11β-Hydroxysteroid Dehydrogenase Type 1 and Glucocorticoid Metabolism in Obesity

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ABBREVIATIONS

3-HOB 3-hydroxybutyrate
11β-HSD1 11beta-hydroxysteroid dehydrogenase type 1
11β-HSD2 11beta-hydroxysteroid dehydrogenase type 2
ACTH adrenocorticotropic hormone
AME apparent mineralocorticoid excess
BMI body mass index
cDNA complementary deoxyribonucleic acid
C/EBP CCAAT/ enhancer binding protein
CRD cortisone reductase deficiency
CRF clinical research facility
CRH corticotrophin releasing hormone
d3E 9,12,12-[^H]3-cortisone
d3F 9,12,12-[^H]3-cortisol
d4F 9,11,12,12-[^H]4-cortisol
DEPC diethyl pyrocarbonate
DHEA dehydroepiandrosterone
DNA deoxyribonucleic acid
E cortisone
ELISA enzyme-linked immunosorbent assay
ER endoplasmic reticulum
F cortisol
FBC full blood count
FFA free fatty acid
ft film thickness
G6P  glucose-6-phosphate
G6Pase  glucose-6-phosphatase
GC-MS  gas chromatography-mass spectrometry
GH  growth hormone
GR  glucocorticoid receptor
GRE  glucocorticoid response element
H6PDH  hexose-6-phosphate dehydrogenase
HABF  hepatic artery blood flow
HbA1C  glycated haemoglobin A1C
HBF  hepatic blood flow
HDL-C  high density lipoprotein-cholesterol
HF-LC  high fat-low carbohydrate
HIV  human immunodeficiency virus
HOMA  homeostasis model of assessment
HPA  hypothalamic-pituitary-adrenal
HPLC  high pressure liquid chromatography
HSL  hormone sensitive lipase
HV  hepatic vein
ICG  indocyanine green
id  internal diameter
IGF-1  insulin-like growth factor-1
IL-1β  interleukin-1β
IL-6  interleukin-6
LC-MS/MS  liquid chromatography- tandem mass spectrometry
LpL  lipoprotein lipase
LSD least significant differences
MF-MC moderate fat-moderate carbohydrate
MR mineralocorticoid receptor
MRI magnetic resonance imaging
mRNA messenger ribonucleic acid
NAD nicotinamide adenine dinucleotide
NADP(H) nicotinamide adenine dinucleotide phosphate
OFN oxygen free nitrogen
OGTT oral glucose tolerance test
PBF portal vein blood flow
PCOS polycystic ovarian syndrome
PCR polymerase chain reaction
PEPCK phosphoenolpyruvate carboxykinase
PPAR peroxisome proliferator activated receptor
PV portal vein
RCF relative centrifugal force
RNA ribonucleic acid
SRM selective reaction monitoring
SNP single nucleotide polymorphism
T2DM type 2 diabetes mellitus
TFTs thyroid function tests
THE tetrahydrocortisone
THF tetrahydrocortisol
TIPSS transjugular intrahepatic porto-systemic shunt
TNFα tumour necrosis factor alpha
<table>
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<tr>
<td>U&amp;Es</td>
<td>urea and electrolytes</td>
</tr>
<tr>
<td>VLCD</td>
<td>very low calorie diet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
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<td>w/v</td>
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PUBLICATIONS ARISING FROM THIS THESIS TO DATE
(February 2008)


* Joint first authors
MEETING ABSTRACTS


type 1 mRNA levels in human adipose tissue. *Proceedings of The Endocrine Society’s 89th Annual Meeting, Toronto, Canada, June 2007. Poster presentation at the annual meeting of the American Endocrine Society (ENDO), Toronto, Canada, 2007*

**Stimson RH,** Wake DJ, Andrew R, Walker BR (2007) Insulin sensitisation by PPARγ-agonists is not mediated through inhibition of glucocorticoid action in humans.

1) *Proceedings of The Endocrine Society’s 89th Annual Meeting, Toronto, Canada, June 2007. Poster presentation at the annual meeting of the American Endocrine Society (ENDO), Toronto, Canada, 2007*

2) *Endocrine Abstracts 13: P282. Poster presentation at the society for endocrinology British Endocrine Society (BES), Birmingham, 2007*

3) *Scottish Medical Journal 52(2): 54-54. Oral communication at the November meeting of the Scottish Society for Experimental Medicine Meeting, Edinburgh, 2006*

DECLARATION

This thesis is the original research of the author unless otherwise stated. Analysis of plasma full blood count, urea and electrolytes, glucose, liver function tests, thyroid function tests, lipids, glycated haemoglobin A1C, insulin and c-peptide in human volunteers in chapters 3 and 6 was performed by staff at the Clinical Biochemistry laboratory, Western General Hospital, Edinburgh. Plasma free fatty acids and 3-hydroxybutyrate were performed by staff at the biochemistry laboratory at the Royal Hospital for Sick Children, Edinburgh. Analysis of the above plasma metabolites and hormones in the diet studies (chapters 4 and 5) was performed by staff at the Rowett Research Institute, Aberdeen. Measurement of plasma RU486 concentrations in chapter 6 was performed by the Wellcome Trust Clinical Research Facility Mass Spectrometry Core staff.

This work has not been submitted previously at this or any other university for a higher degree.

Signature:

Roland H Stimson
ABSTRACT

The recent obesity epidemic has shown that central obesity is associated with insulin resistance, type 2 diabetes mellitus, hypertension and dyslipidaemia (metabolic syndrome), and the presence of these risk factors increase the risk of cardiovascular disease. People with primary glucocorticoid excess (characterised by high circulating plasma cortisol concentrations) develop the metabolic syndrome, however those with simple obesity do not have elevated plasma cortisol. Tissue glucocorticoid levels, however, are also controlled by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which acts to convert inactive cortisone to the active cortisol, thus amplifying local glucocorticoid concentrations. Obese humans have increased 11β-HSD1 messenger RNA levels in subcutaneous adipose tissue but decreased 11β-HSD1 activity in the liver.

Many questions remain unanswered in this field, which this thesis has addressed. The in vivo importance of visceral adipose tissue in cortisol generation by 11β-HSD1 is unknown, and we have used selective vein cannulation with tracer glucocorticoid infusions in humans to examine whether increased visceral adipose 11β-HSD1 activity may increase cortisol concentrations reaching the liver with its resultant adverse metabolic effects. The cause of the dysregulation of 11β-HSD1 in obesity is unknown, and we have utilised tracer glucocorticoid infusions and adipose biopsies in humans, and examined liver and adipose tissue in rats, to determine whether manipulations in dietary macronutrient content regulate 11β-HSD1. We have also examined the potential benefits of glucocorticoid inhibition in the treatment of insulin resistance in humans, and whether PPARγ-agonists, drugs commonly used in the treatment of type 2 diabetes mellitus, may mediate their beneficial metabolic effects partly through inhibition of 11β-HSD1 in adipose tissue in humans.

Our results show that visceral adipose tissue 11β-HSD1 does not significantly contribute to cortisol concentrations reaching the liver in humans, while hepatic 11β-
HSD1 is responsible for most, if not all, of splanchnic cortisol regeneration. We have shown that a high fat-low carbohydrate diet is a potent regulator of hepatic but not subcutaneous adipose 11β-HSD1 activity in humans. Conversely, a high fat diet decreases 11β-HSD1 in liver and adipose tissue in rats. PPARγ-agonists, however, do not acutely regulate 11β-HSD1 in subcutaneous adipose tissue in humans and their insulin-sensitising effects are independent of glucocorticoid action. We have also shown that acute glucocorticoid inhibition does not improve insulin sensitivity in humans. Chronic inhibition of 11β-HSD1 in adipose tissue, however, may be an important tool in the treatment of metabolic disease.
Chapter 1
Introduction

1.1 OBESITY AND THE METABOLIC SYNDROME

The obesity epidemic observed particularly in affluent societies over the past 25 years has been accompanied by a concordant increase in the prevalence of metabolic disease. Obesity, especially central (visceral) in distribution, is associated with type 2 diabetes mellitus (T2DM), hypertension, hypertriglyceridaemia and low plasma levels of high density lipoprotein-cholesterol (HDL-C). These metabolic abnormalities, if present collectively in an individual, have been termed the metabolic syndrome and predict an increased risk of cardiovascular disease (Reaven 2002). Approximately 25% of elderly UK adults are now classified as having the metabolic syndrome (Wild et al 2006), which may translate into increased numbers of cardiovascular deaths in the near future. Insulin resistance is thought to play a key role in the pathophysiology of the metabolic syndrome, although the determinants of insulin resistance are largely unknown. One area which has received much research interest in recent years has been the role of glucocorticoids, as those with primary glucocorticoid excess (Cushing’s syndrome) develop the components of the metabolic syndrome, and it has been postulated that dysregulation of glucocorticoid production or metabolism could link the features of the metabolic syndrome (Bjorntorp 1991; Walker 2006).

1.2 GLUCOCORTICOID EXCESS AND CARDIOVASCULAR DISEASE

Plasma cortisol concentrations under normal circumstances are regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.1). However, in Cushing’s syndrome circulating cortisol levels are elevated, either secondary to endogenous
production from a cortisol- or adrenocorticotrophic hormone (ACTH)-secreting adenoma, or alternatively from exogenous iatrogenic administration to treat inflammatory conditions such as rheumatoid arthritis or asthma. Patients with Cushing’s syndrome, in an environment of caloric excess, develop visceral obesity (Wajchenberg et al 1995; Rockall et al 2003), hypertension, insulin resistance and dyslipidaemia. In addition after surgical cure, total fat mass decreases (Pirlich et al 2002) with a disproportionate reduction in the visceral component (Lonn et al 1994). The glucocorticoid receptor antagonist RU38486 has been used to treat Cushing’s syndrome, and decreases blood pressure, plasma glucose and triglyceride concentrations (Nieman et al 1985; Chu et al 2001). These findings confirm that primary glucocorticoid excess can cause all of the major components of the metabolic syndrome.
The hypothalamic-pituitary-adrenal (HPA) axis

Circulating cortisol levels are tightly controlled by the HPA axis. Corticotrophin-releasing hormone (CRH) secreted from the hypothalamus stimulates the anterior pituitary gland to produce adrenocorticotropic hormone (ACTH). This in turn stimulates the adrenal cortex to produce cortisol. Circulating cortisol then acts directly on both the hypothalamus and pituitary gland to inhibit CRH and ACTH production respectively, maintaining cortisol levels in the desired range.

There is also evidence that glucocorticoid excess leads not only to risk factors for cardiovascular disease, but also to an increased prevalence of cardiovascular disease. Patients with Cushing’s syndrome have a higher prevalence of carotid atherosclerotic plaques, which decrease in size after surgical cure (Faggiano et al 2003). Those on chronic glucocorticoid treatments have also been studied and show increased risk of
cardiovascular disease (Wei et al 2004; Souverein et al 2004; Davis et al 2005). This increased risk is present in a dose-dependent manner and is irrespective of the disease being treated. Although observational data cannot prove causality, it is plausible to infer that glucocorticoids are atherogenic.

1.3 MECHANISMS OF GLUCOCORTICOID ACTION

1.3.1 Glucocorticoid receptor

Glucocorticoids act on intra-cellular glucocorticoid receptors (GR), which are present in most cells and GR mediates the biological effects mainly by acting as a transcription factor (Giguere et al 1986). GR is present in cell cytoplasm bound to many chaperone proteins, of which heat shock protein 90 is considered imperative to allow ligand binding (Pratt & Toft 2003). Once cortisol has bound to GR in the cell cytoplasm, GR undergoes a conformational change and dissociates from its chaperone proteins, dimerizes and moves to the nucleus and binds as a homodimer to glucocorticoid response elements (GRE) or interacts with other transcription factors to either promote or repress messenger RNA (mRNA) transcription of numerous genes (Newton 2000). Hyperactivation of GR can potentially lead to Cushing’s syndrome despite hypocortisolaemia, indicating the adverse metabolic effects of glucocorticoids are mediated through GR (Iida et al 1990). In addition, a single nucleotide polymorphism (SNP) in the GR gene which increases glucocorticoid sensitivity has been shown to be associated with obesity, dyslipidaemia and even coronary artery disease in some studies (Huizenga et al 1998; Lin et al 1999; Lin et al 2003), although these correlations are not reproduced in other cohorts (Echwald et al 2001; Rosmond et al 2001).
1.3.2 Metabolic effects of glucocorticoids

To determine how glucocorticoid excess causes features of the metabolic syndrome, numerous in vitro and in vivo studies have been performed. Glucocorticoids have diverse effects which promote insulin resistance, a cardinal feature of the metabolic syndrome (Andrews & Walker 1999). For example, glucocorticoids both acutely and chronically potently stimulate appetite, particularly for high fat energy dense foods which are especially prevalent in today’s environment (Dallman et al 2004). Glucocorticoids have effects on the liver, increasing hepatic gluconeogenesis in some (Pagano et al 1983; Rooney et al 1993) but not all (Wajngot et al 1990; Nielsen et al 2004a) studies. However, fasting insulin levels are raised by glucocorticoid treatment in these studies, suggesting that increased insulin secretion is required to maintain similar suppression of hepatic gluconeogenesis. Glucocorticoids increase mRNA transcription of two key enzymes involved in hepatic gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) (Sasaki et al 1984; Wang et al 2004) and glucose-6-phosphatase (G6Pase) (Lange et al 1994; Vander Kooi et al 2005).

Glucocorticoids also cause insulin resistance in muscle and adipose tissue. Insulin-stimulated glucose uptake in the muscle is decreased by glucocorticoid treatment in humans (Pagano et al 1983; Perry et al 2003), in part due to reduced translocation of the glucose transporter GLUT4 to the cell surface (Dimitriadis et al 1997; Weinstein et al 1998). Glucocorticoids have direct but complex metabolic effects in adipose tissue, the major organ of lipogenesis and lipolysis. Rates of lipolysis are known to be increased in insulin resistance and obesity (Koutsari & Jensen 2006). In humans, in vivo measurements using microdialysis have shown that glucocorticoids increase subcutaneous adipose tissue lipolysis (Tomlinson et al 2007). Furthermore, insulin resistance caused by glucocorticoids is improved by inhibition of lipolysis (Ekstrand et al 1992). Glucocorticoids regulate transcription of several important enzymes involved in lipolysis in adipose tissue, including increasing hormone sensitive lipase (HSL) expression in rat adipocytes (Slavin et al 1994). In contrast to their effects in the liver, glucocorticoids decrease PEPCK expression and activity in rat adipose
tissue (Meyuhas et al 1976; Nechushtan et al 1987), leading to decreased glyceroneogenesis and higher plasma free fatty acid (FFA) concentrations. However, despite direct induction of lipolysis, glucocorticoids promote fat deposition, particularly in a central distribution. In vitro, glucocorticoids promote pre-adipocyte differentiation into adipocytes in the presence of insulin (Hauner et al 1987), potentially increasing adipocyte numbers. Glucocorticoids also increase lipoprotein lipase (LpL) activity (Fried et al 1993), which hydrolyses circulating triglycerides prior to uptake in adipose tissue. This effect may be greater in visceral rather than subcutaneous adipose tissue, thus promoting central fat accumulation (Rebuffe-Scrive et al 1988).

In addition to promoting insulin resistance, glucocorticoids exert direct adverse effects on the pancreas. In vitro, glucocorticoids directly inhibit insulin release by beta-cells (Lambillotte et al 1997; Ling et al 1998), an effect reproduced in vivo in transgenic mice selectively over-expressing GR in pancreatic β-cells (Delaunay et al 1997). To summarise, in acute times of stress and starvation, glucocorticoids thus function appropriately to increase appetite to promote food seeking behaviour, and to decrease insulin secretion and cause insulin resistance in the liver, adipose tissue and muscle. Thereby, glucocorticoids increase gluconeogenesis and lipolysis in order to appropriately mobilise energy from food stores. However, in chronic glucocorticoid excess these alterations become maladaptive and result in increased appetite and insulin resistance, leading to unfavourable metabolic effects including hyperglycaemia and dyslipidaemia. Moreover, in the presence of a plentiful energy supply with subsequent high circulating insulin concentrations, glucocorticoids have a net lipogenic effect to promote fat deposition in the visceral depot in addition to enhancing lipolysis in some subcutaneous adipose beds, aggravating the adverse metabolic consequences of glucocorticoid excess.
Several studies have examined whether there are abnormalities in circulating glucocorticoid levels in obesity and the metabolic syndrome. Morning fasting plasma cortisol levels are mildly elevated in people with components of the metabolic syndrome, including insulin resistance (Stolk et al 1996; Phillips et al 1998; Walker et al 2000), hypertension (Filipovsky et al 1996; Phillips et al 1998; Walker et al 2000; Phillips et al 2000), and hypertriglyceridaemia (Phillips et al 1998; Walker et al 2000). Furthermore, increased activity of the hypothalamic-pituitary-adrenal (HPA) axis, as judged by the adrenal cortisol secreted in response to ACTH, positively correlates with hypertension and hypertriglyceridaemia (Reynolds et al 2001). Higher 24-hour urinary Cortisol metabolite excretion, a measure of Cortisol secretion rate, also associates with insulin resistance (Walker et al 1998). Elevated plasma cortisol as a result of enhanced Cortisol secretion rate may therefore link some features of the metabolic syndrome. Importantly, however, elevated plasma cortisol does not explain the association of features of metabolic syndrome with central obesity.

Increased HPA axis activity is also observed in people with simple obesity, measured either by corticotrophin-releasing hormone (CRH) or ACTH stimulation tests (Pasquali et al 1993; Solano et al 2001), or by the cortisol secretion rate (Strain et al 1982; Rask et al 2001; Rask et al 2002; Westerbacka et al 2003). However, fasting morning plasma cortisol concentrations have been found to be either low (Strain et al 1982; Hautanen et al 1997; Walker et al 2000; Phillips et al 2000; Jessop et al 2001; Solano et al 2001; Rask et al 2002; Reynolds et al 2003) or normal (Kopelman et al 1988; Weaver et al 1993; Pasquali et al 1993; Yanovski et al 1997) in obese subjects. These contrasting features can be accounted for by increased cortisol clearance. Cortisol is metabolised predominantly in the liver by the A-ring reductases (Figure 1.2), and indeed obesity is associated with increased hepatic 5α- and 5β-reductase activities (Andrew et al 1998). Increased clearance by 5β-reductase is also
seen in people with T2DM (Andrews et al. 2002) and those with fatty liver (Westerbacka et al. 2003), the hepatic manifestation of the metabolic syndrome. In addition, cortisol clearance by 5α-reductase is increased in women with polycystic ovarian syndrome (PCOS) (Stewart et al. 1990a; Tsilchorozidou et al. 2003), a condition associated with insulin resistance. As a result of increased clearance, plasma cortisol levels tend to fall, but intact negative feedback control of the HPA axis leads to a compensatory increase in cortisol secretion and a hyper-responsive HPA axis in response to other stimuli (Figure 1.1). Increased HPA axis activity also results in hyperandrogenism. However, since the net effect is that plasma cortisol is not elevated these alterations in glucocorticoid production rate and clearance do not account for the phenotypic similarities between central obesity and Cushing’s syndrome.
Figure 1.2  Pathways of cortisol regeneration and metabolism

Cortisol (F) and cortisone (E) are metabolised primarily by the A-ring reductases in the liver. F is metabolised by both 5α- and 5β-reductase, while E is solely metabolised by 5β-reductase. The dihydrometabolites are quickly further metabolised by 3α-hydroxysteroid dehydrogenase (3α-HSD) and excreted in the urine predominantly as conjugated forms of the tetrahydrometabolites α-THF, THF and THE.

1.5 REGULATION OF TISSUE GLUCOCORTICOIDS BY THE 11BETA-HYDROXYSTEROID DEHYDROGENASES

While circulating glucocorticoid concentrations are tightly controlled by the HPA axis, tissue glucocorticoid levels are also regulated by an additional mechanism. The 11beta-hydroxysteroid dehydrogenases (11β-HSDs) are enzymes which act to either amplify or decrease active tissue glucocorticoid levels (Figure 1.3). There are two isozymes from separate genes, termed type 1 (11β-HSD1) and type 2 (11β-HSD2) respectively. 11β-HSD2 is highly expressed in the kidney, placenta, colon, and
salivary glands (Rusvai & Naray-Fejes-Toth 1993) and utilises the co-factor nicotinamide adenine dinucleotide (NAD) to convert the active cortisol (Kendall’s compound F) to the inactive cortisone (E) in humans (corticosterone to 11-deoxycorticosterone in rodents) (Brown et al 1993). Glucocorticoids can bind and activate mineralocorticoid receptors (MR) in addition to GR (Arriza et al 1987). 11β-HSD2 is expressed in tissues with high levels of MR, where it functions to prevent MR activation by glucocorticoids and allows selective activation of MR by aldosterone (Funder et al 1988; Edwards et al 1988). Inactivation of 11β-HSD2 by gene mutations leads to the syndrome of apparent mineralocorticoid excess (AME), which presents with hypertension and hypokalaemia but with low aldosterone levels (Ulick et al 1979; Mune et al 1995). Similarly, mice with selective disruption of 11β-HSD2 develop hypokalaemia and hypertension due to endogenous glucocorticoid activation of MR, while plasma aldosterone levels are low (Kotelevtsev et al 1999).
11β-HSD1 is widely distributed in the body, but with high expression in the liver, adipose tissue and certain areas of the brain including the hippocampus (Seckl & Walker 2004). This enzyme is nicotinamide adenine dinucleotide phosphate (NADP(H))-dependent and can potentially act bi-directionally to either increase or decrease tissue cortisol levels (Lakshmi & Monder 1985; Lakshmi & Monder 1988). In vitro, 11β-HSD1 acts as a dehydrogenase when cells are disrupted but in vivo 11β-HSD1 functions predominantly as a reductase, thus amplifying tissue
Once it was known that 11β-HSD1 functioned primarily to increase glucocorticoid levels in tissues such as the liver and adipose tissue, the hypothesis arose that increased 11β-HSD1 activity could be a potential cause of the metabolic syndrome, increasing local glucocorticoid levels without necessarily altering circulating glucocorticoid concentrations (Bujalska et al 1997). This led to strong interest in 11β-HSD1 which consequently has become a research ‘hot topic’ over the past 10 years.

1.6 TRANSGENIC MANIPULATIONS OF 11β-HSD

Transgenic mice have been created to determine whether increased 11β-HSD1 activity can directly cause obesity and the metabolic syndrome. Mice over-expressing 11β-HSD1 selectively in adipose tissue utilising the fatty acid binding protein AP2 promoter have 2-3 fold increased adipose tissue corticosterone levels but low or normal plasma corticosterone concentrations (Masuzaki et al 2001). These mice develop central obesity, insulin resistance, glucose intolerance, dyslipidaemia, and hypertension (Masuzaki et al 2001; Masuzaki et al 2003). These exciting findings showed for the first time that dysregulation of 11β-HSD1 could cause the metabolic syndrome. Mice which selectively over-express 11β-HSD1 in the liver (2 and 5 fold) have also been generated using the ApoE promoter (Paterson et al 2004). These mice develop hypertension, potentially secondary to increased angiotensinogen levels with
resultant activation of the renin-angiotensin-aldosterone system. They develop mild insulin resistance and dyslipidaemia, but interestingly do not become obese, indicating that increased adipose but not hepatic 11β-HSD1 activity is obesogenic.

Mice with selective disruption of 11β-HSD1 have also been created to determine if this is metabolically beneficial. These mice have a normal lifespan and, on a high fat diet, resist hyperglycaemia and weight gain, while they deposit fat in peripheral, not visceral, sites (Kotelevtsev et al 1997; Morton et al 2004a). Their plasma lipid profile is also improved, with decreased plasma triglycerides due to improved lipid oxidation and increased HDL-C (Morton et al 2001). Corticosterone levels are increased in plasma in some strains (Harris et al 2001; Paterson et al 2007), but are decreased in tissues which normally express 11β-HSD1 (Yau et al 2001). Transgenic rodents with tissue-specific deletions of 11β-HSD1 will be important tools to further demonstrate the important metabolic sites for inhibition of glucocorticoid action, although these have not been generated to date. However, transgenic mice have been created which over-express 11β-HSD2 selectively in adipose tissue, again using the AP2 promoter (Kershaw et al 2005). These mice show certain similarities to mice with selective disruption of 11β-HSD1, resisting weight gain and glucose intolerance on a high fat diet. They deposit less fat in subcutaneous and visceral depots, and have increased energy expenditure. The phenotypes of the transgenic mice discussed above support the hypothesis that increased 11β-HSD1 activity can be metabolically disadvantageous and inhibition metabolically beneficial.

1.7 11β-HSD1 ACTIVITY IN OBESITY

After the realisation that 11β-HSD1 functions mainly as a reductase in vivo, coupled with the exciting results from transgenic manipulations, many research studies have examined whether 11β-HSD1 is altered in human obesity.
1.7.1 ‘Whole body’ regeneration of cortisol from cortisone

In order to measure 11β-HSD1 activity in humans, 24 hour urinary collections of cortisol metabolites were formerly considered a good non-invasive method of estimating whole body enzyme activity. Cortisol (F) and cortisone (E) are predominantly metabolised by the hepatic A-ring reductases, 5α- and 5β-reductase before further metabolism by 3α-hydroxysteroid dehydrogenase and excretion predominantly as their tetrahydrometabolites 5α-tetrahydrocortisol (α-THF), 5β-tetrahydrocortisol (THF), and 5β-tetrahydrocortisone (THE) (Figure 1.2). The ratio of cortisol: cortisone metabolites excreted in the urine, (α-THF + THF)/THE, has been used to infer total body 11β-HSD1 activity in many studies. Conversely, the ratio of urinary free cortisol: cortisone has been used to estimate renal 11β-HSD2 activity (Palermo et al 1996;Best & Walker 1997).

Several studies have examined global 11β-HSD1 activity using urinary ratios, but unfortunately the results are inconsistent. The (α-THF + THF)/ THE ratio has been found to be decreased (Stewart et al 1999;Rask et al 2001;Sandeep et al 2005;Wiegand et al 2007), normal (Fraser et al 1999;Reynolds et al 2001;Andrew et al 2002a;Westerbacka et al 2003;Dimitriou et al 2003), or increased (Andrew et al 1998;Rask et al 2002) in obese when compared with normal weight individuals (Table 1.1). In addition, this ratio is not different between people with T2DM when compared to healthy volunteers (Kerstens et al 2000;Andrews et al 2002;Valsamakis et al 2004). Some of the discrepancies observed between these studies may be because of gender differences in total body 11β-HSD1 activity, as the ratio is lower in females (Raven & Taylor 1996; Fraser et al 1999;Toogood et al 2000). However, the main problem with this method of assessment is the lack of specificity for 11β-HSD1 activity, because the ratio is affected by 11β-HSD2, 5α- and 5β-reductase activities in addition to 11β-HSD1. Consequently, alterations in any of these parameters will change the ratio and thus the ‘estimated’ 11β-HSD1 set point. For example, 5α- and/ or 5β-reductase activity are altered by obesity (Andrew et al 1998;Rask et al 2002), gender (Finken et al 1999;Toogood et al 2000) and liver fat content (Westerbacka et al 2003).
Table 1.1  Dysregulation of 11β-HSD1 in human obesity

Summary of the results of the studies performed to date which examined 11β-HSD1 in whole body, liver or adipose tissue in humans, indicating if 11β-HSD1 was increased, unchanged or decreased in obesity.

<table>
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<tr>
<th>Systemic (whole body)</th>
<th>Liver</th>
<th>Adipose</th>
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<tbody>
<tr>
<td><strong>Urinary ratios</strong></td>
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<tr>
<td>Increased (1,2)</td>
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<tr>
<td>Unchanged (3-7)</td>
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<tr>
<td>Decreased (8-11)</td>
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<td><strong>D3-Cortisol generation</strong></td>
<td></td>
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<tr>
<td>Unchanged (10,25)</td>
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**Systemic (whole body)**

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<tr>
<th><strong>Liver</strong></th>
<th><strong>Subcutaneous biopsies</strong></th>
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<tr>
<td>First pass cortisone</td>
<td>Decreased (8,9,12)</td>
</tr>
<tr>
<td>Subcutaneous microdialysis</td>
<td>Unchanged (24)</td>
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**Liver**

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<th><strong>Adipose</strong></th>
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<tr>
<td>Subcutaneous biopsies</td>
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<td>Increased (10)</td>
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**Adipose**

<table>
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<tr>
<th><strong>Visceral biopsies</strong></th>
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<td>Increased (19,21,22,26)</td>
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A more specific method of quantifying total 11β-HSD1 activity has been developed in humans. This has been achieved through intravenous infusions of non-radioactively labelled cortisol, in the form of deuterated 9,11,12,12-²[H]₄-cortisol (Andrew et al 2002b). In vivo, the 9,11,12,12-²[H]₄-cortisol (d4F) is converted by 11β-dehydrogenase predominantly in the kidney, with the resultant loss of the deuterium atom on the 11th carbon, to form 9,12,12-²[H]₃-cortisone (d3E). This is then converted by 11β-HSD1 in the reductase direction with the addition of an unlabelled hydrogen to generate 9,12,12-²[H]₃-cortisol (d3F) (Figure 1.4). Therefore, d4F is irreversibly converted to d3E (d4F cannot be regenerated), while d3F and d3E are interchangeable. Assuming the deuterium atoms do not alter enzyme activity, the ratio of d3F:d4F in steady state can be used to derive the rate of appearance of d3-cortisol, a specific measure of whole body 11β-HSD1 reductase activity. Two studies have examined the effect of obesity on total body 11β-HSD1 activity using this technique, and both have shown no difference (Sandeep et al 2005; Basu et al 2005), while the second study also found no change in those with T2DM. These results suggest more strongly that obesity does not alter whole body 11β-HSD1 reductase activity in humans, and changes in urinary cortisol metabolite ratios are influenced by confounding factors. This lack of difference in whole body 11β-HSD1 suggests that either the enzyme is unchanged in obesity, or that it is altered in a tissue-specific fashion such that an increase in one tissue is balanced by a decrease in another.
Figure 1.4  Quantifying cortisol production using deuterated cortisol

Adapted from (Andrew et al 2005). 9,11,12,12-\textsuperscript{2}H\textsubscript{4}-cortisol (d\textsubscript{4}-cortisol) is converted mainly in the kidney to d\textsubscript{3}-cortisone, with the loss of the deuterium on C\textsubscript{11}. The d\textsubscript{3}-cortisone is then reduced by 11\beta-HSD1, predominantly in the liver and adipose tissue, with the addition of an unlabelled hydrogen to form d\textsubscript{3}-cortisol. Assuming that 11\beta-HSD2, 5\alpha-reductase and 5\beta-reductase metabolise d\textsubscript{4}-cortisol and d\textsubscript{3}-cortisol at the same rate (i.e. the extra deuterium on C\textsubscript{11} does not alter affinity for these enzymes), the differences between d\textsubscript{3}-cortisol and d\textsubscript{4}-cortisol metabolism therefore specifically reflect 11\beta-HSD1 reductase activity.

\begin{center}
\includegraphics[width=0.8\textwidth]{cortisol_metabolism.png}
\end{center}

1.7.2 Tissue-specific dysregulation of 11\beta-HSD1

Tissue-specific dysregulation of 11\beta-HSD1 has been observed in obese Zucker rats, which are leptin-resistant due to a mutation in the leptin receptor. These rats have increased 11\beta-HSD1 activity in omental adipose tissue, but decreased mRNA expression and activity in the liver (Livingstone et al 2000a). Similarly, leptin
deficient mice have decreased hepatic (Liu et al 2003) and increased subcutaneous adipose 11β-HSD1 mRNA levels (Masuzaki et al 2001). However, increased adipose glucocorticoid regeneration is not observed in a polygenic model of obesity in mice, which has decreased adipose and increased hepatic 11β-HSD1 mRNA and activity (Morton et al 2005). Tissue-specific dysregulation of 11β-HSD1 has been recently investigated in humans in several studies.

