MECHANISMS OF STEROID-INDUCED HYPERTENSION IN MAN AND RAT

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Declaration

The work in this thesis contains no material submitted for the award of any other degree or diploma in any university. The work described in this thesis is directly attributable to my own research. Acknowledgement has been made where I have benefitted from collaboration with individuals other than my supervisors.

George J Mangos

June 1999

Foreword

The work presented in this thesis is concerned with mechanisms of steroid-induced hypertension in man and rat. Each chapter has its own introduction and conclusions and can be read independently.

The aim of this thesis is to examine mechanisms by which adrenal steroids raise blood pressure in man and rat. There is large literature describing the physiology of steroid excess in experimental models in rat, sheep and man in ACTH-induced hypertension, dating back to work which commenced in the 1970s at the Howard Florey Institute of Medical Research in Melbourne (Whitworth, 1977). A key feature of this body of research is the dissociation of sodium and water retention from the blood pressure raising effects of ACTH and cortisol in man (Connell, Whitworth et al, 1988).

Some of the mechanisms of ACTH- and steroid-induced hypertension which have been explored during this time include the role of the SNS (Sudhir, Jennings et al, 1989, Tam, Williamson et al, 1997, Macefield, Williamson et al 1998), delineation of the haemodynamic profiles (Connell, Whitworth et al, 1987) and the actions of a variety of vasoactive hormones (Whitworth, van Leeuwen et al, 1984, Connell, Whitworth et al, 1987, Connor, Whitworth et al, 1987).

Since the 1970s, significant discoveries have occurred which have further increased our understanding of cardiovascular pathophysiology. These

include; the understanding of endothelial function - the Nobel prize winning discovery of the endothelial derived relaxing factor in 1980 (Furchgott and Zawadzki, 1980) and the subsequent description of nitric oxide accounting for this factor in 1987 (Palmer, Ferrige et al, 1987), the discovery of endothelin (Yanagisawa, Kurihara et al, 1988), the description of the various natriuretic peptides, the introduction of new classes of antihypertensive agents such as the angiotensin converting enzyme inhibitors and more recently the angiotensin II receptor antagonists and the description of new vasoactive hormones such as parathyroid hypertensive factor (Lewanczuk, Wang et al, 1989) and the dilator adrenomedullin (Kitamura, Kangawa et al, 1993).

In this thesis, I have examined some of the mechanisms by which ACTH and steroid hormones raise blood pressure. In man, I have examined the role of endothelial dilator function in cortisol-induced hypertension and whether the endothelial dysfunction observed in this model of hypertension can be attributed to GR- or MR-mediated effects. In the rat, I have examined whether ACTH-induced hypertension is explicable in terms of ACTH-induced glucocorticoid production (corticosterone) as it is in man (cortisol) (Whitworth, Saines et al, 1984). Further, I have examined the role of the parathyroid glands (and the putative parathyroid hypertensive factor) in ACTH-induced hypertension in the rat.

Acknowledgements

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My supervisors, Professor JA Whitworth and Dr JJ Kelly have provided invaluable support and guidance in the planning, execution and analysis of these studies and to them I am very grateful. I am also indebted to Associate Professor Mark Brown for his supervision of the earlier experiments and his expert advice.

Dr Brian Walker and Professor David Webb collaborated with our group in the human studies and provided expertise in the technical aspects of establishing bilateral strain gauge venous plethysmography in the laboratory at St George Hospital. The human studies described were performed entirely by myself at St George Hospital. I am grateful to Johneen Tierney and Anthony Galuzzo of the Pharmacy, St George Hospital, for their assistance in randomization of the human studies and preparation of drugs. I am also

grateful for technical assistance provided by Jane Lawson, Arlene Robinson, Allison Martin and Paula Williamson in these studies.

I performed the rat studies at St George Hospital. In 1995, I travelled to the University of Alberta, Edmonton, Canada, where I performed a small series of experiments in the laboratory of Dr RZ Lewanczuk, to whom I am grateful (Chapter 8). I am particularly indebted to Steven Turner for his generous assistance with the series of corticosterone experiments (Chapter 6), where he provided assistance with blood pressure and metabolic measurements and analysis of plasma nitrate/nitrite concentration. I am also grateful for the advice and technical assistance provided by Dr Cheng Wen, Dr Ming Li, Tafline Fraser and John Ramunni in these experiments. Novartis generously provided ACTH for use in these experiments.

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I would also like to thank Dr Therese Jacques for allowing me to use the Intensive Care blood gas analyser and Dr Peter Varelis, Nuclear Medicine, St George Hospital, for his assistance with peptides.

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Publications & presentations arising from this thesis

PUBLICATIONS

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Kelly JJ, **Mangos GJ**, Williamson PW, Whitworth JA. Cortisol and hypertension. Clinical and Experimental Pharmacology and Physiology 1998. 25 (suppl): S51 - S56.

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Mangos GJ, Fraser TB, Turner SW, Whitworth JA. The role of the parathyroid glands in adrenocorticotrophin-induced hypertension in the rat. Clinical and Experimental Hypertension 1999.

SUBMITTED

Mangos GJ, Walker BRW, Kelly JJ, Lawson JA, Webb D, Whitworth JA. Cortisol inhibits cholinergic vasodilatation in the human forearm (1999).

IN PREPARATION

Mangos GJ, Walker BRW, Kelly JJ, Lawson JA, Whitworth JA. The effects of fludrocortisone and dexamethasone on forearm blood flow in man (1999).

Mangos GJ, Turner SW, Fraser TB, Whitworth JA. Are the features of adrenocorticotrophin-induced hypertension in the rat explained by the adrenal production of corticosterone ? (in preparation) 1999.

PRESENTATIONS

"Endothelial function in steroid-induced hypertension." Oral presentation at the Annual Scientific Meeting of the Australian and New Zealand Society of Nephrology, Brisbane, March 1999.

"Forearm vascular responses to acetylcholine in glucocorticoid-treated subjects." Oral presentation at the International Society of Hypertension Scientific Meeting in Amsterdam, June 1998.

"Inhibition of cholinergic vasodilatation in the human forearm is not mediated by mineralocorticoid or glucocorticoid receptors." Poster presentations at the St George Hospital Annual Symposium and the High Blood Pressure Research Council Annual Scientific Meeting, Melbourne, November and December 1998.

"Parathyroid hypertensive factor in the SHR." Poster presentation at the International Society of Nephrology Scientific Meeting, Sydney, May 1997.

"Corticosterone administration mimics ACTH-induced hypertension in the rat and is prevented by L-arginine." Poster and oral presentations at the St George Hospital Medical Symposium and the Annual Scientific Meeting of the High Blood Pressure Research Council of Australia, Fremantle, November and December, 1997.

"The role of the parathyroid glands in adrenocorticotrophin-induced hypertension in the rat." Oral presentation at the scientific meeting of the NSW branch of the Australian Society of Medical Research, Sydney NSW, 1996.

"Forearm vascular responsiveness to acetylcholine in cortisol induced hypertension." Oral presentations at the St George Hospital Annual Symposium and the High Blood Pressure Research Council Scientific Meeting, Melbourne, November and December 1996.

Abstract

Models of steroid-induced hypertension in man and rat have been well characterized but the mechanisms by which ACTH and glucocorticoids raise blood pressure are not fully understood. Recently described paracrine (eg endothelial nitric oxide) and humoral (eg PHF) factors may be important in human essential hypertension. These factors were examined in cortisol-induced hypertension in man and ACTH-induced hypertension in the rat respectively. In man, the haemodynamic effects of ACTH can be attributed to the adrenal production of cortisol, but whether the major rodent glucocorticoid corticosterone is responsible for ACTH-induced hypertension in the rat has not been resolved. This question was examined in these studies.

In male volunteers, exogenous cortisol raised blood pressure and suppressed endothelium-dependent vasodilatation, by a mechanism which may be nitric oxide synthase dependent. Although dexamethasone and fludrocortisone also raised blood pressure, attenuation of cholinergic vasodilatation was not observed. From these studies, the data suggest that the effect of cortisol on endothelium-dependent vasodilatation is unique to the endogenous hormone and not reproduced by synthetic agonists of GR or MR. Impaired endothelial vasodilator function may contribute to cortisol-induced hypertension in man.

In the rat, exogenous corticosterone, administered in doses to achieve circulating concentrations similar to those observed in the experimental

model of ACTH excess, reproduced the haemodynamic and some of the metabolic changes which characterize ACTH-induced hypertension. Further, like ACTH-induced hypertension, corticosterone-induced hypertension was prevented by L- but not D-arginine, and this effect was completely prevented by NOLA. It is likely that adrenal corticosterone mediates the hypertensive effects of ACTH excess.

Parathyroidectomy had no significant effect on the rise in blood pressure secondary to ACTH excess. It is unlikely that PHF contributes to the model of ACTH-induced hypertension in the rat. The bioassay for the measurement of PHF could not be reproduced in our laboratory, leaving a question mark about the relevance of this putative factor in hypertension research.

Abbreviations used in this thesis

3β-HSD	3β-hydroxysteroid dehydrogenase
11β-HSD (1 or 2)	11β-hydroxysteroid dehydrogenase type (1 or 2)
ACTH	adrenocorticotrophin
allo-THF	allo-tetrahydrocortisol
AME	apparent mineralocorticoid excess
ANP	atrial natriuretic peptide
AS	aldosterone synthase
B	corticosterone
C11β	11β-hydroxylase
C17α	17α-hydroxylase
C21	21-hydroxylase
CAH	congenital adrenal hyperplasia
CBG	corticosteroid-binding globulin
DBP	diastolic blood pressure
DHEA(-S)	dehydroepiandrosterone(-sulphate)

DNA	deoxyribonucleic acid
DOC(A)	deoxycorticosterone (acetate)
E	cortisone, Kendall's compound E
ECFV	extracellular fluid volume
F	cortisol
GALFs	glycyrrhetinic acid like factors
GR	glucocorticoid receptor(s)
GRE	glucocorticoid response element
GSA	glucocorticoid-suppressible hyperaldosteronism
HSD11B(1 or 2)	gene encoding the 11β-HSD(1 or 2) enzyme
hGR	human glucocorticoid receptor
ICV	intracerebroventricular
iNOS	inducible nitric oxide synthase
K_d	binding affinity
K_M	the Michaelis constant

hGR (α or β)	human glucocorticoid receptor (α or β)
MR	mineralocorticoid receptor
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NOLA	n-nitro L-arginine
NOS	nitric oxide synthase
QC	quality control
PHF	parathyroid hypertensive factor
RFLP	restriction fragment length polymorphism
SBP	systolic blood pressure
SCC	side chain cleavage enzyme (desmolase)
SNS	sympathetic nervous system
THF	tetrahydrocortisol

Errata

v para 2, line 2 “There is large…” should read “There is a large…”

xv line 6, add "GA glycyrrhetinic acid"

p4 para 3, The following should be added to the end of the paragraph, “ACTH stimulates adrenal steroidogenesis and favors the pathway to cortisol and adrenal androgens.”

p20 Table 1.1 should include, under the heading "ACTH-independent", "Food-induced nodular hyperplasia"

p21 para 1, line 9, “… formation. However, the …” should read “… formation. However, most pituitary tumours are monoclonal, a finding against the notion of tonic stimulation by hypothalamic releasing factors (Herman, Fagin et al, 1990).”

p26 para 2, line 8 “incipidus” should read “insipidus”

p48 para 2, line 4 “Morris et al” should read “Brem et al”

p59 para 2, line 9, “(Tam, Williamson et al, 1997)” should read “(Tam, Kelly et al, 1996)”

p55 para 2, line 12 “mmHg” should be omitted

p77 para 2, line 4, “… by pharmacy.” Should read “… by pharmacy, with randomisation achieved by use of a computer random number generator.”

p79 para 1, line 5, the following should be added, “The two methods of measurement of serum cortisol were not formally correlated, as these measurements were primarily for the determination of subject

compliance (ie increase in serum cortisol during cortisol therapy and suppression during dexamethasone therapy). Comparisons of cortisol concentrations between studies were not relevant."

p100 para 4, line 4, “4 %” should read “4^{O} C”

p104 para 1, line 2, "… is 3.2 - 4.9 %." should read "… is, at ACTH concentration 152 pg / ml, 4.9 %, and at ACTH concentration 416 pg / ml, 3.2 %."

p121 line 12, “3.2.1…” should read “3.3.3…”

p180 para 2, line 1, ”… to administered…” should read “…to be administered…”

p188 para 1, line 8, “001” should read “0.001”

p214 para 2, line 3, the following should be added, “I chose the SD rat as a normotensive model, in which parathyroidectomy has previously been shown not to affect blood pressure (Pang and Lewanczuk, 1989). Therefore, any effect of parathyroidectomy on blood pressure in ACTH treated rats, would be an effect of ACTH, possibly mediated by PHF.”

p231 para 2, line 1, “structured” should read “structure”

p272 line 12, additional reference: **Herman V, Fagin J, Gonsky R et al.** Clonal origin of pituitary adenomas. *Journal of Clinical Endocrinology and Metabolism* 1990;71:1427-33.

CHAPTER 1

The role of cortisol in human hypertension

1.1 INTRODUCTION

This chapter will review the literature on cortisol in human hypertension and the mechanisms by which cortisol (and the major glucocorticoid in the rat, corticosterone) are thought to raise blood pressure. It is well established that cortisol is involved in several rare forms of hypertension such as Cushing's disease and apparent mineralocorticoid excess. The role of cortisol in the more common forms of human hypertension, such as essential hypertension and chronic renal failure, is less clear but there is accumulating evidence implicating cortisol as a factor in these forms of hypertension. Much of the understanding of the mechanisms by which glucocorticoids raise blood pressure has come about from experimental models of ACTH- and cortisol-induced hypertension in man and similar models of hypertension in the rat. These mechanisms will be discussed.

1.2 THE ADRENAL GLANDS, STEROID BIOSYNTHESIS AND METABOLISM

1.2.1 History of the adrenal glands

Though the adrenal glands were described and illustrated by the Italian anatomist Bartholomaeus Eustachius (1524-1574) in 1563, the descriptions of the Danish physician Caspar Bartholin the Elder (1585-1629) (Bartholinus,

1611) were the first to receive significant attention because the illustrations of Eustachius were hidden in the Papal library and remained unknown until the early 18th century.

By the beginning of the 19th century it was generally accepted that these glands secreted a substance or substances into the blood (Munro Neville and O'Hare, 1982, Sorkin, 1957). The first detailed histological descriptions of the adrenal glands were given by Ecker in 1846 (Ecker, 1846). In his monograph in 1855, Thomas Addison described the clinical features and autopsy findings of 11 patients with adrenal failure (Addison, 1855). Prompted by these descriptions, Brown-Sequard provided further evidence as to the vital nature of the adrenals in his experiments in 1856. In these studies, adrenalectomy resulted in early death of animals (Brown-Sequard, 1856). However, it was another 80 years before significant progress into characterization of the products of the adrenal cortex occurred. At this time, three groups independently used adrenal cortical extracts to successfully extend the life of adrenalectomized animals (Rogoff and Stewart, 1928, Hartman, Brownell et al, 1928, Swingle and Pfiffner, 1930). Subsequently, Swingle and Pfiffner's cortical extract was used with success in 1930-32 to produce dramatic remissions in cases of Addison's disease.

Richstein and Kendell then isolated over two dozen steroid compounds from beef and hog adrenals. Each of these substances had the basic cyclopentanophenanthrene nucleus. It was paper chromatography that led to

the confirmation of cortisol and corticosterone as the major secretory products of the adrenal cortex in man and other species (Nelson, 1980). In 1932, Cushing's classic monograph *The basophil adenomas of the pituitary body and their clinical manifestations* was published (Cushing, 1932). In the period 1934-38, the major products of the adrenal cortex were prepared.

1.2.2 Biosynthesis of glucocorticoids and mineralocorticoids (Figure 1.1)

Aldosterone is the major steroid secreted by the zona glomerulosa. It has long been recognised that this hormone is largely independent of the adenohypophysis, but influenced by sodium and potassium intake (Haber, Sancho et al, 1975, Himathongkam, Dluhy et al, 1975). The major factors regulating aldosterone secretion are angiotensin II, which is a potent stimulus, and plasma potassium concentration. ACTH has only a minor and transient effect on aldosterone secretion (Fredlund, Saltman et al, 1975).

The zona fasciculata is the only human tissue which produces and secretes cortisol. It is under regulation of pituitary ACTH modulated by ACTH-sensitive 11β-hydroxylase (Tortora and Anagnostakos, 1990, White, 1996).

The function of the zona reticularis is less well characterized. It produces small amounts of adrenal androgens (predominantly DHEA, androstenedione and 11OH-androstenedione), under the regulation of ACTH, not gonadotropins. DHEA and androstenedione are then converted by

extraglandular tissues to the potent androgen, testosterone. The zona reticularis does not clearly differentiate from the zona glomerulosa and zona fasciculata until about the third year of life. In anencephalic infants, the adrenal glands are hypoplastic due to absence of the pituitary gland and inadequate release of ACTH, necessary for the development of the adrenal cortex.

The adrenal medulla produces and secretes catecholamines, adrenaline and noradrenaline into the circulation. It is derived from neural cleft cells (neurectoderm), whereas the adrenal cortex develops from mesoderm (Moore, 1982). In mice with a null mutation for the GR, the adrenal medulla fails to develop, consistent with the notion that this process is dependent upon an intact hypothalamic-pituitary axis (Cole, Blendy et al, 1995).

1.2.3 Cortisol synthesis, secretion, action and metabolism

Cortisol is the biologically active glucocorticoid produced by the human adrenal cortex. It is synthesised from a common precursor of all corticosteroid hormones, cholesterol. The conversion of cholesterol to cortisol consists chiefly of a series of hydroxylations and oxidations (Kannan, 1988). There are five crucial steps (Figure 1.1):

1. conversion of cholesterol to pregnenolone, mediated by cholesterol side-chain-cleavage enzyme (SCC, desmolase),

2. pregnenolone then undergoes two major reactions: the formation of 17-hydroxypregnenolone by 17-hydroxylation (C17α, 17-hydroxylase) or conversion to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD),

3. 17-hydroxyprogesterone is then produced by either 17-hydroxylation of progesterone or from 17-hydroxypregnenolone by 3β-hydroxysteroid dehydrogenase,

4. 11-deoxycortisol (compound S) is then produced by further hydroxylation at the C-21 position of 17-hydroxyprogesterone by 21-hydroxylase (C21),

5. finally, cortisol (compound F) is produced by conversion from 11-deoxycortisol by hydroxylation at the C-11 position. This is mediated by the action of 11β-hydroxylase (C11β).

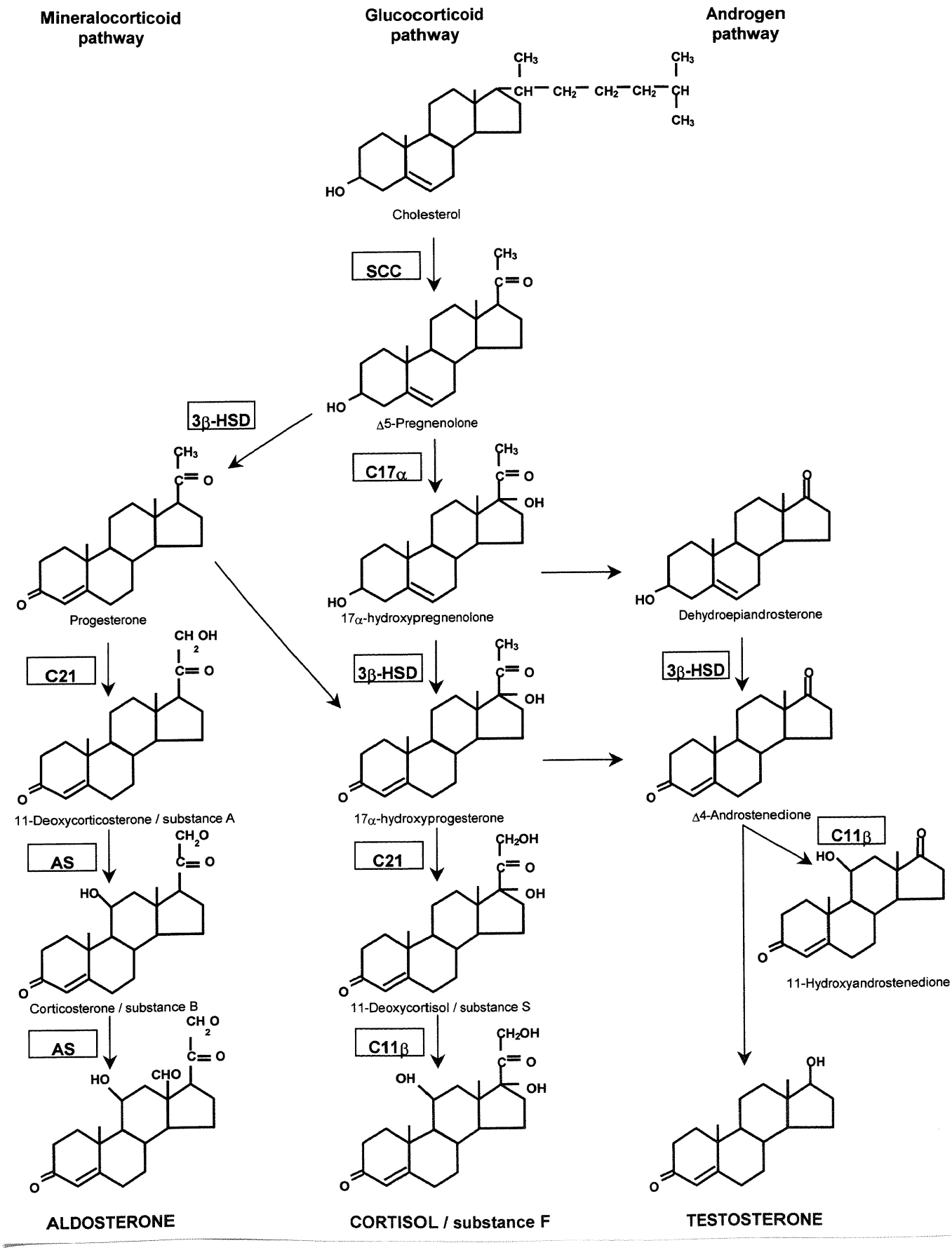


Figure 1.1 Schematic diagram of steroid biosynthesis

As rodents lack 17-hydroxylase (steps 2, 3), hydroxylation of the 11- and 21-carbons results in corticosterone (compound B) circulating as the major glucocorticoid.

1.2.4 Regulation of cortisol secretion

The production of cortisol by the zona glomerulosa is regulated by pituitary ACTH. ACTH itself is a 39 amino acid peptide (Johl, Riniker et al, 1974) produced and secreted from the anterior lobe of the pituitary (Smith, 1930). It is under the control of the hypothalamic hormone corticotrophin releasing factor (CRF). ACTH has a circadian rhythm along with a pulsatile pattern, with peak concentrations occurring in the morning hours (Iranmanesh, Lizarralde et al, 1990). Cortisol is normally secreted in a series of 'spikes', rather than in a steady continuous fashion. Walker and co-workers have reported a seasonal variation in glucocorticoid activity in healthy men, reflected by increased plasma cortisol and tissue sensitivity to glucocorticoids in winter (Walker, Best et al, 1997). Regulation of cortisol secretion is by trophic control of ACTH and by negative feedback by cortisol on both the hypothalamus and the pituitary gland.

Aldosterone, though influenced by ACTH (the effect of ACTH on aldosterone secretion is transient)(Connell, Whitworth et al, 1987), is strongly under the control of angiotensin II. Adrenal sex steroid secretion is under the control of ACTH, though in man the amounts of sex steroids of adrenal origin are small (Tortora and Anagnostakos, 1990).

1.2.5 Steroid receptors and metabolism

GR and MR belong to the super family of DNA-binding nuclear receptors that include thyroid hormone and vitamin D receptors (Mangelsdorf, Thummel et al, 1995). Historically, adrenocorticosteroids were classified according to the physiological responses they evoked. It is now clear that different receptors modulate these vastly different effects.

Specific roles for the GR and MR have recently been demonstrated by the generation of mice with a null mutation for either receptor. GR knockout mice die shortly after birth due to severe lung atelectasis as well as failure of development of the adrenal glands (Cole, Blendy et al, 1995). MR knockout mice die at around day 10 after birth, as a result of a salt-wasting syndrome (Berger, Bleich et al, 1998, Farman, 1999). Survival of the MR -/- homozygotes was prolonged by administration of the exogenous glucocorticoid betamethasone, but only by 17 days, indicating that activated GR cannot replace MR function in aldosterone sensitive cells.

1.2.5.1 The human glucocorticoid receptor

Activation of the GR mediates chronic effects on carbohydrate, protein and lipid metabolism, in a manner opposing many of the effects of insulin. Glucocorticoids increase blood sugar levels, cause protein catabolism, eosinopaenia and redistribution of body fat. GR are expressed in all nucleated normal cells, consistent with the widespread effects of glucocorticoids on metabolism, differentiation and development (Funder, 1996).

The hGR was cloned in 1985 and its gene has been mapped to chromosome 5 (Hollenberg, Weinberger et al, 1985). Two receptor isoforms were described, the active hGRα (777 amino acids) and hGRβ (742 amino acids) which differ in their carboxy terminii. hGRα is a ligand-activated transcription factor, which, when in the hormone bound state, modulates the expression of glucocorticoid-responsive genes by binding to specific DNA sequences. In contrast, hGRβ does not bind glucocorticoids and is transcriptionally inactive (Bamberger, Bamberger et al, 1995). Recently, hGRβ has been shown to act as a negative regulator of activated hGRα. Hormone binding studies have shown that hGRβ does not alter the affinity of hGRα for its ligand. hGRβ is present in nuclear extracts and is able to bind radiolabelled GRE suggesting that its inhibitory effect may be due to competition for GRE target sites (Bamberger, Bamberger et al, 1995). hGRβ may therefore be important as a physiological or pathological regulator of target tissue sensitivity to glucocorticoids (Bamberger, Schulte et al, 1996). More recently, the two isoforms of hGR have been reported to have opposing effects on mineralocorticoid activity (Bamberger, Bamberger et al, 1997). GR distribution within the body is almost ubiquitous (Bonvalet, 1998).

1.2.5.2 The human mineralocorticoid receptor

Mineralocorticoids are mainly involved with sodium and water homeostasis by the epithelial cells of the kidney (in the distal tubule and collecting duct), colon and parotid glands. In contrast to GR, MR are expressed in specific tissues only (Bonvalet, 1998).

The MR was cloned in 1987 (Arriza, Weinberger et al, 1987). It is a 984 amino acid protein expressed both in classical aldosterone-sensitive tissues and other non-epithelial tissues, such as hippocampus and heart (Funder, 1996). Two isoforms of the gene encoding MR have been described. Each isoform is expressed at approximately equivalent levels in the human kidney, colon, sweat glands and cardiomyocytes (Farman, 1999, Zennaro and Lombes, 1998). The functional significance of these isoforms is not known.

1.2.5.2.1 Mineralocorticoid receptor selectivity - a series of filters

The MR has the same affinity for aldosterone and circulating glucocorticoid hormones (cortisol in man, corticosterone in rodents). Because glucocorticoids circulate in concentrations of 2-3 orders of magnitude greater than aldosterone, the MR would be overwhelmingly swamped by glucocorticoids in the absence of mechanisms to exclude them in aldosterone sensitive tissues, leading to mineralocorticoid-excess syndromes and hypertension. Indeed, in non-aldosterone-selective cells, MR are probably occupied by glucocorticoid most of the time (Funder, 1993). A series of selectivity filters modulates access to MR by glucocorticoids leading to hormone-specific and cell-specific aldosterone regulation of cellular functions (Farman, 1999).

1.2.5.2.1.1 MR selectivity: circulating factors influencing MR specificity

Aldosterone normally circulates in the nanomolar range, whereas glucocorticoid hormones may reach micromolar range in stress, ie 10^2-10^3 fold excess. Glucocorticoids are bound to a great extent by CBG (90 %),

reducing the concentration of free hormone by one order of magnitude. In contrast, most aldosterone is not bound to CBG (Farman, 1999).

1.2.5.2.1.2 MR selectivity: metabolism of glucocorticoids

Corticosteroids enter cells by diffusion. Glucocorticoids are prevented from binding to the MR in certain tissues by an enzyme, 11β-HSD2, which metabolizes cortisol to cortisone (in man) or corticosterone to 11-dihydrocorticosterone (in rodents), as discussed in detail below. MR and 11β-HSD2 are found together in high concentration in the distal tubule of the kidney and the colon, ie mineralocorticoid sensitive tissues (Mercer and Krozowski, 1992, Hirasawa, Sasano et al, 1997). Despite the high activity of 11β-HSD2, it is likely that small amounts of glucocorticoids 'escape' or 'leak' from metabolism and may act as ligands for MR (Funder, 1997, Farman, 1999, Funder and Myles, 1996). Little is known about the regulation of 11β-HSD2 activity or its transcription rate and how this could affect mineralocorticoid actions (Farman, 1999). Recently, Alfaidy et al have provided the first evidence for the regulation of the 11β-HSD2 enzyme (in rat cortical collecting duct) by the synergistic action of arginine vasopressin and aldosterone on setting MR selectivity (Alfaidy, Blotchabaud et al, 1997).

Other enzymes may play a similar protective role for MR in other cell types. In the avian intestine, for example, 20β-hydroxysteroid dehydrogenase rather than 11β-HSD2 may be important in MR protection (Vylitova, Miksik et al, 1998). 6β-hydroxylase has been reported to protect the MR in other

mineralocorticoid sensitive tissues (the A6 cell line, derived from the kidney of *Xenopus laevis*) in the absence of 11β-HSD (Morris, Latif et al, 1998). The role of these enzymes on MR selectivity in man is not known.

1.2.5.2.1.3 MR selectivity: restricted expression of the MR

MR have been identified in colonic epithelium and the ducts of sweat and salivary glands in man, where aldosterone also stimulates sodium reabsorption (Hirasawa, Sasano et al, 1997). Both MR and 11β-HSD2 were expressed in colonic and renal distal tubular cells as well as the absorptive epithelia of duodenum, jejunum and ileum and excretory ducts of anal and oesophageal glands. Interestingly, 11β-HSD2 and MR immunoreactivity co-localized in many cells of the respiratory tract and type-II alveolar cells as well as in skin (Hirasawa, Sasano et al, 1997). Other non-epithelial human cells have been shown to express MR, such as cardiac myocytes (which also express 11β-HSD2 activity) (Lombes, Alfaidy et al, 1995) and neurons of the hippocampus (Arriza, Simerly et al, 1988), however, the functional significance of aldosterone action in these cells is largely undetermined (Farman, 1999).

1.2.5.2.1.4 MR selectivity: MR discrimination between ligands

Although glucocorticoid and aldosterone bind to MR with equal affinity, it has recently been reported that steroid:MR complexes behave differently depending on whether the steroid is aldosterone or a glucocorticoid. Aldosterone-MR complexes are more stable than glucocorticoid-MR complexes (Lombes, Kenouch et al, 1994), the efficiency and rate of nuclear

translocation of the steroid-MR complexes are lower with glucocorticoid than with aldosterone (Lombes, Binart et al, 1994) and aldosterone is more efficient than glucocorticoid in preventing MR proteolysis (Couette, Fagart et al, 1996). Together, these observations suggest that the active MR conformation is ligand-dependent (Farman, 1999).

1.2.5.2.1.5 MR selectivity: dimerization of MR

Dimerization of steroid receptors is required for binding to hormone response elements of target genes. As well as homodimers (MR-MR, GR-GR), heterodimers may form, which vary considerably in terms of transactivation potency compared with homodimers (Trapp and Holsboer, 1996). Some groups have reported synergism between MR and GR for transcription, others have shown that MR inhibits GR transcriptional activity through heterodimer formation (Trapp and Holsboer, 1996, Liu, Wang et al, 1995). These differing effects may depend on different cell types and reporter genes used (Farman, 1999).

1.2.5.3 Intracellular mechanisms of GR and MR signalling

Unoccupied GR and MR are held in high affinity for their steroid ligands by heat shock proteins and immunophilins (Funder, 1996). On binding of the steroid to the receptor, these proteins are shed and the steroid-receptor complex migrates to the nucleus. Following dimerization, the complexes then bind to GRE (Trapp and Holsboer, 1996, van Steensel, van Binnendijk et al, 1996, Liu, Wang et al, 1995). Binding of the activated receptor dimer complex to GRE allows the interaction of a complex set of transcription factors required for the initiation of transcription (Funder, 1996).

1.2.5.4 Metabolism of cortisol (Figure 1.2)

Cortisol is metabolized by the reversible conversion to cortisone, an inactive steroid in man. This interconversion occurs in many tissues but the liver and kidney have highest activities (Burton and Turnell, 1968). The major site of cortisone production in man has been shown to be the kidney (Whitworth, Stewart et al, 1989).

1.2.5.4.1 Metabolism of cortisol: the 11β-hydroxysteroid dehydrogenase complex

Two isoforms of 11β-HSD have been described, 11β-hydroxysteroid dehydrogenase, converting cortisol to cortisone (or corticosterone to 11-dehydrocorticosterone in the rat) and an 11-oxo-reductase, converting cortisone to cortisol (or 11-dehydrocorticosterone to corticosterone in the rat)(Monder and Shackleton, 1984).

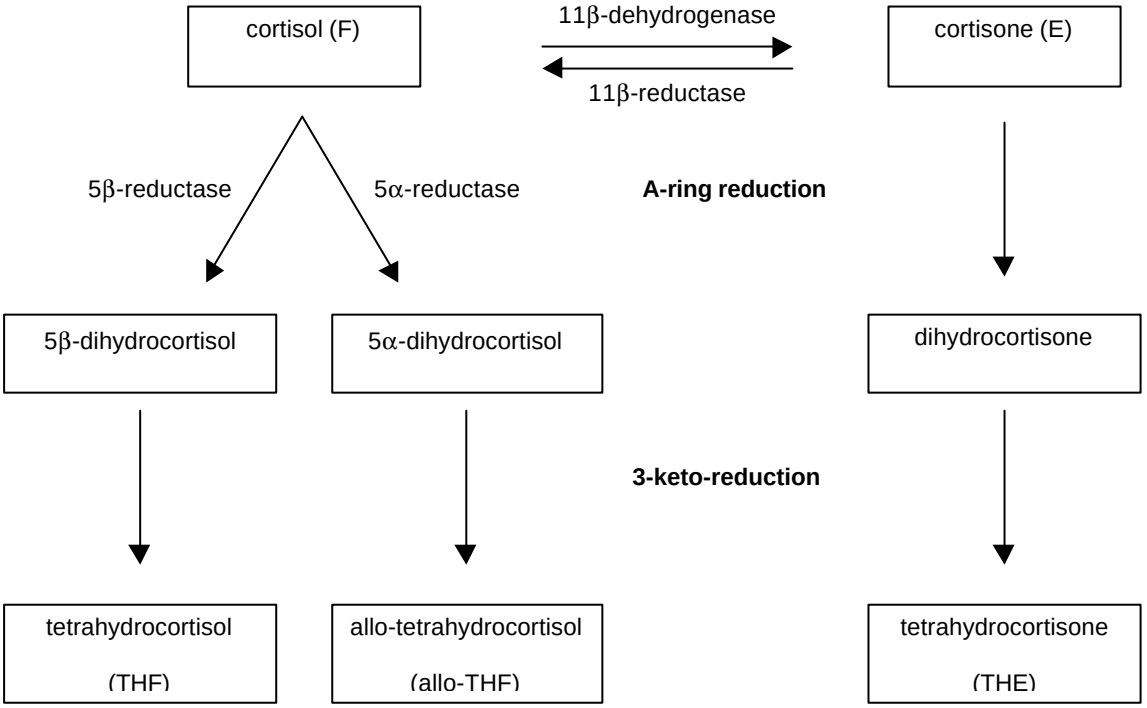


Figure 1.2: Principal urinary metabolites of cortisol and cortisone, from Walker and Edwards, 1994a.

11β-HSD1 is a low-affinity (micromolar range) NADP(H)-dependent enzyme (Krozowski, Provencher et al, 1994) found predominantly in human liver, decidua, lung, gonad, pituitary gland and cerebellum. When purified from the liver, 11β-HSD1 functions only as a dehydrogenase, whereas the recombinant enzyme cloned from cDNA libraries exhibits both 11β-dehydrogenase and oxoreductase activities when expressed in mammalian cells (Agarwal, Monder et al, 1989).

Several lines of evidence suggest that 11β-HSD1 does not confer ligand specificity on the MR (White, 1996). It is expressed at highest levels in the liver, which is not mineralocorticoid sensitive and it is expressed in low levels in human (but not rat) kidney (Tannin, Agarwal et al, 1991). In rat kidney, immunoreactivity to the enzyme is observed in proximal tubules but not distal tubules and collecting ducts (the sites of mineralocorticoid activity). Finally, mutations in the HSD11B1 gene, which encodes 11β-HSD1, are not observed in AME (Nikkila, Tannin et al, 1993). The role of this isoform of 11β-HSD is not clear, but it may amplify glucocorticoid action during the diurnal nadir, drawing upon the substantial circulating levels of 11-keto steroids (Seckl, Chapman et al, 1997).

11β-HSD2 is NAD-dependent and was initially found in distal tubules of the rat kidney (Mercer and Krozowski, 1992) and subsequently in placenta, kidney, colon and salivary glands (Hirasawa, Sasano et al, 1997). 11β-HSD2

has also been found by immunostaining in smooth muscle cells in the arterioles in skin, myocardium, and saphenous vein (Smith, Little et al, 1996). Mazzoochi and co-workers have recently reported 11β-HSD2 activity in human adrenal cortex (Mazzoochi, Rossi et al, 1999). Unlike the type 1 enzyme, 11β-HSD2 has a very high affinity for steroids (K_M in nmol/L range) and very little reductase activity (Rusvai and Naray, 1993). Within the kidney, it has been localised to renal cortical and medullary collecting ducts. Confocal laser microscopy studies have indicated both cytoplasmic and nuclear location for the 11β-HSD2 enzyme within the kidney, with intranuclear staining accounting for up to 40 % of total cellular 11β-HSD2 immunoreactivity (Bujalska, Shimojo et al, 1997). Hence the interaction between mineralocorticoid receptor and its ligand (either cortisol or aldosterone) may be a nuclear rather than a cytoplasmic event. Human 11β-HSD2 was cloned in 1994 (Albiston, Obeyesekere et al, 1994).

The two isozymes do not appear to be closely related. The amino acid sequence of 11β-HSD1 shares only 14 % identity to that of 11β-HSD2 (Albiston, Obeyesekere et al, 1994).

1.3 THE ROLE OF CORTISOL IN HUMAN HYPERTENSION

It is well known that glucocorticoids have a permissive effect on blood pressure. They enhance vascular responsiveness to vasopressors such as catecholamines without necessarily having a direct effect (Axelrod, 1983). Forearm blood flow in Addisonian patients receiving 30 mg hydrocortisone (cortisol) daily increased when the hydrocortisone dose was reduced to 15 mg daily (Dunne, Elliot et al, 1995). Glucocorticoids may suppress vasodilatation induced by bradykinin or histamine (Altura, 1966).

There is increasing evidence for a role for cortisol in the development or maintenance of essential hypertension. This section will discuss the mechanisms proposed by which abnormalities of cortisol production or metabolism may cause or contribute to three rare syndromes of hypertension; Cushing's disease, apparent mineralocorticoid excess and liquorice-induced hypertension. It will also discuss the possible role for cortisol in more common forms of hypertension, such as essential hypertension and chronic renal failure.

The second part of this chapter will discuss the effects of cortisol on some of the important regulatory mechanisms on blood pressure control, learned from experimental models of hypertension in man.

1.3.1 THE ROLE OF CORTISOL IN CUSHING'S SYNDROME

Although William Osler described a case in 1898 resembling Cushing's syndrome, and case reports appeared in the early decades of the twentieth century (including a report by Cushing himself), it was Harvey Cushing who defined the clinical syndrome with his description of 12 cases in his monograph in 1932 (Cushing, 1932). He described a syndrome characterized by truncal obesity, hypertension, weakness, amenorrhoea, hirsutism, striae, oedema, glycosuria, osteoporosis and a basophilic tumour of the pituitary gland.

1.3.1.1 Pathophysiology of Cushing's disease (Table 1.1)

The diagnosis of Cushing's has been broadened since Cushing's description and can be classified into ACTH-dependent or independent. The term Cushing's disease is reserved for those who have hypercortisolism secondary to a pituitary adenoma.

Table 1.1 Classification of Cushing's syndrome

ACTH-dependent (bilateral adrenal hyperplasia)
Pituitary ACTH-dependent Cushing's syndrome (Cushing's disease)
Ectopic ACTH syndrome
Ectopic corticotropin-releasing hormone
ACTH-independent
Adenoma
Carcinoma
Micronodular adrenal hyperplasia
Macronodular hyperplasia
Glucocorticoid administration

The commonest cause of naturally occurring Cushing's Syndrome is Cushing's disease, responsible for some two-thirds of cases (Whitworth, 1994). It occurs most frequently in women of child-bearing age, (female:male ratio 4-8:1). Pituitary adenomas are found in 80 % of cases, 80-90 % of which are microadenomas (<10 mm). Macroadenomas may invade locally and cause sellar enlargement. There is still debate surrounding the mechanisms of Cushing's disease, with some evidence in humans that hypothalamic CRH excess may be responsible for overstimulation of the pituitary and subsequent adenoma formation. However, the weight of evidence in humans views Cushing's disease as a primary pituitary disorder. Adrenal adenomas and carcinomas causing clinically evident Cushing's syndrome are rare. The ectopic ACTH syndrome occurs more frequently in men, and complicates solid tumours such as small-cell carcinoma of the lung, bronchial carcinoid and tumours of neural crest origin. Ectopic CRH production is extremely rare and has been associated with a similar spectrum of diseases (Whitworth, 1994, Danese and Aron, 1994).

1.3.1.2 Clinical features of Cushing's syndrome

Regardless of the cause, in all situations of Cushing's syndrome, symptoms and signs primarily result from the pharmacological effects of excess circulating cortisol (Suzuki, Shibata et al, 1992, Soszynski, Slowinska-Srzednicka et al, 1991). Some manifestations result from increased secretion of adrenal androgens (DHEA, DHEA-S and androstenedione) (Danese and Aron, 1994), which may or may not be ACTH-dependent.

The characteristic features of Cushing's syndrome are truncal obesity with redistribution of facial fat (moon-facies) and 'buffalo hump', facial plethora, hirsutism, purplish striae, bruising and neuropsychiatric disorders commonly occur. Although hypertension is common in this syndrome (75-90 %), overall, it is a rare cause of clinical hypertension, affecting less than 0.1% of the population. Hypertension, however, is a major contributor to cardiovascular morbidity and mortality in Cushing's syndrome. Hypertension is probably not as common in the ectopic ACTH syndrome or when Cushing's syndrome is secondary to exogenous glucocorticoids (about 20 %). In ectopic ACTH syndrome, this may reflect low blood pressure in patients with malignant disease, the fact that the disease duration is quite short or possibly a different steroid profile (Whitworth, 1994, Danese and Aron, 1994).

1.3.1.3 Mechanisms of hypertension and role of cortisol

Studies of hypertension in Cushing's syndrome are limited and the mechanism by which hypertension develops is not fully understood. Hypertension, however, is explicable in terms of cortisol excess (Suzuki, Shibata et al, 1992, Soszynski, Slowinska-Srzednicka et al, 1991) and possibly reflects a combination of factors regulating plasma volume, peripheral vascular resistance and cardiac output, all of which are increased in Cushing's syndrome (Agrest, Finkielman et al, 1974). In Cushing's syndrome, inhibition of steroidogenesis with ketoconazole, or administration of the glucocorticoid receptor antagonist RU486 corrected the hypertension (Chrousos, Laue et al, 1988, Sonino, Boscaro et al, 1991).

Much of the understanding of pathophysiology of Cushing's syndrome has come about through investigation of experimental models of ACTH- and cortisol-induced hypertension in man and in other experimental models as discussed below (see Section 1.3.7).

1.3.1.4 Mechanisms of hypertension examined in Cushing's syndrome

1.3.1.4.1 Exchangeable sodium, plasma volume and cardiac output

In sheep and rat, glucocorticoids are known to shift sodium to the extracellular space (Haack, Mohring et al, 1977, Scoggins, Coghlan et al, 1975). In a cross sectional study of 11 patients with Cushing's disease (seven of whom were hypertensive), however, exchangeable sodium levels were within the normal range (Ritchie, Sheridan et al, 1990). Atrial natriuretic factor levels, a more sensitive index, are generally increased in patients with Cushing's syndrome, and correlate with cortisol levels, but not with blood pressure, suggestive of increased extracellular sodium (McKnight, McCance et al, 1991, Soszynski, Slowinska-Srzednicka et al, 1991, Yamaji, Ishibashi et al, 1988). Cardiac output is increased in Cushing's syndrome (Agrest, Finkielman et al, 1974).

1.3.1.4.2 11β-HSD2 inhibition in Cushing's syndrome

The ratio of cortisol to cortisone is increased in cases of AME (where 11β-HSD2 is deficient) and in subjects with liquorice intoxication (where 11β-HSD2 is inhibited). In patients with Cushing's disease, the urinary THF/THE ratio was increased (Figure 1.2) (Hermus, Hobma et al, 1991), but there were

no significant differences between hypertensive and normotensive patients. Hypokalaemic patients had a higher THF/THE ratio.

In patients with the ectopic ACTH syndrome, plasma cortisol/cortisone ratios have been found to be higher than in other forms of Cushing's syndrome (Walker, Campbell et al, 1992). In this syndrome, other abnormalities of cortisol inactivation have been reported, including the cortisol turnover quotient and the ring A reduction quotient. These features are also observed in AME (Ulick, Wang et al, 1992). Whether the impaired inactivation of cortisol is due to overload of the enzyme or due to inhibition of the enzyme, perhaps by ACTH excess, remains to be determined.

1.3.1.4.3 The renin-angiotensin system in Cushing's syndrome

Glucocorticoids have a profound effect on the renin-angiotensin system. Plasma renin substrate (angiotensinogen) levels are elevated to approximately twice normal in patients with spontaneous or iatrogenic Cushing's syndrome, because of a direct effect of glucocorticoids on hepatic synthesis of renin substrate (Krakoff, 1973, Klett, Ganten et al, 1992). Despite this, activity of the renin-angiotensin system is reported as normal or reduced in Cushing's syndrome (Dalakos, Elias et al, 1978, Ritchie, Sheridan et al, 1990, Saruta, Suzuki et al, 1986). Studies with saralasin, an antagonist of angiotensin II, have variously reported lowering (Dalakos, Elias et al, 1978) or no effect (Vetter, Vetter et al, 1976) on blood pressure. In the study where blood pressure was lowered, subjects were volume deplete, and therefore dependent upon angiotensin II for maintenance of blood pressure. Similarly,

captopril has been reported variably to lower or not lower blood pressure in Cushing's syndrome (Greminger, Vetter et al, 1984).

1.3.1.4.4 Vascular function in Cushing's syndrome

Glucocorticoids exert permissive effects on vascular tone, enhancing pressor effects of circulating factors without overtly causing vasoconstriction. In patients with Cushing's syndrome due to adrenal adenoma, pressor responses to noradrenaline and angiotensin II have been reported to be enhanced (Mendlowitz, Gitlow et al, 1958, Saruta, Suzuki et al, 1986). Cardiac sensitivity to isoprenaline in Cushing's disease was increased in one study, but this was not due to an increase in β-receptor density (Ritchie, Sheridan et al, 1990). Alpha$_2$-adrenoreceptor density and affinity on platelets were normal, and β_2-adrenoreceptor density on lymphocytes was normal but affinity was decreased.

1.3.1.4.5 Endothelial function and circulating factors

In vivo, patients with Cushing's syndrome have been found to have normal or elevated levels of atrial natriuretic factor, normal levels of neuropeptide Y (vasoconstrictor), and decreased urinary excretion of prostaglandin-E_2 and kallikrein (vasodilators) (McKnight, McCance et al, 1991, Soszynski, Slowinska-Srzednicka et al, 1991, Tabarin, Minot et al, 1992, Saruta, Suzuki et al, 1986).

1.3.2 CORTISOL IN THE SYNDROME OF APPARENT MINERALOCORTICOID EXCESS

The syndromes of mineralocorticoid excess in congenital 11β-HSD deficiency and after liquorice administration are similar. Both of these syndromes involve the excessive activation of MR by an ACTH-dependent steroid, cortisol, rather than by the conventional mineralocorticoid agonist. The mechanisms underlying the syndromes are also similar: relative deficiency of 11β-HSD2 enzyme activity, that not only metabolizes cortisol to cortisone but also normally protects the MR from circulating glucocorticoid.

1.3.2.1 Clinical features of congenital AME

AME is an inherited syndrome characterized by the onset of severe hypertension in early childhood or adolescence in association with evidence of mineralocorticoid excess in the kidney with hypokalaemic alkalosis and suppression of the renin-angiotensin-aldosterone axis. Circulating aldosterone and other mineralocorticoids are very low. Patients with AME are often born with intrauterine growth retardation and may have postnatal failure to thrive (White, 1996). Consequences of the severe hypokalaemia include nephrocalcinosis, nephrogenic diabetes incipidus and rhabdomyolysis. Blockade of MR with spironolactone or sodium restriction ameliorates the hypertension, whereas hydrocortisone exacerbates it. Dexamethasone may also attenuate the hypertension. The syndrome of AME is inherited by autosomal recessive mode.

The term Apparent Mineralocorticoid Excess was first coined in 1979 (Ulick, Levine et al, 1979). Case descriptions of the syndrome appeared earlier (New, Levine et al, 1977, Werder, Zachmann et al, 1974) and in retrospect Werder's case was probably the first described. In the patient studied by New and Ulick, there were features of marked mineralocorticoid excess without elevation of circulating steroids but with elevated ratios of urinary cortisol to cortisone metabolites and 5α-reduced to 5β-reduced steroids (Ulick, Ramirez et al, 1977, Funder, 1995). This patient's blood pressure was extremely sensitive to salt and to administered cortisol or adrenocorticotrophin (Oberfield, Levine et al, 1983). This group proposed in 1983 that cortisol was acting as a potent mineralocorticoid in this patient.

The first adult case was described in 1988 by Edwards' group (Ulick, Levine et al, 1979). In this report, hypertension was associated with impaired conversion of cortisol to cortisone, hypokalaemia and suppression of the renin-angiotensin-aldosterone axis. Exogenous dexamethasone produced a natriuresis and potassium retention and the addition of hydrocortisone (cortisol) resulted in marked sodium retention, kaliuresis, suppression of renin and an increase in blood pressure. The authors concluded that the patient had a deficiency of 11β-hydroxysteroid dehydrogenase resulting in the availability of cortisol to act at the type 1 receptor as a mineralocorticoid.

In 1990, Ulick et al described a second congenital syndrome of cortisol-dependent mineralocorticoid excess that they have called AME type II (Ulick,

Tedde et al, 1990, Edwards and Walker, 1993). In this syndrome (in Italian patients), ratios of cortisol to cortisone in plasma and their metabolites in urine are normal. It has been suggested that these patients have a defect in A-ring reduction (Ulick, Tedde et al, 1990). Cortisol half-life and conversion of oral cortisone to cortisol are abnormal, confirming that both 11β-reductase and 11β-dehydrogenase activity are abnormal in AME type 2 (Edwards and Walker, 1993). Urinary free cortisol:cortisone ratios are high in both forms of AME. No mutation in 11β-HSD has yet been reported in AME type II. Dexamethasone is said to be the treatment of choice (Mantero, Palermo et al, 1996).

1.3.2.2 The metabolic defect in apparent mineralocorticoid excess

In 1988, Edwards et al (Edwards, Stewart et al, 1988) and Funder et al (Funder, Pearce et al, 1988) proposed that 11β-HSD protected the non-selective MR from overwhelming stimulation by circulating glucocorticoids, to allow selectivity to aldosterone.

The molecular basis of AME was described by several groups in 1995 as a mutation in the 11β-HSD2 gene (Wilson, Krozowski et al, 1995, Mune, Rogerson et al, 1995, Obeyesekere, Ferrari et al, 1995, Wilson, Harbison et al, 1995). All patients described with AME to date have a mutation in this enzyme. As a result, cortisol half-life in plasma is prolonged from approximately 80 minutes to 120-190 minutes (Ulick, Levine et al, 1979). Urinary steroid metabolite concentrations are abnormal; there are very low levels of cortisone metabolites compared with cortisol metabolites, a

consequence of deficiency of 11β-HSD activity (Figure 1.2). 11-reduction is unimpaired, as labelled cortisone administered to patients is excreted entirely as cortisol and other 11β-reduced metabolites (Shackleton, Rodriguez et al, 1985). The active component of liquorice, glycyrrhetinic acid, inhibits 11β-HSD and therefore the hypertension secondary to liquorice intoxication can be considered a reversible or acquired form of AME (White, 1996).

The prevalence of AME mutations in the general population is low, so this autosomal recessive disease is found mostly in populations in whom inbreeding is relatively high. Six of the kindreds are of Native American origin, however the reason for the relatively high prevalence of this very rare disease in Native Americans is not immediately apparent (White, 1996).

1.3.2.3 Mutations of the 11b-HSD2 enzyme (Figure 1.3)

AME was the third single gene cause of human hypertension described, after glucocorticoid remediable aldosteronism in 1992 and Liddle's syndrome in 1994 (Mantero, Palermo et al, 1996).

The first mutation of 11β-HSD2 was a C to T transition described by Wilson et al (Wilson, Krozowski et al, 1995). The effect of this was the substitution of an arginine residue by cystine, resulting in the so called R337C mutation. Subsequently, this mutant was shown to have no activity in cell free preparations, and a high K_M in whole cells (K_M 1010 nM for R337C mutant compared with K_M 110 for wild-type) (Obeyesekere, Ferrari et al, 1995). Furthermore, R337C activity is less stable than the wild-type activity in intact

cells and the mutant may partially inhibit the wild-type enzyme in heterozygotes (Ferrari, Obeyesekere et al, 1996). As 11β-HSD2 is part of the short-chain alcohol dehydrogenase (SCAD) superfamily of enzymes (Krozowski, 1994) and these enzymes are either dimers or tetramers, it is possible that a heteromeric 11β-HSD2/R337C protein could have a reduced capacity to metabolise cortisol (Ferrari, Obeyesekere et al, 1996). However, until recently, hypertension has not been described in the heterozygote state. In 1997, Li et al described a novel mutation in 11β-HSD2 in a Brazilian kindred (Li, Li et al, 1997). The proband, a 7 year old girl, was found previously to have AME. The father of the proband had hypertension, normal serum potassium, suppressed plasma renin and aldosterone and a moderately elevated urinary cortisol:cortisone ratio. In this kindred, sequence analysis of genomic DNA revealed a novel C1061T point mutation in exon V of the 11β-HSD2 gene. This resulted in an amino acid substitution of alanine by valine at codon 328 of the enzyme protein (A328V). The mutant was devoid of 11β-HSD2 activity.

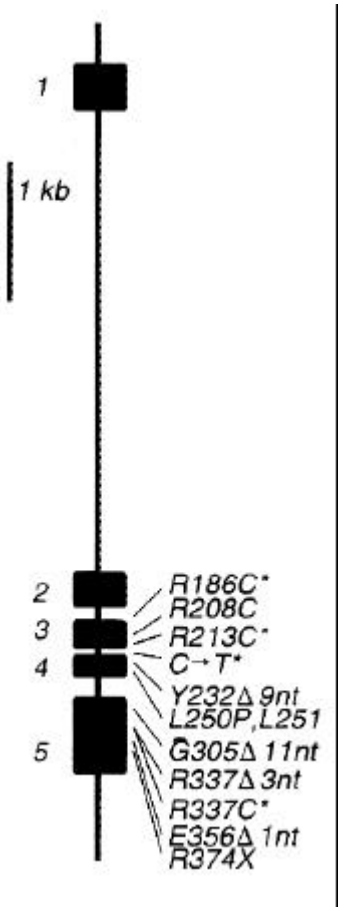


Figure 1.3 Mutations of the HSD11K causing the syndrome of apparent mineralocorticoid excess, from White, 1996.

At least 11 different mutations have now been described in 15 kindreds with AME (White, 1996). Only one of these 11 patients is a compound heterozygote for two different mutations, all other patients have carried homozygous mutations (Kitanaka, Katsumata et al, 1997). The compound heterozygote is an Irish-American whose 11β-HSD2 gene had an 11-base pair deletion giving rise to a premature termination codon and a 9 base pair deletion eliminating the catalytic site.

1.3.2.4 The 11b-HSD2 'knockout' mouse - a model of the syndrome of apparent mineralocorticoid excess

Kotelevtsev et al have recently reported a mouse with targeted disruption of 11β-HSD2 (Kotelevtsev, Brown et al, 1999). Fifty percent of these mice died within 48 hours of birth - these deaths were attributed to complications of profound hypokalaemia. Surviving mice were fertile, but exhibited hypokalaemia, hypotonic polyuria and apparent mineralocorticoid activity of corticosterone. The surviving homozygotes were markedly hypertensive. The most striking structural changes to these mice were hyperplasia and hypertrophy confined to the distal convoluted tubule, presumably a consequence of increased mineralocorticoid activity.

1.3.2.5 Can the hypertension of AME be explained in total by cortisol occupancy of the MR ?

In the 11β-HSD2 deficient state, hypertension has been considered a consequence of MR occupancy and therefore mineralocorticoid excess. Spironolactone, however, has only a partial effect on correction of hypertension in AME, and its effect may not be sustained (Speiser, Riddick et al, 1993, New, Nemery et al, 1989, Edwards and Stewart, 1989). Its effect could be explained by the diuretic effect of spironolactone. Furthermore, hydrocortisone, when administered to patients with AME (Oberfield, Levine et al, 1983) and to normal subjects, has a rapid hypertensive effect, evident within a few days (see section 1.3.6.2). Classical MR agonists, such as aldosterone and deoxycorticosterone, produce profound and rapid urinary sodium retention, but the rise in blood pressure is gradual, appearing over weeks (Kassirer, London et al, 1970). This raises the possibility that other

mechanisms may be involved in cortisol-induced hypertension in AME (Kelly, Mangos et al, 1998).

Data from experimental models of glucocorticoid-induced hypertension in man support this notion. Whitworth et al have shown that cortisol-induced hypertension in man is not dependent upon sodium and water retention, and, indeed, synthetic glucocorticoids devoid of mineralocorticoid activity raise blood pressure in man (Whitworth, Gordon et al, 1989a), as discussed below (section 1.3.6.4). Montrella-Waybill et al (Montrella-Waybill, Clore et al, 1991) have shown that cortisol-induced sodium retention and hypertension are not prevented by MR blockade with spironolactone. Cortisol (240 mg/day) raised blood pressure and produced sodium retention with or without co-administration of the MR antagonist, spironolactone (400 mg/day). Adequacy of MR blockade was determined in another limb of the study where male subjects received fludrocortisone with or without spironolactone. Williamson et al (Williamson, Kelly et al, 1996) examined the effect of MR blockade with spironolactone (400 mg/day) on cortisol-induced hypertension, using the lowest dose of cortisol determined to raise blood pressure in healthy males, 80 mg/day. MR blockade in this study prevented the fall in serum potassium and rise in body weight but not the increase in blood pressure accompanying cortisol treatment. These results are consistent with the notion that glucocorticoids raise blood pressure by mechanisms distinct from their MR-mediated effects and suggest that classical ‘mineralocorticoid effects’ may not be mediated by the MR.

In the context of AME, the above data question the mechanism by which cortisol raises blood pressure in this condition and certainly support the notion that hypertension accompanying AME is not completely explained by MR activation by cortisol and consequent sodium retention.

1.3.3 'ACQUIRED' APPARENT MINERALOCORTICOID EXCESS

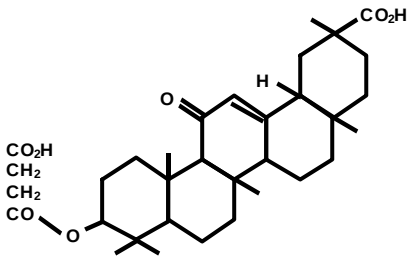
In at least two situations, 11β-HSD can be pharmacologically inhibited to produce syndromes similar to congenital AME. These syndromes can be considered acquired forms of AME as the physiological consequences are similar.

