The role of 11β-Hydroxysteroid Dehydrogenase type 1 and Hepatic Glucocorticoid Metabolism in the Metabolic Syndrome

By

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Abstract

The metabolic syndrome represents a state of insulin resistance, which has drawn great attention due to its public health importance. A quarter of the world's adult population are considered to have the metabolic syndrome (Moadab et al. 2009).

Despite having a striking phenotypic similarity with patients with Cushing's syndrome, those with the metabolic syndrome have normal circulating cortisol levels. It has been proposed that these patients have dysregulated cortisol metabolism with either increased glucocorticoid activation or reduced inactivation at a tissue level. Since this may be amenable to therapeutic manipulation there has been an intense focus on the microsomal enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) which activates cortisone to cortisol *in vivo.*

Through a series of *in vitro* and detailed translational studies, this thesis attempts to investigate hepatic glucocorticoid metabolism with relevance to the pathophysiology of the metabolic syndrome. The hepatic zonation and characterization of 11β-HSD1 was defined. The relation between glucocorticoid metabolism and glucose homeostasis was analysed with relevance to body composition in normal, obese and type 2 diabetic human subjects. Glucose 6 phosphate was identified as a novel direct link between glucose metabolism and the HPA axis. Hepatic glucocorticoid metabolism and its role in the pathogenesis of alcoholic liver disease and non alcoholic fatty liver disease were defined.

To my daughter

Muneera

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They (the angels) said: Be glorified! We have no knowledge saving that which Thou hast taught us. Lo! Thou, only Thou, art the Knower, the Wise.

Al Qur'an Chapter 2 Verse 32

Abbreviations

5αR	5α-reductase
11β-HSD1	11β-Hydroxysteroid Dehydrogenase Type 1
6PG	6-Phosphogluconolactone
A	Dehydrocorticosterone
ACE	Angiotensin Converting Enzyme
ACTH	Adrenocorticotropic Hormone
AME	Apparent Mineralocorticoid Excess
ATII	Angiotensin II
AVP	Arginine Vasopressin
B	Corticosterone
BSA	Bovine Serum Albumin
CBG	Corticosteroid Binding Globulin
CNS	Central Nervous System
CRP	C reactive protein
CVD	Cardiovascular Disease
CT	Computed Tomography
DEXA	Dual-energy X-ray absorptiometry
DHEA	Dehydroepiandrosterone
E	Cortisone
ER	Endoplasmic Reticulum
F	Cortisol
FFA	Free Fatty Acids
G6Pase	Glucose-6-phosphatase
G6PT	Glucose-6-Phosphate Transporter
GCSF	Granulocyte-Colony-Stimulating Factor
GE	Glycyrrhetinic Acid
GI	Glycyrrhizic Acid
GLUT-2	Glucose transporter 2
GR	Glucocorticoid Receptors
GRE	Glucocorticoid Response Element
H6PDH	Hexose-6-Phosphate Dehydrogenase
HDL	High density lipoprotein

hGh	Human Growth Hormone
HMT	Hexamethylenetetramine
HOP	HSP90 Organising Protein
HRP	Horseradish Peroxidise
HSP	Heat Shock Proteins
IL-6	Interleukin-6
IMS	Industrial Methylated Spirit
IR	Infrared
LDL	Low density lipoprotein
MR	Mineralocorticoid Receptors
NADPH	NADP Phosphate
NAFLD	Non-alcoholic Fatty Liver Disease
NDS	Normal Donkey Serum
NF-kB	Nuclear Factor Kappa B
NLS	Nuclear Localization Sequences
NMRS	Nuclear Magnetic Resonance Spectroscopy
P450scc	P450 Side Chain Cleavage Enzyme
PEN	Polyethylene Naphthlate
PEPCK	Phosphoenolpyruvate Carboxykinase
POMC	Pro-Opiomelanocortin
PPAR-γ	Hepatic Nuclear Proliferator-Activated Receptor-γ
PVDF	Polyvinylidine Difluoride
SDS-PAGE	Sodium Docecyl Sulphate Polyacrylamide Gel Electrophoresis
Sgk	Serum and Glucocorticoid Inducible Kinase
siRNA	Small interfering RNA
SNP	Single Nucleotide Polymorphisms
TCA	Tricarboxlylic Acid
TLC	Thin Layer Chromatography
TNFα	Tumour necrosis factor α
UDP	Uridine Diphosphate
UPR	Unfolded Protein Response
VLDL	Very Low Density Lipoprotein
WHO	World Health Organisaton

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1. Introduction

1.1. Overview

The metabolic syndrome represents a state of insulin resistance that is also known either as syndrome X or the dysmetabolic syndrome, which has drawn great attention due to its public health importance. A quarter of the world's adult population are considered to have the metabolic syndrome (Moadab et al. 2009).

The metabolic syndrome collectively characterises a group of closely linked disorders, including obesity, insulin resistance, hypertension, dyslipidaemia, type 2 diabetes and hepatic steatosis, and relates to a loss of insulin sensitivity in key target tissues including adipose, liver and muscle (DeFronzo and Ferrannini 1991;Roden 2004). Additional risk factors include polycystic ovarian syndrome (Anon 2005) and ethnic groups at high risk for type 2 diabetes and coronary artery disease.

Patients with circulating cortisol excess due to increased endogenous production (Cushing's syndrome) or exogenous corticosteroid use develop a classical phenotype which is remarkably similar to the obesity - metabolic syndrome, and which is reversible upon removal of glucocorticoid excess. However, unlike patients with Cushing's syndrome, those with the metabolic syndrome have normal circulating cortisol levels. At a tissue level however, it has been proposed that these patients have dysregulated cortisol metabolism with either increased glucocorticoid activation or reduced inactivation. Since this may be amenable to therapeutic manipulation there has been an intense focus on the microsomal enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) which activates cortisone to cortisol *in vivo.*

This thesis attempts to investigate hepatic glucocorticoid metabolism with relevance to the pathophysiology of the metabolic syndrome.

1.2. The Metabolic Syndrome

The metabolic syndrome describes the clustering of dyslipidaemia, hypertension, glucose intolerance and central adiposity. It was first described by Reaven in 1988 who postulated that insulin resistance was the cause of glucose intolerance, hyperinsulinaemia, increased VLDL, decreased HDL and hypertension (Ferrannini et al. 1997;Reaven 1988). Since then it has been realised that obesity is the usual underlying cause for insulin resistance that leads to the metabolic abnormalities. Hyperuricemia, fatty liver (especially in concurrent obesity) progressing to non-alcoholic fatty liver disease, polycystic ovarian syndrome (in women), and acanthosis nigricans are diseases all closely related to the metabolic syndrome. The metabolic syndrome appears in all ethnic groups, affecting young and old, males and females. The cardiovascular risk associated with abdominal obesity ('apple' distribution) is

greater than with the gluteofemoral ('pear') distribution of body fat seen in women. This may explain why obesity has a more atherogenic effect in men compared with women (McGill, Jr. et al. 2002;Sakkinen et al. 2000). A recent study examining the association of BMI, waist circumference and waist:hip ratio with the risk of death in nearly 360,000 subjects found that after adjustment for BMI, waist circumference and waist:hip ratio were strongly associated with the risk of death (Pischon et al. 2008).

There have been several criteria published for the diagnosis of the metabolic syndrome. The most widely used are the World Health Organisation (WHO) criteria (Alberti and Zimmet 1998): central obesity with a waist: hip ratio above 0.9 for men and 0.85 for women and/or a body mass index (BMI) above 30 kg/m^2, blood pressure above 140/90 mmHg, triglycerides above 1.7 mmol/L, HDL cholesterol <0.9 mmol/L in men and <1 mmol/L in women, glucose fasting or 2 h after a glucose load above 7.8 mmol/L and glucose uptake during hyperinsulinaemic euglycaemic clamp in the lowest quartile for population.

There have been several proposed modifications to the WHO criteria since 1998, most of which have been less strict and have focussed around central obesity as the most important and essential criterion. Most European and American studies have found a prevalence of between 12 and 25% (Ford et al. 2002;Mokdad et al. 2003;Villegas et al. 2003). Asian studies have found a lower prevalence of between 5 – 16% (Gupta et al. 2003). All of these patients are at high risk of developing diabetes and cardiovascular disease (CVD) and the unifying aim is early intervention with intensive lifestyle modification that may ultimately prevent the progression to these deadly and costly diseases (Lakka et al. 2002).

The extremely complex pathophysiology of the metabolic syndrome has only been partially elucidated but is largely accounted for by common factors that reinforce the

interactions between insulin resistance and obesity, and which are also potential targets for therapy. Insulin resistance in these patients appears to be directly linked with ischaemic heart disease (Haffner et al. 1999).

Patients with the metabolic syndrome commonly have a pro atherogenic dyslipidaemia, including low HDL and increased triglycerides and small dense LDL particles (Steinmetz et al. 2001). They also have a pro thrombotic and pro inflammatory state with increased fibrinogen and platelet activator inhibitor (PAI-1) and acute phase reactants, including CRP. These may directly increase the risk of coronary events. CRP is able to destabilize atherosclerotic plaques and cause plaque rupture (Grundy 2002;Morrow and Ridker 2000). PAI-1 which is released from abdominal adipose tissue can increase the thrombotic complications of plaque rupture (Sakkinen et al 2000). The secretory activity of adipose tissue, in particular visceral adipose tissue, contributes to the pro thrombotic and pro inflammatory states of the metabolic syndrome. Secretory products (adipokines) include pro inflammatory cytokines and other inflammatory markers such as TNFα, adiponectin, IL-6, resistin and leptin. Visceral fat also shows increased lipolytic activity resulting in an increase in circulating free fatty acids (FFA) which promotes insulin resistance in many tissues and the development of reservoirs of triglycerides (TG) in liver (via increased FFA delivery to the liver via the portal vein) and muscle. This also increases hepatic insulin resistance and the hepatic output of very low density lipoprotein (VLDL).

1.3. Hepatic glucose and lipid metabolism in health and disease

The liver plays a central role in carbohydrate metabolism. Disordered hepatic carbohydrate metabolism is critically implicated in the pathogenesis of the metabolic syndrome. Hepatic glucose production through the processes of gluconeogenesis and

glycogenolysis represents the principal source of glucose for tissues in the fasting state (Roden and Bernroider 2003). The liver also plays an important role in the regulation of post prandial glucose concentrations.

The aim of hepatic glucose homeostasis is to maintain blood glucose concentrations within a narrow range (4 – 6 mmol/L) despite widely varying inputs from the gastrointestinal tract. In health this is achieved by a precise dynamic equilibrium between glucose utilization and endogenous glucose production (EGP). This involves a series of intracellular metabolic events which are regulated by hormones, in particular glucose and insulin, and metabolites such as glucose, free fatty acids (FFA) and carbon-3 compounds such as alanine, lactate and glycerol.

The recent development of non invasive techniques such as isotopic tracer dilution, as used in this thesis, and nuclear magnetic resonance spectroscopy (NMRS), have allowed detailed assessment of glucose and glycogen metabolism that was previously not easily possible in humans (Radziuk and Pye 2002;Roden et al. 2001).

1.3.1. Applied liver anatomy

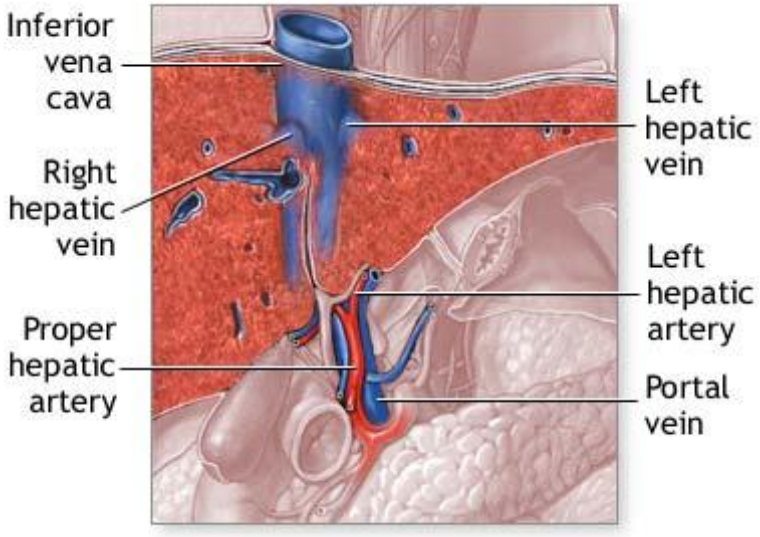


Figure 1-1 The blood supply to and from the liver (image from medline plus online encyclopedia)

Approximately 80% of the blood supply to the liver is received from the portal vein (formed by the superior mesenteric and splenic veins) with the remainder coming from the hepatic artery. As the portal vein drains the intestinal vessels, the liver is well exposed to all the water soluble nutrients absorbed from the gut. Lipid soluble products of digestion are delivered to the liver via the thoracic duct. The pancreatic vein drains into the portal vein directly immediately exposing the liver to insulin and glucagon released from the pancreas. Nearly 50% of the insulin secreted is cleared by the liver before reaching the systemic circulation via the hepatic vein. Hence much higher concentrations of insulin exert an immediate effect upon hepatic cellular receptors than anywhere else via the systemic circulation. This reflects the substantially lower systemic insulin concentration than would be expected from volume dilution alone.

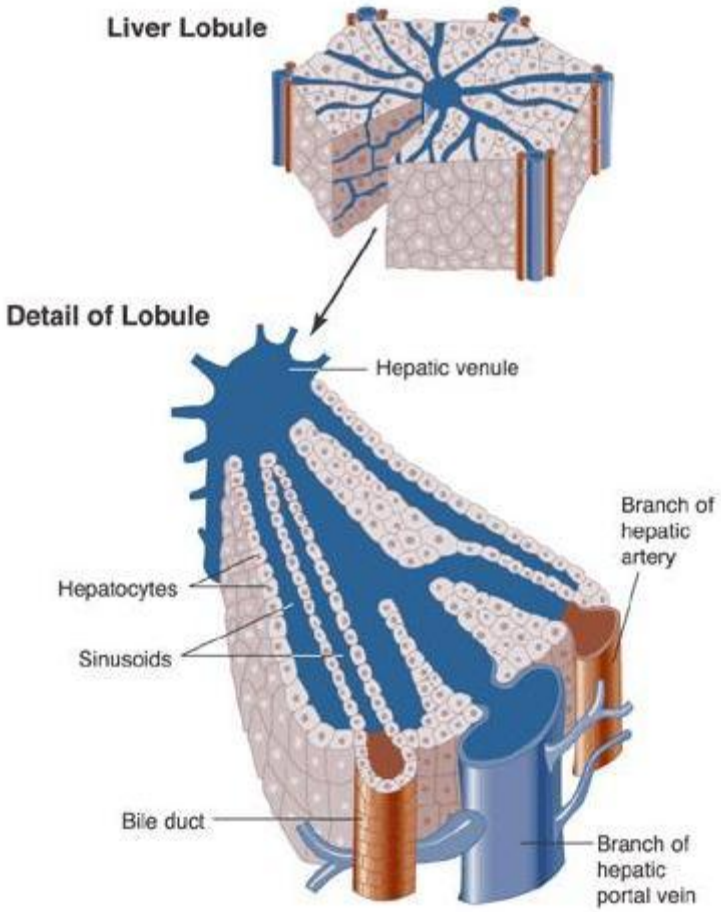


Figure 1-2 A cross section of a liver lobule. Blood enters through the portal triad, passes through the sinusoids and exits through the central vein. (free access image from wikipedia website)

Hepatocytes form plates that are only one or two cells thick separated from each other by large capillary spaces called liver sinusoids. These structures form hexagonal functional units called 'lobules', Figure 1-2. There is very specific metabolic zonation within the liver lobules. Periportal cells are primarily involved in gluconeogenesis, whereas perivenular cells (those closer to the central vein) are more involved with glycolysis and ketogenesis. Each zone is able to adapt and alter the profile of metabolic regulation if needed. Insulin and glucagon differentially regulate

gluconeogenesis and glycolyis, as well as substrate supply to maintain an appropriate hepatic response to blood glucose concentrations (Frayn KN 2003).

1.3.2. Intrahepatic glucose fluxes under normal physiological conditions

1.3.2.1. Glucose uptake

In health the portal venous glucose concentration after a meal may approach 10 mmol/L, much higher than in the systemic circulation. Hepatocytes take up glucose independently of insulin via the low affinity GLUT-2 transporter, which facilitates glucose entry into cells in the presence of high sinusoidal glucose concentrations. Hence, hepatocytes are in a key position to buffer the hyperglycaemic effects of a high carbohydrate meal. It has been proposed that hepatic injury in critical illness leads to hyperglycaemia because of unregulated glucose uptake by the liver. Hepatocytes are not able to rapidly down regulate glucose uptake to protect vital intracellular metabolic functions. Strict control of blood glucose concentration during critical illness protects from hepatic mitochondrial injury and may therefore contribute to improved outcomes clinically (Vanhorebeek et al. 2005).

Once taken up by the liver, glucose is rapidly phosphorylated to glucose-6-phosphate (G6P), by the hepatic specific hexokinase isoform, glucokinase. This reaction is the prelude to glycolysis (resulting in the production of 3-carbon compounds such as lactate and pyruvate). Alternatively the glucose flux can be directed towards the direct pathway of glycogen synthesis via uridine diphosphate (UDP)- glucose, or the pentose phosphate shunt, Figure 1-3.

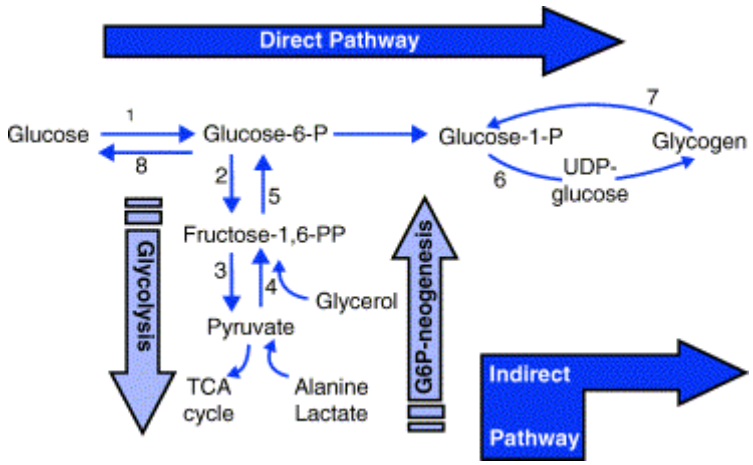


Figure 1-3 *The fate of glucose in the hepatocyte. [1]glucokinase, [2]phosphofructo-1 kinase, [3]pyruvate kinase, [4]phosphoenolpyruvate carboxykinase (PEPCK), [5]fructose-1,6-biphosphatase, [6]glycogen synthase, [7]glycogen phosphorylase, [8]glucose-6-phosphatase (Roden & Bernroider 2003).*

1.3.2.2. Glycolytic flux and gluconeogenesis

Carbon-3 compounds can enter the tricarboxlylic acid (TCA) cycle and undergo further oxidation or serve as substrates for de novo synthesis of glucose and glycogen (G6Pneogenesis and the indirect/gluconeogenic pathway of glycogen synthesis) (Landau 2001). These processes form substrate cycles, a system which allows fine regulation of the direction and rates of flux, by changes in concentration, gene expression and convalent modification of effector enzymes (Dohm and Newsholme 1983;Hue and Bartrons 1984). Gluconeogenesis substrate cycles are controlled by the enzymes phosphoenolpyruvate carboxykinase (PEPCK) and fructose 1,6 biphosphatase, while glycolytic substrate cycles are regulated by pyruvate kinase and phosphofructo-1-kinase, Figure 1-3.

1.3.2.3. Glycogen synthesis and glycogenolysis

Glycogen synthase and glycogen phosphorylase exert another substrate cycle that can be active simultaneously, resulting in glycogen cycling. The effect of this is

negligible in the non diabetic fasting state. Both enzymes are regulated by phosphatases, kinases and allosteric effectors which are dependent on the nutrient and hormonal microenvironment (Bollen et al. 1998;Villar-Palasi and Guinovart 1997). Glycogenolysis requires the action of glycogen phosphorylase and a debranching enzyme to release glucose-1-phosphate which is in equilibrium with G-6-P, Figure 1-3.

1.3.2.4. Glucose-6-phosphatase and glucose release

Glucose-6-phosphatase (G6Pase) catalyses the terminal step resulting in the release of free glucose into the hepatic veins, from either gluconeogenesis or glycogenolysis, Figure 1-3. It catalyses the dephosphorylation of G6P to glucose. G6Pase is expressed in liver and kidney, G6Pneogenesis can result in glucose release from these tissues only. In contrast, skeletal muscle is devoid of G6Pase and therefore cannot release glucose from glycogen despite muscle glycogen depots being four to five fold greater than in liver. Instead lactate is released from skeletal muscle which is shuttled back to the liver via the Cori cycle. G6Pase activity is deficient in patients with Glycogen storage disease Type 1a usually diagnosed by the detection of profound hypoglycaemia in infancy. This condition and its impact upon hepatic glucocorticoid metabolism are further described in chapter 4.

1.3.2.5. Insulin and glucagon

Glucose production by the liver is regulated primarily by insulin, glucagon and hyperglycaemia. Even before there is any carbohydrate induced hyperglycaemia, the cephalic phase of insulin secretion primes the liver to rapidly facilitate the hormonal responses to eating. This coupled with the direct delivery of pancreatic hormones to

the liver sinusoids allows rapid control and response to nutritional stimuli. The ingestion of a mixed meal also results in an increase in plasma glucagon concentration, although a pure glucose load can result in it being unchanged or even decreased. In both situations however, the plasma insulin/glucagon ratio rises due to the more marked rise in plasma insulin (McMahon et al. 1989;Mitrakou et al. 1990).

Under post prandial conditions the portal vein insulin concentration is around 180pM which is almost 3 fold higher than in the systemic circulation. This concentration of insulin is necessary to half maximally stimulate hepatic glycogen production and suppress EGP during periods of hyperglycaemia, much higher than the concentration required in the periphery to stimulate the uptake of glucose by cells (DeFronzo et al. 1983).

During periods of hypoglycaemia, plasma glucagon concentrations rise causing an immediate rise in plasma EGP. Studies using continuous glycogen infusions have shown that the immediate rise in EGP is accounted for by hepatic glycogenolysis. Following this there is a decline in EGP that is explained by a decrease in glycogenolysis due to decreased glycogen stores, and an increase in gluconeogenesis (Magnusson et al. 1995). Glycogen stimulates PEPCK expression and pyruvate carboxykinase activity as well as inhibiting pyruvate kinase and phophofructo-1-kinase (Jiang and Zhang 2003). Even small changes in the portal vein insulin and glucagon concentration affect hepatic glycogen synthesis and glycogenolysis, exerting a fine control on glucose homeostasis.

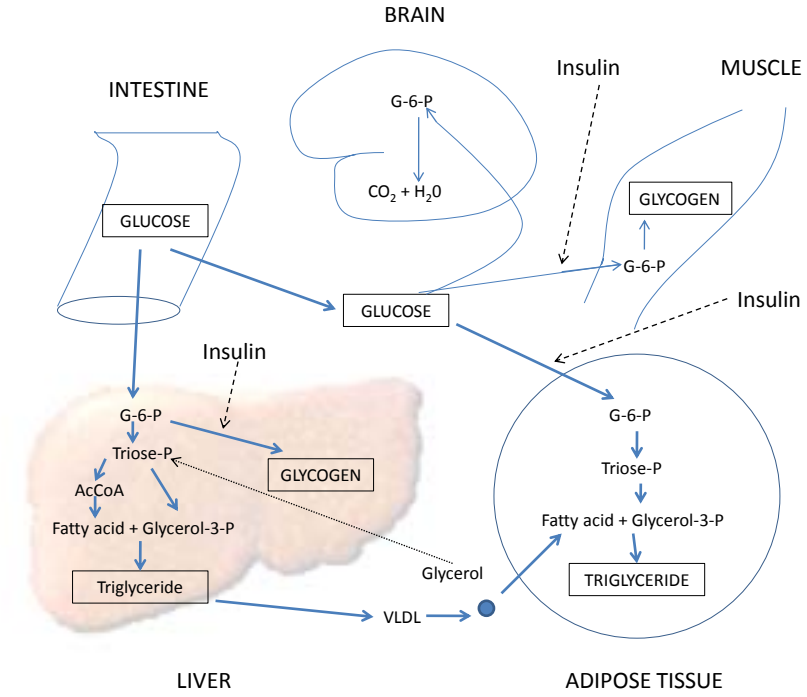


Figure 1-4 Brief overview of postprandial metabolism of glucose

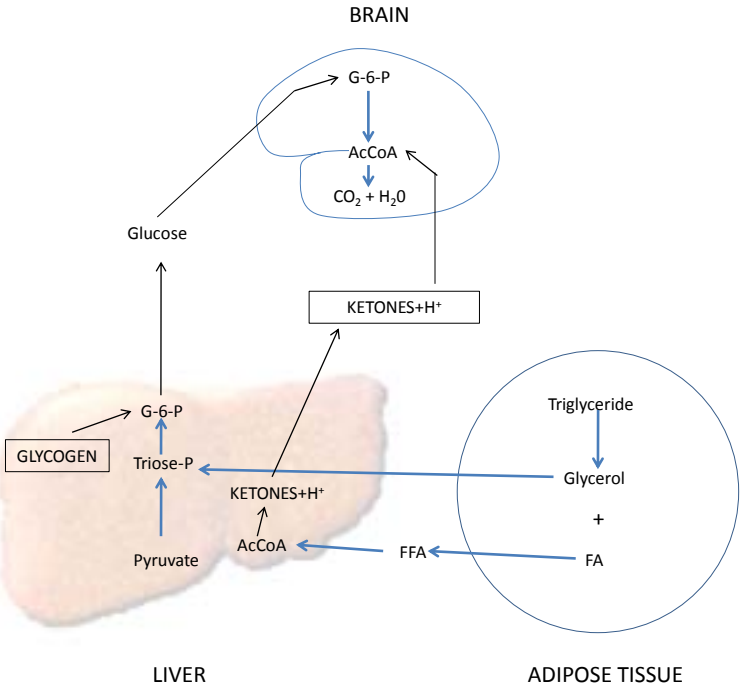


Figure 1-5 Brief overview of intermediary metabolism during fasting

1.3.2.6. Hepatic amino acid metabolism is also linked to carbohydrate and lipid metabolism

A high protein intake in humans can induce glucose intolerance as well as increasing EGP in the fasting state (Linn et al. 1996). Transamination of unbranched amino acids has also been linked to carbohydrate and lipid metabolism. Branched chain amino acids and alanine are often elevated in patients with obesity and insulin resistance. This may result from a change in the secretion of regulatory hormones and the stimulation of gluconeogenesis by amino acids (Felig et al. 1978). Metabolic studies have shown that the rise in EGP as a result of the gluconeogenic effect of post prandial amino acid concentrations, is only unmasked when insulin secretion is impaired. Hence in subjects with normal glucose tolerance, the stimulatory effect of post prandial amino acid concentrations on the secretion of insulin and glucagon balances the increase in gluconeogenesis with no significant effect on glycaemia (Krebs et al. 2003).

1.3.2.7. Hepatic carbohydrate metabolism is closely linked to lipid metabolism

In the fasting state plasma free fatty acid (FFA) concentrations increase and energy is derived from fat oxidation. In insulin resistant states, plasma FFA concentrations correlate with the extent of hyperglycaemia and EGP (Bogardus et al. 1984). The liver is able to synthesise and oxidise fatty acids, but lacks the enzymes necessary to complete the metabolism of the ketone bodies resulting from mitochondrial β-oxidation. The balance between glucose and lipid oxidation is coordinated by the enzyme malonyl-coenzyme A through its ability to inhibit the entry of acetylated fatty acid derivatives into the mitochondria. Fatty acid synthesis is promoted by insulin. Insulin deficiency, with the presence of elevated glucagon levels drives ketogenesis

by promoting β-oxidation, Figure 1-5. This is the mechanism that drives ketogenesis from fatty acids.

Adipocytes act in conjunction with the liver to convert excess glucose to triglyceride for storage. In the liver, triglycerides are formed from glycerol-3-phosphate (from triose phosphate) and fatty acids (from acyl CoA), and are incorporated in VLDL, where they are hydrolysed by lipoprotein lipase. The released FFA are re-esterified with glycerol-3-phosphate derived from glucose that has entered the tissue under the influence of insulin. The resulting triglyceride is stored in adipose tissue, Figure 1-4.

1.3.3. The liver in diabetes mellitus

Insulinopenia in type 1 diabetes results in increased hepatic glucose production that correlates with the degree of fasting hyperglycaemia. Increased generation of gluconeogenic precursors in peripheral tissues further promote gluconeogenesis. Even when exogenous insulin is administered, while hepatic glucose production can decrease to normal, slight defects in hepatic glycogen storage persist (Roden & Bernroider 2003).

In type 2 diabetes too, there is evidence for a reduction in glycogen synthesis, implying that the increase in hepatic glucose output is mainly a result of increased gluconeogenesis. Isotopic tracer dilution studies have shown that there is a loss of autoregulation in patients with fasting hyperglycaemia, as hepatic glucose output rates are either inappropriately normal or elevated by 10-25% (Roden & Bernroider 2003). The increase in hepatic gluconeogenesis is further fuelled by increased rates of lipolysis in adipose tissue, due to impaired insulin action, releasing free fatty acids and glycerol which are subsequently delivered to the liver. Increased hepatic uptake of non-esterified fatty acids also promotes hepatic lipid synthesis as well as increased

hepatic secretion of VLDL, resulting in the typical type 2 diabetic dyslipidaemic phenotype.

In patients with type 2 diabetes, hepatocytes fail to respond adequately to insulin and with lack of suppression of hepatic glucose production. Glucagon levels are also inappropriately non-suppressed. This results in glucose entering the circulation from the intestine as well as from the liver. Defective glucose uptake by muscle and adipose tissue due to insulin resistance adds to the hyperglycaemia resulting in the post prandial elevations in serum glucose concentrations.

1.3.4. Hepatic glucose metabolism in non alcoholic fatty liver disease (NAFLD) and liver cirrhosis

Fatty infiltration of the liver in patients with diabetes was observed as long ago as the 18th century. Before the advent of insulin treatment, marked liver enlargement was seen in young diabetic patients (Malins J 1968). NAFLD represents the liver phenotype of the metabolic syndrome, and covers a spectrum of liver disease ranging from simple hepatic steatosis to steatohepatitis (NASH) and eventually cirrhosis.

NAFLD is strongly associated with visceral obesity, low HDL cholesterol and elevated plasma triglycerides. Hepatic steatosis has been appreciated as a mediator of atherogenesis and many of the factors that increase cardiovascular risk (Targher 2007). Liver fat content has also been shown to be correlated with insulin sensitivity and fasting insulin levels. In fact studies have shown that despite normal fasting endogenous glucose production, patients with NAFLD show a reduction in the insulin stimulated utilization of glucose, similar to patients with Type 2 diabetes. There was also a comparable degree of impairment of insulin-dependent suppression of plasma FFA (Marchesini et al. 2001).

Hepatocellular lipid concentration, measured by ^{1}H-NMRS has been shown to correlate with whole body and hepatic insulin resistance and the insulin dependent suppression of plasma FFA in non diabetic and type 2 diabetic patients. In addition, prolonged infusion of insulin resulted in an increase in hepatocellular lipid concentration (Anderwald et al. 2002a). These observations suggest that increased portal flux of FFA from visceral fat tissue as well as increased portal insulin favours hepatic lipid accumulation. These two factors may be responsible for the hepatic steatosis seen in early NAFLD. Cytokines such as leptin may also play a part in early NAFLD by promoting hepatic triglyceride synthesis and stimulating hepatic insulin signal transduction (Anderwald et al. 2002b).

Cirrhosis of any cause predisposes to the development of hepatocellular carcinoma. This risk is significantly increased with increased BMI and diabetes. Over a third of patients with obesity related cirrhosis develop hepatocellular carcinoma within 2 years (El-Serag et al. 2004). Insulin resistance in patients with cirrhosis of any cause is well documented in the literature. Diabetic patients with chronic liver disease have lower insulin requirements as the disease progresses, and patients with advanced liver disease can often have spontaneous fasting hypoglycaemia. This may reflect reduced hepatic insulin extraction due to vascular shunting in cirrhotic patients. In liver cirrhosis, plasma concentrations of insulin, glucagon, lactate, FFA, and gluconeogenic precursors are increased. Isotopic tracer studies using $^{2}H_2O$ show increased gluconeogenesis and a marginal reduction in glycogenolysis indicating a high degree of glycogen cycling in these patients. The rise in plasma insulin, glucagon and free fatty acid concentrations may all contribute to glycogen cycling in cirrhosis (Petersen et al. 1998;Roden et al. 1996).

Treatment of NAFLD is difficult, although intensive lifestyle management and weight loss appear to help. Insulin sensitising thiazolidinediones such as pioglitazone, which stimulate hepatic nuclear proliferator-activated receptor-γ (PPAR-γ) have been shown to improve certain aspects of NASH histology including steatosis (Belfort et al. 2006). In animal models, the activation of PPAR-α by fibrates also enhances fatty acid oxidation and protects against steatosis.

This thesis includes a detailed study of metabolic phenotype and steroid metabolism in patients with NAFLD. The role of glucocorticoids in the pathophysiology of NAFLD is discussed in chapter 7.

1.4. Adrenal Steroids

The principal hormones secreted by the adrenal cortex in physiologically significant amounts are the glucocorticoids cortisol and corticosterone, the mineralocorticoid aldosterone, and the androgens dehydroepiandrosterone (DHEA) and androstenedione. Cholesterol is the precursor for all adrenal steroid synthesis and is derived primarily from LDL in the circulation, and can also be synthesised *de novo*, in the liver from Acetyl-CoA via the mevalonate pathway. Cytosolic cholesterol is then transferred to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StaR), a 30kDa phosphoprotein which is the hormonally sensitive rate limiting step in steroid biosynthesis in all steroidogenic tissues (Clark et al. 1994;Stocco 2000). The importance of this step is exemplified in congenital lipoid adrenal hyperplasia which is caused by a homozygous mutation in the StAR gene, and characterised by huge lipid accumulation in steroidogenic tissues and severely reduced steroidogenesis (Lin et al. 1995).

The P450 side chain cleavage enzyme (P450scc) catalyses the initial enzymatic step in steroid biosynthesis by converting cholesterol to pregnelonone. Pregnelonone is able to diffuse freely out of the mitochondrion and is made available in the endoplasmic reticulum (ER). A range of enzymes act upon pregnelonone and its products in the ER or the mitochondrion, leading via intermediates to the production of mineralocorticoids, glucocorticoids and androgens, Figure 1-6.

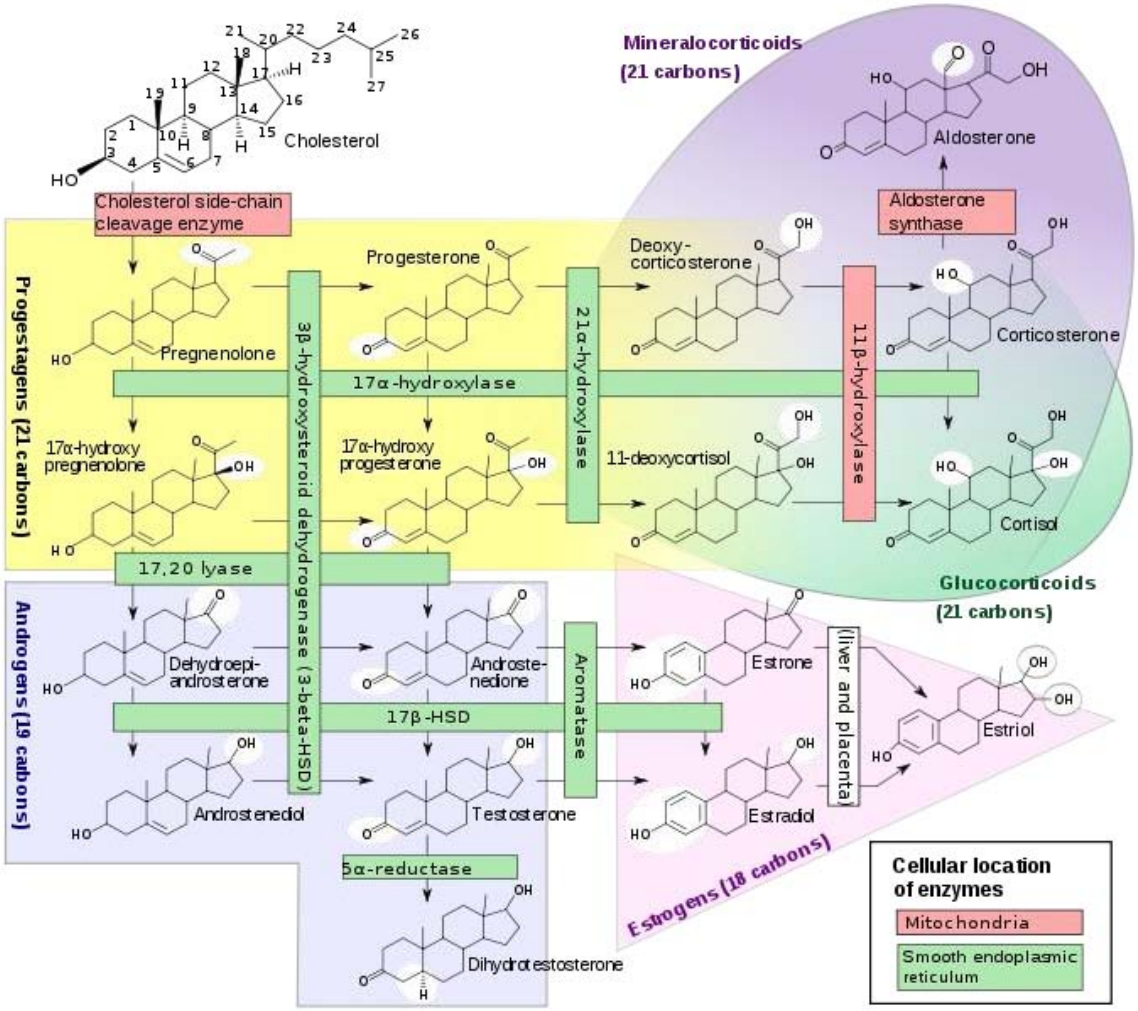


Figure 1-6 Schematic of steroid biosyntheis (open access image from wikipedia website)

The ability of cells within the adrenal cortex to generate specific steroids is critically dependant on the expression of particular enzymes, and there is subspecialisation in

different zones. For example, the synthesis of cortisol and corticosterone from deoxycortisol and deoxycorticosterone relies on the presence of the enzyme 11β-hydroxylase (CYP11B1), whereas the aldosterone production is dependent on the activity and expression of aldosterone synthase (CYP11B2). Aldosterone synthase, but not 11β-hydroxylase is present in the outer zona glomerulosa, mainly responsible for aldosterone production. The inner zona fasciculata and reticularis express 11β-hydroxylase and 17α-hydroxylase and can therefore synthesize corticosteroids sex steroids. In contrast there is no adrenal androgen production in the zona glomerulosa due to the lack of 17α-hydroxylase.

The synthesis of glucocorticoids is regulated by adrenocorticotropic hormone (ACTH) released from the pituitary. ACTH acts to cause an acute increase in the activity of the cholesterol ester hydrolase enzyme and the StAR transport protein. The long term effect of ACTH results in the up regulation of synthesis of all the enzymes involved in steroid biosynthesis. Hence there is a decrease in adrenal function and size with prolonged ACTH deficiency as seen after hypophysectomy, and in conditions such as congenital adrenal hyperplasia due to 21-hydroxylase deficiency there is hypertrophy of the adrenal gland due to prolonged periods of elevated ACTH. ACTH also plays a role in the acute up regulation of aldosterone production and the supply of intermediate molecules for aldosterone synthesis. Aldosterone synthase activity and aldosterone secretion however, is primarily regulated by angiotensin II (ATII) and serum potassium concentration.

The primary glucocorticoid synthesised in humans is cortisol, with corticosterone produced in a lesser amount (PETERSON and PIERCE 1960). Corticosterone is the predominant glucocorticoid generated in mouse and rat, due to the lack of 17α-hydroxylase/lyase in rat adrenal cortex. This difference must be noted when

extrapolating results of rodent studies to humans, as functionally significant differences in the metabolism of cortisol compared to corticosterone may exist.

1.4.1. Glucocorticoid secretion

ACTH secretion from the anterior pituitary stimulates basal glucocorticoid production as well as acute increases in glucocorticoid production in response to stress. ACTH is a single chain, 39 amino acid polypeptide hormone derived from pro-opiomelanocortin (POMC) in the pituitary. Pituitary ACTH production is regulated by the hypothalamic hormone, corticotrophin releasing factor (CRF). Arginine vasopressin (AVP), another hypothalamic hormone is able to potentiate the pituitary ACTH response to CRF.

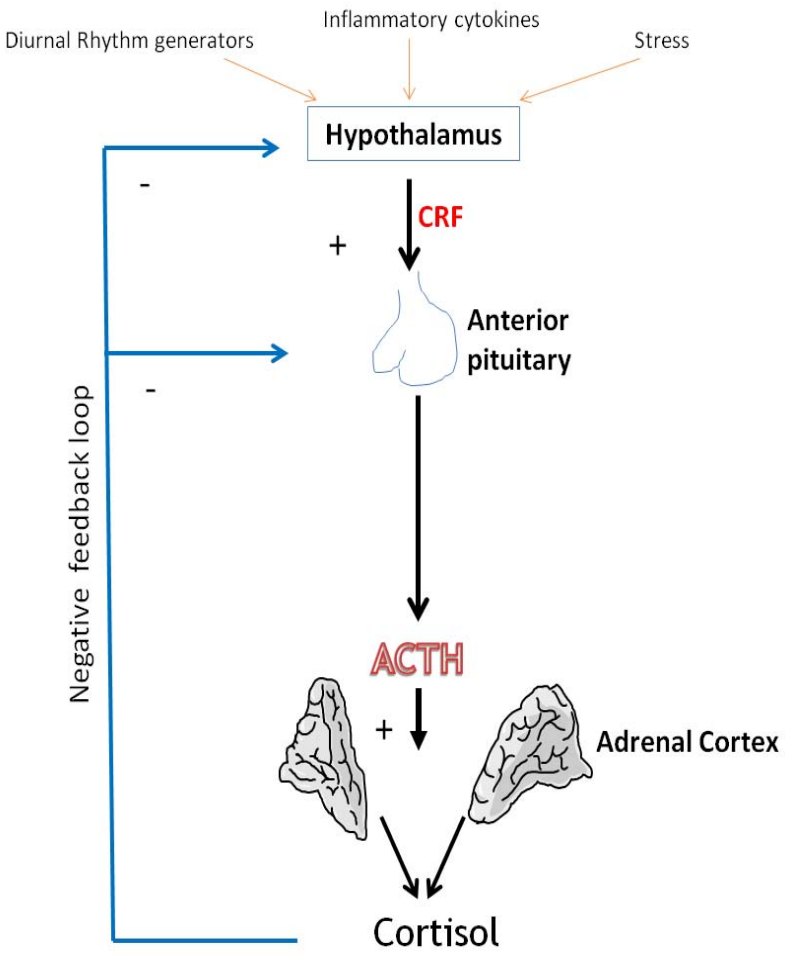


Figure 1-7 Schematic of the hypothalamo-pituitary-adrenal axis. Positive and negative feedback occur at all levels of the axis

Cortisol secretion follows a circadian rhythm in response to irregular bursts of ACTH secretion throughout the day, with the highest secretion rate at around 0800h and a nadir at around 0000h. This circadian rhythm originates from the hypothalamus in response to a number of inputs from other regions of the brain including the suprachiasmatic nucleus (the circadian rhythm generator), from where direct connections to the paraventricular nucleus of the hypothalamus have been demonstrated (Abe et al. 1979;Vrang et al. 1995).

ACTH release is also influenced by a number of other factors including stress and injury, and pro inflammatory cytokines. Pro inflammatory cytokines appear in part to

mediate the stress related cortisol response. In particular, leukemia inhibitory factor (LIF) is needed for the stress driven response that results in pituitary POMC mRNA expression, increased ACTH and glucocorticoid secretion (Chesnokova and Melmed 2000). TNFα and interleukin-6 have been shown to directly stimulate the HPA axis by direct action at the hypothalamic level by stimulating CRH release, as well as direct effects at the level of the pituitary and adrenal (Lyson and McCann 1991;Swain et al. 1997). In contrast, corticosteroids inhibit CRF and ACTH release via negative feedback, as clearly seen in cases of pharmacologic glucocorticoid excess. When administered for a prolonged period there is a dose and time dependant reduction in the ability to generate ACTH which takes a variable amount of time to recover (Henzen et al. 2000), Figure 1-7. These mechanisms allow widely ranging differences in cortisol production rates in response to different conditions. Basal cortisol production rates are around 8-12mg/day which can increase 5-fold when the HPA axis is stimulated by conditions such as trauma or stress (Esteban et al. 1991;Salem et al. 1994).

Cortisol is bound in the circulation to an α-globulin called corticosteroid binding globulin (CBG). There is also a lesser degree of binding to albumin. As with other steroid hormones, only the free (non protein bound) hormone appears to have biological activity. Cortisol and corticosterone are heavily protein bound, such that only 5-10% of these hormones are present as free in the circulation, unbound to protein. CBG binding does vary between corticosteroids. Importantly cortisone binding to CBG is considerably less (~50%) than circulating cortisol which is nearly 95% CBG bound. Of note 11β-dehydrogenated cortisol and cortisone metabolites have much lower binding to CBG (only around 10%). Hence the bioavailability of

these steroids is likely to be significantly greater, for the same circulating level of glucocorticoid, then the parent compound.

1.4.2. Mineralocorticoid secretion

Mineralocorticoids are secreted in a different manner to that of glucocorticoids, Figure 1-8. Angiotensin II (ATII) and extracellular potassium concentrations are the key regulators of aldosterone action. ATII production is reliant upon the release of the enzyme renin from the juxtaglomerular apparatus of the kidney. Renin release is stimulated by a decrease in circulating volume, and / or a reduction in sodium delivery to the distal tubule, which is sensed by the macula densa. A decrease in pressure of the major capacitance vessels also leads to renin release secondary to increased sympathetic stimulation of renal nerves (Gibbons et al. 1984). Renin catalyzes the conversion of the hepatic protein angiotensinogen to Angiotensin, which in turn is converted to ATII by angiotensin converting enzyme (ACE), a reaction which occurs in the lung. ATII acts upon G-protein coupled cell surface receptors of the zona glomerulosa resulting in the stimulation of the enzyme aldosterone synthase and subsequent aldosterone production. The mechanism by which potassium levels affect aldosterone production are not entirely clear, but may be secondary to changes in intracellular calcium concentrations. Low potassium levels stimulate, and high potassium levels inhibit, aldosterone production (Young 1985).

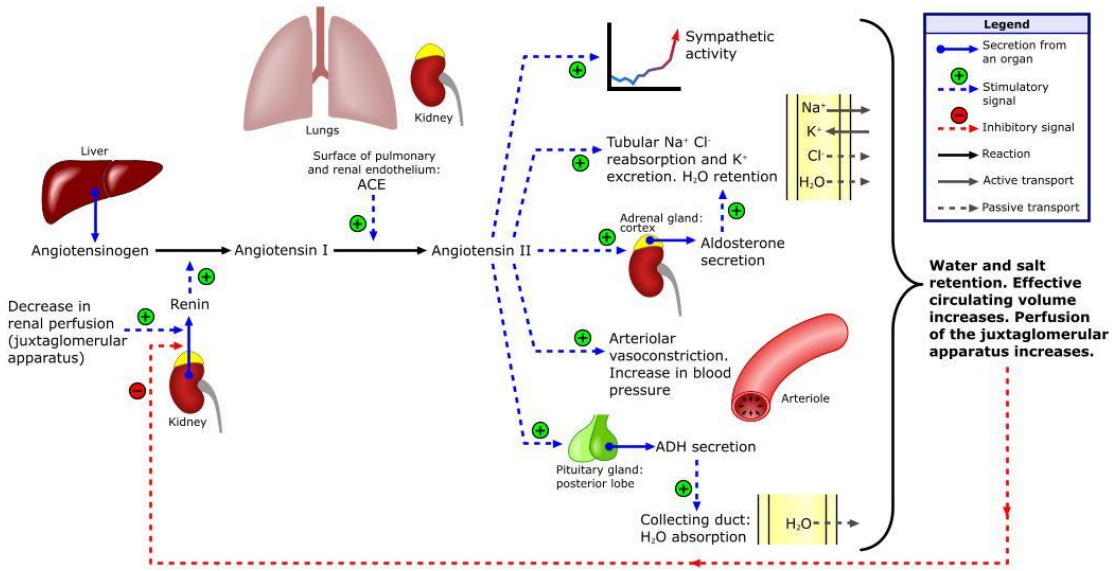


Figure 1-8 Schematic illustrating the renin-angiotensin-aldosterone system (free access article from wikipedia website)

1.4.3. Glucocorticoid metabolism

The major steps in cortisol metabolism are shown in Figure 1-9. Cortisol can be inactivated by conversion to cortisone by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). 11β-hydroxysteroid dehydrogenase is one of the main topics of this thesis and is discussed in detail in section 1.5. Both cortisol and cortisone enter into two main pathways of degradation, the 5α-5β reductases and the 20β-oxoreductase pathways. Dihydrometabolites are quickly reduced to tetrahydrometabolites by 3α- hydroxysteroid dehydrogenase. The tetrahydro-derivatives are then conjugated to glucuronic acid and subsequently excreted in the urine (Cope 1972). The second pathway involves the enzyme 20β-oxoreductase which reduces cortisol and cortisone to their 20β – dihydro derivatives. These derivatives are not known to have any significant glucocorticoid activity, although recent preliminary work has shown that 5α-THB has anti-inflammatory properties similar to those of corticosterone (B) (Yang C. et al. 2009). These reactions predominantly occur in the liver, but also in other tissue to a lesser extent including

adipose tissue, but the contribution of this overall is small compared to hepatic glucocorticoid metabolism (Andrew et al. 1998).

1.4.4. Regulation of Glucocorticoid levels

Cortisol metabolism is critically linked to the set point of the HPA axis. Circulating cortisol levels are maintained as a fine balance between secretion and metabolism. Increased cortisol clearance due to an increase in metabolism results in a compensatory up regulation of ACTH secretion to maintain circulating cortisol levels. A number of factors influence the function of these negative feedback loops (Drouin et al. 1993;Kovacs and Mezey 1987). In particular a functioning glucocorticoid receptor (GR) appears to be essential, as animals with defective GR and GR knockout animals do not suppress ACTH despite chronically high circulating glucocorticoid levels. While these negative feedback loops appear to control glucocorticoid levels in the normal state, it is apparent that central mechanisms can override these controls. For example, during periods of physiological and pathological stress there is an increase in proinflammatory cytokines such as TNFα and interleukin-1β (IL-1β) which cause an increase in cortisol levels via increases in CRF and subsequent increases in ACTH secretion. This altered glucocorticoid negative feedback is imperative in mounting an adequate stress response, as illustrated in cases of adrenal and pituitary disease, where inability to mount a stress response can result in circulatory collapse unless there is pharmacological augmentation of corticosteroid therapy.

Cortisol can be converted to the inactive glucocorticoid cortisone by 11β-hydroxysteroid dehydrogenase type 1, although in vivo the oxo reductase activity of the enzyme predominates, generating active cortisol from inactive cortisone. Type 2

11β-HSD catalyses almost exclusively the conversion of cortisol to cortisone. This ability to interconvert cortisol to cortisone places this enzyme as a pivotal pathway in controlling the local availibility of active cortisol to bind to the GR. It is also able to protect the mineralocorticoid receptor from illicit occupation by cortisol.

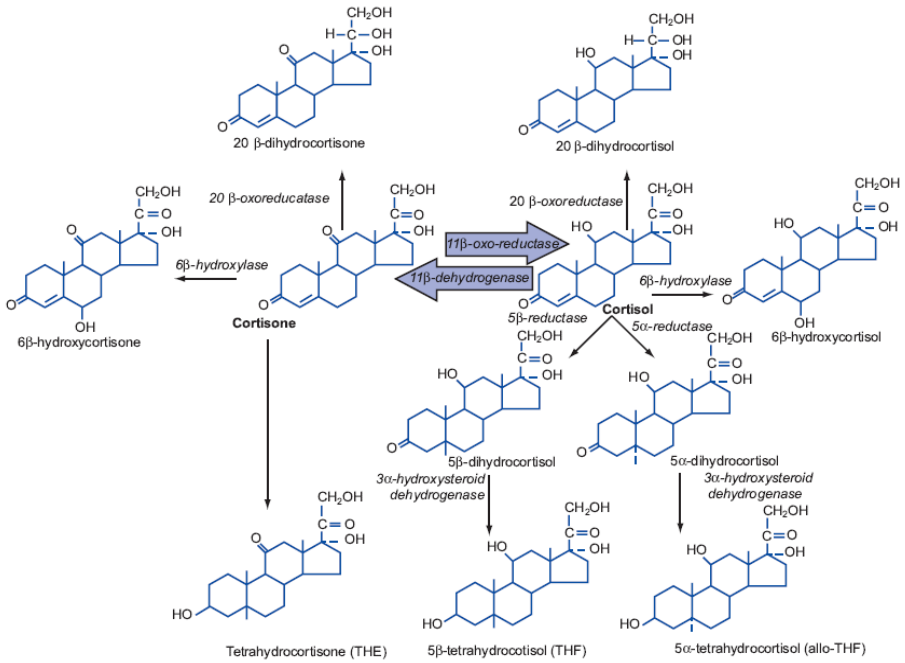


Figure 1-9 The main pathways for glucocorticoid metabolism (Tomlinson and Stewart 2001)

1.4.5. Mechanism of action of corticosteroids

The conventional view is that corticosteroids (cortisol and aldosterone) exert their actions through specific intracellular receptors, glucocorticoid and mineralocorticoid receptors (GR and MR) respectively. More recently, evidence has been presented for the existence of cell surface steroid receptors, and second messengers inside cells that may result in steroid induced non genomic actions (Christ et al. 1999). While there is molecular biological evidence of the existence of these cell surface receptors, detailed information about them is lacking. Steroids are also able to facilitate the action of GABA receptors by binding to $GABA_A$ receptors.

The traditional view that steroids exert stereotyped responses for all ligands has also been put into question by studies that demonstrate that the oestrogen receptor is able to exert a range of actions often opposing, when liganded by molecules with very similar structures (Schaufele et al. 2000). These observations have led to the awareness that a key component of steroid hormone action involves interaction of the ligand with coactivator – corepressor molecules.

1.4.5.1. Corticosteroid receptors

Historically, glucocorticoids were thought to bind exclusively to GR and aldosterone to MR, and regulate carbohydrate and sodium homeostasis respectively. However, following *in-vitro* observations that both receptors bind to glucocorticoids with high affinity, they were classified as Type 1 'high affinity' GR (corresponding to the MR) and Type 2 'low affinity' GR (corresponding to GR). In this thesis the traditional MR and GR classification will be used. Separate receptors for glucocorticoids and mineralocorticoids appear to have occurred via gene duplication late in evolution, explaining why they behave in a similar fashion in some circumstances. This may also reflect the utility of pre receptor mechanisms (section 1.4.7.1) in some tissues to confer further specificity to these receptors. Both receptors have similar ligand binding properties. The MR binds cortisol with an equal affinity to that of aldosterone (K_d for both ~ 1nM) (Arriza et al. 1987). The GR binds cortisol and corticosterone with a K_d between 20 and 40 nM and aldosterone with a K_d of 25 to 65 nM (Marver et al. 1981).

The MR is expressed in target tissues such as epithelia of renal distal tubules, salivary glands and distal colon, as well as within the central nervous system (CNS), in the placenta and foetal tissues, and in bone cells. The GR is widely expressed in tissues

involved in glucose homeostasis, such as liver, adipose tissue and muscle, as well as bone cells and cells in the immune system.

There has been detailed investigation into the actions of glucocorticoid hormones. Steroid binding to the cytosolic GRα receptor results in the activation of the steroid receptor complex involving the dissociation of heat shock proteins HSP90 and HSP70 (Hutchison et al. 1993). The initial dissociation is with HSP70 followed by two HSP 90s. HSP90 organising protein (hop) acts as a scaffold holding the complex together. Once the steroid hormone has been translocated to the nucleus, there is stimulation or repression of gene transcription by binding of the complexes to glucocorticoid response elements in the promoter regions of target genes (Beato et al. 1996). Nuclear localization sequences (NLS) are obscured by chaperone proteins but are exposed on hormone binding which causes the chaperone proteins to dissociate. The NLSs allow protein interaction at the nucleopore enabling transport into the nuclear compartment. There may be additional nuclear chaperone proteins that direct the receptors to specific nuclear sub-compartments (DeFranco 2000).

In addition to the classical GRα, an alternative isoform GRβ can be generated by alternative splicing of the human GR gene, which differs by one amino acid at the C-turminus (Vottero 1999). GRβ may act as a dominant negative regulator of GRα transactivation (Lu and Cidlowski 2004). The GRγ variant has an additional amino acid within the DNA binding domain of the receptor protein that may reduce GR transactivation (Lu & Cidlowski 2004). Critical regions of the receptor responsible for binding and transactivation have been highlighted by naturally occurring mutations in the GR (patients with glucocorticoid resistance) and *in vitro* generated GR mutants (Hollenberg et al. 1987). Many other coactivators and corepressors are

required that may confer tissue specificity of the response in addition to the many hundreds of glucocorticoid responsive genes that have been identified.

Evidence suggests the GR gene expression may be under the control of more than one promoter. Multiple promoters have been identified in human and rat GR resulting in mRNAs for GR with variable untranslated 5' sequences (Breslin et al. 2001). These promoters respond differently to glucocorticoid treatment, and may reflect an additions level of control over GR expression in different tissues.

1.4.5.2. Steroid hormone receptor structure

The typical domains common to steroid hormone receptors are illustrated in Figure 1-10 along with the corresponding domains in the hGR and hMR. The DNA binding domain is a critical component responsible for binding to the double helix. The N-terminal region mediates transactivation functions, and the C-terminal is the ligand binding domain. In reality this model is rather simplistic and activation functions can also be mediated by sequences located throughout the receptor, including NLSs and sequences mediating receptor dimerisation. These features are displayed in GR and MR. The cDNAs encoding the proteins for human GR and MR are highly homologous and share a conserved DNA binding domain with 94% homology and a 57% homology across the ligand binding domain. (Arriza et al 1987;Hollenberg et al 1987). This reflects the promiscuity of ligand binding of aldosterone to the GR and cortisol to the MR. The parts of the complex that are responsible for transrepression of genes are currently unclear. Specificity upon the MR is conferred by the process of pre receptor metabolism of cortisol by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which inactivates cortisol and corticosterone to inactive 11-keto metabolites, allowing aldosterone to bind to the MR (Edwards et al. 1988).

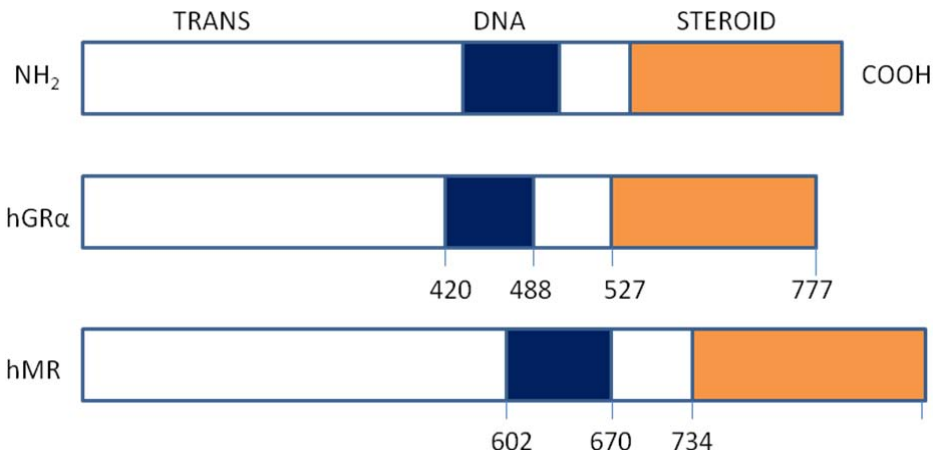


Figure 1-10 Schematic of steroid hormone structure with the N-terminal transactivation, DNA binding and steroid binding domains. The corresponding regions of in the human GR and MR are shown with amino acid numbers.

1.4.5.3. Transactivation of genes by corticosteroid receptors

Transactivation of genes occurs when the GR or MR bind to glucocorticoid response elements (GREs) – specific regulatory sequences of DNA which are usually located near the promoter region of target genes (Beato et al 1996). The classic GRE sequence for GR binding is a partially palindromic structure with the sequence GGTACAnnnTGTTCT (where n is any nucleotide). The consensus sequence for negatively regulated genes is less well conserved. There has been huge investigation into the exact mechanism of transactivation and how the binding of the complex to GREs affects gene transcription. Dimerisation of the steroid-GR complex is required for transactivation. This has been shown *in vitro* and in transgenic dimerisation deficient transgenic animals (Reichardt et al. 1998;Touray et al. 1991).

In addition to interaction of DNA binding and activation domains of the receptor with GREs, gene transcription relies on a number of other vital components. These include general transcription factors and coactivator or corepressors that initiate or repress transcription. Coactivators are thought to be involved in the linking of the hormone

receptor to general transcription factors and in chromatin remodelling during assembly of the transcription complex (Beato et al 1996).

There is no known classic response element for the MR. However MR is able to bind to the GRE and a number of genes appear to be induced both by MR and GR e.g. serum and glucocorticoid inducible kinase (Sgk), a key gene of mineralocorticoid hormone action (Chen et al. 1999). Some genes respond differently depending on whether GR or MR binds to GRE. These differences are likely to be due to differences in the recruitment of coactivators and corepressors. Studies have also shown that GR and MR are able to heterodimerise with each other. These heterodimers appear to have different effects to GR or MR homodimers at the GREs of several genes. For example corticosteroid induced gene transcription of the neuronal serotonin receptor was greater with GR/MR heterodimers than with GR or MR homodimers (Ou et al. 2001).

1.4.5.4. Transrepression of genes by corticosteroid receptors

Transrepression of genes is critical to restrict gene expression so only a subset of genes are expressed in a particular cell type and exert a degree of tissue specificity. The process of transrepression occurs via protein-protein interactions. GR associates with other transcription factors via leucine zipper interactions. Binding of a repressor to the activator can mask its transactivational ability. This type of repression is called quenching, an example of which is the interaction of the GR with members of the bZIP FOS and JUN family of transcription factors, constituting the AP-1 complex. GR acts as a repressor by adjacent binding to the AP-1 factors or tethering to AP-1 factors bound to the composite element, quenching activation by AP-1 (Meyer et al. 1993). This process is of particular importance in the anti-inflammatory effects of

glucocorticoids, by negative regulation of the expression of several genes involved in the inflammatory cascade, by interaction with AP-1 and nuclear factor kappa B (NF-κB). NF-κB is a key regulator of several inflammatory processes and can act as an amplifier of inflammatory pathways, and interaction of GR with NF-κB effects a significant anti inflammatory action. MR is also able to interact with AP-1 and NF-κB, although the relevance of this finding is unclear but may be important in the role of the MR in the pathogenesis of cardiovascular disease (Kolla and Litwack 2000).

1.4.6. Glucocorticoid action

The multiplicity of glucocorticoid function makes it difficult to unravel action at both the cellular and tissue level. Some effects are permissive in nature where glucocorticoids allow the process to occur but do not initiate them directly. Other effects which are difficult to investigate are the specifics of the glucocorticoid response to stress and circadian rhythm, both of which are difficult to reproduce *in-vitro*. At a cellular level glucocorticoids affect cell proliferation, differentiation and cell death/survival. The major effects of glucocorticoids are illustrated in Figure 1-11.

