THE ROLE AND REGULATION OF HEPATIC 11ß-HYDROXYSTEROID DEHYDROGENASE TYPE 1

Pauline Margaret Jamieson
BVM&S

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ABSTRACT

The adrenal steroids are known collectively as corticosteroids and consist of mineralocorticoids and glucocorticoids. They are synthesised in the adrenal cortex, and exert effects on metabolism and biosynthesis in virtually every organ and tissue in the body. The enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD) catalyses the interconversion of the active glucocorticoids (cortisol and corticosterone) with their inactive metabolites (cortisone and 11-dehydrocorticosterone), thus regulating glucocorticoid access to intracellular corticosteroid receptors. 11ß-HSD type 1 is a bi-directional NADP(H)-dependent enzyme *in vitro*, and is expressed in many tissues throughout the body. It is highly expressed in liver, where it is regulated by glucocorticoids, thyroid hormones, oestrogen and growth hormone *in vivo*. However, very little is known about the role of 11ß-HSD-1 in liver, or even its reaction direction, although it is well placed to modulate the action of hepatic glucocorticoid receptors. 11ß-HSD-1 activity is also present in the hippocampus where it may modulate the potentially deleterious effects of glucocorticoids on neuronal survival and function. The purpose of this thesis was to investigate the regulation and function of 11ß-HSD-1 in liver and in the hippocampus.

A culture system was established for rat primary hepatocytes which maintained high levels of 11ß-HSD-1 activity and mRNA expression. I used this system to examine the role and regulation of hepatic 11ß-HSD-1. 11ß-HSD-1 was found to be an exclusive 11ß-reductase (reactivation of inert metabolites), with alterations in pH and cellular NADP/NADPH ratios having little or no effect on reaction direction. Glucocorticoids induced 11ß-HSD-1 and insulin antagonised this effect in primary hepatocytes, indicating their regulation is directly mediated. Growth hormone, oestradiol and thyroid hormone had no effect on 11ß-HSD-1 expression or activity in this system, suggesting their control was either lost in culture or is indirectly mediated. Examination of 11ß-HSD-1 activity in a liver perfusion system by measurement of glucocorticoid metabolism across the intact liver confirmed that 11ß-reduction is the predominant reaction in this organ with approximately 40% conversion of 11-dehydrocorticosterone to corticosterone on a single pass through the liver. These data suggest that hepatic 11ß-HSD-1 activity in liver potentially increases intrahepatic active glucocorticoid levels. Many hepatic enzymes of carbohydrate and fat metabolism are regulated by glucocorticoids, including phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis, and in humans, 11ß-HSD inhibition increases hepatic insulin sensitivity. Therefore I examined the effect of selective and near complete repression

of hepatic 11ß-HSD-1 by oestradiol on glucocorticoid-inducible gene expression in liver. Oestradiol treatment resulted in reduced expression of glucocorticoid-inducible genes, including PEPCK. This effect could not be attributed to a direct action of oestradiol. Furthermore, inhibition of whole body 11ß-HSD by a chemical inhibititor, carbenoxolone, lowered fasting blood glucose. These data suggest that 11ß-HSD-1 plays an important role in potentiating glucocorticoid action in the liver and illustrate the potential to alter gluconeogenesis/insulin sensitivity by manipulating hepatic 11ß-HSD-1 activity.

Examination of the regulation of hippocampal 11ß-HSD-1 demonstrated that intracerebroventricular administration of growth hormone had no effect on 11ß-HSD-1 mRNA expression. This is in direct contrast with the periphery, where growth hormone is a major regulator. Examination of 11ß-HSD-1 activity in the hippocampus and liver in a well-documented model of chronic psychosocial stress in the tree-shrew showed that chronic stress attenuates hippocampal 11ß-HSD-1 activity, whereas excess glucocorticoid administration has no effect. In contrast, in the liver, both chronic stress and glucocorticoid administration decrease activity. In hippocampus, this attenuation of 11ß-HSD-1 appears to be an effect of stress, rather than glucocorticoid excess per se. Attenuated hippocampal 11ß-HSD-1 may reflect an homeostatic response to minimise the adverse effects of prolonged stress or glucocorticoid excess. These data demonstrate that the regulation of 11ß-HSD-1 in the hippocampus differs from that in the periphery, thus indicating that the function of the enzyme is tissue-specific and is as yet undetermined in the hippocampus.

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DECLARATION

I declare that this thesis was written entirely by me, and that all the work presented within it is the results of my own efforts, except for the procedures listed below which are also acknowledged in the text. Where assistance was given with surgical procedures, this is also acknowledged in the text.

This work has not been and is not concurrently submitted for any other degree.

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ABBREVIATIONS

11ß-HSD-1	-	11ß-hydroxysteroid dehydrogenase type 1
11ß-HSD-2	-	11ß-hydroxysteroid dehydrogenase type 2
3ß-HSD	-	3ß-hydroxysteroid dehydrogenase
%	-	percentage
<	-	less than
A	-	11-dehydrocorticosterone
ACTH	-	adrenal corticotrophic hormone
ADX	-	adrenalectomy
AME	-	apparent mineralocorticoid excess
ANOVA	-	analysis of variance
ATP	-	adenosine triphosphate
AVP	-	arginine vasopressin
B	-	corticosterone
bp	-	base pair
BSA	-	bovine serum albumin
cDNA	-	complementary deoxyribonucleic acid
CBG	-	cortisol/corticosteroid-binding globulin
CBX	-	carbenoxolone
CNS	-	central nervous system
cpm	-	counts per minute
CRH	-	corticotrophin releasing hormone
CTP	-	cytidine triphosphate
d	-	day
DEPC	-	diethyl pyrocarbonate
DEX	-	dexamethasone
dH_20	-	distilled water
DNA	-	deoxyribonucleic acid
E	-	cortisone
E_2	-	17ß-oestradiol
EDTA	-	ethylene diamine tetra acetic acid
F	-	cortisol
g	-	gram
GCMS	-	gas chromatography-mass spectrometry
GE	-	glycyrrhetinic acid
GH	-	growth hormone

GR	-	glucocorticoid receptor
HPA axis	-	hypothalamic-pituitary-adrenal axis
HPLC	-	high pressure liquid chromatography
ICV	-	intracerebroventricular
IVC	-	inferior vena cava
kDa	-	kilodaltons
kg	-	kilogram
K_i	-	inhibition constant
m	-	metre
M	-	molar
mmol/L: mM	-	millimolar
mg	-	milligram
min	-	minute
mH_20	-	millipore water
ml	-	millilitre
MR	-	mineralocorticoid receptor
mRNA	-	messenger ribonucleic acid
μg	-	microgram
NAD	-	nicotinamide adenine dinucleotide
NADP	-	nicotinamide adenine dinucleotide phosphate
NADPH	-	nicotinamide adenine dinucleotide phosphate - reduced form
NIDDM	-	non-insulin dependent diabetes mellitus
PEPCK	-	phosphoenolpyruvate carboxykinase
POMC	-	proopiomelanocortin
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
s	-	second
SAMS	-	*S*-adenosylmethionine synthetase
SDS	-	sodium dodecyl sulphate
SEM	-	standard error of the mean
SSC	-	saline-sodium citrate
T_3	-	triiodothyronine (thyroid hormone)
TAT	-	tyrosine aminotransferase
TLC	-	thin layer chromatography
TRIS	-	tris (hydroxymethyl) methyamine
UV	-	ultraviolet
w/v	-	weight per volume

CHAPTER 1

INTRODUCTION

1.1 Adrenocortical Steroids

The steroid hormones are all derivatives of cholesterol and are of two types: those with an intact steroid nucleus including the gonadal steroids (oestrogens, progestins and androgens) and the adrenal steroids, and those in which the B ring of the steroid is broken - these are vitamin D and its derivatives. The adrenal steroids are known collectively as corticosteroids and consist of mineralocorticoids and glucocorticoids. They are synthesised in the adrenal cortex, and exert effects on metabolism and gene expression in virtually every organ and tissue in the body. Corticosteroids exert their effects through two types of receptors - mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). Regulation of corticosteroid levels is achieved at the levels of steroid synthesis and metabolism. Corticosteroids are metabolised in many tissues by enzymes, including 11ß-hydroxysteroid dehydrogenase (11ß-HSD), which specifically regulates levels of active glucocorticoid within the body's tissues.

1.1.1 Adrenal Corticosteroid Biosynthesis

The adrenal cortex is subdivided into three concentric morphologically distinct zones (Arnold et al., 1866). The zona glomerulosa lies just under the capsule and constitutes about 15% of the cortex. It is responsible for the production of the major mineralocorticoid, aldosterone. The zona fasiculata lies inside the glomerulosa, constitutes about 75% of the cortex and together with the innermost cortical layer, the zona reticularis, is responsible for the production of the glucocorticoids cortisol and/or corticosterone, as well as significant amounts of adrenal androgens in some species.

The starting point for adrenocortical steroid synthesis is cholesterol, the basic steroid ring structure of which (Fig. 1.1) is conserved throughout the biosynthetic pathways operating in the adrenal cortex (Fig. 1.2).

The C19 steroids have a keto group attached to C17 and possess weak androgenic activity. Dehydroepiandrosterone and its sulphated form are qualitatively the major products of the human adrenal gland and may function as precursors which are converted to testosterone and oestrogens, largely in the periphery (Nimrod & Ryan, 1975).

Progesterone and 17α-hydroxyprogesterone have a two-carbon side-chain attached to C17, and undergo 21-hydroxylation (Kominami et al., 1980) followed by 11ß-hydroxylation to yield the C21 steroids corticosterone (B) and cortisol (F)

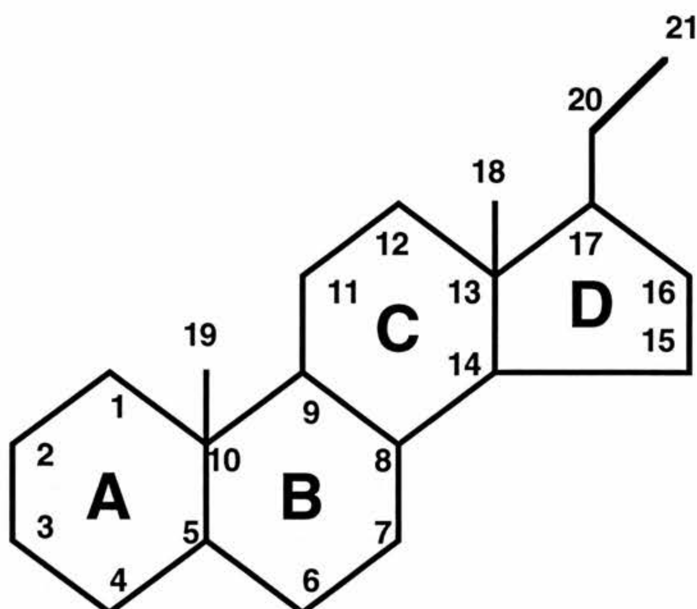


Figure 1.1: The basic steroid ring structure.
The numbering represents the conventional designation of carbon atoms.

respectively. F is the major glucocorticoid in many species including man, but in rodents B predominates. C21 steroids have both mineralocorticoid and glucocorticoid properties with small structural differences responsible for determining which property predominates (Fig. 1.2). Aldosterone - the major mineralocorticoid - possesses an 11,18-hemiacetal bridge, preventing its use as a substrate for 11ß-HSD type 2 (11ß-HSD-2) (section 1.3.1) (Edwards and Hayman, 1991). The synthesis of aldosterone from deoxycorticosterone is catalysed by P-450_{c11} (aldosterone synthase) (Chua et al., 1987), which is found only in the zona glomerulosa and is distinct from the P-450_{c11} involved in the synthesis of glucocorticoids in the zona fasiculata (Chua et al., 1987). The latter enzyme lacks the ability to catalyse the 18-methyl oxidation reaction required for the final step in aldosterone synthesis.

1.1.2 Control of Glucocorticoid Secretion

Glucocorticoid synthesis and release from the adrenal cortex is controlled almost entirely by adrenocorticotrophic hormone (ACTH) released from the anterior pituitary which is in turn regulated by corticotrophin releasing hormone (CRH), arginine vasopressin (AVP) and other agents released from the hypothalamus into the hypophyseal portal circulation in response to neural stimuli (reviewed by Jones

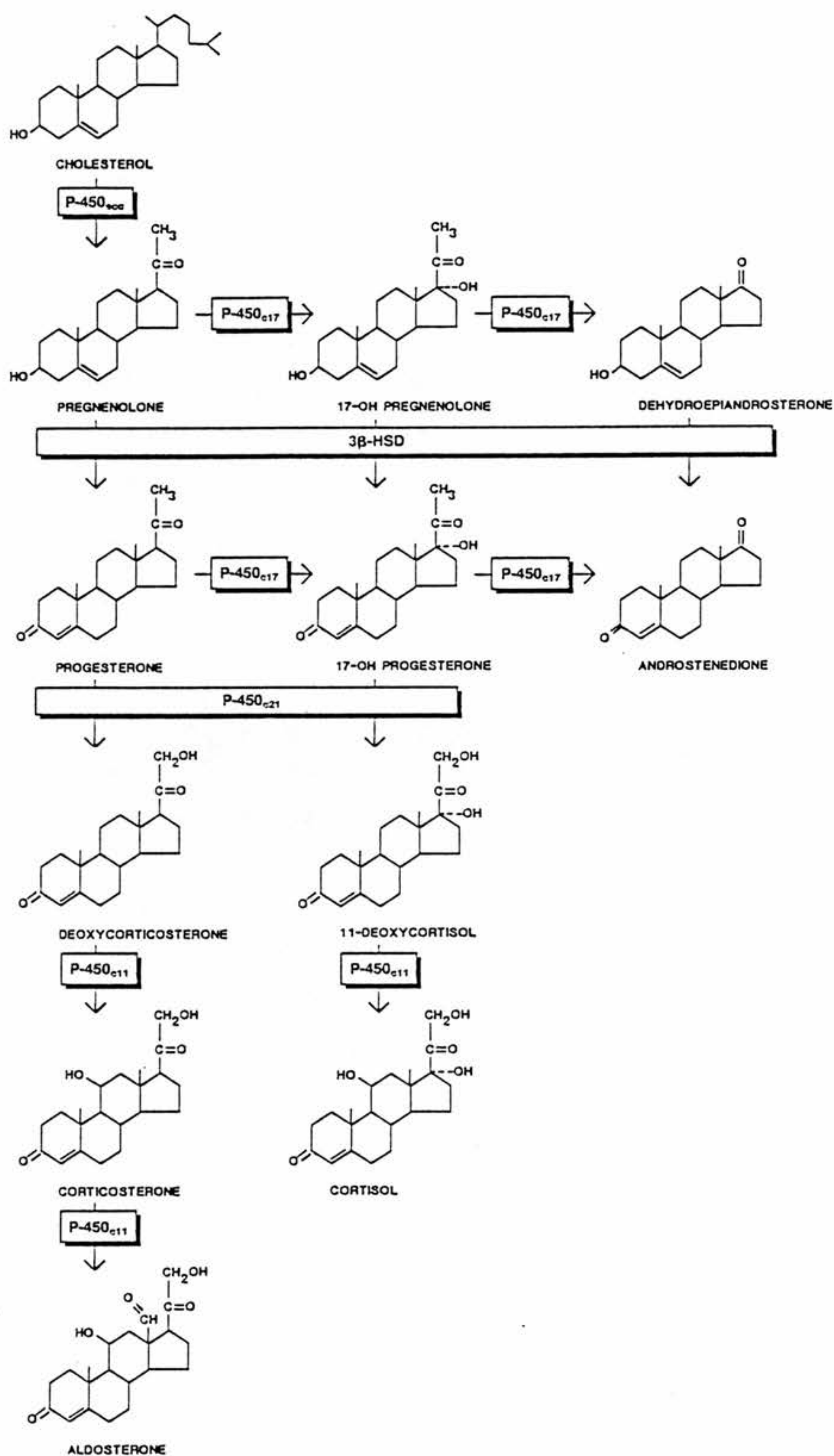


Figure 1.2: Corticosteroid biosynthesis in the adrenal cortex.

The pathways for synthesis of glucocorticoids, mineralocorticoids, adrenal androgens are shown. 3ß-HSD, 3ß-hydroxysteroid dehydrogenase. Taken from Orth et al., 1992.

& Gillham, 1988; Antoni 1993, 1986). The inhibitory effect of glucocorticoids on CRH, AVP and ACTH synthesis and secretion completes the negative feedback loop which regulates their plasma concentration (Fig. 1.3). The system is known as the hypothalamic-pituitary-adrenal axis (HPA axis) and is reviewed in Orth et al., 1992.

ACTH is a 39-amino-acid peptide produced in the anterior pituitary by cleavage and extensive post-translational processing of a large precursor polypeptide, pro-opiomelanocortin (POMC) (Eipper & Mains, 1980). CRH is the major physiological ACTH secretogogue (Vale et al., 1981). It is synthesised by neurones in the parvicellular division of the hypothalamic paraventricular nucleus (Bloom et al., 1982) whose axons project to the median eminence where CRH is secreted into the hypophyseal portal blood. On reaching the anterior pituitary, it binds to cell surface receptors on the corticotrophes (Millan et al., 1987), stimulating ACTH secretion within seconds and also increasing synthesis of POMC mRNA (Lundblad & Roberts, 1988). AVP alone is a weak stimulator of ACTH secretion, but it potentiates the action of CRH (DeBold et al., 1984).

In addition to the homeostatic negative feedback loop controlling glucocorticoid secretion described above, there are other factors known to affect glucocorticoid release. Glucocorticoid and ACTH secretion exhibit a circadian rhythm in humans and other species (Krieger et al., 1971; Veldhuis et al., 1990). This is modified by sleep and light patterns such that in humans, pulse amplitude of ACTH secretion reaches a peak in the few hours before wakening and declines throughout the day to reach a nadir in the evening. Plasma glucocorticoid levels rise and fall in response to the pattern of ACTH release, thus reaching maximal levels in the early morning. The mechanism of this circadian rhythm is unclear. There is probably an endogenous pacemaker in the suprachiasmatic nucleus of the hypothalamus (Szafarczyk et al., 1979), but environmental stimuli play an important role.

Stress stimulates ACTH secretion by increasing hypothalamic secretion of CRF and AVP. Both physical and psychological stresses such as exercise, trauma, surgery, cold exposure, fear and depression activate the HPA axis.

Finally, in addition to the negative feedback inhibition of glucocorticoids on ACTH secretion, they also inhibit POMC gene transcription and hence POMC synthesis (Lundbland & Roberts, 1988), and decrease CRF and AVP mRNA and peptide levels in the paraventricular nucleus of the hypothalamus (Itoi et al., 1987).

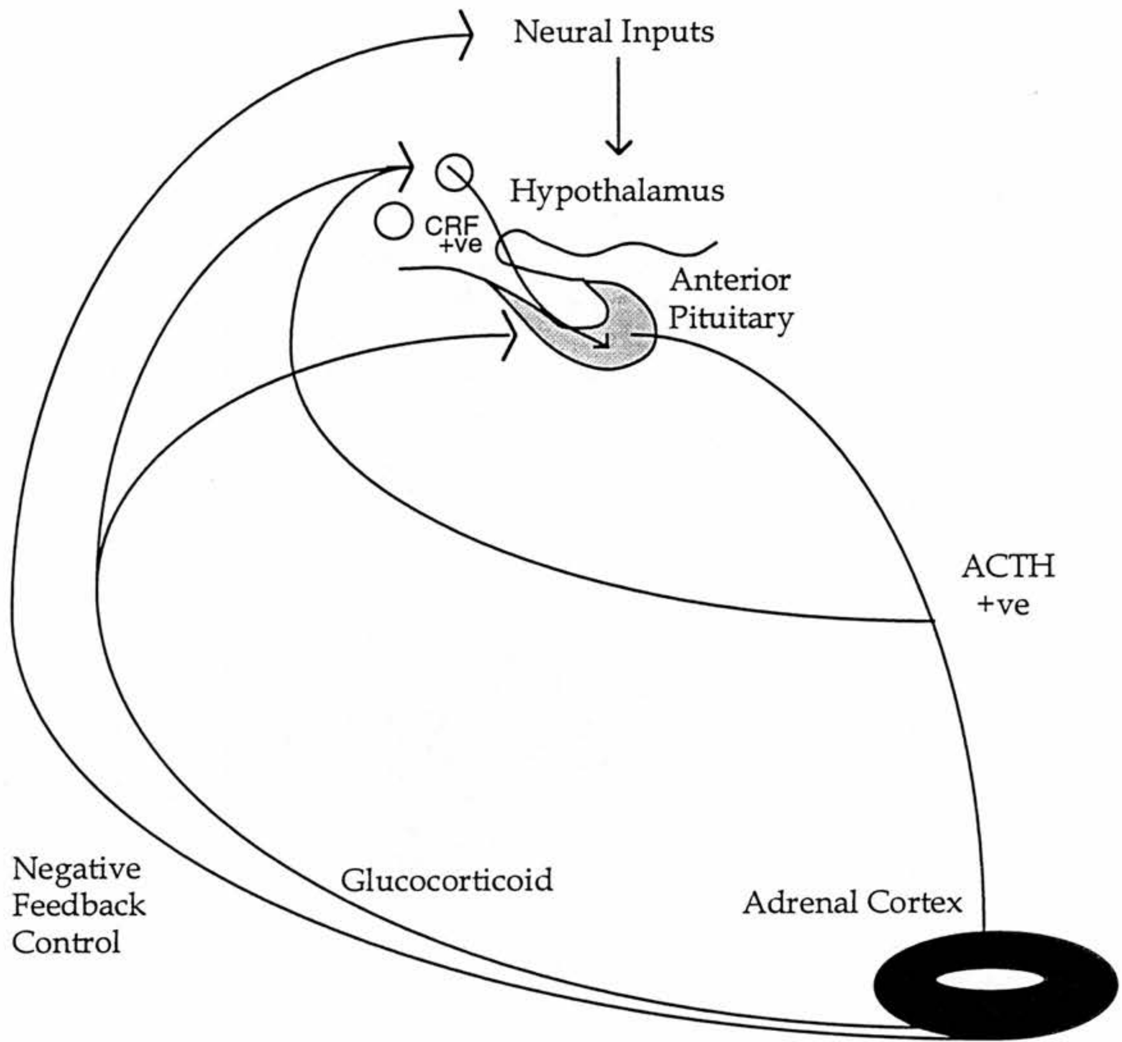


Figure 1.3: The hypothalamic-pituitary-adrenal axis: a schematic outline.
Refer to the text for details. +ve = stimulation.

1.1.3 Corticosteroid Release and Circulation

The primary action of ACTH on the adrenal cortex is to stimulate steroidogenesis (Hall, 1985), thus increasing glucocorticoid secretion and causing circulating glucocorticoids to rise within minutes following ACTH release (Simpson & Waterman, 1983, 1988). In addition, ACTH stimulates adrenal growth, thus maintaining adrenal weight under normal physiological circumstances but resulting in adrenal hypertrophy and hyperplasia if levels are supraphysiological as in pituitary-dependent hyperadrenocorticism, and adrenal atrophy in the absence of ACTH (Gill, 1972).

Corticosteroid hormones are released immediately following synthesis with only small amounts stored in the gland (Dickerman et al., 1984). Circulating steroid hormones are largely bound to plasma proteins. Plasma F and B are largely bound to corticosteroid-binding globulin (CBG) or transcortin (70-80%) with 3-4% circulating free, whilst the remainder is bound to albumin (Dunn et al., 1981; Partridge, 1981; Hammond, 1990). In contrast, only 20% of aldosterone is bound to CBG and 40% to albumin leaving the remaining 40% non-protein bound or free (Dunn et al., 1981; Partridge, 1981). However the total concentration of circulating aldosterone (350 pM) is tiny in comparison with that of glucocorticoid (approximately 500 nM) (Dunn et al., 1981). Steroids are also associated with red blood cells, and erythrocyte-associated cortisol actually exceeds that which is albumin bound or free (Hiramatsu & Nisula, 1987; Hammond et al., 1987). The circulating half-life of F is 50-90 minutes, whilst that of aldosterone is 15-25 minutes.

1.1.4 Glucocorticoid Metabolism

The processes of glucocorticoid metabolism can be divided into hepatic and extrahepatic. The hepatic metabolism of glucocorticoids involves reduction, oxidation, hydroxylation and conjugation (Orth et al., 1992) and is outlined in Fig. 1.4 for F. Reduction of the double bond is important for glucocorticoid inactivation, and it appears this is the most important pathway, and the rate-limiting step in the metabolism of active glucocorticoids (Peterson, 1981). 5ß-reduction predominates over 5α–reduction, although both activities occur resulting in 5α/ß-dihydrocortisol. The 3-keto group is then reduced by 3α/ß-hydroxysteroid dehydrogenases to yield tetrahydrocortisols. 3α-hydroxysteroid dehydrogenase predominates so that the major tetrahydrocortisol is 3α,5ß-tetrahydrocortisol. Extrahepatic metabolism of

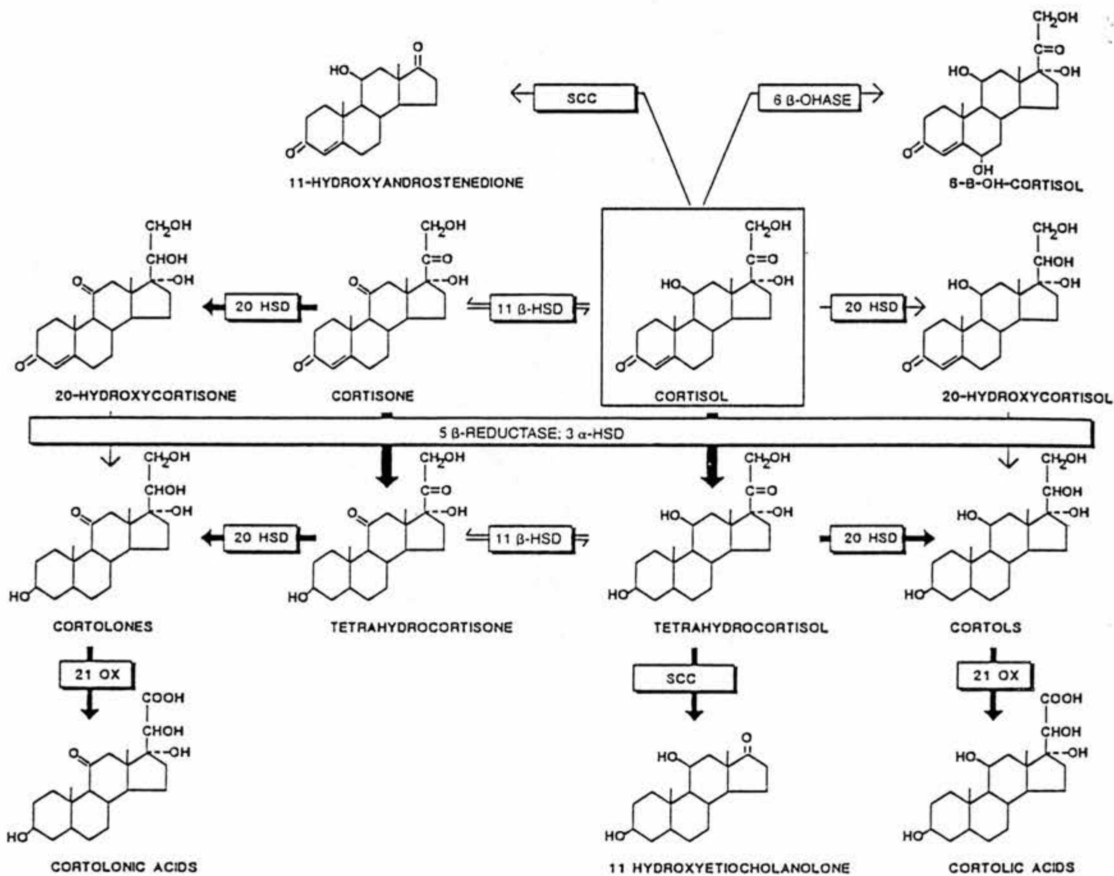


Figure 1.4: The major pathways of cortisol metabolism *in vivo.*
Width of the arrows indicates the relative importance of the pathways under normal conditions. SCC, side chain cleavage activity; 6ß-OHASE, 6ß-hydroxylase; 20 HSD, 20α-hydroxysteroid dehydrogenase; 11ß-HSD, 11ß-hydroxysteroid dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase; 21 OX, 21-oxidase. Taken from Orth et al., 1992.

glucocorticoids is mainly carried out by 11ß-HSD, which is the subject of this thesis (see section 1.3).

1.1.5 Glucocorticoid Action

Glucocorticoids have widespread and varied actions. Their more obvious physiological effects are demonstrated in the disease states of glucocorticoid deficiency and excess, but it should be remembered that they have permissive and modulatory action on the ability of tissues to respond to other hormones and so exert their effects on most, if not every, system in the body.

Glucocorticoid excess, such as occurs in long term exogenous administration or Cushing's disease, results in redistribution of body fat from the periphery to the abdomen, hypertension, impaired glucose tolerance, impaired immune responses and degenerative changes to muscle and connective tissue which are manifest as thin skin, bruising and muscle weakness. Conversely, in corticosteroid deficiency or Addison's disease, the weight loss, hypotension, hypoglycaemia and muscular weakness observed are due in part to lack of glucocorticoids. The effects of glucocorticoids upon fuel homeostasis are discussed in section 1.3. Their other actions are described briefly here.

Glucocorticoids have profound immunosuppressive effects (Graham & Tucker, 1984). They deplete the numbers of lymphocytes in the peripheral circulation by redistributing them from the intravascular compartment to the spleen, lymph nodes, thoracic duct and bone marrow (Facui & Dale, 1974; Yu et al., 1974). They inhibit the phagocytic and cytotoxic functions of macrophages (Rinehart et al., 1982) and their proliferation, as well as that of T-cells and B-cells (Ishii et al., 1983, Gillis et al., 1979; Cupps et al., 1985) and of monocytes. Glucocorticoids inhibit the accumulation of neutrophils at the site of inflammation (Dale et al., 1975) and also inhibit the release of mediators responsible for the inflammatory response. Prostaglandin and kinin synthesis are inhibited by glucocorticoids (Kantrowitz et al., 1975; Hong & Levine, 1976) thus inhibiting the local inflammatory response (Zweifach et al., 1953).

Glucocorticoids affect bone metabolism and connective tissue function. Chronic glucocorticoid excess causes osteopenia. Glucocorticoids inhibit osteoblast function (Frost & Villanueva, 1961; Jowsey & Riggs, 1970) which results in decreased formation of new bone, and increased osteoclast cell numbers (Hahn et al., 1979) which may stimulate bone resorbtion. They also decrease intestinal calcium absorption (Klein et al., 1977) and increase urinary calcium secretion by a decrease

in renal reabsorption (Laake, 1960). The profound myopathy that can result from glucocorticoid excess is due to the catabolic effect on muscle protein and glucocorticoids inhibit fibroblast functions including proliferation (Pratt & Aronow, 1966) and collagen and hyaluronidate synthesis (Sterling et al., 1983; Mapleson et al., 1981). This is manifest clinically as impaired wound healing and friable connective tissues (Leibovich & Ross, 1975).

Glucocorticoid excess is associated with hypertension, often without mineralocorticoid excess, kaliuresis or suppressed plasma renin activity (Krakoff et al., 1975; Saruta et al., 1986). Glucocorticoids have affinity for MR but evidence for this being the mechanism is insubstantial (Grunfeld, 1985). Thus the basis for the hypertension associated with glucocorticoid excess is poorly understood, but may reflect actions on resistance vessels, or on sodium pumps through mechanisms distinct from the Na/K ion exchange.

In glucocorticoid deficiency, there is a defect in free water clearance (Slessor, 1951) associated with increased plasma AVP concentrations (Raff, 1987) and, in rats, an increase in AVP mRNA levels in the paraventricular nucleus of the brain (Davis et al., 1986). There are GR in the AVP-producing cells in the paraventricular nucleus (Uht et al., 1988) and the increase in AVP may contribute to maintaining blood pressure in adrenal insufficiency (Schwartz et al., 1983). Removal of the adrenals with the capsule and zona glomerulosa left intact results in an inability to excrete a salt load (Gaunt et al., 1967 & 1968) and glucocorticoid replacement results in naturesis due in part to induction of secretion and synthesis of atrial naturetic factor from cardiac myocytes (Matsuraba et al., 1987).

Glucocorticoids have a direct effect on sodium transport in the colon (Sandle et al., 1987). There are MR in the colon which regulate ion transport but a glucocorticoid mediated effect has also been demonstrated (Bastl et al., 1987 & 1988). In addition Na^+/K^+-ATPase α_1 and ß-subunit gene expression in the rat descending colon is acutely regulated by the synthetic glucocorticoid dexamethasone (DEX) but not aldosterone (Fuller & Verity, 1990).

Glucocorticoids have multiple effects on the central nervous system (CNS) including modulation of sleep patterns, mood, cognition and reception of sensory input (McEwan, 1979). Over half of patient's with Cushing's syndrome have psychological disturbances, most commonly depression (Cohen, 1980) but euphoria, manic behaviour and even overt psychosis may occur (Christy, 1979). Adrenal insufficiency is linked to depression, apathy and lethargy. The mechanisms of these effects are unclear. Neurones in the CNS contain GR and MR but neuronal responses are often more rapid than is likely to be mediated via classical steroid receptor-gene

interactions. Glucocorticoids are directly neurotoxic (Sapolsky, 1985, 1986, 1992) causing cell death by increasing neuronal vulnerability to a wide range of other insults by inhibiting glucose uptake and potentiating intracellular Ca^{++} release (Virgin et al., 1991; Elliot & Sapolsky, 1993).

Lastly, glucocorticoids have profound effects on many aspects of development. They regulate organ development and stimulate the maturation of many cell types (Ballard, 1979). For example, glucocorticoids stimulate the production of surfactant from type II pneumocytes, a normal developmental process occurring in the foetus before birth (Cole et al., 1995; Mendelson & Boggaram, 1991). Clinically, exogenous glucocorticoids can be employed to accelerate this process when premature birth is imminent (Ballard, 1987). In the developing nervous system, glucocorticoids regulate the differentiation of neural crest epithelium cells into chromaffin cells (Federoff et al., 1988). Glucocorticoid exposure *in utero* retards foetal growth in rats (Benediktsson et al., 1993), is associated with retarded foetal growth in humans (Reinisch et al., 1978) and may increase or decrease placental weight depending on dose and/or timing of the exposure (Gunberg, 1957). Foetal growth retardation has been linked to the risk of developing high blood pressure or ischaemic heart disease in later life in humans (Barker et al., 1989a; 1989b; Barker et al., 1990) and in rats (Benediktsson et al., 1993). Neonatal manipulations can "programme" HPA axis responses, a permanent effect probably attributable to altered GR expression in the brain (LaRocque et al., 1992; Meaney et al., 1993) and brief prenatal glucocorticoid exposure has permanent effects on central noradrenergic activity (Slotkin et al., 1992).

1.1.6 Mineralocorticoid Secretion and Action

Aldosterone secretion is influenced primarily by the renin-angiotensin system and by plasma levels of potassium (Fig 1.5). ACTH and other POMC-derived peptides also have minor regulatory roles (Quinn & Williams, 1988).

Renin is secreted from the juxtaglomerular cells of the renal cortex in response to lowered renal arterial blood pressure, lowered sodium concentration in the tubular fluid and sympathetic nerve stimulation (Gibbons et al., 1984). Angiotensinogen is an α_2 globulin synthesised in the liver and is cleaved by the enzymic action of renin to yield angiotensinogen I (Goodfriend et al., 1984) which has no known biological activity but is converted rapidly to angiotensin II by angiotensin converting enzyme (ACE), mainly in the pulmonary capillary endothelium (Page, 1974; Ryan et al., 1975). Plasma renin levels are the rate limiting

factor in this process (Williams & Dluhy, 1983). Angiotensin II is a powerful vasoconstrictor and stimulates aldosterone synthesis and secretion (Goodfriend et al., 1984) in the adrenal zona glomerulosa. Potassium directly stimulates aldosterone synthesis in the adrenal (McKenna et al., 1978). Renin release is directly inhibited by angiotensin II, hyperkalaemia and atrial naturetic factor (Williams & Dluhy, 1983; Rebuffat et al., 1988), and indirectly by aldosterone mediated tubular sodium reabsorption and plasma volume expansion, thus completing the negative feedback loop.

Aldosterone is the principal physiological mineralocorticoid. Its most important action is in the distal tubules and cortical collecting ducts of the kidney where it promotes active sodium reabsorption in exchange for potassium and hydrogen ions, thus regulating extracellular volume and hence blood pressure. In the sweat glands and salivary glands, aldosterone promotes sodium chloride resorbtion in the primary secretions in exchange for potassium and bicarbonate. This is important physiologically to conserve salts in situations where sweating or salivary loss are excessive (Morris, 1981).

Aldosterone may also have effects on the brain and other non-epithelial tissues where MR are present (Moguilewsky & Raynaud, 1980; Armanini et al., 1985). Intracerebroventricular (ICV) infusion of low concentrations of aldosterone promotes salt appetite and increases blood pressure, an effect not mimicked or blocked by B but largely blocked by aldosterone antagonists (McEwen et al., 1986a; Gomez-Sanchez, 1986).

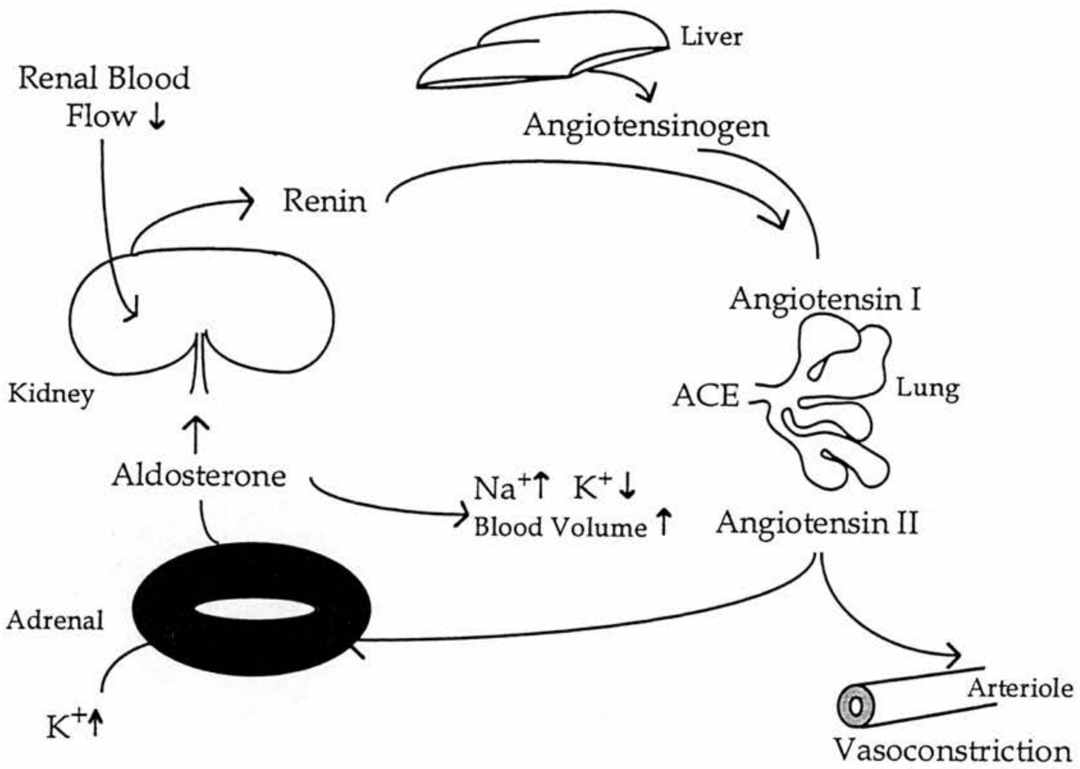


Figure 1.5: The renin-angiotensin-aldosterone system.

A schematic outline. See text for details. ACE, angiotensin-converting enzyme.

1.2 Steroid Receptors

1.2.1 The Steroid Receptor Superfamily

In the classical model, steroid hormones exert their effects by binding to intracellular steroid hormone receptors. These ligand-activated receptors function as nuclear transcription factors which modulate cell function by activating or repressing target genes or gene networks (reviewed by Beato et al., 1996; Ribeiro et al., 1995).

Many of the known steroid receptors have been cloned, and as a result we know a great deal about the structure of steroid receptors. Following cloning of the major rat and human steroid receptors (reviewed by Green & Chambon, 1988; Evans, 1988; Berg, 1989; Beato, 1989; O'Malley, 1990; King 1992), sequence comparisons revealed they belong to a highly homologous family (Fig. 1.6). The steroid receptor superfamily includes GR, MR, thyroid hormone ($TR_{\alpha 1}$, $TR_{\beta 1}$, $TR_{\beta 2}$, *c-erb*$A_{\alpha 2}$), vitamin D (VDR), retinoid (RAR_{α}, RAR_{β}, RAR_{γ}, RXR_{α}, RXR_{β}, RXR_{γ}), progesterone (PR), oestrogen (ER) and androgen (AR) receptors. Other superfamily members have been cloned, but do not have ligands, or the ligands are yet to be identified. These receptors are known as orphan receptors (reviewed by O'Malley, 1990; Mills & Duggan, 1994; Laudet & Adelmant, 1995; Mangelsdorf & Evans, 1995) and it has been suggested their ligands may be indigenous to the cells in which specific orphan receptors are found (O'Malley, 1990).

Steroid receptor proteins have molecular masses of around 65 to 100 kDa. Each receptor protein binds a single steroid molecule, but the receptors usually dimerise when bound to DNA (Perlmann et al., 1990; Kumar & Chambon, 1988; Baniahmad & Tsai, 1993; Yu et al., 1991). Comparative analyses of the cloned receptors reveals a conserved domain structure (Fig 1.6). The most highly conserved domain is the cysteine-rich DNA binding domain (O'Malley & Tsai, 1993; Ribeiro et al., 1995), located towards the centre of the receptor, and within this domain, the most distinctive feature is the zinc finger structure (Freedman, 1993). The DNA binding domain contains a number of highly conserved cysteine residues which co-ordinate two zinc ions tetrahedrally to form two zinc fingers (Freedman et al., 1988; Freedman, 1993; Freedman & Luisi, 1993; Glass, 1994), and the zinc atom is essential for DNA binding (Freedman et al., 1988). The zinc fingers confer specificity for the steroid response element, are required for and stabilise the receptor-DNA interaction (Danielson et al., 1989; Mader et al., 1989; Freedman & Luisi, 1993; Glass, 1994), and are involved in dimerisation of the zinc binding domain (Dahlman-Wright et al., 1991; Luisi et al., 1991). Structurally, each zinc

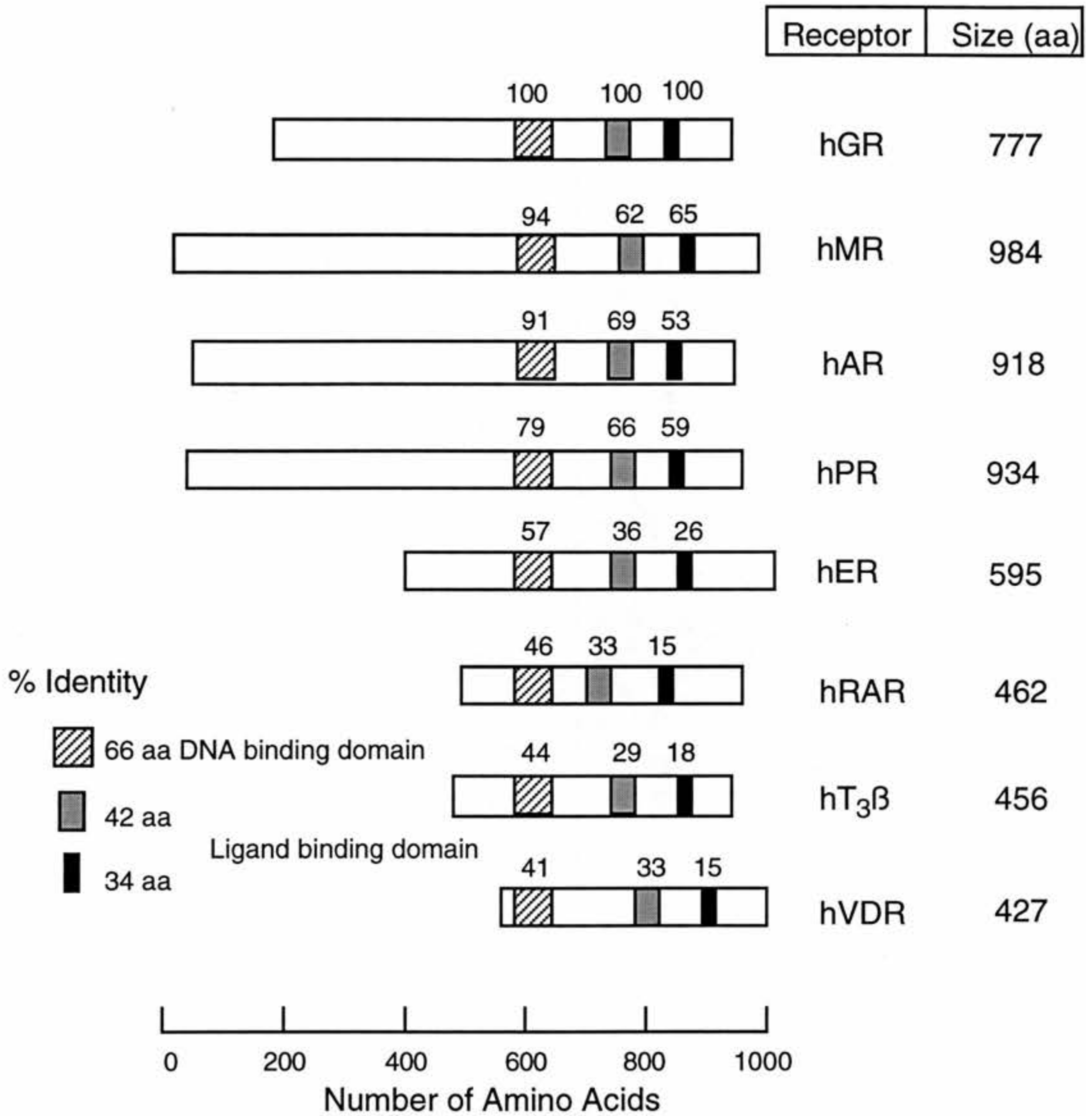


Figure 1.6: The steroid receptor superfamily: Sequence identities.
There are three highly conserved regions, the DNA-binding domain and two regions within the ligand-binding domain. Numbers indicate the percentage identity to GR, which has been arbitrarily assigned 100%.

finger contains a perpendicularly oriented α-helix which extends from the base of the finger. The first (N-terminal) zinc finger helix interacts with DNA in the major groove and confers sequence specificity of binding whilst the second zinc finger, in addition to participating in binding-specificity, may direct subunit interactions and specify alignment of half-sites (Freedman & Luisi, 1993; Glass, 1994). Steroid receptors bind to DNA response elements of only two distinct sequence motifs related to either of the consensus half site sequences, AGAACA or AGGTCA, and DNA specificity appears to be determined by the orientation and relative spacing of these motifs (Freedman & Luisi, 1993).

The ligand-binding domain, located at the C-terminus is also highly conserved throughout all members of the family (O'Malley & Tsai, 1993; Ribeiro et al., 1995). The C-terminus is also associated with a number of functions regulated by ligand-binding, including heat-shock protein dissociation, translocation to the nucleus, ligand-dependent transcriptional-activation, homodimerisation, heterodimerisation and potential protein-protein interactions with other steroid receptors and transcriptional regulatory factors (Baniahmad & Tsai, 1993; Green & Chambon, 1988; Glass, 1994; Parker et al., 1993; Janknecht & Hunter, 1996; Chakravarti et al., 1996; Kamei et al., 1996). In contrast, the N-terminal domain is not conserved and in GR is a major immumoreactive epitope (Carlstedt-Duke et al., 1982). It contains ligand-independent transcriptional activation functions and may interact with other transcriptional activation proteins in complex response elements (Ribeiro et al., 1995).

1.2.2 Glucocorticoid Receptors

Glucocorticoids, like all steroid hormones, are thought to enter cells by passive diffusion (although there is evidence that thyroid hormone (T_3) and retinoic acid entry may involve specific transport processes (Baxter, 1994)) and bind to the C-terminal ligand binding domain of their receptors (Ribeiro et al., 1995). The GR is a single chain polypeptide of about 90 kD, and is associated with several heat shock proteins including heat shock protein 90 (hsp90) (Rexin et al., 1992; Baniahmad & Tsai, 1993; Beato et al., 1996; Pratt, 1993). Glucocorticoid binding leads to the dissociation of hsp90 from the receptor, thus "activating" it (Sanchez et al., 1987; Baniahmad & Tsai, 1993) and enabling it to bind to DNA (Denis et al., 1988; Meshinchi et al., 1990). The activated receptor-hormone complex then translocates to the nucleus - a function dependent on a sequence in the ligand-binding domain

and a sequence in the hinge region between the DNA and ligand-binding domains (Picard & Yamamoto, 1987; Picard et al., 1990; Ylikomi et al., 1992).

Activated GR bind as homodimers to specific regulatory DNA sequences called glucocorticoid response elements (GREs). GREs are frequently located near the promoter region of the target genes (Beato, 1989; Baniahmad & Tsai, 1993) and the GRE is a partially pallindromic structure with the consensus sequence AGAACAnnnTGTTCT (Scheidereit et al., 1983; Strahle et al., 1987). The DNA binding domain of the receptor provides specificity for DNA, but high affinity binding requires additional regions of the receptor (Danielson et al., 1987).

Transcriptional activation and repression by GR are not fully understood. Sequences in the N-terminal domain, the DNA binding domain and the hormone binding domain are all important for efficient transcriptional activation (Beato et al., 1996). One model for transcriptional activation by steroid receptors proposes that the DNA-bound hormone receptor complex interacts with the transcriptional machinery of the cell to form an activation complex (Ptashne, 1986, 1988). Another suggests the DNA-bound hormone receptor complexes realign or displace nucleosomes to allow transcription factor binding to previously inaccessible regions of the target gene promoter (Archer et al., 1991). Glucocorticoids also repress transcription of genes (e.g. POMC, prolactin (Ludbland & Roberts, 1988; Drouin et al., 1987; Sakai, 1988)). However, no clear consensus negative response element has been identified (Beato et al., 1996). Transcriptional repression may be due to competition for common DNA-binding sites, interference of bound receptors with the activity of other transcription factors or the transcriptional machinery (Beato et al., 1996).

1.2.3 Mineralocorticoid Receptors

Mineralocorticoid action is mediated by the classical receptor for aldosterone, MR, which shows a high degree of conservation with GR (Fig 1.6) Curiously, MR binds both glucocorticoids and mineralocorticoids *in vitro* with approximately equal affinity (Krozowski & Funder, 1983; Arriza et al., 1988). *In vivo*, however, MR binds aldosterone selectively in kidney, parotid and colon, but F or B is the physiological ligand in the heart and hippocampus. Cloning of human MR (Arriza et al., 1987) and GR (Hollenberg et al., 1985) demonstrated that the receptors are highly homologous in the DNA binding and ligand binding domains, but distinct in the N-termini. The DNA binding-domain is particularly highly conserved between MR and GR, with 94% homology, and both receptors can bind to the GRE (Freedman, 1993). Isolation of a MR cDNA from a rat brain cDNA library

showed that MR in kidney and brain are identical (Arriza et al., 1988; Patel et al., 1989). Therefore receptor differences cannot confer the tissue-specific ligand selectivity of MR.

Recombinant human MR and GR expressed in mammalian cells are both activated by F and B, and MR is activated at 10-fold lower concentrations than GR (Arriza et al., 1988). Thus MR does not appear to have the intrinsic ability to discriminate between glucocorticoids and mineralocorticoids, and indeed, is a high affinity receptor for glucocorticoids *in vitro*. Arriza et al. (1987, 1988) speculated that both MR and GR may provide responses to glucocorticoids *in vivo*, with MR providing a high-sensitivity, low response function and GR a low-sensitivity, high response function. This model may be particularly relevant to cells where MR and GR are co-expressed, such as hippocampal neurones (Evans & Arriza, 1989).

The aldosterone-selectivity of MR *in vivo*, is provided at least in part, by the enzyme 11ß-HSD-2 (Morris & Souness, 1992), which is co-expressed with the MR intracellularly, and metabolises glucocorticoids to inactivate them. Thus aldosterone, which is not a substrate for the enzyme has sole access to the receptors.

1.3 11ß-Hydroxysteroid Dehydrogenase

11ß-HSD catalyses the reversible conversion of the physiologically active glucocorticoids (F in humans and B in rats) and their inactive 11-dehydro forms (cortisone (E) and 11-dehydrocorticosterone (A)) (Fig. 1.7). The enzyme is thus well placed to act as a tissue specific regulator of glucocorticoid access to intracellular corticosteroid receptors (Monder & White, 1993; Seckl, 1993). 11ß-HSD activity has been described in studies since the 1950s in many body tissues (Amelung et al., 1953; Hubener et al., 1956). However it was not until the 1970s that its importance became apparent, with the recognition of 11ß-HSD deficiency as a clinical syndrome (Werder et al., 1974; New & Levine, 1977; New et al., 1977; Ulick et al., 1977, 1979). To date, two distinct isozymes have been characterised and their encoding cDNAs cloned.

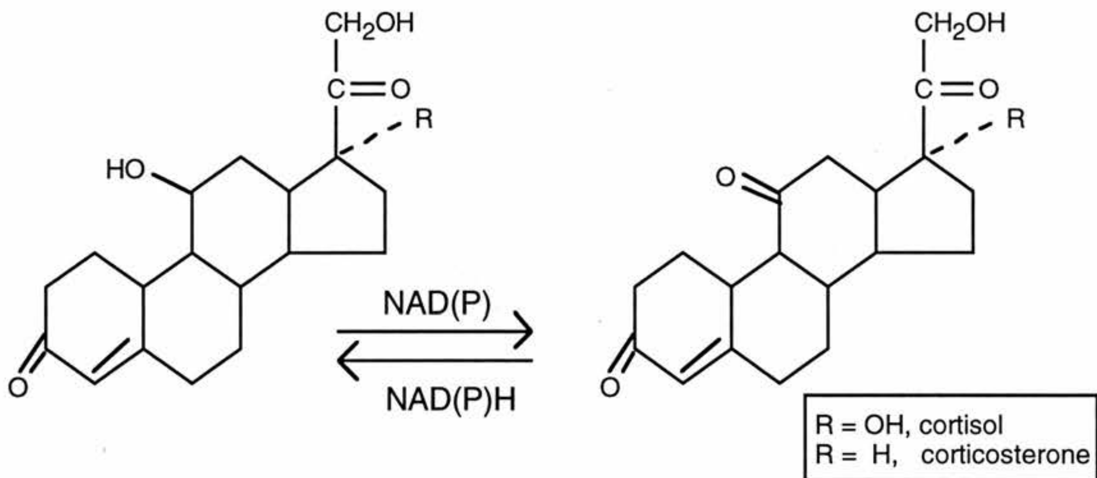


Figure 1.7: The major reactions catalysed by 11ß-HSD in man and rat. 11ß-dehydrogenase activity results in oxidation of the hydroxyl group at C11 to an inactive 11-keto group, whilst 11ß-reductase catalyses the reverse reaction.

1.3.1 11ß-Hydroxysteroid Dehydrogenase Type 2

In 1975, the first case of a rare syndrome of severe hypertension and hypokalaemia, in the absence of detectable plasma renin and low aldosterone was described (Werder et al., 1975). Thus the syndrome became known as "apparent mineralocorticoid excess" (AME). F and ACTH infusion into a AME patient

exacerbated the hypertension and hypokalaemia, whilst spironolactone (an MR antagonist) and DEX (a selective GR agonist) reversed the syndrome (Oberfield et al., 1993). F to E metabolism had previously been demonstrated to be impaired in this syndrome by measurement of urinary metabolites (New & Levine, 1977; New et al., 1977; Ulick et al., 1977, 1979), whilst 11ß-reduction appeared to be unaffected (Ulick et al., 1979; Monder et al., 1986), leading to the proposal that 11ß-dehydrogenase activity was impaired in AME such that intra-renal F levels would be raised sufficiently to occupy and activate MR (Stewart et al., 1988) (Fig 1.8). This hypothesis was supported by studies based on the observation that liquorice ingestion results in similar metabolic abnormalities to AME, with hypertension and kaliuresis (Card et al., 1953; Conn et al., 1968; Stewart et al., 1987), which are again reversible by spironolactone or DEX treatment (Salassa et al., 1962; Hoefnagels & Kloppenborg, 1983) and that administration of glycyrrhetinic acid (GE) - the active component of liquorice - to animals, inhibited renal 11ß-HSD both *in vivo* and *in vitro* (Monder et al., 1989).

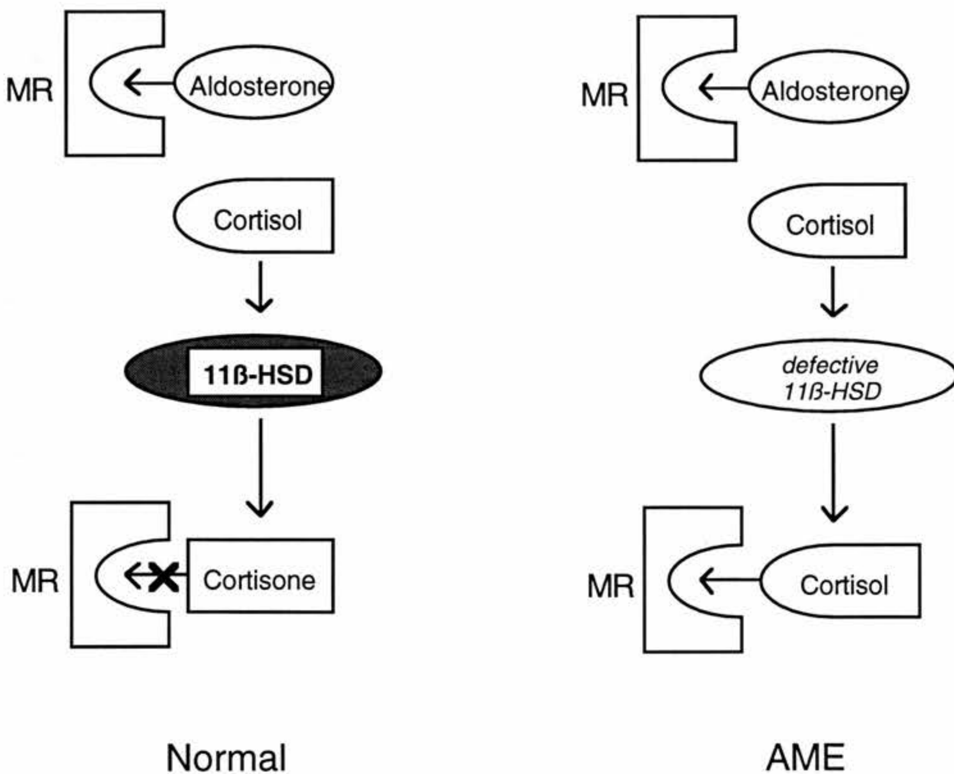


Figure 1.8: 11ß-hydroxysteroid dehydrogenase in AME.
Absence of 11ß-HSD activity results in failure of normal MR protection and allows illicit occupation receptor occupation and activation by F. See text for details.

Thus it was proposed that the *in vivo* selectivity of renal MR (which bind both glucocorticoids and mineralocorticoids with similar affinity *in vitro*) was due to the inactivation of glucocorticoids by 11ß-HSD activity, and this was demonstrated in experimental studies (Edwards et al., 1988; Funder et al., 1988).