1.3.3.1 Cortisol in the syndrome of hypertension of liquorice abuse and carbenoxolone administration (Figure 1.4)

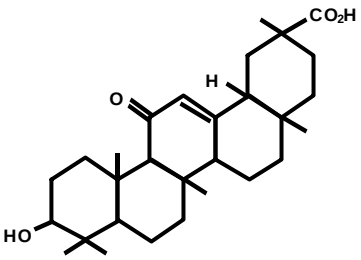
The popular confectionery known as liquorice is produced from the sweet root of the plant Glycyrrhiza glabra (Walker and Edwards, 1994b). The root produces a colourless substance, glycyrrhizin, an enoxolone, which turns black during the process of extraction and is the principle active constituent of liquorice (along with its hydrolytic product, glycyrrhetinic acid). It is also used as an emollient and as a foam stabilizer in fire extinguishers (Microsoft, 1996).

The use of liquorice dates back to at least 1000 BC. Its therapeutic value was evident from writings of the ancient Greeks, Romans and Chinese (Walker and Edwards, 1994b, Davis and Morris, 1991). Liquorice was rediscovered in the fifteenth century and became an established treatment for dyspepsia.

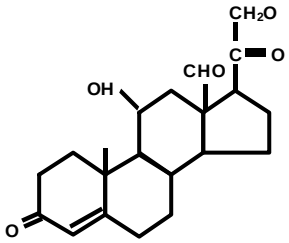
Carbenoxolone, the hemisuccinate derivative of glycyrrhetinic acid, was the most effective medical therapy for peptic ulcer disease until the introduction of cimetidine (Walker and Edwards, 1994b).



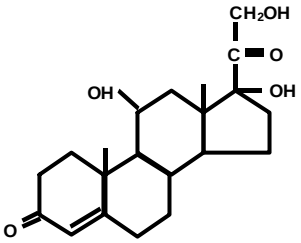
Carbenoxolone



Glycyrrhetinic acid



Aldosterone



Cortisol

Figure 1.4 Chemical structures of carbenoxolone, glycyrrhetinic acid, aldosterone and cortisol.

In the last 60 years, the adverse side-effect profile of liquorice has become apparent. Reevers noted peripheral oedema and breathlessness in patients treated for dyspepsia (Reevers, 1948). Since then, a characteristic syndrome of hypertension with features of mineralocorticoid excess has been reported in habitual consumers of liquorice.

The syndrome resulting from liquorice abuse or therapeutically administered liquorice or carbenoxolone has features in common with primary

aldosteronism. The features include hypertension with sodium retention, hypokalaemic alkalosis, nephrogenic diabetes insipidus, proximal myopathy, tetany and headaches (Walker and Edwards, 1994b). In addition, liquorice abuse may cause oedema and breathlessness, features that are not commonly observed in primary aldosteronism. Carbenoxolone also increases mineralocorticoid receptor activity in the colon.

The syndrome is distinguished from primary aldosteronism because liquorice suppresses aldosterone secretion. Liquorice also suppresses other mineralocorticoids, principally 11-deoxycorticosterone, which allows differentiation from hypertensive forms of CAH. Patients with ectopic ACTH syndrome have measurable levels of 11-deoxycorticosterone and very high urinary free cortisol.

1.3.3.2 Clinical features of liquorice-induced hypertension

The effects of liquorice consumption in normal individuals have been studied since the 1970s. Epstein et al demonstrated that consumption of liquorice in normal subjects suppressed the renin-angiotensin-aldosterone axis and raised urinary cortisol excretion. They showed that doses of glycyrrhizic acid as little as 700 mg/day caused sodium retention, hypokalaemia and resultant hypertension (Epstein, Espiner et al, 1978, Epstein, Espiner et al, 1977a, Epstein, Espiner et al, 1977b). In a prospective study of healthy normal volunteers, Sigurjonsdottir et al gave liquorice 50 or 100 g daily for 4 weeks. In this study, the rise in blood pressure was 3.5 and 6.5 mmHg systolic after 2 and 4 weeks respectively, in association with an increase in the urinary

metabolic ratio (THF+allo-THF:THE) from 1.39 .45 to 2.15 .9. The lower dose of 50 g daily also produced a significant increase in systolic blood pressure of 2.9 and 5.6 mmHg after 2 and 4 weeks respectively (Sigurjonsdottir, Ragnarsson et al, 1995).

Unlike cortisol, liquorice appears to raise blood pressure over weeks rather than hours to days (Sigurjonsdottir, Ragnarsson et al, 1995). In one study, a dissociation between the sodium retention (days) and the blood pressure raising effects of liquorice (weeks) was observed, suggesting that the hypertensive effect of liquorice may not be fully explained by renal mineralocorticoid excess (Sigurjonsdottir, Ragnarsson et al, 1995, Walker, Connacher et al, 1992).

1.3.3.3 Differential diagnosis of liquorice-induced hypertension

The most difficult differential diagnosis is between liquorice ingestion and other forms of low-renin, low-aldosterone, low-11-deoxycorticosterone, hypokalaemic hypertension (Walker and Edwards, 1994b). Liddle's syndrome is characterized by the normalization of hypertension by amiloride but not spironolactone, and surreptitious administration of mineralocorticoid hormones is associated with normal or low urinary free cortisol excretion. In contrast, liquorice-induced hypertension responds to spironolactone (Salassa, Mattox et al, 1962) and induces a mild elevation in urinary free cortisol (Epstein, Espiner et al, 1978). Confirmation of liquorice ingestion can be made by measuring glycyrrhetinic acid in the urine using gas chromatography (Walker and Edwards, 1994b).

1.3.3.4 Cortisol metabolism in liquorice-induced hypertension

Cortisol metabolism is predictably abnormal in patients with liquorice-induced hypertension. Cortisol half-life is prolonged and urinary metabolites of cortisol and cortisone are abnormal. These, however, are difficult to measure and not yet useful in diagnosing individual patients. GA has a long half-life and its effects in patients who stop taking liquorice can persist for months (Walker and Edwards, 1994b).

1.3.3.5 Mechanisms of liquorice-induced hypertension

As liquorice suppresses the secretion of all the recognised mineralocorticoids, it was originally proposed that it contained a direct agonist of the MR. GA was shown to bind to MR in vitro (Armanini, Karbowiak et al, 1983, Ulmann, Menard et al, 1975). However, the in vitro K_d of GA for the MR is relatively low (K_d 2 x 10^{-6} M, compared with K_d for aldosterone of 5 x 10^{-10} M). Hence it seemed unlikely that adequate concentrations for substantial binding would be reached in vivo. Furthermore, mineralocorticoid excess was not observed in adrenalectomized animals treated with liquorice (Card, Mitchell et al, 1953) or in patients with adrenocortical failure. (Borst, Ten Holt et al, 1953). Similarly, the anti-inflammatory properties of liquorice derivatives are not observed in adrenalectomized animals (Khan and Sullivan, 1967). Finally, liquorice–induced mineralocorticoid excess can be reversed in humans by administration of dexamethasone (Hoefnagels and Kloppenborg, 1983), indicating that liquorice requires the presence of an ACTH-dependent product of the adrenal cortex to express its effect and cannot be acting directly on the MR.

Two lines of research provided the clues that led to the explanation for the action of liquorice. Firstly, the cloning of the MR (Arriza, Weinberger et al, 1987) and its expression in vitro revealed that it had equal affinity for cortisol and aldosterone. Moreover, MR in rat hippocampus bind corticosterone (the principal circulating rat glucocorticoid) with equal affinity to that for aldosterone in vivo (Krozowski and Funder, 1983), yet the same receptors are specific for aldosterone in the distal nephron (Sheppard and Funder, 1987). Hence, an alternative mechanism was proposed to confer specificity of MR for aldosterone.

One hypothesis was that extravascular CBG might sequester cortisol or corticosterone in the kidney but not in the hippocampus, which is beyond the blood-brain barrier and therefore not accessible to CBG (Stephenson, Krozowski et al, 1984). This hypothesis was rejected when kidneys of rats deficient in CBG were specific for aldosterone (Sheppard and Funder, 1987, Walker and Edwards, 1994b).

The second line of evidence came from studies of patients with apparent mineralocorticoid excess, deficient in 11B-HSD. Congenital deficiency of 11β-HSD causes the syndrome of AME, as described in detail in section 1.3.2, a syndrome very similar to that induced by liquorice excess.

In healthy volunteers, liquorice-induced mineralocorticoid excess was associated with inhibition of 11β-HSD, confirmed by increased ratio of the metabolites of cortisol:cortisone (THF + allo-THF):THE in urine, and prolonged half-life of ($11\alpha^{3}$H)-cortisol (Stewart, Wallace et al, 1987). Similar observations have been made after the administration of glycyrrhetinic acid (MacKenzie, Hoefnagels et al, 1990) and carbenoxolone (Stewart, Wallace et al, 1990). Both of these substances are inhibitors of rat 11β-HSD in vitro (Monder, Stewart et al, 1989). Their affinity for the enzyme (K_i of 10^{-9} to 10^{-8}M) is much higher than their affinity for MR (Ulmann, Menard et al, 1975, Armanini, Karbowiak et al, 1983).

Walker et al (Walker and Edwards, 1994b) hypothesized a unifying theory that explained both the action of liquorice and the mechanism of mineralocorticoid excess in AME. In their model, MR in the distal nephron have equal affinity for cortisol and aldosterone, but are protected from cortisol by the presence of 11β-HSD, which converts cortisol to cortisone. As aldosterone is protected from the action of 11β-HSD because of its 11-18 hemiacetal structure (Edwards and Hayman, 1991), it gains specific access to MR. When this mechanism is defective, either because of congenital deficiency of 11β-HSD or because of inhibition of the enzyme by liquorice, then intrarenal levels of cortisol rise and cortisol causes inappropriate activation of MR.

This model was simultaneously confirmed by Walker et al in Edinburgh and Funder et al in Melbourne. The Edinburgh group examined in vivo binding of corticosterone and aldosterone in rat kidney, with and without inhibition of 11β-HSD by subcutaneous injection of glycyrrhizic acid (Edwards, Stewart et al, 1988). ^{3}H-corticosterone bound minimally in the kidney in the absence of glycyrrhizic acid, but, after inhibition of 11β-HSD, there was abundant binding in a distribution identical to that of ^{3}H-aldosterone. Furthermore, glycyrrhizic acid had no effect on ^{3}H-aldosterone binding. The Melbourne group used carbenoxolone to inhibit 11β-HSD and also demonstrated glucocorticoid binding to mineralocorticoid receptors in renal cytosol (Funder, Pearce et al, 1988).

1.3.3.6 Mechanisms of carbenoxolone-induced hypertension

It would seem that liquorice and carbenoxolone should have identical metabolic effects, but this is not the case. Oral administration of carbenoxolone to healthy volunteers induced a greater prolongation of half-life of 11α^{3}H-cortisol than did liquorice but the magnitude of sodium retention and elevation of urinary free cortisol was similar. However, carbenoxolone did not reduce the urinary levels of cortisone and did not increase the urinary THF + allo-THF: THE ratio. In addition, despite inducing hypokalaemia, there was no kaliuresis with carbenoxolone (Stewart, Wallace et al, 1987, Stewart, Wallace et al, 1990). This effect does not appear to be due to gastrointestinal potassium loss (Baron, Nabarro et al, 1969).

One explanation for the above has been provided by the hypothesis that carbenoxolone inhibits both 11β-dehydrogenase and 11β-reductase components of 11β-HSD, whereas glycyrrhetinic acid has no effect on 11β-reductase. It has been suggested that a better measure of the inhibitory effect of carbenoxolone on 11β-hydroxydehydrogenation is the urinary free cortisol:cortisone ratio (Ulick, Wang et al, 1993).

1.3.4 THE ROLE OF CORTISOL IN ESSENTIAL HYPERTENSION

Hypertension affects 15-17% of Australian adults and in about 90-95 % of these individuals the hypertension is considered primary or essential. Excess production of adrenal corticosteroids results in high blood pressure and cardiac disease in a number of well defined clinical syndromes, such as Cushing's and Conn's syndromes. These examples of gross glucocorticoid and mineralocorticoid excess raise the possibility that lesser abnormalities of steroid metabolism or action may contribute to more common forms of cardiovascular disease (Connell, Kenyon et al, 1996). There are a number of subgroups of essential hypertensives which may be relevant in this respect. Firstly, the subgroup of low-renin, salt-sensitive hypertension, which contributes to about 30 % of essential hypertension in whites (and a higher percentage in blacks) has features consistent with mineralocorticoid excess (Berretta-Piccola, Davies et al, 1995). Secondly, a proportion of essential hypertensives have central obesity, dyslipidaemia, insulin resistance and are prone to microvascular disease (Syndrome X), characteristics that are also features of Cushing's syndrome.

Cortisol was therefore considered one of the earliest putative mediators in essential hypertension. However, no abnormalities of cortisol secretion rates or circulating cortisol were demonstrated (Vermeulen and Van der Straeten, 1963). In recent years, the complex interactions that modulate tissue sensitivity to cortisol have been understood, and several rare syndromes of abnormal tissue sensitivity to glucocorticoids explained. The possibility has arisen, therefore, that cortisol increases blood pressure in essential hypertension due to abnormalities in tissue sensitivity rather than excess production.

The evidence implicating corticosteroids in the pathogenesis of essential hypertension is preliminary and limited but intriguing. By definition, essential hypertensives do not have biochemical features of Cushing's syndrome, nor do they have obvious signs of mineralocorticoid excess. However, some investigators have noted a positive correlation between blood pressure and body sodium and a negative correlation between blood pressure and potassium in essential hypertension (Berretta-Piccola, Davies et al, 1995), suggestive of a mineralocorticoid effect. Cross-sectional data from the Paris Prospective Study 1 have revealed elevated morning plasma cortisol levels in untreated male hypertensive subjects, particularly so in the lean subgroup (Filipovsky, Ducimetiere et al, 1996).

In 1989, Whitworth et al demonstrated that blood pressure in a group (n=8) of essential hypertensives was lowered by a small dose of dexamethasone (0.5 mg nocte) administered over 4 weeks which had no effect on blood pressure in normotensive individuals (Whitworth, Gordon et al, 1989b). This study suggested a role for the hypothalamic-pituitary axis in contributing to hypertension in these patients. Subsequently, a number of investigators have examined cortisol metabolism in essential hypertensives.

1.3.4.1 The glucocorticoid receptor in essential hypertension

Evidence that variations in the GR gene contribute to essential hypertension has come from observational studies of this locus in humans (Connell, Kenyon et al, 1996). A restriction fragment length polymorphism for the GR locus (following digestion with the enzyme Bcl I) has been described, which gives rise to two alleles, A + a. Watt and co-workers studied a population of young people with contrasting familial predispositions to develop hypertension, the so-called 'Four Corners Study' (Watt, Harrap et al, 1992). Homozygotes for the larger allele (AA) had higher blood pressure scores than homozygotes for the alternative allele (aa). Heterozygotes had intermediate scores. Interestingly, AA homozygotes also had slightly higher plasma cortisol levels. Weaver et al (Weaver, Hitman et al, 1992) have reported that the larger allele (A) was associated with severe hyperinsulinaemic obesity, a phenotypic feature that, in a milder form, is common in patients with essential hypertension (Connell, Kenyon et al, 1996), as well as in Cushing's syndrome.

The above genetic association studies have not investigated the biochemical or physiological changes that may be responsible for an intermediate phenotype in patients with essential hypertension (Connell, Kenyon et al, 1996). Furthermore, a genetic association may represent linkage of the polymorphism to another gene. Connell et al have further examined these associations by measuring glucocorticoid receptor binding characteristics in circulating leukocytes (Connell, Kenyon et al, 1996). They found a trend for lower receptor affinity in subjects homozygous for the A allele (associated with hypertension and obesity in the above studies). There was no significant difference in receptor number or blood pressure in the two homozygous groups.

Connell's group has also examined glucocorticoid action in vitro, by the ability of dexamethasone to inhibit lysozyme production. They reported a trend for the inhibition of lysozyme production to be less sensitive to dexamethasone in AA homozygotes (Connell, Kenyon et al, 1996). In vivo, they measured skin vasoconstrictor responses to the topical application of the synthetic glucocorticoid budesonide. In contrast to the in vitro results above, AA homozygotes showed a greater response to the steroid than aa homozygotes. There is therefore a discrepancy between the in vitro trend for reduced binding to white blood cells and in vivo evidence of increased steroid action in AA homozygotes. This discrepancy is not understood, but may be a consequence of ligand specificity (dexamethasone v budesonide) or tissue specificity (Connell, Kenyon et al, 1996). Similarly, Mulatero et al (Mulatero,

Panarelli et al, 1997) examined glucocorticoid receptor binding characteristics and glucocorticoid responsiveness of human mononuclear cells from hypertensive and normotensive volunteers. They also found impaired binding of cortisol to the GR in the hypertensive group, and that decreased sensitivity to cortisol was associated with suppression of renin. In contrast, Walker et al have recently reported increased glucocorticoid sensitivity in subjects at risk for hypertension and cardiovascular disease (Walker, 1996, Walker, Phillips et al, 1998) and in subjects with essential hypertension (Walker, Best et al, 1996).

1.3.4.2 Sensitivity to glucocorticoids in essential hypertension

Walker et al examined dermal vasoconstrictor responses, GR sensitivity as judged by basal cortisol secretion rates, cortisol response to dexamethasone suppression and cortisol metabolism in a group of controls (n=11) and essential hypertensives (n=11). They reported that essential hypertensives were more sensitive to topical glucocorticoids (both cortisol and beclomethasone) than control subjects. This was associated with prolonged cortisol half life in hypertensive subjects, though this did not correlate with the dermal vasoconstrictor response (Walker, Best et al, 1996). In contrast, Mulatero et al reported reduced affinity of cortisol for the GR in human mononuclear leukocytes from patients with essential hypertension. In addition, in the subgroup of hypertensive patients with low plasma renin activity, mononuclear leukocytes were markedly less sensitive to cortisol than were those from hypertensives with high renin, suggesting that decreased sensitivity to cortisol is associated with renin suppression. They concluded

that this could lead to inappropriate binding of cortisol to MR (Mulatero, Panarelli et al, 1997).

In a study of healthy elderly Dutch subjects (n=216), Huigenza et al (Huizenga, Koper et al, 1998) examined the GR gene for the N363S polymorphism. They found 13 heterozygotes carrying the N363S polymorphism and examined sensitivity to glucocorticoids in these subjects. These subjects had a higher sensitivity to glucocorticoids (as defined by cortisol suppression during the dexamethasone suppression test and insulin response) and a trend to lower bone mineral density in the lumbar spine, compared to controls. Blood pressure was not affected.

In summary, polymorphisms of the gene encoding GR may well be important in determining activity of the hypothalamic-pituitary axis and the familial predisposition to hypertension.

1.3.4.3 The 11b-HSD enzyme system in essential hypertension

11β-HSD2 acts as a dehydrogenase for the conversion of cortisol to cortisone and its deficiency causes AME. Milder abnormalities of this system could contribute to some subsets of essential hypertension, either by allowing exposure of the MR to cortisol or to allow increased cortisol exposure to other potential hypertensinogenic mechanisms. Walker et al (Walker, Stewart et al, 1993) examined this hypothesis in a group of 19 essential hypertensives and matched controls. They reported prolonged cortisol half-life in a subgroup of patients but were unable to demonstrate evidence of altered steroid

metabolism (ie the ratio of cortisol to cortisone in plasma and their metabolites in urine were normal) or mineralocorticoid excess. Cortisol half-life correlated with blood pressure. These results suggested not only a deficit in 11β-HSD activity but also a defect in 11β-reductase activity (as observed following carbenoxolone administration).

This group has postulated that deficiency of 11β-HSD may allow increased exposure of MR to glucocorticoids in extrarenal sites, such as vascular smooth muscle and heart (Walker, Stewart et al, 1991, Walker, Yau et al, 1991). Indeed, Morris et al have reported a bidirectional $NADP^+$ dependent 11β-HSD (ie 11β-HSD1) in rat vascular smooth muscle which is modulated by 11βOH-progesterone. This enzyme and its regulator(s) may well play a role in the vascular effects of glucocorticoids in hypertensive states (Brem, Bina et al, 1997).

In 1995, Soro and co-workers examined urinary corticosteroid metabolites in a group (n=68) of untreated patients with essential hypertension and untreated control subjects (Soro, Ingram et al, 1995). Increased ratios of urinary cortisol to cortisone metabolites as well as increased 5α- to 5β-reductase metabolites were observed (see Figure 1.2), consistent with the notion of reduced 11β-HSD and reduced 5β-reductase activities in essential hypertension.

Litchfield and colleagues recently reported an association between increased urinary free cortisol and salt-resistant essential hypertension (Litchfield, Hunt et al, 1998).

Why 11β-HSD would be deficient in essential hypertension is not known. Walker et al investigated the possibility of endogenous inhibitors of 11β-HSD contributing to deficiency of the enzyme in essential hypertensives and in subjects with syndrome of ectopic ACTH secretion (Walker, Aggarwal et al, 1995). They reported that endogenous GALFs were not ACTH-dependent and had no measurable effect on 11β-HSD in vivo, though they only measured GALF activity on 11β-HSD1. More recently, Takeda et al (Takeda, Miyamori et al, 1996) used urine extracts from hypertensive subjects to inhibit 11β-HSD2 in homogenized human kidney. In their study, endogenous renal 11β-HSD inhibitory factor was significantly increased in patients with low-renin essential hypertension. Further, urinary excretion of this inhibitory factor(s) was positively correlated with urinary sodium excretion, suggesting that sodium may directly or indirectly influence the activity of 11β-HSD by modulating the endogenous renal 11β-HSD inhibitory factor(s). The chemical structure of this putative factor(s) has yet to be described.

Watson et al tested the HSD11B2 locus in essential hypertension (Watson, Bergman et al, 1996). They examined blacks with end stage renal failure attributed to hypertension. They refined the location of HSD11B2 to 16q22.1, and genotyped subjects using the nearest flanking microsatellites (D16S301

and D16S496). They found a significant association between hypertension and the D16S496 microsatellite locus, suggesting that the 16q22.1 chromosome region contains a candidate gene for essential hypertension. This could be HSD11B2 or another gene. It is not clear why this group used ESRF patients and not those with essential hypertension and no other medical disease. Recently, however, Brand et al reported their negative findings on linkage of the 11-βHSD2 gene with essential hypertension (Brand, Kato et al, 1998).

1.3.4.4 Foetal origins of essential hypertension

An interesting association has been noted between low birth weight or low foetal:placental weight ratio and the subsequent risk of developing hypertension. Human epidemiological data have suggested that programming of blood pressure may occur in early stages of life. For example, there is a twofold difference in the death rates from ischaemic heart disease between different areas of England and Wales. Rates are highest in industrial and poorer areas such as North Wales. Though there are regional differences in adult lifestyle, these differences only go a small way to explaining the large differences in death rates (Barker, 1992).

Studies in man and animals have shown that a disproportionately large placenta may be the consequence of maternal undernutrition or the result of anaemia during pregnancy (Beischer, Sivasamboo et al, 1970, Godfrey, Redman et al, 1991). Birth measurements have revealed two groups of babies who develop high blood pressure in adults, those with below average

birth weight and head circumference (growth retardation), and those with above average birth weight and head circumference but below average length (asymmetric growth retardation) (Barker, Godfrey et al, 1992). Hypertension is strongly predicted by low birth weight and a large placenta (Barker, Bull et al, 1990).

Maternal glucocorticoid excess may retard foetal growth. Placental 11β-HSD protects the foetus from the growth-inhibiting effects of maternal glucocorticoids (Beitins, Bayard et al, 1973), thus cortisol concentrations are much lower in the foetal circulation than in the maternal circulation (Edwards, Benediktsson et al, 1993).

In the rat, there is a negative correlation between placental weight and placental 11βHSD activity and positive correlation between birthweight and placental 11βHSD activity. These results suggest that, in the rat, placental 11βHSD may protect the foetoplacental unit against the glucocorticoid effects of corticosterone (Benediktsson, Lindsay et al, 1993, Barker, 1992). It has been suggested that variations in 11β-HSD activity may be one factor influencing foetal growth and ultimately, blood pressure (Edwards, Benediktsson et al, 1993, Benediktsson, Lindsay et al, 1993).

Human studies, however, have failed to demonstrate a correlation between low placental 11β-HSD activity and low birth weight in those who do not suffer from AME (White, Mune et al, 1997).

1.3.5 THE ROLE OF CORTISOL IN HYPERTENSION OF RENAL PARENCHYMAL DISEASE

Hypertension is extremely common in patients with chronic renal failure. About 80 % of those entering a dialysis program have hypertension. It has been recognised for many years that the half-life of cortisol is prolonged in patients with chronic renal failure (Englert, Brown et al, 1958, Bacon, Kenny et al, 1973). Whitworth et al examined the concentration of cortisol and cortisone in 88 patients with chronic renal failure and found an inverse relationship between plasma cortisone and plasma creatinine concentrations, and markedly reduced cortisone concentrations in four anephric patients. They concluded that the kidney is the major site of cortisone production in man and postulated that impaired cortisol metabolism may contribute to the hypertension of chronic renal failure (Whitworth, Stewart et al, 1989).

Hypertension is also a common feature of the nephrotic syndrome, characterized by proteinuria, hypoalbuminaemia and sodium and water retention, often with marked oedema. Whether the sodium and water retention is mediated by an 'overfill mechanism' (ie a defect of primary tubular sodium and water reabsorption) or an 'underfill mechanism' (ie intravascular contraction secondary to hypoalbuminaemia and subsequent secondary aldosteronism) has not been answered. Brown et al demonstrated that captopril had an antihypertensive effect but no effect on sodium excretion in nephrotic patients, and argued that aldosterone was therefore not important in the sodium retention of nephrotic syndrome (Brown, Markandu et al,

1984). Schrier's group, however, examined sodium retention in nephrotic syndrome by administering spironolactone (400 mg daily) to a group (n=5) of nephrotic patients as well as six controls in sodium balance (Shapiro, Hasbargen et al, 1990). A natriuretic response occurred only in the nephrotic patients. Contrary to the study by Brown, his group reported that aldosterone was contributing to sodium retention in nephrotic syndrome.

Conflicting data from the above studies could be explained by another agonist stimulating the MR, such as cortisol. Indeed, in experimental nephrotic syndrome, sodium retention diminishes with adrenalectomy (Kalant, Gupta et al, 1962). Recently, Vogt et al reported reduced 11β-HSD activity in patients with the nephrotic syndrome, as assessed by the ratio of cortisol/cortisone metabolites in urine (Vogt, Dick et al, 1999). These patients all had biopsy proven glomerulonephritis. 11β-HSD deficiency may be a new mechanism for the sodium and water retention in this condition. Unlike AME or the hypertension of liquorice excess, however, the renin-angiotensin axis is not normally suppressed in nephrotic patients, against the notion that cortisol is solely responsible for the overfill observed in many of these patients. Of nine studies of nephrotic syndrome reviewed, elevated plasma renin was seen in approximately 50 % of cases (Dorhout Mees, Geers et al, 1984).

In keeping with the above, a further study in experimental renal disease by Escher et al (Escher, Vogt et al, 1998) examined 11β-HSD activity in the

remaining kidney following nephrectomy in the rat. Twenty four hours post-nephrectomy, there was evidence of reduced 11β-HSD activity, as measured by the ratio of prednisolone:prednisone (ie the ratio was increased). 11β-HSD2 activity in isolated cortical collecting ducts decreased significantly following nephrectomy, suggesting that access of glucocorticoids to GR and MR in the remaining kidney is facilitated after unilateral nephrectomy.

These data may have ramifications for the hypertension commonly observed in nephrotic patients, who may well suffer a variant of 'acquired' AME.

1.3.6 MECHANISMS OF EXPERIMENTAL GLUCOCORTICOID-INDUCED HYPERTENSION IN MAN

Much of the understanding of the mechanisms of hypertension in Cushing's syndrome has come about as a result of experimental models of steroid-induced hypertension in man. Most of these studies were performed in healthy males on approximately 150 mmol of sodium per day. In the studies reported below, placebo had no effects in normal subjects (Whitworth, Scoggins et al, 1992).

1.3.6.1 Adrenal cortisol production accounts for ACTH-induced haemodynamic and metabolic effects

Both exogenous cortisol (Whitworth, Saines et al, 1984) and ACTH (Whitworth, Saines et al, 1983b) raise blood pressure in experimental studies in man. ACTH treatment does not raise blood pressure in Addisonian

women, indicating that ACTH-induced increases in blood pressure are adrenally dependent (Whitworth, Saines et al, 1983b).

ACTH (1 mg/day i/m injection) increased systolic blood pressure by 21 mmHg with no consistent change in diastolic or mean arterial pressure in normal volunteers (Whitworth, Saines et al, 1983b). Cortisol infused intravenously (6-8 mg/hr), at a rate appropriate for conditions of ACTH stimulation, increased systolic blood pressure over a 5 day administration period by 21 mmHg (Whitworth, Saines et al, 1984). Administered orally, cortisol (50 mg every 6h) reproducibly increased systolic blood pressure by 8-20 mmHg (Tam, Kelly et al, 1996, Tam, Williamson et al, 1997) and variably increased MAP (by up to 15 mmHg) (Whitworth, Williamson et al, 1994a, Macefield, Williamson et al, 1998, Williamson, Kelly et al, 1996, Whitworth, Williamson et al, 1994c, Whitworth, Williamson et al, 1994b) mmHg and diastolic blood pressure (by up to 13 mmHg) (Whitworth, Connell et al, 1988, Macefield, Williamson et al, 1998, Whitworth, Williamson et al, 1994c, Whitworth, Williamson et al, 1994b) over five days. Systolic blood pressure and mean arterial pressure were significantly increased by 80 mg but not by 40 mg oral cortisol daily, suggesting the threshold dose for a pressor effect is between 40-80 mg cortisol per day (Williamson, Kelly et al, 1996).

The metabolic effects of ACTH and cortisol administration are similar; body weight gain over the treatment period in association with increased ECFV

and plasma volume, initial urinary sodium retention, increase in plasma sodium and fall in plasma potassium, increase in blood glucose, fall in plasma renin and increase in renin substrate (Connell, Whitworth et al, 1987). Eosinophil count falls in association with increased total white cell count (Whitworth, Saines et al, 1983b).

The above studies suggest that the majority of features of ACTH-induced hypertension in man can be attributed to the adrenal production of cortisol. Hence, pathophysiological mechanisms underlying these models are likely to be common.

1.3.6.2 Timecourse of cortisol-induced increases in blood pressure

The rise in blood pressure accompanying cortisol administration is rapid, observed by 24 hr after commencement of oral cortisol in man (Whitworth, Brown et al, 1995). This should be contrasted with the effects of deoxycorticosterone (Whitworth, Saines et al, 1984) or aldosterone (Dawborn, 1969) administration in humans, in which profound sodium retention is observed, whereas a rise in blood pressure occurs over weeks. Similarly, fludrocortisone, a potent mineralocorticoid, when administered orally, reproducibly results in ‘mineralocorticoid’ effects (rise in body weight in association with fall in serum potassium and sodium retention), but variably raises blood pressure during short term administration (Whitworth, Butkus et al, 1986).

1.3.6.3 The role of dietary sodium in ACTH- and cortisol-induced hypertension

Extreme sodium restriction (15 mmol/day) prevented the rise in ECFV and blunted, but did not prevent the rise in systolic blood pressure due to ACTH in man (ΔSBP ≈10 mmHg) (Connell, Whitworth et al, 1988). Salt loading potentiated the rise in systolic blood pressure (ΔSBP ≈30 mmHg) (Whitworth, Saines et al, 1985). Sodium intake can therefore be considered to modulate ACTH-induced hypertension, as the rise in blood pressure is clearly not dependent on sodium.

1.3.6.4 Role of extracellular fluid volume in ACTH- and cortisol-induced hypertension

Administration of ACTH or cortisol to normal males results in urinary sodium retention in association with an increase in plasma volume, cardiac output and blood pressure (Connell, Whitworth et al, 1987). Plasma albumin and haematocrit fall, probably a dilutional effect. The concentration of ANP increases during both treatments, likely secondary to right atrial distention. Despite this, sodium retention persists during ACTH treatment (Connell, Whitworth et al, 1987).

Expansion of the ECFV is unlikely to be causal to hypertension, however, as administration of synthetic glucocorticoids which do not cause sodium retention or increased plasma volume (prednisolone, methylprednisolone, dexamethasone and triamcinolone) also cause hypertension (Whitworth, Gordon et al, 1989a). As discussed above, sodium restriction (15 mmol/day) blocked the rise in ECFV but not the increase in plasma volume or rise in

blood pressure (Connell, Whitworth et al, 1988). Blockade of the MR with spironolactone prevented the gain in body weight and fall in serum potassium but failed to prevent the rise in blood pressure observed during treatment with cortisol 80 mg daily (Williamson, Kelly et al, 1996). Taken together, these results argue against the sodium and water retaining properties of cortisol being solely responsible for its hypertensive effect.

1.3.6.5 Effects of ACTH and cortisol on cardiac output

Cortisol administration (200 mg/day) increased cardiac output by about 1.1 L/min, as measured by the Fick method or by Doppler technique (Pirpiris, Yeung et al, 1993). There is no consistent change in pulse rate with cortisol or ACTH (Whitworth, Saines et al, 1983b, Whitworth, Saines et al, 1984).

The increase in cardiac output was not essential for the rise in blood pressure, as pre-treatment with the β-adrenoreceptor antagonist atenolol prevented the rise in cardiac output, but not the rise in blood pressure secondary to oral cortisol (Pirpiris, Yeung et al, 1993). Atenolol also prevented the fall in calculated total peripheral resistance secondary to cortisol. The notion that the rise in cardiac output is not essential for the rise in blood pressure is reinforced by studies with dexamethasone, which raises blood pressure in association with an increase in calculated total peripheral resistance, but without an increase in cardiac output (Pirpiris, Sudhir et al, 1992).

1.3.6.6 Effects of ACTH and cortisol on peripheral vascular resistance

In atenolol-treated subjects, the blood pressure rise accompanying oral cortisol is mediated by peripheral resistance (Pirpiris, Yeung et al, 1993). To determine whether the onset of cortisol-induced hypertension is mediated by vasoconstriction, Williamson et al treated male volunteers with felodipine and cortisol (Whitworth, Williamson et al, 1994c). Felodipine (5 mg/day) blocked the increase in calculated peripheral resistance, but had no effect on cortisol-induced hypertension. Hence an increase in peripheral resistance is not essential for development of cortisol-induced hypertension.

1.3.6.7 Effects of ACTH and cortisol on sympathetic nervous system activity

In healthy male subjects, cortisol administration did not increase plasma concentrations of noradrenaline or adrenaline, or neuropeptide Y-like immunoreactivity (Connell, Whitworth et al, 1987). NA spillover and NA uptake were not increased by cortisol, suggesting that overall resting sympathetic nervous system activity was not increased (Sudhir, Jennings et al, 1989). Reflex function tests in cortisol treated subjects (cold pressor, tilt, mental arithmetic and 30% maximal hand grip) have suggested that SNS activity is not increased in subjects with cortisol-induced hypertension, and may be reduced (Tam, Williamson et al, 1997). Similar results were observed in cortisol-treated males in whom pharmacological blockade of the autonomic nervous system was achieved by administration of oral prazosin and intravenous atropine, propranolol and clonidine (Tam, Williamson et al, 1997). Autonomic blockade augmented the rise in DBP, MAP and heart rate secondary to cortisol (SBP also increased during autonomic blockade though

this was not statistically significant), suggesting that the ANS exerts a modulating effect on the blood pressure rise secondary to cortisol.

Recently, Macefield et al have measured muscle sympathetic nerve fibre activity in cortisol-treated subjects. Compared with placebo, MSNA was reduced in cortisol treated males, in keeping with the notion that SNS activity is not increased in cortisol induced hypertension, and may be suppressed (Macefield, Williamson et al, 1998). Regional SNS activity has not been systematically assessed.

Together, the above studies do not support the notion that the SNS is involved with the pathogenesis of cortisol-induced hypertension and suggest that the SNS activity may actually be suppressed by cortisol excess.

1.3.6.8 Effects of ACTH and cortisol on vascular responsiveness

The increased total peripheral resistance characteristic of chronic forms of hypertension is associated with both structural and functional abnormalities in vascular smooth muscle. Structural changes are probably of major importance in patients with long-standing hypertension but are unlikely to play a role in the rise in blood pressure secondary to exogenous corticosteroids in short term experiments.

1.3.6.8.1 Pressor responses to phenylephrine and angiotensin-II in cortisol-treated subjects

At a dose of 200 mg/day, cortisol administration produces a rise in cardiac output in association with reduced calculated peripheral resistance (Sudhir,

Jennings et al, 1989, Pirpiris, Yeung et al, 1993). Resting forearm vascular resistance is unchanged (Sudhir, Jennings et al, 1989). Whitworth et al examined pressor responses to angiotensin II and phenylephrine (an alpha adrenergic agonist) before and after 5 days of ACTH or hydrocortisone (cortisol) (Whitworth, Connell et al, 1986). The effects on blood pressure in response to intravenous infusions of these agents was examined. There was no significant effect of ACTH or cortisol on pressor responses to angiotensin II. Cortisol and ACTH increased pressor responses to phenylephrine infusion at the mid-dose (cortisol) and high-dose (cortisol and ACTH) along with reduction in the threshold for a rise in systolic blood pressure (Whitworth, Connell et al, 1986). It was concluded that these changes were unlikely to cause the rise in blood pressure as ACTH treatment produced greater rises in pressure and cortisol concentrations with changes in pressor responsiveness at high infusion rates only.

In a subsequent study, the pressor effects of angiotensin II and phenylephrine were re-examined in cortisol-treated subjects who also received indomethacin (see below). In this study, the threshold dose for SBP and MAP increases by angiotensin II was examined (1 ng/kg). The initial study (Whitworth, Connell et al, 1986) did not examine threshold doses of angiotensin II, as the lowest dose used was 2 ng/kg, which proved to be pressor. In the indomethacin study, the threshold dose for SBP and MAP rises with angiotensin II was decreased by cortisol (Whitworth, Connell et al, 1988).

To exclude the possible effect of sodium retention as cause of altered vascular responsiveness, pressor effects of angiotensin II and phenylephrine in ACTH-treated subjects on a low sodium (15 mmol/day) diet were investigated (Connell, Fisher et al, 1987). Sodium restriction did not modify the enhanced pressor effect of phenylephrine in ACTH-induced hypertension, suggesting that enhanced pressor sensitivity to catecholamines during ACTH-induced hypertension is not sodium-dependent.

1.3.6.8.2 Pressor responses to cold stimulus and catecholamines

In a further study, vascular responses were examined to cold pressor stimulus to the neck and to intra-arterial infusion of noradrenaline in subjects taking oral hydrocortisone (200 mg daily) (Sudhir, Jennings et al, 1989). There was a marked increase in cold pressor stimulation following cortisol treatment. Cortisol also produced a fall in threshold, a shift to the left of the dose-response relation and a significantly greater average rise in forearm vascular resistance to intra-arterial noradrenaline. Resting nor-adrenaline spillover was unchanged, indicating overall resting sympathetic nervous system activity was not increased. These effects are therefore likely to be due to local postsynaptic effector mechanisms. At the dose of cortisol used in this study, cortisol had both mineralocorticoid and glucocorticoid effects.

1.3.6.8.3 Pressor responses during fludrocortisone- and dexamethasone-induced hypertension

Mineralocorticoids are recognised to increase pressor responses to catecholamines in humans (Raab, Humphries et al, 1950, Schmid, Eckstein

et al, 1966, Phillip, Luff et al, 1983). To further delineate whether the enhanced pressor effects of cortisol are a feature of its glucocorticoid activity, Pirpiris et al examined pressor responses during fludrocortisone- and dexamethasone-induced hypertension (Pirpiris, Sudhir et al, 1992). Although the haemodynamic patterns differed, increased pressor responsiveness was a feature of both mineralocorticoid-and glucocorticoid-induced hypertension in this study.

1.3.6.8.4 Vasodilator function and the nitric oxide system in cortisol-induced hypertension

Vasodilator dysfunction appears to be a common feature of many forms of human hypertension (Takeshita and Mark, 1980, Taddei, Virdis et al, 1993) as well as a feature of diabetes mellitus (Calver, Collier et al, 1992b), dyslipidaemia (Chowienczyk, Watts et al, 1992) and ageing (Gerhard, Roddy et al, 1996). In essential hypertension, the endothelial nitric oxide pathway has been reported abnormal by many investigators (Calver, Collier et al, 1992a, Panza, Casino et al, 1993, Higashi, Oshima et al, 1995, Taddei, Virdis et al, 1997), though at least one group has disputed this finding (Cockcroft, Chowienczyk et al, 1994). There may also be a role for cyclooxygenase-dependent vasoconstrictor substances in the endothelial dysfunction accompanying essential hypertension (Taddei, Virdis et al, 1993, Taddei, Virdis et al, 1997).

The role of vasodilator prostanoids was indirectly examined in experiments measuring pressor responses in cortisol-treated males given indomethacin

(100 mg) (Whitworth, Connell et al, 1988). In this study, the pressor effects of angiotensin II and phenylephrine were enhanced in the cortisol treatment phase of the study, but were not modified by indomethacin, suggesting that the altered pressor responsiveness in cortisol-treated subjects was not mediated by vasodilator prostanoids.

There are some recent data suggesting a role for the nitric oxide system in ACTH- and cortisol-induced hypertension. In the rat, ACTH treatment suppresses concentrations of reactive nitrogen intermediates, NO_2/NO_3. In addition, ACTH-induced hypertension in the rat is prevented by oral supplementation by L-arginine (Turner, Wen et al, 1996), whereas D-arginine has no effect on blood pressure. In healthy males on a nitrate exclusion diet (to minimize confounding effects of dietary nitrate on measurements of reactive nitrogen intermediates), cortisol treatment significantly reduced plasma NO_2/NO_3 (Kelly, Tam et al, 1998).

How glucocorticoids suppress nitric oxide system activity is not known. Kelly et al have reported no effect by cortisol on the plasma concentration of an endogenous inhibitor of NOS, asymmetric dimethyl arginine (Kelly, Tam et al, 1998). Glucocorticoids are known to inhibit the induction of iNOS by endotoxin or interferon-γ and lipopolysaccharide (Radomski, Palmer et al, 1990, Knowles, Salter et al, 1990), but this isoform of NOS has not been implicated in the basal regulation of vascular tone. Glucocorticoids have been shown to limit intracellular arginine and tetrahydrobiopterin availability for

iNOS, both of which are essential factors for the production of nitric oxide; this effect may be relevant to endothelial NOS and therefore NO production in cortisol-induced hypertension (Simmons, Ungureanu-Longrois et al, 1996).

There have been no studies published directly examining vasodilator function and endothelial NOS in experimental glucocorticoid-induced hypertension in man. In this thesis, I have reported results of studies designed to directly examine endothelial vasodilator function in cortisol-treated male subjects.

1.3.6.9 Evidence for a third corticosteroid receptor mediating cortisol-induced hypertension

Whitworth and co-workers proposed a hypertensinogenic class of steroid activity in the sheep, distinct from the actions of MR and GR (Whitworth and Scoggins, 1990). In the rat, the hypertensive effects of ACTH are not blocked by GR or MR antagonists (Li, Wen et al, 1999), and results have been similar in humans treated with cortisol (Clore, Estep et al, 1988, Williamson, Kelly et al, 1996). Clore et al have hypothesized the existence of a third corticosteroid receptor. They have shown that cortisol-induced sodium retention and hypertension are not prevented by MR (Montrella-Waybill, Clore et al, 1991) or GR blockade (Clore, Estep et al, 1988). They postulated the presence of a putative type IV receptor in their studies of A6 epithelial cells. In this model, radiolabelled corticosterone was metabolised to 6β-OH-corticosterone (by 6β-hydroxylase), and this metabolite occupied a third, lower affinity set of nuclear binding sites (Duncan, Grogan et al, 1988). Evidence for a third corticosteroid receptor in man is indirect.

1.3.6.10 Other mechanisms examined in ACTH- and cortisol-induced hypertension in man

Changes to the renin-angiotensin system following cortisol excess are complex. Angiotensinogen (renin substrate) is reproducibly increased, but this hormone is probably not biologically active itself. Plasma renin and aldosterone, however, are (variably) suppressed by oral cortisol or ACTH (Connell, Whitworth et al, 1987). It is generally acknowledged that the renin-angiotensin system plays, at most, a modest role in glucocorticoid-induced hypertension (Whitworth, 1987).

Octreotide, an inhibitor of pancreatic insulin production, profoundly reduced the elevated insulin concentrations observed with cortisol administration, but had no effect on blood pressure (Whitworth, Brown et al, 1995).

Vasodilator peptides such as kallikrein and atrial natriuretic peptide are increased following cortisol (Whitworth, 1992). Recently, there has been interest in the role of the hormone erythropoietin on blood pressure. Hypertension is considered a complication of erythropoietin therapy. In a double-blind study, serum erythropoietin concentrations were increased during cortisol treatment (Martin, Kelly et al, 1998), however, it is not known whether there is any causal role or whether the effect was a paraphenomenon.

1.3.7 ABNORMALITIES OF THE GLUCOCORTICOID RECEPTOR: GLUCOCORTICOID RESISTANCE

The syndrome of primary cortisol resistance was first described in a Dutch family in 1968 (Vingerhoeds, Thijssen et al, 1976). This condition is associated with a reduction in receptor affinity for glucocorticoids with a variable change in receptor number (Lipsett, Tomita et al, 1986, Arai and Chrousos, 1994, Connell, Kenyon et al, 1996). The reduced effect of glucocorticoids leads to increased ACTH drive to the adrenal cortex, leading to increased production of cortisol, androgens and deoxycorticosterone. Hypertension varies between family members, and may reflect other genetic background against which the glucocorticoid receptor abnormality is expressed. Furthermore, because GR action involves homo- and heterodimer formation, the eventual receptor activity will reflect functional activity of the products of both alleles. Similarly, there is phenotypic variation within kindreds with the syndrome of thyroid hormone resistance.

1.3.8 IN VITRO STUDIES OF GLUCOCORTICOID EFFECTS ON VASCULAR SMOOTH MUSCLE

In vitro studies have suggested a number of mechanisms for vascular hyperactivity. These include increased endothelin production by rabbit and rat vascular smooth muscle cells (Kanse, Takahashi et al, 1991), increase in calcium uptake, possibly mediated by an increase in the number of dihydropyridine-sensitive calcium channels (Hayashi, Nakai et al, 1991), increase of $\alpha 1_B$-adrenergic receptors in hamster smooth muscle cells

(Sakaue and Hoffman, 1991) and decrease in ANF-mediated c-GMP formation (which can lead to diminished vasodilatation by c-GMP) (Yasunari, Kohno et al, 1990, Danese and Aron, 1994).

In vitro, glucocorticoids also inhibit the inducible isoform of nitric oxide synthase in porcine aortic endothelial cells (Radomski, Palmer et al, 1990), in addition to inhibition of prostacyclin, as discussed above. Whether the inducible isoform(s) of NOS contribute to blood pressure regulation is not known. However, the locus for the gene encoding iNOS was reported to be linked to hypertension in the Dahl salt-sensitive rat (Deng and Rapp, 1995). Furthermore, the administration of dexamethasone (which suppresses iNOS activity) to Dahl salt-sensitive rats also receiving L-arginine blocked the antihypertensive effect of L-arginine (Chen and Sanders, 1991), suggesting a role for an inducible isoform of NOS in this model (Chen and Sanders, 1993). In a recent report, Chen and coworkers reported reduced iNOS activity in cultured aortic vascular smooth muscle cells of Dahl/Rapp salt-sensitive rats, which increased with addition of L-arginine to the culture medium (Chen, Gladish et al, 1998). Analysis of the iNOS gene identified a single sequence abnormality in the coding region of iNOS that produced an amino acid substitution and restriction fragment length polymorphism, present only in salt sensitive rats. Hence, a mutation of iNOS appears to be associated with hypertension in the Dahl/Rapp salt-sensitive rat.

1.3.9 HYDROXYLASE AND REDUCTASE DEFICIENCIES - THE SYNDROMES OF CONGENITAL ADRENAL HYPERPLASIA

CAH is the inherited inability to synthesize cortisol, as a consequence of mutations in any of four enzymes; 3β-HSD, 21-hydroxylase, 11β-hydroxylase or 17α-hydroxylase (see figure 1.1) or of mutations in the steroidogenic acute regulatory protein required for cholesterol transport into mitochondria (Lin, Sugawara et al, 1995). More than 90 % of cases are due to deficiency of 21-hydroxylase (White, 1996). This enzyme is required for aldosterone and cortisol synthesis, therefore, affected individuals may have hyponatraemia, hyperkalaemia and hypovolaemia and if untreated may progress to shock and death within weeks of birth.

In contrast, the remaining cases of CAH are usually hypertensive forms, usually due to deficiency of 11β-hydroxylase and a few due to 17α-hydroxylase deficiency (White, 1996). These conditions are rare (1 in 200 000 births but up to 1 in 5000 in Moroccan Israelis) autosomal recessive disorders in which steroid intermediates are produced in excess amounts. In 11β-hydroxylase deficiency, there is excess production of 11-deoxycortisol and 11-deoxycorticosterone because of reduced feedback inhibition by glucocorticoids as a result of reduced production. About two thirds of patients with 11β-hydroxylase deficiency are hypertensive, though the mechanism by which hypertension develops is not well understood (White, 1996). In addition, these patients often exhibit signs of androgen excess. This occurs because accumulated cortisol precursors are shunted into the pathway of

androgen biosynthesis. Glucocorticoid administration replaces deficient cortisol and reduces ACTH secretion, and therefore may correct hypertension in these individuals.

In 17α-hydroxylase deficiency, 17-deoxysteroids are synthesized in excessive quantities. Corticosterone acts as a glucocorticoid, and hypertension may develop as a result of excessive 11-deoxycorticosterone (mineralocorticoid) or corticosterone (glucocorticoid). In this condition, there is a lack of sex hormones, which may not become apparent until puberty as prepubertal females appear normal and genetic males can have external genitalia indistinguishable from those of a female (White, 1996).

1.4 SUMMARY - CORTISOL AND HYPERTENSION

Cortisol causes hypertension in Cushing's disease, AME and liquorice abuse and there is evidence that, at the very least, it contributes to some subsets of essential hypertension, the hypertension of renal failure and possibly to nephrotic syndrome in man. Research in recent years has defined abnormalities of cortisol metabolism, at the tissue level, which may help explain just how cortisol may be involved in these hypertensive disorders. At least one enzyme system, 11β-HSD, is critical to the development of hypertension when its activity is abnormal. Other enzyme systems may also be important. Abnormalities of GR or intracellular signalling may be relevant in determining tissue sensitivity to cortisol.

Much information has come from experimental models of ACTH- and cortisol-induced hypertension. Characteristics of various forms of hypertension in which cortisol is causative or is implicated are featured in Table 1.2. The physiological profiles of these models are well defined in man, sheep and rat yet the mechanism by which the hypertension develops is not fully understood. In man, increased responses to vasopressor agents following ACTH- or cortisol has been repeatedly shown, by mechanisms which are not understood. Endothelial vasodilator function has not been directly examined.

Table 1.2: Characteristics of various hypertensive syndromes in which steroids contribute.

	Cortisol-induced hypertension	AME	Liquorice	Essential hypertension
Onset of hypertension	hours	rapid	weeks	slow
Na retention	yes	yes	yes	variable
Response to Na restriction	modulating effect	modulating effect	not reported	variable
Response to spironolactone	not prevented	partial unsustained	yes	yes
Hypokalaemia	yes	yes	yes	no
RAS	suppressed	suppressed	suppressed	variable
SNS	suppressed or unchanged	unknown	unknown	possibly overactive
NO	possibly suppressed	unknown	unknown	possibly suppressed
Cortisol metabolism	normal	prolonged $T_{1/2}$	prolonged $T_{1/2}$	prolonged $T_{1/2}$ reported

CHAPTER 2

Methods

2.1 HUMAN STUDIES

2.1.1 Subjects and ethical approval

To examine the effect of cortisol on endothelial vasodilator function in man and whether any effect is mediated by classical corticosteroid receptors, three studies were conducted.

Healthy male subjects were recruited by advertisement in the St George shire newspaper The Courier, the UNSW student newspaper Tharunka and by leaflets placed on notice boards at St George Hospital. Only subjects aged between 18-40 years were included in these studies. Women were excluded from these studies because of the potential confounding effect of cyclical variation in sex steroids on blood pressure. Care was taken to exclude volunteers with any history of peptic ulcer disease, allergy, asthma, diabetes mellitus, hepatitis, HIV infection or other chronic infectious disease, cardiac disease, hypertension, electrolyte disturbances, renal disease, other endocrine disorders or individuals taking regular medication. To achieve this, each volunteer was screened during an initial visit to St George Hospital. A medical history and physical examination were performed by myself and venous blood was collected from an antecubital vein. The samples were collected and sent to the South Eastern Area Laboratory Service for a full blood count, electrolytes, urea and creatinine, calcium and phosphate, total protein and albumin, liver function tests and for serological tests for hepatitis

B surface antigen and core antibody, hepatitis C antibody and HIV antibody. Volunteers were notified of their blood results when available and enrolled in the study at that stage.

These studies were approved by the South Eastern Sydney Area Health Service Ethics Committee (Approval No 96/09) and by the Committee on Experimental Procedures involving Human Subjects of the University of New South Wales (Approval No 96011). Notification was given to the Therapeutic Goods Administration, Commonwealth Department of Health and Family Services (CTN no 96-0134), and all studies conformed to the guidelines for conduct of human experimentation of the National Health and Medical Research Council of Australia. Each subject gave informed consent prior to commencement in any study. Subjects were remunerated for travel expenses and time. Different individuals were enrolled in each study, however, two subjects completed both the dexamethasone and the fludrocortisone studies. There were eight subjects in the cortisol study, eight in the dexamethasone study and six subjects in the fludrocortisone study.

2.1.2 Protocols for human studies

The three human studies individually examined the effects of five days of cortisol, dexamethasone or fludrocortisone on forearm blood flow and responses to vasodilators. Each was a randomised, double blind, placebo-controlled crossover study where subjects received either placebo or drug in

the first phase of the study then crossed over to the other treatment in the second phase, with FBF measured on day 5 of each phase.

In the cortisol and dexamethasone studies, each phase of the study was 11 days duration (Figure 2.2). There were 3 control days (C1-C3), 5 treatment days (T1-T5) and 3 post-treatment observation days (P1-P3) in each phase. Subjects attended for each of these 11 days (ie 11 experimental days).

In the fludrocortisone study, each phase was 10 days duration with three control days (C1-C3), five treatment days (T1-T5) and two post-treatment observation days (P1, P2). The design of this third study was similar, however, subjects attended for six of the 11 days only (C1, C3, T1, T3, T5, P2). There was a washout period of at least 4 weeks between each phase in each study.

2.1.3 Sodium and diet during the study period

Subjects were asked to maintain a fixed sodium intake of 150 mmol/day from three days prior to and for the duration of each phase of each study, aiming for sodium balance by C1. During this period, they were also asked to abstain from alcohol or prescription medications. During the experimental period, they were asked to maintain a stable lifestyle, with regular sleep and meals and to take a small breakfast prior to each morning visit. To facilitate compliance with dietary restrictions, subjects were educated about dietary

sodium and given written information on sodium content in foods at their initial visit.

2.1.4 Randomisation and blinding

The human studies were double-blinded. The St George Hospital Pharmacy provided the blinded medication. The active steroid was crushed and inserted into a brown capsule. The placebo (lactose) was similarly prepared. The treatments were randomised and labelled "A" or "B" by pharmacy. Subjects received treatment "A" in the first phase, then "B" in the second phase. The subjects were never informed of the order of randomisation, unless they were interested and inquired after the completion of the study.

The code was not broken until after the analysis of forearm data and raw blood pressure data by myself.

2.1.5 HUMAN EXPERIMENTAL PROCEDURES

2.1.5.1 Blood pressure measurement

At 0700-0800 hrs on the experimental days, each subject reported to the Clinical Research Room, St George Hospital. This room is quiet and has a controlled temperature of 22-24^{0}C. The subject was weighed then lay supine for 30 minutes. Blood pressure was recorded in the supine position then after 5 minutes standing, using a Hawksley Random Zero Sphygmomanometer (Hawksley and Sons, England), a device which blinds the observer to the result until after the measurement has been taken. A minimum of three

measurements of blood pressure and pulse were made in each position, until three recordings of mean arterial pressure within 6 mmHg were obtained (mean arterial pressure was calculated as one third the pulse pressure plus the diastolic blood pressure). If the mean arterial pressure varied by more than 6 mmHg, further measurements were performed.

Calf blood pressure was also recorded during the FBF study on T5 using an oscillometric device described below.

2.1.5.2 Metabolic measurements

Subjects were weighed on each experimental day. In the cortisol and dexamethasone studies, venous blood was collected by cannulation of a right antecubital vein after 30 minutes supine recumbency (after blood pressure recording) on experimental days C1, C3, T1, T3, T5 and P2. In the fludrocortisone study, venous blood was collected on days C3 and T5 only. On every blood collecting day, blood was sent for full blood count, electrolytes, urea and creatinine, glucose, calcium, phosphate and albumin to the South Eastern Area Laboratory Service (NSW). On C3 and T5 of all studies, blood was also collected for serum cholesterol, triglycerides and serum cortisol. Supine renin concentration was measured in the fludrocortisone study.

Twenty four hour urine specimens were collected for sodium and potassium excretion and creatinine clearance on C2-3, T4-5 and P2-3 in the cortisol and dexamethasone studies.

In the cortisol study, serum cortisol was measured by radioimmunoassay as described below. In the subsequent studies, autoanalysis of cortisol became available by the South Eastern Sydney Area Laboratory Service so this assay was performed by that laboratory, along with the other biochemistry and haematology.

2.1.5.2.1 ASSAY FOR PLASMA NO_2/NO_3

2.1.5.2.1.1 Principles of the NO_2/NO_3 assay

Total NO_2/NO_3 in plasma was measured by the Griess colorimetric reaction (Boehringer Mannhein, Castle Hill, Sydney).

The principle of the assay is as follows; nitrate is initially reduced to nitrite by nitrate reductase. The concentration of NOx is determined by a colour reaction between nitrite and the Griess reagent. Absorbance is measured by a microplate reader. Background is obtained by treating the samples using 25 % phosphoric acid instead of the complete Greiss reagent.

2.1.5.2.1.2 Performance of the NO_2/NO_3 assay

QC samples were assayed along with the unknown samples. Results for the QC samples were as follows; high QC (40 mmol/l) 32.6 mmol/l, low QC (10 mmol/l) 8.2 mmol/l.

2.1.5.2.1.3 Procedures for the NO_2/NO_3 assay

Briefly, heparinised venous blood was centrifuged at 3000 rpm for 15 min at 4^0C. Plasma was frozen at -20^oC then stored at -80^0C until used. Reduced

NADPH (115 µl, 3 mmol/l), FAD (15 µl, 250 µmol/l) and nitrate reductase (30 µl, 200 units/l) were added to 590 µl of sample in duplicate. Samples were vortexed and incubated at 37^0C for 1 hour. Excessive NADPH was removed by addition of 10 µl LDH (775 mg/ml) and 15 µl sodium pyruvate (516.7 mmol/l) then incubation for 5 min at 37^0C. Samples were then deproteinized by addition of 75 µl zinc sulphate (30 g/l) and microcentrifugation for 5 min at 10000 g. Two hundred eighty microlitres of each sample was then transferred to into a well (in duplicate) with 70 µl Greiss reagent, and allowed to incubate for 10 min at room temperature. After colour had developed, absorbances were read on a microplate reader (Titertek Multiskan MCC; Flow Laboratory, Mclean, VA, USA).

