TISSUE SENSITIVITY TO GLUCOCORTICOIDS IN HYPERTENSION

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Doctor of Medicine

University of Edinburgh

1992

Dedicated to Jane and Michael

Declaration

I hereby declare and affirm that this thesis is entirely my own work and composition. Where I have received technical assistance this is indicated in the text.

Signature Date 16th Dec '92

Abstract

11ß-Hydroxysteroid dehydrogenase catalyses the conversion of cortisol to inactive cortisone. In kidney, the enzyme confers aldosterone-specificity on mineralocorticoid receptors by preventing their occupancy by cortisol. The expression of 11ß-dehydrogenase in many extra-renal sites suggests that it has a wide role in modulating cortisol sensitivity. When renal 11ß-dehydrogenase is defective (as occurs in congenital deficiency and after inhibition of the enzyme by liquorice or carbenoxolone) cortisol-dependent mineralocorticoid excess and hypertension ensue. In this thesis I address the hypotheses that: (i) in addition to its role in the kidney, 11ß-dehydrogenase modulates cortisol sensitivity in vascular smooth muscle; (ii) 11ß-dehydrogenase is regulated physiologically by the hypothalamic-pituitary-adrenal axis; and (iii) deficiency of 11ß-dehydrogenase, associated with increased cortisol sensitivity either in kidney or in blood vessels, contributes to pathophysiology in other forms of hypertension, specifically ectopic ACTH syndrome and essential hypertension.

I show that 11ß-dehydrogenase immunoreactivity and mRNA are localised to vascular smooth muscle cells in rats, and bioactivity is greater in resistance vessels than conduit arteries. In support of a diverse role for the enzyme in modulating vascular tone I demonstrate: (i) potentiation of vasoconstrictor sensitivity to cortisol - and thereby to noradrenaline - in the forearm and dermis of men given enzyme inhibitors or congenitally deficient in 11ß-dehydrogenase; and (ii) attenuation of noradrenaline reactivity by glucocorticoids in rat aorta which is increased following *in vitro* carbenoxolone administration.

By studying *in vitro* affinities of rat vascular 11ß-dehydrogenase for its cofactors I demonstrate its similarity to the hepatic isoform of the enzyme, and distinguish it from that in kidney. Moreover, vascular but not renal 11ß-dehydrogenase is induced by *in vivo* administration of glucocorticoids. By contrast, ACTH is without effect on 11ß-dehydrogenase in adrenalectomised rats, but inhibits the peripheral conversion of cortisol to cortisone in man. By selective venous catheterisation studies I confirm that the major site for this conversion is the kidney. Thus ACTH, in addition to stimulating cortisol synthesis, may stimulate the secretion of an 11ß-dehydrogenase inhibitor from the adrenal, and thereby increase renal sensitivity to cortisol.

In 9 patients with ectopic ACTH syndrome the ratio of plasma cortisol to cortisone was higher than in 17 patients with other forms of Cushing's syndrome, and correlated negatively with plasma potassium concentration. In patients with essential hypertension 11ß-dehydrogenase activity (measured by the half life of ($11\alpha^{3}H$)-cortisol) was deficient in 7 of 20 patients studied. These patients did not have evidence of mineralocorticoid excess. However, dermal vascular sensitivity to cortisol, although not significantly correlated with 11ß-dehydrogenase activity, was increased in the hypertensives.

In conclusion, 11ß-dehydrogenase deficiency may mediate hypertension by more than one mechanism. In ectopic ACTH syndrome inhibition of renal 11ß-dehydrogenase allows cortisol to activate mineralocorticoid receptors and induce the characteristic features of hypokalaemia and hypertension. By contrast, in essential hypertension 11ß-dehydrogenase deficiency is not associated with mineralocorticoid excess, but may underlie an increase in vasoconstrictor sensitivity to cortisol. Thus the significance of defective enzyme-mediated receptor protection in clinical practice is confirmed.

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ABBREVIATIONS

11ß-DH	11ß-dehydrogenase
11ß-OHSD	11ß-hydroxysteroid dehydrogenase (E.C. 1.1.1.146)
11ß-OR	11ß-reductase
ACTH	Adrenocorticotrophic hormone
allo-THF	5α-tetrahydrocortisol
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
BDP	beclomethasone dipropionate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanidine monophoshate
CBG	cortisol binding globulin
DNA	deoxyribonucleic acid
DOC	11-deoxycorticosterone
GALFs	glycyrrhetinic acid-like factors
G-proteins	guanidine nucleotide binding proteins
GC/MS	gas chromatography and mass spectrometry
GE	glycyrrhetinic acid
HPLC	high performance liquid chromatography
KRBG	Kreb's Ringer bicarbonate buffer with glucose
LBNP	lower body negative pressure
L-NMMA	L-N_G-monomethyl-arginine
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NRS	normal rabbit serum
PRA	plasma renin activity
SE	standard error
TBS	tris-buffered saline
THE	tetrahydrocortisone
THF	tetrahydrocortisol

Steroid Nomenclature

Aldosterone	d-11ß,18-epoxy-18,21-dihydroxy-4-pregnene-3,2-dione
allo-Tetrahydrocortisol	5α-pregnane-3α,11ß,17α,21-tetrol-20-one
Androstenedione	4-androstene-3,17-dione
Beclomethasone dipropionate	9-chloro-11ß,17,21-trihydroxy-16ß-methylprega-1,4-diene-3,20-dione 17,21-dipropionate
Corticosterone	11ß,21-dihdroxy-4-pregnene-3,20-dione
Cortisol	11ß,17α,21-trihydroxy-4-pregnene-3,20-dione
Cortisone	17α,21-dihydroxy-4-pregnene-3,11,20-trione
11-Dehydrocorticosterone	11ß,21-dihdroxy-4-pregnene-3,11,20-trione
Dehydroepiandrosterone	5-androsten-3ß-ol-17-one
11-Deoxycorticosterone	21-hydroxy-4-pregnene-3,20-dione
Dexamethasone	9α-fluoro-16α-methyl-11ß,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione
Tetrahydrocortisol	5ß-pregnane-3α,11ß,17α,21-tetrol-20-one
Tetrahydrocortisone	3α,17α,21-trihydroxy-5ß-pregnane-11,20-dione

CHAPTER 1 INTRODUCTION

11ß-HYDROXYSTEROID DEHYDROGENASE AND ENZYME-MEDIATED RECEPTOR PROTECTION

Hypertension is one of the commonest clinical problems in First World Medicine, affecting perhaps 10 % of the population. However, we can identify a cause of "secondary" hypertension in fewer than 5 % of these individuals, leaving > 95 % with "essential" hypertension. It is logical to suppose that the mechanisms which raise blood pressure in the minority with secondary hypertension might contribute to pathophysiology in a more subtle way in the majority. This logic has been pursued, with limited success, for most of the endocrine syndromes which are associated with hypertension [Fraser et al., 1989].

The role of the adrenal cortex in blood pressure regulation was first inferred from the association of adrenocortical insufficiency with hypotension [Addison, 1855], and subsequently when hypertension was recognised as a consequence of excessive secretion of either cortisol in Cushing's syndrome [Cushing, 1912] or aldosterone in Conn's syndrome [Conn, 1955]. Cortisol has therefore been suggested to be a pathophysiological mediator in patients with essential hypertension. This was addressed initially by measurement of production rates of cortisol, which were disappointingly normal [Vermeulen & van der Straeten, 1963]. Nonetheless, evidence has since emerged that the hypophyseal-pituitary-adrenal axis is abnormal in patients with essential hypertension. Firstly, deranged cortisol metabolism was observed in hypertensive patients, who produce relatively large amounts of the polar metabolites of cortisol (6ß- and 20α- hydroxycortisol)[Kornel et al., 1975]. Secondly, the synthetic glucocorticoid receptor agonist dexamethasone was found to lower blood pressure paradoxically in some patients with essential hypertension [Hamilton et al., 1979; Whitworth et al., 1989a], suggesting that in this subgroup the elevation of blood pressure is ACTH-dependent. If it is cortisol which mediates hypertension in these patients then, in the absence of excessive secretion or circulating concentration, it must exert its effect by inducing a more sensitive response in one of its target organs.

Curiously, the target mechanisms which mediate glucocorticoid-induced hypertension remain uncertain. Relevant effects have been observed in kidney, heart, central nervous system, and peripheral blood vessels, but the relative contribution of each

site is unclear (see below). Increased sensitivity to cortisol at any of these sites might increase blood pressure.

From a series of clinical and experimental observations made in this department by Dr Paul Stewart, he and Professor Christopher Edwards uncovered a novel mechanism for cortisol-dependent hypertension [Stewart, 1988a]. Paul's observations concerned the enzyme 11ß-hydroxysteroid dehydrogenase, or the "cortisol-cortisone shuttle", which he demonstrated to play an important role in dictating cortisol sensitivity in the kidney. In this thesis I address the possibility that this mechanism also dictates cortisol sensitivity in vascular smooth muscle, and that its deficiency in either kidney or vasculature could contribute to pathophysiology in common forms of hypertension.

Sensitivity to Glucocorticoids in the Kidney

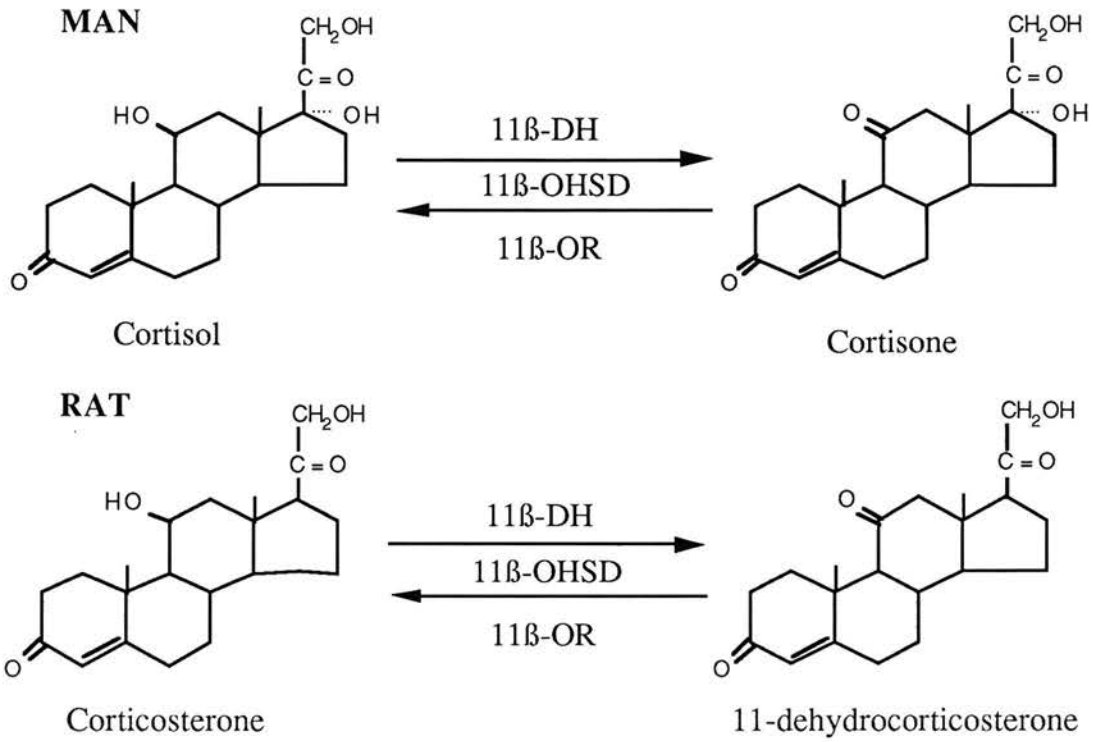
Under the classical model of corticosteroid action, their effects are mediated by binding to cytosolic steroid receptors. The steroid-receptor complex is translocated to the nucleus where it binds to steroid response elements on DNA. These act as promoter genes to influence mRNA transcription and thereby protein synthesis from a number of genes [Gustafsson et al., 1987]. In the kidney aldosterone binds to mineralocorticoid (or type 1) receptors which have a restricted distribution in the distal nephron [Funder et al., 1973a; Rundle et al., 1989], and whose activation results in sodium retention and potassium loss [Fanestil & Park, 1981; Marver, 1984; Funder, 1985]. By contrast cortisol binds to glucocorticoid (or type 2) receptors which have a ubiquitous distribution [Funder et al., 1973b; Gasc et al., 1991] and have more variable effects on tubular function [Fanestil & Park, 1981; Marver, 1984].

It is important in this model that there is little cross-reactivity between glucocorticoid and mineralocorticoid activities (ie that cortisol does not activate mineralocorticoid receptors), because cortisol is present in concentrations 100-1000 fold greater than those of aldosterone and if it did bind to mineralocorticoid receptors they would be permanently fully activated. This specificity of mineralocorticoid receptors for aldosterone has been confirmed *in vivo* [Sheppard & Funder, 1987] and was attributed originally to the intrinsic affinities of mineralocorticoid and glucocorticoid receptors for their physiological ligand. Thus glucocorticoid receptors would have high affinity for some ligands (principally cortisol [=hydrocortisone] in man; also corticosterone, the principal glucocorticoid in rat; and the synthetic steroids dexamethasone, prednisolone, budesonide etc) and mineralocorticoid receptors would

have high affinity for others (principally aldosterone in man and rat; also 11-deoxycorticosterone, and synthetic mineralocorticoids such as fludrocortisone). However, identification of receptors in rat hippocampus which bound corticosterone but not the type 2 ligand dexamethasone raised doubts about this traditional explanation [McEwen et al., 1976]. It emerged that these sites had an identical ligand binding profile to type 1 receptors from kidney, and that both renal and hippocampal type 1 receptors bound corticosterone and aldosterone with equal affinity *in vitro* [Krozowski & Funder, 1983]. Moreover, when cloned, the type 1 and type 2 receptors were found to share remarkable structural and genetic homology in keeping with their promiscuous ligand binding [Arriza et al., 1987]. Thus a mechanism not dependent on intrinsic receptor affinity is required to explain the *in vivo* specificity of renal type 1 receptors for aldosterone over corticosterone, when the same receptors are not specific for aldosterone in hippocampus [Sheppard & Funder, 1987].

Several authors had hinted at the possibility that the aldosterone-specificity of mineralocorticoid receptors might depend on local metabolism or sequestration of cortisol/corticosterone. When Funder, Feldman and Edelman first described the type 1 receptor in kidney they recognised that its affinity for aldosterone, although high, was not enough to explain its specificity when aldosterone levels are so low relative to other corticosteroids [Funder et al., 1973a]. The presence in rat kidney of another binding site, which was unusual in preferring corticosterone above all other ligands, led them to suggest that a type 3 receptor existed [Feldman et al., 1973]. However, a functional effect of type 3 binding was never demonstrated. It was suggested that it represented extra-vascular cortisol binding globulin (CBG), and later that CBG might absorb corticosterone in the kidney but not in hippocampus (beyond the blood-brain barrier) and therefore might prevent the access of glucocorticoids to mineralocorticoid receptors [Stephenson et al., 1984]. However, the CBG hypothesis was untenable after the description of aldosterone specificity in kidneys of 10-day old rats which are deficient in CBG [Sheppard & Funder, 1987]. Diana Marver wrote that the type 3 "receptor" might be an enzyme, and also suggested that preferential metabolism of corticosterone in rat distal tubules might result in specific binding for aldosterone [Marver, 1984]. This remark proved to be prophetic.

Figure 1.1 Principal reactions catalysed by 11ß-hydroxysteroid dehydrogenase in man and rat



11ß-DH = 11ß-dehydrogenase and 11ß-OR = 11ß-reductase activities of the enzyme 11ß-hydroxysteroid dehydrogenase (11ß-OHSD).

11ß-Hydroxysteroid Dehydrogenase

Biochemistry and Distribution

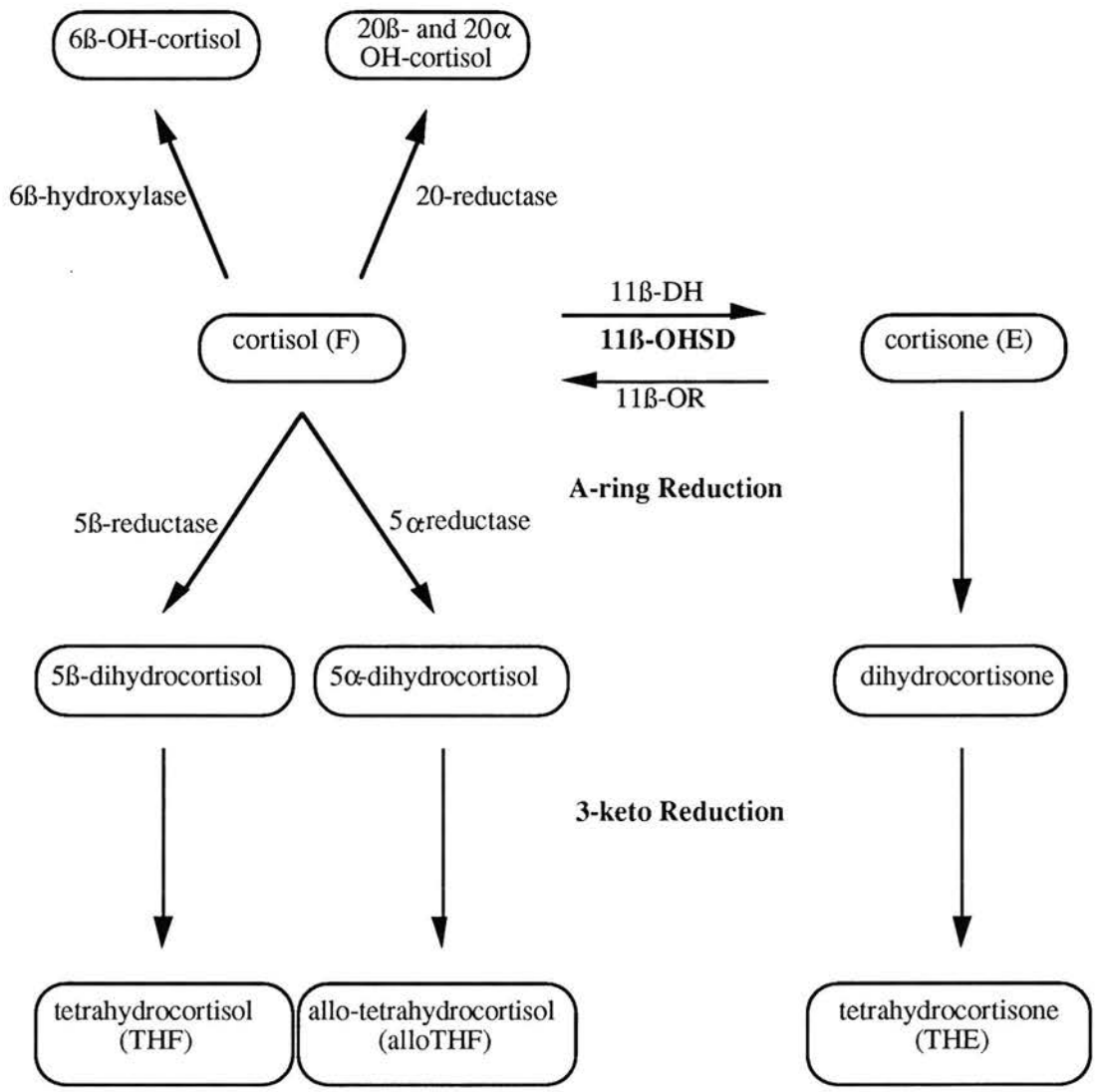
11ß-hydroxysteroid dehydrogenase (11ß-OHSD) is a microsomal enzyme which in man catalyses the reversible inter-conversion of cortisol to the inactive steroid cortisone, and in rats the inter-conversion of corticosterone and 11-dehydrocorticosterone (Figure 1.1). The two activities of the enzyme are separable, an 11ß-dehydrogenase (11ß-DH) catalysing the forward reaction from cortisol to cortisone and from corticosterone to 11-dehydrocorticosterone, and 11ß-reductase (11ß-OR) catalysing the reverse reactions. For many years there was controversy about whether 11ß-DH and 11ß-OR were separate enzymes. Kinetic data indicated the existence of two enzymes in lung [Abramowitz et al., 1982] and liver [Lakshmi & Monder, 1985; 1988] and indeed that liver 11ß-dehydrogenase has distinct kinetic

forms [Monder & Lakshmi, 1989]. However, the argument was recently settled with the cloning of a rat cDNA encoding a protein which catalyses both reactions [Agarwal et al., 1989; 1990]. Nonetheless, congenital deficiency of 11ß-OR in man [Taylor et al., 1984; Phillipou & Higgins, 1985] is dissociated from deficiency of 11ß-DH described below. It seems that although both activities can be expressed from the same gene, the relative activities of the two reactions may vary independently.

Most work on the enzyme has relied on measurement of *in vitro* activity in tissue homogenates or microsomes. In these studies 11ß-DH activity predominates, and the greatest activity was usually reported in liver [Hurlock & Talalay, 1959; Koerner, 1969; Lax et al., 1978; Monder & Shackleton, 1984; Monder & Lakshmi, 1990]. It was widely assumed that the enzyme was one of a variety of integrated hepatic clearance mechanisms which facilitate excretion of cortisol metabolites in urine (Figure 1.2). If this were so then it seems curious that it should be a reversible reaction. In human studies of cortisol clearance, using measurement of urinary steroid metabolites by chromatographic separation [Zumoff et al., 1974] or by radioisotopic labelling [Kowarski et al., 1969; Zumoff et al., 1974], the conversion of cortisol to cortisone was shown to make a substantial contribution to cortisol clearance, though no study has simultaneously quantified the relative contributions of all the pathways illustrated in Figure 1.2 [Brownie, 1992]. However, a discrepancy was found between the *in vivo* half life of ^{14}C-cortisol of ~75 min and that of (11α^{3}H)-cortisol of ~40 min (^{3}H is irreversibly removed from the latter molecule by 11ß-DH), suggesting that reductase conversion of ^{14}C-cortisone back to ^{14}C-cortisol is very active [Hellman et al., 1971]. Cortisol and cortisone therefore represent interchangeable pools of active and inactive steroid respectively. Although total circulating levels of cortisol are higher than those of cortisone, cortisone is not substantially protein bound and free plasma concentrations are approximately equal [Meulenberg et al., 1987].

The assumption that the liver is the principal site of 11ß-DH activity was also called into question by several early findings. Firstly, *in vitro* 11ß-DH activity was found in abundance in kidney [Ganis et al., 1956; Jenkins, 1966]. The contribution of the kidney was emphasised by Hellman and colleagues [Hellman et al., 1971], who estimated from isotopic studies employing renal vein sampling that as much as 10 % of cortisol was subject to 11ß-DH metabolism on single pass through the kidney. Even more avid conversion of cortisol to cortisone was then shown in perfused rat kidney [Reach et al., 1977]. Secondly, in a single experiment in which human liver

Figure 1.2 Principal metabolites of cortisol



was perfused with cortisol, the metabolites of cortisol (cortols) and cortisone (cortolone) were produced in similar amounts and the free steroids were not measurable [Wortmann et al., 1971]. This suggests that the liver rapidly exchanges pools of cortisol and cortisone, and that both pools are cleared to A-ring-reduced metabolites. However, when free cortisol and cortisone were measured in the effluent from a perfused cat liver, it was cortisol which was most abundant, suggesting that in the cat the hepatic equilibrium favours the active steroid, ie that 11ß-OR predominates [Bush, 1969]. More recent experiments suggest that 11ß-OR

also predominates in human liver. Following an oral dose of cortisone peripheral plasma cortisol levels rise but cortisone concentrations do not [Stewart et al., 1990]. This suggests that a substantial proportion of the cortisone delivered to the liver via the portal vein is converted on first pass to cortisol by 11ß-OR, and the rest of the cortisone is probably converted to A-ring-reduced metabolites. Thus not only is cortisol subject to extensive turnover between active and inactive forms, but also the relative concentrations of cortisol and cortisone may vary between organs, with most conversion to cortisone occurring in the kidney and conversion back to cortisol occurring in the liver. I address this question further in Chapter 4.

In addition to expression in liver and kidney, 11ß-OHSD is expressed to some degree in almost all mammalian tissues and also in non-mammals (Table 1.1). Mammalian tissues studied include placenta [Osinski, 1960; Murphy, 1981b], lung [Murphy, 1978; Nicholas & Lugg, 1982; Abramowitz et al., 1982], testis [Phillips et al., 1989], ovary, uterus [Benediktsson et al., 1992], salivary glands, colon [Whorwood et al., 1992], skin [Teelucksingh et al., 1990], adipose tissue, skeletal muscle, brain [Moisan et al., 1990a; 1990b; Monder & Lakshmi, 1990; Lakshmi et al., 1991; Sakai et al., 1992], and vasculature [Kornel et al., 1982; Funder et al., 1989]. The magnitude of the contribution which these other sites make to cortisol/cortisone turnover is unknown.

Physiological Role of 11ß-OHSD

The possibility that the conversion of the active steroid cortisol to its inactive metabolite cortisone may have greater significance than as a simple clearance mechanism for cortisol was first suggested in studies of placental 11ß-OHSD. Human placenta in midgestation has both 11ß-DH and 11ß-OR activities but the fetus has predominantly 11ß-OR activity [Pasqualini et al., 1970]. Placental equilibrium between active and inactive steroids changes during gestation [Giannopoulos et al., 1982], as does enzyme equilibrium in human fetal tissues around parturition [Murphy, 1978; 1981b]. These authors suggested that the shift of equilibrium towards the active steroid in late gestation may have a physiological role since cortisol is involved in the initiation of parturition in some species and is an important factor in normal maturation of lung surfactant [Murphy & Branchaud, 1983].

On this background observations of the clinical syndrome of 11ß-DH deficiency in the last 20 years have provided valuable clues to the physiological role of 11ß-OHSD in the kidney in adults.

Table 1.1 Distribution of 11ß-OHSD in mineralocorticoid and glucocorticoid target organs

Mineralocorticoid targets	Glucocorticoid targets	Potential targets for mineralocorticoids or glucocorticoids
Distal Renal Tubule	Liver	Hippocampus
Salivary Gland	Lung	Vascular Smooth Muscle
Colon	Testis	Heart
Toad Bladder	Proximal and Distal Renal Tubule	
	Cerebellum	
	Pituitary	
	Epidermis	
	Adipose Tissue	
	Skeletal Muscle	
	Placenta	

11ß-Dehydrogenase Deficiency

In 1974 Werder [Werder et al., 1974] described a case of a 3 year old girl with severe hypertension and hypokalaemia. Plasma renin activity was undetectable and mineralocorticoid excess was suspected. However plasma aldosterone was low and no other mineralocorticoids could be identified. Cortisol metabolites as measured by urinary 17-hydroxycorticosteroids were low as was urinary tetrahydrocortisone (THE). After administration of exogenous ACTH it was noted that "surprisingly THE remained low". The significance of this was not understood and it was speculated that an unknown steroid with both mineralo- and gluco-corticoid activity was causing the syndrome. A very similar clinical and biochemical picture was then reported in a patient of Maria New's. They described a defect in the conversion of cortisol to cortisone associated with hypertension and hypokalaemia [New et al., 1977; Ulick et al., 1977; 1979]. Again there was no demonstrable excess of circulating mineralocorticoid and the syndrome was described as one of "apparent mineralocorticoid excess". The link between the hypertension, hypokalaemia and

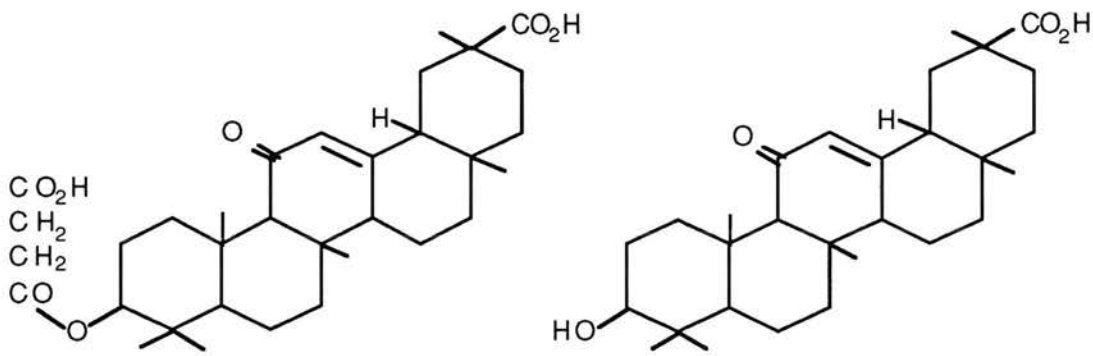
inability to convert cortisol to cortisone remained unexplained though a further 18 cases were reported in children [Shackleton et al., 1980; 1985; Fiselier et al., 1982; Oberfield et al., 1983; Honour et al., 1983; Harinck et al., 1984; Monder et al., 1986; Dimartino-Nardi et al., 1987]. Two families had more than one affected member.

New's group proposed that cortisol was acting as a hypertensive agent [Oberfield et al., 1983] and Shackleton and colleagues [1980] demonstrated a beneficial response to therapy with dexamethasone, as had Werder in the original case. Two possible explanations were advanced for the mineralocorticoid effect of cortisol. In the first they proposed that cortisol had an abnormal affinity for the mineralocorticoid receptor. However, this would mean that there were two genetic defects, one in cortisol-cortisone conversion and the other in the mineralocorticoid receptor. In the second they suggested that the normal metabolism of cortisol to cortisone did not occur in the mineralocorticoid receptor tissue, resulting in accumulation of cortisol and activation of receptors [Oberfield et al., 1983].

In 1984 the oldest patient to have presented with this syndrome, a 20 year old man (GB), was investigated in detail in this department by Professor Edwards [Edwards et al., 1985; Stewart et al., 1988b]. The following features were confirmed: a) normal circulating levels of cortisol but a reduced cortisol production rate; b) elevation of the ratio of urinary metabolites of cortisol (tetrahydrocortisol [THF] + allo-THF) to those of cortisone (THE)(Figure 1.2); c) prolongation of the half life of (11α^{3}H)-cortisol (which is acted on by 11ß-DH to produce ^{3}H-H_2O and unlabelled cortisone); d) intact ability to convert orally administered cortisone to cortisol; e) excess mineralocorticoid activity in kidney (as judged by suppression of plasma renin activity and aldosterone, hypokalaemia, and a very low urinary Na/K ratio) and colon (as measured by subtraction potential difference) which could be reversed with dexamethasone and reproduced by physiological doses of hydrocortisone; and f) no demonstrable excess of any other mineralocorticoid. Family studies suggested that GB's mother also had relative 11ß-DH deficiency, with mild hypokalaemia and hypertension [Stewart et al., 1988b; Shackleton & Stewart, 1990]. In their discussion of this case Stewart and Edwards offered a new hypothesis to explain the syndrome, when they suggested that the normal kidney uses the cortisol-cortisone shuttle to protect mineralocorticoid receptors in the distal nephron from cortisol. Failure to do so results in "Cushing's disease of the kidney" in which intra-renal cortisol levels rise and spill over to occupy type 1 mineralocorticoid receptors [Edwards et al., 1985; Stewart et al., 1988b].

An important step forward in testing this hypothesis followed with the recognition of parallels between the findings in GB and the features of the hypertensive syndrome produced by liquorice. Liquorice abuse had long been associated with hypertension and hypokalaemia which responded to spironolactone [Salassa et al., 1962], and it was thought that glycyrrhetinic acid (GE), the active derivative of liquorice, caused the syndrome by binding directly to renal mineralocorticoid receptors [Ulmann et al., 1975; Armanini et al., 1983]. However, previous studies had shown that the sodium retention was dependent on the presence of cortisol or another ACTH-dependent steroid since it was reversible with dexamethasone [Hoefnagels & Kloppenborg, 1983] and absent in patients without intact adrenal glands [Borst et al., 1953]. Therefore it could not be a direct effect but might be mediated by inhibition of 11ß-DH by liquorice.

Figure 1.3 Chemical structure of liquorice-derived inhibitors of 11ß-OHSD



Carbenoxolone Glycyrrhetinic Acid

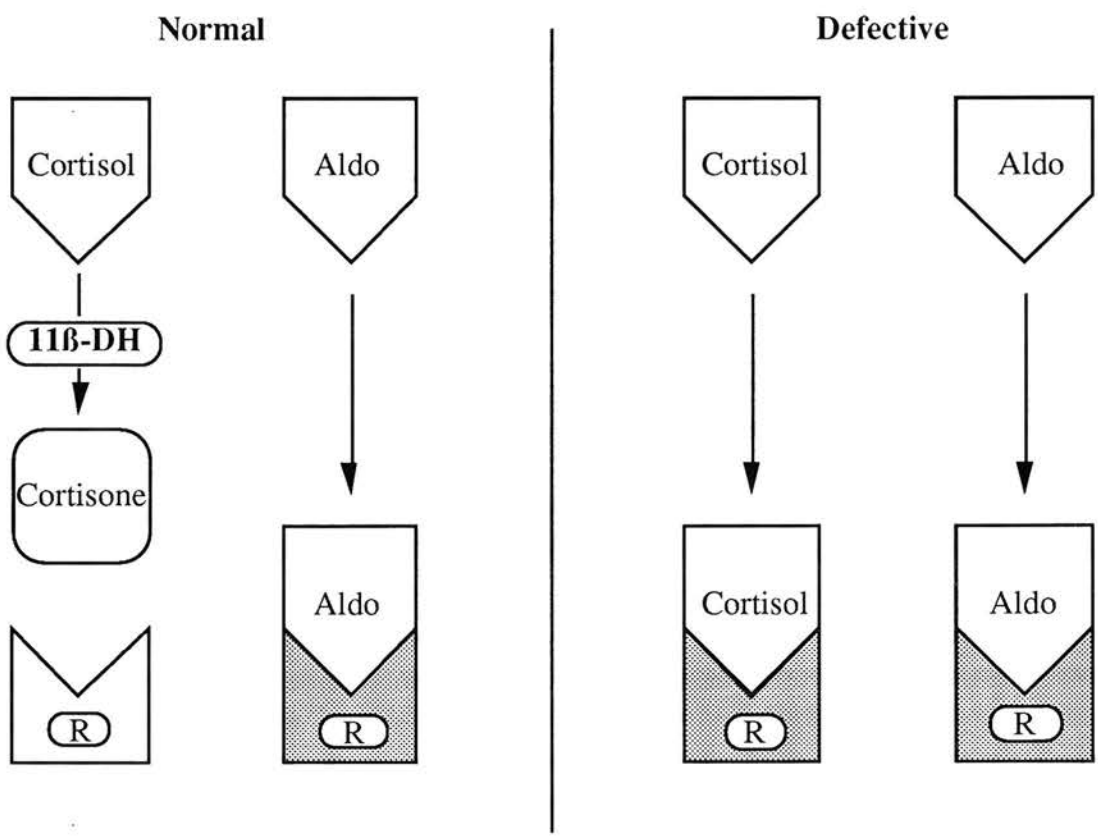
In a metabolic balance study in subjects taking liquorice it was confirmed that sodium retention and kaliuresis were associated with changes in urinary cortisol metabolites and prolongation of the half life of (11α^3H)-cortisol which indicated inhibition of 11ß-DH [Stewart et al., 1987]. Others have confirmed that the effects of confectionary liquorice on cortisol metabolism in man can be reproduced by the administration of either of its principal constituents, GE [MacKenzie et al., 1990] or glycyrrhizic acid (converted to GE *in vivo*)[Kageyama et al., 1992]. Similar findings were observed with the ulcer-healing drug carbenoxolone, which is the hemisuccinate derivative of GE [Monder et al., 1989; Stewart et al., 1990](Figure 1.3).

Furthermore, GE and carbenoxolone competitively inhibit rat 11ß-DH *in vitro* and *in vivo* [Monder et al., 1989] and carbenoxolone potentiated the mineralocorticoid effects of corticosterone in the intact rat [Souness and Morris, 1989] and in toad bladder mucosa [Brem et al., 1989; Gaeggeler et al., 1989]. It thus appears that in both congenital and acquired 11ß-DH deficiency, failure to inactivate cortisol in man or corticosterone in rat is associated with abnormal access of these glucocorticoids to renal mineralocorticoid receptors and hence mineralocorticoid excess.

Specificity of Renal Mineralocorticoid Receptors

The data from congenital and acquired 11ß-DH deficiency provide an alternative explanation for the paradox of aldosterone-specificity of mineralocorticoid receptors in the kidney. This is presented in Figure 1.4. In this model it is accepted that renal mineralocorticoid receptors have equal affinity for cortisol and aldosterone but are protected from the high concentrations of cortisol by the enzyme 11ß-DH, which converts cortisol to cortisone. The 11-18 hemi-acetal structure of aldosterone protects it from the action of 11ß-DH [Edwards & Hayman, 1991], and so aldosterone gains preferential access to the receptors.

Figure 1.4 Enzyme-mediated receptor protection



This hypothesis was confirmed in this department by studying *in vivo* binding of corticosterone and aldosterone in rat kidney, with and without inhibition of 11ß-DH with subcutaneous glycyrrhizic acid [Edwards et al., 1988]. The results were very dramatic, showing by autoradiography that ^{3}H-corticosterone bound minimally in the kidney in the absence of glycyrrhizic acid, but that after 11ß-DH inhibition there was abundant ^{3}H-corticosterone binding in a distribution identical to that of ^{3}H-aldosterone, ie in the distribution of mineralocorticoid receptors. Furthermore, glycyrrhizic acid had no effect on aldosterone binding. These findings were confirmed by John Funder's group in Melbourne who demonstrated that when ^{3}H-corticosterone or ^{3}H-aldosterone were given *in vivo* to rats and the kidneys then removed, there was selective binding of aldosterone to type 1 receptors in the kidney cytosol. However, this selectivity was lost when 11ß-DH was inhibited with carbenoxolone [Funder et al., 1988].

Controversies in the Role of 11ß-OHSD in the Kidney

Cellular Localisation of 11ß-OHSD

The anatomical relationship between 11ß-OHSD and mineralocorticoid receptors in the kidney has been debated. Initial localisation of the enzyme by immunohistochemistry, using a polyclonal antibody raised against purified rat liver 11ß-OHSD [Monder and Lakshmi, 1990], indicated that it was sited in proximal tubules [Edwards et al., 1988]. By contrast mineralocorticoid receptors are predominantly expressed in the distal nephron. This was confirmed by Rundle and colleagues using the same antiserum and also a monoclonal antibody raised against the mineralocorticoid receptor [Rundle et al., 1989] and by Castello and colleagues using a monoclonal antibody to the enzyme [Castello et al., 1989]. The inference was drawn that enzyme-mediated protection of the mineralocorticoid receptor was a "paracrine" mechanism whereby conversion of cortisol to cortisone occurs in one cell and protects mineralocorticoid receptors in another cell.

More recent studies using *in situ* hybridisation indicate that mRNA for 11ß-OHSD is expressed in both proximal and distal renal tubular cells [Stewart et al., 1991; Yau et al., 1991]. This was confirmed by measurement of *in vitro* conversion of corticosterone to 11-dehydrocorticosterone when proximal and distal tubules were separated, either on a density gradient [Edwards et al., 1988], by culture of purified cortical collecting duct cells [Naray-Fejes-Toth et al., 1991], or by microdissection in

rabbit [Bonvalet et al., 1990] and mouse [Bonvalet et al., 1991]. These showed 11ß-DH activity along the length of the nephron which was greatest at sites of highest mineralocorticoid receptor expression. Therefore, enzyme-mediated receptor protection is likely to be an "autocrine" mechanism whereby 11ß-DH and mineralocorticoid receptors are present in the same cell.

