Glucocorticoid Metabolism in Obesity

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Abstract

Primary increased secretion of glucocorticoids causes obesity, and increased cortisol secretion has been noted in subjects with idiopathic obesity, especially if of central distribution. Observations in obese men suggest that peripheral glucocorticoid metabolism may be altered. The Zucker rat model of obesity has been utilised in this thesis to assess glucocorticoid metabolism *in vitro* and *in vivo*, and to study the mechanisms of its dysregulation. Of particular interest was activity of 11β-hydroxysteroid dehydrogenase type 1, which reactivates glucocorticoids and thus increases local glucocorticoid receptor activation eg in liver and adipose tissue.

Obese animals had elevated plasma corticosterone (B), higher urinary B metabolites and heavier adrenal glands when compared with lean rats. They also had increased activity of hepatic 5α-reductase, decreased hepatic 11β-HSD1 and increased renal 11β-HSD2 activity. By contrast, 11β-HSD1 activity was not different in skeletal muscle or subcutaneous fat, and was higher in omental fat of obese animals. Urinary glucocorticoid metabolite profiles, assessed by GCMS, confirmed that metabolism of B by 5α-reductase is increased in obese animals and showed the balance of 11β-HSDs lies in favour of inactive 11-dehydrocorticosterone. Greater inactivation of B by 5α-reductase in liver and 11β-HSD2 in kidney, combined with impaired reactivation by 11β-HSD1 in liver, may decrease local B concentrations in these sites, and increase metabolic clearance rate of glucocorticoids, thus increasing drive to the hypothalamic-pituitary-adrenal axis (HPA). By contrast, increased 11β-HSD1 activity in omental adipose tissue may increase local glucocorticoid receptor activation and promote obesity.

To gain insight into the mechanisms of tissue-specific dysregulation of 11β-HSD1 in obesity, two of the primary regulators of 11β-HSD1 were studied; glucocorticoids which up-regulate, and insulin which down-regulates, 11β-HSD1. Adrenalectomy (ADX) normalised weight gain in obese animals, and furthermore normalised 11β-HSD1 activity in liver and reversed the difference in omental adipose tissue. This highlights that not only are the abnormalities in 11β-HSD1 activity tissue-specific, but the regulation of the enzyme is tissue-specific also. ADX of obese Zucker rats not only removes adrenal hormones, but also normalises many of the metabolic abnormalities, including insulin resistance. To

investigate whether insulin was responsible for the alterations in 11β-HSD1 following ADX, glucocorticoid metabolism was assessed in obese animals that had been sensitised to insulin with either metformin or rosiglitazone. Enhanced insulin sensitivity did not normalise 11β-HSD1 activity, suggesting that insulin is not responsible for dysregulation of 11β-HSD in obese animals, or for changes in enzyme activity following ADX. By contrast, enhanced insulin sensitivity did reverse adrenal hypertrophy, suggesting that insulin may be a key factor contributing to activation of the HPA in obesity, independently of tissue-specific changes in 11β-HSD1.

The alterations in 11β-HSD1 in obesity predict that local glucocorticoid concentrations will vary between tissues, being lower in liver and higher in omental fat. To try and dissect which changes are important to obesity and insulin resistance, animals were treated with carbenoxolone (CBX), an inhibitor of 11β-HSD. CBX had no effect on obesity, and was associated with a worsening of insulin sensitivity. However, inhibition of 11β-HSD1 was only demonstrable *in vitro* in liver and kidney, not adipose tissue or skeletal muscle, and therefore further work is required before the full implications of the results can be assessed.

Finally, the finding of tissue specific alterations in 11β-HSD1 activity in obesity was reproduced in obese humans. 11β-HSD1 activity in homogenates of gluteal subcutaneous adipose tissue obtained by biopsy was higher in obese patients than in lean. By contrast, *in vivo* hepatic 11β-HSD1 activity was impaired in the same obese patients.

In conclusion, 11β-HSD1 activity, and therefore local glucocorticoid concentration, is altered in obesity. Manipulation of this enzyme, particularly in a tissue specific manner, may prove to be a useful therapeutic target in obesity and insulin resistance.

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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, which are also acknowledged in the text.

Surgical procedures were carried out by Dr Chris Kenyon of the Department of Molecular Endocrinology, University of Edinburgh.

Plasmid preparations were carried out by Val Lyons of the Department of Molecular Endocrinology, University of Edinburgh.

5α-Reductase assay was carried out by Dr Greg Jones of the Department of Medical Sciences, University of Edinburgh.

Analyses of human plasma and urine samples were carried out by Inger Arnesjö and Else-Britt Lundström of the Umeå University Hospital, Umeå, Sweden, and Jill Smith of the Department of Medical Sciences, University of Edinburgh.

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

Dawn E.W. Livingstone, Edinburgh, March 2000.

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Chapter One

Introduction

Primary increased glucocorticoid secretion causes obesity, and glucocorticoid secretion is enhanced in both human and rodent obesity (Marin *et al.*, 1992; Pasquali *et al.*, 1993; Andrew *et al.*, 1998; Fraser *et al.*, 1999; Cunningham *et al.*, 1986). However, plasma cortisol is not consistently elevated in obesity, suggesting that peripheral clearance of cortisol may be enhanced (Strain *et al.*, 1982; Rosmond *et al.*, 1998). There are several routes of metabolic clearance of glucocorticoids, including the interconversion of active and inactive glucocorticoids by the isoenzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD). Disruption of glucocorticoid metabolism may alter local, as well as plasma, levels of steroid, as the 11β-HSDs control glucocorticoid action at a tissue level. It has been suggested that alterations in local reactivation of glucocorticoids by 11β-HSD type 1 may be important both in determining metabolic clearance of cortisol and in promoting obesity (Bujalska *et al.*, 1997; Andrew *et al.*, 1998). This is the hypothesis explored in this thesis.

The following chapter reviews glucocorticoid physiology and metabolism, with particular emphasis on the 11β-hydroxysteroid dehydrogenases. Some of the multiple regulators of body weight are then discussed, and the Zucker rat described as an animal model used in many studies of obesity. Finally, evidence for the role of glucocorticoids in obesity is presented and a list of aims of this thesis is laid out.

1.1 Glucocorticoids

Glucocorticoids (cortisol in man and corticosterone in rodents) were originally named for their effects on carbohydrate metabolism. It is now known that they exert an influence on most systems in the body, including the immune system, fluid and electrolyte homeostasis and physiological responses to stress. They are part of a family of steroid hormones that all have the common precursor cholesterol. The basic steroid structure of cholesterol, comprising three cyclohexane rings and one cyclopentane

ring, is conserved throughout the family, with each steroid being given its unique properties by the substitution of chemical groups at various positions on the backbone molecule (Figure 1.1).

1.1.1 Glucocorticoid synthesis

Glucocorticoids are synthesised from cholesterol in the zonae fasiculata and reticularis of the adrenal cortex. Adrenocortical steroid biosynthesis is catalysed by members of the CYP oxidative enzyme family (Figure 1.2), characterised by their absorbence maximum of 450nm (Miller, 1988). The nomenclature of these enzymes has recently been changed and is summarised in Table 1.1.

Rats lack the enzyme CYP17 in the adrenal cortex so steroids cannot be 17-hydroxylated. This is why the major glucocorticoid in the rat is corticosterone rather than cortisol, and also why rats do not synthesise adrenal androgens.

Trivial name	**Previous name**	**Present name**
Cholesterol side chain cleavage	$P450_{SCC}$	CYP11A1
3β-hydroxysteroid dehydrogenase	3βHSD	3βHSDII
17α-hydroxylase / 17,20-lyase	$P450_{C17}$	CYP17
21-hydroxylase	$P450_{C21}$	CYP21A2
11β-hydroxylase	$P450_{C11}$	CYP11B1
Aldosterone synthase	$P450_{C11AS}$	CYP11B2

Table 1.1: Nomenclature of steroidogenic enzymes.

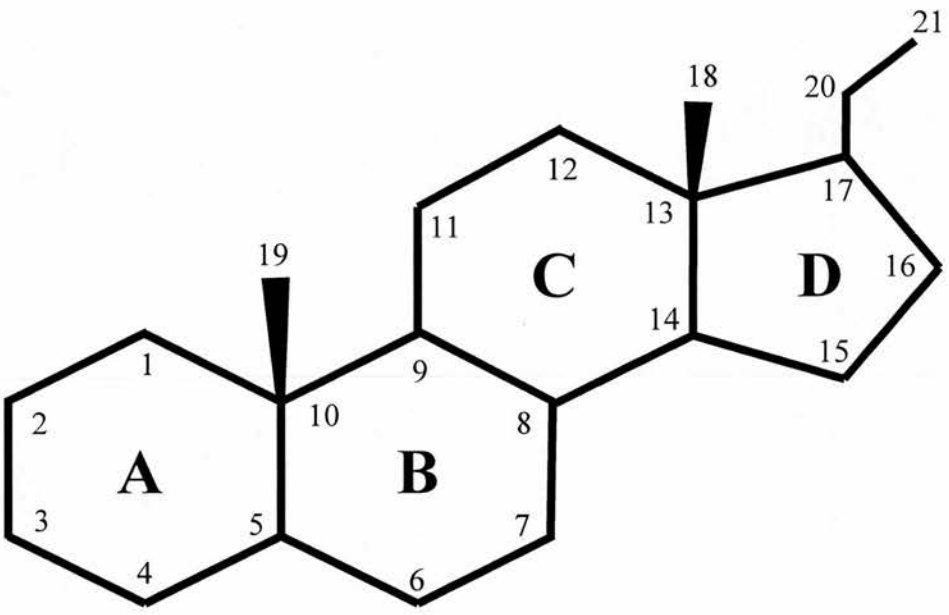

Figure 1.1: The basic steroid ring structure and conventional labeling. The four carbon rings are identified by letters, and the individual carbon atoms by numbers. Chemical groups are designated by the number of the carbon atom to which they are attached.

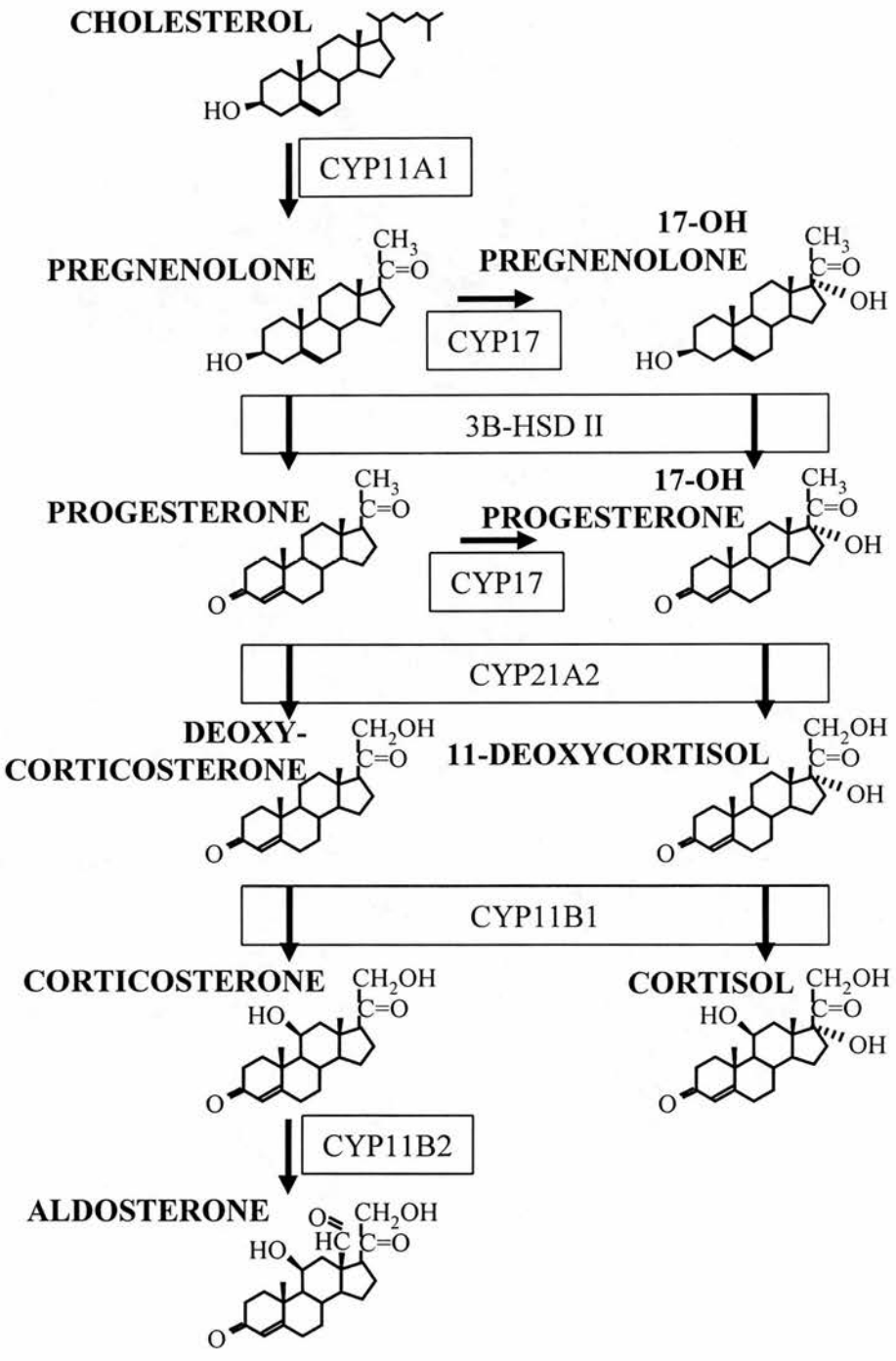

Figure 1.2: The adrenocortical steroid biosynthetic pathway.
The names of the enzymes are shown in the boxes.

1.1.2 Glucocorticoid secretion

The synthesis and release of glucocorticoids from the adrenal cortex is regulated by hormonal interactions among the hypothalamus, pituitary and adrenal, which in turn can be influenced by neural and other stimuli, eg stress. Stimuli to the hypothalamus lead to the release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) into the hypothalamic-hypophyseal portal system. This rapidly leads to adrenocorticotrophic hormone (ACTH) release from the anterior pituitary into the systemic circulation. ACTH is processed from the polypeptide pro-opiomelanocortin (POMC) and acts on the adrenal cortex where it stimulates steroidogenesis and the release of adrenocortical steroids, including glucocorticoids. Glucocorticoids exert an inhibitory effect on CRH and AVP synthesis, POMC processing and secretion of ACTH, to provide a negative feedback loop that helps to maintain physiological plasma glucocorticoid concentrations. This system as a whole is known as the hypothalamic-pituitary-adrenal axis (HPA).

Circulating glucocorticoids are predominantly protein bound. Plasma cortisol and corticosterone circulate largely bound to corticosteroid-binding globulin (CBG) and albumin, with only around 5-10% circulating as free steroid (Hammond *et al.*, 1990). Only free steroid can diffuse across the cell membrane to bind to the intracellular glucocorticoid receptor, so corticosteroids bound to CBG cannot cause glucocorticoid receptor activation. Binding proteins therefore buffer free corticosteroid levels, but they are saturated at high physiological levels so that free steroid levels fluctuate greatly during the diurnal variation in total steroid levels.

1.1.3 Glucocorticoid action

Glucocorticoids exert their effects by diffusing into cells and binding to soluble intracellular receptors. Ligand binding leads to receptor activation, dimerisation and translocation to the nucleus where the glucocorticoid receptor complex can interact with specific DNA sequences known as glucocorticoid response elements (GREs). The receptor dimer interacts with components of the transcription machinery to enhance transcription, and GREs are therefore normally closely associated with the promoter region of target genes (Yamamoto, 1985). Gene transcription can also be repressed by glucocorticoids, eg in the negative feedback loop of the HPA expression of pro-opiomelanocortin (POMC), the precursor to ACTH, is decreased by cortisol via interactions at a negative GRE (Drouin *et al.*, 1993). Glucocorticoids can also inhibit transcription by a second mechanism that does not involve GREs. The receptor dimer can interfere directly with transcription factors to prevent transcription, eg activated glucocorticoid receptors bind to activating protein-1 and inhibit the transcription of genes involved in the immune response (Jonat *et al.*, 1990; Yang-Yen *et al.*, 1990).

Membrane glucocorticoid binding sites have also been described in rodent neurones and synaptic membrane (Liposits & Bohn, 1993; Guo *et al.*, 1995) and in rodent lymphoma and human leukaemic cells (Gametchu *et al.*, 1993; Gametchu *et al.*, 1999). The physiological relevance of these binding sites is still a matter of dispute (see Brann *et al.*, 1995 for a review) but they are thought to have a function in controlling glucocorticoid effects on growth, particularly in lymphoma.

1.1.4 Effects of glucocorticoids

The effects of glucocorticoids are wide-ranging. As well as their many direct effects, they also have permissive and modulatory effects on tissue sensitivity to other hormones, such as catecholamines. The major roles of

glucocorticoids were discovered initially by clinical observation of the effects of adrenal disease. This was later helped by the development of tools such as the steroid biosynthesis inhibitor, metyrapone and the glucocorticoid receptor antagonist, RU486 (mifepristone).

Glucocorticoid excess, eg in Cushing's disease, results in redistribution of body fat to a more central distribution, impaired glucose tolerance, impaired immune responses, hypertension and degenerative changes to muscle and connective tissue. By contrast, in Addison's disease glucocorticoid deficiency leads to weight loss, hypoglycaemia and hypotension.

1.1.4.1 Effects of glucocorticoids on metabolism

Glucocorticoids regulate the metabolism of carbohydrates, protein and fat both in the liver and in the periphery. Glucocorticoids mobilise substrates for hepatic gluconeogenesis by stimulating release of amino acids from skeletal muscle and fatty acids and glycerol from adipose tissue, and also increase expression of gluconeogenic enzymes eg phosphoenolpyruvate carboxykinase (PEPCK; Sasaki *et al.*, 1984). Peripheral glucose uptake and utilisation are inhibited by glucocorticoids (Rizza *et al.*, 1982), partly by decreased translocation of glucose transporters to the cell surface (Weinstein *et al.*, 1995). Glycogen storage is stimulated by glucocorticoids due to activation of glycogen synthase, and decreased metabolism of glycogen by glycogen phosphorylase (Stalmans & Laloux, 1979).

In addition to acutely stimulating lipolysis to provide gluconeogenic substrates, glucocorticoids also promote differentiation of pre-adipocytes to mature adipocytes (Hauner *et al.*, 1987). Site-specific alterations in fat metabolism lead to the characteristic body fat redistribution observed with glucocorticoid excess (Rebuffe-Scrive *et al.*, 1988). The effects of

glucocorticoids on body weight homeostasis are discussed in full in section 1.3.

Glucocorticoid-induced catabolism of muscle to produce gluconeogenic substrates can, in the long term, lead to myopathy. Chronic glucocorticoid excess is also associated with osteopenia and poor wound healing. Glucocorticoids decrease new bone formation and increase osteoclast cell numbers, stimulating bone resorption (Hahn *et al.*, 1979). Both intestinal calcium absorption and renal calcium reabsorption are also impaired by glucocorticoids (Hahn *et al.*, 1979; Laake, 1960). The deleterious effects of glucocorticoids on connective tissue are mediated by an inhibitory effect on fibroblast proliferation and function, and extracellular matrix component synthesis (Pratt & Aronow, 1966).

1.1.4.2 Effects of glucocorticoids on the immune system

Glucocorticoids have profound inhibitory effects on inflammation and the immune system. Trafficking of immune cells is altered by glucocorticoids, with decreased peripheral lymphocyte and monocyte numbers, but increased circulating granulocytes. Apoptosis of lymphoid cells is induced by glucocorticoids and T-cell function and B-cell proliferation are inhibited (Cupps *et al.*, 1985). Glucocorticoids prevent the inflammatory response by inhibiting neutrophil accumulation (Dale *et al.*, 1975) and the phagocytotic and cytotoxic effects of macrophages (Rinehart *et al.*, 1982). They also inhibit synthesis of prostaglandins (Kantrowitz *et al.*, 1975), which mediate the inflammatory response. These functions of glucocorticoids are exploited clinically in the treatment of autoimmune diseases and inflammatory conditions, and in the management of the immune response to transplanted organs.

1.1.4.3 Effects of glucocorticoids on blood pressure

Glucocorticoid excess is associated with hypertension, and deficiency with hypotension, but the mechanisms of these changes in blood pressure are not fully understood. 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). All of these changes could contribute to hypertension, but whether they are sufficient to account for changes in blood pressure is not clear. Paradoxically, glucocorticoids stimulate atrial natriuretic peptide (ANP) synthesis and secretion and potentiate ANP's actions on the kidney to induce salt excretion (Garcia *et al.*, 1985; Hayamizu *et al.*, 1994), which would be expected to lower blood pressure. Many possible mediators of glucocorticoid-induced hypertension have been studied including the sympathetic nervous system, sodium homeostasis and vasodilator hormones, but few of these seem to be important (for a review see Whitworth, 1994). However, the majority of studies have been carried out in animal models, and the studies that have been carried out in humans have shown discrepancies in the effects of different synthetic glucocorticoids on cardiac output and vascular resistance (Pirpiris *et al.*, 1992). The varied responses to synthetic glucocorticoids make it difficult to dissect exactly how cortisol increases blood pressure, but there is increasing evidence that the mechanism may be independent of classical mineralocorticoid or glucocorticoid activity, as receptor antagonists do not block the hypertensinogenic effects of cortisol (Clore *et al.*, 1988; Coghlan *et al.*, 1984).

1.1.4.4 Effects of glucocorticoids on mood and behaviour

Glucocorticoid exhibit a wide range of neuropsychiatric and behavioral effects, but the mechanisms behind these is still unclear. Sleep patterns, mood, cognition and the reception of sensory signals are all affected by glucocorticoids (McEwen *et al.*, 1986). Glucocorticoid excess is

associated with increased rapid eye movement sleep and psychological disturbances, including both mania and depression. Glucocorticoid insufficiency is also associated with psychological disturbances, mainly depression and apathy. The effects of glucocorticoids on the control of appetite and feeding behaviour are discussed in section 1.3. Cells in several parts of the central nervous system (CNS) contain glucocorticoid receptors, but some neuronal responses occur so rapidly that they are unlikely to be mediated by classical transcriptional effects on target genes, and a direct membrane effect is more likely. Glucocorticoids have transcriptional effects in the central nervous system, including neurotoxicity (Sapolsky *et al.*, 1985) and reducing numbers of astrocytes, oligodendrocytes and hippocampal neurones.

1.1.4.5 Effects of glucocorticoids on growth and development

Finally, glucocorticoids have effects on growth and development. Linear skeletal growth is inhibited by glucocorticoid excess in children. The exact mechanism of this effect is not known as neither growth hormone (GH) or insulin-like growth factor 1 (IGF-1) levels are changed (Frantz & Rabkin, 1964), but it seems likely that the direct effects of glucocorticoids on bone and muscle metabolism mentioned earlier have a role to play in this process. Glucocorticoids stimulate the differentiation of many cell types, and their effects on fetal lung maturation are utilised clinically to accelerate lung development in premature babies. They also play a role in the development of the nervous system by regulating the differentiation of neural crest epithelial cells into chromaffin cells, and influence the development of the adrenal medulla (Federoff *et al.*, 1988). Increased glucocorticoid exposure *in utero* retards fetal growth in rats and humans (Benediktsson *et al.*, 1993; Reinisch *et al.*, 1978) and this has been linked with "programming" effects on cardiovascular risk and control of the HPA axis. In rats, it has been shown that perinatal manipulations in glucocorticoid exposure can alter glucocorticoid receptor expression in the brain to permanently alter HPA axis responses (Meaney *et al.*, 1994) and

can alter gluconeogenic enzymes and glucose tolerance (Nyirenda *et al.*, 1998).

1.1.5 Metabolism of glucocorticoids

The metabolism of glucocorticoids involves reduction, oxidation, hydroxylation and conjugation, and the metabolism of cortisol is shown in Figure 1.3. Firstly, cortisol (or corticosterone in the rat) can be interconverted with inactive cortisone (11-dehydrocorticosterone in the rat) by the isozymes of 11β-hydroxysteroid dehydrogenase. The metabolism of cortisol and cortisone then follow similar steps. The rate limiting step in glucocorticoid inactivation is the reduction of the $\Lambda^{4,5}$ double bond in the A ring of the steroid structure. This can be carried out by either 5α- or 5β-reductase, resulting in two isomers differing only in the orientation of the hydrogen atom around the carbon at position 5. Cortisol (corticosterone) can be metabolised by either 5α- or 5β reductase, whereas cortisone (11-dehydrocorticosterone) is only acted on by 5β-reductase. These dihydro-metabolites are then rapidly reduced further by the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD) to form tetrahydro-metabolites. The tetrahydro-metabolites are the most abundant metabolites excreted in the urine, closely followed by the cortols and cortolones, which have been further reduced by 20α-hydroxysteroid dehydrogenase. The oxidation of cortols and cortolones by 21-oxidase results in the production of cortolic and cortolonic acids. Corticosteroids and their metabolites are not very water-soluble, and the final step of glucocorticoid metabolism is conjugation to either glucuronic acid or sulphates, which increases their water-solubility and aids excretion.

Hydroxylation of cortisol to form the water-soluble 6β-hydroxycortisol is normally only a minor route of metabolism, but can become more important in conditions where plasma cortisol levels are high.

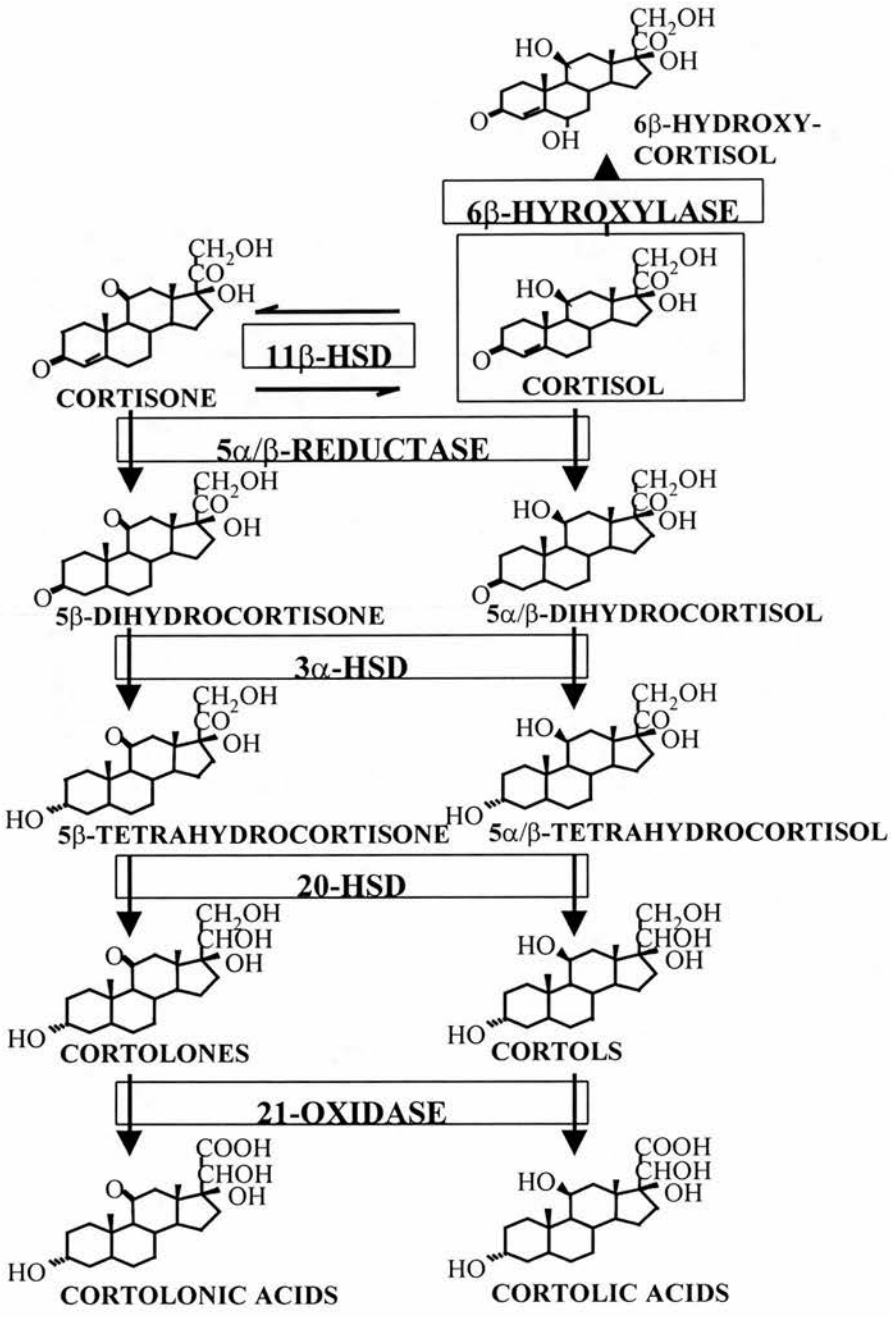

Figure 1.3: Routes of cortisol metabolism *in vivo*.

Enzymes are indicated by the boxes and HSD = hydroxysteroid dehydrogenase. Both 5α- and 5β-reduction of cortisol occur, but cortisone is only acted on by 5β-reductase.

1.2 11β-Hydroxysteroid dehydrogenase

The focus of this thesis is the metabolism of glucocorticoids by the isoenzymes of 11β-hydroxysteroid dehydrogenase, and their physiology is described in this section.

1.2.1 History of 11β-hydroxysteroid dehydrogenase

Liquorice was used as a herbal remedy in ancient times, and was rediscovered in the fifteenth century as a treatment for dyspepsia. The active ingredient in liquorice was identified as glycyrrhetinic acid, and its hemmisuccinate-derivative, carbenoxolone, was the most effective treatment for peptic ulcers before the innovation of the H_2 antagonist cimetidine. Treatment with liquorice derivatives was associated with side effects of breathlessness and peripheral oedema, and more recently it was noted that several patients who consumed large amounts of liquorice developed a syndrome similar to that of primary hyperaldosteronism (Epstein *et al.*, 1977). The patients suffered from the symptoms of mineralocorticoid excess (sodium retention, which leads to oedema, breathlessness, headaches and hypertension, and potassium wasting, which leads to polyuria, proximal myopathy, lethargy and muscle cramps) in the face of low plasma aldosterone levels. It was initially thought that liquorice contained a direct agonist of the mineralocorticoid receptor. Glycyrrhetinic acid does indeed bind to mineralocorticoid receptors in vitro, but with affinity so low (approximately 4,000x lower than the affinity for aldosterone (Armanini *et al.*, 1983) that this is an unlikely mechanism of action. However, liquorice-induced mineralocorticoid effects are dependent on intact adrenocortical function (Borst *et al.*, 1953; Card *et al.*, 1953), and can be inhibited by dexamethasone (Hoefnagels & Kloppenborg, 1983). It was therefore apparent that an ACTH-dependant product of the adrenal cortex was necessary for the effects of liquorice.

The congenital syndrome of apparent mineralocorticoid excess (AME) is remarkably similar to that induced by liquorice consumption (Stewart *et al.*, 1988; Ulick *et al.*, 1979). Patients present with symptoms of mineralocorticoid excess, but have low plasma mineralocorticoid levels. The syndrome is improved by dexamethasone and the mineralocorticoid receptor antagonist spironolactone, and worsened by even physiological doses of cortisol, indicating that an adrenal mineralocorticoid agonist is responsible.

Studies on patients suffering from AME, and on healthy volunteers treated with the liquorice derivative glycyrrhetinic acid to induce AME, showed similar differences in glucocorticoid metabolism. In both groups urinary free cortisol was higher, the ratio of cortisol:cortisone metabolites was higher, and the elimination half life of [^{3}H]-cortisol was longer (Stewart *et al.*, 1988; MacKenzie *et al.*, 1990). The discovery of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) led to the description of the mechanism of AME, and also explained the paradox of mineralocorticoid receptor specificity in the face of equal affinity for glucocorticoids and mineralocorticoids (Arriza *et al.*, 1987).

11β-HSD catalyses the inter-conversion of the active glucocorticoid cortisol and its inactive metabolite cortisone in man (corticosterone and 11-dehydrocorticosterone respectively in the rat). This dehydrogenase activity was co-localised with the mineralocorticoid receptor and it was shown that 11β-HSD inactivates glucocorticoids at these sites, preventing them binding to mineralocorticoid receptor (Edwards *et al.*, 1988; Funder *et al.*, 1988), See Figure 1.4. The 11-18 hemiacetal structure of aldosterone is such that 11β-HSD cannot metabolise it, and it is therefore free to bind to its receptor. It was proposed that AME is due to a lack of this protective mechanism, allowing glucocorticoids to bind to mineralocorticoid receptors.

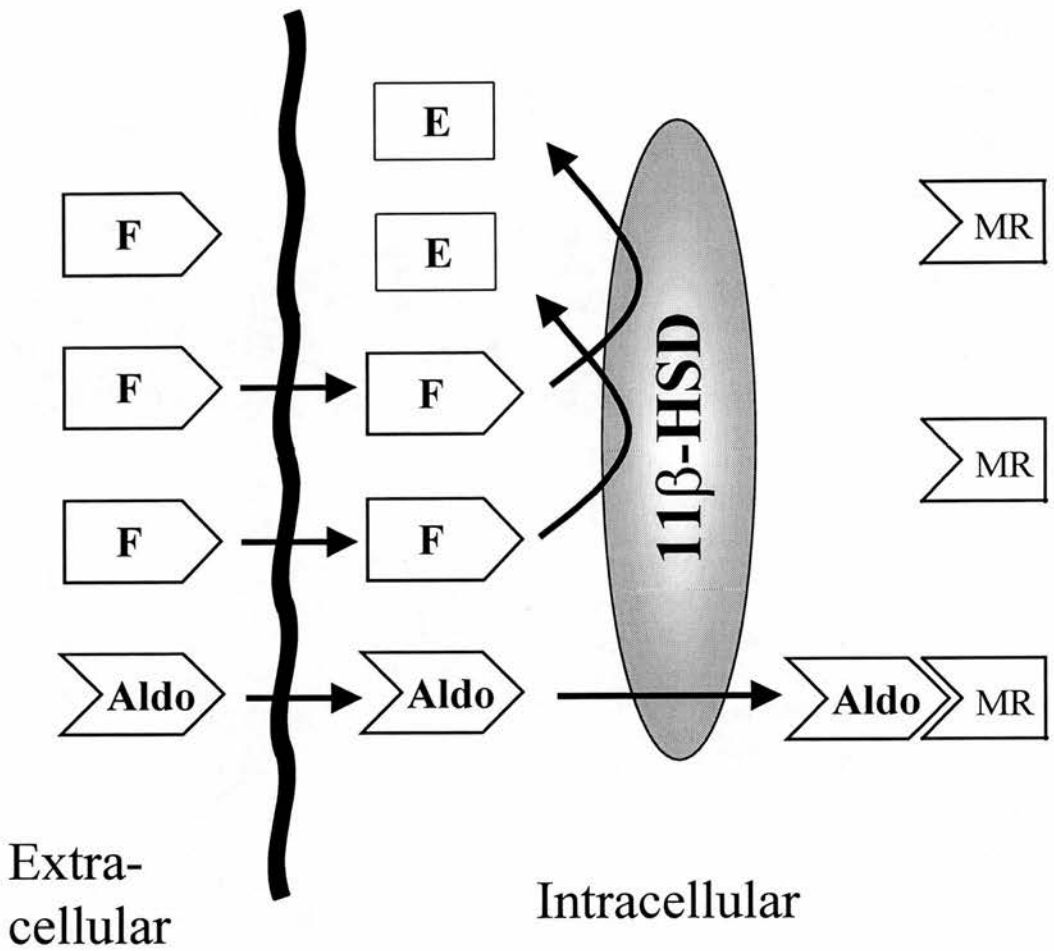

Figure 1.4: 11β-Hydroxysteroid dehydrogenase mediated receptor protection.

E=cortisone; F=cortisol; Aldo=aldosterone; MR=mineralocorticoid receptor.

11β-HSD converts cortisol (which can bind to the mineralocorticoid receptor) to cortisone (which cannot) conferring mineralocorticoid specificity to the mineralocorticoid receptor.