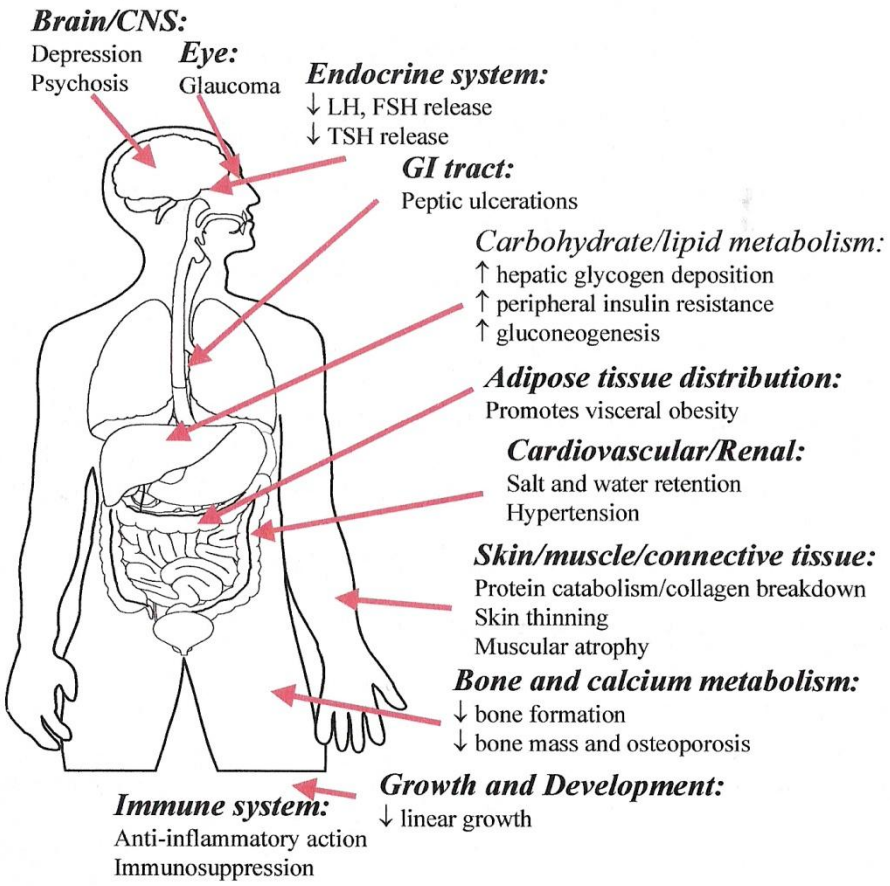


Figure 1-11 Diagram illustrating the effects of glucocorticoids on tissues and organ systems

There is a significant attraction in the development of synthetic glucocorticoids that exert therapeutic effect without the undesired side effects. The discovery of fludrocortisone which has salt retaining activity out of proportion with its glucocorticoid activity was one such achievement. However the development of a steroid anti inflammatory agent devoid of the effects on intermediary metabolism is still awaited. Most of our understanding of glucocorticoid action comes from studying the effects of glucocorticoid excess or deficiency. Excess glucocorticoids leads to a constellation of signs described as Cushing's syndrome. The features of this syndrome are similar regardless of whether the cause is from excess endogenous

glucocorticoid production (from an ACTH secreting pituitary adenoma or a glucocorticoid secreting adrenal adenoma) or exogenous use. Some features are more likely to occur with endogenous Cushing's syndrome due to the production of adrenal androgens and hormones with mineralocorticoid activity (such as deoxycorticosterone). Cushing's syndrome is characterised by truncal obesity, supraclavicular fat pads, buffalo hump, moon face, hirsutism, dark striae, easy bruising, osteoporosis, hypertension and proximal myopathy. Biochemical analysis reveals evidence of impaired glucose tolerance (or overt diabetes) and hypokalaemic alkalosis may also be present. Neutrophil leucocytosis with lymphocytopaenia with decreased eosinophils is also often seen. The features of Cushing's syndrome are described in detail below.

1.4.6.1. Effects of glucocorticoids on carbohydrate, protein and lipid metabolism

The inability of adrenalectomized animals to maintain hepatic glycogen stores was noted in 1927 by Cori (Cori and Cori 1927). Replacement of adrenocortical steroids returned hepatic glycogen stores to normal and reversed hypoglycaemia. Glucocorticoids promote glycogen deposition in the liver by activating glycogen synthase (by promoting its dephosphorylation) and inactivating glycogen phosphorylase (involved in glycogen mobilisation) (Hornbrook et al. 1966;Stalmans and Laloux 1979).

Glucocorticoids are directly able to increase hepatic glucose production. This occurs by activation of key hepatic gluconeogenic enzymes such as glucose-6-phosphatase and PEPCK. Interaction of the glucocorticoid receptor complex with a GRE located in the 5' flanking region of the PEPCK gene mediates glucocorticoid induced gene

transcription (Exton 1979;Magnuson et al. 1987). Glucocorticoids also exert a secondary effect by increasing substrate availability to increase hepatic glucose production by promoting the release of glucogenic amino acids from peripheral tissues such as skeletal muscle, an effect which is particularly apparent when glucocorticoids are replaced in adrenalectomised animals (Exton 1979).

Glucocorticoids also promote hepatic glucose production by exerting a permissive effect upon other gluconeogenic hormones such as adrenaline and glucagon, and increase the sensitivity of target tissues to respond to catecholamines to increase substrate for glucose production. For example, glucocorticoids increase sensitivity of catecholamines to lipolysis in target tissues (Exton 1979). Glycerol and FFA released from lipolysis provides substrate and energy for hepatic glucose production. Glucocorticoids also increase sensitivity to glucagon action but the mechanism for this has not yet been elucidated (Exton 1979).

The effects of glucocorticoids on peripheral glucose utilization further promote the hyperglycaemic state that can often result from glucocorticoid administration. Glucose transport into cells and utilisation by peripheral tissues is inhibited. A decrease in glucose transporters in adipose tissue is associated with decrease in transporter mRNA levels (Garvey et al. 1989).

Glucocorticoids affect lipid metabolism by directly activating lipolysis in adipose, increasing serum FFA, and hence FFA delivery into the circulation. In adrenalectomised animals FFA levels and lipolysis are reduced, and return to normal 2 hours after glucocorticoid administration. The molecular mechanism for this is not yet known but may occur by a permissive effect upon sensitivity to lipolytic hormones such as human growth hormone (hGH) and catecholamines (Fain 1962). The chronic effects of glucocorticoids on lipid metabolism are well known, with the

marked redistribution of body fat. Deposition of fat is seen over the trunk, anterior mediastinum and mesentery, as well as dorsocervial and supraclavicular regions, with sparing of the extremities. The mechanism underlying this central predisposition is not understood but may be related to increased lipogenesis from the hyperinsulinaemia that results from the effects of (HAUSBERGER 1958). Glucocorticoids stimulate adipocyte differentiation and adipogenesis via key differentiation genes including lipoprotein lipase, glycerol-3-phosphate dehydrogenase and leptin (Hauner et al. 1989). Centripetal obesity and weight gain are extremely common in patients with Cushing's syndrome. The deposition of visceral and central adipose tissues provides a useful discriminatory sign in the diagnoses of Cushing's syndrome (Rebuffe-Scrive et al. 1988). Generalised obesity is more common among the general population than in patients with Cushing's syndrome. This predisposition to visceral obesity may also relate to the increased expression of GR and 11β-HSD type 1 (generating active cortisol from inactive cortisone) in omental compared with subcutaneous tissue (Bujalska et al. 1997).

1.4.6.2. Anti-inflammatory actions and effects on immunologic function

Immunologic responses are suppressed by glucocorticoid excess, an observation which provided the stimulus to develop potent glucocorticoids for treatment of a wide range of inflammatory and autoimmune conditions, and organ transplantation. Latent infections such as tuberculosis may be reactivated by glucocorticoid administration (Graham and Tucker, Jr. 1984) and has been reported as a presenting feature of Cushing's disease. Wound infections and poor wound healing are also common with glucocorticoid excess. Numerous effects of glucocorticoids upon the components of the immune and inflammatory response have been documented but the determination

of which are most relevant to the physiological role of glucocorticoids in immunomodulation is yet to be shown.

Glucocorticoids alter the trafficking of immune cells to and from the immune system. Redistribution of lymphocytes from the intravascular compartment to the spleen, lymph nodes, and bone marrow results in an acute reduction in lymphocyte counts (T cells > B cells) (Yu et al. 1974). In addition glucocorticoids act directly on T and B lymphocytes to inhibit immunoglobulin synthesis and stimulate lymphocyte apoptosis (McKay and Cidlowski 1999). Inhibition of cytokine production from lymphocytes is mediated through inhibition of NF-κB by the process of transrepression (section 1.4.5.4). Neutrophil counts increase and eosinophil counts fall rapidly, an effect that historically was used as a bioassay for glucocorticoids.

In addition glucocorticoids exert anti inflammatory effects by inhibiting monocyte differentiation into macrophages, and macrophage phagocytosis and cytotoxic activity. The local inflammatory response is also reduced by preventing the action of histamine and plasminogen activators. Prostoglandin synthesis is impaired by the induction of lipocortins that inhibit phospholipase A2 activity.

1.4.6.3. Effects on skin, musculoskeletal and connective tissue and calcium metabolism

Glucocorticoids cause catabolic changes in muscle, skin and connective tissue. In skin and connective tissue there is inhibition of the synthesis of extracellular matrix components, epidermal cell division, and DNA synthesis. The effects of glucocorticoids on intermediary metabolism in skeletal muscle described earlier (section 1.4.6.1) results in a marked catabolic effect on muscle protein, the basis of the profound myopathy that can result from glucocorticoid excess.

With around 1% of the western population taking chronic glucocorticoid therapy, rising to 2.5% of adults aged 70-79 (van Staa et al. 2000), osteoporosis induced by glucocorticoids is a prominent health issue. Chronic glucocorticoid excess causes osteopenia and osteoporosis by inhibiting osteoblast function (Canalis 1996). A negative calcium balance is also induced by inhibiting intestinal calcium absorption and increasing renal excretion of calcium. This results in increased parathyroid hormone secretion.

Patients with longstanding Cushing's syndrome often have loss of height due to osteoporotic vertebral collapse. Pathologic fractures are also not uncommon. Rib fractures are often painless and heaped up callus formation is seen on radiography. Aseptic necrosis of the femoral and humeral heads is a known feature of high dose corticosteroid treatment and can occur in Cushing's syndrome. Hypercalciuria can cause renal calculi.

1.4.6.4. Effects on salt and water homeostasis and blood pressure control

As discussed in section 1.4.5.2 glucocorticoids have a natural affinity for MR, but the presence of 11β-HSD2 in mineralocorticoid target tissues minimises this action. Glucocorticoids however have a direct effect upon blood vessels, increasing the pressor response to angiotensin II (ATII). Indeed vascular mortality is the major cause of death among patients with untreated Cushing's disease (Boscaro et al. 2001). Glucocorticoids are important in the ability of the kidneys to clear free water. Impaired free water clearance accounts for the hyponatraemia seen clinically with glucocorticoid deficiency (Ahmed et al. 1967;Mandell et al. 1980). This is mediated by AVP- dependant and independent mechanisms. A reduction in glucocorticoid levels results in increased AVP synthesis in the hypothalamus and inappropriately

raised plasma AVP levels for the serum osmolality (Davis et al. 1986). Glucocorticoids also enhance the sensitivity of the collecting ducts to AVP (Davis et al 1986).

1.4.6.5. Effects on mood and the central nervous system

The most evident effect of glucocorticoid excess in the CNS is the effect on mood which can range from mild euphoria to frank psychosis, as well as depression. 50% of patients with Cushing's syndrome have psychiatric abnormalities regardless of cause.

1.4.6.6. Endocrine effects

Glucocorticoids suppress the thyroid axis, via direct inhibition of thyrotropin secretion and inhibition of 5' deiodinase activity (which mediates the conversion of thyroxine to triiodothyronine). Growth hormone secretion is reduced as well as GnRH pulsatility and leutinizing hormone and follicle stimulating hormone release, underlying the amenorrhoea and hypogonadism seen in Cushing's syndrome (Saketos et al. 1993). Gonadal dysfunction is very common in Cushing's syndrome with cortisol directly inhibiting the function of Leydig cells. In females there is menstrual irregularity, hirsutism and acne. These effects reflect the suppression of the pituitary thyroid, and pituitary gonadal axis seen in Cushing's patients.

1.4.6.7. Pseudo-Cushing's Syndrome

Pseudo-Cushing's syndrome represents a condition with all or some of the clinical features of Cushing's syndrome, combined with biochemical evidence of hypercortisolism, and disappearance of the Cushingoid-like features with resolution of

the underlying disorder. Common causes include depression, alcoholism and obesity. Depressed patients with symptoms severe enough to require hospitalization, can have all the hormonal abnormalities of Cushing's syndrome, including the characteristic responses with dynamic testing of the pituitary adrenal axis. Generally, depressed patients do not have the clinical signs of Cushing's syndrome and hence are fairly easy to distinguish. In addition circadian rhythm of ACTH and cortisol secretion is maintained. However the ACTH pulse frequency may be increased with the resulting cortisol pulses being of greater amplitude and duration (Carroll et al. 1976). Patients with Cushing's syndrome however are frequently depressed, and hence careful clinical and biochemical investigation is required.

Alcoholic pseudo-Cushing's is rare and is seen in patients with chronic heavy alcohol intake with clinical and biochemical evidence of chronic liver disease. The pathogenesis of this condition has remained much of a mystery. Some studies have shown that alcohol directly stimulates cortisol secretion. HPA axis function appears to vary depending on whether testing is carried out after acute alcohol consumption, acute withdrawal and long term abstinence (Groote and Meinders 1996). Vasopressin levels are also elevated with decompensated liver disease and may directly stimulate the HPA axis. This condition is discussed in further detail in section 1.5.3.3 and was part of a detailed clinical and *in-vitro* analysis as part of the work for this thesis, described in chapter 5.

A common endocrine referral is the request to exclude an underlying endocrine cause in a patient with obesity. Patients with obesity may have mildly elevated cortisol secretion rates secondary to HPA axis activation (Glass et al. 1981;Ljung et al. 1996). Urinary free cortisol concentrations are only mildly elevated and circulating cortisol levels are invariably normal. This may be related to reduced hepatic cortisone to

cortisol conversion by 11β-HSD1 and increased cortisol clearance by hepatic A ring reductases, resulting in stimulation of the HPA axis (Andrew, Phillips, & Walker 1998;Stewart et al. 1999).

1.4.7. Determinants of tissue sensitivity to glucocorticoids

Studies have failed to show a clear relationship between serum glucocorticoid level and the effect on tissues, suggesting that there are factors intrinsic to tissues that are important in determining tissue sensitivity to glucocorticoids. These differences could occur at a number of levels, including changes in the concentration and affinity of GR or any of the steps involved in the formation of the steroid-receptor complex, to translocation to the nucleus and modulation of transactivation or transrepression of genes. The action of glucocorticoid transporters may also be involved in regulating intracellular glucocorticoid levels. The major part of this thesis relates to the expression and activity of hepatic glucocorticoid metabolising enzymes in health and disease related to the metabolic syndrome. The 11β-HSD enzymes are major candidates for determining tissue sensitivity to glucocorticoids.

While variation in concentration of glucocorticoid receptors may underlie differences in glucocorticoid sensitivity, data suggests that GR variants are not a major factor. Mutations in the GR gene have been associated with glucocorticoid resistance. Numerous GR-gene variants have been identified, as well as single nucleotide polymorphisms (SNP) which are relatively prevalent. In particular, the GRβ-variant has been proposed to influence corticosteroid sensitivity, with evidence derived from the immune system and asthma. Also of relevance are the BcII restriction fragment polymorphism and a Asp363Ser which have been identified to influence the regulation of the HPA axis and are associated with changes in metabolism and

cardiovascular control. These findings further implicate cortisol in the pathophysiology of these disorders. Indeed, it has been proposed that a dysregulation of the HPA axis, especially during stress is a common underlying abnormality in these complex disorders (DeRijk et al. 2002).

Post receptor variation most probably does occur but is difficult to analyse due to sparse knowledge of coactivators and corepressors. Of potential importance are post receptor factors such as AP-1 and NF-κB, and other transcription factors that directly interact with the GR. An example would include the increase in GR-mediated transcription by pro-inflammatory cytokines TNFα and IL-1β seen in several cell types (Costas et al. 1996). Very little is known about the impact of variation in coactivator/corepressors molecules in modulating glucocorticoid action.

1.4.7.1. Pre-receptor metabolism and steroid action

Pre receptor metabolism as a regulator of steroid action has been seen in several tissues, and for several different enzymes. Enzymatic conversion of steroid in the target cell cytoplasm can interconvert hormone between active and inactive forms. Examples of enzymes and steroid receptors involved in pre-receptor metabolism are shown in Table 1-1. One clinical example where pre-receptor hormone metabolism is crucial relates to androgen action, and on the expression of 5α-reductase type 2 to convert testosterone to the more active dihydrotestosterone; lack of this enzyme is a cause of pseudohermaphroditism (Walsh et al. 1974). Androgens are converted to oestrogens by the action of aromatase. Aromatase deficiency has a profound effect on skeletal development, resulting in a eunuchoid habitus with low bone mineral density that is treatable with exogenous oestrogens (Carani et al. 1997).

Enzyme	Steroid Receptor	Function
11β-HSD1	Glucocorticoid	Activation
11β-HSD2	Mineralocorticoid	Inactivation
5α - reductase	Androgen	Activation
17β – HSD, aromatase	Oestrogen	Conversion
1α-hydroxylase	Vitamin D	Activation
24-hydroxylase	Vitamin D	Inactivation

Table 1-1 Steroid receptors and enzymes involved in pre-receptor metabolism with the principle function of each enzyme shown in the final column.

1.5. 11β-hydroxysteroid dehydrogenases

The 11β-HSD enzymes catalyse a number of reactions, the most predominant being the interconversion of cortisol (F) and cortisone (E). This activity has been described in a wide range of tissues (Stewart and Krozowski 1999). Early *in vitro* studies showed significant amounts of 11β-HSD activity in placenta, kidney and liver, although the 'set point' of the enzyme varied between tissues, being predominantly oxidative (F to E) in placenta and kidney and reductase (E to F) in liver (Bush 1969;Jenkins 1966;Osinski PA 1960). Subsequent *in vivo* isotopic studies and clinical studies in patients with renal disease demonstrated that the predominant reaction the kidney was the conversion of cortisol to cortisone (Bush 1969;Hellman et al. 1971;Srivastava et al. 1973). Selective venous catheterisation studies showed significantly lower F/E ratios in renal venous blood compared with peripheral blood. Hepatic venous blood however had much higher F/E ratios, indicating that in the liver the conversion was predominantly of cortisone to cortisol (Walker et al. 1992b). These observations are explained by the activity of two distinct isoenzymes of 11β-HSD, a NADPH dependant type 1 or 'liver' isoenzyme and a NAD dependant

oxidative type 2 or renal isoenzyme. 11β-HSD1 is expressed principally in liver and visceral adipose tissue, as well as brain, bone, muscle, gonad, muscle, eye and other tissues that express GR (Tomlinson et al. 2004b). This 11β-HSD type 1 enzyme was first cloned and sequenced in rats (Agarwal et al. 1989) and humans (Tannin et al. 1991). However it was subsequently realised that this enzyme did not co localise with MR in the kidney (Rundle et al. 1989), nor could it protect the MR from occupation by cortisol. The sequence for the gene of this enzyme was also found to be unchanged in patients suffering from a rare inborn cause of hypertension, the syndrome of apparent mineralocorticoid excess (AME) (Nikkila et al. 1993). These observations formed the basis for the evaluation of a second 11β-HSD isoenzyme (now designated 11β-HSD type 2) which is expressed in mineralocorticoid target tissues (Agarwal et al. 1994;Albiston et al. 1994), and shown to be mutated in individuals with AME (Mune et al. 1995;Whorwood and Stewart 1996;Wilson et al. 1995).

1.5.1. 11β-Hydroxysteroid dehydrogenase type 2

11β-HSD2 is a unidirectional NAD dependant enzyme that converts cortisol to cortisone, Figure 1-12. It has a high affinity for cortisol with a Km of 50 nM (100 times that for 11β-HSD1) and 5nM for corticosterone (Albiston et al 1994;Brown et al. 1993;Stewart et al. 1994).

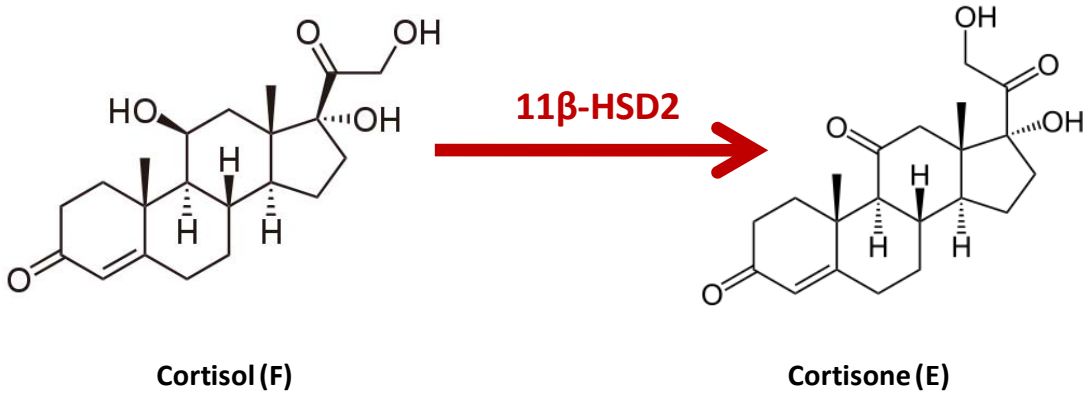


Figure 1-12 The conversion of cortisol to cortisone by 11β-HSD2. This reaction is unidierectional for endogenous glucocorticoids and prednisolone.

The calculated molecular weight for 11β-HSD2 is 44,140 Da, but the purified protein migrates at 44kDa on sodium dodecyl sulphate (SDS) purified gels. The human 11β-HSD2 protein is 405 amino acids long and the cDNA is 5.3kb long, and the gene is located on chromosome 16q22, comprises 5 exons and is 6.2kb in length (Albiston et al 1994).

The enzyme is highly expressed in mineralocorticoid target tissues such as kidney as well as surface epithelial cells in the colon, salivary epithelial cells and skin. In the kidney, expression is found in the principal cells of the collecting ducts in the renal cortex, most of the medulla and the distal convoluted tubule (Krozowski et al. 1995;Whorwood et al. 1995). 11β-HSD2 is also strongly expressed in the human placental trophoblast, which has a powerful ability to inactivate glucocorticoids. Enzyme expression increases to term in human, baboon and rat placenta, but in mouse placenta, expression decreases from day 13 (Brown et al 1993;Krozowski et al 1995;Stewart et al. 1995). The enzyme is also expressed in foetal tissues, and many malignant cell lines. In all situations the enzyme acts as a high affinity dehydrogenase decreasing intracellular corticosteroid levels. 11β-HSD2 is

incorporated into the ER membrane but unlike 11β-HSD1, it has a luminal N-terminus and cytoplasmic C-terminus (Odermatt et al. 1999).

The functional role of 11β-HSD2 in mineralocorticoid target tissues is to protect the mineralocorticoid receptor (MR) from cortisol, in keeping with its expression in a range of mineralocorticoid target tissues. Hence the enzyme has been suggested as a mediator of extracellular fluid volume and blood pressure. Studies have shown a variation in 11β-HSD2 activity in hypertensive subjects compared with controls, with an increased plasma half life of cortisol and urinary (THF+alloTHF)/THE, indicative of decreased 11β-HSD2 activity (Soro et al. 1995;Walker et al. 1993). Genetic studies have shown linkage of microsatellite markers in the 11β-HSD2 gene with salt sensitivity, further suggesting the possible importance of 11β-HSD2 in the regulation of blood pressure (Agarwal et al. 2000). The importance of 11β-HSD2 in MR localization was illustrated by an elegant study which showed that in the presence of 11β-HSD2, MR is localized to the ER, whereas in its absence it is localized to the nucleus (Odermatt et al. 2001). How ligand selectivity is mediated at the molecular level remains somewhat uncertain. In addition, it is unclear why certain synthetic glucocorticoids are not substrates for the enzyme.

1.5.1.1. Regulation of 11β-HSD2 expression and enzyme activity

A number of 11β-HSD2 inhibitors have been identified. Liquorice and its derivatives have been widely studied. Liquorice has been known to cause mineralocorticoid hypertension for many decades, and liquorice excess causes hypertension, hypokalaemia and low renin and aldosterone (Conn et al. 1968;Reevers 1946). It became clear that liquorice itself does not possess intrinsic mineralocorticoid activity, as it failed to provide mineralocorticoid replacement in patients with Addison's

disease or in adrenalectomised animals (BORST et al. 1953;CARD et al. 1953). It was subsequently discovered that liquorice and its derivates have extremely low affinity for the MR, and in fact inhibit 11β-HSD2, inducing a mild form of 'apparent mineralocorticoid excess', section 1.5.1.2. Liquorice administration results in an increase in urinary (THF+allo-THF)/THE ratio, with increased plasma cortisol half life and decreased cortisone levels (Stewart et al. 1987). Glycyrrhizic acid (GI) and its hydrolytic product glycyrrhetinic acid (GE) are the active components of liquorice that competitively inhibit 11β-HSD2 with a K_i of 5-10nM (Albiston et al 1994;Stewart, Murry, & Mason 1994). Carbenoxolone is the hemisuccinate derivative of 18β-GE, and is also a potent 11β-HSD2 inhibitor (Stewart et al. 1990). Unlike GI and GE, carbenoxolone does not cause a major change in the (THF+alloTHF)/THE indicating that it also simultaneously inhibits 11β-HSD1 activity.

Decreased renal 11β-HSD2 activity has been identified as a factor in the cause of pregnancy induced hypertension (Heilmann et al. 2001). This may be due to a placentally derived inhibitor of maternal renal 11β-HSD2, or intrinsically low 11β-HSD2 activity seen in some patients, making them more susceptible to pregnancy induced hypertension.

ACE inhibitors also appear to increase 11β-HSD2 at the enzyme level, thus providing an additional mechanism for the action of ACE inhibitors in reducing blood pressure (Ricketts and Stewart 1999).

1.5.1.2. Syndrome of apparent mineralocorticoid excess

The appreciation of the role of 11β-HSD2 came from the understanding of a rare monogenic inherited cause of hypertension, 'apparent mineralocorticoid excess'

(AME). Defective peripheral conversion of F to E in patients with AME was first suggested by Ulick and colleagues (Ulick et al. 1979). Lack of 11β-HSD2 causes a failure of the protective mechanism preventing illicit MR activation, resulting in cortisol acting as a potent mineralocorticoid. These patients however are not cushingoid, as normal circulating cortisol mechanisms are maintained due to normal negative feedback HPA axis feedback loops, causing a reduction in cortisol secretion rates (Stewart et al. 1988). Approximately 100 cases of AME have been identified worldwide (Shackleton et al. 1985;Ulick et al 1979). AME is characterised by low renin, low aldosterone hypertension and hypokalaemia. Patients present in childhood or adulthood with severe hypertension and hypokalaemia with suppressed aldosterone levels, low plasma renin activity and extended half life of cortisol. The hypertension in AME can be treated with the potassium sparing diuretic, spironolactone.

1.5.2. 11β-Hydroxysteroid dehydrogenase type 1 enzymology and gene structure

11β-HSD1 belongs to the short chain dehydrogenase/reductase SDR superfamily of enzymes. Members of this family have 250 to 400 residues with an N-terminal cofactor binding domain and a centrally located active site. 11β-HSD1 is a bi-directional enzyme that mediates dehydrogenation or reduction at the 11β position of many glucocorticoids, as well as having carbonyl reductase activity (Finckh et al. 2001;Stewart & Krozowski 1999). In contrast to dehydrogenase activity, reductase activity of the enzyme is unstable in vitro (Lakshmi and Monder 1988). However in intact adipocytes and hepatocytes, oxoreductase activity predominates with generation of F from E (Bujalska, Kumar, & Stewart 1997;Kotelevtsev et al. 1997). It was

subsequently shown that reductase activity predominates unless intact cells are disrupted (Duperrex et al. 1993).

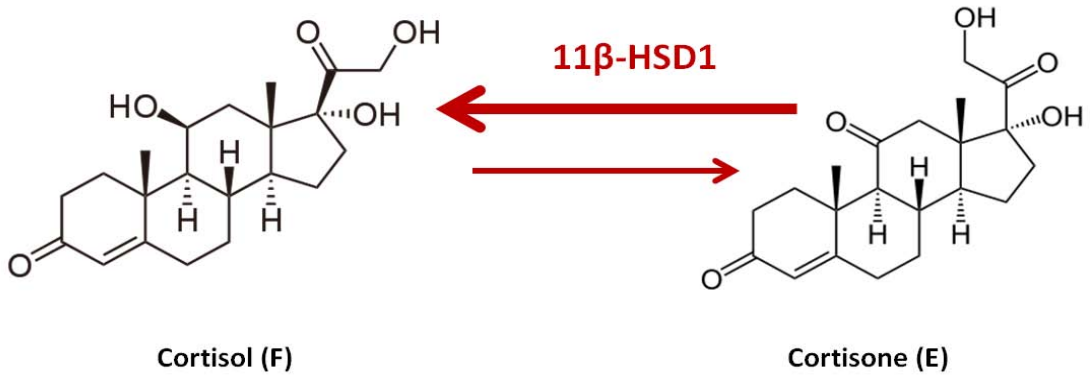


Figure 1-13 11β-hydroxysteroid dehydrogenase type 1 interconverts cortisone and cortisol by modification at the 11β-position.

Kinetic analysis *in vitro* showed that the enzyme has a higher affinity for E (K_m 0.3μM) than F (K_m 2.1 μM), in keeping with predominantly reductase activity *in vivo*, (Tannin et al 1991). In some cell types there may be predominantly 11β dehydrogenase activity according to the physiological or developmental status of the particular cells type. In human omental adipose stromal cells, there is a switch from dehydrogenase to reductase activity upon differentiation to adipocytes (Bujalska et al. 2002). In neuronal cells too, both dehydrogenase and oxoreductase activity have been noted (Jellinck et al. 1999). The location of the 11β-HSD1 enzyme is crucial, facing the inner endoplasmic reticulum lumen, which places in proximity with powerful generators of the reduced co-substrate NADP phosphate (NADPH). Inclusion of a NADPH regeneration system with the enzyme glucose 6 phosphate dehydrogenase allows reductase activity to be regained, suggesting that it is the high level of NADPH present within the ER lumen that allows reductase activity to predominate *in vivo* (Agarwal et al. 1990). Studies have also shown that the reactions are pH dependant, working optimally at pH 7.0 – 7.4 (Walker et al. 2001). The critical role of providing

cofactor to fuel the reductase activity of 11β-HSD1 in the ER is conferred by the enzyme hexose-6-phosphate dehydrogenase (H6PDH) (Draper et al. 2003), as shown in Figure 1-14. This enzyme also constitutes a novel direct link between glucose homeostasis and the hypothalamo-pituitary-adrenal (HPA) axis. Studies relating to hepatic steroid metabolism in glycogen storage disease type 1a are described in detail in chapter 4, and highlight the importance of G6P concentrations in the ER lumen in determining the set point of 11β-HSD1 activity.

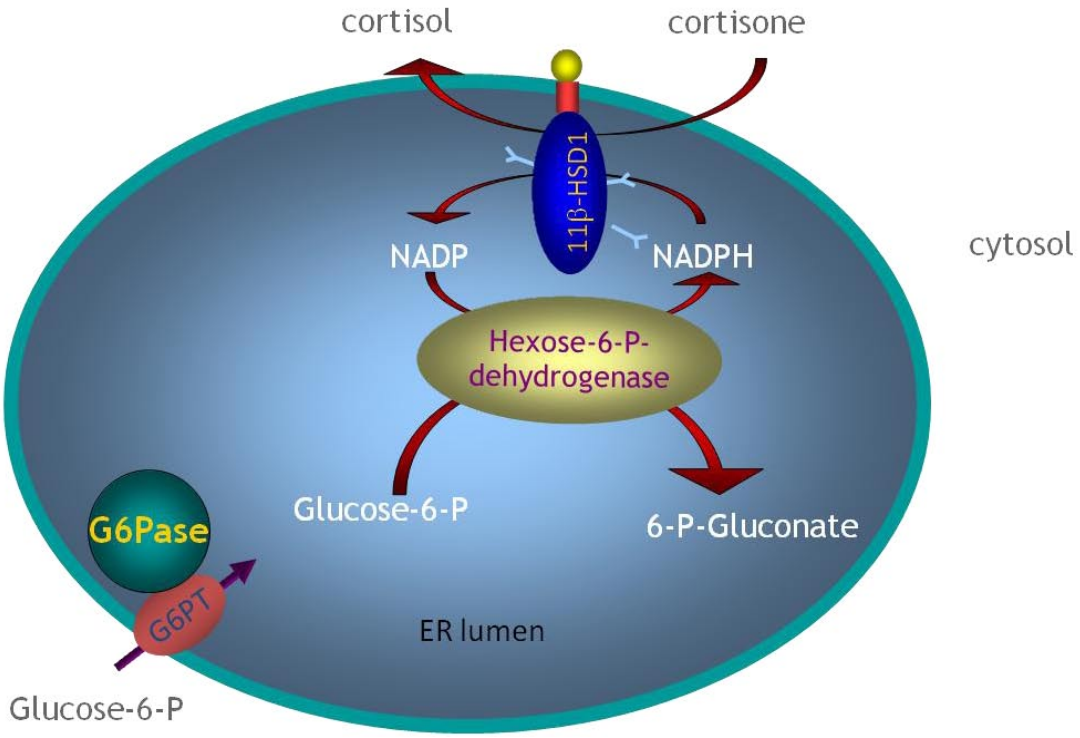


Figure 1-14 Schematic representation of the interaction between 11β-HSD1 and H6PDH in the ER lumen which provdides reductase activity to allow it to function as a reductase (cortisone to cortisol). G6PT, Glucose-6-phosphate translocase; G6Pase, glucose-6-phosphatase; Glucose 6P, glucose-6-phosphate; 6PGluconate, 6-phosphogluconolactonate.

The amino acid sequences of the 11β-HSD1 protein and cDNA were characterised in 1991 (Tannin et al 1991). The gene is located on chromosome 1 and consists of 6

exons, 5 introns, and generates a cDNA encoding a 282 amino acid protein (Figure 1-15). The gene was originally thought to be 9kb long but later studies suggested that the gene was in fact 30kb long, due to a large intron (>25kb) (Draper et al. 2002). Several intronic polymorphisms in the gene have been detected but the coding region remains well conserved.

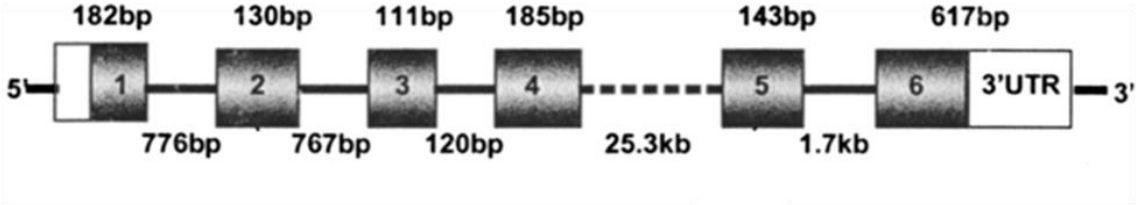


Figure 1-15 Organization of the human 11ß-HSD1 gene. Open boxes indicate the 5'- and 3'-untranslated regions, gray shaded boxes indicate coding exons (1–6), and intervening solid lines indicate introns (Tomlinson et al. 2004) .

There is a high level of sequence homology between species in particular in the cofactor binding region (GASKGIG) and the catalytic site (YSASK). The single hydrophobic N terminal extension preceding the cofactor binding domain anchors the protein in microsomes. The greater part of the 11β-HSD1 protein was found to lie within the ER lumen with a short transmembrane and a five amino acid region on the cytosolic side. Experiments with chimeric proteins where the N terminal regions of 11β-HSD1 and 11β-HSD2 (a cytosolic enzyme) were exchanged, resulted in inverted orientation within the ER and inactive proteins (Odermatt et al 1999). Within the N terminal transmembrane domain are critical charge-specific residues (2 positively charged lysine residues on the cytosolic side and 2 negatively charged glutamate residues) that are crucial to the orientation of the 11β-HSD1 protein in the ER membrane. The lumen of the ER promotes the formation of specific intra-chain disulfide bonds in the 11β-HSD1 protein that are important in the protein structure; disruption of these is detrimental to enzyme activity (Moore et al. 1993;Ozols 1995).

While extracellular glucocorticoid levels can be measured with ease, there is relatively little data regarding the intracellular levels of substrates and products for 11β-HSD1 in the cytoplasm or in the microsome. Any factors that can inhibit the metabolism of the 11β-hydroxyl group will increase glucocorticoid potency. Data indicate the importance of the structural conformation of steroidal compounds as modifications to these can confer specific inhibitory properties. In addition to its important role in glucocorticoid metabolism, 11β-HSD1 mediates the phase 1 biotransformation of carbonyl group bearing foreign compounds including drugs, xenobiotics (Maser and Bannenberg 1994;Oppermann and Maser 2000), and carcinogens exerting a protective detoxifying effect (Maser 1998).

1.5.2.1. 11β-HSD1 inhibitors

A commonly used inhibitor of 11β-HSD1 enzyme are the liquorice derivatives glycyrrhizic acid (GI) and glycyrrhetinic acid (GE), and the hemmisuccinate derivative, carbenoxolone (Jellinck et al. 1993). GE also inhibits 11β-HSD2 with a K_i of 5-10nM (Stewart, Murry, & Mason 1994). These compounds act as non competitive inhibitors at the active site of the enzyme and can also decrease mRNA expression. Studies have shown that carbenoxolone has 11β-HSD1 inhibitory effects *in vivo* (Stewart et al 1990). Bile acids also inhibit 11β-HSD1 activity *in vitro* (Buhler et al. 1994). In particular lithocholic acid and chenodeoxyxholic acid are the most potent. Arylsulfonamidothiazole compounds have been shown to inhibit 11β-HSD1 both *in vivo* and *in vitr*o. The diethylamide derivative was shown to inhibit human 11β-HSD1 with an IC_{50} of 52 nM and an *N*-methylpiperazinamide form (BVT.2733) has been shown to be specific for the mouse 11β-HSD1 enzyme. In a hyperglycaemic mouse strain, BVT.2733 lowered PEPCK and G6Pase mRNA as well

as blood glucose and serum insulin levels raising the exciting possibility of the use of 11β-HSD1 inhibition in the treatment of insulin resistance and diabetes (Alberts et al. 2002). Numerous compounds of various structural classes have since been identified that specifically inhibit 11β-HSD1 (Boyle and Kowalski 2009). Recently reported results using INCB13979, a selective 11β-HSD1 inhibitor showed an improvement in HbA1c and cholesterol in patients with T2D when used in combination with metformin in phase IIb trial (ROSENSTOCK J 2009a).

1.5.2.2. Regulation of expression and activity of 11β-HSD1

11β-HSD1 activity may be regulated at a number of levels. At the enzyme level these would include substrate availability, competitive and non competitive inhibition and allosteric regulation (by other agents or by assembly into more complex units). These have already been discussed in the sections above dealing with enzyme properties (section 1.5.2.). Regulation may also occur at the level of expression, including changes in gene transcription and translation as well as the rate of clearance of the enzyme. Several factors are known to regulate expression, and the most widely investigated include glucocorticoids and cytokines. To summarise, glucocorticoids, C/EBP, peroxisome proliferator-activated receptor γ agonists, and some proinflammatory cytokines (TNFα and IL-1ß) increase 11β-HSD1 expression, whereas GH (acting via IGF-I) and liver X receptor agonists inhibit expression. Other factors, such as sex steroids, insulin and thyroid hormone vary between tissues and between species (Tomlinson et al. 2004). Some of these factors are described in further detail in specific chapters

1.5.3. Tissue expression and activity in normal physiology and pathophysiology of peripheral tissues

Detailed studies have been carried out on 11β-HSD1 in a range of peripheral tissues including liver, adipose tissue, CNS, reproductive tissue and muscle. There have also been studies on kidney, eye and pancreas.

1.5.3.1. 11β-HSD1 in the liver, and regulatory effects on factors affecting insulin sensitivity

11β-HSD1 immuno reactivity is seen in hepatocytes, being particularly intense around the central vein (Ricketts et al. 1998b). In humans the activity of this enzyme activates orally administered cortisone to its biologically potent form. Primary cultures of human and rat hepatocytes and the 2S FAZA cell line indicate exclusive 11-oxoreductase activity (Jamieson et al. 1995;Ricketts et al. 1998a). In rat liver, but not mouse liver, there is a sexually dimorphic pattern of 11β-HSD1 activity and expression, with expression in males being higher than females, which has been linked to the sexually dimorphic pattern of GH secretion (Rajan et al. 1995). In humans, at the age of 4-5 years 11β-HSD1 expression appears to be similar in boys and girls until puberty, when activity decreases in girls (Dimitriou et al. 2003). In studies on elderly patients (Toogood et al. 2000) and GH deficient hypopituitary patients (Weaver et al. 1998), activity is generally higher in men than women.

In clinical studies venous blood draining the liver had increased cortisone to cortisol ratios relative to systemic blood indicating the conversion of cortisone to cortisol *in vivo.* These studies are further supported by studies that used isolated perfused livers where activity was predominantly reductase. Interestingly, while concurrent infusion of carbenoxolone made little difference, seven days pre treatment with carbenoxolone

significantly reduced oxoreductase activity (Jamieson et al. 2000;Walker et al. 1992a). In the rat 2S FAZA cell line reductase activity is inhibited by insulin and IGF-1 and stimulated by dexamethasone (Voice et al. 1996). The promoter region of the rat 11β-HSD1 gene is positively regulated by C/EBPα and to a lesser extent C/EBPβ (Williams et al. 2000). In humans, hyperthyroidism moves the set point of F to E conversion towards E, but this requires a functional thyroid hormone receptor rather than being a function of thyroid hormone *per se* (Whorwood et al. 1993).

There is currently intense research in the role of 11β-HSD1 and insulin resistance. The regulatory role of 11β-HSD1 can occur at the level of adipose tissue (central and visceral obesity), liver (hepatic gluconeogenesis) and muscle. This section focuses on the role of hepatic 11β-HSD1 in this process. An overview of the effect of glucocorticoids on hepatic carbohydrate metabolism has been detailed earlier in this chapter (section 1.4.6.1). Studies on mice with targeted disruption of the 11β-HSD1 gene showed a relative failure to express glucocorticoid-inducible genes in the liver indicating reduced hepatic glucocorticoid generation in these animals. These mice do not display fasting hypoglycaemia, but fasting glucose levels after high fat feeding are significantly lower than in wild type controls. While they have no difference in baseline expression of glucose-6-phosphatase and PECK although upon starvation these key enzymes of hepatic glucose induction fail to be induced (Kotelevtsev et al 1997). The animals also have a less atherogenic lipid profile then wild type, which is thought to be due to increased expression of hepatic enzymes that are involved in fat catabolism (Morton et al. 2001) and provided strong evidence for the role of glucocorticoid metabolism underlying metabolic disease.

Administration of selective 11β-HSD1 inhibitors to rodents for seven days induced a decreased expression of both G6Pase and PEPCK (Alberts et al 2002). Interestingly,

recent studies have shown that the administration of carbenoxolone to diet induced obese and insulin resistant mice, also resulted in a reduction in hepatic GR mRNA levels, which correlated with the suppression of H6PDH and 11β-HSD1 activity (Liu et al. 2008). Studies on healthy humans have supported animal data with a reduction in hepatic glucose production with carbenoxolone administration (Walker et al. 1995). Similar studies on type 2 diabetics showed that the decrease in hepatic glucose production may be due to decreased glycogenolysis rather than a change in hepatic gluconeogenesis (Andrews et al. 2003). Several selective 11β-HSD1 inhibitors are being developed with the aim of lowering intracellular cortisol concentrations in adipose and liver in patients with type 2 diabetes and obesity with promising results (Hughes et al. 2008) (section 1.5.2.1).

While the impact of glucocorticoids on glucose tolerance are well known, studies on subjects with impaired glucose tolerance have failed to show increased hepatic 11β-HSD1 activity when measured by cortisone generation profiles following dexamethasone suppression and oral cortisone acetate, and urine corticosteroid metabolite ratios (Andrews et al. 2002;Rask et al. 2002;Stewart et al 1999). Studies on Type 2 diabetics have had varied results with no difference in 11β-HSD1 activity measured by urine corticosteroid metabolite ratios (Kerstens et al. 2000) and possibly impaired hepatic cortisol generation using cortisol generation curve analysis (Andrews et al. 2002). This may reflect a physiological compensatory mechanism to limit fasting hyperglycaemia and increase hepatic sensitivity by decreasing hepatic cortisol concentrations. Interestingly, transgenic mice over-expressing 11β-HSD1 are hyperinsulinaemic after a glucose load and also have dyslipidaemia and hypertension (Paterson et al. 2004).

H6PDH represents a novel direct link between cellular glucose metabolism, glucocorticoid metabolism and the HPA axis and is described in detail in chapter 4.

1.5.3.2. Hepatic 11β-HSD1 in non alcoholic fatty liver disease

Non alcoholic fatty liver disease (NAFLD) is the hepatic phenotype of the metabolic syndrome. Glucose metabolism in NAFLD has been detailed in section 1.3.4. The possible role of 11β-HSD1 in the pathophysiology of NAFLD is an exciting one as it raises the potential role of treatment with 11β-HSD1 inhibitors, as well as providing insight into the pathogenesis of this condition which remains largely unknown. Of note, 20% of patients with cushings syndrome have NAFLD (Rockall et al. 2003). It is unclear which factors are responsible for the switch from the somewhat benign 'simple hepatic steatosis' to steatohepatitis which can be rapidly progressive to end stage liver disease and cirrhosis. Studies on mice with transgenic overexpression of hepatic 11β-HSD1 (Paterson et al 2004) and adipose selective 11β-HSD1 transgenic mice (described in the next section) (Masuzaki et al. 2001) provided some background to the hypothesis that there may be a net export of cortisol from the omental adipose tissue to the liver via the portal circulation, which in turn would impact on hepatic metabolic phenotype. According to a recent study, this does not appear to be the case. The release of cortisol from adipose tissue and its effect on portal vein cortisol levels was quantified in a study using infusion of a stable isotope of cortisol and simultaneous sampling from the epigastric, portal and hepatic veins and peripheral arterialised blood. There was evidence of significant cortisol release from subcutaneous adipose tissue that may contribute to whole body cortisol regeneration. However splanchnic release of cortisol was accounted for entirely by the liver, and release of cortisol from visceral tissues (gut, pancreas and spleen) into the

portal vein was not detected – speculating that visceral 11β-HSD1 activity is insufficient to influence hepatic glucocorticoid signalling (Stimson et al. 2009). However, an autocrine role for 11β-HSD1 in omental tissue remains a possibility which could still impact on hepatic metabolic phenotype by alterations in portal FFA/adipocytokine delivery.

Clinical studies using healthy male volunteers showed that increased hepatic fat measured by abdominal magnetic imaging resonance scans was not associated with indices of cortisol metabolism, but importantly, these were healthy volunteers with no proven evidence of hepatic steatosis or NAFLD (Westerbacka et al. 2003). Another clinical study did not show any difference in 11β-HSD1 mRNA expression in liver biopsies from different stages of NAFLD, and concluded that 11β-HSD1 gene expression is not involved in the pathogenesis of NAFLD (Konopelska et al. 2009). Detailed investigation into hepatic pre receptor cortisol metabolism in NAFLD was carried out as part of the work for this thesis and is described in detail in chapter 7.

1.5.3.3. 11βHSD1 in alcoholic and other chronic liver diseases

There is now evidence of deranged hepatic cortisol metabolism in NAFLD, alcoholic liver disease as well as chronic liver diseases of other causes. Unlike results from human studies, rats with cirrhosis had reduced hepatic 11β-HSD1 and renal 11β-HSD2 which could be explained by the inhibitory action of bile salts (Escher et al. 1998).

The alcohol-induced pseudo-Cushing's syndrome is an important differential diagnosis of hypercortisolism that is poorly understood (section 1.4.6.7.). In a previous study, urine steroid profile on patients with alcoholic liver disease and non alcoholic chronic liver disease showed a marked increase in urine THF+allo-

THF/THE ratio suggesting either a reduction in renal 11β-HSD2 or an increase in hepatic 11β-HSD1 (Stewart et al. 1993a) suggesting a role for aberrant cortisol metabolism in the process. In a further study, we have further evaluated hepatic glucocorticoid metabolism in patients with alcoholic liver disease (ALD) and non alcoholic/non fatty chronic liver disease (CLD) by measuring F and E concentrations through selective venous sampling of the hepatic, portal, renal and peripheral veins. This showed specifically increased hepatic cortisol generation in alcoholic liver disease. Generation of cortisol in the liver might dampen the inflammatory response and thus limit tissue damage (Ahmed et al. 2008), in a similar theme to that discussed for NAFLD. Indeed, the histological appearances of alcoholic and non alcoholic steatohepatitis are often indistinguishable and may reflect a common response in altered hepatic glucocorticoid metabolism in both diseases. This study formed part of the work for this thesis and is discussed in detail in chapter 5.

1.5.3.4. Adipose tissue

11β-HSD1 is also expressed in adipose tissue and in primary cultures of human omental and subcutaneous adipose stromal cells (Bujalska, Kumar, & Stewart 1997). In cell culture, 11β-HSD1 activity is higher in omental cells the subcutaneous cells and glucocorticoids and proinflammatory cytokines further induce activity in cells from both depots (Tomlinson et al. 2001). Cortisol is essential for adipocyte differentiation and the autocrine generation of cortisol by 11β-HSD1 regulates this process. Inhibition of 11β-HSD1 prevents differentiation of adipose cells (Bujalska et al. 1999). *In vitro* enzyme expression is enough to allow differentiation of omental stromal cells by cortisone alone (Bujalska et al 1999) and there is enzyme induction with differentiation from stromal cells to adipocytes. This appears to be related to a

change in the set point of the enzyme from dehydrogenase in stromal cells to reductase in adipocytes without a significant change in mRNA levels (Bujalska et al 2002). Mice over expressing 11β-HSD1 in adipocytes, by virtue of a 11β-HSD1 cDNA linked to an adipocyte fatty-acid binding protein (aP2) promoter had enhanced adipose tissue differentiation (measured by increased fat cell size) (Figure 1-16) and a viscerally and centrally obese phenotype as well as other key features described later in this section (Masuzaki et al 2001). In rat and human pre adipocytes, glucocorticoids have been seen to exert an anti-proliferative effect which is removed upon inhibition of 11β-HSD1 (Figure 1-16) (Tomlinson et al. 2002b).

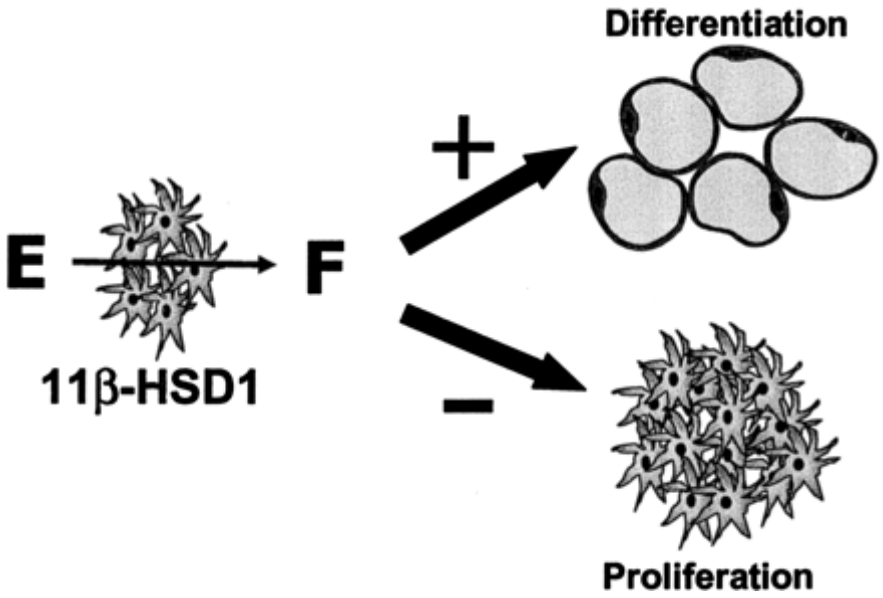


Figure 1-16 In human adipose tissue the autocrine/paracrine generation of cortisol through 11β-HSD1 activity promotes adipose differentiation and inhibits adipocyte proliferation (Tomlinson et al. 2004).

1.5.3.5. Other tissues

11β-HSD1 is also expressed in skeletal muscle and one study has shown that 11β-HSD1 gene expression correlates with markers of insulin resistance, BMI and blood pressure (Whorwood et al. 2002). Another more recent study showed increased 11β-HSD1 expression in myotubes from type 2 diabetic subjects, and the cortisone

induced reduction in glucose uptake was reversed with carbenoxolone (Abdallah et al. 2005).

1.6. Hexose-6-phosphate dehydrogenase, ER redox and metabolic sensing

The enzyme hexose-6-phosphate dehydrogenase (H6PDH) has been known for decades but previously received little attention until it was pushed to the forefront not only as a crucial component of pre receptor glucocorticoid metabolism, but also more recently as a link between the autocrine effects of glucocorticoids and intermediary metabolism. This identified H6PDH as a novel metabolic sensor of the endoplasmic reticulum (ER) linking intracellular hormone signalling and glucose metabolism.

H6PDH is an autosomally linked microsomal enzyme, distinct from the cytosolic sex-linked glucose-6-phosphate dehydrogenase. It uses its own pool of NAD(P)+ and is able to oxidize a variety of phosphorylated hexoses, including glucose-6-sulphate and galactose-6-phosphate. In addition to oxidising G6P to 6-phosphogluconolactone (6PG) it is thought to also hydrolyse 6PG to 6-phosphogluconate, and therefore in effect catalyses the first two steps of the endoluminal pentose phosphate pathway, generating reduced NADPH within the ER (Bublitz and Steavenson 1988), Figure 1-14. The murine enzyme has been proven to have bifunctional activity but this remains to be shown for the human enzyme (Clarke and Mason 2003). H6PDH has been shown to be ubiquitously expressed in nearly all tissues (Hewitt et al. 2005) implying its role as a housekeeping enzyme serving a vital role in redox homeostasis in the ER lumen. Highest activity is observed in steroidogenic cells, liver and renal cells supporting the idea that H6PDH is involved in steroid and drug metabolism.

The protein sequence for H6PDH was first described from rabbit liver microsomes, as a 90 kDa protein with 763 amino acids, unlike cytosolic G6PDH which has a monomer molecular mass of 56kDa (Ozols 1993). The N-terminal region of H6PDH has close homology to cytosolic G6PDH, particularly at the coenzyme and G6P binding sites. The C-terminal 250 amino acids show homology with the N-terminal regions of *Plasmodium falciparum* and *Plasmodiem Berghei* G6PDH proteins, as well as the human 6PGL protein (Clarke et al. 2001;Collard et al. 1999). The human H6PDH gene is localised to chromosome 1p36, spanning 37kb, with 5 exons and 4 introns, that predict a protein of 89kDa (Mason et al. 1999).

While the redox state of the cytosolic pyridine nucleotides is determined by a number of different oxidoreductases, $NADP^+$ reduction in the ER appears to be governed solely by H6PDH. Cotransfection of mammalian cells with expression plasmids encoding 11β-HSD1 and H6PDH consistently increased oxoreductase activity. Substitution with G6PD failed to induce a similar response, demonstrating that NADPH must be generated in the ER lumen to access the active site of 11β-HSD1 (Atanasov et al. 2008;Bujalska et al. 2005). H6PDH has been shown to have direct protein-protein interactions with 11β-HSD1 (Atanasov et al 2008). siRNA experiments which decreased H6PDH expression also decreased oxoreductase activity in intact cells (Bujalska et al 2005). These observations were supported by studies on H6PDH knockout mice. 11β-HSD1 activity in these mice is predominantly dehydrogenase both *in vivo* and *in vitro* (Lavery et al. 2006).

The activity of H6PDH also appears to be governed largely by substrate supply which importantly is sourced via a sole ER membrane protein, the glucose-6-phosphate transporter (G6PT) which is selective for glucose-6-phosphate (Marcolongo et al. 2007;Van and Gerin 2002). This makes the G6PT-H6PDH-11β-HSD1 system an

ideal metabolic sensor of the ER, as minor alterations in the intraluminal [NADPH] / [$NADP^+$] ratio in the ER lumen can cause significant changes in the set point of 11β-HSD1 activity and in turn intracellular glucocorticoid levels. An [NADPH] / [$NADP^+$] ratio of around 9:1 is required for 11β-HSD1 to function as a reductase, implying that the *in vivo* set point of the [NADPH] / [$NADP^+$] redox couple is much more reduced in the ER lumen then the cytosol (Diaz-Flores et al. 2006;Panten and Rustenbeck 2008). Further increases in ER NADPH in turn lead to dramatic increases in 11β-HSD1 driven glucocorticoid generation. Furthermore, H6PDH is a lot more resistant to feedback inhibition by NADPH then cytosolic G6PD (Oka et al. 1981). The redox state of the ER lumen is therefore directly governed by cytosolic G6P concentrations, via the G6PT-H6PDH-11β-HSD1 system. This was demonstrated by studies on patients with glycogen storage disease type 1, described in chapter 4. Further evidence is provided by studies on H6PDH knockout mice which showed fasting hypoglycaemia, low hepatic glycogen content, increased insulin sensitivity and decreased negative feedback on the HPA axis (Lavery et al 2006). These results clearly imply the importance of the G6PT-H6PDH-11β-HSD1 system in the acute metabolic response to feeding with regard to the coupling between metabolic and hormonal responses. Indeed, local 11β-HSD1 activation is needed for the inhibition of glucagon secretion in α-cells of pancreatic islets, further illustrating the direct role of the system in whole body metabolism (Swali et al. 2008).

While the G6PT-H6PDH-11β-HSD1 system is able to act as a physiological metabolic sensor, it also plays a very likely role in the pathophysiology of metabolic states that induce ER stress. Its role in nutrient sensing in insulin sensitive cells has been elegantly illustrated in Figure 1-17 by (Banhegyi et al. 2009). A number of studies have shown that manipulation of the G6PT-H6PDH-11β-HSD1 system by

genetic or pharmacologic means leads to ER stress and UPR, and even apoptosis of various cell types, due to a deficiency of reducing equivalents in the ER lumen (Kardon et al. 2008;Lavery et al. 2008b;Leuzzi et al. 2003). ER stress results in the accumulation of immature proteins which in turn trigger the unfolded protein response (UPR). This leads to numerous downstream effects including apoptosis, insulin resistance and inflammation (Hotamisligil 2005;Zhang and Kaufman 2008). This mechanism is activated by glucose and fatty acids by an unknown mechanism, but may be due to excessive reducing equivalents resulting in an altered redox state in the ER lumen causing ER stress. The ER stress response in essence is a mechanism that is activated to protect the cell from apoptosis, although its activation under conditions chronic excesses of fatty acids and glucose can lead to cellular dysfunction and cell death. Of particular relevance, saturated fatty acids disrupt ER redox homeostasis and cause ER stress and apoptosis in hepatocytes of rats with hepatic steatosis (Wang et al. 2006).

Over-nutrition may therefore be regarded as a cause of excess reducing power in the ER lumen which activates H6PDH via G6PT, resulting in a high endoluminal [NADPH] / [$NADP^+$] ratio and glucocorticoid activation by 11β-HSD1, with the resulting pathophysiological effects. For example, increased 11β-HSD1 activity stimulates pre-adipocyte differentiation, increases expression of lipoprotein lipase, glycerol production and triglyceride synthesis in visceral adipose tissue, contributing to the development of the metabolic syndrome (Bujalska et al. 2008b;Tomlinson et al. 2008a). Increased intracellular glucocorticoid levels results in a number of effects promoting insulin resistance and nearly all the effects of the metabolic syndrome, as detailed in previous sections.

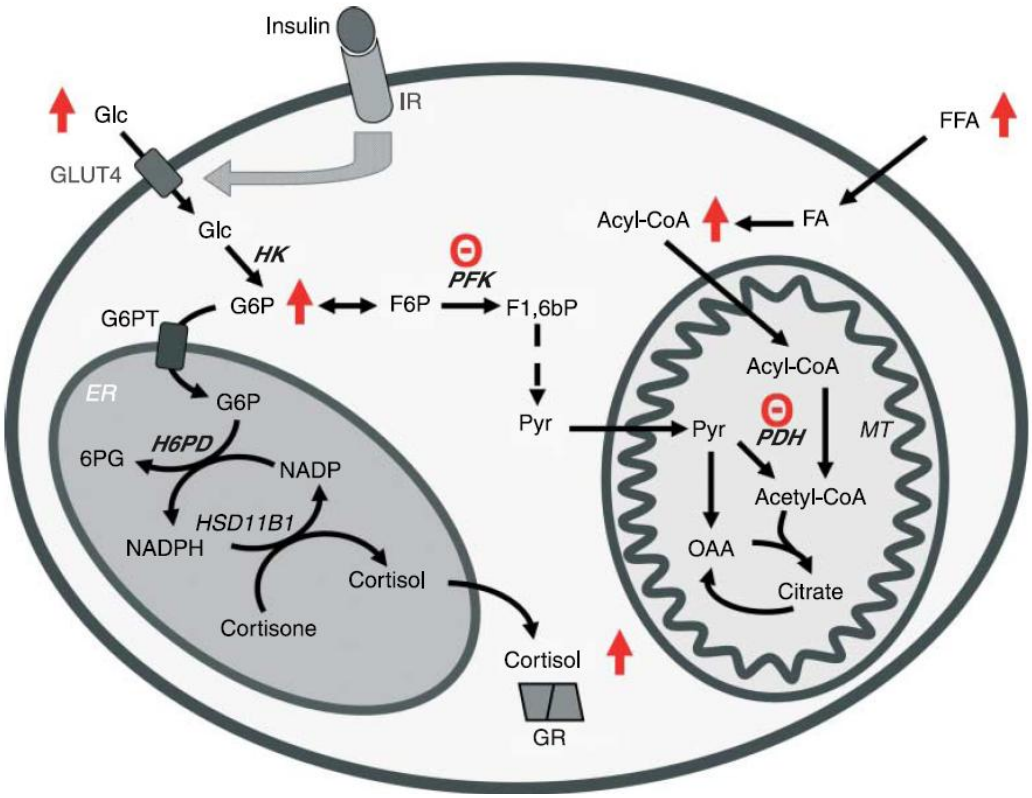


Figure 1-17 *The role of the G6PT–H6PD–HSD11B1 triad in nutrient sensing of insulin-sensitive cells. Insulin stimulates the translocation of GLUT4 into the plasma membrane and hence enhances glucose uptake in muscle and adipose tissue, which results in elevated intracellular glucose-6-phosphate (G6P) level. On the other hand, high level of free fatty acids – according to Randle's 'glucose–fatty acid cycle' – inhibits glucose catabolism at several key steps, most importantly at the level of the pyruvate dehydrogenase (PDH) reaction. As a consequence, glucose-6-phosphate accumulates within the cell. Accumulation of glucose-6-phosphate, in turn, fuels the G6PT–H6PD–HSD11B1 system, which leads to increased prereceptorial activation of glucocorticoids. Thus, oversupply of reducing equivalents – in the form of either carbohydrates or lipids – leads to the enhancement of glucocorticoid activation. Increasing concentrations are indicated by red arrows, and resulting enzyme inhibitions are also indicated in red. Abbreviations: FFA, free fatty acid; F6P, fructose-6-phosphate; F1,6bP, fructose-1,6-bisphosphate; Glc, glucose; G6P, glucose-6-phosphate; GR, glucocorticoid receptor; HK, hexokinase; IR, insulin receptor; MT, mitochondrion; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; Pyr, pyruvate and OAA, oxaloacetate (Banhegyi, Csala, & Benedetti 2009).*

1.6.1. Cortisone reductase deficiency

Cortisone reductase deficiency (CRD) occurs when there is failure of regeneration of cortisol from cortisone via 11β-HSD1. This causes increased cortisol clearance, activation of the HPA axis, and ACTH driven adrenal hyperandrogenism. In males this manifests in early life with precocious pseudopuberty. Females present in midlife with a PCOS phenotype with hirsutism, oligomenorrhea and infertility (Draper et al 2003;Jamieson et al. 1999;Nikkila et al 1993;Phillipov et al. 1996). A biochemical diagnosis of CRD is made by measurement of urinary corticosteroid metabolites reflecting 11β-HSD1 activity – the THF+5αTHF/THE ratio. This ratio is typically lower than 0.1 in affected patients (Tomlinson et al 2004b). HSD11B1 was considered the most likely gene to be mutated in CRD, but no coding mutations directly affecting enzyme activity were identified. However, as the reductase activity of 11β-HSD1 is crucially dependant on the availability of reduced NADPH in the ER lumen, H6PD is clearly also a candidate gene to explain CRD. Previously, sequence variants in *H6PD* and *HSD11B1* in patients with CRD were identified (Draper et al 2003), but large scale population based studies showed that these were polymorphic variants, and not disease causing mutations. The population frequencies of these variants could not explain fully the clinical picture or urinary profiles of CRD patients (Draper et al. 2006;Smit et al. 2007;White 2005). Recently, 4 cases of CRD were reanalysed and inactivating mutations in the *H6PD* gene were identified that were consistent with the steroid biochemistry. Expression and activity assays demonstrated loss of function for all the reported mutations (Lavery et al. 2008a). These data further highlighted the importance of redox homeostasis in the control of cortisol metabolism and the role of H6PDH-11β-HSD1 pathway in the activity of the HPA axis.

1.7. General Aims

On the background presented in the preceding sections the general aim of the work carried out for this thesis was to investigate the impact of hepatic pre receptor glucocorticoid metabolism upon hepatic glucose homeostasis, and hence hepatic and whole body insulin sensitivity in health and disease. The overall hypothesis was that aberrant hepatic pre receptor glucocorticoid metabolism is implicated in the pathophysiology of the metabolic syndrome.

Investigations were planned at the *in vitro* level, to characterise the zonation and expression of hepatic 11β-HSD1 in normal liver and how this compared with diseased liver from patients with NAFLD as well as other histologically analogous liver diseases including alcoholic liver diseases.

A number of clinical investigations were planned in normal patients and those with increasing degrees of insulin resistance (obese non diabetic, type 2 diabetics, NAFLD, and alcoholic liver diseases). The overall aim of these studies was to detect any *in vivo* differences in glucocorticoid metabolism between groups and correlating these differences with markers of metabolic phenotype, and hepatic glucose production by gluconeogenesis.

In-vitro and clinical studies were designed in patients with glycogen storage disease type 1a (G6Pase deficiency) and GSD type 1b (G6P transporter deficiency) to investigate the concept that endoluminal G6P concentrations directly impact upon 11β-HSD1 mediated glucocorticoid generation.

These studies are described in the following chapters with specific aims and hypotheses detailed in relevant sections.