Tissue-Specific Isoforms of 11ß-OHSD in Kidney

In addition to the discrepancy between immunohistochemical and *in situ* hybridisation distribution of the enzyme, a variety of kinetic, immunological and molecular evidence suggests that 11ß-OHSD may be expressed in more than one form, and that the isoform which protects renal mineralocorticoid receptors may differ from that in tissues which are not target sites for aldosterone (Table 1.2). Carl Monder raised two antisera to the purified liver enzyme and performed Western blots which showed different sizes of immunoreactive proteins in different tissues [Monder & Lakshmi, 1990]. The first antiserum (56-125) identified a 34 kD protein in liver, and additional species of 40 kD in kidney, 26 kD in brain, and 47 kD in testis. The second antiserum (56-126) identified the 34 kD protein in all tissues and additional 68 kD proteins in liver and kidney (possibly a dimer) and again a 40 kD protein in kidney. Using the purified rat liver 11ß-OHSD, an 11ß-OHSD cDNA was cloned from a liver cDNA library [Agarwal et al., 1989]. This predicts the amino acid sequence of a protein of 31 kD, but when the cDNA was expressed *in vitro* the resulting 34 kD protein was reduced to 31 kD when de-glycosylated with tunicamycin [Agarwal et al., 1990]. Interestingly this de-glycosylation altered the relative activities of 11ß-DH and 11ß-OR in the expressed protein.

Experiments examining the cofactor preference of 11ß-DH for NADP or NAD also suggest different isoforms in the distal renal tubule. The reduction of tetrazolium to diformazan blue in tissue slices depends on NADPH or NADH generated by 11ß-DH. Blue staining only occurred in distal tubules supplied with NAD rather than NADP [Mercer & Krozowski, 1992]. Tissue-specific cofactor specificity is addressed in detail in Chapter 2. It may contribute to the differences in equilibrium between 11ß-DH and 11ß-OR activity in kidney compared with liver described above.

The rat liver cDNA clone has been used as a probe to study 11ß-OHSD expression in various rat tissues. On Northern blots it cross-hybridised to a single species of mRNA (~1.7 kb) in most sites, except in kidney where at least three other species were detected (1.5 kb, 1.6 kb, and 1.9 kb)[Krozowski et al., 1990], and in colon

where a mRNA of 3.4 kb was found [Whorwood et al., 1992]. More recently cDNA clones have been isolated from a rat kidney library which differ from the liver cDNA clone in their 5' extremity [Krozowski et al., 1992]. One of these has been sequenced and shows an identical open reading frame to the liver cDNA downstream from amino acid Met 27. Interestingly, the amino acid sequence upstream from Met 27 is very hydrophobic and contains a putative signal peptide for membrane insertion. Data from our department, derived from cloning of the 11ß-OHSD rat gene, shows that two isoforms (with and without signal sequence) are generated by alternative promoters and start sites from a single gene [Moisan et al., 1992b]. It remains to be seen whether probes from the two renal isoforms will show differential distribution within rat kidney by *in situ* hybridisation, and whether different isoforms of 11ß-OHSD are concentrated in different sub-cellular fractions.

Table 1.2 Tissue-specific heterogeneity of 11ß-OHSD

	Enzyme species on Western blots * (kD mol wt)		mRNA species on Northern blots † (size in nucleotides)
	Antiserum 56-125	Antiserum 56-126	
Liver	34	34 68	1700
Kidney	34 40	34 40 68	1700 1900 1600 1500
Brain	26	34	1700
Testis	34 47	34	1700
Colon			1700 3400

* Performed with rabbit antisera raised against purified rat liver 11ß-OHSD (Monder & Lakshmi, 1990)

† Performed with cRNA probes transcribed from cDNA encoding rat liver 11ß-OHSD (Krozowski et al., 1990; and for colon, Whorwood et al., 1992)

Recently, a human cDNA clone derived from a testis library has been isolated, and the human 11ß-OHSD gene on chromosome 1 has been partially sequenced [Tannin et al., 1991]. Surprisingly, only one mRNA species was detected in human kidney using this cDNA as a probe. However, in sheep kidney heterogeneity of 11ß-OHSD mRNA has been observed [Yang et al., 1991].

It is possible that tissue-specific 11ß-OHSD isoforms are expressed from a second 11ß-OHSD gene. The mechanism whereby 11ß-OHSD dictates tissue sensitivity to cortisol is analogous to the role of 5α-reductase in dictating sensitivity to testosterone [Fraser, 1990]. Recently, a second gene for 5α-reductase has been cloned [Andersson et al., 1991]. It codes for a second enzyme which differs from the first in its tissue distribution, kinetics, and sensitivity to finasteride inhibition. Furthermore, it is a defect in the second gene which accounts for male pseudohermaphroditism.

Paradoxes in the Influence of 11ß-OHSD on Tubular Electrolyte Transport

The presence of immunoreactive 11ß-OHSD in proximal tubules suggests that, in addition to its autocrine role in the distal renal tubule, the enzyme may have other roles in the kidney. Since mineralocorticoid receptors are not expressed in proximal tubules [Bonvalet et al., 1991] this raises the possibility that local metabolism dictates glucocorticoid activation of proximal type 2 receptors. Our information about the influence of 11ß-OHSD comes mainly from studies of the effects of liquorice and carbenoxolone. Interestingly, there are differences between the renal effects of these two agents (Table 1.3).

A metabolic balance study in man confirmed that carbenoxolone induces antinatriuresis associated with prolonged half life of (11α^{3}H)-cortisol, increased urinary free cortisol excretion and reduced cortisol production rates [Stewart et al., 1990]. Furthermore, carbenoxolone had previously been shown to provoke excessive activation of colonic mineralocorticoid receptors as judged by increased mucosal subtraction potential difference [Tomkins & Edmonds, 1975]. However, carbenoxolone did not reduce levels of plasma cortisone and did not increase the urinary (THF + allo-THF)/THE ratio. In addition, although plasma potassium fell there was no kaliuresis with carbenoxolone [Stewart et al., 1990]. Others have shown this before and excluded increased gastrointestinal potassium loss as the cause [Baron et al., 1969]. This difference has not been clarified by studies in rats since carbenoxolone does induce a kaliuresis in response to corticosterone in this species

Table 1.3 Biochemical features of congenital and acquired 11ß-OHSD deficiency

	Apparent Mineralocorticoid Excess Type 1	Apparent Mineralocorticoid Excess type 2	Liquorice Administration	Carbenoxolone Administration
11ß-OHSD Activity				
11ß-dehydrogenase				
Half life of (11α^{3}H)-cortisol (normal 42 ± 2 min)	131	no data	84.3 ± 3	123 ± 3
11ß-reductase:				
Conversion of oral cortisone to cortisol	Normal	no data	no data	Reduced
Urinary Cortisol Metabolites:				
(THF+allo-THF):THE	Raised	Normal	Raised	Normal
allo-THF:THF	Raised	Normal	Raised	Normal
Secondary Steroid Changes:				
Plasma Cortisone	Reduced	no data	Reduced	Normal
Cortisol Production Rate	Reduced	Reduced	Reduced	Reduced
Urinary Free Cortisol	Raised	Reduced	Raised	Raised
Renal Mineralocorticoid Receptor Activation				
Na balance	Retention	Retention	Retention	Retention
Plasma Renin Activity and Aldosterone	Suppressed	Suppressed	Suppressed	Suppressed
Plasma K	Reduced	Reduced	Reduced	Reduced
K Balance	Kaliuresis	Kaliuresis	Kaliuresis	Urinary K retention

[Souness & Morris, 1989]. In rats the kaliuresis is predominantly mineralocorticoid receptor-mediated since it could be blocked in large part by a type 1 receptor antagonist RU 28318 [Souness & Morris, 1991a] and not by a type 2 antagonist RU38486 [Funder et al., 1990].

In clinical practice we regard kaliuresis as an index of type 1 receptor activation [Conn, 1955], and might conclude from the absence of kaliuresis in man that carbenoxolone does not cause increased exposure of mineralocorticoid receptors to cortisol. It might therefore act by increasing exposure of glucocorticoid receptors. However, this view is probably simplistic.

The type 1 receptor is distributed in distal convoluted tubule, connecting tubule and collecting duct and mediates both sodium retention and kaliuresis [Fanestil & Park, 1981; Marver, 1984]. However, the effects on sodium and potassium may not be mediated by the same mechanism. For example, it is paradoxical that actinomycin D, an inhibitor of protein synthesis, blocked the antinatriuretic effect of aldosterone without affecting its kaliuretic effect [Fanestil & Park, 1981], and type 1 receptor antagonists have been shown to have a disproportionate effect on antinatriuresis compared with kaliuresis [Mills et al., 1962]. This might explain why up to 30 % of patients with Conn's syndrome are normokalaemic [Bravo et al., 1982].

The type 2 receptor is more widely distributed along the nephron and has a less clearly characterised role in which increased glomerular filtration rate and antinatriuresis are most prominent [Fanestil & Park, 1981; Marver, 1984]. However, observations *in vivo* suggest that glucocorticoids exert both antinatriuretic and kaliuretic effects [Johnson et al., 1982; Campen et al., 1983; Whitworth et al., 1984; Connell et al., 1987b] and that only the kaliuretic effect can be prevented by type 2 antagonists [Clore et al., 1988; Montrella-Waybill et al., 1991]. By contrast, the antinatriuresis can be prevented by type 1 antagonists [Mills et al., 1962; Clore et al., 1988].

Moreover, there appears to be promiscuity between mineralocorticoid and glucocorticoid receptors not only in their ligand binding, but also in their activation of some steroid response elements. Thus pure type 2 receptor agonists can induce mineralocorticoid effects in the distal tubule [Naray-Fejes-Toth & Fejes-Toth, 1990]. There are also interactions between type 1 and type 2 agonists, such that type 2 activation may impair the response to aldosterone [Kenyon et al., 1984].

Thus, kaliuresis is far from a *sine qua non* of type 1 receptor activation; it remains to be established whether carbenoxolone-induced antinatriuresis in man is mediated by activation of a receptor other than the mineralocorticoid receptor; and it remains speculative that 11ß-OHSD has a role in regulating access of cortisol to type 2 receptors in the kidney.

There are numerous potential explanations for the discrepancies between liquorice and carbenoxolone in man. Both compounds have a number of biochemical effects which are not related to 11ß-OHSD inhibition [Monder, 1991b]. These include inhibition of prostaglandin synthesis [Baker & Fanestil, 1991], alterations in hepatic aldosterone metabolism [Latif et al., 1990], direct effects on Na/K transport [Baron & Green, 1986], and potentiation of the effects of steroids not subject to 11ß-OHSD metabolism (eg aldosterone, 11-deoxycorticosterone, and pure synthetic glucocorticoid agonists)[Morris & Souness, 1990; Funder et al., 1990]. However, the level of plasma GE attained in the metabolic balance studies was not high enough to cause most of these effects [Stewart et al., 1987; Teelucksingh et al., 1991].

Of greater interest is the observation that carbenoxolone has no effect on the ratio of cortisol to cortisone in plasma or of their metabolites in urine. This is explicable by the hypothesis that in man (but not in rats [Monder et al., 1989]) carbenoxolone inhibits both 11ß-DH and 11ß-OR activities of 11ß-OHSD. This was confirmed by measuring the accumulation of systemic plasma cortisol after oral administration of cortisone, which was reduced after carbenoxolone. By contrast conversion of cortisone to cortisol was not affected by liquorice (author's unpublished data). Carbenoxolone may thus reduce cortisol/cortisone turnover rate without causing a net change in cortisol/cortisone ratios [Stewart et al., 1990]. One might think that absence of net effect on cortisol/cortisone ratio would prevent carbenoxolone from influencing mineralocorticoid receptor activation. However, because 11ß-DH predominates over 11ß-OR in the kidney (see "Biochemistry and Distribution" above) carbenoxolone would increase intra-renal cortisol concentrations. Conversely, in liver inhibition of predominantly 11ß-OR activity would reduce intra-hepatic cortisol concentrations. The net effect would be no change in circulating or urinary levels.

It is tempting to suggest that the inhibition of 11ß-OR by carbenoxolone accounts for the difference in the effects of carbenoxolone and liquorice on urinary potassium excretion. Isolated deficiency of the conversion of cortisone to cortisol has been reported in 3 female patients, 2 of whom were sisters [Taylor et al., 1984; Phillipou

& Higgins, 1985]. In this syndrome the ratio of cortisone to cortisol was elevated and there was ACTH-dependent stimulation of adrenal androgens resulting in hirsutism. Blood pressure and electrolytes were normal. Thus loss of 11ß-OR alone probably has no effect in the kidney. Perhaps of more relevance is the possibility that inhibition of 11ß-OR by carbenoxolone is a marker for differential inhibition of 11ß-OHSD isoforms by liquorice and carbenoxolone. This may explain the difference in potassium excretion if the isoforms were localised in different parts of the tubule where increased corticosteroid receptor activation might have different effects. At the time of writing no-one has been able to demonstrate differences in the inhibitory activity of carbenoxolone and GE in different regions of the nephron.

Regulation of Renal 11ß-OHSD

Despite the extensive literature reporting *in vitro* measurements of 11ß-OHSD activity in many organs there is relatively little information about the factors which might regulate activity, particularly in the kidney. A number of competitive inhibitors of the enzyme have been identified, including endogenous progestogens [Murphy, 1981a], bile acids [Perschel et al., 1991] and several synthetic steroids [Monder & Shackleton, 1984; Buhler et al., 1991](Table 1.4). In cultured fetal lung cells and human skin fibroblasts 11ß-DH activity could be induced by glucocorticoids [Smith et al., 1973; Hammami & Siiteri, 1991] but this was not reproduced in the whole animal [Lugg & Nicholas, 1978]. In liver of intact rats 11ß-DH activity was increased by thyroxine [Koerner & Hellman, 1964] and testosterone [Lax et al., 1978] while oestrogen inhibited it [Lax et al., 1978]. However, in intact baboon placenta oestrogens increased 11ß-DH activity [Baggia et al., 1990]. In keeping with the possibility that a different isoform is responsible for 11ß-DH activity in the kidney, its regulation is not consistent with that in other sites. For example, it varies in a different pattern during ontogeny in early life [Moisan et al., 1992b], and may be increased by oestrogens [Smith & Funder, 1991]. Cloning of the rat 11ß-OHSD gene has identified consensus sequences of response elements for glucocorticoid, thyroid, and oestrogen receptors and for cAMP [Moisan et al., 1992a].

Studies of the regulation of 11ß-OHSD activity in man are hampered by confusion about whether the activity being measured is in kidney or liver. There is good evidence that hypothyroidism is associated with reduced 11ß-DH activity (see below). ACTH may have an extra-adrenal effect to reduce the conversion of cortisol to cortisone [Kornel, 1970]. However, it remains to be seen whether the renal enzyme represents a fixed barrier to cortisol/corticosterone, or whether it might be subject to

Table 1.4 Inhibitors of 11ß-OHSD

Exogenous	Endogenous
Glycyrrhetinic acid	11-OH-progesterone (α or ß)
Glycyrrhizic acid	11-epicortisol
Carbenoxolone	Bile acids
CHAPS	
Ketoconazole	
11-epiprednisolone	

physiological regulation. This possibility is addressed further in the rat in Chapter 2 and in man in Chapter 4.

The Physiological Role of Extra-Renal 11ß-OHSD

Initially it seemed that only tissues with aldosterone selective mineralocorticoid receptors express 11ß-OHSD, and that the non-selectivity of the mineralocorticoid receptor in heart and hippocampus could be explained by lack of the enzyme in these tissues [Edwards et al., 1988]. Thus, bioactivity of 11ß-DH was not demonstrated *in vitro* in initial studies with heart or hippocampus, but there was easily demonstrable activity in specific mineralocorticoid receptor target organs such as salivary gland and kidney. However, recently 11ß-DH bioactivity has been demonstrated in the hippocampus when the *in vitro* reaction is driven in favour of the conversion of corticosterone to 11-dehydrocorticosterone by addition of cofactor NADP [Moisan et al., 1990b]. In addition the enzyme has long been known to be present in tissues which are not thought to be aldosterone targets or to contain mineralocorticoid receptors, many of which have high expression of glucocorticoid receptors (Table 1.1). To explain the presence of 11ß-OHSD in these tissues we need to expand our hypothesis for the modulatory role of enzyme-mediated receptor protection.

The association of 11ß-OHSD with glucocorticoid receptors raises the possibility that 11ß-OHSD modulates access of cortisol to these receptors in many tissues. Dr Paul Teelucksingh first addressed this possibility in this department by employing the skin vasoconstrictor assay, a bioassay previously used to compare the potency of topical synthetic glucocorticoid preparations [McKenzie, 1962a; McKenzie & Stoughton,

1962b]. Cutaneous vasoconstriction has been shown to be a glucocorticoid receptor mediated response since it can be prevented by topical [Marks et al., 1982] or systemic [Gaillard et al., 1985] glucocorticoid receptor antagonists, and Paul confirmed that it was still present in a child with pseudohypoaldosteronism (congenital deficiency of mineralocorticoid receptors). To determine whether 11ß-DH might control the amount of cortisol reaching skin glucocorticoid receptors he carried out a series of studies using this bioassay system. When hydrocortisone (= cortisol) was applied to the forearm overnight under an occlusive dressing, there was minimal vasoconstriction. However, when GE was added to inhibit 11ß-DH there was marked potentiation of the response [Teelucksingh et al., 1990]. He confirmed with immunohistochemistry in human skin and *in vitro* studies of nude mouse skin that 11ß-OHSD is present in the basal epidermal layers [Teelucksingh et al., 1990], though it is uncertain what physiological role it plays there. This avenue of investigation has revealed the potential therapeutic benefit of 11ß-DH inhibition in targeting the local actions of hydrocortisone. It also offers an explanation for the anti-inflammatory properties of topical GE [Adamson & Tillman, 1955] and carbenoxolone [Csonka & Murray, 1971], properties which are absent in adrenalectomised animals [Khan & Sullivan, 1967].

Further evidence that 11ß-OHSD modulates access of corticosteroids to glucocorticoid receptors comes from a recent study of the effects of corticosteroids on the Na/K ATPase α subunit in rat colon [Fuller & Verity, 1990]. It was shown that a glucocorticoid-specific induction of expression of Na/K ATPase could be reproduced by the administration of carbenoxolone, but only in animals with intact adrenals.

In the central nervous system our group and others have described wide distribution of 11ß-OHSD bioactivity, immunoreactivity and mRNA [Moisan et al., 1990b; Monder & Lakshmi, 1990; Lakshmi et al., 1991; Sakai et al., 1992] with very high expression in the cerebellum which also has a high concentration of glucocorticoid receptors [Moisan et al., 1990a]. Dr Jonathan Seckl recently showed that cerebral glucose uptake is influenced by GE administration [Seckl et al., 1991] and that intra-cerebroventricular GE affects the binding of ^{3}H-corticosterone in the rat brain (personal communication).

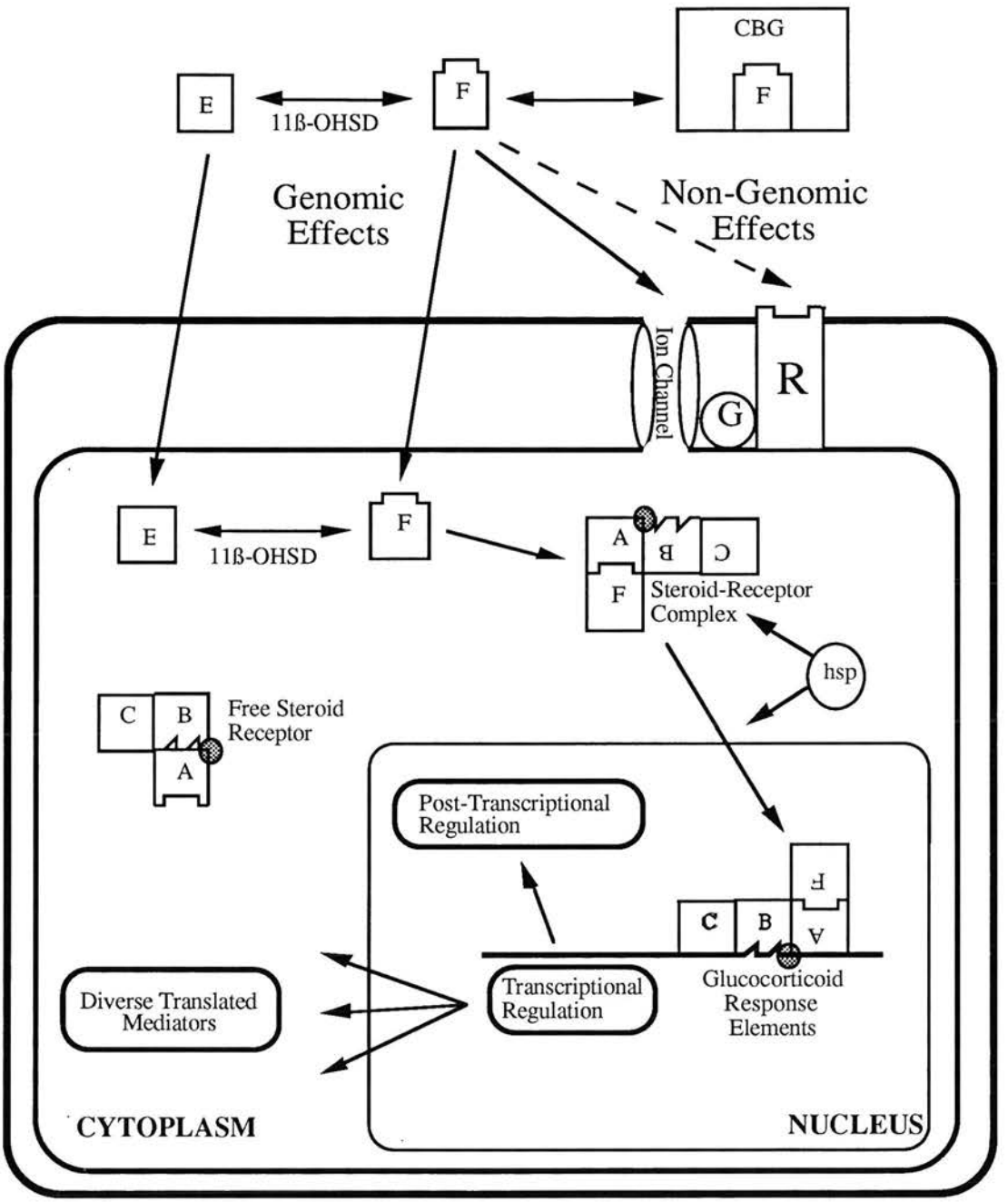
11ß-OHSD is also very active in Leydig cells in the testis. It is known that cortisol inhibits testosterone production in the rat and the observation that 11ß-OHSD expression suddenly increases in the testes of rats as they reach puberty [Phillips et al., 1989] raises the possibility that it plays a role in protecting the adult testis from

excess cortisol exposure. This is confirmed by recent studies showing that carbenoxolone potentiates corticosterone-dependent inhibition of LH-induced testosterone release in isolated Leydig cells [Monder et al., 1991a]. A similar temporal relationship between 11ß-DH activity and function has also been observed in breast tissue [Quirk et al., 1990].

Finally, the potential significance of placental 11ß-OHSD in dictating glucocorticoid exposure of the foetus, originally suggested by Murphy and others (see above), has been highlighted by more recent work. Epidemiological data suggest that individuals with low birth weight and high placental weight are more likely to suffer cardiovascular disease in later life [Barker et al., 1990]. It is possible that glucocorticoid exposure is responsible for this pattern, since administration of glucocorticoids in pregnancy is associated with small babies [Reinisch et al., 1978], large placentae [Gunberg et al., 1957], and hypertension in the offspring [Lindsay et al., 1992]. Moreover, 11ß-DH activity is positively correlated with birth weight and inversely correlated with placental size [Benediktsson et al., 1992]. Further work is in progress in rats to assess whether inhibition of 11ß-DH in pregnant rats will produce large placentae and low-birth-weight offspring who develop hypertension as adults.

Given the wide distribution of its physiological roles, the action of 11ß-OHSD should now be integrated into an up-dated model of factors dictating cortisol sensitivity in each cell, which must also take account of other recent observations. In addition to classical cytosolic steroid receptor activation, it has been suspected that steroids, which are lipid-soluble, interact with cell membranes as they pass through [McEwan, 1991]. This possibility has now crystalised in the recent identification of binding sites in cell membranes for both corticosterone [Orchinik et al., 1991] and aldosterone [Wehling et al., 1991]. These receptors are thought to mediate their effects independently of gene transcription. In addition, the hypothesis [Stephenson et al., 1984] that CBG is involved in dictating cellular sensitivity to cortisol should not be abandoned, since it was recently observed that activated neutrophils release elastase which promotes local dissociation of cortisol from CBG and increases glucocorticoid exposure of the neutrophil [Hammond et al., 1990]. A suggested model for the determinants of cellular sensitivity to cortisol is represented in Figure 1.5.

Figure 1.5 Model of factors which might dictate sensitivity to cortisol in the target cell



A = steroid binding domain; B = DNA binding domain; C = immunomodulatory domain; CBG = cortisol binding globulin; E = cortisone; F = cortisol; G = guanine nucleotide binding protein; hsp = heat shock protein; R = cell surface receptor.

The model of enzyme-mediated receptor protection has parallels in the target organ activity of several other hormones. For most of these local metabolism confers an increased potency on the circulating hormone, contrasting with the protection observed with 11ß-DH. They include the role of 5'-monodeiodinase in converting thyroxine to tri-iodothyronine [Berry et al., 1991], the role of 5α-reductase in converting testosterone to dihydrotestosterone [Andersson et al., 1991], the role of 17ß-hydroxysteroid dehydrogenase in the conversion of oestrone to oestradiol [Stewart & Sheppard, 1992], and the role of aromatase in converting androgens to oestrogens within breast tissue [Aitken et al., 1992]. It seems that for every member of the steroid receptor superfamily there may be a role for local metabolism in determining local ligand concentrations.

Until now it has been possible to test the hypothesis that 11ß-DH "protects" receptors from excessive exposure to active glucocorticoid by examining the effects of 11ß-DH inhibitors. However, there is an alternative role for the enzyme analogous to the role of other "shuttle" systems. While 11ß-DH protects some receptors by inactivation of cortisol to cortisone (eg mineralocorticoid receptors in kidney and glucocorticoid receptors in skin), in other sites 11ß-OR may amplify the exposure of relatively low affinity glucocorticoid receptors by activation of cortisone to cortisol. This might apply in tissues which apparently favour 11ß-OR activity, eg liver. This hypothesis probably explains the apparent effects of the inactive metabolite 11-dehydrocorticosterone in toad bladder cells transfected with rat liver 11ß-OHSD (Bernard Rossier, personal communication) and in intact rat kidney [Souness & Morris, 1991b] when carbenoxolone is present and the 11ß-OHSD equilibrium favours the active steroid.

11ß-OHSD and Blood Pressure Control

One of the most exciting implications of the work in 11ß-OHSD deficiency is the possibility that a similar defect contributes to hypertension in more common clinical syndromes. It is most obvious to seek this association in syndromes where increased activation of mineralocorticoid receptors occurs and might be attributed to cortisol. However, this is not the only mechanism by which cortisol may induce hypertension, nor the only route by which 11ß-DH deficiency may exacerbate it.

Extra-Renal Mechanisms for Cortisol-Induced Hypertension

Despite the fact that Cushing's syndrome is the oldest recognised cause of secondary hypertension, and that the incidence of hypertension in spontaneous Cushing's syndrome may be anything from 50 to 87% [Plotz et al., 1952; Ross & Linch, 1982; Howlett et al., 1986], the mechanisms whereby cortisol raises blood pressure remain poorly understood [Saruta et al., 1986; Fraser et al., 1989]. It has proved hard to identify both the receptors involved and the sites of action of the steroid.

It is clear that type 1 receptor activation can raise blood pressure [Nicholls et al., 1979; Fraser & Padfield, 1985] and that some of the effects of cortisol may reflect spill-over onto these receptors. However, hypertension also occurs after synthetic type 2 receptor agonists when it can be blocked by type 2 receptor antagonists [Grunfeld et al., 1985; Nieman et al., 1985] but not by type 1 antagonists [Grunfeld et al., 1985; Gomez-Sanchez & Gomez-Sanchez, 1991]. Furthermore, only 10% of patients with Cushing's syndrome not due to ectopic ACTH secretion have hypokalaemic alkalosis [Saruta et al., 1986; Howlett et al., 1986], which is a feature in at least 70 % of those with primary adrenal adenomas producing aldosterone [Bravo et al., 1982]. Thus type 2 receptor activation can also contribute to raised blood pressure. It is also possible that some hypertensinogenic effects are mediated by non-classical steroid receptors. These have been proposed to explain the complex cocktail of steroids required to reproduce ACTH-dependent hypertension in sheep [Scoggins et al., 1989].

In attempting to identify the hypertensinogenic site of action of glucocorticoids most attention has focussed on the effect of cortisol on sodium retention in the kidney. However, this does not appear to account for all the effects of glucocorticoids on blood pressure. In the rat dexamethasone-induced hypertension is accompanied by a fall in plasma volume [Tonolo et al., 1988] and in man Cushing's syndrome is not associated with dramatic suppression of the renin-angiotensin-aldosterone axis [Ritchie et al., 1990]. Moreover, in man ACTH-dependent hypertension [Whitworth et al., 1983], which can be reproduced by cortisol [Connell et al., 1987b], is sodium-modified but not sodium-dependent [Whitworth et al., 1985; Connell et al., 1988]. It is associated with only a modest kaliuresis and plasma volume expansion [Connell et al., 1987b]. Several pieces of evidence suggest that actions of cortisol outside the kidney may be equally important. Some of these actions may be influenced by 11ß-OHSD activity.

A hypertensinogenic action of cortisol in the liver was suggested after the measurement of low plasma renin activity but elevated renin substrate (angiotensinogen derived from liver) in glucocorticoid excess, from which it was inferred that bioactive angiotensin II might be elevated and cause vasoconstriction [Krakoff et al., 1975]. However, with accurate measurement of angiotensin II it has become clear that it is not elevated [Connell et al., 1987b; 1988], and that this hepatic effect of glucocorticoids is unlikely to be a significant factor in hypertension.

In the central nervous system corticosteroid receptors are widely distributed and may mediate elevations in blood pressure. However, in rats the intra-cerebroventricular administration of corticosterone tends to lower blood pressure while that of aldosterone raises it [Gomez-Sanchez, 1986; van den Berg et al., 1990]. For glucocorticoid excess to raise blood pressure therefore might require binding to non-classical glucocorticoid receptors. As described above 11ß-OHSD is widely distributed in brain. Elise Gomez-Sanchez recently reported that the elevation in blood pressure in rats given systemic carbenoxolone can be attenuated by simultaneous intra-cerebroventricular administration of a type 1 receptor antagonist, RU28318 [Gomez-Sanchez & Gomez-Sanchez, 1991]. This suggests that activation of mineralocorticoid receptors in brain contributes to the hypertension of 11ß-DH inhibition. Having said that, it remains almost impossible to determine whether defective 11ß-OHSD in the central nervous system contributes to elevated blood pressure in man.

Some of the pressor effect of cortisol may be mediated by an increased cardiac output. Glucocorticoid [Funder et al., 1973c; Seleznev et al., 1978] and mineralocorticoid [Arriza et al., 1987; Funder et al., 1988; Barnett & Pritchett, 1988] receptors have been reported in cardiac muscle. Glucocorticoid-specific modulation of protein synthesis occurs in cardiac cell culture [Fukada, 1980; Nichols et al., 1984b], and mineralocorticoid-specific modulation of RNA polymerase has been demonstrated in isolated cardiac nuclei [Liew et al., 1972]. Increased cardiac output has been demonstrated in corticosteroid excess [Sudhir et al., 1989] and positive inotropic effects of both glucocorticoids and mineralocorticoids have been observed [Sayers & Solomon, 1960; Tanz, 1962], possibly mediated by catecholamines [Cornish et al., 1978]. In addition, mineralocorticoid receptor activation in cardiac fibroblasts may promote myocardial fibrosis [Weber & Brilla, 1991]. However, in the original studies of 11ß-OHSD distribution the presence of the enzyme in heart was not recognised [Edwards et al., 1988].

Finally, some of the hypertensinogenic effect of cortisol may be mediated by actions on vascular smooth muscle cells which determine peripheral vascular resistance. This area is particularly exciting given Paul Teelucksingh's demonstration with the skin vasoconstrictor assay that inhibition of 11ß-DH potentiated the effect of cortisol on dermal blood vessels [Teelucksingh et al., 1990](see above). 11ß-DH activity has been demonstrated in vascular tissue of both rabbit [Kornel et al., 1982] and rat [Funder et al., 1989], and in the latter it has been suggested that the distribution of activity matches the distribution of aldosterone specific corticosteroid receptor binding by the mesenteric vascular bed [Funder et al., 1989].

Clinical Relevance of 11ß-OHSD

In addition to its potential relevance in the pathogenesis of mineralocorticoid hypertension, the appreciation of the diverse potential roles of the enzyme outside the distal nephron implies that deficiency of 11ß-OHSD may be significant in any number of disorders which are associated with "too little" or "too much" local glucocorticoid. Moreover, by inhibiting 11ß-DH we may be able to target local glucocorticoid without incurring systemic effects. In this thesis I limit the discussion to consideration of the relevance of 11ß-OHSD deficiency to hypertension. For a consideration of other potential applications of these principles see Walker & Edwards [1992].

One might suggest that 11ß-OHSD could be relevant in any hypertensive syndrome, but in a handful there are particular reasons to suspect that it has a role.

The Syndrome of Apparent Mineralocorticoid Excess "Type 2"

Ulick and colleagues recently reported a "syndrome of apparent mineralocorticoid excess type 2" in 4 patients with low-aldosterone, low-DOC mineralocorticoid excess exacerbated by ACTH and improved by dexamethasone [Ulick et al., 1990; 1992a; Tedde et al., 1992]. In these patients the total cortisol metabolites in urine are reduced but the ratios of (THF + allo-THF):THE and allo-THF:THF are normal. In this respect the type 2 syndrome is similar to that seen after carbenoxolone and may be caused by congenital deficiency of 11ß-DH in kidney and 11ß-OR elsewhere. In addition, these patients exhibit deficient A-ring reduction of cortisol [Ulick et al., 1992a]. However, two key experiments have yet to be reported in type 2 patients: (i) confirmation that cortisol is the unidentified ACTH-dependent mineralocorticoid by observing the effects of exogenous cortisol on renal sodium and potassium handling;

and (ii) measurement of (11α^3H)-cortisol half life, the most sensitive index of 11ß-DH activity. Without these experiments it remains uncertain whether the findings in the type 2 syndrome are explained by: (i) combined deficiency of 11ß-DH and 11ß-OR, as with carbenoxolone; (ii) deficient A-ring reduction as an alternative failure of normal cortisol clearance in the kidney; or (iii) the presence of another unidentified ACTH-dependent mineralocorticoid.

Hypothyroidism

Hypothyroidism is a reversible cause of hypertension [Streeten et al., 1988]. It is well-documented that in man hypothyroidism is associated with inhibition of 11ß-DH, with elevation of (THF+allo-THF):THE ratio [Hellman et al., 1961] and prolongation of the half life of (11α^3H)-cortisol [Zumoff et al., 1983]. 11ß-OR is also inhibited but to a lesser extent [Ichikawa et al., 1977; Zumoff et al., 1983]. The mechanism of hypothyroid hypertension is disputed. Although suppression of plasma renin activity and aldosterone occurs [Elias et al., 1986] its relevance to pathogenesis has been questioned [Waters, 1978; Richards et al., 1985]. If excessive mineralocorticoid receptor activation does occur then it appears to be limited to the kidney, since colonic potential difference did not suggest mineralocorticoid excess [Elias et al., 1986]. Evidence that hypothyroid hypertension is cortisol-dependent has yet to be reported.

Alcohol Abuse

Alcoholism is another reversible risk factor for hypertension [Saunders et al., 1981]. A study performed in Stockholm showed evidence of impaired 11ß-OHSD activity in alcoholic patients, as shown by increased THF:THE ratio [Cronholm et al., 1985]. However, studies performed in Edinburgh in patients withdrawing from alcohol failed to demonstrate such an abnormality [Edwards & Stewart, 1989]. Alcohol inhibits 11ß-OHSD *in vitro* but when administered to rats *in vivo* does not appear to affect the enzyme (Paul Stewart, personal communication).

Renal Failure

As renal function deteriorates plasma cortisone levels fall, irrespective of the cause of renal failure [Whitworth et al., 1989b]. This suggests that 11ß-DH is destroyed, most likely because of non-specific attrition of cells containing the enzyme. When early work suggested that the enzyme was sited in proximal tubules and played a paracrine role in the protection of mineralocorticoid receptors in the distal nephron

Stewart and Edwards suggested that loss of 11ß-DH might proceed independently of loss of mineralocorticoid receptor target cells. This mechanism could leave mineralocorticoid receptors unprotected from cortisol and contribute to the sodium retention and hypertension of renal failure. On arriving in the department I performed some experiments not included in this thesis in which I examined the effect of dexamethasone on Na/K balance in patients with renal failure. It was disappointing to find an antinatriuresis, such as has been observed previously with glucocorticoids [Clore et al., 1988], rather than the natriuresis seen in congenital 11ß-DH deficiency [Werder et al., 1974; Shackleton et al., 1980; Stewart et al., 1988b; Tedde et al., 1992] and after liquorice [Hoefnagels & Kloppenborg, 1983]. Since then it has emerged that an autocrine rather than paracrine relationship exists between 11ß-DH and renal mineralocorticoid receptors (see above) and therefore loss of 11ß-DH independent of loss of mineralocorticoid receptors is unlikely in the context of the non-specific cellular attrition which occurs in chronic renal failure.

ACTH-Dependent Hypertension

A putative regulator of 11ß-OHSD activity is ACTH, acting extra-adrenally. Administration of ACTH to volunteers produced a rise in the cortisol/cortisone ratio in plasma with virtually no increase in cortisone [Srivastava et al., 1973; MacKenzie et al., 1990]. Given the large capacity of 11ß-DH in kidney [Hellman et al., 1971] it seems unlikely that this is due to saturation of the enzyme by increased substrate. A fall in adrenal secretion of cortisone might be responsible [Srivastava et al., 1973; Burt et al., 1991], but most likely is the hypothesis that peripheral conversion of cortisol to cortisone is decreased by ACTH. This is supported by studies in two Addisonian patients given ACTH in whom the half life of cortisol was prolonged and that of cortisone reduced with a resultant rise in THF:THE ratio [Kornel, 1970]. This hypothesis is explored further in Chapter 4.

The highest levels of bioactive ACTH associated with increased cortisol production occur in the ectopic ACTH syndrome, in which hypertension and hypokalaemia are much more common than in other forms of Cushing's syndrome [Howlett et al., 1986]. ACTH-dependent inhibition of 11ß-OHSD might explain this unusual feature by allowing cortisol to flood renal mineralocorticoid receptors. Experiments to test this hypothesis are presented in Chapter 5.

Essential Hypertension

By comparison with the majority of hypertensive patients who have "essential" hypertension all other hypertensive syndromes are rare. As I discussed at the outset, there is evidence that a sub-group of these patients have hypertension which is dependent on ACTH secretion since it can be suppressed by dexamethasone [Hamilton et al., 1979; Whitworth et al., 1989a]. Furthermore, a number of studies have demonstrated abnormal metabolism of cortisol in essential hypertensives [Kornel et al., 1975]. Defective enzyme-mediated receptor protection provides a mechanism by which abnormal cortisol metabolism could raise blood pressure through over-exposure of corticosteroid receptors to cortisol. This subject is addressed in detail in Chapter 5.

CORTICOSTEROIDS AND VASCULAR TONE

The possible relevance of 11ß-OHSD activity to the control of vascular tone in health and in hypertension interested me from the outset of my work for this thesis. It presented a number of testable hypotheses which tie in many of the themes discussed above in relation to the kidney. It also proved to be more relevant to the role of 11ß-OHSD deficiency in essential hypertension than we had first supposed (see Chapter 5). Interactions between corticosteroids and vascular tone have been the subject of scant review in the literature and in the second section of the Introduction I will present the evidence in detail, in justification of studies of the physiology of 11ß-OHSD in vascular tissues presented in Chapter 2 (biochemistry and distribution of vascular 11ß-OHSD) and Chapter 3 (11ß-OHSD and the control of vascular tone), and to highlight the potential importance of the observations in human hypertensive patients reported in Chapter 5.

Effects of corticosteroids on vascular tone have been recognised since the 1940s. However, despite repeated demonstration of pharmacological effects both *in vitro* and *in vivo*, their physiological significance as regulators of tone in health remains to be established. Evidence that abnormal vascular reactivity is important when adrenal steroid production is deranged is more convincing, and probably contributes to the changes in blood pressure seen in conditions of corticosteroid excess and deficiency. It remains speculative whether the same mechanisms contribute to the pathogenesis of other hypertensive syndromes and of circulatory shock.