11ß-HSD was cloned from the rat liver in 1989 (Agarwal et al., 1989) (section 1.3.2). However, rather than concluding the story, this raised as many questions as it answered. Polyclonal antibody staining demonstrated 11ß-HSD immunoreactivity in the proximal convoluted tubules of the kidney (Edwards et al., 1988; Lakshmi & Monder, 1988; Rundle et al., 1989), whereas MR are located in the distal convoluted tubules and cortical collecting duct cells (Rundle et al., 1989; Farman et al., 1991), and 11ß-HSD activity is found in both microdissected proximal and distal rabbit tubules (Bonvalet et al., 1990). Kinetic studies on the liver-derived 11ß-HSD indicated a μM K_m for F and B (Agarwal et al., 1990) - insufficient to exclude circulating glucocorticoids (present at approximately 500 nM (Dunn et al., 1981)) from MR, which has a K_d for B of approximately 0.5 nM (Arriza et al., 1987; Krozowski & Funder, 1983). The cloned 11ß-HSD cDNA encoded both 11ß-dehydrogenase and 11ß-reductase activities, but AME patients appear to have a selective 11ß-dehydrogenase deficiency, with 11ß-reductase unaffected (Ulick et al., 1979; Monder et al., 1986). Furthermore, genetic analysis of AME patients revealed no gross deletions or rearrangements of the corresponding 11ß-HSD gene (Nikkila et al., 1993; White et al., 1995). Finally, biochemical studies demonstrated a distinct NAD-dependent, high affinity 11ß-HSD isoform in kidney (Mercer & Krozowski, 1992; Rusvai & Naray-Fejes-Toth, 1993) where it co-localised with MR (Rundle et al., 1989, Farnan et al., 1991), and in placenta (Brown et al., 1993). Therefore the weight of evidence suggested a distinct isoform of 11ß-HSD was responsible for MR aldosterone selectivity (Edwards et al., 1989; Funder, 1990, 1993; Stewart & Edwards, 1990).

This isozyme (termed 11ß-HSD-2) has recently been cloned from cDNA libraries made from sheep, human, rat, mouse and rabbit kidney (Agarwal et al., 1994; Albiston et al., 1994; Zhou et al., 1995; Cole TJ, 1995; Náray-Fejes-Tòth & Fejes-Tòth, 1995) and human placenta (Brown et al., 1996). As predicted, 11ß-HSD-2 has a high affinity (K_m in the nM range) for its glucocorticoid substrates, utilises NAD as co-factor, and is an exclusive 11ß-dehydrogenase, thus rapidly inactivating glucocorticoids. 11ß-HSD-2 expression is confined to aldosterone-target tissues (kidney, parotid, colon), the placenta, and a limited number of other sites (Albiston et al., 1994; Whorwood et al., 1995; Brown et al., 1996). Mutations in the 11ß-HSD-2 gene have been identified in AME patients (Mune et al., 1995; Wilson et al.,

1995a, 1995b; Milford et al., 1995; Ferrari et al., 1996) providing conclusive evidence for its function of conferring aldosterone selectivity on MR.

In the placenta, 11ß-dehydrogenase activity provides a barrier to the transfer of active glucocorticoids to the foetus (Beitins et al., 1973; Bernal & Craft, 1981; Giannopoulos et al., 1982; Waddell et al., 1988). 11ß-reduction in placenta is low or undetectable (Osinski, 1960; Kittinger, 1974: Murphy, 1979; Bernal et al, 1980). The proposed role therefore, of 11ß-HSD-2, is to protect the developing foetus from potentially harmful circulating maternal glucocorticoids (Murphy et al., 1974; Munck & Leung, 1977; Slikker et al., 1982), exposure to which may retard foetal growth (Reinisch et al., 1978). Low birthweight is linked to the occurrence of cardiovascular and metabolic disorders in adult life, particularly hypertension (Barker et al., 1989; Whincup et al., 1989; Barker et al., 1990) and non-insulin-dependent diabetes mellitus (NIDDM) (Hales et al., 1991). Placental 11ß-HSD-2 activity correlates positively with birthweight, and negatively with placental weight (Benediktsson et al., 1993) so that individuals with low birthweight and large placentae are those likely to be exposed to the highest levels of maternal glucocorticoid, supporting a role for the enzyme in the pathogenesis of hypertension (Edwards et al., 1993; Seckl, 1994). In keeping with this hypothesis, inhibition of 11ß-HSD in pregnant rats by carbenoxolone (CBX) leads to reduced birthweight and elevated blood pressure in the adult offspring (Lindsay et al., 1996).

However, if 11ß-HSD-2 is an exclusive dehydrogenase restricted to a few target tissues, then which enzyme is responsible for the widely distributed 11ß-HSD activity observed *in vivo*, with high expression in several organs, including the liver, and which enzyme provides hepatic 11ß-reductase activity (Bush, 1969 and Stewart et al., 1990)?

1.3.2 11ß-Hydroxysteroid Dehydrogenase Type 1 - Purification and Cloning

Whether 11ß-dehydrogenase and 11ß-reductase activities were due to the tissue-specific behavior of a single enzyme (Nicholas & Lugg, 1982; Torday et al., 1976; Bush & Mahesh, 1959) or to the expression of two separate enzymes (Abramovitz et al., 1982; Monder & Shackleton, 1984; Lakshmi & Monder, 1985) was a matter of debate for many years. Since 11ß-HSD activity in liver is NADP-dependent, in 1988, Lakshmi and Monder purified 11ß-HSD from rat liver microsomes using NADP-agarose affinity chromatography (Lakshmi & Monder, 1988). The homogeneous enzyme exclusively expressed 11ß-dehydrogenase activity in the absence of 11ß-reductase, supporting the two enzyme hypothesis.

Nevertheless, this did not mean the single enzyme hypothesis had to be rejected as the 11ß-reductase component of 11ß-HSD-1 had been shown to be labile in homogenates and purified microsomes, so that the 11ß-dehydrogenase activity could be studied in the absence of the 11ß-reductase activity (Monder, 1993). Rat liver 11ß-dehydrogenase is a glycoprotein with a monomer molecular weight of 34 kD (Lakshmi & Monder, 1988), is NADP-dependent, and has a Km for F of approximately 17μM and B of 2 μM (Monder & Lakshmi, 1989; Monder et al., 1991).

Production of rabbit-antisera against the purified 11ß-HSD (Monder & Lakshmi, 1990) allowed identification of tissue-specific species of the enzyme by Western analysis, with liver, kidney, testis and lung microsomes containing a single 34 kD protein. Kidney contained an additional 40 kD band and brain an additional 26 kD band. A 68 kD band in liver and kidney was detected - likely to be a dimer of the 34 kD species. The 26 kD and 40 kD species were suggested to represent further isoforms of 11ß-HSD (Monder & Lakshmi, 1990; Monder, 1991b), but could represent differences in post-translational modification or differing transcriptional start sites within the same gene.

The rat "liver type" 11ß-HSD was cloned in 1989 (Agarwal et al., 1989) by screening a rat liver cDNA library with a monospecific anti-serum to 11ß-HSD. The cDNA was 1265 bp long and contained a 861 bp open reading frame predicting a 287 residue polypeptide with a molecular weight of 31 774 kDa. The discrepancy between this, and the 34 kD purified protein is likely to be due to glycosylation of the mature protein at the two putative N-linked glycosylation sites at residues 158-160 and 203-205 (Agarwal et al., 1989).

Expression of the full-length cDNA in Chinese hamster cells (CHO) cells (Agarwal et al., 1989) and human osteosarcoma cells (Agarwal et al., 1990) revealed that in intact cells, the recombinant protein encoded roughly equal 11ß-dehydrogenase and 11ß-reductase activities, in contrast to the exclusive 11ß-dehydrogenation of the purified protein, thus indicating that both activities reside in a single enzyme. More recent studies have demonstrated that in most intact cell types, expression of 11ß-HSD-1 is associated with 11ß-reductase activity, although 11ß-dehydrogenase activity is readily detectable in homogenates from the same cells (Low et al., 1994a; Rajan et al., 1995b; Leckie & Seckl, 1996; Chapter 3).

Northern analysis revealed that the rat "liver type" 11ß-HSD cDNA hybridised to a single mRNA species of approximately 1600-1700 nucleotide (nt) in liver, testis, ovary, lung, vascular tissue and various brain regions (Agarwal et al., 1989; Moisan et al., 1992b), but cross-hybridised with at least four species in rat

kidney (Krozowski et al., 1990; Moisan et al., 1992b). Some, but not all of these could be attributed to variations in the amount of polyadenylation (Krozowski et al., 1990), and so these transcripts were proposed to represent products of a separate 11ß-HSD gene (Krozowski et al., 1990). However, cloning of the rat '"liver type" 11ß-HSD gene demonstrated that the multiple species of renal transcripts all result from products of differential promoter usage (Moisan et al., 1992b).

This isozyme of 11ß-HSD has been designated the type 1 isozyme (11ß-HSD-1), and to date, human, monkey, sheep and mouse homologues have also been cloned (Tannin et al., 1991; Moore et al., 1993; Yang et al., 1992; Rajan et al., 1995a). The nucleotide sequences show a high degree of conservation across species. The human 11ß-HSD-1 gene has been localised to chromosome 1, consists of 6 exons and is at least 9 kb in length (Tannin et al., 1991). 11ß-HSD-1 is a member of the short chain alcohol dehydrogenase (SCAD) superfamily (reviewed by Krozowski, 1992), which includes prokaryotic and eukaryotic enzymes involved in a wide variety of metabolic processes (Baker et al., 1990a; 1990b; Krook et al., 1990; Tannin et al., 1991). 11ß-HSD-1 has highest sequence similarity with the bacterial enzyme 3α, 20ß-HSD (Marekov et al., 1990) which catalyses the reversible oxidation of 3α and 20ß hydroxyl groups of androstane and pregnane derivatives. However, human 11ß-HSD-1 only shares 28.3% sequence homology with 11ß-HSD-2, also a member of the SCAD superfamily (Brown et al., 1996). Members of the SCAD family involved in steroid metabolism include 17ß-HSD and 3ß-HSD, but they are not closely related to 11ß-HSD-1. 11ß-HSD-1 is a microsomal enzyme (Monder & White, 1993), and the characteristics of its tertiary structure can be deduced from comparisons with that of 3α, 20ß-HSD (Ghosh et al., 1991). It is concluded that 11ß-HSD-1 has a single active site capable of catalysing both 11ß-dehydrogenation and 11ß-reduction (Monder, 1993). Protein structure analysis has shown that the bulk of the protein molecule is orientated on the lumenal side of the endoplasmic reticulum and that the catalytic domain is located here (Ozols, 1995).

1.3.3 11ß-Hydroxysteroid Dehydrogenase Type 1 - Tissue-Specific Modulator of Glucocorticoid Receptor Function?

The physiological role of 11ß-HSD-2 has been established in aldosterone-target tissues and placenta. However, despite over 40 years of investigation, the physiological role of 11ß-HSD-1 is still obscure. The highest levels of 11ß-HSD-1 are in the liver and the proximal tubules of the kidney (Monder & Shackleton, 1984). These are tissues where MR are not significantly expressed, but GR are abundant,

and it has therefore been proposed that the role of 11ß-HSD-1 is to modulate glucocorticoid access to intracellular GR in these sites (Moisan et al., 1990b; Teelucksingh et al., 1990; Whorwood et al., 1991). 11ß-HSD-1 is also present in the hippocampus where MR are present, but the physiological ligand is glucocorticoid (Moisan et al., 1990a; Sakai et al., 1992; Lakshmi et al., 1991). The possible role of 11ß-HSD-1 in these tissues is considered below.

Although the highest levels of 11ß-HSD-1 are found in the liver (Monder & Shackleton, 1984), very little is known about the biology of hepatic 11ß-HSD-1. 11ß-reductase activity appears to predominate over 11ß-dehydrogenase activity in liver. The effluent of perfused cat liver contains high levels of F with respect to E (Bush et al., 1969) and in man, oral administration of E leads to a rise in peripheral plasma levels of F, but not E, on first-pass through the liver (Stewart et al., 1990). Thus hepatic 11ß-HSD-1 is likely to potentiate glucocorticoid action in the liver, which is a major target for glucocorticoid action and contains high levels of GR. Many of the hepatic genes encoding key metabolic enzymes are under glucocorticoid regulation (section 1.4), suggesting an important role for 11ß-HSD-1 in this organ.

Pharmacological levels of circulating glucocorticoids directly suppress testosterone secretion from the testis in rats (Saez et al., 1977) through GR mediated actions (Bambino & Hseuh, 1981; Welsh et al., 1982). 11ß-HSD-1 immunoreactivity appears in the testes at puberty in the rat (Philips et al., 1989) and it was therefore hypothesised that the inactivation of B by 11ß-HSD-1 removes this inhibition so that testosterone production and puberty can proceed (Philips et al., 1989). The observations that 11ß-HSD-1 is present only in the Leydig cells and that CBX potentiates B inhibition of testosterone release in Leydig cell cultures (Abayasekara et al., 1990) adds weight to this hypothesis (Monder, 1991). However, 11ß-HSD-1 has recently been shown to be a predominant 11ß-reductase in primary cultures of Leydig cells (Leckie & Seckl, 1996). In addition, 11ß-HSD-1 is not expressed in mouse testis (Rajan et al., 1995a) and both sexes of a transgenic 11ß-HSD-1 "knock-out" mouse recently produced are fertile (Kotelevtsev et al., 1996). Thus the role of 11ß-HSD-1 in the testis is unclear.

11ß-HSD-1 is present in the ovary (Murphy, 1981; Tannin et al., 1991), located in the oocyte and the luteal body (Benediktsson et al., 1992) as are GR (Schreiber et al., 1982). A recent study provided evidence that low 11ß-HSD activity in the granulosa-lutein cells is necessary for a viable pregnancy to occur (Michael et al., 1993a), possibly as corticosteroids inhibit ovarian steroidogenesis (Michael et al., 1993b). Ovarian 11ß-HSD-1 levels fall in metoestrus in rats (Albiston et al., 1995) suggesting 11ß-HSD-1 may play a role in ovarian steroidogenesis and

ovulation. There is however evidence that 11ß-HSD-2 is expressed in the ovary (Roland & Funder, 1996), which confuses the issue as studies based on measurement of 11ß-HSD activity often do not discriminate between the isozymes making retrospective interpretation difficult.

11ß-HSD-1 immunoreactivity has been demonstrated in skin (Teelucksingh et al., 1990) and it has long been known that GE and CBX have an anti-inflammatory effect in cutaneous disorders (Colin-Jones, 1957). It was postulated that 11ß-dehydrogenase inactivated glucocorticoid thus diminishing its effectiveness (Teelucksingh et al., 1990). This was borne out by the observation that the potency of topical application of hydrocortisone in causing GR-mediated cutaneous vasoconstriction (Marks et al., 1982) was potentiated by GE (Teelucksingh et al., 1990). However Hammami and Siiteri paradoxically reported that 11ß-reduction is the primary activity in cultured human skin fibroblasts (Hammami & Siiteri, 1991). The recent cloning of 11ß-HSD-2 has demonstrated that this enzyme is present in skin (Agarwal et al., 1995; Brown et al., 1996). Therefore it is likely 11ß-HSD-1 and 11ß-HSD-2 are expressed in different cell types within the skin, and the overall 11ß-HSD activity in skin will represent a balance between the two activities.

The arterial tree is a target for mineralocorticoids and glucocorticoids (Kornel et al., 1982) and MR and GR are present in the vascular smooth muscle affecting corticosteroid modulation of muscle tone and neural and humoral responsiveness (Kornel et al., 1975; Onoyama et al., 1979). 11ß-HSD activity was reported in the arterial vessels (Kornel et al., 1982; Funder et al., 1989) and 11ß-HSD-1 mRNA localised to the vascular smooth muscle (Walker et al., 1991; Takeda et al., 1994) where it is appropriately located to modulate glucocorticoid access to GR. 11ß-reduction is the primary reaction direction in aortic minces and cultured vascular smooth muscle cells (Brem et al., 1995). The enzyme is primarily in the smaller vessels and therefore well placed to modulate glucocorticoid effects on vascular tone and hence peripheral resistance (Walker, 1994), and recent data employing CBX inhibition of 11ß-HSD supports this (Walker et al., 1994b). The heart also contains a significant amount of 11ß-HSD activity (Funder et al., 1973; Arriza et al., 1987; Walker et al., 1991; Slight et al., 1993), located in the cardiomyocytes where it is co-localised with MR (Lombès et al., 1995), but there is conflicting evidence regarding whether the 11ß-HSD activity is due to the expression of 11ß-HSD-1 (Walker et al., 1991) or 11ß-HSD-2 (Lombès et al., 1995).

11ß-HSD-1 is also expressed in the cortico-medullary region of the rat adrenal, where it may serve a role in maintaining high adrenal medullary glucocorticoid levels which are essential for catecholamine synthesis (Shimojo et al.,

1996). Furthermore, 11ß-HSD-2 mRNA is present in the adrenal in some species (Agarwal et al., 1995; Roland & Funder, 1996; Li et al., 1996; Campbell et al., 1996). However, 11ß-HSD-1 appears to be absent from the human adrenal gland (Shimojo et al., 1996).

The brain is a key target for glucocorticoid action, which, in contrast to the tissues described thus far, is mediated via MR as well as GR (McEwen et al., 1986; de Kloet, 1991; Seckl & Olsson, 1995). The hippocampus expresses a high density of MR, which are occupied by B *in vivo* (de Kloet et al., 1975; Reul & de Kloet, 1985; McEwan et al., 1986; de Kloet, 1991) owing to the intrinsic lack of specificity of MR for substrate (Arriza et al., 1987) (to date, no convincing evidence for the presence of 11ß-HSD-2 in the adult hippocampus has been presented (Albiston et al., 1994; Roland et al., 1995)). As MR bind glucocorticoid with a 10-fold greater affinity than GR, at physiological concentrations, most B will be bound to MR. This provides the basis for the "tonic action of MR" hypothesis of Reul and de Kloet (1985), whereby high MR occupancy provides a baseline sensitive to small changes in glucocorticoid levels for the purpose of monitoring the external environment, whilst GR occupancy is low when circulating glucocorticoid levels are low but increases at the diurnal peak or during stress, and activates negative-feedback systems. A binary response system has also been proposed, where MR and GR do not act independently, but exert synergistic or competitive control (Evans, 1989).

11ß-HSD-1 is expressed in various brain regions, with highest levels in hippocampal, cerebellar and cortical neurones (Moisan et al., 1990a; Lakshmi et al., 1991; Sakai et al., 1992). The high 11ß-HSD-1 activity and mRNA expression in the cerebellum which contains no measurable MR (Moisan et al., 1990b), but well defined GR (Sousa et al., 1989), supports a role for 11ß-HSD-1 in the regulation of glucocorticoid access to GR. 11ß-HSD-1 is co-localised with MR and GR in the hippocampus (Sakai et al., 1992), thereby possibly modulating glucocorticoid access to both types of receptor. Hippocampal 11ß-HSD-1 is induced by chronic glucocorticoid excess or stress (Low et al., 1994c), and as glucocorticoids exert deleterious effects on hippocampal neuronal survival and function (Landfield et al., 1978; Sapolsky et al., 1985, 1986; Sapolsky, 1992), it has been proposed that this induction is a protective mechanism (Monder, 1991; Low et al., 1994c; Seckl & Olsson, 1995). However, recent data has shown that the predominant reaction direction of 11ß-HSD-1 in intact cultured hippocampal cells is reduction (Rajan et al., 1996), thus 11ß-HSD-1 activity is likely to potentiate rather than attenuate the neurotoxic effects of glucocorticoids, as would the glucocorticoid induction of 11ß-

reductase activity. Why this should be the case is unclear, and so the role of 11ß-HSD-1 in the brain is as yet unknown.

1.3.4 11ß-Hydroxysteroid Dehydrogenase Type 1 - Hormonal Regulation

Many of the studies on 11ß-HSD have been carried out by measurement of enzyme activity, which, as mentioned earlier, does not necessarily distinguish between the separate isozymes. Therefore, in tissues where 11ß-HSD-2 is present activity studies must be interpreted cautiously. However, where mRNA expression has been examined, or where 11ß-HSD-1 is the only isozyme present (liver, brain and testis), the interpretation of such studies is much clearer.

In rats, there is sexually dimorphic expression of 11ß-HSD activity in several tissues including liver (Ghraf et al., 1975a; Lax et al., 1978, 1979) and kidney (Ghraf et al., 1975a, 1975b; Smith and Funder 1991) with male rats having higher hepatic and renal 11ß-HSD activity than female rats. This is mediated by sex steroids, as evidenced by the differences between the sexes appearing at puberty and data from studies on the effects of administration and withdrawal of sex steroids (Ghraf et al., 1975a; Lax et al., 1978, 1979). In the liver, sexual dimorphism is due to repression of 11ß-HSD-1 in females (Lax et al., 1978; Low et al., 1993, 1994b) which is mediated by sex steroid determination of patterns of growth hormone GH secretion (see below). In the kidney, oestradiol (E_2) represses 11ß-HSD-1 mRNA expression, but overall 11ß-HSD activity is upregulated (Low et al., 1993, 1994b; Smith & Funder, 1991), suggesting differential regulation of the 11ß-HSD isoforms by E_2 in this tissue. In lung, castration of male rats decreases 11ß-reductase activity (Nicholas & Lugg, 1982), whilst hippocampal 11ß-HSD-1 is not subject to sex hormone control (Low et al., 1993). Therefore sex steroid regulation of 11ß-HSD appears to be tissue-specific.

Sexually dimorphic expression of many hepatic steroid metabolising genes is due to an indirect effect of sex steroids on the secretory profiles of GH (Gustafsson et al., 1983a, 1983b; Wehrenberg & Giustina, 1992), which differ in male and female rats (Saunders et al., 1976; Tannenbaum & Martin, 1976; Eden, 1979). Adult female rats exhibit frequent low amplitude peaks of GH release (1-2 peaks every 2 h), whilst males show lower frequency, high amplitude peaks every 3-4 h, such that levels often fall below the limits of detection. It is these low troughs that differentiate the effects of GH between the sexes. Female rats have approximately 50% of the 11ß-HSD-1 activity levels of male rats (Lax et al., 1978; Low et al., 1993, 1994b). This sexual dimorphism requires the presence of an intact pituitary

(Lax et al., 1978) and is attributable to the actions of E_2 on GH secretory patterns (Low et al., 1994b). However, exogenous E_2 administration to both male and female rats represses hepatic 11ß-HSD-1 activity by up to 95%, and 11ß-HSD-1 mRNA expression to the limits of detection (Low et al., 1993; Chapter 4). In addition, in the hypophysectomised rat, E_2 represses hepatic 11ß-HSD-1, albeit to a lesser extent than in the intact rat (Low et al, 1994b). This suggests that E_2 has an additional regulatory effect on 11ß-HSD-1 in the liver independent from its effects on GH secretory patterns. The lack of regulation of 11ß-HSD-1 by sex steroids in the hippocampus may be due to the failure of peripheral GH to cross the blood-brain-barrier (Low et al., 1994b).

T_3 regulation of 11ß-HSD-1 appears to be not only tissue-specific, but also species-specific. Thus T_3 has been reported to decrease 11ß-HSD-1 activity in male rat liver (Koerner & Hellman, 1964; Lax et al., 1979; Whorwood et al., 1993a), whilst in humans, indirect methods of glucocorticoid metabolism by measurement of urinary steroids, have indicated that 11ß-dehydrogenase activity is increased by T_3 (Hellman et al., 1961; Gordon & Southren, 1977; Zumoff et al., 1983). Hypothyroidism or adrenalectomy (ADX) reverses these responses in both species. Tissue-specificity was demonstrated by a lack of T_3 regulation in the rat kidney (Koerner & Hellman, 1964; Lax et al., 1979; Smith & Funder, 1991; Whorwood et al., 1993a) - a result confounded by the presence of both 11ß-HSD isozymes in this tissue, and the reticuloendothelial system (Dougherty et al., 1960). Lastly, the observation that thyroidectomy increases activity in female rat liver, but decreases it in the male rat (Lax et al., 1979) led to proposals that T_3 controls the available testosterone and thus indirectly influences 11ß-HSD. Overall, the available data are confusing, and whether T_3 has a direct or indirect mechanism of action is still unknown.

It seems likely that glucocorticoids modulate their own metabolism by regulating 11ß-HSD-1. Studies to date have pointed towards a positive regulation of 11ß-HSD-1 by glucocorticoids. Indirect evidence came from the demonstration that the stress of cold exposure or starvation increases 11ß-reductase in lung (Nicholas & Lugg, 1982) and in the same study, ADX led to attenuation of E to F conversion. F increases 11ß-HSD activity in thymocytes (Dougherty et al., 1960). Human foreskin fibroblast cell 11ß-HSD activity is increased by DEX in cell culture (Hammami & Siiteri, 1991), an effect blocked by the GR antagonists RU38486 and dexamethasone mesylate, and insulin antagonises this action. DEX also induces 11ß-HSD activity and 11ß-HSD-1 mRNA in cultured vascular smooth muscle cells, whilst insulin has the opposite effect (Takeda et al., 1995).

In the short- and medium-term, glucocorticoids induce 11ß-HSD activity and 11ß-HSD-1 mRNA expression in the liver and hippocampus *in vivo*, whilst ADX decreases enzyme activity and expression (Moisan et al., 1990c; Low et al., 1994c, Walker et al., 1994). Similar effects have recently been reported in testis, where 11ß-HSD-1 is down-regulated by ADX, and restored by B replacement (Gao et al., 1996). Recently work from this laboratory, using transfection of a liver-derived cell line with plasmids in which 11ß-HSD-1 promoter DNA is fused to a reporter gene, suggests that a positive GRE lies within 1800 base pairs of the transcription start of 11ß-HSD-1 (Voice et al., 1996).

In contrast, 11ß-HSD activity in kidney is unchanged by ADX or DEX treatment (Moisan et al., 1990c; Smith & Funder, 1991). This could represent tissue-specific glucocorticoid regulation of 11ß-HSD-1, or, more likely, represents the distinct regulation of the 11ß-HSD-2 isozyme.

1.4 Hepatic Glucose Metabolism

1.4.1 Effects of Glucocorticoids on Metabolism

The role of glucocorticoids in fuel homeostasis was recognised in 1927 when it was noted that ADX animals cannot maintain hepatic glycogen stores (Cori & Cori, 1927), but that they are restored by corticosteroid administration (Long et al., 1940). Glucocorticoids regulate the metabolism of carbohydrates, fats and proteins in the periphery and in the liver (Fig. 1.9). In the periphery, glucocorticoids mobilise substrate for hepatic gluconeogenesis. They stimulate the release of gluconeogenic amino acids from skeletal muscle (Exton., 1979; Long et al., 1940) and lipolysis in adipose tissue is activated (Fain, 1979), thus releasing fatty acids and glycerol for transport to the liver. Glucocorticoids also have a permissive role in mediating the effects of other gluconeogenic hormones, such as glucagon and catecholamines (Exton, 1979; Friedmann et al., 1967), therefore enhancing their peripheral catabolic effects. Peripheral glucose uptake and utilisation are inhibited by glucocorticoids (Munck, 1962; LeBoeuf, 1962; Fain et al., 1963), partly by the direct inhibition of glucose transport into cells (Olefsky, 1975; Livingston & Lockwood, 1975) due to decreased expression of glucose transporters (Garvey et al., 1989).

The liver contains all the enzymes necessary for the synthesis and degradation of glucose, glycogen and fat (Fig 1.10), and can switch the direction of carbon flow over the metabolic pathways in response to hormonal stimuli and changes in nutritional status (Granner & Pilkis, 1990). These properties are responsible for the central role of the liver in the regulation of fuel metabolism.

1.4.2 The Role of the Liver in Glucose Metabolism

The liver's role in glucose homeostasis is dependent on its ability to switch from net glucose output to net glucose uptake in response to a threshold concentration of blood glucose (Soskin et al., 1938). When a fast is terminated, the circulating concentration of glucose rises, stimulating insulin secretion which promotes uptake and conversion of glucose by the liver into the storage products glycogen and fat (McGarry et al., 1987). However, only a fraction of the glucose taken up by the liver is converted into glycogen (Madison, 1969; Madison et al., 1973), and liver glycogen can be synthesised from carbohydrate-derived sources other than glucose (Cori, 1981). In quantitative terms, glucose-derived lactate rather

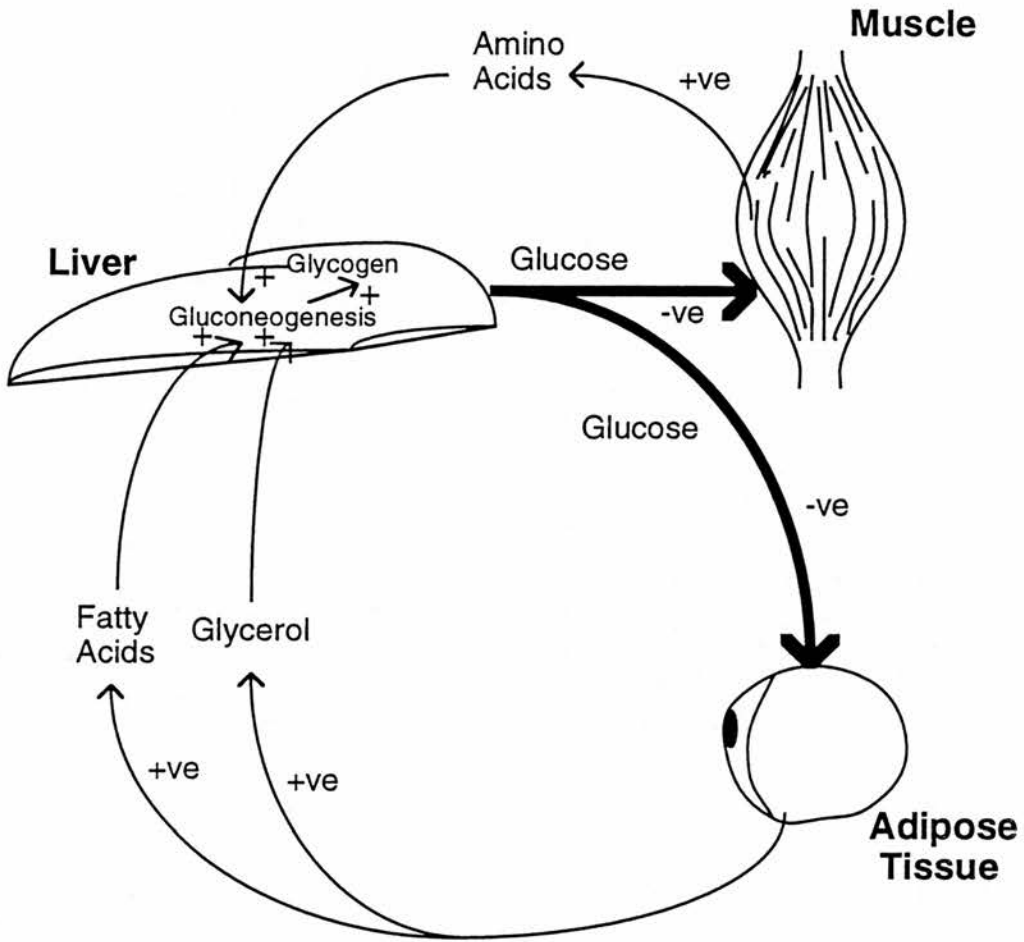


Figure 1.9: Glucocorticoid effects on fuel metabolism in the liver and the periphery. See text for details.
+ve=stimulation, -ve=inhibition.

than glucose itself serves as the precursor for hepatic glycogen synthesis (McGarry et al., 1987).

The liver is, for practical purposes, the sole source of endogenous glucose production. Under fasting conditions, in the short term approximately 75% of hepatic glucose production results from glycogenolysis by glycogen phosphorylase, and the remainder comes from gluconeogenesis (Fig 1.10). In prolonged fasting (24-48 h), hepatic glycogen stores become depleted and gluconeogenesis is the sole source of glucose production (Cryer, 1992). Precursors for hepatic gluconeogenesis are obtained from the breakdown of muscle protein, lipolysis and ketogenesis and transported to the liver in the circulation.

Glycolysis is the metabolic pathway through which glucose is utilised in order to generate ATP and NAD(P)H to fuel cell metabolism (Fig 1.10). The energy needs of the liver are largely provided by the ß-oxidation of fatty acids (Cryer, 1992) under normal conditions, and the glycolytic flux is low except when glucose concentrations are high, so that the main function of hepatic glycolysis is to generate substrate for anabolic pathways.

1.4.3 Hormonal Regulation of Liver Glucose Metabolism

The main glucoregulatory hormones are insulin, glucagon, catecholamines, glucocorticoids and GH. The dominant glucose-lowering hormone is insulin (Service, 1983; Cryer, 1988; Cryer et al., 1989) which is secreted from the ß cells of the pancreas into the portal circulation. It promotes glycogen synthesis and inhibits hepatic glycogenolysis and gluconeogenesis, thus limiting hepatic glucose production in the post-absorptive state, in order to regulate plasma glucose levels (Rizza et al., 1981). It also promotes glucose uptake and utilisation in the periphery.

The glucose-raising hormones act synergistically to effect glucose counterregulation (Shamoon et al., 1981). Glucagon is secreted from pancreatic islet cells and acts solely on the liver under physiological conditions to activate glycogenolysis and gluconeogenesis, increasing hepatic glucose production within minutes (Cryer et al., 1989). Adrenalin also stimulates hepatic glucose production and limits glucose utilisation via both direct (Rizza et al., 1980; Clutter et al., 1988; Diebert & DeFronzo, 1980; Rosen et al., 1983) and indirect mechanisms (limiting insulin secretion (Berk et al., 1985; Clutter et al., 1988) and stimulating glucagon secretion (Gerich et al., 1978; Gray et al., 1980)). Physiological concentrations of GH initially lower plasma glucose, but after a few hours, stimulate glucose production and limit utilisation (MacGorman et al., 1981). Likewise, the

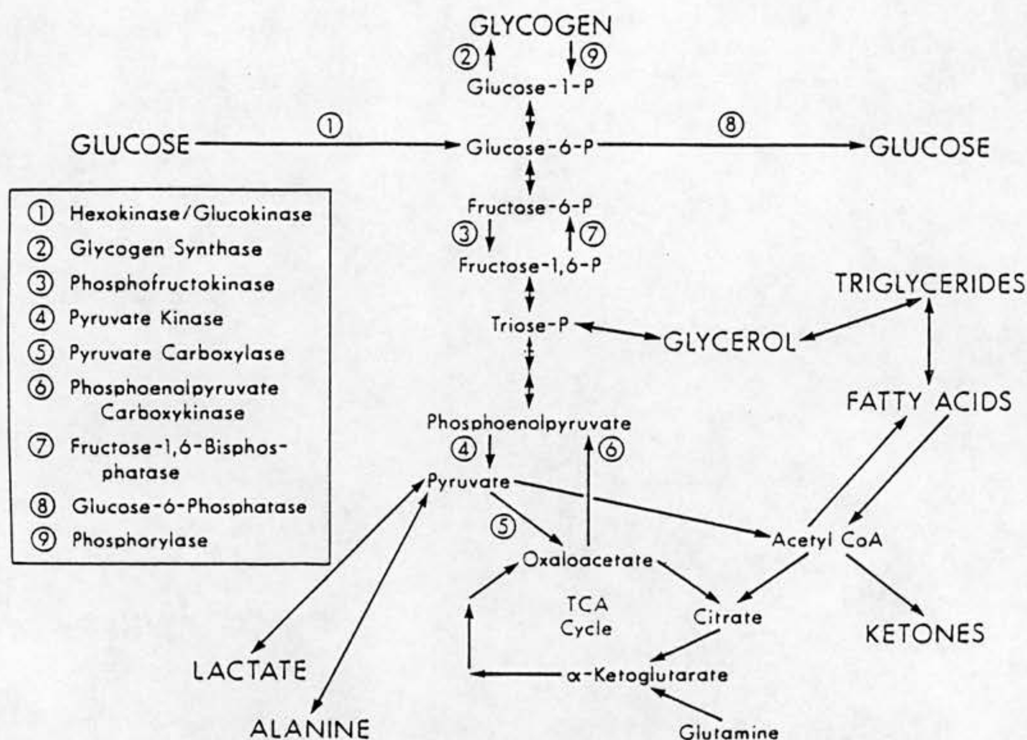


Figure 1.10: Schematic representation of glucose metabolism.
Taken from Cryer, 1992.

hyperglycaemic effect of F is only seen after 2-3 hours (Shamoon et al., 1981). Glycaemic status controls these glucoregulatory hormones such that hypoglycaemia suppresses insulin secretion and stimulates glucagon, adrenalin, GH and F release (Cryer et al., 1989).

Glucocorticoids activate glycogen synthase (Hornbrook et al., 1966; Stalmans & Laloux, 1979) by promoting its dephosphorylation, and inhibit glycogen phosphorylase (Stalmans & Laloux, 1979), thus promoting glycogen synthesis and storage (Fig. 1.10). How glucocorticoids bring about glycogen synthase dephosphorylation is unclear. They may activate a specific hepatic phosphatase or exert an indirect effect through glycogen phosphorylase which is a phosphorylase inhibitor. Glucocorticoids also directly activate and repress the expression of the key hepatic enzymes of glycolysis and gluconeogenesis (reviewed by Exton, 1979).

1.4.4 Regulation of Expression of the Genes of Hepatic Glucose Metabolism

The balance between hepatic glucose production and utilisation is controlled by the movement of substrates through three major cycles operated by the major enzymes of glycolysis and gluconeogenesis (Fig. 1.11). The relative and absolute activities of these key enzymes dictate the net flux and pace of glucose movement through the pathways, and they are therefore the main targets of regulatory mechanisms. The enzymes are subject to both acute and chronic regulation. The acute regulatory effects are mediated by the supply of substrates and hormonally-induced allosteric changes and phosphorylation (reviewed by Hers & Hue, 1983; Pilkis et al., 1988). The principle targets for acute regulation are 6-phosphofructo-1-kinase (PFK-1), fructose 1,6-bisphosphatase (FBPase-1) (regulated by availability of fructose 2,6-bisphosphate (FBP-2)), pyruvate kinase (PK) (hormonally regulated by phosphorylation and dephosphorylation) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2). Chronic effects involve changes in the rates of gene expression and hence protein synthesis, and in some instances by changes in the rate of degradation of specific mRNAs (reviewed by Granner & Pilkis, 1990; Pilkis & Granner, 1992; Lemaigre & Rousseau, 1994). The hormonal and nutritional regulation of the key regulatory enzymes of glycolysis and gluconeogenesis are summarised briefly here.

Conditions favouring gluconeogenesis, e.g. starvation, result in an increase of phosphoenolpyruvate carboxykinase (PEPCK), FBPase-1 and glucose 6-phosphatase (G-6-Pase) activities and a co-ordinate decrease in PK, PFK-1 and glucose kinase (GK) activities (Granner & Pilkis, 1990; Pilkis & Granner, 1992; Lemaigre & Rousseau, 1994). Directly opposing effects are seen upon refeeding, and glycolysis predominates. Glycolysis begins with the efficient conversion of glucose to glucose-6-phosphate (G-6-P) by GK, which is not subject to feedback inhibition and is not altered by post-translational modifications (Granner & Pilkis, 1990). Thus activity is directly related to protein levels. Regulation of the expression of hepatic genes is predominately controlled at the level of mRNA transcription (Derman et al., 1991) and GK gene transcription is potently (20-30 fold) induced by insulin (Sibrowski & Seitz, 1984; Andreone et al., 1989) within minutes (Nouspikel & Iynedjian, 1992). Glucagon (or its intracellular messenger cAMP) inhibits GK transcription, and is the dominant control (Magnusson et al., 1989; Iynedjian et al., 1989). In cultured hepatocytes, glucocorticoids enhance the stimulation of GK gene transcription by insulin (Matsuda et al., 1990).

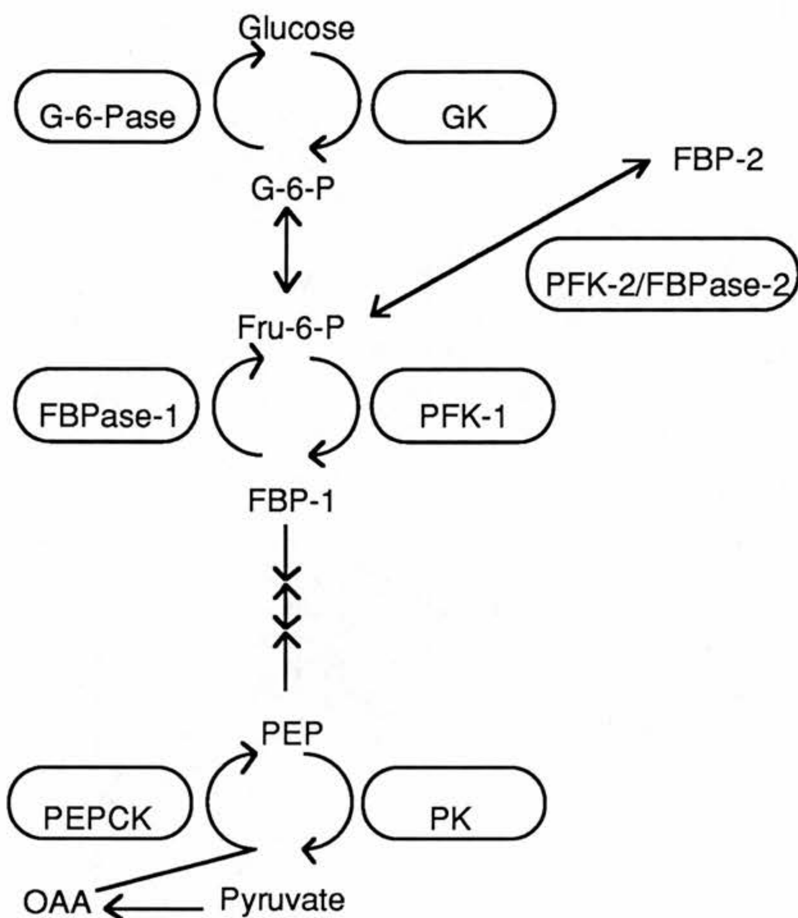


Figure 1.11: Hepatic glycolytic and gluconeogenic pathways.

Key regulatory enzymes and their substrates are shown. GK, glucose kinase; G-6-Pase, glucose 6-phosphatase; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PFK-1, 6-phosphofructo-1-kinase; FBPase-1, fructose-1,6-bisphosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; G-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; FBP-2, fructose 2,6-bisphosphate; FBP-1, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetate.

PFK-2/FBPase-2 is a dual action enzyme which catalyses the synthesis and degradation of FBP-2, which exerts a strongly positive regulation on PFK-1. It therefore can provide a "switch" between glycolysis and gluconeogenesis (Pilkis et al., 1988). PFK-2/FBPase-2 protein levels fall in starvation and diabetes, although mRNA levels do not, suggesting a translational mechanism or enhanced protein degradation may be responsible for the decrease in protein (Colosia et al., 1988; Crepin et al., 1988). Refeeding and insulin restore PFK-2/FBPase-2 protein levels by increasing gene transcription (Colosia et al., 1988). Glucocorticoids directly increase PFK-2/FBPase-2 gene transcription (Marker et al., 1989; Lange et al., 1989; Kummel & Pilkis, 1990), as does insulin at the cessation of fasting (Colosia et al., 1988; Crepin et al., 1988; Miralpeix et al., 1992). Glucocorticoids and insulin act synergistically (Granner & Pilkis, 1990). Their effects require the presence of glucose and are inhibited by glucagon acting through cAMP (Granner & Pilkis, 1990; Espinet et al., 1993). Glucagon also destabilises PFK-2/FBPase-2 mRNA (Rosa et al., 1993). Insulin effects depend on the hormonal context and can antagonise glucocorticoid induction of mRNA synthesis in some situations (Lemaigre et al., 1994).

PFK-1 catalyses the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate which is the first rate-limiting step in glycolysis. Activity is reduced in diabetes and in the fasting state, and mRNA levels are increased upon insulin administration and refeeding respectively (Dunaway et al., 1978; Gehnrich et al., 1988), an effect partially blocked by cAMP. This suggests that PFK-1 gene expression is also under reciprocal control by insulin and cAMP, but this has not been confirmed.

PK is the last key regulatory enzyme in glycolysis and catalyses the conversion of phosphoenolpyruvate into pyruvate. Regulation of gene transcription is very complex. Like PFK-1, PK activity and mRNA are reduced in diabetes and in the fasting state, and levels are restored by insulin administration and refeeding (Vaulont et al., 1986; Noguchi et al., 1985). The stimulatory effect of insulin is slow in onset suggesting induction of another gene product may be required (Vaulont et al., 1986). Glucose also stimulates transcription (Munnich et al., 1987), but neither insulin nor glucose can act alone, they are required together (Decaux et al., 1991) possibly as insulin is required for the production of GK (Vaulont & Kahn, 1994). Glucagon, acting via cAMP, inhibits PK gene transcription and also accelerates mRNA degradation (Vaulont et al., 1986; Decaux et al., 1989). Insulin and glucose on the other hand, stabilise the mRNA (Decaux et al., 1989). Glucocorticoids do not affect the transcription rate, but exert a permissive effect on glucose and insulin

induction by acting at the post-transcriptional level (Vaulont et al., 1986; Decaux et al., 1989).

PEPCK is the most intensely studied gene among those involved in the regulation of glycolysis and gluconeogenesis and its regulation is well characterised. It catalyses the synthesis of phosphoenolpyruvate from oxaloacetate and is generally assumed to be the rate-limiting enzyme of gluconeogenesis in liver (reviewed by Hanson & Garber, 1972). In contrast to the glycolytic enzymes, hepatic PEPCK expression is increased by fasting and is markedly reduced by refeeding (Tilghman et al., 1976). Regulatory effects are largely mediated hormonally, but there is evidence that the dietary response may be partially due to glucose acting independently from insulin, as glucose administration results in a decrease in PEPCK mRNA levels in hepatocyte cultures and in diabetic rats due to both decreased transcription and accelerated degradation (Kahn et al., 1989; Mayer et al., 1991). Insulin rapidly inhibits PEPCK mRNA synthesis and hence protein (Andreone et al., 1982; Granner et al., 1983; Sasaki et al., 1984). Glucagon, acting through cAMP raises PEPCK levels (Wicks et al., 1972; Iynedjian & Hanson, 1977) by stimulation of PEPCK gene expression (Lamers et al., 1982; Granner et al., 1983; Sasaki et al., 1984) and stabilisation of the mRNA (Hod & Hanson, 1988). Glucocorticoids also raise PEPCK levels through increased gene transcription (Sasaki et al., 1984) and mRNA stabilisation (Petersen et al., 1989), and the positive regulation by cAMP and glucocorticoids is additive (Sasaki et al., 1984). Insulin however, inhibits this induction and this is the dominant effect on transcription (Magnuson et al., 1987; Sasaki et al., 1984) under normal circumstances. However, there is evidence that glucocorticoids become the major regulator in diabetes (Friedman et al., 1993). The dominant regulation by insulin may be due to the presence of a complex multi-hormone response unit in the PEPCK gene promoter (Mitchell et al., 1994). This response unit contains an insulin response sequence and GR binding sites, the functions of which can both be modulated by the same regulatory proteins (O'Brien et al., 1994). Such an arrangement affords the potential for complex regulatory patterns.

FBPase-1 has been less intensively studied but its regulation is similar to that of PEPCK. The effects of insulin and cAMP on gene expression are similar to those on PEPCK (Granner & Pilkis, 1990) such that FBPase-1 mRNA is induced by starvation and diabetes and suppressed by insulin (El-Maghrabi et al., 1988). Likewise, G-6-Pase is also induced by starvation and diabetes (Nordlie et al., 1968).

Therefore the effects of hormones on the expression of the regulatory enzymes of hepatic glucose metabolism are consistent with their physiological

actions. Insulin induces the enzymes of glycolysis and represses those of gluconeogenesis. Glucagon has opposite effects. In all cases, negative control is dominant so that cAMP has dominant control of glycolysis and insulin has dominant control of gluconeogenesis. There is a paradox in the role of glucocorticoids, in that they stimulate levels of PEPCK, and are well known for increasing glucose production, but also stimulate GK, a glycolytic enzyme. This reflects their physiological role in fuel metabolism, which is to exert a permissive function on gluconeogenesis when fasting, but also on glucose disposal by glycogen synthesis and glycolysis in the post-prandial state and thus maintain glucose homeostasis.

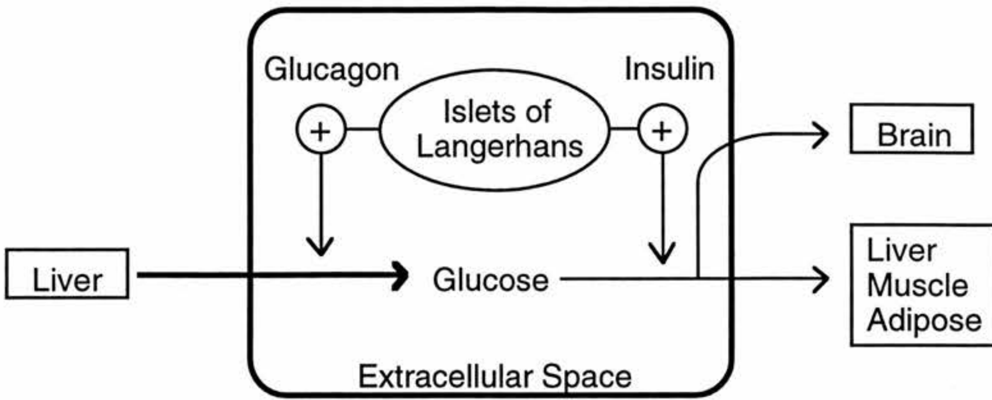
1.4.5 Insulin Resistance and Non-Insulin-Dependent Diabetes Mellitus

Diabetes mellitus is the term used to describe the wide range of hyperglycaemic disorders which occur. It is characterised by the presence of a relative or absolute lack of insulin and is associated with a high incidence of complications, including myocardial infarction, stroke, blindness due to cataract formation and retinopathy, renal failure and gangrene. NIDDM is the most common hyperglycaemic state and the high prevalence of NIDDM means it is a major health concern in all populations.

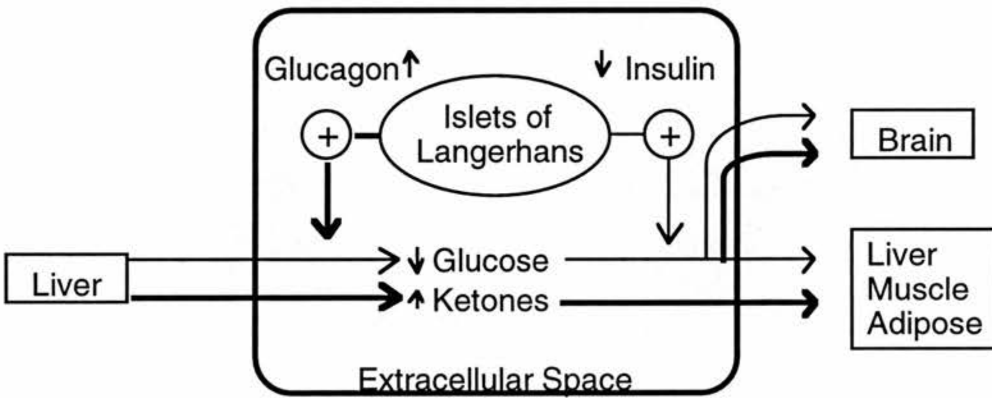
Under normal conditions, insulin and glucagon, secreted from the ß cells and the α cells of the pancreatic Islets of Langerhans respectively, rigorously maintain plasma glucose levels within normal limits (reviewed by Unger, 1981). A fall in glucose concentration elicits a prompt response of glucagon secretion, which together with a simultaneous fall in plasma insulin levels serves to redress the situation and prevent hypoglycaemia, primarily by the stimulation of hepatic glycogenolysis and gluconeogenesis and reduced peripheral glucose utilisation (Fig. 1.12). If starvation is prolonged, a shift towards ketone production occurs. In the case of glucose abundance, glucagon levels fall and there is a prompt release of insulin to facilitate glucose uptake and disposal in the liver and in the periphery (Fig. 1.12).

NIDDM is characterised by persistent fasting hyperglycaemia in the absence of diabetic keto-acidosis due to ß-cell dysfunction (reviewed by Porte, 1990). This results initially in defective first-phase or acute glucose-induced insulin secretion (Porte & Woods, 1990; Seltzer et al., 1967) and a deficiency in glucose-mediated potentiation of other islet non-glucose secretogogues (Halter & Porte, 1978) resulting in a reduced maximal capacity to secrete insulin (Fig 1.13). Paradoxically, the presence of insulin resistance in most patients increases insulin requirements, and

Resting State



Starvation



Post-Prandial

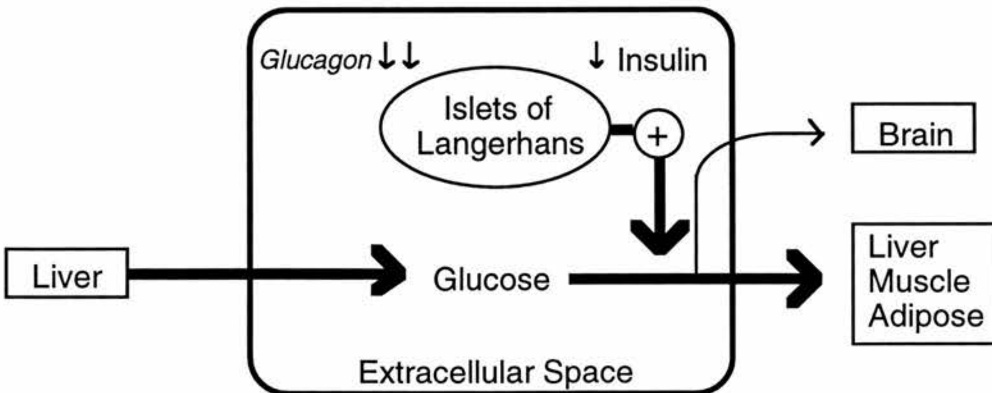


Figure 1.12: Regulation of glucose by insulin and glucagon in the resting state, starvation and in post-prandial conditions.
+ve = stimulation, -ve = inhibition. Adapted from Unger & Foster, 1992.

thus stimulates increased insulin secretion, so that insulin levels may exceed the norm (Perley & Kipnis, 1967). There is still however, an insulin deficiency relative to the requirements for efficient handling of glucose to maintain normoglycaemia (Fig 1.13). In the early stages of pathology, fasting plasma glucose levels are maintained within the normal limits, as basal and second-phase insulin secretion are not severely affected. However, progressive islet cell failure results in rising glucose levels in order to maintain adequate insulin levels by compensating for the defective glucose-stimulation for insulin release (Brunzell et al., 1976). By the time there is overt hyperglycaemia, 75% of the ß cell function has been lost (Halter et al., 1979), and in severe NIDDM the insulin response to glucose may be virtually absent (Brunzell et al., 1976; Pfeifer et al., 1981). Insulin resistance exacerbates the situation by further increasing the requirement of insulin and thus the levels of glucose needed to compensate for defective glucose-stimulation of insulin release (Fig. 1.13). The result is a steady state of glucose overproduction and hyperglycaemia. When the renal threshold for glucose is exceeded, this state of relative insulin deficiency becomes an absolute deficiency, as plasma glucose levels cannot be further elevated to further stimulate insulin release, and there is a requirement for exogenous insulin administration.

Insulin resistance is well documented in NIDDM (reviewed by Olefsky & Kolterman, 1981; Kolterman et al., 1981). It is associated with reduced numbers of insulin receptors on monocytes, erythrocytes and adipocytes (Bar et al., 1976; Gambhir et al., 1978; Olefsky, 1976) which shifts the insulin dose-response curve to the right, therefore increasing insulin requirements and exacerbating the relative insulin deficiency of NIDDM. Insulin resistance is also a feature of obesity (Baron et al., 1991). Whether obesity is a cause or effect of insulin resistance is unclear, as insulin resistance is present in hyperglycaemic states without obesity and vice versa (Baron et al., 1991; DeFronzo et al., 1982). It has been suggested they are both caused by hyperinsulinaemia (Modin et al., 1985), but the specific defect in insulin resistance has not been identified.

The nature of the ß cell lesion in NIDDM has not been precisely determined. It is believed to be, at least in part, due to the decreased activity of the normal glucose transporter in ß cells (Johnson et al., 1990). Pancreatic islet deposits of amyloid are frequently present in NIDDM patients (Clark et al., 1987) which consist primarily of the protein amylin (Cooper et al., 1987). Pro-amylin and pro-insulin processing and mature peptide secretion normally occur together (Johnson et al., 1988) and it has been suggested that abnormal processing of pro-amylin and pro-insulin may contribute to amylin deposition leading to a loss in ß cell mass (Porte &

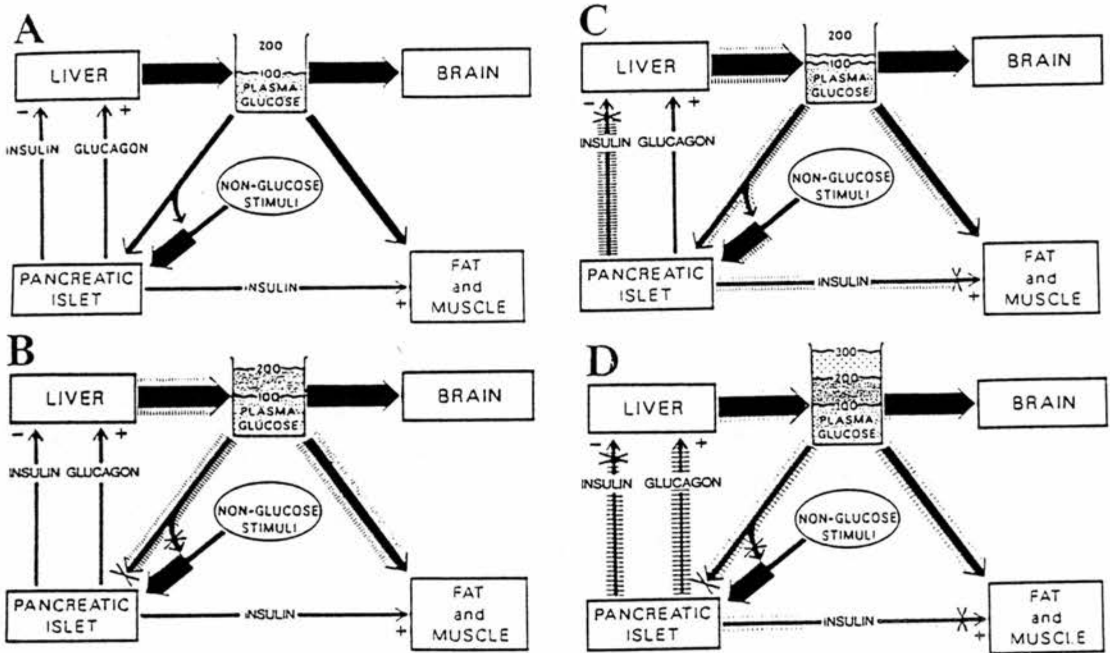


Figure 1.13: Basal glucose regulation in A: normal conditions, B: islet dysfunction C: insulin resistance and D: islet dysfunction with insulin resistance.

B: Glucose stimulation of islet is impaired, leading to increased glucose production and impaired utilisation which elevate plasma glucose to compensate until a new steady-state is reached.

C: Increased glucose production and impaired utilisation leads to increased insulin secretion. Steady state has mild hyperglycaemia and significant hyperinsulinaemia.

D: Insulin resistance requires greatly increased plasma glucose levels to compensate for islet dysfunction.

Taken from Porte, 1991.

Kahn, 1989). This process will be accelerated by the increased requirement for insulin secretion from ß cells in insulin resistance. The relationship between ß cell function and insulin resistance is obscure. Insulin resistance may contribute to ß cell dysfunction by causing beta cell "exhaustion" or direct hyperglycaemic damage (Bonner-Weir et al., 1983). Theoretically, hyperinsulinaemia could induce insulin resistance by the down-regulation of insulin receptors. Amelioration of hyperglycaemia improves ß cell function and reduces insulin resistance (Andrews et al., 1984; Scarlett et al., 1982).

