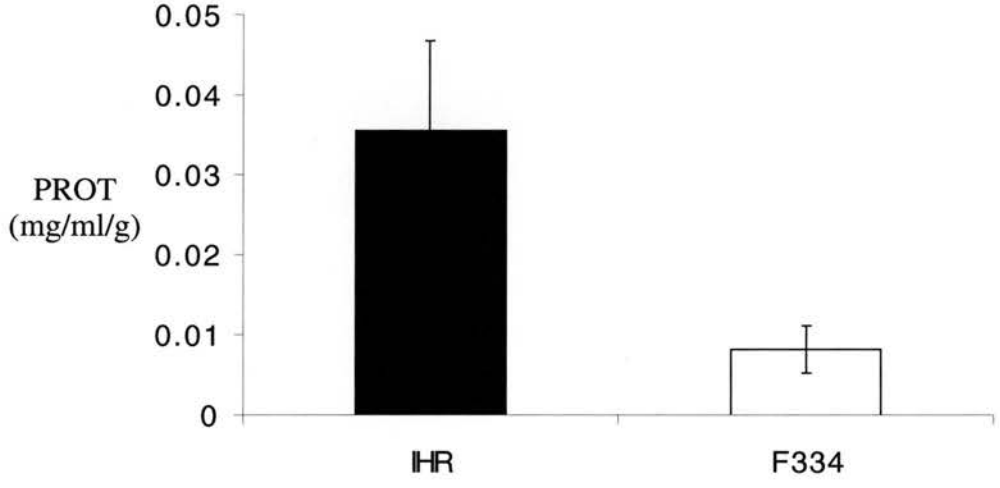

Figure 3.7. Proteinuria Develops After Fourteen Days Of Transgene Induction.

Proteinuria (PROT) adjusted for weight and volume after fourteen days of transgene induction is significantly greater in the IHR group. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

3.3.4 Transgene Induction And The Renin Angiotensin System.

All components of the RAS tested were elevated in the IHR group: Plasma prorenin was elevated 10^3-fold in the IHR group compared to controls from day two onwards (7614 +/- 3647 vs. 6.5 +/- 5.2 ngAngI/ml/hr on day two, p=0.002, figure 3.8). Despite the large elevation in plasma prorenin, circulating active renin in the IHR was only twice the control group level by day eleven (95.67 +/- 7.23 vs. 45.6 +/- 9.63 ngAngI/ml/hr, p=0.036, figure 3.9). This was estimated as predominantly transgene derived for the IHR group by immunoprecipitation using a mouse specific antibody (84 vs. 7 % mouse renin on day eleven, p=0.036). A similar elevation in angiotensin II was recorded which failed to reach significance (280 +/- 101.8 vs. 69.2 +/- 17.4 ng/ml, p=0.09, figure3.10).

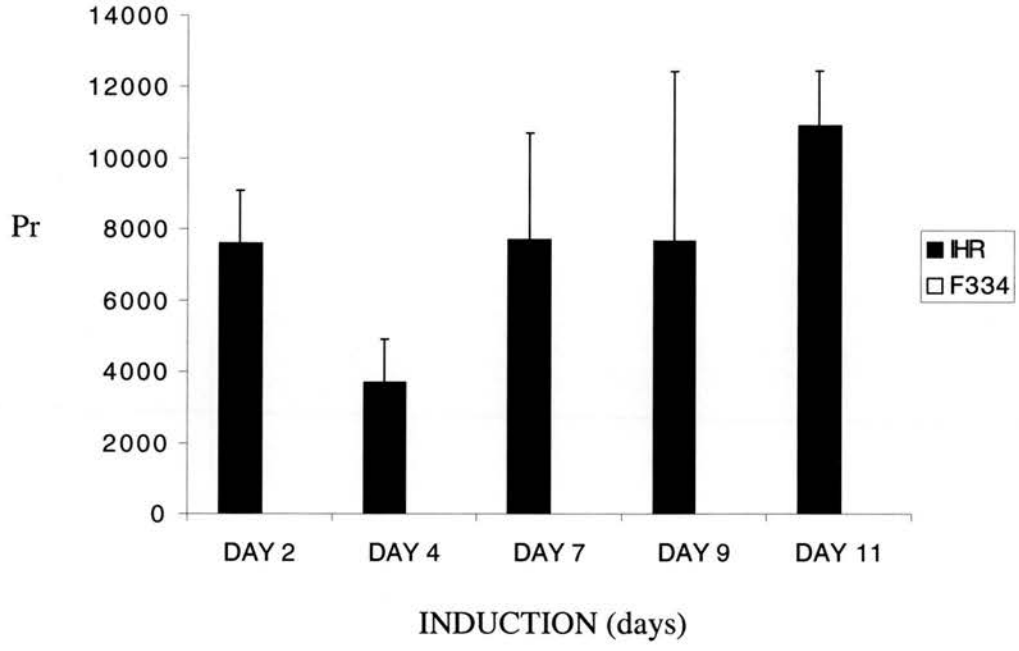

Figure 3.8. Circulating Prorenin Increases By Day Two Of Transgene Induction.

Plasma prorenin (Pr) in ng AngI/ml/hr during transgene induction from two to 11 days. Massive increases over Fisher F334 rats were observed in the IHR group. Values for the Fisher F334 group are not visible on the scale used. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

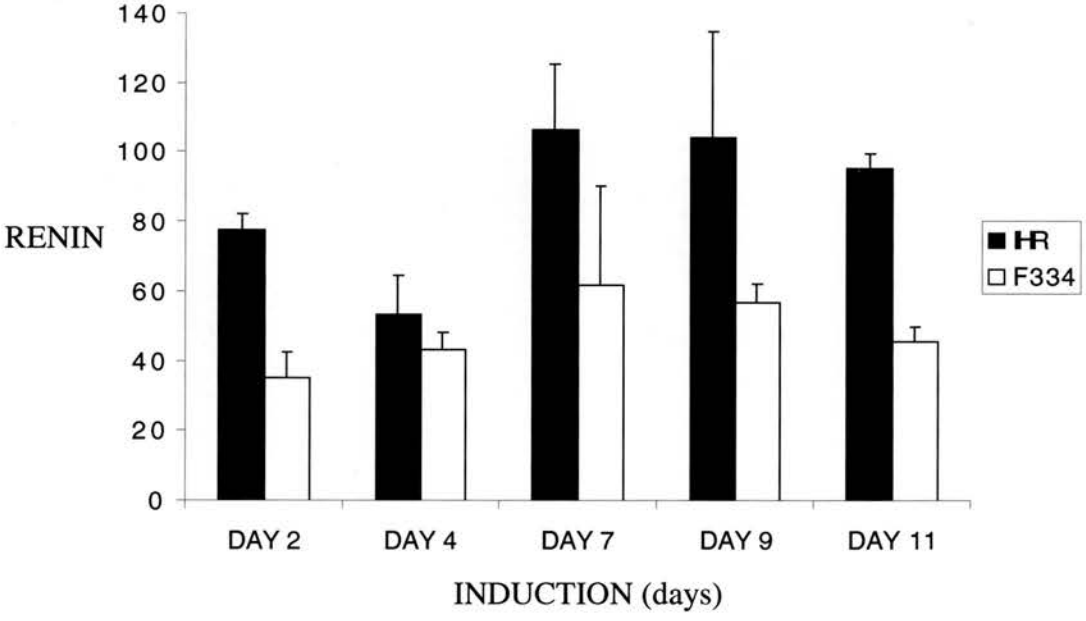

Figure 3.9. Circulating Renin During Transgene Induction.

Plasma active renin in ng AngI/ml/hr during transgene induction from two to 11 days. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

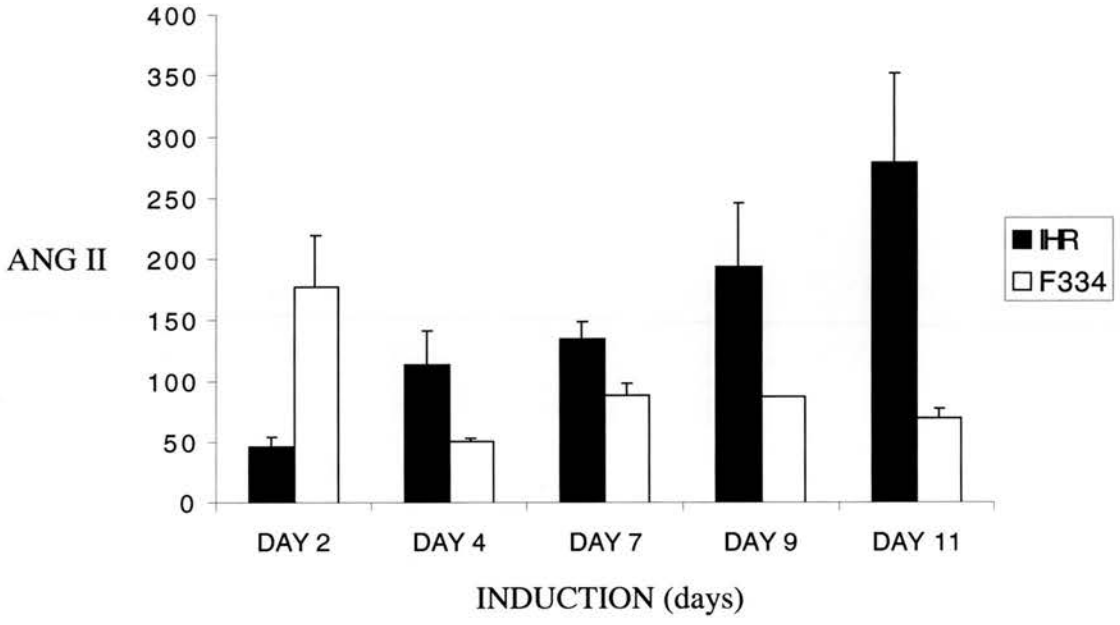

Figure 3.10. Circulating Angiotensin II During Transgene Induction.

Plasma angiotensin II in ng/ml during transgene induction from two to eleven days. IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

Renin staining was seen throughout the kidney within juxta-glomerular cells in the IHR and Fisher F334 rats prior to induction (figures 3.11a and 3.11b). The pattern and intensity of immunohistochemical staining for renin was unaltered by I3C in Fisher F334 control rats. In the IHR, renin staining was absent by day seven of induction and re-emerged weakly on day fourteen (figures 3.11c and 3.11d). These positively stained regions were often in association with areas of afferent arteriolar fibrinoid necrosis, suggesting that renin synthesis was triggered by reduced blood flow in the region of the juxta-glomerular apparatus (figure 3.11d). The suppression of endogenous renin by detection of protein and RNA has been described previously [Kantachuvesiri et al., 2001].

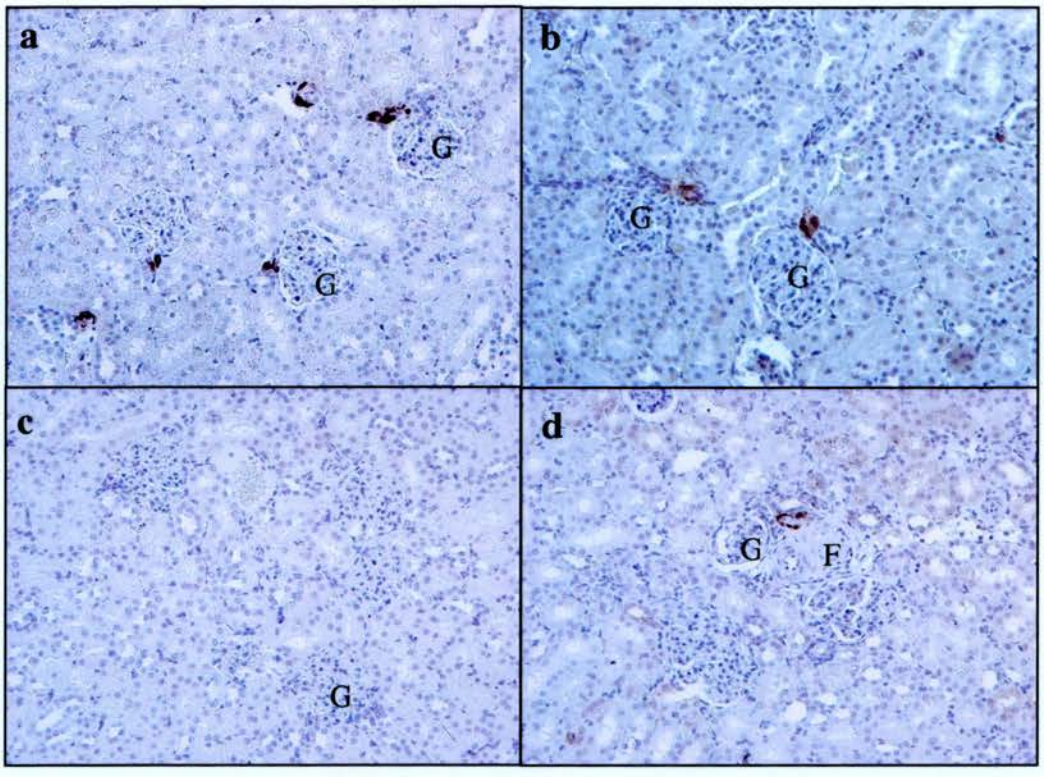

Figure 3.11. Renin Immunostaining.

Renin staining (black) with haematoxylin counter stain. Renin positive cells are seen throughout the cortex of Fisher F334 (a) and transgenic rats prior to induction (b). Seven days post induction no renin protein is detected in the IHR (c). 14 days post induction areas of weak staining are seen in association with fibrinoid necrosis (F, d). G, glomerulus.

3.3.5 Histology.

All organs from Fisher F334 rats after fourteen days of dietary I3C and transgenic rats prior to induction were histologically normal. Fibrinoid necrosis and proliferative endarteritis were present by day seven in the mesentery and heart and by day fourteen in the kidney of transgenic rats. The brain did not undergo vascular remodelling visible

with H&E staining within the two-week period. Histology images of the principal organ affected are shown after fourteen days of induction (figure 3.12).

Differential organ injury has been previously observed in this model and attributed to renovascular and cerebrovascular autoregulation [Kantachuvesiri et al., 2001].

Prior to the onset of fibrinoid necrosis, the only abnormality visible on H&E staining was medial thickening. As discussed later, this is due to vascular smooth muscle cell hyperplasia. The volume of individual vascular smooth muscle cells was not estimated and hence the degree to which hypertrophy contributes to medial enlargement cannot be assessed. Similar to human MH pathology, resistance vessels of around 200μm were most severely affected, with larger vessels being spared (figure 3.12b).

Within the kidney, all pre-glomerular vessels were affected from inter-lobular arteries to afferent arterioles however, those within the renal pelvis were most severely affected suggesting that vessels subject to higher blood pressure under went greater remodelling. No glomerular abnormalities were observed but within the tubulointerstitial compartment patchy distal tubular proliferation, tubular atrophy and necrosis were seen. Distal tubular proliferation may be a response to high circulating aldosterone levels that have been found in this model [Kantachuvesiri, 2000]. The other tubular responses are likely to have occurred as a consequence of ischaemia. Additionally regions of infarction were noted in association with vascular obliteration. Large regions of the interstitium remained intact explaining the preserved renal function in these animals.

Grade IV mesenteric vessels (those closest to the gut wall) were principally affected in the gut (figure 3.12d). No areas of gut infarction were seen probably due to the extensive collateral circulation supplying the gut.

The right ventricle was more severely affected than the left displaying large areas of myocardial necrosis sometimes as early as day four post induction (figure 3.12h). The left ventricle was relatively mildly affected even by day 14 and myocardial necrosis was unusual (figure 3.12f).

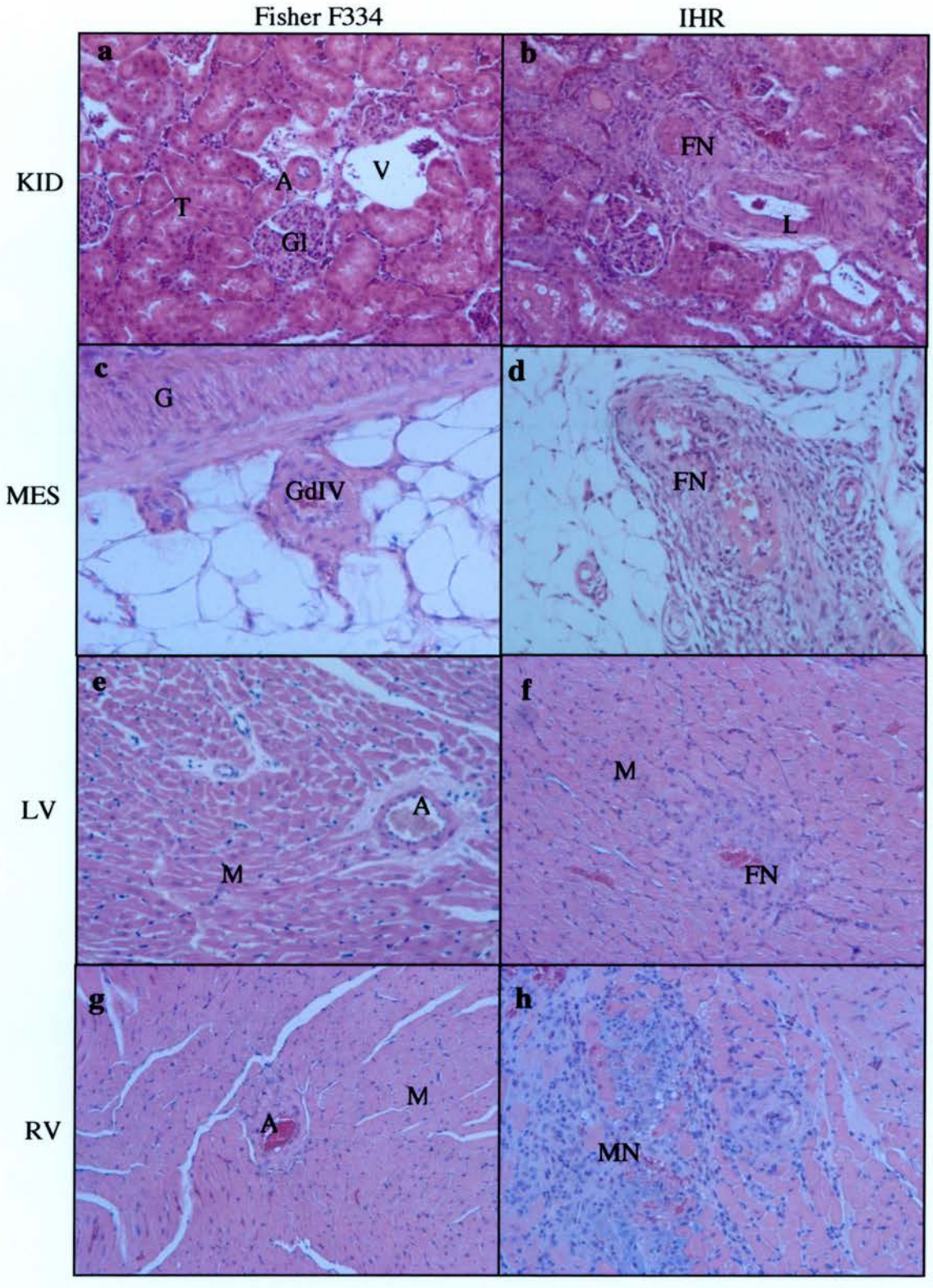

Figure 3.12. H&E Stained Histology After Fourteen Days Of Transgene Induction. Non-transgenic rats have normal histology. Fibrinoid necrosis (FN) develops in transgenic rats. Resistance vessels are principally affected, larger vessels escape injury (b). FN and associated myocardial necrosis is more severe in the right than left ventricle. KID, kidney; MES, mesentery; LV, left ventricle; RV, right ventricle; A, normal artery; L, large interlobular artery; V, vein; Gl, glomerulus; T, tubulointerstitium; GdIV, grade IV mesenteric vessel; F, fat; G, gut; M, normal myocardium; MN, myocardial necrosis.

3.3.6 Changes In Proliferation And Inflammation Precede Fibrinoid Necrosis In The Kidney.

3.3.6.1 Proliferation Precedes The Onset Of Fibrinoid Necrosis.

A low, baseline rate of vascular proliferation was observed within the kidneys of non-transgenic rats and transgenic rats prior to induction. BrDU immunostaining of the kidney, revealed two discrete waves of proliferation occurring in transgenic rats during induction (Figures 3.13 & 3.14). The first began within the adventitia on day two (1.39 +/- 3.08 vs. 0.28 +/- 0.45 positive cells per vessel, $p = 0.02$) and was maximal by day seven (5.43 +/-6.58 vs. 0.27 +/- 0.4 positive cells per vessel, $p < 0.0001$) and the second began within the media on day seven (2.88 +/- 8.92 vs. 0.02 +/- 0.15 positive cells per vessel, $p < 0.0001$). Prior to day seven, there was no increase in S-phase events within the medial or lumenal compartments of transgenic animals. There was a small increase in lumenal proliferation events on day seven (0.31 +/- 0.92 vs. 0.02 +/- 0.15 positive cells per vessel, $p = 0.046$) and proliferation was maximal in all vascular compartments on day seven in the IHR (figure 3.13).

The changes on day two and seven predated the onset of any histological abnormality on H&E staining within the kidney. As stated above the IHR kidney was histologically normal until day seven, beyond this medial thickening was visible and fibrinoid necrosis found on day 14.

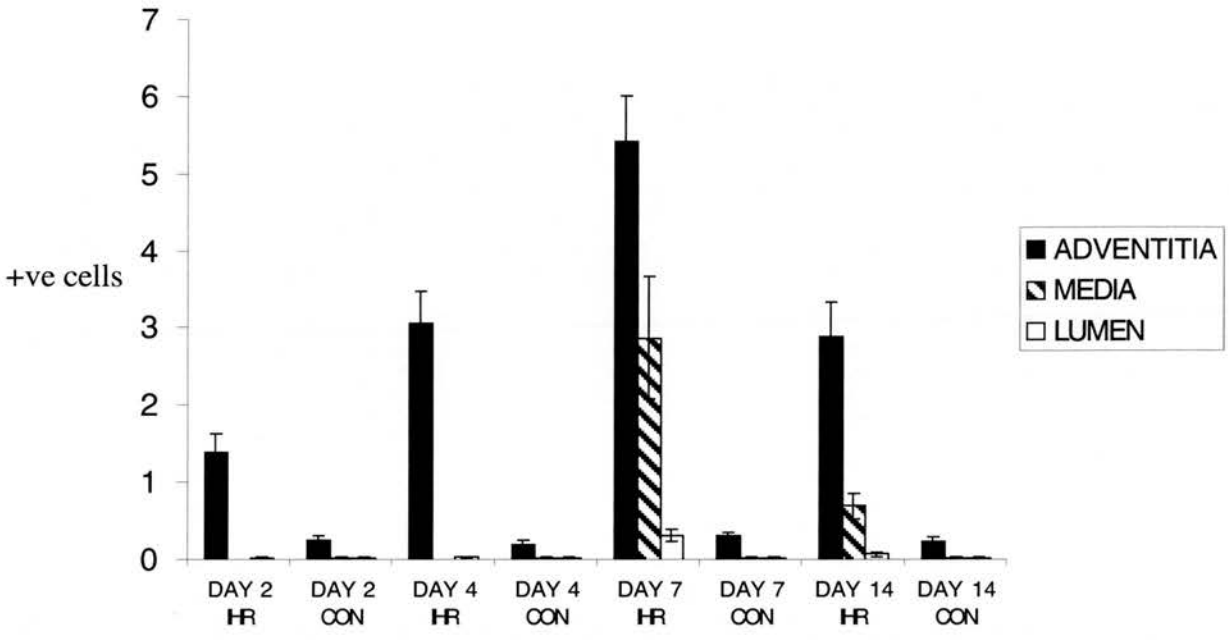

Figure 3.13. Vascular BrDU Immunopositivity Within The Kidney Prior To The Development Of Malignant Hypertension.

Cells identified after immunostaining with anti-BrDU antibody, DAB peroxidase detection and haematoxylin counter staining were counted within the adventitial, medial and endothelial compartments. Transgenic rats have an increase in adventitial cell proliferation from day two and medial and lumenal cell proliferation from day seven compared to non-transgenic controls. IHR, Inducible Hypertensive Rat; CON, Fisher F334 control; +ve cells, positive cells per vessel.

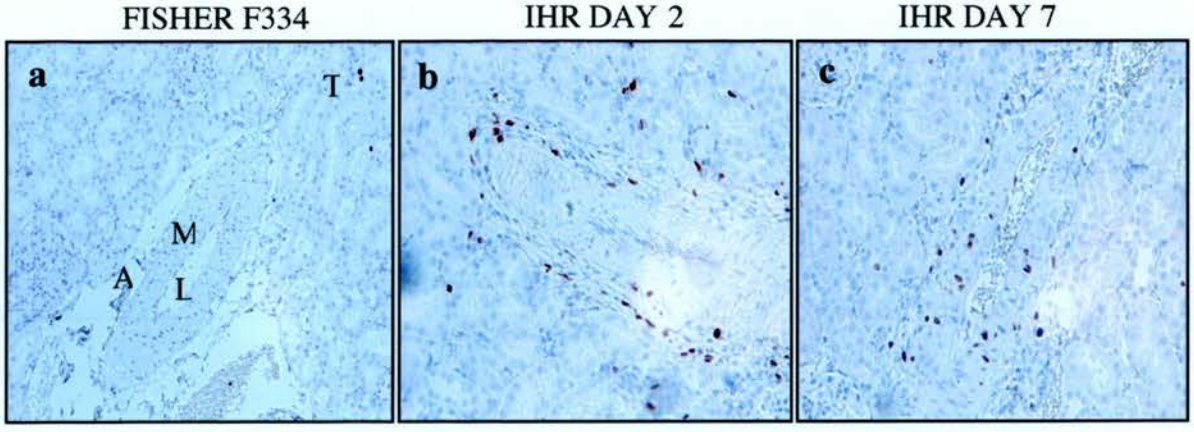

Figure 3.14. Representative Photographs Of BrDU Immunostaining Prior To The Development Of Fibrinoid Necrosis In The Kidney.

BrDU immunostaining (black) of the kidney showing a low level of baseline proliferation within the adventitial (A), medial (M) and lumenal (L) compartments of Fisher F334 rats (a). Two tubular cells are seen having recently divided (T). Adventitial proliferation is seen after two days of induction in the IHR group (b). No proliferation events are seen in the media on day two but are marked by day seven (c). Vessels appear otherwise normal.

3.3.6.2 Determining The Proliferating Cell Type.

Immunostaining with antibodies directed against a variety of cellular antigens, in conjunction with double staining for BrDU or serial sections when staining techniques were incompatible, were employed to identify the proliferating cell type. Adventitial BrDU positive cells stained positive for desmin and BrDU on serial sections consistent with a fibroblast phenotype (Figure 3.15). The adventitial space normally contains fibroblasts and it is therefore likely that these local cells begin to proliferate and are seen in greater numbers in the IHR following induction.

Within the media of transgenic rats, α-smooth muscle actin co-localised with BrDU on day seven of induction consistent with these cells being vascular smooth muscle in origin (figure 3.16).

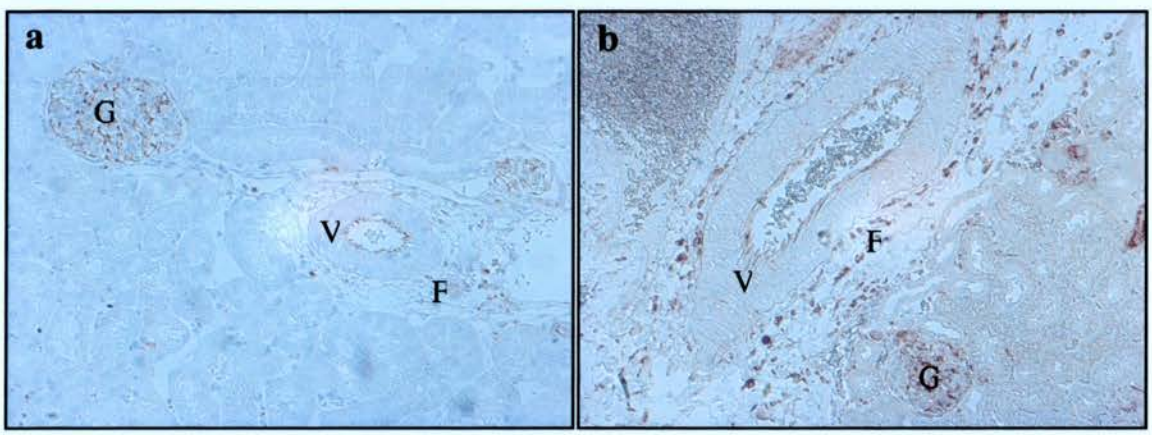

Figure 3.15. Adventitial Fibroblasts Proliferate After Two Days Of Transgene Induction In The IHR.

Fisher F334 kidney illustrating normal desmin staining (brown) within the glomerulus and adventitia (a). Adventitial cells staining positive for desmin are greatly increased by day 2 of induction in the IHR (b). V, normal vessel; G, glomerulus; F, fibroblasts.

