Addendum

p ix:	Heading should be "Symbols and Abbreviations" instead of "Abbreviations".
p 1 line 1:	"An individual is" should be corrected to "individuals are"
p 1 line 24:	"and vice versa'' in this sentence should be deleted and replaced with "while transplantation of a kidney from a normotensive subject into a hypertensive subject ameliorates hypertension".
p 5 line 10:	Replace "glomerular filtration" with "glomeruli".
p 10 Fig 1.2 legend (line 6):	"rennin" should be corrected to "renin".
p 15 line 16:	"surrounds" should be replaced with "is".
p 18 line 20:	Delete "exclusively".
p 47 Heading 2.8:	"Isolation" should be replaced with "Separation".

PARACRINE FACTORS AND REGULATION OF REGIONAL KIDNEY PERFUSION

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Many of life's failures are people who did not realize how close they were to success when they gave up.

Thomas A. Edison

Table of Contents

Summary

Hormonal and neural stimuli can differentially affect renal medullary perfusion (MBF) and cortical perfusion (CBF). This is likely to represent an important regulatory mechanism in long-term blood pressure regulation. The chief aim of the experiments described in this doctoral thesis was to examine the roles of paracrine factors in responses of intrarenal perfusion to hormonal and neural stimuli.

The aim of the experiments described in Chapter 4 was to examine the roles of cytochrome P450 (CYP450) dependent epoxygenase metabolites of arachidonic acid (AA), in responses of intrarenal perfusion to vasoconstrictor agents. We tested the effects of the selective epoxygenase inhibitor N-methylsulfonyl-6-(2 propargyloxyphenyl) hexanamide (MS-PPOH), on responses of intrarenal perfusion to angiotensin II and [Phe^2,Ile^3,Orn^8]-vasopressin in anaesthetized rabbits. [Phe^2,Ile^3,Orn^8]-vasopressin reduced medullary laser Doppler flux (MLDF) but not cortical laser Doppler flux (CLDF) or renal perfusion (RBF), while angiotensin II reduced RBF and CLDF, but not MLDF, after vehicle treatment. MS-PPOH revealed reductions in RBF and CLDF after [Phe^2,Ile^3,Orn^8]-vasopressin but had no significant effects on responses to angiotensin II. MS-PPOH selectively inhibited CYP450 epoxygenase activity in the treated kidneys. The formation of epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acids (DiHETEs) and 20-hydroxyeicosatetraenoic acids (20-HETE) was not affected by incubation of cortical homogenates with [Phe^2,Ile^3,Orn^8]-vasopressin. Thus, vasodilator EETs appear to blunt V_1-receptor-mediated cortical, but not medullary, vasoconstriction. V_1-receptor activation does not increase epoxygenase activity *per se*, but may increase substrate (AA) availability. Our data also indicate that *de novo* synthesis of EETs does not contribute to the responses of intrarenal perfusion to angiotensin II. We also tested the effects of the non-selective CYP450 inhibitor 1-aminobenzotriazole (ABT) and the selective CYP450-dependent ω-hydroxylase inhibitor N-methylsulfonyl-12-12-dibromododec-11-enamide (DDMS) on responses of intrarenal perfusion to hormonal factors. Neither DDMS nor ABT inhibited CYP450 dependent epoxygenase or ω-hydroxylase activity. Thus, we cannot draw conclusions regarding the roles of ω-hydroxylase products of AA in responses of intrarenal perfusion to the vasoactive agents we tested.

The aim of the experiments described in Chapter 5 was to examine the role(s) of cyclooxygenase (COX) products, and the interactions between these and nitric oxide, in modulating renal medullary vasoconstriction induced by renal nerve stimulation. The responses of intrarenal perfusion to renal nerve stimulation were tested in anaesthetized rabbits under control conditions, after COX inhibition, and then after subsequent nitric oxide synthase inhibition. Renal nerve stimulation reduced RBF and CLDF more than MLDF. The COX inhibitor, ibuprofen did not significantly affect these responses suggesting that COX products *per se* do not contribute to the responses of CBF or MBF to stimulation of the renal nerves. Subsequent N^G-nitro-L-arginine (L-NNA) significantly enhanced renal nerve stimulation induced reductions in RBF and CLDF but not MLDF. In contrast, a previous study in our laboratory found that nitric oxide synthase inhibition enhanced renal nerve stimulation-induced reductions in CLDF and MLDF in the presence of an intact COX system. Therefore, while nitric oxide blunts reductions in CLDF to renal nerve stimulation independently of COX, its impact on responses of MLDF may depend on an interaction with COX.

We also examined whether endogenous angiotensin II, via activation of angiotensin II type 1 (AT_1-) receptors and subsequent release of nitric oxide, contributes to the relative insensitivity of MBF to renal nerve stimulation (Chapter 6). The effects of the AT_1-receptor antagonist candesartan, on intrarenal perfusion to renal nerve stimulation, were tested in anaesthetized rabbits. The effects of the nitric oxide synthase inhibitor L-NNA on CLDF and MLDF responses to renal nerve stimulation were also tested, in rabbits pre-treated with candesartan or its vehicle. Candesartan blunted renal nerve stimulation-induced reductions in CLDF and MLDF. L-NNA enhanced CLDF and MLDF responses to renal nerve stimulation in vehicle pre-treated rabbits, but only responses of CLDF in candesartan pre-treated rabbits. These observations suggest that endogenous angiotensin II enhances, while nitric oxide blunts, neurally mediated vasoconstriction in the renal cortical and medullary circulations. This effect of nitric oxide seems to depend on the presence of endogenous angiotensin II in the renal medullary circulation, but not in the cortical circulation.

In conclusion, these data indicate that EETs can selectively modulate responses of CBF to V_1-receptor activation, while nitric oxide and angiotensin II can differentially modulate responses of CBF and MBF to renal nerve stimulation. Future studies should

aim to further elucidate the mechanisms that underlie the differential regulation of intrarenal perfusion by vasoactive factors as disturbances in these mechanisms could contribute to development of hypertension and related cardiovascular diseases.

Declaration

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

[redacted]

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Rajapakse NW, Flower RL, Eppel GA, Denton KM, Malpas SC, and Evans RG. Prostaglandins and nitric oxide in regional kidney blood flow responses to renal nerve stimulation. *Pflugers Archiv-European Journal of Physiology*, (Accepted for publication July 6, 2004)

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Abbreviations

α	Alpha
β	Beta
ω	Omega
<	Less than
>	Greater than
%	Percent change
±	Plus/minus
AA	Arachidonic acid
ABT	1-Aminobenzotriazole
ANOVA	Analysis of Variance
AT_1	Angiotensin II type 1 receptor
AT_2	Angiotensin II type 2 receptor
AVR	Ascending vasa recta
Beats/min	Beats per minute
Ca^{2+}	Calcium
CBF	Cortical blood flow
CLDF	Cortical laser-Doppler flux
COX	Cyclooxygenase
CYP450	Cytochrome P450
DBDD	12,12-dibromododec-11-enamide
DDMS	N-methylsulfonyl-12-12, dibromododec-11-enamide
DPMS	N, methylsulfonyl-15,15-dibromopentadec-14-enamide
df	Degrees of freedom
DiHETEs	Dihydroxyeicosatrienoic acids

DVR	Descending vasa recta
EDHF	Endothelium-derived hyperpolarizing factor
EDTA	Ethylenediamineteraacetic acid
EETs	Epoxyeicosatrienoic acids
EP	PGE_2 receptor
g	Gram(s)
GFR	Glomerular filtration rate
GTN	Glyceryl trinitrate
HET0016	N-hydroxy-N-(4-butyl-2-methylphenyl)formamidine
HETE	Hydroxyeicosatetraenoic acid
H_2O_2	Hydrogen peroxide
Hr	Hour(s)
HR	Heart rate
IC_{50}	50% inhibition dose
IP	PGI_2 receptor
i.v	Intravenous
Kg	Kilogram
L-NNA	N^G-nitro-L-arginine
MAP	Mean arterial pressure
MBF	Medullary blood flow
µg	Microgram(s)
mg	Milligram(s)
µl	Microliter(s)
ml	Mililiter(s)
min	Minute(s)
MLDF	Medullary laser-Doppler flux

mM	Millimole(s)
mmHg	Millimeters of mercury
mRNA	Messenger ribonucleic acids
MS-PPOH	N-methylsulfonyl-6-(2- propargyloxyphenyl) hexanamide
NaCl	Sodium chloride
NADPH	β-nicotinamide adenine nucleotide phosphate, reduced form
$O_2^{\cdot-}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical
PG	Prostaglandin
RIHP	Renal interstitial hydrostatic pressure
RBF	Renal blood flow
SHR	Spontaneously hypertensive rat
V_1	Vasopressin type 1 receptor
V_2	Vasopressin type 2 receptor
WKY	Wistar-Kyoto rat

Chapter One

Literature Review

1.1 Introduction: the kidney in blood pressure control

An individual is defined as having hypertension if their systolic arterial pressure is greater than or equal to 140 mmHg, and/or their diastolic arterial pressure is greater than or equal to 90 mmHg, and/or they are receiving anti-hypertensive pharmacotherapy (10). In the 1999-2000 period it was estimated that approximately 3.69 million Australians aged 25 years and over (30% of the adult population) suffered from hypertension (10). Human essential hypertension is of multi-factorial origin and is a major risk factor for stroke, coronary heart disease, congestive heart failure, peripheral vascular disease and kidney failure (10, 27). In 2002, heart, stoke and vascular diseases accounted for a staggering 50, 294 deaths (37.6% of all deaths) making it the leading cause of death among Australians (10). Coronary heart disease accounted for the highest number of deaths resulting from heart, stroke and vascular diseases (10). In 2000, 755 million dollars were spent on anti-hypertensive drugs sold under the pharmaceutical benefits scheme (10). Given the heavy burden that hypertension and consequent cardiovascular diseases exerts on our society and on most developed countries, it is of great interest to direct research towards developing effective treatment(s) for this condition. It is of equal importance to direct research towards elucidating the underlying causes of essential hypertension, so that effective techniques can be developed to prevent or cure human essential hypertension, rather than just treat its symptoms.

The causes of essential hypertension remain to be elucidated, although there is evidence to suggest that both genetic (124) and psychosocial (115) factors make important contributions. There is also strong evidence that the kidney plays a major role in the pathogenesis of essential hypertension. For example, it has been demonstrated that transplantation of a kidney from a hypertensive subject into a normotensive subject causes hypertension in the recipient and *vice versa* (42). Furthermore, in all experimentally induced hypertensive models, in human essential hypertension, and also

in genetic rat models of hypertension, there is a shift in the pressure diuresis and natriuresis relationship to higher pressures (to the right) (47). Moreover, all effective antihypertensive agents act at least partly by shifting the pressure natriuresis/diuresis relationship to lower levels of arterial pressure (to the left) (47).

It is now over ninety years since Ernest Starling published his work proposing that renal excretion of sodium and water is regulated by the changes in blood volume in capillaries and arteries (47). Although the complex interactions between arterial pressure and renal function were not recognized at the time, Starling seemed to intuitively recognize the role of arterial pressure in sodium and water excretion, which provided the basis to the development of the pressure-natriuresis concept (47). In 1961, Guyton and colleagues first proposed the central role of the kidney in long-term blood pressure regulation. They demonstrated, in a series of experiments, the critical importance of renal control of sodium excretion in the regulation of extracellular fluid volume, and the impact of this on blood pressure control (47). Since then, numerous studies have extensively validated these findings (47). More recent studies have shown that the level of renal medullary blood flow (MBF) is an important determinant of the long-term set point of arterial pressure (48).

The renal medullary circulation seems to play an important role in regulating long-term arterial pressure. Cowley and colleagues have shown that chronic reductions in MBF, induced by medullary interstitial infusion of vasoconstrictor agents in rats, leads to the development of hypertension (47). Conversely, medullary interstitial infusion of vasodilator agents in genetically hypertensive rats increases MBF and ameliorates hypertension (47). The mechanisms underlying the impact of MBF on long-term blood pressure regulation remain a matter of controversy, and this issue will be discussed later in this chapter (see Section 1.2.3 and Figure 1.2). There is considerable evidence that changes in MBF are critical in the pressure diuresis/natriuresis mechanism (47). There is also a body evidence, which suggest that the renal medulla releases a putative vasodepressor substance in response to increased renal arterial pressure (23, 25, 173, 242). Given the profound impact of MBF on long-term blood pressure control, factors that regulate MBF should in turn have a profound effect on the long-term set point of arterial pressure. Previous studies in our laboratory have shown that activation of the renal nerves (69, 70, 93, 94, 126), and vasoactive hormones (71, 76, 184, 185, 206) can

differentially affect MBF and cortical blood flow (CBF). This is likely to represent an important regulatory mechanism in long-term arterial pressure regulation, yet we do not fully understand the mechanisms underlying it.

The global aim of the experiments described in this doctoral thesis was to investigate the roles of paracrine/autocrine factors in responses of regional kidney blood flow to vasoactive hormones and the roles of these factors and the renin angiotensin system in responses of regional kidney blood flow to renal nerve stimulation. In this chapter I will review our current knowledge of the role of MBF in blood pressure control, and the mechanisms underlying the differential regulation of blood flow in the cortex and medulla. First, I will examine the structure of the renal circulation. This will be followed by an assessment of the current knowledge regarding the renal anti-hypertensive mechanisms that are putatively regulated by the medullary microcirculation; pressure natriuresis and the renal medullary depressor hormone. How circulating and locally acting hormones, and the renal nerves, affect regional kidney blood flow, will then be discussed. Finally, the possible mechanisms underlying the differential regulation of CBF and MBF will be examined. In particular, the roles of substances derived from the vascular endothelium and tubular epithelium (eg nitric oxide, prostaglandins and cytochrome P450 (CYP450) metabolites of arachidonic acid (AA)), in modulating regional kidney blood flow responses to sympathetic nerve activity and vasoconstrictor hormones, and also the significance of the interactions between renal sympathetic nerve activity and angiotensin II, will be discussed.

1.2 The renal circulation

1.2.1 Structure of the renal circulation

Approximately 25% of the cardiac output is delivered to the kidney to sustain a high glomerular filtration rate (GFR) (196). A unique feature of the renal circulation is the arrangement of the cortical and medullary circulations. They are in series, because all blood that flows to the renal medulla must first pass through the renal cortex. However, they can also be considered to be virtually in parallel as well, because only efferent

arterioles of the juxtamedullary glomerlui give rise to the medullary circulation (196). (Figure 1.1).

The renal artery arises from the abdominal aorta and branches into progressively smaller arteries; interlobar, arcuate and cortical radial (interlobular) arteries (195). The interlobar arteries ascend within the renal pelvis, follow an arc-like path near the corticomedullary border and give rise to the arcuate arteries. The interlobular arterioles arise from the arcuarte artery and course toward the surface of the kidney (195, 196). Afferent arterioles, which contain one to three layers of smooth muscle cells, branch from the interlobular arteries and supply glomeruli at different levels of the cortex (195). The glomerular capillaries recombine to form the efferent arterioles, which then branch into the peritubular capillary networks and ultimately empty into veins (196).

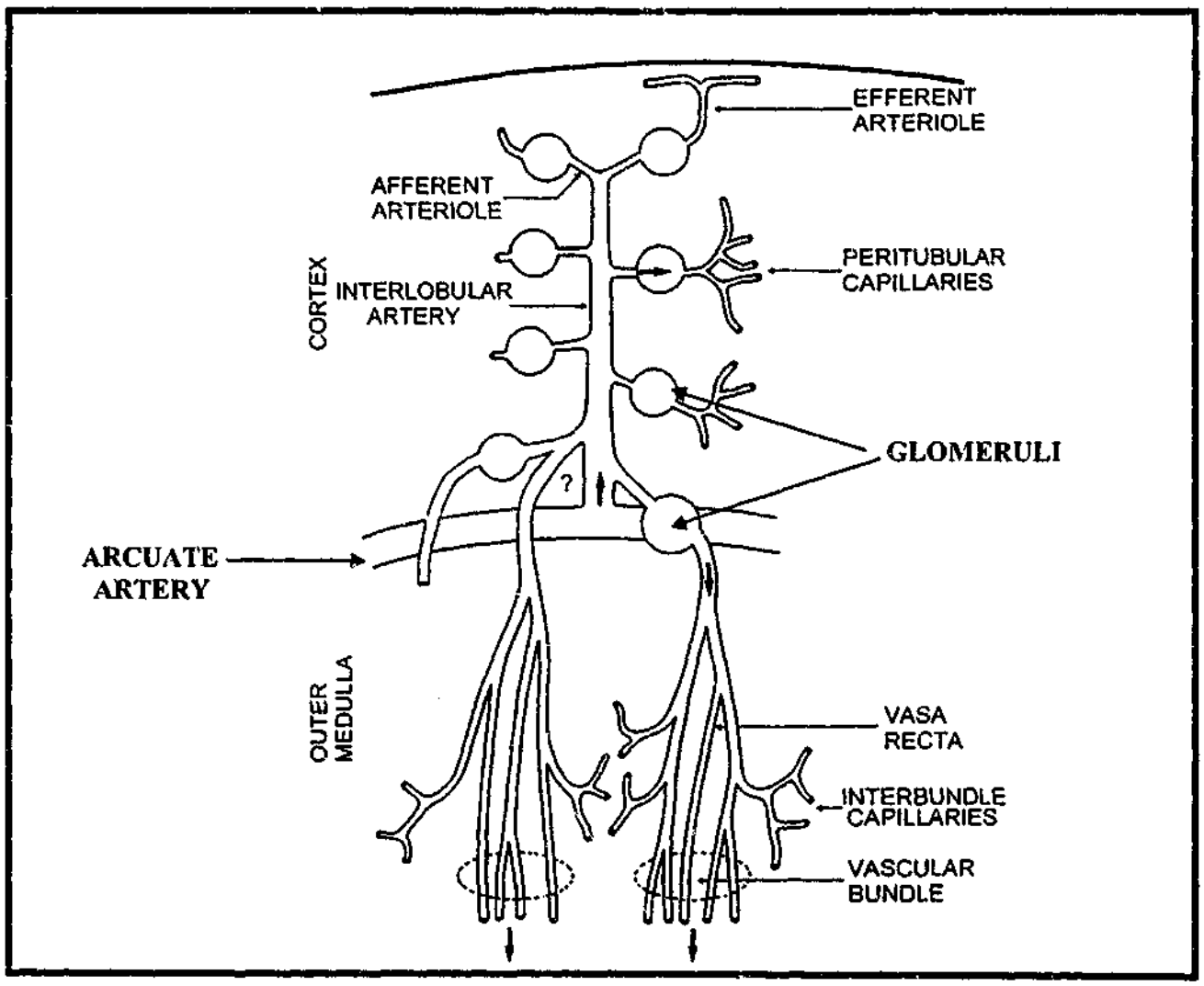

Figure 1.1 Structure of the renal circulation. Modified from (195). The original diagram is from (15).

1.2.2 The renal medullary microcirculation

The medullary circulation, in addition to providing oxygen delivery and waste removal, is responsible for maintaining the corticomedullary gradients of sodium chloride and urea and removing water reabsorbed from the descending limbs of the loops of Henle and the medullary collecting tubules (196). Juxtamedullary efferent arterioles enter the outer medulla and give rise to descending vasa recta (DVR). These are responsible for perfusing the renal medulla. There is also evidence that approximately 10% of juxtamedullary afferent arterioles do not lead to glomeruli, but instead lead directly to efferent arterioles and DVR (see Figure 1.1) (39). Thus, at least a small proportion of MBF might by-pass glomerular filtration (39). Also, since the medullary microcirculation arises exclusively from juxtamedullary arterioles, changes in vascular resistance in juxtamedullary arterioles and downstream vascular elements within the medulla, can theoretically cause large changes in MBF without significantly changing blood flow in the bulk of the cortex (39).

DVR of the inner stripe of the outer medulla run together in vascular bundles (195, 196). Ascending vasa recta (AVR) arising from the inner medulla run close to these DVR while AVR arising from the outer medulla run directly to the cortex (195). This arrangement of the vascular bundles favours the countercurrent exchange between all AVR originating from the inner medulla and DVR which perfuse the outer and inner medulla (196). DVR in the periphery of the vascular bundles give rise to the interbundle capillary plexus and are responsible for perfusion of the nephron segments in the outer medulla, while DVR in the center are responsible for perfusion of the inner medulla. The parallel arrangement of DVR within the vascular bundles theoretically allows hormones and renal nerves to regulate regional perfusion within the medulla (195). For example, increased resistance in the DVR in the periphery of the vascular bundles could theoretically redistribute MBF towards the inner medulla. On the other hand, increased resistance in the DVR in the center could increase blood flow to the outer medullary interbundle region while reducing inner MBF (195). However, to date there is little direct evidence that such redistribution of blood flow within the medulla occurs in practice, although there have been reports of differential changes in inner and outer MBF in response to stimuli such as increased renal perfusion pressure (128) and water deprivation (88). AVR are more abundant and larger than DVR. As a result, single

vessel flow rate progressively reduces from DVR to AVR, allowing efficient countercurrent exchange and trapping of sodium chloride and urea.

1.2.3 The role of the renal medullary microcirculation in blood pressure control

Cowley and colleagues, in an extensive series of experiments, have shown that there is an inverse relationship between the level of MBF and mean arterial pressure (MAP). For example, they demonstrated that chronic infusion captopril, into the renal medulla of spontaneously hypertensive rats (SHR) selectively increased MBF, had little effect on CBF, and reduced MAP. Conversely, chronic infusion of nitro-L-arginine methyl ester into the renal medullary interstitium of conscious rats, selectively reduced MBF, had no effect on CBF and increased MAP (155). Consistent with this latter finding, chronic renal medullary interstitial infusion of a superoxide dismutase inhibiter (diethyldithiocarbamic acid) has been shown to selectively reduce MBF and increase MAP in Sprague-Dawley rats (147). Franchini and coworkers have shown that the medullary microcirculation is highly sensitive to the vasoconstrictor actions of arginine vasopresin mediated via the activation of vasopressin V_1-receptors (88), while activation of V_2-receptors appears to increase MBF (176). Consistent with the hypothesis that the level of MBF plays a key role in regulating the long-term set point of arterial pressure (see Figure 1.2), chronic intravenous infusion of a V_1-receptor agonist, [Phe^2, Ile^3, Orn^8]vasopressin, has been shown to produce sustained hypertension (51) while simultaneous medullary interstitial infusion of a selective V_1- receptor antagonist, has been shown to prevent this effect (51, 239). Hypertension can also be induced by infusion of a selective V_1-receptor agonist, but not arginine vasopressin, into the renal medulla (239). Collectively, these observations suggest that hypertension can be induced through selective activation of V_1-receptors in the renal medullary circulation. These studies provide compelling evidence that a reduction in MBF leads to hypertension while an increase in MBF ameliorates hypertension (47). Although the precise mechanisms by which the level of MBF affects the long-term set point of arterial pressure are yet to be determined, there is evidence that increases in MBF initiate two renal anti-hypertensive mechanisms; pressure diuresis/natriuresis and perhaps also release of a putative renal medullary depressor hormone (47).

1.2.3.1 Pressure diuresis/natriuresis mechanism

The phenomenon of pressure diuresis/natriureis, whereby increased renal perfusion pressure induces an elevation in both water and sodium excretion in the absence of changes in RBF or GFR (48), seems to play a central role in the long-term control of arterial pressure. Although there is some evidence to the contrary (68, 142, 143, 145, 156) experiments conducted in rats (80, 104, 150,156) and dogs (233) indicate that while RBF, CBF and GFR are well autoregulated, MBF is poorly autoregulated. Hence, elevations in MAP can cause parallel elevations in MBF (and so vasa recta capillary hydrostatic pressure), which would in turn cause an increase in renal interstitial hydrostatic pressure (RIHP) and perhaps the release of autocoids such as nitric oxide and prostanoids (25, 47). This increase in RIHP is thought to serve as a global signal to inhibit sodium and water reabsorption (47). In this regard, previous studies have demonstrated that increased RIHP decreased sodium reabsorption in the proximal segments of deep nephrons in rats (96, 213). The precise mechanisms responsible for the reduction in tubular reabsorption of sodium and water with increasing RIHP are not well defined. One suggestion is that increases in RIHP enhance a backleak of sodium chloride via paracellular pathways (48). Although the precise mechanism(s) of pressure natriuresis remain to be determined there is evidence to suggest that changes in intrarenal nitric oxide concentrations in response to changes in arterial pressure play a role in mediating this response (144, 146).

There is also a body of evidence to indicate that MBF is well autoregulated (68, 142, 143, 145, 156). For example, Majid et al, with the use of laser Doppler flowmetry, demonstrated that in anaesthetized dogs, blood flow in the inner (142), mid (143, 145) and outer medullary (142) regions of the kidney were well autoregulated in the face of step-wise changes in renal arterial pressure. Consistent with this, a recent study in our laboratory also found that MBF remained remarkably stable in rabbits when renal perfusion pressure was increased step-wise using an extracorporeal circuit (68). The discrepancies regarding autoregulation of MBF remain difficult to explain. One possible explanation is that volume status of the animal affects the autoregulation capacity of MBF, in that acute volume expansion has been shown to attenuate the autoregulatory capacity of MBF but not CBF (150, 156, 214).

1.2.3.2 The putative renal medullary depressor hormone

The renal medullary interstitial cells may release a putative depressor hormone, in response to an increase in MBF (48). The first piece of evidence for a role of this putative hormone in lowering arterial pressure came from the work of Grollman et al, who demonstrated that ureteral ligation but not ureterocaval anastamoses caused hypertension in dogs (92). Muirhead et al demonstrated that transplants of tissue from the renal medulla (but not renal cortex) were able to reverse hypertension in a number of experimentally induced models of hypertension (174). From the results of these, and a number of other experiments, these authors concluded that renal medullary interstitial cells secrete a lipid hormone with anti-hypertensive properties, which they dubbed medullipin (150).

Christy et al, with the use of an extracorporeal circuit, demonstrated that an intact renal medulla was essential to observe a reduction in arterial pressure in response to increased renal perfusion pressure in both rabbits and dogs (41). When the renal medulla of these animals was chemically destroyed, the hypotensive response to increased renal arterial pressure was abolished (41). Lu and colleagues (134) have shown that chronic infusion of captopril into the renal medullary interstitium of SHR for a duration of five days, increased MBF and reduced MAP. In the first few days of this experiment a reduction in MAP was observed in the absence of significant changes in sodium and water excretion. Thus, actions of the renal medullary depressor hormone could account for the reduction in MAP during this period (23). Further, [Phe^2,Ile^3,Orn^8]-vasopressin induced reductions in MBF have been shown to abolish the depressor effect of increased renal perfusion pressure (24), suggesting that the level of MBF is an important determinant in the release of this putative renal medullary depressor hormone.

Interestingly, Correia et al recently demonstrated in anaesthetized rabbits, that MAP did not decrease in response to increased renal perfusion pressure, if extracellular fluid depletion due to increased urinary salt and water excretion, was prevented (by replacement with compound sodium lactate solution) (44). Likewise, MAP did not decrease after unclipping the renal artery of 1-kidney, 1-clip hypertensive rats, when 154 mM NaCl was infused at a rate equivalent to urine flow (44). These studies provide strong evidence that reductions in MAP observed in response to increased renal arterial pressure are chiefly dependent on the pressure natriuresis/diuresis mechanism rather than

release of 'medullipin' (44). This finding also raises the possibility that the reduction in MAP reported in response to increased renal arterial pressure in previous studies (24, 41, 46, 71) could also chiefly be due to hypovolaemia resulting from pressure natriuresis/diuresis rather than a direct effect of a putative renal medullary depressor hormone (44). Thus, the role of this renal medullary depressor hormone as an antihypertensive mechanism clearly requires further investigation.

Taken together, these data indicate that the renal medullary circulation affects arterial pressure chiefly through the pressure diuresis/natriuresis mechanism, which would act to return arterial pressure to basal values when there is a perturbation in arterial pressure. Figure 1.2 indicates the proposed role of this renal antihypertensive mechanism in the regulation of arterial pressure and the proposed critical role of the medullary microcirculation.

If the hypothesis outlined in Figure 1.2 is correct, then factors that modulate the level of MBF should in turn affect arterial pressure in the long term. An understanding of these factors should therefore provide vital information leading to a greater understanding of blood pressure control and the pathogenesis of hypertension.

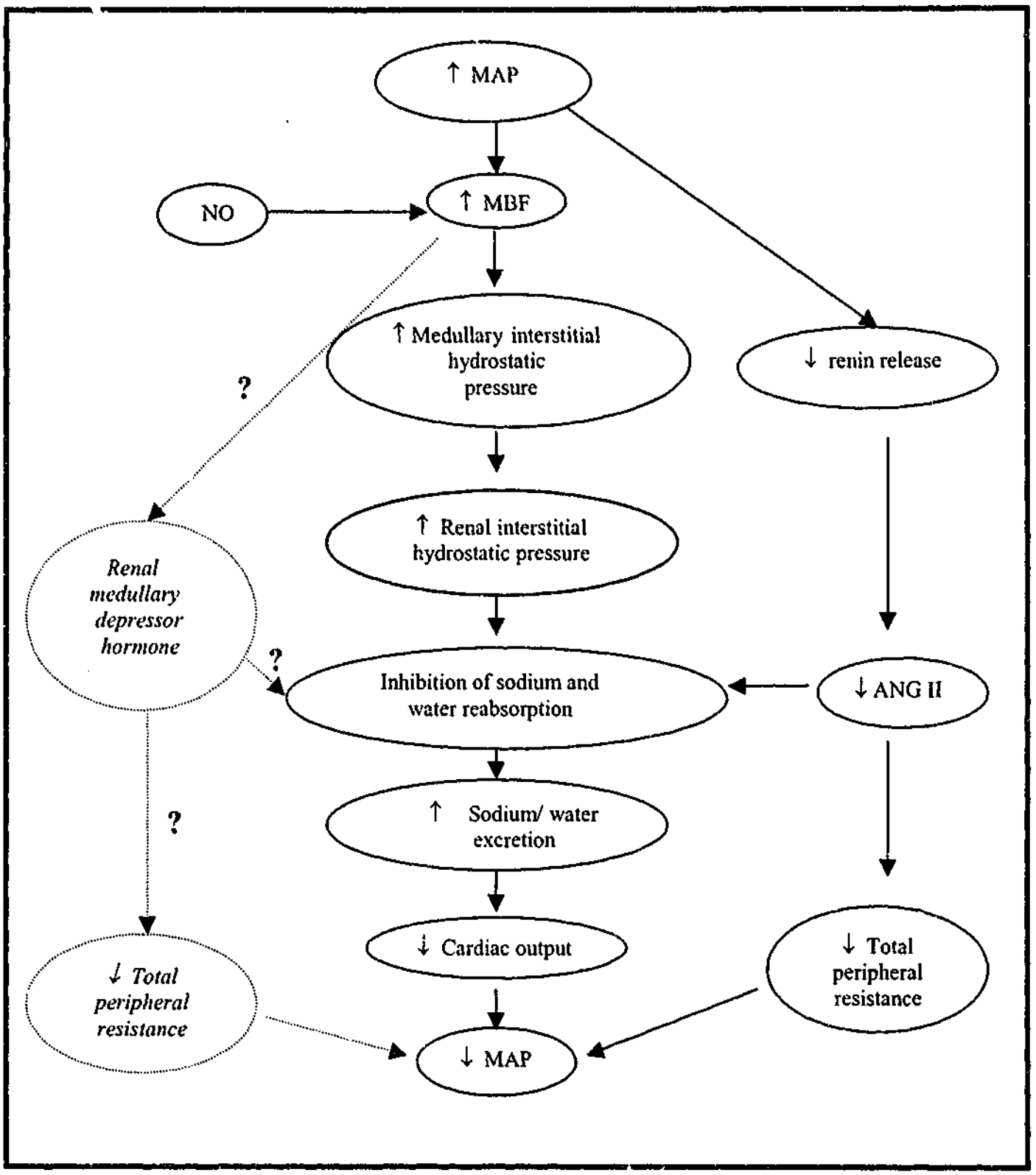

Figure 1.2. Renal antihypertensive mechanisms. An increase in MAP is thought to cause parallel increases in MBF. This increase in MBF is suggested to activate two renal antihypertensive mechanisms; pressure natriuresis and the release of a putative renal medullary depressor hormone, both of which would act to reduce MAP back to its control levels when there is a perturbation in blood pressure. Increased renal perfusion pressure will also inhibit rennin release so reduce the activity of the pro-hypertensive rennin/angiotensin/aldosterone system. (MAP: mean arterial pressure; NO: nitric oxide; ANG II: angiotensin II; MBF: medullary blood flow; ↓: decreased; ↑: increased).

1.3 Effects of hormones, and the renal nerves on intrarenal perfusion

Given the critical role of the renal medullary microcirculation in long-term blood pressure regulation, it is of great interest to investigate the factors that control MBF. There is persuasive evidence that hormonal and neural factors differentially and independently affect blood flow in the renal cortex and medulla. For example, circulating arginine vasopressin and the vasopressin V_1-receptor agonist [Phe^2, Ile^3, Orn^8]-vasopressin selectively reduce MBF but have little effect on CBF (45, 71, 72, 76, 176, 184, 185, 206) In contrast, infusions of noradrenaline (43, 76, 184, 185, 197, 198, 206) and angiotensin II (76, 105, 184, 185, 197, 198, 206) reduce CBF but not MBF. Bolus doses (intravenous or renal arterial) of angiotensin II reduce CBF but increase MBF (71, 72, 180, 185, 206, 252). Electrical as well as reflex mediated stimulation of renal nerves reduces CBF more than MBF (69, 70, 93, 94, 126, 127, 220). In the experiments described in this thesis, we have focused on elucidating the mechanisms underlying the differential control of CBF and MBF by angiotensin II and vasopressin peptides (Chapters 3 and 4) and the renal sympathetic nerves (Chapters 5 and 6). Therefore, the effects of these hormonal and neural factors on regional kidney perfusion are discussed in detail below.

1.3.1 Angiotensin II

Angiotensinogen is synthesized by the liver and secreted into the systemic circulation (208). Once released in to the circulation, angiotensinogen is converted to angiotensin I by the enzyme renin. In the lungs, angiotensin converting enzyme converts angiotensin I to angiotensin II (208). Angiotensin II has diverse actions within the kidney; inhibiting renin release, enhancing sodium reabsorption and promoting renal vasoconstriction (208). In addition, there is a large body of experimental data, which indicate that this hormone is involved in regulating the intrarenal distribution of blood flow (14, 76, 81, 105, 180, 184, 185, 190, 197, 198, 206, 252). However, the findings regarding the effects of angiotensin II on MBF are inconsistent (Table 1.1).

Table 1.1: Responses of regional kidney blood flow and mean arterial pressure to angiotensin II and possible mechanisms underlying the differential control of CBF and MBF by this hormone

Species/ State	Technique used to measure RKBF	Mode of administra -tion	MAP	RBF	CBF	MBF/ PBF*	† Key observation	Refer ence
Anaesthetized dogs	Albumin accumulatio n	Renal arterial infusion	↑	↔	-	↓	Angiotensin II reduced MBF at doses that did not affect total RBF	(81)
Anaesthetized rats	LDF	Intravenous infusion	↑	-	↓	↔	COX inhibition, NOS inhibition or bradykinin receptor antagonism had little effect on responses of CBF and PBF to angiotensin II	(105)
Anaesthetized rats	LDF	Intravenous infusion	↑	-	↓	↑	Inhibition of kallikrein-kinin system, but not COX inhibition, abolished angiotensin II-induced increases in PBF. These authors concluded that increases in PBF to angiotensin II depend on release of kinins but not PGs.	(180)
Anaesthetized rabbits	Local H_2 clearance	Intravenous infusion	↑	↓	↓	An initial reduction (Flow returned to control levels within 3 mins)	NOS inhibition enhanced responses of RBF and CBF, but had little effect on responses of MBF to angiotensin II. These authors concluded that basal nitric oxide buffers angiotensin II-induced renal vasoconstriction. They speculated that angiotensin II might up-regulate the production of medullary prostaglandins to buffer reductions in MBF.	(197)
Anaesthetized rats	Local H_2 clearance	Intravenous infusion	↑	↓	↓	↔	Angiotensin II reduced MBF after COX inhibition, suggesting that under normal physiological conditions vasodilator PGs buffer reductions in MBF in response to angiotensin II	(198)

Table 1.1 (continued)

Species/ State	Technique used to measure RKBF	Mode of administra -tion	MAP	RBF	CBF	MBF/ PBF*	†Key observation	Refer ence
Anaesthetized rabbits	LDF	Intravenous infusion	↑	↓	↓	↑	Transient increase in MBF waned with continued infusion of angiotensin II	(71)
Anaesthetized rats	LDF	Intravenous infusion	↑	↓	-	↑	NOS inhibition abolished angiotensin II-induced increases in PBF suggesting that nitric oxide contributes to this response	(190)
Anaesthetized Rats	LDF	Intravenous infusion	↑	-	-	↑	NOS inhibition abolished angiotensin II-induced increases in PBF. These authors concluded that endogenous nitric oxide contributes to responses of PBF to angiotensin II	(252)
Anaesthetized rabbits	LDF	Renal arterial boluses	↑	↓	↓	An initial reduction and a later increase	COX inhibition abolished angiotensin II-induced increases in MBF suggesting that vasodilator PGs contribute to increases in MBF in response to angiotensin II	(185)
Anaesthetized rabbits	LDF	Renal arterial bolus	↔	↓	↓	An initial reduction and a later increase	NOS inhibition enhanced the initial reduction in MBF and blunted the later increase in MBF, suggesting that nitric oxide contributes to angiotensin II-induced increases in MBF	(206)
Anaesthtized rats	LDF	Intravenous infusion	-	↓	-	↔ inner MBF; ↓ outer MBF	Reductions in inner MBF were revealed after COX inhibition with indomethacin. These authors concluded that PGs buffer angiotensin II induced vasoconstriction in the inner medulla	(13)

Table 1.1 (continued)

↑, increased; ↓, decreased; ↔, no change; -, not examined; PGs, prostaglandins; LDF, laser Doppler flowmetry; RKBF, regional kidney blood flow; MBF, medullary blood flow; CBF, cortical blood flow; PBF, papillary blood flow; RBF, renal blood flow; MAP, mean arterial pressure; COX, cyclooxygenase; NOS, nitric oxide synthase.

* Responses of MBF to angiotensin II are inconsistent between different studies. Although precise reason(s) for these discrepancies remain unknown, it could be due to differences in species, doses of angiotensin II used, mode of administration and/or the techniques used to measure MBF/PBF. In general, however, it seems that in rats and rabbits, MBF is relatively insensitive to the vasoconstrictor effects of angiotensin II, at least partly due to counter regulatory actions of nitric oxide and PGs. This generalization may not hold true in dogs.

† These studies are arranged in chronological order.

Faubert and coworkers, using the albumin accumulation method, found that renal arterial infusion of a 'physiological' dose of angiotensin II reduced papillary plasma flow without changing RBF or GFR in anaesthetized dogs (81). In contrast, a number of studies, utilising a variety of techniques, have demonstrated that angiotensin II can reduce RBF/CBF but not MBF or papillary blood flow in anaesthetized rats (13, 105, 152, 197, 198), and anaesthetized (71) and conscious (76) rabbits. Other studies have shwn that angiotensin II can increase papillary blood flow (180, 252) or MBF (14) in anaesthetized rats. Consistent with these data, recent studies in our laboratory indicated that bolus doses of angiotensin II reduced CBF but caused a biphasic response in MBF with an initial reduction accompanied by a later increase, in anaesthetized rabbits (66, 185, 206). Hence, despite the large number of studies investigating the effects of angiotensin II on the medullary microcirculation, the results seem to be inconsistent. This could be due to a number of confounding factors, including species differences, the differing doses of angiotensin II used, the confounding effects of anaesthesia, and the techniques used to estimate regional kidney blood flow. A further complicating factor in this literature surrounds the observation that inhibitors of angiotensin converting enzyme increase MBF. Rather than reflecting a physiological role of angiotensin II in tonically reducing MBF, it seems more likely that these observations reflect the role of angiotensin converting enzyme in degradation of bradykinin (154), and the profound impact of bradykinin on MBF (114). Neverthless, it seems clear that in rats and rabbits, MBF is relatively insensitive to the vasoconstrictor effects of angiotensin II.

1.3.2 Vasopressin

Previous observations have shown that intravenous infusion of [Phe^2, Ile^3, Orn^8] vasopressin can reduce MBF but not CBF or total RBF in anaesthetized and conscious rabbits (71, 76). These results are in accordance with those of Franchini and associates who demonstrated that small elevations in circulating arginine vasopressin reduce MBF but not CBF in rats (88). In agreement with these findings, our laboratory recently demonstrated that renal arterial injection of the vasopressin V_1-receptor agonist [Phe^2, Ile^3, Orn^8] vasopressin reduced MBF but had little effect on CBF in anaesthetized rabbits (184, 185, 206). These observations, and the fact that V_{1a}-receptor (subset of V_1-receptors) messenger ribonucleic acid (mRNA) is localised to the vascular elements that regulate MBF (afferent and efferent arterioles from the mid cortical to juxtamedullary

region and vasa recta capillaries in the outer medulla) (199), suggests that arginine vasopressin reduces MBF chiefly by activation of V_1-receptors (87, 88).

Renal medullary interstitial infusion of either arginine vasopressin (175) or the vasopressin V_2-receptor agonist, 1-desamino-8-D-arginine vasopressin (175, 200), has been shown to increase MBF in anaesthetized rats pretreated with a selective V_1-receptor antagonist (176). However, as V_2-receptor mRNA has not been observed within the vasculature of the renal cortex or medulla (199), the V_2-receptor dependent increase in MBF is unlikely to be a direct vascular effect (199). V_2-receptor mRNA is localized mainly to tubular elements, particularly to the medullary thick ascending limbs of Henle and collecting ducts (199). There is evidence that vasopressin can cause nitric oxide release from inner medullary collecting duct epithelium (170). Collectively, these data indicate that V_1-receptors mediate vasoconstriction, while V_2-receptors mediate vasodilatation, in the renal circulation.

1.3.3 Noradrenaline and renal sympathetic nerve activation

There is evidence that noradrenaline exerts disparate effects on the renal cortical and medullary circulations. Infusion of noradrenaline reduced CBF but not MBF in both conscious (76) and anaesthetized rabbits (43, 184, 185, 206). Likewise, electrical (69, 70, 93, 94, 126, 220) or reflex mediated stimulation (123, 127) of the renal nerves have been demonstrated to reduce CBF more than MBF in anaesthetized rabbits. Although the precise mechanism(s) responsible for the relative insensitivity of the medullary circulation to noradrenaline and renal nerve stimulation remain to be precisely defined, evidence has been presented to indicate that prostaglandins (198) and nitric oxide (69, 206) are involved (see Sections 1.5 and 1.6).

1.4 Possible mechanisms underlying the differential regulation of CBF and MBF by hormonal and neural factors

The differential control of CBF and MBF by circulating and locally acting hormones, as well as renal sympathetic nerve activity, is likely to represent an important regulatory mechanism in long-term blood pressure control. We are only just beginning to understand the mechanisms underlying it. Differential distribution of pharmacological receptors on vascular elements regulating CBF and MBF, regional variations in

sympathetic innervation density within the kidney, renal vascular geometry, and differential engagement of endothelial- and epithelial-derived paracrine factors are some of the mechanisms that could account for this phenomenon.

1.4.1 Differential distribution of pharmacological receptors

The ability of hormones to differentially regulate CBF and MBF could depend on the pattern of distribution of their pharmacological receptors in vascular elements regulating CBF and MBF. However, as discussed in the following sections, this seems unlikely to fully account for the disparate actions of angiotensin II and vasopressin peptides on regional kidney blood flow.

1.4.1.1 Angiotensin II

There are two major types of angiotensin II receptors, AT_1 and AT_2 (243). Within the kidney, AT_1-receptor protein is localized to the outer medullary proximal tubules and the cortical blood vessels (166). Paxton et al. (202) with the use of immunohistochemical techniques, have identified AT_1-receptor protein in the thick ascending limbs located in the inner stripe of outer medulla. In agreement with these results, Mendelsohn et al. (164) have shown that angiotensin II receptors are localized in the inner stripe of the outer medulla in association with the vasa recta bundles, implying that angiotensin II could directly influence vasa recta function. Furthermore, Pallone (194) observed graded focal constrictions of the vasa recta dissected from the inner stripe of the outer medulla in response to application of angiotensin II and it is suggested that this was due to activation of AT_1-receptors. Other studies have indicated that either angiotensin II receptors, or mRNA for angiotensin II receptors, are distributed in the glomeruli and extraglomerular mesangial cells (113), inner stripe of outer medulla (99), and type 1 interstitial cells in the outer medulla (263).