1.7.2.1 Liver

The conversion of orally administered cortisone to cortisol has been used as a method to estimate hepatic 11β-HSD1 activity by first pass metabolism in humans. It has long been assumed that oral steroids are passively absorbed across the intestinal cells into the portal venous circulation due to their lipophilic nature, however some recent evidence shows that specific organic anion transporters are required for secretion of cortisol from the adrenal cortex (Beery et al 2003). These transporters are present in the intestine (Zair et al 2008), meaning that intestinal absorption of orally administered cortisone may be transporter-dependent. Oral cortisone is metabolised predominantly in the liver (Andrew et al 2005), meaning this test is a reliable estimate of hepatic 11β-HSD1 activity. The results achieved following oral cortisone administration are consistent, showing that hepatic 11β-HSD1 activity is decreased in obese individuals (Stewart et al 1999; Rask et al 2001; Rask et al 2002). Hepatic 11β-HSD1 activity has also been shown to be decreased, albeit to a lesser extent, in people with T2DM (Andrews et al 2002). However, the increased cortisol metabolism by 5α- and 5β-reductase in obesity (see chapter 1.4) will also decrease cortisol generation after cortisone ingestion, which may be a potential confounder of these results.

1.7.2.2 Subcutaneous adipose tissue

Adipose tissue, particularly from the subcutaneous compartment, has been investigated in detail to test for dysregulation of 11β-HSD1 (Table 1.1). There is now a compelling body of evidence that 11β-HSD1 mRNA (Paulmyer-Lacroix et al
2002; Lindsay et al 2003; Wake et al 2003; Engeli et al 2004; Kannisto et al 2004; Nair et al 2004; Desbriere et al 2006; Goedecke et al 2006; Michailidou et al 2007; Paulsen et al 2007; Li et al 2007) and activity (Rask et al 2001; Rask et al 2002; Lindsay et al 2003; Wake et al 2003) are increased in subcutaneous adipose tissue in obesity, with mRNA levels correlating with activity when both are measured (Lindsay et al 2003; Wake et al 2003). However, the activities are measured in vitro in homogenised tissue so may not reliably represent what is occurring in the intracellular environment in vivo. Arterio-venous sampling across the subcutaneous adipose bed has been performed to address this deficit, and shown cortisone extraction across the subcutaneous bed which is most likely due to 11β-HSD1 reductase activity as other enzymes which metabolise cortisone are not present in adipose tissue (Katz et al 1999). In addition, microdialysis infusion of tritiated cortisone showed that 11β-HSD1 reductase activity was increased in obesity in vivo (Sandeep et al 2005). Somewhat surprisingly however, the two studies which have attempted to quantify adipose cortisol concentrations have not found any correlation between 11β-HSD1 mRNA or activity and tissue cortisol levels (Lindsay et al 2003; Wake et al 2003). This may be because analysis techniques are not sensitive enough at present, or that cortisol regeneration by 11β-HSD1 only contributes a small percentage of tissue cortisol compared to that derived from adrenal production. These adipose tissue biopsies are also generally taken in the morning when plasma cortisol concentrations are relatively high, and it may be that the contribution from 11β-HSD1 is more important in the evening or overnight when levels are much lower. Subjects undergoing biopsies may also be stressed, increasing the contribution of plasma cortisol to the intra-adipose measurements.

In addition to being elevated in obesity, 11β-HSD1 mRNA and/or activity correlate with insulin resistance in most of the above studies, although interestingly the presence of type 2 diabetes mellitus does not lead to increased 11β-HSD1 activity over and above any effect of obesity (Andrews et al 2002; Koistinen et al 2004). Whether increased subcutaneous adipose 11β-HSD1 activity is a cause or consequence of insulin resistance is yet to be established. Subcutaneous adipose
tissue 11β-HSD1 has been shown to positively correlate with fasting glucose (Lindsay et al 2003; Goedcke et al 2006) and mean arterial blood pressure (Goedcke et al 2006), although this is not reproduced in all studies while subcutaneous 11β-HSD1 does not correlate with plasma lipids (Wake et al 2003; Michailidou et al 2007).

1.7.2.3 Visceral Adipose Tissue

The results in subcutaneous adipose tissue show that dysregulation of 11β-HSD1 may be an important pathogenic mediator of adverse metabolic disease. Visceral adipose tissue though is often cited as a more important adipose bed, as visceral volume correlates more strongly with insulin resistance (Pouliot et al 1992; Rexrode et al 1998). In addition, free fatty acids and adipokines from visceral adipose tissue are released directly into the portal vein, where they could exert damaging metabolic effects on the liver. Glucocorticoid action may be more important in visceral fat, as the glucocorticoid receptor (GR) is more highly expressed in this site compared to subcutaneous adipose (Rebuffe-Scrive et al 1985; Pedersen et al 1994). Despite the technical difficulty of obtaining visceral adipose tissue in humans, several studies have directly examined 11β-HSD1 in this adipose bed.

11β-HSD1 mRNA and activity are increased in omental compared with subcutaneous adipose vascular stromal cells (Bujalska et al 1997; Bujalska et al 1999), potentially highlighting the importance of 11β-HSD1 in visceral adipose. The effect of obesity has been examined in this depot, and the first study performed did not find any correlation between omental adipose tissue 11β-HSD1 mRNA levels and obesity, indeed 11β-HSD1 activity in cultured omental preadipocytes was decreased in the obese group (Tomlinson et al 2002). This study also found that 11β-HSD1 mRNA and activity in subcutaneous adipose tissue was unaltered in obesity. Two other studies also showed no change in 11β-HSD1 in visceral adipose tissue in obesity (Goedcke et al 2006; Li et al 2007). However, several studies have since showed increased 11β-HSD1 mRNA (Desbriere et al 2006; Mariniello et al 2006; Michailidou et al 2007; Paulsen et al 2007) in visceral adipose tissue in obese
compared with lean people, indicating that 11β-HSD1 may be dysregulated in both subcutaneous and visceral adipose depots in obesity.

Ideally, in vivo measurements would be made to determine if visceral adipose tissue is generating more cortisol in obese individuals. Portal and omental venous cortisol concentrations are not different than peripheral cortisol levels in obese patients (Aldhahi et al 2004; Tarantino et al 2007), although extra-adrenal cortisol production is very difficult to assess during the stress of bariatric surgery. Deuterated cortisol infusions with the additional placement of catheters in the hepatic vein have been used to quantify splanchnic cortisol production by 11β-HSD1 in healthy subjects. These studies have shown that 11β-HSD1 in the splanchnic tissues generates a similar amount of cortisol to the adrenals (Basu et al 2004; Andrew et al 2005). Indirect estimates of the respective contributions of adipose tissue and liver approximate these to be two-thirds from visceral tissues and one-third from the liver (Andrew et al 2005). However, simultaneous portal and hepatic vein cannulation studies in dogs show that the liver and not adipose is responsible for all splanchnic 11β-HSD1 activity (Basu et al 2006a). Unfortunately, no studies have as yet been reported using portal vein cannulation with tracer infusion in humans to accurately quantify visceral adipose tissue cortisol production. One study has examined total splanchnic cortisol production in obesity, and found no change in either obese or diabetic individuals (Basu et al 2005). However, any increased activity in visceral adipose tissue could potentially be counterbalanced by the decreased hepatic 11β-HSD1 activity observed in obesity (Stewart et al 1999; Rask et al 2001; Rask et al 2002).

Although there are reports that 11β-HSD2 is dysregulated in adipose tissue in obesity (Engeli et al 2004; Milagro et al 2007), the transcript levels are extremely low and enzyme activity has yet to be convincingly demonstrated.
1.7.2.4 Skeletal muscle

Any role for 11β-HSD1 in skeletal muscle was initially neglected since expression in rodent muscle is very low. In addition, 11β-HSD1 activity in skeletal muscle was no different between lean and obese Zucker rats (Livingstone & Walker 2003). However, more substantial 11β-HSD1 mRNA and activity is present in human skeletal muscle (Whorwood et al 2001), in both the slow and fast twitch fibres (Jang et al 2006). There are conflicting reports on whether dysregulation of 11β-HSD1 in this tissue could mediate adverse metabolic effects. Although one study showed no association between 11β-HSD1 mRNA levels and body composition (Nair et al 2004), a second found increased 11β-HSD1 mRNA expression in cultured myoblasts in association with BMI, insulin resistance and systolic blood pressure (Whorwood et al 2002). Isolated myotubes from people with T2DM reportedly have increased 11β-HSD1 mRNA levels (Abdallah et al 2005), but another group found decreased skeletal muscle 11β-HSD1 activity in T2DM (Jang et al 2007). In vivo cortisol production by skeletal muscle 11β-HSD1 has been quantified using deuterated cortisol infusions with measurements taken across the leg, which did not find any significant cortisol production by 11β-HSD1 in humans (Basu et al 2005). Further work is needed to elucidate if this enzyme is important and dysregulated in the metabolic syndrome in muscle.

1.8 MECHANISMS OF ALTERED 11β-HSD1

Thus, there is good evidence that dysregulation of 11β-HSD1 can cause metabolic disease in rodents, and that tissue-specific dysregulation of 11β-HSD1 occurs in rodents and humans. It is, however, not known whether obese individuals for example have constitutively ‘switched on’ 11β-HSD1 in adipose tissue from a young age potentially causing predisposition to obesity/metabolic syndrome, or whether this is a secondary effect of obesity. 11β-HSD1 expression and activity in adipose tissue correlates prospectively over 2.5 years with changes in weight and insulin resistance, indicating that 11β-HSD1 may predict rather than result from an adverse
metabolic profile (Koska et al 2006). Recent work has focused on the genetics and on the regulation of 11β-HSD1.

1.8.1 Genetics of 11β-HSD1

Another method of determining whether dysregulation of 11β-HSD1 is a primary cause of obesity and/or the metabolic syndrome is to determine if mutations in the gene are associated with metabolic abnormalities. 11β-HSD1 is encoded by the HSD11B1 gene on chromosome 1, which contains 6 exons and is approximately 30 kilobase pairs in size (Tannin et al 1991; Draper et al 2002). Single nucleotide polymorphisms (SNPs) in the HSD11B1 gene have been reported to be associated with reduced mRNA transcription (Draper et al 2003; de Quervain et al 2004), indicating these mutations may have functional significance. Several common SNPs and altered numbers of microsatellite repeats have been discovered in untranslated and intronic regions of the gene. Most studies have not found a correlation between these variants in HSD11B1 and body composition (Draper et al 2002; Robitaille et al 2004; Nair et al 2004; Franks et al 2004; Smit et al 2007). However, one study found that children homozygous for an intronic polymorphism had increased BMI, although only 11 homozygotes were studied and heterozygotes were no different from controls (Gelernter-Yaniv et al 2003). The evidence on the whole suggests that SNPs in the HSD11B1 gene do not cause alterations in body composition.

Polymorphisms in the HSD11B1 gene have been associated with other features of the metabolic syndrome. One specific SNP (G→A, rs846910) has been associated with insulin resistance, T2DM (Nair et al 2004) and hypertension (Franks et al 2004), although the genotype did not correlate with 11β-HSD1 mRNA transcript levels in either adipose tissue or skeletal muscle in a smaller subset. These results may suggest that polymorphisms in the 11β-HSD1 gene do not determine whether obesity develops, but do determine whether associated metabolic abnormalities will occur if obesity is present.
1.8.1.1 Cortisone Reductase Deficiency

Cortisone reductase deficiency (CRD) is a very rare PCOS-like disorder first reported in 1984 (Taylor et al 1984) which is attributed to congenital lack of 11β-HSD1 activity. CRD most commonly presents in women, and is characterised by hirsutism, hyperandrogenism and oligomenorrhoea (Phillipov et al 1996; Jamieson et al 1999; Biason-Lauber et al 2000). However, one case has been reported in a 6 year old boy who presented with precocious puberty (Malunowicz et al 2003). Although these people do not appear to be protected from developing obesity, they are not invariably overweight or obese. These patients all excrete a very high proportion of their urinary cortisol metabolites in the inactive 11-keto form, and consequently have very low (α-THF+THF)/THE urinary ratios. They are also unable to convert oral cortisone to cortisol. Their inability to regenerate cortisol presumably leads to lower tissue cortisol concentrations and hyperactivation of the HPA axis to normalise plasma cortisol levels, with resultant androgen excess. However, their susceptibility to insulin resistance and other cardiometabolic complications of obesity has not been tested systematically.

It was originally hypothesized that CRD patients would have severe mutations in the HSD11B1 gene. However, sequencing has revealed no mutations in any of these patients (Nikkila et al 1993; Jamieson et al 1999; Nordenstrom et al 1999). However, one group hypothesized that H6PDH, which confers directionality of 11β-HSD1 by generating the co-factor NADPH (Figure 1.5), could be mutated and cause CRD. They discovered that all three of their patients with CRD had polymorphisms in intron 3 of HSD11B1 and intron 5 of H6PD (Draper et al 2003). Thus, it was hypothesized that defects in both genes were required to cause this disorder. However, several recent studies have shown that these polymorphisms are more common, with these specific SNPs found in combination in approximately 3-7% of the general population who lack any biochemical evidence of CRD (White 2005; San Millan et al 2005; Draper et al 2006; Smit et al 2007). Further examination of these patients is needed to unpick the genetic cause of this interesting disease and confirm
the role of H6PDH. Recent work has shown that conditions such as the glycogen storage disorders type 1a and 1b, which dramatically change the availability of the H6PDH substrate glucose-6-phosphate (G6P) required to generate NADPH, cause dramatic changes in 11β-HSD1 reductase activity (Walker et al 2007). Although these patients have other metabolic features not seen in CRD, it shows that other enzyme deficiencies can significantly alter 11β-HSD1 activity.

**Figure 1.5 Co-factor generation and 11β-HSD1 activity**

Glucose-6-phosphate (G6P) enters the endoplasmic reticulum (ER) through the G6P transporter (G6PT) situated in the ER membrane. G6P is converted to 6-phosphogluconate (6-PGL) by hexose-6-phosphate dehydrogenase (H6PDH), in addition generating reduced nicotinamide adenine dinucleotide phosphate (NADPH) during this reaction. The 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme utilises NADPH as co-factor to catalyse conversion of cortisone to cortisol.
1.8.2 Weight Loss and Nutrition

One approach to determine reversibility of up-regulation of 11ß-HSD1 in adipose tissue in obesity is to examine the effect of weight loss in humans. Total body 11ß-HSD1 activity, measured by the urinary (α-THF + THF)/ THE ratio, is unchanged after 10% weight loss on a very low calorie diet (VLCD) (Johnstone et al 2004), but this measurement would not reveal tissue-specific changes. Two studies examining the effects of weight loss on subcutaneous adipose tissue showed 1) no change in cultured abdominal adipocyte 11ß-HSD1 mRNA expression after 5% weight loss (Engeli et al 2004), and 2) no change in mRNA expression in gluteal tissue after 14% weight loss (Tomlinson et al 2004a). These two studies certainly do not show any decrease in 11ß-HSD1 after weight loss, and the latter study in fact found increased 11ß-HSD1 mRNA in isolated adipocytes after weight loss (Tomlinson et al 2004a).

However, there may be confounding effects in weight loss studies. Changes in dietary constituents may have profound effects on 11ß-HSD1, such that the different diets used to achieve weight loss in the above studies may account for any discrepancies. Mice fed a high fat diet show a dramatic decrease in 11ß-HSD1 mRNA and activity in all fat depots after 2-18 weeks (Morton et al 2004b), while a similar decrease in 11ß-HSD1 activity in adipose tissue and liver has been observed in rats (Drake et al 2005). In contrast, a high fat diet increases 11ß-HSD1 activity in the brain, potentially regulating appetite (Densmore et al 2006). In humans, in vivo studies have been performed in humans showing that 11ß-HSD1 responds to nutritional factors. For example, infusion of intralipid (an aqueous suspension of lipid droplets) increases subcutaneous adipose tissue 11ß-HSD1 activity (Wake et al 2006). Moreover, eating a mixed meal acutely increases whole body but not splanchnic 11ß-HSD1 activity measured using deuterated cortisol infusion (Basu et al 2006b), which may be accounted for by changes in subcutaneous adipose tissue or skeletal muscle. Intravenous insulin administration acutely increases whole body 11ß-HSD1 activity, which may account for this effect (Wake et al 2006). However, the more chronic effects of diet on regulation of glucocorticoid metabolism are
unknown. More work is needed to determine whether diet is partly responsible for the dysregulation of 11β-HSD1 in obesity.

### 1.8.3 Hormonal Regulation

11β-HSD1 is regulated by numerous hormones (Tomlinson et al 2004b). Several studies have examined the regulation of 11β-HSD1 in the liver and adipose tissue in an attempt to elucidate which factors may be responsible for the tissue-specific dysregulation of this enzyme in obesity. For example, in vitro, cortisol increases 11β-HSD1 expression and activity in human adipose stromal cells and adipocytes (Bujalska et al 1997; Bujalska et al 1999; Engeli et al 2004). However, plasma cortisol is not elevated in obesity, while patients with Cushing’s syndrome have normal adipose 11β-HSD1 mRNA levels (Mariniello et al 2006). Obesity has been recently termed a ‘pro-inflammatory state’, with increased plasma and adipose tissue cytokine levels (Hotamisligil et al 1995; Diamant et al 2005). The cytokines IL-1β, IL-6 and TNFα increase 11β-HSD1 mRNA or activity in pre-adipocytes or adipocytes but interestingly not in hepatocytes, highlighting tissue-specific regulation (Handoko et al 2000; Tomlinson et al 2001; Friedberg et al 2003). Furthermore, 11β-HSD1 is increased in adipose tissue in patients with human immunodeficiency virus (HIV) treatment-associated lipodystrophy (Sutinen et al 2004), a condition associated with marked intra-adipose inflammation. More work is needed to determine whether increased inflammation in adipose tissue is responsible for the increased 11β-HSD1 in obesity.

Conversely, several hormones and transcription factors decrease 11β-HSD1. Growth hormone (GH), through insulin-like growth factor-1 (IGF-1), decreases whole body 11β-HSD1 activity in humans in vivo and in adipocytes in vitro (Moore et al 1999; Tomlinson et al 2003). The decreased activity in humans is at least partly due to down-regulation of 11β-HSD1 in adipose tissue (Paulsen et al 2006). Although 11β-HSD1 may have an important role in hypothalamic obesity, and obese
individuals tend to have low GH levels, treatment with GH does not decrease fat mass despite lowering 11β-HSD1 in obese subjects (Tomlinson et al 2003). The effects of peroxisome proliferator-activated receptor (PPAR) agonists have also been examined, showing that PPARα-agonists decrease 11β-HSD1 in the liver (Hermanowski-Vosatka et al 2000), while PPARγ-agonists decrease 11β-HSD1 mRNA in vitro and in vivo in rodent adipose tissue (Berger et al 2001; Laplante et al 2003). Indeed, part of the insulin-sensitising effects of PPARγ-agonists may be mediated by down-regulation of 11β-HSD1. Dietary fats are endogenous ligands for the PPAR receptor, thus this may potentially be a mechanism through which changes in diet regulate 11β-HSD1. However, the effects of PPAR agonists on 11β-HSD1 have not yet been reported in humans. The role of insulin in the regulation of 11β-HSD1 is complex. In vitro, insulin has been shown to decrease 11β-HSD1 in rat hepatocytes (Jamieson et al 1995) but does not alter adipose 11β-HSD1 expression (Bujalska et al 1999; Handoko et al 2000). However, in vivo acute intravenous insulin administration in humans has been shown to either increase (Wake et al 2006) or decrease (Sandeep et al 2005) 11β-HSD1 activity in subcutaneous adipose tissue, while increasing total body activity (Wake et al 2006). Obesity is associated with chronically elevated insulin concentrations however, and it remains possible that high insulin levels contribute to the decreased hepatic 11β-HSD1 activity in human obesity. Indeed, hepatic 11β-HSD1 is only minimally decreased in T2DM, a condition of relative insulin insufficiency (Andrews et al 2002).

There are at least 2 different promoters for 11β-HSD1, with the P1 promoter specifically active in lung (Bruley et al 2006). Although separate promoters could allow differential regulation of 11β-HSD1 in distinct tissues, both liver and adipose tissue are under the control of the P2 promoter. The CCAAT/ enhancer binding proteins (C/EBPs), which are transcription factors with specific binding sites in the 11β-HSD1 promoters, increase 11β-HSD1 mRNA expression in hepatocytes (Williams et al 2000), and it is thought that most of the effects of the above hormones and transcription factors are mediated through the C/EBPs. Potentially the
balance of the various C/EBPs may result in the relative activation or repression of 11β-HSD1 expression in different tissues, mediating such tissue-specific regulation.

1.9 INHIBITION OF 11β-HSD1 IN THE METABOLIC SYNDROME

The potential of glucocorticoid excess to cause the metabolic syndrome in humans leads to the hypothesis that inhibiting glucocorticoid action might treat this condition. For example, the antifungal drug ketoconazole inhibits cortisol biosynthesis (Loose et al 1983) and has been used in the treatment of Cushing’s syndrome, although there are no published studies examining this drug in obesity to date. The glucocorticoid receptor antagonist RU38486 for example improves insulin resistance in both high fat fed (Kusunoki et al 1995) and streptozotocin-induced diabetic rats (Bitar 2001), while RU38486 also acutely decreases hepatic gluconeogenesis in humans (Garrel et al 1995). However, the more chronic metabolic effects of RU38486 have not been studied in humans and there is some concern that glucocorticoid receptor antagonism could potentially precipitate an adreno-cortical crisis in times of stress when increased GR activation is required. Moreover, chronic administration of RU38486 induces compensatory activation of the HPA axis, potentially overcoming the blockade.

The powerful effects of transgenic manipulation of 11β-HSD1 and the dysregulation of 11β-HSD1 observed in human obesity have made inhibition of 11β-HSD1 an attractive proposition for the treatment of metabolic disease. 11β-HSD1 inhibitors would have the potential metabolic benefit of decreasing tissue cortisol concentrations chronically, but would also not interfere with acute increases in glucocorticoid secretion by the adrenal or glucocorticoid action in times of stress. In contrast to many areas of drug development, most of the early work in this field was performed in humans. Carbenoxolone, the hemi-succinate derivative of glycyrrhetinic acid, was licensed in humans for the treatment of peptic ulcer disease in the 1950s. Carbenoxolone is an inhibitor of both 11β-HSD1 and 11β-HSD2
activity (Stewart et al 1990b), and has consequently been utilised extensively to study the effects of 11β-HSD1 inhibition in humans. Carbenoxolone improves hepatic insulin sensitivity in lean healthy men (Walker et al 1995) and decreases glycogenolysis in lean men with T2DM (Andrews et al 2003). However, carbenoxolone does not improve insulin sensitivity in obesity (Sandeep et al 2005) or improve glycaemic control in overweight/obese diabetics (Sandeep et al 2004), while its metabolic effects may be limited to the liver as carbenoxolone appears not to inhibit 11β-HSD1 in adipose tissue (Livingstone & Walker 2003; Sandeep et al 2005). This may explain the lack of effect on insulin sensitivity in obesity, as hepatic 11β-HSD1 activity is decreased in these individuals (see section 1.7.2.1). Inhibition of 11β-HSD1 in adipose tissue has greater potential though, as of course this enzyme is increased in adipose tissue in obesity.

Carbenoxolone cannot be used long-term due to concurrent inhibition of 11β-HSD2, leading to hypertension and hypokalaemia, but it proves the potential of 11β-HSD1 inhibition. A number of other compounds with beneficial metabolic effects may also be competitive 11beta-HSD1 inhibitors, although their potency in vivo is not established. These include naturally occurring dietary components such as flavanone (found in fruit and vegetables) (Schweizer et al 2003) and coffee (Atanasov et al 2006), and drugs with other mechanisms of action, such as the 11beta-hydroxylase inhibitor metyrapone (Raven et al 1995; Sampath-Kumar et al 1997). Metyrapone, however, acts principally by preventing cortisol synthesis in the adrenal so that it carries the risk of precipitating adrenocortical crisis during stress. An alternative approach to competitive inhibition of 11beta-HSD1 might be to reduce gene transcription for the enzyme. As discussed earlier, PPARα-agonists such as fibrates used to treat dyslipidaemia decrease 11β-HSD1 mRNA selectively in the liver (Hermanowski-Vosatka et al 2000) while PPARγ-agonists such as thiazolidinediones used to treat T2DM decrease 11β-HSD1 mRNA in adipose tissue (Berger et al 2001; Laplante et al 2003) in rodents. The effects of these drugs on 11β-HSD1 in humans have yet to be examined.
The pharmaceutical industry has recently become highly interested in this area, and developed several novel selective inhibitors of 11β-HSD1 (summary of effects in Table 1.2). For example, the arylsulphonamidothiazole drug BVT.2733 was the first published selective inhibitor of 11β-HSD1 (Barf et al 2002). This compound decreases plasma glucose, insulin, and cholesterol concentrations, while it improves hepatic insulin sensitivity in hyperglycaemic mice (Alberts et al 2002; Alberts et al 2003). BVT.2733 also reduces food intake and weight gain in mice (Wang et al 2006), although inhibition of 11β-HSD1 in adipose tissue has not been reported. Some compounds have been developed which do inhibit adipose 11β-HSD1 activity in rodents, which show extremely promising results. For example, these drugs decrease visceral fat accumulation, by inhibiting lipogenesis and improving β-oxidation in mesenteric fat (Berthiaume et al 2007). In apolipoprotein E knockout mice, selective 11β-HSD1 inhibition decreases cholesterol accumulation in atherosclerotic plaques, showing that these drugs can potentially delay the progression of cardiovascular disease (Hermanowski-Vosatka et al 2005). To date, only a single Phase I study has been published utilising a selective 11β-HSD1 inhibitor in humans which did not examine effects on weight or metabolic parameters, but did demonstrate significant inhibition of 11β-HSD1 in the liver (Courtney et al 2008).

The data in rodents are certainly encouraging, although several potential concerns exist over the use of these inhibitors. Decreasing peripheral regeneration of glucocorticoids may lead to compensatory activation of the HPA axis, as in people with cortisone reductase deficiency. This could potentially cause hyperandrogenism in humans, which was not observed in the mice as they lack the capacity to secrete adrenal androgens. Glucocorticoids are also potent anti-inflammatory agents, and inhibition of 11β-HSD1 may be pro-inflammatory (Gilmour et al 2006). This has not been detected in any of the rodent trials though, while mice with selective disruption of 11β-HSD1 do not develop spontaneous inflammatory disease, but do have prolonged resolution of inflammation (Gilmour et al 2006). Trials of new selective
11β-HSD1 inhibitors in humans are keenly awaited which will answer these questions.

Table 1.2  Metabolic effects of selective 11β-HSD1 inhibition in rodents

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<tr>
<th>Energy Balance and Body Composition</th>
<th>Metabolism</th>
<th>Vascular</th>
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<tr>
<td>↓ food intake (1,2)</td>
<td>Fasting bloods: ↓ insulin (1,4,5)</td>
<td>↓ atherosclerotic plaque progression (1)</td>
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<td>↓ body weight (1,2)</td>
<td>↓ glucose (1,4,5)</td>
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<td>↓ visceral adipose tissue mass (1,3)</td>
<td>↓ cholesterol (1)</td>
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<td>↓ hepatic triglyceride content (3)</td>
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<td>Hyperinsulinaemic Clamp: ↓ hepatic gluconeogenesis (5)</td>
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1.10 11β-HSD1 – CONCLUSIONS

Dysregulation of glucocorticoid production can cause obesity and the metabolic syndrome in rodents, while glucocorticoid excess is associated with increased cardiovascular disease in humans. Although concentrations of circulating glucocorticoids are not elevated in obesity, tissue glucocorticoid production by 11β-HSD1 is altered with decreased activity in the liver and increased 11β-HSD1 in adipose tissue. Increased cortisol regeneration by 11β-HSD1 in visceral adipose tissue may result in higher cortisol levels reaching the liver and exerting adverse metabolic effects, although the relative contributions of visceral adipose and liver to splanchnic cortisol production are unknown. Polymorphisms in the 11β-HSD1 gene do not correlate with obesity, and it appears dysregulation of 11β-HSD1 is secondary to altered gene transcription from other associations with obesity. The causes for this are unknown at present, but variation in dietary macronutrient content is a potential candidate. Whether altered 11β-HSD1 is a primary cause or secondary effect of obesity, inhibition of 11β-HSD1 is an enticing drug target to treat metabolic disease. Selective 11β-HSD1 inhibitors show promising effects in rodents, and the results in humans will be available in the next few years. Selective 11β-HSD1 inhibitors may potentially become a valuable tool in the treatment of the metabolic syndrome.

1.11 HYPOTHESIS AND AIMS

There is now a sizeable body of evidence that obesity is associated with increased 11β-HSD1 in adipose tissue and decreased 11β-HSD1 activity in the liver. However, it is unknown whether increased 11β-HSD1 activity in adipose tissue results in increased cortisol release. In addition, the reasons for dysregulation of 11β-HSD1 are unknown. Recent work in rodents has suggested dietary content may be a powerful regulator of 11β-HSD1, although this has not been examined in humans. No selective
inhibitors of 11β-HSD1 are at present available for use in humans, however some drugs which improve insulin sensitivity may in fact be 11β-HSD1 inhibitors.

The hypotheses in this PhD were as follows:

1) 11β-HSD1 in visceral adipose tissue releases significant amounts of cortisol directly into the portal vein, with increased enzyme activity exposing the liver to increased cortisol concentrations.

2) Changes in dietary macronutrient content regulate 11β-HSD1 and other glucocorticoid metabolising enzymes in humans, such that a Western diet may be responsible for the dysregulation of 11β-HSD1 in obesity in adipose tissue and/or the liver.

3) PPARγ-agonists decrease 11β-HSD1 in humans, and part of their insulin-sensitising effects are mediated through inhibition of glucocorticoid action.

