THE UNIVERSITY *of* EDINBURGH

Title	Hepatic 5α-reduced glucocorticoids : modulators of glucocorticoid receptor activation in obesity
Author	McInnes, Kerry J.
Qualification	PhD
Year	2004

Thesis scanned from best copy available: may contain faint or blurred text, and/or cropped or missing pages.

Digitisation notes:

- Page 87 is missing in the original thesis.

Hepatic 5α-Reduced Glucocorticoids: Modulators of Glucocorticoid Receptor Activation in Obesity

Kerry J McInnes (BSc)

Doctor of Philosophy

University of Edinburgh

October 2003

Abstract

The enzyme steroid 5α-reductase catalyses the reduction of Δ^{4-5} double bonds in a variety of steroid substrates. Preliminary evidence has suggested that 5α-reduction of glucocorticoids is increased in obesity, consistent with the observation that in the face of an enhanced cortisol secretion rate, plasma cortisol levels are not consistently elevated in obesity. The implications of enhanced 5α-reduction of glucocorticoids for glucocorticoid receptor activation are unclear. 5α-Reduced metabolites of other steroids e.g. testosterone and aldosterone have significant affinity for the parent hormone receptor, therefore we hypothesised that 5α-reduced glucocorticoids are glucocorticoid agonists.

In obese versus lean Zucker rats, hepatic 5α-reductase type 1 mRNA expression and protein levels were increased. They also had increased activity of hepatic 5β-reductase activity. By contrast, 3α-hydroxysteroid dehydrogenase mRNA expression was unchanged in obesity. Greater inactivation of corticosterone by A-ring reductases in liver may decrease local corticosterone concentrations in these sites, and increase the metabolic clearance rate of glucocorticoids, thus increasing drive to the hypothalamic-pituitary-adrenal axis (HPA).

To investigate whether 5α-reduced metabolites of corticosterone are glucocorticoid receptor agonists, competition binding studies were carried out. In displacing tritiated dexamethasone from binding sites in hepatocytes from male lean Zucker rats, corticosterone and 5α-tetrahydrocorticosterone (5αTHB) had similar affinities which were greater than 5α-dihydrocorticosterone (5αDHB) and 5β-reduced metabolites. Binding of corticosterone and 5αDHB binding was impaired in obesity whereas 5αTHB binding was unaltered suggesting that 5αTHB may modulate GR activation in obesity.

Activation of glucocorticoid receptors was assessed following transient transfection into HeLa cells with an MMTV-luciferase reporter. By comparison with

i

corticosterone, 5αTHB was active and additive. 5β-Reduced metabolites did not activate glucocorticoid receptors. In addition, in H4IIE cells which express endogenous glucocorticoid receptors, 5αTHB induced tyrosine aminotransferase mRNA expression albeit to a lesser extent than corticosterone. 5αTHB was also found to possess glucocorticoid activity *in vivo* as suppression of plasma ACTH was demonstrated in adrenalectomised lean Zucker rats following i.p. administration of corticosterone or 5αTHB.

We conclude that hepatic A-ring reduction is enhanced in the obese Zucker rat producing increased concentrations of 5αTHB which can bind and activate glucocorticoid receptors. Transcription of glucocorticoid regulated genes in tissues which express 5α-reductases will thus be influenced by intracellular levels of both corticosterone and its 5α-reduced metabolites. Manipulation of this enzyme may prove to be a useful therapeutic target in obesity.

Contents

List of Figures

List of Tables

Abbreviations

3αHSD	3alpha-hydroxysteroid dehydrogenase
3βHSD	3beta-hydroxysteroid dehydrogenase
5αDHB	5-alpha-dihydrocorticosterone
5αDHT	5-alpha-dihydrotestosterone
5αTHB	5-alpha-tetrahydrocorticosterone
5βDHB	5-beta-dihydrocorticosterone
5βTHB	5-beta-tetrahydrocorticosterone
11β-HSD1/2	11β-hydroxysteroid dehydrogenase type ½
A	11-dehydrocorticosterone
ACTH	Adrenocorticotrophic hormone
ADX	Adrenalectomised
AKR	Aldo-keto reductase
ANOVA	Analysis of Variance
ANP	Atrial naturetic peptide
AP1	Activator protein 1
ARE	Androgen regulatory element
as	Antisense
ATP	adenosine triphosphate
AU	Arbitrary units
AVP	Arginine vasopressin
B	Corticosterone
B_{max}	Maximal binding capacity (quantity of ligand required to saturate receptor)
BBS	
bp	base pairs (of nucleic acid)
BSA	Bovine serum albumin
CBG	Corticosteroid-binding globulin
cDNA	Complementary deoxyribonucleic acid
cpm	Counts per minute

CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
dATP	deoxyadenosine triphosphate
DBD	DNA binding domain
dCTP	deoxycytosine triphosphate
DEPC	Diethylpyrocarbonate
Dex	Dexamethsone
dGDP	deoxyguanosine triphosphate
DHA	5-alpha-dihydro-11-dehydrocorticosterone
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyNucleotide triphosphate
DTT	Dithiothreitol
dTTP	deoxythymosine triphosphate
EDTA	Ethylene Diamine Tetra-Acetate
ER	Endoplasmic Reticulum
FSH	Follicle-stimulating hormone
G-6-Pase	Glucose-6-Phosphatase
GABA	Gamma-amino butyric acid
GCMS	Gas Chromatography Mass Spectrometry
GH	Growth hormone
GLUT-4	Glucose Transporter 4
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTE	Glucose tris EDTA
HBSS	
HEPES	(N-[2-Hydroxyethyl] piperazine-N'-[2-[ethanesulphonic acid])
HPA	Hypothalamic-Pituitary-Adrenal Axis
HPLC	High Performance Liquid Chromatography
Hsp	Heat-shock protein

IGF-1	Insulin-like growth factor-1
kb	kilobases
K_d	Dissociation constant (concentration of ligand required to achieve half maximal binding of receptor)
kDa	kiloDaltons
LAGS	Low Affinity Glucocorticoid-binding Sites
LB	Luria-Bertoni
LBD	Ligand-binding domain
LH	Luteinising hormone
LSD	Least square difference
LTR-Luc	Long terminal repeat-Luciferase
MDR1-type P-gp	Multi-drug resistance1-type P-glycoprotein
met	methionine
MMTV	Mouse mammary tumour virus
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	Messenger RNA
MR	Mineralocorticoid receptor
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κB
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEPCK	Phospoenolpyruvate carboxykinase
POMC	Propionomelanocortin
PRE	Progesterone regulatory element
RNA	Ribonucleic acid
RT	Reverse transcription
RU486	Mifepristone
s	sense
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean

SPA	Scintillation proximity assay
SSC	Saline-sodium citrate
TAT	Tyrosine aminotransferase
TBE	Tris boric acid EDTA
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethyl-1-,2-diaminomethane
THA	5-alpha-tetrahydro-11-dehydrocorticosterone
Tris	Tris [hydroxymethyl]-aminomethane
tRNA	transfer RNA
UV	Ultraviolet (light)
v/v	volume/volume
w/v	weight/volume

Declaration

I declare that this thesis was written by me and that the data presented within it is a result of my own work, except the procedures listed below .

Surgical procedures were carried out by Dr Chris Kenyon of the Endocrinology Unit, School of Molecular and Clinical Medicine, University of Edinburgh.

5α-reductase type 1 Western blot was carried out by Dr Dawn Livingstone of the Endocrinology Unit, School of Molecular and Clinical Medicine, University of Edinburgh.

GCMS analysis of samples was carried out by Ms Alison Ayres of the Endocrinology Unit, School of Molecular and Clinical Medicine, University of Edinburgh.

I declare that this work has not been submitted for any other degree.

Kerry J McInnes, Edinburgh,

Acknowledgements

Chapter One

Introduction

The following introductory chapter describes glucocorticoid physiology, with particular emphasis on glucocorticoid metabolism and the role of A-ring reductases. Evidence for the role of glucocorticoids in obesity is discussed and finally, a list of the aims of this thesis is presented.

1.1 Glucocorticoids

Glucocorticoids (corticosterone in rodents and cortisol in humans) were initially named to reflect their effects on carbohydrate metabolism. However, it is now well understood that these hormones are essential for survival and exert an influence on most systems of the body including the immune system, fluid and electrolyte homeostasis and the physiological response to stress.

1.1.1 Steroid Hormone Structure

Glucocorticoids are part of the steroid hormone family, each member of which contains structural modifications of the common precursor molecule, cholesterol. The chemical structure of steroid hormones is based on that of cholesterol, consisting of three cyclohexane rings and one cyclopentane ring (Figure 1.1). Each steroid hormone is given its unique properties by the substitution of chemical groups at various positions on the backbone molecule.

1.1.2 Glucocorticoid Biosynthesis

Glucocorticoid synthesis from cholesterol occurs in the zona fasciculata of the adrenal cortex, with a smaller contribution of the zona reticularis. Steroid hormone biosynthesis is catalysed by members of the cytochrome P450 oxidative enzyme family which are located in the membranes of the mitochondria and endoplasmic reticulum (ER) (Table 1.1), and characterised by their absorbance maximum of 450nm (Miller 1988). The sequence of reactions involved in glucocorticoid biosynthesis is shown in Figure 1.2.
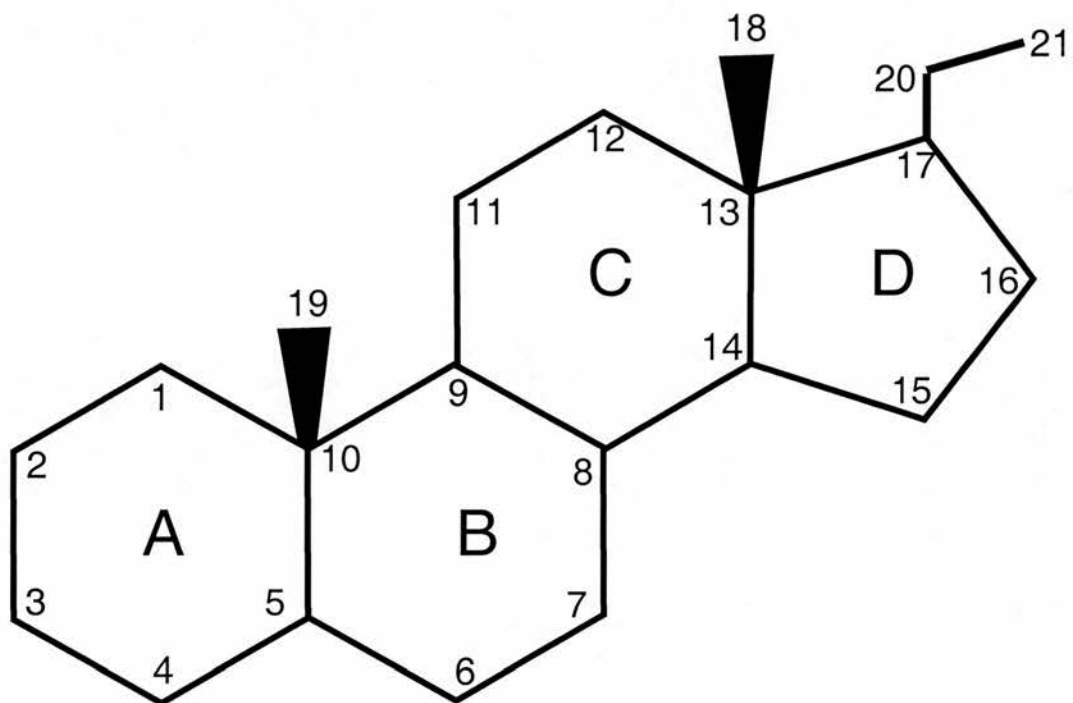
Figure 1.1: The basic steroid ring structure

The four carbon rings are identified by letters and the individual carbon atoms by numbers. Chemical groups are designated by the number of the carbon to which they are attached.
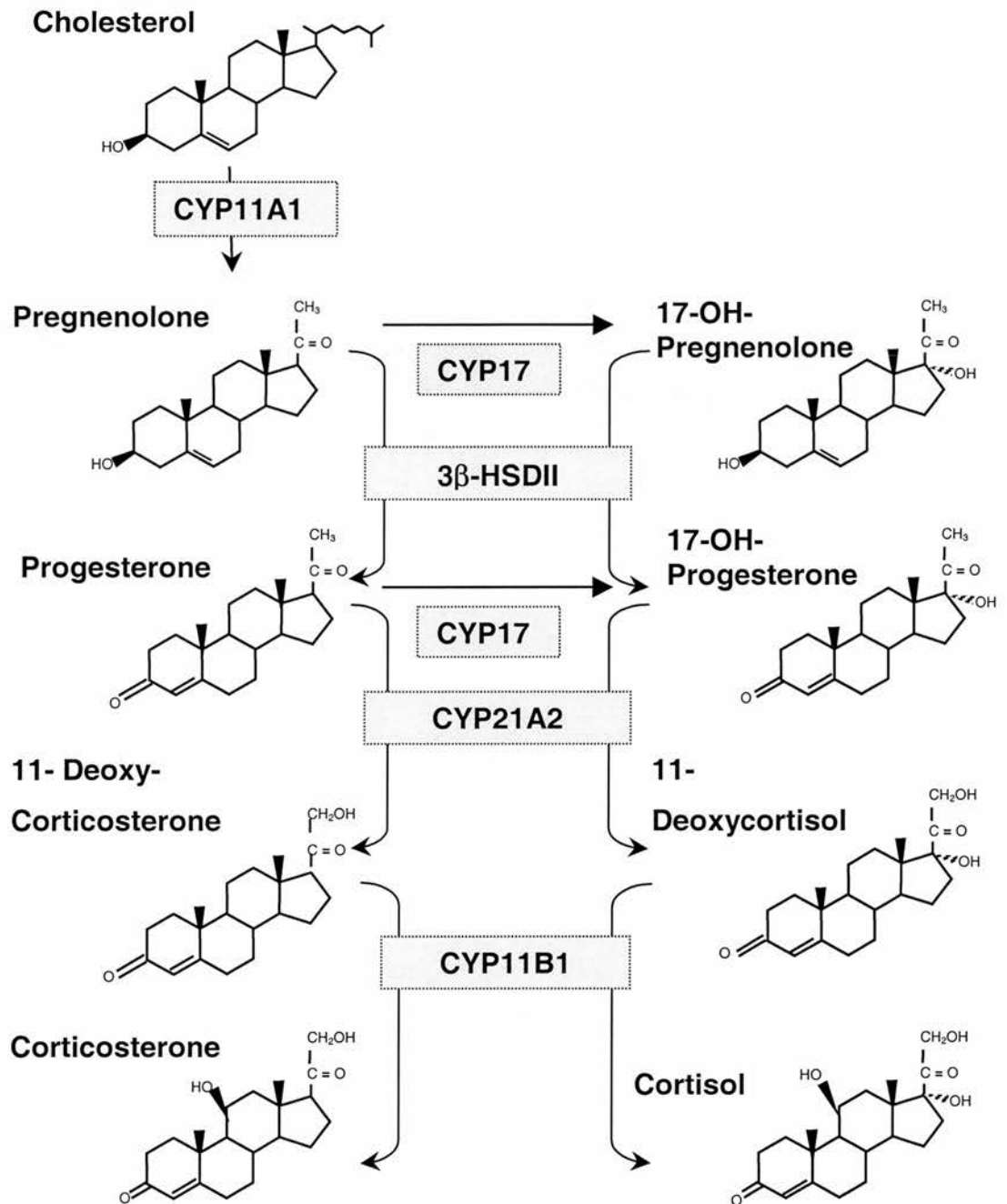
Figure 1.2: Biosynthesis of Glucocorticoids in the Adrenal Cortex

Glucocorticoid biosynthesis from cholesterol in the adrenal cortex is catalysed by CYP oxidative enzymes. The sequence of reactions involved in glucocorticoid biosynthesis is shown, with specific enzyme names indicated in boxes.

General Name	Specific Name	Intracellular Location
Cholesterol side-chain cleavage	CYP11A1	Inner mitochondrial memb.
3β-Hydroxysteroid dehydrogenase	3β-HSDII	Endoplasmic reticulum
*(17α-Hydroxylase)	(CYP17)	(Endoplasmic reticulum)
21-Hydroxylase	CYP21A2	Endoplasmic reticulum
11β-Hydroxylase	CYP11B1	Inner mitochondrial memb.

Table 1.1: Enzymes Involved in Glucocorticoid Biosynthesis

* Does not occur in rat or mouse adrenal.

1.1.3 Glucocorticoid secretion

Glucocorticoid synthesis and release from the adrenal cortex is controlled by hormonal interactions between the hypothalamus, pituitary gland and the adrenal gland (known as the hypothalamic-pituitary-adrenal (HPA) axis), and can in turn be influenced by both neural and chemical stimuli. Stimulation of the hypothalamus (e.g. by stress) results in the release of corticotrophin releasing hormone (CRH) (Vale *et al.* 1981) and arginine vasopressin (AVP) (Lamberts *et al.* 1984; Engler *et al.* 1989) into the hypothalamic-hypophyseal portal capillary system. Stimulation of CRH receptors on corticotrophs of the anterior pituitary results in the rapid release of adrenocorticotrophic hormone (ACTH) into the systemic circulation (Horrocks *et al.* 1990). ACTH is synthesised from the polypeptide pro-opiomelanocortin (POMC) and acts via plasma membrane receptors (melanocortin-2-receptors) on adrenal cells, to stimulate steroidogenesis and hence, the secretion of glucocorticoids.

Glucocorticoids themselves provide a negative feedback loop to regulate their own secretion. They suppress ACTH secretion at the level of the hypothalamus by inhibiting CRH release, and at the level of the pituitary by inhibiting POMC transcription and ACTH synthesis. This feedback loop acts to maintain physiological plasma glucocorticoid levels (De Kloet 1991). The HPA axis of the rat is summarised in Figure 1.3.
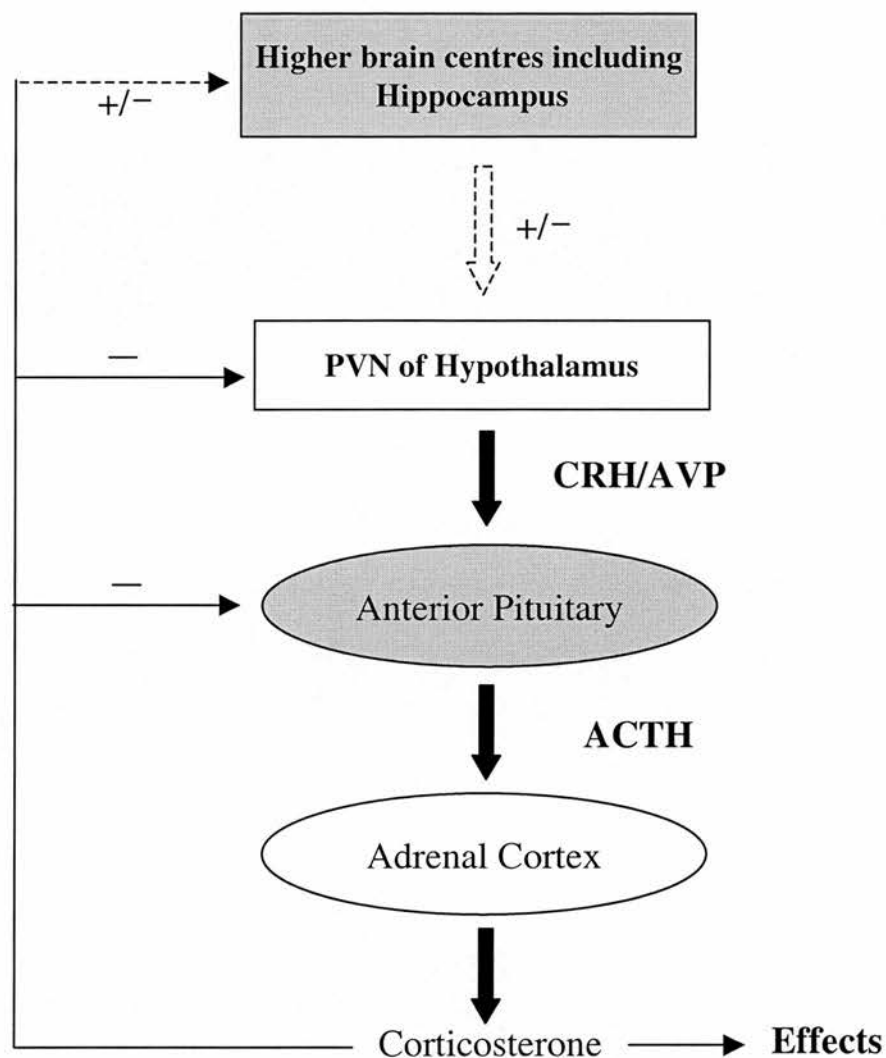
Figure 1.3: The Hypothalamic-Pituitary -Adrenal Axis of the Rat

Corticosterone is secreted by the adrenal cortex in response to the secretion of ACTH by the anterior pituitary, secretion of which is triggered by CRH and AVP release by the PVN of the hypothalamus. Corticosterone has a negative feedback effect upon its own secretion at the hypothalamus and pituitary, and also influences the activity of the neuronal projections to the hypothalamus from higher brain centres.

Key: + up-regulation, -down regulation

In addition to the stimulated release of glucocorticoids described above, there is also a diurnal variation in glucocorticoid secretion. Glucocorticoid secretion follows the pattern of plasma ACTH and there is a major increase in activity before awakening. Thereafter, the release of ACTH and glucocorticoids generally occurs only for brief episodes. After each episode, plasma glucocorticoid levels increase enough to suppress further ACTH release and then fall to reach the set-point of negative feedback control. This diurnal pattern comprising of pulses of ACTH/ glucocorticoid secretion control circulating glucocorticoid levels in the plasma (Horrocks *et al.* 1990; Chrousos & Gold 1998).

1.1.4 Glucocorticoid Action

Circulating glucocorticoids (corticosterone and cortisol) are predominantly bound to corticosteroid-binding globulin (CBG) and albumin, with only 5-10% freely circulating in the plasma (Hammond *et al.* 1990). The lipophilic nature of these hormones allows them to pass freely through the cell membrane. However, only free steroid can diffuse across cell membranes to bind to the intracellular glucocorticoid receptor. Glucocorticoid-binding proteins therefore act to buffer free corticosterone concentrations. However, these proteins can become saturated at high physiological glucocorticoid concentrations, amplifying fluctuations in free steroid levels throughout the period of diurnal variation.

1.1.5 The Glucocorticoid Receptor

1.1.5.1 Background

Endogenous glucocorticoids and their synthetic derivatives act via the glucocorticoid receptor (GR). GR is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and mediates transactivation of target genes by binding sequence specific recognition elements in their promoter region.

When the human GR cDNA was first cloned in 1985 (Hollenberg *et al.* 1985; Weinberger *et al.* 1985) two GR gene splicing products were identified and termed GRα and GRβ. The human GRα and GRβ mRNA both contain exons 1-8, but

contain different versions of exon 9 as a result of alternative splicing. GRα is ubiquitously expressed and is recognised as the classical GR (Hollenberg *et al.* 1985). Most of our understanding of GR physiology comes from the study of this isoform. GRα shows high affinity for dexamethasone, modest affinity for cortisol and corticosterone and low affinity for aldosterone, deoxycorticosterone and the sex steroids. The GRβ isoform, which differs only in the last 15 amino acids, does not bind active glucocorticoids and is transcriptionally inactive on glucocorticoid-response element -containing enhancers (Vottero & Chrousos 1999). GRβ was long dismissed as a cloning artefact however it is expressed at varying levels in a range of tissues (Oakley *et al.* 1996; Oakley *et al.* 1997). Evidence suggests that GRβ acts as a ligand-independent negative regulator of glucocorticoid action by forming GRα/β heterodimers that are incapable of binding co-activators as a GRα homodimer would (Bamberger *et al.* 1995; Oakley *et al.* 1999). However, other groups have not detected this dominant negative inhibitory effect of GRβ (Hecht *et al.* 1997; de Lange *et al.* 1999).

Various GR isoforms are also produced by alternative translation initiation. The major protein product termed GR-A with an apparent molecular mass of 94 kDa represents translation from the first initiator AUG codon. The next downstream start codon (met 27 in humans or met 28 in rodents) results in production of a 91 kDa GR termed GR-B. The shorter GR-B species is nearly twice as efficient in glucocorticoid response element-mediated transactivation as the longer GR-A (Yudt & Cidlowski 2001). The biological significance of this alternative initiation remains unclear. However, the distinct biological activities of these isoforms suggest that they may play a role in the diversity of glucocorticoid response.

The homologous structural organisation of the nuclear receptors is well known. The DNA binding domain (DBD) is centrally located, flanked on the carboxy-terminal by an approximately 250-amino acid ligand-binding domain (LBD) and by a variably sized non-homologous amino-terminal domain (Beato *et al.* 1995).

1.1.5.2 Mechanism of glucocorticoid receptor action

1.1.5.2.1 Ligand binding and dissociation from heat shock proteins

In the absence of ligand, GR resides in the cytosol in association with several other proteins including hsp90 from which it dissociates upon ligand binding (Pratt & Toft 1997). Hsp90 associates with the ligand-binding domain of the receptor and maintains the receptor in a conformation that can bind steroid but is transcriptionally inactive (Cadepond *et al.* 1991). Other chaperone proteins including hsp40 and hsp70 are required for formation of a stable GR-hsp90 complex (Pratt & Dittmar 1998).

The crystal structure of the human GR LBD bound to dexamethasone and a coactivator motif derived from the transcriptional intermediary factor 2 has been recently elucidated (Bledsoe *et al.* 2002). The overall structure of the GR LBD is similar to other nuclear receptor LBDs with 11α-helixes and four β-strands that are folded to create a hydrophobic pocket for the ligand. There are several features unique to the GR LBD including a distinct dimerisation interface that involves the formation of a central hydrophobic intermolecular β-sheet (Bledsoe *et al.* 2002). The crystal structure also reveals an additional charge clamp that determines the binding selectivity of a coactivator and a distinct ligand binding pocket that explains the selectivity of GR for endogenous steroid hormones (Bledsoe *et al.* 2002).

1.1.5.2.2 Phosphorylation and nuclear translocation

The glucocorticoid receptor is phosphorylated in the absence of ligand and becomes hyperphosphorylated after agonist-binding but not after binding of an antagonist (Orti *et al.* 1989). The phosphorylated GR subsequently translocates to the nucleus. However, receptor phosphorylation is not necessary for nuclear translocation since a mouse GR lacking all phosphorylation sites still undergoes nuclear translocation upon ligand activation (Webster *et al.* 1997). Two domains of GR have been implicated in nuclear translocation. The first (NL1) is localised in the C-terminal part of the DBD and is 100% conserved between human and rat GR (Picard & Yamamoto 1987). The LBD inhibits the function of NL1 and this inhibition can be abolished by ligand binding (Cadepond *et al.* 1992). The exact location of the second nuclear

localisation signal is (NL2) is unknown but has been mapped to the LBD (Picard & Yamamoto 1987).

1.1.5.2.3 Dimerisation and DNA binding

Upon ligand binding the GR can form homodimers. This process appears to involve the DNA binding domain (DBD) which consists of two protein loops co-ordinated by a zinc ion, resulting in two zinc fingers which are both followed by an amphipathic α-helix. GR can then bind specific DNA sequences called glucocorticoid-responsive elements (GRE) of target genes to initiate transcription. Several amino acids in the DNA binding domain interact with the DNA to keep GR in the major groove of the DNA α-helix (Luisi *et al.* 1991).

1.1.5.2.4 Transactivation

It is not fully understood how binding of GR to GREs leads to transcription initiation. The basic transcription machinery which consists of RNA polymerase II and other general transcription factors such as TATA box-binding protein must be recruited to the promoter (Beato *et al.* 1989). In addition the transcriptional activity of the GR depends on coactivators that facilitate recruitment of the basal transcription machinery or remodel chromatin (Collingwood *et al.* 1999; Jenkins *et al.* 2001). Optimal transactivation of target genes is dependent on the presence of two domains, τ-1 and τ-2. The function of τ-1 is hormone-independent whereas hormone binding is required for the activity of τ-2 (Hollenberg & Evans 1988). A summary of the events involved in the activity of GR is described in Figure 1.4.

More recent studies investigating the mouse mammary tumour virus long terminal repeat (MMTV-LTR) which contains several GREs have shown that transcriptional activation by GR requires DNA binding, disruption of local chromatin structure and assembly of the initiation complex at a TATA box (Hebbar & Archer 2003).

1.1.5.2.5 Transrepression

GR is also able to transrepress the expression of specific target genes by at least three mechanisms. Firstly, it can bind to DNA sequences called negative GREs in the promoter region of target genes (Cairns *et al.* 1993). Secondly, GR can bind to DNA elements that consist of a non-overlapping GRE and a binding site for a different transcription factor (known as composite GREs) (Pearce & Yamamoto 1993). A third mechanism occurs without binding to DNA. GR inhibits AP-1, the collagenase promoter by direct protein-protein interactions (Jonat *et al.* 1990). In addition GR can repress the activity of NF-κB by physically interacting with the p65/RelA subunit (Ray & Prefontaine 1994) More recent studies have also shown that GR can inhibit p65-induced transcription by interfering with histone acetylation and phosphorylation of RNA polymerase II (Ito *et al.* 2001).

1.1.5.3 Other Glucocorticoid binding sites

Glucocorticoids can bind to a site other than the cytosolic GR. Low affinity glucocorticoid binding sites ("LAGS") have been demonstrated in the microsomal fraction (Ambellan *et al.* 1981) and nuclear envelope (Roszak *et al.* 1990). Glucocorticoids may also be able to act upon membrane bound receptors. Glucocorticoid binding sites have been described in rat neuronal membranes (Liposits & Bohn 1993), rat liver plasma membranes (Allera *et al.* 1980; Lackner *et al.* 1998) and human leukemic cells (Gametchu *et al.* 1999). The physiological relevance of these binding sites remains unclear, but they may be involved in rapid non-genomic actions of glucocorticoids (Borski 2000)
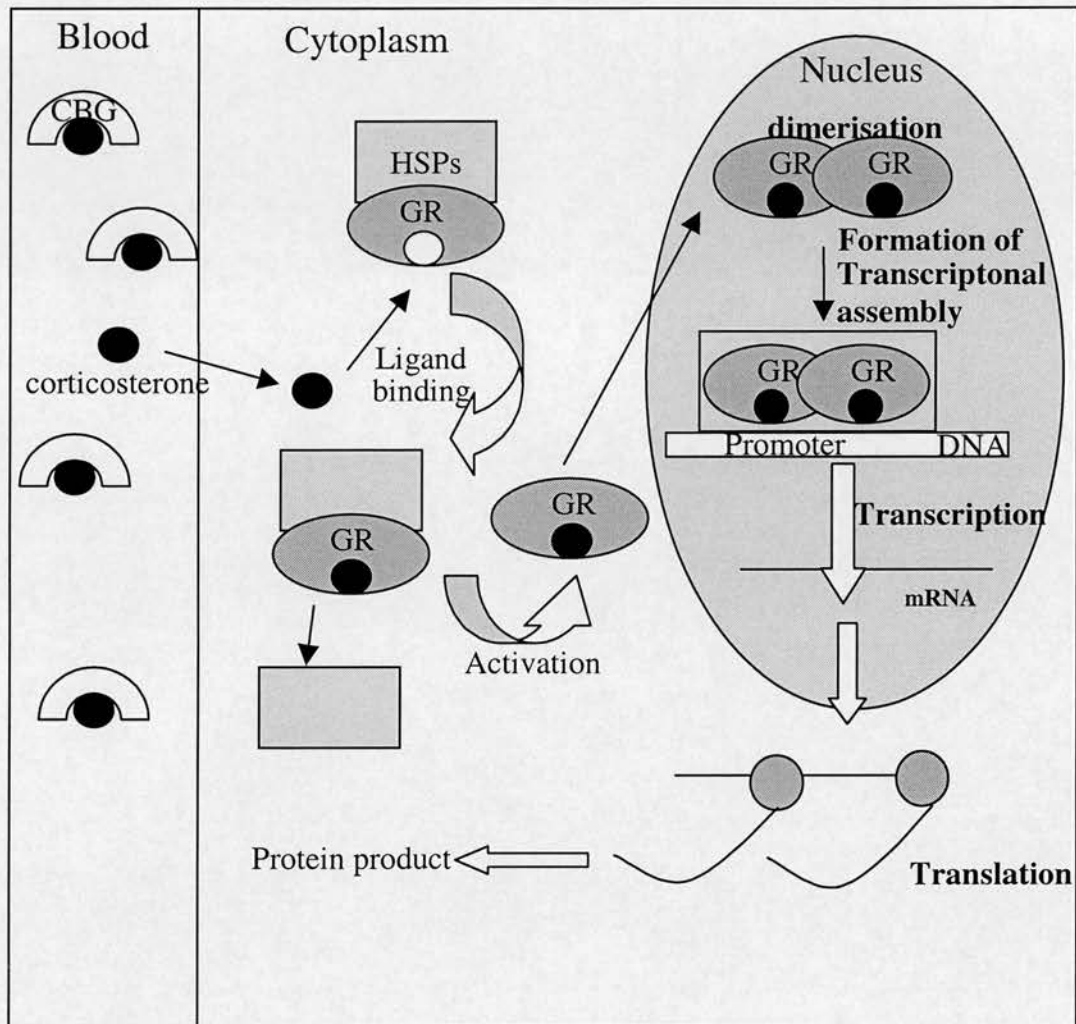
Figure 1.4: Model of glucocorticoid Receptor activity

Free hormone dissociates from the circulating transport protein (CBG), diffuses into the cytosol, and binds to inactive GR. This activates the receptor by causing dissociation of the heat-shock protein complex (HSP). The receptor dimerises and is translocated into the nucleus. Here the activated receptor binds to glucocorticoid-response elements in the promoter region of target genes, and recruits other elements of the transcriptional apparatus of the cell. Transcribed mRNA is translated into protein products by ribosomes in the cytosol.

1.1.6 Glucocorticoid Effects

The roles of glucocorticoid hormones in various physiological systems were initially identified from clinical observations of the consequences of adrenal disease. Glucocorticoid excess (e.g. in Cushing's syndrome) is characterised by redistribution and accumulation of fat in more central depots, impaired glucose tolerance, hypertension, muscle weakness and increased susceptibility to injury (Cushing 1912). In contrast, glucocorticoid insufficiency (e.g. in Addison's disease) is characterised by weight loss, loss of appetite and postural hypotension (Addison 1855). Further insight into the effect of glucocorticoids was aided by the advent of the steroid biosynthesis inhibitor, metyrapone and the glucocorticoid receptor antagonist, mifepristone (RU486).

1.1.6.1 Effects on metabolism

The metabolic effects of glucocorticoids are essentially anabolic in the liver and catabolic in muscle and adipose tissue, the overall effect of which is to increase blood glucose levels. Glucocorticoids increase hepatic glucose production by stimulating the synthesis of enzymes essential in hepatic gluconeogenesis (e.g. phosphoenolpyruvate carboxykinase (PEPCK) (Friedman *et al.* 1997) and glucose-6-phospatase (G6Pase) (Yoshiuchi *et al.* 1998). In addition, glucocorticoids mobilise substrates for hepatic gluconeogenesis by stimulating release of amino acids from skeletal muscle and fatty acids and glycerol from adipose tissue (Exton 1979). Glucocorticoids also inhibit glucose uptake by peripheral tissues by decreasing transport of the GLUT4 transporter to the cell membrane (Weinstein *et al.* 1995), and stimulate glycogen storage by activating glycogen synthase and inhibiting glycogen phosphorylation (Stalmans & Laloux 1979).

Glucocorticoids promote differentiation of pre-adipocytes to mature adipocytes (Hauner *et al.* 1987). Glucocorticoids receptors have been found in isolated adipocytes (Bronnegard *et al.* 1990) and stromal cells (Zhao *et al.* 1995) and they induce several gene products including lipoprotein lipase and glycerol-3-phosphate dehydrogenase (Fried *et al.* 1993). The end result is differentiation of adipose

stromal cells into adipocytes (Hauner *et al.* 1989; Bronnegard *et al.* 1995) and accumulation of lipid. Site-specific alterations in fat accumulation via glucocorticoid-mediated activation of lipoprotein lipase and hormone-sensitive lipase can result in obesity in glucocorticoid excess and may be a result of specific up-regulation of GR (Bronnegard *et al.* 1990).

These various effects of glucocorticoids on metabolism contribute to the maintenance of blood glucose during food deprivation and the mobilisation of extra glucose during stress. These protective effects come at a cost of decreased protein and fat.

1.1.6.2 Effects on the cardiovascular system

Glucocorticoids are needed to maintain normal vascular integrity and responsiveness and to maintain the volume of body fluids. Although the exact mechanisms involved in glucocorticoid-mediated regulation of blood pressure have not been fully elucidated, their effects in the cardiovascular system are wide ranging. Glucocorticoids regulate cardiac output by maintaining contractility and work performance of the heart. In addition, glucocorticoids also play an important role in the regulation of fluid and electrolyte balance. These hormones can directly influence sodium handling (Montrella-Waybill *et al.* 1991), and are essential for the regulation of angiotensinogen production from the liver, AVP production from the hypothalamus and atrial naturetic peptide (ANP) production from cardiac myocytes – each of which can influence sodium/ water handling in the kidney.

Glucocorticoids are therefore essential for the maintenance of normal blood pressure and alterations in circulating glucocorticoid concentrations can cause hypertension (e.g. in Cushing's syndrome) and hypotension (e.g. in Addison's disease). The mechanisms of glucocorticoid-induced hypertension include increased systemic vascular resistance, increased extracellular volume and increased cardiac contractility. Cushing's disease is associated with an enhanced sensitivity to the pressor effects of angiotensin II and noradrenaline and a decrease in the levels of vasodilator prostaglandins (Sartura *et al.* 1986). Observations in mice with a

transgenic deletion of the 11βHSD2 gene (Kotelevtsev *et al.* 1999) have confirmed the role of glucocorticoids in the control of blood pressure and vascular function. 11βHSD2 acts in aldosterone target tissues to protect mineralocorticoid receptors from inappropriate activation by glucocorticoids (Edwards *et al.* 1988). The 11βHSD2 knockout mice are hypertensive and have enhanced vasoconstrictor responses to noradrenaline and 5-hydroxytryptamine and impaired nitric oxide mediated endothelium dependent vasodilatation (Hadoke *et al.* 1999).

1.1.6.3 Effects on the immune system

The reactions to various injuries and foreign substances involve multiple anti-inflammatory and immune responses. Glucocorticoids have profound inhibitory effects on both the early and the late manifestations of inflammation and have therefore been exploited clinically in the treatment of inflammation and autoimmune disease and prevention of rejection of transplanted organs. The anti-inflammatory effects of the glucocorticoids are due to actions on blood vessels, inflammatory cells and inflammatory mediators (Barnes & Adcock 1993).

Glucocorticoids alter the trafficking of cells of the immune system (T- and B-cells, neutrophils, monocytes and granulocytes) resulting in suppression of local inflammatory responses (Dale *et al.* 1975). Glucocorticoids are also able to induce lymphocyte apoptosis (Nazareth *et al.* 1991) and inhibit T-cell cytokine synthesis (Cupps *et al.* 1985), therefore indirectly inhibiting B-cell and macrophage activation and proliferation (Rinehart *et al.* 1982). Glucocorticoids suppress the synthesis of several key substances that mediate the inflammatory response including the prostaglandins and can stimulate synthesis of others that are anti-inflammatory including lipocortins (Goulding & Godolphin 1990).

1.1.6.4 Effects on the central nervous system

Several cell types of the central nervous system (CNS) contain glucocorticoid receptors, including neurones, astrocytes, glial cells and oligodendrocytes. Clinical observations have indicated that glucocorticoids influence cognition, mood, reception of sensory stimuli and sleep patterns by modulating neuronal activity

(McEwen *et al.* 1986). Chronic exposure to glucocorticoids induces neurotoxicity, hypothetically resulting in decreased hippocampal volume and therefore decreased memory function (Sapolsky *et al.* 1985). Psychological disturbances are common in Cushing's disease presenting as initial euphoria but subsequent depression or manic behaviour (Loosen *et al.* 1992). Patients with severe primary depression have an impaired suppression of plasma cortisol following dexamethasone administration which may return to normal after treatment of the depression. Administration of exogenous glucocorticoids is also known to decrease the duration of rapid eye movement sleep (Kreiger 1972). With glucocorticoid deficiency, the senses of taste, hearing and smell are accentuated. The exact mechanisms involved in glucocorticoid-mediated changes in behaviour are unclear.

The non-genomic effects of glucocorticoids are readily apparent in the CNS. These responses (e.g. neuronal hyperpolarisation and suppression of spontaneous electrical activity) are too rapid to be mediated by classical transcriptional effects on target genes, therefore a direct membrane effect is more likely (Orchinik *et al.* 1991).

1.1.6.5 Effects on growth and development

At physiological levels, glucocorticoids are necessary for skeletal growth and are important for the maturation of various foetal organs. They are essential for foetal lung development, including the synthesis of pulmonary surfactant and are used clinically to stimulate lung maturation in premature babies (Ballard 1987). Glucocorticoids are also involved in the maturation of intestinal enzymes.

Glucocorticoid excess is associated with osteopenia (decreased new bone formation and bone resorption) (Weinstein *et al.* 1998) and with impaired calcium and phosphate absorption in the intestine and kidney (Laake 1960; Hahn *et al.* 1979). The combination of these events results in osteoporosis (Reid 2000). Additionally, glucocorticoids have deleterious effects on connective tissue caused by inhibition of fibroblast proliferation and extracellular matrix synthesis (Pratt & Aronow 1966). Excessive glucocorticoid exposure of the foetus *in utero* is also disadvantageous, resulting in retardation of foetal growth (Reinisch *et al.* 1978) which can

'programme' an increased risk of cardiovascular disease in later life. Perinatal manipulation of glucocorticoid exposure in rats has been shown to permanently alter GR expression in the brain, HPA axis responses (Meaney *et al.* 1994), gluconeogenic enzymes, glucose tolerance and blood pressure (Nyirenda *et al.* 1998).

1.1.7 Metabolism of glucocorticoids

The metabolism of glucocorticoids involves reduction, oxidation, hydroxylation and conjugation, and the metabolic pathway of corticosterone is shown in Figure 1.5. The pathway involves the interconversion of corticosterone (B) (cortisol in humans) with its inactive 11-keto metabolite, 11-dehydrocorticosterone (A) (cortisone in humans) by the isozymes of 11β-hydroxysteroid dehydrogenase (11βHSD). The subsequent metabolism of corticosterone and 11-dehydrocorticosterone follow similar paths. The rate-limiting step of glucocorticoid metabolism is the reduction of the $\Delta^{4,5}$ double bond in the A-ring of the steroid structure (the biology of these enzymes is discussed in detail in section 1.2). 5α- and 5β-reductase can both act on corticosterone whereas 11-dehydrocorticosterone is metabolised by 5β-reductase only. These enzymes produce two dihydro-stereoisomers, differing only in the orientation of the additional hydrogen atom at carbon 5. These dihydro-metabolites are then rapidly reduced further by 3α-hydroxysteroid dehydrogenase (3α-HSD) to yield tetrahydro-metabolites, the most abundant urinary glucocorticoid metabolites. Additional reduction by 20α- and 20β-hydroxysteroid dehydrogenases produces cortols and cortolones, which are finally oxidised by 21-oxidase to produce cortolic and cortolonic acids. Glucocorticoids and their metabolites are poorly water-soluble, therefore, conjugation to glucuronic acid or sulphates in the liver increases their water-solubility and decreases their binding to proteins therefore aiding excretion.
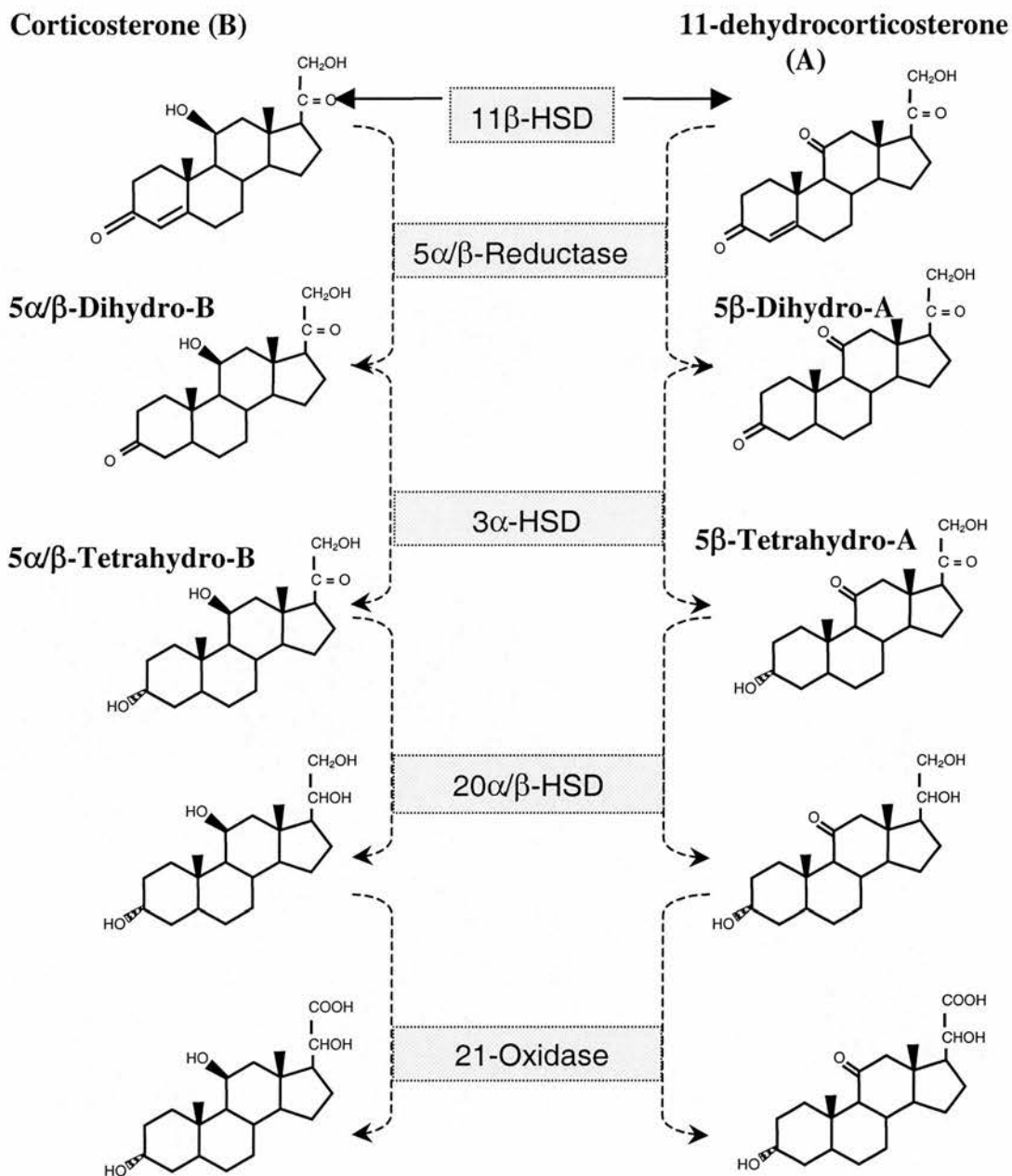
Figure 1.5: Glucocorticoid metabolism

Glucocorticoid metabolism involves reduction, oxidation, hydroxylation, and conjugation, catalysed by the enzymes in the boxes. Both 5α- and 5β-reduction of corticosterone can occur but 11-dehydrocorticosterone is only acted on by 5β-reductase.