As discussed earlier, glucocorticoids antagonise insulin action by inhibiting peripheral glucose utilisation and promoting hepatic glucose secretion (Dinneen et al., 1993; Rooney et al., 1994; Gerich, 1993). In Cushing's syndrome, the glucocorticoid excess often results in overt NIDDM. These effects may be more pronounced in insulin deficiency, since glucocorticoids replace insulin as the major regulator of PEPCK in diabetes (Friedman et al., 1993). Glucocorticoids may play a role in the development of obesity as they increase appetite, decrease brown adipose tissue thermogenesis and increase lipoprotein lipase in adipocytes (Langley & York, 1990; Strack et al., 1995; Ong et al., 1992), and the HPA axis is activated in obesity (Migeon et al, 1963; Marin et al., 1992; Weaver et al., 1993). Therefore glucocorticoids may be involved in the aetiology of obesity, insulin resistance and NIDDM, and they can certainly influence their expression.

AIMS OF THE THESIS

It was the purpose of this thesis to examine the regulation and function of 11ß-HSD-1, primarily in the liver and also in the hippocampus. The main aims were:

1. To establish a culture system for rat primary hepatocytes which maintained high levels of 11ß-HSD-1 activity and mRNA expression, for the examination of hepatic 11ß-HSD-1 function. In particular, 11ß-HSD-1 reaction direction in hepatocytes and the factors which influence it were to be examined, as well as hormonal regulation of 11ß-HSD-1 mRNA expression and activity levels. Glucocorticoid, insulin, GH, E_2 and T_3 regulation of 11ß-HSD-1 were to be examined in this system, to gain information regarding the magnitude (and thus importance), and the mode of action of these hormonal regulatory effects.

2. To establish a liver perfusion system for examination of 11ß-HSD-1 activity by the measurement of glucocorticoid metabolism across the intact liver. 11ß-HSD-1 reaction direction, and a quantitative measurement of hepatic 11ß-HSD-1 activity *in vivo* were to be obtained, as well as a measurement of the efficacy of 11ß-HSD-1 inhibition by CBX in the intact liver.

3. To employ *in vivo* studies in order to gain an insight into the possible implications of glucocorticoid metabolism by hepatic 11ß-HSD-1 for liver function. The effects of manipulating levels of hepatic 11ß-HSD-1 upon glucocorticoid-inducible gene expression were to be used as a marker for effects upon liver function. The consequences of 11ß-HSD-1 manipulation for glucose tolerance and insulin sensitivity were to be examined. In addition, the *in vivo* hormonal regulation of hepatic 11ß-HSD-1 was to be examined in further detail.

4. To examine the effects of chronic psychosocial stress, as opposed to glucocorticoid excess, upon hippocampal 11ß-HSD-1 activity in a well-documented model of chronic psychosocial stress in the tree-shrew. The glucocorticoid and GH (when administered by intracerebroventricular infusion) regulation of 11ß-HSD-1 were to be examined in the rat, in order to gain further insight into the tissue-specific regulation, and possible role of 11ß-HSD-1 in hippocampus.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Sources

General chemicals, unless otherwise specified, were generally purchased from Sigma Chemicals Co., Poole, UK..

Steroid Analysis Materials

The Bio-Rad Protein Assay Kit was purchased from Bio-Rad Laboratories Ltd., Hemel Hampstead, UK.

Bovine Serum Albumin Fraction V was from ICN, High Wycombe, UK.

1,2,6,7 [^{3}H]-corticosterone (specific activity 84 Ci/mmol) was from Amersham International, Aylesbury, UK.

Corticosterone, 11-dehydrocorticosterone, 5α- and 5ß-dihydrocorticosterone, 3α5α–, 3ß5α–, 3α5ß- and 3ß5ß-tetrahydrocorticosterone, glycyrrhetinic acid, NADP, NADPH, NAD, trimethylsilylimidazole and hexamethyldisilazane were from Sigma.

HPLC grade methanol, water, ethyl acetate, acetonitrile, methoxyamine, pyridine, cyclohexane and hexane were from Rathburn Chemical Co., Walkerburn, UK.

TLC plates were from Merck Ltd., Lutterworth, UK.

Cocktail T, chloroform and ethyl acetate were from BDH Ltd. (Merck Ltd.), Lutterworth, UK.

LIPIDEX 500 was from Canberra Packard, U.K.

Tissue Culture Materials

All cell culture plasticware was purchased from Costar Ltd., High Wycome, UK.

Matrigel, CR-Dispase, Rat Tail Collagen Type 1 Gel and Nu-serum were from Collaborative Biomedical Products, Bedford, Massachussets, USA.

DMEM/F12 medium, William's E medium, Nutrient Mixture Ham's F10 medium, Dulbecco's Modification of Eagle's Medium (DMEM), Hanks Balanced Salts (HBSS), Foetal Bovine Serum, Donor Horse Serum, 1x trypsin/EDTA, penicillin, streptomycin, L-glutamine, gentamycin sulphate, amphotericin B, Na pyruvate and sodium bicarbonate were all from GIBCO Laboratories, Paisley, UK.

Collagenase type IV, phosphate buffered saline, epidermal growth factor, linoleic acid, insulin, dexamethasone, thyroid hormone, 17ß-oestradiol, tamoxifen, NaN_3, ascorbic acid and carbenoxolone were from Sigma.

Human growth hormone (Humatrope) was from Eli Lilly & Co. Ltd., Basingstoke, England.

KCN was from Aldrich, Gillingham, UK.

Materials for In Vivo Studies

Surflo cannulae were purchased from Terumo, Belgium.
Venflow cannulae were from Ohneda, Sweden.
Medical grade silastic tubing and silastic sealant were both from Dow Corning Corporation, Midland, Michigan, USA.
Alzet osmotic mini-pumps and introcerebroventricular (ICV) cannulae were from Charles River UK Ltd., Margate, UK.
17ß-oestradiol, dexamethasone and carbenoxolone were from Sigma.
RU38486 was a gift from Rousel-Uclaf, Romaineville, France.
Human growth hormone (Humatrope) was from Eli Lilly & Co. Ltd.

Materials for DNA and RNA Techniques.

Restriction enzymes and buffers were from Promega Ltd, Southhampton, UK.
The 1 kb DNA ladder and agarose were from GIBCO.
Ampicillin, ethidium bromide, citrate-saturated phenol, chloroform:isoamylalcohol, guanidium thiocyanate, ß-mercaptoethanol and herring testicular DNA were from Sigma.
Bactotryptone and bacto yeast extract were from Difco Laboratories Ltd., East Molesey, UK.
[α^{32}P] dCTP (3000 Ci/mmol), Hybond C Extra and Hybond N were from Amersham International.
Random Primed DNA Labelling Kits were from Boehringer Mannheim, Lewes, UK.
NICK columns were from Pharmacia Biotech., St. Albans, UK.
Isopropanol, formaldehyde and butanol were from BDH.

2.2.2 Preparation of routinely used buffers and materials

Kreb's Ringer Bicarbonate Buffer: 118 mM NaCl, 3.8 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4.7H_2O$, and 25 mM $NaHCO_3$ (pH 7.4), gassed with 95% O_2/5% CO_2 for 1 h and supplemented with 0.2% glucose.
Metabolism buffer: 10% glycerol, 300 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.7).
Low pH (pH 5) buffer: 10% glycerol, 300 mM NaCl, 1 mM EDTA, 0.1 M potassium acetate (pH 5.0).

Phosphate buffered saline (PBS): 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl (pH 7.4). Obtained by dissolving 1 tablet in 200 ml dH_2O. Sterilised by autoclaving.

TM Triton: 0.1% Triton X-100 in 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$.

HEPES buffered saline (HBS): 2 x HBS = 50 mM HEPES (pH 7.1), 280 mM NaCl, 1.5 mM Na_2HPO_4.

*Perfusion Buffer I:*10 mM Hepes, 5 mM glucose, 0.05% KCl, 0.001% phenol red, 0.2 mM EDTA, 0.9% NaCl (pH 7.4). All solutions were filter sterilised through a 0.2 µm Millipore filter before addition to the sterile saline. Sterilised by autoclaving.

Perfusion Buffer II: 5 mM $CaCl_2$, 30 mM Hepes, 10 mM glucose, 0.05% KCl, 0.001% phenol red, 0.9% NaCl (pH 7.4). All solutions were filter sterilised through a 0.2 µm Millipore filter before addition to the sterile saline. Sterilised by autoclaving.

Cycling reagent for NADP/NADPH assay: 10 mM isocitric acid, 10 mM $MgCl_2$, 500 µM MTT, 2 mM phenazine ethosulphate, 1 mg/ml BSA in 100 mM Bicine (pH 8.0).

LB (Luria-Bertoni) broth: Per litre: 10g bactotryptone; 5g bacto yeast extract, 10g NaCl. Sterilised by autoclaving.

LB agar: LB broth with 15g agar per litre added prior to sterilisation.

GTE: 50 mM glucose, 25 mM Tris-HCl (pH8), 10 mM EDTA.

K acetate: 3 M potassium, 5 M acetate = 294.42g K acetate and 115g acetic acid made up to 1 litre with dH_2O.

Alkaline-SDS solution: 0.2 M NaOH, 1% SDS.

10x TBE: 108g Tris, 55g boric acid, 4.7g EDTA made up to 1 litre with dH_2O. Sterilised by autoclaving.

20 x SSC: 175.32g NaCl and 88.23g Na citrate made up to 1 litre with dH_2O. Sterilised by autoclaving.

Agarose loading buffer: 0.25% (w/v) bromophenol blue, 0.25% zylene cyanol (w/v), 15% Ficoll (type 100) in dH_2O.

Tris-EDTA (TE): 10 mM Tris-HCl (pH 8), 0.1 M EDTA. Sterilised by autoclaving.

Denaturing solution: 4 M guanidium thiocyanate, 0.025 M Na citrate, 0.5% sarcosyl. 0.1 M ß-mercaptoethanol added immediately prior to use.

Tris-saturated phenol: redistilled phenol mixed with an excess volume of 0.5 M Tris pH 8.0, and the aqueous discarded. Mixed with an excess volume of 0.1 M Tris pH 8.0, and the aqueous discarded three times. Finally, an excess volume of 0.1 M Tris pH 8.0 added.

DEPC-treated H_2O: dH_2O mixed with 0.1% diethylpyrocarbonate and left for 1-24 h before sterilisation by autoclaving.
10x MOPS: 0.2 M MOPS (pH 7.0), 50 mM NaAc, 5 mM EDTA. Sterilised by autoclaving.
Prehybridisation buffer: 0.2 M NaH_2PO_4, 0.6 M Na_2HPO_4, 5 mM EDTA, 6% SDS, 10μg/ml denatured herring testicular DNA.

2.2 Steroid Analysis Techniques

2.2.1 *In Vitro* Assay of 11ß-Dehydrogenase Activity of 11ß-HSD

This assay was used routinely to measure the potential 11ß-HSD enzyme activity in fresh and frozen tissue samples. This assay measures the conversion of B to A in tissue homogenates and does not discriminate between 11ß-HSD-1 and 11ß-HSD-2 activity where there is an abundance of co-factor for both enzymes. Routinely, this assay was performed on fresh tissue samples, but where this was not possible tissue was immediately frozen on dry ice and stored at -70°C until required. All tissues within a single experiment were treated in the same manner.

Tissues were homogenised in Kreb's-Ringer bicarbonate buffer in Dounce tissue homogenisers. Routinely, half a hippocampus was homogenised in 500 µl Kreb's-Ringer buffer, whilst the cerebellum was homogenised in 1 ml. 0.1-0.2g of cerebral cortex, liver, kidney, lung and skeletal muscle were homogenised in 1ml Kreb's-Ringer buffer. Tissues with high 11ß-HSD activity were then diluted 1 in 50 with Kreb's-Ringer buffer. The total protein concentration of each sample was estimated (section 2.2.5), and aliquots of homogenate containing equal amounts of protein were incubated in duplicate with 200 µM NADP and 12 nM [^{3}H]-B in a total volume of 250 µl with Kreb's-Ringer buffer supplemented with 0.2% BSA at 37°C. Preliminary studies carried out to optimise conditions for the assay and the protein concentration employed for each tissue in routine assays was chosen such that the percentage conversion was within the range, 10-50% conversion in a 10 min assay. The exception to this was tree-shrew hippocampus which required incubation for 60 min. This was so that additional protein led to an approximately linear increase in percentage conversion of [^{3}H]-B to [^{3}H]-A. The chosen protein concentration varied between tissues and between species (Fig. 2.1 and Table 2.4). To terminate the assay, 1 ml of ethyl acetate was added to each sample and mixed to separate the steroids from the homogenate. The upper ethyl acetate layer was removed into HPLC tubes and dried down under air at 55°C before analysis by high pressure liquid chromatography (HPLC) (section 2.2.6) to estimate the conversion of [^{3}H]-B to [^{3}H]-A. Enzyme activity was expressed as the percentage conversion to product.

In assays where the 11ß-HSD inhibitor CBX was employed, it was added to the incubate in concentrations from 10^{-9}M to 10^{-4}M in ethanol, as 1% of the total assay volume.

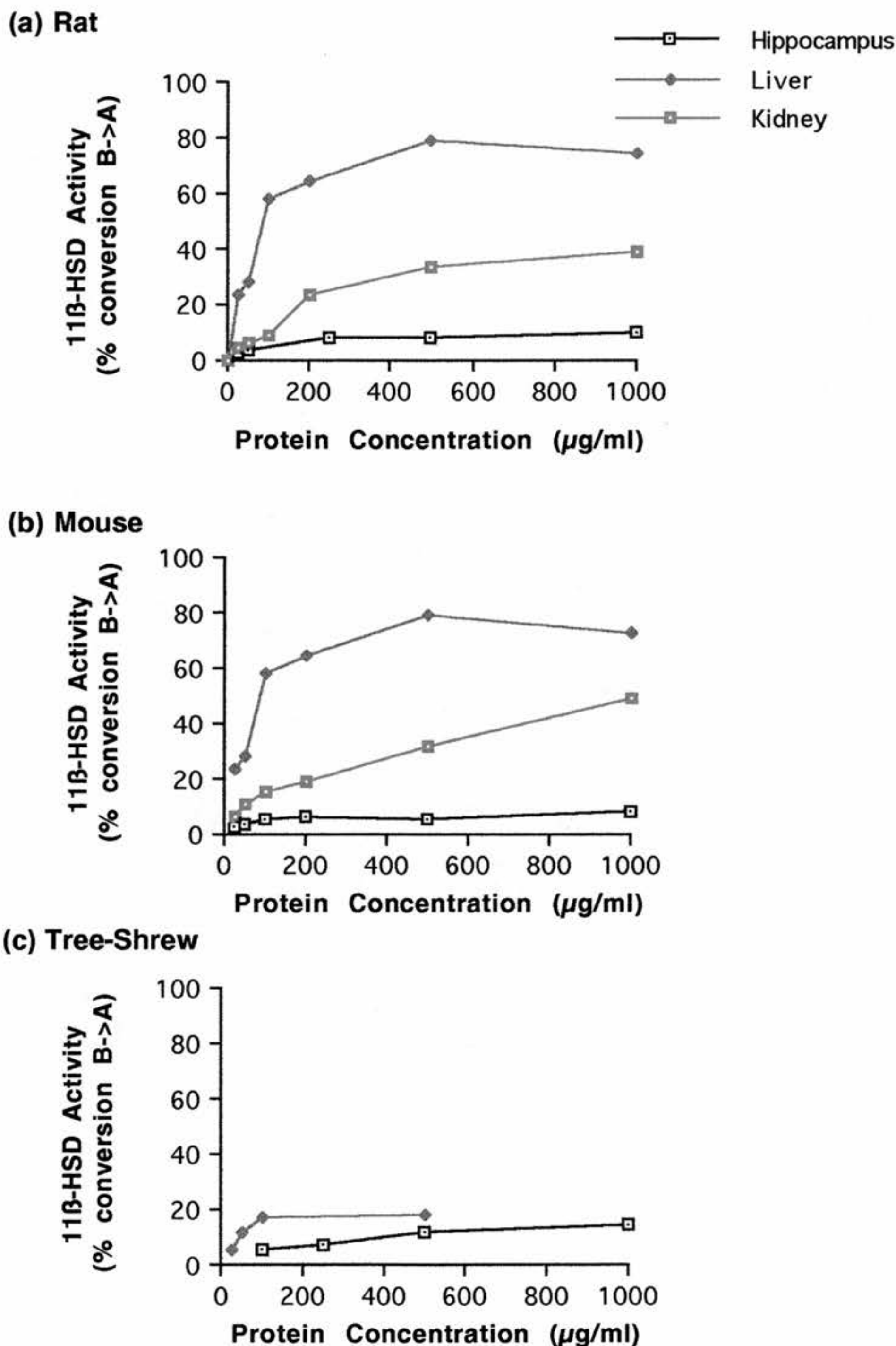


Figure 2.1: 11ß-HSD-1 activity with varying protein concentrations.
11ß-HSD activity in male a) rat b) mouse and c) tree-shrew hippocampus, liver and kidney. 11ß-HSD activity is expressed as % conversion B to A at 37°C for 10 min (60 min for tree-shrew hippocampus) in the presence of 200 µM NADP. Similar data was collected from other tissues and species, and used to select an appropriate protein concentration for routine 11ß-HSD assays.

Species	Tissue	Protein Concentration (μg/ml)
Rat	Hippocampus	1000
	Cerebellum	1000
	Cerebral Cortex	1000
	Liver	50
	Kidney	100
	Skeletal Muscle	1000
Mouse	Hippocampus	1000
	Cerebellum	1000
	Liver	25
	Kidney	200
	Lung	100
	Ovary	1000
Tree-Shrew	Hippocampus	1000
	Liver	100
	Kidney	100

Table 2.1:

Protein concentrations routinely employed in the 11β-HSD dehydrogenase assay.

In assays where 11ß-HSD-2 was to be measured, 200 μM NAD was substituted for NADP, and in assays where detection of possible 11ß-HSD activity was the priority rather than a qualitative analysis, incubation times of up to 2 hours were employed.

2.2.2 In Vitro Assay of 11ß-Reductase Activity of 11ß-HSD

11ß-reductase activity is specific for 11ß-HSD-1 and measures the conversion of A to B in tissue homogenates. This assay was always performed on fresh tissue samples as 11ß-reductase activity is labile and easily lost on freezing.

Essentially, the assay was carried out as for the 11ß-dehydrogenase assay. However, tissues were homogenised in metabolism buffer and duplicate aliquots of homogenate were incubated with 200 μM NADPH, 12 nM [^{3}H]-A and 10% low pH (pH 5) buffer in a total volume of 250 μl with metabolism buffer at 37°C. Routinely, 11ß-dehydrogenase activity was measured in parallel by incubation with 200 μM

NADP and 12 nM [^{3}H]-B in metabolism buffer. Where 11ß-HSD-2 activity was measured within the same experiment, incubation was with 200 µM NADP and 12 nM [^{3}H]-B in metabolism buffer.

2.2.3 Quantitation of 11ß-HSD Activity in Intact Cells

All 11ß-HSD-1 assays in intact hepatocytes were carried out in 35mm dishes. The medium was aspirated and replaced with 2 ml fresh medium containing 2 nM [^{3}H]-B and a quantity of unlabelled B appropriate for the concentration of B required, or 2 nM [^{3}H]-A made as described in section 2.10 and unlabelled A as required. In most experiments, 23 nM unlabelled A or B was added to the medium giving a final concentration of 25 nM A or B. This concentration was chosen so as to reflect moderate to high physiological "free" B levels. Aliquots of medium were removed at various time points as required. Routinely, a 1ml aliquot of medium was removed from the culture medium after 30 min incubation and the remaining 1ml removed after 60 min. In the experiment to determine product formation with time (section 3.3.1), 100 µl aliquots of medium containing 25 nM [^{3}H]-B or 25 nM [^{3}H]-A were removed after 15, 30, 60, 90 and 120 min incubation. Steroids were extracted from the medium with 1 ml of ethyl acetate. The upper ethyl acetate layer was removed into eppendorf tubes, dried down under air at 55°C and subjected to analysis by thin layer chromatography (TLC) (section 2.2.7). Cells were washed twice in fresh medium after each assay and the aspirated medium disposed of as radioactive waste. Assays were repeated at intervals from 4 h to 28 d after plating.

Assays in intact Y1 cells transfected with pSL1 were carried out in 60 mm dishes 24 h after transfection. To measure 11ß-dehydrogenase and 11ß-reductase activities in intact cells, medium was aspirated and replaced with 6 ml fresh growth medium containing 25 nM B (2nM [^{3}H]-B and 23 nM unlabelled B) or 25 nM A (2 nM [^{3}H]-A and 23 nM unlabelled A). 750 µl aliquots of medium were removed from the culture dishes 2, 4, 8 and 24 h later, steroids extracted and analysed by TLC (section 2.2.7).

2.2.4 Quantitation of 11ß-HSD Activity in Cell Homogenates

Seven days after being placed into culture, hepatocytes were harvested from culture dishes by dissolution of the Matrigel basement membrane. The medium was aspirated and 1 ml of CR-Dispase was added to each 35 mm culture dish. Following incubation for 2 h at 37°C, the contents of each dish were transferred to an eppendorf

tube and gently pipetted up and down to ensure complete dissolution of the Matrigel to yield a cell suspension. Cells were sedimented by centrifugation at 50g for 2 min and the supernatant discarded. The pellet was resuspended in 100 µl TM Triton and the total protein concentration of the suspension estimated. 25 µg of cell homogenate protein was incubated for 10 min or 60 min at 37°C with either 200 µM NADP and 12 nM [^{3}H]-B or 200 µM NADPH and 12 nM [^{3}H]-A, in a total volume of 250 µl with Kreb's Ringer buffer supplemented with 0.2% BSA. Steroids were extracted into 1 ml of ethyl acetate, dried down and resuspended in 600 µl of 65:35 methanol: HPLC H_2O and separated by HPLC (section 2.2.6) to estimate the conversion of [^{3}H]-A to [^{3}H]-B or [^{3}H]-B to [^{3}H]-A. Enzyme activity in each direction was expressed as the percentage conversion to product.

To measure 11ß-HSD activity in Y1 cell homogenates, medium was aspirated 48 h after transfection, the cells washed with 3 ml PBS and harvested by scraping into 1 ml PBS and spun down at 14 000 rpm in a bench top centrifuge for 2 min. The cell pellet was resuspended in 100 µl TM Triton, 400 µl Kreb's Ringer buffer was added and the total protein concentration of each cell suspension estimated. 125 µg homogenate protein was incubated for 10 min at 37°C with 100 nM [^{3}H]-B and 5 mM NADP in a total volume of 250 µl with Kreb's Ringer buffer supplemented with 0.2% BSA. Steroids were extracted into ethyl acetate and analysed by HPLC (section 2.2.6).

2.2.5 Protein Estimation

The protein concentrations of tissue and cell homogenates were estimated using the Bio-Rad Protein Assay Kit. Protein standards of 1.0, 0.8, 0.6, 0.4, 0.2 and 0.1 mg/ml were prepared by serial dilution of the Protein Assay Standard II supplied in dH_2O. 20 µl of each protein standard, of each sample and of Kreb's Ringer buffer (blank) was pipetted into an eppendorf tube and 1 ml of Protein Assay Dye Reagent (diluted 1 in 5 with dH_2O and passed through a Whatmann No. 1 Filter Paper) added. The tubes were vortexed and left at room temperature for 15 min before measurement of absorbance at 595 nm by a Shimadzu UV-160A spectrophotometer. The concentration of protein in each sample was estimated from the standard curve.

2.2.6 High Pressure Liquid Chromatography

Following extraction into ethyl acetate, steroids were dried down under air at 55°C in 1 ml HPLC tubes and reconstituted in 600 µl mobile phase (65:35 methanol: water (HPLC grade). A 200 µl aliquot of each sample was injected onto an HPLC column. The HPLC apparatus consists of an auto-injector (Waters 712 Wisp), an HPLC pump (Waters 510), an ultraviolet absorbance detector set at 254 nm for detecting the elution profile of steroid standards, a Waters reverse phase M-Bondapak-C18 (3.9 x 300 mm) HPLC column linked to a radioactivity (ß) detector (Berthold LB506 C1), a scintillation fluid pump (Berthold LB5035) and a computer for analysing and storing data (Berthold HPLC program v. 1.51). The flow rate for mobile phase employed was 1.8 ml/min.

In order to ascertain the elution times of the major hepatic metabolites of A and B, the HPLC elution profiles of steroid standards for the major hepatic metabolites of B were examined by absorbance at 254 nm (Table 2.2). 10^{-9} moles of A or B gave clearly identifiable peaks. However, the 5α– and 5ß- metabolites of B absorb much less efficiently, and so 10^{-6} moles of each of these metabolites was required to give an equivalent peak. Radiolabelled peaks as detected by the on-line ß counter were compared to the elution profiles of the unlabelled steroid standards to identify the products of A or B metabolism in tissue homogenates, cultured hepatocytes or perfused liver. The elution profiles of unlabelled steroid standards were used to identify the peaks of radiolabelled steroid which came off the column and the proportion of total counts in each radioactive peak were used to estimate the conversion of steroids in samples.

2.2.7 Thin Layer Chromatography

Following extraction into ethyl acetate, steroids were dried down under air at 55°C in eppendorf tubes, and resuspended in 100 µl ethanol containing 2.5 mg/ml each of unlabelled A and B. Silica gel coated aluminium TLC plates were divided into lanes of 2cm width to allow application of eight 40 µl samples onto each plate. Following application of the samples, the plates were allowed to dry, and then chromatographed in sealed tanks containing chloroform: 95% ethanol (92:8) for 1 hour, to allow separation of A and B. The steroid bands were identified by fluorescence under ultra-violet light and scraped into scintillation vials. 3 ml liquid scintillant (Cocktail T) was added to each sample and the radioactivity was quantified by a ß-counter. The conversion of $[^3H]$-A to $[^3H]$-B or $[^3H]$-B to $[^3H]$-A

was calculated from the radioactivity in each fraction according to the formula % conversion = [^{3}H]-A or [^{3}H]-B/[^{3}H]-A + [^{3}H]-B. Enzyme activity in each direction was expressed as the percentage conversion to product.

STEROID	PEAK ELUTION TIME (MIN)
corticosterone	6.45
11-dehydrocorticosterone	4.33
5α-dihydrocorticosterone	9.18
5ß-dihydrocorticosterone	8.12
3α5α–tetrahydrocorticosterone	8.24
3ß5α–tetrahydrocorticosterone	8.33
3α5ß-tetrahydrocorticosterone	7.48
3ß5ß-tetrahydrocorticosterone	7.00

Table 2.2:

HPLC elution times of the major metabolites of corticosterone, measured by ultra-violet absorbance at 254 nm. Note the clear separation of 5α and 5ß reduced metabolites from B and A.

2.2.8 Preparation of Samples for Gas Chromatography-Mass Spectrometry

Dried steroid extracts from plasma samples were converted to their methoxime-trimethylsilyl derivatives as described by Shackleton & Honour (1976). 10 ml HPLC grade ethyl acetate was added to tubes for standards and samples, internal standard (500 ng each of deuteriated A ([$^2H_{10}$]-A) and B ([$^2H_9$]-B), a gift from Dr. Ruth Best) was added to each tube and a tube was sealed to serve as the blank. Standards were prepared by adding 100, 200, 300, 400 and 500 ng of A and B in acetonitrile to each of 5 tubes which were then sealed. 1.5 ml of each plasma sample was added to a tube, shaken and sonicated in a sonicating waterbath (Decon) for 10 seconds. The ethyl acetate was evaporated under N_2, and then 50 µl 2% methoxyamine in pyridine was added and the sample heated to 60°C for 30 min. The methoxyamine was evaporated, 50 µl of trimethylsilylimidazole added and the sample heated to 100°C for 2 h. Columns were prepared using pasteur pipettes plugged with glass wool and with 1 ml LIPIDEX 500 added. 3 ml mobile phase (cyclohexane:pyridine:hexamethyldisilazane; 98:1:1) was added, followed by the sample in 2 ml mobile phase. The sample was collected, dried down, and dissolved

in 50 μl hexane for injection into the gas chromatography-mass spectrometry (GCMS) system.

2.2.9 Gas Chromatography-Mass Spectrometry

Samples were subjected to GCMS by Dr. Ruth Best in order to determine their B and A content. The system is described briefly as follows: a Hewlett Packard 5890 gas chromatograph linked to a VG Trio 1000 mass spectrometer (Fisons Instruments, Manchester, UK) was used. The oven temperature was maintained at 50°C for 1 min and then increased by 30°C a minute for 10 min. The injection port was maintained at 290°C and ionisation was carried out in electron impact mode with an electron energy of 70 eV. The source temperature was 200°C and the interface was 280°C. A CP-Sil 5CB column (length 25 m, internal diameter 0.32 mm, film thickness 0.12μm) (Chrompack, London, UK) with column head pressure of 5 psi was used for analysis. Monitoring of the following ions was carried out, using a dwell time of 0.04 seconds: m/z 427, 517 and 548 for derivatised B; m/z 434, 435, 524, 525, 555 and 556 for derivatised deuteriated B; m/z 474 for derivatised A and m/z 484 for derivatised deuteriated A. The ratios of the sum of the peak areas of the ions of the derivatised analyte to those of the derivatised deuteriated standard were used for quantitation.

2.2.10 Synthesis of [^{3}H]-11-Dehydrocorticosterone

[^{3}H]-A was made using homogenised human placenta or placental extract (a gift from Dr. Roger Brown) as described (Brown et al., 1993a). Human placenta is a source of high 11ß-HSD-2 activity (Brown et al., 1993a) with no detectable 11ß-HSD-1 activity and is therefore efficient at producing A with minimal B contamination. Routinely, 100 μl of placental extract or 0.5g of human placenta homogenised in 2 ml 0.1 M Tris-HCl (pH 7.5), was incubated with 1 mM NAD in 0.1 M Tris-HCl (pH 7.5) and 150 mM [^{3}H]-B in a total volume of 5 ml at 37°C for 4 h in a shaking waterbath. Steroids were then extracted into 20 ml ethyl acetate, dried down under air and resuspended in 500 μl ethanol. 1 μl of resuspended steroid was subjected to HPLC (section 2.2.6) to check the purity of the [^{3}H]-A, which was routinely >97%. The volume of ethanol in which the steroid was dissolved was then adjusted to give approximately the same specific activity as the stock [^{3}H]-B. [^{3}H]-A was stored at -20°C for up to several months, but was always checked by HPLC before use as it has a tendency to spontaneously reform [^{3}H]-B.

2.3 Primary Rat Hepatocyte Cultures

2.3.1 Hepatocyte Isolation

Liver perfusions were carried out by Sharon Rossiter of the Biomedical Research Facility, Western General Hospital. Adult male Han Wistar rats were anaesthetised with pentabarbitone (60 mg/kg) injected intraperitoneally and their livers were perfused in situ with collagenase essentially as described by Seglen, 1976. For each liver perfusion, 500 ml of Perfusion Buffer I and 100ml of Perfusion Buffer II was prepared. For each perfusion 33 mg of collagenase type IV was added to 20 ml sterile saline and heated to 37°C for 15 min to dissolve it. It was then filtered into the Perfusion Buffer II. Both Perfusion Buffers were maintained at 40°C in a water bath and connected to a peristaltic pump set at a perfusion rate of 30 ml/min. When the rat was fully anaesthetised, the abdomen was cut open and the intestines retracted to give access to the inferior vena cava (IVC) and the hepatic portal vein. These vessels were blunt dissected from the surrounding abdominal adipose tissue and the portal vein was cannulated (18 gauge x 45 mm Venflow cannula) whilst the IVC was transected. The perfusion system was then attached to the cannula so that the perfusate flowed through the liver and out through the cut end of the IVC. The IVC was then cannulated (18 gauge x 45 mm Venflow cannula) by opening the thoracic cavity and inserting a cannula into the right atrium of the heart and then into the IVC. The lower end of the IVC was then tied off with suture material so that the perfusate flowed out of the IVC cannula. Perfusion Buffer I was pumped through the liver which turned a pale brown as the liver was cleared of blood. This was followed by Perfusion Buffer II, and the liver swelled slightly as collagenase digestion took place.

The liver was removed and dissociated in 100 ml of ice cold Hanks Balanced Salt Solution (HBSS) (Gibco) by teasing apart with tweezers. Hepatocytes were isolated by filtering through a 60 μm filter mesh and then the suspension was transferred into two sterile 50 ml centrifuge tubes and spun at 50g for 2 min in a Fisons MSE Chilspin centrifuge. The liver parenchymal cells sediment in the pellet while the non-parenchymal and dead hepatocytes remain in the supernatant. The supernatant was discarded and the washing procedure repeated a further three times by resuspending the pellet in fresh HBSS and centrifuging. The final pellets were pooled and resuspended in a total of 30 ml HBSS. Cell concentration and viability were determined using a haemocytometer. Yields of 5-10x10^8 cells per liver with 70-75% viability, as determined by trypan blue exclusion, were routinely obtained.

2.3.2 Primary Hepatocyte Culture Conditions

Hepatocytes were plated on dishes coated with Rat Tail Collagen Type 1 Gel or on Matrigel. Dishes were prepared at least 1 d prior to use. Collagen dishes were prepared by placing an even coating of collagen on the dish (0.2 ml for a 35mm dish) and exposing to ammonia vapour for 3 min. The dishes were rinsed with distilled water, allowed to stand for 24 h and then used immediately. Matrigel dishes were prepared according to the thin gel method whereby after overnight thawing in the fridge, Matrigel was applied to the dishes (600 µl for a 35 mm dish, 1.5 ml for a 60 mm dish) which were then placed at 37°C for 30 min to set the gel. Dishes were stored for up to 3 d at room temperature before use. Hepatocytes were routinely maintained in DMEM/F12 medium containing 5% Nu-serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 200 µM L-glutamine, 25 µg/ml gentamycin sulphate, 0.7 µg/ml amphotericin B, 5 µg/ml insulin and 0.1 µM DEX. In preliminary experiments, hepatocytes were also maintained in William's E medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 200 µM L-glutamine, 25 µg/ml gentamycin sulphate, 2 µg/ml insulin, 10 nM DEX, 10 ng/ml epidermal growth factor, 0.2 mg/ml linoleic acid and 20 mM Na pyruvate. Cells were plated at a density of $1x10^6$/35 mm dish in 2 ml medium or $3x10^6$ cells/60 mm dish in 4 ml medium. The medium was aspirated and replaced with fresh medium 4 and 24 h after cells were placed into culture and every 24 to 48 h thereafter. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air for up to 35 d.

2.3.3 Hormonal Manipulation of Hepatocyte Cultures

Test hormones or antagonists were prepared for addition to hepatocyte culture medium as follows;
17β-oestradiol (E_2): A 10^{-5} M solution in ethanol was prepared for addition to medium at a 1 in 1000 dilution to give a final concentration of 10^{-8} M.
triiodothyronine (T_3): 100 mg T_3 was dissolved in 1 ml NaOH and 14 ml of phosphate buffered saline (1x PBS) was added to give a 10^{-2} M solution. This was further diluted in 1x PBS to give a 10^{-6} M working solution which was added to medium at a 1 in 100 dilution giving a final concentration of 10^{-8} M.
growth hormone (GH): 10 i.u. was resuspended in 2 ml diluent (supplied with the GH) for addition to medium at 10 µl/ml giving a concentration of 0.05 i.u./ml.

RU38486: A 10^{-3} M working solution in ethanol was prepared for addition to medium at a 1 in 1000 dilution giving a final concentration of 10^{-6} M.
tamoxifen: A 10^{-3} M working solution in ethanol was prepared for addition to medium at a 1 in 1000 dilution giving a final concentration of 10^{-6} M.
dexamethasone (DEX) : A 10^{-3} M working solution in ethanol was prepared for addition to the medium at a 1 in 10 000 dilution giving a final concentration of 10^{-7} M.
insulin: 100mg insulin was dissolved in 19.8 ml mH_2O and 0.2 ml glacial acetic acid for addition to the medium at a 1 in 1000 dilution to give a final concentration of $1.3x10^{-7}$ M.

11ß-HSD-1 activity in the intact hepatocytes was measured 48 h after plating (section 2.2.3) and then test hormones or antagonists were added to hepatocytes in fresh medium. In some experiments, 48 h after being placed into culture hepatocytes were transferred to medium containing charcoal-stripped Nu-serum. Stripping reduced Nu-serum levels of F, E_2, progesterone, T_3 and T_4 to below detection limits (section 2.3.10). Cells were cultured in medium containing 5% stripped Nu-serum supplemented with 10^{-7} M DEX and/or $1.3x10^{-7}$ M insulin (reflecting the level of glucocorticoid and insulin in control medium containing unstripped Nu-serum), and/or hormones at the concentrations stated above. In all experiments, the medium was aspirated daily and fresh medium and hormones added. In some experiments, 11ß-HSD-1 activity in the hepatocytes was measured at 2 d intervals. All hormones were added to the medium at a concentration chosen so as to be close to physiological levels and antagonists were employed at a concentration appropriate to block the effects of the levels of hormone present (routinely 100x the concentration of hormone). Cells were harvested for mRNA extraction 12 d after the addition of hormones (section 2.8.1).

2.3.4 Measurement of the Effects of Oxidative Stress on Hepatocyte Cultures

48 h after the hepatocytes were placed in culture, medium was aspirated and replaced with fresh medium. The culture dishes were placed in plastic sandwich boxes which had airtight seals of plastic tape. The boxes were then gassed with 2.5% O_2, 21% O_2 for 15 min or not at all, through pasteur pipettes inserted into small holes in the box lids. The holes were then sealed with tape and returned to the incubator for 4 h. The boxes were opened and 1 ml medium from each dish removed for lactate analysis (performed by Professor S.K. Chapman, Department of Chemistry, University of Edinburgh). The remaining medium was aspirated and

replaced with medium containing [^{3}H]-A or [^{3}H]-B for a further assay of 11ß-HSD-1 activity as described in section 2.2.3. The dishes were gassed as before and then incubated for 1 h, before removal of 1 ml medium for lactate analysis and 1 ml medium for [^{3}H]-A and [^{3}H]-B analysis to estimate 11ß-HSD-1 activity in the usual manner.

2.3.5 Metabolic Studies in Hepatocyte Cultures

48 h after being placed in culture, hepatocyte medium was aspirated and replaced with fresh medium containing metabolic inhibitors 5 mM NaN_3 (azide), 1 mM or 10 mM KCN, 10 mM ascorbic acid (Sigma) or vehicle (1% PBS). The concentrations of azide and KCN employed cause inhibition of oxidative phosphorylation in cell culture systems (Sakaida et al., 1992, Prehn et al., 1993, Mason & Sweeny, 1994, Shetty et al., 1993), and 10 mM of ascorbic acid causes an increase in NADP, and therefore an increase in intracellular NADP/NADPH in cell culture systems (Schmidt et al., 1993). Cultures were incubated for 4 h at 37°C in the presence of metabolic inhibitors, the medium was aspirated, and then 11ß-HSD-1 activity assayed in the intact hepatocytes as described in section 2.2.3 in the presence of metabolic inhibitor in the assay medium. Cultures incubated with azide or cyanide were then washed three times in fresh medium without metabolic inhibitor, and returned to the cell culture incubator. 4 d later the intact cells were re-assayed for 11ß-HSD-1 activity in the usual manner to assess cell recovery. For quantitation of NADP and NADPH in hepatocytes treated with azide (5 mM), KCN (1 mM) or 10 mM ascorbic acid, cultures in 60 mm dishes were incubated for 4 h in medium containing the metabolic inhibitors and were then assayed for NADP and NADPH as described in section 2.3.9.

2.3.6 pH Manipulations of Hepatocyte Medium

Medium with a pH of 8.0 was prepared by adjusting the quantity of $NaHCO_3$ added to the medium upon preparation. Medium with a pH of 6.2 was prepared by addition of 1 M HCl to the standard maintenance medium. 48 h after the hepatocytes were placed into culture, medium was aspirated and replaced with fresh medium of the required pH. Cultures were then incubated for 4 h at 37°C, the medium aspirated, and 11ß-HSD-1 activity assayed in the intact cells in usual manner (section 2.2.3) in medium of the appropriate pH.

2.3.7 Inhibition of 11ß-HSD-1 Activity by Carbenoxolone Treatment of Hepatocyte Cultures

48 h after the hepatocytes were placed in culture, medium was aspirated and replaced with fresh medium containing CBX at concentrations from 10^{-9} M to 10^{-4} M or, for controls, 1% ethanol. Cultures were then incubated for 2 h at 37°C, medium aspirated, and 11ß-HSD-1 activity assayed in the intact hepatocytes as described in section 2.2.3, with the appropriate concentration of CBX in the assay medium.

2.3.8 Analysis of the Products of 11-Dehydrocorticosterone Metabolism

To identify the products of A metabolism and to estimate the conversion of [^{3}H]-A to [^{3}H]-B in hepatocyte cultures, 48 h after hepatocytes were placed in culture, medium was aspirated and replaced with fresh medium containing 10 nM [^{3}H]-A and 15 nM unlabelled A. The cultures were then incubated for 1 h at 37°C, and 1 ml of medium was removed. Steroids were extracted into ethyl acetate, dried down and separated by HPLC (section 2.2.6) to identify the products of A metabolism.

2.3.9 NADP and NADPH Extraction and Quantitation from Hepatocytes

Quantitation of NADP and NADPH was carried out according to the enzymatic cycling method, as described by Blomquist and Hakanson (1991).

For assay of NADP, medium was aspirated from the cells, which were then harvested in 1.5 ml ice-cold 500 mM perchloric acid. The addition of perchloric acid resulted in instant dissolution of the Matrigel. The cell suspension was sonicated for 4 min on ice, left on ice for 15 min, transferred to eppendorf tubes and the cell debris pelleted by centrifugation at 5000 rpm for 10 min in a bench top centrifuge. For assay of NADPH, following aspiration of the medium, cells were harvested in 1.5 ml 250 mM NaOH, heated to 60°C for 5 min, and centrifuged at 5000 rpm for 10 min. Thereafter all samples were treated in the same manner. The supernatant was removed from each sample and 0.3 ml 1 M Bicine (pH 8) added and titrated to pH 8 by the addition of 5 M KOH to the NADP samples and of 2 M HCl to the NADPH samples. Standard curves for NADP and NADPH were created by serial dilution of 2.5×10^{-7} M solutions of each cofactor (in 100 mM Bicine, pH 8) to give standards from 0.5 to 2.5×10^{-7} M NADP or NADPH. 0.8 ml of reagent blank (100 mM Bicine,

pH 8), standard or sample was added to 1.0 ml cycling reagent. Cycling was started by the addition of 0.2 ml isocitrate dehydrogenase (1.25 mg/ml in 100 mM Bicine, pH 8) and samples were incubated at room temperature in the dark for 60 min. Absorbance at 570 nm was measured and the concentration of NADP or NADPH in each sample was estimated from the standard curves. The lower detection limits of the assays were 1×10^{-8} M for both NADP and NADPH.

2.3.10 Charcoal Stripping of Nu-serum

0.25% charcoal and 0.0025% dextran T70 were incubated overnight at 4°C whilst stirring, in 0.25 M sucrose, 1.5 mM $MgCl_2$ and 10 mM Hepes (pH 7.4) so the suspension was completely mixed. 50ml aliquots of the charcoal suspension were centrifuged for 10 min at 3000 rpm to pellet the charcoal. The supernatant was discarded and replaced with 50 ml of the Nu-serum to be stripped. This mixture was vortexed, incubated at 4°C whilst stirring overnight, then filtered through a 0.2 μm Millipore filter into a 50 ml centrifuge tube and stored at -20°C until use. Samples of Nu-serum and stripped Nu-serum were analysed by The Department of Clinical Biochemistry, Royal Infirmary of Edinburgh for content of steroid hormones (Table 2.3) to check the efficacy of the stripping procedure. All the steroid hormones assayed were below the limits of detection of the assays employed. However based on the values of oestradiol, the stripping procedure removed >99.8% of steroid.

Hormone	Nu-serum	Stripped Nu-serum
F	45nM	b.d. (< 30nM)
E_2	20 900pM	b.d. (< 37pM)
T_3	1.2nM	b.d. (< 30nM)
T_4	34nM	b.d. (< 30nM)
progesterone	24.8nM	b.d. (< 3nM)

Table 2.3:

Steroid hormone content of Nu-serum before and after charcoal stripping. b.d.= below detection.

2.4 11ß-HSD Activity Studies in Y1 Adrenocortical Cells

2.4.1 Maintenance of Y1 cells in culture

Y1 adrenocortical cells were maintained in vented 75 cm^2 flasks in Nutrient Mixture Ham's F10 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 15% donor horse serum and 10% foetal bovine serum at 37°C in 5% CO_2. Cells were passaged twice per week. Medium was aspirated and the cells washed in 2ml 1x trypsin/EDTA (to remove residual serum-containing medium which inactivates the trypsin/EDTA). 2 ml of trypsin/EDTA was added and the cells incubated at 37°C for 5 min until the cells detached from the flask surface with gentle agitation. 1/4 of the cells were placed in each of 4 fresh 75 cm^2 flasks, 12 ml of culture medium added, and the cells incubated at 37°C in 5% CO_2 until confluent. Three 75 cm^2 flasks of cells were maintained and required for each experiment.

2.4.2 Calcium Phosphate Transfection of Cells

Cells were harvested, resuspended in 10 ml fresh medium and counted using a haemocytometer. Cells were plated at a density of 5 x 10^5 cells per 60 mm cell culture dish (in 6 ml medium) for 11ß-HSD assay in intact cells or 1.5 x 10^6 cells per 100 mm dishes (in 10 ml medium) for assay in cell homogenates. The following day, 1.5 h prior to transfections, the medium was aspirated and replaced with "transfection medium" (Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 200 mM glutamine and 10% foetal calf serum). For each transfection in a 60 mm dish, 300 μl and for each 100 mm dish, 450 μl of 2x HEPES buffered saline was pipetted into a sterile eppendorf tube. In separate tubes, 5 μg of either pSL1 (Low et al., 1994a) or pJ3 (Morgenstern & Land, 1990) was made up to 300 μl (for each 60 mm dish) with 37μl 2 M $CaCl_2$ and filter sterilised mH_2O or 450 μl (for each 100 mm dish) with 55μl 2 M $CaCl_2$ and mH_2O. The prepared DNA solution was added dropwise to the 2x HBS whilst aerating, so that the resulting solution was slightly opaque due to the co-precipitation of DNA with calcium phosphate. The solution was incubated at room temperature for 30 min, vortexed and added dropwise to the plates while swirling to evenly distribute the precipitate, and avoid local acidification of the cells. The cells were then returned to the incubator. "Transfection medium" was replaced after 24 h with Nutrient Mixture Ham's F10 medium as above.

2.5 Liver Perfusion Studies

2.5.1 The Liver Perfusion System

For each perfusion, two 500 ml bottles of Kreb's Ringer buffer supplemented with 0.2% glucose and B (10^{-8}M) or A (from 10^{-9}M to 10^{-6}M as required) were prepared and maintained at 40°C in a water bath whilst constantly bubbled with 95% O_2/5% CO_2. Each bottle was connected to a peristaltic pump set at a perfusion rate of 2 ml/min. Surgery and cannulations were carried out by Sharon Rossiter of the Biomedical Research Facility, Western General Hospital. Adult Han Wistar rats were anaesthetised with pentabarbitone (60 mg/kg) injected intraperitoneally. When the rat was fully anaesthetised, the abdomen was cut open and the vascular bundle supplying the stomach, pancreas and small intestine was tied off with suture material (if this was not done, these organs were perfused and became oedematous). The intestines were retracted to give access to the hepatic artery, the IVC and the hepatic portal vein. These vessels were blunt dissected from the surrounding abdominal adipose tissue and sutures passed underneath them. The hepatic artery was cannulated with a 24 gauge x 0.75 inch Surflo cannula (Terumo, Belgium) and 250 i.u. heparin in 2 ml of Kreb's Ringer buffer was injected to prevent clotting in the artery and to check the catheter was patent. Once blanching of the liver was observed, the catheter was tied in firmly with the suture and connected to one of the perfusion systems with perfusate pumping through at 2 ml/min. Then the IVC was cannulated posterior to the inflow of the hepatic vein with a 18 gauge x 45 mm Venflow cannula (Ohneda, Sweden) which was attached to a length of tubing, and the rat's head was elevated to aid the flow of the perfusate out of the IVC. Finally, the hepatic portal vein was cannulated with a 18 gauge x 45 mm Venflow cannula, flushed with 250 i.u. heparin in 2 ml of Kreb's Ringer buffer and connected to the other perfusion system, with perfusate pumped at 2 ml/min. Thus the perfusate was pumped into the liver through the hepatic artery and the hepatic portal vein, and collected from the hepatic vein via the IVC. Routinely, the perfusion rate was then increased to 7.5 ml/min through each vessel in male rats and 5 ml/min in female rats (as this was the maximum flow rate the hepatic artery could accommodate in females), the preparation was examined for visible leakage and the outflow measured to ensure there were no leaks in the system. At this point, 10^{-9} M [^{3}H]-A or 10^{-9} M [^{3}H]-B was added to the perfusate, and the system was allowed to equilibrate for 10-15 min before collection of samples began. Flow rates were altered from between 0-20 ml/min through each of the vessels. When the flow rates

were altered within a single preparation, the system was allowed to equilibrate for 5-10 min before further collection of samples. 10 ml aliquots of perfusate were collected in duplicate, the steroids extracted into ethyl acetate, dried down and analysed by HPLC (section 2.2.6). Controls consisted of perfusate which had not been passed through the liver. For all samples, the products of A metabolism in the liver were examined and the proportion of steroid recovered was calculated (section 2.2.6).

2.5.2 Inhibition of 11ß-HSD-1 Activity by Carbenoxolone in Perfused Liver

Liver perfusions were carried out in male rats with 10^{-8} M unlabelled A and 10^{-9} M [^{3}H]-A in the perfusate and a flow rate of 7.5 ml/min through both the hepatic artery and the hepatic portal vein. Following equilibration of the preparation, CBX was added at increasing concentrations from 10^{-9} M to 10^{-4} M. When the concentration of CBX was altered, the system was allowed to equilibrate for 5-10 min before further collection of samples. Samples were taken at each concentration, and analysed by HPLC in order to establish the concentration at which 11ß-HSD-1 activity was effectively inhibited.

2.5.3 Analysis of the Products of 11-Dehydrocorticosterone Metabolism

A liver perfusion was carried out in a male rat with 10^{-8} M unlabelled A and 5×10^{-9} M [^{3}H]-A in the perfusate. This was to ensure there was enough radiolabelled steroid in the samples to give clear separation of peaks representing the products of metabolism of A in the perfused liver. The flow rate employed was 7.5 ml/min through the hepatic artery and the hepatic portal vein and 10 ml samples were collected in triplicate. Steroids were extracted into ethyl acetate, dried down and separated by HPLC as described in section 2.2.6. The proportion of steroid recovered was calculated as (cpm recovered in sample/cpm recovered in blank (no homogenate) incubates) x 100%.

2.6 In Vivo Studies

2.6.1 Animal Maintenance

Han Wistar male and female rats and male tree shrews were used for the *in vivo* studies. Rats were maintained under conditions of controlled lighting (lights on from 07.00 to 19.00 h) and temperature (22°C), and had access to water and food ad libitum, except where stated. Following surgery, and during experimental procedures, animals were observed closely, and any animals which appeared to be suffering or in ill health were killed immediately. Keith Chalmers and Andy Wilson of the Biomedical Research Facility, Western General Hospital carried out the day to day care of the rats used in *in vivo* studies, and administered any daily injections throughout the course of the experiments. *In vivo* studies in tree-shrews were carried out at the German Primate Centre (Göttingen, Germany) and details of animal maintenance are described by Fuchs & Schumacher, 1990.

2.6.2 Adrenalectomy ± Oestradiol Treatment

The surgical procedures in this study were carried out with the assistance of Sharon Rossiter and June Noble. Male Han Wistar Rats (200-250g; n=5-10/group) were anaesthetised with 4% halothane, gonadectomised and either bilaterally adrenalectomised or sham-operated through dorsal incisions. Animals were implanted subcutaneously with silicone elastomer capsules (1.95 mm internal diameter, 3.125 mm external diameter) containing 17ß-oestradiol which produce levels of E_2 not significantly different from those found in the plasma of adult female rats (Painson et al., 1992). The capsules were made by sealing one end of a 12 mm length of medical grade silastic tubing with silastic sealant. The sealant was allowed to set overnight, the capsules completely filled with 17ß-oestradiol or left empty and the other end of the capsules sealed. Before implantation, the capsules were incubated overnight in 0.9% NaCl. Some ADX animals were injected subcutaneously with "high dose" DEX (250 µg/kg/day), whilst other ADX and sham ADX animals received vehicle (0.1 ml of 4% ethanol in saline). For animals treated for 42 d with E_2, the capsules were removed and replaced after 21 d. Control animals were implanted with blank capsules. ADX rats were maintained on 0.9% saline to maintain their electrolyte balance. Rats were killed 10, 21 or 42 d after surgery. Tissues were collected for assay of 11ß-HSD activity and extraction of RNA.

2.6.3 Carbenoxolone Administration and Glucose Tolerance Tests

Male Han Wistar Rats (200-250g; n=8-10/group) were injected subcutaneously with either 1 mg, 3 mg or 10 mg of CBX or vehicle (0.2 ml 0.9% saline) daily for 14 d. After 13 d of CBX administration, animals were fasted from 16.00 h until 09.00 h the next day when glucose tolerance tests were performed with the assistance of Keith Chalmers. Rats were weighed and a 2g/kg dose of glucose administered by gavage as a 0.5g/ml solution in water. 100 μl blood samples were taken by tail nick at 0, 30, 60, 90 and 120 min after glucose administration. Blood samples were collected into heparinised eppendorfs (prepared by filling with 1000 i.u./ml heparin and emptying) and immediately placed on ice. As soon as possible (5 min) they were spun at 5000 rpm for 2 min in a bench top centrifuge and the plasma was removed into fresh eppendorfs and placed on dry ice. Plasma samples were stored at -70°C until analysis for glucose and insulin content. The following day, rats were killed. Trunk blood was collected into heparinised tubes, the plasma separated by centrifugation at 5000 rpm for 10 min and stored at -20°C until analysis for K^+, Na^+ and Cl^- content (performed by Dr. Mark Lindsay of the Metabolic Unit, Western General Hospital), and for B and A. Liver was collected for assay of 11ß-HSD activity and extraction of RNA.

2.6.4 Adrenalectomy ± Adrenal Steroid Replacement

Male Han Wistar Rats (200-250g; n=5/group) were bilaterally adrenalectomised or sham-operated through dorsal incisions whilst under halothane anaesthesia. Some ADX animals were injected subcutaneously with high dose DEX (250 μg/kg/day), whilst the other ADX animals and the sham ADX animals received vehicle (0.1 ml of 4% ethanol in saline). ADX rats were maintained on 0.9% saline to maintain their electrolyte balance. Rats were killed 2, 5, 9, 15 or 21 d after surgery. Untouched animals served as controls. Tissues were collected for assay of 11ß-HSD activity and extraction of RNA.

2.6.5 Intracerebroventricular Administration of Growth Hormone

Osmotic mini-pumps were filled with 100 μl of 2.1 μg/μl human GH or vehicle (artificial CSF). These were incubated overnight in 0.9% saline at 37°C in order to allow the rate of pumping to equilibrate at 12 μl/d. Thus 25 μg/d GH was delivered at a constant rate. The following day, male Han Wistar Rats (200-250g;

n=4-5/group) were anaesthetised with 4% halothane. June Noble and Joyce Yau of the Department of Medicine, Western General Hospital assisted with the implantation of introcerebroventricular (ICV) cannulae, which were implanted 1.4 mm to the right of bregma and at a depth of 4.5 mm. Fixing screws were inserted into the skull at 3 mm posterior and at 4 mm lateral to the cannulae and the entire apparatus fixed to the skull with dental cement. The osmotic mini-pumps were implanted subcutaneously in the intrascapular fascia and connected to the cannulae. Rats were killed 5 d later and tissues were collected for extraction of RNA. The osmotic mini-pumps were cut open to check that the contents had been discharged.

2.7 DNA Preparation

2.7.1 Plasmid Vectors

Plasmid	Description	Reference
p11DH	1.2 kb EcoR1 fragment of rat 11ß-HSD-1 cDNA subcloned into pBluescript. Has the capability to maintain a high copy number and contains the ampicillin resistance gene ß lactamase[a].	Agarwal et al., 1989
pJ3	3.5 kb vector containing the ampicillin resistance gene and Simian Virus 40 origin and early promoter. Used as expression vector in mammalian cells.	Morgenstern & Land, 1990
pSL1	1.2 kb EcoR1/St1 fragment of the rat 11ß-HSD-1 cDNA subcloned into pJ3. Used to express 11ß-HSD-1 cDNA in mammalian cells.	Low et al., 1994a
p7S	Coding region of 7S cDNA subcloned into pAT 153.	Balmain et al., 1982
pX3	2.5 kb full-length mouse PEPCK cDNA subcloned into pBluescript KS at EcoR1 site.	Ruppert et al., 1990
pRagU0.3A	305bp Stul/Rsal fragment of rat angiotensinogen cDNA subcloned into the HincII site of pUC19.	Ohkubo et al., 1983
pX2	1.5 kb EcoR1 fragment of mouse SAMS cDNA subcloned into pBluescript KS.	Ruppert et al., 1990
pmcTAT4	1.0 kb EcoR1-BamHI fragment of mouse SAMS cDNA subcloned into pBluescript.	Ruppert et al., 1990
pmTF-1	1.5 kb EcoR1 fragment of mouse transferrin cDNA subcloned into pBluescript.	Ruppert et al., 1990

Table 2.4:

Plasmid vectors. [a] *All these plasmids contain the ampicillin resistance gene.*

2.7.2 Bacterial Transformation

Escherichia coli HB101 cells were made competent for DNA uptake as described by Sambrook et al., 1989. Cells were grown in 100 ml LB (Luria-Bertoni) broth at 37°C in a shaking incubator to mid-log phase (A_{600} = 0.3-0.6). The cells and broth were centrifuged at 3000 rpm in a Beckman J20 rotor for 10 min at 4°C, the pellet resuspended in 10ml ice-cold 0.1 M $CaCl_2$ and left on ice for 10 min. Cells were then pelleted again by centrifugation, and resuspended in 2 ml 0.1 M $CaCl_2$. These competent cells could be stored for up to 4 d at 4°C before transformation.

150 μl of competent cells were mixed with 50ng plasmid DNA in a volume of 10μl TE and left on ice for 10 min. The cells were heat shocked at 42°C for 90 s and then transferred back onto ice for 5 min before spreading onto LB agar plates containing 100 μg/ml ampicillin, using a flame sterilised glass spreader. Plates were incubated at 37°C overnight. Control plates for transformations included a negative control of HB101 cells without added DNA. HB101 cells do not have the ampicillin resistance gene and so do not grow. The positive control consisted of HB101 cells transformed with pGEM3, which contains the ampicillin resistance gene, indicating transformation has taken place. Colonies which grew on the selection media contained cells which had transformed successfully and contained the ampicillin resistance marker. Plates with transformed colonies were stored at 4°C for up to 3 months.

