Intra-Adipose Steroid Metabolism in Human Obesity

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DEDICATION:

To Magnus, Corin and all my supportive family and friends.

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CONTENTS

ABBREVIATIONS:

ACTH	adrenocorticotrophic hormone
AR	androgen receptor
AKR	aldoketoreductase
CNS	central nervous system
CRH	corticotrophin releasing hormone
E	cortisone
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
F	cortisol
FFA	free fatty acid
GH	growth hormone
GR	glucocorticoid receptor
HSL	hormone sensitive lipase
HPA	hypothalamic pituitary axis
H6PDH	hexose-6- phosphate dehydrogenase
HIV	human immunodeficiency virus
11HSD1	11beta- hydroxysteroid dehydrogenase type 1
11HSD2	11beta- hydroxysteroid dehydrogenase type 2
IGF-1	insulin like growth factor type 1
IL-1	interleukin 1
IL-6	interleukin 6
IKK-β	inhibitor of kappa B
LPL	lipoprotein lipase
LXR	liver-x- receptor
MR	mineralocorticoid receptor
NADPH	nicotinamide adenine dinucleotide phosphate
NFκ-B	nuclear factor kappa B
PEPCK	phosphoenolpyruvate carboxykinase
PPAR	peroxisome proliferator activated receptor
TB	tuberculosis
TNFα	tumour necrosis factor alpha

FIGURES:

TABLES

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DECLARATION

This thesis is the original research of the author and has not been previously submitted for a higher degree at this or any other university.

Signed:

Deborah J Wake

ABSTRACT

Glucocorticoid excess causes obesity, dyslipidaemia, insulin resistance and hypertension as seen in Cushing's syndrome. In idiopathic obesity, circulating cortisol levels are not elevated but cortisol metabolism is altered. 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) is an intracellular enzyme that converts inactive cortisone into active cortisol. Data from animals suggests that elevated adipose 11HSD1 may generate more glucocorticoid within adipose tissue and be an important mediator of obesity and the associated metabolic consequences (diabetes, dyslipidaemia and hypertension). This thesis addresses whether adipose 11HSD1 is elevated in *human* obesity, investigates if this occurs through altered transcription, and assesses the downstream impact (changes in downstream target genes and metabolic outcomes). Regulation of 11HSD1 mRNA in animal and cell models (eg by high fat feeding, insulin, and PPAR agonists), and putative control of enzyme direction by NADPH generation (via hexose-6-phosphate dehydrogenase), suggest a dynamic role for 11HSD1 in the adaptive response of adipose to altered nutrition. The importance of these potential regulators in lean and obese humans is also addressed in this thesis.

In subcutaneous adipose biopsies from male and female healthy volunteers from Finland (n=19), Sweden (n=27) and USA (n=35) 11HSD1 activity (in vitro conversion of cortisol to cortisone in presence of NADP) and mRNA (by real time PCR) was increased in association with generalised and 'central' obesity (which is most strongly associated with increased cardiovascular risk), and predicted insulin resistance. However, glucocorticoid receptor mRNA was negatively associated with obesity and insulin resistance. Adipose mRNAs for a number of key functional adipose targets (adiponectin, LPL, HSL, angiotensinogen, resistin, aromatase, PPARγ) were not significantly associated with 11HSD1 expression or activity.

To investigate 11HSD1 regulation, a series of randomised controlled studies in vivo in healthy male volunteers were performed to assess the regulatory effects of insulin (euglycaemic clamp) or lipid (20% Intralipid iv) over 3.5 hours and PPAR agonists (rosiglitazone or fenofibrate for 7 d). Deuterated-cortisol tracer with GCMS analysis was used to measure whole body cortisol turnover and urinary metabolite excretion, and intra adipose microdialysis was used to assess *in vivo* s.c. adipose

11HSD1 activity and directionality. Hyperinsulinaemia increased the rate of appearance of 9,12,12-[^{2}H]$_3$-cortisol in plasma, indicating increased regeneration of cortisol by 11HSD1. Within adipose tissue, the predominant reaction was conversion of cortisone to cortisol rather than cortisol to cortisone; both activities fell during the first hour of hyperinsulinaemia but subsequently increased. Intralipid infusion had no significant effects on deuterated cortisol metabolism, but increased intra-adipose conversion of cortisone to cortisol. The PPARγ agonist rosiglitazone lowered adipose 11HSD1 reductase activity. The PPARα agonist fenofibrate had no effect on adipose 11HSD1 activity although urinary ratios of cortisol/ cortisone metabolites and endogenous cortisol clearance were reduced. These findings may be suggestive of decreased liver 5alpha-reductase activity. There were no significant changes in plasma tracer kinetics with PPAR agonists.

In addition to altered glucocorticoid metabolism, changes in sex steroid metabolizing enzymes dictating oestrogen (aromatase) and androgen (AKR1C2/3) action were found to be associated with fat distribution in s.c biopsies from Swedish (n=27) and Finnish (n=19) volunteers. Further studies to determine the functional relevance of these associations are planned.

These studies implicate dysregulation of 11HSD1 transcription in the pathogenesis of human obesity and the metabolic syndrome although the downstream impact of enzyme dysregulation remains unclear. They also suggest that 11HSD1 is involved in the adaptive response to nutrition, and that alteration in tissue glucocorticoids may contribute to the therapeutic action of PPAR agonists. This understanding of enzyme regulation in humans will allow further studies to dissect the basis of dysregulation in obesity. Inhibition of adipose 11HSD1 remains an intriguing target for the treatment of obesity and its metabolic complications but its physiological importance will best be determined by the development of specific adipose 11HSD1 inhibitors.

Chapter 1

INTRODUCTION

1.1 Obesity and the Metabolic Syndrome

This thesis will examine the role of steroid action in obesity and its metabolic consequences, focusing primarily on the actions of glucocorticoids within adipose. Obesity is set to become the biggest cause of preventable death in the US, and may be the greatest health threat currently facing the western world. The United States are the world leaders with over 50% of the population defined as overweight (Mokdad et al 1999). Scotland is renowned for its poor diet and consequently rates of obesity and cardiovascular disease are some of the worst in Europe. In 1998 nearly 8% of boys and 7% of girls were obese and 62% of men and 54% of women in Scotland were either overweight or obese (Scottish health survey). Childhood obesity has increased approximately seven fold in the last 30 years with associated increases in type 2 diabetes and other complications (Ebbeling et al 2002). Industrialization, urbanization, economic development and market globalisation have resulted in a more sedentary lifestyle and increased refined carbohydrate and fat in the diet. Genetic factors undoubtedly alter the propensity to obesity (Speakman 2004), although monogenic causes of obesity are rare (O'Rahilly et al 2003). The health risks associated with obesity are wide ranging, but include respiratory disease, osteoarthritis, sleep apnoea, cancer, diabetes mellitus and notably cardiovascular and cerebrovascular disease (Fontaine et al 2003). The increased propensity to atheromatous disease is thought to be due to the associated 'metabolic syndrome' or 'syndrome x', defined as the triad of hypertension, insulin resistance and dyslipidaemia (Reaven 1988). Central, as apposed to peripheral or 'gynoid' obesity is particularly associated with increased atherogenic risk (Despres et al 1990).

The exact mechanisms linking obesity and the metabolic syndrome are not fully understood but the role of the adipocyte is emerging as an important one. Adipose tissue produces many metabolically active factors and, although previously considered a benign energy storage organ, should now be considered an endocrine organ in its own right. Adipose tissue metabolism is altered in obesity and may be an important mediator of the associated dysmetabolic phenotype.

1.2 Adipose as an Endocrine Organ

Over recent years, adipose tissue has evoked new interest, emerging as a key metabolic tissue. This was fuelled by the discovery of Leptin in 1994 (Zhang et al 1994), a circulating factor secreted from fat cells shown initially to regulate appetite, and subsequently to have effects on many other aspects of physiology including bone mass, reproductive function and cardiovascular disease (Caro et al 1996). Since then, a spectrum of other 'adipokines' have been discovered including adiponectin, resistin, adiponutrin, acylation stimulating protein (ASP), plasminogen activator inhibitor (PAI-1), angiotensin II, and soluble preadipocyte factor (Cianflone et al 1999;Steppan et al 2001;Stefan & Stumvoll 2002;Diez & Iglesias 2003;Weigle et al 2003;Polson & Thompson 2003;Lee et al 2003). Adiponectin (also called Acrp30, GBP-28, and apM1) is a novel adipose specific protein with a structure similar to collagen VIII and X and complement factor C1q. Circulating levels of adiponectin have been inversely associated with the degree of adiposity, plasma glucose, insulin and triglyceride levels and positively related to insulin sensitivity both in healthy subjects and diabetic patients. This may be mediated in part through increasing tissue fatty acid oxidation (Diez & Iglesias 2003). Resistin is another novel signaling molecule, released from fat cells, and thought to mediate insulin resistance. In mice, administration of anti-resistin antibody improves blood sugar and insulin action and treatment with recombinant resistin impairs glucose tolerance and insulin action (Steppan et al 2001). The important of resistin in human disease however is not yet fully understood. Estrogens (Simpson 2000b), glucocorticoids and inflammatory cytokines are also generated within adipose tissue, and may be elevated in obesity. Insulin resistance may also be controlled by the action of free fatty acids on the liver, draining from visceral fat through the portal circulation. Increased liberation and action of free fatty acids from adipose in obesity, under control of lipases (HSL and LPL) and nuclear receptors (PPAR–γ), may therefore be important regulators of insulin resistance in obesity.

1.3 The Role of Inflammation

The role of 'inflammation' in obesity and cardiovascular disease is particularly topical. Obesity is associated with elevated levels of circulating

inflammatory cytokines (Xydakis et al 2004). Further, increasing inflammatory markers predict the metabolic features (insulin resistance, hypertension and dyslipidaemia) and are independently associated with cardiovascular and cerebrovascular endpoints (Ridker et al 2003;Ridker & Morrow 2003). Inflammatory cytokines including TNF-α, IL-1 and 1L-6 can be generated within adipose tissue although whether these originate from infiltrating immune cells or adipocytes themselves remains controversial. Circulating inflammatory cytokines may regulate pathophysiological processes both locally, e.g. within adipose, and at distal sites such as the endothelium. The hypothalamic pituitary axis (HPA) can also be stimulated by inflammatory cytokines and resultant hypercortisolaemia may be important for resolution of inflammation (Mulla & Buckingham 1999).

1.4 The Neuroendocrine control of Body Weight

Over the past decade there has also been a tremendous increase in the understanding of the molecular endocrine and neural mechanisms underlying control of body weight. Regulation of appetite, feeding and energy balance is complex but in part mediated by circulating hormones and direct neural signals from peripheral tissues, such as adipose and gut. Distension of the stomach and small bowel, insulin release and specific hormonal signals all feedback information to the CNS about feeding. Peptide-YY (PYY) and glucagon-like peptide 1 are hormones co-secreted from the intestine into the circulation following food intake and acutely inhibit appetite (Schwartz et al 2000;Spiegelman & Flier 2001;Saper et al 2002). Ghrelin is thought to perform the opposite role, being released from the stomach and gastrointestinal tract as a 'hunger' signal which is suppressed by food intake (Horvath et al 2001). Adipokines such as leptin provide longer term information from fat tissue about energy balance. The arcuate nuclei and dorsomedial/ paraventricular nuclei in the hypothalamus regulate feeding behaviour, metabolic rate and energy expenditure in response to these peripheral signals (Zigman & Elmquist 2003). Obesity may be associated with dysfunctional neuroendocrine signalling, the most recognised phenomenon being leptin resistance.

1.5 Glucocorticoids in Obesity and the Metabolic Syndrome

This thesis will focus on the role of glucocorticoid metabolism in obesity. Glucocorticoids mediate their tissue effects by activation of intracellular receptors. Cortisol, the principal active glucocorticoid in man, is an important regulator of many physiological pathways, particularly at times of stress or illness. Excessive glucocorticoid exposure either as a result of endogenous overproduction (i.e. Cushing's syndrome) or exogenous administration (in the treatment of inflammatory diseases) results in increased central adiposity, insulin resistance, dyslipidaemia and hypertension. Idiopathic obesity is also associated with these dysmetabolic features, defined as 'the metabolic syndrome'. The phenotype of Cushing's syndrome is reversible upon removal of glucocorticoid excess. It has been proposed that subjects with obesity and the metabolic syndrome have increased glucocorticoid receptor activation, and further that they may be susceptible to therapeutic manipulation of tissue glucocorticoid exposure. In idiopathic obesity, circulating plasma cortisol levels are not elevated, but dysregulation of peripheral cortisol metabolism and excretion and alterations in glucocorticoid feedback (through the HPA axis), may explain the phenotypic similarities with Cushing's syndrome.

1.6 The HPA axis and Cortisol Clearance in Obesity

Idiopathic obesity is associated with increased cortisol secretion indicating activation of the HPA axis. This has variably been attributed to increased long term stress (Schwartz et al 2000) and alterations in HPA axis feedback (Ljung et al 1996;Jessop et al 2001;Di Blasio et al 2003). The HPA axis in obesity is hyper-responsive when stimulated e.g. by acute stress, CRH/ AVP, hypoglycaemia, or a standard meal test (Pasquali et al 1993;Vicennati & Pasquali 2000;O'Rahilly et al 2003). Further, obesity is associated with resistance to glucocorticoid feedback (Jessop et al 2001;Ljung et al 2002) an effect which may be mediated via altered sensitivity of glucocorticoid or mineralocorticoid receptors. Some studies suggest that individuals with common glucocorticoid receptor polymorphisms demonstrate HPA axis abnormalities in addition to a peripheral phenotype of increased adiposity, insulin resistance and hypertension (Bjorntorp et al 1999;Rosmond et al 2000;Van Rossum et al 2002;Ljung et al 2002;Di Blasio et al 2003).

The diurnal rhythm of plasma cortisol is however lost in obesity such that plasma cortisol is higher than in lean subjects during the evening nadir but *lower* than expected during the morning peak (Ljung et al 1996;Rosmond et al 1998;Walker et al 2000). The combination of increased total cortisol secretion with lower plasma cortisol levels during peak secretion suggests that peripheral cortisol clearance is increased in obesity. Over 20 years ago it was observed that obesity is associated with increased cortisol production and clearance rates (Lottenberg et al 1998). Increased glucocorticoid clearance could itself reduce plasma levels and lead to compensatory HPA axis activation.

The enzymes, which may be responsible for increased glucocorticoid clearance in obesity, were identified by analysis of cortisol metabolites in the urine. Increased relative excretion of A-ring reduced metabolites of cortisol was observed (Andrew et al 1998;Fraser et al 1999), attributable to activation of the hepatic A-ring reductase enzymes. These enzymes perform the rate limiting step in the reduction of glucocorticoids (cortisol in man, corticosterone in rats and mice) to tetra-hydro products which are ultimately excreted in the urine (figure 1.1). The A-ring reductase enzymes (namely 5α- and 5β-reductase) are activated in both animal (Livingstone et al 2000) and human obesity (Andrew et al 1998;Fraser et al 1999). The underlying cause of increased enzyme activity is largely unknown but these enzymes may be regulated by nutritional factors such as insulin and lipids, and substrate availability. 5β-Reductase is also a key enzyme involved in cholesterol metabolism and bile acid biosynthesis, which may compete with glucocorticoids.

Despite strong evidence that dysregulation of the HPA axis and glucocorticoid clearance pathways exists in obesity, from first principles manipulation of these pathways for therapeutic benefit is unlikely to be fruitful. Inhibition of cortisol biosynthesis is potentially hazardous due to risk of Addisonian crisis. Further, circulating cortisol levels are already low and any attempt to increase clearance is likely to result in further HPA activation and cortisol and adrenal androgen production. Tissue-specific manipulation of glucocorticoid effects may therefore be a more useful strategy. The 11HSD type 1 enzyme regulates the balance of active and inactive tissue glucocorticoid levels and is dysregulated in obesity; as such it may prove to be an interesting and potent target for manipulation.

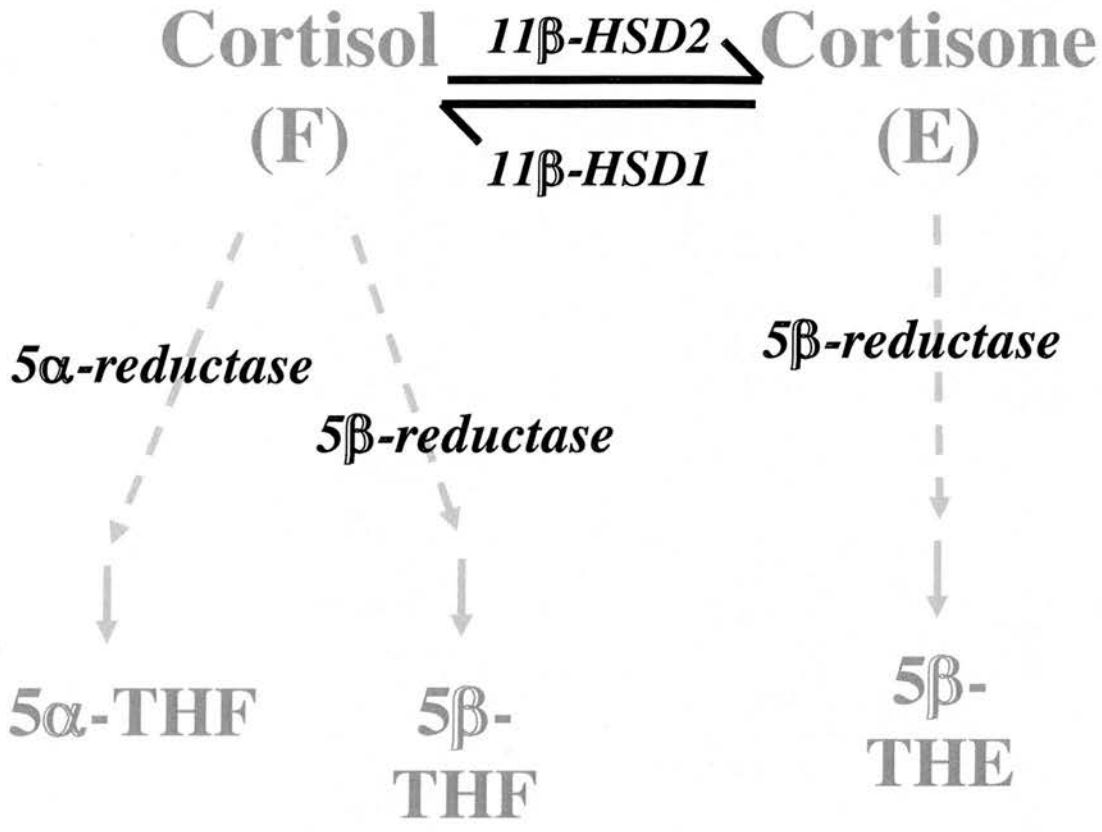

Figure 1.1

A-Ring Reductases.

11HSD1 interconverts cortisol and inactive cortisone. The A-ring Reductases (5α and 5β-Reductase) convert cortisol and cortisone ultimately to tetra-hydro products within the liver. These are ultimately conjugated and excreted in the urine. THF= tetrahydrocortisol, THE= tetrahydrocortisone

1.7 The 11β-Hydroxysteroid Dehydrogenases (11HSDs)

Until relatively recently, tissue glucocorticoid concentrations were thought to be determined exclusively by plasma levels of glucocorticoids and binding proteins, and tissue responses controlled by availability of glucocorticoid receptors. Other hormones, however, also regulate tissue responses through pre-receptor enzymes, co-expressed with the intracellular receptor, which metabolise ligand and control receptor access (e.g. 5α–reductase type 2 for androgens and 5'-monodeiodinase for thyroxine)(Stewart & Sheppard 1992). It is now known that the 11β-hydroxysteroid dehydrogenase enzymes perform this role for glucocorticoid and mineralocorticoid receptors.

The 11β-hydroxysteroid dehydrogenases (11HSDs) were discovered some 50 years ago (Amelung et al 1953), yet their potential as therapeutic targets is only now emerging. These microsomal enzymes interconvert active and inactive glucocorticoids, thus acting as gate keepers for intracellular receptors (White et al 1997;Stewart & Krozowski 1999;Seckl & Walker 2001). By controlling substrate availability (cortisol in humans, corticosterone in rats and mice), they mediate tissue specific glucocorticoid receptor activation, irrespective of circulating plasma cortisol levels. Glucocorticoids are important regulators of patho-physiological processes in many tissues, therefore pharmacological manipulation of these novel enzymes may have wide ranging therapeutic benefits.

There are two 11HSD isozymes (figure 1.2). The type 2 enzyme is an exclusive NAD dependent dehydrogenase, converting active cortisol to inactive cortisone. Its main role is in aldosterone sensitive target tissues (kidney, colon, salivary glands and placenta) as described by Stewart and Edwards and others in the 1980s (Funder et al 1988;Edwards et al 1988). Cortisol circulates in several-fold higher plasma concentrations than aldosterone but both have similar affinity for the non-selective mineralocorticoid receptor. By inactivating cortisol, 11HSD2 prevents flooding of mineralocorticoid receptors leaving free access for aldosterone. This is particularly important in the distal nephron. 11HSD2 was cloned in 1994 (Albiston et al 1994) and since 1995 the rare syndrome of apparent mineralocorticoid excess (SAME) (characterised by hypertension, hypokalaemia and fluid retention due to

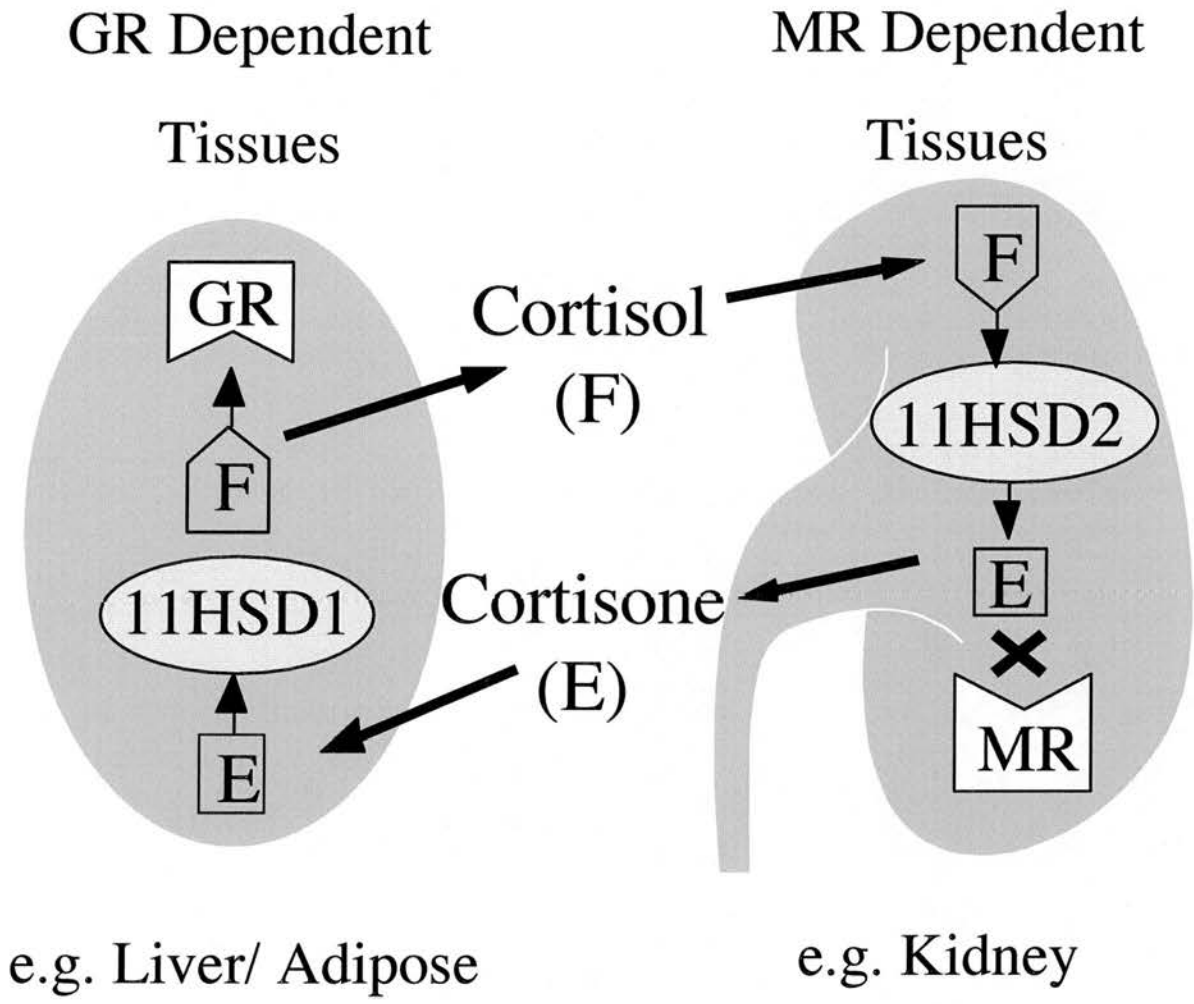

Figure 1.2

Cortisol/ Cortisone generation in MR/ GR Dependent Tissues.
11HSD1 is found in GR sensitive tissues where regeneration of cortisol from cortisone results in activation of GR. 11HSD2 is found in MR sensitive tissues where conversion of cortisol to inactive cortisone prevents illicit occupation of MR receptors. 11HSD1= 11beta-hydroxysteroid dehydrogenase type 1, 11HSD2= 11beta-hydroxysteroid dehydrogenase type 2, F= cortisol, E= cortisone, GR= glucocorticoid receptor, MR= mineralocorticoid receptor.

illicit occupation of mineralocorticoid receptors by cortisol) has been attributed to mutations in the *HSD11B2* gene (Dave-Sharma et al 1998).

11HSD type 1 was poorly understood during these early years, but is now recognized as an abundant and physiologically important enzyme. Mammalian 11HSD1 was first cloned from rat liver cDNA in 1989 (Agarwal et al 1989). Later a library derived from human testis was probed using the rat 11βHSD and further clones identified. Hybridization of the human 11ß-HSD1 cDNA to a human-hamster hybrid panel localized the single *HSD11* gene to chromosome 1 (subsequently refined to chromosome 1q32.2–41) (Tannin et al 1991). The human gene has been designated *HSD11B1* and consists of six exons and five introns.

11HSD1 belongs to the short chain dehydrogenases/reductases family (SDRs), a well established enzyme family of oxidoreductases. This enzyme is microsomal (Ozols 1995), and activity in early purification studies showed bi-directionality in vitro, but with predominant and more stable dehydrogenase activity. Further studies, however, revealed 11ß-HSD1 to act in vivo (and in intact cell systems) as a predominant NADPH dependent reductase (figure 1.3). Thus, 11β-HSD1 performs the opposite role to 11HSD2, i.e. generating cortisol from inactive cortisone (Walker et al 1992;Low et al 1994;Bujalska et al 1997;Katz et al 1999;Seckl & Walker 2001). This striking change in directionality between intact cells and homogenates seems to reflect the specific intracellular localization of 11ß-HSD1 within the lumen of the endoplasmic reticulum (ER), where neighboring enzymes may be powerful generators of the reduced cosubstrate NADP phosphate (NADPH)(Tomlinson et al 2004b).

11HSD1 is found in many tissues including liver, adipose, gonads, brain and vasculature. These tissues are abundant in glucocorticoid rather than mineralocorticoid receptors. If 11HSD1 modulates access of ligand to glucocorticoid receptors in these tissues, then it may have implications in many common diseases which share features of Cushing's syndrome, including idiopathic obesity, diabetes mellitus, polycystic ovary disease and dementia (Seckl & Walker 2001;Tomlinson et al 2004b)

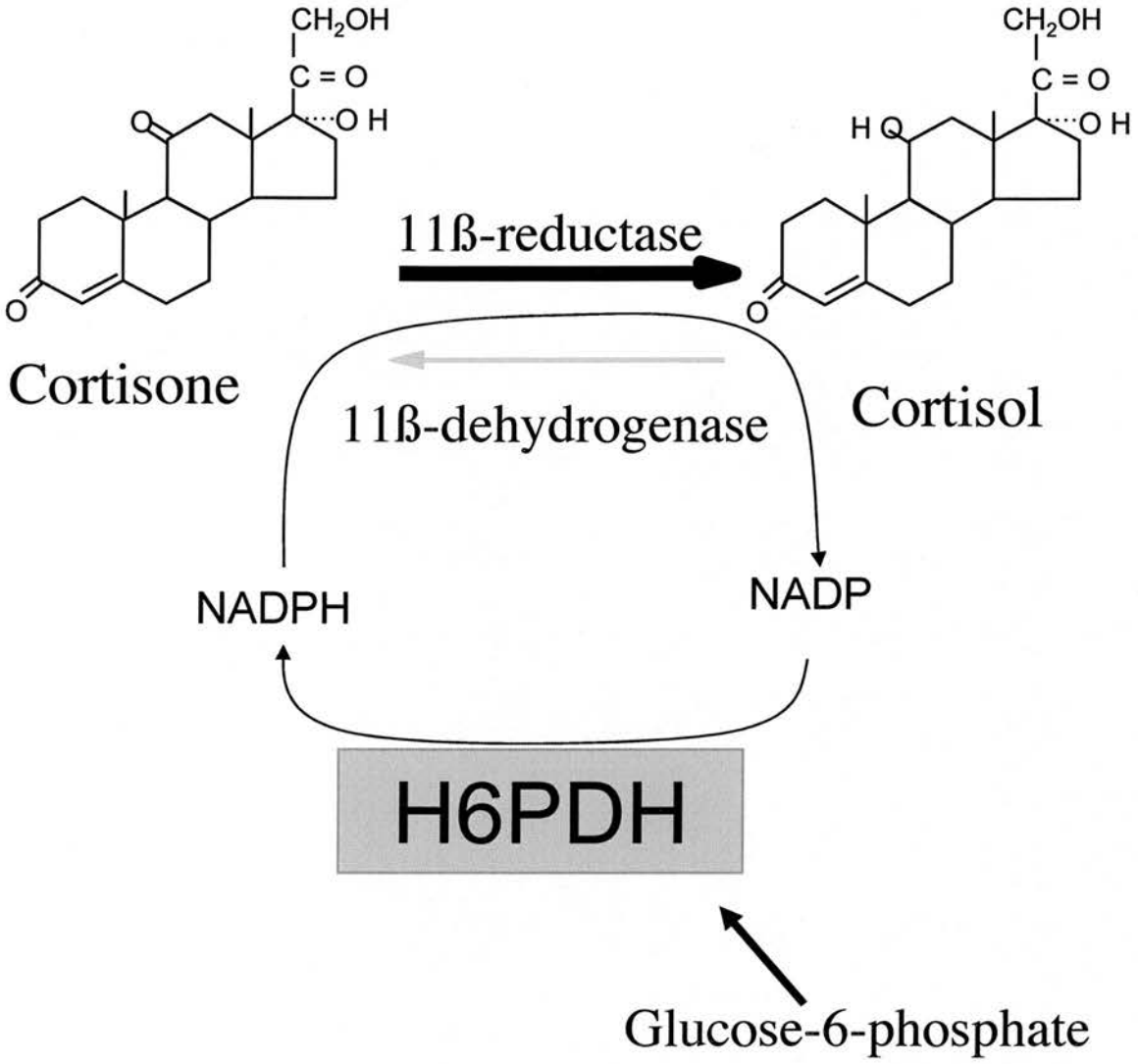

Figure 1.3

Reactions catalysed by 11β-hydroxysteroid dehydrogenase type 1 in humans.

Inactive and active glucocorticoids are interconverted by 11keto-reductase and 11β-dehydrogenase activities. In vivo, Hexose-6-Phosphate Dehydrogenase (H6PDH) generates NADPH which drives reductase activity. Glucose-6-phosphate is the main substrate for H6PDH.

1.8 11HSD1 in the Pathophysiology of Obesity

1.8.1 Lessons from transgenesis

The potential importance of 11HSD1 in obesity has emerged from in vitro studies, in vivo animal models, human studies and perhaps most persuasively by the development of transgenic mice. These mouse models have given insight into the expected human phenotypes resulting from changes in 11HSD1 activity. Striking metabolic consequences have been observed by altering 11HSD1 expression. Adipose specific over-expression of 11HSD1 (under an AP2 promoter) produces a phenotype analogous to the metabolic syndrome (with central obesity, hypertension, insulin resistance and dyslipidaemia) (Masuzaki et al 2001;Masuzaki et al 2003). This is associated with high intra-adipose corticosterone, high levels of plasma leptin, tumour necrosis factor (TNF)-alpha, and adipose lipoprotein lipase (LPL) mRNA. Cortisol is elevated in the portal vein but normal in the peripheral circulation because of reduced adrenal production. Levels of adipose uncoupling protein-1 (UCP-1) mRNA are low, portal flow of triglycerides is increased and angiotensinogen production is elevated which may explain the associated hypertension (Masuzaki et al 2003).

Knockout mice, homozygous for a deleted 11HSD1 allele, are conversely protected from the metabolic consequences of obesity (Kotelevtsev et al 1997;Morton et al 2001). They have lower intra-cellular corticosterone levels despite mildly elevated circulating levels (Harris et al 2001;Yau et al 2001), a favourable lipid profile (lower serum triglycerides, and high HDL cholesterol) and resist hyperglycaemia induced by stress and during high fat feeding. Changes in glucocorticoid dependent gene expression are also seen in the liver, with decreased gluconeogenic enzymes (notably PEPCK) and increased enzymes of lipid oxidation. When rederived against an obesity prone genetic background (C57/Bl6), 11HSD1 -/- mice are further protected from weight gain on a high fat diet (Morton et al 2004b).

Overexpression of 11HSD1 in the liver, alternatively, under an ApoE promoter results in fatty liver, dyslipidaemia, mild insulin resistance and hypertension which again is thought to be mediated by increased angiotensinogen expression. These animals do not however demonstrate increased adiposity (Paterson et al 2004).

The striking phenotype of these transgenic models suggests that 11HSD1 can be a potent pathophysiological force in the development of the metabolic syndrome, and further that there could be wide-ranging therapeutic benefits from 11HSD1 inhibition, including improvements in dyslipidaemia, hypertension, insulin resistance and potentially limitation of weight gain in obesity susceptible individuals (figure 1.4).

1.8.2 Urinary cortisol metabolite profiles

Transgenic models may help to predict human phenotypes, but clinical studies are required to assess in vivo 11HSD1 activity in human obesity. Assessment of 11HSD1 in humans is difficult. Conventionally, urine is examined by mass spectrometry to assess the ratios of cortisol: cortisone metabolites; an elevated ratio may be taken to indicate increased regeneration of cortisol from cortisone by 11HSD1. However, these measurements gave conflicting results, showing variably increased (Andrew et al 1998;Rask et al 2002;Tiosano et al 2003), decreased (Stewart et al 1999;Rask et al 2001), or unchanged (Fraser et al 1999;Reynolds et al 2001) cortisol:cortisone metabolite ratio in relation to parameters of obesity. This suggests that there may be unmeasured confounding influences determining the cortisol:cortisone metabolite ratio, over and above any influence of 11HSD1. Other enzymes such as 11HSD2 and the A-ring reductases (5α- and 5β-reductase) can affect cortisol: cortisone metabolite ratios. A recent study illustrates the importance of these enzymes, since in a group of patients with careful characterization of body fat distribution the cortisol:cortisone urinary metabolite ratio was shown to be more closely associated with accumulation of fat in the liver rather than in adipose tissue; further, the ratio was unrelated to measurements of 11HSD1 in adipose biopsies or to conversion of cortisone to cortisol in vivo, and appeared to reflect mainly an increase in 5β-reductase (Westerbacka et al 2003). Because of these difficulties, urinary measurements should not be used in isolation to assess 11HSD1 activity.

Stable isotope tracers, which allow distinction between cortisol generated from cortisone from that directly secreted by the adrenal or infused, may allow dissection of whole-body 11HSD1 activity specifically but these are laborious techniques (Andrew et al 2002). Moreover, it appears that changes in 11HSD1 in

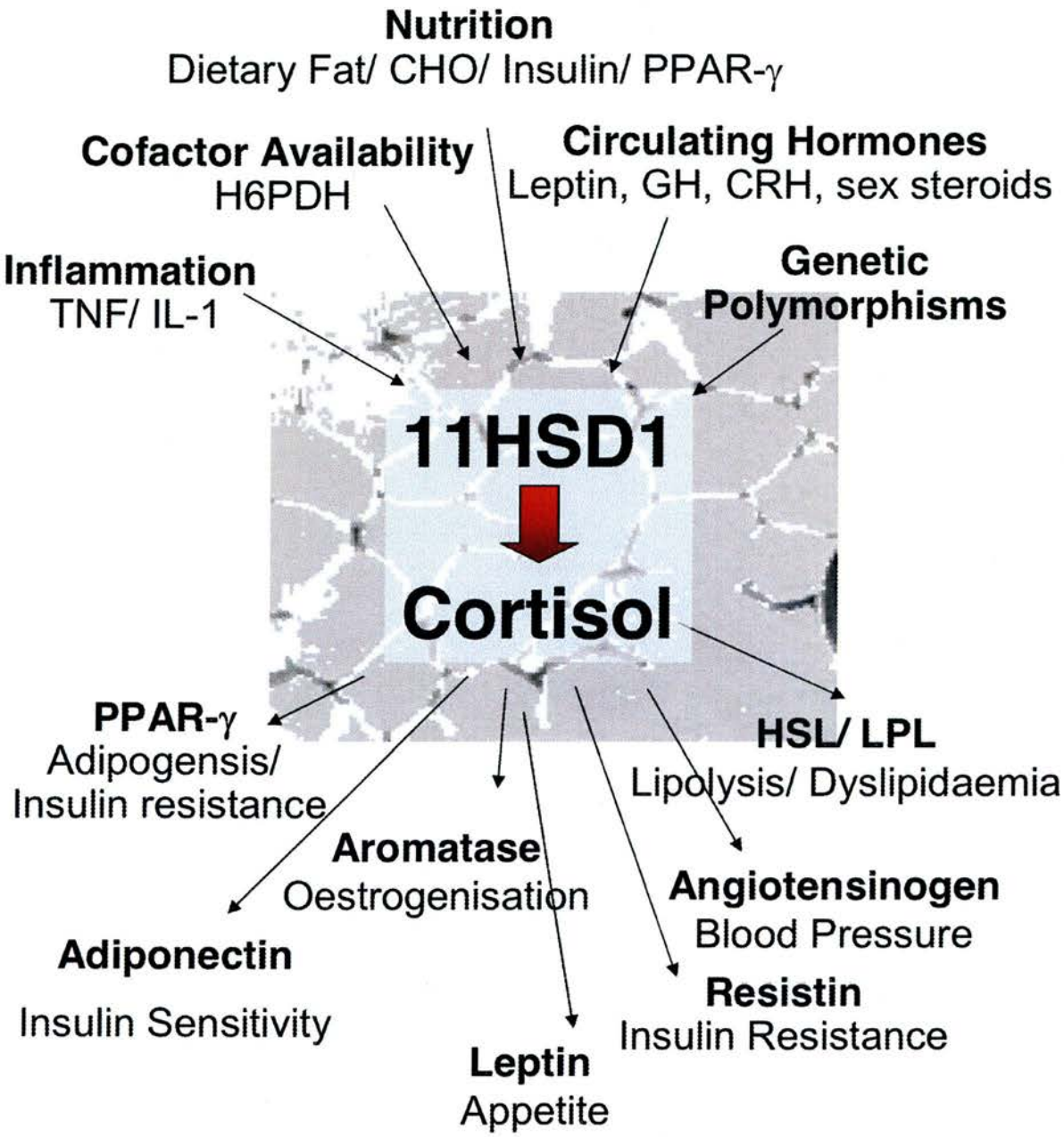

Figure 1.4

A model of the metabolic syndrome mediated by elevated adipose 11β-HSD type 1. Increased adipose 11β-HSD1 in obesity may be secondary to dysregulation by factors described above. Increased cortisol generation will result in activation of GR and downstream genes transcription. This may lead to a pro-atherogenic dysmetabolic phenotype resulting in increased cardiovascular disease

obesity are tissue-specific, so that measurement in isolated circulation (Basu et al 2004) or biopsied tissues may still be essential to understand dysregulation of 11HSD1 in human disease.