AT_2-receptor mRNA has been identified in renal tubules and vasculature including the proximal tubules, collecting ducts, arcuate arteries, afferent arterioles and outer medullary descending vasa recta (166). Although, AT_1- and AT_2-receptors are expressed in vascular elements regulating MBF, previous findings by ourselves (76, 184, 185, 206) and others (105, 197, 198) have demonstrated that MBF is relatively insensitive to the vasoconstrictor effects of this hormone (see Section 1.3.1 and Table 1.1).

1.4.1.2 Vasopressin

To date two types of vasopressin receptors, V_2 and V_{1a}, have been identified within the kidney, and are thought to mediate vasodilatation and vasoconstriction respectively. V_2-receptor mRNA has been identified on renal tubules but not on vascular elements regulating MBF (199). V_{1a}-receptor mRNA is found in mid-cortical to juxtamedullary afferent and efferent arterioles, glomeruli, and outer medullary vasa recta (199). Consistent with these findings, it has been shown repeatedly that vasopressin can reduce MBF in anaesthetized rats and rabbits (see Section 1.3.2). Harrison Bernard and Carmines demonstrated that vasopressin, via V_1-receptor activation, caused vasoconstriction in arcuate and interlobular arteries in the *in-vitro* perfused juxtamedullary rat preparation (97). Surprisingly, infusion of a V_1-receptor agonist has been shown to have considerably less effect on CBF than MBF *in vivo* (45, 71, 76, 184, 185, 206). This relative insensitivity of the cortical circulation to vasopressin is unlikely to be due to lack of V_{1a} receptors in the renal cortex, but could rather be due to the selective release of vasodilator substances such as CYP450 metabolites of AA. We tested this hypothesis in the experiments described in Chapters 3 and 4.

1.4.2 Renal vascular geometry

As shown in Figure 1.1, the arrangement of the renal vascular bed *per se* allows differential regulation of blood flow to the renal cortex and the medulla. The renal medullary microcirculation arises exclusively from the efferent arterioles of juxtamedullary and some mid cortical glomeruli (195). Thus, theoretically, changes in resistance in these efferent arterioles should lead to changes in MBF but not outer CBF (195).

The afferent, and particularly the efferent, arterioles of juxtamedullary glomeruli have larger diameters than the afferent and efferent arterioles of cortical glomeruli (Figure 1.3) (74). Thus, according to the Poiseuille's relationship (resistance of a vessel α 1/(Radius of the vessel)^4), similar reductions in vascular calibre in arterioles of juxtamedullary and cortical glomeruli, should theoretically lead to greater reductions in CBF than MBF. Consistent with this view, previous studies in our research group, using a vascular casting technique, have shown that doses of angiotensin II (56, 57) and endothelin-1 (58) that reduce both cortical and juxtamedullary afferent and efferent arteriole diameters to a similar extent, selectively reduce CBF without affecting MBF.

Thus, the larger vascular calibre of juxtamedullary arterioles (compared with those of cortical glomeruli) seems to provide a structural basis for protection of the renal medullary vasculature from the ischaemic effects of vasoconstrictor agents such as angiotensin II and endothelin-1 (74).

There is also evidence that about 5-10% of juxtamedullary afferent arterioles by-pass the glomeruli and give rise to DVR in the medulla (39). The precise function(s) of these shunts remains to be determined (23). Approximately 20% of the juxtamedullary efferent arterioles supply the inner cortical region while others are responsible for perfusion of the medulla, so differential regulation of blood flow to these two types of glomeruli would in turn lead to differential distribution of blood flow to the inner cortex and the medulla (23).

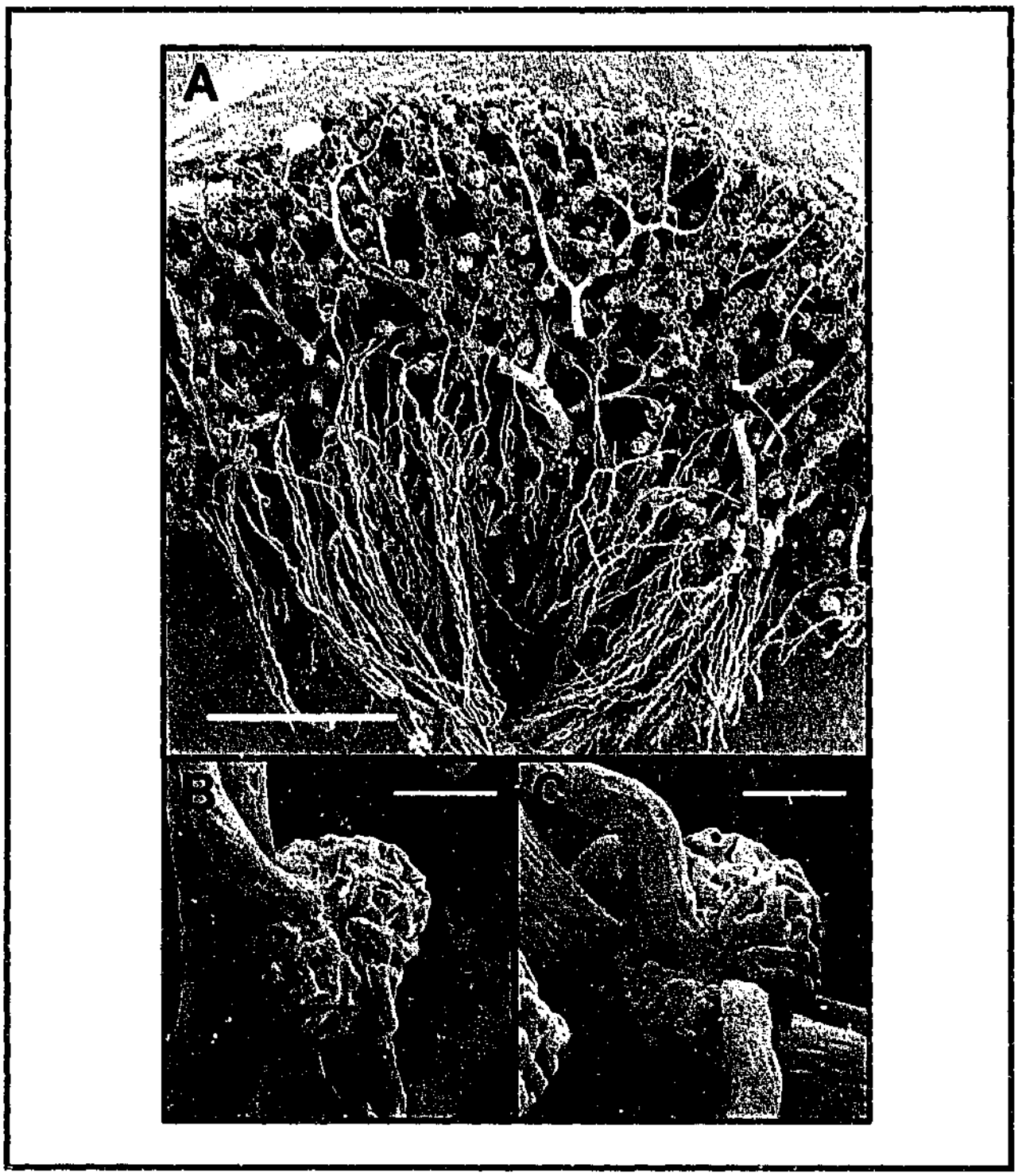

Figure 1.3 **(A)** Resin cast of the renal vasculature of a rabbit, depicting both cortical and medullary vessels (scale bar 1 mm). Note the diameters of cortical peritubular capillaries are considerably less than those of medullary vasa recta. **(B)** Cortical glomeruli showing afferent (upper vessel) and efferent arterioles and the capillary tuft (scale bar 60 μm). **(C)** Juxtamedullary glomeruli showing afferent (upper vessel) and efferent arterioles and the capillary tuft (scale bar 60 μm). Note the larger arteriole diameters of the juxtamedullary compared to the cortical glomerular arterioles, particularly the efferent arterioles. (Taken from (74)).

1.4.3 Regional differences in sympathetic innervation in the kidney

The lumbar splanchnic nerve, the intermesenteric plexus and ganglia of the celiac plexus give rise to renal efferent sympathetic innervation (18). The renal nerves enter the kidney with the vasculature and are observed along the interlobar, arcuate and interlobular arteries, afferent arterioles and outer medullary vasa recta (18). Barajas and Powers (18), with the use of autoradiographic techniques, demonstrated that the renal vasculature is more densly innervated than renal tubules and that vascular elements in the outer medulla are innervated. However, vascular elements in the inner medulla are not innervated (18). Among different vascular segments, the innervation density varies in the following order from highest to lowest: afferent arterioles, efferent arterioles, interlobular arteries, cortical capillaries, and renal veins (18). The innervation density also varies among different tubular segments in the following order from highest to lowest: the proximal tubule, cortical thick ascending limb of Henle, the connecting tubule, the distal convulated tubule, and the collecting duct (18).

Although, vascular elements that regulate MBF are richly innervated (17, 60, 163), previous studies have shown that MBF is relatively insensitive to electrical and reflex mediated stimulation of the renal nerves (see Section 1.3.3). So, mechanisms other than regional variations in sympathetic innervation density within the kidney likely contribute to this relative insensitivity of MBF. One possibility is that paracrine factors from the vascular endothelium and tubular epithelium blunt the vasoconstritor effects of renal sympathetic nerve activity on the medullary circulation, as described in Section 1.6. This issue is the focus of the experiments described in Chapters 5 and 6.

1.4.4 Paracrine factors

There is a large body of evidence to indicate that paracrine factors such as nitric oxide and various AA metabolites act as paracrine and autocrine signaling molecules that modulate responses to vasoactive hormones such as noradrenaline, vasopressin, angiotensin II, and to the activation of the renal nerves (see Sections 1.5 and 1.6 and Table 1.2). Thus, nitric oxide and metabolites of AA could well contribute to the differential regulation of CBF and MBF by renal nerves and hormones. Nitric oxide and AA metabolites also play key roles in regulation of renal and glomerular haemodynamics (106, 179), but these aspects will not be covered in this review as this thesis focuses on the roles of these factors in underlying responses of intrarenal blood

flow to hormonal and neural stimuli. A brief general introduction about the chemistry and biology of nitric oxide and various AA metabolites will be provided, before examining the role(s) of these factors in underlying the differential regulation of CBF and MBF by hormones and renal nerves.

1.4.4.1 Nitric oxide

In 1980, the seminal work of Furchgott and coworkers demonstrated that acetylcholine-induced vasodilatation of blood vessels is dependent on the presence of a substance released by the endothelium, which was later known as the endothelium derived relaxing factor (91). The nature of endothelium derived relaxing factor remained unknown for a long time but growing experimental evidence now confirms that a key endothelium derived relaxing factor is nitric oxide (90, 248). Other endothelial-derived factors such as endothelial derived hyperpolarizing factor (248) and endothelial derived contracting factor (248) have also been described.

Synthesis of nitric oxide

Nitric oxide, a paracrine/autocrine hormone, is synthesised from the terminal guanidino nitrogen atom(s) of the precursor, L-arginine by the enzyme nitric oxide synthase (125) (Figure 1.4).

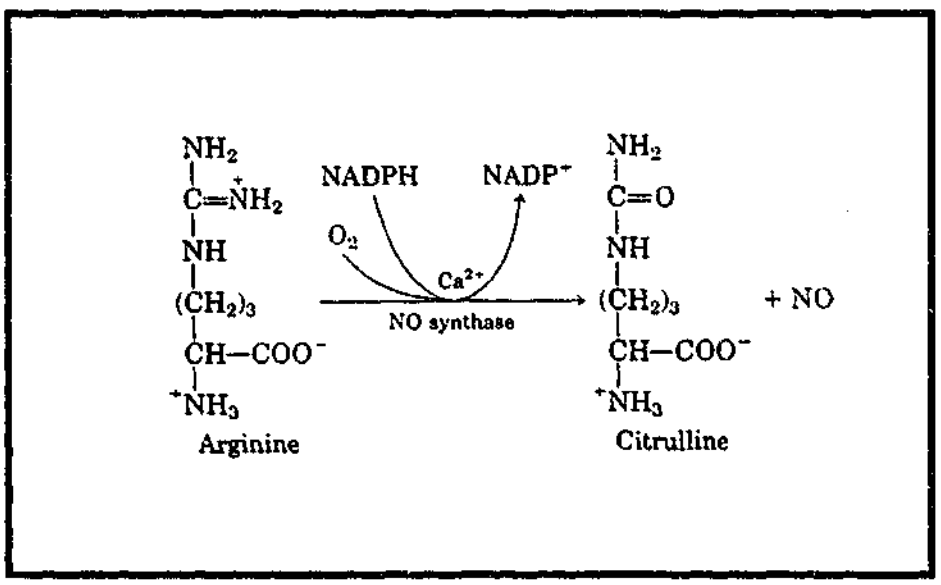

Figure 1.4: Synthesis of nitric oxide from L-arginine. Taken from (125)

Nitric oxide synthase isoforms oxidise the gunidino group of L-arginine, with the help of four cofactors: nicotinamide adenine dinucleotide phosphate, flavin mononucleotide,

flavin adenine dinucleotide, and H_4 biopterin, to form nitric oxide and citrulline. The formation of nitric oxide also requires a porphrin heme group (179). Newly formed nitric oxide diffuses into nearby cells, and binds to the heme moiety of guanylate cyclase and catalyses the production of guanosine 3', 5'-cyclic monophosphate (cGMP), which in turn acts as a signal to cause relaxation of smooth muscle, as well as other actions (125). The actions of nitric oxide are brief, both because it is rapidly degraded, and also because the rate of turnover of cGMP is high (179). Within seconds of nitric oxide synthesis, it is converted into nitrites and nitrates by oxygen and water (179), or to peroxynitrite by superoxide (249). Most of the actions of physiological concentrations of nitric oxide are thought to be mediated via cGMP (179), although there is evidence that nitric oxide interacts with other metallic enzymes, oxygen radicals and other substances (179). For example, a recent study by Alonso-Galicia has shown that nitric oxide inhibits 20-hydroxyeicosatetraenoic acid (20-HETE) production and that this contributes to the vasodilatory actions of nitric oxide (5). L-arginine analogues such as N^G-nitro-L-arginine methyl ester (L-NAME), N^G–nitro-L-arginine (L-NNA) and N^G-monomethyl-L-arginine (L-NMMA) can inhibit the formation of nitric oxide (179) and these agents have been widely used in studies to investigate the roles of nitric oxide in various physiological functions. Nitric oxide has been shown to modulate responses of regional kidney perfusion to hormonal and neural stimuli (179) (see Sections 1.5 and 1.6), and to play a key role in the pressure diuresis/natriuresis mechanism (47) (see Section 1.2.3.1).

Nitric oxide synthase isoforms

Available data suggest the existence of three isoforms of nitric oxide synthase: nitric oxide synthase I (neuronal NOS), nitric oxide synthase II (inducible NOS), and nitric oxide synthase III (endothelial NOS) (179). Nitric oxide synthase I and III are constitutively expressed while nitric oxide synthase II is expressed mainly after transcriptional activation (179). Nitric oxide synthase III has been shown to be expressed in the endothelium of cortical and medullary blood vessels (11, 246). Nitric oxide synthase I is expressed in macula densa cells, the thick ascending limb of Henle's loop, intrarenal neurons and cultured mesangial cells, while nitric oxide synthase II is expressed in mesangial cells and several tubular epithelial cell-types (179). A range of stimuli can mediate the release of this hormone, as depicted in Figure 1.5.

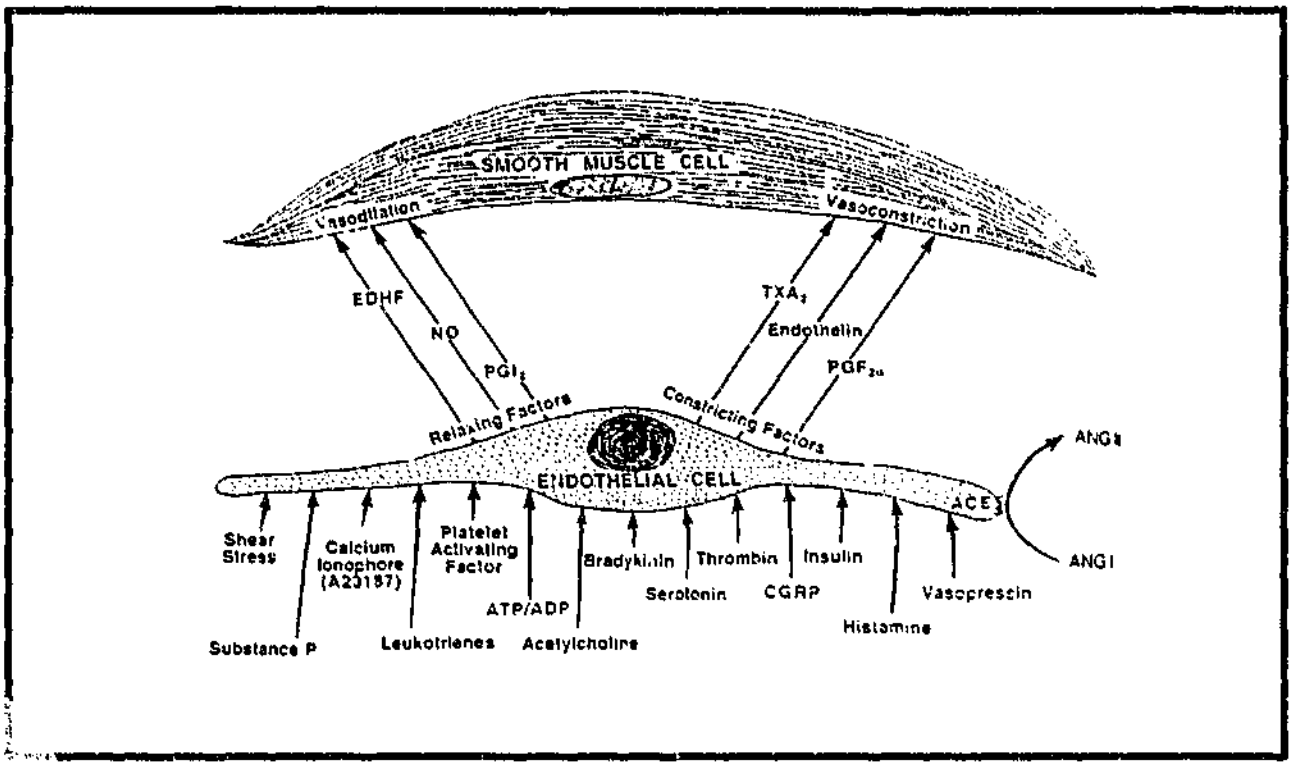

Figure 1.5: Endothelial cells produce nitric oxide and other paracrine agents in response to a variety of stimuli. NO, nitric oxide; TxA_2, thromboxane A_2; PG, prostaglandin; CGRP, calcitonin gene-related peptide; ACE, angiotesin converting enzyme; EDHF endothelial-derived hyperpolarizing factor. Taken from (179).

These stimuli increase the cytosolic calcium (Ca^{2+}) concentrations in endothelial cells via either activation of phospholipase C or by direct opening of Ca^{2+} channels (179). Constitutively expressed nitric oxide synthases are stimulated via a Ca^{2+}/calmodulin dependent pathway (179).

1.4.4.2 AA metabolism

AA is a twenty carbon fatty acid that can be cleaved from membrane phospholipids by phospholipases, to produce a family of biologically active metabolites collectively referred to as 'eicosanoids'(3). Phospholipases present in most types of mammalian cells can cleave AA from membrane phospholipids in response to different stimuli such as peptide hormones, neurotransmitters and other autocoids (3). Three major enzymatic pathways are responsible for converting AA into its biologically active metabolites (Figure 1.6). COX-dependent pathways convert AA acid into prostaglandins, prostacyclins, and thromboxanes whereas the lipoxygenase dependent pathway converts AA into leukotrienes (Figure 1.6) (212). Metabolism of AA by CYP450 enzyme leads to the formation of hydroxyeicosatetraenoic acids (HETEs) and epoxycicosatrienoic

acids (EETs) (Figure 1.6) (212). These eicosanoids have diverse biological functions and have been implicated in the pathophysiology of a number of diseases including diabetes, cancer and hypertension (212).

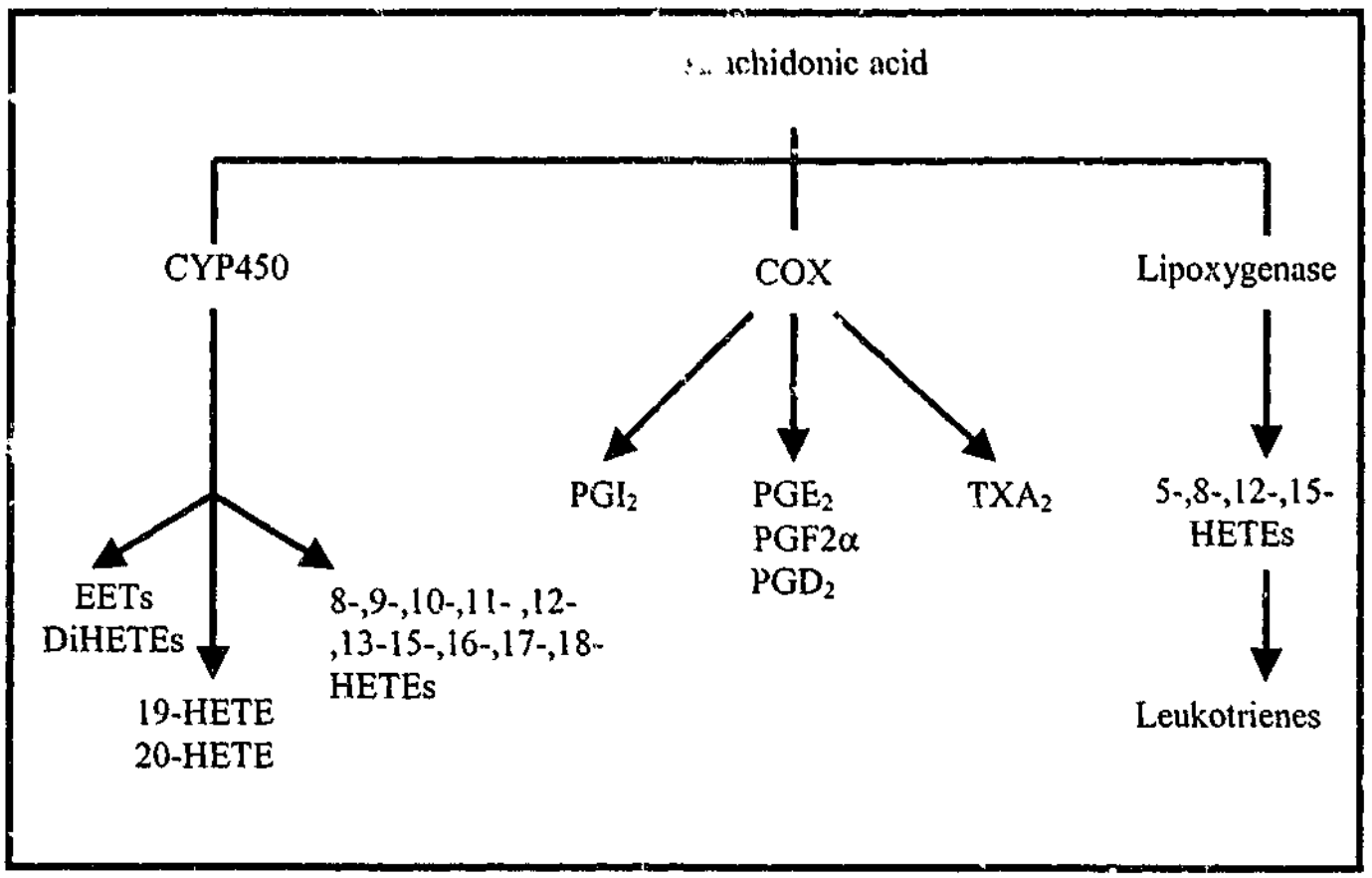

Figure 1.6 Pathways for metabolism of arachidonica acid. Arachidonic acid is metabolized via cyclooxygenase (COX), lipoxygenase, and cytochrome P450 (CYP450) enzymes to prostaglandins (PG), prostacyclin (PGI_2), thromboxane A_2 (TxA_2), or a series of hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs), and dihydroxyeicosatrienoic acids (DIHETEs). Taken from (212).

COX pathway

COX converts AA into vasodilator (PGE_2 and PGI_2) and vasoconstrictor (thromboxane A_2 and PGH_2) prostaglandins. PGE_2 is the predominant COX metab lite in the renal circulation (106). PGE_2 receptors (EP), the most abundant type of prostanoid receptor in the kidney, are distributed in the collecting ducts of the cortex and papilla, and tubules of the outer medulla and cortex (235). PGI_2 is the predominant COX metabolite produced in renal vascular smooth muscle cells and PGI_2 receptors (IP) are distributed throughout the renal cortex and medulla (106). It has been shown that both PGE_2 and PGI_2 can increase RBF and GFR (106). Also, PGE_2 has been shown to contribute to the regulation of water and sodium homeostasis (106).

There is evidence that vasodilator prostaglandins antagonize the effects of vasoactive agents on the renal circulation. For example, COX inhibition has been shown to enhance angiotensin II mediated vasoconstriction of microdissected superficial afferent arterioles (112, 260). Likewise, a number of studies have demonstrated that COX inhibition can augment renal vasoconstrictor responses to noradrenaline and vasopressin both *in vitro* and *in vivo* (9, 16, 107).

Under normal physiological conditions, thromboxane A_2 and PGH_2 are produced in small quantities by the kidney. These vasoconstrictor prostaglandins act on thromboxane/enderperoxide receptors and play a more prominent role under pathophysiological conditions (106).

We recently demonstrated that the COX inhibitor ibuprofen reduced basal MBF when administered alone (185) and also in combination with the lipoxygenase inhibitor esculetin in anaesthetized rabbits (184). In contrast, ibuprofen-treatment did not have a significant effect on basal RBF or CBF suggesting that COX products contribute to the regulation of basal levels of MBF but not CBF (185). Our observations are consistent with the findings of a number of previous studies, the results of which suggest that COX products provide a tonic vasodilator influence within the medullary microcirculation (26, 53, 101, 198).

Lipoxygenase pathway

Lipoxygenase enzymes convert AA to leukotrienes, HETEs and lipoxins (106). Leukocytes, mast cells and macropharges produce these metabolites in response to inflammation (106).

Within the kidney, 12 (S)-HETE and 15-HETE are produced by glomeruli, mesangial cells, cortical tubules, and blood vessels (4, 12, 110). These metabolites contribute to the regulation of renal haemodynamics and glomerular filtration (106). For example, Katoh and colleagues demonstrated that 12(S)-HETE reduced RBF and GFR in rats (116). We recently found that lipoxygenase inhibition increased basal RBF and MBF in anaesthetized rabbits (184). This could reflect the actions of 12-HETE, a vasoconstrictor metabolite of the lipoxygenase pathway, found within the renal vasculature (106).

There is evidence that lipoxygenase products of AA contribute to the renal responses of vasoactive hormones (106). Inhibition of the 12-lipoxygenase enzyme diminished the reduction in RBF in response to angiotensin II in the rat kidney (22). Furthermore, diminished renal arcurate artery vasoconstrictor responses to noradrenaline and potassium chloride have been demonstrated subsequent to lipoxygenase inhibition in the rat kidney (259).

CYP450 pathway

CYP450 enzymes are haeme proteins that require cytochrome c reductase, NADPH/$NADP^+$, and molecular oxygen as cofactors (54, 159). CYP450 dependent metabolism of AA occurs via three types of reactions; (i) olefin epoxidation (ii) allylic oxidation and (iii) oxidation at ω and ω-1 positions (54, 159). Olefin epoxidation gives rise to four regioisomeric EETs and these EETs can in turn be converted to their respective vicinal diols, the dihydroxyeicosatrienoic acids (DHTs) by epoxide hydrolases. Allylic oxidation gives rise to cis-trans conjugated HETEs and oxidation at ω and ω-1 positions gives rise to 20 and 19-HETEs respectively (159). COX can convert 20-HETE to a vasoconstrictor PGH_2 analog (20-OH PGH_2) (161). This product can then be converted to vasodilator (20-OH PGE_2, 20-OH PGI_2) and vasoconstrictor (20-OH thromboxane (Tx) A_2, 20-OH $PGF_{2\alpha}$) metabolites by isomerases (161). CYP450 enzymes are expressed in the renal vasculature and tubules (161, 179). Omata et al. (186) demonstrated that production of 20-HETE occurs in the proximal tubule and thick ascending loops of Henle in SHR. Moreover, medullary thick ascending limbs of the loop of Henle, proximal tubules, cortical collecting ducts and isolated glomeruli have been shown to metabolize AA via CYP450 (205).

CYP450 metabolites can be stored in tissue lipids and released upon demand, in response to different hormonal stimuli (161). It has been documented that membrane bound HETEs and EETs represent a significant reservoir in organs such as kidney, liver and platelets (161). Therefore, functions of these metabolites cannot be fully elucidated by inhibiting their production since this does not inhibit the release of HETEs and EETs from membrane phospholipids. For instance, angiotensin II can release large amounts of HETEs through activation of phospholipase, in the face of inhibition of CYP450 monooxygenase (36). Angiotensin II can also release 20-HETE from rat preglomerular

microvessels (52). On the other hand, there are many studies demonstrating that CYP450 inhibitors can acutely reduce metabolite production (5, 28, 73, 100, 101, 130, 193, 234, 268). Therefore, at least under some conditions CYP450 metabolites of AA must be produced and released on demand.

20-HETE

Members of the CYP450 4A gene family are chiefly responsible for formation of 20-HETE in the kidney (106). CYP450 4A protein is localized to renal arterioles, glomeruli and pericytes of vasa recta capillaries (106). It is postulated that 20-HETE acts predominantly in an autocrine manner (161). 20-HETE has been indicated to be a renal vasoconstrictor (32, 106) although there is evidence to the contrary (38). Caroll et al (38) demonstrated that 20-HETE produces vasodilatation in the isolated perfused rabbit kidney pre-treated with phenylephrine, and this response is thought to be COX-dependent. On the other hand, a number of studies have shown that 20-HETE mediates vasoconstriction in the rabbit afferent arteriole, dog renal arteries, rat juxtamedullary afferent arterioles and isolated perfused rabbit afferent arterioles (106). It has been demonstrated that 20-HETE mediated vasoconstriction of the renal arcuate artery is due to a sustained increase in Ca^{2+} in vascular smooth muscle cells (138). Potassium channel blockade has been shown to abrogate 20-HETE- dependent vasoconstriction of the renal afferent arteriole (267). Also, it has been shown that inhibition of tyrosine kinase diminishes 20-HETE-dependent renal arterial vasoconstriction (236). Collectively these data indicate that 20-HETE-dependent vasoconstriction occurs via activation of tyrosine kinase and subsequent closing of Ca^{2+} activated potassium channels (106).

EETs

There is evidence that members of CYP450 1A, 2B, 2C, 2D, 2E, 2J and 4A gene families can metabolize AA into EETs (106, 212). CYP2C23 is reported to be the main CYP450 epoxygenase enzyme expressed in the rat kidney (103). Ma et al (137) reported that CYP450 2J is highly expressed in the proximal tubule and collecting duct of rats. A membrane-bound receptor has been identified for 14,15 EET in mononuclear cells (258). There is evidence that 11,12-EET activates calcium-dependent potassium channels via stimulatory G proteins in bovine coronary artery smooth muscle (129). This provides further support for the proposition that there are receptors for EETs. Like

20-HETE, EETs are thought to act chiefly in an autocrine/ paracrine manner (106). Previous studies indicate that 11,12 and 14,15 EETs can cause vasodilatation of preglomerular arterioles (108). 5,6- and 8,9-EETs have been shown to cause either vasoconstriction or vasodilatation, and these effects are thought to be COX-dependent (89, 240). Zou et al (266) demonstrated that 11,12 EET dilated dog renal arteries by activating Ca^{2+}-dependent potassium channels. These effects of 11,12 EET are consistent with the notion that it is an endothelium derived hyperpolarising factor (EDHF) (106, 160). In fact, Fisslthaler et al. (85) have identified that 11,12 EET produced by CYP 450 2C is an EDHF in the coronary vasculature.

1.5 Do paracrine factors underlie the differential regulation of CBF and MBF by hormones?

There is evidence that the differential regulation of CBF and MBF by hormonal and neural factors, at least in part, arises from their differential engagement of endothelial/epithelium derived paracrine factors such as nitric oxide and AA metabolites (see Table 1.2).

As discussed earlier, bolus doses of angiotensin II reduce CBF and increase MBF while infusions of angiotensin II reduce CBF but have little effect on MBF (see Section 1.3.1 and Table 1.1). Angiotensin II mediated increases in papillary blood flow were abrogated by pretreatment with the kallikrein inhibitor aprotinin, suggesting that kinin release may contribute to this response (180). More recently, the same group found evidence for a role of nitric oxide in mediating angiotensin II induced increases in papillary blood flow in rats (252). Consistent with this, we have previously shown that nitric oxide synthase inhibition (206) or COX inhibition (185) blunted angiotensin II-induced increases in MBF in anaesthetized rabbits, suggesting that both nitric oxide and metabolites of COX pathways contribute to angiotensin II induced medullary vasodilatation. On the other hand, in anaesthetized rabbits, COX inhibition, lipoxygenase inhibition, nitric oxide synthase inhibition or even combined inhibition of all three systems had little effect on responses of CBF and MBF to renal arterial angiotensin II infusions, suggesting that neither nitric oxide nor metabolites of lipoxygenase and COX pathways fully account for the relative insensitivity of MBF to

intusions of angiotensin II (184, 185, 206). Other studies have shown that COX inhibition augmented angiotensin II-induced vasoconstriction in the rat renal medulla (53, 198, 230). Bądzńska et al (13) recently demonstrated that COX inhibition revelaed reductions in inner MBF in anaesthetized rats. These discrepancies could possibly reflect the presence of different COX products in vascular elements regulating MBF in the rabbit kidney versus the rat kidney.

Previous studies have repeatedly shown that V_1-receptor activation can selectively reduce MBF without having an effect on CBF (see Section 1.3.2). There is evidence that COX metabolites can buffer vasopressin induced reductions in RBF in rats (251). However, we recently demonstrated that inhibition of COX (185), lipooxygenase (184), nitric oxide synthase (206) or combined inhibition of all three pathways (184) had little effect on responses of regional kidney perfusion to [Phe^2,Ile^3,Orn^8]-vasopressin in rabbits. Our data suggest that nitric oxide and metabolites of COX and lipooxygenase pathways do not undelie the relative insensitivity of CBF to V_1-receptor activation. Further, these factors seem to play little role in protecting the renal medullary circulation from the ischaemic effects of V_1-receptor activation.

Renal arterial infusions of noradrenaline reduce CBF more than MBF (see Section 1.3.3). In anaesthetized rats (265) and rabbits (206), nitric oxide synthase inhibition revealed reductions in MBF after noradrenaline. Further, noradrenaline has been shown to increase nitric oxide concentrations within the renal medullary circulation (265). Noradrenaline-induced reductions in RBF and CBF were also enhanced after nitric oxide synthase inhibition (206). Collectively, these data suggest that nitric oxide blunts renal vasoconstrictor responses to noradrenaline, particularly in the medullary circulation. We recently found that COX inhibition has little effect on responses of intrarenal blood flow to noradrenaline in rabbits (185). In contrast, Parekh and Zou demonstrated that COX inhibition enhanced noradrenaline-induced reductions in MBF in rats, suggesting that metabolites of COX pathways buffer noradrenaline-induced vasoconstriction in vascular elements regulating MBF (198). This apparent discrepancy could be due to differences in species and/or route of administration (intravenous *vs* renal arterial in our study). In anaesthetized rabbits, lipoxygenase inhibition enhanced responses of RBF and CBF and tended to enhance responses of MBF to noradrenaline (184). These data suggest that lipooxygenase products antagonize renal vasoconstrictor

responses to this hormone. Alternatively, this could reflect the actions of metabolites of other AA pathways (COX and CYP450), as selective inhibition of the lipooxygenase pathway would increase substrate (AA) availability for CYP450 and COX pathways.

Taken together, previous studies by ourselves and others indicate that regionally selective activation of nitric oxide synthase, lipooxygenase and COX cannot completely account for the diversity of effects of angiotensin II, [Phe^2,Ile^3,Orn^8]-vasopressin and noradrenaline on CBF and MBF. For instance, during nitric oxide synthase inhibition (206), COX inhibition (185), lipoxygenase inhibition (184) or even after combined inhibition of all three systems (184), renal arterial administration of angiotensin II and noradrenaline still reduced CBF more than MBF, while the vasopressin V_1-agonist [Phe^2,Ile^3,Orn^8]-vasopressin still reduced MBF more than CBF. Therefore, these findings suggest that both nitric oxide and prostanoids, contribute to, but do not completely account for, the differential regulation of CBF and MBF by these hormones.

CYP450–dependent AA metabolism is another enzyme cascade that could play a role in the differential regulation of blood flow to the renal cortex and medulla. Kohagura et al demonstrated that miconazole, an inhibitor of CYP450 dependent epoxygenase metabolism, enhanced angiotensin II-mediated vasoconstriction in the isolated microperfused afferent arteriole of the rabbit, suggesting that EETs blunt the vasoconstrictor effects of angiotensin II (119). Inhibition of AA metabolism, on the other hand, has been shown to attenuate vasopressin-induced vasoconstriction in the isolated perfused rat kidney (250). These latter findings suggest that metabolites of AA contribute to vasopressin-induced vasoconstriction in the kidney. These studies certainly provide evidence that CYP450 dependent metabolites of AA participate in renal vascular responses to angiotensin II and vasopressin. However, we are not aware of any studies that have investigated the roles of CYP450 metabolites in modulating responses of regional kidney blood flow to these hormones *in vivo*. This is the focus of the studies described in Chapters 3 and 4 of this thesis. In the experiments described in Chapters 3, we investigated the roles of CYP450 in modulating responses of regional kidney blood flow to vasoconstrictor hormones. We then examined the respective roles of 20-HETE and EETs in contributing to these responses, in the experiments described in Chapter 4.

Table 1.2 Diverse actions, of various vasoactive regulatory factors, on blood flow in the renal cortex and medulla, and proposed mechanisms underlying their differential effects on blood flow in these vascular territories.

Factor	Exogenous		†Physiological		Proposed Mechanisms*	References
	CBF	MBF	CBF	MBF		
A: Vasoconstrictors						
Angiotensin II	↓↓	→ or↑ (rats and rabbits) ↓ (dogs)	↓	→	Nitric oxide and prostaglandins produce AT_1-mediated vasodilatation and blunt vasoconstriction in the medullary circulation, which is opposed by AT_2-receptor activation.	(14, 34, 49, 53, 56, 57, 62, 66, 72, 76, 93, 105, 154, 166, 180, 185, 197, 198, 206, 221, 230, 252, 272)
Noradrenaline	↓↓	↓			α_2-Adrenoceptor mediated nitric oxide release opposes, α_1-mediated vasoconstriction in the medullary circulation.	(49, 76, 185, 197, 198, 206)
Renal sympathetic nerve activity	NA	NA	↓↓	→↓	Nitric oxide blunts MBF responses to renal nerve stimulation. α_2-Adrenoceptor activation blunts responses to renal nerve stimulation in both the cortex and medulla, so this mechanism probably does not explain the insensitivity of MBF to renal nerve stimulation.	(17, 69, 70, 93-95, 120, 123, 126, 127, 220, 230)
20-HETE	ND	ND	↓	↓↓	Unknown, although some of the renal vascular actions of nitric oxide are mediated through inhibition of the synthesis of 20-HETE and EETs	(101, 270)

Table 1.2 (continued)

Factor	Exogenous		†Physiological		Proposed Mechanisms*	References
	CBF	MBF	CBF	MBF		
B: Vasodilators						
Nitric oxide	↑	↑	↑	↑↑	Expression of all 3 isoforms of nitric oxide synthase is particularly prominent within the medulla. Nitric oxide synthase activity and interstitial levels of nitric oxide are greater in the medulla than the cortex.	(49, 62, 69, 101, 114, 151, 153, 157, 197, 206, 272)
Prostaglandins	ND	ND	↑	↑↑	Expression of components of the (vasodilator) prostaglandin system is particularly prominent within the medulla	(1, 2, 13, 53, 101, 154, 185, 198, 216, 221)

↓↓ = Strongly reduced; ↓ = reduced;→↓ = reduced only by intense stimuli, → = virtually insensitive; ↑↑ strongly increased, ↑ increased.
CBF = cortical blood flow; MBF = medullary blood flow; 20-HETE = 20-hydroxyeicosatetraenoic acid; AA = arachidonic acid; ND = not determined; NA = not applicable.

*Differences in vascular geometry between the cortical and medullary circulations predispose MBF to be less sensitive than CBF to any given vasoconstrictor factor (see The role of renal vascular geometry in the control of MBF, Section 1.4.2). †Conclusions regarding the physiological roles of these factors in the control of CBF and MBF come from studies using receptor antagonists, and inhibitors of the synthesis or breakdown of these factors. Modified from (74).

1.6 Do paracrine factors underlie the differential regulation of CBF and MBF by nerves?

There is evidence to suggest paracrine factors derived from the vascular endothelium and tubular epithelium can buffer vasoconstriction induced by stimulation of renal nerves (see Table 1.2). For example, Malmström et al (2001) (148) demonstrated that nitric oxide synthase inhibition augmented renal nerve stimulation-induced global renal vasoconstrictor responses in the pig. Consistent with this, Eppel et al (2003) (69) in our laboratory, recently showed that inhibition of nitric oxide synthase can enhance renal nerve stimulation-induced reductions in RBF, CBF and MBF in anaesthetized rabbits. This latter study also demonstrated that even after nitric oxide synthase inhibition, renal nerve stimulation reduced CBF more than MBF, suggesting that nitric oxide *per se* does not completely account for the relative insensitivity of MBF to neural activation. Evidence has also been presented to suggest a role for vasodilator prostaglandins in buffering renal nerve stimulation-induced vasoconstrictor activity in the renal circulation. For example, inhibition of COX has been shown to enhance, while administration of prostacyclin or PGE_2 has been shown to blunt, renal nerve stimulation-induced renal vasoconstrictor response in rats (109).

There is also evidence that interactions between nitric oxide and prostaglandins in the kidney play an important role in modulating noradrenaline-induced renal vasoconstrictor responses. For example, nitric oxide synthase inhibition has been shown to augment noradrenaline-induced renal vasoconstriction in the isolated perfused rat kidney preparation (262). Inhibition of COX (262) or antagonism of the thromboxane A_2/prostaglandin H_2-receptor (264) antagonism attenuated this vasoconstriction, suggesting that nitric oxide buffers noradrenaline-induced renal vasoconstriction by inhibiting the production and/or actions of vasoconstrictor prostaglandins. On the other hand, nitric oxide synthase inhibition has been demonstrated to reveal renal vasodilator actions of COX products in the rat kidney (19, 20). These latter observations suggest that under normal physiological conditions, nitric oxide might inhibit the production of vasodilator prostaglandins. So, the observation that renal nerve stimulation reduces CBF more than MBF under conditions of nitric oxide synthase inhibition (69) could simply reflect compensatory up-regulation of vasodilator prostaglandins in the renal medulla after nitric oxide synthase inhibition. Therefore, in the experiments described in Chapter

5, and also in a supplementary experiment described in Chapter 7, we examined the roles of COX products, and also the significance of the interactions between nitric oxide and prostaglandins in underlying the relative insensitivity of MBF to sympathetic nerve activation.

1.7 Does angiotensin II protect the renal medulla from the ischaemic effects of renal nerve stimulation?

Angiotensin II has a paradoxical vasodilator effect in the renal medullary microcirculation (see Section 1.3.1 and Table 1.1) This vasodilator effect was blunted after nitric oxide synthase inhibition (206) or after AT_1-receptor antagonism (66) suggesting that angiotensin II, via activation of AT_1-receptors, induces nitric oxide release which in turn causes vasodilatation in vascular elements regulating MBF. In support of this hypothesis, angiotensin II has been shown to increase nitric oxide concentrations in the renal medulla (272). More recently, Dickhout et al. showed that angiotensin II increased nitric oxide concentrations in the isolated medullary thick ascending limbs of Henle's loop (62). Importantly, they showed that angiotensin II-induced increases in nitric oxide concentrations in pericytes of vasa recta were only observed when these vessels were in close proximity to medullary thick ascending limbs (62). These authors concluded that nitric oxide, produced in response to angiotensin II from the medullary thick ascending limbs, diffuses into vascular smooth muscle and antagonizes angiotensin II-mediated vasoconstriction (62). A recent study in our laboratory demonstrated that renal arterial infusion of angiotensin II blunted renal nerve stimulation-induced reductions in MBF, but not CBF, in rabbits (Figure 1.7) (93). Taken together, these studies suggest that angiotensin II, possibly by inducing the release of nitric oxide, can blunt renal nerve stimulation-induced vasoconstriction in the renal medullary circulation. However, we do not yet understand the physiological significance of the roles of endogenous angiotensin II in modulating neurally mediated vasoconstriction in the vascular elements regulating MBF. This is the main focus of experiments described in Chapter 6.