2. Materials and Methods

The techniques described in this chapter are used throughout this thesis but are described here in detail. In each chapter any modifications will be indicated with specific details such as numbers of samples or subjects. Unless otherwise stated, general chemicals were obtained from Sigma-Aldrich, UK, reagents for reverse transcription polymerase chain reactions (RT-PCR) from Applied Biosystems.

This chapter is divided into methods that focus primarily on human tissues and methods used in clinical studies.

2.1. Studies of human tissues

Human liver tissue was obtained from the tissue archive at the Centre for Liver Research, University of Birmingham. Diseased human liver tissue was obtained from

explanted liver from patients undergoing liver transplantation. Normal human liver tissue was derived from donor liver at transplant. Liver histology of all the tissues used was verified from histopathology reports. All tissues were collected with the informed consent of the subjects and all the clinical studies Local Research Ethics Committee (LREC) approval.

2.2. Immunohistochemistry

2.2.1. Principle

Immunohistochemistry allows the detection of specific proteins within cells in intact tissue sections using antibodies specific for the protein of interest. The primary antibodies used are raised in a species distant from the target species allowing the use of labelled anti-species IgG secondary antibodies. Detection is possible via the label conjugated to the secondary antibody. In the work described in this thesis the secondary antibodies was donkey antisheep IgG peroxidase conjugate. Immunohistochemistry reagents were obtained from Binding Site, Birmingham , UK. Optimal concentrations were determined using serial antibody dilutions on standard tissue sections.

2.2.2. Production of the 11β-HSD1 antibody

Hydrophilicity profiles (Hopp 1993;Hopp and Woods 1981) were used to identify likely antigenic sites within the human 11β-HSD1 protein (Tannin et al 1991). One 14 amino acid region was selected, and was synthesised into an eight-branched multiantigenic peptide (Binding Site, Birmingham, UK), which was mixed with Freund's adjuvant and used to immunise a single sheep. An IgG fraction was

prepared from the immune serum by ammonium sulphate precipitation and ion exchange chromatography. Purity of antisera was further enhanced by affinity chromatography using a column coated with the peptide used to immunise the sheep. The IgG fraction of the serum was run down the column to allow specific binding of the anti 11β-HSD1 antibody fraction. The antibodies were then eluted from the column at a low pH. A further Sephacryl S200 gel filtration purification step was performed, to further reduce the presence of non specific antibodies (Binding Site, Birmingham, UK). Immunohistochemistry on standard tissue sections was used to monitor the effects of each purification step. The validation of this antibody has been previously described (Ricketts et al 1998b).

2.2.3. Preparation of tissue sections

For immunohistochemical studies on paraffin sections, tissue was obtained from the Centre for Liver Research, University of Birmingham as above. Tissue was received fixed in 10% Formalin in H_2O. Tissues were then dehydrated through a graded series of ethanol washes (70%, 95% then 100%) and then transferred to xylene for 2 hours. Tissues were embedded in melted paraffin, and then allowed to harden and cool. 5 μm thick sections were cut and transferred to charged microscope slides (Superfrost Plus, BDH, Poole, UK). Slides were ready for use after air drying.

Immunohistochemistry on frozen sections was carried out primarily for the study of NAFLD tissues described in chapter 7. While tissue morphology in cryosections are somewhat inferior to paraffin sections – this method was chosen due to the ease of availability of frozen tissue for analysis.

Freshly dissected tissue had been snap frozen in liquid nitrogen prior to storage in the frozen tissue archive at the Centre for Liver Research, University of Birmingham. Small tissue blocks were received of diseased and normal livers and were transported in liquid nitrogen. Tissues were stored at -80ºC until ready to section.

The tissue sample of interest was mounted on a chuck using O.C.T. (optimal cutting temperature) compound (Tissue-Tek, Ted Pella Inc, USA), trimmed and allowed to temperature equilibrate in the cryostat for 20 minutes prior to cutting. 5µm thick sections were cut and mounted onto charged microscope slides (Superfrost Plus, BDH, Poole, UK). Sections were then fixed in 2 exchanges of 100% acetone, 5 min each, and then dehydrated in 2 changes of 100% IMS for 2 min each. Slides were stored at -20ºC wrapped in foil till use.

2.2.4. Reagents

Antigen retrieval solution - Citrate buffer: 10nM citric acid, 0.05% Tween 20, pH 6.0.

3% methanol hydrogen peroxide: 50ml methanol in a coplin jar, containing 800µl 30% H_2O_2 (for paraffin sections)

0.1% methanol hydrogen peroxide: 50 ml 70% methanol in a coplin jar containing 50µl of 1% H_2O_2 (for cryosections)

Tris Buffered Saline (TBS) pH 7.6 (0.05M):60.5g Trizma base, 31.88g NaCl, 32.63ml 10M HCl made up to 1L with distilled H_2O.

3,3'-diaminobenzidine tetrahydrochloride (DAB) solution: 50mg DAB tablet dissolved in 20 ml TBS. 20µl 30% H_2O_2 added just before use and filtered through whatman paper.

2.2.5. Immunohistochemistry protocol

2.2.5.1. Paraffin sections

For immunohistochemical studies on paraffin sections, tissue was obtained from the Centre for Liver Research, University of Birmingham as above. Tissue was received fixed in 10% Formalin in H_2O. Tissues were then dehydrated through a graded series of ethanol washes (70%, 95% then 100%) and then transferred to xylene for 2 hours. Tissues were embedded in melted paraffin, and then allowed to harden and cool. 5 μm thick sections were cut and transferred to charged microscope slides (Superfrost Plus, BDH, Poole, UK). Slides were ready for use after air drying.

Formalin fixed paraffin embedded liver sections were dewaxed and rehydrated (2 changes of xylene for 2 minutes each; two changes of 100% industrial methylated spirit (IMS) for 2 minutes each). A wax pen was used to draw an area around the section containing the tissue to allow small volumes of solution for incubation with primary and secondary antibody to be used. After washing in tap water the slides were incubated with hydrogen peroxide/methanol for 15 minutes to block endogenous peroxidase activity. Antigen retrieval was enhanced by incubating in an oven at 60ºC for 30 minutes followed by incubation in citrate buffer for 20 minutes in a microwave at medium power. After washing in TBS, non specific antibody binding was blocked with 10% normal donkey serum (NDS) in TBS for 30 min. Sections were then incubated with primary antibody diluted in 10% NDS for 90 minutes at room temperature at a dilution of 1:50 – 1:200. After washing in TBS, sections were incubated with secondary antibody (Donkey anti-sheep IgG conjugated to peroxidase) at a dilution of 1:25, diluted in TBS for 30 min at room temperature, and then

extensively washed. Slides were developed using DAB solution (up to 10 minutes). DAB staining results in a brown precipitate at sites of peroxidase activity. The reaction was stopped by washing in tap water. Cellular nuclei were stained using Mayer's haematoxylin for up to 3 minutes and then washed under running water to remove excess haematoxylin. Sections were then rinsed twice for 5 min in IMS, and then washed twice in xylene. Sections were covered with dibutyl polystyrene, and xylene based mountant.

Controls included incubating with primary antibody in the presence of an excess of immunising peptide (1:100) used at similar concentrations.

2.2.5.2. Cryosections

Slides were removed from -20ºC and allowed to equilibrate to room temperature for 30 min before opening the foil wrapper. Sections were re fixed in 100% acetone for 5 minutes and then air dried. A ring was drawn around the section using a wax pen and placed in a humidified chamber. Endogenous peroxidase was blocked with 0.1% hydrogen peroxide for 20 min and then washed in TBS. Non specific antibody binding was blocked with 10% NDS in TBS for 30 min. Sections were then incubated with primary antibody diluted in 10% NDS for 45 minutes at room temperature at a dilution of 1:50 – 1:200. After washing in TBS, sections were incubated with secondary antibody (Donkey anti-sheep IgG conjugated to peroxidase) at a dilution of 1:25, diluted in TBS for 30 min at room temperature, and then extensively washed. Slides were developed using DAB solution (up to 10 minutes), and then counterstained and mounted as described in section 2.2.5.1.

2.3. mRNA detection and quantification

A number of techniques have been used in this thesis for RNA extraction, and for detection and quantification of mRNA.

2.3.1. Isolation of cellular RNA

Cellular RNA was extracted from snap frozen whole liver tissue chunks using the TriReagent system (Sigma, UK). The chunks were trimmed while frozen and a small volume (~2-3mm^3) was used obtaining high yields of RNA. Liver samples obtained from biopsies of patients with NAFLD were of very small volume (<1mm^3) and RNA extraction was carried out using the Genelute Mammalian Total RNA kit (Sigma). RNA was isolated from laser capture microdissected liver samples using the specialised Ambion RNAqueous-Micro Kit (Applied Biosystems) which is optimized for the purification of total RNA from micro sized samples of tissue. The procedure followed exactly as per manufacturer's protocol for each system used.

2.3.1.1. Measuring RNA concentration

RNA concentration was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). The quality of extracted RNA was also assessed by electrophoresis on 1% agarose gel with 0.15μg/ml ethidium bromide and visualised under UV light. Intact RNA showed clear 18S and 28S bands.

2.3.2. Reverse transcription Polymerase Chain Reaction (PCR)

2.3.2.1. Principle

Reverse transcription PCR is a sensitive technique used to detect small amounts of specific mRNAs within a tissue. Cellular RNA is collected and converted to complimentary (cDNA) using reverse transcriptase enzymes. The cDNA is then used as a template for the amplification of specific sequences. The process utilizes a pair of primers which are complimentary to the 5' end of the sequence for amplification on each of the two strands of cDNA. The primers are then extended by DNA polymerases, with a copy of the strand made after each cycle. The strands are separated by increasing the temperature above the melting point for double stranded DNA, so the template and the generated DNA strand become targets for further annealing of primers. This results in logarithmic amplification of DNA fragments of a size defined by the primers added. Hence, the reaction is specific sequences of target DNA, with capacity for significant amplification and thus detection ability. The DNA polymerases used, unlike mammalian polymerases are able to withstand the high temperatures required for the reaction.

2.3.2.2. Method used for cDNA generation using reverse transcriptase

Reverse transcription of RNA from whole liver samples was performed using a Promega reverse transcription system as previously described (Whorwood et al 1995). In summary, 1µg of total RNA and 0.5µg of random primers in a final volume of 10µl were incubated at 70ºC for 10 min. In a final reaction volume of 20µl made up with nuclease free water, the following were added: 25mM $MgCl_2$ 4µl, 10x RT buffer 2µl,

10mM dNTP 2µl, Ribonuclease inhibitor 0.5µl, 0.5µg/µl random hexamer primers 1µl, 15U avian megablastoma virus reverse transcriptase. The reaction was incubated at room temperature for 10 minutes, 42ºC for 60 min, 95ºC for 5 min, and then 4ºC for 5 min. The cDNA generated was stored at -20ºC.

Reverse transcription of RNA from laser capture microdissected tissues was carried using a One Step Applied Biosystems reverse transcription kit. In a final reaction volume of 20µl with a maximum of 1µg RNA the following were added: 10x RT buffer 1µl, 25mM $MgCl_2$ 2.2µl, 10mM each dNTPs 2µl, Random Hexamers 0.5µl, RNase Inhibitor 0.2µl. The reaction was incubated at 25ºC for 10 min, 37ºC for 60 min, 48ºC for 30 min, 95ºC for 5 min, 4ºC for 5 min, and then stored at -20ºC.

2.3.3. PCR

Several PCR techniques are available based on RT-PCR technology, including non quantitative PCR, semi quantitative PCR and or quantitative 'real time' PCR. 'Real time' PCR was the primary method used in the studies described in this thesis.

2.3.3.1. Principle of real-time quantitative RT-PCR

Real-time analysis is possible by the simultaneous amplification and detection of target RNAs. The technique uses fluorescent dyes that are either incorporated into probes which bind specifically to the nucleic acids (Bustin 2000) of interest or that intercalate specifically with double stranded DNA. The TaqMan assay system developed by Perkin-Elmer-Applied Biosystems was used for studies in this thesis. This system relies on the 5' nuclease activity of DNA polymerase to hydrolyse a hybridisation probe bound to the target nucleic sequence. The hybridisation probe

contains a fluorescent reporter dye at the 5' end and a quencher of fluorescence at the 3' end. Breakdown of the hybridisation probe by the nuclease activity of taq polymerase breaks the reporter-quencher proximity, allowing the unquenched emission of fluorescence that is then detected. An increase in the nucleic acid sequences targeted by the fluorescent probe with each PCR cycle results in a proportional release of fluorescence, due to breakdown of probe and release of reporter. The 3' end of the probe is altered chemically to prevent its extension during PCR cycling. A schematic of this method is illustrated in Figure 2-1.

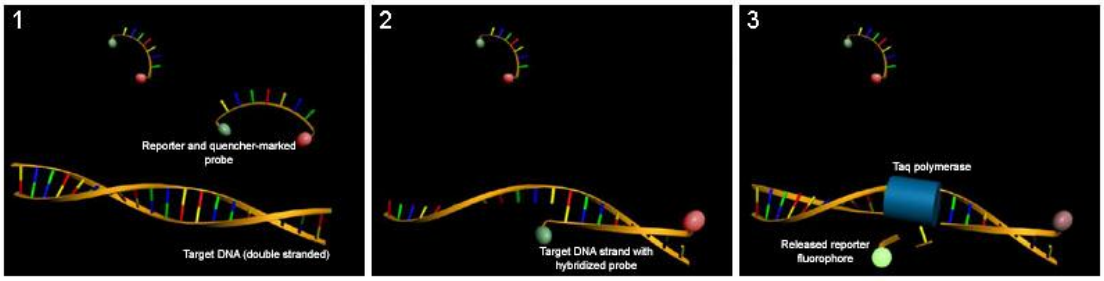


Figure 2-1 *Schematic of the TaqMan assay: Forward and reverse primers define the hybridisation probes that bind within the target sequences.While the probe is intact, emmission of fluorescence is prevented by the close proximity of the reporter with the quencher dye. 5' nuclease activity of DNA polymerase causes displacement of the probe and cleavage of the reporter form the quencher dye resulting in the emission and detection of fluorescence.*

Real-time quantitative PCR was performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, UK).

2.3.3.2. Validation

For each of the primers and probes used, calibration was carried out to ensure linearity of amplification. Using normal human liver cDNA as template, the amplification of the house keeping gene 18S (supplied by the manufacturer) was

examined and compared with the genes of interest. In particular it was confirmed that the 18S values of normal and diseased liver samples was similar. Primer and probe concentrations used were those determined previously by validation techniques in the group.

2.3.3.3. Primers and probes

Primer Express software (Perkin-Elmer Applied Biosystems, UK) had been previously used to design primers and probes for the 11β-HSD1 mRNA and H6PDH and were available for use in the group. These primers were used in the analyses of all whole liver samples:

11β-HSD1 forward primer: 5'-AGGAAAGCTCATGGGAGGACTAG-3'

11β-HSD1 reverse primer 5'-ATGGTGAATATCATCATGAAAAAGATTC-3'

11β-HSD1 hybridisation probe 5'-CATGCTCATTCTCAACCACATCACCAACA-3'

H6PDH forward primer: 5'-AGAACTCGGGACCTTTTTCCA-3'

H6PDH reverse primer: 5'GCGCCACAGCCTGCTT-3'

H6PDH hybridisation probe: 5'-TAAGTAATGGTCCACCCGGTACATCTCCTCC-3'

11β-HSD1 and H6PDH primers used for laser capture microdissected samples were the ready mixed 'assay on demand' (Applied biosystems, UK). Primers for all other genes analysed were purchased as 'assay on demand', detailed below:

HSD11B1: Hs00194153_m1

H6PDH: Hs00188728_m1

Glut-2: Hs00165775_m1

G6Pase-α: Hs 00609178_m1

G6Pase-β: Hs00292720_m1

G6PT: Hs00184616_m1

GR: Hs01005217_m1

CYP2E1: Hs00559368_m1

Alcohol dehydrogenase (ADHB1): Hs00913384_m1

PEPCK1: Hs00159918_m1

PEPCK2: Hs00388934_m1

2.3.3.4. Reagents

TaqMan Universal PCR Master Mix: 3mM $Mn(Oac)_2$, 200μM dNTPs, 1.25U AmpliTaq Gold polymerase, 1.25U Amp Erase UNG.

2.3.3.5. Protocol and data analysis

Real-time PCR was performed in 25μl volumes on 96 well plates, in a reaction buffer containing TaqMan Universal PCR Master Mix, 100nmol TaqMan probe, 900mmol primers, and 25-50ng cDNA. Cycling conditions were 50 ºC for 2 min, 95 ºC for 10 min, and then 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. All reactions were compared with reactions carried out in a separate well for each sample using the 18S control probe (PE Biosystems, UK). Data were analyzed according to the manufacturer's guidelines and were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine dCt values (dCt=Ct of the target gene minus Ct of the internal reference, 18S). Data was analysed at the dCt stage and mean +/- standard error dCt values transformed through the equation [fold increase = 2^{-ddCt}] to give fold change in relative mRNA levels.

Arbitrary units were also used using the transformation [$AU=1000*2^{-dCt}$] to express results obtained.

2.4. Enzyme Assays

Enzyme assays are used to detect the presence of enzymes in cells and tissue as well as to identify and characterize particular enzymes, and investigate substrate specificity and regulation of enzyme activity. The assumptions involved in these experiments and an understanding of the theoretical aspects of enzyme behaviour are fundamental in the interpretation of results obtained.

The Michaelis-Menten model of single substrate reactions was used to approximate the 11β-HSD1 assays used in this thesis, Figure 2-2.

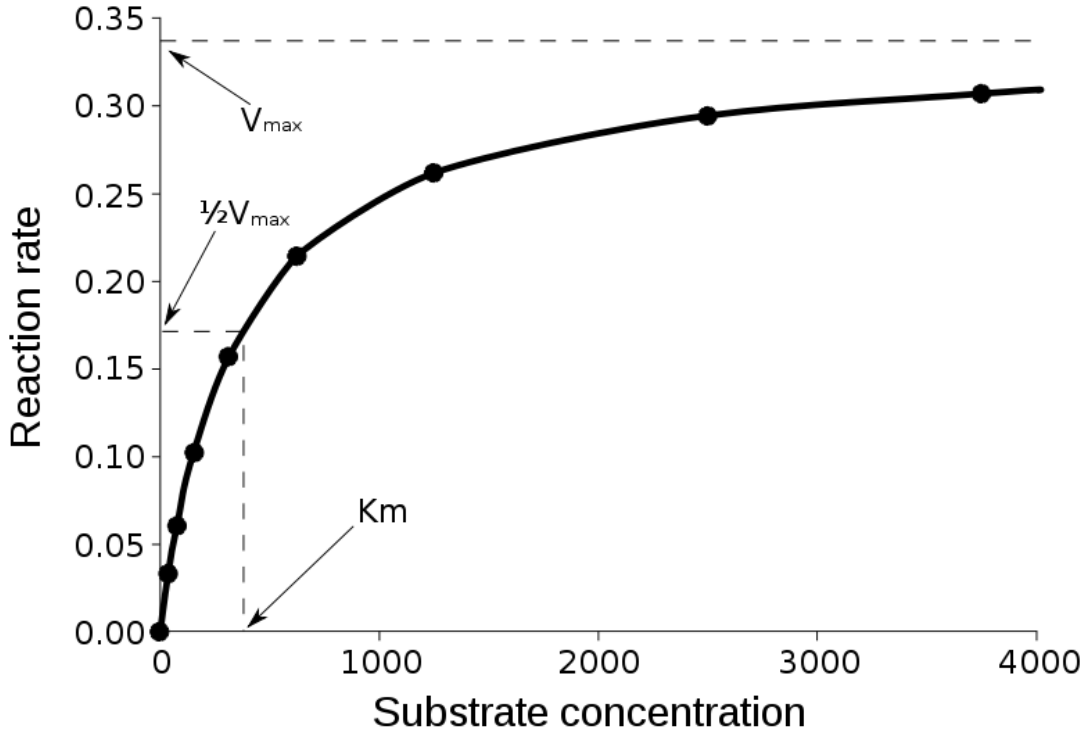


Figure 2-2 Michaelis-Menten saturation curve of an enzyme reaction

Measurement of the initial rate of the reaction over a range of substrate concentrations (denoted [S]), the rate of reaction (*v*) is seen to increase as [S] increases. As [S] gets higher, enzyme [E] saturation occurs and the rate reaches V_{max}, which the maximum rate of the enzyme. If catalysis occurs via rapid reversible formation of the enzyme substrate complex [ES] which only slowly breaks down to regenerate enzyme and new product, then at equilibrium a constant K_m can be defined where K_m=[E][S]/[ES]. Based on this constant, the relationship between reaction velocity, V and [S], where V_{max} is the limiting reaction velocity, can be described as:

$$V = \frac{Vmax[S]}{Km+[S]}$$ (The Michaelis Menten equation)

It follows that when the reaction is at half maximum reaction velocity, ($V = V_{max}/2$), [S] = K_m. Also, when [S] is small compared to K_m, the reaction approximates to $V = V_{max}[S]/ K_m$, thus the reaction would conform to first order kinetics for the enzyme. The V_{max} and K_m value are highly characteristic for different enzymes and can vary significantly between enzymes. For example, enzymes that have 'house-keeping' functions tend to operate closer to their V_{max} concentrations, so that reaction velocity is not dependent on [S]. Enzymes that have a more regulatory role are more likely to operate such that *V* is dependent on [S], with the *in vivo* substrate concentrations likely to be close to the K_m value.

Values determined *in-vitro* for reaction velocities depend on a number of assumptions that may not hold true when applied to the *in vivo* situation. Intracellular substrate concentrations *in vivo* are unknown and therefore the assumption that substrate concentration is higher than enzyme concentration may not hold true. Also, multiple

factors influence enzyme and substrate interaction *in vivo* which cannot be translated to in-vitro studies where E, S and ES are in equilibrium.

2.4.1. Generation of tritiated cortisone (^{3}H-cortisone) for 11β-HSD1 assay

^{3}H-cortisone until very recently was not commercially available, unlike tritiated cortisol (^{3}H-cortisol). An in house method was used to generate ^{3}H-cortisone from ^{3}H-cortisol utilising the high expression and activity of 11β-HSD2 in placenta. In a borosilicate tube 20µl of ^{3}H-cortisol was incubated with 250mg protein from human term placenta, with 500mM NAD in 500µl of 0.1M potassium phosphate buffer, pH 7.4, for 5 hours at 37ºC. Steroids were extracted into 7 ml dichloromethane by shaking the tubes horizontally for 15 min followed by centrifugation at 80 g for 15 min to separate aqueous and organic phases. The aqueous phase was then aspirated and discarded. The organic phase was evaporated to dryness at 50ºC under air in a sample concentrator (Techne, UK). 100µl dichloromethane was added and vortexed to resuspend the steroids. This was then spotted onto a thin layer chromatography (TLC) plate. The plate was run in a TLC chamber that was pre-equilibrated with chloroform:ethanol in a ratio of 92:8 until the solvent front was 5 mm from the top of the plate. A Bioscan 3000 imaging scanner (Lablogic, Sheffield, UK) was used to scan the dried silica plates. Parallel lanes containing 1mg/ml unlabelled cortisone or cortisol were also included on the plate. The plate was viewed under UV light and unlabelled steroids were localised by their fluorescence, allowing tritiated steroids on the plate to be identified. The area within 0.5 cm of the location of the ^{3}H-cortisone was cut from the plate and the steroid was eluted overnight in 300µl ethanol at 4ºC.

The tube was then spun at 100g for 10 min and the liquid phase removed to a new tube. The silica was then resuspended in a further 300µl of ethanol and the process was repeated. The purity of the ^{3}H-cortisone in the ethanol was assessed by further TLC analysis alongside unlabelled steroids. The amount of ethanol was adjusted with addition or evaporation under nitrogen in a sample concentrator to give 100 cpm/µl.

2.4.2. 11β-HSD1 assay

11β-HSD1 assays in this thesis were carried out primarily on liver microsomes, which were prepared in MOPS buffer (section 2.7). Assays were carried out with various concentrations of cortisol (50nM to 1µM) with tracer amounts of ^{3}H-cortisol (specific activity 78.4Ci/mmol, NEN, UK) or cortisone (50nM to 0.5µM) with tracer ^{3}H-cortisone and incubated at 37ºC. Steroids were then extracted from the medium into 10 volumes of dichloromethane and separated by TLC with chloroform : ethanol (92:8) as a mobile phase. The percentage conversion of cortisone to cortisol and cortisol to cortisone was analysed on a Bioscan 3000 Imager. The protein concentration of microsomes added to each tube was measured as described in section 2.5.

2.5. Protein assay

Total protein concentration in liver tissue homogenates were measured using the BioRad RC DC protein assay. This is a colourmetric assay for protein concentration following detergent solubilisation. It is a modified version of the Lowry method (Lowry et al. 1951) and is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent and the subsequent reduction of Folin reagent by

the copper treated protein. The reduction of Folin reagent can produce several reduced species which have a characteristic blue colour, with a maximum absorbance at 750 nm and a minimum absorbance at 405 nm.

2.5.1. Method

Protein assays were carried out in 96 well plates according to the manufacturer's protocol (BioRad, Hemel Hempstead, Herts). 5µl of protein sample or standard per well was added. The range of standards were 0, 0.25, 0.5, 1, 2 mg/ml of bovine serum albumin (BSA). 25µl of solution A, alkaline copper tartrate solution, was added followed by 200µl of solution B, Folin reagent. The solutions were incubated at room temperature for 10 min and the absorbance read at 690nm on a Victor3 1420 multilabel counter (PerkinElmer, Beaconsfield, Bucks).

2.6. Western Blotting

Western blotting is an analytical technique that allows the detection of specific proteins in tissue homogenate or extract (Towbin et al. 1979). Sodium docecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is by far the commonest method used to separate denatured proteins according to molecular weight. The proteins are then transferred to a membrane, usually nitrocellulose or polyvinylidine difluoride (PVDF). The membrane is then blocked with generic proteins, such as milk or bovine serum albumin (BSA), to prevent non specific antibody binding, followed by incubation with the primary antibody that binds to the protein of interest. A conjugated secondary antibody is then directed against the primary antibody used.

Secondary antibody conjugated to horseradish peroxidise (HRP) was used for the western blotting carried out in this thesis. HRP allows detection by catalysing the oxidation of a chemiluminescent substrate producing light, which is captured on photographic film.

2.6.1. Method

SDS-PAGE and protein transfer were carried out using a BioRad protein 3 apparatus and a modified protocol provided with the kit. Between 10-30μg of protein was mixed with the appropriate amount of loading buffer and then heated at 95°C for 5 mins. Samples were loaded on an 8-12% SDS-PAGE gel and run at 200V for 60 – 90 min. Proteins were then transferred onto a nitrocellulose membrane at 100V for 60 min. To check for efficient transfer, membranes were incubated in Ponceau stain with agitation for 2 min, and then rinsed with water until protein bands were visible. Bands should be distinct and well spread out.

Membranes were then blocked in 10 ml of blocking buffer for 60 min with constant agitation, rinsed twice with washing buffer, and incubated in primary antibody overnight at 4°C with constant agitation. Membranes were washed 3 times for 15 min in washing buffer and then incubated with the secondary antibody for 60 min at room temperature, followed by three washes of 15 min each in 50 ml of washing buffer. Chemiluminesence was used for antibody detection; substrate A and B were mixed in equal quantities (0.5 ml each per membrane) and allowed to equilibrate for 5 min. 1ml of substrate was put on each membrane and incubated for 2 min. Membranes were then placed between two sheets of clear plastic and in the dark exposed to

photographic film for 2 sec and 1 h. Photographic film was developed on Compact X4 (Xograph Imaging Systems, Gloustershire, UK).

2.7. Liver microsome preparation

Microsomes are vesicle like structures formed from the endoplasmic reticulum (ER) when cells are broken up in the laboratory. They are not part of the normal ultrastructure of eukaryotic cells. Microsomes are concentrated and purified from other cellular debris by differential centrifugation. At 10,000 g nuclei, unbroken cells and mitochondria sediment out, while fragmented ER, and soluble enzymes remain in solution. At 100, 000 g microsomes are sedimented. Microsomes are a valuable tool for investigating metabolism of compounds, in particular by membrane bound enzymes, and for identifying drug-drug interaction by *in vitro* methods.

2.7.1. Reagents

Sucrose buffer: 0.3M (10.26g) sucrose, 10 mM (121.1 mg) TrisHCl, 0.47 g Hepes, made up to 100 ml with water, pH adjusted to 7.2 with potassium hydroxide. The solution was stored in the fridge and 10ml aliquots were used. 1 EDTA free protease inhibitor tablet was added per 100mls.

MOPS buffer: 0.75 g KCl, 0.12 g NaCl, 0.02g $MgCl_2$, 0.42 g MOPS, pH adjusted to 7.2 with KOH and solution made up to 100 ml with water.

2.7.2. Method

A small chunk (~0.5 cm^3) of snap frozen human/mouse liver or fresh mouse liver was homogenised with an electric homogeniser in 4 volumes of sucrose buffer, followed

by 5 – 10 strokes with a hand held glass dounce homogeniser on ice. The homogenate was then centrifuged at 4°C for 10 min at 1000 g to remove debris. The supernatant was centrifuged at 4°C for 10 min at 12 000 g. The supernatant was then removed to a fresh tube and centrifuged for 4°C for 60 min at 100 000 g (Beckman Ultracentrifuge). The pellet collected contained the microsomal fraction and was resuspended in 1 ml MOPS and the spin was repeated. Finally the pellet was resuspended in 400 – 600 µl with a plastic Pasteur pipette, gently homogenised with 4 – 5 strokes of a hand held 2 ml Dounce homogeniser. A small aliquot was removed to measure protein concentration (section 2.5). 50µl aliquots were snap frozen in liquid nitrogen and stored at -80°C.

2.8. Laser capture microdissection

Laser capture microdissection (LCM) is a specialised technique for isolating highly pure specific cell populations from a heterogeneous tissue section, live cell culture or cytological preparation under direct microscopic visualisation (Emmert-Buck et al. 1996). A variety of downstream applications exist, including DNA genotyping, RNA transcript profiling, generation of cDNA libraries, proteomics, and profiling of signal pathways.

2.8.1. Principle

A number of variations on the basic technique for LCM exist, including infrared (IR) laser platforms, combined IR/UV laser systems and pure UV laser systems. The technique used in this thesis used a UV only laser system (Zeiss P.A.L.M. Microbeam). Using this system almost every kind of biological material can be

microdissected and catapulted directly from glass slide, including archival pathological sections. Optimal performance is obtained from cryosections.

Tissue sections are mounted on glass slides covered with a polyethylene naphthlate (PEN) membrane. The membrane is easily cut and acts as a stabilizing backbone during catapulting. It is 1.35 μm thick and is highly absorptive in the UV-A range, which facilitates laser cutting. Once the cells of interest have been indentified and a region of interest created, the operator activates a software controlled UV laser beam, which is focussed so precisely that a beam accuracy of less than 1 μm can be achieved. The selected specimen is removed and catapulted by a laser pulse in to a collection device (The inner aspect of the cap of a microcentrifuge tube), microdissecting the cells of interest from the heterogeneous tissue section Figure 2-3. The cellular morphology as well as RNA, DNA and proteins remain intact and bound to the membrane. The UV laser system may also be used to ablate unwanted tissue leaving the cells of interest intact.

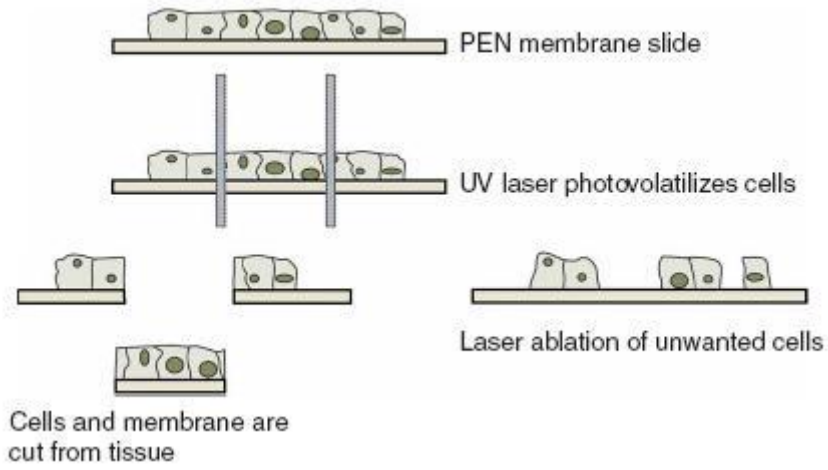


Figure 2-3 Principle of UV laser-capture microdissection. The tissue is mounted on a polyethylene napthalate (PEN) or polyethylene tetraphthalate (PET) membrane. A UV laser can be used to cut away cells of interest or to ablate unwanted tissue, leaving cells of interest intact on the substratum (Espina et al. 2006).

One potential limitation of the UV laser system is that capture of cell with UV-induced damage. These cells are derived from those directly under the UV laser cutting path, and may contribute significantly to the final molecular signal if the number of cells captured at the perimeter of the region of interest is high compared to the total number of microdissected cells in the region of interest.

2.8.2. Method

LCM of normal liver sections was carried for mRNA expression analysis using real-time PCR for 11β-HSD1 localisation studies. The method required a considerable amount of optimisation to ensure good quality tissue sections and a good yield of RNA for successful real-time PCR which has been detailed in chapter 3. A brief description of the overall method is described here. An overview of the process from tissue collection to mRNA expression is illustrated in Figure 2-4.

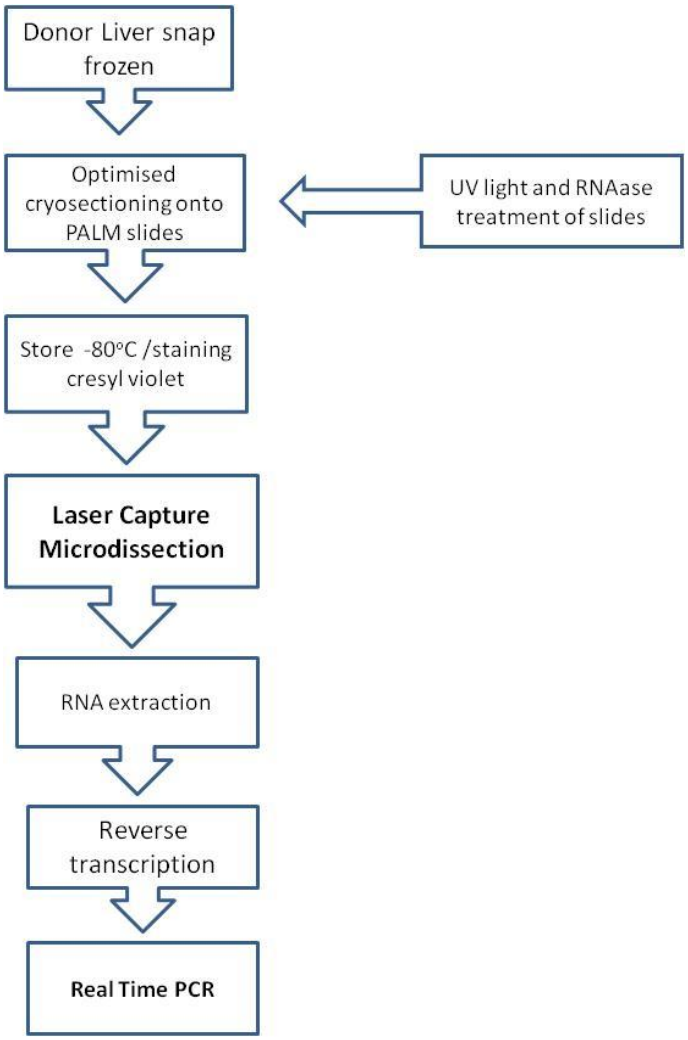


Figure 2-4 Schematic of LCM procedure for mRNA expression studies

2.8.2.1. Specimen preparation

Cryosections of snap frozen liver tissues were carried out as described in section 2.2.3., with a number of modifications as detailed in P.A.L.M. Microlaser Technologies protocols, and described in brief below.

To ensure RNase-free membrane-slides, the slides were dipped for a few seconds into RNase-ZAP (Ambion), followed by two washes in DEPC treated water, and drying at room temperature for up to two hours. To overcome the hydrophobic nature of the

membrane on the PEN slides, the slides were then irradiated with UV light at 254 nm for 30 min. This made the membrane more hydrophilic allowing the sections to obtain better adherence. In addition, irradiation of the slides sterilized and destroyed potentially contaminating nucleic acids which was particularly important for RNA preparation from the captured cells.

Liver tissue cryosections were cut at 8 μm thickness onto PEN membrane slides, air dried for 20 seconds and then fixed in ice cold 100% ethanol. The slides were then placed in a dessicator and stored at -80°C for future use or stained for immediate use for LCM. As OCT media interferes with laser efficiency it was important to remove any remaining media from the slide prior to laser capture. As the OCT compound is easily removed by gentle washing in water, the substance was 'automatically' removed in aqueous staining solutions or diluted ethanol.

RNAase free water and solutions were used for staining and steps involving aqueous solutions were carried out as quickly as possible to reduce the effects of endogenous RNAses in the tissue sections.

Haematoxylin staining was used as recommended; ethanol fixed slides were dipped in distilled water and then dipped in filtered haematoxylin for 3 minutes in an RNase free coplin jar, washed in distilled water and dehydrated in an increasing ethanol series. The stained cryosections were allowed to dry for up to 30 min at room temperature before use. Cresyl violet was an alternative for staining, which only required rehydration with 50% ethanol rather than water.

2.8.2.2. Laser pressure catapulting of samples

20-25 µl of lysis buffer from the Ambion RNAqueous-Micro Kit (Applied Biosystems) was pipetted into the inner ring of the cap of a 0.2 ml microcentrifuge tube. Microbeam Version 2.3-0103 P.A.L.M. RoboSoftware was used to draw regions of interest under microscopic view of the sections. The power and focus of the laser was optimised for fine cutting at the desired magnification and serial regions of interest were cut and catapulted into the tube cap in a single step automated software directed mechanism. For each cell type captured, the total area captured was recorded. For downstream analysis involving RNA work with real time PCR, the recommended area to capture was $0.8 – 1.0 \times 10^6$ µm^2. To control the efficiency of catapulting it was also possible to look into the tube cap with most catapulted areas visualised within the small inner ring of the caps. After microdissection, the sample within the buffer in the tube cap was spun down on a bench centrifuge and then stored for later use or immediately followed by the RNA extraction protocol.

2.8.2.3. RNA extraction from LCM samples

The Ambion RNAqueous-Micro Kit (Applied Biosystems) was used to extract RNA from the LCM samples. The technique followed exactly as per manufacturers protocol, and followed by reverse transcription and real time PCR as described in section 2.3.

2.9. Clinical methods

The bulk of the methods for the clinical study are described in section 6.3. Clinical analyses of whole body hepatic 11β-HSD1 activity, and biochemical assays that were commonly used in the clinical studies described in chapter 4-7 and are described in further detail here.

2.9.1. Cortisone to cortisol generation profile

Measurement of hepatic first pass metabolism of cortisone to cortisol in dexamethasone suppressed individuals was made by determining the cortisol generation profile (CGC). At 2300 h, subjects were given 1 mg of dexamethasone orally to suppress endogenous cortisol production. All subjects attended the Clinical Research Facility at 0900 h the following morning, and after baseline 0900 h measurements of cortisol and adrenocorticotropic hormone, a further 0.5 mg of dexamethasone and cortisone acetate (25 mg) were given orally. Serum cortisol and cortisone concentrations were then measured at 30-min intervals for 240 min. Serum cortisol was assayed as described in section in section 2.9.4.

2.9.2. Measurement of urinary steroids

Urine steroid metabolite analysis was carried out on 24-h urine collections from subjects. All samples were analyzed by gas chromatography/mass spectrometry by Ms Beverley Hughes (Centre for Endocrinology, Diabetes and Metabolism, University of Birmingham), as reported previously (Palermo et al. 1996;Shackleton 1993), measuring free and conjugated cortisol metabolites. Urinary steroid metabolite ratios and total 24-h cortisol metabolite production rates were determined. The

THF+5αTHF/THE, the cortols/cortolones, and the 11OH-androsterone+11OH-etiocholanolone/11-oxoetiocholanolone ratios represent acknowledged markers of global 11β-HSD1 activity, with a high ratio indicating increased 11β-HSD reductase activity. Intra and inter-assay coefficients of variation were <10% for both cortisol and cortisone.

2.9.3. Insulin ELISA

The Mercodia Insulin Elisa kit (Mercodia, Sweden) was used for quantitative determination of serum insulin levels.

2.9.3.1. Principle

The Mercodia Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based in the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. Insulin in the sample reacts with peroxide-conjugated anti insulin antibodies and anti-insulin antibodies bound to the microtitration well during the incubation. A washing step removes the unbound enzyme labelled antibody. The bound conjugate is detected by a reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

2.9.3.2. Sample collection

Blood samples were collected into tubes and centrifuged to separate the serum and stored at -80°C.

2.9.3.3. Method

The ELISA procedure was carried out exactly as per manufacturer protocol (Mercodia, Sweden product no. 10-1113-01).

Each sample was analysed in duplicate. The interassay coefficient of variation was 3.6%.

2.9.4. Serum cortisol measurements

The Coat-a-count cortisol radioimmunoassay (SIEMENS) was used to determine serum cortisol concentrations and were kindly performed by Ms Susan Hughes (Centre for Endocrinology, Diabetes and Metabolism, University of Birmingham).

2.9.4.1. Principle

The procedure is a solid-phase radioimmunoassay, where ^{125}I-labelled cortisol competes for a fixed time with cortisol in the patient sample for antibody sites. As the antibody is immobilized to the wall of a polypropylene tube, decanting the supernatant is sufficient to terminate the competition and isolate the antibody bound fraction of the radiolabelled cortisol. The tube is then counted in a gamma counter which yields a number that converts to a measure of the cortisol present in the sample by way of a calibration curve.

2.9.4.2. Sample collection

Samples were collected by collection into plain tubes without heparin and centrifuged to separate the serum. Ultracentrifugation was carried out to clear lipaemic samples.

2.9.4.3. Method

The protocol was followed exactly as per manufacturer instructions. In summary:

4 plain uncoated tubes (to measure non specific binding) and twelve cortisol Ab-coated tubes were labelled in duplicate for calibration. Additional cortisol Ab-Coated tubes were labelled in duplicate for patient samples.

25µl of the zero calibrator were pipetted into the uncoated tubes and two calibration tubes. 25µl of each calibrator, control and patient sample were pipetted into the bottom of the remaining tubes.

1 ml of ^{125}I-cortisol was added to every tube and vortexed.

The tubes were incubated for 45 minutes at 37°C. The contents of the tubes were then decanted thoroughly using a foam decanting rack and allowed to drain for 2-3 minutes, and struck sharply on absorbant paper to shake off the all the residue droplets.

A gamma counter was used to count for 1 minute.

Intra and inter-assay coefficients of variation were between 4 and 7%

2.9.5. Plasma free fatty acid (FFA) analysis

Plasma FFA were analysed on a COBAS BIO semiautomatic analyser (La Roche, Basel, Switzerland) using a NEFA-C Kit (Alpha Laboratories, UK) as per manufacturer protocol. These samples were analysed by Professor Anton Wagenmakers (Sport and Exercise Science, University of Birmingham).

3. *In vitro* characterisation of hepatic 11β-HSD1 with relevance to hepatic glucose metabolism

The bulk of the *in vitro* work carried out for this thesis has been detailed in relevant chapters underpinning clinical data described. The work described in this chapter details additional *in vitro* work to further characterise 11β-HSD1 in the liver.

3.1. Introduction

3.1.1. Compartmentalised function of the liver in normal physiology

It has become increasingly apparent over the last thirty years or more that most, if not all the functions of the liver are heterogeneously distributed, with the activity of each process varying across the acinus (the functional unit of the liver) (Gebhardt and Mecke 1983;Gumucio 1989), section 1.3.1. This zonation of function is manifested

by phenotypic differences between cells depending on their position within the acinus and appears to be characteristic of all the major metabolic pathways in the liver in normal physiology. Seminal studies demonstrated the acinar distribution of carbohydrate metabolism (Jungermann and Thurman 1992) and nitrogen metabolism (Haussinger et al. 1992), with both processes displaying distinct and prominent compartmentalisation. Zonation of function is particularly apparent in drug metabolism by P450 enzymes (Traber et al. 1989), an important factor in the zonation of hepatocellular damage elicited by many hepatotoxins. There is now a significant volume of evidence suggesting the majority of liver genes are differentially expressed within the acinus in upstream (periportal), or downstream (perivenous) hepatocytes, Table 3-1.

Periportal zone			Perivenous zone	
General metabolism	Drug metabolism		General metabolism	Drug metabolism
Oxidative energy metabolism	Bile acid production		Glycolysis	Monoxygenation (P450)
Fatty acid oxidation	Glutathione production		Lipogenesis	Glutathione conjugation
Gluconeogenesis				Glucuronidation
Ureagenesis				

Table 3-1 Zonation of the major metabolic pathways of the liver (Lindros 1997)

In addition, hepatic zonation has been demonstrated at multiple cellular levels. Morphological studies have demonstrated that not only the number of cells, but also the size of the cell organelles vary along the length of the sinusoid (refer to Figure 1-2 for basic structure of liver lobule). In particular, periportal hepatocytes contain larger and fewer mitochondria whilst perivenous hepatocytes contain larger and more abundant endoplasmic reticulum, (ER). This is an important observation with relevance to the zonation of hepatic microsomal enzymes, in particular 11β-HSD1 expression. Sinusoidal cells such as Kuppfer cells, endothelial cells and stellate cells also display zonation, being more numerous in the periportal region (Sasse et al.

1992). While these gradients of expression within the acinus are very well characterised, knowledge of the control of zonal expression is still lacking. Many studies have shown that in fact, hepatic zonation is dynamic, with only a few exceptions, resulting from differential sinusoidal gradients of oxygen, nutrients and hormones (Jungermann et al. 1982), Table 3-2. Some enzymes are expressed in a strictly limited compartment type of expression, for example, glutamine synthetase which is involved in ammonia detoxification, is located specifically near the terminal hepatic veins and requires specific cell-cell interactions that define the position dependant phenotype (Kuo and Darnell, Jr. 1991), and may result from an imprinting process during the final differentiation and maturation of periportal liver cells (Gumucio 1989).

Modulator	Zonation pattern
Sinusoidal oxygen tension gradient	dynamic gradient
Sinusoidal gradient of hormones and/or receptors	dynamic gradient
Sinusoidal gradient of expression of transcription factors	dynamic gradient
Cell lineage dependent imprinting of positional phenotype	compartment-type/ dynamic
Specific cell-cell or cell-matrix interactions	compartment-type

Table 3-2 Suggested zone modulators of liver gene expression (Lindros 1997)

Of particular importance, are the enzymes involved in hepatic drug metabolism. Indeed, hepatocytes represent 70% of liver mass, and express drug metabolising enzymes (well described for P450 enzymes) at high levels compared to non parenchymal cells. However, depending on their position some are more involved in drug metabolism than others. The highest level of phase I and phase II enzymes are found in the perivenous cells.

3.1.1.1. Enzyme activation and zonation

The question arises whether the enzymes expressed in hepatocytes are activated to the same extent regardless of zonation in the presence of similar concentrations of substrate. Much work has been done on several P450 isoforms that are induced by specific compounds (for example the induction of CYP2E1 by ethanol and acetone). In perivenous hepatocytes expressing these enzymes, enzyme induction occurs more readily than in hepatocytes with low expression of the enzyme (Rich et al. 1989). Hence, the intrahepatic enzyme zonation, which is often attributed to differential expression of enzyme protein, could also result from zonal differences in enzyme specific activity which may be due to differences in allosteric and/or covalent modification. This has been demonstrated for numerous hepatic enzymes including key enzymes involved in hepatic lipogenesis (Evans et al. 1990).

3.1.1.2. Hormonal regulation of zonation

Studies have shown that when hepatocytes are isolated from the perivenous or the pericentral zone and maintained in culture in the presence or absence of hormones, the difference in rates of gluconeogenesis and urea synthesis do not converge, suggesting that there are long acting mechanisms involved in the induction and maintenance of heterogeneity *in vivo* (Poso et al. 1986;Quistorff et al. 1986;Tosh et al. 1988). The effect of altering the endocrine state in vivo on the hepatic zonation of enzyme activity has also been investigated. Hypophysectomised rats show marked reduction in circulating concentrations of glucocorticoids, thyroid hormone and growth hormone, associated with changes in the activity of a number of hepatic enzymes

which are known to be distributed heterogeneously within the acinus (Csanyi and Greengard 1968;Wong and Dunn 1977). Another study showed that, while hypophysectomy caused significant changes in the activities of alanine aminotransferase and pyruvate kinase, the degree of heterogeneity of enzyme activity within the liver acinus, and of the rates of gluconeogenesis and mitochondrial redox state was preserved (Tosh, Alberti, & Agius 1988). Another study investigating the effect of hormones on the activity and zonation of specific P450 enzymes showed that although common hormonally acting elements appear to regulate zonation (e.g., the effect of growth hormone on the spatial expression of CYP2B and 3A), the zonation of other CYP forms appear to be regulated by thus far undefined factors.

3.1.1.3. Hepatic zonation of 11β-HSD1

Previously reported protein expression studies using immunohistochemistry showed 11β-HSD1 expression in hepatocytes in a perivenous distribution, with little or no expression around the portal vein, hepatic artery or bile ducts (Ricketts et al 1998b). The physiological significance of this is not immediately apparent and is in contrast to both the homogeneous, ubiquitous distribution of the hepatic glucocorticoid receptor (Antakly and Eisen 1984), and the heterogeneity of hepatic enzyme expression related to gluconeogenesis (Jungermann & Thurman 1992). The predominant activities in the periportal zone are oxidative energy metabolism with beta-oxidation and amino acid metabolism, ureagenesis, gluconeogenesis, cholesterol synthesis, bile formation and oxidation protection. By contrast, in the perivenous zone glycolysis, lipogenesis, ketogenesis, glutamine formation and biotransformation are the prevalent processes, Table 3-1. Hence, physiologically one would anticipate hepatic 11β-HSD1

expression to localise with functions known to be stimulated by glucocorticoids, including gluconeogenesis (periportal), glycolysis (perivenous) and lipogenesis (perivenous). In particular, promotion of hepatocyte de novo lipogenesis, and inhibition of fatty acid beta oxidation by glucocorticoids would be supported by the perivenous distribution of 11β-HSD1 expression, with a clear role in the pathogenesis of the hepatic fat deposition leading to hepatic steatosis.

3.1.1.4. AIM -1

On this background one of the primary aims of the *in vitro* work was to further establish the zonation of 11β-HSD1 expression in normal human liver using western blotting techniques to investigate protein expression and laser beam microdissection studies to investigate mRNA levels in hepatocytes isolated from different zones in the normal hepatic acinus.

3.1.2. Hepatic 11β-HSD1 expression and activity with relevance to glucose signalling and carbohydrate metabolism in normal and diseased liver

The association of chronic liver disease with impaired glucose metabolism has been recognised for over a century and led to the term ‘hepatogenous diabetes’ being coined (Naunyn 1906). The impairment of glucose tolerance worsens with the progression of chronic hepatitis to liver cirrhosis. The prevalence of impaired glucose tolerance in cirrhotic patients is 60-80%, and of overt diabetes about 15%. As the term CLD encompasses different stages of liver dysfunction and the involvement of a number of intrinsic and extrinsic factors, the exact pathogenic mechanisms underlying

this relationship are not entirely clear. For example, continuous alcohol intake in alcoholic hepatitis, hepatic steatosis in NAFLD, or reduced insulin clearance in portal hypertension, can all directly affect glucose metabolism. Dysregulation of carbohydrate metabolism in non alcoholic fatty liver disease is detailed in section 1.3.4. While alcoholic hypoglycaemia is one of the better known alterations in hepatic glucose metabolism (Freinkel et al. 1963), alcohol excess also induces glucose intolerance (Andersen et al. 1983). The diabetogenic effect of alcohol is more likely to be secondary to an inhibitory effect on insulin secretion rather than effect on peripheral insulin sensitivity. Reports on peripheral insulin resistance measured by euglycaemic clamps have been inconsistent, but studies have consistently shown that alcohol inhibits glucose stimulated insulin secretion (Bunout et al. 1989).

A number of findings have suggested that common mechanisms underly insulin resistance in the entire spectrum of CLD regardless of the cause, and also in the stages heralding the onset of cirrhosis. For example, CLD is characterised by alterations in the growth hormone-insulin like growth factor-1 (GH-IGF-1) axis, with resistance to GH worsening with progressive liver disease (Picardi et al. 2003). These alterations in the GH-IGF-1 axis also significantly alter blood glucose control. In addition, as with other chronic inflammatory conditions, proinflammatory cytokines such as TNFα, IL-1 and IL-6 impair insulin action directly and impact upon the insulin signalling cascade through their effect on the GH-IGF-1 axis.

Given the critical impact of glucocorticoids upon hepatic carbohydrate metabolism (discussed in detail in section 1.4.6.1), dysregulation of pre receptor glucocorticoid metabolism as a common mechanism in the pathogenesis of impaired glucose tolerance in chronic liver disease is an exciting and plausible possibility. An

introduction to key background studies relating to 11β-HSD1 and insulin sensitivity and chronic liver disease are detailed in sections 1.5.3.2., and 1.5.3.3 respectively.

3.1.2.1. AIM-2

On this background another aim of the *in vitro* work was to evaluate hepatic 11β-HSD1 expression and activity in normal liver and in chronic liver diseases (alcoholic liver disease, NAFLD and other non alcoholic chronic liver disease), comparing mRNA expression of 11β-HSD1 with key enzymes of hepatic gluconeogenesis (PEPCK, G6Pase, and the GLUT 2 glucose transporter), and enzymes specifically relating to increased oxidative stress in alcoholic liver disease (CYP2E1 and alcohol dehydrogenase). Current studies are ongoing to compare 11β-HSD1 reductase and dehydrogenase activity assays from microsomal preparations of whole liver samples from normal and diseased groups.

3.2. Methods

3.2.1. Liver samples

Frozen human liver tissue (normal donor liver, or diseased explanted liver) was received from the tissue bank at the Centre for Liver Research, University of Birmingham as described in section 2.1. Thirty samples in total were obtained as follows: 5 normal livers, 9 with severe NAFLD, 7 with alcoholic liver disease, 9 with chronic liver disease (of which 5 had primary biliary cirrhosis, 1 had primary sclerosing cholangtis and one had haemachromatosis). Histology was verified from histopathology reports. The number of samples included in the final analysis for mRNA expression was less than the number stated because there were some livers

with degraded RNA and were hence unsuitable for analysis. Normal liver samples for paraffin sections were also obtained from the above source.

3.2.2. 11β-HSD1 immunohistochemistry in normal and diseased liver

Immunohistochemical analysis of 11β-HSD1 protein expression in paraffin sections of normal liver was carried out as described in section 2.2.

Further immunohistochemistry for 11β-HSD1 was carried out on frozen sections of normal and NAFLD livers. This study is described in detail in chapter 7.

3.2.3. Laser capture microdissection

Laser capture microdissection of hepatocytes from cryosections of normal liver was undertaken. The method is described in section 2.8 in detail and required a considerable amount of optimisation to ensure good quality tissue sections and a good yield of RNA for successful real-time PCR. The important points essential for optimal results, that were not detailed in the manufacturer protocol, or required a change in protocol suggested by Carl Zeiss (Carl Zeiss, Germany) were as follows.

3.2.3.1. Cryosectioning and fixation

Cryosections of normal liver onto PALM membrane slides was associated with a number of difficulties compared with sectioning onto normal glass slides. In particular the sections tended not stick very well onto the membrane surface of the slide. After being mounted on the slide, the ethanol fixation step often resulted in the sections lifting off the slides completely, even when this step was reduced to only 20 – 30 seconds. These difficulties were optimised by altering a number of steps. The slides were always treated with UV light for 30 minutes prior to use, to improve the

adherence of the sections onto the slides as well as reducing the presence of degrading RNAses. The liver blocks were allowed to equilibrate in the cryostat for 30 minutes prior to cutting. Once cut, the slides were allowed to remain opposed to the cut section on the anti roll plate of the cryostat for a few extra seconds and lifted off slowly.

The sections were not fixed in ethanol as recommended but allowed to air dry and then wrapped in foil and stored at -80°C till use or stained straight away prior to laser capture.

3.2.3.2. Staining of cryosections mounted on membrane slides

The staining protocols recommended a hydration step in water for staining in haematoxylin followed by dehydration in ethanol, prior to laser capture. This resulted consistently in sections with extremely poor and distorted morphology (Figure 3-1) that failed to yield good quality RNA, or slides where air bubbles would appear under the section due to the membrane lifting away from the slide and hence making cutting impossible.

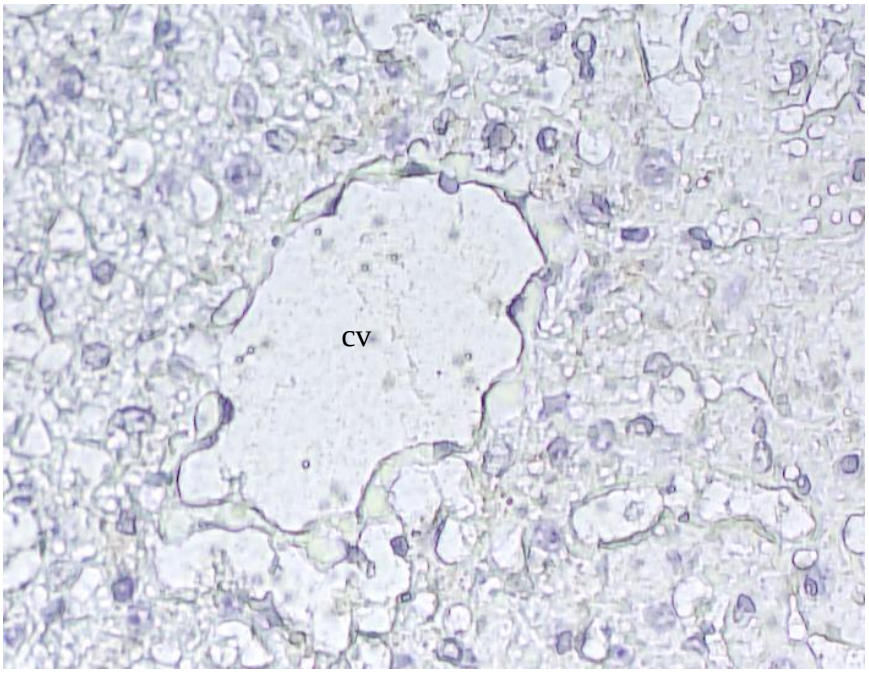


Figure 3-1 Central vein (cv) and surrounding areas on normal human liver cryosection, stained with haematoxylin on membrane coated slide. Note poor distorted morphology.

Hence it was decided to stain in cresyl violet that did not require full hydration of the sections as the cresyl violet stain is diluted in ethanol. This yielded somewhat better results in appearance of the sections and yield of RNA, but the problems with the sections lifting off the slide with ethanol washes persisted.

In collaboration with Dr Mahmood Khan (Department of Immunity and Infection, University of Birmingham) an optimal fixing and staining technique was devised that overcame all the above problems. Sections that had been air dried on the membrane slides were placed on an RNAase inhibitor treated flat surface, and a wax pen was used to draw around the perimeter of the membrane. 300µl of cresyl violet stain was pipetted onto the section and allowed to stain the section over 3 minutes after which excess stain was drained from the slides onto a paper towel. Excess cresyl violet stain was removed by a quick in and out dip in 100% ice cold ethanol, in a coplin jar. The

slides were then allowed to completely air dry (about 5 minutes) before proceeding with laser capture.

3.2.3.3. Morphology of sections on membrane coated slides

While the above staining procedure vastly improved the morphology of the sections, the appearance of the sections was still sub optimal compared with sections on glass slides with a cover slip, Figure 3-2.

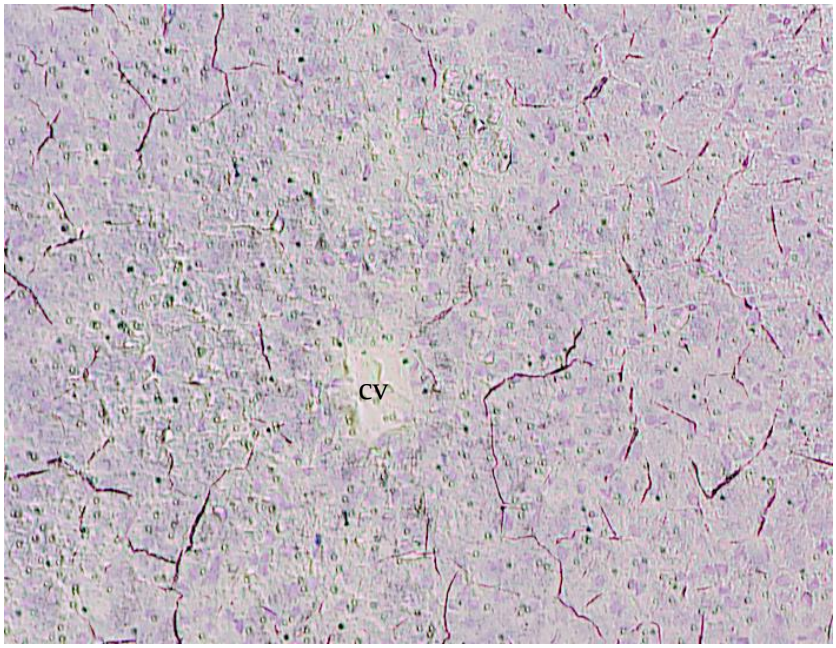


Figure 3-2 *Significantly improved morphology of liver tisue cryosections on membrane coated slide following optimisation of protocol. The image is of a central vein (cv) and surrounding parenchyma. Note effect of ethanol dehydration on fracturing the tissue section in multiple areas.*

While the periportal and perivenous areas were clearly visible the dehydrated sections showed tiny fractures throughout the field of view. In addition the laser capture procedure for each slide needed to be completed within a short time frame to ensure the viability of the RNA. To overcome this problem, investigations were carried for reported techniques to improve morphology further and allow cutting time to be

extended to avoid wastage of slides – each slide required around 2 to 3 hours of cutting time. A fluid cover medium, PALM Liquid Cover Glass (LiquidCoverglass, PALM AG, Bernried, Germany) provides improved morphology and preserves RNA integrity in tissue sections for laser microdissection and pressure catapulting (Micke et al. 2004). This was applied by aerosol to improve morphology and to allow larger tissue areas to be laser pressure-catapulted, and sections were air dried for 5 minutes after application of the medium.

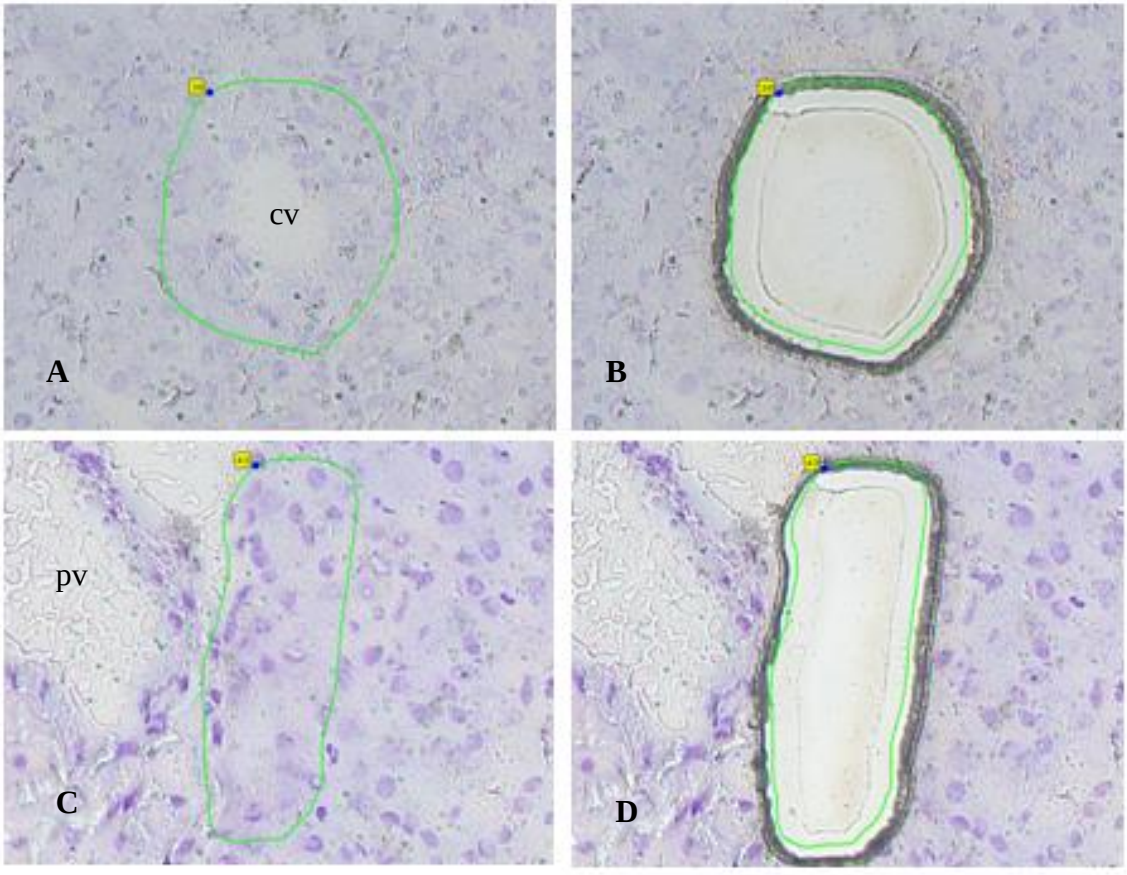


Figure 3-3 *Further improved tissue morphology with the optimised fixing and staining technique with cresyl violet and the use of PALM Liquid Cover Glass. Panels A area drawn around central vein, B appearance follwing cut and pressure catapulting of pericentral area including central vein, C area of periportal hepatocytes marked for cutting, D appearance post cut and pressure catapulting of periportal hepatocytes. (pv - portal vein, cv - central vein).*

The resulting appearance of the tissue sections were much improved and RNA yields were improved with extended cutting times, Figure 3-3.