2.7.3 Plasmid DNA Preparation

A single transformed bacterial colony was transferred from an LB agar plate into 3 ml LB with 100 μg/ml ampicillin and incubated overnight in a rotating tube at 37°C. The overnight culture was added to 500 ml LB with 100 μg/ml ampicillin, and incubated overnight, whilst shaking, in a 2 l flask at 37°C. The resulting culture was centrifuged at 6000 rpm for 5 min at 4°C in a Beckman J14 centrifuge to pellet the cells, and the supernatant discarded. The pellet was resuspended in 12 ml of ice-cold GTE solution, mixed with 24 ml of alkaline-SDS solution and left on ice for 5 min before the addition of 16 ml of ice-cold potassium acetate. The mixture was left on ice for 10 min and centrifuged at 6000 rpm for 10 min at 4°C. It was then strained through sterile gauze to remove the precipitate, and 32 ml isopropanol added to precipitate DNA from the supernatant which was left at room temperature for 30 min. The DNA was recovered by centrifugation at 10 000 rpm in a Beckman J20 centrifuge for 5 min at 4°C. The pellet was dried in an oven at 37°C for 30 min and

resuspended in 1.5 ml TE (pH 8.0) buffer. 1g of CsCl was added for each 1 ml of DNA solution, and dissolved in the DNA solution by warming, 50 μl of ethidium bromide (10 mg/ml) was added, and the resulting solution transferred to 3 ml Beckman Quickseal ultracentrifuge tubes. The tubes were topped up with CsCl solution (100g CsCl plus 100 ml TE) and volume adjusted to balance the tubes for weight, and centrifuged at 70 000 rpm for 20 h at 20°C in a Beckman Optima TLX Ultracentrifuge. The DNA bands (identified by visualisation of the ethidium bromide) were collected with a syringe and 21 gauge needle, transferred to fresh ultracentrifuge tubes, filled as before and centrifuged for 4 h at 100 000 rpm at 20°C. The DNA bands were collected and pooled, and the ethidium bromide was removed by extracting repeatedly with equal volumes of water saturated butanol until both aqueous and organic layers were no longer pink, and then once more. 2 vol of ethanol was added to precipitate the DNA, the mixture vortexed and left at room temperature for 10 min before centrifugation for 5 min at 14 000g. The supernatant was removed and the DNA pellet washed with 70% ethanol. The DNA pellet was dried briefly at room temperature and resuspended in an appropriate volume of TE (usually 1 ml). The concentration and purity of recovered plasmid DNA was estimated spectrophotometrically by measuring the absorbance at 260 nm and 280 nm.

2.7.4 Plasmid DNA Minipreparation

A single transformed bacterial colony was transferred from an LB agar plate into 3 ml LB with 100 μg/ml ampicillin and incubated overnight in a rotating tube at 37°C. 1.5 ml of the overnight culture was centrifuged at 14 000g in a bench top centrifuge for 1 min and the pellet was resuspended in 100 μl ice cold GTE and mixed with 200 μl of alkaline-SDS solution and left on ice for 5-10 min. 150 μl of ice-cold potassium acetate was added and gently mixed before leaving on ice for 5-10 min. The mixture was centrifuged for 5 min to remove denatured chromosomal DNA and cellular proteins. The supernatant was removed and retained, and the DNA extracted from it with an equal volume of Tris-saturated phenol and chloroform:isoamylalcohol (24:1 v/v). The mixture was vortexed, centrifuged for 2 min, and the supernatant transferred to a fresh eppendorf tube. 1 ml of ethanol was added to precipitate the DNA, the mixture vortexed and left at room temperature for 5 min before centrifugation for 5 min. The supernatant was removed and the DNA pellet washed with 70% ethanol. The pellet was dried at room temperature for 15

min and resuspended in 50µl TE (pH 8.0) with DNase-free RNase (20 µg/ml). DNA was stored at -20°C.

2.7.5 Restriction Endonuclease Digestion and Electrophoresis of DNA

Plasmid DNA was digested with 1 unit of restriction enzyme per µg of DNA in 1x restriction buffer and distilled water to the required volume (Table 2.5). Routinely 10 µg of DNA was digested in a total volume of 100 µl for 2 h at 37°C. Complete digestion of DNA was verified by electrophoresis of a 5 µl aliquot of the digest through a 1% agarose gel. Gels were routinely run at 100V with a 1 kb DNA ladder containing DNA fragments from 75 bp to 12 kb as size markers, for as long as was needed to separate DNA fragments enough to allow them to be cut out of the gel. 1% agarose gels (w/v) were prepared by dissolving 0.25g agarose in 25 ml 1x TBE in a microwave oven. The agarose solution was cooled slightly and 1 µl of ethidium bromide (10 mg/ml) was added and mixed by swirling. The solution was poured into a horizontal gel apparatus with a comb in place and left to set. Once set, enough 1x TBE was added to cover the surface of the gel and the electrodes connected to the gel apparatus. 1 µl of agarose loading buffer was added to the DNA sample before loading onto the gel. To isolate DNA fragments from cut plasmid DNA, 3 µl of agarose loading buffer was added to the remaining digest, which was run as above on a low melting point agarose gel in a single large well, and the insert fragment was cut out of the gel using a sterile scalpel blade. The gel was heated at 65°C in an eppendorf tube until it melted, which took 10-30 min. To clean up the DNA, 100 µl Tris-saturated phenol was added, the mixture vortexed, left on dry ice for 5-15 min and centrifuged for 5 min at 14 000g. The upper aqueous phase which contained the DNA was removed and extracted once with 100 µl Tris-saturated phenol, once with 100 µl Tris-saturated phenol and 100 µl chloroform: isoamylalcohol (24:1 v/v) and once with 100 µl chloroform: isoamylalcohol. The DNA was ethanol precipitated by the addition of 0.15 volumes of 5M NaCl and 2.5 volumes of ethanol, left on dry ice for 5-10 min and centrifuged at 14 000g for 5 min. The supernatant was discarded and the DNA pellet left to dry for 10 min at room temperature before resuspention in 20 µl TE. Recovery of DNA was checked by electrophoresis of a 1µl aliquot of the total recovered DNA through a 1% agarose gel as described above.

2.7.6 ^{32}P-Labelling of DNA Fragments

This was accomplished using the method of random priming of DNA which is based on the hybridisation of a mixture of hexanucleotides to the DNA to be labelled (Feinberg & Vogelstein, 1983). Hexanucleotide primers are extended from the 3' OH termini using the Klenow fragment of DNA polymerase 1 in the presence of radiolabelled nucleotide. A random prime DNA labelling kit was used to label DNA fragments prepared as described in section 2.7.5.

Plasmid	Restriction Endonuclease	Encoded cDNA
p11ß-HSD-1	EcoR1	11ß-HSD-1
p7S	EcoR1	7S
pX3	EcoR1	PEPCK
pRagUo.3A	BamH1/Pst1	Angiotensinogen
pX2	EcoR1	SAMS
pmcTAT4	EcoR1/BamH1	TAT
pmTF-2	EcoR1	Transferrin

Table 2.5:
Restriction enzymes used to isolate (or linearise in the case of p7S) fragments of cDNA from plasmids.

Approximately 25 ng of DNA fragment or linearised p7S (1-2 µl) was made up to 9 µl with dH_2O in an eppendorf tube and denatured by boiling for 10 min immediately followed by incubation on ice for 1-2 min. The tube was spun for 10 s in a bench top centrifuge (to bring the contents to the bottom of the tube) and 2 µl hexanucleotide primer mixture, 1µl each of dATP, dTTP and dGTP, 5µl [α^{32}P] dCTP and 1 µl of Klenow added to complete the reaction mixture. The reaction was incubated at 37°C for 2 h and unincorporated [^{32}P]-dCTP removed by passage over a NICK column. The NICK column was washed with 3ml TE and then the reaction mixture was added to the top of the column, 400µl TE run through and the eluate discarded. A further 800µl TE was applied to the column and the eluate collected in an eppendorf tube. This fraction contained the labelled DNA. Unincorporated radioactivity was retained by the column. The probe was heated to 100°C to denature the DNA and routinely, 1 µl of the probe was added to 3 ml Cocktail T to check incorporation of radioactivity in a scintillation counter.

2.8 Northern Analysis of RNA

2.8.1 Extraction of RNA from tissues and cells

Animals were killed by decapitation, tissues immediately dissected on ice, frozen on dry ice and stored at -70°C until required. Hepatocytes in culture were harvested from culture dishes by dissolution of the Matrigel basement membrane. 1ml of CR-Dispase was added to each 35 mm culture dish following aspiration of the medium, and incubated for 2 h at 37°C. The contents of each dish were transferred to an eppendorf tube and gently pipetted up and down to ensure complete dissolution of the Matrigel and yield a cell suspension. Cells were sedimented by centrifugation at 50g for 2 min, the supernatant discarded and extraction of RNA performed immediately.

Total RNA was extracted from brain regions, liver, kidney and cultured hepatocytes by the guanidium thiocyanate method as described by Chomczynski and Sacchi (1987). Routinely, approximately 0.1g of tissue, or the pellet of hepatocytes harvested from one dish, was homogenised in 500 µl of denaturing solution using sterilised Dounce homogenisers. The homogenate was transferred to a 1.5 ml eppendorf tube and 50 µl 0.2M sodium acetate (pH 4) was added to precipitate DNA. The mixture was vortexed and protein was removed by extraction with 500 µl citrate-saturated phenol and 100 µl chloroform:isoamyl alcohol (49:1). The homogenates were vortexed thoroughly and left on ice for 15 min before centrifugation at 14 000g for 20 min. The upper aqueous phase containing the RNA was transferred to fresh tubes, mixed with 200 µl isopropanol to precipitate the RNA, and left at -20°C for at least 1 h. The tube was centrifuged again for 20 min to pellet the RNA, the supernatant removed, and the pellet resuspended in 60 µl denaturing solution and 60 µl isopropanol. The RNA was reprecipitated at -20°C for at least 1 h and the RNA recovered by centrifugation for 10 min. The pellet was washed in 200 µl 70% ethanol, dried briefly at room temperature and resuspended in an appropriate volume of DEPC-treated H_2O. This was generally 30 µl for brain RNA, 50 µl for kidney RNA, 100 µl for liver RNA and 20 µl for cultured hepatocytes, depending on the size of the RNA pellet. RNA concentration and purity were estimated spectrophotometrically by measuring the absorbance at 260 nm and 280 nm. RNA was stored at -20°C for short periods of time prior to use.

2.8.2 RNA Electrophoresis and Capillary Transfer

Total RNA was electrophoresed on 1.2% agarose-2% formaldehyde denaturing gels to fractionate it. For a 100 ml gel, 1.2g of agarose was melted in 88ml DEPC-treated H_2O, allowed to cool slightly before the addition of 2 ml 40% formaldehyde and 10 ml 10x MOPS buffer, then poured into a gel mould with a comb whose tooth size allowed 25-40 μl to be loaded into each well. After setting, the gel was soaked in 1x MOPS buffer in the gel tank for approximately 30 min. Aliquots of total RNA from tissues (20 μg) or cultured hepatocytes (half the total RNA isolated from one dish) were adjusted to a volume of 10 μl by the addition of DEPC-H_2O or by volume reduction in a sample concentrator as required. 10 μl deionised formamide, 2.5 μl formaldehyde and 2.5 μl 10x MOPS were added to each sample to give a total volume of 25 μl and the RNA was denatured by heating at 65°C for 15 min. 1 μl of 10 mg/ml ethidium bromide was added to each 20 μl of loading buffer, and 2 μl of this was added to each RNA sample before loading onto the gel. Gels were routinely run at 100V for 2-8 h depending upon gel size. The gel was soaked in 20x SSC for 15 min, and photographed under UV light before blotting onto a nitrocellulose (Hybond C Extra) or nylon (Hybond N) membrane. The gel was laid onto a wick of Whatman 3MM filter paper draped over an upturned gel mould running into a plastic tray containing 20x SSC. The membrane (cut to exactly the same size as the gel) was placed on top of the gel, 3 sheets of Whatman 3MM filter paper were placed over the membrane and a wad of paper towels on top of this to draw the 20x SSC. A weight balanced on a glass plate was placed on top of the apparatus to secure it, and capillary transfer allowed to take place at room temperature overnight. The efficiency of transfer was checked under UV light, the membrane photographed and then baked between two sheets of Whatman 3MM paper at 80°C for 2 h.

2.8.3 Hybridisation to ^{32}P-Labelled cDNAs

Nitrocellulose and nylon membranes were rehydrated in 20x SSC after baking, rolled up and put into a Hybaid mini hybridisation bottle containing 9 ml pre-hybridisation buffer, taking care to ensure there were no air bubbles between the filter and the bottle. Filters were prehybridised for at least 2 h at 55°C whilst rotating in a Hybaid mini hybridisation oven before addition of the random-primed ^{32}P-labelled cDNA probe, prepared as described in section 2.7.6. The filters were hybridised overnight at 55°C. The following day the probe was disposed of, and the

filters washed in the hybridisation bottle with approximately 50 ml of 1x SSC and 0.1% SDS for a total of 3 washes, each for 20 min at room temperature. This was followed by a stringent wash in 0.3x SSC and 0.1% SDS at 55°C for 15-30 min depending on the level of radioactive signal remaining on the membrane. Filters were then wrapped in clingfilm and exposed to Reflection NEF-495 (Dupont) or Kodak X-OMAT AR (Sigma) autoradiography film at -70°C overnight against an intensifying screen. Films were developed in Kodak D19 developing solution (Ilford) for 2 min, and fixed in a 1 in 5 dilution of Amfix fixative (Champion) for 2 min. Signals were quantified by computer densitometry (Seescan, Cambridge, UK) and filters were re-exposed for time intervals as necessary to ensure that the signal density was within the linear range. They were then rehybridised with 7S or transferrin in exactly the same way as above, to control for RNA loading. If filters were to be rehybridised with another probe, they were stripped by pouring boiling water over them, and the stripping was checked by exposing the filters overnight. Signals were expressed as a ratio of the optical density of the signal of interest to 7S which is a ribosomal RNA found in relative abundance in numerous cells (Balmain et al., 1982) and can therefore be used to control for RNA loading on northern analysis, or as a ratio of the optical density of the signal of interest to transferrin in the experiments described in sections 4.2.1.2 and 4.2.1.3, as expression of transferrin is not altered by E_2 or glucocorticoid.

2.9 Statistics

All data, where statistically analysed as indicated in the text, were compared by Analysis of Variance followed by post hoc tests or by the Student's t-Test, as appropriate. Significance was set at $p<0.05$. Values are expressed as means ± SEM. Where data is expressed as a percentage of control values for clarity, statistical analysis was performed on raw data.

CHAPTER 3

THE FUNCTION AND REGULATION OF 11ß-HSD-1 IN CELL CULTURE

3.1 Introduction

The highest levels of 11ß-HSD-1 are found in the liver (Monder & Shackleton, 1984) which also expresses high levels of glucocorticoid receptors and it has therefore been proposed that the role of hepatic 11ß-HSD-1 is to modulate glucocorticoid access to hepatic GR (Whorwood et al., 1991). 11ß-HSD-1 is bi-directional in tissue homogenates and purified microsomal fractions (Lakshmi & Monder, 1988). However, transfection studies have indicated that the reaction direction of 11ß-HSD-1 varies between clonal cell types, with bi-directional activity in some cells such as CHO cells (Agarwal et al., 1989), but predominant 11ß-reduction (reactivation of inert 11-keto metabolites) in others such as TBM and COS-7 cells (Duperrex et al., 1993; Low et al., 1994a). The reaction direction has been proposed to reflect intracellular co-substrate (NADP/NADPH) ratios, with NADP favouring 11ß-dehydrogenase activity and NADPH favouring 11ß-reduction (Agarwal et al., 1989). pH and glycosylation status have also been implicated in control of direction (Deckx & De Moore, 1966; Agarwal et al., 1989). *In vitro*, acid pH favours reduction and alkaline pH favours dehydrogenase activity, whilst inhibition of glycosylation selectively inhibits dehydrogenase activity leaving reductase activity unaffected (Agarwal et al., 1989). Clearly the cellular context is crucial to the direction of 11ß-HSD-1 enzyme activity.

The reaction direction of 11ß-HSD-1 in liver, which highly expresses this isoform without detectable 11ß-HSD-2 activity or mRNA expression (Brown et al., 1993; Albiston et al., 1994; Brown et al., 1996), is unknown, although indirect evidence suggests that in liver 11ß-reductase predominates. The effluent of perfused cat liver has a high F to E ratio (Bush, 1969), and following the oral administration of E in humans, peripheral plasma levels of F but not E rise (Walker et al., 1992), suggesting that E is activated to F by 11ß-reductase on first pass through the liver. However, the reaction direction of 11ß-HSD-1 in hepatocytes has not been directly addressed.

Hepatic 11ß-HSD-1 is regulated *in vivo* by various hormones, including glucocorticoids, insulin, T_3, GH and sex steroids, in a tissue- and developmentally-specific manner (section 1.3.4). Whether any of these hormonal effects are mediated directly upon hepatocytes remains unknown, and such studies have been hampered by the absence of liver-derived clonal cell systems which express 11ß-HSD-1.

The aim of the work described in this chapter was to establish primary hepatocyte cell cultures optimal for the maintenance of 11ß–HSD-1 activity and mRNA expression, in which to (a) investigate the reaction direction of hepatic 11ß-

HSD-1, (b) investigate the hormonal regulation of hepatic 11ß-HSD-1 and (c) to determine the factors which influence 11ß-HSD-1 reaction direction *in vivo*.

3.2 Results

3.2.1 Optimisation of Culture Conditions for Maintenance of 11ß-HSD Activity and Measurement of Enzyme Direction

In preliminary experiments, carried out by Dr. Karen Chapman and Mrs. Ruth Wallace, primary hepatocytes plated onto either plastic culture dishes or collagen-coated dishes either did not attach well to the dishes or showed loss of 11ß-HSD-1 mRNA expression in otherwise viable primary hepatocyte cultures. This was borne out by two experiments in which I cultured hepatocytes on dishes coated with Rat Tail Collagen Type 1 gel and on dishes coated with Matrigel (section 2.3.2) for comparison of 11ß-HSD-1 mRNA expression. In one experiment, expression was completely lost by 48 h on dishes coated with collagen gel, and in the other, expression persisted at 96 h, but at a lower level than on Matrigel (data not shown). In contrast, maintenance of 11ß-HSD-1 mRNA expression over an extended period of time of at least 14 d was achieved by culturing hepatocytes on Matrigel (a gel matrix extracted from the EHS mouse tumour) (Fig. 3.1). Therefore all further experiments utilised hepatocytes cultured on Matrigel.

A comparison between the maintenance of 11ß-HSD-1 activity in William's E medium which is serum-free and DMEM/F12 medium containing 5% Nu-serum, showed that activity was well maintained in both media and that the predominant reaction direction of 11ß-HSD-1 enzyme activity in intact cells was 11ß-reduction (Fig. 3.2). However, after 15 d in culture, 11ß-HSD-1 activity was higher in cultures maintained in DMEM/F12, and this discrepancy increased over time, so that at 27 d cultures maintained in DMEM/F12 clearly had greater 11ß-HSD-1 activity (Fig. 3.2). In addition, changes in cell morphology were noted. After 4 d, cells cultured in William's E were starting to form aggregates, flattening and enlarging. By 7 d, all these cells were clumped. Cells in DMEM/F12 retained normal morphology with little clumping for up to 28 days and so DMEM/F12 was routinely used in experiments thereafter.

Measurement of enzyme activity in intact cells over 35 days confirmed that the predominant reaction direction was 11ß-reduction and that activity was maintained for up to 28 days (Fig. 3.3). 11ß-dehydrogenase activity was below the limit of detection, except for low levels over the first 2-3 days after plating and after

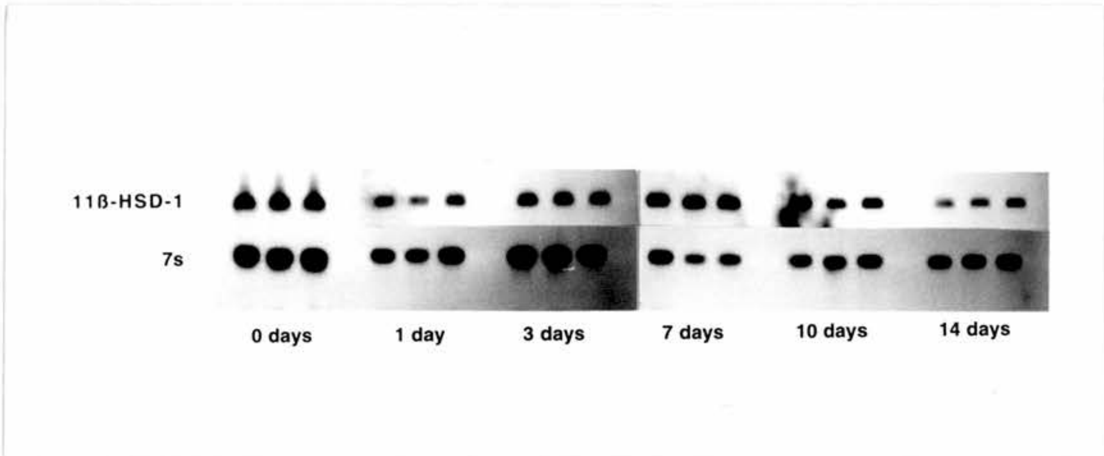


Figure 3.1: 11ß-HSD-1 mRNA expression in primary hepatocyte cultures.
Autoradiograph of a representative northern blot of RNA from freshly isolated hepatocytes (0 days) and from hepatocytes maintained for up to 14 d in culture, probed with 11ß-HSD-1 and 7S RNA cDNAs. Replicates represent RNA extracted from hepatocytes from separate culture dishes from the same culture preparation and are typical of results obtained from several different culture preparations. All samples were electrophoresed on a single northern gel and treated identically thereafter.

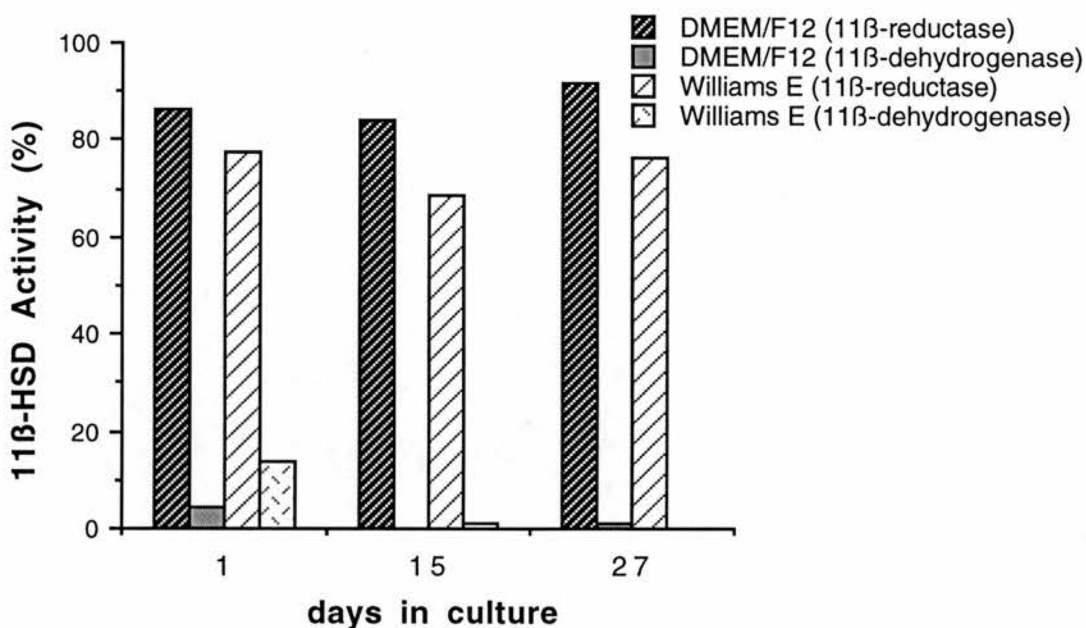


Figure 3.2: 11ß-HSD-1 activity of cultures maintained in DMEM/F12 or Williams E medium.

Hepatocytes were plated on Matrigel basement membrane matrix and maintained for up to 27 d. 11ß-HSD-1 activity was measured at intervals. Activity is expressed as % conversion of A to B (11ß-reductase) or B to A (11ß-dehydrogenase) in the medium overlying the cells after 2 hours. Results are from duplicate samples.

cells had been cultured for more than 28 days (Fig. 3.3). At both these time-points, but not during the intervening period, dead and dying cells were present and by 35 days, some cell loss became apparent.

That 11ß-reduction was the predominant reaction direction held true over a wide range of physiologically relevant substrate concentrations from 10^{-9}M to 10^{-7}M (Fig. 3.4). Product formation was linear with incubation time over at least 1 h (Fig. 3.5) and so measurements of levels of 11ß-HSD-1 activity for comparison were routinely estimated by steroid conversion in the medium at 30 min. Measurement of 11ß-HSD activity in cell homogenates demonstrated that 11ß-dehydrogenase activity (conversion of B to A) is readily detectable under these conditions with $53.9 \pm 0.1\%$ and $93.2 \pm 2.4\%$ conversion after 10 min and 60 min incubation, respectively. In contrast, 11ß-reductase activity (conversion of B to A) appeared to be unstable in homogenates as previously documented (Low et al., 1993; Lakshmi & Monder, 1988) and was undetectable after 60 min incubation.

3.2.2 Products of 11-Dehydrocorticosterone Metabolism in Hepatocyte Cultures

In order to detect products of metabolism other than B and verify that the major product of A metabolism in hepatocyte cultures was indeed B and not a compound which co-chromatographed in TLC analysis in these experiments, steroids present in the medium of cells incubated with [^{3}H]-A were extracted and separated by HPLC. Radiolabelled peaks were compared to the elution profiles of steroid standards of the major hepatic metabolites of A and B (Table 2.3) to identify the products. A representative HPLC trace of the UV absorbance of the steroid standards and of the radiolabelled peaks is shown in Fig 3.6. B was the major product of A metabolism ($86.3\pm0.6\%$) over a 1 h assay, and another smaller peak corresponding to 3ß5α–tetrahydrocorticosterone ($13.7\pm0.6\%$) was identified.

3.2.3 11ß-Reductase Activity Predominates Under Conditions of Oxidative stress, Metabolic Inhibition and Altered pH in Hepatocyte Cultures

11ß-HSD-1 reaction direction has been proposed to reflect intracellular co-substrate (NADP/NADPH) ratios, with NADP favouring 11ß-dehydrogenase activity and NADPH favouring 11ß-reduction (Agarwal et al., 1989). pH has also been implicated in control of direction, *in vitro*, acid pH favours reduction and alkaline pH favours dehydrogenase activity (Deckx & De Moore, 1966; Agarwal et al., 1989). The experiments described below were designed to determine whether

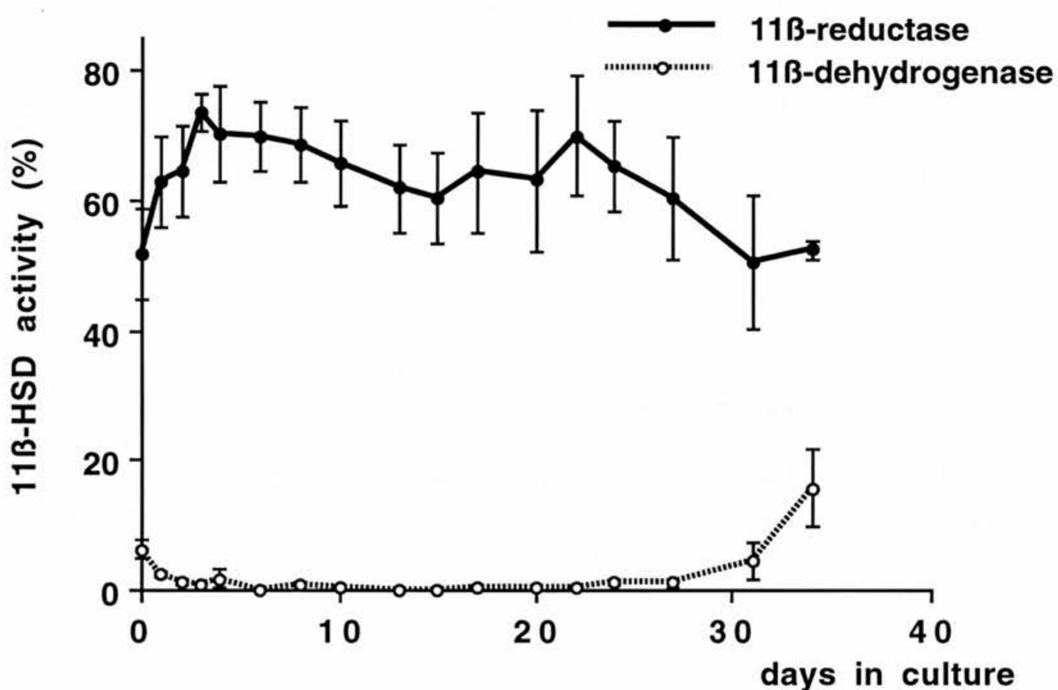


Figure 3.3: 11ß-HSD-1 activity in primary hepatocyte cultures.

Hepatocytes were plated on Matrigel basement membrane matrix and maintained in hepatocyte culture medium as defined in section 2.3.2 for up to 35 days. 11ß-HSD-1 activity was measured at intervals. Activity is expressed as % conversion ± SEM of A to B (11ß-reductase) or B to A (11ß-dehydrogenase) in the medium overlying the cells after 60 min, n=2-6. Results are from 3 separate culture preparations.

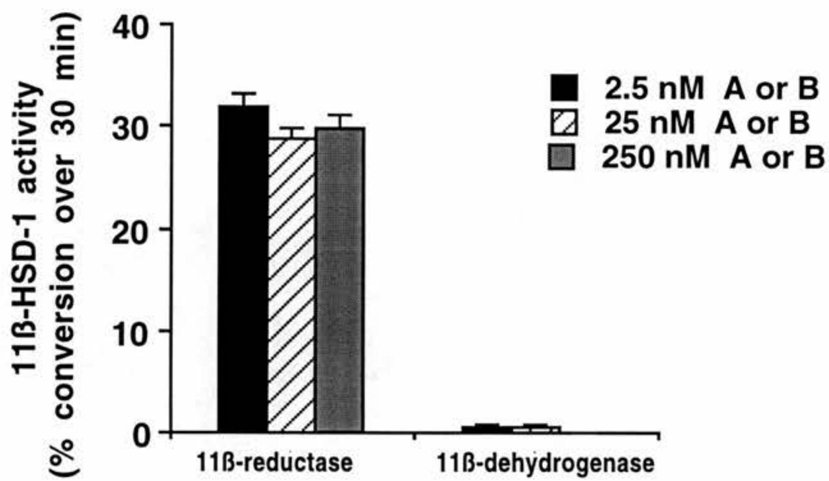


Figure 3.4: 11ß-HSD-1 activity in intact hepatocytes over a range of substrate concentrations.

Activity is expressed as % conversion of A to B (11ß-reductase) or B to A (11ß-dehydrogenase) in the medium overlying the cells after 30 min, n=3.

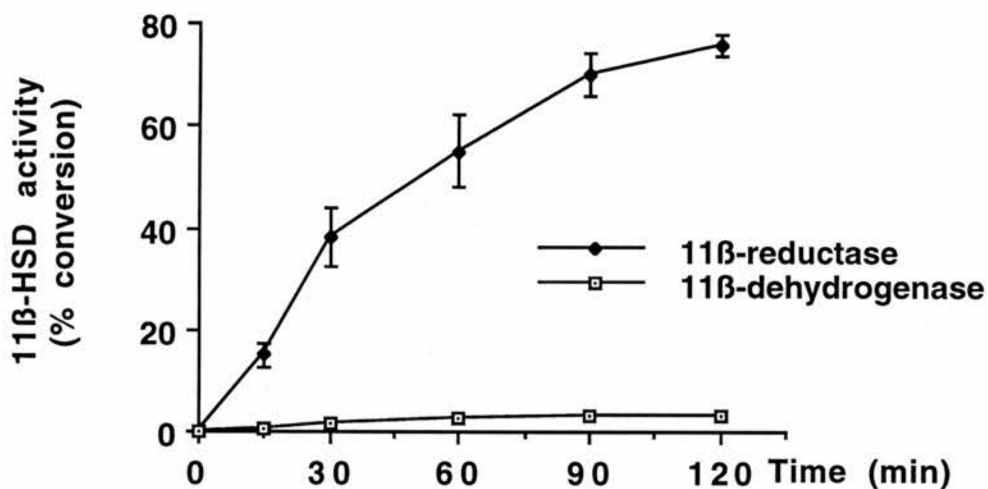


Figure 3.5: Time course of 11ß-HSD-1 activity in intact hepatocytes.
Conversion of substrate to product by 11ß-HSD-1 activity was measured over a time course of 120 min. Activity is expressed as % conversion of A to B (11ß-reductase) or B to A (11ß-dehydrogenase) in the medium overlying the cells.

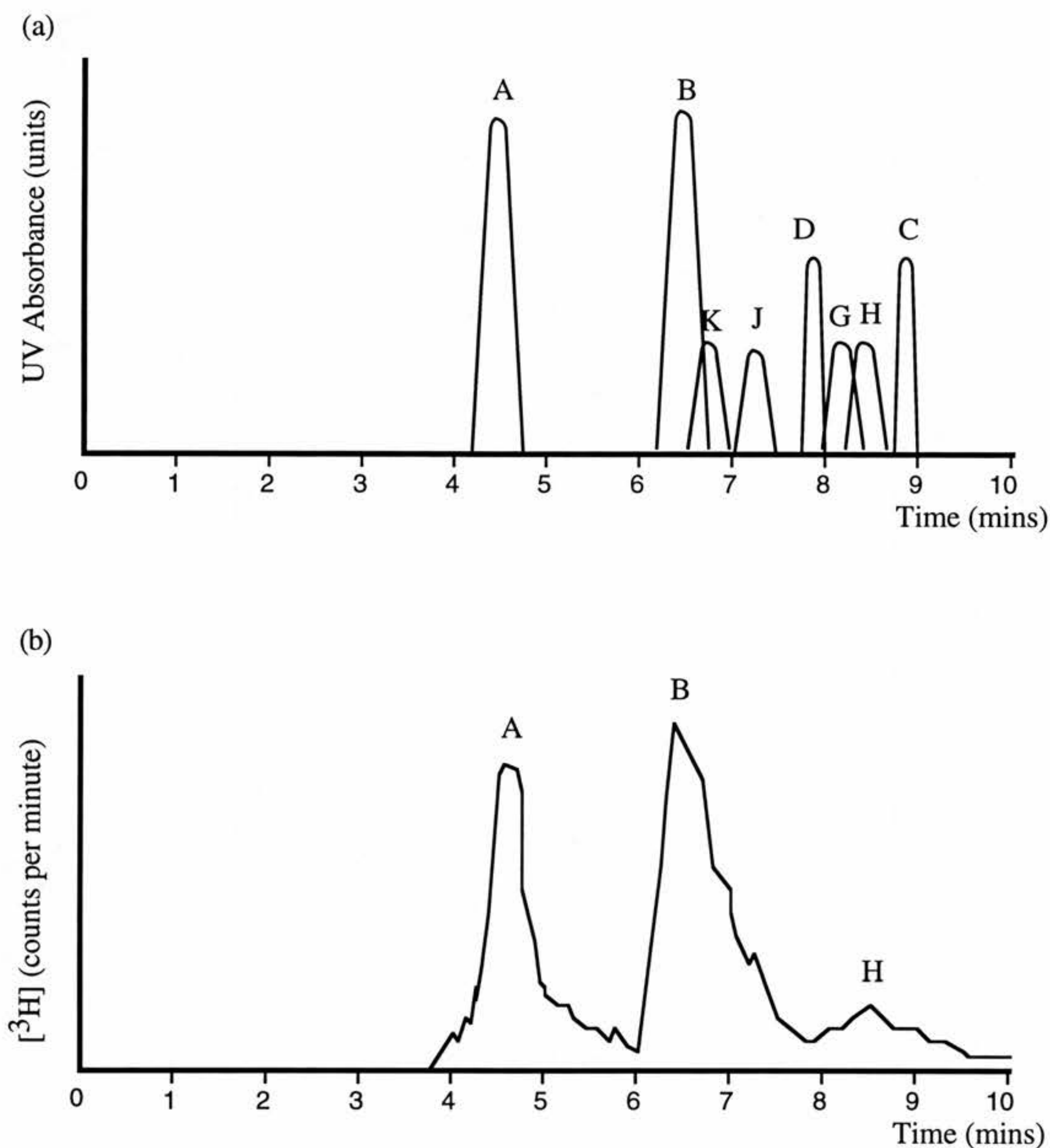


Figure 3.6: Products of 11-dehydrocorticosterone metabolism in hepatocyte cultures.

(a) Elution profiles of steroid standards of the major hepatic metabolites of A and B as measured by UV absorbance at 254nm. A=11-dehydrocorticosterone, B=corticosterone, C=5α-dihydrocorticosterone, D=5ß-dihydrocorticosterone, G=3α5α–tetrahydrocorticosterone, H=3ß5α–tetrahydrocorticosterone, J=3α5ß-tetrahydrocorticosterone, K=3ß5ß-tetrahydrocorticosterone.

(b) Representative HPLC trace of the radiolabelled peaks in an extract of medium from hepatocyte cultures incubated with [^{3}H-A].

alteration of intracellular co-substrate or pH could influence the direction of 11ß-HSD-1 in hepatocyte cultures.

3.2.3.1 Oxidative stress of Hepatocyte Cultures

Oxidative stress in cells results in anaerobic respiration, thus increasing intracellular NADP levels at the expense of NADPH, and lowering the intracellular pH through the production of lactic acid. Thus, whilst on the one hand, the altered co-substrate ratio would be predicted to favour 11ß-dehydrogenation, the change in pH should favour 11ß-reduction.

Hepatocyte cultures were exposed to O_2 concentrations by gassing, as described in section 2.3.4 (that is, 2.5% O_2 or 21% O_2), and incubated in sealed containers to maintain the required O_2 tension, or they were maintained in their normal environment (also 21% O_2) in the cell culture incubator. 4 h later, medium was collected for lactate analysis. The gassed hepatocytes were then re-exposed to the O_2 concentrations as previously for 15 min, sealed, and incubated for a further 1 h during which 11ß-HSD-1 activity was measured in the usual manner. Finally, the lactate concentration in the medium overlying the cells at the end of the experiment was measured (Table 3.1).

Compared to the ungassed controls, hepatocyte cultures exposed to low O_2 or gassed with an equivalent O_2 concentration to their normal environment, showed a trend for the lactate concentration in the medium overlying the cells to be lower than those not gassed at all. Similarly, there was a trend for 11ß-HSD-1 activity to be higher in both the 11ß-reductase and 11ß-dehydrogenase directions in the gassed cells when compared to the control dishes. This experiment was performed only once as an inconclusive result was obtained, possibly due in part to leakage of gasses, or confounding effects due to lactate being a substrate for gluconeogenesis in hepatocytes.

3.2.3.2 Effects of Metabolic Inhibitors on Hepatocyte Cultures

Treatment of 3 day old hepatocyte cultures with 5mM NaN_3, 1mM KCN (both compounds are inhibitors of oxidative phosphorylation in cell culture systems at these concentrations (Sakaida et al., 1992, Prehn et al., 1993, Mason & Sweeny, 1994, Shetty et al., 1993) and hence increase intracellular NADPH levels at the expense of NADP) or 10mM ascorbic acid (which causes an increase in NADP, and therefore an increase in intracellular NADP/NADPH in cell culture systems

	Control (21% O_2)	2.5% O_2	21% O_2
[lactate] 4 h	0.98 M	0.58 M	0.38 M
[lactate] 1 h	0.57 M	0.30 M	0.35 M
11ß-reductase	70.0 %	64.5 %	63.9 %
11ß-dehydrogenase	7.4 %	3.4 %	2.6 %

Table 3.1:

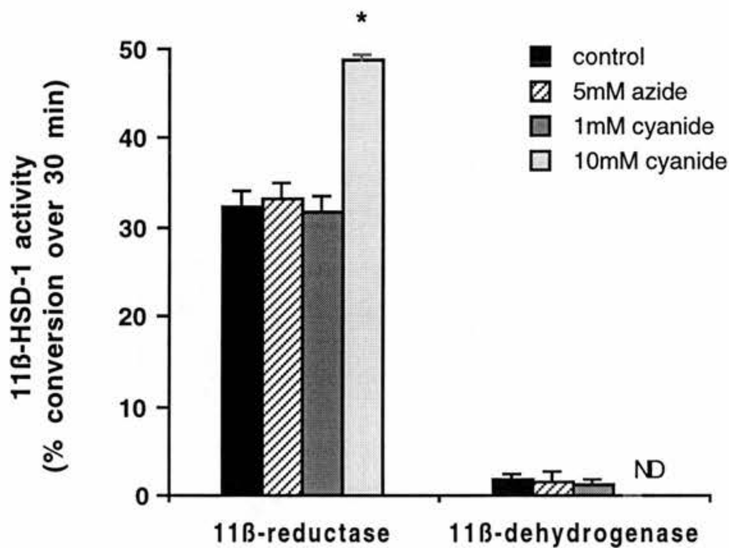
Lactate concentration in the medium overlying the cells and 11ß-HSD-1 activity following exposure to varying concentrations of O_2.
[lactate] 4 h is the concentration of lactate in the culture medium following 4 h incubation. [lactate] 1 h is the concentration of lactate in the culture medium following the 1 h incubation at the end of the experiment (refer to text for details). 11ß-HSD-1 activity is expressed as % conversion of A to B (11ß-reductase) or B to A (11ß-dehydrogenase) in the medium overlying the cells after 60 min. All figures are the average of duplicates from a single experiment.

(Schmidt et al., 1993)) as described in section 2.3.5, did not alter 11ß-HSD activity or direction (Fig. 3.7). Indeed, 11ß-dehydrogenase activity had a tendency to fall below detectable limits with both azide and cyanide treatment. However, KCN at 10mM did increase 11ß-reductase activity, whilst 11ß-dehydrogenation was undetectable (Fig. 3.7).

To order to ascertain that intracellular co-substrate ratios had been altered in hepatocytes, NADP and NADPH concentrations were measured following incubation with sodium azide or KCN (Fig. 3.8). 1mM KCN decreased NADP levels in comparison with extracts from untreated cells, and 5mM azide decreased NADP to levels below detection ($<1x10^{-8}$M), demonstrating that the metabolic inhibitors (at the concentrations used) significantly depleted NADP, whilst having no effect on NADPH levels within the hepatocytes. This represents a large alteration in the intracellular co-substrate ratio (Fig. 3.8).

3.2.3.3 Effects of pH Manipulations on 11ß-HSD-1 in Hepatocyte Cultures

11ß-HSD activity was measured in primary hepatocytes cultured in medium adjusted to pH 6.2, pH 7.4 or pH 8.0. Neither 11ß-reductase activity (conversion of



(b)

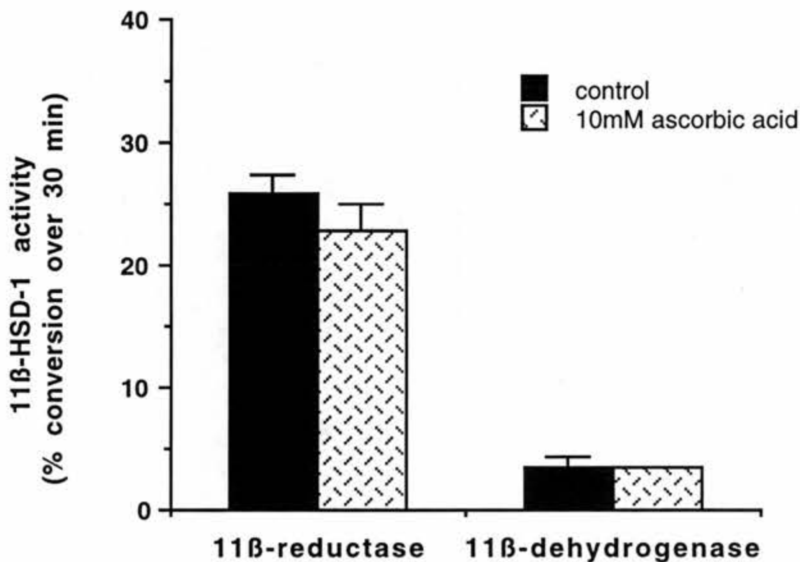


Figure 3.7: Effect of metabolic manipulations on 11ß-HSD-1 activity in hepatocyte cultures.

a) Hepatocytes were incubated for 4 h with 5 mM sodium azide, 1 mM KCN or 10 mM KCN before 11ß-HSD-1 activity was measured. n=7 for 5 mM azide and 1 mM KCN, n=3 for 10 mM KCN. ND=not detectable. Results are from 3 separate culture preparations.

b) Hepatocytes were incubated for 4 h with 10mM ascorbic acid before 11ß-HSD-1 activity was measured. n=6.

Values are expressed as % conversion of A to B (11ß-reductase) or B to A (11ß-dehydrogenase) after 30 min. *P<0.05 vs. control.

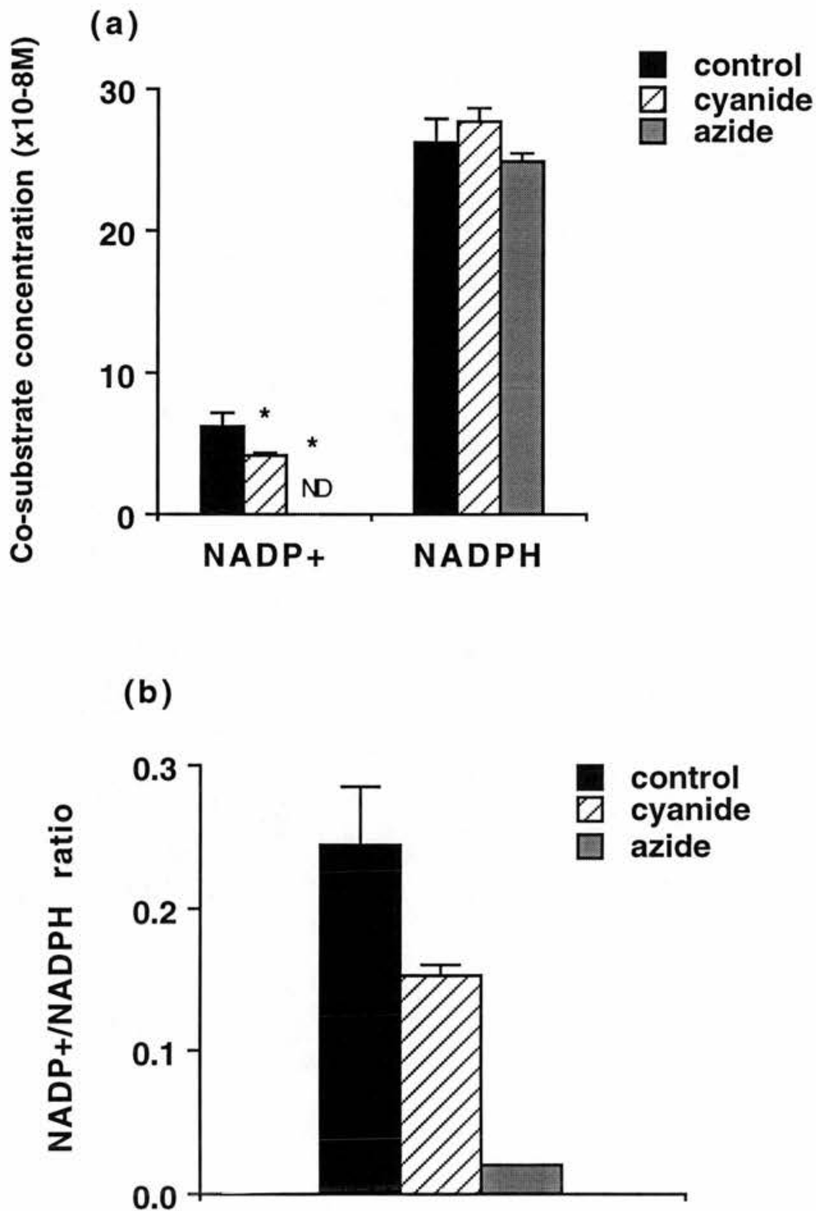


Figure 3.8: Effect of metabolic inhibitors on (a) NADP/NADPH levels and (b) NADP/NADPH ratios in hepatocyte cultures.

Hepatocytes were incubated for 4 hours with 5 mM sodium azide or 1 mM KCN before extraction and quantitation of NADP and NADPH by enzymatic cycling. Values are expressed as (a) the concentration of co-substrate (b) NADP/NADPH ratio in sample extract. ND=not detectable. *$P<0.05$ vs. control.

A to B in 30 min) nor 11ß-dehydrogenase activity (conversion of B to A in 30 min) were altered (Fig. 3.9).

3.2.4 11ß-HSD-1 Expression in Y1 Adrenocortical Cells

To date CHO cells are the only cell type, either of transfected cell lines or primary cells, which display 11ß-dehydrogenase activity in intact cells that is clearly attributable to 11ß-HSD-1 (as opposed to 11ß-HSD-2). CHO cells are steroidogenic, in contrast to other cell lines in which 11ß-HSD-1 has been expressed, which raised the question of whether an intrinsic difference in the cellular localisation of the 11ß-HSD-1 protein in steroidogenic cells might account for the observed difference in reaction direction. To examine the reaction direction of 11ß-HSD-1 in another steroidogenic cell line, Y1 adrenocortical cells (Yasamura et al., 1966) were transfected with the rat 11ß-HSD-1 expression plasmid SL1 (Low et al., 1994a).

Y1 cells transiently transfected with the vector pJ3 expressed no detectable 11ß-reductase activity over a 24 hour assay (Fig. 3.10). However, cells transfected with pJ3 contained clearly detectable endogenous 11ß-dehydrogenase activity (likely to be due to 11ß-HSD-2). In contrast, 11ß-reductase activity was readily apparent in Y1 cells transfected with pSL1; furthermore, 11ß-dehydrogenase activity was reduced by approximately 50% (Fig. 3.10).

When measured in cell homogenates, 11ß-dehydrogenase activity was clearly measurable in homogenates of cells transfected with pSL1 (9.7±1.9% conversion of B to A), whilst in mock transfected cells or cells transfected with pJ3, 11ß-dehydrogenase activity was detectable at much lower levels (2.3±0.2% conversion of B to A).

3.2.5 Hormonal regulation of 11ß-HSD-1 in hepatocytes in culture

Glucocorticoids, T_3, GH and sex steroids all regulate hepatic 11ß-HSD-1 *in vivo* (section 1.3.4). Whether any of these hormonal effects are mediated directly upon hepatocytes remains unknown, and so I have examined the effects of these hormones and of insulin (which regulates 11ß-HSD activity in cultured fibroblasts) on primary cultures of rat hepatocytes *in vitro*.

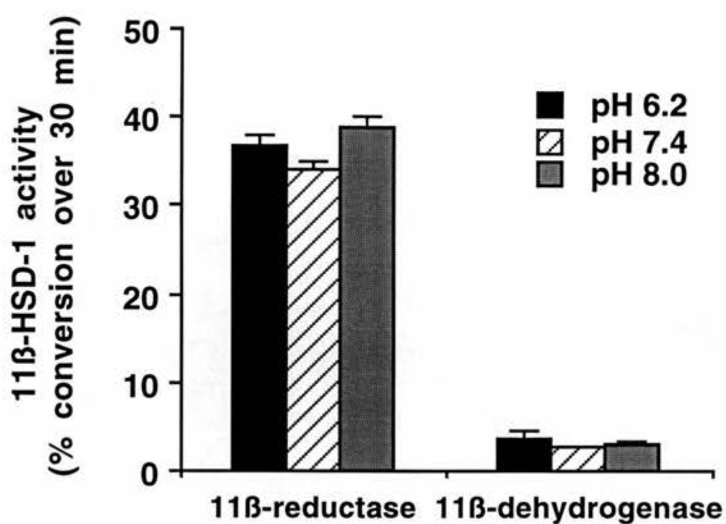


Figure 3.9: Effect of pH on 11ß-HSD-1 activity in hepatocyte cultures. Hepatocytes were incubated with culture media of pH 6.2, pH 7.4 or pH 8.0 for 4 hours before 11ß-HSD-1 activity was measured. Values are expressed as % conversion A to B (11ß-reductase) or B to A (11ß-dehydrogenase) after 30 min.

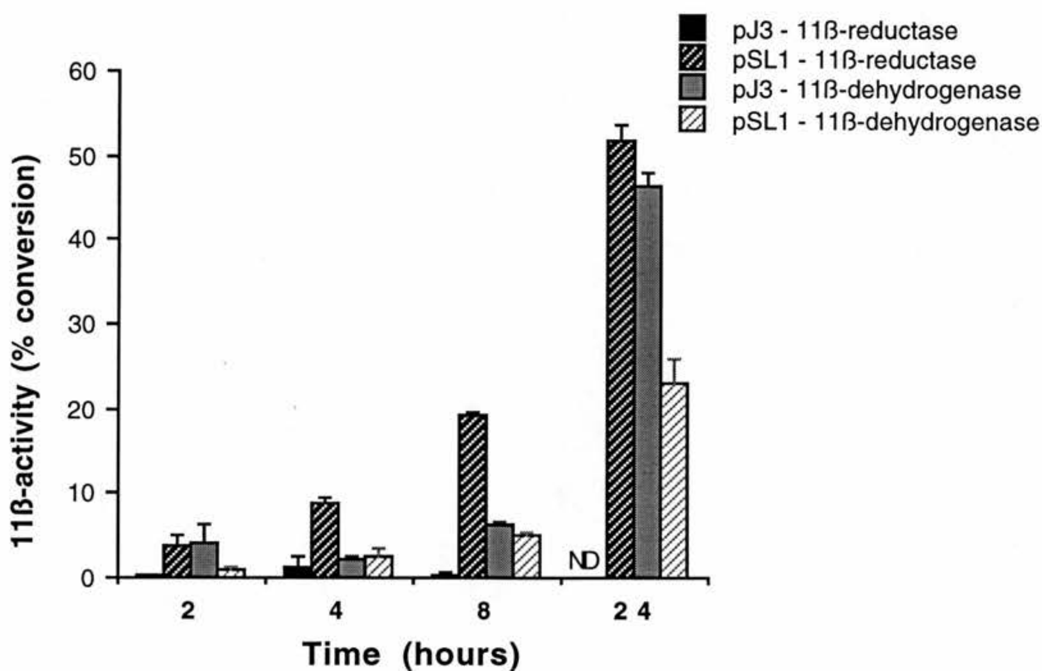


Figure 3.10: 11ß-HSD activity in Y1 adrenocortical cells transiently transfected with 11ß-HSD-1 cDNA.

Activity is expressed as % conversion of A to B (11ß-reductase) or B to A (11ß-dehydrogenase) in the medium overlying the cells. ND=not detectable. Results are the means of triplicate samples.

3.2.5.1 The Effects of Glucocorticoids and Insulin Upon 11ß-HSD-1 Activity and mRNA Expression in Hepatocyte Cultures

In order to examine the effects of glucocorticoid and insulin on 11ß-HSD-1 activity in hepatocytes, Nu-serum was charcoal-stripped to remove endogenous steroids and small peptides before addition to the medium (insulin levels were decreased >5000-fold and F became undetectable - section 2.3.10). 11ß-HSD activity was decreased from 31.2 ± 3.0% to 23.4 ± 0.7% conversion of A to B in hepatocytes cultured for 12 d in medium containing charcoal-stripped Nu-serum. DEX (10^{-7}M) and insulin (1.3×10^{-7}M) were added to medium containing the stripped serum, both singly and in combination, to give concentrations reflecting those in the standard maintenance media. DEX increased both 11ß-HSD activity and 11ß-HSD-1 mRNA expression (Fig. 3.11). In contrast, insulin decreased 11ß-HSD-1 mRNA expression, although enzyme activity was not significantly altered by 12 d treatment with insulin (Fig. 3.11). Insulin also antagonised the increase in 11ß-HSD-1 mRNA by DEX when the hormones were added in combination (Fig. 3.11).

3.2.5.2 The Effects of Oestradiol, Growth Hormone and Thyroid Hormone Upon 11ß-HSD-1 Activity and mRNA Expression in Hepatocyte Cultures

Primary hepatocytes were cultured in the presence of E_2, GH, T_3 or a combination of these hormones, and 11ß-HSD-1 activity was measured every 3 d over a 12 d period. The 11ß-reductase activity of hepatocytes cultured for 12 d in the presence of hormones is shown in Fig. 3.12. No change in 11ß-HSD-1 activity was observed at any time in cells exposed to 10^{-8} M E_2, 0.05 i.u./ml GH, 10^{-8} M T_3 or a combination of E_2 and GH. In addition, 11ß-HSD-1 mRNA expression was unaltered by 12 d treatment with T_3. In case the effects of E_2 were obscured by high endogenous levels in the culture medium of oestrogenic compounds (phenol red is known to have oestrogenic activity at the levels used in normal cell culture medium (Berthois et al., 1986)), the effect of the oestrogen receptor antagonist tamoxifen was examined. Tamoxifen did not have an effect on 11ß-HSD-1 activity in cultured hepatocytes (either 11ß-reductase or dehydrogenase activities) over 12 d (data not shown), indicating that repression of 11ß-HSD-1 activity by high levels of oestrogenic compounds was not confounding experimental results.

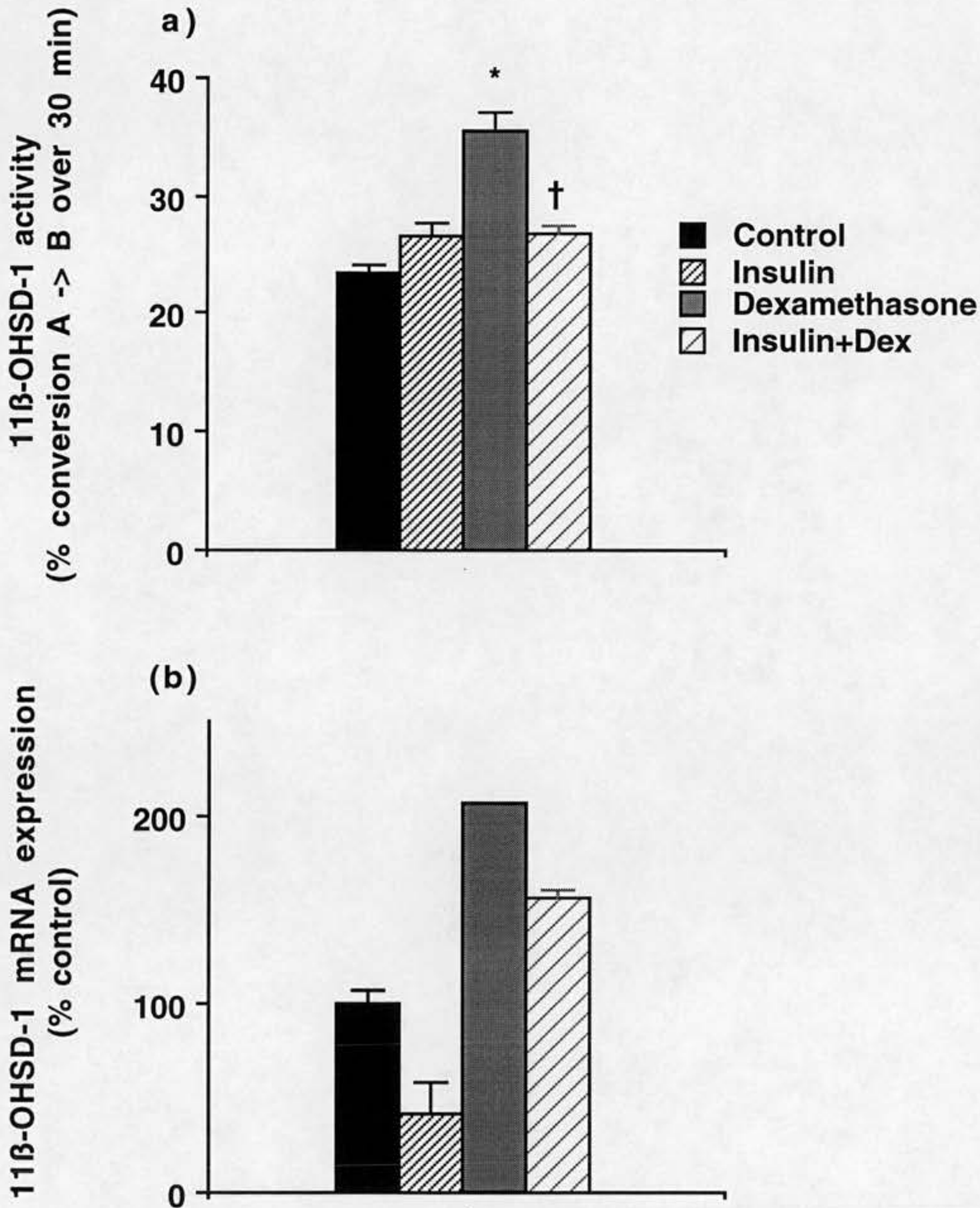


Figure 3.11: Regulation of 11ß-HSD-1 (a) activity and (b) mRNA expression in hepatocyte cultures by dexamethasone and insulin.