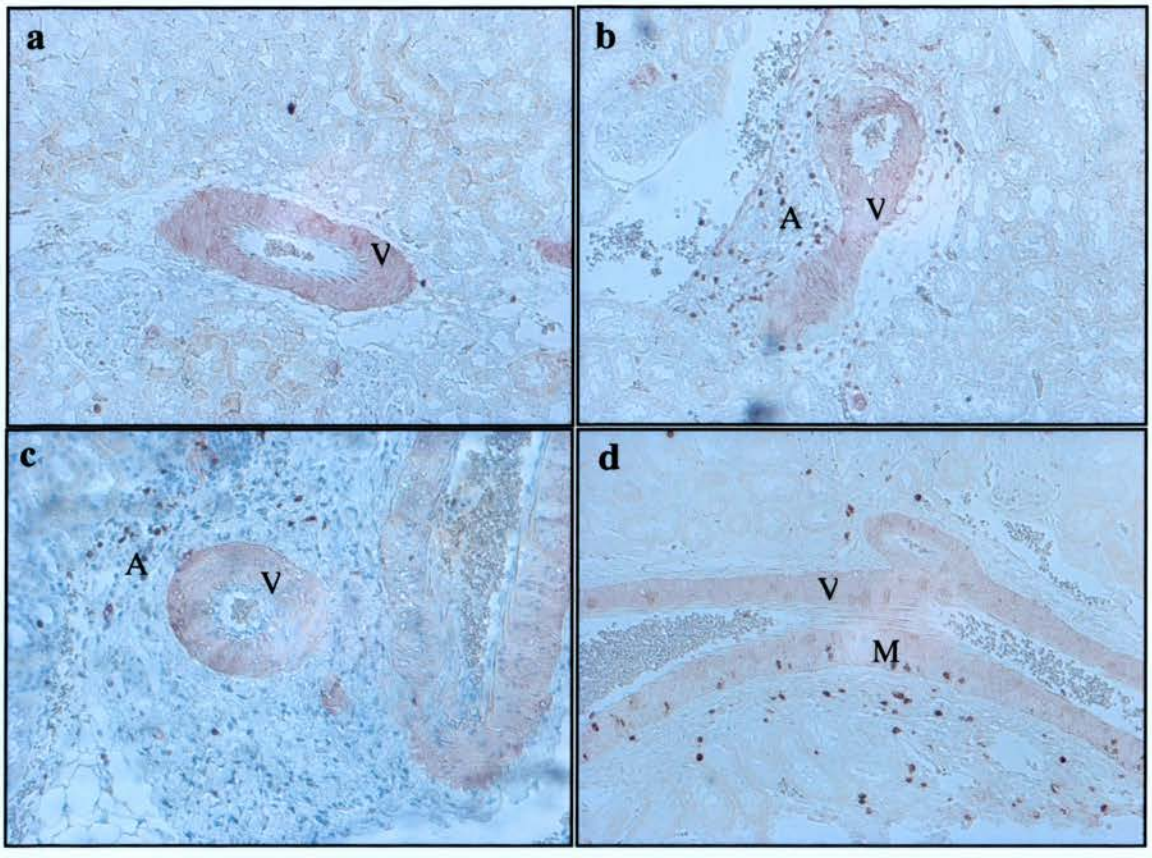

Figure 3.16. Medial Cells Undergoing Proliferation Are Positive For α Smooth Muscle Actin.

Double immunostaining for α smooth muscle actin (αSMA, pink) and BrDU (brown) is shown in the kidney. No proliferation is seen in vessels of Fisher F334 rats (a). By day two of induction in the IHR adventitial proliferation begins (b) and similar changes were found on day four (c). At these time points the vascular media is normal. On day seven proliferation begins within the media and BrDU staining co-localises with αSMA (d). V, vessel; A, adventitial proliferation; M, medial proliferation.

3.3.6.3 Inflammation Precedes The Onset Of Fibrinoid Necrosis.

In the course of identifying the proliferating cell types within the adventitia and media of the kidney by immunostaining, large numbers of inflammatory cells were also identified in the IHR group from day two of induction (figure 3.17). This peri-vascular, mixed inflammatory infiltrate was not seen in Fisher F334 or non-induced transgenic rats. With rare exception, these cells did not co-localise with BrDU confirming that they had not recently under gone S-phase. This response was observed from day two and preceded the onset of vascular smooth muscle cell hyperplasia and fibrinoid necrosis.

There was no evidence histologically of inflammatory cell adherence to the endothelium and migration through the media as would be observed in classical inflammation. However infiltrating cells could be seen adherent to the lumen of veins and capillaries suggesting a path of migration from these low-pressure vascular compartments towards resistance arteries.

The inflammatory cell population was composed of T cells positive for CD3 and macrophages staining for ED-1 (Figure 3.17). Further phenotyping identified the T cells as predominantly T helper in origin with CD4+ cells out-numbering CD8+ cells (data not shown). No B cell component was identified within the infiltrate at early time points however, a few B cells were present following vascular remodelling and necrosis (figure 3.18).

Similar changes in proliferation and inflammation were observed within the mesenteric vasculature, but occurring over a faster time-course consistent with the more rapid onset of vascular injury in this organ (data not shown).

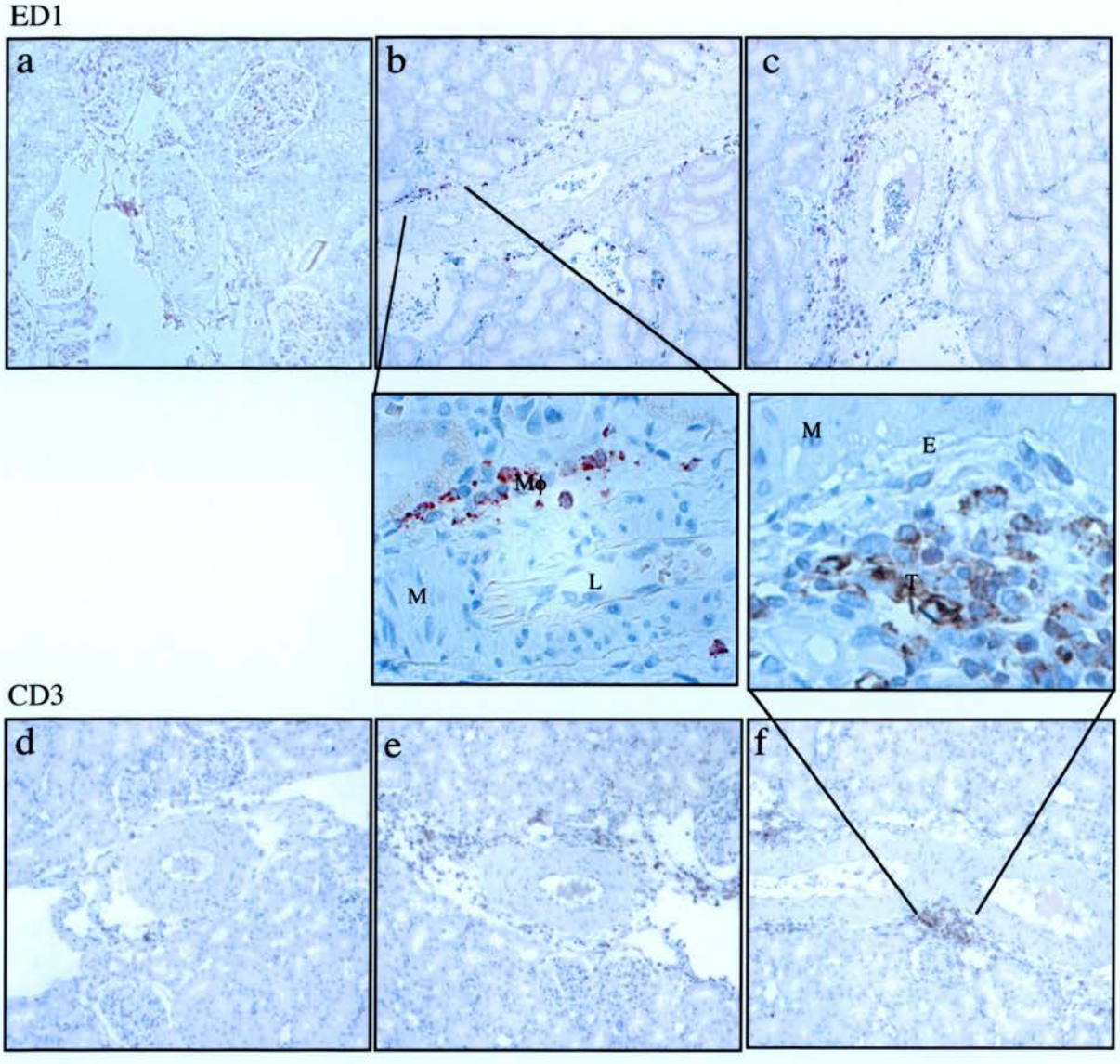

Figure 3.17. A Mixed Inflammatory Peri-Vascular Infiltrate Accumulates On Day Two Of Transgene Induction In The IHR.

CD3 (pan T cell marker) and ED1 (macrophage marker) immunostaining with haematoxylin counter stain. Non-transgenic rats show little positive staining (a and d). An early peri-vascular inflammatory infiltrate of T cell and macrophages is seen in the IHR by day two of induction (b and e). This is more marked by day four of induction (c and f). High power views are annotated. Mϕ, macrophage; M, media; E, external elastic lamina; T, T cell; L, lumen.

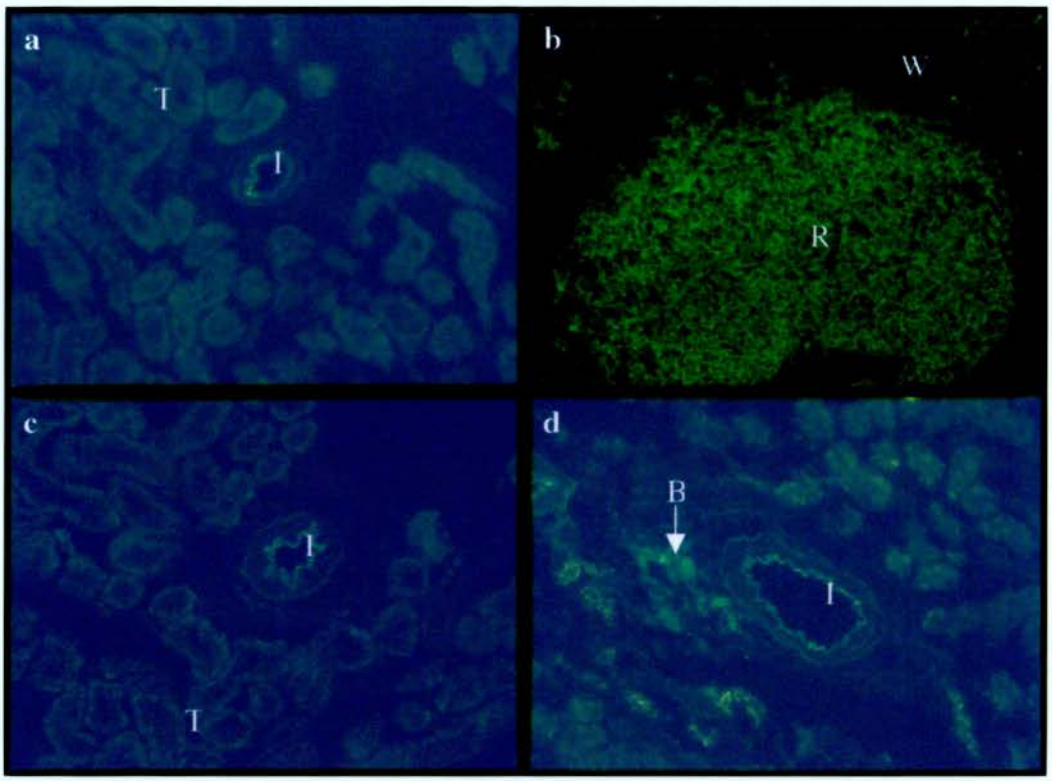

Figure 3.18. Immunofluorescent Staining For B Cells.

Isotype control illustrates background level of autofluorescense (a), strong positive staining in the spleen (b), non-transgenic Fisher F334 kidney (c) and IHR after 14 days of induction (d). T, tubule; I, internal elastic lamina; W, white pulp; R, red pulp; B, B cells.

3.4 DISCUSSION.

This series of timed inductions confirms that the IHR is a robust and highly reproducible model of MH displaying the clinical features of lethargy, weight loss and polyuria together with the development of rapid and severe hypertension with characteristic pathological changes affecting the resistance vasculature of the kidneys, heart and mesentery. In addition, we show that inflammation and discrete waves of proliferation precede vascular injury in this model.

Hypertension developed over a seven-day period, with little inter-animal variation, and diurnal variability was preserved but displayed increased amplitude. The amplitude of animal activity in the IHR group was greater than Fisher F334 controls regardless of the development of hypertension but heart rate differed little from the control and increased moderately during the second week when hypertension was maximal. Such increases in heart rate are observed in human MH and animal models of MH. In man autonomic dysfunction including changes in diurnal variability and rhythm are associated with a poor cardiovascular outcome [Covic et al., 2000; Imai et al., 1999]. Increased variability of blood pressure in hypertension has been described in the 1C2K and Dahl-salt sensitive models of hypertension also without inversion of the rhythm [Mohri et al., 2003; Wallace et al., 1987]. In Dahl salt-sensitive rats the amplitudes of circadian expression changes of three clock genes were decreased peripherally [Mohri et al., 2003]. However, in some SHR strains an inversion has been described with a nocturnal dip in pressure [Minami et al., 1988].

Despite using radio-telemetry, there was no evidence of sudden spikes of hypertension as have been previously described in the developing phase of hypertension in the 1C2K model [Helmchen et al., 1984]. Possibly, these elevations were induced by surgical constriction of the renal artery and secondary vasospasm causing transient hypertension.

Transgene derived circulating prorenin was 10^4 times greater and active renin and angiotensin II approximately double compared to Fisher F334 controls by day eleven of induction. The reasons for the dissociation between very high circulating "inactive" prorenin and lower levels of "active" renin and angiotensin II are unclear. Previously transgene expression was not be detected by RNase protection assay in target organs and immunoprecipitation of circulating mouse renin suggested that the majority was transgene derived [Kantachuvesiri, 1999]. In the kidney, there is appropriate suppression of endogenous renin suggesting that circulating mouse prorenin from the liver is the major source of downstream angiotensin II generation, although low-level transgene expression in extra-hepatic sites cannot be excluded. Prorenin elevation occurred rapidly together with an elevation in blood pressure, and preceded the rise in circulating active renin and angiotensin II suggesting that processing of the transgene at a local, tissue level is responsible in part for the phenotype. It has been suggested that one percent of prorenin can exist in an active form without cleavage via conformational shift [Yamauchi et al., 1990]. If this occurs in the IHR model it would result in significant levels of circulating "active" renin. Initial studies looking at cardiomyocytes in vitro and in vivo using the IHR have suggested that transgene-derived prorenin is elevated and raises the possibility of uptake and activation of prorenin within cardiac

and perhaps other tissues [Peters et al., 2002]. This has been alluded to in other studies where prorenin binding and uptake by vessels and cardiomyocytes via a renin receptor has been suggested [Danser et al., 1994; Prescott et al., 2000; Saris et al., 2001; van Kesteren et al., 1997].

The measurement of circulating levels of the renin-angiotensin system may not accurately reflect activity at a tissue level. Quantification of renin at a tissue level was not undertaken but was performed during earlier studies using tissue homogenates and reported significantly higher levels of renin activity in the spleen, heart and mesentery [Kantachuvesiri, 1999]. High circulating transgene levels make it difficult to exclude contamination from circulating fluid even following perfusion. Many tissues including the heart, kidney and vasculature are able to generate angiotensin II locally and small increases in tissue renin activity could result in local angiotensin II generation [Bader et al., 2001; Muller et al., 1998; Muller and Luft, 1998]. Local angiotensin II generation may be more important in mediating the inflammatory properties of angiotensin II than circulating angiotensin II [Cheng et al., 2005].

As previously reported, the same staggered onset of vascular injury was observed in this series of experiments with the mesentery and heart affected before the kidneys while the brain remained spared [Kantachuvesiri et al., 2001]. Since all vessels of 200-300um in the systemic circulation underwent remodelling including aortic branches and vessels within the spleen and pancreas by seven days (data not shown) and the renal and cerebral circulations occurred later and not at all, it seems likely that the additional autoregulatory capacities of the kidney and brain afford relative protection. All tissues are reported to have local myogenic, endothelial and metabolic mechanisms of

maintaining constant blood flow however, the kidney has additional specialised methods of controlling glomerular blood flow: Neurohumoral pathways, mediated by the sympathetic nervous system, angiotensin II and prostaglandins, and the tubuloglomerular feedback response. In the later, increased sodium chloride delivery to the macular densa, distal to the glomerulus, increases afferent arteriolar tone, reducing glomerular blood flow. Renin secretion is also reduced [Lorenz et al., 1991; Thurau and Schnermann, 1965]. These processes compensate for minute-to-minute blood pressure changes and prevent salt-water loss in the short term and also alter sodium and water balance over a longer time period. Similarly the brain has complex mechanisms to ensure the maintenance of constant blood flow and cerebral vessels are sensitive to the partial pressure of carbon dioxide and pH. The structure of cerebral vessels in rats and humans, also differs from those in the peripheral circulation in that they lack an external elastic lamina and have tight junction endothelial links as part of the blood brain barrier [Lee, 1995]. Consequently, the kidney and brain have additional vascular responses to rising blood pressure and in the case of the brain, protection from circulating factors such as prorenin as well.

There was dissociation between the left and right ventricles with the right sustaining earlier and more severe vascular injury with secondary necrosis and the left undergoing predominantly hypertrophic remodelling with milder vascular injury. The relative protection afforded the left ventricle may be due to the atypical flow of blood through the coronary circulation during the cardiac cycle. Since the left ventricle receives maximal perfusion during diastole, vessels are protected from high systolic pressures. Additionally, vessels within the left ventricle are embedded in thick, supporting

myocardium, which may provide protection from high lumenal pressures. Predominantly right ventricular injury seen in the IHR, was also noted to occur in Wistar rats with renovascular hypertension generated by aortic ligation above the left renal artery. After only four days fibrinoid necrosis and infarction were seen in the right but not left ventricle [Fueyo et al., 1987]. A similar pattern of differential ventricular susceptibility is seen in human disease.

Characteristically in MH, larger arteries are spared. Resistance vessels may be especially susceptible to fibrinoid necrosis because in normotension they are subject to low-pressure, laminar blood flow. In hypertension, developing rapidly without time for adaptation, they are exposed to high-pressure and irregular flow triggering pathological adaptation. The importance of flow is suggested by the observation that fibrinoid necrosis is seen at the branch points of larger vessels.

End-organ dysfunction was mild over the period studied and both the kidney and heart were able to maintain an adequate level of function as marked by the absence of pulmonary oedema and maintenance of creatinine clearance. The presence of proteinuria suggests mild renal dysfunction, which in itself, would be expected to contribute to fibrosis and renal failure given time.

In the IHR early peri-vascular infiltration of inflammatory cells in association with fibroblast proliferation was seen. There is now good evidence that the RAS has an immunomodulatory and mitogenic role principally via angiotensin II and in addition to effects on vasomotor tone and salt-water regulation, angiotensin II has chemoattractant properties for monocytes, upregulates pro-inflammatory transcription factors such as NFkB and is itself secreted by inflammatory cell lines [Brasier et al., 2000; Kranzhofer

et al., 1999a; Ruiz-Ortega et al., 2001b]. The RAS via inflammatory mechanisms, plays an important role in atherosclerotic vascular remodelling [Kranzhofer et al., 1999b; Phillips and Kagiyama, 2002] in which T helper cells and macrophages have been identified as important from early on [Drew and Tipping, 1995b]. Cyclosporin treatment of cholesterol fed rabbits was able to attenuate atheromatous plaque formation in association with reduced T cell numbers suggesting a pathological role for these cells in this model, however human renal transplant patients maintained on cyclosporin are well known to have enhanced atheroma [Drew and Tipping, 1995a].

There is increasing evidence that in hypertensive vascular disease, like atherosclerosis, infiltrating inflammatory cells together with the paracrine RAS play an important role [Fleming, 2000; Montgomery et al., 1998; Unger et al., 1989]. In the Dahl salt sensitive rat and the Goldblatt 1C2K model, fibrinoid necrosis and associated vascular inflammation were observed [Eng et al., 1994; Hampton et al., 1989]. Typically in the 1C2K model the unclipped kidney subject to high systemic pressures displays the greatest injury but monocyte infiltration has also been described in both the unclipped and clipped kidney, protected from hypertension and fibrinoid necrosis, and in association with ICAM-1 expression suggesting classical inflammatory migration of leucocytes through the vascular endothelium [Haller et al., 1997]. In the IHR, inflammatory cells were not adherent to the endothelium and were seen migrating from low-pressure vessels. It is possible that studies conducted later when tissue necrosis was established, observed classical secondary inflammation, which while undoubtedly contributing to ongoing pathology, especially interstitial fibrosis, is not an initiating event.

In one study by Mai *et al*, the pre-hypertensive stage of the 1C2K model was examined in Sprague-Dawley rats but ICAM-1 and LFA-1 expressing infiltrating mononuclear cells were only increased in the perivascular space after 14 days and not prior to the onset of hypertension at four days or at seven days when hypertension was present suggesting that this pro-inflammatory mechanism does not contribute early on in this model [Mai et al., 1996].

Perivascular T helper cell and macrophage populations have also been described in association with collagen I producing fibroblasts within the heart in established hypertensive injury in both the SHR and 1C2K models [Hinglais et al., 1994; Nicoletti et al., 1996]. Again, hypertensive injury was already established when the inflammatory infiltrate was assessed.

In large vessel remodelling caused by angiotensin II or norepinephrine infusions, adventitial macrophage infiltration was observed in association with high levels of MCP-1 mRNA and both were reduced by hydralazine treatment, but no detail of resistance vessels was given [Capers et al., 1997]. The ultimate importance of inflammatory cell infiltration remains unclear but the identification of these cells so early on in the IHR is suggestive of a modulatory role in subsequent vascular remodelling steps.

The RAS plays a key role in proliferative intimal remodelling following polyethylene cuff placement in the large arteries of mice [Akishita et al., 2001]. Akishita *et al* demonstrated intimal expansion together with increased ACE expression and localisation within the neointimal, medial and adventitial compartments of cuffed arteries.

Fibroblast proliferation within the adventitia was seen from as early as day two in the IHR. These cells were desmin positive and did not stain for α-smooth muscle actin excluding a myofibroblast phenotype. The adventitia can no longer be considered an inert support layer to the functionally important medial and endothelial layers. In human atherosclerosis, the adventitial inflammatory infiltrate correlates to the degree of intimal lesion and following angioplasty injury in pigs, proliferating myofibroblasts from the adventitial were found to migrate into the intima, contributing directly to re-stenosis lesions in this model [Wilcox and Scott, 1996; Wilcox et al., 1996]. In rats treated with prazocin for four days, adventitial fibroblast proliferation was seen affecting terminal arterioles. This affect was not seen in vitro, suggesting that chronic vasodilatation and transmitted pressure to the terminal vasculature, rather than the drug itself, was responsible for this response [Price and Skalak, 1998]. In hypertension generated by angiotensin II infusion, there is evidence that adventitial fibroblasts contribute to vascular tone by superoxide generation [Di Wang et al., 1999]. Similar properties of superoxide generation via NADPH oxidase, have been demonstrated in the adventitia of isolated rabbit aortic rings [Pagano et al., 1995]. Nitric oxide production by cells of the adventitia can have an "endothelial-like" role in modulating arterial tone and transfection of eNOS into adventitial fibroblasts can compensate for loss of endothelial NO production [Onoue et al., 1999; Tsutsui et al., 1999]. Hypertension induced by aortic ligation in the rat was associated with a 600% increase in DNA replication and fibroblast accumulation in the adventitia of large vessels [Chatelain and Dardik, 1988] and adventitial fibroblasts stripped from the aorta of SHRs displayed enhanced proliferation and greater basal cyclin p34cdc2 expression in

vitro compared to Wistar-Kyoto fibroblasts [Venance et al., 1993; Zhu et al., 1991]. Adventitial fibroblasts were also sensitive to inflammatory stimuli and upregulated iNOS and nitric oxide levels in response to lipopolysaccharide stimuli in Wistar rats [Zhang et al., 1999].

The presence of a marked adventitial reaction as early as day two following induction, when blood pressure is only minimally elevated, suggests that in the IHR, pressure is not the principal stimulus for this response. Circulating mouse prorenin levels are very high by this time point and local processing of the transgene product may be responsible for this phenomenon.

The co-existence of infiltrating inflammatory cells together with proliferating fibroblasts within the adventitial of the IHR so early on in the pathogenesis of malignant vascular injury is highly suggestive of an important role for this vascular compartment in the subsequent vascular remodelling. Additionally, given that adventitial fibroblasts have been shown to influence the medial compartment by a paracrine route, it is tempting to speculate that the day two and four changes seen in the IHR adventitia are causally linked to the proliferation and subsequent degeneration seen within the media from day seven. The assumption that vascular smooth muscle cells are the proliferating cell type seen within the media on day seven is based on the co-localisation of BrDU and α-smooth muscle actin, consistent with a smooth muscle phenotype. However, the studies performed to date cannot exclude migration and transdifferentiation of another cell type, such as adventitial fibroblasts, into the media.

The results presented in this chapter demonstrate for the first time the early stages in the pathogenesis of malignant vascular injury in the IHR, a model closely resembling the human MH. The identification of discrete stages of inflammatory cell infiltration and proliferation, first within the adventitia and followed by the media, provide a target for more detailed investigation and therapeutic intervention. The chemoattractant factor(s) for inflammatory cell infiltration and mitogenic stimuli for both fibroblast and vascular smooth muscle cell proliferation require identification. Additionally, the role of these events in the subsequent development of cell death and loss of endothelial integrity observed in fibrinoid necrosis and endarteritis proliferans needs to be established.

CHAPTER FOUR

THE EFFECT OF FK506 TREATMENT ON THE DEVELOPMENT OF HYPERTENSION AND VASCULAR INJURY IN THE INDUCIBLE HYPERTENSIVE RAT.

4.1 INTRODUCTION.

Calcineurin is responsible for the dephosphorylation of nuclear factor of activated T cells (NFAT), which in its dephosphorylated state, can translocate into the nucleus and interact with a variety of transcription factors to initiate transcription. Calcineurin itself is activated by calcium-induced calmodulin binding, exposing catalytic sites capable of dephosphorylating serine residues located at the N-terminus of NFAT (figure 4.1) (science 1988 241 202, science 1993 262 750).

FK506, also known as tacrolimus, is able to block the phosphatase activity of calcineurin by complexing with FK506 binding protein (FKBP). It is well established that calcineurin inhibitors can prevent the development of cardiac hypertrophy in a variety of hypertensive rodent models, independent of any blood pressure lowering effects and this has been extensively reviewed [Bueno et al., 2002; Leinwand, 2001; Zhang, 2002]. The effects of calcineurin inhibitors on hypertension and vascular remodelling are less clear.

FK506 is an established immunosuppressant, with a preferential effect on T cells by inhibiting the transcription of several genes including IL-2, required for activation

[Crabtree, 1999; Kay et al., 1989]. The use of this class of drug has revolutionised organ transplantation by enhancing graft survival. Patients tolerate chronic administration of these agents well, although there is an increased incidence of hypertension, diabetes (especially FK506) and nephrotoxicity over time [Curtis, 2002; Morales et al., 2001; Woodward et al., 2003].

Given the identification of an inflammatory infiltrate as the earliest stage in malignant vascular remodelling in the IHR, we used the immunosuppressant calcineurin inhibitor FK506 to determine the effect of immunomodulation on the development of malignant vascular injury.

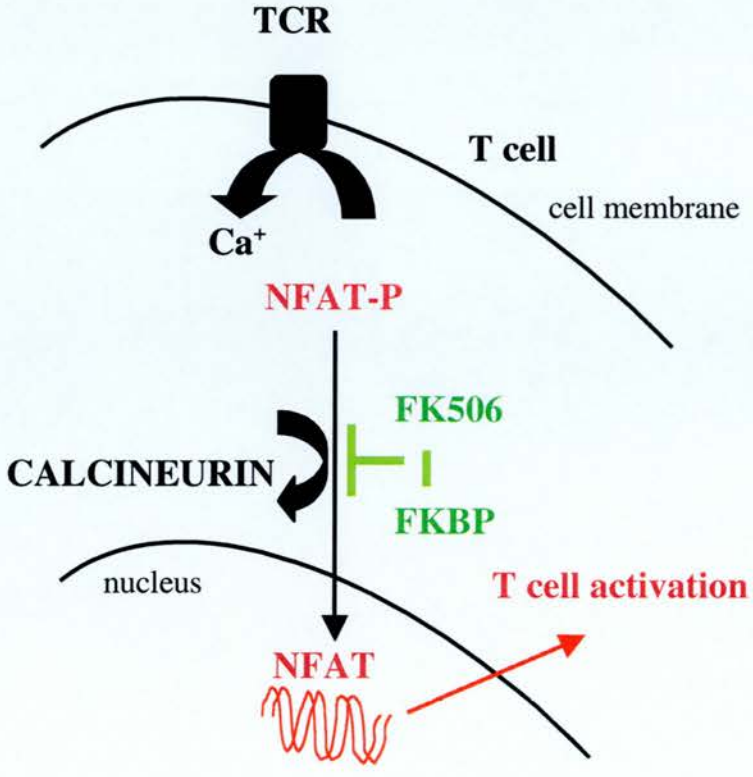

Figure 4.1. Schematic Diagram Of NFAT Activation.

TCR, T cell receptor; Ca^{+}, calcium.

4.2 EXPERIMENTAL DESIGN.

Adult IHR (n=12) and Fisher F334 (n=12) rats were induced using the standard dietary I3C protocol for a total of 14 days. Six animals from each group had daily intraperitoneal injections with water or FK506 suspended in water, at a dose of 1mg/kg/day from three days prior to induction and throughout the 14-day induction period until cull. Animals were weighed on day seven so that the dose of FK506 could be adjusted for any weight change.