Decreased 11β-HSD action could explain the alterations in glucocorticoid metabolism in AME. Urinary free cortisol and cortisol:cortisone ratios would be higher, and the elimination half life of [^{3}H]-cortisol would be longer because cortisol would not be metabolised to cortisone. Renal binding studies showed that glucocorticoids bound only minimally to the kidney. However, in the presence of liquorice derivatives (carbenoxolone or glycyrrhizic acid) glucocorticoids bound to the kidney with the same pattern as mineralocorticoids (Edwards *et al.*, 1988). Furthermore, the glucocorticoid binding to mineralocorticoid receptor was shown to activate the receptor (Souness & Morris, 1989). The evidence for decreased 11β-HSD action being the cause of liquorice-induced apparent mineralocorticoid excess and congenital AME is therefore strong.

1.2.2 Identification of two distinct isoenzymes

As studies on 11β-HSD progressed it become apparent that there was evidence for the existence of more than one 11β-HSD enzyme.

1) In kinetic studies of 11β-HSD purified from the liver, the Km for cortisol and corticosterone was found to be in the μM range (Lakshmi & Monder, 1988). This would not appear to be adequate to protect renal mineralocorticoid receptor from glucocorticoids, which circulate in the nM range. It is also unlikely that this enzyme would be able to compete with the mineralocorticoid receptor for glucocorticoid binding, since the Kd of the mineralocorticoid receptor for glucocorticoids is in the subnanomolar range (Arriza *et al.*, 1987). Further kinetic studies suggested two kinetically distinct isoforms of 11β-HSD in the liver (Lakshmi & Monder, 1985) and in the lung (Abramovitz *et al.*, 1982).

2) Patients with congenital 11β-HSD deficiency maintain the ability to convert an oral dose of cortisone to cortisol, suggesting that only the

dehydrogenase and not the reductase activity of 11β-HSD was affected (Ulick *et al.*, 1979; Stewart *et al.*, 1988).

3) There are differences in the alterations of glucocorticoid metabolism induced by glycyrrhetinic acid and carbenoxolone. In contrast with glycyrrhetinic acid, carbenoxolone does not reduce plasma cortisol levels or urinary tetratrahydrocortisol:tetrahydrocortisone ratios (Stewart *et al.*, 1987; Stewart *et al.*, 1988). This was proposed to be due to carbenoxolone inhibiting both 11β-reductase and 11β-dehydrogenase activity, in contrast to glycyrrhetinic acid inhibiting only 11β-dehydrogenase activity (Walker *et al,* 1994a). This was supported by the observation that carbenoxolone inhibits the formation of cortisol after an oral dose of cortisone, whereas glycyrrhetinic acid does not (Frederich *et al.*, 1995).

4) Immunohistochemical studies found 11β-HSD immunoreactivity and activity were not co-localised in the kidney (Edwards *et al.*, 1988) suggesting that the antibody used was binding to a form of 11β-HSD distinct to that which protects the mineralocorticoid receptor.

11β-HSD was cloned from the rat liver by screening a rat liver cDNA library with anti-serum to 11β-HSD (Agarwal *et al.*, 1989). The cloned 11β-HSD encoded both dehydrogenase and reductase activities, which initially refueled speculation that there may be only one enzyme. Biochemical studies identified a distinct NAD-dependent 11β-HSD isoform in the kidney (Mercer & Krozowski, 1992; Rusvai & Naray-Fejes-Toth, 1993) and in the placenta (Brown *et al.*, 1993). This isoform of 11β-HSD was a much higher affinity enzyme, with a Km in the nM range for glucocorticoids, which seemed more appropriate for its postulated role in the protection of mineralocorticoid receptors. Finally, the question of whether 11β-HSD was one or more enzymes was answered when a second isoform of 11β-HSD (termed 11β-HSD2) was cloned. 11β-HSD2 was initially cloned from the kidney of sheep and man (Agarwal *et al.*, 1994;

Albiston *et al.*, 1994), and human placenta (Brown *et al.*, 1996) and its expression was found to be predominantly confined to aldosterone target tissues, eg kidney and colon, and the placenta (Albiston *et al.*, 1994; Brown *et al.*, 1996).

1.2.3 Role of 11β-HSD2

The function of 11β-HSD2 has been clearly defined. It is an NAD^+-dependent, high affinity, exclusive dehydrogenase, which is colocalised with mineralocorticoid receptors (Rundle *et al.*, 1989). The enzyme converts active glucocorticoids (cortisol in man and corticosterone in the rat) to their receptor inactive 11-keto derivatives (cortisone and 11-dehydrocorticostrone respectively). Mutations in the 11β-HSD2 gene have identified in patients suffering from congenital apparent mineralocorticoid excess (Mune *et al.*, 1995; Wilson *et al.*, 1995a; Wilson *et al.*, 1995b), and liquorice derivatives have been shown to inhibit 11β-HSD activity (Monder *et al.*, 1989; Stewart *et al.*, 1990). The evidence is conclusive that the function of 11β-HSD2 in aldosterone target tissues is to protect the mineralocorticoid receptor from inappropriate activation by glucocorticoids.

The role of 11β-HSD2 in the control of blood pressure and vascular responses was confirmed by observations in mice with a transgenic disruption of the 11β-HSD2 gene (Kotelevtsev *et al.*, 1999). The knock-out mice are hypertensive and also have altered vascular responses. Vasoconstrictor responses to noradrenaline and 5-hydroxytryptamine are enhanced, and nitric oxide mediated endothelium dependent vasodilatation is impaired (Hadoke *et al.*, 1999). This had led to the suggestion that 11β-HSD2 may protect the vasculature from circulating glucocorticoids.

11β-HSD2 is also highly expressed in placenta (Brown *et al.*, 1996) where it is thought to protect the fetus from the much higher circulating levels of

glucocorticoids in the mother (Beitins *et al.*, 1973). Prenatal exposure to excessive glucocorticoids is thought to retard fetal development (Reinisch *et al.*, 1978). The role of 11β-HSD2 in protecting the fetus from maternal glucocorticoids has been supported by the finding that inhibition of 11β-HSD2 in pregnant rats leads to reduced birth-weight of the offspring (Lindsay *et al.*, 1996).

1.2.4 Role of 11β-HSD1

11β-HSD1 is an NADP(H)-dependent lower affinity enzyme that is expressed almost ubiquitously, with the highest levels found in the liver (Monder & Shackleton, 1984). *In vitro,* hepatic 11β-HSD1 reductase activity is labile, and dehydrogenase activity predominates, but in primary cultures of rat hepatocytes the reaction direction is predominantly reductase (Panarelli *et al.*, 1996). *In vivo,* hepatic 11β-HSD1 also acts in the reductase direction, as venous sampling in man has shown the ratio of cortisol to cortisone is increased more than five fold in the hepatic vein compared with the general circulation (Walker *et al.*, 1992). Hepatic 11β-HSD1 therefore regenerates cortisol from cortisone, potentiating glucocorticoid action, and so alterations in 11β-HSD1 would have important implications for glucocorticoid mediated effects. Indeed, transgenic disruption of the 11β-HSD1 gene in mice attenuates activation of glucocorticoid induced gluconeogenic enzymes (Kotelevtsev *et al.*, 1997) and inhibition of 11β-HSD activity by carbenoxolone leads to an improvement in whole body insulin sensitivity in man (Walker *et al.*, 1995). Inhibition of hepatic 11β-HSD1 by chronic oestradiol administration also inhibits the expression of gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (Jamieson *et al.*, 1999). 11β-HSD activity is also found in other metabolically active tissues such as muscle and fat (Bujalska *et al.*, 1997; Napolitano *et al.*, 1998), where it may also affect glucocorticoid action.

The vascular system is a target for both gluco- and mineralocorticoids. 11β-HSD activity was found in arterial vessels, and 11β-HSD1 mRNA localised to vascular smooth muscle (Walker *et al.*, 1991), such that it could modulate glucocorticoid access to the glucocorticoid receptor, and affect vascular tone and hence peripheral resistance. However, the 11β-HSD1 knock-out mouse has normal vasoconstrictor responses to noradrenaline and 5-hydroxytryptamine, and vasodilatation to acetyl choline is unaffected (Hadoke *et al.*, 1999). Therefore the role of 11β-HSD1 in the vasculature remains uncertain.

The brain is a key glucocorticoid target, and glucocorticoid effects are mediated by both gluco- and mineralocorticoid receptors (McEwen *et al.*, 1986; De Kloet, 1991) eg in the hippocampus. It is thought that glucocorticoids bind to mineralocorticoid receptors under normal circumstances, but that when glucocorticoids rise eg at the diurnal peak or with stress, they will bind to classical glucocorticoid receptors (Reul & De Kloet, 1985). 11β-HSD1 is expressed in many brain regions, with highest expression in hippocampal, cerebellar and cortical neurones (Moisan *et al.*, 1990; Lakshmi *et al.*, 1991; Sakai *et al.*, 1992). In primary hippocampal cultures 11β-HSD1 is a reductase (Rajan *et al.*, 1996) and thus potentiates the neurotoxic effects of glucocorticoids (Sapolsky *et al.*, 1985). This may be relevant to neurodegenerative diseases and aging.

The hippocampus, hypothalamus and pituitary all express 11β-HSD1 (Moisan *et al.*, 1990), so there is a potential role for 11β-HSD1 to regulate glucocorticoid secretion via the HPA axis. This is supported by observations in the 11β-HSD1 knock-out mouse, which has adrenocortical hypertrophy, increased sensitivity to ACTH and elevated plasma corticosterone in the diurnal nadir (Kotelevtsev *et al.*, 1997). It has been suggested that these mice have an insensitivity of the HPA to central glucocorticoid inhibition due to lack of amplification of glucocorticoid levels by 11β-HSD1 in key sites of feedback. Impaired activity of 11β-

HSD1 in the periphery could also have an effect on the HPA, by increasing the metabolic clearance rate of cortisol and thus increasing drive to the HPA (Phillipou *et al.*, 1996). Impairment of 11β-HSD2 would be predicted to have the opposite effect, by decreasing the clearance of cortisol. Therefore, both peripheral clearance and central regulation of feedback of glucocorticoids by 11β-HSD may be important.

1.2.5 Regulation of 11β-HSDs

The expression of 11β-HSD1 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). The sexual dimorphism is due to a difference in growth hormone secretion patterns, which is in turn regulated by oestradiol (Low *et al.*, 1994a). Male rats have high amplitude pulsatility to their growth hormone levels, whereas this pulsatility is dampened in female rats leading to more continuous growth hormone levels, and decreased 11β-HSD1 (Low *et al.*, 1993). Exogenous oestradiol administration decreases hepatic and renal 11β-HSD1 mRNA, but has no effect in hippocampus (Low *et al.*, 1993), which is indicative of tissue-specific regulation of the enzyme. Studies in man are limited to analysis of urinary steroid metabolites, and are not conclusive. Increased, normal and decreased 11β-HSD activity have all been reported in women compared with men (Andrew *et al.*, 1998; Stewart *et al.*, 1999; Finken *et al.*, 1999; Fraser *et al.*, 1999).

Thyroid hormone decreases hepatic 11β-HSD1 activity in rats, but has no effect on renal 11β-HSD activity (Whorwood *et al.*, 1993). This could indicate tissue-specific regulation of 11β-HSD, but the result is confounded by the presence of both 11β-HSD1 and 2 in rat kidney. However, in man analysis of urinary glucocorticoid metabolites indicates that 11β-HSD1 activity is increased by thyroid hormone (Hellman *et al.*, 1961; Zumoff *et al.*, 1983).

There is evidence that inflammatory agents such as the interleukins and tumour necrosis factor-α increase 11β-HSD1 activity and expression in kidney cells (Escher *et al.*, 1997). This is thought to be a counterbalance mechanism to limit their pro-inflammatory effects by increasing the activity of anti-inflammatory glucocorticoids. Interleukins 5 and 6 also increase 11β-HSD activity in granulosa cells (Evangelatou *et al.*, 1997), but this activity has not been attributed to a particular isozyme.

Glucocorticoids and insulin have antagonistic effects on 11β-HSD1. Cell culture studies on human fibroblast cells and rat vascular smooth muscle and liver cells all showed induction of 11β-HSD1 by glucocorticoids while insulin had the opposite effect (Hammami & Siiteri, 1991; Takeda *et al.*, 1994; Jamieson *et al.*, 1995). Similar induction of 11β-HSD1 is seen *in vivo* in liver and hippocampus after glucocorticoid treatment, while adrenalectomy resulted in decreased activity and mRNA in the liver (Low *et al.*, 1994b; Walker *et al.*, 1994b). By contrast 11β-HSD activity in the kidney is unaffected by manipulations in glucocorticoids (Smith & Funder, 1991; Walker *et al.*, 1994b), which represents distinct regulation of the 11β-HSD2 isozyme.

As was discussed briefly in section 1.1, glucocorticoids have effects on body weight homeostasis. Since 11β-HSD1 controls local glucocorticoid levels in key target tissues such as liver, muscle and fat, alterations in 11β-HSD1 activity are postulated to be important in obesity. Some of the regulators of body weight are discussed in the following section.

1.3 Regulation of body weight

Body weight is governed by the balance between energy intake, ie food consumption, and energy expenditure, and the centre of energy homeostasis control is thought to be the hypothalamus. Lesion studies carried out in the 1940s and 1950s demonstrated that the ventromedial

hypothalamus is a satiety centre, whereas the lateral hypothalamic area stimulates feeding behaviour (Brobeck *et al.*, 1943; Anand & Brobeck, 1951). More recently, the arcuate nucleus has also been implicated in the regulation of food intake (Mercer *et al.*, 1996; Ollmann *et al.*, 1997; Cheung *et al.*, 1997). The control of body weight regulation is complex, and is an expanding field with new hormones and receptors still being identified.

1.3.1 Leptin

Leptin was recently identified as the peptide hormone product of the *Ob* gene (Zhang *et al.*, 1994), which is mutated in the *ob/ob* obese mouse. It is synthesised in adipose tissue and circulates in plasma at levels correlating to the amount of body fat present (Considine *et al.*, 1995; Lonnqvist *et al.*, 1995). Leptin acts on receptors in the hypothalamus (Tartaglia *et al.*, 1995; Chen *et al.*, 1996) to maintain body weight homeostasis by inducing changes in food intake and energy expenditure (Halaas *et al.*, 1995; Pelleymounter & et al, 1995). Leptin receptors are also expressed in the pancreas where they cause inhibition of glucose stimulated insulin release (Emilsson *et al.*, 1997), and could therefore play a role in the dysregulation of insulin secretion observed in obese states. Although these actions of leptin initially received most interest, the expression of leptin receptors in reproductive tissues and the effects of leptin therapy in restoring fertility in the *ob/ob* mouse (Chehab *et al.*, 1996) has led to studies on the role of leptin in fertility and the onset of puberty. A wide range of other tissues such as liver, lung, heart, kidney and adipose tissue (Tartaglia *et al*, 1995) also express leptin receptors, but their function in these tissues is still to be elucidated.

When leptin was discovered in 1994 and was found to be the satiety factor the obese *ob/ob* mouse lacked (Zhang *et al.*, 1994), it was hoped that human obesity could be explained by a lack of leptin. However, Caro and colleagues proved this hope improbable. The analysis of the leptin gene in

well over one hundred obese people failed to identify a mutation except in one individual (Considine *et al.*, 1995; Considine *et al.*, 1996). One kindred with a functional mutation in the leptin gene has been described (Montague *et al.*, 1997), and initial reports indicate that treatment with recombinant leptin is causing a weight reduction in these individuals (Farooqi *et al.*, 1999). Plasma leptin levels correlate with percentage body fat, even in obesity (Considine *et al.*, 1995; Lonnqvist *et al.*, 1995) which implies that there is not a defect in production of leptin, but that obese humans are resistant to the actions of leptin. There is still hope that leptin treatment may still help obese individuals to lose weight, and indeed the preliminary results of a recent clinical trial indicate this may be the case (Greenberg *et al.*, 1999). However, the weight loss was achieved in this study with the aid of a calorie-controlled diet and very high levels of leptin, and patients were only studied for 24 weeks. Whether these effects will persist, and the long-term effects of leptin treatment remain to be seen.

1.3.2 Neuropeptide Y

Neuropeptide Y (NPY) acts on the hypothalamus and has opposing effects to leptin ie it stimulates appetite and decreases energy expenditure (Clark *et al.*, 1984; Stanley *et al.*, 1986; Billington *et al.*, 1991). Administration of NPY into the paraventricular nucleus of the hypothalamus of normal rats induces an obesity syndrome (Zarjevski *et al.*, 1993), and infusions of anti-NPY serum prevent feeding (Shibasaki *et al.*, 1993). Leptin has been shown to decrease NPY levels independently of effects on food intake and body weight (Schwartz *et al.*, 1996a), which implies the balance of these hormones is important in body weight homeostasis. Glucocorticoids are an absolute requirement for NPY to exert its effects, as NPY has no effect in adrenalectomised animals and replacement of corticosterone leads to a recovery of NPY effects (Stanley *et al.*, 1989).

1.3.3 α-Melanocyte-stimulating hormone

POMC is the precursor for several peptides, one of which is α-melanocyte-stimulating hormone (α-MSH). α-MSH acts through the melanocortin receptor-4 (MC4) to inhibit food intake. There is an endogenous receptor antagonist, agouti-related peptide, over-expression of which leads to obesity (Ollmann *et al.*, 1997). Loss of either POMC, MC4 or agouti peptide, by naturally occuring mutations or gene targeting, are all associated with obesity (Yaswen *et al.*, 1999; Huszar *et al.*, 1997; Lu *et al.*, 1994).

1.3.4 The orexins

Orexin-A and -B were recently identified as the endogenous ligands of an orphan receptor purified from brain (Sakurai *et al.*, 1998). The peptide hormones are specifically expressed by neurones around the lateral hypothalamic area, and intra-cerebro-ventricular injection leads to increased feeding and weight gain (Sakurai *et al.*, 1998). Orexin neurones project widely through the brain and the physiological role of the orexins is therefore thought to be complex.

1.3.5 Glucocorticoids

The focus of this thesis is the role of glucocorticoids in obesity. A possible role for glucocorticoids in the pathogenesis of obesity has long been considered, primarily because chronic glucocorticoid excess promotes accumulation of adipose tissue. Glucocorticoids act on both lipoprotein lipase and hormone-sensitive lipase to cause an accumulation of fat, and promote differentiation of pre-adipocytes to mature adipocytes (Hauner *et al.*, 1987). In man, glucocorticoids increase energy expenditure, but this is accompanied by a larger increase in energy intake, resulting in positive energy balance and therefore weight gain (Tataranni *et*

al., 1996). It has recently been observed in rats that hyperphagia and weight gain are induced by intracerebro-ventricular administration of the synthetic glucocorticoid dexamethasone (Zakrzewska *et al.*, 1999). Glucocorticoids also increase expression of other factors that enhance food intake, including NPY and the NPY receptor (Larsen *et al.*, 1994). The evidence for the involvement of glucocorticoids in obesity is discussed in section 1.5.

1.4 The Zucker rat as a model of obesity

The animal model utilised in the majority of the work in this thesis is the obese Zucker rat, and the physiology of this model is discussed in the following section.

The genetically obese Zucker rat *(fa/fa)* has been commonly used as an animal model of obesity and early type 2 diabetes since it was first described by Lois and Theodore Zucker in 1961 (Zucker & Zucker, 1961). The obese Zucker rat displays a catalogue of metabolic disturbances similar to those observed in the human metabolic syndrome "X". The obese rats are hyperphagic with predominantly visceral obesity (Van Zwieten *et al.*, 1996), which is improved, but not negated, by pair-feeding with lean rats (Cleary *et al.*, 1980). A defect in brown adipose tissue thermogenesis, increasing energy efficiency, contributes to maintenance of obesity in the face of caloric restriction (York *et al.*, 1985). They are also insulin resistant, and have compensatory hyperinsulinaemia (York *et al.*, 1972). There is a sub-strain of obese Zucker rats, known as the Zucker diabetic fatty rats, that are overtly diabetic and suffer β-cell decompensation and failure, and these are used as a model for type 2 diabetes (Tokuyama *et al.*, 1995; Hirose *et al.*, 1996). Obese Zucker rats have altered fat metabolism, including increased synthesis and secretion of very low density lipoproteins (Witztum & Schonfeld, 1979) which may contribute to the high levels of plasma triglycerides observed in these

animals (Barry & Bray, 1969). Whether they are hypertensive has been a matter of debate. It appears that they are mildly hypertensive but whether this reaches statistical significance depends on the number of rats in the study and the precision of the measurements made (Kurtz *et al.*, 1989; Van Zwieten *et al.*, 1996; Alonso-Garcia *et al.*, 1996). Obese Zucker rats also have alterations in the hypothalamic-pituitary adrenal (HPA) axis, which are discussed in section 1.4.3. Both homo-and heterozygous lean Zucker rats are metabolically normal (Bray & York, 1979) and are used as controls in studies involving obese Zucker rats.

1.4.1 Leptin

The primary defect in the obese Zucker rat was recently identified to be a recessive mutation in the leptin receptor gene (Chua *et al.*, 1996). A nucleotide substitution at position 880 (A→C) causes the conversion of amino acid 269 from glutamine in the wild type to proline (Chua *et al.*, 1996). The mutation occurs in an area of β-sheet and proline is known to disrupt β-sheet conformations, so it assumed that the mutation alters the structure of the receptor (Takaya *et al.*, 1996). This mutation occurs in the extracellular domain of the receptor in a region that is conserved between rat, mouse and human leptin receptor sequences and is thought to be involved in cellular transport of the leptin receptor (Chua *et al.*, 1996). The mutant receptor still has the same affinity for leptin as a wild type receptor (Tartaglia & et al, 1995) but is expressed at much reduced level on the cell surface (Rosenblum *et al.*, 1996), supporting the hypothesis that the mutation affects cellular transport. The mutation results in resistance to the actions of leptin and deficient transport of leptin across the blood brain barrier (Wu-Peng *et al.*, 1997).

Obese Zucker rats have been found to be resistant to the actions of leptin in several tissues. Intracerebro-ventricular (icv) administration of leptin to obese Zucker rats has less of a weight reducing effect than observed in

lean Zucker rats (Cusin *et al.*, 1996). Leptin inhibits glucose stimulated insulin secretion in isolated pancreatic islets, and stimulates lipolysis in isolated white fat pads by increasing activity of lipoprotein lipase, but these effects are not observed in obese Zucker rats (Emilsson *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997).

1.4.2 Previous studies in the obese Zucker rat

Obese Zucker rats were studied for more than 30 years before the discovery of leptin, and the identification of their leptin receptor mutation. In this time several hypotheses were put forward to explain their obesity, all of which we now know to be secondary to leptin resistance. Since leptin resistance appears to be a common feature in human obesity, intermediate mechanisms between leptin resistance and obesity are still being studied in an attempt to elucidate alternative therapeutic targets.

Administration of NPY into the hypothalamus of normal rats induces obesity (Zarjevski *et al.*, 1993). In obese Zucker rats, NPY levels within the hypothalamus are higher (Sanacora *et al.*, 1990; McKibbin *et al.*, 1991), and secretion of NPY from the hypothalamus is increased (Dryden *et al.*, 1995), compared with lean animals. However, NPY receptor levels and responses to administration of exogenous NPY are lower in Zucker rats (McCarthy *et al.*, 1991; Brief *et al.*, 1992). Lower receptor numbers in obese animals are thought to be due to down-regulation in the face of chronically high NPY levels, and the reduced response to exogenous NPY may be due to receptor saturation, again due to chronically high NPY levels. Leptin has been shown to decrease NPY levels independently of effects on food intake and body weight (Schwartz *et al.*, 1996b), and it is possible that leptin resistance could contribute to higher NPY levels seen in obese Zucker rats. Whether increased NPY secretion is physiologically important in the face of decreased receptor numbers is still a matter of debate.

The role of the sympathetic nervous system in the obese Zucker rat has also received attention. They have decreased sympathetic tone, which results in defective brown adipose tissue thermogenesis and increased energy efficiency (Onai *et al.*, 1995). The β_3-adrenoceptor is expressed at lower levels in adipose tissue of obese animals (Onai *et al.*, 1995), and it has been shown that the potency of β_3-adrenoceptor agonists is reduced in these animals (Charon *et al.*, 1995). Treatment of obese Zucker rats with low doses of the β_3-adrenoceptor agonist BRL35135A did not alleviate obesity, but did lead to an improvement of hyperinsulinaemia, insulin resistance and hypertriglyceridaemia (Hashimoto *et al.*, 1996). In a more recent study, obesity was attenuated by treatment with a higher dose of BRL35135 (Savontaus *et al.*, 1998). Glucocorticoids play a role in this abnormality, as adrenalectomy restores β_3-adrenoceptor mRNA levels to normal (Onai *et al.*, 1995).

Nitric oxide has also been implicated in the control of food intake in the obese Zucker rat as inhibition of nitric oxide synthase limits both food intake and weight gain (Squadrito *et al.*, 1993). However, when endogenous nitric oxide synthase activity was measured in the hypothalamus, fundus of the stomach and skeletal muscle it was found to be lower in obese rats than lean (Morley & Mattammal, 1996; Young & Leighton, 1998). It has been suggested that nitric oxide synthase may be uncoupled from its normal control mechanisms in obesity, and that inhibitors of nitric oxide synthase may make a useful therapeutic target, but this suggestion remains to be tested.

1.4.3 The HPA axis in obese Zucker rats

The most commonly studied aspect of the obese Zucker rat is the HPA axis, as the evidence suggests that glucocorticoids have an important role to play in the development and maintenance of obesity. Adrenalectomy of young obese rats prevents obesity from developing, and of older rats

attenuates obesity, and these effects are negated in a dose-dependent manner by glucocorticoid replacement (Yukimura *et al.*, 1978; Freedman *et al.*, 1986; Castonguay *et al.*, 1986). Ablation of glucocorticoid action by treatment with a glucocorticoid receptor antagonist also leads to an improvement in obesity (Langley & York, 1990). Both adrenalectomy and glucocorticoid antagonist treatment also lead to an improvement in other metabolic defects in the obese Zucker rat eg plasma insulin and triglyceride levels fall and some aspects of fat metabolism, such as brown adipose tissue thermogenesis are normalised (York *et al.*, 1985; Castonguay *et al.*, 1986; Freedman *et al.*, 1986; Langley & York, 1990).

Studies have shown that obese rats excrete more corticosterone in their urine than lean rats (Cunningham *et al.*, 1986; White *et al.*, 1989), but this is not necessarily associated with increased plasma corticosterone levels. While some reports have described elevated plasma corticosterone levels in obese Zucker rats (Dubuc, 1976; Martin *et al.*, 1978; Fletcher *et al.*, 1986; Guillaume-Gentil *et al.*, 1990; Plotsky *et al.*, 1992; Dryden *et al.*, 1995) other studies, including one where urinary corticosterone excretion was elevated (Cunningham *et al.*, 1986), have reported normal plasma corticosterone levels (Yukimura *et al.*, 1978; Walker *et al.*, 1992). Whether or not hypercorticosteronaemia is reported seems to depend on the sex, age and source of the animals studied, and the time of sampling.

Obese Zucker rats have lower levels of CRH in the hypophyseal-portal circulation (Plotsky *et al.*, 1992) and are less sensitive to CRH (Cunningham *et al.*, 1986). Despite this, plasma ACTH levels are higher in obese animals (Cunningham *et al.*, 1986; Bestetti *et al.*, 1990). Although high circulating ACTH levels would explain increased corticosterone secretion and adrenal hypertrophy there is no clear explanation for increased ACTH in the face of low CRH. Leptin has been reported to decrease CRF secretion (Heiman *et al.*, 1997) and increase POMC expression (the precursor of ACTH) (Thornton *et al.*, 1997).

However, the obese Zucker rat is resistant to the actions of leptin, and so changes in the opposite direction may be predicted.

The sensitivity of corticosterone negative feedback is also equivocal in these animals. The synthetic glucocorticoid dexamethasone has been reported to cause the same degree of adrenal suppression in lean and obese animals (Cunningham *et al.*, 1986; Guillaume-Gentil *et al.*, 1990). By contrast, ACTH is less sensitive to negative feedback by corticosterone in obese animals (Walker *et al.*, 1992). Also, glucocorticoid receptor antagonism fails to induce an increase in plasma AVP or corticosterone levels (Pesonen *et al.*, 1992a; Pesonen *et al.*, 1992b) suggesting dysregulation of the HPA axis.

The magnitude of the stress response in obese Zucker rats is also unresolved. Both normal (Plotsky *et al.*, 1992; Walker *et al.*, 1992) and increased (Bestetti *et al.*, 1990; Guillaume-Gentil *et al.*, 1990; Pacak *et al.*, 1995) pituitary and adrenal responses to stress have been reported. Sexual dimorphism of the stress response was reported by one group (Guillaume-Gentil *et al.*, 1990) but this does not seem to account for the conflicting reports.

1.5 The role of glucocorticoids in obesity

Increased secretion of glucocorticoids (eg in Cushing's syndrome) is associated with obesity; and cortisol secretion is increased in subjects with idiopathic obesity, especially of central or android distribution (Pasquali *et al.*, 1993). It has long been known that total urinary glucocorticoid metabolites are elevated in obese subjects, implying an increase in cortisol secretion rate (Gray *et al.*, 1956; Migeon *et al.*, 1963), and weight loss is accompanied by a fall in glucocorticoid excretion (Migeon *et al.*, 1963). Despite an inferred increase in glucocorticoid secretion, 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; Stewart *et al.*, 1999),

suggesting that metabolism of glucocorticoids must also be increased. Recent studies in man have looked more carefully at the specific urinary glucocorticoid metabolites (Andrew *et al.*, 1998; Stewart *et al.*, 1999; Fraser *et al.*, 1999). These show that metabolism of cortisol by 5α-reductase may be enhanced, and 11β-hydroxysteroid dehydrogenases may be altered in obesity. Specifically, Stewart et al suggest that hepatic reactivation of cortisol may be impaired in obesity as measured by conversion of an oral dose of cortisone to cortisol. This is backed up by a change in urinary glucocorticoid metabolite profiles in this study and one other (Fraser *et al.*, 1999), but urinary profiles must be considered as a less robust assessment of 11β-HSD activity. Urinary steroids give an impression of the "whole body balance" of 11β-IISD activities and, as will be described later in this thesis, tissue specific changes in 11β-HSD activities may be overlooked by this method.

The majority of work linking glucocorticoids with obesity has been carried out in animals. Obese Zucker rats have similar increases in glucocorticoid production rates (White *et al.*, 1989; Cunningham *et al.*, 1986) and have adrenal hypertrophy (Walker *et al.*, 1992; Bestetti *et al.*, 1990), but reports of plasma corticosterone levels are inconclusive. Increased (Dubuc, 1976; Martin *et al.*, 1978; Fletcher *et al.*, 1986; Guillaume-Gentil *et al.*, 1990; (Plotsky *et al.*, 1992) and normal (Yukimura *et al.*, 1978; Walker *et al.*, 1992; Cunningham 1986) plasma corticosterone levels have been reported, as have both normal (Yukimura *et al.*, 1978; Guillaume-Gentil *et al.*, 1990; Walker *et al.*, 1992) and disrupted (Martin *et al.*, 1978; Fletcher *et al.*, 1986) circadian rhythms of corticosterone. Despite this controversy, obesity in animal models has been demonstrated to be glucocorticoid dependent. Adrenalectomy of obese rats and mice prevents development of obesity in young animals and reduces obesity in adult animals, while glucocorticoid replacement induces a dose-dependant recurrence of the obesity (Saito & Bray, 1984; Tokuyama & Himms-Hagen, 1987; Yukimura *et al.*, 1978; Freedman *et al.*, 1986). A similar attenuation of

obesity is observed in rats treated with the glucocorticoid receptor antagonist RU486 (Hardwick *et al.*, 1989; Langley & York, 1990).

Abnormalities of the HPA axis have been described in obese animals, but these findings have not been conclusive and there are many conflicting reports (Guillaume-Gentil *et al.*, 1990; Plotsky *et al.*, 1992; Bestetti *et al.*, 1990). Secretion of corticosterone under the control of the HPA axis is not the only factor affecting glucocorticoid supply to its receptor (Figure 1.5). Metabolic clearance of steroids will also have an impact on circulating corticosterone levels. Any change in metabolic clearance would normally be associated with compensatory changes in glucocorticoid secretion, but if it was accompanied by alterations in the HPA it could result in a failure to maintain normal plasma glucocorticoid levels. Access of glucocorticoids to receptors is also controlled at a tissue level by the steroid metabolising enzymes 11β-HSDs, which interconvert active and inactive glucocorticoids (see section 1.2). Alterations in 11β-HSD activity could results in changes in glucocorticoid availability within a tissue, independently of changes in plasma glucocorticoid levels. Altered 11β-HSD1 activity has been reported in hypertensive, insulin-resistant animal models, such as the Bianchi-Milan and Lyon hypertensive rat strains (Stewart *et al.*, 1993; Lloyd-MacGilp *et al.*, 1999). Specifically, hepatic 11β-HSD1 activity is impaired, which would be predicted to decrease exposure of the liver to glucocorticoids. However, no suggestions have been made as to how this change relates to the pathogenesis of hypertension or insulin resistance, and to date the importance of 11β-HSDs in animal models of obesity has not been assessed

The evidence for a link between glucocorticoids and obesity in both man and animal models is strong. However, the nature of and mechanisms underlying abnormalities of the HPA axis and / or glucocorticoid metabolism in obesity remain unclear.

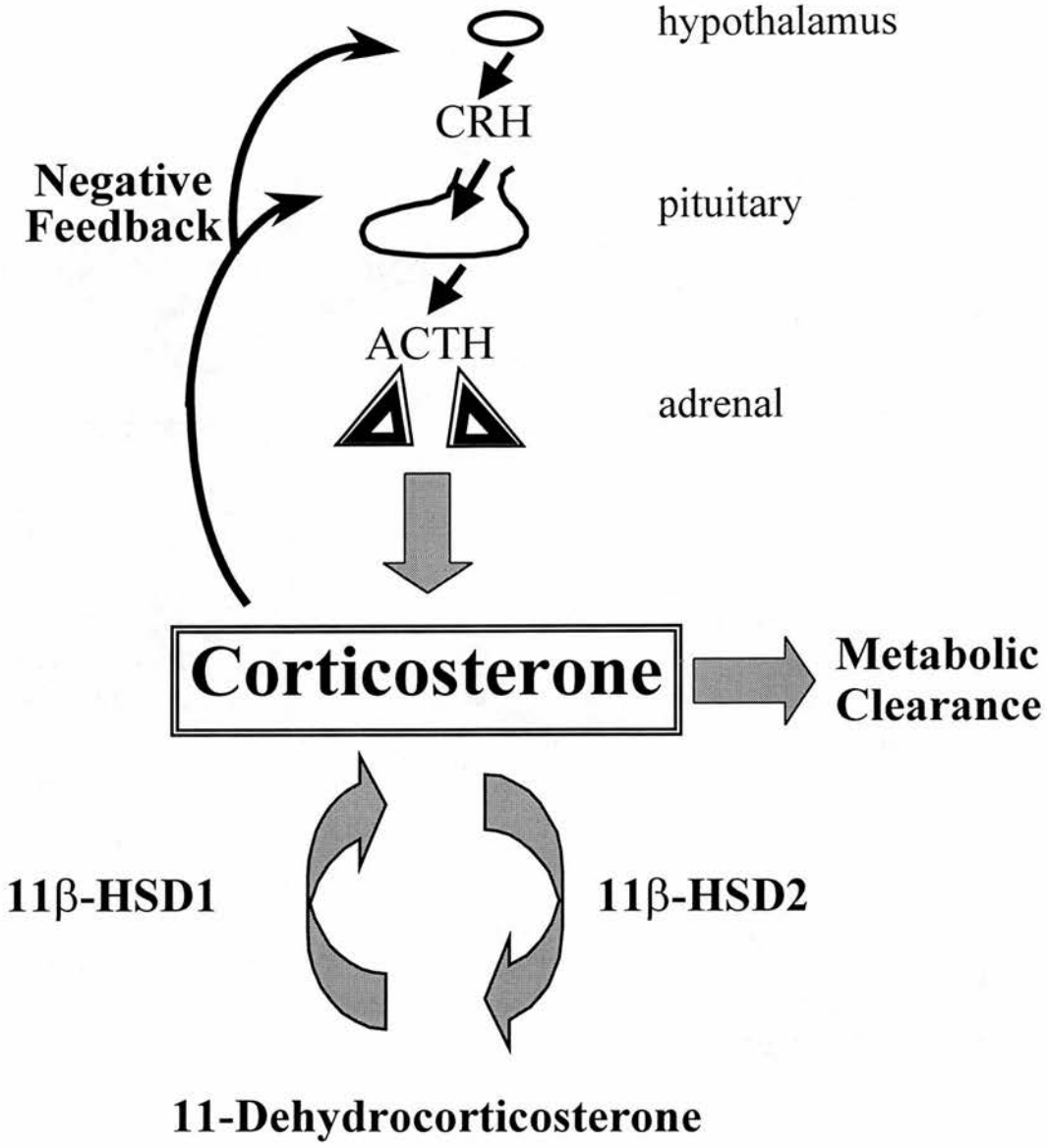

Figure 1.5: Control of availability of glucocorticoids to receptors. CRH=corticotrophin releasing hormone; ACTH=adrenocorticotrophic hormone

1.6 Aims of this thesis

The primary aim of this thesis was to study glucocorticoid metabolism in obesity, to elucidate differences that may account for characteristics of obesity and HPA activation. The majority of the work carried out utilised the Zucker rat model of obesity, described in section 1.4. The following list of aims was addressed in this thesis:

1) To study metabolic clearance of corticosterone in obese Zucker rats by assessing urinary metabolite profiles by GC-MS, necessitating establishment of new methodology, and to confirm inferences made on glucocorticoid metabolising enzyme activities by assessing enzyme activity *in vitro*.