After optimisation of the technique, laser capture microdissection and pressure catapulting was carried out on perivenous and periportal areas of 3 normal livers as described above. Around 60 separate regions were captured for each area per slide (~10^6 μm^2).

3.2.3.4. Sample and primers used for analysis of LCM samples

RNA extraction, reverse transcription and real time PCR was performed as described in section 2.3). To determine zonation of 11β-HSD1 mRNA expression, real time PCR analysis of mRNA expression was carried out for 11β-HSD1 as well as other liver enzymes with known fixed zonation of protein and mRNA expression. CYP2E1 was chosen on the basis of its strong perivenous expression in a 'compartment-type' manner (see section 3.1.1.1) (Ingelman-Sundberg et al. 1988). In addition mRNA expression of the steroid A ring reductases 5α-reductase 1 and 2, and glucocorticoid receptor α were analysed by real time PCR with microdissected samples pooled for the perivenous and periportal areas retrieved from each liver. All reactions were carried out alongside a control reaction with primers specific for 18S rRNA (provided as a pre-optimized mix; Perkin-Elmer, Beaconsfield, Bucks, UK) as an internal reference. All target gene probes were labelled with the fluorescent label FAM, and the 18S probe with the fluorescent label VIC. Details of primers used are detailed in section 2.3.3.3.

3.2.4. mRNA detection and quantification of 11β-HSD1 and genes related to hepatic gluconeogenesis and oxidative stress in normal liver and diseased liver states

Methods are described in detail section 2.3.2.3. HSD11B1, H6PDH, and glucocorticoid receptor α expression was investigated in all groups. The rate limiting enzyme of gluconeogenesis, PEPCK (cytosolic and mitochondrial isoforms) as well as key ER enzymes and proteins related to hepatic glucose output via gluconeogenesis and glycogenolysis: glucose-6-phosphatase (G6Pase), the glucose 6 phosphate transporter, and the hepatic glucose transporter GLUT 2 were analysed. Key enzymes related to oxidative stress in alcoholic liver disease P450IIE1 and alcohol dehydrogenase (ADHB1 isoform) were also compared between groups.

Data was analysed at the dCt stage and mean +/- standard error dCt values transformed through the equation [fold increase = 2^{-ddCt}] to give fold change in relative mRNA levels. Arbitrary units were also to describe the data using the transformation [$AU=1000*2^{-dCt}$] to express results obtained.

3.2.5. Liver microsome preparation

Microsomes were prepared from all the liver samples obtained. The method is described in section 2.4.2.

3.2.6. 11β-HSD1 assay on liver microsomes from normal and diseased groups

The method is described in section 2.4.2. At the time of writing this thesis the reaction conditions for 11β-HSD1 assays on liver microsomes from snap frozen

samples had been optimised. The comparative assays between groups had not yet been performed.

3.3. Results

3.3.1. 11β-HSD1 immunohistochemistry on normal liver

11β-HSD1 immunohistochemistry on paraffin sections of normal human liver showed a perivenous zonal distribution of staining in keeping with previously described results (Ricketts & Stewart 1999). However, repeated experiments also showed a lesser degree of periportal staining with least staining in hepatocytes distant from periportal and perivenous areas. Periportal staining was more prominent when a lower dilution of primary antibody was used. Within the portal triad, hepatic artery staining was not seen. Peri bile duct staining was seen in the paraffin sections but this was an inconsistent finding and was not seen with immunohistochemistry carried out on cryosections, Figure 3-4 and Figure 3-5.

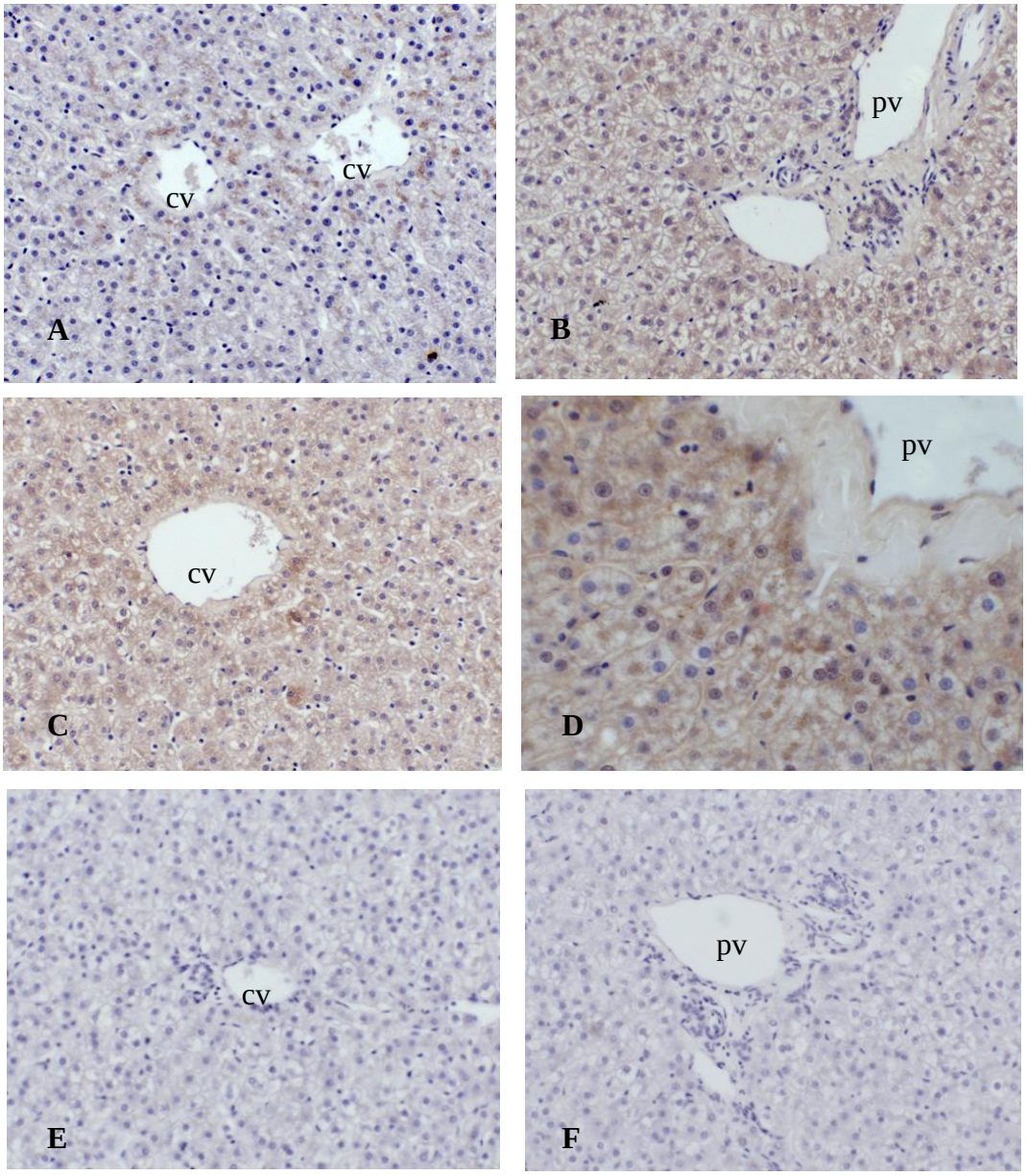


Figure 3-4 *Immunohistochemistry for 11β-HSD1 protein expression in paraffin sections of normal human liver(cv - central vein, pv - portal vein). A 11β-HSD1 antibody at a dilution of 1:300 showing distinct perivenous staining, C perivenous staining with antibody dilution of 1:100 also shows staining in hepatocytes distant from vessels. B-D 11β-HSD1 antibody at a dilution of 1:100. B evidence of periportal staining (not as clearly seen with 1:100 dilution). D x40 magnification of periportal hepatocytes showing increased staining for 11β-HSD1. E-F 11β-HSD1 antibody at a dilution of 1:100 in the presence of an excess neutralising peptide.*

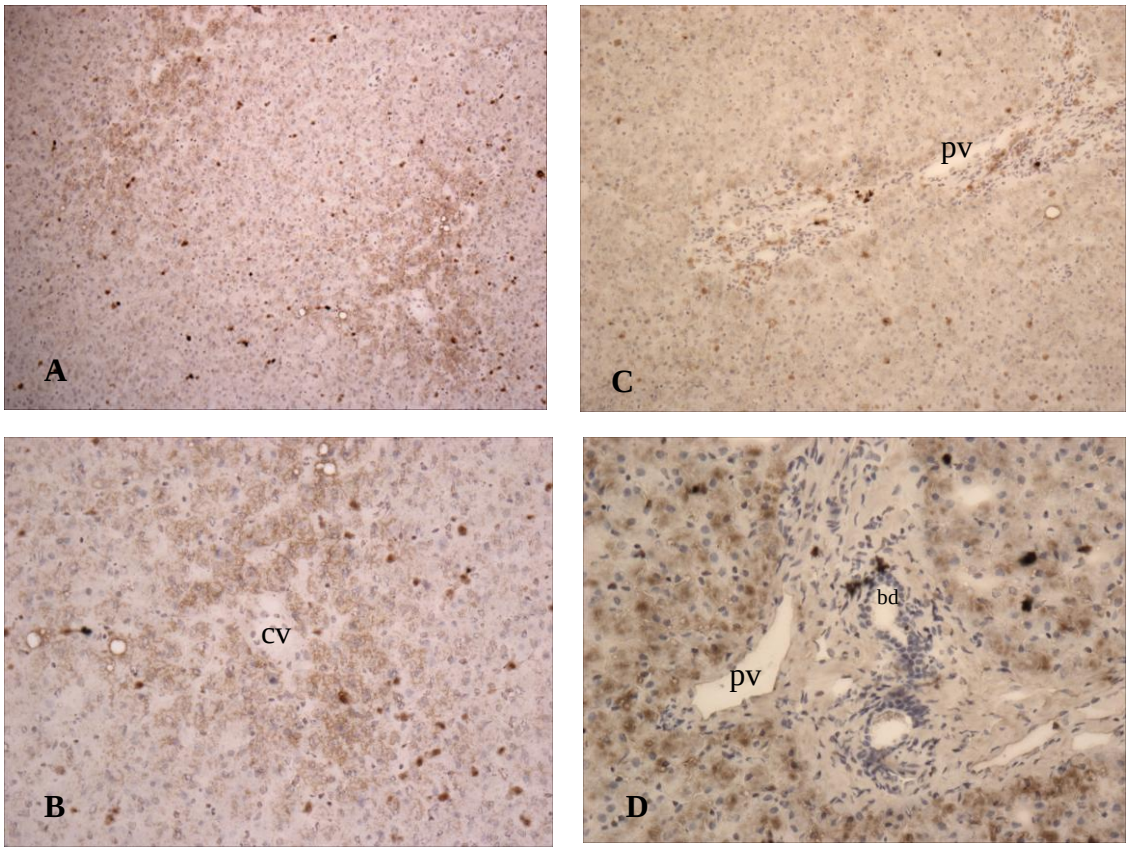
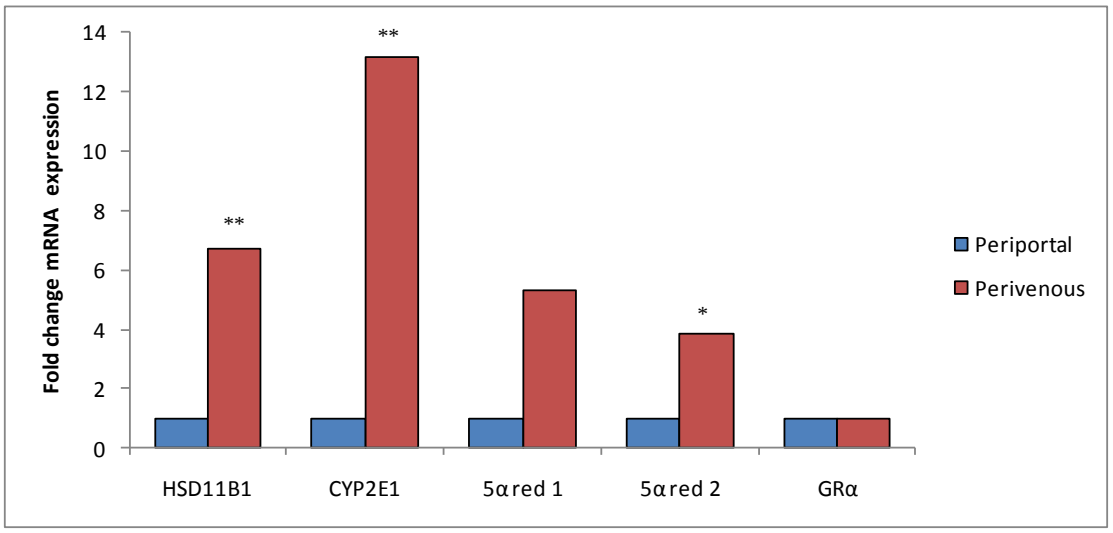


Figure 3-5 *Immunohistochemcal detection of 11β-HSD1expression in cryosections of normal human liver (cv - central vein, pv - portal vein, bd – bile duct). A-C 11β-HSD1 dilution of 1:200. A low power magnification view showing clear perivenous zonation of 11β-HSD1 protein staining. B x20 view of pervinous staining. C low power magnification view showing a suggestion of periportal hepatocyte staining. D 11β-HSD1 dilution of 1:100 showing clear periportal hepatocyte staining and no staining of bile ducts or hepatic artery within portal triad.*

3.3.2. Laser capture microdissection to determine hepatic zonation of 11β-HSD1 mRNA expression

Laser capture microdissection and pressure catapulting was carried out on perivenous and periportal areas of 3 normal livers as described in section 3.2.3.4.

Using 18s as an internal reference, real time PCR analysis of RNA extracted from perivenous and periportal hepatocytes showed significant differences in mRNA expression of genes related to hepatic steroid metabolism. CYP2E1 expression was nearly 13-fold higher in perivenous hepatocytes, supporting widely published data on CYP2E1 zonation. HSD11B1 mRNA expression was 6 fold higher in perivenous hepatocytes compared with periportal hepatocytes. mRNA expression of the A-ring reductases 5α-reductase 1 and 2 (SRD5A1 and SRD5A2) were also higher in the perivenous areas but this achieved statistical significance only for 5α-reductase 2. Glucocorticoid receptor α (NR3C1) mRNA expression was similar in perivenous and periportal areas, in keeping with previously published data of homogenous expression throughout the liver parenchyma (Antakly & Eisen 1984).



	HSD11B1		CYP2E1		5α red 1		5α red 2		GRα	
	pp	pv	pp	pv	pp	pv	pp	pv	pp	pv
dCt	14.7	11.9	11.3	7.6	14.8	12.4	15.5	13.2	15.1	14.7
SE	0.4	1.6	1.2	0.8	1.2	3.0	1.6	2.5	0.1	0.5

Figure 3-6 *mRNA expression (fold change) in periportal hepatocytes compared with perivenous hepatocytes. ** p<0.01,* p<0.05 perivenous vs periportal. Table displaying dCt values and SE of mRNA expression of each gene in periportal (pp) and perivenous (pv) areas.*

3.3.3. Hepatic expression of HSD11B1, H6PDH and key enzymes related to glucose metabolism, and ER stress in normal liver and diseased liver states

HSD11B1 mRNA expression in normal liver compared with diseased liver states are detailed in chapter 7 (non alcoholic steatohepatitis alcoholic liver disease), chapter 5, (other non alcoholic chronic liver diseases),and chapter 4 (glycogen storage disease). These are summarised collectively in Figure 3-7.

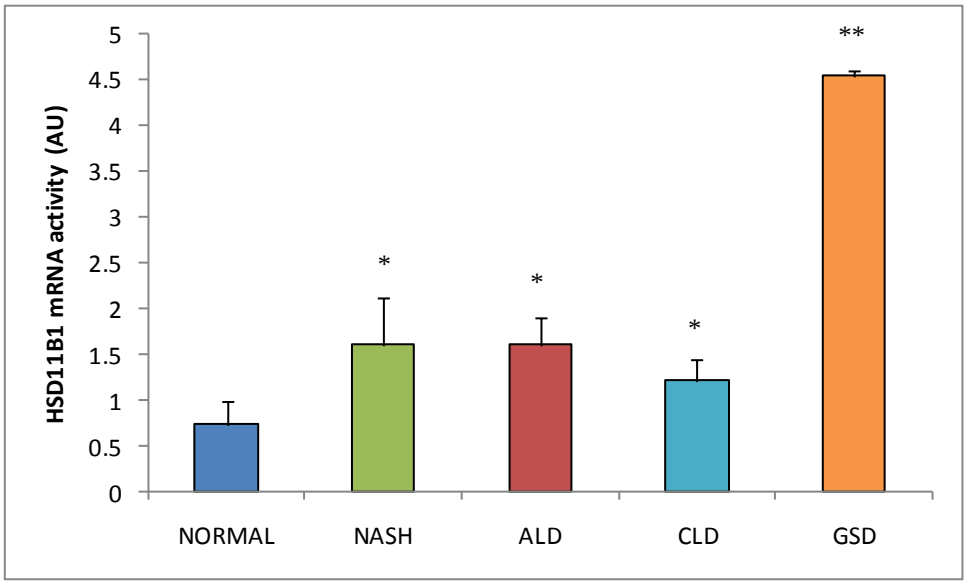


Figure 3-7 *HSD11B1 mRNA expression (expressed in arbitrary units +/- SE) in normal liver and diseased liver states. * p<0.01 compared with normal, ** p<0.001 compared with normal and other diseased liver states. (NASH non alcoholic steatohepatitis, ALD alcoholic liver disease, CLD other non alcoholic chronic liver diseases, GSD glycogen storage disease type 1a).*

3.3.3.1. Glucocorticoid receptor α expression in normal and diseased liver groups

The pattern of expression of glucocorticoid receptor mRNA expression was very similar to that for 11β-HSD1 and was highest in the GSD1a group compared with all other groups. NASH, ALD and CLD liver groups all had higher GRα mRNA expression compared with normal, but statistical significance was achieved in the NASH and CLD groups only.

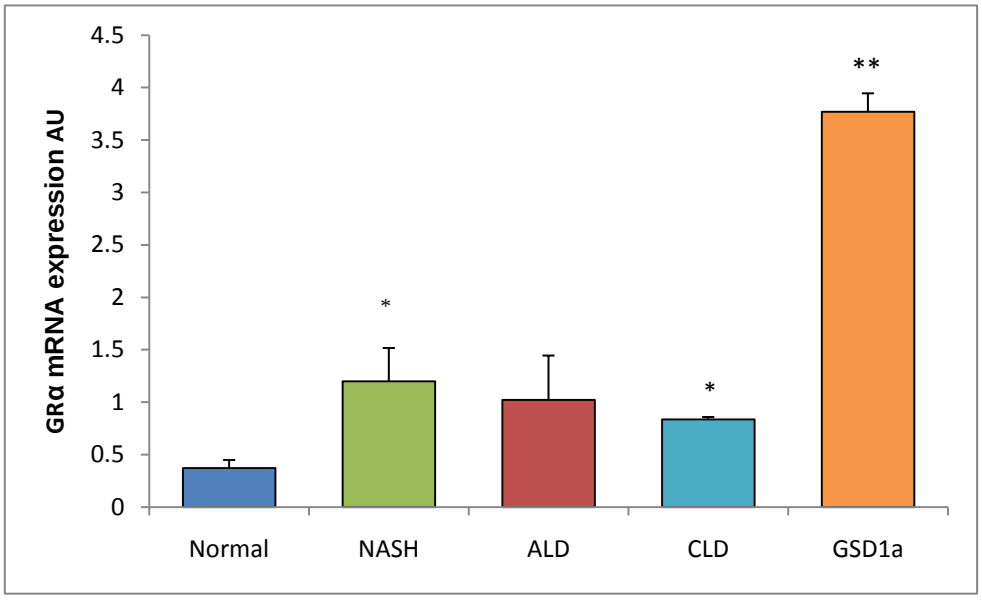


Figure 3-8 *GRα mRNA expression in normal and diseased liver groups; *p < 0.05 compared with normals, **p < 0.01 compared with normals and other diseased liver groups (data expressed as arbitrary units +/- SE).*

3.3.3.2. Hexose-6-phosphate dehydrogenase

H6PDH mRNA expression was investigated in all groups with the exception of glycogen storage disease (the GSD1a tissue had not yet been obtained at the time of this investigation). H6PDH mRNA expression was notably higher in alcoholic liver disease compared with normal as well as NASH liver. There was no significant

difference in mRNA expression of H6PDH between NASH and other non alcoholic chronic liver diseases (CLD) and normal. (dCT +/- SEM: Normal 12.1 +/- 0.4, NASH 12.7 +/- 0.4, ALD 11.3 +/- 0.4, CLD 12.4 +/- 0.5; p <0.05 ALD vs normal and NASH).

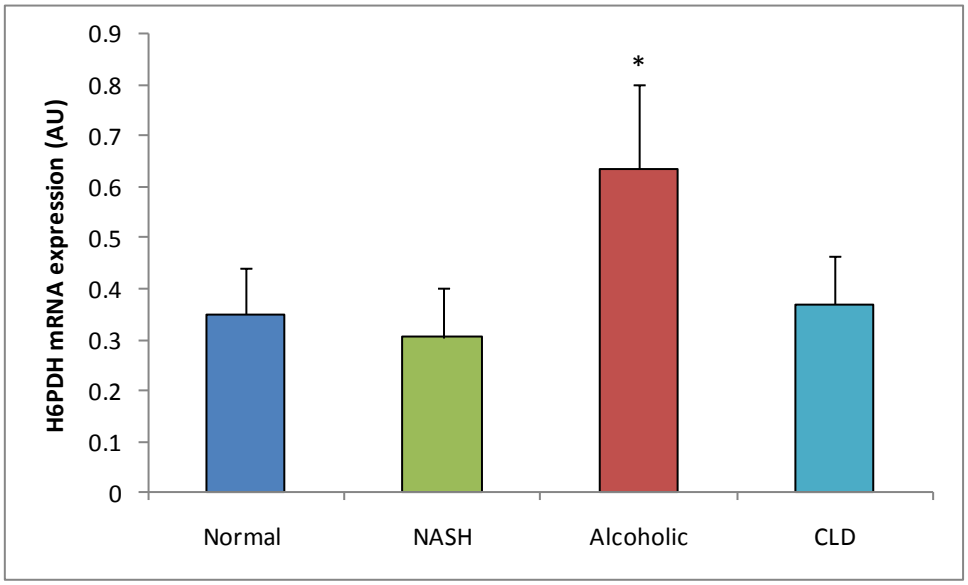


Figure 3-9 *H6PDH mRNA expression in normal liver (NASH), alcoholic liver disease, and other non alcoholic chronic liver diseases (CLD). Data expressed as arbitrary units +/- SE, *p<0.05 compared with normal and NASH group.*

3.3.3.3. mRNA expression of key rate limiting enzymes and proteins governing hepatic glucose output in normal and diseased liver

mRNA expression of both isoforms of PEPCK, PEPCK1 (cytosolic) and PEPCK 2 (mitochondrial) was investigated in normal and diseased liver samples. PEPCK 2 mRNA expression was most notably different in diseased groups compared to normal with significantly higher expression in NASH and ALD groups but no significant difference between normal and CLD groups. PEPCK1 mRNA expression was highest in NASH and ALD groups but the increase seen in the ALD group compared with

normal was not statistically significant : PEPCK1(mean dCt +/- SEM): Normal 6.24 +/- 0.8, NASH 4.6 +/- 0.3, ALD 4.6 +/- 0.3, CLD 6.0 +/- 0.6. PEPCK2: Normal 12.9 +/- 0.5, NASH 10.1 +/- 0.3, ALD 9.6 +/- 0.4, CLD 11.5 +/- 0.6., Figure 3-10.

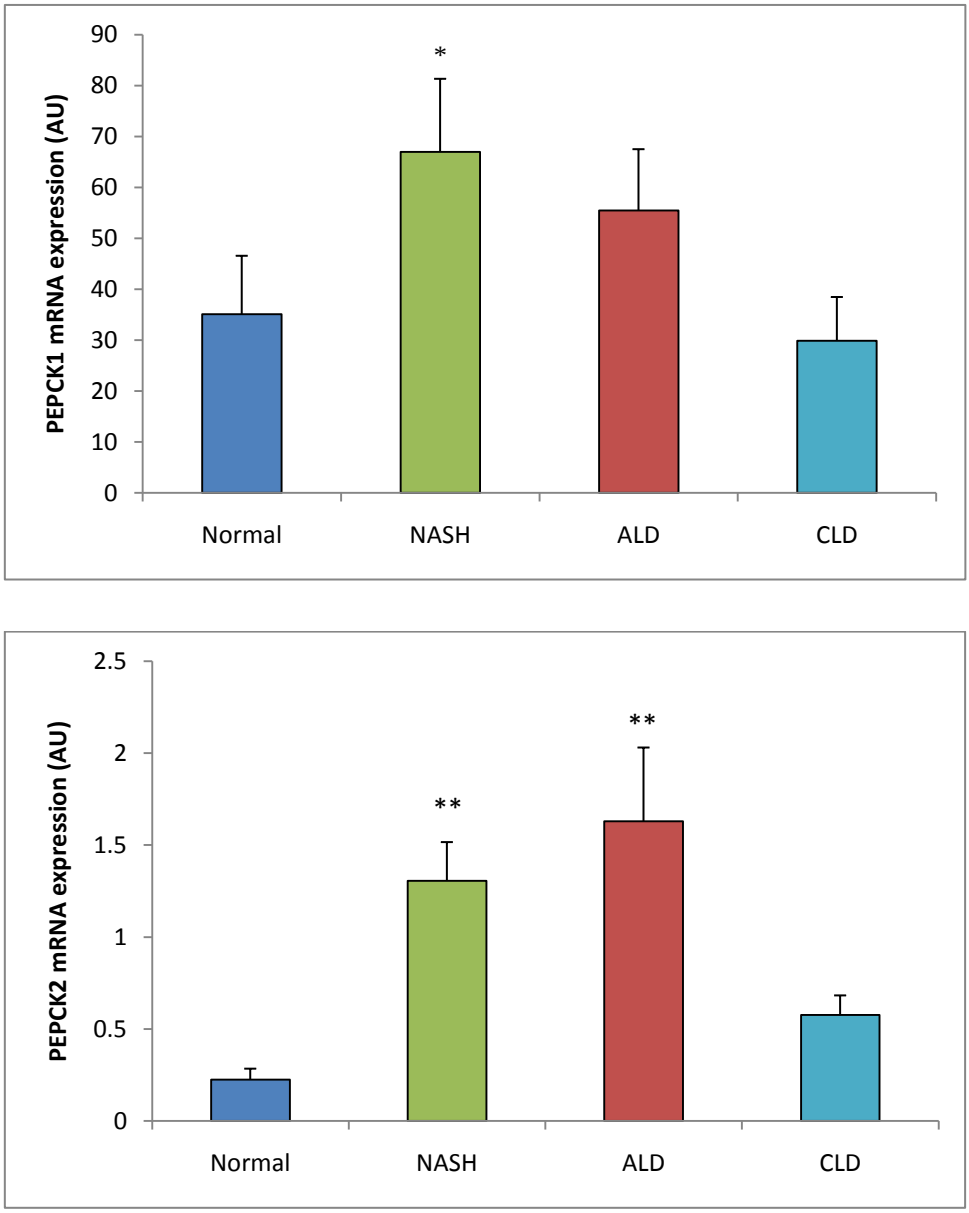


*Figure 3-10 Hepatic expression of PEPCK1 (top panel) and PEPCK2 (lower panel) mRNA expression in normal and diseased liver groups expressed in arbitrary units +/- SE. PEPCK1 expression *p <0.05 NASH compared with normal and CLD groups. PEPCK2 expression **p <0.01 NASH and ALD compared with normal and CLD groups.*

mRNA expression of the glucose 6 phosphate transporter (G6PT) was significantly increased in the ALD group compared with normal. Increased expression was also seen in the NASH group but this did not achieve statistical significance. G6PT (mean dCt +/- SEM): Normal 14.5 +/- 0.6, NASH 13.3 +/- 0.4, ALD 13.1 +/- 0.3, CLD 14.1 +/- 0.6.

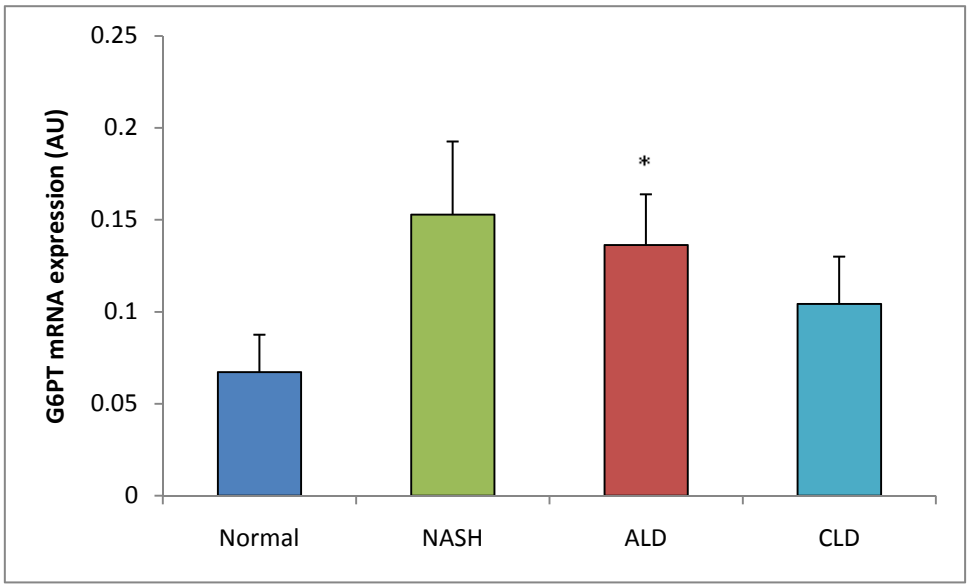


*Figure 3-11 G6PT mRNA expression in normal and diseased liver groups expressed as arbitrary units +/- SE. *p <0.05 ALD compared with normal.*

Glucose 6 phosphatase (G6Pase) α subunit mRNA expression analysis showed very significant induction of mRNA expression in NASH, and to a lesser degree in ALD liver samples. No difference was seen in G6Pase α mRNA expression in CLD compared with normal. G6Pase α (mean dCt +/- SEM): Normal 9.90 +/- 0.7, NASH 7.9 +/- 0.4, ALD 7.7 +/- 0.3, CLD 8.7 +/- 0.5, Figure 3-12. mRNA expression of the β subunit of G6Pase was similar in all groups.

mRNA expression of the hepatic glucose transporter GLUT 2 was significantly increased in all diseased liver groups compared with normal. GLUT 2 (mean dCt +/-

SEM): Normal 10.1 +/- 0.5, NASH 7.9 +/- 0.3, ALD 7.9 +/- 0.3, CLD 8.1 +/- 0.5, Figure 3-13.

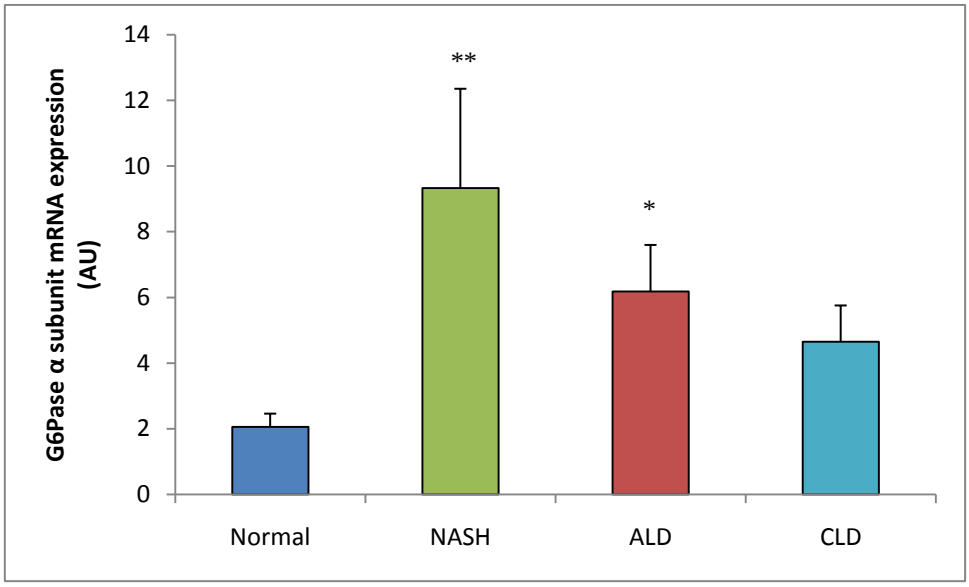


*Figure 3-12 G6Pase α mRNA expression in normal and diseased liver expressed as arbitrary units +/- SE. **p < 0.01 NASH compared with normal,*p < 0.05 ALD compared with normal.*

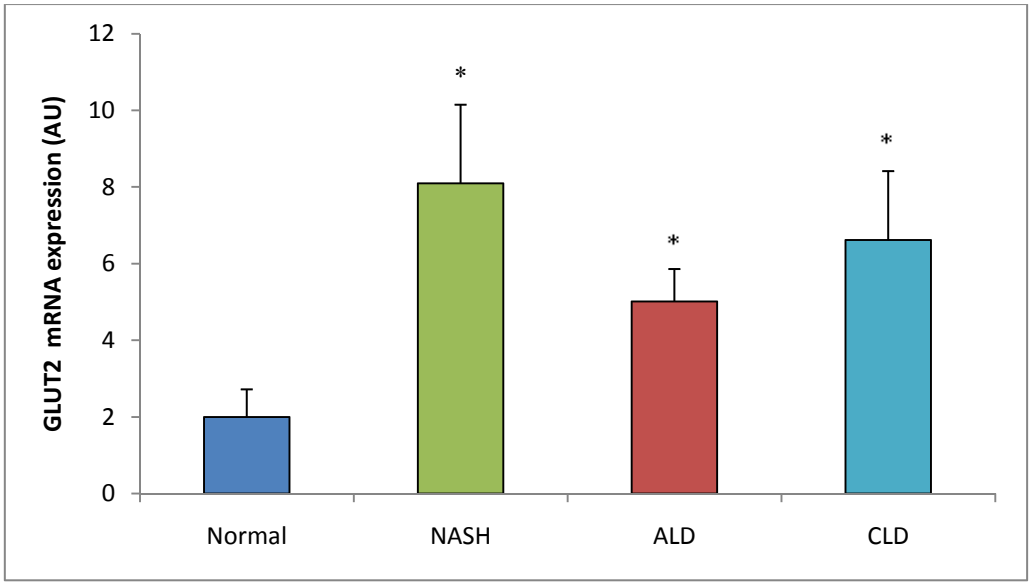


*Figure 3-13 GLUT2 mRNA expression in normal and diseased liver expressed as arbitrary units +/- SE. * p < 0.01 for all diseased groups compared with normal.*

3.3.3.4. Comparative mRNA expression of cytochrome P450IIE1 and alcohol dehydrogenase in normal and diseased liver groups

CYP 2E1 mRNA expression was significantly increased in the NASH liver samples compared with normal. Expression was similar between normal and CLD groups. In the ALD samples CYP2E1 expression appeared to be decreased compared with normal but this was not statistically significant. CYP2E1 (mean dCt +/- SEM): Normal 4.4 +/- 0.6, NASH 3.7 +/- 0.4, ALD 5.1 +/- 0.4, CLD 4.5 +/- 0.5, Figure 3-14. ADHB1 expression was significantly higher than normal in all diseased groups, and highest in the NASH group. ADHB1 (mean dCt +/- SEM): Normal 6.8 +/- 0.7, NASH 4.0 +/- 0.3, ALD 4.6 +/- 0.3, CLD 4.8 +/- 0.6, Figure 3-15.

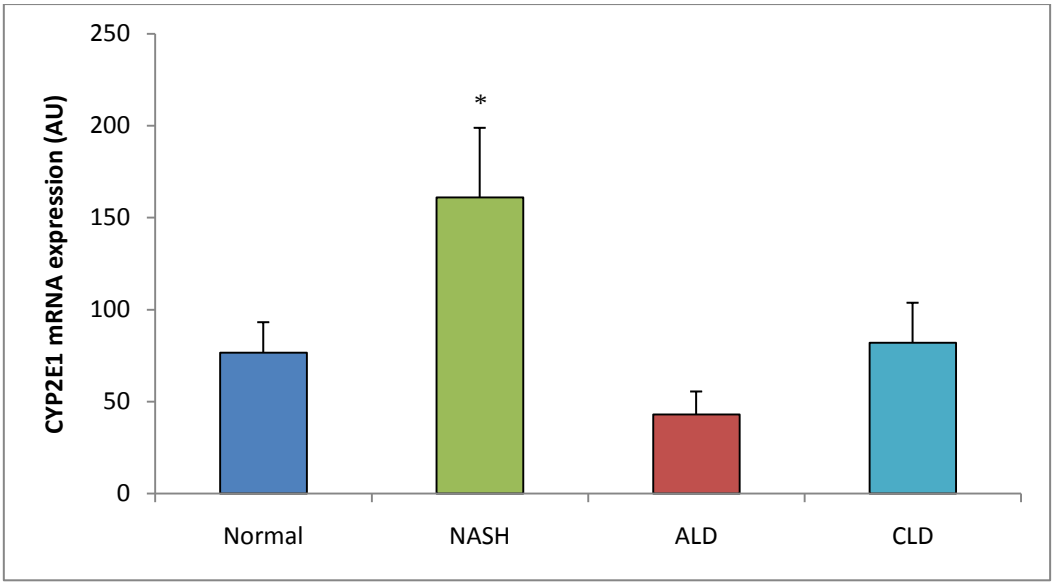


Figure 3-14 *CYP2E1 mRNA expression in normal and diseased liver expressed as arbitrary units +/- SE. * $p < 0.05$ NASH compared with normal and ALD groups.*

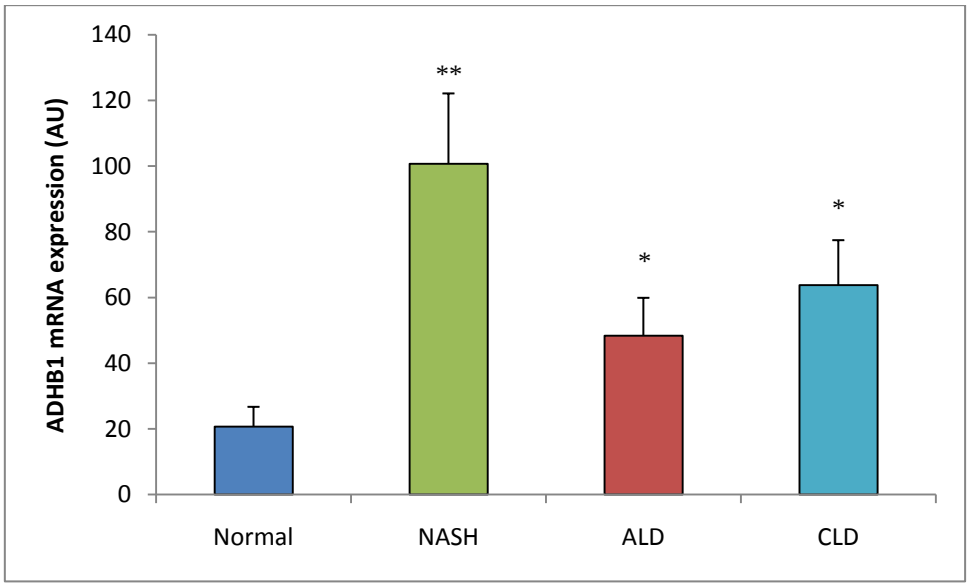


Figure 3-15 *Alcohol dehydrogenase mRNA expression in normal and diseased liver groups expressed as arbitrary units +/- SE. * $p < 0.01$ compared with normal for diseased group compared with normal, ** $p < 0.01$ NASH compared with normal and $p < 0.05$ NASH compared with ALD.*

3.3.4. 11β-HSD1 assay on microsomes from snap frozen normal human liver

Microsomes were prepared from all thirty liver samples from normal and diseased groups with the intention to perform comparative 11β-HSD1 assays between groups. At the time of writing, following the preparation of microsomes, optimisation of the method was carried out using microsomes from snap frozen normal liver (in triplicate) to determine the reaction conditions and concentration of microsomal protein required per assay to yield optimal results. It was decided to use 100μg of microsomal protein per reaction with a one hour incubation at 37°C, with which the percentage of substrate metabolized in each experiment was 10% or less, ensuring that initial rates of metabolism were being measured, Figure 3-16.

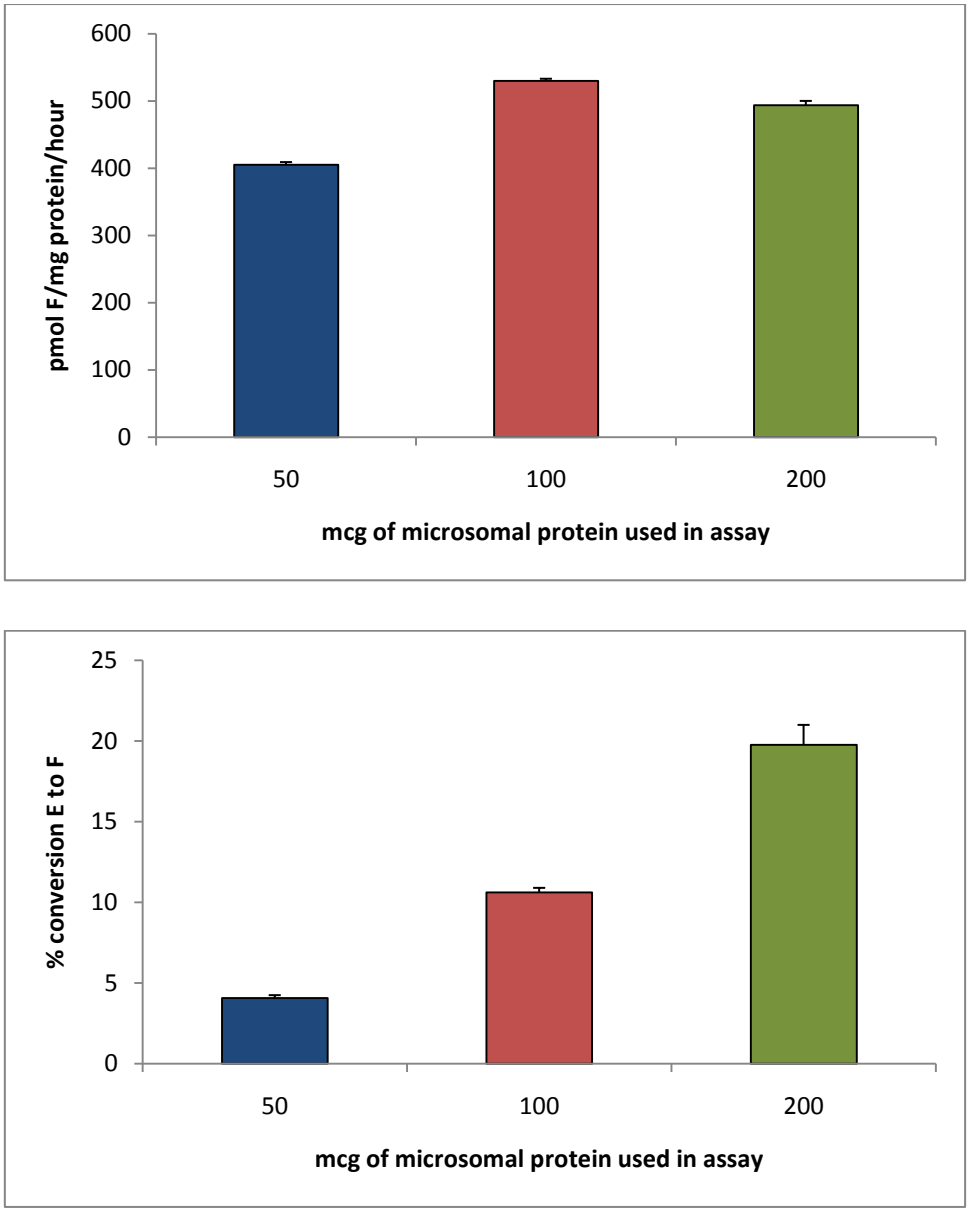


Figure 3-16 *11β-HSD1 reductase activity in liver microsomes from snap fozen normal human liver. Upper panel: Conversion as pmol cortisol/mg microsomal protein/hour in preparations of assay with increasing concentrations of protein used per assay. Lower panel: data illustrated as percentage conversion of E to F with different concentrations of microsomal protein.*

3.4. Discussion

3.4.1. Hepatic zonation of steroid metabolism

The immunohistochemistry and laser capture microdissection studies confirmed the perivenous zonation of 11β-HSD1 protein and gene expression in normal human liver. There was a lesser degree of localisation of 11β-HSD1 to periportal hepatocytes, where protein expression was greater than in the parencymal cells in intermediate areas. Furthermore, there was evidence of preferential perivenous zonation of mRNA expression of the A-ring reductase 5α-reductase 2. Glucocorticoid receptor α mRNA expression was similar between perivenous and periportal areas in keeping with previously published reports of homogenous GR protein expression throughout the liver parenchyma (Antakly & Eisen 1984). The physiological relevance of these findings may be considered with regard to the important effects of glucocorticoids upon the liver.

It has been suggested that the physiological role of hepatic 11β-HSD1 is to ensure adequate activation of low affinity glucocorticoid receptors by the reactivation of cortisone to cortisol. This may be important in maintaining optimal glucocorticoid induced permissive effect upon gluconeogenic hormones. There is considerable evidence to support this hypothesis. Firstly, circulating cortisone concentrations in man are about 50nM, it is not protein bound or subject to circadian rhythm (Walker et al 1992a), hence there is ample cortisone availability for hepatic reactivation to cortisol. Secondly, 11β-HSD1 functions as a reductase in human liver, as reflected by cortisol/cortisone concentrations in the hepatic vein being five-fold higher than in peripheral arterial blood (Walker et al 1992a). In addition, the rapid conversion of

oral cortisone which is delivered to the liver via the portal vein resulting in a rapid rise in serum cortisol levels and a negligible increase in serum cortisone levels (Stewart et al 1990). Carbenoxolone inhibits the conversion of cortisone to cortisol by hepatic 11β-HSD1 (Stewart et al 1990), and results in enhanced whole body insulin sensitivity without impacting on peripheral insulin sensitivity (Walker et al 1995). Hence, hepatic 11β-HSD1 inhibition results in an enhancement of insulin mediated down regulation of hepatic glucose output. In addition, transgenic deletion of 11β-HSD1 in mice results in an absence of 11-dehydrocorticosterone to corticosterone conversion, despite elevated plasma corticosterone concentrations, with impairment of hepatic gluconeogenesis upon starvation (Kotelevtsev et al 1997). While there was clear evidence of hepatic 11β-HSD1 zonation, there was a significantly lesser degree of 11β-HSD1 protein expression throughout the liver parenchyma in intermediate areas. This would support the proposed physiological role for hepatic 11β-HSD1 and would concur with the homogenous expression of GR, to maximise hepatic glucocorticoid exposure. This reflects the importance of this process and its regulation in the maintenance of normal hepatic metabolic phenotype.

While the immunohistochemistry studies did show some periportal zonation of 11β-HSD1 supporting the above observations, the primarily perivenous zonation of 11β-HSD1 would not be entirely in line with expectations. The hypothesis of hepatic pre receptor glucocorticoid activation by 11β-HSD1 impacting upon hepatic gluconeogenesis, and thus being involved in regulatory mechanisms to influence hepatic glucose output in normal physiology and diseased state would predict periportal localisation of hepatic 11β-HSD1 in keeping with the zonation of gluconeogenic enzymes. While it is clear that hepatic 11β-HSD1 activity has an

influence upon hepatic gluconeogenesis, in normal physiology, the impact of hepatic 11β-HSD1 activity upon perivenous metabolic functions may perhaps be more important. Hepatic metabolic functions showing strong perivenous zonation include glycolysis and lipogenesis. The effects of glucocorticoids on hepatic glucose metabolism reflecting the zonation of each process are summarised in Figure 3-17.

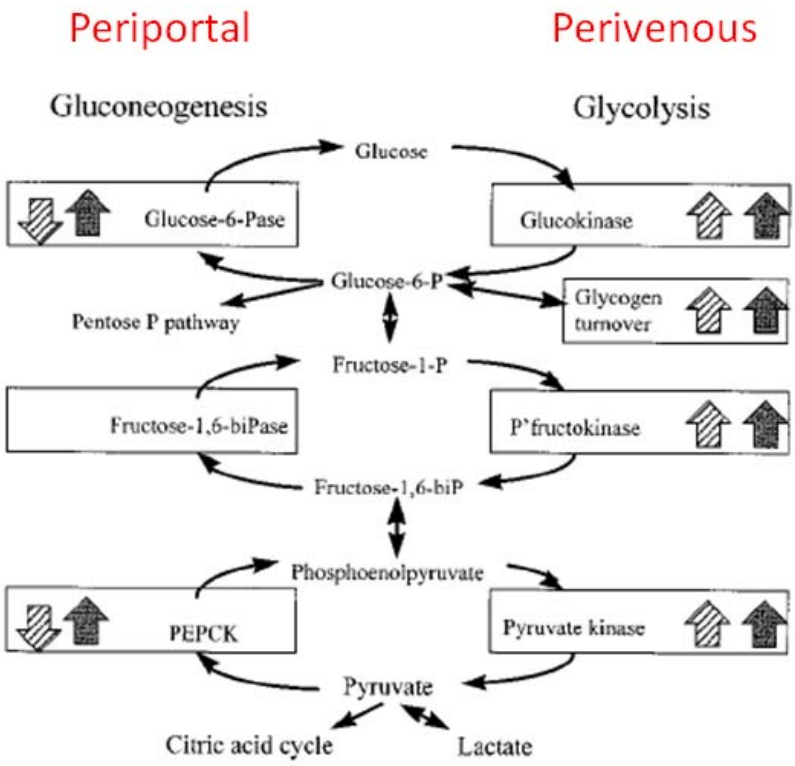


Figure 3-17 Effects of glucocorticoids on hepatic glucose metabolism. The principal metabolic fates of glucose in the liver are shown with the hepatic zonation of each prinicipal pathway. Actions of glucocorticoids (grey arrows) and insulin (striped arrows) are shown either as positive (up arrow)or negative (down arrow) effects. In some respects, insulin and glucocorticoids oppose each others actions, particularly on gluconeogenesis (PEPCK) and release of glucose from G6P . In other respects, insulin and glucocorticoids do not oppose each other, especially in promoting oxidative glycolysis and increasing turnover between G6P and glycogen. Adapted from (Andrews and Walker 1999).

In perivenous localised processes, glucocorticoids promote the actions of insulin including glycolysis and lipogenesis. Importantly insulin receptors display a perivenous zonation in normal liver (Krones et al. 2000). In particular the rate limiting step of glycolysis, the conversion of fructose-1-phosphate to fructose-1-6-bisphosphate by phosphofructokinase (PFK) and the actions of glucokinase (GK) are both stimulated by glucocorticoids (Loiseau et al. 1985) and insulin. However, studies have shown that supraphysiological doses of glucocorticoids decrease hepatic sensitivity to the effects of insulin and increase sensitivity to glucagon (Dirlewanger et al. 2000).

The physiological importance of the high perivenous expression of 11β-HSD1 may reflect a physiological protective mechanism to preserve not only hepatic metabolic phenotype but also whole body insulin sensitivity. Seminal work by Wu et al. showed that mice with hepatic over expression of the key glycolytic enzymes GK or PFK demonstrated increases in hepatic glycolysis and reduced adiposity and body weight in obese mice (Wu et al. 2005). Elevated rates of glycolysis led to a decrease in hepatic glucose production, with a knock-on effect on reducing plasma glucose, and a secondary similar reduction in plasma insulin. This resulted in a relief of inhibition of hyperinsulinaemia on lipolysis in adipose tissue and fatty acid oxidation in muscle. Furthermore, the decrease in hepatic glucose production led to a decrease in whole body glucose disposal, implying a decrease in peripheral glucose utilization (i.e. muscle). This was compensated for by increased rates of fatty acid oxidation in extrahepatic tissues, in particular skeletal muscle, leading to an elevation of energy expenditure. While these effects were similar with either over expression of GK or PFK, GK over expression was also associated with increased hepatic lipogenesis,

while over expression of PFK had the additional effect of causing suppression of food intake and a decrease in the hypothalamic expression of neuropeptide Y – this contributed to a profound reduction in body weight and *decreased* hepatic lipogenesis. Hence, the step at which glycolysis is enhanced has a dramatic influence on whole body energy expenditure and lipid profiles. On this background the effect of 11β-HSD1 driven perivenous glucocorticoid generation would promote glycolysis independently to the effects of insulin. This would be particularly important with increasing insulin resistance or insulin deficiency where local glucocorticoid generation could drive a compensatory increase in glycolysis both at the level of GK and PFK. Any differential effects of glucocorticoids on GK or PFK stimulation would further modulate hepatic lipogenesis in addition to the direct effects of glucocorticoid upon hepatic de novo lipogenesis.

The effect of glucocorticoids on promoting hepatic lipogenesis would also be compatible with the perivenous zonation of 11β-HSD1. A number of studies have shown that hepatic lipogenesis is promoted by physiological concentrations of glucocorticoids (Amatruda et al. 1983). While insulin influences lipogenic enzyme translation (Sun and Holten 1978), glucocorticoids influence lipogenic enzyme transcription (Fritz et al. 1986). Indeed, any beneficial effect of glucocorticoid mediated glycolysis as described above would be offset by the negative effect of glucocorticoids upon hepatic lipid accumulation. The co-localisation of mRNA expression of hepatic A-ring reductases with 11β-HSD1 in perivenous hepatocytes may reflect the importance of the fine tuning of pre-receptor glucocorticoid metabolism in the preservation of a normal hepatic metabolic phenotype, such that regulation of the set point of 11β-HSD1 activity as well as glucocorticoid clearance

by A-ring reductases would optimise the glucocorticoid response to changes in the hepatic metabolic milieu. It would be of considerable interest to explore the zonation of hepatic 11β-HSD1 activity and expression in disease states such as type 2 diabetes, hepatic steatosis and other insulin resistant states, in particular to determine whether there is a dynamic component to zonation of hepatic glucocorticoid metabolism. Indeed studies described in chapter 7 showed generally increased staining for 11β-HSD1 in the histologically disrupted NAFLD cirrhotic liver with periseptal localisation. This would indicate that 11β-HSD1 expression in the liver is indeed dynamic and may alter in response to multiple factors in various hepatic disease states.

3.4.2. Comparative mRNA expression of glucocorticoid and carbohydrate metabolism related genes in normal and diseased liver states

The mRNA expression data from normal and diseased livers showed increased hepatic 11β-HSD1 expression in all types of chronic liver disease studied. These included non alcoholic fatty liver disease, alcoholic liver disease, other non alcoholic chronic liver diseases and glycogen storage disease 1a. mRNA expression of both 11β-HSD1 and GRα were extremely high in the GSD1a liver samples compared with normal and all other diseased liver groups (discussed further in chapter 4). An important difference of the GSD1a sample compared with other diseased livers was the absence of cirrhosis. While most of the other diseased livers showed histological evidence of cirrhosis, there were adequate histological features to suggest the underlying aetiology in all samples chosen for analysis.

If hepatic HSD11B1 gene expression is commonly increased across all chronic liver diseases regardless of underlying aetiology, the physiological relevance of this process is likely to be strongly implicated as cause or effect of common factors unifying chronic liver disease states. With regard to glucocorticoid status in chronic liver disease, it has long been recognised that there is impairment of adrenal glucocorticoid production and often impairment of the HPA axis. For example, chronic hepatic damage due to cholestasis in rats results in decreased hypothalamic CRF (corticotrophin releasing hormone) mRNA and protein expression of corticotrophin – releasing hormone (Swain et al. 1993). In chronic liver disease there is a dissociation of two important functions of the adrenal cortex. A widening of the zona glomerulosa associated with increased aldosterone secretion, and atrophy of the zona fasciculata with a decreased cortisol production rate occurs (Gerdes 1979). This could explain the increased hepatic expression of GRα and 11β-HSD1 mRNA as a compensatory up regulation to maximise hepatic glucocorticoid exposure. In addition inhibition of A-ring reductase activity resulting in decreased glucocorticoid clearance further enhances hepatic glucocorticoid availability, a mechanism seen in non alcoholic steatohepatitis (chapter 7) and cholestasis. In fact bile acids are potent competitive inhibitors and transcriptional regulators of 5β-reduction of glucocorticoids (Mc Neilly and McKenzie2 2005). It is likely that hepatic 11β-HSD1 expression and activity is modulated in conjunction with disease severity as seen in NAFLD where there is evidence of increased glucocorticoid clearance and decreased 11β-HSD1 driven glucocorticoid generation (chapter 7) in hepatic steatosis, with the opposite effect occurring with progression to steatohepatitis. Increased hepatic glucocorticoid generation may occur both at the level of gene expression and protein

expression as well as regulation of the set point of activity of 11β-HSD1. The mechanism may well depend on the underlying aetiology of the chronic liver disease. The role of oxidative stress and ER stress on 11β-HSD1 activity in chronic liver disease merits further investigation. Certainly, chronic liver diseases such as NASH, ALD and even GSD1a all have oxidative stress and ER stress is strongly implicated in pathogenesis. H6PDH mRNA expression was interestingly higher in the ALD group compared with other chronic liver diseases. This may reflect an additional effect of altered ER redox impacting upon 11β-HSD1 in alcoholic liver disease, with H6PDH serving a key role linking steroid metabolism and nutrient sensing (chapter 4). Indeed, ER stress is clearly an important mechanism in the pathogenesis of NAFLD and this may in part be related to its relationship with CYP2E1. CYP2E1 is capable of inducing significant ER protein damage and stress via its catalytic activation of pro-oxidants (Lewis and Roberts 2005) and was increased in the NASH group. This would support the link between non alcoholic fatty liver disease and CYP 2E1, which is associated with insulin resistance and mechanistically with the development of NAFLD (Schattenberg et al. 2005). Insulin resistance and CYP2E1 expression may be interrelated through the ability of CYP2E1-induced oxidant stress to impair hepatic insulin signalling. This may represent another mechanism to worsen the insulin resistance in NAFLD. It may be that the increase in 11β-HSD1 activity seen in NASH (chapter 7) is associated with an NADP+/NADPH ratio in the ER lumen, influenced by the effect of CYP2E1 on ER redox that would favour reductase activity. In ALD, with abstinence from alcohol, hepatic disease progression is associated with a decrease in CYP2E1 (Dilger et al. 1997). Alcohol dehydrogenase (ADH) expression was increased in all groups, and most significantly so in NASH. This too may be

implicated in ER stress. Studies on cardiac myocytes over expressing ADH showed a key role of ADH in cardiac dysfunction, hypertrophy, insulin sensitivity and ER stress (Li and Ren 2008).

Up to 96% of patients with cirrhosis may be glucose intolerant and 30% will have diabetes (Hickman and Macdonald 2007). Diabetes that develops as a consequence of cirrhosis is termed 'hepatogenous diabetes', and is not yet recognised by the World Health Organisation or the American Diabetic Association as a separate entity, although it is distinct from obesity-related type 2 diabetes in that it is much less frequently associated with microangiopathy, and fasting glucose levels are usually normal. Patients with cirrhosis and diabetes have much higher morbidity and mortality from the complications of cirrhosis (El-Serag, Tran, & Everhart 2004;El-Serag and Everhart 2002). The underlying aetiology of chronic liver disease has a strong bearing on the likelihood of the development of 'hepataogenous diabetes'. In particular, NAFLD, alcoholic liver disease, haemachromatosis and hepatitis C are frequently associated with diabetes, which may reflect direct damage to pancreatic β cells (as in alcoholism, and haemachromatosis) or associated with increasing hepatic steatosis and hepatic insulin resistance. Consistent with these observations were the results of the mRNA expression studies on key enzymes and transporter proteins governing hepatic glucose output. Both the NAFLD group and the ALD group had high expression of these genes. Unfortunately data regarding the presence or absence of diabetes in the patients from whom the diseased livers were explanted were not readily available, but these results may reflect a possible higher incidence of diabetes in the NAFLD and ALD groups compared with chronic liver diseases of other causes. G6Pase and the G6P transporter are ER membrane proteins that facilitate the final

step of the gluconeogenic pathway. G6Paseα mRNA was significantly increased in the NASH samples and also to a lesser extent in the ALD samples. In addition there was significantly increased expression of the G6P transporter in the ALD samples. These are important results that may occur secondary to increased hepatic glucocorticoid availability from increased 11β-HSD1 expression and activity, as well as a mechanism to increase G6P substrate availability for H6PDH in ALD where there is increased G6P transporter as well as H6PDH gene expression. This would implicate increased 11β-HSD1 oxoreductase activity as well as increased 11β-HSD1 gene expression in alcoholic liver disease (as demonstrated in the clinical and in vitro studies described in chapter 5), which would have a knock on effect on hepatic glucose output through glucocorticoid stimulated gluconeogenic enzymes. The increase in PEPCK and G6Pase mRNA expression in NASH and ALD compared with both normal and CLD groups may reflect the added effects of glucocorticoid induction on gene transcription of gluconeogenic enzymes in addition to other common mechanisms to increase hepatic glucose output in NASH and CLD. The glucose transporter GLUT 2 mRNA expression was significantly higher in all diseased liver groups and may reflect a compensatory up regulation due to the increased insulin resistance associated with severe chronic liver disease.

Microsomes were prepared from all the liver samples used in these studies, and the conditions for 11β-HSD1 activity assays on these microsomes optimised. Further work will include the comparisons of 11β-HSD1 activity *in vitro* between groups. The in vitro studies described in this chapter provide strong support for the finding described in the translational studies exploring hepatic glucocorticoid metabolism in glycogen storage disease (chapter 4), alcoholic liver disease (chapter 5) and non

alcoholic fatty liver disease (chapter 7). It would also be of great interest to explore the impact of ER stress upon hepatic glucose and glucocorticoid metabolism in chronic liver disease, particularly those with a close association with hepatic steatosis and insulin resistance such as NASH, ALD and chronic hepatitis C.

4. The impact of endoluminal Glucose 6-Phosphate upon 11β-Hydroxysteroid Dehydrogenase Type 1 mediated glucocorticoid generation and Hypothalamo-Pituitary-Adrenal Axis function

4.1. Introduction

A vital function of the liver is to provide glucose during fasting. This occurs through two principal pathways, gluconeogenesis and glycogenolysis, in each case yielding glucose 6-phosphate (G6P) (reviewed in sections 1.3.2.1 - 1.3.2.4), which is then hydrolyzed by glucose-6-phosphatase (G6Pase), more recently described as G6Pase-α (Shieh et al. 2003), to glucose (Chou et al. 2002a). G6Pase-α is a transmembrane protein in the endoplasmic reticulum (ER) with the active enzyme site directed toward

the ER lumen (Chou et al 2002a;Van & Gerin 2002). G6P must be translocated from cytosol to ER via a ubiquitously expressed G6P transporter (G6PT) before hydrolysis can occur.

4.1.1. Glycogen storage disease type 1

Patients and rodent models lacking components of the G6Pase-α, G6PT system emphasize the crucial role of this pathway in maintaining glucose homeostasis in the fasting state. This results in accumulation of glycogen and lipids in gluconeogenic organs including liver, kidney and intestine. The disease is inherited in an autosomal recessive manner. Von Gierke described the first patient with glycogen storage disease type 1 (GSD1) in 1929 from the autopsy reports of two children who had excessive glycogen in their livers and kidneys (Von Gierke 1929). Thirty three years later Cori described six further patients and detected G6Pase deficiency, identifying for the first time a single enzyme defect causing an inborn error of metabolism (CORI and CORI 1952). While they recognised that hepatic GSD causes a heterogenous group of disorders, it remained a mystery why some of these patients had normal G6Pase activity until 1978, when a defect in the intracellular transport of the G6P was identified (Narisawa et al. 1978). In recognition of the original description of the disease the Cori classification was preserved with von Gierke's disease, type 1a caused by G6Pase-α deficiency; and type 1b caused by a G6PT defect (Chen et al. 2003;Van & Gerin 2002). As the product of the G6Pase reaction is free glucose, both type 1a and 1b cause fasting hypoglycaemia, and hepatomegaly as a consequence of glycogen accumulation. The liver in GSD type 1 therefore loses its fundamental function as a glucose homeostatic organ.