To address these hypotheses, we report studies with the following aims:

In chapter 3, I used deuterated cortisol infusions with simultaneous arterialised, hepatic vein and portal vein sampling in men with transjugular intrahepatic porto-systemic shunts (TIPSS) in situ, to address the following questions:

1) Whether the liver generates significant cortisol by 11β-HSD1 reductase activity

2) Whether the visceral tissues (including visceral adipose tissue) generate cortisol by 11β-HSD1 reductase and release cortisol into the portal vein
3) To determine the relative contributions of visceral adipose tissue and liver to splanchnic cortisol regeneration by 11β-HSD1

In chapter 4, we report investigations from two separate clinical studies examining dietary macronutrient content in healthy obese men. Deuterated cortisol infusions, subcutaneous adipose tissue biopsies, and 24 hour urinary collections were performed to address the following questions:

1) Whether dietary fat and carbohydrate content regulate whole body and subcutaneous adipose 11β-HSD1 in humans
2) Whether dietary content alters 5α- and 5β-reductase activity in humans
3) Whether weight loss alters 11β-HSD1 activity

In chapter 5, we report examination into similar dietary macronutrient content in rats with examination of liver and adipose 11β-HSD1 to determine:

1) Whether dietary fat and carbohydrate content regulate hepatic and/ or adipose 11β-HSD1
2) Whether similar effects of dietary manipulation are observed between rodents and humans

In chapter 6, we report investigation of PPARγ-agonists in healthy men to address the following questions:

1) Whether PPARγ-agonists down-regulate 11β-HSD1 in adipose tissue as observed in rodents
2) Whether acute inhibition of glucocorticoid action improves insulin sensitivity
3) Whether improvements in insulin sensitivity by PPARγ-agonists are mediated in part by inhibition of glucocorticoid action

Finally, in chapter 7, we draw conclusions from the results of the above research, highlighting insights into the relative importance of 11β-HSD1, its regulation and potential importance of inhibition in obesity and the metabolic syndrome.
Chapter 2
Materials and methods

2.1 MATERIALS

All chemicals were purchased from Sigma (Poole, UK) unless otherwise indicated. All HPLC grade solvents were glass distilled and purchased from Fisher Scientific (Loughborough, Leicestershire, UK) unless other stated. All non-deuterated steroid standards were purchased from Steraloids (Newport, USA). All radioactively labelled steroids were from Amersham (Buckinghamshire, UK). All sterile needles (Microlance), syringes (Plastipak), and venflons were from Becton Dickinson, Oxford, UK. All microcentrifugre tubes were purchased from Sarstedt (Nümbrecht, Germany). Sources other than these are indicated in parentheses.

Room temperature was 18-20°C.

2.2 COMMONLY USED SOLUTIONS

Sodium acetate buffer – 0.2 mol/l sodium acetate (BDH laboratory supplies, Poole, UK), adjusted to pH 4.6 with 16.7 mol/l glacial acetic acid. Stored at 4°C.

DEPC-treated water – Distilled water (500 ml) mixed with 500 µl (0.1% v/v) diethyl pyrocarbonate (DEPC). Solution allowed to stand for 12-24 hours before autoclaving to inactivate.

Loading buffer – 0.25% w/v bromophenol blue, 0.25% w/v xylene cynol, 15% w/v Ficoll adjusted to volume with DEPC-treated water. Stored at 4°C.

10x TBE buffer – 108 g Tris-base, 55 g boric acid, 40 ml 0.5 mol/l EDTA (pH 8.0), adjusted to 1000 ml with distilled water. Autoclaved before use.
Krebs-Henseleit (Krebs) buffer – 118 mmol/l NaCl, 3.8 mmol/l KCL, 1.19 mmol/l KH₂PO₄, 2.54 mmol/l CaCl₂, 1.19 mmol/l MgSO₄, 25 mmol/l NaHCO₃ (all autoclaved before use), adjusted to 1000 ml with ultrapure water (Milli-Q® Academic system, Millipore, Watford, UK) and to pH 7.4 with 5 mol/l HCl. Stored at 4°C.

2.3 INTRODUCTION TO METHODS

The majority of clinical techniques and laboratory assays used in this thesis were routinely in use in this laboratory. Consequently, I learned and adapted the techniques as required. Some techniques had not been performed in this group previously, i.e. low dose incremental insulin infusion, indocyanine green (ICG) infusion, portal and hepatic vein cannulation, laboratory analysis of endogenous and deuterated cortisol by liquid chromatography/mass spectrometry, and laboratory analysis of ICG. I undertook development of these techniques.

2.4 CLINICAL TECHNIQUES

2.4.1 Subcutaneous abdominal adipose tissue needle biopsy

Subjects were admitted after overnight fast for the biopsies. Participants were placed supine, with the abdominal area exposed. All equipment used in this procedure was sterile. The abdominal area was sterilised with Videne (Adams Healthcare, Leeds, UK), and 5 ml of local anaesthetic (2% lidocaine, Hameln Pharmaceuticals, Gloucester, UK) was injected 5-10 cm lateral to the umbilicus subcutaneously using initially a 23G and subsequently a 18G needle, and allowed to act for 5 minutes. A 14G needle was then attached to a sterile 60 ml luer-lok syringe and passed into the subcutaneous adipose tissue. A vacuum was created using the syringe and approximately 500-1000 mg adipose tissue aspirated into the syringe, using 2-4
passes of the needle. Adipose tissue was evacuated onto sterile gauze and then washed with DEPC-treated water. The adipose tissue was then placed into sterile microcentrifuge tubes and immediately frozen in dry ice, prior to storage at -80°C until analysis.

2.4.2 Intra-venous 9,11,12,12-[2H]4-cortisol infusion

Earlier in vivo work measuring total body 11β-HSD1 activity in humans was performed using 24 hour urinary collections. As described in section 1.7.1, the ratio of cortisol: cortisone metabolites used to derive whole body 11β-HSD1 activity are also influenced by 11β-HSD2 activity and hepatic metabolism by the A-ring reductases. This laboratory has developed a more specific technique for measuring total body 11β-HSD1 activity, utilising the deuterated stable isotope tracer, 9,11,12,12-[2H]4-cortisol (d4-cortisol) (Figure 1.4) (Andrew et al 2002b). This isotopically labelled steroid is converted by predominantly 11β-HSD2 with the loss of the deuterium on the 11th carbon atom to form 9,12,12-[2H]3-cortisone (d3-cortisone). This is then converted exclusively by 11β-HSD1, with the addition of an unlabelled hydrogen, to 9,12,12-[2H]3-cortisol (d3-cortisol). The ratio of d3-cortisol: d4-cortisol in steady state is then used to derive a specific measure of whole body 11β-HSD1 activity.

Participants attended at 0700 (diet studies, chapter 4) or 0800 (portal vein study, chapter 3) after an overnight fast. In the diet studies, 9,11,12,12-[2H]4-cortisol (Cambridge Isotopes, Andover, MA, USA) was intravenously administered into an antecubital vein at 20% w/v molar enrichment with 80% w/v hydrocortisone-21-succinate (Solu-Cortef, Upjohn, West Sussex, UK), initially as a priming bolus of 3.5 mg followed by a continuous infusion at 1.74 mg/hour. In the portal vein study, 9,11,12,12-[2H]4-cortisol was intravenously administered at 40% molar enrichment with 60% hydrocortisone (Calbiochem®, Nottingham, UK). Blood samples were taken from the antecubital vein in the opposite arm pre-infusion and at intervals during the
infusion (see results section for exact timings). In the ad libitum diet study (section 4.2), urine was collected pre-infusion and hourly throughout the infusion, while volunteers were given 250 ml water orally before the infusion and 100 ml hourly during the infusion to aid bladder emptying. Blood samples for cortisol concentrations were collected in lithium heparin tubes and placed on ice, subject to centrifugation at 2000 relative centrifugal force (rcf) at 4°C for 10 minutes, the plasma removed and stored at -20°C until analysis (section 2.5 and 2.6).

2.4.3 Low dose incremental insulin infusion

Standard hyperinsulinaemic-euglycaemic clamps used to examine insulin resistance use doses of insulin which completely suppress endogenous lipolysis, so may miss subtle changes in insulin sensitivity. In order to more accurately assess whole body lipolysis responses to insulin, we adapted a low-dose incremental insulin infusion technique (Krentz & Nattrass 1996). This was performed in chapter 6, immediately after the adipose tissue biopsy (section 2.4.1). Insulin (Actrapid, Novo Nordisk, Crawley, West Sussex, UK) was intravenously infused into the left antecubital vein over a 4 hour period at rates of 0 (0.9% saline only), 10, 33, and 100 mU/kg each for 1 hour. The insulin infusion rates were calculated from previous published data (Hale et al 1986; Krentz et al 1991; Robertson et al 1992) using this technique to achieve suppression of plasma free fatty acids (FFAs) of 20%, 50% and 80% in the final three hours respectively. Venous blood was collected from the right antecubital vein from t+120 (the commencement of 33 mU insulin/kg/hr) every 5 minutes and analysed on an Accu-chek Advantage II blood glucose meter (Roche Diagnostics, West Sussex, UK). Conversion of the venous blood glucose results obtained from the meter to the correct venous glucose (as machine is calibrated for capillary blood) was performed using data supplied by the manufacturer (Table 2.1). A 10% dextrose infusion was used to maintain blood glucose levels between 4.0-4.5 mmol/l. This was commenced at 50 ml/hour (5 g dextrose/hr) when the calculated capillary blood glucose concentration fell below 4 mmol/l. This rate was increased by 50 ml/hr if a glucose reading remained static or fell below the previous value, if this reading was
less than 4.0 mmol/l. Conversely, the dextrose infusion rate was decreased by 50 ml/hr if the calculated venous glucose concentration rose above 4.5 mmol/l. Blood samples were taken to assess intermediate metabolites at regular intervals throughout the insulin infusion, centrifuged at 2000 rcf at 4°C for 10 minutes, the plasma divided into aliquots and stored at -80°C until analysis (section 6.2.4.1).
Table 2.1  Conversion of meter glucose reading obtained from Accu-chek Advantage II meter to whole blood venous glucose

Data derived from Accu-Chek Advantage II Evaluation Report using the equation $y = 1.0981x - 0.2709$.

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<th>Meter glucose reading (mmol/l)</th>
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<td>7.3</td>
</tr>
<tr>
<td>3.6</td>
<td>3.7</td>
<td>5.3</td>
<td>5.5</td>
<td>7.0</td>
<td>7.4</td>
</tr>
</tbody>
</table>
2.4.4 Hepatic blood flow measurement by indocyanine green infusion

Indocyanine green (ICG) infusions were performed in the portal vein study (chapter 3) to assess hepatic blood flow, as ICG is purely extracted by the liver (Cherrick et al 1960). ICG was intravenously infused into an antecubital vein at 0.5 mg/min, as previously described (Andrew et al 2005). Steady state is achieved after 30 minutes (Soons et al 1991), thus during steady state blood samples were taken simultaneously from the arterialised and hepatic vein into 4.7 ml serum clotted gel tubes. Samples were placed on ice for 15 minutes, subjected to centrifugation at 2000 rcf at 4°C for 10 minutes, and serum separated into aliquots. Samples were placed on ice and immediately analysed (section 2.9).

2.5 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS OF DEUTERATED STEROIDS

2.5.1 Extraction and derivatisation of plasma deuterated steroids

Standards of cortisol, cortisone, d4-cortisol and the internal standard epi-cortisol were prepared as 1 mg/ml solutions in methanol. 1.5 ml of each plasma sample was dispensed into 18 ml glass tubes (Corning Life Science, Lowell, MA, USA). 500 ng of epi-cortisol (internal standard) was added to standards and plasma samples. Standards were composed of a blank consisting of epi-cortisol alone, then cortisol, cortisone (30 ng, 50 ng, 100 ng, 200 ng, 300 ng, 400 ng), and d4-cortisol (15 ng, 25 ng, 50 ng, 100 ng, 150 ng, 200 ng) in the respective amounts detailed in parentheses. Chloroform was added to samples and standards in a 10:1 ratio and mixed. The supernatant (mainly plasma lipid) was removed with a pasteur pipette (Fisher Scientific, Loughborough, Leicestershire, UK), and the organic layer transferred to Reactivials (Pierce Chemical Company, Rockford, IL, USA) and evaporated under
oxygen free nitrogen (OFN) at 60°C. Dried samples were then stored at -20°C until derivatisation.

Samples were thawed to room temperature. 50 µl of 2% w/v methoxyamine in dry pyridine was added to all samples, which were capped and incubated at 60°C for 30 minutes, then dried under OFN at 60°C. 50 µl of trimethylsilylimidazole was added to samples, Reactivials capped and incubated at 100°C for 120 minutes. Lipidex 5000 columns were created by plugging pasteur pipettes with silane-treated glass wool (Alltech Associates Inc., Deerfield, IL, USA), then adding Lipidex 5000 (PerkinElmer, Boston, MA, USA) prepared in mobile phase (98% v/v cyclohexane (Rathburn Chemicals (Walkerburn, UK), 1% v/v dry pyridine, 1% v/v hexamethyldisilazane) to each column yielding a bed 1cm in depth. Columns were prepared by washing with 3 x 1 ml of mobile phase. 1ml of mobile phase was added to samples in the Reactivials, mixed and passed through the Lipidex columns. Mobile phase (0.5 ml) was added to Reactivials and passed through Lipidex columns twice, to ensure complete transfer of samples. Finally, 0.5 ml of mobile phase was added to wash the columns. The eluant was collected in 3.5 ml glass vials (MacKay and Lynn, Edinburgh, UK) and dried under OFN at 60°C. The samples were reconstituted in 75 µl decane and 35 µl transferred to GC-MS autosampler vials (Agilent, Berkshire, UK), then capped for analysis.

2.5.2 Extraction of urinary endogenous and deuterated steroids

Standards of α-cortol, β-cortol, α-cortolone, β-cortolone, α-tetrahydrocortisol (α-THF), β-tetrahydrocortisol (β-THF), β-tetrahydrocortisone (β-THE), cortisol, cortisone, and the internal standards epi-cortisol and epi-tetrahydrocortisol (epi-THF) were prepared as 1 mg/ml solutions in methanol. C18 Sep pak cartridges (Waters, Milford, MA, USA) were prepared by passing through 5 ml methanol followed by 5 ml water to waste. 5 µg of epi-cortisol (internal standard for cortisol, d3-cortisol, d4-cortisol, cortisone, d3-cortisone, α- and β-cortol, α- and β-cortolone) and 30 µg epi-
THF (internal standard for α-THF, β-THF, β-THE) were added to standards (in 20 ml water) and 20 ml of urine samples. Standards were composed of a blank containing epi-cortisol and epi-THF, then α-cortol, β-cortol, α-cortolone, β-cortolone, cortisol, cortisone (all 500 ng, 1 μg, 2 μg, 3 μg, 4 μg, 5 μg, 10 μg, 15 μg, 20 μg, 30 μg), α-THF, β-THF, β-THE (all 5 μg, 10 μg, 20 μg, 30 μg, 40 μg, 50 μg, 100 μg, 150 μg, 200 μg, 300 μg) in the respective amounts detailed in parentheses. Standards and urine samples were passed through the C_{18} Sep-pak cartridges, washed with 5 ml of water, and eluted into 3.5 ml glass vials using 2 ml methanol. Samples were then evaporated under OFN at 60°C, then the dried residue stored at -20°C until hydrolysis.

2.5.3 Hydrolysis of urinary conjugated steroids

Two ml of 0.2 mol/l sodium acetate buffer (pH 4.6) and 100 μl β-glucuronidase (98,000 units/ml; Helix pomatia type H-2) were added to samples, capped and incubated at 37°C for 48 hours.

2.5.4 Extraction of hydrolysed steroids

Samples were passed again through the C_{18} Sep pak cartridges, after re-conditioning cartridges with 5 ml methanol followed by 5 ml water. Samples were washed with 5 ml water, and eluted into new 3.5 ml glass vials using 2 ml methanol. Samples were then evaporated under OFN at 60°C. Dried residues were reconstituted in 200 μl water and 3 ml ethyl acetate and subjected to vortexing. The organic supernatant was transferred to Reactivials and solvent evaporated under OFN at 60°C. Dried residues were frozen at -20°C until derivatisation.
2.5.5 Derivatisation of extracted urinary steroids

Derivatisation was performed as described in section 2.5.1 except for the following changes. After the two incubations, 1 ml of mobile phase was added to samples in the Reactivils, mixed and passed through the Lipidex 5000 columns. Mobile phase (0.5 ml) was added to Reactivils and passed through lipidex columns twice, to ensure complete transfer of samples. Finally, 1 ml of mobile phase was added to wash each column. The eluant was collected in 3.5 ml glass vials and solvent evaporated under OFN at 60°C. The samples were reconstituted in 200 µl hexane and 30 µl transferred to GC-MS autosampler vials, then capped for analysis.

2.5.6 Gas Chromatography-Mass Spectrometry Analysis

Analysis was performed on a Voyager GC-MS, consisting of a Finnigan MD 800 Mass Spectrometer and a GC 8000 Top Gas Chromatogram (GC-MS) using a DB17-MS column (15 m length, 0.25 mm id, 0.25 µm ft; J&W Scientific, Folsom, CA). The initial column temperature was 50°C; this was increased to 200°C by 30°C/min and then by 8°C/min to 250°C and then by 6°C/min to 300°C and maintained for 6 minutes. Injection, source and interface temperatures were 280°C, 200°C, and 250°C respectively. Helium was used for the mobile phase using a flow rate of 2.2 ml/min. Total run time per sample was 40 minutes. Ionisation was performed in electron impact mode at 70 eV. Methoxime-trimethylsilyl derivatives of the endogenous steroids, d4-cortisol and its metabolites were measured utilising the ions with the following mass/ charge (m/z) ratios (Figure 2.1):

- Cortisol and epi-cortisol – 605
- d3-cortisol – 608
- d4-cortisol – 609
- Cortisone – 531
d3-cortisone – 534
α-THF, β-THF and epi-THF – 652
d3-α-THF, d3-β-THF – 655
d4-α-THF, d4-β-THF – 656
β-THE – 578
d3-β-THE – 581
α-cortol and β-cortol – 535
α-cortolone and β-cortolone – 449

Compounds were quantified by the ratio of (area under peak of interest)/ (area under internal standard). These ratios were compared against the standard curve for each steroid performed in every assay. The amount of each standard (x axis) was plotted against the peak area ratio (y axis) for each point on the standard curve. A line of best fit was determined in the form of \( y = mx + c \), and if the \( r \) value was deemed acceptable (>0.99) this equation was utilised to calculate the amount of each compound for all samples. An enrichment curve was run in each batch using d4-cortisol: cortisol ratios of 0, 0.025, 0.05, 0.1, 0.15, 0.2, and 0.25 respectively to determine if the efficiency of ionisation of derivatised d4-cortisol was identical to that of unlabelled cortisol.
2.5.7 GC-MS analysis for 24 hour urinary collections

24 hour urinary collections were performed in the ad libitum diet study (section 4.2). The volume of urine voided in 24 hours was used to calculate the total amount of cortisol and its respective metabolites excreted per day. Total urinary metabolites were derived from the sum of cortisol, cortisone, α-THF, β-THF, β-THE, α-cortol, β-cortol, α-cortolone and β-cortolone. Total body 11β-HSD1 activity was measured as the ratio of the predominant cortisol: cortisone metabolites, using the (α-THF + β-THF)/ β-THE ratio (Andrew et al 1998). The ratio of urinary free cortisol: cortisone was used to quantify renal 11β-HSD2 activity (Palermo et al 1996; Best & Walker...
The relative activities of the 5α- and 5β-reductases were measured utilising Ulick's A-ring reduction equations, 5α-THF/ cortisol, 5β-THF/ cortisol, and 5β-THE/ cortisone (Ulick et al 1992), while the balance of 5α- and 5β-reductase activity was measured by the 5α-THF/ 5β-THF ratio (Andrew et al 1998).

2.5.8 GC-MS kinetic analysis for plasma and urine collected during infusion of tracer

Plasma was collected in both ad libitum and isocaloric diet studies during infusion of d4-cortisol (chapter 4), while urine was collected only in the ad libitum study (section 4.2). Peak areas of d3-cortisol were corrected for endogenous mass + 3 cortisol (approximately 6-7% of endogenous cortisol). Results for d4-cortisol were corrected for isotopic interference from endogenous mass + 4 cortisol (approximately 1-2% of endogenous cortisol) and mass + 1 d3-cortisol (approximately 50% of d3-cortisol). Cortisol kinetics were calculated using the mean of 5 measurements obtained in steady state (see Figure 4.2), which was achieved after 180 minutes of the infusion (Andrew et al 2002b). 9,11,12,12-[2H]4-Cortisol (d4-cortisol) is converted by 11β-dehydrogenase to 9,12,12-[2H]3-cortisone (d3-cortisone), which is then reduced exclusively by 11β-HSD1 with the addition of an unlabelled hydrogen to form 9,12,12-[2H]3-cortisol (d3-cortisol) (Figure 1.4). d3-Cortisol and d3-cortisone are reversibly interconverted, whereas d4-cortisol conversion to d3-cortisone is irreversible, so that differences between d3-cortisol and d4-cortisol reflect 11β–HSD1 reductase activity exclusively. The equations used were as follows:

Rate of appearance of unlabelled cortisol = (d4-cortisol infusion rate/ [d4-cortisol/cortisol]) - cortisol infusion rate

Rate of appearance of d3-cortisol = d4-cortisol infusion rate/ (d4-cortisol/d3-cortisol)

d4-Cortisol clearance = d4-cortisol infusion rate/ d4-cortisol concentration
To assess 5α- and 5β-reductase activities, excretion of urinary d4-cortisol metabolites was calculated as an area under the curve of urinary amounts during d4-cortisol infusion.

2.6 LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROMETRY (LC-MS/MS) ANALYSIS OF DEUTERATED STEROIDS

2.6.1 Introduction

The GC-MS method to detect plasma deuterated steroids performed for the diet studies, while accurately detecting d3-cortisol and d4-cortisol concentrations, was not sensitive enough to detect d3-cortisone. While d3-cortisone is not required for kinetic analysis of 11β-HSD1 activity, it is needed to measure 11β-HSD2 activity. We adapted a method of measuring urinary steroids by LC-MS/MS to overcome this problem (Taylor et al 2002). In addition, this method negated the need for derivatisation so decreased the preparation time required. This method was used in the portal vein study (chapter 3) for detection of endogenous and tracer steroids.

2.6.2 Plasma extraction

Plasma extraction was similar to the GC-MS method (section 2.5). Standards of cortisol, cortisone, d4-cortisol and the internal standard epi-cortisol were made up in 1 mg/ml solutions in methanol. 1.5 ml of each thawed plasma sample was pipetted into 18 ml glass tubes. 500 ng of epi-cortisol was added to standards and plasma samples. Standards were composed of a blank consisting of epi-cortisol alone, then cortisol (2 ng, 10 ng, 20 ng, 30 ng, 50 ng, 100 ng, 200 ng, 300 ng, 400 ng), cortisone and d4-cortisol (both 1 ng, 5 ng, 10 ng, 15 ng, 25 ng, 50 ng, 100 ng, 150 ng, 200 ng) in the respective amounts detailed in parentheses. The additional lower points on the
standard curve were added to account for removal of the majority of cortisone by the liver with consequent low concentrations in the hepatic vein. Chloroform was added to samples and standards in a 10:1 ratio and vortexed. The supernatant (mainly plasma lipid) was removed with a pasteur pipette, and the organic layer transferred to 3.5 ml glass vials and evaporated under OFN at 60°C. Samples were reconstituted in 100 μl of mobile phase (60% v/v methanol, 40% v/v 5 mmol/l ammonium acetate) and vortexed to mix. Samples were transferred to LC-MS autosampler vials (Agilent, Berkshire, UK) with a pasteur pipette and capped ready for analysis.

2.6.3 LC-MS/MS analysis

Analysis was performed on a Thermo Finnigan LC-MS/MS, consisting of a TSQ Quantum Discovery Mass Spectrometer and a Surveyor Liquid Chromatogram using a Restek biphenyl column (50 mm length x 4.6 mm id x 5 μm particle size). The column temperature was 25°C. The mobile phase was composed of 60% methanol and 40% 5mM ammonium acetate at injection using an isocratic flow rate of 0.5 ml/min. Total run time was 30 minutes per sample (Figure 2.2). Tandem mass spectrometry was performed to improve product selectivity, while ionisation was achieved by positive electrospray ionisation (ESI). Spray voltage was 3 kV and capillary temperature was 300°C. Solvent was evaporated using nitrogen sheath gas at 60 l/min and auxiliary gas at 5 l/min. Collision was induced in Q2 with a gas pressure of 1.5 mTorr of argon. Cortisol, d3-cortisol, d4-cortisol, cortisone and d3-cortisone were measured utilising the precursor and product ions with the m/z ratios in Table 2.2.
Table 2.2  Ions used to detect endogenous and tracer cortisol compounds by LC-MS/MS

<table>
<thead>
<tr>
<th>Target Compound</th>
<th>Precursor Ion (m/z ratio)</th>
<th>Product Ion (m/z ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortisol and epi-cortisol</td>
<td>363</td>
<td>121</td>
</tr>
<tr>
<td>d3-cortisol</td>
<td>366</td>
<td>121</td>
</tr>
<tr>
<td>d4-cortisol</td>
<td>367</td>
<td>121</td>
</tr>
<tr>
<td>cortisone</td>
<td>361</td>
<td>163</td>
</tr>
<tr>
<td>d3-cortisone</td>
<td>364</td>
<td>164</td>
</tr>
</tbody>
</table>

Compounds were quantified by the ratio of (area under peak of interest)/ (area under internal standard). These ratios were compared against the standard curve for each steroid performed in every assay. An enrichment curve was run in each batch, using d4-cortisol: cortisol ratios of 0, 0.05, 0.1, 0.15, 0.25, 0.5, and 1. The limit of detection for this assay was 1 nmol/l using 1.5ml of plasma. Kinetic analysis of plasma steroids was identical to that for GC-MS derived steroids (section 2.5.8).
Figure 2.2 LC-MS/MS chromatogram measuring endogenous and deuterated cortisol and cortisone in plasma

Chromatograms depicting m/z ratios measuring a) epi-cortisol and cortisol, b) d3-cortisol, c) d4-cortisol, d) cortisone, and e) d3-cortisone. SRM = Selective Reaction Monitoring, showing precursor and product ions.

2.7 IN VITRO 11β-HSD1 ACTIVITY ASSAY

2.7.1 Introduction

This technique was performed in chapters 4, 5 and 6. In vivo, 11β-HSD1 acts as a reductase to convert cortisone to cortisol (11-dehyrocorticosterone to corticosterone in rodents). However, in vitro this reaction direction is reversible, with the dehydrogenase activity predominating. Therefore, this in vitro assay was performed
in the dehydrogenase direction. The activity is directly proportional to total amount of protein present in the reaction, so this technique provides a reliable estimate of 11β-HSD1 protein tissue levels (Livingstone et al. 2000a).

2.7.2 Tissue homogenisation

Tissues were stored at -80°C. Approximately 250 mg of adipose tissue (100 mg of liver) was homogenised in 1 ml Krebs buffer in 2 ml microcentrifuge tubes using a rotor stator homogeniser (Ultra-Turrax® T8, IKA, Staufen, Germany). Adipose homogenates were subject to centrifugation at 300 rcf at 4°C for 5 minutes, and the infranatant transferred to new microcentrifuge tubes.

2.7.3 Protein quantification

Tissue homogenates were quantified using a colorimetric protein assay. Protein standards ranging in concentration from 0-1.2 mg/ml in Krebs buffer were prepared from a bovine serum albumin 10 mg/ml solution (Promega, Madison, WI, USA). Adipose tissue homogenates were diluted 1 in 2 in Krebs buffer, while liver homogenates were diluted 1 in 32 due to their higher protein concentration. Samples and standards were vortexed to mix. 10 µl of diluted homogenate or protein standard was added to 190 µl of 1 in 5 diluted (in distilled water) Bio-Rad protein assay solution (Bio-Rad Laboratories GmbH, Munich, Germany) in duplicate in 96 well plates (BD Flacon, Becton Dickinson, Franklin Lakes, NJ, USA). Samples were shaken to mix, incubated for 30 minutes at room temperature, then the absorbance read at wavelength 590nm on an Optimax microplate reader (Molecular Devices Ltd, Berkshire, UK). Protein concentrations were read off a standard curve (blank, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mg/ml protein) and the mean value of the duplicates recorded. The standard curve was deemed acceptable if the r value was >0.99, while variation between duplicate samples/ standards was accepted if <5%. Samples were then frozen at -20°C until the activity assay.
2.7.4 11β-HSD1 activity assay in tissues

2.7.4.1 11β-HSD1 activity assay in human adipose tissue

Samples were thawed to room temperature. Tissue homogenates were diluted in Krebs buffer to normalise protein concentrations in each sample. Tissue homogenates (see relevant chapters for respective protein concentrations and duration of incubation in each study) were then incubated in duplicate at 37°C in Krebs buffer containing 2 mmol/l nicotinamide adenine dinucleotide phosphate (NADP) as co-factor, 0.2% w/v glucose, and 100 nmol/l cortisol (of which 10nmol/l 1,2,6,7-[3H]4-cortisol) as substrate, in a final reaction volume of 1 ml. Reaction mixtures either without NADP or cortisol were incubated as negative controls. Protein concentrations and incubation times were selected to achieve first order kinetics.

2.7.4.2 11β-HSD1 activity assay in rodent tissues

11β-HSD1 activity assays were performed in rodents identically to the method in humans (section 2.7.4.1), except for the following changes. 100 nmol/l corticosterone (with 10 nmol/l 1,2,6,7-[3H]4-corticosterone) was added as the substrate in the reaction mixture, while the final reaction volume was 500 μl.

2.7.5 Steroid extraction following 11β-HSD1 activity assay

2.7.5.1 Steroid extraction following 11β-HSD1 activity assay from human adipose tissue

Two hundred microlitres of the reaction mixture was removed after incubation, and the reaction stopped with the addition of 2 ml ethyl acetate. Samples were mixed and the organic supernatant transferred into borosilicate glass tubes (Fisher Scientific, Loughborough, Leicestershire, UK) with a pasteur pipette (Fisher Scientific,
Loughborough, Leicestershire, UK). Samples were dried down under OFN at 60°C, and the residue reconstituted in 1 ml mobile phase (65% water, 5% acetonitrile, 30% methanol). Samples were transferred to clear shell HPLC vials (Supelco, Poole, UK) by pasteur pipette, and stored at -20°C until HPLC analysis.

2.7.5.2 Steroid extraction following 11β-HSD1 activity assay from rodent tissues

This was performed identically to the method in humans (section 2.7.5.1), except the mobile phase used to reconstitute the samples was composed of 65% water, 10% acetonitrile and 25% methanol.

2.7.6 HPLC analysis of steroids recovered following 11β-HSD1 activity assay

2.7.6.1 HPLC analysis of steroids recovered following 11β-HSD1 activity assay in humans

Two hundred microlitres of each sample was analysed by HPLC comprising a Waters auto-injector, mobile-phase pump, reverse phase Sunfire C18 5μm (150mm length x 4.6mm id) column (Hertfordshire, UK) in a column heater and a radioactivity monitor (Berthold, Leeds, UK) linked to a scintillation fluid pump. The system was controlled by a computer with Chromelion software (Dionex, Surrey, UK). The flow of mobile phase was set at 1.0 ml/min and the flow rate of scintillant (Gold multi-purpose flow scintillation cocktail, Meridian, Surrey, UK) was set at 2 ml/min to achieve optimal mixing and efficiency of counting. The β radioactive emissions from the tritium cause photoemission from the scintillation fluid which is measured by a photodiode. The column heater was set at 35°C to improve chromatographic resolution and maintain stability of retention times. Radioactive standards were injected at the start of every run to confirm the identity of the peaks. The retention time for cortisone was approximately 27 minutes, and for cortisol approximately 32 minutes, while total run time was 36 minutes (Figure 2.3). The area under the peak was integrated using the Chromelion software. The conversion of 1,2,6,7-[3H]4-cortisol to 1,2,6,7-[3H]4-cortisone was calculated as the area under
the [³H]-cortisone peak / (area under [³H]-cortisone peak + [³H]-cortisol peak), and 11β-HSD1 activity reported as pmol product/ mcg protein/ hour. Routine blank incubations with no tissue were performed for each assay and the conversion in each sample corrected for any apparent conversion in the blank. A variability of <5% between duplicates was considered acceptable, while a minimum of 5% conversion was required to achieve acceptable reproducibility of samples.