2) To quantify 11β-HSD1 activity in glucocorticoid target tissues in obese Zucker rats, to establish whether differences in local glucocorticoid levels could contribute to the obese phenotype. Also to measure expression of glucocorticoid regulated genes, to assess whether any change in enzyme activity relates to a change in glucocorticoid action.

3) To examine possible mechanisms of control of 11β-HSD1 in obesity by making manipulations in obese Zucker rats, with the role of glucocorticoids and insulin of particular interest.

4) To assess the role of 11β-HSDs in obesity, by inhibiting the enzymes with carbenoxolone in obese Zucker rats. Of particular interest were the effects on insulin sensitivity and weight gain.

5) To assess 11β-HSD1 activity in human obesity, and relate this to observations made in the obese Zucker rat, to validate the use of this model for further studies on the regulation of 11β-HSD1 in obesity.

Chapter Two

Materials and Methods

2.1 Materials

Unless otherwise stated, chemicals and reagents were purchased from Sigma, Poole, UK.
All HPLC grade solvents were from Rathburn Chemicals, Walkerburn, UK.
All epi-steroid standards were from Steraloids, Newport, USA.
All radioactivity was from Amersham, Bucks, UK.
Sources other than these are indicated in parenthesis.

2.2 Commonly used buffers and solutions

Acetate buffer: 0.2M sodium acetate, pH4.6. Stored at 4°C.

Alkaline SDS: 0.2M NaOH, 0.1% SDS. Freshly prepared before use.

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

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

Denaturing solution: 4M guanidium thiocyanate, 0.025M Na citrate, 0.5% sarcosyl. β-Mercaptoethanol (0.1M) added immediately prior to use.

DEPC-treated water: Distilled water (500ml) mixed with diethylpyrocarbonate (DEPC; 5 drops) and left for 1 – 24 hours before autoclaving.

Deionised formamide: 15g Amberlite ion exchange resin (BDH, Lutterworth, UK) mixed with 150ml formamide for 1 hour, then filtered twice to remove Amberlite.

GTE: 50mM glucose, 25mM tris[hydroxymethyl]-aminomethane (tris), 10mM EDTA, pH8. Freshly prepared before use.

Kreb's Ringer Bicarbonate Buffer: 118mM NaCl, 3.8mM KCl, 1.19mM KH_2PO_4, 2.54mM$CaCl_2$, 1.19mM $MgSO_4$, 25mM $NaHCO_3$, pH 7.4. Stored at 4°C and supplemented with 0.2% glucose immediately prior to use.

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

Loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 25 % Ficoll made up in DEPC-treated water.

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

10x MOPS buffer: 0.2M 3-[N-morpholino]propanesulfonic acid, 50mM Na acetate, 5mM EDTA, pH7. Autoclaved before use.

Orthophosphate buffer: 40 mM Na_2HPO_4, pH 7.5.

Phosphate buffer: 0.2M NaH_2PO_4, 0.6M Na_2HPO_4, 5mM ethylenediaminetetraacetic acid. Autoclaved before use.

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

20x SSC buffer: 175.32g NaCl, 88.23g Na citrate made up to 1 litre with distilled water. Autoclaved before use.

10x TBE buffer: 56g Tris, 57.5g boric acid, 20ml 0.5M EDTA, made up to 500ml with distilled water. Autoclaved before use.

TE buffer: 10mM Tris-HCl, 1mM EDTA, pH 8. Autoclaved before use.

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

Lean and obese Zucker rats were obtained from Harlan Orlac, Bicester, UK at five weeks-of age. Rats were maintained under controlled conditions of light (lights on 0800 h - 2000 h) and temperature (21-22°C), and allowed free access to standard rat chow (Special Diet Services, Witham, UK) and drinking water. During basal conditions rats were under the care of the animal technicians of the Biomedical Research Facility, Western General Hospital. After surgery and during treatment periods the day to day care of the animals was carried out by me, with the assistance of the animal technicians, during which time the animals were observed closely for signs of ill health. Animals were housed four per cage except when urine collections were being made, when they were housed individually in metabolic cages.

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. The animals were closely monitored until they regained consciousness, and were not returned to their cage until fully conscious. All rats were maintained on 0.9% saline as drinking water to allow the ADX rats to maintain their electrolyte balance. Rats were weighed every second day to follow the progress of weight gain, and were killed 21 days after surgery by decapitation.

2.3.2 Glucose tolerance tests

Rats were fasted from 1700h to 0900h, weighed and a 2g/kg dose of glucose administered by gavage. Blood samples were taken by tail nick at 0, 30 and 120 min after glucose administration. Blood samples were collected into heparinised Eppendorfs, centrifuged at 5000 rpm for 2 min in a bench-top centrifuge and the plasma removed into fresh Eppendorfs. The samples were frozen on dry ice and stored at -20°C. At the end of the glucose tolerance test, rats were allowed free access to food and water.

2.4 Plasma assays

2.4.1 Corticosterone radioimmunoassay

Plasma samples were diluted 1 in 5 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 – 320 nM) to allow construction of a standard curve. Samples and standards were incubated in duplicate with a mixture of [^{3}H]-B (10,000cpm per sample) and B antibody (1 in 10,000 dilution; produced in-house by Dr CJ Kenyon) 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 sealed and incubated overnight. The SPA beads bind to the primary antibody and if the primary antibody is bound to [^{3}H]-B the SPA beads cause scintillation of the radioactive signal. As the concentration of unlabelled B increases there is competition between binding of labelled and unlabelled 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%.

2.4.2 11-Dehydrocorticosterone radioimmunoassay

The 11-dehydrocorticosterone (A) radioimmunoassay (RIA) was carried out in the same way as the B RIA except for the following changes: samples were not diluted as A circulates at much lower levels than B; the standard curve was constructed using A standards; [^{3}H]-A (see below) rather than [^{3}H]-B was used; and an A primary antibody (a gift from Prof Vecsei and Dr Haack, Ruprecht-Karis-Universitat, Heidelberg) was used. The inter- and intra-assay coefficients of variation were <10%.

2.4.3 Preparation of [^{3}H]-11-dehydrocorticosterone

[^{3}H]-A was prepared from [^{3}H]-B using homogenates of rat kidney, which contain high levels of 11β-HSD2 that will readily convert [^{3}H]-B to [^{3}H]-A. Rat kidney also contains 11β-HSD1 that will carry out the reverse reaction, but by using high concentrations of the appropriate cofactor (NAD) almost exclusive dehydrogenase activity was achieved. Kidney homogenate (1mg/ml) was incubated at 37°C with NAD (2mM) and [^{3}H]-B (600nM) in a total volume of 2ml for four hours. The steroids were extracted with 10 volumes of ethyl actetate, dried under oxygen free

nitrogen at 60°C and re-suspended in 100µl of ethanol. A 1µl sample of the re-suspended steroid was analysed by HPLC (see section 2.6.5) to check the purity of [^{3}H]-A, which was routinely >97%. [^{3}H]-A was stored at -20°C and the purity was checked by HPLC before each use as it had a tendency to break down.

2.4.4 Insulin radio-immunoassay

The insulin radioimmunoassay was carried out using a commercially available kit (Amersham, Bucks UK). Plasma samples were diluted 1 in 3 in the supplied buffer before assay. A range of concentrations of insulin was prepared (0.1 – 25 ng/ml) to allow construction of a standard curve. Samples and standards were incubated in duplicate plastic tubes with a mixture of [^{125}I]-insulin (1,000cpm per sample) and insulin primary antibody in buffer (total volume 75µl) at room temperature for 4 hours. Two tubes were prepared with only [^{125}I]-insulin to give the total counts, and two with [^{125}I]-insulin and buffer but no antibody to give the non-specific binding. Amerlex™-M magnetic separation beads (60µl) were then added to each sample and the samples incubated for a further 10min. The samples were centrifuged at 1,000g for 10min at 4°C to pellet the beads, and the supernatant discarded. The tubes were counted in a γ-counter and the concentration of insulin in the samples estimated by comparison with the standard curve. The inter- and intra-assay coefficients of variation were <15% and <10% respectively.

2.4.5 Glucose assay

Plasma samples were incubated in duplicate with glucose hexokinase reagent (Sigma) for 5min. The reagent causes glucose present in the sample to be phosphorylated and NADH to be generated, causing an increase in absorbance at 340nM. The absorbance of the samples at 340nM was measured using a Shimadzu uv/visible recording

spectrophotometer with a reagent blank. If the plasma sample was haemolysed, a plasma blank (prepared by diluting the plasma sample with water) rather than a reagent blank was used to allow correction for the colour in the sample. The amount of glucose in the sample was calculated by comparison with a standard curve (100 – 800 mg/dL glucose). The inter- and intra-assay coefficients of variation were <2%.

2.5 Gas chromatography / mass spectrometry

2.5.1 Preliminary work

There was no existing protocol for the detection of rat urinary glucocorticoid metabolites in our laboratory, so I developed one. The starting point for the development of the assay was the assay currently employed in out laboratory for the analysis of human urinary glucocorticoid metabolites (Best & Walker, 1997). The complexity of the assay necessitates the use of internal standards, which are added to the samples / standards at the beginning of the assay. The standards used in our laboratory for human urine assays are the non-endogenous 11α- (epi) isomers of cortisol and tetrahydrocortisol. These compounds are very structurally similar to the endogenous steroids of interest and behave in a similar manner to the endogenous steroids throughout the assay. A ratio of the signals for the endogenous steroid and the epi-steroid is obtained for the range of standards and a standard curve of the ratios is drawn (epi-cortisol is the internal standard used for cortisol and cortisone and epi-tetrahydrocortisol is the internal standard used for all other cortisol metabolites). The ratios obtained for the samples are then compared to the ratios in the standard curve and the amount of endogenous steroid in the samples calculated. For the rat urine assay it was necessary to use internal standards that were more appropriate for rat steroids, therefore epi-corticosterone (epi-B) and epi-tetrahydrocorticosterone (epi-THB) were used. These compounds had not been used in our laboratory before so

their use had to be validated. First it was determined that these compounds could be detected by gas chromatography / mass spectrometry (GC-MS) and then their fragmentation patterns were analysed to assess which ion was the most appropriate to use (Figure 2.1). Then calibration lines were drawn up for the ratio of endogenous steroid signal and the epi-steroid signal to ensure that in the range used this was linear (a line with an R value from linear regression analysis of ≥ 0.99 was accepted as linear). An example calibration line is shown in Figure 2.2.

One further change to the standard assay was necessary to be able to process rat urine. In the human urine assay steroids are extracted from urine using Sep-pak C18 columns containing 360mg of packing material (Waters, Herts, UK). The rat urine was collected using metabolic cages, and this allowed contamination of the urine with food and faeces. These contaminants necessitated the use of columns with a larger amount of packing material (2g) to be able to extract the urinary steroids with the same efficiency.

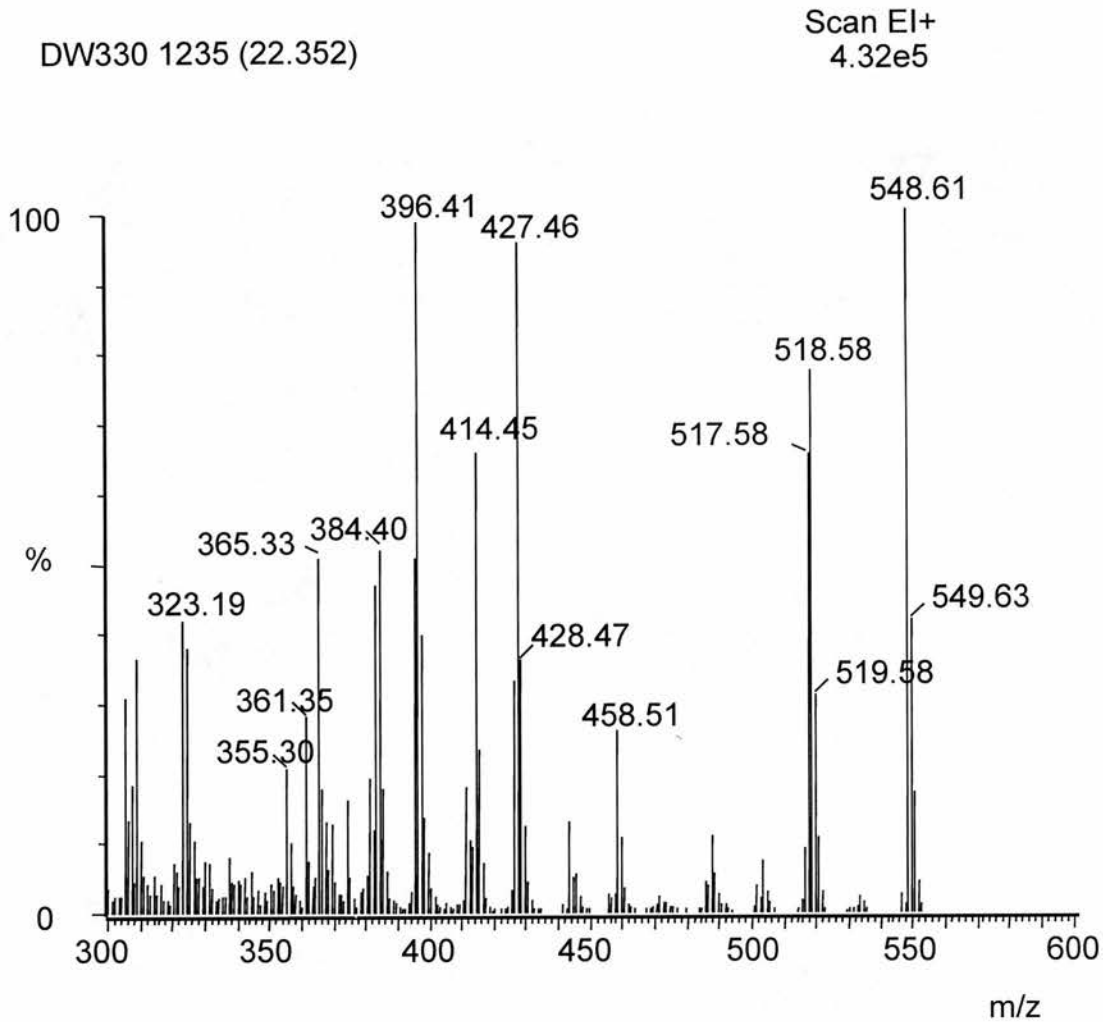

Figure 2.1: Fragmentation pattern of epi-corticosterone derivatised with methoxyamine and trimethylsilylimidazole.

The ion monitored was m/z = 548.

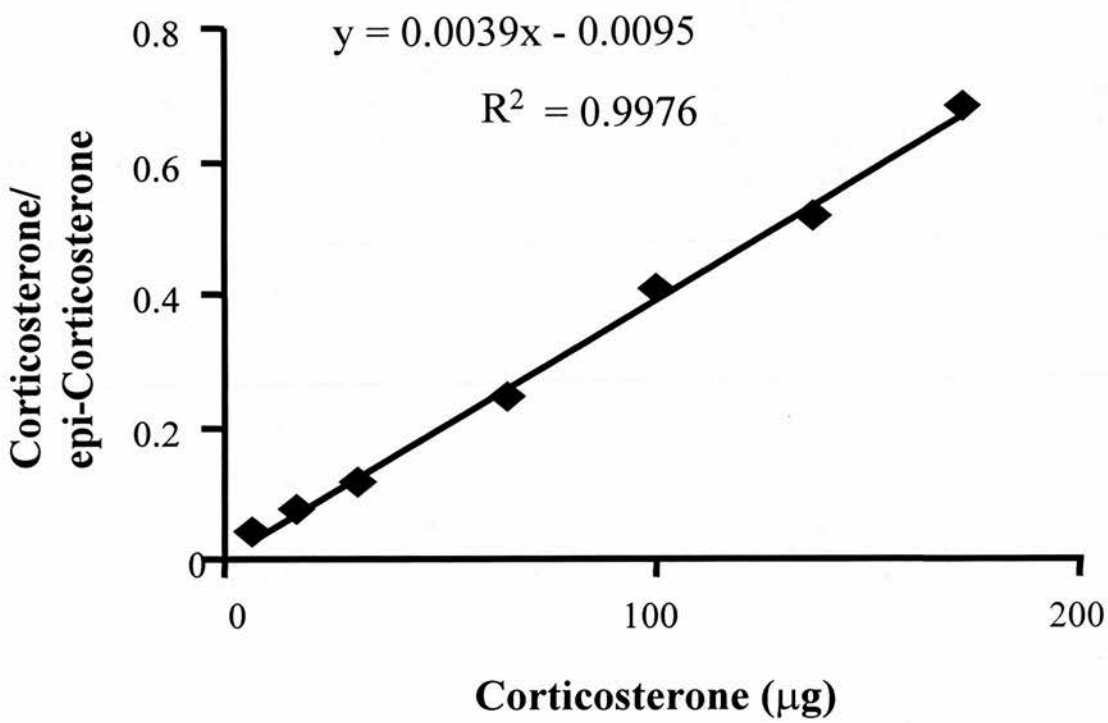

Figure 2.2: Example standard curve for corticosterone by GCMS.

2.5.2 Extraction of steroids from urine

Sep-pak columns were primed by passing through 20ml of methanol then 20ml of water (all solvents used in this assay were HPLC grade). For the standard curve the columns were loaded with 5ml of water, then 10μg of epi-B, 5μg of epi-THB and 100 – 1750 ng of standard steroids in methanol, followed by a further 5ml of water. For the samples the columns were loaded with 5ml of urine, then 10μg of epi-B and 5μg of epi-THB in methanol, followed by a further 5ml of urine. The columns were then washed with 20ml of water, and the steroids eluted with 10ml of methanol. The samples were dried under a stream of oxygen free nitrogen (OFN) at 60°C.

2.5.3 Hydrolysis of steroids

Glucocorticoids are conjugated to glucuronic acid and sulphates to make them more water-soluble before they are excreted. These groups need to be removed to allow derivatisation and good chromatography. Acetate buffer was added to the dry samples and vortexed vigorously to ensure steroids were in solution. β-Glucuronidase enzyme was added and the samples incubated at 37°C for 48 hours. β-Glucuronidase enzyme cleaves the glucuronic acid from the steroid, and also has sulphatase action to remove the sulphate groups.

2.5.4 Extraction of hydrolysate

Once hydrolysis was complete, the steroids were re-extracted. Sep-pak columns were primed as before with 20ml methanol and 20ml water; the hydrolysate was passed through the column; the column washed with 20ml water; and the steroids eluted with 10ml methanol. The methanol was removed by drying the samples under a stream of OFN at 60°C. The steroids were then further extracted by reconstituting the sample in a

mixture of ethyl acetate and water (15:1), vortexing vigorously, removing the organic phase and drying the sample under OFN at 60°C. The aqueous phase was discarded.

2.5.5 Derivatisation of steroids

The steroids underwent two derivatisation steps to mask the polar groups and improve chromatography. Firstly, the ketone groups were replaced by incubating the samples in 2% methoxyamine in dry pyridine at 60°C for 30min. The samples were dried as before, and then incubated with trimethylsilylimidazole at 100°C for 2h to replace the hydroxyl groups. Not all of the available groups on a molecule will be derivatised, for example the ketone group at carbon 20 is not replaced by methoxyamine, possibly because of the stoichiometry of the reaction. The differences in derivatisation aid in the identification of molecules by GC-MS.

2.5.6 Extraction of derivatised steroids

The steroids were extracted from the derivative mixture on lipidex 5000 columns which were prepared by adding 1ml of lipidex 5000 (Canberra Packard, UK) to glass pasteur pipettes loosely plugged with silanised glass wool. The lipidex was prepared in mobile phase (cyclohexane: pyridine: hexamethylsisilazane; 98:1:1) and the columns were washed with 3ml of mobile phase before use. The samples were passed through the column in 2ml of mobile phase, and a further 1ml of mobile phase was passed through to ensure all of the steroid was eluted. The samples were dried under OFN and reconstituted in hexane in preparation for injection into the GC-MS system.

2.5.7 Gas chromatography / mass spectrometry

Steroids were quantified using a Voyager GC-MS system (Finnigan, Manchester, UK) in electron impact mode fitted with a EC-5 capillary column (length 30 metres, internal diameter 0.25mm, film thickness 0.25μm; Alltech, Carnforth, UK). The temperature programme used for the gas chromatography consisted of an initial temperature of 200°C maintained for 1min, temperature increased by 20°C per min for 3min, temperature increased by 2°C per min for 20min and held at 300°C for 2min. This temperature programme was selected to give the best practical separation of ions with the same mass. The mass-spectrometer was programmed to monitor the selected ions with a dwell time of 0.04seconds per ion per cycle. The mass of the ions monitored and the retention times are shown in Table 2.1. The area under each peak was integrated using Masslab software (Fisons, UK); the ratio of each peak to the internal standard calculated; and the concentration of steroid calculated from the calibration line. The intra-assay variability was <10% for all steroids.

Compound	**Ion monitored (m/z)**	**Retention time (min)**
Corticosterone (B)	548	22.070 + 22.245
11-Dehydrocorticosterone (A)	474	21.605
Epi-B	548	21.305 + 21.686
5α-Tetrahydro-B	564	17.963
5β-Tetrahydro-B	564	17.650
5β-Tetrahydro-A	521	17.834
Epi-Tetrahydro-B	564	17.407

Table 2.1: Mass/charge ratio of ion monitored and retention time of that ion for rat urinary glucocorticoid metabolites.

2.6 Enzymology

2.6.1 Tissue preparation

Tissues were routinely frozen 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 4 – 5ml of Kreb's Ringer bicarbonate buffer (KRB) in an Ystral mechanical homogeniser (Scientific Instruments Centre, Liverpool). Homogenates were centrifuged at 500g at 4°C for 5min to allow any small amounts of unhomogenised tissue to sink to the bottom, and the supernatant was removed. Liver microsomes were prepared by repeated centrifugation of the homogenised tissue. Homogenates were centrifuged at 14,000g at 4°C for 20min, and the supernatant was removed and centrifuged at 100,000g at 4°C for 60min. The microsomes appeared as a jelly-like pellet at the bottom of the tube, and were resuspended in 1ml KRB.

2.6.2 Protein estimation

Protein concentration of tissue homogenates and microsomal preparations was determined colorimetrically using a Bio-Rad protein assay kit (Bio-Rad, Hemel Hampstead, UK). A range of protein standards (0.1 – 1.2 mg/ml) was prepared in distilled water from the provided protein standard. Protein assay dye reagent was prepared by diluting the concentrate provided by 1 in 5 with distilled water and then filtering it through a Whatman No 1 filter paper. Diluted protein assay dye reagent (1.96ml) was added to 40µl of either protein standard or appropriately diluted sample in a borosilicate tube, vortexed and left at room temperature for 15 – 30min. The absorbance of the samples at λ=595nm was measured using a Shimadzu uv/visible recording spectrophotometer and the concentration of protein in each sample was estimated from the standard curve.

2.6.3 In vitro *assay of 11β-HSD activity*

In vivo, 11β-HSD1 is a reductase, converting inactive 11-dehydrocorticosterone to corticosterone. However, *in vitro* reductase activity is labile and dehydrogenase activity predominates in tissue homogenates, so 11β-HSD1 activity was quantified by conversion of corticosterone to 11-dehydrocorticosterone. Preliminary studies were carried out for each different tissue to optimise the protein concentration and incubation time to ensure that the percentage conversion was between 10 and 40% (ie not saturated). Aliquots of tissue homogenates at the appropriate concentration (see Table 2.2) were incubated in duplicate at 37°C in Kreb's Ringer bicarbonate buffer containing 0.2% glucose, NADP (2 mM) and [^{3}H]-corticosterone (50 nM) in a total volume of 250μl. Blanks were prepared by incubating [^{3}H]-corticosterone, NADP and buffer with no tissue added. After the incubation period the reaction was stopped by the addition of 10 volumes of ethyl acetate (all solvents used were HPLC grade). The organic phase was removed and evaporated under oxygen free nitrogen at 60°C. Extracts were re-suspended in mobile phase (20% methanol, 30% acetonitrile and 50% water) and stored at -20°C until analysis by high pressure liquid chromatography. The percentage of [^{3}H]-B converted to [^{3}H]-A was corrected for apparent conversion in the blank samples, which was always <3%, and was used as an index of 11β-HSD activity. Where results between different groups of animals were to be compared all the incubations were carried out concurrently, and all samples treated in the same way.

11β-HSD1 activity was assessed in human subcutaneous adipose samples in the same way, but incubations were carried out with [^{3}H]-cortisol, rather than [^{3}H]-corticosterone. The protein concentration and incubation period employed are detailed in Table 2.2.

Tissue	Protein concentration	Incubation period
Kidney	50μg/ml	60 min
Liver	10μg/ml	60 min
Liver microsomes	15μg/ml	10 min
Omental fat	1000μg/ml	60 min
Skeletal muscle	1500μg/ml	60 min
Subcutaneous fat	500μg/ml	60min
Thymus	500μg/ml	60 min
Human subcutaneous fat	750 μg/ml	Up to 30 hr

Table 2.2: Protein concentrations and incubation times employed in 11β-HSD activity assays.

To determine 11β-HSD2 activity in the kidney homogenates incubations were also performed with NAD (2 mM) as cofactor and 10nM [^{3}H]-B in a total volume of 1ml. The percentage conversion of [^{3}H]-B to [^{3}H]-A was corrected for apparent conversion in the blank samples, which was always <3%, and was used as an index of 11β-HSD activity. Where results were to be compared between groups of animals all incubations were carried out concurrently.

2.6.4 In vitro *assay of hepatic 11β-HSD1 kinetics*

To determine hepatic 11β-HSD1 kinetics liver microsomal preparations were incubated as for the 11β-HSD activity assay above, but with the addition unlabelled B (0.3 - 10μM). The percentage conversion of the [^{3}H]-B to [^{3}H]-A was calculated and this percentage conversion applied to the total amount of B present in the assay to give the total conversion of B to A in nmol / min / mg of protein (velocity of the reaction). These values

were used to construct a Lineweaver-Burke plot (1/velocity of reaction vs 1/substrate concentration) to allow calculation of the maximum velocity of the reaction (Vmax) and Michaelis constant of the reaction (concentration of substrate at half maximal velocity; Km).

2.6.5 High-pressure liquid chromatography

A 200µl aliquot of each sample in mobile phase (20% methanol, 30% acetonitrile and 50% water) was injected into the high-pressure liquid chromatography system (HPLC). The HPLC system comprises a Waters (Herts, UK) auto-injector, mobile phase pump, reverse phase µ-Bondapak C18 column in a column heater and a Berthold (Leeds, UK) radioactivity monitor linked to a scintillation fluid pump. The system is controlled by a computer loaded with Winflow software (JMBS Developments, France). The flow rate of the mobile phase was set at 1.5 ml/min and the flow rate of scintillant (Quicksafe Flow 2; Zinsser, Berks, UK) was set at 4.5 ml/min to achieve optimal mixing, and hence efficiency of counting. The column heater was set at 35°C to improve chromatography and maintain stability of retention times. Initially a Waters column (3.9 x 30mm) was used, but this was changed to a Phenomenex (Cheshire, UK) column to achieve better chromatography. Radioactive standards were injected at the start of every batch of samples to confirm the identity of the peaks. The retention time for A was approximately 6 min and for B approximately 8 min, with more than one minute of baseline between the two peaks. The peaks were always less than one minute wide, and the amount of radioactivity used ensured the peak height was at least 50x background. The area under each peak was integrated using the Winflow software, and the proportion of the area of each peak used to quantify the conversion of [^{3}H]-B to [^{3}H]-A, and hence 11β-HSD activity. Routinely blank incubations with no tissue were performed for each assay, and the conversion in each sample was corrected for the apparent conversion in the blank.

The same method was used to quantify [^{3}H]-cortisol and [^{3}H]-cortisone, except the mobile phase used was 25% methanol, 10% acetonitrile and 65% water. The retention time for cortisone was approximately 18 min and for cortisol approximately 20 min.

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

The enzyme 5α-reductase type 1 reduces most $\Delta^{4,5}$ unsaturated steroids with similar efficiency. Activity of the enzyme was quantified in liver homogenates by measuring the conversion of [^{3}H]-testosterone ([^{3}H]-T) to [^{3}H]-5α-dihydrotestosterone ([^{3}H]-5αDHT). Liver homogenates (protein 0.5mg/ml) were incubated in duplicate at 37°C in orthophosphate buffer (40 mM Na_2HPO_4, pH 7.5) with NADPH (1 mM) and [^{3}H]-testosterone (50 nM) in a total volume of 1ml for 10 min. The reaction was stopped by the addition of 10 volumes of HPLC grade ethyl acetate. The organic phase was removed and the ethyl acetate evaporated under oxygen free nitrogen at 60°C. The steroid extracts were re-suspended in ethanol with unlabelled testosterone and 5α-dihydrotestosterone, and separated by thin layer chromatography. The percentage of [^{3}H]-T converted to [^{3}H]-5α-DHT was used as an index of 5α-reductase activity. Where results between different groups of animals were to be compared all the incubations were carried out concurrently, and all samples treated in the same way.

2.6.7 Thin layer chromatography

The steroids were re-suspended in 100μl of ethanol containing 0.5mg/ml each of unlabelled T and 5α-DHT. It was necessary to add unlabelled T and 5α-DHT to enable the visualisation of steroids, as the radioactive steroids were of too low a concentration to be visualised. Polysilicic acid gel impregnated glass fibre sheets were divided into 2cm wide lanes and 40μl of sample was applied to each lane. The steroids were separated in

TLC tanks (mobile phase 9:1 dichloromethane:diethylether) for one hour. The steroid bands were identified using phosphomolybdic acid and were scraped into scintillation vials. The radioactivity in each steroid fraction was quantified by a β-counter using Cocktail T scintillant (BDH Ltd, Lutterworth, UK). The proportion of the radioactivity in each fraction was used to quantify conversion of [^{3}H]-T to [^{3}H]-5α-DHT, and hence 5α-reductase activity.

2.7 Northern analysis of RNA

2.7.1 Extraction of RNA

Tissues were dissected from the animals onto dry ice and stored at -80°C until used. Total RNA was extracted from tissues by the guanidium thiocyanate method of Chomczynski and Sacchi (Chomczynski & Sacchi, 1987). Approximately 100mg of liver samples were homogenised in 500μl denaturing solution using baked Dounce glass homogenisers, and the homogenate transferred to sterile Eppendorf tubes. To remove DNA and protein from the sample 0.2M sodium acetate (pH 4; 50μl), citrate-saturated phenol (500μl) and chloroform:isoamyl alcohol (49:1; 100μl) were added to the homogenate. The mixture was vortexed thoroughly, left on ice for 15min then centrifuged at 14,000g for 20min. The upper aqueous phase containing the RNA was transferred to new Eppendorfs and the lower layers containing DNA and protein were discarded. Isopropanol (200μl) was added to the aqueous phase, the sample left at -20°C for at least one hour to precipitate the RNA and then centrifuged at 14,000g for 20min to pellet the RNA. The supernatant was discarded, the pellet re-suspended in denaturing solution (60μl) and isopropanol (60μl), and the RNA precipitated and pelleted as before. The pellet was washed in 70% ethanol (200μl) and resuspended in DEPC treated water (50μl). The concentration and purity of the RNA were estimated spectrophotometrically by measuring the absorbance at λ=260nm and

280nm. RNA was only used with a Δ260/ Δ280 of between 1.5 and 1.8. RNA was stored at -80°C until used.

2.7.2 RNA electrophoresis and capillary transfer

Total RNA was separated by electrophoresis on a 1.2% agarose formaldehyde denaturing gel. A 100ml gel was prepared by melting 1.2g of agarose in 88ml DEPC-treated water, adding 40% formaldehyde (2ml) and 10x MOPS buffer (10ml), and pouring into a gel mould with appropriately sized combs in place. The gel was allowed to set and was then soaked in 1x MOPS buffer in a gel tank for 30min. RNA was prepared for electrophoresis by aliquoting 20μg of RNA, adding DEPC-treated water to bring the total volume to 10μl, then adding deionised formamide (10μl), 40% formaldehyde (2.5μl) and 10x MOPS buffer (2.5μl) to give a total volume of 25μl. The sample was mixed and the RNA denatured by incubating at 65°C for 15min. Ethidium bromide was added to the loading buffer (1μl per 50μl loading buffer) and 2μl of this mix added to each sample of denatured RNA. The RNA was loaded into the wells on the gel and electrophoresed at 100V for 2 – 4 hours until the front band of the loading buffer was ¾ of the way down the gel. The gel was photographed under UV light (λ=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 Hampstead, 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 the top. A piece of nylon membrane cut to the same size as the gel and pre-wetted in 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 plate of glass 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 transfer was checked by photographing the gel and the membrane under UV light (λ=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. The membranes were stored wrapped in cling-film between two pieces of 3MM paper.

2.7.3 Hybridisation to ^{32}P-labelled cDNA

The nylon membrane was soaked in 20x SSC buffer for 5min, then placed in Hybaid hybridisation bottle containing 3ml 20% SDS and 6ml phosphate buffer that had been warmed to 55°C. To this was added denatured herring testes DNA (10mg/ml; 100µl). The membrane was prehybridised in a Hybaid hybridisation oven at 55°C for at least 2 hours. The ^{32}P-labelled cDNA probe (prepared as described in section 2.8.4) was added to the prehybridisation 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 two washes in wash buffer one at room temperature for 20min, and one wash in wash buffer two at 55°C for 30min. The level of radioactive signal remaining on the membrane was checked, and if this was too high the final wash was repeated. The membrane was wrapped in cling-film and exposed to a Fujifilm imaging screen for six hours and the level of hybridized probe quantified using a Fuji FLA2000 fluorescent image analyzer. The membranes were then rehybridised with glyceraldehydephosphate dehydrogenase (GAPDH) cDNA in the same way to control for RNA loading and transfer. The level of expression of the RNA of interest was then expressed as a ratio of the signal of the RNA of interest to the signal for GAPDH. Membranes were routinely left for at least 4 weeks before reprobing, to allow the original signal to decay, and all the mRNA species probed for were well separated on the membrane. If results between groups of animals were to be compared all the samples were hybridised in the same hybridisation bottle with the same probe.

2.8 Preparation of ^{32}P labelled cDNA probes

2.8.1 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 3000rpm 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 (100µl) were mixed with plasmid DNA (50ng) and left on ice in the fridge for 20min. The cells were heat shocked at 40°C for one minute and placed back on ice. The heat shock and the calcium chloride lead to incorporation of the plasmid DNA into the cells. The cells were spread onto LB agar plates containing 100µg/ml ampicillin, and the plates incubated overnight at 37°C. Only cells that have incorporated the plasmid DNA will grow on the ampicillin plates, as HB101 cells do not have inherent ampicillin resistance.

