The protective role of 11β-HSD1 inhibition in the metabolic syndrome and atherosclerosis

Thesis submitted by Małgorzata Wamil for Doctor of Philosophy.

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Abstract

Obesity is associated with an increased risk of diabetes type 2, dyslipidaemia and atherosclerosis. These cardiovascular and metabolic abnormalities are exacerbated by dietary fats such as cholesterol and its metabolites. High adipose tissue glucocorticoid levels, generated by the intracellular enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) are also implicated in the pathogenesis of obesity, metabolic syndrome and atherosclerosis. Transgenic mice over-expressing 11β-HSD1 selectively in adipose tissue develop the metabolic syndrome whereas 11β-HSD1-/- mice have a 'cardioprotective' phenotype, deriving in part from improved adipose tissue function. Consistent with this, prototypical therapeutic 11β-HSD1 inhibitors ameliorate metabolic disturbances associated with obesity.

11β-HSD1 also inter-converts the atherogenic oxysterols 7-ketocholesterol (7KC) and 7β-hydroxycholesterol (7β-HC). Work presented in the first part of the thesis defines the impact of these alternative substrates on the metabolism of glucocorticoids in adipocyte cell lines (3T3-L1 and 3T3-F442A). 11β-HSD1 catalyses the reduction of 7KC in mature adipocytes leading to accumulation of 7β-HC. Oxysterol and glucocorticoid conversion by 11β-HSD1 was competitive and occurred within a physiologically-relevant IC_{50} range of 450nM for 7KC inhibition of glucocorticoid metabolism. Working as an inhibitor of 11β-HSD1 activity, 7KC decreased the regeneration of active glucocorticoid and limited the process of preadipocyte differentiation. 7-oxysterols did not display intrinsic activation of the glucocorticoid receptor (GR). However, when co-incubated with glucocorticoid, 7KC repressed, and 7β-HC enhanced GR transcriptional activity. The effect of 7-oxysterols resulted from the modulation of 11β-HSD1 reaction direction, at least in transfected HEK293 cells, and could be abrogated by over-expression of hexose 6-phosphate dehydrogenase, which supplies NADPH to drive the reductase activity of 11β-HSD1.

11β-HSD1 inhibition protects from atherosclerosis, yet it is unknown whether it is an effect of alterations in the metabolism of 7-oxysterols. 7KC and 7β-HC did not activate the potential cognate receptor LXRα and FXR/RXR in transactivation

assays. No differential regulation of key gene targets of LXRα, FXR and RORα in the liver and fat depots of high fat fed 11β-HSD1-/- and wild type mice was observed.

To further determine the molecular basis for the metabolically beneficial phenotype of 11β-HSD1-/- mice I analysed global gene expression in subcutaneous and mesenteric adipose tissues of high fat-fed (4 weeks) 11β-HSD1-/- and congenic C57BL/6J mice by microarrays, followed by pathway analysis, gene clustering and realtime-PCR validation of transcripts with >1.5-fold difference between genotypes. 11β-HSD1-/- mice gained less weight and distributed adipose tissue to subcutaneous rather than visceral depots. Broadly, high fat-fed 11β-HSD1-/- mice showed up-regulation of transcripts in subcutaneous fat (70% of 1622 differentially-expressed transcripts), but down-regulation in mesenteric adipose tissue (73% of 849 transcripts). Genes up-regulated in 11β-HSD1-/- subcutaneous adipose were associated with β-adrenergic signaling, glucose metabolism, lipid oxidation, oxidative phosphorylation, MAPK, Wnt/β-catenin, EGF, and PI3K/AKT insulin signaling pathways. Increased subcutaneous fat insulin signaling was confirmed by increased IRS-1 and Akt phosphorylation *in vivo*. Down-regulated genes in 11β-HSD1-/- mesenteric fat were associated with immune cells, NK-kappaB, Jak/Stat, SAPK/JNK, chemokine, toll-like-receptor and Wnt signaling pathways suggesting reduced immune cell infiltration in mesenteric adipose in high fat-fed 11β-HSD1-/- mice. 11β-HSD1 deficiency protects against metabolic disease by increasing peripheral fat insulin sensitivity and through a novel mechanism involving reduction in visceral fat immune/inflammatory cell function.

Data presented in this thesis contribute to the understanding of the role of 11β-HSD1 in adipose tissues in obesity and, potentially, atherosclerosis.

Declaration

I declare that this thesis was written by me and that the data presented represent my own work, with the exceptions listed below:

Adipose tissue dissections and ip insulin and saline injections were performed jointly with Dr. Nicholas Morton.

Cell sizes in subcutaneous and mesenteric fat depot were recorded by Margaret Ross and sectioning were performed by Dr Richardo A. De Sousa Peixoto.

For the genomic analysis samples of RNA were processed by the micro-array team at The Sir Henry Wellcome Functional Genomics Facility (SHWFGF) in Glasgow. The analysis of micro-array experiment was processed joinly with Donald Dunbar and the Bioinformatics Team in the CVS (University of Edinburgh).

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

Malgorzata Wamil

"Imagination is more important than knowledge."

Albert Einstein

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I would like to dedicate this thesis to the memory of my grandfather Waclaw Fudalej. He thaught me to be determined and persistent in perusing my dreams.

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List of abbreviations

-/-	knockout mice model
7KC	7keto-cholesterol
7β-HC	7β-hydroxycholesterol
11βHSD1	11β-hydroxysteroid dehydrogenase type 1
11βHSD1-/-	11β-hydroxysteroid dehydrogenase type 1 knockout mice
11βHSD2	11β-hydroxysteroid dehydrogenase type 2
ABCA1	ATP-binding cassette, subfamily A, member 1
ABCG1	ATP-binding cassette transporter 8
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of Variance
ApoE	Apolipoprotein E
Bmal1	ARNT-like protein 1, brain and muscle
BSA	Bovine serum albumin
BSEP	Bile salt export pump
CEBP	CCAAT enhancer binding protein
Cort	Corticosterone
CTP1	Carnitine palmitoyltransferase 1
Dex	Dexamethasone
11DHC	11 Dehydrocorticosterone
DMEM	Dulbeco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic reticulum
FABP	Fatty acid binding protein
FAS	Fatty acid sythase
FCS	Fetal calf serum
FFA	Free fatty acids
FXR	Farnesoid X receptor
GC	Glucocorticoids
GCMS	Gas Chromatography Mass Spectometry

GLUT	Glucose transporter
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
H6PDH	Hexose-6-phosphate dehydrogenase
HF	High fat
HMGCoA	Hydroxymethylglutaryl-CoA reductase
HPA	Hypothalamic-pituitary adrenal axis
HPLC	High Performance Liquid Chromatography
HSD	Hydroxysteroid dehydrogenase
IBMX	Iso-methylbutyxanthine
IL	Interleukin
l	Litre
LB	Luria-Bertoni
Lep^{ob}	Leptin deficient mice
LXR	Liver X receptor
MARCO	Macrophage receptor with collagenous structure
ml	millilitre
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NBCS	New born calf serum
PBS	Phosphate buffered saline
PEPCK	Phosphoenolpyruvate carboxykinase
PPAR	Peroxisome proliferator-activated receptor
Pref-1	Preadipocyte factor 1
PRKAA2	Protein kinase, AMP-activated, catalytic alpha 2
RORα	Retinoid acid receptor-related orphan receptor α
RXR	Retinoid acid receptor
Sell	Selectin
SEM	Standard error of the mean
SHP	Small heterodimer partner

SREBP	Sterol regulatory element-binding protein
STAT4	Signal transducer and activator of transcription 4
SVF	Stromal vascular fraction
TBP	TATA binding protein
TBE	Trisborate-EDTA
TBP	TATA binding protein
TG	Triglycerides
TNFα	Tumor necrosis factor α
TZD	Thiozolidinedione

List of publications, presentations and awards

Publications

Wamil M, De Sousa Peixoto RA, Dunbar D, Seckl JR, Morton NM. Adipose tissue transcriptome profiling reveals depot-specific protective mechanisms of 11β-HSD1 deficiency. Manuscript in preparation.

Wamil M, Andrew R, Chapman K, Street J, Morton NM, Seckl JR. 7-oxysterols modulate glucocorticoid activity in adipocytes. Endocrinology 2008, Dec; 149(12): 5909-13.

Wamil M., Seckl JR. Inhibition of 11β-hydroxysteroid dehydrogenase type 1 as a promising therapeutic target. Drug Discovery Today. 2007, Jul; 12(13-14):504-520 (review).

Szopa M, **Wamil M**. (2007, May 20). 11β-hydroxysteroid dehydrogenase type 1 inhibitor: a novel therapeutic target in the metabolic syndrome. Diabetologia Praktyczna. 8, 77-83 (review, in Polish).

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CHAPTER 1

Introduction

1 Introduction

The following introductory chapter describes the biology of 11β-HSD1, an enzyme amplifying intracellular glucocorticoid level. Evidence for the role of 11β-HSD1 in the pathogenesis of the metabolic syndrome and atherosclerosis is discussed. Finally, a list of the aims of this thesis is presented.

1.1 Glucocorticoids, obesity and metabolic disease

The prevalence of obesity and its metabolic complications has been increasing rapidly over the last two decades (http://www.cdc.gov/nccdphp/dnpa/obesity/trend/maps/). Obesity is associated with an increased risk of type 2 diabetes, metabolic syndrome, cardiovascular disease, stroke and certain cancers. The World Health Organization has estimated that worldwide 1.6 billion adults are overweight with at least 400 million of them clinically obese (http://www.who.int/mediacentre). These gloomy raw statistics include increasing numbers of children and adolescents, foreshadowing a worsening trend in the future. Given that medical science has always risen to epidemic challenges, the paralleled increase in our understanding of metabolic pathways underlying obesity and its metabolic consequences is not surprising. Whether or not obesity in the absence of its complicating disorders increases mortality remains contentious (Flegal *et al.*, 2005). What is clear is that obesity is a major risk factor for several disorders that are themselves associated with high morbidity and mortality. These include type 2 diabetes, dyslipidaemia, hypertension and cardiovascular disease (which, together with visceral/abdominal obesity, comprise the Metabolic Syndrome), as well as several cancers, respiratory disorders, gallstones, osteoarthritis, depression and anxiety. Whilst prevention of these complications of obesity is a major impetus for research into prevention and treatment, another important if more contentious driving force of the extensive research in the field of obesity is the growing public expectation of a pharmaceutical antidote to our 'obesogenic environment'. Many novel druggable molecular targets have recently been identified. Several have entered drug development and even clinical practice, including rimonabant, a selective endocannabinoid (CB1) receptor antagonist, and exenatide, a glucagon-like peptide-1 (GLP-1) mimetic.

Growing evidence indicates the cooperation between metabolic and inflammatory pathways is disrupted in the pathogenesis of the metabolic complications of obesity (Hotamisligil, 2005). Identification and targeting central molecules involved in integration of metabolic and immune/inflammatory responses appears to have good prospects for a successful therapeutic approach in the metabolic syndrome. The promising reports announcing the benefits of thiazolidinediones, statins and salicylates to control inflammatory processes and to improve metabolic parameters support this notion.

1.1.1 Glucocorticoids

Glucocorticoids are well known ubiquitous hormones playing a key role in modulating immune and inflammatory responses, regulating energy metabolism and cardiovascular homeostasis and the body's responses to stress. Opposing the action of insulin, glucocorticoids stimulate production of glucose, switching the homeostatic balance towards catabolism. Thus, glucocorticoids promote gluconeogenesis but inhibit beta-cell insulin secretion and peripheral glucose uptake (Dallman *et al.*, 1993, Sapolsky *et al.*, 2000). They also increase protein breakdown and lipolysis with consequent fatty acid mobilization. Patients with endogenous or exogenous glucocorticoid excess (Cushing's syndrome) develop visceral obesity, insulin resistance, diabetes type 2, dyslipidemia, hypertension and increased cardiovascular mortality.

The striking similarity of phenotype between rare Cushing's and the common Metabolic Syndrome/idiopathic obesity spectrum has spurred the search for a common underlying mechanism. However, plasma cortisol levels are not notably elevated in simple obesity or the Metabolic Syndrome, at least in the absence of marked complications. It has been hypothesised that tissue-specific differences in glucocorticoid metabolism and hence increased local cellular corticosteroid exposure may explain this apparent paradox. Since most of the features of Cushing's syndrome are reversible by removal of glucocorticoid excess, manipulations reducing cortisol action at a local cellular or tissue level might provide a novel therapeutic strategy for the Metabolic Syndrome.

Glucocorticoids (cortisol in humans and most mammals, corticosterone in rats and mice) are produced by the adrenal cortex and regulated by ACTH under the control of hypothalamic-pituitary-adrenal (HPA) axis. As little as five percent of cortisol circulates free in the plasma, with the majority bound with high affinity corticosteroid-binding globulin, which may act as a transporter of steroid to target cells, as well as lower affinity proteins such as albumin. The production of glucocorticoids is contingent upon the pronounced circadian rhythm (high during the active phase, low during quiescence/sleep) and episodic stressful events which stimulate the HPA axis, considerable variations of free plasma cortisol occur. The dynamic range encompasses very low nanomolar levels at the nadir to low micromolar concentrations during severe stress (Stewart and Krozowski, 1999). Cortisol is believed to diffuse across cell membranes and then binds to cytoplasmic glucocorticoid receptors (GR) and, in some tissues, mineralocorticoid receptors (MR), which then translocate to the nucleus. GR and MR are ligand-gated transcription factors (Dostert and Heinzel, 2004) which regulate a plethora of genes directly or through interactions with other transcription factors (Barnes, 2006a, Barnes, 2006b).

Until recently, it was axiomatic that the major determinant of corticosteroid action was the level of free cortisol in the plasma and the densities of GR and MR in target tissues. However, it has recently become apparent that tissue specific metabolism of glucocorticoids, notably by 11β-hydroxysteroid dehydrogenases (11β-HSDs) alters tissue glucocorticoid levels and hence receptor access. 11β-HSD catalyses the interconversion of non-receptor-binding and therefore inert 11-ketosteroids, cortisone and 11-dehydrocorticosterone (11-DHC), and their receptor-binding active 11-hydroxy forms, cortisol and corticosterone (Fig. 1.1.). Inactive cortisone circulates unbound at around 100nM in humans and therefore its concentration is greater than active cortisol, notably during the diurnal nadir. In rats, 11-DHC levels are also around 50-100 nM, though its levels in mice are lower.

1.2 11β-hydroxysteroid dehydrogenases

Two isoforms of 11β-HSD are known, the products of distinct genes (Stewart and Krozowski, 1999). 11β-HSD2, a high affinity NAD-dependent dehydrogenase, is expressed mainly in mineralocorticoid target tissues (kidney, colon, salivary glands) (van Uum *et al.*, 2004). This distribution reflects its role in protecting intrinsically non-selective MR from activation by cortisol and corticosterone and therefore enabling selective aldosterone binding (Edwards *et al.*, 1988). Additionally, 11β-HSD2 is highly expressed in the placenta and the developing fetus, providing a potent barrier to maternal glucocorticoids (Seckl and Meaney, 2004).

In contrast 11β-HSD1 is a lower-affinity NADP(H)-dependent enzyme, which though bi-directional in purified preparations and tissue homogenates, acts as a predominant 11-ketoreductase in intact cells and organs (Jamieson *et al.*, 2000, Odermatt *et al.*, 2006). 11β-HSD1 is expressed primarily in tissues with high sensitivity to glucocorticoids (liver, adipose tissue, brain, lung) (Bujalska *et al.*, 1997, Seckl and Walker, 2001, Jamieson *et al.*, 1995). 11β-HSD1 is active as a dimer and exhibits cooperative kinetics with cortisone and 11-DHC as substrates (Maser *et al.*, 2002). Thus, 11β-HSD1 dynamically adapts to nanomolar as well as micromolar concentrations of 11-keto steroids. Both isozymes contain an N-terminal membrane-insertion sequence, thus enabling anchoring in the endoplasmic reticulum (ER) (Odermatt *et al.*, 1999). The catalytic moiety of 11β-HSD2 faces the cytoplasm, while 11β-HSD1 is directed into the ER lumen (Odermatt *et al.*, 2006). This has significant implications for cofactor availability (NAD+/NADPH ratio) and potential bi-directionality of 11β-HSD1. The co-localization of 11β-HSD1 in the lumenal surface of the ER membrane with hexose-6-phosphate dehydrogenase (H6PDH), which catalyzes the first two steps of the pentose-phosphate pathway generating NADPH, provides a supply of co-substrate to drive the predominant oxoreductase direction of 11β-HSD1 in intact cells (Banhegyi *et al.*, 2004, Atanasov *et al.*, 2004). H6PDH-/- mice are unable to convert 11-dehydrocorticosterone to corticosterone, but the efficiency of the opposite ‘dehydrogenase’ reaction is unaffected (Lavery *et al.*, 2006).

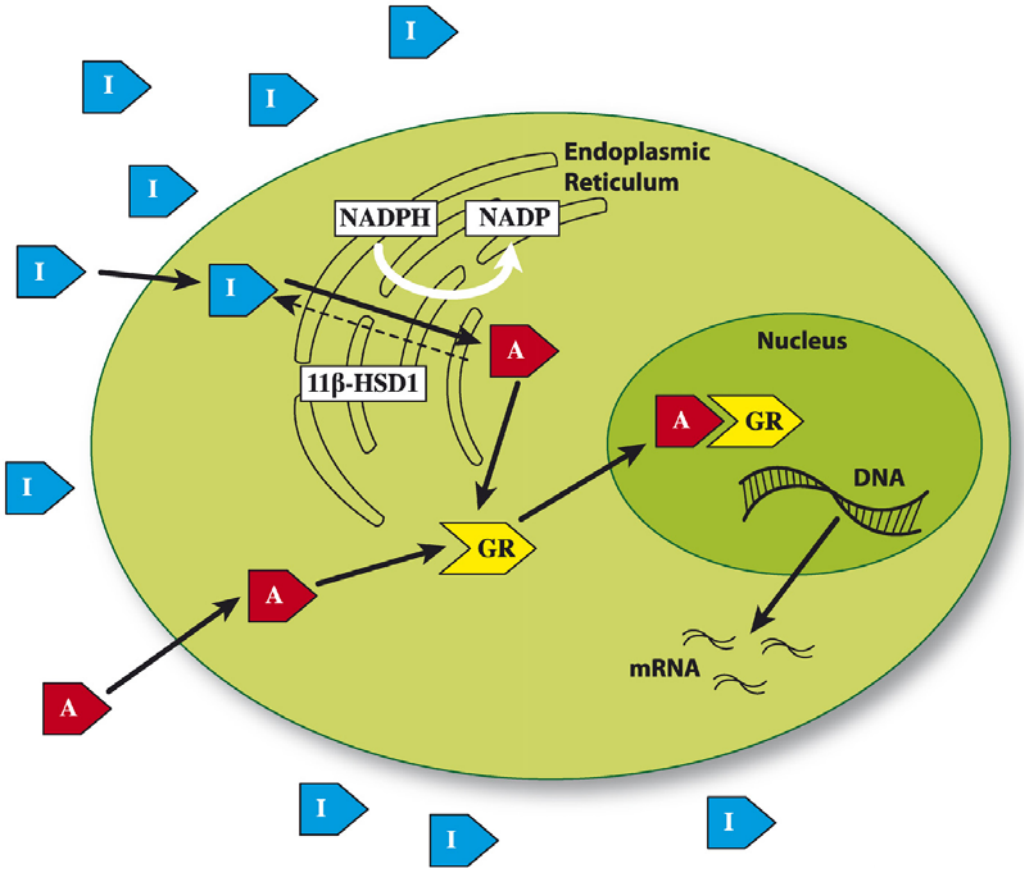

Figure 1.1 Schematic representation of the intracellular 11β-HSD1-dependent regeneration of glucocorticoids.

Glucocorticoids diffuse across cell membrane and bind to glucocorticoid receptors (GR; yellow) in the cytoplasm. When not activated by the ligand, GR are protected from the trafficking to the nucleus by binding to chaperons and forming the complex. Once activated by the ligand, a complex is translocated rapidly into the nucleus where it binds to the promoter region of glucocorticoid-responsive genes and leads to an increase or repression of genes transcription. Since the production of glucocorticoids is contingent upon circadian rhythm, considerable variations of free plasma cortisol occur during diurnal changes. Inactive form of glucocorticoid (blue) circulates unbound, and therefore its concentration is usually greater than active form (red). 11β-HSD1 (acting predominantly as a NADPH-dependent reductase *in vivo*) is located in the endoplasmic reticulum (ER). It interconverts active and inactive glucocorticoids, thus regulating glucocorticoid receptor activation intracellularly.