GSD 1 is one of the few causes of profound hypoglycaemia in newborns, with a rapid drop in blood glucose soon after birth, following the withdrawal of maternal glucose. Seizures, and cyanosis, irritability and life threatening lactic acidosis occur and repeated hypoglycaemia can cause brain damage. Dietary manipulation remains a 'palliative' treatment, but it does reverse the acute complications of hypoglycaemia and lactic acidosis. A number of abnormalities persist despite metabolic control (Moses 2002). The limitations of dietary treatment have become clearer with increased survival of GSD 1 patients. These include growth retardation, hepatomegaly, lactic acidosis, hyperlipidaemia, hyperuricaemia related gout, nephrolithiasis and proteinuria causing renal failure. In adult patients, there is a high risk of pulmonary hypertension, hepatic adenomas with risk of malignant change, progressive renal failure and hypothyroidism (Franco et al. 2005;Moses 2002). There is an increased risk of osteoporosis and fractures, which is unexplained but may be due the restrictive dietary regime, renal calcium loss and high bone turnover (Moses 2002). In addition GSD 1b is has the added complications of neutropenia, and impaired neutrophil function causing recurrent bacterial infections and gut mucosa ulceration and eventually GSD enterocolitis. Also, GSD1b is associated with a higher prevalence of thyroid autoimmunity and hypothyroidism which is rarely seen in GSD1a. The cause of this is also largely unexplained but has been associated with increased amyloidosis in GSD1b patients on chronic therapy with granulocyte-colony-stimulating factor (GCSF). An understanding of the complexities of disruption in cellular homeostasis that results from a single enzyme defect in GSD provides a valuable insight in the interaction between carbohydrate metabolism and other systems. The biochemical and molecular defects underlying GSD 1 are

illustrated in Figure 4-1. As glucose cannot be produced through glycogenolysis, glucose cycling is disrupted in GSD 1 and glycogen accumulates in the liver and kidney. The effects of G6P accumulation on causing increased lactate triglyceride and cholesterol production are reflected in Figure 4-1.

Increased metabolic stress has recently been implicated as a cause of the neutropenia in GSD1b. Neutrophils from G6PT-null (GSD Ib) mice show evidence of increased ER stress, demonstrated by increases in ER chaperones and oxidative stress (Kim et al. 2008). Recent data also suggested that the unique lack of the G6P transporter that blocks glucose production in neutrophils may specifically cause neutropenia in GSD1b. An additional catalytic unit of G6Pase exists, G6Pase-β with properties similar to G6Pase-α. Hence glucose production still occurs in neutrophils of patients with GSD1a albeit at a lower level, and hence netropenia is prevented. In GSD1b, glucose production in neutrophils is absent due to the coupling of the G6P transporter with G6Pase-β (Cheung et al. 2007).

Recombinant mice with global deletion of G6Pase-α (Lei et al. 1996) and G6PT (Van & Gerin 2002) have similar phenotypes with profound hypoglycemia. Characterisation studies showed that G6Pase-/- mice manifest the same phenotype as GSD1a humans with hypoglycemia, growth retardation, hepatomegaly, kidney enlargement, hyperlipidemia, and hyperuricemia. Similar to humans, if an effective dietary therapy is not implemented, the mice die within 4-5 weeks due to severe hepatic and renal disease and metabolic acidosis. The G6PT knockout mouse also mimics human GSD1b including disordered myeloid function and impaired neutrophil trafficking and function (Chen et al 2003). Infusion of an adenoviral vector containing the murine G6Pase gene into a G6Pase deficient mouse resulted in

restoration of 19% of hepatic G6Pase function and, normalisation of glucose, lipid and urate with a decrease in liver and kidney glycogen accumulation to normal levels, improved growth and 100% survival rate (Chou et al. 2002b).

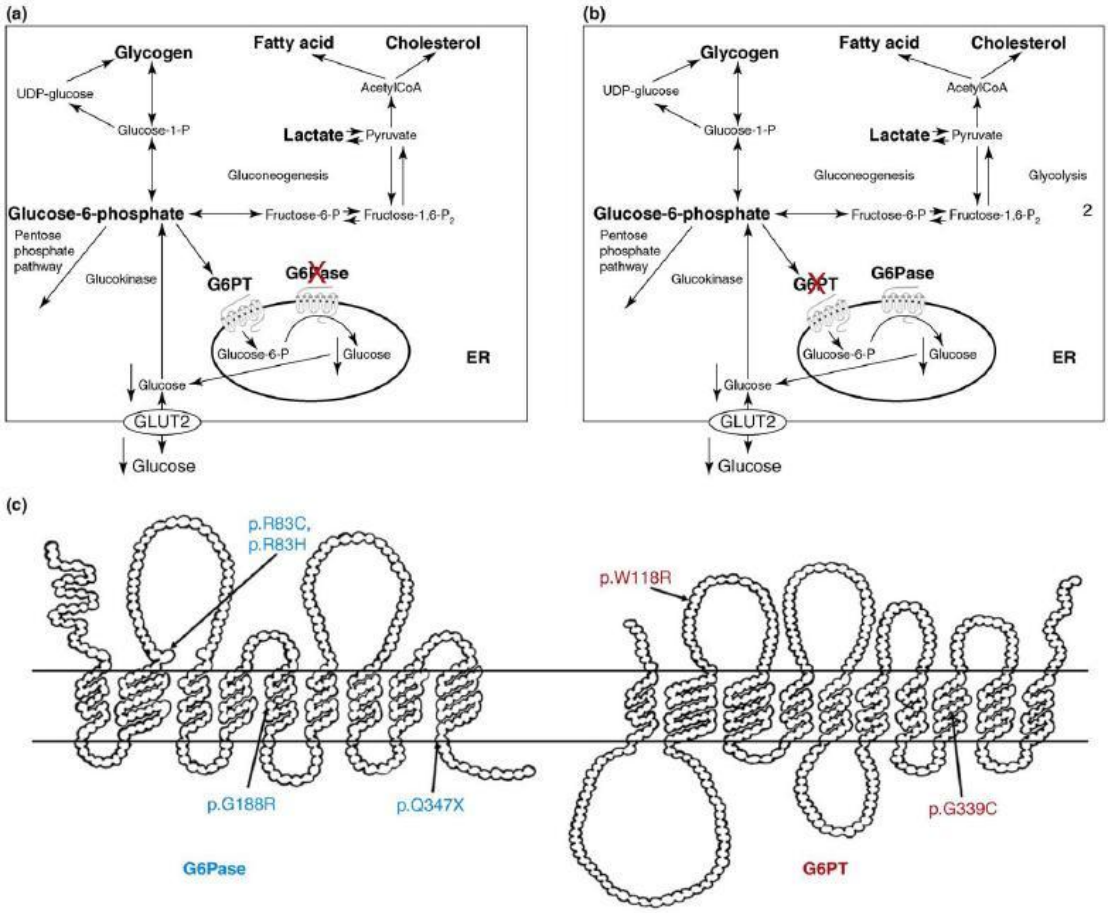


Figure 4-1 *Biochemical and molecular defects underlying GSD I. Glucose cycling is disrupted in glycogen storage disease (GSD I) because glucose cannot be produced through glycogenolysis. Dietary glucose is stored as glycogen, which accumulates in the liver and kidney in GSD I. (a) Biochemical effects of GSD Ia. In the hepatocyte, G6Pase-α deficiency (X) blocks hydrolysis of glucose-6-phosphate (G6P) in the liver, resulting in decreased glucose production and increased lactate, cholesterol and triglyceride production. However, G6Pase-β activity in neutrophils prevents neutropenia in GSD Ia (not shown). (b) Biochemical effects of GSD Ib. In the hepatocyte, G6PT deficiency (X) prevents hydrolysis of G6P with biochemical effects identical to those of GSD Ia. Furthermore, in the neutrophil, G6PT deficiency decreases glucose production, leading to ER stress and neutrophil death. (c) Common missense and nonsense mutations affecting G6Pase (left, blue) and G6PT (right, red) in GSD I (Koeberl et al. 2009) .*

4.1.2. The G6Pase - H6PDH - 11β-HSD1 system

G6Pase- shares its substrate (G6P) with another enzyme within the ER, hexose-6-phosphate-dehydrogenase (H6PDH), that catalyzes the first two steps of an ER-specific "pentose phosphate pathway," *i.e.* both G6P dehydrogenase and 6-phosphogluconolactonase reactions (Hewitt, Walker, & Stewart 2005;Mason et al 1999), illustrated in Figure 4-2 and described in detail in section 1.6.

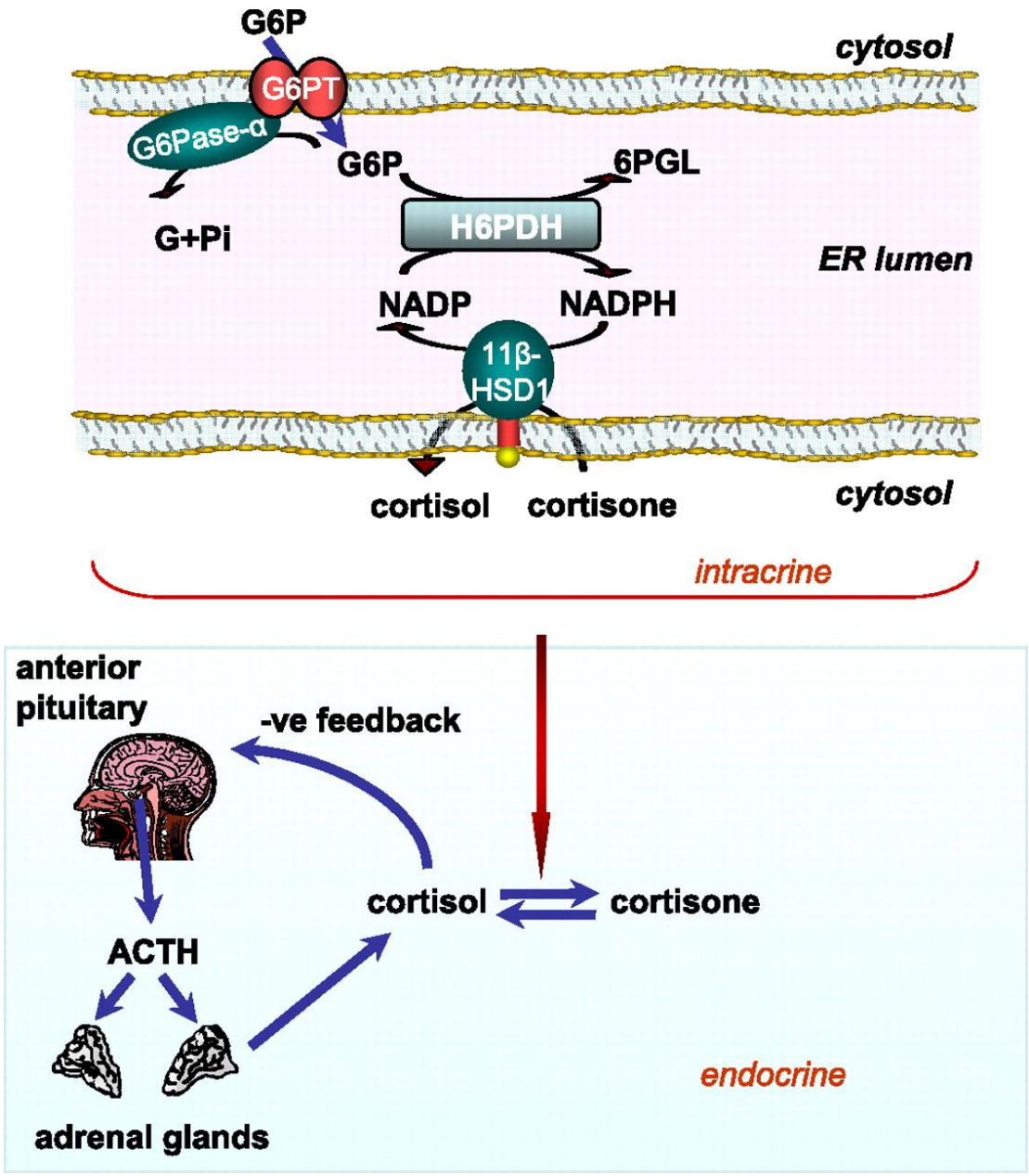


Figure 4-2 The microsomal G6Pase-α, H6PDH and 11β-HSD1 systems and the hypothalamo-pituitary-adrenal axis.

Recent studies have indicated a pivotal link between H6PDH activity in the ER and the control of set point of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), described fully in section 1.5.2. 11β-HSD1 is an ER-bound enzyme (Ozols 1995) catalyzing the interconversion of inactive glucocorticoids (cortisone in humans and

11-dehydrocorticosterone in rodents) and hormonally active glucocorticoids (cortisol and corticosterone) (Tomlinson et al 2004b). The reaction direction, which 11β-HSD1 catalyzes, is determined by the relative abundance of NADP+ and NADPH (Lakshmi & Monder 1988). In its native purified state, 11β-HSD1 acts as a dehydrogenase-inactivating cortisol/corticosterone to cortisone/11-dehydrocorticosterone (11-DHC) (Lakshmi & Monder 1988;Walker et al 2001). However, in the presence of reducing NADPH, generated through H6PDH activity (Clarke & Mason 2003),11β-HSD1 switches to reductase with the generation of active glucocorticoid in key tissues such as liver and adipose (Banhegyi et al. 2004;Bujalska et al 2005;Czegle et al. 2006). In these tissues, this reductase activity has been shown to enhance hepatic glucose output (Kotelevtsev et al 1997) and adipogenesis (Clarke & Mason 2003), respectively.

11β-HSD1 is thus an exciting candidate to explain features of obesity and the metabolic syndrome with selective inhibitors under active development that may prevent and/or reverse diabetes mellitus in obese subjects (Boyle & Kowalski 2009;Tomlinson 2005), described fully in section 1.5.2.1. Previously, it has been shown that H6PDH is an essential requirement for 11β-HSD1 reductase activity; here it was explored whether G6P availability to H6PDH could directly modulate 11β-HSD1 activity, thereby representing a novel pathway linking the metabolism of glucose and glucocorticoids.

4.1.3. Hypothesis

It was hypothesised that inhibition of G6Pase, with resultant reduction in G6P to glucose would increase substrate availability to H6PDH, which in turn would result in increased generation of the cofactor NADPH, driving 11β-HSD1 oxoreductase

activity and generation of active glucocorticoid. The murine 11β-HSD1 enzyme catalyses the conversion of inactive 11-dehydrocorticosterone, to active corticosterone. To test this hypothesis, studies were carried out on normal mouse liver microsomes to elucidate the effects of biochemical inhibition of the G6Pase enzyme on 11β-HSD1 activity. Sodium vanadate was used as a G6Pase inhibitor (Green 1986).

Mouse liver microsomes were prepared from snap frozen livers from 3 normal c57Bl6 male mice aged 10-12 weeks by differential centrifugation as detailed in chapter 2. 11β-HSD1 enzyme activity assays were carried out as described in section 0 Microsomes were assayed in duplicate and incubated for 4 hours either with or without 20μM sodium vanadate.

4.2. Materials and methods

4.2.1. Preparation of Mouse Liver Microsomes

Mouse liver microsomes were prepared from recombinant male mice (n = 3) with global deletion of G6Pase-α, or separately, deletion of G6PT by differential centrifugation as described in detail in section 2.7. The phenotype and background strain of these mice have been previously reported (Chen et al 2003;Lei et al 1996) . All animal experiments had the approval of the Institutional Animal Care and Use Committee and were performed according to procedures approved by that committee.

4.2.2. 11β-HSD1 Enzyme Activity Assays

Microsomes (30 μg) were preincubated at 37 °C for 20 min in Mops buffer with 1 nM of the H6PDH substrate G6P. 11β-HSD enzyme reactions were started by the addition

of 200 nM 11-dehydrocorticosterone/500 nM corticosterone spiked with 20,000 cpm of tritiated 11-dehydrocorticosterone/corticosterone. All experiments were performed in triplicate. After incubation at 37 °C for 30 min, steroids were extracted with dichloromethane, separated by thin layer chromatography using a mobile phase of ethanol and chloroform (8:92), and quantified using a Bioscan 2000 image analyzer (Lablogic, Sheffield, UK) (Lavery et al 2006). The percentage of substrate metabolized in each experiment was 10% or less, ensuring that initial rates of metabolism were being measured. The method used has been described in detail in section 2.4.2.

4.2.3. Immunoblotting

SDS-PAGE was performed by the method of Laemmli (Laemmli 1970) with 10 µg of mouse liver microsomal protein on 11% acrylamide minigels using a Bio-Rad Mini-PROTEAN II apparatus (Bio-Rad). Following electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA). Nonspecific protein binding was blocked by incubating membranes in 20% non-fat milk, 0.1% Tween 20 in phosphate-buffered saline at 25 °C for 1 h. Membranes were then incubated with an in-house raised polyclonal antibody to human H6PDH at a dilution of 1:1000 for 16 h at 4 °C. Following 3 x 10-min washes in phosphate-buffered saline, 0.1% Tween 20, membranes were incubated with secondary antibody (goat anti-rabbit IgG peroxidase-conjugate) at a dilution of 1:25,000 for 1.5 h at room temperature. Bound peroxidase-conjugated IgG was visualized using ECL detection kit (Amersham Biosciences, Buckinghamshire, UK) by exposing membranes to x-ray film (Kodak, France). Following stripping, membranes were reprobed with a polyclonal antibody to

human 11β-HSD1 (1:1000) (Ricketts et al 1998b) in a similar method as above and incubated with goat anti-sheep IgG peroxidase secondary antibody. The method is described in further detail in section 2.6. The immunoblotting was carried out under supervision of Dr Gareth Lavery and Dr Elizabeth Walker.

4.2.4. Analysis of Urine from GSD1a and GSD1b Mice

Urine samples from GSD mice were kindly provided by Dr Janice Chou, National Institute of Health, Bethesda, Maryland.

Urine was collected on filter paper following bladder massage, and samples were pooled from three of each group: wild type (WT), GSD1b, and GSD1a. Three individual pooled samples were collected from WT and GSD1b, and a single pooled sample was analyzed from the GSD1a mice. The filter papers were cut up and eluted with water while vortexing and sonicating. Extracts were subjected to steroid analysis by gas chromatography/mass spectrometry as described previously (Lavery et al 2006;Shackleton 1993;Shackleton 1990) and detailed in section 0. Multiple corticosterone metabolites were found in these analyses. Major metabolites were 6β-hydroxy and 20-dihydro metabolites of corticosterone. Prominent saturated components were hydroxylated (6α - or 11β -) derivatives of "tetrahydro" (3α-, 5α-, or 3α,5β-) or "hexahydro" (additionally reduced at 20α- or 20β-) corticosterone or 11-DHC. The basic structure of each compound and elucidation as a corticosterone or 11-DHC metabolite was easily determined by mass spectral fragmentation, although stereochemistry of the 5-hydrogen and the 20-hydroxyl could not be determined in the absence of authentic compounds. Quantitation was achieved by measuring the total-ion-current response for each peak and relating this to a known amount of internal

standard, assuming an equal mass spectrometry response. For the purpose of this study, excretions of individual 11-oxo and 11β-hydroxy metabolites were separately summed, and the percentage of excretion of each group was calculated.

4.2.5. Patient Studies

Five patients with GSD1a were investigated (two males, three females; mean age ± S.D., 28 ± 1 years). All patients gave formal written consent for the study, which was approved by the local hospital ethics committee and carried out according to the recommendations of the declaration of Helsinki. All patients were diagnosed in early infancy by liver biopsy and G6Pase-α enzyme assay after presenting with severe hypoglycemia.

Urine steroid metabolite analysis was carried out on 24-h urine collections from patients with GSD1a. In addition, we were able to obtain single spot urine samples from two children with GSD1b. All samples were analyzed by gas chromatography/mass spectrometry, as reported previously (Palermo et al 1996;Shackleton 1993), measuring free and conjugated cortisol metabolites (described in further detail in section 2.9.2). Urinary steroid metabolite ratios and total 24-h cortisol metabolite production rates are presented in Table 4-1. GSD1a patients have been compared with 36 healthy controls (mean age ± S.D., 33 ± 8 years; mean body mass index ± S.D., 28.4 ± 5.6 kg/m2). Individual values are presented for the two GSD1b patients, alongside the age-adjusted reference ranges Table 4-1.

At 2300 h, GSD1a subjects were given 1 mg of dexamethasone orally to suppress endogenous cortisol production. All subjects attended the Clinical Research Facility at 0900 h the following morning, and after baseline 0900 h measurements of cortisol and

adrenocorticotropic hormone, a further 0.5 mg of dexamethasone and cortisone acetate (25 mg) were given orally. Serum cortisol and cortisone concentrations were then measured at 30-min intervals for 240 min. Further details of the method are described in section 2.9.1. Results were compared with data from 34 age and body mass index-matched controls from an existing data base of normal controls.

4.2.6. Statistical Analysis

Statistical analysis of comparisons among groups was undertaken using the one-way analysis of variance with Tukey's post hoc testing (for normal distribution) or Mann-Whitney rank sum test (for non-normal distribution). Area under the curve (AUC) analysis was performed using the trapezoidal method. All analyses were performed using the SigmaStat 3.1 software package (Systat Software, Inc. Point Richmond, CA).

4.3. Results

4.3.1. Preliminary studies

Liver microsomal preparations have been extensively used to assess the kinetics of all the enzyme systems analyzed in this study and are considered a representative model system of the ER (Banhegyi et al. 1997;Banhegyi et al 2004;van de Werve et al. 2000;Van & Gerin 2002). In hepatic microsomes from normal mice there was a significant increase in 11β-HSD1 reductase activity in the presence of the G6Pase inhibitor, sodium vanadate (without G6Pase inhibitor: 242 +/- 11, with G6Pase inhibitor 411 +/- 25 pmol/mg/h, mean +/- SEM) Figure 4-3, suggesting that increased microsomal G6P, the substrate for H6PDH does indeed drive increased 11β-HSD1

mediated generation of active glucocorticoid. These results prompted the further clinical studies in patients with GSD1a and 1b and GSD knockout mice.

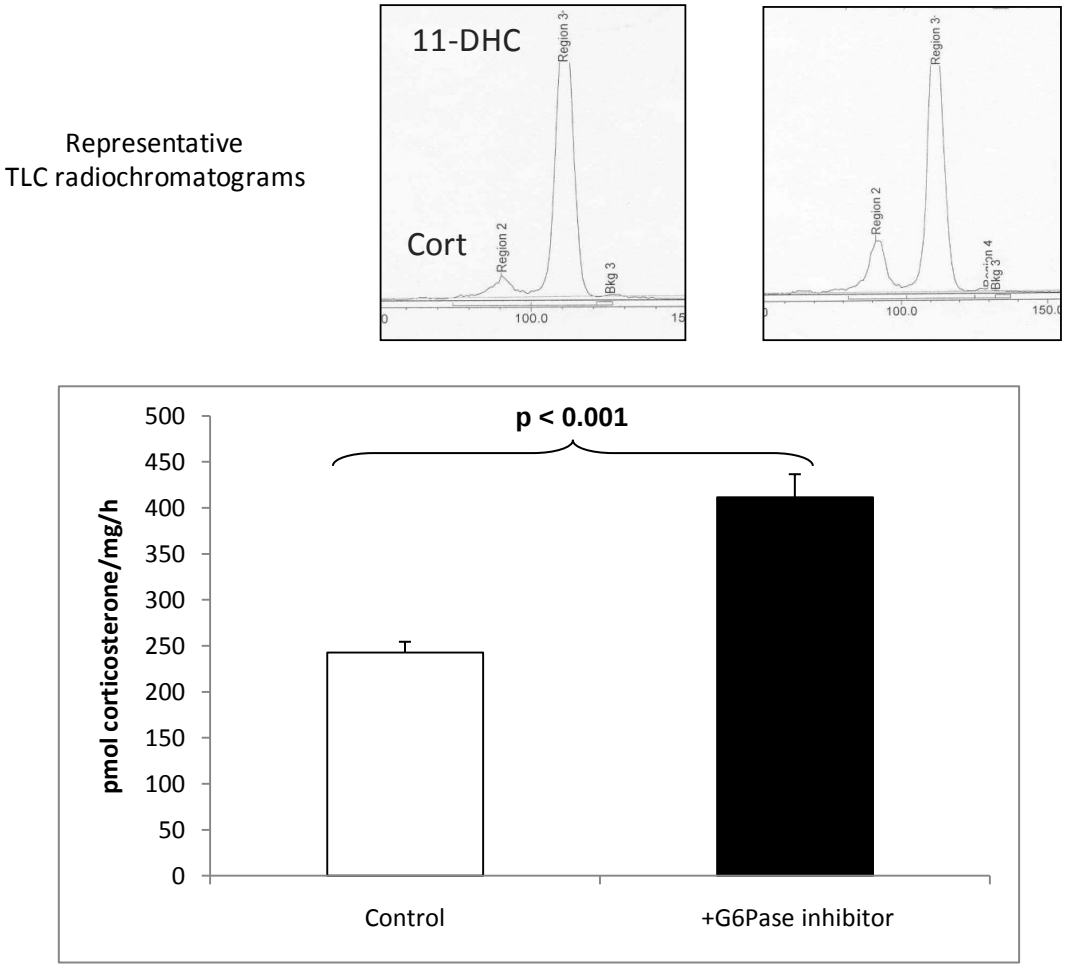


Figure 4-3 *Mean conversion of 11-DHC to corticosterone (+/- SEM) in normal mouse liver microsomes in the presence and absence of G6Pase inhibition with sodium vanadate (pmol/mg/h). The representative TLC radiochromatograms are also shown.*

4.3.2. In Vitro Analysis of 11β-HSD1 Activity and Protein Expression in GSD1b and GSD1a Mice

11β-HSD1 enzyme activity was assessed by examining the 11-reductase (11-DHC to corticosterone) and dehydrogenase (corticosterone to 11-DHC) activity in mouse liver microsomes from WT, GSD1b, and GSD1a animals.

In hepatic microsomes from the WT animals, reductase activity predominated (Figure 4-4A). In liver microsomes of GSD1b animals, lacking G6P, reductase activity was significantly reduced compared with WT animals ($p < 0.001$;Figure 4-4A). H6PDH and 11β-HSD1 immunoreactive protein was similar in WT and GSD1b animals (Figure 4-4B), indicating that changes in the levels of protein expression could not account for the differences in activity.

In the GSD1a mice, both reductase and dehydrogenase activities were reduced when compared with WT animals. This unexpected finding was explained by a reduction in expression of 11β-HSD1 protein in these animals, despite no change in the expression of H6PDH (Figure 4-4B).

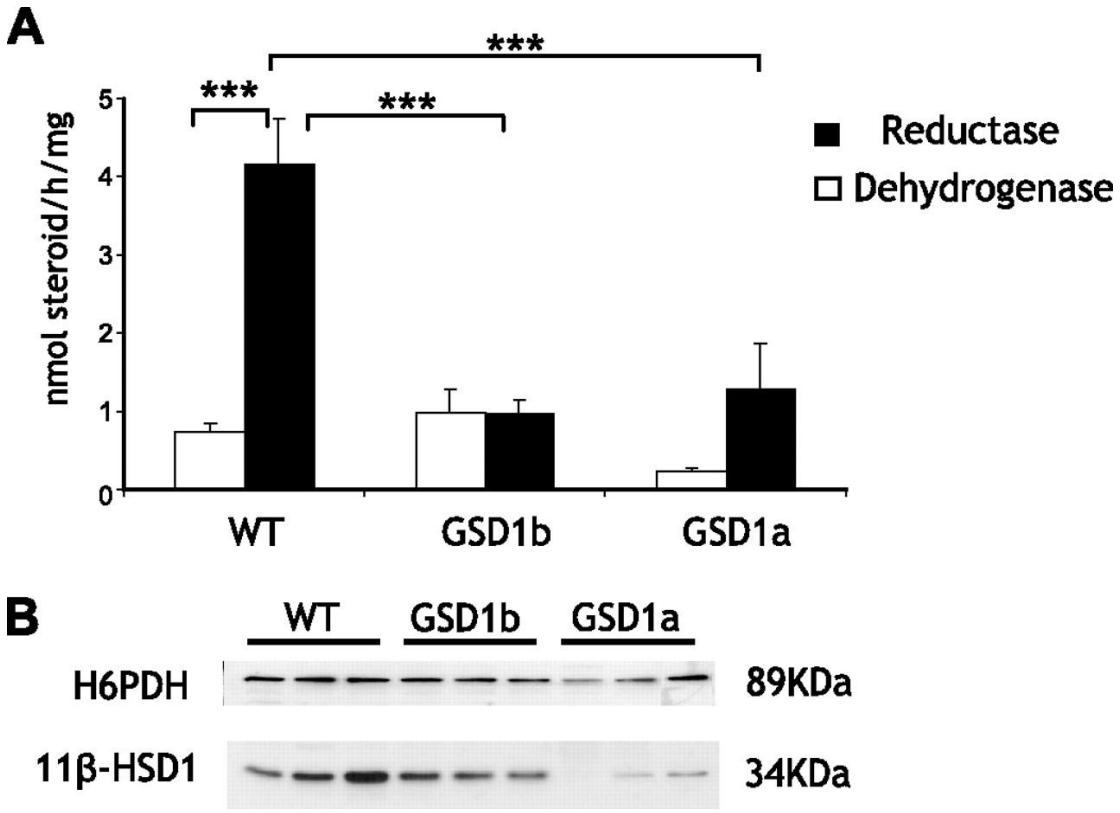


Figure 4-4 *11β-HSD1 reductase and dehydrogenase activity and protein expression from liver microsomes of WT, GSD1b, and GSD1a mice. A, in the WT microsomes, reductase activity (black bars) was significantly higher than dehydrogenase activity (white bars). Reductase activity from GSD1b microsomes was significantly lower when compared with WT and at a similar level to dehydrogenase activity. Microsomes prepared from livers of GSD1a had much lower levels of reductase and dehydrogenase activity when compared with WT, but within these animals, reductase activity still predominates. Values indicate mean activity ± S.E.; n = 3 for each group.* ****, p < 0.001. B, SDS-PAGE Western blot analysis revealed similar expression levels of H6PDH protein across all animals; however, levels of 11β-HSD1 protein were lower in the GSD1a.*

4.3.3. In Vivo Analysis of Corticosterone Metabolism in GSD1b and GSD1a Mice

In vivo assessment of 11β-HSD1 activity in mice was carried out using gas chromatography/mass spectrometry analysis of pooled urine collections (n = 3) from

each group. WT mice were found to excrete almost exclusively 11β-hydroxy metabolites (92.3 ± 3.4%; mean ± S.E.) with only minor amounts of 11-oxo metabolites (Figure 4-5A). The converse was true for the GSD1b mice, where the dominant steroids excreted in the urine were 11-oxo metabolites (92.3 ± 3.4% WT *versus* 60.2 ± 4.6%; GSD1b $p < 0.001$;Figure 4-5A). This pattern of metabolism was mirrored *in vitro*, where the ratio of reductase to dehydrogenase activity obtained from liver microsomal preparations of GSD1b mice was significantly lower than WT (5.21 ± 0.64 WT *versus* 1.13 ± 0.28 GSD1b $p < 0.01$;Figure 4-5B). Results from a single pooled urine sample from GSD1a mice indicated a similar level of 11β-hydroxy metabolites to WT (87.5%;Figure 4-5B). This was consistent with the relative ratio of reductase: dehydrogenase activity seen *in vitro*, being comparable with that of WT animals (5.21 ± 0.64 WT *versus* 5.7 ± 0.7 GSD1a;Figure 4-5B).

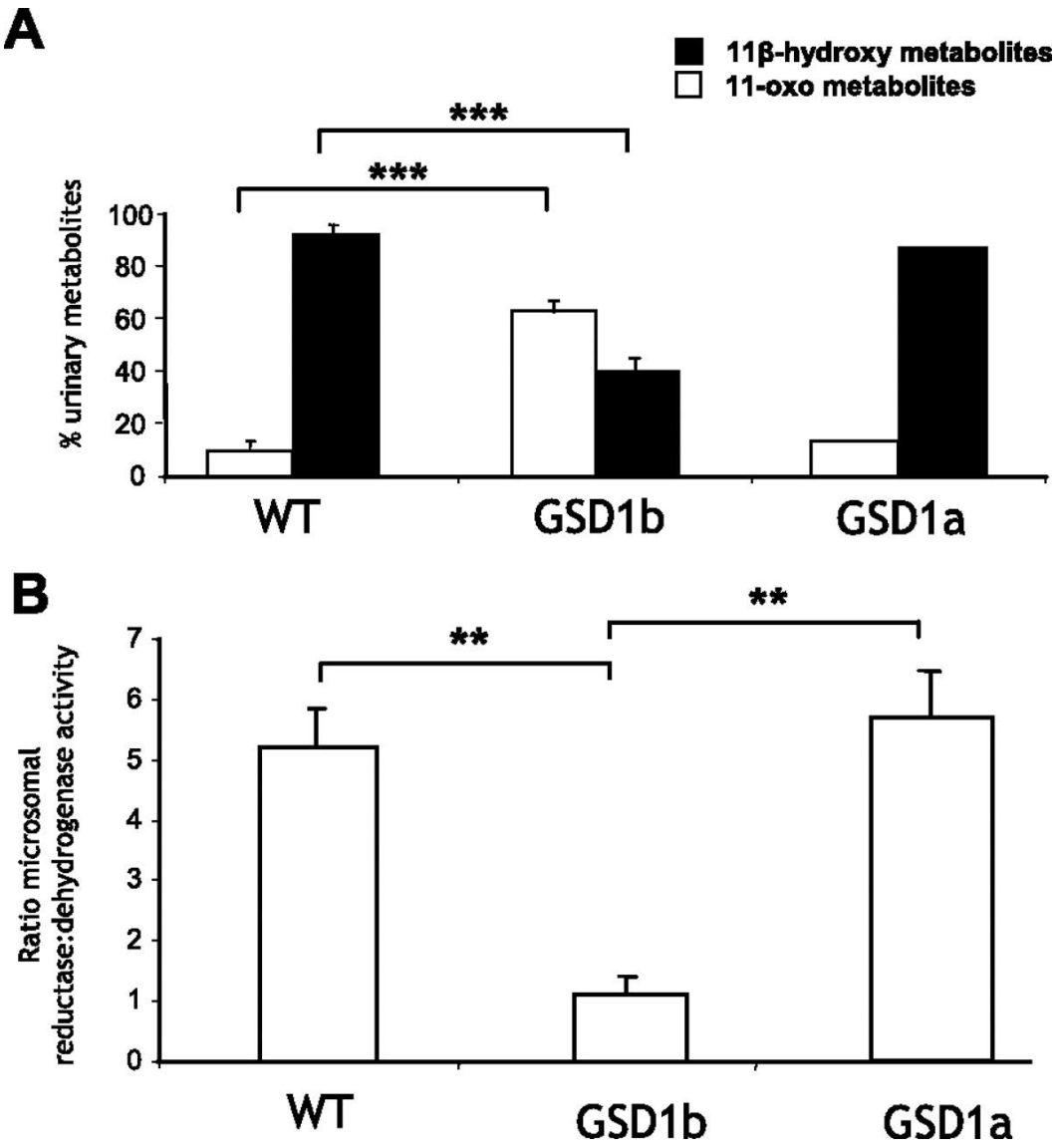


Figure 4-5 *In vivo and in vitro assessment of 11β-HSD1 activity in WT, GSD1b and GSD1a mice. A, comparison of the percentage of urinary metabolites in WT mice with GSD1b and GSD1a revealed that the percentage of 11β-hydroxy metabolites (black bars) were significantly decreased in the GSD1b group when compared with WT. Results from a single pooled sample of GSD1a urine showed a similar profile of metabolites as WT. B, the relative ratio of reductase:dehydrogenase activity from microsomal assays of WT, GSD1b, and GSD1a mice was significantly higher in both the WT and the GSD1a animals when compared with GSD1b. Values indicate mean ± S.E.; **, $p < 0.01$; ***, $p < 0.001$; $n = 3$.*

4.3.4. Analysis of Cortisol Metabolism in patients with GSD1a and GSD1b

In GSD1a patients, total cortisol production rate, as determined from the summation of the metabolite excretion in a 24-h urine collection (Tomlinson et al. 2004a), was significantly decreased Table 4-1. In addition, the THF+5αTHF/THE, the cortols/cortolones, and the 11OH-androsterone+11OH-etiocholanolone/11-oxo-etiocholanolone ratios, all of which reflect global 11β-HSD1 activity, were significantly increased, in keeping with an increased cortisone to cortisol conversion. Absolute levels of THF and THE were significantly lower than in control subjects; however, there was little change in the relative activity of either 5-α reductase or 5β-reductase as measured by the THF/5αTHF and etiocholanolone/androsterone ratios (Figure 4-6), although it is still possible that there is a reduction in both 5α-reductase and 5β-reductase activity. Small numbers of GSD1b patients precluded statistical analysis, but in contrast to patients with GSD1a, a relative decrease in 5α and/or increase in 5β-reductase activity (increased THF/5αTHF ratio) was observed in GSD1b. However, the etiocholanolone/androsterone ratio was within the normal reference range. Importantly, 11β-HSD1 activity, as measured by the THF+5α THF/THE and the cortols/cortolones ratios, was decreased (Table 4-1). These were spot urines from overnight collections, and we were unable therefore to assess 24-h cortisol metabolite secretion rates.

	GSD1a (*n* = 5)	Sex and age matched controls (*n* = 36)	GSD1b (*n* = 2)	Sex and age matched controls (*n* = 15)
Total cortisol metabolites (µg/24 h)	5191 ± 1330**	12502 ± 1106		
THF (µg/24 h)	742 ± 175*	2015 ± 189		
5α-THF (µg/24 h)	724 ± 166	1978 ± 251		
THE (µg/24 h)	856 ± 278**	4303 ± 429		
(THF+5α-THF)/THE	1.94 ± 0.2***	0.96 ± 0.04	0.28, 0.27	0.62–1.71
Cortols/cortolones	0.64 ± 0.1***	0.39 ± 0.02	0.12, 0.11	0.18–0.42
(11OH-andro+11OHetio)/11oxo-etio	13.8 ± 1.0***	3.2 ± 0.2	3.3, 1.9	0.7–6.0
THF/5α-THF	1.08 ± 0.2	1.43 ± 0.19	2.6, 4.47	0.32–1.35
Etio/andro	0.75 ± 0.14	0.94 ± 0.09	0.48, 0.59	0.37–1.08

Table 4-1 *Urinary steroid metabolite analysis: Urine was analyzed for levels of corticosteroid metabolites by GC/MS. 24-h collections were obtained from 36 healthy controls and 5 patients with GSD1a. Data are mean values ± S.E. Only spot urines were available from two children with GSD1b; thus, only individual values are presented. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ vs. control). andro, androsterone; etio, etiocholanolone.*

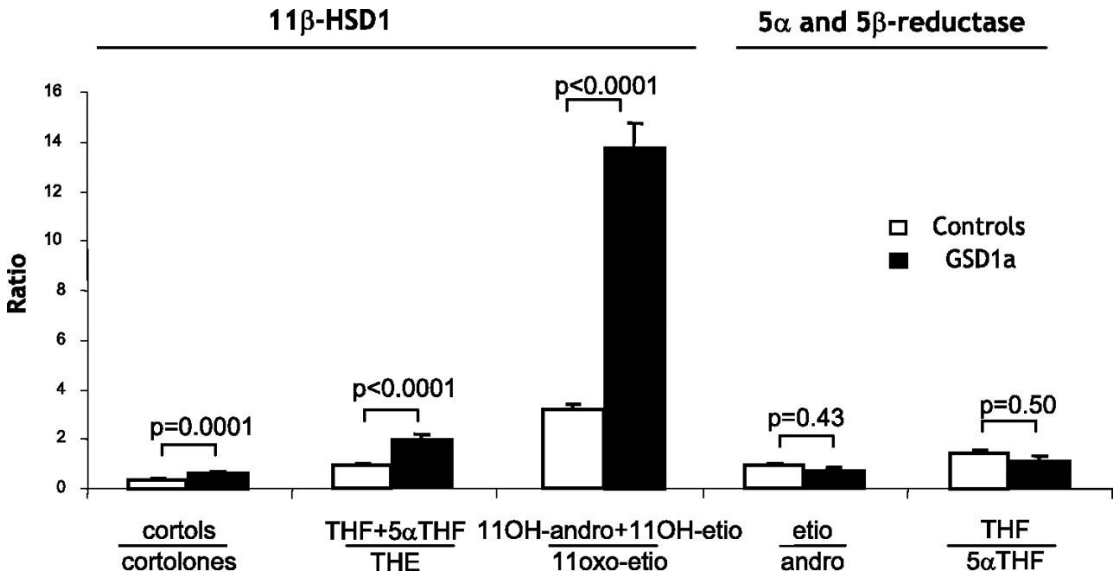


Figure 4-6 ***Urinary steroid metabolite ratios from patients with GSD1a when compared with controls.*** *Markers of 11β-HSD1 activity (cortols/cortolones, THF+5αTHF/THE, 11OH-and+11OH-etio/11-oxo-etio; mean ratio ± S.E.) were significantly increased in the GSD1a patients, whereas indicators of 5α and 5β-reductase activity were unchanged. and, androsterone; etio, etiocholanolone.*

4.3.5. In Vivo Analysis of Cortisol Metabolism in GSD1a Patients, Cortisol Regeneration from an Exogenous Cortisone Challenge

Hepatic 11β-HSD 1 activity, measured as the conversion of orally administered cortisone (25 mg) to cortisol after overnight dexamethasone suppression (Stewart et al 1999), was elevated in the GSD1a group when compared with controls (n = 34) (mean cortisol AUC ± S.E. 248 ± 3 *versus* 75 ± 4 μmol/liter.min, $p < 0.001$;Figure 4-7A). Serial cortisone measurements over the same time course were similar in both control and GSD1a patients (mean AUC 15 ± 3 *versus* 13 ± 5 μmol/liter.min, $p = 0.4$) (Figure 4-7B), suggesting no impact upon renal 11β-HSD2 activity.

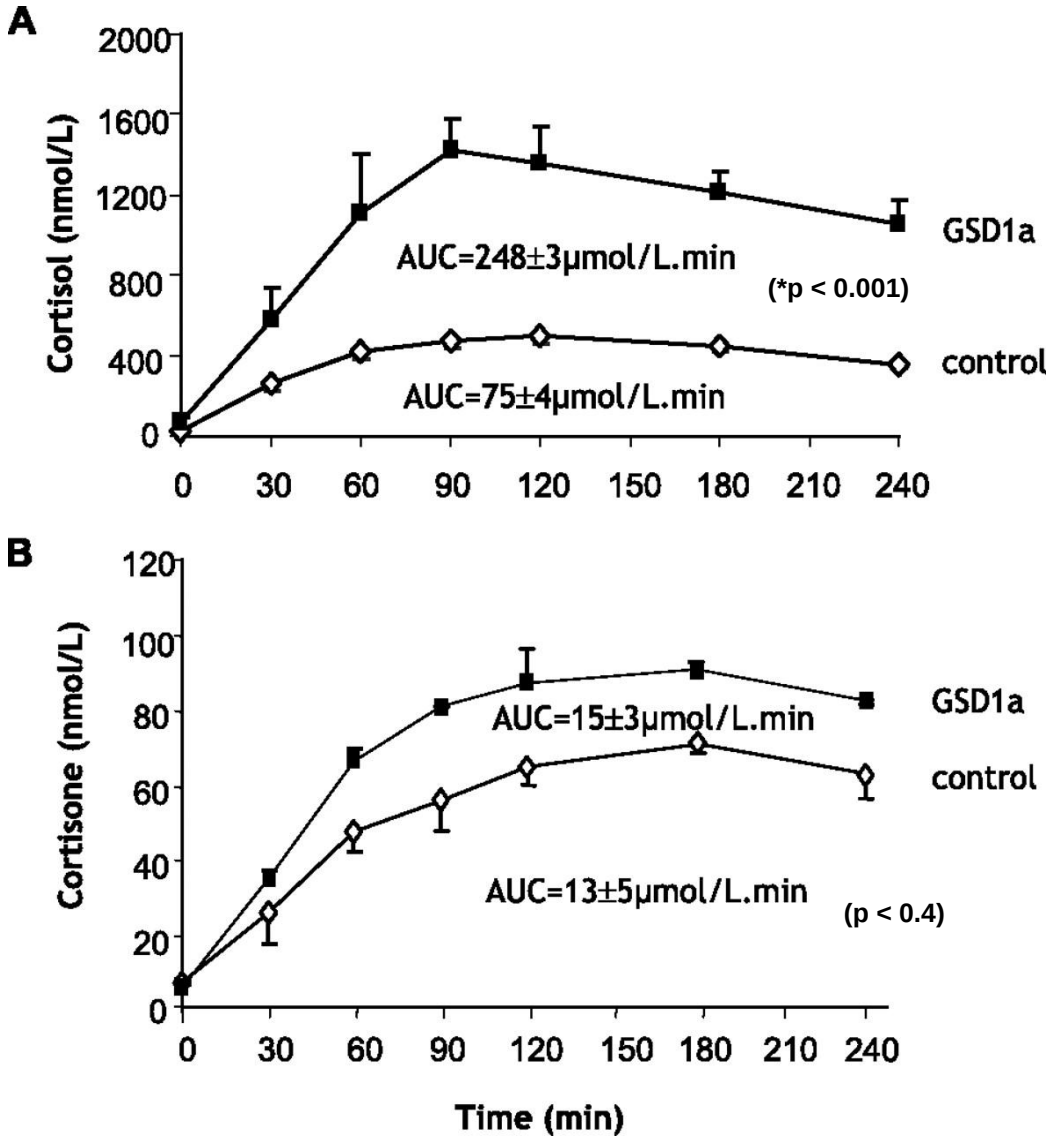


Figure 4-7 ***Serum cortisol and cortisone levels in dexamethasone-suppressed patients with GSD1a following 25 mg of oral cortisone acetate.*** *The data represent the mean ± S.E. of three individuals with GSD1a (two premenopausal females and one male) and compared with data from 34 age and body mass index-matched controls. A, the GSD1a group showed a significant increase in serum cortisol concentrations (mean cortisol 248 ± 3 versus 75 ± 4 µmol/liter.min, p < 0.001), indicating enhanced production of cortisol and therefore increased 11β-HSD1 reductase activity. B, there were no significant changes in serum cortisone concentrations between the controls and the GSD1a group (mean cortisone 15 ± 3 versus 13 ± 5 µmol/liter.min, p = 0.4).*

4.4. Discussion

This work provides compelling evidence for the critical importance of G6P concentrations in the ER lumen in determining the "set point" of 11β-HSD1 activity. It further endorses recent findings of a close functional cooperativity between 11β-HSD1 and H6PDH (Atanasov et al 2008;Banhegyi et al 2004;Bujalska et al 2005) and the existence of a separate pool of NADP/H within the ER (Czegle et al 2006;McCormick et al. 2006;Piccirella et al. 2006).

The studies on humans lacking G6Pase-α (GSD1a) show for the first time an impressive increase in 11β-HSD1 reductase activity *in vivo*, resulting in increased local generation of active glucocorticoid. In these patients, G6P availability to H6PDH is increased, resulting in enhanced NADPH generation for 11β-HSD1. Activity was inferred in humans through an increase in the urinary THF+5αTHF/THE, cortols/cortolones, and 11OH-androsterone+11OH-etiocholanolone/11-oxo-etiocholanolone ratios and increased generation of circulating cortisol following an oral dose of cortisone acetate, with unchanged markers of 5α-reductase and 5α-reductase activity. We and others have extensively utilized the cortisol generation test post-cortisone as a marker of liver 11β-HSD1 activity; a reduced generation of cortisol has been observed in patients with obesity (Stewart et al 1999) and in subjects with apparent cortisone reductase deficiency (Tomlinson et al. 2002a). However, this is the first time an increase in cortisol concentrations following cortisone has been observed. The normal ratio of urinary C11β-hydroxy:C11-oxo metabolites of glucocorticoids in humans is ~1:1, but in mice, this ratio is ~20:1, reflecting a more efficient reductase enzyme (Lavery et al 2006). As a consequence, a further increase

in the percentage of urinary 11β-hydroxy metabolites was not observed in mice lacking G6Pase-α. However, in hepatic microsomes from mice lacking G6Pase-α, absolute levels of both reductase and dehydrogenase activities were lower than WT controls. Since both reductase and dehydrogenase activities were reduced by the same extent, the *ratio* of these activities remained unchanged. This was explained by an overall reduction in expression of 11β-HSD1 protein in the GSD1a liver microsomes. The down-regulation of 11β-HSD1 in the liver of these animals may represent a negative feedback mechanism whereby an attempt is made to increase G6P hydrolysis by limiting the requirement for G6P by H6PDH, although this hypothesis requires further investigation.

Conversely, when delivery of G6P to the ER is compromised, as seen in mice and humans lacking G6PT (GSD1b), a significant impairment of 11β-HSD1 reductase activity was observed. It was not possible to undertake cortisol generation profiles following oral cortisone in two patients with GSD1b, but the urinary THF+5αTHF/THE ratio was reduced by 71% when compared with controls. Similar changes were seen in mice, where the percentage of 11-oxo metabolites fell from 92.3 to 39.8%. Our *in vitro* data indicated that this was a direct result of impaired reductase activity. These data compare favourably with those obtained from our H6PDH knockout mouse. In these animals, we also found that the set point of 11β-HSD1 activity switched from reductase to dehydrogenase with greater dehydrogenase activity evident from liver microsomal preparations and a higher percentage of 11-oxo metabolites present in the urine (Lavery et al 2006).

Glycogen storage disease type 1 is a complex liver disorder, and both GSD1a and GSD1b patients manifest the symptoms of failed G6P hydrolysis, characterized by

growth retardation, hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic academia (Chou et al 2002a). However, there are distinct phenotypic differences between GSD type 1a and 1b, which are not obviously related to G6P metabolism in gluconeogenic tissues, and local alterations in 11β-HSD1 activity may explain some of these differences. The pathogenesis of osteoporosis in GSD1a, which is not present in GSD1b, has been studied in some detail and demonstrates many similarities with glucocorticoid-induced bone loss (Cabrera-Abreu et al. 2004;Rake et al. 2003). Additionally, one unique feature of GSD1b that is not seen in GSD1a is neutropenia (Beaudet et al. 1980;Garty et al. 1996;Gitzelmann and Bosshard 1993;Visser et al. 2002). G6PT is widely distributed, but G6Pase-α is not present in neutrophils (Chou et al 2002a), suggesting that the neutropenia is in some way dependent upon loss of substrate for H6PDH. It remains to be seen whether this is secondary to a reduction in glucocorticoid generation in bone marrow precursors. An important finding that explains why neutropenia specifically occurs in GSD1b is the discovery of an additional catalytic subunit of G6Pase, G6Paseβ, described in detail in section 4.1.1.

However, perhaps the most important clinical implications of these findings relates to the putative role of 11β-HSD1 in the pathogenesis and treatment of patients with obesity-metabolic syndrome. Inhibition of 11β-HSD1 lowers glucocorticoid levels in liver and adipose, thereby reducing hepatic gluconeogenesis and hepatic glucose output and adipogenesis (Bujalska, Kumar, & Stewart 1997;Paterson et al 2004). In each case, the assumption is that 11β-HSD1 reductase activity is inhibited; our data highlight the key role of G6P delivery and H6PDH activity within the ER to direct this activity.

These studies also offer an exciting link between cellular glucose disposal, local glucocorticoid metabolism, and the function of the hypothalamo-pituitary-adrenal axis. During hyperglycemia, cytosolic G6P concentrations increase (Krebs et al. 2001). In addition, under these conditions, free fatty acids limit the availability of G6P (Krebs et al 2001). As a consequence, this would increase G6P availability to the ER lumen, providing substrate for H6PDH, which would, in turn, drive 11β-HSD1 reductase activity and increase local glucocorticoid regeneration. In support of this hypothesis, within 3 h of a mixed meal, both 11β-HSD1 reductase activity (Basu et al. 2006) and total cortisol production rates increase (Basu et al 2006;Brandenberger et al. 1982;Knoll et al. 1984). Furthermore, at a cellular level, the concentration of glucose within cell culture medium has a profound effect on the directionality of 11β-HSD1, omission of glucose leading to decreased 11β-HSD1 reductase activity (Ferguson et al. 1999). This hypothesis may also offer an explanation for observations made in other clinical studies. We have previously observed a failure to down-regulate 11β-HSD1activity (as measured by the urinary THF+5α THF/THE ratio) in obese patients with type 2 diabetes (Valsamakis et al. 2004). In these patients, hyperglycemia, resulting in an increase in cytosolic and endoluminal G6P concentrations, would drive 11β-HSD1 reductase activity and explain the relative increase in the urinary THF+5α THF/THE ratio seen. In addition, it has been reported that selective 11β-HSD1 inhibitors are more efficacious in rodent models of hyperglycemia. This could be explained by the hyperglycemia in these animals increasing reductase activity in key target tissues, thereby making them more responsive to selective 11β-HSD1 inhibition (Alberts et al. 2003). Further studies are

now required to determine the impact of short term fasting and feeding upon glucose trafficking, cortisol metabolism, and specifically, the impact upon 11β-HSD1activity. In summary, human and mouse models of GSD elicit dramatic changes in 11β-HSD1 activity with induction observed in GSD1a and loss of reductase activity in GSD1b. These studies highlight the importance of a novel metabolic pathway involving G6P metabolism via H6PDH and the regulation of redox potential within the ER, linking cellular glucose metabolism to the function of the hypothalamo-pituitary-adrenal axis.

4.5. Further work – in vitro analysis of human GSD1a whole liver

Since the publication of the work described in this chapter (Walker et al. 2007), a female patient who had participated in the clinical study with GSD1a received a liver transplant. Samples of the explanted liver were retained with consent and ethical approval.

4.5.1. Methods

It was planned to investigate 11β-HSD1 gene and protein expression and activity in human GSD1a liver tissue using immunoblotting and enzyme activity assays as described in section 4.2.3.

4.5.1.1. Preparation of human liver microsomes

Microsomes were prepared from normal human whole liver and human GSD1a liver as described in section 4.2.1, and in detail in chapter 2.

4.5.1.2. 11β-HSD1 enzyme activity assays

Microsomes (100 µg) were preincubated at 37 °C for 20 min in Mops buffer with 1 mM of the H6PDH substrate G6P. 11β-HSD enzyme reactions were started by the addition of 50 nM cortisone /50 nM cortisol spiked with 20,000 cpm of tritiated cortisone/cortisol. All experiments were performed in triplicate. After incubation at 37°C for 60 min, steroids were extracted as described in section 4.3.2. These reaction conditions were determined from experiments to optimize reaction conditions using snap frozen whole human liver described in section 3.2.6.

4.5.1.3. Immunoblotting

SDS-PAGE was performed by the method of Laemmli (Laemmli 1970) with 10 µg of human normal liver microsomal protein/ human GSD1a liver microsomal protein described in section 2.6.

4.5.1.4. Real time PCR

11β-HSD1, glucocorticoid receptor and 5α-reductase 2 mRNA expression from 5 normal human liver samples and one GSD1a human liver sample, as described in section 2.3.3.

4.5.2. Results

4.5.2.1. In Vitro Analysis of 11β-HSD1 Activity and Protein Expression in normal human liver and GSD1a human liver

Enzyme activity was assessed by examining reductase (cortisone to cortisol) and dehydrogenase (cortisol to cortisone) activities of 11β-HSD1 in human liver microsomes from normal donor liver and a GSD1a liver.

In hepatic microsomes from normal liver, reductase activity predominated (Figure 4-8A). In the GSD1a liver, both reductase and dehydrogenase activities were reduced when compared with normal, possibly explained by a reduction in expression of 11β-HSD1 protein in GSD1a liver Figure 4-8B.

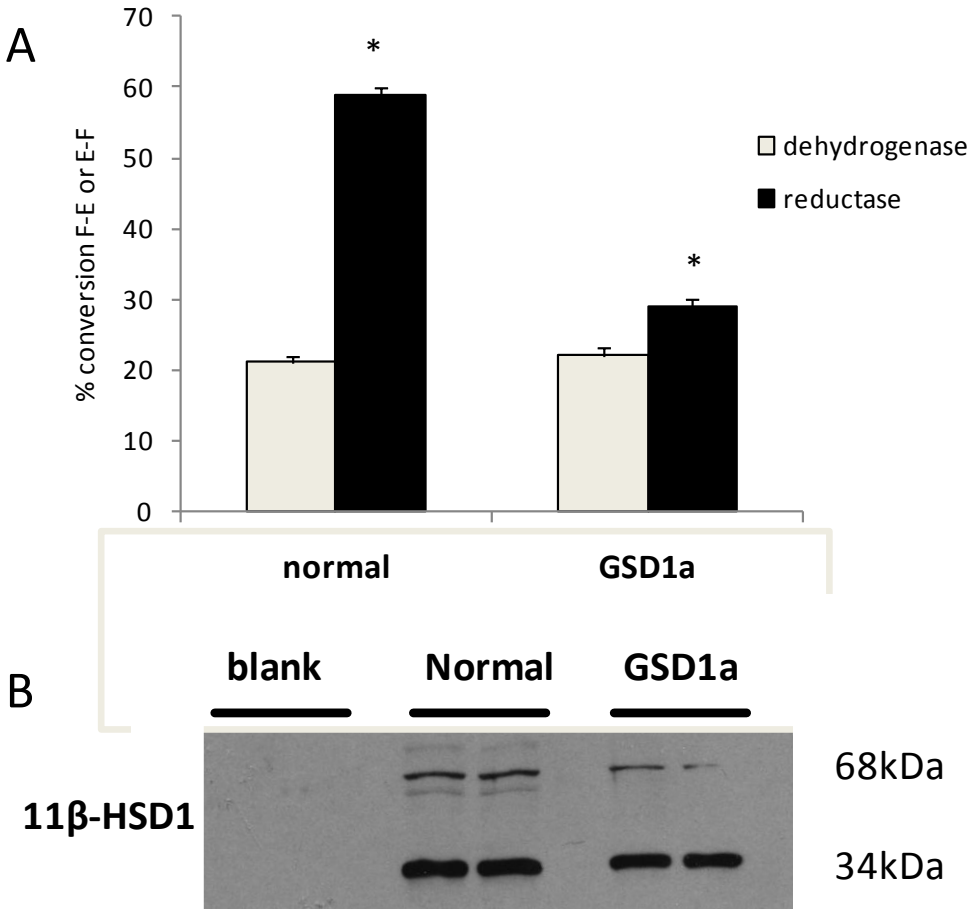


Figure 4-8 *11β-HSD1 reductase and dehydrogenase activity and protein expression from liver microsomes of normal human liver and liver of GSD1a patient. A, in the normal liver microsomes, reductase activity (black bars) was significantly higher than dehydrogenase activity (white bars). Reductase activity from GSD1a microsomes was significantly lower when compared with normal and at a similar level to dehydrogenase activity. Microsomes prepared from livers of GSD1a had much lower levels of reductase and dehydrogenase activity when compared with normal, but reductase activity still predominates. Values indicate mean activity ± S.E.; n = 3 for each group. *, $p < 0.001$. B, SDS-PAGE Western blot analysis lower levels of 11β-HSD1 protein in the GSD1a compared with normal. Bands were seen for the 34kDa protein and less intense bands for the 68kDa protien.*

4.5.2.2. Gene expression studies of steroid metabolism in GSD1a human liver

To put the results in context of changes in gene expression seen in other types of diseased liver, results have been displayed alongside those for NASH diseased liver expression data, chapter 7. Real time PCR analysis on GSD1a human liver showed a dramatic increase in HSD11B1 gene expression when compared with normals and NASH (dCt Normal 25.6+/-0.03, NAFLD 24.6+/-0.05, GSD1a 21.6+/-0.04. GSD1a vs normal $p<0.001$), Figure 4-9A. There was also a significant increase in glucocorticoid receptor gene expression in GSD1a that was significantly greater then normals and the increased expression seen in NASH livers, (dCt Normal 11.67+/-0.36, NAFLD 10.37+/-0.37, GSD1a 8.05+/-0.07. GSD1a vs normal $p<0.001$), Figure 4-8B. This was associated with a very marked decrease in 5α-reductase gene expression in GSD1a that was much greater than the decrease in expression seen in NASH livers, (dCt Normal 10.01+/-0.13, NAFLD 13.3+/-0.01, GSD1a 16.4+/-0.01. GSD1a vs normal $p<0.001$), Figure 4-9C.

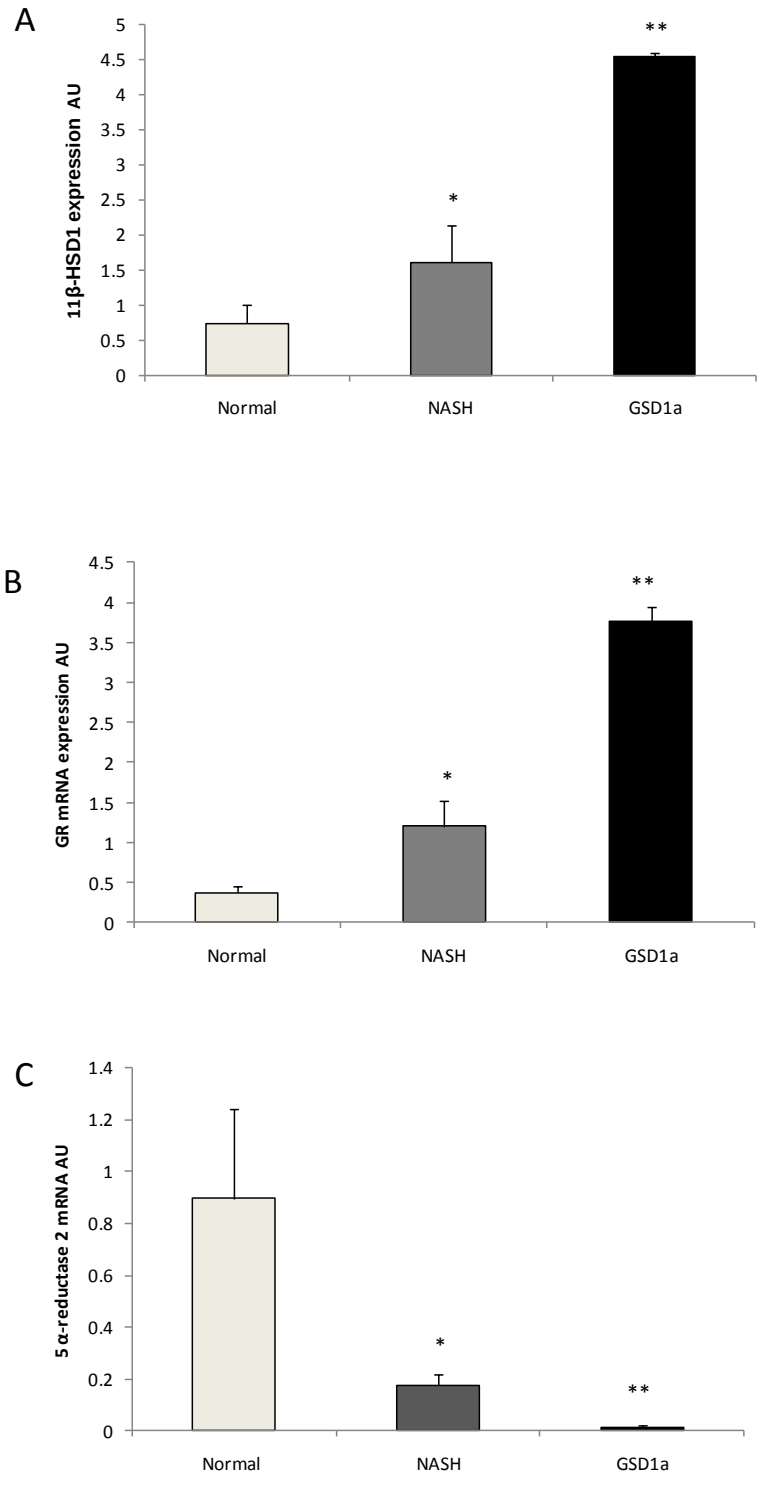


Figure 4-9 *11β-HSD1 (A), Glucorcorticoid receptor (B) and 5α-reductase (C) mRNA expression in human normal liver, NASH liver and GSD1a liver samples expressed in arbitrary units +/- SE. *p<0.01 vs normal, ** p<0.001 vs normal.*

4.5.3. Discussion

These exciting results provide a valuable insight into unravelling the glucocorticoid response in the liver of patients affected with GSD1a. The clinical data for this patient showed increased 11β-HSD1 activity when assessed after an exogenous cortisone challenge as well as on 24 hour urine steroid metabolite analysis. However, western blot analysis showed reduced 11β-HSD1 protein expression in GSD1a liver compared with normal. With the knowledge that G6Pase deficiency increases substrate supply for H6PDH which in turn drives 11β-HSD1 reductase activity, this major shift in the set point of 11β-HSD1 activity may cause negative feedback to down regulate 11β-HSD1 protein expression. However, the change in redox, combined with the increase GR expression may be sufficient to maximise local hepatic glucocorticoid generation as reflected in the clinical data.

Any down regulation of protein expression does not occur at the level of transcription since gene expression data from GSD1a diseased liver showed a marked increase in 11β-HSD1 mRNA expression. This is a curious finding but may be explained on the basis on a number of pathophysiological facts in GSD1a. GSD1a liver disease involves a serious error of metabolism that eliminates the ability of the liver to produce free glucose, hence any compensatory mechanism in an attempt to maximise glucose output will be promoted. This would include increased GR expression and 11β-HSD1 expression and activity, as increased glucocorticoid action would promote expression of gluconeogenic enzymes as well as secondary mechanisms to promote glucose output (section 1.4.6.1), as well as providing a low threshold for local glucocorticoid response during times of severe hypoglycaemia. On the other hand, at the molecular level the effects on substrate supply to H6PDH and resulting increased

glucocorticoid generation via 11β-HSD1 would promote a down regulation in 11β-HSD1 protein expression and activity. Indeed the increased hepatic glucocorticoid activity in GSD1a may contribute to the hepatic lipid accumulation seen in the condition. A third important point is that in adult GSD1a patients there is generally a progressive worsening of chronic liver disease, lipid deposition, with a propensity to develop hepatocellular carcinoma, an observation that bears close similarity to the disease spectrum of NAFLD. Increased hepatic glucocorticoid generation would be favourable to limit hepatic injury in severe GSD1a liver disease as has been argued for non alcoholic steatohepatitis in chapter 7. This observation is supported by the impressive down regulation of 5α-reductase 2 gene expression seen in GSD 1a livers. Indeed, the gene expression data for glucocorticoid action and metabolism in GSD1a follows the same trend as NASH but is much more marked. Urine data from GSD1a patients showed a trend for lower A ring reductase activity in GSD1a but this was not significant. Again this can be argued as part of a protective mechanism to maximise hepatic glucocorticoid availability to limit hepatic injury and perhaps more importantly to allow a better response during periods of hypoglycaemia.