2.8.2 Plasmid DNA preparation

A single transformed bacterial colony was selected from an agar plate and incubated overnight at 37°C in 3ml LB containing 100µg/ml ampicillin. This was then added to 500ml LB containing 100µg/ml ampicillin and incubated overnight at 37°C. The culture was centrifuged at 6,000rpm for 5min at 4°C in a Beckman J14 centrifuge, and the supernatant discarded. The cell pellet was resuspended in 12ml cold GTE buffer, 24ml of cold alkaline SDS was added and the mixture left on ice for 10min. Cold potassium acetate (16ml) was added and the mixture left on ice for a

further 10min before being centrifuged at 6,000rpm for 5min at 4°C in a Beckman J14 centrifuge. The mixture was filtered through 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 10,000rpm for 3min at 4°C in a Beckman J20 centrifuge, and the supernatant discarded. The DNA pellet was resuspended in 2.2ml TE buffer, 2.9g CsCl added and dissolved and 100µl ethidium bromide (10mg/ml) added. The mixture was transferred to Beckman Quickseal ultracentrifuge tubes, tubes topped up with CsCl/TE solution and centrifuged at 70,000rpm 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 21-gauge needle and syringe, transferred to fresh ultracentrifuge tubes, topped up with CsCl/TE solution and centrifuged at 100,000rpm for 4 hours at 20°C. The DNA bands were collected as above and the ethidium bromide was removed by extracting repeatedly with water saturated butanol until the pink colour disappeared. The DNA was precipitated with ethanol (2 volumes) for 10min at room temperature and pelleted by centrifugation for 5min at 14,000g. The supernatant was discarded and the DNA pellet washed with 70% ethanol then resuspended in 1ml TE buffer. The concentration and purity of the DNA was assessed spectrophotometrically using the ratio of absorbances at λ=260 and 280nm.

2.8.3 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 (see Table 2.3). Digestion of the DNA was confirmed by electrophoresis of 3µl of the digest through a 1.2% agarose gel (prepared by dissolving 1.2% agarose in 1x TBE buffer and adding 1µl ethidium bromide (10mg/ml)). The digest was compared with uncut plasmid and a

1kB DNA ladder containing fragments ranging from 75bp – 12kb under UV light at λ=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 (λ=254nm), excised from the gel using a scalpel, purified from the gel using a commercially available kit (Hybaid Recovery DNA Purification kit; Hybaid, Ashford, Middlesex) and resuspended in 50μl DNAase free/RNAase free water. Recovery of the DNA fragment was assessed by electrophoresis of 1μl of the DNA solution through a 1.2% agarose gel as described above.

Fragment	Restriction Enzyme(s)
11β-HSD1	EcoR 1
Tyrosine aminotransferase (TAT)	EcoR 1 and BamH 1
Phosphoenolpyruvate carboxykinase (PEPCK)	EcoR 1
Glyceraldehydephosphate dehydrogenase (GAPDH)	EcoR 1 and Xba 1

Table 2.3 Restriction enzymes used to cut plasmid DNA.

2.8.4 [^{32}P] Labelling of cDNA

A random primed DNA labeling kit was used to label the DNA fragments (Boehringer Mannheim, Lewes, UK). Approximately 25ng of DNA fragment was aliquoted into an Eppendorf, made up to 10μl with DEPC-treated water and denatured at 100°C for 10min. The Eppendorf was

cooled on ice and briefly centrifuged to bring the contents to the bottom of the Eppendorf. Hexanucleotide primer mix (2μl), dATP, dTTP and dGTP (1μl of each), [α^{32}P]-dCTP (4μl) and Klenow (1μl) were added to give a total reaction volume of 20μl and the reaction incubated at 37°C for one hour. Unincorporated radioactivity was removed by passing the mixture over a NICK column (Pharmacia, St.Albans, UK). The NICK column was prepared by washing with 3ml TE then the reaction mixture was applied to the column. The column was washed with 400μl TE, the eluant discarded and the labelled DNA eluted from the column with a further 400μl TE. The activity of the probe was checked by mixing 1μl of probe with 1ml of Cocktail T scintillant and counting in a β-counter. The probe was used if the specific activity was greater than 10,000cpm/μl. The DNA probe was denatured before use by heating to 100°C for 5min.

2.9 Statistics

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

Chapter Three

Glucocorticoid Metabolism in Obese Zucker Rats

3.1 Introduction

Increased secretion of glucocorticoids (eg in Cushing's disease) is associated with obesity; and increased cortisol secretion has been noted in subjects with idiopathic obesity, especially if the obesity is of central distribution (Pasquali *et al.*, 1993). However, the increase in cortisol secretion in idiopathic obesity is not accompanied by an elevation in peak plasma cortisol levels (Rosmond *et al.*, 1998), suggesting that peripheral cortisol metabolism may be enhanced in these subjects. It has been suggested that, in the face of increased clearance of cortisol, obesity may be exaggerated because of increased local reactivation of inactive cortisone to cortisol in adipose tissue by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Bujalska *et al.*, 1997; Andrew *et al.*, 1998). Increased reactivation of cortisol in adipose tissue would be predicted to increase the local concentration of cortisol, independently of circulating cortisol levels, and increase glucocorticoid receptor activation, thus amplifying the actions of glucocorticoids and promoting fat deposition.

As discussed in chapter 1, the obese Zucker rat has been used as an animal model of obesity and non-insulin dependent diabetes for many years (Zucker & Zucker, 1961). The primary abnormality in the obese Zucker rat has been identified as a mutation in the leptin receptor (Chua *et al.*, 1996), which renders these animals resistant to the satiety factor. The obesity in these animals has been shown to be glucocorticoid dependent, as adrenalectomy or treatment with glucocorticoid receptor antagonists attenuate weight gain and associated metabolic abnormalities (Yukimura *et al.*, 1978; Freedman *et al.*, 1986; Castonguay *et al.*, 1986; Langley & York, 1990). The hypothalamic-pituitary-adrenal (HPA) axis has been studied in the obese Zucker rat, with conflicting reports of normal (Yukimura *et al.*, 1978; Cunningham *et al.*, 1986; Walker *et al.*, 1992) and elevated (Dubuc, 1976; Martin *et al.*, 1978; Fletcher *et al.*, 1986;

Guillaume-Gentil *et al.*, 1990; Plotsky *et al.*, 1992; Dryden *et al.*, 1995) plasma corticosterone levels; normal (Cunningham *et al.*, 1986; Guillaume-Gentil *et al.*, 1990) and decreased (Walker *et al.*, 1992) sensitivity to negative feedback}; and normal (Plotsky *et al.*, 1992; Walker *et al.*, 1992) and increased (Bestetti *et al.*, 1990; Guillaume-Gentil *et al.*, 1990; Pacak *et al.*, 1995) pituitary and adrenal responses to stress. Glucocorticoid availability to its receptors is also controlled by metabolic clearance, and at a tissue level by the isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD), as discussed in chapter 1. We have examined glucocorticoid metabolising enzymes in the obese Zucker rat, specifically reactivation of corticosterone by 11β-HSD1 and inactivation of corticosterone by 11β-HSD2 and 5α-reductase. We have also looked at the expression of hepatic glucocorticoid regulated genes, to assess the effect of enzyme changes on glucocorticoid action.

3.2 Methods

Five-week old male lean (*Fa/?*) or obese (*fa/fa*) Zucker rats were obtained from Harlan Orlac, Bicester, and were placed in metabolic cages at eight weeks of age. Animals were allowed to acclimatise to the metabolic cages for one week before a 72-hour urine collection. At nine-weeks of age animals were killed by decapitation at 0900-1100h. Trunk blood was collected and tissues of interest were dissected and either snap-frozen on dry ice or were mechanically homogenised in Krebs Ringer buffer.

Corticosterone and 11-dehydrocorticosterone were measured by radioimmunoassay in plasma prepared from trunk blood samples, and urinary corticosterone metabolites were assessed by GC-MS. 11β-HSD and 5α-reductase activity were estimated by conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone or [^{3}H]-testosterone to [^{3}H]-dihydro-testosterone respectively. mRNA was quantified by Northern blot.

All data are expressed as mean ± standard error. Parametric data were analysed by Student's t-tests and non-parametric data were analysed by Mann-Whitney U tests. N=8 for both groups.

3.3 Results

3.3.1 Body and organ weights

As expected, obese Zucker rats were significantly heavier than lean Fa/? control animals (Table 3.1). In concordance with previous reports they also had heavier adrenal glands, although this difference was not significant when corrected for body weight (Walker *et al.*, 1992). The thymus was also heavier in obese animals, but again this difference was not significant when corrected for body weight.

	Lean (n=8)	Obese (n=8)	P value
Body weight at 9 weeks (g)	226 ± 7	280 ± 6	<0.01
Adrenal weight (mg)	38 ± 2	54 ± 7	0.04
Adrenal / body weight	0.17 ± 0.01	0.20 ± 0.03	0.75
Thymus weight (mg)	457 ± 23	593 ± 37	<0.01
Thymus weight/body weight	2.03 ± 0.09	2.12 ± 0.12	0.66

Table 3.1: Body and organ weights of lean (*Fa/?*) and obese (*fa/fa*) Zucker rats aged 9-weeks.
Data are mean ± SEM. P values are from Student's t-tests.

3.3.2 Plasma glucocorticoid levels

Plasma concentrations of both corticosterone (B) and its inactive metabolite 11-dehydrocorticosterone (A) were significantly elevated in obese animals when compared to lean controls (Figure 3.1).

3.3.3 Urinary glucocorticoid metabolite excretion

The glucocorticoid metabolites measured are shown in Figure 3.2. Obese animals had higher total urinary corticosterone metabolites when compared with lean controls (Figure 3.3). Obese rats exhibited a larger difference in excretion of metabolites of A (greater than 10 fold higher) than of B (3-5 fold higher), and had significantly higher 5α-reduced B (5α-tetrahydro-B; 5α-THB) but not 5β-reduced B (5β-tetrahydro-B; 5β-THB). B was not detected in any of the samples due to technical problems with sensitivity of the GC-MS and an inherently higher limit of detection because of the fragmentation pattern of B.

3.3.4 Glucocorticoid metabolising enzymes[1]

In liver of obese animals 5α-reductase type 1 activity was significantly higher than in lean controls (Figure 3.4). By contrast, hepatic 11β-HSD1 activity, and mRNA expression were significantly lower in obese rats (Figure 3.5). However, 11β-HSD1 activity was not different between groups in skeletal muscle or subcutaneous fat, and was actually higher in omental fat from obese animals (Figure 3.6). Both NADP-dependent (11β-HSD1) and NAD-dependent (11β-HSD2) 11β-HSD activities in the kidney were higher in obese rats than in lean controls (Figure 3.6).

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[1] Measurement of 5α-reductase activity was carried out by Dr Greg Jones, Department of Medical Sciences, University of Edinburgh.

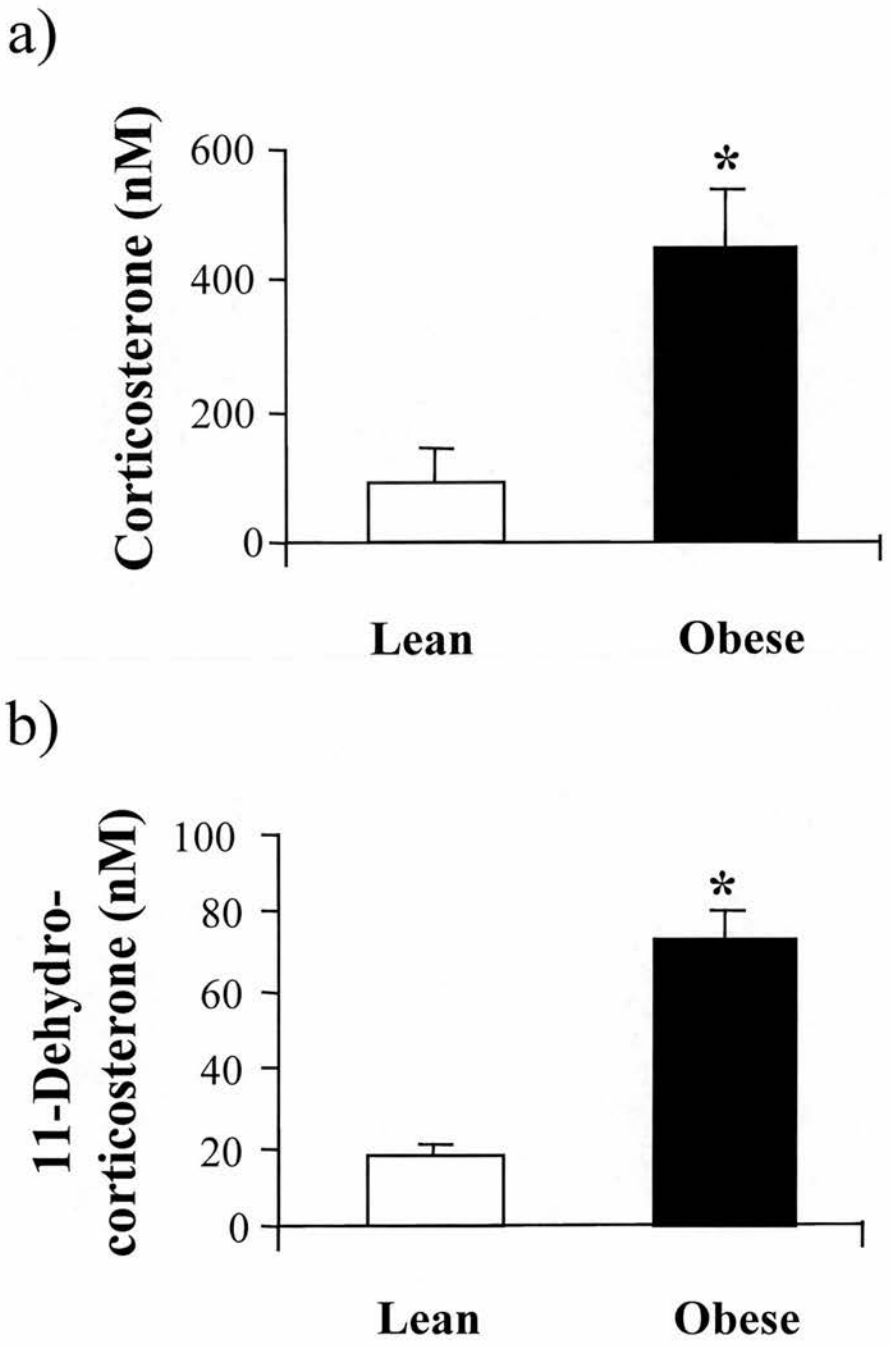

Figure 3.1: Plasma glucocorticoid levels.

Panel a) shows corticosterone and panel b) shows 11-dehydrocorticosterone levels measured in plasma prepared from trunk blood samples.

* denotes $p < 0.05$ from Student's t-tests.

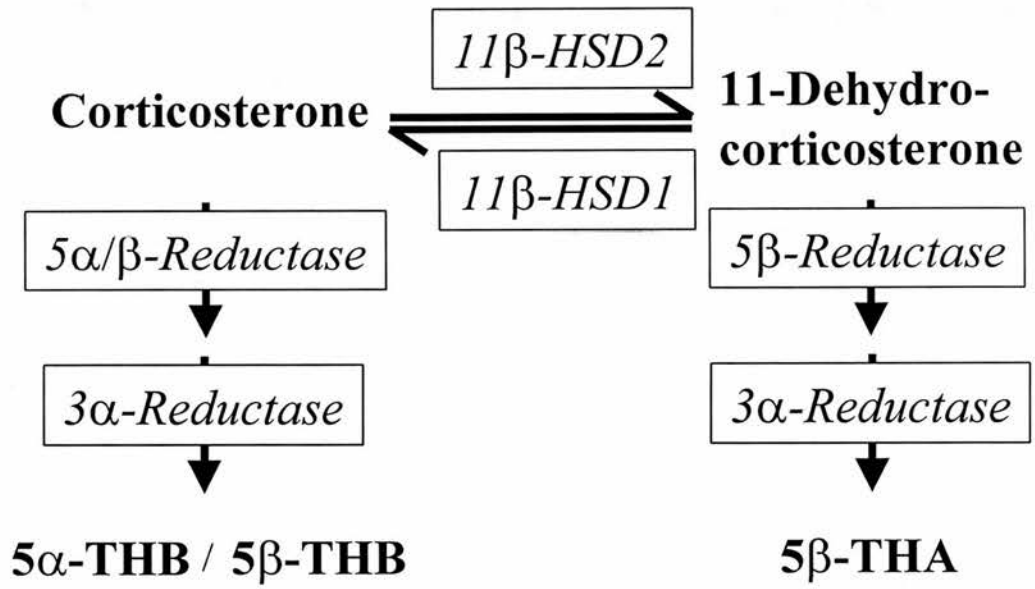

Figure 3.2: Corticosterone metabolites.

Metabolic enzymes are shown in the boxes.

THB = tetrahydrocorticosterone,

THA = tetrahydro-11-dehydrocorticosterone

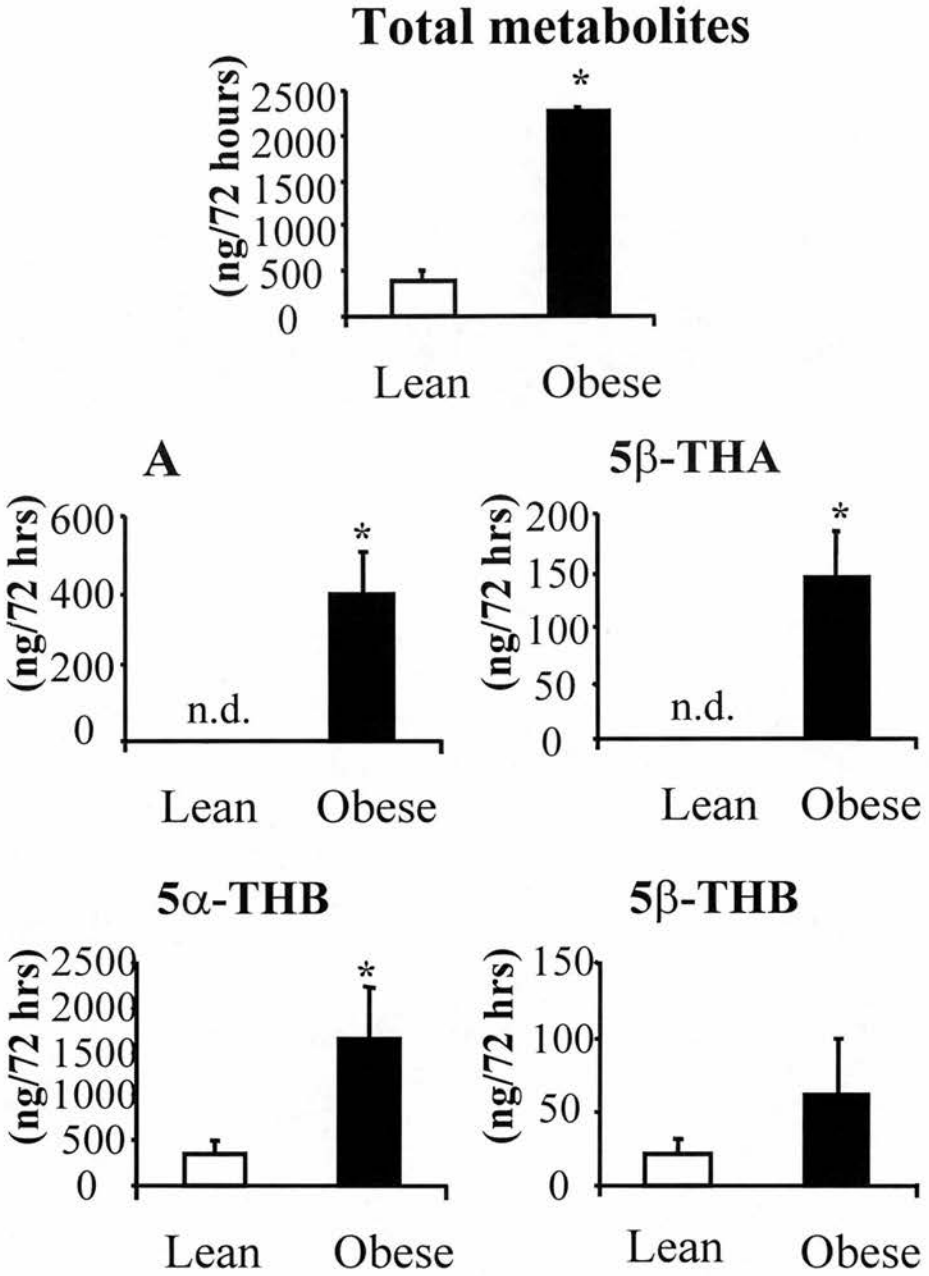

Figure 3.3: Urinary corticosterone metabolites.

Glucocorticoid metabolites were measured by GC-MS in urine collected over a 72h period.

A=11-dehydrocorticosterone; B=corticosterone; THA=tetrahydroA; 5α-THB=5α-tetrahydroB and 5β-THB=5β-tetrahydroB. nd = not detected

Limits of detection were 14ng/72h for THA, and 28ng/72h for A.

* denotes $p < 0.05$ from Mann Whitney U tests.

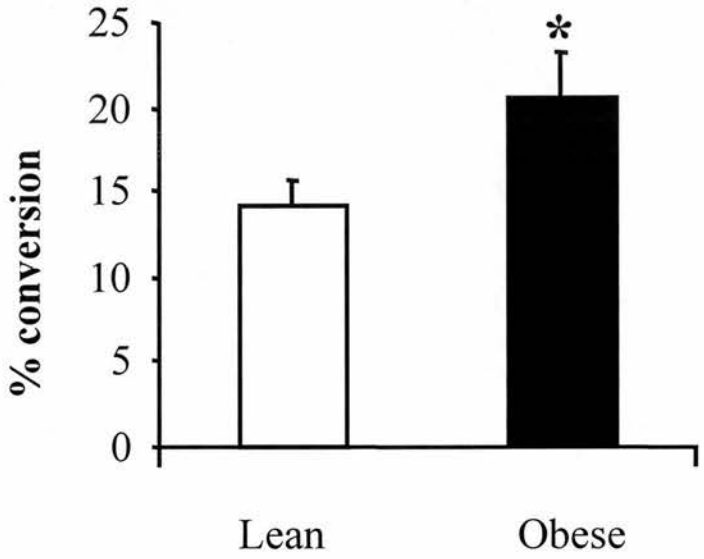

Figure 3.4: 5α-reductase activity.

5α-reductase activity is expressed as percent conversion of testosterone to 5α-dihydrotestosterone.

* denotes $p<0.05$ from Student's t-tests.

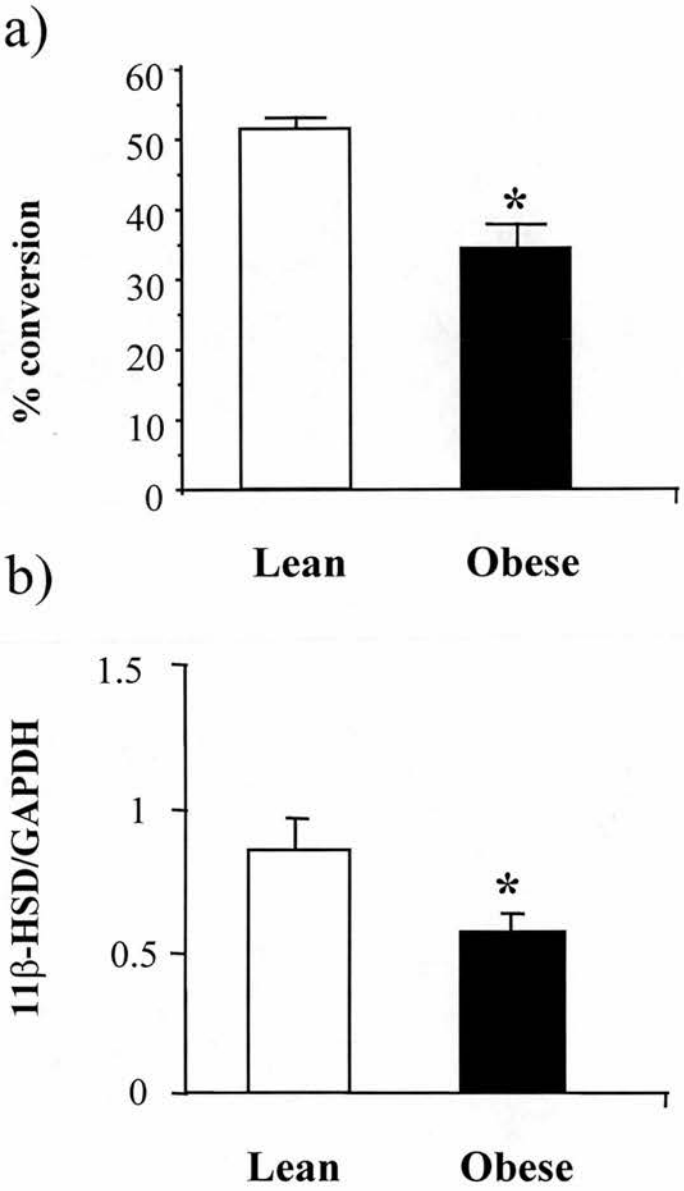

Figure 3.5: Hepatic 11β-HSD activity and expression.

Panel a) shows hepatic 11β-HSD1 activity, measured in tissue homogenates, expressed as percent conversion of corticosterone to 11-dehydrocorticosterone. Panel b) shows hepatic 11βHSD1 mRNA levels measured by Northern blot, expressed as a ratio of the 11β-HSD1 signal to the GAPDH signal.

* denotes $p<0.05$ from Mann Whitney U tests.

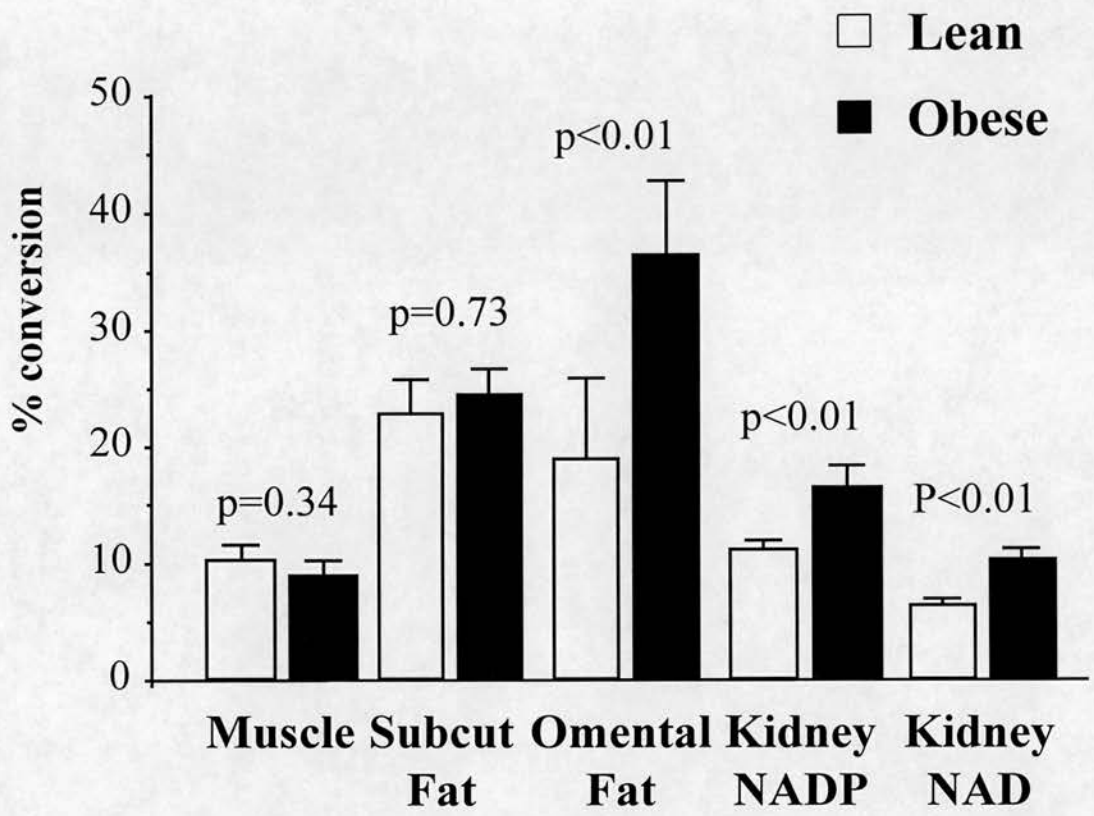

Figure 3.6: 11β-HSD activity in a range of tissues.

11β-HSD activity is expressed as percent conversion of corticosterone to 11-dehydrocorticosterone. All incubations were carried out in the presence of NADP cofactor, except where indicated for kidney when the incubations were carried out with NAD cofactor.

p-values are from Mann Whitney U tests.

3.3.5 Hepatic glucocorticoid regulated gene expression

Hepatic phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression was not different between lean and obese animals (Figure 3.7). In contrast, tyrosine aminotransferase (TAT) expression was significantly higher in livers of obese than lean animals (Figure 3.7).

3.3 Discussion

These data confirm previous reports that obese Zucker rats have heavier adrenal glands (Walker *et al.*, 1992) and increased urinary corticosterone secretion (Cunningham *et al.*, 1986) compared with lean controls. This is the first study to examine excretion of urinary corticosterone metabolites, and link this to tissue enzyme activities. It is also the first study to demonstrate tissue-specific differences in the peripheral metabolism of corticosterone in obese Zucker rats compared with lean controls.

Both adrenal weight and total urinary corticosterone secretion are increased in the obese Zucker rat suggesting chronic activation of the HPA axis, as has been reported in obese humans (Weaver *et al.*, 1993). Adrenal weight is not different between groups when corrected for body weight, but whether merely correcting for body weight in these animals is appropriate is a matter for debate, as the body composition is so different between the lean and obese animals. One possible approach would be to correct organ weight for lean body mass, but unfortunately we were unable to assess this in these animals.

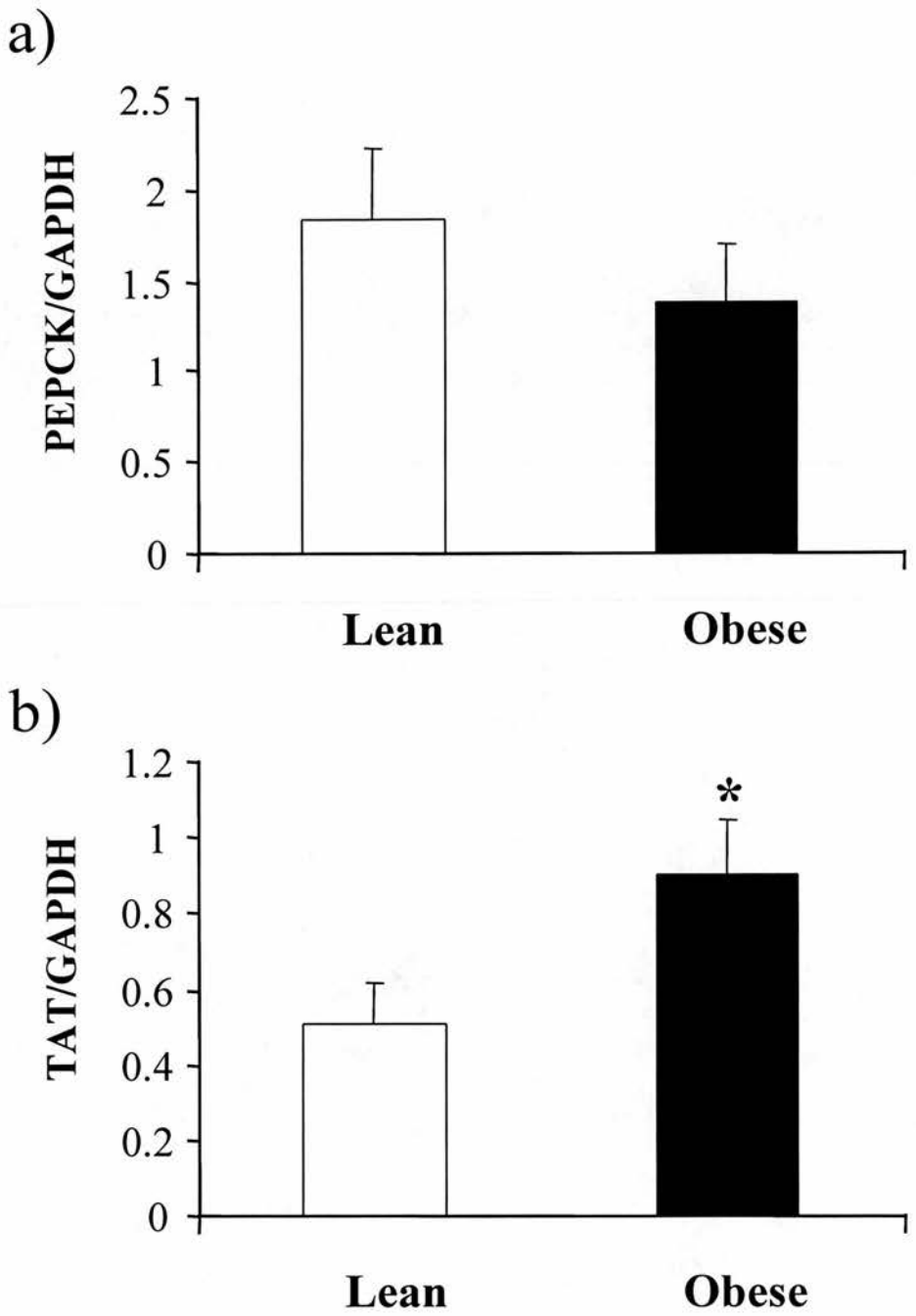

Figure 3.7: Hepatic glucocorticoid regulated gene expression.

Panel a) shows hepatic PEPCK mRNA expression, and panel b) shows hepatic TAT mRNA expression, measured by Northern blot, and expresses as a ratio of the signal of interest to the signal for GAPDH.

* denotes $p<0.05$ from Mann Whitney U tests.

Plasma corticosterone (B) levels were significantly higher in obese rats than in lean controls. Plasma 11-dehydrocorticosterone (A) levels were also higher in obese rats, and this could have important implications. The role of 11β-HSD1 in amplifying glucocorticoid action is dependent on the presence of A, the substrate for the enzyme. The plasma A and B levels presented are total levels, and this does not give a true representation of the free steroid levels. B circulates predominantly bound to proteins, such as corticosteroid-binding-globulin, whereas A is largely unbound, resulting in free A levels that are much higher than free B levels. Thus, variations in A levels could have an impact on tissue glucocorticoid levels by providing substrate for 11β-HSD1 to convert into active B. Therefore, 11β-HSD1 activity and A availability could have a role to play in governing intracellular glucocorticoid levels, independently of circulating B.

The rat liver only expresses the type 1 isozyme of 5α-reductase, which reduces most $\Delta^{4,5}$ unsaturated steroids with similar efficiency (Berman & Russell, 1993). The enzyme assay employed monitored the 5α-reduction of testosterone, but because of the properties of the enzyme this can be used as an estimation of glucocorticoid 5α-reduction. Liver homogenates from obese rats exhibited a higher degree of 5α-reduction of testosterone than liver from lean rats, indicating an increased activity of 5α-reductase, which would be predicted to increase metabolic clearance of B to the inactive 5α-tetrahydroB. When urinary glucocorticoid metabolites were measured this was found to be the case, as obese rats excreted significantly more 5α-tetrahydroB than lean rats.

In contrast with the increased 5α-reductase activity, *in vitro* hepatic 11β-HSD activity was lower in the obese rat than in lean animals, and this was accompanied by lower 11β-HSD1 mRNA levels. Lower 11β-HSD1 activity *in vivo* would be predicted to reduce intracellular glucocorticoid levels, as there would be less reactivation of B from A. This would therefore have the same net effect as higher 5α-reductase activity, as both

would lead to lower tissue levels of active glucocorticoids. However, lower 11β-HSD1 levels were not found in all tissues in the obese Zucker rat. There was no difference in 11β-HSD1 activity between groups in either skeletal muscle or subcutaneous adipose tissue, and indeed 11β-HSD1 activity was actually higher in omental adipose from obese rats compared with controls, which could indicate enhancement of glucocorticoid action in this tissue. 11β-HSD activity was higher *in vitro* in the kidney of obese Zucker rats, suggesting enhanced inactivation of corticosterone *in vivo* to its inert metabolite 11-dehydro-corticosterone. The urinary glucocorticoid metabolite profile indicates an alteration in the balance of 'whole body' 11β-HSDs, favouring 11-dehydro-corticosterone in obese Zucker rats. This suggests that the decreased hepatic 11β-HSD1 activity and increased renal 11β-HSD2 activity predominate over changes in 11β-HSD activity in other tissues.