1.3 11β-HSD1 in obesity and the metabolic syndrome

To test the hypothesis that tissue-specific regulation of 11β-HSD expression and activity contributes to obesity and its metabolic consequences several animal studies have been performed. In leptin-resistant fatty Zucker rats, obesity associates with decreased 11β-HSD1 activity and expression in the liver, but increased 11β-HSD1 in the adipose tissue, notably in visceral fat (Livingstone *et al.*, 2000a). Similar changes have been reported in leptin deficient ob/ob mice (Liu *et al.*, 2003). It is noteworthy that basal 11β-HSD1 levels are higher in peripheral (subcutaneous) than in central (visceral/mesenteric) adipose tissue in such rodent models.

Initial studies of humans, which measured the ratio of cortisol to cortisone metabolites in urine as an indirect index of total body 11β-HSD activity, produced inconsistent results, reporting increased (Tiosano *et al.*, 2003, Rask *et al.*, 2001), decreased (Stewart *et al.*, 1999, Rask *et al.*, 2001) and unchanged (Fraser *et al.*, 1999) urinary '11β-HSD index' values in obesity (Summary of representative human studies in Table 1.1.). Such ratios, however, are inadequate as they may be influenced by other enzymes involved in the metabolism of cortisol (11β-HSD2, 5α- and 5β-reductases, 3α-HSDs, etc). Additionally, opposing, tissue-specific changes in 11β-HSD1 are difficult to dissect with such 'whole body' estimates. More recent studies have attempted various tissue-specific measures in humans to accommodate such concerns. Such work has generally, but not exclusively, suggested that obese humans, as monogenic obesity in rodents, show selective down-regulation of 11β-HSD1 in liver and up-regulation in adipose tissue.

1.3.1 Down-regulation of hepatic 11β-HSD1 in obesity: a potential protective mechanism against diabetes.

Hepatic 11β-HSD1 activity (measured as the conversion of an oral dose of cortisone into cortisol in plasma after 'first pass' metabolism) has been consistently decreased in obesity (Rask *et al.*, 2002, Rask *et al.*, 2001, Stewart *et al.*, 1999). This is not just driven by insulin resistance since down-regulation of hepatic 11β-HSD1 activity is not seen in lean subjects with type 2 diabetes (Valsamakis *et al.*, 2004). The absence of hepatic 11β-HSD1 down-regulation in obese diabetics emphasizes its possible role

in pathogenesis. This raises the intriguing hypothesis that 11β-HSD1 inhibition in obese people who develop impaired glucose tolerance may protect from progression to the type 2 diabetes. In obese rats, the down-regulation of hepatic 11β-HSD1 activity apparently requires elevated circulating glucocorticoid levels and/or weight gain, but not insulin resistance alone since neither thiazolidinediones nor metformin reversed the finding in Zucker rats (Livingstone *et al.*, 2000b). The fundamental mechanism involved thus remains obscure.

1.3.2 Increased glucocorticoid regeneration in adipose tissue: a possible cause of metabolic syndrome.

Increased fat mass in obese subjects is suggested to be associated with increased pre-adipocyte proliferation, differentiation and accumulation of lipid droplets, yet the underpinning mechanisms remain unclear. Hence 11β-HSD1 is highly expressed in fat and its mRNA and activity increases significantly with differentiation of 3T3-L1 pre-adipocytes to mature adipocytes (Napolitano *et al.*, 1998), a new concept of its role as an autocrine regulator of fat mass emerged. Most human studies show increased 11β-HSD1 in subcutaneous fat tissue in obesity (Kannisto *et al.*, 2004, Rask *et al.*, 2001, Rask *et al.*, 2002, Lindsay *et al.*, 2003, Wake *et al.*, 2003, Paulmyer-Lacroix *et al.*, 2002) (Table 1.1.). However, Tomlinson *et al.* (Tomlinson *et al.*, 2002) found no correlation of 11β-HSD1 in human fat tissue with obesity and hypothesised that this may have important implications for the enhancement of pre-adipocytes proliferation. It can not be excluded, however, that even if there is no increase in the enzyme activity per gram of visceral adipose tissue, an increase in the volume of abdominal fat in obese subjects may account for the high cortisol levels delivered by the portal vein to the liver.

It has been suggested that visceral fat is more sensitive to glucocorticoids (higher GR and 11β-HSD1 expression) and therefore it was hypothesised that high ratio of cortisol/cortisone reactivation in visceral adipose tissue may be responsible for the 'Cushing's disease of the omentum'(Bujalska *et al.*, 1997). Introduction of a novel method by Andrew *et al.* (Andrew *et al.*, 2002) using deuterated cortisol tracer infusion enabled measurement *in vivo* of 11β-HSD1 activity in the human splanchnic

bed (Basu *et al.*, 2005) and, separately, in liver to allow an estimate of visceral adipose tissue activity (Sandeep *et al.*, 2005). Such work showed that the splanchnic bed produces approximately one-quarter of the amount of cortisol produced by the adrenal cortex (Basu *et al.*, 2005). Furthermore, one-third of the splanchnic contribution to cortisol production appears to be from liver, with the rest assumed to be largely from visceral fat and other mesenteric sources, at least in healthy subjects (Andrew *et al.*, 2005). Using this technique, Sandeep *et al.* (Sandeep *et al.*, 2005) compared 11β-HSD1 activity in lean and obese subjects. Obese men had no difference in whole-body regeneration cortisol from cortisone, but exhibited greater conversion of [^{3}H]cortisone to [^{3}H]cortisol in abdominal subcutaneous adipose tissue, as measured directly by microdialysis, suggesting again the down-regulation of 11β-HSD1 in liver in obesity. Thus, in obesity whole-body 11β-HSD1 activity is not reliably altered because increased activity of cortisol regeneration in adipose tissue is balanced by the parallel decrease of hepatic activity. The data support the notion that 11β-HSD1 regeneration of glucocorticoids in visceral fat contributes substantially to the concentration of cortisol in the portal vein and therefore is an important determinant of the development of insulin resistance associated with abdominal obesity.

Subjects	Changes in 11β-HSD1 expression/activity in fat and liver.	Correlation with obesity/metabolic syndrome	Method employed	Ref.
16 patients undergoing abdominal surgery	↑ 11β-HSD1 activity in stromal cells from visceral, but not subcutaneous fat.	Increased visceral 11β-HSD1 activity correlates with obesity.	Visceral and sc fat biopsies, stromal cells culture	(Bujalska *et al.*, 1997)
36 patients divided in 3 groups according to BMI	↓ 11β-HSD1 activity in obese subjects	11β-HSD1 inhibition correlates with central obesity.	Urine cortisol metabolites	(Stewart *et al.*, 1999)
34 men with different BMI and insulin sensitivity	↑ 11β-HSD1 in sc fat and ↓ hepatic 11β-HSD1 in obesity	Increased adipose and decreased hepatic 11β-HSD1 correlate with obesity.	Urine cortisol metabolites, sc fat biopsies, oral cortisone acetate test	(Rask *et al.*, 2001)
12 lean and 18 obese patients	↑ 11β-HSD1 mRNA in sc fat in adipocytes and in visceral fat in both adipocytes and stroma from obese patients.	Over-expression of 11β-HSD1 in fat correlates with metabolic disorders linked to obesity.	In situ hybridisation	(Paulmyer-Lacroix *et al.*, 2002)
40 obese women	↑ 11β-HSD1 in fat and ↓ hepatic 11β-HSD1 in obesity ↑ cortisol clearance	Increased 11β-HSD1 in fat, decreased in liver and enhanced inactivation of cortisol by A ring reductases with compensatory activation of the HPA axis correlate with obesity	Urine cortisol metabolites, sc fat biopsies, oral cortisone acetate test	(Rask *et al.*, 2002)
25 unmedicated lean men with glucose intolerance and 25 healthy men	Normal 11β-HSD1 activity in adipose tissue and ↓ hepatic 11β-HSD1 In subjects with impaired glucose intolerance.	Enhanced *in vivo* peripheral tissue sensitivity to glucocortiocids, tissue-specific alterations in 11β-HSD1 activity and increased excretion of A-ring reduced metabolites of cortisol correlate with impaired glucose tolerance without obesity.	Urine cortisol metabolites, Skin beclomethasone test	(Andrews *et al.*, 2002)
32 women undergoing elective abdominal surgery	No difference in adipose 11β-HSD1 mRNA between visceral and sc fat tissue, ↑ 11β-HSD1 activity in visceral preadipocytes.	No correlation of 11β-HSD1 mRNA with obesity. Enhanced preadipocyte proliferation within visceral adipose tissue contribute to increases in visceral fat in obese patients.	Visceral and sc fat biopsies, preadipocytes culture	(Tomlinson *et al.*, 2002)
25 healthy men	↑ adipose 11β-HSD1 activity and increased urinary excretion of 5alpha- and 5β-reduced cortisol metabolites in obesity. Fatty liver is associated with selective increase of 5β-reduced cortisol metabolites	No correlation of visceral fat mass with changes in cortisol metabolism.	Urine cortisol metabolites, sc fat biopsies	(Westerbacka *et al.*, 2003)
12 Caucasian 19 Pima Indian	↑Adipose 11β-HSD1 in obesity	Sc adipose 11β-HSD1 activity correlates with central adiposity and hyperinsulinemia. No difference in enzyme activity between Pima Indians and Caucasians.	Sc fat biopsies	(Lindsay *et al.*, 2003)

16 men and 16 women	↑ Adipose 11β-HSD1 mRNA and activity in obesity	Adipose 11β-HSD1 mRNA and activity correlate with obesity. Leptin but not angiotensinogen and GR were correlated with 11β-HSD1 and obesity.	Sc fat biopsies	(Wake *et al.*, 2003)
33 men with diabetes type 2 38 healthy subjects	No change in 11β-HSD1 activity in diabetic subjects.	Impaired 11β-HSD1 activity correlates with obesity in healthy subjects but not in diabetics. Failure to down-regulate11β-HSD1 activity with diabetes may potentate dyslipidemia, insulin resistance, and obesity.	Urine cortisol metabolites	(Valsamakis *et al.*, 2004)
17 monozygotic twins	↑ Adipose 11β-HSD1 mRNA and protein in obesity	Increased 11β-HSD1 mRNA in sc fat correlates with obesity and insulin resistance.	Sc fat biopsies	(Kannisto *et al.*, 2004)
12 obese subjects on 10-wk very low fat diet	↑ Adipose 11β-HSD1 activity and mRNA in isolated adipocytes	Weight loss increases 11β-HSD1 mRNA in adipose tissue. Decreased 11β-HSD1 activity and expression in obesity may act as a compensatory mechanism to enhance insulin sensitivity.	Urine cortisol metabolites, sc fat biopsies, preadipocyte culture	(Tomlinson *et al.*, 2004)
11 healthy subjects		Splanchnic 11β-HSD1-dependent re-generation of cortisol contributes significantly to the whole body cortisol production. Thus, alterations in splanchnic cortisol production may contribute to visceral obesity.	Hepatic venous and leg catheterization and isotype infusion (*in vivo* splanchnic 11β-HSD1 activity)	(Basu *et al.*, 2004)
6 lean and 6 obese men	↑ In vivo 11β-HSD1 activity in sc fat and unchanged total splanchnic 11β-HSD1 activity in obese men.	Increased 11β-HSD1 activity selectively in adipose tissue correlates with obesity. Obese men are less susceptible than lean men to the insulin-sensitizing effects of carbenoxolone.	Isotype infusion (in vivo splanchnic 11β-HSD1 activity), microdialysis of sc fat	(Sandeep *et al.*, 2005)
9 healthy men	↑ 11β-HSD1 activity in fat after meal.	Hyperinsulinaemia and increased FFA induce acute increases in 11β-HSD1 activity in adipose tissue.	Isotype infusion (*in vivo* splanchnic 11β-HSD1 activity), microdialysis of sc fat	(Wake *et al.*, 2006)
18 healthy subjects	↑ 11β-HSD1 activity following mixed meal	Increased total body cortisol production after an ingestion of a mixed meal does not originate from alter splanchnic cortisol production.	Isotype infusion (*in vivo* splanchnic 11β-HSD1 activity)	(Basu *et al.*, 2006)
70 postmenopausal women	↑ 11β-HSD1 and ↓11β-HSD2 mRNA in sc fat in obese women	Increased 11β-HSD1 mRNA positively correlates with the waist circumference and is associated with decreased insulin sensitivity. No effect of weight loss on the expression of 11β-HSD1 and 11β-HSD2.	Sc fat biopsies	(Engeli *et al.*, 2004)

Table 1.1 Summary of the representative human studies

The table presents an overview of the most significant human studies testing the hypothesis that suggests that changes in 11β-HSD1 expression/activity contribute to obesity and its metabolic complications. It highlights the relationship between the method employed in the study and the corresponding outcomes.

1.3.3 The role of 11β-HSD1 in the pancreas and muscle

11β-HSD1 is expressed in islets of Langerhans isolated from ob/ob mice and also from human pancreas (Davani *et al.*, 2000). 11β-HSD1 is increased in islets of diabetic but not pre-diabetic Zucker rats (Duplomb *et al.*, 2004). Higher levels of 11β-HSD1 mRNA and enzyme activity have been correlated with the appearance of diabetes and are increased further with disease progression (Duplomb *et al.*, 2004). Moreover, in Zucker rats, troglitazone-induced improvement in metabolic abnormalities correlated with a 40% decline in 11β-HSD1 mRNA in the islets (Duplomb *et al.*, 2004). Incubation of islets with 11-dehydrocorticosterone resulted in dose-dependent inhibition of insulin secretion; the effect was reversed by carbenoxolone (Davani *et al.*, 2000). Selective inhibition of 11β-HSD1 or GR antagonist treatment in ob/ob mice attenuated 11-dehydrocorticosterone-induced enhancement of 11β-HSD1 activity (Ortsater *et al.*, 2005). Any fundamental mechanistic importance of these interesting observations remains unclear.

Alongside liver and fat, skeletal muscles are a major target for insulin-mediated glucose uptake. In obese patients presenting with features of metabolic syndrome it has been demonstrated that decreased levels of non-oxidative glucose disposal are determined by impaired insulin action predominantly in skeletal muscle (Thorburn *et al.*, 1990). In human skeletal myoblasts, 11β-HSD1 correlates with insulin sensitivity and blood pressure (Whorwood *et al.*, 2002). Little else is known about the importance of the low levels of 11β-HSD1 in skeletal muscle. In contrast, 11β-HSD1 is more clearly expressed in vascular smooth muscle (Walker *et al.*, 1992). 11β-HSD2 is also present in the endothelium so 11β-HSD1 effects are likely to be very cell-specific. Whilst 11β-HSD1 has no effect on vasoconstrictor/dilator function in healthy vessels (Christy *et al.*, 2003), a role in angiogenesis, typically inhibited by glucocorticoids, has recently been reported (Small *et al.*, 2005). 11β-HSD1 maintains an anti-angiogenic tone *in vivo*. Perhaps in consequence, 11β-HSD1-/- mice have markedly improved myocardial function (ejection fraction) 1 week after experimental infarction despite identical infarct size. Selective 11β-HSD1 inhibition also reduces atheroma formation (Hermanowski-Vosatka *et al.*, 2005), an effect which may include effects in vascular smooth muscle.

1.3.4 Polymorphisms in the gene encoding 11β-HSD1

There are 11 reported cases of deficiency of 11β-HSD1, at least as reported under the term 'cortisone reductase deficiency' (CRD) (Draper and Stewart, 2005). Most of the cases affect women. The clinical presentation resembles polycystic ovarian syndrome (PCOS) with acne, hirsutism, oligo-amenorrhoea and infertility. Obesity has been reported for a few cases (Phillipov *et al.*, 1996). The phenotype is thought to be due to ACTH-mediated androgen excess secondary to the failure to regenerate cortisol which results in feedback activation of HPA axis. Although no gross deletions or re-arrangements in *HSD11B1* have been found in most CRD cases (Biason-Lauber *et al.*, 2000, Nikkila *et al.*, 1993, Nordenstrom *et al.*, 1999), two polymorphisms in complete linkage disequilibrium within intron 3 of *HSD11B1* were described in 3 cases associated with exon 5 polymorphisms of *H6PD* gene (encoding H6PDH) (Draper *et al.*, 2003). Thus, a concept emerged that CRD is caused by a combination of mutations in *HSD11B1* and *H6PD*. However, White (White, 2005) found the co-occurrence of these polymorphisms in normal subjects. San Millan *et al.* also showed that the triallelic genotypes *HSD11B1* 83,557insA and *H6PD* R453Q found in CRD do not always cause CRD but that in their group of patients with PCOS, the *H6PD* gene variant was associated with increased cortisol and 17-hydroxyprogesterone levels (San Millan *et al.*, 2005). Gambineri *et al.* reported that *HSD11B1* 83,557insA contributed to increased cortisol clearance and compensatory adrenal hyperandrogenism in lean women with PCOS but might potentially play a protective role against obesity and dyslipidemia (Gambineri *et al.*, 2006).

1.4 Does elevated adipose 11β-HSD1 cause metabolic disorders?

1.4.1 Transgenic over-expression of 11β-HSD1 models the metabolic syndrome.

To dissect the pathogenic implications of elevated adipose 11β-HSD1 in obesity, transgenic mice with two-to three-fold over-expression of 11β-HSD1 in fat were generated, exploiting the adipocyte fatty acid binding protein (aP2) promoter (Masuzaki *et al.*, 2001) (Table 1.2). These aP2-HSD1 transgenic mice have elevated corticosterone levels in adipose tissue but unaltered plasma concentrations. The mice develop many features of the metabolic syndrome: glucose intolerance and insulin

resistance (exacerbated further by high fat feeding), dyslipidemia, apparent leptin resistance (Masuzaki *et al.*, 2001) and hypertension associated with renin-angiotensin-aldosterone system activation (Masuzaki *et al.*, 2003). Adipokines associated with insulin resistance (resistin, TNF, leptin) are elevated and insulin-sensitising adiponectin is reduced. aP2-HSD1 mice are hyperphagic and obese, predominantly in the visceral fat depot. Expression of the GRα receptor was higher in visceral compared to subcutaneous fat (although unaltered by transgene expression) while the expression of the transgene *HSD11B1* was similar in all fat depots (Masuzaki *et al.*, 2001). The greater effects in visceral adipose may reflect the higher GR and/or higher lipoprotein lipase (LPL) in mesenteric fat depot.

aP2-HSD1 transgenic mice have elevated corticosterone and free fatty acids levels in the hepatic portal vein that drains blood from visceral fat to the liver. To examine the impact of elevated liver glucocorticoids mice over-expressing 11β-HSD1 selectively in the liver under the control of the ApoE promoter have been generated (Table 1.2). ApoE-HSD1 transgenic mice develop mild insulin resistance, fatty liver and dyslipidemia without impairment of glucose tolerance, obesity or changes in adipose distribution (Paterson *et al.*, 2004). Interestingly, ApoE-HSD1 transgenic mice have higher hepatic expression of LXR and PPARα suggesting increased lipid synthesis as well as clearance, and CYP7a, indicating enhanced bile acid synthesis. The mice are also hypertensive, perhaps driven by increased angiotensinogen synthesis in the liver. ApoE-HSD1 mice may model the attenuated metabolic syndrome without obesity, and indeed increased 11β-HSD1 activity in liver is seen in patients with the insulin resistance of myotonic dystrophy (Johansson *et al.*, 2001). In idiopathic steatohepatitis, liver 11β-HSD1 levels are not down-regulated (Westerbacka *et al.*, 2003), but any role of the enzyme in this prevalent problem are speculative.

1.4.2 Is 11β-HSD1 a therapeutic target? 11β-HSD1-/- mice resist the metabolic syndrome.

Elevated 11β-HSD1 levels in adipose tissue in human obesity and the phentoype of two murine models over-expressing 11β-HSD1 in adipose tissue and liver provided evidence that inhibition of glucocorticoid regeneration especially in fat might be a therapeutic target for metabolic syndrome. Since carbenoxolone does not inhibit 11β-

HSD1 in adipose tissue *in vivo* (Livingstone and Walker, 2003, Sandeep *et al.*, 2005), 11β-HSD1-/- mice have offered a unique possibility to model the potential effects of this possible therapeutic strategy. 11β-HSD1-/- mice are viable and healthy (Kotelevtsev *et al.*, 1997), but are unable to convert inert 11-dehydrocorticosterone to corticosterone. To compensate for their decreased production of active glucocorticoids 11β-HSD1-/- mice have adrenal hyperplasia and hyperresponsiveness to exogenous ACTH in vitro and *in vivo* (Harris *et al.*, 2001). 11β-HSD1-/- mice have improved glucose tolerance, plausibly due to decreased activation of key enzymes involved in gluconeogenesis in the liver (PEPCK and glucose-6-phosphatase), but do not show hypoglycemia when fasted (Kotelevtsev *et al.*, 1997). 11β-HSD1-/- mice have reduced triglyceride and NEFA levels, lower hepatic fibrinogen synthesis (reduced hypercoagulability) and raised HDL cholesterol (increased apolipoprotein AI, reduced apolipoprotein CIII), factors associated with a cardioprotective phenotype. These changes are driven by increased lipid β-oxidation (mCPT-I, UCP-2, ACO) and increased PPARα (Morton *et al.*, 2001).