1.2 A-ring reductases

The focus of this thesis is the metabolism of glucocorticoids by A-ring reductases, and their physiology is described in this section

1.2.1 5α-Reductase

1.2.1.1 History of 5α-reductase

A 5α-reductase enzyme was initially characterised in the 1950's in rat liver slices based on its ability to convert deoxycorticosterone to 5α-reduced metabolites (Schneider & Horstmann 1951; Schneider 1952). Subsequent work showed that this enzyme was present in the microsomal fraction and that it utilised reduced pyridine nucleotide (NADPH) as a co-factor (Forchielli & Dorfman 1956; McGuire & Tomkins 1960; McGuire *et al.* 1960). 5α-Reductase catalyses the reduction of $\Delta^{4,5}$ double bonds in a variety of steroid substrates and is thought to have both catabolic and anabolic roles in steroid hormone metabolism. 5α-Reduction of steroids renders their 3-oxo groups more susceptible to reduction by 3α- hydroxysteroid dehydrogenases (3α-HSDs) and to subsequent sulfation and glucuronylation. The latter modifications reduce the affinity of the steroid for binding proteins, make it more hydrophilic, and facilitate its excretion in the urine.

5α-Reductase was thought to participate in the catabolism of steroids and research in the 1960's showed that 5α-reductase would not catalyse the reverse reaction (dehydrogenation) of reduced steroids (Wilson 1975). More attention was focused on 5α-reductase when it was documented that dihydrotestosterone, the 5α- reduced metabolite of testosterone, was a more potent androgen than testosterone in bioassays with prostate and that it could bind preferentially to the androgen receptor (Siiteri & Wilson 1974). Definitive evidence for the key role of 5α-reductase came from developmental and genetic studies that showed that this reaction is crucial for formation of the normal male phenotype during embryogenesis (Siiteri & Wilson 1974) and that absence of steroid 5α-reductase activity underlies a rare form of male pseudohermaphroditism, originally termed pseudovaginal perineoscrotal

hypospadias, in which masculinisation of the developing genitalia is incomplete due to inadequate conversion of testosterone to 5αDHT (Walsh *et al.* 1974; Imperato-McGinley *et al.* 1974).

1.2.1.2. Identification of two distinct isozymes

It was originally assumed that several steroid 5α-reductase isozymes must exist, each of which preferentially using a different steroid substrate (McGuire & Tomkins 1960). In agreement with this notion, steroid 5α-reductase activities with different kinetic properties and pH optima were demonstrated in human tissues and one of these activities was absent in patients with steroid 5α-reductase deficiency (Moore *et al.* 1975). Further insight into the existence of multiple enzymes was hampered by the extreme insolubility of the protein (Moore & Wilson 1972) and many attempts were made to purify 5α-reductase from both rat and human sources.

Expression cloning studies allowed the full length cDNA encoding the rat liver 5α-reductase to be isolated from a *xenopus* library and was subsequently used to identify a human homologue (Andersson & Russell 1990). The human cDNA and its encoded enzyme however exhibited several puzzling features that strongly suggested the existence of a second 5α-reductase gene. 1) The cDNA product was only weakly inhibited by finasteride, a powerful inhibitor of 5α-reductase activity in the prostate, 2) the cDNA produced a 5α-reductase with an alkaline pH optimum compared to the predominant acidic pH optimum enzyme activity of prostate and 3) mutations were not detected in the coding region of the cDNA isolated from patients with 5α-reductase deficiency (Jenkins *et al.* 1992).

A second cDNA was subsequently cloned in cultured human cells (Andersson *et al.* 1991). The two human isozymes designated type 1 and type 2 are approximately 46% identical in sequence (Russell & Wilson 1994). The type 1 isozymes had a broad pH optima that spans the alkaline range (pH 6-8.5) (Normington & Russell 1992) while the type 2 isozyme had a narrow acidic pH optima centred around 5.0 (Andersson *et al.* 1991).

A rat homologue of the second human 5α-reductase has also been cloned and again in this species, the second cDNA encodes a different protein that catalyses the same biochemical reaction as that encoded by the first cDNA (Normington & Russell 1992). 5α-Reductases have also been studied in the mouse and two isozymes have been found which are similar to the rat enzymes and not the human isozymes (Mahendroo *et al.* 1996). The type 2 isozymes of rat and human are more closely related to each other (77% sequence identity) than they are to their type 1 counterparts (<50% sequence identity) and both have an acidic pH optima in contrast to the neutral to basic pH optima of the type 1 isozymes (Normington & Russell 1992). However there are differences in tissue expression of isozymes between species and in their sensitivity to the 4-azasteroid inhibitors.

The rat and human type 1 isozymes differ with respect to their sensitivity to 4-azasteroids in that the human type 1 isozyme is not sensitive to inhibition by these compounds (Thigpen & Russell 1992). This appears to be due to a difference between species in a four amino-acid segment encoded within exon 1 that are thought to define a portion of the substrate-binding domain (Thigpen & Russell 1992). The rat type 2 isozyme is inhibited potently by 4-azasteroid inhibitors with a K_I of 0.46 ± 0.21 nM compared to the rat type 1 isozyme (K_I ; 5.8 ± 0.6 nM) (Normington & Russell 1992).

The human 5α-reductase type 1 isozyme is expressed in liver (Thigpen *et al.* 1993), the skin (Harris *et al.* 1992; Imperato-McGinley *et al.* 1992; Thigpen *et al.* 1993) and kidney (Quinkler *et al.* 2003). 5α-Reductase type 2 predominates in the prostate but is also expressed in the skin and liver (Normington & Russell 1992; Thigpen *et al.* 1993). In the rat, the type 1 enzyme is expressed in both the liver and prostate (Andersson *et al.* 1989) and the type 2 isozyme predominates in male reproductive tissues such as the testis, vas deferens and epidydymis but is not expressed in the liver. In contrast to the situation in human prostate, both isozymes are expressed in the rat ventral prostate with basal epithelial cells expressing the type 1 enzyme and stromal cells expressing the type 2 isozyme (Berman & Russell 1993). A summary

of the properties of the 5α-reductase isozymes of rat and human is shown in Table 1.2.

5α-Reductase type 1 is constitutively expressed in the rat CNS at all stages of brain development and is similar in males and females. The gene expression of the type 2 isozyme is different with expression almost exclusively in late fetal/early post-natal stage (Melcangi *et al.* 1998). 5α-Reductase activity has also been demonstrated in human and rat adipose tissue (Perel *et al.* 1986; Zyirek *et al.* 1987) although it is not known what isozyme is present in fat.

1.2.1.3 The role of 5α-reductase types 1 and 2

The roles of the 5α-reductases have gained most attention with respect to androgen physiology. The significant differences in apparent Km values exhibited by the two isozymes suggested that they may have different physiological functions. The lower Km of the rat type 2 isozyme indicated that this enzyme played an anabolic role in steroid hormone metabolism, thus the type 2 isozyme is responsible largely for the paracrine and autocrine generation of DHT in androgen target tissues. This is supported by the finding that mutations in the human 5α-reductase type 2 gene (Andersson *et al.* 1991) cause developmental abnormalities in DHT-dependent tissues of the embryo termed male pseudohermaphroditism. In contrast, the higher substrate Km value of the rat type 1 isozyme suggested a catabolic role in androgen metabolism, involving the inactivation of testosterone in non-androgen target tissues. The abundant expression of 5α-reductase type 1 in the rat liver supported this role as liver enzymes have a known catabolic role in steroid metabolism.

Mutations in the 5α-reductase type 1 gene have not yet been identified in humans however an induced mutation in the mouse 5α-reductase type 1 gene produced male mice indistinguishable from wild-type counterparts. In contrast, female mice that lack the type 1 enzyme exhibit defects in parturition and fecundity (Mahendroo *et al.* 1996; Mahendroo *et al.* 1997). The fecundity defect was caused by an increase in testosterone being converted into toxic oestrogens by the aromatase enzyme at

	Rat/Mouse		Human	
	Type 1	**Type 2**	**Type 1**	**Type 2**
pH optima	6.0 - 8.5	5.0	6.0 - 8.5	5.0
Localisation	Liver, prostate	Testis, vas deferens, epidydymis, prostate	Liver, skin, kidney	Prostate, skin, liver
Inhibited by finasteride ?	✓	✓	X	✓
Consequence of knockout/mutation	Parturition and fecundity defects in females	None identified	None identified	Pseudoher-maphro-ditim

Table 1.2: Summary of properties of 5α-reductase isozymes of rat, mouse and human.

critical times in pregnancy in the mutant mice, revealing an unexpected but essential function of the type 1 isozyme. The parturition defect was traced to a failure of the uterus to synthesise 5α-reduced androgens in late gestation. The seminal vesicle and ventral prostate are more dependent on the production of DHT for maintenance and growth than any other androgen target tissues (George *et al.* 1991). These tissues express nearly equal levels of both isozymes, thus 5α-reductase type 1 may serve as a back-up generator of DHT. The finding that both isozyme mRNAs are induced by androgens in the regenerating prostate supports this hypothesis. These effects implicate the type 1 isozyme as playing an essential role in maintaining a balance between the levels of oestrogens and androgens.

The parturition defects were not worsened if 5α-reductase type 2 was also knocked-out suggesting that the type 1 enzyme has evolved to play an essential role in the female (Mahendroo & Russell 1999). The female rat liver also expresses 10-20 fold more mRNA (Andersson *et al.* 1989) and activity (Yates *et al.* 1958) than does male liver suggesting that 5α-reduction is a more important pathway of hepatic metabolism in females.

1.2.1.4 Regulation of 5α-Reductase

The expression of steroid 5α-reductase enzyme activity is under hormonal control. The best-studied tissue is the rat ventral prostate. Castration of male rats causes a marked regression in the size and weight of the ventral prostate caused by apoptosis of the lumenal epithelial cells (Raff 1992). Regression can be readily reversed or prevented by administration of testis extracts.

The induction of 5α-reductase enzyme activity in the ventral prostate of castrated rats given testosterone is accompanied by a large increase in the mRNA for both the type 1 and type 2 isozymes (Andersson *et al.* 1989; Normington & Russell 1992). When castrated rats were given the 5α-reductase inhibitor finasteride with testosterone, growth of the prostate gland was blocked and induction of 5α-reductase activity and 5α-reductase mRNA by testosterone was prevented. This regimen did

not prevent DHT-mediated increases in either 5α-reductase activity or mRNA implying that DHT is the active androgen that enhances 5α-reductase type 1 mRNA and activity in the ventral prostate (George *et al.* 1991). This so-called feed-forward regulation is contrary to the usual feedback regulation in which the product of an enzyme negatively regulates expression of the gene.

In adult rat testis, testosterone has been shown to negatively regulate the expression of 5α-reductase type 1. This observation was consistent with the hypothesis that a reduction in testicular testosterone leads to an increase in the metabolism of testosterone to the more potent metabolite, DHT (Pratis *et al.* 2003). Thus, when testicular testosterone levels are low and unable to maintain sperm production, amplification of the androgenic stimulus via conversion of testosterone to DHT would enable continued low levels of spermatogenesis. Negative regulation of 5α-reductase type 1 by testosterone has been demonstrated in other tissues such as liver (Yates *et al.* 1958; Lopez-Solache *et al.* 1996) and adrenal cortex (Lephart *et al.* 1991). Conversely, positive regulation of 5α-reductase type 1 by testosterone and dihydrotestosterone has been elucidated in rat liver (Torres & Ortega 2003). Unlike the 5α-reductase type 1 isozyme, the 5α-reductase type 2 isozyme was not regulated by testosterone in the rat testis. This is in contrast to the above observation in the ventral prostate that androgens positively regulate 5α-reductase type 2, suggesting that the factors that regulate the 5α-reductase isozymes may be isozyme and tissue specific.

5α-Reductase enzymes appear to be controlled by products of the pituitary gland as in hypophysectomised female rats, activity of 5α-reductases are dramatically decreased and steroid hormones no longer influence their activity (Denef 1974; Gustafsson *et al.* 1980). On the other hand replacement of an ectopic adult rat pituitary can restore normal 5α-reductase activity in hypophysectomised female rats (Denef 1974). The pituitary hormones responsible for maintaining hepatic 5α-reductase activity in adult female rats remain to be elucidated. Administration of prolactin to hypophysectomised female rats only produced a slight increase in 5α-

reductase activity (Lax *et al.* 1976). Other investigators have reported that luteinising hormone (LH) and follicle-stimulating hormone (FSH) do not influence 5α-reductase activity although the literature is controversial. In rat testis, some studies showed a positive effect of FSH on 5α-reductase type 1 activity (Nayfey *et al.* 1975; Welsh & Wiebe 1976) whereas others did not (Dorrington & Fritz 1975; Murono & Payne 1979). In a more recent study, Pratis et al showed that FSH positively regulates 5α-reductase type 1 and type 2 in rat testis (Pratis *et al.* 2003). Growth hormone (GH) has also been shown to increase 5α-reductase activity in intact and hypophysectomised male rats and in hypophysectomised-gonadectomised male and female rats, (Rumbaugh & Colby 1980; Mode *et al.* 1981)and in isolated hepatocytes (Miller & Colás 1982). In genital skin fibroblasts that exclusively express 5α-reductase type 2, 5α-reductase activity has been shown to be regulated by DHT and insulin-like growth factor –1 (Horton *et al.* 1993), transforming growth factor-β1 and -β2 (Wahe *et al.* 1993) and activin A (Antonipillai *et al.* 1995).

1.2.2 5β-Reductase and 3α-Hydroxysteroid Dehydrogenase

The enzymes 5β-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) are members of the aldo-keto reductase superfamily. The aldo-keto reductases (AKRs) are an enzyme superfamily of NAD(P)(H)-dependent oxidoreductases with a broad spectrum of physiological roles. Typically, members of the superfamily are monomeric $(\alpha/\beta)_8$-barrel proteins, about 320 amino acids in length, which use NAD(P)(H) to metabolise a range of substrates at an active site containing a catalytic tetrad of a tyrosine, a lysine, an aspartate and a histidine (Jez *et al.* 1997).

The aldo and keto groups of substrates are not involved chemically in the reaction catalysed by 5β-reductase, however the enzyme shares a relatively high homology (approximately 50%) with other members of the AKR superfamily. 5β-Reductase catalyses the reduction of all steroid hormones carrying a 4-ene-3-keto group (Berseus 1967). The presence of the 3-keto group is essential, structurally for the 5β-reductase activity. The enzyme is most abundant in liver and is localised in the cytosol (Onishi *et al.* 1991). The best known activity of 5β-reductase is the formation

of biliary acids, namely cholic and chenodeoxycholic acids (Berseus *et al.* 1965). There is a limited amount of literature on this enzyme with respect to glucocorticoid metabolism, however 5β-reduced glucocorticoids have been shown to be decreased in essential hypertension (Iki *et al.* 1994) and in the Syndrome of Apparent Mineralocorticoid Excess (Monder *et al.* 1986). Altered 5β-reduction of glucocorticoids has also been implicated in obesity (Andrew *et al.* 1998).

3α-HSDs work in concert with the 5α- and 5β-reductases to generate the 3α/5α and 3α/5β-tetrahydrosteroids. In this manner they catalyse the second step in the metabolism of all steroid hormones that contain a Δ^4-3-ketosteroid functionality and serve to protect against circulating steroid hormone excess (Penning *et al.* 1986). The most extensively characterised mammalian 3α-HSD is the enzyme from rat liver. The rat appears to express only one known 3α-HSD isoform whereas at least four human isoforms exist (Khanna *et al.* 1995). Hepatic 3α-HSD also plays a critical step in the synthesis of bile acids and is responsible for the production of 5β-cholestane-3α, 7α-diol, which is a committed precursor of bile acids (Danielsson & Sjovall 1975). In steroid target tissues, the production of 3α/5α and 3α/5β-tetrahydrosteroids is not without consequence. In the human prostate, 3α-HSD can regulate the occupancy of the androgen receptor by androgen. It catalyses the interconversion of the potent androgen 5α-DHT to 3α-androstanediol, a weak androgen (Liao *et al.* 1973) and can therefore regulate androgen-dependent growth of this gland (Taurog *et al.* 1975). Evidence exists in the dog prostate that 3α-HSD may work in concert with 5α-reductase to maintain high levels of DHT (Jacobi *et al.* 1977). In brain, 3α-HSD can regulate the occupancy of the GABA$_A$ receptor by converting 5α-dihydroprogesterone into allopregnanolone, a potent allosteric agonist of the GABA$_A$ receptor. In this way, 3α-HSD can control chloride conductance and regulate the anxiolytic and anaesthetic actions of 3α-hydroxysteroids. Thus, 3α-HSDs can regulate the occupancy of both a nuclear receptor and a membrane bound ion-gated channel and may have profound effects on receptor function (Majewska *et al.* 1986). Glucocorticoids have been shown to increase the transcription of the rat 3α-HSD gene. This effect is mediated by glucocorticoids binding to the occupied GR

which can mediate its effects at both a distal and proximal glucocorticoid response element (GRE) on the 5' flanking region of the 3α-HSD gene (Hou *et al.* 1998). The ability of glucocorticoids to up-regulate 3α-HSD gene expression implies that these steroids can regulate their own metabolism.

1.2.3 11β-hydroxysteroid dehydrogenase type 1

11β-hydroxysteroid dehydrogenase type 1(11β-HSD 1) is a low affinity NADP(H)-dependent enzyme and is expressed in a wide range of tissues including the liver, lung, adipose tissue, gonads and the CNS (Krozowski *et al.* 1990). *In vitro,* hepatic 11β-HSD 1 activity is bi-directional, functioning as both a reductase and a dehydrogenase (Monder & Lakshmi 1989). However, in primary hepatocyte cultures and isolated purfused rat liver reductase activity predominates, suggesting that 11β-HSD 1 reactivates intracellular glucocorticoids in intact tissues (Jamieson *et al.* 1995; Jamieson *et al.* 2000). This is supported by observations from 11β-HSD 1 deficient transgenic mice, which cannot metabolise corticosterone from implanted pellets of 11-dehydrocorticosterone following adrenalectomy (Kotelevtsev *et al.* 1997). Therefore, alterations in hepatic 11β-HSD 1 may play an important role in regulating glucocorticoid-mediated effects. Indeed, transgenic deficiency of 11β-HSD 1 attenuates activation of glucocorticoid induction of gluconeogenic enzymes in fasting animals (Kotelevtsev *et al.* 1997), and inhibition of 11β-HSD 1 with carbenoxolone improves whole body insulin sensitivity in man (Walker *et al.* 1995).

11β-HSD 1 is also expressed in adipose tissue where it functions predominantly as a reductase (Napolitano *et al.* 1998).(Bujalska *et al.* 1999)11β-HSD 1 activity is higher in omental fat compared with subcutaneous fat and can be enhanced by exposure to both glucocorticoids and insulin. It has been suggested that 11β-HSD 1 plays a role in the development of central obesity in situations of glucocorticoid excess (Bujalska *et al.* 1999). Indeed, adipose tissue from obese humans has increased 11β-HSD 1 activity (Rask *et al.* 2002). Also, transgenic mice over-expressing 11β-HSD 1 selectively in adipose tissue develop central obesity which is exaggerated on a high fat-diet (Masuzaki *et al.* 2001).

The brain is a key glucocorticoid target and glucocorticoids regulate developmental, metabolic and neurotransmitter functions in the brain via both glucocorticoid and mineralocorticoid receptors (McEwen *et al.* 1986). Glucocorticoids bind to mineralocorticoid receptors under normal circumstances but when glucocorticoid levels increase e.g. during stress or at the diurnal peak they will bind to classical glucocorticoid receptors (Reul & De Kloet 1985). 11β-HSD 1 is highly expressed in hippocampal neurones and other regions of the CNS (Moisan *et al.* 1992; Sakai *et al.* 1992) where it acts as a reductase to amplify glucocorticoid action (Rajan *et al.* 1995). Studies in 11β-HSD deficient mice support the notion that 11β-HSD 1 in the brain has deleterious effects on cognitive function (Yau *et al.* 2001) and may influence negative feedback regulation of the HPA axis by glucocorticoids (Kotelevtsev *et al.* 1997; Harris *et al.* 2001). Impaired activity of 11β-HSD 1 in the periphery could also have an effect on the HPA axis by increasing the metabolic clearance rate of active glucocorticoid and thus increasing drive to the HPA (Phillipou *et al.* 1996).

The expression of 11β-HSD 1 is sexually dimorphic at least in the rat, with males having higher hepatic and renal enzyme activity than females (Lax *et al.* 1978; Smith & Funder 1991). This sexual dimorphism is due to differences in growth hormone secretion patterns which are regulated by oestradiol (Low *et al.* 1994a). Studies of urinary steroid metabolites in man have not been conclusive with increased, normal and decreased 11β-HSD activity reported in women compared with men (Andrew *et al.* 1998; Stewart *et al.* 1999; Finken *et al.* 1999).

Glucocorticoids increase 11β-HSD 1 expression and activity in both human fibroblast cell cultures and rat vascular smooth muscle cells and hepatocytes *in vitro* (Hammami & Siiteri 1991; Takeda *et al.* 1994; Jamieson *et al.* 1995) *In vivo*, glucocorticoids induce 11β-HSD 1 in liver and hippocampus, whist adrenalectomy decreases hepatic 11β-HSD 1 mRNA and activity (Walker *et al.* 1994; Low *et al.* 1994b).

1.3 The role of glucocorticoids in obesity

The impetus for most studies of glucocorticoids and obesity is the similarities between hypercortisolemic states (e.g Cushing's syndrome) and patients with idiopathic obesity. Idiopathic obesity is associated with elevated urinary free cortisol excretion (Marin *et al.* 1992), impaired suppression of plasma cortisol by dexamethasone (Ljung *et al.* 1996) and enhanced sensitivity of plasma cortisol to exogenous ACTH (Hautanen & Adlercreutz 1993). These abnormalities of the HPA axis are more pronounced and prevalent when the fat is distributed in a central or visceral pattern in both men and women (Pasquali *et al.* 1993). The mechanism of activation of the HPA-axis has not been established. Despite an increase in glucocorticoid excretion rate indicated by an increase in urinary glucocorticoid metabolites (Gray *et al.* 1956; Migeon *et al.* 1963), plasma cortisol levels in obese humans tend to be normal or even low (Migeon *et al.* 1963; Ljung *et al.* 1996; Rosmond *et al.* 1998) implying that the rate of removal of cortisol from the circulation must be increased. This has been confirmed by *in vitro* tracer studies (Strain *et al.* 1980).(Lottenberg *et al.* 1998)

Several studies in man have looked at the levels of specific urinary metabolites in obesity. These showed that 5α-reductase activity may be enhanced in obesity, (Andrew *et al.* 1998; Fraser *et al.* 1999) and that 11β-hydroxysteroid dehydrogenase activity is altered with impaired regeneration of cortisol from cortisone (Stewart *et al.* 1999; Rask *et al.* 2001). The combination of increased cortisol inactivation and impaired liver regeneration of cortisol may lead to a compensatory activation of the HPA axis via loss of the negative feedback signal, to maintain circulating cortisol levels.

Evidence for linking glucocorticoids and obesity has also come from animal studies as several models of obesity have been characterised by increased secretion of glucocorticoids e.g. the ob/ob mouse and the Zucker rat. The obese Zucker rat (*fa/fa*) has frequently been used as an animal model of obesity since its description in the early 1960s (Zucker & Zucker 1961). The phenotypic expression of obesity in the obese Zucker rat is inherited as an autosomal recessive mutation in the leptin

receptor gene (Chua *et al.* 1996). The result of this mutation includes numerous metabolic and endocrine abnormalities (Bray *et al.* 1979) and obese Zucker rats are hyperphagic (Bray & York 1972) and have adrenal hypertrophy (Bestetti *et al.* 1990; Walker *et al.* 1992). The obesity in these animal models has been demonstrated to be glucocorticoid dependent because many of the metabolic and endocrine impairments are normalised or attenuated after adrenalectomy (Freedman *et al.* 1986) or administration of the glucocorticoid receptor antagonist RU486 (Langley & York 1990). Corticosterone replacement reverses the effects of adrenalectomy (Castonguay *et al.* 1986; Freedman *et al.* 1986; Fletcher 1986).

Conflicting results have been reported on the sensitivity of the HPA-axis to the elevated circulating glucocorticoids in obese Zucker rats with reports of normal (Guillaume-Gentil *et al.* 1990), increased (Bestetti *et al.* 1990; Plotsky *et al.* 1992) and decreased (Castonguay *et al.* 1986; Routh *et al.* 1990) sensitivity of the CRH/ACTH secretory system.

It is evident that the nature of and mechanisms underlying the abnormalities of the HPA axis in obesity are unclear and therefore recent studies have focused on factors that control tissue glucocorticoid concentrations.

Access of glucocorticoids to receptors is controlled at a tissue level by the 11β-hydroxysteroid dehydrogenases, which interconvert active and inactive glucocorticoids. Tissue-specific alterations in 11β-HSD1 have been reported in obesity. 11β-HSD1 activity is higher in human visceral compared with subcutaneous adipose tissues (Bujalska *et al.* 1997), and reactivation of cortisol from cortisone is increased selectively in adipose tissue (Rask *et al.* 2001) and decreased in liver of obese humans (Stewart *et al.* 1999; Rask *et al.* 2001). Similar tissue-specific differences were reported in obese Zucker rats (Livingstone *et al.* 2000).

Altered glucocorticoid metabolism by the 11β-hydroxysteroid dehydrogenases in obesity will therefore influence local tissue concentrations of active glucocorticoids independently of circulating glucocorticoid levels.

It is not known if 5α-reductase activity in specific tissues is altered in obesity nor if alterations in 5α-reductase activity can change glucocorticoid availability and receptor activation within tissues. 5α-reductase type-1 and -2 are both capable of metabolising glucocorticoids (Normington & Russell 1992). It is unclear whether one or both of these isozymes are responsible for glucocorticoid metabolism *in vivo* as previous studies investigating glucocorticoid metabolism often report on urinary steroid metabolites and do not identify the isozyme or tissue involved. 5α-Reductase is also expressed in adipose tissue and thus this tissue may be of increased importance as a metabolic site in obesity (Perel *et al.* 1986; Zyirek *et al.* 1987).

1.4 Aims of this thesis

The primary aim of this thesis was to study glucocorticoid metabolism by A-ring reductases in relation to glucocorticoid receptor activation and obesity. The following list of aims was addressed:

- To study localisation and activity of A-ring reductases in glucocorticoid target tissues, necessitating establishment of new methodology.

- To assess whether glucocorticoid receptor binding of A-ring reduced glucocorticoids influences glucocorticoid receptor activation.

- To quantify A-ring reductase expression and activity in glucocorticoid target tissues in obese Zucker rats.

- To determine whether glucocorticoid receptor binding of corticosterone and its A-ring reduced metabolites is altered in obesity.

Chapter 2

Materials and Methods

2.1 Materials

Unless otherwise stated all chemicals, reagents and drugs were purchased from Sigma, Poole, UK. All HPLC grade solvents were purchased from Rathburn Chemicals, Walkerburn, UK. All epi-steroid standards were from Steraloids, Newport, USA. All enzymes for molecular biology were purchased from Promega, Southampton, UK. All radioactively labelled steroids and radioactive isotopes were purchased from Amersham, Little Chalfont, UK. Sources other than these are indicated.

2.2 Buffers and Solutions

Alkaline SDS solution: 0.2M NaOH, 1% w/v SDS

Borate Buffer: 8.25g boric acid, 2.7g NaOH, 3.5ml conc. HCl and 5g BSA made up to 1litre with distilled water, pH 7.4. Stored at -20°C and thawed at room temperature immediately before use.

Box Buffer: 20ml 20xSSC (saline sodium citrate) buffer, 50ml deionised formamide made up to 100ml in DEPC-treated water (see below).

Caesium Chloride/TE solution: 100g CsCl dissolved in 100ml TE buffer (see below).

Calcium-free Buffer: Krebs buffer (see below) without $CaCl_2$.

DEPC-treated water: Distilled water mixed with diethylpyrocarbonate (DEPC; 300µl/ 100 ml), shaken and left for 1-24 hours prior to autoclaving.

Deionised formamide: 150ml Formamide mixed with 15g Amberlite ion exchange resin (MB-6113) (BDH, Lutterworth, UK) for 1 hour, filtered twice to remove Amberlite and stored at -20°C.

1Kb DNA ladder: 20µg 1Kb ladder (Life Technologies, Paisley, UK), in 200µl distilled water with 10% (v/v) loading buffer.

0.5M EDTA (pH 8.0): 800ml water was added to 186.1g $Na_2EDTA.2H_2O$. pH was adjusted to 8.0 with NaOH and the volume adjusted to 1000ml.

GTE: 50mM glucose, 25mM tris (hydroxymethyl)-aminomethane (tris), 10mM EDTA, pH8.0

Hepatocyte incubation buffer: As Krebs buffer (see below) but with 120mM NaCl and 5mM glucose, 10mM lactate, 2mM glutamine, 1mM pyruvate and BSA (RIA grade, Fraction V) (2g/100ml buffer). Dialysed overnight with dialysis tubing, size 1-8/32" (Medicell International Ltd, London, UK) against a large volume (10L) of Krebs buffer and stored at 4°C.

2xHybridisation buffer: 1.2M NaCl, 20mM Tris-HCl, 2x Denhardts, 2mM K_2-EDTA, 0.2mg salmon sperm DNA, 0.2mg yeast tRNA and 2g dextran sulphate made up to 10ml in DEPC-treated water, stored at -20°C.

Krebs'-Ringer Bicarbonate (KRB) Buffer: 118mM NaCl, 3.8mM KCl, 1.19mM KH_2PO_4, 2.54mM $CaCl_2$, 1.19mM $MgSO_4$, 25mM $NaHCO_3$ in distilled water, pH 7.4. Stored at 4°C and supplemented with 0.2% w/v glucose immediately before use.

LB agar: Luria-Bertoni broth with 15g agar per litre broth added before autoclaving.

Loading buffer: 40% sucrose w/ v, 0.25% bromophenol blue (w/v) in distilled water.

Luria-Bertoni broth: 10g bactotryptone, 5g bacto yeast extract, 5g NaCl made up to 1 litre with distilled water and autoclaved immediately.

Luciferase assay buffer: 40mM Tricine, 67mM DTT, 0.2mM $Na_2EDTA.2H_2O$, 2mM $MgSO_4$, 0,25mM coenzyme A, pH 7.8.

Lysis buffer: 25mm Tris phosphate pH 8.0, 2mM DTT, 1% Triton X-100, 10% glycerol.

Molybdate Buffer: 10mM Tris-HCl, 2mM DTT, 1.5mM EDTA, 0.1M sodium molybdate, 10% glycerol, pH 7.2. Stored at 4°C.

10x MOPS buffer: 0.2M 3-(N-morpholino) propanesulfonic acid, 50mM Na acetate, 5mM EDTA, pH 7.0.Autoclaved before use.

4% Paraformaldehyde in 0.1M phosphate buffer: 20mM NaH_2PO_4, 80mM Na_2HPO_4 in 1l DEPC-treated water, heated to 80°C prior to addition of 40g paraformaldehyde. Stirred for 1 hour to dissolve and stored at 4°C.

Phosphate Buffer: 0.2M NaH_2PO_4 0.6M Na_2HPO_4, 5mM EDTA. Autoclaved before use.

Phosphate buffered Saline (PBS): 0.1M phosphate buffer with 137mM NaCl, 2.7mM KCl in distilled water, pH 7.4, autoclaved before use.

5M Potassium Acetate: 60ml of 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml water.

2xPre-hybridisation buffer: 1.2M NaCl, 20mM Tris-HCl, 2x Denhardt's, 2mM K_2-EDTA, 10mg salmon sperm DNA, 0.2mg yeast tRNA made up to 10ml in DEPC-treated water, stored at -20°C.

1x Restriction Digest buffer: 500mM NaCl, 500mm Tris-HCl pH 8.0, 110mM $MgCl_2$, 60mM β-mercaptoethanol, 1mg/ml bovine serum albumin.

10x Reverse Transcription buffer: 0.1M Tris-HCl, 0.5M KCl, 1% Triton X (ready mixed from Promega).

RNase Buffer: 0.5M NaCl, 10mM Tris-HCl, 1mM K_2-EDTA in 10ml distilled water.

20x Saline Sodium Citrate buffer (SSC): 3M NaCl, 0.3M Na citrate in 1l DEPC-treated water, pH 7.0, autoclaved before use.

Sodium Phosphate Buffer: 40mM NaH_2PO_4, 0.32M sucrose, 1mM DTT.

Sucrose buffer: 250mM sucrose, 10mM Hepes, pH7.5.

T4 DNA ligase buffer (1x): 30mM Tris-HCl (pH 7.8), 10mM $MgCl_2$ 10mM DTT, 1mM ATP (ready mixed from Promega).

10xTBE buffer: 0.9M TRIZMA base, 0.9M Boric acid, 20mM K_2-EDTA in distilled water.

TE buffer: 10mM Tris-HCl, 1mM EDTA, pH 7.5, autoclaved before use.

Thermophilic DNA polymerase 10x reaction buffer: 500mM KCl, 100mM Tris-HCl and 1% Triton X (ready mixed from Promega).

5xTranscription optimised buffer: 200mM Tris-HCl, 50mM NaCl, 30mM $MgCl_2$, and 10mM spermidine (ready mixed from Promega).

Wash Buffer One: 1x SSC buffer, 0.1% sodium dodecyl sulphate (SDS) made with DEPC-treated water.

Wash Buffer Two: 0.3x SSC buffer, 0.1% SDS made with DEPC treated water.

2.3 Animal Maintenance

Male lean and obese Zucker rats (Harlan Orlac, Bicester, UK) and male Wistar rats (Charles River, Kent, UK) were obtained at 4-6 weeks of age. Animals were maintained under controlled conditions of light (lights on 0800 h – 2000 h) and temperature (21-22°C), and allowed free access to standard chow (Special Diet Services, Witham, UK) and drinking water.

2.3.1 Surgery

The surgical procedures were carried out by Dr. CJ Kenyon of the Molecular Medicine Centre, Western General Hospital. Six week-old lean and obese Zucker rats were anaesthetised with 4% halothane and either bilaterally adrenalectomised (ADX) or sham-operated through dorsal incisions and the incisions closed with staples. All rats were maintained on 0.9% saline as drinking water to allow the ADX rats to maintain their electrolyte balance.

2.4 Maintenance of cell lines

HeLa (a human cervical cancer cell line) and H4IIE, 2S-FAZA (rat hepatoma cell lines) and HepG2 (a human hepatoma cell line) cells were maintained at 37°C with 5% CO_2, 95% O_2 in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, penicillin /streptomycin (100μg/ml) and L-glutamine (2mM). A summary table of cell lines used is detailed in Table 2.1. Cells were routinely split 1:4 when confluent. To harvest and split cells, they were washed with serum-free DMEM (8ml), then treated with trypsin/EDTA in HBSS (1.5ml) for 1-2min to release the cells from the flask surface, then resuspended in DMEM (6.5ml) with serum. Cells were then diluted as appropriate in DMEM with serum.

Cell line	Animal	Cell type	Reference
H4IIE	Rat	Hepatoma	(Pitot *et al.* 1964)
2S-FAZA	Rat	Hepatoma	(Voice *et al.* 1996)
HEP-G2	Human	Hepatoma	(Knowles & Aden 1980)
HeLa	Human	Cervical adenocarcinoma	(Gey *et al.* 1952)

Table 2.1: Cell lines summary table.

2.5 Enzymology

2.5.1 Preparation of tissue homogenates

Tissues were frozen routinely on dry ice directly after dissection from the animal and stored at -80°C until use. Tissues were roughly dissected while frozen and 0.5-1g of tissue was homogenised in sucrose buffer (4-5ml) with an Ystral mechanical homogeniser (Scientific Instruments Centre, Liverpool, UK). Homogenates were centrifuged at 1000g at 4°C for 10min to allow any small amounts of unhomogenised tissue to sink to the bottom and the supernatant was removed.

2.5.2 Sub-cellular fractionation of liver

Sub-cellular fractions of liver were prepared by differential and sucrose gradient centrifugation of the homogenate as described by Fleischer and Kervina (Fleischer & Kervina 1974). Briefly, rat liver was homogenised in 5 volumes of sucrose buffer. The homogenate was then centrifuged at 1000g x 10 min and the resulting pellet contained the nuclear fraction and plasma membrane. The supernatant was filtered through several layers of cheesecloth and centrifuged at 25000g x 10 min in a Beckman J20 centrifuge. The residue from this second spin is enriched in mitochondria. The supernatant was once again decanted and centifuged at 34000g x 30 min in a Beckman J20 centrifuge. The pellet containing heavy microsomes was resuspended in 0.25M sucrose (2ml) and the supernatant centrifuged at 124000g x 60 min in a Beckman Optima TLX ultracentrifuge . The resulting supernatant was kept

as cytosol and the microsomal pellet resuspended in 0.25M sucrose (2ml). The fractionation process is summarised in Figure 2.1.

2.5.3 Protein Estimation

The protein concentration of tissues and sub-cellular fractions were determined colorimetrically using a Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, UK). A range of protein standards (0.1–1.2mg/ml) was prepared in duplicate in distilled water from the provided protein standard (bovine serum albumin). Protein assay dye reagent was diluted 1:4 in distilled water and filtered through Whatman No. 1 filter paper prior to use. Diluted protein assay dye reagent (1.96 ml) was added to protein standard (40µl) or appropriately diluted tissue homogenate in a borosilicate glass tube, vortexed to mix and left at room temperature for 15 min–1 hour to allow colour development. Absorbance of samples at λ595nm was measured using a Shimadzu UV/ visible recording spectrophotometer and the concentration of protein in each sample was estimated from the standard curve. An example of a standard curve is shown in Figure 2.4.

2.5.4 *In vitro* assay of 5α-reductase activity

The assay conditions to measure the bioactivity of 5α-reductase, 5β-reductase and 3α-hydroxysteroid dehydrogenase were determined. The starting point for the development of the assay was an assay used previously to measure 5α-reduction of testosterone and androstenedione in tissues and cells (Mahendroo *et al.* 2001; Reichert *et al.* 2001).

In our assay, we attempted to quantify the enzymes in liver by measuring the conversion of $[^3H]_4$-corticosterone to $[^3H]_4$-5α-dihydro- and tetrahydro-corticosterone and $[^3H]_4$-5β-dihydro- and tetrahydro-corticosterone. Initially liver homogenates were incubated in duplicate at 37°C in sodium phosphate buffer with NADPH (2mM) and $[^3H]_4$-corticosterone (50nM) in a total volume of 250µl for 4h. The reaction was stopped with 10 volumes of HPLC grade ethyl acetate. The organic phase was removed to a fresh tube and solvent evaporated under oxygen free
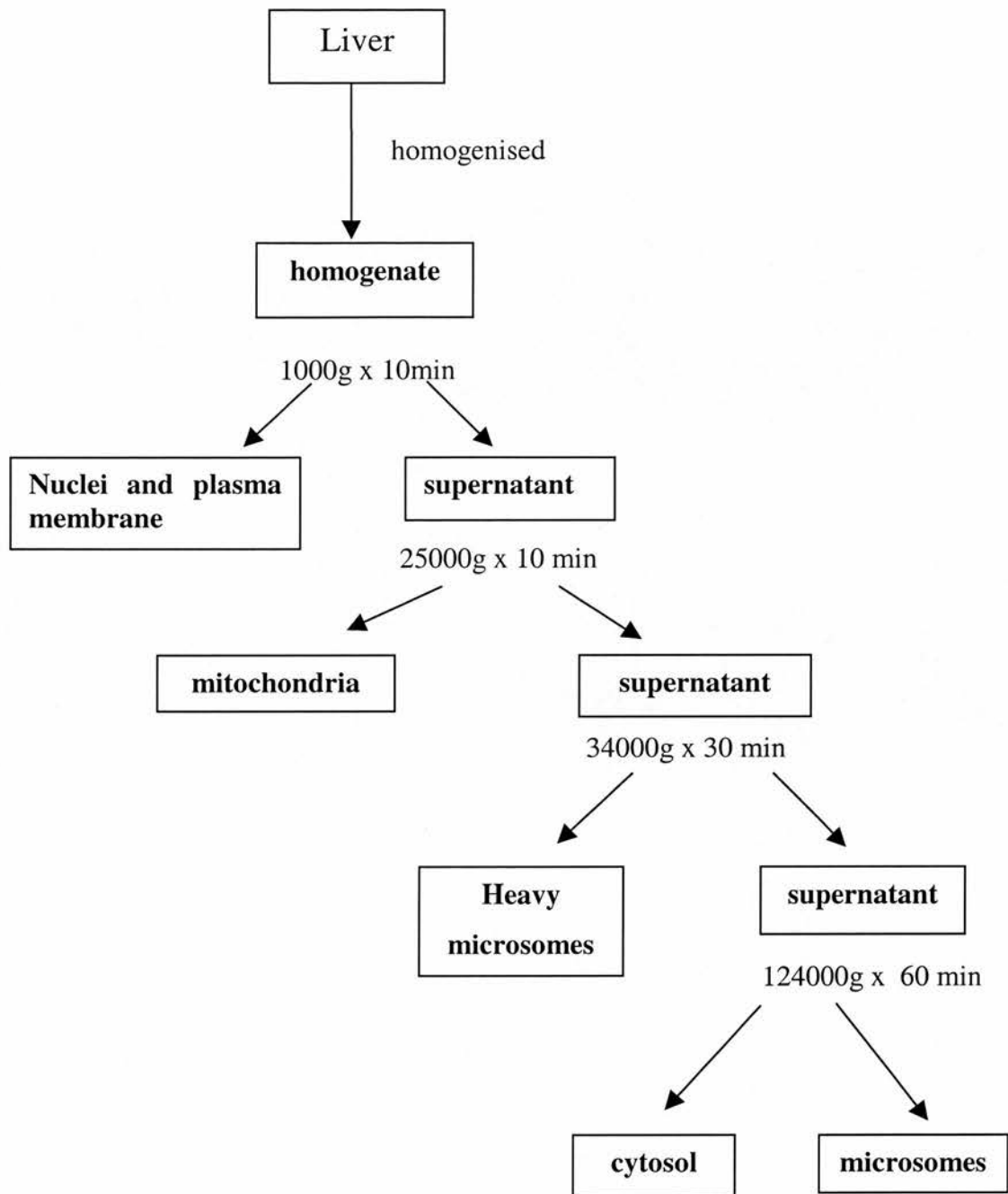
Figure 2.1 : Flow diagram for the subcellular fractionation of rat liver

nitrogen at 50°C. Steroid extracts were-re-suspended in the mobile phase described above and injected into the HPLC system under the optimised conditions described in chapter 2.5.6.

Under these conditions no stable 5α-reductase activity could be detected therefore a series of different tissue preparations were investigated.

Firstly, isolated hepatocytses (2 million cells /ml) were prepared as described in 2.11.1.3 and incubated with NADPH (2mM) and [^{3}H]$_4$-corticosterone(100mM) at 37°C in sodium phospate buffer for 1,2,4,6,and 24h. 5α-reductase bioactivity was observed in these isolated hepatocytes, however this was not very reliable as 5α-reductase activity could not always be measured in this preparation. On many occasions, corticosterone was converted solely to 11-dehydrocorticosterone (A) indicating that the hepatocytes were no longer viable.

5α-Reductase is a microsomal enzyme and therefore we tried the assay under the same initial conditions in microsomes and different sub-cellular fractions of liver including the nuclear fraction, mitochondria, heavy microsomes and cytosol. These were prepared as described in 2.5.2. All assays were carried out with two different tissue concentrations (20μg and 200μg) and aliquots were removed at 10,30,60,120,240 and 360 minutes. The only activity that was detected in any of these preparations was 5β-reductase activity in cytosol which would be expected as it is a cytosolic enzyme. As a positive control, the assay was performed in prostate using corticosterone and testosterone as substrates. 5α-Reduction of testosterone but not corticosterone could be measured in prostate.

This time-course was repeated using Hep-G2, 2S-FAZA, fresh liver, fat and adrenal but still no 5α-reductase activity could be measured.

To try to preserve 5α-reductase activity, buffers used successfully to synthesise reduced metabolites of aldosterone were used. The first buffer included soybean trypsin inhibitor to prevent degradation of the enzyme (Mcdermott *et al.* 1985) and in

the second buffer an NADPH generating system was used as a constituent of the incubation buffer to generate more co-factor for the enzyme (Morris *et al.* 1982).

These buffers were prepared as follows.

1) Sucrose 0.25M, potassium phosphate buffer pH 7.2 (100mM), DTT (5mM) soybean trypsin inhibitor (50µg/ml)

2) Tris-HCl buffer pH 7.4 (50mM), glucose-6-phosphate (50mM), glucose-6-phosphate dehydrogenase (25 units per ml), NADPH (500µM), $MgCl_2$ (5mM).

None of these modifications resulted in detection of 5α-reductase metabolism of corticosterone. A summary of the conditions used is given in Table 2.2.

2.5.5 *In vitro* assay of 5β-reductase activity

Preliminary studies were carried out in order to determine the protein concentrations required to ensure that the percentage conversion of $[^3H]_4$-corticosterone to $[^3H]_4$-3α,5β tetrahydrocorticosterone by each tissue was between 10 and 40% after 240min incubation.

Cytosol at the appropriate concentration (40mg/ml protein) was prepared in duplicate in sodium phosphate buffer, with NADPH (2mM) and $[^3H]_4$-corticosterone (50nM) in a total volume of 250µl. Tissue blanks were prepared in duplicate in sodium phosphate buffer with NADPH and $[^3H]_4$-corticosterone in the absence of cytosol. Samples not containing co-factor were prepared in duplicate in sodium phosphate buffer with $[^3H]_4$-corticosterone and cytosol in the absence of NADPH to ensure the reaction monitored was driven by the presence of NADPH. Samples not containing protein were included to monitor the stability of the substrate under the incubation conditions. Samples were incubated at 37°C for 240min. Following incubation, the reaction was stopped by the addition of ethyl acetate (10 volumes) to each tube, and tubes were vortexed. The organic phase (containing steroids) was removed to a fresh tube and solvent evaporated under oxygen free nitrogen at 50°C.