Hepatocytes were maintained in standard medium (5% Nu-serum) or medium containing 5% charcoal-stripped Nu-serum to which DEX (10^{-7} M) and/or insulin (1.3×10^{-7} M) were added. 11ß-HSD-1 activity and mRNA were quantified after 12 d. Activity is expressed as % conversion A to B (11ß-reductase) after 30 min. mRNA levels are expressed as a percentage of control levels normalised for loading. *$P<0.05$ vs. control, †$P<0.05$ vs. dexamethasone.

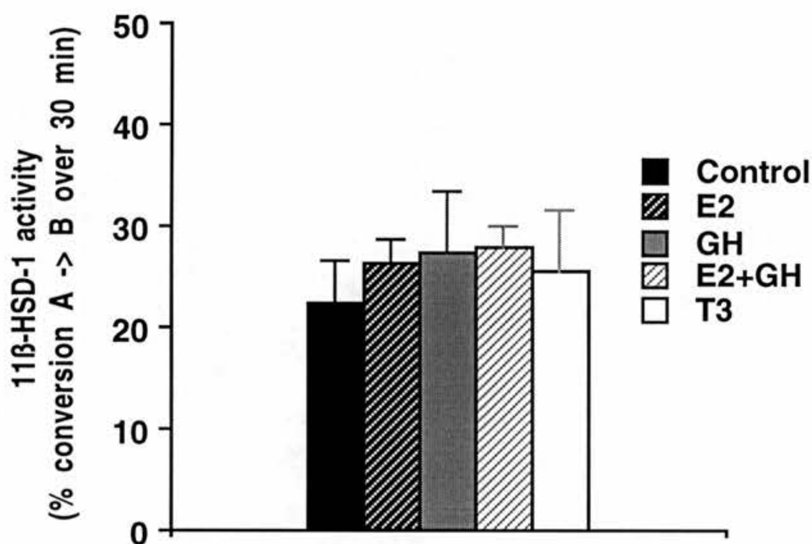


Figure 3.12: Effects of oestradiol, growth hormone and thyroid hormone on 11ß-HSD-1 activity in hepatocyte cultures.

Hepatocytes were maintained in media with E_2 (10^{-8} M), GH (0.05 i.u./ml) or T_3 (10^{-8} M) added. Activity was measured after 12 days. Values are expressed as % conversion A to B (11ß-reductase) after 30 min. Results are from 2 (T_3) or 3 (GH and E_2) separate culture preparations.

3.3 Discussion

I have established conditions for culture of primary rat hepatocyte cultures which maintain 11ß-HSD-1 activity for 4 weeks. Most cell culture conditions are associated with rapid loss of differentiated functions in primary hepatocyte cultures (Clayton et al., 1983, 1985), including loss of 11ß-HSD-1 mRNA expression and enzyme activity. When hepatocytes are dissociated they "turn off" unnecessary differentiated functions and begin to divide, a process perhaps akin to hepatic "regeneration" (Mischoulon et al., 1992; Mooney et al., 1992). Maintenance on a gel matrix (Matrigel) in media containing high levels of glucocorticoids, insulin and other peptides, presumably more closely resembles the milieu of the intact liver and thus allows isolated cells to maintain differentiated functions, including the expression of 11ß-HSD-1. The maintenance of 11ß-HSD-1 expression over an extensive time period makes this system useful for the investigation of the role and regulation of 11ß-HSD-1 in hepatocytes.

Using this cell culture system I demonstrated that 11ß-reduction is the primary activity of 11ß-HSD-1 in intact hepatocytes over a wide range of physiologically relevant substrate concentrations. The major product of A metabolism was B with the only other product identified as 3ß5α-tetrahydrocorticosterone, a further metabolite of B resulting from the activities of 5α-reductase and 3ß-HSD. 11ß-dehydrogenase activity was undetectable or barely detectable. Likewise, intact COS7 cells transiently transfected with 11ß-HSD-1 cDNA show only 11ß-reduction (Low et al., 1994a). However, in homogenates made from liver, primary cultures of hepatocytes or 11ß-HSD-1-transfected COS7 cells, 11ß-dehydrogenase activity is readily detectable (this chapter, Low et al., 1994; Lax et al., 1978; Low et al., 1993b) demonstrating that 11ß-HSD-1 in these cells is clearly capable of 11ß-dehydrogenation under certain conditions. The 11ß-dehydrogenation detectable in hepatocytes in culture over the first 2-3 days after plating and after 28 days, is present at a time where dead and dying cells are visibly present and may be due to enzyme released from dead or dying cells. In contrast, intact CHO cells transiently transfected with 11ß-HSD-1 cDNA show both dehydrogenation and reduction in approximately equal proportions (Agarwal et al., 1989).

The determination of 11ß-HSD-1 enzyme direction *in vivo* has been postulated to be determined by post translational modifications including glycosylation of the 11ß-HSD-1 protein. 11ß-HSD-1 has two putative N-linked glycosylation sites, and inhibition of glycosylation selectively inhibits

dehydrogenase activity leaving reductase unaffected (Agarwal et al., 1989). However, changes in glycosylation cannot explain the exclusive 11ß-reduction observed in intact COS-7 cells transfected with 11ß-HSD-1 cDNA, which nonetheless show 11ß-dehydrogenation when the enzyme is assayed in homogenates of the same transfected cells (Low et al., 1994a). In all cases, 11ß-dehydrogenase activity is readily detectable in homogenates, whereas the equilibrium shifts to favour reduction in most intact cells (this work and Agarwal et al., 1989; Low et al., 1994a, Rajan et al., 1995). Therefore the direction *in vivo* is likely to reflect some aspect of the cellular environment of the protein.

To date CHO cells are the only cell type in which 11ß-HSD-1 exhibits 11ß-dehydrogenase activity in intact cells. CHO cells are steroidogenic, which raised the question of whether a difference in the cellular localisation of the 11ß-HSD-1 protein in steroidogenic cells could account for the difference in reaction direction.

Y1 cells are derived from a mouse adrenocortical tumour (Cohen et al., 1957) and retain many tissue-specific characteristics including steroid synthesis and responsiveness to ACTH (Pierson, 1967; Yasumura et al., 1966). Steroidogenic Y1 adrenocortical cells were found to have intrinsic 11ß-dehydrogenase activity with no 11ß-reductase. However, transient expression of rat 11ß-HSD-1 cDNA in Y1 cells resulted in 11ß-reductase activity which antagonised the intrinsic 11ß-dehydrogenase activity. As expected, 11ß-dehydrogenase activity was readily detectable in cell homogenates. 11ß-HSD-2 is present in the mouse and rat adrenal (Y1 cells are derived from a mouse adrenocortical tumour (Cohen et al., 1957), but is absent from the human adrenal (Shimojo et al., 1996). These results are consistent with the presence of 11ß-HSD-2 in Y1 cells, which is counteracted by expression of 11-HSD-1 encoding 11ß-reductase activity. Thus it seems that in this cell line the 11ß-HSD-1 protein is again predominately a reductase. Why 11ß-HSD-1 in CHO cells demonstrates bi-directional activity remains unclear, but it is most likely a consequence of overexpression of the 11ß-HSD-1 protein in the transient expression system. CHO cells stably transfected with 11ß-HSD-1 show 11ß-reductase activity only (C Leckie & JR Seckl, personal communication). It is likely that transient transfection of 11ß-HSD-1 results in 11ß-HSD-1 protein levels which exceed the cells' ability to localise it correctly, allowing excess mislocalised protein to function as a dehydrogenase.

In principle, co-substrate availability might determine reaction direction, as it indeed does in homogenates, with NADP favouring dehydrogenation and NADPH favouring reduction (Agarwal et al., 1989). Oxidative stress results in anaerobic respiration, thus increasing intracellular NADP levels at the expense of NADPH, and

lowering the intracellular pH through the production of lactic acid. However, oxidative stress did not result in changes in 11ß-HSD-1 reaction direction or activity in primary hepatocytes, and the lactate concentration was decreased in cells exposed to low or normal levels of O_2 when compared to controls. It is possible that problems with gas leakage, compounded with the use of lactate as a substrate for gluconeogenesis in hepatocytes led to the lack of an interpretable result. Thus, the metabolic poisons azide and cyanide were used to put the cells under a greater oxidative stress, in a more easily controllable system. Although treatment with very high concentrations of cyanide (10 mM) did increase 11ß-reductase activity in keeping with the theory that a relative increase in NADPH will favour 11ß-reduction, the large changes in NADP/NADPH ratios achieved with metabolic poisons used here had remarkably little effect on reaction direction. These data suggest that the availability of NADP does not determine reaction direction *in vivo*. However, it remains to be determined whether limiting the availability of NADPH, particularly in the specific subcompartment containing 11ß-HSD-1, could shift the reaction direction in intact cells towards 11ß-dehydrogenation. Similarly, although in cell homogenates pH influences the equilibrium of 11ß-HSD-1 (reduction is favoured at acid pH and dehydrogenation at alkaline pH *in vitro* (Monder & Shackleton, 1984)) there was no alteration in enzyme direction or activity in intact hepatocytes over a range of extracellular pH. However, whether the intracellular pH was altered in this experiment is undetermined, and if it was altered, it still remains possible that within a subcellular compartment pH may be very different to that within the cell as a whole.

11ß-HSD-1 is a membrane-associated protein which shows a microsomal location (Monder & White, 1993), whilst 11ß-HSD-2, an exclusive dehydrogenase which is also a membrane-associated protein shows a different intracellular distribution to 11ß-HSD-1 (Brown et al., 1993). The key determinants of reaction direction probably reflect more than the mere presence of membrane (as this is also present in homogenates) and may be controlled by the sub-organelle microenvironment generated, for example, by neighbouring enzymes. The precise intracellular localisation of both isoforms of 11ß-HSD awaits the development of highly specific antisera suitable for electron microscopical studies.

Previous studies have shown that 11ß-HSD-1 is sexually dimorphic in the liver, with lower levels in females than males (Lax et al., 1978; Low et al., 1993). E_2 markedly attenuates hepatic 11ß-HSD-1 activity and mRNA expression *in vivo*, an effect that is, in part, mediated by GH (Low et al., 1994b). In primary hepatocyte cultures, neither E_2 nor GH affected 11ß-HSD-1 enzyme activity. The failure of GH

to regulate 11ß-HSD-1 in primary hepatocytes in culture might reflect an indirect mechanism of action. However, hepatic enzymes exhibiting sexual dimorphism have been demonstrated to be directly regulated by GH in hepatocyte cultures (Guzelian et al., 1988; Schuetz et al., 1990; Tollet et al., 1990), and so it is more likely to be due to a reduction in the number of GH receptors on the cells, the expression of which are variable in primary culture (Crabb & Roepke, 1987; Niimi et al., 1991). Other sexually dimorphic enzymes which contain a GH-response element in their promoter region lose their GH regulation in hepatocyte culture (Legraverend et al., 1992; Matsunaga et al., 1990; Yoshioka et al., 1990). Therefore, an alternative explanation has been proposed, such that the differences between *in vivo* and *in vitro* GH transcriptional responses may be due to the loss, *in vitro,* of a repressive function (Legraverend et al., 1992). The absence of effects of E_2 and T_3 presumably reflect the indirect nature of their effects upon hepatic 11ß-HSD-1, since loss of their receptors is unlikely and these receptors act directly upon target DNA (Ribeiro et al., 1995). Although sequences resembling E_2 or T_3 response elements are found within the 11ß-HSD-1 gene promoter (Moisan et al., 1992), the data presented here support *in vivo* evidence that the regulation of 11ß-HSD-1 expression by sex steroids, and possibly T_3, is mediated indirectly, in part via their effects on GH secretion. GH secretion is almost totally dependent on T_3 which also markedly increases GH mRNA and protein synthesis (Evans et al., 1992; Spindler et al., 1992; Peake at al., 1973; Hervas et al., 1975; Coiro et al., 1979). Indeed, the latter contention is supported by the sexually dimorphic effects of T_3 on 11ß-HSD-1 in rat liver, with thyroidectomy increasing 11ß-HSD activity in females, but decreasing activity in males (Lax et al., 1979).

In contrast, DEX and insulin clearly regulate 11ß-HSD-1 in hepatocyte cultures. Previous studies have shown that ADX of rats attenuates hepatic 11ß-HSD enzyme activity and also attenuates reactivation of E to F (presumably mediated by hepatic 11ß-HSD-1) (Nicholas & Lugg, 1982); administration of glucocorticoids to ADX rats increases hepatic 11ß-HSD-1 activity and mRNA (Low et al., 1994c). This appears to be a direct effect upon hepatocytes as DEX increased 11ß-HSD-1 activity and mRNA expression in hepatocyte cultures. The regulation of hepatic 11ß-HSD-1 by glucocorticoids is further explored in Chapter 6. The molecular mechanism is unknown, but sequences resembling the GRE have been reported in both human (Tannin et al., 1991) and rat (Moisan et al., 1992b) 11ß-HSD-1 genes, and data from transfection experiments of HepG2 cells with plasmids in which 11ß-HSD-1 promoter DNA is fused to a reporter gene suggest that a GRE lies within 1800 base pairs of the transcription start of 11ß-HSD-1 (Voice et al., 1996). The antagonism by

insulin of DEX-induced 11ß-HSD-1 activity and mRNA expression in cultured hepatocytes reflects similar regulation in cultures of human fibroblasts (Hammami & Siiteri, 1991). This is a common pattern of regulation, and many key metabolic enzymes in the liver are antagonistically regulated by insulin and glucocorticoids (section 1.4.4). The data presented here suggest similar controls apply to hepatic 11ß-HSD-1.

These data are evidence for the predominance of the 11ß-reductase activity of 11ß-HSD-1 in the intact liver. 11ß-HSD-1 is therefore likely to exert effects on glucocorticoid action within the liver via its effects on glucocorticoid metabolism and the possible significance of this is addressed in Chapter 4.

CHAPTER 4

THE ROLE OF HEPATIC 11ß-HSD-1: IN VIVO STUDIES

4.1 Introduction

GR are highly expressed in the liver (Wrange et al., 1979; Govindan et al., 1985), which is therefore a major target for glucocorticoid action. The tissue distribution of 11ß-HSD-1 is similar to that of GR, which has led to the proposal that 11ß-HSD-1 regulates ligand access to GR (Moisan et al., 1990b; Teelucksingh et al, 1990; Whorwood et al., 1991).

In the last chapter, I demonstrated that the predominant reaction direction of 11ß-HSD-1 in hepatocytes is 11ß-reduction, thus potentially increasing local concentrations of active glucocorticoids and potentiating intrahepatic glucocorticoid action. This has important implications for liver physiology. Glucocorticoids regulate the transcription of many of the key hepatic enzymes involved in carbohydrate and fat metabolism (section 1.4.4). It may be that 11ß-reductase activity is necessary to maintain adequate occupation of GR by ligand within the liver during fluctuations in circulating levels of glucocorticoids e.g. during the daily cortisol nadir (section 1.1.2).

Glucocorticoids antagonise the action of insulin (de Feo et al., 1989; Dinneen et al., 1993) within the liver by promoting hepatic glucose secretion (Rooney et al., 1994), an effect mediated in part by promoting the transcription of PEPCK (Friedman et al., 1993; Lamers et al., 1982; Sasaki et al., 1984), the rate-limiting enzyme in gluconeogenesis (reviewed by Hanson & Garber, 1972 and see section 1.4.4). Insulin resistance is associated with a number of diseases including obesity (Baron et al., 1991), NIDDM (Kolterman et al., 1981) and essential hypertension (Welborn et al., 1966; Ferrannini et al., 1987), and there is evidence that glucocorticoid metabolism is abnormal in these conditions (section 1.4.5).

Recent data have suggested that inhibition of 11ß-HSD (both isozymes) by CBX increases fasting hepatic insulin sensitivity in humans *in vivo*, consistent with decreased GR activation. This effect is most likely to be due to the attenuation of hepatic glucocorticoid regeneration by 11ß-reductase activity in the liver counteracting the F antagonism of insulin action (Walker et al., 1995). Thus it can be predicted that decreased 11ß-HSD-1 activity in the liver, by decreasing glucocorticoid action in the liver, will decrease the expression of hepatic glucocorticoid-inducible genes, and increase insulin sensitivity and glucose tolerance in the rat *in vivo.* I have tested this hypothesis in two model systems of decreased hepatic 11ß-HSD-1 activity; attenuation of hepatic 11ß-HSD-1 expression by E_2 administration and inhibition of 11ß-HSD activity by CBX administration. A third potential model for examination of these ideas, achieved by the creation of the

11ß-HSD-1 "knockout" mouse (Kotelevtsev et al., 1996) has been verified as providing the extreme case of a complete deficiency of 11ß-HSD-1.

In the rat liver, 11ß-HSD-1 shows pronounced sexual dimorphism, with two-fold lower activity in females (discussed in section 1.3.4). This effect is attributable to the sex steroid determination of patterns of GH secretion (section 1.3.4). E_2 has an additional tissue-specific repressive effect on 11ß-HSD-1 such that chronic E_2 administration almost completely suppresses hepatic and renal 11ß-HSD-1 expression in both male and female rats (Lax et al., 1978; Low et al., 1993; Low et al., 1994b). This regulation appears to be tissue- and isozyme-specific, as E_2 does not attenuate 11ß-HSD-1 expression in the hippocampus or 11ß-HSD-2 activity in the kidney (Low et al., 1993). In the experiments described in this chapter, I have exploited the selective suppression of hepatic 11ß-HSD-1 by E_2 *in vivo* in order to examine the effect of attenuated hepatic 11ß-HSD-1 on the expression of hepatic glucocorticoid-inducible genes.

CBX is the hemi-succinate derivative of GE (the active constituent of liquorice which inhibits renal 11ß-HSD activity). It has been demonstrated that CBX (Stewart et al., 1990), but not glyccyrhetinic acid (Walker & Edwards, 1994), inhibits hepatic 11ß-reductase *in vivo*. Walker et al. (1995) have shown that CBX administration to humans increases whole body insulin sensitivity, and so I have used CBX to inhibit 11ß-HSD in rats *in vivo* to investigate the effects of inhibition of hepatic 11ß-HSD-1 on insulin sensitivity and glucose tolerance and the expression of PEPCK. The disadvantage of CBX administration, is that it competitively inhibits the activity of both isozymes of 11ß-HSD.

As the proposed model for 11ß-HSD-1 function in liver also predicts that increased hepatic 11ß-HSD-1 activity should lead to increased glucocorticoid levels in the liver, and thus decreased insulin sensitivity, I have examined 11ß-HSD-1 activity in the obese fa/fa Zucker rat (Zucker & Zucker, 1967), which is a well established model of obesity and insulin resistance (Bray, 1977). Obese Zucker rats are hyperinsulinaemic, show reduced glucose tolerance (Rohner-Jeanrenaud et al., 1986) and are hypercorticosteronaemic (Krief & Basin, 1991), all of which are risk factors for the onset of NIDDM in humans (section 1.4.5).

4.2 Results

4.2.1 Effect of Attenuation of Hepatic 11ß-HSD-1 by Chronic Oestradiol Treatment on Hepatic Glucocorticoid Modulated Gene Expression

4.2.1.1 Effect of Chronic Oestradiol Treatment on 11ß-HSD-1 Activity and mRNA Expression

E_2 administration for 10, 21 and 42 d to gonadectomised male rats (section 2.6.2) resulted in marked decreases in hepatic 11ß-HSD activity (Fig. 4.1). However, 11ß-HSD activity was not completely abolished since prolonged (60 min) incubation of liver homogenates from rats given E_2 for 42 d showed detectable (12.2±4.7%) conversion of B to A. 11ß-HSD-1 mRNA expression fell to undetectable levels after 42 d of E_2 treatment (Fig. 4.2) and indeed was undetectable after 21 d treatment (Fig. 4.5) in accordance with previous studies (Low et al., 1993). 11ß-HSD activity in the hippocampus was unaltered by E_2 treatment for 21 or 42 d (Fig. 4.1). The control mRNA, transferrin, which is unaffected by glucocorticoids (Ruppert et al., 1990) was unaffected by E_2 treatment (Fig. 4.2).

4.2.1.2 Effect of Oestradiol on Hepatic Glucocorticoid-Inducible Gene Expression

To examine the effect of attenuated hepatic 11ß-HSD-1 activity on local glucocorticoid action, the expression of hepatic glucocorticoid-inducible genes was measured after 42 d of E_2. Northern analysis was used to measure expression of PEPCK, angiotensinogen, SAMS and TAT mRNA in the livers of control and E_2 treated animals. A representative northern blot of PEPCK is shown in Fig. 4.3. E_2 treatment dramatically decreased PEPCK mRNA whilst leaving transferrin mRNA unaffected (Fig. 4.3). Quantitation of mRNA expression demonstrated that PEPCK and angiotensinogen mRNA expression were significantly reduced by E_2 treatment, whilst SAMS and TAT mRNA expression were not significantly altered, although a downwards trend was noted (Fig. 4.4).

4.2.1.3 Effect of Oestradiol ± Adrenalectomy on Hepatic Glucocorticoid-Inducible Gene Expression

To examine whether these effects of E_2 on hepatic gene expression were mediated directly or were due to attenuated hepatic 11ß-HSD-1 activity resulting in

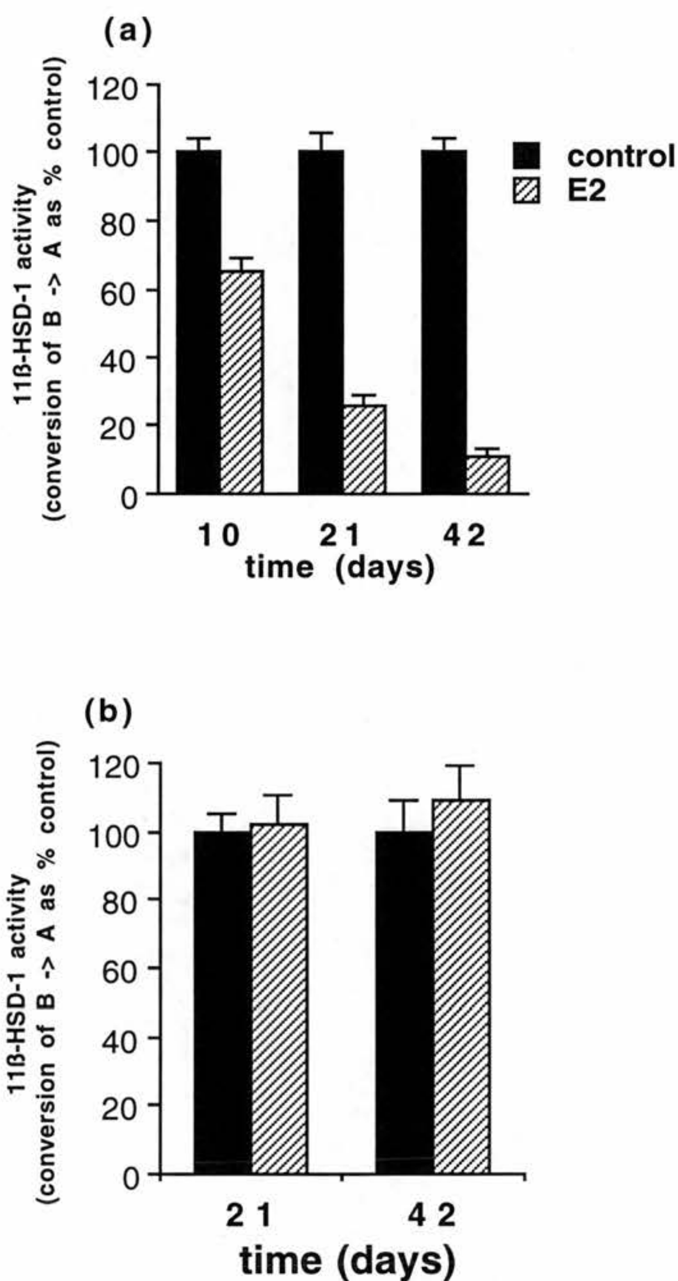


Figure 4.1: Effect of oestradiol administration on 11ß-HSD-1 activity.

E_2 repression of (a) hepatic and (b) hippocampal 11ß-HSD-1 activity. 11ß-HSD-1 activity was assayed in homogenates of liver from gonadectomised male rats receiving E_2 for 10, 21 and 42 d and homogenates of hippocampus from gonadectomised male rats receiving E_2 for 21 and 42 d. Data are expressed as a percentage of activity (±SEM) in control animals receiving vehicle. n=5-10. *$P<0.05$ compared with controls.

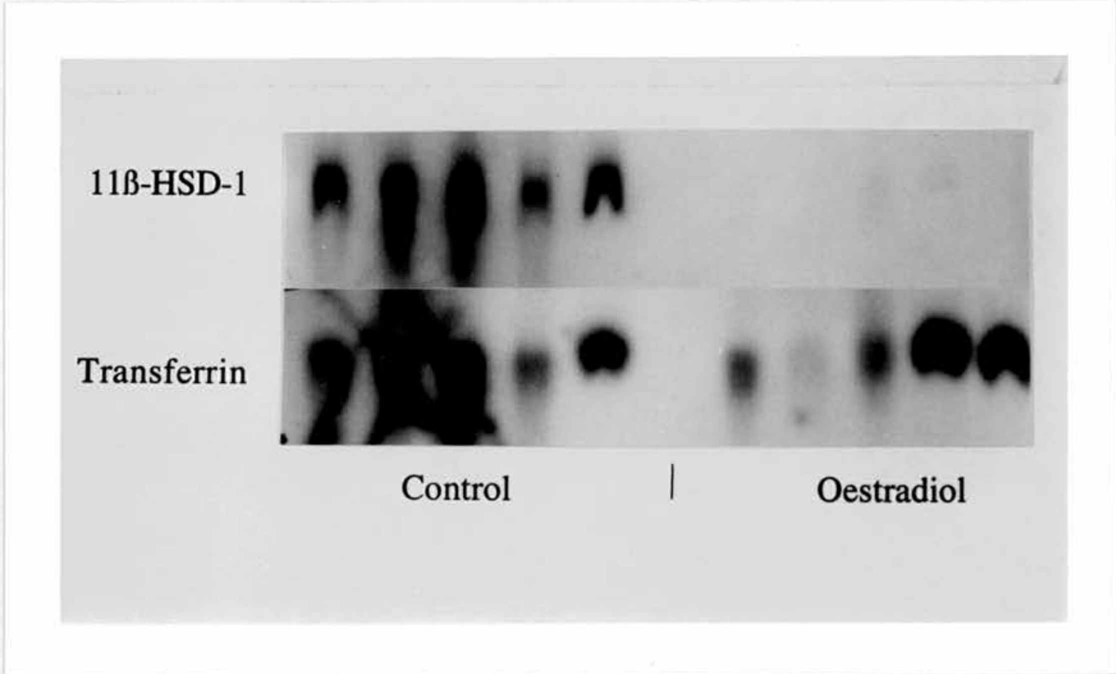


Figure 4.2: Oestradiol repression of hepatic 11ß-HSD-1 mRNA expression in liver. Autoradiograph of northern blots of RNA from the livers of gonadectomised male rats receiving E_2 for 42 days and from controls receiving vehicle. Each lane contains 20 µg total RNA from an individual animal. Hybridised to 11ß-HSD-1 and transferrin cDNAs. No 11ß-HSD-1 mRNA was detectable in RNA from E_2-treated animals.

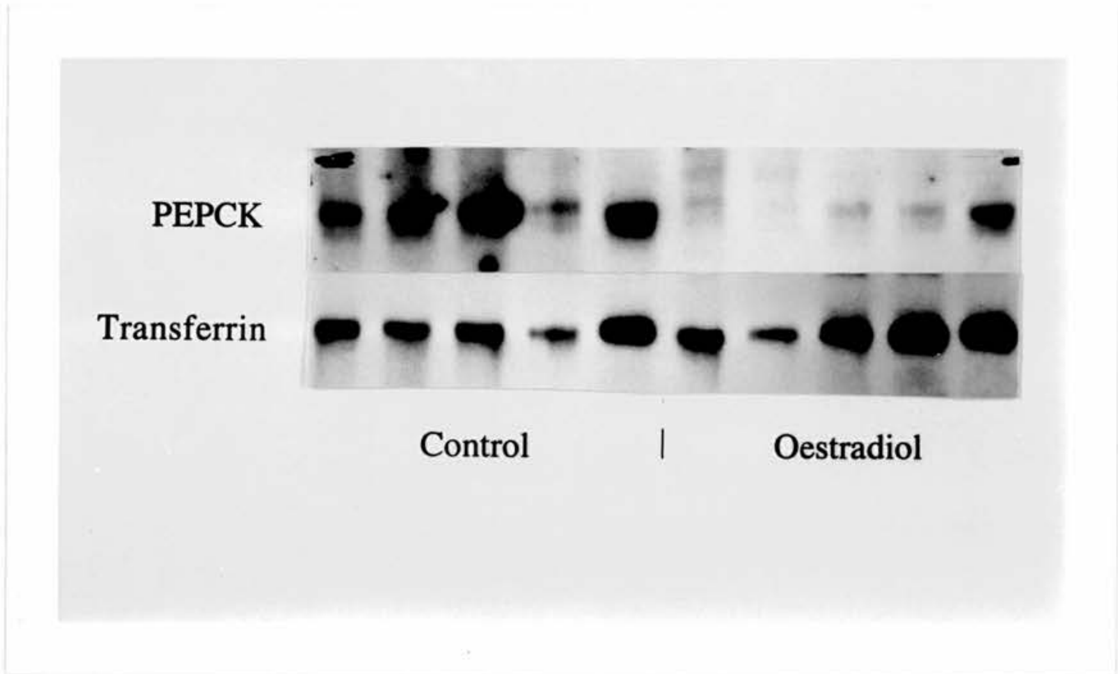


Figure 4.3: Effect of oestradiol on hepatic PEPCK mRNA expression in liver. Autoradiograph of a representative northern blot of RNA from the livers of gonadectomised male rats receiving E_2 for 42 d and from controls receiving vehicle. Each lane contains 20 μg total RNA from an individual animal. Hybridised to PEPCK and transferrin cDNAs.

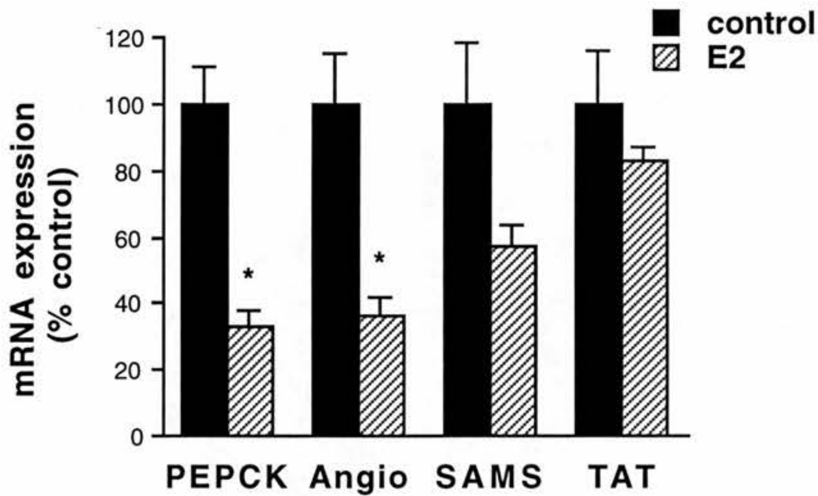


Figure 4.4: Effect of oestradiol on glucocorticoid-modulated hepatic gene expression.

mRNA levels from the livers of male gonadectomised rats receiving E_2 for 42 d and from controls receiving vehicle was quantified by northern analyses. Data are normalised to expression of transferrin, and expressed as a percentage of mRNA levels (±SEM) in control animals. n=5. *$P<0.05$ compared with controls.

altered glucocorticoid action in the liver, the effects of E_2 in ADX animals or sham ADX controls were examined (section 2.6.2). Animals were killed after 21 d. ADX increased hepatic 11ß-HSD-1 gene expression and activity compared with sham ADX controls (Fig. 4.5 and Fig. 4.6). E_2 reduced hepatic gene expression and activity in ADX rats, and although mRNA was higher than E_2-treated adrenally-intact rats, it was reduced compared with untreated controls (Fig 4.6).

Hepatic PEPCK gene expression was decreased after 21 d of E_2 treatment, albeit to a lesser extent than after 42 d (Fig. 4.7 and Fig 4.8). Unsurprisingly, ADX also attenuated hepatic PEPCK gene expression, but in ADX rats E_2 no longer reduced, but indeed increased PEPCK mRNA levels when compared with ADX alone. At this time point, E_2 had no significant effect upon angiotensinogen mRNA expression in adrenally-intact rats, although E_2 did increase angiotensinogen gene expression in ADX rats (Fig. 4.8). Neither SAMS nor TAT mRNA levels were affected by E_2 for 21 d (Fig. 4.8), consistent with the 42 d data. Interestingly, TAT mRNA levels were significantly decreased by ADX and E_2 markedly increased TAT mRNA expression in ADX animals (Fig. 4.8).

4.2.1.4 Effect of Oestradiol on Endogenous Hepatic Metabolism of Corticosterone

In order to determine whether the effects of E_2 upon glucocorticoid-sensitive mRNAs was due to actions upon other pathways of B metabolism in the liver, the metabolism of B in liver homogenates from E_2-treated (42 days) and control animals was measured (Table 4.1). All values are corrected for the values in blank (no homogenate) incubates. There were no differences between the HPLC steroid profiles (section 2.2.6) of the control and E_2-treated sample incubates except in the relative quantities of A and B, and no additional products of B metabolism were detected (Table 4.1). The recovery of [^{3}H]-B as ([^{3}H]-B + [^{3}H]-A) was 109±13% and 107±14% for the control and E_2-treated sample incubates, respectively.

4.2.2 The Effect of Carbenoxolone Administration on 11ß-HSD-1 and Glucose Tolerance

CBX administration for 14 d to intact male rats (section 2.6.3) had no significant effect on hepatic 11ß-HSD enzyme activity *ex vivo* at any of the dosages employed (Fig. 4.9). However 10 mg per day CBX did reduce hepatic mRNA encoding 11ß-HSD-1 (67±6% of control levels, P<0.05). 10 mg per day CBX had no

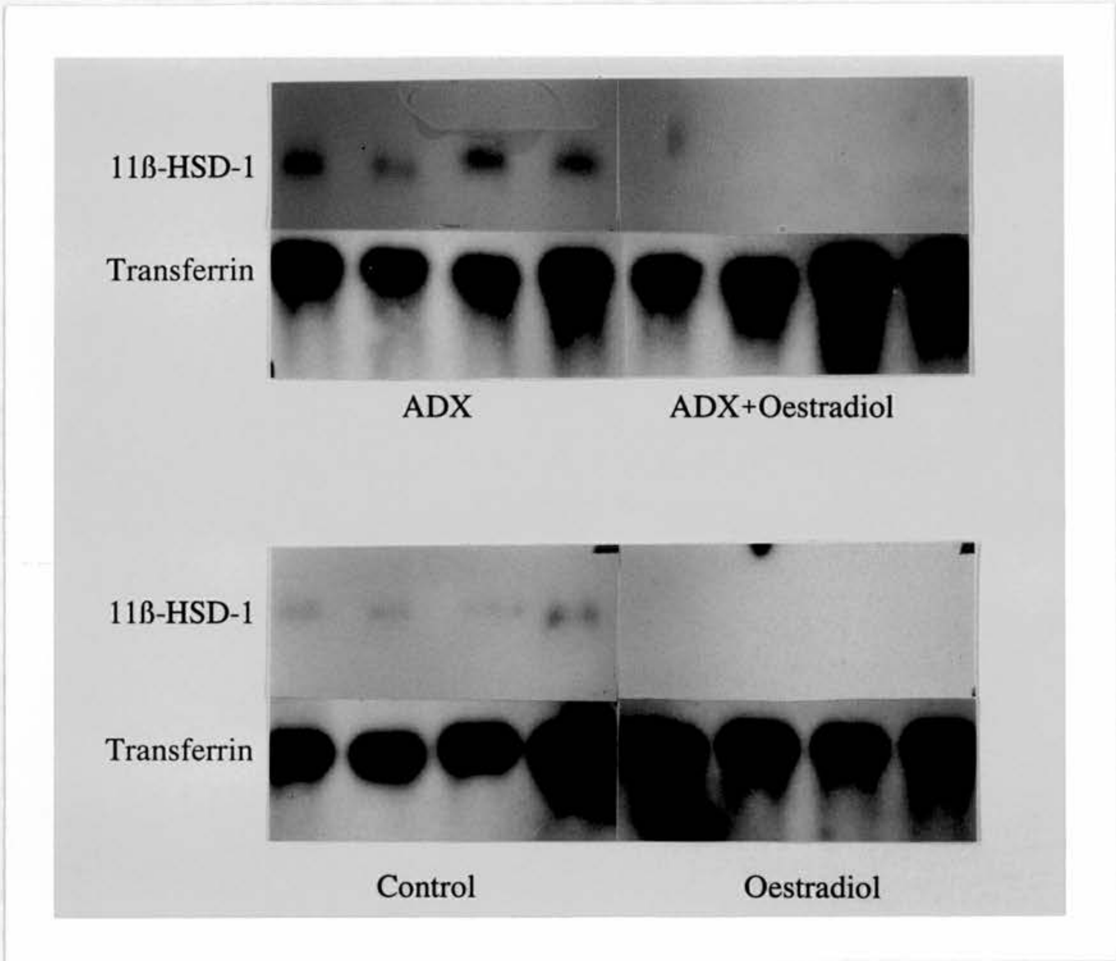


Figure 4.5: Effect of adrenalectomy and oestradiol on hepatic 11ß-HSD-1 mRNA expression.

Autoradiograph of northern blots of RNA from the livers of gonadectomised male rats receiving E_2 or vehicle for 21 d following ADX or sham ADX. Hybridised to 11ß-HSD-1 and transferrin cDNAs. Each lane contains 20 μg total RNA from an individual animal. All samples were electrophoresed on a single northern gel, hybridised on a single blot, exposed to the same film for the same length of time and expressed relative to each other. Differences in background density are due to photography. No 11ß-HSD-1 mRNA was detectable in RNA from E_2-treated animals.

Cont=control, E2=oestradiol, ADX=adrenalectomy,

ADX+E2=adrenalectomy+oestradiol.

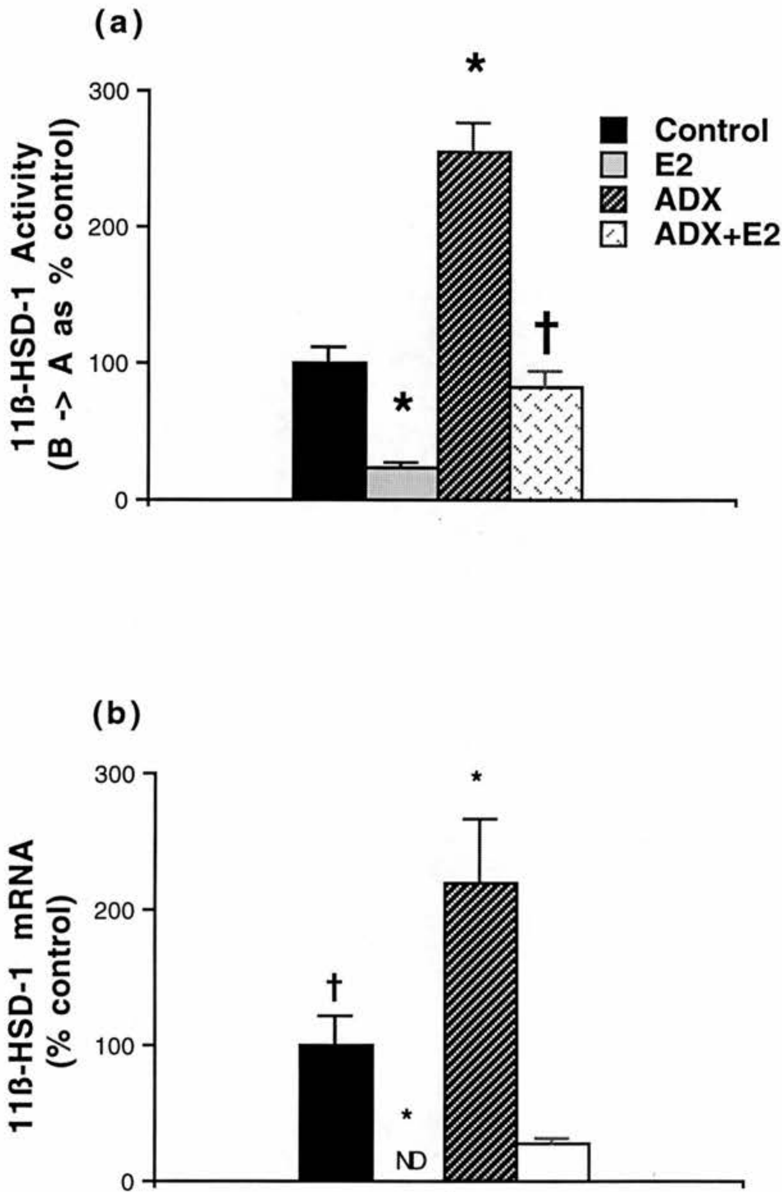


Figure 4.6: Effect of adrenalectomy and oestradiol on hepatic 11ß-HSD-1 activity and mRNA expression.

(a) 11ß-HSD-1 activity was assayed in homogenates and (b) 11ß-HSD-1 mRNA was quantified from the livers of gonadectomised male rats receiving E_2 or vehicle for 21 d following ADX or sham ADX. Data are expressed as a percentage of (a) activity or (b) mRNA levels (normalised to expression of transferrin) ±SEM in control sham ADX animals receiving vehicle.

E2=oestradiol, ADX=adrenalectomy, ADX+E2=adrenalectomy+oestradiol. n=9-10. ND=not detectable. *$P<0.05$ compared with control; †$P<0.05$ compared with ADX.

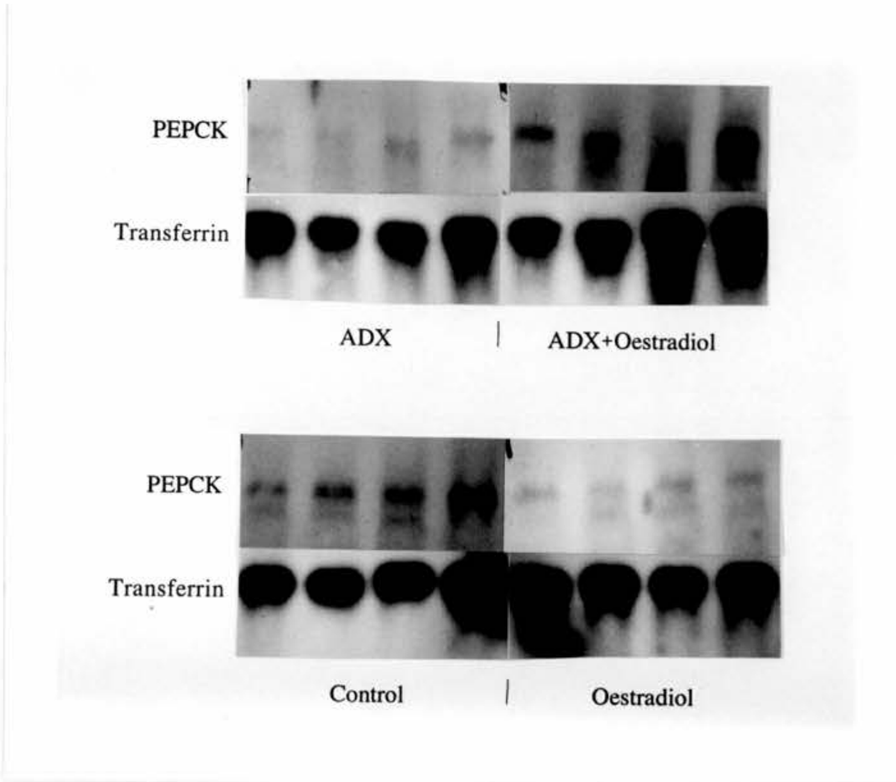


Figure 4.7: Effect of adrenalectomy and oestradiol on hepatic PEPCK mRNA expression.

Autoradiograph of northern blots of RNA from the livers of gonadectomised male rats receiving E_2 or vehicle for 21 d following ADX or sham ADX. Hybridised to PEPCK and transferrin cDNAs. Each lane contains 20 μg total RNA from an individual animal. All samples were electrophoresed on a single northern gel, hybridised on a single blot, exposed to the same film for the same length of time and expressed relative to each other. Differences in background density are due to photography.

Cont=control, E2=oestradiol, ADX=adrenalectomy,

ADX+E2=adrenalectomy+oestradiol.

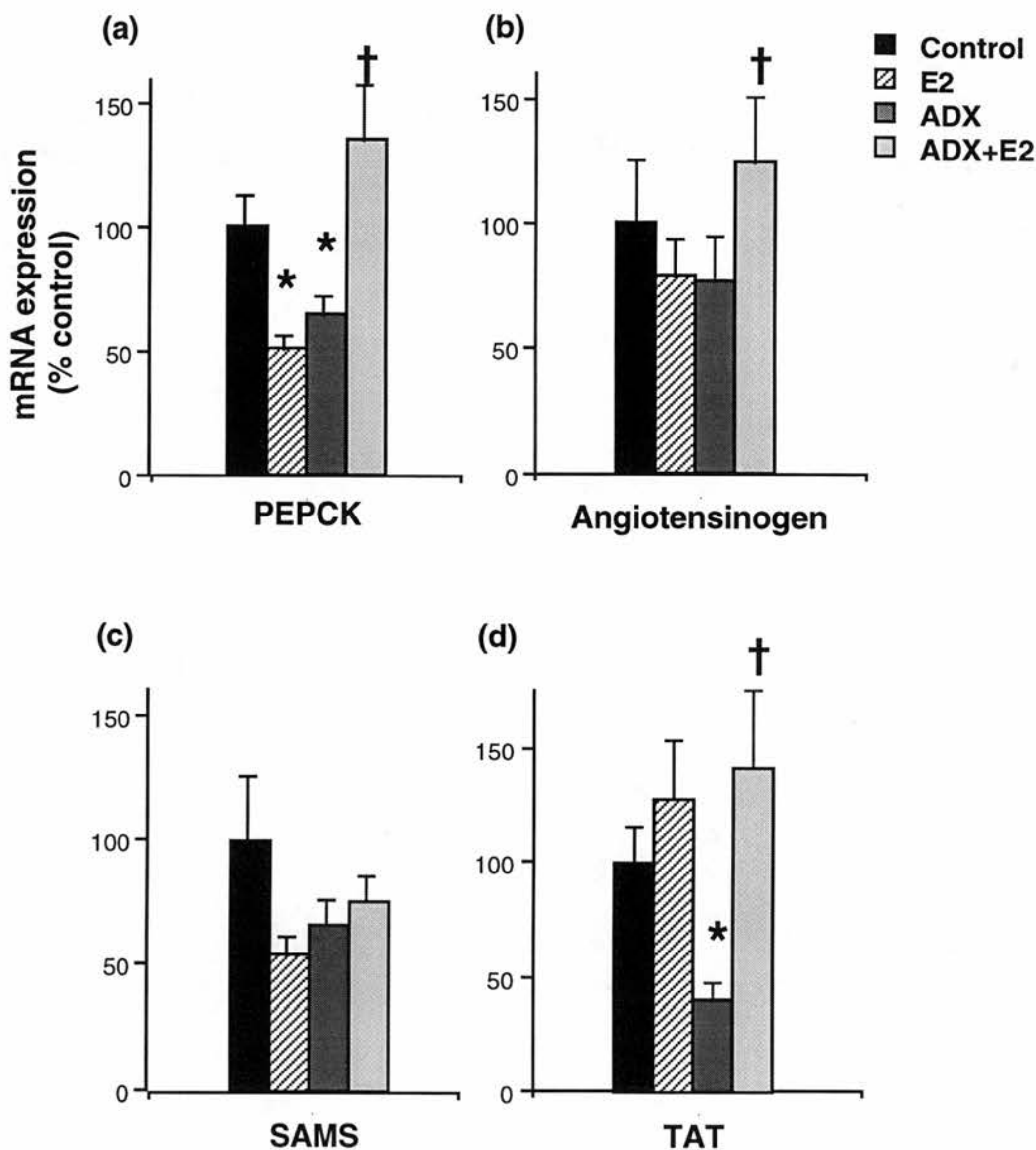


Figure 4.8: The effect of oestradiol on hepatic gene expression is dependent upon adrenal steroids.

Levels of mRNA from the livers of male gonadectomised rats receiving E_2 or vehicle for 21d following ADX or sham ADX was quantified by northern analyses. Data are normalised to expression of transferrin, and expressed as a percentage of mRNA levels (±SEM) in control sham ADX animals receiving vehicle.

E2=oestradiol, ADX=adrenalectomy, ADX+E2=adrenalectomy+oestradiol. n=9-10. *P<0.05 compared with controls; †P<0.05 compared with ADX.

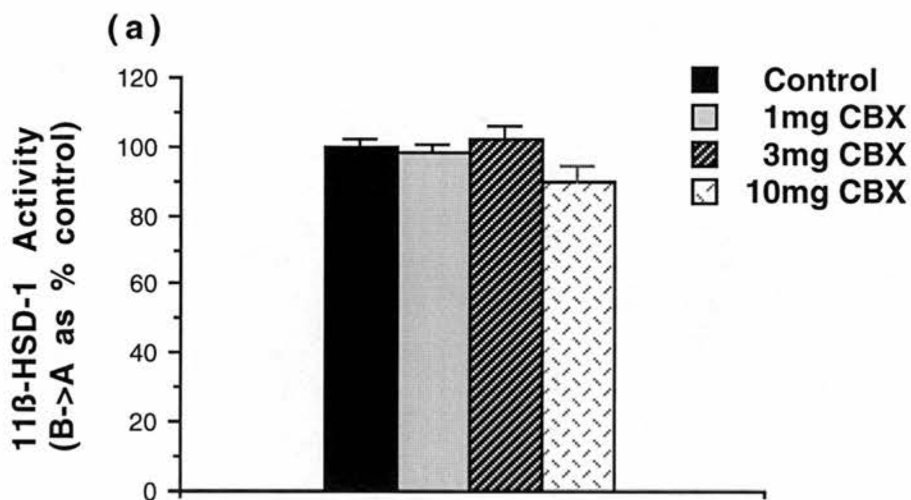


Figure 4.9: Effect of carbenoxolone administration on hepatic 11ß-HSD-1 activity. 11ß-HSD-1 activity was assayed in homogenates of liver from male rats receiving daily injections of 1, 3 or 10mg CBX for 14 days. Data are expressed as a percentage of activity (±SEM) in control animals receiving vehicle. n=8.

Co-factor added	None			NADP			NADPH		
Metabolites	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Control	2.5	97.5	0	28.6	71.4	0	6.7	93.3	0
E2-treated	0	100	0	0.5	99.5	0	0.1	99.9	0

Table 4.1:

Products of corticosterone metabolism in liver homogenates from gonadectomised male rats receiving oestradiol (E_2) for 42 d and control animals receiving vehicle. The relative quantities of metabolites detected are expressed as a percentage of total [^3H]-steroid recovered. A=11-dehydrocorticosterone, B=corticosterone, C=other radiolabelled steroid metabolites (refer to Table 2.3). Note that E_2 attenuates production of A, but that production of other B metabolites remains negligible.

effect on the weight of the rats, when compared to controls receiving vehicle (data not shown).

Administration of 3 mg or 10 mg CBX daily for 14 d to intact male rats resulted in a fall in fasting plasma glucose levels (Fig 4.10). There was no difference in response to an oral glucose load given in a glucose tolerance test (section 2.6.3) in terms of glucose disposal (Fig 4.11). There was no significant difference in fasting plasma insulin concentration (Fig. 4.12) resulting from CBX treatment or in the total amount of insulin secreted in response to the glucose load (Fig. 4.13). However there was a trend for both the level of fasting plasma insulin and the total amount of insulin secreted in response to the glucose load to decrease as the dosage of CBX increased.

Measurement of plasma sodium, chloride and potassium showed that whilst sodium and chloride levels rose as the dosage of CBX increased, potassium levels remained unaltered and were elevated above the expected normal levels (Fig 4.14). This was most likely due to haemolysis of red blood cells during blood collection, which releases potassium into the plasma leading to artificially high levels when measured.

Measurement of plasma A by GCMS (section 2.2.8 and 2.2.9) showed no effect of CBX treatment (Table 4.2).

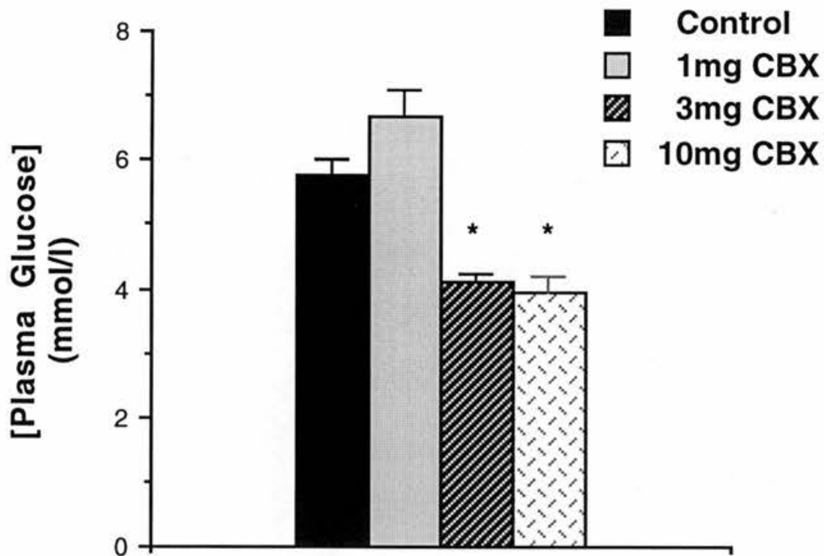


Figure 4.10: Effect of carbenoxolone administration on fasting plasma glucose. Fasting plasma glucose concentration was measured in male rats receiving daily injections of 1, 3 or 10g CBX for 14 d. Data are expressed as mean±SEM. n=6-8. *P<0.05 compared with controls.

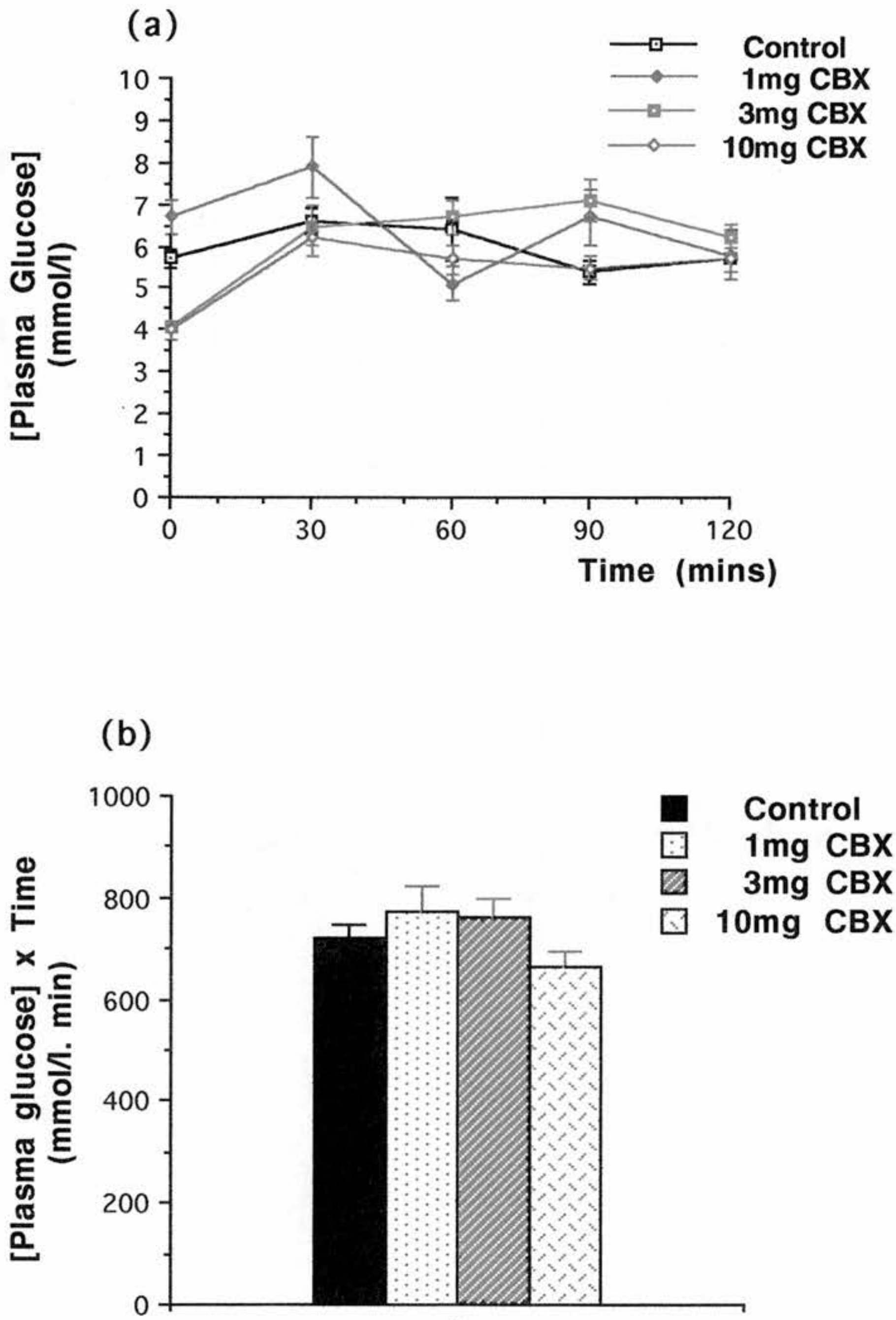


Figure 4.11: Effect of carbenoxolone administration on glucose tolerance. Glucose tolerance tests were carried out in male rats after receiving daily injections of 1, 3 or 10 mg CBX for 14 d. (a) Time course of plasma glucose concentration following an oral glucose load (section 2.6.3). (b) Areas under the curves of plasma glucose concentration plotted against time. Data are expressed as mean±SEM. n=6-8.

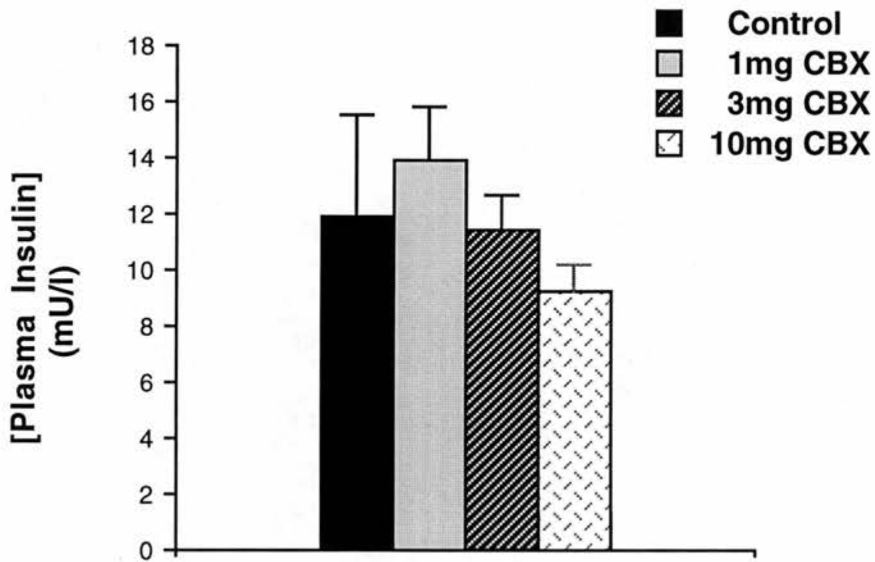


Figure 4.12: Effect of carbenoxolone administration on fasting plasma insulin. Fasting plasma insulin concentration was measured in male rats receiving daily injections of 1, 3 or 10 mg CBX for 14 d. Data are expressed as mean±SEM. n=8.