Moreover, 11β-HSD1-/- mice are insulin-sensitized, notably in adipocytes *in vitro* (Morton *et al.*, 2004b) and adipose tissue *ex vivo* (decreased resistin and TNFα but increased adiponectin and PPARγ) (Morton *et al.*, 2001). A key action of insulin is to stimulate the up-take of glucose into cells by inducing translocation of glucose transporters. Binding of insulin to the receptor induces a conformational changes resulting in the autophosphorylation of a number of tyrosine residues (Van Obberghen *et al.,* 2001) and subsequently leading to the phosphorylation of insulin receptor substrate family IRS proteins and activation a key downstream effector AKT (otherwise known as PKB) (Saltiel and Kahn 2001). The analysis of the phosphorylation sides of these molecules in adipose tissue of 11β-HSD1-/- mice will be discussed in Chapter 5.

Additionally, on a high cholesterol diet, wild-type mice showed a switch in lipoprotein profile from HDL to LDL whereas 11β-HSD1-/- mice have lower plasma cholesterol level and a higher HDL to total cholesterol ratio. On the obesity-prone C57Bl/6J genetic background, high-fat diet-fed 11β-HSD1-/- mice gain significantly

less weight than controls, despite relative hyperphagia. With high fat diet 11β-HSD1-/- mice preferentially gain weight in peripheral rather then in visceral fat depots. It has been suggested increased expression of PPARγ and UCP-2 in 11β-HSD1-/- visceral adipose tissue could explain those changes in accumulation of fat (Morton *et al.*, 2004b, Morton *et al.*, 2001).

To determine whether loss of glucocorticoids specifically in the adipose tissue might protect from the development of metabolic syndrome, mice ectopically over-expressing 11β-HSD2 (the reverse 11β-dehydrogenase enzyme which potently inactivates corticosterone), under the control of the murine aP2 promotor (ap2-HSD2) have been generated (Kershaw *et al.*, 2005) (Table 1.2). Surprisingly, expression and activity of the transgene was higher in subcutaneous than other fat depots. On high fat diet, aP2-HSD2 mice show decreased food intake and increased energy expenditure (Kershaw *et al.*, 2005). They resist weight gain and have improved glucose tolerance and insulin sensitivity (Kershaw *et al.*, 2005). On high fat diet these mice exhibit increased expression of PPARγ, UCP-2, PEPCK and adiponectin, and reduced expression of leptin, TNFα and resistin in adipose tissues (Kershaw *et al.*, 2005). Thus, apart from food intake (see below), aP2-11β-HSD2 mice are phenotypically similar to 11β-HSD1-/- mice emphasizing the importance of adipose tissue as a target for enzyme inhibition.

Although 11β-HSD1-/- mice resist the metabolic consequences of a high fat diet, they show hyperphagia suggesting that 11β-HSD1 plays a role in the central control of food intake and that this predominates over fat-derived signals (Densmore *et al.*, 2006). Normal C57Bl/6 mice fed a high fat diet show a rapid increase in 11β-HSD1 and down-regulation of agouti-related peptide (AgRP) specifically in the arcuate nucleus, but show no increase in food intake to this palatable diet. In the absence of 11β-HSD1 (knockout mice), high fat diet paradoxically up-regulates the orexigenic AgRP mRNA associated with hyperphagia. Thus it has been proposed that induction of 11β-HSD1 in the arcuate serves to constrain intake of palatable energy dense foods by reducing AgRP expression with which the enzyme is co-localized. However, pharmacological glucocorticoid manipulations increase AgRP suggesting

an indirect effect. This might be an opioid pathway since treatment of high fat-fed mice with the opioid antagonist naloxone induces a rise in arcuate AgRP and blocks the rise in 11β-HSD1 (Densmore *et al.*, 2006). The precise anatomy of the circuitry involved remains undetermined.

Transgenic mouse model	Effect on enzyme activity	Phenotype	Ref. No.
aP2-HSD1 transgenic	↑ 11β-HSD1 in adipose tissue	Visceral obesity Insulin resistance Diabetes type 2 Hyperphagia Hypertension Dyslipidemia	(Masuzaki *et al.*, 2003, Masuzaki *et al.*, 2001)
apoE-HSD1 transgenic	↑ 11β-HSD1 in liver	Modest insulin resistance Fatty liver Dyslipidemia Hypertension	(Paterson *et al.*, 2004)
11β-HSD1-/-	No 11β-HSD1 activity	Resistance to diet-induced obesity Peripheral fat redistribution Hyperphagia Absence of hyperglycemia upon starvation and stress challenges ↑ insulin sensitivity HPA axis hyperactivity to stress Adrenal hyperplasia ↑ HDL, ↓ LDL, ↓ TG ↓ fibrinogen Protection from age-related cognitive impairment	(Kotelevtsev *et al.*, 1997, Morton *et al.*, 2001, Morton *et al.*, 2004b, Yau *et al.*, 2001, Harris *et al.*, 2001)
aP2-HSD2 transgenic	↑ 11β-HSD2 predominantly in subcutaneous adipose tissue	Resistance to diet-induced obesity Hypophagia Increased energy expenditure ↑ insulin sensitivity	(Kershaw *et al.*, 2005)

Table 1.2 Transgenic mice models with genetic manipulation of 11β-HSD1.

To identify the tissue-specific role of 11β-HSD1, several transgenic mice models were generated. Overexpression of 11β-HSD1 selectively in adipose tissue was associated with manifestation of phenotypic features resembling full metabolic syndrome. In contrast, 11β-HSD1-/- mice or mice with overexpression of 11β-HSD2 in adipose tissue presented with 'cardioprotective' phenotype.

1.4. How is 11β-HSD1 regulated?

Expression of 11β-HSD1 is regulated by many factors including glucocorticoids, insulin, growth hormone, thyroid and sex hormones, cytokines, PPARα, PPARγ and perhaps other nuclear receptors (Fig.1.2.). *In vivo* in rat liver and hippocampus glucocorticoids have been reported to regulate temporally 11β-HSD1 expression and activity (Jamieson *et al.*, 2000). In human skeletal muscle 11β-HSD1 expression is up-regulated by physiological concentrations of cortisol in a dose-dependent manner (Whorwood *et al.*, 2002). 11β-HSD1 expression and activity is increased with differentiation from pre-adipocytes to mature adipocytes and with differentiation to the adipocyte phenotype in 3T3-L1 cells (Napolitano *et al.*, 1998). Insulin stimulates 11β-HSD1 mRNA and activity through a posttranscriptional mechanism in adipocytes (Balachandran *et al.*, 2008). Pro-inflammatory cytokines such as IL-1β and TNFα have been reported to up-regulate 11β-HSD1 in adipocytes (Tomlinson *et al.*, 2001) and aortic smooth muscle cells (Cai *et al.*, 2001). Growth hormone (GH) inhibits 11β-HSD1, in part through insulin-like growth factor-I (IGF-I) (Moore *et al.*, 1999) and the effect depends on sex-specific pattern of release (Liu *et al.*, 1997). Patients with acromegaly treated with a GH receptor antagonist show changes in cortisol metabolism (Trainer *et al.*, 2001). Furthermore, it has been reported that ligands activating two nuclear receptors at the heart of lipid metabolism, LXR and PPARγ, down-regulate 11β-HSD1 expression and activity *in vitro* and *in vivo* in adipose cells (Berger *et al.*, 2001, Stulnig *et al.*, 2002). Addition of cycloheximide revealed that ongoing protein synthesis is necessary for the effects of either LXR or PPARγ agonists on 11β-HSD1 mRNA, indicating indirect molecular mechanisms. The association of these nuclear receptors with the role of 11β-HSD1 will be discussed in detiles in the introduction to Chapter 4.

In terms of more direct effects, CCAAT/enhancer binding protein alpha (C/EBPα) binds to several sides of 11β-HSD1 promoter and positively regulates its activity in hepatocytes both *in vitro* and *in vivo* (Williams *et al.*, 2000). C/EBPβ (the key mediator of inflammatory and metabolic signaling) also binds and activates 11β-HSD1 transcription following glucocorticoid treatment (Sai *et al.*, 2008). This could potentially explain the link between inflammatory and metabolic pathways. Finally,

it should be pointed out that the regulation of 11β-HSD1 transcription and activity is tissue-specific. Whilst some cells in the lung exploit a distinct promoter (Bruley *et al.*, 2006) this is not the case for adipose and most other tissues. The mechanisms underpinning the strikingly discordant changes in 11β-HSD1 transcription in adipose, liver and other metabolic tissues remains unknown.

1.4.3 High-fat diet induced down-regulation of 11β-HSD1 activity

To gain an insight into the molecular mechanism that drives diet-induced obesity in mice Morton *et al.* (Morton *et al.*, 2004c) studied the expression of 11β-HSD1 in both obesity and metabolic disease-prone (C57Bl/6J) and resistant (A/J) strains. With chronic high fat diet C57Bl/6J mice increased weight and became profoundly hyperinsulinemic and glucose intolerant. A/J mice in contrast resisted these metabolic changes. Intriguingly, high fat diet decreases 11β-HSD1 in all fat depots, whereas in liver the enzyme is unaffected. 11β-HSD1 activity was down-regulated with high fat diet in all adipose depots in both strains. However, A/J mice have lower basal expression and greater down-regulation of 11β-HSD1 by HF in adipose tissues. It was therefore hypothesised that down-regulation of adipose tissue 11β-HSD1 with high fat diet is protective mechanism against metabolically detrimental consequences. These data suggest that individual susceptibility for the development of metabolic disease as a consequence of long term high fat diet exposure is related *inter alia* to the degree of down-regulation of 11β-HSD1 in adipose tissue, it perhaps relates to the genetics of murine strain differences. The molecular explanation for the down-regulation of 11β-HSD1 upon HF diet and its variation with strain is unknown and may relate to polymorphisms of *HSD11B1,* as mooted in humans (San Millan *et al.*, 2005, Robitaille *et al.*, 2004, Nair *et al.*, 2004, Gambineri *et al.*, 2006). Alternatively differences in free fatty acids and lipid products could activate nuclear receptors including LXR and PPARs and consequently variably down-regulate 11β-HSD1.

1.4.4 Regulation of human 11β-HSD1 activity by feeding

While insulin and other hormones exert direct effects on 11β-HSD1 in rodents, the regulatory mechanism in humans is poorly understood. Weight loss has been

reported to increase 11β-HSD1 mRNA in human fat (Tomlinson *et al.*, 2004). Recently it has been hypothesised that in humans 11β-HSD1 may be also involved in acute metabolic response to feeding (Basu *et al.*, 2006, Wake *et al.*, 2006). Sandeep *et al.* provided evidence that insulin induced a rapid down-regulation of adipose 11β-HSD1 in lean but not in obese patients (Sandeep *et al.*, 2005). Failure to reduce adipose tissue cortisol generation by insulin in obese subjects may be a decisive factor determining susceptibility to further obesity. Postprandially, when insulin is secreted, cortisol availability in adipose tissue appears to be controlled independently from the alterations of HPA axis (Wake *et al.*, 2006). Additionally, in obese men, a longer term low-carbohydrate diet enhanced cortisol regeneration by 11β-HSD1 and reduced cortisol inactivation by A-ring reductases in liver (Stimson *et al.*, 2007). Thus, dietary macronutrient composition may contribute to tissue-specific dysregulation of glucocorticoid metabolism in human obesity.

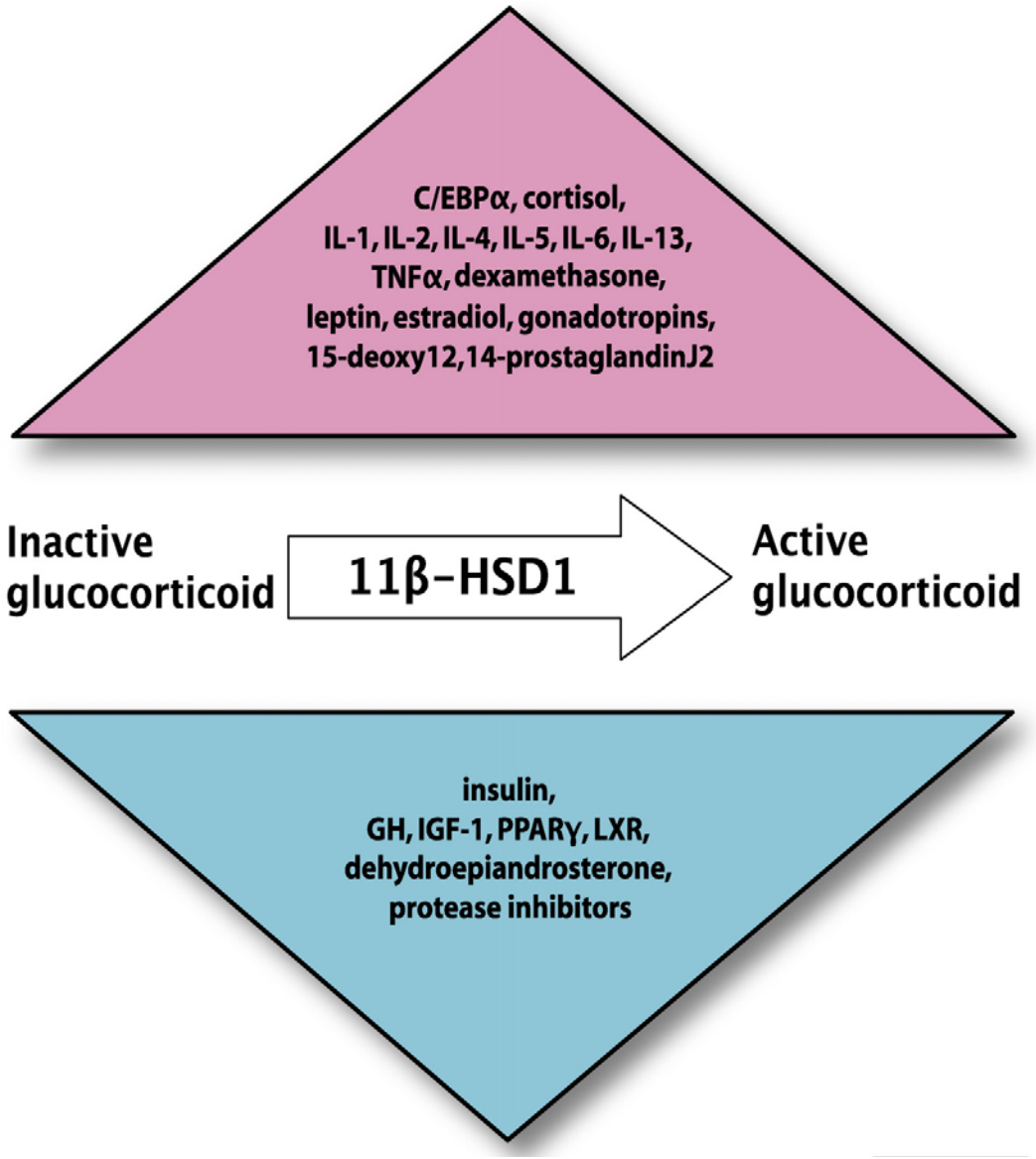

Figure 1.2 Schematic overview of the regulators of 11β-HSD1 activity.

The figure represents an overview of studies that analysed influence of various factors on the up-regulation (pink triangle) and downregulation (blue triangle) of 11β-HSD1 activity. Note: the effect may be tissue- and species-specific.

1.5 The role of 11β-HSD1 in resolution of inflammation

The anti-inflammatory properties of glucocorticoids are well known and these steroids are commonly used in clinical treatment of inflammatory disorders. Notwithstanding this, it has been suggested that while glucocorticoids have immunosuppressive effects in pharmacological doses, at physiological levels they provide a more "immunomodulatory" influence (Sapolsky *et al.*, 2000). Chronic inflammation in such conditions as rheumatoid arthritis and asthma is believed to result from disturbance of the equilibrium between the type-1 (pro-inflammatory) and type 2 (anti-inflammatory) immune responses (McEwen *et al.*, 1997). It has recently been proposed that 11β-HSD1 may be involved in the resolution of inflammation (Chapman *et al.*, 2006b). Reduced 11β-HSD1 has been reported in the synovium in rheumatoid arthritis, suggesting deficient glucocorticoid regeneration may be implicated in pathogenesis (Schmidt *et al.*, 2005). 11β-HSD1 but not 11β-HSD2 is expressed in differentiated macrophages, denritic cells, T and B cells and acts as a reductase (Zhang *et al.*, 2005, Freeman *et al.*, 2005, Thieringer *et al.*, 2001). Moreover, macrophages deficient in 11β-HSD1 are less able to ingest and therefore remove apoptotic neutrophils *in vitro* and *in vivo*, suggesting that deficiency of the enzyme may indeed contribute to the persistence of inflammation, at least in the peritoneum (Gilmour *et al.*, 2006b). Cytokines up-regulate 11β-HSD1 activity in many key cells involved in the inflammatory response including glomerular mesangial cells (Escher *et al.*, 1997, Tetsuka *et al.*, 1999), macrophages (Thieringer *et al.*, 2001, Gilmour *et al.*, 2006b), T, B and dendritic cells (Freeman *et al.*, 2005, Zhang *et al.*, 2005), human fibroblasts derived from synovium, bone marrow and skin (Hardy *et al.*, 2006) and adipocytes (Tomlinson *et al.*, 2001). Perhaps this engenders a local feedback mechanism where pro-inflammatory stimuli enhance re-generation of active glucocorticoids and therefore minimize any tendency to prolong inflammation.

Regulation of 11β-HSD1-mediated re-generation of active glucocorticoids in immune competent cells also appears to act as an autocrine mechanism regulating function. Dendritic cells stimulated by innate immunity signals increase cortisone reductase activity (Freeman *et al.*, 2005), whereas, when activated by adaptive

immune response (CD40 ligation) their capacity to generate active cortisol decreases. Intracellular reactivation of glucocorticoids by 11β-HSD1 has been also demonstrated to provide CD4+, CD8+ and B220+ lymphocytes with an intracrine mechanism controlling their viability (Zhang *et al.*, 2005). Thus, 11β-HSD1-dependent control of glucocorticoid bio-availability inside immune cells may provide a checkpoint attenuating unwanted immune activity. Thus, modulation of 11β-HSD1 activity selectively in immune-inflammatory cells may present as a new therapeutic strategy for chronic inflammatory disease.

1.6 Inhibition of 11β-HSD1 as a therapeutic target

It has been hypothesised that decreasing glucocorticoid activity in adipose tissue and liver might protect against the detrimental metabolic consequences of obesity (Walker and Andrew, 2006). Two principal therapeutic strategies to diminish the exaggerated activation of a receptor may be identified: antagonism of the receptor and/or its signaling pathway or reducing ligand availability, either systemically or locally. Administration of the GR antagonist RU38486 in Cushing's syndrome patients and in db/db mice decreases plasma glucose levels (Friedman *et al.*, 1997, Nieman *et al.*, 1985). This approach is, however, limited by the difficulties of achieving any tissue-specificity and the adverse consequences of general glucocorticoid deficiency (e.g. Addison's disease). Moreover, long-term therapy with GR antagonists leads to compensatory activation of the HPA axis and reversal of the competitive blockade as well as adrenal hyperplasia (Lamberts *et al.*, 1991). Thus, targeting pre-receptor glucocorticoid activation by 11β-HSD1 has some attractions if any compensatory HPA axis activation is incomplete and adrenal stress responses are maintained.

1.6.1 Natural 11β-HSD1 inhibitors

Derivatives of the licorice root (*Glycyrrhiza glabra*), including glycyrrhetinic acid and its synthetic hemisuccinyl ester, carbenoxolone, are potent (IC_{50} nM *in vitro*), but non-specific 11β-HSD inhibitors (Monder *et al.*, 1989). A variety of endogenous steroids and their metabolites (Latif *et al.*, 2005), as well as bile acids such as chenodeoxycholic acid (Diederich *et al.*, 2000), have 11β-HSD inhibitory properties.

Various environmental chemicals and food ingredients also interfere with glucocorticoid metabolism by 11β-HSDs (Schweizer *et al.*, 2003), notably flavanone, 2'-hydroxyflavanone-extracts from fruits and vegetables and coffee extracts (Atanasov *et al.*, 2006). 11β-HSD1 has also recently been reported to catalyze inter-conversion of additional substrates including 7oxy-dehydroepiandrosterone metabolites and 7-oxysterols (Robinzon *et al.*, 2003, Schweizer *et al.*, 2004). Thus, 11β-HSD1-dependent glucocorticoid conversion may perhaps be attenuated by competition for these alternative substrates.