When possible, where results between different groups of animals were to be compared, incubations were carried out at the same time.

Tissue/Cell Preparation	5α-Reductase	5β-reductase activity
Frozen liver homogenate	X	✓
Fresh liver homogenate	X	✓
Fresh liver slices	X	✓
Frozen liver homogenate in Krebs buffer	X	✓
Frozen liver homogenate with NADPH-generating system	X	✓
Microsomes	X	X
Cytosol	X	✓
Nuclear Fraction	X	X
Mitochondria	X	X
Heavy Microsomes	X	X
Isolated Hepatocytes	✓	X
HEP-G2 cells	X	X
2S-FAZA cells	X	X
H4IIE cells	✓	✓
Kidney	X	X
Fat	X	X
Prostate with testosterone	✓	X
Prostate with corticosterone	X	X

Table 2.2: Determination of 5α-reductase and 5β-reductase bioactivity

Table shows different conditions examined in order to measure 5α-reductase and 5β-reductase bioactivity.

$$y = 0.3421 + 0.2893$$
$$R^2 = 0.9943$$
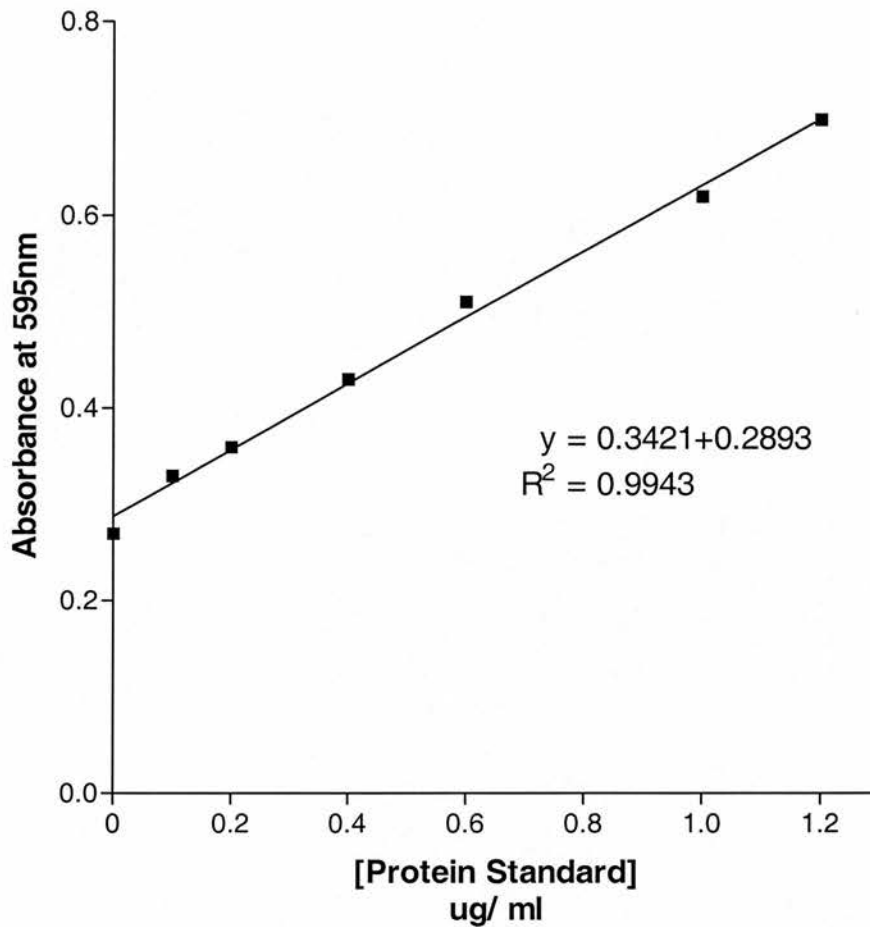
Figure 2.2: Protein Assay Standard Curve

A Bio-Rad protein assay kit was used to colorimetrically determine the protein concentration of tissue homogenates. Absorbance of protein standards of known concentrations (0.1–1.2mg/ ml) at λ595nm was measured using a Shimadzu UV/ visible recording spectrophotometer. A standard curve was produced allowing subsequent estimation of protein concentrations in each tissue homogenate. Samples were prepared in a dilution allowing estimation in the middle range of the curve.

2.5.6 High - Performance Liquid Chromatography

2.5.6.1 Preliminary work

An existing protocol for the measurement of A-ring reductase activity in rat liver using corticosterone as a substrate was not established in our laboratory. In order to measure metabolites of corticosterone by HPLC, separation conditions had to be optimised.

The high-performance liquid chromatography (HPLC) system comprised an auto-sampler and mobile phase pump (Waters, UK), a Symmetry Shield C8 column (15cm diameter, 3.5μM pore size, (Waters, UK), a radioactivity monitor linked to a scintillation fluid pump (Berthold, UK), a dual wavelength absorbance UV detector (Waters, UK) and a column heater/chiller (CIL, France). The system was controlled by the Winflow computer programme (JMBS Developments, France).

Chromatography is a separation technique where component molecules in a sample mixture are transported by a mobile phase through a column of stationary phase material. In our HPLC system, the mobile phase is a liquid solvent system consisting of different percentages of water, acetonitrile and methanol. The solution properties of the liquid mobile phase compete with the retention forces of the column to determine the distribution ratio and hence retention time of the molecules in a sample. The rate of migration of component molecules through the column depends on the distribution of the molecules between the mobile phase and the column. The factors that influence the distribution and therefore retention times are composition of the mobile phase, type and properties of the stationary phase, column temperature and the hydrophobicity/ hydrophilicity of the analyte. Analytes eluting from the column are then detected by a number of characteristics, in this thesis by ultra-violet (UV) and radiochemical detection.

Non-radioactive standards were employed to optimise resolution conditions with an on-line dual wavelength absorbance UV detector. The wavelength of UV detection was determined by the structural characteristics of the analytes. Corticosterone, 11-dehydrocorticosterone and other metabolites with an ene-one moiety in the A-ring of

their steroid structure absorb UV light maximally at around 240-244nm. However this feature is not present in the tetrahydro-metabolites leaving only C-O bonds absorbing at 195nm. This made it necessary to ensure that the composition of the mobile phase was comprised of low UV cut-off solvents. When selecting the solvents for a HPLC mobile phase, the overall solvent strength is adjusted to give a suitable retention by mixing together one weakly eluting solvent with one or more strongly eluting solvents. In reversed-phase HPLC, the weakest solvent and therefore the most commonly used is water. The other solvents are usually chosen from methanol, acetonitrile or tetrahydrofuran. All three of these solvents have low viscosity, a reasonably low toxicity level and a low UV cut-off. Acetonitrile has the lowest UV cut-off all of these, however still has a strong absorbance at this short wavelength and therefore adds to the level of background detected.

Various mobile phases containing different percentages of HPLC grade water, acetonitrile and methanol at different flow rates and column temperatures were investigated to aid resolution of the reduced metabolites.

Our aim was to separate all of the steroids in Table 2.1. Our initial chromatography conditions were as follows based on an existing protocol to separate corticosterone and 11-dehydrocorticosterone only.

- Mobile Phase : water (65%), acetonitrile (10%), methanol (25%)
- Flow rate : 1.5ml/min
- Column temperature : 30°C

These conditions successfully separated most of the steroids, however the most important 5α - and 5β-isomers were not separated. These steroids are stereoisomers and are very similar structurally to each other. The 5α-reduced glucocorticoids are similar in structure to the parent compound corticosterone, complicating the separation process.

Steroid	Retention Time (min)	Resolved
Corticosterone	34.88	✓
11-dehydrocorticosterone (A)	21.98	✓
6α-hydroxycorticosterone	3.17	✓
6β- hydroxycorticosterone	4.28	✓
20β-dihydrocorticosterone	24.33	✓
5α-tetrahydrocorticosterone (5αTHB)	50.18	X
5β-tetrahydrocorticosterone (5βDHB)	51.75	X
5α-dihydrocorticosterone (5αDHB)	57.88	X
5β-dihydrocorticosterone (5βDHB)	57.88	X
Dihydro-11-dehydrocorticosterone (DHA)	49.87	✓
Tetrahydro-11-dehydrocorticosterone (THA)	62.73	✓

Table 2.3: Initial retention times for separation of corticosterone and its metabolites.

Table shows retention times of corticosterone and its metabolites under the following chromatographic conditions : mobile phase : water (65%), acetonitrile (10%), methanol (25%), flow rate 1.5ml/min, column temperature 30°C.

A mobile phase consisting of water (65%), acetonitrile (20%) and methanol (15%) at a flow rate of 0.7ml/min and a column temperature of 40°C allowed resolution of the dihydro-metabolites (Figure 2.3).

A mobile phase of water (60%), acetonitrile (15%) and methanol (25%) at a flow rate of 1.2ml/min and a column temperature of 40°C separated the tetrahydro-metabolites from corticosterone but the retention times for 5α- and 5β-THB were exactly the same as each other (Figure 2.4. (a)). To try to increase the resolution of the tetrahydro-metabolites the flow-rate of the mobile phase was slowed to 0.7ml/min and the column temperature decreased to 10°C. Decreasing the column temperature increases the mobile phase viscosity giving a decreased rate of mass transfer and decreased solubility of the metabolites. The tetrahydo-metabolites maintain their shape and become more rigid exacerbating the small differences in their structures resulting in increased separation with only a 25% overlap between the two stereoisomers, however the run-times were very long (Figure 2.4 (d)).

A mobile-phase modifier, β-cyclodextrin was then added to the mobile phase as previous literature recommended its usefulness in the separation of mixtures of chiral compounds at low temperatures. Cyclodextrins are cyclic chiral carbohydrates composed of six, seven or eight glucopyranose units. The monomers are configured such that the cyclodextrin resembles a hollow truncated cone or barrel-like cavity. The internal lining is essentially hydrophilic composed of methylene and oxygens of the 1,4-glycosidic bonds. When used with reverse phase solvents these chiral-cavity media act by allowing selective occlusion or intercalation of one stereoisomer into chiral cavities in the matrix of the phase, therefore exaggerating the tiny differences between the steroid structures. Addition of β-cyclodextrin to a final concentration of 2mM separated 5α- and 5β-THB by 10 minutes and halved the run-time however the retention time for corticosterone was exactly the same as 5αTHB. Repetition of the same run but with a final concentration of 1mM β-cyclodextrin in the mobile phase resulted in complete separation of the peaks with at least three minutes between each of the steroid retention times (Figure 2.5).
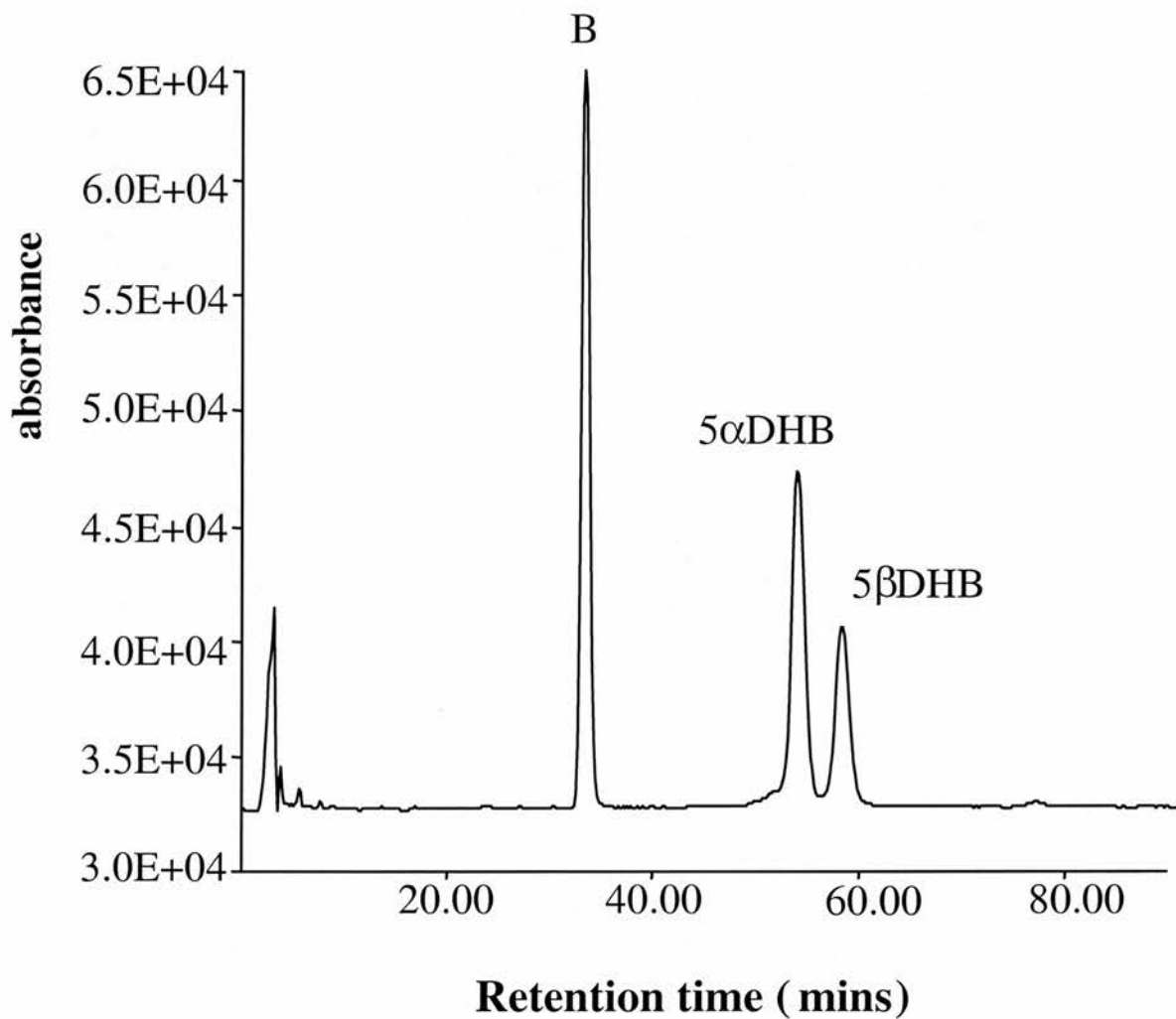
Figure 2.3: Separation of 5α- and 5β-dihydro-metabolites of corticosterone by HPLC

Figure shows separation of 5α- and 5β-dihydro-metabolites of corticosterone by HPLC under the following chromatographic conditions: mobile phase water (65%), acetonitrile (20%), methanol (15%), flow rate: 0.7ml/min, column temperature: 40°C.
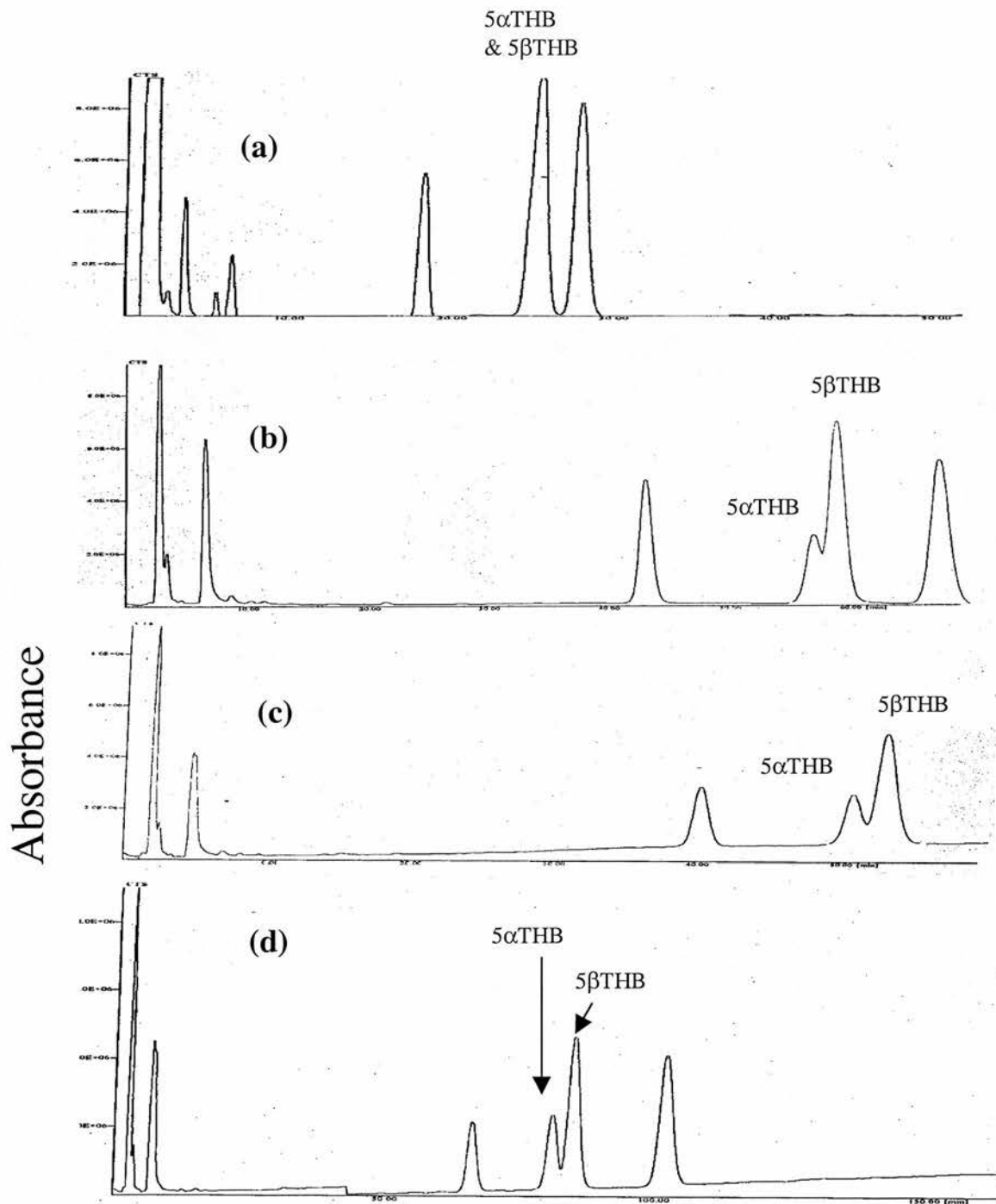
Figure 2.4: Separation of 5α and 5β--tetrahydro-metabolites of corticosterone by HPLC

Figure shows chromatograms of increased separation of 5α-reduced steroids with decreasing temperature and flow rate. The mobile phase for each chromatogram was water; 60%, acetonitrile; 15%, methanol; 25%. Panel (a) flow rate = 1.2ml/min, temp = 40°C. Panel (b) flow rate = 0.7ml/min, temp = 25°C. Panel (c) flow rate = 0.7ml/min, temp = 15°C. Panel (d) flow rate = 0.7ml/min, temp = 10°C
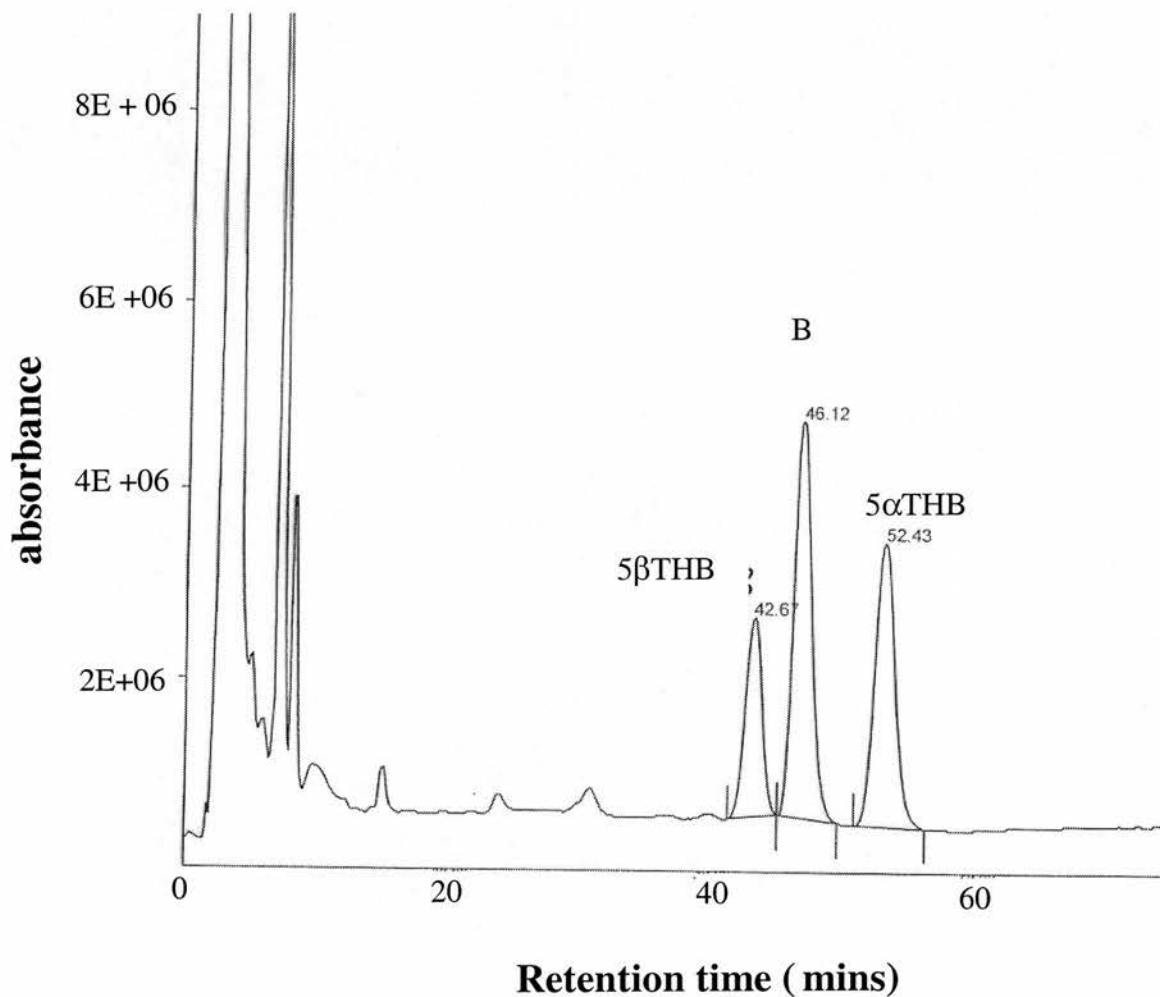
Figure 2.5: Separation of 5α- and 5β- tetrahydro metabolites of corticosterone with β-cyclodextrin

Figure shows chromatogram of separated 5αTHB, 5βTHB and corticosterone UV standards by HPLC upon addition of β-cyclodextrin (1mM) to the mobile phase (water 60%: acetonitrile 15%: methanol 25%), 0.7ml/min, 10°C

2.5.6.2 HPLC conditions for *in vitro* 5β-reductase assay

Steroid extracts from single time point experiments were re-suspended in mobile phase 200μl (60% water, 15% acetonitrile, 25% methanol) containing β-cyclodextrin (1mM). An aliquot (200μl) of each sample was injected into the HPLC system. The flow rate of the mobile phase was 0.7ml/ min and the flow rate of the scintillant (Quicksafe Flow 2; Zinsser, UK) was 1.4ml/ min to achieve optimal mixing and counting efficiency. The column temperature was maintained at 10°C to improve chromatography and maintain stability of retention times. Non-radioactive standards were injected at the start of each batch of samples to confirm peak identity by UV detection (λ 195nm). The approximate retention times for $[^3H]_4$-corticosterone and $[^3H]_4$-3α,5β- tetrahydrocorticosterone were 46min and 42min respectively, with greater than 1min between the two peaks. Peaks were less than 1min 30s wide and peak height was at least 50x background.

Following chromatography, the area under each peak was integrated using the Winflow software to quantify the percentage conversion of $[^3H]_4$-corticosterone to $[^3H]_4$-3α,5β-tetrahydrocorticosterone. Peaks were accepted for quantification if they had areas greater than 2x the background. The percentage conversion in each tissue sample was corrected for the "apparent conversion" occurring in tissue blanks included in each experiment, which was always <4%.

2.6   Gas chromatography / mass spectrometry

2.6.1   Extraction of steroids

2.6.1.1 Extraction from cell medium

Medium was retained at -20°C from H4IIE cells cultured as described in 2.5. Steroids in medium were recovered by solid-phase extraction. Sep-pak C18 cartridges (Water, Herts, UK) were activated using methanol (5ml) followed by water (5ml). Medium was loaded on to the column and the eluant discarded. The column was washed with water (5ml) and steroids eluted in methanol (2ml). The eluant was dried under a stream of oxygen free nitrogen at 60°C and resuspended in

ethyl acetate (2ml) and water (200μl). The organic layer was separated and dried before derivatisation to form methoxime-trimethylsilyl derivatives as described previously (Best & Walker 1997).

2.6.1.2 Extraction from tissues

A solution of methanol containing acetic acid (1%) (10 volumes, w/v) was added together with Wistar rat liver (2g). The sample was then sonicated in an ultrasonic bath for 5 min, left overnight at room temperature and centrifuged at 3000 x g for 5 min. the organic phase was collected and the rest of the extract residue washed again with methanol (20ml) containing acetic acid (1%) and centrifuged. The two organic phases were pooled and evaporated to dryness under a stream of oxygen free nitrogen at 60°C, taken up in 1ml of methanol:water (40:60,v/v) and sonicated for 5min. A clean-up step was performed by solid-phase extraction with Sep-pak C18 cartridges as described in 2.6.1.1. The unconjugated steroid fraction was eluted with 5ml methanol:water (85:15, v/v), filtered through a Millipore PFTE membrane (0.45μm) and dried. The dried samples were then hydrolysed and derivatised as described previously (Best & Walker 1997).

2.6.2 Gas chromatography / mass spectrometry

Gas chromatographic mass spectrometric analysis was performed in electron impact mode using a Polaris Q ion-trap GCMS (Thermofinnigan, UK) as described (Livingstone *et al.* 2000). The identities of steroids were confirmed using analytical standards obtained from Steraloids (Newport, USA). The level of detection for steroids was < 1nM. All solvents were HPLC distilled grade (Rathburn, Walkerburn, UK) and reagents were obtained from Sigma (Poole, UK).

.

2.7 RT-PCR

RT-PCR allows the detection and amplification of specific mRNA species from isolated total RNA. Using Oligo(dT)$_{15}$ primers, the reverse transcriptase enzyme transcribes mRNA into double stranded cDNA – the necessary template for *Taq* polymerase. Subsequent PCR amplification consists of a number of cycles, during which the cDNA template is repeatedly denatured, annealed with target-specific

primers and replicated using *Taq* polymerase. Following 20-40 cycles the amplified DNA can be analysed for size and quantity using gel electrophoresis.

Only RNase free, sterile solutions and equipment were used for RT-PCR in order to prevent degradation of target RNA by exogenous RNases and contamination of reactions with exogenous RNA/ DNA. All reactions were prepared on wet ice unless otherwise stated.

2.7.1 Tissue Preparation

2.7.1.1 Intact Tissues

Liver and prostate were frozen routinely on dry ice immediately after dissection from the animal. Cultures of cell lines at third passage were removed from the culture flask directly into Trizol (7.5ml) and frozen on dry ice. Tissues were stored at -80°C until required.

2.7.2 RNA Extraction

RNA extraction was carried out using TRIzol Reagent (Gibco, UK) - a mono-phasic solution containing phenol and guanidine isothiocyanate. This reagent maintains RNA integrity whilst disrupting cells and dissolving cell components.

2.7.2.1 Homogenization

TRIzol was added per 50-100mg tissue or powdered tissue whilst frozen and samples were homogenized on wet ice using an Ystral mechanical homogeniser (Scientific Instruments Centre, UK). Samples were centrifuged at 12,000 x g at 4°C for 10min to remove insoluble material and small amounts of unhomogenized tissue. The resulting supernatant was removed to a fresh sterile eppendorf.

2.7.2.2 Phase Separation

Following homogenisation, samples were allowed to equilibrate to room temperature (estimated 20°C) then left for 5 minutes to allow complete dissociation of the nucleoprotein complexes. For cell experiments, 1ml aliquots were removed from the

original 7.5ml and the protocol followed as for homogenised tissue. Chloroform (0.2ml per ml TRIzol used in original homogenisation) was added to each sample. Samples were shaken vigorously by hand for 15 seconds then incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 x g at 4°C for 15min resulting in a lower red phenol-containing phase (containing proteins), an interphase (containing DNA and denatured proteins) and an upper aqueous phase containing RNA.

2.7.2.3 RNA Precipitation

The upper aqueous phase from each sample was transferred into a fresh eppendorf and the RNA was precipitated by addition of isopropanol (500µl per ml TRIzol in original homogenisation). Following addition of isopropanol, samples were incubated at room temperature for 10min prior to centrifugation at 12,000 x g at 4°C for 10min. The RNA precipitate forms a visible gel-like pellet on the side of the tube.

2.7.2.4 RNA Wash

Following centrifugation the supernatant was removed and the RNA pellet was washed with ethanol (75% 1ml/ ml TRIzol in original homogenisation). Pellets were vortexed and centrifuged at 7,500 x g at 4°C for 5min.

2.7.2.5 RNA Resuspension

Following the RNA wash, the ethanol was removed and the pellets were briefly air-dried for 5min, being careful not to completely dry out the RNA, which greatly reduces solubility. RNA pellets were dissolved in DEPC-treated water (10-50µl depending on pellet size) by pipeting followed by incubation at 60°C for 10min. For cell experiments all of the pellets from the original 7.5ml were pooled together and then resuspended in DEPC-treated water (20µl). RNA was stored at -80°C until required.

2.7.2.6 RNA Quantification

Before use, RNA was quantified using a GeneQuant RNA/ DNA Calculator (Pharmacia Biotech, Sweden). RNA was diluted 1:25-1:100 in DEPC-treated water

and the optical density at λ260nm (wavelength for RNA) and λ280nm (wavelength for DNA) was determined to assess concentration and purity. RNA was only used with a Δ260/Δ280 of between 1.5 and 1.8.

2.7.3 Reverse Transcriptase Reaction

First strand cDNA synthesis was performed using the Reverse Transcription System (Promega, UK). Total RNA (1µg) was reverse transcribed in a reaction mixture containing MgCl$_2$ (5mM), 1x reverse transcription buffer, dATP, dCTP, dGTP and dTTP (1mM), Rnasin (20U), Oligo(dT)$_{15}$ primers (0.5µg) and AMV-reverse transcriptase (15U) made up to 20µl in DEPC-treated water. Samples were incubated at 42°C for 45min followed by 5min at 95°C then 5min on ice to inactivate enzymes and prevent binding to complementary DNA.

Negative control reactions for each RNA sample were performed in parallel (made up as above but in the absence of AMV-reverse transcriptase) in order to determine genomic DNA contamination. Additionally, a negative control reaction containing water instead of RNA was performed to determine RNA contamination of the Reverse Transcriptase System reagents.

2.7.4 PCR Reactions

cDNA template (5µl) was used in each PCR reaction containing 1x Thermophilic DNA polymerase Reaction Buffer, MgCl$_2$ (1.5mM), dATP, dCTP, dGTP and dTTP (200µM), upstream primer (40pmol), and downstream primer (40pmol) made up to 50µl in DEPC-treated water. One *Taq*Bead (1.25U) was added to each reaction tube before starting the required PCR programme. A negative control reaction containing DEPC-treated water rather than cDNA was prepared in parallel to determine contamination of PCR reagents.

PCRs were carried out on an Eppendorf Mastercycler Gradient (Eppendorf, Germany) with a heated lid. Samples were heated to 95°C for 3min for initial denaturation, then underwent 35 cycles of PCR amplification (denaturation at 95°C for 45s, primer annealing at primer-specific temperature for 30s and elongation at

72°C for 1min 30s). Upon completion of the PCR programme, samples were incubated at 72°C for a further 5min to ensure elongation of products to full length and chilled to 4°C prior to gel electrophoresis. The 5α-Reductase type 1, 2 and 1b primers were gifts from Dr Dawn Livingstone. The 3α-HSD primers have been described previously (Lin *et al.* 1999). To confirm authenticity of PCR products, primers and templates were fully sequenced by Miss Alison Condie (Genetics Core, Wellcome Trust Clinical Research Facility, Edinburgh) using the ABI PRISM® sequencing kit (Applied Biosystems).

Specific primer sequences and PCR conditions are detailed in Table 2.4.

2.8 Real-Time PCR

2.8.1 Tissue preparation

Subcutaneous and omental adipose tissue was removed from lean and obese male Zucker rats at sacrifice and snap frozen.

2.8.2 RNA extraction and Quantification

Total RNA was isolated from frozen subcutaneous and omental adipose tissue by homogenising fat (250mg) in TRIzol (1ml). RNA was purified using RNA aid RNA binding matrix (Anachem, Luton, UK), washed three times, and dissociated by addition of DEPC H_2O/DTT/Rnasin. Total RNA was quantified as described above in 2.7.2.6.

2.8.3 Real -Time PCR reactions

RNA (500ng) was reverse transcribed as described in 2.7.3. cDNA was diluted three times and transcript level quantification for 5α-reductase type 1 was performed with Real Time PCR primer-probe sets using the 7900MT sequence detection system (PE Applied Biosystems, Cheshire,UK) with the following primers and probes: 5'-CTGTTTCCTGACAGGCTTTGC-3'(forward), 5'-GCCTCCCCTGGGTATCTTGT-3' (reverse), and 5'-6-FAM-CAGACCACATCCTGAGGAATCTGAGAAAACC-TAMRA-3' (probe). Rat cyclophillin (PE Applied Biosystems, Cheshire, UK)

59

primers and probes were included in a singleplex reaction to normalise the transcript levels. A standard curve for each primer-probe set was generated in triplicate by serial dilution of cDNA pooled from different animals. Samples for cyclophillin and 5α-reductase type 1 were run in duplicate and the mean values were used to calculate transcript level. Values were calculated as a relative fold change in mRNA from an internal control sample using standard curves. RT negative controls and intron spanning primers were used to examine for genomic DNA and prevent amplification.

2.9 Northern Analysis of mRNA

2.9.1 Extraction of RNA

Total RNA was extracted from liver and H4IIE cells by the TRIzol method as described above (2.7.2).

2.9.2 RNA electrophoresis and capillary transfer

Total RNA was separated by electrophoresis on an agarose formaldehyde denaturing gel (1.2 %). A gel (100ml) was prepared by melting of agarose (1.2g) in DEPC-treated water (88ml), adding formaldehyde (40%) and 10x MOPS buffer (10ml) and pouring into a gel mould with appropriately sized combs in place. RNA was prepared for electrophoresis by aliquoting RNA (20µg), adding DEPC-treated water to bring the total volume to 10µl, then adding deionised formamide (10µl), formaldehyde (2.5µl) and 10x MOPS buffer (2.5µl) to give a total volume of 25µl. The sample was mixed and denatured by incubating at 65°C for 15 min. Ethidium bromide was added to the loading buffer (1µl per 50µl loading buffer) and 2µl of this mix was added to each sample of denatured RNA. The RNA was loaded into the wells on the gel and electrophoresis performed at 80V for 4-6 hours until the front band of the loading buffer was ¾ of the way down the gel. The gel was photographed under UV light (254nm) with as little exposure as possible to minimise RNA damage. The gel was soaked in 20x SSC buffer for 15min before blotting onto a nylon membrane (Zeta-Probe® GT, (Bio-Rad, Hemel Hempstead, UK). A wick of Whatman 3MM filter paper was placed over an upturned gel mould in a plastic tray containing 20x SSC buffer and the gel was placed on top. A piece of nylon membrane cut to the same

size as the gel and pre-wetted in the 20x SSC was smoothed on top of the gel and this was covered with 3 layers of 3MM filter paper and approximately 5cm of paper towels. A glass plate was placed on the top to secure the apparatus and a light weight balanced on the top. Capillary transfer was allowed to take place overnight at room temperature.

The next day, the membrane was washed in 20x SSC to remove any gel and the efficiency of the transfer was checked by photographing the gel and the membrane under UV light (254nm). The membrane was dried between two sheets of 3MM filter paper at 80°C for 2 hours and the RNA cross-linked under UV light.

2.9.3 Hybridisation to ^{32}P-labelled cDNA

The nylon membrane was soaked in 20x SSC buffer for 5min, then placed in an Hybaid hybridisation bottle containing 20% SDS (10ml) and phosphate buffer (20ml) that had been warmed to 55°C. To this was added denatured salmon testes DNA (10mg/ml; 100µl). The membrane was pre-hybridised in an Hybaid hybridisation oven at 55°C for at least 2 hours. The ^{32}P-labelled cDNA probe was added to the pre-hybridisation buffer, and the membrane hybridised with the probe overnight at 55°C. The probe was then disposed of and the membrane rinsed with wash buffer one. This was followed by one wash in wash buffer one at room temperature for 30min, one wash in wash buffer one at 55°C for 30 min and one wash in wash buffer two at 55°C for 30 min. The membrane was wrapped in cling-film and exposed to a Fujifilm imaging screen for 1-48 hours and the level of hybridised probe quantified using a Fuji FLA2000 fluorescent image analyser. The membranes were then rehybridised with U1 cDNA in the same way to control for RNA loading and transfer. The level of expression of the RNA of interest was expressed as a ratio of the signal of the RNA of interest to the signal for U1.

PCR	base position in gene	Primer Sequences	Annealing Temp (°C)	Cycle Number	Product Size (bp)
5α-reductase 1	292-311 748-729	Upstream: 5'-TGA TCC ACT ACG TGC AAA GG -3' Downstream: 5'-AAC TTC TCA TGG TAC CAC TG -3'	48	35	456
5α-reductase 1b	10-29 748-729	Upstream: 5'-AAC TTC TCA TGG TAC CAC TG Downstream: 5'-AAC TTC TCA TGG TAC CAC TG -3'	48	35	738
5α-reductase 2	289-308 856-837	Upstream: 5'-ATG TCC TGC TGG CTC TCT TC -3' Downstream: 5'- AGC AGT TCC TCC ACA GAA AC-3'	48	35	567
5β-reductase	249-268 780-761	Upstream: 5'-AGG TGG CAG AAG GGA AGG TA -3' Downstream: 5'-AGC GAG GTT AGG AGT TCA TC -3'	48	35	512
3α-HSD	252-273 567-547	Upstream: 5'-CTT TGG AGC ACT TTC CAT AGA -3' Downstream: 5'-GTT GCA CAC AGG CTT GTA TTT -3'	48	35	315

Table 2.4: Specific primers and PCR conditions.

2.10 Preparation of ^{32}P and ^{35}S labelled cDNA probes

2.10.1 DNA ligation

Fragments recovered from low melting point agarose gels were ligated using T4 DNA ligase (1-5U) in 1 x supplied buffer in a final volume of 10μl. Typically, 2-4μl (approximately 50ng) of insert DNA was used and 0.5-1μl (approximately 100ng) vector DNA. Reactions were incubated at 4°C overnight before transforming into competent *Eschericia.coli.*

2.10.2 Bacterial Transformation

Eschericia coli HB101 cells were grown in 100ml of Luria-Bertoni (LB) broth at 37°C in a shaking incubator until they reached mid-log phase (A_{600} = 0.3-0.6). They were then centrifuged at 1000 x g for 5min at 4°C, the pellet re-suspended in cold calcium chloride (0.1M; 20ml) and left on ice for between 10min and 2 hours. The centrifugation step was repeated to re-pellet the cells and the cells were re-suspended in cold calcium chloride (0.1M; 2ml). The competent cells were stored on ice in the fridge for up to 3 days before transformation.

Competent cells (200μl) were mixed with plasmid DNA (50ng) and left on ice for 20min. The cells were heat shocked at 42°C for 50s and placed back on ice. The heat shock and the calcium chloride lead to the incorporation of the plasmid DNA into the cells. The cells were spread onto LB agar plates containing ampicillin (100μg/ml), and the plates incubated overnight at 37°C. Only cells that had incorporated the plasmid DNA grew on the plates containing ampicillin, as HB101 cells do not have inherent ampicillin resistance.

2.10.3 Screening of clones

Screening of clones was carried out by small-scale preparation of plasmid DNA followed by appropriate restriction digests and agarose gel elctrophoresis as described later in 2.10.5. Following transformation, single colonies were picked and used to seed 2ml of LB containing ampicillin (100μg/ml). After overnight incubation

in a rotating incubator at 37°C, cultures were transferred into 1.5ml eppendorf tubes, centrifuged in a microcentrifuge at 12000 x g for 1min and the pellets resuspended in GTE (100μl). Fresh 0.2M NaOH/1% SDS (w/v) (200μl) was added, the tubes vortexed and placed on ice for 2 min. 5M potassium acetate (150μl) was then added, the tubes vortexed and placed on ice for 5min. The tubes were centrifuged in a microcentrifuge at 12000 x g for 5min and the supernatant transferred to fresh eppendorf tubes. Chloroform/isoamyl alcohol (24:1) (225μl) and Tris-HCl saturated phenol (225μl) was added, the tubes vortexed and centrifuged for 2min. The supernatant was transferred to a fresh eppendorf tube, 2 volumes of absolute ethanol added and the tubes vortexed prior to incubation at room temperature for 5min. The tubes were centrifuged at 12000 x g for 5min, the supernantant removed with a glass pasteur pipette and the pellet left to air-dry for 10min. The pellet was resuspended in TE (50μl) containing RNAse A (50ng) and stored at -20°C until needed.

2.10.4 Plasmid DNA preparation

A single transformed bacterial colony was selected from an agar plate and incubated for 6 hours in LB (2ml) containing ampicillin (100μg/ml). This was then added to LB (500ml) containing ampicillin (100μg/ml) and incubated overnight at 37°C. The culture was centrifuged at 3500 x g for 5min at 4°C in a Beckman J14 centrifuge, and the supernatant discarded. The cell pellet was resuspended in cold GTE buffer (12ml) and freshly prepared alkaline SDS (24ml). The mixture was shaken vigorously by hand and left on ice for 10min. Cold potassium acetate (5M; 16ml) was added and the mixture left on wet ice for 10 min before being centrifuged at 3500 x g for 5min at 4°C in a Beckman J14 centrifuge. The mixture was filtered through two layers of sterile gauze to remove the precipitate, isopropanol (32ml) was added to the filtrate and the mixture was left at room temperature for 30min to precipitate the DNA. The DNA was pelleted by centrifugation at 7800 x g for 3min at 4°C in a Beckman J20 centrifuge, and the supernatant discarded. The DNA pellet was left to dry. The DNA pellet was resuspended in TE buffer (2.2ml), CsCl (2.95g) added and dissolved and ethidium bromide (100μl, 10mg/ml) added. The mixture was transferred to Beckman Quickseal ultracentrifuge tubes, topped up with CsCl/TE

solution (1g/ml) and centrifuged at 175 000 x g for 20 hours at 20°C in a Beckman Optima TLX ultracentrifuge. The DNA was separated into bands that could be visualised by the pink colour of the ethidium bromide. These DNA bands were removed using a 21gauge needle and syringe, transferred to fresh ultracentrifuge tubes, topped up with CsCl/TE solution (1g/ml) and centrifuged at 356 000 x g for 4 hours at 20°C. The DNA bands were collected as above and the ethidium bromide was removed by extracting repeatedly with isopropanol until the pink colour disappeared. The DNA was transferred to dialysis tubing and dialysed against three changes of TE buffer. The concentration and purity of the DNA was assessed spectrophotometrically using a GeneQuant RNA/ DNA Calculator (Pharmacia Biotech, Sweden). RNA was diluted 1:25-1:100 in DEPC-treated water and the optical density at λ260nm and λ280nm was determined.

2.10.5 Restriction enzyme digestion of plasmid and purification of fragment.

Plasmid DNA (10μg) was digested with the appropriate restriction enzyme (10 units) in 1x restriction enzyme buffer in a total volume of 100μl for 2 hours at 37°C. Digestion of the DNA was confirmed by electrophoresis of 3μl of the digest through a 1.2% agarose gel (prepared by dissolving agarose (1.2g) in 0.5 x TBE and adding ethidium bromide (2μl,(10mg/ml)). The digest was compared with uncut plasmid and a 1kB DNA ladder containing fragments ranging from 75bp-12kb under UV light at 254nm. If the plasmid was sufficiently digested, the remaining digest was electrophoresed in a large single well of a 1.2% low melting point agarose gel (made as above but using low melting point agarose).

The DNA fragment was visualised under UV light, excised from the gel using a scalpel and purified from the gel using a DNA Purification kit (Hybaid, Ashford, Middlesex) and resuspended in DNAase free/RNAase free water (50μl). Recovery of the DNA fragment was assessed by electrophoresis of 1μl of the DNA solution through a 1.2% agarose gel as described above.

2.11 ^{35}S *In Situ* Hybridisation

In situ hybridisation allows the visualisation of the exact cellular and/ or structural location of specific mRNAs (indicating transcription of the corresponding gene) by hybridisation of a ^{35}S-labelled 'antisense' RNA probe to the mRNA of interest. ^{35}S-UTP labelled RNA 'sense' probes of similar length, nucleotide content and specific activity but not complimentary to the gene were included in each experiment in order to assess the specificity of the hybridisation reaction.

Only RNase free, sterile solutions and equipment were used for *in situ* hybridisation experiments in order to prevent degradation of target mRNA by exogenous RNases.

2.11.1 Slide Preparation

Prior to use, glass microscope slides were coated in 3-aminopropyltriethoxysilane in order to prevent section dehiscence. Slides were racked and washed in the following series of solutions; HCl (0.2M) for 3min, DEPC-treated water for 3min, 3-aminopropyltriethoxysilane in acetone (2%; filtered through $NaSO_4$) for 10s, acetone for 3min (twice), and finally DEPC-treated water for 3min. Slides were air-dried for 30-60min before baking at 50°C for 4-16 hours. Dried slides were wrapped in aluminium foil and stored for up to 3 months.

2.11.2 Tissue section preparation

Liver and prostate were routinely frozen on dry ice immediately after dissection from the animal. Tissues were stored at -80°C until required.

Frozen tissue sections were cut using a Leica cryostat (Leica Microsystems, Germany). Tissues frozen at -80°C were placed in the cryostat chamber at -20°C and allowed to equilibrate for approximately 30min. Following equilibration, tissues were embedded in Cryo-m-bed embedding compound (Brights, UK) and positioned in the correct orientation for sectioning. 10μm thick sections of liver and prostate were thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides. Slides with tissue sections were stored at -80°C until required.