1.8.3 Defining tissue-specific changes

Investigation of the tissue-specific dysregulation of 11HSD1 in obesity began with studies of obesity prone, insulin resistant animal models (e.g. obese Zucker rats). These animals have tissue-specific alterations in 11HSD1, notably increased 11HSD1 in visceral fat and reduced 11HSD1 in hepatic tissue (Livingstone et al 2000). Reduced hepatic (Liu et al 2003) and increased adipose (Masuzaki et al 2001) 11HSD1 has also been reported in ob/ob mice. Similar tissue-specific dysregulation was subsequently demonstrated in human studies. Hepatic 11HSD1 activity is reduced in obesity, as judged by impaired conversion of cortisone to cortisol on first pass metabolism through the liver (Stewart et al 1999). This is accompanied by increased 11HSD1 activity in homogenates of sub-cutaneous adipose tissue (Rask et al 2001;Rask et al 2002).

On beginning the work for this thesis, a key question remained concerning whether adipose 11HSD1 upregulation is transcriptional, and the extent to which upregulation in obesity influences intra-adipose cortisol levels and the metabolic consequences of obesity. The model suggested by the Ap2-11HSD1 overexpressing animal is intriguing, but whether this models idiopathic human obesity remains uncertain (Masuzaki et al 2001).

1.8.4 11HSD1 in other insulin resistant syndromes

In addition to dysregulation in patients with idiopathic obesity, it is proposed that the central adiposity of growth hormone (GH) deficient patients and hypothalamic obesity may also be associated with changes in cortisol metabolism and 11HSD1 activation. 11HSD1 is inhibited by GH and IGF-1 in adipose tissue in vitro (Moore et al 1999;Tomlinson et al 2001). In vivo human studies in hypopituitarism and acromegaly suggest that this effect is important (Moore et al 1999;Trainer et al 2001) and have also shown lowering of cortisol:cortisone metabolite ratios by GH therapy in hypopituitary patients (Weaver et al 1994) and in

patients with idiopathic obesity (Tomlinson et al 2003). The latter, however, occurred without any changes in fat mass. As in studies of idiopathic obesity, the difficulty is in the interpretation of urinary cortisol:cortisone metabolite ratios as a reflection of potentially tissue-specific changes.

Abnormalities in cortisol metabolism also exist in patients with type 2 diabetes compared to matched controls, over and above any effect of obesity (Andrew et al 2002;Valsamakis et al 2004). These abnormalities include enhanced central and peripheral sensitivity to glucocorticoids and slight impairment of 11HSD1 activity in the liver, but no difference in adipose 11HSD1.

In polycystic ovary syndrome, urinary cortisol:cortisone metabolite ratios have been reported to be unchanged (Stewart et al 1990) or increased (Rodin et al 1994), but the confounding effect of obesity may account for this.

1.8.5 11HSD1 Genetics

Only a handful of patients with apparent congenital cortisone reductase deficiency (ACRD) exist. These individuals have a defect in cortisone to cortisol conversion that is now thought to be due to combined single allele mutations in 11HSD1 and hexose-6-phosphate dehydrogenase (see also section 1.9.3) (Draper et al 2003). No widespread common functional mutations in the coding region of the type 1 isozyme have been found, however, phenotype-genotype relationships exist with specific 11HSD1 intronic polymorphisms (Draper et al 2002), and polymorphisms in the 5' upstream sequence (Nair et al 2004;Franks et al 2004). The intronic polymorphisms described by Draper et al were associated with obesity related parameters such as waist:hip ratios and hypertension, whereas upstream SNPs in Pima Indians predicted type 2 diabetes, plasma insulin levels and insulin action independently of obesity, and may occur in areas of transcription factor binding sites. Many genetic studies are still ongoing and mutations in the promoter region of 11HSD1 are yet to be fully screened. However, given uncertainties over the specificity of urinary cortisol:cortisone metabolite ratios, linking genotype with phenotype of altered enzyme activity in vivo will be difficult.

1.9 Regulation of Cortisol Metabolism in Obesity

11HSD1 is a highly transcriptionally regulated gene with many transcription factor binding sites in its promoter region (including CEBP and HNF, SF1, AP1 and AP2) (Williams et al 2000). Various regulators have been identified in vitro including glucocorticoids, thyroid hormones, sex steroids, insulin, IGF-1, lipids, leptin, GH, PPAR ligands and cytokines, many of which are altered in obesity (Bujalska et al 1997;Berger et al 2001;Tomlinson et al 2001;Liu et al 2003) (figure 1.4). Here, I will focus on the role of nutritional factors, inflammation, and the potential role of cofactor generation on the regulation of 11HSD1 in human obesity.

1.9.1 Nutritional Regulation

The HPA axis has previously been shown to respond to FFAs, insulin, leptin and high fat feeding. Further studies now suggest that these effects may be secondary to changes in peripheral cortisol metabolism (Dallman et al 1993) and a potential key role for 11HSD1 in the adaptive response to nutritional signals is emerging. 11HSD1 mRNA is regulated in vitro by insulin (Napolitano et al 1998;Handoko et al 2000;Tomlinson et al 2001), and PPAR/ LXR agonists (Berger et al 2001;Stulnig et al 2002). Further, enzyme direction may potentially be controlled by change in glucose flux, as NADPH generation through hexose-6-phosphate dehydrogenase requires glucose-6-phospate as substrate (see section 1.9.3) (Draper et al 2003;Banhegyi et al 2004). In rodents, high fat feeding potently down-regulates hepatic and adipose 11HSD1. Further, 11HSD1 knock-out models resist diet induced visceral obesity (Morton et al 2004a).

Studies in humans have been limited. Weight loss has been shown to increase plasma cortisol/ cortisone ratios and elevate 11HSD1 mRNA in isolated adipocytes (Tomlinson et al 2004a), although this finding is not universal (Engeli et al 2004). 5% weight loss in obese men induced by starvation or very low calorie diet had contrasting effects, with only the latter normalising cortisol clearance but neither apparently affecting 11HSD1 (Johnstone et al 2004) (measured by urinary ratios). Whether changes in cortisol metabolism is due to specific dietary components, changes in weight or mediated through secondary factors such as insulin is unknown. Most recently our group has measured adipose 11HSD1 in man using in vivo intra-

adipose infusion of ^{3}H-cortisone by microdialysis, and shown that hyperinsulinaemia very acutely down-regulates its conversion to ^{3}H-cortisol by 11HSD1; most importantly, obese subjects resist this insulin-dependent down-regulation (Sandeep et al 2005). Dysregulation of adipose 11HSD1 in obesity may therefore potentially be mediated by altered response to nutritional factors.

1.9.2 Inflammatory Regulation

As described in section 1.3, idiopathic obesity is associated with a relative 'inflammatory state', and increases in plasma markers of inflammation such as CRP, IL-6, and IL-1β have been shown to predict the risk of progression to type 2 DM, the risk of subsequent diabetic complications (Streja et al 2003), and to independently predict cardiovascular disease risk (Ridker et al 2003). Anti-inflammatory agents including aspirin and salicylate compounds substantially improve insulin sensitivity in human and animal models (Yuan et al 2001;Hundal et al 2002). This is associated with altered expression of many adipose gene transcripts mediated through activation of NFκ-B and IKK-β signaling pathways. Further, over-expression of NFκ–B in adipose tissue results in an insulin resistant phenotype, which can be reversed by salicylate administration (Yuan et al 2001).

Activation of inflammatory pathways may mediate increased tissue cortisol generation by 11HSD1 in obesity, and a reduction in 11HSD1 may underlie the insulin sensitising effects of anti-inflammatory agents. In-vitro studies have shown that inflammatory cytokines (e.g TNF-α and IL–1) increase tissue 11HSD1 activity in many cell types *in vitro* (Handoko et al 2000;Cai et al 2001;Tomlinson et al 2001;Cooper et al 2001). *In vivo*, inflammatory conditions such as HIV lipodystrophy and TB are associated with increased adipose and hepatic 11HSD1 respectively (Baker et al 2000;Sutinen et al 2004). Further, inflammatory signaling pathways (e.g. NFκ-B) regulate activation of intracellular glucocorticoid receptors (Widen et al 2003) as seen in inflammatory conditions such as rheumatoid arthritis. In obesity, activation of both 11HSD1 and GR in adipose tissue and liver by inflammatory mediators may be detrimental, leading to increased glucocorticoid signaling resulting in an adverse metabolic (pro-atherogenic) phenotype.

1.9.3 11HSD1 regulation by cofactor availability

11HSD1 may also be regulated post-transcriptionally. Here the focus of attention is on the factors determining enzyme directionality. In tissue homogenates and some cultured cells 11HSD1 is a dehydrogenase, converting cortisol to cortisone (Bujalska et al 2002a). The reasons remain unclear, but a recent intriguing hypothesis has been proposed by Stewart and colleagues that the directionality is determined by co-factor availability. 11HSD1 relies on NADPH to function as a reductase (converting inactive 11-keto glucocorticoids to their active 11-hydroxy metabolites) (figure 1.3). Hexose-6–phosphate dehydrogenase (H6PDH) is closely associated with 11HSD1 on the inner endoplasmic reticulum and controls local NADPH availability (Stewart & Krozowski 1999). NADPH generated in this manner may be important for maintaining the predominant reductase activity seen in intact cells in vivo which is lost post homogenization or in culture. Further, alterations in H6PDH expression may be associated with change in 11HSD1 activity and enzyme directionality (Atanasov et al 2004;Banhegyi et al 2004). Combined mutations in H6PDH and 11HSD1 have recently been observed in apparent cortisone reductase deficiency (Draper et al 2002), a rare and hitherto poorly understood condition in which impaired regeneration of cortisol from cortisone and compensatory activation of the HPA axis results in ACTH-dependent adrenal androgen excess (Phillipou et al 1996;Jamieson et al 1999), although other studies suggest these mutations may exist in unaffected individuals (White 2005). Cofactor availability may be an important mechanism mediating acute changes in 11HSD1 activity. Potential regulators of H6PDH remain unknown but as glucose-6-phosphate is the primary substrate for H6PDH, nutritional state and cell glucose flux may be important.

1.10 Adipose 11HSD1: downstream effects

At a cellular level, glucocorticoids are known to be involved in differentiation and proliferation pathways, giving them a speculative role in tumourogenesis and embryological development (Rabbitt et al 2003). In vitro, glucocorticoids are required for the differentiation of stromal cells to mature adipocytes (Hauner et al 1987), and in vivo, 11HSD1 activation may regulate this process. Stromal cells contain 11HSD1 activity which is higher in omental than subcutaneous adipocytes

(Bujalska et al 1999). Commitment to adipogenesis is associated with a switch in 11HSD1 enzyme activity from dehydrogenase to oxoreductase (Bujalska et al 2002b), a process potentially mediated by cofactor availability through H6PDH. Activation of adipose 11HSD1 in obesity may therefore result in increased adipogenesis, thus perpetuating underlying obesity.

Complications of obesity include hypertension, dyslipidaemia and insulin resistance. A number of key genes controlling these metabolic processes are glucocorticoid regulated e.g. angiotensinogen, hormone sensitive lipases, aromatase, glucose transporter 4 and leptin. Increased intraadipose cortisol generation (by 11HSD1) and GR activation may therefore be important in mediating downstream metabolic effects in human obesity. The metabolic impact of altered adipose 11HSD1 is however best assessed by enzyme inhibition.

1.11 11HSD1 Inhibition

Various compounds have been shown to inhibit 11HSDs, but none until recently were selective, potent and drug-like. Liquorice based compounds such as glycyrrhetinic acid or carbenoxolone (previously used as an anti-ulcer treatment) inhibit 11HSDs but select poorly between the different isoforms (type 1 and type 2) (Monder et al 1989). Side effects as a result of 11HSD2 inhibition include hypertension, fluid retention and hypokalaemia. Many other non-specific inhibitors exist including alcohol, bioflavinoids, triterpenoids, polyphenols in cotton seed, grapefruit juice and tea, and environmental agents such as gossypol (Zhang & Wang 1997;Hult et al 1998). In view of the potential wide ranging benefits of type 1 enzyme inhibition, the search for more selective inhibitors of 11HSD1 has been hastening. Chenodeoxycholic acid is a selective inhibitor of the 11HSD type 1 but drug potency is poor (Diederich et al 2000). A new class of both potent and selective inhibitors of 11HSD type 1 is emerging called the arylsulfonamidothiazoles (Barf et al 2002). Also, insulin sensitizing thiazolidinediones (PPAR-γ agonists) may mediate their action in part through inhibition of adipose 11HSD1, an effect demonstrated in cultured adipocytes (Berger et al 2001). Likewise, the lipid lowering agent fenofibrate (a PPAR α agonist), inhibits 11HSD1 in hepatocytes (Hermanowski-

Vosatka et al 2000). A number of these compounds have been employed in in vitro and in vivo studies. The key target tissues examined so far are liver and adipose.

1.11.1 In vitro studies

11HSD1 inhibition in vitro can limit regeneration or synthesis of glucocorticoids and attenuate the biological activity of cortisone on adipose tissue e.g by limiting adipocyte differentiation (Bujalska et al 1999) and aromatase expression (Yang et al 1997).

1.11.2 In vivo studies

Treatment of lean and obese Zucker rats with carbenoxolone results in inhibition of 11HSD1 in the liver but not adipose tissue or skeletal muscle (Livingstone & Walker 2003). It had no effect on weight gain, food intake, or oral glucose tolerance but did result in changes in lipid profile (increased HDL cholesterol). The lack of effect on obesity and insulin sensitivity was attributed to the lack of adipose inhibition. Similar results have been obtained in humans. In healthy human volunteers carbenoxolone improve insulin sensitivity (Walker et al 1995) without a measurable increase in forearm glucose uptake (principally skeletal muscle) and so was attributed to enhanced hepatic insulin sensitivity. Reduced hepatic 11HSD1 activity may result in impaired hepatic gluconeogenesis as a result of modulation of key glucocorticoid responsive gluconeogenic enzymes (e.g. PEPCK). A further study in lean diet controlled type 2 diabetic patients and control healthy volunteers demonstrated reduced glucagon stimulated hepatic glucose production and glycogenolysis in diabetics and reduced total cholesterol in healthy volunteers upon carbenoxolone treatment (Andrews et al 2003), but no increase in glucose uptake. Further Sandeep et al recently showed no improvement in insulin sensitivity in obesity with carbenoxolone attributable to a lack of effect in adipose tissue (Sandeep et al 2005).

Arylsulfonamidothiazoles (Barf et al 2002) show encouraging results in animal studies, with lowering of blood glucose in diabetic mice. These effects seem to be mediated through inhibition of glucocorticoid-dependent expression of PEPCK and glucose-6-phosphate as a result of 11HSD1 inhibition in the liver (Alberts et al

2002;Alberts et al 2003). Hermanowski-Votsaka et al has since shown that selective 11HSD1 inhbition lowered body weight, insulin, fasting glucose, triglycerides, and cholesterol in diet-induced obese mice and lowered fasting glucose, insulin, glucagon, triglycerides, and free fatty acids, as well as improved glucose tolerance, in a mouse model of type 2 diabetes. In addition, inhibition of 11HSD1 slowed plaque progression in a murine model of atherosclerosis (ApoE knock out mice)(Hermanowski-Vosatka et al 2005).

What remains to be established for any human 11HSD1 inhibitors is whether their effects extend beyond the liver and include benefits in adipose tissue. This is especially important in obesity, where liver 11HSD1 levels are already low but adipose 11HSD1 is pathologically elevated. It is intuitive that inhibition of adipose 11HSD1 is therefore likely to give the most therapeutic benefit while any additional inhibition in liver may have limited value. The development of drugs with amphipathic or hydrophobic properties that facilitate sequestration in adipocytes may therefore be particularly useful.

1.11.3 Caveats regarding 11HSD1 inhibitors

11HSD1 is found in many other tissues (e.g. gonads, lung, immune cells, vascular tissues and bone) (Seckl & Walker 2001). Side effects of global inhibition could therefore be wide ranging. Reassuringly, 11HSD1-/- mice are healthy and live a full life span and reproduce normally (Morton et al 2001). Further they maintain an advantageous metabolic phenotype throughout life suggesting that compensatory mechanisms do not over-ride the effects of 11HSD1 loss. A more likely side effect of 11HSD1 inhibition is that of increased adrenal androgen production. This is seen in individuals with apparent cortisone reductase deficiency as a result of reduced peripheral cortisol production, leading to secondary activation of the HPA axis and subsequent activation of adrenal androgen pathways (Phillipov 1998). 11HSD1 inhibition may also affect the response to inflammation and infection. Glucocorticoids are widely known for their anti-inflammatory action, and circulating cytokines (e.g. TNF-α and IL-1) can increased tissue glucocorticoids (via HSD1) (Tomlinson et al 2001) thus limiting the inflammatory response. Further, 11HSD1 may be required for the differentiation of monocytes to macrophages (Thieringer et

al 2001). Altered response to inflammation and infection may therefore be a potential side effect of 11HSD1 inhibition. Ultimately, uncertainties of side effects of therapy can only be answered in trials of 11HSD1 inhibitors.

1. 12 11HSD1; Conclusions

The role of 11HSD1 as a potential therapeutic target in obesity and the associated 'metabolic syndrome' is attractive. Obesity is now reaching epidemic proportions in the western world and health resource implications are enormous. Lifestyle advice rarely results in long term weight reduction, and pharmacological targeting of pathways involved in metabolic consequences (i.e. insulin resistance, dyslipidaemia and hypertension) may be the best option to prevent the otherwise inevitable increased burden of cardiovascular disease. It seems unlikely that the majority of idiopathic obesity is due to an underlying protein or coding defect in 11HSD1, but rather that altered 11HSD1 expression is a result of abnormal gene regulation as a consequence of other metabolic disturbances in obesity. This does not however preclude the usefulness of 11HSD1 as a therapeutic target. Indeed, its physiological importance and tissue specific dysregulation in many common diseases make it a potentially important and novel therapeutic target. With the development of specific inhibitors, the real potential of 11HSD1 inhibition in the metabolic syndrome will soon be discovered.

1.13 Sex Steroids and Obesity

In addition to glucocorticoids, sex steroids (androgens and estrogens) play important roles in determining body fat mass and its distribution, as is evident from predisposition to 'central' ('abdominal' or 'android') obesity in men and 'peripheral' ('gynoid') obesity in women. In both sexes central adiposity is associated with adverse metabolic effects and increased cardiovascular risk (Despres et al 1990), and it has been suggested that it reflects a predominance of androgen action over that of estrogen (Bjorntorp 1997). However, relationships between circulating sex steroids and obesity are not simple. In women, central obesity is associated with increased plasma androgens or free androgen index (Korhonen et al 2003), but in men central adiposity is associated with reduced testosterone levels (Glass et al 1977;Strain et al

1982;Zumoff et al 1990) which rise on weight loss (Kaukua et al 2003). Further, testosterone replacement in mildly deficient men with type 2 diabetes has been reported to improve central obesity and glucose tolerance (Boyanov et al 2003). Moreover, circulating estrogen levels are positively associated with body mass index (BMI) in both post menopausal women (Soler et al 1989), men (Brind et al 1990;Vermeulen et al 2002) and children (Zumoff 1982;Garnett et al 2004). However, the incidence of obesity is higher among post-menopausal women and may be ameliorated by hormone replacement therapy (Tchernof et al 2000).

These conflicting findings may reflect the fact that blood levels do not accurately measure tissue sex steroid action, which as with glucocorticoids, is also controlled by receptor density and by local steroid metabolising enzymes. Estrogen receptors (ERα and ERβ)(Pedersen et al 2001;Dieudonne et al 2004), and androgen receptors (AR) are all present in human adipose tissue, and variations in regional AR (Joyner et al 2002) expression may explain preferential central fat accumulation. Obesity occurs in ER-α knockout mice (Couse & Korach 1999;Heine et al 2000) and in humans is associated with polymorphisms in AR and ER genes (Buemann et al 1997;Zitzmann et al 2003;van Rossum et al 2003;Di Blasio et al 2003;Okura et al 2003;van Rossum & Lamberts 2004). However, whether variations in sex steroid receptor expression in adipose tissue are important in determining variations in body fat and its distribution across the population is unknown.

Steroid metabolising enzymes control tissue sex steroid concentrations and hence ligand availability for intracellular receptors (summarised in Fig 1.5), (Belanger et al 2002), but their role in idiopathic human obesity remains largely unexplored. Intra-adipose aromatase modulates the conversion of androgens to estrogens (androstenedione to estrone, and testosterone to estradiol). Humans with a rare loss of function mutation in the aromatase gene (resulting in predominance of androgens over estrogens) have a metabolic syndrome, abdominal obesity and insulin resistance (Simpson 2000a). Aromatase knockout mice also have marked abdominal adiposity, hepatic steatosis, dyslipidaemia, hyperleptinaemia and hyperinsulinaemia (Jones et al 2000;Jones et al 2001). 5α–Reductases convert androgens (testosterone)

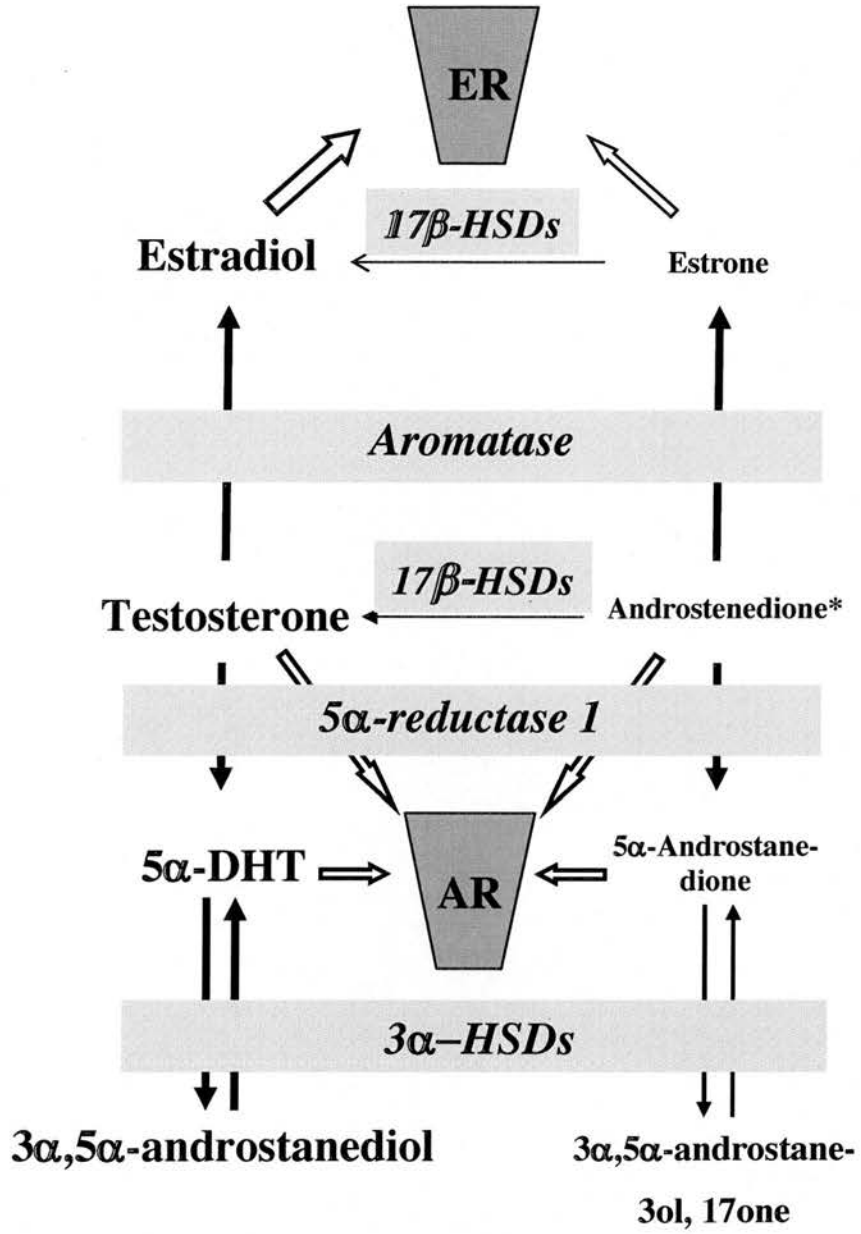

Figure 1.5

Schematic of Tissue Sex Steroid Metabolism

Summary of tissue estrogen and androgen steroid metabolism and receptor activation. Aldoketoreductase enzymes have varying degrees of 3α, 17β and 20α-HSD activity as discussed in sections 1.13 and chapter 6. 3αHSD = 3-alpha hydroxysteroid dehydrogenase, 5αDHT= 5 alpha dihdro-testosterone, 17β-HSDs= 17beta hydroxysteroid dehydrogenase, ER= estrogen receptor, AR= androgen receptor

to 5α-reduced metabolites (5α-dihydro-testosterone) which have increased potency at AR. 5α–Reductase activity is present in adipose (Killinger et al 1990) but the isozyme responsible and its role as a moderator of adiposity is unknown.

New interest is also emerging in the aldoketoreductase family of enzymes which have varying degrees of 3α, 20α and 17β-reductase activity and may therefore be key determinants of glucocorticoid, progesterone, estrogen and androgen metabolism (Penning et al 2000). One isoform, AKR1C type 1, has been studied in human sub-cutaneous and omental adipose tissue and its activity in omental adipose related to degree of central obesity (Blanchette et al 2005). The role of other AKRs in adipose tissue is unknown, but the main effects may be to regulate androgen activation or inactivation in addition to acting in concert with 5α-reductase in the inactivation of dihydro metabolites of pregnene steroids, including androgen, glucocorticoids and progestogens. Although this thesis concentrates on the role of glucococorticoids metabolism in obesity, the final chapter will assess aspects of sex steroid metabolism also.

1.13 Hypothesis and Aims

Whilst evidence for a role of altered glucocorticoid metabolism in obesity is strong, the factors mediating this and the impact on the metabolic phenotype is unclear. Further, many studies detailing regulation and downstream consequences have been performed either in animal models or *in vitro* in cell culture and the impact in idiopathic human obesity is uncertain. Against this background, I aimed to assess whether adipose 11HSD1 is transcriptionally upregulated in human obesity, to determine potential key regulators and to assess impact on downstream gene transcription and metabolic outcomes. In light of the evidence for the importance of tissue glucocorticoid metabolism in obesity, I finally explored whether a similar role exists for key enzymes metabolising sex steroids in adipose tissue. To address this broad agenda, I undertook studies with the following aims:

In **chapter three,** I analysed adipose biopsies from three separate healthy volunteer cohorts from Sweden, Finland and America to answer the following questions:

1. Is adipose 11HSD1 activity elevated in obesity and associated with changes in 11HSD1 mRNA?
2. Does adipose 11HSD1 activity predict tissue cortisol and cortisone levels?
3. Does elevated 11HSD1 activity regulate downstream target genes in adipose?
4. Does elevated adipose 11HSD1 activity predict downstream metabolic phenotypes e.g. degree of insulin sensitivity, hypertension and dyslipidaemia?

In **chapter four,** I described studies performed *in vivo* in healthy volunteers and *in vitro* in primary adipocytes to assess regulation of cortisol metabolism by insulin and lipid to determine:

1. Is adipose 11HSD1 acutely regulated in vivo by acute hyperinsulinaemia or hyperlipidaemia?
2. Is acute regulation of 11HSD1 secondary to changes in enzyme directionality?
3. Is whole body cortisol metabolism and clearance (via 5α- and 5β-Reductase) regulated by nutritional factors?
4. Is regulation of 11HSD1 by insulin/ glucose in vitro secondary to transcriptional or post-transcriptional changes and mediated primarily by glucose flux or insulin signalling?

In **chapter five,** I described studies performed *in vivo* in healthy volunteers to assess regulation of cortisol metabolism by PPAR agonists to determine:

1. Is adipose, liver and 'whole body' 11HSD1 regulated in vivo by PPAR-γ and PPAR-α agonists?
2. Is cortisol clearance regulated by PPAR agonists?

In **chapter six,** I explored the role of sex steroid as mediators of adiposity. Using adipose biopsies from previously described healthy volunteer cohorts (chapter 3), I assess mRNA expression of enzymes and receptors dictating sex steroid action in adipose to determine:

1. Do enzymes and receptors mediating oestrogen and testosterone action in adipose predict metabolic phenotypes, as suggested by genetic models?

2. Are there significant relationships between steroid metabolising enzymes/ receptors and potential regulators and downstream targets?

Finally, in **chapter seven,** I will draw conclusions from the above studies on the regulation, importance and downstream consequences of altered steroid metabolism in obesity.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated, chemicals and reagents were purchased from Sigma, Poole, UK.

All HPLC grade solvents were from Rathburn Chemicals, Walkerburn, UK.

All epi-steroid standards were from Steraloids, Newport, USA.

All radioactivity was from Amersham, Bucks, UK.

Deuterated cortisol tracer (d4F) was obtained from Cambridge Isotope Laboratories (Cambridge, MA).

Lipidex 5000 was obtained from Canberra Packard (Pangourne, UK).

Sep Pak C_{18} cartridges were obtained from Waters Corp (Glasgow, UK).

Sources other than these are indicated in parentheses.

2.2 Commonly used Buffers and Solutions

Acetate buffer: 0.2M sodium acetate, pH 4.6. Stored at 4°C.

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

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

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

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

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

Kreb's (KRB)Buffer: 118mM NaCl, 3.8mM KCL, 1.19mM KH_2PO_4, 2.54mM $CaCl_2$, 1.19mM $MgSO_4$, 25mM $NaHCO_3$ (bubbled with oxygen for 1 hr), pH 7.4. Stored at 4°C and supplemented with 0.2% glucose immediately prior to use.

KRH buffer: 118mM NaCl, 5mM $NaHCO_3$, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4.7H_20$, 25mM HEPES, 2.5mM $CaCl_2$

2.3 Introduction to Methods

All clinical measurements, sample collection and analyses (using the assays described in this chapter) were carried out personally by the author, after appropriate technical advice and training and with the assistance of Wellcome Trust Clinical Research Facility nursing staff. The only exceptions to this were; clinical measurements in Swedish, Finnish and American cohorts (chapter 3 and 6) (carried out in the centre of origin) and measurement of plasma biochemistry (section 2.6.1.) which was carried out in the clinical biochemistry laboratories (Western General Hospital, Edinburgh).

Most methods described were already in routine use within our research group. I therefore learned techniques from experienced individuals and adapted these for use in my studies. Some methods however had to be developed de novo and required considerable personal methodological development. These included i) needle adipose biopsy (2.4.2) ii) intra-adipose analysis of steroid measurements (2.7.3) iii) and mRNA analysis of human adipose. This included personally designing all real time primers and development of a real-time PCR assay for human adipose (2.7.2).

2.4 Clinical Procedures for Human Studies

2.4.1 Recruitment

2.4.1.1 Adipose Biopsy Analysis of overseas cohorts (chapters 3 and 6)

Recruitment, measurement of clinical parameters, and method of adipose biopsy is described in chapter 3, separately for each cohort.

2.4.1.2 In vivo regulation studies (chapters 4 and 5)

Male healthy volunteers between the ages of 20 and 70 were recruited locally, by newspaper advertisement, for in-vivo regulation studies. General criteria for inclusion were: normal thyroid, renal and hepatic function, alcohol intake <28units per week, and no glucocorticoid use in the previous 6 months. Each study had its own BMI criteria (see individual chapters). Local ethical approval (Lothian regional ethics committee) and written consent were obtained for each study. Clinical studies were carried out in the Wellcome trust clinical research facility, Western General Hospital, Edinburgh. If a volunteer 'dropped out' of the study, they were replaced to maintain numbers. Only individuals who had taken part in all study phases were included in the final analysis.

2.4.2 Needle Adipose Biopsy

Needle adipose biopsies were taken for investigation of regulation of cortisol metabolism. Skin laterally to the umbilicus was cleaned with Betadine (SSL, Knutsford, UK) and 5ml local anaesthetic (Lignocaine 1%,Braun UK) infiltrated locally. Sub-cutaneous adipose tissue was extracted using a 14G biopsy needle (Sterican, B. Braun, Melsungen, AG), attached to a sterile 60ml luer lock syringe to provide vacuum. The tissue was then washed with DEPC treated water prior to transfer to a sterile ependorf for immediate freezing on dry ice. This was then stored at -80^{0}C until further use.

2.4.3 Intra-adipose Microdialysis (figure 2.1)

In vivo abdominal sub-cutaneous adipose 11HSD1 activity was assessed using microdialysis (nutritional regulation studies- chapter 4 and 5). Volunteers attended between 0800 and 0900 after an overnight fast. A small area of skin approx

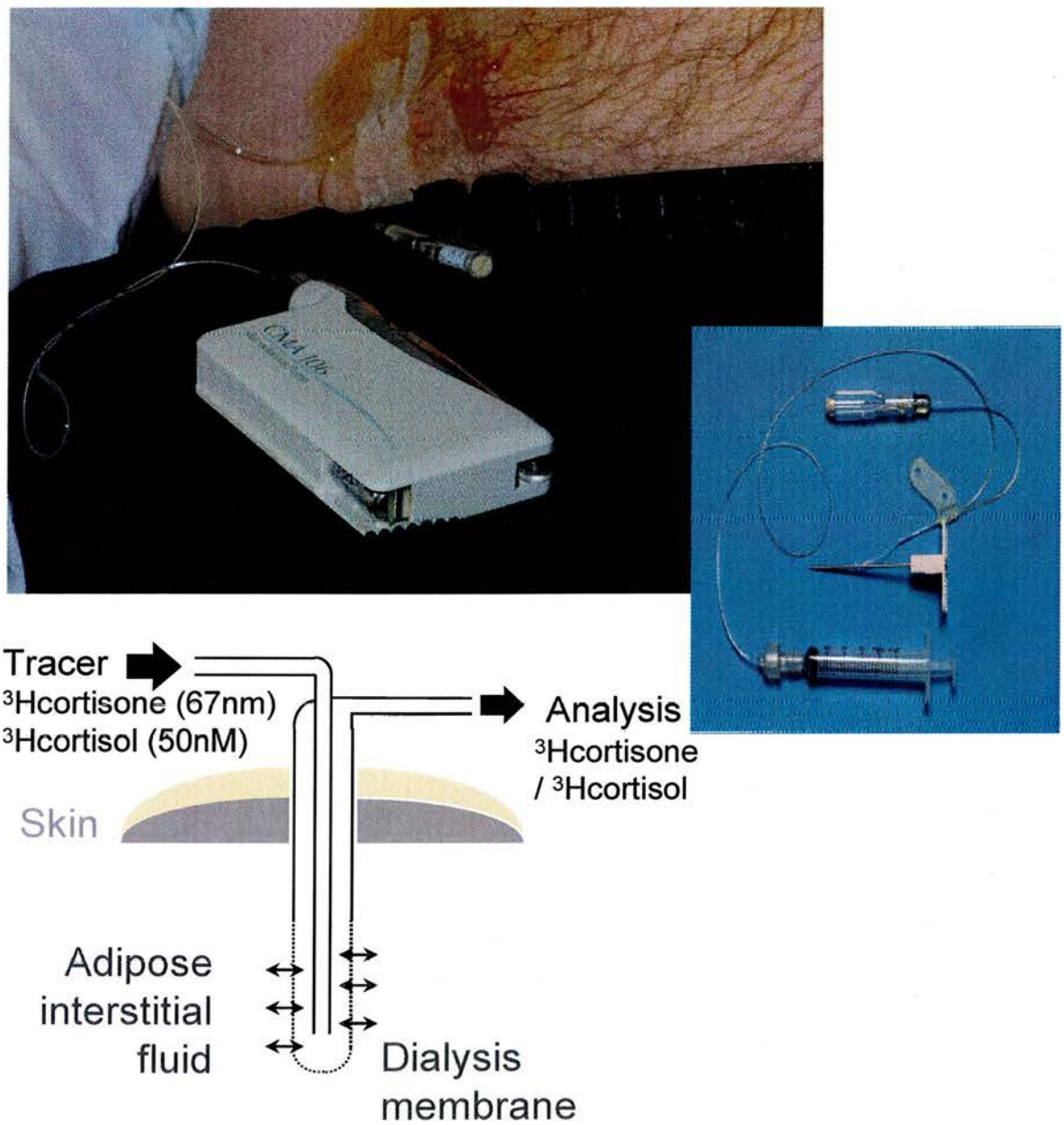

Figure 2.1

Intra-adipose Microdialysis. In vivo 11HSD1 reductase and dehydrogenase activities were assessed by intra-adipose microdialysis (equipment photographed above and diagram below). ^{3}H cortisol/ cortisone infused separately at a rate of 0.3µl/min. The effluent was collected every hour and analysed for % conversion.

10cm laterally to the umbilicus was cleaned with Betadine (SSL, Knutsford, UK) and 5ml local anaesthetic (Lignocaine 1%, Braun, UK) infiltrated locally. A CMA 60 microdialysis cannula (containing 20kD-permeable membranes) (CMA microdialysis, Sweden) was inserted into the subcutaneous adipose. ^{3}H –steroids were diluted in compound sodium lactate sterile solution (300kBq in 2.5ml) prior to infusion. For 11-ketoreductase (RED) and 11β-dehydrogenase (DH) activities, respectively, ^{3}H-cortisone (67 nM) or, in a separate cannula in the opposite side of the abdomen, ^{3}H-cortisol (50 nM) were infused (at 0.3 μl/min) using a CMA 106 microdialysis pump. The effluent was collected at hourly intervals into microvials and tracer cortisol and cortisone were quantified as described in (Sandeep et al 2005) and detailed in section 2.5 below.