Figure 2.3 Chromatogram depicting HPLC measurement of tritiated glucocorticoids from 11β-HSD1 activity assay

Example of a chromatogram obtained from HPLC measurement of 11β-HSD1 activity assay in humans, measuring conversion of 1,2,6,7-[³H]₄-cortisol to 1,2,6,7-[³H]₄-cortisone by online liquid scintillation detection.
2.7.6.2 HPLC analysis of steroids recovered following 11β-HSD1 activity assay in rodents

This was performed in identical fashion to the human 11β-HSD1 activity assays (section 2.7.6.1) except for the following changes. The column temperature was 40°C, while the mobile phase used was 65% water, 10% acetonitrile, and 25% methanol. The mobile phase flow rate was 1.5 ml/min, while the scintillant flow remained 2 ml/min. The retention times for 11-dehydrocorticosterone and corticosterone were approximately 14 and 20 minutes respectively. The conversion of 1,2,6,7-[^3]H]_4-corticosterone to 1,2,6,7-[^3]H]_4-11-dehydrocorticosterone was calculated as the area under the [^3]H]-11-dehydrocorticosterone peak / (area under [^3]H]-11-dehydrocorticosterone peak + [^3]H]-corticosterone peak).

2.8 MESSENGER RNA (mRNA) TRANSCRIPT QUANTIFICATION IN HUMAN AND RODENT STUDIES

2.8.1 Introduction

mRNA transcript quantification in adipose tissue was performed in chapters 4, 5, and 6, while mRNA quantification in the liver was performed only in chapter 5.

2.8.2 Homogenisation of adipose tissue

Tissues were stored at -80°C prior to this procedure. 100 mg of adipose tissue was homogenised in 1 ml Qiazol reagent (Qiagen, West Sussex, UK) using a rotor stator homogeniser (Ultra-Turrax® T8, IKA, Staufen, Germany). Samples were immediately placed on dry ice then stored at -80°C.
2.8.2.1 Homogenisation of liver

This was performed as for the adipose tissue protocol, except 30 mg of liver was homogenised in 600 µl of Buffer RLT (Qiagen, West Sussex, UK).

2.8.3 RNA extraction from adipose tissue

RNA was extracted using the Qiagen RNeasy Lipid Tissue Mini Kit (West Sussex, UK) as per the manufacturer’s instructions. Chloroform (200 µl) was added to the thawed tissue homogenate and mixed thoroughly. The mixture was subjected to centrifugation at 12000 rcf at 4°C for 15 minutes. The supernatant containing the RNA was added to 1 volume of 70% v/v ethanol and transferred to Qiazol mini spin columns. Columns were spun by centrifugation at 8000 rcf at room temp for 30 seconds, and the eluate disposed to waste. Columns were cleaned with wash buffer (containing ethanol to keep RNA bound to columns), and RNA eluted in 30 µl RNase-free water. Samples were divided into 3 microcentrifuge tubes to prevent the need for refreezing and rethawing, and stored at -80°C.

2.8.3.1 RNA extraction from liver

RNA was extracted using the Qiagen RNeasy Mini Kit (West Sussex, UK) as per the manufacturer’s instructions. The liver tissue homogenate was thawed and subjected to centrifugation at 14,000 rcf and the supernatant transferred to a new microcentrifuge tube (approximately 550 µl per sample). The supernatant containing the RNA was added to 1 volume of 70% v/v ethanol and transferred to the Qiazol mini spin columns. Columns were spun by centrifugation at 8000 rcf at room temp for 30 seconds, and the eluate disposed to waste. Columns were cleaned with wash buffer (containing ethanol to keep RNA bound to columns), and RNA eluted in 30µl RNase free water. Samples were divided into 3 microcentrifuge tubes to prevent the need for refreezing and rethawing, and stored at -80°C.
2.8.4 RNA quantification and integrity

RNA was quantified using the Ribogreen quantitation kit (Molecular Probes, Eugene, OR, USA). Sample RNA was diluted in TE buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 7.5) (human adipose tissue diluted 1 in 80, rodent adipose tissue diluted 1 in 400, rodent liver diluted 1 in 1000). Ribogreen reagent® was diluted 1 in 2000 in TE buffer, then 5 µl of each diluted sample was added to 195 µl of the diluted Ribogreen reagent® and incubated for 5 minutes at room temperature in the dark. The results were read off a standard curve (blank, 1, 5, 10, 15, 20, 25, 50 ng RNA/ml) on a Victor2 V-1420 Multilabel HTS counter (PerkinElmer, Waltham, MA, USA), with excitation at 495 nm and emission at 535 nm. The standard curve was deemed acceptable if the r value was >0.99, while variability between duplicates was accepted if <5%. The limit of detection was less than 1 ng/ml.

RNA integrity was checked by agarose gel electrophoresis, containing 1.2% w/v agarose (Lonza, Basel, Switzerland) in 0.5 x TBE solution with 1 µl ethidium bromide (10 mg/ml) per 50 ml. The gel tank and tray were cleaned with RNase Away solution (Molecular BioProducts, San Diego, CA, USA) prior to use, and electrophoresis performed in 0.5 x TBE solution. RNA (1 µl) was added to 10 µl DEPC-treated water and 2 µl loading buffer, placed in wells and run at 100 V for 1 hour. Gels were viewed at 254 nm using a UVIpro system (UVItc, Cambridge, UK) for the presence of distinct 28S and 18S ribosomal bands in an approximate 2:1 ratio to confirm integrity of RNA.

2.8.5 Complementary DNA (cDNA) synthesis by reverse transcription

Five hundred nanograms of RNA from each sample was reverse transcribed using the Promega Reverse Transcription System (Madison, WI, USA). Samples were incubated with 5 mmol/l MgCl₂, 1 mmol/l of each dNTP, 1 x reverse transcription buffer (10 mmol/l Tris-HCl, 50 mmol/l KCl, 0.1% v/v Triton X-100), 500 ng random
primers, 20 units RNasin, and 15 units reverse transcriptase in a standard volume of 20 μl of nuclease-free water. In addition, for each batch of cDNA synthesis, one sample was included in which 500 ng RNA was incubated in the absence of the reverse transcriptase enzyme, while in another RNA was replaced with water in a separate sample with the full reaction mixture (volume replaced with nuclease-free water in both samples). These samples (called –RT and H2O) were used as controls in polymerase chain reaction (PCR) and real time PCR to ensure that genomic or other DNA contamination was not present. Reverse transcription was performed in a G-storm GS1 thermal cycler (Genetic Research Instrumentation Ltd, Essex, UK) using the following conditions:

1) 42°C for 30 minutes (annealing and cDNA synthesis)
2) 95°C for 5 minutes (inactivation of reverse transcriptase and denaturing)
3) 4°C for 5 minutes (to prevent annealing)

The cDNA was stored at -20°C until analysis.

2.8.6 Polymerase chain reaction (PCR) amplification

PCR amplification was used to confirm successful synthesis of cDNA, using human 11β-HSD1 primers (440bp product):

5’-AAAGTGATTGTCACWGGGAGCAAA-3’ (forward)
5’-ATCCARAGCAAACATTGCTTG-3’ (reverse)

(Accession number – NM_005525)

Several samples were chosen at random and 2 μl of cDNA diluted 1 in 5 in nuclease-free water. Five microlitres of diluted cDNA was incubated with 1 x Taqbead Hot Start polymerase (1.25 units/ bead), 1 x PCR buffer (10 mmol/l Tris-HCl (pH 9.0),
50 mmol/l KCl, 0.1% v/v Triton X-100), 1.5 mmol/l MgCl₂, 200 µmol/l of each
dNTP, 40 pmol forward primer, 40 pmol reverse primer adjusted with nuclease-free
water to a final volume of 50 µl (all PCR reagents Promega, Madison, WI, USA).
PCR was performed in a G-storm GS1 thermal cycler using the following conditions:

1) 95°C for 5 minutes (to activate Taq polymerase and denature DNA)
2) 95°C for 30 seconds (denaturing)
3) 55°C for 30 seconds (annealing)
4) 72°C for 1 minute (extension) (stages 2-4 repeated for 29 cycles)
5) 72°C for 5 minutes (final extension)
6) 4°C for 5 minutes (to prevent annealing)

Ten microlitres of PCR products were mixed with 2µl loading buffer and separated
by electrophoresis using a 1.75% w/v agarose/ TBE gel concomitantly with a 1
kilobase DNA ladder.

2.8.7 Real time PCR in human adipose tissue

mRNA transcript quantification was performed using the ABI PRISM 7700
Sequence Detection System (PE Applied Biosystems, Cheshire, UK). 20x primers/
probes mixtures were purchased pre-made from Applied Biosystems (Southampton,
UK) for the following genes, therefore sequences are not available:

18S (ABI number – Hs99999901_s1)
human cyclophilin A (ABI number – Hs99999904_m1)
human 11β-HSD1 (ABI number – Hs00194153_m1)

Other primers/ probes had been previously designed and validated by other users in
the department (Wake et al 2003; Goedecke et al 2006; Wake et al 2007b) to meet the
following specifications: Primers – melting temperature (Tm) 66-68°C, 20-80% GC content, length 9-40 bases, <2°C difference between primers, maximum of 2 G/C’s in the last 5 bases at 3’ end. Probe – Tm 10°C higher than primer Tm, 20-80% GC, length 9-40 bases, no G on the 5’ end, <4 contiguous G’s, more C’s than G’s. All primer/probe sets were designed with at least one primer or probe spanning an intron, to prevent amplification of genomic DNA. Primers were purchased from Applied Biosystems (Southampton, UK), with the following sequences:

**human 5α-reductase Type 1**

5' CGT TTT CTA ATA GGT TTT GGC TTG T 3' (forward),
5' CCC TTG GTA TTT TGT ATC CAG TAT CTC 3' (reverse),
5' 6-FAM- ATAAACATC CATTCAGAT CATATC CTAAGGAATCTCA A- TAMRA 3' (probe)

(Accession number – NM_001047)

**human GR-α**

5' CAT TGT CAA GAG GGA AGG AAA CTC 3' (forward),
5' GAT TTT CAA CCA CTT CAT GCA TAG AA 3' (reverse),
5' 6-FAM- TTT GTC AGT TGA TAA AAC CGC TGC CAG TTC T- TAMRA 3' (probe)

(Accession number – NM_000176)

**human H6PDH**

5' ACC TTC GCA GCC GTC CTA GT 3' (forward),
5' CAA GGC TTT GCC AGA CAT CAG 3' (reverse),
5' 6-FAM- CAC ATT GAC AAC C'T'C CGC TGG GAG G- TAMRA 3' (probe)

(Accession number – NM_004285)
human leptin

5' CAT TTC ACA CAC GCA GTC AGT CT 3' (forward),
5' TGT CTG GTC CAT CTT GGA TAA GGT 3' (reverse),
5' 6-FAM- AAC AGA AAG TCA CCG GTT TGG ACT TCA TTC C- TAMRA 3' (probe)

(Accession number – NM_000230)

human adiponectin

5' TGA CAC CAA CTG ATC ACC ACT AAC T 3' (forward),
5' CCG TAC TGA AAG CCT TTA ATT GAC TT 3' (reverse),
5' 6-FAM- CCT CCT CCA GGC CAA ACA GCC C- TAMRA 3' (probe)

(Accession number – NM_004797)

human resistin

5' CTA ATA TTT AGG GCA ATA AGC AGC ATT 3' (forward),
5' CAC AGG CGG AGC CAC AAG 3' (reverse),
5' 6-FAM- CCC CCG AGG CTT CGC CGT- TAMRA 3' (probe)

(Accession number – NM_020415)

cDNA was diluted 1 in 20 in nuclease-free water, and 2 μl incubated with 1x Taqman mastermix (Applied Biosystems, Cheshire, UK), 6 pmol of each primer, and 2 pmol probe (made up to final volume of 10 μl with nuclease-free water) in 384 well PCR plates with optical heat sealing cover (Applied Biosystems, Cheshire, UK). The plates were subject to centrifugation at 2000 rcf for 1 minute at 4°C to ensure samples were mixed at the base of each well and transferred to ABI PRISM 7700
Sequence Detection for cycling/detection according to the manufacturer's instructions:

1) 95°C for 10 minutes (to activate DNA polymerase)
2) 95°C for 15 seconds (denaturing)
3) 60°C for 1 minute (annealing and extension)
(stages 2 and 3 repeated for 40 cycles)

Quantification was performed using the fluorescent dye FAM as reporter and TAMRA as quencher for all genes studied except for human cyclophilin A, where the fluorescent dye VIC was used as the reporter. FAM was detected using wavelengths of 483 nm for excitation and 533 nm for emission, while VIC was detected using excitation and emission wavelengths of 523 nm and 568 nm respectively.

A standard curve for each primer probe set was generated by serial dilutions (in nuclease-free water) of complementary DNA pooled from all subjects (1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64, 1 in 128). Samples were analysed in triplicate and the mean values of RNA abundance interpolated from the standard curve to calculate transcript levels. Each standard curve was considered acceptable if the r value was >0.99. The variability of RNA abundance between the triplicates was deemed acceptable if less than 10%. Either 18S or the mean of 18S and cyclophilin A (noted in each of the relevant chapters) was used as internal control to normalise abundances of transcript levels. The results are expressed as a ratio to the internal control. Reverse transcriptase negative controls were used to confirm the absence of amplification of genomic DNA.

2.8.7.1 Real time PCR in rodent tissues

mRNA transcript quantification was performed using the Lightcycler 480 Sequence Detection System (Roche Diagnostics, West Sussex, UK). This real time PCR
instrument was recently purchased by the department, so the samples from the rodent diet study described in chapter 5 (which was performed after the two human studies) were analysed on the Lightcycler 480. 18S primers/probe 20x mixture was purchased from Applied Biosystems (Southampton, UK) as for the human studies because the sequences are not species-specific. Other primers/probes had been previously designed and validated by other users in the department (Drake et al 2005), and were purchased from Applied Biosystems with the following sequences:

**rat cyclophilin A**

5' CCC ACC GTG TTC TTC GAC AT 3' (forward),  
5' GAA AGT TTT CTG CTG TCT TTG GAA CT 3' (reverse),  
5' 6-FAM- CAA GGG CTC GCC ATC AGC CGT- TAMRA 3' (probe)  
(Accession number – NM_017101)

**rat 11β-HSD1**

5' TCA TAG ACA CAG AAA CAG CTT TGA AA 3' (forward),  
5' CTC CAG GGC GCA TTC CT 3' (reverse),  
5' 6-FAM- CTG GGA TAA TCT TGA GTC AAG CTG CTC CC- TAMRA 3' (probe)  
(Accession number – NM_017080)

The procedure followed was identical to the human procedure except for the following. cDNA was diluted 1 in 20 in nuclease-free water, and 2 μl incubated with 1x Lightcycler 480 probes master mix (Roche Diagnostics, West Sussex, UK), 6 pmol of each primer, and 2 pmol probe (made up to final volume of 10 μl with nuclease-free water) in 384 well PCR plates sealed using Lightcycler 480 sealing foil (Roche Diagnostics, West Sussex, UK). The plates were centrifuged at 2000 rcf for 1 minute at 4°C to ensure samples were mixed at the base of each well and transferred.
to a Lightcycler 480 for cycling/ detection according to the manufacturer’s instructions:

1) 95°C for 5 minutes (to activate DNA polymerase)
2) 95°C for 10 seconds (denaturing)
3) 60°C for 30 seconds (annealing and extension)
(stages 2 and 3 repeated for 50 cycles)

2.9 QUANTIFICATION OF INDOCYANINE GREEN (ICG)

This was performed in chapter 3, to measure hepatic blood flow. ICG was measured by adapting a previously published method (Burns et al 1991).

2.9.1 Extraction of ICG from serum

Serum samples were kept on ice throughout the procedure. Standards of ICG were prepared in distilled water in solutions representing a series of concentrations (blank, 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 8µg/ml) in 2 ml glass vials (MacKay and Lynn, Edinburgh, UK). Diazepam was used as internal standard (Rappaport & Thiessen 1982), with 2 µg added to each standard solution. Standards were dried down under OFN at 60°C, then reconstituted in 500 µl of the participant’s own serum, obtained prior to the ICG infusion. For samples, 2 µg of diazepam was added to 500 µl of serum. 500 µl of acetonitrile was added to standards and samples, vortexed for 45 seconds to mix, and subject to centrifugation at 2000 rcf at room temperature for 10 minutes to sediment protein. The supernatant was transferred to new 2 ml glass vials and 10 mg of ammonium sulphate crystals added to each vial. Vials were vortexed for 45 seconds, then subject to centrifugation at 2000 rcf for 10 minutes to separate organic and aqueous phases. 300 µl of the organic supernatant containing the ICG was transferred to HPLC vials (Chromacol Ltd, Hertfordshire, UK) and added to 300 µl water. Vials were then capped and immediately analysed.
2.9.2 HPLC measurement of ICG

Detection of ICG and diazepam was performed using a P680 HPLC pump and a PDA-100 photodiode array detector (Dionex, Sunnyvale, CA). 200 µl of standards and samples were injected onto a Nova-pak C18 4µm column (300mm length x 3.9mm id) (Waters, Herts, UK) with a temperature of 35°C, using run times of 34 minutes duration. Analytes were eluted under linear gradient conditions at 1 ml/min, with the following composition:

Initial – 80% water, 12% acetonitrile, and 8% methanol
0-17 minutes – 25% water, 65% acetonitrile, and 8% methanol
17-29 minutes – 80% water, 12% acetonitrile, and 8% methanol
29-34 minutes – 80% water, 12% acetonitrile, and 8% methanol

Standards were injected at the start of every run to confirm the retention time and the identity of the peaks. The retention times were approximately 11 minutes for ICG and 16 minutes for diazepam (Figure 2.4). ICG was detected using a wavelength of 784 nm, with diazepam at wavelength 230 nm. The area under the peaks was calculated using Chromeleon software. The concentration of ICG was determined using the ratio of (area under ICG peak)/ (area under diazepam peak), and interpolated onto the standard curve which was run in each batch. The standard curve was deemed acceptable if the r value was >0.99, while the limit of detection was less than 0.05 mcg/ml.
2.10 OTHER PLASMA/ SERUM BIOCHEMISTRY MEASUREMENTS

Screening blood tests were performed in each of the clinical studies. All subjects had normal full blood count (FBC) (white cell count, haemoglobin, haematocrit and platelets), urea and electrolytes (U&Es) (sodium, potassium, urea, creatinine), glucose, liver function tests (LFTs) (albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, gamma-glutamyltransferase (γGT), bilirubin) and thyroid function tests (TFTs) (thyroid-stimulating hormone (TSH), free thyroxine (T4)).
All the following analytes were measured on an Olympus analyser (Olympus Diagnostics, Co. Clare, Ireland). Serum sodium and potassium were measured by electrical potential generation using an Olympus ISE module. Serum urea, creatinine, bicarbonate, glucose, albumin, ALT, AST, alkaline phosphatase, γGT, total cholesterol, high density lipoprotein-cholesterol (HDL-C) and triglycerides were measured using enzymatic colorimetric methods (Olympus Diagnostics, Co. Clare, Ireland). Serum bilirubin was measured by an enzymatic colorimetric method (Audit Diagnostics, Cork, Ireland). Thyroid-stimulating hormone (TSH) and free thyroxine (T₄) were measured using a chemiluminescent immunoassay (Architect, Abbott Laboratories, IL, USA).

FBC was analysed using a Sysmex XE automated analyser.

Plasma HbA1C was analysed by HPLC, measuring absorbance at 415nm with background correction of refraction at 690nm using a Bio-Rad Variant II HbA1C programme and analyser (Bio-Rad Laboratories, Hertfordshire, UK).

Plasma insulin and c-peptide were measured using chemiluminetic immunoassays on an Advia Centaur analyser (Siemens, Deerfield, IL, USA).

In chapter 6, plasma glucose, free fatty acids and 3-hydroxybutyrate (3-HOB) concentrations were analysed on a Modular P analyser (Roche Diagnostics, West Sussex, UK). Glucose was measured using the hexokinase enzymatic method (Roche), FFAs using an enzymatic colorimetric method (Wako, Neuss, Germany), while 3-HOB was quantitated using a kinetic enzymatic method (Randox, Crumlin, Co. Antrim, UK).
2.10.1 Plasma androgens

2.10.1.1 Androstenedione

Androstenedione was measured in chapter 6 using an ELISA kit as per the manufacturer’s instructions (DRG instruments, Marburg, Germany). Blood was collected in EDTA tubes, subjected to centrifugation at 2000 rcf at 4°C for 10 minutes, plasma placed in microcentrifuge tubes and frozen at -20°C until analysis. Materials were thawed to room temperature, then 20 μl of undiluted samples and standards placed in wells coated with polyclonal anti-androstenedione antibody. Two hundred microlitres of enzyme conjugate (androstenedione bound to horseradish peroxidase) was added to wells, mixed and incubated for 1 hour at 25°C in a rotator (Grant Instruments Ltd, Cambridgeshire, UK). Wells were washed 3 times using 400 μl 1x wash buffer in a microplate washer (Anthos Labtec Instruments, Salzburg, Austria). Tetramethylbenzidine (TMB) (200μl) was added to wells and incubated for 15 minutes at 25°C in a rotator. Stop solution (100 μl of 0.5mol/l sulphuric acid) was added to wells and mixed, then absorbance read on a microplate reader (Dynatech Laboratories, Guernsey, Channel Islands, UK) at 450 nm. A standard curve prepared using a series of concentrations in the range 0.35-34.92 nmol/l was analysed in each batch and used to calculate plasma concentrations. Samples were analysed in duplicate and the mean of these duplicate values was recorded. The standard curve was deemed acceptable if the r value was >0.99, while variability of <5% between duplicates was considered acceptable.

2.10.1.2 Dehydroepiandrosterone (DHEA)

DHEA was measured in chapter 6 using an ELISA kit as per the manufacturer’s instructions (DRG instruments, Marburg, Germany). Blood was collected in EDTA tubes, subject to centrifugation at 2000 rcf at 4°C for 10 minutes, plasma placed in microcentrifuge tubes and frozen at -20°C until analysis. Materials were thawed to room temperature, then 20 μl of undiluted samples and standards placed in wells coated with rabbit anti-DHEA antibody. One hundred microlitres of enzyme
conjugate (DHEA bound to horseradish peroxidase) was added to wells, mixed and incubated for 1 hour at 25°C in a rotator (Grant Instruments Ltd, Cambridgeshire, UK). Wells were washed 3 times using 400 μl 1x wash buffer in a microplate washer (Anthos Labtec Instruments, Salzburg, Austria). Tetramethylbenzidine (TMB) (100 μl) was added to wells and incubated for 15 minutes at 25°C in a rotator. Stop solution (100 μl 0.5mol/l sulphuric acid) was added to wells and mixed, then absorbance read on a microplate reader (Dynatech Laboratories, Guernsey, Channel Islands, UK) at 450 nm. A standard curve prepared using a series of concentrations in the range 1.28-103.8 mmol/l was analysed in each batch and used to calculate plasma DHEA concentrations. Samples were analysed in duplicate and the mean of these duplicate values was recorded. The standard curve was deemed acceptable if the r value was >0.99, while variability of <5% between duplicates was considered acceptable.
Chapter 3
The contribution of visceral adipose tissue and liver to splanchnic cortisol release

3.1 INTRODUCTION

Visceral adipose tissue volume has been shown to correlate with metabolic disease, although it is unproven whether it is causative (Jensen 2006). Adipose 11β-HSD1 activity in vitro was reported to be higher in visceral than subcutaneous adipose tissue, and increased 11β-HSD1 activity in visceral adipose tissue has been hypothesized to be a potential cause of obesity and the metabolic syndrome (Bujalska et al 1997). Products from visceral adipose tissue, including free fatty acids, drain directly into the portal vein (Nielsen et al 2004b), which is the main source of blood supplying the liver. Thus, increased production of cortisol from visceral adipose tissue could lead to increased cortisol delivery to the liver with resultant adverse metabolic effects. Indeed, the transgenic mice selectively over-expressing adipose 11β-HSD1 have 2-3 fold increased corticosterone concentrations in their portal vein (Masuzaki et al 2001). To date, the majority of human studies which have examined visceral adipose 11β-HSD1 have been performed on biopsied tissue examined in vitro. The reports are conflicting, with some showing increased visceral adipose 11β-HSD1 in obesity (Desbriere et al 2006; Mariniello et al 2006; Michailidou et al 2007; Paulsen et al 2007) and some no change (Tomlinson et al 2002; Goedcke et al 2006; Li et al 2007). However, even if 11β-HSD1 activity per volume adipose tissue is unchanged in obesity, the increased visceral fat mass may cause increased cortisol release into the portal vein.

In vivo quantification of cortisol generation by visceral adipose 11β-HSD1 has been more difficult to examine. Two groups have utilised novel stable isotope infusions
(9,11,12,12⁻²[H]₄-cortisol) and selective cannulation of the hepatic vein in healthy people to directly quantify splanchnic cortisol generation. Our group determined that 11β-HSD1 reductase activity in the splanchnic circulation (viscera and liver together) generated 45 nmol of cortisol per minute, which accounted for all of whole body 11β-HSD1 reductase activity (Andrew et al 2005). Furthermore, utilising the oral cortisone test as an indirect measurement of hepatic 11β-HSD1 activity, we estimated that 15 nmol/min of this value is produced by the liver, while the remaining 30 nmol/min is generated by the visceral tissues (including visceral adipose tissue). Basu et al estimated splanchnic cortisol regeneration by 11β-HSD1 at 61 nmol/min, which was similar to cortisol production by the adrenals, and again accounted for the majority of whole body 11β-HSD1 activity (Basu et al 2004). This group further examined splanchnic cortisol production in obesity and type 2 diabetes, but found no change in 11β-HSD1 activity in either of these conditions compared with lean healthy controls (Basu et al 2005). Unfortunately, this study was unable to dissect the relative contributions of the liver and visceral adipose tissue to splanchnic 11β-HSD1 activity, while hepatic 11β-HSD1 activity is known to be decreased in obesity (Stewart et al 1999; Rask et al 2001; Rask et al 2002). Consequently, increased visceral adipose tissue 11β-HSD1 activity may have been missed utilising this approach.

Recently, cannulation of the portal vein, hepatic vein and hepatic artery has been performed in combination with deuterated cortisol infusions in dogs to attempt to quantify the relative contributions of these tissues (Basu et al 2006a). Significant cortisol production via 11β-HSD1 reductase activity was found in the liver, but little if any cortisol was released by the viscera. Although these results suggest that the liver is the major site of cortisol regeneration, they cannot be directly extrapolated to humans, since the dogs were lean and thus probably had little visceral adipose tissue. Two groups have examined portal or omental vein cortisol concentrations in obese humans during abdominal surgery, and found no difference between portal and systemic concentrations (Aldhahi et al 2004; Tarantino et al 2007). However, the stress of major surgery makes it difficult to accurately detect differences in basal
cortisol concentrations and does not discriminate between cortisol produced by 11β-HSD1 or the adrenal.

Cannulation of the portal vein is very difficult in humans not undergoing abdominal surgery, as the only other approach is direct cannulation through the liver. However, patients with cirrhosis and associated portal hypertension often receive treatment in the form of a shunt placed between the portal vein and the hepatic vein, termed a transjugular intrahepatic porto-systemic shunt (TIPSS). This relieves hypertension in the portal system, and also allows direct access to the portal vein through the TIPSS. We hypothesized that both the liver and visceral adipose tissue contribute to splanchnic cortisol release by 11β-HSD1 in humans. In this chapter, we report investigation of deuterated cortisol infusions in individuals with combined cannulation of the portal and hepatic vein.

3.2 METHODS

3.2.1 Participants

4 male volunteers were recruited. Inclusion criteria were as follows: age 20-70 years; body mass index between 20 and 40 kg/m²; a functional TIPSS in situ, placed at least 1 year previously for alcohol-induced cirrhosis with portal hypertension; attending for annual check of TIPSS; no other cause of liver disease; no current alcohol intake; normal full blood count (FBC), renal and thyroid function on biochemical screening; no glucocorticoid therapy in the previous 6 months. Local ethical approval and written informed consent were obtained.

3.2.2 Protocol

Subjects were admitted to the Royal Infirmary of Edinburgh (RIE) the night prior to assessment. Volunteers were given 1mg dexamethasone tablet (Organon
Laboratories, Cambridge, UK) at 11pm to suppress adrenal cortisol production and fasted from this point onwards. At 8am, subjects were transferred to the Clinical Research Facility (CRF). Measurements were taken of their height, weight, waist and hip circumference, fat mass and percentage body fat using an Omron BF-302 bioimpedence monitor (Omron Healthcare, Milton Keynes, UK), and blood pressure using an Omron 705IT monitor (Omron Healthcare, Milton Keynes, UK). Blood measurements were taken for fasting glucose, lipids and HbA1C. 2 x 21G cannulae (BD venflon, Becton Dickinson, Oxford, UK) were placed, one in the right antecubital fossa vein for infusion and the other in a left dorsal hand vein for sampling. The left hand was placed in a box heated to 60°C to achieve arterialisation. The stable isotope tracer deuterated cortisol (9,11,12,12-[H]4-cortisol (Cambridge Isotopes, Andover, MA, USA)) was infused at 40% molar enrichment with 60% hydrocortisone (Calbiochem, Nottingham, UK) at a rate of 1.74mg/ hour for 210 minutes after an initial 3.5mg bolus. Dexamethasone (Organon Laboratories, Cambridge, UK) was concurrently infused at a rate of 240mcg/ hour.