2.11.3 Fixation protocol

Slides were removed from the -80°C freezer and kept on dry ice until the start of the fixation procedure. Slides were fixed in ice cold paraformaldehyde (4%) in phosphate buffer (0.1M) for 10min, rinsed twice in 1x PBS for 5min, acetylated in triethanolamine (0.1M) with acetic anhydride (0.25%) for 10min to prevent loss of signal and rinsed in 1x PBS for 3min. Following dehydration through a series of ethanol solutions (70, 80 and 95% ethanol in DEPC-treated water) slides were air dried for 30min.

2.11.4 Probe templates for *in situ* hybridisation

Target specific RNA probes for rat 5α-reductase type 1, 5α-reductase type 2 and 3α-HSD were produced *in vitro* using T7 or SP6 phage polymerase systems. The T7/ SP6 promoter sequence flanked linear cDNA templates for 5α-reductase type 1 and 5α-reductase type 2 were gifts from Dr Dawn Livingstone. The T7/ SP6 promoter sequence flanked cDNA template for 3α-HSD was synthesised as described previously in 2.9.

2.11.5 Synthesis of ^{35}S-UTP labeled ribo-probes

See Table 2.5 for specific characteristics of probe generation.

Probe	RNA polymerase	Temp (°C)
5α-reductase 1 *s*	T7	37
5α-reductase 1 *as*	SP6	40
5α-reductase 2*s*	T7	37
5α-reductase 2 *as*	SP6	40
5β-reductase *s*	T7	37
5β-reductase *as*	SP6	40
3α-HSD *s*	SP6	40
3α-HSD *as*	T7	37

Table 2.5: Characteristics of Probe Generation for *In Situ* Hybridisation

s = sense, as =antisense

2.11.6 Pre-hybridisation & hybridisation steps

Following fixation, slides were pre-hybridised with 200μl/ slide of 2x pre-hybridisation buffer diluted 1:1 with deionised formamide, at 50°C for 2h. Dampening two layers of Whatman No.3 chromatography paper with box buffer humidified the slide boxes, hence preventing tissue sections from drying out.

For all probes, linear cDNA template (0.5-1μg) was transcribed by incubation at the appropriate temperature for 60-90min with ATP, CTP and GTP (10mM each), ^{35}S-UTP (s.a. 800Ci/ mmol), dithiothreitol (DTT, 10mM), RNase inhibitor (0.5μl), and appropriate polymerase (1μl) in a total volume of 10μl 1x transcription optimised buffer. Following incubation, DNase 1 (RNase free) (1μl) was added and reactions incubated at 37°C for a further 15min to degrade the DNA template, after which probes were placed on ice for 1-5 min and purified using NICK columns (Pharmacia Biotech, Sweden) to remove unincorporated radioactivity. The column was prepared by washing through with TE buffer (3ml). The probe mixture was then applied to the column. The column was washed with TE buffer (400μl) and the initial elutant discarded. Labelled probe was eluted in an additional TE buffer (400μl).

2.11.7 RNase treatment & washes

Following hybridisation, slides were washed three times in 2x SSC for 5min and carefully wiped dry around the sections with lens tissue. RNase A (200μl, 30mg/ ml in RNase buffer) were applied to each slide and slides were incubated at 37°C for 1 hour in humidified boxes (1 layer of Whatman No.3 chromatography paper dampened with RNase buffer) to remove unhybridised probe.

Sense and antisense probes were thawed and added to 2x hybridisation buffer diluted 1:1 in deionised formamide to give a final probe concentration of 20×10^{-6} cpm/ml. Probes were denatured at 90°C for 10min and placed on ice before addition of DTT (10mM). Pre-hybridisation buffer was drained from slides and appropriate probe (200μl) was applied to slides. Slides were hybridised in sealed, humidified boxes at 50°C for an optimum of 16 hours.

Following RNase treatment, slides were washed in 2x SSC at room temperature for 30min, then twice in 0.1x SSC at 60°C for 60min. After washes, slides were dehydrated through a series of ethanol solutions containing ammonium acetate (0.3M) (2min in each of 50, 70 and 90% ethanol) and air-dried.

For each probe, the total activity was estimated by counting 1μl of probe in 1ml PicoFluor 40 scintillant fluid (Canberra Packard, UK) in duplicate in a β-counter (minimum activity required $2x10^5$ cpm/μl). The purity of each probe was determined by running 1-2μl on a urea gel (3.6g urea, 1.32ml acrylamide, 0.1% ammonium persulphate (v/v), 10μl TEMED in 1x TBE) and exposing the gel to Kodak Biomax-MR film (HA West Ltd, Edinburgh, UK), which should produce a single black band on the film when developed. Probes were stored at -20°C until required, for a maximum of 7 days.

2.11.8 Visualisation of Hybridisation

Slides were exposed to Kodak Biomax-MR film for 2-7 days. Afterwards, slides were individually dipped in NTB-2 photographic emulsion (Kodak, UK, diluted 1:1 with DEPC-treated water at 42°C) and exposed in light-tight boxes for 5 days to 12 weeks at 4°C. Slides were developed in D19 solution (HA West Ltd, UK) diluted 1:1 with water at 15°C, fixed in Amfix solution (HA West Ltd, UK) diluted 1:5 with water at 15°C, rinsed in water and counterstained with haematoxylin and eosin.

.

2.12 Glucocorticoid receptor binding assays

2.12.1 Tissue Handling

2.12.1.1 Cytosol preparation

The livers of freshly sacrificed lean male Zucker rats were perfused with saline (20ml) via the hepatic vein to remove blood and the liver was excised. The liver was chopped into small pieces in ice-cold molybdate buffer and then disrupted using an Ystral mechanical homogeniser. Cytosol was prepared by first centrifuging at 20,000

x g for 20 min. at 4°C and then at 105,000 x g for 60 min. at 4°C. The supernatant was then stored on ice.

2.12.1.2 Microsome preparation

Microsomes were prepared as for cytosol (2.11.1.1). After the 105,000 x g spin, the resulting pellet was resuspended in molybdate buffer and stored on ice.

2.12.1.3 Hepatocyte preparation

Rats were anaesthetised with Sagatal (0.3ml) and cannulated via the inferior cava (for waste drainage) and the hepatic vein. Calcium-free buffer was pumped through the liver at a flow rate of 25ml/min and then Krebs buffer containing collagenase type IV was pumped through to break down the contacts between cells. The liver was then removed and transferred to Krebs buffer (100ml) and broken up mechanically. The suspension was rinsed through nylon mesh (aperture 0.125mm) with Krebs Buffer. The remaining cells were centrufuged at 500 x g for 2 min. and then resuspended in Krebs buffer. This centrifugation step was repeated three times. Following this, the cells were resuspended in hepatocyte incubation buffer (30ml). The cell viability was estimated by trypan blue exclusion under light microscope and counted using a haemocytometer.

2.12.2 Binding assays

For competitive binding experiments the incubation medium consisted of 25μl [^{3}H]$_4$ –dexamethasone (specific activity, 91Ci/mmol, 1.5nM final concentration), non-radioactive dexamethasone or corticosterone (25μl, ranging from 0nM to 200μM) or one of the following corticosterone metabolites 5αDHB, 5αTHB, 5βDHB, 5βTHB, A, DHA or THA (25μl, ranging from 0nM to 200μM), cytosol or microsomes (50μl, 4mg/ml) or hepatocytes (2million cells/ml). The assay wells were incubated at 4°C for 24h and all reactions were performed in duplicate. The reaction was terminated by rapid filtration on filtermats pre-soaked in polyethylenimine (0.3% v/v) using a Combi cell harvester (Skatron Instruments) with ice-cold distilled water. The filtermats were left to dry and then encapsulated with Meltilex (heated to approximately 90°C). The radioactivity retained on the filters was counted by a 1450

Microbeta Plus Liquid Scintillation Counter for 5minutes per sample. Results were expressed as percentage dexamethasone bound compared to no competitor and the dissociation constant (Kd) and the total number of binding sites (Bmax) were determined using the non-linear curve-fitting program Radlig.

2.13 Transient transfection studies

2.13.1 Transient transfections

24h before transfection, HeLa cells were seeded on 60mm dishes, allowing three dishes per transfection. Cells were harvested by centrifugation at 500 x g for 5min and resuspended in medium (10ml). Cells were counted using an Improved Neubauer haemocytometer (Hawksley) and if necessary diluted further to give a cell count of 3 x 10^6 cells/ml. 1ml of cells was added to each 60m dish containing 3ml of medium and incubated at 37°C with 5% CO_2.

All solutions used were equilibrated to room temperature prior to use. Medium on cells was replaced with fresh medium (4ml) at least 1hr prior to transfection. For each transfection DNA solutions (300μl total) were set up in triplicate in filter-sterilised water and contained filter-sterilised $CaCl_2$ (2M; 37μl).

The plasmids used were pGEM3 (inert DNA) (Promega), pCH110 (a β-galactosidase expression plasmid which was used as an internal control for transfection efficiency) (Amersham Pharmacia), pSV2L (firefly luciferase gene driven by a mammalian SV40 early promoter which was used as a positive control) (de Wet et al. 1987), pLTR-Luc (full length MMTV LTR driving the expression of a luciferase gene) (Lefebvre et al. 1991) and pRShGR, a human glucocorticoid receptor gene (Lefebvre et al. 1991).

Control DNA solutions consisted of (i) pGEM3 (10μg) and (ii) pSV2L (5μg), pCH110 (1μg) and pGEM3 (4μg) to give a total of 10μg DNA. pCH110 (1μg), RShGR (1μg) and LTR-Luc (5μg) was used in each transfection and the amount of pGEM3 adjusted to give a total of 10μg DNA. DNA solutions were briefly vortexed

to mix. Before transfection (20-30min), DNA solutions were added dropwise to 2 x BBS (300µl) with slow agitation and incubated at room temperature for 20min. After briefly vortexing, the DNA solutions were added slowly to the dishes of cells with agitation. 24 hr after transfection the medium was replaced on the cells with fresh medium (4ml) at least 1hr prior to steroid treatment. To treat cells with steroid, steroid (4µl in ethanol) at the appropriate concentration was added to the cells. 24hr later, cells were harvested for assays. Medium was aspirated, cells were washed with phosphate-buffered saline (3ml) and lysis buffer (300µl) was added to the dishes. After incubation at room temperature for 15min, cells were scraped and pipetted into eppendorf tubes; cell debris was pelleted by centrifugation at 13000 x g for 2min in a microcentrifuge. All transfections were performed using at least two independently prepared plasmid DNAs.

2.13.2 Luciferase assays

Since luciferase activity is labile, luciferase assays were performed in duplicate on the same day that cells were harvested. All solutions were equilibrated to room temperature. Sample (40µl) was added to 2x assay buffer (100µl) with 0.1M ATP (5µl) in a 5ml borosilicate glass tube (Starstedt). Luciferase activity was measured using a Lumat LB9501 luminometer (Berthold) that injected 1mM beetle luciferin (105µl) (Promega, Southampton, UK). Values recorded were the means of the two duplicates.

2.13.3 β-galactosidase assays

β-galactosidase activity was asayed using a Galacto-Light Plus (Applied Biosystems, UK) and all samples were assayed in duplicate. All solutions used equilibrated to room temperature prior to use. Galacton-Plus substrate was diluted 1:100 with Reaction Buffer Diluent to make the reaction buffer which was then dispensed in 67µl aliqouts into 5ml borosilicate tubes. Sample (10µl) was added, left to incubate at room temperature for 15-60min and then assayed using a Lumat LB9501 luminometer which injected Light Emission Accelerator (105µl). Values recorded were the means of the two duplicates.

2.13.4 Data analysis

Data were analysed using a Microsoft Excel spreadsheet. The means of the background luciferase and β-galactosidase activities (those for transfections with pGEM3 only) were subtracted from experimental values and luciferase activity expressed as luciferase activity/β-galactosidase activity, thus controlling for differences in cell number and transfection efficiency. The mean value obtained for empty vector was then set to a value of 1 and mean experimental activities expressed relative to this value. The value obtained with pSV2 luciferase confirmed successful transfection; transfections in which pSV2 luciferase activities were not above that of the empty vector alone were excluded from analysis.

2.14 Examination of glucocorticoid effects on angiogenesis

Aortic rings from 8-10 week old, male C57/Bl6 wild type mice were embedded on steroid free Matrigel (Beckton Dickinson, Bedford, UK) and incubated at 37°C in serum free MCDB 131, with heparin, ascorbic acid, and GA 1000 (all supplied in the EGM-2 bulletkit, Cambrex Biosciences, Berkshire, UK) in the presence or absence of corticosterone (600nM) or 5αTHB (1μM) with/without the glucocorticoid receptor antagonist, RU486 (1μM). Steroids were dissolved in ethanol and subsequently diluted in aqueous solution. The final ethanol content in all culture media was 1-3% v/v. media was changed every 48hours. Angiogenesis was quantified by counting new vessels on day 7 of culture.

2.15 Plasma assays

2.15.1 Adrenocorticotropin (ACTH) radioimmunoassay

The concentrations of ACTH were quantified using a radioimmunoassay kit (Eurodiagnostica BV, Arnhem, The Netherlands). Plasma samples were diluted 1 in 5 in the supplied buffer before assay. A range of concentrations of ACTH were prepared (0-1250pg/ml) to allow construction of a standard curve. Samples and standards were incubated in duplicate in primary sheep anti-rabbit antibody coated plastic tubes with [^{125}I] sheep-anti-ACTH (total volume 100μl) at room temperature for 4 hours. The tubes were then washed twice with supplied wash buffer (5ml) and

counted in a γ-counter. The radio-iodinated sheep IgG recognises the amino terminal region of ACTH. The second antibody reacts non-competitively with the C-terminal region of the ACTH molecule and during incubation both antibodies react with the molecules of the sample. A sandwich type complex is formed and bound to the tube wall. The remaining excess of tracer is removed by washing and the radioactivity measured is directly proportional to the concentration of ACTH in the sample, estimated by comparison with the standard curve. The inter- and intra-assay coefficients of variation were <4%.

2.14.2 Corticosterone radioimmunoassay

Plasma samples were diluted 1 in 10 in borate buffer and denatured at 65° C for 30 min to dissociate corticosterone (B) from proteins. A range of concentrations of B were prepared (0.6-320nM) to allow construction of a standard curve. Samples and standards were incubated with a mixture of $[^3H]_4$-B (10,000cpm per sample) and B antibody (1 in 10,000 dilution) in borate buffer in a total volume of 70μl for 2h. Scintillation proximity assay beads (SPA; Amersham, Bucks, UK) were then added to each sample and the samples were incubated overnight. The SPA beads bind to the primary antibody and if the primary antibody is bound to $[^3H]_4$-B the SPA beads cause scintillation of the radioactive signal. As the concentration of unlabelled B increases there is competition between binding of unlabelled and labelled B to the primary antibody, and the radioactive signal decreases. Samples were counted on a Wallac Microbeta Plus liquid scintillation counter. The concentration of B in each sample was estimated from the standard curve. The inter- and intra-assay coefficients of variation were <10%.

2.15 Statistics

All values are expressed as mean ± standard error. Data were analysed statistically by Student's t-tests or Analysis of variance followed by post-hoc LSD tests for parametric data, or Mann-Whitney U tests for non-parametric data.

Chapter Three

Localisation of A-ring Reductases

3.1 Introduction

Two isozymes of 5α-reductase have been cloned (types 1 and 2). The two isozymes can be distinguished by their pH optima and tissue distribution. The type 1 isozyme has a broad pH optimum that spans the alkaline range (pH 6-8.5), while the type 2 isozyme has a narrow acidic pH optima centred around pH5.0 (Andersson & Russell 1990; Normington & Russell 1992). In the rat, the type 1 isozyme is expressed predominantly in the liver, whereas the type 2 form is associated with androgen sensitive tissues and is primarily responsible for the conversion of testosterone into the more potent androgen, dihydrotestosterone (Andersson *et al.* 1991).

5α-reductase type-1 and -2 are both capable of metabolising glucocorticoids (Normington & Russell 1992). It is unclear whether one or both of these isozymes are responsible for glucocorticoid metabolism *in vivo* as previous studies investigating glucocorticoid metabolism often report on urinary steroid metabolites and do not identify the isozyme or tissue involved. We anticipate that the liver will be the major site of inactivation, hence implicating type 1 at least in the rat. 5α-Reductase is also expressed in adipose tissue and thus this tissue may be of increased importance as a metabolic site in obesity (Perel *et al.* 1986; Zyirek *et al.* 1987). An elongated version of 5α-reductase type 1 (henceforth termed 1b) which differs by 4 amino acids at the N-terminal end has also been reported (Lopez-Solache *et al.* 1996). The exact role of the additional 4 amino acids is unknown, however in transfection studies they confer higher affinity for the substrate to the enzyme, especially for glucocorticoids for which the K_m is decreased 7.5-fold (Lopez-Solache *et al.* 1996). Therefore this form of the enzyme may be responsible for glucocorticoid metabolism by 5α-reductase.

The aims of this chapter were to :

1. Establish methodology to quantify the activity and expression of 5α-reductase type 1 and type 2 in metabolic tissues (i.e liver and adipose) *ex vivo*.
2. Study the cellular distribution of these enzymes.

3.2 Methods

3.2.1 A-ring reductase Expression Studies

3.2.1.1 RT-PCR

Expression of 5α-reductase type 1 mRNA and the reported elongated isoform 5α-reductase type 1b, 5β-reductase and 3α-hydroxysteroid dehydrogenase was investigated in RNA from liver and omental and subcutaneous adipose tisue of male lean Zucker rats (n = 6-8) by RT-PCR as described in chapter 2.7.

3.2.1.2 *In Situ* Hybridisation

Localisation of 5α-reductase type 1 and type 2, 5β-reductase and 3α-hydroxysteroid dehydrogenase mRNA expression was investigated in liver from male Wistar rats (n = 4- 6) using *in situ* hybridisation as described in chapter 2.11. Rat prostate was used as a positive control for 5α-reductase type 2.

3.2.2 Bioactivity assays

5β-reductase activity was measured in rat liver cytosol as described in 2.5.5.

3.2.3 5α- and 5β-reductase activity in liver

The presence of 5α and 5β-reduced metabolites was investigated in Wistar rat liver by GCMS as described in chapter 2.6.

3.3 Results

3.3.1 Detection of A-ring reductase expression by RT-PCR

Expression of mRNA of 5α-reductase type 1, 5β-reductase and 3α-hydroxysteroid dehydrogenase was detected in rat liver (Figure 3.1) and in H4IIE cells (Figure 3.4) 5α-reductase type 2 was not detected in rat liver (Figure 3.2). In omental and subcutaneous adipose tissue, only 5α-reductase type 1 and 3α-hydroxysteroid dehydrogenase were detected (Figure 3.3)

In addition, mRNA encoding the reported elongated version of 5α-reductase type 1 was not detected in rat liver (Figure 3.5).

3.3.2 Localisation of A-ring reductase mRNA expression by *in situ* hybridisation

5α-Reductase type 1 mRNA expression, but not 5α-reductase type 2 mRNA expression, was detected throughout the liver (Figure 3.6). 5α-Reductase type 1 mRNA showed marked zonation in a pattern consistent with expression in the periportal region (Figure 3.7). 5β-reductase and 3α-hydroxysteroid dehydrogenase mRNA expression was detected uniformly throughout the liver. Specific hybridisation was not observed in sections hybridised with sense probes.

3.3.3 5α-Reductase activity

As described in 2.5.4, 5α-reductase activity could not be detected by bioactivity assay in rat liver using corticosterone as a substrate (Table 2.2). However *in vivo* this reaction does occur, since 5α-reduced metabolites of corticosterone were identified in Wistar rat liver (Figure 3.8) and in H4IIE cells by GCMS.

3.3.4 5β-Reductase activity

5β-reductase activity was present in rat liver as indicated by conversion of $[^3H]_4$-corticosterone to $[^3H]_4$-3α,5β-tetrahydrocorticosterone by liver cytosol (Figure 3.9). Consistently there was no conversion of $[^3H]_4$-corticosterone to $[^3H]_4$-3α,5α-tetrahydrocorticosterone by liver cytosol detected therefore the temperature was increased from 10°C to 35°C to enable faster run-times. An additional peak was present in a proportion of the samples. This peak was believed to be the 20β-dihydrocorticosterone metabolite of corticosterone, however a synthetic standard of this steroid was unavailable to allow confirmation of this. To confirm 5β-reductase activity *in vivo*, 5β-reduced metabolites of corticosterone were identified in Wistar rat liver (Figure 3.8) by GCMS.

Figure 3.1: Expression of 5α-reductase type 1, 5β-reductase and 3α-HSD mRNA in rat liver

1µg of total RNA isolated from lean male Zucker liver was used in RT-PCR with specific primers to detect expression of 5α-reductase type 1, 5β-reductase and 3α-HSD. Negative controls included an RT reaction containing no RNA (H₂O) and a reaction carried out in the absence of the RT enzyme for each RNA (-RT).

Figure 3.2: Absence of Expression of 5α-reductase type 2 in rat liver

1µg of total RNA isolated from liver was used in RT-PCR with specific primers to detect expression of 5α-reductase type 2. Negative controls included an RT reaction containing no RNA (H₂O) and a reaction carried out in the absence of the RT enzyme for each RNA (-RT). Prostate was used a positive control.
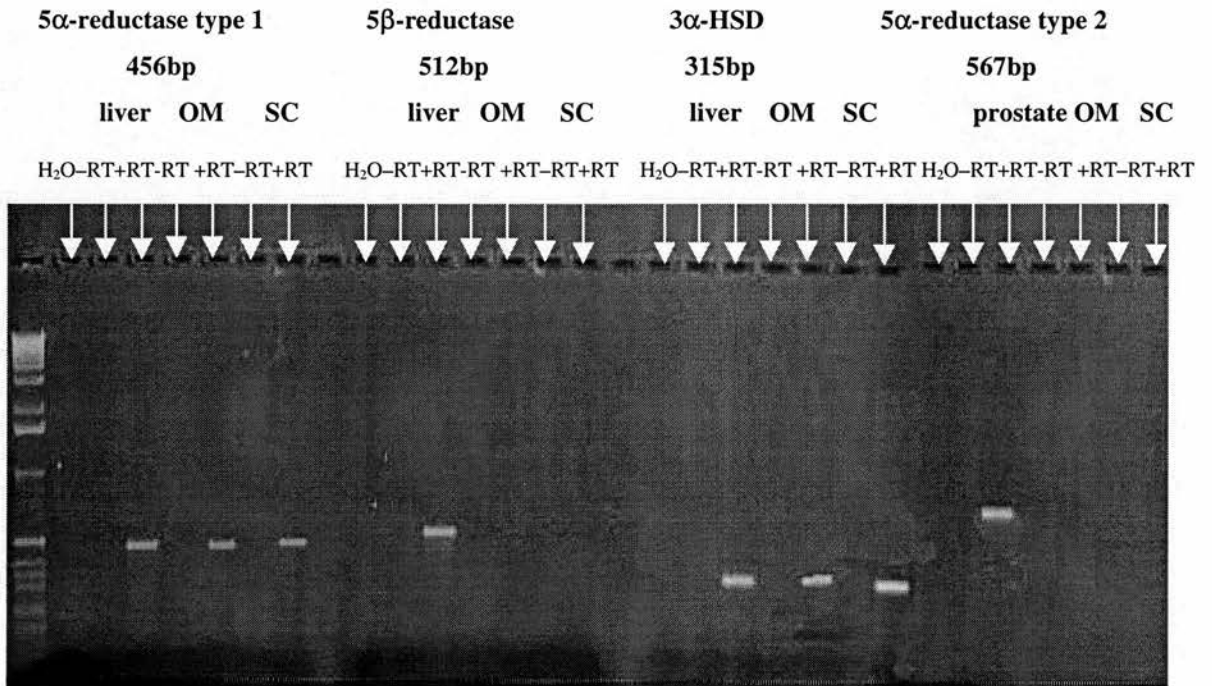
Figure 3.3: Expression of 5α-reductase type 1 and 3α-HSD mRNA in omental and subcutaneous adipose tissue.

1μg of total RNA isolated from lean male Zucker omental (OM) and subcutaneous (SC) adipose tissue was used in RT-PCR with specific primers to detect expression of 5α-reductase type 1 and type 2, 5β-reductase and 3α-HSD. Negative controls included an RT reaction containing no RNA (H₂O) and a reaction carried out in the absence of the RT enzyme for each RNA (-RT). Liver and prostate was used as a positive control.
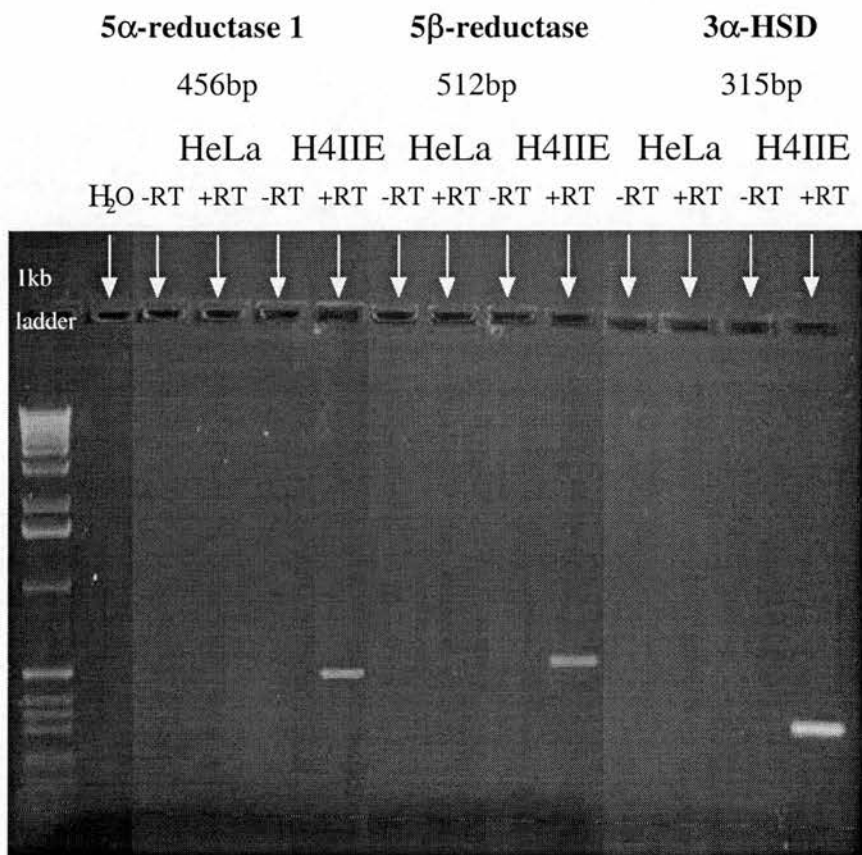
Figure 3.4: Expression of 5α-reductase type 1, 5β-reductase and 3α-hydroxysteroid dehydrogenase in H4IIE cells but not in HeLa cells.

1μg of total RNA isolated from HeLa and H4IIE cells was used in RT-PCR with specific primers to detect expression of 5α-reductase type 1, 5β-reductase and 3α-HSD. Negative controls included an RT reaction containing no RNA (H₂O) and a reaction carried out in the absence of
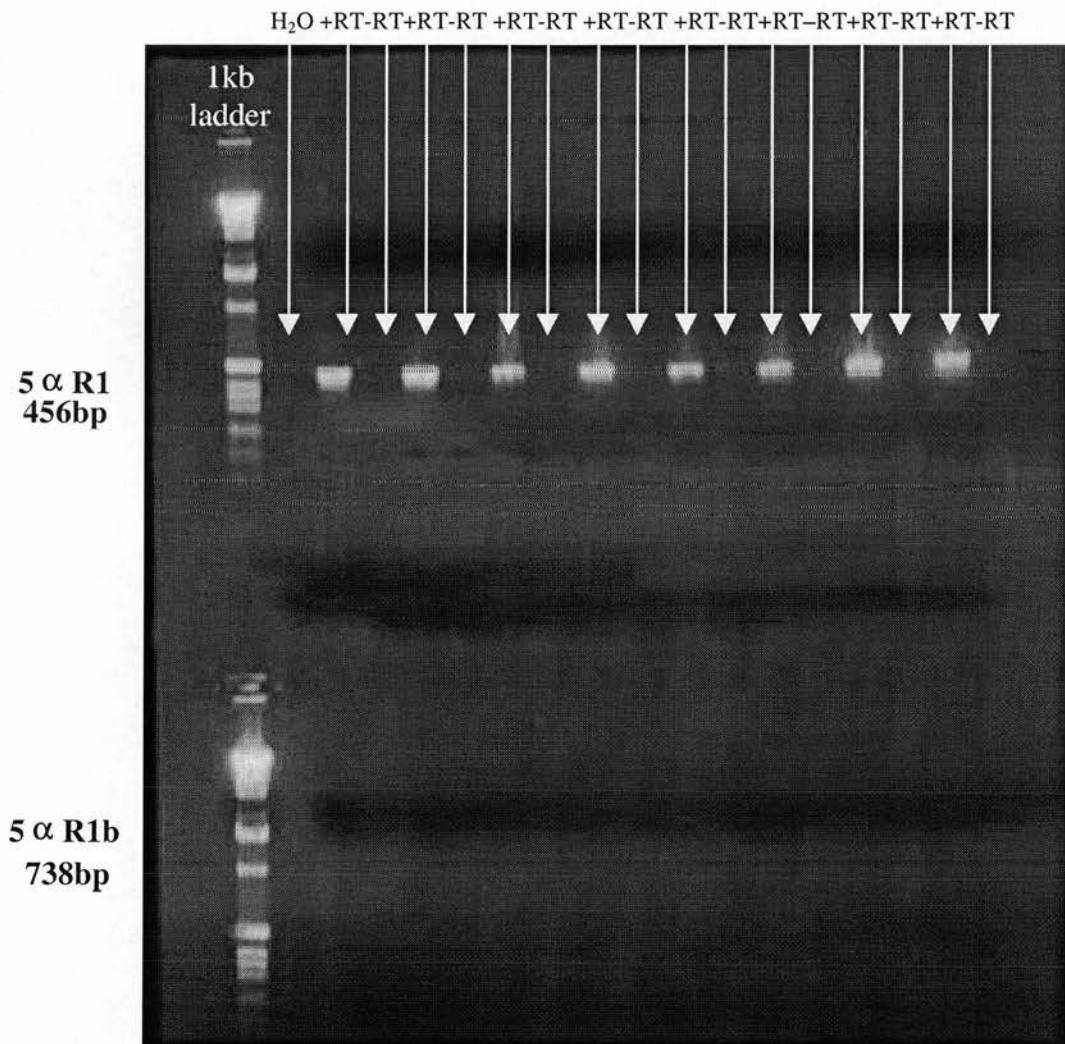
Figure 3.5: Expression of 5α-reductase Type 1 and 5α-reductase Type 1b mRNA in rat liver

Figure shows the presence of 5α-reductase Type 1 but not 5α-reductase Type 1b in lean male Zucker rat liver (n=8). 1μg of total RNA isolated from liver was used in RT-PCR with specific primers to detect expression of 5α-reductase type 1 and 5α-reductase type 1b. Negative controls included an RT reaction containing no RNA (H₂O) and a reaction carried out in the absence of the RT enzyme for each RNA (-RT).
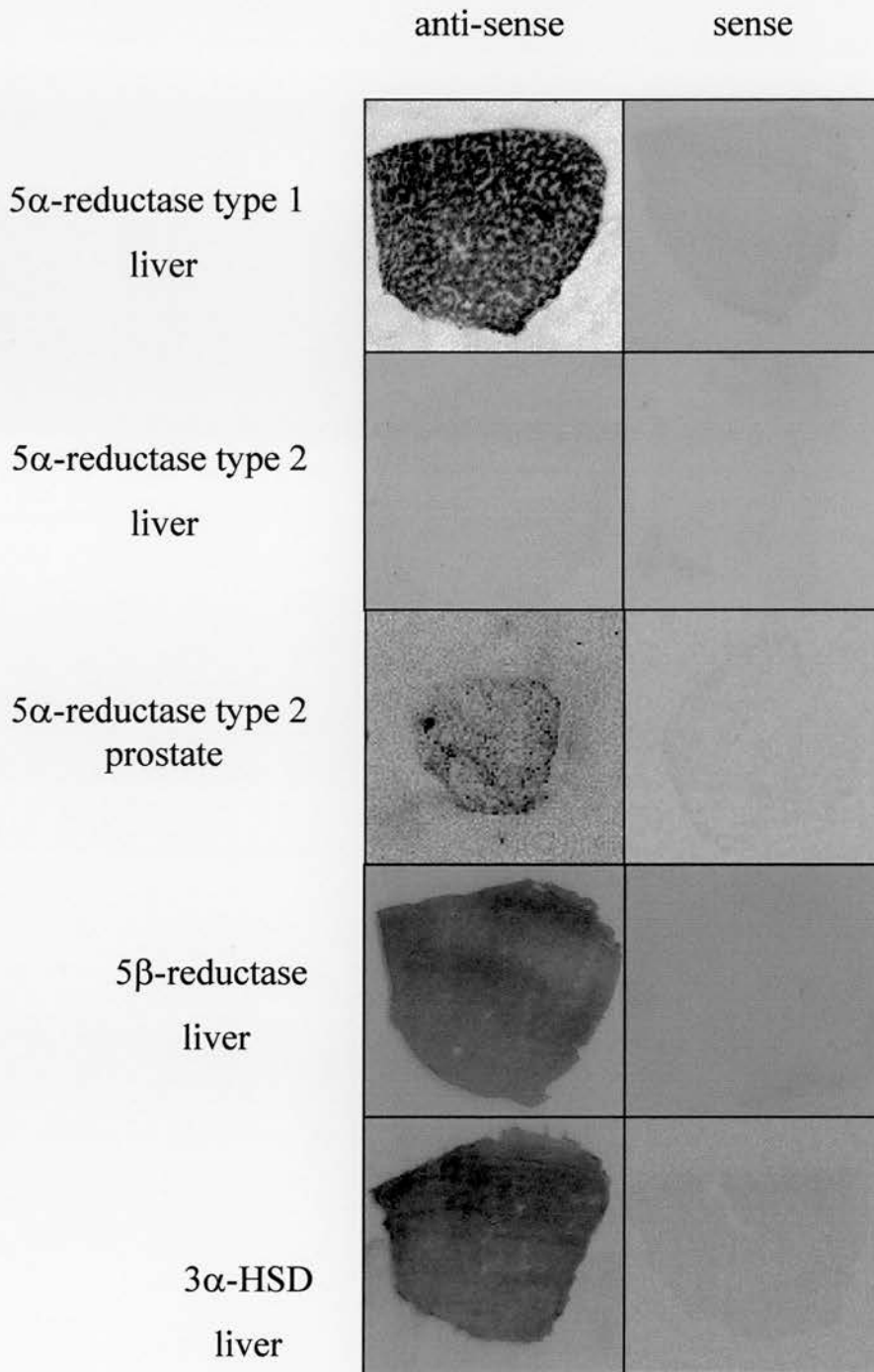
Figure 3.6: A-ring Reductase mRNA Expression in Rat Liver

Anti-sense ^{35}S-labeled ribo-probes (left hand pictures in each panel) specific for rat 5α-reductase type 1 and 2, 5β-reductase and 3α-HSD were used to detect mRNA in 10μm thick sections of liver and prostate from male Wistar rats by *in situ* hybridisation. Sense probes (right hand pictures in each panel) were used to confirm the absence of non-specific hybridisation. Following *in situ* hybridisation, slides were exposed to Kodak autoradiograph film for one-three days.
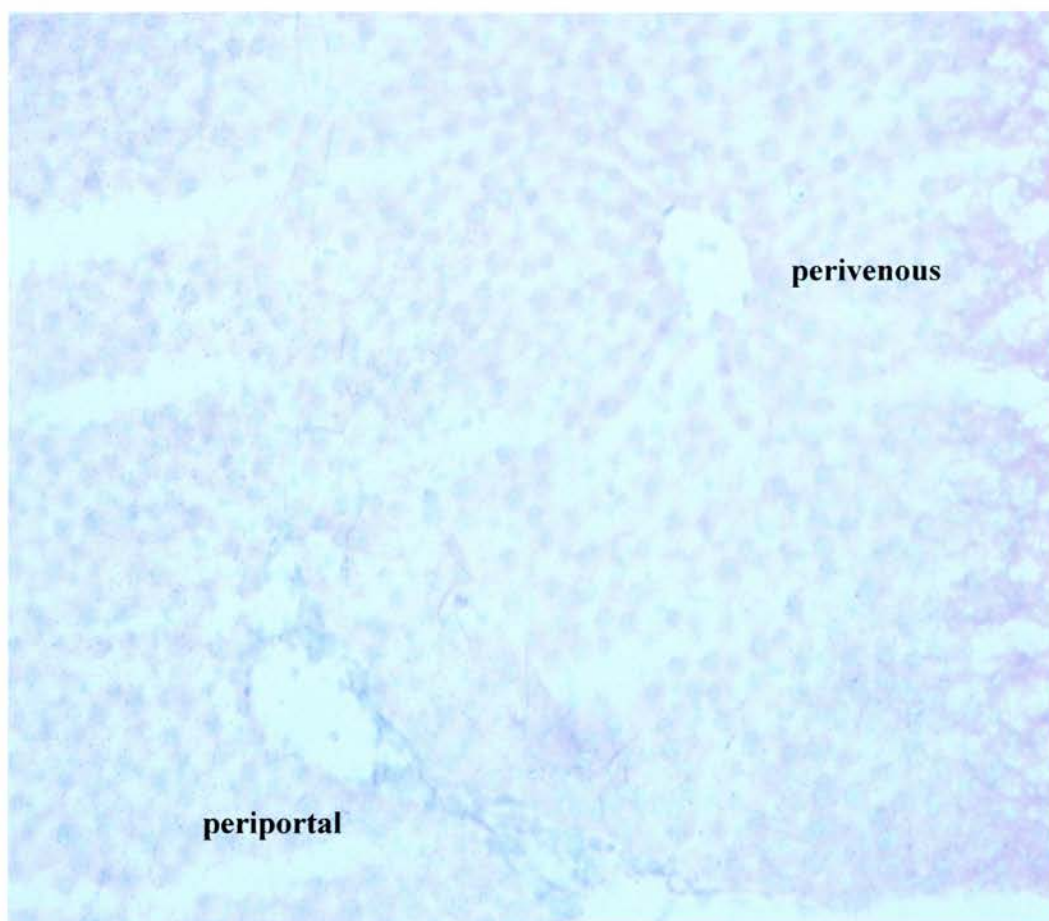
Figure 3.7: Periportal 5α-reductase type 1 distribution

Figure shows representative photomicrograph (original magnification x100) showing *in situ* mRNA hybridisation to frozen sections of rat liver.

Note higher density of silver grains in the periportal area compared to the perivenous area for 5α-reductase type 1 mRNA.
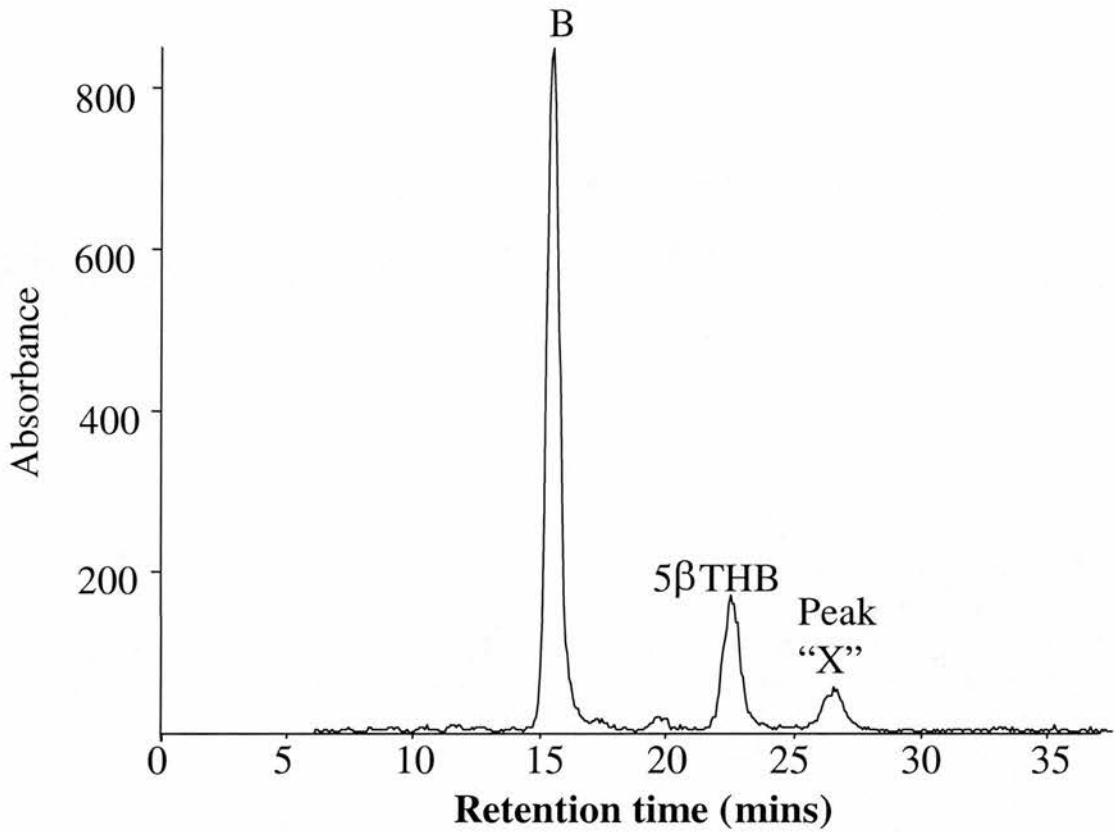
Figure 3.9: Chromatogram of 5β-reductase activity in liver cytosol

Following assay for 5β-reductase activity, steroid extracts were re-suspended in mobile phase consisting of 60% water, 15% acetonitrile, 25% methanol and β-cyclodextrin (1mM) and injected into the HPLC system. The flow rate of the mobile phase was 0.7ml/ min, flow rate of the scintillant was 1.4ml/ min and the column temperature was set to 35°C. The approximate retention times for [³H]₄-corticosterone and [³H]₄-5βTHB were 15.48 min and 23.90 min respectively as shown in the example above.

3.4   Discussion

3.4.1 Bioactivity assays

Having optimised the HPLC conditions for separation of A-ring reduced metabolites of corticosterone, the bio-activity assay conditions remained to be determined. The starting point for the development of the assay was an assay used previously to measure 5α-reduction of testosterone and androstenedione in tissues and cells (Mahendroo *et al.* 2001; Reichert *et al.* 2001).

In our assay we attempted to quantify the enzyme in liver homogenates by measuring the conversion of $[^3H]_4$-corticosterone to $[^3H]_4$-5α-dihydro- and tetrahydro-corticosterone and $[^3H]_4$-5β-dihydro- and tetrahydro-corticosterone as described in section 3.2.2. An assay for 5β-reductase was successfully developed but unfortunately, 5α-reduced metabolites were not detected under these assay conditions. These assay conditions were then repeated using several different time-courses, protein concentrations and co-factor concentrations but 5α-reductase activity could not be detected.

Fresh tissue homogenates and fresh liver slices were then used because the initial assay was carried out with frozen tissue and it was thought that the enzyme might have been degraded as it is known to be extremely labile (Eicheler *et al.* 1995). Again only 5β-reductase activity could be measured.

5α-Reductase is a microsomal enzyme and therefore we tried the assay in microsomes and different sub-cellular fractions of liver including the nuclear fraction, mitochondria, heavy microsomes and cytosol. The only activity that was detected in any of these preparations was 5β-reductase activity in cytosol which would be expected as it is a cytosolic enzyme. As a positive control, the assay was performed in prostate using corticosterone and testosterone as substrates. 5α-Reduction of testosteronebut not corticosterone could be measured in prostate.

To try to preserve 5α-reductase activity, glycerol was added to the tissue sample after homogenisation, and trypsin and protease inhibitors were used in the buffers to prevent degradation of the enzyme. Finally, an NADPH generating system was used as a constituent of the incubation buffer to generate more co-factor for the enzyme. as this had previously been used in synthesis of aldosterone metabolites (Morris *et al.* 1982) and had been used successfully in our laboratory in prostate assays. None of these modifications resulted in detection of 5α-reductase metabolism of corticosterone.

Isolated hepatocytes have been shown to be a model system for studying steroid metabolism (Morais & Wagner 1985). 5α-Reductase activity was measured in isolated hepatocytes, however this preparation was not reliable because on many occasions, 5α-reductase activity was overwhelmed by 11β-HSD1 activity converting corticosterone to 11-dehydrocorticosterone (A). This increased formation of A is recognised as a sign that the hepatocytes are no longer viable (Jamieson *et al.* 1995) and will attenuate 5α-reductase activity because it will have to compete with 11β-HSD1 for substrate. To overcome the problem of cell viability we decided to use a cell-line. After screening several different cell-lines the rat hepatoma H4IIE cell-line was chosen. 5α-Reductase type 1, 5β-reductase and 3α-hydroxysteroid dehydrogenase mRNA were detected in H4IIE cells by RT-PCR. 5α- and 5β-reduced tetrahydro- metabolites of corticosterone could not be detected by UV detection due to low concentrations but were detected by GCMS in H4IIE cells after overnight incubation with corticosterone.

Therefore, we have managed to establish optimal conditions for measuring 5β-reduction of corticosterone in rat liver cytosol using fresh or frozen tissue. In all of our assays, only the tetra-hydro metabolites were detected because 3α-HSD reduces the dihydro-metabolites to tetrahyro-metabolites very rapidly. However, 5α-reductase activity could not be measured in this way. Many studies with 5α-reductase have been hampered previously because of the extreme insolubility of the protein (Moore & Wilson 1972) and its very strong membrane association (Houston *et al.* 1985; Enderle-Scmitt *et al.* 1989). The fact that 5α-reductase activity could be

identified by GCMS and not by UV detection confirms previous reports that 5α-reductase is expressed in low levels because GCMS is a much more sensitive tool than UV-detection.

3.4.2 Isozyme expression

RT-PCR and in situ hybridisation studies have confirmed the previously reported differences in tissue distribution between the different isozymes of 5α-reductase (Normington & Russell 1992). Only the type 1 isozyme was expressed in liver. The expression of the alternative, elongated isoform of 5α-reductase type 1 in rat liver could not be detected by RT-PCR. This alternative isozyme has only been reported once in rat liver (Lopez-Solache *et al.* 1996) therefore it was not possible to include a positive control for the expression of this transcript. It is thought that the reported elongation is an inverted repeat of another part of the sequence and was induced experimentally.

5α-Reduction of steroids has also been identified in adipose tissue however it was not known which isozyme was responsible. Here we confirm that 5α-reductase type 1 and 3α-hydroxysteroid dehydrogenase were expresed in omental and subcutaneous adipose tissue but 5α-reductase type 2 and 5β-reductase were not.