Twenty-four hours prior to cull, all animals were injected with BrDU, using the standard protocol, and placed in metabolic cages for urine collection. Urine volumes for 24 hours were recorded and creatinine assayed.

Systolic blood pressure was assessed using tail cuff plethesmography by two observers and repeated on a second identical experimental group.

An identical experiment was repeated and the animals culled after seven days of induction in order to assess the effect of FK506 treatment earlier on in the development of MH.

At the time of cull, blood was taken to determine creatinine and hence creatinine clearance, plasma renin and angiotensin II. Body weight, left and right ventricular weights and tibial length were recorded and tissues fixed for histological analysis. Liver tissue was snap frozen for RNA extraction and estimation of transgene activation.

4.3 RESULTS.

4.3.1 The Effect Of FK506 On The Course Of Malignant Hypertension.

Transgenic animals induced for fourteen days developed weight loss, polyuria and lethargy typical of MH (Figure 4.2 & 4.3a).

Control animals receiving FK506 gained less weight than water treated controls but were otherwise well. Drug treated transgenics lost more weight than those treated with water alone however this difference did not reach statistical significance. Polyuria was abolished in the FK506 treated transgenic group who exhibited urine outputs comparable to the control group (Figure 4.3a).

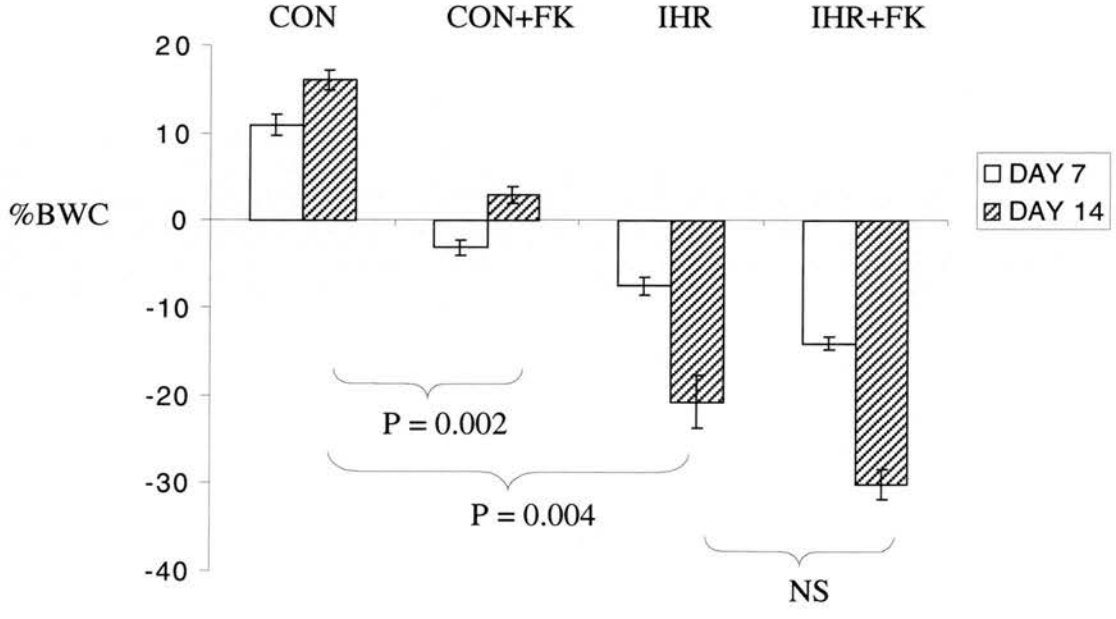

Figure 4.2. Weight Change During Induction With And Without FK506.
Percentage body weight change (%BWC) over seven (open bars) and 14 days (hatched bars) of induction. FK506 treatment associates with significant weight loss in control rats. CON, Fisher F334 control; IHR, inducible hypertensive rat; FK, FK506.

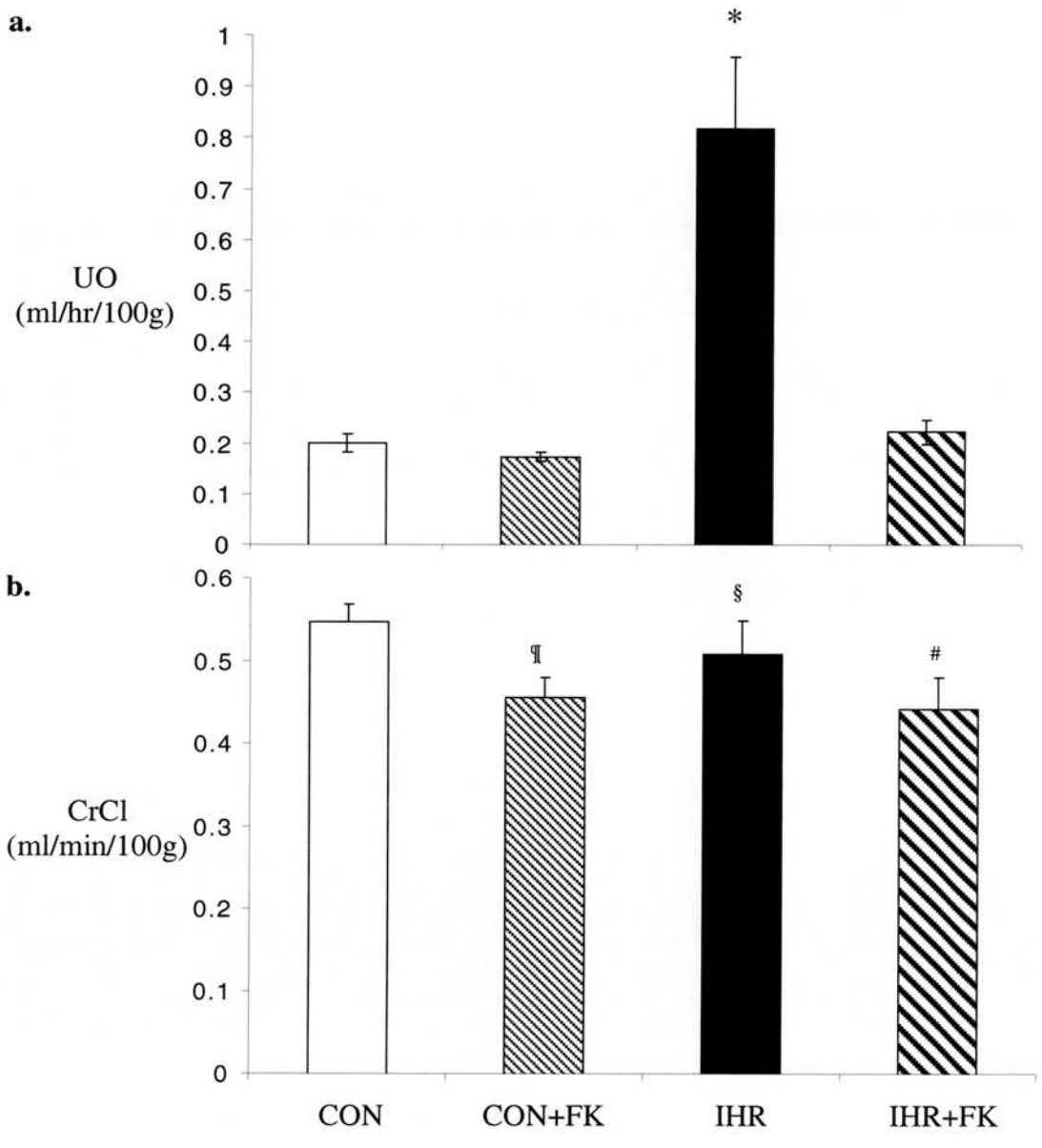

Figure 4.3. Renal Function.

Weight adjusted urine output (UO, a) and creatinine clearance (CrCl, b). Polyuria was abolished in FK506 treated IHRs. There was no significant reduction in creatinine clearance in FK506 treated IHRs. CON, Fisher F334 control; IHR, Inducible Hypertensive Rat; FK, FK506; *p=0.007; ¶p=0.02; §p=NS; #p=NS compared to IHR.

4.3.2 The Effect Of FK506 On The Development Of Hypertension.

Both control groups remained normotensive despite treatment with FK506 and as expected, transgenic rats receiving water injections developed hypertension (figure 4.4). Despite an identical rise in blood pressure until day five, drug treated transgenics failed to sustain this level of hypertension and were equivalent to control groups by day seven.

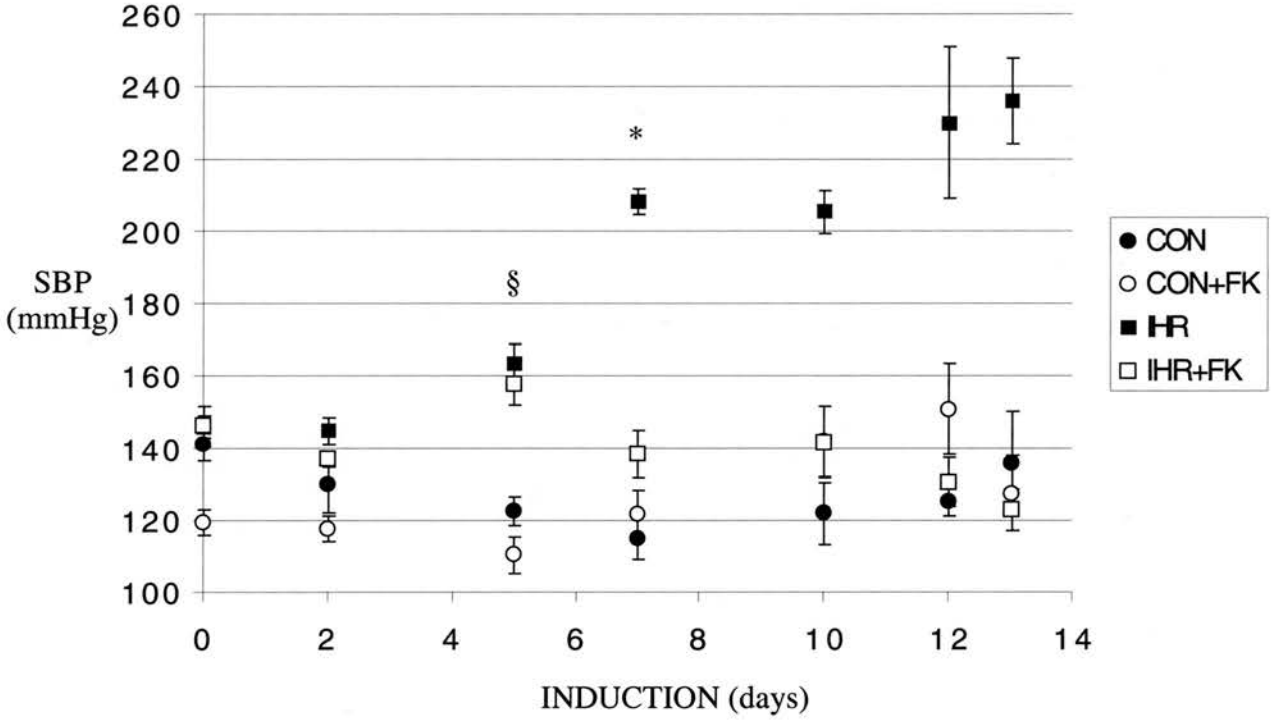

Figure 4.4. The Effect Of FK506 On Hypertension.

Systolic blood pressure (SBP) estimated by tail-cuff plethysmography. * p=0.008 *vs* control; § p=0.008 for both IHR groups *vs* controls; CON, Fisher F334 control; IHR, Inducible Hypertensive Rat; FK, FK506.

4.3.3 The Effect Of FK506 On End-Organ Injury.

There was no difference in creatinine clearance between transgenic animals with or without FK506 treatment following induction for 14 days. A small but significant decrease in weight adjusted creatinine clearance was observed between Fisher F334 rats and Fisher F334 rats treated with FK506. However, the value of the drug treated control group was still within the expected range for normal renal function (Figure 4.3b). Ventricular hypertrophy observed in the water treated transgenic group was not seen in the drug treated transgenic group who were similar to control animals (Table 4.1).

	CON	CON+FK506	IHR	IHR+FK506
LV:Tibial length	1.48	1.29	1.85*	1.56§
RV:Tibial length	3.52	2.52	4.63	3.42#

Table 4.1. Left Ventricular Mass Following Induction With And Without FK506. Ventricular mass expressed as tibial ratios for left (LV) and right (RV) ventricles. p values are compared to the control group unless stated. CON, Fisher F334 control; IHR, Inducible Hypertensive Rat; *p=0.03; §p=0.02 compared to IHR; #p=0.02 compared to IHR.

4.3.4 The Effect Of FK506 On Pathology.

4.3.4.1 Animals Induced For 14 Days.

Control animals had no histological abnormalities in any of the organs studied (Figure 4.5a, 4.6a, 4.6d, 4.6g). Tubular vacuolation and vasculopathy sometimes seen in FK506 toxicity was not observed in either drug treated group (Figure 4.5b).

Transgenic rats receiving water injections had widespread fibrinoid necrosis with associated tissue necrosis affecting the heart, kidney and mesentery as previously observed (Figures 4.5c,d & 4.6b,e,h). Within the mesentery grade IV vessels were most severely affected and the right ventricle of the heart was consistently more diseased than the left. The most striking finding however, was the complete absence of vascular injury and associated end-organ injury, in these organs from the transgenic drug-treated group (Figures 4.5e,f & 4.6c,f,i).

4.3.4.2 Animals Induced For 7 Days.

As with the two-week induction study, control rats had no evidence of histological injury and there was no evidence of FK506 induced toxicity within the kidney or other organs.

The mesenteric and cardiac vessels had under gone fibrinoid necrosis in the water treated transgenic group. The renal vasculature was less severely affected with medial

thickening only seen on H&E staining. As before, vascular injury was absent from all tissues studied in the FK506 treated transgenic group.

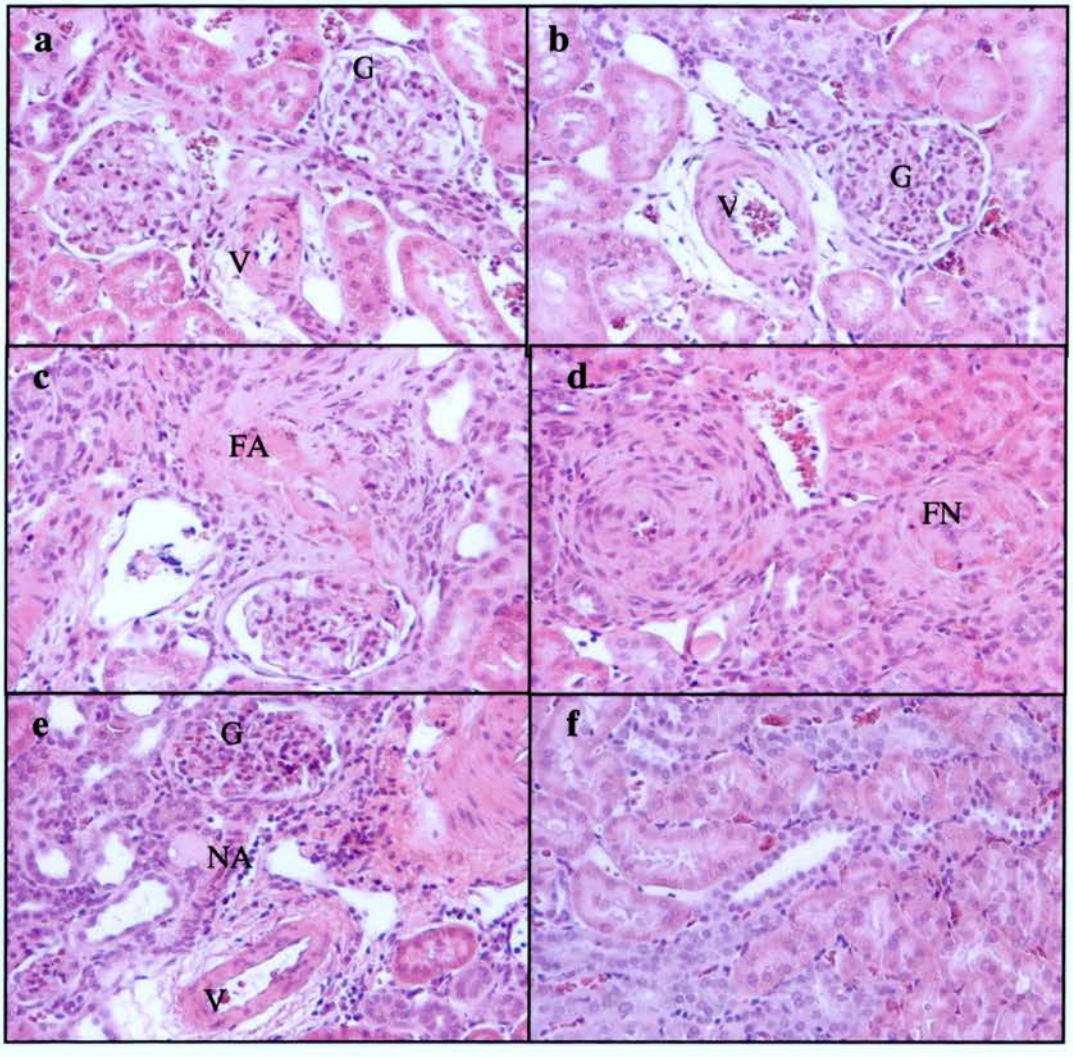

Figure 4.5. H&E histology of the kidney.

Control rats treated with water (a) or FK506 (b) show no abnormality. Water treated transgenics display fibrinoid necrosis (c&d). FK506 treated transgenics do not develop vascular or interstitial injury (e&f). FA, afferent arteriole affected by fibrinoid necrosis; NA, normal afferent artery; FN, fibrinoid necrosis; V, normal vessel; G, normal glomerulus.

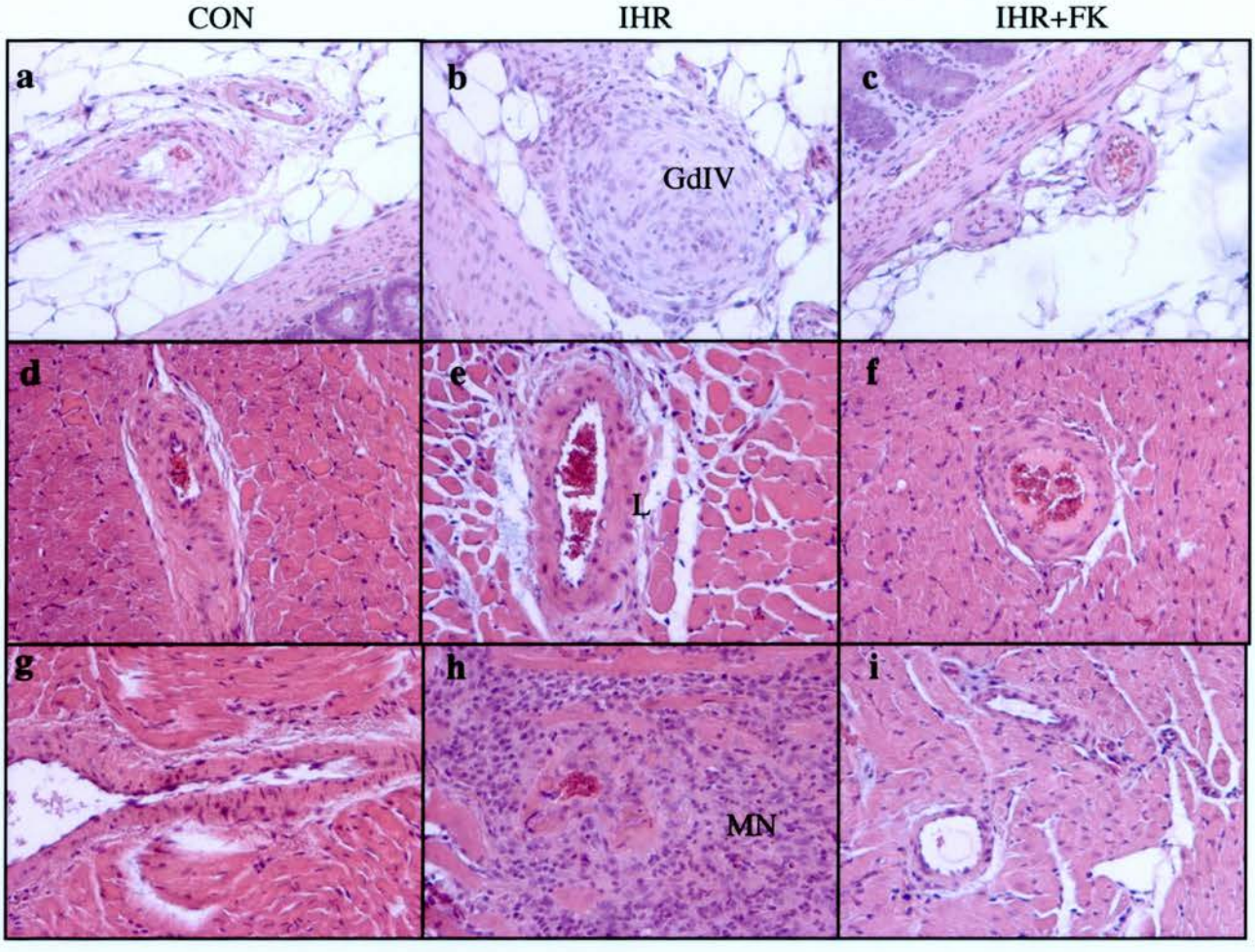

Figure 4.6. H&E histology of Heart And Mesentery.

The mesentery (a-c), left ventricle (d-f) and right ventricle (g-i) are shown. FK506 prevents vascular pathology in transgenic rats. GdIV, fibrinoid necrosis affecting grade IV mesenteric vessels; L, mild disease affecting the left ventricle; MN, fibrinoid necrosis with associated myocardial necrosis within the right ventricle; CON, Fisher F334 control; IHR, inducible hypertensive rat; FK, FK506.

4.3.5 The Effect Of FK506 On Vascular Remodelling Within The Kidney.

4.3.5.1 Animals Induced For 14 Days.

BrDU staining demonstrated a high level of medial proliferation in transgenic water-treated rats when compared to control animals (Figure 4.7a). These cells also stained positive for αSMA, suggesting that they were vascular smooth muscle cells (data not shown). FK506 had no effect on baseline medial vascular smooth muscle cell proliferation and in transgenic animals receiving the drug, proliferation within the media was not greater than control rats.

Staining of the kidney with the pan T cell marker CD3 illustrated a higher baseline level of peri-vascular T cells in the FK506 treated control group. High levels of T cell infiltration were seen in the transgenic water treated group in the context of tissue injury. However, similar levels of T cell infiltration were also seen in the transgenic FK506 treated group despite an absence of vascular injury (Figure 4.7b).

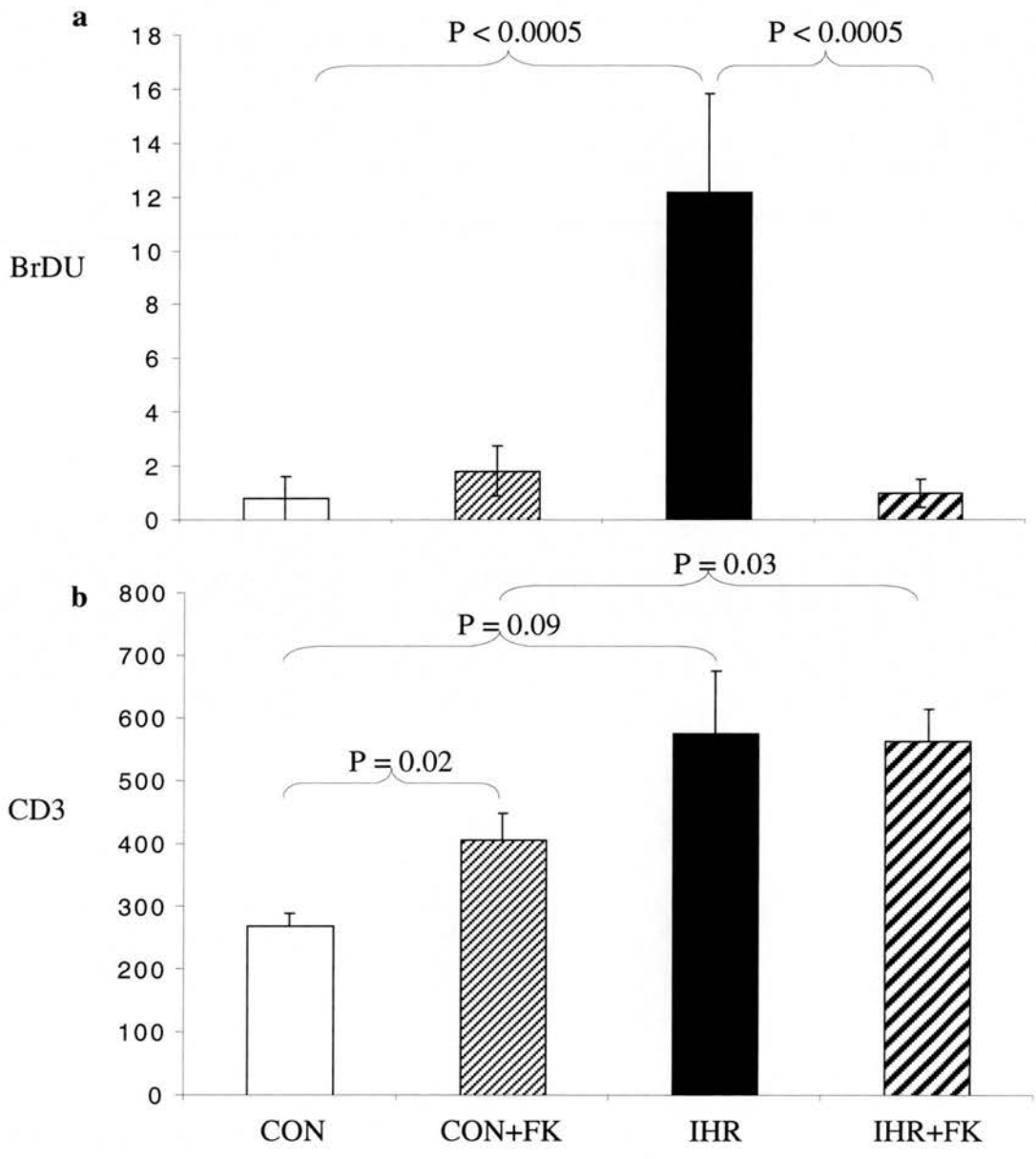

Figure 4.7. Quantification Of BrDU And CD3 Immunopositivity After Fourteen Days Of Induction.

Quantification of BrDU positive medial cells (a) and CD3 positive adventitial cells (b). CON, Fisher F334 control; IHR, Inducible Hypertensive Rat; FK, FK506; BrDU, average number of BrDU positive cells per kidney; CD3, average number of CD3 positive cells per kidney.

4.3.5.2 Animals Induced For 7 Days.

Assessment of vascular remodelling within the kidney on day seven demonstrated similar findings, with regard to medial proliferation, as were found after 14 days of induction (Figure 4.8a). Additionally, CD3 staining showed no increase above baseline at this time point in control animals treated with the drug. There was a significant increase in adventitial T cells in the transgenic group receiving water compared to controls and this increase was not found in the drug treated transgenics at this time-point (Figure 4.8b). Quantification of adventitial macrophage numbers showed a similar trend to T cell infiltration but did not reach statistical significance (Figure 4.8c). Representative immunohistochemistry is shown for BrDU staining (figure 4.9) and CD3 and ED-1 staining (figure 4.10). The distal tubular proliferation observed in the IHR is unaffected by FK506 treatment (figures 4.9e&f). A mild interstitial nephritis is evident by day seven (figure 4.10b) and was more marked by day 14.