Recently the effects of corticosteroids have been studied in cultured vascular cells. These studies exploit our improving understanding of the actions of steroid hormones on their target cells, and better appreciation of the diversity of biochemical signals in the vessel wall which mediate the response to vasoactive stimuli. They include measurements of endothelium-derived factors (which act on neighbouring vascular smooth muscle cells), contractile cell surface receptors, and intra-cellular second messengers (released within the contractile cell in response to activation of surface receptors). Such studies have shown a number of interactions between corticosteroids and newly discovered biochemical messengers. In this section I will assess whether our interpretation of the older evidence that steroids affect vascular tone is influenced by recent studies of their cellular effects, and whether we can now deduce the role of corticosteroids in vascular physiology and pathology. This

represents the background on which vascular 11ß-OHSD activity may play a modulatory role.

Corticosteroid Receptors in Vascular Smooth Muscle

Binding sites with the characteristics of classical glucocorticoid and mineralocorticoid receptors have been identified *in vitro* in homogenates of rabbit [Duval et al., 1977; Kornel, 1981; Kornel et al., 1982; 1983] and bovine aorta [Hayashi & Kornel, 1990], and in cultured vascular smooth muscle cells from rat aorta [Meyer & Nicholls, 1981] and human uterine artery [Scott et al., 1987]. Recently the presence of mineralocorticoid receptors in vascular smooth muscle was confirmed immunohistochemically using an anti-idiotypic monoclonal antibody [Lombes et al., 1990; 1991]. *In vivo* studies have shown binding of [^{3}H]-aldosterone in major vessels of dogs [Hollander et al., 1966] and in rat mesenteric artery [Funder et al., 1989]. These binding sites seem to function as genomic promoters since induction of glucocorticoid-responsive proteins has been demonstrated by 2-dimensional gel electrophoresis in cultures of rat aortic vascular smooth muscle cells [Nichols et al., 1983b; 1984a] and bovine aortic endothelial cells [Nichols et al., 1983a] exposed to a variety of corticosteroids for 24 h. If the effects of corticosteroids on tone are mediated by activation of classical receptors, they should conform to certain characteristics. They typically have a time course from 20 min over several hours, involve induction of expression of glucocorticoid-responsive genes, and can be prevented by inhibitors of protein synthesis (eg actinomycin D or cycloheximide) or by competitive receptor antagonists (eg RU38486 for glucocorticoid receptors and spironolactone for mineralocorticoid receptors)[Gustafsson et al., 1987].

In addition, some effects of corticosteroids may be independent of classical receptors, and of steroid response elements ("non-genomic" effects; see Figure 1.5). These include interactions between the lipid-soluble steroids and cellular lipid bilayer membranes, which induce effects over a faster time course than those resulting from genomic induction. However, this mechanism is hard to confirm since specific antagonists are not available, and it has not yet been examined in vascular tissue. Assuming an autocrine mechanism of 11ß-DH-mediated receptor protection it is hard to invoke a role for a microsomal enzyme in protecting cell surface receptors on the same cell.

Corticosteroid Effects on Vascular Tone

Corticosteroid Insufficiency

The earliest studies highlighting the potential role of corticosteroids in the control of vascular tone examined vessel diameter in the exposed mesenteric vascular arcade of anaesthetised rats. In this model adrenalectomy had a profound vasodilating effect which could be restored by topical administration of adrenocortical extract [Fritz & Levine, 1951; Zweifach et al., 1953]. Other investigators used the pressor response to catecholamine infusion as an indicator of vasoconstrictor sensitivity, and showed loss of pressor response to catecholamines after adrenalectomy in dogs [Goldstein et al., 1950; Ramey et al., 1951]. Interpretation of these studies is limited because they fail to account for the effect of glucocorticoids on cardiac output [Sayers & Solomon, 1960; Cornish et al., 1978] and because these animals also had excision of the adrenal medullae. Persuasive evidence that the changes were attributable to glucocorticoid deficiency came later with demonstration that the pressor response to catecholamines was reduced in rats by administration of the specific glucocorticoid receptor antagonist RU38486 [Grunfeld & Eloy, 1987], and that the effect of adrenalectomy was reversed by replacement with the glucocorticoids dexamethasone [Yagil & Krakoff, 1988] or corticosterone [Darlington et al., 1989] but not by aldosterone [Yagil & Krakoff, 1988].

Studies of vascular response in humans with adrenal insufficiency are scarce because of the need for immediate therapy. While the postural hypotension associated with Addison's disease is in part attributable to depletion of intra-vascular volume, in ACTH deficiency there is often a significant postural fall in blood pressure which is independent of sodium balance and might be attributable to impaired vascular responsiveness. Mineralocorticoids may also be involved in maintenance of tone in man since administration of the mineralocorticoid receptor antagonist canrenoate led to hypotension in man associated with reduced peripheral vascular resistance [Yamakado et al., 1988].

Corticosteroid Excess

In Vitro Studies

A number of investigators have examined the effects of corticosteroids on isolated vessels in organ baths, sometimes with contradictory results. Steroids were added to

baths for short periods of up to 20 min. They had no direct effects when added alone except in one study when a dose of budesonide of 10^{-3} M caused vasoconstriction [Angelo-Khattar & Thulesius, 1983]. In aortae from rabbit [Fowler & Chou, 1961; Kalsner, 1969b; 1969a; Koehler et al., 1979] and dog [Besse & Bass, 1966] 11-deoxycorticosterone, corticosterone, and hydrocortisone at doses of 10^{-5} to 10^{-7} M potentiated the constrictor response to catecholamines but not to other agents such as KCl, angiotensin II, or vasopressin. These findings are broadly in keeping with *in vivo* observations described below. However, in doses of 10^{-4} M corticosteroids caused inhibition of vasoconstriction to all of these agents [Altura & Altura, 1974; Bengtsson, 1981; Angelo-Khattar & Thulesius, 1983]. A dose-dependent biphasic effect was confirmed *in vivo* in studies of aorta and mesenteric artery in rats treated with high and low doses of hydrocortisone and methylprednisolone [Altura & Altura, 1974].

Interpretation of these *in vitro* studies is difficult. Firstly, the dose of steroid was generally higher than would be achieved *in vivo* and inappropriate to the low K_d of classical corticosteroid receptors or to the μM concentrations of steroids found in clinical glucocorticoid excess. Secondly, the time course of the steroid effect observed was often rapid, within seconds or minutes. It seems likely that the *in vitro* results could comprise both classical receptor-mediated events and non-genomic effects of steroids on cell membranes. Which of these responses are relevant *in vivo* is hard to deduce. The major contribution of these studies has been to demonstrate that effects of corticosteroids on vessels do occur, and that *in vivo* effects are therefore not necessarily entirely dependent on secondary changes such as altered renal sodium handling with suppression of angiotensin II production, or effects mediated by steroid actions in the central nervous system.

In Vivo Studies

In Cushing's syndrome due to adrenal adenoma pressor responses to noradrenaline and angiotensin II [Saruta et al., 1986] and digital vasoconstriction to noradrenaline [Mendlowitz et al., 1958] were increased. Systemic administration of cortisol or ACTH to healthy volunteers mimicked these findings except that the response to angiotensin II was not increased [Kurland & Freedberg, 1951; Whitworth et al., 1986]. These effects preceded the rise in blood pressure but took several days to develop [Sambhi et al., 1962]. Evidence is available to suggest that they reflect changes in vascular tone rather than intra-vascular volume or cardiac output. Firstly, the increase in pressor response after oral cortisol was independent of sodium intake

[Connell et al., 1987a]. Secondly, brachial artery infusion of noradrenaline produced a greater fall in forearm blood flow after oral cortisol [Sudhir et al., 1989], and also after dexamethasone, 11-deoxycorticosterone and fludrocortisone [Abboud, 1974].

Local administration of glucocorticoids to specific vascular beds *in vivo* also affects tone. Infusion of hydrocortisone into the brachial artery had no direct effect on blood flow in the hand but potentiated vasoconstriction in response to adrenaline [Ginsburg & Duff, 1958]. Similarly, glucocorticoids cause vasoconstriction when applied to human conjunctiva [Reis, 1960; Lepri & Cristiani, 1964] or skin [McKenzie & Stoughton, 1962b; Teelucksingh et al., 1990].

Broadly similar observations have been reported in animal models. Systemic dexamethasone administration to rats led to an increase in catecholamine vasoconstriction in isolated perfused mesenteric artery [Russo et al., 1990], and increased pressor responsiveness to noradrenaline [Schmid et al., 1967; Handa et al., 1984]. Vasoconstriction has also been observed after local application of glucocorticoids to vessels in rabbit ear [Ashton & Cook, 1952] and rat hind limb [Schomig et al., 1976].

In contrast to the lack of evidence that mineralocorticoid insufficiency affects vascular tone, mineralocorticoid excess has been associated with increased vascular reactivity. This was demonstrated in experimental mineralocorticoid excess in man by increased pressor responses [Raab et al., 1950; Chobanian et al., 1979; Distler et al., 1985] and increased catecholamine sensitivity of forearm vessels [Schmid et al., 1966; Abboud, 1974]. Similarly in animals sensitivity to catecholamines was increased in perfused caudal artery in 11-deoxycorticosterone (DOC)/salt hypertensive rats [Beilin et al., 1970], and in aortae excised from DOC/salt hypertensive rabbits [Kalsner et al., 1971] or aldosterone hypertensive rats [Smith et al., 1987; Jones et al., 1988]. In DOC/salt hypertension the change in vascular reactivity was shown to precede the elevation of blood pressure [Berecek et al., 1980]. However, topical application or infusion of mineralocorticoids had no effect on vascular tone.

Corticosteroid Effects on Vascular Biochemistry

In summary, both glucocorticoids and mineralocorticoids have pharmacological effects which increase vascular tone. Furthermore, glucocorticoids but probably not mineralocorticoids appear to be required for the maintenance of normal tone. These effects are not direct, but rely on permissive potentiation of the response to

catecholamines (particularly noradrenaline) and less consistently to other vasoactive agents studied (eg angiotensin II or vasopressin). By what biochemical mechanisms might these effects be mediated? In order to interact with other vasoactive hormones corticosteroids might influence: a) their local production and/or metabolism; b) their membrane receptors and post-receptor second messengers; or c) the final effector mechanisms mediating contraction. Furthermore, these effects may occur in vascular smooth muscle cells themselves, or in the vascular endothelium.

Some mechanisms of "second messenger" transmission from cell membrane receptor to functional response in vascular smooth muscle are illustrated in Figure 1.6. Each of these elements is a potential target for one of the diverse proteins induced by corticosteroids.

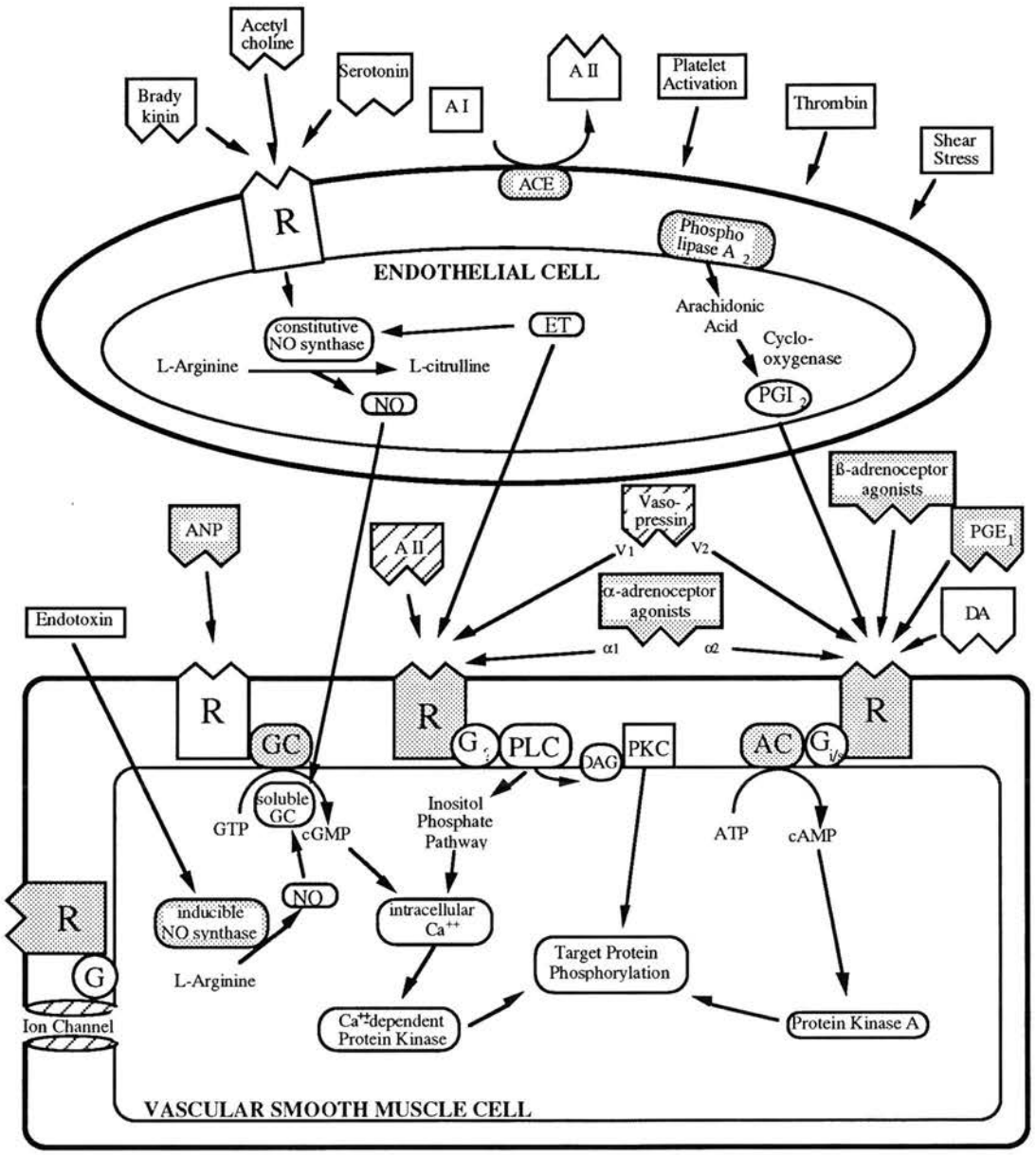
Effects on Vascular Smooth Muscle Cells

Hormone Metabolism

The earliest hypothesis to explain the vascular effects of corticosteroids was that they interfere with catecholamine metabolism thus causing increased local catecholamine concentrations. At high dose *in vitro* corticosteroids inhibited both extra-neuronal reabsorption of noradrenaline [Iversen & Salt, 1970] and catechol-o-methyl transferase metabolism [Kalsner, 1969a; 1969b]. However, in a number of studies *in vivo* these effects could not be shown to be relevant [Kalsner et al., 1971; Schomig et al., 1976; Esler et al., 1981; Sudhir et al., 1989].

In the late '70s and early '80s the importance of the prostaglandin family in vascular biology emerged. Glucocorticoids inhibit phospholipase A_2 and thus reduce production of the vasorelaxant eicosanoids prostaglandin E_1 and prostacyclin [Axelrod, 1983; Flower, 1988]. This action was invoked to explain the inhibition by glucocorticoids of ACTH-induced vasodilatation in rabbit fat pads [Lewis & Piper, 1975] and may also indirectly explain changes in catecholamine sensitivity [Axelrod, 1983]. Glucocorticoid potentiation of catecholamine responses has been mimicked in a number of models by the phospholipase A_2/cyclo-oxygenase inhibitor indomethacin. These include human skin [Bisgaard et al., 1986] and isolated perfused rat hind limb, where the effect of corticosterone was reproduced by indomethacin and abolished by excess arachidonic acid or prostacyclin [Rascher et al., 1980]. The pressor response to noradrenaline in rats was potentiated by both indomethacin and by 0.1 mg/day oral dexamethasone, which reduced urinary PGE_2

Figure 1.6 Potential biochemical signals in the vascular wall affected by glucocorticoids ▨ or mineralocorticoids ▨.



A I = angiotensin I; A II = angiotensin II; AC = adenylate cyclase; ACE = angiotensin converting enzyme; ANP = atrial natriuretic peptide; DA = dopamine; DAG = diacylglycerol; ET = endothelin 1; G = guanine nucleotide binding protein; GC = guanylate cyclase; NO = nitric oxide; PGE_1 = prostaglandin E_1; PGI_2 = prostacyclin; PKC = protein kinase C; PLC = phospholipase C; R = cell surface receptor.

excretion and attenuated the effect of indomethacin [Handa et al., 1984]. Urinary PGE_2 was similarly reduced in patients with Cushing's syndrome [Saruta et al., 1986]. However, other investigators have not been able to confirm a pharmacological effect of glucocorticoids on vascular prostaglandin production *in vivo* [Rogers et al., 1983]. Furthermore, when the dexamethasone given to rats was reduced to a sub-pharmacological dose which was not associated with weight loss but still increased blood pressure, ie 2 μg/day sc [Tonolo et al., 1988], an increased response to noradrenaline in the excised perfused mesenteric circulation was not affected by indomethacin and was not associated with a change in prostacyclin metabolites (6keto-$PGF_{1\alpha}$) in the effluent perfusate [Russo et al., 1990]. Also, the activation of adenylate cyclase by prostaglandin E_1, which usually produces relaxation, was potentiated by dexamethasone in vascular smooth muscle cells [Yasunari et al., 1988]. Thus involvement of prostaglandins remains unproven when physiological concentrations of glucocorticoids are administered *in vivo*.

Receptors and Second Messengers

In membranes from rat aortae the number of α_1-adrenoceptors was decreased by adrenalectomy and restored by dexamethasone replacement [Haigh & Jones, 1990b]. Similarly in cultured rat aortic vascular smooth muscle cells the number of ß-adrenoceptor binding sites was increased by dexamethasone at concentrations appropriate to physiology over 20 hours of incubation [Jazayeri & Meyer, 1988]. In addition to effects on receptor number, in the absence of glucocorticoid the coupling of adrenoceptors to G-proteins was disturbed [Haigh & Jones, 1990b] and the ratio of G_i to G_s subunits fell [Haigh et al., 1990a]. Functional significance of these observations is suggested by studies in vascular smooth muscle cells which confirmed that dexamethasone potentiated production of inositol triphosphate in response to noradrenaline (an α_1-adrenoceptor effect)[Liu et al., 1990], and that concomitant with the increase in ß receptor number there was a greater cAMP response to ß agonists [Jazayeri & Meyer, 1988]. This latter effect was blocked by cycloheximide and RU38486 suggesting that it is mediated through glucocorticoid receptors. Similar effects on angiotensin II receptor number and second messenger induction have been observed with aldosterone [Ullian et al., 1992], and mineralocorticoid receptor antagonists also prevent effects of corticosteroids on noradrenaline-induced inositol phosphate production [Haigh et al., 1991]. Relevance of these studies to the intact animal is suggested by increased generation of inositol monophosphate after incubation with noradrenaline by femoral artery tissue slices

from DOC/salt hypertensive rats [Eid & de Champelan, 1988]. Interestingly, one group described a direct effect of cortisol alone to increase inositol triphosphate concentrations in cultured cells. This was blocked by RU38486, and further increased by noradrenaline [Steiner et al., 1988; 1989]. However, in most laboratories direct effects of steroids on these second messengers have not been observed.

Changes in adrenoceptor number and function are not the only relevant glucocorticoid effects on second messenger systems. Glucocorticoids affect cAMP synthesis induced by other stimuli in other tissues [Davies & Lefkowitz, 1984]. The cAMP responses to dopamine (DA_1) [Yasunari et al., 1989] and prostaglandin E_1 [Yasunari et al., 1988] in cultured vascular smooth muscle cells from renal artery were increased by dexamethasone. These effects were maximal at 24 h, occurred at appropriate concentrations of dexamethasone but not aldosterone, and were inhibited by actinomycin D and cycloheximide [Yasunari et al., 1988; 1989]. The response to forskolin (direct activator of the catalytic unit of adenylate cyclase) was increased, suggesting that the effect of glucocorticoid was not only post-receptor but post-G-protein [Yasunari et al., 1988; 1989]. Phosphodiesterase inhibition was not implicated since isobutylmethylxanthine did not interfere with the potentiation. Increased cAMP would be expected to relax vessel tone, but in the same cells reduced cGMP generation in response to atrial natriuretic peptide (ANP) after dexamethasone provides a possible contractile effect [Yasunari et al., 1990]. Similar insensitivity to ANP was seen in aortae from mineralocorticoid hypertensive rats [Otsuka et al., 1988]. These observations together with the suppression of ANP production by glucocorticoids *in vivo* [Kenyon et al., 1990] suggest that recently recognised vasoactive agents may be highly significant in mediating corticosteroid effects.

Final Common Pathway of Contraction

Early studies to explain the effects of corticosteroids (particularly mineralocorticoids because of the analogy with their effects on mucosal Na/K exchange) focussed on changes in vascular electrolyte permeability. Chronic DOC administration to rabbits was associated with increased Na influx into vascular smooth muscle cells [Kornel, 1981] but *in vitro* administration of aldosterone to pig carotid arteries resulted in a fall in intra-cellular Na [Llaurado et al., 1983]. In rat tail artery aldosterone had two actions: (i) a rapid increase in Na efflux which was not blocked by actinomycin D; and (ii) a slow Na efflux which does appear to be classically mediated since it was blocked by actinomycin D and mineralocorticoid receptor antagonists [Moura &

Worcel, 1984]. These effects may in part result from potentiation of a response to vasopressin [Angeli et al., 1988]. They are not exclusive to mineralocorticoid receptor agonists, but the change in Na/H exchange seen after glucocorticoids [Berk et al., 1988] appears to be mediated by a different ion transporter since it was blocked by both amiloride and bumetanide, in contrast to the mineralocorticoid effect which was only blocked by amiloride [Kornel et al., 1991].

Effects on Endothelial Cells

In recent years the endothelium has been the focus of research attention in vascular biology. A significant advance was the identification of "endothelium-derived relaxant factor" as nitric oxide [Palmer et al., 1987]. In addition to the Ca^{++}-dependent calmodulin-dependent constitutive nitric oxide synthase of endothelium there is another Ca^{++}-independent endotoxin-inducible enzyme abundant in macrophages [DiRosa et al., 1990] and recently described in vascular endothelium [Radomski et al., 1990] and smooth muscle [Knowles et al., 1990; Wood et al., 1990]. This inducible enzyme is inhibited by glucocorticoids [Knowles et al., 1990; DiRosa et al., 1990], an effect which is blocked by glucocorticoid receptor antagonists [Radomski et al., 1990]. A recent *in vitro* study by Moncada's group demonstrated the functional consequences of this effect [Rees et al., 1990]. In untreated rat aortic rings a time-dependent relaxation and loss of response to phenylephrine occurred between 2 and 8 hours after starting the experiment, in rings both with and without endothelium. This was potentiated by nitric oxide synthase substrate (L-arginine), prevented by a nitric oxide synthase inhibitor (L-NMMA), and associated with endotoxin induction of Ca^{++}-independent nitric oxide synthase. Dexamethasone at 10^{-7} M over 8 h prevented this relaxation and loss of response to phenylephrine and inhibited the induction of nitric oxide synthase in vascular smooth muscle. This study was superior to any previous organ bath experiments because the time-course and dose of steroid application were more physiologically relevant (see above). However, the functional significance of this effect is uncertain. Although inhibition of nitric oxide synthase in human forearm caused vasoconstriction [Vallance et al., 1989a], implying that basal nitric oxide production is important, it remains to be seen whether the inducible enzyme makes a significant contribution *in vivo*.

Extensive debate has been conducted on the role of corticosteroids both in the pathogenesis and therapy of circulatory shock. These observations *in vitro* were evoked in this debate by proponents of endotoxin-induced nitric oxide production as a

pathogenetic mediator of circulatory failure in endotoxic shock [Moncada & Palmer, 1991] and in hepatic cirrhosis [Vallance & Moncada, 1991]. They have suggested that inhibition of nitric oxide production by dexamethasone should encourage the use of glucocorticoids in shock, though the *in vitro* data shows prevention rather than reversal of endotoxin-induced nitric oxide synthesis and therapeutic benefit of glucocorticoids in endotoxic shock remains unproven [Lillehei et al., 1964; Altura & Altura, 1974; Hellman & Lundberg, 1985; Chernow & Roth, 1986].

The effects of glucocorticoids on prostaglandin synthesis described above apply similarly to endothelial cells. Glucocorticoids also induce angiotensin converting enzyme in endothelial cells [Mendelsohn et al., 1982]. The importance of the tissue renin-angiotensin system in controlling tone is unknown [Samani, 1991] but corticosteroids may provide a tool to examine it further.

The Significance of Corticosteroids in the Control of Vascular Tone; a Potential Role for 11ß-OHSD

The evidence that corticosteroid receptor activation affects vascular tone is thus persuasive and is now supported by studies showing steroid-receptor mediated effects on vascular biochemistry. However, a confusing variety of biochemical actions of corticosteroids has emerged and it remains difficult to develop a unifying model to explain their role in the control of vascular tone. While the predominant effect when tone has been measured is constriction, glucocorticoids have biochemical actions with the potential for both vasoconstriction (eg nitric oxide synthase inhibition, reduced prostacyclin production, increased phosphoinositide pathway products, increased α-adrenoceptor numbers, decreased cGMP generation) and dilatation (eg increased cAMP responses, increased ß-adrenoceptor numbers). Corticosteroids may also act by non-genomic mechanisms which could potentially oppose their genomic effects (eg vasodilatation at high dose).

The most significant differences between corticosteroids and other vasoactive hormones are their lack of direct effect, their long time course, and their diverse effector mechanisms. These features suggest that corticosteroids can play a role not served by short-acting rapidly metabolised mediators with local actions, in providing a medium-term pattern of vascular responsiveness which is then adapted locally and in the short-term by other hormones. Another recently described vasoactive agent with a relatively long time course is endothelin [Yanagisawa et al., 1988]. Interactions between corticosteroids and endothelin have not been described in the

vessel wall, although in DOC/salt hypertension the clearance rate of endothelin is reduced resulting in increased pressor sensitivity to the peptide [Yokokawa et al., 1990].

It is a characteristic of vascular physiology that responses may be site-specific. It seems most likely that a balance of opposing effects exists with the result depending on the local milieu. Examples include the well-established differential distribution of adrenoceptors in different vascular beds, and the more recently appreciated differences in basal nitric oxide production between arteries and veins [Vallance et al., 1989a; 1989b]. The studies of 11ß-OHSD in the kidney indicate that the activity of the enzyme dictates local sensitivity to cortisol in specific sites. Having found 11ß-OHSD activity in vascular tissue we may have stumbled on one of the mechanisms whereby site-specific modulation of tone occurs in the vascular tree, by differential sensitivity to glucocorticoids. Similar arguments apply to the potential role of 11ß-OHSD in dictating relative contributions of vascular smooth muscle and endothelial cell products in the same vessel. The anatomical and cellular distribution of the enzyme is therefore of primary interest in elucidating its role in the control of vascular tone.

OUTLINE OF THE THESIS

In this thesis I bring together many of the strands alluded to above in order to address a central question: might defective enzyme-mediated receptor protection contribute to pathophysiology in common forms of hypertension? In Chapter 2 I describe the distribution, biochemistry and regulation of 11ß-OHSD in rat vascular smooth muscle and heart. In Chapter 3 I examine the physiological importance of the enzyme in modulating vascular sensitivity to glucocorticoids in rat and in man. In Chapter 4 I present physiological studies in man which address the putative inhibition of the enzyme by ACTH. Finally, in Chapter 5 I provide evidence that deficiency of 11ß-DH occurs in patients with ectopic ACTH syndrome and in a subgroup of patients with essential hypertension, and address the mechanisms whereby failure of enzyme-mediated receptor protection may explain their clinical presentation. These studies provide a new perspective on the mechanisms whereby 11ß-OHSD activity influences blood pressure and demonstrate that its deficiency is indeed a common observation in hypertension.

CHAPTER 2 11ß-HYDROXYSTEROID DEHYDROGENASE IN VASCULAR SMOOTH MUSCLE

I: BIOCHEMISTRY, DISTRIBUTION, AND REGULATION IN THE RAT

DISTRIBUTION OF VASCULAR 11ß-OHSD

In the introductory Chapter I described in detail the potential role of activation of both mineralocorticoid and glucocorticoid receptors in the regulation of vascular tone. 11ß-DH activity was first identified in homogenates of rabbit aorta by Ludwig Kornel [Kornel et al., 1982]. More recently Funder and colleagues [Funder et al., 1989] have shown conversion of corticosterone to 11-dehydrocorticosterone by minced tissue from rat mesenteric artery and aorta, with apparently less 11ß-DH activity in aorta than in mesenteric artery, but the amount of tissue used for incubation was not indicated and the conversion of corticosterone to 11-dehydrocorticosterone not quantified. In order to assess the potential role of 11ß-OHSD in modulating vascular sensitivity to glucocorticoids I first undertook studies to establish its anatomical and cellular distribution in the vascular tree, and to compare the level of enzyme activity in vasculature with that in kidney, where we know that the enzyme plays a central physiological role.

Materials and Methods

11ß-OHSD Activity *In Vitro*

Female Wistar rats weighing 200-250 g were killed by cervical dislocation. Fresh tissues were pooled in ice-cold Krebs Ringer bicarbonate buffer (KRBG containing 118 mM NaCl, 3.8 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM $CaCl_2.2H_2O$, 1.19 mM $MgSO_4.7H_2O$, 25 mM Na HCO_3, 11.1 mM d-glucose; gassed with 95 %O_2/5 %CO_2 to pH 7.4). Vessels were carefully stripped of surrounding fat and connective tissue. Renal cortex was dissected from medulla and capsule. Tissues were homogenised in 2 ml KRBG by 3 x 10 sec bursts in an Ystral homogenizer (Scientific Instrument Centre, Liverpool, UK). Homogenates were centrifuged at 500 x *g* for 5 min to remove debris and the supernatant then assayed for protein colorimetrically (BioRad Laboratories Ltd, UK). Incubations were performed in triplicate at 37 °C in 250 µl containing 1.12 x 10^{-8} M [1,2,6,7-$^3H_4$]-corticosterone (Amersham International, UK; specific activity 84 Ci/mol), ethanol 1 % vol/vol, BSA 0.2 g/dl, and tissue

homogenate at 500 μg protein/ml, and in the presence or absence of exogenous NADP 200 μM. This protein concentration was chosen after preliminary experiments with incubations in duplicate at protein concentrations ranging from 0-2000 μg/ml. In all experiments 3 blank incubations were performed with no homogenate added. After 120 min the incubate was made up to 1 ml with ice-cold KRBG, centrifuged at 1500 x *g* for 15 min, and stored at -70 °C until extraction.

Steroids were extracted with ethyl acetate, dried and re-suspended in ethanol containing 5 mg/ml of unlabelled corticosterone and 11-dehydrocorticosterone (Sigma Chemical Co., UK). Separation was by thin layer chromatography on silica gel plates using chloroform: 95% ethanol (92:8) as solvent. Areas corresponding to steroids were identified under ultraviolet light and scraped into vials before elution with liquid scintillant (Cocktail T, BDH Ltd, UK). Radioactivity was quantitated by counting in a ß counter to an error of < 2 %. 11ß-DH activity is expressed as (cpm for ^{3}H-11-dehydrocorticosterone)÷(cpm for ^{3}H-11-dehydrocorticosterone + cpm for ^{3}H-corticosterone) x 100 %, after correction for blank incubations. Apparent "conversion" in blank incubations was 1.57 ± 0.12 %.

In order to assess the possible conversion of corticosterone to steroids other than 11-dehydrocorticosterone an incubation was performed as above and the steroids were separated by HPLC. After ethyl acetate extraction, steroids were re-suspended in mobile phase of methanol:water (65:35) and separated on a C_{18} Microbondapack reverse phase HPLC column (Millipore Waters, Watford, UK) at a flow rate of 1 ml/min without a gradient. The retention time for corticosterone and for 11-dehydrocorticosterone was established with a Waters 441 UV detector. Retention times were 5.45 - 7.00 min for 11-dehydrocorticosterone and 8.00 - 9.15 min for corticosterone. Fractions were collected at 2.5 min intervals over 25 min and at these retention times. Eluates were dried under nitrogen, suspended in liquid scintillant and radioactivity quantitated in a ß counter. After HPLC recovery was > 90 % when tested with radioactive steroid standards.

To establish whether significant 11ß-OR conversion of 11-dehydrocorticosterone back to corticosterone occurred under these conditions, a further pair of experiments was performed in triplicate as above using ^{3}H-11-dehydrocorticosterone (purified to 94% from supernatant of liver homogenate after incubation with ^{3}H-corticosterone) as substrate and NADPH (200 μM) as cofactor.

Immunohistochemistry

Antisera 56-125 and 56-126 were a kind gift from Dr Carl Monder of the Population Council, New York. They were raised in New Zealand white rabbits inoculated with purified rat liver microsomal 11ß-OHSD [Monder & Lakshmi, 1990]. The immunohistochemistry was performed with technical assistance from Lawrence Brett in the Pathology Department, Western General Hospital, Edinburgh.

Female Wistar rats (n=2) were anaesthetised with pentobarbitone (2 ml/kg ip) and lethally perfused via the left ventricle with 250 ml ice-cold 0.9 % saline followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Tissues were dissected and fixed for 24-48 h in Bouin's fluid before embedding in paraffin. Sections of 4 µm were deparaffinised and rehydrated through graded alcohols. They were exposed to 3 % H_2O_2 for 10 min, washed in tris-buffered saline (TBS), exposed to BSA/TBS, and incubated with 56-125 at 1:50 dilution, 56-126 at 1:100, or normal rabbit serum for 30 min at 20 °C. In addition, a series of dilutions of 56-126 from 1:100 to 1:4000 were used to compare relative absorption in heart and kidney. Immunodetection was with swine anti-rabbit antibody followed by peroxidase anti-peroxidase antibody (Dako, UK). Slides were washed with TBS, exposed to 3,3'-diaminobenzidine/H_2O_2, and washed with water before being counterstained with haematoxylin.

In situ hybridisation

These studies were performed in Dr Jonathan Seckl's laboratory in our department, with assistance from Dr Joyce Yau. Female Wistar rats (250-300 g, n=2) were killed by cervical dislocation and the tissues dissected rapidly on ice, frozen on dry ice, and stored at -85 °C. Sections of 10 µm were cut on a cryostat and mounted on gelatin and poly-L-lysine-coated slides. Sections were fixed in 4 % paraformaldehyde/0.1 M phosphate (pH 7.4), washed twice in 2 x SSC and incubated at 50 °C for 2 h with prehybridisation buffer containing 50 % formamide, 0.6 M NaCl, 10 mM tris-HCl (pH 8.5), 0.02 % ficoll, 0.02 % polyvinylpyrrolidone, 0.1 % BSA, 1 mM EDTA, 0.5 mg/ml denatured salmon sperm DNA, 0.5 mg/ml total yeast RNA, and 0.05 mg/ml yeast tRNA. T3 polymerase (Gibco BRL, UK) was used to transcribe a 598 base pair ^{35}S-labelled antisense cRNA probe from *styI*-linearised pBluescript vector containing the 11ß-OHSD cDNA insert [Agarwal et al., 1989]. As a control T7 polymerase (Promega Ltd, UK) was used to transcribe a sense RNA probe of similar

specific activity. Probes were denatured and added to hybridisation buffer constituted as for prehybridisation buffer except 0.1 mg/ml denatured salmon sperm DNA, 0.05 mg/ml total yeast RNA , dextran sulphate 0.1 g/ml, and 10 mM DTT. After hybridisation overnight at 50 °C using 60 µl per slide at 10^7 cpm/ml, the slides were rinsed in 2 x SSC and treated with RNase A (30 µg/ml) for 30 min at 37 °C. They were washed to a maximum stringency of 0.1 x SSC at 60 °C for 60 min and dehydrated in graded ethanols in 0.3 M Na acetate. Slides were dried in air before being dipped in photographic K5 nuclear emulsion (Ilford, UK), stored for 21 days at 4 °C, developed and counterstained with haematoxylin and eosin.

Statistics

Differences between tissues were tested by ANOVA followed by unpaired *t* tests. Values are quoted as mean ± SE.

Results

11ß-OHSD Activity

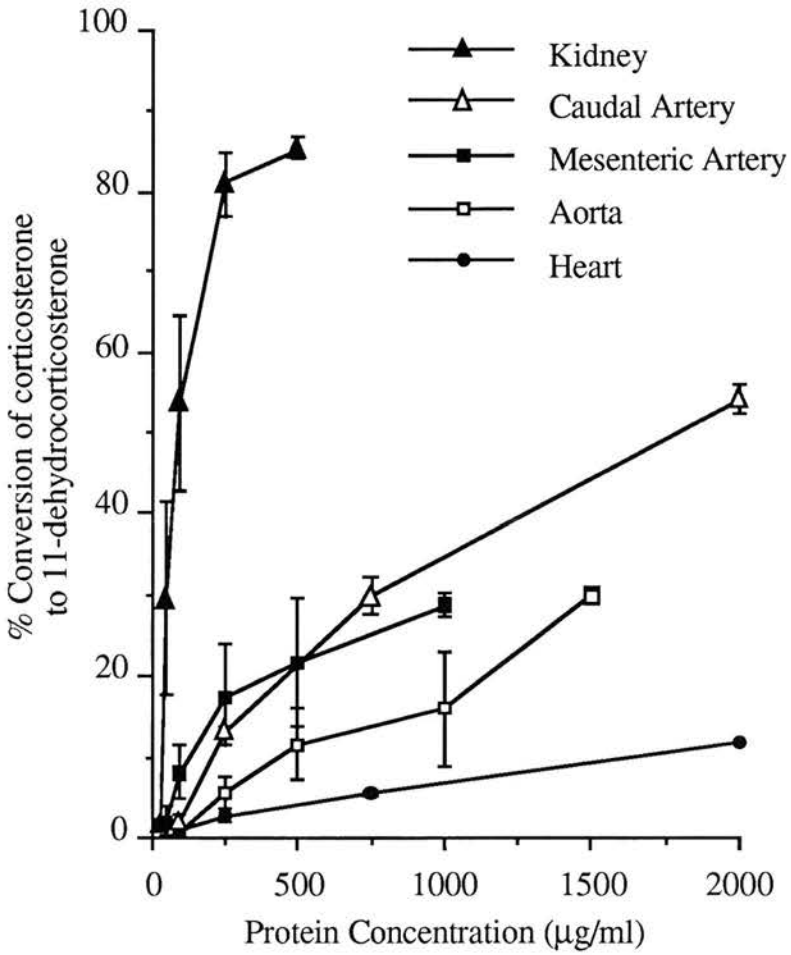
In order to allow comparison of enzyme activity in different tissues, a protein concentration was determined at which the amount of protein was rate-limiting. Results are shown in Figure 2.1 for 2 incubations at different protein concentrations in the presence of exogenous NADP. With vascular tissues detectable 11ß-DH activity continues to increase in a linear fashion with increasing protein well above 500 µg/ml, but with kidney there is a plateau in the curve reflecting consumption of substrate. A protein concentration of 500 µg/ml was chosen as a compromise to achieve measurable activity in vascular tissue and also to allow comparison with kidney.

An experiment was performed to assess possible conversion of corticosterone to other steroids, either extractable with ethyl acetate or non-extractable polar metabolites. The total activity recovered after extraction with ethyl acetate compared with blank incubations was 102.5 ± 6.4 % in the absence of exogenous NADP and 97.4 ± 7.8 % in the presence of NADP. Thus non-extractable steroids do not constitute a major metabolic fate of corticosterone in vascular tissues. Conversion of corticosterone to extractable steroids other than 11-dehydrocorticosterone was examined by HPLC, after which the radioactivity residing in the fractions containing corticosterone and 11-dehydrocorticosterone, when expressed as a % of total radioactivity recovered, was 99.0 ± 0.7 % in the absence of NADP and 98.6 ± 0.6 % in the presence of NADP.

Thus 11-dehydrocorticosterone is the major metabolite of corticosterone in these tissues.