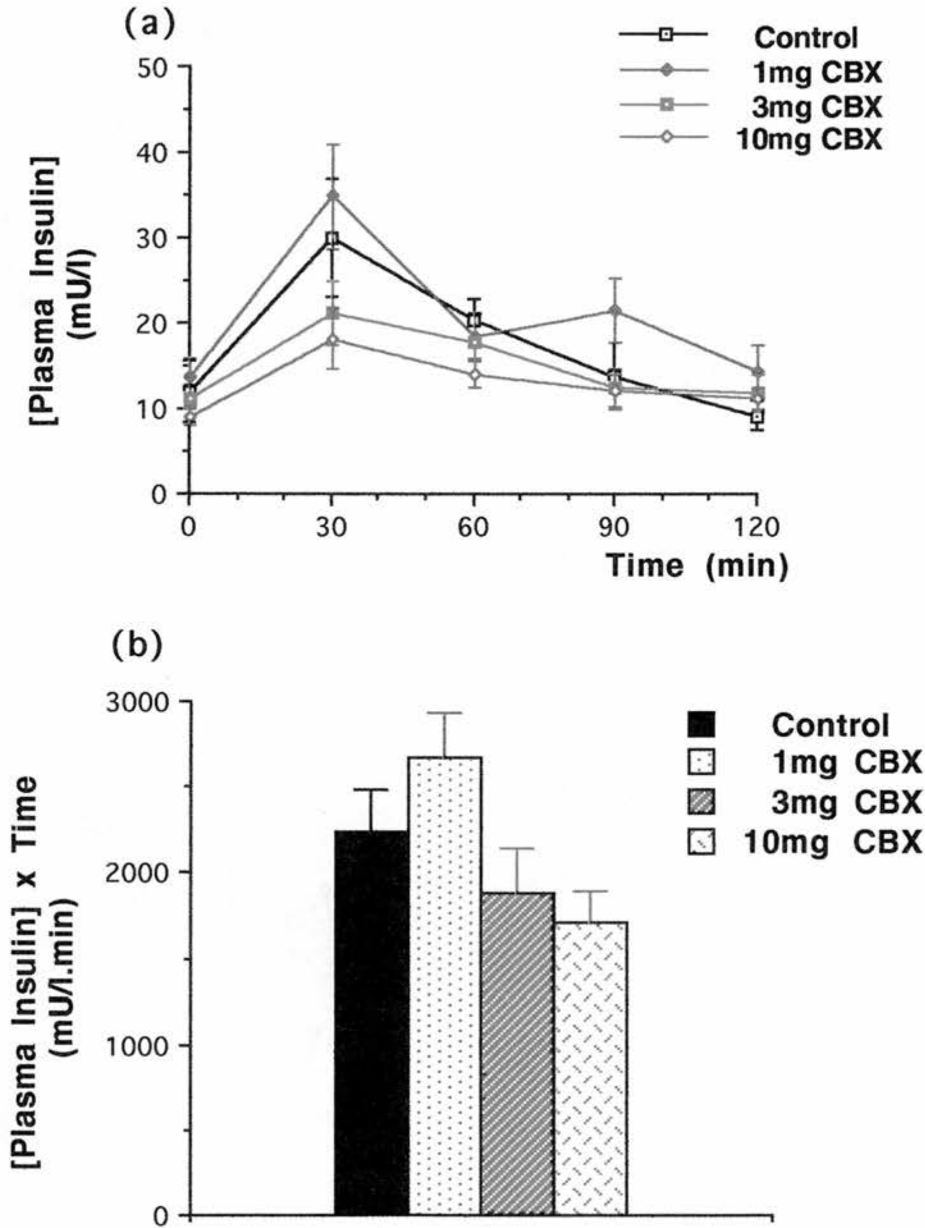


Figure 4.13: Effect of carbenoxolone administration on insulin secretion.
Glucose tolerance tests were carried out in male rats after receiving daily injections of 1, 3 or 10 mg CBX for 14 d. (a) Time course of plasma insulin concentration following an oral glucose load (section 2.6.3). (b) Areas under the curves of plasma insulin concentration plotted against time. Data are expressed as mean±SEM. n=8.

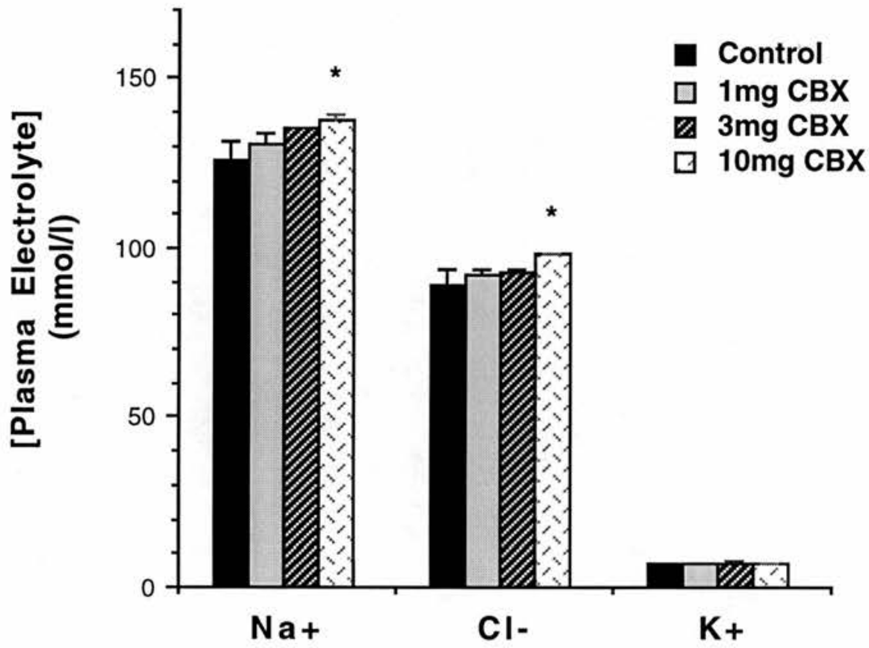


Figure 4.14: Effect of carbenoxolone administration on plasma electrolytes. Plasma electrolytes were measured in male rats after receiving daily injections of 1, 3 or 10 mg CBX for 14 d. Data are expressed as mean±SEM. n=8. *$P<0.05$ compared with controls.

CBX (mg per day)	Plasma A (μg/100ml)
Control	2.9 ± 0.4
1 mg	3.7 ± 0.4
3 mg	2.6 ± 0.5
10 mg	3.4 ± 0.4

Table 4.2:

Plasma levels of 11-dehydrocorticosterone following 14 days of carbenoxolone administration as measured by GCMS.

Data are mean±SEM. n=8.

Furthermore, PEPCK mRNA expression was unaltered by a daily dose of 10 mg carbenoxolone for 14 days (93.8±9.1% of levels in controls receiving vehicle).

4.2.3 11ß-HSD-1 Activity in the Obese Zucker Rat

Measurement of 11ß-HSD activity in the liver and skeletal muscle (quadriceps) of obese fa/fa Zucker rats showed a significant reduction in 11ß-HSD activity when compared to their lean Fa/fa or Fa/Fa littermates in both tissues (Fig 4.15). Neither plasma insulin levels nor B levels showed a correlation with 11ß-HSD activity in the obese Zucker rats (Table 4.3). Therefore in this animal model, hepatic 11ß-HSD-1 activity did not show an association with the NIDDM risk factors of hyperinsulinaemia or hypercorticosteronaemia. Plasma B measurements were carried out by Dr. Chris Kenyon and are reproduced here with kind permission.

4.2.4 11ß-HSD-1 Activity in the 11ß-HSD-1 "Knock-Out" Mouse

Gene targeting of 11ß-HSD-1 was used to obtain homozygous mice with a targeted disruption of the 11ß-HSD-1 gene (Kotelevtsev et al., 1996). Tissues from a female mouse homozygous for the gene disruption were assayed for 11ß-HSD activity and a heterozygote littermate was used as a control (Table 4.4). Negligible 11ß-HSD activity was detectable in liver, brain or lung, all tissues in which 11ß-HSD-1 is highly or moderately expressed, confirming that the homozygous mutant mouse possessed no 11ß-HSD-1 activity. The low level of 11ß-HSD activity remaining in the kidney when assayed for 11ß-HSD-1 dehydrogenase activity with

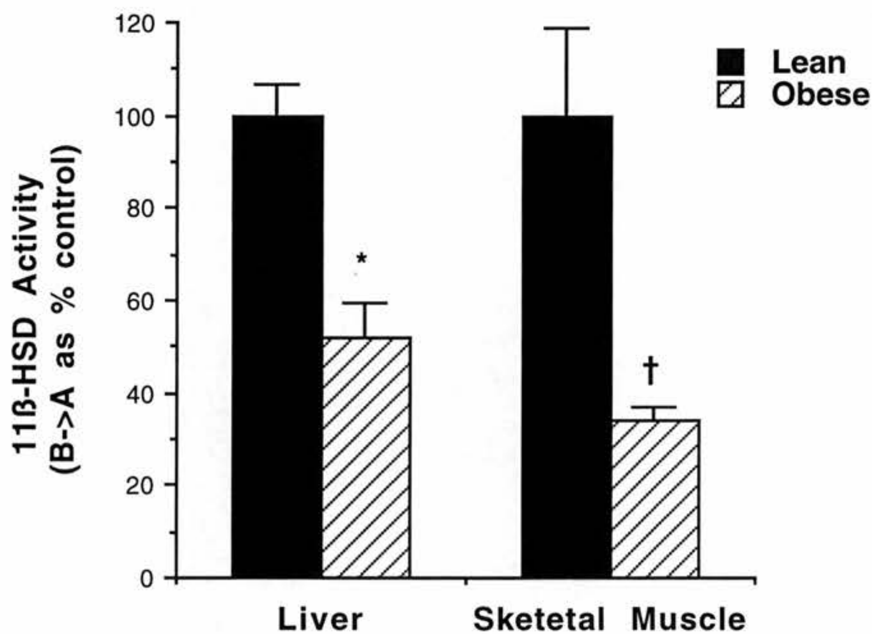


Figure 4.15: 11ß-HSD-1 activity in obese Zucker rats.

11ß-HSD-1 activity was assayed in homogenates of liver and skeletal muscle from obese fa/fa Zucker rats and their lean littermates which served as controls. Data are expressed as a percentage of activity (±SEM) in control animals. n=5-6.

*P<0.001 compared with controls. †P<0.01 compared with controls.

11ß-HSD Activity-Liver	11ß-HSD Activity-Skeletal Muscle	Plasma Insulin (mU/l)	Plasma B (nmol/l)	Body Weight (grams)
Lean				
51.5	85.2	15	87	228
44.9	71.2	27	217	222
43.5	28.8	26	220	200
38.9	37.6	16	46	222
30.8	54.7	5	190	244
Obese				
34.9	25.2	758	741	290
26.7	17.0	742	808	318
19.2	18	345	727	354
18.2	15.8	343	666	372
14.7	19	631	530	340

Table 4.3:

11β-HSD-1 activity in obese Zucker rats and lean controls.

11β-HSD-1 activity (expressed as % conversion B->A) in liver and skeletal muscle, plasma insulin and B, and body weight of each individual animal are presented in each row. Animals are ranked in order of their 11β-HSD-1 activity with the highest activity levels in liver at the top.

Tissue	Activity	"Knock-Out"	Control
Liver	11ß-HSD-1 Dehydrogenase	0	16.4
	11ß-HSD-1 Reductase	0.5	1.4
Brain	11ß-HSD-1 Dehydrogenase	0	17.8
	11ß-HSD-1 Reductase	0.1	12.3
Lung	11ß-HSD-1 Dehydrogenase	1.0	14.5
	11ß-HSD-1 Reductase	0.1	1.1
Kidney	11ß-HSD-1 Dehydrogenase	2.9	2.8
	11ß-HSD-1 Reductase	0.7	0.6
	11ß-HSD-2 Dehydrogenase	47.2	40.8

Table 4.4:

11β-HSD activity in the 11β-HSD-1 "knock-out" mouse.

Tissues from a female mouse homozygous for the mutation were assayed for both 11β-dehydrogenase and 11β-reductase activity of 11β-HSD-1 (sections 2.2.1 and 2.2.2). Kidney was also assayed for 11β-dehydrogenase activity of 11β-HSD-2 (NAD supplied as cofactor). Tissues from a female mouse homozygous for the mutation were assayed for 11β-HSD activity. A heterozygote littermate provided the control.

NADP as cofactor, is likely to be due to 11ß-HSD-2, active in the presence of low levels of endogenous co-factor (NAD).

To examine the dose effect of the gene on 11ß-HSD-1 activity in vivo, 11ß-dehydrogenase of 11ß-HSD-1 was measured in tissues of female mice homozygous for the mutation, heterozygotes and wild-type mice of the parent strain. No consistent dose effect of the gene was observed throughout the tissues. However, in brain and ovary the heterozygotes had decreased activity with respect to wild-type mice (Fig. 4.16).

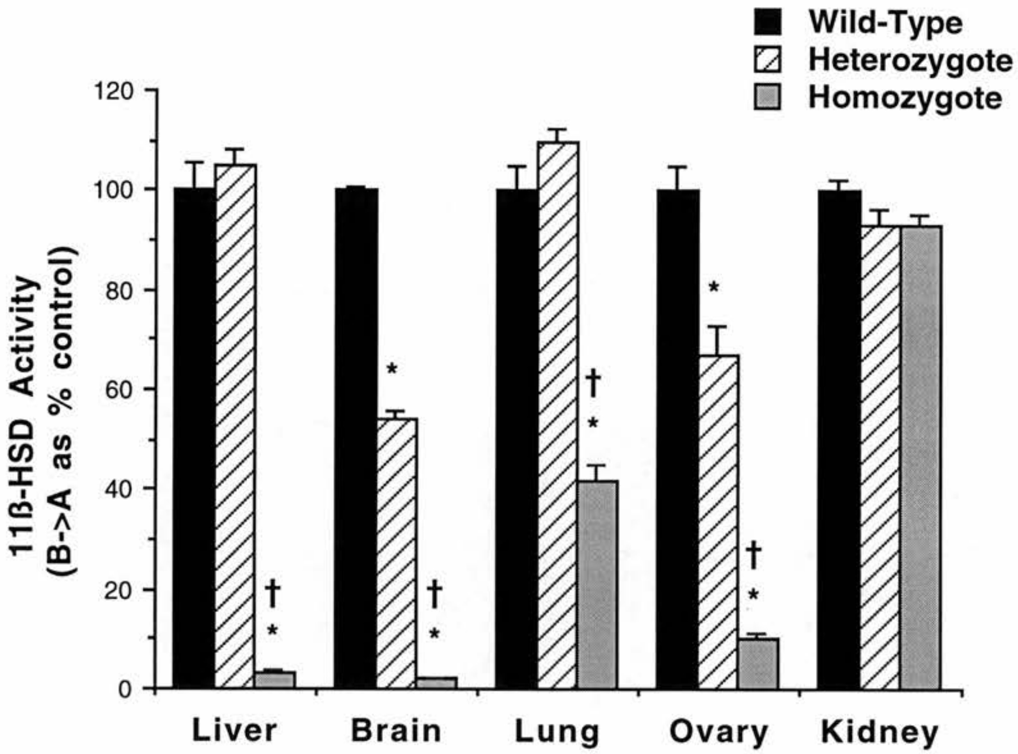


Figure 4.16: 11ß-HSD activity in the 11ß-HSD-1 "knock-out" mouse.

11ß-HSD activity was assayed in homogenates of tissues from female wild-type mice and female mice heterozygous and homozygous for the inactive mutated 11ß-HSD-1 gene. Data are expressed as a percentage of activity (±SEM) in control (wild-type) mice. n=5-8.

*P<0.001 compared with wild-type. †P<0.001 compared with heterozygotes.

4.3 Discussion

E_2 administration for both 21 and 42 d resulted in undetectable hepatic 11ß-HSD-1 mRNA expression and markedly reduced 11ß-HSD activity (although this remained clearly measurable), confirming previous studies (Lax et al., 1978; Low et al., 1993). The discrepancy between mRNA expression and activity might be due to much slower turnover of the 11ß-HSD-1 protein or, more likely, due to the transcription and translation of residual low levels of 11ß-HSD-1 mRNA, undetectable by northern analysis. This effect of sex steroids may be idiosyncratic to the rat (Rajan et al., 1995a and Chapter 6). However it is relatively specific to the liver, since hippocampal 11ß-HSD-1 was unaffected, and previous studies have shown that 11ß-HSD-2 is not attenuated by E_2, in either the kidney or placenta (Low et al., 1993; Low et al., 1994b; Baggia et al., 1990). Thus the advantage of exploiting E_2 down-regulation of hepatic 11ß-HSD-1, as opposed to conventional liquorice-related inhibitors of the enzyme, is that 11ß-HSD-2 is unaffected. The mechanism of E_2 down-regulation of hepatic 11ß-HSD-1, which presumably occurs at the level of transcription, is unclear and appears partly to depend upon sex steroid-determined patterns of GH secretion (Lax et al., 1978; Low et al., 1993; Low et al., 1994b), but E_2 repression of hepatic 11ß-HSD-1 in the hypophysectomised rat, albeit to a lesser extent than in the intact rat, suggests that it has an additional direct regulatory effect (Low et al, 1994b).

Following 42 d of E_2 treatment, hepatic expression of mRNAs encoding PEPCK and angiotensinogen was clearly reduced. Although the alterations in TAT and SAMS expression did not reach significance, they too showed a downwards trend with E_2 treatment. In principle, these effects of chronic E_2 treatment may be explained in several ways: (i) E_2 may act directly to repress hepatic gene expression; (ii) E_2 may alter corticosteroid metabolism in the liver. Reduced hepatic 11ß-HSD-1-mediated reactivation of otherwise inert A is anticipated to reduce expression of these glucocorticoid-sensitive transcripts. Alternatively, 'feminisation' of the male liver may induce 5α-reductase activity (Leblanc & Waxman, 1988) which might be expected to increase B metabolism to inactive products; (iii) there may be other indirect effects of chronic E_2 administration mediated through factors other than 11ß-HSD-1, in a situation analogous with the effects of patterns of GH secretion on the expression of liver enzymes (Lax et al., 1978; Mode, 1993; Low et al., 1994b). At least for PEPCK, TAT and angiotensinogen, direct inhibition by E_2 appears unlikely, since these transcripts are stimulated by E_2 in ADX rats and in other circumstances *in vivo* (this chapter; Eggena & Barrett, 1992; Kunapuli et al., 1987).

Angiotensinogen and SAMS mRNA expression were not significantly affected by E_2 for 21 days (although for SAMS, a downward trend was noted at 42 d when angiotensinogen mRNA had fallen significantly). However, under these experimental conditions, glucocorticoids do not appear to be a potent regulator *in vivo* for angiotensinogen and SAMS, as illustrated by the lack of effect of ADX alone. Thus it is not surprising that attenuation of 11ß-HSD-1 expression by E_2 had little effect on their mRNA expression. This, together with the loss of the attenuating effect of E_2 on PEPCK expression in ADX rats suggests a requirement for glucocorticoids in the process, rather than any other indirect effect. Glucocorticoid induction of TAT gene expression has been well characterised (Ruppert et al., 1990) and ADX reduced TAT mRNA expression. However, up-regulation of TAT by E_2 in ADX animals was greater than for any other transcript, and this action may have opposed the influence of down-regulation of 11ß-HSD-1 in intact animals.

Alterations in the activity of other enzymes in the liver responsible for the metabolism of B is also unlikely to be responsible for the effects on hepatic gene transcription observed in this study since no products of B metabolism other than A were observed in incubates. In particular, there was no observed increase in 5α-reduction of B, despite using the appropriate cofactor (Bhattacharyya et al., 1994) in physiological buffer at physiological temperature and pH. These data suggest that the attenuated expression of hepatic glucocorticoid-sensitive mRNAs observed following E_2 is not due to enhanced metabolism of B in this model.

Glucocorticoids increase expression of PEPCK in the liver (Friedman et al., 1993; Lamers et al., 1982; Sasaki et al., 1984), and so this pattern of changes is compatible with the glucocorticoid control of expression of this gene. The effect of E_2 in intact rats was as potent as ADX alone in attenuating PEPCK mRNA expression. These data are therefore consistent with the effects of E_2 being indirectly mediated via the marked loss of 11ß-HSD-1 (reductase), which thus attenuates intrahepatic glucocorticoid regeneration. Given that E_2 in adrenalectomised rats did not decrease, but indeed increased PEPCK mRNA levels, these data indicate that the 11ß-reductase activity of 11ß-HSD-1 is likely to be of key importance in producing sufficiently high intrahepatic B levels to elevate PEPCK above the minimum levels maintained by basal (non-glucocorticoid) factors. The discrepancy between the E_2-mediated increase in hepatic PEPCK and other transcripts in the complete absence of glucocorticoids, but fall in PEPCK mRNA levels with E_2-mediated repression of 11ß-HSD-1 (and the presumed relative reduction in intrahepatic glucocorticoid levels) may reflect other effects of E_2 occurring via mechanisms which have a qualitative rather than quantitative requirement for glucocorticoids. Similar effects

are seen with the hepatic xenobiotic metabolising enzymes, which are also regulated in opposite directions by sex steroids in the presence or absence of glucocorticoids (Nemoto & Sakurai, 1995), and with the dependence of E_2 receptor expression upon glucocorticoids in some tissues (Ulisse & Tata, 1994).

In vivo F shows a pronounced diurnal variation, whereas E levels vary little and are of similar magnitude to "free" F concentrations during the diurnal maximum (Walker et al., 1992b). In humans, plasma levels of inert E (which is largely unbound to plasma proteins and circulates at levels around 100 nM) are considerably higher than "free" F concentrations during the nadir (perhaps 5 nM compared with 25-50 nM during the diurnal maximum or stress), since most F is tightly bound to CBG (Walker et al., 1992b). Similarly, in the rat, A levels of around 50 nM are found (R Best and JR Seckl, unpublished data and section 4.2.2) again in excess of low nanomolar "free" B levels for most of the day. Thus the potential reservoir of substrate available for 11ß-HSD-1 reductase activity is usually in considerable excess of active free glucocorticoid, and variations in 11ß-reductase activity may therefore be critical in determining intracellular levels of active glucocorticoid. This mechanism would be not dissimilar to that controlling the intracellular levels of active testosterone or T_4 hormone, both of which circulate in a largely inactive form (Stewart & Sheppard, 1992) and are converted to their active forms of dihydrotestosterone and T_3 respectively by intracellular enzymes at the site of action. Thus activation of inert steroid may allow hepatocytes to maintain adequate levels of active 11-hydroxysteroids to ensure glucocorticoid-regulated metabolic functions are not compromised in the face of fluctuating F or B levels during the daily cortisol nadir. This will occur irrespective of circulating F or B levels as the latter will be determined by the activity of the HPA axis (Akana et al., 1992).

There are important implications of the potential influence of 11ß-HSD-1 on liver metabolic processes. PEPCK is the rate-limiting enzyme in gluconeogenesis and its expression is regulated by its rate of gene transcription. Over-expression of PEPCK in transgenic mice increases gluconeogenesis, producing hyperglycaemia and impaired glucose tolerance (Valera et al., 1994). Therefore decreased 11ß-reductase activity resulting in attenuated PEPCK gene transcription would be expected to increase insulin sensitivity. Inhibition of 11ß-HSD-1 with CBX increases hepatic insulin sensitivity in man (Walker et al., 1995) and so in order to further test the hypothesis that hepatic 11ß-reductase activity enhances glucocorticoid action in the liver, with consequent effects upon hepatic insulin sensitivity, the effects of CBX administration upon insulin sensitivity in the rat was examined.

Hepatic 11ß-HSD-1 activity was not altered by CBX administration as measured by the *in vitro* assay technique. However, the concentration of liver protein employed in assay incubates requires an effective tissue dilution of approximately 1 in 5000. Thus the consequent dilution of CBX present in tissue is likely to reduce the concentration to a level below that required for competitive enzyme inhibition. The assay therefore indicates that the CBX administration had no effect on the levels of 11ß-HSD-1 protein present in the liver, but gives no information regarding the extent of 11ß-HSD-1 inhibition *in vivo*. Interestingly, CBX administration decreased 11ß-HSD-1 mRNA expression, in accordance with previously reported transcriptional inhibition of 11ß-HSD-1 mRNA by glycyrrhizic acid (Whorwood et al., 1993b). This discrepancy between protein levels and mRNA levels is likely to be due to the slower turnover of protein *in vivo*. The administration of CBX to rats resulted in a lowering of fasting plasma glucose with a trend (not reaching significance) for fasting plasma insulin to fall also, consistent with an increase in insulin sensitivity. This finding is in accordance with the data of Walker et al. (1995), which demonstrated a trend for CBX to decrease fasting insulin levels without affecting plasma glucose. Likewise, there was a trend - not reaching significance - for insulin secretion to be lower following CBX, with no change in the rate of glucose disposal, which would indicate increased insulin sensitivity. There were no changes in the levels of hepatic PEPCK mRNA expression, or of plasma A, which might be expected to rise in the presence of impaired 11ß-reduction.

Therefore these data are suggestive of CBX effecting an increase in whole body insulin sensitivity. However, the dose of CBX which can be employed in *in vivo* studies is limited due to its side effect of "apparent renal mineralocorticoid excess" consequent upon 11ß-HSD-2 inhibition (as witnessed by the increase in plasma sodium and chloride in this study). In addition, although CBX has a K_i of 3.3 nM for 11ß-HSD activity *in vitro* (Stewart, 1994), the studies in Chapter 5 show that it is a much less efficient inhibitor of hepatic 11ß-HSD-1 *in vivo*. This, together with the presence of an inflammatory reaction and a granular lesion at the injection site of the higher doses of CBX administration, and the likelihood that a high constant level of circulating drug is not achieved by this route of administration, make it probable that inhibition of hepatic 11ß-HSD-1 activity is not optimal for the purposes of this study.

Nevertheless, the results are supportive of a role for 11ß-HSD-1 in the control of insulin sensitivity. Glucocorticoids antagonise insulin action not only in the liver, but also in the peripheral tissues. Whitney (1953) reported a decrease in peripheral glucose utilisation of 40% and an 8% increase in hepatic glucose

production consequent upon F infusion in humans. 11ß-HSD activity is present in skeletal muscle (Monder, 1993) and adipocytes (BR Walker, personal communication; A Napolitano, personal communication) and so a contribution to the increased insulin sensitivity observed following CBX administration from reduced levels of active glucocorticoid in these tissues must be considered. However, high concentrations of CBX in the absence of glucocorticoids antagonised insulin action in adipocytes (Gomez-Capilla et al., 1988). In addition, the data of Walker et al., (1995) demonstrated an increase in whole body insulin sensitivity without increased peripheral uptake of glucose. This, taken together with the data from the experiments of E_2 administration to rats described in this chapter, suggests the most likely explanation is that the main site of action of CBX is within the liver, with reduced intrahepatic glucocorticoid levels resulting in decreased glucocorticoid receptor activation within the liver. It should be mentioned that CBX inhibits other enzymes involved in the metabolism of glucocorticoids, most notably 5ß-reductase (Latif et al., 1990), but inhibition of this enzyme would reduce B metabolism, thus potentially raising intrahepatic glucocorticoid levels which would antagonise insulin action.

The implications of abnormal 11ß-reductase activity for disease are unknown, but in principle, an inappropriate increase in hepatic 11ß-HSD-1 activity could be a contributing factor in syndromes of central insulin resistance including NIDDM (Fery, 1994) as excess activity could increase gluconeogenesis by induction of the gluconeogenic enzymes, and attenuate insulin sensitivity by antagonism of insulin action. However, the measurement of 11ß-HSD-1 activity in the liver and skeletal muscle of the Zucker rat (a well recognised model of obesity and insulin resistance) demonstrated a dramatic reduction in enzyme activity. In polycystic ovary syndrome (a condition characterised by insulin resistance) abnormal ratios of urinary metabolites are consistent with impaired 11ß-reductase activity (Rodin et al., 1994) and, in hypertensive rats, hepatic 11ß-HSD-1 activity and mRNA expression are decreased (Stewart et al., 1993). Therefore, contrary to the predictions of the hypothesis, in syndromes of central insulin resistance, 11ß-HSD-1 is impaired, suggesting the raised insulin levels have mediated the decrease in 11ß-HSD-1. Changes in 11ß-HSD-1 may therefore be a consequence, rather than a cause of these syndromes, and may even represent a physiological adaptation to alleviate the insulin resistance. Therefore it is likely that changes in hepatic 11ß-HSD-1 are consequential rather than causative in the above mentioned syndromes of insulin resistance. However, it remains possible that alterations in hepatic 11ß-HSD-1 activity may be causative of (or exacerbate) insulin resistance under different

conditions. The interactions between 11ß-HSD-1 and insulin activities may be mutual since insulin decreases 11ß-HSD-1 mRNA in hepatocytes in culture (Chapter 3), thus probably decreasing glucocorticoid regeneration and increasing insulin action.

Nevertheless, the potential to alter gluconeogenesis/insulin sensitivity by manipulating 11ß-HSD-1 activity in the liver is of clear therapeutic interest. Enzyme inhibition may provide a useful therapeutic target for manipulating glucose homeostasis. Agents which improve insulin sensitivity (Petrie & Donnelly, 1994) often have a proportionally greater effect as insulin resistance becomes more severe (Nolan et al., 1994; Mimura et al., 1994). This may be the case for the attenuation of hepatic 11ß-reductase activity, particularly as there is evidence that glucocorticoid antagonism of insulin action may also be greater. In obesity the HPA axis is activated in humans (Marin et al., 1992; Weaver et al., 1993) and in the Zucker rat (Krief & Basin, 1991), and tissue sensitivity to glucocorticoids are increased in essential hypertension (Walker at al., 1992c). In addition, glucocorticoids become the major regulator of PEPCK gene transcription during diabetes (Friedman et al., 1993; Gerich, 1993). A more effective inhibitor of 11ß-HSD-1 without the unwanted side effect of 11ß-HSD-2 inhibition may be a useful therapeutic agent, and would certainly aid further investigations.

One useful tool in such studies is likely to be the 11ß-HSD-1 "knock-out" mouse created by Yuri Kotelevtsev working in association with this laboratory (Kotelevtsev et al., 1996). Assay of the tissues of mice homozygous for the mutation in the 11ß-HSD-1 gene demonstrated no 11ß-HSD activity in the brain or in the liver, indicating that both tissues contain this isoform only, at least at levels detectable by the 11ß-HSD-1 activity assay employed. As expected, the kidney contained high levels of 11ß-dehydrogenase activity owing to the high levels of the 11ß-HSD-2 expressed in this tissue. The lower levels of activity present in the ovary and lung are evidence that an isozyme other than 11ß-HSD-1 is present in these tissues. 11ß-HSD activity in the lung was previously assumed to be attributable to 11ß-HSD-1 and the clear reduction of activity in the homozygote when compared to the heterozygote and wildtype mouse indicates 11ß-HSD-1 is certainly present, but clearly this isozyme is not wholly responsible for 11ß-HSD activity in this organ. Likewise, activity is reduced in the ovary when compared to the heterozygote and wildtype mouse. 11ß-HSD-2 has been reported to be expressed in the rat ovary (Roland & Funder, 1996), and in the rat lung (Zhou et al., 1995) and it is therefore likely that this isozyme is responsible for the activity present in these organs in the 11ß-HSD-1 "knock-out" mouse. It has been suggested that 11ß-HSD-2 activity in

the ovary is induced in lutinised cells to prevent the inhibition of LH-stimulated progesterone biosynthesis by glucocorticoids (Michael & Cooke, 1994). The lung is not an aldosterone target tissue and what the role of 11ß-HSD-2 (or of any other 11ß-HSD isozyme which the measured activity represents) in this organ might be is not at all clear.

Obviously, as the "knock-out" mouse has a complete absence of any hepatic 11ß-HSD-1 activity, it will provide a useful model to further examine the role of hepatic 11ß-HSD-1 in liver function and its relationship to glucose homeostasis and insulin sensitivity. Indeed, studies on these aspects of 11ß-HSD-1 in these mice are now underway in this laboratory.

CHAPTER 5

THE FUNCTION OF 11ß-HSD-1 IN PERFUSED LIVER

5.1 Introduction

In Chapter 3, I demonstrated that the predominant reaction direction of 11ß-HSD-1 in intact isolated hepatocytes in culture is 11ß-reduction, thus producing active glucocorticoid from inert substrate. The data presented in Chapter 4 suggest that 11ß-reductase activity in the liver *in vivo* plays an important role in liver physiology by maintaining the expression of key glucocorticoid-regulated hepatic transcripts and I have postulated that the intrahepatic activation of glucocorticoids by 11ß-HSD-1 may allow hepatocytes to maintain adequate levels of active 11-hydroxysteroids to ensure that glucocorticoid-regulated metabolic functions are not compromised in the face of fluctuating F or B levels.

The conversion of A to corticosterone in hepatocyte cultures is approximately 40% after 30 min over a wide range of substrate concentrations, whilst *in vivo*, the substrate is subject to a rapid passage through the liver of a few seconds. Therefore the percentage conversion of substrate to product during one passage through the liver by 11ß-HSD-1, as inferred from the efficiency of conversion observed in hepatocyte culture studies, would be very small and not capable of responding rapidly to decreases in substrate availability *in vivo* in order to maintain levels of active glucocorticoid. However the conditions in cell culture are very different from those that pertain *in vivo*. In the experiments described in Chapter 3, although the substrate was exposed to the cells for a prolonged period, the hepatocyte mass, and hence overall enzyme activity is much less than that *in vivo*. Previous studies have suggested that hepatic 11ß-reductase activity *in vivo* is extremely efficient. The effluent of perfused cat liver has a high F to E ratio (Bush, 1969) and the measurement of the F to E ratio in the plasma of humans by selective venous catheterisation shows that the venous effluent of most organs has a ratio of F to E of around 10:1, whilst in the hepatic vein the ratio is 55:1, consistent with effective 11ß-reduction (Walker et al., 1992b). E administered orally is converted to F by 11ß-reductase on first pass through the liver (Stewart et al., 1990).

In the rat liver, 11ß-HSD-1 shows pronounced sexual dimorphism, with females having approximately 50% of the levels in male rats (Ghraf et al., 1975a; Lax et al., 1978; Low et al., 1993)(discussed in section 1.3.4) as quantified by *in vitro* assay techniques. Whether this is physiologically significant is unknown. In addition, glucocorticoids are subject to a number of metabolic processes within the liver (section 1.1.5) including reduction, oxidation, hydroxylation and conjugation (Orth et al., 1992), and so it is likely that in the intact liver there are multiple

products of the metabolism of A or E entering the liver from the bloodstream or produced within the liver.

Therefore, in order to address these issues, and to gain further insight into the physiological function of 11ß-HSD-1 in the intact liver, I have examined the metabolism of glucocorticoid across the intact perfused rat liver.

5.2 Results

5.2.1 11ß-HSD-1 Activity Across the Intact Perfused Liver

The intact liver, in either male or female rats, was perfused *in situ* with Kreb's Ringer buffer containing either [^{3}H]-A or [^{3}H]-B in order to examine the products of glucocorticoid metabolism in the liver. The flow rate was 7.5ml/min through both the hepatic artery and the hepatic portal vein in male rats and 5ml/min through each vessel in female rats (as this was the maximum flow rate which could be achieved in female rats). The recovery of perfused steroid was 10.6±1.4% from male rat liver and 5.0±0.6% from female rat liver, which represents significantly greater recovery in male rats ($P<0.05$). The recovery of steroid was not flow rate dependent (data not shown). HPLC analysis (section 2.2.6) of the steroid products produced by liver metabolism of A in the intact perfused male rat liver showed that 43.5±5.0% of the total steroid recovered had been converted to B by 11ß-reductase activity (Fig. 5.1). In the female rat liver, this figure dropped significantly to 20.8±2.3%, indicating that on passage through the liver, the total conversion to product in the liver by 11ß-reductase activity in females is approximately half of that seen in males. Hepatic metabolism of A perfused through the liver also produced steroid products other than B, accounting for 15.2±2.7% and 19.4±2.9% of steroid present in the effluent from male or female rat liver, respectively. Measurement of the products of metabolism of B by the intact perfused male rat liver showed that 9.5±0.5% of the total steroid recovered had been converted to A and 22.6±3.1% to other steroids (Fig 5.3).

5.2.2 Effect of Route of Delivery Upon 11ß-HSD-1 Activity in the Perfused Liver

To examine the possible influence of the route of delivery of glucocorticoids upon their metabolism, steroid was perfused through either the hepatic artery, or the hepatic portal vein, or a combination of the two routes, using the livers of male rats. As the proportion of the perfusate delivered through the hepatic artery was increased

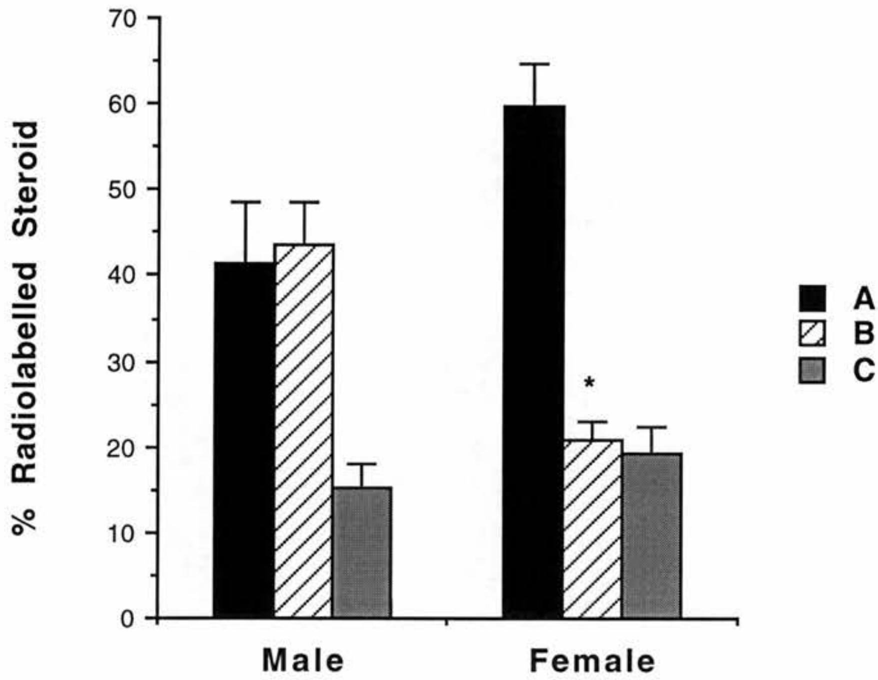


Figure 5.1: 11ß-HSD-1 activity across the intact perfused liver.
The data represents the % of [^{3}H]-labelled steroid recovered from the effluent of livers perfused with 10^{-8}M A as: 11-dehydrocorticosterone (A), corticosterone (B) and other steroids (C).
P<0.01 compared with Male B. n=5.

with respect to the proportion delivered via the hepatic portal vein, the conversion of A to B showed a decreasing trend such that the percentage of steroid recovered as B is significantly lower when 100% of the perfusate is delivered via the hepatic artery than when 100% is delivered by the hepatic portal vein ($P<0.05$) (Fig. 5.2). The percentage of steroid recovered as other steroid products did not change with the route of delivery of the A substrate. In contrast, the proportions of the steroid products recovered when the livers were perfused with B did not alter with changes in the route of substrate delivery (Fig 5.3).

5.2.3 Effect of Substrate Concentration on 11ß-HSD-1 Activity in the Perfused Liver

As the concentration of A perfused through the liver was varied across a wide range of physiologically relevant concentrations, the percentage of steroid recovered as B tended to decrease as the substrate concentration increased (Fig. 5.4), but this was not significant over the range of concentrations employed. The percentage of steroid recovered as steroid products other than A and B did not change with the concentration of A substrate. The total recovery of [^{3}H]-steroid in the effluent was not altered (data not shown).

5.2.4 Carbenoxolone Inhibition of 11ß-HSD-1 Activity in the Perfused Liver

Conversion of A to corticosterone in the intact perfused liver was not significantly inhibited by simultaneous perfusion with CBX until the concentration of CBX reached 10^{-3}M, when the percentage of steroid recovered as B was reduced by 44.5±6% when compared to 10^{-6}M CBX (Fig. 5.5). It was not however significantly different from control values. The percentage of steroid recovered as steroid products other than A and B did not change with the concentration of CBX present in the perfusate. The total recovery of steroid did not change with CBX present in the perfusate (data not shown).

5.2.5 Products of 11-Dehydrocorticosterone Metabolism Across the Perfused Liver

In order to detect steroid products of A metabolism other than B and to verify that the major product recovered in the effluent was indeed B, liver perfusion was carried out in a male rat with 5 x 10^{-9}M [^{3}H]-A in the perfusate, which is five times the amount of radiolabelled steroid normally employed in perfusions. This was to

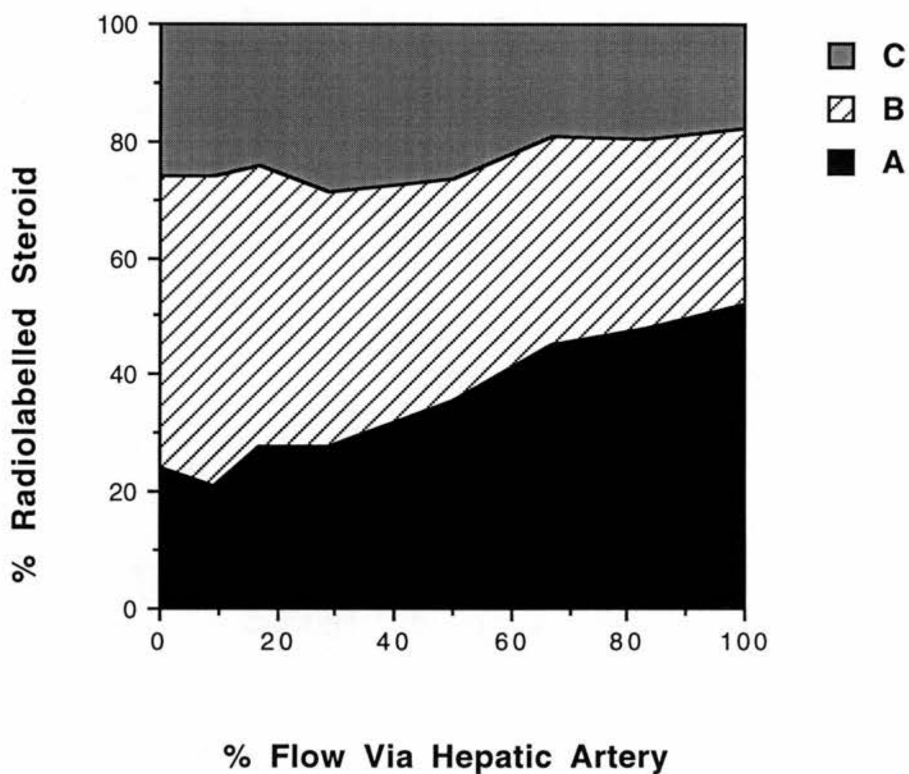


Figure 5.2: Effect of route of delivery upon 11ß-reductase activity in the perfused liver.

The data show the effect of varying the proportion of the perfusate delivered via the hepatic artery to that delivered via the hepatic portal vein from 0 to 100%.

The % of [^{3}H]-labelled steroid recovered from the effluent of male rat livers perfused with 10^{-8}M A is shown as: A=11-dehydrocorticosterone, B=corticosterone, C=other steroids. n=5.

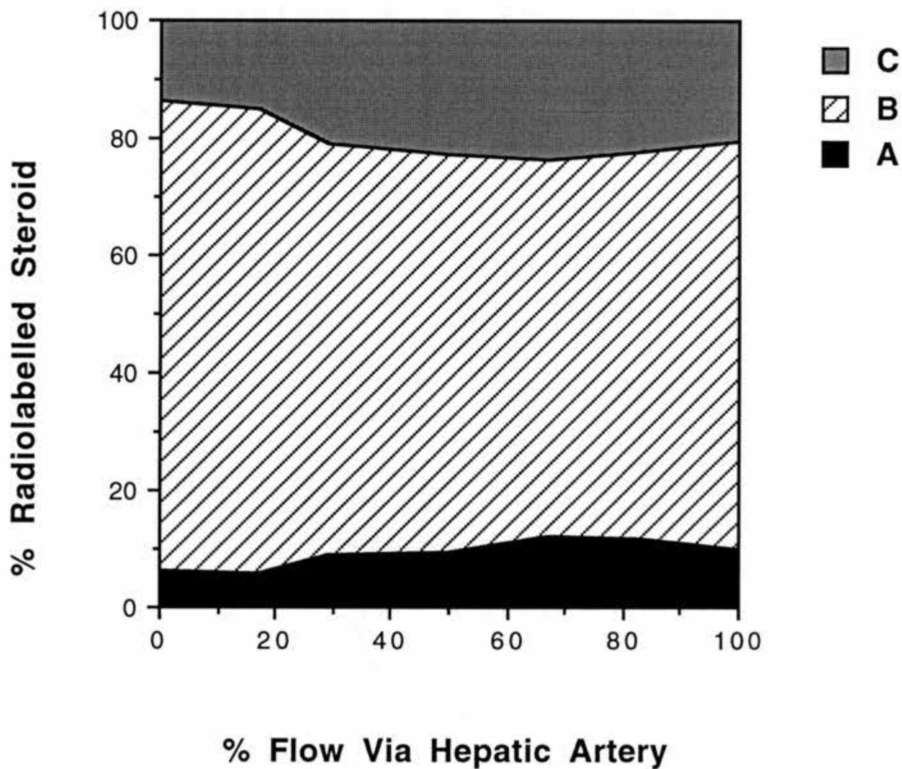


Figure 5.3: Effect of route of delivery upon 11ß-dehydrogenase activity in the perfused liver.
The data show the effect of varying the proportion of the perfusate delivered via the hepatic artery to that delivered via the hepatic portal vein from 0 to 100%.
The % of [^{3}H]-labelled steroid recovered from the effluent of male rat livers perfused with 10^{-8}M B is shown as: A=11-dehydrocorticosterone, B=corticosterone, C=other steroids. n=5.

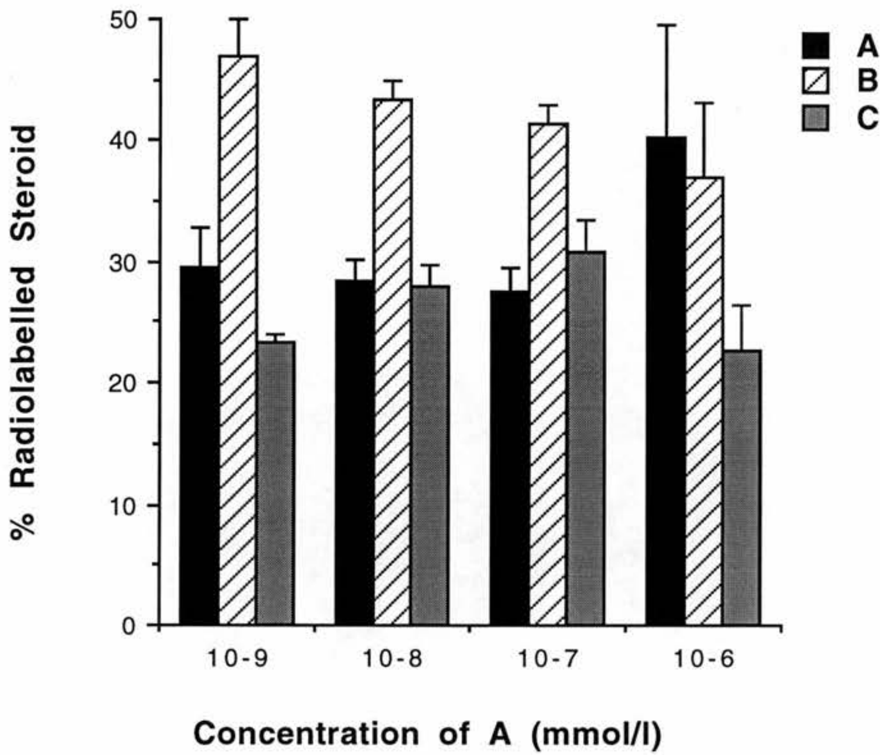


Figure 5.4: Effect of substrate concentration upon 11ß-reductase activity in the perfused liver.

The data shows the % of [^{3}H]-labelled steroid recovered from the effluent of male rat livers perfused with varying concentrations of A as: A=11-dehydrocorticosterone, B=corticosterone, C=other steroids. The flow rate was 7.5ml/min through both the hepatic artery and the hepatic portal vein. n=5.

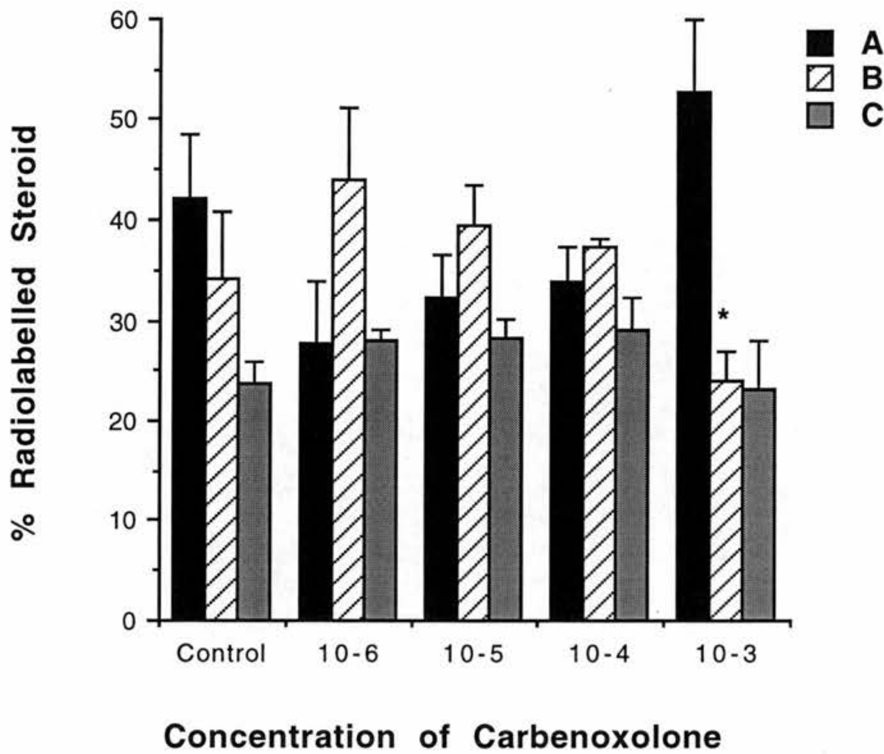


Figure 5.5: Carbenoxolone inhibition of 11ß-reductase activity in the perfused liver. The data represents the % of [^{3}H]-labelled steroid recovered from the effluent of male rat livers perfused with 10^{-8}M A together with various concentrations of CBX as: A=11-dehydrocorticosterone, B=corticosterone, C=other steroids. The flow rate was 7.5ml/min through both the hepatic artery and the hepatic portal vein. $P<0.05$ compared with 10^{-6}M CBX B. n=5.

PEAK ELUTION TIME (MIN)	% OF [^{3}H]-STEROID RECOVERED	STEROID
4.38	27.9±2.8	A
6.43	42.9±0.9	B
8.03	20.1±1.1	?
9.41	8.8±0.7	?

Table 5.1:

Products of 11-dehydrocorticosterone metabolism in the perfused liver.
The HPLC elution times of the radiolabelled peaks of the steroids in the effluent from the intact perfused liver. The elution times were compared to the major hepatic metabolites of B as measured by ultra-violet absorbance at 254nm (Table 2.3) in order to identify the products. The major product of A metabolism was identified as corticosterone. However the other two radiolabelled peaks were not identified.

ensure there was enough radiolabelled steroid in the samples to give clear separation of peaks representing the products of A metabolism in perfused liver. Steroids were separated by HPLC and the radiolabelled peaks were compared to the elution profiles of steroid standards of the major hepatic metabolites of A and B (Table 2.3) to identify the products (Table 5.2). A representative HPLC trace of the UV absorbance of the steroid standards and of the radiolabelled peaks in perfusate is shown in Fig 5.6. The major product of A metabolism was identified as B. However two small peaks accounting for up to 30% of the total radiolabelled steroid recovered did not exactly co-elute with any of the steroid standards examined and so could not be identified.

5.2.5 Carbenoxolone Inhibition of 11ß-HSD-1 in Hepatocyte Cultures

The lack of inhibition of 11ß-reductase activity by CBX in the intact perfused liver is not in accordance with previous accounts of CBX inhibition of 11ß-HSD-1. CBX has a K_i of 10^{-9} to 10^{-8}M for 11ß-HSD-1 activity in tissues homogenates *in vitro* (Monder et al., 1989), and 100mg of CBX administered every 8 h to humans inhibits 11ß-reductase by approximately 50% (Walker et al., 1995; Stewart et al., 1990). Therefore CBX inhibition of 11ß-HSD-1 in primary hepatocyte cultures and in homogenates of rat liver was measured as described in section 2.3.7 and section

2.2.1 respectively. The K_i of CBX in hepatocyte cultures was calculated as approximately 5×10^{-5} M and in liver homogenates *in vitro* as 5×10^{-8} M (Fig. 5.7).

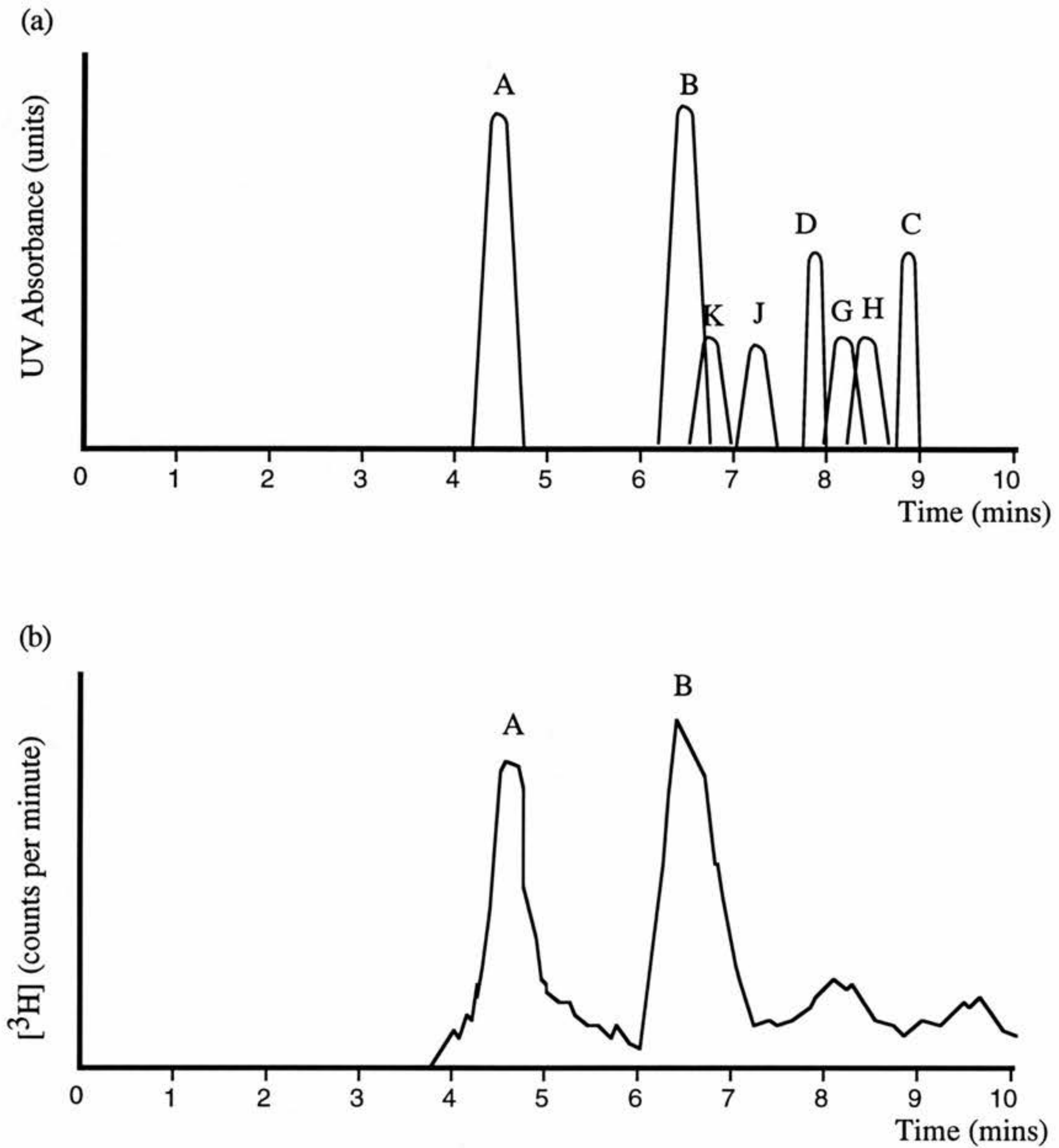


Figure 5.6: Products of 11-dehydrocorticosterone metabolism in the perfused liver.
(a) Elution profiles of steroid standards of the major hepatic metabolites of A and B as measured by UV absorbance at 254nm. A=11-dehydrocorticosterone, B=corticosterone, C=5α-dihydrocorticosterone, D=5ß-dihydrocorticosterone, G=3α5α–tetrahydrocorticosterone, H=3ß5α–tetrahydrocorticosterone, J=3α5ß-tetrahydrocorticosterone, K=3ß5ß-tetrahydrocorticosterone.
(b) Representative HPLC trace of the radiolabelled peaks in an extract of effluent from intact perfused rat liver perfused with [^{3}H-A].

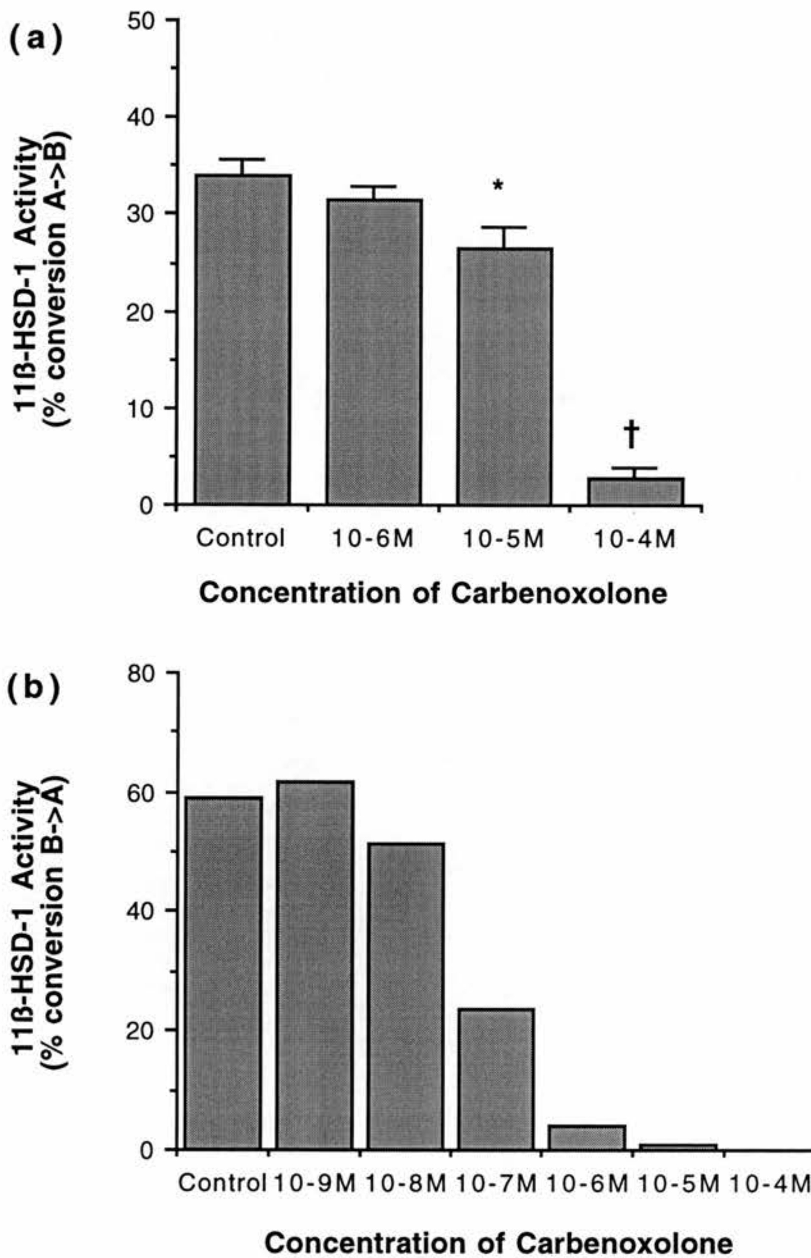


Figure 5.7: Carbenoxolone inhibition of 11ß-HSD-1 in (a) hepatocyte cultures and (b) homogenised liver.