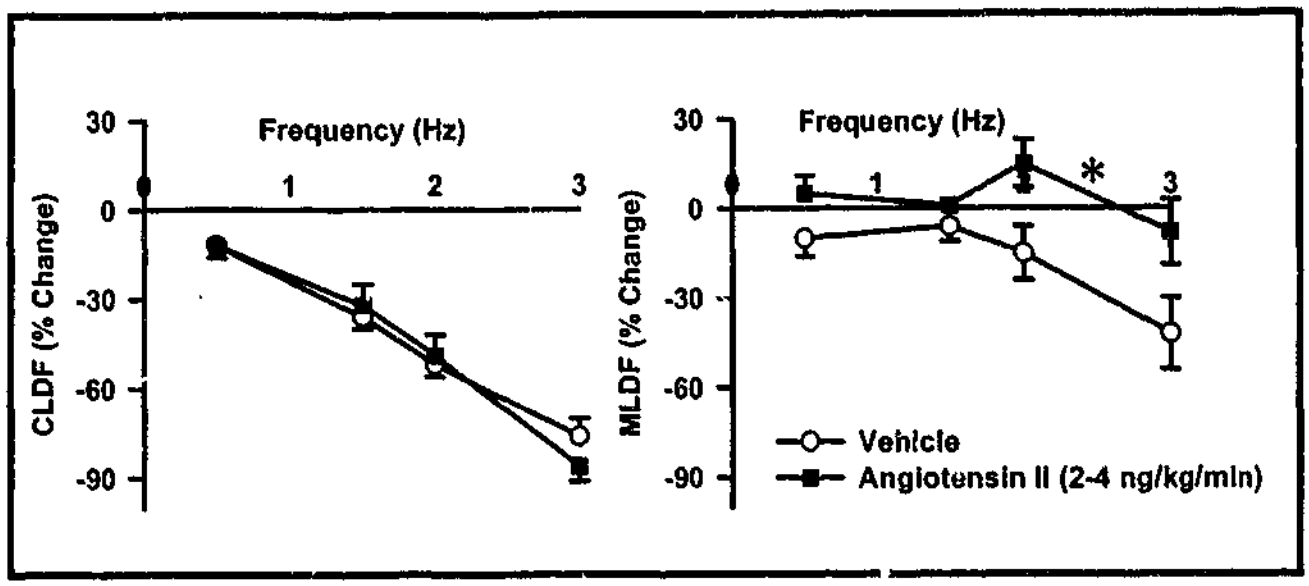

Figure 1.7 The responses of regional renal haemodynamics to electrical stimulation of the renal nerves during infusion of saline vehicle and angiotensin II. *Statistically significant differences in responses to stimulation across all frequencies between vehicle and angiotensin II infusion ($P_{agent} \leq 0.05$). CLDF (cortical laser Doppler flux, an index of blood flow); MLDF (Medullary laser Doppler flux) (Modified from (93)).

1.8 Summary and aims

The role of the renal medullary circulation in long-term regulation of arterial pressure is now well established (47, 48, 50, 150). There is a large body of evidence to suggest that hormonal (45, 46, 71, 76, 180, 184, 185, 197, 198, 206) and neural (69, 70, 93, 94, 126, 220) factors can differentially regulate regional kidney blood flow. This is likely to represent an important regulatory mechanism in long-term blood pressure control yet we are only beginning to understand the mechanisms underlying it. Therefore, the global aim of the experiments described in this doctoral thesis was to examine the roles of paracrine factors in underlying the differential regulation of regional kidney perfusion by hormonal and neural stimuli.

Studies described in Chapters 3 and 4 examined the roles of CYP450 metabolites of AA, in modulating regional kidney blood flow responses to vasoconstrictor hormones. In the experiments described in Chapter 3, the non-selective CYP450 inhibitor, 1-aminobenzotriazole (ABT) was used. In the experiments described in Chapter 4, the roles of EETs were examined using the selective epoxygenase inhibitor, (N-

methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH), and the roles of 20-HETE were examined using the selective ω-hydroxylase inhibitor, N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS).

Studies described in Chapters 5 and 6 investigated the roles of prostaglandins (Chapter 5) and angiotensin II (Chapter 6) and the significance of interactions of these systems with nitric oxide, in modulating responses of CBF and MBF to stimulation of the renal nerves.

Chapter Two

General Methods

2.1 Introduction

The aim of this chapter is to describe the general methods used in the experiments described in this thesis. The specific experimental protocols, and techniques used only once, are described in the relevant experimental chapters (Chapters 3-6).

All experiments were performed using rabbits (Section 2.2), which were surgically prepared (Section 2.3). Renal vascular tone was manipulated either by renal arterial infusion of drugs and hormones (Section 2.3.1) or electrical stimulation of the renal nerves (Section 2.3.2). Total renal blood flow was measured by transit-time ultrasound flowmetry (Section 2.4) and regional kidney perfusion was estimated using laser Doppler flowmetry (Section 2.5). All haemodynamic variables were recorded digitally (Section 2.6). In some studies the ability of renal tissue to metabolise arachidonic acid (AA) via cytochrome P450 (CYP450) pathways was examined (Sections 2.7 and 2.8). To determine the roles of various regulatory factors (eg nitric oxide, products of AA metabolism, angiotensin II) in the control of regional kidney blood flow, specific enzyme inhibitors and pharmacological antagonists were used (Section 2.9). Biological hypotheses were tested using appropriate statistical procedures (Section 2.10).

2.2 Animals

All experiments described in this thesis were performed on male New Zealand White rabbits. They were provided with water *ad libitum* but were fed only once each day from the age of 10 weeks. Their food consisted of a mixture containing pellets, lucerne chaff and oat chaff in the ratio 4:1:1.2 by weight (100 g per day). All experiments were approved in advance by the Monash University Department of Physiology/ Central Animal Services Animal Ethics Committee. These experiments were performed in

accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.3 Surgical procedures

Catheters were placed in the central ear artery and marginal ear vein using a transcutaneous needle puncture technique, under local analgesia (1% Lignocaine; Xylocaine; Astra Pharmaceuticals, North Ryde, NSW, Australia). The ear artery catheters facilitated measurement of arterial pressure, while the ear vain catheters were for infusion of physiological solutions and drugs. General anaesthesia was induced by pentobarbitone sodium (90-150 mg plus 30-50 mg/h; Nembutal: Rhone Merieux, Pinkenba, Queensland, Australia) and was immediately followed by endotracheal intubation and artificial respiration (Model 55-3438 Respirator, Harvard Instruments, MA, USA). The level of anaesthesia was monitored throughout the experiment by testing the toe pinch and cornea reflexes and was adjusted accordingly. A bladder catheter (8-F gauge latex, infant urological Foley catheter; Rush, High Wycombe, Buckinghamshire, UK) was inserted via the urethra prior to commencement of surgery. Surgery was performed on a heated table (Baker Medical Research Institute, Model 165) and oesophageal temperature was maintained between 36-38 °C throughout the experiment with the use of the heated table and a servo controlled infrared lamp (Digi-Sense Temperature Controller, Cole Palmer Instrument Company, Chicago, IL, USA) in experiments where vascular tone was manipulated by renal arterial infusion of vasoconstrictor agents (Chapters 3 and 4). In experiments where renal nerves were stimulated (Chapters 5 and 6) only the heated table was used to maintain the oesophageal temperature between 36-38 °C, as the infrared lamp could damage the renal nerves. Hartmann's solution (compound sodium lactate; Baxter Healthcare Pty Ltd., Toongabbie, NSW, Australia) was infused at a rate of 0.18 ml kg^{-1} min^{-1} during surgery. At the completion of all surgical procedures the rabbit's wounds were covered with gauze soaked in 154 mM NaCl to minimize fluid loss, and the i.v. infusion of Hartmann's solution was repla with a solution containing four parts Hartmann's solution and 1 part 10% vol/vol polygeline (Haemaccel; Hoechst, Melbourne, Victoria, Australia). A 30-60 min equilibration period was allowed before the experimental protocols commenced.

2.3.1 *Insertion of a catheter in the side branch of the left renal artery (Chapters 3 and 4)*

In the experiments described in Chapters 3 and 4 renal vascular tone was manipulated by renal arterial infusion of vasoconstrictor agents. To achieve this, the left kidney was exposed via a left flank retroperitoneal incision and was freed from the peritoneal lining and surrounding fat. The left kidney was then denervated by stripping the nerves from the renal artery. This eliminated any possible confounding effects on renal haemodynamics of changes in renal sympathetic nerve activity. The rabbit was then placed in an upright crouching position and a catheter (single lumen PE10: OD 0.61 × ID 0.28 mm; Datamasters Pty Ltd, Ocean Grove, Victoria, Australia) was placed in a side branch (suprarenolumbar artery) of the renal artery for administration of vasoactive agents. Catheter patency was maintained by a continuous infusion of 154 mM NaCl (20 $\mu l\ kg^{-1}min^{-1}$). The kidney was then placed in a stable cup to facilitate the use of laser Doppler flowmetry (see Section 2.5). The kidney was secured in the cup using gauze (soaked in 154 mM NaCl) packing.

2.3.2 *Preparation for renal nerve stimulation (Chapters 5 and 6)*

In the experiments described in Chapters 5 and 6 renal vascular tone was manipulated by electrical stimulation of post-ganglionic renal sympathetic nerves. To achieve this, the left kidney was exposed via a left flank incision and was placed in a stable cup. The kidney was secured in the cup using gauze (soaked in paraffin oil) packing.The renal nerves running parallel to the left renal artery were then carefully isolated using a microscope and placed across a pair of stimulating electrodes. Therefore, most (but not all) sympathetic nerves innervating the kidney were likely to be in contact with the stimulating electrode. The nerves were then sectioned proximally. A mixture of paraffin oil and petroleum jelly were applied to the nerves throughout the experiment to keep them moist.

Renal nerve stimulation was produced using purpose-written software in the LabVIEW graphical programming language (National Instruments, Austin, TX), which was kindly provided by Associate Professor Simon Malpas form the Department of Physiology, University of Auckland, New Zealand. A supramaximal voltage (3-10 V) was applied in each experiment. This was pre-determined by stimulating the renal nerves with various voltages at 5 Hz for 60 s. The voltage that produced the maximum reduction in RBF

was used to stimulate the renal nerves in all subsequent stimulation sequences. A pulse width of 2 ms was used and either four (Chapter 5: 0.75, 1.5, 3 & 6 Hz) or five (Chapter 6: 0.5, 1, 2, 4 & 8 Hz) different frequencies were applied in random order. Each frequency was applied for 3 min with an 8 min recovery period, except after the highest frequency, where a 10 min recovery period was allowed.

2.4 Measurement of RBF using transit-time ultrasound flowmetry

In all *in vivo* experiments described in this thesis (Chapters 3, 4, 5 and 6), a transit-time ultrasound flow probe (type 2SB, Transonic Systems Inc., Ithaca, NY, USA) was placed around the left renal artery to measure RBF (Figure 2.1). This probe consists of two ultrasound transducers (positioned on each side of the flowprobe body) and an acoustic reflector (245). When the flow probe is placed around a vessel (in our experiments the renal artery), the reflector is positioned midway between the two transducers, on the opposite side of the vessel to where the two transducers are located (245). The downstream transducer emits a wave of ultrasound on electrical excitation. This ultrasound wave travels through the vessel and gets reflected towards the upstream transducer when it reaches the probe reflector. The upstream transducer converts the ultrasound wave into electrical signals and the flowmeter uses these signals to derive the transit-time of the ultrasound wave to travel from one transducer to the other (245). This same transit-receive sequence is then reversed. The ultrasound wave has to travel in the direction opposite to flow in the upstream transit-receive sequence as opposed to same direction of flow in the downstream transit-time receive sequence (245). As a result, the upstream transit time is greater than the downstream transit time. The flowmeter, with the aid of wide-beam ultrasound, measures the volume-flow by subtracting the downstream transit time from the upstream transit time (245).

Transit-time ultrasound flowmetry has many advantages over other techniques for measuring global renal blood flow (eg. electromagnetic flowmetry, continuous wave or pulse-Doppler flowmetry) (64, 75). Firstly, flow measurements are independent of vessel diameter (64). Secondly, unlike electromagnetic flowmetry the zero measurement

remains stable (75). Thirdly, these probes can be used in most *in vivo* experimental conditions without calibration other than that provided by the manufacturer (245). Previous studies have shown that flow measurements made with the use of this technique are largely in agreement with the flow measurements made with the use of electromagnetic flow-probe (98), thermodilution (219) and clearance of [^{14}C]-labelled paraaminohippuric acid (254).

2.5 Measurement of regional kidney perfusion using laser Doppler flowmetry

In all anaesthetized rabbit experiments (Chapters 3, 4, 5 and 6) cortical perfusion (CBF) and medullary perfusion (MBF) were estimated by laser Doppler flowmetry (Moor Instruments Ltd, Millwey, Devon, England). Medullary laser Doppler flux (MLDF) was measured after implanting a needle probe (26 gauge, DP4s) 9 mm below the cortical surface using a micromanipulator (Narishige, Tokyo, Japan) so that its tip would be placed in the "white" inner medullary region of the kidney. A standard plastic straight probe (DP2b) was used to monitor cortical laser Doppler flux (CLDF) (Figure 2.1). This was placed on the dorsal surface of the kidney and held in place with gauze soaked in 154 mM NaCl.

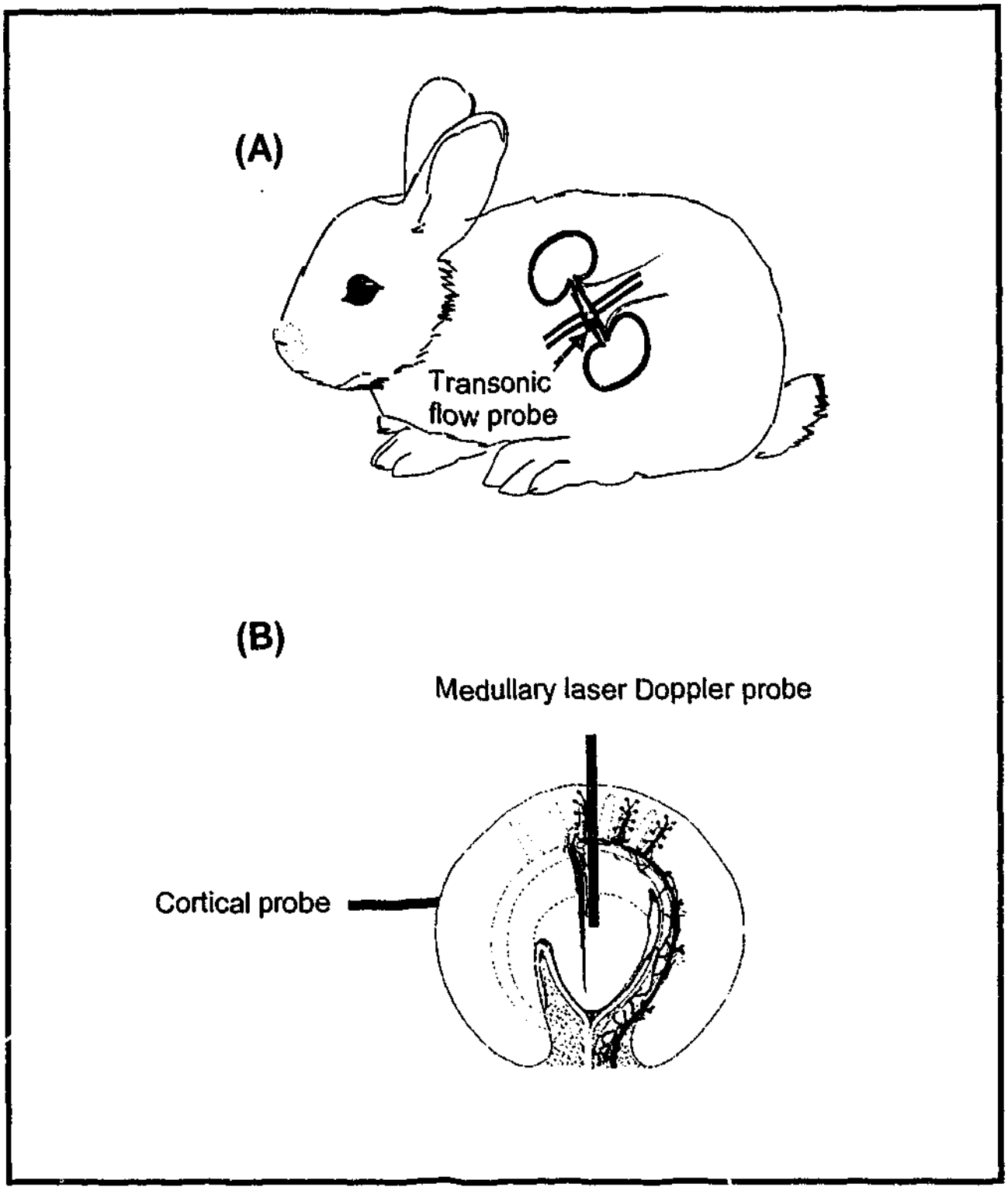

Figure 2.1 **(A)** Anaesthetised rabbit preparation. A transit time ultrasound flow probe was placed around the left renal artery to measure total renal blood flow. **(B)** A needle laser-Doppler flow probe was advanced about 9 mm into the kidney to measure medullary laser Doppler flux and a standard plastic straight probe was placed on the dorsal surface of the kidney to measure cortical laser Doppler flux.

2.5.1 *Laser Doppler flowmetry principle*

A small region of the tissue under study is illuminated by a beam of low intensity monochromatic light emitted by the laser Doppler flowmeter (181). Moving red blood cells within the tissue scatter the laser beam and cause a frequency shift in light proportional to the velocity of the cells. A portion of the light (both that has and has not undergone frequency shifts) is reflected back onto a photo detector inside the flowmeter. The flowmeter then creates a current by mixing the reflected light with different frequencies. This process is termed heterodyne mixing and this is used to determine the Doppler shift power spectrum (209). This is used to derive the velocity and concentration of red blood cells in the tissue under study. The laser Doppler flowmeter measures the flux of red blood cells, which is the product of the velocity and the concentration signal, and which gives an indication of blood flow (209). We measured flux and concentration signals in both the cortex and the medulla in all experiments described in this thesis. However, as described below, the concentration signal is not very meaningful in highly perfused tissues such as the kidney, since this signal is maximal under normal conditions.

Laser Doppler flowmetry has been employed in many previous studies to measure regional kidney perfusion. It is a relatively non-invasive technique, which provides continuous measurements. Furthermore, this technique can be used to estimate perfusion on the surface and/or at different depths of the kidney (as described in our experiments) using a plastic straight probe or a needle probe, respectively (195). Our research group has previously shown that responses of CLDF to the vasoconstrictor endothelin-1 are indistinguishable using these two different probe types (126) indicating that neither the probe type *per se*, nor the haemorrhage associated with insertion of the needle probe, confound observations using this experimental set-up. Furthermore, our research group has previously demonstrated linear relationships between laser Doppler flux and erythrocyte velocity *in vitro* (94) and between RBF and CLDF and MLDF in an isolated and maximally dilated blood perfused kidney (68), using this equipment. These observations are consistent with the findings of other studies demonstrating a linear relationship between laser Doppler flux and erythrocyte velocity (83, 217, 231). However, laser Doppler flowmetry has been shown to be relatively insensitive to changes in the number (volume) of red blood cells in highly perfused tissues such as the kidney (68, 217). Therefore, this technique could be insensitive to changes in blood

flow due to capillary recruitment (68). Other limitations of this technique include the fact that it does not provide measures of absolute flow, and the fact that optical fibres are sensitive to tissue and vessel wall movements (181). In the studies described in this thesis we took great care to avoid motion artefacts by carefully securing the kidney in a stable cup (see Sections 2.3.1 and 2.3.2). Laser Doppler flux measurements are shown to overestimate the actual perfusion of the tissue under study because it incorporates a biological zero which arises chiefly from the Brownian motion of the macro molecules within the interstitium (118). To obtain the correct MLDF and CLDF measurements the background levels of CLDF (7 ± 1 units) and MLDF (14 ± 2 units), recorded after the rabbit was humanely killed by overdose with pentobarbitone (300 mg) and artificial ventilation was stopped, were subtracted from experimental values before data analysis. We chose this option of subtracting background levels of flux after the rabbit was killed and artificial ventilation was ceased in order to eliminate all motion artefacts that might have been present during the course of the experiment.

2.6 Measurement of systemic and renal haemodynamic variables

The ear artery catheters were connected to pressure-transducers to measure arterial pressure (Cobe, Arvarda, CO, USA). The transit-time ultrasound flow probe was connected to a model T208 flowmeter (Transonic Systems) and the laser Doppler flow probes were connected to a laser Doppler flowmeter (DRT4, Moor Instruments). A computer equipped with an analog-to-digital acquisition card (Lab PC$^+$, National Instruments, Austin, Tx) and purpose written software (Universal Acquisition, kindly provided by Associate Professor Simon Malpas from the University of Auckland, New Zealand) provided 2 s means of MAP (mmHg), heart rate (HR, determined from the arterial pressure pulse; beats/min), RBF (ml/min), CLDF (perfusion units), and MLDF (perfusion units).

2.7 Cytochrome P450 (CYP450) dependent ω-hydroxylase and epoxygenase activity

At the end of each *in vivo* experiment described in Chapters 3 and 4, the left kidney of the rabbit was removed, snap frozen in liquid nitrogen and stored at –80 °C for later measurement of the renal metabolism of AA *in vitro*. The left kidney was also removed, under pentobarbitone anaesthesia, from 6 untreated rabbits. We first examined the levels of CYP450 metabolism in the cortex and the medulla of these control rabbit kidneys, by preparing microsomes as described in the following section.

Microsomes were also prepared from the renal cortical tissues of rabbits treated with a mechanism based (suicide) CYP450 inhibitor (eg. 1-aminobenzotriazole). Crude homogenates were prepared from renal cortical tissues of rabbits treated with CYP450 inhibitors of which the mode of inhibition was not definitively known (eg. N-methylsulfonyl-12-12,dibromododec-11-enamide (DDMS), N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH)). This procedure minimized dilution of the inhibitors retained in the tissue.

2.7.1 Microsomal preparation

Renal tissues (the renal cortex, inner and outer medulla of control kidneys and the renal cortex of treated kidneys) were homogenized in 3 volumes of a 10 mM potassium phosphate buffer containing 250 mM sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA). The microsomes were prepared by sequential centrifugation of the homogenate (at 3000 × g for 5 min, 11,000 × g for 15 min and 100,000 × g for 60 min). Microsomal pellets were resuspended in 100 mM potassium phosphate buffer containing 0.5 mM EDTA, 1 mM dithiothreitol, 30% glycerol and 0.1 mM phenylmethylsulfonyl fluoride.

Crude renal homogenates were prepared by homogenizing the kidneys in only 2 volumes of the 10 mM potassium phosphate buffer to minimize dilution of agents that had been infused into the renal artery. The homogenate was then centrifuged at low speed (at 3000 × g for 5 min). An aliquot of the homogenate was taken to determine CYP450 activity as described below.

2.7.2 *AA metabolism*

The renal metabolism of AA was determined by incubating 0.5 mg of microsomal protein or 2 mg of homogenate protein, with ^{14}C-AA (Amersham, Arlington Heights, IL) in 1 ml of a 100 mM potassium phosphate buffer (pH 7.25) containing 10 mM sodium isocitrate, 0.16 U/ml isocitrate dehydrogenase, and 1 mM β-nicotinamide adenine nucleotide phosphate, reduced form (NADPH). The microsome reactions were performed using a saturating concentration of AA (0.1 μCi, 42 μM) whereas the homogenates were studied using a low concentration of substrate (AA 0.1 μCi, 2 μM) to maximize the conversion rate of labeled AA and minimize competition of CYP450 inhibitors with excess substrate. Incubations were carried out in a shaking water bath at 37 °C for 30 min (microsomes) or 60 min (homogenates) in an atmosphere of 100% oxygen. The reaction was terminated with 1 M formic acid and AA metabolites were extracted with 3 ml of ethyl acetate.

2.8 Isolation of AA metabolites using high performance liquid chromatography

The metabolites were separated using a 2 mm × 25 cm C18 reverse phase high performance liquid chromatography column equipped with an online radioactive flow detector (FLO-one/Beta, series A-120; Radiomatic Instruments, Tampa, Florida, USA). A linear solvent gradient ranging from acetonitrile:water:acetic acid (50:50:0.1, vol/vol/vol) to acetonitrile:acetic acid (100:0.1, vol/vol) over 40 min was used to separate the AA metabolites. Rates of product formation are expressed as pmol formed per min per mg protein (pmol $min^{-1}mg^{-1}$). The rate of formation of 20-hydroxyeicosatetraenoic acids (20-HETE) provided a measure of ω-hydroxylase activity, while the rate of formation of epoxyeicosatrienoic acids (EETs) provided a measure of epoxygenase activity. We also measured the rate of formation of dihydroxyeicosatrienoic acids (DiHETEs) to get some indication of epoxide hydrolase activity (which is responsible for converting EETs into DiHETEs) in renal tissue.

2.9 Pharmacological tools

In the studies described in this doctoral thesis we used a range of agents to inhibit nitric oxide synthase (N^G-nitro-L-arginine), cylooxygenase (COX) (ibuprofen), CYP450 (1-aminobenzotriazole), CYP450-dependent epoxygenase activity (N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH), CYP450- dependent ω-hyroxylase activity (N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS)), and to antagonize AT_1- receptors (candesartan). Outlined below are the key features of the pharmacology of these inhibitors.

2.9.1 CYP450 inhibitors

Inhibition of CYP450 enzymes can take place at three stages: (i) the binding of substrates, (ii) the binding of molecular oxygen after the first electron transfer, and (iii) the substrate oxidizing step (188). CYP450 inhibitors can be categorised into three main groups: (i) reversible inhibitors, (ii) irreversible inhibitors, and (iii) inhibitors that form quasi-irreversible complexes with the heme iron atom (188). Reversible inhibitors generally hinder the catalytic activity of the enzyme prior to the oxidization of the substrate whereas most irreversible, quasi-irreversible and mechanism based (suicide) inhibitors render the enzyme inactive during or after the oxidative step (188). 1-aminobenzotriazole (ABT) is a suicide substrate inhibitor (188) frequently used to study the functions of CYP450, and has been shown to be an effective agent for inactivation of P450 enzymes *in vivo* (234). ABT gets converted to benzene, which then interferes with the catalytic heme moiety of the enzyme (188). This agent is a non-selective inhibitor of CYP450 and has been shown to be equally effective at blocking ω-hydroxylase and epoxygenase activity at a dose of 50 mg/kg i.p in the rat (140).

In order to examine the function(s) of a certain metabolite in a particular CYP450 pathway, it is necessary to use more selective pharmacological approaches, to inhibit a selective group of CYP450 products (eg ω-hyroxylase products) without affecting the other pathways simultaneously (161). This is challenging as these enzymes exist in many isoforms and have broad substrate specificity (161). Dr Falck and co-workers in Texas, USA, have recently developed compounds that selectively inhibit CYP450-dependent ω-hydroxylase activity and epoxygenase activity (212). Wang et al have

shown that terminal acetylenic compounds like 6-(2-propargyloxyphenyl) hexanoic acid (PPOH) and MS-PPOH selectively inhibited CYP450 epoxygenase activity with IC_{50} values of 9 and 13 μM respectively, in rat renal microsomes (253). These compounds were shown to have no effect on CYP 450-dependent ω-hydroxylase activity at concentrations up to 50 μM (253). The benzene ring present in the PPOH derivatives is suggested to provide the selectivity for inhibition of AA epoxidation (253). Wang et al demonstrated that the inhibitory activity of PPOH increased with time and was NADPH dependent (253). This suggests that PPOH fits into the category of mechanism based irreversible inhibitors (171, 189).

DDMS, 12,12-dibromododec-11-enamide (DBDD) and N, methylsulfonyl-15,15-dibromopentadec-14-enamide (DPMS) are acyclic dibromide derivatives which selectively inhibit CYP 450-dependent ω-hydroxylase activity (253). The inhibitory activity of these compounds range from highest to lowest in the following order; DBDD = DDMS > DPMS (253). DDMS inhibited CYP450 ω-hydroxylase activity with an IC_{50} of 2 μM whereas its potency as an epoxygenase inhibitor was very low, with an IC_{50} of 60 μM (253). DDMS is suggested to be an effective inhibitor of CYP450-dependent ω-hydroxylase activity (253). In contrast to compounds like PPOH, the inhibitory activity of these compounds has been shown not to be time- or NADPH-dependent, suggesting that the inhibitor activity of these agents could be reversible (253). In this regard, Wang et al have shown that the inhibitory activity of DBDD and DDMS in renal microsomes can be washed out (253).

These acyclic dibromide derivatives (selective ω-hydroxylase inhibitors) as well as terminal acetylenic compounds (selective epoxygenase inhibitors) appear to be effective in blocking CYP450 *in vitro*. These compounds are lipophilic and hence can bind to plasma proteins, so have limited diffusion capacity in tissues. Therefore, these compounds could pose a problem when used under *in vivo* experimental conditions (212). Nevertheless, a number of studies have shown that these drugs effectively inhibited CYP450 metabolism *in vivo* (212).

2.9.2 COX inhibition (Ibuprofen)

In the experiments described in Chapter 5 we used the COX inhibitor ibuprofen, which has been shown to inhibit COX activity with an IC_{50} of 330 μM in microsomes prepared from the cat lung (158). Mayeux et al also demonstrated that ibuprofen had little effect on prostacyclin synthase and thromboxane A_2 synthase activity at concentrations up to 1 mM (158). The plasma half-life of this drug is ≈2 hrs in humans (165). Ibuprofen at a dose of 12.5 mg/kg has been shown to effectively reduce prostaglandin excretion in dogs (201). We recently demonstrated that ibuprofen (12.5 mg/kg + 12.5 mg kg^{-1} hr^{-1}) reduced baseline levels of medullary laser Doppler flux (MLDF) in anaesthetized rabbits in which nitric oxide synthase was inhibited (184). This observation is consistent with previous studies demonstrating that COX inhibition reduces basal MLDF (101, 198). Therefore, it seems likely that this dose of ibuprofen (12.5 mg/kg + 12.5 mg kg^{-1} hr^{-1}) effectively inhibits COX activity in the rabbit kidney.

2.9.3 AT_1- receptor antagonism (Candesartan)

In the experiments described in Chapter 6 we used the AT_1-receptor antagonist candesartan. Candesartan has high affinity for AT_1- (but not AT_2-) receptors (30). The AT_1-receptor binding affinity of candesartan has been shown to be eighty times greater than that of losartan in the rabbit aorta (30). It has a relatively long half-life (≈ 9 hrs) when administered *in vivo* and displays high protein binding capacity (30). Candesartan has also been shown to follow competitive kinetics (84). A recent study in our laboratory demonstrated that candesartan at a dose of 10 μg/kg plus 10 μg kg^{-1} hr^{-1} abolished systemic and renal haemodynamic responses to renal arterial infusion of angiotensin II in anaesthetized rabbits (66).

2.9.4 Nitric oxide synthase inhibitors

Guanidino-substituted analogues of L-arginine (eg. N^{ω}-methyl-arginine, N^{ω}-nitro – arginine, N^{ω}-nitro-arginine methyl ester, N^{G}-nitro-L-arginine) have been used in a vast number of *in vivo* and *in vitro* studies to investigate the roles of nitric oxide in various physiological and pathophysiological functions (168, 179, 247). These compounds (but not their D-enantiomers) are competitive inhibitors, which block the enzyme by competing with L-arginine for nitric oxide synthase binding (247). Addition of L-arginine has been shown to reverse the inhibitory effects of these compounds (168). N^{G}-

nitro-L-arginine methyl ester has been shown to inhibit endothelial nitric oxide synthase in the porcine aorta in a concentration-dependent manner (207). Previous studies in our research group indicate that in rabbits, intravenous administration of N^G-nitro-L-arginine (20 mg/kg + 5 mg kg^{-1} hr^{-1}) provides maximal inhibition of nitric oxide production (55, 77).

2.10 Statistics

A general overview of the statistical tests used in each of the studies described in this thesis is outlined below. These tests are described in detail in the relevant experimental chapters.

All data are expressed as mean ± SE. *P* values ≤ 0.05 were considered statistically significant. Paired or unpaired student's t-tests were only used when there was one pairwise comparison to be made. The EXCEL programme (Microsoft Office 2000) was used to perform these t-tests. Performing multiple pairwise contrasts using t-tests was avoided, since this would greatly increase the chance of making a type 1 error, unless conservative adjustments are made to *P* values using simultaneous or step-down multiple comparison procedures (135). Thus, for data of a factorial nature, biological hypotheses were tested using multiple factor analysis of variance. For data involving repeated measures in the same animal, *P* values were conservatively adjusted using the Greenhouse-Geisser correction to reduce the risk of comparison-wise type-1 error (135). The Greenhouse-Geisser correction is suggested to always overprotect against type 1 error (135). Bonferroni and Dunnett's tests were also performed as appropriate to protect against the probability of making type 1 errors resulting from multiple pairwise contrasts. Dunnett's tests were used when a number of data points were compared with one control data point (horizontal pairwise comparisons) (136). All these tests were performed using the computer software statistical package SYSTAT (255).

Chapter Three

Roles of cytochrome P450-dependent arachidonate metabolism in responses of regional kidney blood flow to hormones

3.1 Summary

Cytochrome P450 (CYP450) enzymes metabolise arachidonic acid (AA) to 20-hydroxyeicosatetraenoic acids (20-HETE) and epoxyeicosatrienoic acids (EETs) via the ω-hydroxylase and epoxygenase pathways, respectively. We tested whether regionally-selective production of these metabolites contributes to the hormonal control of regional kidney perfusion, by testing the effects of renal arterial infusions of the non selective CYP450 inhibitor 1-aminobenzotriazole (ABT), on responses to renal arterial infusions of vasoconstrictors. ABT slightly reduced basal mean arterial pressure (MAP) but did not significantly affect baseline renal haemodynamics. In vehicle treated rabbits, [Phe^2,Ile^3,Orn^8]-vasopressin reduced medullary laser Doppler flux (MLDF) but not cortical laser Doppler flux (CLDF) or total renal blood flow (RBF) while angiotensin II and noradrenaline reduced CLDF and RBF but not MLDF. ABT-treatment did not have a significant effect on any of these responses. Production of 20-HETE, EETs, and dihydroxyeicosatrienoic acids (DiHETEs) in renal cortical microsomes prepared from the kidneys of ABT-treated rabbits were not significantly different from those of vehicle-treated rabbits. These results, therefore, do not allow us to draw conclusions regarding the roles of CYP450 metabolites of AA in modulating the responses of intrarenal blood flow to angiotensin II, noradrenaline and [Phe^2,Ile^3,Orn^8]-vasopressin. The lack of efficacy of ABT under the present experimental conditions may reflect its relatively slow onset of action.

3.2 Introduction

The role of the renal medullary microcirculation in long-term blood pressure regulation is now well established (47). Central to this is the demonstration by Cowley et al of an inverse relationship between the level of renal medullary perfusion (MBF) and the long-term set-point of arterial pressure in rats (47). An understanding of the factors controlling MBF is therefore required.

Previous studies have provided strong evidence that vasoactive hormones can differentially regulate renal MBF and cortical blood flow (CBF). For example, the renal medullary microcirculation is relatively insensitive to the vasoconstrictor effects of renal arterial- and intravenous-administration of noradrenaline (76, 185, 197, 206) and angiotensin II (71, 72, 76, 197, 198, 252). In contrast, the vasopressin V_1-receptor agonist [Phe^2,Ile^3,Orn^8]-vasopressin, or arginine vasopressin itself, appear to selectively reduce MBF at doses that have little effect on CBF or total renal blood flow (RBF) (71, 72, 76, 87). This differential regulation of CBF and MBF by vasoactive hormones likely represents an important regulatory mechanism in long-term blood pressure control, but the mechanisms underlying it remain unknown. Roles for endothelial derived factors such as nitric oxide and prostaglandins have been proposed. This is supported by observations indicating that nitric oxide and/or prostaglandins contribute to angiotensin II-induced increases in MBF (185, 206, 252). Furthermore, there is also good evidence that nitric oxide released in the medullary microvasculature can blunt vasoconstrictor responses to angiotensin II (272) and noradrenaline (206, 265). However, nitric oxide synthase- and cyclooxygenase (COX)-dependent mechanisms cannot completely account for the diversity of effects of vasoactive agents on CBF and MBF. For example, during nitric oxide synthase inhibition (206) or COX inhibition (185), and even after combined inhibition of nitric oxide synthase, COX and lipoxygenase (184), renal arterial administration of angiotensin II and noradrenaline still reduced CBF more than MBF, while the vasopressin V_1-agonist [Phe^2,Ile^3,Orn^8]-vasopressin still reduced MBF more than CBF in anaesthetized rabbits. Another candidate system, which has not yet been investigated in depth, is the cytochrome P450 (CYP450)–dependent arachidonic acid (AA) metabolism cascade.

There is certainly good evidence for roles of CYP450 products of AA in mediating and/or modulating renal vascular responses to angiotensin II (119) and vasopressin (250). However, the role of these factors in underlying the diversity of actions of hormonal factors on regional kidney blood flow remains to be determined.

In the present study, we tested the hypothesis that the relative insensitivity of the medullary microcirculation to angiotensin II-, and noradrenaline-induced vasoconstriction, and the relative insensitivity of the cortical circulation to activation of vasopressin V_1-receptors, arises from differential activation of CYP450 pathways in the renal vasculature. Therefore, we tested the effects of the non-selective CYP450 inhibitor 1-aminobenzotriazole (ABT) on responses of regional kidney blood flow to renal arterial administration of the vasopressin V_1-receptor agonist [Phe^2,Ile^3,Orn^8]-vasopressin, angiotensin II and noradrenaline.

3.3 Methods

3.3.1 Animals

Twelve male, New Zealand White rabbits (2.3 ± 0.06 kg) were used. Animals were provided with water *ad libitum* but were meal fed (78). At the end of the experiment they were killed with an intravenous overdose of pentobarbitone sodium (300 mg). Experiments were approved in advance by the Monash University Department of Physiology/Central Animal Services Animal Ethics Committee.

3.3.2 Surgical preparations

These have been described in detail in Chapter 2, so will only be described briefly here. General anaesthesia was induced and maintained by i.v. pentobarbitone sodium (90-150 mg plus 30-50 mg/h) and was immediately followed by endotracheal intubation and artificial ventilation. An i.v. infusion of Hartmann's solution (compound sodium lactate) was administered at a rate of 0.18 ml $kg^{-1}min^{-1}$ during surgery. The left kidney was exposed via a left flank retroperitoneal incision, and the nerves were stripped from the renal artery and vein. The rabbit was then placed in an upright crouching position and a catheter was placed in a side branch (suprarenolumbar artery) of the renal artery for administration of vasoactive agents. The kidney was placed in a stable cup and a transit-

time ultrasound flow probe was placed around the renal artery to measure RBF. Laser Doppler flow probes were then implanted for measurement of cortical and medullary laser Doppler flux (CLDF and MLDF), as estimates of CBF and MBF respectively. MLDF was measured using a 26 gauge needle probe. A standard plastic straight probe was placed on the dorsal surface of the kidney to monitor CLDF. This probe was secured in place using gauze packing. At the completion of the surgical procedures the intravenous infusion of Hartmann's solution was replaced with a solution containing one part 10% vol/vol polygeline (Haemaccel) and four parts Hartmann's solution. Experimental protocols started 30-60 min later, when systemic and renal haemodynamics were stable.

3.3.3 Recording of haemodynamic variables

Systemic arterial pressure (MAP, mmHg), HR (beats/min) RBF (ml/min), CLDF (perfusion units) and MLDF (perfusion units) were measured throughout the experiment as described in Chapter 2. At the completion of all experiments, the left kidney was removed, snap frozen in liquid nitrogen, and stored at –70 °C for later analysis of CYP450 enzyme activity.

3.3.4 Experimental protocol

Effects of ABT on responses to renal arterial infusions of vasoconstrictors Two groups of rabbits were studied (n = 6 per group). The treatments (renal arterial ABT; 15 mg/kg over 10 min + 5 mg $kg^{-1}h^{-1}$), or vehicle (154 mM NaCl, 3.6 ml/kg over 10 min + 1.2 ml $kg^{-1}h^{-1}$) commenced immediately after the equilibration period and continued until the end of the experiment. Thirty minutes after ABT or vehicle-treatment commenced, a series of renal arterial infusions of vasoconstrictors commenced. Infusions of ascending doses of noradrenaline (20, 60 and 200 ng $kg^{-1}min^{-1}$; 10 min for each dose; Levophed Abbott Australasia Pty. Ltd., Kurnell, N.S.W., Australia), angiotensin II (2, 6 and 20 ng $kg^{-1}min^{-1}$; 10 min for each dose; Auspep, Parkville, Victoria, Australia) and [Phe^2,Ile^3,Orn^8]-vasopressin (3, 10, and 30 ng $kg^{-1}min^{-1}$; 15 min for each dose; Peninsula Laboratories, Belmont, CA, USA) were given in random order. All vasoactive agents were administered in a volume of 20 µl $kg^{-1}min^{-1}$. Each agent was separated by a 20-60 min recovery period, so that all renal haemodynamic variables had returned to their baseline levels before the next infusion commenced. The vasoactive

agents were made-up in the solution containing ABT (or its vehicle) so that these treatments were maintained for the entire experiment. All parts of the protocol were completed in each rabbit. Background levels of CLDF (7 ± 1 units) and MLDF (13 ± 2 units), recorded after the rabbit was humanely killed and artificial ventilation was ceased, were subtracted from experimental values before data analysis.

3.3.5 CYP450 ω-hydroxylase and epoxygenase activity

Microsomes were prepared from the renal cortex of ABT or vehicle treated rabbits as described in Chapter 2. The renal metabolism of AA was determined by incubating 0.5 mg of microsomal protein with a mixture of ^{14}C-AA, 10 mM sodium isocitrate, 0.16 U/ml isocitrate dehydrogenase and 1 mM β-nicotinamide adenine nucleotide phosphate, reduced form (NADPH). Incubations were carried out for 30 min at 37 °C in an atmosphere of 100% oxygen. The reaction was terminated with 1 mM formic acid and AA metabolites were extracted with 3 ml of ethyl acetate. The metabolites were separated using reverse phase high performance liquid chromatography (HPLC) equipped with an online radioactive flow detector. Rates of product formation are expressed as pmol formed per min per mg protein (pmol min^{-1} mg^{-1}). The rate of formation of 20-HETE provided a measure of ω-hydroxylase activity, while the rate of formation of dihydroeicosatrienoic acids (DiHETEs) and EETs provided a measure of epoxygenase activity.

3.3.6 Statistics

Data are expressed as mean ± SE. *P* values ≤ 0.05 were considered statistically significant. To test whether pre-treatment levels of haemodynamic variables during the 10 min control period varied according to the treatment that was to follow, these data were subjected to one-way analysis of variance. Student's unpaired t-test was used to determine whether ABT affected haemodynamic variables differently from vehicle-treatment. Two-way analysis of variance was used to test whether baseline haemodynamic variables during the control period before each renal arterial vasoconstrictor infusion varied according to the vasoconstrictor to be infused. Levels of haemodynamic variables during the final 3 min of control periods and periods during vasoconstrictor infusions, were subjected to repeated measures analysis of variance. The main effect of 'treatment' from a two-way analysis of variance tested whether responses

to the vasoactive agents were affected by ABT. To reduce the risk of comparison-wise type 1 error, *P* values were conservatively adjusted using the Greenhouse-Geisser correction (135). Unpaired students t-tests were used to compare the formation of 20-HETE, DiHETEs and EETs in renal cortical microsomes prepared from kidneys of vehicle-treated rabbits with those of ABT- treated rabbits.

3.4 Results

3.4.1 Baseline levels of haemodynamic variables

The average baseline levels of MAP, HR, RBF, CLDF and MLDF across all 12 rabbits were 70 ± 3 mmHg, 282 ± 3 beats/min, 27 ± 3 ml/min, 383 ± 16 units and 95 ± 6 units respectively. These were similar to values obtained previously under similar experimental conditions in our laboratory (206).