2.4.5. Intra-venous (9,11,12,12-$^{2}H_4$) Cortisol Tracers

I was keen to avoid the pitfalls of previous work which relied on urinary metabolite ratios and ex vivo assays of glucocorticoid turnover as a true measure of 11HSD1 activity. In addition, I wanted to use a technique which would allow accurate and detailed assessment of very acute changes in enzyme regulation in vivo in humans. I therefore adapted an intravenous cortisol tracer technique which had been developed and validated by Andrew et al (Andrew et al 2002). Infusion of intravenous (9,11,12,12-$^{2}H_4$) cortisol (d4F) with subsequent measurement of generated d3E and d3F in the plasma allowed an in vivo assessment of cortisol turnover by 11HSDs (figure 2.2). The rate of appearance of d3F and tracer;tracee ratio give a measurement of whole body 11HSD1 activity. In addition, analysis of tracer glucocorticoid metabolites in the urine allows assessment of A-ring reductase activity.

Subjects attended between 0800 and 0900 after an overnight fast. Subjects were given cortisol/ (9,11,12,12-$^{2}H_4$) cortisol (d4F) [20 atoms percent (AP)] as a bolus injection (3.6mg total), followed by a constant infusion (20AP, 17.4mg/hr). Blood samples were withdrawn from the opposing arm at frequent intervals. Subjects provided a urine sample at baseline and then at intervals throughout the study (see chapter 4 and 5 for exact timings). Volunteers were given water to drink every hour.

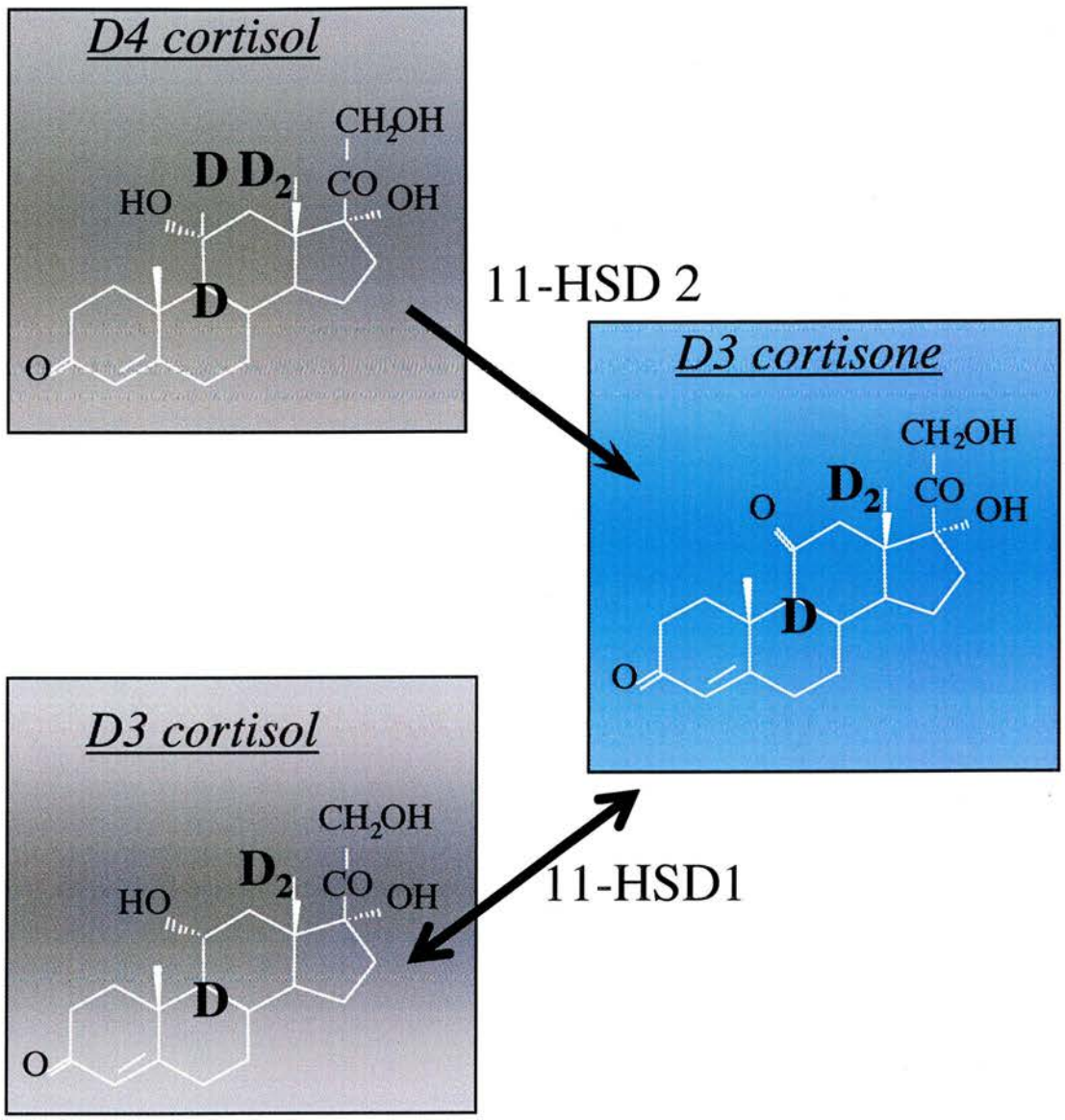

Figure 2.2

Stable Isotope Tracers for 11-HSDs in man

Schematic of metabolism of (9,11,12,12-2H4) cortisol tracer by 11HSDs

Blood samples were maintained on ice, centrifuged and serum extracted. Plasma and urine were stored at -20 °C pending analysis (see section 2.8).

2.4.6 *In vivo* Liver 11HSD1 activity (conversion of oral cortisone to plasma cortisol)

In vivo liver 11HSD1 activity was assessed by measuring conversion of cortisone to cortisol on first pass through the liver after overnight dexamethasone suppression. Volunteers attended fasted at 0900 having taken dexamethasone 1mg (Merck, Sharpe and Dohme Ltd, Herts, UK) at 2300 the evening before, and a venous cannula was inserted to allow repeated blood sampling. Oral cortisone acetate (25mg) (Pantheon UK Ltd, Swindon, UK) was given with water. Plasma samples were collected in lithium heparin tubes (2.6ml), centrifuged at 2380rpm for 10 mins at 4 °C and plasma aliquoted and frozen until analysis for serum cortisol. Samples were taken at time points: 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210 and 240 minutes after oral cortisone.

2.5 Microdialysis Analysis; Steroid Extraction and TLC

The volume of effluent from microdialysis microvials was assessed and made up to 250µl with Ringers Buffer. Ten times volume of ethyl acetate was added and the samples mixed and centrifuged at 1500rpm for 5 mins. The supernatant was removed and dried under oxygen free nitrogen (OFN) at 60 °C. Samples were diluted in 30µl of 1.67mg/ml solution of cold cortisol and cortisone and transferred to TLC plates (DC-Alufolien, Merck, Darmstadt, Germany). Cortisol and cortisone were separated by thin layer chromatography (TLC) over 1 hr using a solvent mix (92mls chloroform and 8mls 95% ethanol) . Cortisol and cortisone bands were identified and marked by viewing under UV. Individual bands were scraped into scintillation vials and ^{3}H-cortisone and ^{3}H-cortisol quantified by addition of 5mls scintillation fluid (Perkin Elmer, Boston, MA) and counting for 30mins in the scintillation (β) counter (Tri-Carb 2100TR, Liquid Scintillation Analyser, Packard, UK). Reductase and dehydrogenase activity was expressed as % conversion per h.

2.6 Plasma Assays

2.6.1. Plasma Cortisol

Plasma cortisol was assessed using ImmunChem cortisol ^{125}I RIA coated tube kit. This containing pre-prepared standards (0mg/dl to 100ug/dl), tubes with impregnated cortisol antibody and cortisol ^{125}I (1ml=approx 45,000cpm). 25ul of standard, control or plasma sample was pipetted into coated tubes. 1ml of cortisol ^{125}I was added to all tubes and vortexed. Samples were incubated for 45 mins at 37 °C. Tubes were placed in gripping racks, decanted and counted in a gamma counter calibrated for ^{125}I (Berthold LB2111, Leeds, UK). A standard curve was produced and sample values derived from the standard curve.

2.6.1. Plasma Insulin, Trigs, Glucose, Cholesterol, HDL, LDL, FFA, C-Peptide, U and Es

Enzyme immunoassays (Eurogenetics Tasah Corp. UK Ltd., Hampton, UK) were used to measure plasma insulin and C peptide. Electrolytes were measured with a Vitras 950 (Ortho Diagnostics, Raritan, NJ), and glucose was determined on a Cabas Mira Plus (Roche, Mannheim, Germany). Triglycerides, total cholesterol, and high density lipoprotein (HDL) cholesterol was measured using ELISA kits (TG, CHOL, and HDLC-plus respectively, Roche). Free fatty acids were measured by a colorimetric technique (Wako, Neuss, Germany).

2.7 Adipose Biopsy Analysis

2.7.1 In vitro 11HSD1 activity assay

2.7.1.1 Tissue preparation

Tissue was routinely frozen on dry ice directly after the adipose biopsy and stored at -80°C until use. Approx 250-500mg of adipose tissue was homogenised in 3ml of KREBs buffer. Homgenates were centrifuged at 300G at 4 °C for 5 min and the infranatant removed.

2.7.1.2 Protein estimation

Protein concentration of tissue homogenates was determined colorimetrically using a Bio-Rad protein assay kit (Bio-Rad, Hemel Hampstead, UK). A range of protein standards (0.1 – 1.2mg/ml) was prepared in distilled water from the provided protein standard. Protein assay dye reagent was prepared by

diluting the concentrate provided by 1 in 5 with distilled water. Diluted protein assay dye reagent (1.96ml) was added to 40ul of either protein standard or appropriately diluted sample in a borosilicate tube, vortexed and left at room temperature for 15 – 30 mins. The absorbance of the samples at λ =595nm was measured using a OPTIMAX tunable microplate reader and the concentration of protein in each sample was estimated from the standard curve.

2.7.1.3 In vitro 11HSD1 activity assay

In vivo, 11HSD1 functions as a predominant reductase (converting inactive cortisone to active cortisol). *In vitro* however, reductase activity is labile and dehydrogenase activity predominates in tissue homogenates. 11HSD1 activity was therefore quantified by the conversion of cortisol to cortisone. Aliquots of adipose homogenate at a concentration of 400ug/ml were incubated in duplicate at 37 °C in Krebs buffer containing 0.25% glucose, NADP (2mM) and [^{3}H]-cortisol (F) (100nM) in a total volume of 1ml. Blanks were prepared by incubating [^{3}H]-cortisol, NADP and buffer with no tissue added. After the incubation the reaction was stopped by the addition of 10 volumes of ethyl acetate (all solvents used were HPLC grade). The organic phase was removed and evaporated under oxygen free nitrogen at 60 °C. Extracts were re-suspended in mobile phase (65% methanol, 10% acetonitrile and 25% water) and stored at -20 °C until analysis by high pressure liquid chromatography.

This in vitro assay for 11HSD1 activity has been used widely in the published literature. In vitro, dehydrogenase activity is a more stable measurement than reductase and reflects protein levels well. We appreciate that no amount of measurement of in vitro enzyme activity in either direction can allow a true interpretation of the in vivo directionality. This issue can only be addressed by in situ measurements as illustrated by the in vivo microdialysis studies in chapters 4 and 5.

2.7.1.4 High Pressure Liquid Chromatography (figure 2.3)

A 100µl aliquot of each sample in mobile phase (65% methanol, 10% acetonitrile and 25% water) was injected into the high-pressure liquid chromatography system (HPLC). The HPLC comprises of a Waters (Herts, UK)

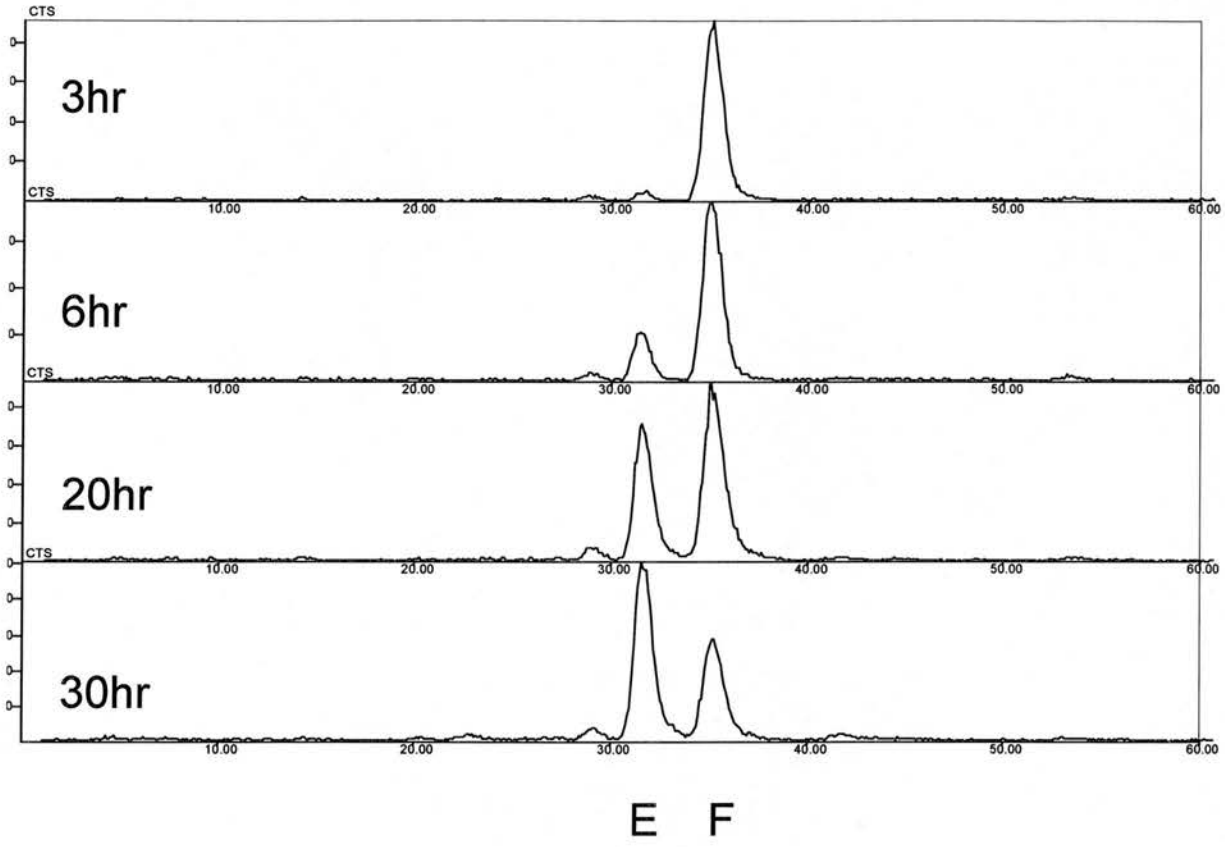

Figure 2.3

HPLC Trace for in vitro Adipose 11HSD1 activity assay

11HSD1 reductase activity was assessed in whole adipose biopsy homogenates after 3,6,20 and 30hr incubations. E (cortisone) and F (cortisol) were separated by HPLC (see figure). % conversion was calculated from area under curve of E and F peaks.

auto-injector, mobile-phase pump, reverse phase μ-Bondapak c18 column in a column heater and a Berthold (Leeds, UK) radioactivity monitor linked to a scintillation fluid pump. The system is controlled by a computer with Winflow software (JMBS Developments, France). The flow of mobile phase was set at 0.7ml/min and the flow rate of scintillant was set at 2.0ml/min. to achieve optimal mixing and efficiency of counting. The column heater was set at 35 °C to improve chromatography 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 (E) was approximately 32 mins, and for cortisol (F) approximately 36 mins. The area under the peak was integrated using the Winflow software, and the proportion of the area of each peak used to quantify the conversion of [^{3}H]-cortisol (F) to [^{3}H]-cortisone (E) and hence 11HSD1 activity. Routine blank incubations with no tissue were performed for each assay and the conversion in each sample was corrected for the apparent conversion in the blank.

2.7.2 mRNA quantification

2.7.2.1 RNA extraction

Tissue was routinely frozen on dry ice directly after the adipose biopsy and stored at -80°C until use. Approximately 250-500 mg of fat was homogenised in 1.5 ml Trizol (registered trademark of Gibco, UK). Samples were centrifuged at 12,000g for 1 min and the infranatant removed. Chloroform was added (250ml per 1ml of Trizol), samples vortexed and left on ice for 15mins. Samples were spun at 12,000rpm at 4 °C for 15mins, and the upper layer removed and transferred to a new sterile eppendorf. RNA was purified using by addition of 20μl of RNAid RNA binding matrix (Anachem, Luton, UK). Samples were washed 3 times in wash buffer matrix (Anachem, Luton, UK), and dissociated by addition of a solution of 89%DEPC H_20/ 10%DTT (10mM) /1%RNAsin (approx 20l). RNA was quantified using spectrophotometric analysis at OD_{260}. RNA was stored at -80°C until further use.

RNA integrity was checked by electropheresis on a 1.2% agarose/ TBE gel containing ethidium bromide 1μl per 50ml gel (figure 2.4a). The gel was made with ultra-pure water and gel tank equipment sterilized by soaking in sodium hydroxide.

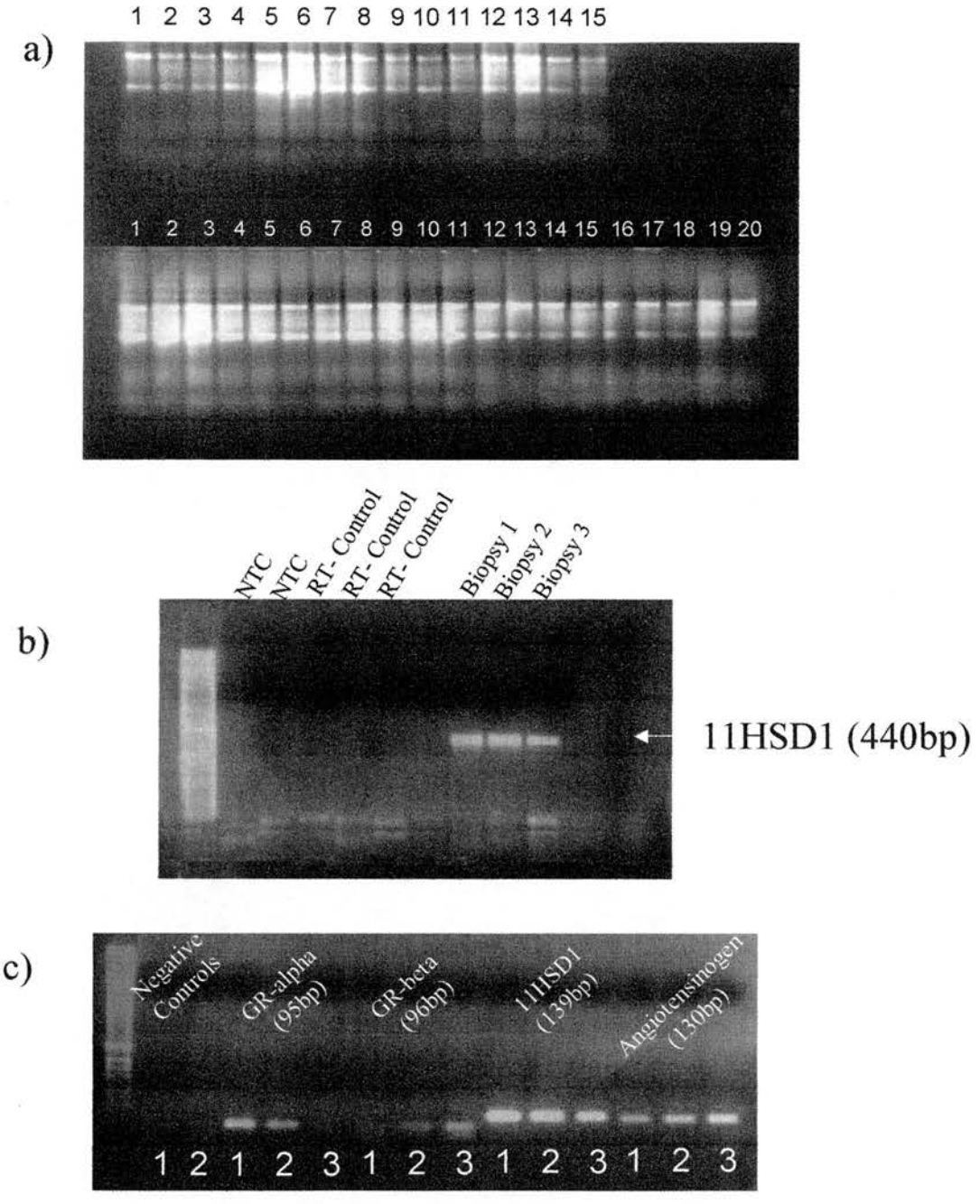

Figure 2.4

RNA/ PCR Gel photographs. a) Shows Adipose Biopsy RNA (1ug) from multiple samples run out on a 1.2% agarose/ TBE gel. b) Shows 11HSD1 mRNA amplification by PCR from human adipose, not present in control samples. c) Shows PCR amplification of adipose biopsy mRNA for GR-α, GR-β, 11HSD1 and Angiotensinogen to check cDNA synthesis and test specificity of primers designed for subsequent real time PCR. NTC= No template control

1μg of RNA with 2μl loading buffer was run at 100V fro 1-1.5hrs. The gel was viewed at 254 (uv) for the presence of clear ribosomal bands and lack of degradation.

2.7.2.2 cDNA synthesis

Oligo dT-primed cDNA was synthesized from 0.5 μg of RNA samples using Promega Reverse Transcription System (Promega, Southampton,UK). 0.5μg RNA was incubated with 5mM $MgCl_2$, 1x RT buffer (10mM Tris-HCL, 50mM KCl, 1% Triton X), 1mM dNTPs, 1u/μl RNase inhibitor, AMV RT (15u/μl) and Oligo(dT) 15 primers (0.5μg) in a total volume of 20μl (made up with NF-water) at 42 °C for 1hr, then 99 °C for 5 mins. For each batch of cDNA synthesis, additional samples (50% chosen at random) (0.5μg) were also incubated in the absence of reverse transcriptase (volume replaced with NF-water). These samples (called RT-) were used as controls in PCR and real time PCR to ensure that genomic DNA contamination (rather than cDNA) is not being amplified.

2.7.2.3 PCR

cDNA was diluted 1 in 4 with nuclease free water and cDNA synthesis checked by PCR using primers for either 11HSD1 or β_2-microglobulin.

11βHSD type 1 primers (440bp product): 5'AAAGTGATTGTCACWGGGAG CAAA-3' (forward) 5'-ATCCARAGCAAACTTGCTTGC-3'

β_2-microglobulin primer 5' - TGT CTT TCA GCA AGG ACT GGT C - 3' (forward) and 5'- TGA TGC TGC TTA CAT GTC TCG AT - 3' (reverse).

PCR was carried out using 5μl of cDNA template, 1x Taqman bead in 1x PCR buffer, $MgCl_2$ (1.5mM), dNTPs (200μM), forward primer (40pmol), reverse primer (40pmol) in a total volume of 50μl (made up with NF-water). (All PCR reagents Promega, Southampton, UK). 10μl of PCR products were mixed with 2μl loading buffer and run on a 1.75% agarose/ TBE gel with 1Kb DNA ladder (figure 2.4b).

PCR was also performed using specific primers (below) for the 4 human isoforms of AKR1C (chapter 6). PCR was performed at 20, 30 and 40 cycles (denaturing at 94 degrees for 2 mins, then for each cycle 45sec denaturing step at 94 degrees, a 45 sec annealing step at 60 degrees and a 2 minute extension step at 72

degrees) to assess comparative expression of the 4 isoforms and 500bp products run on a 1.25% agarose/ TBE gel (fig 6.2).

AKR1C1 5'-dGTAAAGCTTTAGAGGCCAC-3' (F),
5'-dCACCCATGCTTCTTCTCGG-3' (R),
AKR1C2 5'-dGTAAAGCTCTAGAGGCCGT-3'(F),
5'-dCACCCATGGTTCTTCTCGA-3' (R),
AKR1C3 5'-dGTAAAGCTTTGGAGGTCAC-3' (F),
5'-dCACCCATCGTTTGTCTCGT-3'(R),
AKR1C4 5'-dACAGAGCTGTAGAGGTCAC-3'(R),
5'-dCACCCATAGTTTATGTCGT-3'(R),

2.7.2.4 Real time PCR (mRNA quantification)

Transcript level quantification was performed with Real Time PCR primer-probe sets, using the ABI PRISM 7700/ 7900 Sequence Detection System (Applied Biosystems, Southampton, UK). Primers and probes were designed using *Primer Express* Software (Applied Biosystems, Southampton,UK) to meet the following specifications: Primer: Tm 58-60 °C, 20-80% GC, 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, must have more C's than G's. Gene sequences were obtained from PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or Ensembl Genome Browser (http://www.ensembl.org/). All primer/ probe sets were designed with at least one primer or probe spanning an intron, to prevent amplification of genomic DNA.

Primer Sequences (F=forward primer, R=reverse primer)
11HSD1 5'GGAATATTCAGTGTCCAGGGTCAA3'(F),
5'TGATCTCCAGGGCACATTCCT3' (R), and
5'-6-FAM-CTTGGCCTCATAGACACAGAAACAGCCA-TAMRA-3' (probe).
Aromatase 5'CTCATTATCAGCAAGTCCTCAAGTATGT3' (F),
5' GCACTGCAGCCCAAGTTTG3' (R),

5'-6-FAM-CCACATAATGAAGCACAATCATTACAGCTCTCG- TAMRA 3' (probe)

5α-Reductase Type 1 5' CGTTTTCTAATAGGTTTTGGCTTGT3' (F),
5'CCCTTGGTATTTTGTATCCAGTATCTC3' (R),
5'-6-FAM-ATAAACATCCATTCAGATCATATCCTAAGGAATCTCAGAA-TAMRA 3' (probe)

AKR1C2 5'TTCAGAGGAGATGAAAGCCATAGA3'(F),
5'CCTCCATGTTAATATTCATCAGAAAATG3'(R),
5'-6-FAM-TTGACCCTTGATATTTTTGCTGGCCCC-TAMRA3'(probe)

AKR1C3 5'CAGAGGACATGAAAGCCATAGATG3'(F),
5'CCCTCCATGTTAATATTCATCTGAA3'(R),
5'-6-FAM-CCACTATTTTAACAGTGATAGTTTTGCTAGCCACCCTA-TAMRA3'(probe)

GR-α 5'CATTGTCAAGAGGGAAGGAAACTC3'(F),
5'GATTTTCAACCACTTCATGCATAGAA 3'(R),
5'-6-FAM-TTTGTCAGTTGATAAAACCGCTGCCAGTTCT-TAMRA-3' (probe)

GR-β 5'CATTGTCAAGAGGGAAGGAAACTC3'(F),
5'TAACCACATAACATTTTCATGCAT AGAA T3'(R),
5'-6-FAM-TTTGTCAGTTGATAAAACCGCTGCCAGTTCT-TAMRA-3'(probe)

ER–α 5' AAGAGAAGTATTCAAGGACATAACGACTATAT3' (F),
5'CAGCTCTTCCTCCTGTTTTTATCAA3' (R),
5'-6-FAM-TGTCCAGCCACCAACCAGTGCAC-TAMRA 3' (probe)

AR 5' GCGATCCTTCACCAATGTCA3' (F),
5'GCATGCGGTACTCATTGAAAAC3' (R),
5'-6-FAM-CTCCAGGATGCTCTACTTCGCCCCTG-TAMRA3' (probe)

IL-6 5'CCTGAGAAAGGAGACATGTAACAAGA3' (F),
5'GGCAAGTCTCCTCATTGAATCC3' (R),
5'-6-FAM-AAACAACCTGAACCTTCCAAAGATGGCTGAA-TAMRA-3' (probe)

IL-1α 5'TGTCTCTGAATCAGAAATCCTTCTATCA3' (F),
5'GGATGTTTTAGAGGTTTCAGAGATACTCA3'(R),
5'-6-FAM-TGGCCCACTCCATGAAGGCTGC-TAMRA-3'(probe)

TNF-α 5'CCAGGCAGTCAGATCATCTTCTC3' (F),

5'TCAGCTTGAGGGTTTGCTACAA3'(R),
5'-6-FAM-CCCGAGTGACAAGCCTGTAGCCCA-TAMRA-3' (probe)
LPL 5'GAGACTCAGAAAAAGGTGATCTTCTGT3' (F),
5' AGACTTGTCATGGCATTTCACAAA3' (R),
5-6-FAM-CGCAGGTGCCTTTCCTTTCTGCAAA-TAMRA 3' (probe)
HSL 5'CTCAACTCCTTCCTGGAGTTAAGTG3' (F),
5'GGGCCAGTGCTGCTTCAG3' (R),
5-6-FAM-AGCCCATAGCAGAGCCGATGCG-TAMRA 3' (probe)
PPAR-γ 5' TGTCTCATAATGCCATCAGGTTTG3' (F),
5'GCTGGTCGATATCACTGGAGATC3', (R),
5-6-FAM-CCGCCAACAGCTTCTCCTTCTCGG-TAMRA3' (probe)
Angiotensinogen 5'TCTCCCCGGACCATCCA3'(F),
,5'TGCTCAATTTTTGCAGGTTCAG 3'(R),
5'-6-FAM-CCATGCCCCAACTGGTGCTGC-TAMRA3'(probe)
Leptin 5'CATTTCA CACACGCAGTCAGTCT3'(F),
5'TGTCTGGTCCATCTTGGATAAGGT3'(R),
5'-6-FAM-AACAGAAAGTCACCGGTTTGGACTTCATTCC-TAMRA3' (probe)
Resistin 5'CTAATATTTAGGGCAATAAGCAGCATT3'(F),
5'CACAGGCGGAGCCACAAG3'(R),
5'-6-FAM-CCCCCGAGGCTTCGCCGT-TAMRA3' (probe)
Adiponectin 5'TGACACCAACTGATCACCACTAACT3'(F),
5'CCGTACTGAAAGCCTTTAATTGACTT3'(R),
5'-6-FAM-CCTCCTCCAGGCCAAACAGCCC-TAMRA3'(probe)

cDNA was incubated with 1x Taqman mastermix (Applied Biosystems, Cheshire, UK), 25μM primers and 5μM probe (1μl cDNA per 10 μl, made up to volume with NF-water) and transferred to 384 well PCR plates with optical heat sealing cover (Applied Biosystems, Cheshire, UK) . These were centrifuged and transferred to ABI PRISM 7900 Sequence Detection for cycling/ detection as per manufacturers instructions. (50°C, 2 min; 95°C, 10 min; 95°C, 15 sec and 60°C, 1 min, repeated for 40 cycles) (see Figure 2.5 for amplification plots).

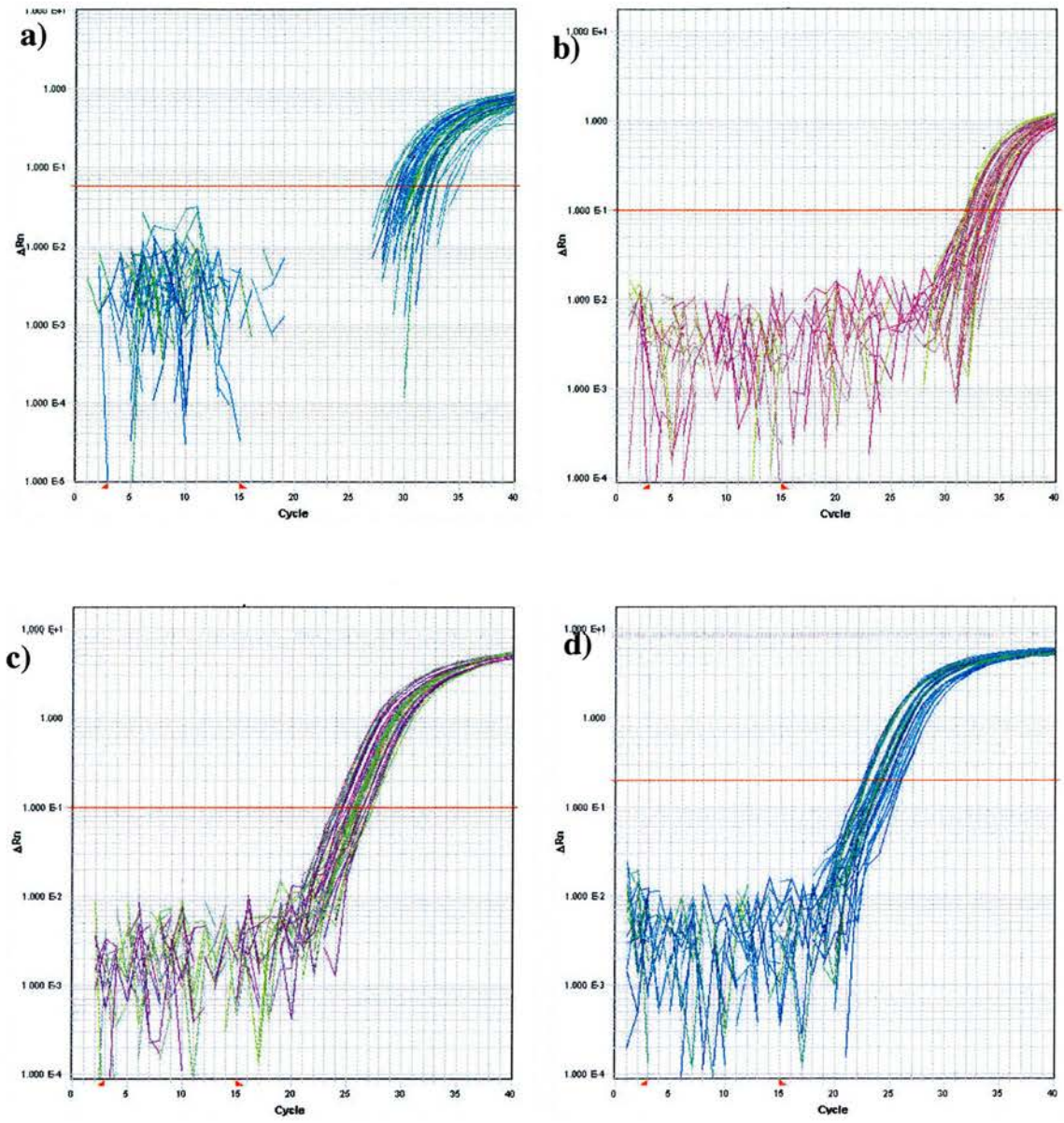

Figure 2.5

mRNA quantification using real time PCR. Amplification plots showing cycle number (ct) v's change in fluorescence (delta Rn) for adipose biopsy mRNAs (Finnish cohort, n=19 men). mRNAs shown are a) Cyclophilin (dye=VIC), b) 5-alpha reductase type 1 (dye=FAM), c) PPAR-γ (dye=FAM), and d) LPL (dye=FAM).

Human cyclophilin A primers/probes (20x) (purchased pre-made from Applied Biosystems, Cheshire, UK) were used to normalize the transcript levels. A standard curve for each primer probe set was generated in triplicate by serial dilution of cDNA pooled from several subjects. Each sample was run in duplicate and the mean values of the duplicates were used to calculate transcript level from the standard curve. The results are expressed as a ratio to cyclophyllin. RT negative controls and intron spanning primers were used to examine for genomic DNA and prevent amplification.

2.7.3 Intra-adipose cortisol/ cortisone levels

2.7.3.1 Steroid Extraction

Following homogenisation in Trizol, the infranatant from the RNA extraction protocol was used to extract steroids. Approximately 0.3 pmol/ml (<1% final tissue concentrations) of 1,2,6,7-$^{3}H_4$-cortisone and 1,2,6,7-$^{3}H_4$-cortisol (Amersham, Little Chalfont, UK) were added to the homogenate as internal standards to correct for steroid extraction efficiency. Samples was centrifuged (300g/ 5mins) to remove the lipid layer and extracted on a sep-pak (Waters (C18 cartridges), Watford, UK), further purified with hexane (1.5ml per sample), dried down and reconstituted in 400µl phosphate buffer and re-extracted with ethyl acetate (4ml). Samples were dried under OFN and stored at -20 °C for further use. Samples were reconstituted in 200µl ethanol; 15µl was removed for F ELISA, and 20µl for E RIA. These aliquots were dried down and reconstituted in 60µl charcoal stripped serum (F ELISA) and charcoal stripped serum/ buffer (Cortisone RIA)(see below). Cortisone and cortisol analysis using these methods produced similar results to RIAs following HPLC separation of cortisone and cortisol. Extraction efficiency for each sample was assessed by recovery of the ^{3}H-steroid. Steroid concentrations are expressed per g of wet weight of adipose tissue after adjustment for extraction efficiency. Steroid extraction efficiency was 28 ± 1% (mean±SEM) (Swedish Cohort), 61 ± 12% (mean±SEM) Pima Cohort.

2.7.3.2 Cortisol ELISA

Intra-adipose cortisol was assessed using a salivary cortisol enzyme immunoassay kit (Salimetrics LLC, State College PA). This contained an antibody coated plate, cortisol standards (0-1.8μg/dl), wash buffer (10x phosphate buffer containing detergents and a non-mercury preservative), diluted 1:10 with deionised water, assay dilutent (phosphate buffered solution containing a pH indicator and a non-mercurial preservative), an enzyme conjugate solution of cortisol labelled with horseradish peroxidase, a ready to use solution of Tetramethylbenzidine (TMB), a 'STOP' solution of sulphuric acid reconstituted in 12.5ml deionised water prior to use. 25μl of standards and samples was pipetted into appropriate wells. Standards and samples were assayed in duplicate. 25μl of assay diluent was used as a 'blank' and non-specific binding was assessed by addition of assay diluent to wells containing no antibody). A 1:1,600 dilution of the conjugate was made and mixed (15μl conjugate and 24ml assay dilutent). 200μl of diluted conjugate was added to all wells using a multi-channel pipette. The plate was mixed on a rotator for 5 mins and incubated at room temperature for 55mins. The plate was washed 4 times with 1x wash buffer using a mechanical plate washer. 200μl of TMB was added to each well using a multichannel pipette. The plate was mixed on a plate rotator for 5 mins and incubated in the dark for a further 25 mins. Finally, 50μl of STOP solution was added with a multi-channel pipette, and mixed for 3 mins on a plate rotator (500rpm). The plate was then read on a plate reader at 450nm (OPTIMAX tunable microplate reader) within 10 mins of adding the STOP solution.

The average optical density (OD) was calculated for each well. The average OD for the NSB was subtracted from the average OD of the zero, standards and unknown. The percentage bound for each standard was calculated by dividing the average OD (B) for the average OD for the zero (BO). A log-linear regression curve was calculated for the standards and results calculated from this.

This assay had good sensitivity and specificity for cortisol (minimum concentration of cortisol that can be distinguished from 0 is <0.007ug/dl. Cross reactivity with other endogenous glucocorticoids or sex steroids was low <1%. (19% cross reactivity with dexamethasone). Further assay details are available at www.salametrics .com.