5. Hepatic glucocorticoid metabolism in alcoholic liver disease and other non alcoholic chronic liver diseases

5.1. Introduction

Alcoholic pseudo-Cushing's syndrome is an important differential in a patient being investigated for Cushing's syndrome, and poses a significant challenge to the physician. These patients usually have phenotypic and biochemical evidence of hypercortisolism, with failure to suppress cortisol following dexamethasone, and loss of the normal circadian rhythm of cortisol secretion. The hypercortisolism resolves within a few days to weeks of stopping alcohol consumption. The aetiology of this condition has remained unknown.

It is difficult to estimate the prevalence of this syndrome for a number of reasons. Firstly, individual case reports and studies have made the diagnosis based on different clinical and biochemical criteria. Secondly, the duration of alcoholism as well as the period of abstinence have both been notoriously difficult to assess and have differing effects on the HPA axis. Acute ethanol loading in healthy non alcoholic and alcoholic subjects has been shown to have a stimulating effect on the HPA axis, increasing ACTH. Chronic alcohol abuse has also been shown to increase ACTH in some but not all patients studied, and usually in the context of alcohol withdrawal. Acute withdrawal from alcohol causes the HPA axis to be stimulated (most likely due to stress), an effect which decreases with the period of maintained abstinence. Interestingly periods as long as 2 - 4 months were required for the clinical signs and symptoms to disappear in some cases. Other factors which commonly affect alcoholics can also stimulate the HPA axis, namely malnutrition and depression. Hence, prevalence figures for alcoholic pseudo-cushing's have ranged from 6 to 40%. In addition, a number of reports have failed to demonstrate any correlation between serum and urine hormone levels, liver function tests and clinical signs - even in alcoholic patients with Cushingoid appearance, normal results have been obtained when the HPA axis was tested (Groote & Meinders 1996).

We have suggested a role for aberrant cortisol metabolism in this process. A normal circulating cortisol level is maintained by a balance between cortisol clearance and secretion. Changes in clearance are very sensitively detected at the hypothalamus-pituitary with up regulation or down regulation of CRF-ACTH secretion accordingly, Figure 5-1.

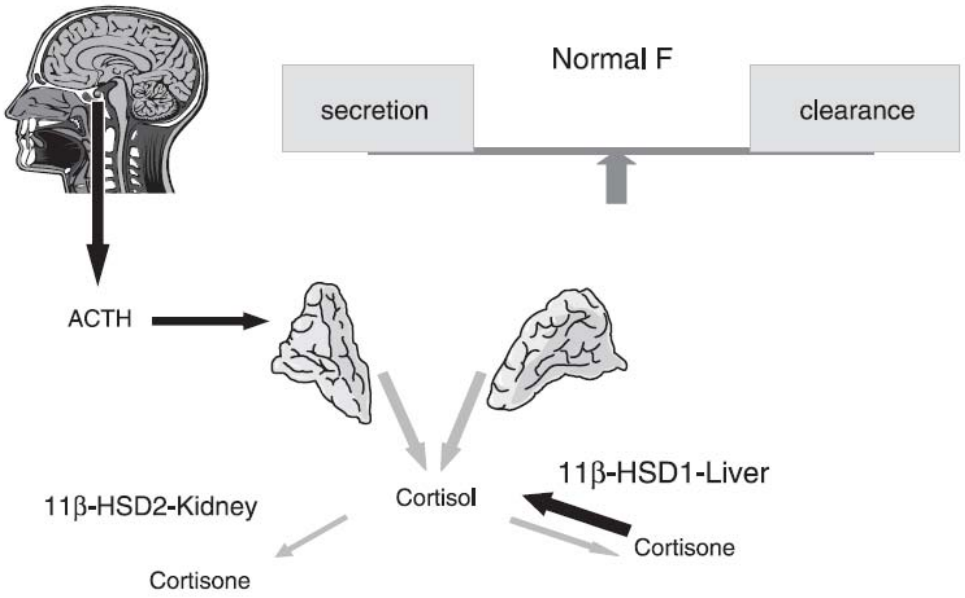


Figure 5-1 Circulating cortisol concentrations reflect a balance between cortisol production and clearance. Cortisol metabolism is predominantly regulated by 11β–HSD's, type 1 in liver converting E to F and type 2 in kidney converting F to E.

In a previous study we have shown higher urinary cortisol : cortisone metabolite ratios in patients with alcoholic liver disease (ALD) compared to patients with chronic liver disease (CLD) of other aetiologies (Stewart et al 1993a). These findings suggested either an increase in 11β-HSD1-mediated oxo-reductase activity or a reduction in 11β-HSD2-mediated dehydrogenase activity. However, impaired metabolism of cortisol in its own right would not cause hypercortisolaemia due to the negative feedback effect on the hypothalamic-pituitary-adrenal axis resulting in decreased ACTH secretion. The study provided evidence that the glucocorticoid excess in patients with alcoholic pseudocushing's reflected a "two-hit" process, i.e. continuing normal cortisol secretion in the face of aberrant cortisol metabolism. In terms of ALD, the obvious target to evaluate is hepatic 11β-HSD1. Hence, in this study the aim was to further evaluate hepatic glucocorticoid metabolism in patients

with ALD and CLD by measuring F and E concentrations through selective venous sampling of the hepatic, portal, renal and peripheral veins. These studies were extended and supported by *in vitro* studies to determine hepatic 11β-HSD1 mRNA levels in patients with ALD, CLD and normal controls (Ahmed et al 2008).

5.2. Materials and methods

5.2.1. Patients studied and Selective Venous Catheterisation

The study was performed with the approval of the local Hospital Ethics committee. Forty seven patients with chronic liver disease were electively admitted to the Liver Unit, Queen Elizabeth Hospital for transjugular liver biopsy or insertion of transjugular intrahepatic portosystemic shunt (TIPS) according to clinical indications and were included in the study after informed written consent. Patients with ALD had a history of significant alcohol consumption (>20 units per day for a minimum of 5 years). Patients with a history of alcohol consumption in the seven days prior to admission or those taking medications that could interfere with the function of the hypothalamic-pituitary-adrenal axis were excluded from the study. Eight patients were excluded from the final analysis for reasons that clearly set them apart from the rest of the group. These included end stage renal failure (n=2), transplant rejection (n=2), Budd Chiari Syndrome (n=1), reverse portal venous flow (n=1), sepsis (n=1), and incomplete sample collection (n=1). The collection of these samples was kindly carried out by Dr Sushma Saksena.

The clinical indications for TIPS were, intraperitoneal variceal bleeding, recurrent ascites, gastric or oesophageal variceal bleeding. Of the 39 patients analysed 20

patients (age 48.1±1.9 years (mean ± SEM), 13 males) had histologically confirmed alcoholic liver disease (ALD) and 19 (age 50.3±3.1 years, 13 males) had chronic liver disease of other aetiologies (CLD), including cryptogenic cirrhosis (n=5), hepatitis C cirrhosis (n=3), hepatitis B cirrhosis (n=2), primary biliary cirrhosis (n=2), chronic hepatitis (n=3), and α -1 anti trypsin deficiency (n=1).

6 otherwise healthy adult patients (3 males) undergoing adrenal sampling for presumed primary aldosteronism were also studied and served as "normal" controls. All had normal liver and renal function tests and no history of liver disease, or evidence of Cushing's syndrome. In each case, selective venous sampling from hepatic, peripheral and right renal vein was undertaken between 10.00h and 12.00h. Peripheral vein samples were used to determine routine biochemistry including liver and renal function tests. There was no significant difference in drug history between patients in the ALD and CLD groups. These samples were kindly provided by Gian Paolo Rossi, University Hospital, Padova, Italy.

5.2.2. Liver Samples used for RNA extraction and Real Time Quantification

Separately, liver samples were obtained from the Liver tissue bank at the Queen Elizabeth Hospital with ethical approval from the local hospital ethics committee. Diseased liver samples were obtained from liver transplant recipients and normal liver samples from donor liver at transplant; 7 patients with alcoholic liver disease, 9 patients with non alcoholic chronic liver disease and 6 normal livers were analysed. Full details of the samples used are described in chapter 3.2.1. All alcoholic patients were abstinent from alcohol for at least 6 months as an eligibility criterion for

transplantation. The underlying diagnosis was verified by histopathology reports. Histological diagnoses in the non alcoholic chronic liver disease group included primary biliary cirrhosis, cryptogenic cirrhosis, primary sclerosing cholangitis, and haemochromatosis.

5.2.3. Measurement of Plasma Cortisol and Cortisone Concentration

Blood was collected in tubes without additive and stored at -20ºC until analysis. Serum cortisol concentrations were measured using an in house direct radioimmunoassay using Guildhay antiserum HPS 631-1G and a cortisol-3-CMO-histamine-(125-I) tracer (Moore et al. 1985). Serum cortisone was measured following the extraction of chloroform using antiserum N-137 and 21 – acetyl – cortisone – 3 CMO- histamine- (125-I) tracer (Wood et al. 1996). Interassay coefficients of variation were less than 8% for serum cortisol values over 30 nmol/L and less than 10% for serum cortisone concentrations over 15 nmol/L. The method is described in further detail in chapter 2.

5.2.4. Real Time PCR

11β-HSD1 mRNA levels were measured by real-time PCR. The method is described in detail in section 2.3.2.

5.2.5. Statistical Analysis

All data were expressed as a comparison of mean absolute serum cortisol or cortisone values obtained from selective venous sampling in the two groups. The mean of the differences in cortisol or cortisone concentrations across the liver or kidney were also compared. Comparisons were made using the Student's t test. Non parametric data

(data for hepatic cortisol generation) were analysed using the Mann Whitney Rank Sum test. A p value of < 0.05 was considered significant.

5.3. Results

5.3.1. Electrolytes and liver function tests in patients with ALD and CLD

Patients in both groups were well matched for severity of liver disease, with similar levels of bilirubin and albumin. The higher alkaline phosphatase level in the CLD group reflected the greater proportion of patients in the CLD group with cholestatic liver disease; Table 5-1. The 6 "normal" patients had normal renal and liver function tests.

Parameter	ALD +/- SE	CLD +/- SE
Urea (mmol/L)	5.2 +/- 0.7	7.2 +/- 1.4
Creatinine (mmol/L)	102 +/- 7.3	111 +/- 28.6
Sodium (mmol/L)	136.8 +/- 1.3	135.3 +/- 1.9
Potassium (mmol/L)	4.1 +/- 0.1	4.1 +/- 0.2
Bilirubin (μmol/L)	107 +/- 29.0	86.0 +/- 20.7
Albumin (g/L)	31.8+/- 1.5	31.3 +/- 5.8
Aspartate Transaminase (U/L)	54 +/- 6.6	172^{A} +/- 50.2
Alkaline Phosphatase (U/L)	218 +/- 25.0	496^{B} +/- 72.5

Table 5-1 Electrolytes and liver function tests in patients with ALD and CLD (shown as mean with standard error). ^{A} *P = 0.01 vs. ALD,* ^{B} *P = 0.0002 vs. ALD*

5.3.2. Cortisone and Cortisol Values from Selective Venous Sampling

Two of the patients in the ALD group, and one of the patients in the CLD group had insufficient samples taken from the renal vein. As anticipated cortisone concentrations in the renal vein were higher than any other vascular bed but no

significant differences were observed between ALD, CLD or normals (ALD 78.4 ± 4.4, CLD 81.5 ± 6.3, normal 79.6 ± 14.3 nmol/L). Peripheral vein cortisone levels were also similar in all three groups indicating that there was no change in cortisone delivery to the liver from the peripheral circulation between groups. There was a trend for lower hepatic vein cortisone levels in the ALD group when compared with CLD but this was not statistically significant (ALD 32.5 ± 3.6, CLD 39.7 ± 4.0 nmol/L, p = 0.09).

Hepatic vein cortisol concentrations were higher in the ALD group (391 ± 42 nmol/L) when compared with CLD (295 ± 35nmol/L, p < 0.05), Figure 5-2**.** There was a trend for higher hepatic vein cortisol concentrations in the ALD group compared with normals, although this was not statistically significant (317 ± 32 nmol/L, p = 0.08), Figure 5-2.

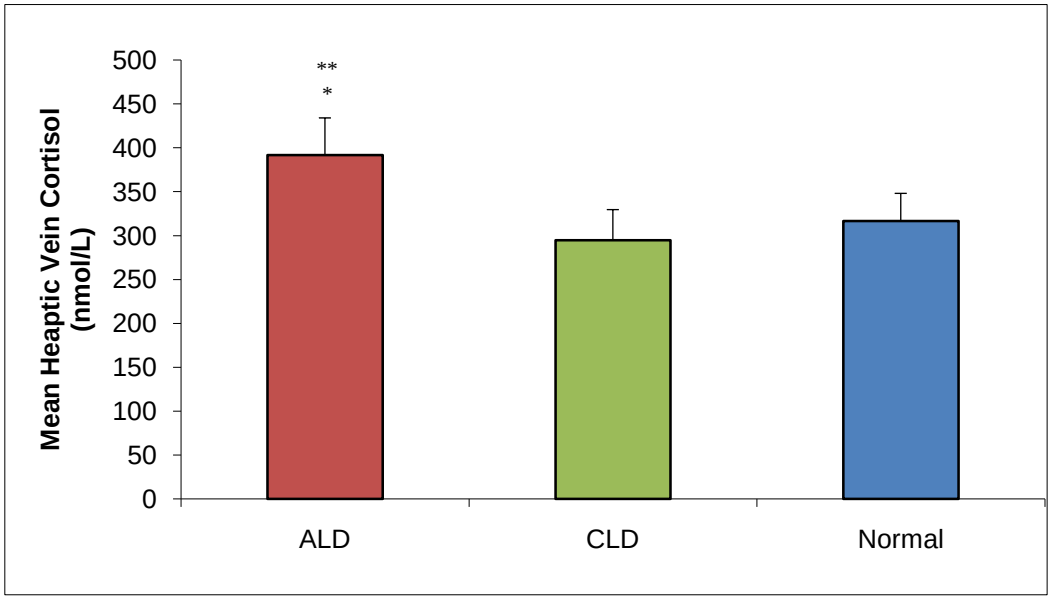


*Figure 5-2 Absolute serum cortisol from hepatic and peripheral veins expressed as mean +/- SE for each group. *ALD vs CLD p < 0.05; ** ALD vs normals p = 0.08.*

When expressed as the difference between the hepatic and peripheral vein cortisol concentration as a surrogate marker for hepatic cortisol production, significantly increased values were observed in ALD (+34.5 ± 21.7 nmol/L) compared to CLD (-21.0 ±18.5 nmol/L, p<0.05) and normals (-19.6 ± 17.2 nmol/L *p* <0.05), Figure 5-3.

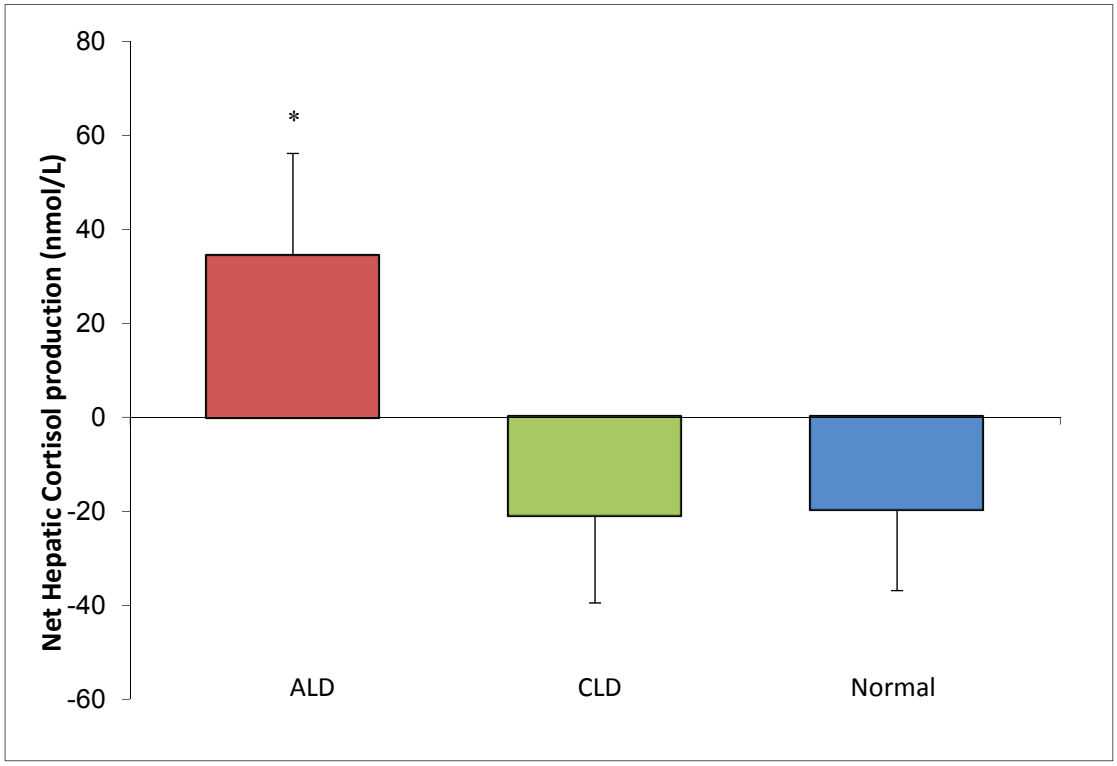


Figure 5-3 *Net hepatic cortisol gradient in ALD vs CLD expressed as Hepatic mean F generation (hepatic vein F – Peripheral vein F) +/- SE. * ALD vs CLD and normal p > 0.05.*

Similarly when the net hepatic cortisone gradient was calculated, as the difference between the hepatic and peripheral vein cortisone concentration, a negative gradient was observed in all three groups with no statistical difference between any group (ALD : -32.4 +/- 4.5, CLD : -23.84 +/- 5.7, Normal : -38.8 +/- 6.89), Figure 5-4.

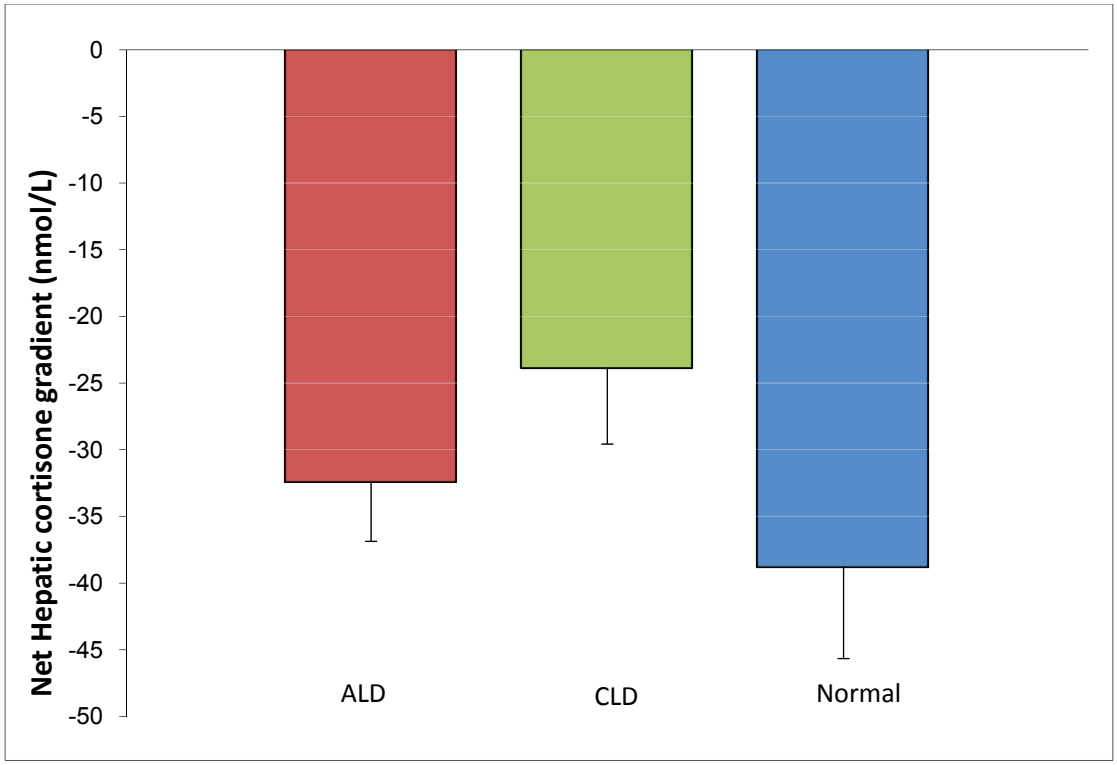


Figure 5-4 *Net hepatic cortisone gradient in ALD, CLD and normals expressed as mean hepatic E generation (hepatic vein E – peripheral vein E) +/- SE. No statistically significant difference observed between groups.*

5.3.3. Gene expression data

Real time PCR analysis of RNA extracted from liver samples showed a 5 fold increase in 11β–HSD1 mRNA expression in liver tissue from patients with alcoholic liver disease compared with normals (ALD : *d*Ct 9.84 +/- 0.27, Normals : *d*Ct 13.48 +/- 1.12, $p < 0.01$) using 18s as an internal reference. There was a lesser though significant 4 fold increase in 11β–HSD1 mRNA expression in patients with non alcoholic chronic liver disease, Figure 5-5. Compared with normal livers there was no significantly increased mRNA expression of H6PDH mRNA expression in the ALD group but not the CLD group (ALD: *d*Ct 11.37 +/- 0.4, CLD: *d*Ct 12.36 +/- 0.4, Normals : *d*Ct 12.15 +/- 0.4, ALD vs normals $p < 0.05$).

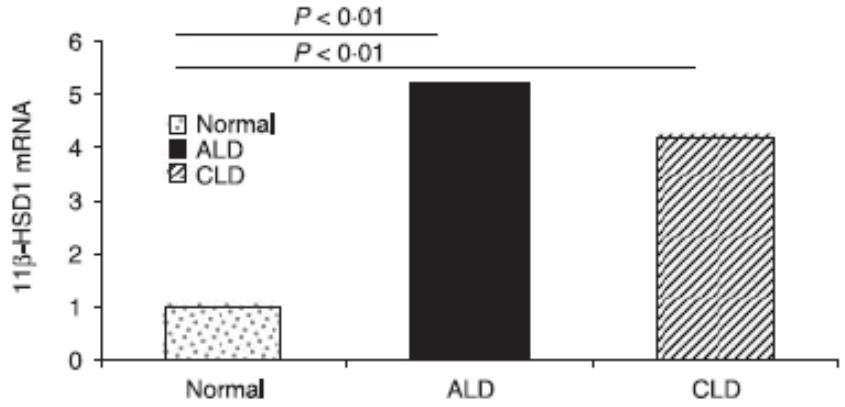


Figure 5-5 11β-HSD1 mRNA expression (fold change)in ALD and CLD vs normal. dCt values +/- SE : Normal 12.0 +/- 0.5; ALD 9.6 +/- 0.3; CLD 9.9 +/- 0.2. ALD vs normal p<0.01, CLD vs normal p<0.01)

5.4. Discussion

This study assessed hepatic 11β-HSD1 and renal 11β-HSD2 activities in patients with and without liver disease by directly measuring E and F in the veins draining these organs. Cortisol concentrations were significantly increased in the hepatic vein of patients with alcoholic chronic liver disease (ALD) compared with non alcoholic chronic liver disease (CLD) and a "normal" control group. In a separate cohort of patients, our in vitro work demonstrated increased 11β-HSD1 mRNA expression in patients with ALD.

As previously reported (Walker et al 1992a;Whitworth et al. 1989), cortisone concentrations were highest in the renal vein in keeping with the abundant expression of 11β-HSD2 in the kidney. However, there were no significant differences in renal function or renal vein cortisone concentrations between the groups, suggesting that 11β-HSD2 activity was similar in both diseased and normal groups; any changes in hepatic vein cortisol levels are unlikely to be due to altered cortisone delivery to the

liver. Although cortisol binding globulin (CBG) was not measured, serum albumin values were normal and similar in both ALD and CLD groups (Table 5-1), suggesting that hepatic synthetic function was not significantly reduced in the diseased groups. Hence, differences in CBG would be an unlikely explanation for the detected difference in cortisol between ALD and CLD groups. Collectively these data suggest that our previous findings of higher urinary cortisol : cortisone metabolites in patients with ALD (Stewart et al 1993a) reflect induction of 11β-HSD1 in the liver rather than inhibition of 11β-HSD2 in the kidney.

This increase in local hepatic generation of cortisol may be explained by a shift in the set point of 11β-HSD1 activity towards greater oxoreductase activity. However, our *in vitro* expression studies data provide compelling evidence for specific induction of hepatic 11β–HSD1 gene expression in alcoholic liver disease. H6PD has been shown to regulate the dehydrogenase / oxoreductase set point of 11β–HSD1 activity (Bujalska et al 2005;Lavery et al 2006), but no changes in the levels of expression of H6PDH mRNA were observed. We observed a negative hepatic cortisol gradient in both the CLD and normal groups (Figure 5-3) and a negative hepatic cortisone gradient in all three groups (Figure 5-4). This may reflect the hepatic metabolism of cortisol and cortisone by enzymes other then 11β–HSD1, such as the A-ring reductases (Morita et al. 2004). While this may be contributing to the present results, the positive hepatic cortisol gradient specifically in the ALD group supported by our in vitro data reflects predominant 11β–HSD1 induction in the ALD group.

We did however also note a significant induction of 11β–HSD1 gene expression in patients with CLD, though to a lesser extent than that observed in the ALD group, yet

no apparent increase in hepatic cortisol production was observed in CLD. It is important to stress that these liver samples were obtained from an alternative patient population; unfortunately ethical constraints prevented liver biopsies being performed in our ALD, CLD and "normal" cohort undergoing catheterisation. There were clear histological differences between the ALD group and the CLD group in the transplant livers studied, but as a whole both groups consisted of patients with end stage liver disease (hence the reason for transplantation), unlike our patients recruited to the clinical study. If 11β–HSD1 gene expression is induced with increasing liver injury, the difference in gene expression between ALD and CLD groups (as clearly seen in the selective venous sampling data and our previous urine results) may become less marked as liver disease progresses and this may account for the lesser difference between the ALD and CLD groups in tissue 11β–HSD1 gene expression when compared to the clinical steroid data. Other possibilities might include a direct effect of alcohol itself. Alcohol has been shown in some studies to directly inhibit 11β-HSD2 (Valentino et al. 1995), but it is unknown whether alcohol directly induces 11β-HSD1 expression. Both isoenzymes of 11β-HSD evolutionally belong to the short chain alcohol dehydrogenase superfamily, so regulation of expression by alcohol would be a reasonable possibility (Krozowski 1994). Alcohol does however, dramatically alter the redox potential within hepatocytes (Jauhonen et al. 1982) and this in itself may influence the expression and enzymatic set point of 11β-HSD1. As a requirement for transplantation, patients in the ALD liver transplant group had been abstinent from alcohol for at least six months, unlike those in the catheterised ALD group who had been abstinent for at least 7 days. It may therefore be possible that the direct effects of alcohol were partially reduced in both groups. Unfortunately we do

not have urinary steroid metabolite data to compare excretion during alcohol intake and at the time of catheterisation.

A number of studies have also shown dysregulation of the HPA axis in patients with CLD. In particular, chronic hepatic damage due to cholestasis in rats results in decreased hypothalamic CRF (corticotrophin releasing hormone) mRNA and protein expression of corticotrophin – releasing hormone (Swain et al 1993). Furthermore, when cholestatic animals are exposed to psychological stress, there is defective activation of the HPA axis with a significant attenuation of circulating glucocorticoid levels (Swain et al 1993). As a group, our CLD patients had significantly increased markers of cholestasis compared with the ALD group. It may be that the increased 11β-HSD1 expression and activity in these patients is offset by the decrease in glucocorticoid release via the HPA axis, unlike the ALD patients where there is ongoing stimulation of the HPA axis (Groote & Meinders 1996).

The induction of 11β-HSD1 in the face of liver injury and inflammation is a novel finding that may well represent a protective effect, with the local generation of cortisol dampening the inflammatory response and hence limiting tissue damage. The hepatoprotective effect of endogenously released glucocorticoids have been previously reported and appear to be caused in part by glucocorticoid-mediated synthesis and release of IL-10 in the liver (Swain et al. 1999). IL-10 is produced by the Kupffer cells in the liver, and has been seen to have a hepatoprotective effect in a number of animal models of acute inflammation. These cells also produce a number of proinflammatory cytokines including IL-6, TNF-α, leukotrienes, PGE_2 and nitric oxide. The production of these cytokines are directly inhibited by glucocorticoids at the level of mRNA stability and gene transcription, as well as through inhibition of

the production of proinflammatory transcription factors such as (NF)-κB and (AP)-1 (Cooper et al. 2001). Previous work on primary cultures of human osteoblasts and human osteosarcoma cell line showed that IL-1β and TNF-α potently induced the expression of 11β-HSD1 (cortisone-cortisol conversion) (Cooper et al 2001). Also of interest in this context, is the enzyme alcohol dehydrogenase (ADH). ADH is an important enzyme in alcohol metabolism having a high affinity for alcohol (low Km 0.2-2 mM) and accounts for essentially all alcohol metabolism when blood and tissue concentrations are low. It is an important first step in alcohol metabolism, in limiting the hepatic injury directly caused by alcohol and appears to be positively regulated by glucocorticoids (Dong et al. 1988). Further studies are required to explore the relationship between hepatic injury, 11β-HSD1 induction and the anti-inflammatory effects of glucocorticoids locally within the liver. Once it is determined whether the harmful effects of 11β-HSD1 induction, both locally in the liver and systemically outweigh any possible protective effect in the liver, the role of selective 11β-HSD1 inhibition may be considered in the treatment of Alcoholic pseudocushings syndrome.
In summary, we have previously suggested that alcoholic pseudo-Cushings might be mediated by a "two-hit" process, firstly impaired cortisol clearance and secondly and specific to alcoholic versus non alcoholic patients, ongoing activation of the HPA axis (Lee et al. 2001;Lee and Rivier 1997;Stewart et al. 1993b) (Figure 5-1). Here we confirm that the impaired cortisol to cortisone metabolism is mediated via the type 1 isozyme of 11β-HSD1 – induction in expression resulting in increased hepatic cortisol production.

In the context of additional studies carried out on non alcoholic liver disease (NAFLD) as part of this thesis in chapter 7, these results are of interest. Alcoholic and non NAFLD are often difficult to distinguish histologically with emphasis placed on history of alcohol intake. It would be plausible to suggest that hepatic pre-receptor glucocorticoid metabolism by 11β-HSD1 is a key player in the pathogenesis of both conditions. While increased cortisol availability may primarily be a protective mechanism to limit hepatic inflammation and injury, it occurs at the expense of hepatic lipid accumulation, and further dysregulation of glucose homeostasis and in turn peripheral insulin resistance in both ALD and NAFLD. It would clearly be of interest to investigate whether hepatic 11β-HSD1 is induced in other types of hepatitis above the induction seen in non inflammatory chronic liver diseases described in this chapter.

11β-HSD1 has emerged as a novel therapeutic target to treat patients with the diabetes mellitus-obesity-metabolic syndrome spectrum, the hypothesis being that inhibition of the enzyme will reduce glucocorticoid concentration locally in liver and fat thereby inhibiting hepatic glucose output and adiposity respectively. As and when inhibitors come on line, it will be of great interest to extend these studies further to hepatic inflammation and specifically as a possible treatment for patients with alcoholic pseudocushing's syndrome.

6. Clinical Study: Expression and activity of 11β-HSD1 and hepatic glucose sensitivity in normal physiology, obesity, type 2 diabetes and non alcoholic fatty liver disease

6.1. Introduction and broad aims

Obesity, in particular central obesity has been closely associated with the development of type 2 diabetes mellitus. The important role played by cortisol in the pathogenesis of obesity and the metabolic complications of insulin resistance has been reviewed (sections 1.4.6.1 and 1.5.3.2.). To summarise, there have been a number of animal and human studies – in obese animals, 11β-HSD1 expression is altered in a tissue specific pattern, with increased expression in adipose tissue and decreased expression in liver (Alberts et al. 2005;Liu et al. 2003;Livingstone et al.

2000;Masuzaki et al 2001). These findings are not however consistent in all animal models of obesity (Morton et al. 2005). In humans, studies which have relied on measurements of urine ratios of cortisol and cortisone metabolites, have yielded inconsistent results (Fraser et al. 1999;Rask et al. 2001;Rask et al 2002;Stewart et al 1999). This may be due to these results being affected by the activity of other enzymes, including 5α and 5β- reductase. It has also been shown using stable isotope studies that the whole body rate of cortisol regeneration is not altered in obesity (Sandeep et al. 2005). Hence altered metabolism in one tissue may be cancelled out by the differences observed in another. Tissue specific studies on cortisol metabolism in humans have detected differences in 11β-HSD1 in obesity. Measurement of hepatic first pass metabolism of cortisone to cortisol in dexamethasone suppressed individuals has consistently shown a decrease in 11β-HSD1 in obesity (Fraser et al 1999;Rask et al 2001;Stewart et al 1999). However, primary cultures from subcutaneous tissue biopsies have consistently showed increased 11β-HSD1 activity and mRNA expression (Kannisto et al. 2004;Lindsay et al. 2003;Paulmyer-Lacroix et al. 2002;Rask et al 2001;Rask et al 2002;Wake et al. 2003;Westerbacka et al 2003). Regulation of 11β-HSD1 in visceral adipose tissue is less clear. In one study where paired fresh omental and subcutaneous fat biopsies were studied, no relationship between 11β-HSD1 and obesity was found (Tomlinson et al 2002b). Stable isotope studies led to the discovery that rates of splanchnic cortisol production in healthy non diabetic individuals exceeded that produced by non splanchnic tissues (e.g. the adrenals), strongly implicating splanchnic cortisone to cortisol conversion via the 11β-HSD1 pathway. However, as there was also a concomitant uptake of cortisol in the splanchnic bed, only a small amount is released by the liver into the systemic

circulation. This observation, that *in vivo* splanchnic cortisol generation is not increased in obesity is a significant one. (Basu et al. 2005). It would suggest that increased cortisol generation in visceral adipose tissue may offset the known decrease in hepatic cortisol generation in obesity. Alternatively, even if there is not a significant increase in visceral adipose cortisol generation, the increase in total visceral adipose volume may be sufficient to increase cortisol delivery to the liver through the portal vein. However, a recent stable isotope study using selective venous sampling showed significant cortisol release from subcutaneous adipose tissue, but cortisol release from visceral tissues in to the portal vein was not detected (Stimson et al 2009), section 1.5.3.2. Despite this, the local production of cortisol in visceral adipose tissue may be significant at an autocrine level, by impacting upon lipolysis portal FFA delivery and hence influencing hepatic metabolic phenotype. Further discussion of the role of 11β-HSD1 in regulating intrahepatic fat in patients with non alcoholic fatty liver disease are described in detail in chapter 7.

An important question is whether increased adipose tissue cortisol generation has an influence on the metabolic abnormalities of obesity and to what extent. While subcutaneous adipose 11β-HSD1 does predict insulin resistance (Lindsay et al 2003;Wake et al 2003), it is difficult to elucidate how much of this is an effect of 11β-HSD1 driven cortisol generation rather than an effect of obesity alone. Studies have assessed endogenous cortisol production and clearance, abdominal fat depots, insulin sensitivity, and adipocyte gene expression before and after weight loss. The data generated supported a model in which increased HPA axis activity in men promotes selective visceral fat accumulation and insulin resistance, with 11β-HSD1 gene expression in subcutaneous fat being a consequence rather than the cause of adiposity

(Purnell et al. 2009). Human studies using selective 11β-HSD1 inhibitors will be a useful test in further establishing the contribution of adipose 11β-HSD1 to the metabolic derangements of obesity. In-vitro studies using selective 11β-HSD1 inhibitors in a transformed human subcutaneous preadipocyte cell line as well as primary cultures of human preadipocytes, documented an increase in 11β-HSD1 activity and expression across adipocyte differentiation. Adipogenesis was blocked in the presence of selective 11β-HSD1 inhibition, implying that 11β-HSD1 is essential for the induction of human adipogenesis (Bujalska et al. 2008a). *In vivo* studies in mice, high-fat diet-induced obesity was accompanied by increased visceral fat preadipocyte differentiation in wild-type but not 11β-HSD1 (-/-) mice. In addition, despite there being comparable levels of mRNA expression in preadipocytes from visceral fat and subcutaneous fat, glucocorticoid reactivation was higher in visceral preadipocytes (De Sousa Peixoto et al. 2008). This would suggest that 11β-HSD1 reductase activity is augmented in mouse visceral preadipocytes where it would promote preadipocyte differentiation contributing to the accumulation of visceral fat in obesity.

The putative role of pre receptor glucocorticoid metabolism in type 2 diabetes is less well established. Detailed discussion of the effects of glucocorticoids on glucose metabolism can be found in section 1.4.6.1 and the background work on the role of 11β-HSD1 in insulin sensitivity (particularly in animal models) in section 1.5.3.2. Studies on human Type 2 diabetics have had varied results but suggest a defect in the down regulation of 11β-HSD1 activity in type 2 diabetes (as seen in obesity), which may potentiate dyslipidaemia, insulin resistance and obesity. No difference in 11β-HSD1 activity measured by urine corticosteroid metabolite ratios was seen in one

study (Kerstens et al 2000) and possibly impaired hepatic cortisol generation using the cortisone to cortisol generation curve analysis when compared with BMI matched controls in another (Andrews et al. 2002). This study also found increased sensitivity to glucocorticoids in type 2 diabetics with lower 9am cortisol levels after dexamethasone suppression, hence it was concluded that although cortisol secretion is normal in type 2 diabetic subjects, it was inappropriately high given the enhanced central and peripheral sensitivity to glucocorticoids. In support of this suggestion, a failure to down-regulate 11β-HSD1 activity compared with BMI matched obese controls (as measured by the urinary THF+5α THF/THE ratio) has been observed in obese patients with type 2 diabetes (Valsamakis et al 2004). In addition isotope studies have shown that splanchnic cortisol production rates did not differ between lean, obese and type 2 diabetic subjects, although splanchnic cortisol uptake was increased in both obese and Type 2 diabetic subjects compared with normals (Basu et al 2005).

Results from studies using 11β-HSD1 inhibition have yielded important results. Carbenoxolone, a non specific 11β-HSD1 inhibitor was found to enhance insulin sensitivity measured during a euglycaemic hyperinsulinaemic clamp in healthy volunteers (Walker et al 1995) Figure 6-1. In type 2 diabetic patients this effect was specific to the liver where glucagon stimulated glucose release was inhibited (Andrews, Rooyackers, & Walker 2003), Figure 6-1. However no beneficial effect was seen in obese non diabetic patients and it was suggested that this may be due to hepatic 11β-HSD1 being already down regulated in obesity (Sandeep et al 2005). This correlated with findings in obese zucker rats where carbenoxolone was ineffective (Livingstone and Walker 2003).

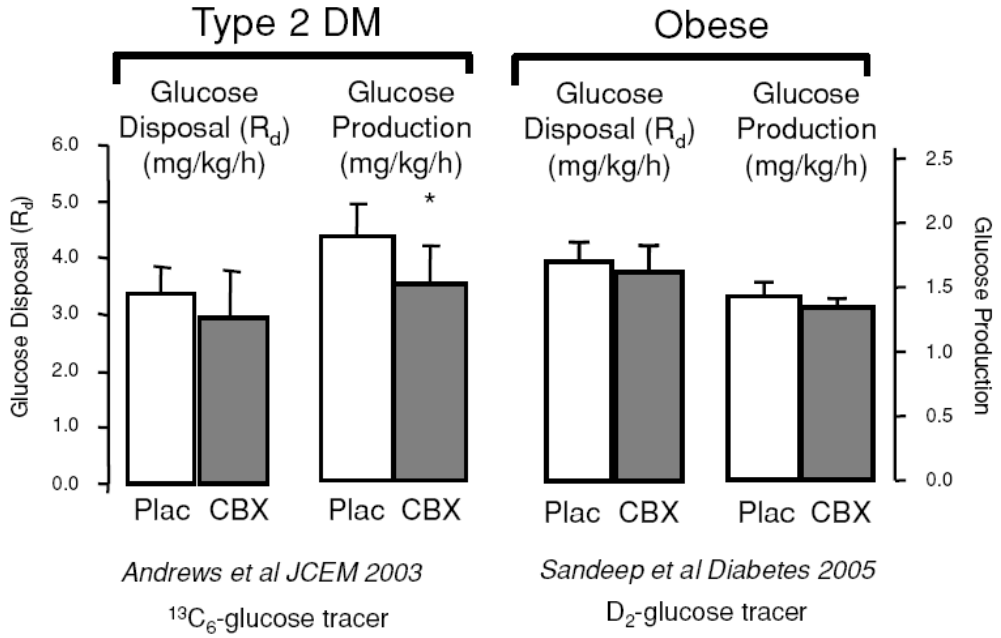


Figure 6-1 *Effects of the 11β-hydroxysteroid dehydrogenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes and obesity. Data are mean ± SEM for glucose disposal and production during a hyperinsulinaemic euglycaemic clamp measured by glucose tracer infusion in six lean patients with type 2 diabetes mellitus and six obese euglycaemic patients treated for 7 days with placebo (Plac) or carbenoxolone (CBX) in randomized double-blind crossover studies (Andrews, Rooyackers, & Walker 2003;Sandeep et al 2005). Carbenoxolone did not enhance peripheral glucose disposal in either group but did decrease hepatic glucose production in lean diabetic patients, consistent with enhanced hepatic insulin sensitivity. This effect in the liver is lost in obese subjects, in whom 11 β-HSD1 is known to be downregulated (Walker and Andrew 2006).*

Recent data have also been presented using selective 11β-HSD1 inhibitors. Using the selective 11β-HSD1 inhibitor INCB13739 in over 300 obese Type 2 diabetic subjects, a significant reduction in haemoglobin A1C (HbA1C), fasting plasma glucose, measures of insulin sensitivity and total cholesterol levels was observed, (section 1.5.2.1.) (ROSENSTOCK J 2009b). These data support numerous animal studies investigating the antidiabetic effects of 11β-HSD1 inhibition, diabetes, dyslipidaemia and atherosclerosis all improved.

6.1.1. Aim

On this background a clinical study was carried out to determine the role of 11β-HSD1 on hepatic gluconeogenesis and total glucose output and correlating measures of 11β-HSD1 activity with body composition, visceral adipose tissue, hepatic gluconeogenesis, and other markers of metabolic dysregulation. Patients with obesity (without diabetes), type 2 diabetes mellitus and non alcoholic fatty liver disease (NAFLD) were compared to normal controls.

The results of these studies form an excellent research platform for the further development of 11β-HSD1 inhibitors.

6.2. Study Group Criteria, Study design and clinical protocols

6.2.1. Inclusion Criteria

Group 1: 20 normal weight healthy volunteers (10 males, 10 females)

Group 2: 20 patients with simple obesity (10 male, 10 females)

Group 3: 20 patients (10 male, 10 females) matched for BMI with group 2 with type 2 diabetes treated with diet or low dose metformin.

AIM of 1,2 and 3: to correlate measures of 11β-HSD1 activity with body composition, body fat distribution and hepatic gluconeogenesis.

Group 4: In collaboration with the regional liver unit, 20 patients admitted for investigation of non alcoholic fatty liver disease (NAFLD).

AIM of group 4: to directly define expression of hepatic 11β-HSD1 and H6PDH in liver biopsy material (compared to normal liver) and correlate with clinical study results from this group. (Results and discussion from this group are described in chapter 7).

No other significant past medical history except as specified in patient groups above

Age 20 – 60 years.

6.2.2. Exclusion Criteria

Poorly controlled hypertension

Cerebrovascular disease

Ischaemic heart disease

Epilepsy

Diabetes (unless specified in patient group)

Liver disease (unless specified within patient group)

Asthma or chronic respiratory disease

Severe Renal impairment

Glucocorticoid therapy in any form within the last 12 months

HRT or hormonal contraception in any form

6.2.3. Sample size calculation

Sample size calculations were performed using the SigmaStat computer package and powered to examine changes in cortisol metabolites (THF, 5α-THF, THE, UFF, UFE) (t-test, power 0.9, alpha 0.05). Using preliminary and published data to anticipate the difference (and its standard deviation) between groups, (obese, lean and obese type 2

diabetics), calculations predicted that we would need 16 patients in each group. To allow for unforeseen drop-outs from the study we planned to recruit 20 patients to each group wherever possible. In reality, the dropout rate was highest in the obese group reflecting the greater total number of patients in that group.

6.2.4. Patient recruitment and ethical approval

Patients were recruited to the study through local sources. These included advertisements in the University and Queen Elizabeth Hospital, Birmingham online newsletters. NAFLD patients were recruited from dedicated outpatient clinics. Letters were sent to all General Practitioners in Birmingham and type 2 diabetics were recruited mainly from them.

Ethical approval for the all aspects of the study including *in vitro* work was obtained from the Solihull local research ethics committee, reference number: *05/Q2706/27*.

6.2.5. Patient information sheet

All patients received an information sheet, (Appendix 1), and gave written informed consent prior to their participation in the study. On completion of the study they received detailed lifestyle advice and all the clinically relevant results from their participation.

6.2.6. Clinical protocol and plan of investigation

Each patient attended the Clinical Research Unit for two separate visits, a brief overview is provided below further details of each test are described in section 6.3.

6.2.6.1. Visit 1

Tests performed: Anthropomorphic measurements, baseline BP, Pulse and serum samples for renal, liver, cholesterol and FBC profile, oral glucose tolerance test (OGTT), deuterated water test.

Day 1 0830 volunteers arrived fasted

Day 1 0845 Patient oriented to ward, general observations (anthropomorphic measurements, baseline BP and pulse)

Day 1 0900 Oral glucose tolerance test and collection of baseline serum samples at time 0.

After OGTT volunteers were allowed to eat and drink normally.

Whole body DEXA scan and CT scan on this day.

Day 1 1800 Volunteers given standardised meal and followed by fast (except water).

Day 1 2300 Baseline venous blood samples and start of Deuterated water test

Day 2 1600 End of investigation on visit 1.

24-hour urine collection for urine steroid metabolite analysis provided to patient to return at next visit.

6.2.6.2. Visit 2 (cortisol generation profile)

Previous night: 1mg dexamethasone taken orally at 2300 previous night (at home) followed by fast.

Day 1 0830 25mg oral cortisone acetate administered

Day 1 0900 serial serum cortisol and cortisone samples for 4 hours

Day 1 1300 Test Complete.

Nursing protocols included in Appendix 2

6.3. Methods

6.3.1. Anthropomorphic measurements and blood pressure

Weight in kilograms was measured to the nearest 0.1kg on a beam balance in subjects without shoes and only light clothing. Height in meters was measured to the nearest 1mm using a stadiometer, and the BMI in kg per square meter was calculated. Waist circumference in centimetres was taken in duplicate with a 6mm wide flexible tape and was measured as the maximum abdominal circumference between the costal margin and the iliac crest. Hip circumference was measured as the distance around the largest area of the hips, usually the widest part of the buttocks. Waist and hip measurements were taken with the subject standing. Sagittal height with the subject supine on a firm examination bed and was measured in centimetres as the maximal distance between the top of the examination table and a spirit level placed horizontally. Supine blood pressure was recorded with an automatic sphygmomanometer using the mean of three measurements.

6.3.2. Whole body composition using dual energy x-ray absorptiometry (DEXA)

Body composition analysis was performed using dual-energy X-ray absorptiometry (DEXA) with a total body scanner (QDR 45OO; Hologic, Bedford, MA). Coefficients of variation for multiple scans were <3%. Total and percentage fat and lean mass were analysed and lean body mass values were used to calculate total body water. The formula of Pace and Rathbun was used to estimate the total body water from lean body mass, where water was found to constitute 72.4% of the fat-free (lean) body mass (Pace and Rathburn 1945).

6.3.3. Computed tomography (CT) scanning to assess intraabdominal fat distribution

6.3.3.1. Scan technique and measurement of liver attenuation

Patients removed any tight abdominal clothing and were scanned in the supine position using a GE Lightspeed scanner (General Electric, Milwaukee, USA). A single slice through the right lobe of the liver of 5mm thickness was obtained. Scout views were used to identify the appropriate slice.

The slice was viewed on liver window settings. A $100mm^2$ region of interest (ROI) was placed over five areas of this section, and the Hounsfield unit of each ROI were recorded. Care was taken not to include any obvious vessels in the chosen ROIs. The mean liver attenuation was calculated from the five liver ROIs. The attenuation of the spleen was measured in the same way using three ROIs per scan (reflecting the smaller area of the spleen on the scan). The CT attenuation characteristics were

recorded as: 1) the mean liver attenuation (liver attenuation <50 HU seen in hepatic steatosis, (Roldan-Valadez et al. 2008)) , 2) the liver-to-spleen (L/S ratio) and 3) as categorical data, being either fatty (L/S ratio of <1) or not fatty (with an L/S ratio of greater the or equal to 1) (Bydder et al. 1981;Piekarski et al. 1980).

6.3.3.2. Abdominal fat distribution analysis

A single 5mm thick slice at the L3 vertebral level was used for the analysis. Care was taken to include the entire skin surface in the field of view. Patients who did not have the entire skin surface included in the field of view were excluded from the fat distribution analysis. At the time of the analysis GE software to carry out the desired function for the analysis had been discontinued, so an alternative commercial software was used that was currently being used for similar purposes for other studies in the radiology department (MeVis PULMO 3D 3.11, MeVIS Research GmbH, Bremen, Germany). A three dimensional analysis was carried out on the scan from which the area was calculated by dividing the volume results by the scan thickness. Total fat area and visceral fat area ROIs were delineated by manually tracing a contour of each region. Fat pixels and therefore fat area were identified with threshold attenuation values between -50 to -250 hounsfield units as described previously (Borkan et al. 1982). The subcutaneous fat area was calculated by subtracting the visceral from total fat area. Data was expressed as 1) total, subcutaneous and visceral fat area, 2) the ratio of visceral to total fat (% visceral fat), 3) the ratio of subcutaneous to total fat (% subcutaneous fat) and 4) the ratio of visceral to subcutaneous fat (V:S ratio).

Figure 6-2 *Screenshot of CT scan slice at level of L3 with region of interest drawn around the entire slice to measure total fat including visceral and subcutaneous (blue line around perimeter of slice). The software automatically selects out areas on the scan that are outside the range of threshold values of -50 to -250.*

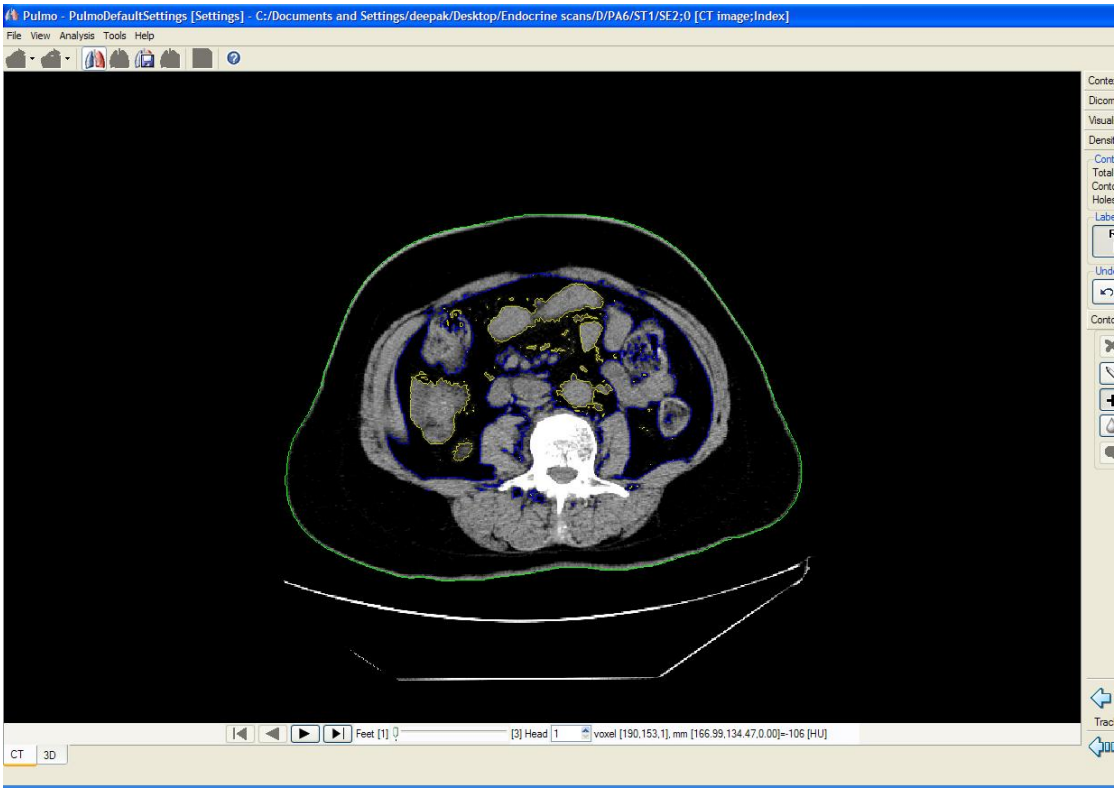


Figure 6-3 *Screenshot of same slice as seen in Figure 6-2 with only visceral fat areas selected automatically by the software after a manual ROI was drawn aound the areas to include visceral fat only (blue markings). Subcutaneous fat area was calculated by subtracting visceral fat area from the total fat area.*

6.3.4. Biochemistry

Patients were studied after an overnight fast and had blood drawn at 0900am on the first day of visit 1 for serum F and E, plasma ACTH, fasting glucose, fasting insulin lipids, free fatty acids (FFA) renal and liver function tests including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (γGT). Glycosylated haemoglobin (HbA1C) was measured in diabetic subjects. Glucose, HbA1C, lipid renal and liver profile and full blood count were measured using standard laboratory methods.

Serum F was assayed using a coat-a-count radioimmunoassay (Diagnostic Products, Los Angeles, CA) as per the manufacturer's guidelines. Interassay coefficient of variation was 3-6% (detailed in full in section 2.9.4).

Insulin was measured using a commercially available colorimetric ELISA (Mercodia, Uppsala, Sweden), section 2.9.3. Plasma FFA were analysed as described in section 2.9.5.

Insulin sensitivity was derived from fasting glucose and insulin data, using the homeostasis model assessment (HOMA) mathematical model (Matthews et al. 1985).

Serum ACTH values had not yet been analysed at the time of writing this thesis.

6.3.5. Clinical assessment of 11β-HSD1 activity

All subjects collected a 24 hour urine collection for urinary steroid metabolite profiles using GC/MS spectrometry as previously reported and described in further detail in section 2.9.2. (Palermo et al 1996;Shackleton 1993). The THF+5α-THF/THE, the cortols/cortolones, and the 11OH-androsterone+11OH-etiocholanolone/11-oxoetiocholanolone ratios represent acknowledged markers of global 11β-HSD1 activity, with a high ratio indicating increased 11β-HSD1 reductase activity. Ratios are presented as well as for total F metabolites and urine F/E ratio (representing renal 11β-HSD2 activity (Palermo et al 1996). The 5αTHF/THF was used as a marker of 5α-reductase activity with a high ratio in the setting of increased absolute levels of urinary 5α-THF indicating increased activity. The urine androsterone/etiocholanolone (An/Et) ratio was also used as a marker of 5α-reductase activity.

Measurement of hepatic first pass metabolism of cortisone to cortisol in dexamethasone suppressed individuals was made by determining the cortisol generation profile (CGC) (described in further detail in section 2.9.1).

6.3.6. Oral glucose tolerance test

Patients underwent a standard 75-g oral glucose tolerance test, with samples taken at 30-min intervals for 120 min for measurement of insulin and glucose.

6.3.7. Deuterated water test

6.3.7.1. Principle

In order to fully define total hepatic glucose production and gluconeogenesis the following quantification methods were used based on published methodologies, (Chandramouli et al. 1997;Landau et al. 1996).

The method depends on the incorporation of hydrogen-2 from $^{2}H_2O$ into glucose. When di-deuterated water ($^{2}H_2O$) is given to a fasting subject, once steady state is achieved, the ratio of enrichment of deuterium bound to C-5 of blood glucose to enrichment in body water is equal to the fraction that gluconeogenesis contributes to glucose production (Landau et al 1996). This happens because the conversion of each molecule of pyruvate to glucose involves the addition of one molecule of hydrogen from body water to C2 of the intermediate phosphoenolpyruvate. This carbon becomes C5 of glucose. The conversion of glycerol to glucose also involves one molecule of hydrogen from body water to be added to C2 of glyceraldehyde-3-phosphate during isomerisation with dihydroxyacetone-3-phosphate. That carbon also becomes C5 of glucose. Therefore, the enrichment in C5 of glucose represents

glucose production from pyruvate and glycerol *i.e*. from all gluconeogenic precursors (see section 1.3.2.2: glycolytic flux and gluconeogenesis), Figure 6-4.

A hydrogen atom from body water bound to the C-2 reflects glucose production via both gluconeogenesis and glycogenolysis. This is because a single molecule of hydrogen is added to C2 of glucose-6-phosphate when fructose-6-phosphate (F6P) is converted to glucose-6-phosphate (G6P) during gluconeogenesis. G6P is also produced as an intermediate during glycogenolysis and equilibrates with F6P, resulting with the H bound to the C2 of G6P being exchanged with that in body water. Enrichment at C5 of glucose does not occur in glycogenolysis; hence the ratio of enrichment at C-5 to that at C-2 provides a measure to the fractional contribution of gluconeogenesis to endogenous glucose production.

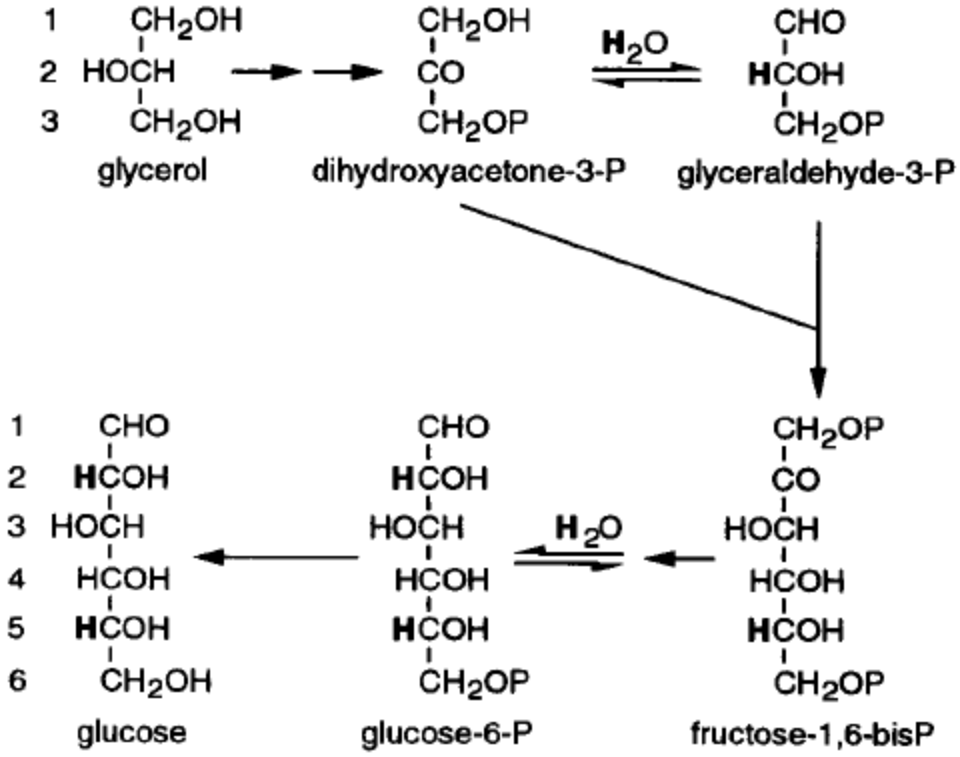


Figure 6-4 Glycerol conversion to glucose and the site of labelling at carbon 2 and 5 of the glucose by deuterium (bold hydrogen) from deuterated water (Landau et al 1996).

To allow the experiments to be performed with a safe and well tolerated dose of $^{2}H_2O$, hydrogen enrichment at C2 and C5 were determined by isolating them in formaldehyde and assaying the formaldehyde mass spectrometry using hexamethylenetetramine (HMT) (Landau et al. 1995;Landau et al 1996). HMT is an adduct of four molecules of ammonia with six molecules of formaldehyde, hence the hydrogen enrichment of glucose is magnified six fold for the assay, Figure 6-5.

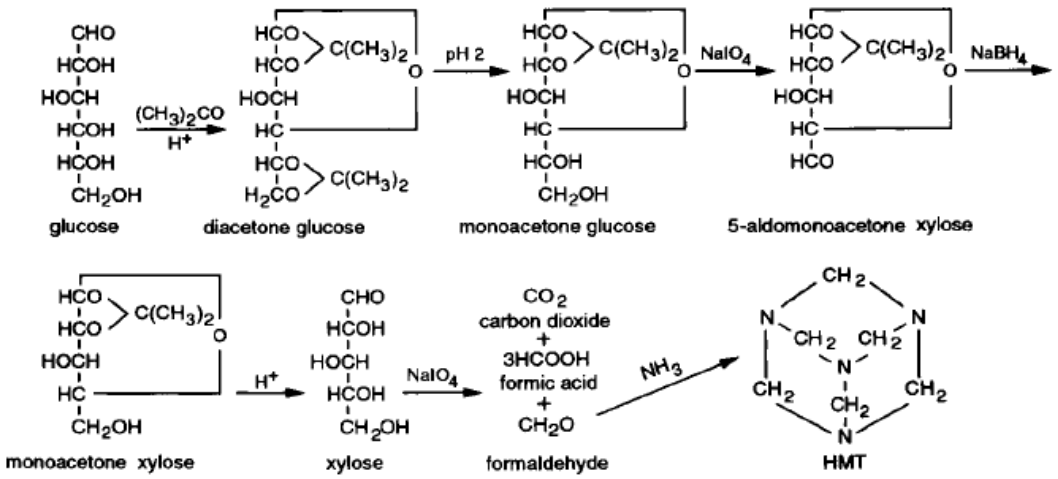


Figure 6-5 Conversion of glucose to xylose and the formation of HMT from formaldehyde from xylose (Landau et al 1996).

The purpose of this part of the study was to determine the fractional contribution of gluconeogenesis to endogenous glucose production in the different patient groups and to relate these to markers of cortisol metabolism, in particular via 11β-HSD1, and known markers of metabolic dysregulation in the metabolic syndrome. This would provide a critical insight into global and hepatic cortisol metabolism with relation to hepatic glucose output in the fasting state.

6.3.7.2. Method

A primed constant intravenous glucose tracer was given to measure total endogenous glucose production, primarily reflecting hepatic glucose production in the overnight fasted state. On the day of the study all subjects were admitted to CRF and a standard meal was ingested at 18.00h – each subject received the same meal (a vegetarian lasagne with salad and bread roll). The meal was followed by a 22 h fast with ad libitum access to drinking water. At 23.00h two mixed venous baseline bloods were

taken. The subjects were then given 2.5g/kg body water of ^2H_2O to drink. Total body water was calculated from lean body mass as detailed in section 6.2. Water ingested ad libitum after 23.00h was enriched 0.05% with ^2H_2O to maintain isotopic steady state in the body water pool. At 06.00h a catheter was placed retrogradely in a hand vein and the hand kept in a hot box at 56°C to arterialize the blood samples. After placement of an antecubital intravenous catheter in the contralateral arm for infusion, a primed (9 µmol per kg) continuous (0.15 µmol per kg per min) infusion of 6,6$^2H_2$glucose (Cambridge Isotopes Laboratories, Andover, MA) was started and continued for 10 hours (06:00h to 16.00h) for determination of rates of glucose production. After 120 min of infusion, blood samples are taken every 60 min for determination of 6,6$^2H_2$ glucose enrichment under basal conditions. Bloods were also taken to be analysed for determination of ^2H enrichment of the H-atoms bound to C2 and C5 of blood glucose and for determination of ^2H enrichment in body water, and free fatty acids at each time point. The tracer infusion and study were stopped after the last blood sample has been obtained at 16.00h. This technique involved collaboration with Professor Anton Wagenmakers (School of Sport and Exercise Sciences, University of Birmingham). A timeline of the sampling protocol for this test is illustrated in Figure 6-6.