3.4.2 Effects of ABT on baseline haemodynamics

Responses of baseline haemodynamics to vehicle- (154 mM NaCl) and ABT-treatment, are shown in Figure 3.1. Levels of MAP, HR, RBF, CLDF and MLDF remained relatively stable after vehicle-treatment. Responses to ABT were indistinguishable from that of vehicle, except for MAP, which fell by - 2 ± 1 mmHg after ABT but not after vehicle (+ 2 ± 2 mmHg change).

We also tested whether the baseline levels of haemodynamic variables, during the control periods before each of the renal arterial vasoconstrictor infusions, varied according to treatment (vehicle or ABT) or the agent to be infused ([Phe^2,Ile^3,Orn^8]-vasopressin, angiotensin II and noradrenaline). Analysis of variance showed no significant effect of treatment ($P_{treatment} \geq 0.15$) or agent ($P_{agent} \geq 0.11$) or interaction between these factors ($P_{treatment*agent} \geq 0.83$).

3.4.3 Effects of ABT on responses to renal arterial infusions of vasoconstrictors

In vehicle-treated rabbits, renal arterial infusions of angiotensin II and noradrenaline had little effect on MAP ($P_{dose} \geq 0.13$) and HR ($P_{dose} \geq 32$). [Phe^2,Ile^3,Orn^8]-vasopressin, caused does-dependent reductions in HR (by 48 ± 5 beats/min at 30 ng kg^{-1}

$^{1}min^{-1}$; P_{dose} = 0.01) but had no significant effect on MAP (P_{dose} = 0.28). Responses of MAP and HR in ABT-treated rabbits were indistinguishable from those in vehicle-treated rabbits. In contrast to systemic haemodynamics, responses of renal haemodynamics to these vasoconstrictor agents were robust and diverse.

In vehicle-treated rabbits [Phe^{2},Ile^{3},Orn^{8}]-vasopressin dose-dependently reduced MLDF (P_{dose} = 0.01) but not RBF or CLDF ($P_{dose} \geq 0.35$). At 30 ng $kg^{-1}min^{-1}$ changes in these variables averaged – 59 ± 5%, + 6 ± 8% and + 5 ± 5% respectively. These responses were not significantly affected by ABT-treatment (Figure 3.2).

In vehicle-treated rabbits, angiotensin II (2-20 ng $kg^{-1}min^{-1}$) dose-dependently reduced RBF (P_{dose} = 0.03) and CLDF (P_{dose} = 0.01) but not MLDF (P_{dose} = 0.73), so that at a dose of 20 ng $kg^{-1}min^{-1}$ changes in these variables averaged –33 ± 10%, -34 ± 11% and +1 ± 11% respectively. ABT-treatment did not significantly affect these responses (Figure 3.2).

In vehicle-treated rabbits noradrenaline (20-200 ng/kg/min) caused dose-dependent reductions in RBF (P_{dose} = 0.03) and CLDF (P_{dose} = 0.05) but not MLDF (P_{dose} = 0.27). At 200 ng $kg^{-1}min^{-1}$ reductions in these variables averaged - 46 ± 14%, - 40 ± 13% and - 21 ± 13% respectively. Similar responses were observed in ABT treated rabbits (Figure 3.2).

3.4.4 CYP450 ω-hydroxylase and epoxygenase activity

The production of 20-HETE, EETs and DIHETEs averaged 142 ± 40, 23 ± 4, 51 ± 11 pmole min^{-1} $protein^{-1}$ respectively, in cortical microsomes prepared from the kidneys of vehicle-treated rabbits and 133 ± 16, 20 ± 4, 31 ± 11 pmole min^{-1} $protein^{-1}$ respectively, in cortical microsomes prepared from the kidneys of ABT-treated rabbits. Thus, the production of 20-HETE, EETs and DiHETEs in renal cortical microsomes did not differ significantly between ABT- and vehicle-treated rabbits (Figure 3.3).

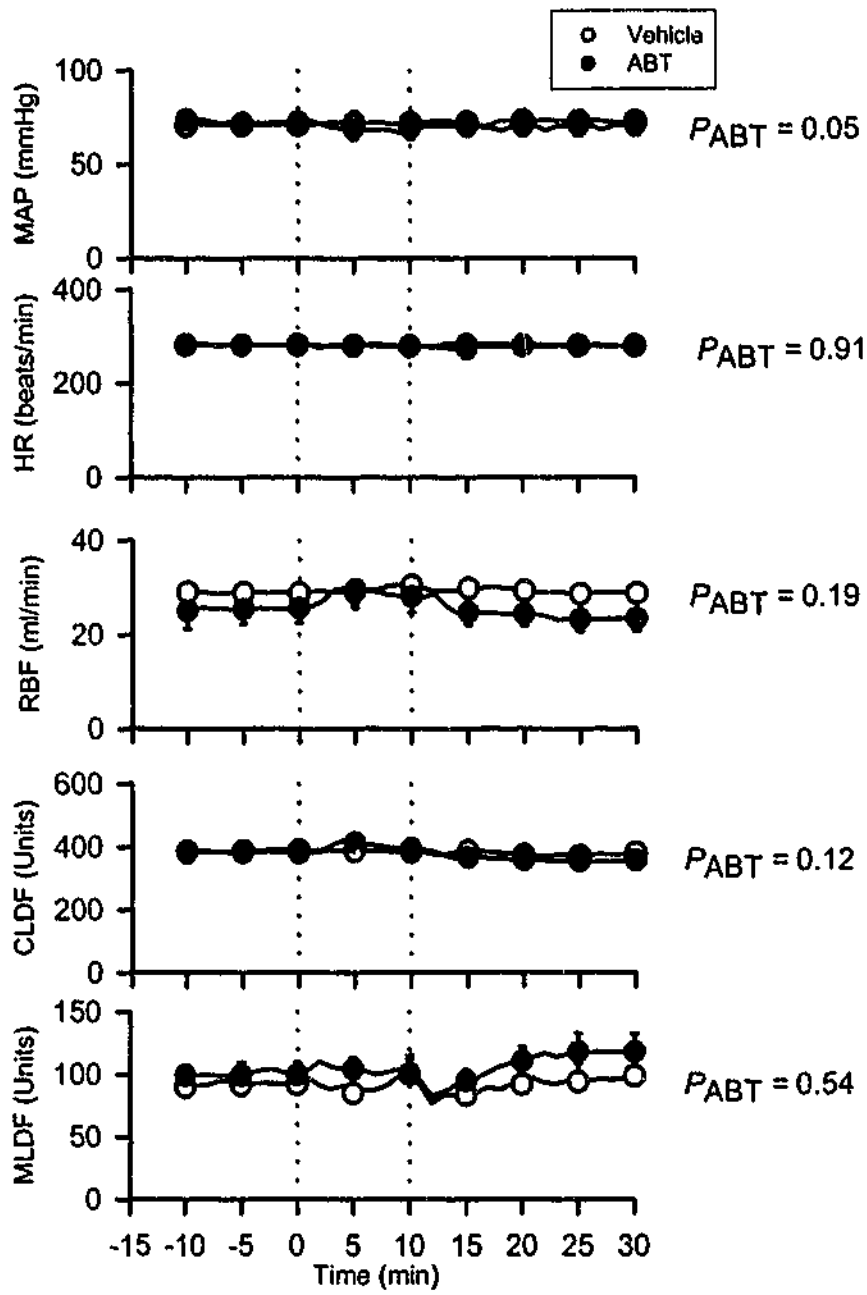

Figure 3.1. Baseline levels of systemic and renal haemodynamic variables before and after commencing renal arterial infusion of 1-aminobenzotriazole (ABT) and its vehicle. The lines indicate 1 min averages for the 6 rabbits in each group. Symbols, ((O) vehicle (154 mM saline) and (•) ABT (1-aminobenzotriazole; 15 mg/kg over 10 min + 5 mg kg^{-1} h^{-1}) and error bars represent the mean ± SE for every 5th one min average. Dotted vertical lines show the period over which the initial 'loading dose' was given. *P* values represent the outcomes of unpaired t-tests, testing whether responses to ABT differed from responses to its vehicle (df = 10). For this purpose the response was defined as the difference between average levels across the 10 min control period, and levels during the period 25 to 30 min after the infusion commenced. MAP, mean arterial pressure. HR, heart rate. RBF, renal blood flow. CLDF, cortical laser Doppler flux. MLDF, medullary laser Doppler flux.

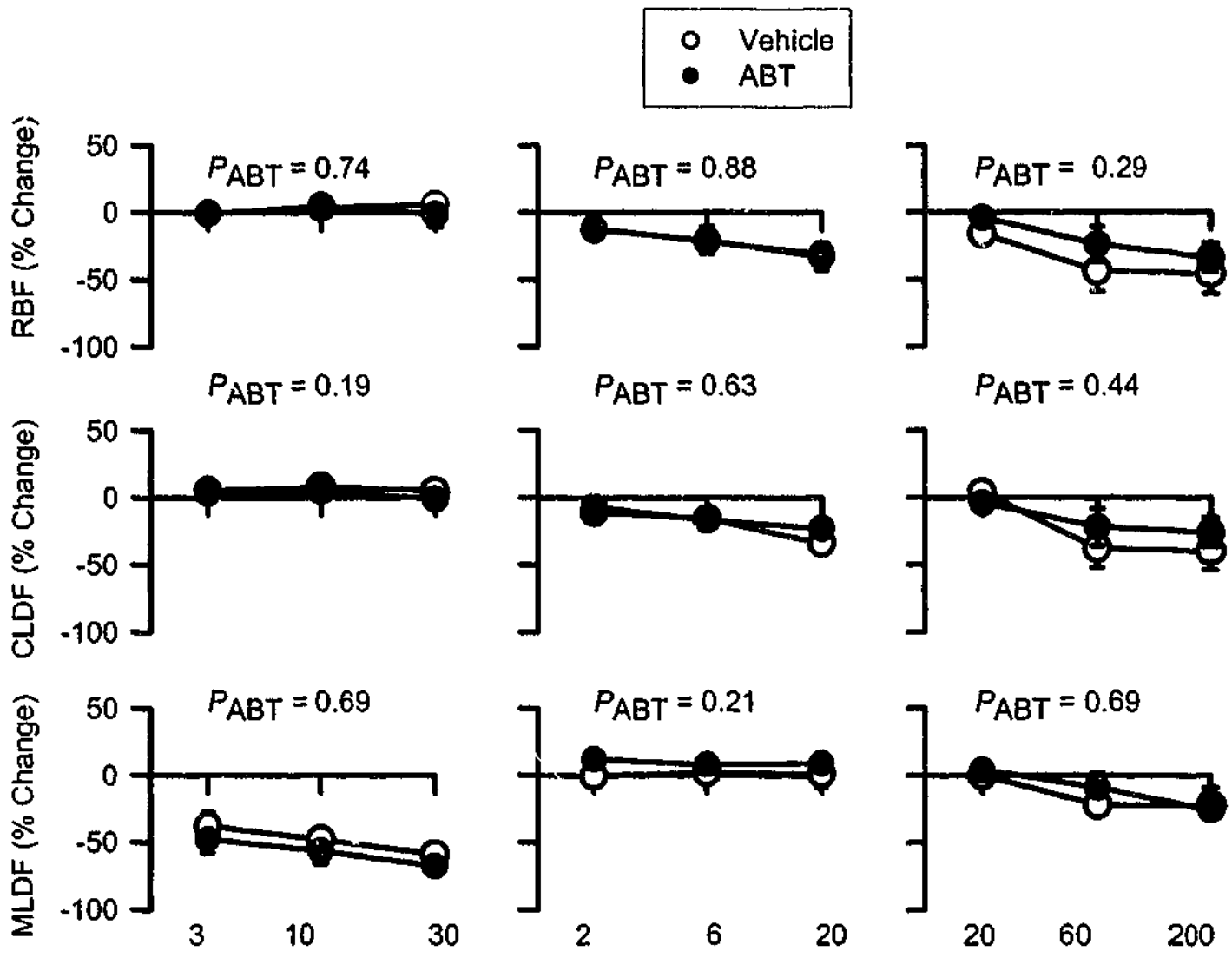

Figure 3.2. Renal haemodynamic responses to renal arterial infusions of [Phe^2,Ile^3,Orn^8]-vasopressin (V_1AG), angiotensin II (AII) and noradrenaline (NA). Symbols indicate mean ± SE of percentage changes observed in the final 3 min of the infusion compared with the pre-treatment levels. (O) vehicle pre-treated rabbits and (•) 1-aminobenzotriazole (ABT) pre-treated rabbits (15 mg/kg + 5 mg kg^{-1} h^{-1}). *P* values indicate the main effect of treatment (P_{ABT}) from repeated measured analysis of variance, testing whether responses to vasoconstrictor agents were affected by ABT treatment (df = 1,10, see Methods). Abbreviations are as for Figure 3.1.

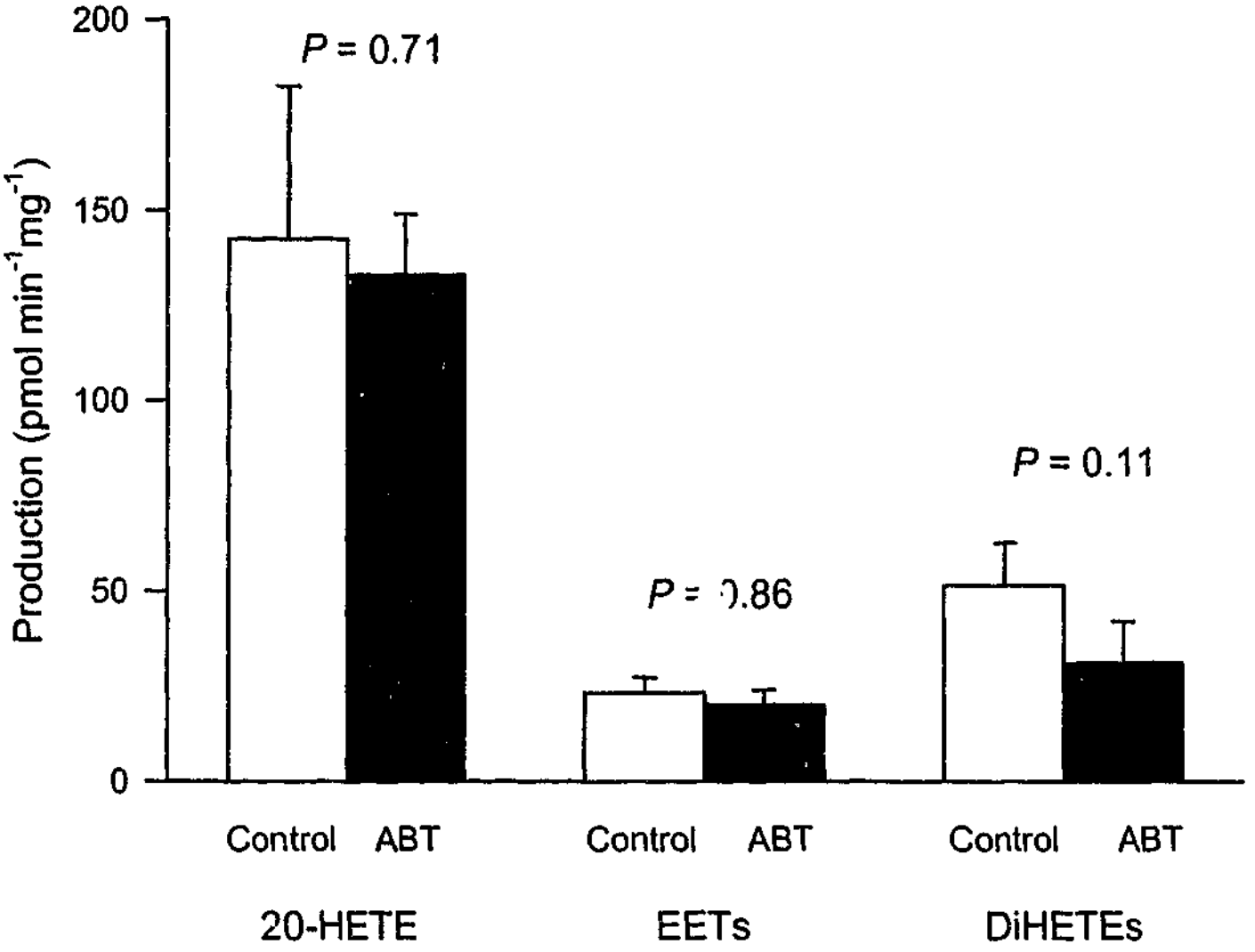

Figure 3.3. Effects of *in vivo* treatment with 1-aminobenzotriazole (ABT) on cytochrome P450-dependent metabolism of arachidonic acid in renal cortical microsomes *in vitro*. Microsomes were prepared using renal cortical tissues of vehicle- (open columns) and ABT- (15 mg/kg plus 5 mg $kg^{-1}h^{-1}$; closed columns) treated rabbits. Columns and error bars represent the mean ± SEM of observations from 6 rabbits in each group. *P* values represent the outcomes of Student's unpaired t-tests, to determine whether *in vivo* treatment with ABT altered the rate of production of 20-hydroxyeicosatetraenoic acid (20-HETE), epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DiHETEs) *in vitro*.

3.5 Discussion

We found that renal arterial infusions of angiotensin II and noradrenaline reduced RBF and CLDF but not MLDF, whereas [Phe^2,Ile^3,Orn^8]-vasopressin reduced MLDF but not CLDF or RBF in vehicle-treated rabbits. These results confirm previous findings in our laboratory indicating that these vasoconstrictors can differentially affect CBF and MBF in the rabbit (45, 71, 72, 76, 185, 206). The main objective of the present study was to investigate whether regionally selective production of CYP450-derived metabolites of AA contributes to this diversity of regional kidney blood flow responses to these vasoactive agents. Our results demonstrated that neither baseline levels of systemic and renal haemodynamics (except for a small reduction in MAP) nor responses of intrarenal blood flow to vasoconstrictor agents were greatly affected by ABT-treatment. However, our results also indicate that ABT-treatment did not produce significant inhibition of CYP450 ω-hydroxylase or epoxygenase activity in microsmes prepared from kidneys of ABT-treated rabbits. Indeed the rate of conversion of exogenous AA to HETEs, EETs and DiHETEs was indistinguishable in cortical microsomes prepared from vehicle- compared with ABT-treated kidneys. Therefore, our results do not allow us to draw conclusions regarding the roles of CYP450 in responses of intrarenal blood flow to vasoconstrictor hormones.

ABT is a suicide-substrate inhibitor of CYP450 enzymes (212). In microsomes prepared from human kidneys, the IC_{50} of ABT for inhibition of 20-HETE production is ~39 μM (167). We originally estimated, based on our measurements of RBF, that the minimum concentration of ABT in renal arterial blood (assuming no re-circulation) was ~1000 μM during the initial 10 min infusion, and ~55 μM for the remainder of the experiment. We presumed that the true levels of the drug in the biophase would far exceed these estimates, since ABT is metabolised and cleared relatively slowly, and binds irreversibly to CYP450 enzymes (244). However, our present data now demonstrate the dose of ABT used in this study (15 mg/kg + 5 mg $kg^{-1}h^{-1}$) was insufficient to inhibit CYP450 ω-hydroxylase or epoxygenase activity in ABT-treated rabbit kidneys. This is at odds with previous findings of Sarkis et al who demonstrated that acute administration of ABT at a dose of 50 mg/kg inhibited the formation of 20-HETE (by

88%) and EETs (by 46%), lowered renal tissue levels of 20-HETE (by 79%), and blunted the pressure natriuresis response in rats (226). Likewise, Lopez et al demonstrated that acute ABT-treatment (50 mg/kg i.v) inhibited the formation of 20-HETE (by ~87%) and EETs (by ~40%) in rats (131). Su et al demonstrated that in rats, maximum inhibition of CYP450 ω- and ω-1 hydroxylase activity occurred within 6 hrs after a single intraperitoneal injection of ABT (50 mg/kg) (234). They also observed a reduction in MAP 4 hrs after administration of this dose of ABT in spontaneously hypertensive rats (SHR) (234). The discrepancy between these previous data and our present results could in part be due to the differences in the amounts of cellular phospholipid pools of CYP450 metabolites in the rabbit kidney versus the rat kidney. 20-HETE and EETs can be pre-stored in membrane phospholipids, and can be released in response to different hormonal stimuli (212). Thus, even in the presence of complete blockade of production of these AA metabolites, they can still contribute to renal vascular responses to vasoactive agents. The rabbit kidney could have greater storage pools of EETs and 20-HETE in tissue phospholipids than the rat kidney which could take days of ABT-treatment to deplete.

Santos et al have shown that chronic administration of ABT (50 mg kg^{-1} day^{-1}; i.p) for five consecutive days reduced the formation of 20-HETE (by 80%) and EETs (by 60%) in the rat kidney (224) suggesting that chronic ABT-treatment can cause greater reductions in EETs formation than acute ABT-treatment. Also, this study demonstrated that urinary excretion of 20-HETE did not significantly reduce until the third day of ABT-treatment (224). After five days of ABT-treatment urinary excretion of 20-HETE reduced by 60%, and over the next five days it reduced by a further 20% (224). 20-HETE excreted in urine may come from cellular phospholipid pools (personal communication; Dr Richard Roman). Other studies have also demonstrated inhibition of CYP450 ω-hydroxylase and epoxygenase activity in renal tissues of rats following chronic ABT-treatment (7, 102). Collectively, these data suggest that although acute ABT-treatment can inhibit the formation of 20-HETE and EETs, chronic ABT-treatment is required to ensure complete inhibition of *de novo* synthesis of CYP450 AA products, and to deplete membrane-bound stores of CYP450 metabolites.

In the present study we commenced testing the responses of intrarenal blood flow to hormonal stimuli, only 30 min after commencing the ABT infusion. According to above mentioned recent studies, 30 min would be barely enough time for ABT to inhibit CYP450 activity. So, in the present study we seemed to have erred by seriously underestimating the time required for ABT to inhibit CYP450 activity in the rabbit kidney. Future studies investigating the renal haemodynamic responses to vasoconstrictor hormones should therefore be tested in animals chronically treated with ABT.

Lopez et al demonstrated that ABT inhibited the formation of 20-HETE and EETs, N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH) inhibited the formation of EETs and N-hydroxy-N-(4-butyl-2-methylphenyl) formamidine (HET0016) inhibited the formation of 20-HETE. However, neither ABT, MS-PPOH nor HET0016 had any significant effects on blood pressure, RBF, glomerular filtration rate or urinary excretion of sodium and water in rats (131). In contrast, renal cortical interstitial infusion of the 20-HETE antagonist WIT002 reduced urinary excretion of sodium and water by 30% (131). These authors have suggested that 20-HETE released from phospholipid pools could be more important in regulating tubular sodium and water reabsorption than basal 20-HETE. Likewise, in the rabbit kidney, 20-HETE (and/or EETs) released from the phospholipid pools could play a more prominent role than basal levels of these metabolites in modulating the responses of intrarenal perfusion to hormones. To definitively test this hypothesis intrarenal responses to hormones need to be tested in animals pre- treated with specific receptor antagonists.

Su et al have demonstrated that ABT dose-dependently reduced the formation of 20-HETE but had little effect on the formation of EETs suggesting that ABT has somewhat less potency as an inhibitor of CYP450 epoxygenase activity (234). In contrast, more recent data indicate that chronic ABT-treatment can significantly reduce the formation of both 20-HETE and EETs in the kidney (7, 102, 140). The reasons for the discrepancy between these findings remain unclear. It could be due to the dose and/or the duration (chronic vs acute) of ABT-treatment in different studies. ABT appears to be selective in inhibiting 20-HETE formation at lower doses (up to 25 mg/kg) but at higher doses it seems to be equally effective in inhibiting EETs production as well (234).

Su et al (234) demonstrated that ABT can reduce MAP chiefly by inhibition of 20-HETE formation in SHR. On the other hand, Hoagland et al recently demonstrated that ABT, but not the selective ω-hydroxylase inhibitor HET0016, reduced MAP in rats, suggesting that ABT reduces blood pressure potentially via inhibition of EET formation (102). However, many EETs are potent vasodilators, and thus inhibition of their production is unlikely to contribute to the blood pressure lowering effect of ABT (102). These latter authors have suggested that the blood pressure lowering effect of ABT could be dependent on its ability to inhibit CYP450 isoforms responsible for producing metabolites other than 20-HETE and EETs (149, 172). In support of this notion, we observed a small, but significant, reduction in MAP in ABT-treated rabbits even in the face of undetectable inhibition of CYP450 dependent ω-hydroxylase or epoxygenase activity by ABT. Thus, our data provide preliminary evidence that the effect of ABT on blood pressure is independent of its ability to inhibit renal CYP450 dependent ω-hydroxylase or epoxygenase activity.

3.6 Conclusions

In summary our present results indicate that the medullary microcirculation is relatively refractory to angiotensin II- and noradrenaline-induced vasoconstriction, and that the cortical circulation is relatively refractory to V_1-receptor mediated vasoconstriction. This differential regulation of regional kidney blood flow by vasoactive hormones is likely to represent an important regulatory mechanism in long-term blood pressure control, but the mechanisms underlying it are ill defined. In previous studies we found that nitric oxide buffers the reductions in MBF during renal arterial infusion of noradrenaline (206) or stimulation of the renal nerves (69). In the current study, we were unable to sufficiently inhibit CYP450 activity in the rabbit kidney with ABT-treatment. Therefore, we are not in a position to draw any conclusions regarding the roles of CYP450 metabolites in modulating the responses of regional kidney blood flow to different vasoconstrictor hormones used in this study. Based on recent literature (102, 224) and personal communications with our colleague Dr Richard Roman (Medical College of Wisconsin, Milwaukee, USA), it seems likely that effective inhibition of CYP450 dependent AA metabolism

(particularly depletion of cellular phospholipid pools of CYP450 metabolites) can only be ensured if this agent is administered chronically, or at least sub-acutely. Our experiment, therefore, should be repeated under conditions of prolonged ABT-treatment. However, selective inhibitors of CYP450 dependent ω-hydroxylase and epoxygenase have recently been synthesized by our colleague Professor John Falck (212), and he has made these available to us. Therefore, in the experiments described in the next chapter, we examined the effects of the selective epoxygenase inhibitor, N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH) and the selective ω-hydroxylase inhibitor, N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS) on responses of intrarenal blood flow to [Phe^2,Ile^3,Orn^8]-vasopressin and angiotensin II.

Chapter Four

Modulation of V_1- receptor-mediated renal vasoconstriction by epoxyeicosatrienoic acids

A modified version of this chapter has been published in the American Journal of Physiology (Regulatory, Integrative and Comparative Physiology) 287:R181-R187, 2004

4.1 Summary

Cytochrome P450 (CYP450) enzymes metabolise arachidonic acid (AA) to 20-hydroxyeicosatetraenoic acids (20-HETE) and epoxyeicosatrienoic acids (EETs) via the ω-hydroxylase and epoxygenase pathways, respectively. We tested whether regionally-selective production of these metabolites contributes to the hormonal control of regional kidney perfusion, by testing the effects of renal arterial infusions of the epoxygenase inhibitor N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH), and the ω-hydroxylase inhibitor N-methylsulfonyl-12-12,dibromododec-11-enamide (DDMS), on responses to renal arterial infusions of vasoconstrictors. Compared with vehicle, neither MS-PPOH nor DDMS affected basal mean arterial pressure, total renal blood flow (RBF), cortical laser Doppler flux (CLDF) or medullary laser Doppler flux (MLDF). After vehicle, [Phe^2,Ile^3,Orn^8]-vasopressin reduced MLDF (up to 62±7%) but not CLDF or RBF. MS-PPOH did not affect responses of MLDF to [Phe^2,Ile^3,Orn^8]-vasopressin, but revealed reductions in RBF (up to 51±8%) and CLDF (up to 59±13%). DDMS did not significantly affect responses to [Phe^2,Ile^3,Orn^8]-vasopressin. After vehicle, angiotensin II reduced RBF (up to 45±10%) and CLDF (up to 41±14%) but not MLDF. DDMS did not affect responses of RBF and CLDF to angiotensin II, but revealed moderate dose-dependent reductions in MLDF (up to 24±14%). MS-PPOH did not significantly affect responses to angiotensin II. Formation of EETs and dihydroxyeicosatrienoic acids (DiHETEs) was 49% lower in homogenates prepared from the renal cortex of MS-PPOH-treated rabbits than from vehicle-treated rabbits. MS-PPOH had no significant effect on the renal formation of 20-HETE. DDMS had no effect on the renal formation of 20-HETE, EETs or DiHETEs. Incubation of renal

cortical homogenates from untreated-rabbits with [Phe^2,Ile^3,Orn^8]-vasopressin (0.2-20 ng/ml) did not affect formation of EETs, DiHETEs or 20-HETE. These results do not support a role for *de novo* EET synthesis in modulating renal haemodynamic responses to angiotensin II. However, EETs appear to selectively oppose V_1-receptor-mediated vasoconstriction in the renal cortex, but not in the medullary circulation, and contribute to the relative insensitivity of cortical blood flow to V_1-receptor activation. The mechanisms underlying the action of DDMS to enhance angiotensin II-induced vasoconstriction in the medullary circulation remain unknown, since this agent did not appear to inhibit CYP450 ω-hydroxylase or epoxygenase activity at the dose we used.

4.2 Introduction

The role of the renal medullary circulation in long-term blood pressure regulation is well established. Numerous studies have reported that intrarenal infusion of vasoactive agents that reduce renal medullary blood flow (MBF) increase arterial pressure, and that pharmacological agents that increase MBF reduce arterial pressure in several models of hypertension (47, 49, 150). However, the factors that control MBF *in vivo* remain to be determined.

There is strong evidence that vasoactive hormones can differentially influence cortical blood flow (CBF) and MBF. For example, MBF appears to be relatively insensitive to the vasoconstrictor effects of angiotensin II (13, 66, 71, 72, 76, 184, 185, 197, 198, 206, 252, 272), whereas vasopressin V_1-receptor agonists selectively reduce MBF at doses that have little effect on CBF or total renal blood flow (RBF) (45, 71, 76, 87, 185, 206). The differential effects of angiotensin II versus vasopressin in the intrarenal control of blood flow likely underlie important mechanisms in long-term regulation of blood pressure and the control of urinary concentrating ability, but the precise mechanism underlying these differences, are unknown. There is evidence that angiotensin II stimulates the release of nitric oxide and prostaglandins in the medullary circulation and that the local release of these vasodilators contributes to the relative insensitivity of MBF to angiotensin II (13, 49, 185, 197, 198, 206, 252, 272). On the other hand, we

have recently obtained evidence that neither nitric oxide nor cyclooxygenase (COX)-derived or lipoxygenase-derived products of arachidonic acid (AA), contribute to the insensitivity of the renal cortical circulation to the V_1-agonist [Phe^2,Ile^3,Orn^8]-vasopressin (184, 185, 206). Importantly, under conditions of nitric oxide synthase blockade (206), cyclooxygenase blockade (185), lipoxygenase blockade (184) or even combined blockade of all three of these enzyme systems (184), renal arterial administration of angiotensin II still reduces CBF more than MBF, whereas [Phe^2,Ile^3,Orn^8]-vasopressin has the opposite effect and reduces MBF more than CBF. Thus, other mechanisms must also contribute to the differential control of CBF and MBF by angiotensin II and vasopressin peptides.

Another candidate system that may modulate reactivity to vasoactive hormones is the cytochrome P450 (CYP450)–dependent AA metabolism cascade. CYP450 products of AA have been shown to mediate and/or modulate renal vascular responses to angiotensin II (119) and vasopressin (250). Furthermore, epoxyeicosatrienoic acids (EETs) are formed in the rabbit kidney (79), and vasopressin stimulates the release of EETs from rabbit renal medullary tissue (229). However, whether EETs and 20-hydroxyeicosatetraenoic acids (20-HETE) modulate the renal vasoconstrictor actions of vasopressin and other hormones *in vivo,* remain to be determined.

The present study, examined the hypothesis that the relative insensitivity of MBF to angiotensin II, and the relative insensitivity of CBF to [Phe^2,Ile^3,Orn^8]-vasopressin, arises from regional differences in the ability of these agents to stimulate the synthesis and/or release of CYP450 epoxygenase and ω-hydroxylase metabolites of AA. Therefore, we examined the effects of the selective CYP450 epoxygenase inhibitor, N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH) (28), and the selective ω-hydroxylase inhibitor, N-methylsulfonyl-12-12,dibromododec-11-enamide (DDMS), on the regional renal blood flow responses to infusion of angiotensin II and [Phe^2,Ile^3,Orn^8]-vasopressin directly into the renal artery of anaesthetized rabbits. We also tested whether MS-PPOH and DDMS were able to selectively reduce epoxygenase and ω-hydroxylase activity following *in vivo* administration, by measuring the metabolism of exogenous AA in homogenates prepared from the kidneys of vehicle-treated, MS-PPOH-treated and DDMS-treated rabbits. The results suggest that in

rabbits *in vivo*, *de novo* synthesis of EETs buffers the vasoconstrictor effects of V_1-receptor agonists in the renal cortical circulation.

4.3 Methods

4.3.1 Animals

Experiments were performed on 18 male, New Zealand White rabbits (2.73 ± 0.06 kg).

4.3.2 Surgical preparations

These have been described in detail in Chapter 2, so will only be described briefly here. The rabbits were anaesthetized with i.v. pentobarbitone sodium (90-150 mg plus 30-50 mg/h) and this was immediately followed by endotracheal intubation and artificial ventilation. An i.v. infusion of Hartmann's solution (compound sodium lactate) was administered at a rate of 0.18 ml $kg^{-1}min^{-1}$ during surgery. The left kidney was exposed via a left flank retroperitoneal incision, and denervated by stripping the nerves from the renal artery and vein. The rabbit was then placed in an upright crouching position and a catheter was placed in a side branch (suprarenolumbar artery) of the renal artery for administration of vasoactive agents. Catheter patency was maintained by a continuous infusion of 154 mM NaCl (20 µl $kg^{-1}min^{-1}$). The kidney was placed in a stable cup and a transit-time ultrasound flow probe (type 2SB) was placed around the renal artery to measure total renal blood flow (RBF). CBF and MBF were estimated by laser Doppler flowmetry. Medullary laser Doppler flux (MLDF) was measured after implanting a needle probe (26 gauge, DP4s) 9 mm below the cortical surface using a micromanipulator. A standard plastic straight probe (DP2b) was used to monitor cortical laser Doppler flux (CLDF). This probe was secured in place using gauze packing. At the completion of the surgical procedures the intravenous infusion of Hartmann's solution was replaced with a solution containing one part 10% vol/vol polygeline (Haemaccel) and four parts Hartmann's solution. Experimental protocols started 30-60 min later, when systemic and renal haemodynamics were stable.

4.3.3 Recording of haemodynamic variables

Systemic arterial pressure (MAP, mmHg), HR (beats/min) RBF (ml/min), CLDF (perfusion units) and MLDF (perfusion units) were measured throughout the experiment as described in Chapter 2. At the completion of all experiments, the left kidney was removed, snap frozen in liquid nitrogen, and stored at –70 °C for later analysis of CYP450 enzyme activity.

4.3.4 Experimental protocol

Effects of MS-PPOH and DDMS on responses to renal arterial infusions of vasoconstrictors After surgery and a 30-60 min equilibration period, MS-PPOH (2 mg/kg plus 1.5 mg $kg^{-1}h^{-1}$) , DDMS (2 mg/kg plus 1.5 mg $kg^{-1}h^{-1}$) or vehicle (45% hydroxypropyl-β-cyclodextran; Sigma Chemical Company, St Louis, MO; 0.16 ml/kg + 1.2 ml $kg^{-1}h^{-1}$) was infused into the renal artery. Thirty minutes later the responses to graded renal arterial infusions of angiotensin II (2, 6 and 20 ng $kg^{-1}min^{-1}$; 10 min for each dose; Auspep, Parkville, Victoria, Australia) and [Phe^2,Ile^3,Orn^8]-vasopressin (3, 10, and 30 ng $kg^{-1}min^{-1}$; 15 min for each dose; Peninsula Laboratories, Belmont, CA, USA) were determined in random order. At the end of each experiment the left kidney was removed, frozen in liquid nitrogen and stored at –80 °C for later measurement of the renal metabolism of AA *in vitro*. Background levels of CLDF (16 ± 1 units) and MLDF (14 ± 2 units), recorded after the rabbit was humanely killed and artificial ventilation was ceased, were subtracted from experimental values before data analysis.

4.3.5 CYP450 ω-hydroxylase and epoxygenase activity

Microsomes were prepared from the renal cortex and medulla of 6 untreated rabbits. Briefly, the renal cortex and inner and outer medulla were homogenized in 3 volumes of a 10 mM potassium phosphate buffer containing 250 mM sucrose and 1 mM ethylenediamineteteraacetic acid (EDTA). The microsomes were prepared by sequential centrifugation of the homogenate (at 3000 × g for 5 min, 11,000 × g for 15 min and 100,000 × g for 60 min). Microsomal pellets were resuspended in 100 mM potassium phosphate buffer (pH = 7.25) containing 0.5 mM EDTA, 1 mM dithiothreitol, 30% glycerol and 0.1 mM phenylmethylsulfonyl fluoride.

Renal homogenates rather than microsomes were prepared from the kidneys of the rabbits treated *in vivo* with MS-PPOH, DDMS or its vehicle, since it remains to be determined whether MS-PPOH and DDMS act as competitive, noncompetitive or mixed mode inhibitors of epoxygenase and ω-hydroxylase activity respectively. Thus, these kidneys were homogenized in only 2 volumes of the 10 mM potassium phosphate buffer to minimize dilution of MS-PPOH and DDMS retained in the tissue, and centrifuged at low speed (at 3000 × g for 5 min). An aliquot of the homogenate was taken to determine the CYP450 activity as described below.

The renal metabolism of AA was determined by incubating 0.5 mg of microsomal protein or 2 mg of homogenate protein with ^{14}C-AA (Amersham, Arlington Heights, IL) in 1 ml of a 100 mM potassium phosphate buffer (pH 7.25) containing 10 mM sodium isocitrate, 0.16 U/ml isocitrate dehydrogenase, and 1 mM β-nicotinamide adenine nucleotide phosphate, reduced form (NADPH). The microsome reactions were performed using a saturating concentration of AA (0.1 μCi, 4^ μM) whereas the homogenates were studied using a low concentration of substrate (AA, 0.1 μCi, 2 μM) to maximize the conversion rate of labeled AA and minimize competition of MS-PPOH and DDMS with excess substrate.

Incubations were carried out in a shaking water bath at 37 °C for 30 min (microsomes) or 60 min (homogenates) in an atmosphere of 100% oxygen. The reaction was terminated with 1 M formic acid and AA metabolites were extracted with 3 ml of ethyl acetate. The metabolites were separated using reverse phase high performance liquid chromatography equipped with an online radioactive flow detector as previously described (270). Rates of product formation are expressed as pmol formed per min per mg protein (pmol $min^{-1}mg^{-1}$). The rate of formation of 20-HETE provided a measure of ω-hydroxylase activity, while the rate of formation of dihydroxyeicosatrienoic acids (DiHETEs) and EETs provided a measure of epoxygenase activity.

4.3.6 Statistics

Data are expressed as mean ± SE. *P* values ≤ 0.05 were considered statistically significant. To test whether pre-treatment levels of haemodynamic variables during the

10 min control period varied according to the treatment that was to follow, these data were subjected to one-way analysis of variance. Student's unpaired t-test was used to determine whether MS-PPOH and DDMS affected haemodynamic variables differently from vehicle-treatment. Two-way analysis of variance was used to test whether baseline haemodynamic variables, during the control period before each renal arterial vasoconstrictor infusion, varied according to the vasoconstrictor to be infused. Levels of haemodynamic variables during the final 3 min of control periods and periods during vasoconstrictor infusions, were subjected to repeated measures analysis of variance. The main effect of 'treatment' from a two-way analysis of variance tested whether responses to the vasoactive agents were affected by MS-PPOH and DDMS. To reduce the risk of comparison-wise type 1 error, P values derived from within-subject factors in this 'repeated–measures' design were conservatively adjusted using the Greenhouse-Geisser correction (135). Dunnett's tests were used to compare the formation of 20-HETE, DiHETEs and EETs in renal cortical microsomes prepared from kidneys of vehicle-treated rabbits with those of MS-PPOH- and DDMS-treated rabbits.

4.4 Results

4.4.1 Baseline levels of haemodynamic variables

The average baseline levels of MAP, HR, RBF, CLDF and MLDF across all 18 rabbits were 77 ± 2 mmHg, 274 ± 4 beats/min, 31 ± 2 ml/min, 384 ± 31 units and 67 ± 8 units respectively. These were similar to values obtained previously under similar experimental conditions in our laboratory (206; Chapter 3). Vehicle-treatment increased MAP (13 ± 3 %), and reduced HR (6 ± 1%) and RBF (10 ± 3 %) but had little or no effect on CLDF (3 ± 3 %) or MLDF (1 ± 8 %). Responses to DDMS and MS-PPOH were not significantly different to those of the vehicle-treatment (Figure 4.1).

We tested whether the baseline levels of haemodynamic variables during the control periods before each of the renal arterial vasoconstrictor infusions differed according to treatment (vehicle, DDMS or MS-PPOH) or the agent (angiotensin II or [Phe^2,Ile^3,Orn^8]-vasopressin) to be infused. Analysis of variance showed no significant effect on MAP, RBF, CLDF and MLDF of treatment ($P_{treatment} \geq 0.11$) or agent ($P_{agent} \geq 0.11$), or interaction between these factors ($P_{treatment*agent} \geq 0.22$). HR, however, was

slightly (15 ± 9 beats/min) before angiotensin II infusion than before [Phe^2,Ile^3,Orn^8]-vasopressin infusion (P_{agent} = 0.04).

4.4.2 *Effects of MS-PPOH and DDMS on responses to renal arterial infusions of vasoconstrictors*

A comparison of the effects of [Phe^2,Ile^3,Orn^8]-vasopressin on renal haemodynamics in rabbits treated with vehicle, DDMS and MS-PPOH is presented in Figure 4.2. In vehicle-treated rabbits, infusion of the V_1-agonist dose-dependently reduced MLDF but it had no effect on RBF or CLDF (Figure 4.2). The V_1-agonist did reduce HR (by -17 ± 2% at 30 ng kg^{-1}min^{-1}) but did not significantly affect MAP (data not shown). In contrast, [Phe^2,Ile^3,Orn^8]-vasopressin dose-dependently reduced RBF and CLDF in MS-PPOH-treated rabbits. For example, at a dose of 30 ng kg^{-1} min^{-1} RBF and CLDF fell by -51 ± 8% and -59 ± 13% respectively in MS-PPOH-treated rabbits, but changed only by -13 ± 15% and +3 ± 9% in vehicle-treated rabbits. The MLDF response to [Phe^2,Ile^3,Orn^8]-vasopressin was not significantly altered by MS-PPOH (Figure 4.2). Thus, responses of MLDF to [Phe^2,Ile^3,Orn^8]-vasopressin were significantly greater than those of CLDF in vehicle-treated rabbits, but not in MS-PPOH-treated rabbits. DDMS had no significant effect on RBF, CLDF or MLDF responses to [Phe^2,Ile^3,Orn^8]-vasopressin (Figure 4.2).

In vehicle-treated rabbits, angiotensin II dose-dependently reduced RBF and CLDF but it had no significant effect on MLDF (Figure 4.2) or MAP or HR (data not shown). At the highest dose studied (20 ng kg^{-1}min^{-1}) RBF was reduced by -45 ± 10% and CLDF was reduced by -41 ± 14% but MLDF was unaffected (+1 ± 10%). Neither MS-PPOH- nor DDMS-treatment significantly affected the responses of RBF and CLDF to angiotensin II. In contrast, DDMS-treatment significantly enhanced reductions in MLDF after angiotensin II so that at a dose of 20 ng kg^{-1}min^{-1} MLDF was reduced by -24 ± 14%. MLDF responses to angiotensin II also tended to be enhanced after MS-PPOH, but this was not statistically significant (Figure 4.2).

4.4.3 *CYP450 ω-hydroxylase and epoxygenase activity*

Because the renal metabolism of AA is highly influenced by genetics and environmental conditions, we first characterized the renal metabolism of AA in microsomes prepared from the renal cortex and medulla of the strain of rabbits used in this study. These

results are presented in Figure 4.3. Basal production of 20-HETE, EETs and DiHETEs averaged 120 ± 16, 18 ± 3 and 43 ± 9 pmole $min^{-1}mg\ protein^{-1}$ respectively, in the renal cortex. The production of 20-HETE was 59 ± 9% less in microsomes prepared from the medulla of these rabbits, whereas production of EETs and DiHETEs was not significantly different from that in the cortex.