The combination of increased 5α-reductase, increased renal 11β-HSD2 and decreased hepatic 11β-HSD1 activities together predict an increase in the glucocorticoid metabolic clearance rate, which in normal circumstances would result in a compensatory increase in corticosterone production (Walker & Edwards, 1994a). This mechanism could contribute to activation of the HPA and adrenocortical hypertrophy in obesity. However, it does not explain why trough plasma corticosterone levels are elevated in obese rats. Abnormalities of central control of the HPA have been sought previously in these animals but responses to glucocorticoid feedback and stressful stimuli have been variably reported as normal, increased or decreased (Guillaume-Gentil *et al.*, 1990; Plotsky *et al.*, 1992; Bestetti *et al.*, 1990). Moreover, it remains unclear whether leptin resistance alone could explain activation of the HPA, since there may be opposing effects of increased neuropeptide Y (NPY) and decreased pro-opiomelanocortin (POMC) expression (Schwartz *et al.*, 1996b; Inoue, 1988; Thornton *et al.*, 1997). One possible explanation for central HPA activation is that obese rats have similarly reduced 11β-HSD1 expression

in sites responsible for glucocorticoid negative feedback in the brain (hippocampus, hypothalamus and anterior pituitary). Reduced 11β-HSD1 activity would be predicted to decrease intracellular glucocorticoid levels, and would thus reduce the brain's impression of circulating glucocorticoid levels. This would decrease negative feedback, and cause activation of the HPA. In support of this theory, mice with transgenic disruption of the 11β-HSD1 gene (Kotelevtsev *et al.*, 1997) have increased plasma corticosterone levels which have been attributed to a lack of 11β-HSD1 in sites responsible for negative feedback.

The observed changes in glucocorticoid metabolism also predict changes in peripheral corticosteroid receptor activation. Enhanced inactivation (by 5α-reductase) and impaired reactivation (by 11β-HSD1) of glucocorticoids in the liver predicts lower intracellular corticosterone concentrations. Decreased glucocorticoid exposure would normally be associated with enhanced insulin sensitivity and decreased gluconeogenesis, as in 11β-HSD1 knockout mice (Kotelevtsev *et al.*, 1997), so it may be that reduced hepatic glucocorticoid exposure in the obese Zucker rat represents a compensatory mechanism which limits the metabolic complications of obesity. By contrast, 11β-HSD1 activity was normal in other glucocorticoid targets (skeletal muscle and subcutaneous adipose tissue) in obese rats so the proposed compensatory mechanism may not operate in all tissues. Moreover, 11β-HSD1 activity was elevated in omental fat in obese rats and this predicts increased local glucocorticoid receptor activation, and may promote obesity. In addition to the higher 11β-HSD1 activity in omental fat there is also an increase in the availability of the substrate for reactivation in obese animals (ie A), which could further increase local corticosterone levels and obesity.

An interesting anomaly to the activated HPA is the fact that obese Zucker rats had heavier thymuses. The thymus is an organ that undergoes glucocorticoid-induced regression, and thus lower thymus mass would

normally be associated with higher circulating glucocorticoid levels. Obese Zucker rats actually had significantly heavier thymuses in the face of much higher plasma glucocorticoid levels. This emphasises that circulating plasma levels of glucocorticoids are not necessarily indicative of tissue glucocorticoid levels. Thymus mass was not significantly different between groups when corrected for body weight, but again, the validity of correcting thymus weight for body weight is debatable.

The expression of hepatic glucocorticoid regulated genes was also examined in obese Zucker rats to get an impression of the net effect of the higher plasma corticosterone and the alterations in metabolism of corticosterone on glucocorticoid action. Phosphoenolpyruvate carboxykinase (PEPCK) is the rate-limiting enzyme in the gluconeogenic pathway. Expression of PEPCK is up-regulated in response to glucocorticoids and down-regulated in response to insulin (Sasaki *et al.*, 1984), both of which are altered in obese animals. There was no difference in PEPCK mRNA levels between obese and lean animals, and there are several possible explanations for this. It is well established that obese Zucker rats are insulin resistant, and may therefore have defective insulin mediated down-regulation of PEPCK, ie increased PEPCK expression. Thus, any decrease in glucocorticoid action could merely negate the increase in PEPCK due to insulin resistance, leading to no net change in PEPCK. Alternatively, PEPCK expression is affected by the feeding state of the animal, with subtle differences being revealed by fasting in prenatally programmed rats and 11β-HSD1 knockout mice (Nyirenda *et al.*, 1998; Kotelevtsev *et al.*, 1997). Since the animals in this study were not fasted, it is possible that subtle alterations in PEPCK expression could have been overlooked.

Tyrosine amino transferase (TAT) converts amino acids into substrates for gluconeogenesis, and is central to glucocorticoid-induced gluconeogenesis. TAT is up-regulated by glucocorticoids (Omrani *et al.*, 1980), but

regulation by insulin seems to be transient (Krett *et al.*, 1983). Hepatic TAT mRNA levels were higher in obese rats than in lean controls, indicating that these animals had a higher level of glucocorticoid receptor activation. This does not discount the above theory that the change in hepatic 11β-HSD1 is compensatory, and acts to limit the metabolic complications of obesity. It may be that the lower hepatic 11β-HSD1 in the obese Zucker rat is compensatory, but cannot completely negate the greatly higher plasma B levels, and without lower 11β-HSD the animals would display even more striking metabolic abnormalities.

The effect of altered 11β-HSD1 activity on glucocorticoid action in omental fat will obviously be a key observation. This could be examined in the same way as for liver, ie examining the expression of glucocorticoid regulated genes. Unfortunately, due to time constraints, this issue has not been addressed in this thesis.

The mechanism of dysregulation of 5α-reductase and 11β-HSD activities in obesity has not been addressed by this study. With respect to 11β-HSD1 we have shown that hepatic mRNA levels are reduced, suggesting that gene transcription is altered in obese animals. It is unlikely that the altered 11β-HSD activities are secondary to activation of the HPA, since chronic glucocorticoid excess is associated with up-regulation (not down-regulation) of hepatic 11β-HSD1 activity and expression (Low *et al.*, 1994b). 11β-HSD1 is regulated by many other factors, including some which are altered in obesity, such as growth hormone, insulin, TNFα, and gonadal steroids (Low *et al.*, 1994a; Hammami & Siteri, 1991; Escher *et al.*, 1997). By contrast, 5α-Reductase type 1 and 11β-HSD type 2 do not appear to be highly regulated enzymes. Further studies will be required to try and elucidate the mechanism behind altered peripheral glucocorticoid metabolism in obesity.

Finally, the differences in metabolism of glucocorticoids in obese Zucker rats mirror observations that have been made in obese humans. It has been reported by our research group and others that obese men and women excrete more cortisol as 5α-reduced metabolites (Andrew *et al.*, 1998; Fraser *et al.*, 1999). Detailed dynamic tests and arteriovenous sampling have shown that hepatic 11β-HSD1 activity is impaired in obese men (Stewart *et al.*, 1999) but adipose 11β-HSD1 is either maintained or marginally increased in obesity (Katz *et al.*, 1999). The results presented in this chapter demonstrate that the obese Zucker rat will be a useful model in which to explore the mechanisms of disrupted glucocorticoid metabolism, its impact on body weight regulation, and its potential for therapeutic manipulation.

Chapter Four

Effects of Adrenalectomy of Obese Zucker Rats

4.1 Introduction

The results presented in the previous chapter describe alterations of peripheral glucocorticoid metabolism in the obese Zucker rat that are analogous to those described in human obesity (Andrew *et al.*, 1998; Fraser *et al.*, 1999; Stewart *et al.*, 1999).

The aim of this study was to gain insight into the possible mechanisms of dysregulation of glucocorticoid metabolism by 11β-HSD1 in obese Zucker rats. Two important regulators of 11β-HSD1 are glucocorticoids, which are associated with chronic up-regulation of both activity and expression (Low *et al.*, 1994b) and insulin, which down-regulates 11β-HSD1 expression (Hammami & Siiteri, 1991; Jamieson *et al.*, 1995). As discussed previously, glucocorticoids are thought to be pivotal to the phenotype of the obese Zucker rat. To investigate whether glucocorticoids have a role in the alterations of glucocorticoid metabolism observed in the obese Zucker rat we assessed the effect of adrenalectomy on 11β-HSD1 activity in lean and obese Zucker rats.

4.2 Methods

Groups of eight 6-week old male obese and lean Zucker rats were either adrenalectomised or sham-operated through dorsal incisions under halothane anaesthesia and maintained on 0.9% saline drinking water for three weeks. At age 9-weeks animals were decapitated at 0900-1100h, trunk blood collected, and tissues dissected and either snap-frozen on dry ice or mechanically homogenised in Krebs Ringer buffer.

Corticosterone was measured by radioimmunoassay in plasma prepared from trunk blood. 11β-HSD activity was estimated by conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone, and hepatic enzyme kinetics were determined by assessing enzyme activity at a range of

substrate concentrations. Levels of mRNA were assessed by northern blot hybridisation.

All data are expressed as mean ± standard error. Parametric data were analysed by Student's t-test for paired data or Analysis of Variance followed by post hoc tests (ANOVA). Non-parametric data were analysed by Mann-Whitney U tests. N=8 for all groups except lean adrenalectomised group where n=7.

4.3 Results

4.3.1 Body and organ weights

Obese sham-operated (sham) Zucker rats were heavier than lean sham-operated animals at age 9-weeks (Figure 4.1), and adrenalectomy was associated with lower body weight in both lean and obese animals. The amount of weight gained by each animal in the three-week period following surgery was also compared. Obese sham animals gained more weight than lean sham animals (Figure 4.1), and this difference was attenuated by adrenalectomy. Adrenalectomy reduced weight gain in both lean and obese animals.

Obese sham rats had heavier adrenal glands than lean animals (Figure 4.2), but this was not significant when corrected for body weight. Adrenal glands were not found postmortem in any of the adrenalectomised animals. Thymus weight was also greater in obese sham operated animals when compared with lean (Figure 4.3), but not if corrected for body weight, and the same differences were observed in adrenalectomised animals. Adrenalectomy did not affect absolute thymus weight, but thymus weight corrected for body weight was greater in adrenalectomised animals.

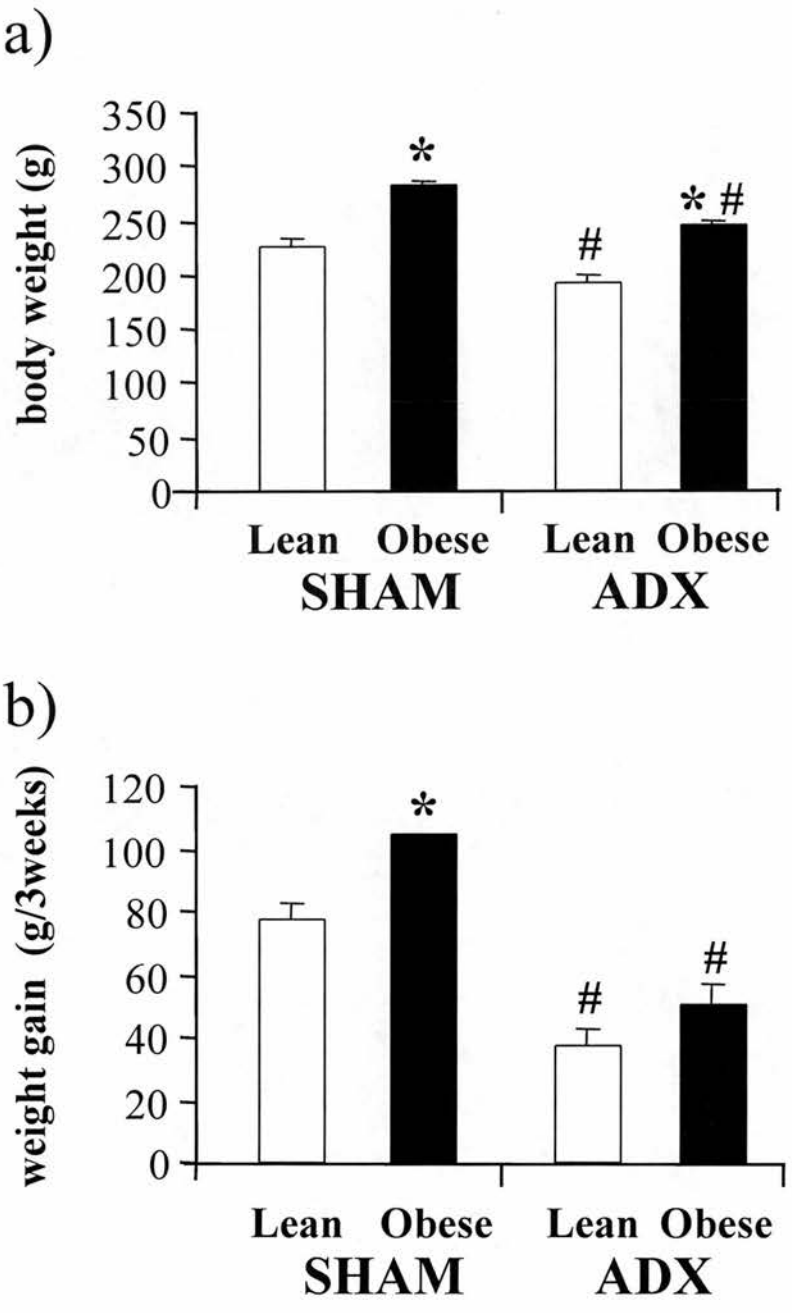

Figure 4.1: Body weights and weight gain.

Panel a) shows body weight at 9-weeks of age and panel b) shows weight gained in the 3-week period following surgery.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same surgery group. # denotes $p<0.05$ compared with sham-operated animals of the same phenotype.

Sham denotes sham-operated, and ADX denotes adrenalectomised, animals.

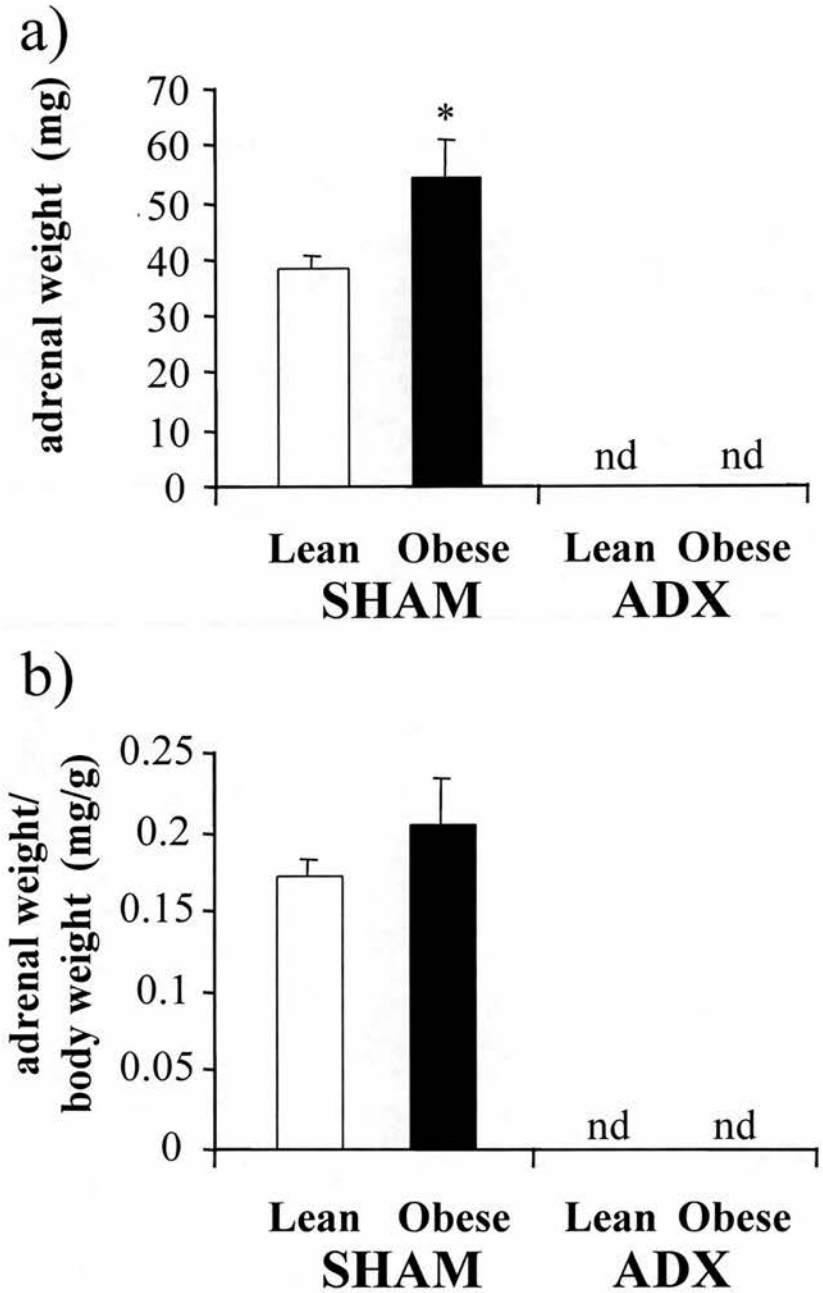

Figure 4.2: Adrenal weights.

Panel a) shows adrenal weight at 9-weeks of age and panel b) shows adrenal weight corrected for body weight.

* denotes $p<0.05$ from Student's t-test. nd denotes not detected.

Sham denotes sham-operated, and ADX denotes adrenalectomised, animals.

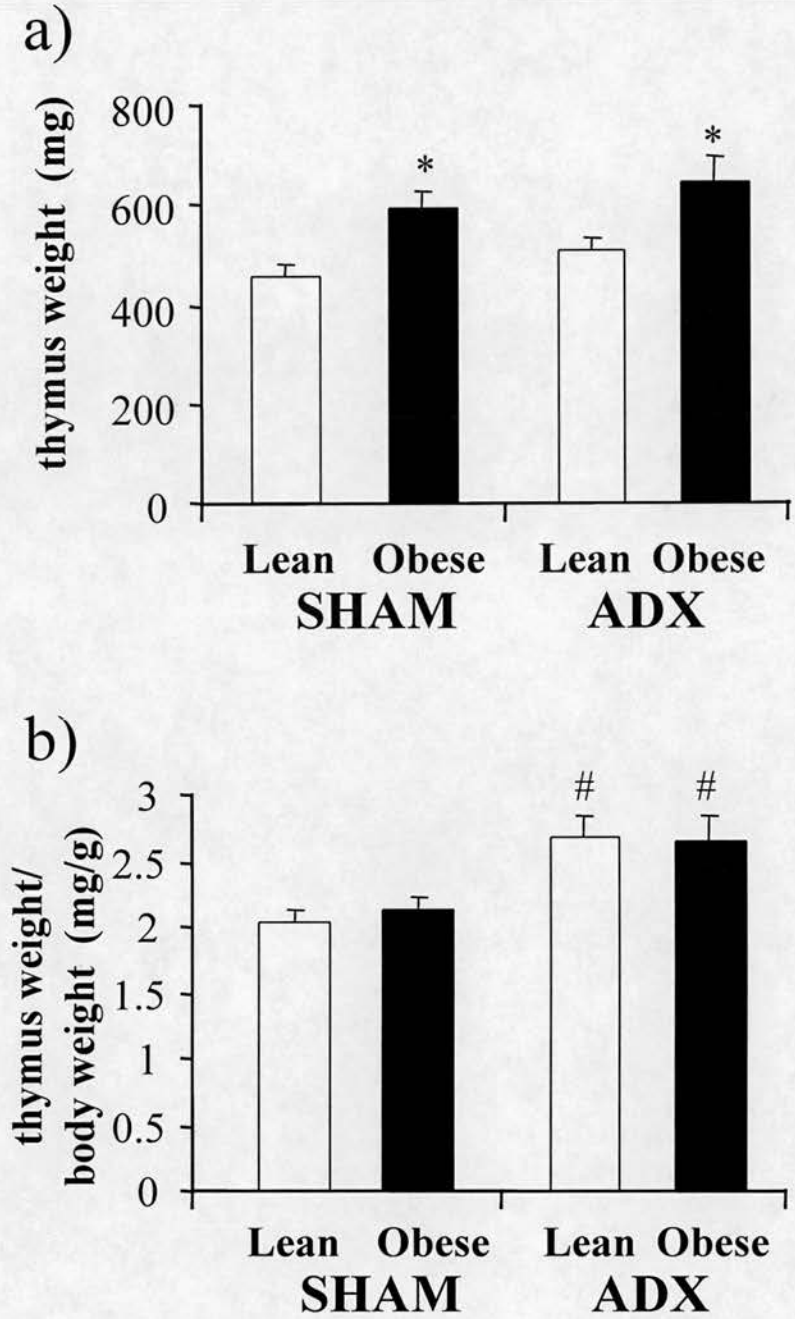

Figure 4.3: Thymus weights.

Panel a) shows thymus weight at 9-weeks of age and panel b) shows thymus weight corrected for body weight.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same surgery group. # denotes $p<0.05$ compared with sham-operated animals of the same phenotype.

Sham denotes sham-operated, and ADX denotes adrenalectomised, animals.

4.3.2 Plasma glucocorticoid levels

Obese rats had higher plasma levels of corticosterone (Figure 4.4) than lean rats. Both lean and obese adrenalectomised animals had no detectable plasma corticosterone.

4.3.3 Hepatic 11β-HSD1 kinetics and mRNA expression

Kinetic constants for 11β-HSD1 were calculated from the Lineweaver-Burke linear transformation of the Michaelis-Menten equation (Lineweaver & Burk, 1934) for each individual animal. A Lineweaver-Burke plot constructed from the mean data is shown in Figure 4.5 for illustration. The Michaelis constant (Km) was not different between lean and obese animals and was unaffected by adrenalectomy (Figure 4.5). Maximum velocity (Vmax) values for obese animals were approximately half those for lean animals (Figure 4.5). Adrenalectomy normalised Vmax in obese animals but had no effect in lean animals.

Hepatic 11β-HSD1 mRNA levels were lower in the liver of obese sham animals than lean (Figure 4.6). This difference was attenuated by adrenalectomy, but adrenalectomy *per se* increased 11β-HSD1 expression in both lean and obese animals.

4.3.4 11β-HSD1 activity in omental fat

11β-HSD1 activity in omental fat tended to be higher in obese than lean rats, but this was not statistically significant (Figure 4.7). Adrenalectomy tended to decrease omental fat 11β-HSD1 activity in obese rats, but increase it in lean rats, such that after adrenalectomy enzyme activity was lower rather than higher in obese animals.

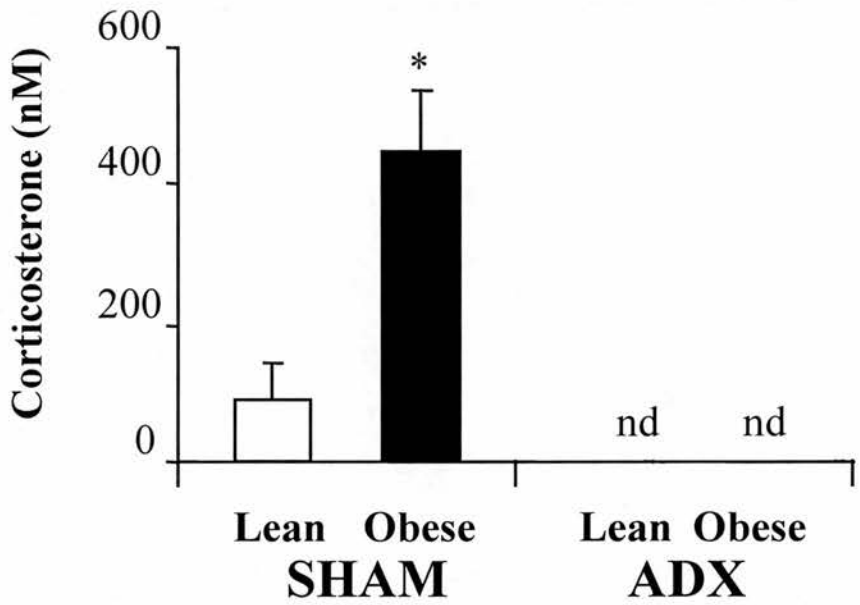

Figure 4.4: Plasma corticosterone levels

nd denotes not detected: limit of detection was 6.25nM.

* denotes $p<0.05$ from Student's t-test.

Sham denotes sham-operated, and ADX denotes adrenalectomised, animals.

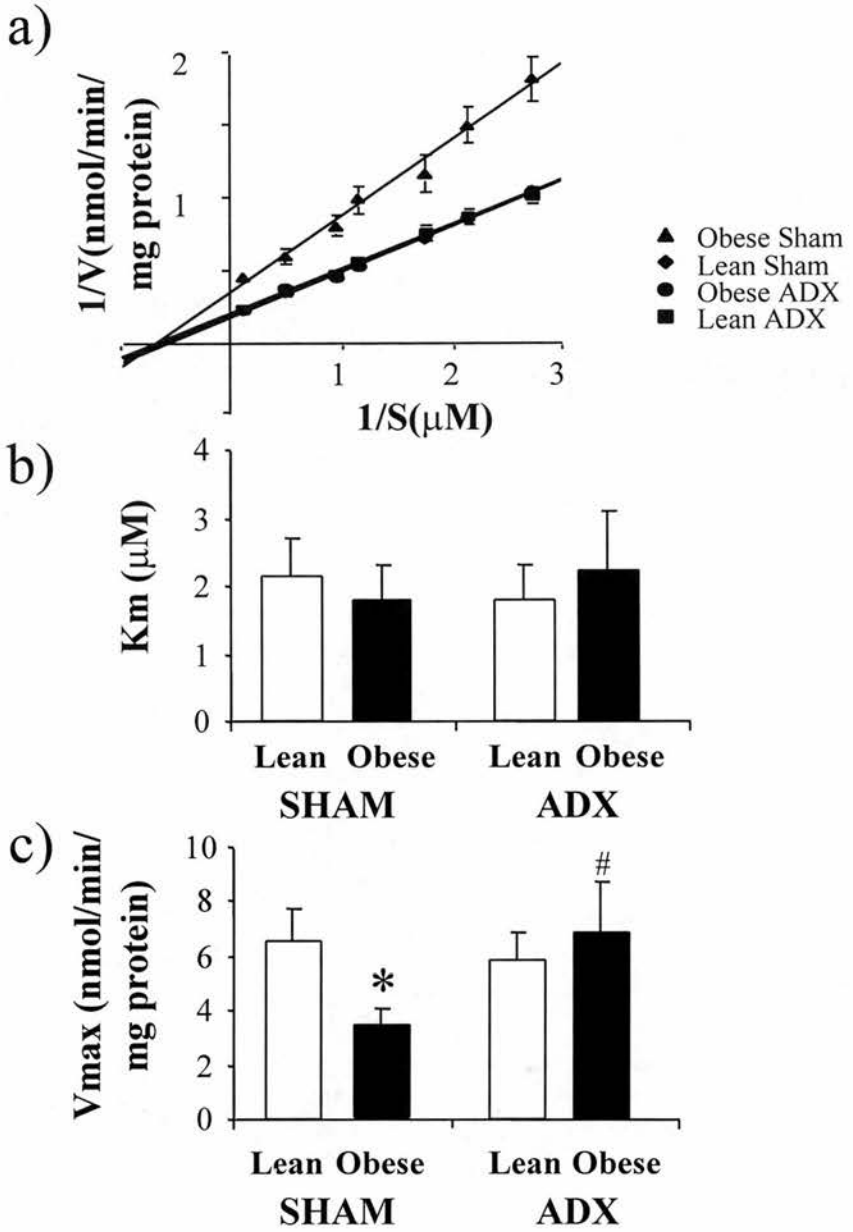

Figure 4.5: Hepatic 11β-HSD1 kinetics

Panel a) shows Lineweaver-Burke plots for summarized data. Km (panel b) and Vmax (panel c) were calculated from Lineweaver-Burke plots for each individual animal.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese sham-operated animals. # denotes $p<0.05$ compared with sham-operated animals of the same phenotype.

Sham denotes sham-operated, and ADX denotes adrenalectomised, animals.

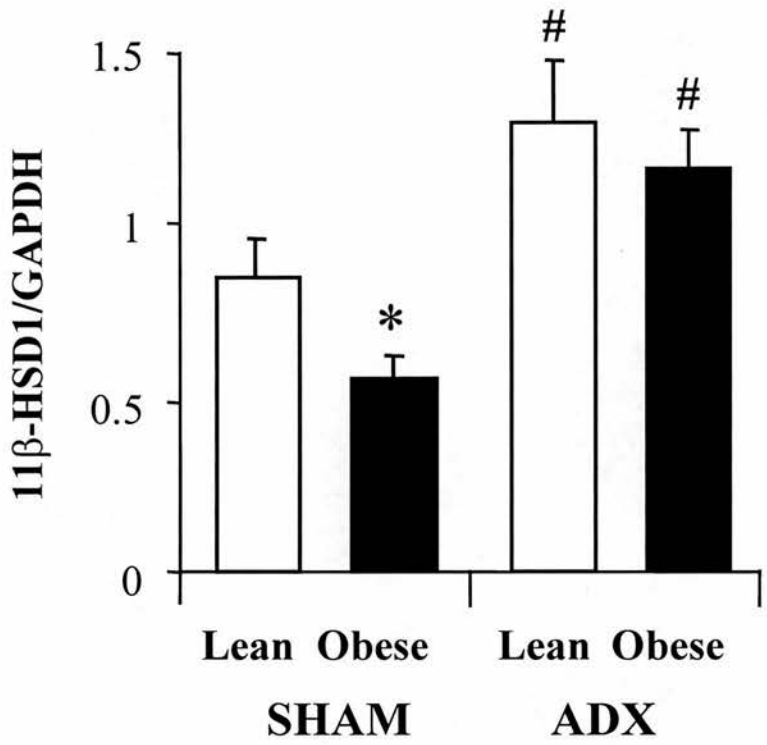

Figure 4.6: Hepatic 11β-HSD1 mRNA expression

11β-HSD1 mRNA levels are expressed as a ratio of the 11β-HSD1 signal to the GAPDH signal.

Data were analysed by Mann-Whitney U tests. * denotes $p<0.05$ comparing lean and obese sham-operated animals. # denotes $p<0.05$ compared with sham-operated animals of the same phenotype.

Sham denotes sham-operated, and ADX denotes adrenalectomised, animals.

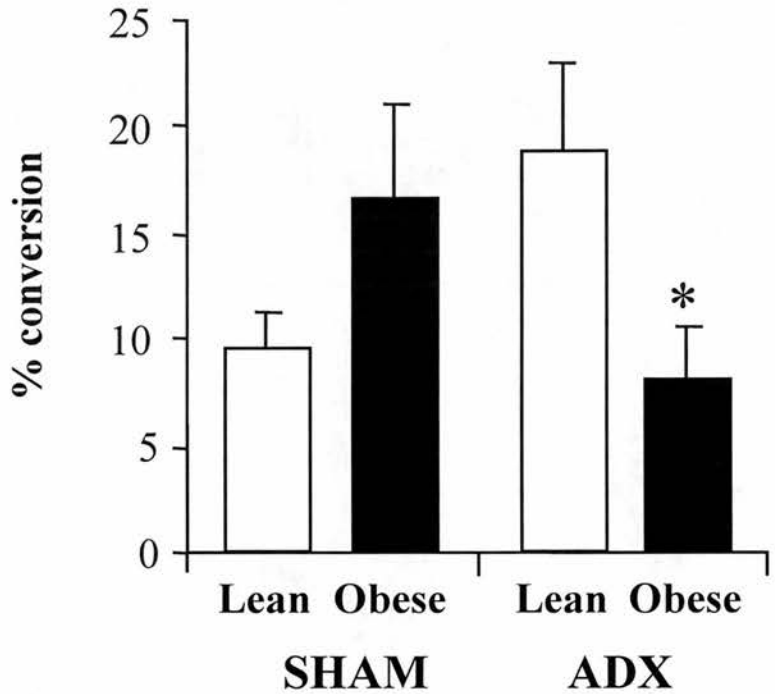

Figure 4.7: 11β-HSD1 activity in omental fat

11β-HSD1 activity is expressed as percent conversion of corticosterone to 11-dehydrocorticosterone.

Data were analysed by Mann-Whitney U tests. * denotes $p<0.05$ comparing lean and obese animals in the same surgery group.

Sham denotes sham-operated, and ADX denotes adrenalectomised, animals.

4.3.5 Hepatic glucocorticoid regulated gene expression

Hepatic PEPCK mRNA levels were not different between lean and obese animals (Figure 4.8). Adrenalectomy had no effect on PEPCK mRNA levels in either lean or obese animals.

In contrast, as found in chapter 3, hepatic TAT mRNA levels were higher in obese animals than lean (Figure 4.8). Adrenalectomy had no effect on TAT mRNA in lean animals, but caused a decrease in TAT expression in obese animals, such that there was no difference between lean and obese adrenalectomised animals.

4.4 Discussion

This study confirms the observation that adrenalectomy normalises weight gain in obese Zucker rats (Yukimura *et al.*, 1978; York & Godbole, 1979; Freedman *et al.*, 1986). It is the first study, however, to describe the nature of the defect in hepatic 11β-HSD1 in the obese Zucker rat, and to examine the effects of adrenalectomy on the tissue specific dysregulation of 11β-HSD1.

As before, obese animals were found to have heavier adrenal glands than lean animals, although this was not significant after correction for body weight. The validity, or otherwise, of correcting organ weights for body weight in animals with such different body compositions was also discussed in Chapter 3, and it was decided to present results corrected for body weight for the sake of completeness. Animals from all groups were checked for the presence of adrenals to confirm that adrenalectomy was complete. Successful adrenalectomy was also confirmed by plasma corticosterone measurements. Sham obese Zucker rats had higher plasma corticosterone levels than lean rats, but corticosterone was not detected in any of the adrenalectomised animals.

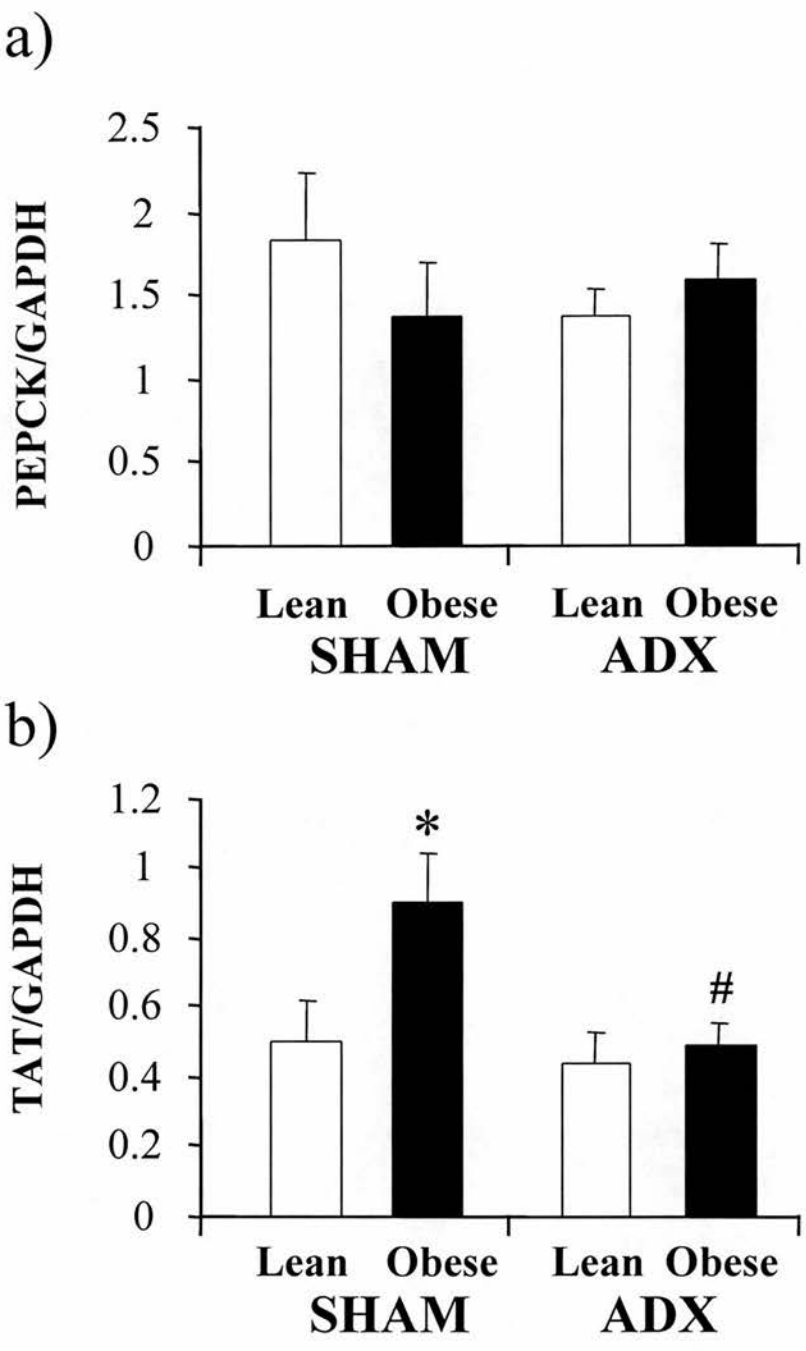

Figure 4.8: Hepatic glucocorticoid regulated gene expression

PEPCK (panel a) and TAT (panel b) mRNA levels are expressed as a ratio of their signal to the GAPDH signal.