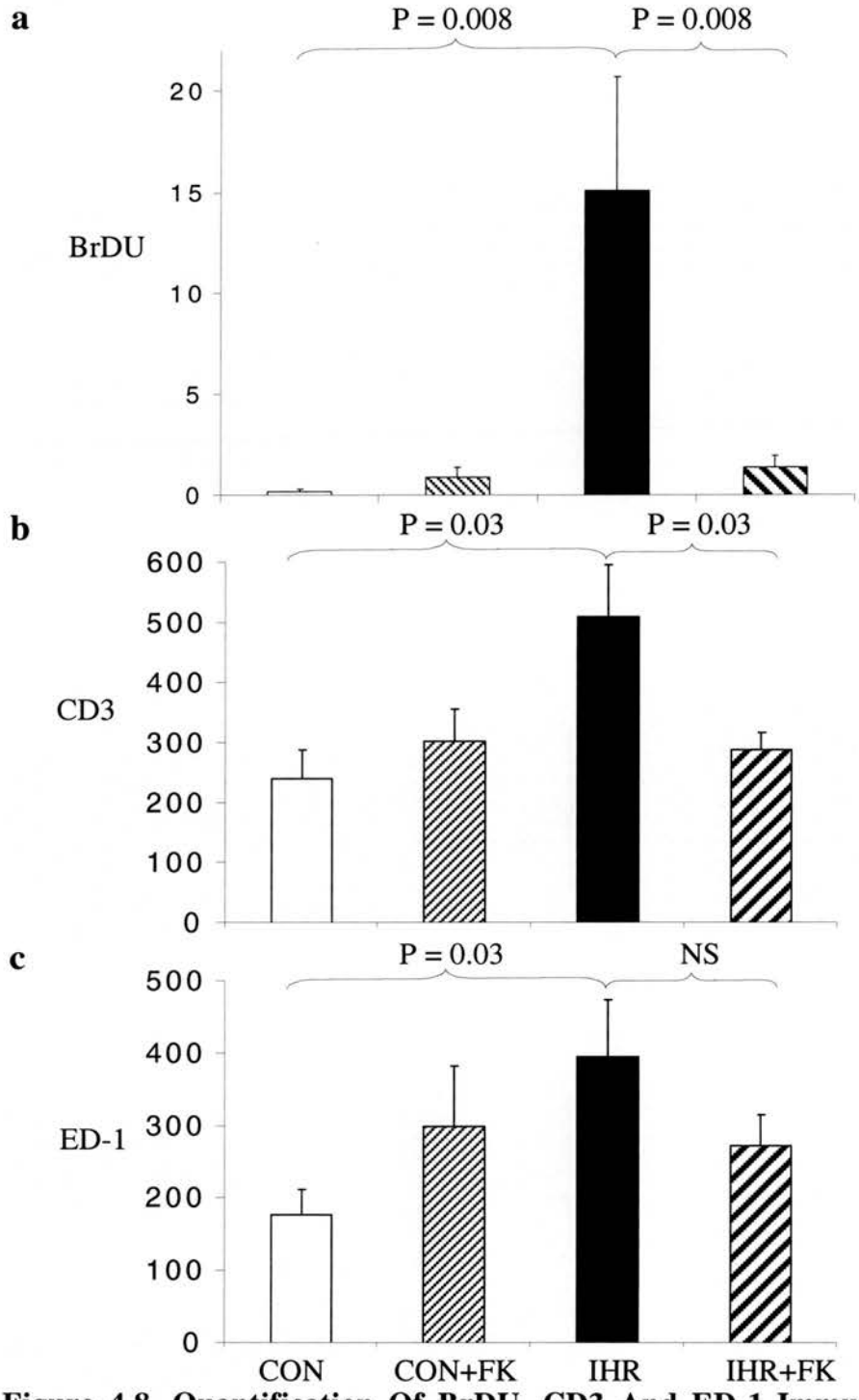

Figure 4.8. Quantification Of BrDU, CD3 And ED-1 Immunopositivity After Seven Days Of Induction.

Quantification of BrDU positive medial cells (a), T cells (b) and macrophages (c) in the adventitia. CON, Fisher F334 control; IHR, Inducible Hypertensive Rat; FK, FK506; BrDU, average number of BrDU positive cells per kidney; CD3, average number of CD3 positive cells per kidney; ED-1, average number of ED-1 positive cells per kidney.

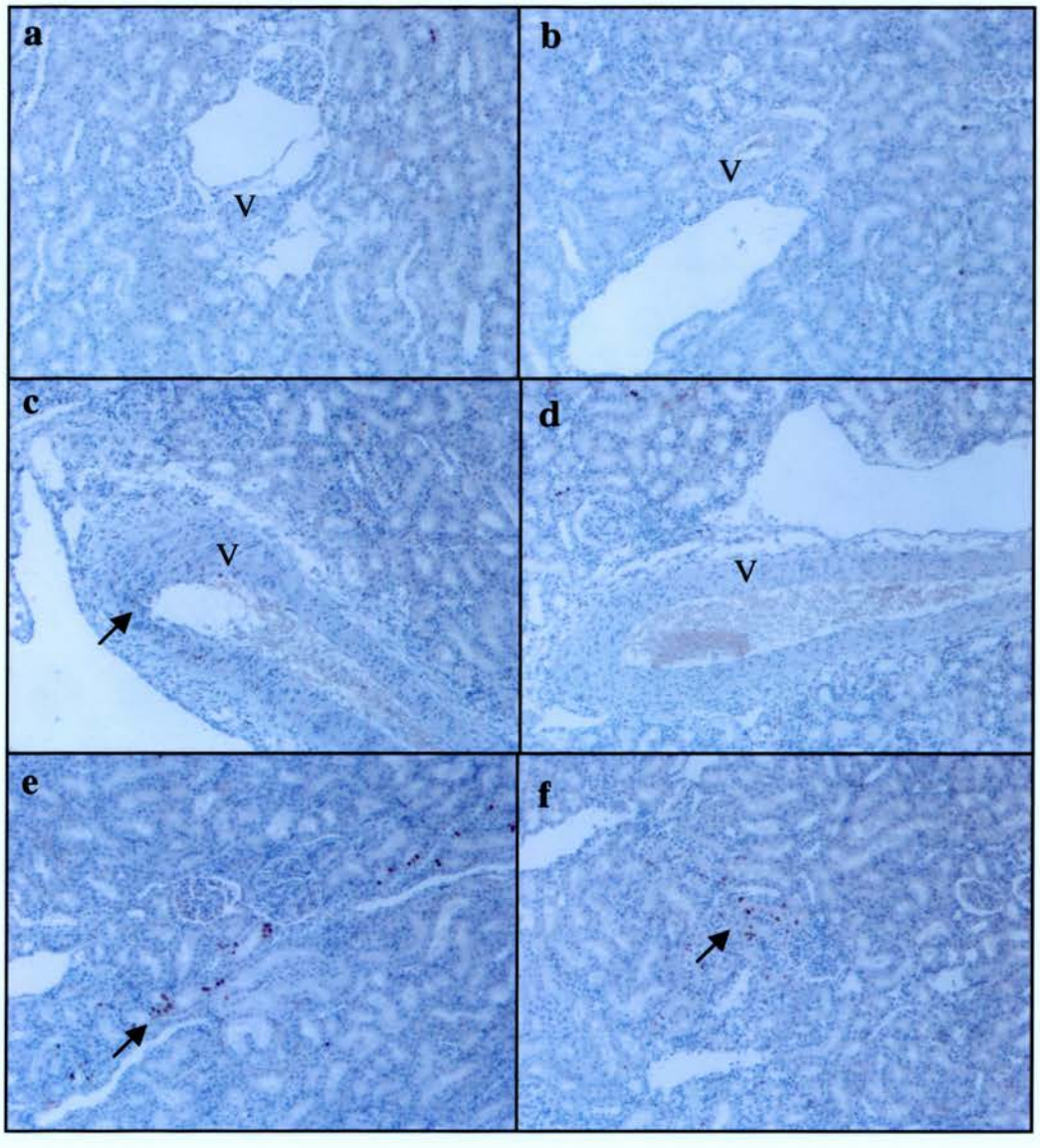

Figure 4.9. Representative BrDU Immunohistochemistry After Seven Days Of Induction.

BrDU staining (brown) in the kidney. Fisher F334 (a), Fisher F334+FK506 (b) and IHR+FK506 vessels (d) have no positive medial cells. IHR sections display BrDU positive cells within the media (c, arrow). IHR (e) and IHR+FK506 (f) show distal tubular proliferation (arrowed). V, vessel.

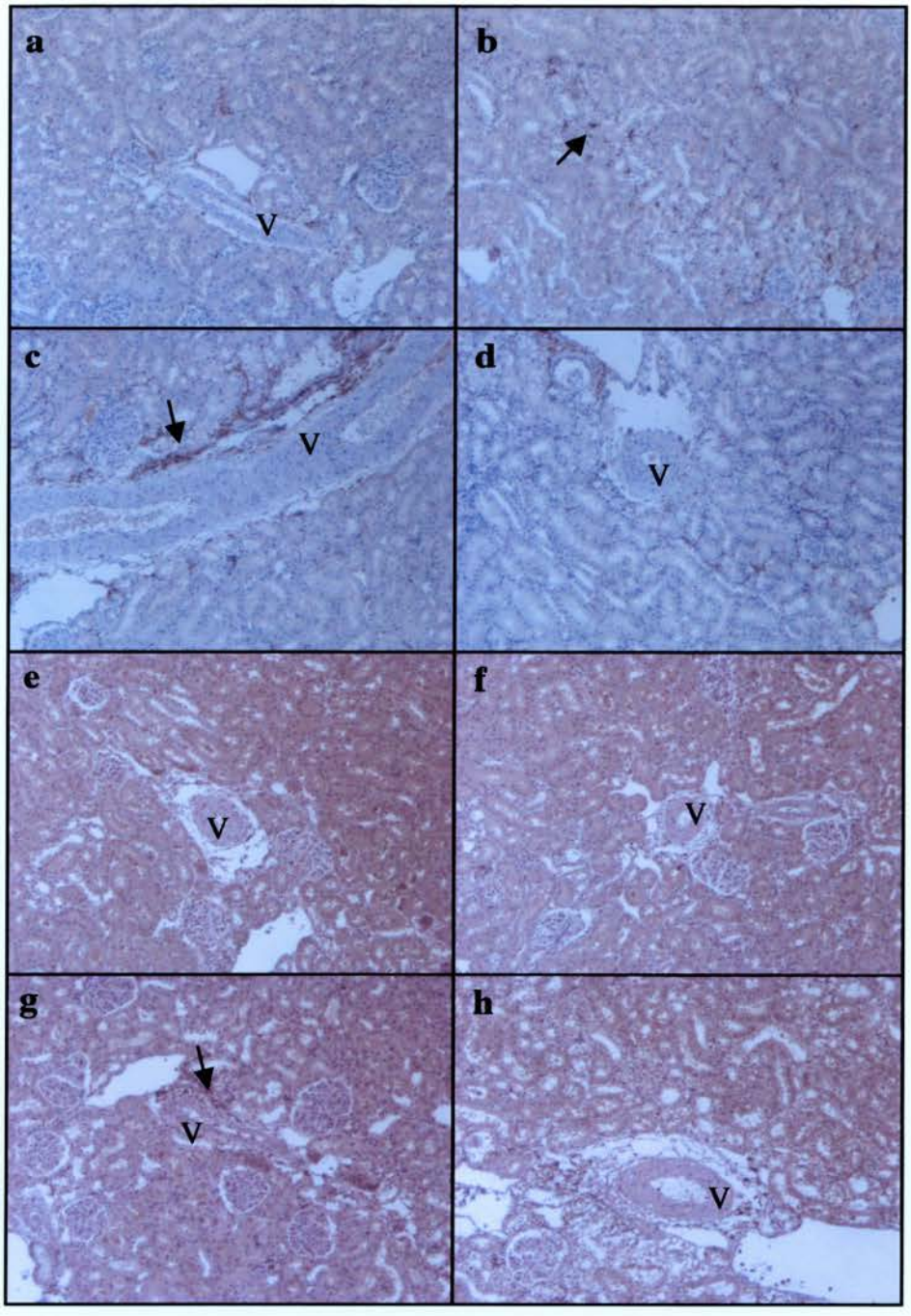

Figure 4.10. Representative CD3 And ED-1 Immunohistochemistry After Seven Days Of Induction.

CD3 staining (a-d brown) and ED-1 staining (e-h brown) in the kidney. Fisher F334 vessels (a) have low numbers of adventitial CD3 positive cells. Fisher F334 treated with FK506 show a mild interstitial nephritis (b, arrow). IHRs show high numbers of peri-vascular CD3 positive cells (c, arrow). IHR treated with FK506 display low levels of CD3 positive cells (d). A similar pattern is seen for ED-1. Fisher F334 control (e); Fisher F334 + FK506 (f); IHR (g); IHR + FK506 (H); V, vessel.

4.3.6 The Effect Of FK506 On Transgene Activation.

Circulating prorenin was elevated in both transgenic groups. Immunoprecipitation confirmed that this was mouse in origin and as such, transgene derived. For all components of the RAS, this elevation was greatest in the drug treated transgenics (figure 4.11a-c). To determine if this occurred at the level of transgene expression, real time rtPCR was performed. As expected no expression of mouse renin was detected above background in the control groups. Transgenic rats receiving FK506 had a higher level of expression than the water treated group suggesting that FK506 was capable of inducing transgene expression (figure 4.11d).

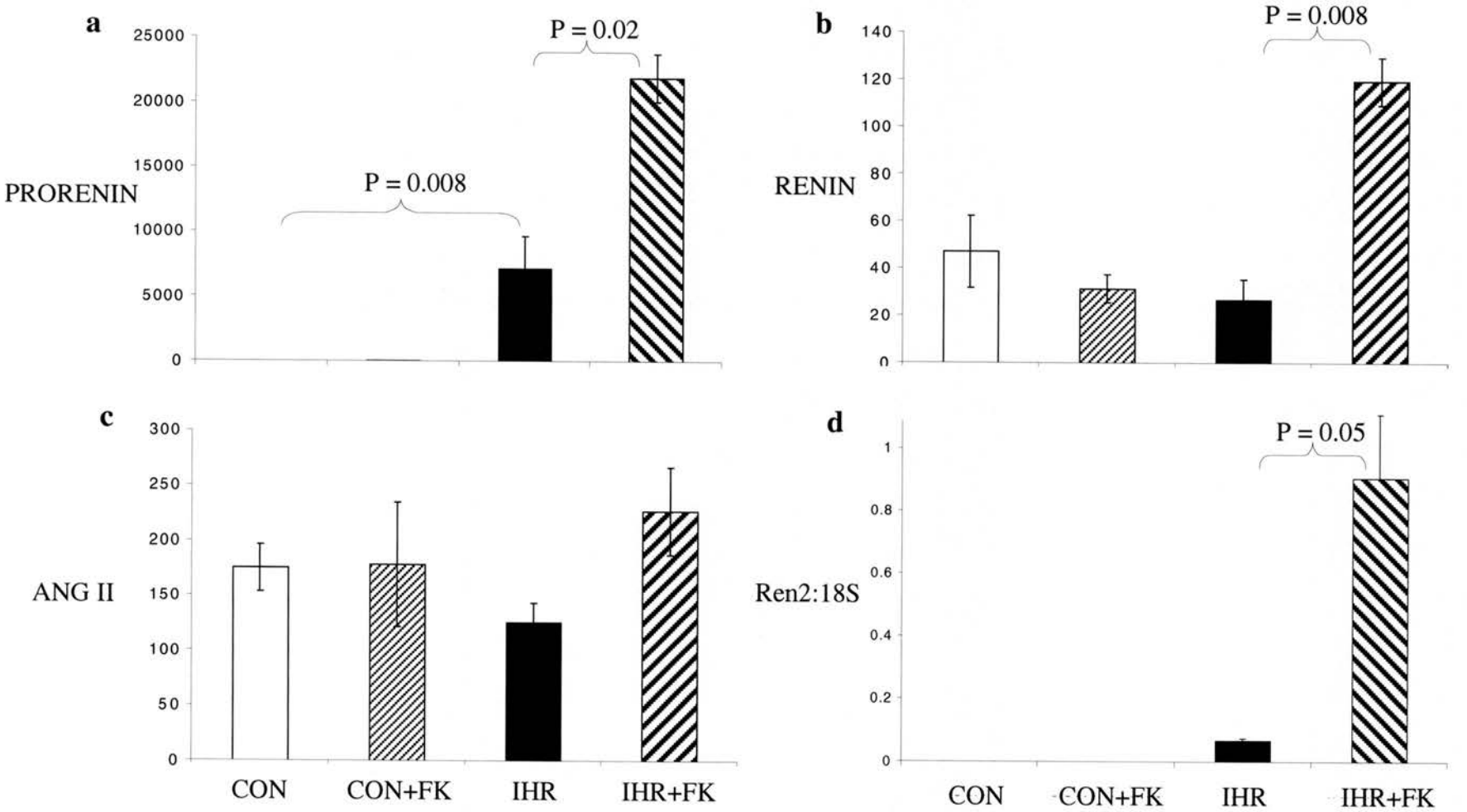

Figure 4.11. Circulating Renin Angiotensin System After Fourteen Days Induction.

Plasma levels of prorenin (a), renin (b) in ng/AngI/ml/hr and angiotensin II (c) in ng/ml are shown. Hepatic renin expression calculated by rtPCR is shown relative to 18S expression (d). CON, Fisher F334 control; IHR, Inducible Hypertensive Rat.

4.4 DISCUSSION.

The results presented in this chapter demonstrate that FK506 can abolish the development of hypertension and vascular remodelling leading to fibrinoid necrosis and end-organ injury in the IHR model.

The finding that hypertension was prevented in FK506-treated animals was unexpected, particularly since in toxicity, calcineurin inhibitors are strongly associated with the development of hypertension in both humans and rodents [Rabkin et al., 2002; Takeda et al., 1999]. In rats four weeks of FK506 at a dose of 5mg/kg/day (five times the dose used here) resulted in a 50% elevation of blood pressure associated with reduced eNOS but increased ET-1 expression and was inhibited using an endothelin A antagonist [Takeda et al., 1999]. Additionally, FK506 and cyclosporin have been used in numerous rodent models of hypertension to investigate cardiac hypertrophy and with few exceptions, were capable of reducing hypertrophy but without lowering blood pressure [Lim et al., 2000; Luo et al., 1998; Murat et al., 2000; Shimoyama et al., 1999; Shimoyama et al., 2000]. In a few examples blood pressure was attenuated, but never normalised [Meguro et al., 1999; Mervaala et al., 2000; Oie et al., 2000]. In one comparable study [Mervaala et al., 2000], using a double transgenic rat model expressing the human angiotensinogen and renin genes constitutively, cyclosporin treatment resulted in a reduction in end-organ injury and hypertension. The effects seen here were less marked than in our own study possibly due to the constitutive nature of this model. The other studies were performed using mice where the measurement of blood pressure was complicated by anaesthesia.

The inhibition of hypertension observed in our study was not complete since on day five IHRs regardless of FK506 treatment, were equally hypertensive and it was only beyond this point that the drug-treated group became normotensive. This finding was confirmed using two identical experimental groups at different times and by two separate observers. Day five is prior to significant vascular remodelling of the systemic peripheral vasculature in the IHR and it is possible that the early (prior to the establishment of vascular remodelling) and late (when remodelling has occurred) mechanisms for sustaining hypertension differ in this model. FK506 was unable to influence the early phase possibly mediated by vasoconstriction, however the effect of FK506 on established hypertension was not tested since vascular remodelling was prevented from developing.

There are features of the IHR that are unique when compared to existing hypertensive rodent models driven by the renin-angiotensin system, that may account for our dramatic findings: Because of the inducible nature of the transgene, hypertension can be generated at will, on a normal cardiovascular background, allowing any intervention to target the early stages in the pathogenesis of MH. As such, the pre-emptive treatment used here, may have covered a critical, responsive period in the development of MH. Additionally, circulating prorenin is very high in the IHR, while other down-stream components of the renin-angiotensin system including angiotensin II, the traditional effecter molecule of the system, were not significantly elevated. Although circulating levels do not elude to tissue activity, we cannot exclude that prorenin in such supra-physiological quantities, was not able to exert some effect involved in the development

of MH in the IHR, that is responsive to FK506. However, the role of prorenin in the development of hypertension remains highly controversial.

The pathological findings clearly demonstrated a complete absence of fibrinoid necrosis in the IHR group treated with FK506 in all tissues examined. Assessment of vascular remodelling is consistent with the day seven medial vascular smooth muscle cell proliferative response having not taken place. This was still evident after fourteen days of induction (figures 4.7 & 4.8) and suggests that medial remodelling in the form of vascular smooth muscle cell hyperplasia is required for the later development of necrotic vascular injury. Since circulating transgene levels were actually higher in FK506-treated rats, medial remodelling may have failed to occur either because of the absence of hypertension at this time-point, or as a consequence of the failure of earlier inflammatory cell accumulation in the adventitia. The adventitial fibroblast proliferative response known to occur from day two, was also reduced on day seven and it can be assumed, did not occur at this earlier time point (figure 4.8).

The adventitial T cell infiltrate was reduced to control levels on day seven in the transgenic group treated with FK506, suggesting that FK506 was capable of preventing the accumulation of these cells. There was a similar trend in the macrophage population at this time point suggesting inter-dependence between these two cell types or macrophage susceptibility to FK506. However, following fourteen days of induction, both control and transgenic rats treated with FK506 had a significant increase in T cell infiltration above non-drug treated controls. In these animals treated with FK506 for a total of seventeen days, we observed a mild interstitial nephritis, likely to explain the apparent rise in peri-vascular inflammation at this time point.

T cell and monocyte/macrophage populations are known to express components of the renin-angiotensin system including AT1, AT2 and ACE and angiotensin II-induced activation of T cells and monocyte activation may occur via calcineurin-dependant pathways [Conboy et al., 1999; Costerousse et al., 1993; Kunert-Radek et al., 1994; Lazarus et al., 1994; Nataraj et al., 1999; Okamura et al., 1999; Rohrbach and Conrad, 1991; Weinstock and Blum, 1984]. Additionally, FK506 can also modulate non-lymphoid cell types such as fibroblasts via NFκB and this has been proposed as a mechanism contributing to its' nephrotoxicity [Muraoka et al., 1996].

Inhibitors of calcineurin have a high degree of T cell specificity, thought to derive from wide variations in intracellular calcineurin concentrations. Concentrations of 10nM FK506 were sufficient to inhibit 70% of calcineurin activity in lymphocytes that contain only 5000 molecules of calcineurin per cell. However the same dose would inhibit only 4% of calcineurin activity within cardiomyocytes or hippocampus cells which contain 200,000 molecules per cell [Crabtree, 1999; Klee et al., 1998]. Given this, T cells should be the first cell-type to succumb to the effects of calcineurin inhibition, and as such, may be responsible for the inhibition of vascular smooth muscle cell proliferation occurring later. Consequent upon this lack of vascular remodelling, sustained hypertension would not develop, as total peripheral resistance would remain unchanged.

While it is established that immune dysfunction associates with hypertension in rats and humans [Khraibi, 1991], it is uncertain if this occurs as a primary or secondary event. Immune modulation in the form of thymectomy or administration of cyclophosphamide, has been shown to improve hypertension in some spontaneously

hypertensive rat stains [Khraibi et al., 1984; Khraibi et al., 1987]. Bush *et al* demonstrated that the macrophage MCP-1 receptor CCR2 was in part required for the development of angiotensin II-induced end-organ injury, but not hypertension, in mice [Bush et al., 2000]. Also the development of subsequent salt-sensitive hypertension induced by renal injury caused by L-NAME administration to rats was prevented by the co-administration of mycophenolate mofetil (MMF), a strong inhibitor of lymphocyte proliferation. Although MMF had no effect on L-NAME induced hypertension it did reduce the numbers of infiltrating T cells and angiotensin II expressing cells in the interstitium and subsequent remodelling proposed to mediate salt-sensitivity [Quiroz et al., 2001]. MMF also prevented salt-sensitive hypertension induced by angiotensin II infusion in rats [Rodriguez-Iturbe et al., 2001]. In proliferative vasculopathies such as atherosclerosis and post angioplasty re-stenosis, the contributory role of inflammation in their pathogenesis is accepted [Simon et al., 2000; Tellides et al., 2000].

While it is attractive to attribute the effects of FK506 solely to an inhibition of T cell activation, it is unlikely to be the complete story. Although FK506 is widely and effectively used in humans as an immunosuppressant, and as mentioned above, is relatively specific in its ability to inhibit T cell activation, calcineurin is a highly conserved, ubiquitous signalling pathway, present in many cell-types [Klee et al., 1998]. As such, it is difficult to exclude that other cell types have not mediated, wholly or partially, these effects.

Additionally, non-calcineurin dependant pathways of FK506 activity have now been identified, opening up additional mechanisms of action. TGFβ has been implicated as one possible mediator of nephrotoxicity caused by the drug, and treatment of mice with

an anti-TGFβ1 antibody was able to reduce histological injury [Khanna et al., 1999]. The immunomodulatory effects of TGFβ were in part attributable to IL-2 inhibition [Brabletz et al., 1993] and TGFβ administered by plasmid gene transfer was capable of enhancing murine cardiac graft survival [Qin et al., 1994]. It has also been proposed that cyclosporin and FK506 can inhibit the JNK and p38 members of the MAPK family, also involved in IL-2 induction independently of calcineurin via AP-1, and this combined calcineurin and MAPK blockade results in their powerful inhibition of T cell activation [Matsuda et al., 2000]. FK506 has also been shown in vitro, to alter the transcription of mineralocorticoid receptors and inhibit aldosterone-induced sodium transport, which could explain the relative mineralocorticoid insensitivity of patients treated with this drug [Deppe et al., 2002; Rokaw et al., 1996]. In the IHR where circulating aldosterone is elevated and may contribute to pathology, this mechanism of FK506 action could be important. Thus, non-calcineurin pathways could in theory, have mediated the effects seen in this study and via multiple cell types and mechanisms.

Vascular smooth muscle cells should be considered as a potential target for FK506, as inhibiting their proliferation would undoubtedly alter vascular remodelling. We found that FK506 did not alter baseline vascular smooth muscle cell proliferation in Fisher F334 controls suggesting that at this dose, FK506 was unable to alter vascular smooth muscle cell turnover (figures 4.7 & 4.8).

At the doses used in this study, FK506 did not induce hypertension and was not functionally or histologically nephrotoxic and trough FK506 levels were performed on two animals and found to be six and seven μg/L, well within the non-toxic range for

humans. Fisher F334 rats treated with FK506 did display weight loss consistent with drug toxicity but weight loss seen in the transgenic group treated with FK506 when compared to the non-drug treated transgenic animals was minimal, and as such, unlikely to have influenced any weight-adjusted data.

Transgene induction was enhanced by FK506, which is metabolised by *Cyp3a3,* via increased hepatic transgene expression. Possibly, FK506 can induce *Cyp1a1* directly or alternatively, *Cyp3a3* may act indirectly on *Cyp1a1* to enhance expression. Throughout the study period food intake was observed daily and found to be equivalent between the transgenic groups. Consequently, IHRs treated with FK506 were subject to higher circulating levels of the renin-angiotensin system than transgenic rats receiving vehicle injections.

In summary, hypertension and vascular remodelling were prevented in the IHR by FK506 treatment. This is the first time that hypertension and end-organ injury have been abolished using FK506 in an animal model of hypertension and these striking results may in part, be mediated by the inducible nature of the IHR facilitating pre-emptive treatment. However, given the data from other studies where only minimal reductions in blood pressure were reported, our results may pertain specifically to the IHR model. The initial data suggests that the potent effects of FK506 may be due to the early inhibition of infiltrating inflammatory cells, suggesting that they are an important, early stage in the pathogenesis of vascular remodelling and hypertension in the IHR.

CHAPTER FIVE.

THE ROLE OF MACROPHAGES IN VASCULAR REMODELLING IN A MOUSE MODEL OF MALIGNANT HYPERTENSION.

5.1 INTRODUCTION.

The identification of inflammatory cells early on during the development of hypertensive vascular remodelling in the IHR raises the possibility that the salutary effects of FK506 treatment were mediated via immunomodulation. Given the limitations at present in targeting inflammatory cells experimentally in the rat, a hypertensive mouse, resembling the IHR, was developed to investigate the role of inflammation in hypertensive vascular remodelling.

A transgenic approach was not adopted due to time limitations and to avoid the need to cross breed with "knockout" strains in future experiments. Angiotensin II infusion was chosen since it can induce hypertension and end organ injury, associated with fibrinoid necrosis and inflammation in rats [Wiener et al., 1996]. Similarly to the IHR, pathology is also associated with high levels of circulating angiotensin II.

Mice display greater sensitivity to blockade of the RAS than rats, but are more resistant to the effects of exogenous angiotensin II, requiring much higher doses to achieve a similar response. Twenty-five times the dose used to illicit a modest effect in the rat, causes in no detectable response in mice. (20-500ng/kg/min will achieve a range of

effects in rats *versus* greater than 1000ng/kg/min needed to achieve an effect in mice). In part this is due to differences in angiotensin II catabolism and receptor regulation [Cassis et al., 2004; Cholewa et al., 2005; Daugherty and Cassis, 2004]. Cassis *et al* showed that similar infusions of angiotensin II resulted in higher circulating levels of the peptide and its' catabolic fragments in mice compared to rats and that infusion was associated with significantly reduced receptor density in the kidney. This combination of accelerated catabolism and enhanced receptor down regulation could be responsible for relative insensitivity to angiotensin II seen in mice. Angiotensin II infusion also increases AT2 receptor expression within the renal cortex. Specific inhibition of AT2 using PD-123,319, increased angiotensin II induced hypertension, suggesting AT2 plays a protective role [Wesseling et al., 2005].

Peri-aortic macrophage infiltration is associated with angiotensin II infusion in mice and can be attenuated, together with CCR2 expression, by sFlt-1 gene transfer suggesting that angiotensin II induced vascular inflammation is mediated by VEGF [Zhao et al., 2004]. Studies using a CCR2 knockout mouse suggested that the expression of this receptor on macrophages was critical for large vessel hypertrophy and macrophage recruitment during low dose angiotensin II infusion [Bush et al., 2000; Ishibashi et al., 2004]. However, less is known regarding the effects of exogenous angiotensin II on resistance vessel remodelling in mice.

The CD11bDTR mouse model expresses the human diphtheria toxin (DT) receptor under control of the CD11b promoter [Cailhier et al., 2005]. Since the mouse DT receptor (hbEGF) binds DT poorly, expression of the human receptor conveys toxin-sensitivity [Cha et al., 2003; Naglich et al., 1992]. Additionally, restricting expression

to cells of macrophage and granulocyte lineage permits a cell targeting strategy. In this model, DT conveniently does not deplete neutrophil, T cell or B cell numbers [Cailhier et al., 2005; Duffield et al., 2005]. DT specific cell ablation has also been used to target hepatocytes and cardiomyocytes [Akazawa et al., 2004; Saito et al., 2001]. 20ng/g of DT administered intraperitoneally results in ablation of 99% of peritoneal and 60% of renal macrophages by 24 hours. The effects on circulating monocyte/macrophage populations are less clear. This conditional transgenic, macrophage ablation model was used to investigate the effects of depleting resident peritoneal macrophages on the development of hypertensive vascular remodelling, induced by angiotensin II, in mesenteric vessels.