1.6.2 Studies with 'natural' 11β-HSD inhibitors

Studies using the prototypic drug, carbenoxolone, showed hepatic insulin sensitization in lean healthy subjects (Walker *et al.*, 1995) and patients with type 2 diabetes (Andrews *et al.*, 2003), but not obese men (Sandeep *et al.*, 2005) (Table 1.3). The insulin sensitization appears due reduced hepatic glucose production and glycogenolysis rather than any effect on peripheral glucose uptake, perhaps because carbenoxolone fails to inhibit 11β-HSD1 in adipose tissue in rats or humans (Sandeep *et al.*, 2005, Livingstone and Walker, 2003). This raises the notion that a successful inhibitor for the treatment of obese patients with diabetes type 2/metabolic syndrome should be effective in adipose tissue. Interestingly, a recent study on healthy women with normal BMI reported significant changes in circumference and thickness of the superficial thigh fat layer after one month treatment with topical glycyrrhetinic acid (Armanini *et al.*, 2005). Such non-selective licorice-based compounds also potently inhibit 11β-HSD2 causing renal sodium retention, hypertension and hypokalaemia (Stewart and Edwards, 1991, Walker *et al.*, 1992, Schuster *et al.*, 2006). They also have effects on other short-chain dehydrogenases, such as 15-prostaglandin dehydrogenase (Schuster *et al.*, 2006) and on gap junctions (Sagar and Larson, 2006), though these effects occur at higher concentrations than 11β-HSD inhibition.

1.6.3 Novel compounds selectively inhibiting 11β-HSD.

Biovitrum first reported selective 11β-HSD1 inhibitors (Table 3). Their arylsulphonamidothiazole compounds efficiently inhibit 11β-HSD1 both *in vitro* and

in vivo and showed impressive isoform-specificy (>200fold selectivity over human and murine 11β-HSD2) (Barf *et al.*, 2002b). BVT.2733 has an IC_{50} of 96nM for mouse 11β-HSD1 (Alberts *et al.*, 2002b). *In vivo* BVT.2733 lowers plasma glucose and insulin in various hyperglycemic mice (ob/ob, db/db and KKAy), reduces hepatic expression of PEPCK and G-6-P mRNAs and therefore decreases hepatic glucose production (Alberts *et al.*, 2003b). In accordance to observations in humans, inhibition of 11β-HSD1 achieves beneficial glucose lowering only in diabetes type 2 mice models but doesn't alter plasma glucose levels in controls (Alberts *et al.*, 2003b). Moreover, BVT.2733 decreased cholesterol, free fatty acids and triglyceride levels, replicating the phenotype of 11β-HSD1-/- mice.

Merck's compound 544 (Table 1.3.) also shows selective inhibition of 11β-HSD1 activity (Hermanowski-Vosatka *et al.*, 2005). The drug lowers body weight and appetite in diet-induced obesity in mice. In parallel to previous findings, compound 544 also lowers fasting glucose, improves insulin resistance, glucose tolerance and serum lipids in a mouse model of diabetes type 2. Intriguingly, it has a pronounced atheroprotective effect in vulnerable ApoE-/- mice (Hermanowski-Vosatka *et al.*, 2005). Furthermore, RU486 showed very similar effects to the 11β-HSD1 inhibitor, suggesting that reducing the intracellular glucocorticoid signal accounted for the efficacy of compound 544.

Inhibitor	Animal studies	Human studies	Carbohydrate metabolism	Lipid metabolism	CNS effect	Proposed application	Disadvantages
Carbenoxolone	(Livingstone and Walker, 2003)	(Sandeep *et al.*, 2005, Sandeep *et al.*, 2004, Tomlinson *et al.*, 2007, Rauz *et al.*, 2003, Andrews *et al.*, 2003, Walker *et al.*, 1995)	↑ insulin sensitivity ↓ hepatic gluconeogenesis	↓ cholesterol ↓ lipolysis	Protection from age-related cognitive impairment.	• Obesity • Diabetes type 2 • Cognitive impairment • Glaucoma	• No penetration to fat tissue • Requires addition of amiloride
Compound 544 Merck	(Hermanowski-Vosatka *et al.*, 2005)	no	↑ insulin sensitivity ↓ fasting glucose ↑ glucose tolerance test ↓ food intake	↓ triglyceride ↓ cholesterol ↓ free fatty acids ↓ adipose tissue mass	Not tested	• Atherosclerosis • Diabetes type 2 • Obesity	• No human studies
BVT.2733 Biovitrum	(Alberts *et al.*, 2003b, Barf *et al.*, 2002b, Alberts *et al.*, 2005, Alberts *et al.*, 2002b)	no	↑ insulin sensitivity ↓ hepatic gluconeogenesis ↓ circulating glucose ↓ circulating insulin	↓ triglyceride ↓ cholesterol ↓ free fatty acids	Not tested	• Diabetes type 2	• No human studies • No changes in body weight • Only small decrease of food intake

Table 1.3. Prototypic 11β-HSD1 inhibitors.

The table summarises results of the early pharmaceutical programs targeting 11β-HSD1. It presents a short description of the efficacy of non-selective and prototypic selective 11β-HSD1 inhibitors with the particular focus on their effects on the carbohydrate and lipid metabolism.

1.6.4 A viable drug target for cognitive impairment?

Glucocorticoids play myriad important functions in the central nervous system. They are involved in neurotransmission, cellular metabolism, neuronal division and survival (Woolley *et al.*, 1990, Swaab *et al.*, 2005, Landfield *et al.*, 1978). Chronic elevation of glucocorticoids, as in Cushing's syndrome, is associated with affective, cognitive and even psychotic disorders (Swaab *et al.*, 2005). Accumulating evidence suggest that cognitive impairments with aging associate with elevated glucocorticoid levels in rodents and humans (Meaney *et al.*, 1995). Indeed, maintenance of low glucocorticoid level throughout life, either via neonatal 'programming' of tighter HPA axis control or by adrenalectomy with low-dose glucocorticoid replacement in mid-life, prevent the emergence of cognitive deficits with age (Meaney *et al.*, 1988, Landfield *et al.*, 1981).

11β-HSD1 is widely expressed in brain (Moisan *et al.*, 1990), notably in hippocampus, cerebellum and neocortex suggesting its potential involvement in such processes as memory and learning. The enzyme is also expressed, albeit at lower levels, in the hypothalamus and anterior pituitary indicating a role in neuroendocrine control (Seckl, 1997). 11β-HSD1 is maintained in primary cultures of hippocampal cells, where it is a reductase and inhibited by carbenoxolone. The enzyme acts *in vivo* to amplify the known neuro-endangering actions of glucocorticoids, potentiating excitatory aminoacid-induced neurotoxity (Rajan *et al.*, 1996, Ajilore and Sapolsky, 1999). This appears to be important *in vivo*, as aged 11β-HSD1-/- mice resist the usual cognitive impairments seen in aged wild type mice [129]. *In situ* hybridization studies in post-mortem human brain confirmed the expression of 11β-HSD1 but not 11β-HSD2 in hippocampus, prefrontal cortex and cerebellum (Sandeep *et al.*, 2004). In two small, randomized, double-blind, placebo-controlled, crossover studies, carbenoxolone improved verbal fluency in healthy elderly men and verbal memory in patients with diabetes type 2 (Sandeep *et al.*, 2004). Similar finding have been reported in rodents (Yau *et al.*, 2001).

Finally, a potential link between cognition and metabolism should be highlighted. Since 11β-HSD1-/- mice are insulin sensitized and have an atheroprotective lipid

profile, it might be anticipated that the neuroprotective effect of the enzyme inhibitors could be secondary to metabolic and vascular effects. Chronic hyperglycemia in type 2 diabetes indeed associates with mild cognitive impairments (Strachan *et al.*, 1997). Polymorphisms in *HSD11B1* gene have been linked to diabetes type 2 and hypertension, at least in Native Americans, and a rare polymorphism (rs846911-C/A) has been correlated with an increased risk of Alzheimer's disease (de Quervain *et al.*, 2004). However, in a study of 194 participants of the Scottish Mental Survey the common variants did not associate with cognitive impairment with ageing and the rare polymorphism was not detected (Deary *et al.*, 2006). However, although carbenoxolone enhances insulin sensitivity in healthy young volunteers (Walker *et al.*, 1995) and patients with diabetes type 2 (Andrews *et al.*, 2003), in the elderly cognition studies there were no effects on indices of glycaemic control or serum lipids. The potentially synergistic effects of 11β-HSD1 inhibition on the brain and metabolism appear propitious, but the locus of action of selective 11β-HSD1 inhibitors needs to be defined.

1.6.5 Topical 11β-HSD1 inhibitors for the treatment of glaucoma

Raised intraocular pressure is a well-recognized feature of Cushing's syndrome. Topical or systemic glucocorticoid administration results in rise in intraocular pressure and subsequently may cause iatrogenic glaucoma. Intraocular pressure is kept under control by the balance between the production and outflow of aqueous humour. Mammalian ocular tissues involved in the regulation of aqueous humour express 11β-HSD1 (Stokes *et al.*, 2000). In the support of the hypothesis that 11β-HSD1 enhanced activity may be important for the etiology of glaucoma systemic administration of carbenoxolone to healthy volunteers resulted in a reduction of intraocular pressure (Rauz *et al.*, 2003). Selective 11β-HSD1 inhibitors administered topically are a potential novel therapy for glaucoma.

1.6.6 New perspective for the treatment of glucocorticoid-induced osteoporosis

Glucocorticoids play an important role in human osteoblast differentiation, proliferation and matrix mineralization (Cooper *et al.*, 1999, Eijken *et al.*, 2005, Eijken *et al.*, 2006). Clinical use of glucocorticoids is associated with the

development of osteoporosis. Although young 11β-HSD1-/- mice have normal bones (Justesen *et al.*, 2004), this may not be pertinent to elderly humans. 11β-HSD1 is expressed in human bone and cultured osteoblasts and is regulated by pro-inflammatory cytokines and glucocorticoids (Cooper *et al.*, 2000, Cooper *et al.*, 2002). Moreover, 11β-HSD1 is regulated in differentiation dependent manner in osteoblasts (Eijken *et al.*, 2005): non-differentiated pre-osteoblasts have low alkaline phosphatase activity but increased 11β-HSD1 activity; the opposite features are found in differentiated osteoblasts. Interestingly, in primary cultures of human osteoblasts 11β-HSD1 activity increases with age of the patient, suggesting a link with the age-related incidence of osteoporosis (Cooper *et al.*, 2002). The level of 11β-HSD1 activity in human bone correlates with susceptibility of glucocorticoid (prednisone)-induced changes on bone turnover, although this is perhaps expected since inert prednisone is converted by 11β-HSD1 to active prednisolone (Cooper *et al.*, 2003). Inhibition of 11β-HSD1 by carbenoxolone suppresses bone resorption markers (Cooper *et al.*, 2000). The role of 11β-HSD1 inhibitors upon human bones *in vivo* requires further study.

1.7 11β-HSD1 inhibition and atherosclerosis.

Treatment of ApoE-/- mice, which are susceptible to 'western' diet induced atheroma, with 11β-HSD1 inhibitors protects against the development of atherosclerosis (Hermanowski-Vosatka *et al.*, 2005). Since a decrease in atherosclerosis is more pronounced than might be expected by the serum lipid-lowering effect, it raises the possibility that the additional factors may play a role. The inflammatory component of atherogenesis involves monocyte accumulation, differentiation into macrophages and foam cell formation. As atherosclerotic lesions advance, macrophages increasingly take up oxidized lipoproteins and vascular wall cells secrete inflammatory cytokines, growth factors and adhesion molecules which together contribute to the formation of advanced lesions. 11β-HSD1 is expressed in both vascular smooth muscle cells, perhaps endothelial cells (Cai *et al.*, 2001, Hatakeyama *et al.*, 2001), differentiated macrophages (Gilmour *et al.*, 2006b, Thieringer *et al.*, 2001), and is up-regulated by inflammatory cytokines providing a local mechanism to control inflammation (Cai *et al.*, 2001). However, by analogy

with the peritoneum, deficiency of 11β-HSD1 should exacerbate inflammation, potentially worsening disease. Alternatively 11β-HSD1 in inflammatory cells may impact upon their infiltration, proliferation and differentiation, which may be enhanced by glucocorticoids in the physiological range (Chapman *et al.*, 2006a). In this case, inhibition of the enzyme might be advantageous in the developing atherosclerotic lesion.

Another important possible indication for 11β-HSD1 inhibitors is associated with the ability of glucocorticoids to enhance angiogenesis (Small *et al.*, 2005). *In vitro* and *in vivo* studies demonstrated that 11β-HSD1, by locally amplifying glucocorticoid action in the vessel wall, tonically represses angiogenic responses. Moreover, seven days after coronary artery ligation 11β-HSD1-/- mice show increased vascularisation in the infarcted myocardium, associated with partial protection against myocardial dysfunction (Small *et al.*, 2005). Effects of 11β-HSD1 inhibition on pathological neovascularisation, for instance in the diabetic eye, and in cancers and metastasis remain to be determined, but are potential toxicities.

1.7.1 A novel role of 11β-HSD1 in the metabolism of oxysterols.

One of the alternative pathways of bile acid synthesis, initiated by sterol 27-hydroxylase (CYP27; 27-OHase) and characterized in a syndrome called cerebrotendinous xanthomatosis (Fujiyama *et al*., 1991), involves the conversion of 7KC to 7β-HC. This syndrome, clinically presenting with early development of atherosclerosis, is caused by a mutation in 27-hydroxylase and is associated with an increased level of 7KC in the plasma. Hence it has been hypothesised that dietary 7KC is metabolised in the liver exclusively by 27-OHase. However, contrary to expectations, when Lyons *et al.* constructed 27-OHase-/- mice they detected increased metabolism of 7KC compared with wild- type controls (Lyons *et al.*, 2002). This suggested the existence of an additional pathway of 7KC metabolism in the liver. This was supported by Song *et al.* who confirmed that an $NADP^+$-dependent 7α-hydroxycholesterol dehydrogenase in hamster microsomes generating 7KC as a product belonged to the 11β-hydroxysteroid dehydrogenase family and had high specificity for corticosterone, cortisol and 7α-hydroxycholesterol (Song *et al*.,

1998). It was likely this was the major hepatic route of metabolism of dietary 7KC. Furthermore, a non-specific 11β-HSD1 inhibitor (carbenoxolone) caused almost complete inhibition of 7-oxysterol metabolite formation in hepatic microsomes (Hult *et al.*, 2004). The novel role of 11β-HSD1 in the metabolism of 7-oxysterols showed high species-specific dependence of products (Schweizer *et al.*, 2004, Maeda *et al.*, 2002, Song *et al*., 1998). Schweizer *et al.* demonstrated the stereo-specific inter-conversion of 7KC and 7β-HC by rat and human 11β-HSD1 (Fig.1.3), whereas the hamster enzyme inter-converted 7α-, 7β-HC and 7KC (Schweizer *et al*., 2004).

7-oxygenated forms of cholesterol are present in the plasma (Corradini *et al.*, 2005, Iuliano *et al.*, 2003, Zieden *et al.*, 1999), with much higher concentrations in foam cells and atherosclerotic plaques (Schroepfer, 2000). Thus, 11β-HSD1 plays an apparent role in the clearance of highly atherogenic 7KC to more soluble 7β-HC (Schweizer *et al.*, 2004). Whether or not loss of this reaction contributes to the beneficial effects of 11β-HSD1 inhibition in atherosclerotic plaque formation remains unclear. In this thesis I tested the hypothesis that these cholesterol metabolites might act through liver X receptor (LXR) or farnesoid X receptor (FXR) pathways. The review of literature describing the role of these nuclear receptors in cholesterol and lipid metabolism and their hypothetical association with 11β-HSD1-dependent conversion of oxysterols will be discussed in detiles in the introduction to Chapter 4.

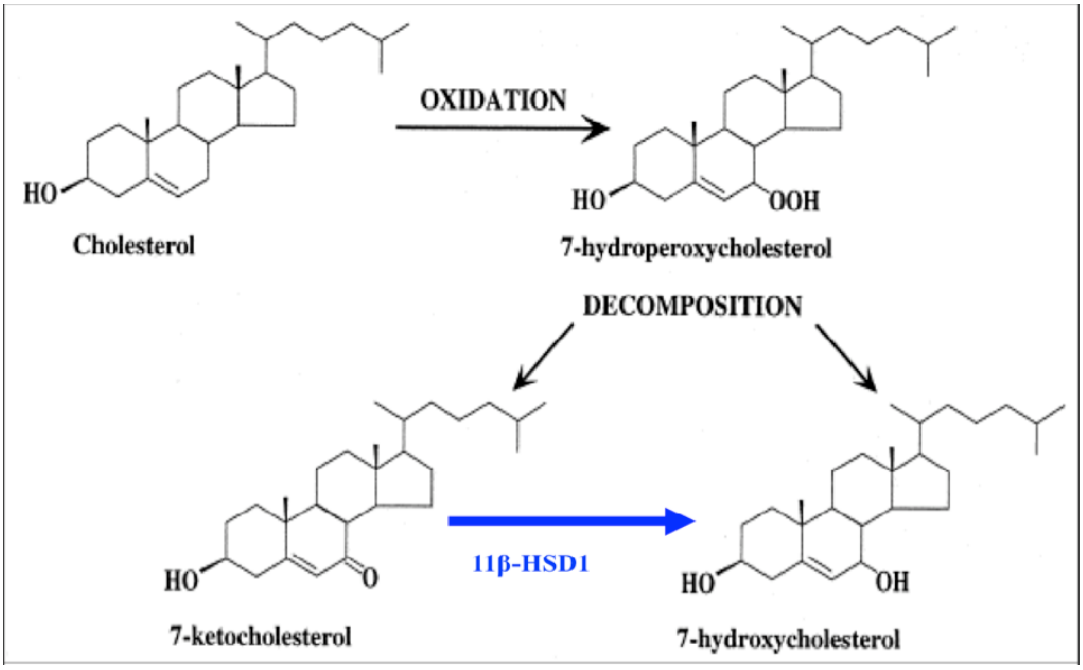

Figure 1.3 Schematic representation of 11β-HSD1-dependent conversion of 7KC to 7β-HC.

7-hydroxycholesterol is a major product of cholesterol peroxidation, however, being very unstable it undergoes a rapid conversion to 7KC and 7β-HC. 11β-HSD1 dependent conversion of 7KC to 7β-HC is the first example showing that these highly atherogenic oxysterols could be additionally produced in enzymatic reactions.

1.8 Aims

The primary aim of this thesis was to study the fat depot-specific effects of 11β-HSD1 deficiency on adipose tissue gene expression. Given recently reported novel role of 11β-HSD1 in the metabolism of 7-oxysterols the possible impact of non-glucocorticoid mediated pathways was investigated.

The following list of aims was addressed:

1) To establish whether 11β-HSD1 converts 7-oxysterols in adipocytes and to study the mutually competitive metabolism of glucocorticoids and oxysterols.

2) To investigate the potential implication of 11β-HSD1 in activation of LXR and FXR dependent pathways.

3) To map the key pathways downstream of 11β-HSD1 in both peripheral (subcutaneous) and visceral (mesenteric) adipose depots using micro-array and bio-informatics techniques.

4) To characterize depot-specific mechanisms of 11β-HSD1 deficiency leading to peripheral fat redistribution and protecting from insulin resistance on HF feeding.

CHAPTER 2

Materials and Methods

2 Materials and Methods

2.1 Introduction

Unless otherwise stated all chemicals, reagents and drugs were purchased from Sigma-Aldrich, UK. 7KC, 7β-HC and chenodeoxycholic acid (CDCA) were from Steraloids, [1,2,6,7-H^3]-corticosterone, [1,2,6-H^3]-KC from Amersham Pharmacia Biotech (Aylesbury, UK). 11-[1,2,6,7-H^3]-Dehydrocorticosterone was prepared as described previously though conversion of [1,2,6,7- H^3]-corticosterone by 11β-HSD 2 from rat placenta (Brown *et al.*, 1993). All HPLC grade solvents were purchased from Rathburn Chemicals (Walkerburn, UK). All enzymes for molecular biology were purchased from Promega (Southampton, UK). Anti-IRS-1 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Anti-Akt, Anti-Phospho-Akt (Ser 473), Anti-Phospho-Tyrosine and Anti-rabbit and Anti-mouse IgG were from Cell Signaling Technology (Beverly, MA, USA). Protein A-Sepharose was from Amersham Biosciences (Little Chalfont, UK). NuPAGE 4-12% Bis-Tris Gel and nitrocellulose Membrane were purchased from Invitrogen (Paisley, UK). Human recombinant insulin was purchased from Eli Lilly and Co. (Windlesham, UK). Routine reagents were obtained from Sigma Aldrich (Suffolk, UK) unless specified elsewhere.