The effects of *in vivo* infusion of MS-PPOH and DDMS, on the metabolism of AA by renal cortical homogenates, are presented in Figure 4.4. Production of EETs and DiHETEs was 38% and 65% less respectively, and total epoxygenase activity was 49% less, in cortical homogenates prepared from the kidneys of rabbits treated with MS-PPOH than in those from vehicle-treated rabbits. In contrast, ω-hydroxylase activity was similar in homogenates prepared from the kidneys of rabbits treated with vehicle and MS-PPOH. The production of 20-HETE, EETs and DiHETEs in cortical tissue homogenates from DDMS-treated rabbits did not differ from that in homogenates from vehicle-treated rabbits (Figure 4.4).

The effects of [Phe^2,Ile^3,Orn^8]-vasopressin on CYP450 activity *in vitro* are shown in Figure 4.5. Addition of [Phe^2, Ile^3,Orn^8]-vasopressin (0.2, 2 and 20 ng/ml) had no significant effects on the formation of EETs, DiHETEs or 20-HETE by cortical homogenates *in vitro*

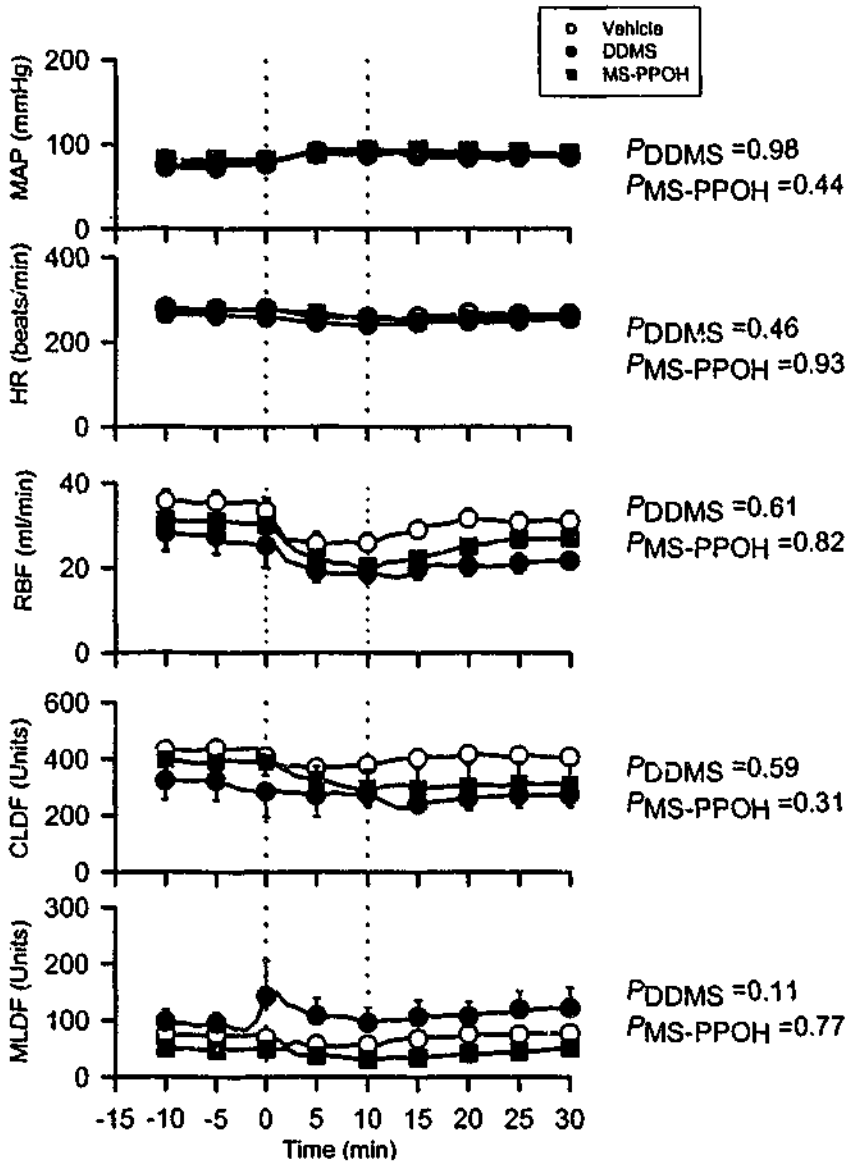

Figure 4.1. Baseline levels of systemic and renal haemodynamic variables before and after commencing renal arterial infusions of N-methylsulfonyl-12,12,dibromododec-11-enamide (DDMS), N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH) and vehicle. Lines indicate 1 min averages of each variable. Symbols (O, n = 6; vehicle, 45% *w/v* hydroxypropyl β cyclodextran); (•, n = 6; DDMS, 2 mg/kg + 1.5 mg $kg^{-1}h^{-1}$); (■, n = 6; MS-PPOH, 2 mg/kg + 1.5 mg $kg^{-1}h^{-1}$) and error bars indicate mean ± SE for every 5th min. Dotted vertical lines show the period over which the initial 'loading dose' was administered. *P* values indicate the results of unpaired t-tests, testing whether responses to DDMS and MS-PPOH differed from the responses to vehicle treatment (df =10). For this purpose, the response was defined as the difference between average levels across the 10 min control period, and levels during the period 25 to 30 min after the infusion commenced. MAP, mean arterial pressure. HR, heart rate. RBF, renal blood flow. CLDF, cortical laser Doppler flux. MLDF, medullary laser Doppler flux.

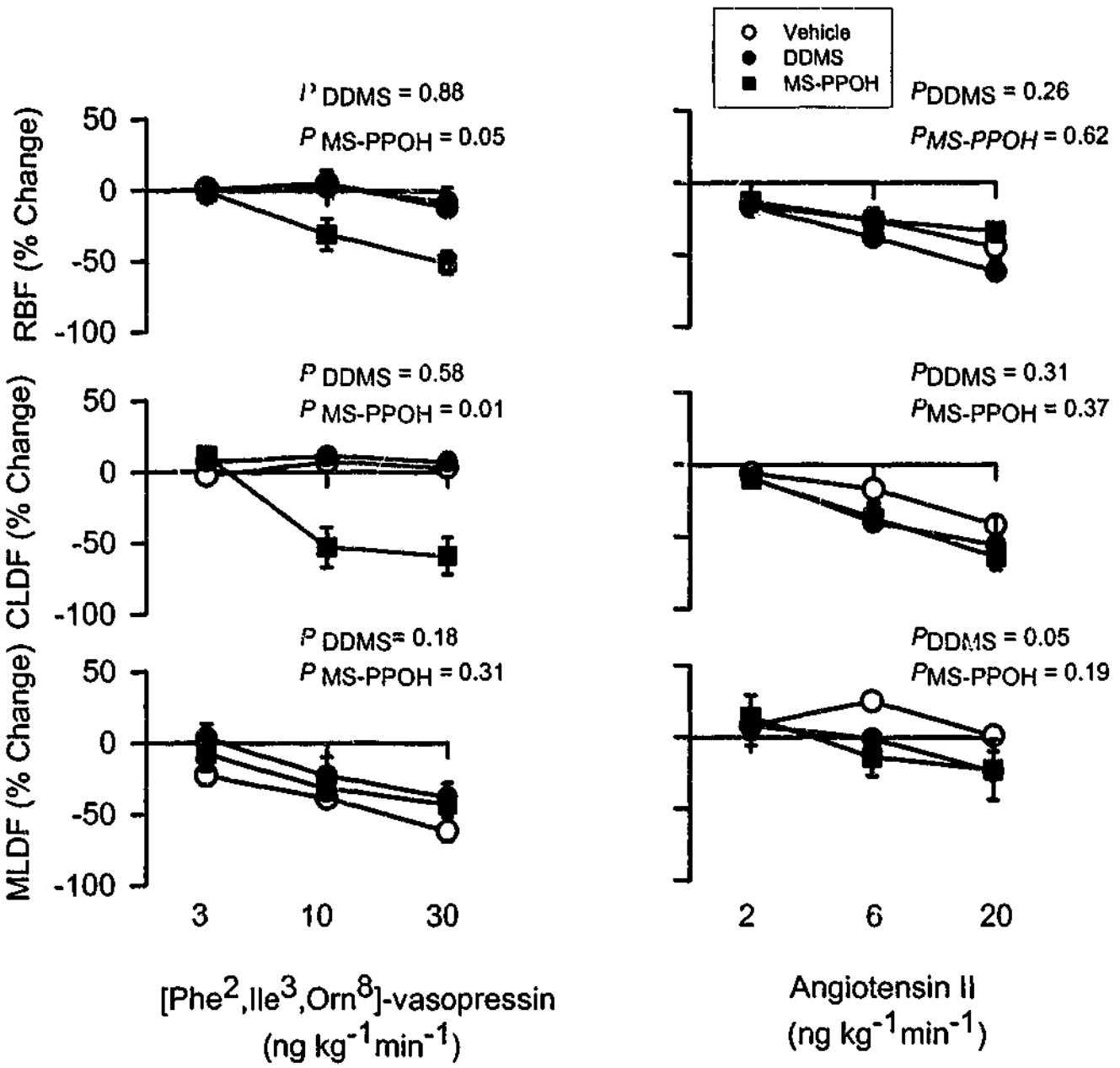

Figure 4.2 Renal haemodynamic responses to renal arterial infusions of (A) [Phe^2,Ile^3,Orn^8]-vasopressin and (B) angiotensin II. Symbols indicate mean ± SE of percentage changes observed in the final 3 min of the infusion compared with the pre-treatment levels. (O) Vehicle pre-treated rabbits; (•) N-methylsulfonyl-12,12,dibromododec-11-enamide (DDMS) pre-treated rabbits (2 mg/kg + 1.5 mg kg^{-1}h^{-1}); and (■) N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH) pre-treated rabbits (2 mg/kg + 1.5 mg kg^{-1}h^{-1}). *P* values indicate the main effect of treatment from repeated measures analysis of variance (df = 1, 9-10). These *P* values test whether responses to the vasoconstrictor agents were affected by DDMS or MS-PPOH treatments

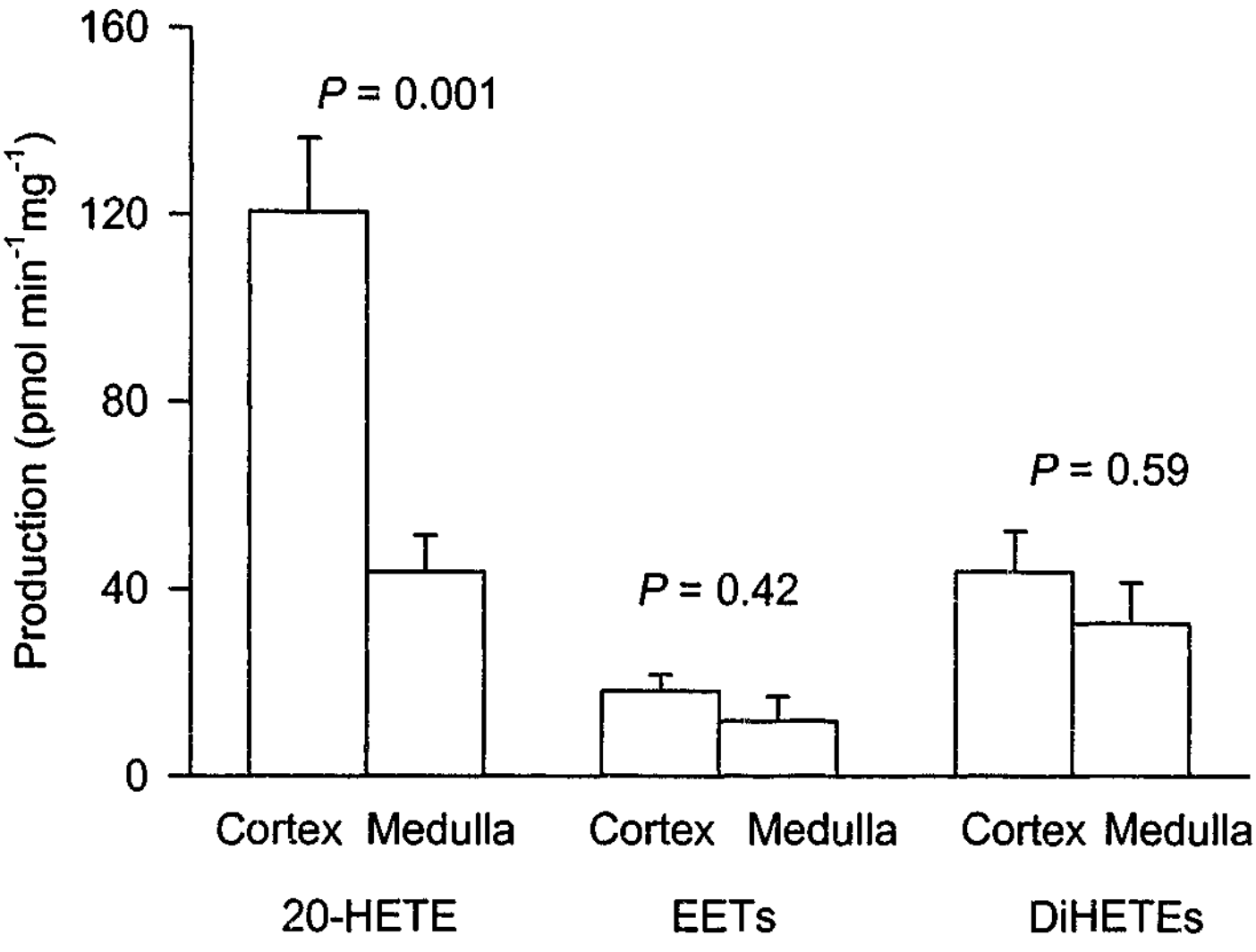

Figure 4.3 The rate of production of 20-hydroxyeicosatetraenoic acid (20-HETE), epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DiHETEs) in rabbit renal cortical and medullary microsomes. Microsomal preparations were incubated with a mixture of [^{14}C]-arachidonic acid (0.1 μCi) and NADPH (1 mM) for 30 min at 37 °C. Arachidonic acid metabolites were extracted with ethyl acetate and water. High performance liquid chromatography was used to separate the arachidonic acid metabolites as described in Methods. Columns and error bars represent the mean ± SE of observations from 6 kidneys from separate rabbits. *P* values represent the outcomes of Student's paired t-tests, contrasting production of the various metabolites in cortical and medullary microsomes.

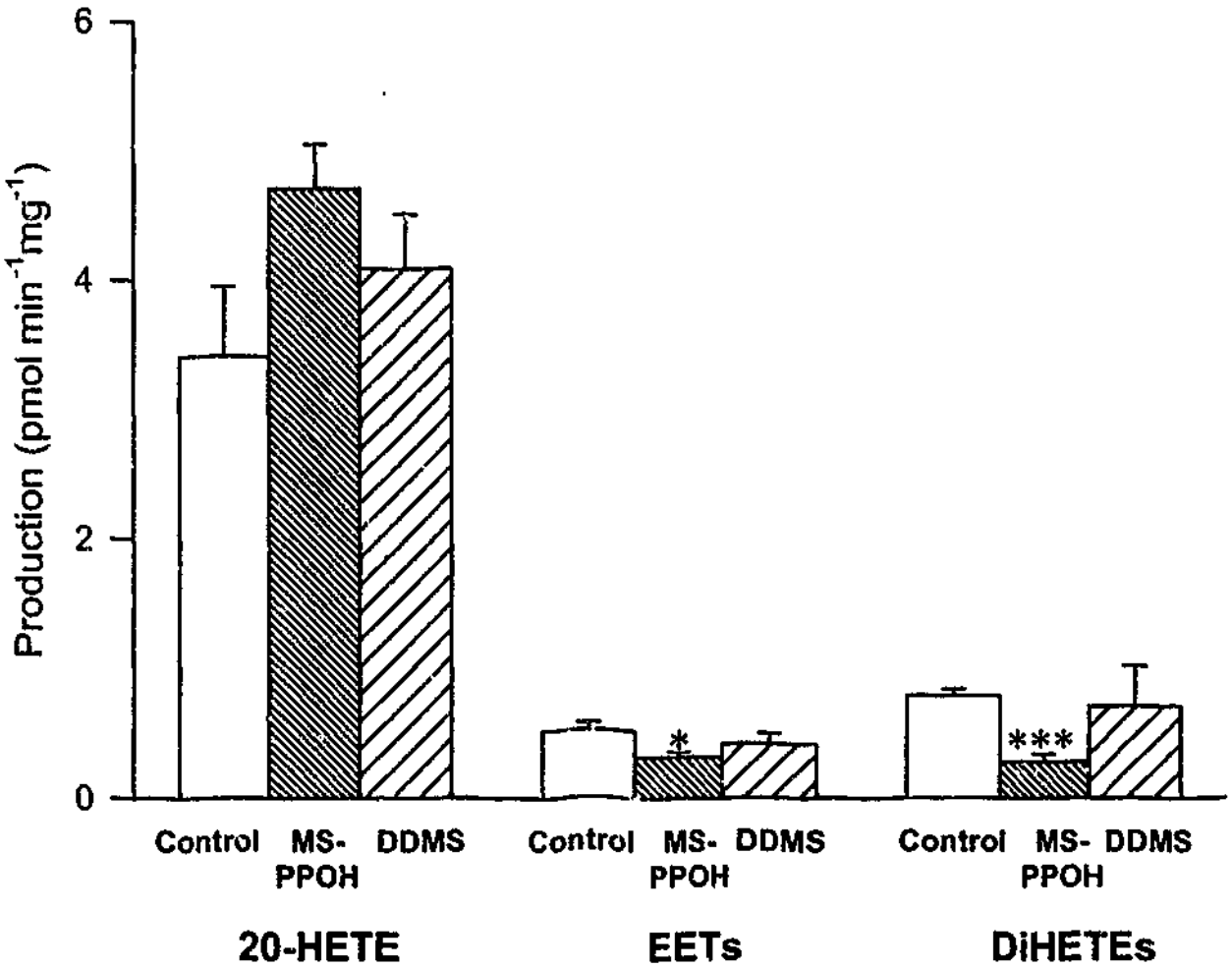

Figure 4.4. Effect of N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH) and N-methylsulfonyl-12-12,dibromododec-11-enamide (DDMS) on cytochrome P450-dependent metabolism of arachidonic acid. Homogenates were prepared (as described in Methods) using renal cortical tissues of vehicle-, MS-PPOH- (2 mg/kg plus 1.5 mg $kg^{-1}h^{-1}$) and DDMS- (2 mg/kg plus 1.5 mg $kg^{-1}h^{-1}$) treated rabbits. Columns and error bars represent the mean ± SE of observations from 6 rabbits in each group. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ indicate the outcomes of Dunnetts tests, to determine whether *in vivo* treatment with MS-PPOH altered the rate of production of 20-hydroxyeicosatetraenoic acids (20-HETE), epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DiHETEs) *in vitro*.

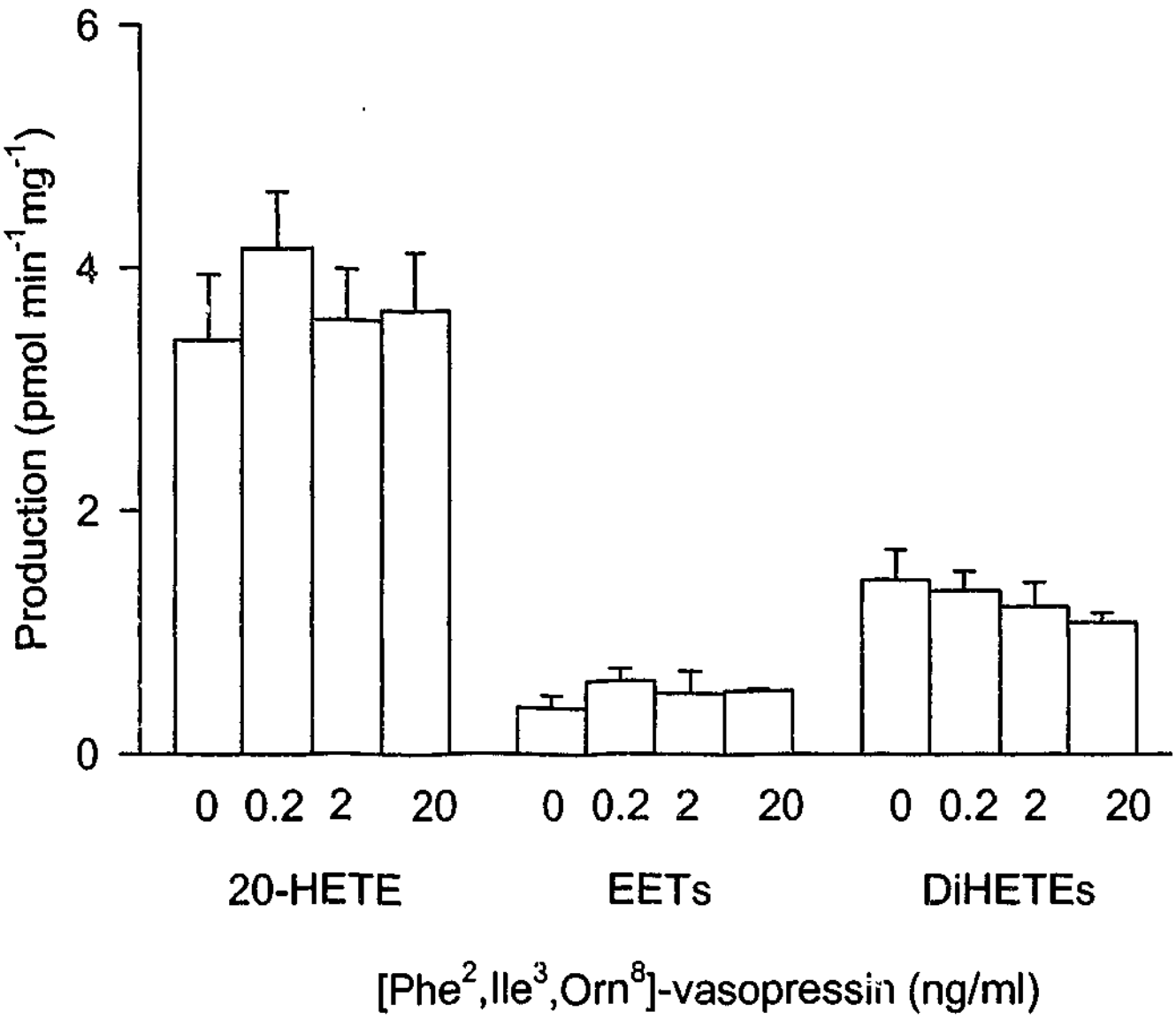

Figure 4.5. The effects of [Phe^2,Ile^3,Orn^8]-vasopressin (0, 0.2, 2 and 20 ng/ml) on cytochrome P450 dependent metabolism of arachidonic acid in microsomes prepared from rabbit renal cortex. Homogenates were prepared as described in Methods. These samples were incubated with a mixture of ^{14}C-arachidonic acid (0.1 μCi), NADPH (1 mM) and [Phe^2,Ile^3,Orn^8]-vasopressin (0, 0.2, 2 and 20 ng/ml). Columns and error bars represent the mean ± SE of observations from 6 kidneys from separate rabbits. Dunnett's tests showed that formation of 20-hydroxyeicosatetraenoic acids (20-HETE), epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DiHETEs) was not significantly altered at any concentration of [Phe^2,Ile^3,Orn^8]-vasopressin.

4.5 Discussion

This study examined whether regional differences in the formation and release of EETs and 20-HETE contributes to differences in the regional kidney blood flow responses to angiotensin II and [Phe^2,Ile^3,Orn^8]-vasopressin in rabbits. The results indicate that inhibition of epoxygenase activity with MS-PPOH markedly enhances the sensitivity of the renal cortical circulation to the vasoconstrictor actions of the V_1-agonist [Phe^2,Ile^3,Orn^8]-vasopressin. In contrast MS-PPOH had no significant effect on the changes in MLDF elicited by the V_1-agonist, nor did it affect the responses to a different vasoconstrictor agonist, angiotensin II. These results suggest that products of CYP450 epoxygenation buffer V_1-receptor-mediated reductions in CBF but not MBF, and so in large part account for the relative insensitivity of the cortical circulation to V_1-mediated vasoconstriction. Our results are consistent with previous observations indicating that neither nitric oxide (206), nor products of cyclooxygenase- (185) or lipoxygenase- (184) dependent AA metabolism make major contributions to the relative insensitivity of the cortical circulation to the vasoconstrictor effects of V_1-receptor activation.

We were unable to demonstrate effective inhibition of 20-HETE or EETs formation in the homogenates prepared from kidneys of DDMS-treated rabbits. Our finding is consistent with a recent report from Roman and colleagues, showing that DDMS is not effective in inhibiting CYP450 dependent ω-hydroxylase metabolites under *in vivo* experimental conditions (102). DDMS binds to plasma proteins and thus has limited diffusing capacity when administered via a blood borne route (212). Therefore, we are unable to draw conclusions regarding the roles of CYP450 dependent ω-hydroxylase metabolites in responses of regional kidney blood flow to angiotensin II and [Phe^2,Ile^3,Orn^8]-vasopressin. Surprisingly, DDMS revealed a small but significant reduction in MLDF in response to angiotensin II even in the face of undetctable inhibition of CYP450 ω-hydroxylase (and epoxygenase) activity. One interpretation of this observation is that DDMS can inhibit the formation of AA metabolites (other than 20-HETE and EETs), which buffer angiotensin II-induced vasoconstriction in the medullary circulation. This hypothesis remains to be definitively tested.

We are not aware of any previous studies, examining the effects of selective inhibition of epoxygenase activity, on renal vasoconstrictor responses to angiotensin II *in vivo*. Our observation, that MS-PPOH did not significantly affect renal haemodynamic responses to angiotensin II, does not allow us to exclude roles for EETs in modulating renal haemodynamic responses to this peptide, because 20-HETE and EETs can be pre-stored in membrane phospholipids, and released in response to hormonal stimuli (212). There is also evidence that angiotensin II stimulates the release of P450 metabolites of AA from rabbit isolated perfused kidneys (37) and that EETs modulate the vasoconstrictor responses of *in vitro* perfused afferent arterioles of rabbits (119) and rats (107) *in vitro*. Nevertheless, the results of the present study indicate that the renal haemodynamic effects of angiotensin II *in vivo* in rabbits are not modulated by the *de novo* synthesis of EETs.

Measurements of CYP450 activity (as assessed by the production of EETs and DiHETEs from AA) in renal cortical homogenates prepared from the rabbits studied *in vivo* indicated that the dose of MS-PPOH was sufficient to selectively reduce CYP450 epoxygenase activity *in vitro*. Indeed, it may be that our *in vitro* assay underestimated the degree of epoxygenase inhibition *in vivo*, since preparation of the homogenates necessitated considerable dilution of the MS-PPOH within the kidney. As expected, MS-PPOH had no effect on the renal production of 20-HETE. These findings are consistent with those of previous *in vivo* (28) and *in vitro* (253) studies of rat kidney indicating that MS-PPOH is a selective epoxygenase inhibitor that is effective at blocking this pathway *in vivo* at doses comparable to those used in the present study.

We also characterized the production of EETs, DiHETEs and 20-HETE in the kidneys of the rabbits used in this study, since previous studies have indicated that there are large differences in renal AA metabolism and CYP450 isoform expression between species, and even between strains within a single species, and that the production of these compounds is dependent on diet and other environmental conditions (33, 162, 184, 261). The results indicate that the rate of formation of EETs and 20-HETE in renal cortical microsomes of the rabbits used in the present study was similar to that reported in the rat when studied under similar conditions (7) and much higher than that seen in

mice (240). Our present data also indicate that CYP450 ω-hydroxylase activity is 2-4 times greater in the renal cortex than outer medulla of the rabbit, similar to what has generally been reported in studies of rats (7, 240, 271).

Although our experiment provides no information regarding the precise identity of the AA epoxygenase products that appear to blunt V_1-receptor mediated cortical vasoconstriction, 11,12-EET, 8,9-EET and 5,6-EET must be prime candidates, since these (or their derivatives) have been demonstrated to be potent vasodilators of the renal circulation in rats and rabbits under *in vitro* conditions (35, 203, 266). In contrast, other studies have shown these EETs to produce vasoconstriction when infused directly into the renal artery (182, 240), and have also provided evidence that endogenous EETs (or their metabolites) at least partly mediate renal vasoconstrictor responses to nitric oxide synthase inhibition in rats (182). Most evidence suggests that the vascular actions of EETs depend heavily on the relative activity of downstream metabolic pathways (eg cyclooxygenase), which seem capable of converting EETs both into vasoconstrictor (240) and vasodilator (35) products. Thus, the apparent discrepancy between studies identifying vasoconstrictor (182, 240) and vasodilator (present study) roles of EETs within the kidney *in vivo* might reflect differential activation of these downstream metabolic pathways under different experimental conditions.

Our results also do not allow us to determine whether V_1-receptor activation stimulates release of EETs within the cortex, or whether basal EET production is sufficient to blunt V_1-mediated cortical vasoconstriction. However, the latter scenario seems unlikely, given our observation of similar CYP450 epoxygenase activity in cortical compared with medullary microsomes. The hypothesis that V_1-receptor activation increases *de novo* synthesis of EETs is further supported by the observation that vasopressin increases release of CYP450 metabolites in the isolated perfused rat kidney, and that this effect is blunted by treatment with CYP450 inhibitors (187, 250).

We also found that [Phe^2,Ile^3,Orn^8]-vasopressin had little or no effect on CYP450 epoxygenase or ω-hydroxylase activity *per se*. Thus, our results are consistent with the hypothesis that V_1-receptor activation increases EET production in the cortex by

increasing substrate availability. This hypothesis remains to be tested, but is at least consistent with the established effect of vasopressin to stimulate phospolipase A_2 activity in vascular smooth muscle (31, 111). Regardless of the precise mechanism involved, our observations implicate CYP450-dependent EET/DiHETE formation as an important counter-regulatory vasodilator mechanism, buffering renal vasoconstrictor responses to V_1-receptor activation in the cortex, but not in vascular elements controlling MBF.

MS-PPOH had little effect on resting systemic and renal haemodynamics. Similarly, our research group previously found in anaesthetized rabbits that renal arterial infusion of the non-selective CYP450 inhibitor 17-octadecynoic acid, had no significant effects on RBF (73), and others have shown little effect of miconazole, a selective epoxygenase inhibitor, on RBF (192, 193). Thus, these data do not support a role for EETs in control of basal renal vascular tone.

4.6 Conclusions

The present study confirms previous findings (13, 37, 49, 66, 76, 87, 184, 185, 197, 198, 206, 252, 272) that the medullary microcirculation is refractory to angiotensin II-induced vasoconstriction and that the cortical circulation is refractory to V_1-receptor-mediated vasoconstriction. This differential effect of vasoactive hormones on regional kidney perfusion likely represents an important regulatory mechanism in long-term blood pressure control, but we are only just beginning to understand the mechanisms underlying it. Our present results indicate that EETs do not contribute to the relative insensitivity of the medullary circulation to angiotensin II, which has been previously attributed to the actions of nitric oxide and prostaglandins (13, 49, 185, 197, 198, 206, 252, 272). However, the relative insensitivity of the renal cortical circulation to V_1-mediated vasoconstriction appears to be largely attributable to the actions of epoxygenase products of AA.

Chapter Five

Prostaglandins and nitric oxide in regional kidney blood flow responses to renal nerve stimulation

A modified version of this chapter has been accepted for publication in Pflugers Archive: European Journal of Physiology

5.1 Summary

Electrical renal nerve stimulation reduces total renal perfusion (RBF) and cortical perfusion (CBF) more than medullary perfusion (MBF) under control conditions and even after nitric oxide synthase inhibition. Therefore, we examined the roles of cyclooxygenase (COX) products, and of interactions between these and nitric oxide synthase, in regional kidney perfusion responses to renal nerve stimulation, by testing responses to renal nerve stimulation in anaesthetized rabbits before and after sequential treatment with ibuprofen and N^G-nitro-L-arginine (L-NNA). Under control conditions, renal nerve stimulation (at 6 Hz) reduced RBF (80 ± 4%), CBF (82 ± 5%) and to a lesser extent MBF (37 ± 8 %). Ibuprofen did not significantly affect these responses, suggesting that COX products have little net role in modulating renal vascular responses to renal nerve stimulation. Subsequent administration of L-NNA after ibuprofen pre-treatment enhanced renal nerve stimulation-induced reductions in RBF ($P \leq 0.001$) and CBF ($P = 0.02$) but not MBF ($P = 0.7$). This contrasts with our previous finding under conditions of intact COX activity, where L-NNA greatly enhanced responses of MBF to renal nerve stimulation (69). Thus, while nitric oxide blunts responses of CBF to renal nerve stimulation independently of COX, its impact on responses of MBF may depend on an interaction with COX, perhaps through inhibiting production of vasoconstrictor COX products.

5.2 Introduction

There is compelling evidence that renal medullary blood flow (MBF) profoundly influences tubular sodium and water handling, and hence the long-term set-point of arterial pressure (47, 150). In rats and rabbits, medullary laser Doppler flux (MLDF, an index of blood flow) is less responsive to both electrical (69, 93-95, 126, 220) and reflex mediated (120, 123, 127) activation of the renal nerves, than is total renal blood flow (RBF) or cortical laser Doppler flux (CLDF, an index of blood flow), even though vascular elements that regulate medullary perfusion are richly innervated (17, 60, 163). The differential regulation of cortical blood flow (CBF) and MBF by sympathetic activiy could be an important regulatory mechanism in long-term blood pressure regulation, yet the mechanisms underlying it are poorly understood.

One plausible hypothesis is that vasodilator substances, released from the vascular endothelium and tubular epithelium, blunt the impact of renal sympathetic nerve activity on the medullary microcirculation. This notion is supported indirectly by observations that nitric oxide synthase blockade can augment reductions in MLDF in response to exogenous noradrenaline (206, 265), and enhance global renal vasoconstrictor responses to sympathetic nerve stimulation (148). Recently, our group provided direct support for this hypothesis, by showing in anaesthetized rabbits that nitric oxide synthase blockade can enhance renal nerve stimulation-induced reductions in MLDF (69). However, even after nitric oxide synthase blockade, renal nerve stimulation still reduced CLDF more than MLDF, suggesting that nitrergic mechanisms are not solely responsible for the differential sensitivity of CBF and MBF to renal nerve stimulation. One possibility is that vasodilator prostaglandins also act to blunt the impact of renal nerve stimulation on MBF. In support of this, Inokuchi and Malik have shown that inhibition of prostaglandin formation enhanced, while administration of PGI_2 or PGE_2 blunted, the renal vasoconstrictor response to renal nerve stimulation in anaesthetized rats (109).

There is also considerable evidence of interactions between nitric oxide and prostaglandins in the kidney (19, 20, 132, 222, 264). Depending on the experimental

conditions, nitric oxide has been shown to activate cyclooxygenase (COX) (223, 262), and also to inhibit its expression and/or activity (121, 191, 238) *in vitro*. A number of studies have provided data consistent with the idea that nitric oxide inhibits production of COX products within the kidney (19, 20, 262, 264), although the functional consequences of this effect seems to depend on the balance between production of vasodilator (eg prostaglandin I_2 and E_2) and vasoconstrictor (eg prostaglandin H_2 and thromboxane A_2) products under the specific experimental conditions. For example, COX inhibition (262), and thromboxane A_2/prostaglandin H_2-receptor antagonsim (264), attenuates nitric oxide synthase inhibition-induced potentiation of noradrenaline-induced vasoconstriction in the rat isolated perfused kidney, probably because the effects of nitric oxide synthase blockade under these experimental conditions are mediated through stimulation of release of vasoconstrictor COX products. In contrast, acute nitric oxide synthase blockade reveals renal vasoconstrictor responses to non-specific COX blockade (2) or selective COX-2 blockade (20) in the rat kidney *in vivo*, probably because nitric oxide synthase blockade stimulates release of vasodilator prostaglandins under these experimental conditions. Thus, the inability of nitric oxide synthase blockade to enhance renal nerve stimulation-induced reductions in MLDF to the extent that they match reductions in CLDF (69), might simply reflect compensatory up regulation of vasodilator prostanoids after nitric oxide synthase blockade. Alternatively, vasoconstrictor prostanoids in the medulla might even partly mediate the effect of nitric oxide synthase blockade to enhance renal vasoconstrictor responses to renal nerve stimulation.

Therefore, in the present study we examined the roles of prostaglandins, and also the significance of interactions between prostaglandins and nitric oxide, in modulating renal nerve stimulation-induced vasoconstriction in the renal medulla. We tested responses of RBF, CLDF and MLDF to renal nerve stimulation after COX inhibition alone and after simultaneous inhibition of both COX and nitric oxide synthase. Because nitric oxide synthase inhibition reduced basal levels of RBF, CLDF and MLDF and increased basal mean arterial pressure (MAP), we also tested the effects of co-infusion of a nitric oxide donor to restore baseline haemodynamic variables to their level before nitric oxide synthase inhibition.

5.3 Methods

5.3.1 Animals

Twenty four male New Zealand White rabbits (mean weight 2.63 ± 0.01 kg) were used in this study.

5.3.2 Surgical preparations

These procedures have been outlined in Chapter 2. Briefly, rabbits were anaesthetized with pentobarbitone sodium (90-150 mg plus 30-50 mg/h) and were artificially ventilated. The rabbits received an intravenous infusion of Hartmann's solution (compound sodium lactate) at a rate of 0.18 ml $kg^{-1}min^{-1}$ during surgery to replace fluid losses. Esophageal temperature was maintained between 36-38 °C throughout the experiment. The left kidney was exposed via a left flank incision and was placed in a stable cup. The renal nerves running parallel to the left renal artery were then carefully isolated and placed across a pair of stimulating electrodes. The nerves were then sectioned proximally. A transit-time ultrasound flow probe (type 2SB) was placed around the renal artery to measure RBF. A needle-type laser Doppler flow probe (26 gauge) was inserted approximately 9 mm into the kidney with the use of a micromanipulator for measurement of MLDF and a standard plastic laser Doppler probe (DP2b) was placed on the surface of the kidney to measure CLDF. At the completion of all surgical procedures the i.v. infusion of Hartmann's solution was replaced with a solution containing four parts Hartmann's solution and 1 part 10% vol/vol polygeline (Haemaccel). A 30-60 min equilibration period was allowed before the experimental protocol commenced.

5.3.3 Recording of haemodynamic variables

MAP (mmHg), heart rate (HR, beats/min), RBF (ml/min), CLDF (perfusion units), and MLDF (perfusion units) were measured throughout the experiment as described in Chapter 2. Background levels of CLDF (6 ± 2 units) and MLDF (15 ± 3 units), recorded after the rabbit was humanely killed by overdose with pentobarbitone (300 mg), and artificial ventilation was ceased, were subtracted from experimental values before data analysis.

5.3.4 Experimental protocol

This study was designed in a manner that allowed us to compare the effects of different treatment regimens on intrarenal blood flow responses to renal nerve stimulation, with the respective control responses in the same rabbit. This within-animal design allowed us to eliminate the confounding effects of variations in the responses of individual rabbits to renal nerve stimulation.

Four groups of rabbits were studied (n = 6 per group). All groups were subjected to three renal nerve stimulation sequences (see below). The first period of renal nerve stimulation served as the control response for each group. Thus, the first group of rabbits served as the 'time' and 'vehicle' control. This group of rabbits received a vehicle-treatment (154 mM NaCl: 1 ml/kg + 1 ml $kg^{-1}h^{-1}$; i.v. until the end of the experiment [vehicle for ibuprofen]) after the first stimulation sequence. Twenty minutes after commencing the vehicle infusion, responses to renal nerve stimulation were re-tested. After this stimulation sequence, and a further 5 min equilibration period, rabbits in this group received another vehicle-treatment (154 mM NaCl; 4 ml/kg + 1 ml $kg^{-1}h^{-1}$; i.v. until the end of the experiment [the vehicle for N^G-nitro-L-arginine (L-NNA)]). Eight minutes after commencing the vehicle infusion these rabbits received an additional vehicle infusion (154 mM NaCl, 50 μg $kg^{-1}min^{-1}$; i.v. until the end of the experiment [this was the vehicle for glyceryl trinitrate (GTN)]). Responses to renal nerve stimulation were re-tested 25 min after starting this vehicle infusion. Rabbits in groups 2, 3 and 4 were subjected to similar procedures but, as described below, they received the relevant treatment regimens. Thus, the second group of rabbits received the COX inhibitor, ibuprofen (12.5 mg/kg + 12.5 mg $kg^{-1}h^{-1}$; Sigma Chemical Company, St Lois, MO, USA) after the first stimulation sequence and vehicle infusions for L-NNA (154 mM NaCl; 4 ml/kg + 1 ml $kg^{-1}h^{-1}$) and GTN (154 mM NaCl, 50 μl $kg^{-1}min^{-1}$) after the second stimulation sequence. This experimental sequence allowed us to determine the roles of prostaglandins in modulating responses of intrarenal blood flow to renal nerve stimulation. The third group of rabbits received ibuprofen (12.5 mg/kg + 12.5 mg $kg^{-1}h^{-1}$) after the first stimulation period and the nitric oxide synthase inhibitor, L-NNA (20 mg/kg + 5 mg $kg^{-1}h^{-1}$; Sigma Chemical Company, St Louis, MO, USA) and the vehicle for GTN (154 mM NaCl, 50 μg $kg^{-1}min^{-1}$) after the second stimulation sequence. This experimental sequence allowed us to determine whether interactions

between nitric oxide and prostaglandins can modulate responses of intrarenal blood flow to renal nerve stimulation. The fourth group of rabbits received ibuprofen (12.5 mg/kg + 12.5 mg $kg^{-1}h^{-1}$) after the first stimulation sequence and both L-NNA (20 mg/kg + 5 mg $kg^{-1}h^{-1}$) and G (8-22 µg $kg^{-1}min^{-1}$; David Bull Laboratories, Mulgrave, Vic., Australia) after the second stimulation sequence. The dose of GTN was titrated to restore MAP to its level before administration of L-NNA. This experimental sequence allowed us to determine whether the effects of L-NNA could be abolished by co-infusion of GTN to restore resting nitrergic vasodilator tone (Figure 5.1).

Group 1	**Stimulation sequence 1**	Vehicle for ibuprofen 20 min	**Stimulation sequence 2**	Vehicle for L-NNA 17 min	Vehicle for GTN 8 min	**Stimulation sequence 3**
Group 2	**Stimulation sequence 1**	**Ibuprofen 20 min**	**Stimulation sequence 2**	Vehicle for L-NNA 17 min	Vehicle for GTN 8 min	**Stimulation sequence 3**
Group 3	**Stimulation sequence 1**	**Ibuprofen 20 min**	**Stimulation sequence 2**	**L-NNA 17 min**	Vehicle for GTN 8 min	**Stimulation sequence 3**
Group 4	**Stimulation sequence 1**	**Ibuprofen 20 min**	**Stimulation sequence 2**	**L-NNA 17 min**	**GTN 8 min**	**Stimulation sequence 3**

Figure 5.1 Schematic representation of the experimental protocol. Rabbits in group 1 received vehicle infusions (154 mM NaCl) after stimulation sequences 1 and 2. The second group of rabbits received ibuprofen (12.5 mg/kg + 12.5 mg $kg^{-1}h^{-1}$) after stimulation sequence 1 and vehicle infusions after stiulation sequence 2. The third group of rabbits received ibuprofen after stimulation sequence 1, and N^G-nitro-L-arginine (L-NNA; 20 mg/kg + 5 mg $kg^{-1}h^{-1}$) plus vehicle infusion for glyceryltrinitrate (GTN) after stimulation sequence 2. The fourth group of rabbits received ibuprofen after stimulation sequence 1 and L-NNA plus GTN after stimulation sequence 2. Once commenced, all infusions were continued until the end of the experiement.

5.3.5 *Electrical nerve stimulation*

Renal nerve stimulation was produced using purpose-written software in the LabVIEW graphical programming language. A supramaximal voltage (3-10 V) was applied in each experiment. This was pre-determined by stimulating the renal nerves with various voltages at 5 Hz for 60 s. The voltage that produced the maximum reduction in RBF was used to stimulate the renal nerves in all subsequent stimulation sequences. A pulse width of 2 ms was used and four different frequencies (0.75, 1.5, 3 & 6 Hz) were applied in random order. Each frequency was applied for 3 min with an 8 min recovery period, except after 6 Hz, where a 10 min recovery period was allowed.

5.3.6 *Statistics*

All data are expressed as mean ± SE. $P \leq 0.05$ was considered statistically significant. Baseline levels of systemic and renal haemodynamic variables were calculated by averaging the 30 s control periods, immediately prior to each stimulus train, across all four frequencies in each renal nerve stimulation sequence. Analysis of variance (ANOVA) was used, to test (i) whether mean baseline levels observed during stimulation sequences 2 and 3 were different from those observed during stimulation sequence 1 in the control group of rabbits (n = 6; P_{Time}), (ii) whether average baseline levels across all 24 rabbits differed according to the treatment that was to follow (P_{Group}), and (iii) whether each of the treatments (ibuprofen, L-NNA and L-NNA + GTN) affected baseline haemodynamic variables (P_{Treat}).