2.7.3.3 Direct Cortisone RIA

Serum cortisone assays were purchased from Immunovation Ltd (Southampton, UK) containing antiserum and ^{125}I RIA. Buffer was made fresh prior to each assay by the addition of BSA (5g/L), sodium azide (1g/L) to Tris-HCl 50mmol/L at pH=7.5. Standards (0-500nmol/L) of cortisone in assay buffer were prepared in batches and frozen at -20 °C. Steroids were extracted as above and samples reconstituted in charcoal stripped serum. 450µl assay buffer was added to 50µl of samples and 400µl assay buffer with 50µl stripped serum added to 50µl of standards. Steroid were extracted using 5ml chloroform, the upper layer aspirated and duplicate 1ml aliquots taken into glass tubes and dried down under oxygen free nitrogen (OFN). To the dried extracts we added 300µl of Tracer (10nCi/300µl in assay buffer) and 100µl of antiserum N137 (in 500 dilution in assay buffer), then vortexed the samples and left them at room temperature for 2hrs. 100µl of Sac-Cel (Donkey anti-rabbit) Ab (Wellcome diagnostics, IDS, Boldon, UK) was added and the samples incubated for a further 30 mins at room temperature. Finally 1ml water was added, the samples centrifuged, the supernatant aspirated and the tubes counted in a gamma counter (Berthold LB2111, Leeds, UK). This assay was had good specificity for cortisone (cross reactivity with cortisol, cortisone, engogenous cortisol metabolites, all <0.1%.; cross reactivity with prednisolone= 20%).

2.8 Gas Chromatography/ mass spectrometry

2.8.1 Urinary steroid examination (endogenous steroid and tracer analysis)

2.8.1.1Preliminary work

Urine samples were collected from volunteers during cortisol tracer studies and as 24 hour collections. The total sample volume was noted and aliquots of 20ml frozen at -20°C for further analysis. Internal standards (epi-cortisol (epi-F) and epi-tetrahydrocortisol (epi-THF)) and standard curve ranges for α–THF, tetrahydrocortisone (THE), α-cortol, β-cortol, α-cortolone, and β-cortolone were prepared.

2.8.1.2 Extraction of steroids from urine

Internal standard (5μg Epi-F and 30μg Epi-THF) was added to urine (20ml) and standard curve samples before passing through a Sep-Pak C_{18} column [conditioned with methanol (5ml), then water (5ml)]. The column was washed with 5ml water and samples and eluted with 2ml methanol. Samples were dried under OFN.

2.8.1.3 Hydrolysis of steroids

Samples were incubated with 2ml 0.2M Sodium Acetate and 100μl β-Glucoronidase, mixed and incubated for 48 hours at 37 °C

2.8.1.4 Extraction of hydrolysate

Sep-Paks were reconditioned with 5ml methanol and 5ml H_20 and samples passed through, washed with 5ml water and eluted with 2ml methanol. Samples were dried under OFN. The dried layer was reconstituted in 200μl water and 3ml ethyl acetate and the organic layer removed and dried under OFN at 60 °C.

2.8.1.5 Derivatisation of steroids

50μl of 2% methoxyamine in dry pyridine was added and the samples evaporated to dryness at 60 °C. 50μl of trimethlsilylimidazole was added and samples incubated at 100°C for 2hrs.

2.8.1.6. Extraction of derivatised steroids

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

2.8.2 Plasma Steroid extraction (tracer studies)

2.8.2.1 Steroid extraction from plasma

Plasma (1.5ml) containing epi-cortisol (1μg) as an internal standard was shaken with chloroform (15ml). The upper layer (plasma residue) was removed with a Pasteur pipette, and the organic layer transferred to clean tube. The organic layer was evaporated to dryness under OFN at 60 °C in reactivials.

2.8.2.3 Derivatisation of steroids

Plasma steroids were derivatised by addition of 50μl 2% methoxyamine in dry pyridine to each sample. Vials were capped and heated at 60 °C for 30mins then evaporated to dryness under OFN at 60 °C. 50μl of trimethylsilylimidazole was added to each sample and incubated for 2hrs at 100 °C. Samples were passed through freshly prepared Lipidex columns as described above by addition of 1ml, followed by 2x 500μl mobile phase (cylohexane:hexamethyldisilazane:pyridine (98:1:1)). The eluant was collected and dried under OFN at 60 °C. Samples were reconstituted in 100μl decane and a 30μl aliquot transferred to GC vials for analysis.

2.8.3 Gas chromatography/ mass spectrometry

Analysis was performed using a 'Voyager', consisting of a Finnigan MD 800 Mass Spectrometer, GC 8000 Top Gas Chromatogram (GC/MS) using a DB17-MS column (15m, 0.25mm id, 0.25μm ft; J&W Scientific, Folsom, CA). The initial 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 min. Injection, source and interfact temperatures were 280, 200, 250 °C respectively. Ionization was performed in electron impact mode at 70eV. The following ions were monitored to allow analysis of methoximetrimethylsilyl (MO-TMS) derivative of endogenous steroid and the tracer and its metabolites: cortisol m/z 605; d4F, m/z 609; d3F m/z 608; cortisone m/z 531; d3 cortisone, m/z 534; 5β and 5αTHF m/z 652; d4-5β and 5αTHF m/z 656; d3-5β and 5αTHF m/z 655; THE, m/z 578; d3-THE, m/z 581 . Epi-cortisol (plasma and urine) and epi-tetrahydrocortisol (urine) were used as internal standards. Peaks of interest were quantified by the ratio of (area under the peak)/ (area under neighbouring internal standard). Ratios were compared against standard

curves for each steroid included in every assay batch. For tracer studies, the enrichment of total steroid with tracer was determined as the APE (i.e. the amount of tracer divided by the sum of the amount of tracer and tracee, expressed as a percentage), and this value was corrected for any naturally occurring m+3 and m+4 isotopic components (identified in analysis of samples taken before tracer infusion).

2.8.3.1 Gas chromatography/ mass spectrometry result analysis

i) Unlabelled endogenous steroid analysis

Cortisol, cortisone, THF, αTHF, THE, α-cortol, β-cortol, α-cortolone, β- and cortolone were measured in 24 hour urine collections for a number of studies (regulation of 11βHSD by PPAR agonists/ inflammatory cytokines; chapters 4 and 5). The balance between 11HSD activities in all tissues was assessed as the ratio of (5β-THF + 5α-THF)/THE. Renal 11β-HSD type 2 activity was assessed as urinary cortisol/cortisone ratio (Palermo et al 1996;Best & Walker 1997). The balance of 5α- and 5β-reductases was assessed by the ratio 5β-THF/5α-THF (Andrew et al 1998). Relative 5α- and 5β-reduction of cortisol was also assessed by Ulick's A-ring reduction quotients, 5α-THF/cortisol, 5β-THF/cortisol and 5β-THE/cortisone (Ulick et al 1992).

ii) Tracer sample analysis: in vivo kinetics (figure 2.2)

Cortisol and cortisone, 9,11,12,12-$^{2}H_4$ cortisol (d4F),9,12,12-$^{2}H_4$ cortisol (d3F) and 9,12,12 $^{2}H_4$ cortisone (d3E) were detected in plasma, and their major metabolites (unlabelled THF and αTHF, d4- and d3THF and αTHF, unlabelled THE and d3THE) were also measured in urine over the infusion period.

Rates of appearance of endogenous cortisol were calculated as [(rate of D4-cortisol infusion)/(D4-cortisol:cortisol ratio)]-(rate of infusion of cortisol)-(rate of infusion of D4-cortisol). Clearance of cortisol and of D4-cortisol were calculated as (infusion rate)/(steady state concentration). Rate of appearance of D3-cortisol was calculated as (rate of D4-cortisol infusion)/(D4-cortisol:D3-cortisol ratio).

Urine deuterated steroid excretion rates were calculated for each hour as (concentration of deuterated steroid) x (volume of urine) (Andrew et al 2002).

2.9 In Vitro Primary Cell Cultures (chapter 4; glucose/ insulin regulation)

2.9.1 Primary adipocyte culture preparation

Fresh epididymal adipose tissue was isolated from male wistar rats (age 8-11 months). This was transferred into sterile containers containing KRH buffer (with adenosine (0.2uM) and 1% bovine serum albumin (BSA) (RIA grade, fraction V, Sigma, UK) added) at 37 °C (approx 4ml KRH per g fat). Collagenase digestion was performed using 2mg collagenase (Worthington biochemical corporation, Lakewood, NJ) per 1ml buffer. The tissue was cut into tiny pieces using sterile scissors and the tissue incubated in a shaking water bath at 37 °C for 30minutes (shaken vigorously after 15mins). Tissue was filtered through mesh and washed in x3 in buffer (centrifuged at 800rpm for 8mins between washes). Adipocytes were removed with a syringe, transferred to a sterile container, and reconstituted in buffer to make an approximately 30% adipocyte solution. 300µl adipocyte solution was added to 200µl solution containing appropriate concentrations of glucose/ insulin/ and steroids (see chapter 4).

2.9.2 In vitro assessment of 11HSD1 dehydrogenase and reductase activities

To assess dehydrogenase and reductase activity in vitro, adipocytes were incubated in the presence of either corticosterone (B) or 11-dehydrocorticosterone (A) respectively (mixture of 25nM 'cold' steroid and 2.5nM ^{3}H- steroid). ^{3}H-A was made for this assay by incubating placental homogenate in KRB buffer in the presence of 25nM NAD and 50µl ^{3}H-B for 4hr at 37 °C. The purity of ^{3}H-A was assessed by HPLC and always >99%. At the end of the incubation period, adipocyte incubations were transferred to sterile Eppendorfs and spun at 13,000rpm for 1 min. The lower layer (buffer) was transferred to glass tubes, and steroids extracted using 1ml ethyl acetate (solvent layer transferred to a new tube and dried under OFN). Trizol (1ml) was added to the remaining adipocytes and frozen on dry ice for RNA extraction later. A and B were separated by TLC as discussed above (see section 2.4) using cold A and B standards rather than E and F.

2.9.3 Assessment of glucose uptake

1ml of a 30% adipocyte preparation was incubated for 15mins at 37 °C in glucose free KRH buffer. 10nM 'cold' 2-deoxy-glucose and 1:400 dilution of stock ^{3}H-2-deoxyglucose (1.59TBq/mmol/ 49.0Ci/mmol) was added and samples incubated for a further 3 minutes. 3 x 200µl aliquots were taken into 0.5ml eppendorf tubes and 200µl corning oil added. Samples were spun for 1 min at 13,000rpm and the cell pellet transferred to a scintillant vial with 1ml 1% Triton (Sigma, UK). After 30 mins, 3ml of scintillant (Perkin Elmer, Boston, MA) was added and samples counted in the β scintillation-counter.

2.9.4 Reagents for assessment of glucose/ insulin

Glucose was diluted in KRH buffer to make final assay concentrations of 5.6mM (termed 'normal' glucose) or 22.4mM (termed 'high' glucose). Human 'Actrapid' Insulin (Novo-Nordisk, Crawley, UK) was stored at 4^{0}C in a stock concentration of 500nM. This was diluted in buffer to give a final assay concentration of 5nM.

2.9.5 mRNA quantification

RNA extraction and mRNA quantification was carried out using real time PCR as discussed above. Primers and probes used for amplification of rat 11HSD1 were:

11HSD1 5'TCATAGACACAGAAACAGCTTTGAAA3'(F),
5'CTCCAGGGCGCATTCCT3' (R),
5'-6-FAM- CTGGGATAATCTTGAGTCAAGCTGCTCCC -TAMRA-3' (probe).
Transcript levels were normalised using cyclophilin:
CYLOPHILIN 5'CCCACCGTGTTCTTCGACAT3'(F),
5'GAAAGTTTTCTGCTGTCTTTGGAACT3' (R),
5'-6-VIC-CAAGGGCTCGCCATCAGCCGT-TAMRA-3' (probe).

2.10 Statistics

Statistical methodology for each study is discussed in the appropriate sections of chapters 3-6. Statistical analyses were carried out using STATISTICA software version 6.1 (Stat Soft Inc, Tulsa, *USA).*

Chapter 3

ADIPOSE GLUCOCORTICOID METABOLISM IN OBESITY AND THE METABOLIC SYNDROME

3.1 Introduction

The potential importance of 11HSD1 in obesity and the metabolic syndrome is illustrated by experiments in rodents. In obese Zucker rats (Livingstone et al 2000), 11HSD1 activity is increased selectively in adipose tissue and decreased in the liver. Selective over-expression of 11HSD1 in white adipose tissue under the AP2 promoter/enhancer results in central obesity, dyslipidaemia, and insulin resistance (Masuzaki et al 2001). Conversely, homozygous 11HSD1 'knockout' mice are protected from features of the metabolic syndrome (Kotelevtsev et al 1997).

Whether similar tissue-specific dysregulation of 11HSD1 occurs in human obesity has been controversial. Conversion of oral cortisone to cortisol on first pass metabolism in the liver is consistently impaired in obese individuals (Stewart et al 1999;Rask et al 2001;Virkamaki et al 2001;Rask et al 2002;Tiosano et al 2003), indicating impaired hepatic 11HSD1. More conventional measurements of in vivo 11HSD activities, such as urinary ratios of cortisol/cortisone metabolites, have been unhelpful in resolving this controversy (Andrew et al 1998;Stewart et al 1999;Fraser et al 1999;Reynolds et al 2001;Rask et al 2001;Rask et al 2002;Seppala-Lindroos et al 2002). There is further uncertainty concerning the impact of altered adipose 11HSD1 on downstream target genes and its role as a mediator of increased adiposity and the associated metabolic complications in humans. Intra-adipose metabolism of glucocorticoids may also be dependent on H6PDH (which provides cofactor for 11HSD1), and 5α- reductase which in addition to its role as an activator of androgens, metabolises glucocorticoids. 5α-Reduced steroids were traditionally considered inactive excretion products, but have now been shown to have activity at the Glucocorticoid Receptor (McInnes et al 2004).

In this chapter, we report investigations using subcutaneous adipose biopsies from three different cohorts from a) America (12 Caucasian (7 male, 5 female) and 19 Pima Indian (10 male, 9 female)), b) Finland (n=19 male) and c) Sweden (n=27, 14 male, 13 female) (Rask et al 2001;Rask et al 2002). Adipose biopsies were

analysed for 11HSD1 activity, 11HSD1, GR, H6PDH and 5α–reductase type 1mRNA, intra-adipose cortisol/ cortisone levels and glucocorticoid-dependent gene transcripts. Any difference in procedures from those described in chapter 2 is highlighted here.

The initial study on Pima Indian and Caucasian male volunteers addressed whether 11HSD1 is transcriptionally upregulated and altered by ethnicity. The Finnish study generated detailed measurements of adiposity by MRI and allowed a clearer assessment of the associations of 11HSD1 with body fat distribution. Finally, the Swedish study extended these observations into women and addressed the impact on downstream targets. Each of these studies is individually detailed below.

3.2 Subcutaneous Adipose 11β- Hydroxysteroid Dehydrogenase Type 1 Activity and mRNA levels; Association with Adiposity and Insulinaemia in Pima Indians and Caucasians

3.2.1 Introduction

In this study, I examined 11HSD1 activity, mRNA levels of 11HSD1 and GR and tissue cortisol and cortisone levels in subcutaneous adipose tissue biopsies from 12 Caucasian (7 male, 5 female) and 19 Pima Indian (10 male, 9 female) non-diabetic subjects aged 28 ± 7.6 years (mean ±SD, range 18-45). We investigated the cross sectional relationship of human adipose biopsy measurements to metabolic variables. We examined the hypotheses that i) 11HSD1 activity, mRNA levels and tissue cortisol levels are positively related to indices of adiposity, and ii) 11HSD1 activity, mRNA and tissue cortisol levels are positively related to indices of insulin resistance and hyperglycemia. We further assessed whether there are ethnic differences in 11HSD1 activity by examining these relationships in both Pima Indians, a group with marked propensity to both obesity and type 2 diabetes, and Caucasians.

3.2.2 Methods

3.2.2.1 Subjects

Pima and Caucasian volunteers were admitted to the metabolic ward of the Clinical Diabetes and Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (Phoenix, AZ). Subjects were recruited by newspaper advertisement or by community based recruiters. While subjects are not formally matched for adiposity, an effort was made to recruit Caucasian subjects across a similar range of adiposity as in the Pima group. All subjects were determined to be in good health by medical history, physical examination and laboratory screening tests. None was taking medication. Exclusion criteria included smoking, alcohol or drug abuse, and diabetes, according to a 75g oral glucose tolerance test (Paterson et al 2004). Upon admission subjects were placed on a weight maintenance diet (50% carbohydrate, 30% fat, 20% protein) calculated on the basis of body weight and adjusted to maintain body weight within ± 1%. Body composition, including % body fat (%fat) was assessed by dual energy x-ray absorptiometry (DPX-1: Lunar, Madison, WI)(Tataranni & Ravussin 1995). The circumference of the waist was measured supine at the level of the umbilicus. Fat biopsies were obtained after a 12 hour overnight fast, between 0830h and 1000h. Procedures for biopsy have been described previously (Rosmond et al 1998). In brief, subcutaneous abdominal adipose tissue was removed from the periumbilical region by percutaneous needle biopsy under local anaesthesia (lidocaine 1%). Biopsies were frozen immediately at –70°C.

The study was approved by the ethics committee of the National Institute of Diabetes and Digestive and Kidney Diseases and by the Tribal Council of the Gila River Indian Community. Subjects provided written informed consent.

3.2.2.2 *Analytical Measurements*

The plasma glucose concentration was measured by the glucose oxidase method (Beckman Glucose Analyser: Beckman, Fullerton CA) and insulin by radioimmunoassay (Concept 4: ICN Biomedicals, Costa Mesa, CA). Homeostasis Model Assessment Insulin Resistance Index (HOMA-IR) was calculated as previously described (Gorzelniak et al 2002).

3.2.2.3 Biopsy Analysis

11HSD1 activity, 11HSD1 and GRα mRNA and tissue cortisol and cortisone were analysed as described in chapter 2 (section 2.7). Recovery of tracer ^{3}H-steroid for tissue cortisol and cortisone measurements averaged 61 ± 12%.

3.2.2.4 Statistical Analysis

All values are represented as mean ± SD unless otherwise indicated. Statistical analyses were performed using the procedures of the SAS Institute (Cary, NC, USA). Relationships between 11HSD1 activity and adipose or metabolic variables were assessed either by Pearson correlation (using area under the curve for the 11HSD1 reaction) or, where multiple covariates were assessed, by general linear modeling. Throughout this chapter, where data were not normally distributed, these were log transformed and distribution plots re-assessed to ensure a normal distribution had been achieved. Where log transformation has been performed this is indicated in the relevant tables (e.g. fasting insulin, 11HSD1 mRNA and tissue cortisol). Where 11HSD1 activity is modeled as the dependent variable, separate time points of the 11HSD1 assay (3, 6, 20, 30 hours) were fitted in a repeated measures design.

3.2.3 Results

3.2.3.1. Baseline characteristics and relation of adipose 11HSD1 activity to mRNA levels and tissue cortisol

Measures of adiposity (BMI, %fat, waist circumference), fasting plasma glucose and insulin were comparable between the Pima and Caucasian groups (Table 3.1). Neither fasting plasma insulin nor HOMA-IR differed between Pimas and Caucasians even after adjustment for sex, and %fat or BMI (data not shown). Ten subjects (Caucasian 2 males, 1 female; Pima 3 males, 4 females) had impaired glucose tolerance (IGT)(World Health Organisation 2004). All other subjects were normal glucose tolerant (NGT). While glucose at 2 hours was higher in those with IGT, other baseline variables were similar (%fat, BMI, waist, all P>0.5 vs. NGT) including fasting glucose (NGT 4.79±0.39 mmol/l; IGT 5.06±0.61 mmol/l; P=0.2).

11HSD1 activity (area under curve for adipose biopsies at 3, 6, 20 and 30 hours of incubation) was significantly associated with mRNA levels (Figure 3.1: r=

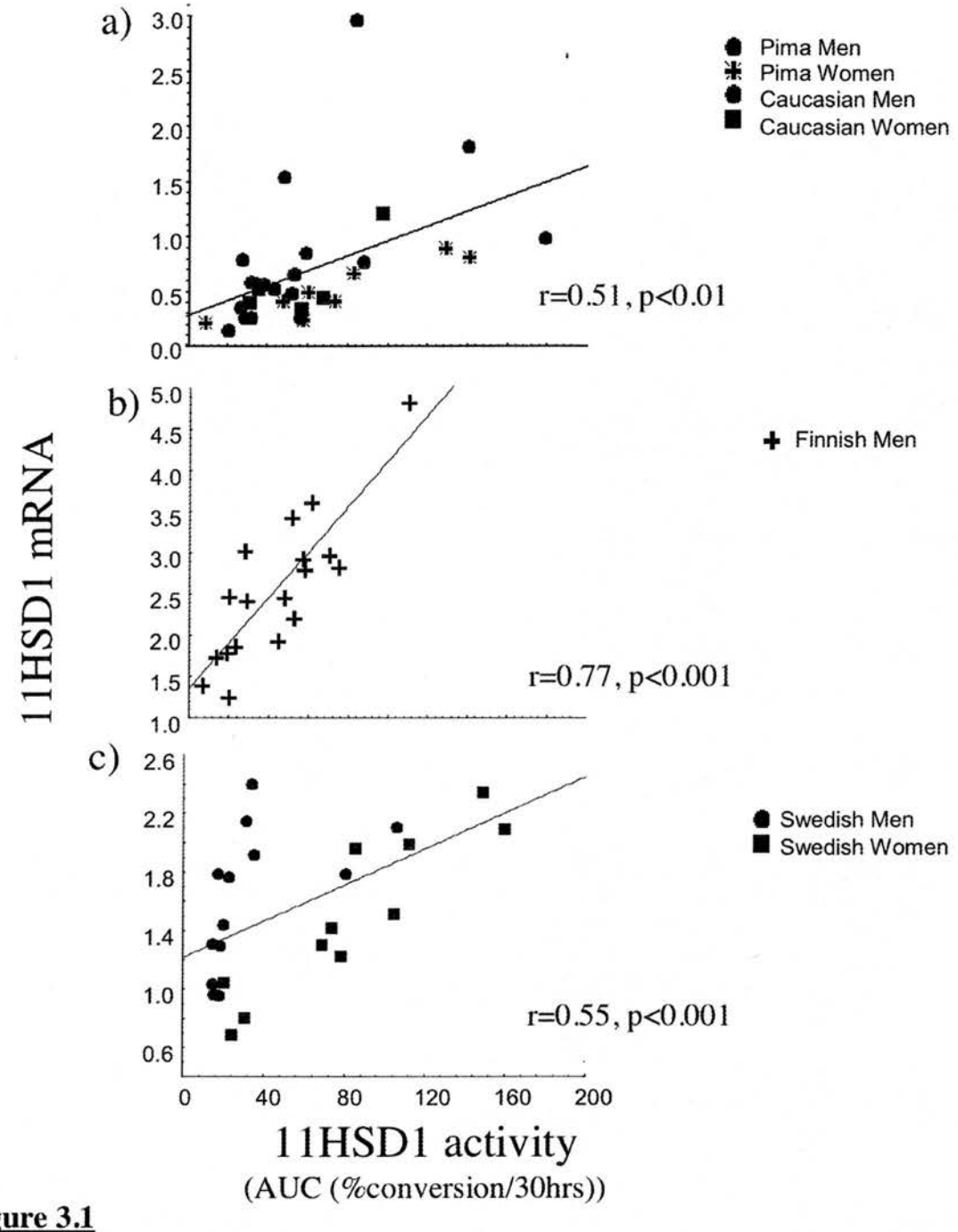

Figure 3.1

Associations between 11HSD1 activity and mRNA

11HSD1 mRNA levels and in-vitro activity correlate in subcutaneous adipose biopsies in a)American, b) Finnish, c) Swedish Cohorts. 11HSD1 mRNA levels were analysed by real time PCR and expressed as a ratio against cyclophyllin mRNA. 11HSD1 activity is calculated as area under the curve of % conversion of cortisol to cortisone after incubation for 3, 6, 20 and 30 h.

0.51, P=0.003). This relationship was consistent across all of time points of the activity assay (3, 6, 20, 30 hours; r=0.47, 0.49, 0.56, 0.60, respectively; P<0.01 for all). When considered separately the relation of 11-HSD1 activity and mRNA levels was present in both Pima Indians (r= 0.58, P=0.01) and Caucasians (r= 0.69, P=0.02).

Intra-adipose concentrations of cortisol (median[inter-quartile range]: 14.2[9.4-25.8] nmol/kg) and cortisone (14.8 [5.0-21.4]nmol/kg) were similar (ratio of intra-adipose cortisol: cortisone 1.4[0.5-2.9]). There were no significant relationships of 11-HSD1 activity (r=0.13, P=0.49) or mRNA (r=0.30, P=0.11) to intra-adipose cortisol. Intra-adipose cortisol: cortisone ratio showed no relationship to 11HSD1 activity or mRNA (assessed by Spearman correlation r=0.07 and -0.05, respectively, P>0.5 for both).

3.2.3.2 Relationship of adipose 11HSD1 to obesity and metabolic variables

In all subjects combined, 11HSD1 activity was significantly associated with BMI, %fat, waist circumference, fasting plasma glucose, fasting plasma insulin and HOMA-IR (Table 3.2, Figure 3.2). When Pima Indians and Caucasians were considered separately, positive associations between 11HSD1 activity and BMI, %fat, waist, fasting plasma glucose, fasting plasma insulin and HOMA-IR were all present (with r >0.5 and P<0.05) although this was not significant for %fat (r=0.52, P=0.09) and glucose (r=0.43, P=0.18) in the isolated Caucasian group. In general, similar but somewhat weaker relationships were observed with adipose 11HSD1 mRNA (Table 3.2). To dissect confounding effects in these relationships, 11HSD1 activity was modeled against continuous variables by repeated ANOVA. Either fasting insulin (P=0.02) or HOMA-IR (P=0.0002) remained significant predictors of 11HSD1 activity in models also including %fat, sex, ethnicity (Pima vs Caucasian) and waist circumference. Similarly either fasting insulin (P=0.02) or HOMA-IR (P=0.01) were significant predictors of 11HSD1 mRNA (in models also including %fat, sex, ethnicity and waist circumference). By contrast, in these models, measures of adiposity (%fat or waist circumference) were not significant predictors of 11HSD1 activity or mRNA (P>0.2 for all), indicating that the stronger relationships were with insulin sensitivity rather than adiposity.

Table 3.1: American Cohort; Subject Characteristics

	Pima	Caucasian	P
N (male, female)	19 (10,9)	12 (7,5)	
Age (years)	29.5 ± 7.4	27.6± 8.1	0.51
BMI (kg/m^2)	34.6± 6.7	36.6± 8.6	0.47
%fat	35.5± 5.6	34.1± 7.7	0.57
Waist (cm) (n=17/10)	43.3± 3.9	42.6± 5.7	0.70
Fasting glucose (mg/dl)	90± 9	87± 7	0.41
2 hour glucose (mg/dl)	126± 27	134± 30	0.46
Fasting insulin (mU/l)	45 [38-52]	38 [32-51]	0.40
11HSD1 activity (%.hours) $^{\alpha}$	464± 345	496± 257	0.78
11HSD1 mRNA$^{\beta}$	0.56 [0.40-0.94]	0.41 [0.24-0.60]	0.07
Tissue cortisol (nmol/kg)	14.2 [9.3-22.4]	16.0 [10.7-43.4]	0.54
Tissue cortisone (nmol/kg)	15.3 [7.9-26.4]	10.2 [4.4-20.7]	0.50

Characteristics of Pima and Caucasian volunteers. Data are expressed as mean± SD or median [inter quartile range]. P= unpaired t test or Wilcoxon respectively.

$^{\alpha}$ 11HSD1 activity- Area under curve for conversion cortisol to cortisone at 3,6,20 and 30 hours.

$^{\beta}$ expressed as ratio of 11HSD1 to cyclophilin.

Table 3.2: American Cohort; Correlations of 11-HSD1 activity and mRNA *vs.* metabolic variables

	Activity	mRNA	Adipose Cortisol
BMI	**0.68**	**0.34**	*0.29*
%fat	**0.48**	*0.15*	*0.22*
waist (n=27)	**0.52**	*0.26*	*0.21*
Fasting glucose	**0.43**	*0.19*	*-0.28*
2 hour glucose	*0.08*	*0.09*	*-0.19*
Fasting insulin	**0.60**	**0.42**	**0.37**
HOMA-IR	**0.70**	**0.46**	*0.30*

Pearson correlation (*r*) of adipose biopsy 11HSD1 activity (expressed as area under curve for bioassay at 3,6,20 and 30 hours) or mRNA (normalized to cyclophilin as reference standard) *vs.* metabolic variables with adjustment for sex. Values in bold are significant at P<0.05.

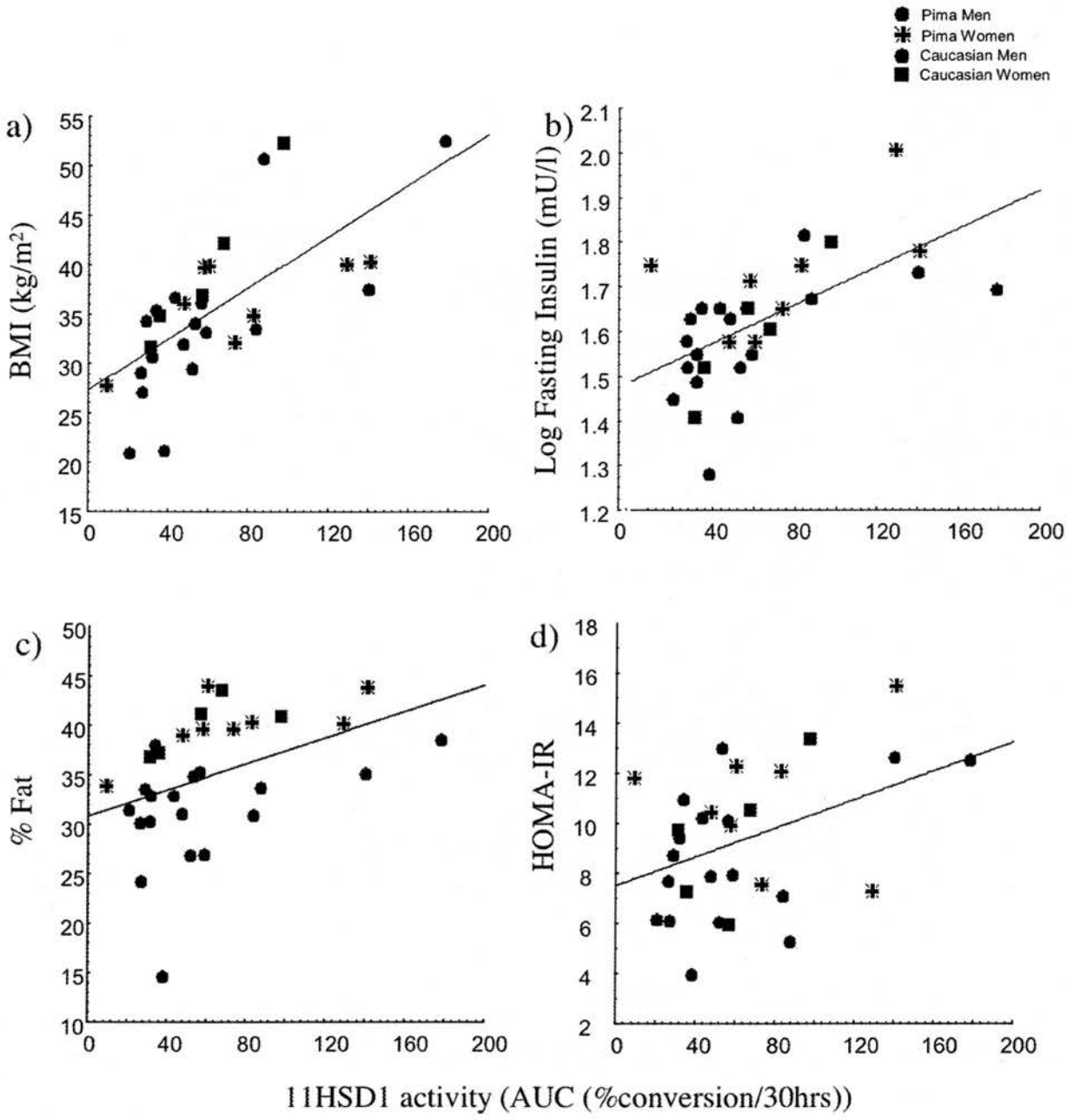

Figure 3.2

American Cohort; Adiposity and metabolic variables *vs.* 11HSD1 activity

Relationship of 11-HSD1 activity to metabolic variables in 31 subjects (see also Table 3.2). 11-HSD1 activity is defined as the area under the curve for bioassay of 11-HSD1 adipose biopsies at 3,6,20 and 30 hours of incubation.

Ethnicity was not significantly related to 11HSD1 activity as a single predictor (P=0.53) or in the above models. Overall Caucasians tended to have higher 11-HSD1 mRNA levels as a single predictor (Table 3.1, P=0.07) and this was significant in the above models (P=0.0007 in model with sex, %fat, waist and fasting insulin; P=0.0005 in model with sex, %fat, waist and HOMA-IR).

No difference in 11HSD1 activity or mRNA was found in individuals with IGT vs. NGT (P=0.71) even after adjustment for other covariates (sex, %fat, ethnicity; P for effect of glucose tolerance status =0.68). In multivariate models fasting glucose was not related to mRNA or 11HSD-1 (data not shown).

Gender was not related to 11HSD1 activity or mRNA either as a single predictor (P=0.73, P=0.11 respectively) or after inclusion of %fat, fasting insulin or HOMA-IR as additional predictors.

3.2.3.3. Relation of intra-adipose cortisol and cortisone to obesity and metabolic variables

Correlations between intra-adipose cortisol and anthropometric or metabolic variables were generally positive, but only significant in the case of fasting insulin (Table 3.2). Intra-adipose cortisol was not significantly related to fasting insulin or HOMA-IR in multivariate models (including %fat , gender and waist). Neither intra-adipose cortisone nor cortisol:cortisone ratios correlated with anthropometric or metabolic variables (data not shown). There were no ethnic differences in intra-adipose cortisol or cortisone levels.

3.2.3.3. Relation of GRα to obesity and metabolic variables

GRα mRNA was not significantly associated with central (WHR =-0.25) or generalised (BMI=-0.07, %Fat=-0.18) obesity.

3.3. Body Fat Distribution and Cortisol Metabolism in Healthy Finnish Men

3.3.1 Introduction

This study addresses in detail the relationships between cortisol metabolism and body fat distribution, in a group of healthy men in whom body fat distribution and associated metabolic parameters have been carefully characterised, and include assessment of liver fat content by magnetic resonance imaging (MRI) spectroscopy and visceral fat mass by cross-sectional MRI. Accumulation of triglyceride stores in selected adipose and extra-adipose sites is recognised increasingly as a determinant of insulin sensitivity and its attendant cardiovascular risk. Metabolically adverse sites for fat accumulation include visceral fat, skeletal muscle and liver (Bjorntorp 1991;Virkamaki et al 2001;Seppala-Lindroos et al 2002). Glucocorticoid excess (as in Cushing's syndrome), results in accumulation of fat in selected adipose tissue depots, especially in the face, nape of the neck, and visceral compartments (Ross & Linch 1982). Anecdotal reports also suggest that glucocorticoid excess drives triglyceride accumulation within the liver (Itoh et al 1997;Nanki et al 1999;Dourakis et al 2002). In this study we assess intra-adipose glucocorticoid metabolism, by assessment of 11HSD1 activity and mRNAs for 11HSD1, GR, H6PDH, 5α-Reductase Type 1 and downstream target genes (LPL, HSL, PPAR-γ, adiponectin, resistin, angiotensinogen, aromatase and leptin) in subcutaneous adipose biopsies. We related these measurements to anthropometric parameters of obesity and insulin sensitivity.

3.3.2 Methods

3.3.2.1 Subjects

Men were recruited from occupational health services in Helsinki. The subjects did not use any drugs and were healthy as judged by history and physical examination, negative serology (for hepatitis A, B or C, or of autoimmune hepatitis), and the absence of clinical features of inborn errors of metabolism or a history of use of toxins or drugs associated with steatosis, except for moderate alcohol consumpation (<20 g/day). Alcohol consumption was assessed by detailed history and laboratory markers [serum γ-glutamyltranspeptidase, the aspartate

aminotransferase (AST)/alanine aminotransferase (ALT) ratio, and mean corpuscular volume]. Written informed consent was obtained. The experimental protocol was approved by the ethical committee of the Helsinki University Hospital.

3.3.2.2 Body composition

Height, weight, waist and hip circumferences, and whole body composition by bioimpedance plethysmography (Bio-Electrical Impedance Analysis System, Model #BIA-101A, RJL Systems,MI) were measured after overnight fasting as previously described (Tukkainen et al 2002). In addition, body fat distribution was measured in the liver by MRI proton spectroscopy using methylene signal intensity and in the abdomen by cross-sectional MRI using data from sixteen 10 mm slices (Tukkainen et al 2002).

3.3.2.3 In vivo insulin sensitivity of glucose production and utilization

At 0800 h after an overnight fast, blood pressure was recorded and basal blood samples were obtained for the measurements in Table 1, and subjects underwent a euglycaemic hyperinsulinaemic clamp as previously described (DeFronzo et al 1979;Seppala-Lindroos et al 2002). Glucose production and disposal were measured by infusion of [3-^{3}H]glucose in a primed (20 μCi) continuous (0.2 μCi/min) fashion for a total of 360 min (Ryysy et al 2000). After 120 minutes, insulin was infused in a primed-continuous (0.3 mU/kg·min) fashion. Plasma glucose was maintained at 5 mmol/l (90 mg/dl) until 360 min using a variable rate infusion of 20 % glucose. Glucose kinetics and % suppression of free fatty acids from baseline were calculated during the final 60 min of the clamp. Serum free insulin concentrations were measured using radioimmunoassay (Pharmacia Insulin RIA kit, Pharmacia, Uppsala, Sweden) after precipitation with polyethylene glycol. Plasma glucose concentrations were measured in duplicate with the glucose oxidase method using a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Serum free fatty acids were measured using a fluorometric method. Serum lipids were measured as previously described (Tukkainen et al 2002).

3.3.2.4 Intra-adipose cortisol metabolism

A randomly selected subgroup of subjects who consented to providing a biopsy reattended between 0800-1200 h on another day, when a ~500 mg subcutaneous adipose biopsy was obtained under local anaesthesia through a 2-3 cm incision from the para-umbilical anterior abdominal wall. Subcutaneous fat was frozen immediately in two aliquots in liquid nitrogen. Adipose 11HSD1 activity, mRNA for 11HSD1 and downstream targets was measured as described in chapter 2.

3.3.2.5 Statistical analyses

Areas under curves for 11HSD1 activity in adipose in vitro were calculated using the trapezoidal rule. In all sections of this chapter Variables were log transformed if necessary to obtain a normal distribution, as indicated in the tables. Pearson correlation analyses were performed to identify associations. Confounding between associations was addressed using forward stepwise mutliple linear regression analysis with variables entering the final model if F>1.0. All data are shown as mean±standard error of mean. A p-value less than 0.05 was considered statistically significant.