2.2 Buffers and Solutions

Alkaline SDS solution	0.2M NaOH, 1% w/v SDS
Borate Buffer	8.25g boric acid, 2.7g NaOH, 3.5ml conc. HCl and 5g BSA made up to 1litre with distilled water, pH 7.4. Stored at -20°C and thawed at room temperature immediately before use.
C buffer	63g glycerol, 8.77g NaCl, 186mg EDTA, 3,03g Trizma base in 500ml dH_2O. pH adjusted to 7.7
Caesium Chloride/TE solution	100g CsCl dissolved in 100ml TE buffer (see below)
Calcium-free Buffer	Krebs buffer (see below) without $CaCl_2$
DEPC-treated water	Distilled water mixed with diethylpyrocarbonate (DEPC; 300μl/ 100 ml), shaken and left for 1-24 h prior to autoclaving

1Kb DNA ladder	20μg 1Kb ladder (Life Technologies, Paisley, UK), in 200μl distilled water with 10% (v/v) loading buffer
0.5M EDTA (pH 8.0)	800ml water was added to 186.1g $Na_2EDTA.2H_2O$. pH was adjusted to 8.0 with NaOH and the volume adjusted to 1000ml
GTE	50mM glucose, 25mM tris (hydroxymethyl)-aminomethane (tris), 10mM EDTA, pH8.0
5M K acetate	3M potassium, 5M acetate. 60 ml of K acetate and 11.5 ml of glacia acetic acetate were diluted in 28.5 ml depH_2O
Krebs'-Ringer Bicarbonate (KRB) Buffer	118mM NaCl, 3.8mM KCl, 1.19mM KH_2PO_4, 2.54mM $CaCl_2$, 1.19mM $MgSO_4$, 25mM $NaHCO_3$ in distilled water, pH 7.4. Stored at 4°C and supplemented with 0.2% w/v glucose immediately before use
LB agar	Luria-Bertoni broth with 15g agar per litre broth added before autoclaving
Loading buffer	40% sucrose w/ v, 0.25% bromophenol blue (w/v) in distilled water
Luria-Bertoni broth	10g bactotryptone, 5g bacto yeast extract, 5g NaCl made up to 1 litre with distilled water and autoclaved immediately
Luciferase assay buffer	40mM Tricine, 67mM DTT, 0.2mM $Na_2EDTA.2H_2O$, 2mM $MgSO_4$, 0,25mM coenzyme A, pH 7.8
Lysis buffer	25mm Tris phosphate pH 8.0, 2mM DTT, 1% Triton X-100, 10% glycerol
Oil Red O	0.5g of oil red O in 100 ml isoproplanolol
4% Paraformaldehyde in 0.1M phosphate buffer	20mM NaH_2PO_4, 80mM Na_2HPO_4 in 1l DEPC-treated water, heated to 80°C prior to addition of 40g paraformaldehyde. Stirred for 1 hour to dissolve and stored at 4°C
Phosphate Buffer	0.2M NaH_2PO_4 0.6M Na_2HPO_4, 5mM EDTA. Autoclaved before use
Phosphate buffered Saline (PBS)	0.1M phosphate buffer, 0.0027M KCl, 0.137M NaCl in distilled water, pH 7.4, autoclaved before use. Obtained by dissolving 1 tablet per 200 ml water (Sigma)
10x Reverse Transcription buffer	0.1M Tris-HCl, 0.5M KCl, 1% Triton X (ready mixed from Promega)
RNase Buffer	0.5M NaCl, 10mM Tris-HCl, 1mM K_2-EDTA in 10ml distilled water
20x Saline Sodium Citrate buffer (SSC)	3M NaCl, 0.3M Na citrate in 1l DEPC-treated water, pH 7.0, autoclaved before use
Sodium Phosphate Buffer	40mM NaH_2PO_4, 0.32M sucrose, 1mM DTT
Sucrose buffer	250mM sucrose, 10mM Hepes, pH7.5

10xTBE buffer:	0.9M Tris, 0.9M Boric acid, 20mM EDTA in distilled water
Tris-EDTA (TE) buffer	10mM Tris-HCl, 1mM EDTA, pH 7.5, autoclaved before use
Thermophilic DNA polymerase 10x reaction buffer	500mM KCl, 100mM Tris-HCl and 1% Triton X (ready mixed from Promega)
5xTranscription optimised buffer	200mM Tris-HCl, 50mM NaCl, 30mM $MgCl_2$, and 10mM spermidine (ready mixed from Promega)

Table 2.1 List of buffers and solutions

2.3 Animals

Male C57BL/6J mice (Charles River, UK) were obtained at 4-6 weeks of age. Animals were housed in 7.696 litre (16cm wide, 37cm long and 13cm high) cages and maintained under controlled conditions of light (12-h light/120h dark cycle: lights on 07:00h–20:00h) and temperature (21-22°C), and allowed free access to drinking water and standard chow (Special Diet Services, Witham, UK) or specifically designed high fat diets (see below). Mice homozygous for a targeted disruption of the 11β-HSD1-/- gene have been described elsewhere (Kotelevtsev *et al.*, 1997). The disrupted 11β-HSD1 allele (originally on a 129/OLA background) has been rederived onto a C57BL/6J background. All experiments conformed to ethical codes of the University of Edinburgh and Home Office (UK) regulations according to the Animals (Scientific Procedures) Act 1986. Experiments using animals were performed under the Prof. Jonathan Seckl's Programme Licence and my own Personal Licence (2004-2009). As a requirement of the Home Office I obtained appropriate qualifications by attending the Animal Handling Course organized by the University of Edinburgh in September 2004.

2.3.1 Genotyping

Genomic DNA was extracted from tails of 11β-HSD1-/- mice using Tissue Nucleospin Macherey-Nagel kit (Dueren, Germany) according to the manufactures instructions. DNA was diluted 1:50 in water. 2µl of diluted DNA were added per tube for PCR reaction. Three following primers were used: oligo ex13f1: 5'-TTC TTC GTG TGT CCT ACA GG-3', oligo ex13r1: 5'-CCC GCC TTG ACA ATA

AAT TG-3', Yuri2oligo4: 5'-CAC TGC ATT CTA GTT GTC GTT TGT CC-3'. PCR was performed according to the following protocol: 34 cycles of denaturation in 95º for 5 min, annealing in 53º for 1 min and extension in 72º for 3 min.

2.3.2 High fat diet experiments

11β-HSD1-/- mice and their congenic controls were fed a chow or high fat diet (research diet-R12331-w/sucrose and 58% of calories as saturated fat-hydrogenated coconut oil, previously optimized for weight gain and insulin resistance (Black *et al.*, 1998)) for 4 or 10 weeks, as indicated for particular experiment. Animal weight and food intake were recorded weekly.

2.3.3 Animal sacrifice and harvesting of tissues.

At the end of study period animals were fasted for 6 h to obtain plasma for fasting glucose and insulin measurements. In all experiments mice were killed by decapitation in an adjacent room at around 2:00 pm within 1 min of disturbing each cage to minimize stress to the animals. Trunk blood was collected in EDTA-tubes and plasma prepared by centrifuged (13000rpm, 10mins, 4ºC) which was then snap frozen on dry ice in Eppendorf tubes, and stored at -80ºC. White adipose tissue (WAT) was dissected *post mortem* from thigh and axillary (subcutaneous) and mesenteric depots (intra-abdominal) then frozen rapidly in liquid nitrogen for insulin signaling studies or on dry ice for RNA extraction.

2.3.4 Plasma assays

2.3.4.1 Plasma insulin measurement

Fasting insulin concentrations in plasma was measured using Ultra sensitive rat insulin ELISA kit (Crystal Chem, IL, USA). This assay works according to principles of Enzyme-Linked ImmunoSorbent Assay. In the first phase a sample of mouse plasma containing insulin was applied on a microplate 96 well plate coated with guinea pig anti-insulin antibody and incubated for 2 h in 4ºC to allow binding. Unbound material was removed by washing in washing buffer. In the second reaction the horseradish peroxidase-conjugated anti-insulin antibody was bound to the guinea pig anti-insulin antibody/mouse insulin complex immobilized on the microplate well.

Again the excess of material was removed by washing. In the final step the bound horseradish peroxidase conjugate was detected by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution. The insulin concentration was determined by measuring the absorbance at a wavelength 450nm subtracting wavelength: 630nm and calculating the insulin concentration according to the standard curve.

Reagent supplied in the kit: antibody-coated microplate, mouse insulin standard, anti-insulin enzyme conjugate stock solution, enzyme conjugate diluent, enzyme substrate solution, enzyme reaction stopping solution, sample diluent, washing buffer stock solution. A standard curve was constructed by preparing serial solutions from rat insulin stock solution (10000 pg/ml) by dilution in Diluent 2 and represented a range of concentrations of insulin (0, 156, 313, 625, 1250, 2500, 5000, 10000 pg/ml).

2.3.4.2 Plasma glucose measurement

Quantification of plasma glucose was performed using Infinity™ glucose hexokinase reagent (ThermDMA, CO, USA), which works according to the hexokinase/glucose-6-phosphate method and enables the measurement of produced NADPH proportionally to the amount of glucose in the sample. Glucose concentration was determined according to the standard curve. 2µl of plasma were diluted in 200µl reagent and incubated for 3 minutes in 37ºC. The absorbance was measured in 340nm.

2.3.4.3 Plasma corticosterone measurement in radioimmunoassay (RIA).

Samples were diluted 1:10 in borate buffer and denaturated by incubation for 30 minutes at 75ºC. Assays were performed in 96-well plates. 20µl of sample and 50µl of mix containing labeled tracer antigen (^{3}H-corticosterone diluted in borate buffer and anti-corticosterone antibody) were added per well. Plates were left to equilibrate for 1h at room temperature. In order to separate antibody-bound from free tracer, 50µl of SPA beads (Amersham, UK) was added per well and left for 24 h incubation at room temperature. Radioactivity was detected by β-counter (Liquid Scintillation Analyzer) and calculated according to the standard curve.

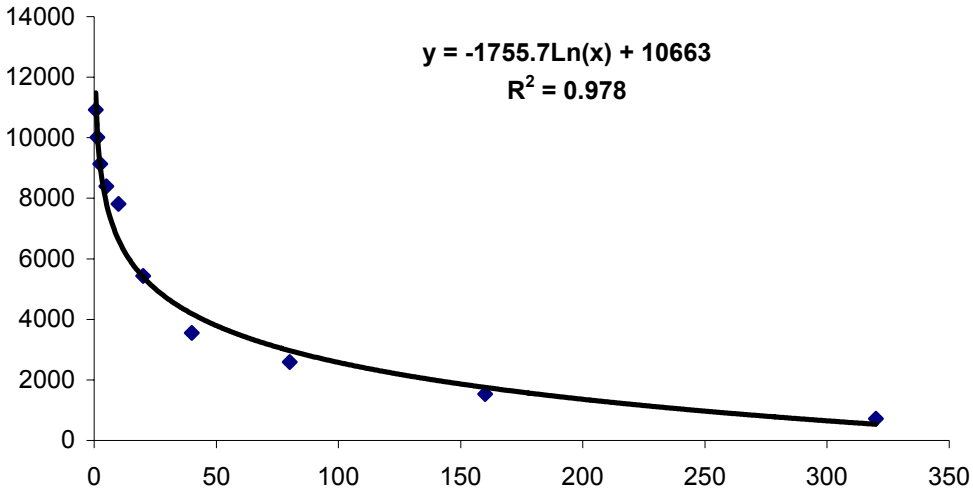

Figure 2.1 Example of the standard curve for corticosterone measurement in radioimmunoassay (RIA).

X axis represents concentration of corticosterone in nM, Y axis represents average measurement of radioactivity counts measured by β-counter.

2.4 Cell cultures

2.4.1 Reagents

Dulbecco's Modification of Eagle's Medium (DMEM)	GIBCO, Invitrogen (Life Technologies)
New Born Calf Serum (NBCS)	GIBCO, Invitrogen (Life Technologies)
Foetal Bovine Serum (FCS)	GIBCO, Invitrogen (Life Technologies)
1x trypsin/EDTA	GIBCO, Invitrogen (Life Technologies)
penicillin/streptomycin	GIBCO, Invitrogen (Life Technologies)
L-glutamine	GIBCO, Invitrogen (Life Technologies)
Bovine Serum Albumin (BSA)	GIBCO, Invitrogen (Life Technologies)
60 mm plate dishes w/poly-D-lysine	Becton and Dickinson
6 well plastic plates	Becton and Dickinson
6 well plastic dishes w/collagen I	Becton and Dickinson
12 well plastic dishes w/collagen I	Becton and Dickinson
75 cm^2 cell culture flasks	Cornig BV (Costar labs)
5, 10, 25 ml sterile pipettes	Cornig BV (Costar labs)
15, 50 ml Falcon tubes	Greiner Bioone
0.4 Trypan Blue (w/v)	Sigma-Aldrich Company Ltd
Insulin	Sigma-Aldrich Company Ltd
Dexamethasone	Sigma-Aldrich Company Ltd
Iso-butylmethylxanthine (IBMX)	Sigma-Aldrich Company Ltd
Rosiglitasone	Sigma-Aldrich Company Ltd
Poly-D-lysine	Sigma-Aldrich Company Ltd

Table 2.2 List of reagents used for cell cultures.

2.4.2 Cells

Murine 3T3-F442A cells were a gift from Prof. Bruce Spiegelman's Laboratory (Harvard University, USA). The original stock was passaged into various flasks and stored in liquid nitrogen. Murine 3T3-L1 cells were purchased from American Type Cell Collection (ATCC, LGC Promochem, UK) passaged into various flasks and stored in liquid nitrogen for future usage. HEK 293 (human embryonic kidney) cells were a gift from Dr Scott Webster (Univerity of Edinburgh, UK).

2.4.3 Maintenance of cells in culture

3T3-F442A and 3T3-L1 cells were maintained in 75 cm^2 flasks at 37°C with 5% CO_2, 95% O_2 in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated new born calf serum, penicillin /streptomycin (100μg/ml) and L-glutamine (2mM). Cells were passaged every two days (until 70% confluent). HEK 293 cells were cultured in 75 cm^2 flasks coated with poly-D-lysine in DMEM supplemented with 10% heat-inactivated foetal calf serum, penicillin /streptomycin (100μg/ml) and L-glutamine (2mM). Cells were routinely split 1:4 when confluent.

To harvest and split cells, they were washed with serum-free DMEM (8ml), then treated with trypsin/EDTA (5ml) for 1-2 min to release the cells from the flask surface. Trypsin was inactivated by the addition of culture medium containing serum and the cells diluted into new flasks or into culture plates.

2.4.4 Differentiation of 3T3-F442A and 3T3-L1 preadipocytes into adipocytes

Preadipocytes were differentiated in 6 or 12 well plates coated with collagen I (BD Bioscience). $1x10^5$ cells per well in DMEM supplemented with 10% heat-inactivated foetal calf serum, penicillin /streptomycin (100μg/ml) and L-glutamine (2mM) were plated. Two-day post-confluent medium from the culture of 3T3-F442A cells was changed for the differentiation medium (DMEM supplemented with 10% fetal bovine serum (FBS), antibiotics, glutamine) with the addition of 5μg/ml insulin. The media were changed every second day. Experiments were performed on day 7 to 10 of differentiation.

3T3-L1 cells were differentiated as previously described (Stulnig *et al.*, 2002). Two days post-confluent cells were incubated for two days in DMEM medium enriched with 10% FBS, 1% antibiotics, 1% glutamine, 0.5 μM dexamethasone, 200 μM 3-isobutyl-1-methylxanthine and 1μg/ml insulin and then for three days in medium supplemented with 1μg/ml insulin. Experiments were performed on day eight.

2.4.4.1 Oil Red O staining

On day 7 cells were stained with Oil Red O and hematoxyline-ammonium water to visualize the lipid droplets and justify the end point of differentiation. The medium was aspirated and cells were washed with PBS and fixed with 4% paraformaldehyde. 6 ml of Oil Red O stock solution were added to 4 ml depH_2O and the solution was filtered through a Whatman filter paper. 1ml of Oil Red O solution was added per 1 well of culture 6-well plate, incubated for 15-30 min and then washed with water. In further experiments the detection of lipid droplets as an indicator of differentiation was determined by phase contrast microscopy.

2.4.4.2 Trypan Blue staining

Cells pellets were ressuspended in 1ml of PBS. 10µl of cells were added to the same volume of 0.4% Trypan Blue for 5min. 5µl was then transformed to a haemocytometer with a pipette by capillary action with cover-slip in place. Stained (blue) and not stained (transparent) cells were counted in the 16 sub-squares in each of the haemocytometer chambers. Staining indicated dead cells and the ratio was calculated dividing this number by total cell number and multiplying by 100. To calculate total cell number cells were counted according to the following equation: number of cells/ml=(average count/square)x(dilution factor)x10^4(chamber conversion factor).

2.5 Enzymology

2.5.1 11β-HSD1 activity assay

The 11β-HSD1 activity assays were performed by incubating intact cells in 12-well plates with 1ml of serum-free culture media containing 200nM corticosterone (oxo-reductase) and 5nM [1,2,6,7-H^3]- corticosterone as a tracer or the same concentrations of 11-dehydrocorticosterone (dehydrogenase) and 11-[1,2,6,7-H^3]-dehydrocorticosterone. The incubation was carried out in serum- and steroid-free medium in 37˚C for 4 h. After incubation, media was transferred to a glass tube and steroids were extracted with 3ml of ethyl acetate. The aqueous phase was removed and organic phase was evaporated. The dried etheric extracts were then resuspended in 45µl of ethanol and transferred to silica gel-precoated plastic sheets (Merck,

Darmstadt, Germany) for thin-layer chromatographic separation of precursor and product in the solvent system chloroform:ethanol (92:8 by vol) (BDH Laboratory Supplies, Poole, Dorset, UK) and quantitated after 48 h incubation using a phosphorimager (Fuji FLA-2000, Raytek Scientific Ltd, Sheffield, UK). All experiments were carried out in triplicate. Enzymatic activity was expressed as percentage total radioactivity [(product cpm/substrate cpm+product cpm)x100] after correction for values from control (no cell) incubations.

2.5.2 Conversion of oxysterols

A well established protocol for the measurement of glucocorticoid conversion in the 11β-HSD1 activity assay was used in the primary development of the 7-oxysterol conversion assay. 3T3-F442A fully-differentiated adipocytes were incubated with 7KC diluted 1:1000 from ethanol stocks in the medium to final concentrations of 5μM, 500nM and 50nM. Different time points ranging from 1 to 24 h were tested. Initially, I attempted to detect the potential product of 7KC conversion in the medium (by analogy to glucocorticoids) using a previously established method of HPLC analysis with UV detector (Wamil *et al.*, MSc project 2005, University of Edinburgh, unpublished). 40μl of each terminated enzymatic reaction was analyzed for conversion by HPLC with a C18 column (Symmetry Shield, 15cm, 4.6mm, 5μm pore size, Waters, UK). The system was controlled by the Winflow computer programme (JMBS Developments, France). Individual experiments were carried out in duplicates with single data points. The mobile phase was acetonitrile/water (90:10). The 7KC and 7β-HC absorption were measured at 241nm and 195nm, respectively. The amount of product formed was determined by comparison to standard curves of authentic 7-oxo-cholesterol derivatives using 5α-cholestanol as an internal standard.

The following methods of oxysterol extraction were tested:

1) Steroids in medium were recovered by solid-phase extraction. Samples of culture medium with 7KC from incubation experiments, control medium and standard media with 7-KC and 7β-HC were purified through Sep-pak C18 cartridges (Water, Herts, UK) according to the manufacture instructions. Columns were washed with 5ml of

methanol and 5ml HPLC grade water prior to sample injection and with 5ml water after sample application. 2 ml of methanol was used to elute the extracted oxysterols. The eluant was dried under a stream of oxygen-free nitrogen at 60°C and resuspended in 2ml petroleum ether and 200µl water. This step was repeated 3 times. The upper layers were evaporated and resuspended in 500µl of mobile phase (95% acetonitrile, 5% water) and prepared for HPLC analysis.
2) Samples of culture medium with 7KC from incubation experiments, control medium and standard media with 7-KC and 7β-HC were mixed with 3 volumes of following solvents: petroleum ether, methanol, hexane: iso-proplanolol (2:3 v/v), dichloromethane, centrifuged and the upper layers (the bottom layer in case of dichloromethane) were taken into new tubes, evaporated and prepared for HPLC analysis.

As little as 1-10% of oxysterols added to the medium, either in standards or in tested samples, was recovered using the above methods. Secondly, I tested the efficiency of oxysterol extraction by increasing the volume of solvents used as an organic phase. The recovery from the medium was improved to 50-70% by using 6 volumes of dichloromethane.