(a) Activity is expressed as % conversion A to B (11ß-reductase) in the medium overlying the cells after 30 min. n=3-5. Results are from 3 separate culture preparations. *P<0.05 vs. control, †P<0.001 vs. control.

(b) 11ß-HSD-1 activity was assayed in homogenates of male rat liver. 11ß-HSD-1 activity is expressed as % conversion B to A at 37°C for 10 min in the presence of 200μM NADP. Values are the mean of duplicate incubations from a representative experiment.

5.3 Discussion

Examination of the metabolism of A and B across the intact perfused rat liver demonstrated that 11ß-HSD-1 activity is present and measurable in this system. This makes the liver perfusion model extremely useful for investigation of hepatic 11ß-HSD-1 as it allows examination of the function and the physiological role of the enzyme in the isolated organ, and provides a system for easy manipulation of conditions within the liver without the problems which beset *in vivo* experiments. The studies described in Chapter 4 of this thesis provide a valuable insight into the physiological role of hepatic 11ß-HSD-1, but are complicated by the lack of specificity of the methods employed to manipulate hepatic 11ß-HSD-1. These methods either have additional actions in other tissues or on other physiological processes, (e.g. CBX inhibition of renal 11ß-HSD-2 and of 11ß-HSD-1 in tissues other than liver), or are limited by the restricted access to the organ of interest. This, for example, enforced the estimation of hepatic insulin sensitivity by measurement of whole body glucose levels in the experiments in Chapter 4. This liver perfusion system overcomes some of these obstacles and allows more detailed examination of liver physiology in relation to 11ß-HSD-1.

Over 40% of A is converted to active B in the intact liver, reinforcing the evidence that 11ß-reduction is the primary activity of hepatic 11ß-HSD-1. However, in contrast to the data from Chapter 3 demonstrating its near exclusive 11ß-reduction in hepatocyte cultures, and from the other studies on 11ß-HSD-1 function in intact cells which demonstrate predominant 11ß-reduction (Low et al., 1994a; Agarwal et al., 1989; Rajan et al., 1995b), on passage through the intact liver, approximately 10% of B is converted to A. The reasons for this are not clear, but there are three possible explanations. Firstly, the equilibrium reached by 11ß-HSD-1 may differ in intact liver, as opposed to hepatocyte culture conditions. The equilibrium position of 11ß-HSD-1 will be determined by substrate availability, product inhibition and co-factor availability, all of which will differ *in vivo* from the *in vitro* situation. However, 11ß-reduction is the favoured direction and still predominates in the experiments described in this chapter (although in vivo 11ß-dehydrogenase is significant). Studies in humans *in vivo*, which have estimated hepatic 11ß-HSD-1 activity by indirect methods in the presence of endogenous steroids (Walker et al., 1995), would not have detected a small amount of 11ß-dehydrogenase activity in the presence of a predominance of 11ß-reductase activity. In contrast to the studies in hepatocyte cultures (Chapter 3), the cellular environment of hepatocytes *in vivo* is dynamic, with constant fluxes in substrate and product concentrations dependent

upon delivery and removal via the circulation, and on the processes of steroid metabolism within the liver. The experiments described in Chapter 4 indicated that cofactor availability is unlikely to be important in determining 11ß-HSD-1 reaction direction, but did not succeed in limiting the availability of NADPH, which could, in theory, shift the reaction direction in intact cells towards 11ß-dehydrogenation. Thus, if the 11ß-dehydrogenase activity is indeed due to the action of 11ß-HSD-1, then the precise factors which may determine reaction direction are still to be identified.

Secondly, the 11ß-dehydrogenation observed could be an experimental artefact. 11ß-dehydrogenase is readily demonstrable in homogenates of tissues or cells containing the 11ß-HSD-1 protein (Chapter 3; Low et al., 1993; Low et al., 1994b), and so in a compromised tissue such as a perfused liver, 11ß-HSD-1 protein may be released from its cellular context and carry out the dehydrogenation reaction. However, if this was the case, it would be expected that 11ß-dehydrogenation would increase over the course of the experiment, which it does not, making this an unlikely explanation.

Lastly, the bi-directionality of 11ß-HSD activity in the whole liver may be due to the presence of another enzyme catalysing the 11ß-dehydrogenation reaction. 11ß-HSD-2 is the only other enzyme known to have 11ß-dehydrogenase activity and it is not present in the liver parenchyma (Brown et al., 1993; Albiston et al., 1994; Brown et al., 1996). However its presence in the bile ducts (ZS Krozowski, personal communication) may account for the 11ß-dehydrogenation of glucocorticoid observed following perfusion through the whole liver.

The conversion of A to B increased as the proportion of substrate delivered via the hepatic portal vein increased with respect to the hepatic artery. The hepatic artery normally delivers some 20% of the volume of blood delivered via the hepatic portal vein to the liver (Blomley et al, 1995), and so this route of flow will have a smaller total volume capacity and therefore a faster passage time through the liver. However, this does not appear to be a purely mechanical phenomenon as the proportion of A converted to other steroid products did not vary to a similar extent and 11ß-dehydrogenation did not vary with route of substrate delivery. Although the total passage time through the hepatic circulation will be less for perfusate delivered via the hepatic artery, the passage time through the actual hepatic lobule will be the same regardless of the route, as the both routes merge at the level of the lobular circulation (Bloom & Fawcett, 1975). The functional significance of this difference in metabolism with route of delivery, if any, is not clear as both are afferent blood vessels and so deliver substrate to the hepatic lobule in the periportal zone (Bloom &

Fawcett, 1975). Closer examination of the distribution of 11ß-HSD-1 within the liver, particularly with respect to its intralobular localisation may yield an explanation for this discrepancy.

In the rat, A circulates at levels of around 50 nM (R Best and JR Seckl, unpublished data and section 4.2.2) and a large portion of this is unbound to plasma proteins. Thus the lack of effect of A concentration on the percentage of substrate to product conversion by 11ß-reductase in the perfused liver when concentrations of up to 1µM A were employed, indicates that the enzyme has a huge reserve capacity. Supraphysiological A levels do not saturate the enzyme, in agreement with what would be predicted from the µM Km of the enzyme (Monder & Lakshmi, 1989; Monder et al., 1991), and so physiological variations in substrate levels are unlikely to affect hepatic 11ß-reductase function.

The rate of blood flow through the male rat liver is normally approximately 1.75 ml/min/g (Angus et al., 1995), and so the rate of flow employed in these experiments (normally 15 ml/min) is not very different to the physiological flow rate. There was approximately 40% conversion of A to active B on a single pass through the male rat liver in these experiments, whilst in female rats, the conversion was approximately half this amount, consistent with measurements *in vitro* (Lax et al., 1978; Low et al., 1993). This is on a single pass through the liver. Therefore, whether the difference in hepatic levels of 11ß-HSD-1 between the sexes is of physiological significance under normal conditions must remain in question, since such avid generation of active glucocorticoid within the liver may ensure that sufficient intrahepatic glucocorticoid levels to ensure that glucocorticoid dependent hepatic functions are not compromised are always maintained. Nevertheless, changes in hepatic 11ß-HSD-1 activity will alter the equilibrium reached such that intrahepatic glucocorticoid levels will be higher when 11ß-HSD-1 activity is greater. Given the antagonism of insulin action by glucocorticoids in the liver (de Feo et al., 1989; Dinneen et al., 1993; Rooney et al., 1994), this would have implications for insulin sensitivity, particularly in situations of insulin deficiency, either relative (NIDDM) or absolute (insulin-dependent diabetes mellitus). Glucocorticoid action may be more pronounced in situations of insulin deficiency, since F replaces insulin as the major regulator of PEPCK in diabetes (Friedman et al., 1993).

The efficiency of conversion of A to B suggests that a substantial reduction in 11ß-HSD-1 activity within the liver would be needed in order to effect changes in insulin sensitivity in a normal individual. Nevertheless, the potential for quantitative alterations in hepatic 11ß-HSD-1 is large, and as discussed in Chapter 4, means of improving insulin sensitivity by attenuation of hepatic 11ß-HSD-1 may have a

proportionally greater effect in situations of insulin resistance. However, in this perfusion model, CBX, which is the most potent inhibitor of 11ß-HSD-1 available at present, showed itself to be extremely ineffective. At 10^{-3}M, CBX forms a very viscous solution, which apart from making it a difficult concentration to employ *in vivo*, probably poorly accesses its proposed (intracellular) site of action. At this concentration, CBX also inhibits other steroid metabolising enzymes, most notably 5ß-reductase (Latif et al., 1990), and activates both MR and GR (Armanini et al., 1983), adding to the impracticalities of employing it as a selective 11ß-HSD-1 inhibitor. In hepatocyte cultures, $5x10^{-5}$ M CBX effectively inhibited 11ß-HSD-1, presumably due to easier access to the hepatocytes. This may account for the effects of CBX on insulin sensitivity when administered *in vivo* (Walker et al., 1995, Chapter 4), where continuous administration of CBX may allow an equilibrium to become established over some time as the drug gains access to hepatocytes ("drug loading"). Alternatively, the decrease in 11ß-HSD-1 mRNA expression following CBX administration *in vivo* (section 4.2.3) may be the mechanism by which CBX decreases hepatic 11ß-HSD-1 activity, or a combination of these mechanisms may operate. Therefore a greater effect of CBX on 11ß-HSD-1 inhibition may be observed in perfused livers from animals pre-treated with CBX. Information on the metabolism of CBX is scant but it may be that hepatic metabolism of the drug is an important causative factor in its lack of efficiency in 11ß-HSD-1 inhibition in the intact liver. That aside, these data show that the concentration of CBX required to inhibit 11ß-reductase in intact cells is at least 100 times that required in homogenates, and is even greater in the whole organ over a short time period. This highlights again the need for a potent and selective inhibitor for 11ß-HSD-1 in order to carry out further studies, and as a potential therapeutic agent.

More detailed information on the fate of the glucocorticoid entering the perfused liver in these experiments is required in order to validate the model. Up to 95% of the steroid delivered in the perfusate was sequestered within the liver. *In vivo,* at physiological concentrations, 90-97% of circulating F is protein-bound, 93% of which is bound to CBG (Dunn et al., 1981; Partridge, 1981). The concentration of steroid employed in the liver perfusions was chosen as an approximation of the concentration of "free" hormone circulating in the plasma, as the intracellular concentration of a hormone, and thus the amount available for metabolism by 11ß-HSD-1, is proportional to the concentration of free hormone in the plasma and not to that of the plasma protein bound hormone (Mendel, 1989). The perfusate was therefore protein-free. However, this means that a large proportion of the glucocorticoid in the perfusate will bind when exposed to binding-proteins. CBG is

produced primarily in the liver (Brien, 1981) and it is therefore likely that some B will be sequestered within the liver by CBG. CBG may also bind A, but at a much lower level. Thus there will probably be a quantitatively lesser sequestration of A. In addition, B will bind within the liver to intracellular GR. DEX is not bound by CBG (Pugeat et al., 1981), but has high affinity for GR and so could be used to estimate glucocorticoid associated with GR over the course of a perfusion. Perfusate containing plasma protein may lessen the glucocorticoid loss within the liver, although hormone enters tissues exclusively from the pool of free hormone following spontaneous dissociation from the binding proteins in the circulation (Mendel, 1989) and so excess protein binding may limit diffusion of hormone into the liver. If this was the case, an excess of free hormone would have to be ensured. However, given that hepatic uptake of F is several times greater than the amount of free F in plasma and that the uptake from protein-free solutions by the perfused rat liver is high enough to account for this uptake (i.e. uptake of protein-bound F is not necessary) (Mendel et al., 1989), protein-binding in the perfusate may not be such a problem.

However, the binding capacity of CBG and GR is finite, and although the proportion of steroid recovered increased slightly over the first few minutes of a perfusion, the majority continued to be removed from the perfusate for the length of the experiment. There are two other likely possibilities for the fate of the large quantities of glucocorticoid unaccounted for. Sequestration of steroids into the biliary system may be considerable, and *in vivo*, they would enter the enterohepatic circulation, and return to the liver in the portal blood following intestinal reabsorption. Cannulation of the major bile ducts or of the duodenum may allow some estimation of steroid entering this pathway. The majority of steroid however, is likely to be present in the perfusion effluent. Following hepatic metabolism (the main pathways of which are outlined in Fig. 1.4), a large proportion of glucocorticoid metabolites undergo conjugation with glucuronic acid or sulphate in order to render them more water soluble and aid excretion (Orth et al., 1992). Glucuronidation predominates and is catalysed by isozymes of UDP glucuronosyl transferase in the endoplasmic reticulum (Siest et al., 1987) and most 3α5ß-tetrahydro metabolites are excreted as glucuronides (Orth et al., 1992). Most 3ß-hydroxysteroids undergo sulphation in the cytosol (Orth et al., 1992). All such conjugated products, being water soluble, are not recovered by the methods employed in these experiments. Hydrolysis of conjugated products in the samples, followed by non-polar solvent extraction with ethyl acetate will yield information on

the proportion of steroid in this fraction, and the nature of the products of the hepatic metabolism.

The other products of A and B metabolism detected by HPLC analysis were not identified. They were not the major dihydro- or tetrahydro- metabolites of corticosterone. The proportion of these products did not vary with alterations in 11ß-HSD-1 activity suggesting they are not dependent on the production of corticosterone, and so are likely to be the result of alternative pathways of A metabolism. Glucocorticoids are subject to hepatic metabolism via a number of pathways (section 1.1.5 and Fig. 1.4), which are not distinct, and so the ultimate fate of an intermediate metabolite is indeterminate. Determination of the identity of the other products of A and B metabolism will form the basis for further studies.

Notwithstanding these problems, these preliminary experiments have provided useful information regarding the nature of hepatic 11ß-HSD-1, and with further refinement the liver perfusion model will prove an extremely useful tool in investigation of the function of 11ß-HSD-1 by virtue of its ability to permit access to the isolated liver. This will mean more convenient sampling and easier manipulation of the hepatic environment than has previously been possible and therefore allow more detailed investigations into the physiology of hepatic 11ß-HSD-1.

CHAPTER 6

THE IN VIVO REGULATION OF 11ß-HSD-1

6.1 Introduction

The majority of studies on hormonal regulation of 11ß-HSD have focused on measuring changes in enzyme activity, and the methods employed do not necessarily distinguish the effects of 11ß-HSD-1 and 11ß-HSD-2. Therefore, this should be kept in mind when drawing conclusions from any studies on tissues which may contain both isozymes. However the liver does not express 11ß-HSD-2 (Brown et al., 1993; Albiston et al., 1994; Brown et al., 1996), and to date 11ß-HSD-2 has not been demonstrated to be present in the adult hippocampus, and so data from studies on these tissues as described in this chapter are likely to pertain to the type 1 isozyme. 11ß-HSD-1 is regulated *in vivo* by various hormones, including glucocorticoids, insulin, sex steroids, GH and T_3, in a tissue- and developmentally-specific manner (section 1.3.4). The experiments described in this chapter pertain to the hormonal regulation of 11ß-HSD-1 in the liver and the hippocampus *in vivo*, and are in the main preliminary studies aimed towards further unravelling the complex hormonal control of this enzyme.

11ß-HSD regulation by T_3 has been reported to differ between species as well as the sexes, with raised T_3 levels increasing the conversion of F to E in humans and thyroidectomy reversing this effect (Gordon & Southren 1977; Zumoff et al., 1983). However, indirect estimation of enzyme activity in humans by the measurement of plasma or urinary metabolites do not take account of 11ß-HSD-2 activity, and, in contrast, thyroid hormone decreases enzyme activity in rat liver (Whorwood et al., 1993a), whilst thyroidectomy increases activity in female rat liver but decreases it in the male rat (Lax et al., 1979; Whorwood et al., 1993a). Thus the studies are inconclusive and contradictory. One of the problems with studies of T_3 regulation in the rat, is that GH secretion is almost totally dependent on T_3 (Evans et al., 1992; Spindler et al., 1992; Peake at al., 1973; Hervas et al., 1975; Coiro et al., 1979). Therefore the sexually dimorphic effects of T_3 on 11ß-HSD-1 in rat liver may be mediated indirectly via GH, whilst an additional direct effect of T_3 may or may not be operating simultaneously. Studies employing thyroidectomy to model T_3 deficiency in the rat are complicated by loss of the parathyroid and thyroid C-cells in addition to the thyroid follicle cells, whilst studies employing antithyroid drugs such as methimazole and propylthiouracil inhibit T_3 production from T_4 inconsistently. and may be associated with side-effects (Horvath et al., 1989; Rutgers et al., 1990; Chiba et al., 1990).

An elegant mouse model has been created in which the T_3 producing thyroid follicle cells have been specifically and completely ablated, to provide a model of

selective and complete T_3 deficiency (Wallace et al., 1991). In these mice, the coding region of the herpes simplex type 1 virus thymidine kinase gene coupled to the promoter of the bovine thyroglobulin gene was introduced into the mouse genome. As the viral thyroglobulin promoter directed expression of the thymidine kinase gene mainly in the thyroid gland and the testes (Al Shawi et al., 1991), this meant that administration of the antiviral agent glanciclovir effectively ablated thyroid follicle cells whilst leaving the parathyroid gland and the C-cells of the thyroid intact. This completely deprived the mice of T_3 (Wallace et al., 1991). I have investigated the T_3 regulation of 11ß-HSD-1 in these mice, thus avoiding the complications of surgical thyroidectomy and hypothyroidism achieved by drug administration.

Hepatic 11ß-HSD-1 is sexually dimorphic in rats, with females showing approximately half the activity of males (Lax et al., 1978; Low et al., 1993) due to suppression of activity in the female by the sexually dimorphic pattern of GH secretion (Low et al., 1994b). Whether hepatic 11ß-HSD-1 is sexually dimorphic in other species is unknown, but hepatic steroid and drug metabolism shows less pronounced sexual dimorphism in humans than in rodents (Pfaffenberg & Horning, 1977; MacLeod et al., 1979). Therefore I have measured 11ß-HSD-1 activity in the tissues of male and female mice in order to ascertain whether 11ß-HSD-1 is sexually dimorphic, clearly an important factor to be taken into consideration when interpreting results from the thyroid-ablated mice.

Previous studies have reported glucocorticoid induction of 11ß-HSD activity, both in the liver *in vivo* (Low et al., 1994; Walker at al., 1994) and in cell culture *in vitro* (Hammami & Siiteri, 1991 and Chapter 3). It has been shown that ADX of rats attenuates hepatic 11ß-HSD-1 enzyme activity and also attenuates reactivation of E to F (presumably mediated by hepatic 11ß-HSD-1) (Nicholas & Lugg, 1982) whilst administration of glucocorticoids to ADX rats increases hepatic 11ß-HSD-1 activity and mRNA (Low et al., 1994c). However in Chapter 4 (section 4.2.1.3) I showed that ADX increased 11ß-HSD-1 activity and mRNA expression with respect to sham ADX controls, an observation repeated on four separate occasions. In addition, glucocorticoid regulation of 11ß-HSD-1 appears to be tissue-specific, since neither ADX, nor glucocorticoid administration affect 11ß-HSD-1 mRNA levels, or enzyme bioactivity in the rat kidney (Moisan et al., 1990c; Smith & Funder, 1991). Chronic glucocorticoid excess or stress increase 11ß-HSD-1 mRNA and enzyme bioactivity in the hippocampus (Moisan et al., 1990c; Low et al., 1994c). However, the reasons for this are unclear, as chronic glucocorticoid excess is detrimental to neuronal function and survival (Landfield et al., 1978; Sapolsky et al., 1985, 1986; Sapolsky,

1992), and this would potentiate their neurotoxicity. Therefore I have further investigated glucocorticoid control of 11ß-HSD by examining the effects of ADX and glucocorticoid replacement over a time-course of 21 days in liver, kidney and hippocampus in the rat.

Various experimental manipulations of circulating glucocorticoid levels, including exogenous corticosteroid administration and a range of stressors, have demonstrated deleterious effects on neuronal morphology and survival (Kerr et al., 1991; Landfield & Eldridge, 1994; Mizoguchi et al., 1992; Watanabe et al., 1992). Most of the studies on the effects of stress in the hippocampus have employed noxious stressors or physical restraint, but not all stressors have the same physiological effects. For example, Komesaroff and Funder (1994) have demonstrated that plasma adrenaline levels rise in fear-induced stress but not in hypoglycaemic stress. Psychological, or emotional stress is of particular interest as it considered to be an important element in the development of various neuropsychiatric diseases such as insomnia, anxiety disorders and depression (Checkley, 1992; Paykel, 1978) and studies on prolonged social stress in monkeys have provided evidence for a link between the degree of stress experienced and the extent of hippocampal pyramidal neuron damage (Uno et al., 1989).

One well-characterised experimental model for studying the effects of psychosocial stress is the tree-shrew (Flügge et al., 1992; Fuchs & Flügge, 1995; Fuchs et al., 1993; Jöhren et al., 1994). Male tree shrews establish stable dominant/ subordinate relations in the laboratory, and stress is only experienced by the subordinate animal (von Holst, 1977) with no negative physiological effects on the dominant individual (Fuchs et al., 1993; von Holst, et al., 1983). Stress in the subordinate animal results from the cognitive interpretation of the continuous visual presence of the dominant individual, not from physical interactions and exertions during fighting (von Holst, 1977; von Holst et al., 1983; Rabb & Stortz, 1976) making it a suitable model for studying the neuroendocrine responses to psychosocial stress. Subordinate tree-shrews exhibit disturbed feeding patterns, sleeping disturbances, alterations in circadian rhythm and clear behavioural depression. Endocrine responses include constant hyperactivity of the HPA axis and the sympathoadrenal system (Fuchs, 1984; Fuchs et al., 1992, 1993; von Holst, 1977), whilst in the hippocampus, $\alpha 2$-adrenoreceptors, GR mRNA and CRH-binding sites are decreased (Flügge et al., 1992; Fuchs & Flügge, 1995). Many of these reactions are comparable to the symptoms observed in clinically depressed patients. Therefore, I have examined the regulation of 11ß-HSD-1 activity in the hippocampus by psychosocial stress, glucocorticoids and growth hormone, in this

model, in order to see if there are changes in the enzyme's activity, thus indicating a role for hippocampal 11ß-HSD-1 in psychosocial stress.

6.2 Results

6.2.1 Thyroid Hormone Regulation of Hepatic 11ß-HSD-1 in the Mouse

RNA used in these experiments was donated by Dr. Helen Wallace of the Institute of Cell and Population Biology, University of Edinburgh (Wallace et al., 1991). 11ß-HSD-1 was quantitated in the livers of transgenic mice in which the thyroid follicle cells had been specifically ablated. The vehicle for glanciclovir (administration of which effectively ablated the thyroid follicle cells) was PBS. Some thyroid ablated mice received daily T_4 replacement injections (250µg/kg) and some received 12 hourly GH injections (5 mg/kg). Controls were mice equivalent to the transgenic mice in background (Wallace et al., 1991).

I quantified 11ß-HSD-1 mRNA expression in these transgenic mice (Fig. 6.1). In female mice, thyroid ablation did not alter 11ß-HSD-1 mRNA expression, and GH replacement had no effect. However T_4 replacement significantly reduced 11ß-HSD-1 expression when compared to controls and to thyroid ablated mice (Fig 6.1). In male mice, 11ß-HSD-1 mRNA expression followed the same pattern, but numbers were not large enough to allow statistical analysis (data not shown).

6.2.2 Sexual Dimorphism of 11ß-HSD-1 Activity in the Mouse

Measurement of 11ß-HSD activity in the liver, hippocampus and kidney of male and female CBA/B6 F1 mice (section 2.2.1) showed no differences between the sexes (Fig. 6.2).

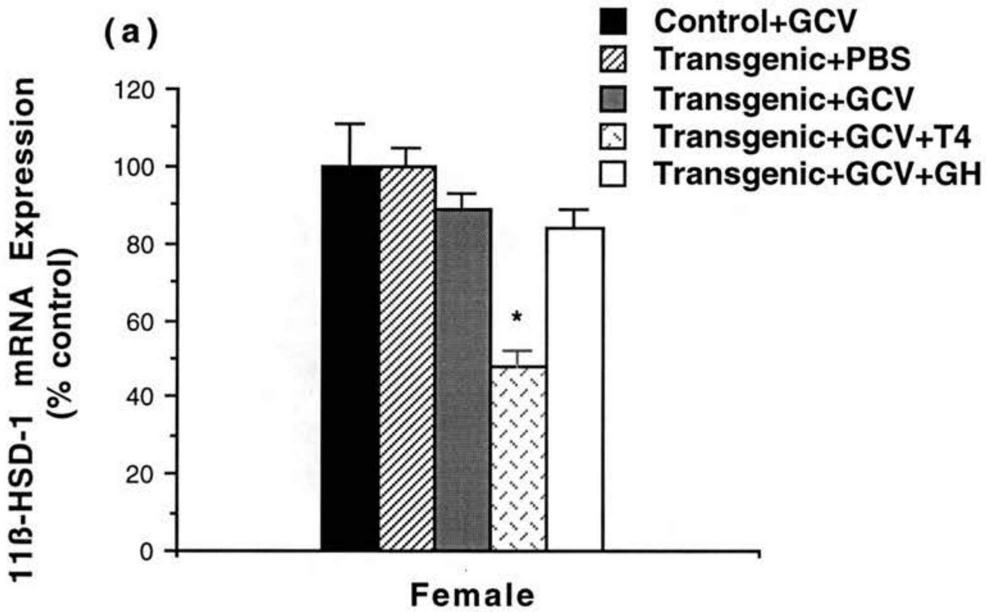


Figure 6.1: Thyroid hormone regulation of hepatic 11ß-HSD-1 in the mouse. Quantification by northern analyses of 11ß-HSD-1 mRNA levels from the livers of female non-transgenic (Control+GCV) and transgenic female mice following thyroid ablation (Transgenic+GCV) ± T_4 or GH replacement or sham ablation (Transgenic+PBS). Data are expressed as a percentage of mRNA levels (±SEM for females, n=3-5) in control animals. GCV=glanciclovir, PBS=phosphate buffered saline (vehicle), T4=thyroxine, GH=growth hormone.
*P<0.01 compared with all other columns.

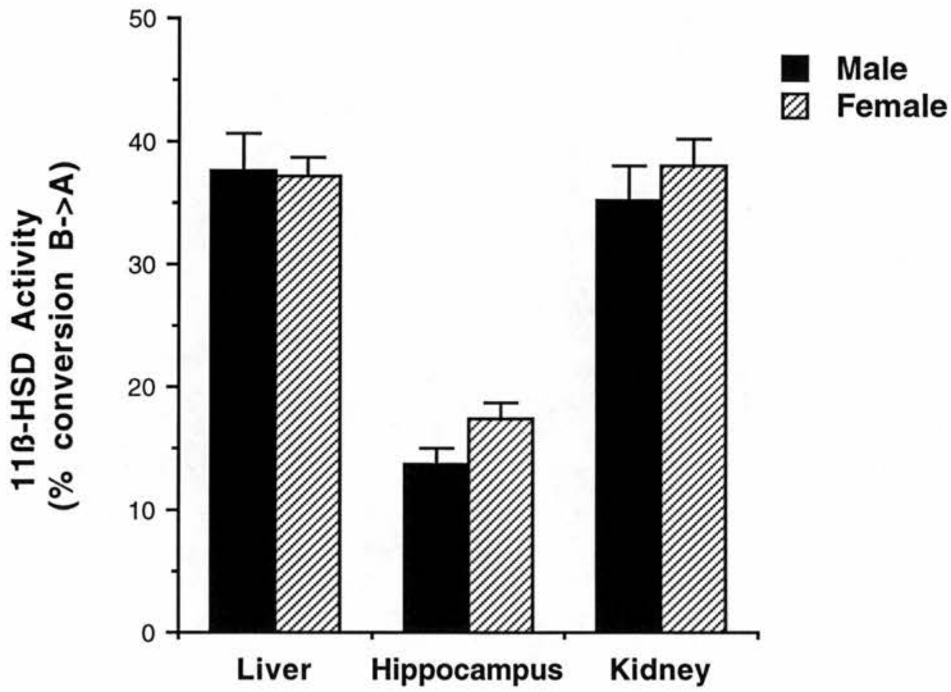


Figure 6.2: Lack of Sexual dimorphism of 11ß-HSD activity in mice.
11ß-HSD-1 activity was assayed in homogenates of tissues from male and female mice. Data are expressed as mean±SEM. n=9-10.

6.2.3 The Effects of Adrenalectomy and Glucocorticoid Replacement Upon 11ß-HSD-1

The experiments described in section 4.2.1.3 reproducibly showed an increase in hepatic 11ß-HSD-1 activity in ADX animals when compared to sham ADX controls in contrast to previous studies reporting glucocorticoid induction of 11ß-HSD-1 in the liver *in vivo* (Low et al., 1994; Walker at al., 1994) and in cell culture *in vitro* (Hammami & Siiteri, 1991 and Chapter 3). A possible reason for this discrepancy is the longer time course of adrenal manipulations in the experiments in section 4.2.1.3 (21 d as opposed to 1-10 d in previous studies). Therefore the time course of the effects of adrenal manipulations upon 11ß-HSD activity and 11ß-HSD-1 mRNA expression in rat liver, hippocampus and kidney was examined in more detail. Male rats were either adrenalectomised or sham-operated and some ADX animals received glucocorticoid replacement in the form of high dose DEX. 11ß-HSD activity and 11ß-HSD-1 mRNA expression were measured 2, 5, 9, 15 or 21 d after surgery. Untouched animals served as controls. A representative northern blot is shown in Fig. 6.3.

Sham ADX resulted in a continuing decrease in both 11ß-HSD-1 activity and mRNA expression in the liver over 21d (Fig. 6.3 and Fig. 6.4). The decrease in 11ß-HSD-1 mRNA levels preceded the decrease in activity. There was no recovery in 11ß-HSD-1 activity or mRNA levels over this time period. ADX resulted in a quicker decrease in 11ß-HSD-1 activity and mRNA than did sham ADX, such that both were minimal and significantly less than control levels at 2 d (Fig. 6.4). 11ß-HSD-1 activity then recovered, reaching control levels by 9 d, whereas mRNA levels remained at approximately 50% of control levels over the time course examined, confirming the observations in section 4.2.1.3. ADX together with DEX also resulted in a significant decrease in hepatic 11ß-HSD-1 levels apparent by 2 d (Fig. 6.4). There was no recovery in mRNA levels over 21 d. 11ß-HSD-1 activity however did not alter, except perhaps for a fall in the ADX with DEX group at the end of the time course, with activity at 21 d less than that at 5 d. At 21 d, hepatic 11ß-HSD-1 activity is less following DEX treatment than after ADX alone, and sham ADX animals have lower activity and mRNA expression than either of the other two groups.

Hippocampal 11ß-HSD-1 activity fell following sham ADX, and remained significantly lower than control until at least 9 d (Fig. 6.5). ADX resulted in changes in 11ß-HSD-1 activity in the hippocampus which were significant between each sequential time point. Activity fell following ADX, reaching a low point at 9 d, and

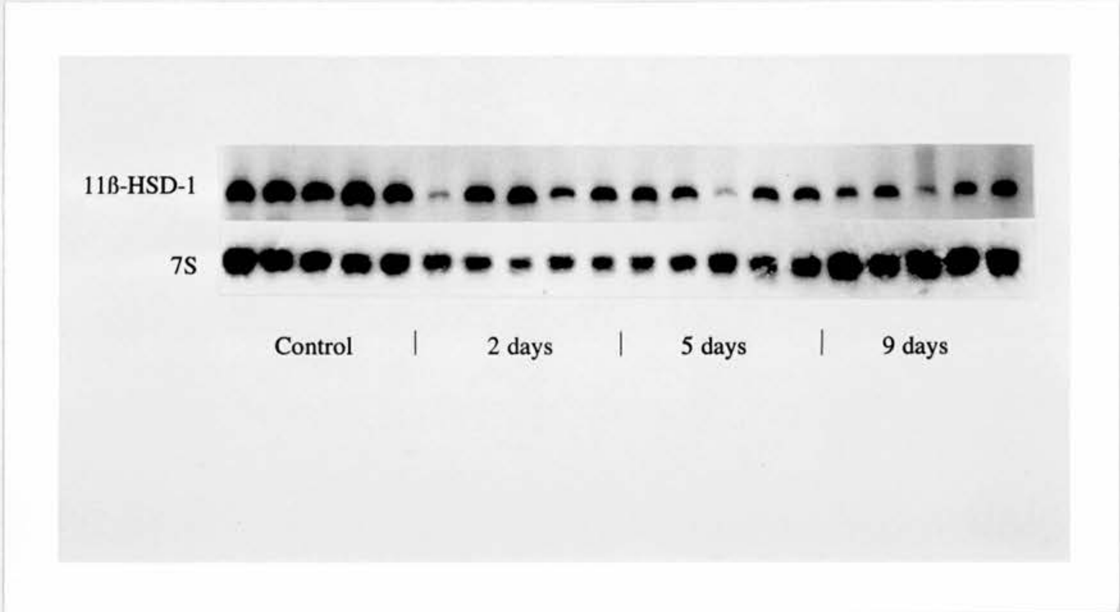


Figure 6.3: Time course of the effect of sham adrenalectomy upon hepatic 11ß-HSD-1 mRNA expression.

Autoradiograph of northern blots of RNA from the livers of male rats at various time points following sham ADX. Controls are untouched animals. Hybridised to 11ß-HSD-1 and 7S cDNAs.

then recovering, but were still significantly less than control at 21 d. ADX followed by DEX led to a slow decline in hippocampal 11ß-HSD-1 reaching significance at 15 d. There was no difference in activity between the groups at any time point. Neither was there any difference in 11ß-HSD-1 mRNA levels between or within treatments (Fig. 6.5), probably owing to large standard errors, but there was a trend for 11ß-HSD-1 in the hippocampus to fall following all manipulations and then to recover over the time course.

11ß-HSD activity in the kidney (reflecting the presence of both 11ß-HSD-1 and 11ß-HSD-2 activities) was unaffected by sham ADX (Fig. 6.6). There was, however, a small but significant decrease in renal 11ß-HSD activity following ADX, with the lowest level at 5 d. Levels were back to control values by 9 d. Likewise, ADX followed by DEX showed a small drop in activity at 2 d. At 21 d, both ADX and ADX followed by DEX resulted in 11ß-HSD activity levels less than in the control group. In contrast, both sham ADX and ADX resulted in a dramatic decrease in renal 11ß-HSD-1 mRNA expression (Fig. 6.6) which did not recover over the time courses examined.

6.2.4 The Effects of Chronic Stress and Glucocorticoid Administration Upon 11ß-HSD Activity in the Tree-Shrew

I examined alterations in 11ß-HSD activity in the hippocampus, liver and kidney in a well-documented model of chronic psychosocial stress in the tree-shrew, a species close to the primate lineage. All the *in vivo* manipulations in tree-shrews were carried out at the German Primate Centre (Göttingen, Germany). Details of the experimental design of this model are described by Fuchs et al. (1992). Briefly, the opaque partition between the cages of pairs of male tree-shrews unknown to each other were removed leading to territorial competition. Once stable dominant/subordinate relationships had been established, the partitions were replaced by transparent wire mesh, so that although each animal in a pair was caged separately, the subordinate males were subjected to a continuing psychosocial stress. The dominant male served as a control. Additional tree-shrews received excess F (~6 mg/day) in the drinking water. After 28 d the animals were killed and 11ß-HSD activity was assayed in tissue homogenates of liver, hippocampus and kidney (Fig. 6.7). In the hippocampus, chronic psychosocial stress led to an attenuation of 11ß-HSD-1 activity whereas excess cortisol alone had no effect. In contrast, both chronic stress and cortisol alone decreased hepatic 11ß-HSD-1 activity. No changes in 11ß-HSD activity were observed in kidney.

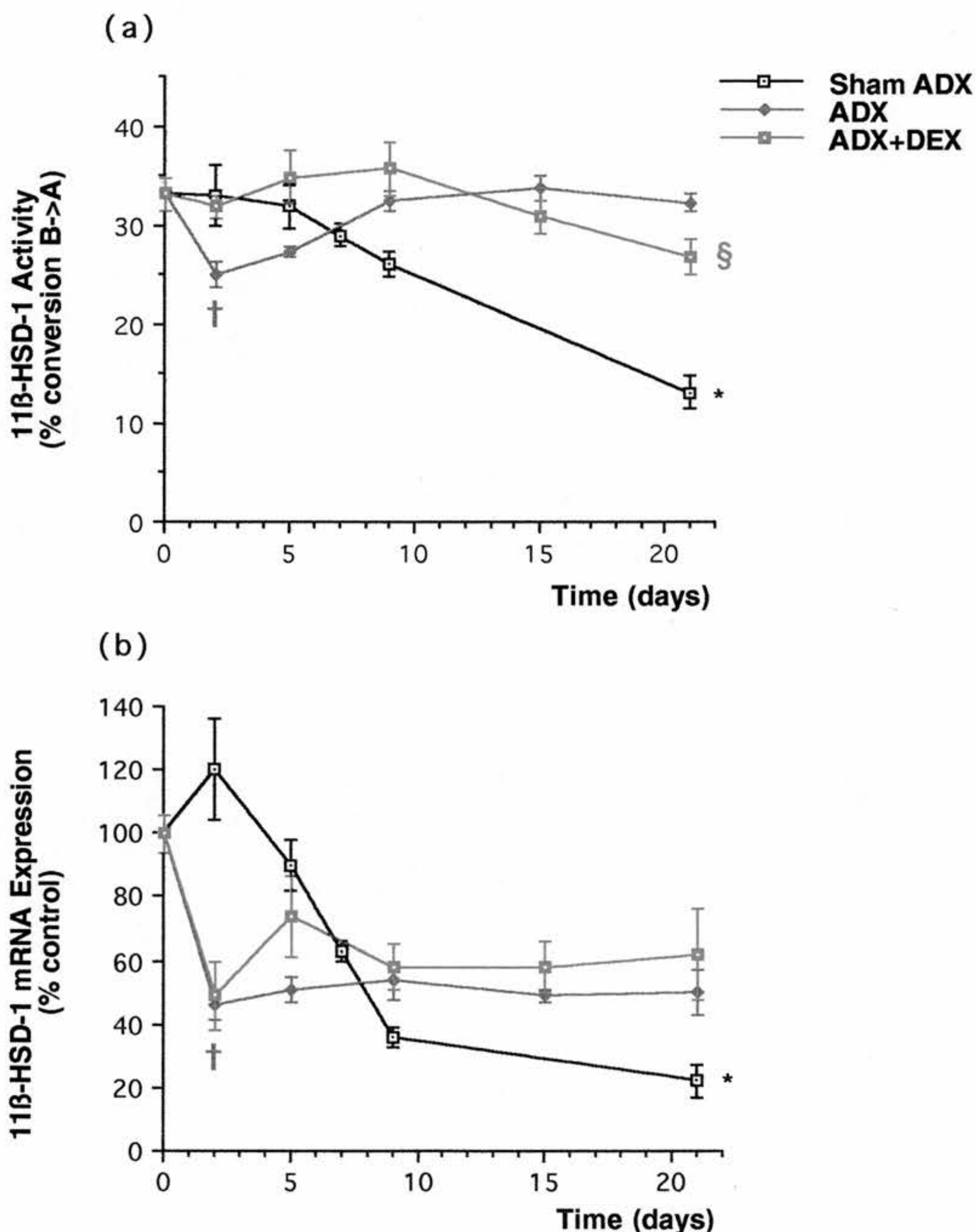


Figure 6.4: Time course of the effect of adrenalectomy and dexamethasone replacement upon hepatic 11ß-HSD-1.

(a) 11ß-HSD-1 activity and (b) 11ß-HSD-1 mRNA levels were quantified from the livers of male rats at various time points following ADX, ADX with DEX or sham ADX. Controls are untouched animals and are represented by the values on the y-axis at the 0 d time point. Data are expressed as (a) % conversion B to A (±SEM) or (b) percentage of mRNA levels (±SEM) in untouched control animals. n=5.

*$P<0.001$ compared with control; †$P<0.01$ compared with control; §$P<0.05$ compared with control.

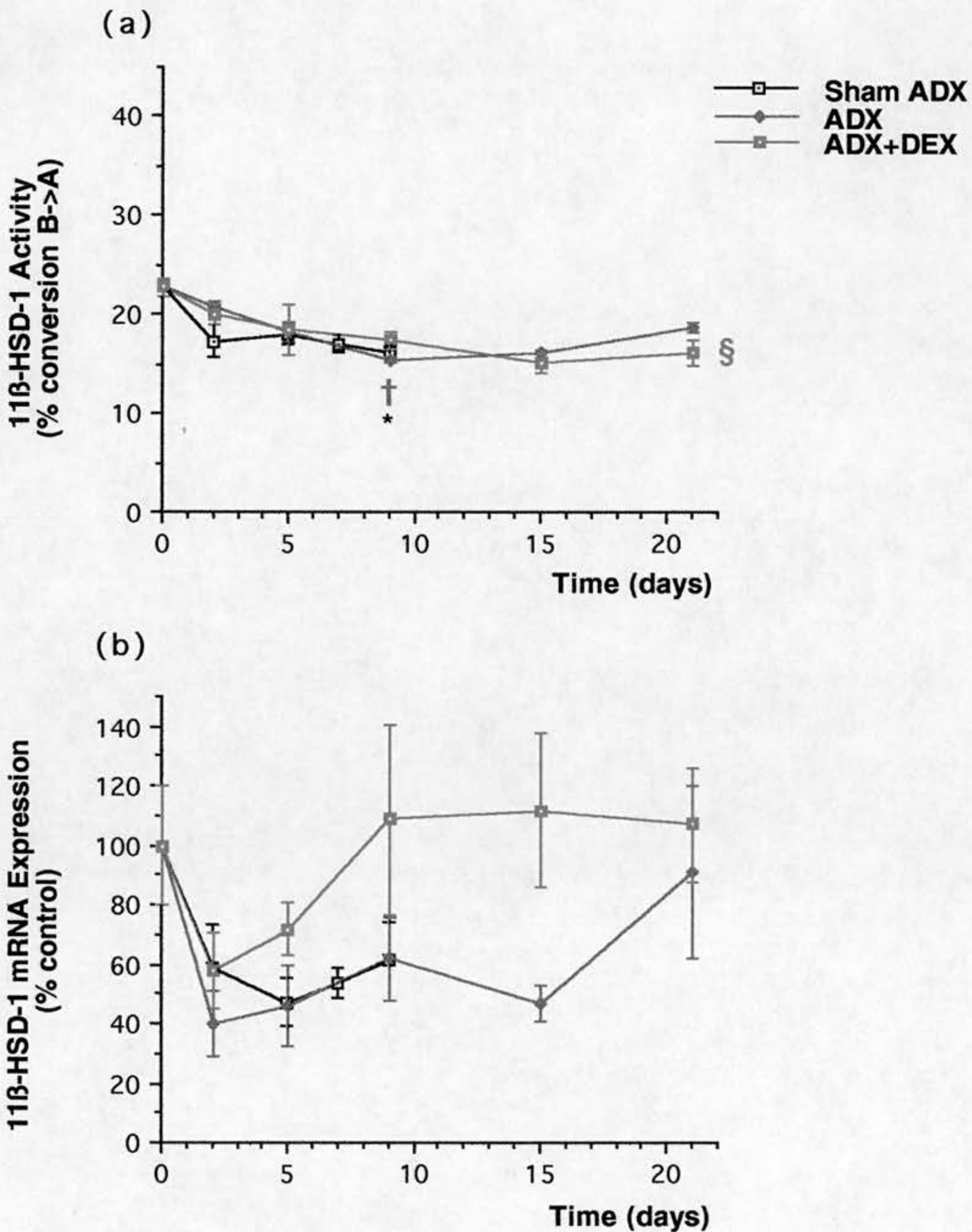


Figure 6.5: Time course of the effect of adrenalectomy and dexamethasone replacement upon hippocampal 11ß-HSD-1.

(a) 11ß-HSD-1 activity and (b) 11ß-HSD-1 mRNA levels were quantified from the hippocampi of male rats at various time points following ADX. ADX with DEX or sham ADX. Controls are untouched animals and are represented by the values on the y-axis at the 0 d time point. Data are expressed as (a) % conversion B to A (±SEM) or (b) percentage of mRNA levels (±SEM) in untouched control animals. n=5.

*$P<0.01$ compared with control; †$P<0.001$ compared with control; §$P<0.05$ compared with control.

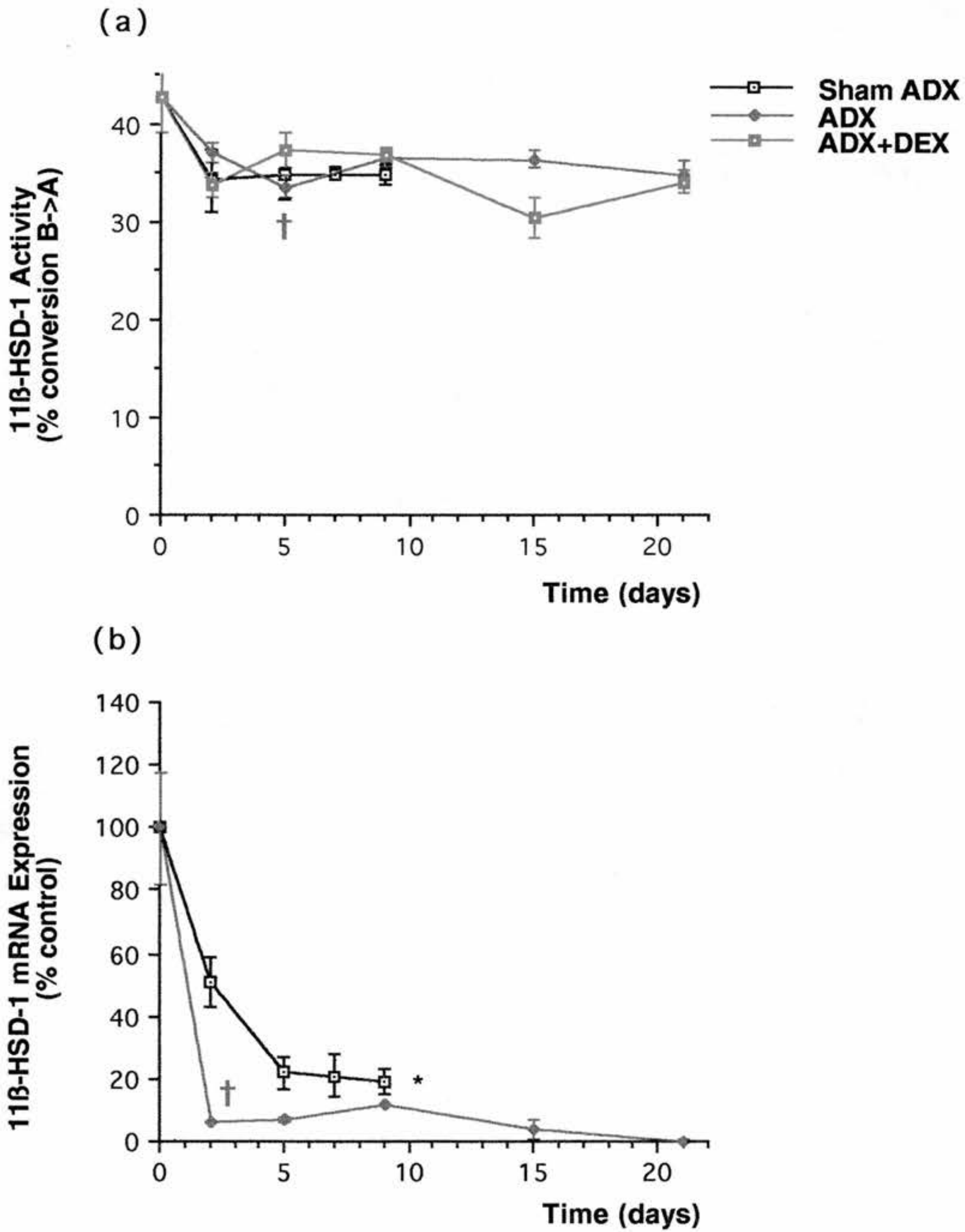


Figure 6.6: Time course of the effect of adrenalectomy and dexamethasone replacement upon renal 11ß-HSD.

(a) 11ß-HSD activity and (b) 11ß-HSD-1 mRNA levels were quantified from the kidneys of male rats at various time points following ADX, ADX with DEX or sham ADX. Controls are untouched animals and are represented by the values on the y-axis at the 0 d time point. Data are expressed as (a) % conversion B to A (±SEM) or (b) percentage of mRNA levels (±SEM) in untouched control animals. n=5.
*P<0.001 compared with control; †P<0.001 compared with control.

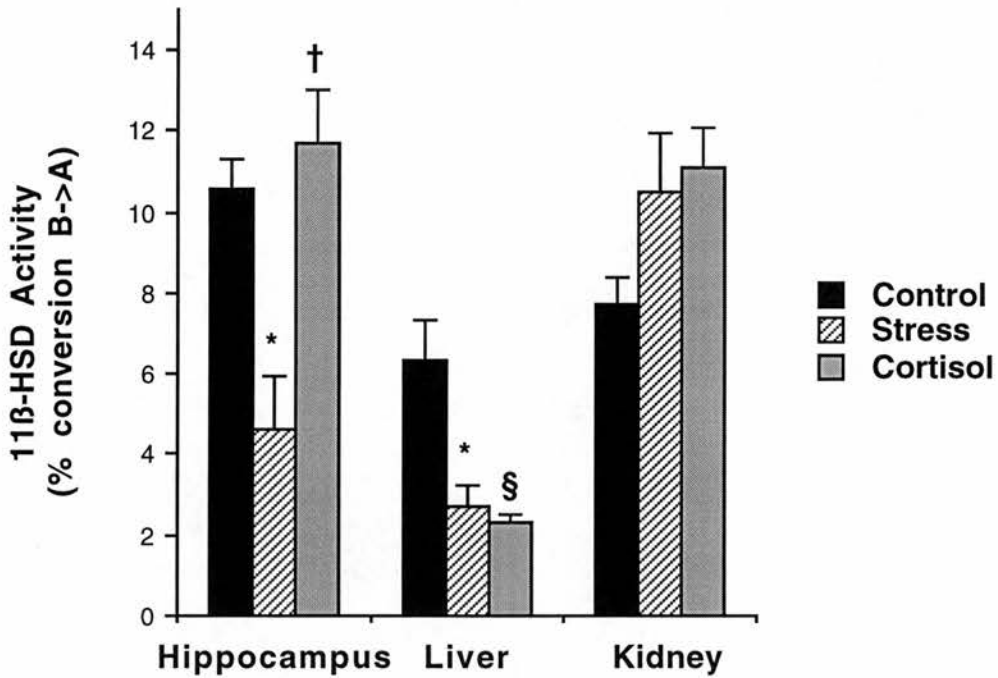


Figure 6.7: The effects of chronic stress and glucocorticoid administration upon 11ß-HSD activity in the tree-shrew.

11ß-HSD activity was assayed in homogenates of hippocampus, liver and kidney from male tree-shrews following 28 days psychosocial stress or F administration. Data are expressed as % conversion B to A (±SEM). n=4-8.

*$P<0.01$ compared with control; †$P<0.01$ compared with stress. §$P<0.05$ compared with control.

6.2.5 Effect of Introcerebroventricular Administration of Growth Hormone on 11ß-HSD-1 mRNA Expression in the Brain

Hippocampal 11ß-HSD-1 is not sexually dimorphic in rats, nor can it be altered with sex steroid or GH manipulations (Low et al., 1993; Low et al., 1994b), and it was proposed that this was due to the failure of GH to cross the blood-brain-barrier, which is impervious to most small peptides. In order to test this hypothesis, GH was continuously infused into the lateral ventricle of the brains of male rats for 5 days (section 2.6.5) This had no effect upon 11ß-HSD-1 mRNA expression in the hippocampus, cerebellum or cerebral cortex (Fig. 6.8). It did however significantly reduce 11ß-HSD-1 mRNA expression in the liver and kidney of the same animals (Fig 6.8).

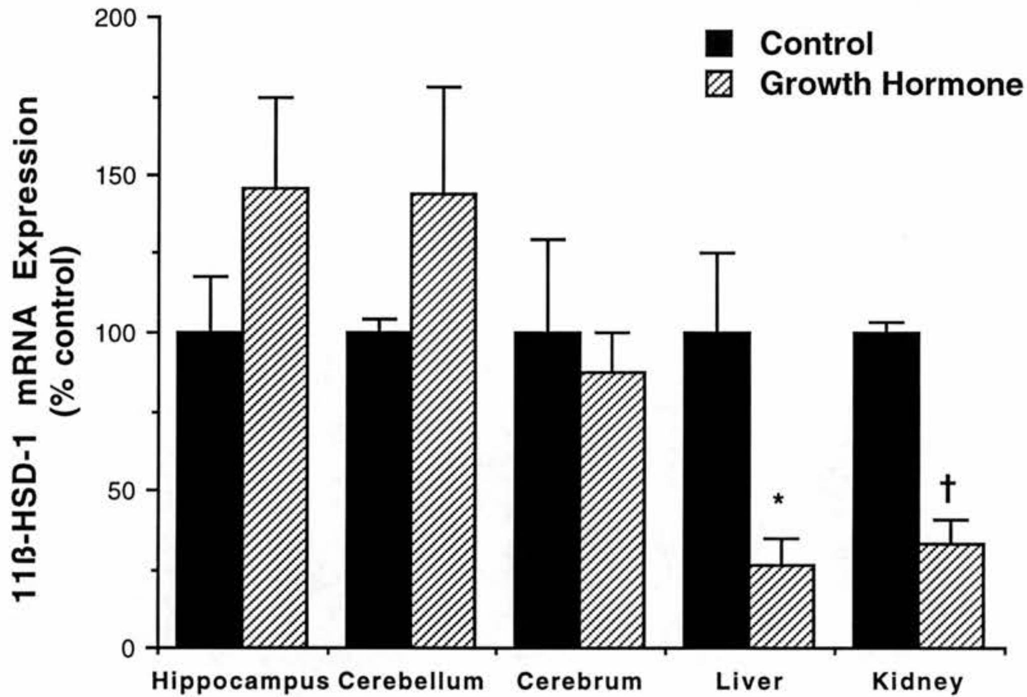


Figure 6.8: Effect of introcerebroventricular administration of growth hormone upon 11ß-HSD-1 mRNA in brain.

11ß-HSD activity was assayed in homogenates of brain regions, liver and kidney of male rats following 5 days continuous ICV infusion of growth hormone. Data are expressed as % of control levels±SEM. n=4-5.

*P<0.05 compared with control; †P<0.001 compared with control.

6.3 Discussion

11ß-HSD-1 activity has previously been described in several mouse tissues (Burton & Tufnell, 1967), and the recent creation of a transgenic 11ß-HSD-1 "knock-out" mouse (Kotelevtsev et al., 1996) means that the regulation of 11ß-HSD-1 in mouse is of interest, particularly in the liver, as preliminary data from these mice show a decrease in fasting levels of glucose and a lack of induction of hepatic PEPCK and G-6-Pase in response to starvation (YV Kotelevtsev et al., personal communication). Measurement of murine hepatic 11ß-HSD-1 activity revealed that in contrast to the rat (Lax et al., 1978; Low et al., 1993), there is no sexual dimorphism. In addition, there is no sexual dimorphism in hepatic 11ß-HSD-1 mRNA expression as quantified by northern analysis (Rajan et al., 1995a). Sexually dimorphic expression of 11ß-HSD-1 in the rat is mediated by sex-dependent patterns of GH secretion (Low et al., 1994b). The GH-mediated sexual differentiation of the liver (Gustafsson et al., 1983a; Gustafsson et al., 1983b; Roy & Chatterjee, 1983; Mode, 1993) is not peculiar to the rat and operates in mice also (Norstedt & Palmiter, 1984). Although some differences are smaller than in the rat, they are still large (Norstedt & Palmiter, 1984), suggesting that sexual-dimorphism of hepatic 11ß-HSD-1 in mice, if present, would be detectable by the 11ß-HSD-1 bioassay. Nevertheless, it is possible that blunting of a sexual-dimorphism of hepatic 11ß-HSD-1 in mice may render it undetectable. That these effects of GH are due to a direct action on the hepatocyte has been demonstrated by studies in primary rat hepatocyte cultures (Guzelian et al., 1988; Tollet et al., Liddle et al., 1992) and gene expression is controlled at the transcriptional level (Legraverend et al., 1992; Sundseth et al., 1992). Whether there is a GH-responsive element on the 11ß-HSD-1 gene is not known, but as there is sexually dimorphic expression of 11ß-HSD-1 mRNA in the mouse kidney (less than 50% in females compared with males) (Rajan et al., 1995a), it is unlikely the differences in the pattern of expression of rat and mouse 11ß-HSD-1 in liver is due to differences in gene structure, but rather due to tissue-specific differences in regulation of gene transcription. Possible explanations include specificity of response conferred by the existence of subpopulations of the GH receptor, as suggested by the existence of several mRNA species, epitope mapping studies and possible receptor-membrane proteins interactions (Smith et al., 1989; Barnard et al., 1985; Stred et al., 1990), or differences in the intracellular signalling pathways of GH action which involve a cascade of reactions and complex interactions (reviewed by Postel-Vinay & Kelly, 1996; Postel-Vinay & Finadori, 1996).

This species-specificity of sexually dimorphic hepatic 11ß-HSD-1 expression raises the question of whether it is sexually dimorphic in humans, where differences in steroid metabolism may have implications for disease as discussed in Chapter 4. Hepatic steroid and drug metabolism shows less pronounced sexual dimorphism in humans than in rodents (Pfaffenberg & Horning, 1977; MacLeod et al., 1979; Schmucker et al., 1991), probably due, at least in part, to there being only subtle sex differences in patterns of GH secretion (Ho et al., 1987). However GH administration to GH-deficient children does alter hepatic drug metabolism (Redmond et al., 1980). Therefore direct extrapolation of data obtained in animal studies to humans is not possible, and it is likely that sexual dimorphism of 11ß-HSD-1 will be of a lesser magnitude, if not absent, in humans. Until hepatic 11ß-HSD-1 expression has been measured in humans, the significance of sexually dimorphic expression in general mammalian biology must remain questionable.

The complete absence of T_3, achieved by the specific ablation of thyroid follicle cells in transgenic mice, had no effect on hepatic 11ß-HSD-1 mRNA expression and it can be concluded that T_3 does not regulate 11ß-HSD-1 in mouse liver. T_3 stimulates transcription of the GH gene (Evans et al., 1992; Spindler et al., 1992) and in hypothyroidism, GH secretion is almost totally abolished (Spindler et al., 1992; Hervas et al., 1975). As discussed in Chapter 4, the failure of T_3 to regulate 11ß-HSD-1 in primary rat hepatocyte cultures is likely to reflect an indirect nature of action, possibly via its effects on GH secretion, and this is supported by previous studies in rats reporting sexually dimorphic effects of T_3 on the liver, with thyroidectomy increasing 11ß-HSD activity in females, but decreasing activity in males (Lax et al., 1979; Whorwood et al., 1993a). In the light of the lack of GH-mediated sexual dimorphism of 11ß-HSD-1 in mouse liver, the absence of T_3 regulation in mice is consistent with the idea that it is GH-mediated. T_3 replacement in thyroid-ablated mice down-regulated 11ß-HSD-1 mRNA for reasons that are not clear, but the circulating T_4 levels achieved by replacement are approximately 50% higher than in control mice (although T_3 levels were not higher than controls) (Wallace et al., 1991). Excess T_3 may attenuate 11ß-HSD-1 expression via a "threshold" mechanism, whereby under normal physiological conditions T_3 levels do not affect 11ß-HSD-1 expression, but upon exogenous administration of T_3, an excess of hormone is repressive. This may be akin to the E_2 repression of hepatic 11ß-HSD-1 in the rat, where exogenous E_2 administration results in a dramatic decrease in enzyme levels far in excess of that seen in normal females (Low et al., 1993). It is interesting to note that there are sequences resembling a T_3 response element/E_2 response element half-site, which are found within the 11ß-HSD-1 gene

promoter (Moisan et al., 1992), and to speculate that T_3 and E_2 may perhaps exert their effects by repression through these sequences.