Systemic and renal haemodynamic responses to renal nerve stimulation were determined by comparing the levels of each variable during the last 30 s of each stimulation period with the control values during the 30 s immediately prior to stimulation. These data are expressed as percentage changes from control. We used ANOVA (factors comprising group, rabbit, frequency and treatment) to test (i) whether renal nerve stimulation had frequency-dependent effects on systemic and renal haemodynamics under control conditions ($P_{Frequency}$), (ii) whether the various treatments (vehicle, ibuprofen, ibuprofen + L-NNA or ibuprofen + L-NNA + GTN) affected responses to renal nerve stimulation within each group (P_{Treat}) and (iii) whether responses to renal nerve stimulation of RBF and CLDF differed from those of MLDF (P_{Region}). Thus, our key biological hypotheses, that the various treatments affected

baseline haemodynamics, and responses to renal nerve stimulation, were tested in a 'within' animal fashion.

5.4 Results

5.4.1 Baseline haemodynamics

Responses to vehicle alone and ibuprofen Baseline levels of MAP, HR, RBF, CLDF and MLDF during the initial control stimulation sequence, when averaged across all 24 rabbits, were 77 ± 2 mmHg, 263 ± 5 beats/min, 30 ± 2 ml/min, 322 ± 12 units and 102 ± 9 units respectively. These did not differ significantly according to the treatment that was to follow ($P_{Group} \geq 0.39$). In the group of rabbits that received only the vehicle treatments (group 1), these variables remained stable across the course of the experiment except for CLDF ($P_{Time} = 0.01$) which was 11± 3 % and 17 ± 3% less respectively, during stimulation sequences 2 and 3 than during stimulation sequence 1 (Table 5.1A). Baseline levels of RBF followed a similar pattern, but this trend was not statistically significant ($P_{Time} = 0.06$). Ibuprofen reduced baseline levels of RBF (-11 ± 4%), CLDF (-7 ± 3%) and MLDF (-23 ± 9%) but did not significantly affect MAP or HR (Table 5.1B).

Responses to NOS blockade in ibuprofen pre-treated rabbits In ibuprofen pre-treated rabbits, haemodynamic variables were not significantly affected by vehicle-treatment, with the exception of a small reduction in HR (-15 ± 5%) (Table 5.1C). L-NNA alone significantly increased MAP (+ 43 ± 7%), and reduced HR (-10 ± 4%), RBF (-28 ± 3%) and MLDF (- 52 ± 8 %) but not CLDF (Table 5.1D). Baseline levels of all measured variables after L-NNA + GTN were similar to those observed before this treatment was administered (Table 5.1E).

5.4.2 Responses to renal nerve stimulation

Responses under control conditions Analysis of responses to renal nerve stimulation during the initial (control) stimulation sequence in all 24 rabbits demonstrated frequency-dependent reductions in RBF (-80 ± 4% at 6 Hz; $P_{Frequency} < 0.001$) and CLDF (-82 ± 5% at 6 Hz; $P_{Frequency} < 0.001$). Renal nerve stimulation tended to reduce

MLDF (-37 ± 8% at 6 Hz; $P_{Frequency} = 0.07$) but across the full frequency range this did not reach statistical significance, probably because of the lack of response to frequencies ≤ 1.5 Hz. Thus, as we have shown previously (127) renal nerve stimulation causes greater reductions in RBF and CLDF than MLDF ($P_{Region} < 0.001$). There was also a slight increase in MAP (5 ± 1% at 6 Hz) but no change in HR during renal nerve stimulation (Figure 5.2).

Effects of vehicle and ibuprofen: In vehicle-treated rabbits (group 1) responses of RBF, CLDF and MLDF to renal nerve stimulation remained stable over the course of the experiment ($P_{Treat} \geq 0.41$) (Figure 5.3). Ibuprofen (groups 2, 3 and 4) tended to slightly enhance renal nerve stimulation-induced reductions in CLDF ($P_{Treat} = 0.06$) but had little or no effect on the responses of RBF or MLDF (Figure 5.4).

Effects of L-NNA and L-NNA + GTN in ibuprofen pre-treated rabbits In ibuprofen pre-treated rabbits, responses of RBF, CLDF and MLDF to renal nerve stimulation were not significantly altered by administration of the vehicles for L-NNA + GTN ($P_{Treat} \geq 0.21$; Figure 5.5A). In contrast, L-NNA augmented renal nerve stimulation induced reductions in RBF ($P_{Treat} \leq 0.001$) and CLDF ($P_{Treat} = 0.02$) to renal nerve stimulation. For example, 6 Hz stimulation reduced RBF and CLDF by -82 ± 6% and -80 ± 10% respectively after L-NNA, but only by -70 ± 9 and -69 ± 10 % respectively before L-NNA (Figure 5.5B). Responses of MLDF to renal nerve stimulation were not significantly affected by L-NNA. Responses of RBF, CLDF and MLDF after L-NNA + GTN were indistinguishable from those before this treatment (Figure 5.5C). Regardless of the treatment administered (ibuprofen, ibuprofen + L-NNA or ibuprofen + L-NNA + GTN), renal nerve stimulation always reduced RBF and CLDF more than MLDF ($P_{Region}<0.001$).

Table 5.1 Mean baseline levels of systemic and renal haemodynamic variables

	MAP (mmHg)	HR (beats/min)	RBF (ml/min)	CLDF (units)	MLDF (units)
A: Effects of vehicle treatments (Group 1, n = 6)					
Control	80 ± 3	266 ± 8	30 ± 4	336 ± 24	77 ± 19
Vehicle 1	79 ± 3	265 ± 8	26 ± 3	298 ± 17*	83 ± 22
Vehicle 2	76 ± 3	258 ± 9	25 ± 3	277 ± 21*	68 ± 14
B: Effects of Ibuprofen (Groups 2-4, n = 18)					
Control	77 ± 2	262 ± 6	27 ± 2	300 ± 10	92 ± 13
Ibuprofen	76 ± 3	253 ± 6	23 ± 2*	278 ± 12*	71 ± 13*
C: Effects of vehicle in ibuprofen pre-treated rabbits (Group 2, n = 6)					
Control	81 ± 6	264 ± 7	27 ± 4	305 ± 23	73 ± 20
Vehicle	78 ± 5	249 ± 8*	26 ± 4	292 ± 24	68 ± 13
D: Effects of N^G-nitro-L-arginine in ibuprofen pre-treated rabbits (Group 3, n = 6)					
Control	74 ± 4	244 ± 13	21 ± 2	272 ± 14	96 ± 26
L-NNA	104 ± 2***	220 ± 12*	15 ± 2*	259 ± 19	38 ± 6*

Table 5.1 (Continued)

	MAP (mmHg)	HR (beats/min)	RBF (ml/min)	CLDF (units)	MLDF (units)
E: Effects of N^G-nitro-L-arginine plus glyceryl trinitrate after ibuprofen pre-treatment (Group 4, n = 6)					
Control	74 ± 2	251 ± 9	19 ± 2	257 ± 22	78 ± 8
L-NNA + GTN	72 ± 4	249 ± 10	17 ± 3	233 ± 26	64 ± 24

Data indicate mean ± SE. MAP, mean arterial pressure; HR, heart rate; RBF, renal blood flow; CLDF, cortical laser Doppler flux; MLDF, medullary laser Doppler flux. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for comparison with respective control levels, derived from specific hypothesis testing within analysis of variance.

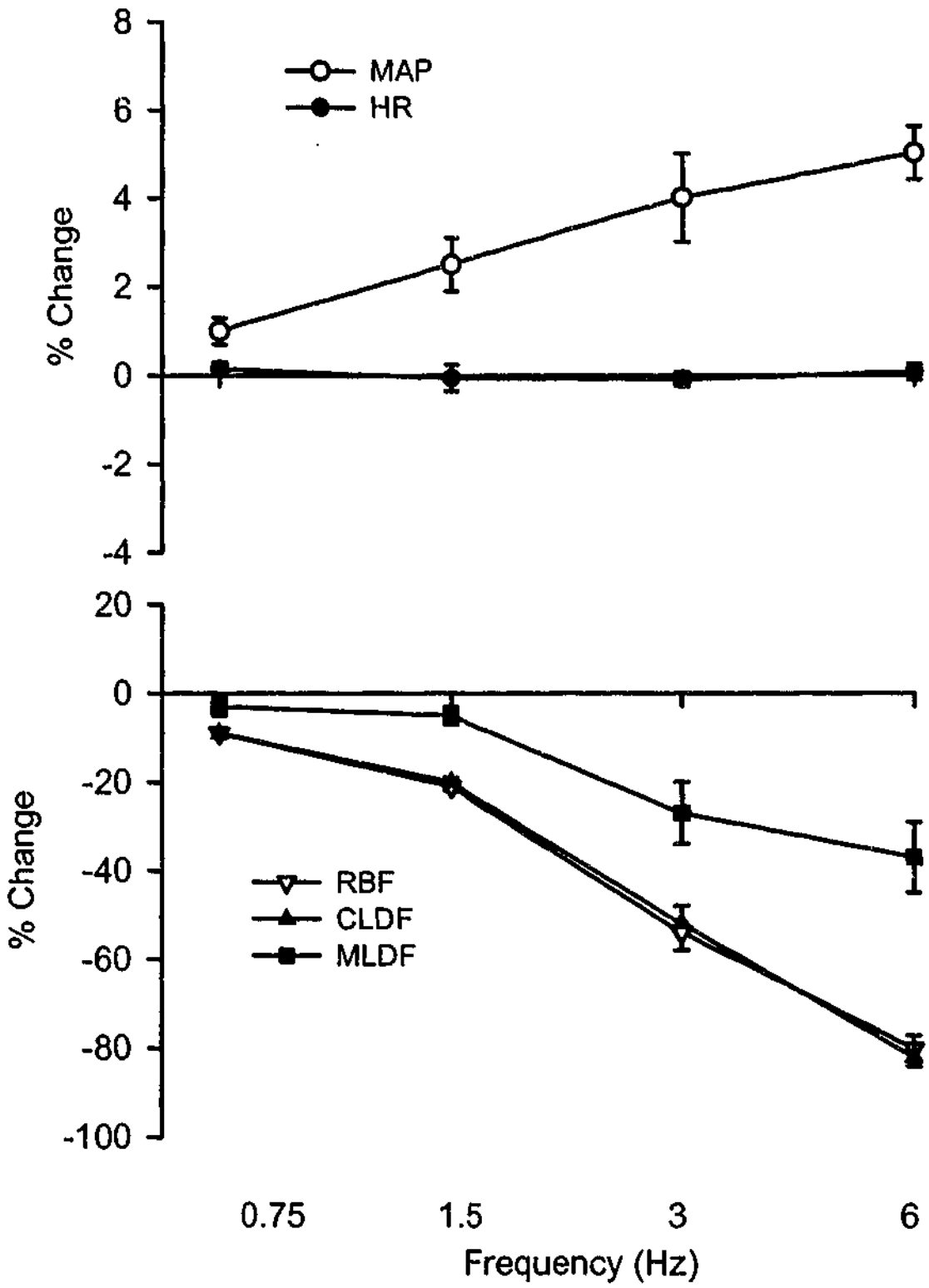

Figure 5.2 Responses of (A) systemic- and (B) renal-haemodynamic variables during the initial sequence of renal nerve stimulation. Symbols and error bars indicate the mean ± SE of the average percentage changes of each variable during the last 30 s of each stimulus train, compared with last 30 s immediately prior to stimulation, in all 24 rabbits. MAP = mean arterial pressure, HR = heart rate, RBF = renal blood flow, CLDF = cortical laser Doppler flux, MLDF = medullary laser Doppler flux.

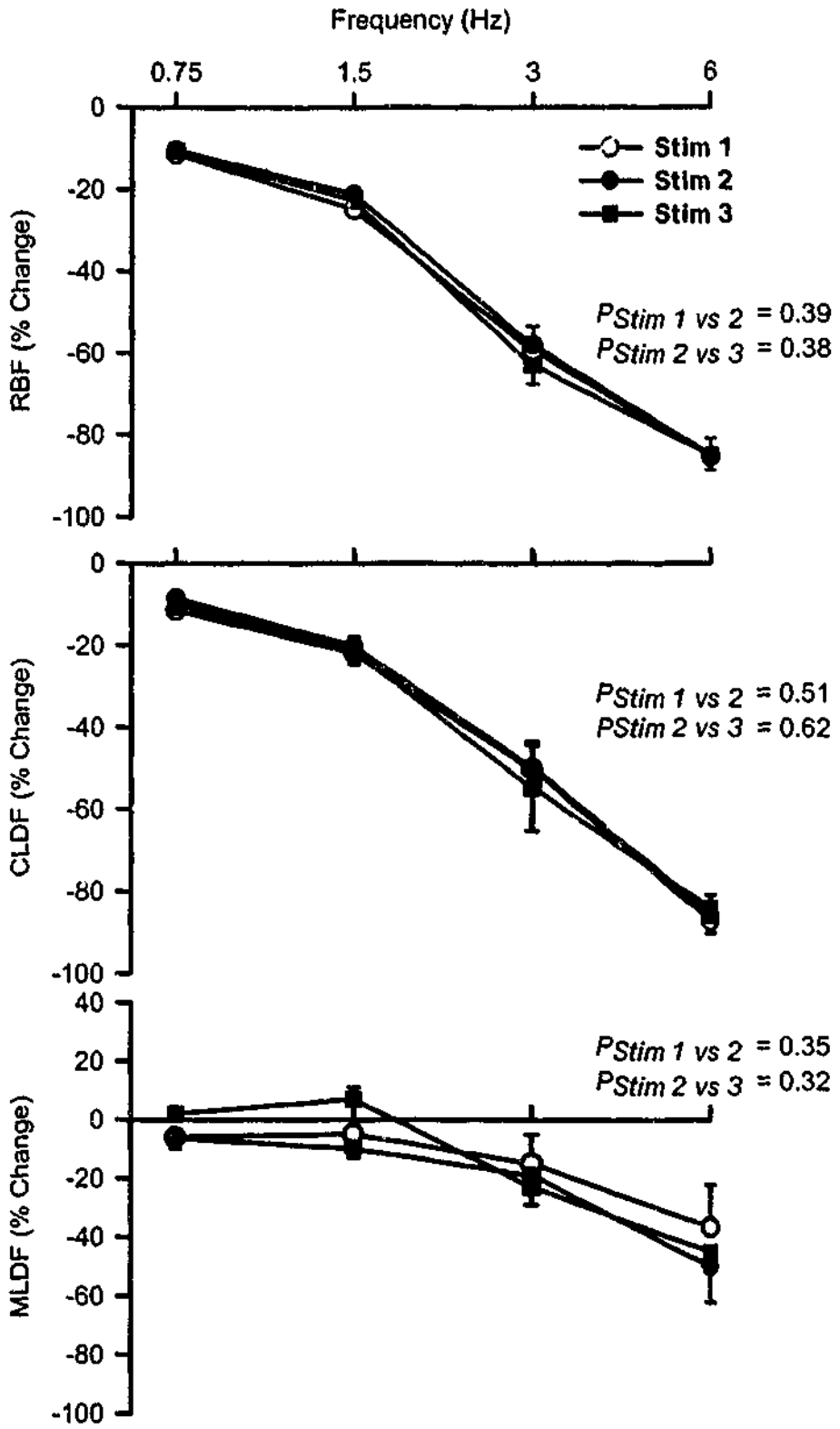

Figure 5.3 Effects of vehicle treatments on the responses of renal haemodynamics to renal nerve stimulation. Symbols and error bars indicate mean ± SE of data from 6 rabbits (Group 1). *P* values indicate the outcomes of partitioned analysis of variance, testing whether renal haemodynamic responses during stimulation sequences 2 (•) and 3 (■) (after the vehicle treatments) differed from those observed during the initial stimulation sequence (o). Stim = stimulation. Other abbreviations as for Figure 5.2.

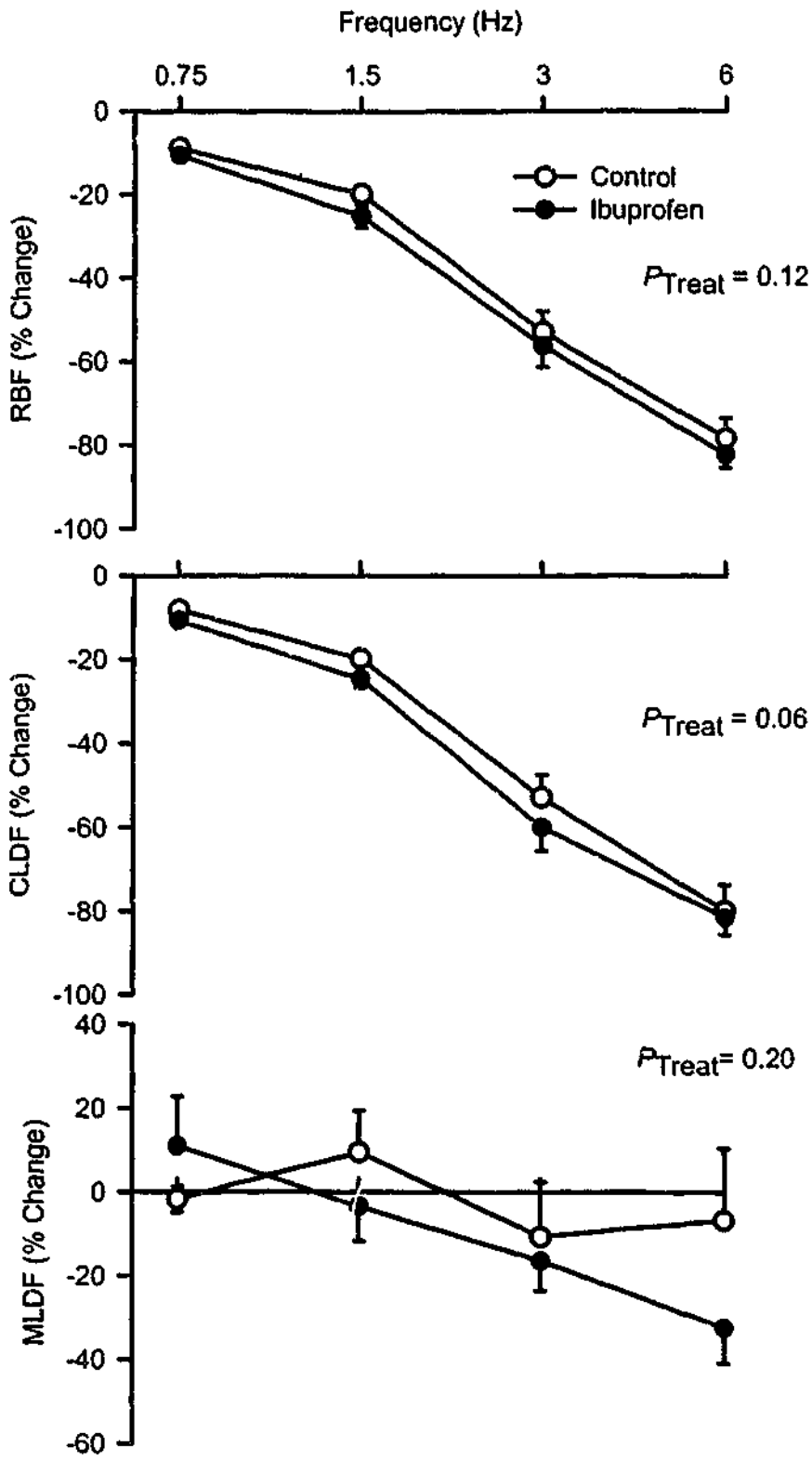

Figure 5.4 Effects of ibuprofen (12.5 mg/ kg + 12.5 mg $kg^{-1}h^{-1}$) treatment on responses of renal haemodynamics to renal nerve stimulation. Symbols and error bars indicate mean ± SE of data from 18 rabbits (Groups 2, 3 and 4). *P* values indicate the outcomes of analysis of variance testing whether renal haemodynamic responses after ibuprofen (stimulation sequences 2; •) differed from those observed before ibuprofen was administered. (stimulation sequence 1; o). Abbreviations are as for Figure 5.3.

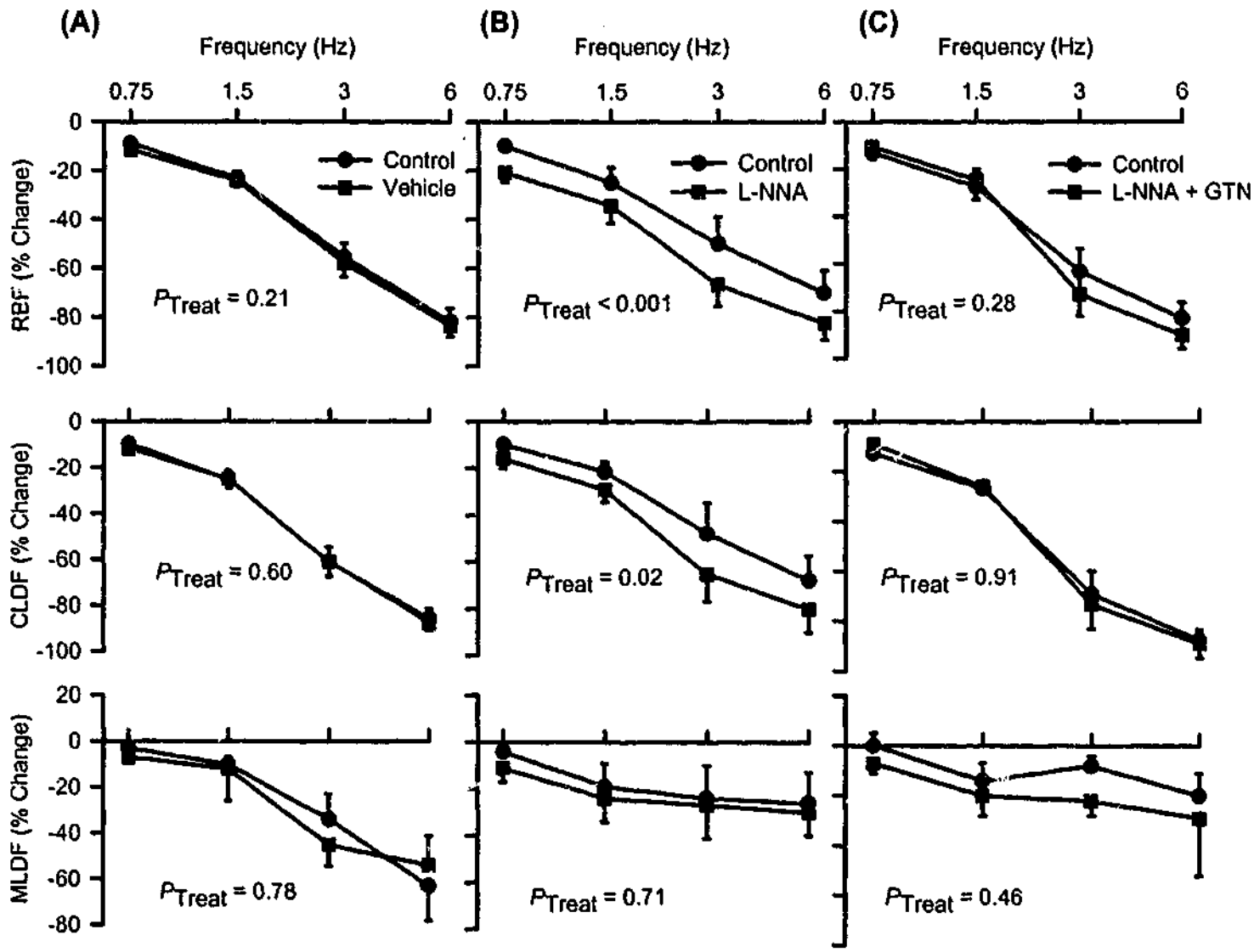

Figure 5.5 Responses to renal nerve stimulation in rabbits pre-treated with ibuprofen, before and after (A) vehicle treatment (154 mM NaCl; 4 ml/kg + 1 ml $kg^{-1}h^{-1}$; group 2) (B) N^G-nitro-L-arginine (L-NNA; 20 mg/kg + 5 mg $kg^{-1}h^{-1}$; group 3) and (C) L-NNA plus glyceryl trinitrate (8 – 22 μg kg^{-1} min^{-1}; group 4). Symbols and error bars indicate mean ± SE of data from 6 rabbits. P_{Treat} values indicate the outcomes of analysis of variance testing whether renal haemodynamic responses after the various treatments (stimulation sequence 3; ■) differed from those observed before these treatments (stimulation sequence 2; •). Abbreviations are as for Figure 5.3.

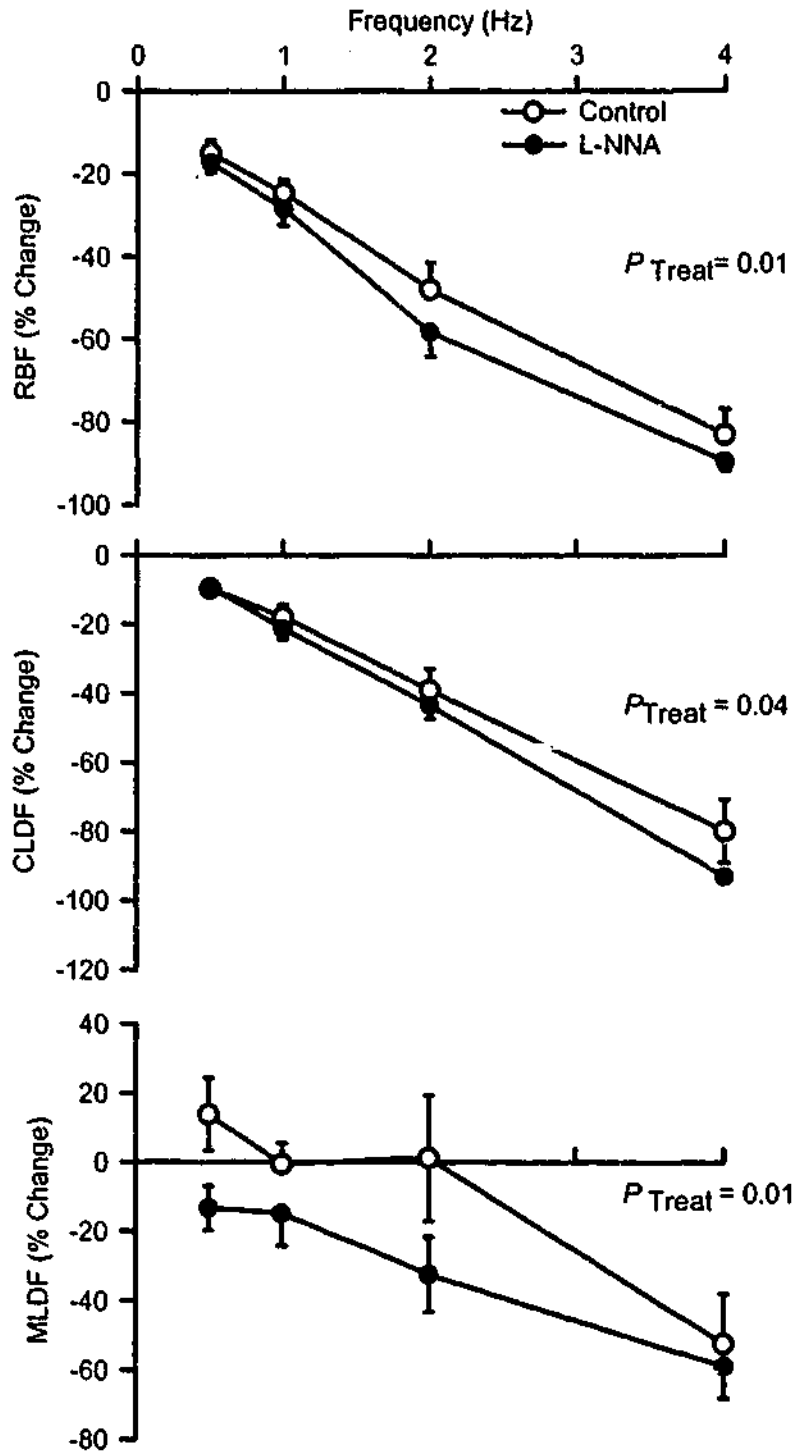

Figure 5.6 Effects of N^G-nitro-L-arginine (L-NNA; 20 mg/kg + 5 mg kg^{-1} h^{-1}) on responses of renal haemodynamics to renal nerve stimulation. Symbols and error bars indicate mean ± SE of data from 6 rabbits. P_{Treat} values indicate the outcomes of analysis of variance testing whether renal haemodynamic responses during stimulation sequence 2 (•) differed from those observed during the initial stimulation sequence (o). Abbreviations are as for Figure 5.3. Data taken from a previous published study from our laboratory (69).

5.5 Discussion

Previous studies have provided compelling evidence that renal nerve stimulation reduces renal CBF more than MBF (69, 93-95, 126, 220). However, the mechanisms underlying this phenomenon remain largely unknown. MBF also appears to be less sensitive than CBF to the effects of exogenous noradrenaline, and this appears to be largely due to a counter-regulatory role of nitric oxide, released in response to activation of α_2-adrenoceptors (206, 265). Consistent with this, we have recently shown in the experimental model used in the current studies, that nitric oxide synthase blockade enhances responses of MLDF to renal nerve stimulation (69). However, renal nerve stimulation still reduced CLDF considerably more than MLDF after nitric oxide synthase blockade alone, indicating that other mechanisms also contribute to the relative insensitivity of MBF to activation of the renal sympathetic nerves. In the current study we aimed to determine whether products of COX also contribute to this phenomenon, and by testing the effects of nitric oxide synthase-inhibition on a background of COX inhibition, explore the possibility that nitric oxide and prostaglandins interact in modulating regional kidney blood flow responses to renal nerve stimulation.

We had three major findings. Firstly, the COX inhibitor ibuprofen did not significantly alter responses to renal nerve stimulation, suggesting little net role for prostanoids in modulating renal vascular responses to activation of the sympathetic nerves under our experimental conditions. Secondly, we found that under conditions of prior COX blockade, administration of L-NNA to block nitric oxide synthase enhanced responses of RBF and CLDF to renal nerve stimulation. This is consistent with our previous finding that nitric oxide synthase blockade alone augments RBF and CLDF responses to renal nerve stimulation (Figure 5.6) (69). Thus, nitric oxide appears to blunt renal nerve stimulation-induced vasoconstriction in the renal cortex in a manner independent of COX. In contrast, our third major finding was that after prior COX blockade, we could not detect an effect of nitric oxide synthase blockade on responses of MLDF to renal nerve stimulation. This observation contrasts directly with those of our previous study under conditions of intact COX activity, where the most prominent effect of nitric oxide synthase blockade was to enhance responses of MLDF to renal nerve stimulation

(Figure 5.6) (69). Collectively, these findings raise the intriguing possibility that the impact of nitric oxide synthase blockade on responses of MLDF to renal nerve stimulation are at least partly mediated through products of COX (see below).

Our finding that ibuprofen treatment *per se* did not significantly affect responses to renal nerve stimulation suggests that prostaglandins play little role in physiologically antagonising renal nerve stimulation-induced vasoconstriction in the rabbit kidney *in vivo*. This accords with our previous observations in the rabbit, showing no significant effect of COX blockade on responses to renal arterial boluses or infusions of noradrenaline (184, 185). However, our data in the rabbit contrast with those in anaesthetized rats, in which COX blockade enhances renal vasoconstrictor responses to both renal nerve stimulation and exogenous noradrenaline (109, 198). Taken together, these data suggest that a true species difference exists, in the role of prostaglandins in modulating responses of the kidney to adrenergic stimuli, between rabbits and rats.

As outlined previously (see Introduction), there is considerable evidence for complex interactions between the prostaglandin and nitric oxide systems in the kidney. We hypothesised that up-regulated release of vasodilator prostanoids within the medullary microvasculature after nitric oxide synthase inhibition might explain why renal nerve stimulation still reduced MLDF less than CLDF under these experimental conditions (69). We can now confidently reject this hypothesis, because even after combined treatment with L-NNA and ibuprofen, renal nerve stimulation reduced CLDF significantly more than MLDF.

Taken at face value, our present observation, that L-NNA did not significantly affect responses of MLDF to renal nerve stimulation when given after prior COX blockade (Figure 5.5B), is at odds with the results of our previous study, in which L-NNA alone greatly enhanced renal nerve stimulation-induced reductions in MLDF (Figure 5.6) (69). Our present findings might simply reflect the considerable variability of responses of MLDF to renal nerve stimulation. On the other hand, it might also reflect an important interaction between nitric oxide and prostaglandins in the neural control of MBF. Importantly, we must consider the fact that COX catalyses the synthesis of

vasoconstrictor prostaglandins (eg H_2) and thromboxanes (eg A_2), as well as vasodilator products (eg prostacyclin). Zhang and colleagues found in the rat isolated perfused kidney, that although nitric oxide synthase blockade alone enhanced noradrenaline-induced vasoconstriction, this effect was markedly attenuated by concomitant COX blockade (262) or antagonism of thromboxane A_2-prostaglandin H_2 (TP) receptors (264). These observations were interpreted to suggest that nitric oxide normally inhibits COX activity within the kidney, so that nitric oxide synthase blockade enhances the production of vasoconstrictor prostanoids in response to adrenergic stimuli. Such a mechanism might also explain the apparent contradiction between our present (Figure 5.5B) and previous (Figure 5.6) findings. Thus, nitric oxide might blunt renal nerve stimulation-induced reductions in MBF by inhibiting production of vasoconstrictor prostaglandins/thromboxanes. Ms Rebecca Flower in our laboratory (in collaboration with me) recently performed a supplementary experiment to test this hypothesis (see Chapter 7 for details of this study).

Consistent with our previous studies (69, 206), we found that intravenous infusion of GTN, at a dose titrated to restore MAP to its control level, also restored RBF, CLDF and MLDF to their control levels. These observations indicate that this technique allows restoration of resting nitrergic vasodilator tone under conditions of nitric oxide synthase blockade. However, this does not necessarily mean that local levels of nitric oxide within the vasculature are normalised by this treatment regimen. Nevertheless, the fact that the effects of L-NNA, on responses to renal nerve stimulation, of RBF and CLDF in the current study, and of RBF, CLDF and MLDF in our previous study (69), were abolished by this GTN infusion, provides important information to aid in interpreting our experiments. One possibility is that the effects of L-NNA on responses to renal nerve stimulation occur secondarily to reduced RBF or increased arterial pressure. However, this seems unlikely, since we have recently shown that moderate changes in renal vascular tone *per se* have little impact on the responsiveness of the renal vasculature to renal nerve stimulation (93). Furthermore, ibuprofen reduced resting levels of RBF, CLDF and MLDF, yet did not significantly affect the responsiveness of these variables to renal nerve stimulation. The pressor effect of L-NNA is also unlikely to account for its influence on responses to renal nerve stimulation, since increased renal perfusion pressure appears to blunt the responsiveness of the renal circulation (at least

the cortical circulation) to renal nerve stimulation, rather than enhance it (95). Thus, it seems likely that L-NNA enhances responses to renal nerve stimulation through its direct action to inhibit nitric oxide synthesis. The fact that co-infusion of GTN abolishes these effects raises the possibility that they are due to removing the influence of basal levels of nitric oxide, rather than abolition of nitric oxide released in response to renal nerve stimulation. However, further validation of the GTN infusion technique, including measurements of nitric oxide levels *in vivo*, are required before these observations can be definitively interpreted.

A major advantage of our experimental approach, is that our observations are not confounded by the differences, between individual rabbit preparations, in responses to renal nerve stimulation. These differences probably arise partly due to variability in our success, between preparations, in isolating all sympathetic nerves innervating the kidney. Importantly, the results from rabbits in group 1 show that responses to renal nerve stimulation are extremely stable across the course of the experiment.

A number of limitations of our current study need to be borne in mind. The first relates to our use of laser Doppler flowmetry, which in highly perfused tissues such as the kidney is essentially a measure of erythrocyte velocity rather than bulk blood flow *per se* (68, 94). However, most evidence suggests that changes in medullary perfusion are mediated mainly by changes in blood velocity within vasa recta capillaries rather than recruitment of vasa recta (see 68 for discussion), although this issue has not been studied in depth. Thus, future investigations should apply methods for studying the control of medullary perfusion at the capillary level to the problems described herein. A second limitation relates to the fact that nitric oxide can affect responses to renal nerve stimulation at pre-junctional sites (139). Our present experimental approach does not allow this effect to be studied independently of post-junctional effects on nitric oxide. On the other hand, there is evidence that inhibition of prostaglandins has little effect on the release of noradrenaline in response to reflex activation of renal nerves in dogs (183). Thirdly, both nitric oxide synthase- and COX blockade altered resting RBF, CLDF and/or MLDF, and nitric oxide synthase blockade increased MAP. As we have already argued (see above) previously published data from our laboratory indicate that

these changes in systemic and renal haemodynamics are unlikely, in themselves, to account for the impacts of these treatments on responses to renal nerve stimulation. However, our present conclusions might be different if they were based on the effects of renal nerve stimulation on absolute rather than the percentage changes in renal haemodynamic variables. We argue that percentage changes rather than absolute changes reflect the functional state of the renal vasculature, because Poiseuille's relationship (flow $\propto$ pressure $\times$ radius^4) predicts that a given percentage change in radius will cause the same proportional change in flow at any given pressure, regardless of resting vascular calibre (see (95)).

5.6 Conclusions

In conclusion, the results of the present study indicate that under physiological conditions, basal production of COX products has little net impact on renal vascular responses to activation of the renal sympathetic nerves, and so does not contribute to the relative insensitivity of MBF to renal nerve stimulation. As we have shown previously (69), nitric oxide synthase blockade enhances responses to renal nerve stimulation. Without prior COX blockade, the effect of nitric oxide synthase blockade is particularly prominent for MLDF (although responses of RBF and CLDF are also enhanced) (Figure 5.6) (69), suggesting that nitric oxide plays an important role in underlying the relative insensitivity of MBF to activation of the renal sympathetic nerves. After prior COX blockade, nitric oxide synthase blockade still enhanced responses of RBF and CLDF to renal nerve stimulation, but we could detect no effect on responses of MLDF to renal nerve stimulation (Figure 5.5B). Collectively, these observations are consistent with a role of vasoconstrictor products of COX in mediating the effects of nitric oxide synthase-blockade on responses of MLDF (but not CLDF) to nerve stimulation. It seems that nitric oxide normally blunts nerve stimulation-induced vasoconstriction in both the renal cortex and the medulla. In the renal cortex, this effect appears to be mediated independently of COX. In contrast, the impact of nitric oxide on responses of MBF to renal nerve stimulation may be mediated through inhibition of production of vasoconstrictor prostaglandins and thromboxanes, which would otherwise enhance the response of MBF to sympathetic activation. Regardless, these mechanisms cannot fully

account for the relative insensitivity of the medullary circulation to sympathetic activation. Future studies should therefore focus on the possible involvement of other mechanisms, involving non-COX products of arachidonic acid metabolism (106), or regional differences within the kidney in sympathetic neuroeffector function.

Chapter Six

Endogenous angiotensin II and nitric oxide in neural control of intrarenal blood flow

A modified version of this chapter was submitted to American Journal of Physiology (Regulatory, Integrative and Comparative Physiology) (Manuscript number R-00477-2004)

6.1 Summary

Renal sympathetic nerve stimulation reduces renal cortical perfusion (CBF) more than medullary perfusion (MBF). Nitric oxide buffers renal nerve stimulation-induced vasoconstriction in the renal cortex and medulla, but the precise mechanisms underlying the relative insensitivity of MBF to renal nerve stimulation remain unknown. We investigated the role(s) of the renin-angiotensin system, and the significance of interactions between angiotensin II and nitric oxide, in responses of regional kidney perfusion to renal nerve stimulation in pentobarbitone anaesthetized rabbits. Under control conditions, renal nerve stimulation (0.5 - 8 Hz) reduced RBF (-89 ± 3% at 8 Hz) and CBF (-90 ± 2 % at 8 Hz) more than MBF (-55 ± 5 % at 8Hz) in a frequency dependent manner. Angiotensin II type 1 (AT_1) receptor antagonism with candesartan blunted renal nerve stimulation-induced reductions in total renal blood flow (RBF) (P_{Treat} = 0.03), CBF (P_{Treat} = 0.007) and MBF (P_{Treat} = 0.04). Nitric oxide synthase inhibition with N^G-nitro-L-arginine (L-NNA) enhanced RBF (P_{Treat} = 0.003), CBF (P_{Treat} = 0.001) and MBF (P_{Treat} = 0.03) responses to renal nerve stimulation. In rabbits pre-treated with candesartan, L-NNA enhanced renal nerve stimulation induced reductions in RBF (P_{Treat} = 0.04) and CBF (P_{Treat} = 0.007) but not MBF (P_{Treat} = 0.66). These results suggest that endogenous angiotensin II enhances, while nitric oxide blunts, neurally mediated vasoconstriction in the renal cortex and medulla. In the renal medulla, but not the cortex, the influence of nitric oxide seems to depend on the presence of endogenous angiotensin II.

6.2 Introduction

Renal medullary blood flow (MBF) plays a key role in the regulation of long-term blood pressure, mainly through its effects on tubular sodium and water handling (47, 48, 50, 150). Therefore, factors that regulate MBF should in turn have a profound effect on the long-term set-point of arterial pressure. There is good evidence that neuronal stimuli can differentially regulate renal cortical blood flow (CBF) and MBF. For example, electrical as well as reflex mediated stimulation of the renal nerves has been shown to cause greater reductions in total renal blood flow (RBF) and cortical laser Doppler flux (CLDF, an index of blood flow) than medullary laser Doppler flux (MLDF) (69, 70, 93, 120, 123, 126, 127, 220). This differential regulation of regional kidney blood flow by the renal nerves is likely to represent an important regulatory mechanism in long-term blood pressure control, yet we are only beginning to understand the mechanisms underlying it. One key factor seems to be nitric oxide, since inhibition of nitric oxide synthase enhances neurally meditated renal vasoconstriction, particularly within the medulla (69).

Another factor that might modulate responses of intrarenal blood flow to renal nerve stimulation is angiotensin II, since renal nerve stimulation can increase renin release from the juxtaglomerular apparatus, and so formation of angiotensin II in the kidney (117). However, at first sight this would seem an unlikely candidate for a mechanism blunting responses to renal nerve stimulation in the medulla, since angiotensin II type 1 (AT_1) receptor antagonism has been shown to blunt renal nerve stimulation-induced reductions in RBF in anaesthetized dogs (67, 237) and rats (61). Most evidence suggests that this is chiefly due to angiotensin II-induced facilitation of noradrenaline release from nerve terminals in response to renal nerve stimulation (232, 237). Collectively, these data indicate that endogenous angiotensin II, by activation of AT_1-receptors, facilitates neurally mediated renal vasoconstriction, at least when one considers total RBF.

However, angiotensin II has also been shown to have vasodilator effects in the renal medullary circulation. Angiotensin II can increase medullary nitric oxide concentration (272) and MBF (252) and these phenomena may be linked through the mechanism of

'tubulovascular nitric oxide cross talk' (62). In anaesthetized rabbits, the MLDF response to renal arterial boluses of angiotensin II is biphasic, consisting of an initial reduction followed by a later increase (66, 206). This increase in MLDF is blunted by nitric oxide synthase-inhibition (206) or AT_1-receptor antagonism (66) suggesting that angiotensin II-induced release of nitric oxide within the medullary circulation depends on activation of AT_1-receptors. Intriguingly, our research group recently found that renal arterial infusion of angiotensin II, at a dose that did not affect basal MLDF, greatly blunted renal nerve stimulation induced reductions in MLDF but not CLDF (Figure 1.7) (93). Collectively, these data have generated the hypothesis that angiotensin II modulates responses of the renal vasculature to renal nerve stimulation in a regionally specific manner. Thus, in vascular elements regulating MBF (juxtamedullary arterioles and descending vasa recta), angiotensin II might stimulate nitric oxide release, which could in turn blunt neurally mediated vasoconstriction.

In the current study we investigated the physiological significance of this phenomenon. We tested the hypothesis that endogenous angiotensin II, via activation of AT_1-receptors, contributes to the differential impact of renal nerve stimulation on regional kidney blood flow by blunting neurally-mediated vasoconstriction in the medullary vasculature. We also tested whether prior AT_1-receptor antagonism can alter the impact of inhibition of nitric oxide synthase on responses to renal nerve stimulation, which would indicate a direct interaction between these systems in modulating neural regulation of intrarenal blood flow.

6.3 Methods

6.3.1 Animals

Twenty male New Zealand White rabbits (mean weight 2.66 ± 0.08 kg) were used in this study.