2.1.5.2.2 ASSAY FOR SERUM CORTISOL

2.1.5.2.2.1 Principles of the cortisol assay

Serum cortisol was measured by radioimmunoassay technique (Amerlex Cortisol RIA Kit, Johnson & Johnson Clinical Diagnostics Ltd, UK). Cortisol is released from CBG by a chemical in the tracer reagent. Total cortisol in the sample is then free to compete with radiolabelled ^{125}I-cortisol for a limited number of binding sites on a sheep anti-cortisol antibody bound to polymer particles. The antibody-bound complex is separated by centrifugation followed by decanting of the supernatant. The amount of tracer bound is inversely proportional to the concentration of cortisol present.

2.1.5.2.2.2 Cortisol assay procedure

All standards, controls and samples were assayed in duplicate. A standard curve was generated by the measurement of known concentrations of cortisol supplied with the kit (standards) from 0 to 1665 nmol/l. Low (75-118 nmol/l), mid (492-639 nmol/l) and high controls (718-1007 nmol/l) were included and assayed as unknowns. Concentration of cortisol was determined in subjects' samples from this standard curve, generated by computer using custom software on a log/logit curve.

Fifty microlitres of standard, control or sample was pipetted into labelled polypropylene tubes. Two hundred microlitres of ^{125}I-labelled cortisol derivative was added to each tube ('total' tubes were then capped and set aside). Two hundred microlitres of antibody suspension was then dispensed into all tubes. The tubes were vortexed, incubated at 37^{0}C for one hour then centrifuged at 3000 rpm for 15 min. The supernatant was decanted and the tubes allowed to drain completely. Each tube was then counted in a gamma counter for one minute and counts recorded automatically.

2.1.5.2.2.3 Performance of the cortisol assay

Sensitivity of this assay is defined as the concentration two standard deviations from the zero standard when twenty replicates are determined. The sensitivity is approximately 2.8 nmol/l. Intra-assay coefficient of variation is 4.3 - 5.8 % and inter-assay coefficient of variation is 7.7 - 8.9 %. Results from the control samples were as follows; low 87 nmol/l, mid 441 nmol/l*, high 738 nmol/l (* below range).

2.1.5.3 Forearm venous occlusion plethysmography

2.1.5.3.1 Principles of the technique

Venous occlusion plethysmography has been used to study forearm blood flow for more than 80 years (Benjamin, Calver et al, 1995). Initially, it required cumbersome water jackets, but with the advent of mercury-in-rubber strain gauges, it has become widely used to study mechanisms of human vascular control.

Venous occlusion plethysmography measures total forearm blood flow, of which, under resting conditions, blood flow through skeletal muscle is the bulk (50-70%), the remainder being flow through skin (Benjamin, Calver et al, 1995). The hands must be excluded from the circulation (typically by wrist cuffs inflated to suprasystolic pressures), as blood flow through the hand is predominantly through the skin. The principle of the technique is that the rate of flow in the forearm vascular bed is proportional to the increase in circumference accompanying occlusion of venous return at the level of the mid-humerus (Whitney, 1953). Arrest of forearm venous return is usually achieved by inflating a cuff around the upper arm to 40 mmHg for 10 seconds, a manoeuvre that does not affect arterial inflow or pressure (Wilkins and Bradley, 1946). The rate of swelling of the forearm can be measured by water displacement or, more conveniently, can be calculated from changes of forearm circumference, as a percentage increase from baseline by means of a strain gauge placed about a third of the way between the elbow and wrist. The principle is that the mercury within the strain gauge increases its

resistance to direct current on stretching, and that the change in resistance is proportional to the change in length of the gauge.

This technique estimates the total flow in the forearm, not the flow immediately beneath the gauge (Benjamin, Calver et al, 1995). Flow is expressed per unit volume of forearm, usually as millilitres per 100 ml forearm per minute. This technique has been shown to be accurate and is often used as the standard against which other methods are judged (Roddie and Wallace, 1979).

The relationship between the rate of increase in forearm volume and arterial flow is true only when the forearm veins are not fully distended. Once the veins are full, the pressure will rise and blood will escape under the congesting cuff (Benjamin, Calver et al, 1995). Furthermore, if the pressure in the veins reaches 40 mmHg, arterial inflow may be attenuated (Wilkins and Bradley, 1946).

2.1.5.3.2 Forearm blood flow v calculated vascular resistance

Although some authors prefer to report forearm vascular resistance (derived from perfusion pressure / blood flow), many consider this derived term an unreliable and unjustified expression (Benjamin, Calver et al, 1995). Vascular resistance is not comparable to the resistance in a direct current system, as described by Ohm's Law. The formula used to calculate vascular resistance ignores the impedance element due to the alternating current component. Furthermore, this formula is applicable to laminar flow of a Newtonian fluid

through a fixed resistance under a steady driving pressure. Blood is not considered a Newtonian fluid and is driven through a distensible system by a pulsatile pressure (Benjamin, Calver et al, 1995).

2.1.5.3.3 The contractile state of vascular smooth muscle

Although vasoactive drugs affect the contractile state of circular smooth muscle, rather than directly affecting flow or resistance, it is flow that we can conveniently measure and, if arterial perfusion pressure does not change significantly, it provides a reasonable estimate of the state of contraction of smooth muscle. Changes in arterial perfusion pressure will affect the contractile state. This technique therefore requires every effort to be made to ensure that arterial pressure does not vary during the course of the study. To achieve this, the surroundings are temperature controlled, quiet, and subjects should be comfortable, relaxed and given adequate time to settle prior to any measurements being taken.

2.1.5.3.4 Ratio of flow in the arms

To compensate for small changes in sympathetic outflow and blood pressure, simultaneous measurement of flow in both arms is employed. In the absence of intervention, the ratio of flow in the two arms approaches unity and stays constant even if blood flow alters markedly in response to changes in systemic arterial pressure or sympathetic arousal (Benjamin, Calver et al, 1995, Greenfield and Patterson, 1954). Hence, expressing the results in terms of the ratio of flow in the two arms provides an internal control, uses all the available data, minimises variation and gives reproducible results.

2.1.5.3.5 Local administration of drugs into the brachial artery

Because the systemic administration of drugs may lead to central effects, hormonal responses, changes in sympathetic output and alterations in blood pressure, changes in forearm blood flow are difficult to interpret. The infusion of drugs into the brachial artery overcomes these problems, as the dose required to produce large changes in forearm blood flow may be 100-1000 times smaller than those required to produce systemic effects. Practically, this is achieved by a catheter placed in the brachial artery at the level of the antecubital fossa. The use of a very fine needle (27 gauge) allows this to be done safely and with minimal risk of endothelial trauma. Use of such a small needles precludes the measurement of intra-arterial pressure, which requires a needle of about 18 gauge. This measurement is seldom indicated, however, unless calculation of calculated resistance is required. It is appropriate to intermittently measure arterial pressure throughout the study to demonstrate that no systemic changes have occurred.

2.1.5.3.6 Protocols for forearm venous occlusion plethysmography in these studies

Forearm blood flow was measured on treatment day 5 of each phase of each study using an identical protocol for each study (Figure 2.3). This protocol was adopted from that used by Professor David Webb's group in the UK (University of Edinburgh). All solutions were infused at a rate of 1 ml/min.

The 2 3/4 hour protocol can be summarised as follows;

1. 30 min run-in infusion of normal saline
2. 3 x 6 min infusions of the first drug (ACh or SNP, randomised), in increasing concentration
3. 18 min saline washout infusion
4. 3 x 6 min infusions of the second (alternate) drug, in increasing concentration
5. 18 min saline washout
6. 6 min infusion of L-NMMA
7. repeat 3 x 6 min infusions of the first drug, in increasing concentration
8. 18 min saline washout
9. repeat 6 min infusion of L-NMMA
10. repeat 3 x 6 min infusions of the second drug, in increasing concentration

This protocol allowed the investigation of endothelium-dependent vasodilator responses, by the infusion of acetylcholine, and endothelium-independent vasodilator responses by the infusion of sodium nitroprusside, a nitric oxide donor. The use of incrementally increasing doses allowed the construction of dose-response curves.

L-NMMA is a methyl analogue of L-arginine and a potent competitive inhibitor of all isoforms of nitric oxide synthase. The infusion of L-NMMA, followed by

repeat infusions of ACh and SNP, immediately thereafter, allowed the determination of the component of cholinergic vasodilatation attributable to endothelial nitric oxide.

2.1.5.3.6.1 PROCEDURES ON TREATMENT DAY 5

2.1.5.3.6.1.1 Equipment and setup

After supine and erect blood pressure and pulse measurements, weighing and collection of venous blood, the subject had a 15 min break during which he was asked to empty his bladder. The subject then lay supine on a comfortable bed. The environment was maintained as close as possible to 24^{o}C. The arms were inclined at 30 degrees to improve venous drainage and rested comfortably at the level of the wrist on pillows for support. Blood pressure cuffs were applied at the level of the mid-humerus bilaterally, supplied by an automated rapid cuff inflator (D.E. Hokanson, Incorporated, Bellevue, Washington USA). A sponge support, 2.5 cm thick, was placed between the olecranon and the bed to support the arm and minimise communication between the upper arm cuff and the bed below. Wrist cuffs were applied, and were inflated to 200 mmHg from one min prior to and during the recording period by a similar rapid cuff inflator to exclude hand circulation from the measurements (Figure 2.4).

The forearms were marked 7 cm distal to the olecranon and the circumference of the forearm at that point documented. A mercury-in-silastic strain gauge (D.E. Hokanson, Incorporated, Bellevue, Washington USA), 2 cm in length less than the forearm circumference, was applied to each

forearm at this mark (Figure 2.5). The same size gauge was used in each of the two studies (ie, placebo and treatment). These gauges were connected to a dual channel strain gauge plethysmograph capable of electronic calibration (Vasculabs SPG16, Mediasonics, Mountain View, California, USA). The analogue signal outputs from this device were converted to digital signal by an MP100 analogue to digital converter (MP 100, Biopac Systems, Goleta, California, USA) which communicated via a serial port to a personal computer running a data acquistion software package (Acqknowledge version 2.1).

2.1.5.3.6.1.2 Brachial artery cannulation

After all cuffs were applied and secured, a 27 gauge stainless steel sterile needle (Becton Dickinson and Company, Franklin Lakes, NJ, USA) was introduced under 1% lignocaine anaesthesia into the left brachial artery at the level of the antecubital fossa. Patency was maintained by infusion of physiological saline 0.9% (Baxter Healthcare Ltd) at a rate of 1 ml/min.

2.1.5.3.6.1.3 Calibration of the system

Ideally, calibration of the system would be achieved by simulating a 1% stretch of the gauges using reference cylinders of exact circumferences. The gauges, however, are temperature sensitive and must be allowed to equilibrate to the forearm temperature prior to any recordings occurring. In practice, electronic simulation of a 1 % stretch is delivered by generation of a square wave by the bridge amplifier device. This is then registered by the data acquisition software and the bridge amplifier zeroed and calibrated to 1 % stretch accordingly.

2.1.5.3.6.1.4 Forearm blood flow data acquisition

The upper arm congesting cuffs were intermittently inflated to 40 mmHg for three minutes then deflated. Occlusion of venous return resulted in congestion of the forearm, stretching of the strain gauges and produced a curve for each arm on the computer screen. Multiple measures were taken during three minute periods and the slopes of the final five recordings in each period averaged to determine the forearm blood flow. The slope of the curve analyzed was the most linear portion of the initial part of the curve. An example is shown below. The software package provides the calculations for gradient, required for calculation of FBF.

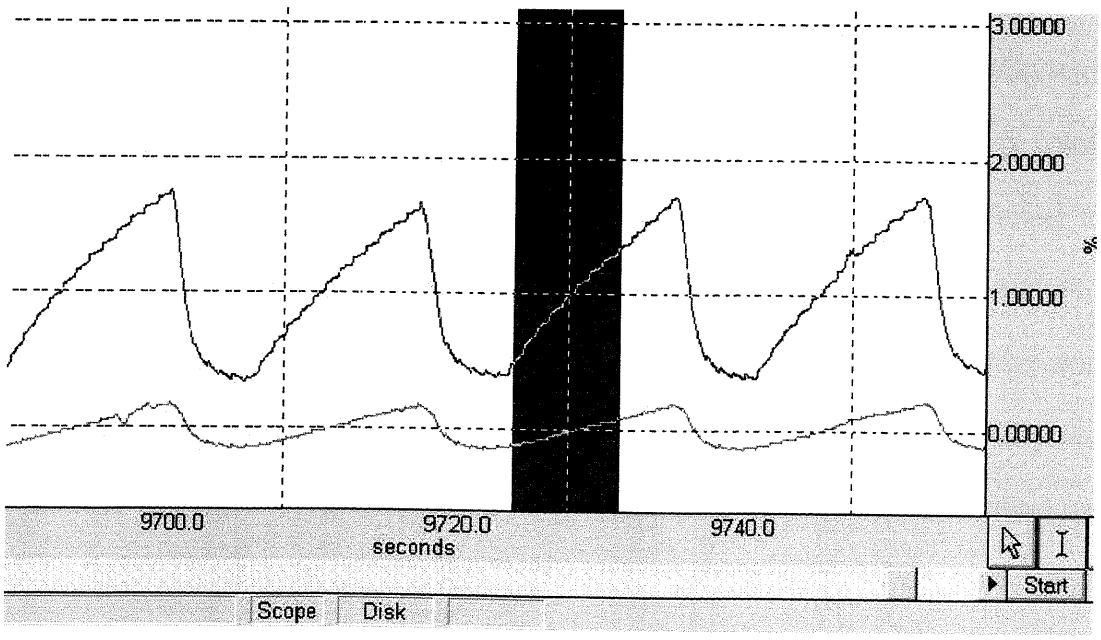


Figure 2.1 An example of the Acqknowledge data acquisition software package. The y-axis represents % increase in strain gauge length (forearm circumference), and the x-axis, time (sec). The infused arm (left) is blue and the non-infused is red. The steeper gradient of the blue curve is due to intra-arterial infusion of SNP 3 mcg / min in this example. The highlighted part is the linear section used for analysis.

Forearm blood flow was derived as the ratio of flow in the infused arm to that of the control arm, expressed as the percentage change from baseline, as below;

$$\% \text{ increase FBF} = \frac{\text{FBF (infused)} - \text{FBF (infused baseline)}}{\text{FBF (non-infused)}} * 100$$

Blood pressure was measured every 10 minutes in the right calf using a semi-automated oscillometric sphygmomanometer (Critikon Inc., Tampa, Florida, USA).

2.1.5.3.6.2 Pharmacological agents

All drugs to be infused were prepared on the morning of the study. ACh was reconstituted with physiological saline from 20mg vials (Miochol 20mg, Iolab Pharmaceuticals, CIBA Vision, Castle Hill, NSW). It was serially diluted to produce solutions of 3.75, 7.5 and 15 mcg/ml. SNP powder was reconstituted in 5 % dextrose solution from 50 mg vials (Sodium Nitroprusside for Injection B.P. 50mg, David Bull Laboratories, Victoria, Australia) and serially diluted to produce solutions of 0.3, 3 and 10 mcg/ml. L-NMMA was purchased as lyophilised powder in 1 g vials (Clinalfa, Switzerland or Oxonon, San Francisco, USA). It was reconstituted in physiological saline then aliquoted into vials containing 20 mg / 2 ml and stored at -20^{o}C until required. On the morning of the forearm study, L-NMMA was thawed and diluted to produce a solution of 20 mg / 20 ml. These solutions were drawn up into 20 ml syringes

for subsequent intra-arterial injection via an infusion pump (Terumo Medical Corp., Maryland, USA).

Forearm blood flow was allowed to stabilise during a 30 minute run-in infusion of saline. Then, cumulative dose-response curves were produced with infusions at 1 ml/min of ACh 3.75, 7.5 and 15 mcg/ml and SNP 0.3, 3 and 10 mcg/ml, as indicated in Figure 2.3 and described above. The order of drug administration was randomised between subjects.

2.1.5.3.6.3 Validation of bilateral forearm venous occlusion plethysmography

To determine the reproducibility of the system, baseline FBF was measured in 6 healthy individuals (3 male, 3 female) on two occasions. Inter-study coefficient of variation was calculated. Subjects lay supine and the above protocol was observed, except for cannulation of the brachial artery, which was omitted. For each limb, the coefficient of variation was calculated by multiplying the standard deviation of the two measurements divided by the mean of the measurements by 100 %. The coefficient of variation between measurements of baseline FBF on different days was 10.3 % for the left arm and 14.75 % for the right arm.

2.2 RAT STUDIES

2.2.1 Experimental Animals

Male Sprague Dawley, Wistar Kyoto and Spontaneously Hypertensive rats were used in these studies. Females were not used to remove any effect of

gender on outcomes. They were purchased from the Animal Resources Centre, Western Australia where the genetic status of the inbred strains is examined twice yearly (Animal Resources Centre, 1995). The animals were specific pathogen free. Animals were housed in the Animal House, St George Hospital in plastic cages with wire grill covers through which the animals could interact with the environment. In all studies, animals were allowed 7 days to acclimatise to the environment prior to the commencement of experimental procedures. The studies described were approved by the Animal Care and Ethics Committee of the University of New South Wales (Approval numbers 93/117, 95/8 and 97/50) and conformed to the Guidelines of the National Health and Medical Research Council of Australia.

Up to four rats per cage were allowed. In this facility, temperature was controlled (20-22^{0}C) and a 12 hour light/dark cycle was maintained (0600/1800 hrs). Animals were allowed free access to standard rat food (sodium content 0.2 %, Doust and Rabbidge, Sydney, Australia) and tap water, unless otherwise described (see below).

2.2.2 DRUG, FOOD AND WATER PREPARATION

2.2.2.1 ACTH (tetracosactrin) preparation

ACTH, administered in the parathyroidectomy study (see Chapter 7), was kindly donated by Novartis (tetracosactrin 1mg/ml, Synacthen Depot, Novartis, NSW). This preparation is a synthetic polypeptide composed of the first 24 of 39 amino acids contained in the naturally occurring ACTH

molecule. The suspension was drawn into a 1 ml syringe and injected subcutaneously via a 25 gauge needle. The dose administered was 500 mg/kg/day at 0800 hours as a once daily dose, from treatment day 0 to 10.

2.2.2.2 Corticosterone preparation

Corticosterone, used in the corticosterone study (see Chapter 6) (Corticosterone, Sigma Chemicals, St Louis, Missouri, USA) was reconstituted from a vial containing 500 mg of lyophilised powder into 1 ml ethanol then 24 ml polyethylene glycol 400 (PEG 400, Sigma Chemicals, St Louis, Missouri) to produce a suspension of 20 mg corticosterone/ml polyethylene glycol. To maximize solubility, the solution was allowed to stir on a magnetic stirrer for at least 8 hours prior to injection into an animal.

2.2.2.3 L-arginine and D-arginine in food

Ten kg of ground standard rat food (Doust and Rabbidge, Sydney, Australia) was mixed with 60 g of either L-arginine (Sigma Chemicals, St Louis, Missouri, USA) or D-arginine (Novabiochem, Clinalfa, NSW). Enough tap water was added to produce a soft paste (≈ 2 L) that could be moulded by hand into a flat cake (about 2-3 cm thick) onto a tray. The cakes of prepared food were dried in an air drier over 48-72 hours then broken into appropriate size chips for the animal cages. The prepared food was stored (sealed) at room temperature until required.

2.2.2.4 N-Nitro-L-arginine in drinking water

Preliminary data from animals treated with corticosterone 20 mg/kg/day demonstrated an average weight (on control day 4) of 288 g and average

water intake during corticosterone treatment of 31 ml/day, with no significant change during treatment (see Study 2, Chapter 6). The dose of NOLA administered was calculated from previous data in ACTH-induced hypertension (Li, Dusting et al, 1992), approximately 30 mg/kg/day. A solution of 308.6 mg NOLA per litre water was prepared and administered as the drinking water from treatment days 0 to 10 for group 5 of Study 3 (see Chapter 6). This group was included to examine the combined effects of NOS inhibition and oral L-arginine on corticosterone-induced hypertension.

2.2.2.5 Calcium chloride in drinking water

Calcium chloride (Univar, Ajax Chemicals, NSW) was added to deionised water to produce a 1 % solution.

2.2.3 Indirect systolic blood pressure measurement

In conscious animals, the indirect tail cuff technique was used for the measurement of systolic blood pressure. This system was custom made for the laboratory at St George Hospital by SDR Clinical Technology, Sydney (Figures 2.7 & 2.8).

At 0800 hrs on blood pressure days, animals were transferred from their cages to the indirect blood pressure system in a separate, quiet room. Conscious rats were restrained in clear semicircular tubes on a warming plate (38-40^{o}C, SDR Clinical Technology, Sydney) for 15 minutes prior to any measurements (Fig 2.8). This allowed vasodilatation of the tail artery, necessary for the detection of a satisfactory oscillatory pulse wave. An

inflatable pneumatic cuff controlled by an electrosphygmomanometer (Narco Biosystems, California, USA) was placed over the tail and distal to this a pulse pressure microphone (Narco Biosystems, California, USA) was secured with adhesive tape (Leukopor, Biersdorf AG, Hamburg, Germany).

The pulse signal from the microphone was amplified by a PA100 amplifier (SDR technology, Sydney) and subsequently processed by a BIOPAC MP100 processor (analogue to digital converter, Biopac Systems Inc., Goleta, California, USA). Data was acquired by an Apple Macintosh Computer running Hyperrat V2.1 software (SDR Technology, Sydney).

The tail cuff was inflated to 250 mmHg and deflated and SBP was defined as the re-appearance of the pulse signal during the deflation, as observed on the computer screen. A minimum of four inflation/deflation cycles were performed and the first result was discarded. If SBP varied by more than 10 mmHg further recordings were performed. The mean of four recordings within 10 mmHg was accepted as the SBP.

The training process to familiarise the animals with the mornings' procedures occurred during the control phases of these studies. Animals were usually restless during the initial morning session but rapidly became accustomed to the procedure and often slept through the recordings, usually about 30 minutes in duration.

This system could not technically be calibrated against the 'gold standard' of a column of mercury. The blood pressure was determined by a pressure sensor in the electrosphygmomanometer which delivered the air for cuff inflation, and this was further confounded by an algorithm in the software which was limited by its design and the power of the hardware. Therefore, absolute systolic blood pressure recordings were subject to a number of errors. To minimize possible variance in day-to-day recordings (most relevant in these studies), most likely a result of air leakage from the cuffs or tubing system, the delivered maximal air pressure was recorded by a modified mercury sphygmomanometer each morning prior to any actual blood pressure recordings (typically 242 mmHg). If this varied by more than 4 mmHg from 242 mmHg, a small leak somewhere in the high pressure system could usually be detected and was repaired prior to any data collection.

2.2.4 Metabolic measurements

In the 10 and 14 day experiments (Chapters 6 & 7), metabolic measurements were made on alternate days. The cages (Mascot Wire Co., Sydney, Australia, Figure 2.9) individually housed animals for periods of 24 hours They had wire sides to allow interaction with the environment. Food intake, water intake and urine output were measured and the animals were weighed on these days. Urine was collected in a modified 30 ml syringe, the volume measured and an aliquot stored (-20^{0}C) for subsequent electrolyte analysis. The collection of urine was not necessarily complete, as the animals were not catheterised, and some urine may have bypassed the collecting chute.

Similarly, some food may have been chewed and spat out. Nevertheless, the change in these parameters over time was the most important parameter in these studies and hence a control group was always used for comparison.

Water was provided in modified 50 ml syringes and the deficit from the provided 50 ml at the end of a 24-hour period accepted as the 24-hour intake. Animals were weighed on an electronic balance (Mettler BB 2400, Mettler-Toledo, Grifensee, Switzerland).

2.2.4.1 Urinary Na & K measurements by flame photometry

Twenty four hour urinary electrolyte excretion was measured in the ACTH/PTx study. A flame photometer (IL643 AutoCal Flame Photometer, Instrument Laboratory, Massachusetts, USA) was used to assay samples in duplicate. The principle of flame photometry is that alkali metals, when exposed to a flame of sufficient temperature, will be forced into an excited state. As these states are unstable, the molecules revert back to their initial state, in doing so, a photon of energy at a frequency specific to each metal is discharged. This can be detected by a photodetector and the amount of metal present (Na, K or Li) is proportional to the intensity of the light emitted.

Aliquots from twenty four hour urine specimens were defrosted and centrifuged at 3000 rpm. The supernatant was used for analysis. Because of the limitations of the range of the IL643, the urine was diluted. One hundred microlitres of urine was added to 900 microlitres of deionised water to make a 1 in 10 solution.

Pooled urine from previous studies was used as a quality control. Twenty millilitres of the pooled urine was mixed and centrifuged at 3000 rpm for 15 minutes. The supernatant was withdrawn and aliquoted into 1 ml samples. This specimen was analyzed every ten true urine samples to determine the interassay variablility (CV for Na 1.6 %, K 1.3 %). The flame photometer was recalibrated with a known control (Na 100 mmol/l, K 5 mmol/l) every ten samples. Urine samples were analyzed in duplicate and the mean calculated.

2.2.4.2 Whole blood ionized calcium measurement

One ml of whole blood collected at sacrifice was drawn into an arterial blood gas sampler (QS 50, Radiometer Copenhagen, Radiometer Pacific, Lane Cove NSW) which contained 50 IU of electrolyte balanced lithium/sodium heparin coated on a fibre disc. Use of balanced heparin minimised any effect of heparin on ionised calcium concentration. This blood was then analyzed by a blood gas analyser (Radiometer Copenhagen ABL 700 series, Radiometer Pacific, Lane Cove NSW) which provided measurements of ionised calcium, sodium, potassium and blood glucose, by ion specific electrodes.

2.2.5 SURGICAL PROCEDURES

2.2.5.1 Bilateral parathyroidectomy procedure

General anaesthesia was attained with intraperitoneal sodium pentobarbitone (60 mg/kg). Rats were placed on a warming pad (surface temperature 38-40^{o}C) immediately after induction and were warmed during surgery until early

recovery. Via a midline ventral incision of the neck, sharp dissection exposed the strap muscles and midline blunt dissection through these revealed the thyroid gland attached to the trachea. The parathyroid glands were visible, under a dissecting microscope, bilaterally on the anterolateral surface of each lobe of the thyroid gland (one on each lobe). After ligation of visible large vessels supplying the parathyroid glands (6/0 chromic suture) each parathyroid gland was removed by combination of sharp and blunt dissection. Haemostasis was secured and the neck wound closed with interrupted 3/0 silk sutures. Tissue specimens were immediately placed in mercuric formalin for subsequent histological analysis.

2.2.5.2 Histology of parathyroid tissue

Fixed tissue was prepared with assistance of the Histology Laboratory, South Eastern Area Laboratory Services, St George Hospital. Specimens were initially mounted in paraffin, then 4 micron sections were made by a microtome. These sections were transferred to clean glass slides, dried, and stained by standard haematoxylin and eosin technique.

2.2.5.3 Analysis of parathyroid tissue

Specimens were analyzed for evidence of parathyroid tissue, defined by the presence of tissue with characteristic features of parathyroid tissue: dark staining nuclei, clear cytoplasm, etc, often surrounded by some thyroid tissue (Figure 2.10). Presence of such tissue was required for histological confirmation of parathyroidectomy. As the parathyroid bed was not examined at autopsy for evidence of residual parathyroid tissue, further evidence of complete parathyroidectomy was necessary and hence biochemical

measurement of circulating immunoreactive parathyroid hormone was made at sacrifice, as described below.

2.2.5.4 Definition of parathyroidectomy (Figure 2.10)

Total parathyroidectomy was defined as evidence of parathyroid tissue excised bilaterally and reduced PTH concentration at sacrifice. Animals with evidence of inadequate parathyroidectomy (ie unilateral or no parathyroid tissue) were excluded from analysis.

2.2.6 Sacrifice procedures

On treatment day 10 (of the ACTH parathyroidectomy study and Study 3 of the corticosterone experiments) or treatment day 14 (of Studies 1 & 2 of the corticosterone experiments), all animals were sacrificed. After indirect blood pressure measurements, animals were administered the last subcutaneous injection of ACTH, corticosterone or sham and then anaesthetised with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). In the event of inadequate anaesthesia, defined by failure to sleep, twitching or the presence of withdrawal reflexes, further pentobarbital was administered, in boluses of 6 mg subcutaneously.

The carotid artery was then catheterised as described below (see Section 8.2.3). Arterial blood was withdrawn from this catheter into 10 ml syringes and immediately transferred to either plain chilled glass tubes and allowed to clot at 4 %, chilled tubes containing lithium heparin 7 IU/ml or chilled tubes containing EDTA. Once clotted, serum was centrifuged at 3000 rpm then

transferred to polypropylene tubes. Anticoagulated plasma was immediately centrifuged at 3000 rpm at 4^0C and transferred to polypropylene tubes. All specimens were stored at -70^0C.

The animals were then administered a lethal dose of intra-arterial pentobarbital sodium. After death, the abdominal and thoracic cavities were opened and the kidneys, adrenal glands and heart were dissected out and weighed.

2.2.6.1 PTH ASSAY AT SACRIFICE

PTH was measured at sacrifice in the parathyroidectomy study. Animals were sacrificed under anaesthesia and exsanguinated via a catheter in the carotid artery. Clotted blood was centrifuged at 4^0C and serum stored at -70^0C and later assayed for PTH by immunoradiometric assay (IRMA, rat PTH, Nichols Institute Diagnostics).

2.2.6.1.1 Principles of PTH assay

This assay is a two-site IRMA in which intact (1-84) and N-terminal (1-34) PTH are measured. Two different goat antibodies to the N-terminal region (1-34) of rat PTH have been purified by affinity chromatography. One of the antibodies is immobilised onto a plastic bead to capture the PTH molecules and the other antibody is radiolabelled for detection. A sample containing rat PTH is incubated simultaneously with an antibody coated bead and the radioiodine (^{125}I) labelled antibody. Both intact (1-84) and N-terminal PTH (1-34) contained in the sample are immunologically bound by both the

immobilised antibody and the radiolabelled antibody to form a "sandwich" complex.

At the end of the incubation period, the bead is washed to remove any unbound labelled antibody and other components. The radioactivity bound to the bead is then measured in a gamma counter. The radioactivity of the antibody complex is directly proportional to the concentration of rat PTH (limitations of assay [PTH] 1-1250 pg/ml).

2.2.6.1.2 Performance characteristics of PTH assay.

The sensitivity of the assay as defined by the 95% confidence limit on 20 duplicate determinations of the 0 pg/ml standard is 1.0 pg/ml. Intra-assay coefficient of variation is 4.2 % and inter-assay coefficient of variation is 4.5 %. Results from the control samples were as follows; low (32-48 pg/ml) 44 pg/ml, high (270-490 pg/ml) 385 pg/ml.

2.2.6.1.3 PTH assay procedure

All standards, controls and samples were assayed in duplicate. A standard curve was generated by the measurement of known concentrations of PTH supplied with the kit (standards) from 0 to 200 pg/ml PTH. Concentration of PTH was measured in rat samples from this standard curve, as generated by computer using custom software on a log/logit curve.

Two hundred microlitres of standard, control or sample was pipetted into labelled polypropylene tubes. One hundred microlitres of radioiodine-labelled rat PTH was added to each tube. After vortexing all tubes, one bead was

added to each tube, and then allowed to incubate for 20 hours. The contents of each tube was aspirated and washed three times by dispensing 2.0 ml of wash solution then completely aspirating the contents. Each tube was then counted in a gamma counter for one minute and counts recorded automatically.

2.2.6.2 ACTH ASSAY AT SACRIFICE

To examine the effects of low dose subcutaneous infusion of corticosterone on the hypothalamic-pituitary axis, plasma ACTH was measured at sacrifice.

2.2.6.2.1 Principles of the ACTH assay

Plasma concentration of ACTH was measured at sacrifice in Study 1 of the corticosterone studies (see Chapter 6) by radioimmunoassay (ACTH Double Antibody, Diagnostics Products Corporation, California). In the procedure, ^{125}I-labelled ACTH competes with ACTH in the rat plasma for antibody sites. After incubation, free ACTH is separated from bound ACTH by addition of a precipitating solution, containing goat anti-rabbit gamma globulin (second antibody). The precipitate (containing bound ACTH and ^{125}I-ACTH) is centrifuged to a pellet and counted in a gamma counter. The contained radioactivity is inversely proportional to the ACTH concentration in the rat sample. A standard curve is generated by the measurement of known concentrations of ACTH supplied with the kit (standards) from 0 to 1250 pg/ml ACTH. Low (22-38 pg/ml) and high (330-470 pg/ml) controls were included. Concentration of plasma ACTH was measured in rat samples from this standard curve, as generated by computer using custom software on a log/logit curve (Nuclear Medicine, St George Hospital, Sydney).

2.2.6.2.2 Performance of the ACTH assay

The intra-assay coefficient of variation of this procedure, in the range of ACTH concentrations achieved, is 3.2 - 4.9 %. The inter-assay coefficient of variation is 6.4 - 9.7 %. Results from the control samples were as follows; low 25 pg/ml, high 361 pg/ml.

2.2.6.2.3 ACTH assay procedure

All standards, controls and samples were assayed in duplicate. Plasma (EDTA) collected on the sacrifice day was used. Each sample was assayed in duplicate. One hundred microlitres of each standard, each control and of each sample of rat plasma was pipetted into appropriately labelled polypropylene tubes. One hundred microlitres of ACTH antiserum was added to the tubes containing standards or rat plasma, and the tubes were vortexed. The tubes were left to incubate for 60 minutes at room temperature. One hundred microlitres of ^{125}I ACTH was then added to all tubes which were again vortexed. The tubes were then incubated for a further 16 hours at 4^0C. One millilitre of the second antibody (precipitating solution) was then added and the tubes vortexed.

All tubes were then centrifuged at 3000 g for 15 minutes. The supernatant was decanted off, leaving a firm plug at the base of each tube. The tubes were then counted for one minute each in a gamma counter.

2.2.6.3 CORTICOSTERONE ASSAY AT SACRIFICE

2.2.6.3.1 Principles of corticosterone radioimmunoassay

This is a solid-phase radioimmunoassay in which ^{125}I-labelled rat corticosterone competes for a fixed time with corticosterone in the sample for antibody sites (Coat-a-Count, Diagnostic Products Corporation, California). The antibody is bound to the wall of a polypropylene tube, so decanting the supernatant terminates the competition and isolates the antibody bound fraction of radio-labelled corticosterone.

Eight standards (0-200 ng/ml) are provided for the generation of a standard curve. Concentration of corticosterone in rat samples is achieved using this curve, as described above. Two standards were also assayed as controls.

2.2.6.3.2 Performance characteristics of rat corticosterone assay

The intra-assay coefficient of variation of this procedure is 4.0-4.3 %. The inter-assay coefficient of variation is 4.8-5.8 %. Results from the control samples were as follows; low (50 ng/ml) 50 .3 ng/ml, high (2000 ng/ml) 1808 ng/ml.

2.2.6.3.3 Corticosterone radioimmunoassay procedure

Standards, controls and samples were assayed in duplicate. Fifty millilitres of each standard, control and sample were pipetted into prepared polypropylene tubes. One millilitre of ^{125}I rat corticosterone was added to each tube and the tubes were vortexed. All tubes were then incubated at

room temperature for two hours. The tubes were then decanted and counted for one minute in a gamma counter.

2.3 Statistics and data presentation

All data are presented as mean SEM. All statistical analyses were performed using the software package Systat 7.0 (SPSS Inc., Chicago, Illinois, USA) on a personal computer. All data were initially analyzed for distribution by the Lilliefors test. In these studies, only very occasional sets of data were of non-parametric distribution, therefore, statistical techniques employed were for parametric data.

In the human and rat studies, data from repeated measures experiments were analyzed for changes over time by one-way Repeated Measures Analysis of Variance, with the Greenhouse-Geiser estimation accepted as the probability value for disproof of the null hypothesis. The F-test used in this analysis factors in correction for multiple tests. This test was used with blood pressure and heart rate, weight and other metabolic parameters which were repeatedly measured during the experimental period. Where it was necessary to compare two groups to examine for differences between groups over repeated measures, a two way RMANOVA was used. Where appropriate, the Student t-test was used to compare data between groups at a single timepoint or paired data within groups between two timepoints. Where multiple t-tests were used, the Hochberg correction was applied to the

results. Other metabolic parameters measured on two occasions only were compared by the Student t-test.

Forearm blood flow data were screened for parametric distribution as described above. The dose-response curves to vasodilators were analyzed using the technique of Calver et al (Calver, Collier et al, 1992a). The area under the curve was calculated by the trapezoidal rule. These areas were compared by the Student t-test. Differences in baseline FBF and flow before and after LNMMA were also analyzed by the Student t-test.

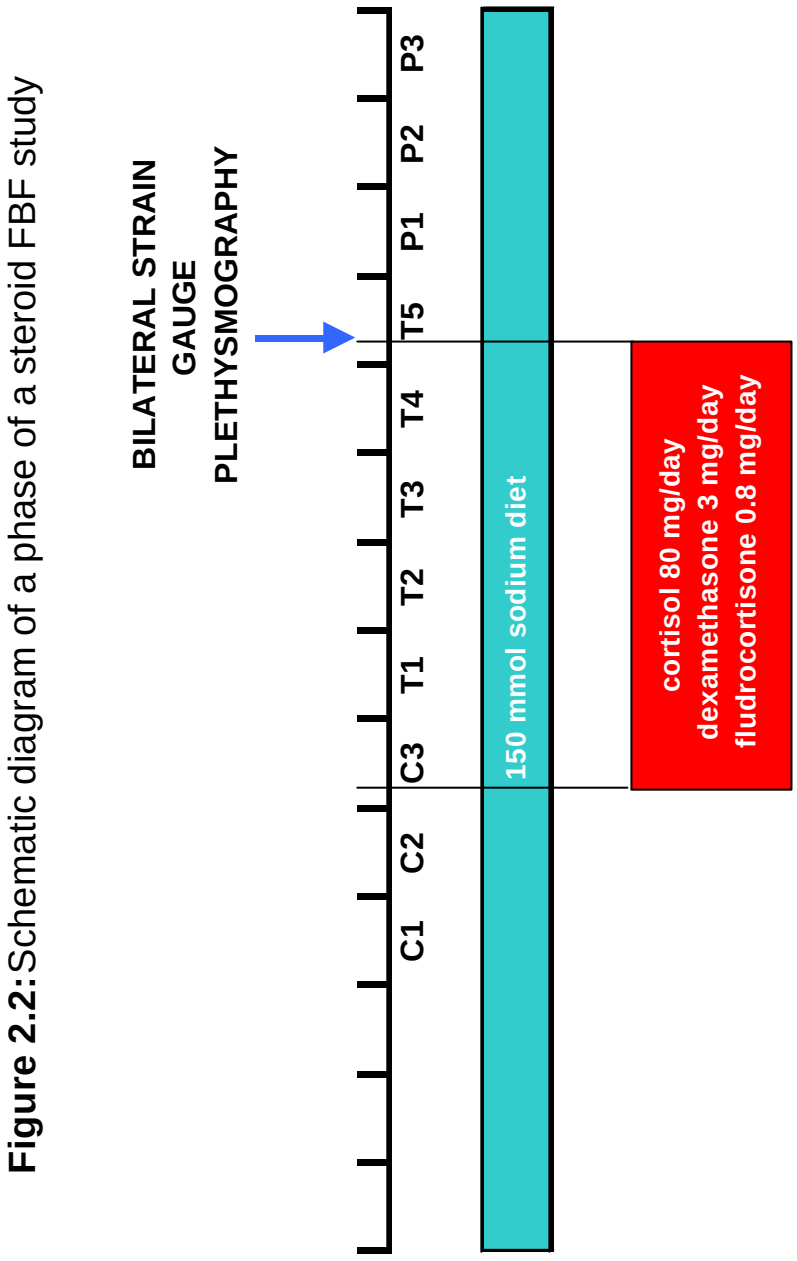


Figure 2.2: Schematic diagram of a phase of a steroid FBF study

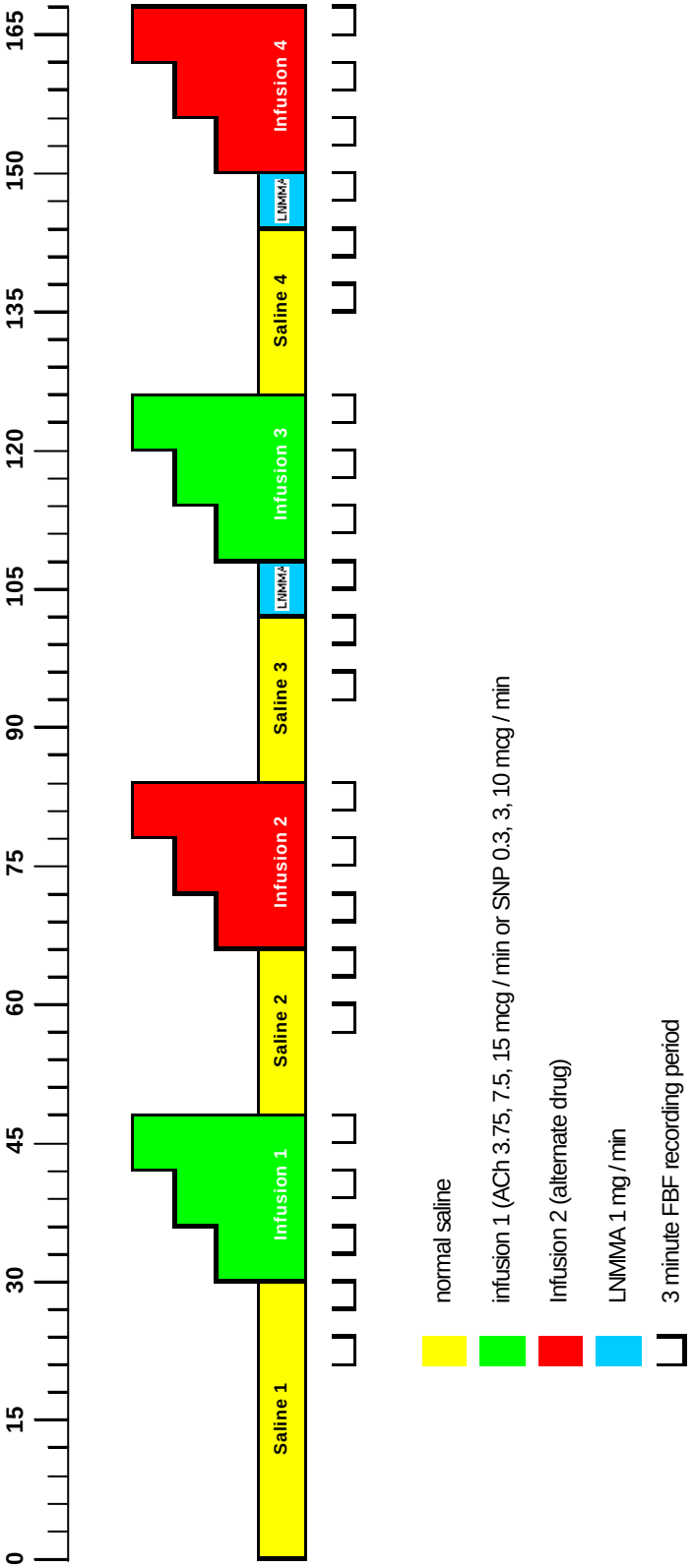


Figure 2.3: Bilateral forearm venous plethysmography protocol

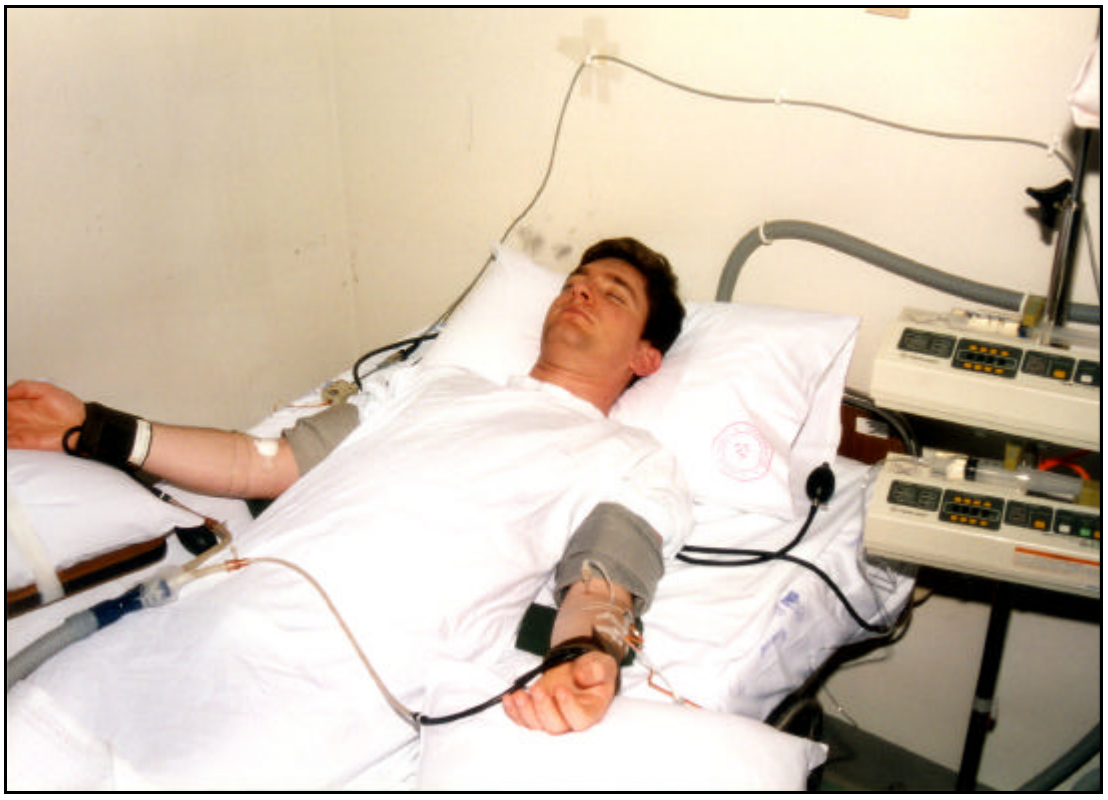


Figure 2.4 Bilateral forearm venous plethysmography. The subject is lying flat with the forearms elevated. Arterial infusion is controlled by a syringe pump(s), visible on the right side of the picture.

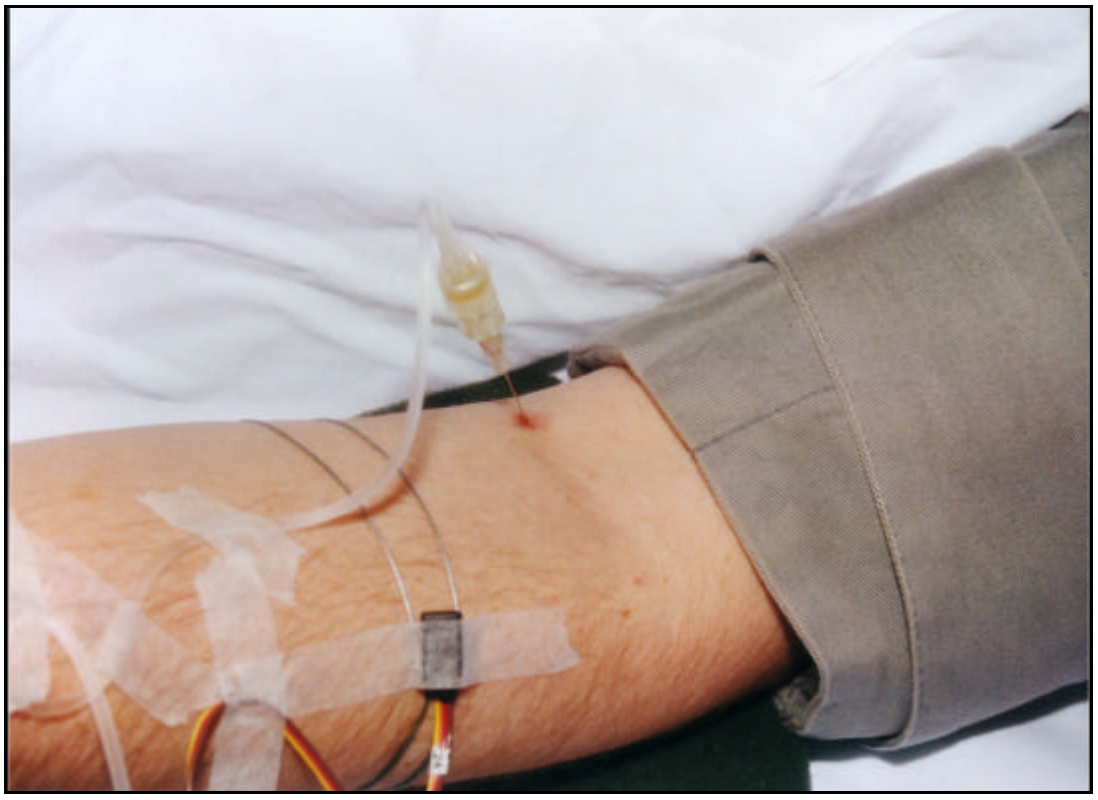


Figure 2.5 Close-up view of mercury-in-silastic strain gauge applied to left forearm and 27 g needle in the left brachial artery. Venous occluding cuff has been applied around the arm at the mid-humerus level.

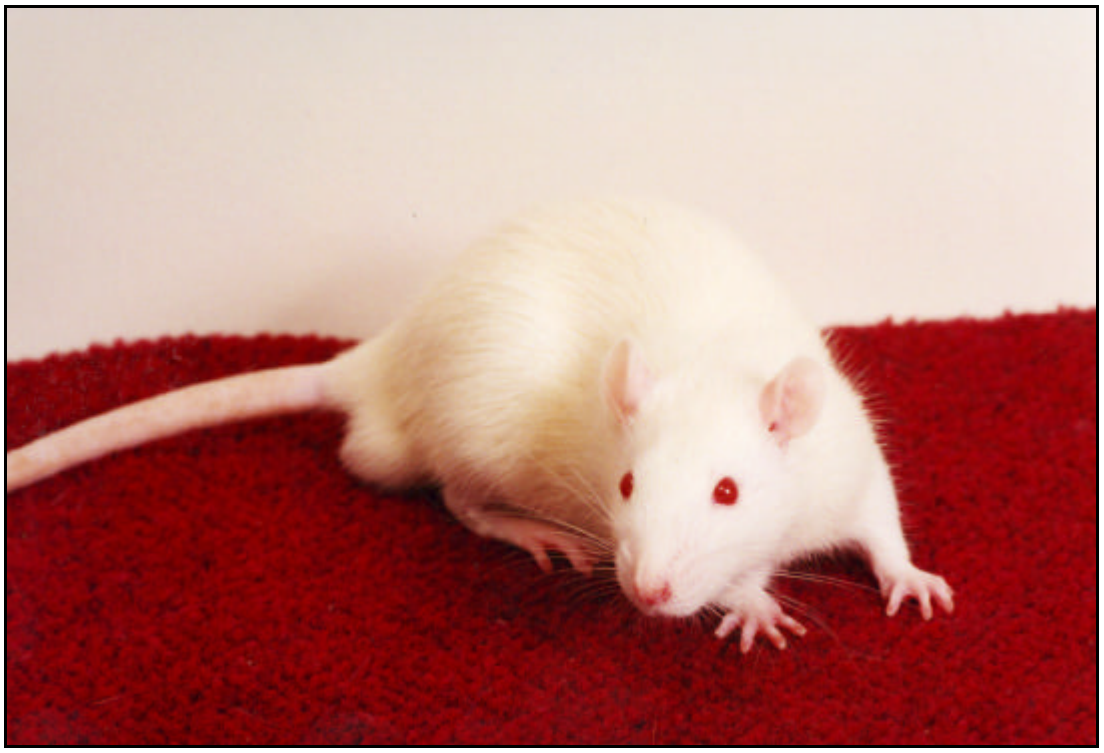


Figure 2.6 Male Sprague Dawley rat.

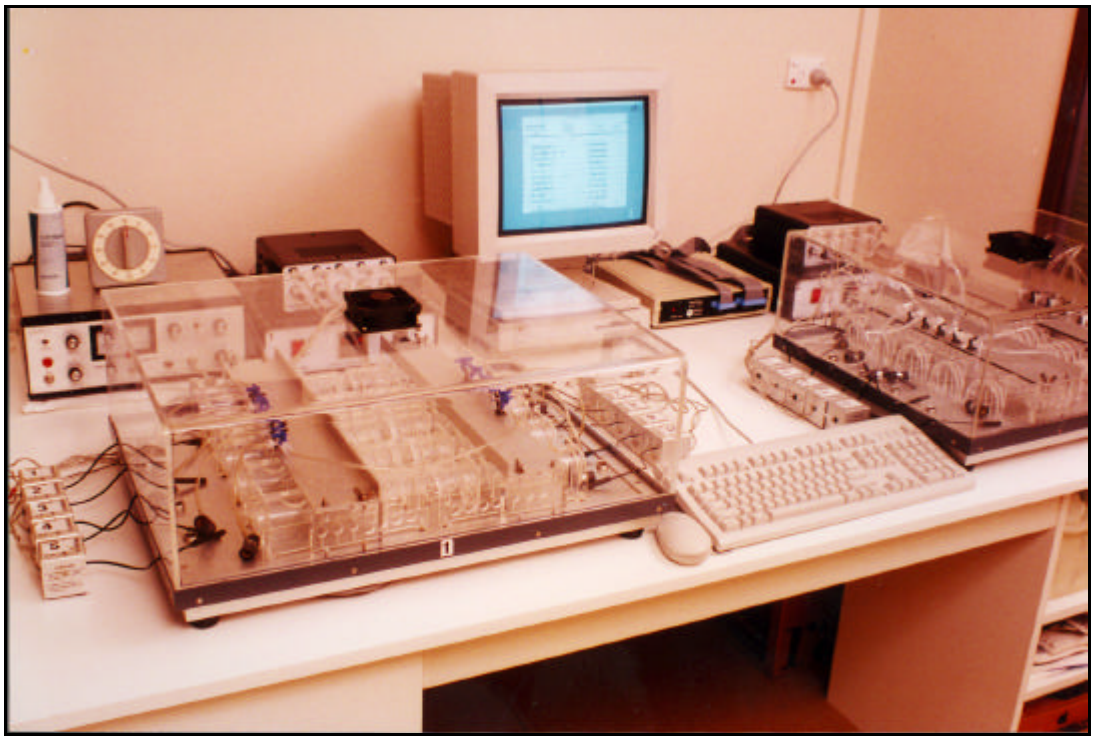


Figure 2.7 Tail-cuff systolic blood pressure apparatus.

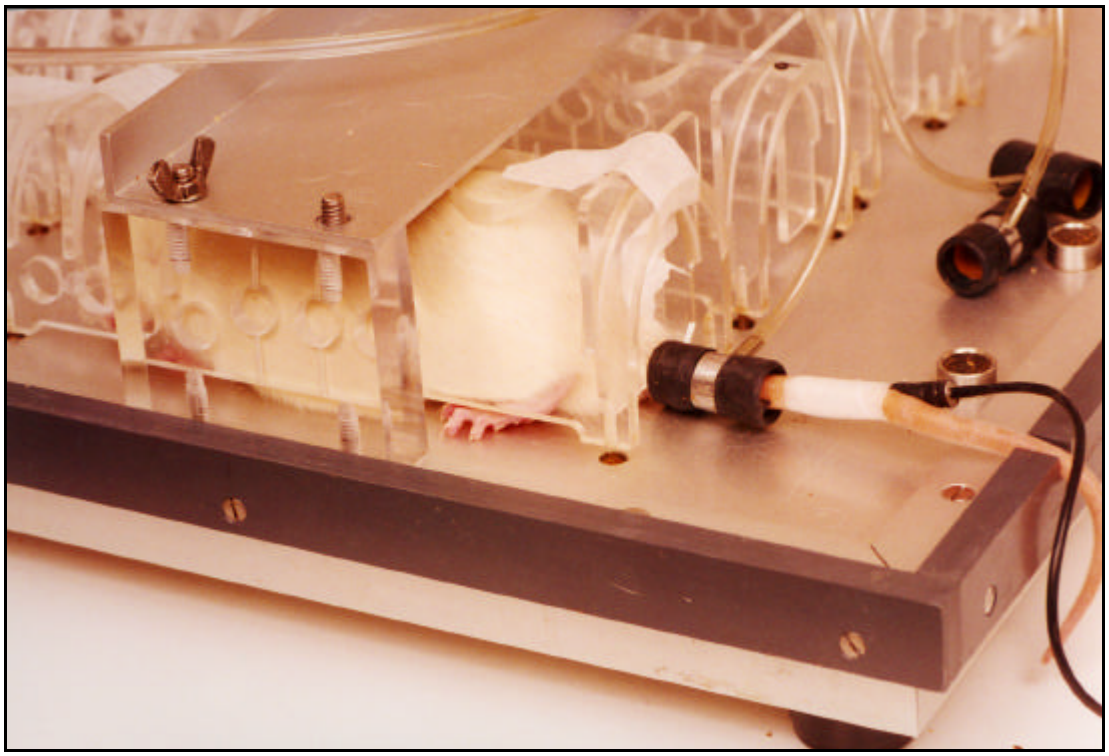


Figure 2.8 Rat in restraining tube with cuff and pulse pressure microphone attached on warming plate.

Figure 2.9 Twenty four hour metabolic cages.

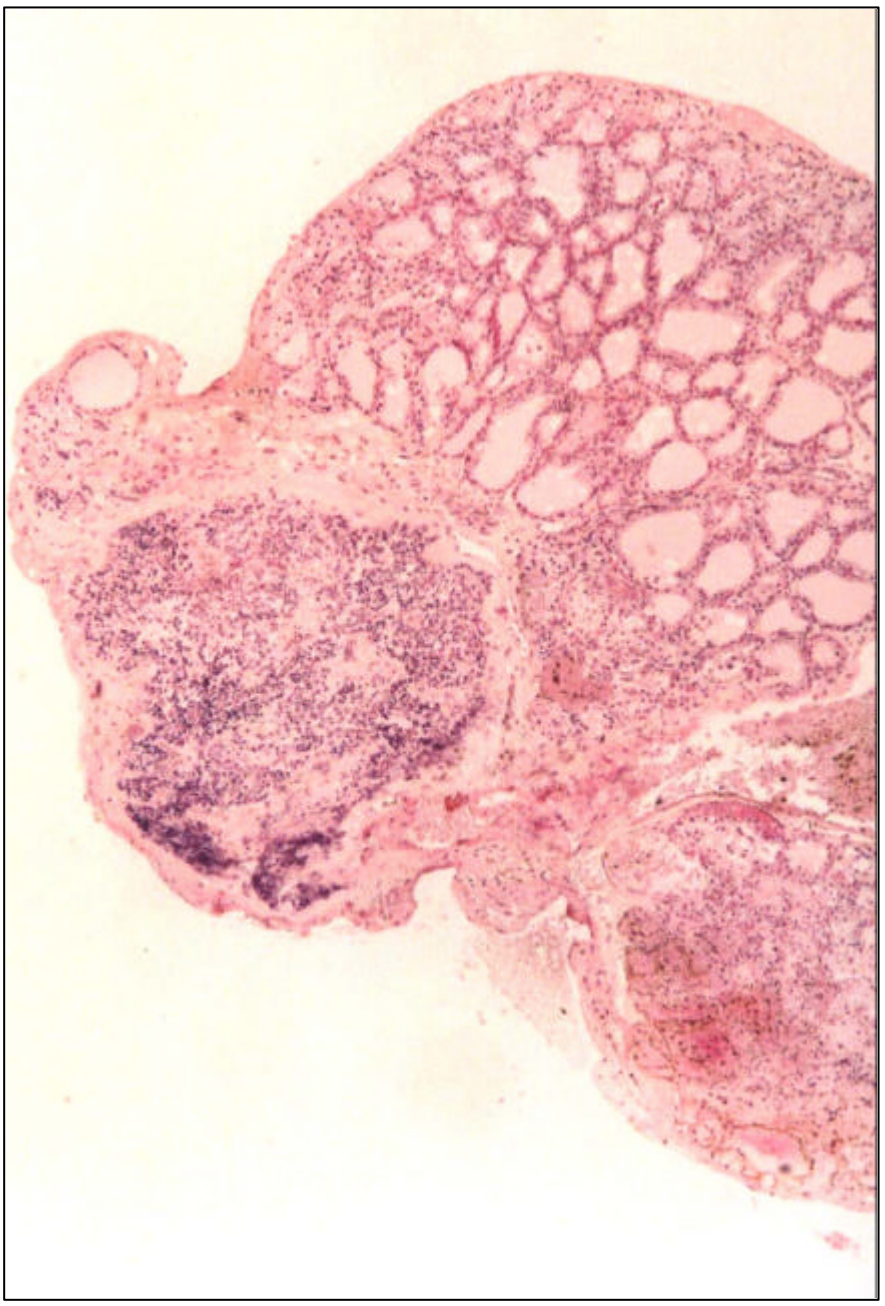


Figure 2.10 Histological section of rat parathyroid tissue (left) with attached thyroid gland.