Experiments to measure the conversion of 11-dehydrocorticosterone to corticosterone showed that under these incubation conditions there was no significant 11ß-OR activity in any of the tissues studied.

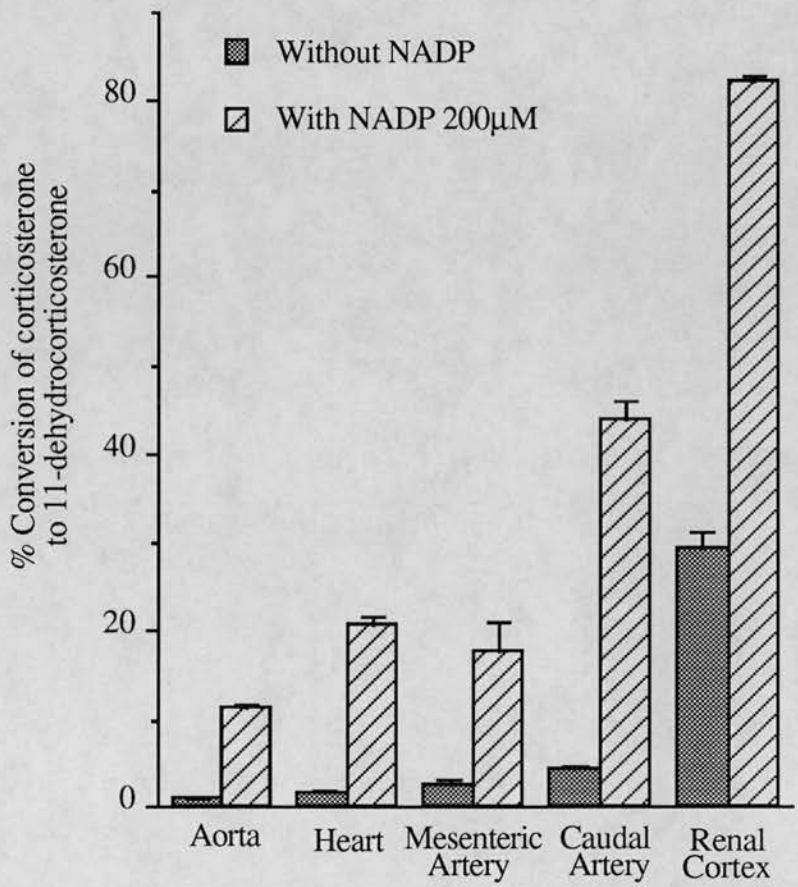
Figure 2.1 Effect of increasing protein concentration on 11ß-DH activity in vascular tissues and renal cortex in the presence of 200 µM NADP



The conversion of corticosterone to 11-dehydrocorticosterone was quantitated in homogenates from aorta, superior mesenteric artery, caudal artery and heart, and compared with conversion in renal cortex. The results from 3 experiments, each with the pooled tissues of 4 animals incubated in triplicate, are shown in Figure 2.2. 11ß-

DH activity was demonstrated in all tissues studied. Without NADP vascular activity was just detectable and there were significant differences ($p < 0.05$) between vascular sites [caudal artery (4.2 ± 0.2 %) > mesenteric artery (2.5 ± 0.7 %) = heart (1.67 ± 0.2 %) > aorta (0.79 ± 0.2 %)]. All of these were lower than renal cortex (29.4 ± 1.8 %; $p < 0.001$). NADP increased conversion in all tissues ($p < 0.001$), and the hierarchy was maintained ($p < 0.001$) except that mesenteric artery and aorta were not significantly different in this comparison [renal cortex (82.4 ± 0.4 %) > caudal artery (43.9 ± 2.1 %) > heart (20.6 ± 1.0 %) = mesenteric artery (17.7 ± 3.1 %) = aorta (11.4 ± 0.4 %); heart > aorta].

Figure 2.2 Relative 11ß-DH activity in vascular tissues and renal cortex



Immunohistochemistry

In aorta, mesenteric artery, and caudal artery there was specific cytoplasmic 11ß-OHSD immunoreactivity in smooth muscle cells throughout the vessel wall (Figure

2.3 A-F). There was also staining of the adventitia of the vessels and of fat cells in the mesentery, but there was no staining of endothelium. In the heart (Figure 3 G,H) there was staining of cardiac muscle cells and of smooth muscle in coronary arteries. Staining was heavier near the endocardium than on the pericardial surface. The signal intensity in heart was of similar magnitude to that in kidney, since both disappeared at antibody dilutions between 1:400 and 1:1000. Both antisera gave similar results.

In situ hybridisation

Specific 11ß-OHSD mRNA expression was localised to vascular smooth muscle in aorta and mesenteric artery (Figure 2.4 A-D) and was also found in cardiac muscle (Figure 2.4 E,F). Sense probes showed little or no hybridisation under the stringent conditions used.

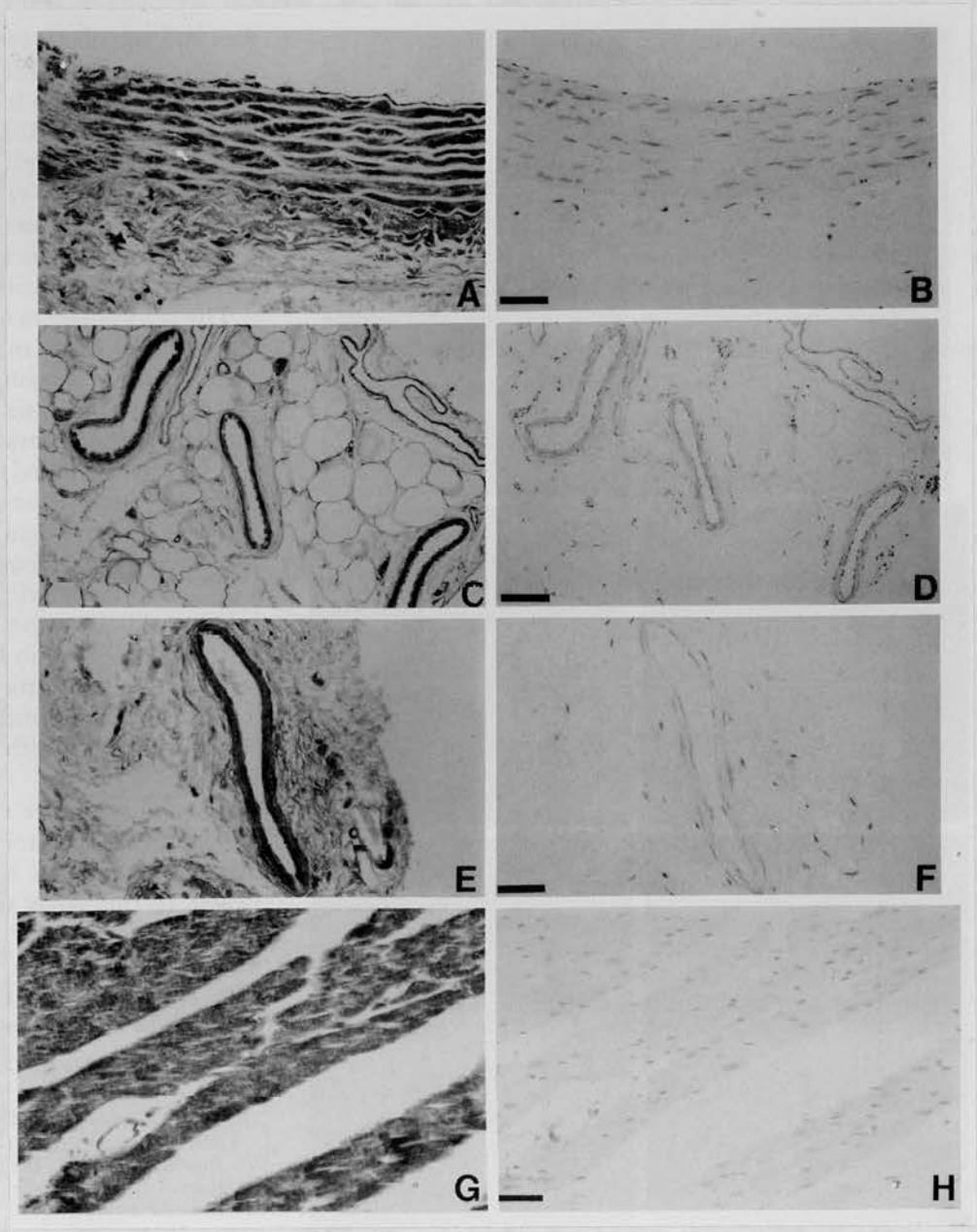
Conclusions

These studies demonstrate 11ß-DH bioactivity, and 11ß-OHSD immunoreactivity and mRNA expression in cardiac and vascular smooth muscle cells. 11ß-DH bioactivity has been identified previously in these tissues [Kornel et al., 1982; Funder et al., 1989], but no quantitative studies have been performed and the cellular localisation of the enzyme was not known. I have shown that 11ß-DH makes the greatest contribution to *in vitro* metabolism of corticosterone in these tissues, and that there are quantitative differences in enzyme activity between different sites.

Greater 11ß-DH activity was found in caudal artery and mesenteric artery than in aorta without NADP, and greater activity in caudal artery than other vessels with NADP. This suggests that 11ß-OHSD is most active in smaller vessels. It is thus appropriately sited to modulate tone in resistance beds and thereby influence blood pressure.

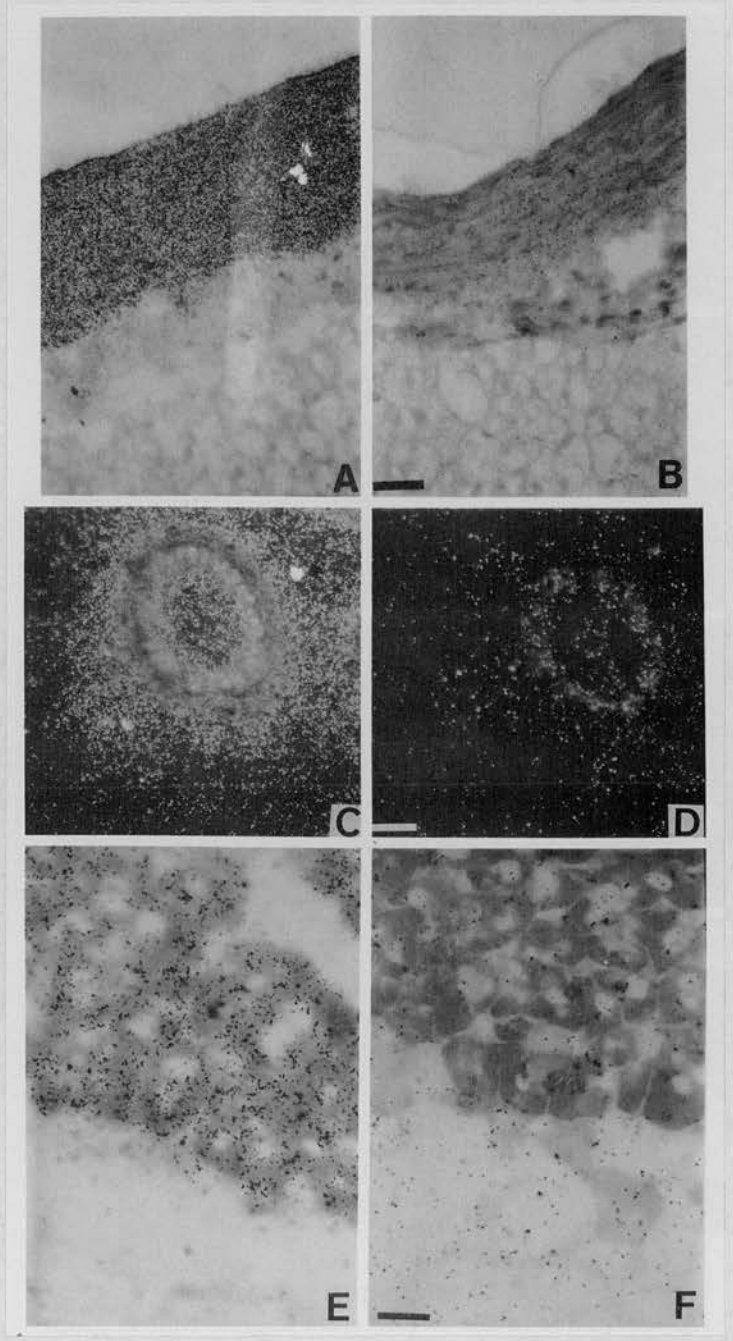
Both 11ß-OHSD immunoreactivity and mRNA were localised to smooth muscle cells and not endothelium in heart and blood vessels. Previously, 11ß-OHSD antigen and mRNA have only been reported in cardiac extracts [Monder & Lakshmi, 1990; Krozowski et al., 1990]. These workers failed to detect 11ß-OHSD mRNA in extracts from mesenteric vascular arcade [Krozowski et al., 1990], but I have demonstrated hybridisation at this site. It is likely that *in situ* hybridisation is the more sensitive technique. This localisation within contractile cells suggests that, if 11ß-OHSD modulates the effects of glucocorticoids on tone, then it does so by an autocrine rather than a paracrine mechanism.

Figure 2.3 Immunohistochemical localisation of 11ß-OHSD in vascular tissues



Sections were counterstained after incubation with antiserum 56-126. Controls were incubated with normal rabbit serum (NRS). For x40 bar is 100μm and for x20 bar is 200μm. **A** aorta (x40) with 56-126 **B** aorta (x40) with NRS **C** mesenteric artery (x20) with 56-126 **D** mesenteric artery (x20) with NRS **E** caudal artery (x40) with 56-126 **F** caudal artery (x40) with NRS **G** heart including coronary artery (x40) with 56-126 **H** heart (x40) with NRS.

Figure 2.4 *In situ* hybridisation for 11ß-OHSD mRNA in vascular tissues



Hybridisation was performed with an antisense cDNA probe. Controls were hybridised with a sense probe. Bar is 100µm for x40 and 40µm for x100. **A** aorta, antisense (x40) **B** aorta, sense (x40) **C** mesenteric artery, antisense (x40); dark field with some refraction artefact around the luminal surface **D** mesenteric artery, sense (x40); dark field with refraction artefact around lumen **E** heart, antisense (x100) **F** heart, sense (x100).

Despite the abundance of 11ß-OHSD immunoreactivity and mRNA expression in vascular tissues, the conversion of corticosterone to 11-dehydrocorticosterone in these sites was low relative to that in renal cortex, even when 11ß-DH was "driven" by the addition of NADP. This might reflect lower abundance of 11ß-OHSD, reduced *in vitro* stability of the enzyme in vasculature, or the possibility that vascular-specific isoforms of 11ß-OHSD exist with different kinetic properties from that in kidney. I was not able to demonstrate 11ß-OR activity under these conditions, but it would be wrong to conclude that 11ß-DH predominates *in vivo*, since the two activities of 11ß-OHSD vary in their stability [Abramowitz et al., 1982; Lakshmi & Monder, 1985; 1988; Monder & Lakshmi, 1989]. It may be that the low 11ß-DH activity in vascular tissue, in the face of apparently high immunoreactivity and mRNA expression, reflects abundant 11ß-OR which is too labile for bioactivity to be demonstrable *in vitro*. As discussed in Chapter 1, predominant 11ß-OR activity *in vivo* does not preclude the possibility that 11ß-OHSD modulates access of steroids to receptors in vasculature. For example, 11ß-OHSD could act to maintain high local corticosterone concentrations thus ensuring adequate exposure of glucocorticoid receptors and maintaining vascular sensitivity to catecholamines.

For these reasons it was important to characterise the biochemistry of the enzyme in vascular tissue in greater detail, to establish whether it resembled most the isoform expressed in liver or that in kidney.

IN VITRO KINETICS OF THE 11ß-OHSD ISOFORM EXPRESSED IN VASCULAR SMOOTH MUSCLE

The isoforms of 11ß-OHSD in kidney can be distinguished from that in liver by differences in antigenicity, protein size, and size of mRNA expressed, as well as differences in the equilibrium between active and inactive steroid (see Table 1.2). It has also been suggested that the K_m of 11ß-DH for cortisol is lower in kidney than it is in liver [Naray-Fejes-Toth & Fejes-Toth, 1991], although this observation may depend on differences between the incubation conditions used by different investigators. Finally, experiments examining the conversion of tetrazolium to diformazan blue in renal tubules suggested that an NAD-dependent "11ß-$OHSD_2$" may be expressed in the kidney [Mercer & Krozowski, 1992]. The same group have cloned one of the additional mRNA species in kidney which codes for a protein without the first 26 amino acids of hepatic 11ß-OHSD [Krozowski et al., 1992]. This is a hydrophobic putative membrane insertion sequence immediately adjacent to which is the cofactor binding domain of the enzyme. A change in the N-terminus might therefore account for altered cofactor binding affinity of the protein. I examined the kinetic properties of 11ß-DH in vascular smooth muscle and compared it with kidney and other tissues.

Methods

Substrate Affinity

The apparent K_m and V_{max} for an enzyme can be calculated from a reciprocal plot of substrate concentration vs velocity of product accumulation [Lineweaver & Burk, 1934]. It is important for these calculations to ensure that the velocity of reaction is as close to initial, or saturated, velocity as possible. Tissues were collected from female Wistar rats. Incubations were performed as described above in the presence of NADP (200 µM), except that different protein and substrate concentrations and shorter time periods were used. Protein concentrations were chosen from experiments similar to that shown in Figure 2.1, such that the protein concentration was on the linear part of the curve of protein concentration vs % conversion. These were: 1000 µg/ml for mesenteric artery and aorta; 500 µg/ml for heart and caudal artery; and 100 µg/ml for renal cortex. Time of incubation was chosen from preliminary experiments in which the reaction was terminated with ethyl acetate at 1, 2.5, 5, 7.5, 10, and 30 min. All reactions remained linear up to at least 10 min, so

this interval was chosen for the velocity measurements. ^{3}H-Corticosterone substrate concentrations were 2 x 10^{-7}, 1 x 10^{-7}, 3.3 x 10^{-8}, 2 x 10^{-8}, 1 x 10^{-8}, 8 x 10^{-9}, 5 x 10^{-9}, and 2 x 10^{-9} M. Experiments were performed 3 or 6 times at each substrate concentration and the mean used for Lineweaver-Burk analysis by simple linear regression.

Cofactor Affinity

Tissues were obtained from non-pregnant female Wistar rats (250 - 300 g; n = 3 - 5), placentae at 19 days gestation, or testes from male rats of the same weight. To separate proximal and distal renal tubules [Edwards et al., 1988], diced renal cortex was digested at 37 °C for 3 x 30 min in KRBG with 2 % BSA, 600 units/ml collagenase (type IV, Sigma) and 0.1 % w/v hyaluronidase (Worthington). Tubules were separated by unit gravity sedimentation on a Ficoll gradient (1 - 12 % Ficoll, molecular weight 4 x 10^{5}, in KRBG). Proximal and distal tubules were counted in a series of fractions by their morphology in a haemocytometer, and the viability of the tubules confirmed by Trypan blue exclusion. Fractions were combined to give suspensions enriched with proximal and distal tubules for homogenisation.

Incubations were in duplicate as in previous experiments in the presence of NAD or NADP at 0, 5, 25, and 125 μM. Protein concentrations were: 100 μg/ml for liver, testis, lung, renal cortex and tubules; 500 μg/ml for placenta and hippocampus; 650 μg/ml for mesenteric artery; and 1000 μg/ml for aorta, heart, and colon.

All experiments were repeated three times. Affinity constants for each cofactor in each site were not calculated on the grounds that the absolute values are not meaningful without purification of the enzyme from each site. Instead, the effect of each cofactor in each tissue was assessed by single-factor ANOVA, and the effects of NAD and NADP compared by two-way ANOVA. Results are mean ± SE.

Results

Substrate Affinity

Results are shown in Table 2.1. The differences in apparent affinity for corticosterone are not dramatic, being less than one order of magnitude. Therefore, no distinction can be made between the enzyme in vascular tissue and in renal cortex on the basis of substrate affinity.

Table 2.1 Apparent K_m and V_{max} for 11ß-DH in homogenates of vascular tissues and renal cortex

	K_m	V_{max}
Renal Cortex	3.7×10^{-8}	1.9×10^{-9}
Mesenteric Artery	1.3×10^{-8}	7.2×10^{-9}
Aorta	1.6×10^{-8}	4.3×10^{-9}
Caudal Artery	4.7×10^{-9}	2.6×10^{-8}
Heart	8.0×10^{-9}	6.0×10^{-9}

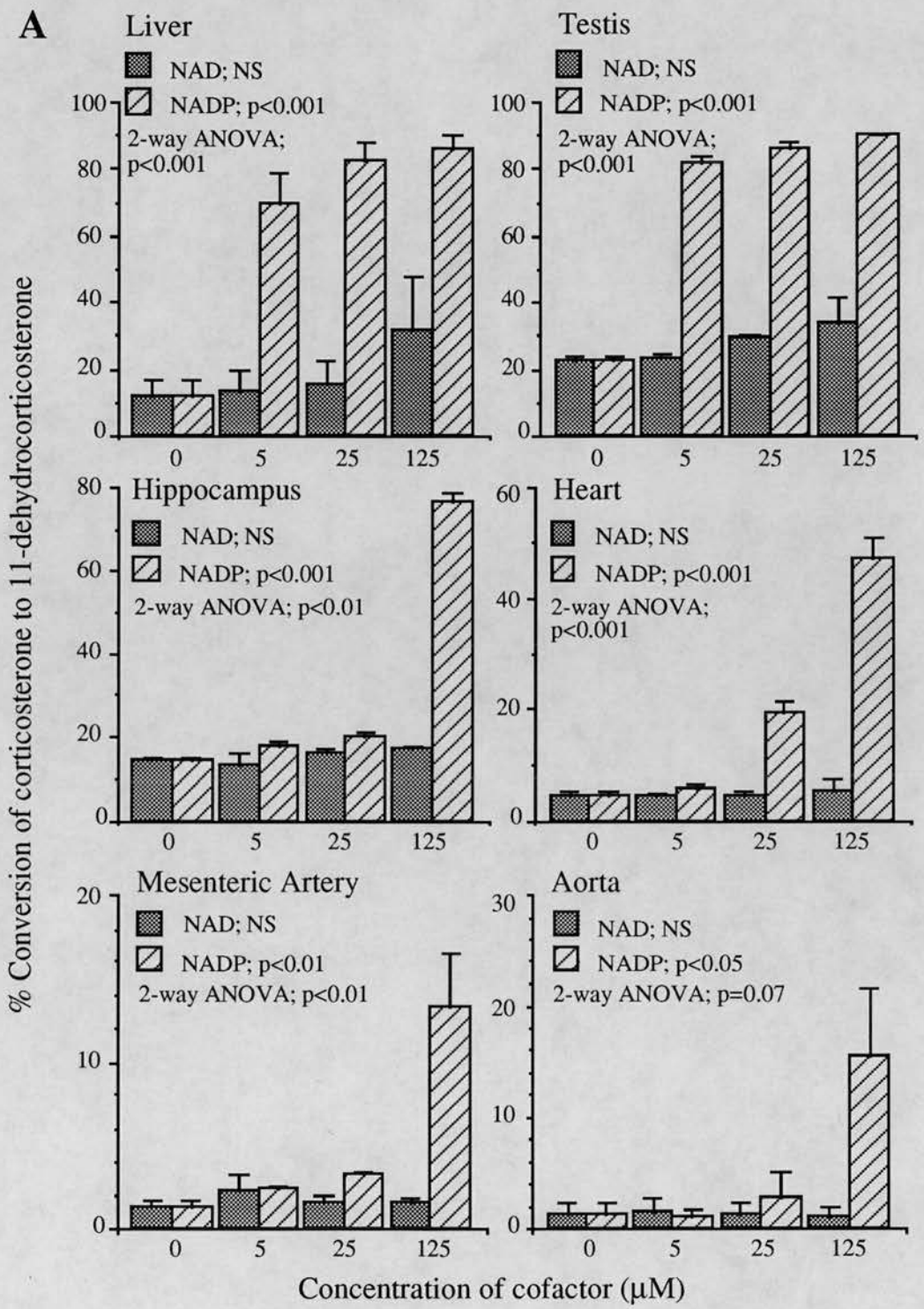
Cofactor Affinity

In liver, testis, hippocampus, heart, mesenteric artery and aorta NAD had no significant effect and NADP produced a concentration-dependent increase in activity (single factor ANOVA $p < 0.01$ except aorta $p < 0.05$)(Figure 2.5A).

In lung, placenta and colon NAD had a significant effect (single factor ANOVA $p < 0.01$) but not as great as NADP (two-way ANOVA shown in Figure 2.5B).

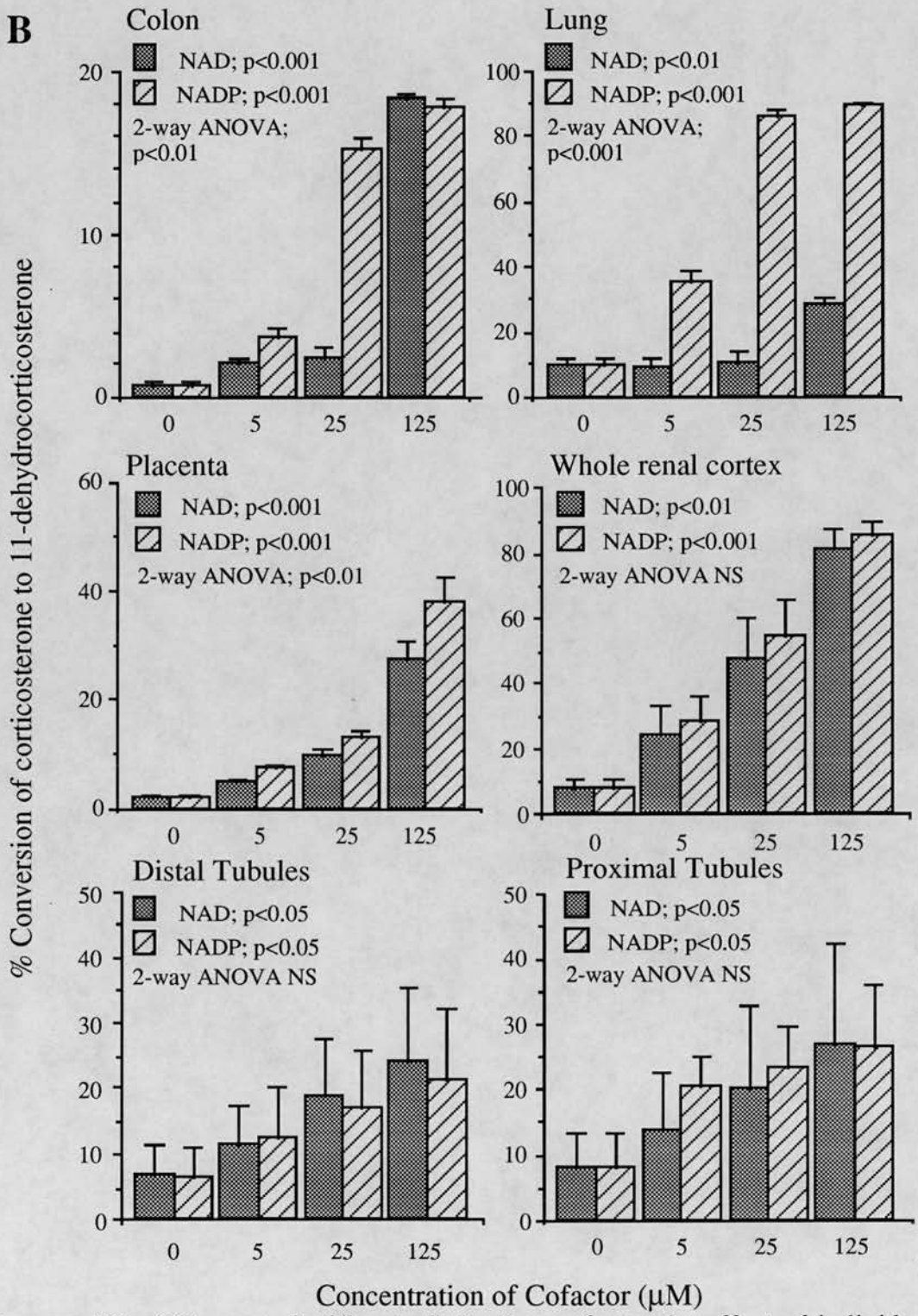
Only in renal cortex did the effect of NAD match that of NADP (Figure 2.5B; both single-factor ANOVA $p < 0.001$; two-way ANOVA not significant). Tubular fractionation achieved enrichment of 68 % for proximal tubules and 100 % for distal tubules. No difference was apparent between these fractions.

Figure 2.5 Effect of NADP and NAD on 11ß-DH activity in homogenised rat tissues: **A** Tissues in which NAD had no effect.



Bars are SE. NS = not significant. "p" values refer to the effect of individual cofactors in each tissue (analysed by single-factor ANOVA) and to the comparison between the two cofactors by two-way ANOVA.

Figure 2.5 Effect of NADP or NAD on 11ß-DH activity in homogenised rat tissues: **B** Tissues in which NAD increased activity.



Bars are SE. NS = not significant. "p" values refer to the effect of individual cofactors in each tissue (analysed by single-factor ANOVA) and to the comparison between the two cofactors by two-way ANOVA.

Conclusions

Substrate affinities were not different between vascular tissues and kidney, suggesting either that all 11ß-OHSD isoforms in these tissues have the same substrate binding site, or that any additional isoforms expressed in kidney with different substrate affinities are masked by a more abundant isoform. Therefore measurement of substrate affinity without purifying the relevant isoforms does not appear to be a useful tool in delineating different enzyme species.

However, several of the tissues can be distinguished by their affinity for cofactors. These studies illustrate a novel principle which highlights the potential significance of the expression of tissue-specific 11ß-OHSD isoforms: that heterogeneity of 11ß-OHSD is reflected in tissue-specific differences in cofactor utilisation by the enzyme. Most sites which are distinguished by the utilisation of NAD (kidney, colon, and placenta) correspond to those in which isoforms have been identified in studies of protein and mRNA size which differ from those found in liver (Table 1.2). The exceptions are lung (which uses NAD but is in other respects similar to the liver isoform; however, the effect of NAD in lung is relatively minor) and testis (which has additional proteins identified on Western blotting but does not use NAD). Thus on Western blots the polyclonal antisera to hepatic 11ß-OHSD bind proteins with molecular weights of 34 kD and 68 kD in most sites (including liver, lung, and colon), and additional species with molecular weights of 40 kD in kidney, 26 kD in brain, and 47 kD in testis [Monder & Lakshmi, 1990]. Western blots for 11ß-OHSD in vascular tissues and placenta are not available. On Northern blots 11ß-OHSD cDNA hybridises to mRNA of 1700 nucleotides in most tissues (including liver, lung, hippocampus, and testis), and to additional species of 1500, 1600 and 1900 nucleotides in kidney [Krozowski et al., 1990], 3400 nucleotides in colon [Whorwood et al., 1992], and larger species in placenta (Susan Low, personal communication). Northern blots are not yet available for vascular tissues.

Furthermore, it is plausible to suggest that these kinetic differences between isoforms are reflected in different functioning of the enzyme. The utilisation of NAD broadly corresponds to the abundance of aldosterone-specific mineralocorticoid receptors. Thus NAD is used in kidney and colon (where aldosterone-specific mineralocorticoid receptors abound) but not in hippocampus (where mineralocorticoid receptors are present but are not aldosterone-specific [Sheppard & Funder, 1987]), nor in liver or testis (where mineralocorticoid receptors are absent or in low abundance). This

relationship would be even more convincing if we could demonstrate differences between cofactor utilisation in proximal and distal renal tubules. The failure to do this may reflect inadequate purity of separation, which is intrinsic to the density gradient technique, and this negative result does not exclude differential distribution of the NAD-dependent isoform within the kidney. Indeed more recent observations of the kinetics of 11ß-DH purified from isolated rabbit cortical collecting duct cells (ie mineralocorticoid receptor-containing cells) confirm that this enzyme utilises NAD almost exclusively [Rusvai et al., 1992]. In lung [Krozowski & Funder, 1981] and heart [Barnett & Pritchett, 1988] mineralocorticoid receptors have been identified *in vitro* but their binding specificity *in vivo* has not been determined. Finally, corticosteroid receptors in the placenta are predominantly type 2, though in this organ the putative functional role of the enzyme is not as an autocrine protector of local receptors but rather as a barrier preventing maternal glucocorticoids gaining access to the fetus [Murphy, 1981; Baggia et al., 1990].

Finally, the pattern of utilisation of NAD corresponds to the equilibrium between active and inactive steroid in different sites. Thus the liver enzyme is not NAD-dependent and has predominant 11ß-OR activity, while the kidney enzyme is NAD-dependent and has predominant 11ß-DH activity. We might now suggest that this kinetic difference may depend on the response to the cofactor milieu, and accounts in turn for the diverse function of the enzyme in relation to receptors in different sites.

On this background, the kinetic profile of 11ß-OHSD in vascular smooth muscle raises some contradictions. In rat mesenteric artery, which is the only vascular site so far examined, mineralocorticoid receptors do display aldosterone-specificity *in vivo* [Funder et al., 1989]. Thus the hypothesis presented above would predict that the vascular enzyme would be NAD-dependent and function as a predominant 11ß-DH. That it does not respond to NAD raises the possibility that 11ß-OHSD in vascular smooth muscle is a functional hybrid between liver (predominantly 11ß-OR; NAD-independent; possible amplification of cortisol exposure of glucocorticoid receptors) and kidney (predominantly 11ß-DH; NAD-dependent; avid inactivation of cortisol to protect mineralocorticoid receptors). Perhaps its hybrid kinetics permit the vascular enzyme to perform different roles in different vascular beds? This putative diversity of function is addressed in more detail in the following Chapter.

IN VIVO REGULATION OF 11ß-OHSD IN VASCULAR SMOOTH MUSCLE

In addition to the potential role of intra-cellular cofactors in dictating the equilibrium point of 11ß-OHSD [Nicholas & Lugg, 1982; Monder et al., 1991] it seems likely that the expression of the enzyme is under physiological control. The enzyme contributes to two separate physiological axes: the "hypophyseal-pituitary-adrenal" axis which dictates activity of glucocorticoids; and the "renin-angiotensin-aldosterone" axis, which relies on 11ß-OHSD for its integrity. The putative regulators of the enzyme which I chose to address are therefore ACTH, glucocorticoids, and sodium intake. The possible influence of androgens, oestrogens and thyroid hormones were discussed in Chapter 1 and I have not examined these further.

Salt diet has been addressed by others in liver, lung and kidney. They reported that low sodium intake caused elevated mRNA expression in the liver only, and that bioactivity of the enzyme was not affected in any site [Krozowski et al., 1990], but they do not show their data. If this manipulation has an effect on the enzyme then it would prompt a series of experiments to examine the effects of angiotensin II, aldosterone and potassium. I therefore repeated this experiment in a variety of tissues.

ACTH seems an unlikely direct regulator of 11ß-OHSD, since we usually think that its effects are mediated by adrenal ACTH-dependent steroids. However, in two patients with Addison's disease Kornel found that ACTH administration prolonged the half life of ^{14}C-cortisol and shortened the half life of ^{14}C-cortisone [Kornel, 1970]. He suggested that this represented an extra-adrenal effect of ACTH to inhibit 11ß-DH. This mechanism might allow amplification of the tissue response to glucocorticoids under ACTH stimulation. However, it remains possible that his patients had some residual adrenal function and that this effect was due to secretion of an adrenal inhibitor of the enzyme. These possibilities are addressed more fully in consideration of the control of renal 11ß-OHSD in man in Chapter 4. In this Chapter I examine the effects of ACTH in adrenalectomised rats.

Glucocorticoids induce 11ß-OHSD activity in cultured cells [Smith et al., 1973; Lugg & Nicholas, 1978; Hammami & Siiteri, 1991]. Putative glucocorticoid response elements are present in the 5' sequences of the rat gene [Moisan et al., 1992a]. The effects of *in vivo* administration of synthetic glucocorticoid receptor agonists on 11ß-

OHSD bioactivity and mRNA expression have been reported previously by others from this department for kidney and hippocampus [Moisan et al., 1991; Low et al., 1992]. Their results show that dexamethasone induces 11ß-OHSD expression in hippocampus but not in kidney when given in high pharmacological doses (50 µg/day for 10 days). This tissue-specificity has provided one of the arguments in favour of multiple isoforms of the enzyme. In the following experiments I wished to test: (i) whether vascular tissues express an isoform which is regulated by glucocorticoids; (ii) whether the effect of dexamethasone could be demonstrated at doses which are equivalent to physiological glucocorticoid levels (ie do not cause profound cachexia); and (iii) whether the effect of dexamethasone is attributable to suppression of ACTH levels and loss of ACTH-dependent inhibition of the enzyme.

Methods

Effect of Sodium Intake on 11ß-DH Activity

Groups of 3 female Wistar rats (200-250 g) were given *ad libitum* drinking water containing 0 % Na Cl (low salt), 1 % Na Cl (normal salt), and 3 % Na Cl (high salt) for 7 days before sacrifice by cervical dislocation. Fluid consumption and body weight was similar in the three groups. Tissues from each animal were dissected, homogenised, and incubated as above in triplicate with ^{3}H-corticosterone in the presence of 200 µM NADP for 120 min. Protein concentrations were 100 µg/ml for renal cortex and liver, and 1000 µg/ml for heart and aorta.

Results were compared by single-factor ANOVA. All results are mean ± SE.

Effect of Adrenalectomy, Dexamethasone Replacement, and ACTH on 11ß-DH Activity

Male Wistar rats were adrenalectomised or sham operated and had subcutaneous miniosmotic pumps (Alzet, UK) inserted under pentobarbitone anaesthesia. Surgery was performed by June Noble, senior technician in our Animal Unit. Animals were given 0.9 % NaCl to drink until sacrifice by decapitation on day 9.

ACTH (1-24; Acthar gel, Rorer Pharmaceuticals; 20 µg/d) or oil vehicle were administered daily at 0900 h by subcutaneous bolus.

Dexamethasone (2 µg/d or 50 µg/d) or 0.9 % NaCl vehicle was given continuously by subcutaneous pump.

At sacrifice truncal blood was collected for radioimmunoassay of ACTH (performed by Dr John Bennie in the MRC Brain Metabolism Unit, Edinburgh [Fink et al., 1988]). Data from 1 sham and 1 adrenalectomised animal treated with dexamethasone 50 μg/d were excluded from analysis because of inappropriate changes in body weight and plasma ACTH suggesting surgical error and pump failure respectively.

Tissues from each animal were dissected, homogenised, and incubated as above in duplicate with ^{3}H-corticosterone in the presence of 200 μM NADP for 120 min. Protein concentrations were 50 μg/ml for liver, 100 μg/ml for renal cortex, 500 μg/ml for hippocampus, and 1000 μg/ml for aorta and heart.

Results were compared by single-factor ANOVA. All results are mean ± SE.

Results

Effect of Sodium Intake on 11ß-DH Activity

Results are shown in Figure 2.6. Salt intake had no effect on 11ß-DH activity in any tissue studied.

Effect of Adrenalectomy, Dexamethasone Replacement, and ACTH on 11ß-DH Activity

Adrenalectomy produced a non-significant fall in 11ß-DH activity in hippocampus and aorta and had no effect in renal cortex, liver, or heart (Figure 2.7).

After adrenalectomy low-dose dexamethasone replacement (2 μg/d) had no effect on 11ß-DH activity in any tissue. High-dose dexamethasone (50 μg/d) increased 11ß-DH activity in hippocampus, aorta and heart, but had no effect in renal cortex and liver (Figure 2.8).

Exogenous ACTH (1-24) had no effect on dexamethasone induction of 11ß-DH activity in any tissue (Figure 2.9) despite achieving ACTH levels in animals receiving dexamethasone similar to those in untreated adrenalectomised rats (Table 2.2).