Previous studies have reported glucocorticoid induction of 11ß-HSD activity in the liver (Low et al., 1994c; Walker et al., 1994), hippocampus (Moisan et al., 1990c; Low et al., 1994c, Walker et al., 1994), testis (Gao et al., 1996) and in cell culture (Chapter 3; Takeda et al., 1995; Hammami & Siiteri), whilst ADX decreases enzyme activity and mRNA expression. In contrast, the data in Chapter 4, section 4.2.1.3 demonstrated a marked increase in hepatic 11ß-HSD-1 activity and mRNA expression following ADX with respect to sham ADX controls 21 days post-operatively, and this observation was reproduced on several occasions. Previous studies examined changes in hepatic 11ß-HSD activity and mRNA expression over 1-10 days (Low et al., 1994c; Walker et al., 1994). That the discrepancy might reflect the longer duration of ADX was considered a possible explanation, and prompted the closer examination of the time course of the control of 11ß-HSD-1 gene expression by glucocorticoids.

All of the treatments showed changes in hepatic 11ß-HSD-1 activity of a lesser magnitude than in the encoding mRNA, but following approximately the same pattern, presumably reflecting the slower turnover of the 11ß-HSD-1 protein, as previously reported (Low et al., 1994c). ADX resulted in an initial decrease in 11ß-HSD-1 activity, returning to control levels over approximately 10 days, which is in accordance with the findings in previous studies (Low et al., 1994c; Walker et al., 1994). What is interesting, is that the discrepancy between 11ß-HSD-1 activity and mRNA at 21 days observed in the experiments in Chapter 4, and confirmed in these experiments, is in fact due to the marked down-regulation of 11ß-HSD-1 in sham-operated animals. DEX administration to ADX animals resulted in a slow decline in 11ß-HSD-1 activity, such that at 21 days it is less than in ADX animals receiving vehicle. These effects are complicated, but may be partially explained by the proposal that the response of 11ß-HSD-1 gene expression to removal of glucocorticoids is an initial decrease of mRNA transcription followed by a compensatory up-regulation to restore levels towards normal, mediated by a distinct mechanism consequent upon glucocorticoid depletion. ADX-associated induction of hepatic 11ß-HSD-1 is perhaps more in accordance with its proposed role to regenerate active glucocorticoids. Thus in chronically-ADX animals, the increase in 11ß-reductase may be an attempt to compensate for the lack of intrahepatic glucocorticoid to sustain crucial glucocorticoid-regulated functions. Long-term DEX administration will obviate the need for 11ß-HSD-1 generation of glucocorticoid, which may explain the decline in activity. Alternatively, the opposing effects of

ADX and ADX with DEX could be mediated via their opposing effects on ACTH secretion. ADX increases ACTH levels, whilst DEX administration inhibits secretion. The decrease in 11ß-HSD-1 activity and mRNA (also observed in the kidney) resultant upon sham ADX remains to be explained, but does not appear to be directly mediated by glucocorticoid hormone action.

Neither ADX, nor glucocorticoid administration affect 11ß-HSD bioactivity in the rat kidney (Moisan et al., 1990c; Smith & Funder, 1991 and this data). This is due to the presence of the 11ß-HSD-2 isozyme whose glucocorticoid regulation is distinct from that of 11ß-HSD-1 in this tissue, since 11ß-HSD-1 mRNA levels are dramatically attenuated upon glucocorticoid depletion.

In contrast with previous short- to medium-term studies (Moisan et al., 1990c; Low et al., 1994c, Walker et al., 1994), chronic glucocorticoid excess did not increase 11ß-HSD-1 bioactivity in the hippocampus. Again, 11ß-HSD-1 activity fell following ADX and then levels returned towards normal, whilst with DEX administration to ADX animals, activity stayed repressed, indicating a similar pattern of glucocorticoid regulation to that in liver. The glucocorticoid regulation of 11ß-HSD-1 in the hippocampus is worthy of special consideration for two reasons. Firstly, the hippocampus has a crucial role in the HPA axis stress response feedback by inhibiting CRH neurosecretion and modulating the circadian rhythm of glucocorticoid secretion (reviewed by Jacobson & Sapolsky, 1991; Whitnall, 1993) and secondly, hippocampal neurones are extremely sensitive to changes in glucocorticoid concentrations. The hippocampus is rich in both MR and GR (McEwan et al., 1986; Reul & De Kloet, 1985) and so 11ß-HSD-1 has the potential to play a major role in modulating glucocorticoid action in this tissue (Moisan et al., 1990a; Lakshmi et al., 1991). Glucocorticoid excess compromises hippocampal cell function and survival and results in pyramidal neuronal loss (Landfield et al., 1978; Sapolsky et al., 1985, 1986; Sapolsky 1985, 1992; Landfield & Eldridge, 1991). Therefore reasons for regeneration of glucocorticoid by 11ß-HSD-1 reductase activity (Rajan et al., 1995b) are not clear. However, chronic glucocorticoid depletion following ADX also leads to neuronal dysfunction and possibly death in the dentate gyrus granule cells (Sloviter et al., 1989; Gould et al., 1991) and it may be that the function of hippocampal 11ß-HSD-1 is to ensure the provision of the optimal level of active glucocorticoid required for maintenance of essential neuronal functions from the pool of circulating A when glucocorticoid levels are low (e.g. during the diurnal nadir), whilst negative-feedback provides rapid responses to avoid over-exposure to glucocorticoids under normal circumstances. Glucocorticoid-mediated regulation of hippocampal 11ß-HSD-1 may help to ensure this protection

of neurones. This situation is analogous to that proposed in the liver and would explain the similarities in the patterns of glucocorticoid regulation observed in these tissues.

11ß-HSD-1 activity in the hippocampus of tree-shrews is down-regulated by psychosocial stress to approximately 50% of control levels. In contrast, excess cortisol administration has no effect on levels of activity. The increase in hippocampal 11ß-HSD-1 in rats by arthritic stress appears to depend in part on an adrenal product (Low et al., 1994c) and the induction of 11ß-HSD-1 in primary rat hippocampal cultures by dexamethasone (Rajan et al., 1995b) provides evidence for direct glucocorticoid induction. This is supported by the presence of sequences resembling the GRE which have been reported in both human (Tannin et al., 1991) and rat (Moisan et al., 1992b) 11ß-HSD-1 genes, and data from transfection experiments of HepG2 cells with plasmids in which 11ß-HSD-1 promoter DNA is fused to a reporter gene suggest a GRE lies within 1800 base pairs of the transcription start of rat 11ß-HSD-1 (Voice et al., 1996). However, differences in the effects of stress and of exogenous glucocorticoid administration on hippocampal neuronal morphology have been previously observed in rats (Stein-Behrens et al., 1994), and prolonged (28 days) psychosocial stress in tree-shrews results in an increase in the optical density of the nucleoplasm in pyramidal neurons (indicating a change in the chromatin structure), and a reduction in the number of branch points and dendritic length of neurones, but no reduction in pyramidal neuron density or evidence of histopathological changes (Fuchs et al., 1995). This is in contrast with previous studies in rats and suggests that stress per se may exert effects on the hippocampus via mechanisms distinct from glucocorticoids which may explain why hippocampal damage similar to that observed in rats is not observed in humans on long-term glucocorticoid therapy (Hall, 1990). These differential effects may include the regulation of 11ß-HSD-1, such that the effects of glucocorticoids may depend on other neuroendocrine factors (such as sympathoadrenal activity or the expression of CRH-binding sites) which are wide open to speculation at present.

In the liver, both stress and glucocorticoid administration attenuate 11ß-HSD-1 activity, implying there is a direct negative regulation by glucocorticoids in this tissue. The regulation in both liver and hippocampus are in agreement with the proposed role of 11ß-HSD-1 to potentiate glucocorticoid action within the tissues where it is expressed. The lack of effect of stress or glucocorticoid regulation on renal 11ß-HSD activity again reflects the expression of 11ß-HSD-2 in this organ.

Therefore the glucocorticoid regulation of 11ß-HSD-1 is complicated and tissue-specific. Presumably the pattern of regulation within a tissue reflects the

function of the enzyme within that tissue. Further information regarding the regulation of 11ß-HSD-1 from molecular studies on the mechanisms governing gene transcription, coupled with studies on enzyme function, will help to aid the understanding of these regulatory patterns.

Hippocampal 11ß-HSD-1 is not sexually dimorphic in rats, nor can it be altered with sex steroid or GH manipulations (Low et al., 1993; Low et al., 1994b), and it was proposed that this was due to the failure of GH to cross the blood-brain-barrier, which is impervious to most small peptides. Therefore it was a surprise to find that 11ß-HSD-1 mRNA expression was not regulated by GH in the brain. That the GH used in this experiment was biologically active was confirmed by the striking down-regulation of 11ß-HSD-1 mRNA in the periphery (liver and kidney), typical of the effects of continuous female-pattern GH secretion on 11ß-HSD-1 expression mimicked here by the continuous ICV infusion of GH. This also suggests that GH has crossed the blood brain barrier. This may be due to the fact that the levels of GH achieved within the brain by this experiment are likely to be supraphysiological, and may overcome the capacity of the blood brain barrier to prevent passage of GH across it. Why the regulation of 11ß-HSD-1 in brain should be different from the periphery is not clear. It could be analogous to the situation in the liver, where GH-mediated sexual differentiation is species-specific as discussed above, or it may be due to the presence of subpopulations of the GH receptor (Smith et al., 1989; Barnard et al., 1985; Stred et al., 1990). It could be due to differences in the intracellular signalling pathways of GH action in different cells, or mediated via the interaction of the GH signalling pathway with a variety of transcription factors which may regulate 11ß-HSD-1 and may be differentially expressed between tissues. It could also be due to lower expression levels of the GH receptor in neurons in comparison to hepatocytes, although some specificity of response would still be expected.

The studies in this chapter have further characterised the hormonal regulation of 11ß-HSD-1. Some of this data is preliminary. They are suggestive rather than conclusive of the patterns of 11ß-HSD-1 regulation by these hormones, and have emphasised the complexity of this regulation. The widely varying effects of hormones on 11ß-HSD-1 between tissues and between species make interpretation of the data available on hormonal regulation difficult in some cases. However as the function of the enzyme becomes better understood, the interpretation of these data and the significance of 11ß-HSD-1 regulatory patterns will become clearer.

CHAPTER 7

DISCUSSION

The experiments presented in this thesis were carried out in order to gain an insight into the biological role of the enzyme, 11ß-HSD-1. This was prompted by the existence of the large body of knowledge concerning the distribution and modulation of expression of the enzyme, but a lack of understanding regarding its biological importance. The co-localisation of 11ß-HSD-1 expression with GR in many tissues throughout the body has led to suggestions that 11ß-HSD-1 has a central role in the regulation of corticosteroid access to GR (Moisan et al., 1990b; Teelucksingh et al., 1990; Whorwood et al., 1991) and possibly to MR in the brain (where glucocorticoid is the physiological ligand (McEwan et al., 1986; de Kloet, 1991; Seckl & Olsson, 1995)). Thus 11ß-HSD-1 would mediate the expression of glucocorticoid functions in tissues where it is co-localised with GR. As the highest expression of 11ß-HSD-1 is in the liver (Monder & Shackleton, 1984), which is also a major target for glucocorticoid action, the studies in this thesis concentrated predominantly on this organ.

The experiments described in Chapter 3 were designed to examine 11ß-HSD-1 activity and expression in cell culture and addressed some fundamental aspects of 11ß-HSD-1 function. The enzyme functions predominantly as a 11ß-reductase in intact hepatocytes. That this was relevant to intact liver was confirmed by the examination of 11ß-HSD-1 activity in the perfused liver as described in Chapter 5. Previously, interpretation of studies on 11ß-HSD-1 were based on the premise that the enzyme activity is reversible (Agarwal et al., 1989; Monder & White, 1993), and that different tissues may exhibit either predominantly 11ß-reductase activity or 11ß-dehydrogenase activity, depending on their specific requirements for low or for high glucocorticoid levels, or, alternatively that 11ß-HSD-1 was capable of rapid shifts in reaction direction as required, in order to maintain a constant intracellular glucocorticoid concentration in the face of fluctuations in the circulating levels of hormone. In fact, enzyme direction in intact hepatocytes proved refractory to a wide range of physiological and physiochemical manipulations designed to favour 11ß-dehydrogenation, and taken together with other recent studies on enzyme function in intact cells and organs (Stewart et al., 1990; Leckie & Seckl, 1996; Hammami & Siiteri, 1991; Brem et al., 1995; Rajan et al., 1996), it is looking increasingly likely that 11ß-HSD-1 functions largely as an 11ß-reductase *in vivo*, and so we must review our understanding of the proposed physiological actions of 11ß-HSD-1.

On the basis of the evidence that hepatic 11ß-HSD-1 is an 11ß-reductase (thus generating active glucocorticoids within the liver), I examined the possibility that 11ß-HSD-1 modulates glucocorticoid actions within the liver in the experiments described in Chapter 4. The results point strongly towards 11ß-HSD-1 activity

playing a fundamental role in maintaining hepatic glucocorticoid-regulated functions, in particular glucose homeostasis, as evidenced by the effects on hepatic gene expression of metabolic enzymes whose expression is known to be glucocorticoid-modulated. The data are also suggestive of an ability of enzyme activity to modulate hepatic insulin sensitivity. The study by Walker et al., (1995) demonstrating an increase in insulin sensitivity upon CBX inhibition of 11ß-HSD in humans, reinforces the hypothesis that reduced intrahepatic glucocorticoid regeneration by inhibition of 11ß-HSD-1 activity may increase insulin sensitivity *in vivo*.

Obviously this is a target for further studies. The primary hepatocyte culture system for study of 11ß-HSD-1 described in Chapter 3 will be a useful model for exploring these ideas. It provides a system for easy manipulation of 11ß-HSD-1 and for examination of parameters of hepatic insulin sensitivity including glucose uptake and net glycogen synthesis, as well as measurements of glucocorticoid-sensitive gene transcription. Likewise, the liver perfusion system (Chapter 5) provides a model in which to examine these indicators of insulin sensitivity and will permit manipulation of physiological conditions *in vivo,* whilst allowing examination of the effects of manipulations in the isolated organ of interest.

Clearly a method of down-regulation of hepatic 11ß-HSD-1 or inhibition of enzyme activity is crucial in furthering these investigations. The oestradiol attenuation of 11ß-HSD-1 expression employed in the experiments in Chapter 4 is extremely effective, but subject to criticism as E_2, in common with most hormones, has many effects on a wide range of physiological functions including regulation of other enzymes involved in glucocorticoid metabolism (Leblanc & Waxman, 1988). Therefore a more specific manipulation is required. At the moment, the most effective pharmacological inhibitor of 11ß-HSD-1 available is CBX. However, as demonstrated by its poor efficacy of inhibition in intact hepatocytes and in perfused liver, it is far from adequate in this respect, and also lacks specificity of action as it inhibits other enzyme activities, most notably 11ß-HSD-2 and 5α-reductase (Walker & Edwards, 1994; Latif et al., 1990). Hopefully a more specific and more effective inhibitor will be found. Other compounds are known to inhibit 11ß-HSD activity, including a number of endogenous compounds (Hierholzer et al., 1991; Bühler et al., 1994) which may direct us towards suitable candidates.

A specific 11ß-HSD-1 inhibitor would be a invaluable tool in investigations of the enzyme's physiological role not only in liver, but in all the organs where it is expressed. However the creation of an 11ß-HSD-1 "knockout" mouse (Kotelevtsev et al., 1996) has provided us with a model of complete enzyme inhibition. Studies

regarding the status of these animals with respect to glucose tolerance, insulin sensitivity and the effects of dietary and glucocorticoid manipulations upon these functions are already in progress.

The physicochemical and hormonal manipulations of 11ß-HSD-1 in hepatocyte cultures and hormonal manipulations *in vivo* (Chapter 6) indicated that alterations in the biological activity of the enzyme are probably mediated primarily via down-regulation at the transcriptional level and not through alterations in protein activity or degradation. This results in a slow decline in 11ß-HSD-1 activity when it is down-regulated as evidenced by these experiments and those of Low et al. (1993, 1994b, 1994c). Presumably this is due to the long half-life of the protein which appears to be in the order of days. There is however, the potential for a rapid increase in activity when transcription and hence protein synthesis is stimulated. This coupled with the function of 11ß-HSD-1 to generate active glucocorticoids by its 11ß-reductase activity, points towards a crucial role for the enzyme in the maintenance of glucocorticoid levels within a given tissue to above a minimum level required for optimal tissue function. It is unlikely to be concerned with acute adaptations to glucocorticoid excess. This is in accordance with the role, proposed here, for hepatic 11ß-HSD-1 to maintain glucocorticoid-regulated functions in the liver during times of low glucocorticoid availability. The precise role of 11ß-HSD-1 in the brain (and indeed in the wide variety of other tissues in which it is expressed but which have not been considered in this thesis), is yet to be determined, but a similar function to protect neurons from the adverse effects of relative glucocorticoid deficiency is conceivable. The long-term stress and/or glucocorticoid-mediated regulation of 11ß-HSD-1 in liver and in hippocampus are in accordance with this hypothesis.

The hormonal regulation of 11ß-HSD-1 expression is obviously extremely complex. It is species- and tissue-specific, and, at least in the case of hippocampal 11ß-HSD-1, modulated by factors that are as yet unknown. Further understanding of the mechanisms governing regulation of 11ß-HSD-1 expression is most likely to come from molecular studies on the cloned 11ß-HSD-1 gene (Agarwal et al., 1989). Studies on the 11ß-HSD-1 promoter to identify DNA sequences required for responses to regulatory hormones and proteins and perhaps sequences associated with tissue-specific factors, will yield information on the regulation of the gene. Tissue-specific intracellular messenger signalling systems could provide an additional level of control and studies concentrating on these aspects of 11ß-HSD-1 biology, alongside physiological studies both *in vivo* and in cell culture, will help to further understanding of the biological functions of this enzyme.

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11β-Hydroxysteroid Dehydrogenase Is an Exclusive 11β-Reductase in Primary Cultures of Rat Hepatocytes: Effect of Physicochemical and Hormonal Manipulations*

PAULINE M. JAMIESON, KAREN E. CHAPMAN†, CHRISTOPHER R. W. EDWARDS, AND JONATHAN R. SECKL

University of Edinburgh, Department of Medicine, Western General Hospital, Edinburgh, Scotland EH4 2XU

ABSTRACT

11β-Hydroxysteroid dehydrogenase (11βHSD) catalyzes the conversion of corticosterone to inert 11-dehydrocorticosterone, thus regulating glucocorticoid access to intracellular receptors. The type 1 isoform (11βHSD-1) is a bidirectional NADP(H)-dependent enzyme *in vitro* and is highly expressed in liver, where it is regulated by glucocorticoids, thyroid hormones, estrogen, and GH *in vivo*. In humans *in vivo*, enzyme inhibition alters glucose homeostasis, an effect thought to be mediated in the liver. However, detailed investigation of the biology of 11βHSD-1 in liver, its function, regulation, and indeed even reaction direction, has been hampered by the lack of clonal hepatic cell lines that express 11βHSD-1. Studies of nonhepatic cell lines have suggested that 11βHSD-1 is directly regulated by hormones, and transfection of nonhepatic cell lines has shown that reaction direction varies between cell types, possibly reflecting intracellular cosubstrate ($NADP^+$/NADPH) ratios or pH.

To investigate reaction direction and gene regulation of 11βHSD-1 in hepatocytes, we defined conditions for primary culture of adult rat hepatocytes that maintain high 11βHSD-1 messenger RNA expression. In intact primary hepatocytes over a wide range of steroid concentrations (2.5–250 nM), 11β-reduction was the predominant reaction direction [33.5 ± 0.5% conversion of 11-dehydrocorticosterone (25 nM) to corticosterone after 30 min], with undetectable 11β-dehydrogenation. However, homogenates of hepatocyte cultures showed plentiful 11β-dehydrogenase activity. Treatment of hepatocyte cultures with the metabolic inhibitors sodium azide (5 mM) and KCN (1 mM) altered cellular $NADP^+$/NADPH ratios from 0.244 ± 0.042 in controls to 0.020 ± 0.001 and 0.152 ± 0.009, respectively, but had no effect on 11β-reductase or 11β-dehydrogenase activity. High concentrations of KCN (10 mM) modestly increased 11β-reductase activity (32.4 ± 1.7% to 48.8 ± 0.5%), whereas 11β-dehydrogenation remained at the limit of detection. Manipulation of culture medium pH (6.2–8.0) had no effect on enzyme activity. Dexamethasone (10^{-7} M) induced hepatocyte 11β-reductase activity from 23.4 ± 0.7% to 35.5 ± 1.5% and 11βHSD-1 messenger RNA expression (207% rise), an action inhibited by insulin (10^{-7} M). GH, estradiol, and T_3 had no effect.

These results demonstrate that 11βHSD-1 functions as an 11β-reductase in intact rat hepatocytes in culture. The cosubstrate ratio only weakly affects reaction direction. Glucocorticoid and insulin regulation of hepatic 11βHSD-1 is directly mediated, but other hormonal controls are either lost in culture or mediated indirectly. This primary hepatocyte culture system will allow investigation of the control of 11β-reductase activity and its implications for glucocorticoid-regulated hepatic functions. (*Endocrinology* **136:** 4754–4761, 1995)

The 11β-HYDROXYSTEROID dehydrogenase (11βHSD) catalyzes the reversible conversion of physiologically active corticosteroids [cortisol in humans and corticosterone (B) in rats] to inactive 11-dehydro forms [cortisone and 11-dehydrocorticosterone (A)], thus acting as a tissue-specific regulator of glucocorticoid access to intracellular corticosteroid receptors (1, 2). At least two distinct isoforms exist, 11βHSD-1, originally purified from liver (3), and 11βHSD-2, recently characterized in kidney and placenta (4, 5). The best-documented physiological role for 11βHSD is found in the distal nephron, where the enzyme ensures aldosterone-selective access *in vivo* to otherwise nonspecific mineralocorticoid receptors by rapidly inactivating glucocorticoids (6, 7). The isoform responsible, 11βHSD-2, is a high affinity, NAD-dependent, exclusive 11β-dehydrogenase (5, 8) whose expression is restricted to mineralocorticoid target tissues, placenta, and a limited number of other tissues. Congenital deficiency of this enzyme or inhibition by licorice or its derivatives allows illicit occupation of mineralocorticoid receptors in the distal nephron by glucocorticoids, producing sodium retention and hypertension (9, 10).

By contrast, 11βHSD-1 is widely distributed, with the highest levels in liver and proximal tubules of kidney [although it is absent from the distal nephron (6)], tissues that also express high levels of glucocorticoid receptors. It has been proposed that the role of 11βHSD-1 is to modulate glucocorticoid access to these sites (11–13). 11βHSD-1 has been purified from rat liver, antisera raised, and an encoding complementary DNA (cDNA) isolated (3, 14, 15). The human, monkey, sheep, and mouse homologs have also been cloned (16–19). 11βHSD-1 has a lower affinity for steroid substrates (K_m, 2 μM for corticosterone) than the type 2 isoform, is NADP(H) dependent, and is bidirectional in tissue homogenates and purified microsomal fractions (3). However, transfection studies have indicated that the reaction direction of 11βHSD-1 varies between clonal cell types, with

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Address all correspondence and requests for reprints to: Miss P. M. Jamieson, University of Edinburgh, Department of Medicine, Western General Hospital, Edinburgh, Scotland EH4 2XU.

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bidirectional activity in some cells [*e.g.* Chinese hamster ovary cells (15)], but predominant 11β-reduction (reactivation of inert 11-keto metabolites) in others [*e.g.* TBM and COS-7 (20, 21)]. Reaction direction has been proposed to reflect cosubstrate ($NADP^+$/NADPH) ratios, with $NADP^+$ favoring 11β-dehydrogenase activity, and NADPH favoring 11β-reduction (22). Glycosylation status and pH have also been implicated in the control of direction (22, 23). However, changes in glycosylation cannot explain exclusive 11β-reduction in intact COS-7 cells transfected with 11βHSD-1 cDNA, which nonetheless shows 11β-dehydrogenation when the enzyme is assayed in homogenates of the same transfected cells (21). Clearly, cell context is crucial to the direction of 11βHSD-1 enzyme activity.

The reaction direction of 11βHSD-1 in liver [which highly expresses this isoform without detectable 11βHSD-2 (4)] is unknown, although indirect evidence suggests that in liver, 11β-reductase predominates. Thus, the effluent of perfused cat liver has a high cortisol to cortisone ratio (24). Moreover, after oral administration of cortisone in humans, peripheral plasma levels of cortisol, but not cortisone, rise (25), suggesting that cortisone is activated by 11β-reductase on its first pass through the liver. However, the reaction direction of 11βHSD-1 in hepatocytes has not been directly addressed.

11βHSD-1 is regulated *in vivo* by various hormones, including glucocorticoids, insulin, thyroid hormone (T_3), GH, and sex steroids, in a tissue- and developmentally specific manner. Hepatic 11βHSD-1 is sexually dimorphic in rats, with females showing lower activity than males (26, 27). This is due to suppression of activity in the female by estradiol (E_2), an effect largely mediated indirectly via the effects of sex steroids on the pattern of pituitary GH secretion (26–28). In rat liver and cultured human skin fibroblasts, glucocorticoids up-regulate enzyme activity, an effect that may be antagonized by insulin, at least in cultured fibroblasts (29, 30). Thyroid hormone has been reported to decrease enzyme activity in rat liver, whereas thyroidectomy increases activity in female rat liver, but decreases it in that of the male rat (31, 32). Whether any of these hormonal effects are mediated directly upon hepatocytes remains unknown, and such studies have been hampered by the absence of liver-derived clonal cell systems that express 11βHSD-1.

The aims of this study were, therefore, 1) to establish primary hepatocyte cell cultures optimal for the maintenance of 11βHSD-1 activity and messenger RNA (mRNA) expression, 2) to determine whether the direction of 11βHSD-1 enzyme activity in hepatocytes can be manipulated by alterations in NADP/NADPH ratios or pH, and 3) to examine the hormonal regulation of 11βHSD-1 in cell culture.

Materials and Methods

Hepatocyte isolation

Male Han Wistar rats were anesthetized with pentabarbitone (60 mg/kg), and their livers were perfused *in situ* with collagenase, essentially as described by Seglen (33). The liver was perfused *in situ* with a calcium-free buffer, followed by a solution containing 0.0275% collagenase type IV (Sigma Chemical Co., Poole, UK) in 5 mM $CaCl_2$, 30 mM HEPES, 10 mM glucose, 0.05% KCl, 0.001% phenol red, and 0.9% NaCl. The dissociated liver was removed, and hepatocytes were isolated by filtration through a 60-μm filter mesh. Parenchymal cells were separated from nonparenchymal and dead hepatocytes by repeated low speed centrifugation. Yields of 5–10 × 10^8 cells/liver with 70–75% viability, as determined by trypan blue exclusion, were routinely obtained.

Cell culture conditions and hormonal manipulations

Hepatocytes were plated on dishes coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) in Dulbecco's Modified Eagle's Medium-Ham's F-12 medium (GIBCO, Paisley, UK) containing 5% Nu-serum (Collaborative Biomedical Products), 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM L-glutamine, 25 μg/ml gentamycin sulfate, 0.7 μg/ml amphotericin B (all from GIBCO), 5 μg/ml insulin (Sigma), and 0.1 μM dexamethasone (Sigma). Cells were plated at a density of 1 × 10^6/35-mm dish in 2 ml medium (for 11βHSD assays) or 3 × 10^6 cells/60-mm dish in 4 ml medium (for $NADP^+$/NADPH quantitation). The medium was replaced every 24 h after cells were placed in culture. Cultures were maintained at 37 C in a humidified atmosphere containing 5% CO_2 and 95% air. Test hormones or antagonists were added to hepatocytes in fresh medium 48 h after plating at the following concentrations; E_2, 10^{-8} M (Sigma); T_3, 10^{-8} M (Sigma); GH, 0.05 IU/ml (Eli Lilly Co., Basingstoke, UK); RU38486, 10^{-6} M (gift from Roussel-UCLAF, Romaineville, France); and tamoxifen, 10^{-6} M (Sigma). In some experiments, 48 h after being placed into culture hepatocytes were transferred to medium containing charcoal-stripped Nu-serum, prepared as previously described (34), and analyzed for steroid hormones to check the efficiency of the procedure. Stripping reduced Nu-serum levels of cortisol, E_2, progesterone, T_3, and T_4 to below detection limits. Cells were cultured in medium containing stripped Nu-serum supplemented with dexamethasone (10^{-7} M) reflecting the level of glucocorticoid in control medium, insulin (1.3 × 10^{-7} M), and hormones, as described above, where stated. The medium was changed daily, and fresh hormones were added. All hormones were added to the medium at a concentration chosen to be close to physiological levels, and antagonists were employed at concentrations sufficient to block the effects of the hormone present. Twelve days after the addition of hormones, the cells were harvested for mRNA extraction.

Quantitation of 11βHSD activity

In intact cells. Cells were first assayed for enzyme activity 48 h after being placed into culture and then every 3–4 days thereafter. Medium was aspirated and replaced by 2 ml fresh medium containing 2 nM [^{3}H]B (SA, 84 Ci/mmol; Amersham International, Aylesbury, UK) and a quantity of unlabeled B appropriate for the concentration of B required or 2 nM [^{3}H]A made as previously described (4), and unlabeled A as required. In most experiments, 23 nM unlabeled A or B was added to the medium, giving a final concentration of 25 nM A or B. This concentration was chosen as to reflect moderate to high physiological free steroid levels. [^{3}H]A was at least 97% pure, as estimated by HPLC, and was resuspended to give the same specific activity as [^{3}H]B. In most experiments, 1-ml aliquots of medium were removed from the culture medium after 30 and 60 min of incubation. In the experiment shown in Fig. 3b, the determination of product formation with time, 100-μl aliquots of medium containing 25 nM [^{3}H]B or 25 nM [^{3}H]A were removed after 15, 30, 60, 90, and 120 min incubation. Steroids were extracted with ethyl acetate, dried, resuspended in ethanol, separated by TLC, and estimated by scintillation counting. Enzyme activity in each direction was expressed as the percent conversion to product. Cells were washed twice in fresh medium after each assay. Assays were repeated at intervals from 4 h to 28 days after plating.

In cell homogenates. Seven days after being placed in culture, cells were harvested from culture dishes by dissolution of Matrigel basement membrane. One milliliter of CR-Dispase (Collaborative Biomedical Products) was added to each 35-mm culture dish after aspiration of the medium and incubated for 2 h at 37 C to yield a cell suspension. Cells were sedimented by centrifugation at 50 × *g* for 2 min, and the supernatant was discarded. The pellet was resuspended in 100 μl TM Triton [0.1% Triton X-100 in 20 mM Tris-HCl (pH 7.5), and 2 mM $MgCl_2$], and the total protein concentration of the suspension was estimated colorimetrically (Bio-Rad protein assay kit, Hemel Hampstead, UK). Twenty-five micrograms of cell homogenate were incubated with 200 μM NADP and

12 nM [^{3}H]B or with 200 μM NADPH and 12 nM [^{3}H]A in a total volume of 250 μl with Krebs-Ringer buffer supplemented with 0.2% glucose and 0.2% BSA for 10 or 60 min at 37 C. Steroids were extracted with ethyl acetate and separated by HPLC. Enzyme activity in each direction was expressed as the percent conversion to product.

Metabolic inhibition and pH manipulation

Medium was aspirated and replaced with fresh medium containing 5 mM sodium azide (Sigma) or KCN (Aldrich, Gillingham, UK) at either 1 or 10 mM or with medium of the appropriate pH. The concentrations of metabolic poisons employed are well recognized to cause inhibition of oxidative phosphorylation in cell culture systems. For pH manipulations, medium with the appropriate pH was prepared by adjustment of the quantity of sodium bicarbonate (GIBCO) added to the medium upon preparation or by addition of HCl to achieve a medium of pH 6.2. Cells were preincubated for 4 h before assaying 11βHSD activity, as described above, or quantitation of $NADP^+$/NADPH.

$NADP^+$ and NADPH extraction and quantitation

Quantitation of $NADP^+$ and NADPH was carried out according to the enzymatic cycling method, as described by Blomquist and Hakanson (35). For $NADP^+$, cells were harvested in 1.5 ml ice-cold 500 mM perchloric acid, sonicated for 4 min on ice, left on ice for 15 min, and pelleted (5000 rpm; 10 min). For NADPH, cells were harvested in 1.5 ml 250 mM sodium hydroxide, heated to 60 C for 5 min, and centrifuged. Bicine (0.3 ml 1 M; Sigma), pH 8, was added to the supernatant of each sample and titrated to pH 8. Reagent blank (100 mM bicine, pH 8), standard, or sample was added to 1.0 ml cycling reagent (10 mM isocitric acid, 10 mM magnesium chloride, 500 μM dithiothreitol, 2 mM phenazine ethosulfate, and 1.0 g/liter BSA in 100 mM bicine, pH 8). Cycling was started by the addition of 0.2 ml isocitrate dehydrogenase (1.25 mg protein/ml; Sigma). Samples were incubated at room temperature in the dark for 60 min, absorbance at 570 nm was measured, and $NADP^+$ and NADPH were estimated from standard curves. Standards were $0.5–2.5 \times 10^{-7}$ M $NADP^+$ or NADPH in 100 mM bicine, pH 8. The detection limit was 1×10^{-8} M for both NADP and NADPH.

Extraction and analysis of mRNA

mRNA was extracted from freshly isolated (uncultured) hepatocytes and from hepatocytes at varying time points after being placed into culture. Cells were harvested from culture dishes as described above, and total RNA was extracted from cells by the guanidinium thiocyanate method, as described by Chomczynski and Sacchi (36), and separated on a 1.2% agarose gel containing 2% formaldehyde. RNA was blotted onto nitrocellulose membranes (Hybond-N, Amersham International); prehybridized in 6 ml phosphate buffer (0.2 M NaH_2PO_4, 0.6 M Na_2HPO_4, and 5 mM EDTA), 3 ml 20% sodium dodecyl sulfate (SDS), and 100 μg denatured herring testicular DNA (Sigma) for 2 h at 55 C; and hybridized at 55 C overnight in the same solution containing rat 11βHSD-1 cDNA labeled with [^{32}P]deoxy-CTP using a random primed DNA labeling kit (Boehringer Mannheim UK, Lewes, UK). Three 20-min washes were carried out at room temperature in 1 × SSC (0.3 M NaCl and 0.03 M sodium citrate)-0.1% SDS followed by a stringent wash at 55 C for 30 min in 0.3 × SSC-0.1% SDS. Filters were exposed to autoradiographic film for 1–4 days (adjusted to ensure that the signal density was within the linear range). Filters were rehybridized with similarly labeled 7S RNA cDNA probes to control for loading, as previously described (27, 28). Optical density was determined using a computer-driven image analysis system (Seescan, Cambs, UK).

Statistics

Experimental points are the mean ± SEM of three to seven replicates from the same culture preparation or different cultures, as indicated in the figure legends. Data were compared by analysis of variance and Newman-Keuls *post-hoc* tests, as appropriate. Significance was set at $P < 0.05$. Data are the mean ± SEM.

Results

Measurement of enzyme direction and maintenance of 11βHSD activity

In preliminary experiments, most cell culture conditions, using several different media and either plastic culture dishes or collagen-coated dishes, were associated with either variable cell viability or the loss of 11βHSD-1 activity and mRNA expression from otherwise viable primary hepatocyte cultures (data not shown). However, maintenance of 11βHSD-1 mRNA expression over an extended period of time up to 35 days was achieved by culturing hepatocytes on Matrigel (a gel matrix extracted from the Englebreth-Holm-Swarm mouse tumor; Fig. 1 and data not shown). Toward the end of this time, some cell loss became apparent. Measurement of enzyme activity in intact cells over this time demonstrated that the predominant reaction direction was 11β-reduction (Fig. 2). This held over a wide range of physiologically relevant substrate concentrations (Fig. 3a). Product formation was linear with incubation time over at least 1 h (Fig. 3b). 11β-Dehydrogenase activity was below the limit of detection, except for low levels over the first 2–3 days after plating and after cells had been cultured for more than 28 days (Fig. 2). At both of these time points, but not during the intervening period, dead and dying cells were present. Measurement of 11βHSD activity in cell homogenates demonstrated that 11β-dehydrogenase activity (conversion of B to A) is readily detectable under these conditions, with 53.9 ± 0.1% and 93.2 ± 2.4% conversion after 10 and 60 min of incubation, respectively. In contrast, 11β-reductase activity (conversion of B to A) appeared to be unstable in homogenates, as previously documented (27), and was undetectable after 60 min of incubation.

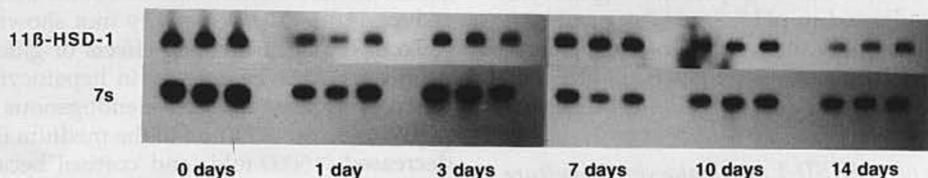

FIG. 1. 11βHSD-1 mRNA expression in primary hepatocyte cultures. Autoradiograph of a representative Northern blot of RNA from freshly isolated hepatocytes (0 days) and from hepatocytes maintained for up to 14 days in culture, probed with 11βHSD-1 and 7S RNA cDNAs. Replicates represent RNA extracted from hepatocytes from separate culture dishes from the same culture preparation. All samples were electrophoresed on a single Northern gel and treated identically thereafter, and are typical of results obtained from several different culture preparations.

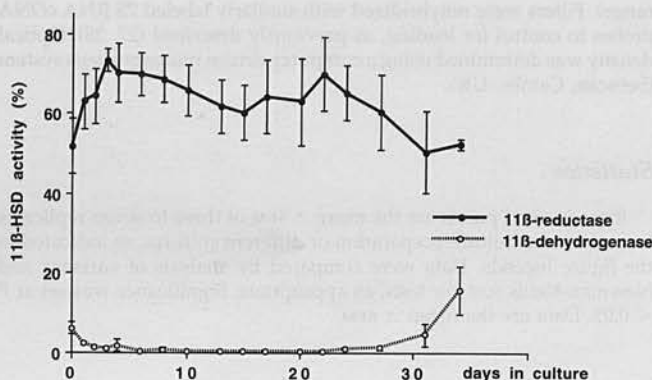

FIG. 2. 11βHSD-1 activity in primary hepatocyte cultures. Hepatocytes were plated on Matrigel basement membrane matrix and maintained in Dulbecco's Modified Eagle's Medium-Ham's F-12 medium with 5% Nu-serum for up to 35 days. 11βHSD-1 activity was measured at intervals. Activity is expressed as the percent conversion (±SEM) of A to B (11β-reductase) or B to A (11β-dehydrogenase) in the medium overlying the cells after 60 min (n = 2–6). Results are from three separate culture preparations.

Effect of alteration of intracellular cosubstrate ratio on 11βHSD-1 reaction direction in primary hepatocytes in culture

Treatment of 3-day-old hepatocyte cultures with sodium azide (5 mM) or KCN (1 mM) did not alter 11βHSD activity or direction. Indeed, 11β-dehydrogenase activity had a tendency to fall below detectable limits with both azide and cyanide treatment. Increasing the KCN to 10 mM increased 11β-reductase activity, whereas 11β-dehydrogenation was undetectable (Fig. 4).

To ascertain that azide and cyanide had altered intracellular cosubstrate ratios in hepatocytes, $NADP^+$ and NADPH concentrations were measured after 4-h incubation with either KCN or sodium azide. KCN (1 mM) decreased $NADP^+$ levels compared with those in extracts from untreated cells, and 5 mM azide decreased $NADP^+$ to levels below the detection limit ($<1 \times 10^{-8}$ M), demonstrating that the metabolic inhibitors significantly depleted $NADP^+$ availability, but had no effect on NADPH levels within the hepatocytes at the concentrations used (Fig. 5a). This represents a large alteration in the intracellular cosubstrate ratio (Fig. 5b).

Effect of pH on reaction direction of 11βHSD-1 in hepatocytes in culture

11βHSD activity was measured in primary hepatocytes cultured in medium adjusted to pH 6.2, pH 7.4, or pH 8.0. Neither 11β-reductase activity (conversion of A to B in 30 min) nor 11β-dehydrogenase activity (conversion of B to A in 30 min) was altered (Fig. 6).

Hormonal regulation of 11βHSD-1 in hepatocytes in culture

11β-Reductase activity of 11βHSD-1 was measured in primary hepatocytes cultured in the presence of E_2, GH, T_3, or a combination of hormones; activity was measured every 3 days over a 12-day period. No change in 11βHSD activity

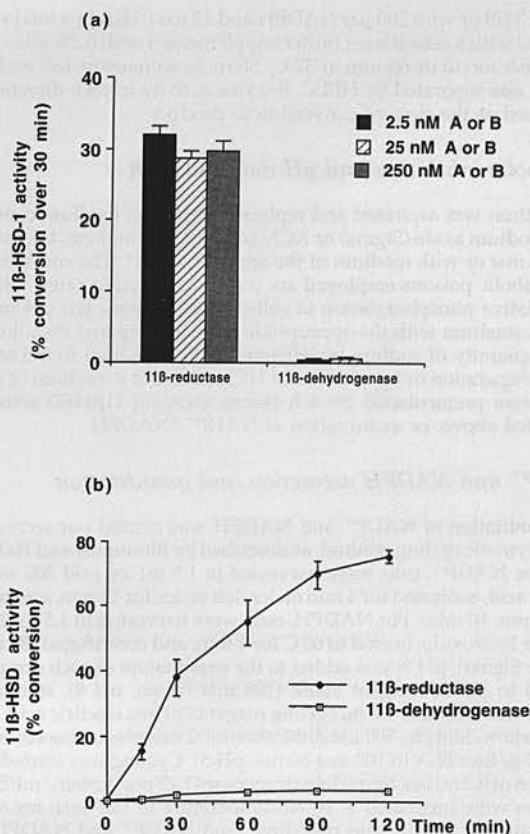

FIG. 3. 11βHSD-1 is a predominant reductase in intact hepatocytes. a, 11βHSD-1 activity was measured in hepatocytes over a range of substrate concentrations. Activity is expressed as the percent conversion of A to B (11β-reductase) or B to A (11β-dehydrogenase) in the medium overlying the cells after 30 min (n = 3). b, Conversion of substrate to product by 11βHSD-1 activity was measured over a time course of 120 min. Activity is expressed as the percent conversion of A to B (11β-reductase) or B to A (11β-dehydrogenase) in the medium overlying the cells.

was observed at any time in cells exposed to 10^{-8} M E_2, 0.05 IU/ml GH, 10^{-8} M T_3, or a combination of E_2 and GH (Fig. 7). T_3 also did not alter 11βHSD-1 mRNA expression after 12 days (data not shown). In case the effects of hormones were obscured by high endogenous levels in the culture medium, the effects of the estrogen receptor antagonist tamoxifen and the glucocorticoid receptor antagonist RU38486 (mifepristone) were examined. Neither antagonist had a consistent effect on 11βHSD activity in cultured hepatocytes (either 11β-reductase or dehydrogenase activities) over 12 days (data not shown), although there was a trend for RU38486 to reduce 11β-reductase activity (not shown).

To examine further the effects of glucocorticoid and insulin on 11βHSD-1 activity in hepatocytes, Nu-serum was charcoal stripped to remove endogenous steroids and small peptides before addition to the medium (insulin levels were decreased >5000-fold, and cortisol became undetectable). 11βHSD activity was decreased by charcoal stripping of the Nu-serum from 31.2 ± 3.0% to 23.4 ± 0.7% conversion of A to B. Dexamethasone (10^{-7} M) and insulin (1.3×10^{-7} M) were added to medium containing the stripped serum, singly and in combination, to concentrations reflecting those in the stan-

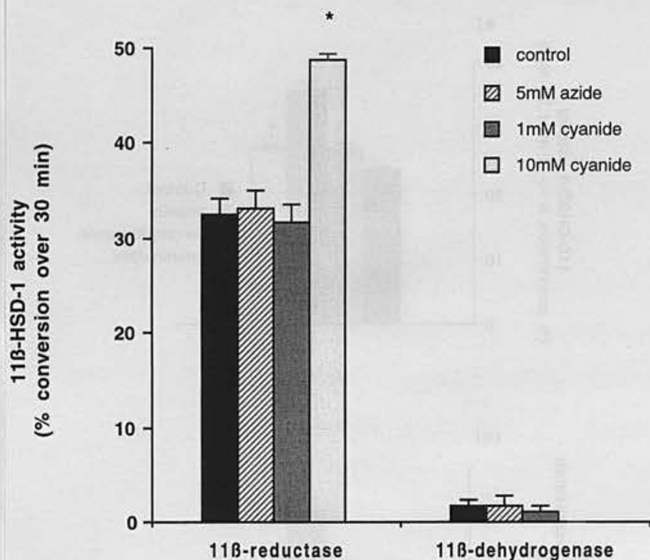

FIG. 4. Effects of metabolic inhibitors on 11βHSD-1 activity in hepatocyte cultures. Hepatocytes were incubated for 4 h with 5 mM sodium azide, 1 mM KCN, or 10 mM KCN before 11βHSD-1 activity was measured. Values are expressed as the percent conversion of A to B (11β-reductase) or B to A (11β-dehydrogenase) after 30 min (n = 7 for 5 mM azide and 1 mM KCN; n = 3 for 10 mM KCN). *, $P < 0.05$ *vs.* control. Results are from three separate culture preparations.

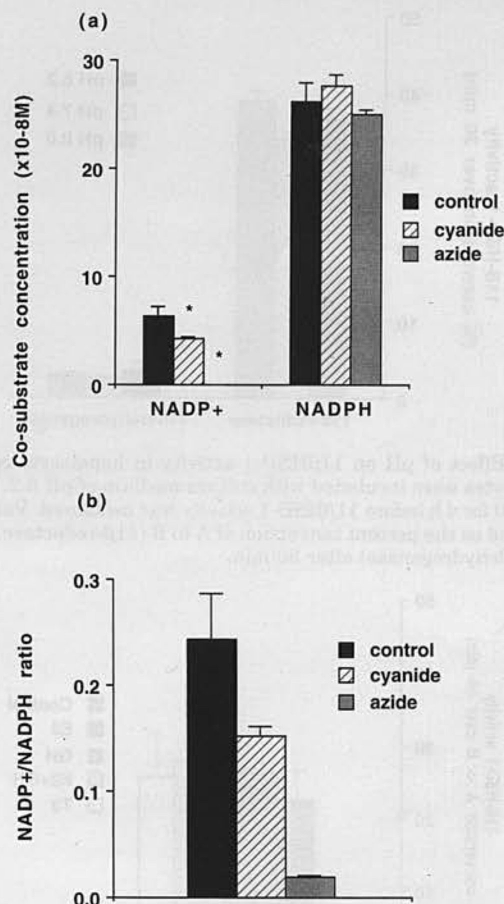

FIG. 5. Effect of metabolic inhibitors on $NADP^+$/NADPH levels (a) and $NADP^+$/NADPH ratios (b) in hepatocyte cultures. Hepatocytes were incubated for 4 h with 5 mM sodium azide or 1 mM KCN before extraction and quantitation of $NADP^+$ and NADPH by enzymatic cycling. Values are expressed as the concentration of cosubstrate (a) and the $NADP^+$/NADPH ratio (b) in sample extract. *, $P < 0.05$ *vs.* control.

dard maintenance medium. Dexamethasone increased both 11βHSD activity and 11βHSD-1 mRNA expression, whereas insulin decreased 11βHSD-1 mRNA expression, although enzyme activity was not significantly altered over the 12-day period. Insulin also antagonized the increase in 11βHSD-1 mRNA by dexamethasone when the hormones were added in combination (Fig. 8). Where effects of hormones or antagonists in the standard maintenance medium were not significant but suggested a trend, the experiments were repeated in medium containing charcoal-stripped serum. T_3 again had no significant effect in medium containing stripped Nu-serum (data not shown).

Discussion

We have developed primary rat hepatocyte cultures that maintain 11βHSD-1 gene expression for several weeks. These cells show exclusively 11β-reductase activity over a wide range of substrate concentrations. Reaction direction is little affected by alterations in the intracellular NADP/NADPH ratio or pH. 11β-Reductase activity is increased by dexamethasone, an effect antagonized by insulin, but no direct effects of estrogen, T_3, or GH were detected. These data are evidence for the predominance of the 11β-reductase activity of 11βHSD-1 in the intact liver and indicate that unknown factors, presumably related to cell type and possibly intracellular localization, determine the predominant reaction direction of 11βHSD-1 *in vivo*.

Most cell culture conditions are associated with the rapid loss of differentiated functions in primary hepatocyte cultures (37, 38), including the loss of 11βHSD-1 mRNA expression and enzyme activity. Presumably, when hepatocytes are dissociated they "turn off" unnecessary differentiated functions and begin to divide, a process perhaps akin to hepatic "regeneration" (39, 40). Maintenance on a gel matrix in medium containing high levels of glucocorticoids, insulin, and other peptides, presumably more closely resembles the milieu of the intact liver and its portal circulation and, thus, allows isolated cells to maintain differentiated functions, including expression of 11βHSD-1.

11β-Reduction is the primary activity of 11βHSD-1 in intact hepatocytes, with 11β-dehydrogenase activity undetectable or barely detectable. Likewise, intact COS7 cells transiently transfected with 11βHSD-1 cDNA show only 11β-reduction. However, in homogenates of liver, primary cultures of hepatocytes, or 11βHSD-1-transfected COS7 cells, 11β-dehydrogenase activity is readily detectable (21, 26, 27). In contrast, intact Chinese hamster ovary cells transiently transfected with 11βHSD-1 cDNA show both dehydrogenation and reduction (15). The determination of enzyme direction *in vivo* is unlikely to be due to glycosylation or other posttranslational modifications, as 11β-dehydrogenase activity in all cases is readily detectable in homogenates,

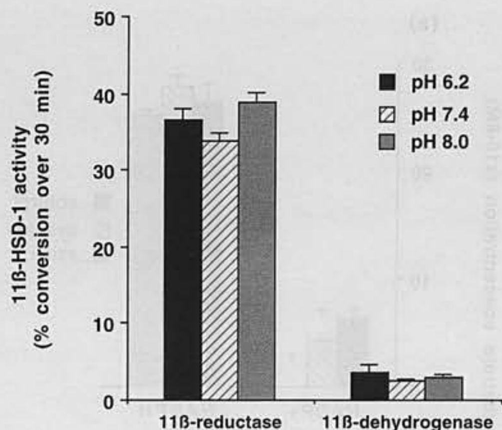

FIG. 6. Effect of pH on 11βHSD-1 activity in hepatocyte cultures. Hepatocytes were incubated with culture medium of pH 6.2, pH 7.4, or pH 8.0 for 4 h before 11βHSD-1 activity was measured. Values are expressed as the percent conversion of A to B (11β-reductase) or B to A (11β-dehydrogenase) after 30 min.

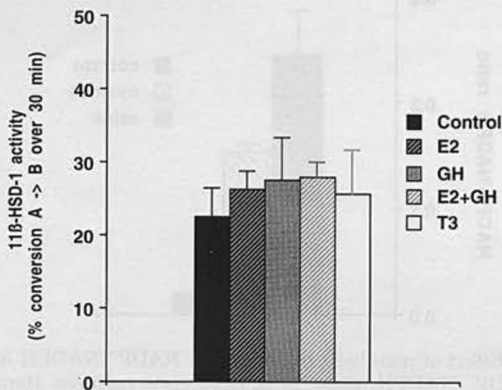

FIG. 7. Effects of addition of E_2, GH, and T_3 to hepatocyte cultures on 11βHSD-1 activity. Hepatocytes were maintained in medium with E_2 (10^{-8} M), GH (0.05 IU/ml), or T_3 (10^{-8} M) added. Activity was measured after 12 days. Values are expressed as the percent conversion of A to B (11β-reductase) after 30 min. Results are from two (T_3) or three (GH and E_2) separate culture preparations.

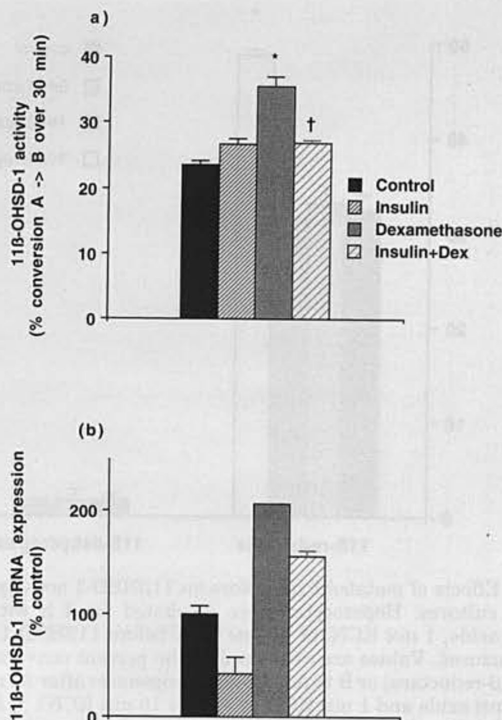

FIG. 8. Regulation of 11β-OHSD-1 activity (a) and mRNA expression (b) in hepatocyte cultures by dexamethasone and insulin. Hepatocytes were maintained in standard medium (5% Nu-serum) or medium containing 5% charcoal-stripped Nu-serum to which dexamethasone (10^{-7} M) and/or insulin (1.3×10^{-7} M) were added. Activity and mRNA were quantified after 12 days. Activity is expressed as the percent conversion of A to B (11β-reductase) after 30 min. mRNA levels are expressed as a percentage of control levels normalized for loading. * $P < 0.05$ *vs.* control; †, $P < 0.05$ *vs.* dexamethasone.

whereas the equilibrium shifts to favor reduction in most intact cells (this work and Refs. 15 and 21). Therefore, the direction *in vivo* is likely to reflect some aspect of the cellular environment of the protein. In principle, cosubstrate availability might determine reaction direction, as indeed it does in homogenates, with $NADP^+$ favoring dehydrogenation and NADPH favoring reduction. Although treatment with very high concentrations of cyanide (10 mM) did increase 11β-reductase activity, in keeping with the theory that a relative increase in NADPH will favor 11β-reduction, the large changes in $NADP^+$/NADPH ratios achieved with the metabolic poisons used here had remarkably little effect on reaction direction. These data suggest that neither the ratio of $NADP^+$/NADPH nor the availability of $NADP^+$ determines the reaction direction *in vivo* (23). However, it remains to be determined whether limiting the availability of NADPH, particularly in the specific subcompartment containing 11βHSD-1, could shift the reaction direction in intact cells toward 11β-dehydrogenation. Similarly, although in cell homogenates, pH influences the equilibrium of 11βHSD-1 [reduction is favored at acid pH and dehydrogenation at alkaline pH *in vitro* (22)], there was no alteration in enzyme direction or activity in intact hepatocytes over a range of extracellular pH values. Thus, it is unlikely that variations in pH determine 11βHSD-1 reaction direction, although it remains possible that within a subcellular compartment pH may be very different from that within the cell as a whole. 11βHSD-1 is a membrane-associated protein that shows a microsomal location (1); 11βHSD-2, which is an exclusive dehydrogenase, is also a membrane-associated protein, but shows a different intracellular distribution to 11βHSD-1 (4). The key determinants of reaction direction probably reflect more than the mere presence of membrane (as this is also present in homogenates) and may be controlled by the suborganelle microenvironment generated, for example, by neighboring enzymes. The precise intracellular localization of both isoforms of 11βHSD awaits the development of highly specific antisera suitable for electron microscopic studies.

Previous studies have shown that 11βHSD-1 is sexually dimorphic in the liver, with lower levels in females than males. E_2 markedly attenuates hepatic 11βHSD-1 activity and mRNA expression *in vivo*, an effect that is in part mediated by GH (28). In primary hepatocyte cultures, neither E_2 nor GH affected 11βHSD-1 enzyme activity. The failure of

GH to regulate 11βHSD-1 in primary hepatocytes in culture might reflect an indirect mechanism of action or may be due to a reduction in the number of GH receptors on the cells in culture. The absence of effects of E_2 and T_3 presumably reflects the indirect nature of their effects on hepatic 11βHSD-1, as loss of their receptors is unlikely, and these receptors act directly on target DNA. Although, sequences resembling estrogen or thyroid hormone response elements are found within the 11βHSD-1 gene promoter (41), the data presented here support *in vivo* evidence that the regulation of 11βHSD-1 expression by sex steroids, and presumably thyroid hormones, is mediated indirectly. Indeed, the latter contention is supported by the sexually dimorphic effects of thyroid hormones on rat liver, with thyroidectomy increasing 11βHSD activity in females, but decreasing activity in males (31).

In contrast, dexamethasone and insulin clearly regulate 11βHSD-1 in hepatocyte cultures. Previous studies have shown that adrenalectomy of rats attenuates hepatic 11βHSD enzyme activity as well as reactivation of cortisone to cortisol (presumably mediated by hepatic 11βHSD-1); administration of glucocorticoids to adrenalectomized rats increased hepatic 11βHSD-1 activity and mRNA (29). This appears to be a direct effect on hepatocytes, as dexamethasone increased 11βHSD-1 activity and mRNA expression in hepatocyte cultures. The molecular mechanism is unknown, but preliminary data from transfection experiments of HepG2 cells with plasmids in which 11βHSD-1 promoter DNA is fused to a reporter gene suggest that a glucocorticoid response element lies within 3700 base pairs of the transcription start of 11βHSD-1 (Chapman, K. E., M. Voice, R. Wallace, and V. Lyons, unpublished data). The antagonism by insulin of dexamethasone-induced 11βHSD-1 activity and mRNA expression in cultured hepatocytes reflects similar regulation in cultures of human fibroblasts (30). Many key metabolic enzymes in the liver are antagonistically regulated by insulin and glucocorticoids, and the data presented here suggest that similar controls apply to hepatic 11βHSD-1.

Intriguingly, many hepatic enzymes involved in carbohydrate and fat metabolism are controlled by glucocorticoids, including phosphoenolpyruvate carboxykinase, the rate-limiting enzyme in gluconeogenesis. 11β-Reduction of A or cortisone in liver will increase local concentrations of active glucocorticoids, potentiating glucocorticoid action regardless of circulating cortisol or B levels (the latter will be determined by the activity of the hypothalamic-pituitary-adrenal axis). In this regard, it is interesting that cortisol shows a pronounced diurnal variation, whereas cortisone levels vary little and are similar to free cortisol concentrations (25). Thus, activation of inert cortisone or A may allow hepatocytes to maintain crucial glucocorticoid-regulated metabolic functions during the daily cortisol nadir. The implications of abnormal 11β-reductase activity for disease are unknown, but in principle, excess activity may increase gluconeogenesis and attenuate insulin sensitivity, producing insulin resistance/noninsulin-dependent diabetes mellitus. Indeed, recent data suggest that 11βHSD inhibitors increase central (hepatic) insulin sensitivity in humans, presumably by attenuating glucocorticoid regeneration in the liver (42). The primary hepatocyte system described here will assist further examination of these ideas.

Acknowledgment

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