6.3.2 Surgical preparations

These surgical procedures have been described in detail in Chpater 2, so will only be described briefly here. Rabbits were anesthetized with pentobarbitone sodium and were artificially ventilated. The left kidney was exposed via a left flank incision and placed in

a stable cup. The nerves running parallel to the left renal artery were carefully isolated and placed across a stimulating electrode. The nerves were then sectioned proximally to the electrode. Paraffin oil was used to keep the nerves moist throughout the experiment. RBF was measured using a transit-time ultrasound flow probe (type 2SB) placed around the renal artery. A needle-type laser Doppler flow probe (26 gauge, DP4s) was advanced approximately 9 mm into the kidney with the use of a miromanipulator to measure MLDF. A standard plastic laser Doppler flow probe was placed on the dorsal surface of the kidney to measure CLDF (DP2b). After completion of all surgical procedures the infusion of Hartmann's solution was replaced with a solution containing four parts Hartmann's solution and one part 10% vol/vol polygeline (Haemaccel). The experimental protocol commenced after a 30-60 min equilibration period.

6.3.3 Experimental protocol

We designed this study in a manner that allowed us to compare the effects of different treatment regimens, on intrarenal blood flow responses to renal nerve stimulation, with the respective control responses in the same rabbit (see below). This within-animal design allowed us to eliminate the confounding effects resulting from variations in responses of intrarenal blood flow to renal nerve stimulation in different rabbits.

Three groups of rabbits were studied (n = 6 per group, except group 2 where n = 8). Rabbits in all three groups were subjected to three renal nerve stimulation sequences (see below). In all these groups, the first renal nerve stimulation sequence served to define control responses. The first group of rabbits served as the time- and vehicle-control. In this group of rabbits, the vehicle for candesartan (154 mM NaCl: 1 ml/kg + 1 ml kg^{-1} h^{-1}) was administered after the first stimulation sequence, and this infusion was continued for the duration of the experiment. Twenty minutes after commencing this infusion, rabbits were subjected to a second stimulation sequence. After a 10 min equilibration period, following completion of the second stimulation period, rabbits of this group received the vehicle for N^G-nitro-L-arginine (L-NNA; 154 mM NaCl: 4 ml/kg + 1 ml^{-1} kg^{-1} h^{-1}), which was also infused for the remainder of the experiment. Responses to renal nerve stimulation were re-tested 25 min after commencing this vehicle infusion. Rabbits in groups 2 and 3 were subjected to similar procedures, but as described below they received the relevant treatment regimens. Thus, the second group

of rabbits received the vehicle for candesartan (154 mM NaCl: 1 ml/kg + 1 ml kg^{-1} h^{-1}) after the first stimulation sequence and L-NNA (20 mg/kg + 5 mg kg^{-1} h^{-1} i.v.; Sigma Chemical Company, St Lois, MO, USA) after the second stimulation sequence. This experimental sequence allowed us to determine the role of endogenous nitric oxide in modulating responses of regional kidney perfusion to renal nerve stimulation, in the presence of an intact renin-angiotensin system. The third group of rabbits received candesartan (10 μg/kg + 10 μg $kg^{-1}h^{-1}$ i.v.; Astra Zeneca, Switzerland) after the first stimulation sequence and L-NNA after the second stimulation sequence. This experimental sequence therefore allowed us to determine whether endogenous angiotensin II (acting via AT_1-receptors) modulates responses of intrarenal perfusion to renal nerve stimulation. It also allowed us to determine whether the effects of endogenous nitric oxide are dependent on activation of AT_1-receptors by endogenous angiotensin II. Ten minutes after completing the third stimulation sequence, rabbits in all three groups received three i.v bolus doses of angiotensin II (10, 100 and 1000 ng/kg) in ascending order. A 5-10 min recovery period was allowed between each dose of angiotensin II (Figure 6.1).

6.3.4 Electrical nerve stimulation

The LabVIEW graphical programming language (National Instruments, Austin, TX) was used to provide computer generated stimulation of the renal nerves. The voltage that produced the maximum reductions in RBF, CLDF and MLDF were determined in each rabbit by stimulating the renal nerves with various voltages (3-10 V) at 5 Hz for 60 s. This pre-determined voltage was used in all subsequent stimulation periods in that rabbit. Each stimulation sequence consisted of five, 3 min stimulus trains (0.5, 1, 2, 4 and 8 Hz; 2 ms pulse duration). These frequencies were applied in random order. Recovery periods of 8-10 min were allowed between each stimulus train.

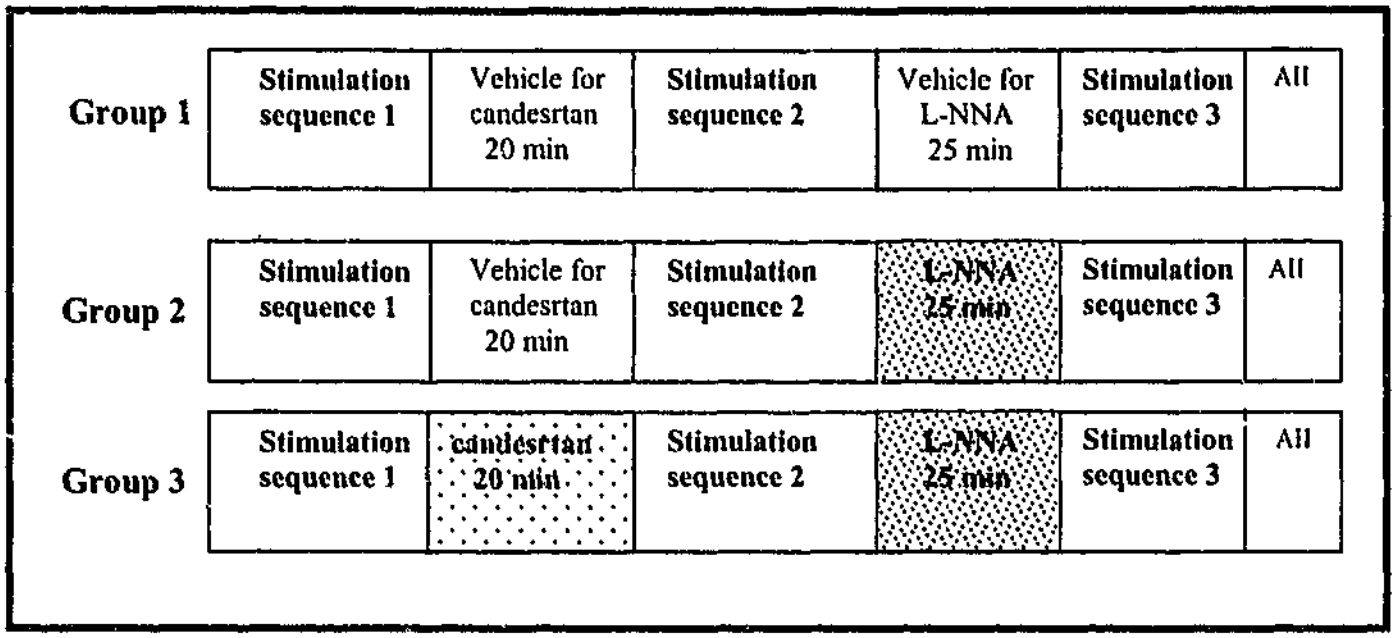

Figure 6.1 A schematic diagram of the experimental protocol. Rabbits in group 1 received vehicle infusions (154 mM NaCl) after stimulation sequences 1 and 2. Rabbits in group 2 received vehicle for candesartan after stimulation sequence 1 and N^G-nitro-L-arginine (L-NNA; 20 mg/kg + 5 mg kg^{-1} h^{-1}) after stimulation sequence 2. Rabbits in group 3 received candesartan (10 μg/kg + 10 μg $kg^{-1}h^{-1}$) after stimulation sequence 1 and L-NNA after stimulation sequence 2. Once commenced, all infusions were continued until the end of the experiment. Rabbits in all three groups received three i.v bolus doses of angiotensin II (AII; 10, 100 and 1000 ng/kg), ten minutes after completing the third stimulation sequence.

6.3.5 Recording of haemodynamic variables

The ear artery catheters were connected to pressure transducers to measure arterial pressure (Cobe, Arvarda, CO, USA). RBF was measured by connecting the transit-time ultrasound flow probe to a model T208 flowmeter (Transonic systems) and CLDF and MLDF were measured by connecting the laser Doppler flow probes to a laser Doppler flowmeter (DRT4, Moor instruments). A computer equipped with an analog-to-digital acquisition card (Lab PC^+, National Instruments, Austin, TX) and purpose written software (Universal Acquisition, University of Auckland, New Zealand) provided 2 s averages of mean arterial pressure (MAP; mmHg), heart rate (HR, determined from the arterial pressure pulse; beats/min), RBF (ml/min), CLDF (perfusion units), and MLDF (perfusion units). The values of CLDF (7 ± 1 units) and MLDF (9 ± 1 units), during the

60 s immediately after the rabbit was humanely killed by overdose with pentobarbital (300 mg), and artificial ventilation was ceased were subtracted from the values obtained during the experiment, before data analysis was performed.

6.3.6 Statistics

All data are expressed as mean ± SE. *P* values ≤ 0.05 were considered statistically significant. Baseline levels of systemic and renal haemodynamic variables were calculated by averaging the 30 s control periods immediately prior to each stimulation train, across all five frequencies in each renal nerve stimulation sequence. Systemic and renal haemodynamic responses to renal nerve stimulation were determined by comparing the levels of each variable, during the last 30 s of each stimulation period, with the control values during the 30 s immediately prior to stimulation. We used analysis of variance (ANOVA) to test whether baseline levels of haemodynamic variables, and responses to renal nerve stimulation, differed (i) between the three experimental groups during the control period (P_{Group}) and (ii) during stimulation sequence 2 compared with stimulation sequence 1, and during stimulation sequence 3 compared with stimulation sequence 2 within each treatment group (P_{Treat}). We also tested whether responses of MLDF to renal nerve stimulation differed from those of CLDF (P_{Region}). Therefore, we tested our key biological hypotheses, that the various treatments affected baseline haemodynamics and responses to renal nerve stimulation, in a 'within'subject fashion.

For analysis of responses to exogenous angiotensin II, levels of haemodynamic variables during the final 30 s of the control periods, and periods of maximum reductions and /or increases after the angiotensin II boluses, were subjected to repeated measures ANOVA. We tested whether angiotensin II had dose-dependent effects in the vehicle-control group (P_{Dose}), and whether each of the treatments affected these responses (P_{Group}). *P* values from within-subject factors in repeated measures analyses were conservatively adjusted using the Greenhouse-Geisser correction (135).

6.4 Results

6.4.1 *Baseline haemodynamics*

During the first (control) stimulation sequence, baseline levels of MAP, HR, RBF, CLDF and MLDF, when averaged across all 20 rabbits, were 72 ± 2 mmHg, 245 ± 4 beats/min, 27 ± 2 ml/min, 264 ± 10 units and 67 ± 5 units respectively. These variables did not vary according to the treatment that was to follow ($P_{Group} \geq 0.1$). In vehicle-treated rabbits (group 1) baseline levels of all measured variables remained stable across the course of the experiment (Table 6.1A). In the rabbits of group 2, vehicle-treatment was accompanied by little change in MAP, HR, RBF, and CLDF, but there was a significant reduction in MLDF (-14 ± 4%; $P_{Treat} = 0.02$). Subsequent L-NNA treatment increased MAP (30 ± 9%), and reduced HR (-14 ± 4 %), RBF (-22 ± 4 %), CLDF (-13 ± 2%) and MLDF (-25 ± 5 %) (Table 6.1B). In the rabbits of group 3, candesartan alone increased RBF (43 ±12 %) and CLDF (16 ± 3 %), but had little effect on MLDF or HR. Candesartan also tended to reduce MAP (-7 ± 3%) but this did not reach statistical significance. Subsequent administration of L-NNA reduced HR (-10 ± 2 %), RBF (-35 ± 8 %), CLDF (-19 ± 5 %) and MLDF (-36 ± 6 %), but did not significantly alter MAP (11 ± 5 % increase) (Table 6.1C).

6.4.2 *Responses to renal nerve stimulation*

Responses to renal nerve stimulation under control conditions During the initial stimulation sequence, responses of RBF, CLDF and MLDF to renal nerve stimulation did not differ significantly between the three treatment groups, so these data were pooled. When averaged across all 20 rabbits, renal nerve stimulation caused frequency-dependent reductions in RBF (-89 ± 3 % at 8 Hz; $P_{Frequency} < 0.001$), CLDF (-90 ± 2 % at 8 Hz; $P_{Frequency} < 0.001$), and MLDF (-55 ± 5 % at 8 Hz; $P_{Frequency} < 0.001$). Renal nerve stimulation induced significantly greater reductions in CLDF than in MLDF ($P_{Region} < 0.001$). Also, renal nerve stimulation caused a slight but significant increase in MAP (7 ± 1 % at 8 Hz) but had little effect on HR (Figure 6.2).

Effects of vehicle, L-NNA and candesartan In the rabbits of group 1, responses of RBF, CLDF and MLDF to renal nerve stimulation remained remarkably stable across

the three stimulation sequences ($P_{Treat} \geq 0.18$) (Figure 6.3). In the rabbits of group 2, responses of RBF, CLDF and MLDF to renal nerve stimulation were similar during stimulation sequences 1 and 2 (after vehicle-treatment) ($P_{Treat} \geq 0.24$) (Figure 6.4). In contrast, renal nerve stimulation-induced reductions in RBF ($P_{Treat} = 0.003$), CLDF ($P_{Treat} = 0.001$) and MLDF ($P_{Treat} = 0.03$) were enhanced after L-NNA treatment (Figure 6.4). Enhancement of responses of MLDF to renal nerve stimulation was observed at frequencies ≤ 2 Hz. For example, 2 Hz renal nerve stimulation reduced RBF, CLDF and MLDF by - 34 ± 5%, - 31 ± 2 % and - 2 ± 6 % respectively, prior nitric oxide synthase-blockade, and by - 43 ± 5%, -39 ± 5% and - 23 ± 5% respectively, after nitric oxide synthase-blockade. In rabbits from group 3, renal nerve stimulation-induced reductions in RBF ($P_{Treat} = 0.03$), CLDF ($P_{Treat} = 0.007$) and MLDF ($P_{Treat} = 0.04$) were blunted after candesartan treatment (Figure 6.5). For example, 4 Hz renal nerve stimulation reduced RBF, CLDF and MLDF by - 81 ± 5%, -85 ± 5 % and -68 ± 4% respectively under control conditions, but only by -71 ± 7%, -71± 7% and – 45 ± 5% respectively, after candesartan-treatment. Subsequent administration of L-NNA enhanced renal nerve stimulation-induced reductions in RBF ($P_{Treat} = 0.04$), and CLDF ($P_{Treat} = 0.007$) but not MLDF ($P_{Treat} = 0.66$), so that at 4 Hz reductions were – 80 ± 6%, - 85 ± 3% and – 47 ± 4% respectively (Figure 6.5). Renal nerve stimulation-induced reductions in CLDF were always greater than those of MLDF, regardless of the treatment (vehicle, L-NNA, candesartan or candesartan + L-NNA) administered ($P_{Region} < 0.001$).

6.4.3 Responses to bolus doses of angiotensin II

In vehicle-treated rabbits (Group 1), intravenous angiotensin II caused dose-dependent reductions in RBF and CLDF ($P_{Dose} \leq 0.01$). The response of MLDF to bolus angiotensin II was biphasic, consisting of an initial reduction (during the first 20 s of the response) followed by a later increase (during the period 20-70 s after the bolus). Also, angiotensin II dose-dependently increased MAP (by 21 ± 3 mmHg at 1000 ng/kg; $P_{Dose} = 0.003$) but had little effect on HR. Compared to vehicle-treated rabbits, in rabbits treated with L-NNA (Group 2) angiotensin II produced greater reductions in CLDF ($P_{Treat} = 0.03$) and tended to produce greater reductions in RBF ($P_{Treat} = 0.07$) and MLDF ($P_{Treat} = 0.09$). Compared to rabbits treated with L-NNA alone, in rabbits treated with candesartan plus L-NNA (Group 3), angiotensin II-induced reductions in RBF ($P_{Treat} = 0.004$), CLDF (0.001) and MLDF ($P_{Treat} = 0.002$), and also angiotensin II-

induced increases in MAP ($P_{Treat} < 0.001$) were significantly reduced. Angiotensin II-induced increases in MLDF were virtually abolished by combined candesartan and L-NNA treatment (Figure 6.6).

Table 6.1 Mean baseline levels of systemic and renal haemodynamic variables

	MAP (mmHg)	HR (beats/min)	RBF (ml/min)	CLDF (units)	MLDF (units)
A: Effects of vehicle treatments (Group 1, n = 6)					
Control	71 ± 3	247 ± 11	31 ± 2	285 ± 17	76 ± 13
Vehicle 1	67 ± 3	243 ± 11	36 ± 1	272 ± 8	70 ± 15
Vehicle 2	69 ± 4	240 ± 11	37 ± 1	261 ± 9	79 ± 23
B: Effects of N^G-nitro-L-arginine (Group 2, n = 8)					
Control	76 ± 4	230 ± 9	23 ± 4	269 ± 21	60 ± 3
Vehicle 1	75 ± 5	230 ± 9	23 ± 1	269 ± 9	51 ± 1*
N^G-nitro-L-arginine	95 ± 5**	195 ± 9**	17 ± 1*	218 ± 10*	36 ± 2*
C: Effects of candesartan plus N^G-nitro-L-arginine (Group 3, n = 6)					
Control	70 ± 2	244 ± 7	24 ± 3	273 ± 11	61 ± 14
Candesartan	64 ± 2	243 ± 9	34 ± 5*	313 ± 18**	58 ± 11
N^G-nitro-L-arginine	72 ± 5	220 ± 10**	21 ± 3**	256 ± 26**	34 ± 6**

Data indicate mean ± SE. MAP, mean arterial pressure; HR, heart rate; RBF, renal blood flow; CLDF, cortical laser Doppler flux; MLDF, medullary laser Doppler flux. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ indicate the outcomes of analysis of variance, testing whether baseline levels observed during stimulation sequence 2 differed from those observed during stimulation sequence 1 and whether baseline levels observed during stimulation sequence 3 differed from those observed during stimulation sequence 2, within each group of rabbits

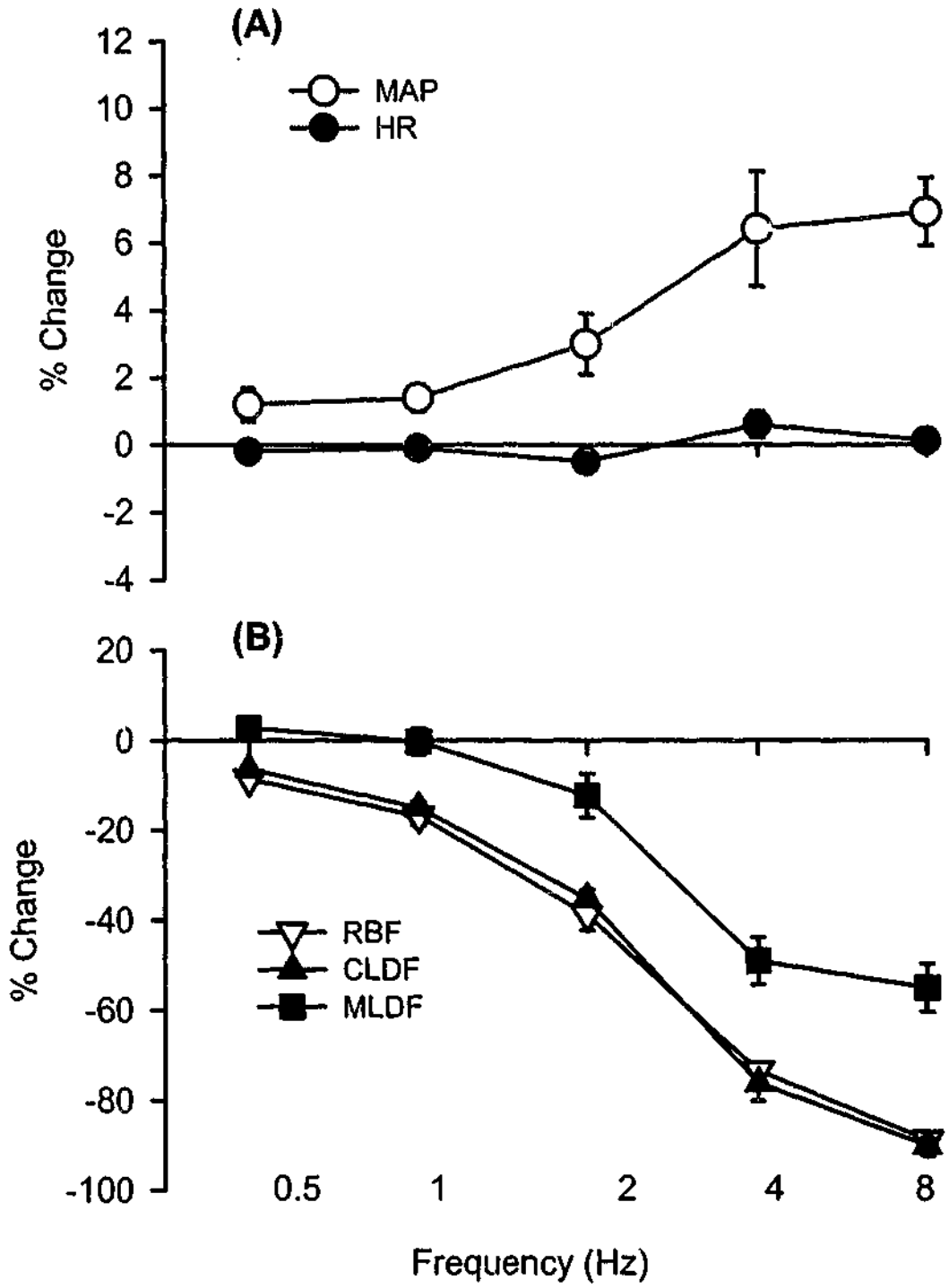

Figure 6.2 Responses of (A) systemic- and (B) renal-haemodynamic variables during the initial (control) renal nerve stimulation sequence. Symbols and error bars indicate the mean ± SE of the average percentage changes of each variable during the last 30 s of each stimulus train, compared with last 30 s immediately prior to stimulation, in all 20 rabbits. MAP = mean arterial pressure, HR = heart rate, RBF = renal blood flow, CLDF = cortical laser Doppler flux, MLDF = medullary laser Doppler flux.

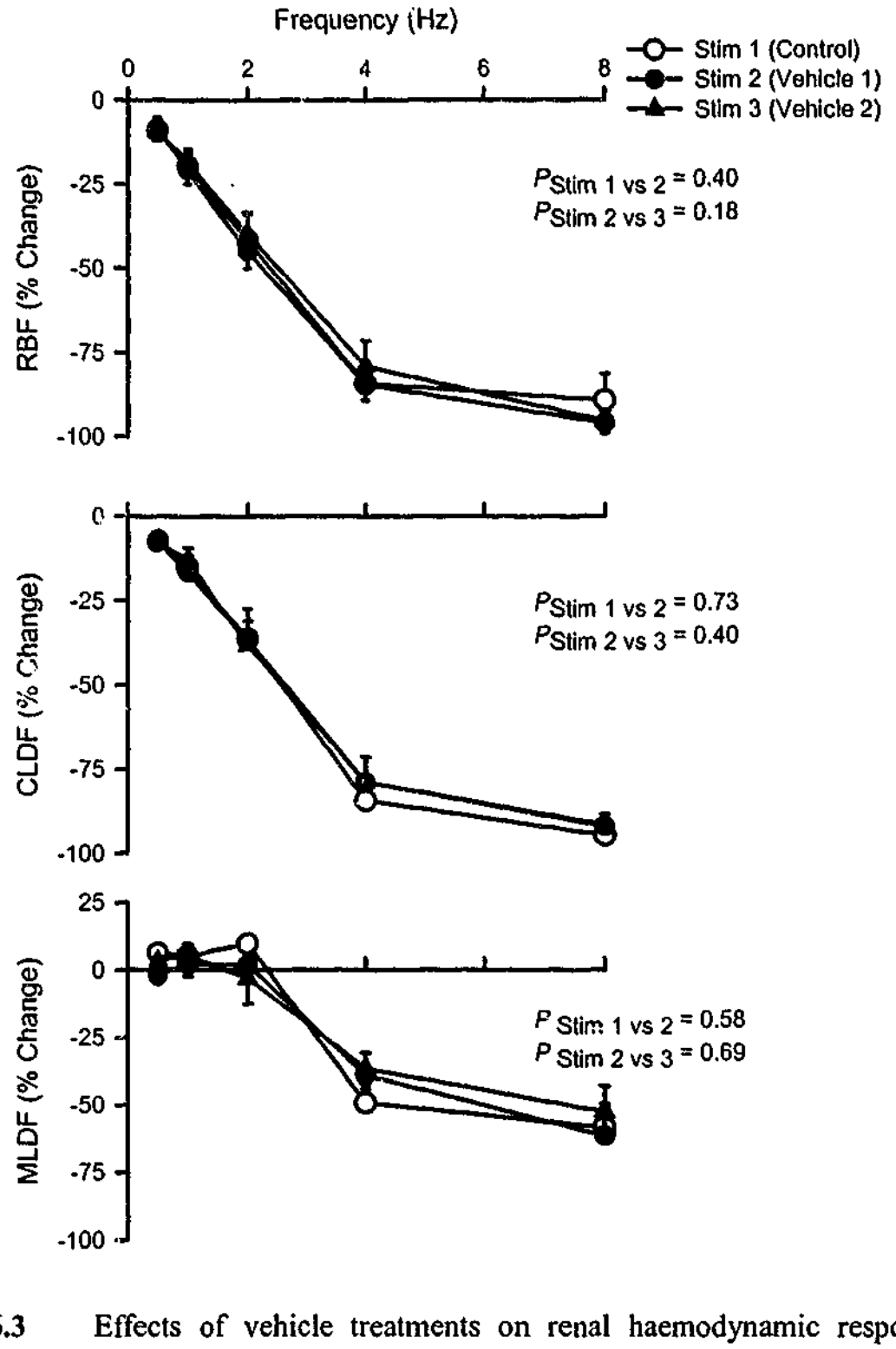

Figure 6.3 Effects of vehicle treatments on renal haemodynamic responses to stimulation of the renal nerves. Symbols and error bars indicate mean ± SE of data from rabbits in group 1 (n = 6). *P* values indicate the outcomes of analysis of variance, testing whether renal haemodynamic responses during stimulation sequence 2 (•) differed from those observed during the initial stimulation sequence (o), and whether responses during stimulation sequence 3 (▲) differed from those observed during stimulation sequence 2. Stim = Stimulation. Other abbreviations as for Figure 6.2.

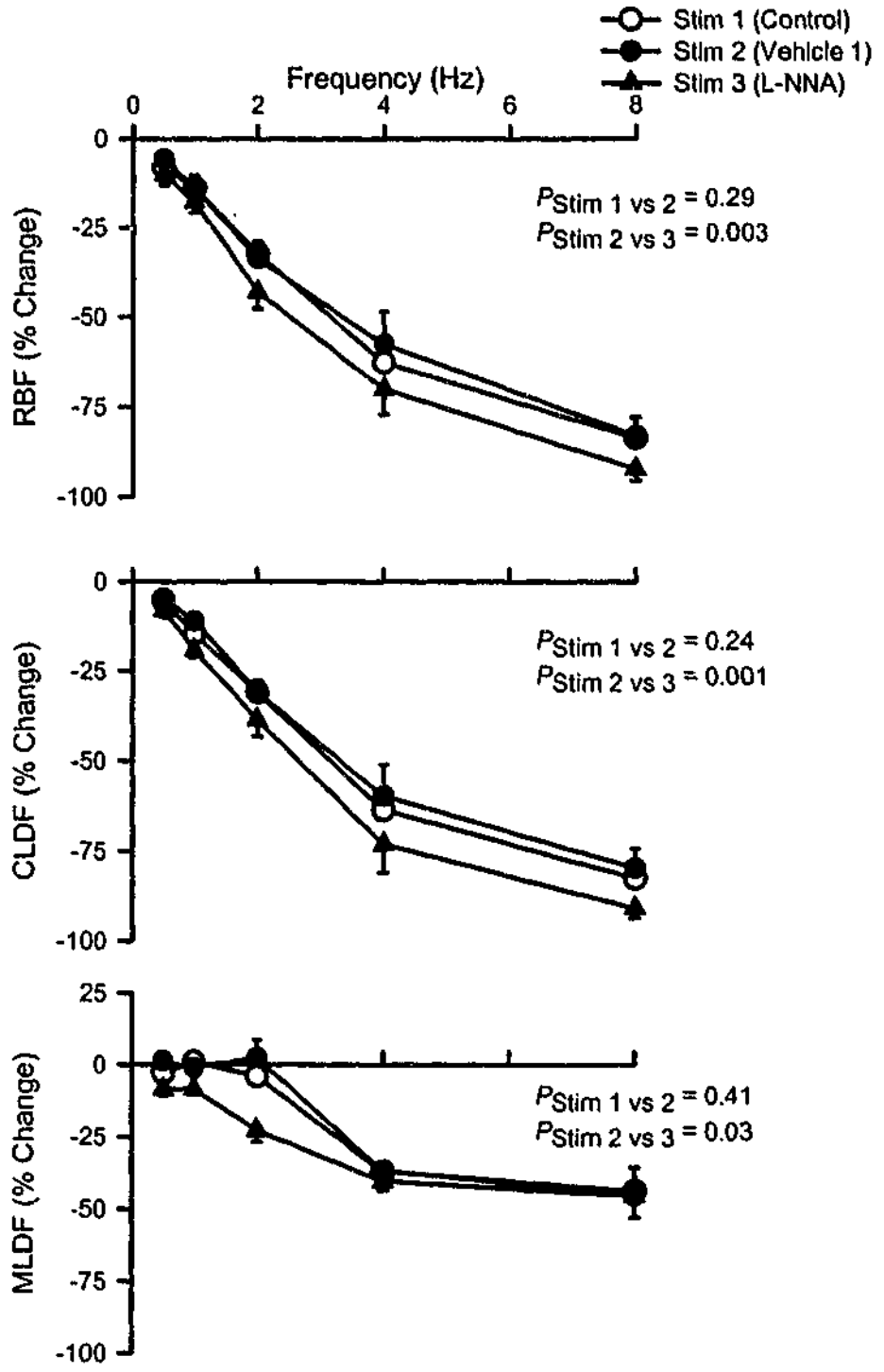

Figure 6.4 Effects of N^G-nitro-L-arginine (L-NNA; 20 mg/kg + 5 mg $kg^{-1}h^{-1}$) on renal hemodynamic responses to renal nerve stimulation. Symbols and error bars indicate mean ± SE of data from rabbits in group 2 (n = 8). *P* values indicate the outcomes of analysis of variance testing whether renal hemodynamic responses during stimulation sequence 2 (•; performed after commencing vehicle-treatment) differed from those observed during the initial stimulation sequence (o), and whether renal hemodynamic responses during stimulation sequence 3 (▲; performed after commencing L-NNA-treatment) differed from those observed during stimulation sequence 2. Abbreviations as for Figure 6.3.

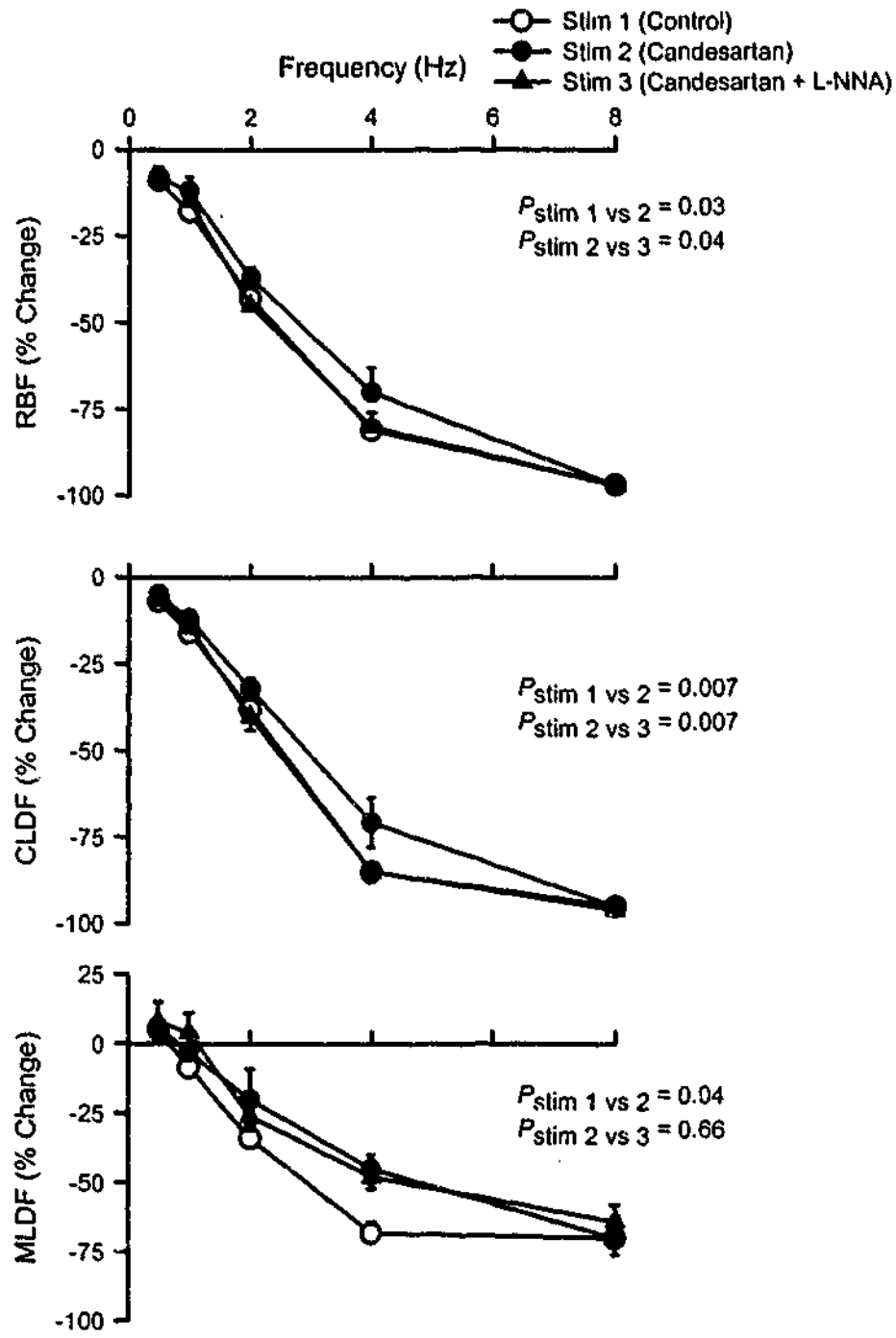

Figure 6.5 Effects of candesartan (10 μg/kg + 10 μg $kg^{-1}h^{-1}$) and subsequently N^G-nitro-L-arginine (L-NNA; 20 mg/kg + 5 mg $kg^{-1}h^{-1}$) on renal hemodynamic responses to renal nerve stimulation. Symbols and error bars indicate mean ± SE of data from rabbits in group 3 (n = 6). *P* values indicate the outcomes of analysis of variance testing whether responses during stimulation sequence 2 (•; performed after commencing candesartan-treatment) differed from those observed during the initial stimulation sequence (o), and whether responses during stimulation sequence 3 (▲; performed after commencing L-NNA treatment) differed from those observed during stimulation sequence 2. Abbreviations as for Figure 6.3.

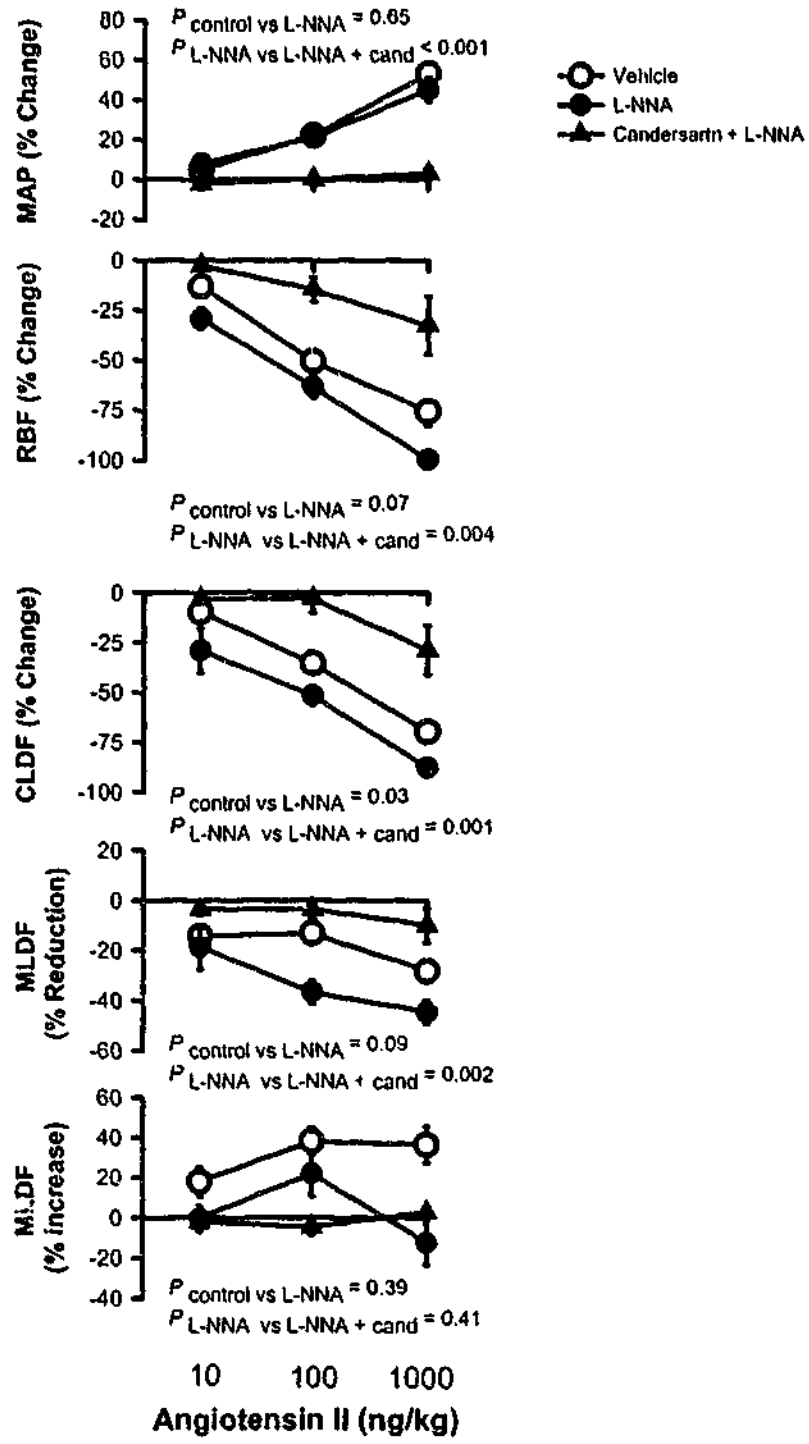

Figure 6.6 Responses to intravenous bolus doses of angiotensin II in rabbits treated with vehicle only (o; Group 1; n = 6), N^G-nitro-L-arginine (•; Group 2; n = 8; L-NNA; 20 mg/kg + 5 mg $kg^{-1}h^{-1}$), or candesartan (10 μg/kg + 10 μg $kg^{-1}h^{-1}$) plus L-NNA (▲; Group 3; n = 6). *P* values indicate the outcomes of analysis of variance testing whether responses to angiotensin II differed in rabbits treated with L-NNA compared with vehicle-treated rabbits, and in rabbits treated with candesartan plus L-NNA compared with those treated with L-NNA only. MAP = mean arterial pressure, HR = heart rate, RBF = renal blood flow, CLDF = cortical laser Doppler flux, MLDF = medullary laser Doppler flux.

6.5 Discussion

The aim of our study was to investigate the roles of endogenous angiotensin II acting at AT_1-receptors, and the significance of interactions between nitric oxide and angiotensin II, in the differential regulation of regional kidney blood flow by renal nerve stimulation. As shown previously by ourselves (69, 93, 94, 126), and others (220), renal nerve stimulation reduced CLDF more than MLDF. Consistent with our previous findings (69), nitric oxide synthase-inhibition alone enhanced neurally mediated renal vasoconstriction, particularly at low frequencies within the medulla. Thus, endogenous nitric oxide appears to play a key role in the mechanisms underlying the relative insensitivity of the medullary circulation to low frequency renal nerve stimulation. Our present data also demonstrate that AT_1- receptor antagonism with candesartan can blunt renal nerve stimulation-induced reductions in RBF and CLDF. This confirms and extends previous studies of the effects of AT_1 antagonists on responses of global RBF to renal nerve stimulation (61, 67, 237, 257), and suggests that endogenous angiotensin II, acting at AT_1-receptors, augments renal nerve stimulation-induced vasoconstriction in vascular elements regulating CBF.

There are two major novel findings of the present study. Firstly, the AT_1-receptor antagonist candesartan blunted responses of MLDF to renal nerve stimulation. We conclude from this that the dominant role of endogenous angiotensin II, with regard to neural control of MBF, is to enhance vasoconstrictor responses to renal nerve stimulation within the medullary circulation. Secondly, we found that prior AT_1-receptor antagonism abolished the ability of L-NNA-treatment to enhance MLDF responses to renal nerve stimulation, while leaving its effects on responses of RBF and CLDF intact. Therefore, in the renal medullary, but not the cortical, circulation the ability of endogenous nitric oxide to blunt neurally-mediated vasoconstriction appears to depend upon tonic activation of AT_1-receptors. Thus, within the medullary circulation, endogenous angiotensin II appears to have actions to both enhance and blunt neurally mediated vasoconstriction, with the former action predominating.

Renal sympathetic nerve activation (via stimulation of intrarenal β-adrenergic receptors) can increase renin release and probably also angiotensin II concentrations in the kidney

(117). Therefore, the blunted CLDF and MLDF responses to renal nerve stimulation observed in candesartan treated rabbits could simply reflect abolition of the direct vasoconstrictor activity of angiotensin II, produced in response to renal nerve stimulation. However, this seems unlikely as previous studies have shown that angiotensin converting enzyme inhibitors and receptor antagonists have little effect on the renal vasoconstrictor response to noradrenaline (117). There is also a body of evidence indicating that angiotensin II facilitates the release of noradrenaline from adrenergic nerve terminals (232, 237). Thus, our present observations may reflect the ability of angiotensin II to potentiate the release of noradrenaline from nerve terminals in both the cortical and medullary vasculature of the rabbit kidney. This hypothesis was not tested in the present study and merits further investigation.

As we have shown previously (69), nitric oxide synthase-inhibition enhanced renal nerve stimulation-induced reductions in RBF, CLDF and MLDF under conditions of an intact renin-angiotensin system. Our major novel finding was that nitric oxide synthase-inhibition enhanced renal nerve stimulation-induced reductions in CLDF, but not MLDF, in rabbits pre-treated with candesartan. This finding suggests that the influence of endogenous nitric oxide on neurally mediated vasoconstriction in the medullary circulation depends on activation of AT_1-receptors by endogenous angiotensin II. Collectively, these data are consistent with the view that angiotensin II can release nitric oxide in the renal medullary vasculature (272) which in turn blunts adrenergic vasoconstriction within the medullary circulation (69, 206, 265). Our present data, indicate that this effect is physiologically relevant, since it is induced by endogenous angiotensin II acting at AT_1-receptors. The corollary of this is that angiotensin II acts to blunt responses of MBF to renal nerve stimulation, through the release of nitric oxide. However, our present results indicate that this action is normally obscured by the dominant effect of endogenous angiotensin II to enhance reductions in MBF to renal nerve stimulation.

Our present observations are also consistent with our previous finding that renal arterial infusions of angiotensin II can blunt reductions in MLDF in response to renal nerve stimulation in anaesthetized rabbits (93). The apparent discrepancy between our present observation, that candesartan blunted responses of RBF, CLDF and MLDF to renal nerve stimulation, and our previous observation that exogenous angiotensin II either had

no significant effect (RBF and CLDF) or blunted (MLDF) responses to renal nerve stimulation, deserves comment. One possible explanation relates to differences in bioavailability between exogenous angiotensin II, which is rapidly degraded in the kidney (half life ~ 16 secs) (29), and that of the more stable candesartan (half-life ~ 9 hrs) (30). Thus, intravenously administered candesartan would likely reach similar concentrations throughout the kidney, including vascular smooth muscle and its associated sympathetic innervation. In contrast, angiotensin II infused into the renal artery would reach relatively high concentrations within the vascular and tubular lumen, and so be able to stimulate nitric oxide release from endothelial and/or epithelial cells (62). However, the vascular endothelium and the interstitium would act as barriers for diffusion of exogenous angiotensin II from sites within the vascular and tubular lumen to vascular smooth muscle (and its associated sympathetic innervation).