Having determined the presence of 5α-reductase, 5β-reductase and 3α-HSD in rat liver, it was important to confirm the specific cellular localisation of these enzymes. *In situ* hybridisation was used to investigate expression of A-ring reductases in the liver at the level of gene transcription. The expression of 5α-reductase showed marked zonation with high expression in the periportal zone of the liver. The expression of many hepatic proteins is dependent on their relative position within the liver acinus. While hepatocytes in different parts of the liver acinus appear similar, cells located in the periportal region have a functional capacity different from those surrounding the central vein, leading to "metabolic zonation" of the liver (Jungermann & Kietzmann 1996). These differences are due to gradients in

concentrations of substrates and hormones, related to the passage of blood from the periportal to the perivenous zone.

Functions of the liver which are zonally distributed include glucose metabolism, amino acid utilisation, bile formation and xenobiotic metabolism e.g PEPCK is the rate-limiting enzyme of gluconeogenesis, and is expressed at higher levels in the periportal than the perivenous zones of the liver (Bartels *et al.* 1993). Insulin receptor protein is predominantly located perivenously (Krones *et al.* 2000) and glucose uptake and glycolysis occur perivenously (Lindros 1997). The glucocorticoid receptor has been found to be homogenously (Antakly & Eissen 1984) or periportally distributed (Nyirenda *et al.* 1998) .

5β-reductase and 3α-HSD did not show any marked zonation of mRNA expression. The best known activity of 5β-reductase is the formation of bile acids (Berseus 1967). 3α-HSD is the major bile acid-binding protein and is involved in the *de-novo* synthesis and metabolism of bile acids (Danielsson & Sjovall 1975). Bile acid processing has been shown to be greater in the pericentral than in the periportal region of the liver, therefore the uniform expression of 5β-reductase and 3α-HSD probably reflects their roles in bile acid synthesis and metabolism in addition to their role in steroid hormone metabolism.

We have confirmed that 5α-reductase type 1 but not 5α-reductase type 2 is expressed in liver and adipose tissue. 3α-hydroxysteroid dehygrogenase is also expressed in these tissues. We have shown that 5α-reductase type 1, 5β-reductase and 3α-hydroxysteroid dehygrogenase can work *in vivo* as measured by GCMS. However, although we can measure mRNA expression levels, activity of these enzymes can not always be measured.

Chapter Four

Activation of the Glucocorticoid Receptor by 5α-Reduced Glucocorticoids

4.1 Introduction

The rate-limiting step in glucocorticoid inactivation is the reduction of the $\Delta^{4.5}$ double bond in the A-ring of the steroid structure. This can be carried out by either 5α- or 5β-reductase. The resultant dihydro-metabolites are then reduced further by 3α-hydroxysteroid dehydrogenase (3α-HSD) to form tetrahydro-metabolites. As discussed in chapter 1, two genes encoding 5α-reductase have been identified in rats (Normington & Russell 1992) and these isozymes are referred to as 5α-reductase Type 1 and Type 2. 5α-reductase Type 1 predominates in androgen independent tissues such as the liver and skin, whereas 5α-reductase Type 2 is the predominant isozyme in reproductive tissues (Andersson *et al.* 1991).

5α-reduction of glucocorticoids has been accepted as a pathway of irreversible inactivation. However, 5α-reduced metabolites of other steroids have been shown to be more potent in activity than their parent hormone. 5α-Reductase Type 2 catalyses the conversion of testosterone to dihydrotestosterone (DHT) which is a more potent androgen than testosterone and can bind preferentially to androgen receptors and activate them (Siiteri & Wilson 1974). Aldosterone metabolism also results in active metabolites. Aldosterone is converted to 5α-dihydroaldosterone and this is subsequently converted to 3α, 5α-tetrahydroaldosterone. Experiments with rat kidney has demonstrated that these metabolites possess mineralocorticoid activity (Kenyon *et al.* 1985; Gorsline *et al.* 1986). The progesterone metabolites, 5α-dihydroprogesterone and allopregnanolone are also active and allopregnanolone binds to the non-nuclear GABA$_A$ receptor (Rapkin *et al.* 1997).

Rat liver contains two distinct types of glucocorticoid binding sites; 1) the high affinity glucocorticoid receptor (GR) which is located in the cytosol and in the cell nucleus (Antakly & Eissen 1984) and 2) the low affinity glucocorticoid binding sites (LAGS) which are present in the microsomal fraction (Ambellan *et al.* 1981) and in the nuclear envelope (Kaufmann & Shaper 1984; Roszak *et al.* 1990).

The aims of this chapter were to:

1) determine whether 5α- and 5β- reduced metabolites of corticosterone can bind to GR.

2) determine whether 5α- and 5β- reduced metabolites of corticosterone can activate GR *in vitro*, *ex vivo* and *in vivo*.

4.2 Methods

4.2.1 Assay of glucocorticoid receptor binding activity

The binding affinities of the A-ring reduced metabolites of corticosterone for the glucocorticoid receptor were determined by competition binding experiments as described in chapter 2.12. The ability of the A-ring reduced metabolites of corticosterone to displace tritiated dexamethasone from binding sites in isolated hepatocytes, microsomes and cytosol from lean male Zucker rats was determined by incubation with increasing concentrations of the potential competing steroids.

4.2.2 Glucocorticoid receptor activation in cell lines

HeLa or H4IIE cells were cultured as described in section 2.4.

a) For assessment of glucocorticoid receptor activation, HeLA cells were transfected with human GR and a luciferase reporter gene linked to the mouse mammary tumour virus long terminal repeat (MMTV-LTR) which contains several GREs by calcium phosphate coprecipitation as described in chapter 2.13. Three different transfection experiments were carried out as follows:

1) To determine whether A-ring reduced metabolites could activate GR, transfected HeLa cells were incubated with 1µM steroid.

2) To examine the dose-response relationships of corticosterone and 5αTHB, transfected HeLa cells were incubated with increasing concentrations of steroid (0-1µM).

3) To determine whether 5αTHB was a full or partial agonist, transfected HeLa cells were incubated with 5αTHB alone or in combination with corticosterone (50nM).

b) Induction of tyrosine aminotransferase (TAT) – (a gene whose transcription is induced by activated GR, (Granner & Hargrove 1983)) mRNA, was investigated in

H4IIE cells. Cells were treated overnight with steroid (1µM) with/without the glucocorticoid receptor antagonist RU486 (1µM). Total RNA was prepared using the TRIzol® method as described in chapter 2.7.2 and separated on a 1.2% agarose formaldehyde denaturing gel and mRNA was quantified by Northern blot as described in chapter 2.9.

4.2.3 Metabolic transformation of steroids

Metabolism of steroids by HeLa and H4IE cells was examined by GCMS as described in chapter 2.6.

4.2.4 *Ex vivo* glucocorticoid activity of 5αTHB

The ability of 5αTHB to suppress angiogenesis in C57/Bl6 mouse aortic rings was examined ex vivo as described in chapter 2.14.

4.2.5 *In vivo* glucocorticoid activity of 5αTHB

An experiment was carried out to assess in vivo glucocorticoid activity of 5αTHB. In this experiment 1) suppression of ACTH was measured and 2) hepatic TAT and PEPCK mRNA expression was measured.

Groups of six, 6-8 week old male lean Zucker rats were bilaterally adrenalectomised through dorsal incisions under halothane anaesthesia and maintained on 0.9% saline drinking water for one week before steroid treatment. Corticosterone, 5αTHB (5mg/kg body weight) or vehicle (saline with 10% ethanol and 10% DMSO) were administered by i.p. injection at 0900h.

1) Blood samples were taken on ice by tail-tip at 0,60 and 120 min post-injection and plasma separated immediately and stored at -80°C. Plasma ACTH and corticosterone concentrations were determined by radioimmunosassay as described in chapter 2.15.

2) Rats were sacrificed 6h post-injection and livers removed, snap-frozen and stored at -80°C. Total RNA was prepared using the TRIzol® method as described in

chapter 2.7.2 and separated on a 1.2% agarose formaldehyde denaturing gel and mRNA was quantified by Northern blot as described in chapter 2.9.

4.2.6 Statistics

All data are expressed as mean ± standard error and data were statistically analysed by Analysis of Variance followed by LSD post-hoc tests, n=6 for all groups.

4.3 Results

4.3.1 Glucocorticoid receptor binding in isolated hepatocytes

In isolated hepatocytes from male lean Zucker rats, the 5α-reduced metabolites, 5α-DHB and 5α-THB displaced tritiated dexamethasone with a similar affinity as corticosterone. The Kd value for 5α-THB (258 ± 78nM) was not significantly different from that of corticosterone (158 ± 43nM) (Table 4.1 & Figures 4.1 and 4.2). The 5β-reduced metabolites were ineffective in displacing dexamethasone at these concentrations (5β-DHB, Kd 7798 ± 1901nM, 5β-THB, Kd 3590 ± 802nM). 11-Dehydrocorticosterone and its 5α-reduced metabolites, 5α-DHA and 5α-THA did not displace dexamethasone from its binding sites.

4.3.2 Glucocorticoid receptor binding in cytosol

In cytosol, 5αDHB (Kd, 133 ± 77.5 nM) bound with a similar affinity as corticosterone (Kd, 161 ± 109 nM) (Table 4.1 & Figure 4.2), however the Kd value for 5αTHB was much higher than for binding in hepatocytes and was significantly different from that of corticosterone (5αTHB; Kd, 8580 ± 4230nM). The 5β-reduced metabolite, 5βDHB (Kd, 384 ± 174 nM) also displaced tritiated dexamethasone with a similar affinity as corticosterone but 5βTHB was again ineffective at these concentrations (Kd, $5628. \pm 2019$ nM).

4.3.3 Glucocorticoid receptor binding in microsomes

In the microsomal fraction, 5αDHB displaced tritiated dexamethasone (Kd, 205 ± 12nM) with a greater affinity than corticosterone (5αDHB; Kd, $153 \pm 16.$ nM vs corticosterone; Kd, 246 ± 29 nM) and 5α-THB displaced tritiated dexamethasone with a similar affinity as corticosterone. The Kd value for 5α-THB (154 ± 43nM) was not significantly different from that of corticosterone (Table 4.1 & Figure 4.2). The 5β-reduced metabolites were again ineffective at these concentrations (5βDHB; Kd, 5740 ± 1900 nM, 5βTHB; Kd, 8275 ± 4058 nM).

4.3.4 Activation of Glucocorticoid Receptors in Transfected HeLa cells.

In HeLa cells, transiently transfected with human GR and a luciferase reporter construct linked to the MMTV-LTR, both corticosterone and 5α-reduced corticosterone metabolites stimulated luciferase activity. The 5β-reduced metabolites of corticosterone did not stimulate luciferase activity (Figure 4.3). None of the steroids induced luciferase activity in the absence of transfected GR (Figure 4.3).

The dose response curves for 5αTHB and corticosterone were different at low concentrations with 5αTHB eliciting a lesser response at lower concentrations than corticosterone (Figure 4.4). At 1μM concentrations, the responses of the two steroids were not different although it was not possible to measure maximal activation since cell death was observed at higher concentrations (>2μM) of active steroids.

To test whether 5αTHB was a full or partial agonist at glucocorticoid receptors, 5αTHB was added to the incubation medium with corticosterone. The resulting luciferase activity was not different from the sum of the activities when each steroid was added on its own, indicating full agonist activity (Figure 4.5).

4.3.5 Induction of tyrosine aminotransferase mRNA in H4IIE cells.

To confirm that 5α-reduced glucocorticoids activate GR and to test the effect on endogenous gene expression, the amount of mRNA encoding the liver-specific glucocorticoid-inducible gene, TAT was quantified in liver-derived H4IIE cells after 16h of incubation with steroids. TAT mRNA expression was induced by 5αDHB and 5αTHB albeit to a lesser extent than by corticosterone (29% and 46% response of corticosterone at maximum respectively) (Figure 4.6). Incubation of H4IIE cells with the GR antagonist RU486 had no effect alone and prevented TAT induction by 5αTHB (Figure 4.7).

4.3.6 Metabolic transformation of steroids

By GCMS, corticosterone was not detected in the medium from HeLa or H4IIE cells to which 5αTHB had been added and in HeLa cells 5αTHB was not detected in the medium to which corticosterone had been added. The level of detection for steroids was < 1nM.

4.3.7 Inhibition of angiogenesis *ex vivo*

Both corticosterone and 5αTHB inhibited angiogenesis (Figure 4.8 and 4.9). The glucocorticoid receptor antagonist RU486 did not significantly affect angiogenesis on its own, but eliminated the angiostatic effects of both corticosterone and 5αTHB (Figure 4.8)

4.3.8 Suppression of plasma ACTH *in vivo*

Basal plasma ACTH levels were not different between groups and were high, consistent with prior adrenalectomy (Figure 4.10 (a)). Within one hour following administration of steroid, plasma ACTH levels had been significantly suppressed in B-treated animals compared to a small spontaneous reduction in vehicle treated animals. 5α-THB also suppressed ACTH levels although this was of similar magnitude as suppression by corticosterone, it was not apparent until 2 hours after steroid treatment by which time, ACTH in vehicle treated animals had fallen from baseline.

Plasma corticosterone levels were significantly increased in the corticosterone-treated animals by 1 hour post-injection and remained elevated by 2 hours post-injection. At 6 hours, plasma corticosterone levels in these animals had returned to baseline. Plasma corticosterone levels in vehicle and 5αTHB–treated animals remained low and constant throughout the experiment (Figure 4.10 (b)).

4.3.9 Effect of 5αTHB on hepatic TAT and PEPCK *in vivo*

Injection of corticosterone or 5αTHB did not alter hepatic PEPCK (corticosterone; p=0.77, 5αTHB; p=0.6) (Figure 4.11) and TAT (corticosterone; p=0.35, 5αTHB; p=0.76) (Figure 4.12) mRNA expression.
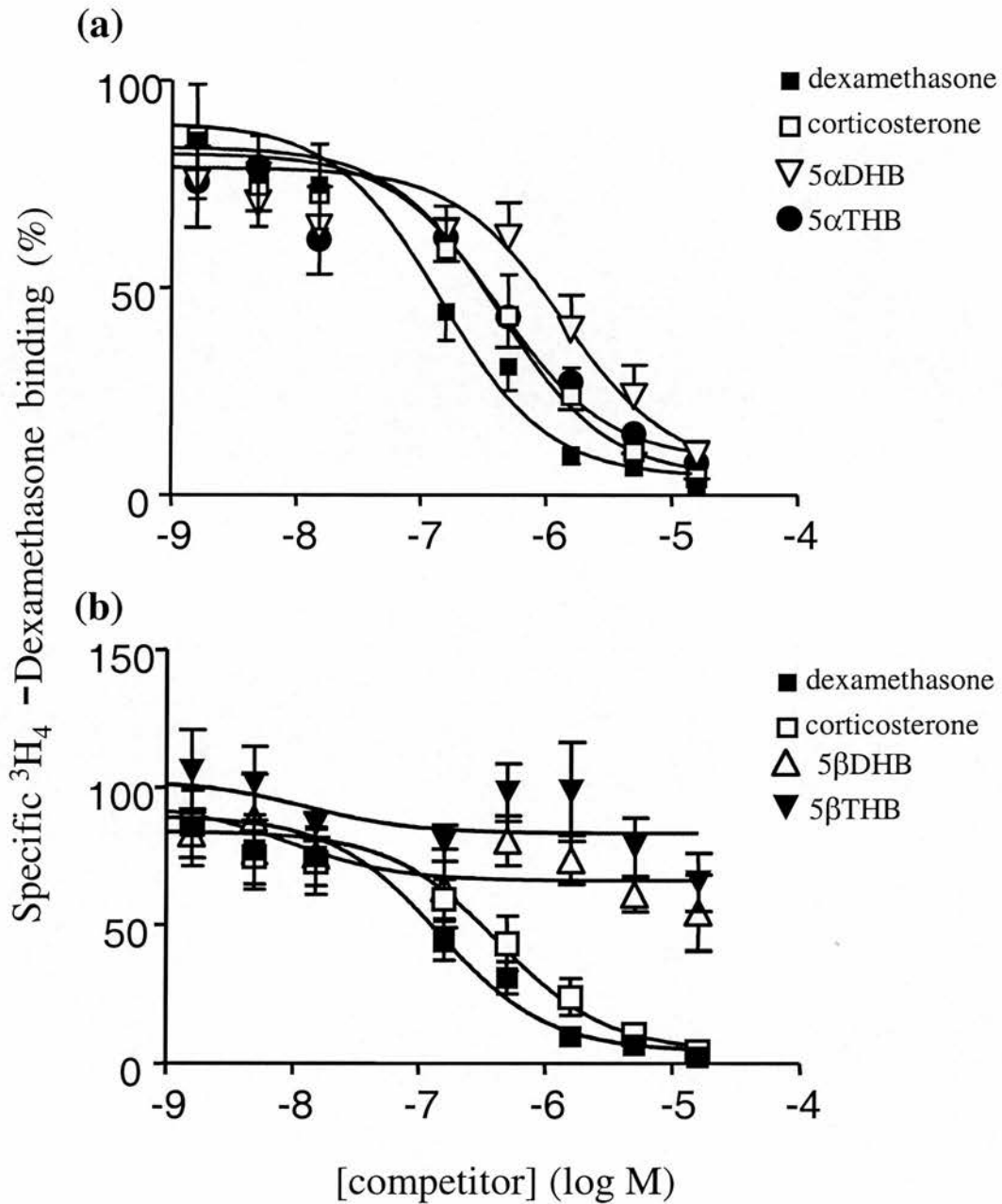
Figure 4.1: Glucocorticoid receptor binding in isolated hepatocytes from male lean Zucker rats

Figures depict displacement of tritiated dexamethasone in isolated hepatocytes by increasing concentrations of competitor steroids. Curves were fitted by Graphpad Prism. Data are mean ± SEM, n=6.

(a) Displacement of tritiated dexamethasone in isolated hepatocytes by increasing concentrations of dexamethasone, corticosterone, 5αDHB and 5αTHB.

(b) Displacement of tritiated Dexamethasone in isolated hepatocytes by increasing concentrations of dexamethasone, corticosterone, 5βDHB and 5βTHB.
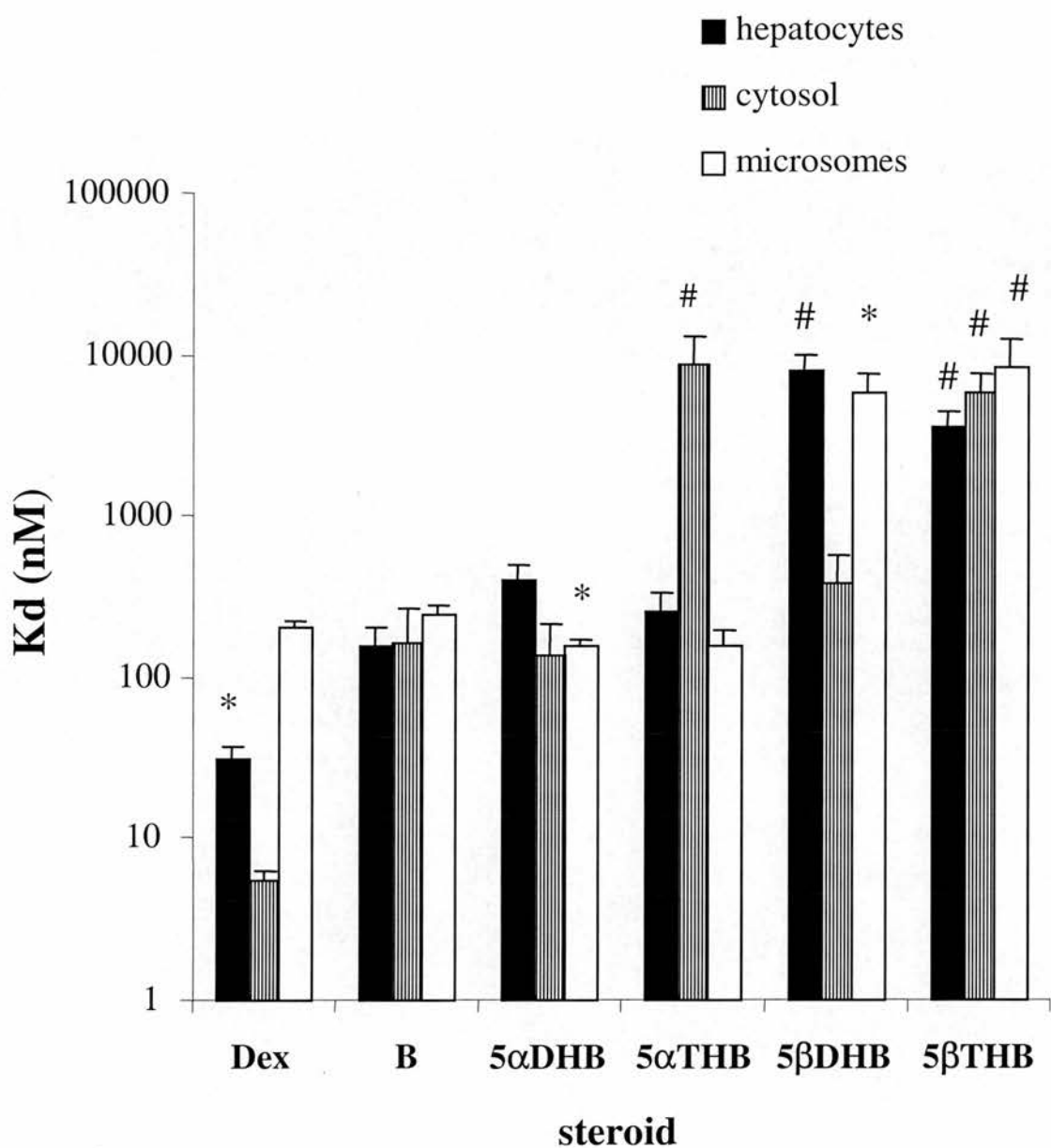
Figure 4.2: Glucocorticoid binding in isolated hepatocytes, cytosol and microsomes from liver of male lean Zucker rats

Figure shows binding affinities of glucocorticoids in isolated hepatocytes, cytosol and microsomes from liver of lean Zucker rats. Data are mean ±SEM, n=6, *p < 0.02 vs B, #p < 0.005 vs B

Steroid	Hepatocytes		Cytosol		Microsomes	
	Kd (nM)	p vs B	Kd (nM)	p vs B	Kd (nM)	p vs B
Dex	37±8	0.002	5.48±0.64	0.18	205±11.8	0.22
B	153±79	-	161±109	-	246±29.4	-
5αTHB	268±78	0.33	8580±4230	0.0005	154±42.9	0.107
5αDHB	336±42	0.02	133±77.5	0.84	153±16.4	0.019
5βTHB	4484±1313	0.001	5628±2019	0.015	8275±4060	0.004
5βDHB	9656±3230	0.001	384±174	0.29	5740±1900	0.016

Table 4.1: Binding affinities of glucocorticoids in isolated rat hepatocytes, cytosol and microsomes.

Data are mean ± SEM, n=6. p vs B indicates whether the Kd for the steroid is significantly different from that of the parent hormone corticosterone.
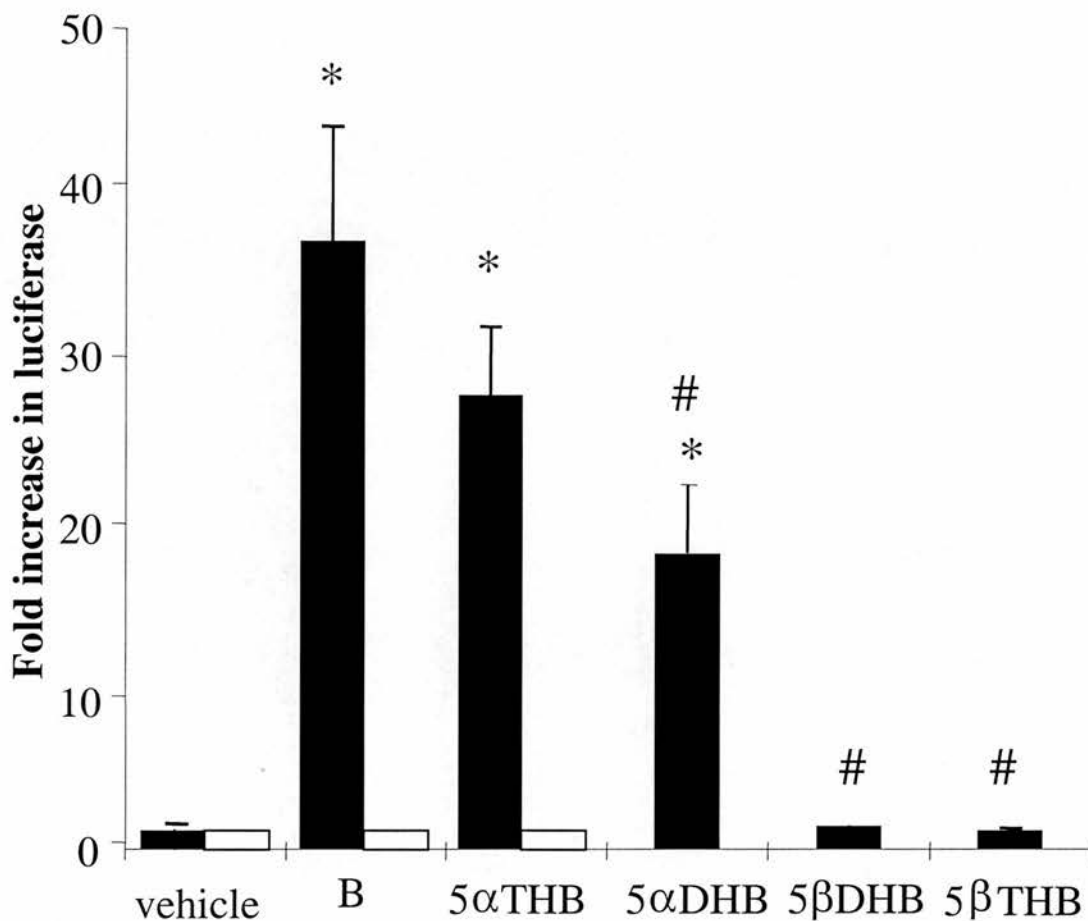
Figure 4.3: Effects of corticosterone metabolites in HeLa cells transfected with human GR and a luciferase reporter linked to the MMTV–LTR.

Effects of individual steroids, showing fold-increases in luciferase activity compared with basal luciferase activity (vehicle). Steroids were incubated at 1μM of B, 5αTHB, 5α-DHB, 5βDHB or 5βTHB. Data shown in open bars represents responses observed in the absence of transfected GR. Data are mean ±SEM, *p<0.05 vs basal luciferase activity, #p<0.01 vs B, n =6.
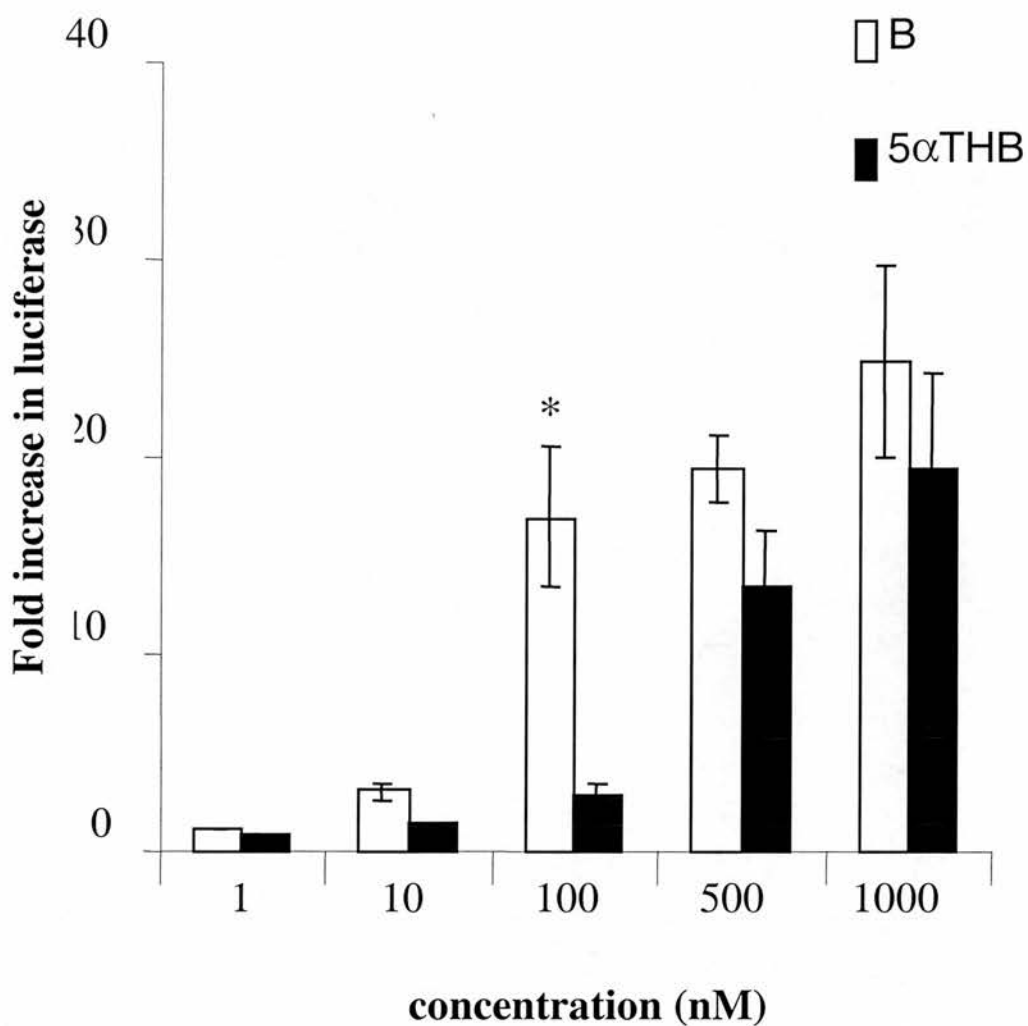
Figure 4.4: Effects of corticosterone metabolites in HeLa cells transfected with human GR and a luciferase reporter linked to the MMTV-LTR.

Figure shows dose response curve for B and 5αTHB. Corticosterone (B) and 5α-tetrahydrocorticosterone (5αTHB) were incubated alone at the final concentrations shown. *p<0.05 vs 5α-THB. Data are mean ± SEM, n=6.
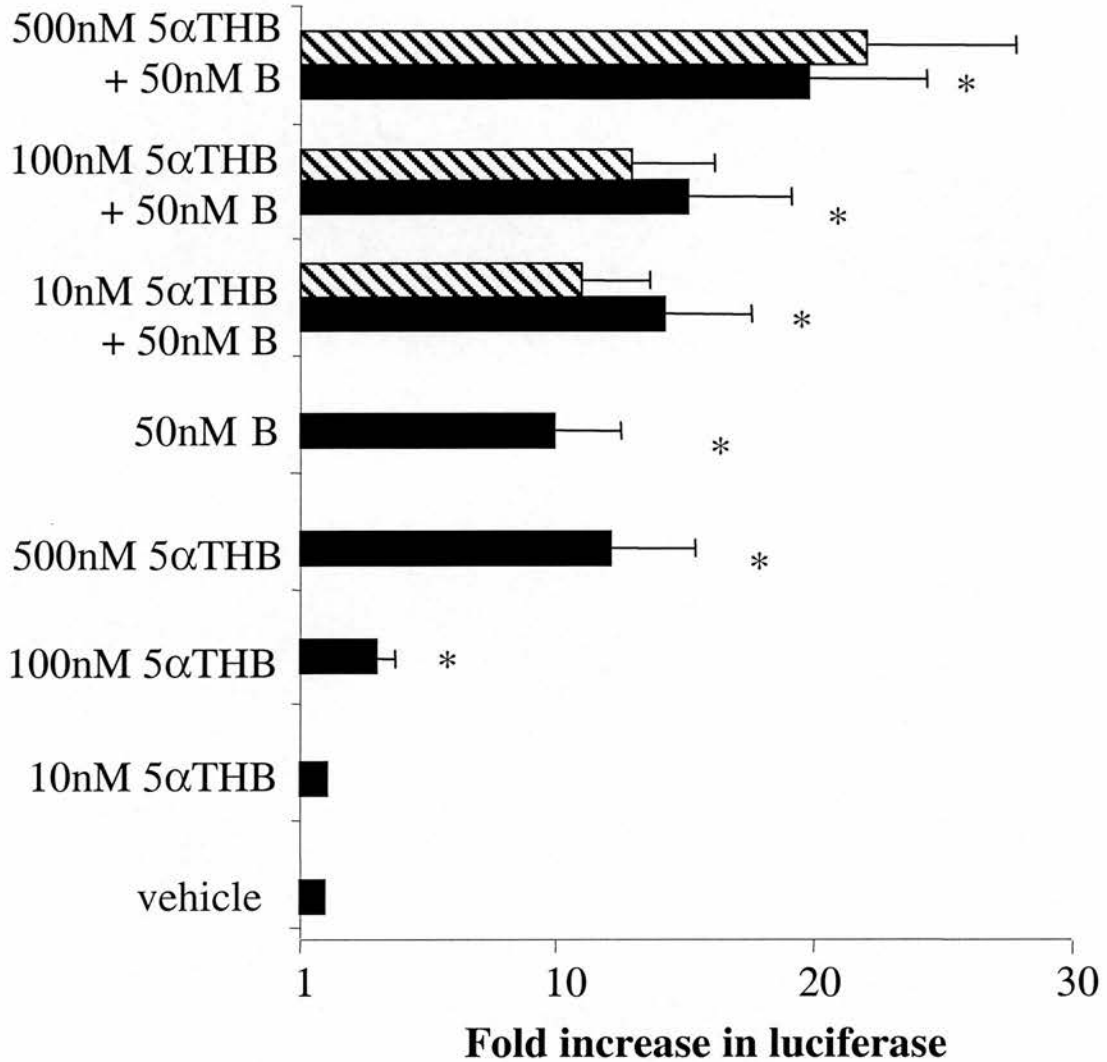
Figure 4.5: Effects of corticosterone metabolites in HeLa cells transfected with human GR and a luciferase reporter linked to the MMTV-LTR.

Additive effect of corticosterone (B) and 5αTHB showing fold-increases in luciferase activity compared with basal luciferase activity (vehicle). All cells were transfected with both the MMTV-luciferase and the GR constructs. B and 5α-THB were incubated alone or in combination at the concentrations shown. For combined steroids, striped bars indicate the predicted luciferase induction, calculated by adding the responses for each steroid when incubate alone. Filled bars indicate the observed actual luciferase induction. There was no difference between predicted and observed induction, consistent with full rather than partial agonist activity of each steroid. *p<0.05 vs no added steroid. Data are mean ± SEM, n=6.
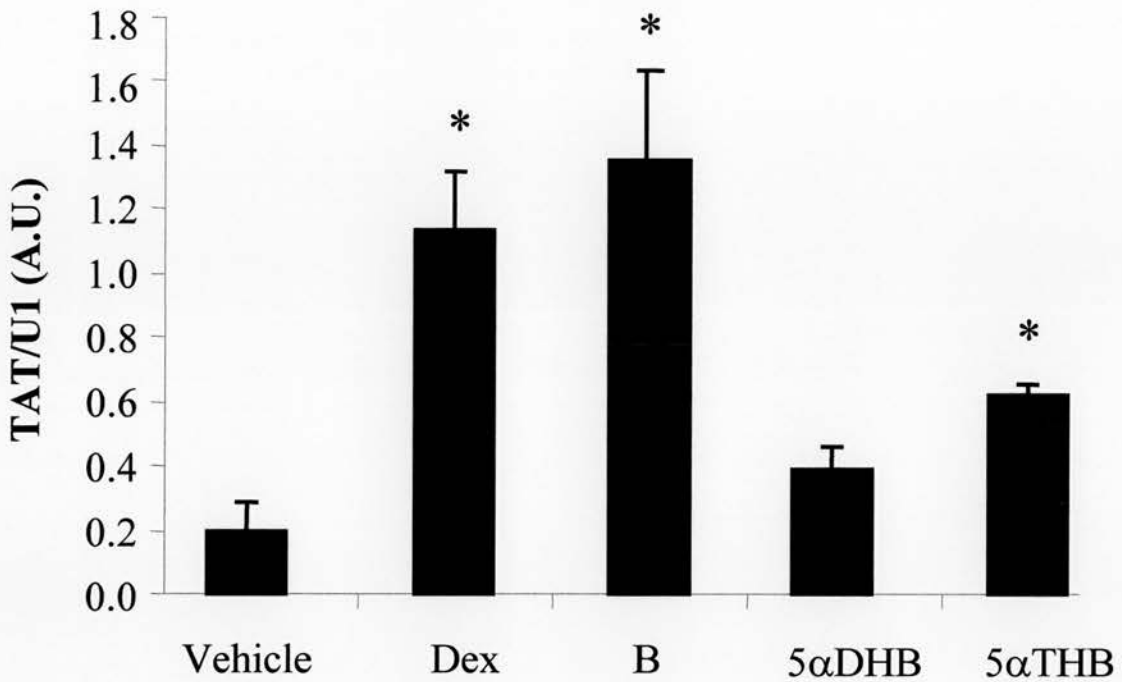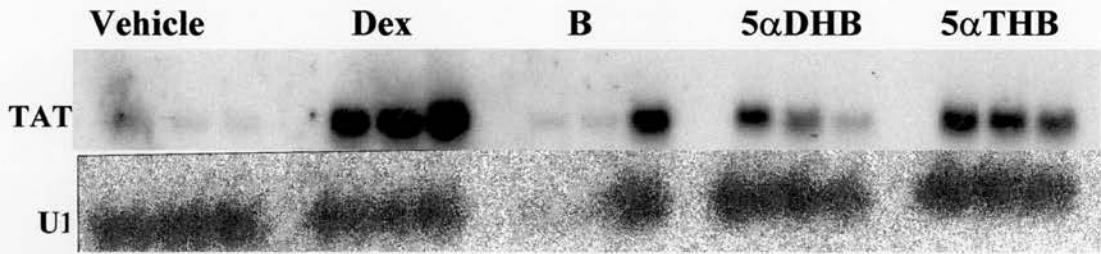
Figure 4.6: Induction of tyrosine aminotransferase (TAT) mRNA by corticosterone metabolites in H4IIE liver-derived cell line

Figure shows TAT mRNA expression was measured by Northern blot in H4IIE cells after 16h incubation with 1μM steroid: corticosterone (B), 5αTHB or 5α-DHB. Values are corrected for loading with U1. A representative blot from one experiment is shown. Data are mean ±SEM, n=6 *p<0.05 vs vehicle.
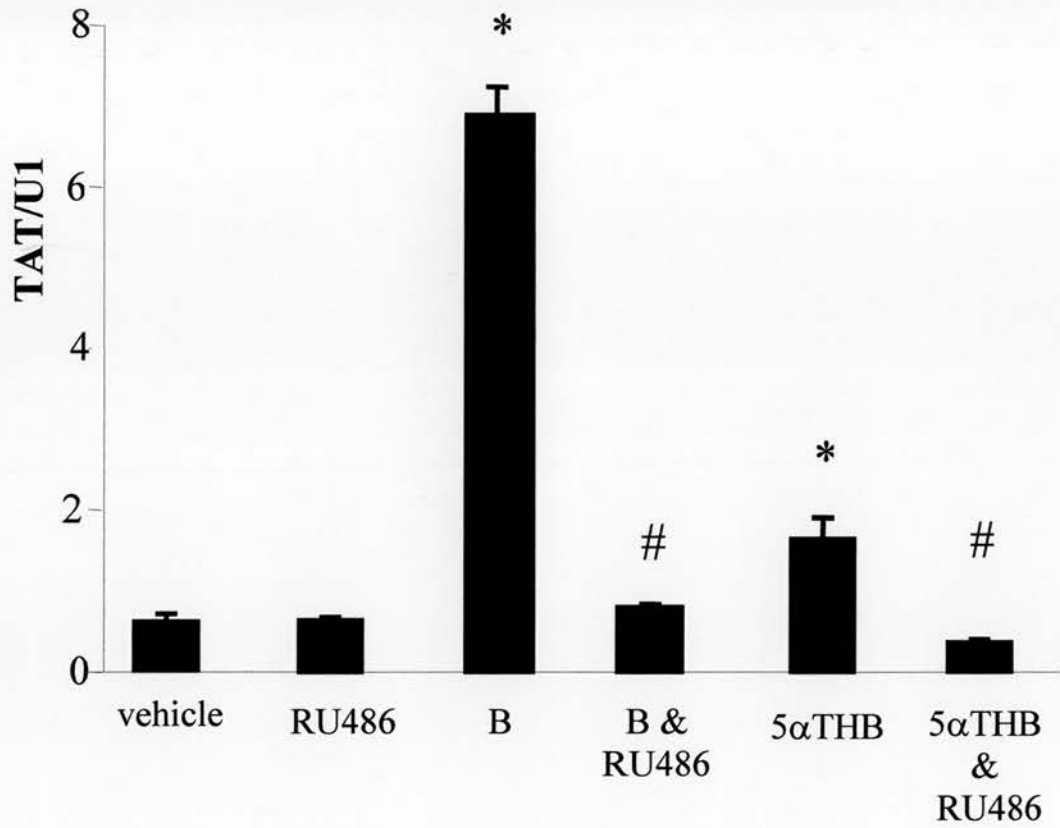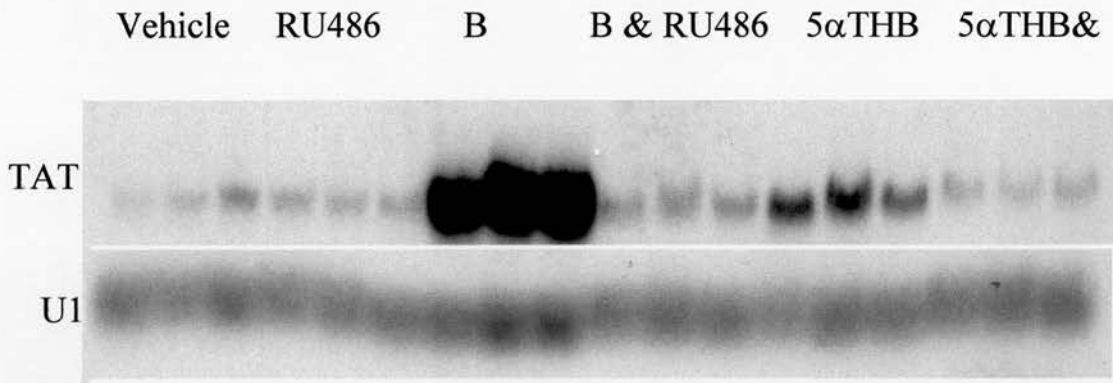
Figure 4.7: Induction of tyrosine aminotransferase (TAT) mRNA by corticosterone metabolites in H4IIE liver-derived cell line

Figure shows the effect of the glucocorticoid receptor antagonist RU486 on TAT mRNA induction by B and 5αTHB. A representative blot from one experiment is shown. Data are mean ± SEM, n=6. *p<0.05 vs vehicle, #p<0.01 vs steroid alone.
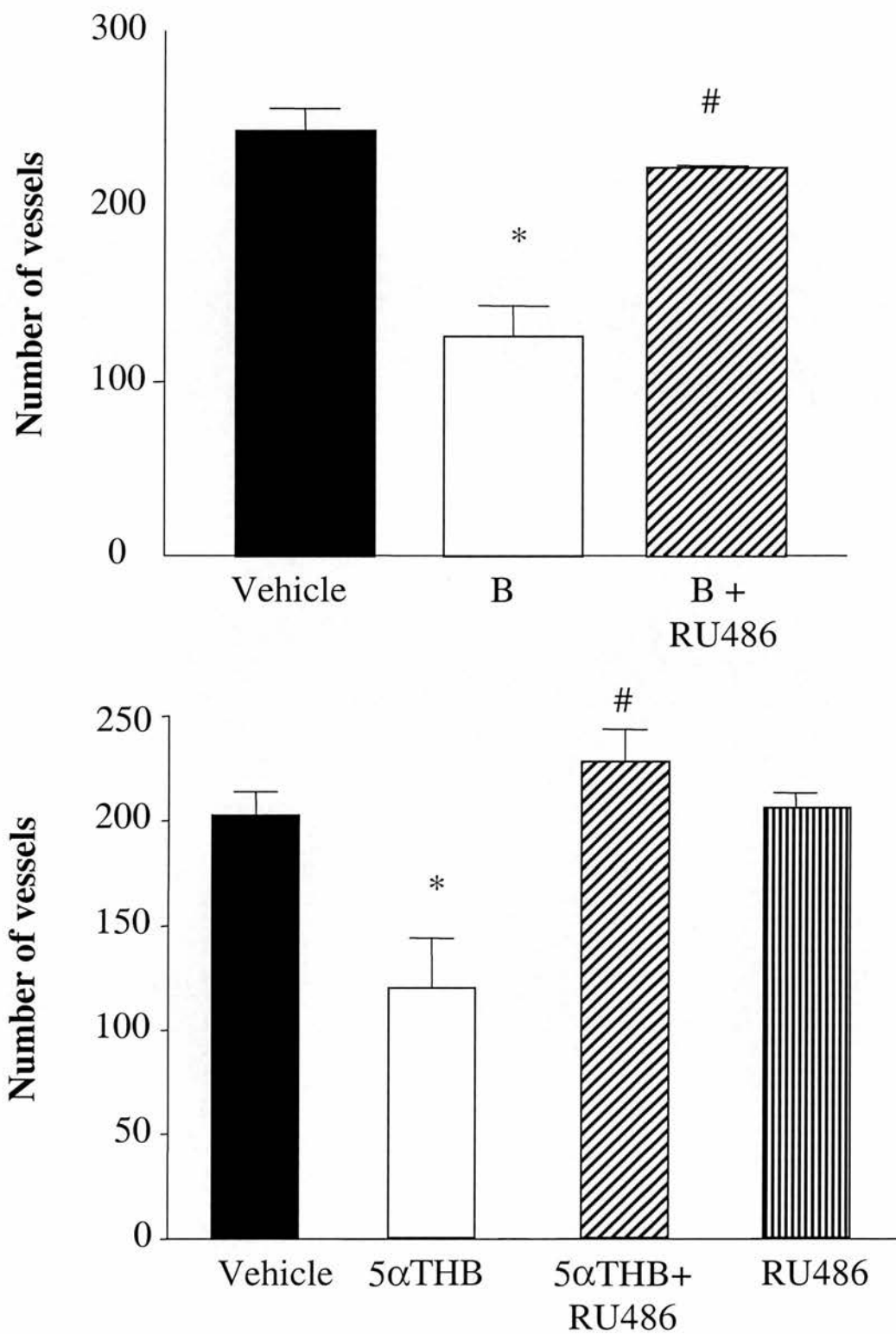
Figure 4.8: Inhibition of angiogenesis by corticosterone and 5αTHB