CHAPTER 3

Endothelial vasodilator function in experimental cortisol-induced hypertension in man

3.1 INTRODUCTION

Corticosteroids are well known to increase pressor activity of vascular tissue. In addition to the permissive effect of cortisol on blood pressure (Axelrod, 1983), an excess of cortisol is associated with increased vascular responsiveness to phenylephrine (Whitworth, Connell et al, 1986) and angiotensin II (Whitworth, Connell et al, 1988) in healthy male subjects. The mechanisms by which this occurs are unknown, but may include a compensatory response to suppression of the sympathetic nervous system (Connell, Whitworth et al, 1987, Sudhir, Jennings et al, 1989, Macefield, Williamson et al, 1998) and the renin-angiotensin system respectively (Connell, Whitworth et al, 1987).

Vasoconstriction to topical cortisol or beclomethasone is enhanced in essential hypertensives (Walker, Best et al, 1996). In the syndrome of AME and in normal individuals, the effect of excess cortisol on blood pressure is rapid, suggesting a mechanism distinct from MR mediated sodium and water retention in the renal tubule (Stewart, Corrie et al, 1988).

In the model of ACTH-induced hypertension in the rat, Turner et al have demonstrated that dietary supplementation with L-arginine but not D-arginine prevents the onset of hypertension (Turner, Wen et al, 1996). This suggests a role for the nitric oxide system in ACTH-induced hypertension and, by

extension, in cortisol-induced hypertension (Kelly, Tam et al, 1998). To examine endothelial function and relevance of the nitric oxide system to cortisol-induced hypertension in humans, I measured the vasodilator responses to intra-arterial acetylcholine in the forearm vascular bed in subjects treated with oral cortisol for five days.

3.2 METHODS

3.2.1 Subjects

Eight healthy non-smoking normotensive male volunteers without contraindications to corticosteroid therapy were studied. Each subject gave written informed consent. Each subject was asked to maintain a fixed sodium intake (150 mmol/day) from two days prior and for the duration of each phase of the study. Details of subject selection have been described in section 2.1.2.

3.2.2 Study design (Figure 2.2)

This study was a two-phase, randomised, placebo controlled, double blind crossover study comparing placebo with cortisol (hydrocortisone acetate, Alphapharm, Australia or Merck, Sharp and Dohme, UK) 20 mg 6 hourly by mouth for five days. Subjects attended the Clinical Research Room at St George Hospital after a light breakfast at a similar time on each morning of the study period. On each morning, supine and erect blood pressure and pulse measurements were made and the subjects were weighed. On the last treatment day of each phase (ie placebo or cortisol treatment day 5), forearm

blood flow measurements were performed as described in section 2.1.5.3.6. Treatment phases were separated by wash out periods of at least four weeks.

A right antecubital vein was cannulated every second day with the subject recumbent and blood collected for measurement of electrolytes, urea and creatinine, albumin, glucose, calcium, phosphate, haematocrit, haemoglobin, leucocyte count, platelet count (routine automated methods) and cortisol concentration (Amerlex Cortisol RIA, Amersham UK). Twenty four hour urine collections were obtained on control days 2-3, treatment days 4-5 and post-treatment observation days 2-3 for measurement of electrolyte excretion and creatinine clearance.

3.3 RESULTS

3.3.1 Effects of cortisol on blood pressure and heart rate (Figures 3.1 & 3.2)

Pooled control supine and erect SBP and DBP were similar between treatment phases (Figure 3.1). There was no change in supine or erect systolic or diastolic blood pressure with placebo treatment (supine 113 2 / 62 4 to 113 2 / 58 5 mmHg; erect 106 3 / 69 2 to 104 4 / 66 3 mmHg, PC to T5). Cortisol treatment increased supine systolic ($p < 0.05$, RMANOVA) and erect systolic ($p < 0.05$, RMANOVA) blood pressure with no effect on diastolic blood pressure in either position (supine 112 3 / 59 3 to 118 2 / 61 4 mmHg; erect 106 4 / 68 1 to 116 4 / 71 3 mmHg, PC

to T5). There was no consistent change in heart rate on either treatment (Figure 3.2).

3.3.2 Metabolic effects of cortisol (Figure 3.3 and Tables 3.1 & 3.2)

Serum cortisol concentration increased with cortisol treatment (397 44 to 845 64 nmol/L, $p < 0.001$, C to T5) and was unaffected by placebo (429 ± 17 to 364 29 nmol/L). Cortisol treatment increased body weight, white cell count, absolute neutrophil count, platelet count and serum glucose and reduced eosinophil count as previously reported (Whitworth, Saines et al, 1984). There were no changes to urinary sodium or potassium excretion, serum sodium, potassium, albumin, cholesterol and triglyceride concentrations on either treatment.

3.2.1 Forearm blood flow after five days of placebo or cortisol (Table 3.4 and Figures 3.4 and 3.5)

Baseline FBF was not affected by cortisol or by LNMMA (Table 3.4).

ACh infusion resulted in dose-dependent increases in FBF in both placebo and cortisol phases of the study (Figure 3.4). Cholinergic vasodilatation was impaired in the cortisol phase of the study compared with placebo (925 108 v 666 101 units, AUC placebo v cortisol, $p < 0.05$). LNMMA inhibited cholinergic vasodilatation in the placebo phase of the study (925 108 to 636 132 units, AUC placebo pre- v post-LNMMA, $p < 0.05$), but had no effect on cholinergic vasodilatation in the cortisol treatment phase (666 101 to 673 ± 132 units, AUC cortisol pre- v post-LNMMA, p=n.s).

SNP infusion produced dose-dependent increases in FBF which were similar in both phases of the study (1062 119 v 933 117 units, AUC placebo v cortisol. p = n.s). Further, vasodilatation to SNP was not altered by LNMMA in the placebo phase (1062 119 to 1020 181 units, AUC placebo pre- v post-LNMMA, p = n.s) or in the cortisol phase (933 ± 119 to 1005 ± 136 units, AUC cortisol pre- v post-LNMMA, p = n.s)(Figure 3.5).

3.4 DISCUSSION

This study shows that cortisol inhibits cholinergic vasodilatation in the human forearm circulation. The effect of cortisol was similar to the effect of LNMMA in the placebo treatment phase, suggesting that the nitric oxide synthase-dependent component of the cholinergic vasodilator response was suppressed by cortisol. This was not due to an effect of cortisol on vascular smooth muscle as vasodilator responses to the nitric oxide donor, SNP, were similar between cortisol and placebo phases. These results imply that cortisol excess induces endothelial dysfunction which may contribute to this model of hypertension.

Cholinergic vasodilatation is mediated, at least in part, by endothelial NOS. In vitro, glucocorticoids do not influence endothelial NOS expression, although they do inhibit inducible NOS (Radomski, Palmer et al, 1990). Inducible NOS, however, has not been recognised as an important regulator of basal vascular tone. In this study, plasma nitrate/nitrite concentrations tended to be

lower on treatment day 5 in the cortisol phase, though this did not reach statistical significance ($p < 0.20$). Kelly et al have recently shown that plasma nitrate/nitrite concentrations are reduced in cortisol-treated male subjects on a fixed nitrate diet, in a protocol very similar to the present study (Kelly, Tam et al, 1997). Dietary nitrates have significant effect on circulating concentrations of total plasma nitrate/nitrite, which may explain the NOx results of this study, where the subjects were not limited in their consumption of nitrate. NOx concentrations are an indirect marker of NO system activity, and should be interpreted carefully, especially where there are conflicting data of NO system activity (Baylis and Vallance, 1998). In the present study, the effect of cortisol on cholinergic vasodilatation was in accordance with the observed indirect metabolic evidence of reduced NO system activity.

It may be inferred that the effect of cortisol on cholinergic dilatation may be dependent on inhibited endothelial NOS, however indirect mechanisms (eg altered prostanoid synthesis) cannot be excluded. Prostanoids do not contribute directly to forearm cholinergic dilatation in health (Noon, Walker et al, 1998), however, there is evidence for interaction between prostacyclin and NOS activity in vitro and in other vascular beds (Gambone, Murray et al, 1997, Wang and Diamond, 1997). Glucocorticoids are known to inhibit prostacyclin production (Axelrod, 1983), however, Whitworth et al have previously demonstrated that co-administration of indomethacin does not alter pressor responsiveness in cortisol subjects (Whitworth, Connell et al, 1988). Alternatively, the effect on cholinergic dilatation may be an indirect

effect of altered sensitivity to noradrenaline or angiotensin II, both of which are affected by glucocorticoids (Sudhir, Jennings et al, 1989, Whitworth, Connell et al, 1986) and have actions on endothelium as well as vascular smooth muscle. Finally, glucocorticoids may reduce endothelial NO production by mechanisms other than NOS inhibition. Glucocorticoids have been demonstrated to inhibit synthesis of tetrahydrobiopterin which is a necessary co-factor for maximal activity of all NOS isoforms (Hattori, Akimoto et al, 1997, Axelrod, 1983).

These changes in vascular responsiveness occurred in the context of a small but significant increase in blood pressure during five days of cortisol treatment accompanied by glucocorticoid (hyperglycaemia, leucocytosis and eosinopaenia) and mineralocorticoid (increased body weight) effects consistent with those previously documented (Whitworth, Saines et al, 1984). These changes can not be explained by the rise in blood pressure per se, because the control arm, to which the infused arm was compared, was subject to the same arterial pressure, and all vasodilator responses were reported as the ratio of flow in the infused/non-infused arm (ie any effect of perfusion pressure on the forearm vasculature was symmetrical and therefore cancelled out by the use of flow ratios rather than absolute flows). Placebo treatment did not change blood pressure, plasma cortisol concentrations or other metabolic parameters.

This study did not determine whether the effect on FBF was mediated by GR or MR. To examine this, further studies using pure agonists of GR and MR have been reported in the subsequent chapters.

3.5 CONCLUSIONS

These data support the hypothesis that impaired endothelium-dependent vasodilatation is important in glucocorticoid-induced hypertension, as suggested by recent human and animal observations. Plasma nitrate/nitrite concentrations are reduced in cortisol-treated human males (Kelly, Tam et al, 1997). In the Sprague Dawley rat, L-arginine supplementation prevents ACTH-induced hypertension (Turner, Wen et al, 1996). Further evidence of altered NO system activity has been reported in corticosterone-induced hypertension in the rat, in Chapter 6 of this thesis. No matter whether the effect of cortisol is direct or indirect, these data also raise the possibility that enhanced vascular action of glucocorticoids may contribute to endothelial dysfunction and to the pathophysiology of essential hypertension (Walker, Phillips et al, 1998, Phillips, Barker et al, 1998).

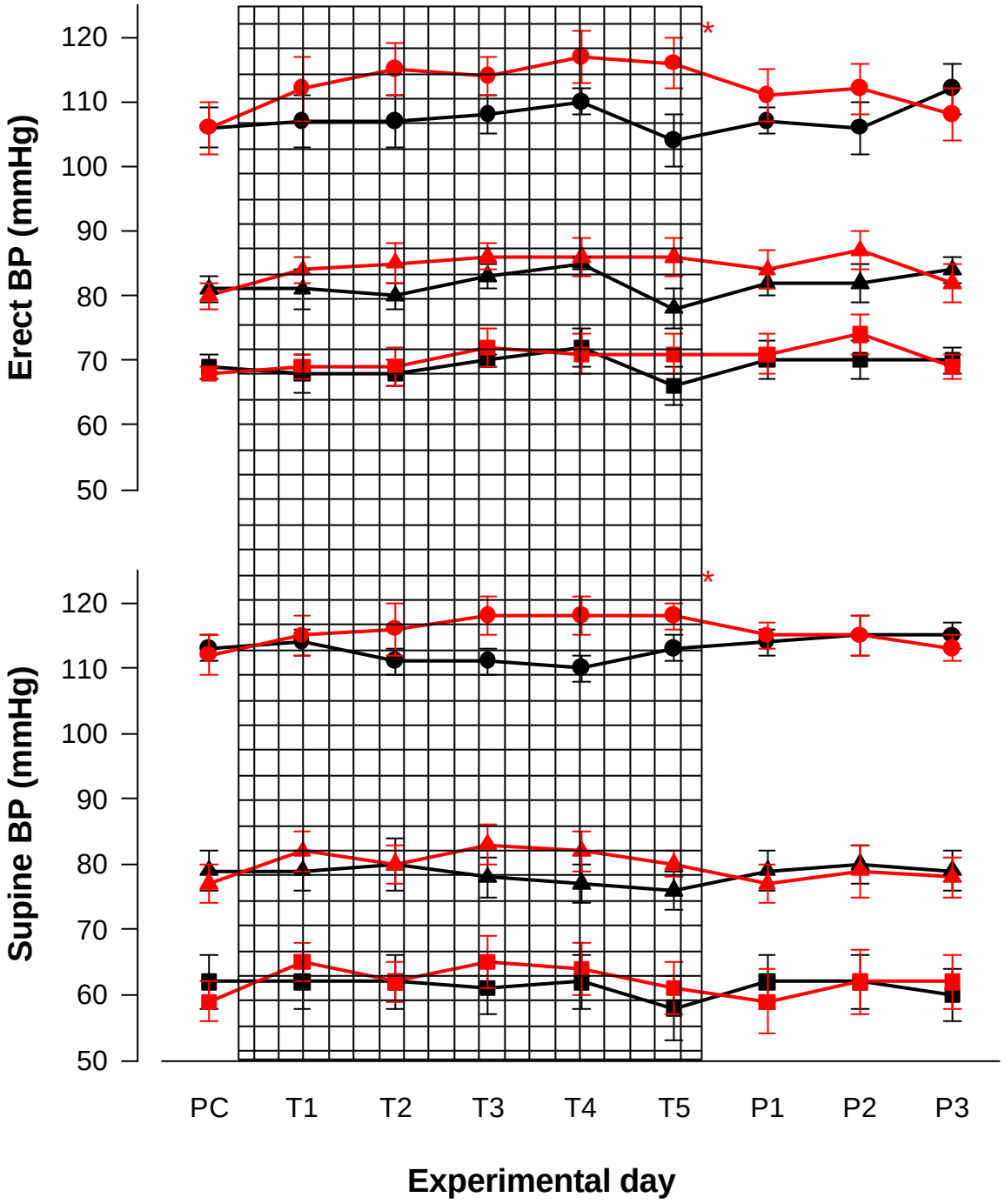


Figure 3.1 Supine and erect blood pressure in subjects (n = 8) treated with placebo (black) or cortisol 80 mg/day (red). Hatched area indicates treatment period. ● = SBP, ▲ = MAP, ■ = DBP. PC = pooled control. * $p<0.05$ for changes over time by one-way RMANOVA.

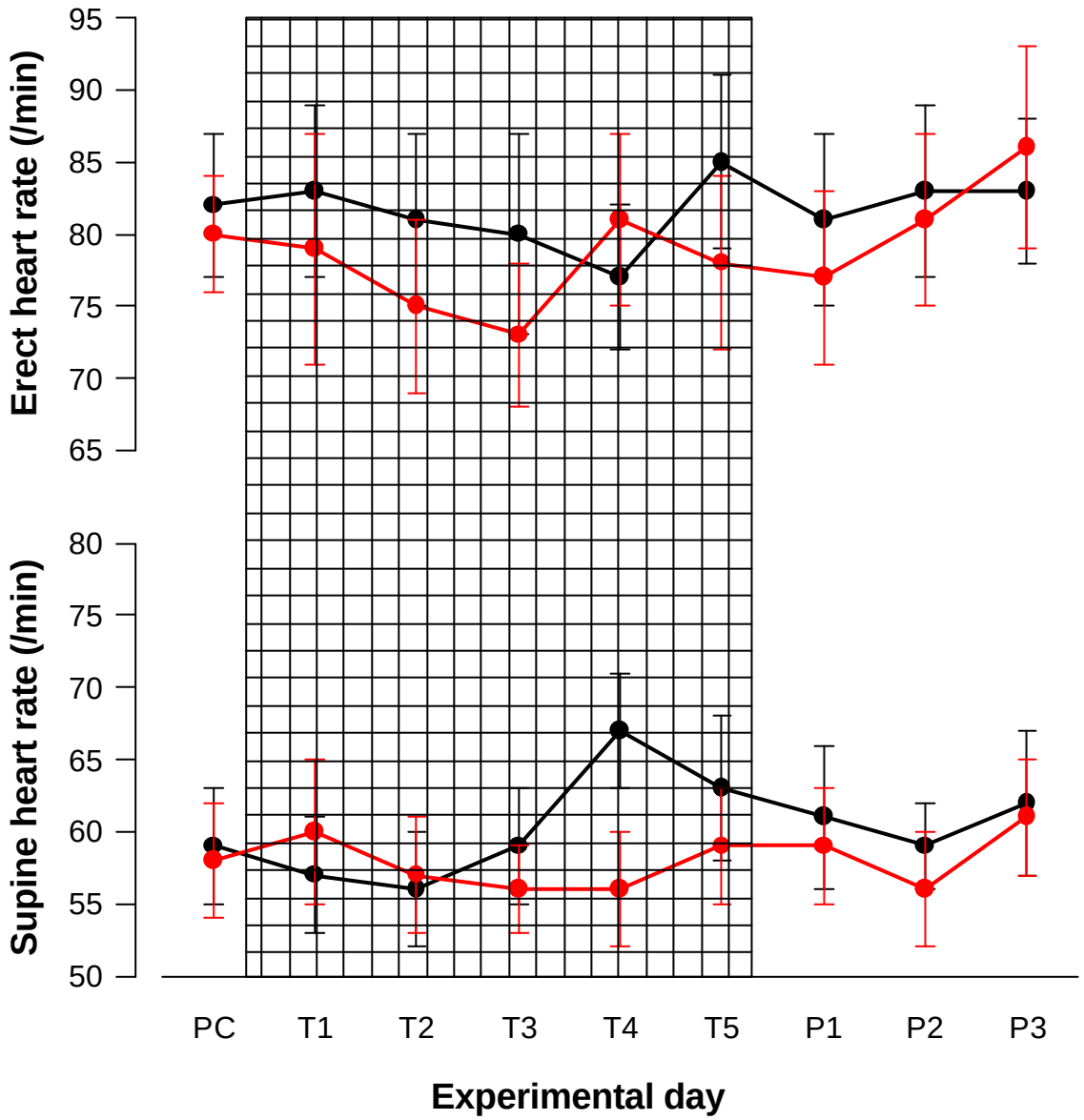


Figure 3.2 Effect of placebo (black) or cortisol 80 mg/day (red) on heart rate over 5 days in healthy male volunteers (n = 8). Hatched area indicates treatment period. No significant changes were observed.

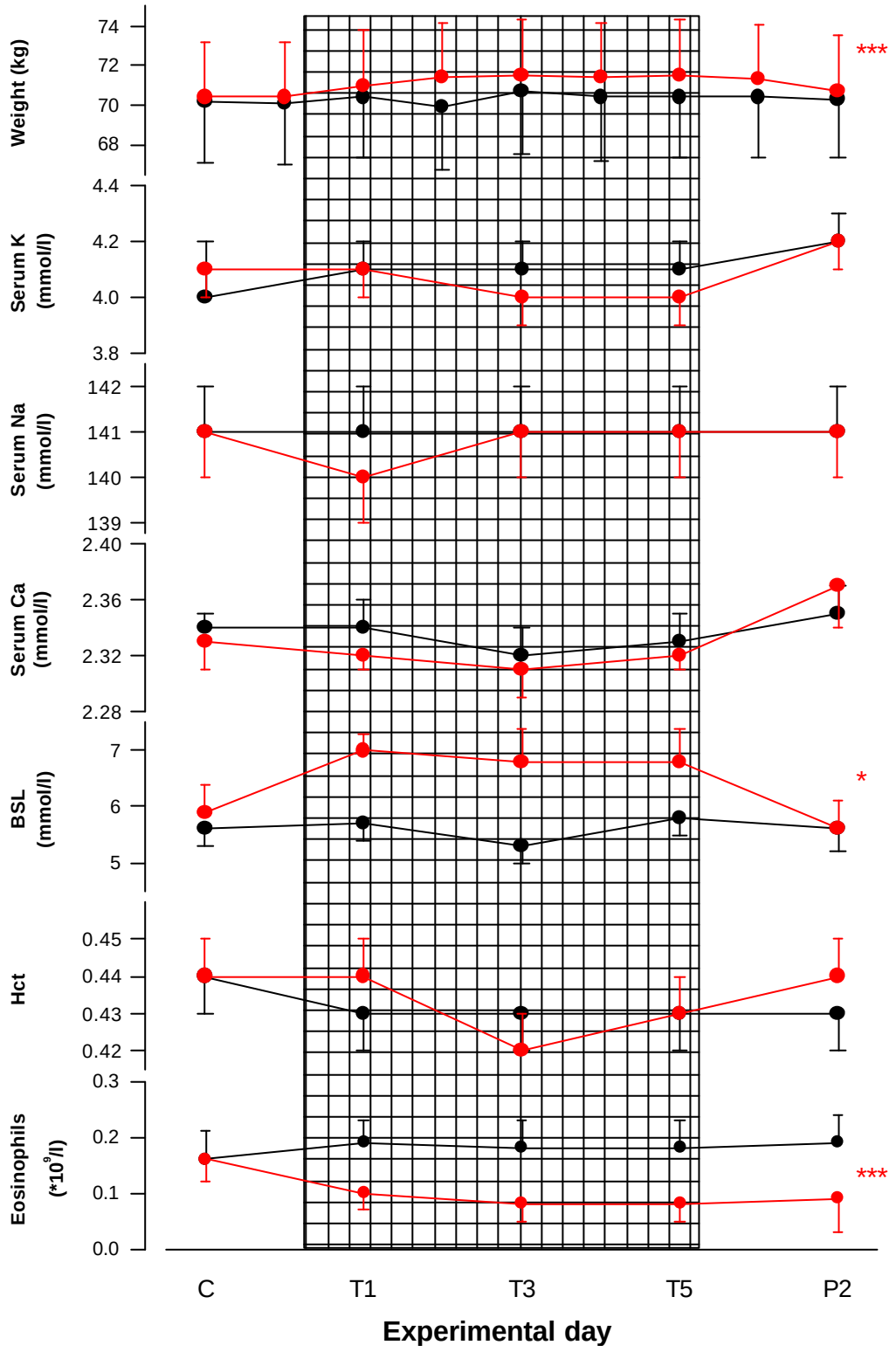


Figure 3.3 Metabolic effects of placebo (black) or cortisol 80 mg/day (red) over 5 day treatment period. Hatched area represents treatment period. C = control measurement. * p<0.05, *** p<0.001 for changes within groups by RMANOVA.

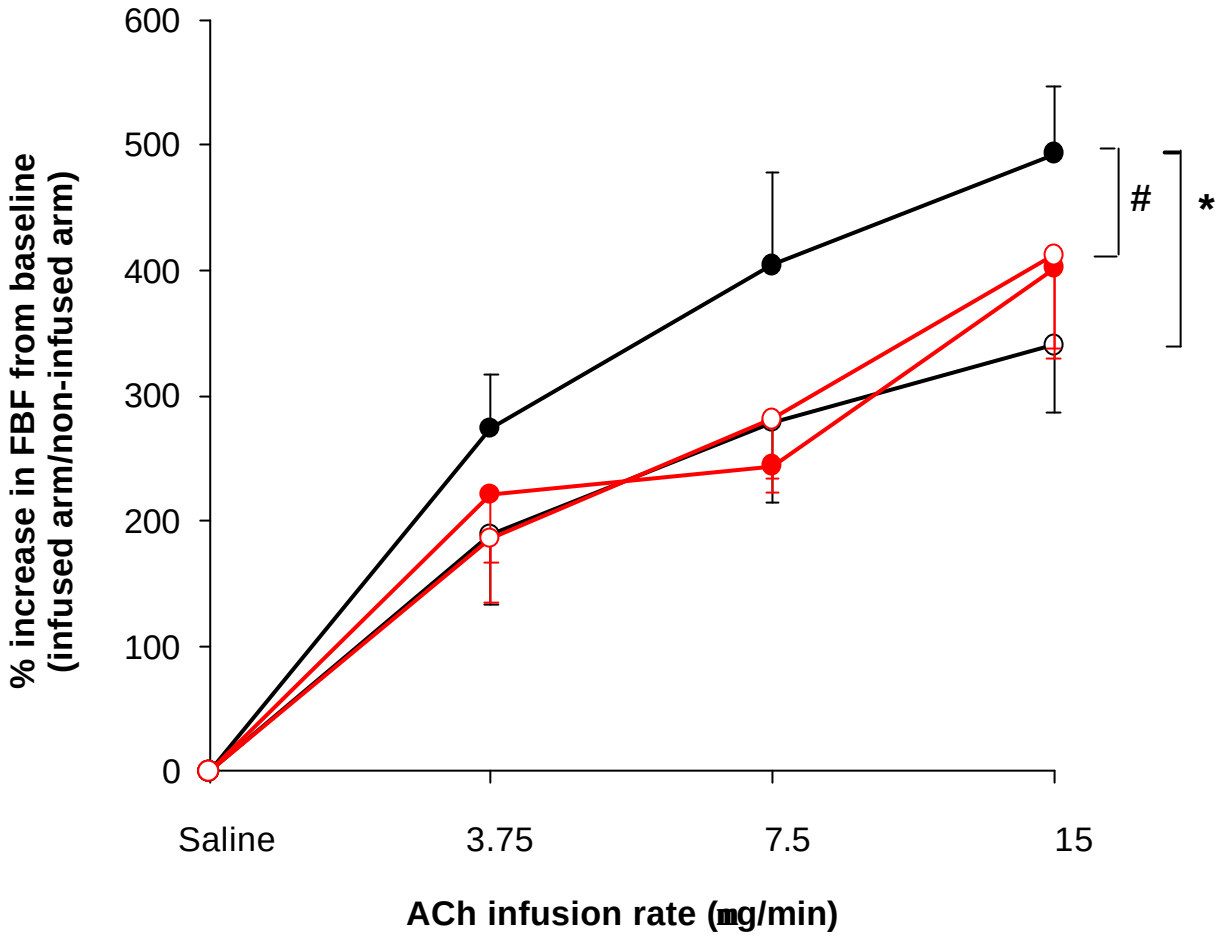


Figure 3.4 Forearm blood flow responses to increasing doses of ACh on day 5 of placebo (black) or cortisol 80 mg/day (red) treatments pre-LNMMA (closed symbols) and post-LNMMA (open symbols). The area under the dose response curves between placebo and cortisol treatments to both ACh and SNP were compared pre- and post-LNMMA. * Indicates a difference between pre- and post-LNMMA cholinergic responses in placebo treated subjects, $p<0.05$, and # indicates that the dilator response to ACh was suppressed in the cortisol phase (cortisol v placebo, $p<0.05$).

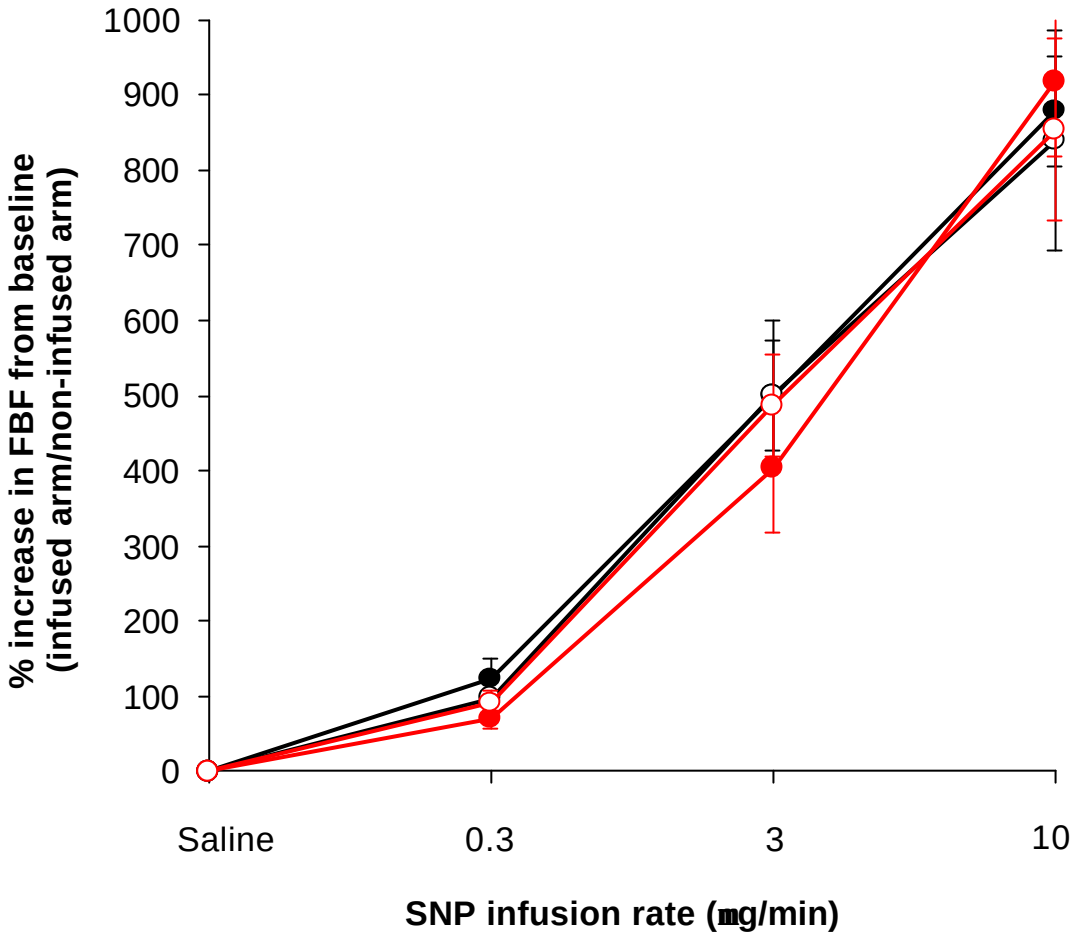


Figure 3.5 Forearm blood flow responses to increasing doses of SNP on day 5 of placebo (black) or cortisol 80 mg/day (red) treatments pre-LNMMA (closed symbols) and post-LNMMA (open symbols). The area under the dose response curves between placebo and cortisol treatments were compared pre- and post-LNMMA.

Table 3.1 Metabolic effects of placebo for 5 days in healthy male subjects (n=8)

	Control	Treatment day 5	p value
Body weight (kg)	70.2 ± 3.1	70.4 ± 3.2	n.s
Serum Na (mmol/l)	141 ± 1	141 ± 1	n.s
Serum K (mmol/l)	4 ± 0.2	4.1 ± 0.1	n.s
Albumin (g/l)	44 ± 1	44 ± 1	n.s
Ca (corrected) (mmol/l)	2.34 ± 0.01	2.33 ± 0.02	n.s
PO_4 (mmol/l)	1.1 ± 0.1	1 ± 0.1	n.s
BSL (mmol/l)	5.6 ± 0.3	5.8 ± 0.3	n.s
Cholesterol (mmol/l)	4.5 ± 0.3	4.5 ± 0.2	n.s
Triglycerides (mmol/l)	1.8 ± 0.4	1.6 ± 0.3	n.s
Cr (mmol/l)	0.1 ± 0.01	0.1 ± 0.01	n.s
Urea (mmol/l)	5 ± 0.4	4.7 ± 0.3	n.s
Haematocrit	0.441 ± 0.008	0.435 ± 0.006	n.s
Platelets (*10^9/l)	246 ± 14	239 ± 13	n.s
Leukocytes (*10^9/l)	5.8 ± 0.7	5.9 ± 0.7	n.s
Neutrophils (*10^9/l)	3.3 ± 0.5	3.3 ± 0.4	n.s
Eosinophils (*10^9/l)	0.16 ± 0.05	0.18 ± 0.05	n.s
Serum cortisol (nmol/l)	429 ± 17	364 ± 29	n.s
NO_2/NO_3 (mmol/l)	29.8 ± 5.3	30.5 ± 6.2	n.s

Table 3.2 Metabolic effects of cortisol (80 mg/day) for 5 days in healthy male subjects (n=8)

	Control	Treatment day 5	p value
Body weight (kg)	70.4 ± 2.8	71.4 ± 2.8	< 0.001
Serum Na (mmol/l)	141 ± 1	141 ± 1	n.s
Serum K (mmol/l)	4.1 ± 0.1	3.9 ± 0.1	n.s
Albumin (g/l)	43 ± 1	43 ± 1	n.s
Ca (corrected) (mmol/l)	2.33 ± 0.02	2.32 ± 0.01	n.s
PO_4 (mmol/l)	1 ± 0.1	1 ± 0.01	n.s
BSL (mmol/l)	5.9 ± 0.5	6.8 ± 0.6	0.05
Cholesterol (mmol/l)	4.5 ± 0.4	4.5 ± 0.3	n.s
Triglycerides (mmol/l)	1.5 ± 0.1	1.4 ± 0.1	n.s
Cr (mmol/l)	0.1 ± 0.01	0.09 ± 0.01	< 0.05
Urea (mmol/l)	5 ± 0.5	4.5 ± 0.3	n.s
Haematocrit	0.431 ± 0.007	0.426 ± 0.009	n.s
Platelets (*10^9/l)	220 ± 11	244 ± 10	< 0.01
Leukocytes (*10^9/l)	5.1 ± 0.3	7.7 ± 0.7	< 0.01
Neutrophils (*10^9/l)	2.7 ± 0.3	5.4 ± 0.6	< 0.01
Eosinophils (*10^9/l)	0.16 ± 0.04	0.08 ± 0.03	< 0.01
Serum cortisol (nmol/l)	397 ± 45	845 ± 64	< 0.001
NO_2/NO_3 (mmol/l)	20.9 ± 2.1	15.8 ± 2.4	n.s

Table 3.3 Effects of cortisol (80 mg/day) or placebo on electrolyte excretion and creatinine clearance (n=8).

	Placebo			Cortisol		
	C2-3	**T4-5**	**P2-3**	**C2-3**	**T4-5**	**P2-3**
Urine volume (ml/24 hr)	1450 ± 183	1956 ± 233	1456 ± 170*	1229 ± 145	1694 ± 145	1623 ± 157
Na excretion (mmol/24 hr)	163 ± 25	171 ± 20	156 ± 14	144 ± 22	203 ± 23	185 ± 25
K excretion (mmol/24 hr)	69 ± 9	62 ± 5	68 ± 7	60 ± 9	54 ± 9	75 ± 16
Na:K excretion	2.63 ± 0.43	2.87 ± 0.36	2.4 ± 0.2	2.77 ± 0.53	4.1 ± 0.42	3.17 ± 0.65
Cr clearance (ml/sec)	1.6 ± 0.2	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.1

RMANOVA employed to analyse changes over time within groups over three repeated measures. * = significant (quad
trend at $p < 0.05$.

Table 3.4: Effects of cortisol (80 mg/day) or placebo on baseline forearm blood flow pre- and post-LNMMA (n=8)

		Supine BP (mmHg)	Basal FBF (ml/100 ml/min)	LNMMA FBF (ml/100ml/min)
Placebo	**Infused**	127/55	4.3 ± 0.5	4.9 ± 0.6
	Non-infused		4.1 ± 0.5	4.2 ± 0.6
Cortisol	**Infused**	131/55	4.1 ± 0.4	5 ± 0.8
	Non-infused		4.1 ± 0.4	4.2 ± 0.3

Blood pressure was recorded by oscillometric method as described. There was no difference in basal FBF between cortisol and placebo treatments. In this study, LNMMA did not produce significant vasoconstriction from baseline FBF after five days of either treatment.

CHAPTER 4

Endothelial vasodilator function in experimental dexamethasone-induced hypertension in man

4.1 INTRODUCTION

Dexamethasone-induced hypertension is a well characterized model of hypertension in the rat. Evidence from several studies suggests that dexamethasone-induced hypertension varies in its underlying pathophysiological mechanism compared with that produced by ACTH in rat or cortisol in man. Li et al have shown that dexamethasone-induced hypertension in rat, unlike ACTH-induced hypertension, is not prevented by the co-administration of oral L-arginine (Li, Fraser et al, 1997). Saruta's group have provided evidence implicating the renin-angiotensin system as a factor in the development of hypertension in dexamethasone treated rats, at variance with pathophysiological features of ACTH-induced hypertension in man or in rat (Saruta, 1996). Collectively, these studies suggest that ACTH-induced hypertension in the rat cannot be explained solely by GR activation.

In man, dexamethasone-induced hypertension has been less well studied. Whitworth and coworkers examined the features of pure glucocorticoid hypertension in healthy male subjects who received synthetic steroids for 5 days. Prednisolone (40 mg/day), methylprednisolone (32 mg/day), triamcinolone (40 mg/day) and dexamethasone (8 mg/day) each raised SBP and DBP in association with glucocorticoid effects but no mineralocorticoid effects or increase in plasma volume (Whitworth, Gordon et al, 1989a). This

study clearly demonstrated that the hypertensive effects of these synthetic steroids in man were not dependent on mineralocorticoid activity.

Pirpiris et al examined pressor responses to dexamethasone (3mg/day) in a study comparing fludrocortisone- and dexamethasone-induced hypertension with cortisol induced hypertension, specifically investigating the pressor effects of these drugs (Pirpiris, Sudhir et al, 1992). Dexamethasone increased MAP with no change in cardiac output or body weight. Cold pressor responses were increased, as were forearm vascular responses to intra-arterial noradrenaline and angiotensin II.

In the previous chapter, I reported the effects of cortisol on endothelial vasodilator function. These results were consistent with the notion that cortisol impairs endothelium-dependent vasodilatation, and that a mechanism dependent on nitric-oxide synthase may be involved. To determine whether this effect is mediated by GR or MR, I performed a second study, examining the effects of dexamethasone on FBF.

4.2 METHODS

4.2.1 Subjects

Eight healthy non-smoking normotensive male volunteers (21-39 yrs) without contraindications to corticosteroid therapy were studied. Each subject gave written informed consent. Each subject was asked to maintain a fixed sodium intake (150 mmol/day) from two days prior and for the duration of each phase

of the study. Details of subject selection have been described in Section 2.1.2.

4.2.2 Study design

This study was a two-phase, randomised, placebo controlled, double blind crossover study comparing placebo with dexamethasone (Dexmethsone, Rhone Poulenc Rorer, NSW, Australia) 1 mg 8 hourly by mouth for five days. Subjects attended the Clinical Research Room at St George Hospital after a light breakfast at a similar time on each morning of the study period. On each morning, supine and erect blood pressure and pulse measurements were made and the subjects were weighed. On the last treatment day of each phase (ie placebo or dexamethasone treatment day 5), forearm blood flow measurements were performed as described in section 2.1.7. Treatment phases were separated by wash out periods of at least four weeks.

A right antecubital vein was cannulated every second day with the subject recumbent and blood collected for measurement of electrolytes, urea and creatinine, albumin, glucose, calcium, phosphate, haematocrit, haemoglobin, leucocyte count, platelet count and cortisol concentration (routine automated methods). Twenty four hour urine collections were obtained on control days 2-3, treatment days 4-5 and post-treatment observation days 2-3 for measurement of electrolyte excretion and creatinine clearance.

4.3 RESULTS

4.3.1 Effects of dexamethasone on blood pressure and heart rate (Figures 4.1 & 4.2)

Pooled control supine and erect SBP and DBP were similar between treatment phases (Figure 4.1). There was no change in supine or erect systolic or diastolic blood pressure with placebo treatment (supine 114 3 / 66 2 to 117 4 / 69 2 mmHg; erect 115 3 / 75 2 to 114 4 / 75 3 mmHg, PC to T5). Dexamethasone treatment increased supine systolic ($p < 0.05$, RMANOVA), erect systolic ($p < 0.01$) and erect diastolic ($p < 0.01$) blood pressure (supine BP 114 4 / 64 2 to 119 4 / 61 3 mmHg; erect BP 112 4 / 72 3 to 117 4 / 77 2 mmHg (T4), PC to T4/T5). Blood pressure, however, was similar between placebo and dexamethasone treatment phases (Figure 4.1). There were no consistent changes in heart rate on either treatment (Figure 4.2).

4.3.2 Metabolic effects of dexamethasone (Figure 4.3 and Tables 4.1 & 4.2)

Placebo treatment had no significant effect on metabolic parameters (Table 4.1). Serum cortisol concentration was suppressed by dexamethasone treatment (316 49 to 25 3 nmol/L, $p < 0.001$, C to T5) and was unaffected by placebo (308 37 to 331 28 nmol/L). Dexamethasone treatment increased body weight, white cell count, absolute neutrophil count, platelet count and reduced serum potassium, NO_2/NO_3 concentration and eosinophil

count. In this study, there was no significant increase in serum glucose, though there was a trend for an increase in glucose concentration which peaked on treatment day 3 (Figure 4.3). Urinary sodium excretion increased throughout the dexamethasone phase of the study, however, sodium excretion during control and treatment periods were similar (p=0.2, t-test). There were no changes to potassium excretion, creatinine clearance, serum sodium, potassium, calcium, phosphate, albumin, cholesterol and triglyceride concentrations on either treatment.

4.3.3 Forearm blood flow after five days of placebo or dexamethasone (Table 4.4 and Figures 4.4 and 4.5)

Baseline FBF was reduced by dexamethasone, achieving statistical significance in the infused arm (5.74 ± 0.65 v 4.29 0.47 ml/100ml/min, placebo v dexamethasone, infused arm, $p < 0.01$) (Table 4.4). Baseline FBF in the infused arm was not significantly altered by LNMMA.

ACh infusion resulted in dose-dependent increases in FBF in both placebo and cortisol phases of the study (Figure 4.4). Cholinergic vasodilatation was similar in the placebo and dexamethasone phases of the study (562 211 v 484 169 units, AUC placebo v dexamethasone). LNMMA infusion had no significant effect on cholinergic vasodilatation in the placebo phase of the study, though there was a trend to attenuation by LNMMA (562 211 to 425 180 units, AUC placebo pre- v post-LNMMA, $p = 0.12$) LNMMA infusion had no effect on cholinergic vasodilatation in the dexamethasone phase of

the study (484 169 to 407 149 units, AUC dexamethasone pre- v post-LNMMA).

SNP infusion produced dose-dependent increase in FBF which were similar in both phases of the study (458 119 v 580 87 units, AUC placebo v dexamethasone, p = n.s). Vasodilatation to SNP was not altered by LNMMA in the placebo phase (458 119 to 328 102 units, AUC placebo pre- v post-LNMMA, p = n.s) or in the dexamethasone phase of the study (580 ± 87 to 536 ± 70 units, AUC dexamethasone pre- v post-LNMMA, p = n.s)(Figure 4.5).

4.4 DISCUSSION

In this study, cholinergic vasodilatation was not impaired by dexamethasone, suggesting that the previously reported effect of cortisol on endothelial function is not mediated by GR.

Dexamethasone administration resulted in glucocorticoid effects, such as suppression of serum cortisol and eosinopaenia, but was also associated with weight gain and an initial fall in serum potassium. There was an increase in serum glucose, but this rise was not statistically significant. The weight gain during the treatment period suggests sodium and water retention, but there were no consistent features of MR activity, such as hypernatraemia or fall in haematocrit, which would suggest volume expansion. Diuresis and natriuresis are the more recognised short term effects of dexamethasone

(Whitworth, Gordon et al, 1989a, Field, Stanton et al, 1984, Nakamoto, Suzuki et al, 1991, Handa, Kondo et al, 1984). The initial fall in serum potassium may possibly be explained by transient, renal tubular, flow-dependent, kaliuresis (Field, Stanton et al, 1984). Twenty four hour electrolyte excretion was not measured in the early treatment days (T1-3), however, to confirm this.

Montrella-Waybill et al have previously shown that blockade of either GR by RU38486 or MR by spironolactone (400 mg/day) did not prevent cortisol-induced sodium retention (Montrella-Waybill, Clore et al, 1991), suggesting that cortisol induced sodium retention occurs by a renal mechanism distinct from its classical GR and MR activity. In this study, dexamethasone could be mediating sodium retention by a similar mechanism. In the study by Pirpiris et al, however, using the same dose of dexamethasone (3 mg/day), weight did not change throughout the experimental period (Pirpiris, Sudhir et al, 1992).

Dexamethasone administration resulted in a small but significant rise in systolic blood pressure over the five treatment days. However, interpretation of data from this study is limited by the failure of dexamethasone to raise blood pressure above levels measured on treatment day 5 of placebo. This can at least partly be explained by the trend to lower blood pressure in the dexamethasone control period compared with the placebo control period (Figure 4.1). Interpretation of the results obtained in this study may be

improved by a repeat study using a higher dose of dexamethasone to obtain a more marked increase in blood pressure.

It is of interest that dexamethasone reduced basal FBF in the infused limb and to a (non-significant) lesser degree in the non-infused limb. This is in keeping with Pirpiris' data (Pirpiris, Sudhir et al, 1992) in man and from studies in the rat (Handa, Kondo et al, 1984, Saruta, 1996) and dog (Nakamoto, Suzuki et al, 1991), suggesting that hypertension accompanying synthetic glucocorticoid excess is associated with vasoconstriction. Data from the plethysmography studies were technically satisfactory with both vasodilator drugs producing dose-dependent increases in FBF. LNMMA, however, failed to significantly suppress the cholinergic vasodilator response in this study. This suggests a degree of endothelial dysfunction in the group studied. Consistent with this is the comparison of the cholinergic responses between the cortisol study group (Figure 3.4) and the dexamethasone study group (Figure 4.4). Cholinergic responses are clearly greater in the first study. These differences may be explained by the greater age of subjects in this study (mean age 30 yrs, range 21-39 yrs) compared with the cortisol study (mean age 26 years, range 20-31 yrs) suggesting a degree of endothelial dysfunction in the dexamethasone study group (Gerhard, Roddy et al, 1996). Other factors in these studies were constant; smokers were excluded, and no subjects had hypercholesterolaemia, diabetes mellitus or other medical illness which may account for endothelial dysfunction.

Unlike cortisol, dexamethasone failed to attenuate cholinergic vasodilatation. This suggests that the effect of cortisol on endothelial vasodilatation is independent of the GR. Alternatively, the effect of cortisol may have been secondary to the rise in blood pressure. This has been discussed previously and is thought to be unlikely (Chapter 3). Some investigators have questioned whether endothelial dysfunction accompanying essential and other chronic forms of human hypertension is a secondary rather than primary event (Calver, Collier et al, 1992a, Forte, Copland et al, 1997, Vanhoutte, 1996, Taddei, Virdis et al, 1998). If this were the case, suppressed endothelium-dependent vasodilatation would be expected, proportional to the rise in blood pressure, in all models of human hypertension. Although this hypothesis could explain forearm blood flow data from the present study, results from Chapter 5 are inconsistent with the notion.

Despite the failure of dexamethasone to suppress cholinergic vasodilatation in the present study, plasma NOx was suppressed by dexamethasone. As an indirect marker of NOS activity, reduced NOx suggests reduced NO system activity, but interpretation of this result must be limited. NOx concentrations do not necessarily reflect vascular NOS activity, and, as discussed earlier, dietary nitrates may influence plasma concentrations of nitrate. Nevertheless, the result is interesting because dexamethasone-induced hypertension in the rat is not prevented by L-arginine, suggesting that this animal model of hypertension is not associated with reduced NO system activity. From the

present study, evidence is lacking supporting the notion that hypertension secondary to dexamethasone administration is due to nitric oxide deficiency.

4.5 CONCLUSIONS

In summary, results from this experiment show that although dexamethasone appeared vasoactive, its administration did not affect endothelium-dependent or independent vasodilatation. Taken together with the previous study, these results suggest that the effect of cortisol on endothelium-dependent vasodilatation is independent of GR.

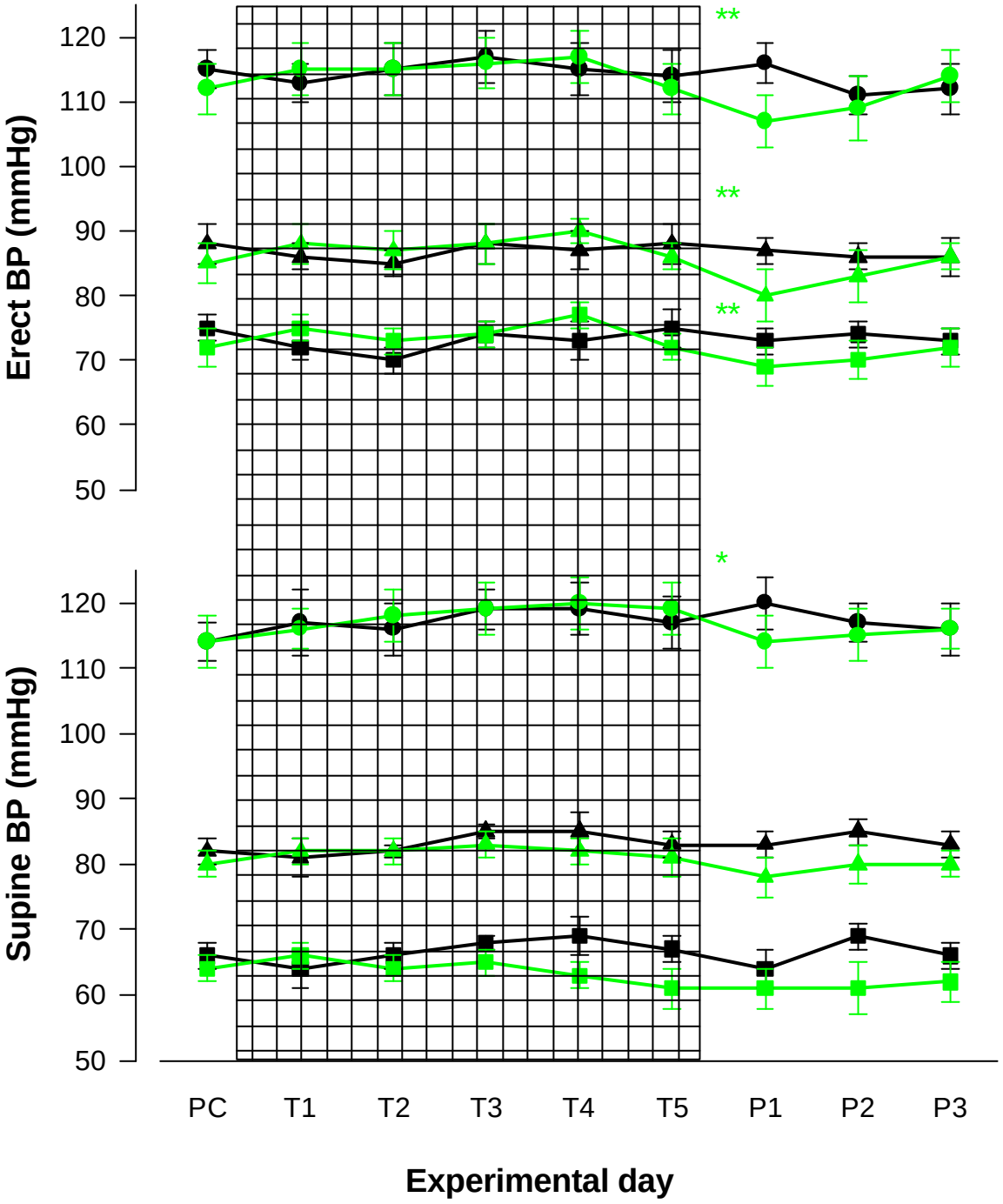


Figure 4.1 Supine and erect blood pressure in subjects (n = 8) treated with placebo (black) or dexamethasone 3 mg/day (green). Hatched area indicates treatment period. ● = SBP, ▲ = MAP, ■ = DBP. PC = pooled control. * $p < 0.05$, ** $p < 0.01$ by one-way RMANOVA.

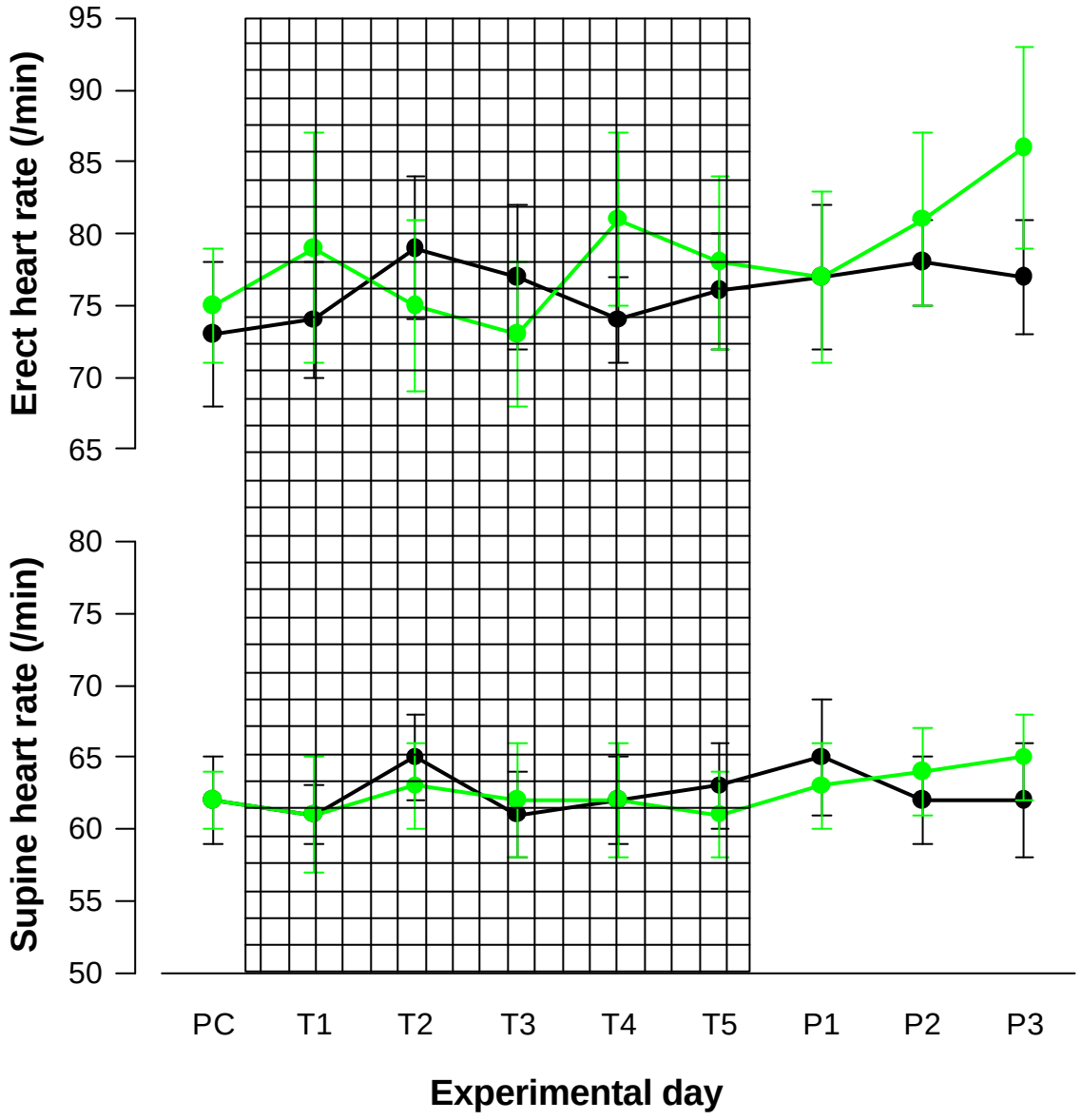


Figure 4.2 Effect of placebo (black) or dexamethasone 3 mg/day (green) on heart rate over 5 days in healthy male volunteers (n = 8). No significant changes over time were observed.

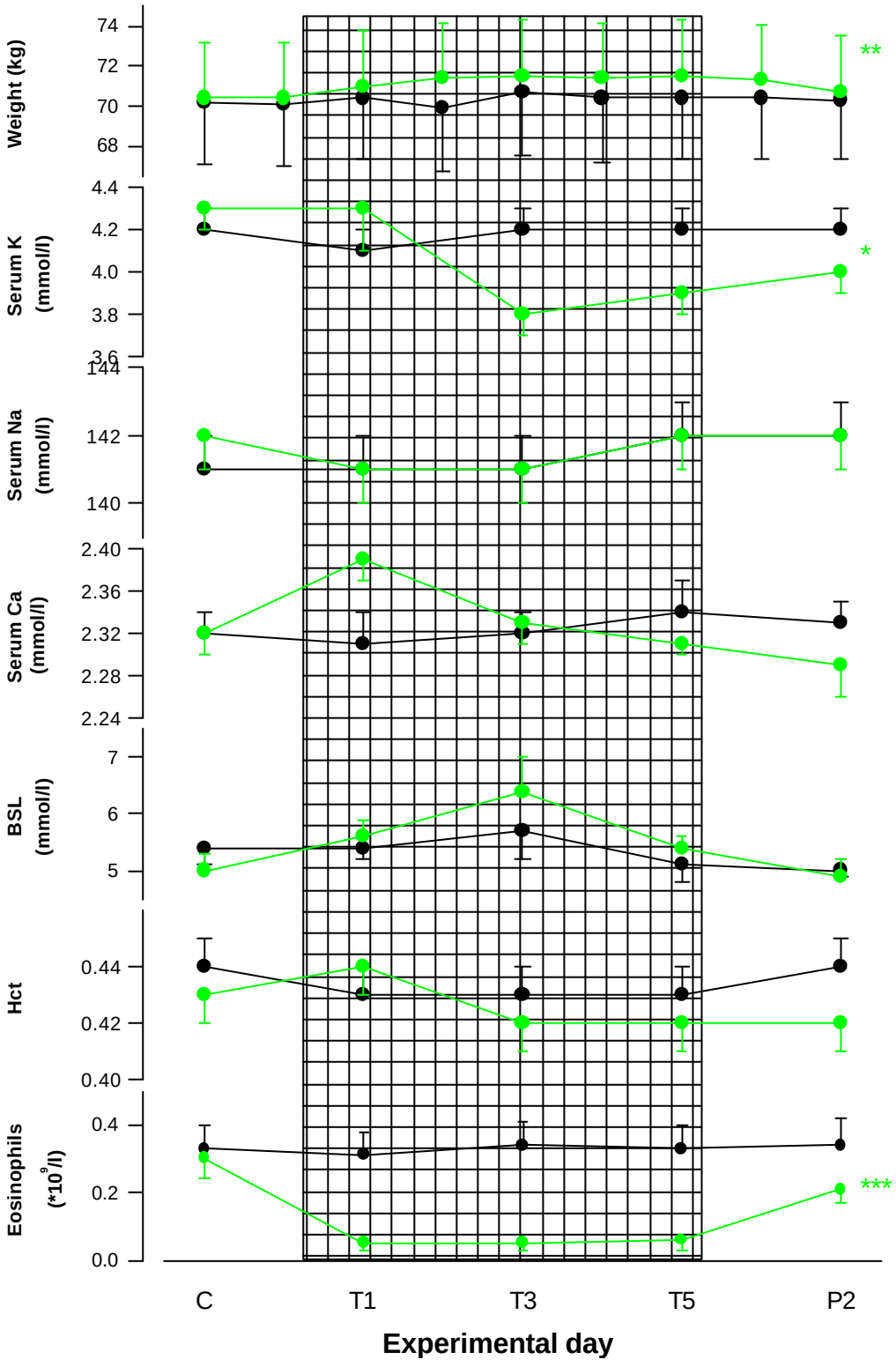


Figure 4.3 Metabolic effects of placebo (black) or dexamethasone 3 mg/day, (green) over 5 day treatment period (n = 8). Hatched area represents treatment period. C = control measurement. * $p < 0.05$, *** $p < 0.001$ for changes within groups over time by RMANOVA.