Subjects were then transferred to the interventional radiology suite. Volunteers were sedated with 5mg intravenous midazolam (Roche Products Limited, Welwyn Garden City, UK), then 5ml of 2% lidocaine (Hameln Pharmaceuticals, Gloucester, UK) was injected subcutaneously in the skin overlying the right internal jugular vein. The internal jugular vein was cannulated and a 5F pigtail catheter (Cordis, Berkshire, UK) passed into the TIPSS under x-ray guidance. After confirming patency of the TIPSS, the catheter was positioned in the portal vein for sampling. A 5F vertebral catheter (Merrit Medical, Lanarkshire, UK) was then placed in a separate hepatic vein (Figure 3.1) for sampling. Volunteers then returned to the CRF. At 120 minutes, an indocyanine green (ICG) (Pulsion Medical, Middlesex, UK) infusion was commenced into the antecubital vein at a rate of 30mg/ hour. Blood samples were taken from the portal, hepatic and arterialised veins as in Figure 3.2. Samples taken during steady state (180-210 minutes) were used for kinetic analyses.
Figure 3.1  Hepatic blood supply and placement of cannulae for sampling

Blood from the viscera (e.g. visceral adipose tissue, gut, spleen) drains into the portal vein (PV), which is the major blood supply to the liver in addition to the hepatic artery (HA). Blood is drained from the liver by the left (LHV), central (CHV), and right hepatic veins (RHV). In cirrhosis with portal hypertension, a transjugular intrahepatic porto-systemic shunt (TIPSS) is placed to connect the PV to the CHV, thus bypassing the liver and reducing portal blood pressure. In this study, one catheter was passed down the CHV, through the TIPSS into the PV for sampling portal blood, while a second catheter was positioned in the RHV for sampling hepatic venous blood. Arrows in the respective veins and artery depict the direction of blood flow.
Figure 3.2  Study protocol

Diagram depicting the duration of infusion and blood samples obtained from the arterialised, hepatic and portal cannulae on the study day. ICG = indocyanine green; X = plasma samples taken from respective cannulae for measurement of endogenous and tracer cortisol; F = serum samples obtained simultaneously from arterialised and hepatic vein cannulae for measurement of hepatic blood flow.

<table>
<thead>
<tr>
<th>Night before 23:00h, 1 mg oral dexamethasone</th>
<th>cortisol/ d4-cortisol (60:40), bolus 3.5 mg + 1.74 mg/h IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dexamethasone 240 mcg/ hour IV</td>
</tr>
<tr>
<td></td>
<td>ICG 30mg/ hour IV</td>
</tr>
<tr>
<td></td>
<td>Portal venous catheter</td>
</tr>
<tr>
<td></td>
<td>Hepatic venous catheter</td>
</tr>
</tbody>
</table>

Artery       X       X       X       XXXXXXXX
Hepatic Vein FF       FFFFFFF
Portal Vein  X       X       XXXXXXXX

Duration of infusion (minutes)

3.2.3 Laboratory analyses

Plasma FBC, U&Es, TFTs, glucose, lipids and HbA1C were measured as previously described (section 2.10).
Plasma cortisol, d3-cortisol, d4-cortisol, cortisone and d3-cortisone were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in section 2.6.

Serum ICG was measured as described in section 2.9.

3.2.4 Calculation of hepatic blood flow

The concentration of ICG in mg/ litre in the artery (ICG[A]) and hepatic vein (ICG[HV]) in steady state were used to calculate hepatic blood flow (HBF) (Leevy et al 1962). At steady state, which occurs after 30 minutes of continuous infusion (Soons et al 1991), the ICG infusion rate equals the rate of extraction which was 0.5 mg/ minute. The equation used was as follows:

\[ \text{HBF (litres/ min)} = \frac{\text{extraction rate}}{(\text{ICG[A]} - \text{ICG[HV]}) \times 1/ (1 - \text{haematocrit})} \]

3.2.5 Cortisol kinetic analysis

Cortisol kinetics were calculated using the mean of 7 measurements obtained during steady state. The rate of appearance of cortisol and d3-cortisol were calculated as described previously (section 2.5.8).

The equations used to determine tissue cortisol release secondary to 11β-HSD1 are based on the principles as recommended by Robert Wolfe (Wolfe R.L. & Chinkes 2005). Tissue A-V appearance of a substance (in this instance either unlabelled cortisol or d3-cortisol) is calculated by:

\[ \text{total appearance} – \text{influx} \]
Total appearance is calculated by:

\[ \text{Blood flow} \times \text{arterial concentration} \times \text{tracer: tracee ratio [artery]} \div \text{tracer: tracee ratio [vein]} \]

Influx is calculated as:

\[ \text{Blood flow} \times \text{arterial concentration} \]

Using this equation structure, we have substituted d4-cortisol as the tracer and either cortisol or d3-cortisol as the respective tracee, and used these equations to determine tissue appearance of cortisol and d3-cortisol.

Splanchnic cortisol (equation 1 below) and d3-cortisol (equation 2) generation by 11β-HSD1 (from visceral tissues and liver combined) was calculated as previously described (Andrew et al 2005):

1) \[ \text{cortisol[A]} \times \text{HBF} \times \frac{(\text{d4-cortisol[A]} \div \text{cortisol[A]})}{(\text{d4-cortisol[HV]} \div \text{cortisol[HV]})} - (\text{cortisol[A]} \times \text{HBF}) \]

2) \[ (\text{d3-cortisol[A]} \times \text{HBF} \times \frac{(\text{d4-cortisol[A]} \div \text{d3-cortisol[A]})}{(\text{d4-cortisol[HV]} \div \text{d3-cortisol[HV]})}) - (\text{d3-cortisol[A]} \times \text{HBF}) \]

Splanchnic cortisol generation was also calculated using the equation derived by Basu et al (Basu et al 2004):

3) \[ \frac{(\text{HBF} \times (\text{d4-cortisol[A]} - \text{d4-cortisol[HV]}))}{(\text{d4-cortisol[A]} \div \text{cortisol[A]})} - (\text{HBF} \times (\text{cortisol[A]} - \text{cortisol[HV]})) \]
Cortisol and d3-cortisol generation by the visceral tissues alone was calculated by:

4) \( \frac{\text{cortisol}[A] \times \text{PBF} \times \left( \frac{\text{d4-cortisol}[A]}{\text{cortisol}[A]} \right)}{\left( \frac{\text{d4-cortisol}[\text{PV}]}{\text{cortisol}[\text{PV}]} \right)} - \left( \text{cortisol}[A] \times \text{PBF} \right) \)

5) \( \frac{\text{d3-cortisol}[A] \times \text{PBF} \times \left( \frac{\text{d4-cortisol}[A]}{\text{d3-cortisol}[A]} \right)}{\left( \frac{\text{d4-cortisol}[\text{PV}]}{\text{d3-cortisol}[\text{PV}]} \right)} - \left( \text{d3-cortisol}[A] \times \text{PBF} \right) \)

In these equations PBF represents the portal blood flow. However, only the HBF is directly measured, so the portal blood flow has to be estimated. In health, the portal vein accounts for approximately 70-80% of the blood travelling through the liver, with the hepatic artery responsible for the remaining 20% (Zoli et al 1999). In cirrhosis, portal blood flow decreases with a compensatory increase in hepatic artery blood flow (HABF) (Richter et al 2000; Lautt 2007). Placement of a TIPSS also decreases portal blood flow to the liver with a further increase in HABF (Patel et al 2001). Consequently, as little as 10% of HBF may originate from the portal vein (Menzel et al 1997). In order to account for this unknown, we have modelled a range of PBF between 10-80% of the HBF, and concordantly for the HABF to range between 90-20% of HBF.

Cortisol and d3-cortisol generation across the liver was calculated by:

6) \( \frac{\left( \text{cortisol}[A] \times \text{HABF} \right) + \left( \text{cortisol}[\text{PV}] \times \text{PBF} \right)}{\left( \text{HABF} \times \text{HBF} \right)} \times \frac{\left( \left( \frac{\text{d4-cortisol}[A]}{\text{cortisol}[A]} \right) \times \left( \frac{\text{PBF}}{\text{HBF}} \right) \times \left( \frac{\text{d4-cortisol}[\text{PV}]}{\text{cortisol}[\text{PV}]} \right) \right)}{\left( \frac{\text{d4-cortisol}[\text{HV}]}{\text{cortisol}[\text{HV}]} \right)} - \left( \text{cortisol}[A] \times \text{HABF} \right) + \left( \text{cortisol}[\text{PV}] \times \text{PBF} \right) \)
\begin{align*}
7) & \quad (d_3\text{-cortisol}[A] \times HABF) + (d_3\text{-cortisol}[PV] \times PBF) \times ((HABF/ HBF) \times (d_4\text{-cortisol}[A]/ d_3\text{-cortisol}[A])) + ((PBF/ HBF) \times (d_4\text{-cortisol}[PV]/ d_3\text{-cortisol}[PV]))) / (d_4\text{-cortisol}[HV]/ d_3\text{-cortisol}[HV]))
\quad - ((d_3\text{-cortisol}[A] \times HABF) + (d_3\text{-cortisol}[PV] \times PBF))
\end{align*}

The following equations used by Basu et al to calculate liver and visceral $11\beta$-HSD1 reductase activity in dogs (Basu et al 2006a) were used to compare against the results obtained using the above equations:

\begin{align*}
8) & \quad \text{Liver cortisol extraction ratio} = \frac{(d_4\text{-cortisol}[A] \times HABF) + (d_4\text{-cortisol}[PV] \times PBF) - (d_4\text{-cortisol}[HV] \times HBF)}{(d_4\text{-cortisol}[A] \times HABF) + (d_4\text{-cortisol}[PV] \times PBF)}

9) & \quad \text{Visceral cortisol extraction ratio} = \frac{(d_4\text{-cortisol}[A] - d_4\text{-cortisol}[PV])}{d_4\text{-cortisol}[A]}

10) & \quad \text{Net visceral cortisol balance} = \text{total cortisol}[A] - \text{total cortisol}[PV] \times PBF

11) & \quad \text{Net liver cortisol balance} = (\text{total cortisol}[A] \times HABF) + (\text{total cortisol}[PV] \times PBF) - (\text{total cortisol}[HV] \times HBF)

12) & \quad \text{Liver cortisol uptake} = (\text{total cortisol}[A] \times HBF) \times \text{liver cortisol extraction ratio}

13) & \quad \text{Visceral cortisol uptake} = (\text{total cortisol}[PV] \times PBF) \times \text{visceral cortisol extraction ratio}
\end{align*}
14) Liver cortisol production = liver cortisol balance – liver cortisol uptake

15) Visceral cortisol production = visceral cortisol balance – visceral cortisol uptake

In the above equations total cortisol equals the sum of cortisol + d3-cortisol + d4-cortisol concentrations. Cortisone and d3-cortisone generation across the viscera was measured by the following equations:

16) \((\text{cortisone}[\text{PV}] - \text{cortisone}[\text{A}]) \times \text{PBF}\)

17) \((\text{d3-cortisone}[\text{PV}] - \text{d3-cortisone}[\text{A}]) \times \text{PBF}\)

3.2.6 Statistical analysis

SPSS version 14 was used for all analyses. Measurements of multiple variables in individuals were analysed by repeated measures ANOVA with post-hoc testing using Fisher’s least significant differences test. Tests of one variable to determine differences from zero were performed using the one-sample t test. p<0.05 was considered significant.

3.3 RESULTS

3.3.1 Subject characteristics

Data are presented as mean ± SEM. The 4 participants’ anthropometric and biochemistry measurements are shown in Table 3.1. Three of the 4 subjects had no
other medical conditions except cirrhosis, while 1 subject had type 2 diabetes mellitus (subject 1). Three of the 4 subjects were prescribed regular medications – proton pump inhibitors were used by 3 of the participants while insulin, metoprolol, spironolactone, furosemide and aspirin were taken each by 1 subject.

Table 3.1 Participant anthropometry and fasting biochemistry.

Data are mean ± SEM. BMI = body mass index; HbA1C = glycated haemoglobin A1C.

<table>
<thead>
<tr>
<th></th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>52.9</td>
<td>38.6</td>
<td>56.3</td>
<td>55.8</td>
<td>50.9 ± 4.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.9</td>
<td>33.0</td>
<td>34.2</td>
<td>29.1</td>
<td>32.3 ± 1.1</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>31.1</td>
<td>26.1</td>
<td>36.4</td>
<td>28.8</td>
<td>30.6 ± 2.6</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>31.2</td>
<td>29.2</td>
<td>44.7</td>
<td>24.7</td>
<td>32.5 ± 4.3</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>13.6</td>
<td>5.6</td>
<td>6.6</td>
<td>5.3</td>
<td>7.8 ± 2.0</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>9.9</td>
<td>5.1</td>
<td>5.5</td>
<td>4.7</td>
<td>6.3 ± 1.2</td>
</tr>
<tr>
<td>Total plasma cholesterol (mmol/l)</td>
<td>2.9</td>
<td>4.4</td>
<td>3.7</td>
<td>6.1</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/l)</td>
<td>1.9</td>
<td>0.5</td>
<td>0.9</td>
<td>0.8</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>
3.3.2 Plasma cortisol and cortisone during deuterated cortisol infusion

Adrenal cortisol production was suppressed by the dexamethasone in all participants, as indicated by fasting morning plasma cortisol concentrations of $15 \pm 5$ nmol/l. Likewise, fasting plasma cortisone concentrations were $11 \pm 2$ nmol/l. Steady state was achieved between 180 and 210 minutes of the deuterated cortisol infusion (Figure 3.3).
Figure 3.3  Plasma measurements during deuterated cortisol infusion

Data are mean ± SEM for n=4 during deuterated cortisol infusion, with plasma samples from arterialised (filled squares), portal (open diamonds) and hepatic (filled triangles) cannulae. a) Plasma cortisol concentrations. b) Plasma d4-cortisol/ cortisol enrichment. c) d4-cortisol/ d3-cortisol ratio. Steady state was achieved between 180 and 210 minutes, with less than 4% variation observed in d4-cortisol enrichment.

Duration of infusion (mins)

<table>
<thead>
<tr>
<th>Duration</th>
<th>Artery</th>
<th>Portal Vein</th>
<th>Hepatic vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>210</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Plasma unlabelled and tracer cortisol and cortisone measurements at steady state are shown in Table 3.2. Plasma cortisol concentrations were no different between the artery, hepatic vein or portal vein. d3-Cortisol concentrations were increased and cortisone and d3-cortisone concentrations decreased in the hepatic vein, indicating substantial hepatic 11β-HSD1 reductase activity and cortisone extraction. Conversely, d4-cortisol concentrations, which cannot be regenerated, were lower in the hepatic vein indicating significant hepatic cortisol metabolism. However, d3-cortisone levels were significantly increased and there was a trend for increased cortisone (p=0.051) in the portal vein compared to the artery, consistent with visceral dehydrogenase activity.

Table 3.2 Steady state concentrations

Data are mean ± SEM for n=4 participants. The mean data from 7 samples obtained between 180 and 210 minutes was used to calculate steady state concentrations. Comparisons were made by repeated measures ANOVA with post-hoc testing with Fisher’s least significant differences test. * p<0.05 compared to artery, °p<0.05 compared to portal vein.

<table>
<thead>
<tr>
<th>Site of sampling</th>
<th>Artery</th>
<th>Portal Vein</th>
<th>Hepatic Vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol (nmol/l)</td>
<td>186 ± 17</td>
<td>176 ± 22</td>
<td>180 ± 18</td>
</tr>
<tr>
<td>d3-Cortisol (nmol/l)</td>
<td>59 ± 4</td>
<td>56 ± 4</td>
<td>65 ± 6 °</td>
</tr>
<tr>
<td>d4-Cortisol (nmol/l)</td>
<td>71 ± 9</td>
<td>67 ± 11</td>
<td>57 ± 6 °</td>
</tr>
<tr>
<td>Cortisone (nmol/l)</td>
<td>75 ± 6</td>
<td>91 ± 6</td>
<td>22 ± 16 °</td>
</tr>
<tr>
<td>d3-Cortisone (nmol/l)</td>
<td>36 ± 4</td>
<td>45 ± 4 °</td>
<td>9 ± 8 °</td>
</tr>
</tbody>
</table>
### 3.3.3 Whole body and splanchnic 11β-HSD1 reductase activity

The rate of appearance of cortisol (Ra cortisol) and d3-cortisol (Ra d3-cortisol) in the artery, specific measures of whole body 11β-HSD1 reductase activity, was 36.3 ± 3.7 and 26.9 ± 1.8 nmol/ min respectively. Cortisol and d3-cortisol generation across the splanchnic bed was 13.5 ± 3.1 and 8.0 ± 1.7 nmol/ minute, calculated using equation 1 and 2 respectively (Table 3.3). This was discovered to be secondary to cortisol generation by 11β-HSD1 entirely by the liver, while no significant cortisol release from 11β-HSD1 reductase activity was observed by the viscera. Modelling for portal venous flow to range from 10 to 80% of hepatic blood flow did not significantly alter these results (Table 3.3). Splanchnic cortisol and d3-cortisol release were compared with hepatic cortisol and d3-cortisol release respectively using the paired t test. There was no significant difference between hepatic and splanchnic cortisol (p=0.8) or d3-cortisol release (p=0.5), confirming the liver was responsible for all of splanchnic cortisol release.
Table 3.3  Cortisol generation by 11β-HSD1 across the splanchnic tissues

Cortisol kinetics measuring 11β-HSD1 reductase activity across the splanchnic tissues (viscera plus liver), and the viscera and liver individually. The number in brackets denotes the equation used from the methods for each respective variable. Data presented as mean ± SEM. For equations 4–7, portal (and hepatic artery) blood flow is unknown, thus portal blood flow is modelled to range from 10 (1st number) to 80% (2nd number) of the total hepatic blood flow. Comparisons of each variable against zero were calculated using the one sample t-test. * p<0.05, ** p<0.01.

<table>
<thead>
<tr>
<th></th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic blood flow</td>
<td>0.26</td>
<td>0.54</td>
<td>0.54</td>
<td>0.26</td>
<td>0.40 ± 0.08**</td>
</tr>
<tr>
<td>(litres/ min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splanchnic cortisol</td>
<td>9.6</td>
<td>7.0</td>
<td>20.2</td>
<td>17.4</td>
<td>13.5±3.1*</td>
</tr>
<tr>
<td>release (nmol/ min) (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splanchnic d3-cortisol</td>
<td>6.6</td>
<td>3.9</td>
<td>11.1</td>
<td>10.6</td>
<td>8.0±1.7*</td>
</tr>
<tr>
<td>release (nmol/ min) (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splanchnic cortisol</td>
<td>7.0</td>
<td>6.5</td>
<td>17.0</td>
<td>12.6</td>
<td>10.8±2.5*</td>
</tr>
<tr>
<td>release (nmol/ min) (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visceral cortisol release</td>
<td>0.08 – 0.63</td>
<td>0.26 – 2.09</td>
<td>-0.38 – -3.07</td>
<td>0.05 – 0.40</td>
<td>0.00±0.14 – 0.01±1.09</td>
</tr>
<tr>
<td>(nmol/ min) (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visceral d3-cortisol</td>
<td>0.09 – 0.68</td>
<td>0.21 – 1.69</td>
<td>-0.12 – -0.94</td>
<td>0.03 – 0.20</td>
<td>0.05±0.07 – 0.41±0.55</td>
</tr>
<tr>
<td>release (nmol/ min) (5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic cortisol release</td>
<td>9.3 – 7.8</td>
<td>6.6 – 4.5</td>
<td>20.7 – 24.4</td>
<td>17.2 – 16.2</td>
<td>13.5±3.3 – 13.2±4.5*</td>
</tr>
<tr>
<td>(nmol/ min) (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatic d3-cortisol</td>
<td>6.4 – 5.1</td>
<td>3.7 – 2.1</td>
<td>11.3 – 12.6</td>
<td>10.5 – 9.9</td>
<td>8.0±1.8 – 7.4±2.4*</td>
</tr>
<tr>
<td>release (nmol/ min) (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Cortisol release by 11β-HSD1 separately by the liver and viscera, when calculated using the equations used by Basu et al (Basu et al 2006a), was in agreement with the above measurements. Again modelling portal blood flow to account for 10-80% of hepatic blood flow, total cortisol production by the liver (equation 14) was significant at $17.2 \pm 3.9 - 17.1 \pm 4.9 \text{ nmol/ min}$ ($p<0.05$). Liver total cortisol uptake (equation 12) was also significant, measuring $20.4 \pm 4.9 - 17.2 \pm 8.3 \text{ nmol/ min}$ ($p<0.05$), indicating substantial hepatic cortisol metabolism. Net liver cortisol balance (equation 11) was consequently no different from zero, measuring $-3.1 \pm 1.9 -0.2 \pm 3.4 \text{ nmol/ min}$. However, total cortisol production by the viscera (equation 15) was not different from zero, measuring $0.1 \pm 0.2 -0.4 \pm 1.2 \text{ nmol/ min}$ ($p=0.8$). Visceral cortisol uptake (equation 13) was similarly not significant at $0.4 \pm 0.6 -3.0 \pm 4.5 \text{ nmol/ min}$, meaning that net visceral cortisol balance (equation 10) measured $-0.4 \pm 0.4 -3.4 \pm 3.1 \text{ nmol/ min}$ and was not different from 0 ($p=0.4$).

Cortisone generation across the viscera (equation 16) measured $0.6 \pm 0.3 - 5.1 \pm 2.3 \text{ nmol/min}$ which was not significant ($p=0.12$ versus 0), modelling for portal flow to account for 10-80% of hepatic blood flow. However, there was a trend for d3-cortisone generation across the viscera (equation 17), measuring $0.3 \pm 0.1 - 2.7 \pm 1.1 \text{ nmol/min}$ ($p=0.09$ versus 0).

3.4 DISCUSSION

These data show for the first time the relative contribution of the viscera and liver to splanchnic cortisol production in humans. In all 4 of our participants, there was substantial splanchnic cortisol and d3-cortisone generation by 11β-HSD1 reductase activity in agreement with previous work (Basu et al 2004; Andrew et al 2005). In these individuals, the liver was the primary source of splanchnic cortisol regeneration, while there was no demonstrable cortisol release from 11β-HSD1 activity from the visceral tissues. While these results do not support our hypothesis, they are in agreement with the study by Basu et al which found that the liver was
completely responsible for splanchnic cortisol regeneration in dogs (Basu et al 2006a). Furthermore, the participants were all overweight or obese, with a mean total fat mass of 32.6 kg. Thus, it is highly unlikely that these subjects did not have enough visceral adipose tissue to release significant cortisol into the portal vein.

These results refute the hypothesis that increased 11β-HSD1 activity in visceral adipose tissue in humans is likely to cause increased cortisol concentrations in the portal vein. It would appear that cortisol release by visceral adipose 11β-HSD1 is insufficient to significantly impact on portal vein concentrations. Mean cortisol release with lower and upper 95% confidence intervals was 0.00 nmol/min (-0.43, 0.44) assuming portal blood flow accounted for 10% of hepatic blood flow in these volunteers, meaning that cortisol release by the viscera is at most 0.44 nmol/min which accounts for only 3% of splanchnic cortisol release in these volunteers. These results suggest that my supervisors’ previous model (Andrew et al 2005), in which they suggested that two thirds of splanchnic cortisol production was from the viscera and one third from liver, requires revision. In the original study, hepatic 11β-HSD1 activity was determined by measuring the conversion of orally administered cortisone to cortisol by first pass metabolism. Crucially, concentrations of cortisone reaching the liver after oral administration were estimated on the basis of estimates of bioavailability derived from the appearance of cortisol. A linear relationship between cortisone concentration and cortisol production was then assumed and this was extrapolated to the conditions observed in steady state. However, steady state portal vein cortisone concentrations could only be estimated and these were assumed to be lower than arterial concentrations in proportion to visceral 11β-HSD1 activity. In light of the results obtained here, it appears likely that cortisol generation by the liver at steady state was underestimated. Having now discovered significant cortisone production in the viscera it appears that portal vein cortisone concentrations in steady state in the previous study were underestimated. As a result, our model determined that liver 11β-HSD1 activity in steady state could only account for one-third of splanchnic 11β-HSD1 activity, and hence two-thirds of splanchnic 11β-HSD1 activity arose from the viscera. However, in the current study we observed higher
cortisone concentrations in the portal vein compared with the artery and no significant 11β-HSD1 reductase activity from the viscera. Therefore, the concentration of cortisone reaching the liver is higher than in the artery, meaning cortisol generation by hepatic 11β-HSD1 at steady state is higher than we had originally predicted. Accounting for this in our original model dramatically decreases the relative proportion of splanchnic 11β-HSD1 activity attributed to the viscera.

However, the volunteers studied here have liver disease and the above results may not be representative of visceral and liver cortisol generation in normal health. Indeed, cortisol metabolism predominantly occurs in the liver and is decreased in patients with cirrhosis (Tyler 1957; Kawai et al 1985), while extrahepatic cortisol metabolism by 11β-HSD2 may also be reduced in cirrhosis (Frey 2006). The decreased cortisol inactivation though is accompanied by reduced total cortisol urinary metabolites (Vogeser et al 1998), indicating that cortisol secretion is appropriately decreased in this setting, although the reduced cortisol inactivation may lead to loss of diurnal rhythm with elevated levels later in the day (Johnston et al 1982). However, it seems likely that the hepatic contribution to cortisol generation would be affected more than the adipose contribution, so it is no less surprising that visceral cortisol release was not observed. The blood supply to the liver is also altered in these participants, as the portal vein usually provides approximately 80% of the hepatic blood flow, with the hepatic artery accounting for the remainder (Zoli et al 1999). These participants have cirrhosis of the liver and a TIPSS in situ, which means portal blood flow will be dramatically reduced. The true proportion of portal blood flow supplying the liver is unknown, therefore we have modelled for portal blood flows to range between 10-80% of hepatic blood flow to account for this unknown variable. However, altering this parameter did not significantly alter the result that cortisol was not released from the viscera tissues.

Although we have sampled blood from the portal vein as a means to measure visceral adipose tissue cortisol production, the portal vein drains blood from other internal organs, which includes the gut, pancreas, and spleen. Thus, differences in cortisol
metabolism between the artery and portal vein (across the viscera) cannot be assumed to arise solely from visceral adipose tissue. However, our results suggest that none of these organs release cortisol by \(11\beta\)-HSD1 reductase activity. A proportion of blood in the portal circulation is shunted away from the portal vein in patients with liver disease via anastomoses with the systemic circulation (Bosch et al 1992), although this should occur less in patients with TIPSS in situ. In addition, portal blood flow can be reversed (hepatofugal flow) in some cirrhotic patients (Rector, Jr. et al 1988), which would dilute portal measurements with systemic blood, although in these patients portal flow was assessed during the placement of the portal venous catheter and determined to be flowing towards the liver in all patients. However, it is highly unlikely that venous blood from the respective internal organs (e.g. visceral adipose tissue) is shunted away from the portal vein to a greater extent than other organs, so that the blood reaching the portal vein is a mixture of the visceral tissues. Thus, the ratio of \(d_3\)-cortisol/ \(d_4\)-cortisol should still be increased if visceral adipose tissue is releasing cortisol into the portal vein, which we did not find in this study. However, the proportion of portal venous blood arising from visceral fat is unknown, and it remains possible that venous drainage from the other visceral tissues such as the gut dilutes out any observable change in \(d_3\)-cortisol/ \(d_4\)-cortisol ratios from visceral fat, which could only be confirmed by cannulation of one of the omental veins during the infusion.

Our results though do not suggest that \(11\beta\)-HSD1 is unimportant in visceral adipose tissue. Increased adipose \(11\beta\)-HSD1 reductase activity with resultant elevated local cortisol levels may be an important regulator of many metabolic processes, for example in promoting pre-adipocyte differentiation and increasing visceral adipose mass (De Sousa Peixoto et al 2008). Further work is required examining intra-adipose cortisol levels to determine the metabolic consequences of local cortisol excess.

Interestingly, steady state plasma \(d_3\)-cortisone concentrations were significantly increased in the portal vein compared with the arterialised samples, and there was a
strong trend for cortisone to do the same (Table 3.2). This was coupled with a trend for visceral d3-cortisone production and non-significantly decreased cortisol, d3-cortisol and d4-cortisol concentrations, supporting the presence of 11β-hydroxysteroid dehydrogenase activity in the viscera. It seems likely that this could be from the enzyme 11β-HSD2, which is expressed in the gut and the vessel wall and functions as an exclusive dehydrogenase (Funder et al 1988; Hadoke et al 2006), although it is also possible that 11β-HSD1 may be functioning in the dehydrogenase direction in visceral adipose tissue (Bujalska et al 2002). More in vivo work will be required to examine where this visceral dehydrogenase activity arises, potentially involving cannulation of the omental and mesenteric veins during infusion of stable cortisone tracer.

In our previous study using hepatic vein cannulation in healthy volunteers, total body cortisol regeneration by 11β-HSD1 was measured at 36 nmol/min with splanchnic cortisol production 45 nmol/min (Andrew et al 2005). Total body cortisol regeneration was very similar in this study measuring 37 nmol/min, however, splanchnic cortisol production was much less at 15 nmol/min. A reduction in absolute production rate may not be unexpected as these volunteers have abnormal liver function which may result in decreased hepatic 11β-HSD1 activity. More surprising is the fact that splanchnic cortisol production only accounted for one third of whole body 11β-HSD1 activity. However, measurement of hepatic blood flow (HBF) was dependent on clearance of ICG which potentially may not be accurate. Indeed, HBF measured 0.4 ± 0.1 litres/min in our 4 volunteers, compared with 1.2 ± 0.1 litres/min in our original study in healthy volunteers (Andrew et al 2005). It is possible that ICG extraction is reduced to a greater extent than hepatic blood flow in these individuals, leading to an underestimation of HBF in these individuals, with concordant underestimation of hepatic cortisol generation by 11β-HSD1. Alternatively, if these results are correct this may suggest that subjects with cirrhosis up-regulate 11β-HSD1 in other unmeasured tissues such as subcutaneous adipose, muscle or the brain to compensate for decreased splanchnic cortisol production.
To conclude, we have demonstrated considerable splanchnic cortisol generation by 11β-HSD1 in people with chronic liver disease. The liver, not visceral adipose tissue, is the major site of cortisol release in these individuals, indicating that visceral adipose tissue does not release cortisol into the portal vein. These results suggest that the liver is solely responsible for splanchnic cortisol release into the circulation, and that increased visceral adipose tissue mass would not lead to increased cortisol concentrations in the portal vein.
Chapter 4

Dietary regulation of glucocorticoid metabolism in human obesity

4.1 INTRODUCTION

The cause for dysregulation of cortisol metabolism, and in particular for the tissue-specific dysregulation of 11β-HSD1, in obesity is unknown. Epidemiology and short term intervention studies suggest that factors which predict adverse outcomes, over and above the effect of obesity, include dietary content of fat and carbohydrate (Foster et al 2003; Yancy, Jr. et al 2004; Griel & Kris-Etherton 2006; Halton et al 2006). There is evidence that the hypothalamic-pituitary-adrenal (HPA) axis responds to macronutrient intake, so that changes in circulating cortisol may mediate adverse effects of diet (Dallman et al 2004). In addition, metabolism of cortisol in extra-adrenal tissues may be responsive to dietary macronutrient content.

Recent experiments suggest that glucocorticoid metabolism is influenced by dietary macronutrients. In rats, high fat overfeeding recapitulates the combination of decreased 11β-HSD1 and increased 5β-reductase in the liver observed in human obesity (Drake et al 2005). Conversely, high fat overfeeding in mice (Morton et al 2004b) and rats (Drake et al 2005) decreases 11β-HSD1 mRNA and activity in adipose tissue. In humans, weight loss induced with a very low calorie diet decreases excretion of 5α- and 5β-reduced cortisol metabolites (Johnstone et al 2004) and may alter adipose 11β-HSD1 expression (Engeli et al 2004; Tomlinson et al 2004a). However, none of these experiments has dissected effects of individual dietary macronutrient components from alterations in fat mass. In acute studies either a mixed meal (Basu et al 2006b) or infusions with insulin (Wake et al 2006) increase whole body 11β-HSD1 activity, suggesting that dietary content may influence 11β-HSD1 over and above any effect of weight loss or gain.
In this chapter, we report investigations from two separate studies examining dietary regulation of glucocorticoid metabolism in humans. Both studies were performed in collaboration with Drs Alexandra Johnstone and Gerald Lobley from the Division of Obesity and Metabolic Health, Rowett Research Institute (RRI), Aberdeen, Scotland. For these studies, participants were resident in the human nutrition unit at the RRI, and I visited Aberdeen to perform all of the clinical assessments of glucocorticoid metabolism at the intervals described in the methods sections.