Vessels were counted after 7 days of incubation with steroids at the concentrations indicated. Data are mean ± SEM, n=6 for the number of vessels indicated in each column, *p =0.02 vs vehicle, #p = 0.001 vs steroid alone.
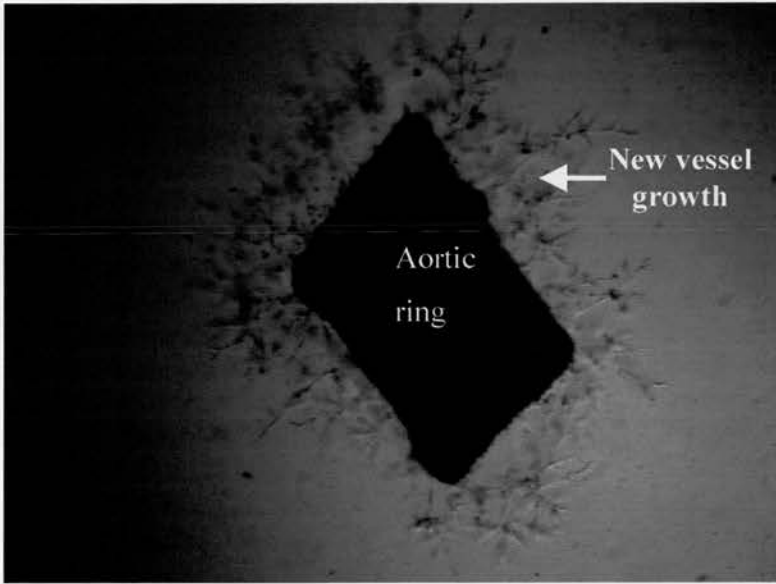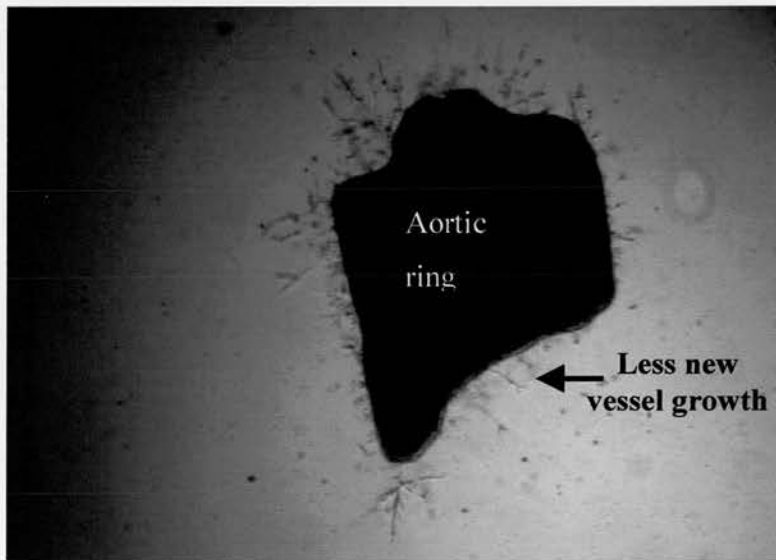
Figure 4.9 Inhibition of angiogenesis by 5αTHB

Figure shows aortic ring (original magnification x 50) after 7 days in culture (a) without 5αTHB and (b) with 5αTHB (1μM). Note fewer new vessels growing around the main aortic ring with 5αTHB treatment.
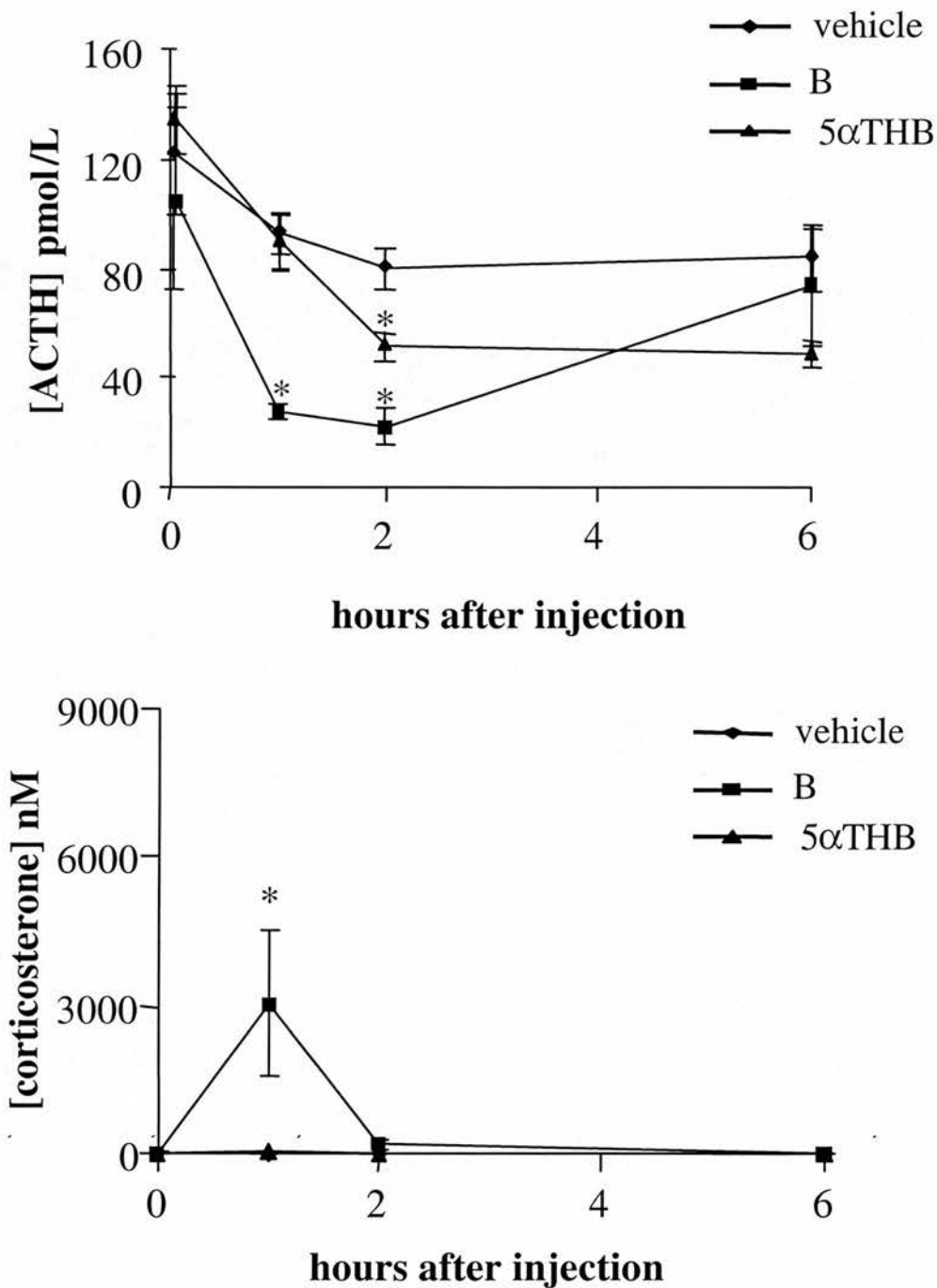
Figure 4.10: Suppression of plasma ACTH by B and 5αTHB *in vivo*

Panel (a). Plasma ACTH was measured in lean male Zucker rats treated with vehicle or 5mg/kg B or 5αTHB by i.p injection. Data are mean ± SEM, *p<0.001 vs vehicle, n=6 in each group.
Panel (b). plasma corticosterone (B) was measured in lean male Zucker rats treated with vehicle or 5mg/kg corticosterone or 5αTHB by i.p injection. Data are mean ± SEM, *p<0.05 vs vehicle, n=6 in each group.
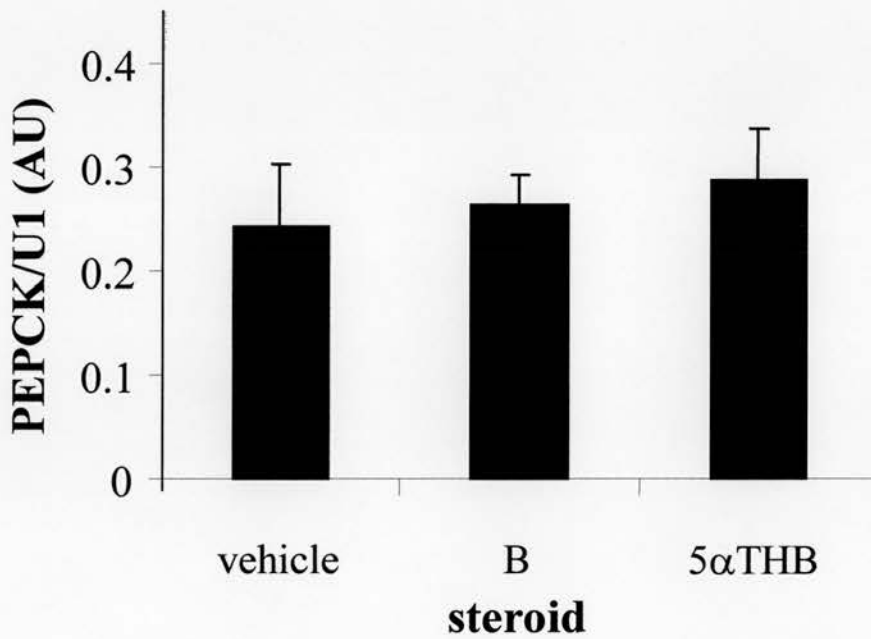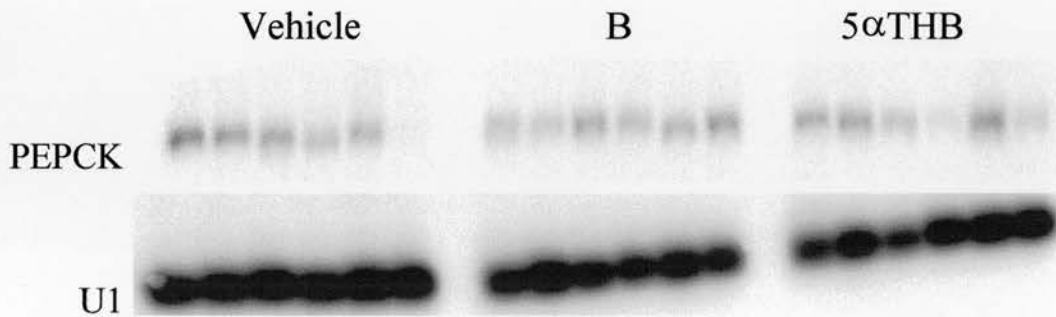
Figure 4.11: Absence of induction of phosphoenolpyruvate carboxykinase (PEPCK) mRNA by corticosterone and 5αTHB in rat liver

Figure shows PEPCK mRNA expression measured by Northern blot in lean male Zucker rats treated with vehicle or 5mg/kg B or 5αTHB by i.p injection. Values are corrected for loading with U1 Data are mean ±SEM, n=6 in each group.
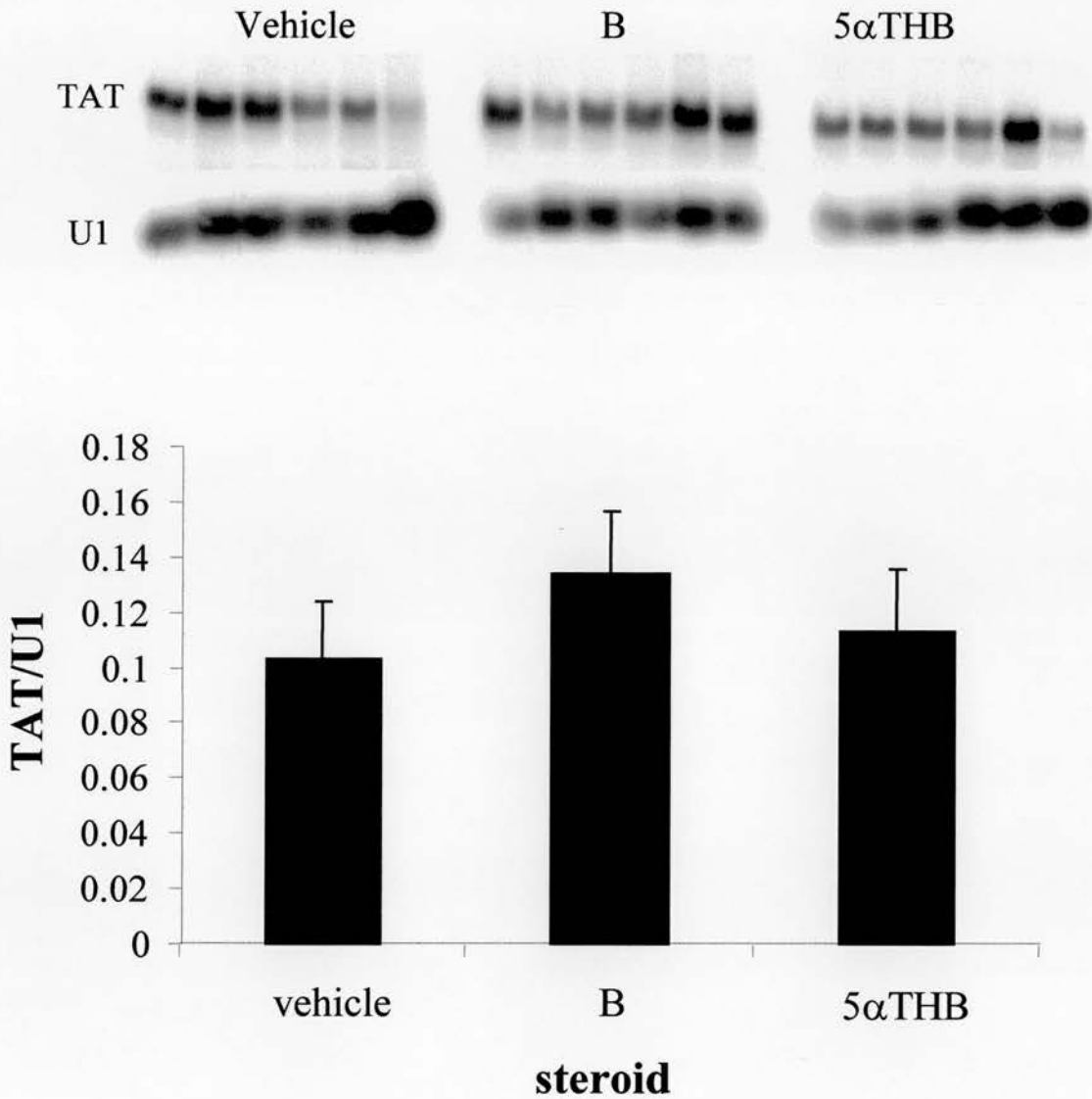
Figure 4.12: Absence of induction of tyrosine aminotransferase (TAT) mRNA by corticosterone and 5αTHB in rat liver

Figure shows TAT mRNA expression measured by Northern blot in lean male Zucker rats treated with vehicle or 5mg/kg B or 5αTHB by i.p injection. Values are corrected for loading with U1. Data are mean ±SEM, n=6 in each group.

4.4 Discussion

Our studies demonstrate that 5α-reduced glucocorticoids are able to compete with dexamethasone for its binding sites in hepatocytes with affinity similar to that of corticosterone. In two cell models we have shown that this binding is associated with the induction of transcription mediated by GR activation. Further 5αTHB acts as a glucocorticoid *ex vivo* and *in vivo,* as judged by its ability to inhibit angiogenesis and to suppress the hypothalamic-pituitary adrenal axis. This study is the first to report the agonist properties of 5αTHB at GR although previous studies have been carried out to investigate the 5α-dihydro products of cortisol (Baxter & Tomkins 1971; Carlstedt-Duke *et al.* 1977).

Our initial receptor binding studies in isolated hepatocytes demonstrated that 5α-reduced glucocorticoids bind to the dexamethasone binding sites with a Kd of similar magnitude to that of corticosterone. The affinity of 5αTHB was greater than that of the 5α-dihydrometabolite and in contrast 5β-reduced metabolites show limited binding. The ability of 5α- but not 5β- reduced metabolites to bind to GR is due probably to differences in stereochemistry around the A/B ring junction. Previous studies of dexamethasone binding in cytosol, nuclear envelopes and plasma membranes have shown similarly that binding is sensitive to the configuration at the C5 position (Roszak *et al.* 1990). The A/B ring junction of the 5β stereoisomers is "cis" and therefore skewed relative to the plane through rings B, C and D, whereas the 5α orientation is "trans" and hence has a structure akin to corticosterone. Similar stereospecificity in favour of 5α- rather than 5β- metabolites has been observed for binding to the androgen receptor (Lefebvre & Morante 1982).

Glucocorticoids can bind to a site other than the cytosolic GR in hepatocytes. Low affinity glucocorticoid binding sites ("LAGS") have been demonstrated in the microsomal fraction (Ambellan *et al.* 1981) and nuclear envelope (Roszak *et al.* 1990). Melville et al have shown that LAGS have a preference for 5α-rather than 5β-reduced steroids (Melville *et al.* 1998) however, the nature and function of LAGS is

uncertain. Therefore the following experiments were designed to separate the classical cytosolic GR and microsomal LAGS.

Firstly we investigated steroid binding in the cytosolic fraction. The presence of the classical GR was confirmed by a decreased Kd for dexamethasone in this fraction compared to the Kd obtained for dexamethasone in the isolated hepatocytes. However, compared to the isolated hepatocytes 5αTHB did not bind whereas both 5αDHB and 5βDHB displaced dexamethasone with similar Kd values as corticosterone. This implies that in the cytosol, the binding site is not sensitive to the configuration at the C5 position and that the 3α-position is more important in determining binding affinity.

Secondly we investigated steroid binding in the microsomal fraction. The expected binding pattern was observed with dexamethasone with higher Kd values observed in the microsomes compared to cytosol. The affinities of LAGS for dexamethasone and corticosterone were in accordance with previous publications (Melville *et al.* 1998) confirming that our system was working. We observed the same stereo-specificity in microsomes as in the hepatocytes with both of the 5α-reduced metabolites displacing dexamethasone with the same affinity as corticosterone. However, in this fraction 5αTHB binding was greater than 5αDHB.

Therefore we have shown that 5αDHB binds to the cytosolic GR whereas 5αTHB binds with the same affinity as corticosterone in the microsomal fraction but not in the cytosol. This suggested that 5αTHB was actually binding to microsomal LAGS and not the classical cytosolic GR. It was therefore surprising that in further experiments 5αTHB was more potent than 5αDHB in activating cytosolic GR.

In a transient transfection system, luciferase expression was placed under control of the mouse-mammary tumour virus long terminal repeat (MMTV-LTR) promoter linked to a glucocorticoid response element (GRE). In order to induce translation of luciferase, ligand-activated GR must dimerise and associate with the GRE. The results obtained indicate that the 5α-reduced metabolites were able to induce

117

hGRrshα-mediated luciferase activity and that this response was dependent completely on the presence of GR. However 5αDHB did not stimulate luciferase activity to the same extent as 5αTHB.

In a second model, H4IIE cells, activation of endogenous GR was measured by induction of endogenous TAT mRNA expression. Expression of TAT mRNA is controlled by numerous factors, including an upstream positive GRE (Grange et al. 2001). Transcriptional up-regulation of TAT was observed in the presence of 5αTHB and this was prevented by the addition of the GR antagonist, RU486. 5αDHB did not induce TAT mRNA expression significantly above basal levels.

Taken together with the results from the binding experiments, these data suggest that 5αTHB binding to microsomal LAGS can activate cytosolic GR. This binding can mediate transcription of target genes in the same way as steroid binding to the cytosolic GR, although to date, LAGS has only been associated with non-genomic effects of steroid hormones (Falkenstein et al. 2000). To further examine if steroid binding to microsomal LAGS can activate cytosolic GR, it would be advantageous to carry out a time-course experiment with our transfection system as it would be predicted that this pathway would take longer than the traditional mechanism of nuclear translocation. In addition it would be interesting to carry out the hepatocyte binding experiments with female rats as they do not have LAGS (Omrani et al. 1983) and therefore it would be predicted that 5αTHB would not bind in these cells.

A determinant for the reduced receptor-binding activity of 5αTHB for the glucocorticoid receptor in cytosol could be the redox status of the cytosol preparation. In the absence of added reducing agents, glucocorticoid receptors are maintained in their reduced, steroid binding form by an NADPH-dependent and thioredoxin-dependent thiol-disulphide exchange system (Grippo et al. 1983). All cytosol solutions contain some free thiol (–SH) groups, a condition that favours the formation of an intramolecular disulphide bond between a vicinally spaced pair of cysteine -SH groups which blocks the steroid binding domain of the receptor (Miller et al, 1988). The ability of thiol modifications to block steroid binding depends on

the structure of the steroid but is independent of the biological activity of the steroid (Zakula & Moudgil 1991). However addition of the reducing agent, DTT and molybdate, which stabilises the complex between hsp90 and the 16-kDa fragment of the hormone binding domain containing the thiols (Simons *et al.* 1989) to the buffer used should have prevented disulphide bonds forming.

Another reason for the reduced binding of 5αTHB in the cytosol could be that 3α-hydroxysteroid dehydrogenase which converts 5αDHB into 5αTHB is freely reversible and its direction is driven by prevailing steroid and co-factor concentrations. At physiological pH, the oxidation reaction is favoured by 3-fold (Penning *et al.* 1997) and it has been reported that 3α-hydroxysteroid dehydrogenase acts in the reverse direction when the cell is disrupted. (Hardy *et al.* 2000). In our binding experiments the experimental conditions favour the reverse reaction as the pH is 7.2, 5αTHB is added in excess and the cell is disrupted. Therefore in experiments where the cells were incubated with 5αTHB, 5αTHB may have been converted back into 5αDHB and the apparent low binding capacity observed here in cytosol with 5αTHB could actually reflect 5αDHB binding.

In our transfection system, the dose-response curves for corticosterone and 5αTHB were different with an apparent shift to the right for 5αTHB indicating that 5αTHB is a weaker agonist. At a concentration of 1μM, corticosterone and 5αTHB produced the same induction in luciferase activity and the effects of 5αTHB were additive with those of corticosterone, suggesting that 5αTHB is a full agonist. A full agonist can produce maximal effects and has high efficacy, which is the ability once bound to initiate changes which lead to effects. It was not possible to compare maximal activation of 5αTHB to that of corticosterone in this system since cell death was observed at the higher concentrations of glucocorticoids that would have been necessary (>2μM). However a concentration of 1μM is 20-100 times higher than reported Kd values and therefore all glucocorticoids should saturate GR and produce a maximal effect (Chen *et al.* 1994).

In this study, compared to corticosterone, the magnitude of the response observed with 5αTHB in H4IIE cells was smaller than that observed in the transfected HeLa cells. In studying different glucocorticoids, Jaffuel et al, 2001 showed that trans – activation of a luciferase reporter gene in HeLa cells correlated well with trans-activation of tyrosine aminotransferase in rat hepatoma (HTC) cells. However, trans-activation potencies and relative binding affinities were not always interrelated (Jaffuel et al. 2001). It is not uncommon for a given steroid to afford different dose-response curves in different cells, possibly due to unequal amounts of metabolism, serum binding proteins and non-specific binding to cells. The ratio of co-activator to co-repressor is now known to be a defining ingredient for the dose-response curve for agonist steroids (Szapary et al. 1999). Variations in the levels of co-activators and co-repressors in different cells have been reported, providing an attractive mechanism for explaining the cellular variations in response to B and 5αTHB (Misiti et al. 1998).

The binding experiments carried out in this study were performed at 4°C whereas the gene expression studies were carried out at 37°C, temperature at which cellular metabolisms must be taken into account. Thus, the observation that 5αTHB does not induce tyrosine aminotransferase mRNA to the same extent as corticosterone could be explained by selective inactivation of 5αTHB in H4IIE cells compared to HeLa cells. We have shown by RT-PCR that H4IIE cells contain 5α - and 5β-reductase as well as 3α-HSD and are therefore probably more metabolically active than HeLa cells as would be expected from a hepatic cell line. We have shown by GCMS that metabolic transformation of steroids did not occur in HeLa cells but metabolism of corticosterone to 5αTHB was observed in H4IIE cells. However the reverse reaction was not observed and therefore the efficacy of 5αTHB was not due to production of corticosterone.

Glucocorticoids inhibit angiogenesis in chronic inflammatory conditions for example rheumatoid arthritis (Colville-Nash & Scott 1992) and in models of rat aortic rings, administration of supraphysiological concentrations of cortisol inhibit new vessel formation (Jaggers et al. 1996). Studies in our laboratory have previously shown that

physiologically relevant concentrations of corticosterone can act via glucocorticoid receptors to inhibit angiogenesis (Small *et al.* 2003). Here we show that 5αTHB can also inhibit angiogenesis to the same extent as corticosterone and that this angiostatic effect of 5αTHB is mediated via GR as it is blocked by the GR antagonist RU486.

Finally, we showed that 5αTHB adminstration *in vivo* induced a classical negative feedback effect on the hypothalamic-pituitary-adrenal axis. It appears that in suppressing ACTH, 5αTHB has a slower onset of effect than corticosterone. This could relate in part to different CNS availabilty of 5αTHB compared to corticosterone. Although 5αTHB is minimally protein-bound in plasma (Murphy *et al.* 1963), its access across the blood brain barrier, and susceptibility to export from the CNS by multidrug resistance P-glycopreoteins is unknown.

Dubrovsky *et al* showed that 5α -DHB can easily penetrate the blood-brain barrier as a higher percentage of neurons responded to i.v. injection of 5αDHB compared to i.v. injection of corticosterone (Dubrovsky *et al.* 1985). Access of corticosterone to mouse and human brain is not hampered by MDR1-type P-gp (Karssen *et al.* 2001) because corticosterone lacks a 17-hydroxyl group which in addition to an 11-hydroxyl group has been shown to determine the ability of steroids to be transported by MDR1-type P-gp (Bourgeois *et al.* 1993). 5αTHB also lacks a 17-hydroxyl group and therefore is probably minimally, if at all, transported. Stereoisomerism around carbon 5 of the steroid ring structure is also important for steroids to be recognised by MDR1-type P-gp. An investigation to determine the effect of progesterone and its metabolites on MDR1-type P-gp showed that metabolites in the 5β-pathway but none in the 5α-pathway could enhance activity of MDR1-type P-gp in adrenal glands (Ichikawa-Haraguchi *et al.* 1993)It is therefore unlikely that 5αTHB is a substrate for MDR1-type P-gp and will not be pumped out of the brain.

Another explanation for the different time courses is that corticosterone acts through both mineralocorticoid (MR) and glucocorticoid receptors. The MR contributes more to early "shut-off" after stress and may account for the rapid effect of corticosterone

(Ratka *et al.* 1989). Corticosterone binds to MR with a higher affinity than GR in the brain (De Kloet *et al.* 1975). It is not known if 5αTHB can bind to MR, however 5αDHB has been shown not to possess any mineralocorticoid activity (Sekihara *et al.* 1978).

A surprising observation is that by 6h post-injection, 5αTHB further suppressed ACTH secretion whereas suppression by corticosterone had ceased. It appears that corticosterone is being cleared more rapidly than 5αTHB by hepatic metabolism although it would be expected that 5αTHB would be cleared more quickly as it is a stage further in the metabolic pathway. Corticosterone may also be metabolised by 5α-reductase and 3α-HSD in the brain (Melcangi *et al.* 1993). It may also be that 5αTHB dissociates from its receptor slower then corticosterone.

If endogenously present at high enough concentrations, 5α-reduced glucocorticoids will contribute to GR activation in sites where 5α-reductases are expressed. Many hepatic enzymes involved in carbohydrate and fat metabolism are controlled by glucocorticoids e.g TAT and PEPCK. 5α- Reduction of corticosterone in liver will increase local concentrations of active glucocorticoids, potentiating glucocorticoid action regardless of circulating corticosterone levels. In our *in vivo* experiment administration of corticosterone or 5αTHB had no effect on the expression of TAT or PEPCK. TAT gene expression is under composite hormonal control, being activated by glucocorticoids and glucagon (via cAMP) (Hashimoto *et al.* 1984) and repressed by insulin (Moore & Koontz 1989; Ganss *et al.* 1994). Other groups carrying out similar studies in Sprague-Dawley rats have found differences in TAT mRNA expression after injection of glucocorticoid (prednisolone) within three hours however the dose administered was 10 times higher than the dose used in our study. The differences in our result may also reflect strain difference between Zucker rat and Sprague-Dawley rats.

Whether activation of GR by 5α-reduced glucocorticoids are of significant clinical importance in conditions in which alterations in 5α-reductase activity have been

implicated such as obesity (Andrew et al, 1998) and polycystic ovary syndrome (Stewart *et al.* 1990) remains to be determined (Fassnacht *et al.* 2003).

Chapter Five

A-ring Reductases in Obesity

5.1 Introduction

Obesity is of increasing prevalence but is poorly understood. There is increasing evidence of genetic causes of obesity bringing about metabolic changes which influence weight gain and feeding behaviour however these only account for a small proportion of cases.

Features of the Metabolic Syndrome (insulin resistance, centripetal obesity, hypertriglyceridaemia and hypertension) are remarkably similar to those observed in subjects with Cushing's Syndrome, where the primary defect is excess cortisol secretion as a consequence of a tumour (Cushing 1912; Walker & Edwards 1992). This has led to the hypothesis that subtle abnormalities in glucocorticoid metabolism and tissue sensitivity to glucocorticoids may contribute to the development of the Metabolic Syndrome and obesity in the general population. In central obesity, total glucocorticoid turnover and production are increased and circulating cortisol levels are often lower than normal (Marin *et al.* 1992; Haytanen & Adlercreutz 1993; Ljung *et al.* 1996; Walker *et al.* 2000).

The mechanisms whereby glucocorticoid metabolism is altered in obesity are poorly understood. One explanation is that peripheral clearance of glucocorticoids is enhanced, driving activation of the Hypothalamic-Pituitary-Adrenal axis and subsequent excess glucocorticoid production. It has previously been reported that excretion of A-ring reduced glucocorticoid metabolites, in particular the 5α-reduced metabolites, are increased in urine of obese men and women (Andrew *et al.* 1998; Fraser *et al.* 1999; Rask *et al.* 2001; Rask *et al.* 2002) and also in obese Zucker rats (Livingstone *et al.* 2000). These changes will only affect tissues where the metabolic enzymes are expressed. Urinary steroid metabolite profiles do not identify which tissue or isozyme is involved, however we predicted that the liver would be the major site of metabolism given that this is where most metabolites are synthesised. The results described in chapter three of this thesis confirm that 5α-reductase type 1 is the only isozyme of 5α-reductase expressed in rat liver and fat therefore we

hypothesised that it is this enzyme that is responsible for the increased clearance of glucocorticoids in obesity.

Hepatic glucocorticoid receptor binding has also been reported to be altered in obesity with glucocorticoid receptors from obese rats displaying lower binding affinities for corticosterone than in lean rats (Nelson *et al.* 1998). This may be a consequence of abnormal regulation of GR transcription (Jenson *et al.* 1996) or a decrease in receptor integrity. In addition, we hypothesised that competition for GR between corticosterone and 5α-reduced corticosterone may be responsible for the reduced binding affinity of corticosterone in obesity.

To investigate the activity of 5α-reductase in obesity we used the obese Zucker rat. Many studies have addressed the question of central and peripheral target tissue sensitivity to glucocorticoids in genetically obese Zucker rats (Zucker and Zucker, 1961). The primary defect in the obese Zucker rat is a recessive mutation in the leptin receptor (Chua *et al.* 1996). Leptin is a hormone that regulates food intake and energy balance (Zhang *et al.* 1994). The obesity in these animals has been shown to be glucocorticoid dependent, as adrenalectomy or treatment with glucocorticoid receptor antagonists have been shown to normalise weight gain and associated metabolic abnormalities (Yukimura *et al.* 1978; Castonguay *et al.* 1986; Freedman *et al.* 1986; Langley & York 1990). Conflicting results have been reported on the sensitivity of the hypothalamic-pituitary-adrenal axis in obese Zucker rats with data supporting normal (Guillaume-Gentil *et al.* 1990), increased (Bestetti *et al.* 1990; Plotsky *et al.* 1992)or decreased (Castonguay *et al.* 1986; Plotsky *et al.* 1992) sensitivity to the inhibitory effects of corticosterone. Similarly there are reports of increased (Bestetti *et al.* 1990; Plotsky *et al.* 1992) and decreased (Nakaishi *et al.* 1990; Routh *et al.* 1990; Plotsky *et al.* 1992) responses to stress in these animals.

The aims of this chapter were to :

1) determine the expression and activity of A-ring reductases in obesity

2) examine glucocorticoid-receptor binding of A-ring reduced metabolites in lean and obese Zucker rats.

3) determine if the presence of A-ring reduced metabolites may account for the changes in GR binding of corticosterone previously reported in obesity.

5.2 Methods

5.2.1 A-ring reductase Expression Studies

5.2.1.1 RT-PCR

Expression of 5α-reductase type 1 and the reported elongated isoform 5α-reductase type 1b was investigated in liver from male lean and obese Zucker rats (n=8 per group) by RT-PCR as described in chapter 2.7.

5.2.1.2 Real Time PCR

Expression of 5α-reductase type 1 was investigated in subcutaneous and omental fat from lean and obese Zucker rats (n=6 per group) by Real Time PCR as described in chapter 2.8.

5.2.1.3 Northern Blots

5α- and 5β -reductase and 3α-HSD mRNA expression was quantified in liver from male lean and obese Zucker rats (n=6-8 per group) by Northern blot as described in chapter 2.9.

5.2.1.4 Western Blots[1]

5α-reductase type 1 protein levels were determined in liver from male lean and obese Zucker rats (n=6 per group) by Western Blot. Briefly, Liver microsomes were prepared by repeated centrifugation in 50mM Tris; 5 mM EDTA; 20mM Sodium Molybdate; 0.25 sucrose with Complete™ Protease Inhibitor (Roche Diagnostics, East Sussex, UK). Proteins (30μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to ECL nitrocellulose membrane (Amersham Biosciences, Bucks, UK). Non-specific binding was blocked with 5% blotting grade non-fat dried milk (Bio-Rad Laboratories, Herts, UK) in 20mM Tris; 137mM NaCl; 0.1% Tween 20 and the membrane hybridised with 5α-reductase type

---

[1] 5α-reductase type 1 Western blot was carried out by Dr Dawn Livingstone, Endocrinology Unit, University of Edinburgh

1 antibody (Eicheler *et al.* 1995). Specific binding was visualised using the Amersham ECL system and quantified by optical densitometry. eQuivalent loading of protein was confirmed by Ponceau Red staining of the membrane.

5.2.1.5 In Situ Hybridisation

Localisation of 5α-reductase type 1 mRNA expression was investigated in liver from male lean and obese Zucker rats (n=3) using *in situ* hybridisation as described in chapter 2.11.

5.2.2 5β-Reductase Activity Studies

5β -Reductase activity was investigated in liver from male lean and obese Zucker rats ($n = 8$ per group). HPLC analysis determined the percentage conversion of $[^3H]_4$-corticosterone to $[^3H]_4$-3α,5β-tetrahydrocorticosterone in cytosol at known protein concentrations as detailed in chapter 2.5. All animals were aged 9 weeks.

5.2.3 Glucocorticoid Binding Experiments

For glucocorticoid binding experiments, hepatocytes were prepared from six-eight-week old male lean and obese Zucker rats (n=6 per group) as described in chapter 2.12.1.3. Competition binding experiments were performed as described in chapter 2.12.2

5.2.4 Statistics

All data are expressed as mean ± standard error and data were analysed statistcally by one-way ANOVA followed by LSD post-hoc tests except the 5β-reductase activity assay which was analysed by Student's t-test followed by LSD post-hoc tests.

5.3 Results

5.3.1 A-ring reductase expression

5α-Reductase type 1a but not the reported elongated version of 5α-reductase type 1 was identified by RT-PCR in livers of lean or obese Zucker rats (Figure 5.1). In liver of obese versus lean animals, 5α-reductase type 1 mRNA and protein was increased (Northern blot, 0.58 ± 0.12 vs 0.24 ± 0.03 AU, $p<0.01$, Figure 5.2 (a); Western blot, 0.70 ± 0.05 vs 0.4 ± 0.07 relative optical density, $p<0.005$, Figure 5.2 (b)). A typical Western blot run is shown in Figure 5.3.

Qualitatively, the distribution pattern of hepatic expression of 5α-reductase type 1 was not different between lean and obese Zucker rats (Figure 5.4).

In subcutaneous adipose tissue of Zucker rats there was no significant difference in mRNA expression of 5α-reductase type 1 between lean and obese rats ($p=0.71$) (Figure 5.5 (a)). In omental adipose tissue 5α-reductase type 1 mRNA levels were significantly decreased in obese animals compared to lean ($p<0.01$) (Figure 5.5 (b)).

By contrast, hepatic 5β-reductase mRNA expression was not different between lean and obese animals (Figure 5.6(b)). 3α-HSD mRNA expression was not different between lean and obese animals (Figure 5.7).

5.3.2 A-ring reductase activity

5β-reductase activity was increased in obese rats compared to lean ($p<0.05$) (Figure 5.6 (a)). Note that 5α-reductase activity could not be measured (see chapter 3.3.3)

5.3.3 Glucocorticoid receptor binding

Corticosterone ($p=0.02$) and 5α-DHB ($p=0.06$) were weaker competitors for dexamethasone in hepatocytes from obese compared with lean rats whereas no difference was observed with 5α-THB (Figure 5.8 and Table 5.1). Bmax for

dexamethasone binding was higher in obese versus lean animals (223 ± 47 vs 87 ± 21 fmols/million cells, p=0.03).
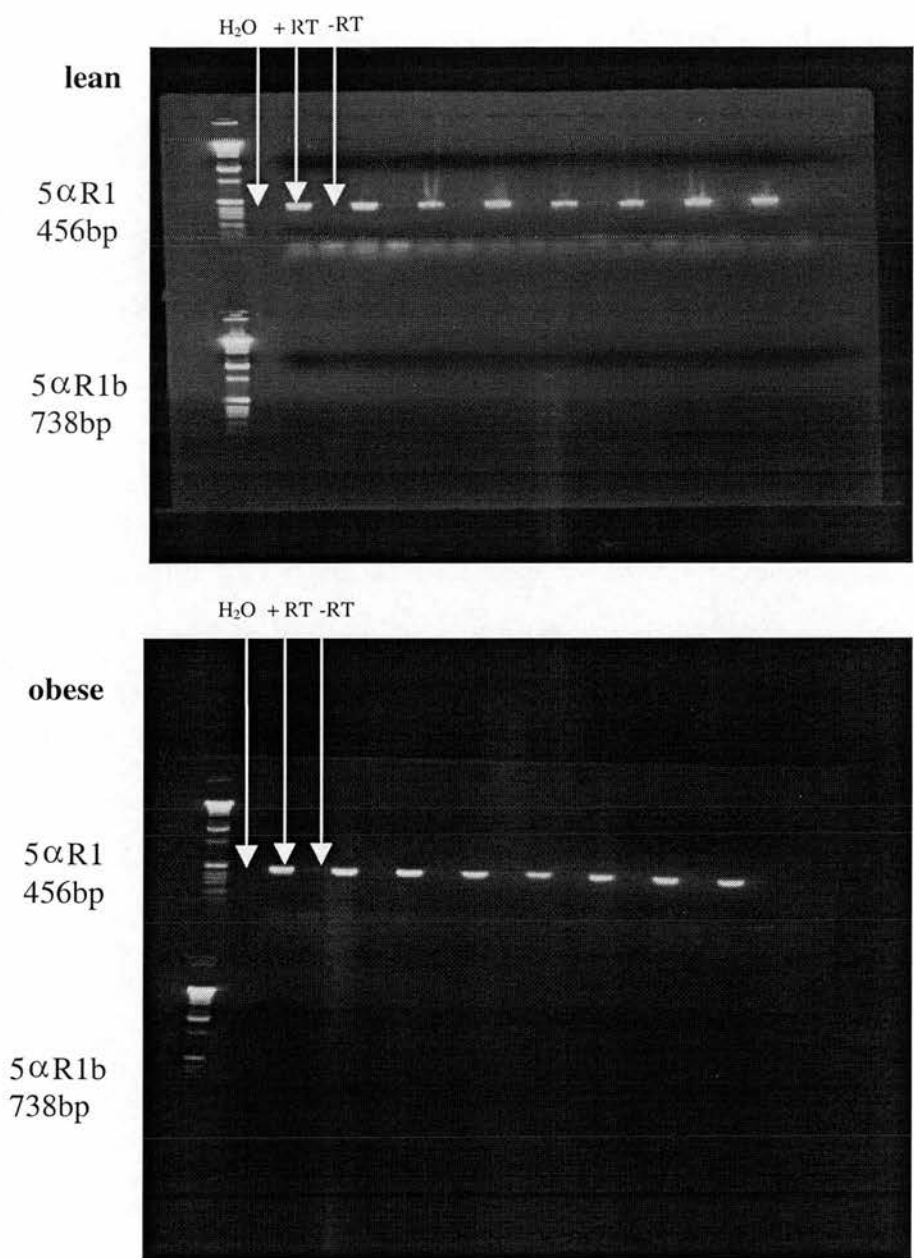
Figure 5.1: Expression of 5α-reductase type 1 and 5α-reductase type 1b in rat liver

1μg of total RNA isolated from lean and obese male Zucker rat liver (n=8 per group) was used in RT-PCR with specific primers to detect expression of 5α-reductase type 1b. Negative controls included an RT reaction containing no RNA (H₂O) and a reaction carried out in the absence of the RT enzyme for each RNA (-RT) (n = 8 per group).

(a)

(b)
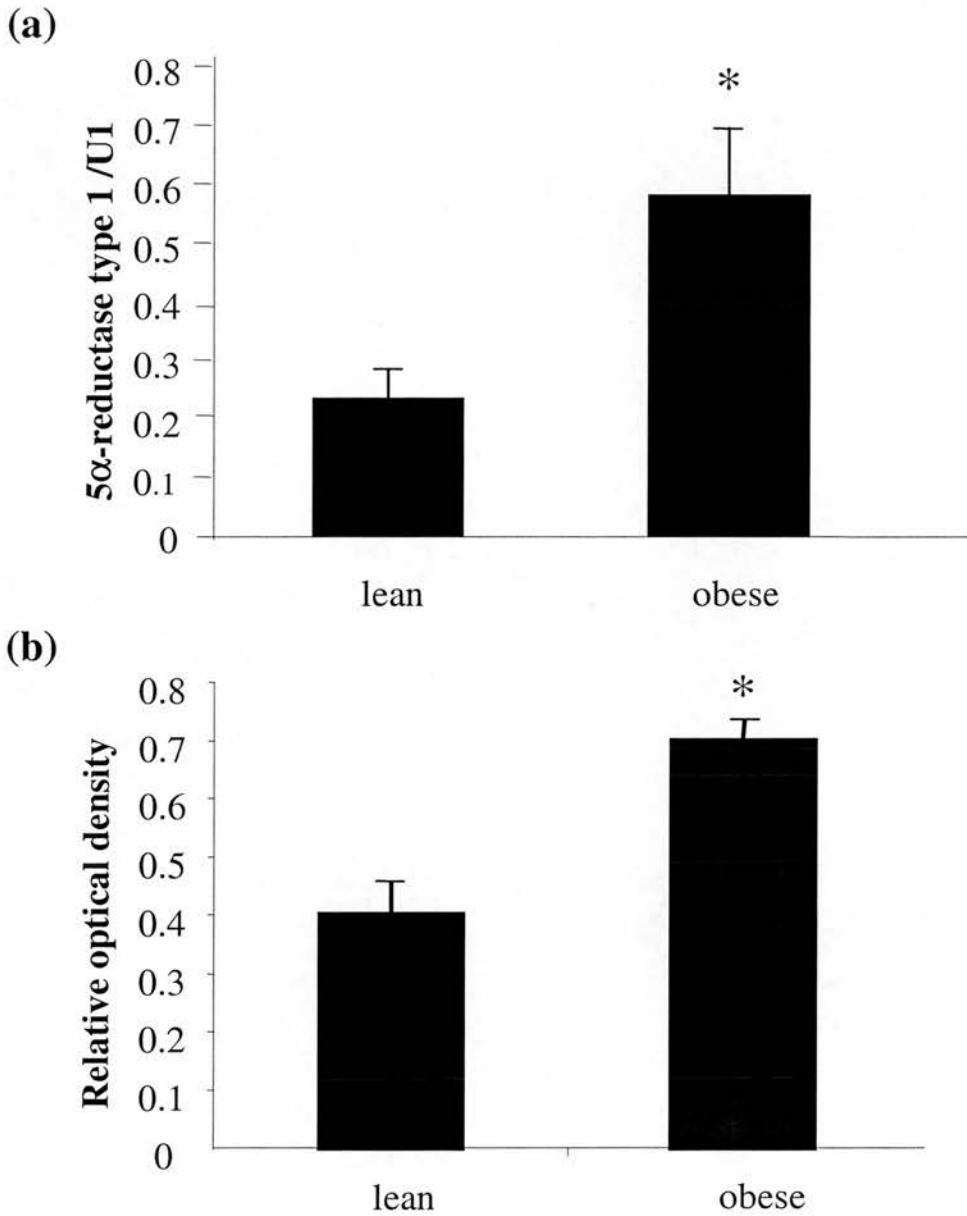
Figure 5.2: Hepatic 5α-reductase type 1 mRNA and protein expression

Figure shows a) 5α-reductase type 1 mRNA measured by Northern blot in liver from lean and obese Zucker rats. Values are corrected for loading with U1, *p<0.005. b) 5α-Reductase type 1 protein levels measured by Western Blot in liver from lean and obese Zucker rats, *p=0.008. Data are mean ± SEM, n=6 per group.

133
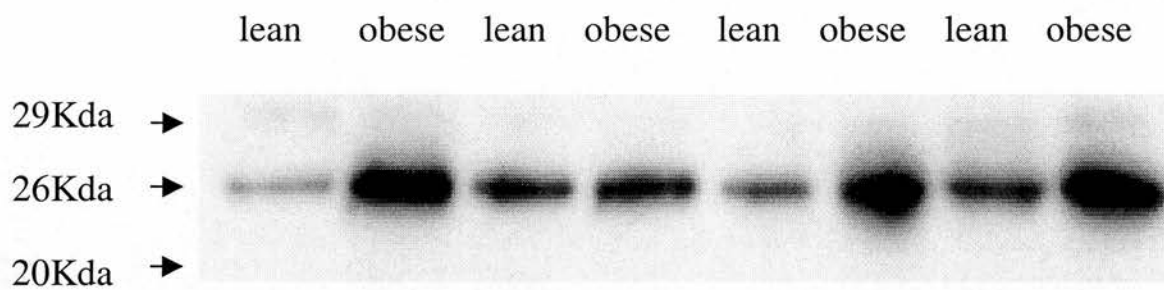
Figure 5.3: Western blot analysis of liver microsomes from lean and obese Zucker rats.