3.3.3 Results

3.3.3.1 Subject characteristics

Participating men were aged 22-57 years with a wide range of body mass index (20.6 – 35.6 kg/m^2), waist/hip ratio (0.85 – 1.1), whole body fat (11.2 – 31.0 %), visceral fat (1423 – 7025 cm^3), subcutaneous abdominal fat (1302-4650 cm^3), and liver fat (1 - 41%). Associations between body composition variables are shown in Table 3.3. Data are shown for subjects above or below the median for each measurement of body composition; however, statistical comparisons were by Pearson correlations. Body composition did not change significantly with increasing age, but subjects with generalised or visceral obesity tended to be older. Increased whole body fat was associated with higher body mass index and liver fat content. Increased visceral fat was associated with higher body mass index. However, waist/hip ratio correlated more strongly with MRI measurements of subcutaneous abdominal fat mass (r=0.39, p=0.05) than with visceral fat mass (r=0.20, p=0.35).

3.3.3.2 Associations of cortisol metabolism and body composition

Results of Pearson correlations are in Table 3.3b). Increasing whole body fat was associated with increased 11HSD1 activity in adipose tissue with non-significant trends for increased adipose 11HSD1 mRNA. Similar associations of glucocorticoid parameters were observed with waist circumference, waist/hip ratio, and subcutaneous abdominal fat by MRI (data not shown). In contrast, visceral fat mass was not associated significantly with any indices of cortisol metabolism. In men with higher liver fat content, there was no difference in adipose 11HSD1 activity or mRNA.

For the variables for which associations were identified in Pearson correlation analyses in Table 3.3, we explored the relative influence of fat accumulation in different sites. We performed stepwise multiple regression analyses of indices of cortisol metabolism with % body fat, visceral fat, subcutaneous abdominal fat, and liver fat as independent variables.Values are beta correlation coeffients (r). Whole body % fat was consistently the best predictor of indices of increased adipose 11HSD1 activity ($r=1.23$, $p<0.01$). Liver fat accumulation was independently associated with decreased adipose 11HSD1 ($r= -0.63$, $p<0.05$). When corrected for the effect of total body fat and liver fat, visceral fat mass was *inversely* associated with adipose 11HSD1 activity ($r=-0.24$, p=NS).

3.3.3.5 Adipose Biopsy analysis; associations with parameters of obesity, insulin resistance, and downstream targets

Adipose 11HSD1 activity and mRNA were closely correlated ($r=0.77$, $p<0.001$, figure 3.1). Results are summarised in table 3.4. Generalised obesity was associated positively with 11HSD1 activity, 11HSD1 mRNA (trend only), H6PDH mRNA and negatively with GRα mRNA. Central obesity (waist hip ratio) was similarly associated with 11HSD1 activity and GRα mRNA. GRα mRNA was negatively associated with log fasting insulin, and positively associated with Angiotensinogen mRNA. 5α–reductase type 1 mRNA was positively associated with mediators of lipolysis/ adipogenesis (HSL, LPL and PPAR-γ), and aromatase.

Tables 3.3: Finnish Cohort; a) Subject characteristics according to body composition, b) Inter-correlations between body composition and subject characteristics

a)	Body Fat		Visceral fat		Liver fat[1]	
	Low (<21.1%)	High (≥21.1%)	Low (<3035cm^3)	High (≥3035cm^3)	Low (<10%)	High (≥10%)
N (for adipose biopsy)	12 (9)	13 (9)	14 (10)	11 (8)	11 (9)	14 (9)
Age (y)	39.3 ± 3.1	44.0 ± 3.1	39.1 ± 2.7	45.1 ± 3.5	44.2 ± 3.1	39.9 ± 3.1
Body mass index (kg/m^2)	23.9 ± 0.59	28.8 ± 1.1	25.0 ± 0.8	28.3 ± 1.3	26.5 ± 1.4	26.4 ± 1.0
Waist/Hip Ratio	0.95 ± 0.02	0.98 ± 0.01	0.94 ± 0.02	0.98 ± 0.01	0.96 ± 0.02	0.96 ± 0.02
Body fat (%)	17.4 ± 0.88	25.6 ± 0.79	20.3 ± 1.6	23.3 ± 0.9	20.2 ± 1.6	22.7 ± 1.3
Liver fat (%)[1]	*8.9 ± 2.3	17.5 ± 4.0	16.8 ± 3.9	9.0 ± 2.2	3.1 ± 0.7	21.4 ± 2.9
Visceral fat (cm^3)	2462 ± 254	3565 ± 390	2138 ± 135	4178 ± 315	3104 ± 285	2982 ± 412
Subcut abdominal fat (cm^3)	2338 ± 229	3426 ± 343	2534 ± 296	3374 ± 336	2867 ± 385	2933 ± 300
Adipose biopsy results:						
11HSD1 activity (%.h)	283 ± 60	389 ± 75	303 ± 59	377 ± 82	360 ± 84	312 ± 53
11HSD1 mRNA (ratio to cyclophyllin)	1.15 ± 0.02	1.17 ± 0.02	1.14 ± 0.02	1.19 ± 0.02	1.18 ± 0.02	1.14 ± 0.02

b)	Total Body Fat (%)	Visceral Fat (cm^3)	Liver Fat (%)
Age	0.16	0.36	-0.19
BMI	**0.74*** **	**0.45***	0.25
WHR	**0.42***	0.20	0.11
%Body Fat		0.3	**0.53****
Liver Fat (%)	**0.53****	-0.21	
Visceral Fat (cm3)	0.30		-0.21
Subcut abdominal fat (cm3)	**0.76****	0.22	0.23
Adipose biopsy results:			
11HSD1 activity	**0.52***	0.16	0.08
11HSD1 mRNA	0.32	0.35	-0.14

Footnotes to tables 3.3 a) Data are mean ± SEM, b) Results are Pearson correlation coefficients: * $p<0.05$; ** $p<0.01$; *** $p<0.00$

Table 3.4; Finnish Cohort; Associations between mediators of adipose glucocorticoid metabolism with in vivo measurements and potential glucocorticoid regulated target mRNAs

	11HSD1 activity (AUC F to E)	11HSD1 mRNA	H6PDH mRNA	GRα mRNA	5α Reductase Type 1 mRNA	Intra-adipose Cortisol	Intra-adipose Cortisone
In vivo Measurements:							
BMI	0.17	-0.11	0.28	**-0.59****	-0.19	-0.26	-0.12
% Fat	**0.52***	0.26	**0.51***	**-0.66****	-0.11	-0.39	0.07
Waist Hip Ratio	**0.47***	0.28	0.18	**-0.69****	-0.11	-0.01	0.38
Fasting plasma glucose	**0.51***	0.21	0.12	**-0.61****	-0.26	-0.27	0.10
Log fasting plasma Insulin	0.07	-0.16	0.16	**-0.60****	-0.33	0.11	-0.01
Plasma HDL cholesterol	-0.16	0.05	-0.41	0.24	0.01	0.06	0.23
Systolic Blood Pressure	0.08	0.15	-0.14	-0.21	-0.06	-0.16	0.17
Target Adipose mRNAs:							
Angiotensinogen	-0.15	-0.06	-0.01	**0.41***	0.20	-0.09	-0.40
Leptin	-0.01	0.20	0.19	-0.21	0.07	**-0.49***	-0.38
HSL	0.17	0.30	0.13	-0.11	**0.67****	-0.26	-0.18
LPL	-0.01	0.18	-0.12	0.24	**0.72****	-0.16	-0.35
PPAR-γ	-0.06	0.09	-0.15	0.28	**0.71****	-0.12	-0.39
Adiponectin	0.24	0.04	-0.08	0.08	-0.07	-0.13	-0.27
Resistin	0.40	0.34	-0.15	-0.04	0.08	0.06	0.35
Aromatase	0.17	0.27	0.00	0.05	**0.64***	-0.40	-0.32

Data are standardized beta coefficients after adjustment in multiple regression for the influence of gender for all, and also BMI for Adipose downstream targets * $p<0.05$, ** $p<0.01$

3.4 Local and Systemic Impact of Transcriptional Upregulation of 11β-Hydroxysteroid Dehydrogenase Type 1 in Adipose Tissue in Human Obesity in Healthy Swedish Volunteers

3.4.1 Introduction

In this Swedish cohort of men and women, previous investigations had shown increased *ex vivo* 11HSD1 activity in subcutaneous adipose tissue associated with *in vivo* obesity and insulin resistance (Rask et al 2001;Rask et al 2002). Using these biopsies, we investigated this further by measuring 11HSD1 mRNA and mRNAs for other mediators of glucocorticoid metabolism (H6PDH and 5α-Reductase type 1), and putative glucocorticoid regulated adipose target gene expression (angiotensinogen, leptin, HSL, LPL, PPAR-γ adiponectin, restitin, aromatase). We have further examined the impact on intra-adipose cortisol concentrations, and systemic measurements of cortisol metabolism.

3.4.2 Methods

3.4.2.1 Subjects

Subjects were recruited from a population-based study in northern Sweden, the WHO-conducted MONICA Project. From an original random sample of 2815 women and men, 41 Caucasian women and 40 men were selected to represent high and low fasting insulin concentrations and a wide range of BMI. Investigations in these groups have been reported previously (Rask et al 2001;Rask et al 2002). A subgroup of these subjects, selected at random (n=16 females, n=16 males), attended in the morning after an overnight fast. After local anaesthetic injection of prilocaine (10mg/ml Citanest, Astra, Södertalje, Sweden) in the skin area to the right of the umbilicus, approximately 1.5cm^2 sc fat was excised through a 2-3cm incision. Subcutaneous fat was frozen immediately in two aliquots at –70°C.

Diabetes mellitus, liver, renal and thyroid disease were excluded by routine laboratory tests. In the pre- and peri-menopausal women, investigations were performed in the follicular phase of the menstrual cycle (5-10d after starting menstruation). These studies were approved by the ethics committee of Umea University Hospital, and written informed consent was obtained.

3.4.2.2 Previous Clinical and Biochemical Measurements

Measurements, as described previously, (Rask et al 2001;Rask et al 2002) included baseline anthropometry, blood pressure and body composition. Insulin sensitivity was measured using the euglycaemic hyperinsulinaemic clamp technique. Cortisol metabolites were measured in 24 hour urine samples by gas chromatography and electron impact mass spectrometry. Liver 11HSD1 activity was assessed in vivo by conversion of oral cortisone to cortisol (measure in plasma) on first pass through the liver. Subjects took oral dexamethasone (3.5ug/kg body weight) at 2300h, fasted overnight and attended at 0830h for intravenous cannulation and oral cortisone acetate 25mg. Plasma samples were taken over 4 hours to measure kinetics of cortisol appearance as previously described (Rask et al 2001;Rask et al 2002).

3.4.2.3 Biopsy Analysis

Adipose 11HSD1 activity, adipose mRNAs, intra-adipose cortisol and cortisone levels were measured as described in chapter 2. Extraction efficiency for intra-adipose cortisol/ cortisone was assessed by recovery of the ^{3}H-steroid. Steroid extraction efficiency was 28.4% ± 1.2% (mean±SEM).

3.4.2.4 Statistics

Data are mean ± SEM unless otherwise stated. Where indicated, data were naturally log transformed to obtain a normal distribution for parametric testing. Areas under the curve for 11HSD1 activity in vitro and conversion of cortisone to cortisol in vivo were calculated using the trapezoidal rule. Results in men and women were compared by Student's t-tests. Multiple regression analyses were employed to adjust for the influence of gender. To assess relationships between adipose mRNAs, adjustment was also made for BMI, where appropriate. Standardised beta coefficients (SBC) are presented. $P<0.05$ was considered to indicate statistical significance.

3.4.3 Results

3.4.3.1 Subject Characteristics

Characteristics of participants are shown in Table 3.5. Men had lower % body fat, lower urine cortisol/cortisone metabolite ratios, HDL cholesterol, lower angiotensinogen and leptin mRNA levels and higher waist hip ratios than women. H6PDH, 5α-Reductase Type 1, HSL, LPL and aromatase mRNA levels were higher in men (Table 3.5).

3.4.3.2 Adipose 11HSD1

Adipose 11HSD1 activity and mRNA levels were closely associated with each other (Figure 3.1) and with parameters of obesity and insulin resistance (Table 3.6, Figure 3.3). To minimize potential confounding by effects of gender, relationships with obesity and other metabolic variables were adjusted for gender in multiple regression analyses. Further, BMI was chosen as the measurement of obesity in multiple regression because it was distributed similarly between men and women (unlike waist circumference, waist/hip ratio, or % fat). Results are shown in Table 3.6. Higher 11HSD1 activity was associated with both obesity and fasting hyperinsulinaemia, while higher 11HSD1 mRNA was significantly associated with obesity alone. In multiple regression analyses, the influence of BMI on adipose 11HSD1 activity was adjusted for insulin sensitivity and vice versa; associations of BMI and insulin sensitivity with 11HSD1 activity or mRNA could not be shown to be independent of each other in these models (data not shown). Neither 11HSD1 activity nor mRNA associated with in vivo urinary cortisol/cortisone metabolites ratios. 11HSD1 mRNA was positively associated with aromatase mRNA expression (0.37, $p<0.05$).

3.4.3.2 Adipose H6PDH/ 5α-Reductase Type 1 mRNAs

Results are summarised in table 3.6. H6PDH mRNA was positively associated with HDL cholesterol and systolic blood pressure but no other parameters of obesity or insulin sensitivity. H6PDH was positively associated with mRNA expression of Leptin, HSL, Adiponectin and Resistin. 5α-Reductase Type 1 mRNA was negatively associated with WHR but no other parameters of generalized obesity or insulin resistance.

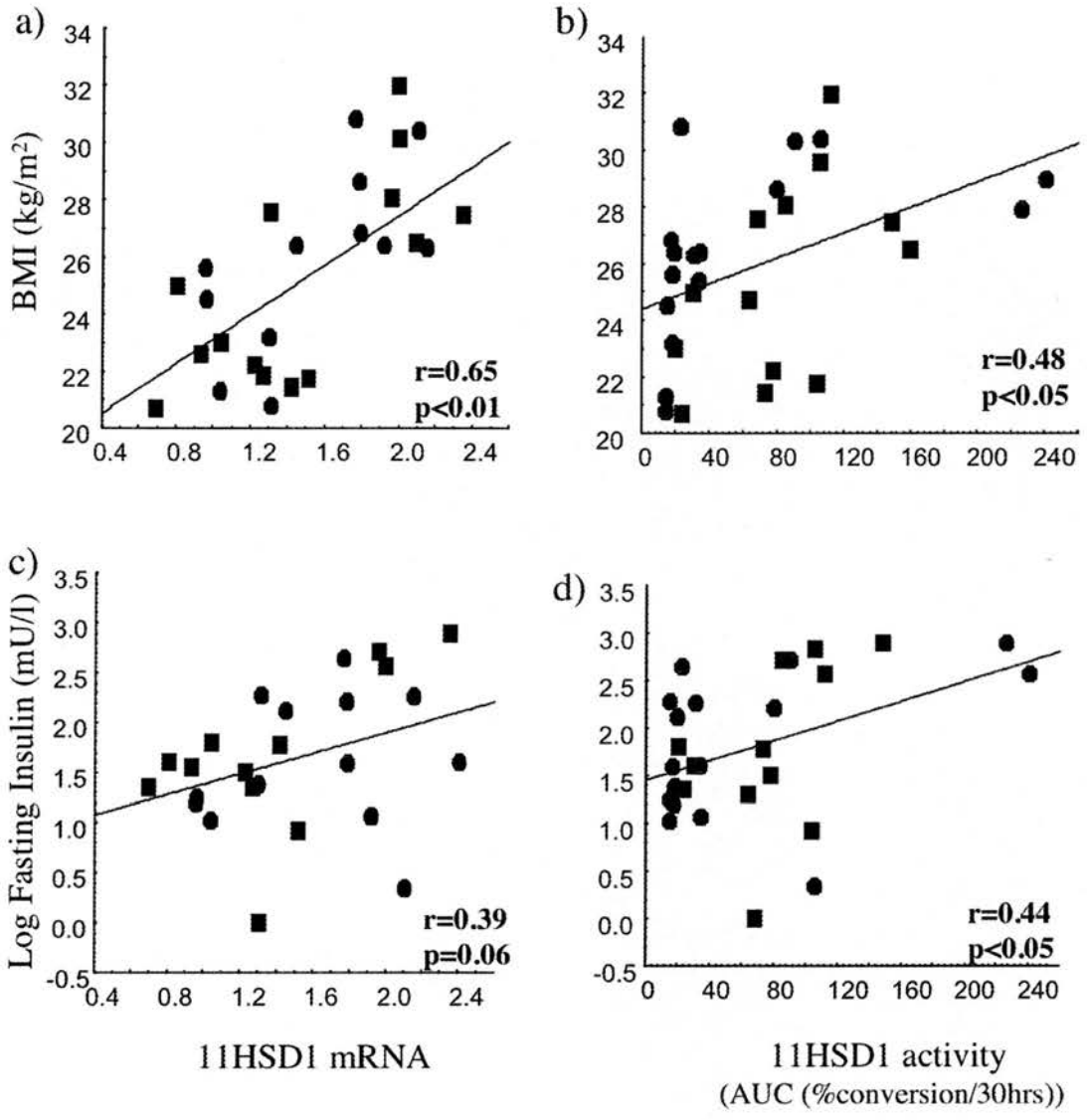

Figure 3.3

Swedish Cohort; Adiposity and metabolic variables vs. 11HSD1 activity and mRNA

Associations of 11HSD1 mRNA (a and c) or 11HSD1 activity (b and d) with Body Mass Index (BMI; a and b) or fasting plasma insulin (c and d) in subcutaneous adipose biopsies in men (open circles, n=13) and women (filled squares, n=14). 11HSD1 mRNA levels were analysed by real time PCR and expressed as a ratio against cyclophyllin mRNA. 11HSD1 activity was calculated as area under the curve of % conversion of cortisol to cortisone after incubation for 3, 6, 20 and 30 h.

Table 3.5: Swedish Cohort; comparison of male and female subjects

	Male (n=16)	Females (n=16)
Age (y)	53.4±2.5	56.8±2.8
BMI (kg/m^2)	26.5±0.8	25.3±0.9
Waist:Hip Ratio	**0.9±0.01****	**0.9±0.02****
% Fat	**26.9±0.9***	**31.74±1.6***
Fasting 0900h blood results:		
Glucose (mmol/l)	4.7±0.1	4.5±0.2
Insulin (mU/l)	7.8±1.3	7.4±1.5
Triglycerides (mmol/l)	1.8±0.4	1.3±0.1
Total cholesterol (mmol/l)	5.2±0.2	5.9±0.4
HDL cholesterol (mmol/l)	**1.1±0.08***	**1.4±0.09***
Cortisol (nmol/l)	293±30	369±28
Insulin sensitivity index (euglycaemic clamp)[a]	6.7±0.8	7.9±1.0
Systolic blood pressure (mmHg)	135±5	139±6
Diastolic blood pressure (mmHg)	82±3	81±4
Urine THFs/THE[b]	**1.2±0.1****	**1.92±0.2****
Conversion of oral cortisone to plasma cortisol (area under curve 0-240nm.min)	109104±9990	125630±7023
Intra-Adipose Measurements:		
Adipose 11HSD1 activity (area under curve 0-30%. h) (n=29, 16m,13f)	61.3±18.3	45.5±12.1
Intra-adipose cortisol (nmol/kg) (n=27, 13m, 14f)	157±37	81±26
Intra-adipose cortisone (nmol/kg) (n=27, 13m, 14f)	293±111	162±43
Adipose mRNAs (n=27, 13m, 14f):		
11HSD1	1.61±0.13	1.47±0.14
H6PDH	**3.00±0.6***	**1.61±0.3***
GRα	1.1±0.1	1.12±0.04
5α-Reductase Type 1	**1.34±0.2***	**0.68±0.1***
Angiotensinogen	**0.84±0.2***	**1.43±0.3***
Leptin	**0.86±0.12***	**1.32±0.2***
HSL	**2.1±0.2***	**1.53±0.2***
LPL	**0.77±0.07***	**0.56±0.04***
PPAR-γ	1.13±0.2	0.9±0.1
Resistin	1.24±0.3	0.65±0.1
Adiponectin	1.01±0.2	0.71±0.1
Aromatase	**2.14 ±0.4***	**1.11±0.2***

Data are mean ± SEM [a] mg glucose.kg^{-1}.min^{-1} per mU/l of insulin x 100 [b] THFs= 5αtetrahydrocortisol + 5βtetrahydrocortisol: THE=tetrahydrocortisone * $p<0.05$ **$p<0.01$ between men and women by Student's t tests

Table 3.6: Swedish Cohort; associations between mediators of adipose glucocorticoid metabolism with in vivo measurements and potential glucocorticoid regulated target mRNAs.

	11HSD1 activity (AUC F to E) (n=29)	11HSD1 mRNA (n=27)	H6PDH mRNA (n=27)	GRα mRNA (n=27)	5α Reductase Type 1 mRNA (n=27)	Intra-adipose Cortisol (n=27)	Intra-adipose Cortisone (n=27)
In vivo Measurements:							
BMI	**0.48***	**0.65****	-0.15	-0.18	-0.08	-0.34	-0.27
% Fat	0.19	**0.43***	0.03	-0.08	-0.22	-0.11	-0.31
Waist Hip Ratio	0.09	0.34	-0.06	-0.07	**-0.42***	0.01	-0.27
Fasting plasma glucose	0.14	0.14	0.20	-0.14	-0.14	-0.17	-0.18
Log fasting plasma Insulin	**0.44***	0.39	0.12	-0.10	-0.15	-0.26	-0.25
Fasting plasma triglycerides	0.17	0.11	0.11	0.03	-0.14	-0.23	-0.39
Plasma HDL cholesterol	-0.25	-0.27	**0.48***	0.23	0.07	0.01	0.18
Insulin sensitivity (euglycaemic clamp)	-0.27	-0.25	-0.07	0.37	0.06	0.49	0.39
Systolic Blood Pressure	-0.00	0.03	**0.50****	-0.11	-0.17	-0.07	-0.15
Urinary THFs/THE[b]	-0.13	0.32	0.12	0.13	0.19	-0.00	0.03
Conversion of oral cortisone to plasma cortisol (log)	**-0.40***	-0.34	0.23	0.08	-0.16	**0.53***	0.37
9am Plasma Cortisol	-0.05	0.08	-0.05	0.14	-0.24	0.41	0.28
Adipose Downstream Target mRNAs:							
Angiotensinogen	-0.01	-0.23	0.01	0.19	-0.05	0.12	0.04
Leptin	0.36	0.16	**0.71****	0.05	0.02	0.17	0.40
HSL	-0.39	-0.08	**0.59****	**0.51***	-0.15	-0.05	0.06
LPL	-0.40	-0.2	0.29	**0.50***	-0.03	0.13	0.08
PPAR-γ	0.05	0.01	0.27	0.23	0.08	-0.19	-0.13
Adiponectin	-0.11	-0.10	**0.77****	0.29	0.14	0.01	-0.05
Resistin	-0.21	-0.13	**0.46***	0.29	0.26	-0.13	-0.03
Aromatase	0.14	**0.37***	0.35	0.31	0.04	-0.12	-0.11

[b] THFs= 5αtetrahydrocortisol + 5βtertahydrocortisol: THE=tetrahydrocortisone Data are standardized beta coefficients after adjustment in multiple regression for the influence of gender for all, and also BMI for Adipose downstream targets * $p<0.05$, ** $p<0.01$

Table 3.7 Associations between steroid metabolising enzymes and their potential downstream target genes (all mRNAs unless otherwise stated).

	Angiotensinogen	Leptin	HSL	LPL	PPAR-γ	Resistin	Adiponectin	Aromatase
11HSD1 activity	-0.01	0.16	**-0.29***	-0.01	0.08	-0.13	0.1	0.11
mRNAs								
11HSD1	-0.11	0.18	0.04	0.19	0.15	-0.00	-0.02	0.28
5α-Reductase	0.09	0.06	0.14	**0.49****	**0.36***	0.17	0.02	0.17
H6PDH	0.00	**0.36***	**0.33****	-0.03	0.06	0.26	0.17	0.18
GRα	0.32	-0.1	0.19	0.28	0.32	0.17	0.14	-0.09

Results are expressed using combined data from Swedish and Finnish cohorts. $^{\$}$= available in Swedish cohort only. r= standardised beta coefficient calculated using multiple regression analysis, adjusted for gender, study cohort and BMI. *=$p<0.05$, **=$p<0.01$.

3.4.3.4 Glucocorticoid Receptor mRNA

Glucocorticoid receptor alpha (GR-α) was the predominant isoform in human adipose (mean 26.7 cycles in real time PCR versus 37.5 cycles for GR-β). GR-α mRNA showed no significant correlations with parameters of obesity or hyperinsulinaemia (although trends were towards inverse correlations)(Table 3.6). To test a possible interaction between 11HSD1 and GRα expression in predicting obesity and insulin resistance, multiple regression analyses were performed. These did not suggest any interaction and did not attenuate the relationships shown in Table 3.6 (data not shown). GR-α mRNA was positively associated with HSL and LPL mRNAs.

3.4.3.3 Intra-Adipose Cortisone and Cortisol

Tissue cortisol and cortisone concentrations are shown in Table 3.5. Values were log transformed for analysis. Neither correlated with adipose 11HSD1 enzyme activity or mRNA. Higher intra-adipose cortisol was associated with higher plasma cortisol levels after oral cortisone administration (Table 3.6), and non-significantly with cortisol levels at 0900 (SBC=0.41, p=0.06) and after overnight dexamethasone (SBC=0.34, p=0.18).

3.4.4 Combined results from Finnish and Swedish Cohorts

Data from Swedish and Finnish Cohorts were combined to further assess relationships between determinants of glucocorticoid metabolism and downstream targets. The American cohort was not included as there was not enough cDNA in this group to measure downstream target mRNAs. Combining the cohorts results in increased subject numbers (power) and allowed us to assess which relationships are most robust and remained consistent across cohorts. Results are summarised in table 3.8. Main findings were as follows. 11HSD1 activity and mRNAs for 11HSD1 and aromatase were positively associated with generalised obesity (BMI or %fat). 11HSD1 mRNA was also associated with central obesity (waist-hip ratio). GR-α mRNA was negatively associated with all aspects of obesity (BMI, %fat and waist hip ratio). Gender differences shown are discussed in section 3.4.3.1. We found differences in mRNA expression between cohorts (higher 11HSD and lower HSL

Table 3.8 Combined Analysis of Swedish and Finnish Samples; gender and cohort differences in adipose mRNAs and associations with anthropometry

		Swedish Men (n=14)	Swedish Women (n=13)	Finnish Men (n=18)	BMI	%Fat	WHR
Steroid Metabolising Enzymes:	11HSD1 activity	61.31±18.30	45.54±12.09	43.5±6.05	**0.36***	0.13	0.20
	11HSD1	1.61±0.13	1.47±0.14	**2.58±0.20****	0.30	**0.35***	**0.35***
	Aromatase	2.14±0.42	**1.11±0.2***	1.07±0.19	**0.35***	0.19	0.06
	5α Reductase T 1	1.34±0.23	**0.68±0.13***	1.26±0.20	-0.13	-0.14	-0.22
	H6PDH	3.00±0.62	**1.61±0.29***	2.51±0.53	0.09	0.20	0.07
Steroid Receptors:	GR-α	1.10±0.07	1.12±0.04	**0.97±0.04***	**-0.41****	**-0.28***	**-0.30***
	ER-α	1.11±0.25	1.14±0.32	2.05±0.51	0.10	0.06	0.04
	AR	1.56±0.33	**0.63±0.07****	0.68±0.06	-0.22	0.05	0.10
Other adipose genes:	Angiotensinogen	0.84±0.15	**1.43±0.25***	1.30±0.18	-0.10	-0.09	-0.13
	Leptin	0.86±0.12	**1.32±0.15***	1.29±0.24	0.25	0.20	-0.01
	HSL	2.10±0.18	**1.53±0.16***	**0.96±0.11****	-0.29	-0.07	-0.19
	LPL	0.77±0.07	**0.56±0.04***	0.99±0.19	-0.25	-0.18	-0.26
	PPAR-γ	1.13±0.23	0.90±0.08	0.88±0.13	-0.05	-0.10	-0.25
	Resistin	1.24±0.32	0.65±0.12	0.82±0.12	-0.15	0.05	-0.12
	Adiponectin	1.01±0.19	0.71±0.07	1.96±0.31	-0.17	-0.11	-0.07
	TNF-α	1.49±0.26	1.58±0.62		0.15	-0.02	0.24
	IL-1α	2.37±0.83	2.44±1.18		0.11	-0.11	-0.23
	IL-6	1.66±0.31	3.33±1.10	2.94±1.16	0.03	0.02	0.14

Data are mean ± SE for the mRNA transcript (expressed as ratio to cyclophyllin) and 11HSD1 activity (expressed as % conversion (calculated as combined area under curve for 3, 6, 20 and 30hr incubation)). Gender differences were tested in the Swedish cohort only and cohort differences in males only using Student's t tests. Associations with parameters of obesity were tested in multiple regression using combined data from Swedish and Finnish cohorts; results are standardised beta coefficient adjusted for gender and study cohort. *$p<0.05$, **=$p<0.01$.

and GRα mRNAs), which may reflect methodological rather than biological differences, since real time PCR on each cohort was performed in separate batches, using a relative standard curve of diluted cDNA pooled from within each cohort, so that, although cohorts may differ in apparent absolute mRNA levels, this may reflect a systematic difference in the standard curves in the two cohorts rather than selective differences in these mRNAs. For this reason multiple regression analysis adjusted for differences between cohorts (in addition to gender) where appropriate in multiple regression analysis of combined cohort data.

3.5 Discussion

3.5.1 Adipose Glucocorticoid Metabolism in Human Obesity

A number of recent studies have supported a potential role of glucocorticoid metabolism by adipose tissue in lipid and glucose homeostasis. In these cross sectional studies we confirm clear associations of subcutaneous adipose 11HSD1 activity and mRNA levels with indices of obesity, and show additional relationships with metabolic variables reflecting insulin sensitivity.

The concept of tissue-specific dysregulation of 11HSD1 activity in human obesity is now supported by a series of studies. It arose from observations that conversion of oral cortisone to plasma cortisol is impaired in obese men (Stewart et al 1999;Rask et al 2001) consistent with impaired hepatic 11HSD1, yet urinary ratios of cortisol/cortisone metabolites are highly variable (Andrew et al 1998;Stewart et al 1999;Fraser et al 1999;Rask et al 2001;Rask et al 2002) suggesting compensatory differences in extra-hepatic 11HSD activity. The concept was supported by increased 11HSD1 activity in adipose of Zucker obese rats and ob/ob obese mice associated with reduced 11HSD1 activity in livers in these animals (Livingstone et al 2000). Our findings of increased 11HSD1 in adipose in human obesity are consistent with a parallel study by Paulmyer-Lacroix et al (Paulmyer-Lacroix et al 2002) and have subsequently been confirmed by others (Engeli et al 2004;Kannisto et al 2004). The current data confirm the magnitude of this increase in 11HSD1 activity, demonstrate a close relationship of 11HSD1 activity and mRNA from the same biopsies, as has previously been seen in studies in rodents (Livingstone et al 2000),

and support higher 11HSD1 activity and gene transcription in adipose tissue in obesity. By contrast one study, examining biopsy material obtained during intra-abdominal surgery, has recently reported no relationship of 11HSD1 mRNA levels in subcutaneous adipocytes to BMI and an inverse relationship of 11HSD1 activity (in cultured preadipocytes) to BMI (Tomlinson et al 2002). There are notable differences in the studies. 11HSD1 activity and mRNA may have been altered by the stress of major surgery. 11HSD1 activity was measured in cells cultured to confluence rather than from the primary biopsy material, as in our study. The close relationship of mRNA and 11HSD1 activity in all three studies gives support to our results reflecting a true measure of adipose enzyme activity.

The Finnish study gives a more detailed description of altered cortisol metabolism in association with regional fat accumulation. Increased abdominal subcutaneous adipose 11HSD1 (which predicts increased cortisol regeneration from cortisone within fat) and increased total cortisol metabolite excretion were associated with a higher proportion of whole body fat but not with preferential fat accumulation in either visceral, subcutaneous abdominal, or hepatic depots.

In humans, it has been hypothesised that there is a syndrome in which increased intra-adipose generation of cortisol by 11HSD1 in visceral fat leads to central obesity and the metabolic syndrome, in what has been described as 'Cushing's disease of the omentum' (Bujalska et al 1997). We observed however that adipose 11HSD1 seemed to be associated more with generalised than central obesity and used detailed MRI measurement in the Finnish study to further address this paradox. This confirmed a lack of association with visceral adipose accumulation and strong associations with generalised fat accumulation.

Studies in primary culture suggest that 11HSD1 is more active in visceral than subcutaneous adipocytes (Bujalska et al 1999),although this may not hold in freshly isolated tissue (Paulmyer-Lacroix et al 2002), and there is as yet no direct evidence that visceral 11HSD1 is increased in obesity (Tomlinson et al 2002). Rather, it is studies of subcutaneous adipose tissue, like these, which have consistently shown increased 11HSD1 activity and mRNA in obese subjects (Rask et al 2001;Paulmyer-Lacroix et al 2002;Rask et al 2002;Wake et al 2003;Lindsay et al 2003) predicting either generalised (body mass index or total % body fat) or central (waist/hip ratio or

waist circumference) obesity. However, waist circumference and waist/hip ratio provide imprecise indices of visceral fat accumulation. Indeed, in the current data waist/hip ratio was more closely related to abdominal subcutaneous fat than visceral fat (Table 3.3). One previous small study used CT scans of the abdomen to quantify visceral fat but was not definitive as it reported only a lack of association between visceral/subcutaneous fat ratio with the urinary cortisol/cortisone metabolite ratio in patients receiving cortisol replacement therapy (Tiosano et al 2003); this urinary ratio is an inadequate indicator of 11HSD1 activity. Thus, whether variations in subcutaneous adipose 11HSD1 are associated with variations in visceral fat accumulation has not been examined previously. The current results show that it is total body fat, not visceral fat mass, which is associated with increased subcutaneous adipose 11HSD1 activity. Increased adipose 11HSD1 activity in human obesity may be invoked as a cause of 'Cushing's disease of the adipose' but not yet of 'Cushing's disease of the omentum'.

In mice with transgenic over-expression of 11HSD1 in adipose, plasma corticosterone levels are unaltered but intra-adipose corticosterone levels are elevated ~2-fold (Masuzaki et al 2001). To test whether the adverse metabolic consequences of increased adipose 11HSD1 could similarly be attributed to increased intra-adipose generation of cortisol we extracted glucocorticoids from adipose biopsies. Assessment of adipose regeneration of cortisol from cortisone in vivo is difficult; a previous attempt using arteriovenous sampling in large numbers of patients showed a strong trend that was not quite statistically significant (Katz et al 1999). The lack of relationship between adipose 11HSD1 and concentrations of intra-adipose cortisol or cortisone may be explained in a number of ways. Firstly, extraction of steroids from human adipose biopsies is technically demanding and application to small samples is novel. Second, variations in plasma cortisol, including those relating to the stress of the biopsy may contribute to the adipose pool. (Unfortunately concomitant plasma samples were not available to allow assessment of this). In the transgenic mice with adipose 11HSD1 overexpression, samples were obtained during the diurnal nadir of glucocorticoid secretion (Masuzaki et al 2001), when 11HSD1 is putatively more important in maintaining intra-cellular glucocorticoid levels (Seckl & Walker 2001). In these studies, biopsies were obtained during the diurnal peak of cortisol secretion

when the influence of circulating cortisol may be more important. In that light we consider our examination of intra-adipose cortisol exploratory and clearly far from definitive. Nevertheless, the American study did show positive relationships between intra-adipose cortisol and most anthropometric and metabolic indices of obesity and insulin resistance. In the Swedish study this was not the case but intra-adipose cortisol correlated with plasma cortisol levels taken at other times suggesting that measurements may have been influenced primarily by stress at the time of taking the biopsy. These relationships deserve further exploration to establish if they can be attributed to variations in adipose 11HSD1 and/or to increased plasma cortisol, which has been observed in subjects with the metabolic syndrome (Phillips et al 1998;Rosmond et al 1998;Walker et al 2000). Finally these studies give no indication of the mechanisms leading to elevated adipose 11HSD1 in the metabolic syndrome. We explore potential regulators of 11HSD1 which may mediate dysregulation in obesity through further *in vivo* studies in chapters 4 and 5.

3.5.2 Additional Determinants of Glucocorticoid Action; GR, H6PDH and 5α-Reductase

Sub-cutaneous adipose GRα mRNA was negatively associated with parameters of generalised and central obesity in all studies, but this association was only significant in the Finnish cohort. Reduction in GR may limit glucocorticoid signaling and downstream metabolic consequences of increased 11HSD1 in obesity. The differences in 11HSD1/ GR balance between cohorts cannot be explained by gender alone, and may reflect different genetic or environmental backgrounds or minor differences in the biopsy protocols. To get a feel for the overall balance of 11HSD1 vs GR, we combined data form the Swedish and Finnish studies (n=46). In this combined dataset, central adiposity (measured by waist:hip ratio) was positively associated with 11HSD1 mRNA ($r=0.35$, $p<0.05$) and negatively associated with GRα mRNAs ($r=-0.30$, $p<0.05$) in subcutaneous adipose tissue. A difference in waist:hip ratio of 0.1 was associated with a ~34% increase in 11HSD1 mRNA and a ~10% reduction in GRα.

H6PDH generates NADPH, a cofactor required for 11HSD1 reductase activity to convert cortisone into cortisol (Draper et al 2003;Banhegyi et al 2004), and is co-

localised with 11HSD1 in the lumen of the endoplasmic reticulum. Recent data suggest an intimate association between these enzymes which may explain why 11HSD1 functions predominantly as a reductase in vivo but is bi-directional when liberated from its intracellular environment. Complete loss of H6PDH, such as has been described in the rare syndrome of 'cortisone reductase deficiency' results in markedly impaired 11HSD1 reductase activity (Draper et al 2003). However, whether subtle variations in H6PDH provide physiological control over cortisol generation remains to be established. To date, H6PDH has not been studied extensively in adipose tissue and its regulation is uncertain. In the Finnish cohort, H6PDH mRNA was positively associated with %Fat and in the Swedish cohort with potential glucocorticoid targets genes Leptin, HSL, Adiponectin and Resistin. Whilst intriguing, this should be interpreted with caution as these findings were not consistent across cohorts. Nonetheless these associations suggest that H6PDH may be more than a constitutive supplier of cofactor. Indeed, its expression has been shown to vary according to adipocyte differentiation state, emphasising that its potential importance deserves further dissection.