Data were analysed by Mann-Whitney U tests. * denotes $p<0.05$ comparing lean and obese sham-operated animals; # denotes $p<0.05$ compared with sham-operated animals of the same phenotype.

Sham denotes sham-operated, and ADX denotes adrenalectomised, animals.

In this study hepatic 11β-HSD1 was examined more closely to determine the nature of the decrease in activity. Kinetic analysis revealed that the Km of 11β-HSD1 for corticosterone was not different between lean and obese sham-operated animals, indicating that there is no difference in the affinity of the enzyme for its substrate. The Vmax calculated for obese animals, however, was approximately half that of the Vmax calculated for lean animals, indicating that the capacity of the enzyme is reduced in obese animals. This explanation is supported by the observation that 11β-HSD1 mRNA levels are lower in obese animals. Adrenalectomy is associated with normalisation of hepatic kinetics in obese animals, ie an increase in Vmax with no change in Km. These changes in enzyme kinetics are paralleled by changes in 11β-HSD1 mRNA levels, with adrenalectomy increasing 11β-HSD1 mRNA in obese animals. One obvious explanation for this might be that the hypercorticosteronaemia in the obese animals is responsible for the dysregulation of hepatic 11β-HSD1. However, there is an inherent difficulty with attributing the results of this study to one particular factor, as adrenalectomy is not a simple manipulation. As well as removing circulating corticosterone and other adrenal steroids, adrenalectomy of obese Zucker rats is associated with normalisation of many of their metabolic abnormalities, including insulin resistance (Castonguay *et al.*, 1986; Freedman *et al.*, 1986). Replacement of glucocorticoids restores the metabolic abnormalities in a dose-dependant manner (Freedman *et al.*, 1986). It is therefore difficult to directly address the role of glucocorticoids in isolation in the obese Zucker rat, as removal, and subsequent replacement of glucocorticoids affect many of the metabolic abnormalities observed. However, hypercorticosteronaemia does not seem to be a likely explanation for alterations in hepatic 11β-HSD1, in the light of evidence that chronic glucocorticoid exposure is associated with up-regulation of hepatic 11β-HSD1 activity in normal rats (Low *et al.*, 1994b). An alternative explanation for the effect of adrenalectomy is that reversal of hyperinsulinaemia is important, as insulin down-regulates 11β-HSD1

activity (Hammami & Siiteri, 1991; Jamieson *et al.*, 1995). This issue is addressed in chapter 5.

The alterations in 11β-HSD1 in omental fat in obese Zucker rats are qualitatively different from those in liver. The current results confirm increased 11β-HSD1 activity in omental fat from obese animals, albeit that this was a non-significant trend ($p=0.1$) in the sham-operated animals. After adrenalectomy, this difference was reversed, so that omental fat 11β-HSD1 activity was lower in obese animals. Relatively little is known about regulation of 11β-HSD1 expression in rat adipose tissue, but experiments in human cultured cells suggest that both glucocorticoids and insulin may up-regulate activity (Bujalska *et al.*, 1997). However, our observation that adrenalectomy up-regulates 11β-HSD1 activity in omental fat of lean animals, by contrast with the lack of effect in liver of lean Zucker rats, reinforces previous observations that regulation of this enzyme is tissue specific (Low *et al.*, 1994b) and suggests that it is unlikely that chronic hypercorticosteronaemia is responsible for up-regulation of 11β-HSD1 in obese Zucker rats.

As in the previous chapter, hepatic glucocorticoid-regulated gene expression and thymus mass were assessed to try to gain an impression of the effects of altered glucocorticoid metabolism on glucocorticoid action. In this study there was again no difference found in expression of hepatic phosphoenolpyruvate carboxykinase (PEPCK; the rate limiting enzyme in gluconeogenesis) mRNA between lean and obese animals. Adrenalectomy might have been expected to cause a decrease in PEPCK expression, particularly in obese animals, as both the removal of glucocorticoids, which normally stimulate PEPCK, and an improvement in insulin sensitivity would be predicted to decrease PEPCK expression. However, adrenalectomy had no effect on PEPCK mRNA levels in either lean or obese animals.

In contrast, another gluconeogenic enzyme, tyrosine aminotransferase (TAT), was induced in the livers of obese Zucker rats. Adrenalectomy reduced the expression of TAT mRNA in obese animals to a level not different from lean animals, while having no effect on TAT mRNA expression in lean animals. This would suggest that hypercorticosteronaemia may be responsible for the induction of TAT expression. However, glucocorticoids do not seem to be necessary for basal expression of TAT, as adrenalectomy had no effect on TAT expression in lean animals. It is noteworthy that hepatic TAT expression follows the same pattern of regulation as hepatic 11β-HSD1, albeit that the changes are in the opposite direction.

The thymus is an organ that undergoes glucocorticoid-induced regression, and so thymus mass can be used as an index of glucocorticoid action. As was found in chapter 3, obese animals had significantly heavier thymuses than lean animals. Adrenalectomy did not cause a significant increase in thymus mass in either lean or obese animals, and obese adrenalectomised animals had significantly heavier thymuses than lean adrenalectomised animals. This indicates that the thymus of the obese Zucker rat undergoes less regression than the thymus of the lean Zucker rat, whether or not the animals have been adrenalectomised. The results had a different pattern when corrected for body weight, in that adrenalectomy caused a significant rise in thymus mass in both lean and obese animals, but there was no difference between lean and obese groups.

In conclusion, the alterations in 11β-HSD1 in liver and fat in the obese Zucker rat described in Chapter 3 are both reversed by adrenalectomy. The regulation of 11β-HSD1 by adrenalectomy is tissue specific, as an increase in 11β-HSD1 is observed in the liver and a decrease is observed in fat. The exact mechanism behind these changes is unclear, as there are many effects of adrenalectomy, including an improvement in insulin resistance.

Chapter Five

Effects of Improved Insulin Sensitivity in Obese Zucker Rats

5.1 Introduction

In the previous chapter tissue-specific alterations in regulation of 11β-HSD1 following adrenalectomy of Zucker rats was described. While glucocorticoids could have a role in this regulation, the exact mechanism is not certain, as adrenalectomy normalises many of the metabolic abnormalities observed in the obese Zucker rat, including insulin resistance (Yukimura *et al.*, 1978; Freedman *et al.*, 1986; Castonguay *et al.*, 1986). Insulin is an important regulator of 11β-HSD1 (Hammami & Siiteri, 1991; Jamieson *et al.*, 1995) and therefore could be implicated in the alteration to 11β-HSD1 following adrenalectomy. To investigate this possibility we conducted a study in which obese Zucker rats were treated with insulin-sensitising drugs.

The drugs employed in this study were the biguanide oral hypoglycaemic agent, metformin, and the thiazolidinedione insulin sensitising agent, rosiglitazone (BRL 49653)[2]. Both of these drugs enhance insulin sensitivity, but have different mechanisms of action. Metformin has been used in the management of type II diabetes for many years, but its precise mode of action is not defined. Metformin improves hepatic and peripheral insulin sensitivity, without affecting insulin secretion and is also thought to improve plasma lipid profiles (Dunn & Peters, 1995). By contrast, rosiglitazone acts as an agonist at the peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ agonists have been shown to increase peripheral glucose uptake while decreasing insulin secretion and gluconeogenesis in a variety of animal models of type II diabetes, while having no effect on normal animals (Fugita *et al.*, 1983; Fujiwara *et al.*, 1988; Stevenson *et al.*, 1990).

[2] Rosiglitazone was a kind gift from SmithKline Beecham

5.2 Methods

Groups of eight 6-week old male lean and obese Zucker rats were treated with either metformin (43mg/kg body weight) or rosiglitazone (1mg/kg body weight) at 0900h daily. Drugs were dissolved in water and administered by gavage, and a matched volume of water (1ml/kg body weight) was administered to the control groups. Animals were weighed daily to allow accurate dosing with drugs, and to follow the progress of weight gain. After two weeks of treatment animals underwent an oral glucose tolerance test which consisted of an overnight fast followed by an oral glucose load of 2g/kg body weight administered by gavage at 0900h. Blood samples were taken at 0, 30 and 120 min after the glucose bolus. At nine weeks of age, after three weeks of treatment, animals were decapitated at 0900-1100h, trunk blood collected and tissues dissected and either snap-frozen on dry ice or mechanically homogenised in Krebs Ringer Bicarbonate buffer.

Corticosterone was measured by radioimmunoassay in plasma prepared from trunk blood samples. Plasma prepared from blood samples taken during the oral glucose tolerance tests was analysed for glucose, using a hexokinase assay kit, and insulin, using a radioimmunoassay. 11β-HSD activity was estimated by conversion of [^{3}H]-corticosterone to [^{3}H]-11-dehydrocorticosterone.

All data are expressed as mean ± standard error, and data were analysed by Analysis of Variance followed by post hoc tests (ANOVA). N=8 for all groups.

5.3 Results

5.3.1 Body and organ weights

There were no differences in weight between any of the lean groups or any of the obese groups at the start of the treatment period, and all the obese groups were slightly heavier than all the lean groups. Obese Zucker rats in all treatment groups gained more weight in the three week treatment period when compared with lean Zucker rats (Figure 5.1). Neither of the treatments had any effect on weight gain in lean animals, but rosiglitazone caused a small but significant increase in weight gained by obese animals, when compared with vehicle treated obese animals. This difference in weight gain was paralleled by changes in food intake (Figure 5.1).

Adrenal weight was higher in obese vehicle treated animals than in lean (Figure 5.2) but, as previously, this was not significant when corrected for body weight. Both metformin and rosiglitazone normalised adrenal weight in obese animals while having no effect on adrenal weight in lean animals. When adrenal weights were corrected for body weight both treatments caused adrenal weight to fall in obese animals, but again had no effect on lean animals.

Thymus weight was also higher in obese vehicle treated animals than in lean (Figure 5.3) but again, this was not significant when corrected for body weight. Neither metformin nor rosiglitazone had any effect on thymus weight in either lean or obese animals.

5.3.2 Oral glucose tolerance tests

Results from the glucose tolerance tests are shown in Figure 5.4. Obese vehicle treated animals exhibited hyperglycaemia in the fasting state and 120min after glucose bolus when compared with lean rats.

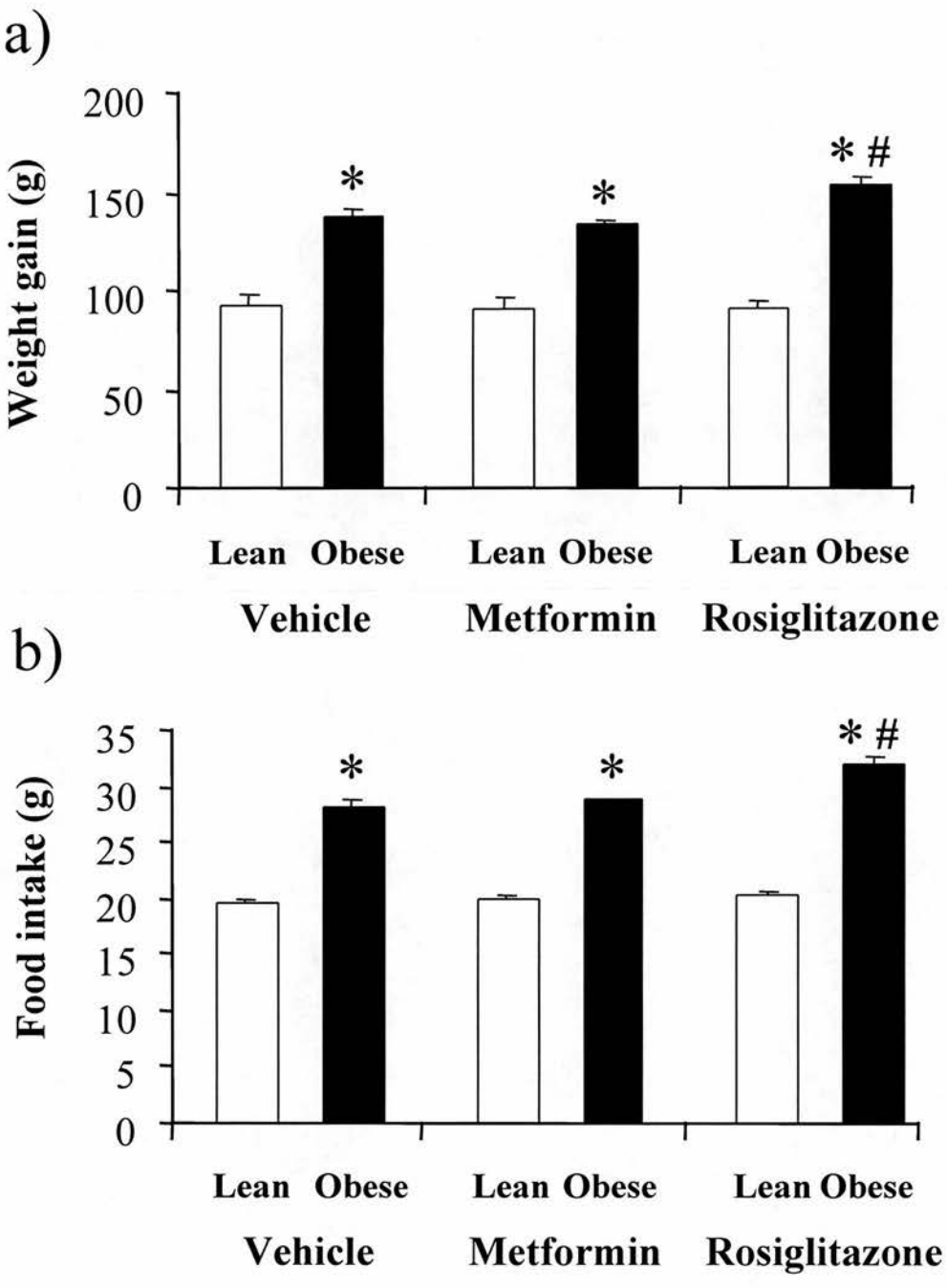

Figure 5.1: Weight gain and food intake during treatment period.

Panel a) shows amount of weight gained in the three-week treatment period and panel b) shows average food intake per animal per day during the three-week treatment period.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

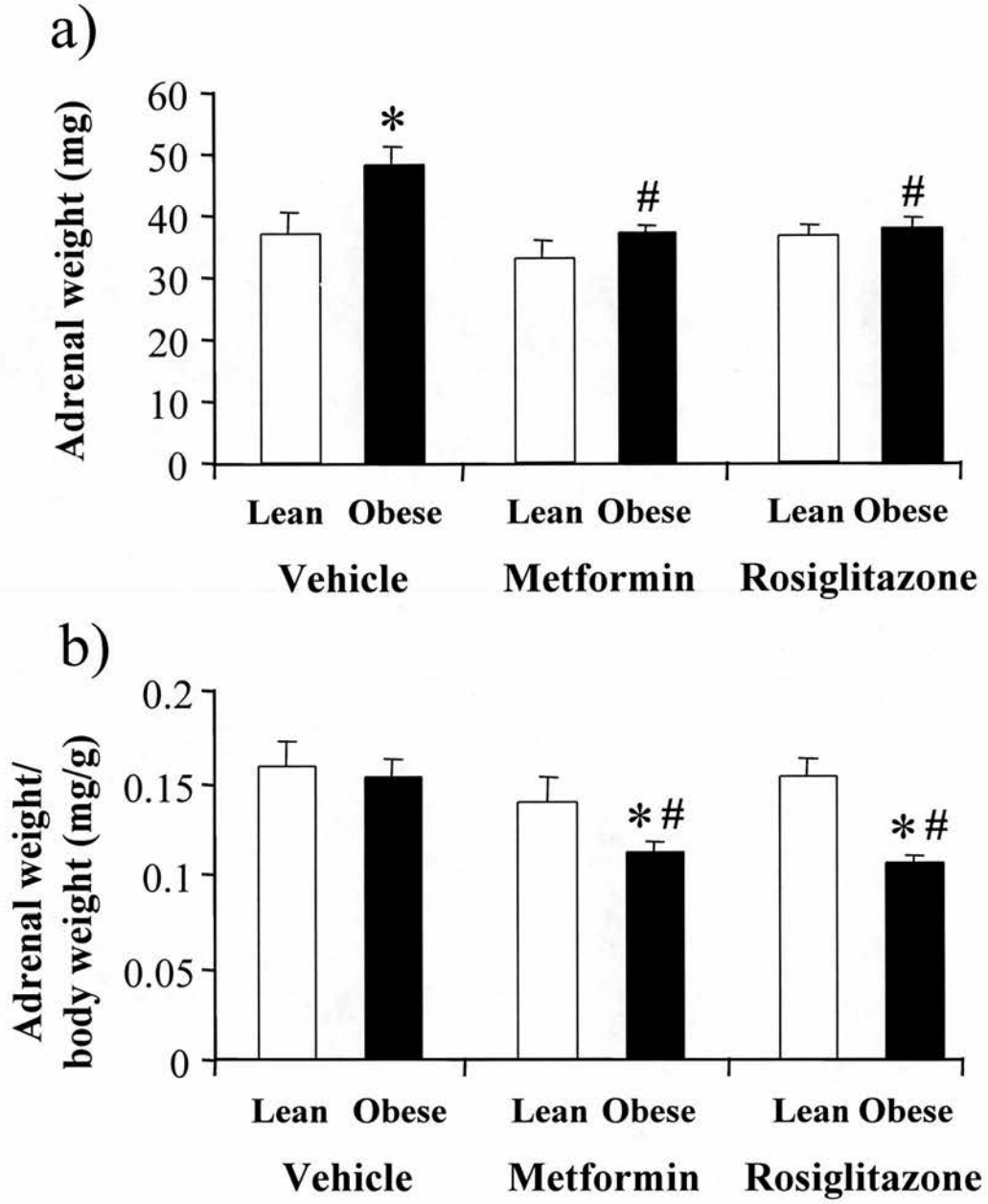

Figure 5.2: Adrenal weights.

Panel a) shows adrenal weight and panel b) shows adrenal weight corrected for body weight.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

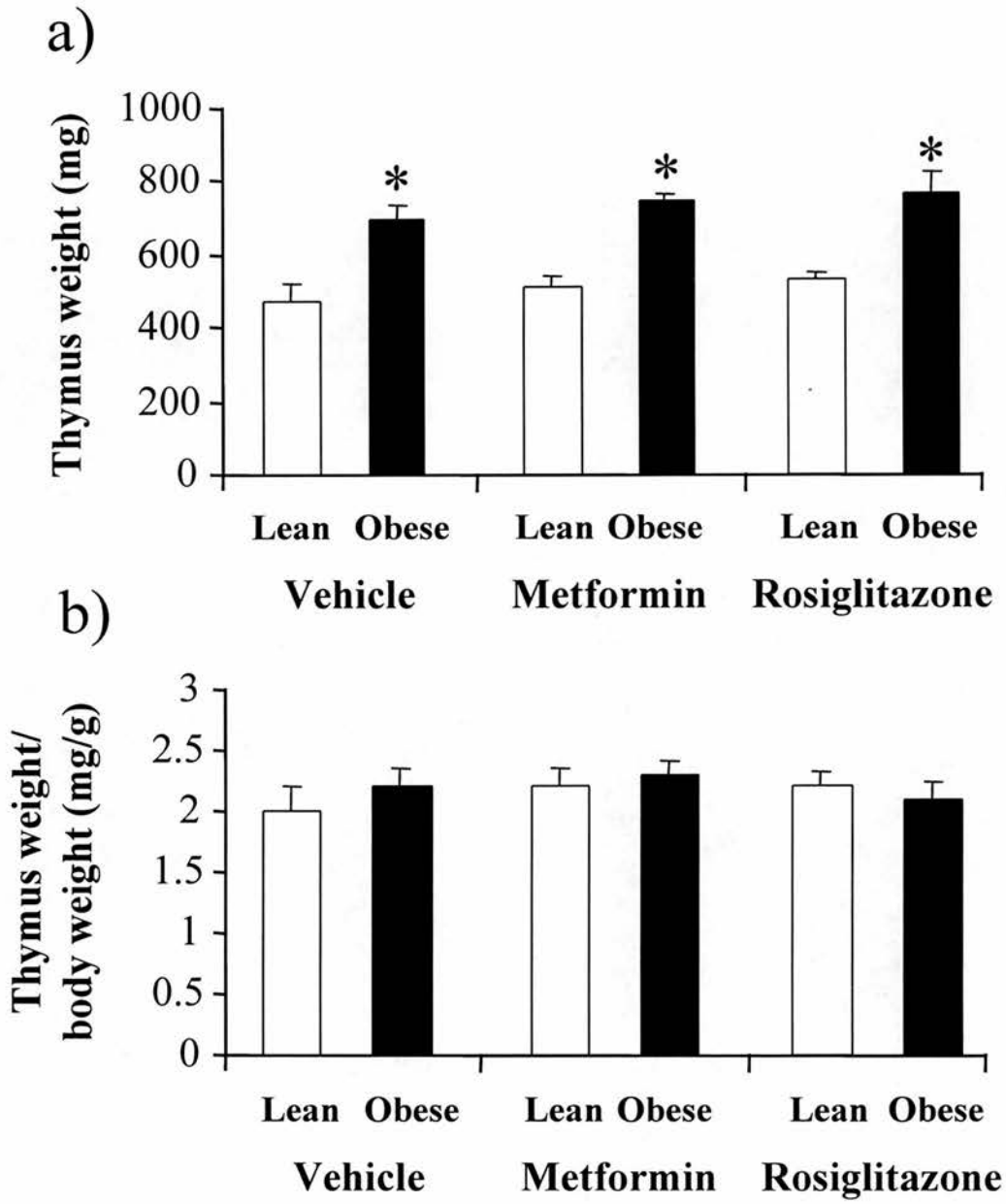

Figure 5.3: Thymus weights.

Panel a) shows thymus weight and panel b) shows thymus weight corrected for body weight.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

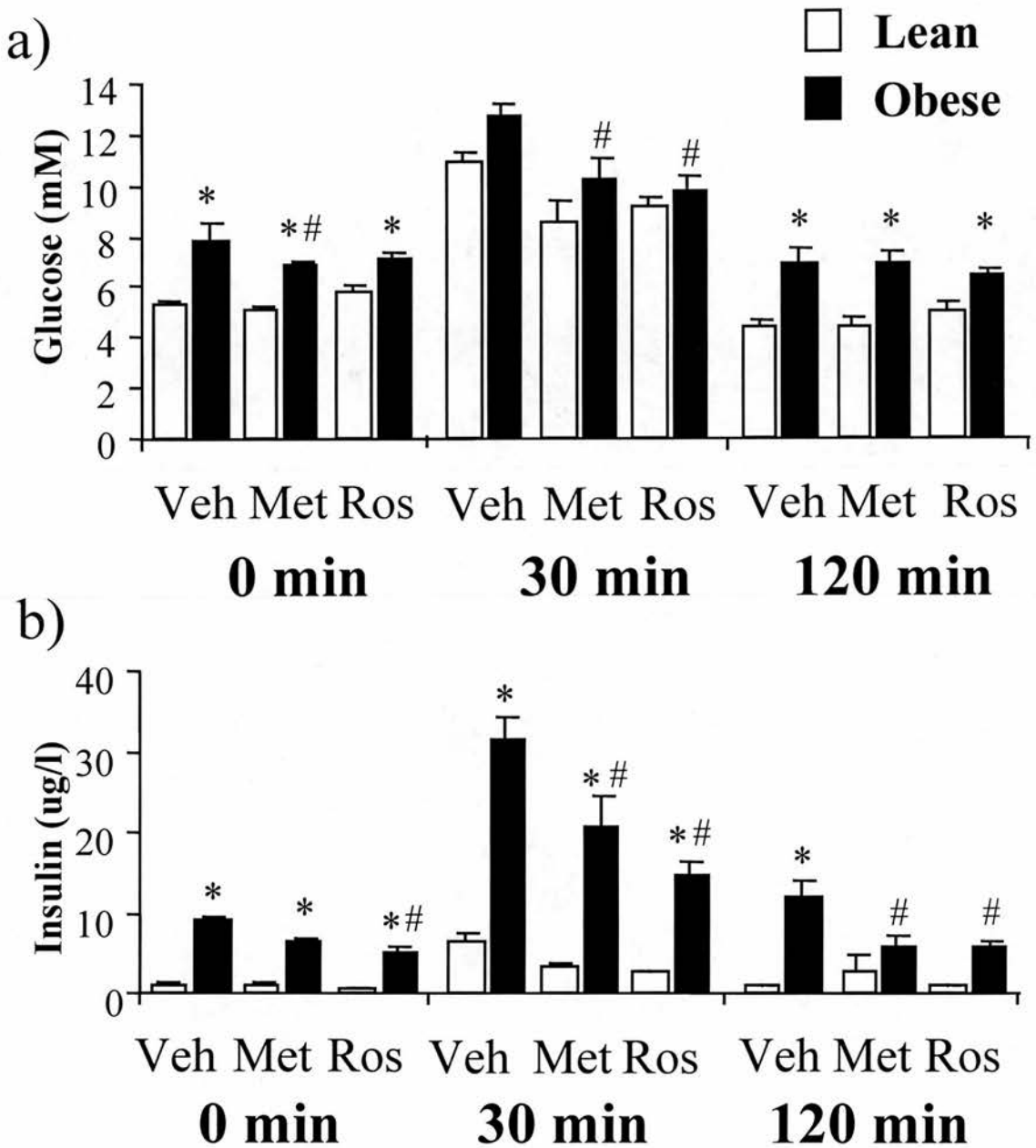

Figure 5.4: Oral glucose tolerance tests.

Panel a) shows glucose levels and panel b) shows insulin levels in plasma prepared from tail-nick blood samples taken 0, 30, and 120min after oral glucose load.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

Veh denotes vehicle treated animals, met denotes metformin treated animals and ros denotes rosiglitazone treated animals.

Insulin levels were markedly higher in obese animals at all time points during the glucose tolerance test (Figure 5.4). Neither metformin nor rosiglitazone had any effect on plasma glucose or insulin in lean rats. In obese rats the effects on plasma insulin were more striking than the effects on plasma glucose. Metformin decreased both fasting and 30min plasma glucose levels, and decreased 30 and 120min insulin levels, whereas rosiglitazone decreased only the 30min glucose level, but reduced plasma insulin at all time points.

5.3.3 Plasma corticosterone levels

Plasma corticosterone levels were rather variable in this experiment, so that trends rather than statistically significant differences were observed. The variability probably reflects poorly controlled stress at the time of decapitation, as this experiment was carried out in another department's animal unit due to the refurbishment of our own unit. It seems that obese vehicle-treated rats had higher plasma corticosterone than lean rats (Figure 5.5). Neither metformin nor rosiglitazone had any effect on plasma corticosterone in lean animals, but rosiglitazone tended to normalise plasma corticosterone in obese animals.

5.3.4 11β-HSD activities

In vehicle treated animals, hepatic 11β-HSD1 activity was lower, and omental fat 11β-HSD1 activity was higher, in obese rats when compared with lean (Figure 5.6). Neither metformin nor rosiglitazone altered hepatic or omental fat 11β-HSD1 activity.

11β-HSD1 activity in subcutaneous fat was not different between lean and obese vehicle treated animals. Metformin treatment caused an increase in 11β-HSD1 activity in lean animals, but had no effect in obese animals. By

contrast, rosiglitazone caused a decrease in 11β-HSD1 activity in obese animals, while having no effect in lean animals.

There was also no difference in 11β-HSD1 activity between lean and obese vehicle treated animals in skeletal muscle. Metformin had no effect on 11β-HSD1 activity in lean animals, but there was a small, although not statistically significant decrease in 11β-HSD1 activity in obese animals. Rosiglitazone caused a rise in 11β-HSD1 activity in both lean and obese animals.

Renal NAD- and NADP-dependent 11β-HSD activity was higher in obese vehicle treated Zucker rats than in lean, although this was not statistically significant for NAD-dependent activity. Neither metformin nor rosiglitazone had any effect on renal 11β-HSD activity, and enzyme activity was higher in all obese treatment groups than in lean.

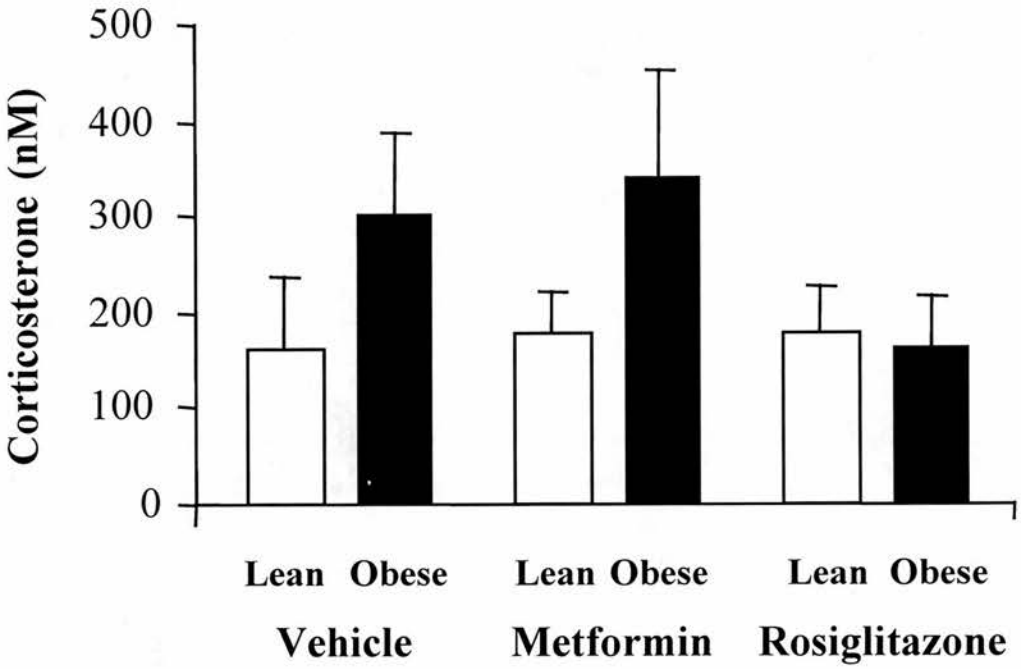

Figure 5.5: Plasma corticosterone levels.

Corticosterone levels were measured in plasma prepared from trunk blood samples.

There were no statistically significant differences between groups.

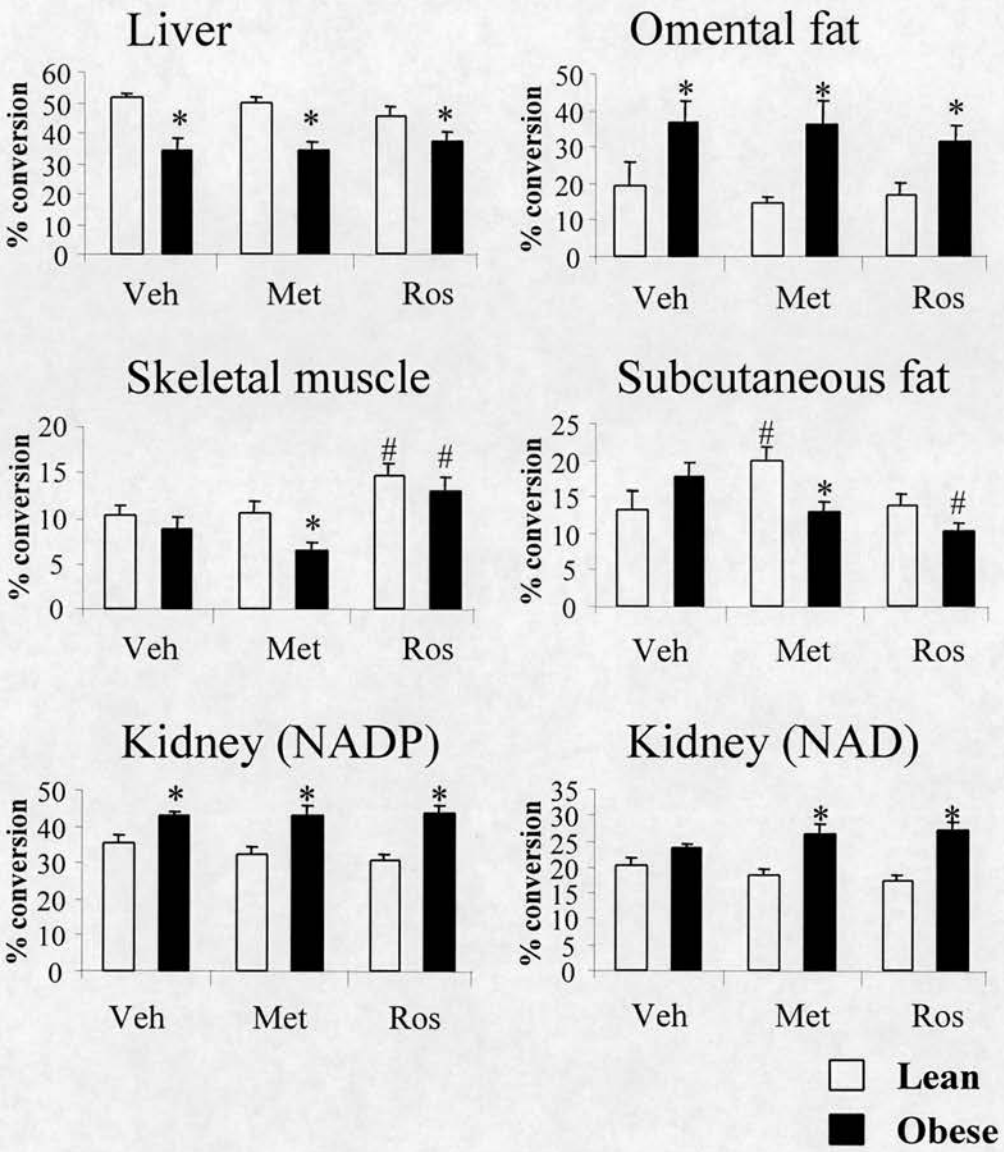

Figure 5.6: 11β-HSD activity in tissue homogenates.

11β-HSD activity is expressed as percent conversion of corticosterone to 11-dehydrocorticosterone. All incubations were carried out in the presence of NADP cofactor, except where indicated for kidney when the incubations were carried out with NAD cofactor.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

Veh denotes vehicle treated animals, met denotes metformin treated animals and ros denotes rosiglitazone treated animals.

5.4 Discussion

These data confirm previous reports that obese Zucker rats have impaired glucose tolerance and insulin resistance (York *et al.*, 1972), and reiterate the tissue-specific alterations in peripheral glucocorticoid metabolism described in the previous chapters of this thesis. This study was designed to examine the possible contribution of insulin to the alterations in 11β-HSD activity in the obese Zucker rat, and utilised two different insulin-sensitising treatments. The results show that neither insulin-sensitising treatment normalised aberrant 11β-HSD1 activity in liver or fat, but both treatments reversed adrenal hypertrophy in obese rats.

While metformin had no effect on either food intake or body weight, rosiglitazone treatment caused an increase in both food intake and weight gain in obese animals, but had no effect in lean animals. This effect of rosiglitazone has been reported previously, and is independent of leptin and neuropeptide Y (Wang *et al.*, 1997), but the mechanism is still unknown.