5.2 EXPERIMENTAL DESIGN.

Angiotensin II was infused into FVB/NJ and C57Bl/6 mice. The CD11bDTR model, described above, is on an FVB/NJ background. C57Bl/6 mice were included since they have good breeding characteristics and strains carrying targeted disruption of genes are commonly maintained on this background. The concentration of angiotensin II infused was initially titrated (0.5, 1.0 and 2.0μg/g/day *vs* 0.9% saline) to determine the amount of peptide required to generate a MH phenotype for future experiments. This was defined histologically by the presence of fibrinoid necrosis. A dose of 2.0μg/g/day was determined to be effective for both strains, although FVB/NJ mice developed fibrinoid necrosis over a shorter time scale. Angiotensin II or 0.9% saline were administered continuously via ALZET 2002 mini osmotic pumps implanted subcutaneously under light general anaesthesia. Animals were culled by CO_2 inhalation and cervical dislocation, blood was taken by cardiac puncture and tissues fixed for histology. Male mice (n=5 per group) were used throughout due to their greater responsiveness to angiotensin II infusion and to avoid sexual dimorphism [Xue et al., 2004]. C57Bl/6 mice were housed within the Biological Resource Facility at the Western General Hospital, Edinburgh. Due to their location within the Hugh Robson Building at the University of Edinburgh, tail cuff plethysmography was not feasible on FVB/NJ or CD11bDTR mice, since transfer of equipment between sites was prohibited.

The following experiments were performed (figure 5.1):

1. Two groups of FVB/NJ mice (total ten animals) received either 2.0μg/g/day angiotensin II or 0.9% saline infusion for ten days.

2. Four groups of CD11bDTR mice on an FVB/NJ background (total 20 animals) received either 2.0μg/g/day angiotensin II or 0.9% saline infusion for nine days. Either DT (20ng/g) or 0.9% saline was injected on days two, four and six. Since striking fibrinoid necrosis was observed in experiment 1, a nine-day infusion period was chosen to reduce the toxicity to the mice from DT exposure. Mr Spike Clay, being adequately licensed and desensitised, carried out the injections of DT.
3. Two groups of C57Bl/6 mice received either 2.0μg/g/day angiotensin II or 0.9% saline infusion for seven, nine or 14 days (total 30 animals). Tail cuff plethysmography was used to measure blood pressure in the 14-day group. Recordings were taken daily for one week prior to the beginning of the experiment to familiarise the mice to the procedure. The shortest infusion time was chosen to examine vascular remodelling, and principally macrophage infiltration, prior to the development fibrinoid necrosis.

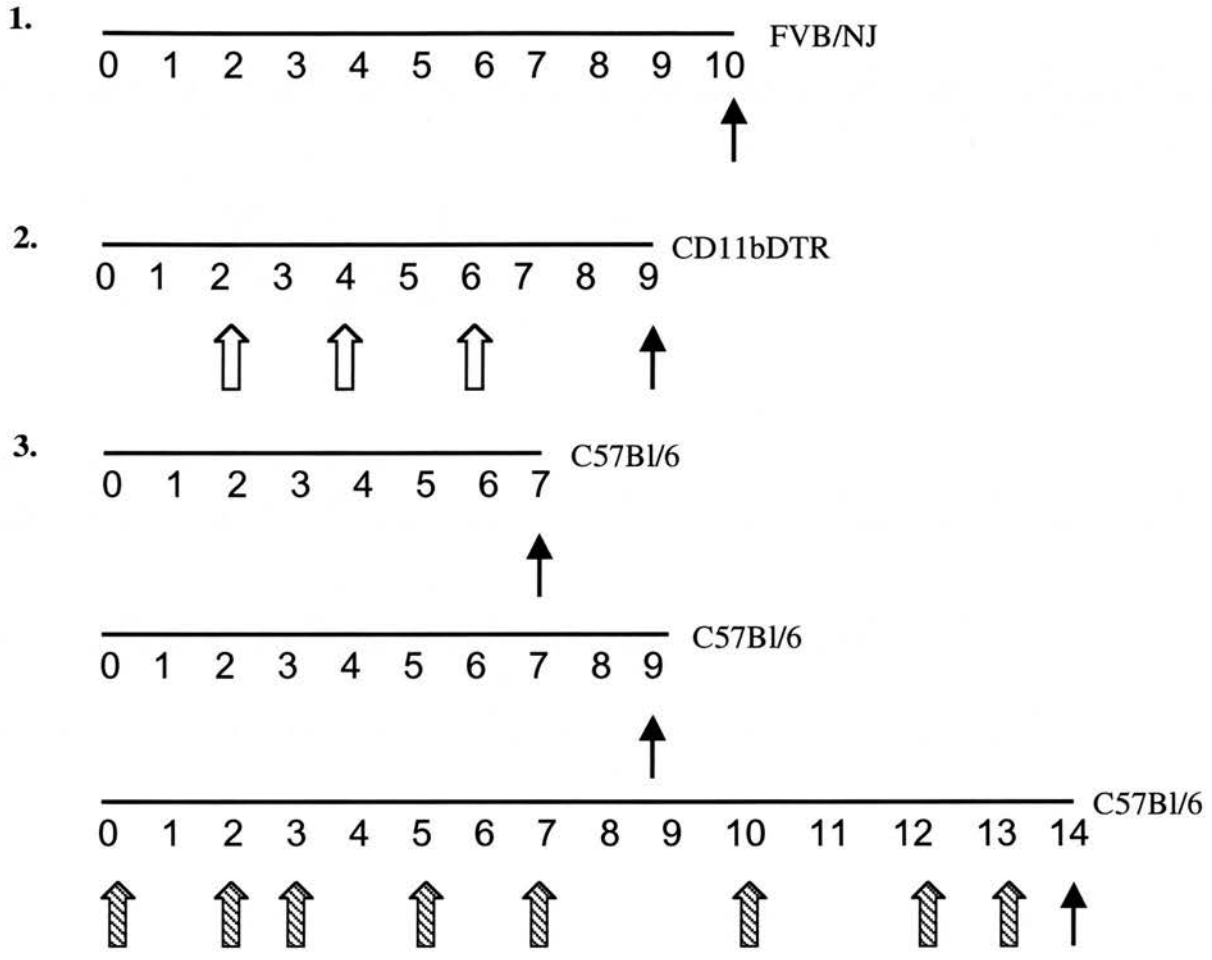

Figure 5.1. Experimental Timelines For Angiotensin II Infusion Into Mice. 2μg/g/day angiotensin II or 0.9% saline infusions are represented by the solid black line for each experiment (1-3). The mouse strain is shown at the end of each timeline. Cull dates (black arrow), blood pressure recordings (hatched arrow) and diphtheria toxin (20ng/g) or 0.9% saline injection (open arrow) are shown beneath the timescale in days.

5.3 RESULTS.

5.3.1 The Effect Of Macrophage Depletion On The Development Of Vascular Injury In Mice.

The CD11bDTR mouse model was used to determine the role of macrophages in the development of vascular remodelling induced by angiotensin II. Initially angiotensin II was infused into FVB/NJ mice, since the CD11bDTR model is on this background. A ten-day infusion resulted in fibrinoid necrosis in the heart and mesentery (figure 5.2). Mesenteric vessels were of particular interest since macrophage depletion is greatest here. As in the IHR, fibrinoid necrosis was most severe in grades III and IV mesenteric vessels (figure 5.2).

Angiotensin II infusion was also accompanied by significant weight loss and an increase in cardiac mass index indicative of left ventricular hypertrophy (table 5.1).

	SALINE	**ANGII**
% WC day 10	4.9±1.6	-14.0±0.3 *
CI day 10	5.37±0.21	6.45±0.23 *

Table 5.1. Angiotensin II Induces Weight Loss And Cardiac Hypertrophy In FVB/NJ Mice.

* $p<0.05$; CI:cardiac mass index; %WC:percentage weight change; AngII:angiotensin II.

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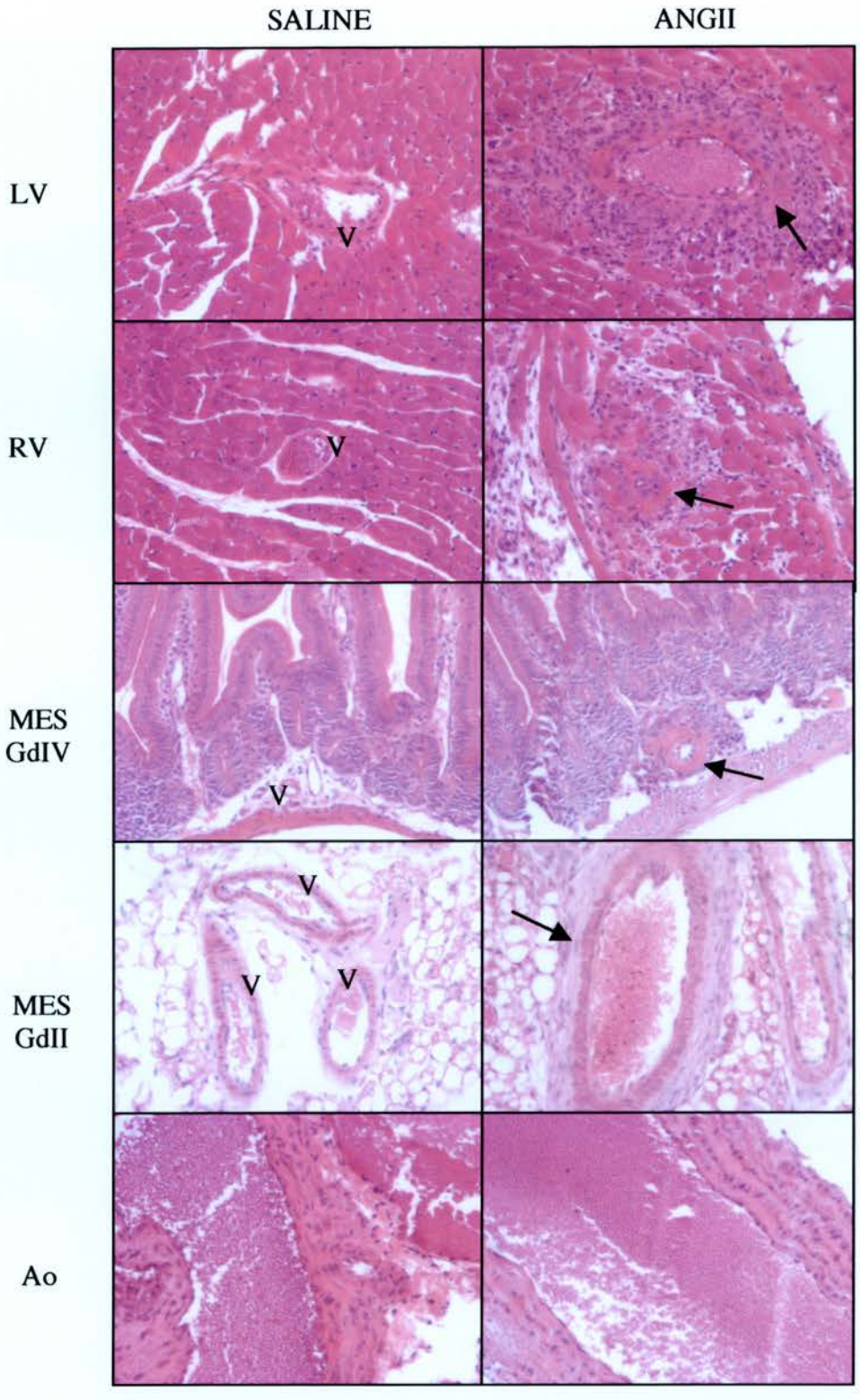

Figure 5.2. Effect Of Angiotensin II Infusion For 10 Days On FVB/NJ Mice.

H&E histology showing fibrinoid necrosis in the cardiac and mesenteric vessels (arrows). Pathology was restricted to resistance vessels with large vessel sparing. AngII:angiotensin II; RV:right ventricle; LV:left ventricle; MES:mesentery; Ao:aorta; Gd II/IV:grade two/four mesenteric vessel; V:normal vessel.

To determine the effect of macrophage depletion on the development of vascular remodelling, ALZET pumps were inserted into CD11bDTR mice and DT administered as detailed above. Sacrifice had to be carried out earlier than scheduled on day six due to the poor condition of mice receiving DT and no injection of DT was given on day six as planned. Only mice administered DT appeared unwell, irrespective of whether they received angiotensin II or saline from the mini-pump, so that DT appeared to be the factor responsible for the condition of the mice.

Angiotensin II induced weight loss and this was greater if DT was given as well. However DT alone was able to induce weight loss, suggesting that administration of DT in this model was associated with significant systemic toxicity (table 5.2). There was a tendency for cardiac mass index to be increased by day six of angiotensin II infusion. Given the weight loss associated with DT administration cardiac index should be interpreted with caution but overall, cardiac hypertrophy induced by angiotensin II appears unaffected by DT administration (table 5.2).

	SALINE	**ANGII**	**SALINE+DT**	**ANGII+DT**
% WC	2.6±0.5	-6.5±1.0 *	-14.2±1.2~	-18.7±1.3**/^{NS}
CI	5.32±0.13	5.82±0.07~~	5.45±0.25	6.06±0.20^{NS}

Table 5.2. Effect Of Angiotensin II On Weight And Cardiac Index In CD11bDTR Mice .

* p<0.05, saline *vs* angiotensin II; ~ p<0.05, saline *vs* saline+DT; ** p<0.05, angiotensin II+DT *vs* angiotensin II; ^{NS} not significant angiotensin II+DT *vs* saline+DT; ~~ not significant saline *vs* angiotensin II; CI:cardiac mass index; %WC:percentage weight change; AngII:angiotensin II; DT:diphtheria toxin.

DTR mice infused with angiotensin II but not exposed to DT, developed fibrinoid necrosis within the heart, mesentery and to a lesser extent kidney, as expected (figures 5.3-5.5). Administration of DT to CD11bDTR mice infused with angiotensin II resulted in the abolition of vascular remodelling within the mesentery (figure 5.3). However, DT had no effect on vascular remodelling within the kidney or heart (figures 5.4 & 5.5). There was evidence of histological toxicity in the DT treated group infused with saline, but no vasculopathy. Altered myocardial repair was observed in the angiotensin II + DT group. Classical myocardial necrosis, in association with infiltrating inflammatory cells and fibroblasts, was not seen. Instead, the cellular response to myocyte death was absent and matrix deposition was reduced (figure 5.6).

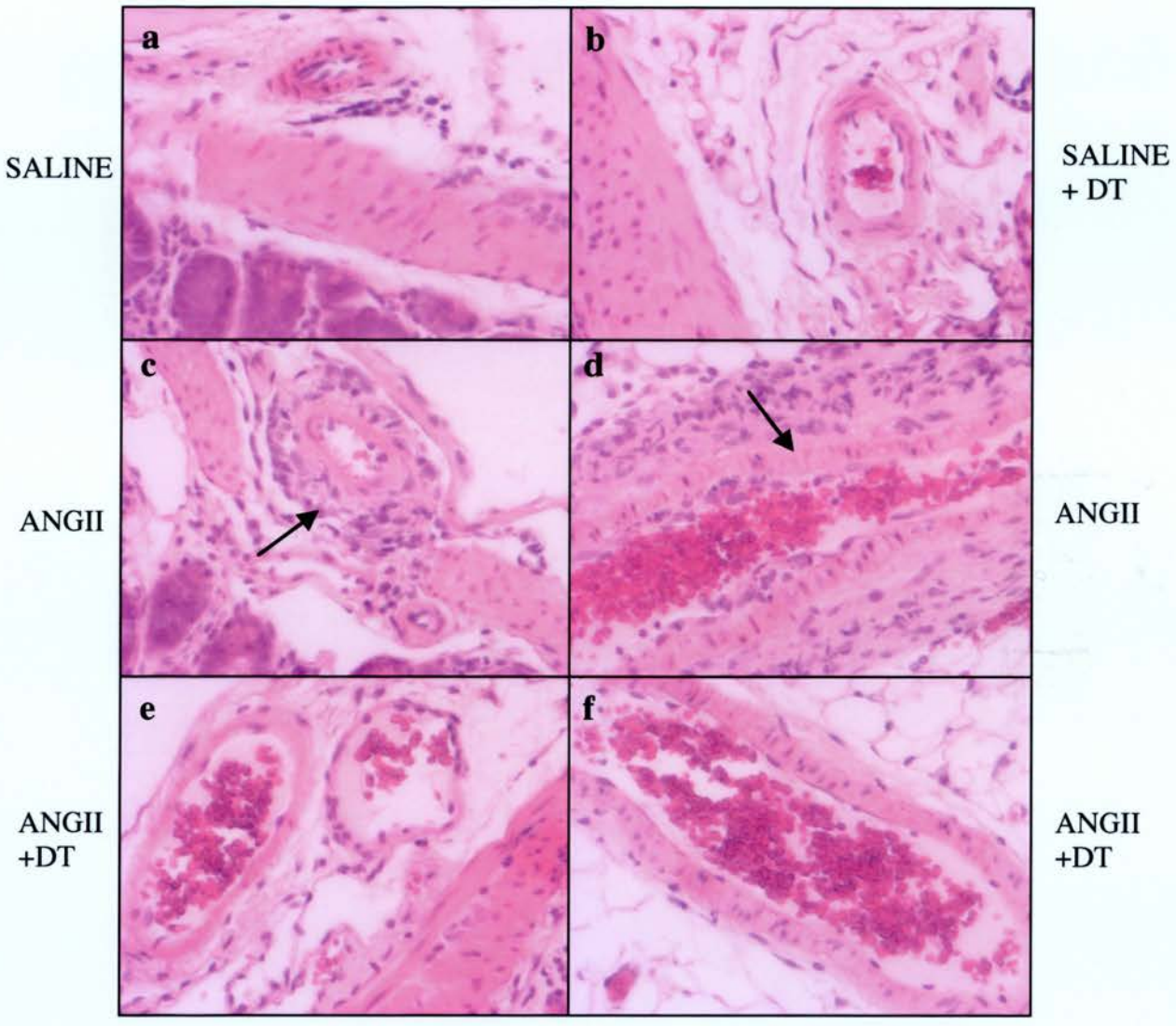

Figure 5.3. Effects Of Angiotensin II Infusion And DT Treatment On Mesenteric Vessels.

Administration of DT alone was not associated with obvious histological toxicity (b). Angiotensin II infusion generated fibrinoid necrosis (c & d, arrows). Vessels appear normal following DT treatment (e & f). ANGII:angiotensin II; DT:diphtheria toxin.

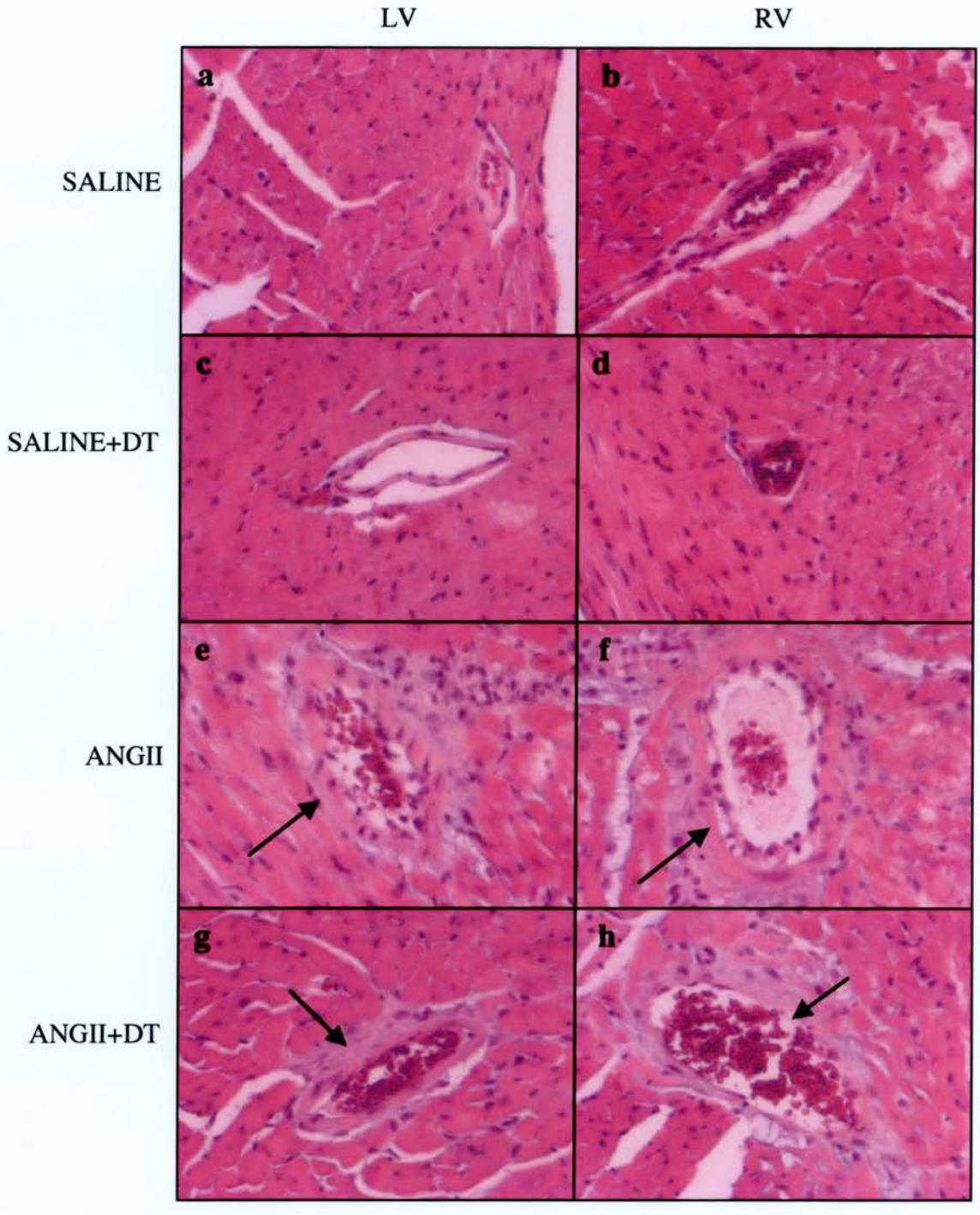

Figure 5.4. Effects Of Angiotensin II Infusion And DT Treatment On Cardiac Vessels.

DT treatment was not associated with myocardial injury or vasculopathy (c&d). Angiotensin II infusion resulted in fibrinoid necrosis as expected (e&f, arrows). This was unaffected by DT treatment (g&h, arrows). ANGII:angiotensin II; DT:diphtheria toxin; RV:right ventricle; LV:left ventricle.

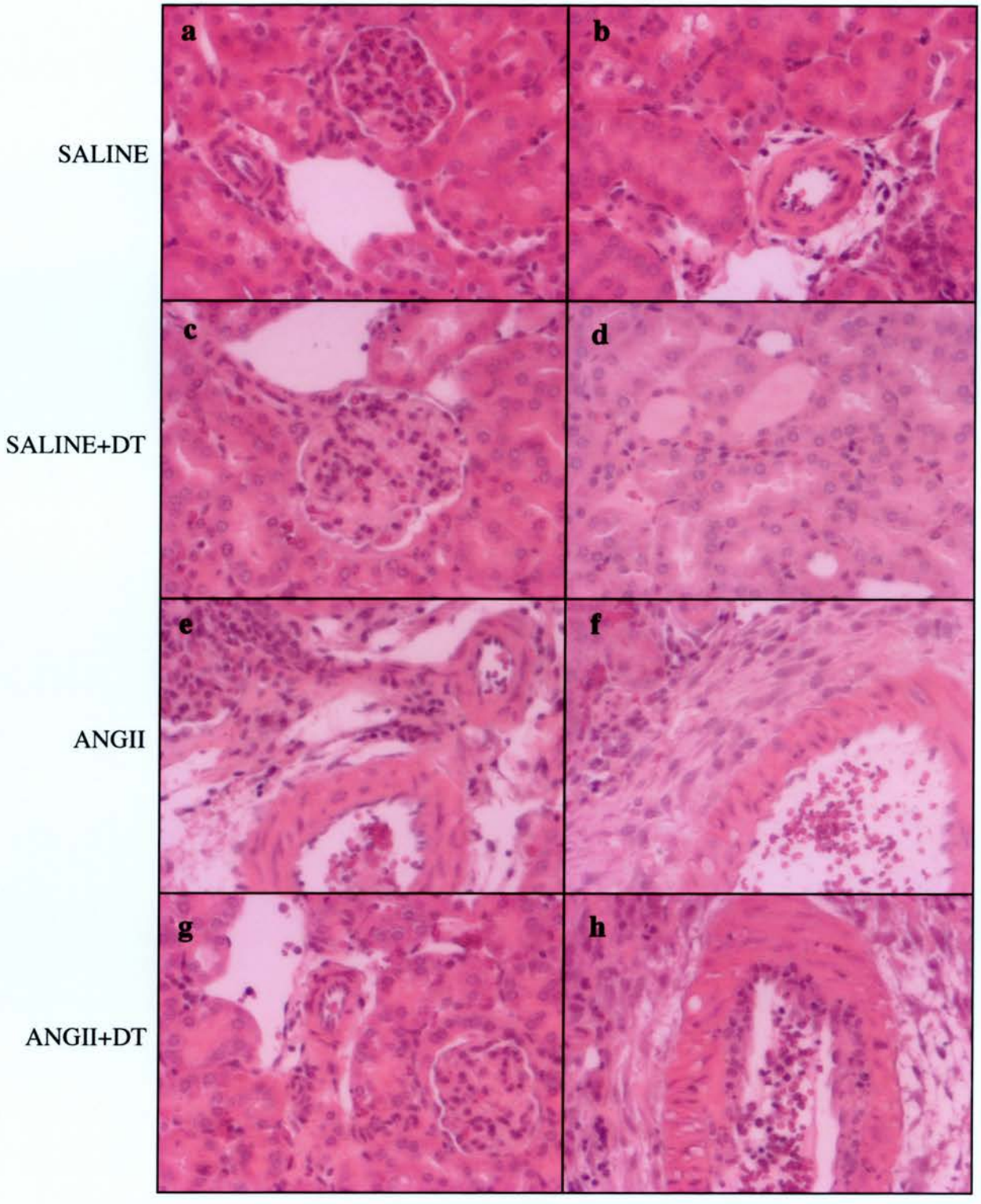

Figure 5.5. Effects Of Angiotensin II Infusion And DT Treatment On Renal Vessels.

DT administration resulted in renal tubular toxicity (d) but no vasculopathy (c).Angiotensin II induced mild to moderate changes in renal vessels (e & f) which were unaffected by DT treatment (g & h). ANGII:angiotensin II; DT:diphtheria toxin.

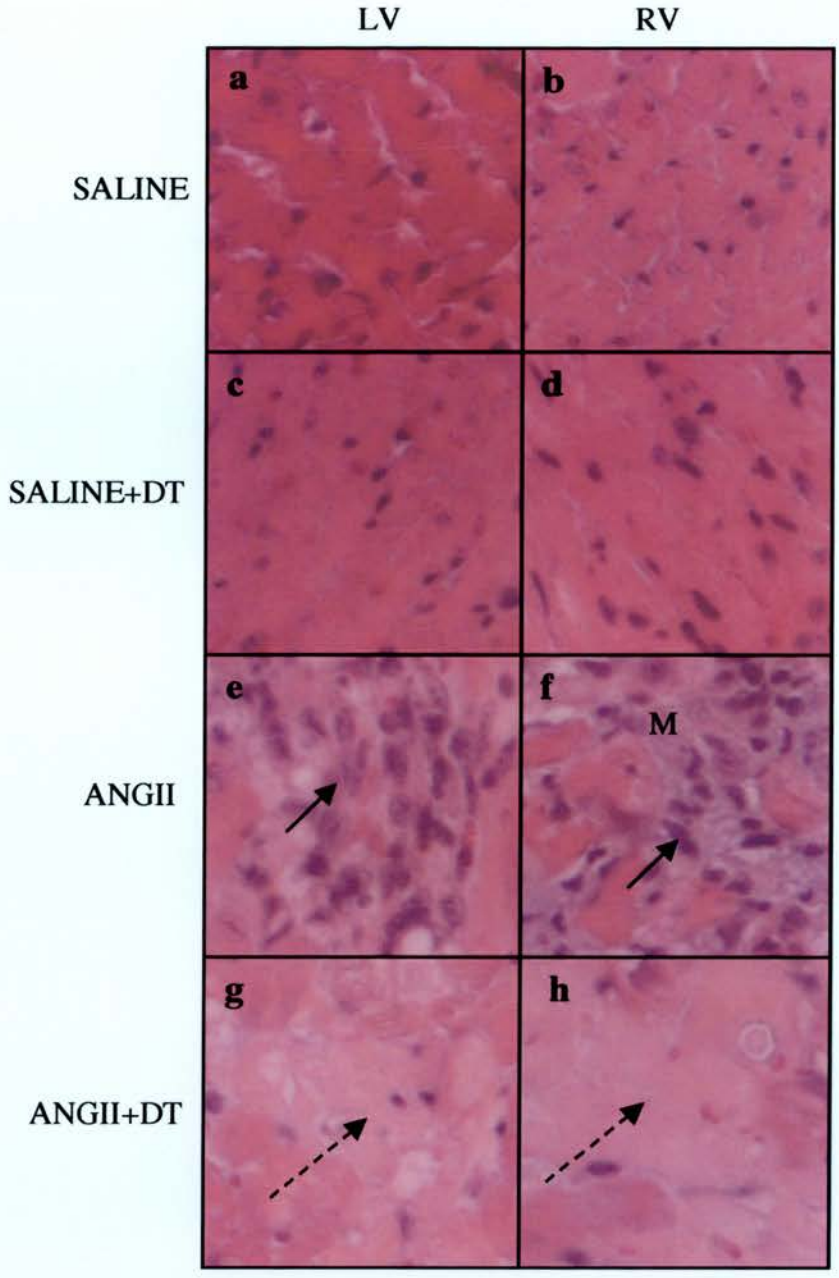

Figure 5.6. Effects Of Angiotensin II Infusion And DT Treatment On Myocardial Injury.