4.2 AD LIBUTUM MANIPULATION OF DIETARY MACRONUTRIENT CONTENT IN OBESE MEN

4.2.1 Introduction

This study was carried out to determine if dietary macronutrient content alters peripheral glucocorticoid metabolism in humans, as was suggested by the rodent dietary studies discussed above. In particular, we explored the hypotheses that manipulating dietary fat and carbohydrate content regulates adipose tissue and whole body cortisol regeneration by 11β-HSD1, and cortisol inactivation by the hepatic A-ring reductases. We also explored the hypothesis that dietary macronutrient content is responsible for the dysregulation of 11β-HSD1 observed in obesity, and whether weight loss altered 11β-HSD1 activity. Subjects were recruited as part of a weight loss programme, and were individuals highly motivated to lose weight.

4.2.2 Methods

4.2.2.1 Participants

Obese healthy males aged 20-65 years with body mass index (BMI) 30-40 kg/m² were recruited to a residential study at the Human Nutrition Unit, Rowett Research
Institute, Aberdeen. Volunteers suffered no chronic medical conditions, drank less than 28 units alcohol per week, were on no regular medication or any special diets, had not used oral or topical glucocorticoids in the preceding 6 months, and had no abnormalities detected by physical examination or laboratory blood tests (full blood count, urea and electrolytes, liver function, glucose, thyroid function). Written informed consent and local ethical committee approval were obtained.

4.2.2.2 Protocol

20 men were recruited but only 17 completed the full protocol (BMI 35.1 ± 0.9 kg/m², age 38 ± 10 years). Three participants withdrew due to dislike of the diets. Subjects were commenced on a fixed intake weight maintenance diet for 3 days (i.e. ‘baseline’) and then randomised to receive either 4 weeks (days 4-31) of an high fat-low carbohydrate diet (HF-LC) or a moderate fat-moderate carbohydrate diet (MF-MC). A further 3 day weight maintenance period (days 32-34) preceded the second 4 week diet (days 35-62) in a crossover design (Figure 4.1). The relative proportions of energy from each macronutrient group in each meal offered to participants were as follows: maintenance – protein 13%, fat 30%, carbohydrate 57%; HF-LC – protein 30%, fat 66%, carbohydrate 4%; and MF-MC – protein 30%, fat 35%, carbohydrate 35%.
Figure 4.1  Ad libitum diet protocol

Participants were randomised to receive either the HF-LC or the MF-MC for diet 1, then received the other diet in a crossover design.

The proportions of different fats in each diet were similar: HF-LC – saturated 40%, mono-unsaturated 44%, poly-unsaturated 16%; and MF-MC – saturated 44%, mono-unsaturated 40%, poly-unsaturated 16%. Diets and meals were energy isodense (1315 kcal/kg) and food was available ad libitum (Johnstone et al 2008). Food offered and food left uneaten were weighed and recorded to generate dietary intake data in Table 4.1.

4.2.2.3 Clinical measurements

Food intake and body weight were monitored daily. At the beginning and end of each diet phase, blood was obtained after overnight fast for insulin, glucose, and lipid concentrations, while body composition was measured by a 4 compartment model (Fuller et al 1992) utilising whole body plethysmography (BodPod® Body Composition System, Life Measurement Instruments, Concord, Connecticut, USA) and deuterated water dilution. Bone mineral content was measured by dual energy X-Ray absorptiometry at baseline (Norland XR-36, Mark II-high speed pencil beam scanner, Norland Corporation, Wisconsin, USA).
Glucocorticoid production and metabolism were assessed at the end of each 4 week dietary phase. Subjects provided a 24 h urine sample then fasted overnight. At 0700 h, approximately 500-1000 mg of subcutaneous peri-umbilical adipose tissue was obtained under local anaesthetic by needle biopsy, as described in section 2.4.1. Intravenous cannulae were sited in antecubital veins of each arm for infusion and sampling. After a 3.5 mg priming dose, 9,11,12,12-[2H]4-cortisol (d4-cortisol, Cambridge Isotopes, Andover, MA) was infused at 20% molar percent excess in hydrocortisone-21-succinate (Solu-Cortef, Upjohn, West Sussex, UK) at 1.74 mg/hour for 4 hours as described in section 2.4.2. Plasma was collected pre-infusion (T-5) and at timepoints T+60, +120, +180, +195, +210, +225 and +240 minutes during the infusion. Urine aliquots were collected hourly.

**4.2.2.4 Laboratory analyses**

Plasma and urinary cortisol, cortisone, d3-cortisol, d3-cortisone, d4-cortisol and urinary cortisol metabolites were analysed by electron impact gas chromatography/mass spectrometry as described in section 2.5.

Adipose tissue samples were analysed for mRNA by real time PCR as described in section 2.8. Gene transcript levels of 11β-HSD1, GRα, H6PDH, adiponectin, leptin, resistin, and 5α-reductase type 1 were quantified using 18S and cyclophilin A as internal controls. 11β-HSD1 activity was measured in the dehydrogenase direction to estimate total 11β-HSD1 protein as described in section 2.7. Briefly, 560 µg/ml total protein was incubated with 2 mmol/l NADP, 0.2% glucose and 100 nmol/l cortisol (of which 10 nmol/l 1,2,6,7-[3H]4-cortisol) at 37°C for 24 hours. Conversion to 1,2,6,7-[3H]4-cortisone was measured by HPLC with on line scintillation detection.

Analysis of plasma insulin, glucose and lipids was performed by staff at the RRI in Aberdeen. Insulin was measured by ELISA (Mercodia, Uppsala, Sweden). Glucose was measured by the glucose oxidase method (Labmedics, Salford, UK) and lipids with enzymatic colorimetric kits (Labmedics, Salford, UK) on a Kone discrete
automated clinical analyser (Kone Oyj, Espoo, Finland). The homeostasis model of assessment of insulin resistance index (HOMA-IR) and β-cell function (HOMA β-cell) were calculated as previously described (Matthews et al 1985).

4.2.2.5 Cortisol kinetic analyses

Cortisol kinetics were calculated using the mean of 5 measurements obtained in steady state (Figure 4.2). The rate of appearance of cortisol and d3-cortisol were calculated as described in section 2.5.8.

Excretion of urinary d4-cortisol metabolites was calculated as an area under the curve during d4-cortisol infusion to assess 5α- and 5β-reductase activities.

In the 24 h urine samples, assessment of cortisol metabolite excretion was calculated as described in section 2.5.7. Total deuterated metabolite excretion was calculated as the sum of d3-5α-tetrahydrocortisol, d3-5β-tetrahydrocortisol, d4-5α-tetrahydrocortisol, d4-5β-tetrahydrocortisol and d3-5β-tetrahydrocortisone.

4.2.2.6 Power calculations

Power calculations were performed on the primary outcome measure, dietary regulation of adipose 11β-HSD1 mRNA expression. Estimating an intra-subject standard deviation of 0.08 from previous paired adipose biopsies in Caucasian males (unpublished data), a sample size of 17 results in over 90% power to detect to p<0.05 a 10% change in 11β-HSD1 mRNA expression. This compares favourably with the rodent data, in which dietary changes decreased 11β-HSD1 mRNA by approximately 50% (Morton et al 2004b).
4.2.2.7 Statistical analysis

SPSS version 14 was used for all statistical analyses. All parameters were normally distributed by Kolmogorov-Smirnov testing. Comparisons between 2 diets were analysed by paired t tests. Repeated measures ANOVA was used for 3 or more measurements with post-hoc testing using Fisher’s least significant differences test. p<0.05 was considered significant. Data are presented as mean ± SEM.

4.2.3 Results

4.2.3.1 Energy intake, body composition and biochemistry

Energy intakes on both HF-LC and MF-MC were markedly reduced compared with the weight maintenance diet at baseline (p<0.001) (Table 4.1). However, energy intakes on the HF-LC diet were 154 kcal/day lower compared with the MF-MC diet (p<0.05), and weight loss was correspondingly greater (6.3 ± 2.2 vs 4.4 ± 2.6 kg, p<0.01). Differences in loss of fat mass were not statistically significant (5.1 ± 0.5 vs 4.1 ± 0.4 kg, p=0.08).

Fasting plasma glucose, HOMA-IR, cholesterol and triglyceride levels were reduced by both diets compared with baseline (Table 4.1). Plasma insulin concentrations were decreased on the HF-LC diet compared to baseline (p<0.001) and MF-MC (p<0.01). Plasma glucose and HOMA-IR were reduced more markedly on the HF-LC diet, while plasma cholesterol levels were decreased more on the MF-MC diet. The HOMA β-cell function was lower on the HF-LC compared to baseline.
Table 4.1  Energy intake and changes in anthropometry and biochemistry

Data are mean ± SEM. HF-LC = high fat-low carbohydrate diet. MF-MC = moderate fat-moderate carbohydrate diet. HOMA = Homeostasis Model of Assessment. IR = insulin resistance index. Comparisons by repeated measures ANOVA with posthoc Fisher's LSD tests: *p<0.05, **p<0.01, ***p<0.001 compared with baseline. *p<0.05, **p<0.01, ***p<0.001 compared with MF-MC diet.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>HF-LC</th>
<th>MF-MC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy Intake</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total kcal/day</td>
<td>2829 ± 60</td>
<td>1753 ± 112**</td>
<td>1907 ± 124***</td>
</tr>
<tr>
<td>Carbohydrate kcal/day</td>
<td>1480 ± 31</td>
<td>89 ± 7***</td>
<td>654 ± 39***</td>
</tr>
<tr>
<td>(% of total)</td>
<td>(52%)</td>
<td>(5%)</td>
<td>(34%)</td>
</tr>
<tr>
<td>Fat kcal/day</td>
<td>998 ± 22</td>
<td>1157 ± 74***</td>
<td>697 ± 48###</td>
</tr>
<tr>
<td>(% of total)</td>
<td>(35%)</td>
<td>(66%)</td>
<td>(37%)</td>
</tr>
<tr>
<td>Protein kcal/day</td>
<td>351 ± 7</td>
<td>503 ± 34###</td>
<td>549 ± 38###</td>
</tr>
<tr>
<td>(% of total)</td>
<td>(13%)</td>
<td>(29%)</td>
<td>(29%)</td>
</tr>
<tr>
<td><strong>Anthropometry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>111.1 ± 3.2</td>
<td>101.7 ± 2.8***</td>
<td>103.8 ± 3.2###</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>35.1 ± 0.93</td>
<td>32.1 ± 0.9***</td>
<td>32.8 ± 1.0###</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>41.1 ± 2.4</td>
<td>33.0 ± 2.6###</td>
<td>34.3 ± 2.8###</td>
</tr>
<tr>
<td><strong>Fasting plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>10.5 ± 0.5</td>
<td>6.1 ± 0.5###</td>
<td>9.5 ± 0.9</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.9 ± 0.1</td>
<td>5.3 ± 0.1###</td>
<td>5.6 ± 0.1*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.7 ± 0.2</td>
<td>1.4 ± 0.1###</td>
<td>2.4 ± 0.3*</td>
</tr>
<tr>
<td>HOMA β-cell</td>
<td>92 ± 9</td>
<td>74 ± 8*</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.7±0.2</td>
<td>4.8±0.2###</td>
<td>4.4±0.2###</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.0±0.3</td>
<td>1.1±0.1###</td>
<td>1.0±0.1###</td>
</tr>
</tbody>
</table>
4.2.3.2 Deuterated cortisol infusions

Morning fasting plasma cortisol concentrations were not different between the HF-LC and MF-MC diets (Figure 4.2). Steady state was achieved during the final hour of the deuterated cortisol infusions. Plasma cortisol was higher at steady state on the HF-LC diet (266 ± 15 vs 224 ± 12 nmol/l, p<0.001), consistent with a strong trend for slower d4-cortisol clearance rate (0.37 ± 0.03 vs 0.39 ± 0.03 litres/min, p=0.05). Plasma cortisone concentrations were unchanged between the two diets either before the infusion or in steady state.
Figure 4.2  Plasma measurements during deuterated cortisol infusion

Data are mean ± SEM for n=17 during High Fat-Low Carbohydrate diet (HF-LC, filled squares) and Moderate Fat-Moderate Carbohydrate diet (MF-MC, open squares). a Total plasma cortisol (lines) and cortisone (dotted lines). b d4-Cortisol/ cortisol enrichment. c d4-Cortisol/ d3-cortisol ratio.
At steady state, the rates of appearance of unlabelled cortisol and d3-cortisol, measures of whole body 11β-HSD1 activity, were higher on the HF-LC than the MF-MC diet (Figure 4.3).

**Figure 4.3 Whole body 11β-HSD1 activity**

Data are mean ± SEM for n=17 during High Fat-Low Carbohydrate diet (HF-LC, filled columns) and Moderate Fat-Moderate Carbohydrate diet (MF-MC, open columns). Comparisons by paired t tests (ad libitum study): *p<0.05, **p<0.01 compared with MF-MC diet. a Rate of appearance of unlabelled cortisol (Ra cortisol). b Rate of appearance of d3-cortisol (Ra d3-cortisol).

Urinary metabolites excreted during the d4-cortisol infusions are shown in Table 4.2. In concordance with the lower d4-cortisol clearance, total deuterated metabolite excretion was significantly reduced on the HF-LC diet (p<0.01). This was due to lower excretion of both 5α- and 5β- reduced tetrahydrometabolites.
Table 4.2  Urinary excretion of deuterated steroids during d4-cortisol infusion

Data are mean ± SEM. HF-LC = high fat-low carbohydrate diet. MF-MC = moderate fat-moderate carbohydrate diet. Comparisons by paired t tests. *p<0.05, **p<0.01 compared with MF-MC diet.

<table>
<thead>
<tr>
<th>Cortisol metabolites</th>
<th>HF-LC (n=17)</th>
<th>MF-MC (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d4-5α-tetrahydrocortisol</td>
<td>82.6 ± 14.9*</td>
<td>103.1 ± 17.2</td>
</tr>
<tr>
<td>d4-5β-tetrahydrocortisol</td>
<td>42.2 ± 5.1**</td>
<td>49.4 ± 5.5</td>
</tr>
<tr>
<td>d3-5β-tetrahydrocortisone</td>
<td>72.9 ± 7.1**</td>
<td>104.1 ± 12.0</td>
</tr>
<tr>
<td>d3-5α-tetrahydrocortisol</td>
<td>86.3 ± 15.0</td>
<td>98.1 ± 16.6</td>
</tr>
<tr>
<td>d3-5β-tetrahydrocortisol</td>
<td>37.5 ± 4.0</td>
<td>42.2 ± 4.1</td>
</tr>
<tr>
<td>Total deuterated metabolites</td>
<td>321.5 ± 37.3**</td>
<td>396.9 ± 48.0</td>
</tr>
</tbody>
</table>

4.2.3.3 24 hour endogenous urinary cortisol metabolite excretion

Total urinary excretion of endogenous cortisol metabolites decreased on each diet from baseline (Table 4.3). Over and above this, total metabolites were 24% lower on the HF-LC diet compared with the MF-MC diet. These differences were due to lower excretion of both 5α- and 5β-reduced metabolites, with no difference in the 5α-THF/5β-THF ratio. Urinary free cortisol and cortisone levels and the ((5α-THF + 5β-THF)/5β-THE) ratio were not different between the diets.
Table 4.3 24 hour urinary excretion of endogenous cortisol metabolites

Data are mean ± SEM. HF-LC = high fat-low carbohydrate diet. MF-MC = moderate fat-
moderate carbohydrate diet. Comparisons by repeated measures ANOVA with posthoc
Fisher’s LSD tests: *p<0.05, **p<0.01, ***p<0.001 compared with baseline. *p<0.05,
"p<0.01 compared with MF-MC diet. 1Total endogenous metabolites calculated as the sum
of 5α-THF, 5β-THF, 5β-THE, cortols and cortolones.

<table>
<thead>
<tr>
<th>Cortisol metabolites (µg in 24 h)</th>
<th>Baseline (n=17)</th>
<th>HF-LC (n=17)</th>
<th>MF-MC (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-tetrahydrocortisol (5α-THF)</td>
<td>2953 ± 420</td>
<td>1114 ± 201***</td>
<td>1760 ± 275###</td>
</tr>
<tr>
<td>5β-tetrahydrocortisol (5β-THF)</td>
<td>2085 ± 344</td>
<td>1109 ± 231###</td>
<td>1426 ± 234#</td>
</tr>
<tr>
<td>5β-tetrahydrocortisone (5β-THE)</td>
<td>5520 ± 917</td>
<td>2835 ± 541**</td>
<td>3791 ± 634#</td>
</tr>
<tr>
<td>Total metabolites ¹</td>
<td>15854 ± 2525</td>
<td>7441 ± 1402###*</td>
<td>10073 ± 1558#</td>
</tr>
<tr>
<td>Cortisone</td>
<td>118 ± 10</td>
<td>93 ± 10</td>
<td>96 ± 9</td>
</tr>
<tr>
<td>Cortisol</td>
<td>167 ± 13</td>
<td>142 ± 15</td>
<td>141 ± 13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ratios</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol/Cortisone</td>
<td>1.45 ± 0.06</td>
<td>1.59 ± 0.08</td>
<td>1.48 ± 0.05</td>
</tr>
<tr>
<td>(5α-THF+5β-THF)/5β-THE</td>
<td>0.93 ± 0.05</td>
<td>0.78 ± 0.06</td>
<td>0.88 ± 0.09</td>
</tr>
<tr>
<td>5α-THF/5β-THF</td>
<td>1.45 ± 0.13</td>
<td>1.11 ± 0.15*</td>
<td>1.32 ± 0.13</td>
</tr>
<tr>
<td>5α-THF/cortisol</td>
<td>16.7 ± 1.76</td>
<td>7.8 ± 1.24###**</td>
<td>12.2 ± 1.65#</td>
</tr>
<tr>
<td>5β-THF/cortisol</td>
<td>11.9 ± 1.12</td>
<td>7.3 ± 1.01###**</td>
<td>9.6 ± 1.05##</td>
</tr>
<tr>
<td>5β-THE/cortisone</td>
<td>45.0 ± 4.13</td>
<td>30.1 ± 4.07###**</td>
<td>38.1 ± 4.28</td>
</tr>
</tbody>
</table>
4.2.3.4 Adipose tissue mRNA and 11β-HSD1 activity

Transcript levels of the internal standards 18S and cyclophilin A did not vary between HF-LC and MF-MC diets (488 ± 88 vs 383 ± 31 arbitrary units (AU)). Transcript levels of resistin were too low in adipose tissue to be reliably detected. 11β-HSD1 mRNA levels were not different between the HF-LC and MF-MC diets (1.00 ± 0.09 vs 1.15 ± 0.10 AU), while the other genes of interest were also unchanged (Figure 4.4). In addition, 11β-HSD1 activity was no different between the HF-LC and MF-MC diets (3.32 ± 0.30 vs 3.25 ± 0.26 pmol product/mcg protein/hour).

Participants lost approximately 5% of their body weight on the second diet (4.8 ± 0.7 kg). There was no difference in either 11β-HSD1 mRNA (1.00 ± 0.09 vs 0.92 ± 0.08 AU) or 11β-HSD1 activity (3.26 ± 0.29 vs 3.31 ± 0.27 pmol product/ mcg protein/hr) from the end of diet 1 to the end of diet 2.
Figure 4.4  mRNA transcript levels in subcutaneous adipose tissue on HF-LC and MF-MC diets

Data are mean ± SEM. Comparisons were performed using paired t tests. The mean of 18S and cyclophilin A was used as the internal control. mRNA transcript levels are expressed as ratio to the internal control (IC), which are normalised to 1 on the HF-LC diet. 11β-HSD1 = 11β-hydroxysteroid dehydrogenase type 1; GRα = glucocorticoid receptor α; H6PDH = hexose-6-phosphate dehydrogenase; 5αR1 = 5α-reductase type 1.
4.3 ISOCALORIC MANIPULATION OF DIETARY MACRONUTRIENT CONTENT IN OBESE MEN

4.3.1 Introduction

The ad libitum study showed that the HF-LC diet increased total body 11β-HSD1 activity, without altering subcutaneous adipose 11β-HSD1. However, the decreased energy intake and subsequent increased weight loss in HF-LC compared with MF-MC were potential confounders of the results. In addition, the ad libitum study did not elucidate if the HF-LC diet increased or the MF-MC decreased 11β-HSD1 from baseline, or the timescale of these effects. Consequently, an isocaloric study was performed to answer these questions. This was a smaller study, powered using the results of the ad libitum study to detect the same differences in cortisol kinetics in plasma but not to detect differences in clearance or in urinary d4-cortisol metabolite excretion.

Following the observation of decreased d4-cortisol clearance and increased endogenous cortisol production rate in the ad libitum study, further salivary samples were collected each week to look for variations in diurnal cortisol on the different diets.

4.3.2 Methods

4.3.2.1 Participants

The entry criteria were identical to the ad libitum study (section 4.2.2.1).
4.3.2.2 Protocol

Six men were recruited and all completed the protocol (mean BMI 38.0 ± 1.3 kg/m², age 41 ± 5 years), which was identical to the ad libitum study (section 4.2.2.2) except for the following differences. In the isocaloric study, (i) participants were provided with food with a fixed energy intake of 2000 kcal/day; (ii) a longer weight maintenance period of 7 days was adopted at the start in order to obtain detailed cortisol measurements at baseline; and (iii) no weight maintenance period was employed between the two diet phases (Figure 4.5).

Figure 4.5  Isocaloric diet protocol

Participants were randomised to receive either the HF-LC or the MF-MC for diet 1, then received the other diet in a crossover design.

4.3.2.3 Clinical measurements

Measurements of food intake, anthropometry, and biochemistry were performed as in the ad libitum study (section 4.2.2.3).

Additional measurements of glucocorticoid production and metabolism were obtained in the isocaloric study. The d4-cortisol infusions were performed at the end of the 7-day weight maintenance phase (‘baseline’), and after 1 and 4 weeks of each diet, although urinary collections were not performed during the infusions as the
study was not powered for this outcome. 24 hour urines were also not collected in this study. The subcutaneous adipose tissue biopsies were obtained at baseline and after 4 weeks of each diet. In addition, salivary samples were collected into Salivette tubes (Sarstedt, Nümbrecht, Germany) four times a day (0800, 1200, 1800, and 2200 h) each week for measurement of diurnal variation in cortisol concentrations. Salivette tubes were then subject to centrifugation at 2000 rcf at 4°C for 10 minutes and the saliva transferred to eppendorfs, then frozen at -80°C until analysis.

4.3.2.4 Laboratory analyses

Analyses for plasma biochemistry, deuterated cortisol infusions and adipose mRNA transcript levels were performed as in the ad libitum study (section 4.2.2.4). Adipose 11β-HSD1 activities were not measured in the biopsies.

Salivary cortisol was analysed using a high sensitivity ELISA kit (Salimetrics, PA, USA) as per the manufacturer’s instructions. Samples and standards were thawed to room temperature, and 25 μl of each placed on a 96-well plate coated with monoclonal antibodies to cortisol. Samples were analysed in duplicate. 15 μl of 1:1600 enzyme conjugate (containing cortisol bound to horseradish peroxidase) diluted in distilled water was added to wells, and incubated at 500 rpm for 1 hour at 25°C in a rotator (Grant Instruments Ltd, Cambridgeshire, UK). The wells were then washed 4 times with 300 μl 1x wash buffer using a microplate washer (Anthos Labtec Instruments, Salzburg, Austria). 200 μl of tetramethylbenzidine (TMB) was added to wells and incubated in the dark at 500 rpm for 30 minutes at 25°C in a rotator. 50 μl of stop solution (2 mol/l sulphuric acid) was added to wells and mixed, then absorbance read on a plate reader (Dynatech Laboratories, Guernsey, Channel Islands, UK) at 450 nm. A standard curve prepared using a series of concentrations in the range 0.33-82.77 nmol/l was analysed in each batch and used to calculate salivary cortisol concentrations. The mean of the duplicate values was recorded. The standard curve was deemed acceptable if the r value was >0.99, while variability of <5% between duplicates was considered acceptable.
4.3.2.5 Cortisol kinetic analysis

Cortisol kinetics were calculated as described in section 2.5.8.

4.3.2.6 Power calculations

Power calculations were performed on the primary endpoint in this study, rate of appearance of d3-cortisol as a measure of whole body 11β-HSD1 activity, using the results from the ad libitum study. Using the intra-subject standard deviation of 1.81, a sample size of 6 resulted in an 80% power to detect a 20% change in whole body 11β-HSD1 activity.

4.3.2.7 Statistical analysis

All parameters were normally distributed by Kolmogorov-Smirnov testing. Comparisons between multiple variables in individuals was performed using repeated measures ANOVA with post-hoc testing by Fisher's least significant differences test. Comparisons between the ad libitum and isocaloric studies were made by unpaired t tests. $p<0.05$ was considered significant. Data are presented as mean ± SEM.

4.3.3 Results

4.3.3.1 Energy intake, body composition and biochemistry

Despite being served food of the same energy content, energy intake was slightly lower (66 kcal/day) on the HF-LC diet after correction for food that was not consumed (Table 4.4). Weight loss during each phase was greater on the HF-LC compared to the MF-MC diet ($7.2 \pm 2.3$ vs $4.7 \pm 1.0$ kg, $p<0.05$), but there was no difference in loss of fat mass ($5.8 \pm 0.3$ vs $4.5 \pm 0.6$ kg, $p=0.12$). Over the two diets, from baseline to the end of diet 2, participants lost approximately 10% of their body
weight (119 ± 6 vs 107 ± 5 kg), which compares with the 10% weight loss achieved by participants during the ad libitum study from baseline to the end of diet 2 (111 ± 3 vs 100 ± 3 kg).

Fasting plasma insulin and glucose concentrations (both p<0.01) and HOMA-IR (p<0.05) were reduced from baseline only by the HF-LC diet. The HOMA β-cell function index was reduced similarly by both diets. Total plasma cholesterol and triglyceride levels were no different between HF-LC and MF-MC diets (Table 4.4).

Participants in the ad libitum and isocaloric studies had similar characteristics at baseline except that those in the isocaloric study had higher fasting insulin, HOMA-IR (both p<0.05) and HOMA β-cell index (p<0.01) and lower fasting plasma glucose (p<0.05). The difference in energy intake between HF-LC and MF-MC diets in the isocaloric study tended to be less than in the ad libitum study (66 ± 12 vs 154 ± 27 kcal/ day, p=0.06). Despite this, the increased weight loss on the HF-LC compared to MF-MC was no different between isocaloric and ad libitum studies (2.5 ± 0.8 vs 2.0 ± 0.6 kg, p=0.7).
Table 4.4  Energy intake and changes in anthropometry and biochemistry in isocaloric study

Data are mean ± SEM. HF-LC = high fat-low carbohydrate diet. MF-MC = moderate fat-moderate carbohydrate diet. HOMA = Homeostasis Model of Assessment. IR = insulin resistance index. Comparisons by repeated measures ANOVA with posthoc Fisher’s LSD tests: *p<0.05, **p<0.01, ***p<0.001 compared with baseline. *p<0.05, **p<0.01, ***p<0.001 compared with MF-MC diet.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>HF-LC</th>
<th>MF-MC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy Intake</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total kcal/day</td>
<td>3014 ± 55</td>
<td>1930 ± 8**</td>
<td>1996 ± 9**</td>
</tr>
<tr>
<td>Carbohydrate kcal/day</td>
<td>1514 ± 28</td>
<td>83 ± 1***</td>
<td>692 ± 3***</td>
</tr>
<tr>
<td>(% of total)</td>
<td>(50%)</td>
<td>(4%)</td>
<td>(35%)</td>
</tr>
<tr>
<td>Fat kcal/day</td>
<td>1115 ± 20</td>
<td>1282 ± 6***</td>
<td>736 ± 8**</td>
</tr>
<tr>
<td>(% of total)</td>
<td>(37%)</td>
<td>(66%)</td>
<td>(37%)</td>
</tr>
<tr>
<td>Protein kcal/day</td>
<td>381 ± 7</td>
<td>564 ± 3***</td>
<td>566 ± 2***</td>
</tr>
<tr>
<td>(% of total)</td>
<td>(13%)</td>
<td>(29%)</td>
<td>(28%)</td>
</tr>
<tr>
<td><strong>Anthropometry</strong></td>
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</tr>
<tr>
<td>Weight (kg)</td>
<td>118.6 ± 6.0</td>
<td>108.3 ± 4.3#</td>
<td>110.5 ± 5.8##</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>38.0 ± 1.3</td>
<td>34.7 ± 1.2##</td>
<td>35.4 ± 1.2##</td>
</tr>
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<td>Fat Mass (kg)</td>
<td>49.6 ± 4.4</td>
<td>41.4 ± 4.1##</td>
<td>42.3 ± 4.6##</td>
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<td><strong>Fasting plasma</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (mU/L)</td>
<td>16.6 ± 3.3</td>
<td>7.3 ± 1.4##</td>
<td>9.2 ± 1.0</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
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<td>4.8 ± 0.1**</td>
<td>5.2 ± 0.1</td>
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<td>HOMA-IR</td>
<td>4.1 ± 0.9</td>
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<td>2.1 ± 0.2</td>
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<td>HOMA ß-cell</td>
<td>168 ± 28</td>
<td>117 ± 22*</td>
<td>113 ± 17*</td>
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<td>Total cholesterol (mmol/l)</td>
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<td>4.0±0.5**</td>
<td>3.8±0.5**</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.5±0.2</td>
<td>1.0±0.1*</td>
<td>1.0±0.1</td>
</tr>
</tbody>
</table>
4.3.3.2 Deuterated cortisol infusions

Morning plasma cortisol levels were higher at baseline on the subjects initial visit compared to the MF-MC diet after 4 weeks (382 ± 46 vs 239 ± 36 nmol/l, p<0.05), but were no different between HF-LC and the other two diets. Steady state was achieved in the final hour of the deuterated infusions (Figure 4.6). At steady state, plasma cortisol concentrations were unchanged between baseline, 4 weeks of HF-LC or MF-MC (258 ± 28 vs 285 ± 33 vs 263 ± 26 nmol/l). d4-cortisol clearance was similarly unchanged between all three diets (0.35 ± 0.05 vs 0.39 ± 0.04 vs 0.36 ± 0.04 l/min). Both morning fasting and steady state plasma cortisone levels were unchanged between diets (data not shown).
Figure 4.6 Plasma measurements during deuterated cortisol infusion in isocaloric study

Data are mean ± SEM for n=6 during baseline (open circles), High Fat-Low Carbohydrate diet (HF-LC, filled squares) and Moderate Fat-Moderate Carbohydrate diet (MF-MC, open squares). Comparisons between diets for plasma cortisol by repeated measures ANOVA followed by post-hoc Fisher's LSD test. * p<0.05 vs baseline. a Total plasma cortisol. b d4-Cortisol/ cortisol enrichment. c d4-Cortisol/ d3-cortisol ratio.
After 4 weeks, rates of appearance of unlabelled cortisol and d3-cortisol were higher on HF-LC diet than MF-MC diet (Figure 4.7). This was explained by increases from baseline during HF-LC and not by any change from baseline with weight loss on the MF-MC diet. The increase in rate of appearance of unlabelled cortisol was statistically significant within 1 week of beginning the HF-LC diet, whereas the rate of appearance of d3-cortisol was tending to increase in 1 week but was statistically significant at 4 weeks. Furthermore, there was no change in rate of appearance of d3-cortisol (a specific measure of whole body 11β-HSD1 activity) from baseline to the end of diet 2 (14.5 ± 1.3 vs 15.2 ± 0.7 nmol/min), despite 10% loss of body weight.
Figure 4.7 Whole body 11β-HSD1 activity in ad libitum and isocaloric studies

Data are mean ± SEM for n=17 (ad libitum study) and n=6 (isocaloric study) during High Fat-Low Carbohydrate diet (HF-LC, filled columns) and Moderate Fat-Moderate Carbohydrate diet (MF-MC, open columns). Comparisons by paired t tests (ad libitum study) or repeated measures ANOVA followed by post-hoc Fisher’s LSD tests (isocaloric study): *p<0.05, **p<0.01 compared with MF-MC diet. ¢p<0.05, ¢¢p<0.01 compared with baseline. 2a Rate of appearance of unlabelled cortisol. Ra cortisol was higher on the HF-LC diet in both studies, and increased from baseline at both 1 and 4 weeks only on the HF-LC diet. 2b Rate of appearance of d3-cortisol. Ra d3-cortisol was higher in both studies after 4 weeks.
4.3.3.3 Adipose mRNA transcript levels

18S and cyclophilin A transcript levels did not change between diets (data not shown). Again, transcript levels of resistin were too low in adipose tissue to be reliably detected. 11β-HSD1 mRNA was unchanged between baseline, HF-LC and MF-MC diets (Figure 4.8). Leptin mRNA levels were decreased on the HF-LC diet from baseline (p<0.01). 11β-HSD1 mRNA transcript levels were unchanged from baseline to the end of diet 2 (1.00 ± 0.13 vs 0.98 ± 0.24) despite 10% weight loss.