Position of molecular weight markers are shown on the left hand side. In lean and obese Zucker rat liver microsomes, a protein with an apparent molecular weight of 26Kda is detected.
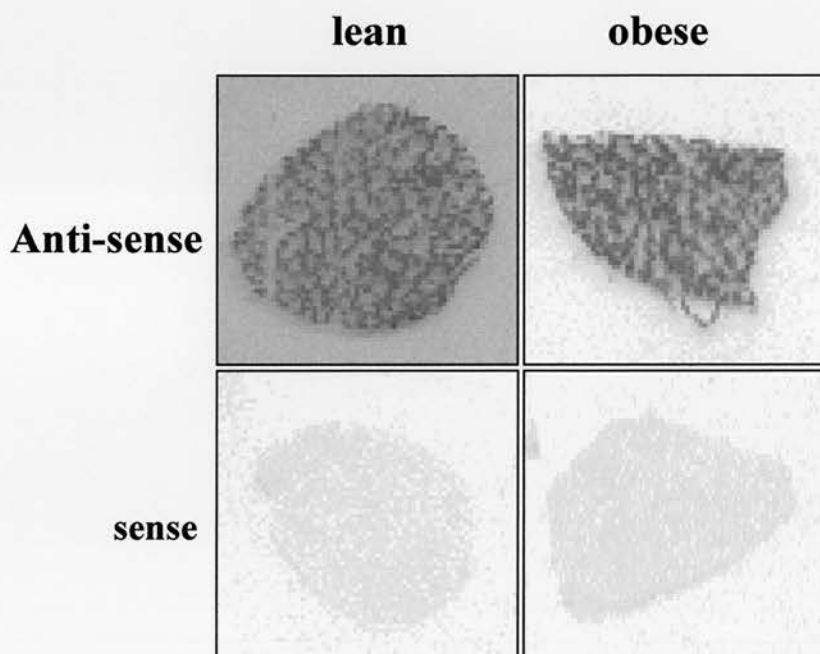
Figure 5.3: 5α-Reductase mRNA Expression in Lean and Obese Zucker Rat Liver

Anti-sense ^{35}S-labeled ribo-probes (top pictures in each panel) specific for rat 5α-reductase type were used to detect mRNA in 10μm thick sections of liver from male lean and obese Zucker rats by *in situ* hybridisation. Sense probes (bottom pictures in each panel) were used as a control for non-specific hybridisation. Following *in situ* hybridisation, slides were exposed to Kodak autoradiograph film for one-three days.

(a) subcutaneous
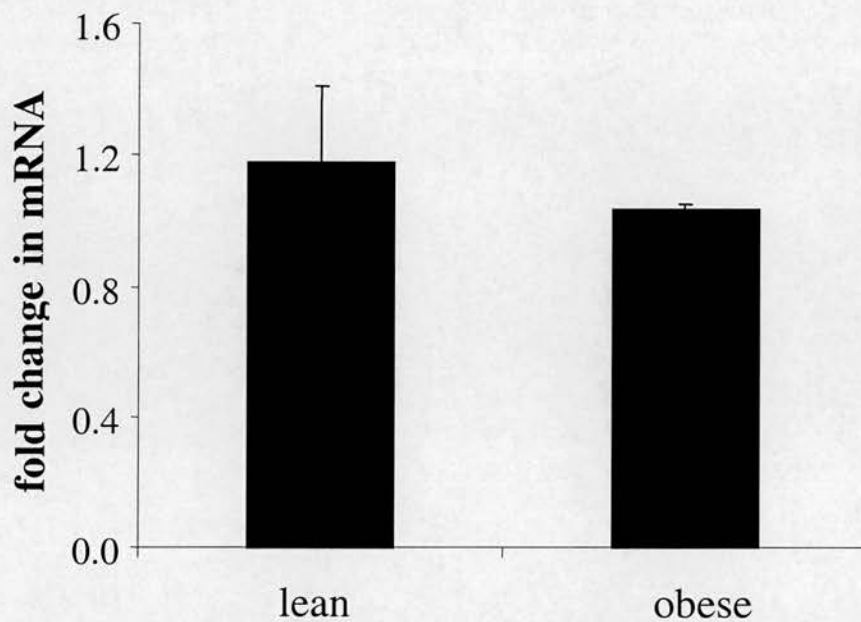
(b) omental
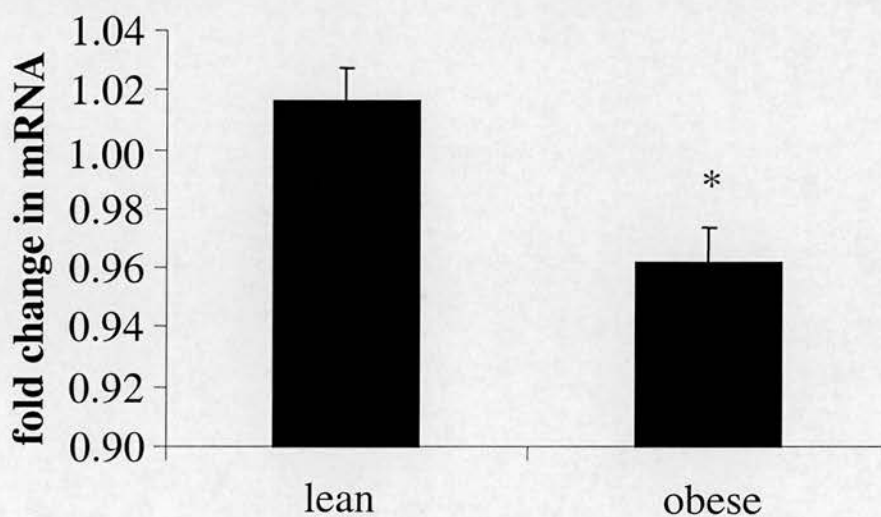
Figure 5.5: 5α-Reductase type 1 mRNA expression in lean and obese Zucker rat subcutaneous and omental adipose tissue

Figure shows relative fold change in 5α-Reductase type 1 mRNA expression measured by Real Time PCR in (a) subcutaneous and (b) omental adipose tissue from lean and obese Zucker rats. Data are mean ± SEM, *p<0.01, n=6 per group.

(a)

(b)
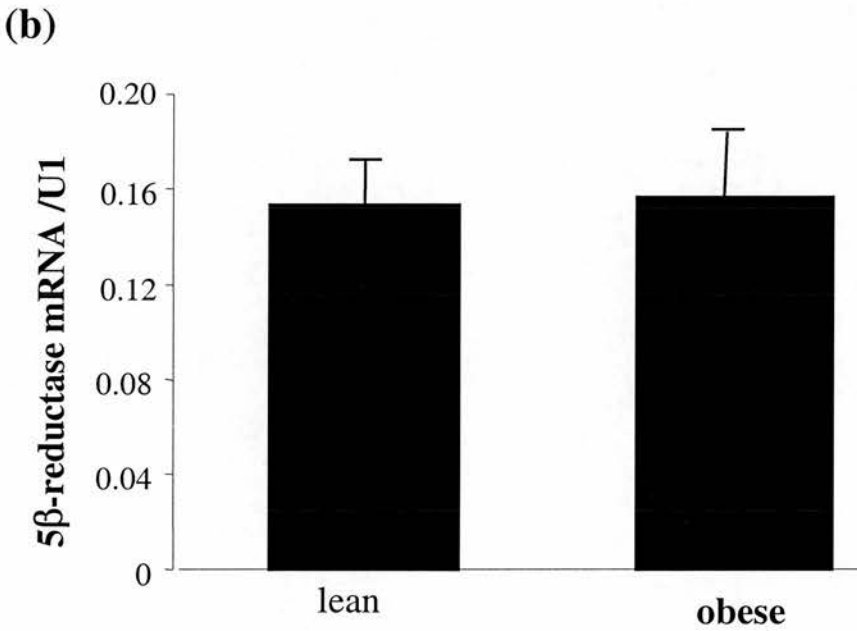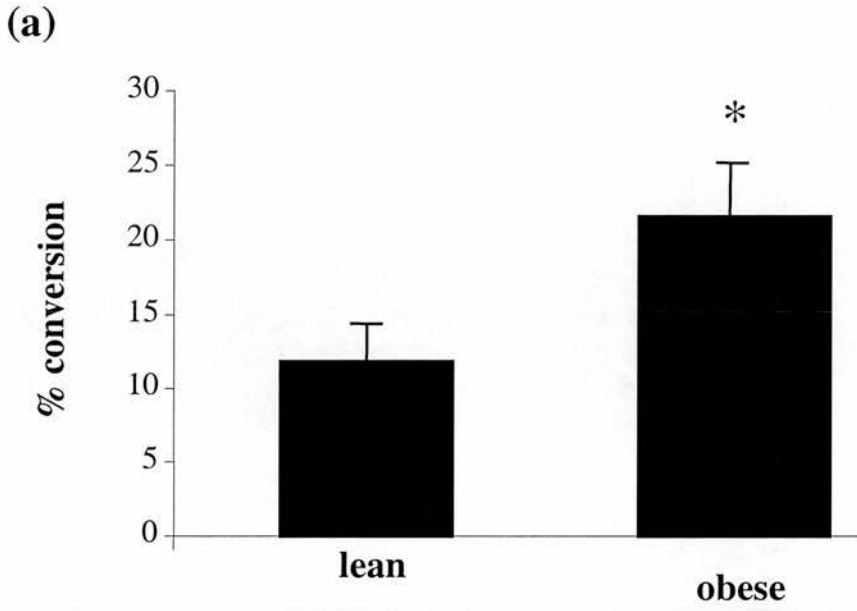
Figure 5.6: 5β-reductase (a) activity and (b) mRNA expression

Figure shows 5β-reductase activity and mRNA expression in liver from lean and obese Zucker rats. mRNA values are corrected for loading with U1. Data are mean ± SEM, *p<0.05, n=6 per group.
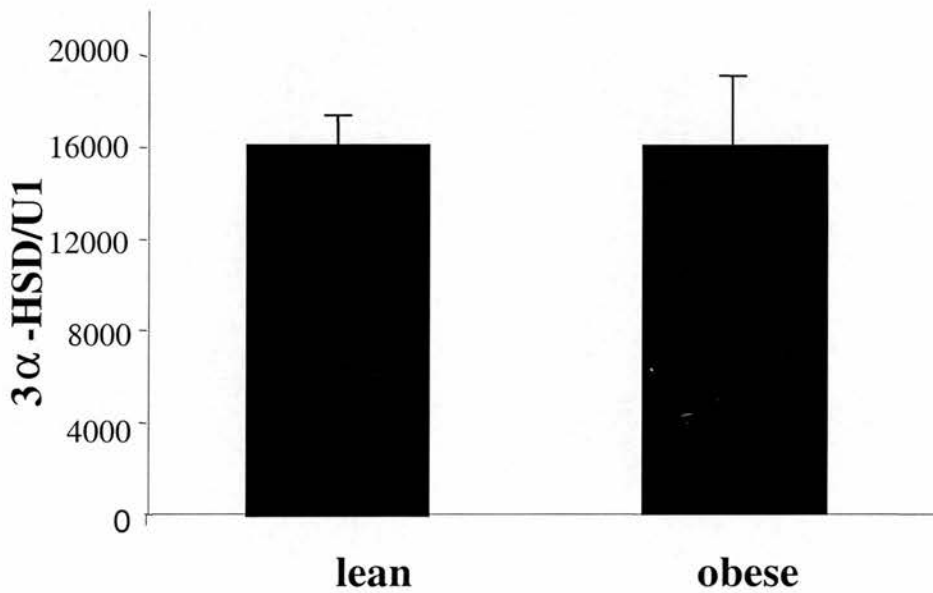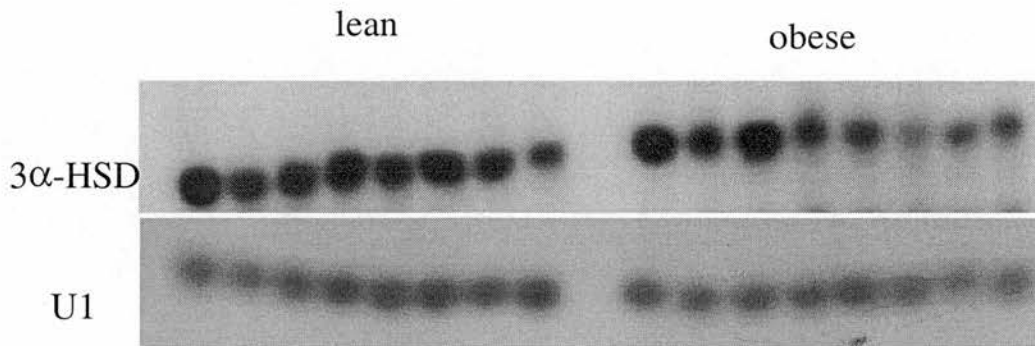
Figure 5.7: 3α-HSD mRNA expression

Figure shows 3α-HSD mRNA expression measured by Northern blot in liver from lean and obese Zucker rats. Values are corrected for loading with U1. Data are mean ± SEM, n =8 per group.
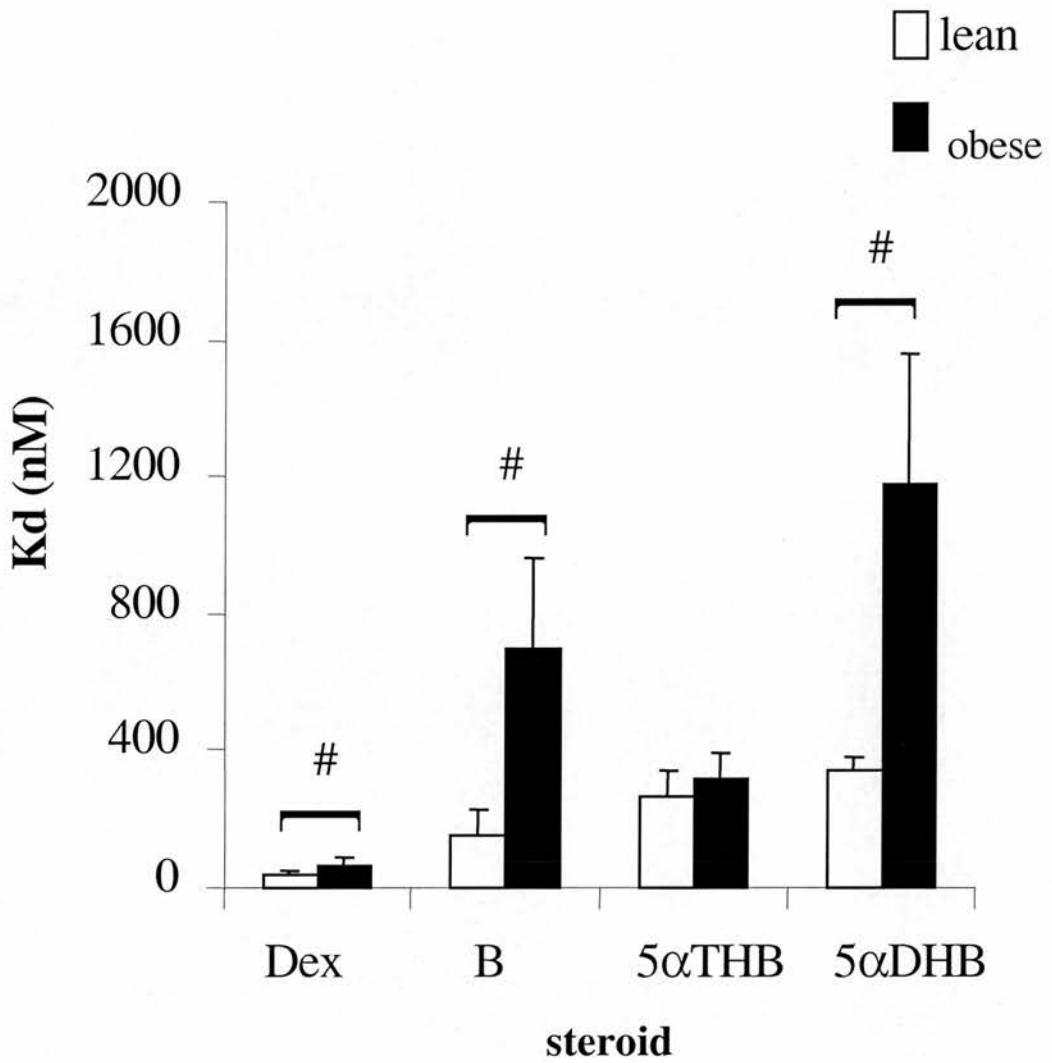
Figure 5.8: Binding coefficients of glucocorticoids in isolated hepatocytes

Figure shows Kd values of glucocorticoids in isolated hepatocytes from lean versus obese Zucker rats. Data are mean ± SEM. #p≤0.06 lean vs obese, n=6 per group.

Glucocorticoid	Lean	Obese	p value
Dexamethasone	37±8	67±16	0.02
B	153±79	698±263	0.005
5α-THB	268±78	312±84	0.44
5α-DHB	336±42	1180±377	0.06
5β-THB	4484±1313	5072±1436	0.26
5β-DHB	9656±3230	11333±4101	0.33

Table 5.1 Binding coefficients (Kd, nM) of glucocorticoids in hepatocytes from lean and obese Zucker rats.

Data are mean ± SEM, n=8-10 per group.

5.4 Discussion

These data demonstrate that the previously observed increased urinary excretion of A-ring reduced metabolites of corticosterone in obese Zucker rats is associated with increased expression of hepatic but not adipose A-ring reductases. In particular, both 5α-reductase type 1 mRNA and protein levels were increased in liver of obese animals. In addition hepatic 5β-reductase activity was increased in obese rats compared to lean although the mRNA was not. Increased hepatic A-ring reductase expression and activity predicts and explains an increase in the metabolic clearance of corticosterone, which may contribute to HPA activation and adrenocortical hypertrophy in obesity. In this situation, the HPA axis may behave differently under certain conditions, e.g to stress and diurnal variation, parameters that are altered in obese animals and humans.

It is likely that this up-regulation of hepatic 5α-reductase type 1 explains the increased excretion of 5α-reduced glucocorticoids in obese Zucker rats and that similar mechanisms operate in human obesity. The contribution of the 5α-reductase type 2 isozyme cannot be ruled out in obesity as it is expressed in human liver as well as 5α-reductase type 1. In addition, the observed increases in metabolites of 5α- and 5β- reductase are probably not due to increased expression of hepatic 3αHSD as the mRNA expression for this enzyme was not changed between lean and obese Zucker rats. Adipose tissues also have the ability to metabolise glucocorticoids by 5α-reductase (Perel *et al.* 1986). While 5α-reductase type 1 could be detected in fat, the expression levels were much greater in liver suggesting a more significant role for this enzyme in hepatic tissue. We have shown by Real Time PCR that 5α-reductase mRNA levels were unchanged in subcutaneous adipose tissue but decreased in omental adipose tissue of obese Zucker rats indicating that regulation of 5α-reductase may be tissue specific as has been shown for 11β-hydroxysteroid dehydrogenase type 1 (Livingstone *et al.* 2000).

We investigated also the expression of the alternative, elongated isoform of 5α-reductase type 1 in liver of lean and obese Zucker rats since it was reported to have

higher affinity for glucocorticoids (Lopez-Solache *et al.* 1996). It was therefore possible that this gene product may have been dysregulated in obesity. This isoform could not be detected in lean or obese liver by RT-PCR and therefore cannot explain the up-regulation of 5α-reduction in obesity. Since this clone has only been expressed by one group it was not possible to include a positive control for the expression of this transcript.

In addition to its role in the metabolic clearance of glucocorticoids, 5β-reductase plays a major role in cholesterol homeostasis by catalysing the catabolism of cholesterol to bile acids. Thus, dysregulation of 5β-reductase may contribute to altered lipid and bile acid metabolism in obesity.

Metabolism of glucocorticoids by 5β-reductase was also increased in obese Zucker rats as observed by bioactivity assay. This is consistent with increased excretion of 5β-reduced glucocorticoid metabolites in rodent (Livingstone *et al.* 2000) and human (Stewart *et al.* 1999; Fraser *et al.* 1999; Rask *et al.* 2001; Rask *et al.* 2002) obesity. The increase in 5β-reductase activity was not reflected in the levels of hepatic mRNA expression. One explanation could be that a second isoform of 5β-reductase may exist; however, such a gene has not been identified to date (Kondo *et al.* 1994). Alternatively, the mRNA of 5β-reductase may be degraded more rapidly than its protein. 5β-Reductase is a unique member of the aldo-keto reductase family in that it has a very long 3' non-coding region containing AT-rich sequences and frequent ATTTA motifs. It is well known that these motifs are present in mRNA species that are degraded rapidly and diurnally regulated (Shaw & Kamen 1986).

The enhanced metabolism of glucocorticoids by hepatic 5α- reductase may also have a more direct effect on glucocorticoid target tissues. The data presented in chapter 4 of this thesis has shown that 5α-reduced glucocorticoids can activate glucocorticoid receptor mediated transcription of genes involved in gluconeogenesis, proteolysis and lipolysis. This may explain why in obese Zucker rats, in the face of decreased hepatic 11β-hydroxysteroid dehydrogenase type 1 activity (Livingstone *et al*, 2000),

142

an enzyme that regenerates corticosterone in the liver, symptoms of glucocorticoid excess e.g impaired fasting glucose and lipid profile are still apparent.

In chapter 3, data was presented that 5α-reductase type 1 was present in greater abundance in the periportal zone of the liver. Therefore 5α-reduced steroids would be expected to exert their greatest effects on processes mediated in this area of the liver. This pattern of expression may allow 5α-reduced steroids to exert a greater effect on GR as the hepatic distribution of GR has been reported to be also periportal (Nyirenda *et al.* 1998).

There have been several reports in the literature suggesting that binding of corticosterone to GR is impaired in the liver in obesity (Langley & York 1992; Nelson *et al.* 1998). This again presents a paradox of impaired efficacy of corticosterone in the face of evidence of increased glucocorticoid-receptor mediated effects.

Interestingly, unlike dexamethasone, corticosterone and 5αDHB the binding of 5αTHB was not impaired in obesity. Therefore up-regulation of 5α-reductase activity will generate increased amounts of competing steroid which could contribute to the higher Kd values observed with corticosterone in obesity.

5α-Reductase type 1 in the liver but not in fat is upregulated in obesity generating increased quantities of active glucocorticoid. The liver is protected from the adverse effects of glucocorticoids in obesity to some degree by decreased GR expression and binding capacity and also by decreased 11β-hydroxysteroid dehydrogenase type 1 activity. However the ability of 5αTHB to bind is not impaired in obesity, thus in the face of decreased concentrations of local corticosterone, 5α-reduced glucocorticoids may stimulate GR –mediated events. It is not clear what factors cause this up-regulation of 5α-Reductase type 1 in obesity but after carrying out some very preliminary studies it appears that 5α-Reductase type 1 may be regulated by corticosterone via the glucocorticoid receptor. This is a similar finding to previous work in mice that has shown that transcriptional control of 5α-reductase is regulated

by dihydrotestosterone binding to the androgen receptor in the male brain and by progesterone binding to the progesterone receptor in the female brain via a progesterone and androgen regulatory element (m5α-R2 PRE/ARE) (Matsui *et al.* 2002). It is therefore possible that 5α-reductase type 1 may contain a glucocorticoid response element (GRE) recognisable by the glucocorticoid receptor. Further studies are required to elucidate the mechanisms underlying the upregulation of 5α-reductase in obesity.

Chapter 6

Conclusions

6.1 5α-reduced glucocorticoids and the glucocorticoid receptor

5α-reduction of glucocorticoids had been accepted as a pathway of irreversible inactivation however data presented in this thesis show that 5α-reduced glucocorticoids are GR agonists. When activation of glucocorticoid receptors by these steroids was assessed 5αTHB was more potent than 5αDHB suggesting that 5αTHB can activate classical cytosolic GR and can mediate GR-dependent gene transcription.

6.2 A-ring reductases in obesity

Preliminary evidence suggested that A-ring reduction of glucocorticoids was increased in obesity however the tissues and isozymes involved were not known. In obese versus lean Zucker rats, hepatic 5α-reductase type 1 mRNA expression and protein levels were increased. They also had increased activity of hepatic 5β-reductase. By contrast, 3α-hydroxysteroid dehydrogenase mRNA expression was unchanged in obesity. Conversely, 5α-reductase type 1 mRNA expression was decreased in omental adipose tissue and unchanged in subcutaneous fat. These data demonstrate that the previously observed increased urinary excretion of A-ring reduced metabolites of corticosterone in obese Zucker rats is associated with increased expression of hepatic but not adipose A-ring reductases.

Binding of corticosterone was impaired in obesity whereas 5αTHB binding was unaltered suggesting that 5αTHB may modulate GR activation disproportionately in obesity. The differences in glucocorticoid binding observed in obese Zucker rats may reflect differences in distribution of LAGS between cytosol and microsomal fractions. This again brings into question the importance of LAGS in GR induced processes in the liver as to date it is not known to what extent microsomal binding contributes to glucocorticoid tone in normal physiological conditions or in pathophysiology.

6.3 Physiological relevance of alterations in 5α-reductase

The data from this thesis suggest that 5α-reductase could play an important role in maintaining glucocorticoid "tone" in the face of low corticosterone levels. It is apparent that 11β-hydroxysteroid dehydrogenase type 1 and 5α-reductase type 1 may be reciprocally regulated and it is therefore possible that under normal physiological conditions they are maintained in a state of equilibrium with each other. In pathophysiological conditions such as obesity, it appears that the balance is tipped in favour of 5α-reductase at least in the liver. Corticosterone levels are decreased in the liver of obese Zucker rats due to impaired 11β-hydroxysteroid dehydrogenase type 1 activity, therefore an increase in 5α-reductase activity will act to preserve glucocorticoid action in several ways (Figure 6.1).

Firstly, an increase in 5α-reductase activity would be predicted to enhance peripheral glucocorticoid clearance rate, lower plasma glucocorticoid levels and induce compensatory activation of the HPA axis resulting in subsequent corticosterone production. Secondly, increased 5α-reductase activity will increase production of 5αTHB which activates transcription of glucocorticoid-regulated genes via binding to GR. 5αTHB may therefore act to maintain glucocorticoid action when corticosterone levels are low.

The regulatory factors that cause this imbalance in enzyme activities in obesity have not yet been identified and further research is needed to elucidate the underlying mechanisms of dysregulation.

6.4 Future Studies

The work presented in this thesis has provided a basis for future work aimed at confirming the role of 5α-reductase in obesity.

The H4IIE hepatoma cell-line has proved a useful tool in this thesis for the study of

glucocorticoid-mediated transcription of genes and also for study of the regulation of 5α-reductase type 1. This cell-line could be used further to study the role and regulation of 5α-reductase by incubating the cells with finasteride or by manipulating insulin/IGF-1 concentrations.

The characterisation of a functional green-fluorescent protein (GFP) – glucocorticoid receptor (Hager *et al.* 2000) will also allow further study of 5αTHB binding to GR. The unliganded GFP-GR is found completely in the cytoplasm whereas the hormone activated receptor is located almost exclusively in the nucleus. Nuclear translocation of this intrinsically fluorescent receptor is dramatic and easily monitored in real-time *in vitro* therefore transfection of this receptor into cells with subsequent 5αTHB treatment would reveal whether 5αTHB can bind to the traditional cytosolic glucocorticoid receptor and cause nuclear translocation.

5α-Reductase activity is sexually dimorphic with activity levels in female rats 3-10 times greater than in male rats (Colby 1980). All of the experiments in this thesis have been carried out in male rats therefore repetition of the experiments in female rats or in gonadectomised male rats may reveal the role of the sex hormones in regulation of 5α-reductase. Another reason for carrying out experiments in female rats is that female rats do not contain microsomal LAGS (Omrani *et al.* 1983). Therefore repetition of the competition binding experiments in female rats would confirm whether 5αTHB is having its effect via binding to microsomal LAGS as binding should not be observed in hepatocytes or microsomes from these animals.
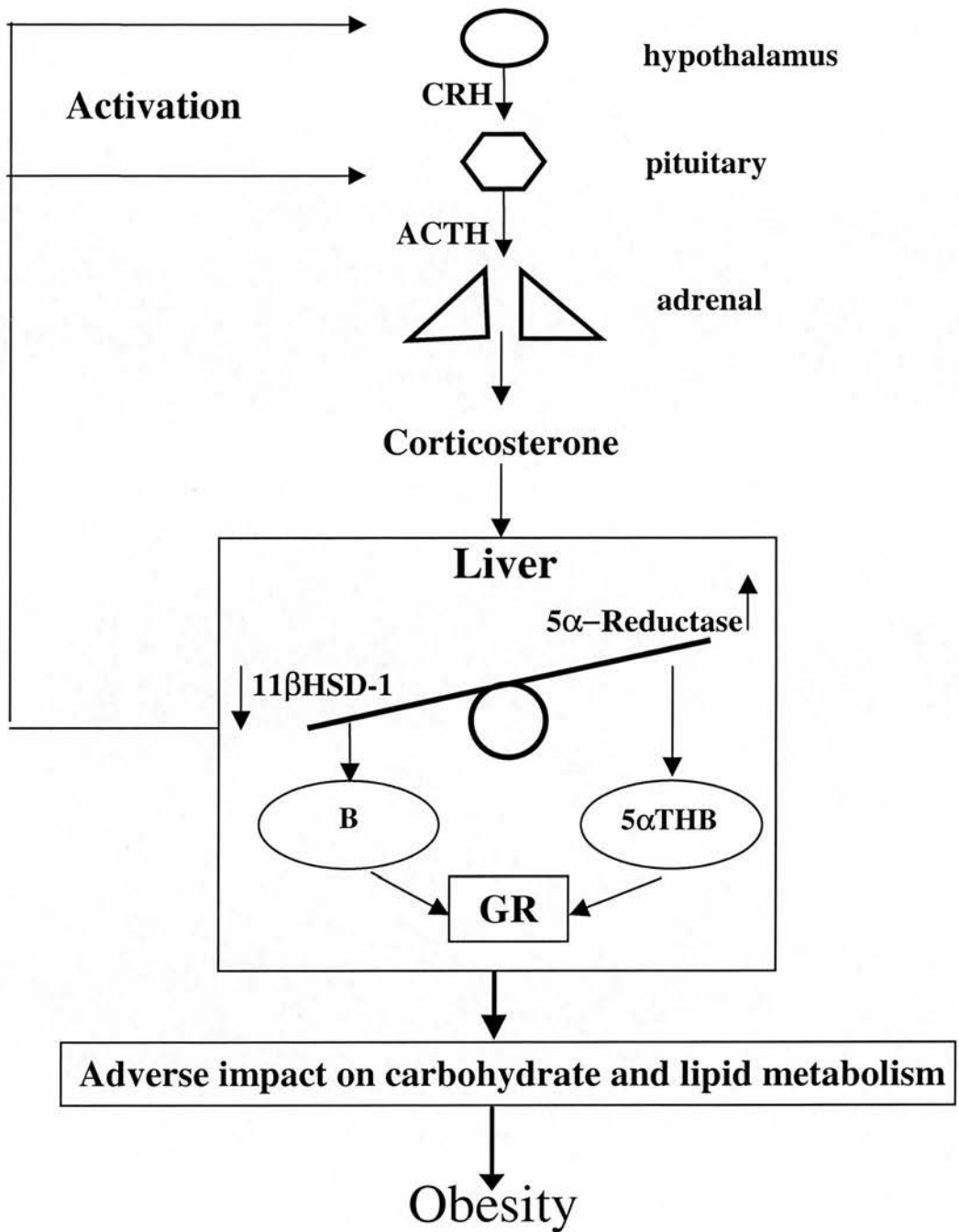
Figure 6.1: Proposed mechanism for the role of 5α-reductase in obesity

5α-reductase and 11β-hydroxysteroid dehydrogenase type 1 are regulated in opposite directions in glucocorticoid target tissues so that when one is decreased the other is increased. This will have important implications for activation of the HPA-axis and subsequent corticosterone production and also on the activation of GR-mediated transcription of genes.

Pharmacological manipulation would also be advantageous. However, to date a specific 5α-reductase type 1 inhibitor does not exist although a dual 5α-reductase type 1 and type 2 inhibitor is being developed. The advent of a specific liver targeted inhibitor would probably be of most use and may have therapeutic potential in the treatment of obesity as inhibiting 5α-reductase will result in corticosterone being metabolised by 5β-reductase to the inactive 5β-reduced metabolites.

The use of transgenic knock-out and over-expressor models will prove particularly useful tools in identifying regulators of 5α-reductase. The 11β-hydroxysteroid dehydrogenase type 1 knockout mouse (Koteletsev *et al.* 1997) and the 5α-reductase type 1 knock-out mouse (Mahendroo *et al.* 2001) will enable confirmation of the reciprocal relationship between 11β-hydroxysteroid dehydrogenase type 1 and 5α-reductase type 1. Similarly, transgenic mice over- expressing 11β-HSD 1 in adipose tissue have been created (Masuzaki *et al.* 2001) which could be used to determine if 5α-reductase type 1 is down-regulated when 11β-hydroxysteroid dehydrogenase type 1 is up-regulated.

6.5 Summary

We conclude that hepatic A-ring reduction is enhanced in the obese Zucker rat producing increased concentrations of 5αTHB which can bind and activate glucocorticoid receptors. Transcription of glucocorticoid regulated genes in tissues which express 5α-reductases will thus be influenced by intracellular levels of both corticosterone and its 5α-reduced metabolites. Manipulation of this enzyme may prove to be a useful therapeutic target in obesity.

References

Addison T 1855 On the constitutional and local effects of disease of the supra-renal capsules. In London: Highley.

Allera A, Rao GS & Breuer H 1980 Specific interaction of corticosteroids with components of the cell membrane which are involved in the translocation of the hormone into the intravesicular space of purified rat liver plasma membrane vesicles. *Journal of Steroid Biochemistry* **12** 259-266.

Ambellan E, Swanson M & Davidson A 1981 Glucocorticoid binding to rat liver microsomal fractions in vitro. *Journal of Steroid Biochemistry* **14** 421-428.

Andersson S, Berman DM, Jenkins EP & Russell DW 1991 Deletion of steroid 5α-reductase 2 gene in male pseudohermaphroditism. *Nature* **354** 159-161.

Andersson S, Bishop RW & Russell DW 1989 Expression cloning and regulation of steroid 5α-reductase, an enzyme essential for male sexual differentiation. *Journal of Biological Chemistry* **264** 16249-16255.

Andersson S & Russell DW 1990 Structural and biochemical properties of cloned and expressed human and rat steroid 5α-reductases. *Proceedings of the National Academy of Sciences USA* **87** 3640-3644.

Andrew R, Phillips DIW & Walker BR 1998 Obesity and gender influence cortisol secretion and metabolism in man. *Journal of Clinical Endocrinology and Metabolism* **83** 1806-1809.

Antakly T & Eissen HJ 1984 Immunocytochemical localisation of GR in target cells. *Endocrinology* **115** 1984-1989.

Antonipillai I, Wahe M, Yamamoto J & Horton R 1995 Activin and inhibin have opposite effects on steroid 5α-reductase activity in genital skin fibroblasts. *Endocrinology* **107** 99-104.

Ballard PL 1987 Glucocorticoid regulation of lung maturation. *Mead Johnson Symposium of Perinatal Developmental Medicine* 22-27.

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

Barnes PJ & Adcock IM 1993 Anti-inflammatory actions of steroids: molecular mechanisms. *Trends in Pharmacological Sciences* **14** 436-441.

Bartels H, Freimann S & Jungermann K 1993 Predominant periportal expression of the phosphoenolpyruvate carboxykinase gene in liver of fed and fasted mice, hamsters and rats studied by *in situ* hybridisation. *Histochemistry* **99** 303-309.

Baxter JD & Tomkins GM 1971 Specific cytoplasmic glucocorticoid hormone receptors in hepatoma tissue culture cells. *Proceedings of the National Academy of Sciences USA* **68** 932-937.

Beato M, Chalepakis G, Schauer M & Slater EP 1989 Interaction of steroid hormone receptors with the transcription initiation complex. *Journal of Steroid Biochemistry* **32** 737-747.

Beato, M., Herrlich, P., and Schütz, G. Steroid hormone receptors: many actors in search of a plot. Cell 83, 851-857. 1995.
Ref Type: Abstract

Berman DM & Russell DW 1993 Cell-type-specific expression of rat steroid 5α-reductase isozymes. *Proceedings of the National Academy of Sciences USA* **90** 9359-9363.

Berseus O 1967 Conversion of cholesterol to bile acids in rat: purification and properties of a Δ^4-3-ketosteroid 5β-reductase of rat liver. *European Journal of Biochemistry* **2** 493-502.

Berseus O, Danielsson H & Kallner A 1965 Synthesis and metabolism of cholest-4-ene-7α,12α-diol-3-one and 5β-Cholestane-7α,12α-diol-3-one. Bile acids and steroids 153. *Journal of Biological Chemistry* **240** 2396-2401.

Best R & Walker BR 1997 Additional value of measurement of urinary cortisone and unconjugated cortisol metabolites in assessing the activity of 11β-hydroxysteroid dehydrogenase *in vivo*. *Clinical Endocrinology (Oxford)* **47** 231-236.

Bestetti GE, Abramo F, Guillaume-Gentil C, Rohner-Jeanrenaud F, Jeanrenaud B & Rossi GL 1990 Changes in the hypothalamo-pituitary-adrenal axis of genetically obese *fa/fa* rats: a structural, immunocytochemical and morphometrical study. *Endocrinology* **126** 1880-1887.

Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH & Xu HE 2002 Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* **110** 93-105.

Borski RJ 2000 Nongenomic membrane actions of glucocorticoids in vertebrates. *Trends in Endocrinology and Metabolism* **11** 427-436.

Bourgeois S, Gruol DJ, Newby RF & Rajah FM 1993 Expression of an *mdr* gene is associated with a new form of resistance to a dexamethasone-induced apoptosis. *Molecular Endocrinology* **7** 840-851.

Bray GA, Sipols AJ & Woods SC 1979 Hypothalamic and genetic obesity in experimental animals:an autonomic and endocrine hypothesis. *Physiological Reviews* **59** 719-809.

Bray GA & York DA 1972 Studies on food intake of genetically obese rats. *American Journal of Physiology* **223** 176-179.

Bronnegard M, Arner P, Hellstrom L, Akner G & Gustafsson J-A 1990 Glucocorticoid receptor messenger ribonucleic acid in different regions of human adipose tissue. *Endocrinology* **127** 1689-1696.

Bronnegard M, Reynisdottir S, Marcus C, Stierna P & Arner P 1995 Effect of glucocorticoid treatment on glucocorticoid receptor expression in human adipocytes. *Journal of Clinical Endocrinology and Metabolism* **80** 3608-3612.

Bujalska IJ, Kumar S, Hewison M & Stewart PM 1999 Differentiation of adipose stromal cells: the roles of glucocorticoids and 11beta-hydroxysteroid dehydrogenase. *Endocrinology* **140** 3188-3196.

Bujalska IJ, Kumar S & Stewart PM 1997 Does central obesity reflect "Cushing's disease of the omentum"? *Lancet* **349** 1210-1213.

Cadepond F, Gask JM, Delahaye F, Jibard N, Schweizer-Groyer G, Segard-Maurel I, Evans RM & Baulieu EE 1992 Hormonal regulation of the nuclear localisation signalsof the human glucocorticosteroid receptor. *Experimental Cell Research* **201** 99-108.

Cadepond F, Schweizer-Groyer G, Segard-Maurel I, Jibard N, Hollenberg SM, Giguere V, Evans RM & Baulieu EE 1991 Heat shock protein 90 as a critical factor in maintaining glucocorticosteroid receptor in a nonfunctional state. *Journal of Biological Chemistry* **266** 5834-5841.

Cairns C, Cairns W & Okret S 1993 Inhibition of gene expression by steroid hormone receptors via a negative glucocorticoid response element: evidence for the involvement of DNA-binding and agonistic effects of the antiglucocorticoid/antiprogestin RU486. *DNA and Cell Biology* **12** 695-702.

Carlstedt-Duke J, Gustafsson J-A, Gustafsson SA & Wrange O 1977 Interactions of Corticosterone, 5α -Dihydrocorticosterone and Dexamethasone with Proteins in Rat-Liver Cytosol. *European Journal of Biochemistry* **73** 231-238.

Castonguay TW>, Dallman MF & Stern JS 1986 Some metabolic and behavioural effects of adrenalectomy on obese Zucker rats. *American Journal of Physiology* **251** R923-R933.

Chen D, Kohli K, Zhang S, Danielsen M & Stallcup MR 1994 Phenylalanine-780 near the C-terminus of the mouse glucocorticoid receptor is important for ligand binding affinity and specificity. *Molecular Endocrinology* **8** 422-430.

Chrousos GP & Gold PW 1998 A healthy body in a healthy mind - and *vice versa* the damaging power of "uncontrollable" stress. *Journal of Clinical Endocrinology and Metabolism* **83** 1842-1845.

Chua SC, Chung WK, Wu-Peng XS, Zhang Y, Liu SM, Tartaglia L & Leibel RL 1996 Phenotypes of mouse diabetes and fatty rat due to mutations in the OB (leptin) receptor. *Science* **271** 994-996.

Colby HD 1980 Regulation of hepatic drug and steroid metabolism by androgens and estrogens. *Advances in sex hormone research* **4** 27.

Collingwood TN, Urnov FD & Wolffe AP 1999 Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. *Journal of Molecular Endocrinology* **23** 255-275.

Colville-Nash PR & Scott DL 1992 Angiogenesis and rheumatoid arthritis; pathogenic and therapeutic implications. *Annals of Rheumatic Disease* **51** 919-925.

Cupps TR, Gerard TI, Falkoff RJM, Whalen G & Fauci AS 1985 Effects of in vitro corticosteroids on B cell activation, proliferation and differentiation. *Journal of Clinical Investigation* **75** 754-761.

Cushing H 1912 *The pituitary body and its disorders*. Philadelphia and London: Lippincott.

Dale DC, Fauci AS, Guerry D & Wolss SM 1975 Comparison of agents producing a neutrophilic leucocytosis in man: hydrocortisone, prednisone, endotoxin and etiocholanone. *Journal of Clinical Investigation* **56** 808-813.

Danielsson H & Sjovall J 1975 Bile-acid metabolism. *Annual Review of Biochemistry* **44** 233-253.

De Kloet ER 1991 Brain corticosteroid receptor balance and homeostatic control. *Frontiers in Neuroendocrinology* **12** 95-164.

De Kloet ER, Wallach G & McEwan BS 1975 Differences in corticosterone and dexamethasone binding to rat brain and pituitary. *Endocrinology* **96** 598-609.

de Lange P, Koper JW, Brinkmann AO, de Jong FH & Lamberts SW 1999 Natural variants of the beta isoform of the human glucocorticoid receptor do not alter sensitivity to glucocorticoids. *Molecular and Cellular Endocrinology* **153** 163-168.

de Wet JR, Wood KV, de Luca M, Helinski DR & Subramani S 1987 Firefly luciferase gene-structure and expression in mammalian cells. *Molecular and Cellular Biology* **7** 725-737.

Denef L 1974 Effect of hypophysectomy and pituitary implants at puberty on the sexual differentiation of testosterone metabolism in rat liver. *Endocrinology* **94** 1577.

Dorrington JF & Fritz IB 1975 Cellular localisation of 5α-reductase and 3α-hydroxysteroid dehydrogenase in the seminiferous tubule of the rat testis. *Endocrinology* **96** 879-889.

Dubrovsky B, Williams D & Kraulis I 1985 Effects of corticosterone and 5α-dihydrocorticosterone on brain excitability in the rat. *Journal of Neuroscience Research* **14** 117-128.

Edwards CRW, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, DeKloet ER & Monder C 1988 Localisation of 11β-hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* **ii** 986-989.

Eicheler W, Seitz J, Steinhoff M, Forssmann WG, Adermann K & Aumüller G 1995 Distrinution of rat hepatic steroid 5α-reductase 1 as shown by immunohistochemistry. *Endocrinology and Diabetes* **103** 105-112.

Enderle-Scmitt U, Volck-Badouin E, Scmitt J & Aumuller G 1989 Solubilization of nuclear steroid 5α-reductase from rat ventral prostate. *Biochemica et Biophysica Acta* **987** 21-28.

Engler O, Pham T, Fullenon MJ, Ooi G, Funder JW & Clarke IJ 1989 Studies of the secretion of corticotropin releasing factor and arginine vasopressin into hypophyseal portal circulation of the conscious sheep. *Neuroendocrinology* **49** 367-381.

Exton JH 1979 Regulation of gluconeogenesis by glucocorticoids. In *Glucocorticoid hormone action*, pp 535-546. Eds JD Baxter & GC Rousseau. New York: Springer-Verlag.

Falkenstein E, Tillmann HC, Christ M, Feuring M & Wehling M 2000 Multiple actions of steroid hormones - a focus on rapid, non-genomic effects. *Pharmacological Reviews* **52** 513-555.

Fassnacht M, Schlenz N, Schneider SB, Wudy SA, Allolio B & Arlt W 2003 Beyond Adrenal and Ovarian Androgen Generation: Increased Peripheral 5α-Reductase Activity in Women with Polycystic Ovary Syndrome. *Journal of Clinical Endocrinology and Metabolism* **88** 2760-2766.

Finken MJJ, Andrews RC, Andrew R & Walker BR 1999 Cortisol metabolism in healthy young adults: sexual dimorphism in activities of A-ring reductase but not 11-hydroxysteroid dehydrogenases. *Journal of Clinical Endocrinology and Metabolism* **84** 3316-3321.

Fleischer S & Kervina M 1974 Subcellular Fractionation of Rat Liver. *methods in enzymology* **31** 6-41.

Fletcher JM 1986 Effects of adrenalectomy before weaning and short- or long-term glucocorticoid administration on the genetically obese Zucker rat. *Biochemical Journal* **238** 459-463.

Forchielli E & Dorfman RI 1956 Separation of 5alpha and 5beta-hydrogenases from rat liver homogenates. *Journal of Biological Chemistry* **223** 443-448.

Fraser R, Ingram MC, Anderson NH, Morrison C, Davies E & Connell JMC 1999 Cortisol effects on body mass, blood pressure and cholesterol in the general population. *Hypertension* **33** 1364-1368.

Freedman MR, Horwitz BA & Stern JS 1986 Effect of adrenalectomy and glucocorticoid replacement on development of obesity. *American Journal of Physiology* **250** R595-607.

Fried SK, Russell CD, Grauso NL & Brolin RE 1993 Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. *Journal of Clinical Investigation* **92** 2191-2198.