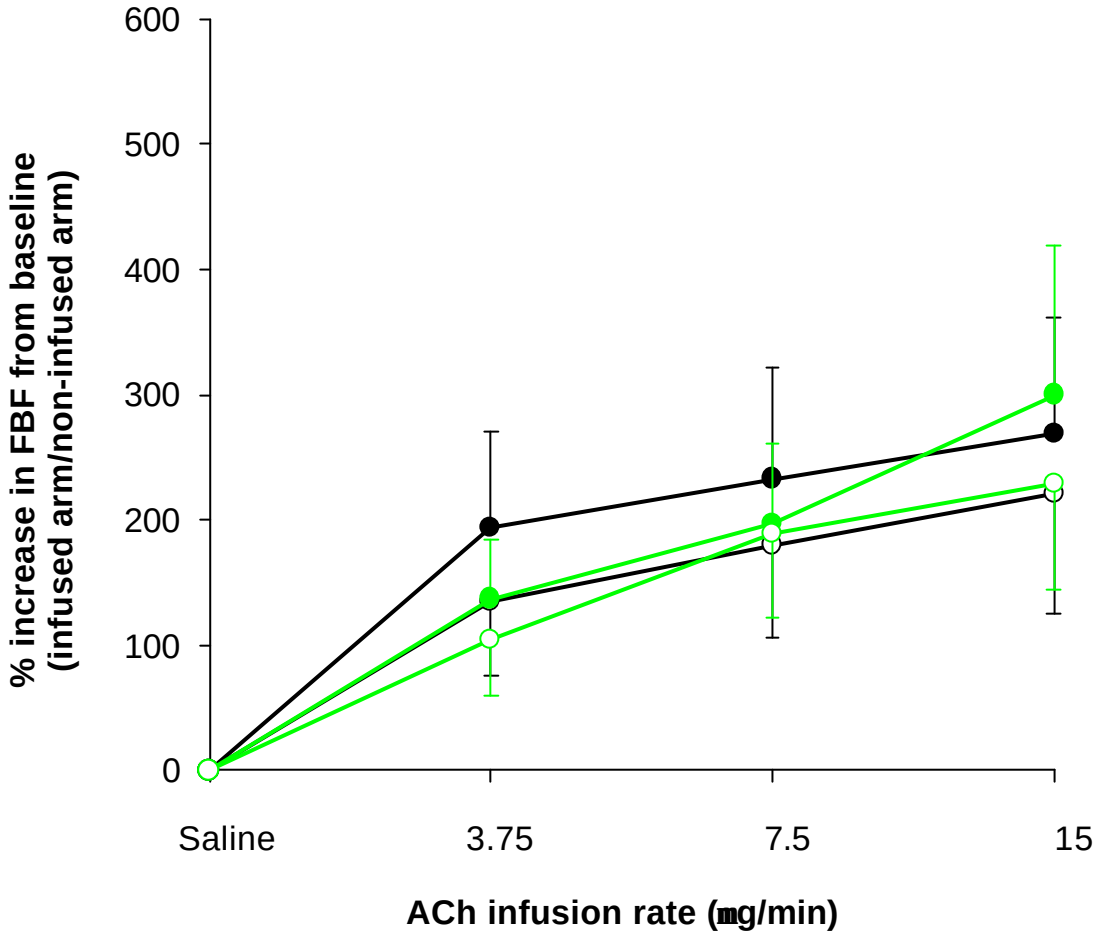


Figure 4.4 Forearm blood flow responses (n = 8) to increasing doses of ACh on day 5 of placebo (black) or dexamethasone (green) pre-LNMMA (closed symbols) and post-LNMMA (open symbols). The area under the dose-response curves between placebo and dexamethasone treatments to both ACh and SNP were compared pre- and post-LNMMA. There were no significant differences between the cholinergic vasodilator responses in either treatment phase.

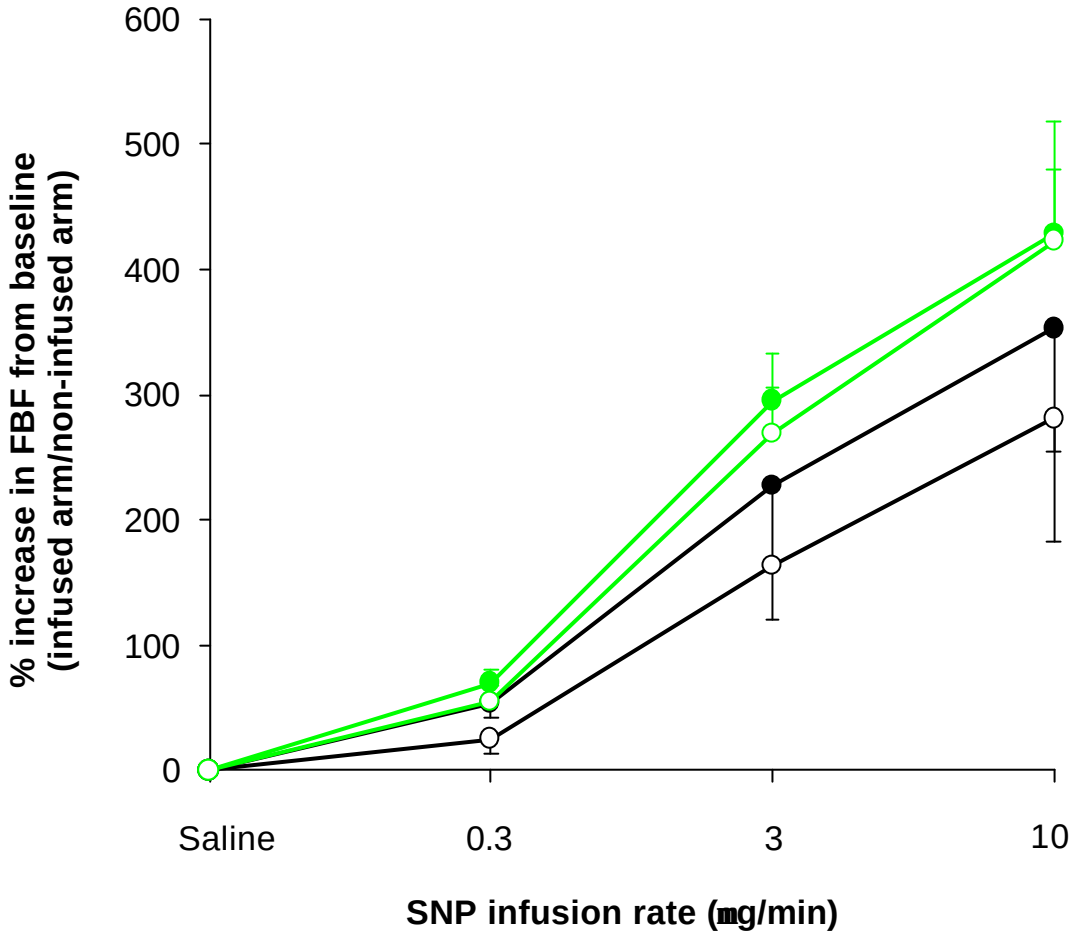


Figure 4.5 Forearm blood flow responses (n = 8) to increasing doses of SNP on day 5 of placebo (black) or dexamethasone (green) pre-LNMMA (closed symbols) and post-LNMMA (open symbols). The area under the dose-response curves between placebo and dexamethasone treatments was compared pre- and post-LNMMA.

Table 4.1: Metabolic effects of placebo for 5 days in healthy male subjects (n=8)

	Control	Treatment day 5	p value
Body weight (kg)	76.1 ± 3.7	75.9 ± 3.8	n.s
Serum Na (mmol/l)	141 ± 1	142 ± 1	n.s
Serum K (mmol/l)	4.2 ± 0.1	4.2 ± 0.1	n.s
Albumin (g/l)	42 ± 1	42 ± 1	n.s
Ca (corrected) (mmol/l)	2.32 ± 0.02	2.34 ± 0.03	n.s
PO_4 (mmol/l)	1.1 ± 0.1	1.1 ± 0.1	n.s
BSL (mmol/l)	5.4 ± 0.3	5.1 ± 0.3	n.s
Cholesterol (mmol/l)	4.5 ± 0.3	4.2 ± 0.2	n.s
Triglycerides (mmol/l)	1.1 ± 0.1	1.1 ± 0.1	n.s
Cr (mmol/l)	0.09 ± 0.01	0.09 ± 0.01	n.s
Urea (mmol/l)	5.2 ± 0.4	5.8 ± 0.4	< 0.05
Haematocrit	0.437 ± 0.008	0.426 ± 0.008	n.s
Platelets (*10^9/l)	212 ± 10	203 ± 7	n.s
Leukocytes (*10^9/l)	4.9 ± 0.4	5.2 ± 0.4	n.s
Neutrophils (*10^9/l)	2.6 ± 0.3	2.9 ± 0.3	n.s
Eosinophils (*10^9/l)	0.33 ± 0.07	0.33 ± 0.07	n.s
Serum cortisol (nmol/l)	308 ± 37	331 ± 28	n.s
NO_2/NO_3 (mmol/l)	23.2 ± 2.5	20 ± 2.1	n.s

Table 4.2: Metabolic effects of dexamethasone (3 mg/day) for 5 days in healthy male subjects (n=8)

	Control	Treatment day 5	p value
Body weight (kg)	75.6 ± 3.7	76.4 ± 3.7	< 0.05
Serum Na (mmol/l)	142 ± 1	142 ± 1	n.s
Serum K (mmol/l)	4.3 ± 0.1	3.9 ± 0.1	< 0.05
Albumin (g/l)	42 ± 1	43 ± 1	n.s
Ca (corrected) (mmol/l)	2.32 ± 0.03	2.31 ± 0.02	n.s
PO_4 (mmol/l)	1.1 ± 0.1	1.2 ± 0.01	n.s
BSL (mmol/l)	5 ± 0.4	5.4 ± 0.2	n.s
Cholesterol (mmol/l)	4.5 ± 0.3	4.5 ± 0.2	n.s
Triglycerides (mmol/l)	1 ± 0.1	1.1 ± 0.2	n.s
Cr (mmol/l)	0.09 ± 0.01	0.09 ± 0.01	n.s
Urea (mmol/l)	5.6 ± 0.4	4.7 ± 0.4	< 0.01
Haematocrit	0.43 ± 0.009	0.419 ± 0.008	n.s
Platelets (*10^9/l)	210 ± 9	231 ± 12	< 0.01
Leukocytes (*10^9/l)	5.3 ± 0.4	7.7 ± 0.5	< 0.01
Neutrophils (*10^9/l)	3 ± 0.4	5.2 ± 0.4	< 0.001
Eosinophils (*10^9/l)	0.3 ± 0.06	0.06 ± 0.03	< 0.01
Serum cortisol (nmol/l)	316 ± 49	25 ± 3	< 0.001
NO_2/NO_3 (mmol/l)	24.3 ± 2.8	14.1 ± 1.5	< 0.01

Table 4.3: Effects of dexamethasone (3 mg/day) or placebo on electrolyte excretion and creatinine clearance (n=8)

	Placebo			Dexamethasone		
	C2-3	**T4-5**	**P2-3**	**C2-3**	**T4-5**	**P2-3**
Urine volume (ml/24 hr)	1384 ± 198	1449 ± 214	1309 ± 170	1439 ± 267	1797 ± 303	1655 ± 198
Na excretion (mmol/24 hr)	113 ± 8	148 ± 17	145 ± 21	141 ± 13	160 ± 15	216 ± 17*
K excretion (mmol/24 hr)	83 ± 19	75 ± 11	63 ± 8	74 ± 10	62 ± 14	94 ± 17
Na:K excretion	1.79 ± 0.36	2.28 ± 0.39	2.63 ± 0.52	2.11 ± 0.25	3.69 ± 0.89	3.38 ± 1.01
Cr clearance (ml/sec)	1.5 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.1

RMANOVA employed to analyse changes over time within groups over three repeated measures. * = significant (linear) trend at $p < 0.05$.

Table 4.4: Effects of dexamethasone (3 mg/day) or placebo on baseline forearm blood flow pre- and post-LNMMA (n=8)

		Supine BP (mmHg)	Basal FBF (ml/100 ml/min)	LNMMA FBF (ml/100ml/min)
Placebo	**Infused**	136±4 / 56±2	5.7 ± 0.7	6.1 ± 0.9
	Non-infused		5.4 ± 0.6	7 ± 1
Dexamethasone	**Infused**	133±2 / 59±2	4.3 ± 0.47*	5.3 ± 0.6
	Non-infused		4.6 ± 0.4	6 ± 0.6

Blood pressure was recorded by oscillometric method as described. Dexamethasone reduced baseline FBF in the in the infused arm ($p<0.01$) with a similar trend in the non-infused arm ($p=0.08$). In this study, LNMMA did not produce significant vasoconstriction from baseline FBF after five days of either treatment.

CHAPTER 5

Endothelial function in experimental fludrocortisone-induced hypertension in man

5.1 INTRODUCTION

Fludrocortisone (9-α-fluorocortisol(one), fluorohydrocortisol) is a halogenated derivative of cortisol, with very potent mineralocorticoid activity. The chemical name (of the acetate) is 9α-fluoro-11β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione 21 acetate. Although it has 10-15 times the glucocorticoid potency of cortisol, the mineralocorticoid activity clearly predominates (Goldfien, Laidlaw et al, 1955). It is used clinically in the management of Addison's disease (Goldfien, Laidlaw et al, 1955), chronic orthostatic hypotension (Hickler, Thompson et al, 1959) and has also been used in hyporeninaemic hypoaldosteronism.

Swingle et al showed that fludrocortisone can completely reverse adrenal deficiency in dogs and restore fluid and electrolyte status (Swingle, Baker et al, 1955, Swingle, Brannick et al, 1957). In sheep, fludrocortisone was equipotent with aldosterone on renal type I MR and with dexamethasone on type II GR (Coghlan, Butkus et al, 1979).

Most, but not all, studies of the effects of fludrocortisone in man report an increase in blood pressure (Distler and Philipp, 1976, Nicholls, Ramsay et al, 1979, Martin, Zipser et al, 1981, Whitworth, Saines et al, 1983a). Whitworth et al examined the blood pressure and metabolic effects of fludrocortisone at 0.15 mg and 1.5 mg/day over 5 days in man. At the high doses, systolic

blood pressure and body weight rose and plasma potassium, urinary sodium excretion and plasma renin concentration were reduced (Whitworth, Saines et al, 1983a). At the lower doses, however, similar metabolic effects were observed in the absence of changes in blood pressure. Thus, fludrocortisone has mineralocorticoid effects in man at a dose that does not alter blood pressure in the short term.

Distler and co workers showed that fludrocortisone administered at a dose of 0.8 mg/day increased mean arterial pressure within one week and this increase was a consequence of increased cardiac output. By six weeks, however, the increase was due to increased peripheral resistance in association with reduced plasma noradrenaline but increased reactivity to exogenous noradrenaline (Distler, Philipp et al, 1979). Increased pressor responses to cold, noradrenaline and angiotensin II have also been reported by Pirpiris et al (Pirpiris, Sudhir et al, 1992) in subjects treated with fludrocortisone (0.3 mg/day) over 7 days. In this short-term study, fludrocortisone increased mean arterial pressure (by 9 mmHg) and cardiac output but peripheral resistance was reduced.

The mechanism underlying increased pressor responses in mineralocorticoid hypertension has not been determined. Endothelial vasodilator function in this model in man has not previously been examined. The aim of the present study was to examine the effect of oral fludrocortisone on forearm blood flow and endothelial responses to acetylcholine.

5.2 METHODS

5.2.1 Subjects

Six healthy non-smoking normotensive male volunteers (19-40 yrs) without contraindications to corticosteroid therapy were studied. Each subject gave written informed consent. Each subject was asked to maintain a fixed sodium intake (150 mmol/day) from two days prior and for the duration of each phase of the study. Details of subject selection have been described in section 2.1.2.

5.2.2 Study design

This study was a two-phase, randomised, placebo controlled, double blind crossover study comparing placebo with fludrocortisone (fludrocortisone acetate, Florinef, Bristol-Myers Squibb, Victoria, Australia) 0.2 mg 6 hourly by mouth for five days. Subjects attended the Clinical Research Room at St George Hospital after a light breakfast at a similar time on the mornings of control days 1 & 3, treatment days 1,3 & 5 and post-treatment observation day 2. On each morning, supine and erect blood pressure and pulse measurements were made and the subjects were weighed. On the last treatment day of each phase (ie placebo or fludrocortisone treatment day 5), forearm blood flow measurements were performed as described in section 2.1.7. Treatment phases were separated by wash out periods of at least four weeks.

A right antecubital vein was cannulated on control day 3 and treatment day 5 with subjects recumbent and blood collected for measurement of electrolytes, urea and creatinine, albumin, glucose, calcium, phosphate, haematocrit, haemoglobin, leukocyte count, platelet count, cortisol and plasma renin concentrations.

5.3 RESULTS

5.3.1 Effects of fludrocortisone on blood pressure and heart rate (Figures 5.1, 5.2 & 5.3)

Pooled control supine and erect systolic and diastolic blood pressure were similar between treatment phases (Figure 5.1). Pooled control supine MAP was higher in the placebo phase (84 1 v 80 ± 1 mmHg, $p < 0.05$, placebo v fludrocortisone PC supine MAP). There was no change in supine or erect systolic or diastolic blood pressure with placebo treatment (supine 114 2 / 70 2 to 113 3 / 69 2 mmHg; erect 106 3 / 69 2 to 103 3 / 68 2 mmHg, PC to T5). Fludrocortisone treatment increased supine systolic ($p < 0.05$, RMANOVA), erect systolic ($p < 0.01$), erect diastolic ($p < 0.01$) and erect mean arterial pressure ($p < 0.01$) (supine BP 111 3 / 65 1 to 116 3 / 71 2 mmHg; erect BP 104 4 / 68 4 to 112 5 / 71 4 mmHg, PC to T5). There were no consistent changes in heart rate on either treatment (Figure 5.2).

5.3.2 Metabolic effects of fludrocortisone (Tables 5.1 & 5.2)

Placebo treatment had no significant effect on metabolic parameters (Table 5.1). Serum cortisol concentration was slightly suppressed by fludrocortisone (311 56 to 231 43 nmol/L, p = 0.05, C to T5) and was unaffected by placebo (307 38 to 273 45 nmol/L). Plasma renin was suppressed by fludrocortisone ($p < 0.01$) and unchanged by placebo. Fludrocortisone increased body weight and serum sodium and reduced serum potassium, albumin, haematocrit and plasma NO_2/NO_3 concentration. There were no changes to blood sugar, cholesterol and triglyceride concentrations and differential white cell count on either treatment.

5.3.3 Forearm blood flow after five days of placebo or fludrocortisone (Table 5.3 and Figures 5.4 and 5.5)

Baseline FBF was not altered by fludrocortisone (Table 5.3). Baseline FBF in the infused arm was not significantly altered by LNMMA.

ACh infusion resulted in similar dose-dependent increases in FBF in placebo and fludrocortisone phases of the study (Figure 5.4) (612 195 v 963 284 units, AUC placebo v fludrocortisone, p=n.s). LNMMA tended to suppress the cholinergic vasodilator response in both phases of the study though this effect did not achieve statistical significance (612 195 to 311 97 units, ACh AUC placebo pre- v post-LNMMA, p = 0.07, 963 ± 284 to 790 ± 221, ACh AUC fludrocortisone pre v post-LNMMA, p = 0.08). The cholinergic

vasodilator response was greater following LNMMA in the fludrocortisone phase compared with the placebo phase ($p < 0.05$).

SNP infusion produced dose-dependent increases in FBF which were similar in both phases of the study (502 131 v 743 123 units, AUC placebo v fludrocortisone. p = n.s). Vasodilatation to SNP was not altered by LNMMA in the placebo phase (502 131 to 426 92 units, AUC placebo pre- v post-LNMMA, p = n.s) or in the fludrocortisone phase of the study (743 ± 123 to 572 ± 102 units, AUC fludrocortisone pre- v post-LNMMA, p = n.s)(Figure 5.5).

5.4 DISCUSSION

In this study, fludrocortisone increased blood pressure in association with metabolic changes of mineralocorticoid excess over the five day treatment period. Cholinergic vasodilatation in the forearm was not suppressed by fludrocortisone, but tended to be (not significantly) increased, and was greater following the administration of LNMMA than in the placebo treatment phase. In view of the well described effects of mineralocorticoids on pressor responses these results are surprising and discordant with the effects of cortisol on cholinergic vasodilatation, reported in Chapter 3. The results described in this study may represent a modulating response of endothelium to an increased contractile sensitivity in a state of mineralocorticoid excess.

Increased cholinergic vasodilatation has rarely been described in hypertensive syndromes. Bockman et al (Bockman, Jeffries et al, 1992) reported increased endothelial nitric oxide production in isolated mesenteric artery rings from DOCA-salt rats, and postulated a modulating effect of endothelium, masking the enhanced pressor sensitivity of the smooth muscle. Almost all other hypertensive models in man and rat have been associated with either no change in endothelium-dependent responses or suppression of endothelium-dependent vasodilatation (Hishikawa, Nakaki et al, 1993, Panza, Quyyumi et al, 1990, Calver, Collier et al, 1992a, Panza, Casino et al, 1993, Panza, Casino et al, 1994, Vanhoutte, 1996). In the present study, FBF cholinergic responses in the fludrocortisone phase tended to be greater than in placebo, but this was not statistically significant when compared with placebo. This effect did not appear to be nitric oxide dependent, as suppression with LNMMA was minimal and FBF was significantly greater when cholinergic responses were compared following LNMMA between fludrocortisone and placebo phases. Further, metabolites of nitric oxide were reduced in plasma, suggestive (but in no way conclusive) of reduced nitric oxide system activity. These responses were not due to changes in vascular smooth muscle sensitivity, as responses to SNP were similar. The mechanisms possibly involved include a compensatory effect of endothelium as described above, mediated by another endothelium dependent substance, such as prostacyclin, or a non-specific effect of fludrocortisone on metabolism of the vasodilator, ACh. ACh is largely

metabolised by cholinesterase (Bruning, Chang et al, 1996), and inhibition of this enzyme could prolong cholinergic exposure to vascular endothelium.

Martin et al studied the effect of indomethacin treatment on the blood pressure and metabolic effects of fludrocortisone in man (Martin, Zipser et al, 1981). Blood pressure increased to a greater extent in indomethacin treated subjects, with no difference in weight gain. Vascular prostanoids may have played a modulating role in fludrocortisone hypertension in these subjects.

Mineralocorticoid hypertension has been considered cardiac output mediated in the early phase (with reduced peripheral resistance) then peripheral resistance mediated over the longer term (Distler and Philipp, 1976). It is possible that enhanced endothelium-dependent vasodilatation is responsible for this vasodilated state in the early phases of this form of hypertension, suggested in this study. Indeed, flow mediated vasodilatation in the human forearm is at least partly nitric oxide mediated (Joannides, Haefeli et al, 1995).

5.5 CONCLUSIONS

Regardless of the mechanism, results from this study argue against the notion that endothelium-dependent vasodilatation is suppressed as a consequence of hypertension. Taken together with the cortisol and dexamethasone studies, the results suggest that the effect of cortisol on endothelium-dependent vasodilatation is unique and not mediated by MR.

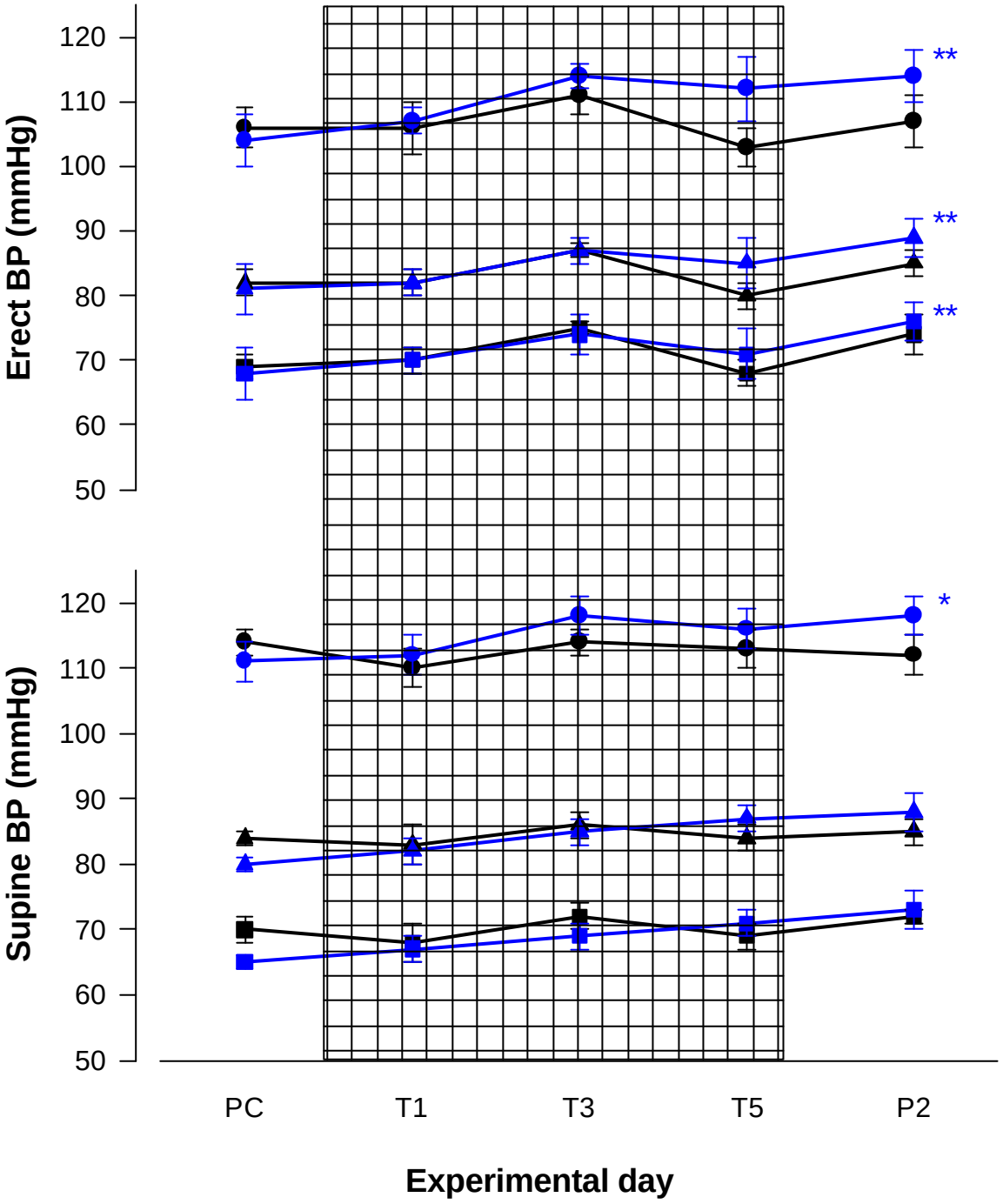


Figure 5.1 Supine and erect blood pressure in subjects (n = 6) treated with placebo (black) or fludrocortisone 0.8 mg/day, (blue). Hatched area indicates treatment period. ● = SBP, ▲ = MAP, ■ = DBP. PC = pooled control. * $p < 0.05$, ** $p < 0.01$ for linear or quadratic trends by one-way RMANOVA.

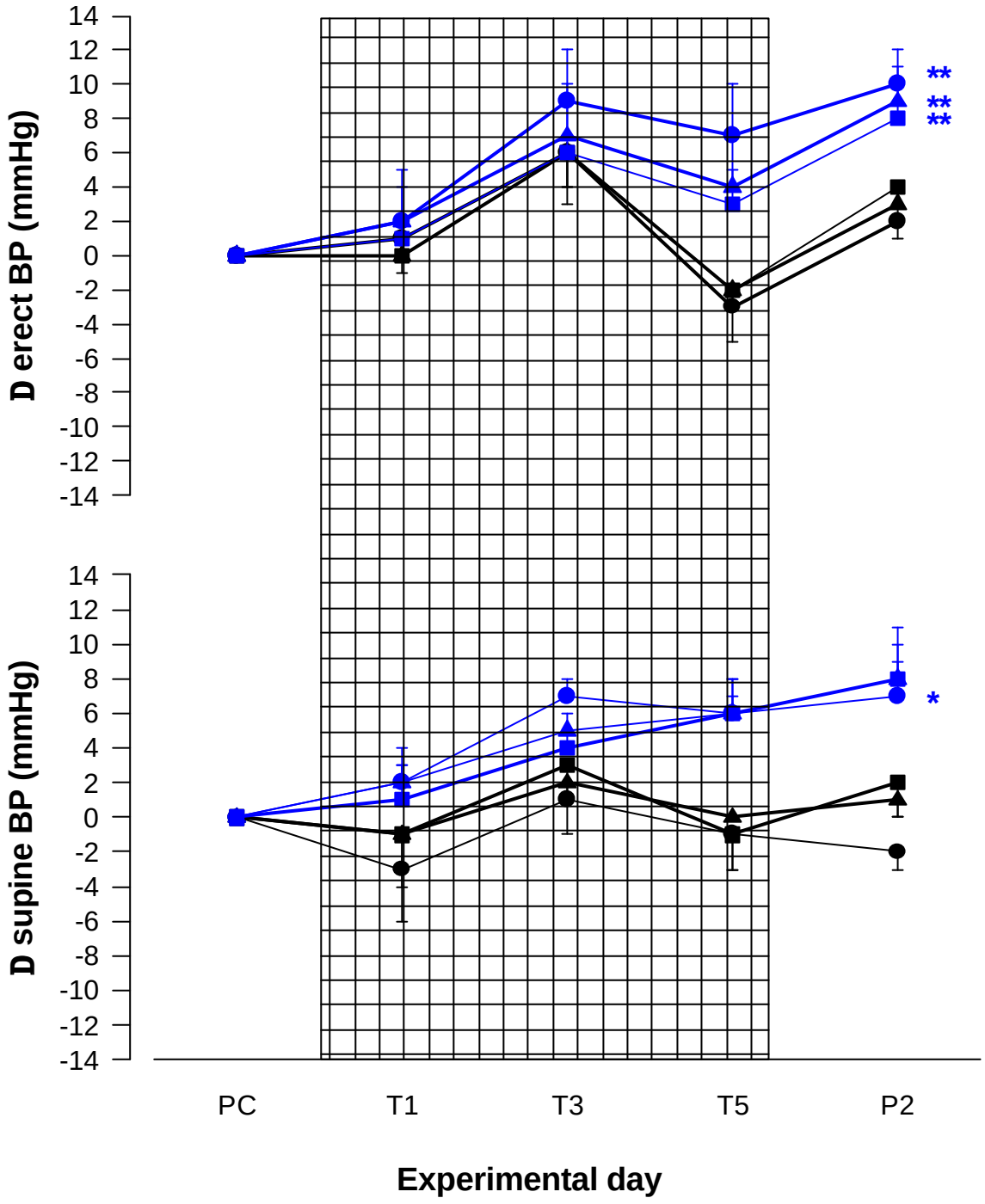


Figure 5.2 Changes in erect and supine blood pressure in subjects (n = 6) treated with placebo (black) or fludrocortisone 0.8 mg/day (blue). ● = SBP, ▲ = MAP, ■ = DBP. PC = pooled control. Changes are from PC. * $p < 0.05$ (for supine SBP), ** $p < 0.01$, for linear or quadratic trends by one-way RMANOVA.

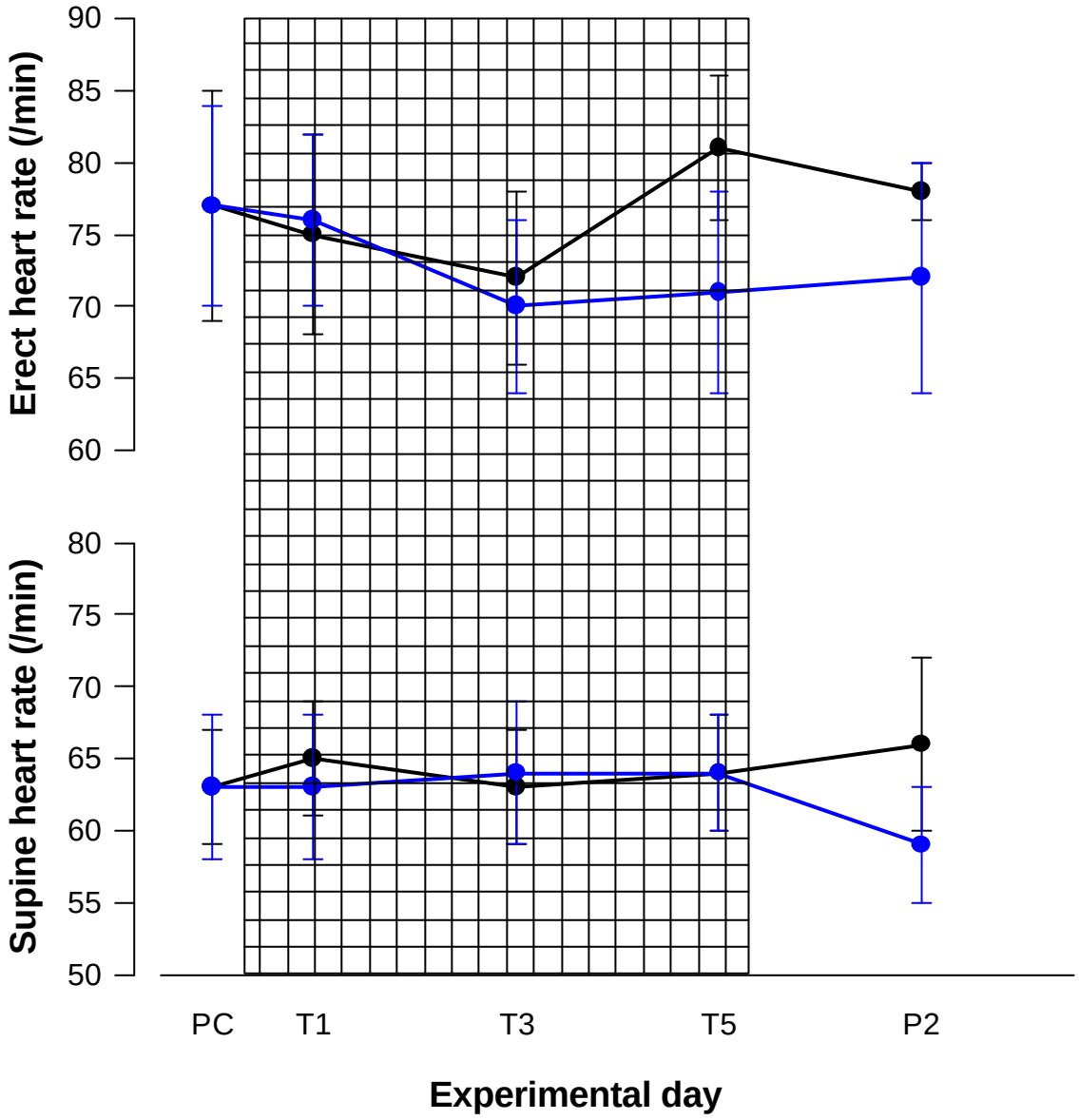


Figure 5.3 Effect of placebo (black) or fludrocortisone 0.8 mg/day (blue) on heart rate over 5 days in healthy male volunteers (n = 6). No significant changes over time were observed.

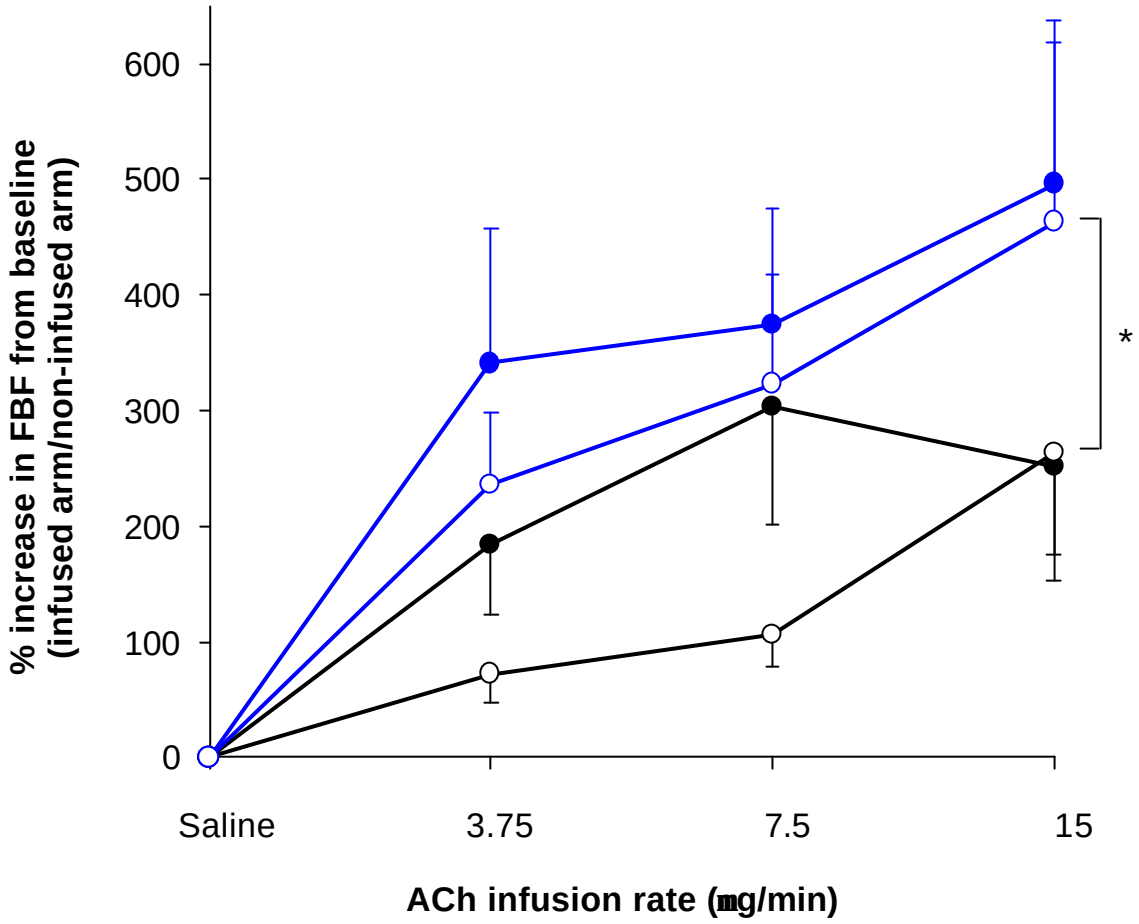


Figure 5.4 Forearm blood flow responses to increasing doses of ACh on day 5 of placebo (black) or fludrocortisone 0.8 mg/day (blue) pre-LNMMA (closed symbols) and post-LNMMA (open symbols) (n = 6). The area under the dose-response curves between placebo and fludrocortisone treatments to ACh was compared pre- and post-LNMMA. Responses to ACh were greater post-LNMMA in fludrocortisone-treated than in placebo treated subjects * $p < 0.05$.

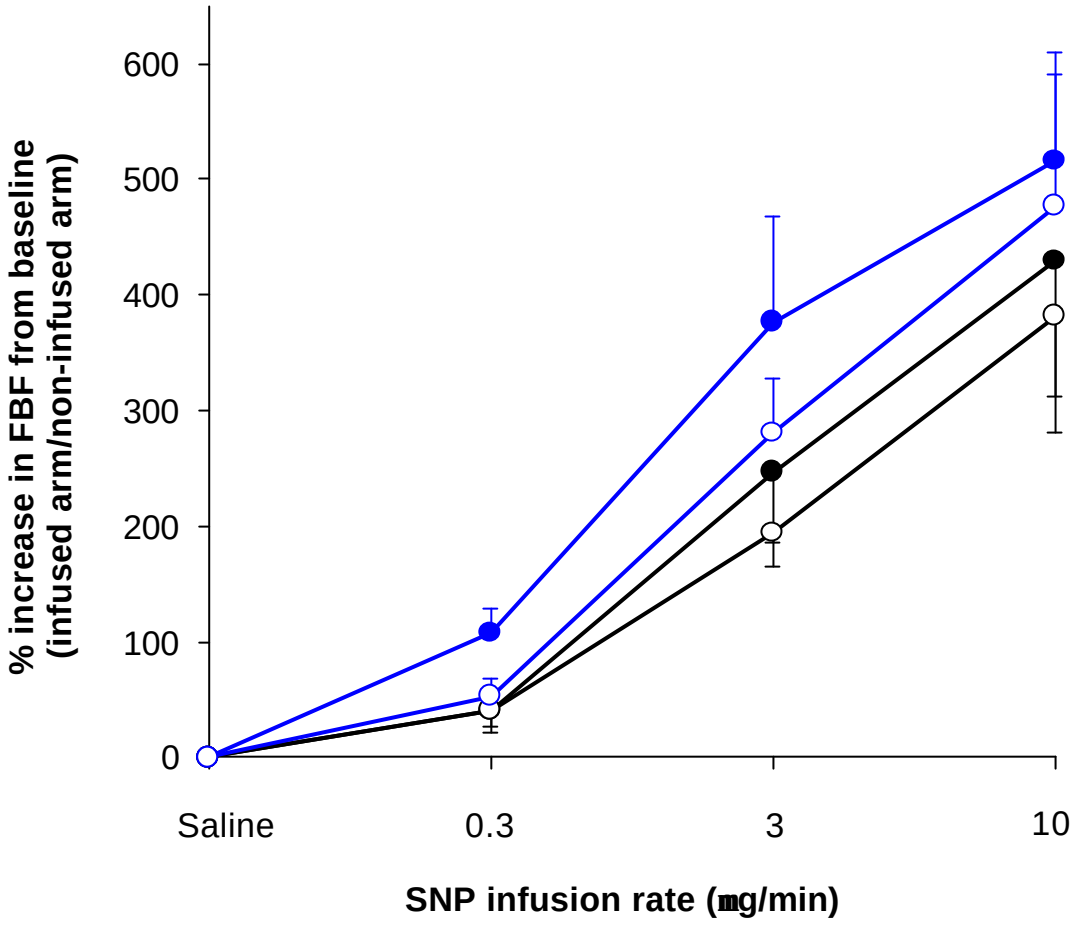


Figure 5.5 Forearm blood flow responses to increasing doses of SNP on day 5 of placebo (black) or fludrocortisone 0.8 mg/day (blue) pre-LNMMA (closed symbols) and post-LNMMA (open symbols) (n = 6). The area under the dose-response curves between placebo and fludrocortisone treatments was compared pre- and post-LNMMA. The dose-response curves did not significantly differ from each other.

Table 5.1 Metabolic effects of placebo for 5 days in healthy male subjects (n=6)

	Control	Treatment day 5	p value
Body weight (kg)	75.6 ± 3.6	75.7 ± 3.8	n.s
Serum Na (mmol/l)	142 ± 1	141 ± 1	n.s
Serum K (mmol/l)	4.3 ± 0.1	4.1 ± 0.1	n.s
Albumin (g/l)	43 ± 1	42 ± 1	n.s
Ca (corrected) (mmol/l)	2.29 ± 0.3	2.27 ± 0.02	n.s
PO_4 (mmol/l)	1.1 ± 0.1	1.1 ± 0.1	n.s
BSL (mmol/l)	4.9 ± 0.2	4.9 ± 0.2	n.s
Cholesterol (mmol/l)	4.2 ± 0.5	4.2 ± 0.5	n.s
Triglycerides (mmol/l)	1 ± 0.2	1 ± 0.2	n.s
Cr (mmol/l)	0.09 ± 0.01	0.09 ± 0.01	n.s
Urea (mmol/l)	5.3 ± 0.4	5.7 ± 0.8	n.s
Haematocrit	0.431 ± 0.003	0.419 ± 0.007	n.s
Platelets (*10^9/l)	201 ± 8	199 ± 10	n.s
Leukocytes (*10^9/l)	3.7 ± 0.3	3.9 ± 0.4	n.s
Neutrophils (*10^9/l)	1.8 ± 0.2	1.8 ± 0.2	n.s
Eosinophils (*10^9/l)	0.2 ± 0.04	0.26 ± 0.05	n.s
Serum cortisol (nmol/l)	307 ± 38	273 ± 45	n.s
NO_2/NO_3 (mmol/l)	20.4 ± 3.1	18.4 ± 2.5	n.s
Plasma renin activity (pg/ml)	16.9 ± 4.1	13.6 ± 1.7	n.s

Table 5.2 Metabolic effects of fludrocortisone (0.8 mg/day) for 5 days in healthy male subjects (n=6)

	Control	Treatment day 5	p value
Body weight (kg)	75.9 ± 3.7	77.6 ± 3.7	< 0.05
Serum Na (mmol/l)	142 ± 1	145 ± 1	< 0.01
Serum K (mmol/l)	4.4 ± 0.1	3.7 ± 0.1	< 0.001
Albumin (g/l)	42 ± 1	39 ± 1	< 0.001
Ca (corrected) (mmol/l)	2.29 ± 0.02	2.27 ± 0.03	n.s
PO_4 (mmol/l)	1.06 ± 0.08	0.99 ± 0.05	n.s
BSL (mmol/l)	4.6 ± 0.2	5.1 ± 0.2	n.s
Cholesterol (mmol/l)	4.2 ± 0.6	3.7 ± 0.3	n.s
Triglycerides (mmol/l)	0.9 ± 0.2	0.8 ± 0.1	n.s
Cr (mmol/l)	0.09 ± 0.01	0.08 ± 0.01	n.s
Urea (mmol/l)	5.2 ± 0.4	4.5 ± 0.6	n.s
Haematocrit	0.414 ± 0.009	0.385 ± 0.008	< 0.01
Platelets (*10^9/l)	198 ± 6	201 ± 9	n.s
Leukocytes (*10^9/l)	4.4 ± 0.5	4.4 ± 0.6	n.s
Neutrophils (*10^9/l)	2.3 ± 0.4	2.6 ± 0.5	n.s
Eosinophils (*10^9/l)	0.23 ± 0.08	0.18 ± 0.05	n.s
Serum cortisol (nmol/l)	311 ± 56	231 ± 43	= 0.05
NO_2/NO_3 (mmol/l)	28.5 ± 4.3	16.4 ± 3	< 0.01
Plasma renin activity (pg/ml)	11.7 ± 1.7	3.2 ± 0.5	< 0.01

Table 5.3 Effects of fludrocortisone (0.8 mg/day) or placebo on baseline forearm blood flow pre- and post-LNMMA (n=6)

		Supine BP (mmHg)	Basal FBF (ml/100 ml/min)	LNMMA FBF (ml/100ml/min)
Placebo	**Infused**	122±8 / 53±4	7.1 ± 2.4	5.6 ± 0.1
	Non-infused		4.7 ± 0.5	5.1 ± 0.8
Fludrocortisone	**Infused**	135±5* / 61±3	5.3 ± 1.2	6.1 ± 1.3
	Non-infused		5.9 ± 1.1	6.6 ± 1.2

Blood pressure was recorded by oscillometric method as described. Calf SBP was significantly higher in the fludrocortisone phase compared with placebo. Fludrocortisone administration had no significant effect on baseline FBF. In this study, LNMMA did not produce significant vasoconstriction from baseline FBF after five days of either treatment. * $p < 0.05$.

CHAPTER 6

Can the features of exogenous ACTH excess in the rat be explained by the adrenal production of corticosterone ?

6.1 INTRODUCTION

ACTH excess raises blood pressure in man, sheep, dog and rat though the mechanism of the hypertension may not be similar between species (Whitworth, Saines et al, 1983b, Scoggins, Allen et al, 1979, Lohmeier, 1982, Whitworth, Hewitson et al, 1990). In man, for example, the metabolic and haemodynamic features of ACTH-induced hypertension could be precisely reproduced by the oral or intravenous administration of cortisol, the major circulating glucocorticoid, at doses that produced plasma concentrations seen with ACTH treatment (Whitworth, Saines et al, 1984, Connell, Whitworth et al, 1987). ACTH does not raise blood pressure in humans with adrenal failure (Whitworth, Saines et al, 1983b). Hence, the blood pressure raising effects of ACTH in man can be attributed to the stimulated adrenal production of cortisol.

In sheep, intravenous administration of ACTH, at a rate as low as 1 μg/kg/day, raises blood pressure with increases in blood pressure observed within 8-10 hours (Scoggins, Coghlan et al, 1982). The associated metabolic changes include initial (24-48 hours) urinary sodium retention, hypernatraemia and hypokalaemia without any change in urinary potassium excretion and an increase in urine output and water intake (Scoggins, Coghlan et al, 1974). The metabolic effects of ACTH could be reproduced

exactly by the infusion of the five major ovine adrenal steroids; aldosterone, cortisol, 11-deoxycortisol, corticosterone and deoxycorticosterone. In contrast to man, however, the hypertensive effect of ACTH was not reproduced by this combination, unless 17α-hydroxyprogesterone and 17α20α-dihydroxyprogesterone were included (Coghlan, Denton et al, 1976). Because neither of these steroids exhibited classical glucocorticoid or mineralocorticoid activity in vitro or in vivo, a hypertensinogenic class of steroid hormone action was proposed (Soding, Coghlan et al, 1983, Butkus, Congiu et al, 1982).

In the male SD rat, Whitworth et al examined the features of exogenous ACTH excess in 1989 (Whitworth, Hewitson et al, 1990). ACTH (0.5 mg/kg/day) increased SBP by approximately 27 mmHg over the treatment period of 14 days. In addition, ACTH produced metabolic effects including decrease in body weight, increased water intake and urine output, increased plasma sodium and urinary sodium excretion and decreased plasma potassium and increased urinary potassium excretion. ACTH increased adrenal, renal, cardiac and brain weights with no significant effects on vascular morphology. The adrenal zona glomerulosa was abolished in the ACTH-treated animals. In addition, adrenalectomy prevented the metabolic and haemodynamic effects of ACTH, confirming the adrenal dependence of the above in ACTH-induced hypertension.

ACTH-induced hypertension in the rat is characterized by increases in blood pressure observed by day two of treatment (Turner, Wen et al, 1996, Turner, Wen et al, 1998). The threshold dose for the development of the hypertension has been reported as 1 μg/kg/day (Turner, Wen et al, 1998). In association with the hypertension and metabolic changes, serum corticosterone concentration was elevated in animals treated with 50-500 μg/kg/day of ACTH.

Unlike dexamethasone-induced hypertension in the rat, ACTH-induced hypertension was prevented by the co-administration of oral L-, but not D-arginine, suggesting a role for the nitric oxide pathway in this model (Turner, Wen et al, 1996, Li, Fraser et al, 1997). Recently, Li et al have shown that ACTH-induced hypertension in the rat is not prevented by type 1 receptor blockade (by RU486) or type II receptor blockade (by spironolactone) (Li, Wen et al, 1999). Data from this and a previous study (Li, Wong et al, 1994) are consistent with notion that ACTH raises blood pressure by mechanisms that are, at least in part, independent of classical mineralocorticoid and glucocorticoid activities.

Wen and co-workers examined the regional haemodynamics of this model of hypertension by use of a transonic flowmeter (Wen, Fraser et al, 1998). ACTH increased cardiac output and renal vascular resistance but did not affect calculated total peripheral resistance or hindquarter or mesenteric blood flow. The effect of the addition of dietary L-arginine supplementation

was to augment the rise in cardiac output, prevent the rise in renal vascular resistance and prevent the hypertension.

It is not known, however, whether ACTH-induced hypertension in the rat can be explained by adrenal production of corticosterone, the major circulating glucocorticoid in the rodent. Turner et al have shown a clear dose-response relationship between ACTH administered over 10 days and corticosterone (Turner, Wen et al, 1998). A number of other studies have examined the relationship between ACTH, corticosterone, aldosterone, other steroids and hypertension in the rat. Vazir and co-workers administered ACTH (10 IU/day, 100 μg/day) to male SD rats for 16 days and measured blood pressure and the concentrations of 5 major steroids; corticosterone, 18-OH-corticosterone, 18-OH-DOC, aldosterone and DOC. In this study, ACTH markedly suppressed adrenal production of aldosterone and 18-OH-corticosterone and increased corticosterone and 18-OH-DOC production, in association with a rise in systolic blood pressure of around 40 mmHg (Vazir, Whitehouse et al, 1981). The authors concluded that aldosterone and 18-OH-corticosterone were unlikely to mediate ACTH-induced hypertension and that 18-OH-DOC and corticosterone were likely to be contributing. In this study, sodium intake had no effect on ACTH-induced increases in blood pressure or sodium balance. Freeman and co-workers, however, found that low-dose ACTH infusion (1 IU/day) over 7 days increased both aldosterone and corticosterone in association with positive (but reduced when compared to controls) sodium and potassium balance (Freeman, Davis et al, 1980). The

discrepancies in the above studies probably relate to the transient effect of ACTH on aldosterone excretion.

Earlier, Haack et al had examined the differences between corticosterone and DOCA hypertension in the rat (Haack, Mohring et al, 1977). Both steroids raised systolic blood pressure to a similar degree, about 20 mmHg over 5 days. Whereas DOCA induced renal sodium and water retention, corticosterone caused negative sodium and water balance. Both drugs increased extracellular fluid volume and plasma volume, suggesting that corticosterone shifted fluid from the intracellular to extracellular compartment. In this study, the authors chose a dose of corticosterone (40 mg/kg/day) that induced negative sodium and water balance and increased serum corticosterone concentration to high physiologic (742 ng/ml) levels, after a single subcutaneous injection.

A number of steroids have been suggested in the mechanism of adrenal regeneration hypertension, an ACTH-dependent model, eg DOC, corticosterone, 19-nor-DOC, but the precise mechanism of the hypertension is not understood (Grekin, Dale et al, 1972, Scoggins, Denton et al, 1984).

Hence there are many detailed studies on ACTH-dependent adrenocortical steroid hormone production and ACTH-induced hypertension in the rat, and much data on the sodium-dependent model of DOC hypertension. Whether the adrenal production of corticosterone accounts for the features of ACTH-

induced hypertension in the rat has not been resolved. The aim of this experiment was to address this issue.

A dose-response study was initially performed. To further characterize this model of hypertension, rats were treated with corticosterone in addition to oral L- or D- arginine. In a final experiment, a combination of oral L-arginine, NOLA and corticosterone were administered to determine the effects of a competitive antagonist of nitric oxide synthase on corticosterone-induced hypertension.

6.2 METHODS

The general methods used in this study have been described in Chapter 2.

6.2.1 STUDY 1: subcutaneous infusion of low dose corticosterone by mini-osmotic pump - dose-response study

In this experiment, the effect of low dose infusion of subcutaneous corticosterone was examined in the male SD rat. The kinetics of corticosterone were determined from published data (see section 2.1.7), and corticosterone infused at calculated dose rates appropriate to achieve blood concentrations of corticosterone similar to those found under conditions of ACTH stimulation (Turner, Wen et al, 1996). Forty male SD rats were randomly divided into four groups;

Group 1: - Sham (vehicle): - PEG 400 0.5 microlitres/hour by subcutaneous mini-osmotic pump.

Group 2: - Low dose corticosterone infusion: - corticosterone 6.75 μg/hr in PEG 400 by subcutaneous mini-osmotic pump.

Group 3: - Mid-dose corticosterone infusion: - corticosterone 11.63 μg /hr in PEG 400 by subcutaneous mini-osmotic pump.

Group 4: - High dose corticosterone infusion: - corticosterone 16.5 μg /hr in PEG 400 by subcutaneous mini-osmotic pump.

6.2.1.1 Study 1: experimental protocol

Blood pressure and metabolic parameters were measured for 4 control days (C1-4). The pumps were surgically implanted on treatment day 0 (T0), after the measurement of systolic blood pressure (ie three control measurements of systolic blood pressure, C1, C3 & T1). Blood pressure and metabolic parameters were then measured on alternate days for a further 14 days. Animals were allowed a recovery day after surgery, therefore there are no data for metabolic parameters for T1, ie the 24 hour period following the surgery. Animals were sacrificed on T14 and blood collected for corticosterone and ACTH assays and a kidney, adrenal and the heart were weighed.

6.2.1.2 Subcutaneous mini-osmotic pumps

In the pilot corticosterone study, 4 groups of SD rats were administered corticosterone by mini-osmotic pump implanted subcutaneously (Alzet Model

2002, ALZA Corporation, California USA). The length of these pumps is 3 cm and the mean volume of the lot was 226 μl. The mean pumping rate was 0.51 μg per hour. This pump is designed to expel 0.5 μg per hour for 14 days.

The dose of corticosterone to administered was determined by the metabolic clearance rate for corticosterone in the rat, and the known concentration of corticosterone achieved in ACTH-induced hypertension in the rat. The metabolic clearance rate is defined as follows;

MCR (μl/min)= plasma concentration / production rate

Several authors have reported the MCR of corticosterone in the rat, however, there is a wide variation between strains and sexes (White, Corll et al, 1989, Woodward, Hervey et al, 1991, Waddell and Atkinson, 1994). The MCR falls during infancy (Leeper, Schroeder et al, 1988) and may also decrease with fasting (Woodward, Hervey et al, 1991) and possibly pregnancy (Waddell and Atkinson, 1994). In this study, I used the MCR reported by Woodward et al, in non-fasted crossed Wistar/Lister male rats, approximately the same weight as those I used (MCR = 0.5 ml/min) (Leeper, Schroeder et al, 1988).

The aim of the pilot study was to achieve circulating concentrations of corticosterone similar to those achieved under conditions of adrenal stimulation, as described in ACTH-induced hypertension (Turner, Wen et al,

1998). At a dose of ACTH of 50 μg/day, serum corticosterone was increased to about 800 ng/ml, from a baseline of approximately 350 ng/ml in association with a rise in systolic blood pressure of about 20 mmHg (Turner, Wen et al, 1998). Using a desired concentration of corticosterone of 800 ng/ml and MCR = 0.5 ml/min, the calculated secretion rate from the mini-osmotic pumps was 0.58 mg/day and 8.06 mg over 14 days.

The amount of corticosterone administered, however, was limited by the solubility of corticosterone in the solvent, and the maximum internal volume of the available mini-osmotic pumps (226 μgs). Polyethylene glycol (average molecular weight 400, Sigma chemicals) was used as the solvent because of its considerable ability to dissolve steroids and that it has been shown to be a satisfactory solvent for these pumps. The maximum solubility of corticosterone in polyethylene glycol is 26 g/L (Will, Cortright et al, 1980). Corticosterone powder (Sigma chemicals) was dissolved as follows: 200 mg of corticosterone was dissolved in 7.692 mls of polyethylene glycol with a magnetic stirrer and heated to 60^{o}C (Will, Cortright et al, 1980). This produced a (saturated) corticosterone solution of 26 mg/ml, which was used for the high dose group (group 4). Two hundred twenty microlitres of this solution containing 5.2 mg corticosterone was injected carefully into each of 10 mini-osmotic pumps for the highest dose group. The remaining solution was then diluted conveniently to produce two lower dose groups. Group 1 received the vehicle, polyethylene glycol only. The remainder of the mini-osmotic pumps were filled with the appropriate solution.

As this study resulted in no measurable haemodynamic effects attributable to the drug and no significant effect on serum corticosterone concentration, in the next series of experiments the doses of corticosterone administered (subcutaneously) were considerably higher, determined by doses previously published which increased blood pressure (Haack, Mohring et al, 1977).

6.2.2 STUDY 2: Subcutaneous administration of corticosterone by injection - dose-response study

As there were no discernable haemodynamic or metabolic effects of corticosterone in study one, the doses of corticosterone administered in this study (10-40 mg/kg/day) overlapped those used by Haack et al in 1977 (20 - 40 mg/kg/day) to produce a plasma corticosterone concentration of approximately 750 ng/ml (Haack, Mohring et al, 1977); ie similar to that achieved under conditions of ACTH stimulation (ACTH 50 μg/kg/day) (Turner, Wen et al, 1998). Forty male SD rats were randomly divided into four groups as follows;

Sham (n=10): - vehicle (PEG 400) 0.5 ml/kg/day by s/c injection in divided doses.