Figure 2.6 Effect of 7 days of controlled sodium intake on 11ß-DH activity in renal cortex, liver, heart and aorta

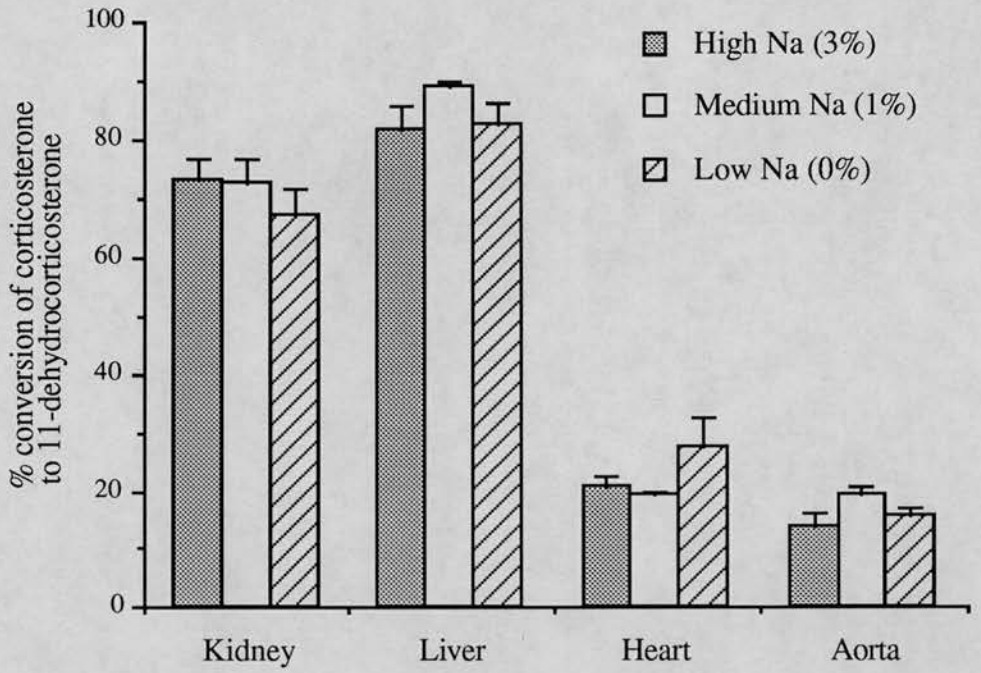


Table 2.2 Body weights, and plasma ACTH concentrations in animals treated by adrenalectomy/sham surgery and given dexamethasone ± ACTH

	[ACTH] pg/ml	Body weight day 1 (g)	Body weight day 9 (g)
Sham	84 ± 41	208.5 ± 11.0	251.2 ± 11.0
ADX + vehicle	3644 ± 376	210.2 ± 9.9	225.6 ± 8.0
ADX + DEX 2 µg/d		212.7 ± 5.7	225.3 ± 2.7
ADX + DEX 2 µg/d + ACTH 20 µg/d		211.3 ± 12.7	235.3 ± 17.0
ADX + DEX 50 µg/d	13 ± 13	202.4 ± 9.5	165.2 ± 8.0
ADX + DEX 50 µg/d + ACTH 20 µg/d	3478 ± 570	202.3 ± 5.9	163.0 ± 5.1

ADX = adrenalectomy; DEX = dexamethasone

Figure 2.7 Effect of adrenalectomy on 11ß-DH activity in liver, renal cortex, aorta, heart and hippocampus

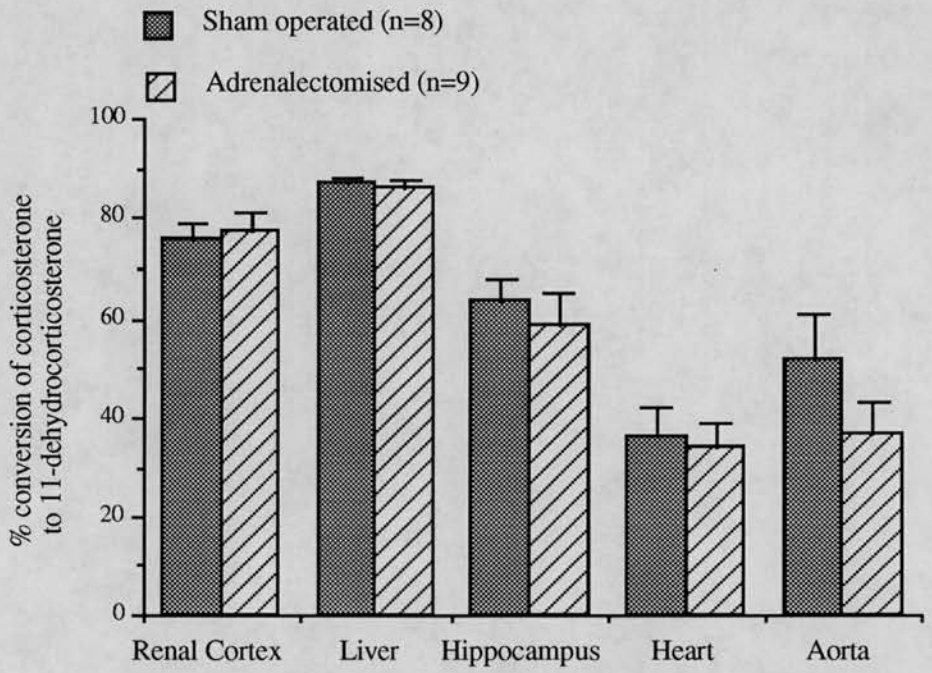


Figure 2.8 Effect of *in vivo* administration of dexamethasone on 11ß-DH activity in liver, renal cortex, aorta, heart and hippocampus of adrenalectomised rats

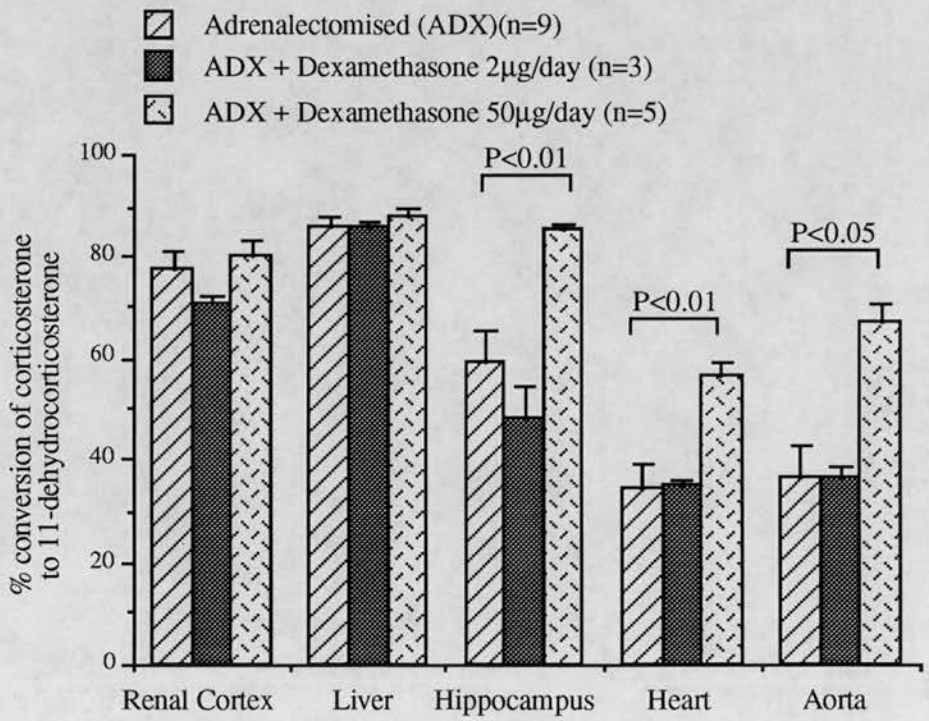
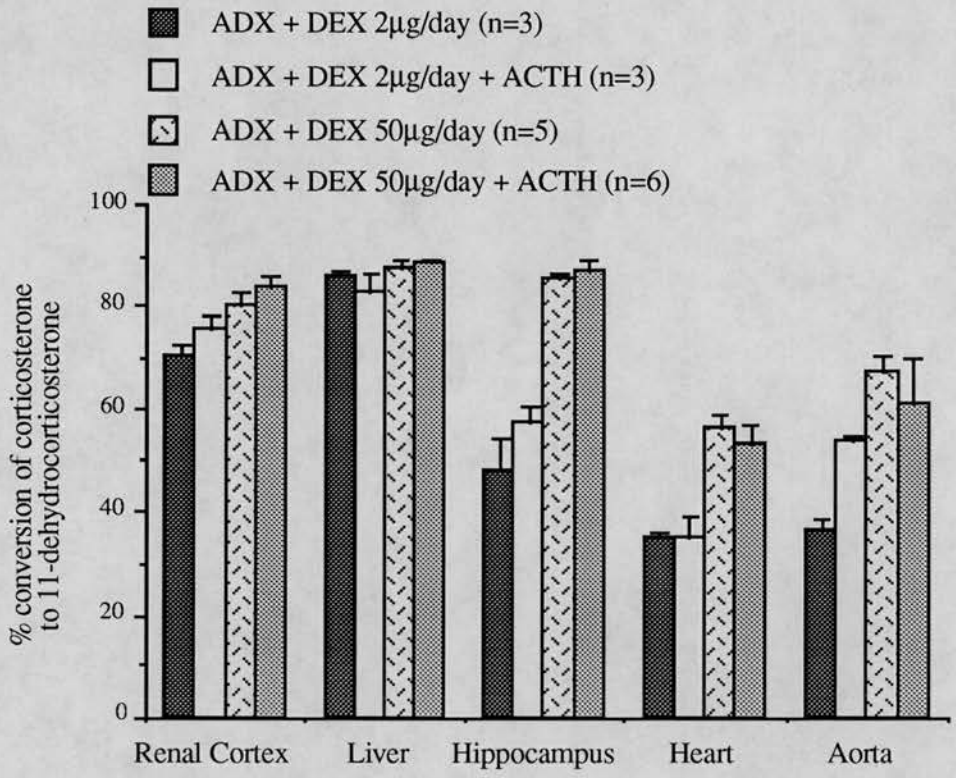


Figure 2.9 Effect of *in vivo* ACTH administration on dexamethasone induction of 11ß-DH activity in liver, renal cortex, aorta, heart and hippocampus



ADX = adrenalectomised; DEX = dexamethasone

Conclusions

If the renin-angiotensin-aldosterone axis regulates 11ß-OHSD activity then this did not emerge from the experiment with manipulation of salt diet described here. The negative results in liver and kidney should be interpreted with caution because the basal 11ß-DH activity was relatively high, leaving relatively little scope for enzyme induction to be apparent. However, given the consistency with other reports [Krozowski et al., 1990] and the negative results in other tissues, I elected not to pursue this further.

The present results confirm that dexamethasone induces 11ß-DH activity in hippocampus. By contrast in renal cortex, as shown by others [Moisan et al., 1991; Low et al., 1992], and also in liver there was no demonstrable change. However, it is interesting that there was no significant change in hippocampus after adrenalectomy or after low dose dexamethasone replacement. This dose of dexamethasone is a

reasonable approximation to physiological glucocorticoid levels in the rat, but is certainly not inadequate, since it does not make animals lose weight but does produce elevations in blood pressure [Tonolo et al., 1988]. It may be that within physiological ranges of glucocorticoid concentration the effects on 11ß-DH activity are not relevant.

The tissue specificity of glucocorticoid induction is difficult to interpret and again is subject to the limitation that basal 11ß-DH activity was relatively high in the tissues in which no induction was seen. However, there is some teleological logic in these results. In tissues where 11ß-DH is induced (hippocampus, aorta and heart) this could limit the response to a surge of glucocorticoid secretion. In tissues where induction does not occur (liver and renal cortex) 11ß-DH may either be constantly fully active as a barrier to glucocorticoids (eg kidney), or may remain inactive to facilitate maximal receptor exposure when adrenal secretion is increased (eg liver).

Finally, if ACTH inhibits 11ß-OHSD (discussed above) then it is possible that apparent induction of enzyme activity by dexamethasone is mediated indirectly by suppression of ACTH secretion. However, administration of ACTH with dexamethasone did not reverse the dexamethasone induction of 11ß-DH activity, thus ACTH does not act by an extra-adrenal mechanism to inhibit the enzyme and does not account for the effect of dexamethasone in the rat. This result is discussed in the interpretation of the effects of ACTH on 11ß-DH activity in man in Chapter 4.

SUMMARY

In this Chapter I have described the expression and distribution of 11ß-OHSD in vascular smooth muscle and cardiac muscle. I have confirmed that it is appropriately sited to modify the vascular response to glucocorticoids, and observed that several of its characteristics *in vitro* and *in vivo* distinguish the vascular enzyme from that in other tissues. The next Chapter describes experiments in which I examine the hypothesis that manipulation of 11ß-OHSD activity influences vascular tone in rat and man.

CHAPTER 3 11ß-HYDROXYSTEROID DEHYDROGENASE IN VASCULAR SMOOTH MUSCLE

II: SIGNIFICANCE IN THE CONTROL OF VASCULAR TONE IN MAN AND RAT

The stimulus for the experiments presented in the previous Chapter was the preliminary evidence that 11ß-OHSD activity might influence vascular tone. This came firstly from experiments in human skin showing that the vasoconstrictor response to cortisol, a glucocorticoid receptor-mediated phenomenon [Marks et al., 1982; Gaillard et al., 1985], was potentiated by inhibition of 11ß-DH with topical glycyrrhetinic acid [Teelucksingh et al., 1990]. However, it was not clear where glycyrrhetinic acid has its effect because immunostaining for 11ß-DH in skin is present in both dermal vascular smooth muscle and epidermis [Teelucksingh et al., 1990]. Secondly, it was demonstrated that mineralocorticoid receptors in rat mesenteric vascular arcade bind aldosterone in preference to corticosterone [Funder et al., 1989], suggesting that the enzyme in vascular smooth muscle may also be required to protect mineralocorticoid receptors, analogous to its role in the distal nephron. On the other hand, the kinetic data presented in Chapter 2 emphasise the differences between the enzyme in vasculature and that in kidney.

This Chapter describes experiments which address the physiological relationship between 11ß-DH activity and vascular sensitivity to glucocorticoids. The most consistent effect of corticosteroids on blood vessels is potentiation of their sensitivity to noradrenaline rather than any direct effect on tone (see Chapter 1). I therefore began by testing the hypothesis that loss of 11ß-DH activity in human blood vessels results in increased vascular sensitivity to cortisol and hence to noradrenaline. I examined the effects of oral 11ß-DH inhibitors on vascular responses to these agents in healthy subjects, and repeated the studies in our patient with the syndrome of apparent mineralocorticoid excess type 1 (GB), who remains the only adult case of congenital 11ß-DH deficiency to have been reported [Stewart et al., 1988].

THE INFLUENCE OF 11ß-OHSD ON VASCULAR SENSITIVITY TO CORTISOL AND NORADRENALINE IN MAN

Methods

All subjects gave written informed consent. Local Ethics Committee approval was obtained. Cannulae were inserted using local anaesthesia with 1 % lignocaine. Studies were performed on *ad libitum* sodium intake. Noradrenaline was measured by radioenzymatic assay [Ball et al., 1981] by Dr Gordon Inglis (MRC Blood Pressure Unit, Western Infirmary, Glasgow). Blood pressure was measured using a Takeda semi-automated sphygmomanometer (A&D UA-751)[Jamieson et al., 1990]. Mean arterial pressure was calculated as (diastolic pressure) + (pulse pressure ÷ 3).

Drug Preparations

Liquorice was a kind gift from Geo. Bassett's Ltd, UK. Carbenoxolone sodium tablets were obtained from Biorex Labs, UK and crushed into 50 mg capsules. Placebo was lactose in identical capsules. For skin vasoconstriction studies cortisol (as hydrocortisone-21-acetate; Sigma, UK) and beclomethasone dipropionate (BDP; Steraloids, UK) were dissolved in 95 % ethanol; 5 % H_2O. For brachial artery infusions cortisol (as hydrocortisone-21-succinate; Solucortef, Upjohn, UK) was dissolved in 0.9 % NaCl, and noradrenaline (Levophed, Winthrop Labs, UK) diluted in a solution containing 1 mM ascorbic acid and 0.9 % NaCl. The ascorbic acid was added to prevent oxidation of noradrenaline.

Skin Vasoconstrictor Assay Method

This was performed as previously described [Teelucksingh et al., 1990]. The more potent synthetic glucocorticoid BDP was used as a positive control, because it is protected from 11ß-DH metabolism by its 9α-chloro group. Twelve solutions containing cortisol (0.1, 0.3, 1, 3, 5, and 10 mg/ml) or BDP (0.1, 0.3, 1, 3, 5, and 10 µg/ml) were prepared on the morning of the test. In the afternoon (1600-1700 h) 12 squares of 7 x 7 mm were outlined on the volar aspect of the subject's forearm with silicone grease. The squares had 10 µl of steroid solution applied, with a different solution for each square. The order of application was randomised and double-blind. After evaporation the site was occluded with Saran wrap (Dow, UK) which was removed at 0800 h the following morning. Intensity of dermal vasoconstriction for each square was assessed at 1, 2, 3, 4, 6 and 8 h later by a

blinded observer using a visual analogue scale from 0-3 . The response to each steroid solution was expressed as the sum of scores obtained over time for that square (max = 18 units). The response to cortisol and BDP in each subject was represented by the area under the dose-response curve and designated the "blanching score" for each drug (maximum = 180 units.$\mu g.ml^{-1}$ for BDP and 180 units.$mg.ml^{-1}$ for cortisol).

Forearm Blood Flow Method

I performed these studies in Dr David Webb's laboratory in the Clinical Research Centre, Western General Hospital. Before starting the experiments subjects rested supine for at least 30 min. Room temperature was maintained at 27 ± 1 °C. The left brachial artery was cannulated with a 27 standard-wire-gauge steel cannula (Cooper's Needle Works, Birmingham, UK). Vehicle, noradrenaline or cortisol solutions were infused at a constant rate of 1 ml/min. At least 30 min elapsed between arterial cannulation and the start of experimental recordings. Forearm blood flow was measured in both arms using venous occlusion plethysmography with temperature-compensated indium/gallium-in-silastic strain gauges [Whitney, 1953; Seidelin et al., 1991]. During recording periods the hand circulation was excluded by inflation of wrist cuffs to 200 mmHg and flows were measured for 10 s in every 15 s by repeated inflation of upper arm cuffs to 40 mmHg. Recording periods lasted for 4 min (when lower body negative pressure (LBNP) was not required) or 6 min (when LBNP was applied during the second 3 min). The interval between recording periods was at least 6 min. After each recording period the gauges were calibrated on-limb and blood pressure was measured in the right arm. Data was collected and analysed on a Macintosh micro-computer. The mean of the final five measurements from each recording period was used for analysis.

Effects of infusion are represented by the percentage change in forearm blood flow calculated as:

$$\left(\frac{I_d}{NI_d} - \frac{I_v}{NI_v}\right) \Big/ \frac{I_v}{NI_v} \times 100\%$$

where I and NI represent measured blood flows in the infused and non-infused arms respectively during periods of drug (d) and vehicle (v) administration. Using this calculation the non-infused arm acts as a control for non-specific variations in blood

flow [Greenfield & Patterson, 1954]. Forearm vascular resistance is derived from (mean arterial pressure in mmHg)/(flow in ml.100ml^{-1}.min^{-1}).

When LBNP was required it was applied, as described previously [Zoller et al., 1972; Seidelin et al., 1991], for the second half of the recording period. Subjects rested supine in a plastic-covered steel cage enclosing the lower limbs and hips and sealed around the waist above the level of the anterior superior iliac spines. Suction was applied using a vacuum pump to produce a constant 20 cm H_20 negative pressure (compared with atmospheric). The alteration from atmospheric pressure was both applied and relieved rapidly. This degree of LBNP induces a sympathetically-mediated reflex which reduces forearm blood flow without measurable effect on heart rate or blood pressure 2 min after application [Seidelin et al., 1991].

Study 1: Effect of 11ß-DH Inhibitors on Vascular Sensitivity to Cortisol

(i) In Dermal Vascular Bed

Six healthy subjects (3 male, 3 female) aged 22-33 yr (mean = 26) had skin vasoconstrictor assays performed before and after 7 days of oral liquorice (200 g daily).

(ii) In Forearm Vascular Bed

Six healthy males aged 26-32 yr (mean = 29) had forearm blood flow studies on 2 occasions in random order, either with or without pre-administration of 7 days oral liquorice (200 g daily). Infusions were with vehicle for 12 min, then cortisol at 200 µg/min for 30 min, and finally vehicle again for a 36 min washout period. Recordings were made with and without LBNP during the first 6 of every 12 min.

Study 2: Vascular Sensitivity to Cortisol in Congenital 11ß-DH Deficiency

Our patient with apparent mineralocorticoid excess [Stewart et al., 1988] was described in Chapter 1. He was aged 25 yr at the time of this study. His maintenance therapy was dexamethasone 0.25 mg at 0900 h and 0.75 mg at 2300 h, frusemide 40 mg at 0900 h, and captopril 25 mg 12 hrly. Plasma cortisol was undetectable, supine plasma renin activity 5.4 ng Angiotensin I.ml^{-1}.h^{-1} (normal range 0.3-1.5), plasma aldosterone 380 pmol/l (30-440), and blood pressure 127/72 mmHg. His last doses of frusemide, captopril and dexamethasone were 30 h, 16 h, and 6 h respectively

before the experiments. He had both the skin vasoconstrictor assay and intra-arterial cortisol infusion performed as described above.

Study 3: Effect of 11ß-DH Inhibitors on Vascular Sensitivity to Noradrenaline

(i) In Forearm Vascular Bed and (ii) During Systemic Noradrenaline Infusion

Carbenoxolone was used for 11ß-dehydrogenase inhibition in this study in part to allow a placebo-controlled double-blind design, in part because it may be a more potent inhibitor of 11ß-DH than liquorice (see Table 1.3), and in part to avoid the gastrointestinal side effects of liquorice. Six healthy males aged 26-33 yr (mean = 30) attended on 2 occasions after 7 days of oral carbenoxolone (100 mg 8 hrly) or placebo, in random double-blind order. A 21 gauge cannula was inserted in a right ante-cubital vein for blood sampling. Intra-arterial infusions were with vehicle for 12 min followed by incremental doses of noradrenaline (12 min each at 10, 20, 40, and 80 ng/min). LBNP was not applied in this study and recordings were made during the last 4 min of each infusion.

Immediately after the intra-arterial study the cannula was removed. A 21 gauge venous cannula was sited in the left arm and infused with incremental doses of noradrenaline (5 min each at 1, 2, 4, and 8 µg/min). Blood pressure was recorded in the right arm at 90 sec intervals.

For measurement of plasma noradrenaline concentrations 6 ml blood was withdrawn from the right arm cannula into Lithium Heparin at 4 °C: at the beginning of the experiment; during the highest dose of the intra-arterial infusion; and during systemic infusion with 2 and 8 µg/min noradrenaline. Plasma was stored at -70 °C until assayed.

Statistics

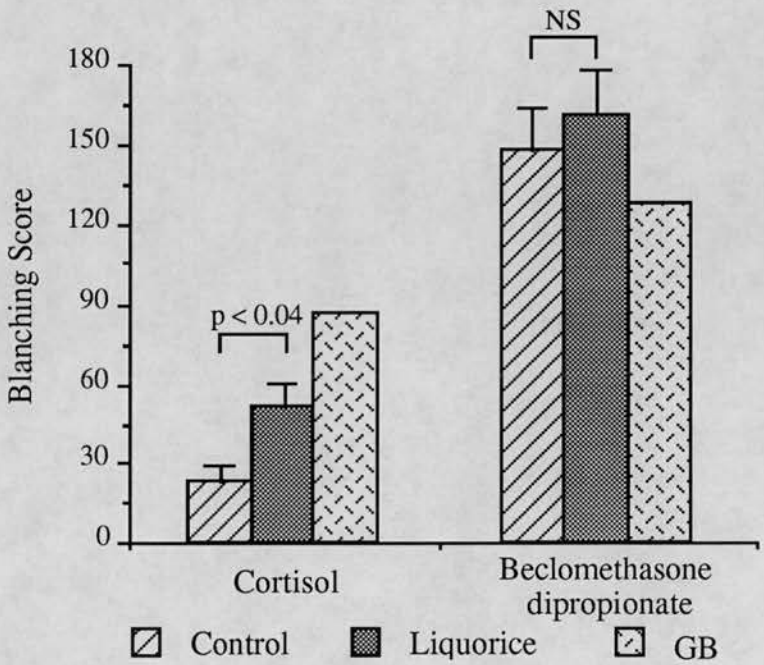
Results are given as mean ± SE. For intra-arterial cortisol infusion time-points were compared by paired multi-factorial ANOVA. For noradrenaline infusions dose-response curves were compared by multiple regression with "% change in flow" or "mean arterial pressure" as the dependent variable and "dose noradrenaline" and "pre-treatment" (placebo versus carbenoxolone; assigned values of 0 and 1) as controlling variables. Paired two-tail Student's *t* tests were used for two-group comparisons.

Results

Dermal Vasoconstrictor Sensitivity to Cortisol

Results are shown in Figure 3.1. Oral liquorice potentiated the intensity of vasoconstriction in response to topical cortisol ($p < 0.04$) but not BDP. Dermal vasoconstriction in GB was greater after cortisol and less after BDP compared with healthy subjects either with or without liquorice.

Figure 3.1 Effect of congenital and acquired 11ß-DH deficiency on dermal vasoconstrictor sensitivity to cortisol and beclomethasone dipropionate

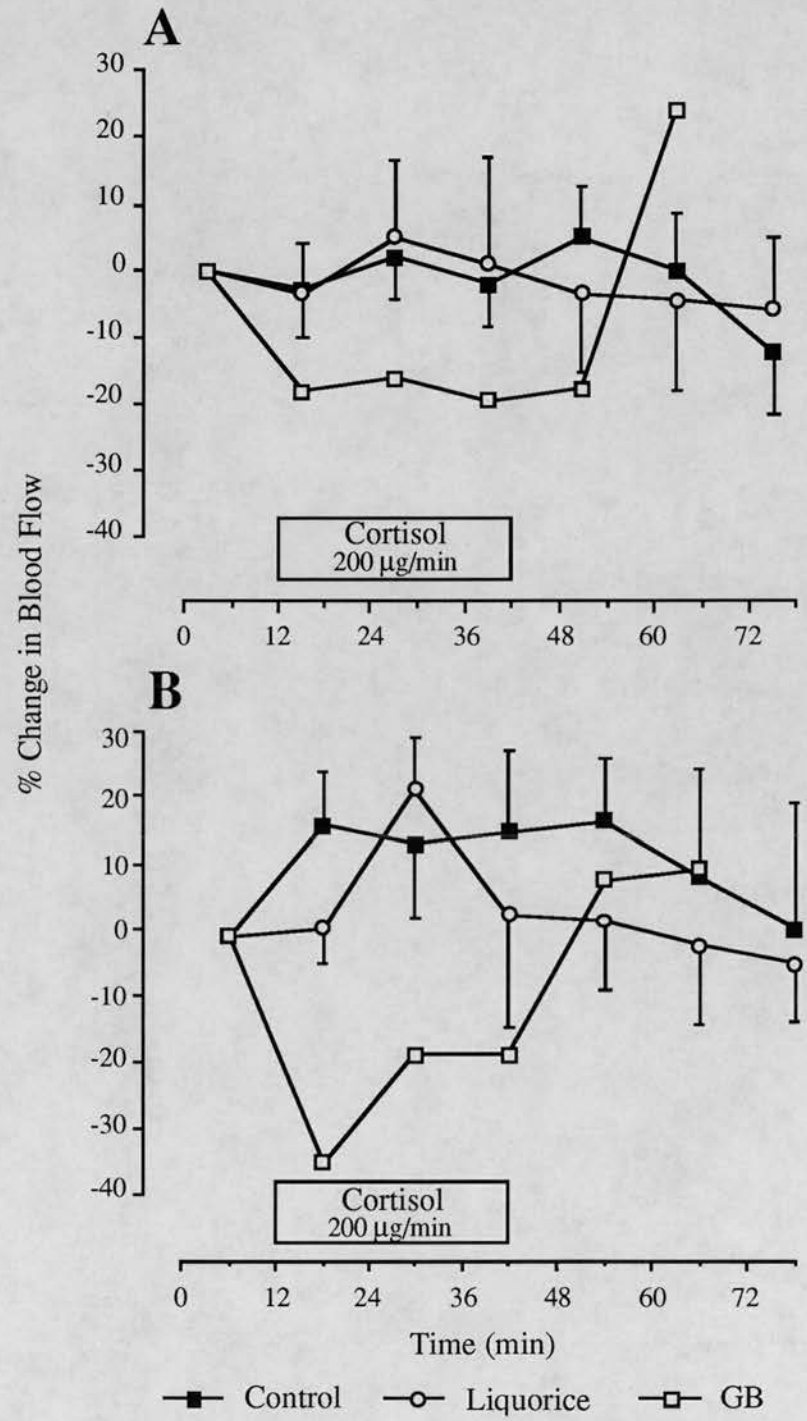


GB is our patient with the syndrome of apparent mineralocorticoid excess type 1. Bars are SE. NS = not significant.

Forearm Vascular Sensitivity to Intra-Arterial Cortisol ± LBNP

Results are shown in Table 3.1 and Figure 3.2. Resting blood flow, mean arterial pressure, derived forearm vascular resistance, and degree of vasoconstriction in response to LBNP at the start of the infusions were not affected by liquorice and were not remarkable in GB (Table 3.1). During cortisol infusions there was no

Figure 3.2 Effect of congenital and acquired 11ß-DH deficiency on forearm blood flow during intra-arterial cortisol infusion



GB is our patient with the syndrome of apparent mineralocorticoid excess type 1. Recordings were made with (**Figure 3.2B**) and without (**Figure 3.2A**) the application of lower body negative pressure (LBNP). Results are expressed as % change from baseline of ratio: (flow in the infused arm)/(flow in non-infused arm). Bars are SE.

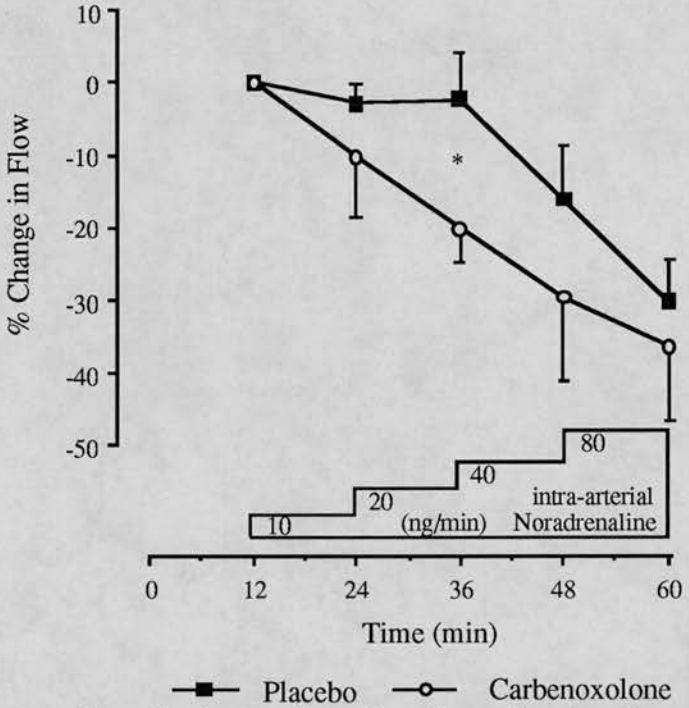
confounding systemic effect since neither mean arterial pressure, nor blood flow in the non-infused arm, changed significantly. In healthy subjects, either with or without liquorice, cortisol infusion affected neither forearm flow without LBNP (Figure 3.2A) nor vasoconstriction in response to LBNP (Figure 3.2B). However, in GB there was vasoconstriction during cortisol infusion which was exaggerated by LBNP. This was followed, during the washout period, by relative vasodilatation in the absence of LBNP.

Vascular Sensitivity to Noradrenaline

Results for intra-arterial noradrenaline infusions are shown in Tables 3.1 and 3.2 and Figure 3.3. Resting blood flow, mean arterial pressure, and derived forearm vascular resistance at the start of the infusions were not affected by carbenoxolone (Table 3.1). During infusions noradrenaline had no confounding systemic effect since neither mean arterial pressure nor blood flow in the non-infused arm changed significantly. Furthermore, in neither study did plasma noradrenaline levels rise in the contra-lateral arm during intra-arterial infusions (Table 3.2). Carbenoxolone caused potentiation of noradrenaline-induced forearm vasoconstriction (Figure 3.3; $p < 0.01$).

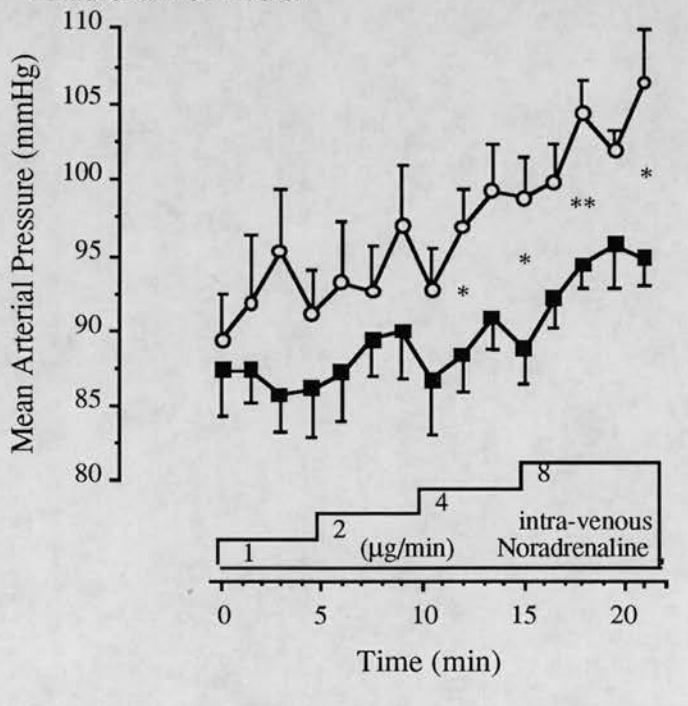
Results for systemic noradrenaline infusions are shown in Table 3.2 and Figure 3.4. After carbenoxolone the levels of noradrenaline in the contralateral arm were significantly lower during infusion with 8 μg/min noradrenaline (Table 3.2). Even so, carbenoxolone potentiated the pressor response to noradrenaline (Figure 3.4; $p < 0.001$).

Figure 3.3 Effect of carbenoxolone on forearm blood flow during intra-arterial noradrenaline infusion



Results are expressed as % change from baseline of ratio: (flow in the infused arm)/(flow in non-infused arm). Bars are SE. Comparison of curves by multiple regression $p < 0.01$. Paired Student's t tests: * = $p < 0.03$.

Figure 3.4 Effect of carbenoxolone on blood pressure during intra-venous noradrenaline infusion



Bars are SE. Comparison of curves by multiple regression $p < 0.001$. Paired Student's t tests: * = $p < 0.05$; ** = $p < 0.01$.

Table 3.1 Forearm blood flow and blood pressure at the beginning of each experimental infusion

		Study 1		Study 2	Study 3	
mean ± SE		Control	Liquorice	GB †	Placebo	Carbenoxolone
Forearm Blood Flow without LBNP* ($ml.100ml^{-1}.min^{-1}$)	Infused arm	4.3 ± 0.8	4.5 ± 2.1	3.6	2.2 ± 0.3	3.3 ± 0.8
	Non-infused arm	3.2 ± 0.4	3.3 ± 1.0	3.5	2.5 ± 0.3	2.8 ± 0.6
Forearm Blood Flow with LBNP* ($ml.100ml^{-1}.min^{-1}$)	Infused arm	3.8 ± 0.8	3.3 ± 1.1	3.1		
	Non-infused arm	3.4 ± 0.7	2.9 ± 0.7	2.7		
Blood Pressure (mmHg)	Systolic	128 ± 4	124 ± 4	144	140 ± 3	134 ± 3
	Diastolic	71 ± 1	74 ± 1	104	72 ± 4	76 ± 2
	Mean Arterial Pressure	90 ± 3	91 ± 1	117	95 ± 3	95 ± 2

* LBNP = lower body negative pressure
† GB is our patient with the syndrome of apparent mineralocorticoid excess type 1

Table 3.2 Plasma noradrenaline levels during noradrenaline infusions

nM; mean ± SE	Placebo	Carbenoxolone	Pre-infusion vs intervention*		Placebo vs Carbenoxolone†
			Placebo	Carbenoxolone	
Pre-infusion	1.17 ± 0.22	1.33 ± 0.28			NS
Intra-arterial infusion at 80 ng/min	1.48 ± 0.25	1.25 ± 0.23	NS	NS	NS
Intra-venous infusion at 2 µg/min	1.98 ± 0.37	2.23 ± 0.62	NS	NS	NS
Intra-venous infusion at 8 µg/min	6.12 ± 0.79	3.87 ± 0.62	$p < 0.01$	$p < 0.01$	$p < 0.04$

* Changes within experiments were compared by single-factor ANOVA ($p < 0.001$ for both placebo and carbenoxolone) and then by paired Student's *t* tests as shown. NS = not significant.

† Comparison between experiments was by paired 2-way ANOVA ($p < 0.01$ for placebo vs carbenoxolone) and then by paired Student's *t* tests at each dose as shown.

Conclusions

These studies in man show that: (i) cortisol-induced dermal vasoconstriction is increased in congenital 11ß-DH deficiency and after oral liquorice; (ii) cortisol-induced forearm vasoconstriction occurs in congenital 11ß-DH deficiency (when it is potentiated by the sympathetic stimulus of LBNP) but not in healthy volunteers either before or after liquorice; and (iii) both forearm vasoconstriction induced by intra-arterial noradrenaline and pressor response induced by systemic noradrenaline are potentiated by carbenoxolone. This is consistent with 11ß-DH modulating the access of cortisol to receptors in blood vessels, and thereby influencing their sensitivity to noradrenaline.

A number of points complicate the interpretation of these data. Firstly, in GB there was cortisol-induced forearm vasoconstriction which was not reproduced in normal volunteers by liquorice. It must be acknowledged that the interpretation of GB's responses is compromised to some extent by his long-term therapy with dexamethasone and angiotensin converting enzyme inhibitors. Nonetheless, the consistency of the results of topical vasoconstriction between congenital and acquired 11ß-DH deficiency support the contention that the same mechanism is responsible. The difference in forearm responses possibly reflects the severity of 11ß-DH deficiency in GB. The half life of ($11\alpha^{3}H$)-cortisol, which measures 11ß-DH activity, was 42 ± 2 min in 19 controls, prolonged to 131 min in this patient [Stewart et al., 1988] and 123 min after carbenoxolone [Stewart et al., 1990], but was only 85 min after liquorice [Stewart et al., 1987] (see Table 1.3).

Secondly, is it possible that the renal effects of 11ß-DH inhibition account for these results? For example, increased plasma volume consequent on renal sodium retention might increase vascular sensitivity. However, previous investigators have shown that, although volume expansion due to administration of mineralocorticoids increases forearm vasoconstrictor sensitivity [Abboud, 1974] and volume expansion due to salt loading increases pressor responses to noradrenaline [Pusterla et al., 1988], salt loading (with suppressed plasma renin activity and aldosterone) leads to relative relaxation of vessels in the forearm [Abboud, 1974]. Since endogenous mineralocorticoid secretion is suppressed when 11ß-DH is inhibited it is unlikely that our findings in the forearm are secondary to renal sodium retention. Similarly, elevated blood pressure alone might increase vascular sensitivity. However, blood pressure was no different after 7 days of carbenoxolone or liquorice. Furthermore,

although GB had a history of hypertension and had higher blood pressure on the day of this study, he had been normotensive on therapy during the three years beforehand.

Thirdly, it may be important that both liquorice and carbenoxolone have actions additional to their effect on 11ß-DH. As discussed in Chapter 1, they inhibit a number of enzymes which metabolise steroids [Monder, 1991] and prostaglandins [Baker & Fanestil, 1991], and may act directly on steroid receptors [Armanini et al., 1983] and cell membranes [Baron & Green, 1986]. However, it is unlikely that these effects are relevant because similar abnormal vascular responses were found in congenital 11ß-DH deficiency, and because previous experiments have shown that plasma concentrations of liquorice derivatives at the doses used in these experiments are not high enough to produce other biochemical effects [Stewart et al., 1987; Teelucksingh et al., 1991].