Friedman JE, Sun Y, Ishizuka T, Farrell CJ, McCormack SE, Herron LM, Hakimi P, Lechner P & Yun JS 1997 Phosphoenolpyruvate carboxykinase (GTP) gene transcription and hypoglycemia are regulated by GC's in genetically obese db/db transgenic mice. *Journal of Biological Chemistry* **272** 31475-31481.

Gametchu B, Chen F, Sackey F, Powell CS & Watson CS 1999 Plasma-membrane resident glucocorticoid receptors in rodent lymphoma and human leukemia models. *Steroids* **64** 107-119.

Ganss R, Weih F & Schütz G 1994 The cyclic adenosine 3',5'-monophosphate- and the glucocorticoid-dependent enhancers are targets for insulin repression of tyrosine aminotransferase gene transcription. *Molecular Endocrinology* **8** 895-903.

George FW, Russell DW & Wilson JD 1991 Feed-forward control of prostate growth: Dihydrotestosterone induces expression of its own biosynthetic enzyme, steroid 5α-reductase. *Proceedings of the National Academy of Sciences USA* **88** 8044-8047.

Gey GO, Coffman WD & Kubicek MT 1952 Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Research* **12** 264-265.

Gorsline J, Harnik M, Tresco PA & Morris DJ 1986 Hypertensinogenic activities of ring-A-reduced metabolites of alsdosterone. *Hypertension* **8** I-187-I-190.

Goulding NJ & Godolphin Jl 1990 Anti-inflammatory lipocortin I production by peripheral blood leucocytes in response to hydrocortisone. *Lancet* **1** 1418.

Grange T, Cappabianca L, Flavin M, Sassi H & Thomassin H 2001 *In vivo* analysis of the model tyrosine aminotransferase gene reveals multiple sequential steps in glucocorticoid receptor action. *Oncogene* **20** 3028-3038.

Granner D & Hargrove JL 1983 Regulation of the synthesis of tyrosine aminotransferase: the relationship to mRNA-TAT. *Molecular and Cellular Biochemistry* **53** 113-128.

Gray CH, Sunnon JB, Pond MH & Simpson SL 1956 Steroid studies in normal and adipose children. *Journal of Clinical Endocrinology* **16** 473.

Grippo JF, Tienrunroj W, Dahmer MK, Housley PR & Pratt WB 1983 Evidence that the endogenous heat-stable glucocorticoid receptor activating factor is thioredoxin. *Journal of Biological Chemistry* **258** 13658.

Guillaume-Gentil C, Rohner-Jeanrenaud F, Abramo F, Bestetti GE, Rossi GL & Jeanrenaud B 1990 Abnormal regulation of the hypothalamic-pituitary-adrenal axis in the genetically obese *fa/fa* rat. *Endocrinology* **126** 1873-1879.

Gustafsson J-A, Mode A, Norstedt G, Hökfelt T, Sonnenschein C, Eneroth P & Skett P 1980 The hypothalamo-pituitary-liver axis: a new hormonal system in control of hepatic steroid and drug matabolism. *Biochemical Actions of Hormones* **7** 47.

Hadoke PWF, Kotelevtsev YV, Williams BC, Seckl JR, Mullins JJ & Walker BR 1999 Influence of 11β-hydroxysteroid dehydrogenases (11HSDs) on vascular function: studies in mice with transgenic deletion of 11-HSD type 1 or type 2. *Proceedings of the Endocrine Society.*

Hager GL, Lim CS, Elbi C & Baumann CT 2000 Trafficking of nuclear receptors in living cells. *Journal of Steroid Biochemistry and Molecular Biology* **74** 249-254.

Hahn TJ, Halstead LR, Teitelbaum SL & Hahn BH 1979 Altered mineral metabolism in glucocorticoid induced ostopenia: efect of 25-hydroxyvitamin D administration. *Journal of Clinical Investigation* **64** 21-28.

Hammami MM & Siiteri PK 1991 Regulation of 11β -hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action. *Journal of Clinical Endocrinology and Metabolism* **73** 326-334.

Hammond GL, Smith CL, Paterson NAM & Sibbald WJ 1990 A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. *Journal of Clinical Endocrinology and Metabolism* **73** 326-334.

Hardy DO, Ge R-S, Catterall JF, Hou Y-T, Penning T & Hardy MP 2000 Identification of the Oxidative 3α–Hydroxysteroid dehydrogenase activity of Rat Leydig cells as Type II Retinol Dehydrogenase. *Endocrinology* **141** 1608-1617.

Harris G, Azzolina B, Baginsky W, Cimis G, Rasmusson GH, Tolman RL, Raetz CRH & Ellsworth K 1992 Identification and slective inhibition of an isozyme of steroid 5α-reductase in human scalp. *Proceedings of the National Academy of Sciences USA* **89** 10787-10791.

Harris HJ, Koteletsev Y, Mullins JJ, Seckl JR & Holmes MC 2001 Intracellular regeneration of glucocorticoids by 11B-hydroxysteroid dehydrogense (11B-HSD)-1 plays a key role in regulation of the hypothalamic-pituitary-adrenal axis: Analysis of 11B-HSD-1-deficient mice. *Endocrinology* **142** 114-120.

Hashimoto S, Schmid W & Schütz G 1984 Transcriptional activation of the rat liver tyrosine aminotransferase gene by cAMP. *Proceedings of the National Academy of Sciences USA* **81** 6637-6641.

Hauner H, Entenmann G & Wabitsch M 1989 Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *Journal of Clinical Investigation* **84** 1663-1670.

Hauner H, Schmidt P & Pfieffer E-F 1987 Glucocorticoids and insulin promote the differentiation of human adipocyte precursor cells into fat cells. *Journal of Clinical Endocrinology and Metabolism* **64** 832-835.

Hautanen A & Adlercreutz H 1993 Altered adrenocorticotropin and cortisol secretion in abdominal obesity:implications for the insulin resistance syndrome. *Journal of Internal Medicine* **234** 461-469.

Haytanen A & Adlercreutz H 1993 Altered adrenocorticotropin and cortisol secretion in abdominal obesity: implications for the insulin resistance syndrome. *Journal of Internal Medicine* **234** 461-469.

Hebbar PB & Archer TK 2003 Chromatin remodelling by nuclear receptors. *Chromosoma* **111** 495-504.

Hecht K, Carlstedt-Duke J, Stierna P, Gustafsson J-A, Bronnegard M & Wikstrom AC 1997 Evidence that the beta-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor. *Journal of Biological Chemistry* **272** 26659-26664.

Hollenberg SM & Evans RM 1988 Multiple and cooperative transactivation domains of the human glucocorticoid receptor. *Cell* **55** 899-906.

Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG & Evans RM 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318** 635-641.

Horrocks PM, Jones AF, Ratcliffe WA, Holder G, White A, Holder R, Ratcliffe JG & London DR 1990 Patterns of ACTH and cortisol pulsatility over twenty-four hours in normal males and females. *Journal of Clinical Endocrinology and Metabolism* **32** 127-134.

Horton R, Pasupuletti V & Antonipillai I 1993 Androgen induction of steroid 5α-reductase may be mediated via insulin-like growth factor-1. *Endocrinology* **133** 447-451.

Hou Y-T, Lin HK & Penning TM 1998 Dexamethasone regulation of the rat 3α-Hydroxysteroid/dihydrodiol dehydrogenase gene. *Molecular Pharmacology* **53** 459-466.

Houston B, Chisholm GD & Habib FK 1985 Solubilisation of human prostatic 5α-reductase. *Journal of Steroid Biochemistry* **22** 461-467.

Ichikawa-Haraguchi M, Sumizawa T, Yoshimura A, Furukawa T, Hiramoto S, Sugita M & Akiyama S 1993 Progesterone and its metabolites: the potent inhibitors of the transporting activity of P-glycoprotein in the adrenal gland. *Biochemica et Biophysica Acta* **1158** 201-208.

Iki K, Miyamori I, Hatakeyama H, Yoneda T, Takeda Y, Takeda R & Dai Q 1994 The activities of 5β-reductase and 11β-hydroxysteroid dehydrogenase in essential hypertension. *Steroids* **59** 656-660.

Imperato-McGinley J, Gautier T, Cai L-Q, Yee B, Epstein J & Pochi P 1992 The androgen control of sebum production. Studies of subjects with dihydrotestosterone deficiency and complete androgen insensitivity. *Journal of Clinical Endocrinology and Metabolism* **76** 524-528.

Imperato-McGinley J, Guerrero L, Gautier T & Peterson RE 1974 Steroid 5α-reductase deficiency in men: an inherited form of male pseudohermaphroditism. *Science* **186** 1213-1217.

Ito K, Jazrawi E, Cosio B, Barnes PJ & Adcock IM 2001 p65-activated histone acetyltransferase activity is repressed by glucocorticoids: mifepristone fails to recruit HDAC2 to the p65-HAT complex. *Journal of Biological Chemistry* **276** 30208-30215.

Jacobi GH, Moore RJ & Wilson JD 1977 Characterisation of the 3α–hydroxysteroid dehydrogenase of dog prostate. *Journal of Steroid Biochemistry* **8** 719-723.

Jaffuel D, Roumestan C, Balaguer P, Henriquet C, Gougat C, Bousquet J, Demoly P & Mathieu M 2001 Correlation between different gene expression assays designed to measure trans-activation potencies of systemic glucocorticoids. *Steroids* **66** 597-604.

Jaggers DC, Collins WP & Milligan SR 1996 Potent inhibitory effects of steroids in an in vitro model of angiogenesis. *Journal of Endocrinology* **150** 457-464.

Jamieson PM, Chapman KE, Edwards CRW & Seckl JR 1995 11β-Hydroxysteroid dehydrogenase is an exclusive 11β-reductase in primary cultures of rat hepatocytes: effect of physiochemical and hormonal manipulations. *Endocrinology* **136** 4754-4761.

Jamieson PM, Walker BR, Chapman KE, Rossiter S & Seckl JR 2000 11β-Hydroxysteroid dehydrogenase type 1 is a predominant 11β-reductase in the intact perfused rat liver. *Molecular Endocrinology* **165** 685-692.

Jenkins BD, Pullen CB & Darimont BD 2001 Novel glucocorticoid receptor coactivator effector mechanisms. *Trends in Endocrinology and Metabolism* **12** 122-126.

Jenkins EP, Andersson S, Imperato-McGinley J, Wilson JD & Russell DW 1992 Genetic and pharmacological evidence for more than one human steroid 5a-reductase. *Journal of Clinical Investigation* **89** 293-300.

Jenson M, Kilroy G, York DA & Braymer D 1996 Abnormal regulation of hepatic glucocorticoid receptor mRNA and receptor protein distribution in the obese Zucker rat. *Obesity Research* **4** 133-143.

Jez JM, Bennett MJ, Sclegel BP, Lewis M & Penning TM 1997 Comparative anatomy of the aldo-keto reductase superfamily. *Biochemical Journal* **326** 625-636.

Jonat C, Rahmsdorf HJ, Park KK, Cato AC, Gebel S, Ponta H & Herrlich P 1990 Antitumour promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* **62** 1205-1215.

Jungermann K & Kietzmann T 1996 Zonation of parenchymal and nonparenchymal metabolism in liver. *Annual review of Nutrition* **16** 179-203.

Karssen AM, Meijer OC, van der Sandt CJ, Lucassen PJ, De Lange ECM, De Boer AG & De Kloet ER 2001 Multidrug resistance p-glycoprotein hampers the access of cortisol but not of corticosterone to mouse and human brain. *Endocrinology* **142** 2686-2694.

Kaufmann SH & Shaper JH 1984 Binding of dexamethasone to rat liver nuclei in vivo and in vitro. evidence for two distinct binding sites. *Journal of Steroid Biochemistry* **20** 699-708.

Kenyon CJ, Saccoccio NA, Harnik M & Morris DJ 1985 The effects of Long-term Infusions of the Reduced derivatives of Aldosterone on Water and Electrolyte Metabolism. *The Adrenal Gland and Hypertension* **27** 209-214.

Khanna M, Qin K-N, Wan RW & Cheng K-C 1995 Substrate specificty, gene structure, and tissue-specific distribution of multiple human 3α-hydroxysteroid dehydrogenases. *Journal of Biological Chemistry* **270** 20162-20168.

King AE, Melville J, Nelson SM, Panarelli M, Benediktsson R & Kenyon CJ 1998 regulation of low affinity glucocorticoid binding site (LAGS) in relation to high affinity glucocorticoid receptor properties. *Journal of Endocrinology Supplement* **156** 286.

Knowles BB & Aden DP 1980 Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* **209** 497-499.

Kondo K-H, Kai M-H, Setoguchi Y, Eggertsen G, Sjoblom P, Setoguchi T, Okuda K-I & Bjorkhem I 1994 Cloning and expression of CDNA of human Delta4-3-oxosteroid 5β-reductase and substrate specificity of the expressed enzyme. *European Journal of Biochemistry* **219** 357-363.

Koteletsev Y, Holmes MC, Burchell A, Houston PM, Scholl D, Jamieson PM, Best R, Brown RW, Edwards CRW, Seckl JR & Mullins JJ 1997 11B-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proceedings of the National Academy of Sciences USA* **94** 14924-14929.

Kotelevtsev YV, Brown RW, Fleming S, Edwards CRW, Seckl JR & Mullins JJ 1999 Hypertension in mice lacking 11β-hydroxysteroid dehydrogenase type 2. *Journal of Clinical Investigation* **103** 683-689.

Kotelevtsev YV, Holmes MC, Burchell A, Houston PM, Scholl D, Jamieson PM, Best R, Brown RW, Edwards CRW, Seckl JR & Mullins JJ 1997 11β-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity and stress. *Proceedings of the National Academy of Sciences USA* **94** 14924-14929.

Kreiger DT 1972 The central nervous system and Cushing's disease. *Mount Sinai Journal of Medicine* **39** 416-422.

Krones A, Kietzmann T & Jungermann K 2000 Perivenous localization of insulin receptor protein in rat liver, and regulation of its expression by glucose and oxygen in hepatocyte cultures. *Biochemical Journal* **348** 433-438.

Krozowski ZS, Stuchberry S, White PC, Monder C & Funder JW 1990 Characterisation of 11β-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinology* **127** 3009-3013.

Laake H 1960 The actions of corticosteroids on the renal absorption of calcium. *Acta Endocrinologica (Copenhagen)* **34** 60-64.

Lackner C, Daufeldt S, Wildt L & Allera A 1998 Glucocorticoid-recognising and effector sites in rat liver plasma membrane.Kinetics of corticosterone uptake by isolated membrane vesicles. Specificity and stereospecificity. *Journal of Steroid Biochemistry and Molecular Biology* **64** 69-82.

Lamberts SWJ, Verleun T, Oosterom R, DeJong P & Hackeng WHL 1984 Corticotropin releasing factor and vasopressin exert a synergistic effect on adrenocorticotropin release in man. *Journal of Clinical Endocrinology and Metabolism* **58** 298-303.

Langley SC & York DA 1990 Effects of antiglucocorticoid RU486 on development of obesity in obese *fa/fa* Zucker rats. *American Journal of Physiology* **259** R539-544.

Langley SC & York DA 1992 Glucocorticoid receptor numbers in the brain and liver of the obese Zucker rat. *International Journal of Obesity and Related Metabolic Disorders* **16** 135-143.

Lax ER, Ghraf R & Schriefers H 1978 The hormonal regulation of hepatic microsomal 11β-hydroxysteroid dehydrogenase activity in the rat. *Acta Endocrinologica (Copenhagen)* **89** 352-358.

Lax ER, Ghraf R, Schriefers H, Hermann M & Petutschnigk D 1976 Regulation of the activities of the enzymes involved in the metabolism of steroid

hormones in the rat liver. The effect of administration of anterior hypophyseal hormones and gonadotrophin preparations to hypophysectomised rats. *Acta Endocrinologica (Copenhagen)* **82** 774.

Lefebvre P, Berard DS, Cordingley MG & Hager GL 1991 Two regions of the mouse mammary tumour virus long terminal repeat regulate the activity of its promoter in mammary cell lines. *Molecular and Cellular Biology* **11** 2529-2537.

Lefebvre YA & Morante SJ 1982 Binding of dihydrotestosterone to a nuclear-envelope fraction from the rat liver. *Biochemical Journal* 225-230.

Lephart ED, Simpson ER & Trzeciak WH 1991 Rat adrenal 5α-reductase mRNA content and enzyme activity are sex-hormone dependent. *Journal of Molecular Endocrinology* **6** 163-170.

Liao S, Liang T, Fang S, Castañeda E & Shao T-C 1973 Steroid structure and Androgenic activity. *Journal of Biological Chemistry* **248** 6154-6162.

Lin HK, Chien-Fu H, Moore M & Penning T 1999 Genomic structure of rat 3α-hydroxysteroid/dihydriol dehydrogenase (3 α-HSD/DD, AKR1C9). *Journal of Steroid Biochemistry and Molecular Biology* **71** 29-39.

Lindros KO 1997 Zonation of cytochrome P450 expression, drug metabolism and toxicity in liver. *Genetics of Pharmacology* **28** 191-196.

Liposits Z & Bohn MC 1993 Association of glucocorticoid receptor immunoreactivity with cell membrane and transport vesicles in hippocampal and hypothalamic neurones of the rat. *Journal of Neuroendocrine Research* **35** 14-19.

Livingstone DEW, Jones GC, Smith K, Jamieson PM, Andrew R, Kenyon CJ & Walker BR 2000 Understanding the role of glucocorticoids in obesity: tissue specific alterations of corticosterone metabolism in obese Zucker rats. *Endocrinology* **141** 560-563.

Ljung T, Andersson B, Bengtsson B, Bjorntorp P & Marin P 1996 Inhibition of cortisol secretion by dexamethasone in relation to body fat distribution; a dose-response study. *Obesity Research* **4** 277-282.

Loosen PT, Chambliss B, DeBold CR, Shelton R & Orth DN 1992 Psychiatric phenomenology in Cushing's disease. *Pharmacopsychiatry* **25** 192-198.

Lopez-Solache I, Luu-The V, Seralini G & Labrie F 1996 Heterogeneity of rat type 1 5α-reductase cDNA: cloning, expression and regulation by pituitary implants and dihydrotestosterone. *Biochemica et Biophysica Acta* **1305** 139-144.

Lottenberg SA, Giannella-Neto D, Derendorf H, Rocha M, Bosco A, Carvalho SV, Moretti AE, Lerário AC & Wajchenberg BL 1998 Effect of fat distribution on the pharmacokinetics of cortisol in obesity. *International Journal of Clinical Pharmacology and Therapeutics* **36** 501-505.

Low SC, Chapman KE, Edwards CRW, Wells T, Robinson ICAF & Seckl JR 1994a Sexual dimorphism of hepatic 11β-hydroxysteroid dehydrogenase in the rat: the role of growth hormone patterns. *Journal of Endocrinology* **143** 541-548.

Low SC, Moisan M-P, Edwards CRW & Seckl JR 1994b Glucocorticoids regulate hippocampal 11β-hydroxysteroid dehydrogenase activity and gene expression in vivo in the rat. *Journal of Neuroendocrinology* **6** 285-290.

Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR & Sigler PB 1991 Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352** 497-505.

Mahendroo MS, Cala KM, Hess DL & Russell DW 2001 Unexpected virilisation in male mice lacking steroid 5α-reductase enzymes. *Endocrinology* **142** 4652-4662.

Mahendroo MS, Cala KM, Landrum CP & Russell DW 1997 Fetal death in mice lacking 5α-reductase type 1 caused by estrogen excess. *Molecular Endocrinology* **11** 917-927.

Mahendroo MS, Cala KM & Russell DW 1996 5α-reduced androgens play a key role in murine parturition. *Molecular Endocrinology* **10** 380-392.

Mahendroo MS & Russell DW 1999 Male and female isoenzymes of steroid 5α-reductase. *Reviews of Reproduction* **4** 179-183.

Majewska MD, Harrison NL, Schwartz RD, Barker JL & Paul SM 1986 Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* **232** 1004-1007.

Marin P, Darin M, Amemiya T, Andersson B, Jern S & Bjorntorp P 1992 Cortisol secretion in relation to body fat distribution in obese premenopausal women. *Metabolism* **41** 882-886.

Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR & Flier JS 2001 A transgenic model of visceral obesity and the metabolic syndrome. *Science* **294** 2166-2170.

Matsui D, Sakari M, Sato T, Murayama A, Takada I, Kim M, Takeyama K & Kato S 2002 Transcriptional regulation of the mouse steroid 5α-reductase type II gene by progesterone in brain. *Nucleic Acids Research* **30** 1387-1393.

Mcdermott MJ, Freiberger M, Latif S & Morris DJ 1985 The synthesis of reduced metabolites of aldosterone by subcellular fractions of rat kidney;effects of antimineralocorticoids. *Journal of Steroid Biochemistry* **23** 503-509.

McEwen BS, De Kloet ER & Rostene W 1986 Adrenal steroid receptors and actions in the nervous system. *Physiological Reviews* **66** 1121-1188.

McGuire JS, Hollis VW & Tomkins GM 1960 Some characteristics of the microsomal steroid reductases (5α) of rat liver. *Journal of Biological Chemistry* **235** 3112-3116.

McGuire JS & Tomkins GM 1960 The heterogeneity of Δ^4-3-ketosteroid reductases (5α). *Journal of Biological Chemistry* **235** 1634-1638.

Meaney M, Tannenbaum B, Francis D, Bhatnagar S, Shanks N, Viau V, O'Donnel D & Plotsky PM 1994 Early environmental programming of hypothalamic-pituitary-adrenal responses to stress. *Seminars in Neuroscience* **6** 247-259.

Melcangi RC, Celotti F, Castano P & Martini L 1993 Differential localisation of the 5α-reductase and the 3α-hydroxysteroid dehydrogenase in neuronal and glial cultures. *Endocrinology* **132** 1252-1259.

Melcangi RC, Poletti A, Cavarretta I, Celotti F, Colciago A, Magnaghi V, Motta M, Negri-Cesi P & Martini L 1998 The 5α-reductase in the central nervous system: expression and modes of control. *Journal of Steroid Biochemistry and Molecular Biology* **65** 295-299.

Melville J, King AE, Watson DE, Kenyon CJ & Benediktsson R 1998 The hepatic low affinity glucocorticoid binding site: a steroid metabolising enzyme? *Journal of Endocrinology Supplement* **156** p286.

Migeon CJ, Green OC & Eckert JP 1963 Study of adrenocortical function in obesity. *Metabolism* **12** 718-739.

Miller MA & Colás AE 1982 Multihormonal control of microsomal 5α-reductase activity in cultured adult female rat hepatocytes. *Endocrinology* **111** 136-143.

Miller WL 1988 Molecular biology of steroid hormone biosynthesis. *Endocrine Reviews* **9** 295-318.

Misiti S, schomburg L, Yen PM & Chin WW 1998 Expression and hormonal regulation of coactivator and corepressor genes. *Endocrinology* **139** 2493-2500.

Mode A, Norstedt G, Simic B, Eneroth P & Gustafsson J-A 1981 Continuous infusion of of growth hormone feminises hepatic steroid metabolism in the rat. *Endocrinology* **108** 2103-2108.

Moisan M-P, Edwards CRW & Seckl JR 1992 Ontogeny of 11β-hydroxysteroid dehydrogenase in rat brain and kidney. *Endocrinology* **130** 400-404.

Monder C & Lakshmi V 1989 Evidence for kinetically distinct forms of corticosteroid 11β-dehydrogenase in rat liver microsomes. *Journal of Steroid Biochemistry* **32** 77-83.

Monder C, Shackleton CHL, Bradlow HL, New MI, Stoner E, Iohan F & Lakshmi V 1986 The syndrome of apparent mineralocorticoid excess: its association with 11β-dehydrogenase and 5β-reductase deficiency and some consequences for corticosteroid metabolism. *Journal of Clinical Endocrinology and Metabolism* **63** 550-557.

Montrella-Waybill M, Clore JN, Schollwerth AC & Watlington CO 1991 Evidence that high dose cortisol-induced sodium retention in man is not mediated by the mineralocorticoid receptor. *Journal of Clinical Endocrinology and Metabolism* **72** 1060-1066.

Moore PS & Koontz JW 1989 Insulin-mediated regulation of tyrosine aminotransferase in rat hepatoma cells: inhibition of transcription and inhibition of enzyme degradation. *Archives of Biochemistry and Biophysics* **275** 486-495.

Moore RJ, Griffin JE & Wilson JD 1975 Diminished 5α-reductase activity in extracts of fibroblasts cultured from patients with familial incomplete male pseudohermaphroditism. *Journal of Biological Chemistry* **251** 7168-7172.

Moore RJ & Wilson JD 1972 Localisation of the reduced nicotinamide adenine dinucleotide phosphate: Δ^4-3-ketosteroid 5α-oxidoreductase in the nuclear membrane of the rat ventral prostate. *Journal of Biological Chemistry* **247** 958-967.

Morais JA & Wagner JG 1985 Steroid metabolism in isolated rat hepatocytes. *European journal of drug metabolism and pharmacokinetics* **10** 295-307.

Morris DJ, Kenyon CJ, Latif S, Mcdermott MJ, Goodfriend TL & . 1982 The possible biological role of alsosterone metabolites. *Hypertension* **5** I-35-I-40.

Murono EP & Payne AH 1979 Testicular maturation in the rat. *In vivo* effect of gonadotropins on steroidogenic enzymes in the hypophysectomised immature rat. *Biology of Reproduction* **20** 911-917.

Murphy BP, Engelberg W & Pattee CJ 1963 hhjhjhjhj. *Journal of Clinical Endocrinology* **23** 293-300.

Nakaishi S, Nakai Y, Fukata J, Naito Y, Usui T & Imura H 1990 Immunoreactive corticotropin-releasing hormone levels in brain regions of genetically obese Zucker rats. *International Journal of Obesity* **14** 951-955.

Napolitano A, Voice MW, Edwards CW, Seckl JR & Chapman KE 1998 11beta-Hydroxysteroid dehydrogenase 1 in adipocytes: Expression is differentiation-dependent and hormonally regulated. *Journal of Steroid Biochemistry and Molecular Biology* **64** 251-260.

Nayfey SN, Coffey JC, Hansson V & French VS 1975 Maturational changes in testicular steroidogenesis: hormonal regulation of 5α -reductase. *Journal of Steroid Biochemistry* **6** 329-335.

Nazareth LV, Harbour DV & Thompson EB 1991 Mapping of the human glucocorticoid receptor for leukemic cell death. *Journal of Biological Chemistry* **266** 12976-12980.

Nelson SM, Panarelli M, Walker BR & Kenyon CJ 1998 Reduced affinity of hepatic glucocorticoid receptors for corticosterone in obese Zucker rats: effects of adrenalectomy. *Journal of Endocrinology Supplement* **156** 286.

Normington K & Russell DW 1992 Tissue distribition and kinetic characteristics of rat steroid 5α-reductase isozymes. *Journal of Biological Chemistry* **267** 19548-19554.

Nyirenda MJ, Lindsay RS, Kenyon CJ, Burchell A & Seckl JR 1998 Glucocorticoid exposure in late gestation permanently programmes rat hepatic phosohoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. *Journal of Clinical Investigation* **101** 2174-2181.

Oakley RH, Jewell CM, Yudt MR, Bofetiado DM & Cidlowski JA 1999 The dominant negative activity of the human glucocorticoid receptor β isoform. Specificity and mechanisms of action. *Journal of Biological Chemistry* **274** 27857-27866.

Oakley RH, Sar M & Cidlowski JA 1996 The human glucocorticoid receptor β isoform. Expression, biochemical properties and putative function. *Journal of Biological Chemistry* **271** 9550-9559.

Oakley RH, Webster JC, Sar M, Parker Jr CR & Cidlowski JA 1997 Expression and subcellular distribution of the β–isoform of the human glucocorticoid receptor. *Endocrinology* **138** 5028-5038.

Omrani GR, Furukawa H, Sherwood JA & Loeb JN 1983 [^{3}H]-Dexamethason binding by rat liver microsomes: effects of age, sex and adrenal ststus. *Endocrinology* 178-186.

Onishi Y, Mitsuhide N, Shimosato T & Okuda K 1991 Molecular cloning and and sequence analysis of cDNA encoding Δ^4-3-ketosteroid 5β-reductase of rat liver. *FEBS Letters* **283** 215-218.

Orchinik M, Murray TF & Moore FL 1991 A corticosteroid receptor in neuronal membranes. *Science* **252** 1848-1850.

Orti E, Mendel DB, Smith LI & Munck A 1989 Agonist-dependent phosphorylation and nuclear dephosphorylation of glucocorticoid receptors in intact cells. *Journal of Biological Chemistry* **266** 7549-7555.

Pasquali R, Cantobelli S, Casimirri F, Capelli M, Bortoluzzi L, Flamia R, Labate AMM & Barbara L 1993 The hypothalamic-pituitary-adrenal axis in obese women with different patterns of body fat distribution. *Journal of Clinical Endocrinology and Metabolism* **77** 341-346.

Pearce D & Yamamoto KR 1993 Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. *Science* **259** 1161-1165.

Penning TM, Bennett MJ, Smith-Hoog S, Schlegel BP, Jez JM & Lewis M 1997 Structure and function of 3α-hydroxysteroid dehydrogenase. *Steroids* **62** 101-111.

Penning TM, Smithgall TE, Askonas LJ & Sharp RB 1986 Rat liver 3α-hydroxysteroid dehydrogenase. *Steroids* **47** 221-247.

Perel E, Daniilescu D, Kindler S, Kharlip L & Killinger DW 1986 The formation of 5α-reduced androgens in stromal cells from human breast adipose tissue. *Journal of Clinical Endocrinology and Metabolism* **62** 314-318.

Phillipou G, Palermo M & Shackleton CHL 1996 Apparent cortisone reductase deficiency; a unique form of hypercortisolism. *Journal of Clinical Endocrinology and Metabolism* **81** 3855-3860.

Picard D & Yamamoto KR 1987 Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO Journal* **11** 3333-3340.

Pitot HC, Peraino C, Morse PA & Potter VR 1964 Hepatomas in tissue culture compared with adapting liver in vivo. *National Cancer Institute Monograph* **13** 229-245.

Plotsky PM, Thrivikraman KV, Watts AG & Hauger RL 1992 Hypothalamic-pituitary-adrenal axis function in the Zucker obese rat. *Endocrinology* **130** 1931-1941.

Pratis K, O'Donnell L, Ooi GT, Stanton PG, McLachlan RI & Robertson DM 2003 Differential regulation of rat testicular 5α-reductase type 1 and 2 isoforms by testosterone and FSH. *Journal of Endocrinology* **176** 393-403.

Pratt WB & Aronow L 1966 The effect of glucocorticoids on protein and nucleic acid synthesis in mouse fibroblasts growing in vitro. *Journal of Biological Chemistry* **241** 5244-5250.

Pratt WB & Dittmar KD 1998 Studies with purified chaperones advance the understanding of the mechanism of glucocorticoid receptor-hsp90 heterocomplex assembly. *Trends in Endocrinology and Metabolism* **9** 244-252.

Pratt WB & Toft DO 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocrine Reviews* **18** 306-360.

Quinkler M, Bumke-Vogt C, Meyer B, Bähr V, Oeklers W & Diederich S 2003 The human kidney is a progesterone-metabolizing and androgen-producing organ. *Journal of Clinical Endocrinology and Metabolism* **88** 2803-2809.

Raff MC 1992 Social controls on cell survival and cell death. *Nature* **356** 397-400.

Rajan V, Chapman KE, Lyons V, Jamieson PM, Mullins JJ, Edwards CRW & Seckl JR 1995 Cloning, sequencing and tissue-distribution of mouse 11beta-hydroxysteroid dehydrogenase-1 cDNA. *Journal of Steroid Biochemistry and Molecular Biology* **52** 141-147.

Rapkin A, Morgan M, Goldman L, Brann D, Simone D & Matiesh V 1997 Progesterone metabolite allopregnanolone in women with premenstrual syndrome. *Journal of Steroid Biochemistry* **90** 709-714.

Rask E, Olsson T, Soderberg S, Andrew R, Livingstone DEW, Johnson O & Walker BR 2001 Tissue-specific dysregulation of cortisol metabolism in human obesity. *Journal of Clinical Endocrinology and Metabolism* **86** 1418-1421.

Rask E, Walker BR, Soderberg S, Livingstone DEW, Eliasson M, Johnson O, Andrew R & Olsson T 2002 Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11B-hydroxysteroid dehydrogenase type 1 activity. *Journal of Clinical Endocrinology and Metabolism* **87** 3330-3336.

Ratka A, Sutanto WS, Bloemers M & De Kloet ER 1989 On the role of brain mineralocorticoid (type1) and glucocorticoid (type II) receptors in neuroendocrine regulation. *Neuroendocrinology* **50** 117-123.

Ray A & Prefontaine KE 1994 Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proceedings of the National Academy of Sciences USA* **91** 752-756.

Reichert W, Michel A, Hartmann RW & Jose J 2001 Stable expression of human 5α-reductase type II in COS1 cells due to chromosomal gene integration: a novel tool for inhibitor identification. *Journal of Steroid Biochemistry and Molecular Biology* **78** 275-284.

Reid IR 2000 Glucocorticoid-induced osteoporosis. *Baillieres Clinical and Endocrinology Metabolism* **14** 279-298.

Reinisch JM, Simon NG, Karow WG & Gandelman R 1978 Prenatal exposure to prednisone in humans and animals retards intrauterine growth. *Science* **202** 436-438.

Reul JMHM & De Kloet ER 1985 Two receptor systems for corticosterone in rat brain: Microdistribution and differential occupation. *Endocrinology* **117** 2505-2511.

Rinehart JJ, Sagone AL, Balcerzak SP, Ackerman GA & LoBuglio AF 1982 Effects of corticosteroids on human monocyte function. *New England Journal of Medicine* **292** 236-241.

Rosmond R, Dallman MF & Bjorntorp P 1998 Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. *Journal of Clinical Endocrinology and Metabolism* **83** 1853-1859.

Roszak AW, Lefebvre YA, Howell GM & Codding PW 1990 Structural requirements for the binding of dexamethasone to nuclear envelopes and plasma membranes. *Journal of Steroid Biochemistry and Molecular Biology* **37** 201-214.

Routh VH, Murakami DM, Stern JS, Fuller CA & Horwitz BA 1990 Neuronal activity in hypothalamic nuclei of obese and lean Zucker rats. *International Journal of Obesity* **14** 879-891.

Rumbaugh RC & Colby HD 1980 Is growth hormone the pituitary feminizing factor mediating the actions of estradiol on hepatic drug and steroid metabolism? *Endocrinology* **107** 719.

Russell DW & Wilson JD 1994 Steroid 5α-reductase: Two Genes/Two Enzymes. *Annual Review of Biochemistry* **63** 25-61.

Sakai RR, Lakshmi V, Monder C & McEwen BS 1992 Immunocytochemical localisation of 11B-hydroxysteroid dehydrogenase in hippocampus and other brain regions of the rat. *Journal of Neuroendocrinology* **4** 101-106.

Sapolsky RM, Krey LC & McEwen BS 1985 Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. *Journal of Neuroscience* **5** 1222-1227.

Sartura T, Suzuki H, Handa M, Igarashi Y, Kondo K & Senba S 1986 Multiple factors contribute to the pathogenesis of hypertension in Cushing's syndrome. *Journal of Clinical Endocrinology and Metabolism* **62** 275-279.

Schneider JJ 1952 Conversion of deoxycorticosterone to four allopregnane metabolites by rat liver in vitro. *Journal of Biological Chemistry* **199** 235-244.

Schneider JJ & Horstmann PM 1951 Effects of incubating deoxycorticosterone with various rat tissues. *Journal of Biological Chemistry* **191** 327-338.

Sekihara H, Island dP & Liddle GW 1978 New mineralocorticoids:5α-dihydroaldosterone and 5α-dihydro-11-deoxycorticosterone. *Endocrinology* **103** 1450-1452.

Shaw G & Kamen R 1986 A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46** 659-667.

Siiteri PK & Wilson JD 1974 Testosterone formation and metabolism during male sexual diferentiation in the human embryo. *Journal of Clinical Endocrinology and Metabolism* **38** 113-125.

Simons JSS, Sistare FD & Chakraborti PK 1989 Steroid binding activity is retained in a 16-kDa fragment of the steroid binding domain of glucocorticoid receptors. *Journal of Biological Chemistry* **264** 14493.

Small GR, Hadoke PWF, Dover AR, Mullins JJ, Seckl JR & Walker BR 2003 Glucocorticoids inhibit angiogenesis in mouse aorta: a novel role for 11β-hydroxysteroid dehydrogenase type 1 in modifying vascular structure. *in press*.

Smith RE & Funder JW 1991 Renal 11β-hydroxysteroid dehydrogenase activity: effects of age, sex and altered hormonal status. *Journal of Steroid Biochemistry and Molecular Biology* **38** 265-267.

Stalmans W & Laloux M 1979 Glucocorticoids and hepatic glycogen metabolism. In *Glucocorticoid hormone action*, pp 518-533. Eds JD Baxter & GC Rousseau. New York: Springer-Verlag.

Stewart PM, Boulton A, Kumar S, Clark PMS & Shackleton CHL 1999 Cortisol metabolism in human obesity: impaired cortisone - cortisol conversion in subjects with central adiposity. *Journal of Clinical Endocrinology and Metabolism* **84** 1022-1027.

Stewart PM, Shackleton CHL, Beastall GH & Edwards CRW 1990 5α-reductase activity in polycystic ovary syndrome. *Lancet* **335** 431-433.

Strain GW, Zumoff B, Strain JJ, Levin J & Fukushima D 1980 Cortisol production in obesity. *Metabolism* **29** 980-985.

Szapary D, Huang Y & Stoney Simons S 1999 Opposing effects of corepressor and coactivators in determining the dose-response curve of agonists, and residual agonist activity of antagonists, for glucocorticoid receptor-regulated genes. *Molecular Endocrinology* **13** 2108-2121.

Takeda Y, Miyamori I, Yoneda T, Ito Y & Takeda R 1994 Expression of 11β-hydroxysteroid dehydrogenase mRNA in rat vascular smooth muscle cells. *Life Sciences* **54** 281-285.

Taurog JD, Moore RJ & Wilson JD 1975 Partial characterisation of the cytosol 3α-hydroxysteroid: NAD(P)$^+$ oxidoreductase of rat ventral prostste . *Biochemistry* **14** 810-817.

Thigpen AE & Russell DW 1992 Four-amino acid segment in steroid 5α-reductase 1confers sensitivity to finasteride, a competitive inhibitor. *Journal of Biological Chemistry* **267** 8577-8583.

Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell JD & Russell DW 1993 Tissue distribution and ontogeny of steroid 5α-reductase isozyme expression. *Journal of Clinical Investigation* **92** 903-910.

Torres JM & Ortega E 2003 Precise quantification of 5α-reductase type 1 mRNA by RT-PCR in rat liver and its positive regulation by testosterone and

dihydrotestosterone. *Biochemical & Biophysical Research Communications* **308** 469-473.

Vale WW, Spiess S, Rivier C & Rivier J 1981 Characterisation of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* **213** 1394-1397.

Voice MW, Seckl JR, Edwards CRW & Chapman KE 1996 11β-hydroxysteroid dehydrogenase type 1 expression in 2S FAZA hepatoma cells is horminally regulated: a model system for the study of hepatic glucocorticoid metabolism. *Biochemical Journal* **317** 621-625.

Vottero A & Chrousos GP 1999 GC receptor β: view I. *Trends in Endocrinology and Metabolism* **19** 333-338.

Wahe M, Antonipillai I & Horton R 1993 Effects of transforming growth factor β and epidermal growth factor on steroid 5α-reductase activity in genital skin fibroblasts. *Molecular and Cellular Endocrinology* **98** 55-59.

Walker BR, Connacher AA, Lindsay RM, Webb DJ & Edwards CRW 1995 Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. *Journal of Clinical Endocrinology and Metabolism* **80** 3155-3159.

Walker BR & Edwards CRW 1992 Cushing's syndrome. In *The adrenal gland*, edn 2, pp 289-318. Ed VHT James. New York: Raven Press.

Walker BR, Soderberg S, Lindahl B & Olsson T 2000 Independent effects of obesity and cortisol in predicting cardiovascular risk factors in men and women. *Journal of Internal Medicine* **247** 198-204.

Walker BR, Williams BC & Edwards CRW 1994 Regulation of 11 β-hydroxysteroid dehydrogenase activity by the hypothalamic-pituitary-adrenal axis in the rat. *Journal of Endocrinology* **141** 467-472.

Walker C-D, Scribner KA, Stern JS & Dallman MF 1992 Obese zucker (*fa/fa*) rats Exhibit Normal Target Sensitivity to Corticosterone and Increased Drive to Adrenocorticotropin during the Diurnal Trough. *Endocrinology* **131** 2629-2637.

Walsh PC, Madden JD, Harrod MJ, Goldstein JL, MacDonald PC & Wilson JD 1974 Familial incomplete pseudohermaphroditism, type2 . Decreased dihydrotestosterone formation in pseudovaginal perineoscrotal hypospadias. *New England Journal of Medicine* **291** 944-949.

Webster JC, Jewell CM, Bodwell JE, Munck A, Sar M & Cidlowski JA 1997 Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. *Journal of Biological Chemistry* **272** 9287-9293.

Weinberger C, Hollenberg SM, Ong ES, Harmon JM, Brower ST, Cidlowski JA, Thompson EB, Rosenfeld MG & Evans RM 1985 Identification of human glucocorticoid receptor complementary DNA clones by epitope selection. *Science* **228** 740-742.

Weinstein RS, Jilka RL, Parfitt AM & Manolagas SC 1998 Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by GC's. Potential mechanisms of their deleterious effects on bone. *Journal of Clinical Investigation* **102** 274-282.

Weinstein SP, Paquin T, Pritsker A & Haber RS 1995 Glucocorticoid-induced insulin resistance: dexamethasone inhibits the activation of glucocse transport in rat skeletal muscle by both insulin- and non-insulin related stimuli. *Diabetes* **44** 441-445.

Welsh MJ & Wiebe JP 1976 Sertoli cells from immature rats:*in vitro* stimulation of steroid metabolism by FSH. *Biochemical & Biophysical Research Communications* **69** 936-941.

Wilson JD 1975 Dihydrotestosterone formation in cultured human fibroblasts. *Journal of Biological Chemistry* **250** 3498-3504.

Yates FE, Herbst AL & Urquhart J 1958 Sex difference in rate of ring A reduction of 4-3-keto-steroids in vitro by rat liver. *Endocrinology* **63** 887-902.

Yau JL, Noble JM, Kenyon CJ, Hibberd CJ, Koteletsev Y, Mullins JJ & Seckl JR 2001 Lack of tissue glucocorticoid reactivation in 11beta-hydroxysteroid dehrdrogenase type 1 knockout mice ameliorates age-related learning impairments. *Proceedings of the National Academy of Sciences USA* **98** 4716-4721.

Yoshiuchi I, Shingu R, Nakajima H, Hamaguchi T, Horikawa Y, Yamasaki T, Oue T, Ono A, Miyagawa JI, Namba M, Hanfusa T & Matsuzawa Y 1998 Mutation/polymorphism scanning of glucose-6-phosphastase gene promoter in noninsulin-dependent diabetes mellitus patients. *Journal of Clinical Metabolism* **83** 1016-1019.

Yudt MR & Cidlowski JA 2001 Molecular identification and characterisation of A and B forms of the glucocorticoid receptor. *Molecular Endocrinology* **15** 1093-1103.

Yukimura Y, Bray GA, Wolfsen AR & . 1978 Some effects of adrenalectomy in the fatty rat. *Endocrinology* **103** 1924-1928.

Zakula Z & Moudgil VK 1991 Interaction of rat liver glucocorticoid receptor with a newly synthesised antisteroid ZK 98299. *Biochemica et Biophysica Acta* **1092** 188.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. *Nature* **372** 425-432.

Zhao Y, Mendelson CR & Simpson ER 1995 Characterisation of the sequences of the human CYP19 (aromatase) gene that mediate regulation by glucocorticoids in adipose stromal cells and fetal hepatocytes. *Molecular Endocrinology* **9** 340-349.

Zucker LM & Zucker TM 1961 Fatty, a new mutation in the rat. *Journal of Heredity* **52** 275-278.

Zyirek M, Flood C & Longcope C 1987 5α-reductase activity in rat adipose tissue. *Proceedings of the Society for Experimental Biology and Medicine* **186** 134-138.

Publications from this thesis

Papers

5α-reduced glucocorticoids: Novel endogenous activators of the glucocorticoid receptor. Kerry J McInnes, Christopher J Kenyon, Karen E Chapman, Dawn EW Livingstone, Brian R Walker and Ruth Andrew. Submited 2003.

Enhanced Hepatic A-ring Reduction of Glucocorticoids in Obese Zucker rats: Regulation by Insulin. Dawn EW Livingstone, Kerry J McInnes, Brian R Walker and Ruth Andrew. Submitted 2003.

Abstracts

Oral Abstract

McInnes KJ, Livingstone DEW, Kenyon CJ, Chapman KE, Walker BR and Andrew R. 5α-reduced Glucocorticoid Metabolites: Novel Endogenous Activators of Glucocorticoid Receptors (GR). OC21, Endocrine Abstracts, Vol.5, March 2003.

Poster Abstracts

McInnes KJ, Livingstone DEW, Kenyon CJ, Chapman KE, Walker BR, Andrew R. Hepatic 5α-reduced Glucocorticoid Metabolites: Novel Endogenous Activators of Glucocorticoid Receptors. P3-220, The Proceedings of the Endocrine Society's 85th Annual Meeting, June 2003.

Andrew R, Livingstone DEW, McInnes KJ, Kenyon CJ, Walker BR. Generation of Locally Active Glucocorticoid Metabolites in the Liver in Obesity. John Scrimgeour Lectureship, University of Edinburgh, May 2003.

McInnes KJ, Kenyon CJ, Hardie DE, Livingstone DEW, Walker BR, Andrew R. 5α-reduced Glucocorticoids: Novel Modulators of Glucocorticoid Receptors in Obesity. P2-510 The Proceedings of the Endocrine Society's 84th Annual Meeting, June 2002.

McInnes KJ, Kenyon CJ, Hardie DE, Livingstone DEW, Walker BR, Andrew R. 5α-reductase and Glucocorticoid Action in Obesity. The Scottish Medical Journal. In Press

Livingstone DEW, McInnes KJ, Walker BR, Andrew R. Enhanced Hepatic A-ring Reduction of Glucocorticoids in Obesity: Regulation by Glucocorticoids and Insulin. P257. Endocrine Abstracts, Vol3, March 2002.

Livingstone DEW, McInnes KJ, Walker BR, Andrew R. Dysregulation of Hepatic A-ring Reduction of Glucocorticoids in Obesity. P1-384, The Proceedings of the Endocrine Society's 83rd Annual meeting, June 2001.