Corticosterone 10 (n=10): - corticosterone 10 mg/kg/day in PEG 400 by s/c injection in divided doses.

Corticosterone 20 (n=10): - corticosterone 20 mg/kg/day in PEG 400 by s/c injection in divided doses.

Corticosterone 40 (n=10): - corticosterone 40 mg/kg/day in PEG 400 by s/c injection in divided doses.

6.2.2.1 Study 2: experimental protocol

There were 4 control (C1-C4) and 15 treatment (T0-T14) days. Systolic blood pressure and metabolic parameters were measured on alternate days, and subcutaneous administration of either sham or corticosterone began after systolic blood pressure recordings on T0, ie there were three control days of blood pressure measurement. At sacrifice, organs (heart, a kidney and an adrenal) were weighed and serum collected for corticosterone estimation and total concentration of the end products of nitric oxide metabolism, NO_2/NO_3 (NOx).

6.2.3 STUDY 3: Effects of L-arginine, D-arginine and NOLA on corticosterone - induced hypertension

L-arginine is an essential substrate for the gas, nitric oxide. Nitric oxide, now recognised to play a critical role in blood pressure regulation as well as many other diverse physiological processes, is produced by the enzymatic action of several widely distributed nitric oxide synthases. D-arginine does not act as a substrate for this group of enzymes. Several methylated derivatives of L-arginine have been reported to act as competitive antagonists for the nitric oxide synthases, including NOLA. I hypothesized that L- but not D- arginine would prevent the rise in blood pressure produced by corticosterone, and that NOLA would reverse the L-arginine effect. To examine the effects of L-arginine, D-arginine and L-arginine + NOLA on corticosterone - induced hypertension, sixty male SD rats were randomly divided into 5 groups as follows;

Sham (n=15) - vehicle (PEG 400) 0.5 ml/kg/day by s/c injection in divided doses.

Corticosterone 20 mg/kg/day (n=15): - corticosterone 20 mg/kg/day in PEG 400 by s/c injection in divided doses.

Corticosterone + L-arginine (n=10): - corticosterone 20 mg/kg/day in PEG 400 by s/c injection in divided doses + oral L-arginine 0.6 % in food.

Corticosterone + D-arginine (n=10): - corticosterone 20 mg/kg/day in PEG 400 by s/c injection in divided doses + oral D-arginine 0.6 % in food

Corticosterone + L-arginine + NOLA (n=10): - corticosterone 20 mg/kg/day in PEG 400 by s/c injection in divided doses + oral L-arginine 0.6 % in food + NOLA ≈30 mg/kg/day in the drinking water.

6.2.3.1 Study 3: experimental protocol

There were four control (C1-C4) and 11 treatment (T0-T10) days. In this experiment, animals were sacrificed on T10 rather than T14 because of data obtained from study two, demonstrating no significant change in blood pressure from T10 to T14, ie blood pressure plateaued at T10. Systolic blood pressure and metabolic parameters were measured on alternate days. Subcutaneous injections of either sham or corticosterone commenced on the morning of T0, following measurement of systolic blood pressure (ie three control measures of systolic blood pressure). At sacrifice, blood was collected for measurement of plasma NOx and organs collected to be weighed.

Normal rat food was substituted during the treatment period (treatment days 0 to 10) for the L-arginine chow (groups 3 & 5) or the D-arginine chow (group 4) in Study 3 of the corticosterone experiments (see Chapter 6). Tap water was used for the corticosterone study. For group 5 of Study 3, NOLA was added to the water as described in section 2.2.2.4.

6.3 RESULTS

6.3.1 RESULTS - Study 1: Effect of low dose continuous infusion of corticosterone - dose - response study

6.3.1.1 Systolic blood pressure (Figure 6.1)

Baseline systolic blood pressures (pooled control of C1, C3 and T0) were similar between the four groups. Sham infusion (group 1) significantly increased systolic blood pressure over the treatment period (122 1 to 132 ± 3 mmHg, $p < 0.05$, ANOVA). There was a similar effect in each of the other groups, as systolic blood pressure increased in the low dose infusion group (123 1 to 134 2 mmHg, $p = 0.06$), the mid-dose group (124 2 to 135 4 mmHg, $p<0.0.01$) and the high dose group (122 1 to 134 4 mmHg, $p < 0.05$). There was no significant effect of corticosterone beyond the effect attributable to polyethylene glycol.

6.3.1.2 Metabolic effects of subcutaneous corticosterone infusion (Table 6.1)

Minipumps were explanted at sacrifice and examined to ensure malfunction had not occurred.

In the sham treated group, there was an increase in body weight from 263 ± 3 to 327 5 g ($p < 0.001$) with no change in 24 hour water intake or urine volume. There was a small but significant rise in food intake over the treatment period, from 24 1 to 30 1 g (T11) and 28 1 g (T13), $p < 0.01$.

Corticosterone treatment had no significant effect on the increase in body weight in any of the treatment groups. There was also no change in urine output secondary to corticosterone. There was a transient increase in water intake in the low-dose corticosterone infusion group on treatment day 9 (33 ± 2 (PC) to 38 2 ml / 24 hr (T9), $P < 0.05$) which was not sustained on T11 or T13. This effect on water intake was not observed in the mid- or high- dose corticosterone infusion groups, in which there was also no change in water intake. Food intake increased slightly in the low dose group (24 2 to 29 1 g / 24 hr, $P < 0.01$) with no significant changes recorded in the mid- and high dose corticosterone treated groups.

6.3.1.3 Serum corticosterone and plasma ACTH concentrations (Figure 6.2)

There was a trend for reduction in ACTH concentration at increasing infusion rates of corticosterone, suggesting partial suppression of the hypothalamic-pituitary axis. Similarly, the serum corticosterone concentration was slightly but significantly lower in the higher dose infusion group (474 28 v 371 25 ng/ml, group 1 v 4, $p < 0.05$ by ANOVA and t-test).

6.3.1.4 Organ weights at sacrifice (Table 6.2)

As illustrated in Table 5.3, there were no effects of low dose corticosterone on the weight of the adrenal, kidney or heart.

6.3.2 RESULTS - Study 2: Effect of corticosterone by subcutaneous injection - dose response study

6.3.2.1 Systolic blood pressure (Figures 6.3 & 6.4)

Pooled control systolic blood pressures between the four groups were similar. Systolic blood pressure increased over the treatment period in each of the four groups; sham group (116 3 to 128 4 mmHg, PC to T14, $p < 0.05$, RMANOVA); corticosterone 10 group (121 2 mmHg to 138 3 mmHg, $p < 0.001$), corticosterone 20 group (117 2 to 145 4 mmHg, $p <$ 001) and corticosterone 40 group (122 2 to 151 4 mmHg, $p < 0.001$).

The was a significant dose-dependent effect of corticosterone on systolic blood pressure, above that of sham injection (Fig 6.4, $p < 0.01$ for sham v corticosterone 20 group and $p < 0.05$ for sham v corticosterone 40 group, RMANOVA). There was no statistical difference over the treatment period in the sham v corticosterone 10 groups. Systolic blood pressures in groups 3 & 4 overlap suggesting that 20 mg/kg/day of corticosterone is at the top of the dose response curve.

Significant increases in systolic blood pressure were observed as early as treatment day 2 in corticosterone groups 20 and 40 ($p < 0.01$, $p < 0.001$, PC v T2, groups 3 & 4 respectively by Student's t-test). The rise in blood pressure plateaued by treatment day 10, with no significant change in blood pressure from T10 to T14 in any group.

6.3.2.2 Metabolic parameters (Table 6.3)

6.3.2.2.1 Body weight

Body weight increased in the sham group (300 4 to 342 5 g, PC to T13, $p < 0.001$, RMANOVA). There was also an increase in weight in the corticosterone 10 group (302 5 to 314 5 g, $p < 0.01$), which was significantly attenuated compared with the increase in the sham group ($p < 0.001$, sham v corticosterone 10, RMANOVA). Body weight fluctuated in the corticosterone 20 group, accounting for a cubic trend by analysis of variance, with no significant change by T13 from the control weight (294 5 to 287 7 g, PC v T13, t-test, p=n.s). Animals in the corticosterone 40 group lost weight during the treatment period (307 5 to 262 5 g, PC v T13, $p < 0.001$). The fall in weight in the corticosterone 40 rats was evident by T3 (307 5 g to 290 4 g, PC v T3, t-test, $p < 0.001$).

6.3.2.2.2 Twenty four hour water intake

Twenty four hour water intake fell slightly but significantly in the sham group (33 1 to 30 1 ml/24hr, PC to T13, $p < 0.05$, RMANOVA). There were no significant linear changes in any of the corticosterone treated groups. There was, however, a slight dip in water intake in the corticosterone 20 group on days T3 and T5 resulting in a quadratic trend for this parameter by analysis of variance.

6.3.2.2.3 Twenty four hour urine output

Twenty four hour urine output was unchanged in the sham and corticosterone 10 groups but increased as a result of treatment with

corticosterone 20 and 40 mg/kg/day (4 1 to 9 1 ml/24 hr, p < 0.001 and 5 1 to 13 1 . ml/24 hr, p < 0.01 respectively, RMANOVA). The effect of corticosterone on urine output was evident by T3 in the corticosterone 40 group (5 1 ml to 9 1 ml/24 hr, PC v T3, p < 0.05, t-test) and by T7 in the corticosterone 20 group (4 1 to 8 1 ml/24 hr, PC v T7, P < 0.05, t-test).

6.3.2.2.4 Twenty four hour food intake

There were fluctuations in food intake in each of the four groups, achieving statistical significance in the sham, corticosterone 10 and 40 groups by repeated measures analysis of variance. In the sham and corticosterone 10 groups, there was no linear change in food intake from the control period to the last treatment day (29 1 to 30 1 g/24 hr, PC to T13, p=n.s and 28 1 to 30 1 g/24 hr, p=n.s by t-test respectively). Food intake fell slightly in the corticosterone 40 group (31 1 to 27 1 g/24 hr, p < 0.05, t-test).

6.3.2.2.5 Serum corticosterone and plasma NOx at sacrifice (Table 6.4)

Serum corticosterone at sacrifice was 364 25 ng/ml in the sham group. There were dose-dependent changes in serum corticosterone, with reduction in the concentration of corticosterone in the corticosterone 10 group (364 ± 25 v 261 21 ng/ml, P < 0.01), no change in the corticosterone 20 group (376 37 ng/ml) and increased concentration in the corticosterone 40 group(364 25 v 502 20 ng/ml, P < 0.001).

Plasma NOx was 11.3 mmol/l in the sham group at sacrifice. There was trend for lower plasma NOx in all corticosterone treated groups, though this

achieved statistical significance in the comparison of sham with the corticosterone 20 group (11.3 v 7.7 0.9 mmol/l, $p < 0.05$) only.

6.3.2.2.6 Organ weights at sacrifice (Table 6.5)

There was no significant effect of corticosterone on the heart or kidney mass after 14 days. The adrenal gland, however, was of smaller mass in each corticosterone treated group, compared with sham ($p < 0.05$).

6.3.3 RESULTS - Study 3: Effects of L-arginine, D-arginine and NOLA on corticosterone -induced hypertension

6.3.3.1 Systolic blood pressure (Figure 6.5)

In this experiment, sham injection had no significant effect on systolic blood pressure over the treatment period (126 ± 2 to 129 ±2 mmHg, PC to T10, .p = n.s, RMANOVA) Corticosterone 20 mg/kg/day increased systolic blood pressure (126 ± 2 to 139 ± 3 mmHg, $P < 0.001$) and this effect was completely prevented by the addition of L-arginine to the food (131 ±3 to 131 ± 2 mmHg, p = n.s). The addition of D-arginine did not prevent corticosterone-induced increases in blood pressure (129 ± 3 to 142 4 on T8 and 135 ± 3 mmHg on T10, $P < 0.01$). The addition of NOLA to the drinking water blocked the effect of L-arginine and amplified the rise in blood pressure due to corticosterone (130 ± 3 to 171 ± 6 mmHg, $p < 0.001$).

6.3.3.2 Metabolic parameters (Table 6.6)

6.3.3.2.1 Body Weight

Body weight on C4 was similar for all groups. Body weight increased over the treatment period in the sham group (283 2 to 314 4 g, C4 to T9, $p < 0.001$, RMANOVA). Body weight was unchanged in the corticosterone 20 (284 2 to 285 4 g, p=n.s) and corticosterone + L-arginine (295 5 to 299 6 g, p=n.s) groups. There was a fall in body weight in the corticosterone + D-arginine group (290 5 to 280 5 g, $p < 0.01$) and the corticosterone + L-arginine + NOLA group (289 2 to 264 3 g, $P < 0.001$). The addition of NOLA significantly amplified the fall in weight during the treatment period (B 20 + D-arg v B 20 + L-arg + NOLA, $p < 0.001$).

6.3.3.2.2 Twenty four hour water intake

Twenty four hour water intake was similar in all groups in the control period. There were no changes in water intake in the corticosterone 20 group (31 1 to 33 2 ml/24 hrs, PC to T9, p=n.s, RMANOVA) or in the corticosterone + D-arginine group (32 2 to 36 3 ml/24 hr, p=n.s). Water intake slightly increased in the sham group (30 1 to 34 1 ml/24 hrs, $p < 0.05$) and in the corticosterone + L-arginine group (30 1 to 36 2 ml/24 hrs, $p < 0.05$) and to a larger extent in the corticosterone + L-arginine + NOLA group (26 2 to 37 3 ml/24 hrs, $p < 0.001$).

6.3.3.2.3 Twenty four hour urine output

Pooled control values for 24 hour urine output were dissimilar between groups ($p < 0.001$, ANOVA). There was no change in urine volume in the

sham group (8 1 to 9 1 ml/24 hrs, PC to T9, p = n.s, RMANOVA). In each corticosterone treated group, there was a significant linear increase in urine volume over the treatment period; corticosterone 20 (6 1 to 10 1 ml/24 hrs, $p < 0.001$), corticosterone + L-arginine (11 1 to 14 1 ml/24 hrs, $p < 0.05$), corticosterone + D-arginine (7 ± 1 to12 ± 1 ml/24 hrs, $p < 0.01$) and corticosterone + L-arginine + NOLA (5 ± 1 to 13 ± 2 ml/24 hrs, $P < 0.001$).

6.3.3.2.4 Twenty four hour food intake

Food intake during the control period varied between groups ($P < 0.001$, ANOVA). There was no change in food intake in the sham group (23 ± 1 to 20 ± 1 g/24 hrs, PC to T9, p=n.s, RMANOVA). In the corticosterone 20 and the corticosterone + L-arginine groups, food intake fell during treatment (23 ± 1 to 20 ± 1 g/24 hrs, $p < 0.001$ and 27 ± 1 to 21 ± 1 g/24 hrs, $p < 0.05$ respectively). There was a transient fall in food intake in the corticosterone + D-arginine group (22 ± 1 to 21 ± 1 g/24 hrs, $p < 0.001$) and the corticosterone + L-arginine + NOLA groups (19 ± 1 to 19 ± 1 g/24 hrs, $p < 0.001$) accounting for quadratic trends in these groups.

6.3.3.2.5 Plasma NOx at sacrifice (Table 6.7)

Plasma NOx concentrations tended to be lower in all steroid treated groups but were only significantly lower than sham in the group treated with corticosterone + L-arginine + NOLA.

6.3.3.2.6 Organ weights at sacrifice (Table 6.8)

There was a trend to reduction in adrenal mass at sacrifice in corticosterone-treated animals ($p < 0.05$, ANOVA), which was not statistically significant

after Hochberg's correction. There were no consistent trends in kidney or heart mass in the corticosterone-treated animals.

6.4 DISCUSSION

Exogenous corticosterone (40 mg/kg/day) raised blood pressure by approximately 30 mmHg over 14 days and produced metabolic effects including a fall in body weight, increase in urine output and a small reduction in food intake with no significant change in water intake. At this dose, serum corticosterone concentration (approximately 500 ng/ml) was higher than that at sacrifice in sham treated rats (approximately 360 ng/ml) and similar to that in ACTH-treated rats (between 5-50 micrograms ACTH /kg/day) in previous studies (Turner, Wen et al, 1998). The magnitude and time course of the rise in blood pressure, the metabolic effects and the increase in corticosterone concentration are similar to those achieved during exogenous ACTH administration suggesting that corticosterone is at least partly responsible for ACTH-induced hypertension in the rat. Not all of the features of ACTH excess were observed, however, as water intake did not significantly increase. This is probably because ACTH excess results in the secretion of many adrenal steroids (see section 6.1) with various metabolic effects, whereas, in this experiment, I administered only corticosterone.

In study one, low dose infusion of corticosterone had no measurable effect on blood pressure or metabolic parameters. Sham infusion with PEG, as well as the three doses of corticosterone, raised systolic blood pressure to a

similar degree, by a mechanism that has not been explored. In numerous previous reports of ACTH-induced hypertension in the SD rat, however, saline injection had no effect on systolic blood pressure (Li, Wen et al, 1999, Whitworth, Hewitson et al, 1990, Wen, Fraser et al, 1998, Turner, Wen et al, 1996), so the effect of PEG is probably a true physiological effect, rather than an effect related to growth or stress during the experiment.

It is likely that insufficient corticosterone was infused rather than there being a complete failure of absorption from the subcutaneous tissue, as subcutaneous injection raised blood pressure (in Studies 2 & 3). However, there may have been incomplete absorption of the drug from the subcutaneous space confounding the results. Failure of the minipump is unlikely as there were no pumps which had evidence of pump failure during the experiment. The value used to calculate the infusion rate was considered the most reasonable of those data published, however, the MCR for corticosterone has reported to vary widely, as discussed in section 6.2.1.2. It is of interest to note a reduction in serum corticosterone concentration in association with a trend to reduced ACTH concentration at increasing infusion rates in this experiment. Whether this reflects simple biological variation or whether there was partial suppression by the infused drug on the hypothalamic-pituitary axis, at the level of the hypothalamus, is not clear from the available data.

In study 2, sham injection again raised systolic blood pressure (by 12 mmHg). There was a definite dose-dependent effect of corticosterone on blood pressure as is evident in Figure 6.4. It is also clear from this figure that the increase in blood pressure in the corticosterone 20 and 40 groups was similar. Systolic blood pressure had peaked by T10, and possibly earlier. For these reasons, corticosterone 20 mg/kg/day over 10 days was administered in Study 3.

The increase in blood pressure was observed by the first measurement after corticosterone treatment had commenced, ie treatment day 2. This is typical of glucocorticoid-induced hypertension, in which the onset of hypertension is rapid, as compared with mineralocorticoid hypertension, which may take weeks to develop.

The metabolic effects of corticosterone injection included reduction in body weight, increased urine output and inconsistent changes in food intake. The fall in body weight occurred only in the group which received 40 mg/kg/day corticosterone, with attenuation of weight gain occurring in the 10 mg/kg/day group and fluctuation in weight in the group which received 20 mg/kg/day. Increases in urine output were observed in the corticosterone 20 and 40 mg/kg/day groups, with no corresponding increase in water intake. Hence, at least some of the weight loss in the corticosterone groups could be attributed to negative water balance.

In Study 2, corticosterone-treated animals had dose-dependent increases in serum corticosterone concentration. The concentration of corticosterone in sham treated animals, however, was similar to that in the corticosterone 20 group. This can probably be explained by the stress that the animals were subjected to on sacrifice day. After blood pressure measurement, the rats were injected with the respective drug, then anaesthetised with intraperitoneal pentobarbital, then the neck was opened and carotid artery cannulated prior to the rat being killed by exsanguination. It is possible that the control (sham) rats had a significant stress response, which the steroid treated rats were unable to mount, due to a suppressed hypothalamic-pituitary axis. Indeed, the baseline corticosterone concentration in unstressed rats from other reports is of the order of 20-130 ng/ml, significantly lower than the concentrations in these studies (Akana, Cascio et al, 1985, Haack, Mohring et al, 1977).

Plasma NOx, metabolites of nitric oxide, were slightly but not significantly reduced in two of three corticosterone-treated groups, and there was a significant reduction in NOx in the corticosterone 20 group, compared with sham. This is similar to data from other studies of ACTH-induced hypertension in the rat (Turner, Wen et al, 1996) and from human studies of cortisol-induced hypertension and in keeping with the notion that suppression of the nitric oxide system contributes to glucocorticoid-induced hypertension (Kelly, Mangos et al, 1998).

In Study 3, corticosterone-induced increases in blood pressure were prevented by the co-administration of L-, but not D-arginine. Turner et al reported similar findings in ACTH-induced hypertension in the rat in 1996 (Turner, Wen et al, 1996). Li et al, however, were unable to prevent dexamethasone-induced hypertension in the rat using a similar protocol. It appears that ACTH and dexamethasone may raise blood pressure by differing mechanisms. Saruta et al have reported evidence implicating a key role for angiotensin II in dexamethasone-induced hypertension (Saruta, 1996). The renin-angiotensin system does not appear to contribute to ACTH-induced hypertension (see section 2.3.6.10).

The effect of arginine supplementation was stereospecific in this experiment and therefore the implication is that production was restored by the increased availability of substrate for nitric oxide synthase. L-arginine, however, may lower blood pressure by other proposed mechanisms, including suppression of the renin-angiotensin system and possibly by acting as a scavenger for radicals involved in the inactivation of nitric oxide, allowing existing nitric oxide a longer half life (MacAllister, Calver et al, 1995). Co-administration of L-arginine in food and NOLA in drinking water resulted in a profound increase in blood pressure, beyond that due to corticosterone itself. This effect has also been observed in ACTH-induced hypertension in the rat, and probably reflects overwhelming competitive inhibition of nitric oxide synthase by NOLA (Li, Dusting et al, 1992). The reduced NOx concentration in this group supports this notion.

In summary, the haemodynamic features of ACTH-induced hypertension were reproduced by corticosterone excess, with concentrations of corticosterone similar to those achieved in studies of exogenous ACTH administration. These include dose-dependent hypertension, reversed by L- but not D- arginine and a profound inhibition of the antihypertensive effect of L-arginine by NOLA. In addition, the dose-dependent metabolic effects of corticosterone on body weight and urine output were similar to those observed in ACTH-induced hypertension, as were the effects of corticosterone on plasma NOx.

6.5 CONCLUSIONS

This study shows that it is likely that adrenal production of corticosterone accounts for the features of ACTH-induced hypertension in the rat. Thus the major adrenal glucocorticoid (cortisol in man and corticosterone in the rat) appears to be responsible for ACTH-induced hypertension in both species.

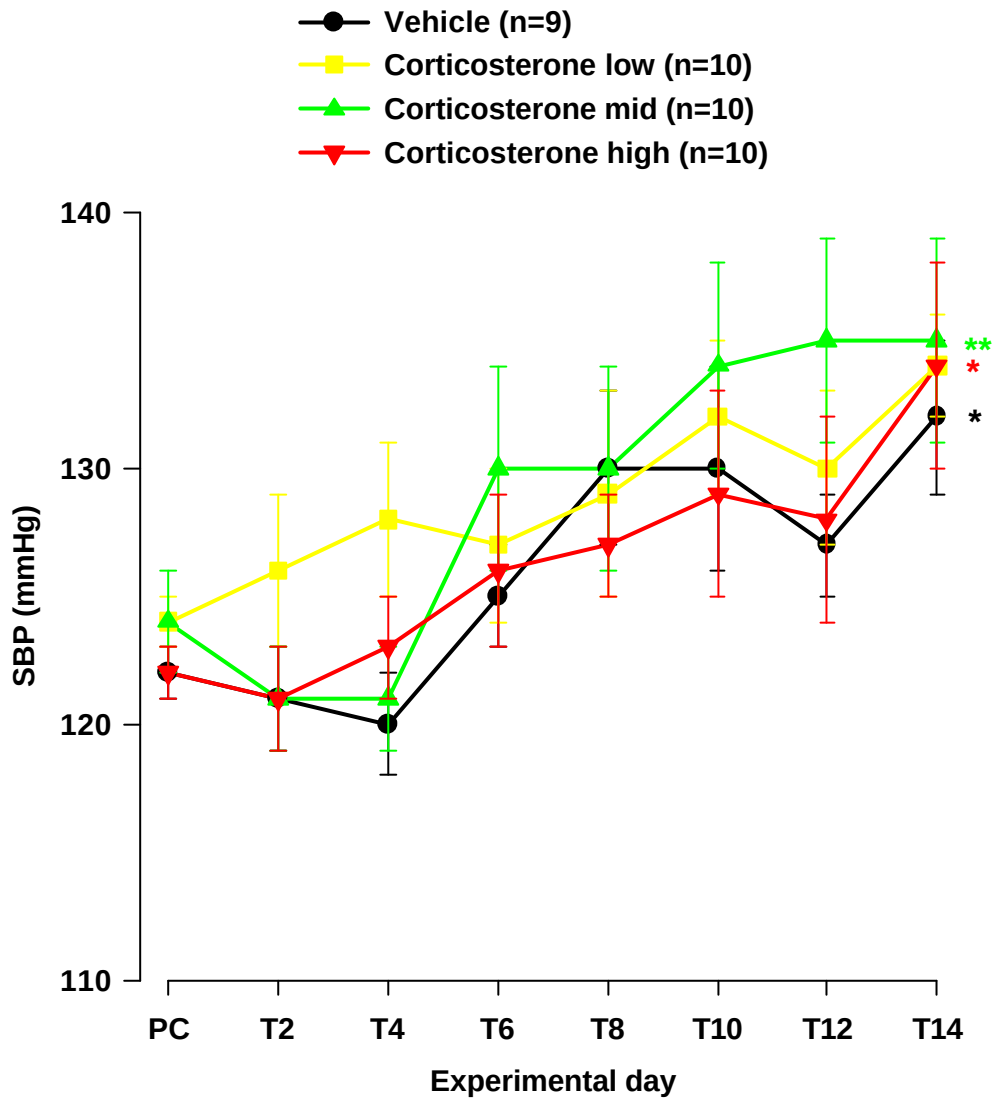


Figure 6.1 (Study 1) Systolic blood pressure of male SD rats treated with sham infusion or low, mid- or high dose corticosterone infusion by subcutaneous miniosmotic pump over fourteen treatment days (* p<0.05, ** p<0.01, RMANOVA).

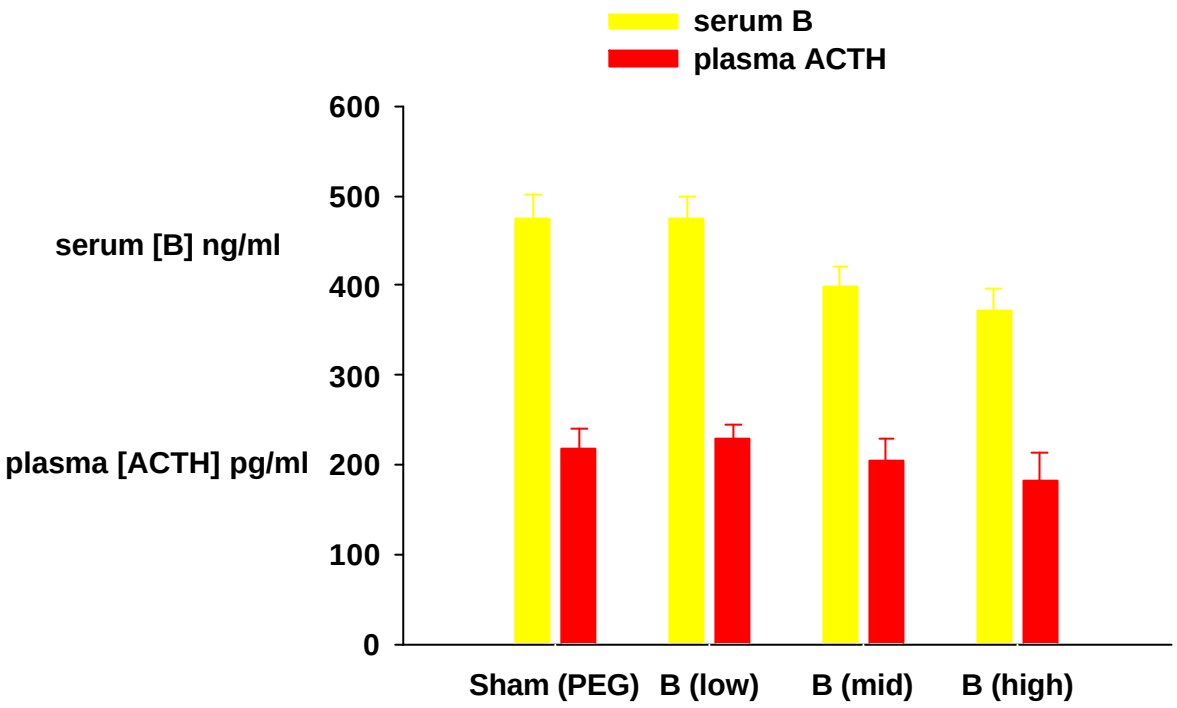


Figure 6.2 (Study 1) Effect of sham infusion and low, mid- and high dose corticosterone infusion on serum corticosterone and plasma ACTH concentrations at sacrifice.

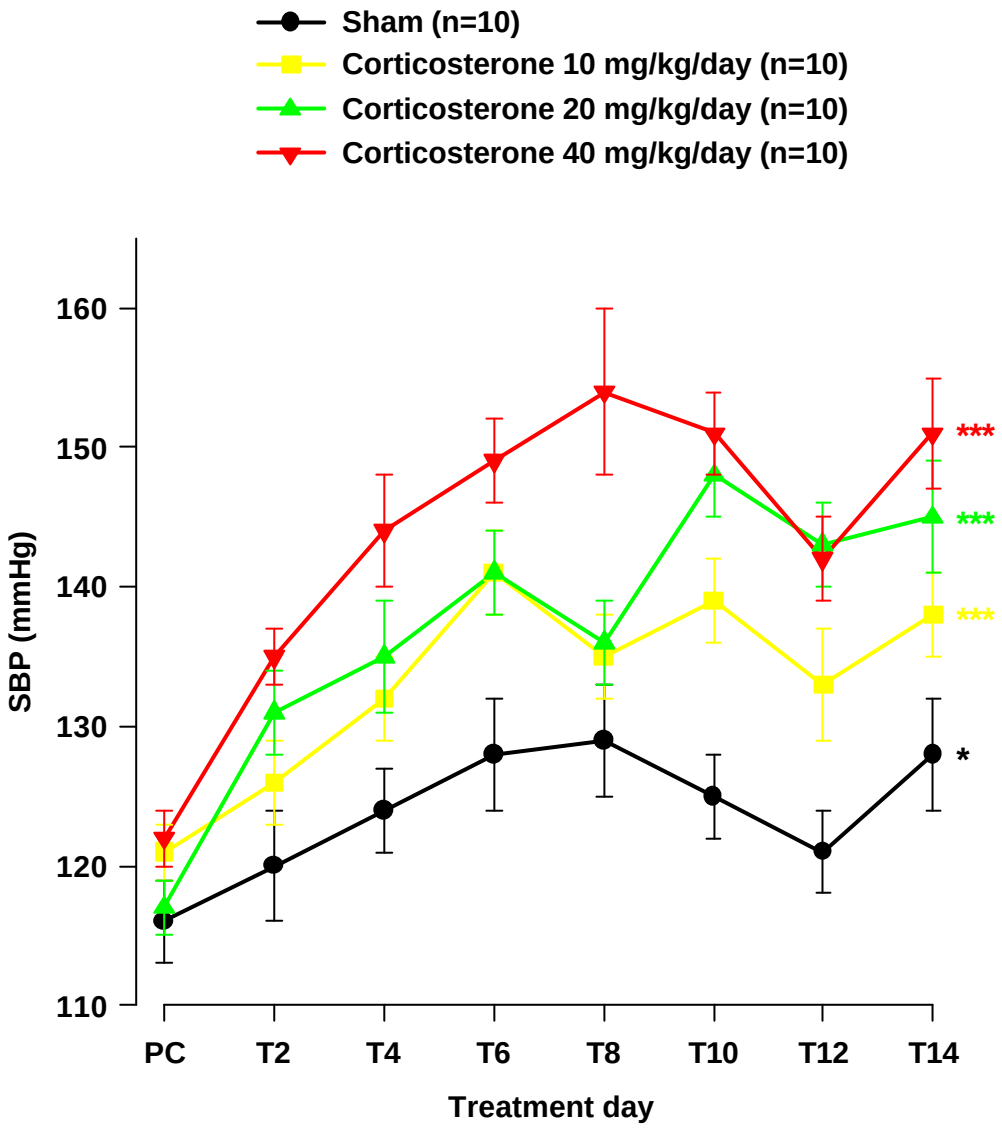


Figure 6.3 Haemodynamic effects of sham (PEG) or corticosterone (10, 20 or 40 mg/kg/day) by subcutaneous injection (* p<0.05, *** p<0.001 by one-way RMANOVA).

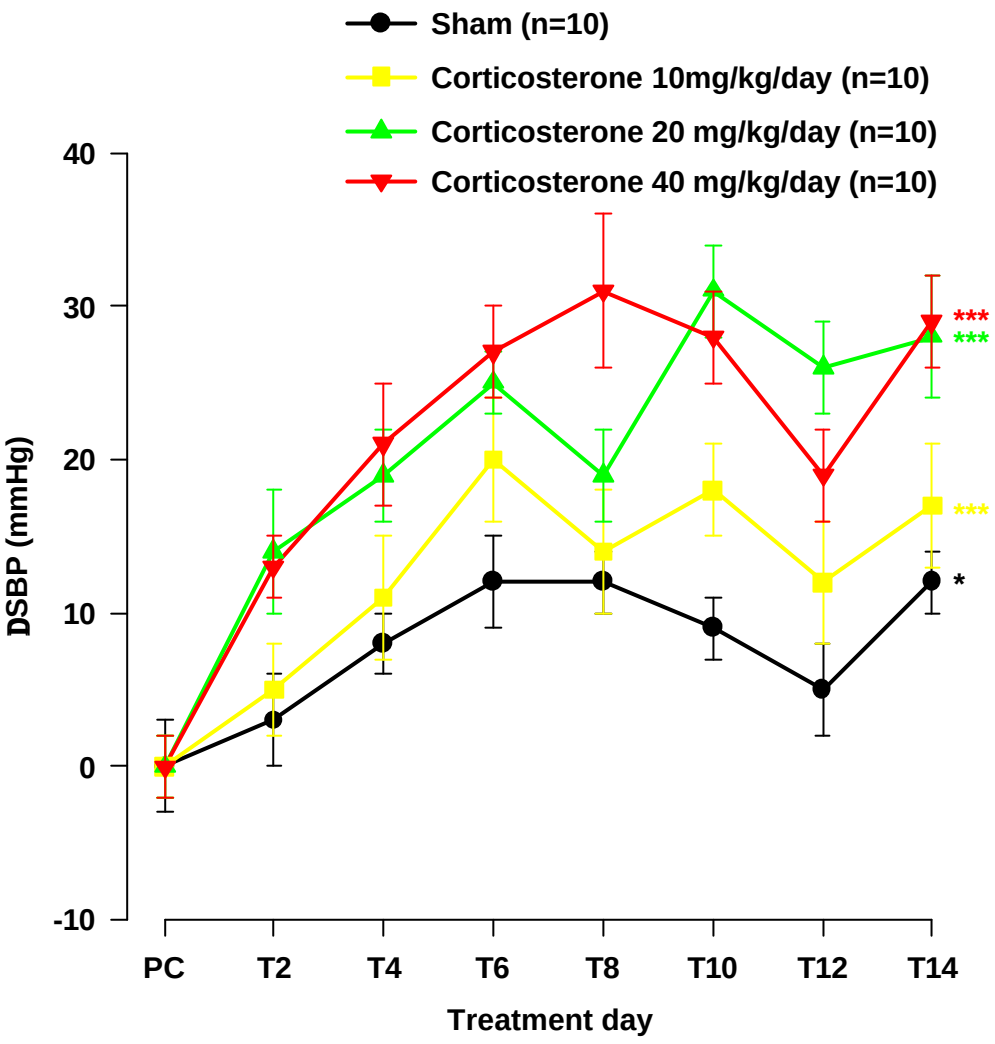


Figure 6.4: Haemodynamic effects of sham (PEG) or corticosterone (10, 20 or 40 mg/kg/day) by subcutaneous injection, plotted as change in SBP from PC (* p<0.05, *** p<0.001 by one-way RMANOVA).

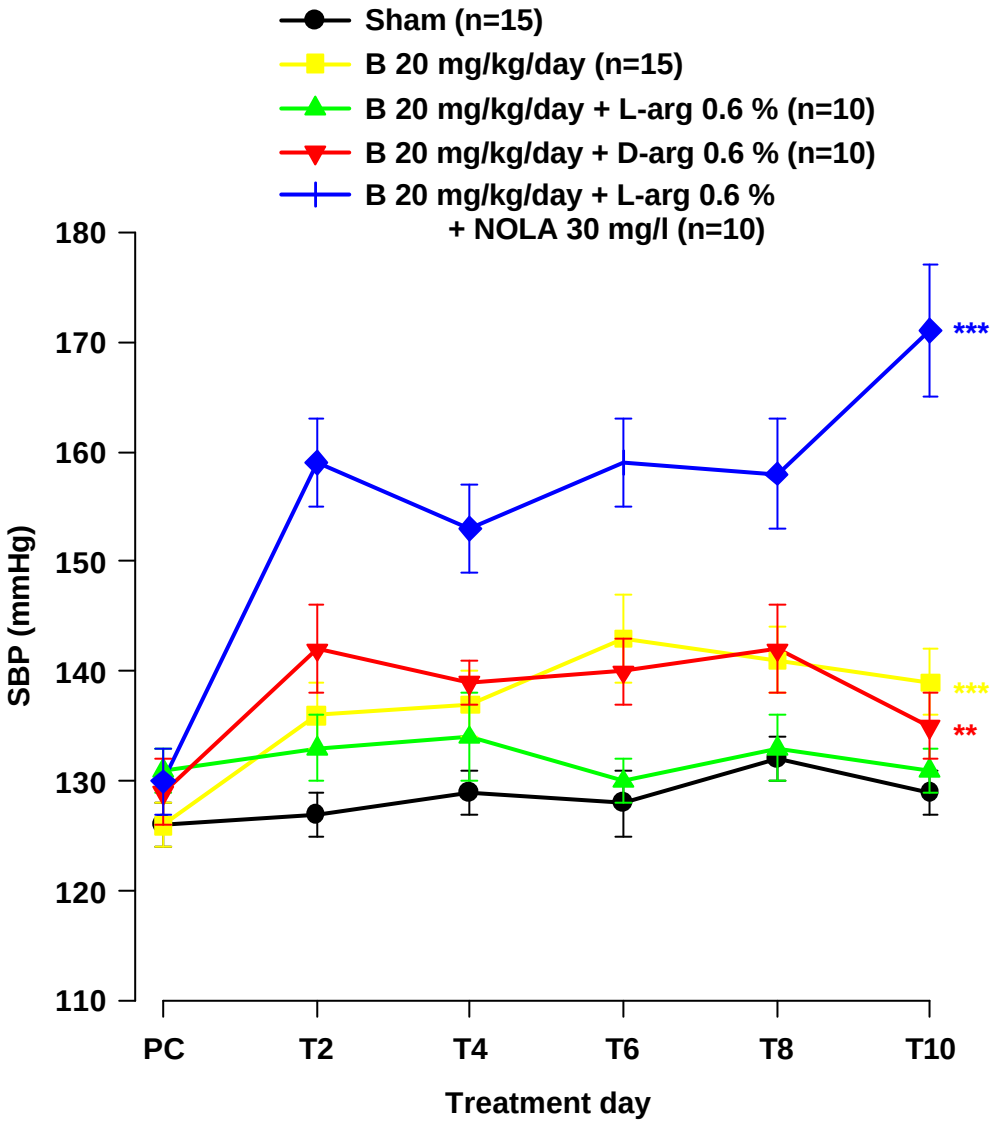


Figure 6.5 Effects of L-arginine, D-arginine or L-arginine + NOLA on corticosterone-induced hypertension.

Table 6.1 Metabolic effects of low dose corticosterone infusion in the male SD rat (n=39).

		C4	T3	T5	T7	T9	T11	T13	RMANOVA
Weight (g)	**Group 1**	263 ± 3	283 ± 4	293 ± 4	300 ± 5	309 ± 4	318 ± 4	327 ± 5	p < 0.001
	Group 2	272 ± 5	290 ± 5	299 ± 5	308 ± 6	318 ± 6	325 ± 6	333 ± 6	p < 0.001
	Group 3	268 ± 3	284 ± 4	294 ± 4	301 ± 5	309 ± 5	318 ± 5	325 ± 5	p < 0.001
	Group 4	262 ± 7	283 ± 4	293 ± 5	299 ± 4	308 ± 4	316 ± 5	325 ± 5	p < 0.001
		PC							
Water intake (ml)	**Group 1**	30 ± 1	30 ± 2	31 ± 2	30 ± 1	33 ± 1	32 ± 2	32 ± 2	n.s
	Group 2	33 ± 2	33 ± 1	35 ± 2	33 ± 1	38 ± 2	35 ± 1	34 ± 1	p < 0.05
	Group 3	35 ± 1	33 ± 2	35 ± 1	30 ± 3	36 ± 1	35 ± 1	33 ± 1	n.s
	Group 4	33 ± 2	29 ± 2	35 ± 1	29 ± 2	34 ± 2	33 ± 2	33 ± 2	n.s
Urine volume (ml)	**Group 1**	6 ± 1	8 ± 1	8 ± 1	7 ± 1	7 ± 1	8 ± 1	7 ± 1	n.s
	Group 2	8 ± 1	6 ± 1	7 ± 1	7 ± 1	7 ± 1	8 ± 1	7 ± 1	n.s
	Group 3	7 ± 1	7 ± 1	8 ± 1	7 ± 1	7 ± 1	8 ± 1	8 ± 1	n.s
	Group 4	6 ± 1	7 ± 1	8 ± 1	7 ± 1	8 ± 1	8 ± 1	8 ± 1	n.s
Food intake (g)	**Group 1**	24 ± 1	25 ± 2	27 ± 1	28 ± 1	29 ± 1	30 ± 1	28 ± 1	p < 0.01
	Group 2	24 ± 2	27 ± 1	29 ± 1	30 ± 1	32 ± 1	30 ± 1	29 ± 1	p < 0.01
	Group 3	27 ± 2	26 ± 1	29 ± 1	30 ± 1	30 ± 1	30 ± 2	26 ± 1	n.s
	Group 4	24 ± 2	25 ± 1	29 ± 1	27 ± 2	29 ± 2	28 ± 1	26 ± 2	n.s

Group 1 (n=9) sham (PEG), Group 2 (n=10) B 0.162 mg/24 h, Group 3 (n=10) B 0.279 mg/24 h, Group 4 (n=10) B 0.396 mg/24 h

Table 6.2 Organ weights in rats infused with subcutaneous low dose corticosterone (n=39)

	Adrenal (g)	Heart (g)	Kidney (g)
Sham (n=9)	0.04 ± 0.01	1.06 ± 0.03	2.34 ± 0.06
Corticosterone low (n=10)	0.04 ± 0.01	1.07 ± 0.03	2.32 ± 0.05
Corticosterone mid (n=10)	0.04 ± 0.01	1.07 ± 0.04	2.4 ± 0.07
Corticosterone high (n=10)	0.04 ± 0.01	1.03 ± 0.03	2.31 ± 0.06

Table 6.3 Metabolic effects of subcutaneous corticosterone injection (10, 20 & 40 mg/kg/day) in the male SD rat (n=40).

		C4	T1	T3	T5	T7	T9	T11	T13	RMANOVA
Weight (g)	**Sham**	300 ± 4	308 ± 3	314 ± 4	323 ± 4	323 ± 4	329 ± 4	335 ± 5	342 ± 5	p < 0.001
	B 10	302 ± 5	308 ± 4	301 ± 4	307 ± 4	302 ± 4	304 ± 4	307 ± 4	314 ± 5	p < 0.01
	B 20	294 ± 5	302 ± 5	287 ± 5	284 ± 6	280 ± 6	278 ± 6	281 ± 6	287 ± 7	p < 0.01 ##
	B 40	307 ± 5	311 ± 5	290 ± 4	280 ± 4	267 ± 4	262 ± 4	261 ± 4	262 ± 5	p < 0.001
		PC								
Water intake (ml)	**Sham**	33 ± 1	33 ± 1	33 ± 1	32 ± 1	31 ± 1	31 ± 1	28 ± 2	30 ± 1	p < 0.05
	B 10	33 ± 2	33 ± 2	31 ± 1	33 ± 2	33 ± 1	32 ± 1	32 ± 1	33 ± 2	n.s
	B 20	31 ± 1	32 ± 1	28 ± 1	28 ± 1	33 ± 2	31 ± 2	31 ± 1	32 ± 1	p < 0.05 #
	B 40	35 ± 1	32 ± 1	28 ± 1	31 ± 4	40 ± 3	35 ± 2	36 ± 1	35 ± 3	n.s
Urine volume (ml)	**Sham**	5 ± 1	4 ± 1	6 ± 1	5 ± 1	5 ± 1	5 ± 1	6 ± 1	7 ± 1	n.s
	B 10	5 ± 1	5 ± 1	5 ± 1	5 ± 1	6 ± 1	7 ± 1	6 ± 1	8 ± 2	n.s
	B 20	4 ± 1	4 ± 1	6 ± 1	5 ± 1	8 ± 1	6 ± 1	7 ± 1	9 ± 1	p < 0.001
	B 40	5 ± 1	5 ± 1	9 ± 1	10 ± 3	11 ± 2	12 ± 1	12 ± 1	13 ± 1	p < 0.01
Food intake (g)	**Sham**	29 ± 1	33 ± 1	31 ± 1	33 ± 2	33 ± 1	32 ± 1	30 ± 1	30 ± 1	p < 0.01 #
	B 10	28 ± 1	34 ± 1	28 ± 1	32 ± 1	31 ± 1	32 ± 1	31 ± 1	30 ± 1	p < 0.01 #
	B 20	29 ± 1	35 ± 1	27 ± 1	29 ± 2	32 ± 1	33 ± 2	30 ± 1	30 ± 2	n.s
	B 40	31 ± 1	36 ± 2	24 ± 1	24 ± 1	29 ± 2	30 ± 2	29 ± 1	27 ± 1	p < 0.001

Sham (n=10): PEG 0.5 ml/kg/day, B 10 (n=10): corticosterone 10 mg/kg/day, B 20 (n=10): corticosterone 20 mg/kg/day, B 40 (n=10): corticosterone mg/kg/day. P values indicate statistical significance by RMANOVA as linear changes over time. ## = quadratic trend.### = cubic trend.

Table 6.4 Concentrations of serum corticosterone and plasma NOx at sacrifice in corticosterone-treated rats (n=40).

	Corticosterone (ng/ml)	NOx (mmol/l)
Sham (n=10)	364 ± 25	11 ± 1
Corticosterone 10 (n=10)	261 ± 21*	8.4 ± 1.2
Corticosterone 20 (n=10)	376 ± 37	7.7 ± 0.9*
Corticosterone 40 (n=10)	502 ± 20**	9.3 ± 1.4

* $p < 0.05$, ** $p < 0.01$, respective group v sham

Table 6.5 Organ weights of rats injected with subcutaneous corticosterone 10, 20 and 40 mg/kg/day (n=40)

	Adrenal (g)	Heart (g)	Kidney (g)
Sham (n=10)	0.05 ± 0.01	1.06 ± 0.05	2.22 ± 0.05
Corticosterone 10 (n=10)	0.03 ± 0.01*	1.15 ± 0.05	2.31 ± 0.03
Corticosterone 20 (n=10)	0.02 ± 0.01*	1.04 ± 0.05	2.23 ± 0.06
Corticosterone 40 (n=10)	0.02 ± 0.01*	1.07 ± 0.03	2.33 ± 0.06

* $p < 0.05$ respective group v sham, after Hochberg's correction.

Table 6.6 Metabolic effects of subcutaneous corticosterone ± L-arginine, D-arginine or L-arginine + NOLA (n=60).

		C4	T1	T3	T5	T7	T9	RMANOVA
Weight (g)	**Sham**	283 ± 2	292 ± 2	299 ± 2	304 ± 3	310 ± 4	314 ± 4	p < 0.001
	B 20	284 ± 2	288 ± 2	285 ± 2	285 ± 3	284 ± 3	285 ± 4	n.s
	B 20 + L-arg	295 ± 5	299 ± 5	297 ± 5	301 ± 6	298 ± 6	299 ± 6	n.s
	B 20 + D-arg	290 ± 5	289 ± 5	284 ± 5	283 ± 5	282 ± 5	280 ± 5	p < 0.01
	B 20 + L-arg + NOLA	289 ± 2	281 ± 3	275 ± 3	267 ± 3	265 ± 3	264 ± 3	p < 0.001
		PC						
Water intake (ml	**Sham**	30 ± 1	30 ± 1	35 ± 2	33 ± 1	34 ± 2	34 ± 1	p < 0.05
	B 20	31 ± 1	30 ± 1	30 ± 1	30 ± 1	31 ± 1	33 ± 2	n.s
	B 20 + L-arg	30 ± 1	31 ± 1	32 ± 1	35 ± 2	35 ± 2	36 ± 2	p < 0.05
	B 20 + D-arg	32 ± 2	34 ± 4	32 ± 2	36 ± 4	34 ± 2	36 ± 3	n.s
	B 20 + L-arg + NOLA	26 ± 2	23 ± 1	28 ± 2	32 ± 3	37 ± 3	37 ± 3	p < 0.001
Urine volume (ml	**Sham**	8 ± 1	7 ± 1	9 ± 1	9 ± 1	9 ± 1	9 ± 1	n.s
	B 20	6 ± 1	8 ± 1	10 ± 1	9 ± 1	11 ± 1	10 ± 1	p < 0.001
	B 20 + L-arg	11 ± 1	14 ± 1	15 ± 1	14 ± 1	14 ± 1	14 ± 1	p < 0.05
	B 20 + D-arg	7 ± 1	10 ± 1	10 ± 1	10 ± 1	10 ± 1	12 ± 1	p < 0.01
	B 20 + L-arg + NOLA	5 ± 1	9 ± 1	10 ± 1	11 ± 2	12 ± 2	13 ± 2	p < 0.001
Food intake (g)	**Sham**	23 ± 1	22 ± 2	24 ± 1	22 ± 1	21 ± 1	20 ± 1	n.s
	B 20	23 ± 1	27 ± 2	21 ± 1	21 ± 1	20 ± 1	19 ± 1	p < 0.001
	B 20 + L-arg	27 ± 1	24 ± 2	25 ± 1	24 ± 1	23 ± 1	21 ± 1	p < 0.05
	B 20 + D-arg	22 ± 1	19 ± 1	18 ± 1	20 ± 1	20 ± 1	21 ± 1	p < 0.001 #
	B 20 + L-arg + NOLA	19 ± 1	14 ± 1	14 ± 1	17 ± 1	18 ± 1	19 ± 1	p < 0.001 #

Sham (n=15) PEG 0.5 ml/kg/day, B 20 (n=15) B 20 mg/kg/day, B 20 + L-arg (n=10) B 20 mg/kg/day + L-arginine 0.6 %, B 20 + D-arg (n=10) B 20 mg/kg/day + D-arginine 0.6 %, B 20 + L-arg + NOLA (n=10) B 20 mg/kg/day + L-arginine 0.6 % + NOLA 30 mg/L. P-values represent linear changes over the treatment period. # = quadratic trend.

Table 6.7 Concentration of plasma NOx at sacrifice in rats treated with subcutaneous corticosterone 20 mg/kg/day ± L-arginine, D-arginine or L-arginine + NOLA (n=60)

	NOx (mmol/l)
Sham (n=15)	8.2 ± 0.7
B 20 (n=15)	7.1 ± 0.5
B 20 + L-arg (n=10)	7.6 ± 0.4
B 20 + D-arg (n=10)	7.1 ± 1.3
B 20 + L-arg + NOLA (n=10)	4.7 ± 0.7 **

Sham (n=15) PEG 0.5 ml/kg/day, B 20 (n=15) B 20 mg/kg/day, B 20 + L-arg (n=10) B 20 mg/kg/day + L-arginine 0.6 %, B 20 + D-arg (n=10) B 20 mg/kg/day + D-arginine 0.6 %, B 20 + arg + NOLA (n=10) B 20 mg/kg/day + L-arginine 0.6 % + NOLA 30 mg/L. ** $p < 0.01$, group v sham, t-test after Hochberg's correction.

CHAPTER 7

The role of the parathyroid glands in ACTH-induced hypertension in the rat

7.1 INTRODUCTION

The parathyroid glands have been implicated in the development of genetic and mineralocorticoid hypertension in the rat. Bilateral parathyroidectomy attenuated the development of hypertension in the spontaneously hypertensive rat (SHR) and deoxycorticosterone-acetate-saline (DOCA-salt) rat (Gairard, Berthelot et al, 1982) and transplantation of SHR parathyroid glands into the normotensive Sprague-Dawley (SD) rat increased blood pressure (Pang and Lewanczuk, 1989). A putative circulating substance, parathyroid hypertensive factor (PHF), was detected in plasma from SHR (Lewanczuk, Wang et al, 1989) and subsequently in Dahl-S (Lewanczuk and Pang, 1993) and DOCA-salt (Lewanczuk and Pang, 1991) rats and may act as a potentiator for other pressor hormones (Pang, Benishin et al, 1994). PHF was absent from plasma of renal artery clip (2K1C) (Lewanczuk and Pang, 1991) and Dahl-R (Lewanczuk and Pang, 1993) rats. PHF is suggested to contribute to the low-renin salt–sensitive subset of essential hypertension in man (Resnick, Lewanczuk et al, 1993).

Exogenous ACTH excess produces hypertension in man, sheep, rat and dog (Scoggins, Denton et al, 1984). Hypertension develops with rapid onset in the rat with SBP increases observed by day two of treatment (Turner, Wen et al, 1996). The hypertension is adrenally dependent (Whitworth, Hewitson et al, 1990) and probably mediated by the adrenal glucocorticoid corticosterone, as

I have demonstrated in Chapter 6. The metabolic effects of ACTH excess in the rat include increases in water intake, urine output and plasma sodium concentration and reduction in plasma potassium concentration (Whitworth, Hewitson et al, 1990). Despite some features of mineralocorticoid excess, ACTH-induced hypertension in the rat is not a classical salt-sensitive model as it develops in animals on normal or low sodium intake and is not amplified by high sodium intake (Vazir, Whitehouse et al, 1981), (Li and Whitworth, 1992).

To determine whether the parathyroid glands contribute to ACTH-induced hypertension, I performed a study examining the effects of exogenous ACTH in parathyroidectomised SD rats.

7.2 METHODS

7.2.1 Animals and Protocols

Details regarding animals and housing are discussed in Chapter 2 (section 2.1)

Rats were allowed 7 days to acclimatise after arrival during which time they had free access to deionised water and standard rat food (sodium content 0.2%, Doust and Rabbidge, Sydney). Deionised water was used as drinking water to reduce confounding effects of unmeasured dietary cations on the development of ACTH-induced hypertension and was initially offered to all animals.

On day 7, bilateral parathyroidectomy was performed on animals in groups 3 & 4 (see below) as described in section 2.1.5.1 and these rats were allowed 6 days to recover. Adequacy of parathyroidectomy was determined histologically.

Because of the risk of profound hypocalcaemia following total parathyroidectomy, calcium was added to the drinking water of parathyroidectomised animals. The aim was to use the minimal concentration of calcium required to prevent tetany, as calcium supplementation has been shown to lower blood pressure in a number of experimental models of hypertension (Hatton and McCarron, 1994). In a pilot study of parathyroidectomy (n=3), animals did not drink a 2% solution of calcium chloride, but did tolerate a 1 % solution. This solution was given to the parathyroidectomy rats (Groups 3 and 4) from 1 day prior to parathyroidectomy until the end of the experiment. The 1% calcium chloride solution was also given to Group 5 to examine the effect of oral calcium supplementation on the development of ACTH-induced hypertension, independent of parathyroidectomy (ie as a positive control).

There were 4 control (C1-C4) and 11 treatment (T0-T10) days. Prior to the commencement of the control period, rats were randomly assigned to one of five treatment groups:

Group 1:- (sham): 1 ml/kg of 0.9% saline per day subcutaneously

Group 2:- (ACTH): injected with 0.5 mg/kg of tetracosactrin per day subcutaneously (ACTH, Synacthen Depot, Novartis)

Group 3:- (saline/PTx/$CaCl_2$): parathyroidectomised animals injected with 0.9% saline per day subcutaneously and maintained with calcium chloride in drinking water

Group 4:- (ACTH/PTx/$CaCl_2$): parathyroidectomised animals injected with 0.5 mg/kg of tetracosactrin per day subcutaneously and maintained with calcium chloride in drinking water

Group 5:- (ACTH/$CaCl_2$): animals administered both calcium chloride in drinking water and 0.5 mg/kg of tetracosactrin per day subcutaneously to examine the effect of calcium supplementation on hypertensive effects of ACTH.

After exclusion of rats with inadequate parathyroidectomy (see below), the numbers in each group were: Group 1: n=8, Group 2: n=8, Group 3: n=7, Group 4: n=10 and Group 5: n=8.

Systolic blood pressure and metabolic parameters were measured on alternate days. Urine was collected for measurement of sodium and potassium excretion. Treatment with either ACTH or sham commenced after blood pressure measurements were made on the morning of T0 (ie three control measurements of blood pressure - C1, C3, T0). At sacrifice, blood was collected for sodium, potassium and ionised calcium concentrations as well as parathyroid hormone concentration.

7.3 RESULTS

7.3.1 Adequacy of parathyroidectomy (Table 7.1)

Forty-five rats were used and four were subsequently excluded because of evidence of inadequate parathyroidectomy. Three rats had histological evidence of unilateral parathyroidectomy with parathyroid hormone concentrations similar to the sham group (30, 44 and 39 pg/ml, sham 54 pg/ml) and the remaining rat had no parathyroid tissue histologically and the parathyroid hormone concentration was 45 pg/ml. All other parathyroidectomised rats had histological evidence of bilateral parathyroidectomy and reduced parathyroid concentrations at sacrifice (Gp 3 [PTH]=10 pg/ml {range 6-14 pg/ml}, Gp 4 [PTH]=10 pg/ml {range 4-15 pg/ml}).

7.3.2 Effects of ACTH PTx on systolic blood pressure (Figure 7.1)

Pooled control measurements of systolic blood pressure were similar in the five groups (Group 1 (n=8): 114 4 mmHg, Group 2 (n=8): 119 3 mmHg, Group 3 (n=7): 118 4 mmHg, Group 4 (n=10): 119 3 mmHg, Group 5 (n=8): 121 3 mmHg). ACTH-treatment increased systolic blood pressure in the ACTH-treated rats (ΔSBP 27 5 mmHg, $p<0.01$), ACTH/PTx/$CaCl_2$ (ΔSBP 21 6 mmHg, $p<0.01$) and ACTH/$CaCl_2$ group (ΔSBP 19 4 mmHg, $p<0.05$). There was no change in SBP in the sham rats or the saline/PTx/$CaCl_2$ group. The increases in SBP in the three ACTH-treated groups (Gps 2, 4 & 5) were not significantly different from each other.

7.3.3 Metabolic Parameters (Table 7.2)

7.3.3.1 Body weight

Body weight was lower prior to treatment in parathyroidectomised animals compared with the sham group (saline/PTx/$CaCl_2$ 303 6 g, ACTH/PTx/$CaCl_2$ 296 7 g, compared with sham 323 4 g, $p<0.05$, $p<0.01$ respectively). Body weight increased during the treatment period in the saline-treated rats and decreased in the ACTH-treated animals.

7.3.3.2 Water intake

Pooled control values of 24-hour water intake were similar in all groups. Water intake increased in ACTH-treated rats and did not change in the saline-treated animals. The increase in water intake was greatest in the ACTH group (Δ WI 40 ml/day, T9-PC) compared with the ACTH groups

that drank 1% $CaCl_2$ solution (ACTH/PTx/$CaCl_2$ ΔWI 25 ml/day, Gp 2 v 4, $p<0.05$ and ACTH/$CaCl_2$ ΔWI 25 ml/24 hr, Gp 2 v 5, $p<0.05$).