Optimization of assay conditions:
In order to investigate whether the observed decrease in the concentration of 7KC was caused by degradation, the radio-labeled form of 7KC was used in following experiments. Firstly, quantification of the amount of radioactivity in the medium and in the cellular pellet (after different time points of incubation with 7KC) was performed using Liquid Scintillation Analyzer. This showed that the measured decline of radioactivity in the medium could be explained by its accumulation in the cellular pellet. Moreover, there was no adherence of oxysterols, either to plastic or to glass pipettes and plates. It was confirmed that [1,2,6-H^3]-KC had been transported through the cell membrane in a linear manner over the time course (Fig. 3.2.). Interestingly, after 24h incubation all radioactivity counts were accumulated in the cellular pellet with very little remaining in the medium (<17%). Based on these findings, the optimal conditions of oxysterol recovery from cell lysates were

established. Extraction from lysis buffer using the ethyl acetate as an organic phase showed the highest rate of recovery of added radioactive 7KC (90%). Conditions for TLC analysis of metabolites using the solvent system 2:3 (v/v) hexane: ethyl acetate to separate radio-labeled 7KC and the product of its conversion-7βHC were also established.

Experiments were performed according to the following protocol:

Conversion of [1,2,6-^{3}H]-KC was studied in 3T3-F442A cells. Culture medium containing substrate 7KC (5μM, 500nM), including 0.5 μCi [1,2,6-^{3}H]-KC, was added to each well. Stocks of sterols were diluted in 100% ethanol and used in 1:1000 dilutions in culture media. Aliquots were removed at 1, 2, 5 and 24 h. All incubations were in triplicate. Incubation was for 24 h at 37ºC in a humidified tissue culture incubator gassed with 95% air-5% CO_2. Media from individual wells were then pipetted into glass tubes, to which 6ml dichloromethane was added and the samples were vortexed for 1min. Cells were washed with 1ml PBS, centrifuged for 5 min at 2000rpm and resuspended in an appropriate volume (100-300 μl) of lysis buffer (300 mM NaCl, 50mM Tris-HCl, 1mM EDTA, 5% glycerol, 5% Triton X-100, pH 7). Lysates were incubated for 10 min at 37˚C. Oxysterols were extracted by adding 3 ml of ethyl acetate to the lysis buffer. The organic phase was removed to the fresh tube and solvent evaporated under oxygen free nitrogen at 50°C. Oxysterol extracts were resuspended in mobile phase consisting of water (15%) and acetonitrile (85%) and injected into the HPLC system with radioactivity detector (LD509 Berthold absorbance detector), using a Sun Fire C_{18} column (15 cm, 4.9 μm particle size; Waters, Macclesfield, UK). A flow rate of 1.0ml/min and a column temperature of 40°C allowed the best resolution of the metabolites. Non-radioactive 7-oxysterol standards were employed to optimize resolution conditions with an on-line dual wavelength absorbance UV detector in HPLC using C18 column (Symmetry Shield, 15cm, 4.6mm, 5μm pore size, Waters, UK). The wavelength of UV detection was determined by the structural characteristics of the analytes similar to the previous conditions (Wamil et al, 2005, MSc manuscript, University of Edinburgh, unpublished). The percent conversion of substrate to product was calculated by the measurement of peak areas. Assays were performed in duplicate and also without

cells as controls. 7β-HC and 7KC were detected at retention times 28 and 32 minutes, respectively. No additional products of 7KC conversion were observed, either by HPLC or by TLC. The complete inhibition of the 7-oxysterol conversion by the selective 11β-HSD1 inhibitor was used as a control for autoxidation during sample preparation. Additionally, in samples, in which 11β-HSD1 has been heat inactivated, no product of the reaction was present.

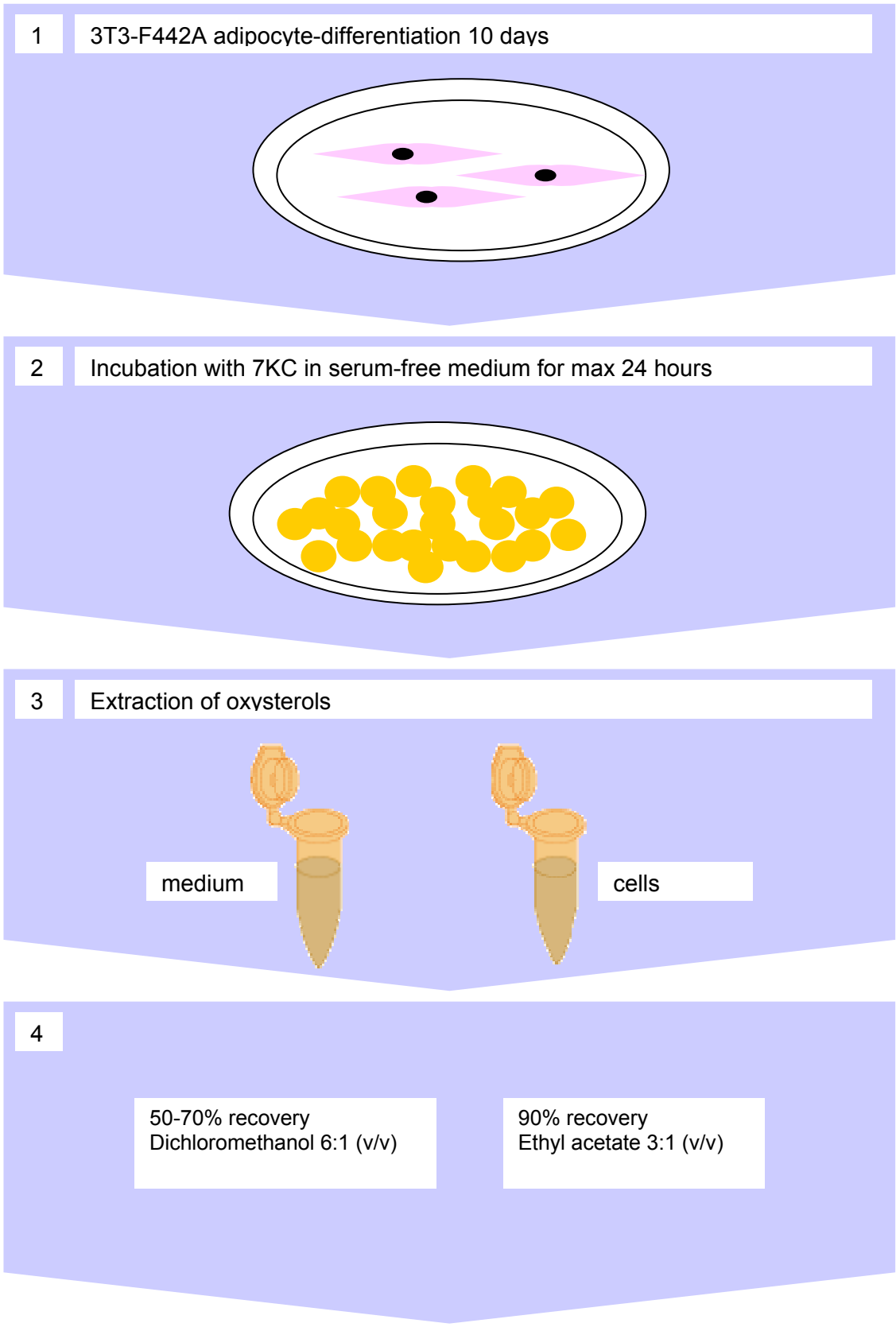

Figure 2.2. Schematic representation of the protocol used for experiments investigating 7-oxysterol conversion in adipocytes.

2.6 Recombinant DNA techniques

2.6.1 Bacterial transformation

For cloning of DNA, Escherichia coli HB101 cells (Promega, Southampton, UK) were grown in 100ml of Luria-Bertoni (LB) broth at 37°C in a shaking incubator until they reached mid-log phase. They were then centrifuged at 1000 x g for 5min at 4°C (Heraeus, Germany), the pellet re-suspended in 20ml cold calcium chloride (0.1M) and left on ice for between 10min and 2h. The centrifugation step was repeated to re-pellet the cells and the cells were re-suspended in 2ml cold calcium chloride (0.1M). The competent cells were stored on ice at 4˚C for up to 4 days before transformation.

200µl of competent cells was mixed with 50ng plasmid DNA and left on ice for 20-30 min. To incorporate the plasmid DNA into the competent cells they were subjected to 42°C for 50s and placed back on ice for 2-3 min. The cells were spread onto LB agar plates containing ampicillin (100µg/ml) to allow only the grow of colonies containing incorporated DNA. The plates were left to dry and then they were incubated for 16 h at 37°C. Plates with transformed colonies were stored at 4˚C.

2.6.2 Plasmid DNA preparation

Following transformation, single colonies were picked and used to seed 5ml of LB containing ampicillin (100µg/ml). After 8h incubation in a rotating incubator at 37°C, cultures were transferred into 500ml LB broth (containing 100µg/ml ampicillin) and shaking overnight in a 2l flask at 37°C. The resulting culture was pelleted by centrifugation at 6000 rpm for 5min at 4°C (Heraceus, Germany) and the pellets resuspended in ice-cold GTE (12ml) mixed with 24ml of fresh alkaline-SDS and incubated on ice for 5 min before the addition of 16ml of ice-cold 5M K acetate. The mixture was centrifuged at 6000 rpm for 10min. The mixture was filtered through two layers of sterile gauze into fresh centrifuge pots containing 32ml isopropanol and left at room temperature for 30min to precipitate the DNA. DNA was recovered by centrifugation at 10000 rpm in a Beckman J20 rotor for 5min at 4˚C. The pellet was dried and resuspended in 2.2ml TE buffer, 2.95g of CsCl was

added and dissolved and then ethidium bromide (100µl, 10mg/ml) was added. The mixture was transferred to Beckman Quickseal ultracentrifuge tubes, topped up with 1g/ml CsCl/TE solution and the volume adjusted to balance tubes. They were then centrifuged at 70000 rpm for 17h at 20°C in a Beckman J20 rotor. The DNA was separated into bands that could be visualised by the pink colour of the ethidium bromide. These DNA bands were removed using a 21gauge needle and syringe, transferred to fresh ultracentrifuge tubes, topped up with 1g/ml CsCl/TE solution and centrifuged at 100000 rpm for 4h at 20°C. The DNA bands were collected as above and the ethidium bromide was removed by extracting repeatedly with equal volumes of isopropanol until the pink colour disappeared. The supernatant was removed and the pellet was washed with 70% ethanol. The DNA was transferred to dialysis tubing and dialysed against three changes of TE buffer. The concentration and purity of the recovered plasmid DNA was measured spectrophotometrically using a GeneQuant RNA/DNA Calculator (Pharmacia Biotech, Sweden). DNA was diluted 1:100 in DEPC-treated water and the optical density at λ260nm and λ280nm was determined. Plasmids were stored at -20°C.

2.7 RNA extraction and analysis

2.7.1 Materials

RNA extraction	
RNeasy Lipid Tissue Midi kit	Qiagen
Trizol	Invitrogen
Rnase Plus Bio 101 matrix	Anachem
RNA wash concentrate	Anachem
Dithiothreitol (DTT)	Sigma-Aldrich Company Ltd
Reverse Transcriptase	Invitrogen
SuperScript III kit	Invitrogen
Dnase amlification Grade	
PCR reaction	Promega
1.5mM $MgCl_2$	Promega
AmpliTaq Gold	Applied Biosystems
Taqman Real Time Assays	Invitrogen
Oligonucleotid primers for RT-PCR	Invitrogen
dNTPs	
396 well plates	Promega
Ultra pure Rnase, DNase free water	Sigma

Table 2.3 List of reagents used for RNA extraction.

2.7.2 Primers and probes for real time PCR were designed by Applied Biosystems (Table 2.4).

Gene	Assay ID
11β-HSD1	Mm00476182_m1
ABCA1	Mm00442646_m1
ABCG1	Mm00446249_m1
Actin	Mm00607939_s1
Angiotensinogen	Mm00599662_m1
Bmal1	Mm00441730_m1
BSEP	Mm00445168_m1
CD8	Mm01182107_g1
CTP1	Mm00487200_m1
FAS	Mm00662319_m1
FABP3	Mm02342494_m1
GLUT4	Mm00436615_m1
HGCoAR	Mm01282499_m1
Hsp6	Mm01176578_m1
IL7R	Mm00434295_m1
Leptin	Mm00434759_m1
MARCO	Mm00440265_m1
PEPCK	Mm00440636_m1
PPARγ	Mm00440945_m1
PRKAA2	Mm01264790_m1
Rev-erbα	Mm00441730_m1
Sell	Mm00441291_m1
SHP	Mm00442278_m1
SREBP1c	Mm00550338_m1
SREBP2	Mm01306292_m1
STAT4	Mm00448890_m1

Table 2.4 TaqMan gene expression assays (Applied biosystems, UK)

2.7.3 RNA extraction from tissues and cells

Tissues were collected from mice and frozen on dry ice immediately after dissection and stored at –80˚C until required. To obtain the best quality material for micro-array experiments RNA from adipose tissues was extracted using RNeasy Lipid Tissue Midi kit according to the manufacture's instructions (Qiagen, UK).

In order to extract RNA from cells, the medium was removed, and cells were washed with 1ml of PBS and 1ml of Trizol (per well of a 6 well plate). Cells were resuspended by pipetting up and down, collected in eppendorfs and immediately frozen on dry ice. Due to the inherent instability of RNA, special care was applied when working with it to avoid degradation by ribonucleases.

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

2.7.3.1 Homogenization

Trizol was added and samples were homogenized using an Ystral mechanical homogeniser (Scientific Instruments Centre, UK) at maximal speed. To remove insoluble material and small amounts of unhomogenized tissue samples were centrifuged at 12000 rpm at 4°C for 10min The supernatant was removed to a fresh eppendorf.

2.7.3.2 Phase Separation

Following homogenisation, samples were allowed to equilibrate to room temperature then left for 5 minutes to allow complete dissociation of the nucleoprotein complexes. For cell experiments, apart from homogenisation the same protocol was followed as for homogenised tissue. 0.3ml chloroform per 1ml Trizol was added to each sample and vortexed for 15s. Samples were left on ice for 15 min and then centrifuged at 12000 rpm at 4°C for 15min resulting in a lower red phenol-containing phase (containing proteins), an interphase (containing DNA and denatured proteins) and an upper aqueous phase containing RNA.

2.7.3.3 RNA Precipitation

The upper aqueous phase from each sample was transferred into a fresh eppendorf and the RNA was precipitated by addition of 30µl RNaid Plus Bio 101 matrix (Anachem, UK). To allow binding of RNA to the RNA matrix samples were shaken at room temperature for 3min and then incubated at room temperature for 10min and then centrifugated at 12000 rpm at 4°C for 10min.

2.7.3.4 RNA Wash

Following centrifugation the supernatant was removed and the RNA pellet was washed with RNA wash concentrate diluted by the addition of 30% v/v absolute ethanol. The wash was repeated three times.

2.7.3.5 RNA Resuspension

Following the RNA wash, the ethanol was removed and the pellets were briefly air-dried for 5min. RNA pellets were dissolved in 20µl DEPC-treated water containing 10mM DTT and 400U/ml RNAse inhibitor and incubated at 55°C for 12min. Samples were vortexed after 6 min of incubation. Then samples were immediately centrifuged 13000 rpm at 4°C for 2min. Supernatant was aliquoted in three eppendorfs (6µl) and stored at -80°C.

2.7.3.6 RNA quality and integrity

RNA concentration and purity was assessed using a GeneQuant RNA/ DNA Calculator (Pharmacia Biotech, Sweden). RNA was diluted 1:100 in DEPC-treated water and the optical density at wavelength 260nm and 280nm was determined to assess concentration and purity. RNA was only used with a $\lambda 260/\lambda 280$ of between 1.6 and 1.9. Integrity of RNA was assessed by elecrophoresis in a 1% agarose/TBE gel containing 500ng/µl ethidium bromide. Visualization showed two clear bands corresponding to 18S and 28S rRNA.

2.7.4 Reverse transcriptase reaction

The Super Script III First-Strand Synthesis System for RT-PCR has been used to synthesize cDNA from total RNA. The enzyme is used to synthesize cDNA at a temperature range of 42-55ºC. In order to remove all traces of genomic DNA contamination from RNA samples containing 1µg RNA were treated with DNase I, Amplification Grade (Invitrogen, UK), which digests single- and double-stranded DNA to oligodeoxyribonucleotides. Samples were then immediately preceded for first strand cDNA synthesis. Reactions containing 0.5µg Oligo$(dT)_{20}$ primers (a more specific priming method which hybridize to 3'poly(A) tails), 1mM dNTP and the whole samples of RNA resulted from previous reaction were heated at 65°C for 15min followed by 5min on ice to inactivate enzymes. A master mix was performed containing 5x First Strand Buffer (250mM Tris-HCl, 375mM KCl, 15mM $MgCl_2$), 0.1mM DTT, 40U RNaseOUT and 200U SuperScript III. RNaseOUT Recombinant Ribonuclease Inhibitor, also included in the enzyme mix, is an RNase inhibitor protein that safeguards against the degradation of target RNA due to ribonuclease contamination of the RNA preparation. 7µl was added to each sample. Reactions were carried out in Eppendorf thermocycler. Samples were incubated for 55min at 50°C. Reaction was terminated by heating for 10min at 75°C. Finally, to remove the RNA template from the cDNA:RNA hybrid molecule after first-strand synthesis 1µl (2 U) of E. coli RNase H were added and samples were incubated for 20min at 37°C. Negative controls containing DEPC-H_2O instead of enzyme or omitting RNA were run in parallel to determine contamination of PCR reagents.

2.7.5 PCR reactions

PCRs were carried out on an Eppendorf Mastercycler Gradient (Eppendorf, Germany) with a heated lid. The Invitrogen AmpliTaq system was used for PCR during this project. Reactions were carried out in 0.5 ml reaction tubes in a total volume of 20µl containing 2µl of cDNA, 2µl of 10x AmpliTaq buffer, 2µl 1.5 mM $MgCl_2$, 0.1µl of dNTP, 1U AmpliTaq and 13.4µl of DEPC-H_2O. Samples were heated to 95°C for 3min for initial denaturation, then underwent 35 cycles of PCR amplification (denaturation at 95°C for 45s, primer annealing at primer-specific

temperature for 30s and elongation at 72°C for 1min 30s). Upon completion of the PCR programme, samples were incubated at 72°C for a further 5min to ensure elongation of products to full length and chilled to 4°C prior to gel electrophoresis.

2.7.6 Quantitative Real Time PCR

mRNA expression was quantitated by Light Cycler ® 480 thermocycler (Roche, UK). Reactions were carried out in 396 well plates. The commercially available TaqMan® gene expression assays for the selected genes were purchased from Applied Biosystems. Triplicate reactions were carried out for each sample. Each reaction contained per 10µl of total volume: 2µl of diluted cDNA, 5µl of Roche Probe Master Mix, 2.5µl DEPC-H_2O and 0.5µl appropriate assay. Mouse TATA box binding protein (TBP; Applied Biosystems, UK) assay was used to normalise the transcript levels for all chosen genes. In preliminary experiments TBP, 18S and actin were used to confirm the relevance of internal standard. Hence the expression levels of TBP were in the same range of concentrations as most of chosen genes and did not show variations in relation to genotype and tissue TBP was used as an internal standard. A standard curve for each primer-probe set was generated in triplicate by serial dilution of cDNA pooled from all samples tested. This curve was then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations (Fig. 2.3.). Samles of cDNA were diluted 1:20 in water to ensure that the level of experession of the gene of interest will be in the middle of the standatr curve. Samples for TBP and tested genes were run in triplicates and the mean values were used to calculate transcript level. Values were calculated as a relative fold change in mRNA from an internal control sample using standard curves.