Mechanism of Increased Vascular Responses

The present results are consistent with many others *in vivo* and *in vitro* in which corticosteroids increased the response to noradrenaline. This effect is common to agonists of both mineralocorticoid and glucocorticoid receptors (see Chapter 1). In the kidney it is predominantly mineralocorticoid receptors which are protected by 11ß-DH [Edwards et al., 1988; Funder et al., 1988]. However, cortisol-induced dermal vasoconstriction is mediated by glucocorticoid receptors [Marks et al., 1982; Gaillard et al., 1985]. Therefore in vascular tissue it is, at least in part, the access of cortisol to glucocorticoid receptors which is controlled by 11ß-DH.

In addition, it is possible that some effects of steroids are mediated by cell surface receptors [Orchinik et al., 1991; Wehling et al., 1991]. These would act more rapidly than classical receptors because they are not dependent on gene induction. Activation of surface receptors could account for the rapid vasoconstriction after intra-arterial cortisol in GB. By contrast, the time course of the dermal response potentiated by liquorice is consistent with gene induction, being maximal at 12-15 hours [Trikam & Morton, 1985].

The mechanism linking steroid receptor activation and increased response to noradrenaline is uncertain. As discussed in Chapter 1, one possible explanation is glucocorticoid-dependent inhibition of extra-neuronal noradrenaline uptake [Iverson & Salt, 1970] and catechol-O-methyltransferase [Kalsner, 1969]. However, there is

no evidence that these effects are important *in vivo* [Sudhir et al., 1989]. In this study noradrenaline clearance was if anything increased by carbenoxolone, as judged by noradrenaline levels during infusions. Perhaps of greater relevance are recent observations, described in Chapter 1, of the influence of corticosteroids on adrenoceptor numbers and second messenger sensitivity.

Relevance to Blood Pressure Regulation

The contribution which increased vascular tone makes to the hypertension of 11ß-DH deficiency is difficult to quantify. After liquorice there is a dissociation between sodium retention (which occurs in the first few days and reaches equilibrium within 10 days) and elevated blood pressure (which occurs only after chronic administration [Farese et al., 1991]). This delay is longer than the few days in which blood pressure rises after administration of exogenous mineralocorticoids [Nicholls et al., 1979; Fraser & Padfield, 1985]. Therefore the rise in blood pressure may be independent of renal mineralocorticoid excess. In the present study, despite increased sensitivity to noradrenaline, resting forearm vascular resistance was no different after 7 days of carbenoxolone and blood pressure had not risen. This is consistent with findings during dexamethasone administration to rats, when increased vascular sensitivity [Russo et al., 1990] and pressor response [Handa et al., 1984] to noradrenaline preceded the rise in blood pressure.

In summary the studies in man support the hypothesis that 11ß-DH activity dictates vascular sensitivity to cortisol and thereby to noradrenaline. In order to confirm that this effect is mediated by increased access of glucocorticoids to receptors in vascular smooth muscle cells, and not by a secondary neurogenic or cardiovascular reflex, I have applied the principles of the studies in man to experiments with isolated rat vessels.

INFLUENCE OF 11ß-DH INHIBITORS ON VASCULAR RESPONSES TO NORADRENALINE IN RAT AORTIC STRIPS

The influence of corticosteroids on vascular tone has been investigated most extensively in rats. Many of these experiments examined the effects of corticosteroid insufficiency or excess *in vivo* on vascular reactivity *ex vivo*. This offers little advantage over the experiments in man described above. A second approach has been to infer the mechanism of a change in tone by studying biochemical changes in cultured vascular smooth muscle cells. However, it remains to be seen whether biochemical observations correspond with function *in vivo*. A third approach has been to administer corticosteroids to vessels in isolated preparations *in vitro*, where observed effects cannot be mediated indirectly by plasma volume expansion, neurogenic reflexes, or hypertension. Unfortunately, many of these studies have been performed at doses of steroids and time courses which are irrelevant to classical steroid-induced transcription-dependent effects (see Chapter 1). The most common mistakes have been to use too high a concentration and too short a time course. In the experiments which follow I re-examine the effect of corticosterone on noradrenaline sensitivity in rat aortic strips, and assess the effect of 11ß-DH inhibition with carbenoxolone.

Methods

Aortic Strips in Organ Baths

I was assisted in these experiments by Kathleen Sang, an Honours B.Sc. student working under my supervision. Male Wistar rats (250-300 g) were killed by decapitation. When required they were adrenalectomised under fentanyl anaesthesia between 2 and 7 days before the experiment. Surgery was performed by June Noble, and truncal blood collected for assay of plasma corticosterone to confirm its success. After decapitation aortae were carefully dissected from the animal and connective tissue removed. They were cut helically and the endothelium removed by abrasion. Strips measuring 15 x 3 mm were mounted on transducers with silk sutures in a 5 ml organ bath chamber which was gassed continuously with 95 % O_2 / 5 % CO_2. Resting tension was adjusted to 2 g by internal calibration. The chamber was perfused at 5 ml/min with KRBG (same composition as in Chapter 2 except without albumin) containing steroids ± carbenoxolone. Both the chamber and perfusate were warmed to 37 ^{o}C by water jackets.

At the end of 2 or 5 h the perfusion was stopped. A cumulative dose-response curve to noradrenaline stimulation was performed by adding 50 µl of each of a series of stock solutions to the chamber. Contractions plateaued after 3-5 min and the next dose of noradrenaline was added. Noradrenaline (Arterenol, Sigma) was diluted fresh each day in 0.9 % NaCl containing 1 mM ascorbic acid to give final chamber concentrations from 10^{-12} M to 10^{-4} M. At the end of the noradrenaline dose response a maximal contraction was elicited by adding 100 µl of KCl to the chamber to a final concentration of 50 µM.

All steroids and inhibitors were obtained from Sigma, except RU38486 (a glucocorticoid receptor antagonist [Gaillard et al., 1984]) which was obtained from Roussel UCLAF (Paris, France). Steroids were prepared in ethanol solutions and added to the perfusate at ≤ 0.01 % final ethanol concentration. Carbenoxolone was diluted directly in KRBG. The dose of steroids used was an approximation to their K_d for classical type 2 glucocorticoid receptors or, in the case of spironolactone and RU38486, for their K_i for steroid binding. The exception was carbenoxolone. The apparent K_i for 11ß-DH in microsomal preparations is ~10^{-9} M [Monder et al., 1989], but the data in Figure 3.5 illustrate that in homogenates, and by inference in whole aortic strips, it is significantly higher. This experiment was performed as in Chapter 2 with a protein concentration of 500 µg/ml and in the presence of 200 µM NADP and concentrations of carbenoxolone diluted in KRBG as shown. In order to ensure adequate 11ß-DH inhibition in aortic strips a carbenoxolone concentration of 10^{-5} M was employed.

Dose-response curves were compared by two-way repeated measure ANOVA. Results are mean ± SE and all experiments were performed on at least 6 aortic strips.

Results

Typical dose-response curves to noradrenaline are shown in Figure 3.6. Basal tone after 2 h was not significantly different in all preparations therefore none of the steroid treatments were directly vasoactive. The most dramatic differences were apparent in the maximum contraction obtained rather than in the ED_{50} for noradrenaline. For that reason, and to simplify visual comparison of multiple dose-responses, the data are presented as maximum contraction achieved rather than showing the whole curve for each drug. Nonetheless, the statistics refer to ANOVA of the whole curve.

Figure 3.5 Inhibition of 11ß-DH activity in homogenised rat aorta by carbenoxolone

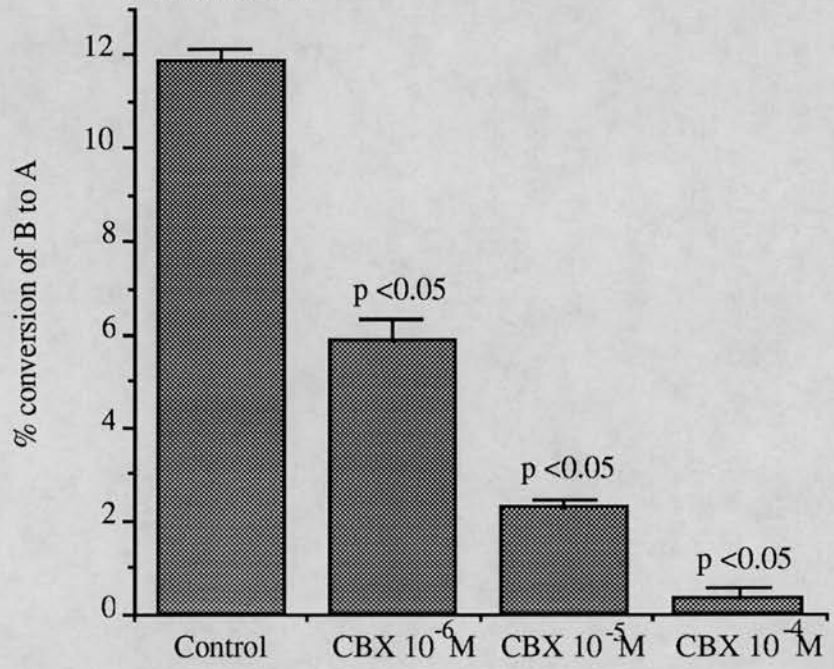
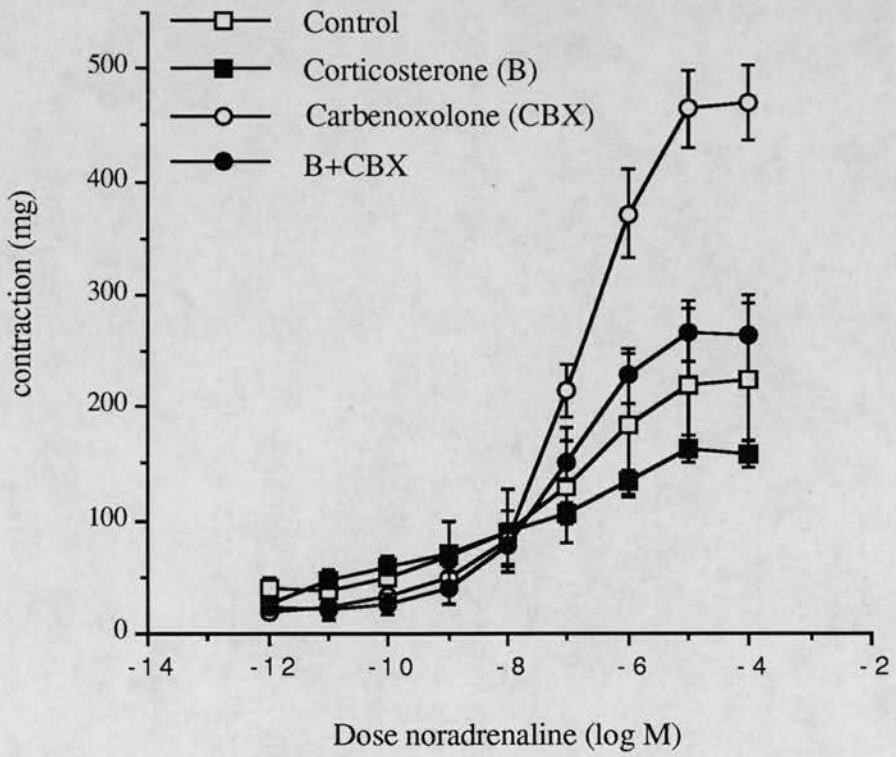


Figure 3.6 Dose-response to noradrenaline in rat aortic strips



Strips obtained from non-adrenalectomised rats were treated *in vitro* for 5 h with steroids shown.

All changes observed in noradrenaline sensitivity were also reflected in responses to KCl, so that results are all presented as absolute values of mg contraction rather than as % of maximal contraction with KCl. The efficacy of removal of endothelium was confirmed in preliminary experiments which showed vasoconstriction in response to acetyl choline (10^{-6} M).

Non-adrenalectomised Rats

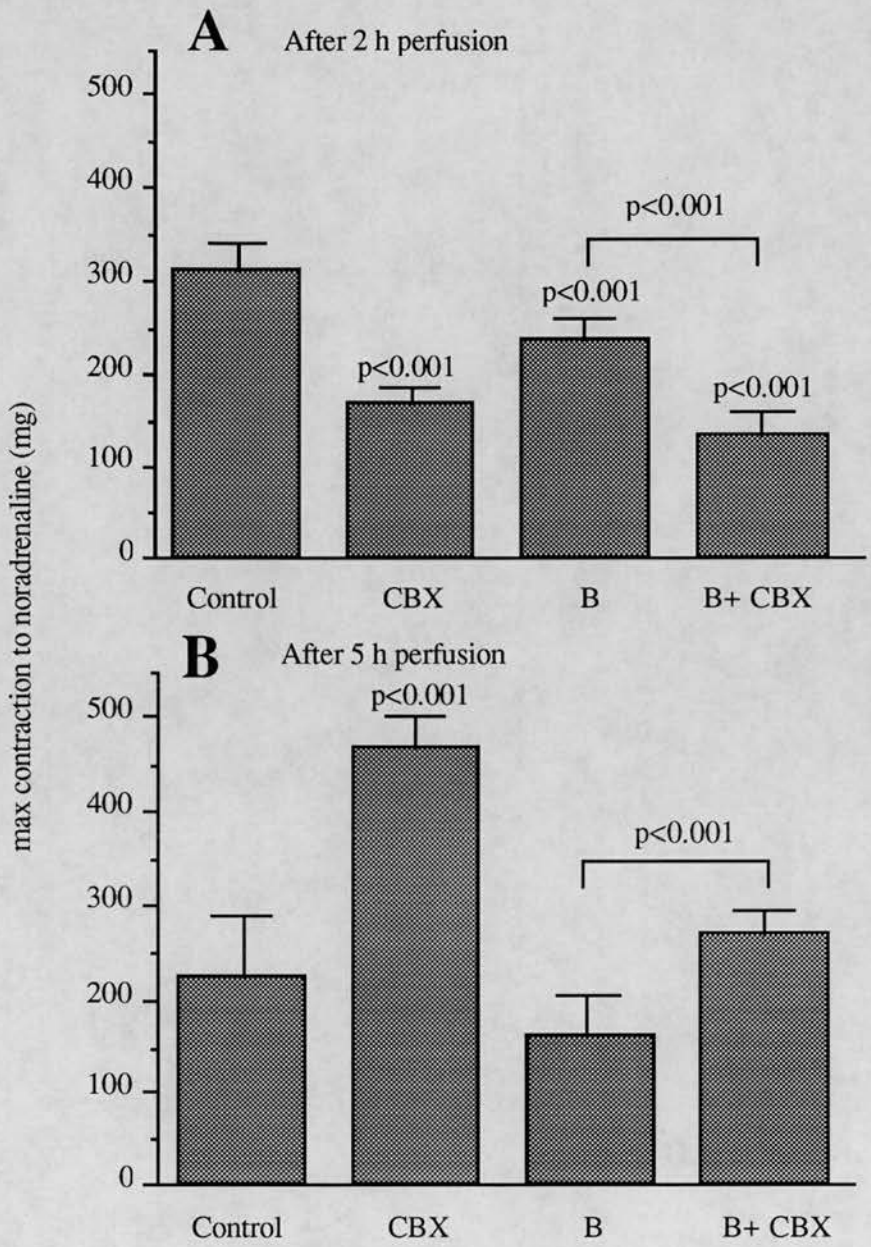
Results are shown in Figure 3.7. After perfusion for 2 h both corticosterone (10^{-7}M) and carbenoxolone (10^{-5}M) reduced sensitivity to noradrenaline ($p < 0.001$), and the combination of the two was indistinguishable from carbenoxolone alone. However, when the experiments were repeated with perfusion for 5 h, carbenoxolone potentiated the response to noradrenaline ($p < 0.001$) but corticosterone produced a non-significant reduction of response. The combination of corticosterone and carbenoxolone for 5 h was associated with responses similar to controls, significantly lower than carbenoxolone alone ($p < 0.001$), but significantly greater than corticosterone alone ($p < 0.001$).

Adrenalectomised Rats

Results are shown in Figure 3.8, after perfusion for 2 h. The response to noradrenaline was not significantly different in aortae from adrenalectomised compared with non-adrenalectomised rats. Carbenoxolone produced a dose-dependent increase in the response to noradrenaline. Corticosterone had no significant effect. The combination of carbenoxolone with corticosterone produced a reduction in response, which was much lower than carbenoxolone alone ($p < 0.001$) and also lower than corticosterone alone ($p < 0.04$). 11-Dehydrocorticosterone (10^{-7}M) had a non-significant potentiating effect when added alone and when added with carbenoxolone was not different from carbenoxolone alone.

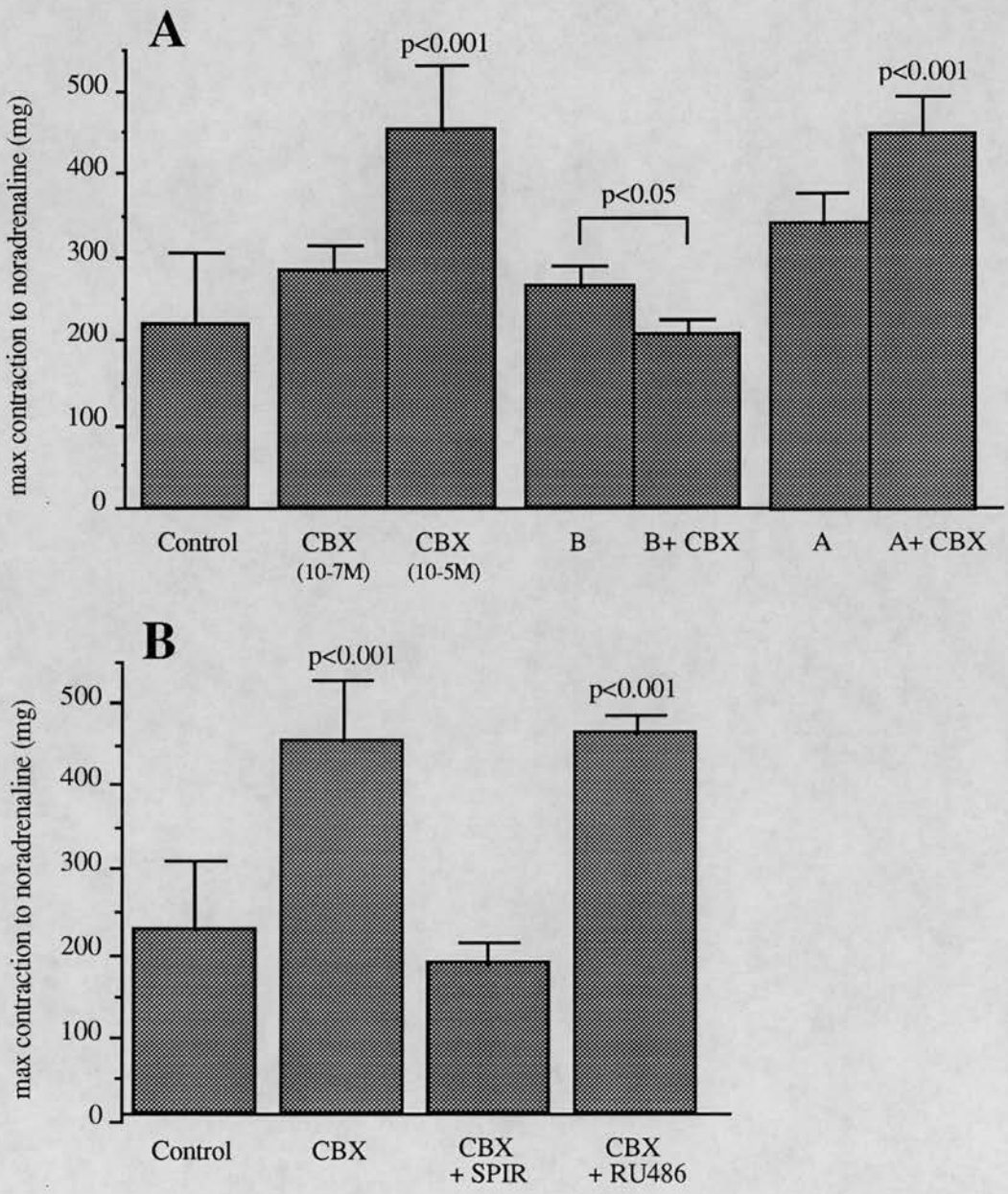
The potentiating effect of carbenoxolone was not affected by RU38486 (10^{-6}M) but was blocked by spironolactone (10^{-5}M). RU38486 and spironolactone had no significant effects when added alone.

Figure 3.7 Maximum contraction to noradrenaline in aortic strips from non-adrenalectomised rats



B = corticosterone (10^{-7}M); CBX = carbenoxolone (10^{-5}M). p values without brackets refer to comparison with control data.

Figure 3.8 Maximum contraction to noradrenaline in aortic strips from adrenalectomised rats



A: manipulation of 11ß-DH activity with carbenoxolone (CBX) and the response to corticosterone (B; 10^{-7}M) and 11-dehydrocorticosterone (A; 10^{-7}M); **B**: effect of competitive antagonists of glucocorticoid (RU38486; 10^{-6}M) and mineralocorticoid (spironolactone = SPIR; 10^{-5}M) receptors on the response to carbenoxolone (10^{-5}M). p values without brackets refer to comparison with control data. All perfusions were for 2 h.

Conclusions

The original explanation offered for the mineralocorticoid hypertension induced by liquorice and carbenoxolone was that these agents bind directly to mineralocorticoid receptors and activate them [Ulmann et al., 1975; Armanini et al., 1983]. *In vivo* in man sufficiently high concentrations of liquorice and carbenoxolone are not achieved and their direct effects are probably irrelevant [Teelucksingh et al., 1991]. However, in aorta *in vitro*, in order to ensure inhibition of 11ß-DH, I have used much higher concentrations of carbenoxolone. Similar conditions prevailed in previous studies on lymphocytes *in vitro* when a change in sodium efflux was demonstrated with mM concentrations of carbenoxolone [Baron & Green, 1986]. The current studies show that carbenoxolone potentiates the response to noradrenaline at 10^{-5}M but not significantly at 10^{-7}M. Potentiation only occurred in the absence of corticosterone (either when the animals had been adrenalectomised, or when endogenous corticosterone had been washed out for 5 h from the preparation of aortae from non-adrenalectomised animals). This effect of carbenoxolone is probably mediated by mineralocorticoid receptors since it can be blocked by spironolactone. The observation that contraction to KCl is also affected in the same direction as the response to noradrenaline suggests that carbenoxolone affects the final common pathway of contraction, rather than its effects being limited to a single second messenger pathway.

In the presence of corticosterone (either present endogenously in the first 2 h following removal from the non-adrenalectomised animals, or added at physiological concentrations to preparations from adrenalectomised animals), the effect of carbenoxolone was dramatically different. Thus in aortae from adrenalectomised animals, although corticosterone had little effect alone, when added with carbenoxolone there was attenuation of response relative to both corticosterone alone and to carbenoxolone alone. Similarly, in aortae from non-adrenalectomised animals the potentiation of response by carbenoxolone after 5 h was substantially blunted by corticosterone, albeit that in this preparation the net effect when carbenoxolone and corticosterone were combined remained a modest potentiation of response. These data are consistent with carbenoxolone opening access for corticosterone to receptors which promote relative vasodilatation, ie attenuate the response to noradrenaline. Thus 11ß-DH may act physiologically to protect these receptors from corticosterone and thereby influence vascular tone.

The identity of the contraction-attenuating receptors activated by corticosterone is not clear, but it is unlikely that they are mineralocorticoid receptors since we suspect that mineralocorticoid receptor activation by carbenoxolone results in potentiation of the response to noradrenaline. Furthermore, they are unlikely to be glucocorticoid receptors because in a similar preparation Rees and colleagues [1990] showed that the glucocorticoid receptor agonist dexamethasone potentiated the response to noradrenaline over a similar time-course. It may be that the attenuating effect of corticosterone is mediated by a third class of receptor in vascular smooth muscle. In rabbit and bovine aorta Kornel and colleagues have demonstrated intra-cellular binding sites with higher affinity for cortisol than for dexamethasone, in addition to binding sites with characteristics of mineralocorticoid and glucocorticoid receptors [Kornel et al., 1982; Hayashi & Kornel, 1990]. These may represent intra-cellular CBG or binding to 11ß-OHSD. Alternatively, they may be site-specific corticosteroid receptors which are normally protected from glucocorticoids by 11ß-DH. To identify the receptor which mediates corticosterone-induced attenuation in the presence of carbenoxolone I have attempted to combine these two agents with spironolactone or RU38486, but have found that these combinations result in very poor responses in the vessels. It is hard to establish the mechanism of this loss of response, but it probably relates to non-specific effects of high concentrations of several lipid-soluble agents on cell membranes.

Finally, 11-dehydrocorticosterone was without effect either with or without carbenoxolone, suggesting that 11ß-OR does not act in rat aorta to amplify exposure to glucocorticoids by converting the pool of inactive steroid to active corticosterone.

SUMMARY

In this Chapter I have demonstrated that manipulation of 11ß-DH activity and congenital deficiency of 11ß-DH are associated with changes in vascular sensitivity to glucocorticoids and catecholamines in man and rat. In human dermal and forearm vessels this increases vascular reactivity; is probably mediated by glucocorticoid receptors; and might contribute to the pathophysiology of hypertension. By contrast, in rat aorta 11ß-DH inhibition leads to relative loss of noradrenaline responsiveness, mediated by an unidentified receptor. There are several possible explanations for this discrepancy: (i) there may be differences between species; (ii) there may be site-specific differences between vascular beds; or (iii) the removal of the endothelium from the rat aortae may be significant.

The first possibility is difficult to test. Although there are different effects of enzyme inhibitors on potassium balance and 11ß-OR activity in man and rat (see Chapter 1), we have no evidence that the physiological role of the enzyme in the kidney is different between species. Furthermore, enzyme-mediated receptor protection is a phylogenetically well-conserved mechanism, since it has been demonstrated in toad bladder mucosa [Gaeggeler et al., 1989; Brem et al., 1989].

The second possibility is testable. As I stressed in conclusion in Chapter 1, the action of glucocorticoids in blood vessels may offer site-specific regulation dependent on the local milieu. In Chapter 2 this possibility was emphasised by the apparent hybrid character of the 11ß-OHSD in the vessel wall: having the kinetic parameters of the liver enzyme in the functional setting of the renal enzyme. The significance of site-specificity could be corroborated by two experimental strategies: (i) to demonstrate in another rat vascular bed that inhibition of enzyme activity is associated with increased responsiveness to noradrenaline, as seen in man; and (ii) to demonstrate in aortic vascular smooth muscle cells that a vasodilator second messenger is increased when corticosterone is administered together with carbenoxolone, while this does not occur in cells from another rat vascular bed. At the time of writing I am investigating the effects of corticosterone and carbenoxolone on: (i) noradrenaline responsiveness in perfused rat superior mesenteric arcade; and (ii) cAMP generation in vascular smooth muscle cells in primary culture from rat aorta and mesentery.

Finally, the role of the endothelium in mediating the response to corticosteroids is unclear. I chose to study rat aortic strips without endothelium because 11ß-OHSD

immunoreactivity and mRNA expression are not present in endothelial cells (see Chapter 2). Furthermore, previous studies with dexamethasone have shown that the presence or absence of the endothelium makes no difference to the effect on noradrenaline sensitivity [Rees et al., 1990]. However, from the present data I cannot exclude the possibility that changes in endothelium-derived paracrine mediators (either increased release of a promoter of vasoconstriction or decreased release of a promoter of vasodilatation) are responsible for the effect of cortisol in man when 11ß-DH is inhibited or deficient. In the rat aorta, when 11ß-DH is inhibited, the absence of such an endothelial effect might allow a less potent attenuating effect of corticosterone in the vascular smooth muscle cells to predominate.

Thus although the mechanism is complex, these data confirm that the expression of 11ß-OHSD in vascular smooth muscle cells is functionally significant, and suggest that the enzyme plays diverse site-specific roles within the vascular tree. In investigating the role of 11ß-OHSD deficiency in human hypertension it will clearly be important to consider the possible contribution made by increased vascular tone.

CHAPTER 4 REGULATION OF 11ß-HYDROXYSTEROID DEHYDROGENASE BY ACTH IN MAN

The factors which regulate 11ß-OHSD activity are poorly understood. A number of observations led me to address the possibility that ACTH might influence enzyme activity. Firstly, Ludwig Kornel showed that ACTH infusion in two Addisonian patients was associated with a prolonged half life of ^{14}C-cortisol and a reduced half life of ^{14}C-cortisone, suggesting that ACTH acts extra-adrenally to inhibit 11ß-DH [Kornel, 1970]. Secondly, others had shown that infusion of exogenous ACTH does not increase plasma cortisone concentrations despite a rise in cortisol [Srivastava et al., 1973; MacKenzie et al., 1990]. Finally, work with which I was involved examining the activity of 11ß-OHSD in bovine adrenocortical cells in primary culture showed that basal synthesis of cortisone is substantial, but that ACTH does not stimulate the secretion of cortisone after 60 min [Burt et al., 1991], suggesting that in the presence of ACTH the proportion of cortisol converted to cortisone is reduced. (We have since shown that this apparent inhibition is less pronounced when ACTH is administered for 6-24 h [Williams et al., 1992], perhaps reflecting induction of adrenal 11ß-OHSD expression by glucocorticoids).

I first addressed the hypothesis that ACTH inhibits 11ß-DH by an extra-adrenal mechanism in an experiment in adrenalectomised rats presented in Figure 2.9. Under these conditions ACTH (1-24) had no measurable effect on the enzyme. By contrast, administration of ACTH to non-adrenalectomised rats resulted in increased 11ß-DH activity in the kidney *in vitro* [Stewart et al., 1989], but this may reflect glucocorticoid induction of the enzyme. In order to establish whether ACTH has an inhibitory effect on 11ß-DH activity when the adrenals are intact I turned my attention to man and undertook the following experiments in healthy volunteers.

To test the hypothesis that ACTH inhibits 11ß-DH requires measurement of enzyme activity in subjects with varying plasma cortisol concentrations. The most sensitive measurement of cortisol-cortisone conversion is the half life of (11α^3H)-cortisol [Stewart et al., 1987; 1988; 1990]. However, the affinity of the labelled steroid for 11ß-DH is less than that of endogenous cortisol because of its primary isotope effect [Hellman et al., 1971]. Therefore, the technique is not valid to compare cortisol inactivation as endogenous plasma cortisol concentrations change. A more conventional method is the measurement of urinary metabolites of cortisol and cortisone by gas chromatography and mass spectrometry. However, this did not

detect 11ß-OHSD deficiency after carbenoxolone administration [Stewart et al., 1990] and is a laborious procedure. For these reasons I chose to measure cortisol/cortisone ratios in peripheral blood as an index of 11ß-DH activity. This is as sensitive as the measurement of urinary metabolites in 11ß-DH deficiency [Stewart et al., 1987; 1990](Table 1.3), and it offers the advantage that the estimation of 11ß-DH activity in single plasma samples allows more detailed examination of the physiology of the enzyme. In order to relate changes in peripheral cortisol/cortisone ratios to changes in renal 11ß-DH activity it was also necessary to identify the major sources of plasma cortisone in man using selective venous catheterisation.

Methods

Local Ethical Committee approval and written informed consent were obtained. Plasma cortisol was assayed directly by the method of McConway and Chapman [1986] using a sheep antiserum from the Scottish Antibody Production Unit (Carluke, UK). Plasma cortisone was assayed after ethyl acetate extraction and HPLC separation with an in-house rabbit antiserum [Whitworth et al., 1989].

Selective Venous Catheterisation

Blood samples were obtained by Dr Cheok Song in the Cardiology department, from 24 patients undergoing routine left and/or right heart catheterisations for ischaemic or valvular heart disease. Positioning of the catheter was confirmed radiographically for each site. Sampling was not simultaneous.

Circadian Blood Sampling

Seven healthy males on no medication aged 26-35 yr had a 21 gauge cannula inserted in a forearm vein at 1200 h. They drew their own samples at 4 hrly intervals during waking hours for the following 24 h.

Insulin Tolerance Tests

Three healthy males on no medication aged 28, 29 and 32 yr had a 21 gauge cannula inserted in a forearm vein and were injected with insulin (0.15 units soluble human insulin / kg body weight) at 0900 h. Samples were drawn at 15 min intervals for 105 min. Plasma glucose fell to < 2.0 mM and hyperadrenergic symptoms and signs were noted in all cases.

Infusions of ACTH and Cortisol

Five healthy males on no medication aged 26-33 yr attended at 0900 h on 2 occasions after oral dexamethasone (1 mg at midnight and 0.5 mg at 0800 h). Bilateral forearm cannulae were inserted, and one was infused on one occasion with 1-24 ACTH (Synacthen, Ciba, UK) and on the other occasion with cortisol (hydrocortisone-21-succinate, Solucortef, Upjohn, UK), both diluted in 0.9 % NaCl. ACTH infusion was for 10 min each at 0.1, 0.25, 0.5, 1, 3, and 6 μg/min followed by 30 min at 12 μg/min. Cortisol infusion was for 10 min each at 50, 100, 140, 180, 220, 260, and 300 μg/min followed by 20 min at 400 μg/min. Samples were drawn from the non-infused arm at 10 min intervals. One subject only attended for cortisol infusion, so that 50 data points are available for cortisol and 40 for ACTH infusions.

Statistics

Results are expressed as mean ± SE. Student's unpaired two-tail *t* test was used to compare data from two groups. Multiple groups were compared by single-factor ANOVA followed by Fisher's PLSD test. Interval data in the circadian and insulin tolerance studies were compared by repeated measure ANOVA and Fisher's PLSD. Relationships between continuous variables were assessed by linear regression, and differences between these relationships assessed by multiple regression (where necessary assigning non-continuous variables values of 0 and 1).

Results

Principal Site of Cortisone Production

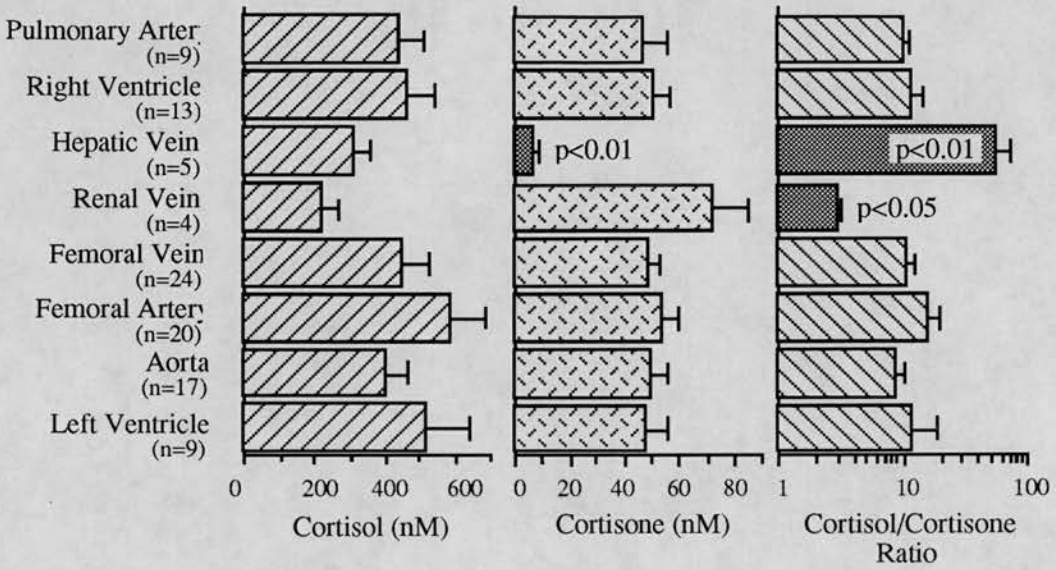
Results of selective venous catheterisation are shown in Figure 4.1. Plasma cortisol was not different between sites. However, plasma cortisone was low in hepatic vein ($p < 0.01$) and cortisol/cortisone ratio was low in renal vein ($p < 0.05$) and high in hepatic vein ($p < 0.01$).

Response of Cortisol and Cortisone to Endogenous ACTH

(i) Circadian Rhythm

Results are shown in Figure 4.2. Plasma cortisol peaked as expected at 0800 h and reached a nadir at 2400 h (repeated measure ANOVA $p < 0.001$). Plasma cortisone showed no significant circadian rhythm.

Figure 4.1 Concentrations of cortisol and cortisone, and cortisol/cortisone ratio, in plasma from selective venous catheterisations



Bars are SE.

(ii) Insulin Tolerance Tests

Results are shown in Figure 4.3. A significant rise in plasma cortisol in response to hypoglycaemic stress was accompanied by a non-significant fall in mean plasma cortisone.

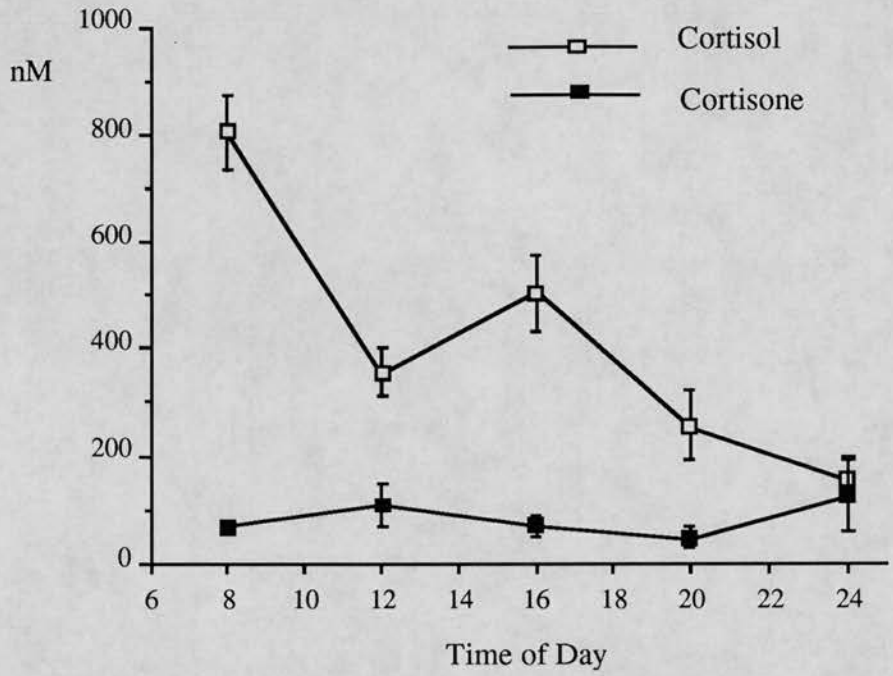
(iii) Dexamethasone Suppression

Dexamethasone (given in preparation for the study with cortisol and ACTH infusion) resulted in a non-significant fall in mean basal plasma cortisone from 77 ± 16 nM to 28 ± 11 nM ($p = 0.16$) on the first study morning when plasma cortisol was suppressed from 514 ± 68 nM to 37 ± 3 nM ($p < 0.001$).

Response of Plasma Cortisol and Cortisone to Exogenous ACTH

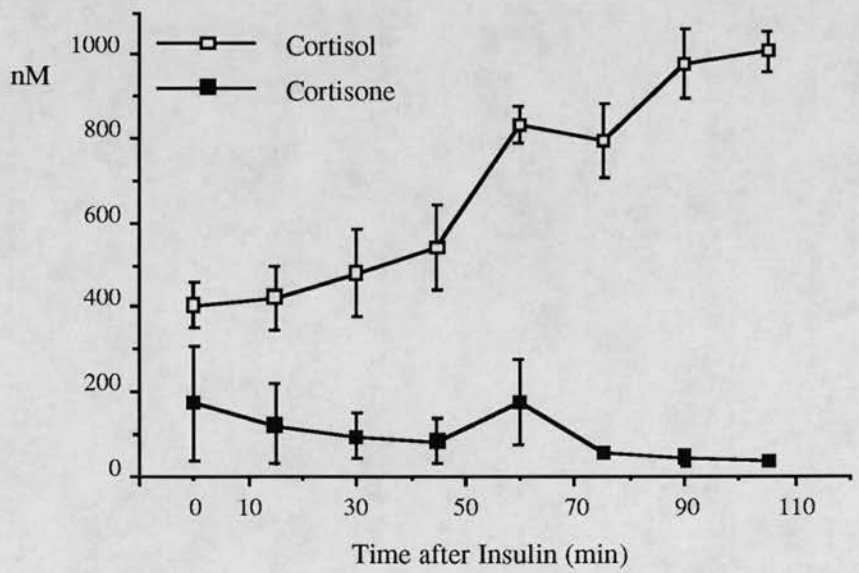
The relationships between plasma cortisol and cortisone during infusion with cortisol and ACTH are shown in Figure 4.4. Infusion of cortisol resulted in a significant rise in cortisone ($R^2 = 0.18$, $p < 0.002$), but infusion with ACTH had no effect ($R^2 = 0.005$, $p > 0.6$). Multiple regression showed a significant difference between cortisol and ACTH infusions ($p < 0.03$).