Previous in vitro and in vivo studies have demonstrated the presence of 5α-reductase activity (but have not assessed mRNA) in adipose tissue, which does not differ with adipose site and does not appear to be potently regulated by glucocorticoids (Killinger et al 1990). We have demonstrated that adipose 5α–reductase activity can be attributed to the type 1 isozyme but its expression was not consistently associated with generalised or central adiposity. 5α-reductase type 1 is traditionally considered as an androgen-activating enzyme (chapter 6), but is most highly expressed in liver where it makes a major contribution to metabolism of cortisol. Recent data show that the 5α-reduced metabolites of glucocorticoids are not inert, but in fact can bind and activate GR both in vitro and in vivo (McInnes et al 2004). Thus, it is possible that there is a component of glucocorticoid action in adipose which is associated with increased 5α-reductase type 1 activity (discussed further in chapter 6). It may therefore be either glucocorticoid or androgen receptor activation which account for the links between 5α-reductase type 1 and downstream targets (LPL and PPARγ) (see 3.5.3). There is a risk, particularly in the inter-analysis of adipose mRNAs, that the results are subject to bias from multiple statistical

testing. Bonferroni corrections were not used here, but we accept that results with a marginal p values should be interpreted with caution. We have therefore concentrated in our discussion on findings that were consistently found in all cohorts or with highly significant results suggesting a 'real' effect.

3.5.3 Downstream Effects of Altered Adipose Glucocorticoid Metabolism

Angiotensinogen is produced in the liver and adipose tissue. Animal studies suggest its production is glucocorticoid-regulated and contributes to obesity-induced hypertension (Masuzaki et al 2001;Massiera et al 2001;Hainault et al 2002;Gorzelniak et al 2002;Paterson et al 2004), but human studies show inconsistent relationships of adipose angiotensinogen expression with obesity (Van et al 2000a;Van et al 2000b;Giacchetti et al 2002). We found that subcutaneous adipose angiotensinogen levels are higher in females (consistent with well recognized oestrogenic regulation) but show no relationship with adipose 11HSD1, or parameters of obesity or blood pressure. Visceral adipose tissue was not assessed in this study and may be the more abundant adipose source of angiotensinogen. GRα mRNA was associated with angiotensinogen mRNA, but only in the Finnish cohort.

Adipose leptin synthesis and secretion is increased by exogenously administered glucocorticoids in vitro and in vivo (Leal-Cerro et al 2001). Adipose specific transgenic overexpression of 11HSD1 in mice leads to substantial increases in adipose leptin mRNA and serum leptin (Masuzaki et al 2001). We demonstrated higher adipose leptin mRNA levels in women. This gender difference is well recognised and thought to be androgen dependent (Soderberg et al 2001). Leptin mRNA was positively associated 11HSD1 activity and mRNA levels but was not significant after correction for BMI.

In vitro studies suggest that aromatase is glucocorticoid regulated. There was a positive trend between 11HSD1 and aromatase in these studies but this was not significant when adjusted for BMI. Aromatase is further discussed in chapter 6.

Glucocorticoids regulate lipolysis and adipogenesis in vitro (Ringold et al 1986;Ottosson et al 1994;Samra et al 1998;Anderson et al 2002;Dang et al 2002;Palin et al 2003). The principal correlations here were of H6PDH with leptin and HSL, and of 5α-reductase 1 with LPL and PPARγ (these relationships persist

after combining the Swedish and Finnish datasets ($r=0.49$, $p<0.01$ and 0.36, $p<0.05$ respectively). Interestingly, H6PDH expression was associated with mRNA expression of mediators of insulin sensitivity/ resistance, adiponectin and resistin. As this relationship was only present in the Swedish cohort however its importance is uncertain.

3.6 Summary

Obesity is associated with widespread changes in glucocorticoid metabolism, namely activation of the HPA axis, flattening of the diurnal cortisol rhythm, increased cortisol clearance and reduced hepatic cortisol activation (via 11HSD1). We now demonstrate changes in adipose glucocorticoid metabolism, namely increased 11HSD1 activity and mRNA levels in generalised obesity which is associated with insulin resistance in both men and women. Whether these relationships persist in visceral adipose, which is arguably the most metabolically active fat depot, remains unknown. The importance of adipose 11HSD1 as a functional mediator of metabolic complications also remains uncertain. These studies suggest that the downstream impact of cortisol generation in adipose may be limited by compensatory changes in GR expression. Further although these studies show clear associations between 11HSD1 and measures of insulin resistance, it has been difficult to identify specific glucocorticoid regulated downstream target mRNAs or to prove causality in these relationships. The real impact on glucocorticoid-dependent processes in vivo, will undoubtedly best be assessed by the use of new technologies such as microarray and the development of specific inhibitors of 11HSD1 (Alberts et al 2002).

Chapter 4

ACUTE REGULATION OF CORTISOL METABOLISM BY METABOLIC SIGNALS: INSULIN AND LIPID

4.1 Introduction

Chapter 3 clearly demonstrates increased adipose 11HSD1 in obesity, but does not speculate on the cause of up-regulation. In the next 3 chapters, we explore potential regulators of tissue 11HSD1 activity. Regulation of 11HSD1 mRNA in animal and cell models by high fat feeding (Morton et al 2004a), insulin (Napolitano et al 1998;Handoko et al 2000;Tomlinson et al 2001), and PPAR/ LXR agonists (Berger et al 2001;Stulnig et al 2002), and putative control of enzyme direction by NADPH generation (by hexose-6-phosphate dehydrogenase)(Banhegyi et al 2004), suggest a key role for 11HSD1 in the adaptive response of adipose to altered nutrition. Further, maladaptive dysregulation may underlie increased adipose 11HSD1 in human and rodent obesity. However, 11HSD1 regulation is tissue-specific, and has not been studied in human adipose in vivo. Further, the metabolic clearance rate for cortisol is increased in obese subjects (Andrew et al 1998;Rask et al 2002). Our group has attributed this to increased A-ring reduction of cortisol by 5β- and 5α-reductase enzymes. A-ring reductases may also be regulated by nutritional factors (Tsilchorozidou et al 2003) and 5β-Reductase, a key enzyme also involved in cholesterol and bile acid biosynthesis, is thought to be regulated by substrate availability.

In this study we speculate that 11HSD1 may be acutely responsive to changes in dietary factors and mediate cellular responses to eating. This is supported by evidence that dietary lipid regulates adipose 11HSD in short term animal studies (Morton et al 2004b) and in recent studies within our laboratory insulin appears to cause very acute down-regulation of adipose 11HSD1 (Sandeep et al 2005). On this background, we assessed acute regulation of cortisol metabolism by nutritional factors in vivo, in 9 healthy male volunteers specifically in response to hyperinsulinaemia, and hyperlipidaemia; both being likely regulators as suggested by the background evidence. Adipose 11HSD1 activity was determined by intra-adipose microdialysis, and whole body cortisol metabolism and liver A-ring

reductases by intra-venous (9,11,12,12-$^2H_4$) cortisol tracers (as described in chapter 2).

This chapter specifically aimed to determine i) Is adipose 11HSD1 acutely regulated in vivo by hyperinsulinaemia and hyperlipidaemia and mediated by changes in enzyme directionality? ii) Is whole body cortisol metabolism and clearance (via 5α- and 5β-Reductase) regulated by nutritional factors? and iii) Is regulation of 11HSD1 by insulin and glucose transcriptional or post-transcriptional? The later aim is assessed using in vitro studies in primary adipocyte cultures.

4.2. Methods

4.2.1 Study Design & Protocol (figure 4.1)

Nine healthy men (BMI 20-30kg/m^2) were recruited as discussed in chapter 2. Volunteers participated in a random-order 3-phase crossover single-blind study comparing cortisol metabolism during: (i) intravenous insulin and glucose infusion; (ii) intravenous lipid infusion; and (iii) placebo saline infusion. The study order was randomized and balanced and phases were separated by 7-14 days. In order to study the acute time course of effects of hyperinsulinaemia and hyperlipidaemia, (9,11,12,12-$^2H_4$) cortisol tracers were infused to steady state before these manipulations were introduced. The use of intravenous deuterated cortisol tracers was developed by Andrew et al (Andrew et al 2002) and the justification for use as a mean of in vivo assessment of glucocorticoid metabolism in these acute regulation studies is discussed in section 2.4.5.

In each phase, volunteers attended the Clinical Research Facility after overnight fast. To avoid discomfort from prolonged fasting, subjects were given a standard light breakfast at 7.00am, in anticipation that any post-absorptive effects would have passed before steady state tracer measurements were made and interventions began ~5 hours later. Three intravenous cannulae were inserted: one for sampling arterialized blood was placed in a dorsal hand vein kept under a heated pad; an antecubital vein was cannulated in each arm for infusions. After cleaning with Betadine (SSL, Knutsford, UK) and injection of local anaesthetic (5mls Lignocaine 1%, Braun, UK), two microdialysis cannulae (containing 20kDa-permeable membranes, CMA Microdialysis, Sweden) were inserted in abdominal subcutaneous

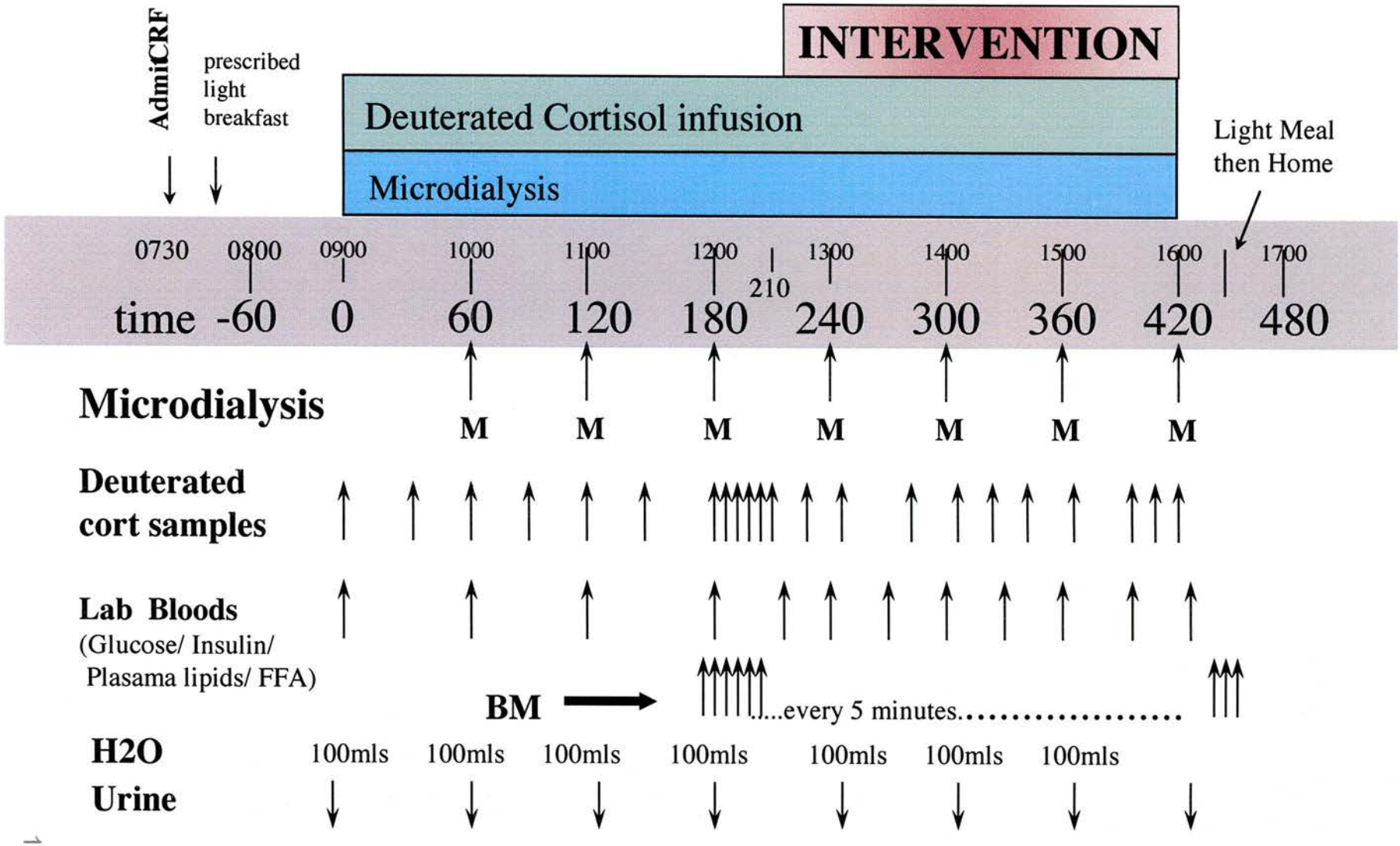

Figure 4.1 Study protocol for assessment of acute regulation of cortisol metabolism by metabolic signals

fat, approximately 10cm lateral to the umbilicus on each side, as previously described in chapter 2.

At time zero (~8.30am) a primed intravenous infusion of cortisol (20% 9,11,12,12-$^{2}H_4$-cortisol and 80% hydrocortisone-21-succinate) was commenced (3.6 mg priming bolus then continuous 1.74 mg/h infusion)(Andrew et al 2002;Basu et al 2004;Basu et al 2005;Sandeep et al 2005;Andrew et al 2005). Through separate microdialysis cannulae (Sandeep et al 2005), intra-adipose infusions were commenced of (i) 1,2,6,7-$^{3}H_4$-cortisol (50 nmol/l) and (ii) 1,2-$^{3}H_2$-cortisone (67 nmol/l) at rates of 0.3 ul/min. After 3.5 hours, a further intravenous infusion was commenced with either: (i) soluble human insulin (0.8 mU/kg/min) with variable rate 20% dextrose infusion to maintain arterialised blood glucose of 5 mmol/l; (ii) Intralipid 20% (Intralipid^{TM}, Kabivitrum Inc., California and Stockholm)(30 ml/h for 15 min then 50 ml/m^2/h); or (iii) 0.9% saline as placebo control (same rate as Intralipid). Measurements continued for a further 3.5 hours. Volunteers were given 200mls of water to drink every hour to encourage regular bladder emptying.

Arterialized blood glucose was measured every 5 min using a glucometer (Roche Accu-Check, Advantage 2). Arterialized blood samples were obtained at intervals indicated in the Figures (summarized in Figure 4.1), plasma was separated promptly and stored at -80C. Urine was collected at baseline and then every hour for analysis of tracer steroid metabolism, the volume recorded and aliquots stored at -20C. Microdialysis microvials were changed every hour and dialysate stored at -80C.

4.2.2 Laboratory assays

Isotopomers of 9,11,12,12-$^{2}H_4$-cortisol (D4-cortisol), 9,12,12-$^{2}H_3$-cortisone (D3-cortisone) and 9,12,12-$^{2}H_3$-cortisol (D3-cortisol) were measured in plasma and urine, and cortisol kinetics calculated as described in sections 2.4.5 and 2.8. Microdialysis cannulae were changed hourly and % conversion of cortisone to cortisol (reductase activity) or cortisol to cortisone (dehydrogenase activity) per hour was calculated (section 2.4.3). Insulin, glucose, triglycerides and free fatty acids were measured in plasma (see section 2.6).

4.2.3 Statistics

Sample size calculations were based on the main outcome measure (rate of D3-cortisol generation). Standard deviations (4.2 nmol/min) were taken from previously published work using this technique (Andrew et al 2002). A sample size of 9 has 80% power to detect a 22% change in enzyme activity to $p<0.05$. This is in keeping with expected levels of change shown in previous in vitro and in vivo experiments (>50% dowregulation in 11HSD1 activity following insulin intervention).

In analysis of tracer and microdialysis data where repeated measures analysis was undertaken, there were very few missing time points. Where a single time point was missing an average value was calculated using preceding and following values. For other data (not repeated measures), missing data points resulted in exclusion of the case from paired comparison of that variable.

Data are presented as mean ± SEM. The time course of effects of insulin and Intralipid on each measurement was examined by repeated measures ANOVA using 'placebo corrected' data (i.e. values at each time point for insulin or Intralipid were subtracted from the values for saline infusion for each participant). Where ANOVA identified effects of infusion, post-hoc paired t tests were performed to identify differences in absolute values. This approach corrects for inter-subject variability in a similar manner to a 'paired' repeated measures ANOVA, which cannot be performed in Statistica, and is a method commonly used in crossover drug studies. It allows investigation of acute changes in time course which would not be adequately represented by paired comparisons of summary statistics (such as steady state values or areas under curve).

To reduce the variance of kinetic parameters derived from deuterated-steroid measurements, values were averaged for four measurements taken during steady state in the final 30 minutes of the run-in period (180-210 min) and for three measurements taken in the final 40 minutes of each infusion (380-420 min).

4.3 Results

4.3.1 Participants

The subjects were aged between 24 and 68 years (mean ± SEM, 41 ± 4.3), with body mass index 19.7-30.2 kg/m^2 (25.5 ± 1.2), Waist:Hip Ratio 0.8-1.1 (0.9 ± 0.03), and systolic blood pressure 106-168 mmHg (125 ± 6.2).

4.3.2 Effects of infusions on plasma fuel substrates and insulin (Figure 4.2)

Following a light breakfast 90 minutes before the start of the study, insulin and glucose concentrations returned to baseline values within 120 minutes. Insulin infusion from 210 minutes resulted in raised plasma insulin, and reduced free fatty acid levels, while plasma glucose levels were maintained as intended at ~5.0 mmol/l with dextrose infusion. Intralipid infusion from 210 minutes resulted in raised plasma triglyceride and free fatty acid levels and no change in plasma glucose or insulin levels.

4.3.3 Whole body cortisol metabolism

See Table 4.1 and Figure 4.3 and 4.4. Endogenous cortisol production declined during the study day, reflected in a rise in D4-cortisol enrichment, although the downward trend in total unlabelled plasma cortisol concentrations during D4-cortisol infusions was not statistically significant. As previously described (Andrew et al 2002;Sandeep et al 2005), D4-Cortisol enrichment and D4-cortisol:D3-cortisol ratios were in steady state at the end of the run-in period (ie values did not change between 180 and 210 min). Thereafter, insulin and dextrose infusion increased the rate of appearance of plasma D3-cortisol in plasma (reflected in lower D4-cortisol:D3-cortisol ratios) and tended to increase the appearance of endogenous cortisol (ANOVA p=0.07; reflected in lower D4-cortisol enrichment). Intralipid infusion did not significantly alter any of the measurements of deuterated cortisol metabolism. Neither insulin not Intralipid infusion altered clearance rates of cortisol and D4-cortisol.

As previously described (Andrew et al 2002), urinary deuterated cortisol metabolite excretion does not reach steady state even during 7 hours of infusion, so excretion rates were highest in the final hour of measurement (Table 4.1). However, neither insulin nor Intralipid infusion altered urinary deuterated cortisol metabolite excretion.

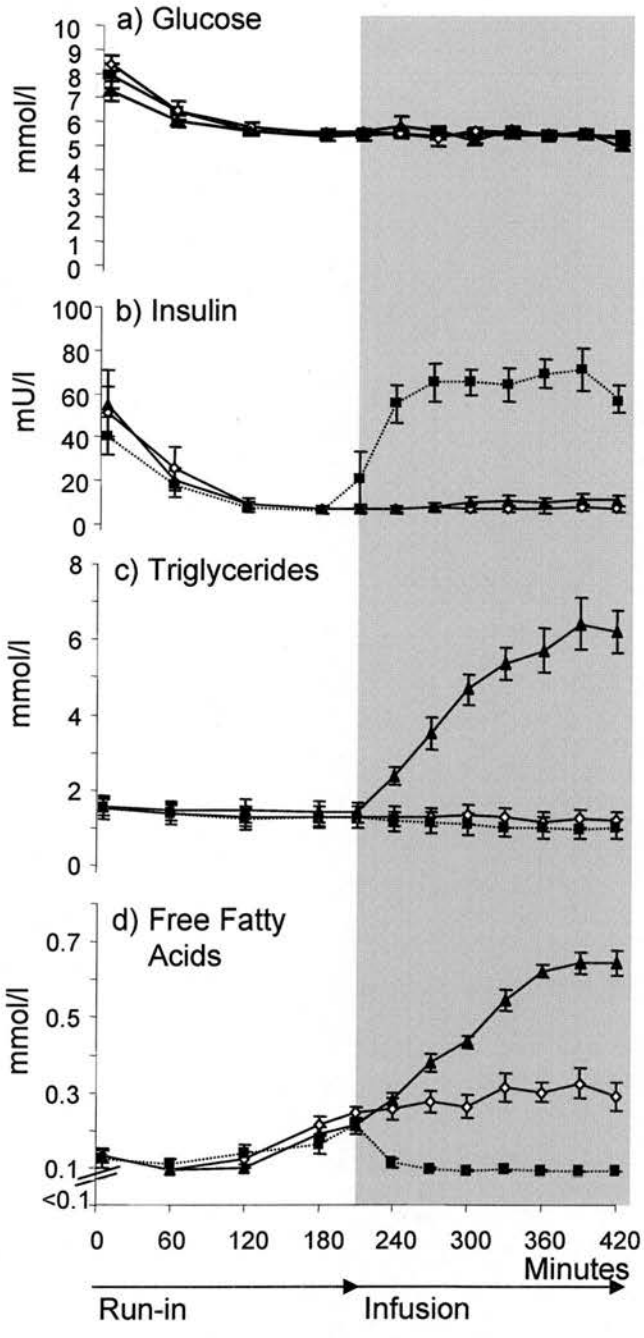

Figure 4.2

Effects of insulin and Intralipid infusion on plasma fuel substrates and insulin Data are mean ± SEM for measurements during a 3.5 hour 'run-in' tracer infusion followed by 3.5 infusion with saline (open diamonds, solid line), insulin (filled squares, dotted line) or Intralipid (filled triangles, solid line). Insulin infusion increased plasma insulin and suppressed plasma free fatty acid concentrations (ANOVA, both $p<0.01$). Intralipid infusion increased plasma triglycerides and free fatty acid concentrations (ANOVA, $p<0.01$) without affecting plasma insulin. There were no differences in plasma glucose between the groups.

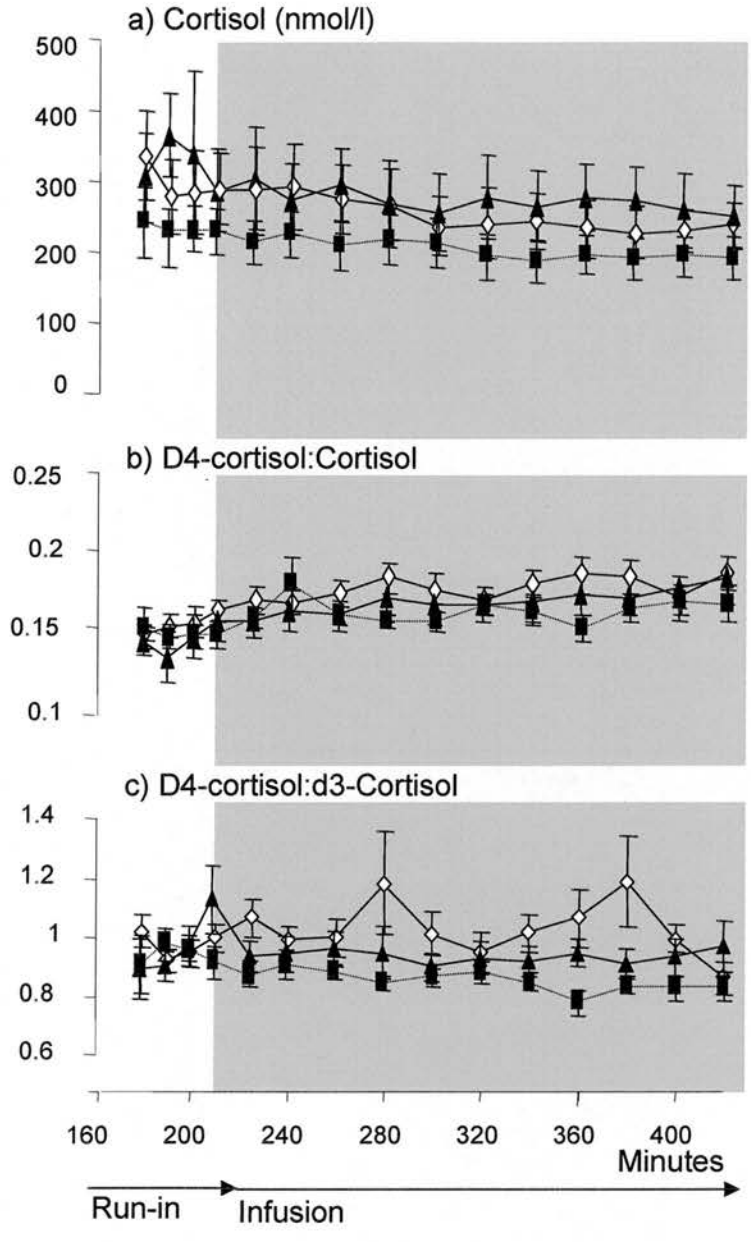

Figure 4.3

Effects of insulin and Intralipid infusion on plasma cortisol and deuterated-cortisol enrichment Data are mean ± SEM for measurements during a 3.5 hour 'run-in' tracer infusion followed by 3.5 infusion with saline (open diamonds, solid line), insulin (filled squares, dotted line) or Intralipid (filled triangles, solid line). Plasma cortisol concentrations were not significantly affected by insulin or Intralipid infusions. Statistical testing of deuterated-cortisol metabolism was performed on kinetic parameters shown in Table 4.1 rather than on 'raw' enrichment data.

Table 4.1
Insulin and Lipid Regulation: $^2H_4$-Cortisol kinetics

	Saline		Insulin		Intralipid	
	Run-in	**Infusion**	**Run-in**	**Infusion**	**Run-in**	**Infusion**
Plasma kinetics[1]						
Total cortisol (nM)	285 ± 52	232 ± 33	230 ± 39	192 ± 32	298 ± 57	263 ± 49
Cortisol clearance (ml/min)	299 ± 61	326 ± 47	340 ± 58	452 ± 122	280 ± 52	351 ± 96
D4-cortisol clearance (ml/min)	400 ± 62	406 ± 71	521 ± 77	643 ± 204	410 ± 62	409 ± 88
Rate of appearance of endogenous cortisol (nmol/min)	23.9 ± 4.8	12.7 ± 4.5	33.1 ± 7.0	21.5 ± 6.5	34.2 ± 8.5	16.7 ± 7.2
Rate of appearance of D3-cortisol (nmol/min)	16.8 ± 0.7	16.7 ± 1.1	16.8 ± 0.7	19.3 ± 0.8 ***	16.7 ± 1.0	17.7 ± 1.3
Urine excretion[2]						
D4-5α-tetrahydrocortisol (μg/h)	7.8 ± 1.8	19.2 ± 3.4	12.1 ± 2.2	17.1 ± 2.4	12.3 ± 3.3	25.0 ± 7.0
D4-5β-tetrahydrocortisol (μg/h)	8.6 ± 1.7	19.0 ±3.8	13.9 ± 2.3	15.7 ± 3.5	11.9 ± 2.2	19.1 ± 3.4
D3-5β-tetrahydrocortisone (μg/h)	18.5 ± 3.0	56.0 ± 6.9	26.1 ± 6.4	46.1 ± 8.4	23.6 ± 2.9	49.8 ± 7.1
Sum of deuterated cortisol metabolites (μg/h)[3]	54.3 ± 7.2	162.4 ± 15.4	82.3 ± 13.7	137.5 ± 15.2	75.9 ± 8.1	158.7 ± 19.0

Data are mean ± SEM. Post-hoc comparisons were made only for variables for which repeated measures ANOVA revealed effects of insulin or Intralipid infusion; differences between saline infusion and insulin or Intralipid infusion were then tested by paired *t* tests. ***p<0.001

[1] For plasma measurements, 'Run-in' data describe the mean of 4 measurements in the final 30 min of the 3.5 h D4-cortisol infusion before the introduction of saline, insulin or Intralipid infusions. 'Infusion' data describe the mean of 3 measurements in the final 40 min of the 3.5 h during which saline, insulin and Intralipid were infused.

[2] For urine measurements, 'Run-in' data describe measurements in the final 60 min of the 3.5 h D4-cortisol infusion before the introduction of saline, insulin or Intralipid infusions. 'Infusion' data describe measurements in the final 60 min of the 3.5 h during which saline, insulin and Intralipid were infused.

[3] Sum of urine metabolites = D4-5α-tetrahydrocortisol + D4-5β-tetrahydrocortisol + D3-5β-tetrahydrocortisone + D3-5α-tetrahydrocortisol + D3-5β-tetrahydrocortisol

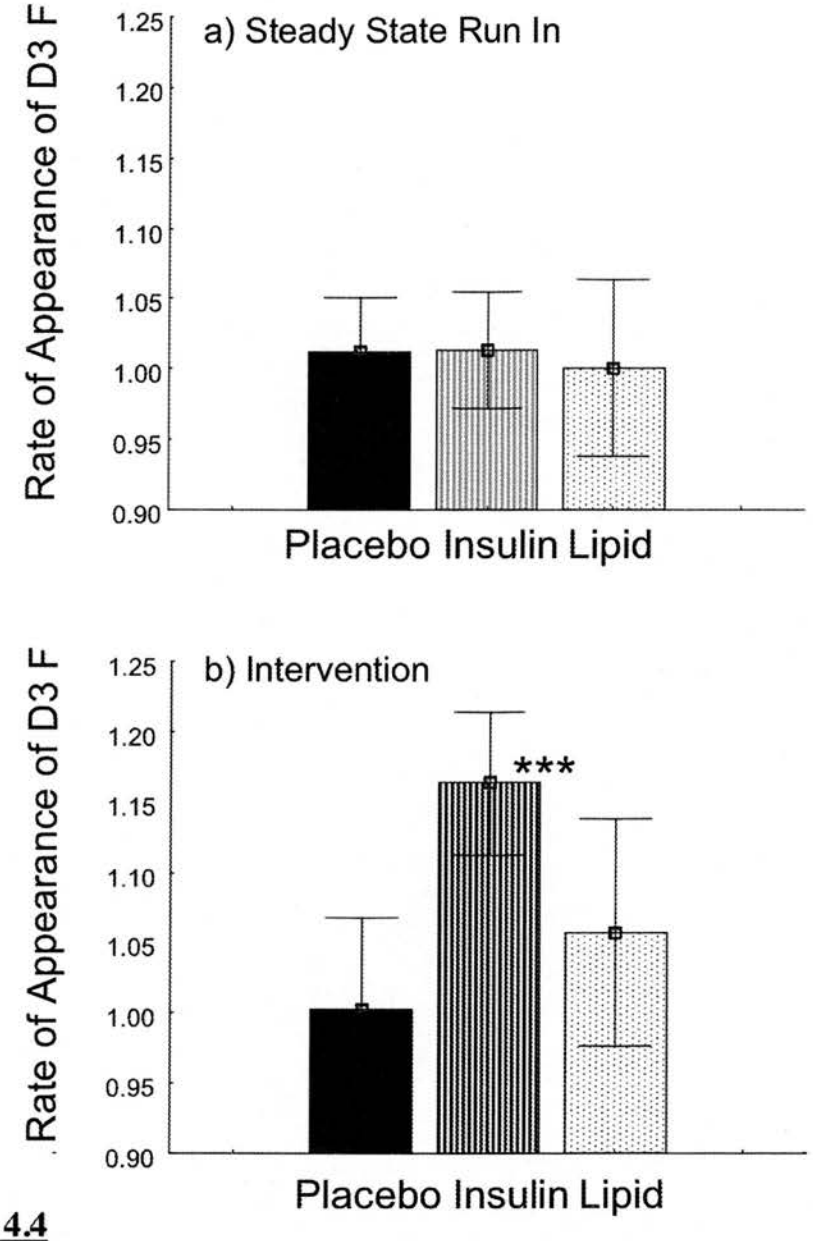

Figure 4.4

Effects of acute hyperinsulinaemia and hyperlipidaemia on rate of appearance of plasma d3F after infusion of intravenous d4F. Data are mean ± SEM for measurements of rate of appearance of d3F generated from infused d4F in plasma of 9 participants before and during a 3.5 hour infusion of saline (placebo), insulin (euglycaemic clamp) and lipid (20% Intralipid) infusions. Steady state values were calculated from the mean of four measurements between 180 and 210 min of D4-cortisol infusion. The intervention values are taken as a mean of 3 measurements in the final 40 minutes of the intervention period.

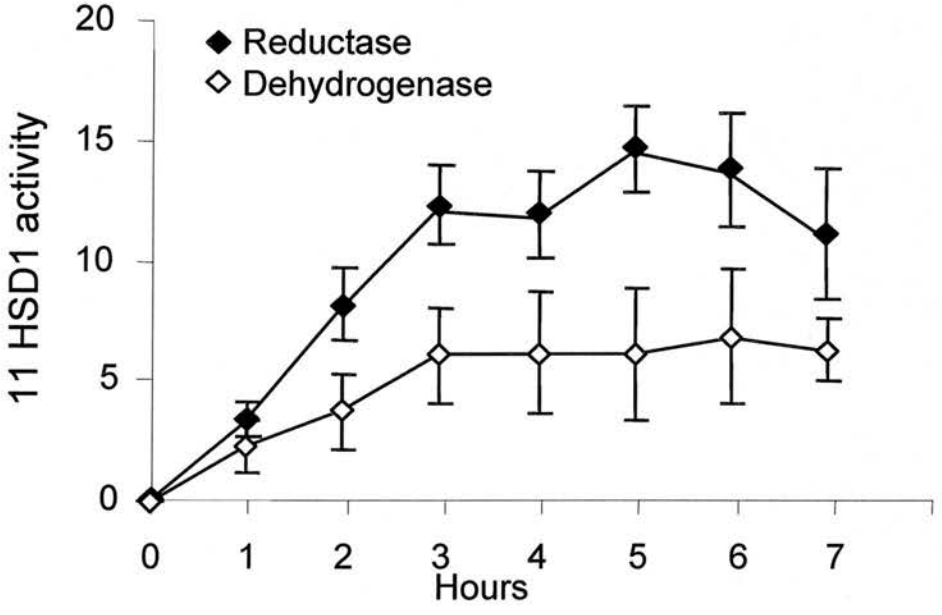

Figure 4.5

***In Vivo* 11HSD1 reductase vs dehydrogenase activity in adipose tissue.** Data are mean ± SEM for measurements from two microdialysis cannulae within abdominal subcutaneous adipose tissue in 9 participants during the saline infusion phase alone. In one cannula, 3H-cortisone was infused and results are expressed as % conversion to 3H-cortisol (reductase activity, filled diamonds). In the other cannula, 3H-cortisol was infused and results are expressed as % conversion to 3H-cortisone (dehydrogenase activity, open diamonds). Reductase activity was greater than dehydrogenase activity (ANOVA $p<0.02$).

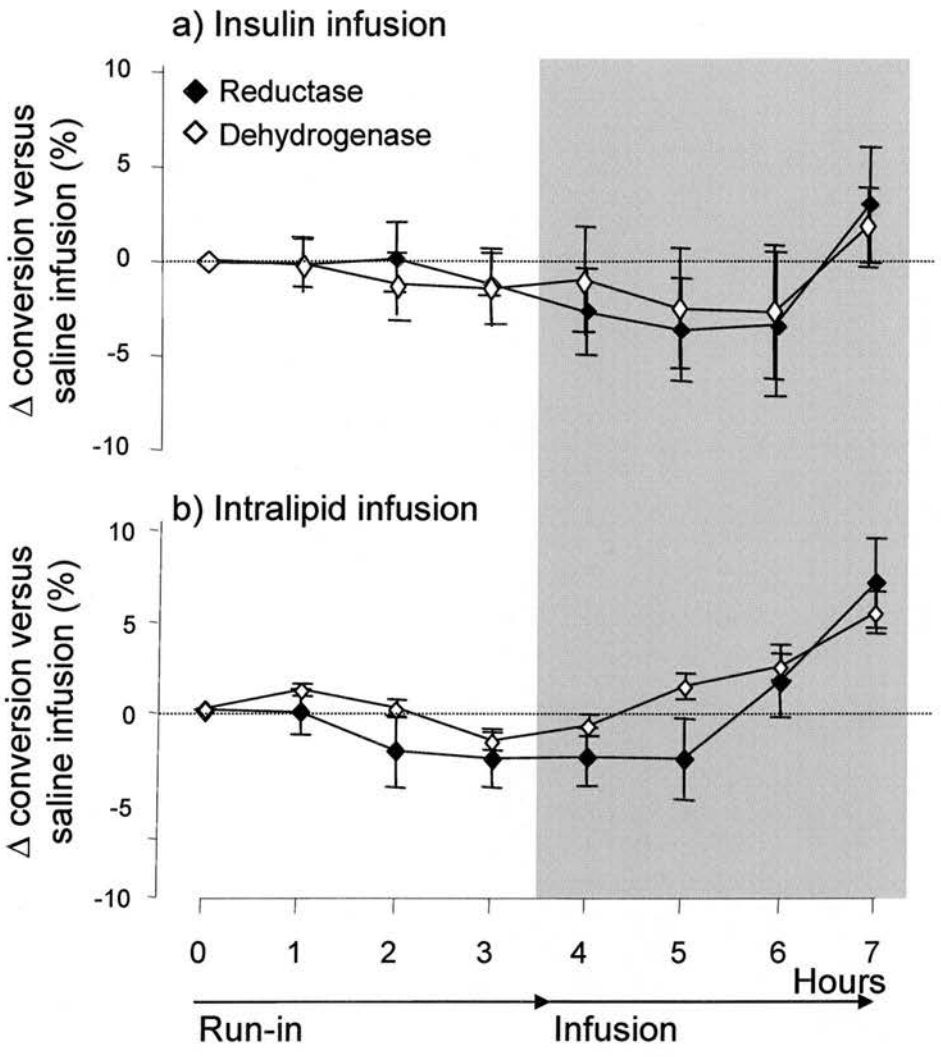

Figure 4.6

Effects of insulin and Intralipid infusion on 11HSD1 activities in subcutaneous adipose tissue. Results are from a 3.5 hour 'run-in' tracer infusion followed by 3.5 infusion a) insulin or b) Intralipid. Data are mean ± SEM of 'placebo-corrected' values, i.e. the difference between the value for each participant during saline infusion from the results during insulin or Intralipid infusion. Absolute values during saline infusion are shown in Figure 4.5a. Differences in conversion of 3H-cortisone to 3H-cortisol (reductase activity) are in filled diamonds and differences in conversion of 3H-cortisol to 3H-cortisone are in open diamonds. By repeated measures ANOVA, insulin altered reductase ($p<0.05$) but not dehydrogenase activity, and Intralipid altered both reductase ($p<0.01$) and dehydrogenase ($p<0.03$) activities.

4.3.4 Adipose 11HSD1 Activity (microdialysis)

During infusion of ^{3}H-steroid, 11HSD1 reductase activity (conversion of cortisone to cortisol) exceeded dehydrogenase activity (conversion of cortisol to cortisone) as measured during the placebo infusion (Figure 4.5). During acute hyperinsulinaemia in a euglycaemic clamp, reductase activity tended to fall during the first hour but increased by 3.5 h (Figure 4.6, ANOVA $p<0.05$); dehydrogenase activity showed similar changes which were not statistically significant. Intralipid infusion increased both reductase (ANOVA $p<0.01$) and dehydrogenase activities (ANOVA $p<0.03$).