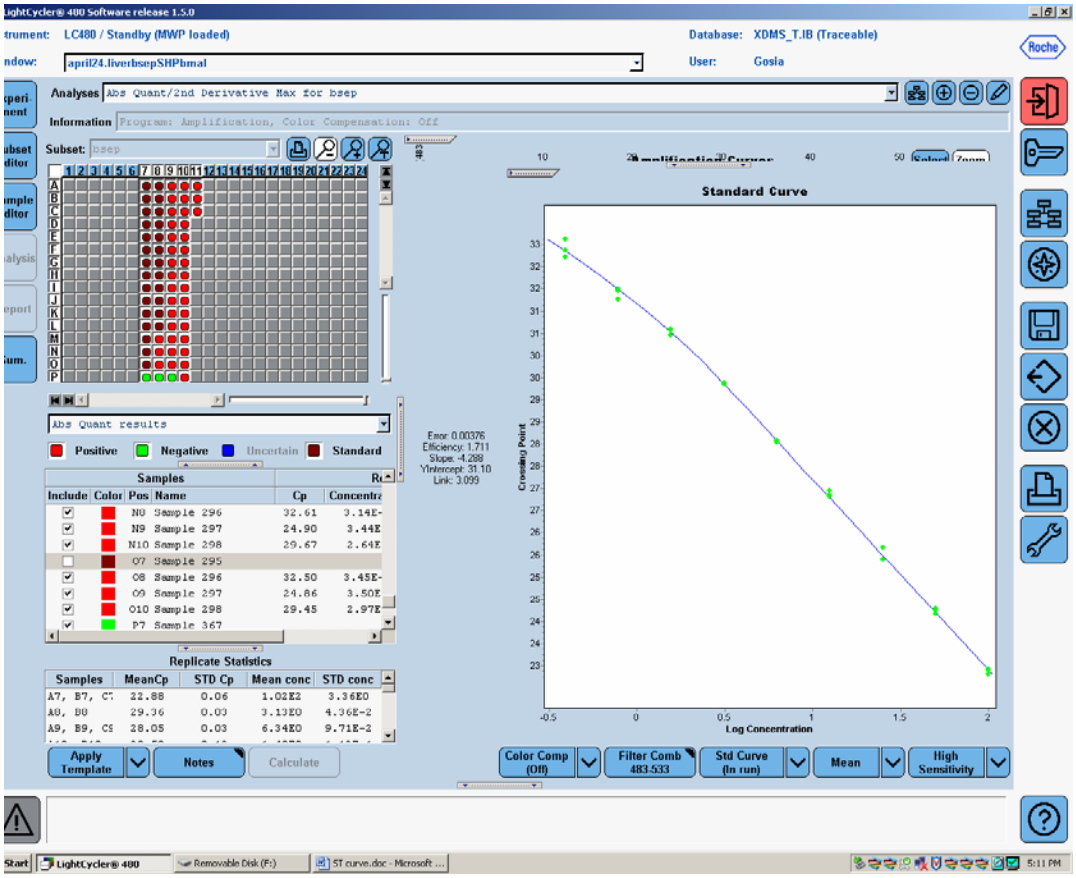

Figure 2.3 An example of a qRT-PCR standard curve.

2.7.7 Transient transfections

The plasmids used were pGEM3 (inert DNA) (Promega, UK), pKC275 (a β-galactosidase expression plasmid which was used as an internal control for transfection efficiency) (Amersham Pharmacia, UK), pLTR-Luc (full length MMTV LTR driving the expression of a luciferase gene) (Prima *et al.*, 2000) and pEGFP-GR (Prima *et al.*, 2000) a rat glucocorticoid receptor, CMX-Gal4-hLXRa, (an expression construct containing the LXR-α ligand binding site; amino acids 166-447), pMH100X4–thymidine kinase–luc (pMH100X4–TK–luc), a luciferase reporter construct (Willy *et al.*, 1995), human FXR (Dupont Pharmaceutical, France) expression constructs, 3XR-FXRE-pTA-Luc (containing 3 copies of FXRE from murine I-BABP) (Zhang *et al.*, 2003).

2.7.7.1 GR, LXRα and FXR activation in cell lines.

Transient transfections were carried out using the Gene Juice Transfection Reagent (Novogen, UK). Cells were seeded at $2x10^5$ per well in 6 well plates coated with poly-D-lysine (Sigma, UK) a day before transfections. DNA solutions were briefly vortexed to mix and added dropwise to serum free 100µl OPTIMEM mixed with 4µl Gene Juice Transfection Reagent with slow agitation and incubated at room temperature for 15min. Then they were added slowly dropwise to the dishes of cells. After overnight incubation, medium was replaced for charcoal-stripped FCS medium and cells were transfected with a total 1µg of DNA comprising 0.1µg pEGFP-GR (Prima *et al.*, 2000), 0.5µg pLTR-Luc (Prima *et al.*, 2000) and 0.1µg pKC275 (internal control). Alternatively, for LXRα activation, cells were transfected with 0.1µg CMX-Gal4-hLXRa, an expression construct LXR-α, 0.5µg TK-MH100X4-Luc reporter plasmid (a kind gift from Prof. David J. Mangelsdorf, Southwestern Medical Center, Dallas, Texas) and 0.5µg pKC275 (Pawar *et al.*, 2002, Willy *et al.*, 1995). For FXR activation assays, cells were transfected with 0.1µg pS65 hFXR, 0.1µg hRXRα (a gift from Dr Karen Chapman, University of Edinburgh), 0.1µg FXRE-pTA-Luc and 0.5µg pKC275. After overnight incubation steroids and oxysterols were added for 16-24 h before harvesting and lysis of cells. GR was activated with 11-DHC and corticosterone (100nM-1000nM), LXRα with 20µM 22(R)-hydroxycholesterol and 250nM T0901317 and hFXR/RXRα with

chenodeoxycholic acid (20 and 40μM). 24h later, cells were harvested for assays. Medium was aspirated, cells were washed with 1ml PBS and 200μl lysis buffer was added to the dishes. After incubation at room temperature for 15min, cells were scraped and pipetted into eppendorf tubes; cell debris was pelleted by centrifugation at 13000 rpm for 2min in a microcentrifuge. All transfections were carried out in triplicates and mean ratio of luciferase/β-galactosidase activities were calculated.

2.7.7.2 Luciferase assays

Luciferase assays were performed in duplicate on the same day that cells were harvested. 40μl sample was added to 100μl 2x assay buffer with 5μl 0.1M ATP in a 96 well plate. Luciferase activity was measured using a Lumat LB9501 luminometer (Berthold) that injected 105μl 1mM beetle luciferin (Sigma, UK). Values recorded were the means of the duplicates.

2.7.7.3 β-galactosidase assays

β-galactosidase activity was assayed using a Galacto-Light Plus (Applied Biosystems, UK). Galacton-Plus substrate was diluted 1:100 with Reaction Buffer Diluent to make the reaction buffer which was then dispensed in 67μl aliqouts into 96 well plate. 10μl sample was added, left to incubate at room temperature for 15-60min and then assayed using a Lumat LB9501 luminometer which injected 105μl Light Emission Accelerator. Data were analysed using a Microsoft Excel spreadsheet. To standardize the transfection efficiency, the relative light units obtained in the luciferase assay were divided by optical density obtained in the β-galactosidase assay. Presented values of fold luciferase increase were the means of two duplicates of luciferase activity/β-galactosidase activity. The mean value obtained for empty vector was then set to a value of 1 and mean experimental activities expressed relative to this value.

2.7.8 GR trafficking

pEGFP-rGR and NGFP-GR (positive control) were obtained from P. Lefebvre (Inserm, Little Cedex, France)(Prima *et al.*, 2000). HEK 293 cells stably transfected with human 11β-HSD1 were kindly provided from Dr Scott Webster

(Endocrinology, University of Edinburgh). Cells were grown on poly-D-lysine-coated glass slides in 6 well plates containing 2ml serum-stripped medium. Cells were transfected as described above with 1µg EGFP-GR per well and incubated for 48h. Cells were then incubated for an appropriate time with 7-oxysterols and glucocorticoids. After washing the cells with 1ml PBS and fixation with 4% paraformaldehyde cells were stained with DAPI for nuclear visualization. The localization of GFP-GR was detected by fluorescence and confocal microscopy (LSM510, Zeiss, Oberkochen, Germany). The EGFP was excited with the 488 nm line from an argon laser.

2.8 Genomics

2.8.1 Concept

DNA micro-arrays are used to measure simultaneously changes in the level of expression of many genes. Depending on the chip type, the entire genome or clusters of genes representing the chosen pathways can be interrogated. A traditional micro-array chip is a library of microscopic DNA probes (approximately 200microns in size) attached to glass, plastic or silicon chip. Other types of micro-array chips are also used to detect SNPs in the genome, to perform chromatin immunoprecipitation based on CHIP-on chip technology or comparative genomic hybridizations.

The most important applications for the use of micro-array technology include:

1) Genes expression profiling
2) Pharmacogenomics
3) Toxicology screening
4) Diagnostics

2.8.2 Design

Experimental design has to include consideration of sufficient biological replicates according to the expected biological variance. Replicates of the same RNA aliquot or the same treatment can additionally improve precision. Noteworthy probes of cDNA representing the same gene or a particular sequence are spotted on the chip in

replicates. Thus, the careful planning needs to be employed in order to draw biologically valuable conclusions.

A scheme of micro-array experiment design.

A) Biological question
B) Experimental design
C) RNA extraction
D) Quality control- Bioanalyser system Agilent Technologies
E) Target sample preparation and hybridization
F) Image acquisition (scan of a chip)
G) Image quantification
H) Background correction
I) Normalization
J) Data analysis and interpretation
K) Verification of results by RT-PCR and Western blotting
L) Biological interpretation

2.8.3 Data analysis

The most common use of the micro-array experiment is to extract differentially expressed genes from a large number of genes presented on a chip by comparison between two experimental conditions (transgenic versus wild-type animal in our experiment). However, the identification of differentially regulated genes in this simple experimental setup may be complicated by high biological variability and the consequent "noisiness" of the data. Thus, selection between the biologically relevant changes and false positive findings is one of to the most challenging steps in micro-array studies. Regarding these issues, a number of obstacles could be identified. The choice of the most reliable method of data-mining and statistical analysis is usually underestimated. Most currently used programs use basic statistics and give a similar outcome when applied to the set of micro-array data. Significantly, the level of rejection of the borderline genes may differ between the methods. This can have

serious consequences if it is acknowledged that biological relevance may not correspond directly to the statistical significance or the highest fold changes but rather to the direction of changes in functional gene classes. Thus, in my project analysis of the data was performed using two different approaches. The first technique was based on the calculation of rank products (RP) from replicate experiments (Breitling *et al.*, 2004a, Breitling *et al.*, 2004b, Breitling *et al.*, 2004c, Breitling and Herzyk, 2005b, Breitling and Herzyk, 2005a). This approach was designed at the Sir Henry Wellcome Functional Genomics Facility at University of Glasgow (http://www.gla.ac.uk/functionalgenomics/). According to its designers, using RP can lead to a sharp reduction in the number of replicate experiments needed to obtain reproducible results (Breitling *et al.*, 2004a, Breitling *et al.*, 2004b, Breitling *et al.*, 2004c, Breitling and Herzyk, 2005b, Breitling and Herzyk, 2005a). Furthermore, the results were also analysed using a basic statistical approaching (the Limma tool on BioConductor: http://www.bioconductor.org). The results are presented as lists of differentially-regulated genes.

2.8.4 Pathway analysis

Micro-array experiments usually generate long lists of differentially regulated genes but they provide few clues as to how those changes can be biologically relevant in establishing a given phenotype. This also turned out to be the case in my experiment. If the understanding of the results is limited to the knowledge of the investigator and his/her current ideas, the exploration of the data can be significantly reduced. Additionally, another issue appeared. I had to overcome an intuitive concept that interesting genes would show the highest fold changes. My data set contained only a limited number of genes with high fold changes. These were especially related to metabolically important genes. Thus, I decided to address these problems by focusing on all functional gene classes similarly regulated in this particular experimental setup, rather than on single genes. Both techniques used for the data-mining provided tools that enabled pathway analysis. Interactive Group Analysis offers the functional classes analysis based on the GeneOntology (http://www.geneontology.org).

Additionally, a newer approach of genome-wide, micro-array data analysis using a network-based method was used (Calvano *et al.*, 2005, Cobb *et al.*, 2005, Forster *et al.*, 2003). I used a web-based tool developed by Ingenuity System Inc. (http://www.ingenuity.com), which provides a modeling of networks representing transcriptional interactions among significantly changed genes. Every gene interaction in the network is supported by published scientific articles. Moreover, apart from known interactions providing a framework, the software enables creation of novel associations among significantly up-regulated and down-regulated genes. The software also allows to analyze the whole pool of differentially changed genes with respect to known functions and canonical pathways as well as to extract variability between whole comparisons (http://www.ingenuity.com).

2.8.5 Micro-array experiment

RNA samples were used for target preparation and subjected to hybridizations to an Affymetrix Mouse Genome 430 2.0 GeneChips. Samples were processed by the micro-array team at The Sir Henry Wellcome Functional Genomics Facility (SHWFGF) in Glasgow. RNAs were processed through standard Affymetrix protocols (http://www.affymetrix.com). Samples of RNA extracted from paired samples of whole subcutaneous and mesenteric adipose tissue of 5 11β-HSD1-/- and 5 congenic C57Bl6/J control mice were hybridized to 20 Affymetrix Mouse Genome 430 2.0 GeneChips, and data were extracted through the GCOS software. CEL files were made available for further data processing. CEL files for all 20 chips were imported into the Affy package of BioConductor (http://www.bioconductor.org/packages/bioc/), where they were processed (background subtraction and normalization) with the Robust Multichip Average (RMA) algorithm. Differential expression was determined by linear modeling using the Limma tool on BioConductor, followed by multiple detection correction by the Benjamini and Hochberg FDR method (www.bioconductor.org). Once expression data were processed, they were exported in text format and imported into a MySQL database. In addition, annotation data for the genes were obtained from NetAffx (http://www.affymetrix.com/analysis/index.affx). A web accessible front-end query tool was built that allows querying of the data by expression data (normalised

expression, fold-changes, p-values) and by sequence annotation (gene title and symbol, Entrez gene ID, Affymetrix ID, and Gene Ontology data). Once the query is performed, and table with the results is presented, along with several hyperlinks to data in on-line databases (Entrez gene, PubMed, MGI, iHOP). Although data quality control was good (Fig.2.4. and 2.5.), there appeared be "biological noise", especially in the 11β-HSD1-/- group. High variability did not correlate with the physiological measurements of glucose/insulin. Due to this only a small number of differentially regulated genes reached statistical significance and the following analysis was based predominantly on the significance of pathways. In this case, WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt, Vanderbilt University, USA) and the Ingenuity Pathways Analysis (IPA) program (http://www.ingenuity.com/index.html) were used to further analyze the gene cluster functions that were significantly regulated. The micro-array data set with associated > 1.5-fold differential expression levels between genotypes in the subcutaneous and mesenteric adipose tissue was imported into IPA. This set of genes was used as the reference set for function and canonical pathway analysis statistical calculations in IPA.

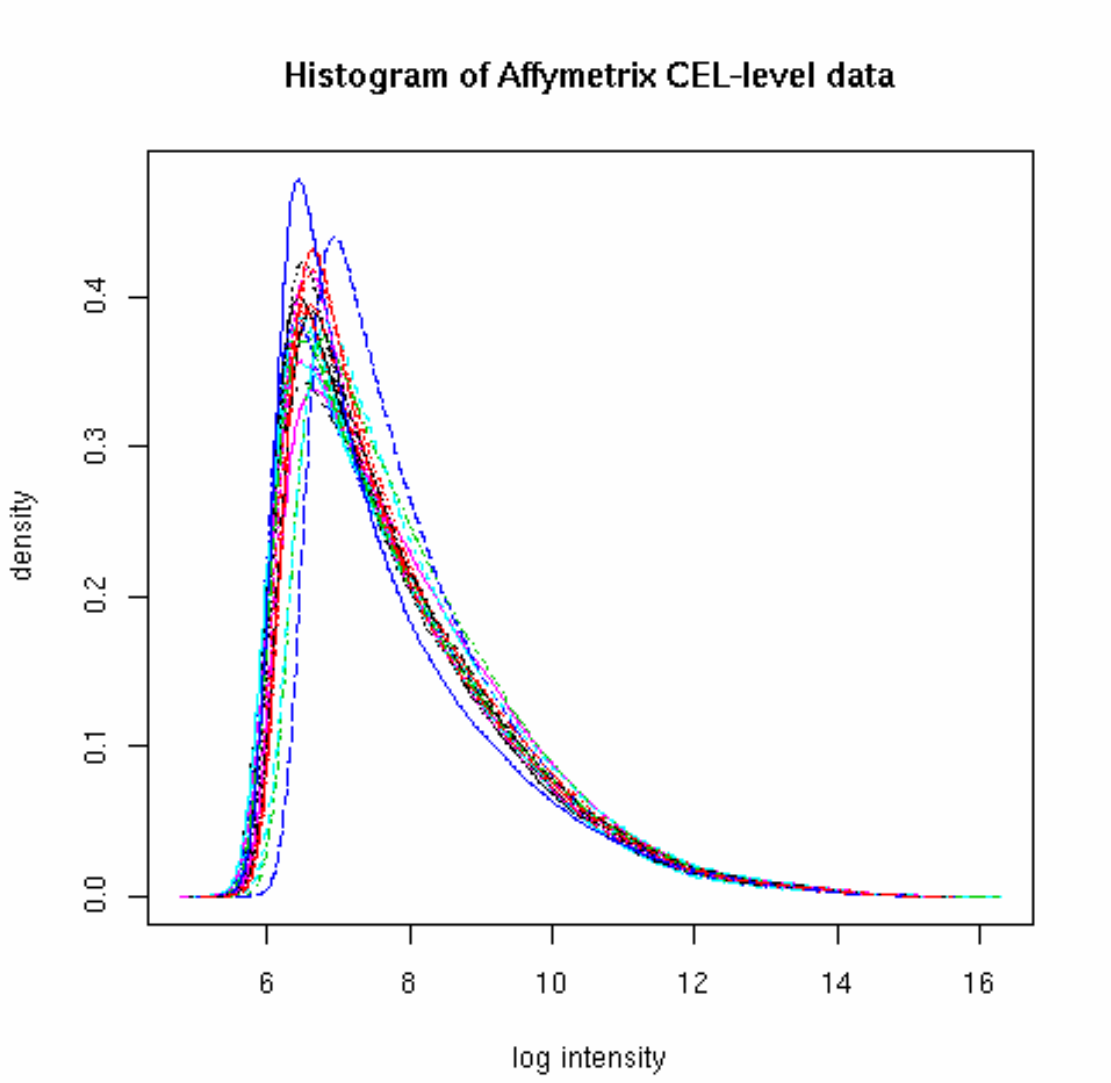

Figure 2.4 Histiogram of Affymetrix CEL-level data.

The graph shows the distribution of intensities of probes across the chips. Each chip is depicted as one line (a different colour for each one). The Y axis shows the dencity of proportion of probes that have the log intensity shown on the X axis. This helps to spot chips with a high background often indicating problems with processing. Histiograms of log intensities are diagnostic measurements used to assess the quality of the arrays. No spatial artefacts are apparent in these arrays.

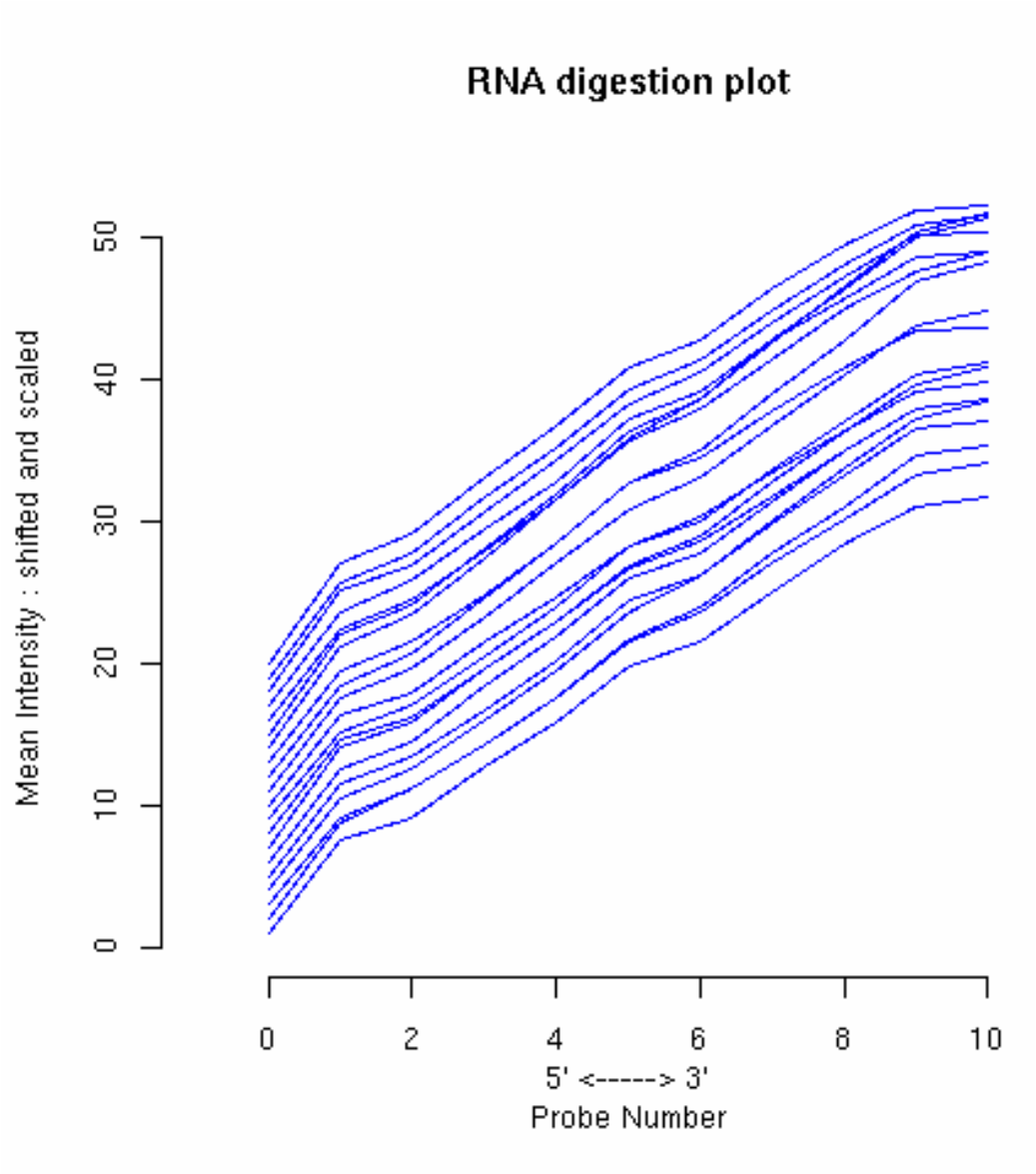

Figure 2.5 RNA degradation plot (Affy package on BioConductor) of chips.