Angiotensin II infusion resulted in widespread myocardial necrosis (e & f)with associated cellular infiltration (solid arrows) and matrix deposition (light purple, M). DT treatment alone was not associated with myocardial injury (c & d). DT treatment with angiotensin II resulted in reduced matrix deposition and cellular response to myocardial injury (dashed arrows). ANGII:angiotensin II; DT:diphtheria toxin; RV:right ventricle; LV:left ventricle.

5.3.2 Angiotensin II Infusion Induced Hypertension And End-Organ Injury In C57Bl/6 Mice.

C57Bl/6 mice infused with 2μg/g/day angiotensin II for up to 14 days appeared well throughout. ALZET pump insertion was well tolerated and mice moved about normally following the procedure. Fibrinoid necrosis was present throughout the resistance vasculature, including mesenteric, cardiac and para-aortic vessels, of mice after nine days of angiotensin II (figure 5.7). Renal vessels demonstrated mild or absent pathology even after 14 days of angiotensin II infusion suggesting, as in the IHR model, that glomerular autoregulation afforded some protection from vascular injury. Comparatively, pathology was more severe in the FVB/NJ strain than C57Bl/6.

Significant weight loss was present by seven days and cardiac enlargement after 14 days (table 5.3).

	SALINE	**ANGII**
% WC day 7	3.4±1.1	-5.5±1.3 *
% WC day14	2.9±0.6	-6.4±1.5 *
CI day 9	6.01±0.32	6.87±0.31
CI day 14	5.12±0.11	7.33±0.31 *

Table 3. Angiotensin II Induces Weight Loss And Cardiac Hypertrophy In C57Bl/6 Mice.

* $p<0.05$; CI:cardiac mass index; %WC:percentage weight change; AngII:angiotensin II.

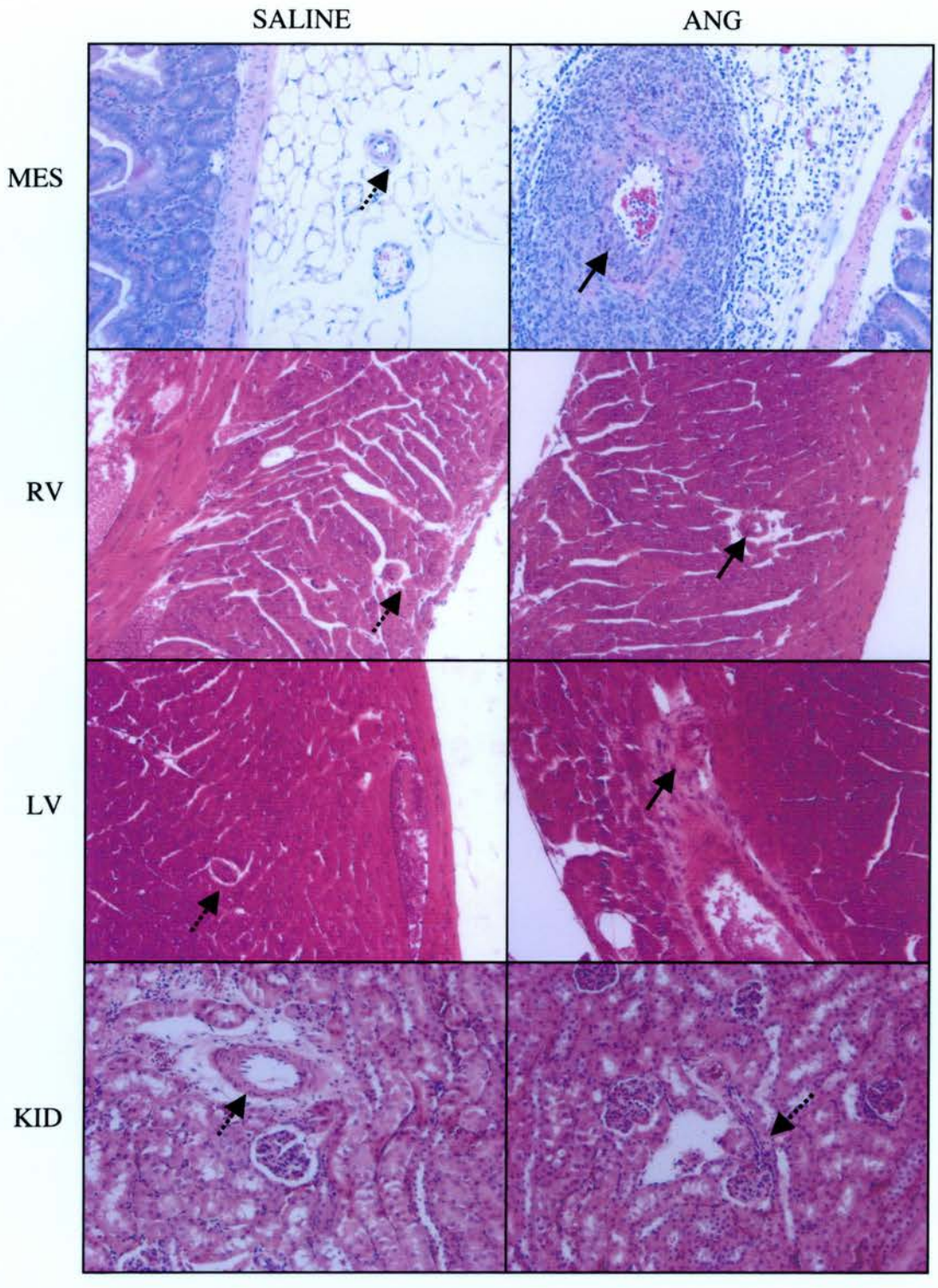

Figure 5.7. Angiotensin II Induces End Organ Injury In C57Bl/6 Mice After Nine Days.

H&E stained tissue is shown. Fibrinoid necrosis was present in the mesenteric and cardiac vessels. Resistance vessels within the kidney were only mildly affected. MES:mesentery; RV:right ventricle; LV:left ventricle; KID:kidney; ANGII:angiotensin II 2μg/g/day; solid arrow:fibrinoid necrosis; dashed arrow:normal vessel.

Systolic blood pressure, estimated by tail cuff plethysmography, was significantly elevated after two days of angiotensin II infusion (figure 5.8). Blood pressure rose steadily to reach a mean of 201.5+/-5.8mmHg *vs* 134.5+/-5.4mmHg in the control group by day 13. The systolic pressure of mice infused with normal saline remained between 118mmHg and 135mmHg on average.

As expected, plasma renin concentration and active renin were reduced by angiotensin II administration (figure 5.9). Circulating inactive renin (prorenin) was unaffected by 14 days of 2μg/g/day angiotensin II infusion.

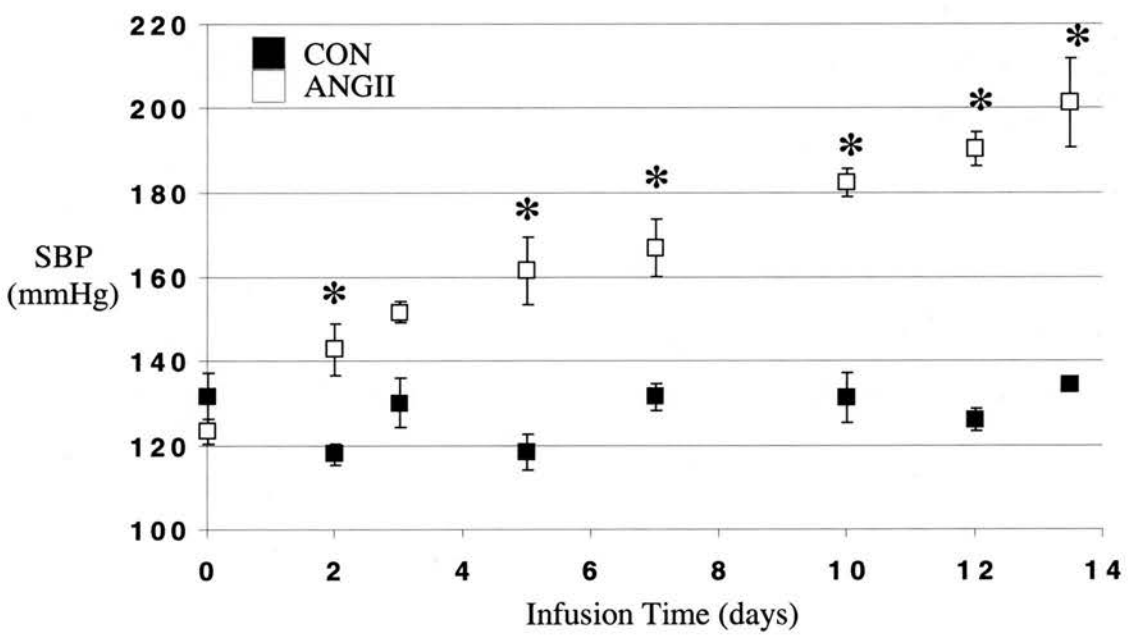

Figure 5.8. Angiotensin II Induces Severe Hypertension In C57Bl/6 Mice.

Tail cuff readings from Harvard apparatus. Systolic pressure reached 200mmHg after 14 days of Angiotensin II 2μg/g/day. Mice infused with saline remained normotensive. * $p<0.05$. ANGII:angiotensin II; CON:saline; SBP:systolic blood pressure.

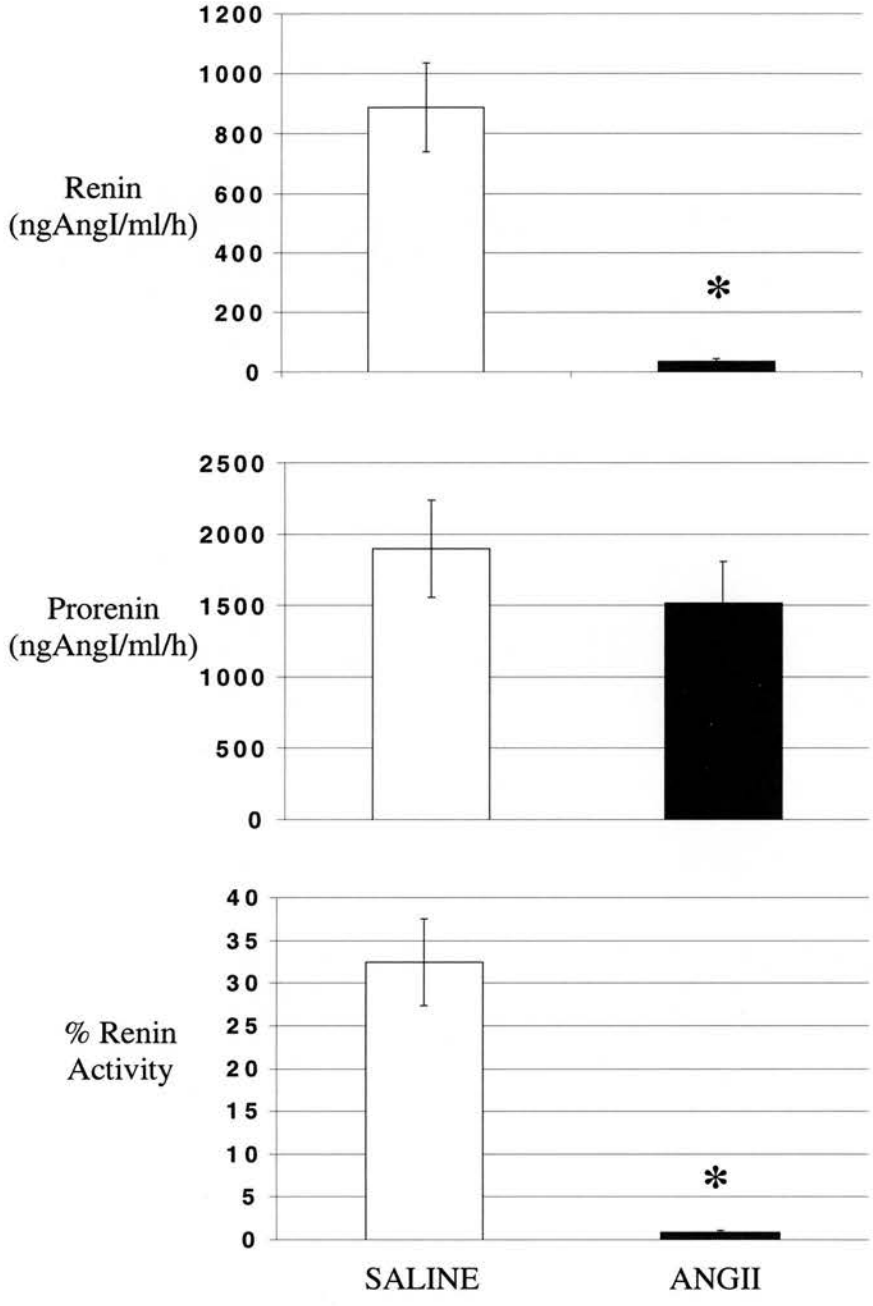

Figure 5.9. Circulating Renin In C57Bl/6 Following 14 Days Angiotensin II Infusion.

Renin and renin activity were suppressed by angiotensin II infusion. *$p<0.05$.

5.3.3 Peri-Vascular Inflammation Preceded Fibrinoid Necrosis Induced By Angiotensin II Infusion.

To determine if inflammation was present prior to the development of fibrinoid necrosis, C57Bl/6 mice were infused with 2μg/g/day angiotensin II for seven days. Mesenteric vessels were studied since they are susceptible to fibrinoid necrosis and easily visualised. H&E staining revealed a thickened perivascular cuff in the adventitial layer. Endothelial and medial vascular layers appeared intact, confirming that fibrinoid necrosis had not yet developed (Figure 5.10b). F4/80 staining revealed large numbers of macrophages within the adventitial cuff similar to the IHR model (figure 5.10d).

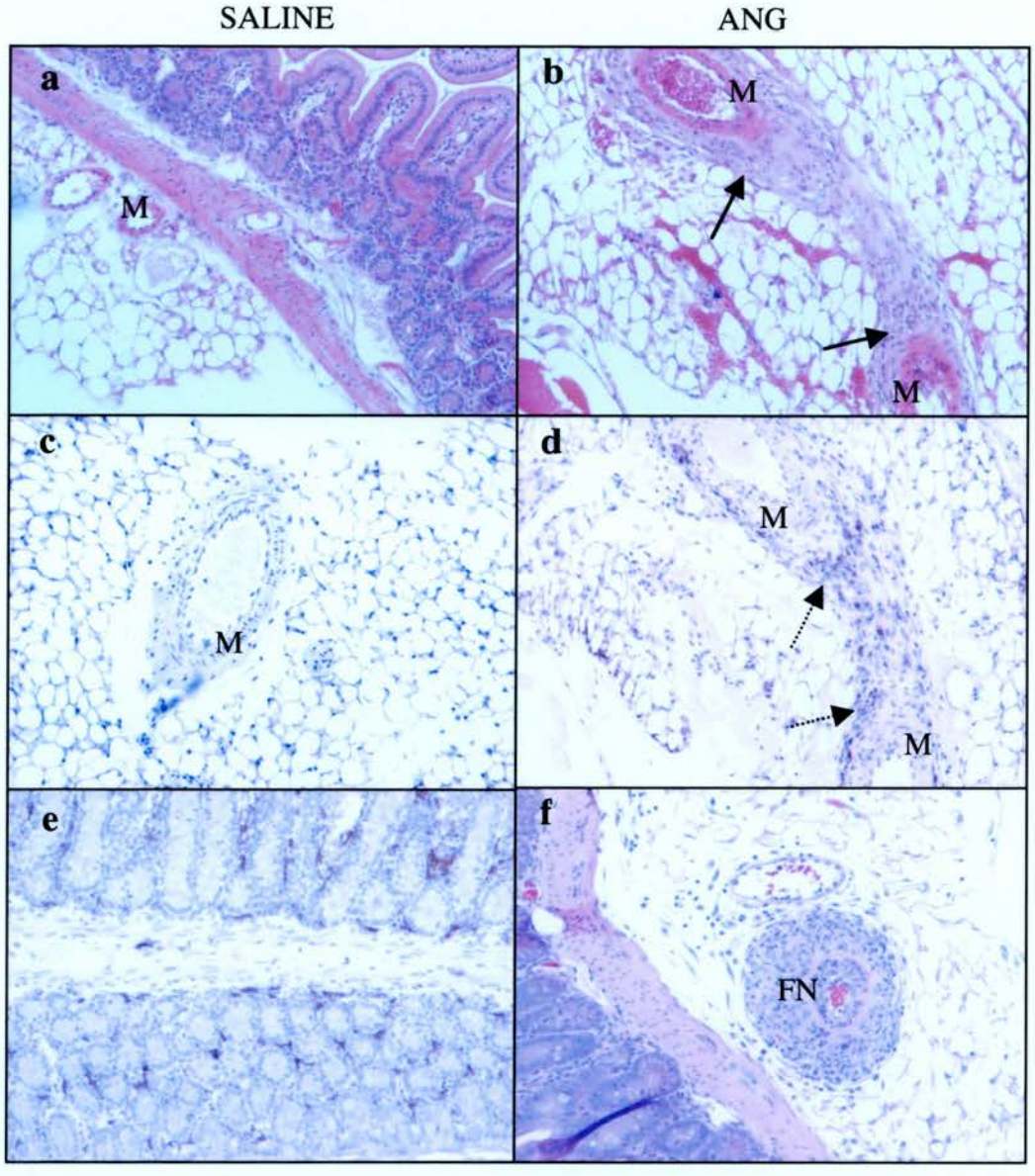

Figure 5.10. Angiotensin II Induces Inflammation Prior To Fibrinoid Necrosis In C57Bl/6 Mice.

Mesenteric vessels are shown following angiotensin II infusion at 2μg/g/day. H&E staining (a,b,f) shows a perivascular cuff surrounding vessels following seven days of angiotensin II infusion (b, solid arrows). F4/80 staining (brown, c,d,e) demonstrates macrophages within the cuff in a serial section (d, dashed arrows). Few macrophages surround normal vessels in control mice (c). A normal pattern of F4/80 staining was seen in control gut (e). Established fibrinoid necrosis after nine days of angiotensin II infusion (f). M:intact media; ANGII:angiotensin II infusion; FN:fibrinoid necrosis.

5.4 DISCUSSION.

Severe hypertension accompanied by fibrinoid necrosis of resistance vessels and cardiac hypertrophy developed in C57Bl/6 and FVB/NJ mice following administration of exogenous angiotensin II at a dose of 2μg/g/day. As such, angiotensin II infusion can be adapted to provide a good model of MH in the mouse. The use of ALZET mini osmotic pumps is cheap, fast, reproducible and transferable to different mouse models and strains permitting use in various experimental settings.

Peri-vascular macrophage infiltration was found in resistance vessels of C57Bl/6 mice infused with angiotensin II prior to the development of fibrinoid necrosis. These findings are similar to those seen in the IHR model and suggestive of a role for inflammatory cells in the development of fibrinoid necrosis. In order to determine the importance of macrophages in the pathogenesis of fibrinoid necrosis, angiotensin II was infused into CD11bDTR mice where macrophages can be conditionally ablated by administration of DT. The results of this experiment need to be interpreted cautiously due to the toxicity induced by DT. Additionally, premature termination of the experiment, required tissues to be collected in formalin rather than methacarn and as such, they were unsuitable for F4/80 staining. Similarly, FACS analysis of blood for circulating monocyte/macrophages was not performed as planned. Given these considerations, the results of this preliminary experiment are interesting and warrant confirmation and further investigation. The most striking finding was an absence of vascular remodelling within the mesentery of the DT-treated angiotensin II infusion group. In contrast vascular remodelling was unaffected in the kidney and heart. Since

macrophage ablation is most effective in the peritoneal cavity where DT is injected (99% versus 60% ablation in the kidney), this may account for these findings. If so, this is the first account implicating macrophages directly in the hypertensive remodelling of resistance vessels.

The beneficial effects of non-specific immunomodulation on vascular responses to injury, including hypertension, have been discussed in chapter four. However, the effect of macrophage depletion on vascular remodelling is less clear, and has not been well described in relation to small vessel hypertensive injury. Macrophage depletion using liposomal bisphonsphonates (LBP) including alendronate and clodronate, can alter large vessel response to injury: For example, LBP administered intravenously reduced neointimal formation in balloon-injured carotid arteries in rats and rabbits [Danenberg et al., 2003a; Danenberg et al., 2002]. Also neointimal hyperplasia and stenosis were reduced post angioplasty and stent insertion by LBP in hypercholestrolaemic rabbits [Danenberg et al., 2003b]. However, LBP administration can be toxic and macrophage depletion incomplete with associated reduction in neutrophil numbers [Feith et al., 1997]. Targeting macrophages using anti-macrophage sera is also associated with unwanted effects [Loewi et al., 1969].

CCR2 knockout mice, deficient in the monocyte/macrophage MCP-1 receptor, show reduced accumulation of vascular macrophages and neointimal formation following femoral artery injury [Egashira et al., 2002; Roque et al., 2002]. In an elegant experiment, leucocytes from CCR2 knockout mice were transplanted into wild-type mice then subject to angiotensin II infusion. The aortic accumulation of macrophages was reduced and medial hypertrophy attenuated implicating circulating macrophages

directly via a CCR2/MCP-1 pathway, in the development of angiotensin II induced large vessel remodelling [Ishibashi et al., 2004].

It is possible that the dramatic effects seen in the mesentery were not the result of macrophage depletion. The proximity of mesenteric vessels to DT administration may have led to local effects not seen in other tissues or the development hypertension may have been affected by DT treatment. The effect of DT on angiotensin II-induced hypertension was not determined. However the development of histologically identical pathology in the heart and kidney of mice infused with angiotensin II, regardless of DT administration, suggests that significant hypertension did develop despite DT administration. Given the protective effects of glomerular autoregulation, a drop in blood pressure would be expected to spare renal before mesenteric vessels.

Undoubtedly mice receiving DT suffered toxic effects independent of angiotensin II administration resulting in the early termination of the study. Although vasculopathy was not observed in DT treated mice infused with saline. Failure of vascular remodelling within mesenteric vessels occurring due to generalised toxicity on multiple cell types at the site of DT administration cannot be excluded. Duffield *et al* also used CD11bDTR mice and did not experience the same problems with toxicity despite using a higher DT dose, either 10ng/g I.V. or 25ng/g I.P. [Duffield et al., 2005]. Three injections were administered 24 hours apart and the mice sacrificed on day three. Our mice received injections (20ng/g) on day two and four and became unwell on the forth day following DT. The more concentrated dosing schedule used by the authors, may account for this difference or possibly DT toxicity was exacerbated by some aspects of our experiment. The procedural stress of osmotic pump insertion or occult infection

could have influenced the effects of DT. CD11bDTR mice were not housed in pathogen free conditions and as such, infection cannot be excluded.

DT treated mice infused with angiotensin II displayed a striking lack of cellular response to myocardial necrosis which typically accompanies fibrinoid necrosis within the heart. Despite this, the cardiac mass index was increased suggesting hypertensive cardiac remodelling had occurred. Macrophages are involved in coordinating cellular responses to injury and scar formation, as well as resolution of scarring, in a number of tissues and disease settings [Duffield et al., 2005; Leibovich and Ross, 1975]. Most data were obtained from in vitro studies due to the difficulties in selectively targeting macrophages already discussed. The depletion of macrophages within the heart may have been responsible for this effect, rather than other actions or toxicity of DT. Depletion of macrophages using LBP reduced the inflammatory cell infiltrate and collagen deposition in a mouse model of lung fibrosis [Zhang-Hoover et al., 2000]. Similar reductions in mesangial matrix deposition were found in a rat anti-thy-1 nephritis model [Westerhuis et al., 2000]. The effect of macrophages on fibrosis may be mediated in part by transforming growth factor beta (TGF-β) induced collagen deposition [Friedman, 2005; Matsuoka and Tsukamoto, 1990]. Duffield *et al* used the CD11bDTR model to examine the effects of macrophage depletion during the development and resolution of carbon tetrachloride induced liver fibrosis [Duffield et al., 2005]. Depletion during injury resulted in a reduction in macrophages and myofibroblast-like cells expressing TGF-β1 and matrix deposition (elastin and collagen III) in response to tissue injury, similar to the myocardial response observed here. Duffield and colleges went on to show that during resolution of liver fibrosis

macrophage depletion slowed down scar clearance, demonstrating the complex and opposing roles of these cells in vivo.

MCP-1 is upregulated in areas of myocardial infarction and macrophages are believed to play an important role in coordinating fibroblast proliferation and neovascularization [Frangogiannis et al., 2002]. Recently, reduced macrophage and myofibroblast infiltration, osteopontin-1 expression and altered post-infarction remodelling were demonstrated in MCP-1 knockout mice [Dewald et al., 2005]. Given the importance of understanding and minimizing myocardial necrosis for therapeutic gain, the possibility that macrophage depletion alters myocardial response to injury warrants further investigation.

Fibrinoid necrosis was also induced in FVB/NJ mice after only six days of angiotensin II infusion, suggesting greater susceptibility of this strain to angiotensin II induced end organ injury. A similar tendency towards more severe injury in FVB/NJ *vs* C57Bl/6 has also been noted in a model of large vessel (carotid) remodelling in terms of intima-media thickness and plasminogen activator levels [Korshunov and Berk, 2004; Korshunov et al., 2004]. In a carotid ligation model, vascular smooth muscle cell proliferation was strain dependant [Harmon et al., 2000]. Despite the identification of inter-strain variation and the potential for inter-breeding to identify susceptibility loci, no candidate genes have been identified to date. It is possible that differences in renin genes could have contributed to the strain variation observed here in response to angiotensin II infusion. Mice are polymorphic at the renin locus probably due to gene duplication. The C57Bl/6 stain carries a single *Ren1*^{c} gene while FVB/NJ mice carry two-genes, *Ren1*^{d} and *Ren2*. Conflicting comparative data exists regarding one and two-

gene strains [Hansen et al., 2004; Lum et al., 2004] . However, Wang *et al*, compared renin genes on comparative genetic backgrounds by backcrossing 129Ola (two gene) and C57Bl/6 mice to generate N_{5-6}(129Ola/C57Bl/6) strains homozygous at either the *Ren1*^{c} or *Ren1*^{d}*/Ren2* locus. Mice carrying two genes had higher baseline blood pressure and 100-fold higher plasma renin concentration/activity, enhanced salt-sensitivity and enhanced pressor response and cardiac hypertrophy to DOCA-salt [Wang et al., 2002]. These findings strongly suggest that *Ren2*, produced mostly from the submaxillary gland, is physiologically relevant and less responsive to feedback mechanisms.

In summary, a MH phenotype was successfully created in two mice strains by high-dose angiotensin II infusion. This model resembled the IHR model in that inflammatory cell infiltration occurred early in vascular remodelling. Initial results targeting these cells using a conditional macrophage ablation model resulted in the abolition of vascular remodelling within the mesentery, where ablation is greatest, and altered the response to myocardial injury.

CHAPTER SIX

THE CREATION OF A NOVEL, INDUCIBLE STROKE PHENOTYPE BY SUPPLEMENTING SALT-WATER DURING THE DEVELOPMENT OF MALIGNANT HYPERTENSION IN THE INDUCIBLE HYPERTENSIVE RAT.

6.1 INTRODUCTION

Cerebrovascular disease is the third leading cause of mortality in the Western world, with approximately 2400 people per million, per year, suffering a stroke. By the end of one year, 700 of these will be dead and less than half able to live independently with a considerable associated health and economic burden [Hankey and Warlow, 1999; Leys et al., 2002]. Beyond the relative risk to an individual, stroke cannot be predicted and patients often present with an established neurological deficit. MRI imaging can identify regions of potentially reversible ischaemia from infarcted tissue, the so called "ischaemic penumbra", but treatment approaches with anti-platelet or thrombolytic agents have to date, been disappointing and prevention remains the best cure.

In man the risk of ischaemic or haemorrhagic stroke is strongly associated with hypertension. The risk of stroke doubling for every 7.5mmHg rise in diastolic pressure [1996]. The brain is especially susceptible to the rapid rises in blood pressure that

characterise accelerated hypertension. This may overcome cerebral autoregulation resulting in fibrinoid necrosis, infarction, cerebral oedema and secondary haemorrhage [Fisher, 1968; Fisher, 1982; Lassen and Agnoli, 1972].

It is believed that small vessel fibrinoid necrosis is important in the pathogenesis of hypertensive small vessel stroke and other stroke subtypes [Lammie, 2002]. Since blood vessels are capable of withstanding high pressures without leak and cerebral vessels do not display the signs of medial hypertrophy commonly seen in the systemic circulation, how hypertension causes stroke remains unclear. [Dickinson, 2001; Dickinson and Thomson, 1960; Margolis and Sadowsky, 1976]. But once cerebral autoregulation is overcome and pressure is transmitted to the micro-circulation, breakdown of the blood-brain barrier can occur resulting in microvascular and cerebral injury.