Figure 4.8  mRNA transcript levels in adipose tissue on baseline, HF-LC and MF-MC diets

Data are mean ± SEM. Comparisons were performed by repeated measures ANOVA with post-hoc testing by Fisher’s LSD test. ##, p<0.01 compared with baseline. The mean of 18S and cyclophilin A was used as the internal control. mRNA transcript levels are expressed as ratio to the internal control (IC), which are normalised to 1 on the baseline diet. 11β-HSD1 = 11β-hydroxysteroid dehydrogenase type 1; GRα = glucocorticoid receptor α; H6PDH = hexose-6-phosphate dehydrogenase; 5αR1 = 5α-reductase type 1.
4.3.3.4 Salivary cortisol levels

Results from baseline and the end of each diet are shown in Figure 4.9. There were no differences in salivary cortisol or its diurnal variation on either diet. Similar results were obtained after 1, 2 and 3 weeks of each diet (data not shown). There was no effect of weight loss on salivary cortisol levels (data not shown).

Figure 4.9  Diurnal salivary cortisol levels

Data are mean ± SEM for n=6 at baseline (open circles) and after 4 weeks of High Fat-Low Carbohydrate diet (HF-LC, filled squares) and Moderate Fat-Moderate Carbohydrate diet (MF-MC, open squares). Comparisons were performed by repeated measures ANOVA with clock time as a dependent variable and post-hoc testing by Fisher’s LSD test. Mean salivary cortisol levels fell progressively throughout the day (p<0.05). No significant difference was observed between baseline, HF-LC and MF-MC diet.
These data show for the first time that chronic manipulations of dietary macronutrient composition influence glucocorticoid metabolism in humans, independently of changes in weight. In obese men, a high fat-low carbohydrate diet increased whole-body regeneration of cortisol by 11β-HSD1 and reduced the rate of inactivation of cortisol by 5α- and 5β-reductases. As in previous studies (Westerbacka et al 2003; Sandeep et al 2005), discrepancy between cortisol regeneration measured during dynamic testing and the more conventional index of 24 h urinary endogenous cortisol/cortisone metabolite ratios (Table 4.3) reflects the confounding effects of 5α- and 5β-reductase activities on ratios of steroids excreted in urine. Participants were recruited as part of a weight loss study that examined the impact of carbohydrate intake (and, inversely, fat consumption) when offered satiating diets high in protein. Energy consumption was lower during both high fat-low carbohydrate (HF-LC) and moderate fat-moderate carbohydrate (MF-MC) diets than at baseline, but the increased 11β-HSD1 on HF-LC was independent of differences in total energy consumption: the same differences were observed also under fixed feeding (approximately isocaloric) conditions. Differences in cortisol metabolism were also independent of weight loss: 11β-HSD1 activity was similar on the MF-MC diet compared with baseline despite substantial weight loss. Moreover, at least for unlabelled cortisol production rate, the effect of HF-LC had already reached its maximum after just 1 week of the 4 week diet, when weight loss was minimal (approximately 2 kg).

Low carbohydrate intake appears to be the key factor responsible for alterations in glucocorticoid metabolism. Protein intake was similar between the HF-LC and MF-MC diets. Compared with baseline intake, fat intake was only marginally higher (~160 kcal/day) on the HF-LC diet, while carbohydrate intake was substantially lower (~1400 kcal/day). However, carbohydrate intake was also lower than baseline on the MF-MC diet (~800 kcal/day) without measurably affecting glucocorticoid metabolism, so that either there is a threshold of reduced carbohydrate intake which
mediates the effect, or glucocorticoid metabolism is responsive to relative rather than absolute changes in macronutrients. A threshold effect appears more likely, and is supported by fasting insulin concentrations (Tables 4.1 and 4.4), which were decreased by HF-LC but not MF-MC in both studies. Indeed, there is evidence that reductions in insulin concentrations may directly mediate the effects of the HF-LC diet on glucocorticoid metabolism. Insulin down-regulates 11β-HSD1 expression in hepatocytes (Jamieson et al 1995), while insulin sensitisation of obese rats with PPARγ-agonists or metformin ameliorates the changes in hepatic 5α- and 5β-reductases (Livingstone et al 2005). In humans, hyperinsulinaemia is independently associated with alterations in urinary cortisol metabolite excretion reflecting 5α- and 5β-reductase activity (Tsilchorozidou et al 2003). Thus, lowering of insulin concentrations would be predicted to enhance hepatic 11β-HSD1 and lower hepatic 5α- and 5β-reductase activities. However, additional studies will be required to dissect the pathways mediating the influence of macronutrient content on cortisol metabolism, and it remains possible that other unmeasured effects of the different diets are important.

Most of whole-body cortisol regeneration by 11β-HSD1 occurs in the splanchnic circulation (Basu et al 2004;Andrew et al 2005), and the liver is the major source of 5α- and 5β-reduced cortisol metabolites. Consistent with actions predominantly in the liver, we did not find effects of diet on 11β-HSD1 mRNA or activity or 5α-reductase mRNA in subcutaneous adipose tissue. In these obese men, therefore, the low carbohydrate diet reverses the increase in metabolic clearance of cortisol (Strain et al 1982;Lottenberg et al 1998), increase in 5α- and 5β-reductase (Andrew et al 1998) and decrease in hepatic 11β-HSD1 (Stewart et al 1999;Rask et al 2001) previously described in obesity. In response to a reduction in metabolic clearance rate for cortisol, the HPA axis made appropriate compensation to reduce cortisol secretion rate (as measured by total 24 h urinary glucocorticoid metabolites (Zumoff et al 1974)) and maintain normal circulating cortisol concentrations and diurnal variation in cortisol (Figure 4.8). Our results are in keeping with a previous study (Tomlinson et al 2004a), which showed that weight loss per se had no effect on
indices of liver 11β-HSD1 activity. These data support the concept that increased cortisol production rate in obesity is explained by enhanced peripheral metabolic clearance of cortisol, and suggest that alterations in hepatic cortisol metabolism may result from a high carbohydrate diet and associated elevated insulin concentrations, rather than from obesity per se. Indeed, there is some evidence that lack of an appropriate elevation in insulin in obesity is associated with failure to down-regulate hepatic 11β-HSD1, as appears to occur in obese patients with diabetes mellitus (Valsamakis et al 2004).

The lack of effect of dietary manipulation on 11β-HSD1 in adipose tissue might seem at odds with recent data in mice, which shows that overfeeding with a high saturated fat diet down-regulates 11β-HSD1 in adipose tissue but not in liver (Morton et al 2004b). However, in the current studies the total fat intake was not substantially increased between baseline and HF-LC diets, the fat content was a mixture of saturated, mono- and poly-unsaturated forms, and subjects were losing rather than gaining weight, so that the paradigms are not comparable. Our data are consistent with previous studies which examined subcutaneous adipose 11β-HSD1 during weight loss in obese humans. One group found no change in abdominal 11β-HSD1 mRNA after 5% weight loss (Engeli et al 2004), while another observed no difference in gluteal adipose tissue after 14% weight loss, although 11β-HSD1 mRNA expression in isolated gluteal adipocytes was increased (Tomlinson et al 2004a). It appears that the mechanisms maintaining elevated intra-adipose 11β-HSD1 expression in adipose tissue in obesity differ from those altering hepatic cortisol metabolism and are resistant to dietary manipulation, perhaps suggesting a ‘constitutive activation’ of adipose 11β-HSD1 in obesity.

In conclusion, extra-adrenal regeneration of cortisol is responsive to the macronutrient content of the diet, and may modulate the metabolic response to specific macronutrients. Thus, chronic changes in dietary macronutrients may be a primary driver for altered hepatic cortisol metabolism and compensatory activation of the HPA axis in obesity. In contrast, subcutaneous adipose 11β-HSD1 is
unaffected by macronutrient restriction in obese men. The effects in liver may be mediated by the degree of hyperinsulinaemia, suggesting that foods with differing glycaemic index may have contrasting effects on cortisol metabolism. The increase in 11β-HSD1 activity, and hence intra-hepatic cortisol concentrations, caused by a ketogenic low carbohydrate diet has implications for the efficacy of different dietary strategies in reversing the metabolic consequences of obesity.
Chapter 5

Dietary regulation of 11β-HSD1 in rats

5.1 INTRODUCTION

Recent work has shown that 11β-HSD1 is regulated by dietary content. An ad libitum high fat diet (58% of total calories vs 11% on control diet) for 2 and 18 weeks in mice decreased 11β-HSD1 mRNA and activity in subcutaneous, visceral and epididymal adipose tissue but had no effect on hepatic 11β-HSD1 (Morton et al 2004b). Similarly, an ad libitum high fat diet (45% versus 10% control diet) for 3 weeks in rats decreased 11β-HSD1 activity in adipose tissue and liver, although these changes were no longer observed after 20 weeks duration (Drake et al 2005). Dietary regulation appears to be tissue specific, as a high fat diet increased 11β-HSD1 mRNA in the brain (Densmore et al 2006).

In the previous chapter I described how a high fat-low carbohydrate (HF-LC) diet (66% fat, 4% CHO, 30% protein) increased total body 11β-HSD1 activity compared to a moderate fat-moderate carbohydrate (MF-MC) diet (35% fat, 35% CHO, 30% protein) in humans. However, subcutaneous adipose tissue 11β-HSD1 was unaltered by these diets and the altered activity was hypothesized to be secondary to increased 11β-HSD1 regeneration in the liver. This work, particularly with regards to 11β-HSD1 in adipose tissue, did not fit with the hypothesis derived from the work in rodents (Morton et al 2004b; Drake et al 2005), however the results suggested that the low carbohydrate component was the likely key driver of the increased whole body 11β-HSD1 activity in humans. The human research (HF-LC) diets had a similar proportion of dietary fat to the rodent studies, but far higher protein and lower carbohydrate content. In addition, the rodent dietary fat content was mainly saturated
fat, unlike in the human studies which was a mixture of mono-unsaturated, poly-
unsaturated and saturated fat.

Therefore, we felt it was inappropriate to make direct comparison between the
different results obtained in experiments in different species. We hypothesized that a
HF-LC diet in rats would increase hepatic 11β-HSD1 while not altering adipose 11β-
HSD1, as observed in humans. To examine this, we collaborated with Dr Gerald
Lobley at the Rowett Research Institute (RRI), Aberdeen, to perform a dietary study
using diets similar in macronutrient content to the research diets utilised in our
human research study described in chapter 4. A diet-induced obesity model was used
to match the obese human volunteers who participated in the original human study.
The experiment was performed at the RRI and tissues were provided to me at the end
of the study for analysis.

5.2 METHODS

5.2.1 Animals and protocol

Full details of the protocol have been described previously (Lobley et al 2007). 48
male Hooded Lister rats were weaned at 19 days of age onto a stock (control) diet
(CRM (pellets), Special Diet Services, Witham, Essex, UK). The composition of this
diet according to caloric energy content was carbohydrate (CHO) 69%, protein 22%,
fat 9%. After 9 days (t=0 weeks), the rats were randomly divided into 2 groups, the
first (n=12) remaining on the control diet while the second (n=36) were fed a 37% fat
diet (CHO 47%, protein 16%, fat 37%) (Figure 5.1). The rats were fed ad libitum on
these diets for 22 weeks. The 37% fat and subsequent diets described in this chapter
were developed by staff at the RRI. Rats were housed in a 12: 12 hour light – dark
cycle (0700-1900 h light), and all procedures were approved by the animal ethics
committee of the Rowett Research Institute under the UK Animals (Scientific
Procedures) Act 1996.
Figure 5.1  Study protocol

Rats were initially placed on ad libitum control or 37% fat diets for 22 weeks. Rats on the 37% fat diet were further divided into 3 separate diets for the remaining 8 weeks of the experiment. Respective dietary macronutrient content is shown as percentage total caloric energy intake. Oral glucose tolerance tests (OGTT) were performed at 20 and 28 weeks, while magnetic resonance imaging (MRI) scans were performed at 22 and 30 weeks. CHO = carbohydrate; HF-LC = high fat-low carbohydrate; MF-MC = moderate fat-moderate carbohydrate.

During this period energy intake was recorded daily, the rats were weighed twice weekly, and body composition was determined at 22 weeks using a MRI scanner (EchoMRI, Houston, Texas, USA). An oral glucose tolerance test was performed after 20 weeks at 8am after overnight fasting, using gavage of a 50% glucose solution equivalent to 2g glucose/ kg body weight. Blood samples (0.25ml) were taken by tail tip prior to gavage then at 7, 15, 30, 60, 90 and 120 minutes.
At 22 weeks, rats from the 37% fat group were subdivided into 3 further groups (n=12), based on their percentage body fat from the MRI scan to ensure rats of equal adiposity were in each group. One group (n=12) remained on the 37% fat diet, a second (n=12) was placed on a ketogenic high fat-low carbohydrate (HF-LC) diet (CHO 7%, protein 37%, fat 56%), while a third (n=12) was commenced on a moderate fat-moderate carbohydrate (MF-MC) diet (CHO 27%, protein 37%, fat 36%) (Figure 5.1). All three groups were fed ad libitum for a further 8 weeks. The rats on the ad libitum control diet remained on this until the end of the study.

At week 28, a second OGTT was performed, identical to the previous one at 20 weeks. Body composition was similarly measured by MRI scan at 30 weeks. After 30 weeks, the rats were decapitated in the fed state at 10am. Organs were immediately removed, including the liver and peri-renal adipose tissue and frozen at -80°C until analysis.

5.2.2 Laboratory measurements

Plasma insulin and glucose were analysed as described previously (Lobley et al 2007).

Liver and peri-renal adipose tissue were analysed for 11β-HSD1 mRNA transcript levels as described in section 2.8. 18S and cyclophilin A were used as internal controls.

Liver and adipose tissue were analysed for 11β-HSD1 activity in the dehydrogenase direction to estimate total 11β-HSD1 protein as described in section 2.7. Briefly, 500 μg/ml total protein of adipose tissue homogenate was incubated with 2mmol/l NADP, 0.2% glucose and 100 nmol/l corticosterone (of which 10 nmol/l 1,2,6,7-
[\textsuperscript{3}H\textsubscript{4}-corticosterone (Amersham, Berkshire, UK)] at 37°C for 2 hours. Conversion to 1,2,6,7-[\textsuperscript{3}H\textsubscript{4}]-11-dehydrocorticosterone was measured by HPLC with on line scintillation detection. For liver, the procedure was identical except 2 µg/ml total protein of liver homogenate was incubated with the above concentrations of NADP, glucose and corticosterone for 4 hours.

5.2.3 Power calculations

A n of 12 were chosen to give 85% power to detect to p<0.05 a difference in mRNA of 15% between diets, using a group standard deviation of 0.15 from previous dietary work in rodents (Morton et al 2004b).

5.2.4 Statistical analysis

All statistical analysis was performed using SPSS version 14. All parameters were normally distributed by Kolmogorov-Smirnov testing. Comparison of measurements between 3 or more diets was performed using one way ANOVA with post-hoc testing using Fisher's least significant differences test. Comparison of variables between 2 diets was performed using a Student's t test. p<0.05 was considered significant. Data are presented as mean ± SEM.

5.3 RESULTS

1 animal died prematurely from the control group and was therefore excluded from the analysis.
5.3.1 Anthropometry and biochemistry after first dietary intervention (22 weeks)

Anthropometry and biochemistry have been described in detail previously (Lobley et al 2007). Briefly, the 37% fat diet successfully induced an obese phenotype. The rats fed the 37% fat diet were heavier than the control-fed rats (649 ± 5 vs 580 ± 9 grams, p<0.001), due to increased whole body fat mass (169 ± 4 vs 86 ± 6 grams, p<0.001) with no significant difference in lean mass (444 ± 3 vs 448 ± 5 grams). This increased fat mass was predominantly due to increased daily energy intake (83 ± 1 vs 78 ± 1 kcal/day, p<0.01).

During the OGTT, fasting insulin concentrations were higher on the 37% fat diet, while glucose and insulin during the OGTT were increased on the 37% fat diet (Figure 5.2).
Figure 5.2  Plasma measurements during OGTT at 20 weeks

Data reproduced from Lobley et al (Lobley et al 2007). Data presented as mean ± SEM for control (n=12, open squares and 37% fat (n=36, filled squares). Blood was taken for measurement of a) plasma insulin and b) glucose before (t=0) and 7, 15, 30, 60, 90, and 120 minutes after ingestion of 2g/kg glucose oral gavage. Comparisons between groups by Student’s t test. * p<0.05, ** p<0.01, *** p<0.001 vs control.
5.3.2 Anthropometry and biochemistry after second dietary intervention (30 weeks)

Rats in the control group were compared solely with rats in the 37% fat group, conversely rats in the 37% fat, HF-LC and MF-MC groups were compared with each other. Rats on the 37% fat diet continued to gain more weight than those on the control diet from weeks 22-30 (25 ± 3 vs 11 ± 4 grams, p<0.05). From the rats switched from the 37% fat diet after 22 weeks, the HF-LC gained weight (15 ± 5 grams) while the MF-MC group lost weight (-25 ± 6 grams, p<0.001 vs 37% fat and HF-LC groups). The weight gain was due to increased fat mass in the 37% fat and HF-LC groups (19 ± 2 and 21 ± 3 grams respectively), and due to increased lean mass in the control group (8 ± 3 grams). The MF-MC group lost fat mass (-21 ± 5 grams). Mean energy intake was highest in the rats fed the HF-LC diet (Table 5.1).
Table 5.1  Dietary intakes during weeks 23-30

Data presented as mean ± SEM for n=11 for control diet, and n=12 for 37% fat, high fat-low carbohydrate (HF-LC) and moderate fat-moderate carbohydrate (MF-MC) diets. All variables presented were significantly different between all 4 diet groups (p<0.001) except total energy intake between MF-MC and control (p=ns).

<table>
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<th>37% Fat</th>
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<th>MF-MC</th>
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<tbody>
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<tr>
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<td><strong>Carbohydrate</strong></td>
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<td>intake (kcal/ day)</td>
<td>46.5 ± 0.7</td>
<td>34.2 ± 0.5</td>
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<tr>
<td>(kcal/ day)</td>
<td>14.9 ± 0.2</td>
<td>12.0 ± 0.2</td>
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<td><strong>Fat intake</strong></td>
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<tr>
<td>(kcal/ day)</td>
<td>6.1 ± 0.1</td>
<td>26.9 ± 0.4</td>
<td>46.1 ± 0.9</td>
<td>25.1 ± 0.4</td>
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<td><strong>Total energy</strong></td>
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<tr>
<td>intake (kcal/ day)</td>
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</tr>
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</table>

In the OGTT at 28 weeks, fasting insulin and glucose were higher in the 37% fat compared with the control group, as was total glucose area under the curve during the OGTT (p<0.001) (Figure 5.3). When comparing groups originally on the 37% fat diet, fasting glucose was lower in the HF-LC group compared with the 37% fat and MF-MC groups, while fasting insulin was lower on the HF-LC and MF-MC diets compared with the 37% fat group. Total insulin area under the curve during the OGTT was lower on the MF-MC diet compared with the 37% fat group (p<0.05).
Figure 5.3  Plasma measurements during OGTT at 28 weeks

Data reproduced from Lobley at al (Lobley et al 2007). Data presented as mean ± SEM for control (n=11, open squares), 37% fat (n=12, filled squares), HF-LC (n=12, filled triangles), and MF-MC (n=12, filled circles). Blood was taken for measurement of a) plasma insulin and b) glucose before (t=0) and 7, 15, 30, 60, 90, and 120 minutes after ingestion of 2g/kg glucose oral gavage. Comparison between control and 37% fat by Student’s t test. Comparisons between 3 groups by one way ANOVA with post-hoc testing as appropriate using Fisher’s LSD test. * p<0.05, ** p<0.01, *** p<0.001 37% fat vs control. # p<0.05 HF-LC vs 37% fat. $ p<0.05, $$ p<0.01 MF-MC vs 37% fat.

a) Plasma Insulin

b) Plasma Glucose

Duration of test (minutes)
5.3.3 11β-HSD1 mRNA and activity in liver and adipose tissue

The mean of 18S and cyclophillin A was used as the internal control. In the liver, 11β-HSD1 mRNA levels were no different between the control and 37% fat diets (Figure 5.4a). However, 11β-HSD1 mRNA was decreased on HF-LC and MF-MC diets compared with the 37% fat diet. However, 11β-HSD1 activity was decreased on the 37% fat diet compared with control (Figure 5.4b). In concordance with the mRNA data, there was a trend for decreased hepatic 11β-HSD1 activity on the HF-LC and MF-MC diets compared with the 37% fat diet. There was no difference in hepatic 11β-HSD1 mRNA or activity between HF-LC and MF-MC diets.

11β-HSD1 mRNA and activity in peri-renal adipose tissue were significantly decreased on the 37% fat versus control diet (Figures 5.4c and d). However, the HF-LC and MF-MC diets did not alter either 11β-HSD1 mRNA or activity compared to 37% fat diets. 11β-HSD1 in adipose tissue was not different between HF-LC and MF-MC diets.
Figure 5.4  Hepatic and peri-renal adipose 11β-HSD1

Data presented as mean ± SEM for control (n=11, white columns), 37% fat (n=12, black columns), high fat-low carbohydrate (HF-LC) (n=12, grey columns), and moderate fat-moderate carbohydrate (MF-MC) diets (n=12, striped columns). a) Hepatic 11β-HSD1 mRNA levels. b) Hepatic 11β-HSD1 activity. c) Peri-renal adipose 11β-HSD1 mRNA levels. d) Peri-renal adipose 11β-HSD1 activity. The mean of 18S and cyclophillin A was used as internal control (IC) for mRNA data. 11β-HSD1 mRNA transcript levels are expressed as ratio to IC, normalised to 1 for the control diet. Comparison between control and 37% fat diets was performed using Student’s t tests. Comparison between 37% fat, HF-LC and MF-MC diets by one way ANOVA with post-hoc testing using Fisher’s least significant differences test. * p<0.05, ** p<0.01, *** p<0.001 37% fat vs control. # p<0.05, ## p<0.01 versus 37% fat diet.
5.4 DISCUSSION

These data show that a 37% high fat diet for 30 weeks in rats decreases 11β-HSD1 mRNA and activity in peri-renal adipose tissue and decreases 11β-HSD1 activity in the liver. This is consistent with the previous work in rodents (Morton et al 2004b; Drake et al 2005), although this is the longest time course reported to date. Unlike the previous study in rats which reported only regulation of 11β-HSD1 activity by diet (Drake et al 2005), we found regulation of 11β-HSD1 mRNA in addition to activity. Furthermore, dietary regulation of 11β-HSD1 persisted over the 30 weeks rather than being attenuated with prolonged high fat feeding as in the previous work (Drake et al 2005), although of course we did not measure whether down-regulation of 11β-HSD1 was more pronounced earlier in the study.

The ad libitum 37% fat diet worked successfully as a diet-induced obesity model, and had similar macronutrient content to the average ‘Western’ human diet, although the maize oil used had lower saturated fat (10-12%) and higher mono-unsaturated (39-42%) and poly-unsaturated (43-47%) fat content (Lobley et al 2007). Consequently, the rats placed on the second dietary intervention were obese in concordance with the human volunteers described in chapter 4. The adipose tissue results in the rats were consistent with those from the human study, showing that 11β-HSD1 was not different between high fat-low carbohydrate (HF-LC) and moderate fat-moderate carbohydrate (MF-MC) diets. Increasing the fat content further from 37% to 56% (the HF-LC diet) did not further alter adipose 11β-HSD1, indicating there may be a threshold effect of dietary fat content. This may potentially be the reason why no difference in adipose tissue 11β-HSD1 mRNA levels were observed between HF-LC and MF-MC diets in the human study, as the fat content on the lower fat MF-MC diet was still considerable at 35%. In the original rodent studies, dietary fat content in the control diets was only 10-11% of total energy content. In addition, we were only able to study peri-renal adipose tissue as the other adipose beds were unavailable to us, so we do not know if dietary regulation of 11β-
HSD1 is identical in all adipose sites, although dietary regulation of 11β-HSD1 has been observed in all studied adipose sites in mice (Morton et al 2004b).

The results in the liver are more difficult to interpret. Hepatic 11β-HSD1 was unaltered by the HF-LC compared with the MF-MC diet in rats. These results differ from the human diet studies, showing that low carbohydrate intake does not increase hepatic 11β-HSD1 in rats. However, hepatic 11β-HSD1 was regulated by dietary content, as 11β-HSD1 mRNA was decreased (with a trend for reduced 11β-HSD1 activity) by both HF-LC and MF-MC diets compared with the 37% fat diet. Both groups had lower fasting insulin levels compared to 37% fat, potentially secondary to the lower carbohydrate content of these diets. In addition, weight loss may be partially responsible for the decreased fasting insulin concentrations on MF-MC. Therefore, these results do not support the hypothesis that higher insulin concentrations decrease hepatic 11β-HSD1 in obesity.

What then is the cause of the decreased hepatic 11β-HSD1 on the HF-LC and MF-MC diets? Differences in macronutrient content may be directly responsible, for example the lower carbohydrate and/or increased protein content of both these diets. However, these conclusions can only be made tentatively. Of note, absolute energy intakes may be important. Indeed, energy intakes were different between all the diets, in part because the diets were not energy isodense (Table 5.1). Weight gain was also different between diets, particularly on the MF-MC diet where the rats lost weight despite respective energy intakes above the control group. The reasons for weight loss in the MF-MC group are unclear, however this could be a potential confounder as weight loss may alter 11β-HSD1 (Engeli et al 2004; Tomlinson et al 2004a) although this has never been examined previously in rodents. However, the unaltered 11β-HSD1 between HF-LC and MF-MC diets suggest changes in weight of these magnitudes do not influence 11β-HSD1 per se. It is also possible that the liver is not the site responsible for the increased whole body 11β-HSD1 activity observed in the human study, although it is most likely as the liver is the major site of whole body 11β-HSD1 reductase activity (Basu et al 2004; Basu et al 2006a). Further work is
required in humans to determine if hepatic 11β-HSD1 activity is regulated by dietary content and to dissect the contribution of variations in carbohydrate intake.

To conclude, we have shown that a high fat diet decreases adipose 11β-HSD1 mRNA and activity, and hepatic 11β-HSD1 activity in rats. Unlike in humans, a high-fat low carbohydrate diet does not increase hepatic 11β-HSD1, indeed lower carbohydrate content may in fact decrease hepatic 11β-HSD1 further. This work suggests that dietary regulation of glucocorticoid metabolism differs between rodents and humans. Further work is required to dissect the mechanisms through which manipulations in dietary content regulate 11β-HSD1 in rodents and humans.
Chapter 6

Regulation of 11β-HSD1 by peroxisome proliferator-activated receptor (PPAR) γ-agonists and the role of glucocorticoids in their insulin-sensitising effect

6.1 INTRODUCTION

The previous two chapters have shown that dietary content regulates 11β-HSD1 in humans and rodents, although the effects observed appear to be different in the two species. One proposed mechanism for the down-regulation of 11β-HSD1 by a high fat diet in rodents is through direct activation of peroxisome proliferator-activated receptor-γ (PPARγ) (Morton et al 2004b). These receptors are not only activated by dietary fats, but also by certain drugs, for example the thiazolidinediones (PPARγ-agonists) (Semple et al 2006). These PPARγ-agonists act principally in the adipose tissue and alter adipokine secretion, increase insulin sensitivity, promote adipocyte differentiation, and alter body fat distribution in favour of subcutaneous rather than visceral fat accumulation. In several respects, the actions of PPARγ-agonists are the inverse of those of glucocorticoids. In excess, glucocorticoids promote an atherogenic lipid profile, decrease insulin sensitivity, and alter fat distribution in favour of visceral fat accumulation (Dallman et al 2004).

Recent data suggest that the effects of PPARγ-agonists might be mediated, at least in part, by a reduction in glucocorticoid receptor (GR) activation by 11β-HSD1. PPARγ-agonists, including rosiglitazone, reduce 11β-HSD1 mRNA levels and activity in murine 3T3-L1 adipocyte-derived cells, in epididymal adipose tissue of db/db mice after 11 days treatment (Berger et al 2001), and in adipose tissue in rats after 3 weeks administration (Laplante et al 2003). Reducing 11β-HSD1 activity by a similar magnitude has been achieved with selective enzyme inhibitors and results in
enhanced insulin sensitivity, weight loss, and an atheroprotective lipid profile in several mouse models of diabetes and obesity (Alberts et al 2002; Hermanowski-Vosatka et al 2005). However, the extent to which a reduction in 11β-HSD1 activity, and thereby in intracellular glucocorticoid concentrations, accounts for the beneficial metabolic effects of PPARγ-agonists is unknown. In one study, rosiglitazone had no effect on liver or adipose 11β-HSD1 activity in Zucker rats (Livingstone et al 2000b), although it did alter other pathways of intra-hepatic glucocorticoid metabolism (Livingstone et al 2005). Another study showed that improvements in insulin sensitivity by rosiglitazone were similar in rats which had been adrenalectomised or sham-operated (Berthiaume et al 2004), suggesting that the insulin-sensitising effect of PPARγ-agonists is independent of glucocorticoid availability.