4.4 Discussion

These data demonstrate acute changes in cortisol metabolism in healthy humans in response to manipulation of fuel substrate availability. The increase in cortisol generation by 11HSD1 within peripheral tissues following the introduction of hyperinsulinaemia or hyperlipidaemia suggests that adjustment of intracellular cortisol concentration may be a key component of the physiological response to feeding.

Hyperinsulinaemia produced a highly statistically significant increase in whole-body regeneration of D3-cortisol by 11HSD1. This observation is novel since previous *in vivo* data are scarce, and those which rely upon measurement of urinary cortisol metabolites (Kerstens et al 2000) probably have insufficient sensitivity and specificity to confirm or refute any effect of insulin on 11HSD1. In one previous study D4-cortisol infusion was undertaken before and after hyperinsulinaemia (Basu et al 2004), but no norm-insulinaemic control group was included, and effects on splanchnic D3-cortisol production were not reported in detail, although there was a small increase in D3-cortisol generation in the leg. Recent studies using arteriovenous sampling have suggested that the major source of this extra-adrenal production of cortisol is the splanchnic circulation (Basu et al 2004), with contributions from both liver and other visceral tissues (most likely visceral adipose tissue)(Andrew et al 2005). Regeneration of cortisol in non-splanchnic tissues has been detected, for example in the leg (Basu et al 2005), where it may still play an important role in determining local concentrations of cortisol, even if substantial cortisol is not released into the circulation. In the current experiments, however, we

found only a small effect of insulin locally within subcutaneous adipose tissue (Figure 4.6). Investigations from our lab have previously shown that acute hyperinsulinaemia induces a rapid, but temporary, fall in intra-adipose cortisol generation in lean but not obese men (Sandeep et al 2005). Here, the immediate fall in 11HSD1 activity with hyperinsulinaemia was of smaller magnitude (Figure 4.6), perhaps because the subjects had not fasted overnight in advance of the introduction of hyperinsulinaemia. This was followed by a minor increase in 11HSD1 activity, which is unlikely to contribute substantially to the increase in whole body D3-cortisol generation. These observations suggest that the acute effects of insulin on cortisol regeneration occur in splanchnic rather than peripheral tissues. They do not allow discrimination of effects in liver versus visceral adipose tissue, since it is quite possible that regulation of 11HSD1 differs in subcutaneous versus visceral adipose tissue, as illustrated by the lack of evidence for increased 11HSD1 in visceral adipose tissue in obese subjects (Tomlinson et al 2002;Aldhahi et al 2004), despite compelling evidence for increased enzyme expression in subcutaneous adipose tissue (Rask et al 2001;Paulmyer-Lacroix et al 2002;Rask et al 2002;Wake et al 2003;Lindsay et al 2003;Engeli et al 2004;Kannisto et al 2004) (see chapter 3).

Intralipid infusion also influenced cortisol metabolism. However, in contrast with insulin, Intralipid induced substantial changes in subcutaneous adipose tissue 11HSD1 without altering systemic measurements of deuterated-cortisol metabolism. There was no accompanying change in serum insulin concentrations. These observations suggest a specific effect on 11HSD1 in extra-splanchnic tissues, specifically subcutaneous rather than visceral adipose tissue, where fatty acid turnover is high (Nielsen et al 2004) and the contribution of cortisol regeneration to the systemic circulation is probably low (Basu et al 2004). This may be mediated directly by changes in fatty acid flux and concentration within the adipose cells but this possibility has not been tested *in vitro*.

The mechanism of the effect of insulin and lipid infusion has not been tested here. *In vitro,* insulin has been reported to down-regulate 11HSD1 expression and activity in a number of cells, including hepatocytes, fibroblasts and adipose cells (reviewed in (Tomlinson et al 2004b)). However, the *in vitro* effects may be confounded by the influence of insulin on cellular differentiation in cell culture.

Given the very acute effect of insulin observed in our previous study (Sandeep et al 2005) we suspected a post-transcriptional effect. Stewart and colleagues have recently proposed that variations in supply of NADPH cofactor for 11HSD1 by the enzyme hexose-6-phosphate dehydrogenase may limit the capacity for reductase activity (regenerating cortisol from cortisone), allowing a switch in favour of dehydrogenase inactivation of cortisol by 11HSD1 (Hewitt et al 2005). We did find dehydrogenase activity within subcutaneous adipose tissue *in vivo*, as previously reported *ex vivo* (Bujalska et al 2002a), but insertion of the microdialysis cannulae causes significant local trauma so that it is possible that this reflects 11HSD1 favouring dehydrogenase activity only when it is dissociated from hexose-6-phosphate dehydrogenase in damaged tissues. The magnitude of dehydrogenase activity in adipose tissue *in vivo* might be assessed better with arteriovenous sampling studies. Importantly, there was no evidence from our data that acute regulation of 11HSD1 by insulin or Intralipid infusion is due to a switch in enzyme direction (from reductase to dehydrogenase or vice versa) since enzyme activities changed in parallel (Figure 4.6). Indeed, the time course of the Intralipid effect, being maximal at the end of a 3.5 hour infusion, is consistent with transcriptional regulation of availability of 11HSD1 protein, albeit that free fatty acid concentrations were also rising steadily during the infusion (Figure 4.2).

Although the effects on regeneration of cortisol were the most obvious in this study, there may also be effects of insulin and lipids on other enzymes which metabolise cortisol. Basu et al. have reported that insulin increases splanchnic cortisol uptake (Basu et al 2004) while in the current study, in the face of increased peripheral regeneration of cortisol during hyperinsulinaemia there was no decrease in cortisol clearance rates. This suggests that insulin increases removal of cortisol by enzymes other than 11HSD1. The pathway responsible was not identified, since rates of excretion of deuterated-cortisol metabolites in urine were not increased by insulin (Table 4.1). Whether the increase in plasma cortisol which follows ingestion of food (Follenius et al 1982) has a component determined by altered cortisol metabolism has therefore not been resolved here.

These data have important implications in physiology and pathology. Physiologically, they highlight that the rate of local regeneration of cortisol is

dynamic rather than static, and may allow acute control of glucocorticoid action over and above alterations in the hypothalamic-pituitary-adrenal axis. In pathology, the contrasting effects of insulin and lipid infusions on 11HSD1 in different tissues highlights the capacity for tissue-specific regulation, which presumably underlies the tissue-specific dysregulation which accompanies obesity (Rask et al 2001). Indeed, we have shown that obese humans resist acute regulation of adipose 11HSD1 by hyperinsulinaemia (Sandeep et al 2005), while obesity-prone strains of mice resist down-regulation of adipose 11HSD1 by high fat feeding (Morton et al 2004b). The current studies also illustrate that detailed dissection of cortisol metabolism within individual tissues in humans is now not only possible using these contemporary tools, but likely to yield novel insights into glucocorticoid signaling in health and disease.

4.5 Regulation of Adipose 11HSD1 by Insulin and Glucose *in vitro*

4.5.1 Introduction

To further assess the cellular mechanisms behind insulin and glucose regulation of 11HSD1 in adipocytes, in vitro studies in primary rat adipocytes were performed. I assessed whether changes in 11HSD1 activity were associated with changes in mRNA and/or changes in enzyme directionality, suggesting post transcriptional modification via H6PDH as discussed above. In addition, the contribution of insulin vs glucose as a regulator of 11HSD1 was assessed.

4.5.2 Methods

Primary adipocyte cultures were freshly prepared from adult male wistar rat epididymal fat (see section 2.9). These were incubated in the presence or absence of insulin and with varying concentrations of glucose, to form four groups; NILG= no insulin, low glucose, NIHG= No insulin, high glucose, ILG= insulin, low glucose, IHG= insulin, high glucose (see below):

11HSD1 reductase and dehydrogenase activities were assessed by separate incubations with either 11-dehydrocorticosterone or corticosterone respectively, assayed at 1 hour and 3.5 hours. Trizol was added to the remaining adipocytes, mRNA extracted and quantified as discussed (section 2.9). Glucose uptake in the

presence and absence of insulin was assessed (see section 2.9.3) All assays were completed in duplicate. The whole experiment was repeated 6 times on different days with different animals.

	Insulin Conc	Glucose Conc
No Insulin, Low Glucose (NILG)	Nil	5.6mM
No Insulin, High Glucose (NIHG)	Nil	22.4mM
Insulin, Low Glucose (ILG)	5nM	5.6mM
Insulin, High Glucose (IHG)	5nM	22.4mM

4.5.2.1 Statistics

All data were placebo corrected and assessed by ANOVA to determine differences between groups and the individual role of insulin and glucose as a determinant of change by post hoc analysis. Data was further analysed using Students t-tests where discussed.

4.5.3 Results

4.5.3.1 Glucose Uptake

Insulin resulted in increased glucose uptake into adipocytes ($p<0.05$).

4.5.3.2 11HSD1 Enzyme Directionality

After 1hr culture under basal conditions (no insulin, low glucose), reductase activity is significantly greater than dehydrogenase (15.9 ± 1.1 vs 10.3 ± 0.9, $p<0.002$, by students t-test), but no significant difference was found at 3.5hrs (19.0 ± 1.8 vs 18.1 ± 2.1, $p=0.34$).

4.5.3.3 Insulin and Glucose Regulation

There were significant reductions in 11HSD1 reductase activity in the presence of either insulin or high glucose at 1 hour. Insulin alone (without hyperglycaemia) increased 11HSD1 reductase activity at 3.5hours (figure 4.7).There was no difference in dehydrogenase activity between groups after at any time point (figure 4.8) There was no differences in 11HSD1 mRNA expression after one hour, but 11HSD1 mRNA was increased after 3.5 hours, in the presence of both insulin

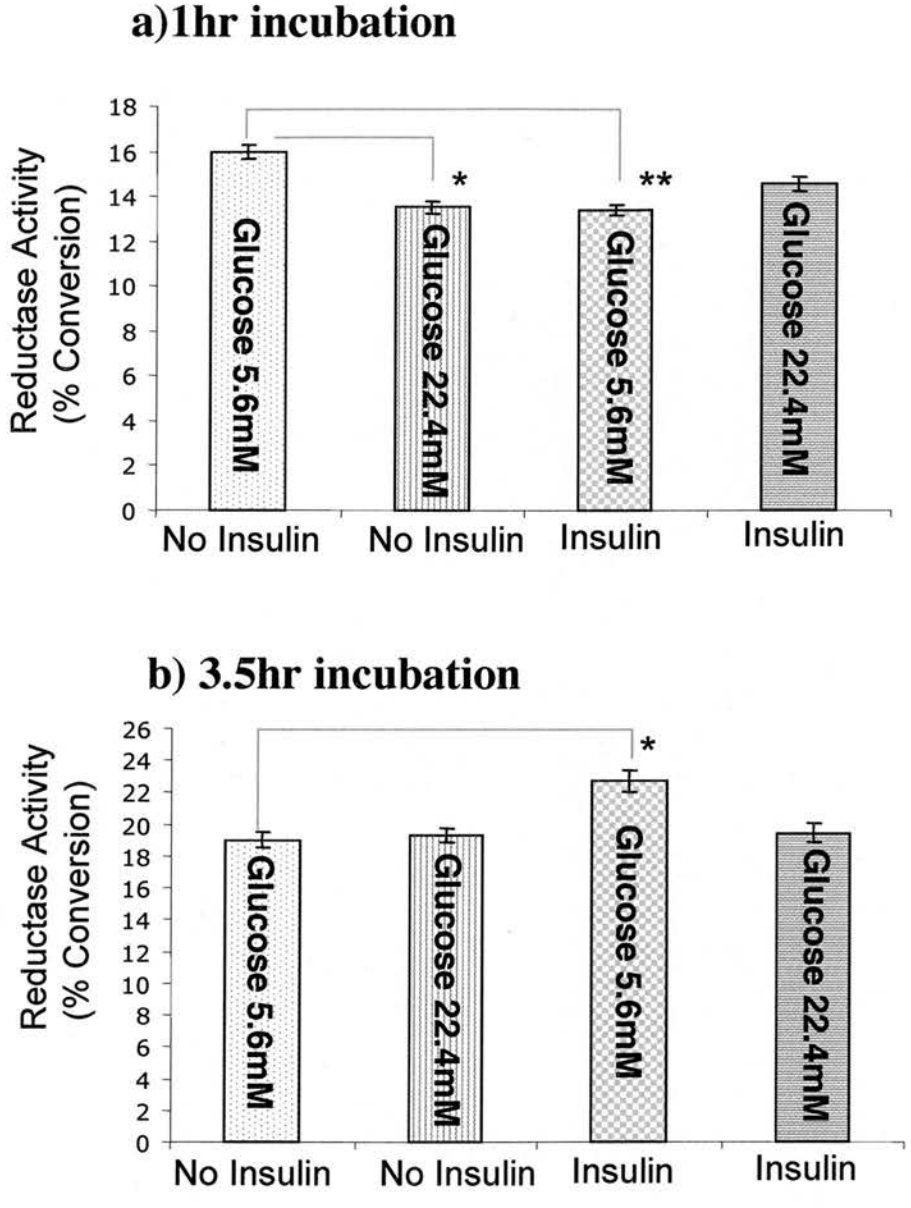

Figure 4.7

Acute changes in 11HSD1 reductase activity in primary rat adipocytes with insulin and glucose after a) 1 hour of incubation and b) 3.5 hour of incubation. There were significant differences between all groups (assessed by ANOVA) a) $p=0.026$ at 1hr and b) $p=0.025$ at 3.5 hrs. Values shown are *=$p<0.05$, **$p<0.01$ when compared by t-test with the control group (No Insulin, Low Glucose).

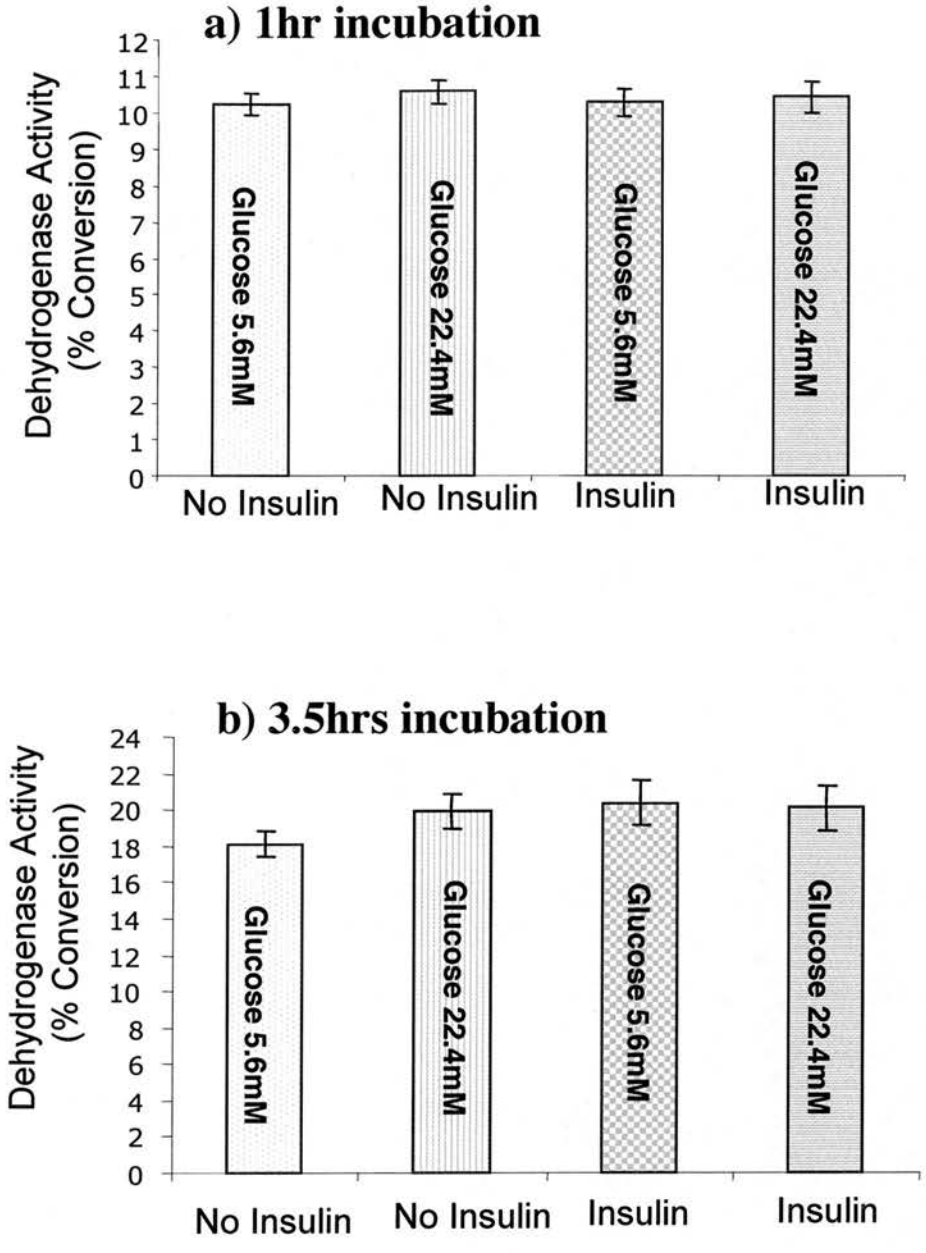

Figure 4.8

Acute changes in adipose 11HSD1 dehydrogenase activity in primary rat adipocyte cultures by insulin and glucose after a) 1hour of incubation and b) 3.5 hours of incubation. There was no significant changes in 11HSD1 dehydrogenase activity found between group at any time points.

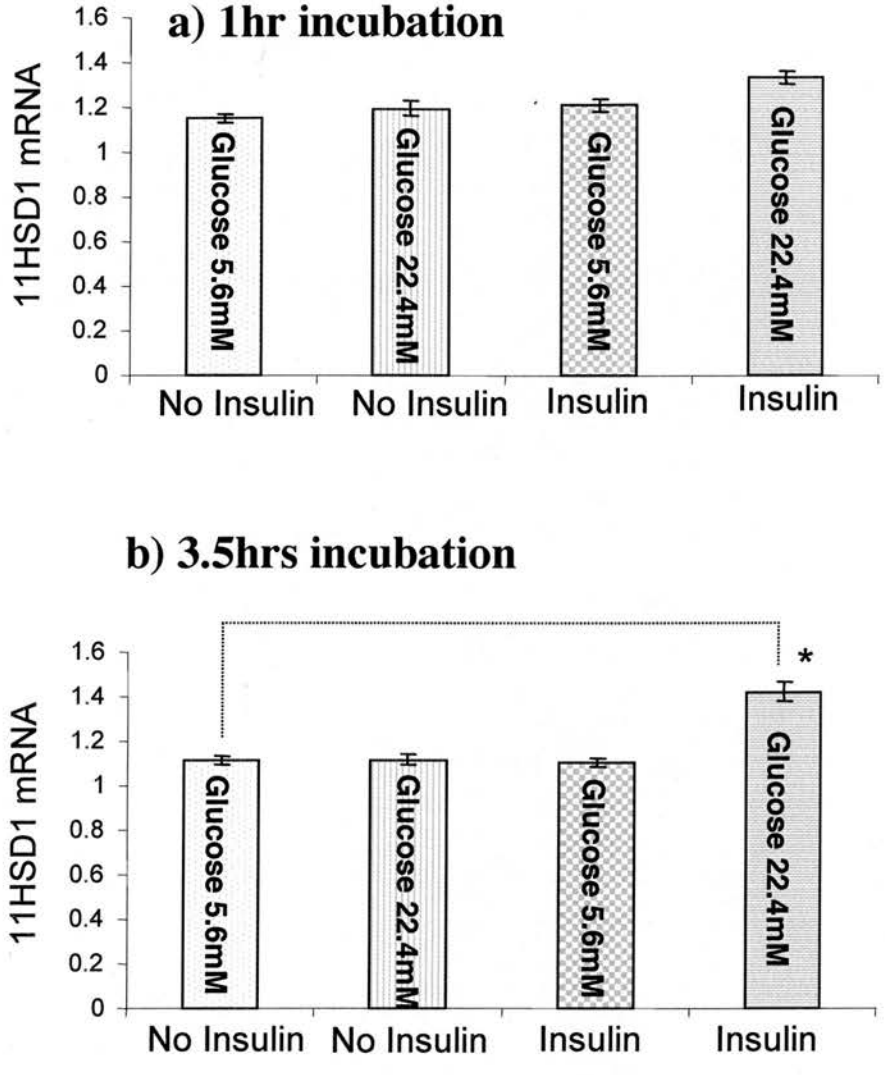

Figure 4.9

Acute changes in 11HSD1 mRNA in primary rat adipocytes with insulin and glucose

There were no differences in mRNA expression at 1 hour. There were significant differences in 11HSD1 mRNA between groups at 3.5 hours (ANOVA $p<0.00$). Post hoc analysis showed that this could be attributed to independent changes in both glucose ($p=0.006$) and insulin ($p=0.04$). Values shown are $^{**}p<0.01$ when compared by t-test with the control group (No Insulin, Low Glucose).

and hyperglycaemia together (Figure 4.9). In all groups combined, 11HSD1 reductase activity was associated with changes in mRNA after 3.5hrs ($r=0.41$, $p=0.005$), but not after 1 hr ($r=0.16$, $p=0.28$).

4.5.4 Discussion

In vitro regulation of 11HSD1 with insulin and glucose using rat adipocytes in primary culture generally mimicked in vivo findings with an initial fall then subsequent rise in 11HSD1 reductase activity at 1hr and 3.5hrs respectively. The initial reduction in 11HSD1 reductase activity was not associated with changes in mRNA or enzyme directionality and appeared to be regulated by both insulin and glucose; suggesting a post transcriptional process driven by 'glucose flux'. Increased 11HSD1 reductase activity at 3.5hrs occurred only in the low glucose/ high insulin group, whereas increased mRNA was significantly increased in the high insulin/ high glucose group only (i.e different groups). Across all samples there was, however, a significant correlation between 11HSD1 activity and mRNA at 3.5 hours ($p<0.005$) which was not seen at 1hr. Although speculative, this suggests that transcriptional regulation may have a role in mediating changes in enzyme activity at the later time point, although clearly these preliminary studies are far from conclusive. In support of *in vivo* findings, these studies do not suggest that the enzyme directionality is acutely modulated by changes in cofactor availability. Had time permitted, I would have assessed regulatory pathways in more detail, using inhibitors of transcription, post transcriptional modification and insulin signalling and by in vitro manipulation of H6PDH. In addition, I suggested earlier in this chapter that 11HSD1 up-regulation in adipose with intravenous lipid followed a time course compatible with transcriptional upregulation and would also have liked to have determined this using in vitro studies or by mRNA analysis of human adipose biopsies.

Regulation of adipose 11HSD1 by lipid and dietary fat may alternatively be mediated indirectly. One potential mechanism is through peroxisome proliferator-activated receptors (PPARs) acting as cellular signallers of free fatty acid flux. The potential regulatory role of PPAR agonists on 11HSD1 is discussed further in the next chapter.

Chapter 5
REGULATION OF CORTISOL METABOLISM BY PPAR AGONISTS

5.1 Introduction

In chapter 4, we demonstrated regulation of cortisol metabolism by two key nutritional factors; insulin and lipid. Acute upregulation of adipose 11HSD1 by lipid contrasts with reports of chronic down regulation with high fat feeding in animal models (Morton et al 2004b). The exact mechanisms underlying nutritional regulation of 11HSD1 remain unknown, but PPARs (as an important intracellular lipid signaling pathway) may be implicated. PPAR agonists can downregulate 11HSD1 in adipose (PPAR-γ) (Berger et al 2001) and liver (PPAR-α) (Hermanowski-Vosatka et al 2000) respectively in animal and cell models, although their effect in-vivo in humans is unknown. In addition, PPAR agonists are used therapeutically; PPAR-γ agonists (glitazones or thiazolidinediones) are adipose insulin sensitizing agents used in type 2 diabetes whilst PPAR-α agonists (fibrates) work within liver as lipid lowering drugs. It is an intriguing possibility that lowering of intra-cellular cortisol levels via 11HSD1 is a key mechanism by which PPAR agonists mediate their downstream therapeutic effects thus limiting the metabolic consequences of obesity. The main endogenous ligands for PPARs are free fatty acids and PPAR activation may underlie regulation of 11HSD1 with high fat diet and short term lipid exposure.

To assess this further, we assessed *in vivo* in 9 healthy male volunteers the effects of short term PPAR agonists on whole body glucocorticoid turnover and excretion, and tissue specific regulation of 11HSD1 within adipose and liver.

5.2 Methods (see chapter 2 and 4 for details)

Under ethical approval (Lothian regional ethics committee), 9 healthy men (BMI 20-35kg/m^2) participated in a 3-phase double-blind crossover study comparing 1 week of oral rosiglitazone (Avandia 4 mg daily), or fenofibrate (Lipantil Micro 200 mg daily) with placebo (once daily tablet). The study order was randomised and balanced. Phases were separated by 2-week washout. At the end of each treatment

phase volunteers attended on two consecutive days for the following measurements (discussed in detail in chapter 2):

i) 24 h urine collection for measurement of cortisol metabolites by GCMS (during the 24 hours prior to day 1)
ii) plasma lipids, insulin, glucose after overnight fast (day 1)
iii) $^{2}H_{4}$-cortisol steady state infusion to measure 'whole-body' 11HSD1 activity (day 1)
iv) infusion of ^{3}H-cortisone by microdialysis into abdominal subcutaneous adipose tissue to measure adipose 11HSD1 activity (day 1) and
v) oral 'cortisone to cortisol' test to measure hepatic 11HSD1 activity (after overnight dexamethasone suppression) (day 2).

5.2.1 Statistics

Sample size calculations were employed to calculate the participant number required. The primary end point in this study was changes in 11HSD1 activity. The sample size was calculated to give 80% power to detect differences in systemic, hepatic and adipose 11-HSD activity of >20%, based on variance obtained with all of these measurements in previous studies. This magnitude of change, equivalent to an increase of plasma cortisol from 300 nM to 360 nM is more than enough to be biologically important, according to data from our large epidemiological studies associating cortisol with insulin resistance (Reynolds et al 2001).

In analysis of tracer and microdialysis data, when repeated measures analysis was undertaken, there were very few missing time points. Where a single time point was missing an average value was calculated using preceding and following values. For other data (not repeated measures), missing data points resulted in exclusion of the case from paired comparison of that variable.

Data are presented as mean ± SEM. To reduce the variance of kinetic parameters derived from deuterated-steroid measurements, values were averaged for five measurements taken during steady state in the final 45 minutes of the five hour infusion to calculate plasma kinetics (255-300 min) (see chapter 2). Plasma data was further analysed using paired t-tests to establish differences between placebo and treatment phases. The time course of effects of fenofibrate and rosiglitazone

treatment on adipose 11HSD1 (microdialysis) was examined by repeated measures ANOVA using 'placebo corrected' data (i.e. values at each time point for fenofibrate or rosiglitazone were subtracted from the values for the same time point in the placebo phase for each participant). (Justification of this method of analysis is discussed in section 4.2.3). Microdialysis values presented were calculated from area under the curve (AUC) during the 5 hour period.

5.3 Results

5.3.1 Metabolic Effects; plasma markers (Table 5.1)

Treatment with fenofibrate resulted in lower plasma cholesterol, a trend to lower serum triglycerides, but no significant changes in plasma glucose or fasting insulin. Treatment with Rosiglitazone resulted in no significant changes in plasma cholesterol, fasting triglycerides, fasting glucose, or fasting insulin.

5.3.2 Cortisol Tracer Kinetics (figure 5.1 and 5.2 and table 5.2)

As previously described (Andrew et al 2002;Sandeep et al 2005), plasma D4-Cortisol enrichment and D4-cortisol:D3-cortisol ratios were in steady state at the end of the study. The rate of appearance of plasma D3-cortisol and cortisol measured over the steady state period did not change as a result of fenofibrate or rosiglitazone treatment. The clearance rates for cortisol appeared to be reduced by fenofibrate, but not rosiglitazone. Clearance of D4-cortisol was not significantly changed by either drug.

Tracer cortisol metabolites were measured in the urine during the last hour of the plasma tracer infusion. There were non significant reductions in 5α-reduced urinary metabolites with fenofibrate but no significant changes in the urinary excretion of tracer cortisol metabolites with either fenofibrate or rosiglitazone.

5.3.3 Adipose 11HSD1

The PPARα agonist fenofibrate had no significant effect on adipose 11HSD1 reductase activity (AUC; 44.1 ±9.6 vs 49.3 ± 7.1 (placebo), p=0.13 by repeated measures ANOVA). The PPARγ agonist rosiglitazone however lowered reductase activity significantly (AUC; 45.5 ± 6.6 vs 53.9 ±8.4 (placebo) p=0.01 by repeated measures ANOVA) (figure 5.3).

Table 5.1

PPAR Regulation: Metabolic measurements and urinary metabolites

	Placebo	Fenofibrate	Rosiglitazone
Plasma Measurements:			
Fasting Glucose (mmol/l)	5.2 ± 0.2	5.2 ± 0.2	5.1 ± 0.2
Fasting Insulin (mU/l)	7.7 ± 2.7	7.9 ± 2.2	7.2 ± 1.3
Total Cholesterol (mmol/l)	4.6 ± 0.3	**4.2 ± 0.3***	4.8 ± 0.3
Fasting Triglycerides (mmol/l)	1.4 ± 0.3	1.0 ± 0.2$^{\alpha}$	1.6 ± 0.3
Urinary Metabolite Excretion over 24 hours (μg/ 24 hours):			
5α-THF	1057 ± 190	1171 ± 334	1691 ± 334$^{\alpha}$
5β- THF	1851 ± 367	1762 ±289	2301 ± 616
5β-THE	2943 ± 620	3012 ±520	3601 ± 972
Cortisol	85.1 ±12.9	80.6 ± 10.1	85.8 ± 16.2
Cortisone	120.4 ± 17.0	113.6 ± 13.2	131.1 ± 22.9
Sum Total Metabolites	5850 ±997	5944 ± 991	7594 ± 1719
Urinary Ratios:			
11HSD1 (THF+5αTHF/ THE)	1.1 ± 0.1	**0.99 ± 0.07***	2.1 ± 1.0
5α Reductase (5αTHF/F)	14.8 ± 3.2	13.8 ± 3.2	25.1 ± 7.2
5β Reductase (5βTHF/F)	23.3 ± 5.0	22.0 ± 2.6	24.2 ± 3.9
5β Reductase (5βTHE/E)	24.9 ± 3.9	27.0 ± 3.5	24.4 ± 4.2
11HSD2 (F/E)	0.72 ± 0.05	0.73 ± 0.04	0.65 ± 0.03
5α/ 5β Ratio (5αTHF/5βTHF)	0.79 ± 0.2	0.68 ± 0.16	2.2 ± 1.5

Data are mean ± SEM. *$p<0.05$, $^{\alpha}p<0.1$ by paired Student's t tests compared with placebo. THF= tetrahydro-cortisol, THE= tetrahydro-cortisone, F=cortisol, E= cortisone. Sum Total Metabolites= 5β-THF + 5α-THF +5β-THE

Table 5.2
PPAR Regulation: $^{2}H_4$-Cortisol kinetics

	Placebo	Fenofibrate	Rosiglitazone
Steady state plasma calculations:			
F Clearance (L/ min)	0.19 ± 0.02	**0.17 ±0.02***	0.21 ± 0.03
d4F Clearance (L/ min)	0.37 ± 0.06	0.35 ± 0.08	0.43 ± 0.09
Ra F (nmol/min)	50.7 ± 14.4	54.8 ± 16.4	67.01 ± 16.0
Ra d3F (nmol/min)	16.3 ± 1.6	16.07 ±2.6	16.6 ±2.8
Urinary labeled steroid excretion in steady state (t 240-300) (nmol/min):			
D4-cortisol	0.12 ± 0.02	0.09 ± 0.02	0.10 ±0.02
D4-5α-tetrahydrocortisol	1.33 ± 0.29	1.03 ± 0.21	0.91 ± 0.14
D4-5β- tetrahydrocortisol	1.57±0.24	1.54 ±0.21	1.42 ± 0.19
D3-cortisone	0.21 ±0.01	0.18 ± 0.02	0.22 ± 0.01
D3-5β-tetrahydrocortisone	1.95 ± 0.14	2.04 ±0.3	2.05 ± 0.34
D3-cortisol	0.27 ±0.07	0.23 ±0.05	0.18 ± 0.02
D3-5α-tetrahydrocortisol	1.05±0.19	0.85 ±0.17	0.75 ± 0.11
D3-5β-tetrahydrocortisol	1.14 ± 0.15	1.17 ± 0.15	1.01 ±0.11
D3-THFs/D4-THFs	0.77 ± 0.02	0.80 ±0.03	0.77 ± 0.03
D3-THE/D4-THFs	0.76 ±0.11	0.85 ± 0.14	0.84 ± 0.07
D3-THFs/D3-THE	1.12 ± 0.11	1.1 ± 0.13	0.97 ± 0.08

Data are mean ± SEM. *$p<0.05$, $^{\alpha}p<0.1$ by paired Student's t tests compared with placebo. For plasma measurements, kinetics were calculated using the mean of all measurements between 255 and 300 minutes of the 5 h D4-cortisol tracer infusion. For urinary data values were calculated on urine over the last 1 hour of the tracer infusion.

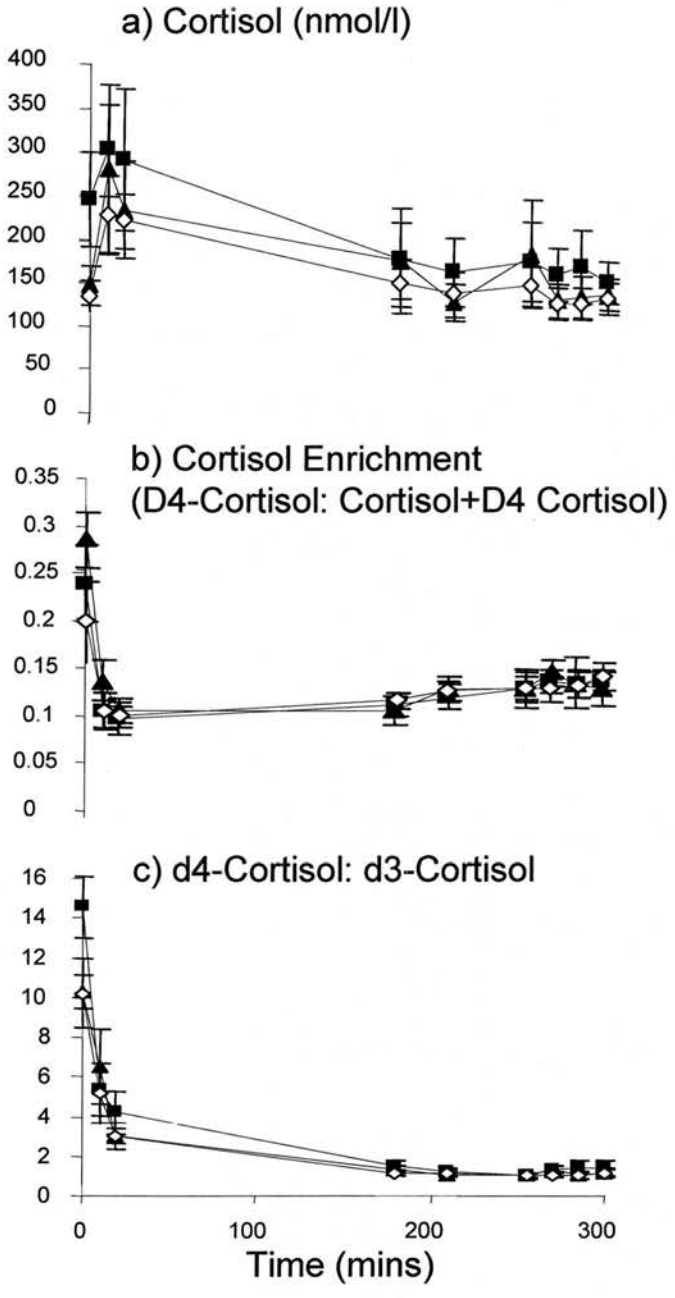

Figure 5.1

Effects of PPAR agonists on in vivo cortisol kinetics. Data are mean ± SEM. for measurements during a 5 hour tracer infusion followed treatment with PPAR agonists/ placebo. Placebo (open diamonds, solid line), fenofibrate (filled squares, dotted line) or rosiglitazone (filled triangles, solid line).

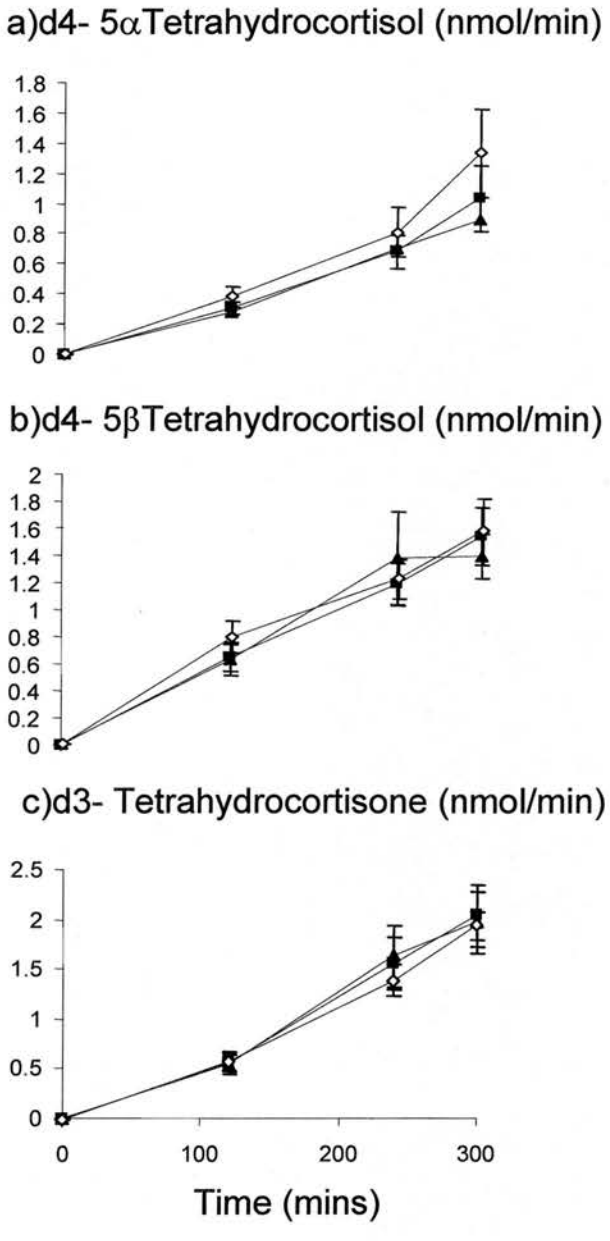

Figure 5.2

Effects of PPAR agonists on in vivo cortisol urinary tracer metabolite excretion. Data are mean ± SEM. for measurements during a 5 hour tracer infusion followed treatment with PPAR agonists/ placebo. Placebo (open diamonds, solid line), fenofibrate (filled squares, dotted line) or rosiglitazone (filled triangles, solid line).