Both metformin and rosiglitazone enhanced insulin sensitivity in obese, but not in lean, Zucker rats, as demonstrated by the results of the oral glucose tolerance tests. However, the mechanism of their action is probably different, with metformin lowering fasting glucose and acting principally on hepatic gluconeogenesis, while rosiglitazone may have more important effects on glucose uptake in adipose tissue and skeletal muscle.

Having established that both treatments have insulin sensitised the obese Zucker rats, and had no effect on the lean animals, we could then look at the effects of these changes on glucocorticoid metabolism. 11β-HSD1 activity in vehicle treated rats followed the same pattern that has been described in previous chapters of this thesis, with obese animals having

lower 11β-HSD1 activity in the liver and higher 11β-HSD1 activity in omental fat, when compared with lean controls. Neither metformin nor rosiglitazone caused any change in 11β-HSD1 activity in either liver or omental fat from lean or obese animals. The pattern for renal 11β-HSD2 activity was similar, with vehicle treated obese animals having higher enzyme activity than lean animals, and neither of the insulin-sensitising treatments having any effect. The most striking effect of the insulin-sensitising treatments on 11β-HSD activity was the effect of rosiglitazone in skeletal muscle. This increase in 11β-HSD1 activity would be predicted to increase local glucocorticoid levels, and thus antagonise insulin action, negating some of the insulin-sensitising action. Interestingly, this effect occurred in both obese animals, which have been insulin-sensitised, and in lean animals, in which there is no effect of rosiglitazone on insulin sensitivity. This raises the possibility that this is a direct effect of the drug, rather than a consequence of insulin-sensitisation, and re-emphasises that there are tissue-specific differences in 11β-HSD1 regulation. It would therefore be interesting to observe whether the effects of rosiglitazone on insulin sensitivity are enhanced when combined with 11β-HSD1 inhibition.

Mechanisms for activation of the HPA axis in obesity are poorly understood. The results presented in chapter 3 of this thesis support the possibility that enhanced peripheral clearance of glucocorticoids resulting from enhanced 5α-reductase and impaired hepatic 11β-HSD1 activities may be an important stimulus. However, both metformin and rosiglitazone normalised the adrenal hypertrophy in obese Zucker rats while having no effect on the altered hepatic 11β-HSD1 activity. In addition, rosiglitazone tended to normalise plasma corticosterone levels, albeit that this is contrary to a previous report where rosiglitazone increased plasma corticosterone in obese Zucker rats (Wang *et al.*, 1997). This suggests that insulin-sensitising agents may exert effects on other glucocorticoid metabolising enzymes, such as 5α-reductase, or may

indicate effects of insulin resistance or hyperinsulinaemia on central control of the HPA axis. Despite the alteration in plasma corticosterone with rosiglitazone treatment, neither of the insulin-sensitising agents altered thymus mass. This reinforces the point that circulating glucocorticoid levels are not necessarily indicative of local glucocorticoid action.

In conclusion, insulin-sensitisation does not normalise the alterations in 11β-HSD activity described in the obese Zucker rat. This suggests that the mechanism underlying abnormal 11β-HSD1 activity does not relate to hyperinsulinaemia, and that reversal of altered 11β-HSD1 activity following adrenalectomy is not related to reversal of insulin resistance. Alternative mediators of tissue-specific dysregulation of 11β-HSD, such as growth hormone (Low *et al.*, 1994a) or gonadal steroids (Low *et al.*, 1993), may be more important in obesity. However, hyperinsulinaemia may be a key factor contributing to activation of the HPA axis in obesity independently of tissue-specific changes in 11β-HSD1.

Chapter Six

Effects of Carbenoxolone Treatment in Obese Zucker Rats

6.1 Introduction

In chapter 3, tissue-specific alterations in the activity of 11β-HSD in obese Zucker rats were described. Down-regulation of 11β-HSD1 in the liver is predicted to attenuate local glucocorticoid action, and may enhance metabolic clearance of glucocorticoids. By contrast, enhanced 11β-HSD1 in omental fat may maintain local glucocorticoid concentrations and promote lipogenesis. This study was designed to examine the contribution that these changes in enzyme activity may make to obesity and insulin resistance.

Inhibition of 11β-HSD with carbenoxolone treatment in healthy adult men increases hepatic insulin sensitivity and decreases glucose production (Walker *et al.*, 1995), and lack of 11β-HSD1 in transgenic mice has similar effects (Kotelevtsev *et al.*, 1997). We therefore postulated that inhibition of 11β-HSD might improve insulin sensitivity in obese Zucker rats. It also seemed possible that 11β-HSD1 inhibition would improve the obesity in these animals, by decreasing local glucocorticoid levels and attenuating the stimulus to lipogenesis. Zucker rats were treated with carbenoxolone, an inhibitor of 11β-HSD (Stewart *et al.*, 1990), to test these hypotheses.

6.2 Methods

Groups of eight 6-week old male lean and obese Zucker rats were treated by gavage with either carbenoxolone (50mg/kg body weight) or a matched volume of water (1ml/kg body weight) at 0900h daily. Animals were weighed daily to allow accurate dosing with carbenoxolone, and to follow the progress of weight gain. After two weeks of treatment animals underwent an oral glucose tolerance test which consisted of an overnight fast followed by an oral glucose load of 2g/kg body weight administered by gavage at 0900h. Tail-nick blood samples were taken 0, 30 and 120

min after the glucose bolus. At nine weeks of age, after three weeks of treatment, animals were decapitated at 0900-1100h, trunk blood collected and tissues dissected and either snap-frozen on dry ice or mechanically homogenised in Krebs Ringer buffer

Corticosterone was measured in plasma prepared from trunk blood samples by radioimmunoassay. Plasma prepared from blood samples taken during the oral glucose tolerance tests was analysed for glucose, using a hexokinase assay kit, and insulin, using a radioimmunoassay. 11β-HSD activity was estimated by conversion of ^{3}H-corticosterone to ^{3}H-11-dehydrocorticosterone.

All data are expressed as mean ± standard error, and data were analysed by Analysis of Variance followed by post hoc tests (ANOVA). N=8 for all groups.

6.3 Results

6.3.1 Body and organ weights

Vehicle treated obese Zucker rats gained more weight in the three-week treatment period and had higher food consumption than lean animals (Figure 6.1). Carbenoxolone treatment had no effect on food intake or body weight, in either lean or obese animals.

Adrenal weight was higher in obese vehicle treated animals than in lean (Figure 6.2), but not when corrected for body weight. Carbenoxolone treatment had no effect on adrenal weight in lean animals, but caused a decrease in adrenal weight in obese animals so that they were not different to lean animals. When corrected for body weight, obese carbenoxolone treated animals had lighter adrenals than lean carbenoxolone treated animals.

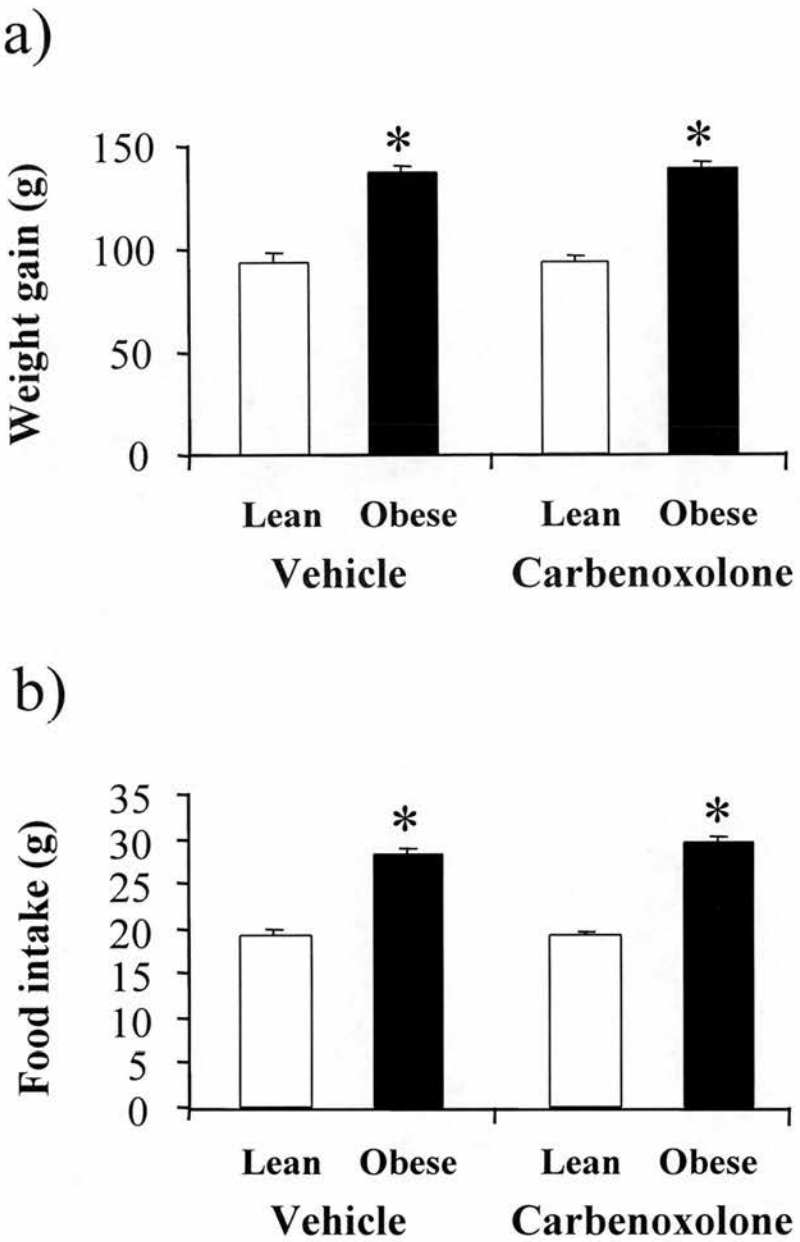

Figure 6.1: Weight gain and food intake during treatment period.

Panel a) shows amount of weight gained in the three-week treatment period and panel b) shows average food intake per animal per day in the three-week treatment period.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

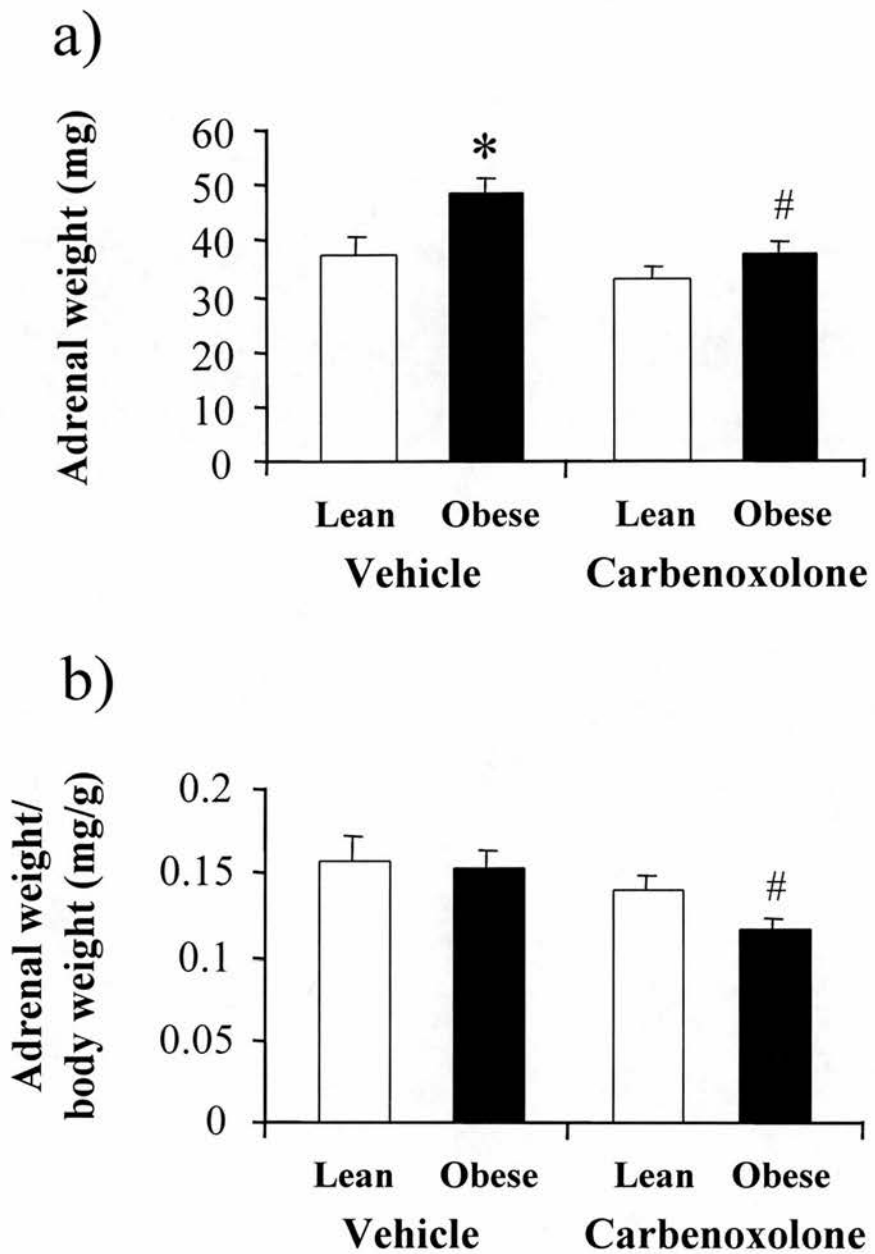

Figure 6.2: Adrenal weights.

Panel a) shows adrenal weight and panel b) shows adrenal weight corrected for body weight.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

Thymus weight was also higher in obese vehicle treated animals than in lean (Figure 6.3), but again not when corrected for body weight. Carbenoxolone treatment had no effect on thymus weight in obese animals, but caused a slight increase in thymus mass in lean animals. When corrected for body weight, carbenoxolone had no effect on thymus weight in obese animals, but caused an increase in thymus mass in lean animals.

6.3.2 11β-HSD activity in tissue homogenates

In this study the same tissue profile of 11β-HSD1 activity was found in the vehicle treated animals as has been described in the previous chapters. Carbenoxolone inhibited hepatic 11β-HSD1 and renal 11β-HSD2 activity in both lean and obese animals (Figure 6.4). However, there was no demonstrable inhibition of 11β-HSD1 activity by carbenoxolone in skeletal muscle homogenates, or in homogenates of subcutaneous or omental fat.

6.3.3 Plasma corticosterone levels

Plasma corticosterone levels were variable in this experiment. This study was carried out in the same animal unit as the study using rosiglitazone and metformin, and the variability probably reflects uncontrolled stress at the time of decapitation. There was a trend for plasma corticosterone to be higher in the obese animals than in the lean (Figure 6.5), and carbenoxolone treatment tended to increase plasma corticosterone in both lean and obese animals.

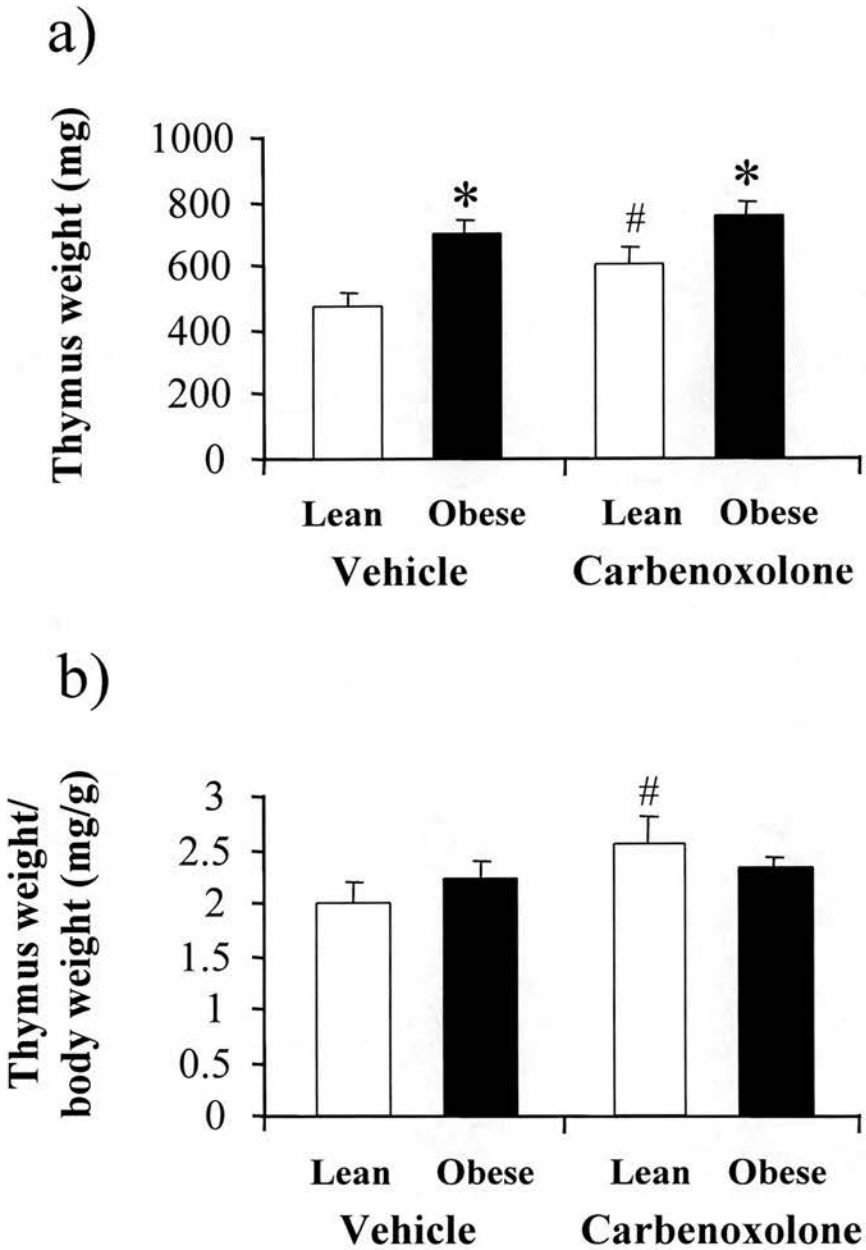

Figure 6.3: Thymus weights.

Panel a) shows thymus weight and panel b) shows thymus weight corrected for body weight.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

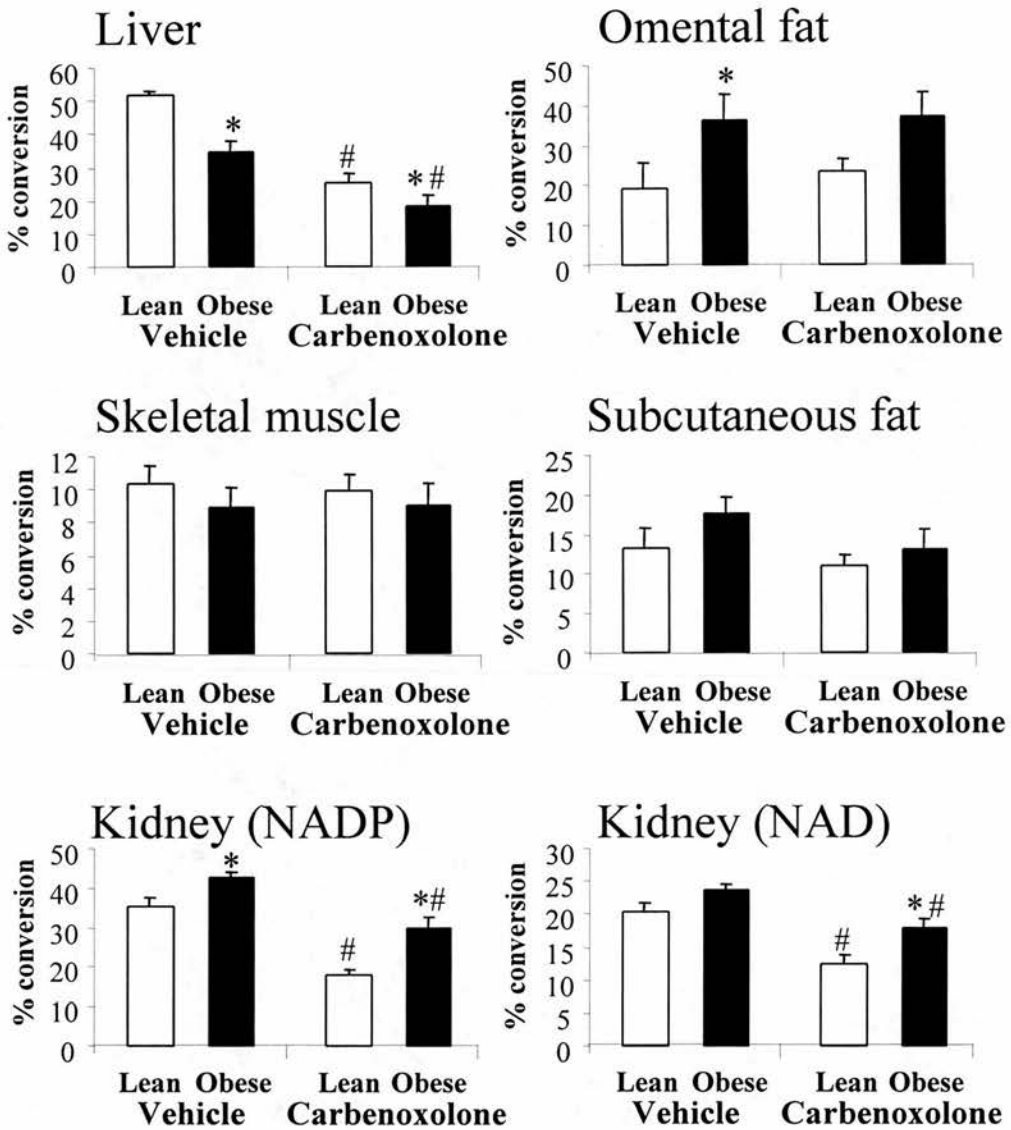

Figure 6.4: 11β-HSD activity in tissue homogenates.

11β-HSD activity is expressed as percent conversion of corticosterone to 11-dehydrocorticosterone. All incubations were carried out in the presence of NADP cofactor, except where indicated for kidney, when the incubations were carried out with NAD cofactor.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

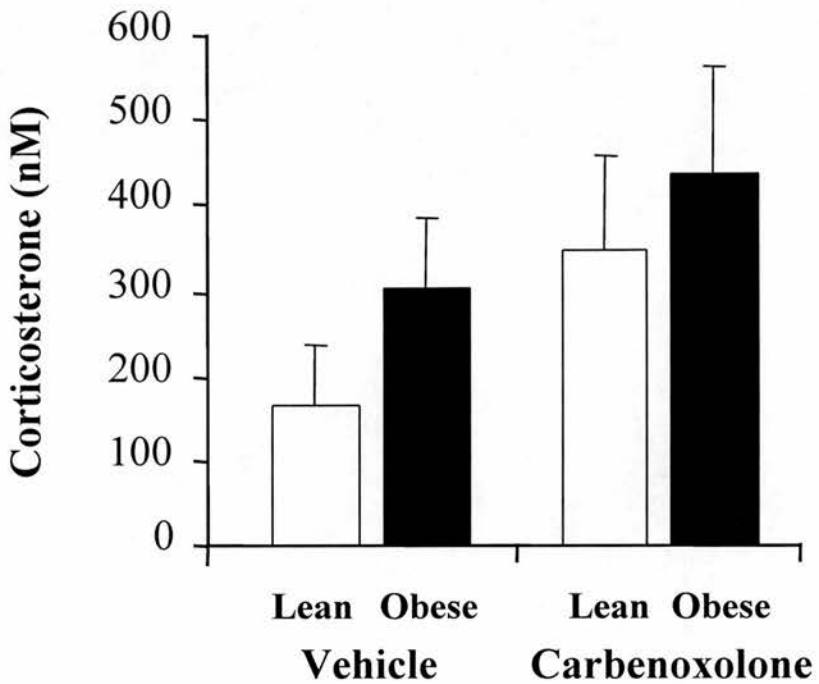

Figure 6.5: Plasma corticosterone levels.

Corticosterone levels were measured in plasma prepared from trunk blood samples.

There were no statistically significant differences between groups.

6.3.4 Oral glucose tolerance tests

Results from the glucose tolerance tests are shown in Figure 6.6. Obese vehicle treated rats had fasting hyperglycaemia and were also hyperglycaemic 120min after glucose bolus, when compared with lean rats. Insulin levels were markedly higher in obese animals at all time points during the glucose tolerance test. Carbenoxolone treatment had no significant effect on plasma glucose levels at any time point. By contrast, carbenoxolone increased plasma insulin in the fasting state and 30min after glucose in both lean and obese animals, and at 120min in obese animals only.

6.4 Discussion

The results presented in this chapter suggest that carbenoxolone treatment of obese Zucker rats did not improve obesity or insulin sensitivity, and in fact exacerbated insulin resistance. However, inhibition of 11β-HSD was not demonstrable *in vitro* in all tissues, and 11β-HSD is not the only enzyme inhibited by carbenoxolone.

Inhibition of 11β-HSD1 by carbenoxolone treatment was evident in tissue homogenates of liver and kidney, but not in skeletal muscle or adipose tissue. It is notoriously difficult to demonstrate *in vitro* inhibition of 11β-HSD by carbenoxolone treatment, but chronic carbenoxolone has been reported to inhibit transcription of 11β-HSD1 in liver and kidney (Whorwood *et al.*, 1993). This raises the possibility that the enzyme in muscle and adipose tissue is being inhibited *in vivo* by carbenoxolone, but that the effects are not transcriptional and therefore are difficult to reproduce *in vitro*. An alternative explanation is that the distribution of carbenoxolone varies between tissues and that the drug did not access muscle and adipose tissue to the same extent as liver and kidney.

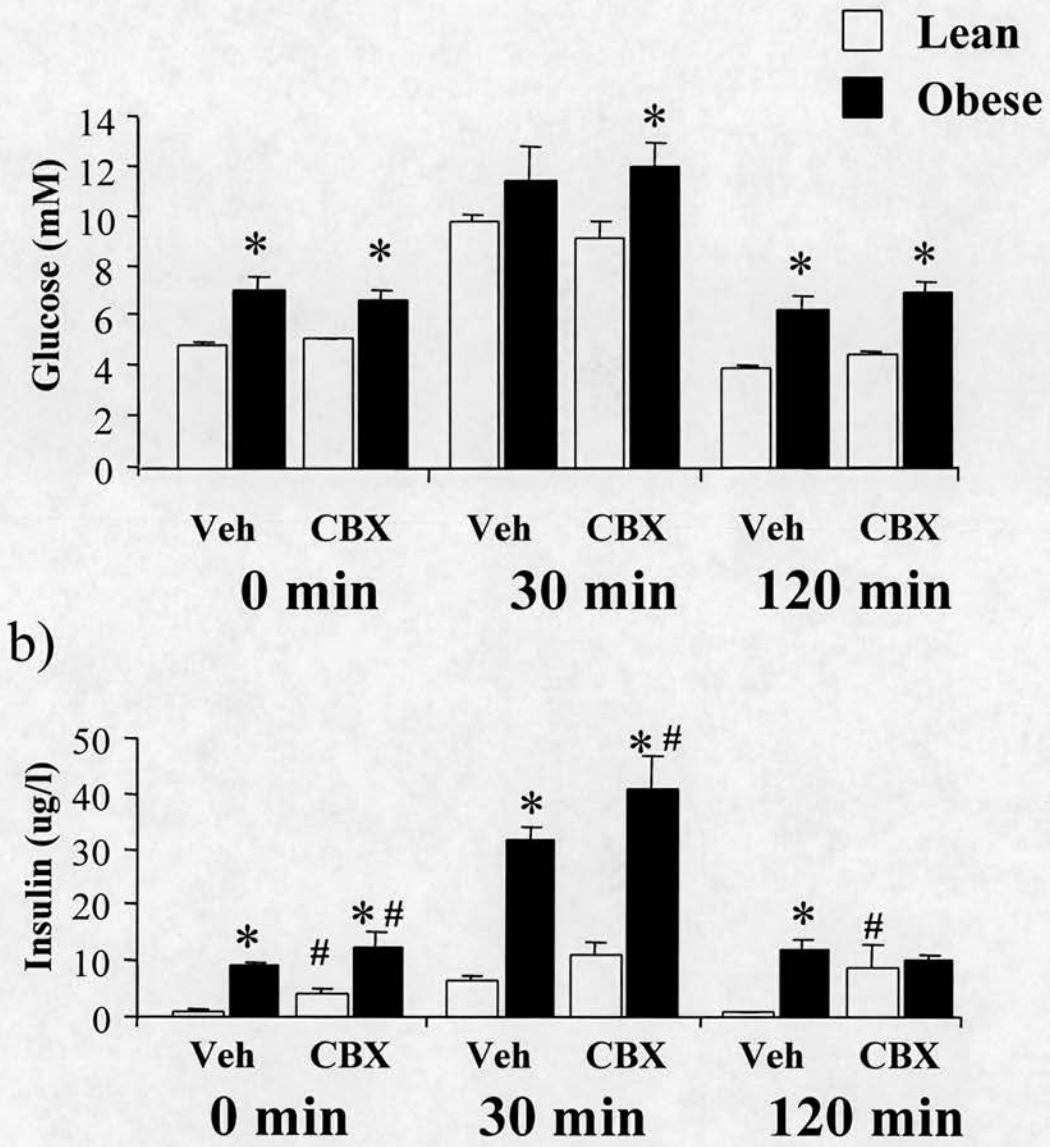

Figure 6.6: Oral glucose tolerance tests.

Panel a) shows glucose levels and panel b) shows insulin levels in plasma prepared from tail-nick blood samples taken 0, 30 and 120min after oral glucose load.

Data were analysed by ANOVA. * denotes $p<0.05$ comparing lean and obese animals in the same treatment group. # denotes $p<0.05$ compared with vehicle treated group of the same phenotype.

Veh denotes vehicle treated animals and CBX denotes carbenoxolone treated animals.

If the latter were the case, tissue specific inhibition of the enzyme would need to be taken into account when interpreting the results. The issue could be resolved by measuring carbenoxolone concentrations within the tissues, and work is in progress in our laboratory to establish such an assay. It is also planned to quantify 11β-HSD mRNA levels to establish whether the changes in enzyme activity observed reflect transcriptional effects.

Carbenoxolone had no effect on adrenal size in lean animals, but reduced adrenal size in obese animals and had no significant effect on plasma corticosterone. Attenuated HPA activity (as assessed by decreased adrenal weight) in the face of no drop in plasma corticosterone suggests that the net clearance of corticosterone must also be attenuated. As well as inhibiting metabolism of corticosterone by 11β-HSD2, carbenoxolone inhibits other glucocorticoid metabolising enzymes, including 5β-reductase (Latif *et al.*, 1990). Inhibition of both 11β-HSD2 and 5β-reductase would decrease metabolic clearance of corticosterone.

However, despite inhibition of hepatic 11β-HSD1 and attenuation of HPA activation, no normalisation of weight gain was observed in obese animals. There are several explanations for this. Firstly, enhanced 11β-HSD1 activity in omental fat may be important to maintaining obesity, and the lack of effect of carbenoxolone on body weight may be due to the enzyme not being demonstrably inhibited in this tissue. Secondly, as suggested in chapter 3, attenuated hepatic 11β-HSD1 may be compensatory, so that further inhibition of 11β-HSD1 activity cannot add to this effect. Thirdly, it may be that neither altered 11β-HSD1 nor activated HPA is causally linked to obesity. The last explanation seems unlikely in light of the growing body of evidence discussed in chapter 1.5 linking HPA activation, glucocorticoids and obesity.

The most striking result from this study was the effect of carbenoxolone on insulin sensitivity. Inhibition of 11β-HSD1 in the liver would cause a decrease in glucocorticoid receptor activation, and would therefore be predicted to enhance insulin action, as has been reported in man (Walker *et al.*, 1995). However, the results of this study suggest that, if anything, carbenoxolone treatment impaired insulin sensitivity in both lean and obese animals. In chapter 3 it was suggested that attenuated hepatic 11β-HSD1 activity in obese Zucker rats may be compensatory to increased plasma corticosterone. It may be that, as already suggested, this compensation is maximal, and further inhibition of 11β-HSD1 activity has no additional effect. Alternatively, carbenoxolone also inhibits metabolic clearance of corticosterone by 5β-reductase (Latif *et al,* 1990) and this would tend to increase local corticosterone levels, possibly offsetting any beneficial effect of inhibited reactivation of corticosterone by 11β-HSD1. The net effect of carbenoxolone on intrahepatic corticosterone levels will therefore depend on the balance between decreased reactivation by 11β-HSD1 and decreased inactivation by 5β-reductase. Studying glucocorticoid regulated gene expression would help to establish whether hepatic glucocorticoid receptor activation is increased or decreased by carbenoxolone. Urinary glucocorticoid metabolite profiles would enable us to assess the effect of carbenoxolone on different steroid metabolising enzymes, but unfortunately this was not possible in the course of this experiment. It would be interesting to examine the effects of specifically inhibiting hepatic 11β-HSD1, for example using oestradiol (Low *et al.*, 1993; Jamieson *et al.*, 1999), on insulin sensitivity.

The lack of specificity of carbenoxolone could also explain the inconsistency between the human and rodent data, as it is possible that the drug inhibits different enzymes to varying degrees in the different species. Another explanation for the discrepancy could be the circumstances under which insulin sensitivity was assessed. Euglycaemic hyperinsulinaemic clamp studies were used to assess insulin sensitivity in men treated with

carbenoxolone (Walker *et al*, 1995) and it is possible that the effects observed in man are only apparent when insulin is clamped at a very high level.

In conclusion, carbenoxolone treatment of Zucker rats had no effect on either food intake or weight gain but did impair insulin sensitivity in both lean and obese animals. This is in contrast with a previous report of carbenoxolone enhancing insulin sensitivity and the finding that 11β-HSD1 knock-out mice have attenuation of activation of gluconeogenic enzymes, and some possible explanations for these discrepancies have been discussed. However, full interpretation of the data presented should be reserved until the tissue distribution of carbenoxolone, and its effects on other steroid metabolising enzymes, can be clarified.

Chapter Seven

11β-HSD in Human Obesity

7.1 Introduction

The results presented in chapter 3 describe increased metabolic clearance of corticosterone, and tissue-specific differences in metabolism of corticosterone by 11β-HSD1 in the Zucker rat model of obesity. The urinary glucocorticoid metabolite profiles support *in vitro* measurements of glucocorticoid metabolising enzymes, and the results are also in concordance with published data for urinary glucocorticoid metabolite profiles in obese men (Andrew *et al.*, 1998; Stewart *et al.*, 1999), validating the use of obese Zucker rats as a model of human obesity. Previous studies have attempted to examine adipose tissue 11β-HSD1 activity in man. However, these have been limited to cell culture work on adipose stromal cells (Bujalska *et al.*, 1997), and arteriovenous sampling, which gives highly variable results (Katz *et al.*, 1999). The aim of this study was to assess whether obese men exhibit similar tissue-specific alterations in 11β-HSD1 activity.

7.2 Methods

Participants in the study were recruited from members of the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) study of Northern Sweden (Soderberg *et al.*, 1999). Approval of Umeå University Hospital ethics committee and written informed consent were obtained. Thirty-four men were selected from the MONICA population to provide a group with a wide range of body composition and fasting plasma insulin levels. Hepatic 11β-HSD1 activity was assessed by conversion of oral cortisone to cortisol after suppression of endogenous cortisol production with dexamethasone. Patients also collected a 24-hour urine sample for analysis of cortisol metabolites by GC-MS, and a sub-group of 13 patients consented to a subcutaneous fat biopsy taken from the gluteal/buttock region. The patients who had biopsies performed did not differ significantly to the study population in general. The clinical portion of the

study took part in Sweden, including the biopsies. The fat biopsy was frozen immediately at –70°C and transported on dry ice. The fat was mechanically homogenised in Krebs buffer and 11β-HSD1 activity was assessed by conversion of [^{3}H]-cortisol to [^{3}H]-cortisone.

Body mass index (BMI) was normally distributed in the sample of men. Indices of obesity were therefore analysed as continuous variables in regression analyses, but are presented in Tables and Figures in tertiles of BMI for clarity. Urinary cortisol metabolites were normalised by logarithmic transformation before analysis, but are presented as untransformed data for clarity. Area under the curve was calculated according to the trapezoidal rule, and centered cumulative response was used to compensate for different baseline hormone levels.