Only one group has examined whether PPARγ-agonists regulate 11β-HSD1 in humans and found no effect, measuring adipose tissue mRNA after 12 weeks of pioglitazone treatment in obese and overweight people with type two diabetes (Bogacka et al 2004). Since 11β-HSD1 expression is increased in adipose tissue in obese humans (Rask et al 2001; Wake et al 2003), and 11β-HSD1 in obese mice is more sensitive to PPARγ-agonists (Berger et al 2001), it is possible that obese subjects might respond differently from lean subjects.

My predecessor, Dr Deborah Wake, had previously investigated the effect of 7 days administration of the PPARγ-agonist rosiglitazone in healthy volunteers on whole body, hepatic and subcutaneous adipose 11β-HSD1 (Wake et al 2007a). This was performed using deuterated cortisol infusions, oral cortisone to cortisol conversion tests, and subcutaneous microdialysis infusions of tritiated cortisone respectively. 7 days rosiglitazone had no effect on whole body or hepatic 11β-HSD1, but there was a minor decrease in subcutaneous adipose 11β-HSD1 activity. To validate this observation, we aimed to assess changes in adipose 11β-HSD1 mRNA and activity. Transcriptional regulation of 11β-HSD1 by PPARγ-agonist treatment in rodents had been observed after 11-21 days, thus a longer period of rosiglitazone administration
was used than the previous study. In addition, we aimed to test whether reductions in glucocorticoid signalling mediate the insulin-sensitising effects of rosiglitazone: consequently the drug was administered for 5 weeks to ensure that insulin sensitisation would be measurable (Tonelli et al 2004). In animals, the most striking change in adipose 11β-HSD1 mRNA was described following PPARγ-agonist administration to obese db/db mice (Berger et al 2001), so that in this study participants with a wide range of body mass indices were recruited to establish whether obesity modifies the effects of rosiglitazone on 11β-HSD1 in humans.

6.2 METHODS

6.2.1 Participants

Twelve male healthy volunteers were recruited. Inclusion criteria were: age 20-70 years; body mass index between 20-26 kg/m² (lean) or 30-40 kg/m² (obese); normal renal, hepatic and thyroid function on biochemical screening; alcohol intake <28 units per week; no chronic disease (including impaired fasting glucose or impaired glucose tolerance); on no medication and no glucocorticoid therapy in the previous 6 months. Local ethical approval and written informed consent were obtained.

6.2.2 Protocol

The height and weight of each participant was measured prior to entry into the study (baseline). A two-phase double-blind randomised balanced crossover study was performed, comparing 35 days (5 weeks) treatment with oral rosiglitazone 4 mg daily versus placebo (Table 6.1). Phases were separated by a 14 day washout period. Subjects attended for measurements on days 28 and 35 during each phase. On one of these visits they received a ‘glucocorticoid blockade’ combination of tablets and on the other occasion placebo tablets, in double-blind randomised order.
‘Glucocorticoid blockade’ was achieved by administration of the GR antagonist RU38486 (mifepristone, Exelgyn, Henley-on-Thames, UK) and, to prevent rebound hypercortisolaemia, the cortisol biosynthesis 11β-hydroxylase inhibitor metyrapone (Metyrapone, Alliance Pharmaceuticals, Chippenham, UK). Subjects took 400mg RU38486 and 1g of metyrapone at 2200h the night before and at 0700h on the morning of the study. This dose of RU38486 has previously been shown to reduce plasma triglycerides (Ottosson et al 1995) and achieve substantial drug levels in adipose tissue (Heikinheimo et al 1994).

Table 6.1  Study protocol

*Indicates double-blinded randomisation to either active drug or placebo

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<thead>
<tr>
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<th>Day 27</th>
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<th>Day 34</th>
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<tr>
<td>Add 400mg RU38486 and 1g metyrapone OR placebo night before and morning of assessment *</td>
<td>Assessment of insulin sensitivity and adipose 11β-HSD1</td>
<td>Add placebo OR 400mg RU38486 and 1g metyrapone night before and morning of assessment *</td>
<td>Assessment of insulin sensitivity and adipose 11β-HSD1</td>
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WASH-OUT PERIOD FOR 2 WEEKS

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<th>Day 27</th>
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<tr>
<td>Add 400mg RU38486 and 1g metyrapone OR placebo night before and morning of assessment *</td>
<td>Assessment of insulin sensitivity and adipose 11β-HSD1</td>
<td>Add placebo OR 400mg RU38486 and 1g metyrapone night before and morning of assessment *</td>
<td>Assessment of insulin sensitivity and adipose 11β-HSD1</td>
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6.2.3 Clinical measurements

On each assessment day (days 28 and 35 of each phase, 4 in total), subjects attended the Wellcome Trust Clinical Research Facility at the Western General Hospital in Edinburgh at 0800 h after overnight fast. Subjects had height and weight measured at each visit. Body fat was measured by an Omron BF-302 bioimpedence monitor (Omron Healthcare, Milton Keynes, UK) and blood pressure by an Omron 705IT monitor (Omron Healthcare, Milton Keynes, UK). Cannulae were inserted in both antecubital fossae for infusion and sampling. Fasting bloods were taken at the same time on each visit for lipids, cortisol, ACTH, androstenedione, DHEA, glucose and insulin. Approximately 500-1000 mg of subcutaneous peri-umbilical adipose tissue was obtained under local anaesthetic by needle biopsy, as described in section 2.4.1. Subsequently, an incremental low-dose insulin infusion was performed, infusing saline for one hour, then insulin at 0.01 IU/kg body weight for 1 h, 0.033 IU/kg/h for 1 h, and finally 0.1 IU/kg/h for 1 h as described in section 2.4.3. Venous blood was taken every 60 minutes and plasma stored at -80°C for measurement of glucose, insulin, C-peptide, free fatty acids, glycerol and 3-hydroxybutyrate (3-HOB). 10% dextrose was infused as required to maintain blood glucose between 4.0 and 4.5 mmol/l.

6.2.4 Laboratory analyses

6.2.4.1 Plasma assays

Insulin, c-peptide, glucose, free fatty acids and 3-HOB concentrations were measured as described previously (section 2.10).

Glycerol levels were analysed using an enzymatic colorimetric kit (Sigma, Poole, UK) on a rotor centrifuge spectrophotometer (Cobas Fara, Roche, West Sussex, UK). 25 µl of samples, blanks and standards were incubated with 175 µl free glycerol
Glucose and lipid profile were determined on a Cobas Mira Plus (Roche, Mannheim, Germany), using enzymatic colorimetric kits from Roche for triglycerides and total cholesterol. RU38486 concentrations were quantified by gas chromatography/mass spectrometry (Homer et al 2006). Plasma dihydroepiandrosterone (DHEA) and androstenedione concentrations were measured as described in section 2.10.1. Plasma cortisol was measured by ELISA (DRG Instruments, Marburg, Germany) as per the manufacturer’s instructions, using the same principle as used in the measurement of plasma androgens (section 2.10.1). Plasma ACTH was analysed using a two-site ELISA (Biomerica Inc, CA, USA) as per the manufacturer’s instructions. Blood was collected in EDTA tubes, subject to centrifugation at 2000 rcf for 10 minutes at 4°C and stored at -80°C until analysis. Samples and materials were thawed to room temperature, and samples and standards were added to wells coated with streptavidin. 2 different antibodies were simultaneously incubated with the samples in these wells (one bound to biotin and one bound to horseradish peroxidase) for 4 hours in a rotator (Grant Instruments Ltd, Cambridgeshire, UK) at room temperature. Samples were washed five times using the 1 x wash buffer in a microplate washer (Anthos Labtec Instruments, Salzburg, Austria). Tetramethylbenzidine (TMB) was added to wells and incubated for 30 minutes at room temperature in a rotator. Stop solution (0.5 mol/l sulphuric acid) was added then absorbance read on a microplate reader (Dynatech Laboratories, Guernsey, Channel Islands, UK) at 450 nm.

6.2.4.2 Adipose tissue biopsies

RNA was extracted from whole adipose tissue and mRNA amplified and quantified by real-time PCR as described in section 2.8. mRNA transcript levels of 11β-HSD1, GRα, H6PDH, adiponectin, leptin and resistin were expressed as a ratio against 18S
as internal control. Cyclophilin A was measured to be used as a second internal control as in the diet studies, however transcript levels increased with rosiglitazone treatment (77 ± 13 vs 52 ± 8 arbitrary units, p<0.05) and so were not used for normalisation of the data.

To estimate total 11β-HSD1 protein, activity was measured in the dehydrogenase direction which is the more stable in vitro, as described in section 2.7. Briefly, 250 mg tissue was homogenised in Krebs buffer. 750 μg/ml total protein was incubated with 2 mmol/l NADP, 0.2% glucose and 100 nmol/l cortisol (of which 10 nmol/l 1,2,6,7-[3H]4-cortisol) and incubated at 37°C for 30 hours. Aliquots were withdrawn at 3, 6, 20 and 30 hours and conversion to 1,2,6,7-[3H]4-cortisone was measured by HPLC with on line scintillation detection.

6.2.5 Power calculations

Rosiglitazone resulted in a 50% decrease in 11β-HSD1 mRNA expression in rodents (Berger et al 2001). Estimating an intra-subject standard deviation of 0.08 from previous paired adipose biopsies in Caucasian males (unpublished data), a sample size of 12 results in a 90% power to detect to p<0.05 a 10% change in 11β-HSD1 mRNA expression, which was the primary outcome.

6.2.6 Statistical analyses

Results are shown as mean ± SEM. P<0.05 was considered statistically significant. For measurements in one individual between two groups, comparison was made by paired t tests. For single measurements in multiple paired groups, comparison was by repeated-measures ANOVA with post-hoc Fisher’s LSD tests as appropriate. For dual simultaneous interventions (i.e. with rosiglitazone and glucocorticoid blockade) effects of each intervention were tested by factorial ANOVA with post-hoc Fisher’s
LSD tests as appropriate; to test variability in response to intervention, median body mass index was used to define subgroups, and differences between subgroups tested in a factorial ANOVA model.

6.3 RESULTS

6.3.1 Anthropometry and biochemistry

Participants were aged 41 ± 2.4 years with body mass indices of 29.6 ± 1.7 kg/m² (range 20.3 - 39.6). Body weight was not different between baseline, placebo or rosiglitazone phases (93.4 ± 5.3 vs 93.8 ± 5.5 vs 93.7 ±5.7 kg, respectively). Similarly, fat mass was unchanged between placebo and rosiglitazone phases (25.0 ± 3.0 vs 24.8 ± 3.1 kg). Systolic and diastolic blood pressure was unaltered by rosiglitazone treatment. However, systolic BP decreased on combined rosiglitazone and GC blockade versus rosiglitazone alone (128 ± 3 vs 135 ± 3 mmHg, p<0.05), although there was no difference between GC blockade and placebo (133 ± 3 vs 137 ± 3 mmHg). Diastolic BP was unchanged by GC blockade.

Effects of rosiglitazone and glucocorticoid blockade on baseline biochemical measurements are shown in Table 6.2. GC blockade was successful and led to a compensatory increase in ACTH. Plasma DHEA and androstenedione concentrations were increased by GC blockade. Plasma triglycerides were increased by rosiglitazone and decreased by GC blockade. Plasma cholesterol (Table 6.2), HDL-cholesterol and LDL-cholesterol (data not shown) were unchanged between phases.
Table 6.2  Effects of ‘glucocorticoid blockade’ with RU38486 and metyrapone on plasma biochemistry at 0800 h in the presence and absence of rosiglitazone

Results are mean ± SEM for 12 participants. Differences were tested by repeated measures ANOVA followed, as appropriate, by post-hoc Fisher’s LSD tests. **p<0.01, ***p<0.001 versus placebo without glucocorticoid blockade. $p<0.05, $$p<0.01, $$$p<0.001 versus rosiglitazone without glucocorticoid blockade.

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<tr>
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<th>Without glucocorticoid blockade</th>
<th>With glucocorticoid blockade</th>
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<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Rosiglitazone</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.7± 0.4</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.4 ± 0.2</td>
<td>1.8 ± 0.2***</td>
</tr>
<tr>
<td>ACTH (pmol/l)</td>
<td>2.4 ± 0.5</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>9.6 ± 1.3</td>
<td>11.1 ± 1.6</td>
</tr>
<tr>
<td>Dehydroepiandrosterone (nmol/l)</td>
<td>42 ± 7</td>
<td>37 ± 7</td>
</tr>
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</table>

6.3.2 Adipose tissue mRNA and 11β-HSD1 activity

18S mRNA transcript levels did not vary between the 4 phases (data not shown). Transcript levels of resistin were too low in adipose tissue to be reliably detected so results are not shown. Rosiglitazone alone or GC blockade alone had no effect on mRNA levels of 11β-HSD1, GRα, H6PDH, or adiponectin (Figure 6.1). GC blockade however, increased leptin mRNA in the presence of rosiglitazone and tended to increase leptin mRNA in the absence of rosiglitazone (p<0.06).
Figure 6.1  Effects of rosiglitazone on subcutaneous adipose tissue mRNA levels in the presence and absence of ‘GC blockade’ with RU38486 and metyrapone

Results are mean ± SEM for 12 participants. mRNA levels are expressed as arbitrary units compared with a standard curve from a pooled sample and corrected for 18S as housekeeping gene internal control. Ratios normalised to 1 for placebo. 11β-HSD1 = 11β-hydroxysteroid dehydrogenase type 1. GRα = glucocorticoid receptor α. H6PDH = hexose-6-phosphate dehydrogenase. Open bars, placebo; solid bars, rosiglitazone alone; hatched bars, glucocorticoid blockade alone; and checkered bars, rosiglitazone with glucocorticoid blockade. Differences were tested by repeated measures ANOVA followed, as appropriate, by post-hoc Fisher’s LSD tests. **p<0.01, versus placebo without glucocorticoid blockade.
$^{11}\beta$-HSD1 activity was measured after 3, 6, 20, and 30 hours (Figure 6.2). Rosiglitazone did not alter $^{11}\beta$-HSD1 activity versus placebo. Conversely, GC blockade decreased $^{11}\beta$-HSD1 activity in the presence of rosiglitazone and tended to decrease activity in the absence of rosiglitazone. $^{11}\beta$-HSD1 mRNA and activity were highly correlated ($r^2 = 0.49$, $p<0.01$).

**Figure 6.2  Effects of rosiglitazone with and without GC blockade on subcutaneous adipose tissue $^{11}\beta$-HSD1 activity**

Results are mean ± SEM for 12 participants. $^{11}\beta$-HSD1 activity measured by conversion of cortisol to cortisone. Differences were tested by repeated measures ANOVA followed, as appropriate, by post-hoc Fisher's LSD tests. $^5p<0.05$, $^{55}p<0.01$ for effect of GC blockade in the presence of rosiglitazone.
6.3.3 Plasma metabolites and hormones throughout incremental insulin infusion

In the absence of glucocorticoid blockade, rosiglitazone lowered free fatty acids, glycerol and C-peptide concentrations before and during incremental insulin infusion (Figure 6.3). Rosiglitazone did not alter plasma insulin, glucose, cortisol or 3-hydroxybutyrate concentrations achieved, or dextrose infusion rates, during the low-dose incremental insulin infusion.

Following glucocorticoid blockade, plasma concentrations of RU38486 were undetectable in the placebo phase and 6.2 ± 0.6 μmol/l at 0800 h on the morning of study, comparable with previous studies (Heikinheimo et al 1989). Glucocorticoid blockade lowered plasma cortisol throughout the infusion, as observed at baseline (Figure 6.3a); these effects were not modified by rosiglitazone. In the presence or absence of rosiglitazone, glucocorticoid blockade had no effect on biochemical responses to insulin infusion (Figure 6.3).
Figure 6.3 Metabolic effects of rosiglitazone in the presence and absence of ‘glucocorticoid blockade’ with RU38486 and metyrapone

Results are mean ± SEM for 12 participants. Data were analysed by factorial repeated measures ANOVA, with values at each insulin dose as the dependent variables and presence or absence of rosiglitazone and glucocorticoid blockade as independent variables. Square symbols for rosiglitazone and circle symbols for its placebo; closed symbols for glucocorticoid blockade and open symbols for its placebo. Insulin infusion induced statistically significant effects on all variables (p<0.001) except plasma cortisol. The interaction of dose of insulin with rosiglitazone was significant for free fatty acids (p<0.03) and glycerol (p<0.001) and showed a strong trend for C-peptide (p<0.06). Glucocorticoid blockade reduced plasma cortisol but had no influence on the response to insulin, and there were no significant interactions between glucocorticoid blockade and rosiglitazone treatment.

Post-hoc comparisons at each time point by Fisher’s LSD tests were undertaken for free fatty acids, glycerol and C-peptide only: *p<0.05, **p<0.01 for effect of glucocorticoid blockade with and without rosiglitazone; *p<0.05, **p<0.01 for effect of rosiglitazone in the absence of glucocorticoid blockade; ***p<0.01 for effect of rosiglitazone in the presence of glucocorticoid blockade.
6.3.4 Lean vs obese subgroup analysis

To address the possibility that rosiglitazone only alters 11β-HSD1 in obese individuals, participants were divided into those with body mass indices < 27 kg/m² (n=5, mean 23.9 ± 1.0 kg/m²) or >27 (n=7, mean 33.7 ± 1.6 kg/m²). Insulin sensitisation by rosiglitazone, as measured by a decrease in FFAs, was confined to the obese group (Figure 6.4). Plasma glycerol and C-peptide were similarly decreased by rosiglitazone in the obese group, while glucocorticoid blockade had no effect on biochemical responses to insulin infusion (data not shown). In the obese group, rosiglitazone versus placebo did not alter adipose tissue 11β-HSD1 mRNA (1.03 ± 0.34 vs 1.00 ± 0.25 AU; placebo normalised to 1) or activity (3.3 ± 0.6 vs 2.8 ± 0.6 pmol product/ mcg protein/ hour). Similarly, there was no effect of rosiglitazone in the lean group (data not shown).
Figure 6.4  Metabolic effects of rosiglitazone in the presence and absence of ‘glucocorticoid blockade’ with RU38486 and metyrapone on FFAs in lean and obese groups

Results are mean ± SEM for 12 participants. Data were analysed by factorial repeated measures ANOVA, with values at each insulin dose as the dependent variables and presence or absence of rosiglitazone and glucocorticoid blockade as independent variables. Square symbols for rosiglitazone and circle symbols for its placebo; closed symbols for glucocorticoid blockade and open symbols for its placebo. Post-hoc comparisons at each time point by Fisher’s LSD tests; *p<0.05, for effect of rosiglitazone in the absence of glucocorticoid blockade.

![Diagram a) FFAs in lean group](image)

![Diagram b) FFAs in obese group](image)

Dose of insulin infused in previous hour (mU/kg)
6.4 DISCUSSION

PPARγ is expressed mainly in adipose tissue, while rosiglitazone reduces 11β-HSD1 expression principally in adipose tissue in rodents (Berger et al 2001; Laplante et al 2003). In concordance with this, the pilot 7 day study in humans suggested that rosiglitazone may decrease 11β-HSD1 activity in subcutaneous adipose tissue, with no effect on whole body or hepatic 11β-HSD1 (Wake et al 2007a). However, 5 weeks of rosiglitazone therapy did not change 11β-HSD1 mRNA or enzyme activity in adipose tissue. This was not due to lack of effect of rosiglitazone, as demonstrated by significant suppression of fasting and insulin-induced plasma free fatty acids, glycerol and C-peptide concentrations. In addition, improvement in insulin sensitivity by rosiglitazone, as measured by fasting and insulin-induced suppression of free fatty acid and glycerol concentrations, was equally effective in the presence or absence of ‘glucocorticoid blockade’. These data are consistent with previous experiments in rats showing that metabolic responses to rosiglitazone are not influenced by adrenalectomy (Berthiaume et al 2004) and suggest that, contrary to inferences from the literature (Berger et al 2001; Laplante et al 2003), changes in intra-adipose cortisol regeneration or glucocorticoid receptor activation do not mediate acute effects of PPARγ agonists in humans.

This study was conducted in non-diabetic subjects with a wide range of body mass index, while rosiglitazone is used in the treatment of subjects with type 2 diabetes mellitus. Although type 2 diabetes per se does not alter adipose 11β-HSD1 activity (Andrews et al 2002), it is conceivable that patients with diabetes respond differently to PPARγ agonists, particularly since the insulin sensitising effect of these agents is greatest amongst the most insulin-resistant patients (Semple et al 2006). However, in this study we did not find any down-regulation of adipose 11β-HSD1 mRNA even in the most obese participants, who exhibited the greatest insulin-sensitising response.
The protocol for 'glucocorticoid blockade', using a combination of RU38486 and metyrapone, was based upon previous work demonstrating effective inhibition of glucocorticoid negative feedback and lowering of serum triglycerides (Ottosson et al 1995) and basal hepatic glucose output (Garrel et al 1995). We achieved similar serum concentrations of RU38486, increased ACTH and adrenal androgen levels, consistent with successful glucocorticoid receptor antagonism. In addition, metyrapone prevented any compensatory hypercortisolaemia and indeed plasma cortisol concentrations were lower with glucocorticoid blockade. No detailed evaluation of metabolic responses to this form of glucocorticoid blockade has been published previously, but on the basis of previous studies with similarly acute administration of glucocorticoid receptor agonists, we anticipated that glucocorticoid blockade would decrease serum triglycerides (Ottosson et al 1995), free fatty acids (Samra et al 1996), ketone bodies (Schade et al 1978), glycerol (Divertie et al 1991), and C-peptide and increase glucose infusion rate during low-dose insulin infusion. Effects on intra-adipose mRNA levels were more difficult to predict, since relatively little research has been conducted in vivo in humans. Further, we predicted that these effects would be smaller during rosiglitazone administration, if rosiglitazone reduced intra-adipose cortisol concentrations. However, the only effects of glucocorticoid blockade that we observed were a reduction in triglycerides, an increase in intra-adipose leptin mRNA levels, and a reduction in adipose 11β-HSD1 activity, all paradoxically more obvious during rosiglitazone treatment. The lack of effect of glucocorticoid blockade alone makes the incremental effects during rosiglitazone therapy more difficult to interpret. Different results may have been obtained if it were possible to administer glucocorticoid blockade for a longer period, but these results call into question the acute physiological role of glucocorticoids in modifying fatty acid metabolism.

11β-HSD1 may be important also in visceral adipose tissue, where we were unable to make direct measurements. It is unlikely that visceral adipose tissue 11β-HSD1 was altered as we would have expected changes in responses to glucocorticoid blockade. However, additional studies will be required to clarify the effects of long-term
PPARγ-agonist administration on visceral adipose 11β-HSD1. Although the primary focus of these experiments was on cortisol metabolism, we also assessed cortisol concentrations in blood, knowing that PPARγ-agonists might influence ACTH secretion (Heaney et al 2002). Morning plasma cortisol concentrations though were unaffected by rosiglitazone.

There is evidence from 11β-HSD1 knockout mice that loss of glucocorticoid receptor activation leads to up-regulation of PPAR expression (Morton et al 2001), potentially increasing sensitivity to PPAR agonists. Thus, PPAR agonists and 11β-HSD1 inhibitors may yet have synergistic effects if administered concurrently. However, we conclude that, in contrast with findings in rodents, PPARγ-agonists do not acutely regulate 11β-HSD1 in humans, and the direct insulin-sensitising effect of PPARγ-agonists appears to be independent of glucocorticoid action. We also conclude that, in humans, PPARγ is very unlikely to mediate the effects of dietary regulation of 11β-HSD1.
Chapter 7

Conclusions

Obesity is the fastest growing preventable cause of premature morbidity and mortality in the world. Obesity is associated with metabolic disease (hypertension, diabetes, dyslipidaemia), which increases the risk of developing cardiovascular disease. People with primary glucocorticoid excess also develop obesity with metabolic disease, and links between these conditions have been sought. The enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which locally generates cortisol in predominantly the liver and adipose tissue, is dysregulated in human obesity and has been hypothesized to be a mediator of adverse metabolic disease. 11β-HSD1 is decreased in the liver but increased in adipose tissue in obese humans, although the significance or causes are unknown.

Against this backdrop, we aimed to determine if increased local cortisol regeneration in visceral adipose tissue would result in increased cortisol concentrations in the portal vein supplying the liver. Our work in men with transjugular intrahepatic portal-systemic shunts (TIPSS) in situ has shown that the liver is responsible for the majority if not all of splanchnic cortisol release, and that visceral adipose tissue 11β-HSD1 reductase activity does not significantly contribute to portal vein cortisol concentrations in these individuals. These data do not support the hypothesis that increased 11β-HSD1 activity in visceral adipose tissue would cause hepatic insulin resistance via increased hepatic cortisol concentrations. However, increased visceral adipose 11β-HSD1 may increase local cortisol concentrations with resultant autocrine and/or paracrine effects and lead to deleterious metabolic effects. Future in vivo work in humans is required to measure local cortisol concentrations in the different adipose beds in obesity, once techniques have improved sufficiently to measure this accurately. The d4-cortisol infusion in this setting would quantify the contribution and potentially importance of adipose 11β-HSD1 to local cortisol concentrations.
concentrations, and examine whether increased local cortisol regeneration via 11\(\beta\)-HSD1 is an important mediator of metabolic dysfunction. The role of hepatic 11\(\beta\)-HSD1 in obesity needs to be unpicked further in humans, with access to this tissue in health presenting a major challenge. Arterio-venous cannulation as used in this study does not quantify local cortisol concentrations, thus new techniques must be developed in order to overcome the lack of available tissue for study. New imaging modalities such as magnetic resonance spectroscopy in combination with labelled cortisol may allow quantification of local cortisol concentrations in vivo or measure local binding of cortisol to GR, to determine if hepatic cortisol levels and/or GR activation are altered in obesity.

One of the most important causes of the recent obesity epidemic is altered dietary macronutrient content. We have shown for the first time that diet is a powerful regulator of 11\(\beta\)-HSD1 activity in humans. We found that a high fat-low carbohydrate (HF-LC) diet increased whole body 11\(\beta\)-HSD1 activity by 20% compared to a moderate fat-moderate carbohydrate (MF-MC) diet. Furthermore, this was not due to subcutaneous adipose 11\(\beta\)-HSD1, and probably reflects increased cortisol regeneration in the liver. These effects were reproduced on ad libitum and isocaloric intakes, indicating that the macronutrient content was key. The results suggested that low carbohydrate content was the primary mediator of this regulation. Furthermore, the HF-LC diet decreased cortisol metabolism by the hepatic 5\(\alpha\)- and 5\(\beta\)-reductases, indicating that this diet would cause increased hepatic cortisol concentrations by decreasing cortisol metabolism in addition to increasing cortisol regeneration. Insulin concentrations were significantly lower on the HF-LC diet, suggesting that hyperinsulinaemia may be responsible for the decreased hepatic 11\(\beta\)-HSD1 and increased hepatic 5\(\alpha\)- and 5\(\beta\)-reductase activities observed in obesity. These results are in keeping with the theory that decreased hepatic 11\(\beta\)-HSD1 activity is a consequence of obesity rather than a cause. Dietary regulation of 11\(\beta\)-HSD1 and 5\(\alpha\)- and 5\(\beta\)-reductases may be an important adaptive mechanism to limit hepatic glucocorticoid availability and insulin resistance in humans.
In order to dissect the above dietary regulation of 11β-HSD1, we examined these diets in rats. In concordance with previous work in rodents, we discovered that an obesity-inducing 37% fat diet recapitulated the decreased hepatic 11β-HSD1 observed in human obesity, but caused a decrease in adipose 11β-HSD1 opposite to that seen in humans. Furthermore, the HF-LC and MF-MC diets decreased hepatic 11β-HSD1 compared with the 37% fat diet but had no effect on adipose 11β-HSD1. However, no differences in hepatic and adipose 11β-HSD1 were found when comparing the HF-LC and MF-MC diets, unlike in humans. These results suggest that dietary regulation of 11β-HSD1 in rodents is different to humans, as low carbohydrate content did not alter hepatic 11β-HSD1.

The final chapter examined the role of PPARγ-agonists on 11β-HSD1 in human adipose tissue, which have been shown to decrease 11β-HSD1 mRNA in adipose tissue in rodents. PPARγ has also been suggested as a potential mechanism for mediating the effects of a high fat diet on adipose 11β-HSD1 in rodents. Again, our results differed from the rodent data, as 5 weeks of PPARγ-agonist treatment did not alter subcutaneous adipose tissue 11β-HSD1 in humans. Furthermore, the insulin-sensitising effect of rosiglitazone was equally effective in the presence or absence of glucocorticoid blockade, meaning that PPARγ-agonists do not mediate these effects through inhibition of glucocorticoid action.

The differences in nutritional signalling between humans and rodents are not necessarily surprising, as these two species have evolved in vastly different environments, although HPA axis responses are similar between the species, reflecting the acute changes in energy metabolism required by all species in response to stress and starvation. However, changes in nutrition between humans and rodents over tens of thousands of years of evolution have led to different nutritional signalling pathways. For long periods of history, humans were essentially hunter-gatherers potentially requiring periods of energy storage (anabolic state) and periods of famine (catabolic state). In this environment, regulation of tissue glucocorticoids by dietary content may be, in combination with insulin, an important determinant of
the balance between energy storage and availability. In times of high carbohydrate intake, a decrease in hepatic glucocorticoid levels would potentially improve insulin sensitivity and promote hepatic glycogen storage. Concordantly, in times of lower carbohydrate intake, increased hepatic glucocorticoid concentrations would increase hepatic insulin resistance promoting gluconeogenesis. It is possible that adipose 11β-HSD1 and thus cortisol concentrations are increased at lower fat intakes than the in the above studies in humans, potentially increasing lipolysis at times of starvation to promote energy mobilisation. However, insulin may be a more important determinant of adipose energy storage and mobilisation. The complex regulation of glucocorticoid metabolism by dietary content requires more investigation, and our studies demonstrate that rodents may not be a good model for this research. In rodents, downregulation of adipose 11β-HSD1 by high fat feeding may decrease lipogenesis as a protective mechanism to prevent excessive weight gain, which would decrease their ability to escape from predators. Further work is required to dissect the effects of insulin from dietary content on glucocorticoid metabolism, potentially utilising diets of identical macronutrient content but with different glycaemic indices to alter insulin secretion.

To conclude, this thesis has shown that the liver is responsible for all of splanchnic cortisol regeneration by 11β-HSD1 in TIPSS patients, although it is unknown if this is similar in healthy humans. We have discovered that hepatic 11β-HSD1 is regulated by a low carbohydrate diet in obese men, and that increased insulin concentrations may be responsible for the altered hepatic cortisol metabolism in obesity. However, neither these manipulations in dietary macronutrient content nor PPARγ-agonists alter subcutaneous adipose tissue 11β-HSD1 in humans. The causes of increased adipose 11β-HSD1 in obesity humans remain unknown at present and more work is now required, including into other factors such as inflammation. In addition, this work shows that the results from rodent models do not always translate into humans, so other models may be required to dissect the complex dysregulation of 11β-HSD1 in obesity.
Bibliography