7.3.3.3 Urine output

Pooled control values of 24-hour urine output were similar for all groups. Urine output increased in ACTH treated but not in saline treated rats. The largest increase observed was in the ACTH treated group (ΔUO 23 ml/24 hr). The ACTH/PTx/$CaCl_2$ animals had a smaller increase in urine output (ΔUO 10 ml/24 hr, T9-PC, Gp 2 v 4, $p<0.01$) as did the ACTH/$CaCl_2$ rats (ΔUO 18 ml/24 hr, T9-PC, Gp 2 v 5, $p = 0.05$).

7.3.3.4 Food intake

Pooled control values for food intake were similar in all groups. There was no significant change over time in any group in daily food intake.

7.3.3.5 Twenty four hour sodium and potassium excretion (Table 7.3)

Pooled control values for sodium and potassium excretion were similar between groups. Sodium excretion increased in the ACTH/$CaCl_2$ group (0.53 to 1.11 mmol / 24 hr, PC to T9, $p < 0.05$, RMANOVA) and tended to rise in the ACTH group (0.57 to 1.34 mmol / 24 hr. $p = 0.08$) with no changes observed in the sham, saline/PTx/$CaCl_2$ or ACTH/PTx/$CaCl_2$ groups.

There were no consistent trends in potassium excretion.

7.3.3.6 Plasma electrolytes at sacrifice (Table 7.1)

Plasma sodium concentration was increased in Gp 4 (ACTH/PTx/$CaCl_2$) compared with the sham group. ACTH treatment lowered plasma potassium

(Gps 2, 4 & 5) compared with sham. Plasma ionised calcium was decreased in parathyroidectomised rats (Gps 3 & 4) compared with sham despite calcium chloride supplementation. There was a small but non-significant reduction (after correction for multiple tests) in ionised calcium in non-parathyroidectomised ACTH-treated rats (Gp 2) compared with sham ($p = 0.04$, Gp 1 v 2), which was not corrected by calcium chloride supplementation in the ACTH/$CaCl_2$ group ($p = 0.03$, Gp 1 v 5).

7.4 DISCUSSION

Parathyroidectomy did not prevent ACTH-induced hypertension or metabolic effects in the male SD rat. These results suggest that the neither the parathyroid glands nor PHF are involved in the genesis of hypertension this model.

Neither saline injection in normal rats nor in parathyroidectomised rats had any significant effect on SBP over the treatment period. These results are consistent with those of Pang et al who have shown that parathyroidectomy does not affect BP in the normotensive SD rat (Pang and Lewanczuk, 1989). ACTH treatment increased SBP and produced metabolic effects (hypokalaemia, fall in body weight and increase in urine output and water intake) as previously reported (Turner, Wen et al, 1996). Parathyroidectomy did not alter the development of hypertension in ACTH-treated rats. I was careful to exclude rats with any evidence of residual functioning parathyroid tissue, by parathyroid hormone assay and histological analysis. Parathyroid

hormone concentrations in the parathyroidectomised animals were greater than zero, and may be explained by limitations of IRMA when very low levels of binding occur (ie less than 20 pg/ml), allowing significant non-specific binding by other constituents of serum. Also, the assay detects intact and fragments of parathyroid hormone, so low-level binding of fragments is not unexpected. Although the remaining thyroid bed was not examined histologically for residual parathyroid tissue at sacrifice, it is unlikely that any of the parathyroidectomised rats had functioning parathyroid tissue, given the above histological and biochemical evidence and the marked decrease in ionised calcium in these animals (Table 7.1). Similarly, it is unlikely that any residual circulating parathyroid product(s) had an influence on the blood pressure in this study, as the pressor effects of PHF are reported to occur between 30-70 minutes post-injection and resolve by 90 minutes (Lewanczuk, Wang et al, 1989), and, a fall in blood pressure has been observed as early as 48 hours post-parathyroidectomy in the SHR (Pang and Lewanczuk, 1989). Therefore, any PHF, if present in my SD rats, would only be biologically active in the initial hours post-parathyroidectomy and be unlikely to contribute to hypertension developing ten days later. Further, PHF has not been reported in the normotensive SD rat (Lewanczuk, Wang et al, 1989).

The parathyroid glands, therefore, are not necessary for the development of hypertension in this model. This is in contrast to effect of parathyroidectomy in DOCA-salt rats and in the SHR (Gairard, Berthelot et al, 1982), where

parathyroidectomy attenuated the onset of hypertension. In established SHR hypertension, parathyroidectomy has been shown to lower blood pressure (Pang and Lewanczuk, 1989). Each of the latter models are salt-sensitive whereas there is evidence that this may not be the case for ACTH-induced hypertension in the rat. Li et al examined the effects of a diet high in sodium (1% NaCl in water) compared with normal sodium intake (tap water) on ACTH-induced hypertension. The high sodium supplement did not alter the blood pressure rise observed secondary to ACTH, but markedly increased fluid intake, urine volume and urinary sodium excretion and some rats became oedematous (Li and Whitworth, 1992). In contrast, Vazir et al (Vazir, Whitehouse et al, 1981) examined the effect of severe sodium restriction (< 20 micromols / day) which had no effect on ACTH-induced increases in blood pressure. Hence, it appears that ACTH-induced hypertension in the rat is not a classical salt-sensitive model of hypertension. As PHF has been implicated in salt-sensitive hypertension in man and rat, the above studies may explain why parathyroidectomy had no effect on ACTH-induced hypertension in this study.

The increases in systolic blood pressure in the ACTH/PTx and the ACTH/$CaCl_2$ groups over the treatment period were slightly but not significantly attenuated compared with ACTH treatment alone. Calcium supplementation rather than parathyroidectomy was probably responsible for this small effect, as the systolic blood pressure profile in the calcium-supplemented parathyroidectomised and non-parathyroidectomised rats was

similar. An inverse relationship between dietary calcium and blood pressure has been described in many experimental models of hypertension, including the SHR (Lewanczuk, Chen et al, 1990), DOCA-salt dog (Bravo and Kageyama, 1994) and rat (Lin, Saito et al, 1994), Dahl-S (Kang, Cregor et al, 1990) and renal artery clip (Kageyama, Suzuki et al, 1987) rat models. In ACTH-hypertensive sheep, however, dietary calcium supplementation did not alter plasma calcium concentrations or blood pressure (Tresham, McGuire et al, 1988). Liu et al were unable to prevent DOCA-salt hypertension in rats fed a high (0.8%) calcium diet (Liu, Birchall et al, 1994), however most studies of prevention of hypertension with calcium supplementation have used higher amounts of dietary calcium (Hatton and McCarron, 1994). I was careful to choose a level of calcium intake that would prevent tetany in the parathyroidectomised animals but avoid the confounding effect of dietary calcium on blood pressure. Nevertheless, the excess dietary calcium in these groups may have attenuated the hypertensive effects of ACTH. A number of physiological mechanisms have been proposed to explain how calcium supplementation lowers blood pressure in experimental hypertension (Hatton and McCarron, 1994). These include reduction in vascular smooth muscle contractility, increased sodium, potassium-ATPase activity, suppression of PHF release, increases in calmodulin and calcitonin gene-related peptide concentrations, reduction in sympathetic nervous system activity and calcium-induced natriuresis.

The rats treated with ACTH and supplemented with calcium in the drinking water also had smaller increases in water intake and urine output compared with rats receiving ACTH alone. This may partly be explained by the unpleasant taste of calcium in water. The rats treated with ACTH and calcium supplementation (Groups 4 & 5) lost more body weight over the treatment period compared with those that drank deionised water, despite the observation that food intake was similar in all groups, raising the possibility that animals in Groups 4 & 5 may have been relatively volume deplete. As fluid balance was measured second daily, complete water balance could not be determined. This observation could explain the small attenuation in the rise in systolic blood pressure in these two groups.

This study suggests a trend towards lower plasma ionised calcium in ACTH-treated animals. This phenomenon has previously been reported in low-renin essential hypertension in man (Resnick, Laragh et al, 1983) though the mechanism is not understood. Pang et al have hypothesised that it may be explained by the effect of PHF on L-type calcium channels, allowing an influx of calcium into vascular smooth muscle cells (Pang, Benishin et al, 1994). I was unable to measure PHF in this study (see next chapter).

7.5 CONCLUSIONS

In summary, I was unable to demonstrate involvement of the parathyroid glands in ACTH-induced hypertension in the rat. The role of the parathyroids

and PHF experimental and human hypertension is still unclear hence the structural characterisation of this putative hormone is eagerly awaited.

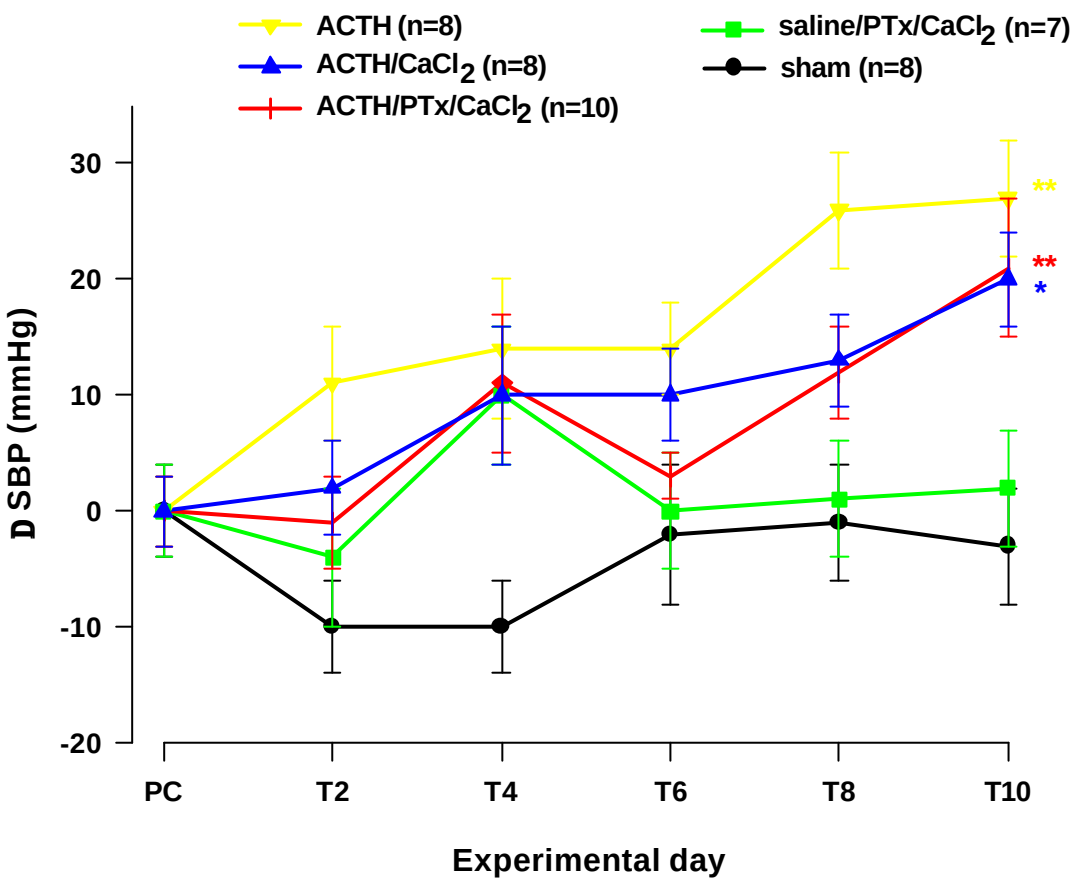


Figure 7.1 Change in systolic blood pressure from pooled control (PC) to tenth treatment day (T10). * $p < 0.05$, ** $p < 0.01$ by RMANOVA.

Table 7.1 Serum parathyroid hormone, plasma sodium, potassium and ionised calcium at sacrifice (n=41)

	Parathyroid hormone (pg/ml)	Ionised calcium (mmol/l)	Sodium (mmol/l)	Potassium (mmol/l)
Sham (n=8)	54 ± 7	1.32 ± 0.01	139.9 ± 1	3.9 ± 0.2
ACTH (n=8)	33 ± 5	1.25 ± 0.03	140.1 ± 1.5	2.5 ± 0.1**
Saline/PTx/CaCl (n=7)	10 ± 1**	0.93 ± 0.07**	142 ± 0.4	3.9 ± 0.1
ACTH/PTx/CaCl (n=10)	10 ± 1**	0.9 ± 0.02**	143.5 ± 0.5*	2.5 ± 0.2**
ACTH/CaCl (n=8)	40 ± 7	1.26 ± 0.02	142.4 ± 0.5	2.8 ± 0.1**

* $p < 0.05$, ** $p < 0.01$, respective group v sham, after Hochberg's correction for multiple tests.

Table 7.2 Metabolic effects of sham, ACTH, saline/PTx/CaCl, ACTH/PTx/CaCl and ACTH/CaCl in the male SD rat (n=41)

		C4	T1	T3	T5	T7	T9	RMANOVA
Weight (g)	**Sham**	323 ± 4	330 ± 6	335 ± 6	342 ± 7	348 ± 8	352 ± 8	p < 0.001
	ACTH	317 ± 5	308 ± 6	298 ± 6	293 ± 8	296 ± 9	288 ± 9	p < 0.01
	Saline/PTx/CaCl	303 ± 6	307 ± 7	316 ± 8	319 ± 9	330 ± 9	337 ± 10	p < 0.01
	ACTH/PTx/CaCl	296 ± 7	296 ± 7	270 ± 8	260 ± 8	252 ± 7	244 ± 7	p < 0.001
	ACTH/CaCl	309 ± 4	306 ± 7	290 ± 7	281 ± 7	273 ± 9	267 ± 7	p < 0.001
		PC						
Water intake	**Sham**	30 ± 3	32 ± 3	24 ± 2	28 ± 4	28 ± 3	31 ± 3	n.s
(ml / 24 hr)	**ACTH**	27 ± 2	29 ± 2	32 ± 4	50 ± 3	55 ± 4	67 ± 3	p < 0.001
	Saline/PTx/CaCl	28 ± 3	31 ± 3	31 ± 3	33 ± 3	34 ± 3	33 ± 3	n.s
	ACTH/PTx/CaCl	28 ± 3	26 ± 3	31 ± 3	41 ± 3	47 ± 2	53 ± 3	p < 0.001
	ACTH/CaCl	29 ± 3	26 ± 3	29 ± 3	36 ± 5	45 ± 6	54 ± 4	p < 0.001
Urine volume	**Sham**	4 ± 1	4 ± 1	4 ± 1	6 ± 2	4 ± 1	5 ± 1	n.s
(ml / 24 hr)	**ACTH**	4 ± 1	8 ± 1	14 ± 3	20 ± 2	28 ± 2	27 ± 2	p < 0.001
	Saline/PTx/CaCl	3 ± 1	5 ± 1	4 ± 1	3 ± 1	5 ± 1	6 ± 1	n.s
	ACTH/PTx/CaCl	4 ± 1	4 ± 1	7 ± 1	9 ± 1	9 ± 1	14 ± 2	p < 0.001
	ACTH/CaCl	5 ± 1	6 ± 1	6 ± 1	10 ± 2	16 ± 4	23 ± 3	p < 0.001
Food intake	**Sham**	29 ± 2	32 ± 2	28 ± 1	29 ± 3	29 ± 3	31 ± 1	n.s
(g / 24 hr)	**ACTH**	27 ± 1	29 ± 1	24 ± 2	27 ± 2	32 ± 2	30 ± 2	n.s
	Saline/PTx/CaCl	30 ± 2	31 ± 1	31 ± 3	30 ± 2	32 ± 1	32 ± 1	n.s
	ACTH/PTx/CaCl	33 ± 3	32 ± 2	26 ± 4	34 ± 3	33 ± 2	33 ± 2	n.s
	ACTH/CaCl	29 ± 2	31 ± 2	23 ± 2	28 ± 3	30 ± 2	29 ± 2	n.s

Sham (n=8) saline 1 ml/kg/day, ACTH (n=8) ACTH 0.5 mg/kg/day, saline/PTx/CaCl (n=7) parathyroidectomy + saline 1 ml/kg/day + 1% CaCl in water, ACTH/PTx/CaCl (n=10) parathyroidectomy + ACTH 0.5 mg/kg/day + 1% CaCl in water, ACTH/CaCl (n=8) ACTH 0.5 mg/kg/day + 1% CaCl in water. P-values represent significant linear changes over time by RMANOVA.

Table 7.3 Effects of sham, ACTH, saline/PTx/$CaCl_2$, ACTH/PTx/$CaCl_2$ and ACTH/$CaCl_2$ on sodium and potassium excretion in the male SD rat (n=41)

		C4	T1	T3	T5	T7	T9	RMANOVA
Sodium excretion (mmol / 24 hr)	**Sham**	0.603 ± 0.11	0.58 ± 0.13	0.498 ± 0.09	0.847 ± 0.34	0.477 ± 0.07	0.593 ± 0.24	n.s
	ACTH	0.556 ± 0.06	0.87 ± 0.1	1.107 ± 0.21	0.96 ± 0.16	1.005 ± 0.08	1.358 ± 0.21	n.s
	Saline/PTx/CaCl	0.35 ± 0.07	0.573 ± 0.09	0.665 ± 0.19	0.401 ± 0.09	0.481 ± 0.11	0.574 ± 0.1	n.s
	ACTH/PTx/CaCl	0.373 ± 0.05	0.28 ± 0.11	0.379 ± 0.09	0.427 ± 0.09	0.447 ± 0.09	0.813 ± 0.2	n.s
	ACTH/CaCl	0.528 ± 0.1	0.608 ± 0.09	0.448 ± 0.07	0.666 ± 0.18	0.637 ± 0.17	1.708 ± 0.18	$p < 0.05$
Potassium excretion (mmol / 24 hr)	**Sham**	1.24 ± 0.12	1.107 ± 0.09	1.066 ± 0.34	1.455 ± 0.55	0.974 ± 0.08	1.095 ± 0.1	n.s
	ACTH	1.102 ± 0.13	1.418 ± 0.16	1.604 ± 0.17	1.151 ± 0.14	1.136 ± 0.13	1.134 ± 0.18	n.s
	Saline/PTx/CaCl	0.924 ± 0.1	1.279 ± 0.14	1.302 ± 0.23	0.816 ± 0.07	1.116 ± 0.28	1.264 ± 0.29	n.s
	ACTH/PTx/CaCl	1.035 ± 0.12	0.982 ± 0.17	0.931 ± 0.13	1.001 ± 0.11	0.703 ± 0.06	1.025 ± 0.13	n.s
	ACTH/CaCl	1.278 ± 0.16	1.316 ± 0.13	1.038 ± 0.06	1.038 ± 0.23	0.977 ± 0.15	1.433 ± 0.18	n.s

Sham (n=8) saline 1 ml/kg/day, ACTH (n=8) ACTH 0.5 mg/kg/day, saline/PTx/CaCl (n=7) parathyroidectomy + saline 1 ml/kg/day + 1% CaCl in water, ACTH/PTx/CaCl (n=10) parathyroidectomy + ACTH 0.5 mg/kg/day + 1% CaCl in water, ACTH/CaCl (n=8) ACTH 0.5 mg/kg/day + 1% CaCl in water. P-values represent significant linear changes over time by RMANOVA.

CHAPTER 8

Rat bioassay for the detection of parathyroid hypertensive factor

8.1 INTRODUCTION

Parathyroid hypertensive factor (PHF) is a putative humoral factor secreted from the parathyroid glands. Initially described in SHR (Lewanczuk, Wang et al, 1989), it has subsequently been reported in the Dahl-S (Lewanczuk and Pang, 1993) and deoxycorticosterone-saline (DOCA-salt) (Lewanczuk and Pang, 1991) models of hypertension and in normotensive rats transplanted with SHR parathyroid glands (Pang and Lewanczuk, 1989). When SHR plasma is injected into an anaesthetised normotensive rat, a characteristic delayed hypertensive response has been described, beginning at approximately 30 minutes, peaking at 45 minutes and returning to baseline by 70 minutes. This has been attributed to PHF activity and the magnitude of this response defines the rat PHF bioassay (Pang and Lewanczuk, 1989). The bioassay is the only technique available to measure PHF and has been used in a number of studies from a group in Canada (Pang, Shan et al, 1996).

PHF has been partially purified but its structured has not been published (Benishin, Lewanczuk et al, 1994). Plasma PHF-like activity has been detected in humans with salt sensitive hypertension (Resnick, Lewanczuk et al, 1993), in 30 % of patients with type II diabetes mellitus and in over 70 % of cancer patients compared with less than 10% of normal individuals (Pang, Shan et al, 1996). As it may play a role in other diseases of altered calcium

metabolism, it has also been labelled Parathyroid Hypercytocalcic Factor (Pang, Shan et al, 1996).

The aim of the present study was to establish the rat bioassay required for the measurement of PHF. I anticipated measuring PHF activity in ACTH-induced hypertension in the rat (Chapter 7) and in steroid-induced hypertension in man (Chapters 3-5).

8.2 METHODS

8.2.1 Experimental protocols

Direct blood pressure was continuously measured in unconscious rats in the bioassay. Five series of experiments were performed, due to difficulties in detection of PHF. A number of anaesthetic techniques were employed to examine the effect of anaesthesia on blood pressure and the PHF response (Series 1-4). Because of difficulties in establishing this bioassay in these experiments, a series of bioassays were also performed in the laboratory of the original authors in Edmonton, Canada. These results are described (Series 5).

Methods used were those described in the literature (Lewanczuk, Wang et al, 1989). I was assisted with advice by the original author of the PHF bioassay, Dr Richard Lewanczuk. I chose SHR plasma as the ‘gold standard’ hypertensive plasma, SD and WKY plasma as normotensive control plasma and SD and WKY animals as the normotensive rats into which sample

plasma was injected, as previously described (Lewanczuk, Wang et al, 1989).

8.2.2 Preparation of sample plasma

Hypertension was confirmed in donor SHR by the tailcuff method, as described in Chapter 2.2.3 (SBP 179 $\pm$ 4 mmHg, mean SEM). Plasma samples of donor SHR, SD and WKY rats were prepared for subsequent injection into normotensive rats as follows; under sodium pentobarbital anaesthesia (60 mg/kg Nembutal® (Boehringer Ingelheim, Sydney) by intraperitoneal (i.p.) injection) a carotid artery was cannulated with polyethylene tubing (internal diameter (i.d.) 0.58 mm, outer diameter (o.d.) 0.96 mm, Critchley Electrical Products, NSW) and the blood volume was collected into chilled tubes containing lithium heparin (7 U/ml). Samples were immediately centrifuged at 4^{o}C for 15 minutes at 3000 rpm and then dialysed for 24 hours at 4^{o}C against 0.9% saline (Spectra-por CE dialysis membrane, molecular weight cutoff 1000 Dalton, Spectrum, Texas). Plasma was stored in polypropylene tubes at -70^{o}C until use.

8.2.3 Surgical and technical aspects of the PHF bioassay (Figures 8.1, 8.2)

Anaesthetised male SD or WKY rats were placed on a warming pad (38^{o}C) and remained there throughout the recording period. Via a midline incision, the right carotid artery was exposed, the nerves surrounding the artery were separated away by blunt dissection and the artery was cannulated with heparinised (50 U/ml) polyethylene tubing (i.d. 0.58 mm, o.d. 0.96 mm,

Critchley Electrical Products, Auburn, NSW). The right jugular vein was catheterised with heparinised polyethylene tubing for injection of plasma. Carotid arterial pressure was continuously monitored by a pressure transducer (Cobe, Lakewood, California, 80215 USA). Data were recorded by an Apple Macintosh computer running AcqKnowledge V2.1 which calculated mean arterial pressure (MAP) in real time. The system was calibrated against a modified mercury sphygmomanometer (0 & 200 mmHg calibrations).

Following stabilisation of MAP (within 5 mmHg for 5 minutes), prepared sample plasma of either SD, WKY or SHR (at room temperature) was slowly injected into the jugular catheter over a 2 minute period. Plasma was prepared as described above but in additional experiments plasma was immediately frozen and stored at -70^0C before use. MAP was then recorded for a further 90 minute period to determine PHF activity.

PHF activity was defined according to the original description as a delayed sustained (>5 minutes) rise in MAP, occurring 30-70 minutes after intravenous plasma injection, expected to peak at about 45-50 minutes and return to baseline by 90 minutes (Lewanczuk, Wang et al, 1989). If a delayed rise in MAP occurred and either plateaued or fell slightly (but not to baseline) within 70 minutes this was still considered a PHF response.

MAP was considered unstable if it oscillated ≥ 10 mmHg per minute or ≥ 20 mmHg within 5 minutes.

8.2.4 Techniques used and modifications made to the PHF bioassay

In Series 1 below, the PHF bioassay was to be reproduced as per the exact description of the original authors (Lewanczuk, Wang et al, 1989). Because of failure to reproduce the 'PHF response', Series 2-5 report a number of modifications to the original described technique.

8.2.4.1 Series 1: pentobarbital sodium by intraperitoneal injection (Nembutal®)

Fourteen male SD rats (358 4g) were anaesthetised with a single dose of intraperitoneal (i.p.) sodium pentobarbital (60 mg/kg). Anaesthesia was maintained throughout the recording period if necessary with additional doses (6-9 mg i.p.) to maintain adequate anaesthesia defined by absence of withdrawal reflexes to pain (pinch to heel pad or ear prick) and absence of whisker movement during respiration.

8.2.4.2 Series 2: pentobarbital sodium by intravenous infusion (Nembutal®)

Ten male SD rats (360 g) were anaesthetised with a single dose of sodium pentobarbital (40-60 mg/kg i.p.) and maintained unconscious by titrated intravenous infusion of sodium pentobarbital.

8.2.4.3 Series 3: halothane anaesthesia

In order to test the effects of other forms of anaesthesia on BP as an independent factor involved in this bioassay, 14 male SD rats (440 g) were

anaesthetised with halothane 2-2.5% in oxygen 2 L/minute administered by a conical apparatus applied to the nose and mouth. Following induction of anaesthesia, animals were maintained with halothane 1-1.5% in oxygen 1-1.5 L/min.

8.2.4.4 Series 4: Canadian brand pentobarbital sodium (Somnotol®)

To determine whether the brand of pentobarbital influenced the apparent bioassay result, 25 male SD rats were anaesthetised with a single i.p. dose (60 mg/kg) of a Canadian brand of sodium pentobarbital (Somnotol®, MTC Pharmaceuticals, Ontario, Canada, as used by the group who described PHF originally - personal communication) and maintained anaesthetised as required with additional doses (6-9 mg i.p.) according to the above criteria. Some of these bioassays were performed after I returned from The University of Alberta, with undialysed, filtered plasma (see below).

8.2.4.5 Series 5: PHF bioassays performed at the University of Alberta, Edmonton, Canada

Because I was unable to reproduce the PHF bioassay despite a number of modifications to the original description and advice from Dr R Lewanczuk, I travelled to the University of Alberta, Edmonton, Canada, where I was invited by Dr RZ Lewanczuk to attend his laboratory and refine the bioassay technique. The anaesthetic technique used in this series was similar to that of Series 1, however, Somnotol was used rather than Nembutal. Further, plasma was prepared without dialysis and filtered through a 0.45 mm cellulose membrane (Millex-HA Ultracleaning Filter Unit, Millipore).

8.3 RESULTS

Blood pressure responses to injected SHR plasma are summarised in Table 8.1.

8.3.1 Intraperitoneal pentobarbital anaesthesia in the PHF bioassay (Figure 8.3)

All results in this series of experiments were uninterpretable because of unstable blood pressure, as defined above, following injection of plasma. MAP oscillated in 10 rats, fell and continued to fall in three and increased and continued to rise in one. All animals required top-up anaesthesia during the 90 minute recording period.

8.3.2 Effect of intravenous infusion of pentobarbital in PHF bioassay (Figure 8.4)

Because of MAP instability in the above experiments, intravenous infusion of pentobarbital was used for maintenance of anaesthesia. Dose-response studies in three animals defined the satisfactory dose rate as 20-60 mg/kg/hr pentobarbital, though there were fluctuations in MAP over 90 minutes in each of these animals.

SHR plasma (1.6 .0 ml, mean volume SEM) was injected in seven rats. MAP was unstable in two rats. PHF bioassay activity was positive in three (MAP rose 15, 18 & 24 mmHg) and MAP fell in two rats (-3 & -13 mmHg).

8.3.3 Effect of inhaled halothane anaesthesia on PHF bioassay (Figure 8.5)

Halothane provided anaesthesia with virtually no fluctuation in MAP (only 1/17 MAPs unstable). Two rats were injected with WKY (control) plasma (1.2, 2.0 ml) and the PHF response in each of these was negative (MAP fell 4 & 11 mmHg). Fourteen rats were injected with SHR plasma (mean volume 1.8 .1 ml). Of these, the PHF response was positive in four rats (MAP rose 5, 6, 6 & 8 mmHg), there was no response in three rats, a negative response occurred in six rats (MAP fell -15 mmHg) and MAP became unstable in one rat.

8.3.4 Effect of filtration of crude donor plasma and use of Canadian brand of pentobarbital (Somnotol®) on PHF bioassay (Figure 8.6)

Somnotol provided general anaesthesia with almost no effect on MAP. After injection of filtered undialysed SHR plasma (n=16, mean volume 1.8 .0 ml), the PHF response was positive in six (MAP rose 13 mmHg), MAP fell in five by 7 mmHg and MAP became unstable in five. Following injection of filtered undialysed (control) WKY plasma (n=9, mean volume 1.8 .1 ml), the PHF response was positive in three (MAP rose 7, 11, & 13 mmHg), MAP fell in two by 8 & 17 mmHg and MAP became unstable in four animals.

8.3.5 Results of PHF bioassays performed at the University of Alberta, Edmonton, Canada (Figure 8.7)

Despite the guidance and expertise of the discoverer of PHF and his group, I was unable to reproduce the PHF response using the bioassay in Edmonton. The technique was the same as in Series 4. After injection of filtered, undialysed SHR plasma (n=8), the PHF response was positive in four (MAP rose 5, 5, 5 & 10 mmHg), negative in one (MAP fell 15 mmHg), there was no change in MAP in one and two results were unstable. Following injection of WKY plasma (n=4), there was no change in two, one response was negative and one response unstable.

8.4 DISCUSSION

Despite careful attention to the details of these experiments, I have been unable to reproduce the PHF bioassay. I have used the technique of the group who described PHF, modified this to correct for MAP fluctuations associated with some forms of anaesthesia and further modified our method as suggested by the original authors themselves (personal communications). Despite this, the PHF response as described above was not consistently observed; in fact, the classic response i.e. BP rising at 30 min, plateauing smoothly and falling to baseline was never observed. Furthermore, following injection of SHR plasma, the number of rats exhibiting a BP rise was similar to the number in which BP fell.

I have addressed problems which may have resulted in failure of the bioassay due to technical reasons before concluding the absence of a PHF-like factor in the SHR I studied. Initially, anaesthesia was a problem, as baseline MAP was frequently unstable in association with evidence of lightening anaesthesia during the recording period. Lin et al (Lin, Saito et al, 1994) also reported awakening of rats by 90 minutes after plasma injection when performing this bioassay on DOCA-salt treated rats, leading them to omit MAP data recorded more than 60 minutes following injection of plasma. This is at odds with the original description of PHF which required that MAP should return to baseline or at least plateau for the response to be considered a true PHF response.

To address the problems of anaesthetic I used both intravenous pentobarbital and inhalation anaesthesia. Unfortunately, intravenous anaesthesia also produced unstable baseline BP and variation in the depth of anaesthesia. While small intravenous bolus doses of pentobarbital would re-anaesthetise the rat, this usually resulted in substantial changes in MAP.

Halothane provided satisfactory anaesthesia with stable MAP in most assays but the PHF response was more frequently 'negative' than positive. One possible confounding factor with the use of halothane is its reported ability to cause both vasoconstriction by mobilising calcium from intracellular stores and vasodilatation by inhibiting calcium activation of contractile proteins (Akata and Boyle, 1995).

Following correspondence with the original authors, I further modified the assay by using the same brand of pentobarbital as Pang's group, which may affect the duration of anaesthesia and by filtration of injected plasma to remove particulate matter (personal communications). The difference in quality of anaesthesia observed with Somnotol may be due to differences in purity of the drug or of the solvent. Even under these conditions, the PHF response could not be obtained reproducibly, with six positive and five negative BP responses observed after injection of SHR plasma.

The bioassays performed at the University of Alberta were also unreliable, for reasons that are unclear. All care was taken to perform the surgery carefully. SHR plasma from confirmed hypertensive animals was shipped to Canada from the St George Hospital, stored at -20^0C. Other SHR plasma from animals at the University of Alberta was also assayed. None of these results yielded a 'classic PHF response'.

The original description of PHF suggested it was involved several types of hypertension (Pang, Shan et al, 1996). However, the PHF bioassay has been reproduced infrequently in published literature outside of Canada. Lin's group measured PHF-like activity in DOCA-salt treated rats using a modification of the assay as described above, but did not use SHR plasma as a positive control (Lin, Saito et al, 1994). Recently, Tikkanen et al reported PHF-like activity in plasma of SHR on high (2.6%) but not normal (0.3%) salt diets

(Tikkanen, Teravainen et al, 1997). In the present study, SHR developed significant hypertension on a normal (0.2%) salt diet but PHF activity was absent and together these reports argue against PHF being a causative factor for hypertension in the SHR.

8.5 CONCLUSIONS

In summary, I have been unable to establish the PHF bioassay despite several modifications to address possible confounding factors. I await the development of a more reliable and reproducible technique for the measurement of PHF together with elucidation of its structure. In the absence of this information I am uncertain whether PHF exists and I question the role of PHF in the development of hypertension in the SHR.

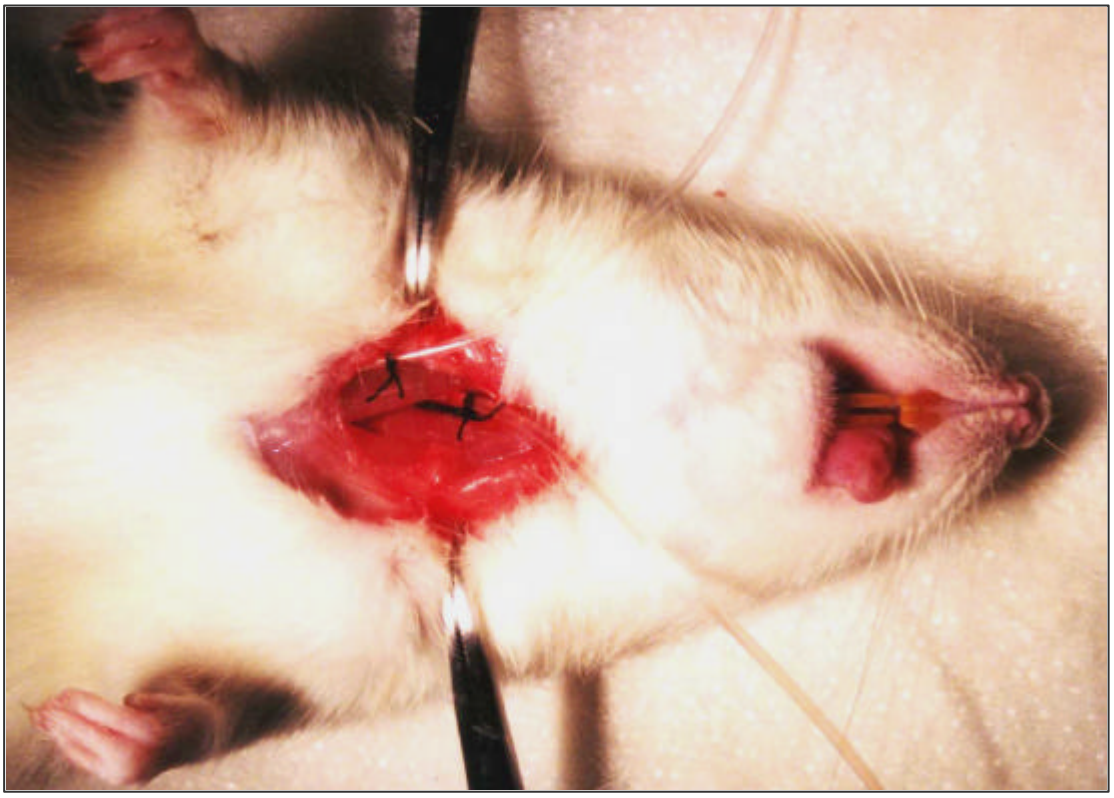


Figure 8.1 PHF bioassay. Catheters in the common carotid artery and jugular vein in an anaesthetised rat.

Figure 8.2 Direct BP monitoring setup for PHF bioassay. (a) pressure transducer, (b) PA-100 amplifier, (c) Biopac MP-100, (d) Apple Macintosh running Acqknowledge data acquisition software.

Figure 8.3 Example of MAP recorded over 90 minutes in an animal from Series 1. Uninterpretable results due to oscillations in blood pressure.

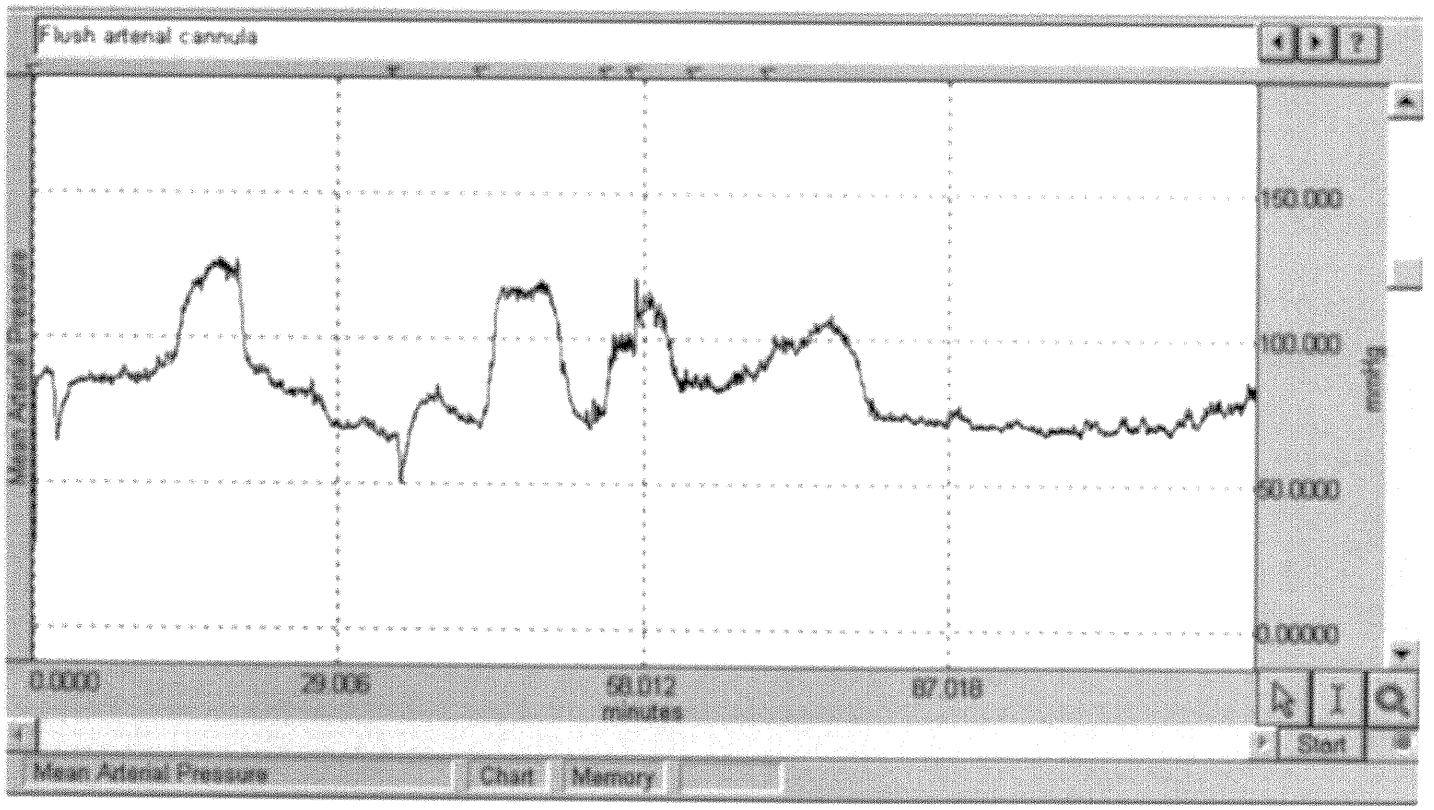


Figure 8.4 Effect of intravenous infusion of pentobarbital sodium on MAP and the PHF response. Both positive and 'negative' PHF responses were observed.

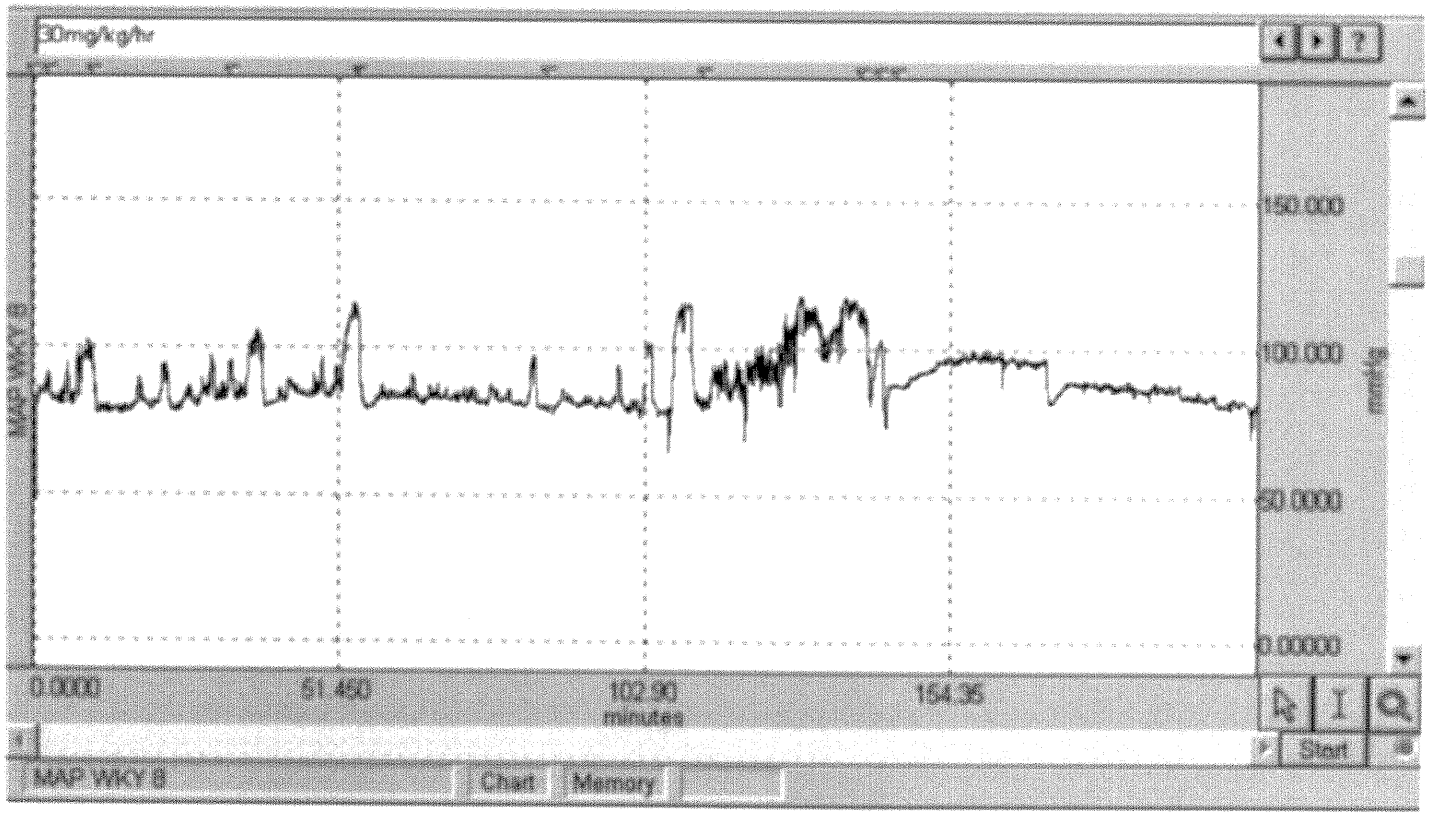
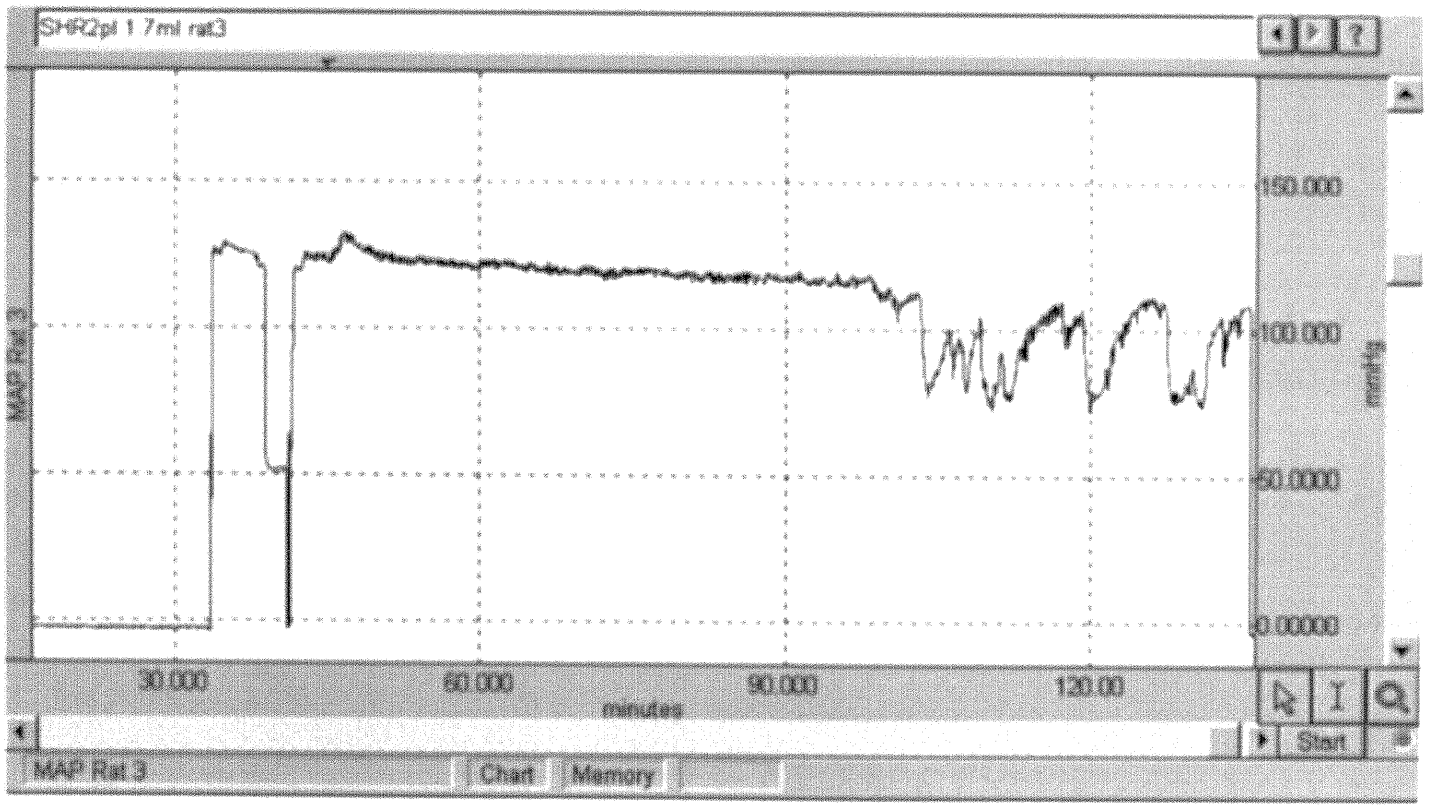


Figure 8.5 Effect of inhaled halothane on baseline MAP and the PHF response. Stable baseline blood pressure but PHF response not reproducibly observed.

Figure 8.6 Effect of Canadian brand pentobarbital sodium anaesthetic and crude plasma on the PHF bioassay. Non-reproducible results.



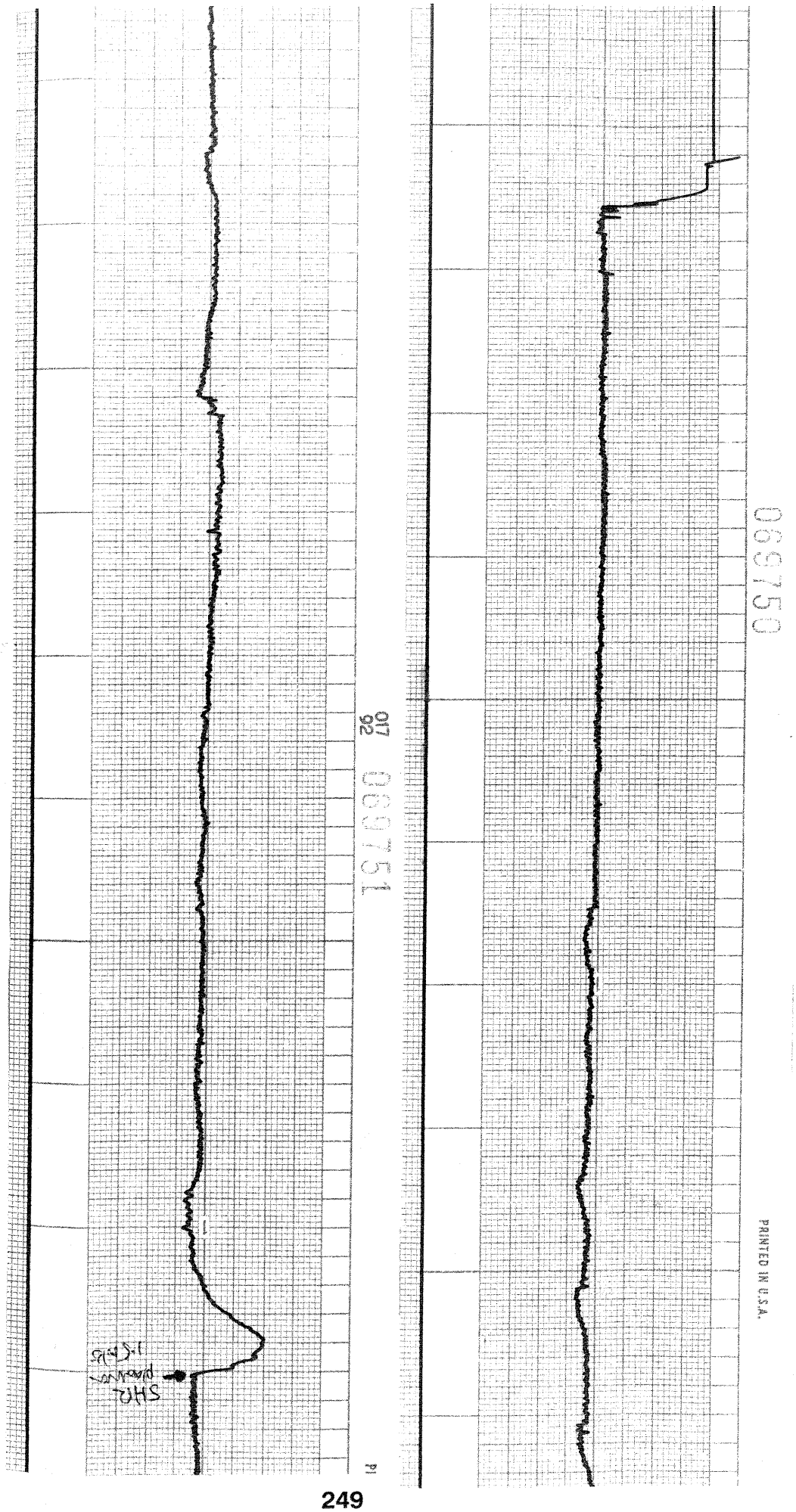


Figure 8.7 Results of a bioassay performed in Canada. No consistent PHF response observed. (y-axis 5 mmHg/mm, x-axis 2 min/5 mm). SHR plasma injected at arrow.

Table 8.1 Blood pressure responses in anaesthetised normotensive male rats following injection of SHR plasma.

	Nembutal i.p. (n=14)	Nembutal i.v. (n=7)	Halothane (n=14)	Somnotol i.p. (n=16)	Canada (n=12)
Positive (PHF-like responses)	0	3	4	6	4
Fall in MAP	0	2	6	5	1
No change	0	0	3	0	1
Unstable BP	14	2	1	5	2

CHAPTER 9

Summary

9.1 SUMMARY

In this thesis I have explored a number of mechanisms which may be relevant to glucocorticoid-induced hypertension in man and rat. Further understanding of the glucocorticoid-mediated human hypertensive syndromes is required. The existing dogma has been that glucocorticoid-induced hypertension is explained by glucocorticoid occupancy of the mineralocorticoid receptor, with consequent salt and water retention leading to volume expansion, increase in cardiac output and rise in blood pressure. Data from experimental sheep, human and rat studies do not support the notion that glucocorticoid-induced hypertension is mediated by the mineralocorticoid receptor or salt and water retention and repeatedly these studies suggest that there may be other mechanisms by which glucocorticoids raise blood pressure. In the sheep, ACTH-induced hypertension was not reproduced by infusion of the major ovine corticosteroids, an experimental result which gave rise to the notion of a 'hypertensinogenic' class of steroid activity. In man and rat, antagonism of either the glucocorticoid or mineralocorticoid receptor did not prevent glucocorticoid-induced hypertension.

Review of the literature reveals accumulating evidence for a role for adrenal glucocorticoids in the maintenance of blood pressure in man. Abnormalities

of glucocorticoid production, receptor sensitivity or metabolism may contribute to variations in blood pressure within a population. Further insights are likely to arise from the generation and phenotyping of genetically altered rodents with mutations for various known components of the pathway of glucocorticoid production and metabolism.

Experimental cortisol-induced hypertension in man remains incompletely understood. In this thesis, I have reported the effects of cortisol on endothelium-induced vasodilatation. Oral cortisol over 5 treatment days raised blood pressure and induced endothelial dysfunction, which appeared to be nitric oxide synthase dependent. Despite this, experimental hypertension induced by agonists of the glucocorticoid and mineralocorticoid receptors respectively, was not associated with a similar defect in endothelial vasodilator function. These results suggest that the effect of cortisol on endothelium-dependent vasodilatation is not simply explained by actions on classical glucocorticoid and mineralocorticoid receptors in man.

ACTH-induced hypertension in the rat has been well characterized physiologically though the adrenal steroid mediating the effects of ACTH in this model has not been determined. In this thesis, I have reported results of studies of corticosterone-induced hypertension in the rat. At the doses used, corticosterone administration increased serum corticosterone concentrations

to levels equivalent to those observed in ACTH-induced hypertension. Corticosterone administration reproduced the haemodynamic features and some of the metabolic features of ACTH excess in the rat, suggesting that corticosterone, the major glucocorticoid in the rat, is responsible for ACTH-induced hypertension in the rat. This is similar to the human model of ACTH-induced hypertension, which is explained by the adrenal production of cortisol, the major human glucocorticoid.

Parathyroid hypertensive factor has been identified in plasma from rats with DOCA hypertension, a mineralocorticoid-excess model of hypertension. In ACTH-treated rats, however, prior parathyroidectomy did not prevent or significantly attenuate the increase in blood pressure secondary to exogenous ACTH excess in these studies. Further, I was unable to establish the bioassay to measure PHF, despite using a range of experimental conditions and assistance from the original authors of this putative substance. These results cast doubt over the relevance of this factor to experimental hypertension, and indeed, human hypertension.

In conclusion, despite the recognition for over 50 years that steroids may cause hypertension, the mechanisms by which glucocorticoids raise blood pressure remain incompletely understood. Further studies into the mechanisms of experimental hypertension, focussing on the regulation and

effects of glucocorticoids on the vasculature, are likely to assist in understanding the relevance of cortisol to blood pressure regulation and the contribution of abnormalities of cortisol production and metabolism to essential hypertension in man.

Bibliography

Addison, T. On the Constitutional and Local Effects of Disease of the Supra-renal Capsules. 1855. London, Highley.

Agarwal AK, Monder C, Eckstein B, White PC. Cloning and expression of rat cDNA encoding corticosteroid 11 beta-dehydrogenase. *Journal of Biological Chemistry* 1989;264:18939-18943.

Agrest A, Finkielman S, Elijovich F. Hemodynamics of arterial hypertension in Cushing's syndrome. (Spanish). *Medicina* 1974;34:457-462.

Akana SF, Cascio CS, Shinsako J, Dallman MF. Corticosterone: narrow range required for normal body and thymus weight and ACTH. *American Journal of Physiology* 1985;249:t-32

Akata T, Boyle WA. Volatile anesthetic actions on contractile proteins in membrane- permeabilized small mesenteric arteries. *Anesthesiology* 1995;82:700-712.

Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS. Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme. *Molecular & Cellular Endocrinology* 1994;105:R11-R17.

Alfaidy N, Blotchabaud M, Bonvalet JP, et al. Vasopressin potentiates mineralocorticoid selectivity by stimulating 11-beta hydroxysteroid

dehydrogenase in rat collecting duct. *Journal of Clinical Investigation.* 1997;100:2437-2442.

Altura BM. Role of glucocorticoids in regulation of local blood flow. *American Journal of Physiology* 1966;211:1393-1397.

Animal Resources Centre WA: *A User's Guide to ARC animals and products*, Canning Vale, 1995:

Arai K, Chrousos GP. Hormone-nuclear receptor interactions in health and disease. Glucocorticoid resistance. *Baillieres Clinical Endocrinology & Metabolism* 1994;8:317-331.

Armanini D, Karbowiak I, Funder JW. Affinity of liquorice derivatives for mineralocorticoid and glucocorticoid receptors. *Clinical Endocrinology* 1983;19:609-612.

Arriza JL, Simerly RB, Swanson LW, Evans RM. The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron* 1988;1:887-900.

Arriza JL, Weinberger C, Cerelli G, et al. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* 1987;237:268-275.

Axelrod L. Inhibition of prostacyclin production mediates permissive effect of glucocorticoids on vascular tone. Perturbations of this mechanism contribute

to pathogenesis of Cushing's syndrome and Addison's disease. *Lancet* 1983;1:904-906.

Bacon GE, Kenny FM, Murdaugh HV, Richards C. Prolonged serum half-life of cortisol in renal failure. *Johns Hopkins Medical Journal* 1973;132:127-130.

Bamberger CM, Bamberger AM, de CM, Chrousos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. *Journal of Clinical Investigation.* 1995;95:2435-2441.

Bamberger CM, Bamberger AM, Wald M, Chrousos GP, Schulte HM. Inhibition of mineralocorticoid activity by the beta-isoform of the human glucocorticoid receptor. *Journal of Steroid Biochemistry & Molecular Biology* 1997;60:43-50.

Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocrine Reviews* 1996;17:245-261.

Barker DJ. The fetal origins of adult hypertension. *Journal of Hypertension - Supplement* 1992;10:S39-S44.

Barker DJ, Godfrey KM, Osmond C, Bull A. The relation of fetal length, ponderal index and head circumference to blood pressure and the risk of hypertension in adult life. *Paediatric & Perinatal Epidemiology* 1992;6:35-44.

Barker DJP, Bull AR, Osmond C, Simmons SJ. Fetal and placental size and the risk of hypertension in adult life. *British Medical Journal* 1990;301:259-262.

Baron JH, Nabarro JD, Slater JD, Tuffley R. Metabolic studies, aldosterone secretion rate, and plasma renin after carbenoxolone sodium. *British Medical Journal* 1969;2:793-795.

Bartholinus, C. Anatomicae Institutiones Corporis Humani. Wittenberg.