The effects of salt and water supplementation on the development of hypertension and end-organ injury is complex. Where salt sensitivity exists, the addition of salt results in worsening hypertension and end-organ injury. For example in spontaneously hypertensive stroke prone rats, the addition of 4% sodium chloride to the diet resulted in worse hypertension, a more rapid onset of stroke (between 12 and 20 weeks of age) and worsening renal and cardiac injury. But there are theoretical reasons why replacement of salt water lost through natriuresis may improve hypertension by reducing inappropriate RAS activation. Offering rats with 1C2K hypertension 0.9% saline was able to postpone the development of MH possibly by this mechanism [Mohring et al., 1976]. Hypertension and pathology were not improved by 0.9% saline in an aortic ligation model of MH [Rojo-Ortega et al., 1979].

In the IHR model the cerebral circulation is spared during the first fourteen days of induction. In order to examine the effect of salt and salt-water loading on the course of malignant vascular remodelling with specific reference to the cerebral circulation, we induced the IHR for 14 days with either free access to 0.9% saline in addition to water or with a compulsory high salt diet.

6.2 EXPERIMENTAL DESIGN

In the first experimental group, Fisher F334 rats and IHRs were induced for 14 days using 0.3% w/w I3C mixed with standard powdered diet containing 0.32% sodium chloride with free access to drinking water. This group was offered an additional bottle of 0.9% saline, so that they had the choice of consuming either saline or water. A second, simultaneous, transgenic group was induced with water alone. Fluid intake was estimated by daily weighing of the water bottles.

This study was repeated using identical treatment groups housed continuously in metabolic cages for fourteen days to allow urine volume to be measured daily. Daily urine aliquots were collected, spun to remove debris and stored at $-80^{o}C$ for analysis. A further repeat of this experiment was performed following radiotelemetry insertion, by standard protocol, and allowing a one week recovery time to measure intra-arterial pressure.

A second study was performed in which Fisher F334 and transgenic animals were induced with a compulsory high salt diet (4% w/w sodium chloride) with free access to water. Fisher F334 and transgenic animals were also induced simultaneously on a 0.32% sodium diet. Saline was not offered. The group size for all experiments was six except the telemetry and metabolic cage studies where the group size was four.

Animals were killed by CO_2 inhalation followed by cervical dislocation and blood was obtained by cardiac puncture and organs fixed in formalin. Whole brain was snap frozen and stored at $-80^{o}C$ for RNA extraction.

6.3 RESULTS.

6.3.1 Clinical Outcome.

All Fisher F334 control animals gained weight and remained well throughout the studies. Transgenic animals induced without access to saline lost weight and had reduced food intake but did not show signs of distress. Transgenic rats with access to saline were less well from day five onwards with reduced movement, deterioration in coat condition and displayed the greatest weight loss after fourteen days (figure 6.1).

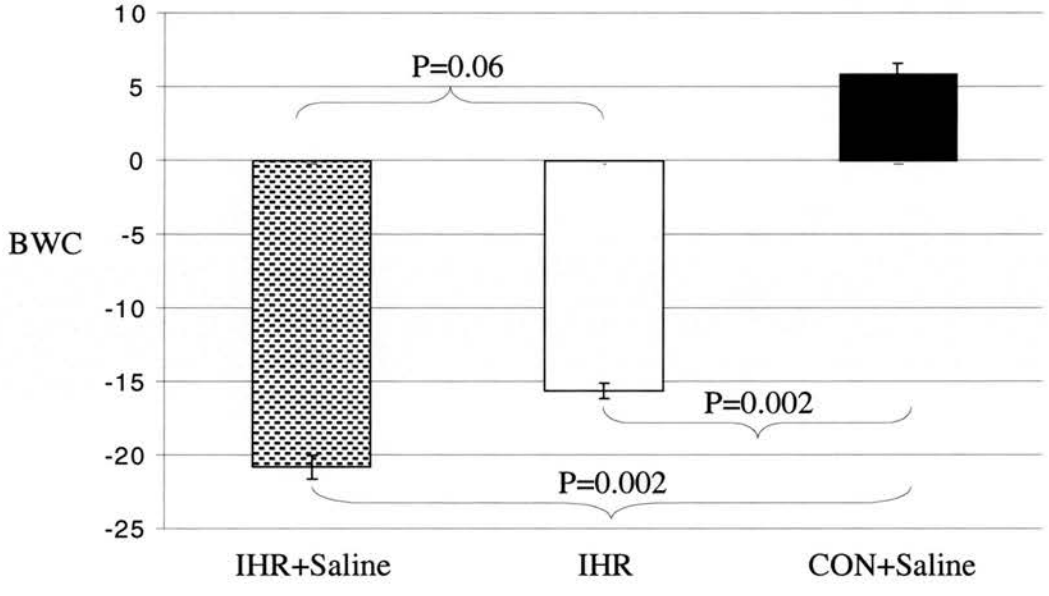

Figure 6.1. Body Weight Change During Induction With And Without Saline.
Percentage body weight change, BWC; IHR, inducible hypertensive rat; CON, Fisher F334 control.

Between days seven and ten transgenic animals offered saline displayed motor hyper-responsiveness to noise and movement stimuli and an unsteady gait. One of the six animals was observed to have a generalised seizure on day nine and was culled. Cerebral oedema was found at post mortem. The condition of the remaining five animals from this group improved from day ten onwards and they were culled as planned, on day fourteen. Repetition of this experiment to include a total of 14 animals offered saline did not result in additional early deaths and with the exception of this single animal all transgenic animals offered saline survived until day 14.

6.3.2 Fluid Balance.

Fisher F334 control animals consumed very little saline by choice (figure 6.2). The small volume recorded was felt to have resulted from a combination of evaporation and spillage. Salt appetite in rats is influenced by strain and it has been previously documented that Fisher rats have a low salt appetite [Breslin et al., 1995; Moore and Lux, 1998].

Transgenic rats with access to water alone displayed increased consumption from day four onwards at the same time that polyuria, presumed secondary to pressure natriuresis, developed (figure 6.2).

Those with access to saline drank avidly from day two consuming large volumes. From day 12 consumption increased further and in some instances animals consumed their body weight in fluid (figure 6.2). Regardless of the total volume of fluid consumed, the

ratio of saline to water remained approximately 7:3, illustrating a strong preference for saline in induced IHRs.

Cumulative fluid balance demonstrated a positive fluid balance in the saline drinking transgenic group over the 14-day experimental period (figure 6.2).

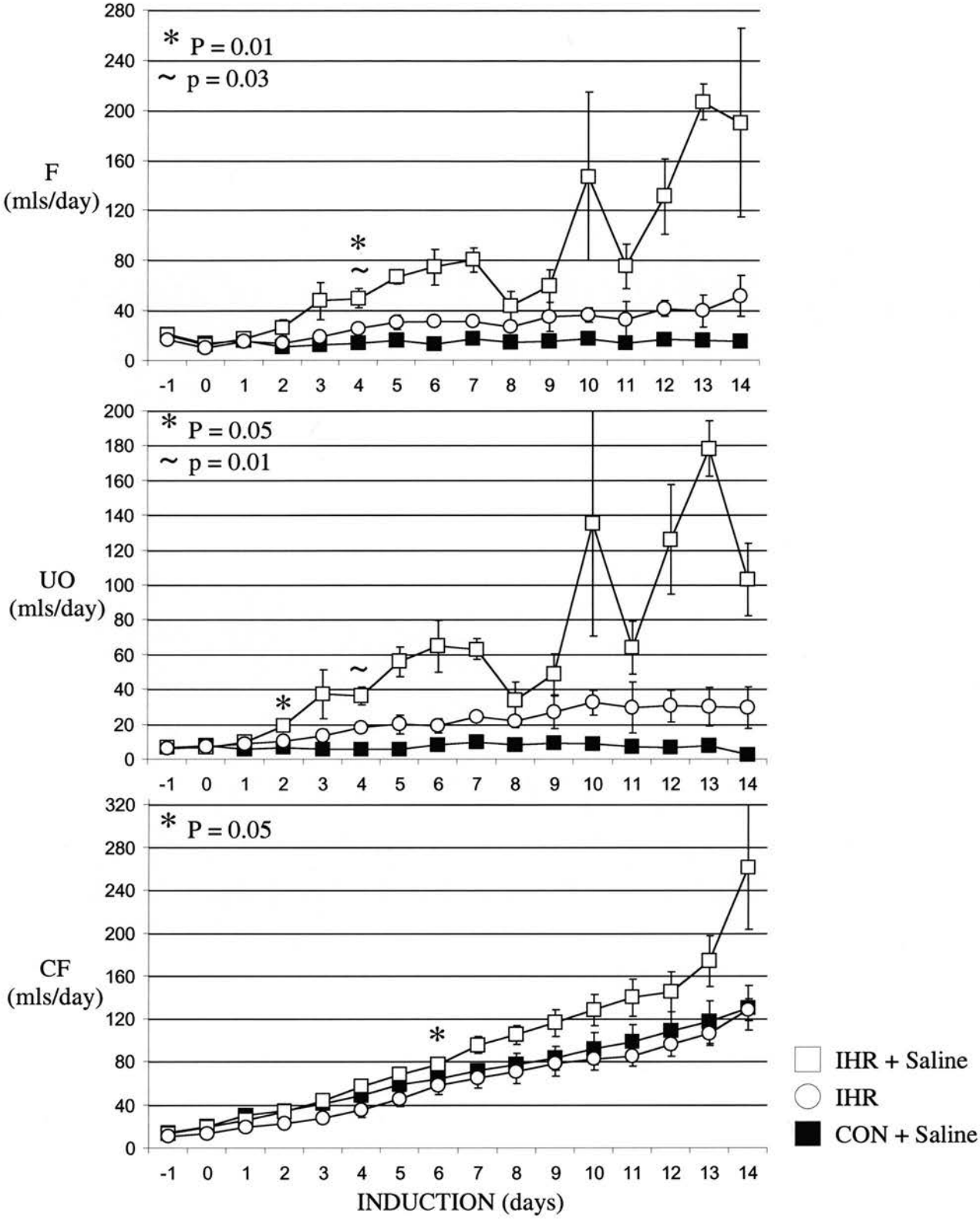

Figure 6.2. Fluid Balance During Induction With And Without Saline Choice.

F, fluid intake; UO, urine output; CF cumulative fluid balance; IHR, inducible hypertensive rat; CON, Fisher F334 control; * = IHR+saline *vs.* CON; ~ = IHR *vs.* CON.

6.3.3 Electrolytes, Renal Function And Haematology.

Sodium, urea and plasma osmolarity were lower in both induced transgenic groups on day fourteen, in keeping with either haemodilution or volume expansion. This difference was greatest in the saline drinking group (figure 6.3). There was a tendency towards hyperkalaemia in the transgenic groups possibly due to intravascular haemolysis known to occur in this model. Renal function estimated by creatinine clearance calculated on the final 24 hour urine volume was normal for all groups (0.10 vs. 0.27 vs. 0.19 ml/min/100g; F334 + saline vs. IHR + saline vs. IHR). The relative increase in clearance for the saline drinking transgenic group was most likely to reflect hyperfiltration consequent upon both hypertension and volume expansion.

No significant differences in haemoglobin, haematocrit, total white cell count or platelet count were detected between the experimental groups (table 6.1).

	CON+SALINE	IHR	IHR+SALINE
White Cell Count ($X10^9$/l)	4.85+/-2.28	4.23+/-1.69	4.54+/-1.81
Haemoglobin (g/dl)	15.1+/-0.96	16.2+/-1.36	14.6+/-2.07
Haematocrit (%)	42.7+/-2.69	45.3+/-3.79	41.1+/-5.38
Mean Cell Volume (fl)	49.7+/-0.22	49.2+/-1.46	51.1+/-2.48
Platelet Count ($X10^9$/l)	618+/-128	633+/-89	598+/-61

Table 6.1. Haematology During Induction With And Without Saline.
No significant differences were found.

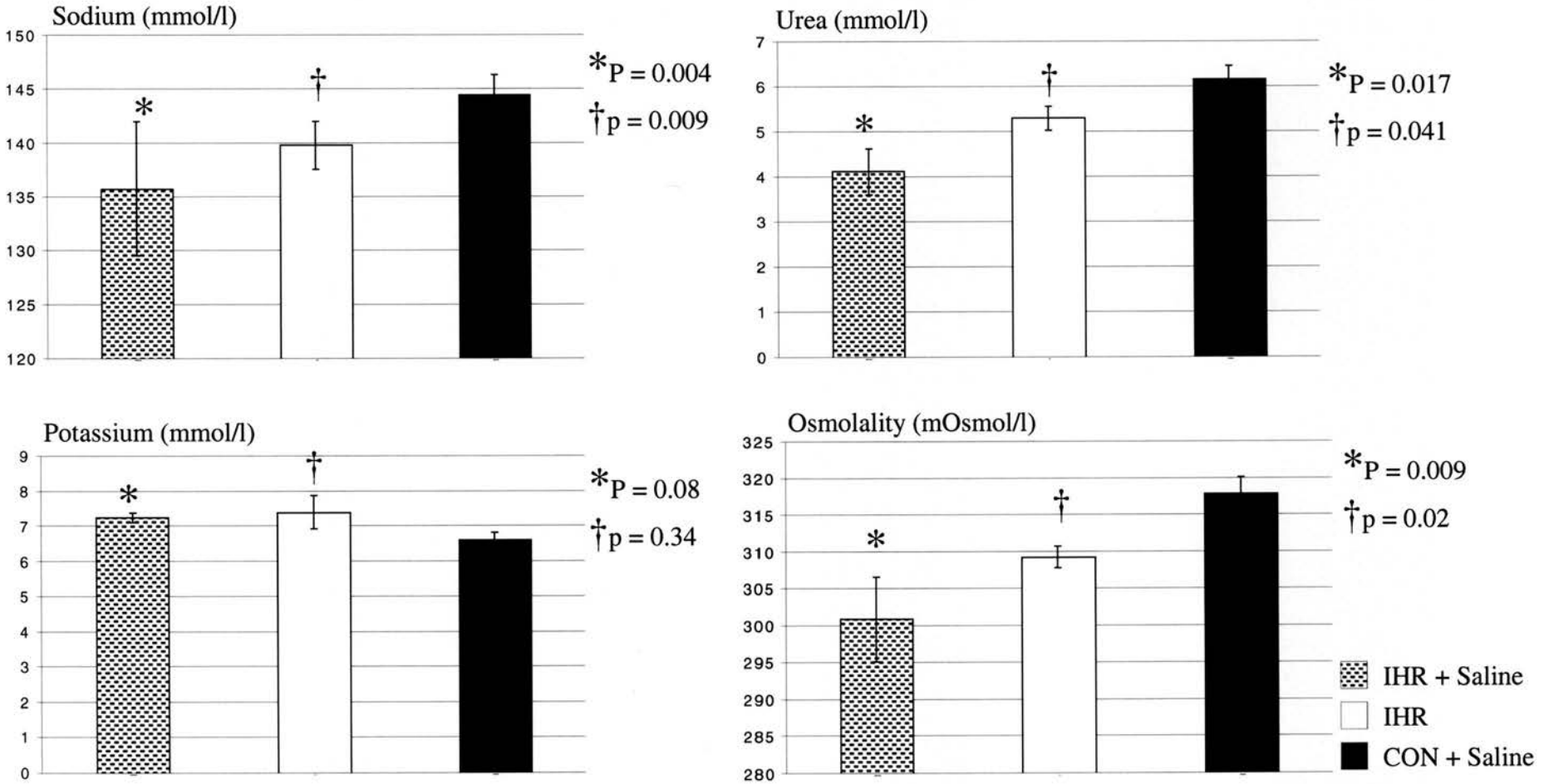

Figure 6.3. Serum Urea And Electrolyte Changes During Induction With And Without Saline Choice.

IHR, inducible hypertensive rat; CON, Fisher F334 control.

6.3.4 Transgene Induction & The Circulating Renin Angiotensin System.

All components of the RAS were elevated in transgenic animals drinking only water compared to controls. The majority of prorenin was transgene derived (mouse origin). Plasma renin and prorenin levels were elevated to a lesser extent in transgenics consuming saline and circulating angiotensin II levels were no different to control animals (figure 6.4).

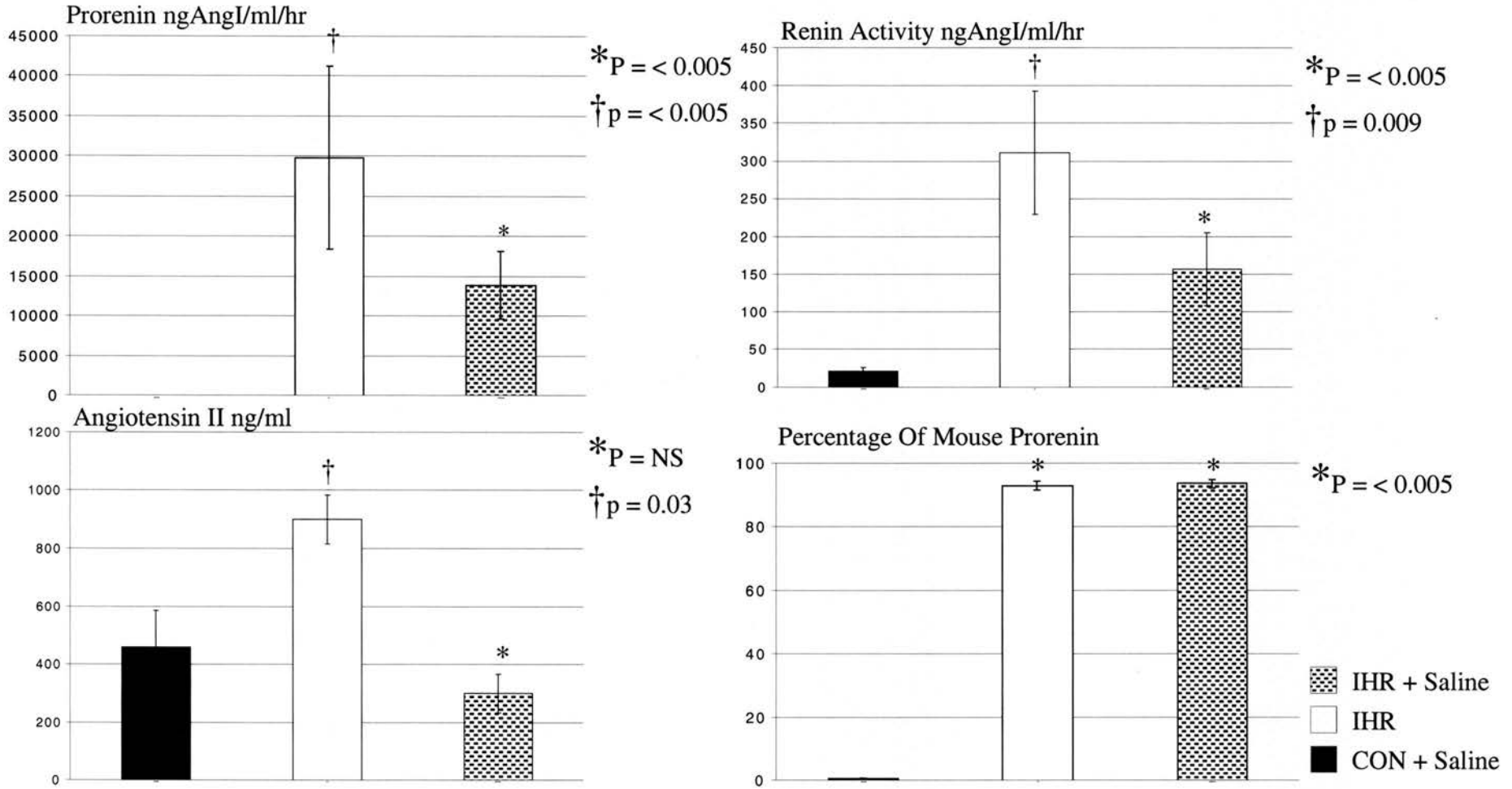

Figure 6.4. Circulating Renin Angiotensin System Levels During Induction With And Without Saline Choice.

IHR, inducible hypertensive rat; CON, Fisher F334 control.

6.3.5 Pathology.

All organs examined were macroscopically and histologically normal in the Fisher F334 control group offered saline (figure 6.5 & 6.6).

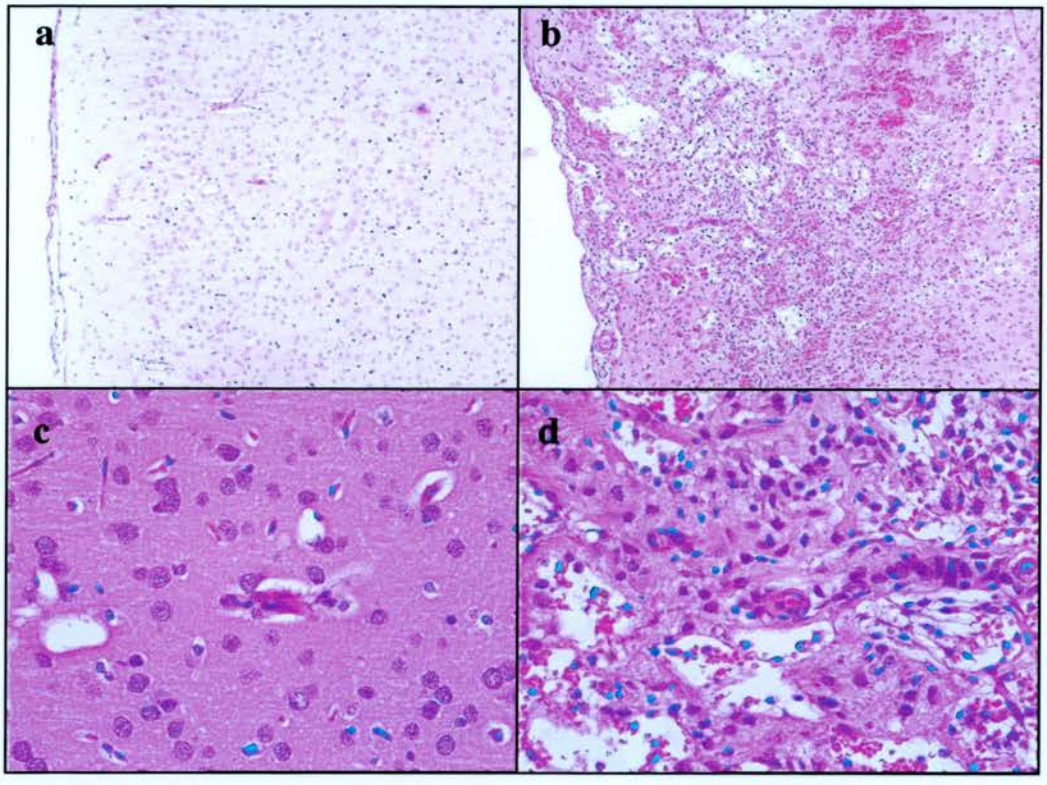

Figure 6.5. Brain Histology.

Fisher F334 rats (a low power & c high power) display normal brain structure. IHR offered saline develop cerebral infarction with secondary haemorrhage (b low power & d high power).

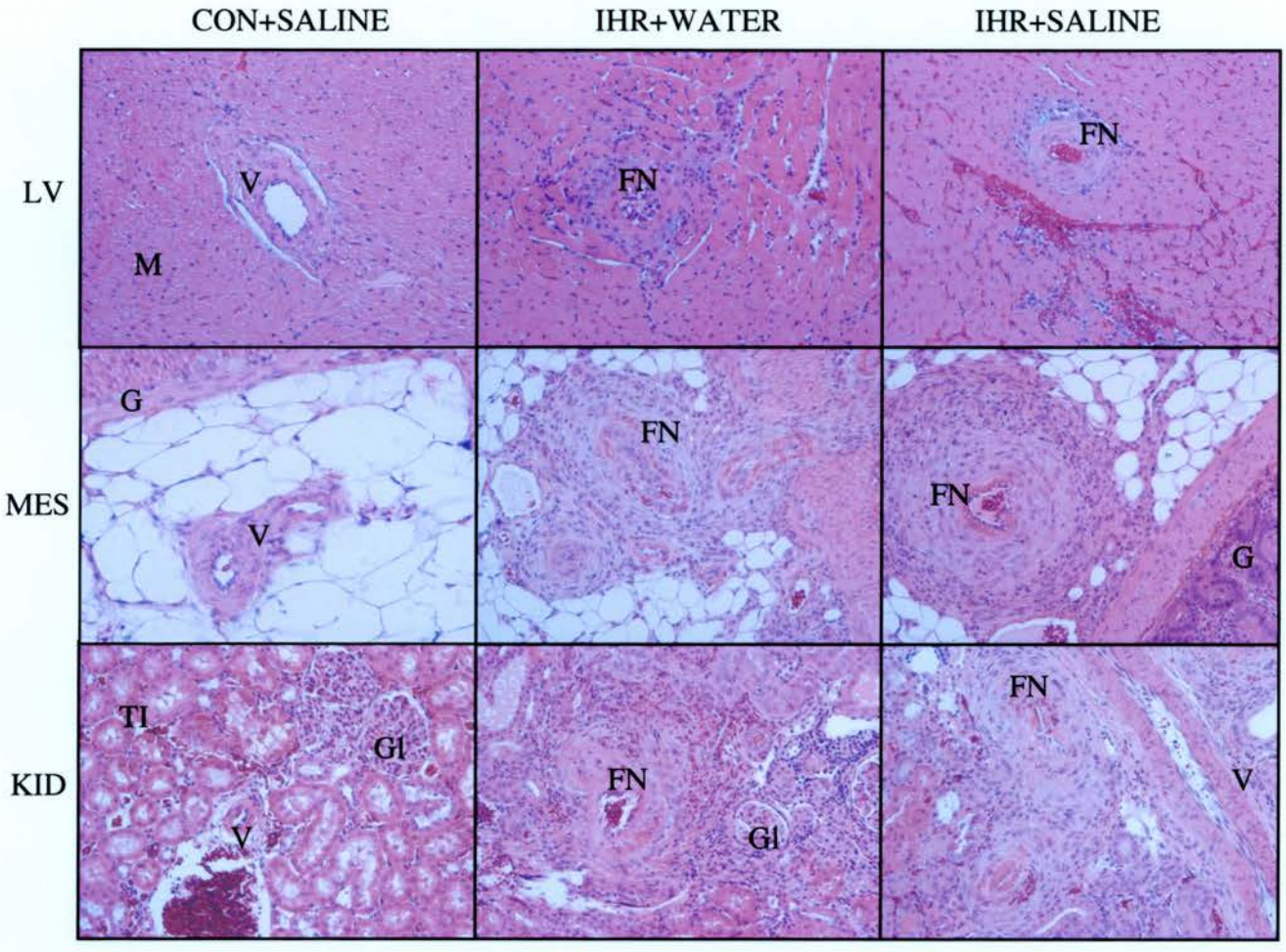

Figure 6.6. Renal, Mesenteric And Myocardial Histology.

H&E histology of the left ventricle (LV), mesentery (MES) and kidney (KID). Fisher F334 (CON) rats are normal. IHRs induced for fourteen days have a similar level of malignant hypertensive injury regardless of saline consumption. M=myocardium, V=vessel, FN=fibrinoid necrosis, G=gut, TI=tubulointerstitium, Gl=glomerulus.

Transgenic animals induced with water alone had evidence of cardiac hypertrophy assessed by total heart to body weight ratio. Both start and end weight ratios are included since both are influenced by changes in body weight during the experimental period and a tendency for cardiac mass to increase with falling body weight (figure 6.7a & 6.7b).

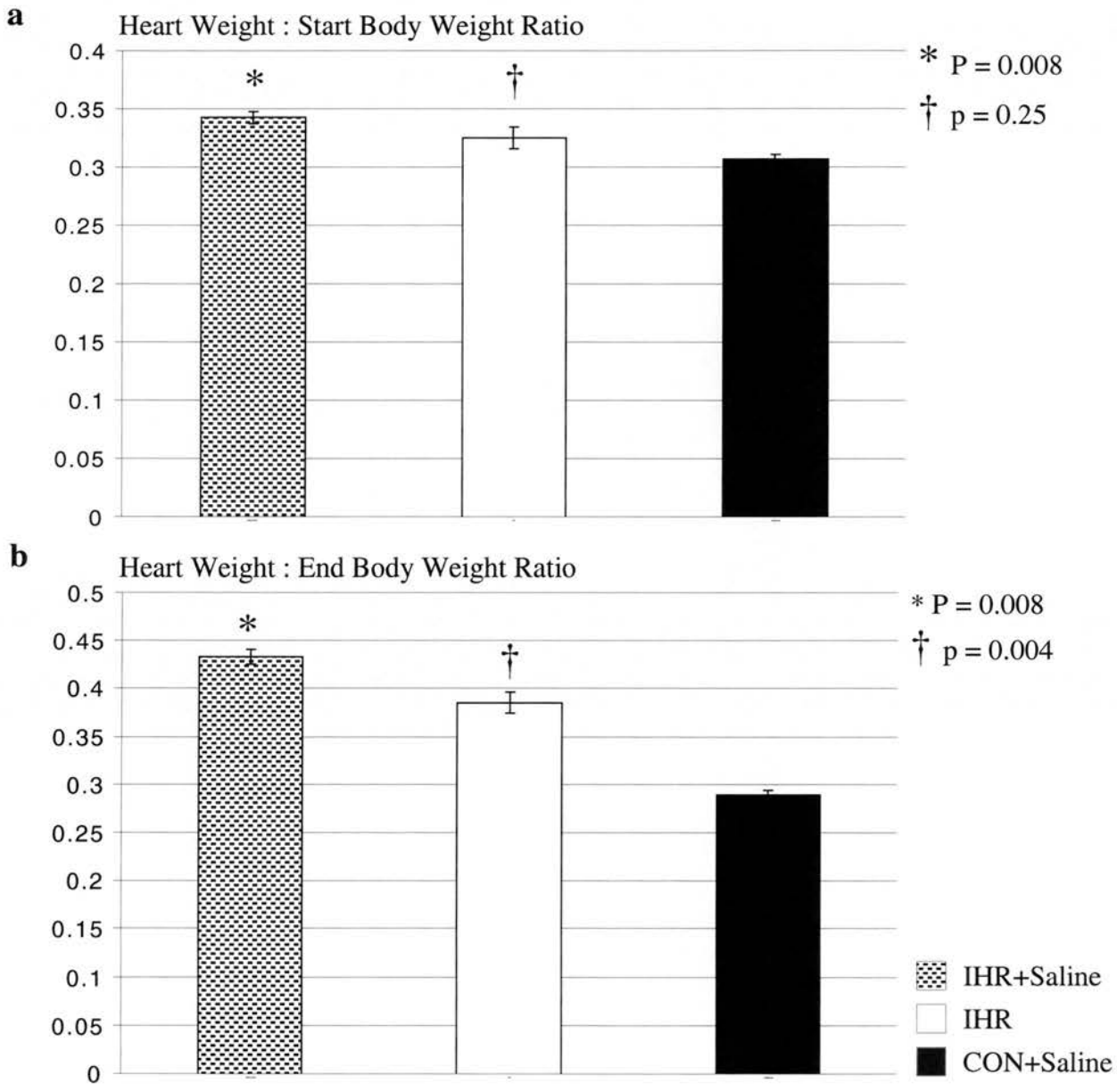

Figure 6.7. Cardiac Hypertrophy.