Figure 4.2 Circadian variations in plasma cortisol and cortisone



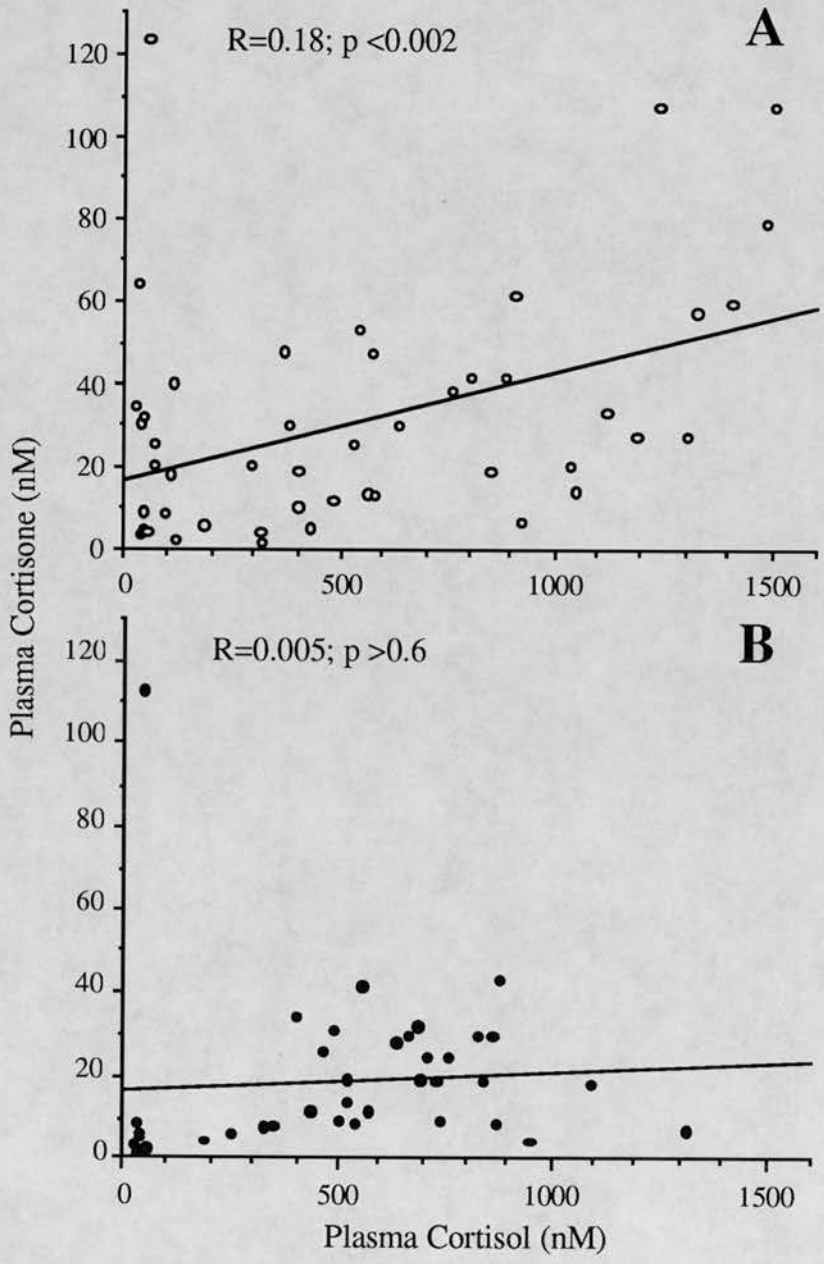
Bars are SE. By repeated measure ANOVA the variations in cortisol were significant ($p < 0.001$) while those in cortisone were not.

Figure 4.3 Response of plasma cortisol and cortisone to insulin-induced hypoglycaemia



Bars are SE. By repeated measure ANOVA cortisol rose significantly ($p < 0.001$) while cortisone showed a non-significant fall following insulin.

Figure 4.4 Plasma cortisol and cortisone concentrations during infusion with cortisol (**A**) or ACTH (1-24)(**B**)



The results shown are for linear regression analysis. Multiple regression showed a significant difference between the curves ($p < 0.03$).

Conclusions

The present data confirm that the major site of peripheral conversion of cortisol to cortisone is the kidney rather than the liver, a finding which was suggested by the observation of reduced plasma cortisone levels in patients with chronic renal failure [Whitworth et al., 1989]. Furthermore, it confirms the hypothesis presented in Chapter 1 that the activity of 11ß-OHSD in the liver is predominantly 11ß-OR.

One possible source of cortisone which was not assessed is the adrenal cortex. We were not the first to show that cortisone is produced by isolated adrenocortical cells [Whitehouse et al., 1967], and previous studies in man have shown a cortisone concentration gradient from adrenal vein to peripheral vein of 2.9:1 [Bailey & West, 1969; Dazord et al., 1972]. Our data from bovine cells show that acute ACTH stimulation may be associated with an increased secretion of cortisol relative to cortisone from the adrenal [Burt et al., 1991] and this might also be relevant to the observations of acute changes in cortisol/cortisone ratio presented here.

Acute increases in endogenous ACTH levels, during circadian rhythm and insulin-induced hypoglycaemic stress, were associated with an increase in cortisol/cortisone ratio. This might be explained if: (i) ACTH, or an ACTH-stimulated adrenal precursor steroid, inhibits the adrenal production of cortisone acutely; (ii) increased cortisol concentrations of this magnitude saturate 11ß-DH and clearance of cortisol is no longer first order; or (iii) ACTH, or an ACTH-stimulated steroid, inhibits the peripheral conversion of cortisol to cortisone in the kidney. These possibilities were dissected in the study presented in Figure 4.4 which demonstrates that, when adrenal cortisone production is suppressed, over a range of plasma cortisol concentrations from 0 to 1500 nM 11ß-DH is not saturated since plasma cortisone levels increase. By contrast, similar cortisol concentrations produced by ACTH do not elevate cortisone levels, implying that ACTH, or an ACTH-dependent factor, inhibits the peripheral conversion of cortisol to cortisone. The failure of exogenous ACTH to raise plasma cortisone levels has been described before [Srivastava et al., 1973; MacKenzie et al., 1990] but this is the first study to distinguish between saturation and inhibition of 11ß-DH. Interestingly, endogenous ACTH does induce a circadian rhythm in salivary cortisone [Katz & Shannon, 1969]. This suggests that ACTH-dependent inhibition of 11ß-DH may be tissue-specific.

Given that the kidney is the major site of peripheral 11ß-DH activity, we can infer that increased ACTH concentrations are not only associated with increased adrenal cortisol secretion, but also cause an increase in renal sensitivity to cortisol. This raises the possibility that in physiological circumstances when ACTH is high, cortisol may act as a mineralocorticoid. This might be important in dictating circadian rhythms of sodium excretion and blood pressure [Millar-Craig et al., 1978].

At present the mechanism of ACTH-dependent 11ß-DH inhibition is obscure. Kornel's hypothesis of an extra-adrenal action of ACTH may be flawed because both of the Addisonian patients he studied could have had some residual adrenocortical function [Kornel, 1970]. In intact rats, chronic administration of ACTH resulted in increased *in vitro* renal 11ß-DH activity [Stewart et al., 1989]. By contrast, in adrenalectomised rats ACTH had no measurable effect on the enzyme (Figure 2.9). These results probably reflect glucocorticoid-dependent induction of 11ß-OHSD expression in the longer term (Figures 2.7 - 2.9), but they raise the possibility that ACTH does not inhibit 11ß-DH in the rat. Thus the most likely explanation for the present observations in man is that the effect of ACTH is mediated by an ACTH-dependent corticosteroid which inhibits 11ß-DH, and which may not be produced in rat adrenal. Many steroids are competitive inhibitors of 11ß-DH [Monder & Shackleton, 1984](Table 1.4), including several ACTH-dependent 17-hydroxylated progestogens not produced by rat adrenal.

This mechanism might even explain the paradox that in sheep the hypertension induced by ACTH administration cannot be reproduced by infusion of the major glucocorticoid and mineralocorticoid hormones, but is reproduced with the addition of 17α-hydroxyprogesterone and 17,20α-dihydroxyprogesterone, steroids which are devoid of intrinsic corticosteroid agonist activity [Scoggins et al., 1989]. These progestogens may act as ACTH-dependent inhibitors of 11ß-DH, thus potentiating the hypertensinogenic action of cortisol. Similarly, an effect of ACTH to increase mineralocorticoid receptor sensitivity to cortisol might explain why kaliuresis induced by exogenous cortisol in man appears to be mediated by glucocorticoid receptors, while ACTH-induced kaliuresis is mediated by mineralocorticoid receptors [Clore et al., 1988]. However, the relevance to ACTH-dependent hypertension in man remains uncertain since, by contrast with sheep, it seems that ACTH-dependent hypertension can be reproduced by cortisol alone in man [Connell et al., 1987].

It is possible that ACTH-dependent inhibitors of 11ß-DH have already been measured, albeit inadvertently, when Morris and colleagues demonstrated that extracts of human urine have inhibitory activity for 11ß-DH *in vitro* [Morris et al., 1992]. The excretion of these factors increases during pregnancy, suggesting that they may be progesterone metabolites or precursors. I am currently investigating whether manipulation of ACTH secretion affects the levels of extractable urinary inhibitory activity in man.

Finally, the observation of ACTH-dependent inhibition of 11ß-DH may be important in a clinical syndrome in which ACTH levels are grossly elevated and hypokalaemic alkalosis is a common feature: the ectopic ACTH syndrome. The possibility that 11ß-DH deficiency is important in this and other hypertensive syndromes is addressed in Chapter 5.

CHAPTER 5 11ß-HYDROXYSTEROID DEHYDROGENASE DEFICIENCY IN HYPERTENSIVE SYNDROMES

In the preceding chapters I have described the elucidation and implications of the mechanism of 11ß-OHSD-mediated protection of corticosteroid receptors. I have illustrated how hypertension may be associated with 11ß-DH deficiency, not only because of increased cortisol-dependent mineralocorticoid receptor activation in distal renal tubules, but also because of increased sensitivity to cortisol (and thereby to noradrenaline) in blood vessels. Furthermore, I have demonstrated that 11ß-DH may be impaired, not only in the congenital syndrome of apparent mineralocorticoid excess and after administration of the 11ß-DH inhibitors liquorice and carbenoxolone, but also as a result of endogenous ACTH-dependent inhibition. In the present chapter I address the clinical implications of these observations by measuring 11ß-DH activity in hypertensive patients.

The most obvious syndromes in which 11ß-DH deficiency may contribute to pathophysiology are those in which cortisol is suspected to mediate mineralocorticoid excess. This applies in the rare syndrome of apparent mineralocorticoid excess type 2 (see Chapter 1) and also in a much more common syndrome, that of ectopic secretion of ACTH. However, given the diversity of mechanisms described in this thesis by which 11ß-DH activity may influence blood pressure, it is also possible that enzyme deficiency is important in syndromes in which mineralocorticoid excess is not a feature, but in which cortisol is suspected to mediate the hypertension. This applies in a subgroup of patients with essential hypertension.

ECTOPIC ACTH SYNDROME

Ectopic ACTH secretion can be distinguished from other causes of Cushing's syndrome by a high incidence of hypokalaemic alkalosis (> 90 % compared with 10 % in Cushing's syndrome of other aetiologies)[Howlett et al., 1986]. This probably reflects increased secretion of an endogenous steroid with high affinity for mineralocorticoid receptors but its identity remains uncertain. It is not aldosterone which is invariably suppressed [Christy & Laragh, 1961]. It is widely supposed that either corticosterone [Cost, 1963b] or 11-deoxycorticosterone (DOC) [Crane & Harris, 1966; Schambelan et al., 1971] are responsible. However, some hypokalaemic patients have normal corticosterone and DOC levels [Christy & Laragh,

1961] while corticosterone and DOC are both raised in normokalaemic patients with pituitary-dependent Cushing's syndrome [Ritchie et al., 1990]. In fact, it is the secretion rate of cortisol which correlates best with the degree of mineralocorticoid excess [Christy & Laragh, 1961]. For cortisol to account for the mineralocorticoid excess would require breaching of the protection which 11ß-DH normally confers on mineralocorticoid receptors. The conclusion in Chapter 4 that ACTH indirectly inhibits 11ß-DH suggests that this could occur. In patients who have the highest ACTH secretion rates, ie those with ectopic rather than pituitary ACTH excess, this effect might be of sufficient magnitude to provoke cortisol-dependent mineralocorticoid excess.

In the following studies 11ß-DH activity is estimated using the peripheral plasma cortisol/cortisone ratio in 26 patients with Cushing's syndrome of various aetiologies. Having confirmed a defect in cortisol inactivation in patients with ectopic ACTH syndrome, I then assessed the relative contributions of 11ß-DH deficiency, and plasma DOC and corticosterone concentrations in predicting the mineralocorticoid status of these patients.

Patients and Methods

Local Ethical Committee approval and written informed consent were obtained. Plasma cortisol and cortisone were assayed as in Chapter 4. Urine free cortisol was measured with the Amerlex kit (Amersham, UK). DOC and corticosterone were measured by Dr Robert Fraser, MRC Blood Pressure Unit, Glasgow Western Infirmary, using radioimmunoassays after extraction and partial purification by paper chromatography [Fraser et al., 1975]. ACTH was measured by two-site immunoradiometric assay [White et al., 1990] by Dr Anne White, Hope Hospital, Manchester.

Samples were drawn after 5 min sitting between 0900 h and 1000 h from: (i) 20 healthy subjects (18 males) on no medication aged 19-62 yr (mean 36 yr); (ii) 12 hypopituitary patients (8 males) on oral hydrocortisone replacement for at least 12 months (from 7.5 to 30 mg daily in divided doses) aged 27-61 yr (mean 47 yr); (iii) 13 patients (9 males) on hydrocortisone replacement for primary adrenal insufficiency aged 18-80 yr (mean 52 yr); and (iv) 26 patients with Cushing's syndrome described in Table 5.1. Fifteen of the 26 patients with Cushing's syndrome were recruited at the Queen Elizabeth Hospital, Birmingham by Dr Paul Stewart.

All patients diagnosed as having pituitary-dependent Cushing's disease had > 50 % suppression of urinary free cortisol after high dose oral dexamethasone (2 mg 6 hrly for 48 h) and, in the subjects tested (n=11), a > 100 % increase in ACTH following 100 μg iv corticotrophin releasing hormone. All but two of the patients with ectopic ACTH secretion had no suppression of urinary free cortisol with high dose dexamethasone (two patients had no dynamic test performed but had very high plasma cortisol and hypokalaemia). Histological confirmation was obtained in 14/15 patients with pituitary adenoma and in all those with ectopic ACTH secretion. Patients with adrenal adenomas both had undetectable ACTH and radiological evidence of a unilateral adrenal mass. Histological confirmation was obtained in one of these patients.

Statistics

Results are expressed as mean ± SE. Multiple groups of subjects were compared by single-factor ANOVA followed by Fisher's PLSD test. Relationships between continuous variables were assessed by linear regression, and differences between these relationships assessed by multiple regression (where necessary assigning non-continuous variables values of 0 and 1).

Results

Cortisol/Cortisone Equilibrium

Results are shown in Table 5.1 and in Figure 5.1 for the levels of plasma cortisol, cortisone, and cortisol/cortisone ratios. 0900-1000 h plasma cortisol in patients with pituitary-dependent and non-ACTH-dependent Cushing's syndrome were not significantly higher than in controls but plasma cortisol was higher in patients with ectopic ACTH syndrome ($p < 0.05$). Patients on hydrocortisone replacement for hypopituitarism also had high plasma cortisol ($p < 0.05$), reflecting either differences in the timing of sampling after their morning dose of oral hydrocortisone or inappropriately high doses prescribed for these patients. However, patients on hydrocortisone replacement for primary adrenal insufficiency had plasma cortisol levels within the normal range.

Plasma cortisone levels were more variable, and not significantly different between groups.

Table 5.1 Patients with Cushing's syndrome

Sex	Age (yr)	Histology	Plasma cortisol (nM)	Plasma cortisone (nM)	Plasma K^+ (mM)	Urine free cortisol (nmol/day)	Plasma ACTH (pM)
Pituitary-dependent Cushing's syndrome							
F	57	Pituitary adenoma	606	226	3.3	722	2
F	54	Pituitary adenoma	663	66	3.5	3106	2
M	47	Pituitary adenoma	713	81	3.5	1854	19
F	49	Pituitary adenoma	782	48	4.4	1050	13.6
F	21	Pituitary adenoma	979	55	4.1	490	11.6
F	31	Pituitary adenoma	601	40	4.2	1650	5.8
F	41	Pituitary adenoma	864	55	3.8	530	5.8
F	37	Pituitary adenoma	590	48	4.1	504	28.4
M	44	Pituitary adenoma	1357	48	4.3	2500	20.5
F	40	Pituitary adenoma	512	55	3.9	3800	3.2
F	24	Pituitary adenoma	1236	40	4.1	2369	10.0
F	41	Pituitary adenoma	611	133	4.2	770	3.8
F	33	see †	641	120	4.4	3600	4.5
F	56	Pituitary adenoma	632	133	4.4	720	9.9
M	50	Pituitary adenoma	271	146	no data	700	10

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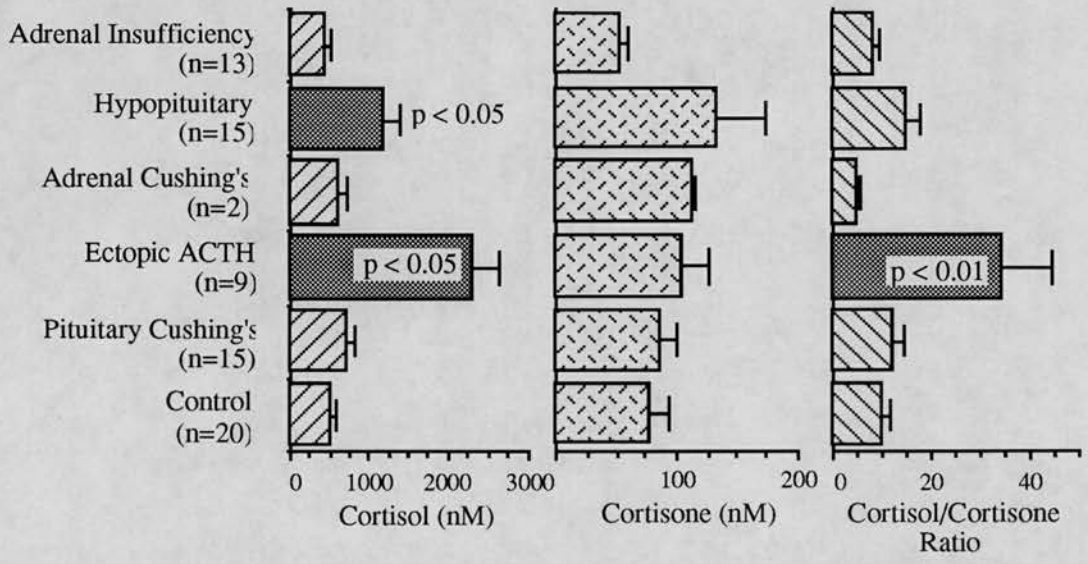
Sex	Age (yr)	Histology	Plasma cortisol (nM)	Plasma cortisone (nM)	Plasma K^+ (mM)	Urine free cortisol (nmol/day)	Plasma ACTH (pM)
Ectopic ACTH syndrome							
F	57	SCLC	2760	243	1.9	15090	57
M	71	SCLC	2084	60	3.1*	29053	64
F	62	Pancreatic oat cell carcinoma	837	81	2.0	6700	151
F	46	SCLC	2604	42	1.6	6250	22
F	41	Thyroid medullary carcinoma	3843	40	2.5	1200	57
F	55	SCLC	1479	60	1.9	no data	17
M	53	Thyroid medullary carcinoma	1471	194	2.7	6500	18
F	63	SCLC	3311	67	1.5	no data	no data
M	68	SCLC	2285	146	1.9	6300	45
Primary adrenal Cushing's syndrome							
F	28	Adrenal Adenoma	516	113	4.3	1668	undetect -able
F	60	(died; no post mortem)	737	116	3.5*	561	undetect -able

SCLC = small cell carcinoma of lung

* = on K^+-sparing diuretic

† This patient had normal pituitary radiology and no adenoma was seen at trans-sphenoidal surgery, but radical hypophysectomy resulted in cure.

Figure 5.1 0900-1000 h plasma cortisol and cortisone concentrations and cortisol/cortisone ratio in patients with disorders of ACTH secretion and controls.



Bars are SE.

The most striking differences emerged from examination of the cortisol/cortisone ratios (Figure 5.1). These were much higher in the ectopic ACTH group than in any other, including hypopituitary patients who had similar, albeit short-term, elevations in plasma cortisol. Furthermore, cortisol/cortisone ratios were lowest in the two patients with non-ACTH-dependent Cushing's syndrome.

11ß-OHSD, Corticosterone and DOC as Determinants of Hypokalaemia

The levels of plasma cortisol/cortisone ratio, corticosterone and DOC in patients with Cushing's syndrome are shown in Figure 5.2. There is overlap between the groups in all parameters. In general those patients with high cortisol/cortisone ratios also have high corticosterone and DOC levels. However, some patients in the ectopic ACTH group with normal cortisol/cortisone ratios have very high plasma corticosterone. No patients were identified with low DOC and corticosterone levels and a high cortisol/cortisone ratio.

Regression analyses were performed to correlate plasma potassium concentrations (in patients who were not on diuretics) with: plasma cortisol, cortisone, cortisol/cortisone ratio, DOC, corticosterone, ACTH and urinary free cortisol. No significant correlations were found when the patients with ectopic ACTH syndrome or with pituitary-dependent Cushing's syndrome were considered alone. Results when all

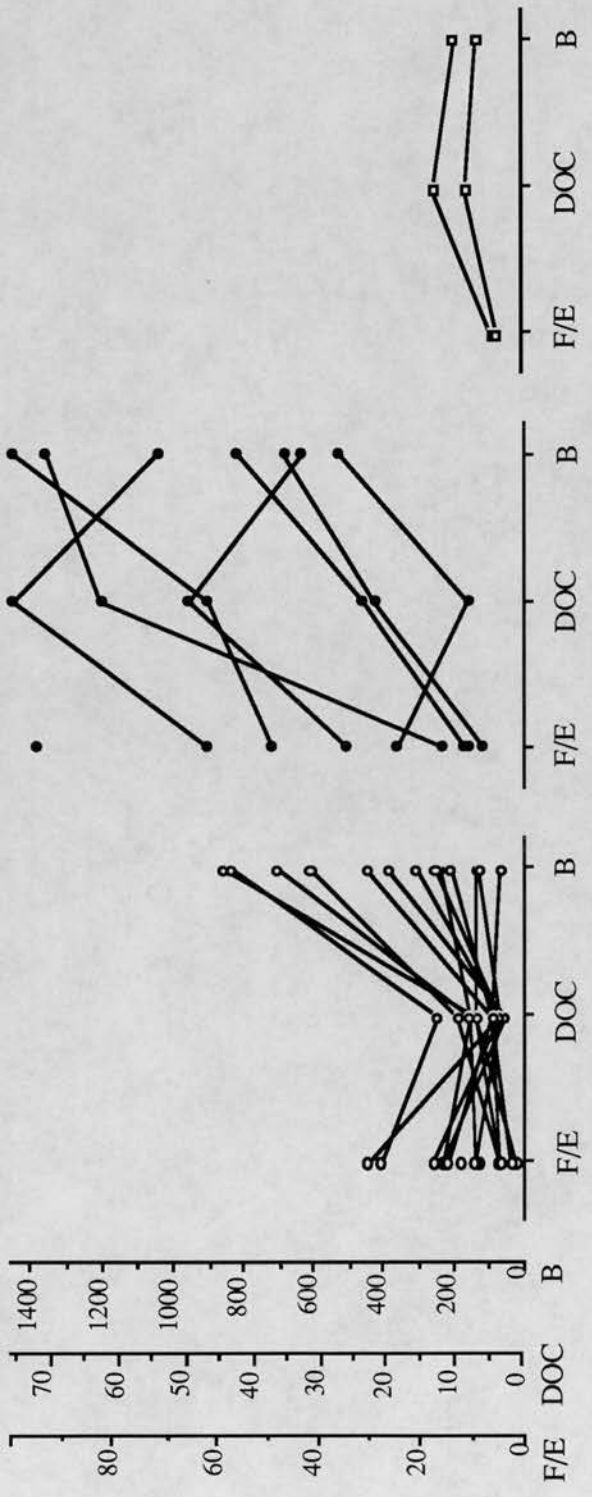
patients were included are shown in Table 5.2. The severity of Cushing's syndrome (assessed by ACTH concentration; urinary free cortisol; and plasma cortisol) was negatively correlated with plasma potassium, reflecting the fact that cortisol secretion was higher in the group with ectopic ACTH syndrome (Table 5.1). Furthermore, plasma corticosterone, DOC and cortisol/cortisone ratios all correlated negatively with plasma potassium. However, in a multiple regression analysis none of these correlations was independent, therefore the level of no single steroid predicted the degree of hypokalaemia.

Table 5.2 Correlations between ACTH and steroid concentrations and the degree of hypokalaemia

	Linear Regression†		Multiple Regression
	R value	p value	p value
ACTH	-0.63	0.002	0.08
Urinary Free Cortisol	-0.70	0.0004	0.96
Plasma Cortisol	-0.75	0.0001	0.06
Plasma Cortisone		0.4	0.89
Cortisol/Cortisone Ratio	-0.45	0.03	0.19
Plasma Corticosterone	-0.74	0.0003	0.81
Plasma DOC	-0.76	0.0002	0.17

† Patients on K^+-sparing diuretics were excluded from this analysis (1 adrenal adenoma, 1 ectopic ACTH and 1 pituitary adenoma). In addition within each row the following data were excluded: 2 ectopic ACTH and 2 pituitary adenomas for whom insufficient plasma samples were available for corticosterone and 11-deoxycorticosterone assays; 2 ectopic ACTH for whom urinary free cortisol was unavailable; and 1 ectopic ACTH for whom plasma ACTH was unavailable.

Figure 5.2 Plasma cortisol/cortisone ratio, corticosterone concentration, and 11-deoxycorticosterone concentration in patients with Cushing's syndrome.



F/E = cortisol/cortisone ratio; B = corticosterone concentration in ng/100ml; DOC = 11-deoxycorticosterone concentration in ng/100ml. The lines link the matching results for each patient. Two patients with ectopic ACTH syndrome and 2 with pituitary-dependent disease had insufficient sample to measure B and DOC.

Conclusions

These data illustrate that cortisol inactivation by 11ß-DH is less complete in patients with ectopic ACTH secretion than in those with other forms of Cushing's syndrome. These patients generally have cortisol concentrations outwith the range studied in the experiment presented in Figure 4.4, and their failure to inactivate cortisol by this route may reflect a combination of ACTH-dependent inhibition and saturation of renal 11ß-DH.

A number of observations in older literature lend weight to this interpretation. Chromatographic techniques have been used in attempts to differentiate the pattern of urinary metabolites in different forms of Cushing's syndrome. Although not often discussed in detail several of these studies measured the ratio of urinary metabolites of cortisol (THF and allo-THF) to those of cortisone (THE) (Figure 1.2). Two consistent abnormalities were observed in Cushing's syndrome: decreased 11ß-DH activity (increased ratio of [THF+allo-THF]/THE)[Cost, 1963a; Phillipou, 1982]; and decreased A-ring reduction by 5α-reductase (increased ratio of etiocholanolone/androstenedione [Jailer et al., 1959; Moolenaar & Van Seters, 1971; Phillipou, 1982] and of THF/allo-THF [Cost, 1963a; Phillipou, 1982]). The same pattern was found in patients with chronic stress [Gold et al., 1958; Ichikawa, 1966], and in volunteers and patients receiving exogenous ACTH [Jailer et al., 1959; Cost, 1963c]. The 5α-reductase defect correlated with ACTH secretion, since it was most obvious in ectopic ACTH, less so in pituitary-dependent disease and least in adrenal adenomas [Moolenaar & Van Seters, 1971]. It could also be reproduced by administration of the ACTH-dependent precursor, dehydroepiandrosterone [Kirschner & Lipsett, 1964]. In this respect the mechanism may be very similar to that which I propose in Chapter 4 for ACTH-dependent inhibition of 11ß-DH, ie inhibition of the enzyme by an ACTH-dependent steroid. However, the correlation between ACTH secretion, diagnosis, and 11ß-DH activity has not been examined previously. Most studies failed to separate patients according to their aetiology and, until recently, (THF + alloTHF)/THE ratios had only been reported in two patients with definite ectopic ACTH syndrome whose results were not separated from those of 4 patients with pituitary-dependent Cushing's disease [Phillipou, 1982]. Very recently, Ulick and colleagues [1992b] reported two more patients with ectopic ACTH syndrome who had deficient cortisol clearance both by 11ß-OHSD and by A-ring reduction (measured by urinary metabolite profiles). It is concluded that deficient cortisol inactivation is responsible for the hypokalaemia in these patients. Unfortunately, these authors do

not acknowledge that the abnormalities have been described before in Cushing's syndrome, and do not have any data for comparison from normokalaemic patients with other causes of the syndrome.

Conventionally, hypokalaemic alkalosis in ectopic ACTH syndrome has been attributed to increased secretion of DOC [Schambelan et al., 1971] or corticosterone [Cost, 1963b]. Both of these steroids mediate mineralocorticoid excess in other circumstances, including 11ß-hydroxylase deficiency, 17-hydroxylase deficiency [Schambelan et al., 1971], and adrenal tumours [Crane & Harris, 1966; Hogan et al., 1977; Ishikawa et al., 1988]. However, the absolute levels of DOC in these patients, although above the normal range, are orders of magnitude lower than the levels of cortisol and corticosterone (Figure 5.2). Thus the contribution of DOC to activation of mineralocorticoid receptors is probably insignificant. By contrast, corticosterone levels are substantially elevated in the patients with ectopic ACTH syndrome. Corticosterone is also a substrate for 11ß-DH [Monder & Shackleton, 1984], and its increased concentration may reflect inhibition of 11ß-DH in addition to increased adrenal corticosterone secretion.

11ß-DH deficiency offers an alternative mechanism for the hypokalaemic alkalosis of ectopic ACTH syndrome. In this study 11ß-DH is impaired in ectopic ACTH syndrome and not in other forms of Cushing's syndrome, and the degree of enzyme impairment correlates with the degree of hypokalaemia. However, there is also considerable overlap between groups for cortisol/cortisone ratios, suggesting some heterogeneity in the degree of 11ß-DH impairment. Furthermore, cortisol/cortisone ratio is not an independent predictor of mineralocorticoid excess (Table 5.2).

It seems most likely that a combination of factors is responsible for hypokalaemic alkalosis in these patients. Moreover, it is possible that the impaired 11ß-DH activity and increased DOC secretion have more than an additive effect in inducing kaliuresis. This possibility is raised by Morris's observations in rats that carbenoxolone not only increases renal mineralocorticoid receptor sensitivity to corticosterone and cortisol [Souness & Morris, 1989] but also increases sensitivity to DOC and aldosterone [Morris & Souness, 1990]. The mechanism of this interaction is unknown, but it raises the possibility that the loss of 11ß-DH activity in ectopic ACTH syndrome may potentiate the response not only to cortisol and corticosterone, but also to DOC.

ESSENTIAL HYPERTENSION

Not only is Cushing's syndrome associated with hypertension in approximately 75% of patients [Fraser et al., 1989], but circumstantial evidence suggests that cortisol is a significant mediator in some patients with essential hypertension. This comes principally from the observation that dexamethasone therapy is associated with a fall in blood pressure in some essential hypertensives [Hamilton et al., 1979; Whitworth et al., 1989a]. Furthermore, although circulating concentrations and secretion rates of cortisol are not elevated [Vermeulen & Van der Straeten, 1963], cortisol metabolism is abnormal resulting in increased excretion of polar derivatives of cortisol (non-A-ring-reduced 20-hydroxy and 6-hydroxysteroids)[Kornel et al., 1975]. These abnormalities might all be explained if 11ß-DH is deficient in these patients.

Methods

Patients and Subjects

Twenty patients with no clinical or biochemical features of secondary hypertension were recruited from Dr Paul Padfield's Hypertension Clinic and compared with 19 normal controls. All were Caucasian. Exclusion criteria were: alcohol intake > 4 units/day; abnormal liver, renal or thyroid function on biochemical screening; body weight > 120 % of predicted; a history of depressive illness; previous corticosteroid therapy; and in controls only, a first degree relative with hypertension. Female subjects were studied on day 5 of the menstrual cycle if pre-menopausal. All but 4 patients were studied either before the institution of anti-hypertensive therapy or after therapy had been discontinued for at least 6 weeks. The patients studied during continuing therapy had demonstrable complications of hypertension, either left ventricular hypertrophy or a history of cerebrovascular disease, and were included to avoid bias towards mild disease. The matching criteria for hypertensives and controls are shown in Table 5.3. Local Ethical Committee approval and written informed consent were obtained.

Half Life of (11α^3H)-cortisol

(11α^3H)-cortisol is acted on by 11ß-dehydrogenase to produce ^3H-H_2O and unlabelled cortisone. Preparation and use of this isotope has been described previously [Hellman et al., 1971; Ulick et al., 1979; Stewart et al., 1988]. The subject was supine with an ante-cubital iv cannula *in situ* for 30 min before blood was

withdrawn for baseline investigations and blood pressure measured by the mean of 3 recordings from a Copal automatic sphygmomanometer. At 0900 h an iv bolus of 1.20-1.54 MBq of (11α^{3}H)-cortisol containing 0.7 mg of cortisol diluted in 15 ml 2 % ethanol/water was injected over 20 s. Sequential 10 ml blood samples were collected in Lithium Heparin at 15-30 min intervals for 120 min, centrifuged at 4 °C, and the plasma stored at -20 °C. Plasma and the ^{3}H-H_2O collected from it after sublimation were both counted in Picofluor-30 scintillant (Packard Canberra, UK) to an error of < 2 % and corrected for quench.

From these data 11ß-dehydrogenase activity can be estimated either: (i) as the rate of accumulation of ^{3}H-H_2O product (calculated by linear regression as the slope of [(dpm/ml for ^{3}H-H_2O)/(dpm/ml for plasma) x 100%] versus [time]); or (ii) as the rate of disappearance of (11α^{3}H)-cortisol substrate (calculated as a half life by linear regression from the elimination phase between 45 and 120 min of a plot of log[(dpm/ml for total radioactivity in plasma)-(dpm/ml for ^{3}H-H_2O)] versus [time]). In order to choose between these two methods we examined their mathematical precision. There was excellent correlation between ^{3}H-H_2O accumulation rate and half life of (11α^{3}H)-cortisol ($R = 0.57$; $p < 0.0001$). However, the mathematical fit of individual ^{3}H-H_2O accumulation rates was less precise ($R \geq 0.77$) than the first order elimination curves for the half life of (11α^{3}H)-cortisol ($R \geq 0.96$). For this reason the half life of (11α^{3}H)-cortisol was chosen as the summary statistic reflecting 11ß-dehydrogenase activity.

This method may be subject to artefact if (11α^{3}H)-cortisol is converted to other steroids which retain the ^{3}H label but are not metabolised by 11ß-dehydrogenase. This was addressed by extracting steroids with ethyl acetate [Whitworth et al., 1989b] from plasma samples taken at +80 or +90 min from 15 of the controls and 17 of the hypertensives. The steroids were separated by reverse phase HPLC using a C18 µBondapack column (Millipore-Waters) with mobile phase of methanol:water (1:1) at flow rate 1.3 ml/min. The elution of ^{3}H-steroids was monitored in 3 s fractions for 25 min by on-line radioactive detection using a Berthold Z1000 cell with scintillant flow rate of 3.9 ml/min. The integrated counts within peaks were analysed by Berthold HPLC software on a PC. The radioactivity eluting with cortisol averaged 98 % of the total extracted radioactivity and no other consistent peaks were observed in the chromatograms. Moreover, the extracted radioactivity eluting with cortisol averaged 84 % of the radioactivity in plasma attributed to (11α^{3}H)-cortisol in the calculation of half lives. The extraction efficiency of cortisol by ethyl acetate is 90 %, therefore 93

% of radioactivity in the non-aqueous plasma fraction was extracted by ethyl acetate and eluted with cortisol on HPLC. Thus, conversion of (11α^3H)-cortisol to steroids which retain the ^{3}H label averaged 2 % for ethyl acetate extractable steroids, and 7 % for non-extractable steroids. Furthermore, neither of these errors correlated with the half life of (11α^3H)-cortisol, therefore they were not considered to compromise the interpretation of our results.

Finally, the primary isotope effect of ^{3}H (which makes the affinity of (11α^3H)-cortisol for the 11ß-dehydrogenase active site lower than that of endogenous cortisol (Hellman *et al.* 1971)) dictates that apparent 11ß-dehydrogenase activity will be artefactually reduced when endogenous cortisol levels are very high. In the present study endogenous plasma cortisol concentrations were similar in the groups of subjects studied, and did not correlate with the half life of (11α^3H)-cortisol. Thus the primary isotope effect was not a determinant of the differences observed.

Other Assays

Radioimmunoassays were performed as described in Chapter 4 for cortisol and cortisone. Plasma renin activity (PRA)[Haber et al., 1969], aldosterone (Coat-a-Count kit; Diagnostic Products Corporation, USA), and electrolytes (ion-selective method on a Beckman CX3) were measured by Susan Walker in our Metabolic Unit. ACTH [Nicholson et al., 1984], dehydroepiandrosterone sulphate (DHEA-S) and androstenedione [Semple et al., 1988] were measured by Dr Christina Gray in the Department of Clinical Biochemistry, Glasgow Royal Infirmary.

Aliquots of 24 h urine collections were stored and transported at -20 ^{o}C before analysis of urinary steroid metabolites by gas chromatography/mass spectrometry (GC/MS), performed by Dr Cedric Shackleton in Oakland, California [Shackleton et al., 1985].

Cortisone assay was not available for the first 20 subjects in the study, and for them full urinary metabolite profiles were also not available, though the ratios of the major cortisol metabolites were quantified.

Sequential Dexamethasone Suppression

Ten patients and 5 controls, as shown in Table 5.3, were given oral dexamethasone in increasing doses ranging from 100 µg to 3 mg at 2400 h on 5 occasions, separated by at least 48 h. The following morning they lay supine from 0830 h to 0900 h before

blood pressure recording and withdrawal of blood for measurement of plasma for cortisol, PRA, aldosterone, Na^+, and K^+. An aliquot of urine was collected for Na^+ and K^+ assay. Sodium intake was *ad libitum*. Subjects on anti-hypertensive therapy were included only in the analysis of cortisol and blood pressure.

Skin Vasoconstrictor Assay

Eleven patients and 11 controls had this test and are described in Table 5.3. The technique is as described in Chapter 3 with minor modifications. The steroids applied were: beclomethasone dipropionate (BDP)(Steraloids, UK) at 0.1, 0.3, 1, 3, 5, 10, 20, 40, 70, and 100 μg/ml; and hydrocortisone-21-acetate (Sigma, UK) at 0.1, 0.3, 1, 3, 5, and 10 mg/ml. Intensity of vasoconstriction for each square was assessed at 1, 2, 3, 4, and 5 h after removal of the occlusive dressing. The effect of each dose was expressed as the sum of the readings over 5 h (maximum = 15 units) and the response to each drug represented by the area under the dose-response curve (maximum = 150 units.10μg.ml^{-1} for BDP and 150 units.mg.ml^{-1} for hydrocortisone).