Baylis C, Vallance P. Measurement of nitrite and nitrate levels in plasma and urine - what does this measure tell us about the activity of the endogenous nitric oxide system. *Current Opinion in Nephrology & Hypertension* 1998;7:59-62.

Beischer NA, Sivasamboo R, Vohra S, Silpisornkosal S, Reid S. Placental hypertrophy in severe pregnancy anaemia. *Journal of Obstetrics & Gynaecology of the British Commonwealth* 1970;77:398-409.

Beitins IZ, Bayard F, Ances IG, Kowarski A, Migeon CJ. The metabolic clearance rate, blood production, interconversion and transplacental passageof cortisol and cortisone in pregnancy near term. *Pediatric Research* 1973;7:509-519.

Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CR. Glucocorticoid exposure in utero: new model for adult hypertension. *Lancet* 1993;341:339-341.

Benishin CG, Lewanczuk RZ, Shan J, Pang PK: Purification and structural characterization of parathyroid hypertensive factor. *Journal of Cardiovascular Pharmacology* 1994;23:Suppl 2:S9-13

Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. *Hypertension* 1995;25:918-923.

Berger S, Bleich M, Schmid W, et al. Mineralocorticoid receptor knockout mice: pathophysiology of Na+ metabolism. *Proceedings of the National Academy of Sciences of the United States of America* 1998;95:9424-9429.

Berretta-Piccola C, Davies DL, Brown JJ, et al. The relation of arterial pressure with plasma and body electrolytes is similar in Conn's syndrome and essential hypertension. *Clinical Science* 1995;63:89s-92s.

Bockman CS, Jeffries WB, Pettinger WA, Abel PW. Enhanced release of endothelium-derived relaxing factor in mineralocorticoid hypertension. *Hypertension* 1992;20:304-313.

Bonvalet JP. Regulation of sodium transport by steroid hormones. *Kidney International* 1998;S-S

Borst JGG, Ten Holt SP, DeVries LA. Synergistic action of liquorice and cortisone in Addison's and Simmond's disease. *Lancet* 1953;657

Brand E, Kato N, Chatelain N, et al. Structural analysis and evaluation of the 11 beta-hydroxysteroid dehydrogenase type 2 (11 beta-HSD2) gene in human essential hypertension. *Journal of Hypertension* 1998;16:1627-1633.

Bravo EL, Kageyama Y. Dietary calcium supplementation prevents the development of hypertension in deoxycorticosterone-salt-treated dogs. *Journal of Cardiovascular Pharmacology* 1994;23:Suppl 2:S27-30.

Brem AS, Bina RB, King T, et al. 11-beta-OH-progesterone affects vascular glucocorticoid metabolism and contractile response. *Hypertension* 1997;30:449-454.

Brown-Sequard E. Recherches exp imentales sur la physiologie et la pathologie des capsules surr ales. *Arch G M* 1856;8:385.

Brown EA, Markandu ND, Sagnella GA, Jones BE, MacGregor GA. Lack of effect of captopril on the sodium retention of nephrotic syndrome. *Nephron* 1984;37:43-48.

Bruning TA, Chang PC, Kemme MJ, Vermeij P, Pfaffendorf M, van ZP. Comparison of cholinergic vasodilator responses to acetylcholine and methacholine in the human forearm. *Blood Pressure* 1996;5:333-341.

Bujalska I, Shimojo M, Howie A, Stewart PM. Human 11 beta-hydroxysteroid dehydrogenase: studies on the stably transfected isoforms and localization of the type 2 isozyme within renal tissue. *Steroids* 1997;62:77-82.

Burton AF, Turnell RW. 11-Dehydrocorticoids in tissues of mice. *Canadian Journal of Biochemistry* 1968;46:497-502.

Butkus A, Congiu M, Scoggins BA, Coghlan JP. The affinity of 17 alpha-hydroxyprogesterone and 17 alpha, 20 alpha-dihydroxyprogesterone for classical mineralocorticoid or glucocorticoid receptors. *Clinical and Experimental Pharmacology and Physiology* 1982;9:157-163.

Calver A, Collier J, Moncada S, Vallance P. Effect of local intra-arterial NG-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. *Journal of Hypertension* 1992a;10:1025-1031.

Calver A, Collier J, Vallance P. Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *Journal of Clinical Investigation.* 1992b;90:2548-2554.

Card WI, Mitchell W, Strong JA. Effects of liquorice and its derivatives on salt and water metabolism. *Lancet* 1953;663

Chen PY, Gladish RD, Sanders PW. Vascular smooth muscle nitric oxide synthase anomalies in dahl/rapp salt-sensitive rats. *Hypertension* 1998;31:918-924.

Chen PY, Sanders PW. L-arginine abrogates salt-sensitive hypertension in Dahl/Rapp rats. *Journal of Clinical Investigation.* 1991;88:1559-1567.

Chen PY, Sanders PW. Role of nitric oxide synthesis in salt-sensitive hypertension in Dahl/Rapp rats. *Hypertension* 1993;22:812-818.

Chowienczyk PJ, Watts GF, Cockcroft JR, Ritter JM. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet* 1992;340:1430-1432.

Chrousos GP, Laue L, Nieman LK, et al. Glucocorticoids and glucocorticoid antagonists: lessons from RU 486. *Kidney International - Supplement* 1988;26:S18-S23

Clore JN, Estep H, Ross-Clunis H, Watlington CO. Adrenocorticotropin and cortisol-induced changes in urinary sodium and potassium excretion in man: effects of spironolactone and RU-486. *Journal of Clinical Endocrinology and Metabolism* 1988;67:824.

Cockcroft JR, Chowienczyk PJ, Benjamin N, Ritter JM. Preserved endothelium-dependent vasodilatation in patients with essential hypertension. *New England Journal of Medicine* 1994;330:1036-1040.

Coghlan JP, Butkus A, Denton DA, McDougall JG, Scoggins BA, Whitworth JA. Blood pressure and metabolic effects of 9-alpha-fluoro-hydrocortisone in sheep. *Clinical & Experimental Hypertension* 1979;1:629-648.

Coghlan JP, Denton DA, Fan JS, McDougall JG, Scoggins BA. Hypertensive effect of 17alpha, 20alpha-dihydroxyprogesterone and 17alpha-hydroxyprogesterone in the sheep. *Nature* 1976;263:608-609.

Cole TJ, Blendy JA, Monaghan AP, et al. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes and Development* 1995;9:1608-1621.

Connell JM, Fisher BM, Davidson G, Fraser R, Whitworth JA. Effect of sodium depletion on pressor responsiveness in ACTH- induced hypertension in man. *Clinical and Experimental Pharmacology and Physiology* 1987;14:237-242.

Connell JM, Kenyon CJ, Ingram M, et al. Corticosteroids in essential hypertension: multiple candidate loci and phenotypic variation. *Clinical and Experimental Pharmacology and Physiology* 1996;23:369-374.

Connell JM, Whitworth JA, Davies DL, Lever AF, Richards AM, Fraser R. Effects of ACTH and cortisol administration on blood pressure, electrolyte metabolism, atrial natriuretic peptide and renal function in normal man. *Journal of Hypertension* 1987;5:425-433.

Connell JM, Whitworth JA, Davies DL, Richards AM, Fraser R. Haemodynamic, hormonal and renal effects of adrenocorticotrophic hormone in sodium-restricted man. *Journal of Hypertension* 1988;6:17-23.

Connor JM, Whitworth JA, Inglis GC, Kenyon CJ, Lever AF, Fraser R. Changes in body electrolytes, renal function, blood pressure and pressor responsivenessduring development of ACTH and cortisol excess in man, in

Mantero F, Vescei P (eds): *Corticosteroids and Peptide Hormones in Hypertension*. New York, Raven Press; 1987:179-190.

Couette B, Fagart J, Jalaguier S, Lombes M, Souque A, Rafestin-Oblin ME. Ligand-induced conformational change in the human mineralocorticoid receptor occurs within its hetero-oligomeric structure. *Biochemical Journal* 1996;315:421-427.

Cushing H. The basophil adenomas of the pituitary body and their clinical manifestations (Pituitary basophilism). *Bulletin of the Johns Hopkins Hospital* 1932;50:137.

Dalakos TG, Elias AN, Anderson GHJ, Streeten DH, Schroeder ET. Evidence for an angiotensinogenic mechanism of the hypertension of Cushing's syndrome. *Journal of Clinical Endocrinology & Metabolism* 1978;46:114-118.

Danese RD, Aron DC. Cushing's syndrome and hypertension. *Endocrinology & Metabolism Clinics of North America* 1994;23:299-324.

Davis EA, Morris DJ. Medicinal uses of licorice through the millennia: the good and plenty of it. *Molecular & Cellular Endocrinology* 1991;78:1-6.

Dawborn JK. The effect of prolonged intravenous infusion of aldosterone in a normal human subject. *Medical Journal of Australia* 1969;1:1079-1083.

Deng AY, Rapp JP. Locus for the inducible, but not a constitutive, nitric oxide synthase cosegregates with blood pressure in the Dahl salt- sensitive rat. *Journal of Clinical Investigation.* 1995;95:2170-2177.

Distler A, Philipp T. On the mechanism of mineralocorticoid induced hypertension: effects of 9αfluorohydrocortisone on haemodynamics in man., in Kaufman W, Krause DK (eds): *Central Nervous Control of Sodium Balance - Relations to the Renin-Angiotensin System.* Stuttgart, George Thieme; 1976:16.

Distler A, Philipp T, Luth B, Wucherer G. Studies on the mechanism of mineralocorticoid-induced blood pressure increase in man. *Clinical Science* 1979;57:Suppl-305s

Dorhout Mees EJ, Geers AB, Koomans HA. Blood volume control and sodium retention in the nephrotic syndrome: a controversial pathophysiological concept. *Nephron* 1984;36:201-211.

Duncan RL, Grogan WM, Kramer LB, Watlington CO. Corticosterone's metabolite is an agonist for Na+ transport stimulation in A6 cells. *American Journal of Physiology* 1988;255:t-48

Dunne FP, Elliot P, Gammage MD, et al. Cardiovascular function and glucocorticoid replacement in patients with hypopituitarism. *Clinical Endocrinology* 1995;43:623-629.

Ecker, A. Der feinere bau der nebennieren beim menschen und den wirbelthierclassen. Braunschweig . 1846.

Edwards CR, Benediktsson R, Lindsay RS, Seckl JR. Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? *Lancet* 1993;341:355-357.

Edwards CR, Stewart PM, Burt D, et al. Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. *Lancet* 1988;2:986-989.

Edwards CR, Walker BR. Cortisol and hypertension: what was not so apparent about "apparent mineralocorticoid excess". *Journal of Laboratory and Clinical Medicine* 1993;122:632-635.

Edwards CRW, Hayman A. Enzyme protection of the mineralocorticoid receptor: Evidence in favour of the hemi-acetal structure of aldosterone., in Bonvalet JP, Farman N, Lombes M (eds): *Aldosterone: Fundamental Aspects*. London, John Libbey Eurotext; 1991:67.

Edwards CRW, Stewart PM. Defective cortisol:cortisone shuttle: Novel mechanism for the genesis of hypertension., in Mantero F (ed): *The adrenal and hypertension: from cloning to clinic. Sereno symposium.* Raven Press.; 1989:345

Englert JE, Brown H, Wallach S, Simons EL. Metabolism of free and conjugated 17-hydroxycorticosteroids in subjects with uraemia. *Journal of Clinical Endocrinology and Metabolism* 1958;18:36-45.

Epstein MT, Espiner EA, Donald RA, Hughes H. Effect of eating liquorice on the renin-angiotensin aldosterone axis in normal subjects. *British Medical Journal* 1977a;1:488-490.

Epstein MT, Espiner EA, Donald RA, Hughes H. Liquorice toxicity and the renin-angiotensin-aldosterone axis in man. *British Medical Journal* 1977b;1:209-210.

Epstein MT, Espiner EA, Donald RA, Hughes H, Cowles RJ, Lun S. Licorice raises urinary cortisol in man. *Journal of Clinical Endocrinology & Metabolism* 1978;47:397-400.

Escher G, Vogt B, Beck T, Guntern D, Frey BM, Frey FJ. Reduced 11-beta-hydroxysteroid dehydrogenase activity in the remaining kidney following nephrectomy. *Endocrinology* 1998;139:1533-1539.

Farman N. Molecular and cellular determinants of mineralocorticoid selectivity. *Current Opinion in Nephrology and Hypertension* 1999;8:45-51.

Ferrari P, Obeyesekere VR, Li K, Andrews RK, Krozowski ZS:. The 11 beta-hydroxysteroid dehydrogenase type II enzyme: biochemical consequences of the congenital R337C mutation. *Steroids* 1996;61:197-200.

Field MJ, Stanton BA, Giebisch GH. Differential acute effects of aldosterone, dexamethasone, and hyperkalemia on distal tubular potassium secretion in the rat kidney. *Journal of Clinical Investigation.* 1984;74:1792-1802.

Filipovsky J, Ducimetiere P, Eschwege E, Richard JL, Rosselin G, Claude JR. The relationship of blood pressure with glucose, insulin, heart rate, free fatty acids and plasma cortisol levels according to degree of obesity in middle-aged men. *Journal of Hypertension* 1996;14:229-235.

Forte P, Copland M, Smith LM, Milne E, Sutherland J, Benjamin N. Basal nitric oxide synthesis in essential hypertension. *Lancet* 1997;349:837-842.

Fredlund P, Saltman S, Catt KJ. Aldosterone production by isolated adrenal glomerulosa cells: stimulation by physiological concentrations of angiotensin II. *Endocrinology* 1975;97:1577-1586.

Freeman RH, Davis JO, Fullerton D. Chronic ACTH administration and the development of hypertension in rats. *Proceedings of the Society for Experimental Biology & Medicine* 1980;163:473-477.

Funder J, Myles K. Exclusion of corticosterone from epithelial mineralocorticoid receptors is insufficient for selectivity of aldosterone action: in vivo binding studies. *Endocrinology* 1996;137:5264-5268.

Funder JW. Mineralocorticoids, glucocorticoids, receptors and response elements. *Science* 1993;259:1132-1133.

Funder JW. Apparent mineralocorticoid excess. *Endocrinology & Metabolism Clinics of North America* 1995;24:613-621.

Funder JW. Mineralocorticoid receptors and glucocorticoid receptors. *Clinical Endocrinology* 1996;45:651-656.

Funder JW. Aldosterone, salt and cardiac fibrosis. *Clinical & Experimental Hypertension* 1997;19:885-899.

Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* 1988;242:583-585.

Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980;288:373-376.

Gairard A, Berthelot A, Schleiffer R, Pernot F. Parathyroidectomy significantly decreases hypertension in spontaneously hypertensive and deoxycorticosterone plus saline treated rats. *Canadian Journal of Physiology and Pharmacology* 1982;60:208-212.

Gambone LM, Murray PA, Flavahan NA. Synergistic interaction between endothelium-derived NO and prostacyclin in pulmonary artery: potential role for K+ATP channels. *British Journal of Pharmacology* 1997;121:271-279.

Gerhard M, Roddy M, Creager SJ, Creager MA. Age progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. *Hypertension* 1996;27:849-853.

Godfrey KM, Redman CW, Barker DJ, Osmond C. The effect of maternal anaemia and iron deficiency on the ratio of fetal weight to placental weight. *British Journal of Obstetrics & Gynaecology* 1991;98:886-891.

Goldfien A, Laidlaw JC, Haydar NA, Renold AE, Thorn GW. Fluorohydrocortisone and chlorohydrocortisone, highly potent derivatives of compound F. *New England Journal of Medicine* 1955;252:415-421.

Greenfield ADM, Patterson GC. Reactions of the blood vessels of the human forearm to increases in transmural pressure. *Journal of Physiology* 1954;125:508-524.

Grekin RJ, Dale SL, Gaunt R, Melby JC. Steroid secretion by the enucleated rat adrenal: measurements during salt retention and the development of hypertension. *Endocrinology* 1972;91:1166-1171.

Greminger P, Vetter W, Groth H, et al. Captopril in Cushing's syndrome. *Klinische Wochenschrift* 1984;62:855-858.

Haack D, Mohring J, Mohring B, Petri M, Hackenthal E. Comparative study on development of corticosterone and DOCA hypertension in rats. *American Journal of Physiology* 1977;233:F403-F411

Haber E, Sancho J, Re R, Burton J, Barger AC. The role of the renin--angiotensin--aldosterone system in cardiovascular homeostasis in normal man. *Clinical Science & Molecular Medicine - Supplement* 1975;2:49s-52s.

Handa M, Kondo K, Suzuki H, Saruta T. Dexamethasone hypertension in rats: role of prostaglandins and pressor sensitivity to norepinephrine. *Hypertension* 1984;6:t-41

Hartman FA, Brownell KA, Hartman WE, Dean GA, MacArthur GC. The hormone of the adrenal cortex. *American Journal of Physiology* 1928;86:853

Hatton DC, McCarron DA. Dietary calcium and blood pressure in experimental models of hypertension. A review. *Hypertension* 1994;23:513-530.

Hattori Y, Akimoto K, Nakanishi N, Kasai K. Glucocorticoid regulation of nitric oxide and tetrahydrobiopterin in a rat model of endotoxic shock. *Biochemical & Biophysical Research Communications* 1997;240:298-303.

Hayashi T, Nakai T, Miyabo S. Glucocorticoids increase Ca2+ uptake and [3H]dihydropyridine binding in A7r5 vascular smooth muscle cells. *American Journal of Physiology* 1991;261:t-14

Hermus A, Hobma S, Pieters G, van dC, Smals A, Kloppenborg P. Are the hypokalaemia and hypertension in Cushing's disease caused by apparent mineralocorticoid excess?. *Hormone & Metabolic Research* 1991;23:572-573.

Hickler RB, Thompson GR, Fox LM, Hamlin JT. Successful treatment of orthostatic hypotension with 9-alpha-fluorohydrocortisone. *New England Journal of Medicine* 1959;261:788-791.

Higashi Y, Oshima T, Ozono R, Watanabe M, Matsuura H, Kajiyama G. Effects of L-arginine infusion on renal haemodynamics in patients with mild essential hypertension. *Hypertension* 1995;25:898-902.

Himathongkam T, Dluhy RG, Williams GH. Potassim-aldosterone-renin interrelationships. *Journal of Clinical Endocrinology & Metabolism* 1975;41:153-159.

Hirasawa G, Sasano H, Takahashi K, et al. Colocalization of 11 beta-hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human epithelia. *Journal of Clinical Endocrinology & Metabolism* 1997;82:3859-3863.

Hishikawa K, Nakaki T, Suzuki H, Kato R, Saruta T. Role of L-arginine-nitric oxide pathway in hypertension. *Journal of Hypertension* 1993;11:639-645.

Hoefnagels WHL, Kloppenborg WC. Antimineralocorticoid effects of dexamethasone in subjects treated with glycyrrhetinic acid. *Journal of Hypertension* 1983;1 (suppl 2):313-315.

Hollenberg SM, Weinberger C, Ong ES, et al. Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* 1985;318:635-641.

Huizenga NA, Koper JW, De LP, et al. A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. *Journal of Clinical Endocrinology & Metabolism* 1998;83:144-151.

Iranmanesh A, Lizarralde G, Short D, Veldhuis JD. Intensive venous sampling paradigms disclose high frequency adrenocorticotropin release

episodes in normal men. *Journal of Clinical Endocrinology & Metabolism* 1990;71:1276-1283.

Joannides R, Haefeli WE, Linder L, et al. Nitric oxide is responsible for flow dependent dilatation of human peripheral conduit arteries in vivo. *Circulation* 1995;91:1314-1319.

Johl A, Riniker B, Schenkel-Hulliger L. Identity of structure of ovine and bovine ACTH: correction of revised structure of the ovine hormone. *FEBS Letters* 1974;45:172-174.

Kageyama Y, Suzuki H, Arima K, Saruta T. Oral calcium treatment lowers blood pressure in renovascular hypertensive rats by suppressing the renin-angiotensin system. *Hypertension* 1987;10:375-382.

Kalant N, Gupta DD, Despointes R, Giroud CJP. Mechanisms of edema formation in experimental nephrosis. *American Journal of Physiology* 1962;202:91-96.

Kang JS, Cregor MD, Smith JB. Effect of calcium on blood pressure, platelet aggregation and erythrocyte sodium transport in Dahl salt-sensitive rats. *Journal of Hypertension* 1990;8:245-250.

Kannan CR. The Glucocorticoid Hormones, in Kannan CR (ed): *The Adrenal Gland*. New York, Plenum Publishing; 1988:1-29.

Kanse SM, Takahashi K, Warren JB, Ghatei M, Bloom SR. Glucocorticoids induce endothelin release from vascular smooth muscle cells

but not endothelial cells. *European Journal of Pharmacology* 1991;199:99-101.

Kassirer JP, London AM, Goldman DM, Schwartz WB. On the pathogenesis of metabolic alkalosis in hyperaldosteronism. *American Journal of Medicine* 1970;49:315

Kelly JJ, Mangos GJ, Williamson PM, Whitworth JA. Cortisol and hypertension. *Clinical and Experimental Pharmacology and Physiology* 1998;25:S51-S56

Kelly JJ, Tam SH, Lawson J, Whitworth JA. The nitric oxide system in cortisol-induced hypertension in humans. *Nephrology* 1997;3 (Supplement 1):525(Abstract)

Kelly JJ, Tam SH, Williamson PM, Lawson J, Whitworth JA. The nitric oxide system and cortisol-induced hypertension in humans. *Clinical and Experimental Pharmacology and Physiology* 1998;25:945-946.

Khan MH, Sullivan FM. The pharmacology of carbenoxolone sodium, in Robson JM, Sullivan FM (eds): *Symposium on Carbenoxolone Sodium.* London, Butterworths; 1967:5

Kitamura K, Kangawa K, Kawamoto M, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochemical & Biophysical Research Communications* 1993;192:553-560.

Kitanaka S, Katsumata N, Tanae A, et al. A new compound heterozygous mutation in the 11-beta hydroxysteroid dehydrogenase type 2 gene in a case of apparent mineralocorticoid excess. *Journal of Clinical Endocrinology & Metabolism* 1997;82:4054-4058.

Klett C, Ganten D, Hellmann W, et al. Regulation of hepatic angiotensinogen synthesis and secretion by steroid hormones. *Endocrinology* 1992;130:3660-3668.

Knowles RG, Salter M, Brooks SL, Moncada S. Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochemical & Biophysical Research Communications* 1990;172:1042-1048.

Kotelevtsev Y, Brown RW, Fleming S, et al. Hypertension in mice lacking 11β-hydroxysteroid dehydrogenase type 2. *Journal of Clinical Investigation.* 1999;103:683-689.

Krakoff LR. Measurement of plasma renin substrate by radioimmunoassay of angiotensin. I. Concentration in syndromes associated with steroid excess. *Journal of Clinical Endocrinology & Metabolism* 1973;37:110-117.

Krozowski Z. The short-chain alcohol dehydrogenase superfamily: variations on a common theme. *Journal of Steroid Biochemistry & Molecular Biology* 1994;51:125-130.

Krozowski ZS, Funder JW. Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid

specificity. *Proceedings of the National Academy of Sciences of the United States of America* 1983;80:6056-6060.

Krozowski ZS, Provencher PH, Smith RE, Obeyesekere VR, Mercer WR, Albiston AL. Isozymes of 11 beta-hydroxysteroid dehydrogenase: which enzyme endows mineralocorticoid specificity? *Steroids* 1994;59:116-120.

Leeper LL, Schroeder R, Henning SJ. Kinetics of circulating corticosterone in infant rats. *Pediatric Research* 1988;24:595-599.

Lewanczuk RZ, Chen A, Pang PK. The effects of dietary calcium on blood pressure in spontaneously hypertensive rats may be mediated by parathyroid hypertensive factor. *American Journal of Hypertension* 1990;3:Pt 1):349-53

Lewanczuk RZ, Pang PK. Parathyroid hypertensive factor is present in DOCA-salt but not two-kidney-one-clip hypertensive rats. *American Journal of Hypertension* 1991;4:Pt 1):802-5

Lewanczuk RZ, Pang PK. The occurrence of parathyroid hypertensive factor (PHF) in Dahl rats. *American Journal of Hypertension* 1993;6:758-762.

Lewanczuk RZ, Wang J, Zhang ZR, Pang PK. Effects of spontaneously hypertensive rat plasma on blood pressure and tail artery calcium uptake in normotensive rats. *American Journal of Hypertension* 1989;2:26-31.

Li A, Li KX, Marui S, et al. Apparent mineralocorticoid excess in a Brazilian kindred: hypertension in the heterozygote state. *Journal of Hypertension* 1997;15:t-402

Li M, Dusting GJ, Whitworth JA. Inhibition of NO synthesis has an additive effect on hypertension induced by ACTH in conscious rats. *Clinical and Experimental Pharmacology and Physiology* 1992;19:675-681.

Li M, Fraser T, Wang J, Whitworth JA. Dexamethasone-induced hypertension in the rat: effects of L-arginine. *Clinical and Experimental Pharmacology and Physiology* 1997;24:730-732.

Li M, Wen C, Fraser T, Whitworth JA. Adrenocorticotrophin-induced hypertension: effects of mineralocorticoid and glucocorticoid receptor antagonism. *Journal of Hypertension* 1999;17:419-426.

Li M, Whitworth JA. ACTH hypertension in the rat: role of sodium chloride. *Clinical and Experimental Hypertension* 1992;14:567-585.

Li M, Wong KS, Martin A, Whitworth JA. Adrenocorticotrophin-induced hypertension in rats. Role of progesterone and digoxin-like substances. *American Journal of Hypertension* 1994;7:59-68.

Lin CM, Saito K, Tsujino T, Yokoyama M. Calcium supplementation inhibits the expression of parathyroid hypertensive factor in DOCA-salt hypertensive rats. *American Journal of Hypertension* 1994;7:201-204.

Lin D, Sugawara T, Strauss JF, et al. Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* 1995;267:1828-1831.

Lipsett MB, Tomita M, Brandon DD, De VM, Loriaux DL, Chrousos GP. Cortisol resistance in man. *Advances in Experimental Medicine & Biology* 1986;196:97-109.

Litchfield WR, Hunt SC, Jeunemaitre X, et al. Increased urinary free cortisol - a potential intermediate phenotype of essential hypertension. *Hypertension* 1998;31:569-574.

Liu DT, Birchall I, Hewitson T, Kincaid-Smith P, Whitworth JA. Effect of dietary calcium on the development of hypertension and hypertensive vascular lesions in DOCA-salt and two-kidney, one clip hypertensive rats. *Journal of Hypertension* 1994;12:145-153.

Liu W, Wang J, Sauter NK, Pearce D. Steroid receptor heterodimerization demonstrated in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 1995;92:12480-12484.

Lohmeier TEKPR. Chronic effect of ACTH and cortisol excess in arterial pressure in normotensive and hypertensive dogs. *Hypertension* 1982;4:652

Lombes M, Alfaidy N, Eugene E, Lessana A, Farman N, Bonvalet JP. Prerequisite for cardiac aldosterone action. Mineralocorticoid receptor and 11 beta-hydroxysteroid dehydrogenase in the human heart. *Circulation* 1995;92:175-182.

Lombes M, Binart N, Delahaye F, Baulieu EE, Rafestin-Oblin ME. Differential intracellular localization of human mineralocorticosteroid receptor

on binding of agonists and antagonists. *Biochemical Journal* 1994;302:191-197.

Lombes M, Kenouch S, Souque A, Farman N, Rafestin-Oblin ME. The mineralocorticoid receptor discriminates aldosterone from glucocorticoids independently of the 11 beta-hydroxysteroid dehydrogenase. *Endocrinology* 1994;135:834-840.

MacAllister RJ, Calver AL, Collier J, et al. Vascular and hormonal responses to arginine: provision of substrate for nitric oxide or non-specific effect? *Clinical Science* 1995;89:183-190.

Macefield VG, Williamson PW, Wilson LR, Kelly JJ, Gandevia SC, Whitworth JA. Muscle sympathetic vasoconstrictor activity in hydrocortisone-induced hypertension in humans. *Blood Pressure* 1998;7:215-222.

MacKenzie MA, Hoefnagels WH, Jansen RW, Benraad TJ, Kloppenborg PW. The influence of glycyrrhetinic acid on plasma cortisol and cortisone in healthy young volunteers. *Journal of Clinical Endocrinology & Metabolism* 1990;70:1637-1643.

Mangelsdorf DJ, Thummel C, Beato M, et al. The nuclear receptor superfamily: the second decade. *Cell* 1995;83:835-839.

Mantero F, Palermo M, Petrelli MD, Tedde R, Stewart PM, Shackleton CH. Apparent mineralocorticoid excess: type I and type II. *Steroids* 1996;61:193-196.

Martin A, Kelly JJ, Williamson PM, Mangos GJ, Whitworth JA. Serum erythropoietin concentrations in cortisol-induced hypertension. (abstract). *Journal of Hypertension* 1998;12:S37.

Martin K, Zipser R, Horton R. Effect of prostaglandin inhibition on the hypertensive action of sodium-retaining steroids. *Hypertension* 1981;3:622-628.

Mazzoochi G, Rossi GP, Neri G, Malendowicz LK, Albertin G, Nussdorfer GG. Identification of active type II 11 beta-hydroxysteroid dehydrogenase (11 beta HSD2) expression in the human adrenal cortex (ADC). (abstract). *Hypertension* 1999;32:799.

McKnight JA, McCance DR, Roberts G, Sheridan B, Atkinson AB. Basal and saline-stimulated plasma atrial natriuretic hormone in Cushing's syndrome. *Acta Endocrinologica* 1991;124:7-11.

Mendlowitz M, Gitlow S, Nagano M. Work of digital vasoconstriction produced by infused norephrine in Cushing's syndrome. *Journal of Applied Physiology* 1958;13:252-256.

Mercer WR, Krozowski ZS. Localization of an 11 beta hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. *Endocrinology* 1992;130:540-543.

Microsoft. Microsoft Encarta 97 Encyclopedia. 1996. United States, Microsoft Corporation (CD Rom).

Monder C, Shackleton CH. 11 beta-Hydroxysteroid dehydrogenase: fact or fancy?. *Steroids* 1984;44:383-417.

Monder C, Stewart PM, Lakshmi V, Valentino R, Burt D, Edwards CR. Licorice inhibits corticosteroid 11 beta-dehydrogenase of rat kidney and liver: in vivo and in vitro studies. *Endocrinology* 1989;125:1046-1053.

Montrella-Waybill M, Clore JN, Schoolwerth AC, Watlington CO. Evidence that high dose cortisol-induced Na+ retention in man is not mediated by the mineralocorticoid receptor. *Journal of Clinical Endocrinology and Metabolism* 1991;72:1060-1066.

Moore KL. The Urogenital System, in Moore KL (ed): *The Developing Human*. Philadephia, W. B. Saunders Company; 1982:255-295.

Morris DJ, Latif SA, Rokaw MD, Watlington CO, Johnson JP. A second enzyme protecting mineralocorticoid receptors from glucocorticoid occupancy. *American Journal of Physiology - Cell Physiology* 1998;43:C1245-C1252

Mulatero P, Panarelli M, Schiavone D, et al. Impaired cortisol binding to glucocorticoid receptors in hypertensive patients. *Hypertension* 1997;30:1274-1278.

Mune T, Rogerson FM, Nikkila H, Agarwal AK, White PC. Human hypertension caused by mutations in the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. *Nature Genetics* 1995;10:394-399.

Munro Neville A, O'Hare MJ. Historical Aspects, in *The Human Adrenal Cortex*. Hampshire, Springer-Verlag; 1982:2-7.

Nakamoto H, Suzuki H, Kageyama Y, et al. Characterization of alterations of hemodynamics and neuroendocrine hormones in dexamethasone induced hypertension in dogs. *Clinical and Experimental Hypertension* 1991;13:587-606.

Nelson DH. The Secretion of the Adrenal Cortex and Steroid Biosynthesis, in Nelson DH (ed): *The Adrenal Cortex: Physiological Function and Disease*. Philadelphia, W.B.Saunders; 1980:10-23.

New MI, Levine LS, Biglieri EG, Pareira J, Ulick S. Evidence for an unidentified steroid in a child with apparent mineralocorticoid hypertension. *Journal of Clinical Endocrinology & Metabolism* 1977;44:924-933.

New MI, Nemery RL, Chow DM, et al. Low renin hypertension of childhood., in Mantero F (ed): *The adrenal and hypertension: from cloning to clinic. Sereno Symposium.* Raven Press; 1989:321

Nicholls MG, Ramsay LE, Boddy K, Fraser R, Morton JJ, Robertson JI. Mineralocorticoid-induced blood pressure, electrolyte, and hormone changes, and reversal with spironolactone, in healthy men. *Metabolism: Clinical & Experimental* 1979;28:584-593.

Nikkila H, Tannin GM, New MI, et al. Defects in the HSD11 gene encoding 11 beta-hydroxysteroid dehydrogenase are not found in patients with

apparent mineralocorticoid excess or 11-oxoreductase deficiency. *Journal of Clinical Endocrinology & Metabolism* 1993;77:687-691.

Noon JP, Walker BR, Hand MF, Webb DJ. Impairment of forearm vasodilatation to acetylcholine in hypercholesterolaemia is reversed by aspirin. *Cardiovascular Research* 1998;38:480-484.

Oberfield SE, Levine LS, Carey RM, Greig F, Ulick S, New MI. Metabolic and blood pressure responses to hydrocortisone in the syndrome of apparent mineralocorticoid excess. *Journal of Clinical Endocrinology and Metabolism* 1983;56:332-339.

Obeyesekere VR, Ferrari P, Andrews RK, et al. The R337C mutation generates a high Km 11 beta-hydroxysteroid dehydrogenase type II enzyme in a family with apparent mineralocorticoid excess. *Journal of Clinical Endocrinology & Metabolism* 1995;80:3381-3383.

Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987;327:524-525.

Pang PK, Benishin CG, Shan J, Lewanczuk RZ. PHF: the new parathyroid hypertensive factor. *Blood Pressure* 1994;3:148-155.

Pang PK, Lewanczuk RZ. Parathyroid origin of a new circulating hypertensive factor in spontaneously hypertensive rats. *American Journal of Hypertension* 1989;2:Pt 1):898-902

Pang PKT, Shan JJ, Lewanczuk RZ, et al. Parathyroid hypertensive factor and intracellular calcium regulation. *Journal of Hypertension* 1996;14:1053-1060.

Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Role of endothelium derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation* 1993;87:1468-1474.

Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Impaired endothelium-dependent vasodilation in patients with essential hypertension: evidence that the abnormality is not at the muscarinic receptor level. *Journal of the American College of Cardiology* 1994;23:1610-1616.

Panza JA, Quyyumi AA, Brush JEJ, Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *New England Journal of Medicine* 1990;323:22-27.

Phillip T, Luff B, Wucherer G, Distler A. Mechanisms of mineralocorticoid induced hypertension in man., in Kaufman W, Wambach HA, Meurer KA (eds): *Mineralocorticoid Hypertension*. New York, Springer-Verlag; 1983:129-143.

Phillips DIW, Barker DJP, Fall CHD, et al. Elevated plasma cortisol concentrations - a link between low birth weight and the insulin resistance syndrome. *Journal of Clinical Endocrinology & Metabolism* 1998;83:757-760.

Pirpiris M, Sudhir K, Yeung S, Jennings G, Whitworth JA. Pressor responsiveness in corticosteroid-induced hypertension in humans. *Hypertension* 1992;19:Pt 1):567-74

Pirpiris M, Yeung S, Dewar E, Jennings GL, Whitworth JA. Hydrocortisone-induced hypertension in men. The role of cardiac output. *American Journal of Hypertension* 1993;6:287-294.

Raab W, Humphries J, Lepeschkin E. Potentiation of pressor effects of norepinephrine and epinephrine in man by deoxycorticosterone acetate. *Journal of Clinical Investigation.* 1950;29:1404

Radomski MW, Palmer RM, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 1990;87:10043-10047.

Reevers F. Behandeling van uleus ventriculi in uleus duodenimet succus liquoritiae. *Ned Tijdschr Geneeskd* 1948;92:2968

Resnick LM, Laragh JH, Sealey JE, Alderman MH: Divalent cations in essential hypertension. Relations between serum ionized calcium, magnesium and plasma renin activity. *New England Journal of Medicine* 1983;309:888-891.

Resnick LM, Lewanczuk RZ, Laragh JH, Pang PK. Parathyroid hypertensive factor-like activity in human essential hypertension: relationship

to plasma renin activity and dietary salt sensitivity. *Journal of Hypertension* 1993;11:1235-1241.

Ritchie CM, Sheridan B, Fraser R, et al. Studies on the pathogenesis of hypertension in Cushing's disease and acromegaly. *Quarterly Journal of Medicine* 1990;76:855-867.

Roddie IC, Wallace WFM. Methods of assessment of the effects of drugs on the arterial system in man. *British Journal of Clinical Pharmacology* 1979;7:317-323.

Rogoff JM, Stewart GN. Studies on adrenal insufficiency in dogs. *American Journal of Physiology* 1928;84:860

Rusvai E, Naray FT. A new isoform of 11 beta-hydroxysteroid dehydrogenase in aldosterone target cells. *Journal of Biological Chemistry* 1993;268:10717-10720.

Sakaue M, Hoffman BB. Glucocorticoids induce transcription and expression of the alpha 1B adrenergic receptor gene in DTT1 MF-2 smooth muscle cells. *Journal of Clinical Investigation.* 1991;88:385-389.

Salassa RR, Mattox VR, Rosevear JW. Inhibition of mineralocorticoid activity of licorice by spironolactone. *Journal of Clinical Endocrinology* 1962;22:1156

Saruta T. Mechanism of glucocorticoid-induced hypertension. *Hypertension Research* 1996;19:1-8.

Saruta T, Suzuki H, Handa M, Igarashi Y, Kondo K, Senba S. Multiple factors contribute to the pathogenesis of hypertension in Cushing's syndrome. *Journal of Clinical Endocrinology & Metabolism* 1986;62:275-279.

Schmid PG, Eckstein JW, Abboud FM. Effect of 9-α-fludrohydrocortisone on forearm vascular responses to norepinephrine. *Circulation* 1966;34:625

Scoggins BA, Allen KJ, Coghlan JP, et al. Haemodynamics of ACTH-induced hypertension in sheep. *Clinical Science* 1979;57:Suppl 5:333s-336s

Scoggins BA, Coghlan JP, Denton DA, et al. The role of adrenocortical hormones in ACTH-induced hypertension. *Clinical and Experimental Pharmacology and Physiology* 1975;119-122.

Scoggins BA, Coghlan JP, Denton DA, et al. Metabolic effects of ACTH in the sheep. *American Journal of Physiology* 1974;226:198-205.

Scoggins BA, Coghlan JP, Denton DA, Mason RT, Whitworth JA. A review of mechanisms involved in the production of steroid induced hypertension with particular reference to ACTH dependent hypertension., in Mantero F, Biglieri EG, Edwards CRW (eds): *Endocrinology of Hypertension.* London and New York, Academic Press; 1982:41

Scoggins BA, Denton DA, Whitworth JA, Coghlan JP. ACTH dependent hypertension. *Clinical and Experimental Hypertension* 1984;6:599-646.

Seckl JR, Chapman KE. The 11-beta hydroxysteroid dehydrogenase system, a determinant of glucocorticoid and mineralocorticoid action -

medical and physiological aspects of the 11-beta hydroxysteroid dehydrogenase system. *European Journal of Biochemistry* 1997;249:361-364.

Shackleton CH, Rodriguez J, Arteaga E, Lopez JM, Winter JS. Congenital 11 beta-hydroxysteroid dehydrogenase deficiency associated with juvenile hypertension: corticosteroid metabolite profiles of four patients and their families. *Clinical Endocrinology* 1985;22:701-712.

Shapiro MD, Hasbargen J, Hensen J, Schrier RW. Role of aldosterone in the sodium retention of patients with the nephrotic syndrome. *Nephron* 1990;10:44-48.

Sheppard K, Funder JW. Mineralocorticoid specificity of renal type I receptors: in vivo binding studies. *American Journal of Physiology* 1987;252:t-9

Sigurjonsdottir HA, Ragnarsson J, Franzson L, Sigurdsson G. Is blood pressure commonly raised by moderate consumption of liquorice? *Journal of Human Hypertension* 1995;9:345-348.

Simmons WW, Ungureanu-Longrois D, Smith GK, Smith TW, Kelly RA. Glucocorticoids regulate inducible nitric oxide synthase by inhibiting tetrahydrobiopterin synthesis and L-arginine transport. *Journal of Biological Chemistry* 1996;271:23928-23937.

Smith P. Hypophysectomy and a replacement therapy in the rat. *American Journal of Anatomy* 1930;45:205-274.

Smith RE, Little PJ, Maguire JA, Stein-Oakley AN, Krozowski ZS. Vascular localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme. *Clinical and Experimental Pharmacology and Physiology* 1996;23:549-551.

Soding P, Coghlan JP, Denton DA, Graham WF, Humphery TJ, Scoggins BA. The effect of ACTH on the blood clearance rate of aldosterone, cortisol, 17 alpha-hydroxyprogesterone and 17 alpha, 20 alpha-dihydroxy-4-pregnen-3-one in the sheep. *Journal of Steroid Biochemistry* 1983;18:173-177.

Sonino N, Boscaro M, Paoletta A, Mantero F, Ziliotto D. Ketoconazole treatment in Cushing's syndrome: experience in 34 patients. *Clinical Endocrinology* 1991;35:347-352.

Sorkin SZ. The adrenals before Addison. *Journal of Vascular Research* 1957;24:1238

Soro A, Ingram MC, Tonolo G, Glorioso N, Fraser R. Evidence of coexisting changes in 11 beta-hydroxysteroid dehydrogenase and 5 beta-reductase activity in subjects with untreated essential hypertension. *Hypertension* 1995;25:67-70.

Soszynski P, Slowinska-Srzednicka J, Kasberlik-Zaluska A, Zgliczynski S. Endogenous natriuretic factors: atrial natriuretic hormone and digitalis-like substance in Cushing's syndrome. *Journal of Endocrinology* 1991;129:453-458.

Speiser PW, Riddick LM, Martin K, New MI. Investigation of the mechanism of hypertension in apparent mineralocorticoid excess. *Metabolism* 1993;42:843

Stephenson G, Krozowski Z, Funder JW. Extravascular CBG-like sites in rat kidney and mineralocorticoid receptor specificity. *American Journal of Physiology* 1984;246:t-33

Stewart PM, Corrie JE, Shackleton CH, Edwards CR. Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. *Journal of Clinical Investigation.* 1988;82:340-349.

Stewart PM, Wallace AM, Atherden SM, Shearing CH, Edwards CR. Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11 beta-hydroxysteroid dehydrogenase activity in man. *Clinical Science* 1990;78:49-54.

Stewart PM, Wallace AM, Valentino R, Burt D, Shackleton CHL, Edwards CRW. Mineralocorticoid activity in liquorice: 11β-hydroxysteroid dehydrogenase comes of age. *Lancet* 1987;821-824.

Sudhir K, Jennings GL, Esler MD, et al. Hydrocortisone-induced hypertension in humans: pressor responsiveness and sympathetic function. *Hypertension* 1989;13:416-421.

Suzuki H, Shibata H, Murakami M, Nakamoto H, Kondo K, Saruta T. Case report: hypertension in Cushing's syndrome. *American Journal of the Medical Sciences* 1992;303:329-332.

Swingle WW, Baker C, Eisler M, Le Brie SJ, Brannick LJ. Efficacy of 9-Alpha-halp steroids for maintenance of adrenalectomized dogs. *Proceedings of the Society for Experimental Biology & Medicine* 1955;88:193-196.

Swingle WW, Brannick LJ, Barlow AF. Effect of 2 methyl 9-alpha fluorohydrocortisone on internal distribution of fluids and electrolytes of fasted adrenalectomized dogs. *Proceedings of the Society for Experimental Biology & Medicine* 1957;94:156-159.

Swingle WW, Pfiffner JJ. An aqueous extract of the suprarenal cortex which maintains the life of bilaterally adrenalectomized cats. *Science* 1930;71:321

Tabarin A, Minot AP, Dallochio M, Roger P, Ducassou D. Plasma concentration of neuropeptide Y in patients with adrenal hypertension. *Regulatory Peptides* 1992;42:51-61.

Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Cyclooxygenase inhibition restrores nitric oxide activity in essential hypertension. *Hypertension* 1997;29:t-9

Taddei S, Virdis A, Ghiadoni L, Salvetti A. The role of endothelium in human hypertension. *Current Opinion in Nephrology & Hypertension* 1998;7:203-209.

Taddei S, Virdis A, Mattei P, Salvetti A. Vasodilation to acetylcholine in primary and secondary forms of human hypertension. *Hypertension* 1993;21:929-933.

Takeda Y, Miyamori I, Iki K, et al. Endogenous renal 11 beta-hydroxysteroid dehydrogenase inhibitory factors in patients with low-renin essential hypertension. *Hypertension* 1996;27:197-201.

Takeshita A, Mark AL. Decreased vasodilator capacity of forearm resistance vessels in borderline hypertension. *Hypertension* 1980;2:610-616.

Tam SH, Williamson PM, Kelly JJ, Whitworth JA. Autonomic blockade amplifies cortisol-induced hypertension in man. *Clinical and Experimental Pharmacology and Physiology* 1997;24:31-33.

Tam ST, Kelly JJ, Williamson PM, Whitworth JA. Reflex sympathetic function in cortisol induced hypertension in humans. *Clinical & Experimental Hypertension* 1996;19:479-493.

Tannin GM, Agarwal AK, Monder C, New MI, White PC. The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. *Journal of Biological Chemistry* 1991;266:16653-16658.

Tikkanen I, Teravainen T-L, Mervaala E, Karppanen H. Delayed increase in blood pressure induced by spontaneously hypertensive rat plasma after high sodium intake. *Blood Pressure* 1997;6:188-191.

Tortora GJ, Anagnostakos NP. The Endocrine System, in Dollinger EA, Ginsberg R, Farrell TR (eds): *Principles of Anatomy and Physiology*. New York, Harper & Row; 1990:495-541.

Trapp T, Holsboer F. Heterodimerization between mineralocorticoid and glucocorticoid receptors increases the functional diversity of corticosteroid action. *Trends in Pharmacological Sciences* 1996;17:145-149.

Tresham JJ, McGuire P, Coghlan JP, Whitworth JA, Scoggins BA. The effects of calcium and vitamin D on blood pressure in conscious sheep. *Clin Exp Hypertension* 1988;10:1085-1106.

Turner SW, Wen C, Li M, Fraser TB, Whitworth JA. Adrenocorticotrophin dose-response relationships in the rat - haemodynamic, metabolic and hormonal effects. *Journal of Hypertension* 1998;16:593-600.

Turner SW, Wen C, Li M, Whitworth JA. L-arginine prevents corticotropin-induced increases in blood pressure in the rat. *Hypertension* 1996;27:184-189.

Ulick S, Levine LS, Gunczler P, et al. A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *Journal of Clinical Endocrinology & Metabolism* 1979;49:757-764.

Ulick S, Ramirez LC, New MI. An abnormality in steroid reductive metabolism in a hypertensive syndrome. *Journal of Clinical Endocrinology & Metabolism* 1977;44:799-802.

Ulick S, Tedde R, Mantero F. Pathogenesis of the type 2 variant of the syndrome of apparent mineralocorticoid excess. *Journal of Clinical Endocrinology and Metabolism* 1990;70:200-206.

Ulick S, Wang JZ, Blumenfeld JD, Pickering TG. Cortisol inactivation overload: a mechanism of mineralocorticoid hypertension in the ectopic adrenocorticotropin syndrome. *Journal of Clinical Endocrinology & Metabolism* 1992;74:963-967.

Ulick S, Wang JZ, Hanukoglu A, Rosler A. The effect of carbenoxolone on the peripheral metabolism of cortisol in human patients. *Journal of Laboratory and Clinical Medicine* 1993;122:673-676.

Ulmann A, Menard J, Corvol P. Binding of glycyrrhetinic acid to kidney mineralocorticoid and glucocorticoid receptors. *Endocrinology* 1975;97:46-51.

van Steensel B, van Binnendijk E, Hornsby CD, et al. Partial colocalization of glucocorticoid and mineralocorticoid receptors in discrete compartments in nuclei of rat hippocampus neurons. *Journal of Cell Science* 1996;109:787-792.

Vanhoutte PM. Endothelial dysfunction in hypertension. *Journal of Hypertension - Supplement* 1996;14:S83-S93

Vazir H, Whitehouse BJ, Vinson GP, McCredie E. Effects of prolonged ACTH treatment on adrenal steroidogenesis and blood pressure in rats. *Acta Endocrinol* 1981;97:533-542.

Vermeulen A, Van der Straeten M. Adrenal cortical function in benign essential hypertension. *Journal of Clinical Endocrinology and Metabolism* 1963;23:574-578.

Vetter W, Vetter H, Beckerhoff R, Redlich B, Cottier P, Siegenthaler W. The effect of saralasin (1sar-8-ala-angiotensin II) on blood pressure in patients with Cushing's syndrome. *Klinische Wochenschrift* 1976;54:661-663.

Vingerhoeds AC, Thijssen JH, Schwarz F. Spontaneous hypercortisolism without Cushing's syndrome. *Journal of Clinical Endocrinology & Metabolism* 1976;43:1128-1133.

Vogt B, Dick B, Gankam V, Frey FJ, Frey BM. Reduced 11 beta-hydroxysteroid dehydrogenase activity in patients with the nephrotic syndrome. *Journal of Clinical Endocrinology & Metabolism* 1999;84:811-814.

Vylitova M, Miksik I, Pacha J. Metabolism of corticosterone in mammalian and avian intestine. *General & Comparative Endocrinology* 1998;109:315-324.

Waddell BJ, Atkinson HC. Production rate, metabolic clearance rate and uterine extraction of corticosterone during rat pregnancy. *Journal of Endocrinology* 1994;143:183-190.

Walker BR. Abnormal glucocorticoid activity in subjects with risk factors for cardiovascular disease. *Endocrine Research* 1996;22:701-708.

Walker BR, Aggarwal I, Stewart PM, Padfield PL, Edwards CR. Endogenous inhibitors of 11 beta-hydroxysteroid dehydrogenase in hypertension. *Journal of Clinical Endocrinology & Metabolism* 1995;80:529-533.

Walker BR, Best R, Noon JP, Watt GC, Webb DJ. Seasonal variation in glucocorticoid activity in healthy men. *Journal of Clinical Endocrinology & Metabolism* 1997;82:4015-4019.

Walker BR, Best R, Shackleton CH, Padfield PL, Edwards CR. Increased vasoconstrictor sensitivity to glucocorticoids in essential hypertension. *Hypertension* 1996;27:190-196.

Walker BR, Campbell JC, Fraser R, Stewart PM, Edwards CR. Mineralocorticoid excess and inhibition of 11 beta-hydroxysteroid dehydrogenase in patients with ectopic ACTH syndrome. *Clinical Endocrinology* 1992;37:483-492.

Walker BR, Connacher AA, Webb DJ, Edwards CR. Glucocorticoids and blood pressure: a role for the cortisol/cortisone shuttle in the control of vascular tone in man. *Clinical Science* 1992;83:171-178.

Walker BR, Edwards CR. Licorice-induced hypertension and syndromes of apparent mineralocorticoid excess. *Endocrinology & Metabolism Clinics of North America* 1994a;23:359-377.

Walker BR, Edwards CR. Licorice-induced hypertension and syndromes of apparent mineralocorticoid excess. *Endocrinology & Metabolism Clinics of North America* 1994b;23:359-377.

Walker BR, Phillips DIW, Noon JP, et al. Increased glucocorticoid activity in men with cardiovascular risk factors. *Hypertension* 1998;31:891-895.

Walker BR, Stewart PM, Padfield PL, Edwards CRW. Increased vasoconstriction to glucocorticoids in essential hypertension. 11β-hydroxysteroid dehydrogenase deficiency revisited (abstract). *Journal of Hypertension* 1991;9:1082-1083.

Walker BR, Stewart PM, Shackleton CH, Padfield PL, Edwards CR. Deficient inactivation of cortisol by 11 beta-hydroxysteroid dehydrogenase in essential hypertension. *Clinical Endocrinology* 1993;39:221-227.

Walker BR, Yau JL, Brett LP, et al. 11 beta-hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. *Endocrinology* 1991;129:3305-3312.

Wang W, Diamond SL. Does elevated nitric oxide production enhance the release of prostacyclin from shear stressed aortic endothelial cells?. *Biochemical & Biophysical Research Communications* 1997;233:748-751.

Watson BJ, Bergman SM, Myracle A, Callen DF, Acton RT, Warnock DG. Genetic association of 11 beta-hydroxysteroid dehydrogenase type 2 (HSD11B2) flanking microsatellites with essential hypertension in blacks. *Hypertension* 1996;28:478-482.

Watt GCM, Harrap SB, Foy CJW, et al. Abnormalities of glucocorticoid metabolism and the renin-angiotensin system: A four corners approach to the identification of genetic determinants of blood pressure. *Journal of Hypertension* 1992;10:473-482.

Weaver JU, Hitman GA, Kopelman PG. An association between Bcl 1 restriction fragment length polymorphism of the glucocorticoid receptor locus and hyperinsulinaemia in obese women. *Journal of Molecular Endocrinology* 1992;9:295-300.

Wen C, Fraser T, Li M, Whitworth JA. Hemodynamic profile of corticotropin-induced hypertension in the rat. *Journal of Hypertension* 1998;16:187-194.

Werder E, Zachmann M, Vollmin JA. Unusual steroid excretion in a child with low renin hypertension. *Research in Steroids* 1974;6:385-389.

White BD, Corll CB, Porter JR. The metabolic clearance rate of corticosterone in lean and obese male Zucker rats. *Metabolism: Clinical & Experimental* 1989;38:530-536.

White PC. Inherited forms of mineralocorticoid hypertension. *Hypertension* 1996;28:927-936.

White PC, Mune T, Rogerson FM, Kayes KM, Agarwal AK. Molecular analysis of 11 beta-hydroxysteroid dehydrogenase and its role in the syndrome of apparent mineralocorticoid excess. *Steroids* 1997;62:83-88.

Whitney RJ. The measurement of volume changes in human limbs. *Journal of Physiology* 1953;121:1-27.

Whitworth, JA. Steroids and hypertension in the sheep. 1977. Howard Florey Instiute of Experimental Physiology and Medicine. Thesis.

Whitworth JA. Mechanisms of glucocorticoid-induced hypertension. *Kidney International* 1987;31:1213-1224.

Whitworth JA. Adrenocorticotrophin and steroid-induced hypertension in humans. *Kidney International - Supplement* 1992;37:S34-7.

Whitworth JA. Cushing's Syndrome and Hypertension, in Swales JD (ed): *Textbook of Hypertension*. Oxford, Blackwell Scientific Publications; 1994:893-903.

Whitworth JA, Brown MA, Kelly JJ, Williamson PM. Mechanisms of cortisol-induced hypertension in humans. *Steroids* 1995;60:76-80.

Whitworth JA, Butkus A, Coghlan JP, et al. 9-alpha-Fluorocortisol-induced hypertension: a review. *Journal of Hypertension* 1986;4:133-139.

Whitworth JA, Connell JM, Gordon D, Scoggins BA. Effects of indomethacin on steroid-induced changes in pressor responsiveness in man. *Clinical and Experimental Pharmacology and Physiology* 1988;15:305-310.

Whitworth JA, Connell JM, Lever AF, Fraser R. Pressor responsiveness in steroid-induced hypertension in man. *Clinical and Experimental Pharmacology and Physiology* 1986;13:353-358.

Whitworth JA, Gordon D, Andrews J, Scoggins BA. The hypertensive effect of synthetic glucocorticoids in man: role of sodium and volume. *Journal of Hypertension* 1989a;7:537-549.

Whitworth JA, Gordon D, McLachlan-Troup N, Scoggins BA, Moulds RW. Dexamethasone suppression in essential hypertension: effects on cortisol and blood pressure. *Clinical and Experimental Hypertension* 1989b;11:323-335.

Whitworth JA, Hewitson TD, Ming L, et al. Adrenocorticotrophin-induced hypertension in the rat: haemodynamic, metabolic and morphological characteristics. *Journal of Hypertension* 1990;8:27-36.

Whitworth JA, Saines D, Scoggins BA. Blood pressure and metabolic effects of cortisol and deoxycorticosterone in man. *Clinical and Experimental Hypertension* 1984;6:795-809.

Whitworth JA, Saines D, Scoggins BA. Potentiation of ACTH hypertension in man with salt loading. *Clinical and Experimental Pharmacology and Physiology* 1985;12:239-243.

Whitworth JA, Saines D, Thatcher R. Differential blood pressure and metabolic effects of 9 alpha- fluorocortisol in man. *Clinical and Experimental Pharmacology and Physiology* 1983a;10:351-354.

Whitworth JA, Saines D, Thatcher R, Butkus A, Scoggins BA. Blood pressure and metabolic effects of ACTH in normotensive and hypertensive man. *Clinical and Experimental Hypertension* 1983b;5:501-522.

Whitworth JA, Scoggins BA. A 'hypertensinogenic' class of steroid hormone activity in man? *Clinical and Experimental Pharmacology and Physiology* 1990;17:163-166.

Whitworth JA, Scoggins BA, Andrews J, Williamson PM, Brown MA. Haemodynamic and metabolic effects of short term administration of synthetic sex steroids in humans. *Clinical and Experimental Hypertension* 1992;14:905-922.

Whitworth JA, Stewart PM, Burt D, Atherden SM, Edwards CR. The kidney is the major site of cortisone production in man. *Clinical Endocrinology* 1989;31:355-361.

Whitworth JA, van Leeuwen BH, Hannah MC, Scoggins BA, Thatcher R. Effect of ACTH administration on urinary kallikrein excretion in man. *Clinical and Experimental Pharmacology and Physiology* 1984;11:87-90.

Whitworth JA, Williamson PM, Brown MA, Colman P. Hyperinsulinemia is not a cause of cortisol-induced hypertension. *American Journal of Hypertension* 1994a;7:562-565.

Whitworth JA, Williamson PM, Brown MA, Morris MJ. Neuropeptide Y in cortisol-induced hypertension in male volunteers. *Clinical and Experimental Pharmacology and Physiology* 1994b;21:435-438.

Whitworth JA, Williamson PM, Ramsey D. Hemodynamic responses to cortisol in man: effects of felodipine. *Hypertension Research* 1994c;17:137-142.

Wilkins RW, Bradley SE. Changes in arterial and venous blood pressure and flow distal to a cuff inflated on the human arm. *American Journal of Physiology* 1946;147:260-269.

Will PC, Cortright RN, Hopfer U. Polyethylene glycols as solvents in implantable osmotic pumps. *Journal of Pharmaceutical Sciences* 1980;69:747-749.

Williamson PM, Kelly JJ, Whitworth JA. Dose-response relationships and mineralocorticoid activity in cortisol-induced hypertension in humans. *Journal of Hypertension - Supplement* 1996;14:S37-S41

Wilson RC, Harbison MD, Krozowski ZS, et al. Several homozygous mutations in the gene for 11 beta-hydroxysteroid dehydrogenase type 2 in patients with apparent mineralocorticoid excess. *Journal of Clinical Endocrinology & Metabolism* 1995;80:3145-3150.

Wilson RC, Krozowski ZS, Li K, et al. A mutation in the HSD11B2 gene in a family with apparent mineralocorticoid excess. *Journal of Clinical Endocrinology & Metabolism* 1995;80:2263-2266.

Woodward CJ, Hervey GR, Oakey RE, Whitaker EM. The effects of fasting on plasma corticosterone kinetics in rats. *British Journal of Nutrition* 1991;66:117-127.

Yamaji T, Ishibashi M, Yamada A, et al. Plasma levels of atrial natriuretic hormone in Cushing's syndrome. *Journal of Clinical Endocrinology & Metabolism* 1988;67:348-352.

Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;332:411-415.

Yasunari K, Kohno M, Murakawa K, Yokokawa K, Takeda T. Glucocorticoids and atrial natriuretic factor receptors on vascular smooth muscle. *Hypertension* 1990;16:581-586.

Zennaro MC, Lombes M. Mineralocorticoid receptor isoforms. *Current Opinion in Endocrinology and Diabetes* 1998;5:183-188.