IHR, inducible hypertensive rat; CON, Fisher F334.

In concordance with previous findings fibrinoid necrosis within the kidney, mesentery and heart was observed affecting the resistance vasculature of around 200μm in diameter. As observed before, areas of micro-infarction were present within the kidney and heart. The brain parenchyma, cerebral vasculature and pulmonary vasculature were normal. There was no pulmonary oedema suggestive of cardiac failure. Branches of the aorta within the thorax of around 200μm also demonstrated fibrinoid necrosis.

Transgenic animals drinking saline had loss of abdominal fat pads in keeping with their marked weight loss. Vascular injury of similar severity was also observed affecting the heart, mesentery and kidney (figure 6.6). The kidney from one animal in this group also had widespread tubular apoptosis reflective of poor health at the time of cull. Additionally, all surviving five animals had cerebral oedema. Three out of the five surviving animals had areas of macroscopic haemorrhage visible on the surface of the brain. Histological areas of cerebral infarction scattered throughout the cortex with associated areas of secondary haemorrhage (figures 6.5 & 6.8).

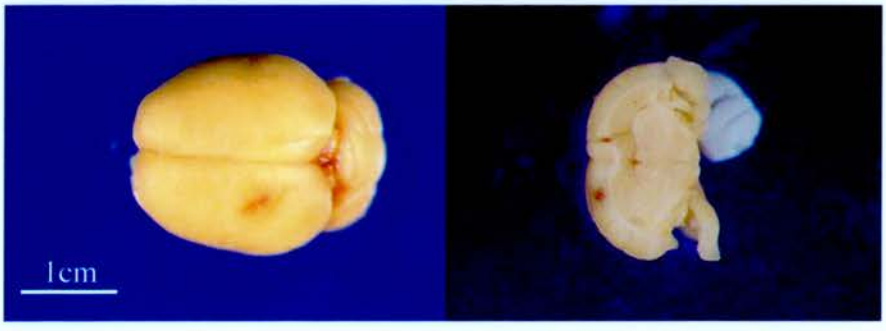

Figure 6.8. Brain Pathology.

A brain from an IHR offered saline illustrating an area of haemorrhage.

Cerebral infarction was estimated to have occurred within two days of death. The brains were examined by Prof. Stewart Fleming, renal pathologist and Prof. Alistair Lammie, neuropathologist.

Immunostaining for renin within the kidney confirmed the presence of the protein particularly in association with afferent artery injury and ischaemia, in both transgenic groups regardless of saline consumption. However, staining was less intense than in Fisher F334 animals (figure 6.9).

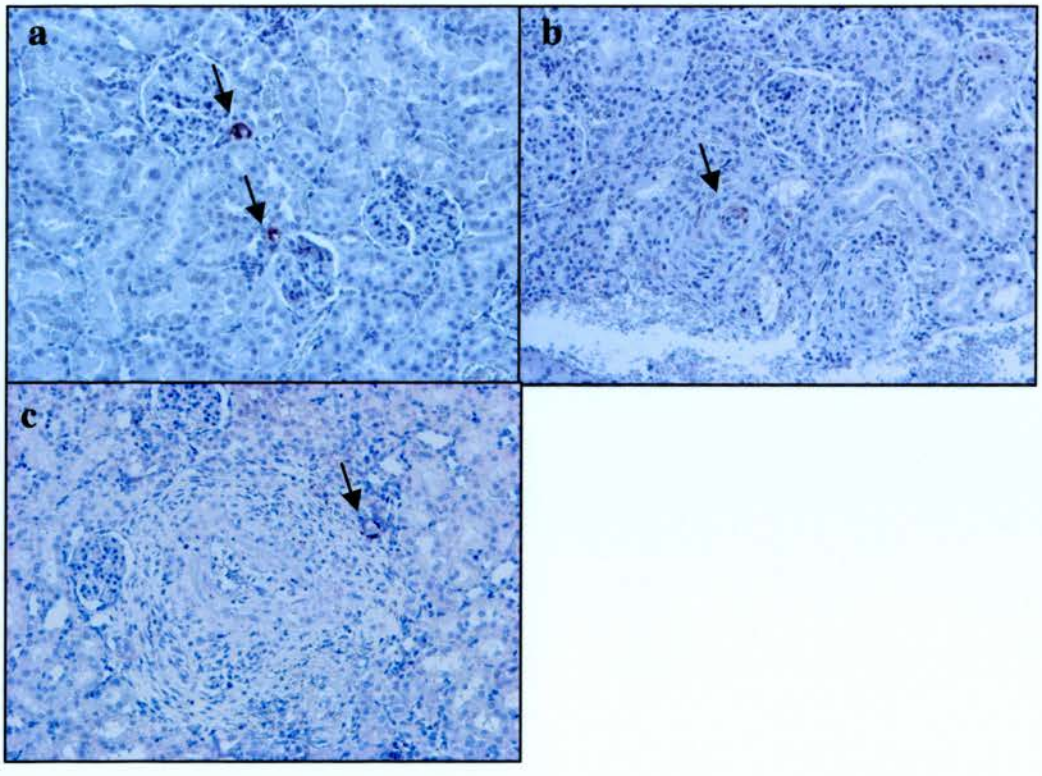

Figure 6.9. Renin Immunostaining.

Fisher F334 control (a), IHR offered water (b) and IHR offered saline (c). Renin staining (brown) is indicated by arrows.

6.3.6 Telemetry Data

Mean arterial pressure is shown for all groups including Fisher F334 rats offered water (figure 6.10). Both Fisher control groups displayed normal blood pressure and diurnal variation regardless of the availability of saline. Despite positive fluid balance in the saline drinking transgenic group there was no difference in either rate of blood pressure rise or overall level reached between those offered saline or not. This was also true for systolic, diastolic and pulse pressures (data not shown).

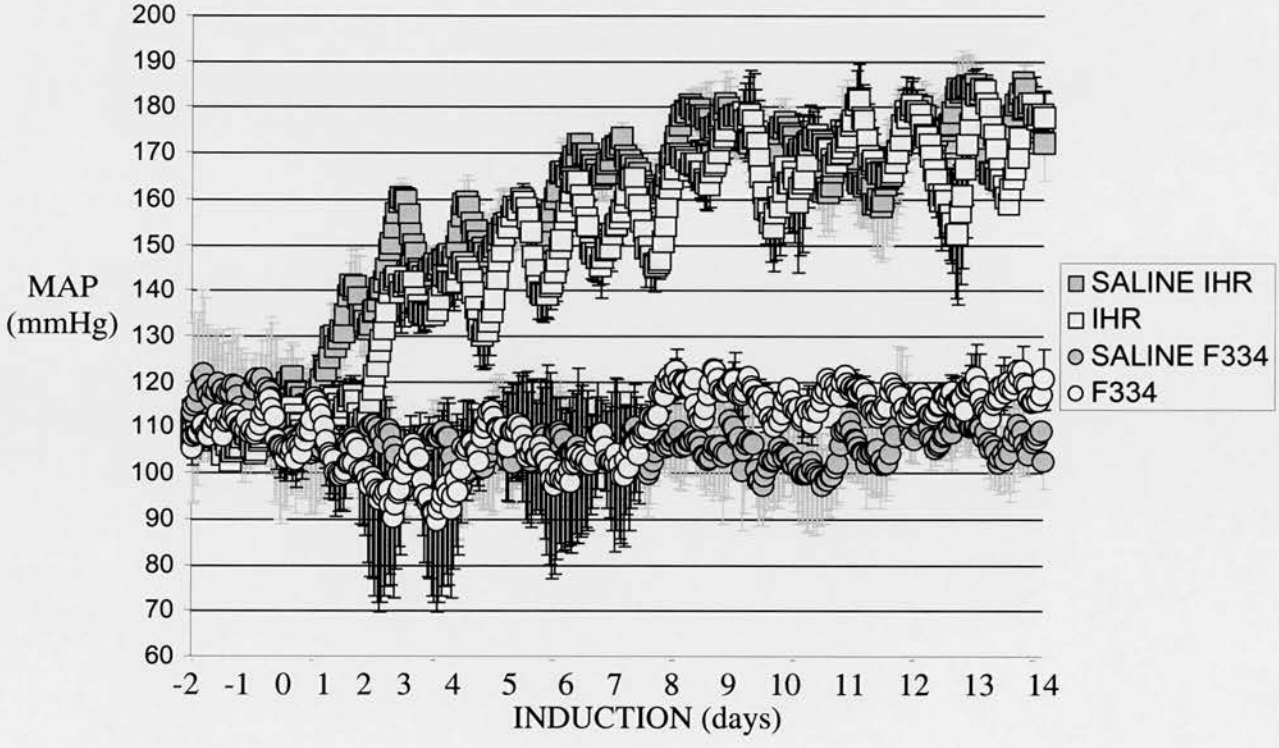

Figure 6.10. Telemetered Mean Arterial Blood Pressure (MAP) During Induction With And Without Saline.

IHR, Inducible Hypertensive Rat; F334, Fisher F334 control.

6.3.7 Transgene Expression In The Brain.

Analysis by rtPCR using prorenin primers could not detect transgene expression in the brain of IHRs following fourteen days of induction.

6.3.8 Effect Of A Compulsory High Salt Diet.

In the experimental group receiving a compulsory high salt diet during induction, no difference in behaviour or weight change was seen when compared to the transgenic group induced with a standard sodium diet. Animals receiving the high salt diet had systemic vascular injury comparable to animals induced on a standard sodium diet and there was no evidence of cerebral oedema or infarction in the high salt group. Fluid consumption and blood pressure was not estimated for the high salt experimental group.

6.4 DISCUSSION

Systemic vascular injury and associated end organ damage seen in the IHR model of MH was unaffected by either a high salt diet or saline consumption. Neither was renal function, estimated by creatinine clearance, impaired despite a high salt load in those animals with free access to saline. In models of hypertension with reduced renal mass such as following unilateral nephrectomy and treatment with DOCA, a 1% salt diet is sufficient to induce malignant vascular injury [Gavras et al., 1975]. It is possible that preserved renal mass in the IHR prevented a worsening of vascular injury despite salt loading. However, a novel neurological phenotype with cerebral oedema and cortical infarction was generated by saline supplementation, but not by high salt diet alone.

Importantly, there was no difference in systemic hypertension between the saline drinking and non-saline drinking transgenic groups measured by radiotelemetry, despite an increase in cumulative fluid balance. As such, hypertension has been dissociated from cerebral lesions in the IHR, allowing dissection of those factors mediating cerebrovascular injury in animals with equivalent hypertension and systemic vascular injury. Stroke was established (Prof. Stewart Fleming, pathologist) to have occurred within 48 hours of death hence the onset of injury is tightly predictable in this model.

Neurological lesions previously observed in the SHRSP model of stroke have been described as cortical infarction with or without haemorrhage. Fibrinoid degeneration within the vascular media is seen in keeping with the severe hypertension observed in this model. The neurological lesions seen in the saline loaded IHR are histologically identical to those of the SHRSP. We did not see fibrinoid necrosis and multiple serial

sections were not performed, however, it is likely that fibrinoid necrosis occurred in association with the cerebral lesions, as previous studies documenting hypertensive lacunar infarcts in man, required serial sections to demonstrate vascular lesions associated with areas of infarction [Fisher, 1968].

Fisher F334 rats did not consume saline by choice. Salt appetite is subject to strain variation and it has been previously demonstrated that the Fisher F334 strain has a low salt appetite when salt replete [Breslin et al., 1995; Moore and Lux, 1998]. Saline drinking by transgenic animals began at 48 hours, prior to the onset of significant hypertension or polyuria in IHRs not offered saline, suggesting that it was not a response to volume depletion as a consequence of pressure induced natriuresis. However transgene expression occurs early on in the IHR and it is well documented that increases in salt and water appetite can be stimulated by activation of the central RAS. For example, direct intra-ventricular injection of angiotensin II or renin will stimulate immediate drinking behaviour in rats [DiNicolantonio et al., 1982; Fitts et al., 2000; Fitzsimons, 1998]. Additionally, local inhibition with angiotensin II receptor antagonists abolishes this drinking response. Although we have not assessed the renin-angiotensin system in the brain in detail, analysis by RNase protection [Kantachuvesiri, 1999] and real time rtPCR on whole brain homogenates, have suggested that transgene-derived mouse prorenin is not expressed in the brain. Circulating angiotensin II could be responsible for the drinking behaviour by stimulating central sodium appetite centres [Fitts and Thunhorst, 1996; Fitzsimons, 1998]. Alternatively prorenin, expressed in high levels in the IHR, or active renin, might be able to enter the brain under pathological conditions [Chiaraviglio, 1976]. Either way, it seems likely that saline

consumption occurs via a neurological mechanism not yet fully elucidated in this model. Despite the highest level of detection, we cannot completely exclude that low levels of transgene production are not occurring in the IHR brain and directly contributing to brain pathology.

Cerebral oedema was consistently found in all IHRs consuming saline and is likely to be responsible for the illness observed in this group between days five and ten of induction. The improvement in their condition from day ten onwards may have resulted from cerebral adaptation to hypertension and volume overload. Saline consumption resulted in a lower plasma osmolarity and a greater positive fluid balance than seen in transgenic animals not offered saline and as such it is likely that volume overload contributed to this phenotype.

Two theories have arisen in humans to explain cerebral infarction occurring in association with hypertension: Forced dilatation and vasospasm. In the former pressure is transmitted to the normally low-pressure microvasculature with resulting tissue injury by dilatation of resistance vessels unable to cope with the increase in pressure, resulting in capillary leak. In the latter, inappropriate constriction of the resistance vasculature in response to pressure directly causes ischaemic injury. The assessment of cerebrovascular tone is beyond the scope of this study but identical hypertension with increased total body fluid in the saline-versus water-drinking IHR group would favour a "forced dilatation" mechanism of overcoming cerebral autoregulation. The presence of cerebral oedema also suggests that blood brain barrier breakdown is occurring. Early fluid overload on a background of rapidly rising blood pressure may exhaust cerebral autoregulatory reserve, generate cerebral oedema and initiate vascular injury and

thrombotic stroke. MH also associates with coagulopathy via endothelial activation, volume depletion and intravascular haemolysis, which would predispose to thrombosis. The loss of cerebral blood flow autoregulation has been described prior to the development of stroke in spontaneously hypertensive stroke prone rats. Over-perfusion could result in endothelial damage causing blood brain barrier breakdown as well as facilitating fibrinoid necrosis. Pressure dependant arterial constriction was reduced prior to stroke, but did not account fully for the degree of loss of autoregulation.

Previous investigators and clinical experience has demonstrated that salt and water depletion (for example following diuretic use) in hypovolaemic hypertension, especially with high circulating renin, may be harmful. These conditions include MH and pre-eclampsia. Additionally evidence exists that saline supplementation of animals and humans with MH may be beneficial in some settings [Dauda et al., 1973; Gavras et al., 1975; Mohring et al., 1976; Palomaki and Lindheimer, 1970; Robinson, 1958].

Morhing *et al* suggested that renin upregulation seen within the un-clipped kidney in the 1C2K model was important for the development of MH and fibrinoid necrosis [Mohring et al., 1975]. Following renal artery constriction in this model, circulating renin derived from the ischaemic kidney rises, resulting in suppression of renin from the un-clipped kidney. This early suppression is clearly appropriate in the face of high circulating renin, but is lost later for reasons that are not fully understood. They suggested that this inappropriate renin production may be contributory to the pathology based on the observation that animals offered a choice of 0.9% saline prior to the development of MH consumed saline avidly and failed to develop fibrinoid necrosis or

the increase in plasma angiotensin II, compared to those without access to saline [Mohring et al., 1976].

In the IHR model we also observe the reappearance of endogenous rat renin from day 10 onwards within the juxtaglomerular cells of both kidneys, following an initial suppression (figure 6.9). Contrary to the findings of Mohring *et al*, we do not observe a suppression of endogenous rat renin at the protein level within the kidney in the IHR offered saline. In fact, despite large volumes of saline consumption there are no observable differences in histological injury within the renal, mesenteric or cardiac circulations. Neither was renal function affected. There was a reduction in the circulating components of the RAS explained in part, by haemodilution. Local kidney renin production may have been influenced by saline consumption but was not assessed in this study.

Additionally despite similar levels of saline consumption in the 1C2K model and the IHR, no cerebral pathology was described in the former. Although neurological deficit was not specifically looked for, the authors performed daily observation of their animals such that signs of cerebral infection or oedema would be unlikely to have been missed.

We cannot entirely explain these opposing findings however the following considerations may contribute: Firstly, there is wide inter-strain variation in both the response to angiotensin II and also to salt depletion. Fishers are known to be high renin animals and susceptible to renal injury and malignant vascular injury specifically. The 1C2K model described above was performed on an out-bred Sprague-Dawley background. Secondly, the IC2K model is generated with an associated reduced renal

mass not found in the IHR model. It is described that reduction in renal mass predisposes to hypertensive injury in animal models of hypertension and also in humans. Thirdly, as stated above, we cannot exclude that transgene production is not occurring in the IHR brain and directly contributing to neurological pathology. However our findings highlight the potential dangers of saline administration and volume expansion in the clinical setting.

In summary, dietary salt and water intake alone were manipulated in the IHR to generate a novel, inducible model of stroke. Cortical infarction developed over a predictable time period and salt-water overload did not influence the development of fibrinoid necrosis or hypertension systemically. Dissociation of the systemic vascular response from cerebrovascular injury will permit comparative study of animals with equivalent hypertension, genetic background and systemic features of hypertension with or without stroke.

CHAPTER SEVEN.

DISCUSSION AND CONCLUSIONS.

A poor understanding of both the pathogenesis of MH and the influence of genetic and environmental factors, contributes to the high morbidity and mortality still observed in this condition. While drug treatment can be highly efficacious, it is often initiated late, after end-organ injury is established, and is difficult to optimise due to heterogeneity of individual patient response. Additionally, recurrence is high if medication is discontinued since anti-hypertensive agents do not influence the underlying condition. A clearer understanding of the pathogenesis and factors that govern patient susceptibility to end-organ injury will be required to develop new treatments and to optimise pre-existing therapies.

Vascular remodelling is central to ischaemic end-organ injury in MH, as it is in other conditions including essential hypertension, atherosclerosis, post-angioplasty and stent re-stenosis. Over the past decade it has become apparent that inflammation plays a central role in many of these conditions and in an experimental setting, immunomodulatory therapies have been used with effect.

MH is characterised by fibrinoid necrosis affecting resistance vessels throughout the body. Due to late presentation of this disease in humans and a lack of predictable animal models, little is known of the development of this pathology. This thesis describes for the first time, the vascular changes that occur during the development of accelerated hypertension using the IHR model and has identified inflammation as well

as proliferation as early events pre-dating fibrinoid necrosis. The identification of a timetable of cellular responses occurring during vascular remodelling could allow these events to be targeted experimentally. The earliest response, two days after transgene induction and preceding vascular smooth muscle cell proliferation and vascular remodelling, was fibroblast proliferation (presumably from the resident population) together with mixed inflammatory cell infiltration. Both these events occurred within the adventitial layer. Inflammatory cells and fibroblasts have been implicated in other types of proliferative vascular remodelling, suggesting that vessels may have a similar set of responses to injury, orchestrated by these cells. By implication, understanding the mechanisms involved in one disease may have wider relevance.

The molecular pathways responsible for initiating and continuing these cellular responses are still to be elucidated in the IHR, but have been determined, in part, in other conditions. Analysis of differential expression data from micro-dissected vessels or whole organ extracts from the IHR could reveal relevant changes in gene expression during induction. Similar early adventitial reactions have also been described in other types of vascular injury including post angioplasty re-stenosis, angiotensin II-enhanced atherosclerosis and hypertensive remodelling induced by aortic ligation [Chatelain and Dardik, 1988; Daugherty et al., 2000; Wilcox et al., 1996]. In human atherosclerosis, the extent of adventitial inflammation is related to the extent of intimal disease [Wilcox and Scott, 1996]. Our findings support the idea that the adventitia is an important vascular layer and probably involved in initiating pathological vascular remodelling in some settings.

Adventitial fibroblasts play an important role in the response to balloon-induced injury in porcine coronary arteries: Fibroblast proliferation (up to one week post injury) and differentiation into α-smooth muscle actin expressing "myofibroblasts" (three to fourteen days post injury) occurred early [Shi et al., 1996c]. Additionally, adventitial fibroblasts upregulated procollagen I and TGFβ1 [Zalewski and Shi, 1997]. The latter may provide the differentiation signal for these cells [Shi et al., 1996b]. Selective labelling of proliferating fibroblasts suggested that in addition to differentiation, adventitial fibroblasts migrated through the medial compartment and contributed directly to neointimal formation [Shi et al., 1996a]. The timescale of injury in the IHR did not permit tight labelling of proliferating adventitial cells with BrDU and radiolabelling with agents such as tritiated thymidine was not feasible. Consequently, we cannot exclude that the proliferating, desmin expressing, adventitial fibroblast population in the IHR did not differentiate and migrate in response to transgene activation.

Although the identification of α-smooth muscle actin expressing cells of fibroblast origin is well documented, additional difficulties present themselves when studying this population within the vascular compartment where myocytes co-exist [Desmouliere et al., 1992]. Identification of cells of fibroblast origin has relied on spatial changes (i.e. adventitial location), cytoskeletal markers (such as smooth muscle actin) and ultrastructural characteristics. Better markers will be required to fully explore the contribution of these cells in vascular remodelling.

Despite detailed investigation into the mechanism of angiotensin II induced vascular smooth muscle cell proliferation it remains poorly understood. The mechanism is in

part independent of blood pressure since mesenteric remodelling following angiotensin II infusion in rats was not inhibited by restoration of normotension with hydralazine [Griffin et al., 1991]. Angiotensin II, but not noradrenaline, induced medial hypertrophy and hypertension associated with superoxide production via NADH/NADPH oxidase activation, which was inhibited by the AT1 receptor antagonist Losartan [Laursen et al., 1997; Rajagopalan et al., 1996]. Vascular smooth muscle cell proliferation induced by balloon injury and angiotensin II infusion, was found to be dependant on vascular smooth muscle cell derived bFGF in larger vessels but not resistance vessels where macrophages were implicated [Lindner et al., 1991; Lindner and Reidy, 1991; Su et al., 1998b].

However, cellular heterogeneity within the medial compartment of some arteries raises the possibility that non-muscle cells within the media may contribute to vascular remodelling. These cells share a fibroblast phenotype and display increased adherence and proliferation in vitro when compared to neighbouring vascular myocytes [Holifield et al., 1996]. This medial heterogeneity suggests that inter-species differences in vascular responses could be greater than anticipated. This is exemplified by the relative contributions of angiotensin II to vascular remodelling induced by balloon injury, endarterectomy and stenting in the rat verses pigs and baboons: In the former, neointimal formation is reduced by AT1 blockade. This does not occur in higher species [Hanson et al., 1991; Huckle et al., 1996]. Additionally, the MERCATOR study group found no beneficial effect of ACE inhibition on human post-angioplasty re-stenosis [1992].

Increasingly, it is appreciated that in diseases once thought of as non-inflammatory, inflammation can play an important role [Nathan, 2002]. Cellular responses to a given inflammatory stimulus are complex and there is an increasing awareness of heterogeneity of response within cell populations. For example macrophages can exhibit different types of activation and response even within the same location [Minto et al., 2003; Wilson et al., 2004].

Pre-emptive treatment of the IHR with the immunomodulatory agent FK506 resulted in the abolition of vascular remodelling and surprisingly, hypertension. Despite a high degree of T cell specificity, the pleiotropic effects of this agent make it impossible to attribute these effects solely to an immune mechanism. Indeed it is possible that the anti-hypertensive effects of FK506 in this model were responsible for its salutary effects.

Either way, the identification of mechanisms responsible for this effect will yield a better understanding of the pathogenesis of MH in the IHR.

Monoclonal antibody treatments used in mice have been applied in the rat to investigate immune mediated injury by targeting specific cytokines or whole cells. Anti-CD8 antibodies have been used in rat models of glomerulonephritis, mercury chloride-induced autoimmunity, myasthenia gravis and thryroiditis to prevent and sometimes treat established disease [Cohen et al., 1990; Kawasaki et al., 1992; Kiely et al., 1996; Reynolds et al., 2002; Zhang et al., 1995]. More specifically, T cell activation in a rat model of Goodpastures disease (autoimmune nephritis) was treated with anti-CD154 directed against CD40L on T helper cells to block interaction with CD40 on antigen presenting cells. The development of crescentic nephritis was prevented [Reynolds et

al., 2004]. Anti-CD40L treatment of LDL receptor deficient mice fed a high fat diet reduced atherosclerotic plaque formation by two-thirds [Mach et al., 1998]. TNFα produced by macrophages can stimulate MCP-1, IL-1β and TGFβ production and anti-TNFα treatment reduced renal injury in the Goodpastures model [Khan et al., 2005]. Such therapies are associated with toxicity but considerably less than older polyclonal, anti-sera preparations. Monoclonal therapies such as rituximab (anti-CD20 which targets B cells) and basiliximab (anti-CD25 the IL-2 receptor expressed on activated T cells) are increasingly used in human immune-based disease. Applying these techniques to the IHR model would allow the role of T cells (and other inflammatory cells) to be more selectively investigated.

Treatment with liposomal bisphonsphonate preparations aimed at targeting macrophages has been used in models of large vessel injury (post angioplasty or stent) to demonstrate a causal role for these cells in the pathogenesis [Danenberg et al., 2003a; Danenberg et al., 2003b]. However the use of these agents can be associated with toxicity and they may lack cell specificity.

Given the lack of cell-specific drugs available for use in the rat at the present time, it was decided to develop a model of MH in the mouse to access the wider range of immunomodulatory techniques available in this species. This was done in a straightforward way by continuous, subcutaneous angiotensin II infusion using ALZET mini-osmotic pumps. The technique is simple, cheap and transferable and resulted in the development of vascular injury histologically identical to that seen in the IHR and humans with MH. Additionally the time scale and extent of hypertension was similar between the IHR and mouse infusion model.

Infusion onto two strains of mice uncovered differences in genetic susceptibility to vascular injury induced by angiotensin II.

Macrophage ablation using CD11b-targeted diphtheria toxin sensitivity in the CD11bDTR mouse resulted in the abolition of vascular injury in the mesentery where ablation is greatest. No effect on vascular remodelling was seen in the heart or kidney where the reduction in resident macrophages is known to be lower. But the possibility of macrophage involvement in hypertensive myocardial remodelling was suggested by an altered cellular response to myocardial necrosis. The experiment was complicated by toxicity associated with DT injection such that interpretation of the data is difficult. However, the greater susceptibility of the FVB/NJ strain to MH (compared to C57Bl/6) could be exploited to shorten the duration of DT treatment required, since established vascular injury was present on day six in this strain.

By generating a model of hypertensive vascular remodelling in the mouse, strains with targeted gene disruption could be used, without cross breeding, to investigate multiple areas of interest. Since the role of circulating inflammatory cells may be important, the use of bone marrow transplantation from a knockout animal into the wild type, following marrow ablation, would permit the study of a single protein an one cell type while avoiding the problems of 'adaptation' sometimes raised with knockout strains. This technique was used to examine the role of the macrophage MCP-1 receptor, CCR2, in large vessel hypertensive, inflammatory changes [Ishibashi et al., 2004].

Allowing the IHR free access to salt-water during transgene induction, resulted in the generation of a novel model of small vessel, ischaemic stroke, phenotypically similar to the SHRSP rat strains. Additionally the time of onset of ischaemic injury was

predictable due to the inbred background. It is hoped that this model will be useful in the study of ischaemic brain injury.

In conclusion, this thesis describes the vascular remodelling that occurs during the development of MH in the IHR. Inflammation and proliferation of fibroblasts were identified as early events and the abolition of pathology resulting from FK506 treatment, suggests that inflammation is central to vascular remodelling in the IHR. To overcome the difficulties of targeting immunomodulatory therapies in the rat, a mouse model was developed using angiotensin II infusion. Initial experiments to ascertain the effect of macrophage depletion in this model have suggested an important role for these cells. This model, together with the IHR, could be used to further investigate the molecular mechanisms involved in hypertensive vascular remodelling.

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