Statistics

Hypertensive and control groups were compared by two-tailed unpaired Student's *t* tests for quantitative data and contingency tables with chi-squared tests for qualitative data. For ACTH and DHEA-S assays, for which some results were below the lower limit of detection, comparison was by Mann-Whitney U. The distribution of half life of (11α^{3}H)-cortisol in each group was assessed using kernel density estimates by the method of Silverman [1981]. Analysis of factors influencing half life and of the effect of increasing doses of dexamethasone was by multiple regression analysis in which qualitative data were assigned values of 0 and 1. Results are described as mean ± SE throughout. Analyses were performed with assistance from Dr Bill Adams in the Medical Statistics Unit, University of Edinburgh.

Table 5.3 Matching criteria for subjects in the three limbs of the essential hypertension study

	Controls	Essential Hypertensives			Dexamethasone Suppression Subjects		Skin Vasoconstrictor Assay Subjects	
		All	long half life	normal half life	Control	Hypertensive	Control	Hypertensive
Number of subjects	19	20	7	13	5	10	11	11
Age (yr)	42.7 ± 3.0	47.6 ± 2.6	51.3 ± 3.8	45.5 ± 3.4	44.2 ± 6.1	49.4 ± 4.4	49.6 ± 3.6	49.6 ± 3.9
Sex	3F 16M	8F 12M	4F 3M	4F 9M	3F 2M	5F 5F	3F 8M	5F 6M
Family History (first degree relative)	None	11 Yes 9 No	3 Yes 4 No	8 Yes 5 No	None	4 Yes 6 No	None	4 Yes 7 No
Systolic BP (mmHg)	126 ± 3[a]	166 ± 6[b]	176 ± 9	160 ± 7	126 ± 7[e]	163 ± 10[f]	128 ± 5[i]	165 ± 9[j]
Diastolic BP (mmHg)	74 ± 2[c]	100 ± 3[d]	96 ± 4	103 ± 4	73 ± 5[g]	97 ± 5[h]	77 ± 3[k]	98 ± 5[l]
Creatinine Clearance (ml/min)	107 ± 15 (n=9)	91 ± 8 (n=11)	85 ± 11 (n=5)	96 ± 11 (n=6)				

Comparison between hypertensives and their normotensive controls and between hypertensives with half life of ($11\alpha^3$H)-cortisol ≥ 62.4 min ("long half lives") with those with half life < 62.4 min ("normal half lives"). Results expressed as mean ± SE. Significance levels: $p < 0.001$ for *a* versus *b* and *c* versus *d*; $p < 0.01$ for *i* versus *j* and *k* versus *l*; $p < 0.05$ for *e* versus *f* and *g* versus *h*..

Results

11ß-OHSD Activity

Half life of (11α³H)-cortisol

See Figure 5.3. Half lives for controls showed a normal distribution of 42.3 ± 2.3 min. In the hypertensives the distribution was bimodal ($p < 0.05$) with an overall mean of 53.2 ± 3.6 min. Seven individuals were identified with half lives > 2 SD above the control mean, ie ≥ 62.4 min. These 7 had mean half lives of 72.5 ± 2.9 min, while the rest of the hypertensives had a distribution very similar to controls of 42.9 ± 2.0 min. Other parameters described below have been illustrated separately for the hypertensives with half lives ≥ 62.4 min compared with hypertensives with half lives < 62.4 min, but statistical comparison is by multiple regression analysis. Clinical data for patients with long half lives are presented in Table 5.4. Apart from a relatively high prevalence of hypertension during pregnancy in this group, there were no other remarkable clinical features.

Multiple regression analysis with half life as the dependent variable and diagnosis, blood pressure, age, sex, and family history as controlling variables, confirmed that half lives were significantly longer in hypertensives ($p < 0.05$). The only other variable with a significant interaction was mean arterial blood pressure which correlated positively with half life ($p < 0.02$).

Figure 5.3 Half lives of (11α³H)-cortisol in essential hypertension

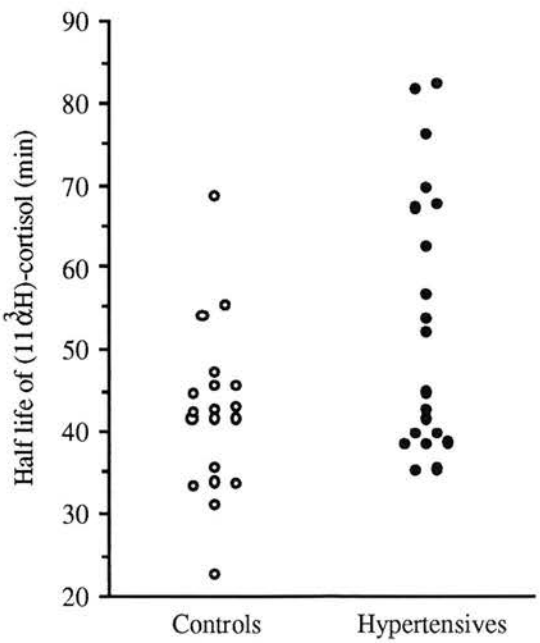


Table 5.4 Clinical details of hypertensives with prolonged half lives of (11α^3H)-cortisol

Half life (11α^3H) -cortisol (min)	Age (yr)	Sex	Family history of hypertension	Known duration of hypertension (mo)	Blood pressure at presentation* (mmHg)	Previous anti-hypertensive medication	Current anti-hypertensive medication	Body weight (kg)	Plasma creatinine (μmol/l)	Other information
62.4	57	F	No	3	182/108	Yes	Enalapril 20 mg daily	57	85	Hemiparesis Nulliparous
67.3	54	F	Yes	144	210/110	Yes	No	59	68	Hypertensive in pregnancy
67.9	59	M	No	24	198/112	Yes	No	76.5	100	
69.6	32	M	Yes	1	188/118	No	No	99	80	
76.2	54	M	No	24	210/110	Yes	No	78.5	102	
81.6	43	F	Yes	1	220/140	No	No	105	110	Obese Hypertensive in pregnancy
82.5	60	F	No	8	208/108	Yes	Captopril 50mg 8hrly Frusemide 40 mg daily	65	90	Hypertensive in pregnancy

* blood pressure at time of study rather than blood pressure at presentation was used for statistical analyses.

Table 5.5 Cortisol and its metabolites in essential hypertension

	Controls	Essential Hypertensives		
		All	Long half life	Normal half life
(i) Plasma at 0900 h				
Cortisol (nM)	418 ± 78 (n=19)	504 ± 52 (n=19)	610 ± 112 (n=7)	442 ± 47 (n=12)
Cortisone (nM)	73 ± 37 (n=9)	80 ± 46 (n=11)	136 ± 100 (n=5)	34 ± 3.9 (n=6)
ACTH (mU/l)	2.4 ± 0.02 (n=9)	3.0 ± 0.4 (n=10)	2.9 ± 0.4 (n=4)	3.0 ± 0.6 (n=6)
Androstenedione (nM)	5.1 ± 1.8 (n=9)	4.6 ± 0.4 (n=9)	5.0 ± 0.5 (n=4)	4.2 ± 0.5 (n=5)
DHEA-S (μM)	3.3 ± 0.6 (n=9)	2.9 ± 0.4 (n=9)	3.2 ± 0.5 (n=4)	2.7 ± 0.7 (n=5)
(ii) 24 h Urine				
Urinary Free Cortisol (nmol/24 h)	282 ± 25[a] (n=19)	179 ± 25[b] (n=19)	201 ±53 (n=7)	166 ± 26 (n=12)
Cortisol Production Rate (THF+allo-THF+THE+cortols+ cortolones)(μg/24 h)	8049 ± 1050 (n=9)	8593 ± 799 (n=10)	9218 ± 674 (n=5)	7968 ±1492 (n=5)
THF:allo-THF ratio	1.70 ± 0.24 (n=19)	1.68 ± 0.56 (n=18)	1.71 ± 0.23 (n=7)	1.62 ± 0.17 (n=11)
(THF + allo-THF):THE ratio	1.10 ± 0.04 (n=19)	1.17 ± 0.08 (n=18)	1.26 ± 0.14 (n=7)	1.11 ± 0.09 (n=11)
Ring A reduction constant (THF + allo-THF):F	26.9 ± 3.1 (n=9)	33.8 ± 3.5 (n=10)	39.4 ± 5.3 (n=5)	28.2 ± 3.3 (n=5)

Comparison between hypertensives and their normotensive controls and between hypertensives with half life of ($11\alpha^3$H)-cortisol ≥ 62.4 min ("long half life") with those with half life < 62.4 min ("normal half life"). Plasma cortisone assay and full urinary metabolite profile for assessment of cortisol production rate were not available for early subjects in the study, nor did these subjects have ACTH or adrenal androgens measured. Results expressed as mean ± SE. Significance level: $p < 0.01$ for *a* versus *b*.

Results are shown in Table 5.5. Plasma cortisol and cortisone were not different between hypertensives and controls nor between hypertensives with prolonged half life and those with normal half life. Urinary free cortisol by radioimmunoassay was lower in hypertensives than controls ($p < 0.01$). There were no differences between groups in urinary metabolite profiles. Specifically, there was no increase in the ratio of metabolites of cortisol (THF + allo-THF) to those of cortisone (THE), no reduction in THF:allo-THF ratio, no fall in cortisol production rate estimated by summation of urinary metabolites [Zumoff et al., 1974], and no fall in ring A reduction constant [Ulick et al., 1992a] in the hypertensives. Normal adrenal cortisol production was also reflected in normal ACTH and adrenal androgen levels.

Table 5.6 Indices of mineralocorticoid receptor activation in essential hypertension

	Controls	Essential Hypertensives		
		All	Long half life	Normal half life
Plasma Na (mM)	140 ± 0.8 (n=9)	141 ± 0.8 (n=11)	141 ± 1.4 (n=5)	141 ± 0.9 (n=6)
Plasma K^+ (mM)	4.0 ± 0.1 (n=19)	3.9 ± 0.1 (n=16)	4.2 ± 0.2 (n=5)	3.7 ± 0.1 (n=11)
Plasma Renin Activity (ng/ml/h)	0.6 ± 0.1 (n=19)	0.9 ± 0.2 (n=16)	0.9 ± 0.3 (n=5)	0.9 ± 0.3 (n=11)
Plasma Aldosterone (pM)	415 ± 68 (n=19)	508 ± 74 (n=16)	447 ± 191 (n=5)	536 ± 72 (n=11)
Urine Na excretion (mmol/24 h)	180 ± 25 (n=9)	185 ± 17 (n=11)	177 ± 33 (n=5)	192 ± 19 (n=6)
Urine K excretion (mmol/24 h)	73 ± 8 (n=9)	70 ± 8 (n=11)	73 ± 17 (n=5)	68 ± 8 (n=6)

Comparison between hypertensives and their normotensive controls and between hypertensives with half life of (11α-^{3}H)-cortisol ≥ 62.4 min ("long half life") with those with half life < 62.4 min ("normal half life"). Plasma Na and urinary electrolytes were not assessed in earliest subjects in the study. Four hypertensives taking therapy were excluded from this analysis. Results expressed as mean ± SE. No differences between groups reached statistical significance.

Mineralocorticoid Receptor Activation

Under Basal Conditions

Results are shown in Table 5.6. None of the differences between groups were statistically significant.

Acute Response to Dexamethasone

Dexamethasone produced a dose-dependent fall in 0900 h plasma cortisol ($p < 0.0001$) which was independent of diagnosis (hypertensive versus control), age, half life, family history or sex (Figure 5.4). The resultant plasma cortisol after dexamethasone correlated with the urinary Na^+/K^+ ratio in the 0900 h aliquot of urine, such that a higher dose of dexamethasone and lower plasma cortisol were associated with lower urinary Na^+/K^+ ratio ($R = 0.59$; $p < 0.01$). This relationship was independent of diagnosis, but urine Na^+/K^+ ratio fell more markedly with increasing half life ($p < 0.01$). The fall in Na^+/K^+ ratio comprised both antinatriuresis and kaliuresis, neither of which were significant alone. There was no relationship between plasma cortisol after dexamethasone and resultant blood pressure or plasma Na^+, K^+, PRA, or aldosterone.

Glucocorticoid Receptor Sensitivity

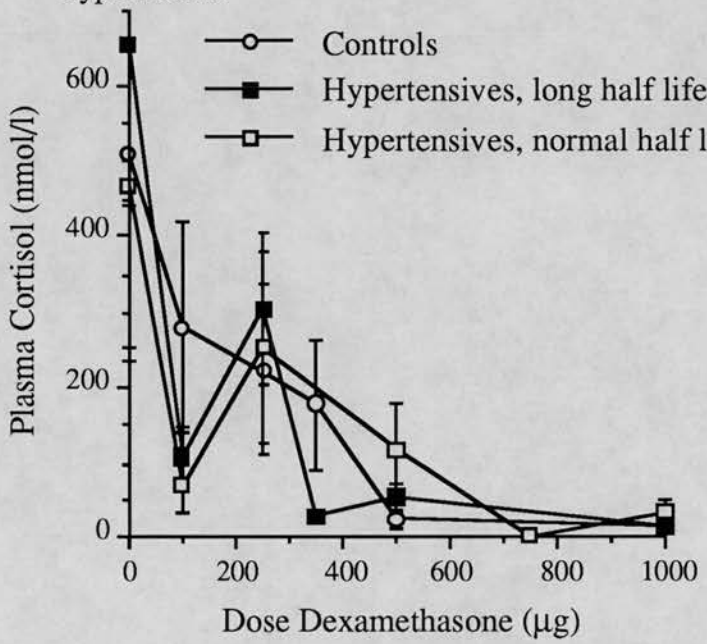
Skin Vasoconstrictor Assay

The response to both steroid preparations was greater in hypertensives than controls (Figure 5.5). Differences were: a) for hydrocortisone, 31.9 ± 4.8 units for hypertensives versus 16.6 ± 3.6 for controls ($p < 0.02$); and b) for BDP, 100.0 ± 6.6 versus 75.1 ± 9.5 ($p < 0.04$). By transforming this data to square roots of the values the difference in variance between readings of high and low intensity was eliminated. After this transformation the difference between hypertensives and controls was no greater for hydrocortisone than for BDP. Half life of ($11\alpha^3H$)-cortisol did not correlate with the degree of vasoconstriction to either drug.

Sequential Dexamethasone Suppression

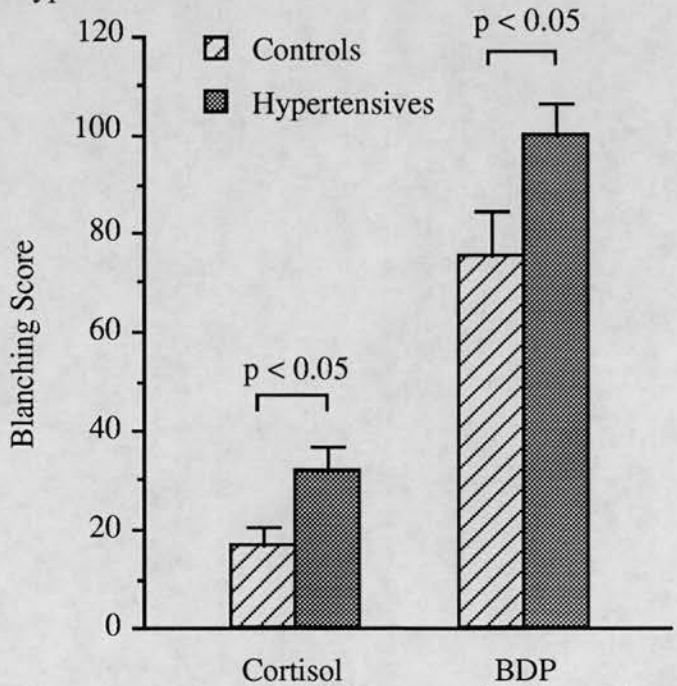
As described above, there were no differences between groups in the suppression of plasma cortisol by dexamethasone (Figure 5.4).

Figure 5.4 Suppression of plasma cortisol by dexamethasone in essential hypertension



Effect on 0900 h plasma cortisol of sequential doses of oral dexamethasone at 2400 h in normotensive controls (n = 5), and hypertensives divided by their half life of ($11\alpha^3$H)-cortisol into "long half life" (≥ 62.4 min, n = 4) and "normal half life" (< 62.4 min, n = 6). Plasma cortisol levels at doses from 1 to 3 mg were all suppressed below 30 nM and are not shown. Results are mean ± SE.

Figure 5.5 Skin vasoconstrictor response to glucocorticoids in essential hypertension



Intensity of dermal vasoconstriction in 11 hypertensives and 11 controls following topical application of beclomethasone dipropionate (BDP) and cortisol (hydrocortisone-21-acetate). Results are mean ± SE.

Conclusions

These data show that 11ß-DH activity, when measured by half life of (11α^3H)-cortisol, is reduced in a subgroup of patients with essential hypertension. This finding has been reproduced very recently in another study of essential hypertensives in whom the ratio of cortisol/cortisone metabolites was elevated in urine and plasma following administration of 4-^{14}C-cortisol (Kornel & Margolis, 1992). It had also been suggested in an older un-controlled study in which urinary and plasma cortisol metabolites were measured in hypertensive patients [Lewicka et al., 1991]. The magnitude of the abnormality (with a maximum of 82.5 min, mean for all hypertensives of 53.2 min, and mean for those hypertensives outwith 2 SD of normal of 72.5 min) compares with half lives of 84.3 ± 5 min after liquorice [Stewart et al., 1987], 123 ± 3 min after carbenoxolone [Stewart et al., 1990], and 131 min in our patient with congenital enzyme deficiency [Stewart et al., 1988](Table 1.3). As after carbenoxolone, and in type 2 apparent mineralocorticoid excess syndrome, plasma cortisone and ratios of urinary metabolites were normal. As discussed in Chapter 1 this suggests that both 11ß-DH and 11ß-OR activities are deficient.

The half life technique does not locate 11ß-OHSD deficiency in essential hypertension to a particular organ. Recent evidence for the existence of multiple tissue-specific isoforms of 11ß-OHSD (see Chapters 1 and 2) makes it possible that a tissue-specific defect in the enzyme accounts for the defect in essential hypertension. The proportion of injected (11α^3H)-cortisol metabolised by the kidney was estimated to be as little as 10 % in original studies employing renal vein sampling [Hellman et al., 1971], so there is scope for other organs to make a significant contribution. When 11ß-OHSD deficiency does affect the kidney urinary free cortisol is usually high, and plasma cortisone levels low (Table 1.3). In the present study urinary free cortisol was lower in hypertensives than controls, plasma cortisone was no different, and neither were different in the subgroup with prolonged half lives of (11α^3H)-cortisol. These data suggest that in essential hypertension the defect in 11ß-OHSD is extra-renal.

By contrast with all previously recognised syndromes of 11ß-DH deficiency, patients with essential hypertension and 11ß-OHSD deficiency did not have hypokalaemia or low-renin, low-aldosterone hypertension, and the administration of dexamethasone resulted in antinatriuresis rather than reversal of mineralocorticoid excess. Therefore their hypertension is unlikely to be due to excessive activation of renal mineralocorticoid receptors by cortisol. Whether increased renal mineralocorticoid

receptor activation occurs in any patients with essential hypertension is uncertain. Although 20-30 % of patients have low levels of plasma renin activity, it is by no means clear that this reflects expanded plasma volume or total body sodium [Fraser & Padfield, 1985]. The identification of patients who might have excess renal mineralocorticoid activity is made more difficult by the paradox that up to a quarter of patients with proven primary hyperaldosteronism have normokalaemia [Bravo et al., 1982]. Significantly, in neither of the studies of essential hypertensives in which dexamethasone was shown to decrease blood pressure was it suggested that these patients' hypertension was dependent on renal mineralocorticoid receptor activation, since those subjects who responded to dexamethasone in the first study did not have lower PRA than those who did not respond [Hamilton et al., 1979], and in the second study successful therapy was associated with a fall in plasma renin activity [Whitworth et al., 1989a]. Therefore, to explain both the efficacy of dexamethasone and the relevance of 11ß-OHSD deficiency in essential hypertension requires a hypertensinogenic mechanism independent of renal mineralocorticoid receptors.

As illustrated in Chapters 2 and 3, an alternative mechanism exists whereby 11ß-DH deficiency might contribute to hypertension: by increased sensitivity to cortisol in vascular smooth muscle. Others have assessed vasoconstrictor sensitivity to glucocorticoids in essential hypertensives indirectly, and demonstrated greater potentiation of the response to catecholamines by glucocorticoids in hypertensives [Mendlowitz et al., 1961; Solomon et al., 1965]. In our hypertensive patients we found increased direct vasoconstrictor sensitivity to both cortisol and beclomethasone dipropionate.

However, the mechanism of the increase in dermal vascular glucocorticoid sensitivity is unclear. From these data it cannot be attributed to 11ß-OHSD deficiency since it was not specific to steroids metabolised by 11ß-DH (BDP has a 9α-chloro group protecting it from the enzyme) and there was no correlation with half life of (11α^{3}H)-cortisol. On the other hand the statistical power of such a correlation is limited by the small number of subjects studied (n = 5) in whom abnormal 11ß-OHSD activity had been demonstrated. The design of this study included BDP as a positive control [Teelucksingh et al., 1990], anticipating that the response to this steroid would be the same in hypertensive and normotensive subjects. No non-steroid agent is available with a comparable time course which would serve as a control. Given that a difference was observed with BDP the result with cortisol must be interpreted with caution. It may be that glucocorticoids should be added to the long list of vasoactive

agents which induce a non-specifically greater response in vessels exposed to high blood pressure [Doyle et al., 1959; Panza et al., 1990]. However, in almost all experimental circumstances corticosteroids are not directly vasoactive and their effect depends on modulation of responses to other agents. A non-specific influence of hypertension on vascular responsiveness might increase basal sensitivity to direct vasoactive agents, but seems less likely to increase the glucocorticoid-dependent increment of response. Thus the difference between hypertensives and controls is unlikely to be a consequence of the hypertension alone, and may indeed contribute to the abnormal sensitivity to other vasoactive agents.

Corticosteroid-induced dermal vasoconstriction is mediated by glucocorticoid receptors [Marks et al., 1982; Gaillard et al., 1985]. As an alternative to abnormal cortisol metabolism, the increased sensitivity in the hypertensives may be explained if they express glucocorticoid receptors with higher intrinsic affinity for cortisol. This possibility is supported by a recent observation in the Edinburgh Ladywell family blood pressure study that a larger allele of the glucocorticoid receptor gene is more frequent in young people who are genetically predisposed to hypertension [Watt et al., 1992]. However, when functionally abnormal glucocorticoid receptors have been demonstrated in association with hypertension, they have been relatively resistant (rather than relatively sensitive) to activation by cortisol [Brandon et al., 1989], and the hypertension is probably due to increased secretion of DOC. Moreover, in the rare syndrome of congenital generalised glucocorticoid receptor hypersensitivity blood pressure is normal [Iida et al., 1990]. This probably reflects increased hypothalamic-pituitary glucocorticoid sensitivity and compensatory reduction in cortisol production rate. Thus, to induce cortisol-dependent hypertension glucocorticoid receptors of increased sensitivity would have to be distributed in peripheral tissues and not in hypothalamus and pituitary. Furthermore, if abnormal glucocorticoid receptor sensitivity explains the effect of dexamethasone in lowering blood pressure in some essential hypertensives [Hamilton et al., 1979; Whitworth et al., 1989a], then the receptor abnormality must increase the response to cortisol but not to dexamethasone, otherwise dexamethasone would also act peripherally to induce hypertension.

Glucocorticoid sensitivity to dexamethasone in the hypothalamus-pituitary was measured in the current study by suppression of plasma cortisol. The normal results suggest that abnormal glucocorticoid sensitivity in the skin is tissue-specific and/or steroid-specific, ie that it either does not include glucocorticoid receptors in the hypothalamus-pituitary and/or does not include the response to dexamethasone. If

both of these inferences are true then the present results provide an explanation for both cortisol-dependent hypertension (mediated by increased peripheral vascular sensitivity in the face of normal cortisol production rate) and suppression of the hypertension by dexamethasone (to which the peripheral glucocorticoid receptors are not hypersensitive). Site-specific 11ß-DH deficiency remains a most plausible explanation for this pattern, but a large number of subjects may be required to confirm it.

Finally, in the studies reported above I have not addressed the mechanism of 11ß-OHSD deficiency in essential hypertension. ACTH-dependent steroids are not measurably secreted in excess in this condition [Vermeulen & Van der Straeten, 1963], a conclusion underlined once more by the normal urinary steroid metabolite profile in this study. Thus the mechanism for inhibition of 11ß-DH which I propose in Chapter 4 and in ectopic ACTH syndrome does not seem relevant. However, in collaboration with Dr David Morris (in Providence, Rhode Island) we have examined the possibility that the urine of these subjects contains an increased amount of "glycyrrhetinic acid-like factors" (GALFs), ie endogenous inhibitors of the enzyme which he originally detected in urine of pregnant women [Morris et al., 1992]. We extracted the urines with Sep-Paks and methanol and added an aliquot of the extract to a preparation of rat liver and kidney microsomes incubated with ^{3}H-corticosterone. After incubation the conversion to ^{3}H-11-dehydrocorticosterone was measured and the extent of inhibition compared with a glycyrrhetinic acid standard curve. In the small number of subjects studied to date (n = 20) we have found significant amounts of GALF, and their excretion rates correlated positively with blood pressure and with the half life of (11α^{3}H)-cortisol, and negatively with plasma aldosterone concentration. However, the nature of these inhibitory compounds is unknown and their significance as an explanation for 11ß-OHSD deficiency in essential hypertension remains to be established.

SUMMARY

In this chapter I have demonstrated deficient 11ß-OHSD activity in two clinical syndromes of hypertension. The first is ectopic ACTH syndrome, in which 11ß-DH rather than 11ß-OR is deficient; the defect affects the kidney; and there is a correlation between the impaired inactivation of cortisol to cortisone and the degree of hypokalaemia. Thus, in this syndrome deficiency of 11ß-DH may allow cortisol to activate mineralocorticoid receptors in the kidney. The second is essential hypertension, in which both 11ß-DH and 11ß-OR are deficient; the defect probably lies outwith the kidney; and the extent of enzyme deficiency bears no relation to indices of mineralocorticoid receptor activation. In this condition vascular sensitivity to glucocorticoids is increased, and although this does not correlate with the extent of enzyme deficiency in the small numbers studied, it remains possible that these two observations are related. Thus circumstantial evidence suggests that dysfunction of 11ß-OHSD-mediated receptor protection contributes to clinical hypertension by mechanisms dictating either sodium balance or peripheral vascular resistance.

CHAPTER 6 CONCLUDING COMMENTS

The hypothesis of enzyme-mediated receptor protection for renal mineralocorticoid receptors was coined in a clinical context [Edwards et al., 1985; Stewart et al., 1987; 1988]. In the few years which have elapsed since it was confirmed in the rat [Edwards et al., 1988; Funder et al., 1988], there has been a remarkable explosion of research activity which has focussed principally on the basic science of 11ß-OHSD expression and distribution in rat kidney and elsewhere. The most significant advances have been the recognition that 11ß-OHSD may modulate access of cortisol to receptors in many extra-renal sites, and that different isoforms of the enzyme may be responsible for its diverse functions.

In parallel with these observations, I have addressed a principally clinical question: might defective enzyme-mediated receptor protection contribute to pathophysiology in common forms of hypertension? It rapidly became clear that the potential role of the enzyme in vascular smooth muscle, and similarly in the central nervous system, could not be ignored when considering the mechanisms of hypertension in 11ß-OHSD deficiency. The effect of corticosteroids on the central nervous system remains difficult to study in man. However, the hypothesis that 11ß-OHSD regulates cortisol sensitivity in the vascular tree lent itself to clinical investigation in health and disease.

The studies presented in Chapter 2 are most significant for their description of the anatomical and cellular localisation of 11ß-OHSD in vascular smooth muscle. The simultaneous identification of multiple isoforms of the enzyme in the kidney and liver [Monder & Lakshmi, 1990; Krozowski et al., 1990; 1992; Mercer & Krozowski, 1992; Moisan et al., 1992a] stimulated me to examine the kinetic characteristics of 11ß-DH in vascular tissues. The affinity for substrate does not discriminate vascular from renal 11ß-DH. However, on the grounds of utilisation of cofactors, the vascular enzyme is kinetically similar to the hepatic isoform and distinguishable from the renal enzyme. These data presaged those of Naray-Fejes-Toth and her colleagues [Rusvai et al., 1992] who showed that 11ß-OHSD purified in the microsomal fraction of isolated cortical collecting duct cells utilises NAD almost exclusively over NADP, and is therefore distinct from 11ß-OHSD elsewhere in the kidney. This may have important implications for the relative activity of 11ß-DH and 11ß-OR at each site, and therefore may be crucial in the local physiological role of the enzyme. In collaboration with Dr Jonathan Seckl and Susan Low we are currently performing Northern blots to identify the size of mRNA for 11ß-OHSD expressed in vascular smooth muscle. With

Dr Simon Walker and Moira Nicol at the Royal Infirmary, Edinburgh, we are performing Western blots with Carl Monder's antisera. Preliminary data from these experiments confirm that the vascular enzyme is similar to the liver in molecular size and in the size of mRNA from which it is probably translated.

What remains unclear is whether NAD utilisation is a marker for avid inactivation of cortisol/corticosterone. In other words, might the failure of 11ß-OHSD from vascular tissue to utilise NAD prevent it from "protecting" local corticosteroid receptors? At present we cannot reliably measure the equilibrium between 11ß-DH and 11ß-OR *in vivo*, and *in vitro* measurements are unlikely to reflect the balance *in vivo*. I am currently setting up a system for perfusion of rat mesenteric artery in which we hope to establish the relative activities and relate them to effects on vascular responsiveness. However, from data in Chapter 3, we already know that 11-dehydrocorticosterone was inactive in rat aortic strips, suggesting that avid 11ß-OR activity does not occur in that preparation.

The studies on regulation of 11ß-DH in rat presented in Chapter 2 principally serve to underline an observation made by others that in some tissues the enzyme is induced by glucocorticoids, and to demonstrate that the vasculature is one of these sites. In no site have consistent changes in activity resulted from manipulation of sodium intake. These data also suggest that glucocorticoid regulation may not be physiologically significant in the sites where it occurs, since it can only be demonstrated with pharmacological doses of dexamethasone. Finally, they show that the effect of dexamethasone is not mediated indirectly by suppression of ACTH.

The demonstration of 11ß-OHSD expression in vascular smooth muscle invited the next series of experiments, presented in Chapter 3, in which I showed that inhibition of 11ß-DH activity and congenital 11ß-DH deficiency are associated with increased vascular sensitivity to cortisol in man. This result highlights the significance for cardiovascular regulation of the observation that topical glycyrrhetinic acid increases the vasoconstrictor response to cortisol in skin [Teelucksingh et al., 1990].

Associated with the increased sensitivity to cortisol in man were increased vasoconstrictor and pressor responses to noradrenaline. It is presumed that this is secondary to increased cortisol exposure, which we know can produce these effects [Sudhir et al., 1989]. However, confirmation of this presumption would be difficult in man. For that reason I elected to pursue the same hypothesis in an isolated rat vessel where the interactions between glucocorticoids (in this case corticosterone) and

noradrenaline could be examined without confounding systemic influences. The results in rat aorta confirm that inhibition of 11ß-DH results in a corticosterone-dependent change in sensitivity, but paradoxically this is an attenuation rather than potentiation of the response to noradrenaline. This may reflect site-specific differences in the expression of corticosteroid receptors and of their target second messenger systems. I am currently addressing this possibility in studies of vascular smooth muscle cells in primary culture and in studies of another isolated rat vascular preparation, the perfused mesenteric artery. In these models I hope to be able to measure binding to both mineralocorticoid and glucocorticoid receptors in vascular smooth muscle cells and relate these to 11ß-OHSD function.

From a clinician's point of view, the justification of the intense research interest in 11ß-OHSD lies in the results presented in Chapter 5. Here I chose two clinical syndromes of hypertension: in the first, ectopic ACTH syndrome, there was good reason to suspect cortisol-dependent mineralocorticoid excess, but the mechanism whereby 11ß-OHSD might be involved was unclear; and in the second, essential hypertension, the pathophysiological mechanisms were not understood, but there was evidence to suggest that cortisol is a mediator in some patients, and there would be immense benefit from elucidating the cause of hypertension even in a small fraction of this enormous population.

The hypothesis that ectopic ACTH syndrome includes 11ß-DH deficiency led me to perform the initial experiments in healthy volunteers presented in Chapter 4. I showed that increased ACTH secretion is associated with decreased inactivation of cortisol to cortisone. The selective venous catheterisation studies confirm that the most likely site for this action is the kidney. Taken together with the failure of ACTH to influence 11ß-DH bioactivity in adrenalectomised rats (Chapter 2) I suggest that there are ACTH-dependent endogenous factors secreted from the adrenal which inhibit the enzyme. Thus a mechanism exists whereby 11ß-DH activity may be inadequate in ectopic ACTH syndrome. Its significance is confirmed in Chapter 5, in which patients with ectopic ACTH syndrome are shown to have increased plasma cortisol/cortisone ratios and corticosterone levels compared with patients with other forms of Cushing's syndrome. However, this abnormality is not universal in the hypokalaemic patients, and is not separable from the increased secretion of DOC and corticosterone, which might also contribute to mineralocorticoid excess.

To clarify the relative contribution of ACTH-dependent 11ß-DH inhibition versus increased secretion of corticosterone and DOC to mineralocorticoid receptor activation

in these syndromes will require a study in patients whose cortisol concentrations are not so dramatically elevated that they might saturate 11ß-DH in the kidney. This should be achieved in a study currently underway with the Respiratory Physicians in which we are correlating indices of 11ß-DH activity, DOC and corticosterone secretion and mineralocorticoid excess in patients with small cell carcinoma of lung (many of whom will have sub-clinical ectopic ACTH syndrome) and in patients with other bronchial tumours.

Finally, in Chapter 5 I have assessed the activity of 11ß-DH and related it to cortisol sensitivity in the kidney and in dermal blood vessels in patients with essential hypertension. The evidence for 11ß-DH deficiency in this syndrome comes from the half lives of (11α^3H)-cortisol, the most sensitive index of 11ß-DH activity in man. It seems that enzyme deficiency in this syndrome differs from that in all other conditions of 11ß-DH deficiency, including ectopic ACTH syndrome, because the defect not only affects both 11ß-DH and 11ß-OR activities but also appears not to affect the isoform of 11ß-OHSD expressed in the kidney. The increased vascular sensitivity to glucocorticoids which I demonstrate in the hypertensives is therefore all the more exciting, since it could represent an alternative link between 11ß-DH deficiency and hypertension. However, in studies to date I have not confirmed a relationship between abnormal enzyme activity and abnormal sensitivity to glucocorticoid, perhaps because of inadequate numbers of subjects with reduced 11ß-DH activity.

We are only just beginning to address the mechanism of 11ß-OHSD deficiency in patients with essential hypertension. Indeed, cloning of the human gene on chromosome 1 has yet to result in identification of a mutation in any patient with congenital 11ß-DH deficiency [Dr Perrin White, personal communication], so that this syndrome also remains in a sense unexplained. One experimental starting point in this area is the ability to measure glycyrretinic acid-like factors (GALFs) in human urine. Clearly a great deal of experimental time will need to be devoted to characterising the nature of these factors, but this task may provide clues to the pathogenesis of 11ß-DH inhibition both in essential hypertension and in ACTH excess. Having established David Morris's assay for GALFs in Edinburgh I am now examining their physiological regulation and clinical distribution in greater detail.

It will important to increase the numbers of hypertensive subjects identified with the phenotypic abnormality of 11ß-OHSD deficiency. Having shown a prolonged half life of (11α^3H)-cortisol in this group it is ethically difficult to proceed with a larger screening study which involves radioisotopic exposure. For that reason we have

arranged for the synthesis of a deuterated cortisol compound which we can measure by GC/MS. This approach has been employed with some success by Ulick's group [Linberg et al., 1991]. We will then seek to correlate half life of 9α,11α,12α,12ß-$^{2}H_4$-cortisol, excretion rate of GALFs, and vasoconstrictor sensitivity in two populations of untreated individuals to whom we have access: (i) middle-aged adults taking part in a screening programme with ambulatory blood pressure monitoring at Ferranti's in Edinburgh; and (ii) young subjects in the Ladywell Family blood pressure study who have defined genetic predisposition to hypertension [Watt et al., 1992]. Only when larger scale studies are complete will we be confident that the enzyme has a pathophysiological role in essential hypertension.

The elucidation of the mechanism of 11ß-OHSD-mediated receptor protection has laid down a challenge to biochemists and molecular biologists to detail its operation at a cellular level; to physiologists to understand its significance and regulation in each organ; and to clinicians to determine its potential importance in the pathogenesis of hypertension. In this thesis I describe work which contributes at each of these levels. In addressing the potential significance of 11ß-OHSD activity in the control of blood pressure, and of its deficiency in common hypertensive conditions, I have brought the story of 11ß-OHSD full circle: from serendipitous clinical observation to testable hypotheses on the laboratory bench, and finally to application in clinical populations. We can be confident that future manipulation of the "hypothalamic-pituitary-adrenal-11ß-OHSD" axis will reap considerable clinical reward.

ACKNOWLEDGEMENTS

This research was performed between August 1989 and June 1992 with support from the Medical Research Council of Great Britain, the British Heart Foundation, the Clinical and Biomedical Research Committee of the Scottish Home and Health Department, and the Sir Stanley and Lady Davidson fund.

I am grateful to all those mentioned in the text who performed laboratory assays. My special thanks go to Jill Campbell, who worked most closely with me and whose meticulous technical skills I greatly admire. I also thank Brent Williams and Jonathan Seckl for their collaboration and advice. Many others not mentioned in the text have made significant contributions: Dan Burt, Greta Proven and Barbara Beattie who kept the department in smooth working order; Gerry Kane and June Grieve who helped me with clinical studies; the staff in the Metabolic Unit, Medical Illustration Department, and Animal Unit who were always extremely helpful; and my close friends in the department who made the "low points" bearable.

I also thank the healthy volunteers and patients, especially GB, who willingly gave of their time, blood and urine.

I have been privileged to work in a department with a world-wide reputation in this field. It was Paul Stewart's original work which was the starting point for the story, and it has been a particular pleasure to collaborate with him now that he is in Birmingham. My final, and most sincere, thanks are reserved for Professor Christopher Edwards. Despite his increasingly heavy administrative load, he remains the driving force behind the work of a large multi-disciplinary team investigating many aspects of this enzyme. It was he who originally encouraged me to consider this period of research as long ago as 1986, and I am deeply grateful for his continuing encouragement and support.

PUBLICATION OF DATA FROM THIS THESIS

Original Papers

1 Walker BR, Yau JL, Brett LP, Seckl JR, Monder C, Williams BC & Edwards CRW (1991) 11ß-hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. Endocrinology, 129: 3305-3312.

The abstract of this paper is included in the 1992 edition of the American Heart Association's Council for High Blood Pressure annual review entitled "Advances in Hypertension" edited by Frohlich ED & Kotchen TA. JB Lippincott, New York.

2 Walker BR, Connacher AA, Webb DJ & Edwards CRW (1992) Glucocorticoids and blood pressure: a role for the cortisol/cortisone shuttle in the control of vascular tone in man. Clinical Science, 83: 171-178.

3 Walker BR, Campbell JC, Williams BC & Edwards CRW (1992) Tissue-specific distribution of the NAD^+-dependent isoform of 11ß-hydroxysteroid dehydrogenase. Endocrinology, 131: 970-972.

4 Walker BR, Campbell JC, Fraser R, Stewart PM & Edwards CRW (1992) Mineralocorticoid excess and inhibition of 11ß-hydroxysteroid dehydrogenase in patients with ectopic ACTH syndrome. Clinical Endocrinology, 37: 483-492.

This paper is accompanied by a Commentary by Dr John Funder in the same issue; pp 481-482.

Review Articles

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