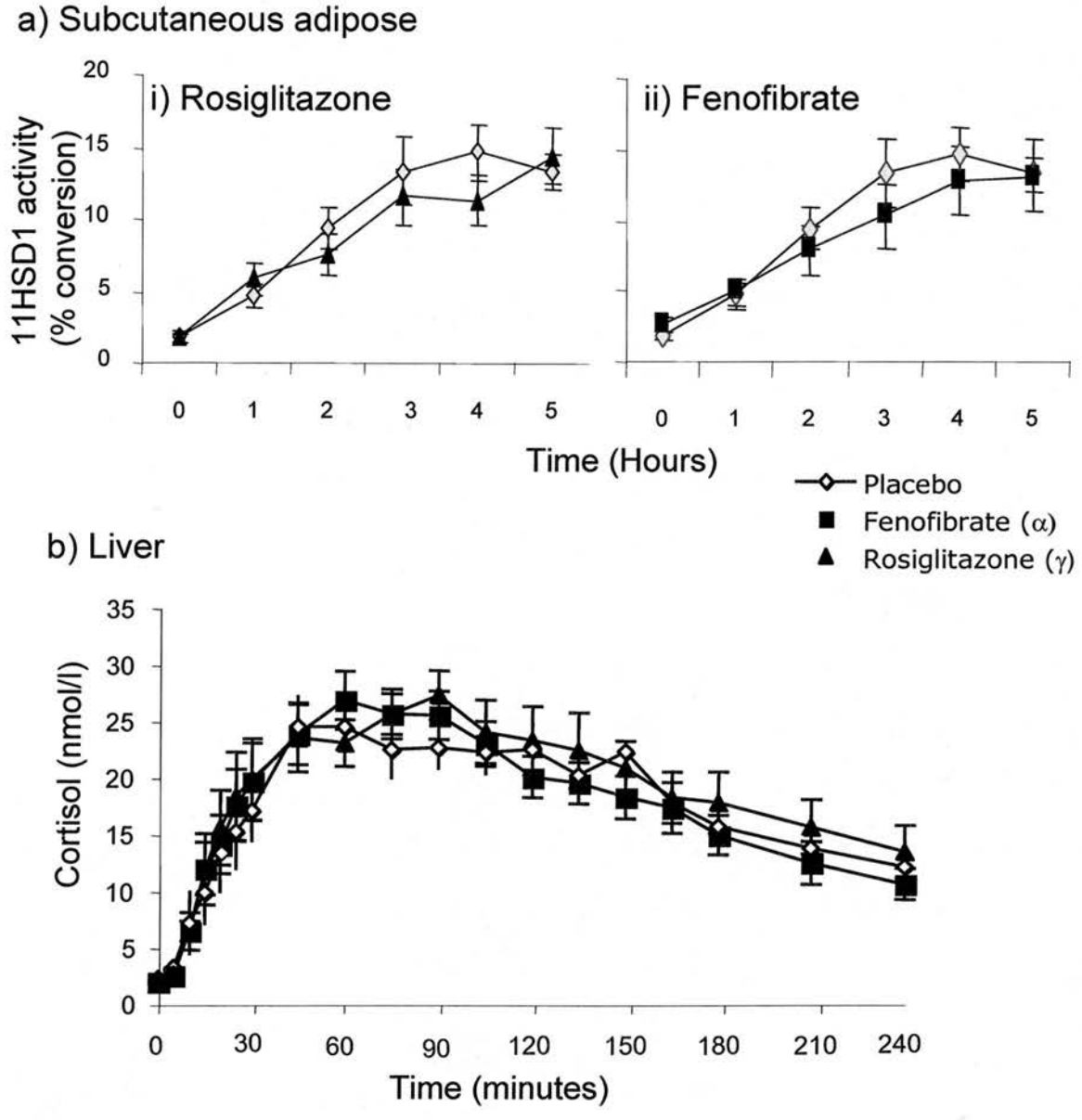

Figure 5.3

Effects of PPAR agonists on 11HSD1 reductase activity in adipose and liver

Data are mean ± SEM. Adipose 11HSD1 reductase activity was reduced by rosiglitazone (ANOVA,$p<0.01$), but not fenofibrate. Adipose 11HSD1 activity measured by hourly conversion of ^{3}H-cortisone using *in vivo* microdialysis. Liver 11HSD1 activity was assessed by conversion of oral cortisone to plasma cortisol. No significant difference seen with Rosiglitazone or Fenofibrate.

5.3.4 Liver 11HSD1; oral cortisone to cortisol test

Appearance of cortisol in the plasma following oral cortisone was analysed and kinetic parameters calculated using Kinetica^{TM} software (Innaphase Corporation, PA, USA) (figure 5.3). Neither PPARα agonist fenofibrate nor PPARγ agonist rosiglitazone had a significant effect on any of the kinetic parameters measured (Rate of appearance, area under curve, maximum cortisol concentration (CMax), elimination constant (Kel), half life or clearance rates (Cl)). There were trends towards lower clearance parameters with Rosiglitazone (higher Kel; 6.5 ± 2.3 vs 5.5 ± 2.9, p=0.13 and reduced Cl; 116.4 ± 3.93 vs 176.19 ± 15.54, p=0.17)

5.3.5 Urinary Cortisol Metabolites (24 hour urine collection) (table 5.1)

In urine collected over the 24 hour period immediately prior to admission to the research facility, there was a trend towards higher 5α-THF production with rosiglitazone but no other difference in the total excretion of urinary cortisol (tetra-hydro) metabolites or excretion of any other individual metabolite (all p>0.1). There was no difference in the relative 5α and 5β reduction of cortisol as assessed by Ulick's A-ring reduction quotients (section 2.6.3) but the ratio of endogenous THFs to THE (indicative of total 11HSD1 activity) was lowered by fenofibrate (table 5.1).

5.4 Discussion

Cortisol generation within adipose tissue may be important in mediating adipogenesis, insulin resistance, dyslipidaemia and hypertension as is demonstrated by the obese and dysmetabolic phenotype of adipose specific 11HSD1 overexpressing mice (Masuzaki et al 2001). Further, obese humans have elevated 11HSD1 within sub-cutaneous adipose which correlates well with the degree of insulin resistance (Lindsay et al 2003). Rosiglitazone is an insulin sensitising agent used in the treatment of type 2 diabetes. Previous in vitro studies demonstrate reduced 11HSD1 following short term PPARγ agonist therapy (Hermanowski-Vosatka et al 2000). Here, we demonstrate a small but significant reduction in 11HSD1 activity in vivo in subcutaneous adipose tissue in man with oral PPARγ agonists. We speculate that lowering of adipose 11HSD activity (and intra-adipose

cortisol) may potentially contribute to the action of thiazolidinediones, altering transcription of insulin sensitising genes via glucocorticoid receptors.

PPARγ is an important mediator of cellular response to dietary fat via circulating free fatty acids (FFAs) and a major regulator of adipogenesis. High fat feeding in rodents results in reduced adipose 11HSD1 and obesity prone rodent strains resist downregulation of adipose 11HSD1 by dietary fat (Morton et al 2004a;Morton et al 2004b). The underlying mechanism is unclear but we can now speculate that changes in 11HSD1 may be secondary to PPARγ activation via FFAs. Reduced cortisol generation during periods of increased free fatty acid flux may impact on lipolytic response and limit adipogenesis.

In this study we saw no change in whole body plasma glucocorticoid turnover with PPARγ activation. Plasma tracer measurements give a net effect of whole body change, and it is possible that decreased 11HSD1 in adipose is being balanced by upregulation in another tissue (e.g. liver) with no measurable change in net turnover. Alternatively, this may be because small changes in sub-cutaneous adipose 11HSD1 do not significantly contribute to the circulating glucocorticoid pool. Indeed, Basu et al (Basu et al 2004) showed that splanchnic cortisol generation accounts for the majority of whole body regeneration, suggesting that subcutaneous generation may be important locally but not systemically.

11HSD1 within liver regulate keys gluconeogenic enzymes such as PEPCK. Overexpression of 11HSD1 in the liver under an ApoE promoter results in fatty liver, dyslipidaemia, mild insulin resistance and hypertension which is thought to be mediated by increased angiotensinogen expression (Paterson et al 2004). PPARα is found mainly within liver and is the target for fenofibrate, a therapeutic lipid lowering agent, although downstream targets of the drug are poorly understood. Fenofibrate lowers liver 11HSD1 in animal studies (Berger et al 2001) and we speculated that this may contribute to its therapeutic action. In this *in vivo* study in man, fenofibrate resulted in reduced clearance of endogenous cortisol but no change in plasma tracer turnover or clearance kinetics. The impact on 11HSD1 specifically within the liver remains unclear. Although no major effect was seen on conversion of oral cortisone to plasma cortisol, the urinary ratio of THFs: THE was decreased. This might be indicative of either reduced liver 11HSD1 or altered hepatic A-ring

reductase activities. 5α metabolites did appear to be reduced by fenofibrate during tracer infusion, although this did not reach significance. Decreased 5α-reductase activity could ceratianly explain both these findings i.e. changes in urinary ratios and reduced cortisol clearance observed with fenofibrate treatment.

These studies suggest that acute regulation of cortisol generation, particularly within adipose, may have important physiological and therapeutic consequences. However, the magnitude of these changes, in the absence of systemic differences in 11HSD1, is at the threshold of sensitivity of the techniques we are using. 11HSD1 is emerging as a potentially important regulator of cellular response to dietary fat and FFA flux. Alterations in tissue cortisol levels via 11HSD1 may mediate important downstream effects via manipulation of GR-sensitive gene targets within tissues even without measurable impact on circulating glucocorticoid levels, as seen here.

Whether PPAR agonists truly require glucocorticoid signalling for their action is however unclear. We have set up further studies to test this hypothesis, looking at the effects of PPARγ agonists in the presence and absence of glucocorticoid receptor inhibitor (RU486). This will determine the role of glucocorticoid signalling in the therapeutic action of PPAR agonists and also, by assessment of mRNAs in adipose biopsies, allow determination of potential downstream glucocorticoid targets mediating insulin sensitivity.

Chapter 6

ADIPOSE SEX STEROID METABOLISM AS A DETERMINANT OF FAT ACCUMULATION AND DISTRIBUTION

6.1 Introduction

In addition to the effect of glucocorticoids, sex steroids (androgens and estrogens) also play an important role in determining body fat distribution. Central adiposity, which is associated with increased cardiovascular risk, is thought to reflect a predominance of androgens over estrogens (Bjorntorp 1997) as is evident from predisposition to 'central' ('abdominal' or 'android') obesity in men and 'peripheral' ('gynoid') obesity in women. As with glucocorticoids, sex steroid action in adipose is mediated by intra-cellular receptors for oestrogens (ERα and ERβ)(Pedersen et al 2001;Dieudonne et al 2004), and androgens (AR). Aromatase and 5α-reductase enzymes also function in a similar manner to 11HSD1 controlling pre-receptor ligand availability for oestrogen and androgen receptors respectively (section 1.13).

In addition, the aldo-ketoreductase enzymes (AKRs) are now emerging as potentially important regulators of progesterone, glucocorticoid and most significantly androgen metabolism in adipose. Several isoforms have been studied in human sub-cutaneous and omental adipose tissue. AKR1C type 1 (20α-HSD) in omental adipose tissue has been related to the degree of central obesity (Blanchette et al 2005). Conversely, AKR1C3 (i.e. 17β-HSD5, the predominant 17β-HSD isozyme in human adipose tissue) (Quinkler et al 2004) in subcutaneous rather than visceral adipose tissue has been associated with generalised obesity (Quinkler et al 2004). The significance of other human 17β-HSD isozymes is less clear: types 1-3 have not been detected by all investigators (Corbould et al 1998;Quinkler et al 2004) and type 4 is present at lower abundance than type 5 (Quinkler et al 2004). AKR1C2 (3α-HSD3) is also expressed in human adipose tissue but its role is uncertain (Quinkler et al 2004) Adipose sex steroid metabolizing enzymes are summarized in schematic – figure 1.5

Against this background, we have extended our previous studies of 11HSD1 in adipose tissue from healthy male and female adults with well-characterised body fat distribution as discussed in chapter 3 (Rask et al 2001;Westerbacka et al 2003),

by examining mRNAs in these biopsies for sex steroid receptors (ERα, AR) and enzymes dictating local activation of estrogens (aromatase), and androgens (5α-reductase type 1). Further, we assessed for the presence of the 4 AKR isoforms in adipose and quantified mRNA for the 2 predominant aldoketoreductase isoforms (AKR1C2 and AKR1C3). To understand likely pathways of dysregulation of these enzymes and receptors, and their likely impact on metabolic complications of obesity, we also measured mRNAs for potential regulating factors (IL-1, 1L-6, TNF-α) and key steroid-regulated target genes (adiponectin, resistin, LPL, HSL, PPAR-γ, angiotensinogen and leptin).

6.2 Methods

6.2.1 Subjects

We analysed adipose tissue from two previously described cohorts: 14 men and 13 women from Sweden (Rask et al 2001;Rask et al 2002;Wake et al 2003) and 18 men from Finland (Westerbacka et al 2003) (see sections 3.3.2 and 3.4.2 for details of recruitment, clinical measurements, and procedures).

6.2.2 Adipose Biopsy analysis

mRNA was extracted and processed as described in chapter 2. Transcript level quantification was performed with specific Real Time PCR primer-probe sets using the ABI PRISM 7700/ 7900 Sequence Detection System for Aromatase, 5α-Reductase Type 1, AKR1C2, AKR1C3, ER–α, AR, IL-6, IL-1α , TNF-α , LPL, HSL, PPAR-γ, Angiotensinogen, Leptin , Resistin, Adiponectin, AKR1C2 and AKR1C3.

5α-reductase type 2 is typically found in cutaneous and genital tissue and has a major role in development of an external masculinised phenotype. The type 1 enzyme, however, is found in many peripheral tissue and has a role in both glucocorticoid and androgen metabolism. We therefore assessed for the presence of the type 1, but not the type 2, enzyme. There are two estrogen receptors in adipose (ERα and ERβ). We did not assess ERβ because, although it is present in adipose, ERα is by far the predominant isoform and is thought to be the major receptor dictating adipose oestrogenic effects (Dieudonne et al 2004).

PCR was also performed using specific primers (below) for the 4 human isoforms of AKR1C in pooled cDNA from each cohort (see section 2.5.2.3) at 20,30 and 40 cycles and run on a 1.25% agarose/ TBE gel for analysis.

6.2.3 Statistics

T-tests were performed to examine differences between genders and cohorts. Relationships between biopsy measurements and anthropometric parameters in the combined cohorts were examined by multiple regression analysis and corrected for cohort and gender (both coded as 0 or 1). Real time PCR on each cohort was performed in separate batches, using a relative standard curve of diluted cDNA pooled from within each cohort, so that, although cohorts may differ in apparent absolute mRNA levels, this may reflect a systematic difference in the standard curves in the two cohorts rather than selective differences in these mRNAs. For this reason multiple regression analysis adjusted for differences between cohorts where appropriate. Inter-relationships between mRNAs were examined using multiple regression analysis to correct for cohort, gender (both coded as 0 or 1), and BMI. Results are presented as standardised beta-coefficients (r).

6.3 Results

6.3.1 Gender differences and other potential confounders

Participants were aged 54.5± 2.0 years (range 33-73) with BMI 25.9 ± 0.6 kg/m^2 (range 20.7- 32.0) in the Swedish cohort and 41.8± 2.5 years (range 22- 57) with BMI 26.4 ± 0.9 kg/m^2 (range 20.6- 35.6) in the Finnish cohort. The cohorts differed in age, percentage fat (29.0± 0.06 Sweden vs 21.5± 1.37 Finland, $p<0.01$) and waist:hip ratio (0.90± 0.01 Sweden vs 0.97± 0.02 Finland, $p<0.01$) .

Comparisons by gender and between cohorts is shown in Table 6.1. In the Swedish cohort, aromatase, 5α–reductase type 1, AR, LPL and HSL mRNAs were higher in men than women. Leptin and angiotensinogen mRNAs were higher in women. There were no gender differences in expression of other mRNAs. As discussed in section 6.2.3, although cohorts differed in apparent absolute mRNA levels for LPL and HSL, this may reflect a systematic difference in the realtime PCR standard curves in the two cohorts rather than selective differences in these mRNAs.

Table 6.1 Gender and cohort differences in adipose mRNAs and associations with anthropometric measurements

		Swedish		Finnish			
		Male (n=14)	Female (n=13)	Male (n=18)	BMI	%Fat	WHR
Steroid Metabolising Enzymes:	Aromatase	2.14±0.42	**1.11±0.2***	1.07±0.19	**0.35***	0.19	0.06
	5α-Reductase Type 1	1.34±0.23	**0.68±0.13***	1.26±0.20	-0.13	-0.14	-0.22
	AKR1C2	1.75±0.55	1.97±0.41	2.29±0.49	0.05	-0.07	**0.28***
	AKR1C3	1.87±0.77	1.23±0.19	2.90±1.18	0.22	0.18	**0.38****
Steroid Receptors	ER-α	1.11±0.25	1.14±0.32	2.05±0.51	0.10	0.06	0.04
	AR	1.56±0.33	**0.63±0.07****	0.68±0.06	-0.22	0.05	0.10
Other adipose genes	Angiotensinogen	0.84±0.15	**1.43±0.25***	1.30±0.18	-0.10	-0.09	-0.13
	Leptin	0.86±0.12	**1.32±0.15***	1.29±0.24	0.25	0.20	-0.01
	HSL	2.10±0.18	**1.53±0.16***	**0.96±0.11****	-0.29	-0.07	-0.19
	LPL	0.77±0.07	**0.56±0.04***	0.99±0.19	-0.25	-0.18	-0.26
	PPAR-γ	1.13±0.23	0.90±0.08	0.88±0.13	-0.05	-0.10	-0.25
	Resistin	1.24±0.32	0.65±0.12	0.82±0.12	-0.15	0.05	-0.12
	Adiponectin	1.01±0.19	0.71±0.07	1.96±0.31	-0.17	-0.11	-0.07
	TNF-α	1.49±0.26	1.58±0.62		0.15	-0.02	0.24
	IL-1α	2.37±0.83	2.44±1.18		0.11	-0.11	-0.23
	IL-6	1.66±0.31	3.33±1.10	2.94±1.16	0.03	0.02	0.14

Data are mean ± SE for the mRNA transcript (expressed as ratio to cyclophyllin). Gender differences were tested in the Swedish cohort only (shown as * in the Swedish females column) and cohort differences in males only (shown as * in the Finnish males column) using Student's t tests. Associations with parameters of obesity were tested in multiple regression using combined data from Swedish and Finnish cohorts; Data are shown as standardised beta coefficients adjusted for gender and study cohort. *$p<0.05$, **=$p<0.01$.

6.3.2. Presence of Aldoketoreductase

AKR1C2 was the predominant isoform in subcutaneous human adipose, followed by AKR1C3 and then AKR1C1 (20α(3α)-HSD). AKR1C4 mRNA was not detected (figure 6.1). Real Time PCR quantification of the 2 predominant isoforms revealed ~32-fold higher AKR1C2 than AKR1C3 transcript expression (23.5± 0.16 vs 28.5± 0.23 cycles), with both being higher in abundance than mRNA for aromatase (35.9± 0.17 cycles).

6.3.3. Predictors of body fat and its distribution

See Table 6.1 and figure 6.2. To examine associations with obesity, multiple regression analysis was employed with adjustment for potential confounding by gender and cohort. BMI was positively associated with adipose aromatase mRNA. Percentage body fat showed a strong trend for an association with aromatase mRNA. Neither 5α–reductase type 1, ERα nor AR mRNAs were associated with generalised obesity.

In contrast, central adiposity (measured by waist:hip ratio) was not correlated with aromatase mRNA in subcutaneous adipose tissue. In addition, central obesity was associated with higher mRNA levels for both AKR1C2 and AKR1C3. There was no overall relationship between waist:hip ratio and any other mRNAs.

6.3.4. Relationships between determinants of steroid hormone action and their potential regulators and target genes

Inter-relationships between mRNAs for steroid metabolising enzymes and their receptors were sought by multiple regression adjusting for gender, cohort and BMI. Results are shown in Table 6.2. Broadly, there were rather few correlations observed, although AKR1C3 and AKR1C2 isoform mRNAs correlated with each other.

Inflammatory cytokine expression (IL-1α, IL-6, TNF-α) was not related to body fat or its distribution (Table 6.1). To examine relationships between determinants of steroid hormone action and inflammatory cytokines, adipokines and key metabolic genes in adipose, multiple regression analyses were performed with adjustment for the effects of cohort, gender and BMI (see Table 6.3). Of note, ERα mRNA strongly

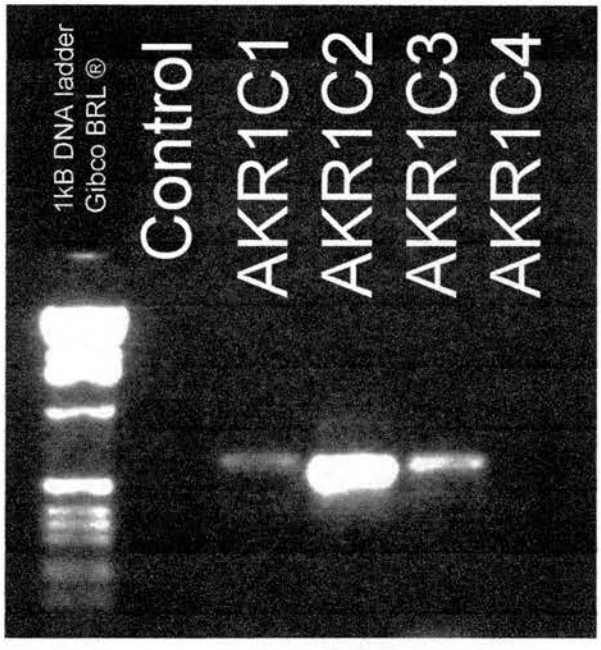

Figure 6.1

Aldoketoreductase Isoforms in Human Adipose

PCR results using specific primers for the four human 3alpha HSD genes, AKR1C1 (h20a (3α)-HSD), AKR1C2 (type 3, 3α-HSD), AKR1C3 (type 2, 3α-HSD), AKR1C4 (type 1, h3α-HSD). All PCR products are 500bp. Subcutaneous adipose cDNA was pooled from both Swedish and Finnish cohorts and PCR products generated at 40 cycles. A single RT- control is shown, but both water and RT- controls were assessed for all genes (all negative).

Table 6.2 Inter-associations between adipose mRNAs for steroid metabolising enzymes and receptors

	5α-Reductase	AKR1C2	AKR1C3	ERα	AR
Aromatase	0.17	-0.20	-0.07	0.18	0.08
5α-Reductase		-0.11	0.09	0.10	-0.05
AKR1C2			**0.46****	-0.1	0.20
AKR1C3				-0.12	0.26
ERα					0.04
AR					

Results are expressed using combined data from Swedish and Finnish cohorts. $available in Swedish cohort only. Data are standardised beta coefficients calculated using multiple regression analysis, with dependent variable in the top row and independent variable in the left column adjusted for gender, study cohort (both coded as 0 and 1) and BMI. **=p<0.01.

Table 6.3 Associations between mRNAs for steroid metabolising enzymes and receptors and their potential regulators and downstream target genes

	Angiotensinogen	Leptin	HSL	LPL	PPAR-γ	Resistin	Adiponectin	TNFα$	IL-1α$	IL-6
mRNAs										
Aromatase	-0.04	0.07	0.13	0.26	0.25	-0.15	-0.05	0.08	0.28	0.09
5α-Reductase	0.09	0.06	0.14	**0.49****	**0.36***	0.17	0.02	0.38	**0.72****	0.02
AKR1C2	-0.14	-0.10	0.07	-0.13	0.00	0.06	0.24	-0.00	-0.18	0.00
AKR1C3	0.01	-0.11	0.08	-0.09	-0.02	0.05	0.04	0.08	-0.08	-0.08
ERα	0.19	**0.62****	**0.30***	0.15	0.11	0.04	0.03	**0.85****	0.35	**0.91****
AR	-0.16	0.21	0.05	-0.13	-0.01	0.17	0.2	0.03	0.05	0.00

Results are expressed using combined data from Swedish and Finnish cohorts. $= available in Swedish cohort only. Data are standardised beta coefficients calculated using multiple regression analysis, adjusted for gender, study cohort and BMI. *=p<0.05, **=p<0.01.

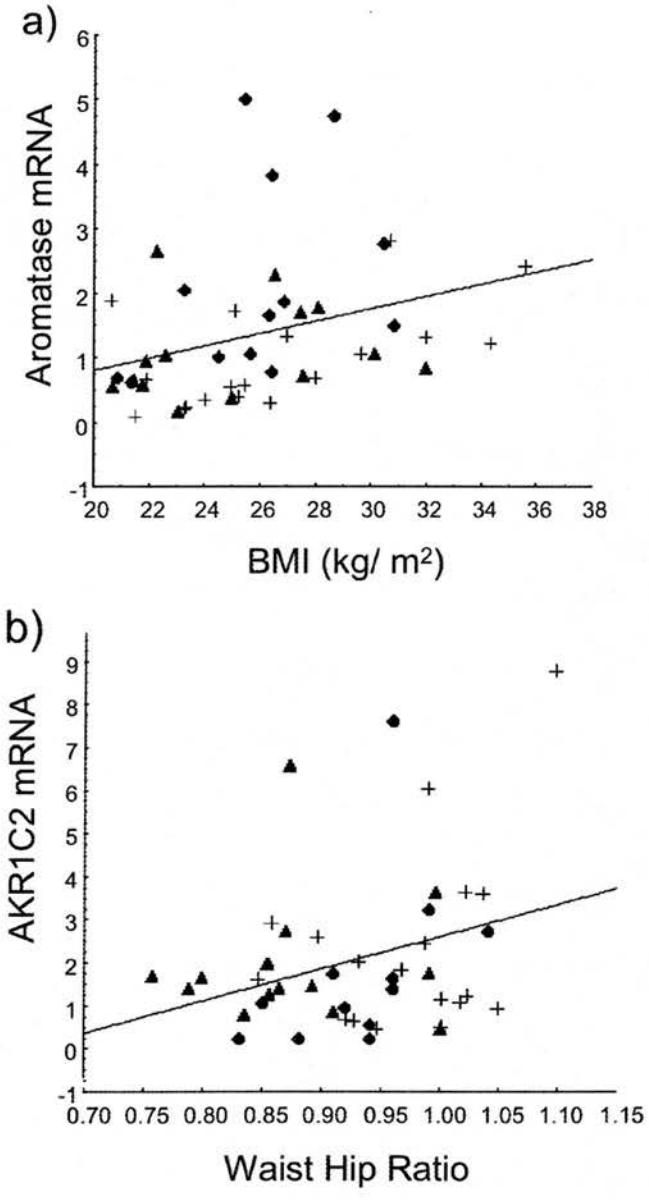

Figure 6.2

Associations of obesity with mRNA for (a) aromatase and (b) AKR1C2 in subcutaneous adipose tissue

Results are shown for filled diamonds=Swedish men, filled triangles=Swedish women, and crosses=Finnish men. Correlations are shown in Table 1.

positively correlated with IL-6 and TNF-α mRNA. IL-1α was associated with 5α-reductase 1 mRNA.

Regarding metabolic genes in adipose tissue, findings were rather diverse. HSL and leptin mRNAs correlated with ERα mRNA. 5α-Reductase 1 mRNA correlated positively with LPL and PPARγ mRNAs. Neither resistin nor adiponectin mRNAs correlated with any mRNAs for determinants of steroid signaling.

6.4 Discussion

These studies address the associations of variations in sex steroid hormone signalling in subcutaneous adipose tissue with body fat accumulation and distribution, and intra-adipose gene expression, in humans. Subjects represented a common range of body fat mass, rather than being morbidly obese or lean. The major findings were that increasing generalised obesity is associated with increased mRNA for the estrogen-generating enzyme aromatase. Conversely, central fat distribution (as measured by waist/hip ratio) was associated with no difference in aromatase, but increased AKR1C2 and AKR1C3

Genetic deficiency of aromatase in human and animal models results in central obesity (Jones et al 2000;Simpson 2000a;Jones et al 2001) while a gain of function mutation in aromatase causes a female body habitus and gynaecomastia in a male patient (Shozu et al 2003). In a previous smaller study, aromatase mRNA in omental adipose tissue was inversely correlated with BMI, while aromatase mRNA in subcutaneous adipose was not related to obesity, but the latter observation was restricted to just 8 participants (Corbould et al 2002). Our findings suggest that increased subcutaneous adipose aromatase, generating more local ER ligands and consuming potential AR ligands, may favour peripheral fat deposition rather than intra-abdominal deposition in subjects who gain weight. This may underlie raised plasma estrogen levels in idiopathic obesity (Zumoff 1982).

It is more difficult to deduce the consequences of alterations in aldoketoreductases in adipose tissue. The four human AKR1C isoforms share extensive sequence homology but have varying degrees of 3, 17 and 20 keto-reductase/dehydrogenase activity, and tissue specific distributions. AKR1C4 is almost entirely liver specific and probably responsible for much of the 3α reduction

of glucocorticoids and other 5α- and 5β-reduced pregnene steroids (Penning et al 2000) so it is unsurprising that we did not detect its mRNA in human adipose. However, confirming a previous report (Quinkler et al 2004), we found high expression of AKR1C2 and AKR1C3 in human sub-cutaneous adipose. These isoforms were expressed at higher levels than the AKR1C1 isoform, which has recently been associated with obesity by another group (Blanchette et al 2005) but has a primary role in 20α-reduction of progesterone. Extrapolation from their roles in prostate suggests that the type 2 and 3 isoforms can increase AR ligands within adipose tissue, since they convert 3α-androstanediol to 5α-DHT (AKR1C2) and androstenedione to testosterone (AKR1C3) (Figure 1.5). However, these isoforms can also catalyse 3α-reduction and inactivation of 5α-DHT. Previous studies of their net catalytic activity in human adipose tissue have been inconsistent, with reports of predominant androgen inactivation (Blouin et al 2003) and activation (Quinkler et al 2004). This merits further investigation, including measurements of steroid interconversions in vivo, but our results are consistent with the hypothesis that increased adipose AKR1C3 may increase androgen action (acting as 17β-HSD type 5 – Figure 1.5) and hence promote central obesity, but that this effect may be offset by increased androgen inactivation by AKR1C2 (acting as 3α-HSD type 3 – Figure 1.5).

Previous in vitro and in vivo studies have demonstrated the presence of 5α-reductase activity (but not mRNA) in adipose tissue (Killinger et al 1990). We have demonstrated that adipose 5α–reductase activity can be attributed to the type 1 isozyme but its expression was not associated with generalised or central adiposity. However, 5α-reductase type 1 mRNA was associated with LPL and PPARγ mRNAs, which might be explained by effects of 5α-reduced androgens on AR. 5α–Reduced metabolites of glucocorticoids acting through GR (McInnes et al 2004) may also contribute to these associations. It is possible that these results, particularly in the inter-analysis of adipose mRNAs, may be subject to bias from multiple statistical testing. Bonferroni corrections were not used here, but we accept that results with borderline significance should be interpreted with caution. We have therefore concentrated in our discussion on findings that were consistently found in all cohorts or with highly significant p values suggesting a 'real' effect.

In the analysis of inter-relationships between adipose tissue mRNAs we found that inflammatory cytokine mRNAs were associated with some of the determinants of sex steroid action (5α-reductase 1 and ERα mRNAs), independently of obesity and gender. Although in vitro regulation of these cytokine genes has not been reported in detail in human adipose, and the likely direction of causality remains uncertain, it is notable that estrogen may modulate the expression of several pro-inflammatory cytokines and serum levels of IL-6 are decreased in ERα knockout mice (Lindberg et al 2001). Estrogen may also regulate leptin mRNA and production (Ainslie et al 2001;Okura et al 2003) and leptin regulates proinflammatory immune responses to inflammatory stimuli (Loffreda et al 1998).

We also correlated mRNAs for gene products involved in steroid signalling with some of those regulating adipose metabolic function. Androgens and estrogens regulate lipolysis, adipogenesis and leptin in vitro (Ringold et al 1986;Machinal-Quelin et al 2002;Anderson et al 2002;Dang et al 2002;Bourguiba et al 2003;Palin et al 2003). The principal correlations were, again, with ERα and 5α-reductase 1 (Table 6.3). However, the genes that were measurably different in obese subjects (ie aromatase, AKR1C2 and 3) were not correlated with gene products determining adipose metabolism. Further, the striking effects of estrogen on angiotensinogen mRNA in animal models (Stavreus-Evers et al 2001) were not reflected in these human studies. Thus, the local impact within the adipose tissue of altered steroid metabolism in obesity remains unproven.

Although it has been speculated that it is intra-abdominal adipose where steroid signalling is most influential, and it is possible that different regulation of enzymes occurs in the visceral bed, it is not possible to obtain biopsies of this tissue under non-stressed non-anaesthetised conditions and so our studies have been restricted to subcutaneous adipose tissue. It may be that quite different dysregulation of steroid signalling occurs in intra-abdominal adipose tissue (Montague et al 1998), as suggested in previous studies (Corbould et al 2002;Quinkler et al 2004). Clearly, correlations within samples in cross-sectional studies cannot test causality, but they provide important circumstantial evidence for the likely pathways in which alterations in steroid signalling are involved. Further studies of protein levels and using enzyme inhibitors will ultimately be required.

In conclusion, these data suggest that there is dysregulation of enzymes regulating the availability of ligands for ER (aromatase) and AR (AKR1C2 and 3) in subcutaneous adipose tissue in human obesity. These may interact to determine body fat distribution during weight gain. It is clear that studying circulating hormone levels is no longer sufficient, and exciting insights are to be gained from understanding the complex intracrinology of steroid action in adipose tissue. The challenge now is to translate these findings into measurements of steroid metabolism in vivo.

Chapter 7

CONCLUSIONS

Obesity is the fastest growing health threat to face the Western World. The specific role of adipose tissue as a generator of hormonal factors and cytokines with both local and distal action is emerging and adipose endocrinology may play a major role in the pathogenesis of the metabolic syndrome. Glucocorticoid excess, as in Cushing's syndrome, causes central obesity, hypertension, insulin resistance and dyslipidaemia. Local generation of glucocorticoids within tissues may be mediated by 11HSD1 and both obesity prone animal models and transgenic manipulation of 11HSD1 suggested a key role in obesity and the associated dysmetabolic phenotype. On this background, this thesis assessed the impact of local steroid generation particularly within adipose in human obesity, and aimed to determine key regulators and downstream targets.

These studies support previous animal work and demonstrate transcriptional up-regulation of 11HSD1 in sub-cutaneous adipose tissue in human obesity. This finding was confirmed in men and women and across different populations (in Finland, Sweden and America), but was not altered by ethnicity, as determined in American Pima Indians. Adipose 11HSD1 was clearly associated with adiposity (generalised rather than visceral fat accumulation) and downstream features of the metabolic syndrome, notably insulin resistance. We could not, however, demonstrate a clear relationship between 11HSD1 and potential downstream glucocorticoid target genes. Also, glucocorticoid receptor mRNA was downregulated in subcutaneous adipose in obesity which may attenuate the downstream impact of active generated steroid. These studies however simply assessed relationships by cross sectional analysis of mRNAs. Determination of downstream impact would be best assessed by modulation of 11HSD1 activity in dynamic *in vivo* studies.

11HSD1 is altered in a tissue specific manner in generalised idiopathic obesity (increased in adipose and reduced in liver), suggesting that the enzyme is being dysregulated at a local level. Potential regulating factors that are altered in obesity include cytokines and nutritional signals, and both animal and in vitro studies suggest that 11HSD1 is involved in the adaptive response to nutrition.We assessed regulation in healthy male volunteers to metabolic signals demonstrating adipose

specific changes in glucocorticoid generation secondary to acute hyperlipidaemia, hyperinsulinaemia and short term PPAR agonists. Changes in local cortisol production may regulate key adipocyte functions, such as control of lipolysis, adipogenesis and adipokine production. Adipose 11HSD1 may be an important mediator of cellular responses to dietary fat and carbohydrate intake, although whether these regulatory mechanisms are altered in obesity remains to be determined. Further, alterations in tissue glucocorticoids may contribute to the therapeutic action of PPAR agonists, used clinically as insulin sensitizing and lipid lowering agents. Increased systemic cortisol generation was demonstrated with hyperinsulinaemia but could not be fully explained by local changes in subcutaneous adipose 11HSD1. It is likely that this is due to increased 11HSD1 in another tissue source such as visceral adipose tissue or liver. Increased peripheral generation of cortisol by hyperinsulinaemia may contribute to the phenomenon of post prandial hypercortisolaemia, previously attributed to changes in hypothalamic pituitary signaling.

Our *in vivo* studies demonstrate changes in local cortisol generation without corresponding impact on circulating cortisol levels, suggesting that subcutaneous adipose has a limited role in systemic glucocorticoid generation. Indeed Basu et al and Andrew et al (Basu et al 2004;Andrew et al 2005) suggested that splanchnic, rather than subcutaneous adipose may be the greater source of whole body cortisone to cortisol conversion. Sub-cutaneous adipose 11HSD1 may however retain a very important local role mediating key adipocyte processes through intra-cellular regulation of downstream glucocorticoid target genes.

Although the main focus of this thesis was glucocorticoid metabolism in obesity, we briefly extended our studies to determine whether abnormalities were also found in key enzymes controlling sex steroid generation within adipose. The role of testosterone and estrogen in determining adipose tissue distribution is evident by gender differences, but circulating sex steroid levels do not consistently determine adipose deposition in population studies. We therefore speculated that tissue steroid metabolism may have a key role.

These studies demonstrate that mRNA for testosterone metabolizing enzymes, AKR1C2 and 3 was associated with central adiposity, whilst mRNA for

the estrogen generating enzyme aromatase was associated with generalised obesity. 11HSD1 is emerging as a key mediator of local glucocorticoid response. We now speculate that there is a very similar role for these key sex steroid metabolizing enzymes in determining local androgen and eostrogen action, although further dynamic and functional studies are undoubtedly required.

To conclude, these studies implicate dysregulation of 11HSD1 transcription in the pathogenesis of human obesity and the metabolic syndrome although the downstream impact of enzyme dysregulation remains unclear. These studies have identified some key enzyme regulators in vivo in humans. Understanding normal enzyme regulation in humans will allow further studies to dissect the basis of dysregulation in obesity. The widespread metabolic benefits of transgenic manipulation and enzyme inhibition in rodents suggest an important role for 11HSD1. Inhibition of adipose 11HSD1 remains an intriguing target for the treatment of human obesity and its metabolic complications but its physiological importance will be best determined by the development of specific human adipose 11HSD1 inhibitors.

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