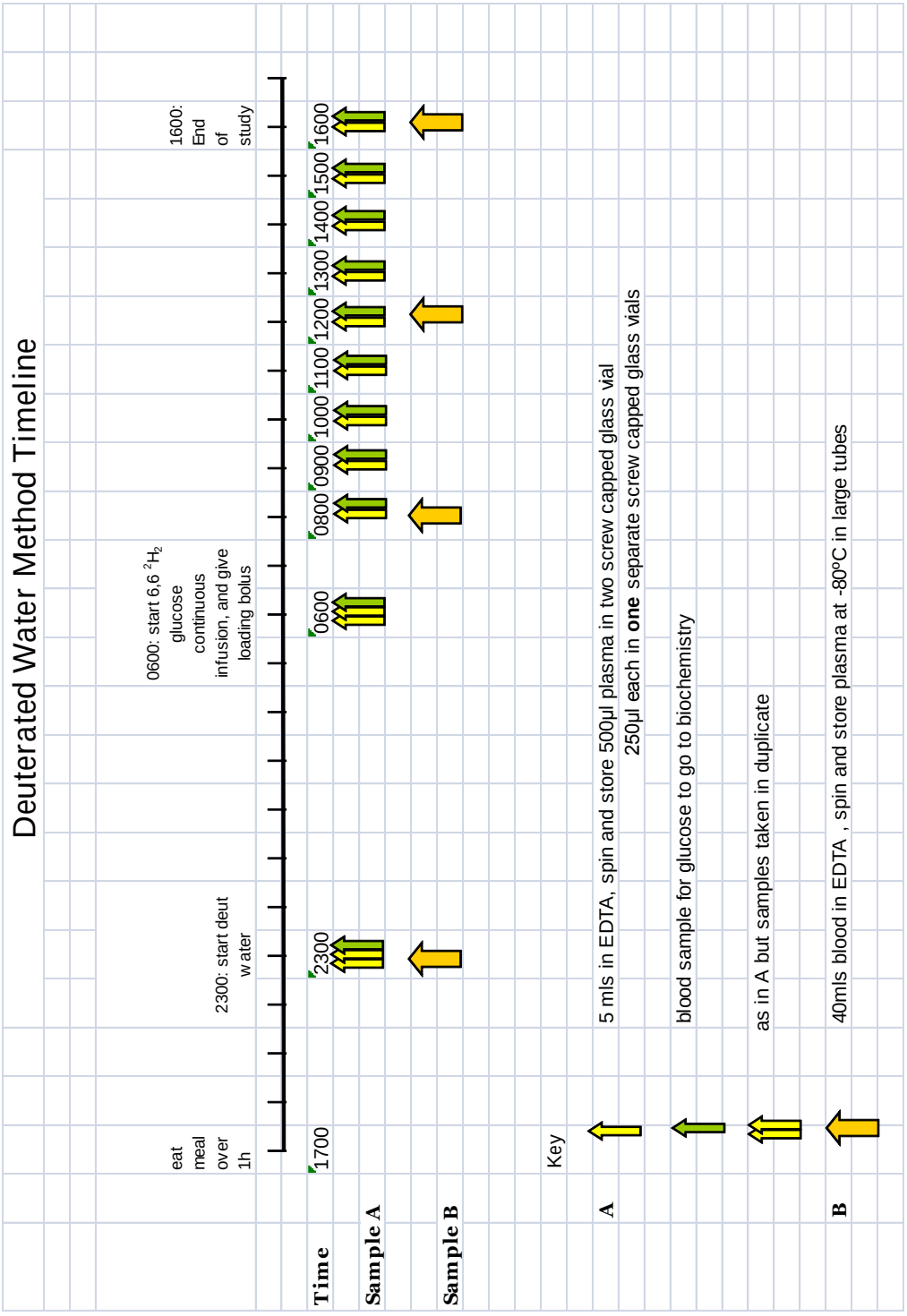


Figure 6-6 Timeline of sample collection for deuterated water test

6.3.8. Statistical analysis

Results are expressed as mean +/- SE in the case of normally distributed data or median (interquartile range) in the case of non-Gaussian distribution. Statistical analyses comparing groups of subjects were undertaken using the Student's t test and Mann-Whitney U test in the case of non Gaussian distribution of the variables. To test the association between different variables, Spearman correlation analyses were performed. A *P*-value of < 0.05 was considered to be significant. The SPSS 17.0 statistical software was used for statistical analysis (SPSS, Inc., Chicago, IL).

6.4. Results

6.4.1. Study population descriptive statistics

Parameters from the complete groups of normal weight healthy volunteers, obese, and type 2 diabetic cohorts are shown in Table 6-1. The obese cohort was closely BMI matched with the diabetic group.

	Normal (1)		Obese (2)		Diabetic (3)		*P* value		
	mean	SEM	mean	SEM	mean	SEM	2 vs 1	3 vs 1	3 vs 2
N	21		36		22				
BMI (kg/m^2)	21.9	0.7	32.1	0.9	33.5	1.5	**<0.001**	**<0.001**	0.4
Waist:Hip ratio	0.88	0.02	0.91	0.01	0.96	0.01	0.2	**0.002**	**0.01**
pulse (bpm)	66	2	70	2	72	3	0.2	0.1	0.7
Systolic blood pressure (mmHg)	118	3	133	4	145	3	**0.009**	**<0.001**	**0.04**
Diastolic blood pressure (mmHg)	73	2	76	2	84	2	0.5	**0.002**	**0.04**
Total % body Fat (from DEXA)	24	1.7	37	1.3	34	1.6	**<0.001**	**<0.001**	0.2
Lean body mass (kg)	47	2	53	2	55	5	0.09	0.1	0.7
sag height (cm)	18.9	0.4	22.9	0.6	24.2	0.9	**<0.001**	**<0.001**	0.2
Glucose tolerance test and HOMA-IR									
fasting glucose (mmol/L)	5.0	0.4	4.8	0.1	8.5	0.7	0.5	**<0.001**	**<0.001**
OGTT glucose AUC (mmol/L.120min)	709	41	761	26	1591	98	0.3	**<0.001**	**<0.001**
Fasting insulin (mIU/L)	5.9	1.5	9.5	1.8	13.4	1.9	0.2	**0.004**	0.1
OGTT Insulin AUC (mIU/L.120min)	4125	455	7734	814	5542	793	**0.003**	0.1	**0.07**
HOMA-IR	1.3	0.3	2.0	0.4	4.8	0.7	0.2	**<0.001**	**<0.001**
Baseline serum biochemistry									
HBA1C (%)	.	.	.	.	7.5	0.4			
Na (mmol/L)	141	0.4	140	0.4	140	0.6	0.05	0.1	0.9
K (mmol/L)	4.1	0.1	6.2	2.0	4.2	0.0	0.4	0.4	0.4
Urea (mmol/L)	4.7	0.3	5.4	0.3	5.1	0.3	0.1	0.4	0.4
Creat (µmol/L)	86	3.2	89	2.6	82	5.4	0.4	0.5	0.2
Ca (mmol/L)	2.3	0.0	2.2	0.0	2.2	0.0	0.7	0.8	0.9
Alb (g/L)	44	1.2	44	0.5	43	0.5	1.0	0.6	0.4
TP (g/L)	72	1.1	72	0.6	72	0.9	0.7	0.6	0.7
ALP (u/L)	148	6.3	163	8.0	162	13.1	0.2	0.3	1.0
AST (iU/L)	23	1.6	22	0.9	25	1.5	0.5	0.3	**0.047**
Bilirubin (µmol/L)	11	1.2	9	0.7	9	0.6	0.1	0.2	0.7
γGT (iu/L)	23	6.9	22	2.2	41	7.1	0.8	0.1	**0.004**
Total cholesterol (mmol/L)	4.5	0.2	6.7	1.3	4.6	0.2	0.2	0.8	0.2
HDL cholesterol (mmol/L)	1.6	0.1	1.4	0.1	1.2	0.1	0.2	**0.008**	**0.03**
Triglycerides (mmol/L)	0.8	0.1	1.3	0.1	2.1	0.5	**0.002**	**0.02**	**0.08**
ALT (iu/L)	21.6	2.2	23.7	2.3	28.8	2.8	0.5	0.05	0.2
Hb (g/dL)	13.2	0.3	13.6	0.3	13.8	0.3	0.4	0.2	0.7
WCC (x10^9/L)	21.7	16.2	6.1	0.3	6.7	0.5	0.2	0.4	0.3
Plts (x10^9/L)	225	20	242	10	222	17	0.4	0.9	0.3
fasting FFA (µmol/L)	286	38	328	17	506	51	0.2	**0.004**	**0**
CT measured body fat distribution									
Liver attenuation (HU)	64.3	1.63	63.3	1.40	54.2	2.37	0.6	**0.001**	**0.001**
Spleen attenuation (HU)	54.8	0.71	55.4	0.54	55.3	0.61	0.5	0.6	0.9
L/S ratio	1.18	0.03	1.15	0.03	0.97	0.04	0.4	**0.001**	**0.001**
% visceral fat	33.7	3.70	33.2	3.21	49.7	3.10	0.9	**0.002**	**0.001**
% s/c fat	66.0	3.67	66.7	3.21	50.3	3.10	0.9	**0.003**	**0.001**
V:S ratio	0.66	0.17	0.64	0.13	1.09	0.14	0.9	0.06	**0.02**

Table 6-1 Study population descriptive statistics for normal, obese and diabetic groups (values expressed as mean +/- SEM or median (interquartile range)). Significant P values in bold.

6.4.2. Cortisol metabolism in normal, obese and type 2 diabetic subjects

	Normal (1)		Obese (2)		Diabetic (3)		*P* value		
	mean/ *median*	SEM	mean/ *median*	SEM	mean/ *median*	SEM	2 vs 1	3 vs 1	3 vs 2
N	21		36		22				
Baseline serum 9am Cortisol (nmol/L)	359	72	239	30	346	37	***0.08***	0.86	**0.04**
Serum cortisol generation profile (CGC)									
Baseline cortisol post dex suppression (nmol/L)	12.8	2	23.3	5	16.1	2	0.17	0.29	0.28
Peak cortisol (nmol/L)	603	56	602	33	632	34	0.99	0.64	0.55
Area under the curve (nmol/L.240min)	*105469*	*51986*	*84469*	*38994*	*100323*	*30003*	0.73	0.92	0.59
24 hr urine steroid anaysis									
Urine Total F metabolites (μg/24hrs)	7741	817	9042	854	10385	892	0.34	**0.04**	0.31
THF	*1149*	*806*	1571	166	1752	157	0.17	**0.01**	0.47
5α-THF	*1084*	*1504*	*1444*	*1433*	1570	300	0.89	0.17	0.29
THE	*2578*	*1715*	*4123*	*4283*	*2649*	*2215*	0.44	***0.07***	0.29
α-cortol	256	27	*328*	*196*	369	33	0.24	**0.01**	0.15
β-cortol	*465*	*356*	*454*	*373*	*460*	*350*	0.34	0.70	0.54
α-cortolone	*958*	*85*	*1273*	*108*	*1466*	*135*	***0.07***	**0.00**	0.28
β-cortolone	*555*	*54*	*612*	*57*	*701*	*50*	0.53	***0.06***	0.30
F:E	0.60	0.04	*0.65*	*0.15*	*0.62*	*0.21*	0.74	0.14	0.16
(THF+5αTHF):THE	*0.85*	*0.31*	*0.96*	*0.34*	*0.94*	*0.44*	0.82	0.93	0.61
cortols:cortolones	0.49	0.02	*0.40*	*0.09*	*0.38*	*0.06*	**0.04**	**0.00**	0.44
11βOHEt+11βOHAn:11OXOEt	*2.91*	*2.04*	*2.57*	*1.47*	*2.50*	*1.52*	**0.03**	0.17	0.42
5αTHF:THF	*1.02*	*1.16*	*0.79*	*0.46*	*0.83*	*0.61*	0.26	0.81	0.45
An:ET	*1.63*	*1.35*	*1.20*	*0.50*	*0.94*	*0.84*	0.13	0.48	0.54

Table 6-2 Data reflecting cortisol metabolism in normal, obese and type 2 diabetic cohorts. Significant differences are displayed in bold. Notable differences that did not quite achieve significance are displayed in bold italics. [values expressed as mean +/- SEM or (in italics) median (interquartile range)]

Data reflecting cortisol metabolism in the normal, obese and type 2 diabetic subjects is shown in Table 6-2. 9am baseline serum cortisol levels were significantly lower in the obese group when compared with both normal and diabetic groups. There was no difference in 9am cortisol between diabetic and normal weight subjects.

Area under the curve calculations from serum cortisol generation profiles after 25mg of cortisone acetate and dexamethasone suppression were used as an *in-vivo* marker of hepatic 11β-HSD1 activity. There was no significant difference observed between groups although the cortisol generation AUC was highest in the normal group and lowest in the obese group, a trend which matched the urine cortisol metabolite ratios for 11β-HSD1 activity. The peak cortisol level was highest in the diabetic group, and higher in obese subjects compared with normals, but these differences also did not achieve significance. Values for the final two time points (180 min and 240 min) showed a significant difference between normal and obese groups Table 6-2, Figure 6-7.

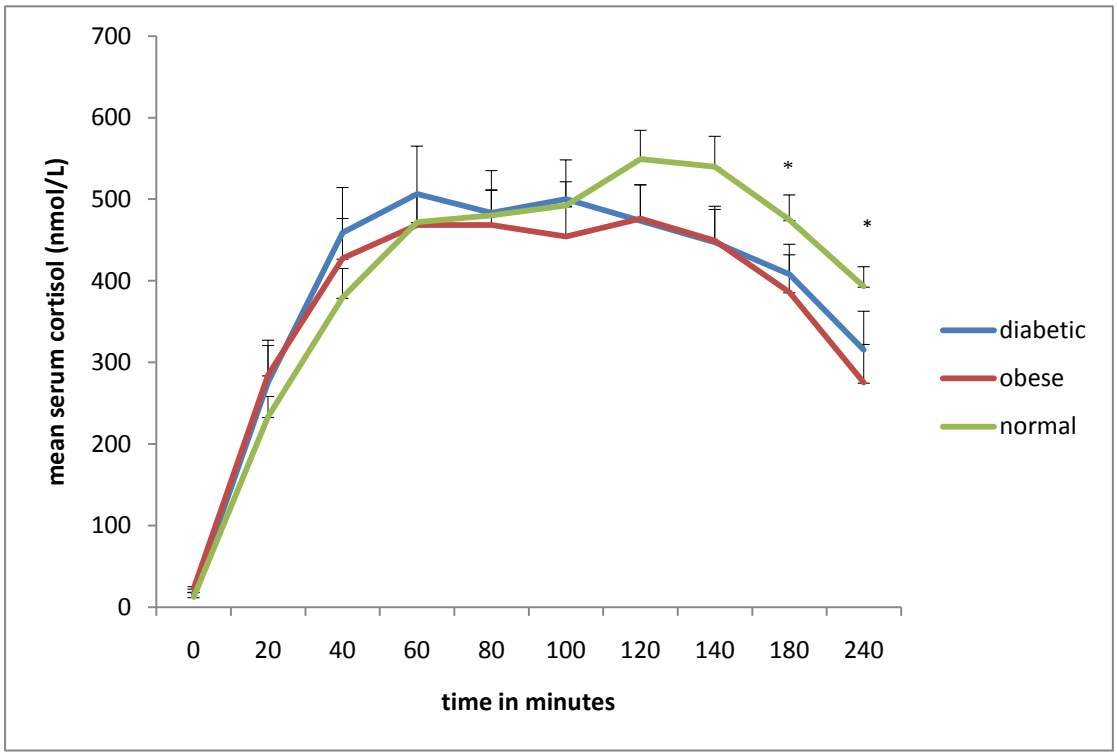


Figure 6-7 *Serum cortisol generation profiles follwoing 25mg cortisone acetate in dexamethasone suppressed subjects in normal, obese and diabetic cohorts (mean +/- SEM).(* $p<0.05$ normal vs obese).*

24 hour urine steroid metabolite analysis did reveal some interesting differences between groups. Urine total F metabolites were highest in the diabetic group. This difference was significant when compared with the normal cohort, but not when compared with BMI matched controls (the obese group). Total F metabolites were higher in the obese group compared with normals, although the difference was not statistically significant. These results show evidence of HPA axis activation in Type 2 diabetes, Figure 6-8.

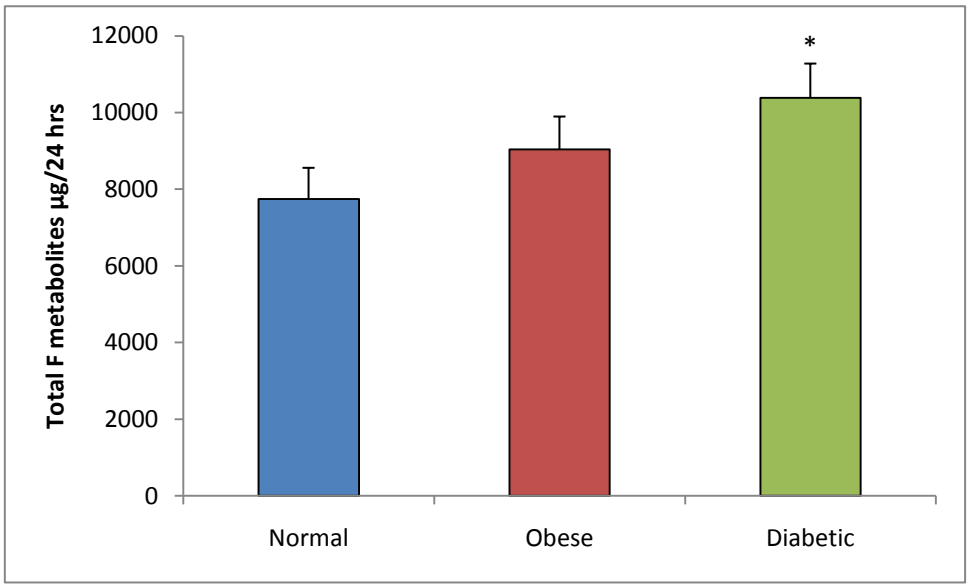


Figure 6-8 24 hour urine total cortisol metabolites (mean +/- SEM) in normal, obese and diabetic cohorts. ($p < 0.05$ diabetic vs normal).*

Urine F:E ratios were not different between groups implying that renal 11β-HSD2 activity was similar and hence did not impact any results reflecting 11β-HSD1 driven cortisol metabolism between groups (as cortisone (substrate) availability for 11β-HSD1 would be assumed to be unchanged between groups), Figure 6-9.

Urinary markers of 11β-HSD1 driven cortisol generation were the urinary steroid metabolite ratios: THF+5α-THF/THE, cortols/cortolones, and 11OH-androsterone

+11OH-etiocholanolone/11-oxoetiocholanolone. As displayed in Figure 6-9, the widely used urinary marker for 11β-HSD1 activity - the THF+5α-THF/THE ratio, was similar for all three groups.

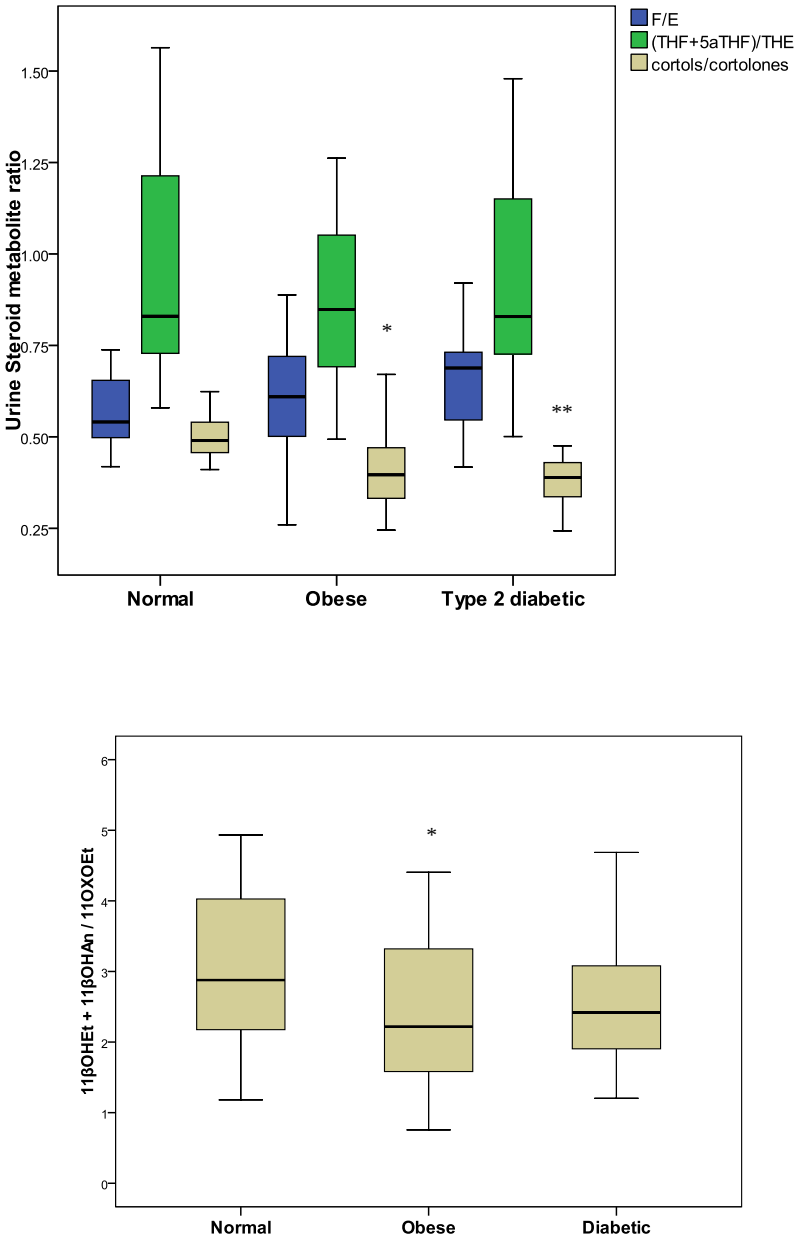


Figure 6-9 *Urine steroid metabolite ratios displayed as boxplots with median and interquartile range. (*obese vs normal p<0.05,** diabetics vs normal p<0.01).*

When compared with normal, the urine cortols/cortolones ratio was significantly lower in both the obese and the diabetic groups. The 11OH-androsterone+11OH-etiocholanolone/11-oxoetiocholanolone ratio was significantly lower in the obese group compared with the normal group Figure 6-9. There was no significant difference in these ratios between diabetic and the obese group, who were BMI matched.

Markers of cortisol clearance via the A-ring reductases include the urinary 5αTHF/THF and An/Et ratios. While there was a trend for these ratios to be lower in the obese and diabetic groups compared with normal, these were not statistically significant.

The observed differences were unaltered after matching for sex between groups.

Two important observations from this analysis were, 1) HPA axis activation in the type 2 diabetic group with significantly increased total cortisol metabolites and significantly higher 9am cortisol compared with the BMI matched obese group; 2) some evidence of suppression of 11β-HSD1 driven glucocorticoid metabolism in the obese cohort compared with normal. There was also a suggestion of loss of suppression of 11β-HSD1 activity with the highest peak cortisol in the cortisol generation profile compared and higher total AUC of the cortisol generation profile in the type 2 diabetic group compared with obese non diabetic (neither of these differences however achieved statistical significance).

6.4.3. Body fat distribution analysis and lean body mass

The markers of body fat distribution used in the study were waist:hip circumference ratio, sagittal height, total % body fat measured by DEXA and CT measured intrahepatic fat, visceral and subcutaneous fat area Table 6-1.

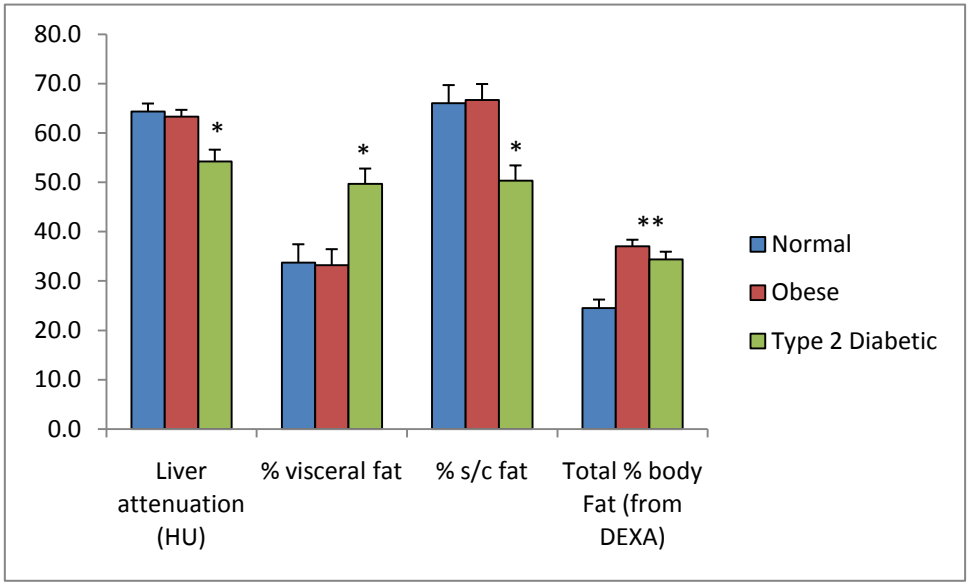


Figure 6-10 Fat distribution analysis using CT measured liver attenuation (lower attenuation implying increased intrahepatic fat), % visceral and subcutaneous fat (measured by CT), and % total body fat measured by DEXA +/- SEM, (p<0.01 diabetic vs obese and normal, ** p<0.01 obese vs normal and diabetic vs normal).*

Despite having similar BMI compared with obese non diabetic patients, and total % body fat (calculated from DEXA), patients with diabetes had a significantly higher waist:hip ratio, and CT measured %visceral and lower % subcutaneous fat. This was accompanied by significantly increased CT measured intrahepatic fat in diabetics compared with obese (BMI matched) controls. Interestingly, when obese and normal weight healthy volunteers were compared there were no significant differences in waist:hip ratio or CT measured % visceral, % subcutaneous and intrahepatic fat,

despite significantly increased BMI, total % body fat and sagittal height in the obese group, Figure 6-10.

With non contrast enhanced CT, as used in this analysis, a liver attenuation of less than 50 is seen in overt steatosis and a reduction of 1.6HU is observed for approximately each milligram of triglycerides deposited per gram of liver tissue (Roldan-Valadez et al 2008). In quantitative evaluations, a liver-to-spleen attenuation ratio with a cut-off value of 1.1 has been proposed to exclude moderate steatosis (Roldan-Valadez et al 2008). Based on this criterion, the diabetic group had radiological evidence of hepatic steatosis.

6.4.3.1. Lean body mass

The link between lean body mass and insulin resistance has been described previously (Brochu 2008). While the lean body mass (calculated from DEXA) was highest in the diabetic group compared with obese and normal groups, the difference between groups did not achieve statistical significance. However a strong relationship between lean body mass and cortisol metabolism and HPA axis function was observed in all three groups, section 6.4.7.2.

6.4.4. Baseline serum biochemistry

In comparison with the BMI matched obese group, diabetics had significantly lower HDL cholesterol, and higher triglycerides and free fatty acids (FFA). A difference in total cholesterol was not seen which would reflect statin treatment in the diabetic group (which was stopped three days before participation in the study). When the obese and normal cohorts were compared, there was no difference in total cholesterol,

HDL cholesterol and fasting FFA, although fasting serum triglycerides were significantly higher in the obese group Table 6-1.

Baseline renal function and full blood count were similar in all three cohorts. However from the liver function tests, serum AST and γGT were significantly higher in the diabetics compared with obese patients. Serum ALT was also higher in the diabetic group but this did not achieve statistical significance. Of importance in this observation was that the mean serum AST, ALT and γGT were still within normal reference range values for all groups, including diabetics. None of the patients with diabetes had any suggestion on liver function tests of the possibility of hepatic steatosis or other liver abnormality, although there was radiological evidence of early hepatic steatosis in this group, section 6.4.7.1.

6.4.5. Glucose homeostasis (oral glucose tolerance test) and HOMA-IR

As expected, fasting glucose was highest in the diabetic group, and also at each subsequent time point during the OGTT. The OGTT glucose AUC was highest in the diabetic group and lowest in the normal group although the increase in the obese group compared with normal did not achieve statistical significance, Table 6-1, Figure 6-11.

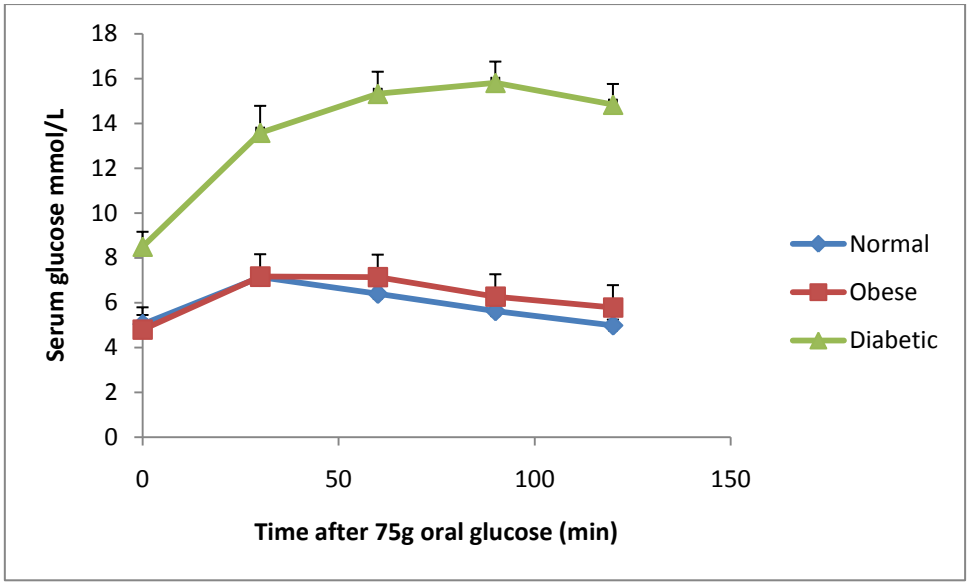


Figure 6-11 75g Oral glucose tolerance test glucose profiles in normal, obese and diabetic cohorts. Mean serum glucose +/- SEM at each time point

Fasting insulin levels were also highest in the diabetic group (normals 5.9 +/- 1.5, obese 9.5 +/- 1.8, diabetic 13.4 +/- 1.9 miU/L +/- SEM) Table 6-1. Obese patients also had a higher fasting insulin level compared with normal subjects but this was not statistically significant. The OGTT insulin AUC however, was highest in the obese group and this was significantly higher than in the normal and diabetic cohorts, Figure 6-12 and Figure 6-13.

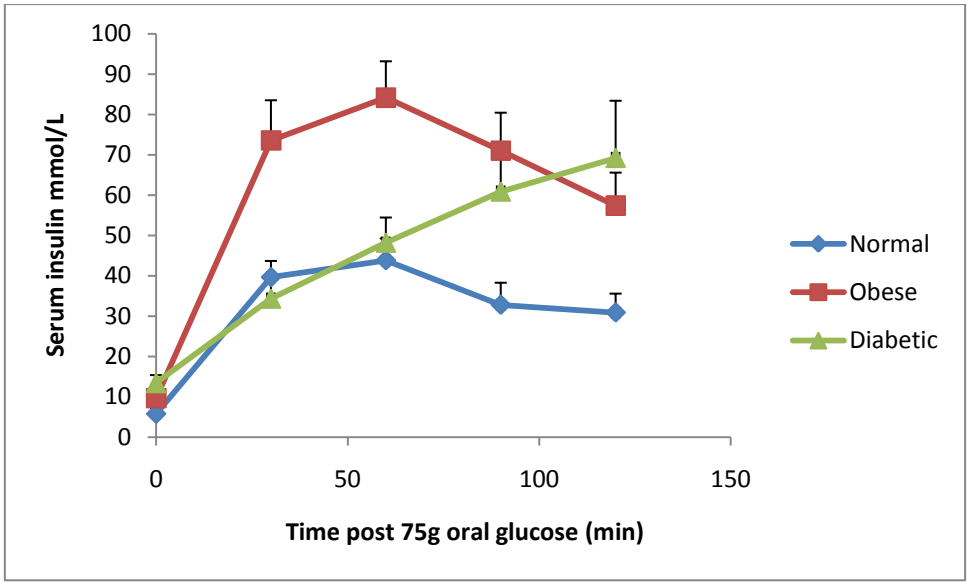


Figure 6-12 *Insulin profile during oral glucose tolerance test in normal, obese and type 2 diabetic cohorts. Data expressed as mean serum insulin at each time point +/- SEM.*

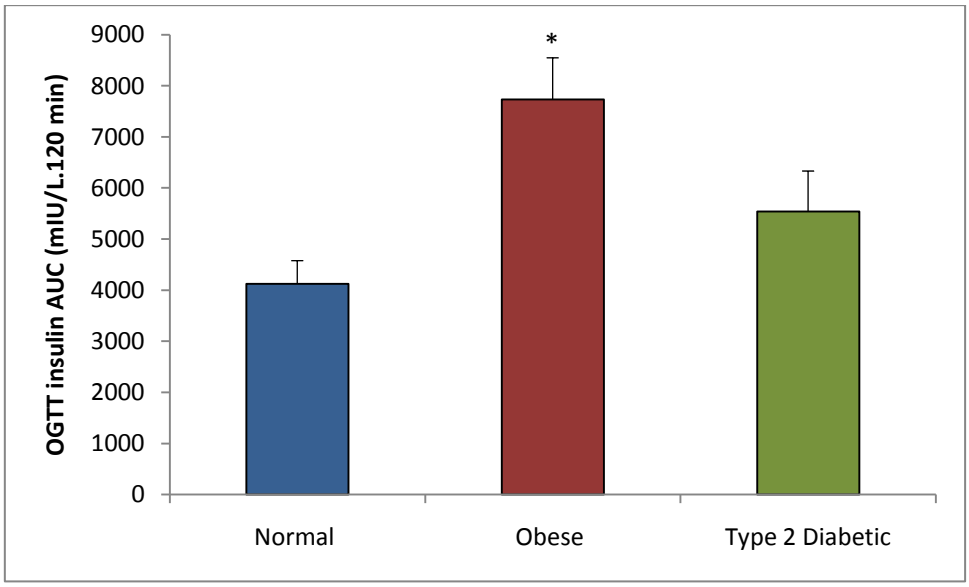


Figure 6-13 *Total area under the curve of insulin profile following 75g oral glucose tolerance test in normal, obese and type 2 diabetic cohorts +/- SEM (* $p < 0.01$ obese vs normal and obese vs diabetics).*

6.4.6. Endogenous glucose production (EGP) with prolonged fasting in normal, obese and diabetic cohorts

Glucose production, i.e., the rate of appearance (R_a) of glucose in μmol/kg body wt/min was set equal to the enrichment of the [6,6-$^2H_2$]glucose divided by the moles percent excess enrichment at C6 of blood glucose and the rate of infusion of the [6,6-$^2H_2$]glucose and then minus that rate. While it was possible to obtain samples from most of the subjects in each cohort, it was only possible to obtain results for total glucose production. The fraction of glucose produced by gluconeogenesis was to be obtained by calculating the enrichment of the hydrogen bound to C-5 to that of C-2. This part of the analysis has not yet been possible due technical difficulties with analysis using the equipment currently available. It is hoped that these results may be obtainable in the near future.

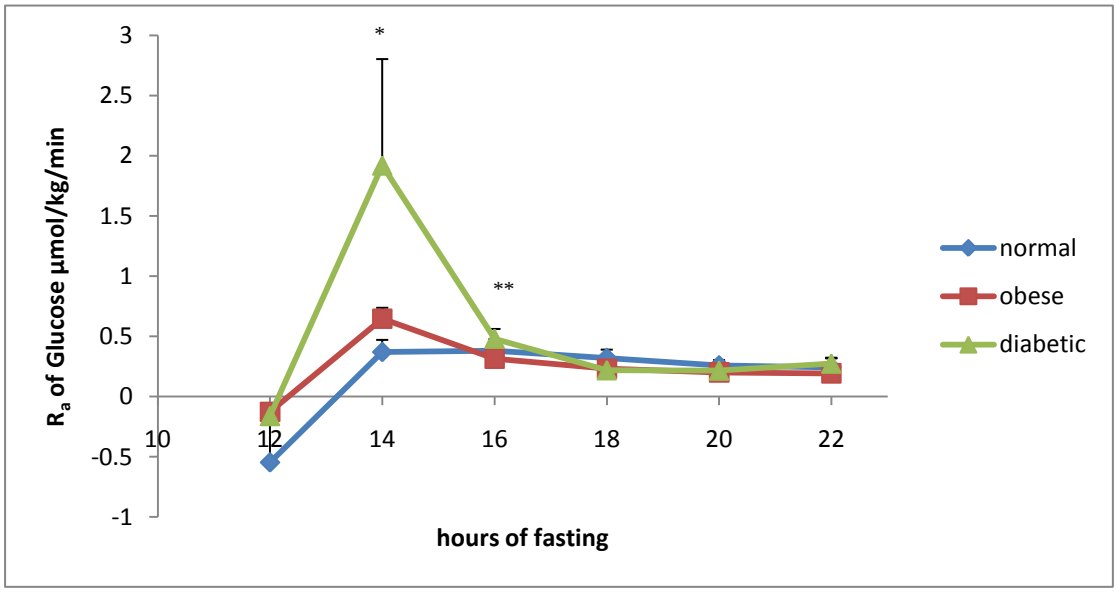


*Figure 6-14 Mean rate of appearance of glucose (μmol/kg/min +/- SEM) at selected timepoints during fast during the [6,6-2H2] glucose infusion which was started at the 12h timepoint of the fast. *diabetic vs obese p <0.01, diabetic vs normal p<0.05, **diabetic vs obese p<0.05.*

EGP results were obtained from 3 normal patients, 18 obese, and 8 type 2 diabetic patients, illustrated in Figure 6-14. The samples from the rest of the patients were yet to be analysed at the time of writing this thesis.

As displayed in Figure 6-14 endogenous glucose production i.e. the rate of appearance (R_a) of glucose was significantly higher in the diabetic groups at the 14h (μmol/kg/min +/- SEM: normal 0.37 +/- 0.1, obese 0.65 +/- 0.09, diabetic 1.92 +/- 0.8; diabetic vs obese $p<0.01$, diabetic vs normal $p<0.05$) and 16h time points (μmol/kg/min +/- SEM: normal 0.38 +/- 0.09, obese 0.31 +/- 0.025, diabetic 0.48 +/- 0.08; diabetic vs obese $p<0.05$). This was followed by a steady state of glucose production that was not significantly different between groups.

The R_a of glucose at the 14h time point was tightly correlated with hepatic 11β-HSD1 activity (AUC of cortisol generation profile ($R=0.92$, $p<0.05$) specifically in the diabetic group.

6.4.7. Correlation of body composition and biochemical parameters with markers of glucocorticoid metabolism

6.4.7.1. Body fat distribution

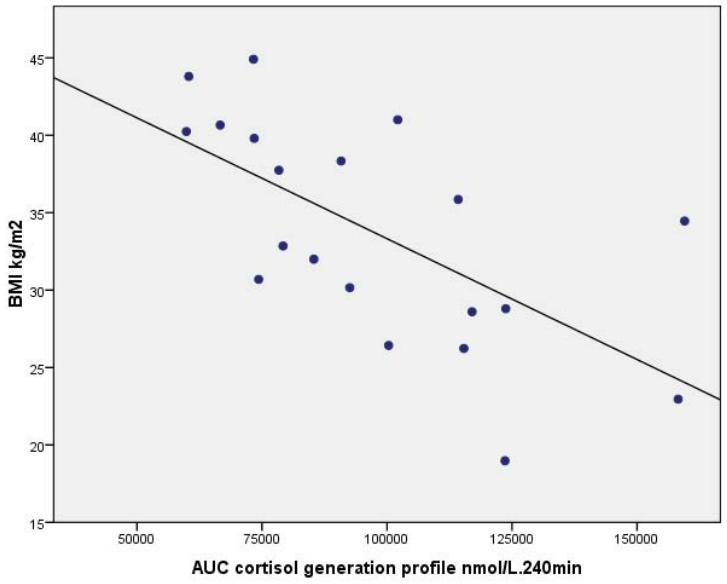


Figure 6-15 Association between body mass index (R=0.6, p < 0.01), and hepatic 11β-HSD1 activity in type 2 diabetic subjects.

In the diabetic cohort, BMI was strongly negatively correlated with hepatic 11β-HSD1 activity (AUC from cortisol generation profile), Figure 6-15.

Despite the significant differences in subcutaneous and visceral fat distribution in the diabetic group, there was no consistent relationship of CT measured visceral fat and subcutaneous fat with glucocorticoid markers in any group. Surprisingly, waist:hip ratio, which is considered a more crude measure of visceral fat then when measured by CT correlated negatively with hepatic 11β-HSD1 activity (AUC from cortisol generation profile) (R=0.5, p <0.05).

6.4.7.2. Lean body mass

For the entire study population, (excluding the NAFLD cohort from the analysis), there was a strong negative correlation of lean body mass with hepatic 11β-HSD1 activity (AUC from cortisol generation profile) (R = -0.5, p <0.001), Figure 6-16. There was also a strong positive correlation of lean body mass with total urinary cortisol metabolites (R = 0.6, p <0.001), Figure 6-17.

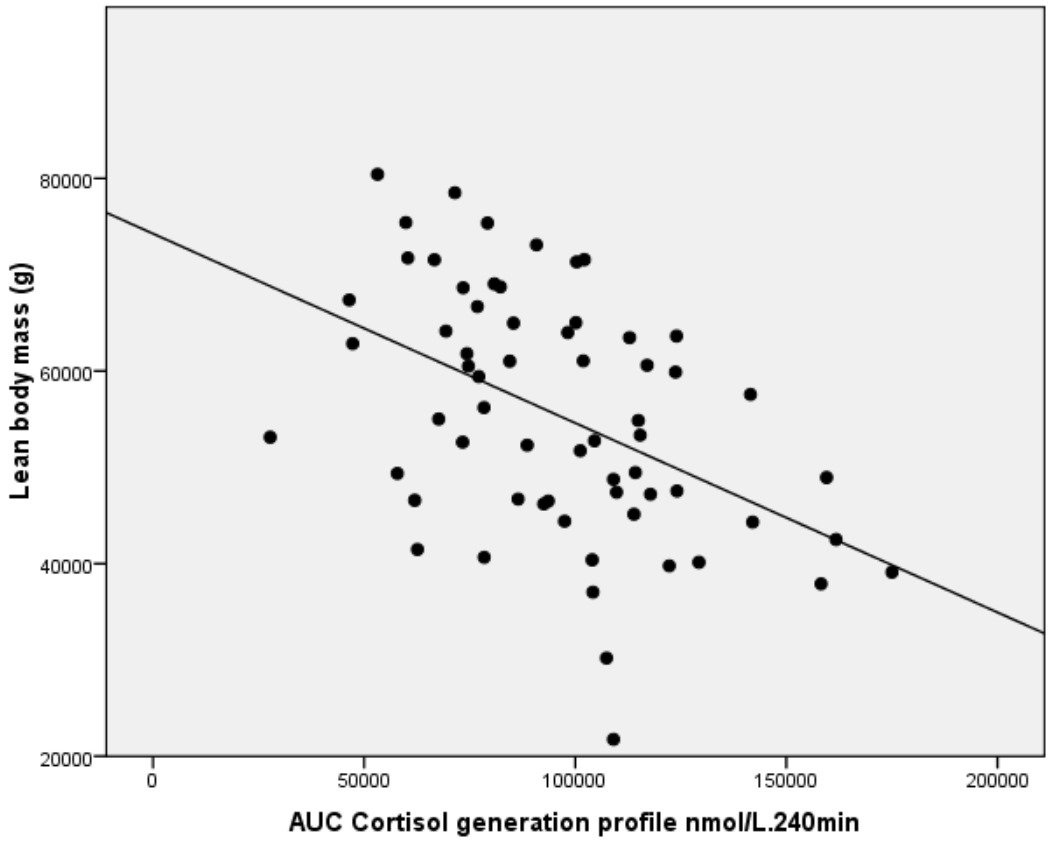


Figure 6-16 Correlation of lean body mass with hepatic 11β-HSD1 activity in normal, obese and diabetic patients, R= -0.5, p <0.001.

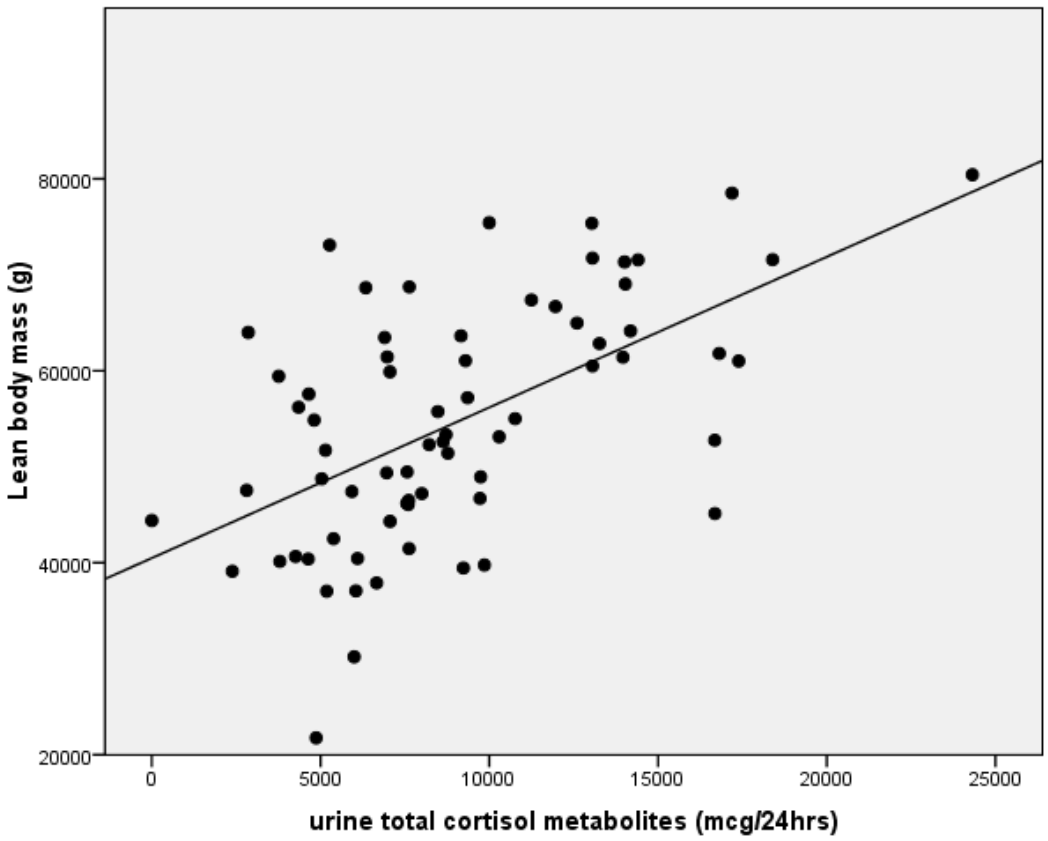


Figure 6-17 Correlation of lean body mass with 24hr urine total cortisol metabolites, R = 0.6, p <0.001.

On further analysis of these relationships at subgroup level, it was discovered that the correlation between lean body mass and hepatic 11β-HSD1 activity was not present in the normal group, but was present in the obese and diabetic groups, with the correlation being stronger in the diabetic group compared with the obese group, Table 6-3. The correlation between lean body mass and urine total cortisol metabolites held true for all three groups when analysed at subgroup level.

		Lean body mass vs hepatic 11β-HSD1 activity
Normal	Pearson Correlation	-0.21
	Sig. (2-tailed)	0.43
	N	16
Obese	Pearson Correlation	**-.585****
	Sig. (2-tailed)	**0.001**
	N	29
Diabetic	Pearson Correlation	**-.723****
	Sig. (2-tailed)	**0.001**
	N	18

Table 6-3 Correlation of lean body mass with hepatic 11β-HSD1 in normal, obese and diabetic groups.

6.4.7.3. Serum fasting free fatty acids

Fasting FFA in normal subjects *negatively* correlated with hepatic 11β-HSD1 activity (AUC from cortisol generation profile) ($R= -0.8$, $p<0.05$). In obese subjects, fasting FFA were strongly associated with 5α-reductase driven cortisol clearance, being positively associated with the urine 5αTHF/THF and An/ET ratios ($R=0.4$, $p<0.05$ and $R= 0.5$, $p<0.01$ respectively) as well as total cortisol metabolites ($R=0.5$, $p<0.01$), which were higher in the obese group compared with normals. In contrast fasting free FFA in the diabetic group were *positively* correlated with hepatic 11β-HSD1 activity. These results showed a change in hepatic glucocorticoid metabolism in relation to FFA with increasing states of insulin resistance. Hence, in normal subjects higher fasting serum FFA was associated with a down regulation of hepatic 11β-HSD1 activity, whereas in obese subjects the association was with increased glucocorticoid

clearance, and in the diabetics there was a switch in the association with higher serum FFA being associated with increased hepatic 11β-HSD1 activity.

6.4.7.4. Correlation of glucose and insulin markers with glucocorticoid metabolism

The observed differences between groups for fasting and post oral glucose insulin and glucose profiles were associated with markers of adrenal glucocorticoid production and glucocorticoid metabolism. In the diabetic group there was a strong *negative* correlation between fasting insulin and hepatic 11β-HSD1 activity measured by serum cortisone generation profile (R= -0.6, p<0.01), Figure 6-18, as well as the peak serum cortisol achieved during the test (R= -0.5, p<0.05).

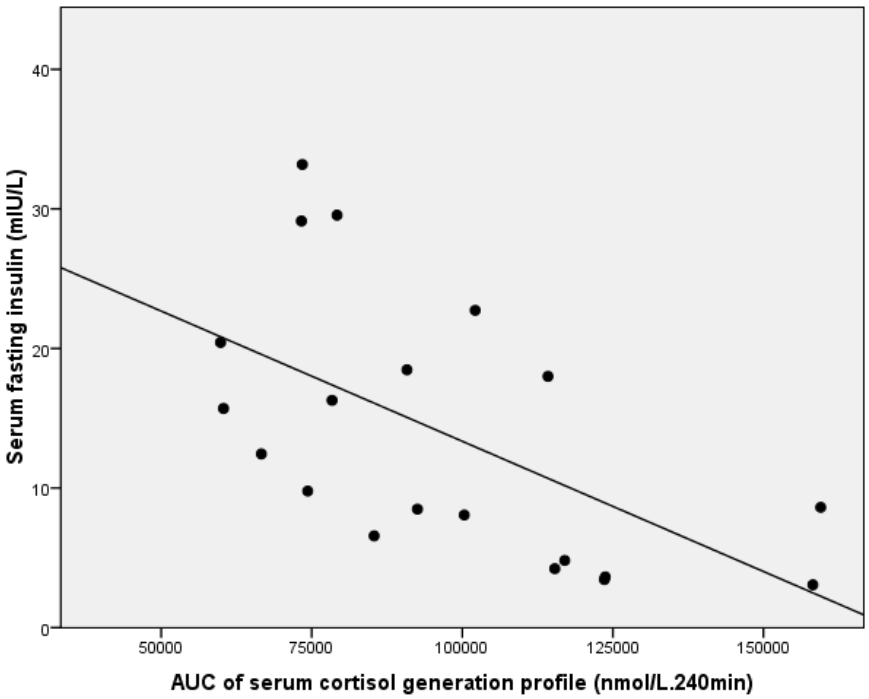


Figure 6-18 Association between serum fasting insulin and hepatic 11β-HSD1 activity measured by serum cortisol generation profile after 25mg oral cortisone acetate in type 2 diabetic subjects (R=0.6, p<0.01).

Furthermore, the insulin values at all other time points significantly correlated negatively with the peak cortisol in the cortisol generation curve in the diabetic group. The total insulin AUC from the OGTT insulin profile also correlated negatively with hepatic 11β-HSD1 activity measured by serum cortisol generation profile (R= -0.5, $p<0.05$) in the diabetic group.

Endogenous glucose production in the diabetic group was significantly associated with increased 11β-HSD1 activity at the time points significantly higher than normal and obese groups, (section 6.4.6).

HOMA-IR was, as expected, highest in the diabetic group and lowest in the normal group. Compared with the BMI matched obese group HOMA-IR was significantly higher in the diabetic group (mean HOMA-IR +/- SEM): normal 1.3 +/- 0.3, obese 2.0 +/- 0.4, diabetic 4.8 +/- 0.7; diabetic vs obese and diabetic vs normal $p<0.01$). In the diabetic group, HOMA-IR was negatively associated with hepatic 11β-HSD1 activity measured by serum cortisol generation profile (R= -0.5, $p<0.05$), as would be expected from the association of fasting insulin with 11β-HSD1 activity in the diabetic group.

6.4.8. Multivariate regression analysis

On the background of the strong univariate negative correlation between fasting insulin (in diabetics) and lean body mass (in obese patients) with hepatic 11β-HSD1, and the relationship between fasting FFA and glucocorticoid metabolism, multivariate linear regression modelling was undertaken to further explore the contribution of cortisol metabolism to these variables. Multivariate forward regression models were created using the following as measures: fasting insulin, fasting glucose,

CT measured liver attenuation (indicative of liver fat content), and lean body mass, each as the dependant variable in separate models. The possible explanatory variables used were: markers of HPA axis function and cortisol metabolism, total cholesterol, triglycerides, fasting FFA, intrahepatic fat (CT liver attenuation and liver:spleen ratio, not used in the model with liver fat as the dependant variable), liver enzymes (γGT, AST, ALT, bilirubin), total % fat (from DEXA), %visceral fat, %subcutaneous fat, and lean body mass (except in the model with lean mass as the dependant variable).

6.4.8.1. Regression models with fasting insulin as the dependent outcome variable

Variation in fasting FFA, HDL cholesterol, and urine F/E ratio explained 72% (R^2 = 0.72) of the variance in fasting insulin, Table 6-4.

	Unstandardized B Coefficients	Std. Error	*P* value
fasting FFA	0.03	0.006	0
HDL cholesterol	-9.27	2.52	0.002
urine F/E ratio	11.28	4.44	0.023

Table 6-4 Regression model with fasting insulin as the outcome measure for all subjects in normal, obese and diabetic groups.

To further explore the relationship between fasting insulin and cortisol metabolism, fasting FFA were omitted from the analysis. In this situation a number of other variables became independently associated with fasting insulin: γGT, urine cortol/cortolones (a marker of 11β-HSD1 activity), % body fat and lean body mass, the variation of which explained 70% (R^2 = 0.70) of the variation in fasting insulin Table 6-5.

	Unstandardized B Coefficients	Std. Error	*P* value
γGT	0.13	0.03	0
cortols/cortolones	-16.31	6.46	0.018
%Fat	0.25	0.07	0.001
Lean body mass	0.14	0.06	0.022

Table 6-5 Regression model with fasting insulin as the outcome measure for all subjects in normal, obese and diabetic groups, with fasting FFA removed from the analysis.

These two models suggest that the close relationship seen between fasting insulin and 11β-HSD1 activity is influenced by a contribution from serum FFA and is associated with body composition, in particular total % fat and lean body mass.

6.4.8.2. Regression model with fasting glucose as the outcome variable

Serum creatinine, urine THF+5α-THF/THE (a urine marker of 11β-HSD1) and post dexamethasone suppressed baseline cortisol independently predicted serum fasting glucose with variance in these three variables explaining 98% (R^2 = 0.98) of the variation in fasting glucose Table 6-6.

	Unstandardized B Coefficients	Std. Error	*P* value
Creat	0.11	0.01	0
Urine (THF+5aTHF)/THE	4.52	0.54	0.001
Baseline serum cortisol post dex suppression	-0.08	0.01	0.005

Table 6-6 Regression model with fasting glucose as the outcome measure for all subjects in normal, obese and diabetic groups

These relationships suggest a key role of 11β-HSD1 in fasting glucose homeostasis in addition to the known influence of HPA axis function on fasting glucose.

6.4.8.3. Regression model with CT measured liver attenuation (a marker of intrahepatic fat) as the outcome variable

The urine 5α-THF/THF, and An/ET ratios (markers of cortisol clearance via A-ring reductases), serum triglycerides, total protein and serum potassium independently predicted serum liver attenuation with variance in these variables explaining 100% (R^2 = 1) of the variation in liver attenuation with high significance Table 6-7.

	Unstandardized B Coefficients	Std. Error	*P* value
urine 5aTHF/THF	-13.58	0.11	0
serum triglycerides	-5.54	0.03	0
total protein	0.74	0.01	0
serum potassium	3.49	0.09	0.001
urine An/ET	-0.58	0.09	0.023

Table 6-7 Regression model with liver fat attenuation on CT as the outcome measure for all subjects in normal, obese and diabetic groups

The results of this model strongly support the significance of the finding of increased cortisol clearance via A-ring reductases with increasing liver fat as shown in the NAFLD studies described in chapter 7, as well as the close association of CT liver attenuation with hepatic triglyceride levels.

6.4.8.4. Regression model with lean body mass as the outcome variable

The urine cortols/cortolones ratio (a urine marker of 11β-HSD1 independently predicted serum liver attenuation with variance in urine cortols/cortolones explaining 34% (R^2 = 0.34) of the variation in lean body mass with no other variables from the list of explanatory variables used being associated in the relationship.

	Unstandardized B Coefficient	Std. Error	*P* value
urine cortols/cortolones	-61.557	22.304	0.015

Table 6-8 Regression model with lean body mass as the outcome measure for all subjects in normal, obese and diabetic groups

6.5. Discussion

The similarities between patients with Cushing's syndrome and the metabolic syndrome have focussed interest in the role of glucocorticoids in the pathophysiology of insulin resistance and type 2 diabetes, and 11β-HSD1 has emerged as novel candidate both in the pathogenesis and treatment of the metabolic syndrome.

The results of this detailed analysis have revealed a complex and definite role, both of adrenal glucocorticoid production and pre receptor glucocorticoid metabolism as a mechanism to promote normal metabolic phenotype, as well as in the pathophysiology of insulin resistance. Many of these results have endorsed previous findings, but the relationship of hepatic tissue specific 11β-HSD1 with lean body mass, insulin sensitivity, and hepatic glucose output form novel results that provide an exciting reflection into the role of 11β-HSD1 in the pathophysiology of type 2 diabetes and support the putative role of selective 11β-HSD1 inhibition to preserve and promote a favourable metabolic phenotype.

While circulating cortisol concentrations are invariably normal in obesity and type 2 diabetes, changes in HPA axis activity and sensitivity in these subjects have been reported with stress, dexamethasone and other stimuli (Duclos et al. 2001;MIGEON et al. 1963;Pasquali et al. 1993). This work has shown increased sensitivity to glucocorticoids in type 2 diabetics with lower 9am cortisol levels after dexamethasone suppression compared with BMI matched obese subjects as previously reported

(Andrews et al 2002). While baseline 9am cortisol remained within the normal range in the diabetic cohort it was significantly higher in this group when compared with the obese and normal cohorts. Total urinary cortisol metabolites were also significantly elevated in the type 2 diabetics, and together these observations imply HPA axis activation despite the increased sensitivity to glucocorticoids. Furthermore, there was an overall lack of inhibition of hepatic 11β-HSD1 in diabetics compared with BMI matched obese controls, with the highest peak cortisol generated during cortisone generation profile following 25mg cortisone acetate and the total area under the curve generated being higher than in non diabetic obese subjects. However while there was a lack of inhibition of whole body 11β-HSD1 with increasing BMI in diabetics as reported in a previous study based on urine glucocorticoid metabolite ratios reflecting whole body 11β-HSD1 activity (Valsamakis et al 2004), there was clear evidence of inhibition of hepatic 11β-HSD1 with increasing BMI in diabetics in this study, Figure 6-15. Increased hepatic triglyceride content is associated with decreased 11β-HSD1 activity (Westerbacka et al 2003). Type 2 diabetes is associated with impaired hepatic triglyceride clearance (Curtin et al. 1996). Our diabetic subjects had increased serum triglycerides compared with normal and obese controls, with radiological evidence of increased hepatic fat (CT liver attenuation is directly related to hepatic triglyceride content, section 6.4.3). Hence, increased hepatic triglyceride content may decrease hepatic 11β-HSD1 activity. This is an important observation as it endorses the model described in the NAFLD cohort of the study (chapter 7) where hepatic steatosis specifically inhibited hepatic glucocorticoid generation and promoted hepatic GC clearance.

6.5.1. Fat distribution, body composition, and cortisol metabolism

Visceral fat has been established as a major contributor to the pathogenesis of the metabolic syndrome. A summary of published work related to visceral fat and 11β-HSD1 and expression are discussed in section 6.1. Surprisingly the results from the study did not uncover associations between CT measured visceral fat and 11β-HSD1 activity, despite it being significantly higher in the diabetic group. However there were significant associations of cortisol metabolism with waist:hip ratio, a more crude index of visceral fat and increased metabolic risk. In the diabetic group it was associated with the AUC of the cortisol generation profile and hence hepatic 11β-HSD1 activity. This was an interesting observation in light of recent studies which have shown that visceral fat cortisol production does not contribute to splanchnic glucocorticoid delivery to the liver, section 6.1. Instead it may be the increased FFA delivery to the liver and its potential effect upon liver fat accumulation resulting in increased 5α-reductase mediated hepatic glucocorticoid clearance as a protective effect as described above. Indeed, intrahepatic fat and not visceral fat has been shown to be linked with the metabolic complications of obesity (Fabbrini et al. 2009).

Perhaps the most exciting and novel finding of this study relate to the association of lean body mass with hepatic glucocorticoid metabolism and HPA axis function. Lean body mass was highest in the diabetic group of the study but not significantly so. Univariate analysis revealed a strong negative correlation between lean body mass and hepatic 11β-HSD1 activity and a strong positive correlation with urine total cortisol metabolites. Subgroup level analysis revealed that the relationship with hepatic 11β-HSD1 activity was not present in the normal group but was present and more significant in the diabetic group compared with obese. Furthermore, multivariate

regression model analysis for the entire study population confirmed whole body 11β-HSD1 activity to be the sole factor, from all the variables analysed that independently predicted with lean body mass. These findings would support *in vitro* studies where, at the tissue specific level, 11β-HSD1 has been shown to regulate glucocorticoid mediated insulin resistance in skeletal muscle (Morgan et al. 2009). Skeletal muscle in type 2 diabetes is resistant to insulin action, with a significant reduction in the ability of insulin to increase muscle glucose disposal. This action is mediated by the GLUT-4 transporter. The translocation of GLUT-4 to the cell surface in response to insulin and to other stimuli is inhibited in the presence of glucocorticoids (Coderre et al. 1996;Oda et al. 1995). The positive association of HPA axis function and lean mass in the diabetic group may therefore contribute negatively upon skeletal muscle insulin resistance. The specific relationship between lean body mass and specific hepatic 11β-HSD1 activity is a curious one – by what mechanism does hepatic 11β-HSD1 impact skeletal muscle? This may be speculated upon from the inferences drawn from the characterisation of zonation of hepatic 11β-HSD1 described in chapter 3. The perivenous zonation of hepatic 11β-HSD1 would promote increased glycolysis with up regulation of activity or expression of 11β-HSD1. Increased hepatic glycolysis has been elegantly demonstrated as a mechanism by which skeletal muscle mass and insulin resistance may be reduced (described in detail in section 3.4.1) (Wu et al 2005). This may underly the negative correlation seen between lean body mass and hepatic 11β-HSD1 activity, and may reflect a protective mechanism to limit whole body insulin resistance. It would therefore be of interest to investigate the impact of selective 11β-HSD1 inhibition upon skeletal muscle insulin resistance.

6.5.2. Associations of cortisol metabolism with insulin sensitivity and glucose output

Insulin resistance may reflect impaired insulin dependent down-regulation of hepatic glucose release (gluconeogenesis) and / or an impairment in the insulin mediated increase in peripheral glucose uptake. Which is the more important factor in the metabolic syndrome remains controversial, but the effect of glucocorticoids appears to include both of these variables (Rizza et al. 1982;Rooney et al. 1993). With respect to hepatic pre receptor glucocorticoid activation promoting insulin resistance, from the results of this study, dysregulation of hepatic 11β-HSD1 appears to provide critical link in the insulin resistance and glucose intolerance associated with type 2 diabetes. Whole body 11β-HSD1 activity for the entire study population independently predicted fasting glucose in multivariate regression model analysis, and was unmasked as predictor of fasting insulin under the influence of free fatty acids.

While fasting insulin levels were highest in the diabetic group, both fasting glucose and endogenous glucose production in the fasting state were significantly higher in the diabetic group, reflecting the impairment of the insulin-dependent down regulation of hepatic glucose release in diabetes. The increase in endogenous glucose production in type 2 diabetics was specifically associated with increased hepatic 11β-HSD1 activity, whereas fasting insulin was strongly linked with decreased hepatic 11β-HSD1 activity. The effect of hepatic glucocorticoid generation in promoting endogenous glucose production in diabetes can be explained on the basis of the local effects of glucocorticoid on hepatic glucose output. The inverse correlation between fasting insulin and hepatic 11β-HSD1 activity would be a favourable one during fasting hyperinsulinaemia in insulin resistance as the additional contribution to

hepatic glucose output via local glucocorticoid stimulation would be reduced as a result. It would be interesting to investigate whether this association is maintained with increasing beta cell dysfunction in type 2 diabetes where lower fasting insulin levels would be associated with increased hepatic 11β-HSD1 activity with further stimulation of hepatic glucose output. Indeed, glucocorticoids inhibit insulin secretion from pancreatic β cells (Delaunay et al. 1997;Ling et al. 1998) and recent work has excitingly shown that local regeneration of glucocorticoid via 11beta-HSD1 within alpha cells regulates glucagon secretion and in addition may act in a paracrine manner to limit insulin secretion from β cells (Swali et al 2008).

Insulin may also indirectly promote hepatic 11β-HSD1 mediated cortisol generation via inhibition of glucose-6-phosphatase (G6Pase). Studies have shown selective tonic inhibition of G6Pase catalytic subunit, but not G6P transporter, gene expression by insulin in vivo (Hornbuckle et al. 2001). Any degree of inhibition of G6Pase, with normal G6P transporter activity would in turn lead to increased G6P within the ER lumen. This would cause increased 11β-HSD1 driven cortisol generation from cortisone secondary to increased cofactor delivery by the H6PDH enzyme which uses G6P as a substrate. This link between HPA axis function and glucose metabolism has been fully explored in the glycogen storage disease type 1 studies described in chapter 4. On this background it would be of interest to explore whether hepatic 11β-HSD1 activity is lower in type 2 diabetic patients with evidence of marked beta cell dysfunction, where fasting insulin levels would be lower.