7.3 Results

7.3.1 In vivo *measurements of cortisol metabolism*[3]

Increasing obesity was associated with differences in urinary cortisol metabolites (Table 7.1). Obese men excreted relatively more cortisol as derivatives of cortisone (5β-tetrahydrocortisone; THE) than cortisol (5α- and 5β-tetrahydrocortisol; 5α- and 5β-THF), and tended to have higher excretion of 5α- than 5β-THF.

Hepatic 11β-HSD1 activity measured *in vivo* was impaired in obesity, as measured by area under the curve for plasma cortisol concentrations after oral cortisone (Figure 7.1). Endogenous glucocorticoid production was suppressed with a dose of dexamethasone given the night before testing, and by the cortisone given during testing. This is shown by plasma

[3] The *in vivo* study was carried out in Sweden by Drs Eva Rask, Tommy Olsson, Stefan Södeberg and O Johnson. Analysis of plasma and urine was carried out by Inger Arnesjö, Else-Britt Lundström, and Jill Smith. Analysis of the biopsy samples was carried out by me.

corticosterone concentrations, which fell during the time course of the test. Therefore, the difference in plasma cortisol concentrations between groups can only be explained by differences in conversion of cortisone to cortisol.

7.3.2 In vitro *11β-HSD1 activity in subcutaneous adipose tissue*

By contrast with their lower hepatic 11β-HSD1 activity, obese men had substantially higher 11β-HSD1 activity in subcutaneous adipose tissue (Figure 7.2). Under the conditions used there was no evidence of conversion of cortisol to any other metabolites than cortisone.

	Lowest tertile of BMI (n=11)	Middle tertile of BMI (n=11)	Highest tertile of BMI (n=12)	p for trend
Body Mass Index (kg/m^2)	22.9±1.4	26.4±0.7	31.7±4.0	
Total urinary cortisol metabolites (mg/day)	11.3±1.5	12.8±1.6	15.3±1.0	0.01
5α-Tetrahydrocortisol (α-THF)	2.15±0.29	2.06±.0.24	3.10±.0.32	0.06
5β-Tetrahydrocortisol (β-THF)	2.81±0.27	3.29±0.42	2.59±0.29	0.82
Tetrahydrocortisone (THE)	4.44±0.58	4.92±0.78	7.73±0.85	0.006
(5αTHF+5βTHF) /THE	1.18±0.08	1.29±0.17	0.87±0.28	0.06
5αTHF/5βTHF	0.78±0.09	0.71±0.09	1.13±0.10	0.10

Table 7.1: Subject classification and urinary cortisol metabolite measurements

Data are mean ± SEM of untransformed data. p-values are from regression analysis, performed on log transformed data.

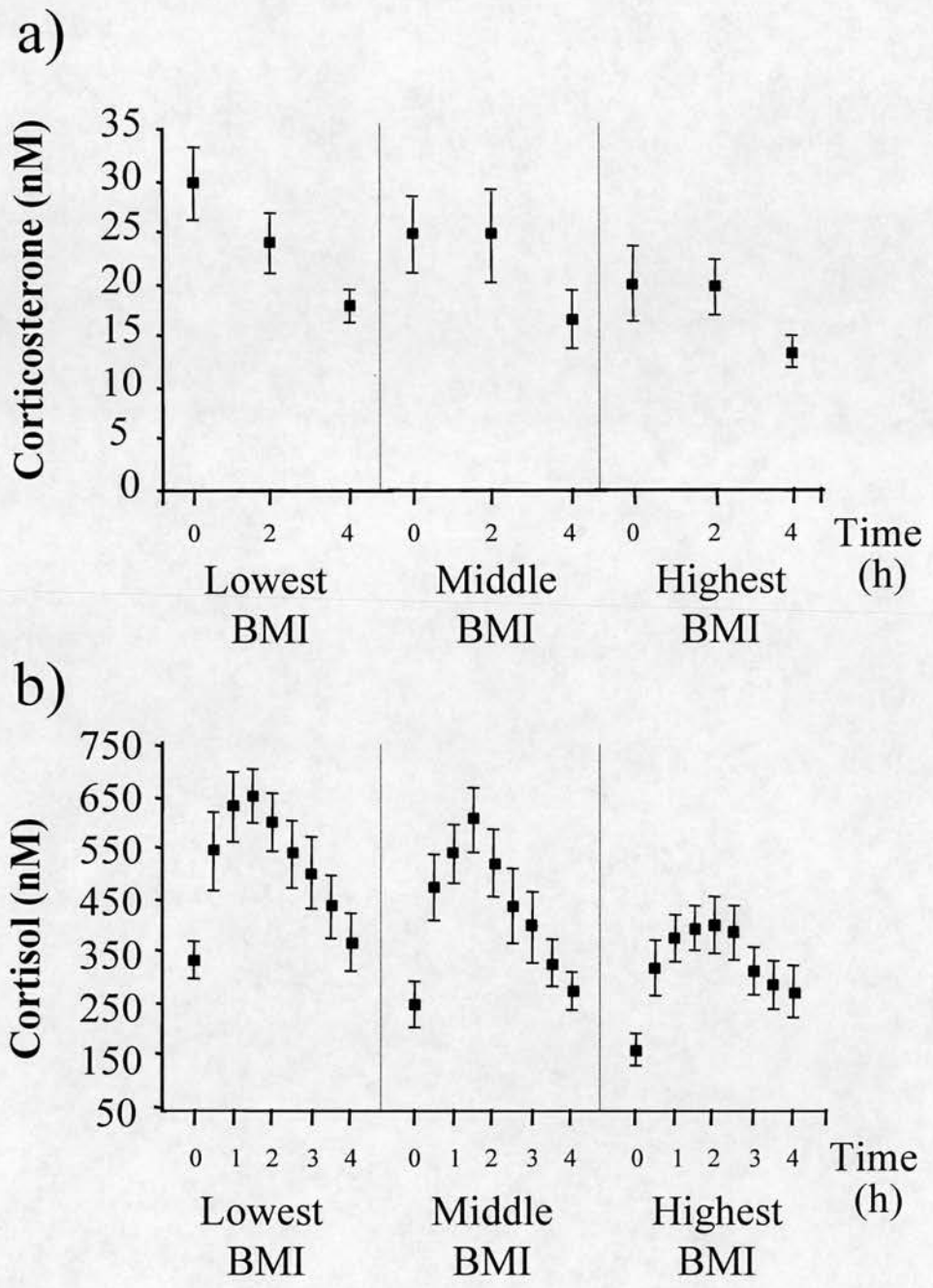

Figure 7.1: Plasma cortisol after oral cortisone

Endogenous plasma cortisol was suppressed, as demonstrated by plasma corticosterone in panel a), and production of cortisol after an oral dose of cortisone was measured, panel b). Data are mean ± SEM for subjects from the lowest, middle and highest tertiles of BMI. Comparison of responses by regression analysis of area under the curve show a negative correlation, R=-0.512, p=0.002.

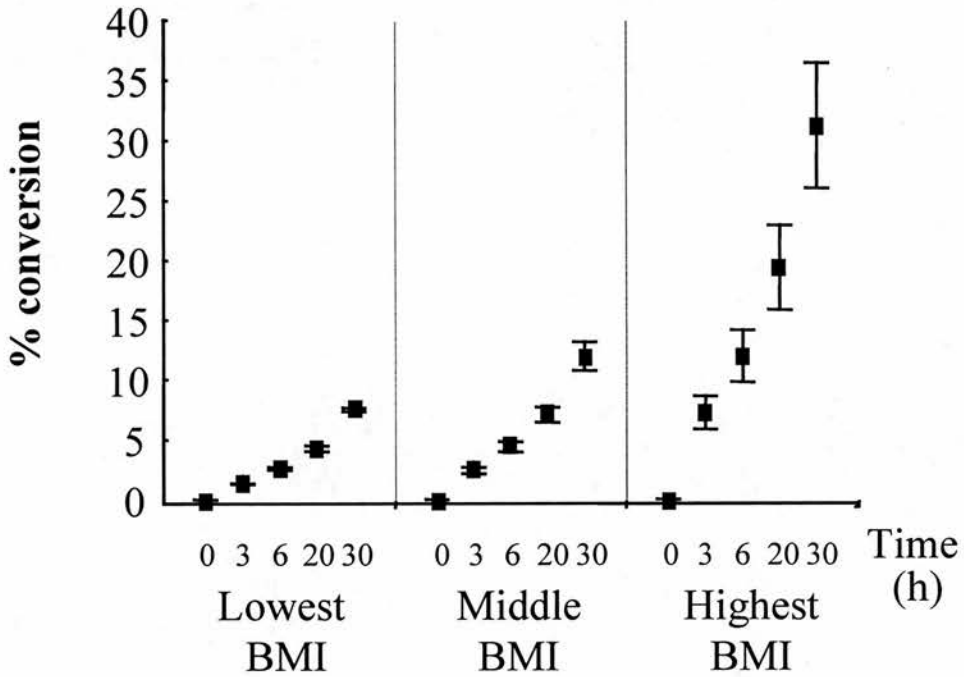

Figure 7.2: *In vitro* 11β-HSD1 activity in subcutaneous adipose tissue

Data are mean ± SEM for subjects from the lowest (n=4), middle (n=6) and highest (n=3) tertiles of BMI. Comparison of responses by regression analysis of area under the curve show a positive correlation, R=0.617, p=0.025.

7.4 Discussion

The urinary cortisol metabolite profiles described in this study confirm previous reports of altered peripheral metabolism of cortisol in obesity due to a combination of enhanced 5α-reductase activity (Andrew *et al.*, 1998) and impaired 11β-HSD1 activity in liver (Stewart *et al.*, 1999). This study has also described tissue-specific alterations in 11β-HSD1 activity that may not be revealed by urinary metabolite analysis alone.

In this study, the balance of 11β-HSD activity in the whole body, as assessed by urinary cortisol metabolites, favours cortisone. This could be explained by decreased 11β-HSD1 activity in the liver, and the results of the oral cortisone-cortisol conversion test suggest this may be the case. Urinary cortisol/cortisone metabolite ratios are not always decreased in obesity (Andrew *et al.*, 1998; Stewart *et al.*, 1999), and this may be explained by tissue-specific disruption of 11β-HSD1. Tissue activity of 11β-HSD1 has been difficult to address in man, with studies limited to cell culture (Bujalska *et al.*, 1997) and variable techniques such as arteriovenous sampling (Katz *et al.*, 1999). The availability of fresh subcutaneous adipose samples in this study has allowed the direct measurement of 11β-HSD1 activity. Obese men had substantially higher 11β-HSD1 activity in subcutaneous fat, predicting enhanced reactivation of cortisone to cortisol in adipose tissue, and promotion of obesity. Thus, obese men appear to exhibit similar tissue-specific differences in 11β-HSD1 activity as those described in obese Zucker rats in previous chapters. These results further validate the use of the obese Zucker rat as a model in which to study the mechanisms of altered glucocorticoid metabolism, and reinforce the potential value of specific inhibitors of 11β-HSD1 to limit weight gain in obesity.

Chapter Eight

Conclusions

In the introduction to this thesis a list of aims was drawn up. These aims have been addressed and the following conclusions reached.

8.1 Glucocorticoid metabolism in obesity.

Glucocorticoid metabolism has been examined in the Zucker rat model of obesity. Clearance of corticosterone by hepatic 5α-reductase and renal 11β-HSD2 is enhanced in obese animals, and reactivation of corticosterone by hepatic 11β-HSD1 is impaired. These changes will result in increased metabolic clearance of corticosterone, which may contribute to HPA activation in obesity. By contrast, 11β-HSD1 activity is normal in skeletal muscle and subcutaneous adipose tissue, and is enhanced in omental adipose tissue. Increased 11β-HSD1 activity would be predicted to raise local corticosterone levels and glucocorticoid receptor activation, and may promote obesity. These results validate the use of urinary steroid metabolite profiles to assess enzyme activity, as changes in urinary steroids were paralleled by changes in enzyme activity measured *in vitro*.

The results described in chapter 7 of this thesis indicate that obesity in man may be associated with similar tissue specific differences in 11β-HSD1 activity, with enzyme activity impaired in the liver, but enhanced in fat. This provides further evidence that the obese Zucker rat is a valid model in which to study glucocorticoid metabolism in obesity. This model was therefore used to try and dissect the mechanism(s) underlying dysregulation of 11β-HSD1 in obesity, and to determine the physiological effects of alterations in 11β-HSD1.

8.2 Mechanisms of dysregulation of 11β-HSD1 in obesity

The obese Zucker rat displays a catalogue of metabolic abnormalities, and we were interested in whether any of these could contribute to the

alterations in 11β-HSD1 described in chapter 3. Two of the primary regulators of 11β-HSD1 are glucocorticoids and insulin, both of which are elevated in the obese Zucker rat. The role of adrenal steroids in the dysregulation of 11β-HSD1 was assessed by studying adrenalectomised animals, and the role of insulin by studying animals treated with insulin sensitising agents.

Adrenalectomy attenuated weight gain in obese animals to a level not different from lean animals, and normalised hepatic 11β-HSD1 kinetics. The abnormality in omental fat 11β-HSD1 activity was reversed by adrenalectomy, with activity falling in obese animals and rising in lean animals. This highlights that not only are the abnormalities in 11β-HSD1 activity tissue-specific, but their regulation is also tissue-specific. Since adrenalectomy also improves insulin sensitivity, it is possible that the effects of adrenalectomy are mediated by changes in insulin. However, in light of the results described in chapter 5, this seems unlikely. While both insulin-sensitising agents improved insulin sensitivity, they had no effect on 11β-HSD1 activity in either liver or omental fat. Both metformin and rosiglitazone normalised adrenal hypertrophy in obese animals, and rosiglitazone tended to decrease plasma corticosterone levels, suggesting that hyperinsulinaemia may contribute to HPA activation in obesity. These results suggest that dysregulation of 11β-HSD1 in obesity is not related to insulin sensitivity, and normalisation of enzyme activity by adrenalectomy is not due to changes in insulin sensitivity.

8.3 Physiological relevance of alterations in 11β-HSD1

The tissue specific alterations in 11β-HSD1 activity described in chapter 3 would be predicted to decrease local glucocorticoid levels in liver and increase them in omental fat. To assess the effect of decreased hepatic 11β-HSD1 activity on glucocorticoid activity, the expression of glucocorticoid regulated genes was measured. Hepatic tyrosine

aminotransferase (TAT) expression, which is induced by glucocorticoids, was higher in obese animals than in lean. This suggests that 11β-HSD1 activity is not decreased enough to compensate fully for the elevated plasma corticosterone levels in obese animals. Due to time constraints, glucocorticoid regulated gene expression has not been measured in fat. We can therefore only speculate that enhanced 11β-HSD1 activity will lead to increased activity of glucocorticoids in this tissue.

To ascertain the importance of alterations in 11β-HSD1 activity to obesity and insulin resistance, Zucker rats were treated the 11β-HSD inhibitor, carbenoxolone. Inhibition of 11β-HSD1 by carbenoxolone treatment was demonstrated *in vitro* in liver and kidney, but not in adipose tissue or skeletal muscle. Despite normalising adrenal hypertrophy and inhibiting hepatic 11β-HSD1 activity, carbenoxolone treatment did not attenuate weight gain in obese animals, and did not improve insulin resistance. In order to clarify the interpretation of this study some further work is required to determine whether carbenoxolone inhibited 11β-HSD1 in all tissues, and the effect of carbenoxolone on other steroid metabolising enzymes.

In summary, tissue specific alterations in glucocorticoid metabolism have been demonstrated in both human and rodent obesity, and the Zucker rat has been described as a suitable model in which to study the mechanisms and implications of these changes. The mechanisms behind altered 11β-HSD1 are still not clear, but appear to relate to a product of the adrenal gland, and not to insulin sensitivity. However, insulin may have a role in HPA activation in obesity, as improved insulin sensitivity results in normalisation of adrenal hypertrophy. Finally, the importance of enhanced adipose tissue 11β-HSD1 activity to the maintenance of obesity, and its potential for therapeutic manipulation is still a key issue to be addressed.

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Publications From This Thesis

Full papers

1) Understanding the role of glucocorticoids in obesity: tissue specific alterations of corticosterone metabolism in obese Zucker rats. (2000)
DEW Livingstone, GC Jones, K Smith, PM Jamieson, R Andrew, CJ Kenyon, BR Walker.
Endocrinology, **141** (2) pp560-563.

2) Mechanisms of dysregulation of 11β-hydroxysteroid dehydrogenase type 1 in obese Zucker rats. (2000)
DEW Livingstone, CJ Kenyon, BR Walker.
In preparation

3) The ebb and flow of cortisol in obesity: high tide in subcutaneous fat. (2000)
E Rask, T Olsson, S Soderberg, R Andrew, DEW Livingstone, O Johnson, BR Walker.
In preparation

Abstracts

1) Impaired hepatic 11β-hydroxysteroid dehydrogenase type 1 in the obese Zucker rat: implications for glucocorticoid metabolism in obesity. (1997)
DE Watson, CJ Kenyon, PM Jamieson, S Nelson, BR Walker.
Journal of Endocrinology; 155 (suppl); OC3.

2) Tissue specific differences in 11β-hydroxysteroid dehydrogenase in Zucker obese rats. (1998)
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Journal of Endocrinology; 156 (suppl); OC42.

3) Altered glucocorticoid metabolism in Zucker obese rats mimics human obesity. (1999)
DEW Livingstone, K Smith, CJ Kenyon, BR Walker, R Andrew.
Journal of Endocrinology; 160 (suppl); P209.

4) Enhanced glucocorticoid metabolism in Obese Zucker rats: a novel explanation for activation of the HPA in obesity. (1999)
DEW Livingstone, GC Jones, K Smith, CJ Kenyon, R Andrew, BR Walker.
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5) Mechanisms of dysregulation of 11β-hydroxysteroid dehydrogenase type 1 in obese Zucker rats. (2000)
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Understanding the Role of Glucocorticoids in Obesity: Tissue-Specific Alterations of Corticosterone Metabolism in Obese Zucker Rats*

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ABSTRACT

The role of glucocorticoids in obesity is poorly understood. Observations in obese men suggest enhanced inactivation of cortisol by 5α-reductase and altered reactivation of cortisone to cortisol by 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1). These changes in glucocorticoid metabolism may influence corticosteroid receptor activation and feedback regulation of the hypothalamic-pituitary-adrenal axis (HPA). We have compared corticosterone metabolism *in vivo* and *in vitro* in male obese and lean Zucker rats, aged 9 weeks (n = 8/group). Steroids were measured in 72-h urine and 0900 h trunk blood samples. 5α-Reductase type 1 and 11βHSD activities were assessed in dissected tissues. Obese animals were hypercorticosteronemic and excreted more total corticosterone metabolites (2264 ± 623 *vs.* 388 ± 144 ng/72 h; $P = 0.003$), with a greater proportion being 5α-reduced or 11-oxidized. 11-Dehydrocorticosterone was also elevated in plasma (73 ± 9 *vs.* 18 ± 2 nM; $P = 0.001$) and urine (408 ± 111 *vs.* <28 ng/72 h; $P = 0.01$). In liver of obese rats, 5α-reductase type 1 activity was greater (20.6 ± 2.7% *vs.* 14.1 ± 1.5%; $P < 0.04$), but 11βHSD1 activity (maximum velocity, 3.43 ± 0.56 *vs.* 6.57 ± 1.13 nmol/min/mg protein; $P = 0.01$) and messenger RNA levels (0.56 ± 0.08 *vs.* 1.03 ± 0.15; $P = 0.02$) were lower. In contrast, in obese rats, 11βHSD1 activity was not different in skeletal muscle and sc fat and was higher in omental fat (36.4 ± 6.2 *vs.* 19.2 ± 6.6; $P = 0.01$), whereas 11βHSD2 activity was higher in kidney (16.7 ± 0.6% *vs.* 11.3 ± 1.5%; $P = 0.01$).

We conclude that greater inactivation of glucocorticoids by 5α-reductase in liver and 11βHSD2 in kidney combined with impaired reactivation of glucocorticoids by 11βHSD1 in liver may increase the MCR of glucocorticoids and decrease local glucocorticoid concentrations at these sites. By contrast, enhanced 11βHSD1 in omental adipose tissue may increase local glucocorticoid receptor activation and promote obesity. (*Endocrinology* **141:** 560–563, 2000)

INCREASED SECRETION of glucocorticoids (*e.g.* in Cushing's syndrome) is associated with obesity, and cortisol secretion is increased in subjects with idiopathic obesity, especially of central distribution (1). However, peak plasma cortisol levels are not elevated in idiopathic obesity (2), suggesting that peripheral metabolism of cortisol may be enhanced. We recently reported that inactivation of cortisol to 5α-tetrahydrocortisol is enhanced in obese humans (3). Alternatively, increased activation of glucocorticoid receptors in obesity could result from raised local, rather than systemic, glucocorticoid concentrations. We and others have suggested that obesity is exaggerated because of increased reactivation of inactive cortisone into cortisol in adipose tissue by 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) (3, 4).

Obese Zucker rats are leptin resistant due to a homozygous point mutation in the leptin receptor gene (5). Glucocorticoids seem to be important in the development of their obesity, as adrenalectomy or glucocorticoid receptor antagonists attenuate weight gain and associated metabolic abnormalities (6, 7). Because of their relevance to local glucocorticoid levels and MCR, we have now examined glucocorticoid-metabolizing enzymes in Zucker rats. Specifically, we have measured inactivation of corticosterone by the enzymes 5α-reductase type 1 in liver and 11βHSD type 2 in kidney. We have also assessed reactivation of corticosterone by 11βHSD1.

Materials and Methods

Animals

Groups of eight 5-week-old male obese and lean Zucker rats (Harlan Orlac, Bicester, UK) were characterized by phenotype, maintained under controlled conditions of light (lights on, 0800–2000 h) and temperature (21 C) and allowed free access to standard rat chow (Special Diet Services, Witham, UK) and drinking water. At 8 weeks of age, animals were acclimatized in metabolic cages for 4 days before 72-h urine collection. At 9 weeks of age, they were decapitated between 0900–1100 h, and trunk blood was collected. Tissues were dissected, and whole adrenals were blotted dry and weighed. Portions of other tissues were snap-frozen on dry ice or mechanically homogenized in Krebs-Ringer buffer (118 mM NaCl, 3.8 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM$CaCl_2$, 1.19 mM $MgSO_4$, and 25 mM $NaHCO_3$, pH 7.4).

Measurement of endogenous steroids

Plasma corticosterone and 11-dehydrocorticosterone were measured in trunk blood by RIAs. Urinary steroids were extracted and derivatised from a 10-ml aliquot for each animal using methods previously de-

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scribed for human urine (8), except that epicorticosterone and epitetrahydrocorticosterone (Steraloids, Newport, RI) were used as internal standards and 2-g/12-cc Sep-Pak cartridges (Waters Corp., Herts, UK) were used. Steroids were quantified using a Voyager gas chromatograph-mass spectrometer (Finnigan, Manchester, UK) in electron impact mode fitted with a EC-5 capillary column (30 m; id, 0.25 mm; film thickness, 0.25 μm; Alltech, Carnforth, UK).

Measurement of enzyme activities

In vivo 11βHSD1 is a reductase, converting inactive 11-dehydrocorticosterone to corticosterone. However, *in vitro* dehydrogenase activity predominates, so we quantified 11βHSD1 activity by conversion of corticosterone to 11-dehydrocorticosterone. Hepatic 11βHSD1 kinetics were determined by preparing microsomes from tissue homogenate (by centrifugation; 14,000 × *g* for 20 min, supernatant was removed and centrifuged at 100,000 × *g* for 60 min, pellet was resuspended in Krebs-Ringer buffer). The protein concentration was determined colorimetrically. Microsomal preparations (15 μg/ml protein) were incubated in duplicate at 37 C in Krebs-Ringer buffer containing 0.2% glucose, NADP (2 mM), [^{3}H]corticosterone (50 nM), and unlabeled corticosterone (0.3–10 μM). After 10 min, steroids were extracted with ethyl acetate, the organic phase was evaporated under nitrogen, and extracts were resuspended in mobile phase (20% methanol, 30% acetonitrile, and 50% water). Steroids were separated by HPLC using a reverse phase μ-Bondapak C_{18} column (Phenomenex, Cheshire, UK) and were quantified by on-line liquid scintillation counting.

11βHSD1 activity was also measured in homogenates of liver (10 μg/ml protein), quadriceps skeletal muscle (1.5 mg/ml), sc lumbar fat (0.5 mg/ml), and omental fat (1 mg/ml) by the same method, except that incubations were performed for 60 min in the presence of 100 nM [^{3}H]corticosterone. Conditions were optimized for each tissue to ensure first order kinetics. 11βHSD activity was measured in kidney homogenates by the same method (50 μg/ml protein). In addition, to detect 11βHSD type 2 rather than type 1 activity, incubations were performed with NAD (2 mM) rather than NADP as cofactor and 10 nM [^{3}H]corticosterone.

5α-Reductase type 1 activity was assessed in liver, as previously described in prostate (9), by measuring the metabolism of testosterone to 5α-dihydrotestosterone. Liver homogenate (0.5 mg/ml protein) was incubated in duplicate at 37 C in orthophosphate buffer (40 mM Na_2HPO_4, pH 7.5) with NADPH (1 mM) and [^{3}H]testosterone (50 nM) as a substrate. After 10 min, steroids were extracted with ethyl acetate, the organic phase was evaporated under nitrogen, and extracts were resuspended in ethanol with unlabeled testosterone and 5α-dihydrotestosterone and separated by TLC. Fractions containing testosterone and 5α-dihydrotestosterone were identified with phosphomolybdic acid, scraped from plates, and counted in Cocktail T liquid scintillant (BDH, Dorset, UK).

Radiolabeled steroids were obtained from Amersham Pharmacia Biotech (Aylesbury, UK). Solvents were HPLC glass-distilled grade from Rathburn Chemicals (Walkerburn, UK). Other chemicals were purchased from Sigma (Poole, UK).

Quantification of messenger RNA (mRNA) by Northern blot

Total mRNA was extracted from snap-frozen liver samples, and 20 μg were separated by electrophoresis. The RNA was blotted onto a Bio-Rad Laboratories, Inc. Zeta-Probe nylon membrane (Richmond, CA), and 11βHSD1 mRNA was identified as previously described (10). Hybridized probe was quantified using a Fuji Photo Film Co., Ltd. FLA2000 fluorescent image analyzer (Tokyo, Japan). Membranes were rehybridized with a glyceraldehyde-3-phosphate dehydrogenase probe using the same method, to control for differences in mRNA loading and transfer.

TABLE 1. Comparison of corticosteroids in lean and obese Zucker rats

	Lean (n = 8)	Obese (n = 8)	*P*
BW at 9 weeks (g)	226 ± 7	280 ± 6	<0.001
Adrenal wt (mg)	38 ± 2	54 ± 7	0.04
Adrenal/body wt	0.17 ± 0.01	0.20 ± 0.03	0.75
Plasma (nM)			
Corticosterone	78 ± 48	446 ± 91	0.002
11-Dehydrocorticosterone	18 ± 2	73 ± 9	0.001
Urine (ng/72 h)			
Corticosterone (B)	ND	ND	
11-Dehydrocorticosterone (A)	ND	408 ± 111	0.01
5β-TetrahydroB	20 ± 11	60 ± 21	0.53
5α-TetrahydroB	369 ± 114	1653 ± 562	0.06
5β-TetrahydroA	ND	144 ± 42	0.001
Sum of B metabolites	388 ± 114	2264 ± 623	0.003

Data are the mean ± SEM. ND, Not detected. *P* values were determined by Mann-Whitney U tests. Limits of detection: 14–28 ng/72 h (urine) and 6.25 nM (plasma).

Results

In obese rats, plasma corticosterone concentrations and total urinary corticosterone metabolites were elevated compared with those in lean Zucker rats (Table 1). Obese rats exhibited a larger difference in excretion of metabolites of 11-dehydrocorticosterone (>10-fold higher) than of corticosterone (3- to 5-fold higher), and a more substantial difference in 5α-reduced rather than 5β-reduced metabolites of corticosterone compared with lean rats. Adrenal glands were heavier in obese animals, but this was not statistically significant after correction for body weight.

In liver of obese rats 5α-reductase type 1 activity was increased (20.6 ± 2.7% in obese *vs.* 14.1 ± 1.5% in lean; $P < 0.04$). Hepatic 11βHSD1 activity (Fig. 1) and mRNA levels

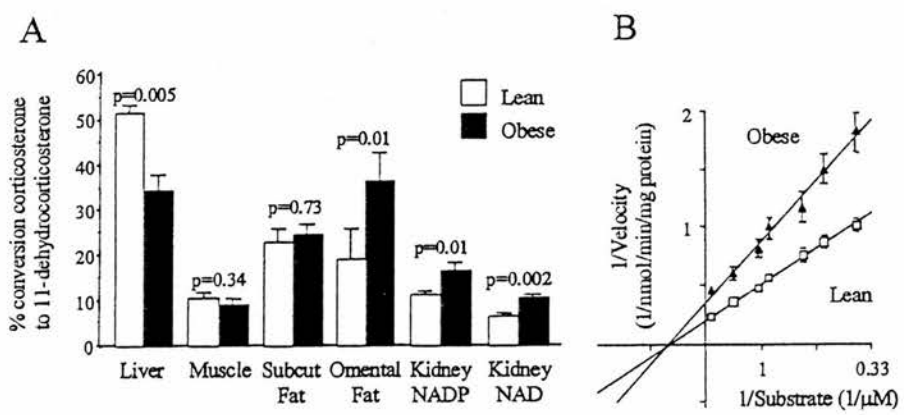

FIG. 1. Data are the mean ± SEM for eight lean (*open symbols*) and eight obese (*closed symbols*) Zucker rats. *P* values are for Mann-Whitney U tests. A shows 11βHSD activity in homogenates of each tissue incubated with NADP, except where indicated. B shows Lineweaver-Burke plots for summarized data from lean and obese liver microsomes. K_m values (2.15 ± 0.55 μM in lean *vs.* 1.80 ± 0.53 μM in obese; $P = 0.31$) and maximum velocity (V_{max}) values (6.57 ± 1.13 nmol/min·mg protein in lean *vs.* 3.43 ± 0.56 nmol/min·mg protein in obese; $P = 0.01$) were calculated for each individual animal.

(11βHSD1/glyceraldehyde-3-phosphate dehydrogenase ratio, 0.56 ± 0.08 in obese *vs.* 1.03 ± 0.15 in lean; $P = 0.02$) were also markedly lower in obese rats, but there was no difference in K_m for corticosterone (Fig. 1). However, 11βHSD1 activity was not different between groups in skeletal muscle or sc fat and was higher in omental fat from obese rats (Fig. 1). In the kidney, both NADP-dependent (11βHSD1) and NAD-dependent (11βHSD2) 11βHSD activities were greater in obese rats (Fig. 1).

Discussion

These data confirm previous reports that obese Zucker rats have heavier adrenal glands (11) and increased urinary free corticosterone excretion (12) compared with lean controls, but this is the first study to examine the excretion of urinary corticosterone metabolites. We also show that there are tissue-specific differences in peripheral metabolism of corticosterone in obese Zucker rats compared with their lean controls.

There are two isozymes of 5α-reductase, but only the type 1 isozyme is expressed in rat liver (13). This isozyme reduces most $\Delta^{4,5}$ unsaturated steroids with similar efficiency, in contrast to the type 2 isozyme, which modulates androgen receptor activation by converting testosterone to active 5α-dihydrotestosterone (14). In obese Zucker rats we found an increase in urinary excretion of 5α-reduced corticosterone metabolites and increased activity of 5α-reductase in liver.

Interconversion of active corticosterone with inactive 11-dehydrocorticosterone (or cortisol and cortisone, respectively, in man) is catalyzed by the isozymes of 11βHSD. 11βHSD type 2 is NAD dependent, catalyzes the dehydrogenase (inactivating) reaction, and is expressed in tissues such as distal nephron, where it protects mineralocorticoid receptors from inappropriate activation by glucocorticoids (15). In obese Zucker rats, we found higher renal NAD-dependent 11βHSD activity *in vitro*, predicting enhanced inactivation of corticosterone. 11βHSD type 1 is NADP(H) dependent and is expressed in a wider range of tissues, including liver, adipose tissue, and skeletal muscle, where it is usually a reductase, reactivating glucocorticoids and maintaining local activation of glucocorticoid receptors (16). 11βHSD1 activity and mRNA levels were decreased in liver of obese animals, whereas affinity for corticosterone was not different. By contrast with liver, 11βHSD1 activity was higher in omental adipose tissue from obese rats, but was not different in skeletal muscle and sc adipose tissue. The overall balance between 11βHSD activities in different tissues was assessed by excretion of corticosterone metabolites in urine. In obese Zucker rats we found differences in urinary metabolites consistent with an alteration in overall balance of whole body 11βHSDs toward inactive 11-dehydrocorticosterone, suggesting that decreased hepatic 11βHSD1 and increased renal 11βHSD2 predominate over changes in other tissues.

The combination of increased 5α-reductase, increased renal 11βHSD2, and decreased hepatic 11βHSD1 activities predict an increased glucocorticoid MCR. In normal circumstances this would result in a compensatory increase in corticosterone production (17). This mechanism could contribute to activation of the hypothalamic-pituitary-adrenal axis (HPA) and adrenocortical hypertrophy in obesity. However, it does not explain why trough plasma corticosterone levels are elevated in obese rats. Abnormalities of central control of the HPA have been sought previously in these animals, but responses to glucocorticoid feedback and stressful stimuli have been variably reported as normal, increased, or decreased (18–20). Moreover, it remains unclear whether leptin resistance alone could explain activation of the HPA, as there may be opposing effects of increased neuropeptide Y and decreased POMC expression (21–23). If obese rats have similarly reduced 11βHSD1 expression in brain and pituitary, this may explain central HPA activation. Mice with transgenic disruption of the 11βHSD1 gene (16) have increased plasma corticosterone levels, which have been attributed to decreased 11βHSD1 (and hence local corticosterone levels) in sites responsible for negative feedback (hippocampus, hypothalamus, and anterior pituitary).

The observed changes in glucocorticoid metabolism also predict changes in peripheral corticosteroid receptor activation. Enhanced inactivation (5α-reductase) and impaired reactivation (11βHSD1) of glucocorticoids in the liver predict lower intracellular corticosterone concentrations. Decreased glucocorticoid exposure would normally be associated with enhanced insulin sensitivity and decreased gluconeogenesis, as in 11βHSD1 knockout mice (16), so it may be that reduced hepatic glucocorticoid exposure in the obese Zucker rat represents a compensatory mechanism that limits the metabolic complications of obesity. By contrast, 11βHSD1 activity was normal in other glucocorticoid targets (skeletal muscle and sc adipose tissue) in obese rats, so the proposed compensatory mechanism may not operate in all tissues. Moreover, 11βHSD1 activity was elevated in omental fat in obese rats, and this predicts higher local glucocorticoid receptor activation and may promote obesity.

The current study does not address the mechanism of dysregulation of 5α-reductase and 11βHSD activities in obesity. With respect to 11βHSD1 we have shown that there is no difference in affinity for corticosterone in obese Zucker rats, and that hepatic mRNA levels are reduced, suggesting that gene transcription is altered in obese animals. It is unlikely that the altered 11βHSD activities are secondary to activation of the HPA, as chronic glucocorticoid excess is associated with up-regulation (not down-regulation) of hepatic 11βHSD1 activity and expression (24). 11βHSD1 is regulated by many other factors, including some that are altered in obesity, such as GH, insulin, tumor necrosis factor-α, and gonadal steroids (25–27). By contrast, 5α-reductase type 1 and 11βHSD type 2 do not appear to be highly regulated enzymes. Further studies will be required to elucidate these mechanisms.

Finally, the differences in metabolism of glucocorticoids in obese Zucker rats mirror observations in obese humans. We and others have reported that obese men and women excrete more cortisol as 5α-reduced metabolites (3, 28). Detailed dynamic tests and arteriovenous sampling have shown that hepatic 11βHSD1 activity is impaired in obese men (29), but adipose 11βHSD1 is either maintained or marginally increased in obesity (30). The present studies demonstrate that the obese Zucker rat will be a useful model in which to

explore the mechanisms of disrupted glucocorticoid metabolism, its impact on body weight regulation, and its potential for therapeutic manipulation.

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