A major advantage of our experimental approach using a 'within animal' design is that our results are not confounded by between-rabbit variations in responses to renal nerve stimulation. Importantly, our results do indicate that the responses to renal nerve stimulation are very stable across the course of the experiment (Figure 6.3).

There are some limitations of this study. Firstly, there is evidence that nitric oxide can inhibit release of noradrenaline from pre-junctional sites in response to renal nerve stimulation (139). Thus, enhanced responses of RBF, CLDF and MLDF to renal nerve stimulation in rabbits pre-treated with L-NNA could be due to the facilitated release of noradrenaline, to a post-juctional effect to enhance responses to noradrenaline, or both. Our present results do not allow us to identify the precise mechanisms by which nitric oxide, or endogenous angiotensin II, modulate renal haemodynamic responses to renal nerve stimulation. Secondly, L-NNA reduced baseline levels of RBF, CLDF and MLDF and candesartan increased baseline levels of RBF and CLDF. Therefore, the effects of candesartan and L-NNA on responses of intrarenal blood flow to renal nerve stimulation could be secondary to the effects of these treatments on baseline levels of systemic and renal haemodynamics. However, this seems unlikely as previous studies in our laboratory have indicated that changes in renal vascular tone *per se* (93), and changes in arterial pressure similar to those produced by L-NNA and candesartan in the current study (70) have little impact on responses of regional kidney blood flow to renal nerve

stimulation. Moreover, even large increases in renal perfusion pressure, produced using an extracorporeal circuit preparation in anaesthetized rabbits, have little impact on the responses of MLDF to RNS (95).

6.6 Conclusions

In conclusion, our present results suggest that endogenous angiotensin II enhances renal nerve stimulation-induced vasoconstriction in vascular elements regulating both CBF and MBF. Our data confirm that endogenous nitric oxide buffers neurally mediated vasoconstriction in the renal cortical and medullary circulations. In the renal medulla, but not the cortex, this effect of endogenous nitric oxide seems to depend on activation of AT_1- receptors by endogenous angiotensin II. Thus, endogenous angiotensin II appears to produce competing effects to both enhance and blunt neurally-mediated reductions in MBF. However, under physiological conditions, the action of endogenous angiotensin II to enhance renal nerve stimulation-induced vasoconstriction in the medullary circulation appears to predominate.

Chapter Seven

General Discussion

7.1 Introduction

I have already discussed the significance of the findings of experiments described in this thesis with respect to the relevant literature in Chapters 3-6. In this chapter, the significance of these findings will be discussed in a more broader physiological context, paying particular attention to the implications of my work for future investigations of the regulation of regional kidney perfusion.

7.2 Synopsis of experimental aims and findings

7.2.1 Roles of cytochrome P450-dependent arachidonate metabolism in responses of regional kidney perfusion to hormones

The aim of the experiments described in Chapter 3 was to examine the roles of arachidonic acid (AA) metabolites produced via cytochrome P450 (CYP450) mono-oxygenase pathways, in modulating regional kidney blood flow responses to angiotensin II, [Phe^2,Ile^3,Orn^8]-vasopressin and noradrenaline. CYP450 enzymes metabolize AA to 20-hydroxyeicosatetraenoic acids (20-HETE) and epoxyeicosatrienoic acids (EETs). We tested the effects of renal arterial infusion of the non-selective inhibitor 1-aminobenzotriazole (ABT) on regional kidney blood flow responses to renal arterial vasoconstrictor infusions. In vehicle-treated rabbits, [Phe^2,Ile^3,Orn^8]-vasopressin reduced medullary laser Doppler flux (MLDF) but not total renal blood flow (RBF) or cortical laser Doppler flux (CLDF) while angiotensin II and noradrenaline reduced RBF and CLDF but had little effect on MLDF. ABT-treatment had no significant effect on any of these responses. Biochemical analysis of the treated kidneys revealed that ABT, at the dose given, failed to significantly inhibit CYP450-dependent arachidonate metabolism. Therefore, we were unable to draw any conclusions regarding the roles of CYP450 enzymes in responses of regional kidney perfusion to angiotenisn II, noradrenaline and [Phe^2,Ile^3,Orn^8]-vasopressin.

7.2.2 *Roles of CYP450-dependent epoxygenase and ω-hydroxylase metabolites of AA in responses of regional kidney perfusion to hormones*

We then turned our attention to more potent and selective inhibitors of CYP450 dependent AA metabolism. In the experiments described in Chapter 4 we examined the effects of renal arterial infusions of the selective epoxygenase inhibitor N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (MS-PPOH), and the selective ω-hydroxylase inhibitor N-methylsulfonyl-12-12-dibromododec-11-enamide (DDMS) on regional kidney blood flow responses to angiotensin II and [Phe^2,Ile^3,Orn^8]-vasopressin. As in the experiments described in Chapter 3, [Phe^2,Ile^3,Orn^8]-vasopressin reduced MLDF but not CLDF or RBF, while angiotensin II reduced RBF and CLDF, but not MLDF, after vehicle-treatment. MS-PPOH had no significant effect on MLDF responses to [Phe^2,Ile^3,Orn^8]-vasopressin, but revealed reductions in RBF and CLDF. DDMS had little effect on responses to [Phe^2,Ile^3,Orn^8]-vasopressin. DDMS revealed moderate dose-dependent reductions in MLDF to angiotensin II but had no significant effects on RBF and CLDF responses. MS-PPOH-treatment had no significant effects on responses to angiotensin II. Biochemical analysis of the treated kidneys revealed that MS-PPOH-treatment selectively inhibited the production of EETs, but DDMS-treatment did not significantly reduce the production of 20-HETE. Furthermore, the formation of EETs, dihydroxyeicosatrienoic acids (DiHETEs) or 20-HETE was not affected by incubation of cortical homogenates with [Phe^2,Ile^3,Orn^8]-vasopressin. Our findings therefore suggest that vasodilator EETs blunt V_1-receptor-mediated cortical, but not medullary, vasoconstriction. Indeed, after MS-PPOH-treatment [Phe^2,Ile^3,Orn^8]-vasopressin infusion produced similar reductions in CLDF and MLDF, indicating that the relative insensitivity of cortical blood flow (CBF) to V_1-receptor activation can be entirely accounted for by this mechanism. We found that [Phe^2,Ile^3,Orn^8]-vasopressin had little effect on CYP450 enzyme activity *per se*, so our data are consistent with the notion that V_1-receptor activation enhances the liberation of AA, the substrate for CYP450 epoxygenase. Our data also indicate that *de novo* synthesis of EETs does not contribute to the responses of regional kidney blood flow to angiotensin II. Because neither DDMS nor ABT (Chapter 3) inhibited 20-HETE production under our experimental conditions, we cannot draw conclusions regarding the roles of ω-hydroxylase products of AA in regional kidney blood flow responses to the vasoactive agents we tested.

7.2.3 *Prostaglandins and nitric oxide in regional kidney blood flow responses to renal nerve stimulation*

The aim of the experiments described in Chapter 5 (Prostaglandins and nitric oxide in regional kidney blood flow responses to renal nerve stimulation) was to examine the role(s) of cyclooxygenase (COX) products, and the interactions between these and nitric oxide synthase, in modulating renal medullary vasoconstriction induced by renal nerve stimulation. A recent study in our laboratory demonstrated that nitric oxide synthase inhibition enhances responses of MLDF to renal nerve stimulation (69). However, even after nitric oxide synthase blockade, renal nerve stimulation induced greater reductions in CLDF than in MLDF, suggesting that nitrergic mechanisms do not completely account for the differential impact on CLDF and MLDF of renal nerve stimulation (69). In the experiments described in Chapter 5, we tested the responses of regional kidney perfusion to renal nerve stimulation in anaesthetized rabbits under control conditions, after COX inhibition, and then after subsequent nitric oxide synthase inhibition. Renal nerve stimulation induced reductions in RBF and CLDF, and to a lesser extent MLDF. The COX inhibitor ibuprofen did not significantly affect these responses. In contrast, subsequent N^G-nitro-L-arginine (L-NNA) treatment enhanced renal nerve stimulation induced reductions in RBF and CLDF but not MLDF. These data suggest that COX products *per se* do not contribute to the responses of CLDF and MLDF to stimulation of the renal nerves. On the other hand, endogenous nitric oxide appears to blunt renal nerve stimulation-induced vasoconstriction in the renal cortex. This effect of nitric oxide seems to be independent of the activity COX metabolites, as enhanced renal nerve stimulation-induced reductions in CLDF were also observed after nitric oxide synthase inhibition alone, in a previous study (69). In the present study, L-NNA had little effect on renal nerve stimulation-induced reductions in MLDF in ibuprofen pre-treated rabbits. Contrary to this observation, Eppel et al in our laboratory demonstrated that nitric oxide synthase inhibition can enhance MLDF responses to renal nerve stimulation in the presence of an intact COX system (69). As outlined in the Discussion section of Chapter 5, one interpretation of these findings is that endogenous nitric oxide blunts the vasoconstrictor effects of renal nerve stimulation in the vascular elements regulating MBF, chiefly by inhibition of vasoconstrictor prostaglandins. As described below, Ms Rebecca Flower in our laboratory, in collaboration with me, recently performed a supplementary study to test this hypothesis.

Surgical preparations These procedures have been outlined in detail in Chapter 2. All surgical procedures were identical to those described for the experiments in Chapter 5. Briefly, rabbits were anaesthetized with pentobarbitone sodium and were artificially ventilated. The left kidney was exposed via a left flank incision and was placed in a stable cup. The renal nerves running parallel to the left renal artery were then carefully isolated and placed across a pair of stimulating electrodes. The nerves were then sectioned proximally. A transit-time ultrasound flow probe was placed around the renal artery to measure RBF. A needle-type laser Doppler flow probe was inserted approximately 9 mm into the kidney with the use of a micromanupulater for measurement of MLDF and a standard plastic laser Doppler probe was placed on the surface of the kidney to measure CLDF.

Experimental protocol We designed this study in a manner which allowed us to compare the effects of different treatment regimens on intrarenal blood flow responses to renal nerve stimulation with the respective control responses in the same rabbit (as for the experiments described in Chapter 5).

The rabbits were subjected to three renal nerve stimulation periods (as described in Chapter 5). The first sequence of renal nerve stimulation served as the control response. Rabbits received the nitric oxide synthase inhibitor, L-NNA (20 mg/kg + 5 mg $kg^{-1}h^{-1}$) after the first stimulation sequence and ibuprofen (12.5 mg/kg + 12.5 mg $kg^{-1}h^{-1}$) after the second stimulation sequence (see Figure 7.1). This experimental sequence allowed us to test whether effects of L-NNA on responses of CLDF and MLDF to renal nerve stimulation depend on the presence of COX metabolites.

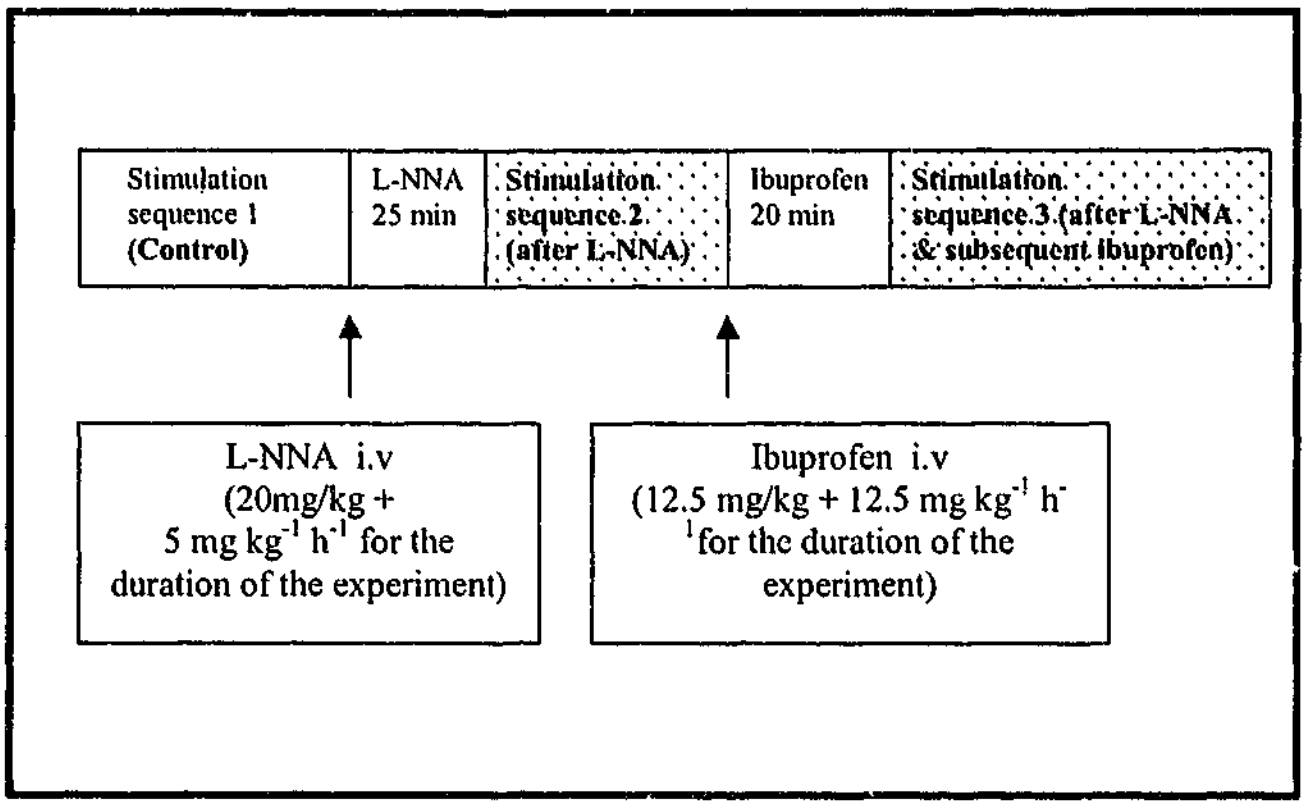

Figure 7.1 The experimental protocol for the study investigating the effects of N^G-nitro-L-arginine (L-NNA) and subsequent treatment with ibuprofen on responses of intrarenal blood flow to renal nerve stimulation in anaesthetized rabbits (n = 6). The voltage that produced the maximum reduction in renal blood flow was determined in each rabbit by stimulating the renal nerves with various voltages (3-10 V) at 5 Hz for 60 s. This pre-determined voltage was applied in all subsequent stimulation periods in that rabbit. Each stimulation sequence consisted of four, 3 min stimulus trains (0.75, 1.5, 3 and 6 Hz; 2 ms pulse duration). These frequencies were applied in random order. Recovery periods of 8-10 min were allowed between each stimulus train.

Effects of L-NNA and subsequent ibuprofen on baseline haemodynamic variables

L-NNA increased MAP (34 ± 9%), and reduced HR (-13 ± 4%) but did not significantly reduce RBF (-11 ± 5%) or CLDF (-1 ± 4%) (Table 7.1). MLDF tended to decrease (-24 ± 14%) after L-NNA treatment but this did not reach statistical significance (P_{Treat} = 0.06). Subsequent ibuprofen treatment did not significantly affect MAP, HR or MLDF, but reduced CLDF (-13 ± 4%) and tended to reduce RBF (-25 ± 16%; P_{Treat} = 0.06) (Table 7.1).

Table 7.1. Mean baseline levels of systemic and renal haemodynamic variables

	MAP (mmHg)	HR (beats/min)	RBF (ml/min)	CLDF (units)	MLDF (units)
Effects of N^G-nitro-L-arginine (L-NNA) and subsequent ibuprofen (n = 6)					
Control	70 ± 3	249 ± 10	28 ± 3	308 ± 29	80 ±12
L-NNA	91 ± 3***	210 ± 12**	25 ± 3	299 ± 26	55 ± 5
L-NNA + ibuprofen	94 ± 3	211 ± 17	22 ± 2	264 ± 25*	49 ± 8

Data indicate mean ± SE. MAP, mean arterial pressure; HR, heart rate; RBF, renal blood flow; CLDF, cortical laser Doppler flux; MLDF, medullary laser Doppler flux. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ indicate the outcomes of analysis of variance, testing whether baseline levels observed during stimulation sequence 2 differed from those observed during stimulation sequence 1 and whether baseline levels observed during stimulation sequence 3 differed from those observed during stimulation sequence 2, within each group of rabbits (P_{Treat}).

Effects of L-NNA and subsequent ibuprofen on responses to renal nerve stimulation L-NNA augmented renal nerve stimulation-induced reductions in RBF ($P_{Treat} = 0.002$), CLDF ($P_{Treat} = 0.03$) and MLDF ($P_{Treat} = 0.03$). For example, stimulation of the renal nerves at 1.5 Hz reduced RBF, CLDF and MLDF by -47 ± 6%, -50 ± 10% and -20 ± 6% respectively after L-NNA, but only by -36 ± 4%, -43 ± 7% and -10 ± 9% respectively before L-NNA (Figure 7.2). Subsequent ibuprofen treatment tended to blunt the responses to renal nerve stimulation at 1.5 Hz. However, when analyzed across all four frequencies, ibuprofen had little effect on the responses of RBF, CLDF and MLDF to renal nerve stimulation ($P_{Treat} \geq 0.12$) (Figure 7.2). Regardless of the treatment administered (L-NNA, or L-NNA plus ibuprofen), renal nerve stimulation always reduced RBF and CLDF more than MLDF ($P_{Region} < 0.001$).

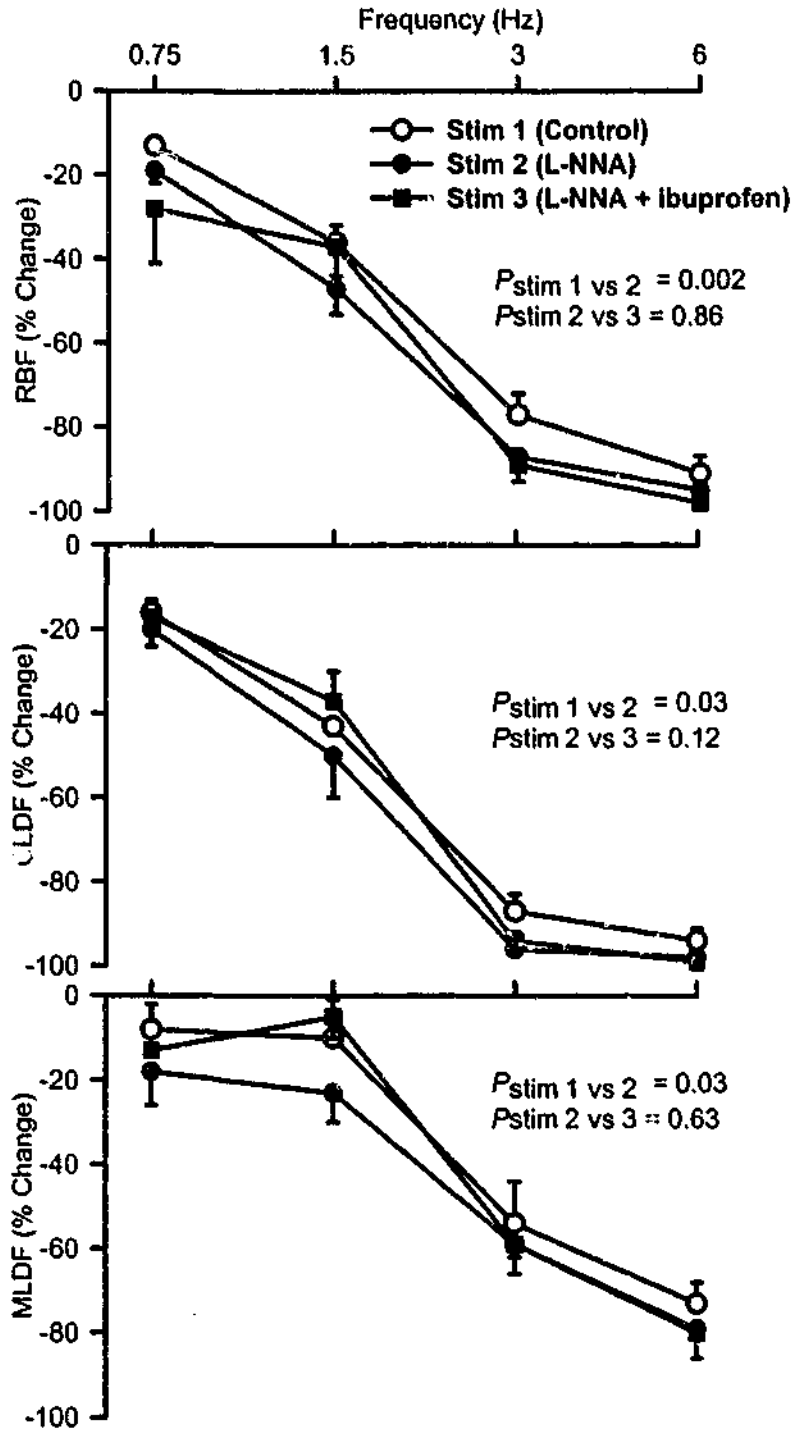

Figure 7.2. Effects of N^G-nitro-L-arginine (L-NNA; 20 mg/kg + 5 mg $kg^{-1}h^{-1}$) and subsequently ibuprofen (12.5 mg/kg + 12.5 mg $kg^{-1}h^{-1}$) on renal haemodynamic responses to renal nerve stimulation. Symbols and error bars indicate mean ± SE of data from 6 rabbits. *P* values indicate the outcomes of analysis of variance testing whether renal haemodynamic responses during stimulation sequence 2 (•; performed after commencing L-NNA-treatment) differed from those observed during the initial stimulation sequence (o), and whether renal haemodynamic responses during stimulation sequence 3 (■; performed after commencing ibuprofen treatment) differed from those observed during stimulation sequence 2.

The results of this experiment provide little support for the hypothesis that nitric oxide buffers renal nerve stimulation-induced medullary vasoconstriction by inhibiting the production of vasoconstrictor prostaglandins. If this hypothesis were true then one would expect COX inhibition to blunt the effects of L-NNA on responses of MLDF to renal nerve stimulation. Unfortunately, our results, at least in this respect, are equivocal. Thus, ibuprofen did appear to blunt the effects of L-NNA on responses of MLDF to 1.5 Hz stimulation, but not at other frequencies. Across the full range of stimulation frequencies tested, there was certainly no significant effect of ibuprofen on responses of MLDF to renal nerve stimulation ($P = 0.63$). However, our results at least allow us to more confidently reject the hypothesis that up-regulated production of vasodilator prostaglandins accounts for the relative insensitivity of the medullary circulation to renal nerve stimulation under conditions of nitric oxide synthase inhibition. Thus, we can confidently conclude that, while endogenous nitric oxide contributes to the relative insensitivity of the medullary circulation to neurally-mediated ischaemia, other mechanisms are also involved. Our present results provide strong evidence that these 'other mechanisms' do not include vasodilator prostaglandins, at least under the conditions of our experiment. The reason(s) that we could not detect an effect of L-NNA on responses of MLDF to renal nerve stimulation in ibuprofen pre-treated rabbits (Chapter 5) is not clear. This could be due to the inability of the laser Doppler technique to consistently detect small changes in MLDF. Because basal MLDF is lower than CLDF, the laser Doppler technique provides a lower signal to noise ratio when measuring the blood flow in the renal medulla. This will increase the coefficient of variation and make it more difficult to detect effects in the renal medullary microcirculation.

7.2.4 Endogenous angiotensin II and nitric oxide in neural control of intrarenal blood flow

The aim of the experiments described in Chapter 6 was to examine the possibility that endogenous angiotensin II, via activation of AT_1-receptors and subsequent release of nitric oxide, contributes to the relative insensitivity of MBF to renal nerve stimulation. We first tested the effects of the AT_1-receptor antagonist candesartan, on intrarenal blood flow responses to renal nerve stimulation. We also tested the effects of the nitric oxide synthase inhibitor L-NNA, with and without prior AT_1-receptor antagonism, on CLDF and MLDF responses to renal nerve stimulation. Candesartan blunted renal

nerve stimulation-induced reductions in CLDF and MLDF, suggesting that endogenous angiotensin II augments vasoconstrictior responses to renal nerve stimulation in both the cortical and medullary circulations. As previously shown (69), nitric oxide synthase inhibition enhanced CLDF and MLDF responses to renal nerve stimulation in control rabbits. Nitric oxide synthase inhibition enhanced responses of CLDF, but not MLDF, in rabbits pre-treated with candesartan. Collectively, our observations suggest that angiotensin II contributes to, while nitric oxide (as shown before) blunts, renal nerve stimulation-induced vasoconstriction in the renal cortical and the medullary circulations. This effect of nitric oxide seems to depend on the presence of endogenous angiotensin II in the renal medullary circulation, but not the cortical circulation.

7.3 Broader significance and future directions

7.3.1 CYP450 metabolites of AA in responses of intrarenal blood flow to hormones

The roles of CYP450 metabolites of AA in the regulation of renal, as well as pulmonary, cardiac and vascular function, remained relatively unknown until quite recently, partly because of limited availability of selective inhibitors of CYP450 enzyme cascades (212). We found that acute ABT-treatment (15mg/kg + 5 mg kg^{-1} h^{-1}) did not have a significant effect on CYP 450-dependent ω-hydroxylase or epoxygenase activity in the rabbit kidney (see Chapter 3). Recently, Santos et al demonstrated that chronic ABT-treatment (50 mg/kg *i.p* for five days) significantly reduced the formation of 20-HETE (by 80%) and EETs (by 60%) and blunted the pressure natriuresis mechanism in rats (224). These authors found that significant reductions in urinary excretion of 20-HETE were not observed until the third day of ABT-treatment (224), suggesting that chronic ABT-treatment is required to deplete the cellular phospholipid pools of CYP450 metabolites. Therefore, the study described in Chapter 3 should be repeated in rabbits chronically treated with ABT (50 mg/kg i.v) (224), to test the hypothesis that CYP450 metabolites of AA modulate responses of intrarenal blood flow to vasoactive hormones.

Dr John Falck (University of Texas Southwestern Medical Centre, Dallas, Texas, USA) provided us the selective epoxygenase inhibitor, MS-PPOH and the selective ω-hydroxylase inhibitor, DDMS. We decided that it would be more useful to first examine intrarenal blood flow responses to hormones in rabbits treated with DDMS and MS-

PPOH, before examining these responses in rabbits chronically treated with ABT. Due to the time restrictions of my PhD candidature I could not perform the latter part of this experiment. Our observation, that the selective epoxygenase inhibitor (MS-PPOH) revealed reductions in RBF and CLDF in response to V_1-receptor activation, predicts that chronic ABT- treatment should also have a similar effect on responses of RBF and CLDF to [Phe^2,Ile^3,Orn^8]-vasopressin. We examined the roles of EETs in modulating CLDF and MLDF responses to only two vasoconstrictor hormones ([Phe^2,Ile^3,Orn^8]-vasopressin and angiotensin II). Future studies should investigate the roles CYP450-dependent ω-hydroxylase and epoxygenase metabolites in modulating the responses of intrarenal blood flow to other vasoactive factors such as noradrenaline, endothelin-1, bradykinin and acetylcholine.

7.3.2 Novel approaches in examining the roles of CYP450 metabolites in regulation of intrarenal perfusion and blood pressure

At the time we performed the study described in Chapter 4, MS-PPOH and DDMS were the only selective CYP450 inhibitors available to us. We were unable to demonstrate effective inhibition of 20-HETE production in rabbit kidneys treated *in vivo* with DDMS. Our observation is consistent with those of Hoagland et al, who also reported that in their hands, DDMS does not effectively inhibit the production of 20-HETE *in vivo* (102). DDMS binds to plasma proteins and this limits its capacity to diffuse into target tissues to inhibit CYP450 metabolites of ω-hydroxylase (212). Therefore, DDMS should be effective in inhibiting products of ω-hydroxylase pathway *in vitro* but not *in vivo* when administered via a blood-borne route (212). More recent studies have shown that N-hydroxy-N – (4-butyl-2-methylphenyl)formamidine (HET-0016) is a highly selective inhibitor of the ω-hydroxylase pathway (102, 167). This compound is reported to selectively inhibit 20-HETE formation at very low concentrations (< 10 nM) (212) and to have little effect on other AA metabolite pathways (COX, lipooxygenase and CYP450 dependent epoxygenase) at concentrations up to 1 μM (167). Another major advantage of this compound (over DDMS) is that it dissolves in saline (102). Therefore, future studies aimed at investigating the role(s) of CYP450 dependent ω-hydroxylase metabolites in responses of intrarenal blood flow to vasoactive hormones or activation of the renal nerves should employ this new compound. Analogues of 20-HETE are available (212). The most effective 20-HETE analogue, 20-hydroxyeicosa-6(Z),15(Z),-dienoic acid appears to act as a 20-HETE antagonist (6), but this compound is

reported to be effective only when used under *in vitro* experimental conditions as it binds to plasma proteins (212). At present, it is difficult to selectively inhibit a production of a single metabolite of the ω-hydroxylase pathway due to the high homology among CYP4504A isoforms which are responsible for converting AA into various ω-hydroxylase metabolites (161). One sensible approach to overcome this problem would be to use specific antisense oligonucleotides directed against specific CYP450 isoforms responsible for production of 20-HETE. This approach appears to be effective in examining the roles of this metabolite under *in vivo* experimental conditions (161, 212).

We demonstrated that MS-PPOH is an effective inhibitor when used under *in vivo* experimental conditions. However, our data do not allow us to define the precise metabolite(s) of the epoxygenase pathway that are responsible for buffering the vasoconstrictor effects of V_1-receptor activation in the renal cortical circulation. Future studies could further delineate the epoxygenase metabolites responsible for antagonising V_1-receptor mediated cortical vasoconstriction by testing the CBF responses to [Phe^2,Ile^3,Orn^8]-vasopressin in animals pre-treated with a compound that inhibits the conversion of EETs to DiHETEs (eg trichloropropropylene oxide or 4-phenyl-chalcone oxide) (212). Future studies should also investigate whether V_1-receptor activation can increase the release of AA and/or EETs from cellular lipids in the renal cortex. This could be performed by measuring the levels of endogenous EETs and AA in renal cortical tissues incubated with a V_1-receptor agonist *in vitro*. Selective receptor antagonists for different epoxygenase metabolites should be useful in studying the specific roles of different EETs in underlying the differential regulation of regional kidney blood flow by nerves and hormones. These compounds await development (212). Other approaches that could be useful in examining the role(s) of specific CYP450 metabolites include over expression of specific proteins using gene transfer technology, use of specific antisense oligonucleotides directed against specific CYP450 isoforms, and use of gene knockout mice (161). Also, there is evidence that production of CYP450 metabolites is altered in different animal models of hypertension (32, 141). Future studies should attempt to identify CYP450 genes involved in regulation of renal and glomerular haemodynamics and blood pressure under normal physiological conditions, as polymorphisms and mutations in these genes could contribute to the development of human hypertension (177). In a recent review article on 20-HETE and

the kidney, McGiff and Quilley (161) stated that *"This area of research is in the logarithmic phase of its development, offering boundless opportunities to biological scientists. The challenges are great, the intellectual rewards are greater"*. This is indeed true and future investigations should attempt to further characterize the roles of CYP450 metabolites in regulation of regional renal perfusion and blood pressure with the use of the aforementioned novel biochemical and pharmacological tools.

7.3.3 Pathophysiological implications of modulation of V_1-receptor mediated cortical vasoconstriction by EETs

Vasopressin is an antidiuretic hormone which plays a central role in promoting water retention when plasma osmolarity is high, as would occur under conditions of haemorrhage and dehydration (208). Physiological increases in vasopressin concentrations (eg during mild dehydration) have been shown to selectively reduce MBF without affecting CBF (87). Under these conditions, a selective reduction in MBF would enhance water reabsorption chiefly through improving counter-current exchange mechanisms in medullary vasa recta (208). Sustaining RBF and CBF at normal physiological levels would ensure that parallel reductions in glomerular filtration rate (GFR) (and therefore sodium excretion) do not occur under conditions of dehydration. Our findings indicate that EETs can buffer reductions in RBF and CBF mediated by V_1-receptor activation under normal physiological conditions. Thus under pathophysiological conditions of insufficient formation of EETs, V_1-receptor activation could reduce RBF and possibly GFR.

There is evidence that conversion of EETs to DiHETEs is markedly increased during the developmental phase of hypertension in spontaneously hypertensive rats (SHR) (187). It is suggested that DiHETEs have considerably less potent vasodilator potency than EETs (212). The metabolism of EETs to DiHETEs is suggested to be a key mechanism for removing EETs from the biophase, particularly since DiHETEs do not get incorporated into membrane lipids as readily as EETs (86). Thus, during the developmental phase of hypertension in SHR the pools of lipid-stored EETs should be low (86). There is also evidence that plasma vasopressin concentrations are higher than normal in patients with severe hypertension and in various animal models of hypertension (241). Under these conditions of low concentrations of EETs and high concentrations of vasopressin, tonic V_1-receptor activation could cause chronic

reductions in RBF and GFR. In fact, vasopressin has been shown to induce greater reductions in RBF in SHR than in Wistar-Kyoto rats (WKY) under control conditions and after inhibition of COX (82). This suggests that vasodilator prostaglandins do not account for the differential sensitivity of the renal circulation to vasopressin between these two strains. Also, 6-week old SHR have been shown to have a lower RBF and GFR than age-matched WKY (63). In fact, reductions in RBF and GFR and sodium and water retention coincides with the rapid developmental phase of hypertension in SHR (21, 63) suggesting that chronic reductions in RBF and GFR could be central to the pathogenesis of hypertension. Our findings suggest that low levels of lipid-stored pools of EETs in SHR could contribute to the increased sensitivity of RBF to vasopressin in this strain of rats. This hypothesis merits investigation in the future.

Information regarding CYP450 activity in the human kidney under physiological and pathophysiological conditions is very limited. Formation of CYP450 metabolites has been shown to be enhanced in a patient with hypertension (228) but more data are clearly required to improve our understanding of the roles of CYP450 metabolites in development of human essential hypertension.

7.3.4 Paracrine factors and responses of intrarenal blood flow to renal nerves

Our data indicate that prostaglandins do not modulate responses of CBF and MBF to renal nerve stimulation, but that nitric oxide does (see Chapter 5). However, renal nerve stimulation reduced CLDF more than MLDF even after combined inhibition of nitric oxide synthase and COX. Therefore, other paracrine factors such as metabolites of CYP450 and lipoxygenase pathways could play key roles in protecting the renal medullary circulation from the ischaemic effects of renal nerve stimulation. We are not aware of any previous studies that have directly addressed this issue. We previously found that esculetin, an inhibitor of lipoxygenase pathways, enhanced responses of RBF and CLDF, and tended to enhance responses of MLDF, to noradrenaline. These data suggest that lipoxygenase metabolites buffer noradrenaline induced renal vasoconstriction (184). Future studies should therefore test the role(s) of CYP450 and lipoxygenase metabolites of AA in modulating responses of intrarenal blood flow to stimulation of the renal nerves.

7.3.5 *Does angiotensin II protect the renal medulla from the ischaemic effects of neurally mediated vasoconstriction?*

We demonstrated that L-NNA enhances responses of MBF to renal nerve stimulation in vehicle-treated rabbits, but not in rabbits pre-treated with the AT_1-receptor antagonist candesartan (see Chapter 6). One plausible interpretation of this observation is that the effects of endogenous nitric oxide on MBF responses to renal nerve stimulation depend on the presence of endogenous angiotensin II. Further studies are required to confirm this interpretation of our current data, and to further delineate the interactions between angiotensin II and nitric oxide in protecting the renal medullary circulation from neurally mediated vasoconstriction. In particular, it would be useful to determine whether the ability of exogenous angiotensin II, to blunt responses of MLDF to renal nerve stimulation (93), depends on an intact nitric oxide synthase. Experiments are already underway in our laboratory to examine this proposition.

Also, future studies should focus on the pathophysiological implications of this unusual action of angiotensin II, in protecting the renal medullary circulation from the ischaemic effects of renal nerve stimulation. These studies should examine intrarenal blood flow responses to renal nerve stimulation under conditions of increased levels of endogenous angiotensin II (eg. 2-kidney 1-clip hypertension) to test whether the sensitivity of MBF to renal nerve stimulation would be reduced or augmented, under these experimental conditions. These experiments are also now underway in our laboratory.

Recently, Dickhout et al, using microtissue strips of the vascular bundles of the outer medullary vasa recta of rats, demonstrated that angiotensin II can increase nitric oxide concentrations in (i) isolated medullary thick ascending limbs and (ii) pericytes of isolated vasa recta. However, pericite nitric oxide concentration only increased when they were closely associated with medullary thick ascending limbs (62). These authors concluded that within the outer medulla, angiotensin II induces nitric oxide formation in the tubular epithelium (but not in the vascular endothelium) and that nitric oxide diffuses from the tubular epithelium to vascular smooth muscle to buffer angiotensin II induced vasoconstriction (62). These findings challenge the generally held view that nitric oxide is chiefly released from the vascular endothelium. It would be of interest to repeat this experiment by Dickhout and colleagues (62) using rabbit outer medullary

vasa recta to determine whether this so called 'tubulovascular vascular nitric oxide cross talk' occurs in the rabbit renal medullary vasculature as well.

7.3.6 Angiotensin II receptors and intrarenal perfusion

Angiotensin II can exert vasoconstrictor effects in the cortical and medullary circulations (184, 185, 206). In addition, angiotensin II can also exert a paradoxical vasodilator effect in the renal medullary circulation. Duke et al (66) investigated the contribution of AT_1- and AT_2-receptors to angiotensin II-induced vasodilator and vasoconstrictor effects in the renal medullary and cortical circulations. These authors tested the responses of CLDF and MLDF to exogenous angiotensin II in either vehicle-, candesartan- (AT_1-receptor antagonist), PD123319- (AT_2-receptor antagonist), or combined candesartan plus PD123319-treated rabbits. They found that candesartan abolished, while PD123319 enhanced angiotensin II-induced reductions in RBF and CLDF (66). Candesartan also abolished the effects of PD123319 on responses of RBF and CLDF to angiotensin II (66). These observations suggest that AT_2-receptor mediated vasodilatation can blunt AT_1-receptor mediated vasoconstriction in the cortical circulation (66). Furthermore, Duke et al (66) observed that PD123319 revealed dose-dependent increases in MLDF in response to angiotensin II, which were abolished by candesartan treatment suggesting that AT_2-receptor activation can counteract AT_1-receptor mediated medullary vasodilatation.

More recently, Duke et al have shown that the AT_2-receptor antagonist PD123319 can increase basal MLDF by about 20% in the non-clipped kidney of rats with 2-kidney, 1-clip hypertension, and that this effect was abolished by AT_1-receptor antagonism with candesartan (Ms Lisa Duke; personal communication). Thus, in this model of pathophysiological increases in circulating and intrarenal angiotensin II (178), AT_2-receptor activation appears to blunt AT_1-mediated vasoconstriction. Taken collectively, my data (Chapter 6), those of Duke et al, and those of Dickhout et al (62) have led us to generate the hypothesis that AT_2-receptor activation limits AT_1-receptor-mediated nitric oxide release from thick ascending limbs of Henle's loop, and that during conditions of activation of the renin/angiotensin system (eg renovascular hypertension and sympathetic activation), angiotensin II-induced nitric oxide release will blunt reductions in MBF induced by extrinsic vasoconstrictor factors (eg nerves and hormones). This hypothesis is yet to be tested.

7.3.7 Pathophysiological implications of the actions of angiotensin II

Formation of reactive oxygen species such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) is increased in genetic and experimentally induced models of hypertension as well as in human essential hypertension (211, 249). Hypertension itself has also been suggested to induce the formation of reactive oxygen species leading to a vicious cycle (211). The outer medullary region of the kidney has been shown to have high concentrations of mitochondrial enzymes and nitcotinamide adenine dinucleotide oxidase that produce reactive oxygen species (269). Reactive oxygen species can react with and inactivate nitric oxide and thereby blunt nitric oxide-mediated vasodilatation. (211). So low bioavailability of renal nitric oxide observed in hypertension (122) could result from increased production of reactive oxygen species within the kidney. Increased renal sympathetic nerve activity is also observed in various animal models of hypertension and in human essential hypertension (59) and this in turn can increase renin release and probably also angiotensin II concentrations in the kidney (117). Angiotensin II can increase $O_2^{\cdot-}$produced by medullary thick ascending limbs of the loop of Henle *in vitro* (169). Under normal physiological conditions high bioavailability of nitric oxide could protect the renal medullary circulation from the ischaemic effects of $O_2^{\cdot-}$ (169). In contrast, under pathophysiological conditions of hypertension, increased production of reactive oxygen species could reduce the bioavailability of nitric oxide rendering the medullary circulation susceptible to the vasoconstrictor effects of $O_2^{\cdot-}$ (169).

We speculate that under these conditions of high circulation and intrarenal levels of angiotensin II, and low bioavailability of nitric oxide, the vasoconstrictor component of angiotensin II (which enhances renal nerve stimulation-induced reductions in MBF) would be potentiated, while the 'vasodilator' component of angiotensin II (which buffers neurally-mediated vasoconstriction in the medullary circulation) would be diminished. Therefore, in animal models of hypertension and in human essential hypertension, the sensitivity of the medullary circulation to vasoconstrictor effects of renal nerves could be greatly enhanced which could potentially lead to chronic reductions in MBF. This hypothesis remains to be tested. However, there is evidence that the sensitivity of the medullary circulation to the vasoconstrictor activity of angiotensin II is greatly enhanced in SHR (65) and Lyon hypertensive rats (225). This could contribute to the relatively low levels of MBF observed in SHR (compared with

WKY rats) (215) which in turn could increase sodium and water retention (by shifting the pressure natriuresis relationship to the right) and thereby contribute to the development of hypertension. Long- and short-term enalapril (angiotensin converting enzyme inhibitor) treatment has been shown to shift the pressure natriuresis relationship to the left in SHR, but the precise mechanism(s) by which this occurs remain unknown. One possibility is that ACE-inhibition could increase MBF by elevation of nitric oxide bioavailability, which buffers angiotensin II-induced reductions in MBF. In support of this notion, Dukacz et al. have shown that nitric oxide synthase inhibition can cause greater reductions in MBF in response to angiotensin II in SHR treated chronically with enalapril, than in control SHR. This suggests that chronic ACE-inhibition increases the basal levels of MBF in SHR by increasing renal nitric oxide bioavailability, and that this in-turn buffers angiotensin II-induced reductions in MBF (65) .

7.4 Measurement of regional renal perfusion in humans

Recent advances in technology have made it possible to measure regional kidney perfusion in humans. Some of the techniques that could be used to measure regional renal blood flow in humans include positron emission tomography (8, 133), computed tomography (133, 218), and magnetic resonance imaging (40, 133, 204, 210, 256). Roberts et al, using magnetic resonance imaging, estimated that CBF and MBF averaged 278 ± 55 ml.100 $g^{-1}\cdot$ min^{-1} and 55 ± 25 ml. 100 $g^{-1}\cdot$ min^{-1} respectively, in seven human subjects (210). However, conventional magnetic resonance imaging techniques have been reported to be of limited use, particularly if there is low perfusion in the kidney, since these techniques have a low signal to noise ratio (227). A recent study demonstrated that use of dynamic susceptibility-weighted gradient-echo imaging in combination with an intravascular ultra small particle iron oxide contrast agent, can be used for measurement of regional renal perfusion in humans, even when blood flow to the kidney is reduced by more than fifty percent (227). With the use of these novel techniques, future studies should aim to characterize the role(s) of CYP450 metabolites of AA, angiotensin II and nitric oxide in regulation of intrarenal perfusion and glomerular haemodynamics in hypertensive patients. Findings of these studies could be useful in developing effective treatment(s) for human essential hypertension.

7.5 Conclusions

The role of the renal medullary circulation in long-term blood pressure regulation is now well established. We and others have previously shown that hormonal and neural factors can differentially regulate CBF and MBF. This likely represents an important regulatory mechanism in long-term blood pressure control but we are only beginning to understand the mechanisms underlying it. The findings of the experiments described in this thesis suggest that (i) EETs underlie the relative insensitivity of the cortical circulation to V_1-receptor activation. (ii) neither prostaglandins *per se*, nor interactions between prostaglandins and nitric oxide, modulate responses of intrarenal perfusion to renal nerve stimulation and (iii) angiotensin II, and interactions between angiotensin II and nitric oxide, play important roles in modulating responses of MBF to renal sympathetic nerve activation. More studies are clearly required to further delineate the mechanisms that underlie the differential control of CBF and MBF by various hormonal and neural stimuli. An understanding of how these mechanisms operate under normal physiological conditions is of great interest, as failure of these mechanisms could be central to development of hypertension and other related cardiovascular diseases.

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