There was some evidence of suppression of hepatic 11β-HSD1 activity in obese non diabetic subjects. This may be regarded as a favourable protective response, as has been demonstrated previously, including studies on obese animals which have shown

increased adipose but reduced hepatic 11β-HSD1 (Alberts et al 2005;Liu et al 2003;Livingstone et al 2000;Masuzaki et al 2001). The loss of this protective response may predict the onset of glucose intolerance and diabetes, and may well relate to body fat distribution as detailed below.

6.5.2.1. Free fatty acids (FFA) and insulin sensitivity with relevance to cortisol metabolism

FFA are an important link between obesity, insulin resistance and type 2 diabetes (Boden 1999). As expected, FFA were significantly elevated in obese subjects compared with normal controls, and in the diabetic group compared both with obese and normal controls. Multivariate regression modelling confirmed that serum FFA independently predicted fasting insulin and the impact of 11β-HSD1 on fasting insulin was strongly influenced by FFA. This finding was associated with clear differences in the relationship between fasting serum FFA and hepatic glucocorticoid metabolism in the different cohorts. In the normal group FFA was negatively associated with hepatic 11β-HSD1 activity. Low FFA levels in this group in conjunction with this association would serve well to protect hepatic metabolic phenotype. In the obese cohort serum fasting FFA correlated with increased 5α reductase mediated cortisol clearance. Increased FFA delivery to the liver from the splanchnic circulation in obesity may have a direct effect upon the liver to increase 5α reductase mediated cortisol clearance to preserve hepatic phenotype and limit hepatic lipid accumulation. This would be supported by similar findings in hepatic steatosis, an early stage of NAFLD, the hepatic phenotype of the metabolic syndrome chapter 7. It is however important to note the results of a study which showed that specific aliphatic

unsaturated fatty acids inhibited 5α-reductase (Liang and Liao 1992). In diabetic subjects fasting free fatty acids correlated with increased hepatic 11β-HSD1 activity. This in addition to the HPA axis activation seen in diabetics would have a deleterious effect by further promoting glucocorticoid mediated hepatic glucose output and hepatic fat accumulation as seen in the diabetic cohort, as well as promoting adipose tissue lipolysis, further increasing FFA delivery to the liver.

The total serum cholesterol in the diabetic group was not easy to interpret perhaps due to the lasting effects of statin therapy in that group, despite patients having stopped therapy three days before participating in the study. However, glucocorticoids are known to exert and anti-atherosclerotic effect possibly by limiting local inflammation in the endothelium (Hadoke et al. 2009), and 11β-HSD1 inhibition has been shown to limit the progression of atherosclerosis in mice (Hermanowski-Vosatka et al. 2005). Further investigations are required to clarify the link between glucocorticoid excess and cardiovascular events and to determine the mechanism through which glucocorticoid treatment inhibits atherosclerosis/restenosis. This will provide greater insights into the potential benefit of selective 11β-HSD1 inhibitors in treatment of cardiovascular disease.

Collectively, these data support the hypothesis of a reduction in 11β-HSD1 activity may act as a protective mechanism to prevent increasing adiposity and increased hepatic glucose output with advancing obesity. This adaptive mechanism is altered in obesity associated type 2 diabetes both at the tissue specific level in liver, muscle and adipose as well as at the level of HPA axis function, and may therefore be implicated in the underlying pathogenesis of the disease. The importance of glucocorticoid metabolism in lean mass and hence skeletal muscle insulin resistance has also been

discussed. This work provides support for the observed and predicted effects of selective 11β-HSD1 in the treatment of the metabolic syndrome and its multifactorial effects.

7. Cortisol metabolism and hepatic expression of 11β-hydroxysteroid dehydrogenase type 1 in patients with non alcoholic fatty liver disease

7.1. Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and is now the commonest liver problem of the western world, and the leading cause of cryptogenic cirrhosis. NAFLD represents a spectrum of liver disease ranging from simple and reversible hepatic steatosis, to non alcoholic steato-hepatitis (NASH) where there is evidence of inflammation and liver cell death. It may progress to cirrhosis with liver failure, and hepatocellular carcinoma. NASH cirrhosis is rapidly becoming the leading indication for liver transplantation.

Critically, the histological diagnosis at presentation predicts prognosis in these patients. Those with simple fatty liver at presentation only have a 2% risk of progressing to end stage cirrhosis in a 20 year period. However when there is evidence of steatohepatitis or fibrosis, the risk of developing cirrhosis is up to 50% in a 2 year period (de Alwis and Day 2008).

The pathogenesis of NAFLD is poorly understood but several factors are thought to be important, including, insulin resistance, obesity and type 2 diabetes; 90% of patients with NAFLD cirrhosis having obesity and/or diabetes mellitus.

Patients with the metabolic syndrome share many phenotypic features with Cushing's syndrome (such as hypertension, central obesity, insulin resistance and impaired glucose tolerance). Indeed, 20% of patients with Cushing's syndrome have NAFLD (Rockall et al 2003), and there are a number of reports that implicate excess glucocorticoid (GC) in hepatic triglyceride accumulation (Dourakis et al. 2002;Nanki et al. 1999). Glucocorticoids promote steatosis by directly stimulating hepatic de novo lipogenesis and FFA utilization (Dolinsky et al. 2004;Hellerstein 1999;Norrheim et al. 1990), and by a secondary effect on adipose tissue, promoting lipolysis within omental fat, resulting in increased portal free fatty acid (FFA) delivery to the liver (Baxter and Forsham 1972). Cushing's syndrome is rare and the vast majority of patients with the obesity-metabolic syndrome have normal circulating cortisol levels. However, local GC concentrations within key metabolic target tissues are controlled at the pre-receptor level through a series of enzymes; 11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1), interconverts hormonally inactive cortisone (E) to active cortisol (F) and, 5α and 5β reductase which inactivate cortisol. There is increasing evidence for the role of 11β-HSD1 in the pathophysiology of the metabolic

syndrome through its amplification of GC responses in key insulin target tissues, resulting in visceral obesity and other features of the metabolic syndrome (Bujalska, Kumar, & Stewart 1997;Masuzaki et al 2001;Morton et al 2001).

We have proposed that dysregulation of hepatic GC metabolism may be critical in the progression of NAFLD and that increased regeneration (11β-HSD1) or decreased clearance (5α-reductase) contributes to the hepatic phenotype. We have therefore performed a detailed characterisation (*in vivo* and *ex vivo)* of GC metabolism in patients with NAFLD compared with BMI matched controls. A number of cross sectional studies have reported the association of NAFLD with chronic, subclinical activation of the hypothalamo-pituitary-adrenal (HPA) axis in humans (Konopelska et al 2009;Targher et al. 2006;Westerbacka et al 2003). None of these studies however have undertaken a detailed analysis of hepatic pre receptor cortisol metabolism in patients with NAFLD.

7.2. Methods

7.2.1. Subjects

Clinical studies were carried out on 16 patients recruited from hospital clinics. All patients had chronically elevated liver enzymes and evidence of hepatic steatosis on ultrasound. The diagnosis of NAFLD was made on histological analysis of biopsies after exclusion of other possible aetiological factors (alcohol intake of >20g/day, viral and autoimmune hepatitis and hepatototoxic drugs). Renal function was normal and none were taking any drugs known to interfere with the HPA axis (glucocorticoids,

anticonvulsants, oestrogen treatment). 5 patients had type 2 diabetes (2 diet controlled, 3 treated with metformin)

32 healthy age, sex and BMI matched control volunteers recruited were by local advertisements. All had normal liver function biochemistry (AST, γGT, ALT, ALP and bilirubin).

Separately, liver samples were obtained from the liver tissue archive at the Centre for Liver Research, University of Birmingham (section 2.1) and were used for gene and protein expression studies. All diagnoses were verified by histological analysis (NASH n=5, normal transplant donor liver n=5). None of the donor organs were obtained from institutionalized persons.

7.2.2. Clinical Studies

The study and protocol received approval from the local ethics committee and written informed consent was obtained from all participants. The NAFLD subjects formed the fourth patient group of the clinical study.

The clinical methods were as detailed in sections 6.3.1 - 6.3.6. and included anthropometric measurements, whole body composition using DEXA, CT measurements of fat distribution, whole body and hepatic 11β-HSD activity and oral glucose tolerance testing. Biochemical analysis was carried out as described in section 6.3.4.

7.2.3. Real Time PCR

11β-HSD1 mRNA levels were measured by real-time PCR. The method is described in section 2.3 in detail.

7.2.4. Immunohistochemistry

Immunohistochemistry for 11β-HSD1 was carried out on five explanted livers with severe NASH and five normal donor livers. The method has been described in section 2.2.5.2 in detail.

7.2.5. Statistical Analysis

Data are presented as means ± SE unless otherwise stated. Area under the curve (AUC) analysis was performed using the trapezoidal method. For comparison of single variables, *t* tests were used (or nonparametric equivalents where data were not normally distributed). Regression analyses were performed using Pearson correlations; where more than one variable was considered, multiple linear regression analysis was used. Analysis was performed using SPSS Statistics 17.0 software.

7.3. Results

7.3.1. Clinical and biochemical characteristics of participants

Metabolic phenotype and hepatic cortisol metabolism was characterised in patients with histologically proven NAFLD (n=16 of whom 7 had steatosis only and 9 had NASH) compared to age and BMI-matched controls (n=32). As expected, intra-hepatic fat (measured by liver:spleen attenuation ratio (L:S) on CT) was higher in the NAFLD group compared to controls (L:S 0.81± 0.08 vs. 1.13±0.04, $p<0.01$). Fasting serum free fatty acids (FFA) were significantly increased in the NAFLD group and correlated with intrahepatic fat measured by CT ($r = 0.4$, $p<0.05$). Baseline clinical and biochemical characteristics of both groups are presented in Table 7-1.

Variable	NAFLD patients	Control subjects	*P* values
age (years)	47 ± 2.6	47 ± 2.0	NS
BMI kg/m^2	36.0 ± 2.4	32.4 ± 0.9	NS
sag height (cm)	25.2 ± 1.2	23.3 ± 0.6	NS
%Fat	33.1 ± 2.0	38.5 ± 1.1	<0.05
Liver:spleen attenuation ratio	0.81 ± 0.08	1.13 ± 0.04	<0.01
Creat (μmol/L)	91.5 ± 3.0	89.5 ± 3.0	NS
γGT (iu/L)	103 ± 33	24 ± 2.6	<0.01
Total cholesterol (mmol/L)	5.4 ± 0.3	6.8 ± 1.5	NS
HDL cholesterol (mmol/L)	1.2 ± 0.1	1.4 ± 0.1	<0.05
Triglycerides (mmol/L)	2.2 ± 0.3	1.4 ± 0.13	<0.01
ALT (iu/L)	63.0 ± 10.6	25.1 ± 3.0	<0.01
ALP (u/L)	191 ± 19	173 ± 8	NS
AST (iU/L)	45.2 ± 5.7	22.7 ± 1.1	<0.01
fasting FFA (μmol/L)	422 ± 35	336 ± 17	<0.05
fasting glucose (mmol/L)	5.6 ± 0.4	4.7 ± 0.1	<0.05
Systolic blood pressure (mmHg)	134 ± 2.7	136 ± 3.7	NS
Diastolic blood pressure (mmHg)	78 ± 3.0	78 ± 2.5	NS

Table 7-1 *Baseline clinical characteristics of patients with NAFLD and controls. Data are presented as means ± SE. *CT measured liver:spleen attenuation ratio (mean ± SEM). Lower ratio implies increased intrahepatic fat. Whole body fat measured by DEXA.*

7.3.2. Urinary steroid metabolite analysis

24h urinary steroid metabolite analysis by GC/MS demonstrated increased cortisol clearance with higher 5αR activity in patients with hepatic steatosis only, (5αTHF/THF ratio, controls 0.80 ± 0.07, steatosis 1.31 ± 0.21, NASH 0.90 ± 0.30; steatosis vs obese: $p < 0.05$), Figure 7-1A. 5α-, and not 5β-reduced metabolites were increased in the steatosis group, Figure 7-1B. Absolute values are presented in Table 7-2. Interestingly, in both NAFLD and control groups there was a correlation between FFA and hepatic cortisol clearance as measured by the urinary 5αTHF/THF ratio ($r = 0.4$, $p<0.05$), Figure 7-2.

Steroid (µg/24h)	controls	steatosis	NASH
Cortisol	71 ± 7	78 ± 8	112 ± 20[a]
Cortisone	120 ± 11	127 ± 20	162 ± 39
THE	3426 ± 346	5626 ± 554	3447 ± 326[c]
THF	1624 ± 169	1973 ± 199	1759 ± 231
5α-THF	1324 ± 178	2500 ± 417[a]	1429 ± 300
α-cortol	316 ± 27	379 ± 42	496 ± 90[a]
β-cortol	457 ± 36	533 ± 102	515 ± 97
α-cortolone	1303 ± 111	1899 ± 206[a]	1789 ± 256
β-cortolone	621 ± 57	834 ± 112	641 ± 91
Total F metabolites	9266 ± 857	13949 ± 1075[a]	10351 ± 984[d]
Ratios			
F/E	0.61 ± 0.03	0.65 ± 0.06	0.74 ± 012
(THF+5αTHF)/THE	0.89 ± 0.04	0.81 ± 0.06	0.96 ± 0.13
cortols/cortolones	0.43 ± 0.02	0.33 ± 0.02[a]	0.42 ± 0.04
5αTHF/THF	0.84 ± 0.07	1.31 ± 0.22[a]	0.93 ± 0.3
An/ET	1.11 ± 0.10	1.99 ± 0.31[b]	1.55 ± 0.6

Table 7-2 Urinary steroid metabolites and ratios in patients with steatosis, NASH and control patients. Mean absolute values are shown (µg/24h) +/- SEM. [a]$p < 0.05$ vs controls, [b]$p < 0.01$ vs controls, [c]$p < 0.05$ vs steatosis, [d]$p < 0.01$ vs steatosis.

In addition, total cortisol metabolites were significantly increased in patients with steatosis consistent with increased glucocorticoid production rate, Table 7-2, Figure 7-1C. The urinary THF+5αTHF/THE ratio was lower in the steatosis group and elevated in the steatohepatitis group but this did not reach statistical significance.

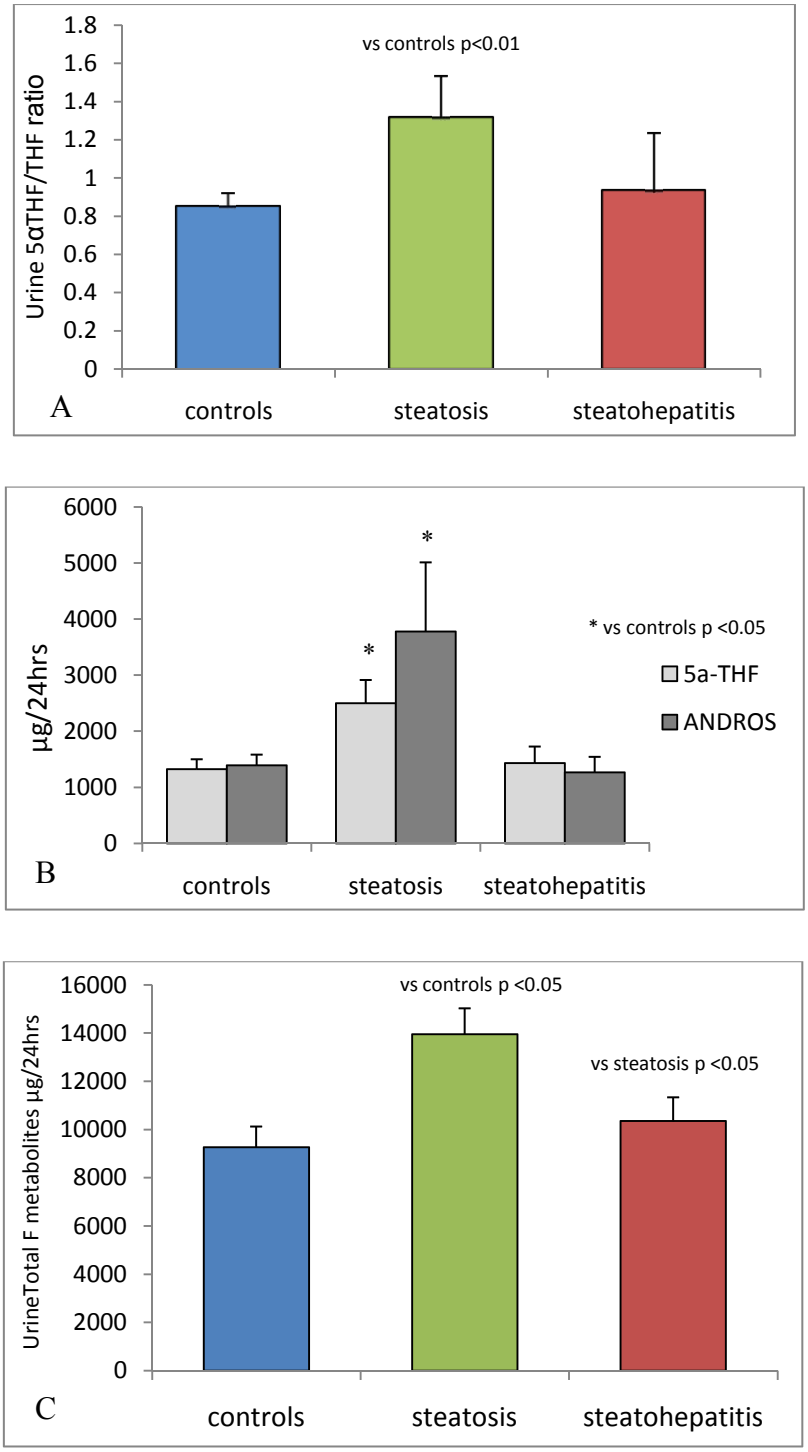


Figure 7-1 *5α-reductase activity as depicted by the urinary 5αTHF/THF ratio (mean ± SEM) (A), total 24 Urine 5α-reduced metabolites (mean ± SEM) (B), total 24hr Urine F metabolites (mean ± SEM) (C), in patients with steatosis and steatohepatitis compared with controls.*

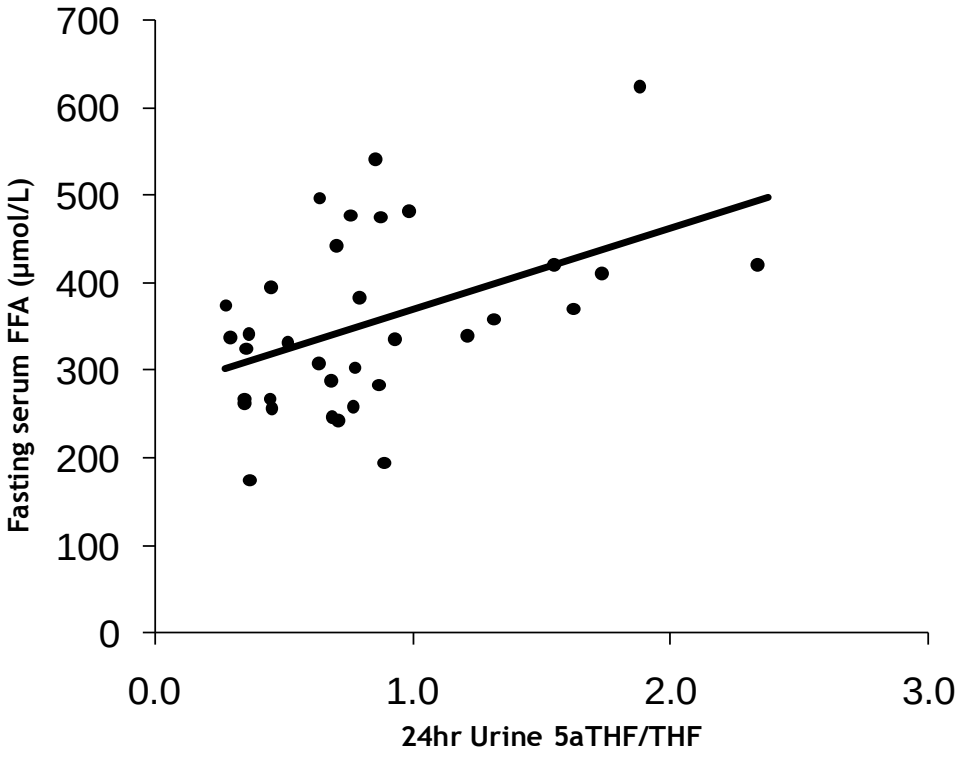


Figure 7-2 Serum fasting FFA in NAFLD patients and controls correlates with 5α-reductase activity (R=0.4, p<0.05).

However the cortols/cortolones ratio, which also reflects 11β-HSD1 activity, was significantly reduced in the steatosis group, Table 7-2, Figure 7-3. Importantly the urine UFF/UFE ratio was similar between groups indicating that there was no difference in extrahepatic 11β-HSD2 activity, Table 7-2.

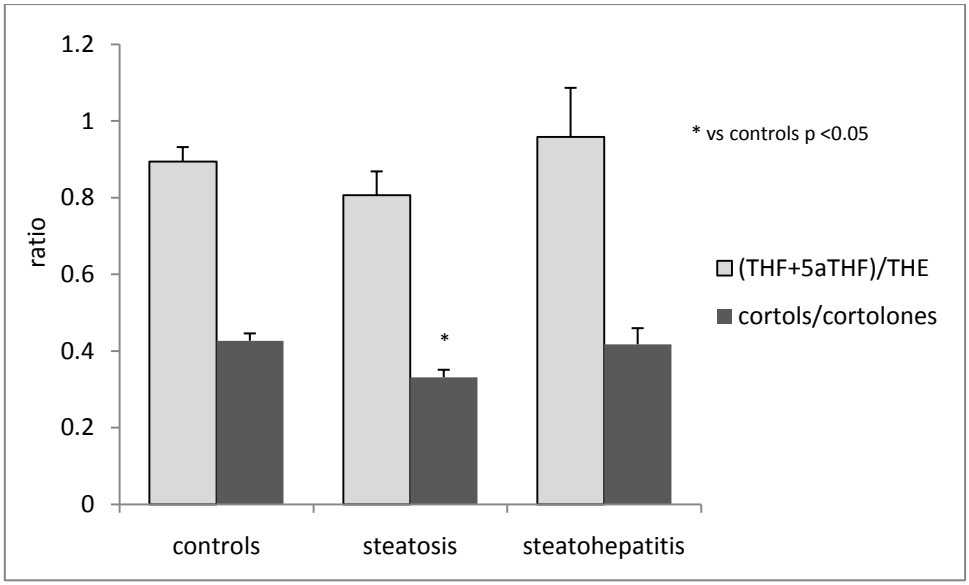


Figure 7-3 *11β-HSD1 activity assessed by 24hr urine cortols/cortolones and 5αTHF+THF/THE ratios (mean ± SEM) in patients with steatosis and steatohepatitis compared with controls.*

7.3.3. Cortisol Generation Profiles

Endorsing the urinary steroid metabolite data, cortisol generation from oral cortisone was decreased in patients with steatosis compared with controls Figure 7-4. In contrast, patients with steatohepatitis had significantly increased cortisol generation consistent with increased hepatic 11β-HSD 1 activity compared with controls and patients with steatosis, (AUC cortisol: controls 382 ± 22, steatosis 304 ± 27, NASH 496 ± 22 μg/L.min; steatosis vs obese $p < 0.05$, NASH vs obese $p < 0.05$, NASH vs steatosis $p < 0.01$), Figure 7-4.

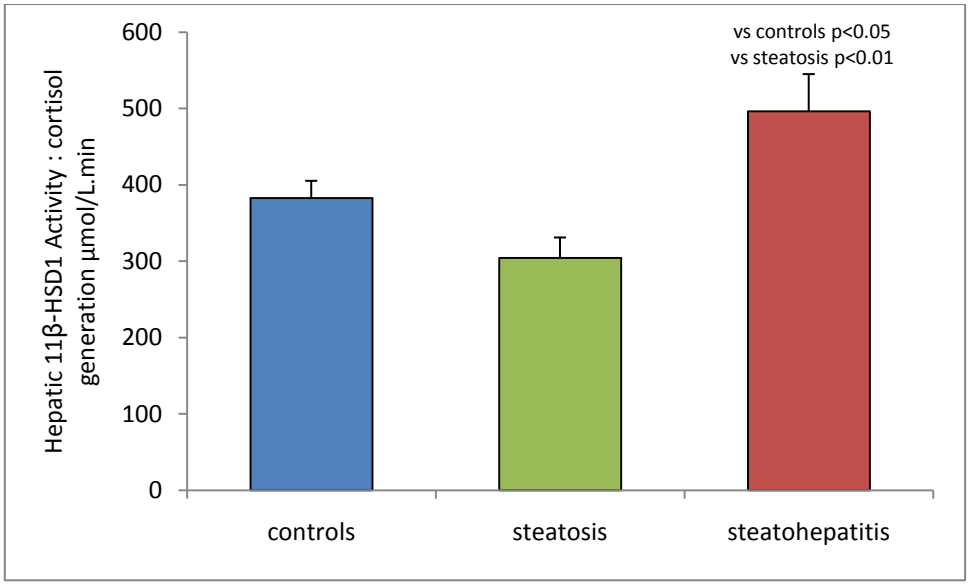


Figure 7-4 *Hepatic cortisol generation measured by cortisol generation profiles (mean AUC ± SEM) in patients with steatosis and steatohepatitis compared with controls.*

7.3.4. 11β-HSD1 expression studies

11β-HSD1 mRNA expression in explant livers from patients with NASH was significantly higher compared with normal controls (dCT NASH 9.65 ± 0.29 vs 11.96± 0.29, $p < 0.01$ NASH vs normal), Figure 7-5.

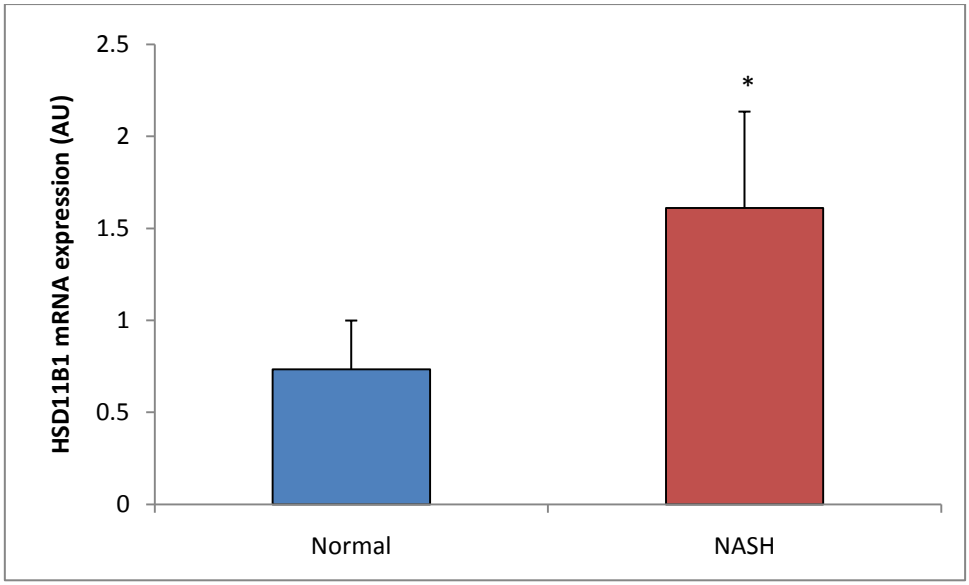


Figure 7-5 Real time PCR mRNA expression data for 11β-HSD1 on whole liver samples from 5 normal patients and 5 NASH patients expressed as arbitrary units +/- SE.

These results were supported at the protein level by immunohistochemistry; liver parenchymal staining for 11β-HSD1 was increased in NASH livers compared with normals. Specifically, intense staining was seen in the periseptal areas in particular at limiting plate. There was also staining within the portal/septal inflammatory cells with a morphology in keeping with macrophages, Figure 7-6 A-E.

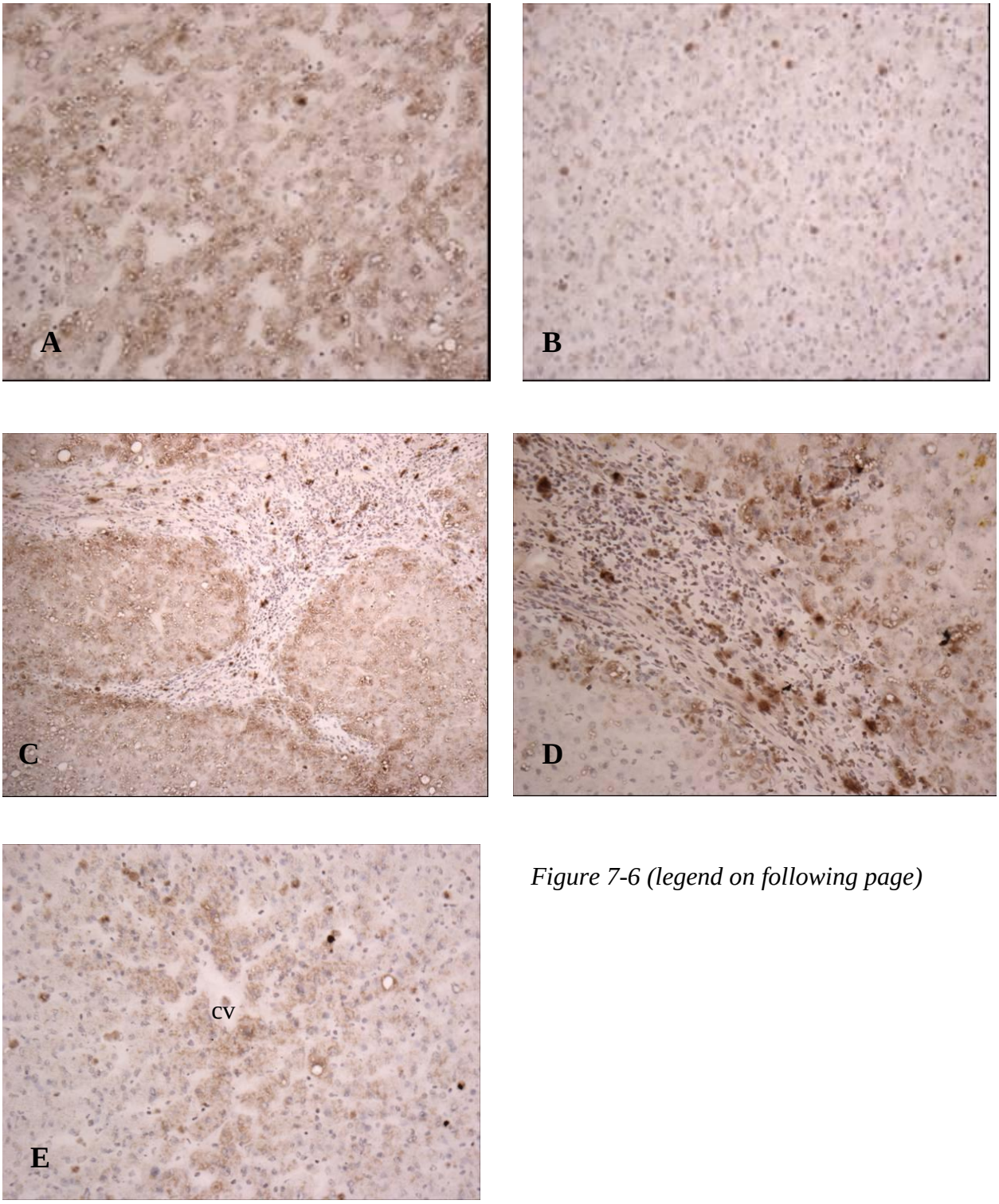


Figure 7-6 (legend on following page)

Figure 7-6 *11β-HSD 1 protein expression in liver sections from patients with severe NASH compared to normal controls. There was generally increased staining for 11β-HSD1 throughout the liver parenchyma in (A) NASH samples compared with (B) Normal liver x20. (C) Increased staining at the limiting plate in peri-septal areas in NASH x10 (D) Increased staining at the limiting plate in peri-septal areas and in portal/septal inflammatory cells with a morphology in keeping with macrophages in NASH x20 (E) Peri-central zonation of 11β-HSD1 in normal liver x20 (cv – central vein).*

7.4. Discussion

Results from this study have defined hepatic glucocorticoid metabolism in patients with the full spectrum of NAFLD, illustrated in Figure 7-7.

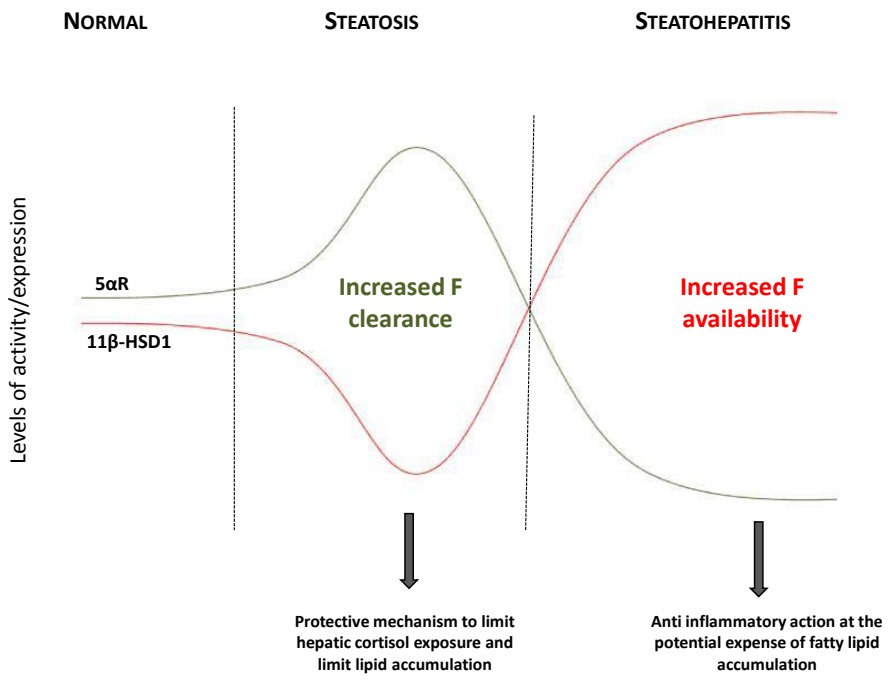


Figure 7-7 *Schematic: Hepatic glucocorticoid metabolism and its modulation in response to disease progression in NAFLD*

In the early stages of NAFLD, characterized by hepatic steatosis alone, hepatic cortisol clearance predominates, driven by increased 5αR activity, decreased cortisol

generation by 11β-HSD1 and consequent increased adrenal glucocorticoid production. With disease progression and worsening inflammation and liver injury, there is induction of hepatic 11β-HSD1 expression and activity with increased hepatic glucocorticoid generation, Figure 7-7. Collectively these results provide a crucial insight into the pathophysiology of the NAFLD disease spectrum.

The first suggestion of a role of 11β-HSD1 in NAFLD came with the observation that transgenic mice over expressing 11β-HSD1 in adipose tissue develop the full phenotype of the metabolic syndrome including hepatic steatosis. Conversely 11β-HSD1 knockout mice are protected from many of these features including hepatic steatosis (Masuzaki et al 2001;Morton et al 2001). Transgenic mice overexpressing the 11β-HSD1 gene selectively in the liver under the transcriptional control of the human apoE gene, exhibit fatty liver with increased hepatic triglyceride accumulation and impaired hepatic lipid clearance (Paterson et al 2004). Furthermore 11β-HSD1 inhibition reduced hepatic triglyceride concentration by nearly 30% and increased in vivo hepatic fat oxidation and expression of related genes in rats fed an obesogenic diet (Berthiaume et al. 2009), reflecting the detrimental effect of glucocorticoids upon liver function by impacting on a number of pathogenic mechanisms.

We postulate that the increase in hepatic cortisol clearance by 5α-reductases, and decreased 11β-HSD1 driven hepatic cortisol generation in hepatic steatosis is a protective mechanism to preserve hepatic metabolic phenotype by limiting hepatic cortisol exposure and reduce glucocorticoid induced deleterious effects. These include hepatic de novo lipogenesis, β-oxidation and increased glucose output all of which worsen hepatic and peripheral insulin resistance. Indeed, recent work from our group has shown a reduction in 5α-reductase activity with weight loss in otherwise healthy

obese subjects (Tomlinson et al. 2008b). Women with polycystic ovarian syndrome who are characterized by a susceptibility to the metabolic syndrome also have increased 5α-reductase activity and adrenocortical drive (Vassiliadi et al. 2009). Both these groups of patients have an increased propensity to develop NAFLD, and these findings may represent a protective hepatic mechanism to limit hepatic lipid accumulation and dysregulation of glucose homeostasis, thus preserving hepatic insulin sensitivity, with a knock on effect of limiting extrahepatic insulin resistance. Our data support previously published findings of a subtle activation of the HPA axis in patients with NAFLD, (Konopelska et al 2009;Targher et al 2006). This may reflect the increased clearance of hepatic glucocorticoid by the 5α-reductase enzymes as shown in our steatosis patients.

By contrast, increased hepatic glucocorticoid availability is a favourable protective mechanism to limit further hepatic inflammation and injury. Immunologic responses are suppressed by glucocorticoid excess, an observation which provided the stimulus to develop potent glucocorticoids for treatment of a wide range of inflammatory and autoimmune conditions, and organ transplantation. Indeed, tissue inflammation is known to activate the HPA axis through release of cytokines into the circulation and the stimulation of vagal afferents, an interaction that has been termed the 'neuroimmune axis'(Turnbull and Rivier 1999). Elevated plasma cytokines have been demonstrated in patients with chronic hepatic inflammation, and chronic exposure to these cytokines has been shown to lead to dysregulation of the HPA axis (Turnbull & Rivier 1999). Indeed rats with cholestatic liver injury show defective HPA axis activation in response to psychological stress, with an attenuation of glucocorticoid release into the circulation compared with control animals (Swain et al

1993). Our urinary data also reflect decreased HPA axis activation in patients with NASH compared with steatosis. Hence, an inherent mechanism to increase local glucocorticoid availability by increasing 11β-HSD 1 activity and expression would be a crucial response in an attempt to limit further liver inflammation. Specific induction of 11β-HSD1 activity was seen but only in patients with steatohepatitis. In livers with NASH increased staining of 11β-HSD1 was seen in the peri septal areas, specifically opposed to areas of pathological fibrogenesis and inflammation. The appearances would be consistent with a secreted factor from the inflammatory infiltrate inducing 11β-HSD1 in the adjacent hepatocytes. Studies on other tissues, including synovium from patients with rheumatoid arthritis (Hardy et al. 2008), human and rodent colitis (Zbankova et al. 2007), aortic smooth muscle cells (Dover et al. 2007), and granulosa cells in the inflammatory response to ovulation (Rae et al. 2004), all show a consistent picture of induction of cell specific 11β-HSD1 gene expression in response to pro inflammatory cytokines, TNFα and IL-1β being the most commonly implicated (Cooper et al 2001). The molecular mechanism by which 11β-HSD1 is induced in response to cytokines is not entirely clear, but key transcription factors of the C/EBP family play a crucial role (Chapman et al. 2009;Williams et al 2000).

There was also intense staining of 11β-HSD1 in portal/septal inflammatory cells with morphology in keeping with macrophages in NASH livers. Clearly local amplification of glucocorticoids by macrophages in the chronic inflammatory infiltrate would dampen the inflammatory response and promote macrophage phagocytosis of inflammatory cells. In vitro models of acute inflammation showed the dynamic regulation of 11β-HSD1 in human monocyte macrophages by pro resolution

mechanisms - 11β-HSD1 was down regulated following phagocytosis of apoptotic neutrophils (Gilmour et al. 2006).

Simple hepatic steatosis is a relatively benign entity in the NAFLD disease spectrum with only a 2% risk of developing progressive disease in a twenty year period. However, the presence of fibrosis or inflammation at diagnosis is associated with a risk of developing NASH cirrhosis of up to 50% in a two year period (de Alwis & Day 2008). The factors implicated in the crucial switch between simple steatosis and NASH are not entirely clear. Increased liver fat is pivotal to inflammation in NAFLD, and thus the increased supply of free fatty acids to the liver, associated with adipose tissue insulin resistance and obesity is a key factor in the development of hepatic inflammation in NAFLD. Our data show significantly increased fasting serum FFA in patients with NAFLD compared with BMI matched controls which correlated with intrahepatic fat. Adipose tissue insulin resistance may occur in obesity in part through the infiltration of macrophages which release pro inflammatory cytokines such as TNFα, IL-6 and IL1β (Weisberg et al. 2003). Once FFA are taken up by the liver, as well as being oxidized and stored as triglyceride, they activate the transcription factor NFκB, a key regulator of gene transcription of proinflammatory cytokines, adhesion molecules, and chemokines (Feldstein et al. 2004). What results is a cycle of hepatic injury and inflammation. The cytokines released from hepatocytes, in particular TNFα activate classic inflammatory cells, as well as Kupffer cells which generate more cytokines. These cytokines further contribute to hepatic oxidative stress by promoting FFA oxidation, which enhances the hepatic injury that occurs by cytokine driven hepatocyte apoptosis and necrosis (Cai et al. 2005).

An important question that arises from this work is whether 11β-HSD1 activity and expression is induced in other types of chronic hepatitis. Our work on alcoholic liver disease (ALD) showed significant induction of 11β-HSD1 expression in explanted livers with ALD, with a smaller but significant induction in a mixed group of (non alcoholic, non fatty, and non infectious) severe liver disease (CLD). This was supported by clinical data from selective venous sampling that showed net cortisol generation specifically in patients with ALD but not CLD (Ahmed et al 2008), chapter 5. The histological appearance of severe NASH, ALD and hepatitis C are relatively similar – all involving a degree steatohepatitis. It would therefore be plausible to suggest a role for 11β-HSD1 induced local glucocorticoid production in the pathophysiology of severe chronic hepatitis and would certainly merit further investigation.

This work has defined hepatic glucocorticoid metabolism in progressive NAFLD, which can be summarized into two distinct phases of altered regulation of hepatic cortisol metabolism; increased hepatic cortisol clearance in steatosis, and increased hepatic cortisol regeneration in NASH. Failure to regulate in this way may worsen the phenotype of liver disease i.e. drive hepatic steatosis or unchecked progressive hepatic inflammation. Hence the therapeutic benefit of 11β-HSD1 inhibition may critically depend on the histological stage of NAFLD.

8. Conclusions and future studies

The work described in this thesis has established a pivotal role of hepatic glucocorticoid metabolism by 11β-HSD1 in normal physiology, insulin resistant states and chronic metabolic liver diseases, with particular relevance to hepatic and whole body insulin sensitivity.

In vitro studies characterized the zonation of hepatic 11β-HSD1 mRNA and protein expression in detail, confirming perivenous zonation. By inference, the impact of hepatic 11β-HSD1 on hepatic glycolyis, and the resulting impact on whole body insulin sensitivity is a novel suggestion of the physiological role of hepatic 11β-HSD1, and may explain many of the observations from the clinical study comparing normal, obese and diabetic cohorts. This is in contrast to the intense focus of numerous published and ongoing studies on the importance and impact of 11β-HSD1

on hepatic glucose output via gluconeogenesis and represents an exciting new paradigm that warrants further investigation.

Hepatic pre receptor glucocorticoid metabolism resulting in changes in hepatic glucocorticoid activation and/or glucocorticoid clearance was found to be implicated in a range of liver diseases and may thus be associated with the dysregulation of insulin sensitivity seen in these conditions. In particular, hepatic glucocorticoid metabolism was found to be abnormal in each histological stage of non alcoholic fatty liver disease (NAFLD) (the hepatic phenotype of the metabolic syndrome). In alcoholic liver disease there was clear evidence of increased hepatic glucocorticoid activation and 11β-HSD1 gene expression, that may be important in defining the cause of the alcoholic 'pseudocushings' state.

Glucose 6 phosphate was defined as a direct link between glucose and glucocorticoid metabolism and the HPA axis and formed a seminal publication; the concept of ER nutrient sensing with respect to glucose and cortisol metabolism is now a major focus of research activity.

Clinical studies comparing glucose and glucocorticoid metabolism in normal, obese and type 2 diabetic subjects revealed new data with relevance to the importance of lean body mass and insulin sensitivity and a strong association with HPA axis function and hepatic 11β-HSD1 activity. In addition the association of hepatic lipid accumulation with suppression of hepatic 11β-HSD1 and stimulation of 5α-reductases in insulin resistant states without any clinical suggestion of hepatic steatosis was confirmed, strongly endorsing the findings of the NAFLD study.

Collectively, the data presented in this thesis provide support for the role of selective 11β-HSD1 inhibition for the treatment of insulin resistant states and type 2 diabetes.

However, important questions arise with regard to the use of these agents in the presence of inflammatory liver disease, in particular non alcoholic steatohepatitis, where increased hepatic glucocorticoid activation, and decreased glucocorticoid clearance serve as a protective response to limit worsening hepatic inflammation and injury. In addition the predicted impact of these inhibitors upon hepatic glycolysis and skeletal muscle insulin resistance warrants further investigation.

In addition to the above, this work has led to the possibility of a number of future studies. It would be of great importance to fully define the physiological role of the perivenous distribution of hepatic 11β-HSD1 and its relevance upon glycolytic pathways and the resulting impact upon whole body and in particular skeletal muscle insulin sensitivity. An initial study would include the comparison of the activity and expression of key glycolytic enzymes in normal and 11β-HSD1 knockout mice.

It would be of significant interest to characterise hepatic zonation of 11β-HSD1 in progressive stages of NAFLD, as well as other chronic liver diseases (such as alcoholic liver disease and chronic hepatitis C) that are strongly associated with insulin resistance and histologically bear close resemblance with NAFLD. This would be allied with studies to define the localisation of 11β-HSD1 within inflammatory cells in hepatitis and the factors involved in the activation of this process. In normal liver it would be of interest carry out *in vitro* studies to investigate the presence of 11β-HSD1 in hepatic stellate cells, and its possible role in stellate cell activation.

In addition, clinical studies to define hepatic glucocorticoid metabolism *in vivo*, in progressive stages of NAFLD would be important. Animal models of NAFLD would be a useful tool to explore the role of selective 11β-HSD1 inhibition in progressive

stages of the disease. On the basis of the results of the NAFLD study, a novel role for selective 5α-reductase inhibition would be as a therapy specifically for steatohepatitis and other forms of chronic hepatitis. Its aim would be to reduce hepatic glucocorticoid clearance, and hence maximise endogenous hepatic glucocorticoid to limit inflammation. Its role if proven would be very significant in its ability to exert a tissue specific increase in hepatic glucocorticoid without the multiple undesirable effects of systemic glucocorticoid therapy.

Further clinical studies in type 2 diabetes are warranted to examine hepatic and whole body glucocorticoid metabolism in progressive disease. This study and many others previously reported specifically chose diet controlled diabetics or those with early disease. It would be important to extend these investigations to diabetic subjects with increased states of insulin resistance and type 2 diabetics with evidence of beta cell failure. Technically these studies would be more challenging but would provide important information of the detrimental or beneficial effect of 11β-HSD1 driven glucocorticoid activation and hepatic glucocorticoid clearance by A-ring reductases. Results from the stable isotope studies quantifying gluconeogenesis in normal and diseased states, and its relationship with hepatic and whole body glucocorticoid metabolism carried out as part of the work for this thesis will provide key results in this area.

The studies defining glucose 6 phosphate as a novel regulator of 11β-HSD1 driven glucocorticoid activation lead to many important further studies, many of which have already led to early findings being published by other investigators. These would include a detailed definition of the role H6PDH and 11β-HSD1 in ER redox and ER nutrient sensing. Further investigation of glycogen storage diseases type 1a and 1b

may implicate 11β-HSD1 in the pathogenesis of a number of unexplained complications of these diseases. In particular the role of excess glucocorticoid activation in bone as a contributing cause of osteoporosis in GSD1a, and the absence of 11β-HSD1driven local glucocorticoid production in bowel in GSD 1b patients who have a specific propensity to develop inflammatory bowel disease. It would also be of interest to investigate the role of the hepatic glucocorticoid excess seen in GSD type 1a in the significant hepatic lipid accumulation that occurs in the disease.

The results described in this thesis form key data with clear clinical importance and relevance in the application of 11β-HSD1 inhibition. Further studies will follow that will provide a greater depth of understanding and offer new possibilities for the treatment of patients with the metabolic syndrome, a serious global public health concern.

List of Journal Publications arising from this Thesis

*Ahmed, A., Saksena, S., Sherlock, M., Olliff, S.P., Elias, E., & Stewart, P.M. 2008. Induction of hepatic 11beta-hydroxysteroid dehydrogenase type 1 in patients with alcoholic liver disease. *Clin.Endocrinol.(Oxf)*, 68, (6) 898-903 available from: PM:18031327

*Walker, E.A., Ahmed, A., Lavery, G.G., Tomlinson, J.W., Kim, S.Y., Cooper, M.S., Ride, J.P., Hughes, B.A., Shackleton, C.H., McKiernan, P., Elias, E., Chou, J.Y., & Stewart, P.M. 2007. 11beta-Hydroxysteroid Dehydrogenase Type 1 Regulation by Intracellular Glucose 6-Phosphate Provides Evidence for a Novel Link between Glucose Metabolism and Hypothalamo-Pituitary-Adrenal Axis Function. *J.Biol.Chem.*, 282, (37) 27030-27036 available from: PM:17588937 (JOINT FIRST AUTHOR publication)

*Copy of publication appended after references section

The work described in Chapter 7 was submitted to Hepatology in January 2010:

A Ahmed, T Brady, C Brown, P Guest, A Wagenmakers, P Newsome, S Hubscher, E Elias, D Adams, J.W.Tomlinson, P.M.Stewart. Cortisol metabolism and hepatic expression of 11β-hydroxysteroid dehydrogenase type 1 in patients with non alcoholic fatty liver disease

Appendices

Appendix 1: Patient information sheet for clinical study

Appendix 2: Nursing protocols for clinical study

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University Hospital Birmingham NHS
NHS Trust

Novel Targets in the Metabolic Syndrome: The Role of 11βHSD Type 1 and H6PDH

Information for Study Participants

Investigators : Professor PAUL M STEWART, *Consultant Physician and Professor of Medicine and Endocrinology, University of Birmingham*
Dr ADEEBA AHMED, *Clinical Research Fellow, University of Birmingham*

Thank you for taking the time to read this leaflet. We would like to invite you to be part of this study. If there is any further information that you would like then please don't hesitate to contact us.

What is the study about?

The link between obesity and sugar diabetes is well established and is recognised as an increasingly common health hazard. We have come up with a mechanism that might explain this link – an enzyme (11βHSD1) present in liver and fat that can generate a hormone called cortisol that directly predisposes to obesity and diabetes. This study will address the role of this enzyme and additional regulating enzymes specifically in the liver and their contribution to the development and progression of diabetes. Additional clinical studies will use novel methods to measure the release of sugar from the liver in patients with obesity, sugar diabetes and fatty liver and to compare this with normal volunteers. It will provide the key research basis for the development of a new treatment for obesity-related diabetes – '11βHSD1 inhibitors'. This would potentially have a huge impact in the management of diabetes and in particular delay or prevent the progression to treatment with insulin.

What will I have to do?

The study involves two periods of investigations. The first visit involves tests carried out over the course of one and a half days (involving an overnight stay), and a second visit from 9am to 1pm. The 2 periods of investigations will usually be separated by about a week, but can be suited to your convenience.

On the first day of the study, having arrived fasting from midnight the previous night, the first investigation will be commenced - a Glucose Tolerance Test. This test will tell us how well your body handles sugar and whether you have diabetes. It involves 3 blood samples taken from the cannula (a tiny plastic tube, which allows blood samples to be drawn without the need to use further needles for each sample) inserted into a vein in one of your arms – one while you are fasting, and two over the course of the next hour after having a sugary drink. After this test you may eat and drink normally. The same cannula will be used to take all the blood samples for the day.

This will be followed by a DEXA scan and CT scan of a small region of the abdomen. These scans are to help us calculate your total amount of body fat and the amount of fat in and around your liver. These investigations are painless and should be complete before 5pm.

At 6pm you will be given a 'standardised' meal – where we will exactly calculate the amount of carbohydrate, fat and protein according to your body weight. After this meal you will be fasting till 4pm (at the very latest) the following day – but can drink as much water as you wish. The fast may end sooner pending the results of our preliminary investigations. The water you will drink will contain a 'marker' so we can trace the passage of sugar and water in your liver. At 11pm, 2 blood samples will be taken from the cannula in your arm. At 6am the next morning, a small cannula will be inserted in back of the hand or lower forearm of the other arm, and your hand will be placed in a box which will keep it warmer then body temperature. Hourly blood samples will be taken from this cannula while a sugar solution is pumped at a very slow rate through the cannula in your free arm. This investigation will tell us exactly how your liver handles sugar and how much sugar it produces on its own while you are fasting. You may eat and drink normally after the test is completed.

Immediately prior to the second part of the study we ask you to complete a 24-hour urine collection (alternatively this can be done while you are in hospital during the first visit), and we will also give you a small dose of a steroid tablet that you will have to take at 11pm the evening before you attend for the second part of the study. On the first morning of the second part of the study we give you 2 further doses of the steroid tablet. Over the next 4 hours we take regular blood samples every 20 minutes from a cannula inserted into one of the veins in your arm. This test will tell us how well you activate this steroid in the liver. All the doses of steroid used in this study are small and have a short duration of action; within 12 hours they are completely out of the system. Using these doses there are no short or long term side effects

With your permission we will inform your GP that you are taking part in this study. If we detect any abnormality during the study we will inform you straight away and refer you to your GP for further treatment.

We will offer financial reimbursement to cover travel expenses and inconvenience as we appreciate that this study requires a great deal of your time during the 2 periods of investigation.

What are the benefits?

This study will help us to determine how well the body and in particular the liver is able to handle sugar and relate this to measures of activity of the two enzymes we are studying (11 beta hydroxysteroid dehydrogenase type 1 and hexose 6 phosphate dehydrogenase) . You will receive valuable information about your body fat distribution and sugar metabolism. You will also receive valuable lifestyle advice, and if you are overweight you will receive advice with regards to the lifestyle changes that will aid healthy and long term weight loss.

This is an important study that will tell us the crucial details of steroid metabolism. It will also serve as the platform and scientific basis for the mechanism of action and effectiveness of drugs that are currently in development. These drugs are likely to be used in the treatment of conditions including diabetes and obesity.

What are the risks?

The DEXA and CT scans involve the use of X rays. The amount of radiation received is similar to the 'background radiation' you would normally receive from the atmosphere over about 6-8 weeks. There are no other significant risks associated with taking part in this study.

What if I do not want to take part?

If having read this leaflet you feel you do not want to take part then you need take no further action. Importantly this will not affect your future medical care in any way.

What happens to the information?

All the information is entirely confidential. The only people who have access to the information are those who are directly involved with the study. With your permission we would like to inform your GP that you are taking part in this study.

Who else is taking part?

We are aiming to recruit 20 volunteers from different groups between the ages of 20 and 65.

Group 1: 20 healthy volunteers (10 males, 10 females), non diabetic, non obese.

Group 2: 20 volunteers with simple obesity (10 male,10 females).

Group 3: 20 volunteers (10 male, 10 females) matched for body mass index with group 2, with type 2 diabetes (diet controlled or on low dose metformin only).

Group 4: In collaboration with the regional liver unit, 20 patients admitted for investigation of 'fatty liver' with and without a history of alcohol ingestion will be studied.

Group 5: A small number of known patients with 'Cortisone Reductase Deficiency'.

Unfortunately, if you have a history of epilepsy, poorly controlled high blood pressure, steroid treatment, stroke, or if you are taking the oral contraceptive pill you will not be able to take part. In addition, if you are pregnant or planning pregnancy you will not be allowed to take part.

What if something goes wrong?

It is most unlikely that anything will go wrong. We will provide you with names and numbers of people who you will be able to contact in the unlikely event that anything does go wrong.

What happens at the end of the study?

At the end of the study the results will be published in medical journals

What if I have more questions or do not understand something?

If you do have any further questions or concerns then feel free to contact us (see below).

What happens now if I decide to take part?

If you have decided that you would like to take part then we will make an appointment for you to come up to our research facility where you will need to sign a consent form and we can then begin the study.

What happens if I change my mind during the study?

You are free to leave the study at any point for any reason. You do not have to tell us why you wish to leave the study and importantly it will not affect your future medical care in any way.

Contact name and number:

Dr. Adeeba Ahmed, Division of Medical Sciences, Institute of Biomedical Research, Queen Elizabeth Hospital, Birmingham. B15 2TT.

University Hospital Birmingham NHS
NHS Trust

If you would like to be part of this study or if you would like more information then please return this slip in the envelope provided or contact Dr Adeeba Ahmed at the email address / telephone number above.

Name ……Signature……Date……...

I would like / I would not like [delete as applicable] to be part of the study entitled '11β-hydroxysteroid dehydrogenase type 1 and Hexose 6 Phosphate Dehydrogenase in the metabolic syndrome'

Address……
……
……
……
Daytime Tel. ……..Fax. ……...……..
E-mail……..

Name:	Hospital No:
Subject No:	

Flow sheet

Visit 1 (overnight stay) date_____

Glucose Tolerance Test	**Sign**
• Dr Ahmed to clerk patient and consent for trial and obtain physical examination	
• Obtain prescription for, IV labelled glucose, deuterated water, (both supplied by Dr Ahmed) heparinised saline (dexamethasone, cortisone acetate on out patient prescription for visit 2) and sign dexa form and CT scan form	
• Check patient has fasted overnight • Order lunch (patient choice) • Phone kitchen and ask for Linda McCartney vegetarian lasagne from the 24 hour fridge as arranged for dinner	
• Complete page 1 and 2 of CRF including • BP, pulse, temp and oxygen saturations • height, weight and BMI • hip and waist measurements • Allergies, medical conditions, family history of diabetes • medications	
• Cannulate and take baseline bloods 1, 2a 6 and 7 (4 purple, 2 yellow, 1 grey,2 red)	
• Give 75g oral glucose	
• Take bloods 2a + 7 (1 grey, 1 red) at 30, 60, 90 and 120 minutes post glucose	
30 mins time =	
60 mins time =	
90 mins time =	
120 mins time =	
• Feed patient. DO NOT REMOVE CANNULA, PLEASE FLUSH WITH HEPARINISED SALINE	
• Patient to have dexa scan in the morning and CT scan if possible	
• Whilst patient is having dexa scan please measure saggital height and record on CRF. This is done once pt is lying flat on dexa table by placing spirit level (in box file) on pts stomach and measuring the distance from the table to the spirit level	
• Send letter to GP (from study file on computer, adding pt details)	

Name:	Hospital No:
Subject No:	

Deuterated Water Test

• 17.00 – 17.40 give patient standardised meal. **FAST FROM END OF MEAL EXCEPT WATER UNTIL END OF TEST APPROX 22 HOURS!**	
• **23.00 Baseline 1** • take bloods 2b, 4, 5 (1 grey, 2 purple, 4 large purple)	
• 23.00 give deuterated water, all water ingested from this time must contain the deuterated additive	
• **05.40 Cannula inserted into hand which is then placed into hot box at 60ºC and left for 20 minutes prior to blood sampling so the sample is an arterial sample taken at 06.00**	
• **06.00 Baseline 2** • 2b, 4, (1 grey, 2 purple)	
• Cannula to anticubital fossa to be used for IV infusion of 5% glucose containing tracer. To run for 10 hours, **start at 06.00**	
• 5 minutes from start of infusion give loading bolus over 30 seconds	
• Time infusion started =	
After 2 hours take hourly bloods from time infusion started **Remember to put patients' hand back into the hot box for 20 minutes prior to taking blood sample**	
+ 2 hour time = bloods 2b, 3 5 (1 grey, 1 purple, 4 large purple)	
+ 3 hours time = bloods 2b, 3 (1 grey, 1 purple)	
+ 4 hours time = bloods 2b, 3 (1 grey, 1 purple)	
+ 5 hours time = bloods 2b,3 (1 grey, 1 purple)	
+ 6 hours time = bloods 2b, 3, 5 (1 grey, 1 purple, 4 large purple)	
+ 7 hours time = bloods 2b, 3 (1 grey, 1 purple)	
+ 8 hours time = bloods 2b, 3 (1 grey, 1 purple)	
+ 9 hours time = bloods 2b, 3 (1 grey, 1 purple)	
+ 10 hours time = bloods 2b, 3, 5 (1 grey, 1 purple, 4 large purple)	
• Stop infusion, remove cannulas, feed patient	
• Give patient TTO for dexamethasone for visit 2	
• Issue patient with plain 24 hour urine bottle to bring back at visit 2	
• Arrange for visit two for within one weeks time if possible at 09.00	

Visit 2 Date _____

Cortisol Generation Profile

• Make sure patient has taken 1 mg of dexamethasone at **23.00** the previous night and 0.5 mg at **09.00** this morning	
• Collect urine save and decant as protocol	

Name:	Hospital No:
Subject No:	

• Patient may have CT scan today if it was not possible at first admission	
• Check patient fasted from 23.00 the previous night	
• **08.30**insert cannula into anticubital fossa vein	
• Take baseline bloods 6 (1 red top)	
• **09.00** (1 hour after administration of dexamethasone, 25mg of cortisone acetate is administered orally)	
• Bloods 6 (1 red top) to be obtained after the following times	
20 minutes time =	
40 minutes time =	
60 minutes time =	
80 minutes time =	
100 minutes time =	
120 minutes time =	
140 minutes time =	
180 minutes time =	
240 minutes time =	
• Patient to be given food and drink	
• Remove cannula and discharge	
• Patient given £40 expenses from Dr Ahmed on completion and asked to sign receipt form	

This flow sheet accurately represents the study protocol

Signed (research fellow) _____ Date_____

Processing of Samples

Bloods 1

Test	Bottle	Instructions	Distribution
U & Es, LFTs, cholesterols GGT	2 yellow tops	On biochemistry form tick renal, liver, bone, write on total chol, HDL, Trigs, ALT,GGT and tick fasting	Send to biochemistry
FBC	1 purple top	On haematology form tick FBC	Send to haematology
ACTH	1 purple top on ice	**Spin within 20 minutes** for 3000 rpm for 10 minutes at 4ºC	Aliquot into as many microcentrifuge tubes as possible Label with study no, subject no, date and ACTH, store in -80ºC
Genotyping	2 purple tops	**Do not spin**	Store 2 purple tops in -80ºC freezer, label with study no, subject no, date and DNA

Bloods 2a

Test	Bottle	Instructions	Distribution
glucose	1 grey top	On biochemistry form tick glucose and write on GTT **Add either 0, +30, +60 ,+90 or +120 to grey tops**	Send to biochemistry in 1 form

Bloods 2b

Test	Bottle	Instructions	Distribution
glucose	1 grey top	On biochemistry form tick glucose and write on 'deuterated water test' **Add date and time from infusion to grey tops i.e. baseline1, baseline 2, + 2 +3 +4 etc**	Send to biochemistry in 1 form

Bloods 3

Test	Bottle	Instructions	Distribution
Total glucose production	1 purple top	Spin 2200 rpm for 10 mins at 4ºC	Aliquot 500ul in 0518 glass bottle and 250ul in another 0518 glass bottle Label as study no, subject no, date, time from start of infusion (i.e. 1 hour, 2 hr etc) + **glucose** Aliquot another 250ul into glass bottle and label as above but with **FFA**

Bloods 4

Test	Bottle	Instructions	Distribution
Total glucose production	2 purple tops	Spin 2200 rpm for 10 mins at 4ºC	Aliquot 500ul in 2 0518 glass bottles and 250ul in another 2 0518 glass bottles Label as study no, subject no, date, baseline 1 (23.00) or baseline 2 (06.00) and **glucose** Aliquot another 250ul into glass bottle and label as above but with **FFA**

Bloods 5

Test	Bottle	Instructions	Distribution
Plasma	4 **large** purple tops (supplied by Dr Ahmed)	Spin 2200 rpm for 10 mins at 4ºC	Aliquot plasma between large 0518 glass bottles Label as study no, subject no, date, time from start of infusion (i.e. baseline 1(23.00) + 1 hour, 6 hr or + 10 hr) + **plasma**

Bloods 6

Test	Bottle	Instructions	Distribution
Cortisol and cortisone	1 red top	Clot at room temp for 30 minutes before spinning Spin at 3000 rpm for 10 minutes at 4ºC	Divide between microcentrifuge tubes Label as study no, subject no, date time (i.e. 0, 20, 40 etc) and cortisone

Bloods 7

Test	Bottle	Instructions	Distribution
Insulin	1 red top	Place in fridge for 30 minutes before spinning Spin at 3000 rpm for 10 minutes at 4ºC	Divide between microcentrifuge tubes Label as study no, subject no, date time (i.e. 0, 30,60,90,120) and insulin

24 hour urine collection – use plain bottle

Instructions	Storage
Measure total volume and decant into 3 20ml universal containers	Label with study no, subject no, date, Total Volume and freeze at -80ºC

Papers written by Adeeba Ahmed

Induction of hepatic 11β-hydroxysteroid dehydrogenase type 1 in patients with alcoholic liver disease
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11 β Hydroxysteroid Dehydrogenase Type 1 Regulation by Intracellular Glucose 6-Phosphate Provides Evidence for a Novel Link between Glucose Metabolism and Hypothalamo-Pituitary-Adrenal Axis Function
The Journal of Biological Chemistry Vol. 282, No. 37, pp. 27030–27036, September 14, 2007

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