The plot shows the output from the AffyRNAdeg function on the Affy package. RNA degradation plot shows the mean expression from the 5' to the 3' end of the mRNA. Every chip is represented with a single line. In an ideal situation the lines would be flat, but it is usually not the case. If the lines are not flat, the slopes and profiles should be as similar as possible. If all lines have similar slopes, the *in vitro* transcription has worked similarly across all samples.

2.9 Insulin signaling *in vivo*

12-week-old male 11β-HSD1-/- and congenic C57Bl/6J mice were divided into four groups (n=6) with similar body weight and assigned to receive two kinds of diet: standard chow diet or HF diet (as described above) for 10 weeks. At the end of diet period mice were fasted for 6h and then injected i.p. with 0.75mU/g humulinS or saline. Blood samples were taken before and after fasting for glucose and insulin measurements. At 15min after the injection subcutaneous and mesenteric fat depots and liver were dissected and snap frozen in liquid nitrogen and stored at -80. Tissues were homogenized in ice-cold lysis buffer (50mM Tris, pH 7.4, 0.27M sucrose, 1mM Na-orthovanadate, pH 10, 1mM EDTA, 1mM EGTA, 10 mM Na β-glycerophosphate, 50mM NaF, 5mM Na pyrophosphate, 1% (w/v) Triton X-100, 0.1% (v/v) 2-mercapto ethanol, 1 tablet of complete TM protease inhibitor (Roche, UK). Extracts were centrifuged at 13,000 rpm for 20min to remove insoluble material. The supernatants were used for Western blotting.

2.9.1 Protein concentration measurements.

Protein concentrations in tissue homogenates were determined by the method of Bradford (protein assay kit; Sigma-Aldrich, UK). Assay is based on the colorimetric method and the protein concentrations in each tissue homogenate is estimated according to the standard curve based on serial dilutions of bovine serum albumin standards (0.1–1.2mg/ml). Measurements were performed at λ595nm using a spectrophotometer. Samples were prepared in a dilution allowing estimations in the middle range of the standard curve.

2.9.2 Western blotting

For immunoprecipitations, 1mg of supernatant was mixed with 2μg IRS-1 per tube and protein A-Sepharose (Amersham, UK). The precipitated proteins or whole-tissue extracts were denatured with Laemmli sample buffer containing 100mM dithiothreitol by heating in a boiling water bath for 5min, after which they were subjected to SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen, UK) in MOPS SDS Running Buffer (Invitrogen, UK). For total extracts, 50μg of proteins were used. Proteins were transferred onto nitrocellulose membranes (Invitrogen, UK) at 150mA

over 1h 15min in Transfer Buffer (Invitrogen, UK), blocked for 2h at room temperature in 5% (wt/vol) skimmed milk/Tris-buffered saline (50 mmol/l Tris-HCl, pH 7.6, and 150 mmol/l NaCl) supplemented with 0.1% (vol/vol) Tween 20, and probed overnight at 4˚C with antibodies against total AKT (1:1000), or phospho-AKT Ser^{473} (1:1000) or phosphor-IRS-1 (1:2,000). Nitrocellulose membranes were washed three times in Tris-buffered saline/0.1% (vol/vol) Tween 20 for 10min before incubation with either HRP–anti-mouse IgG (1:5,000) or HRP–anti-rabbit (1:1,000). Protein signals were visualized using enhanced chemiluminescence for 1min, wrapped in Saran Wrap and exposed to Amersham HyperfilmTH ECL autoradiographic film (Amersham, UK). The x-ray film was developed using a Konica SRX-101A X-ray processor (Konica Corporation, Japan).

2.10 Fat cell size measurement

Mean diameter of mature adipocytes in every group was measured by counting 20 randomly selected areas in sections from mesenteric and subcutaneous fat depot of 11β-HSD1-/- and control mice on chow and HF diet (10 weeks).

2.11 Statistical analysis

Data were analysed using a Microsoft Excel spreadsheet and expressed as means ± SEM accompanied by the indicated number of independent experiments. All statistical analyses were performed using GraphPad prism (Version 3) and SigmaStat software. Initially, the analysis of distribution normality was performed using the Shapiro-Wilk *W* test integrated in GraphPad prism. The groups were then compared using parametric tests: Student's t-test or a one-way or two-way ANOVA, as stated, with Newman-Keuls and Bonferroni post hoc tests. $p<0.05$ was considered as significant.

CHAPTER 3

7-oxysterols modulate glucocorticoid activity in adipocytes

3 7-oxysterols modulate glucocorticoid activity in adipocytes

3.1 Introduction

Obesity is a known risk factor of cardiovascular diseases, yet the molecular link between increased fat mass and atherosclerosis is still unknown. It has been estimated that adipose tissue stores over half of total body cholesterol, mainly as free cholesterol (Krause and Hartman, 1984) and this proportion increases with the hypertrophy of adipocytes (Le Lay *et al.*, 2001). Adipocytes also remove serum oxidized low density lipoproteins (oxLDL) through scavenger receptors (CD36, OLR1, SR-B1) (Krause and Hartman, 1984, Chui *et al.*, 2005), a major source of atherogenic 7-oxysterols. Insulin, a critical regulator of adipocyte biology, promotes lipids storage and up-take of cholesterol (Tondu *et al.*, 2005). It has been observed that adipocyte cholesterol levels increase in proportion to the triglyceride content (Kovanen *et al.*, 1975, Schreibman and Dell, 1975) and that cholesterol-depletion in adipocytes causes perturbation in fatty acids and glucose metabolism (Pohl *et al.*, 2004, Le Lay *et al.*, 2001). Rates of adipocyte lipolysis, a process that increases with the insulin resistance of hypertrophic obesity, are also linked to cholesterol efflux (Verghese *et al.*, 2007). Thus, the ability of adipocytes to store and metabolise cholesterol may be crucial for the regulation of the whole body lipid/cholesterol homeostasis and thereby atherosclerosis risk. Hypertrophied adipocytes from 2 obese rodent models showed elevated mRNA levels of HMG CoA reductase and the LDL receptor suggesting that these cells are cholesterol deficient and have increased cholesterol biosynthesis and uptake in proportion with icreased fat cell size (Boizard *et al.*, 1998, Le Lay *et al.*, 2001). Thus, adipose tissue is a potential sump for harmful cholesterol metabolites and these protective qualities are lost during insulin resistant obesity.

Patients with glucocorticoid excess (Cushing's syndrome) develop visceral obesity, insulin resistance, diabetes type 2, dyslipidemia and have an increased risk of cardiovascular mortality. Tissue-specific differences in local cellular glucocorticoid exposure may explain the Cushing's-like features of idiopathic obesity/metabolic syndrome in the absence of elevated plasma cortisol levels (Seckl and Walker, 2004). One contention is that the 11-keto-reduction of inert cortisone (11-

dehydrocorticoisterone in mice and rats) to active cortisol (corticosterone) by the activity of the intracellular enzyme 11β-HSD1 is elevated specifically in adipose of obese humans and rodents (Seckl and Walker, 2004, Livingstone *et al*., 2000a, Livingstone *et al*., 2000b, Rask *et al*., 2002) . Transgenic mice over-expressing 11β-HSD1 selectively in adipose tissue develop many features of the metabolic syndrome, including glucose intolerance, insulin resistance, dyslipidemia and hypertension (Masuzaki *et al.*, 2001) whereas 11β-HSD1-/- mice are protected from these deleterious effects upon high fat feeding (Morton *et al*., 2001, Morton *et al*., 2004b). Crucially, 11β-HSD1-/- mice also exhibit an atheroprotective phenotype, with raised HDL cholesterol (Morton *et al*., 2001). Further, treatment of atherosclerosis-prone ApoE-/- mice with an 11β-HSD1 inhibitor attenuates atherogenesis (Hermanowski-Vosatka *et al.*, 2005). Taken together these data suggest a role for elevated adipose 11β-HSD1 levels driving adipocyte hypertrophy and insulin resistance that, together with consequent downstream vascular (Hermanowski-Vosatka *et al.*, 2005) and hepatic lipid and cholesterol handling effects (Morton *et al*., 2001, Morton *et al.,*2004b) , is potentially proatherogenic.

It was reported recently that 11β-HSD1 inter-converts 7-ketocholesterol (7-KC) and 7β-hydroxycholesterol (7β-HC) in the liver (Schweizer *et al.*, 2004). 7KC, followed by 7β-HC are the most abundant oxysterols in oxLDL (Brown *et al.*, 1997) and their concentrations correlate with atherosclerosis risk (Ferderbar *et al.*, 2007). Whilst present in nanomolar concentrations in the plasma under physiological conditions (Corradini *et al.*, 2005, Iuliano *et al.*, 2003, Zieden *et al.*, 1999), these 7-oxygenated metabolites are found in micromolar concentrations in human foam cells, atherosclerotic plaques and plasma of dyslipidemic patients (Schroepfer, 2000). 7-oxysterol levels in adipose tissue have not been reported.

Given the key role of adipose tissue in modulating whole body cholesterol homeostasis (Pohl *et al.*, 2004, Le Lay *et al.*, 2001) we hypothesised that metabolism of these oxysterols by 11β-HSD1 might provide a novel link between obesity, adipose glucocorticoid action and atherogenesis.

Aims:

1. To investigate whether differentiated 3T3-F442A adipocytes metabolise 7-oxysterols and whether this reaction is 11β-HSD1–dependent.
2. To study the competitive nature of glucocorticoid and 7-oxysterol metabolism in cultured adipocytes.
3. To investigate whether 7-oxysterols activate glucocorticoid receptors and/or interfere with the GR translocation.
4. To investigate the impact of 7-oxysterols on the differentiation of adipocytes.

3.2 Methods:

3.2.1 11β-HSD1 activity in fully differentiated adipocytes.

3T3-F442A cells were cultured and differentiated according to the standard protocol as described in section 2.4.3 and 2.4.4.

An initial validation experiment was performed to confirm previously described preferential reductase direction of 11β-HSD1 (Napolitano *et al.*, 1998). The enzyme activity assay in fully-differentiated 3T3-F442A adipocytes was measured by thin layer chromatography (TLC) as described in section 2.5.1. Two time points (1h and 3h) were chosen based on previous reports.

3.2.2 Accumulation of $[^3H]_3$-7KC in adipocytes measured by β-counter.

Initially, based on the well established protocol of 11β-HSD1 activity for glucocorticoid conversion (as described in chapter 2 in section 2.5.1.), I attempted to detect the potential product of 7KC conversion in the culture medium. Although I significantly improved recovery of oxysterols from the medium using 6 volumes of dichloromethane instead of ethyl acetate I detected only decreasing amounts of 7KC over time and no product in the medium. To solve problems with the detection of 7-oxysterols in the culture medium 3T3-F442A adipocytes were incubated with radio-labeled 7KC. Several methods of extraction were tested in the preliminary stage of the study as described in details in section 2.5.2.

Radioactivity was measured in the medium and in the cellular pellet using a β-counter as described. After 24 h incubation all radioactivity counts were accumulated in the cellular pellet. Based on these findings, I established the optimal conditions of oxysterol recovery from both medium and cell lysates.

3.2.3 Metabolism of [3H]3-7KC in adipocytes.

The conversion of 7KC to 7β-HC was investigated using high performance liquid chromathography (HPLC). 7KC and 7β-HC were extracted from the culture medium and lysed cells using the new established method described in details in section 2.5.2 The selective 11β-HSD1 inhibitor (Merck, compound 544 (Hermanowski-Vosatka *et al.*, 2005)) was used as a control.

3.2.4 11β-HSD1 activity assays

3.2.4.1 Glucocorticoid conversion measured by TLC (section 2.5.1)

3.2.4.2 7-oxysterol conversion measured by HPLC (section 2.5.2)

11β-HSD1 activity in fully-differentiated intact 3T3-F442A adipocytes was assayed in the medium to which appropriate substrate with radio-labeled tracer was added. For the enzyme competition assays, the 2 substrates (11-DHC and 7KC) were added in the medium: one in a constant concentration and the other in a range of concentrations. TLC and HPLC (as described in section 2.5) were used for measurement of 11β-HSD1-dependent glucocorticoid and 7-oxysterols metabolism, respectively.

3.2.5 Glucocorticoid receptor studies

3.2.5.1 Transient transfection with GR or/and H6PDH

HEK 293(11β-HSD1) cells were cultured as described in section 2.4.2.
Two different transfections experiments were carried out as follow:

1) For assessment of glucocorticoid receptor activation, HEK 293(11β-HSD1) cells-stably transfected with human 11β-HSD1, were transfected with human GR and a luciferase reporter gene linked to the mouse mammary tumour virus long terminal repeat (MMTV-LTR) containing several GREs as described in section 2.7.7.

Transfected cells were incubated with active (corticosterone) or inactive (11-DHC) glucocorticoid and/or with 7-oxysterols.

2) HEK 293(11β-HSD1)+GR were co-transfected with H6PDH to test whether the observed modulation of GR activity depends on the availability of co-factor.

3) Experiments were performed on HEK293+GR cells (not transfected with 11β-HSD1) as controls.

3.2.5.2 GR translocation

To investigate whether 7-oxysterols may influence GR trafficking HEK 293 and HEK 293(11β-HSD1) cells were transfected with EGFP-GR (rat) as described in section 2.7.7. Images of the living cells were captured with fluorescent microscopy. Representative experiments were additionally examined under confocal microscopy. Untreated cells showed the GR-associated fluorescence only in the cytoplasm (negative control). The use of 10^{-8} cortisone for 60min triggered a complete transfer of the EGFP-GR fluorescence from the cytoplasm to the nuclei (positive control). Two different experiments were performed:

a) To exclude the inhibitory effect of 7KC on 11β-HSD1-dependent production of a GR ligand HEK293(11β-HSD1) cells were co-incubated with 7KC and 11-DHC. Cells incubated with 11-DHC were used as a positive control. Different time-points and concentrations were tested.
b) To investigate whether the incubation with 7KC or 7β-HC caused translocation of EGFP-GR to the nucleus HEK293 cells were incubated with either of two 7-oxysterols in various concentrations.

3.2.6 Modulation of 11β-HSD1 reaction direction (HEK293 cells)

To investigate whether 7-oxysterols influence the direction of glucocorticoid conversion 11β-HSD1 activity assays were performed as described in section 2.5.1.

3.2.7 3T3-L1 differentiation

3.2.7.1 Oil Red O staining (section 2.4.4.1.)

The differentiation of adipocytes was assessed visually by the Oil Red O staining, which stains all neutral lipid and lipid moieties.

3.2.7.2 qRT-PCR

Expression of glucocorticoid-regulated genes (PEPCK and angiotensinogen) were investigated in RNA isolated from 3T3-L1 cells by qRT-PCR as described in section 2.7.6. TATA binding protein mRNA levels were used as an internal control.

3.2.8 Statistics

Individual experiments were conducted in triplicates and the mean was used for statistical analysis. All data are presented as mean ± standard error of the mean (SEM) and were statistically analysed by Student's t-test or one-way or two-way ANOVA, as appropriate. Bonferroni or Newman-Keuls post hoc tests were used for multiple comparisons. All analysis was performed using Sigma Stat or Graphpad Prism (Version 3). *$p<0.05$ was set as significant.

3.3 Results

3.3.1 11β-HSD1 in differentiated 3T3-F442A adipocytes works predominantly as a reductase.

11β-HSD1 has been described as a low-affinity NADP-dependent dehydrogenase/oxoreductase (Napolitano *et al.*, 1998, Bujalska *et al.*, 2002). Depending on the availability of the cofactor 11β-HSD1 inter-converts glucocorticoids and 7-oxysterols in liver microsomes (Schweizer *et al.*, 2004). In order to establish conditions for the conversion of 7-oxysterols in cultured adipocytes I tested the 11β-HSD1 dehydrogenase and reductase activities for the conversion of glucocorticoids in differentiated 3T3-F442A adipocytes and confirmed that in our *in vitro* model the dehydrogenase reaction was negligible. After 4 h of incubation with 11-DHC only the product of the reaction was detected in the medium (Fig. 3.1.).

3.3.2 Differentiated 3T3-F442A adipocytes accumulate 7-oxysterols.

To investigate whether 11β-HSD1 metabolises oxysterol substrate in adipocytes I examined conversion of $[3H]_3$-7KC to 7β-HC in differentiated 3T3-F442A adipocytes. After 24 h incubation, most of the added radioactivity was detected in the cellular fraction, with <17% remaining in the medium (Fig. 3.2.), suggesting sequestration of oxysterols in the adipocytes rather than balanced influx/efflux. Radioactivity was measured in whole samples of medium and resuspended cellular pellets without performing additional steps of oxysterol extraction.

HPLC analysis of oxysterols extracted from the lysed cells showed two peaks with the retention time: 28 and 32minutes, corresponding to 7β-HC and 7KC respectively (Fig. 3.3.). Thus, in the adipocytes the metabolism of 7-oxysterols was exclusively through 11β-HSD1 and contrary to that, which occurs in other tissues (liver, macrophages) 7β-HC was not metabolised further. After 24h ~ 73% of 7KC was converted to 7β-HC (Fig. 3.3), indicating a predominant reductase effect on oxysterols (similar to glucocorticoid conversion [21]). Consistent with previous observations after 24 h incubation no 7-oxysterols were detected in the medium.

11β-HSD1 specificity for the conversion of 7KC to 7β-HC in the differentiated adipocytes was confirmed using a selective 11β-HSD1 inhibitor (compound 544, Merck, (Hermanowski-Vosatka *et al.*, 2005)) (Fig. 3.4.).

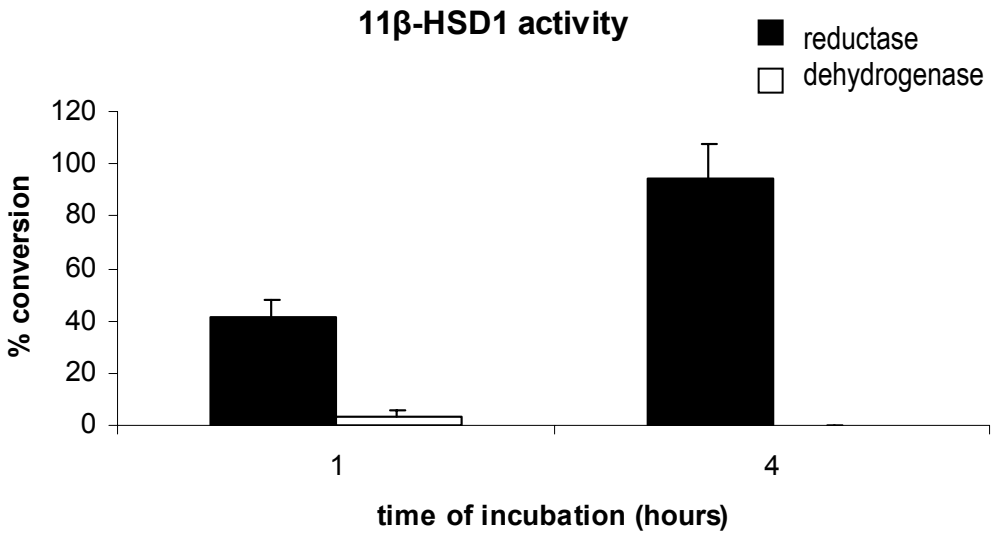

Figure 3.1 11β-HSD1 activity towards glucocorticoids.

To measure the 11β-HSD1 activity fully-differentiated 3T3-F442A adipocytes were incubated with 200nM 11-DHC and 5nM of $[3H]_4$-11-DHC as a tracer (reductase assay; black bars) for 1 or 4 hours. The same concentrations were used when cells were incubated with corticosterone (dehydrogenase assay; white bars). Reductase and dehydrogenase activities were measured in triplicate in 3 independent experiments. Activity is expressed as percent conversion of substrate to product. 11β-HSD1 worked predominantly as a reductase in fully-differentiated adipocytes. Data represent mean± SEM percent conversion.

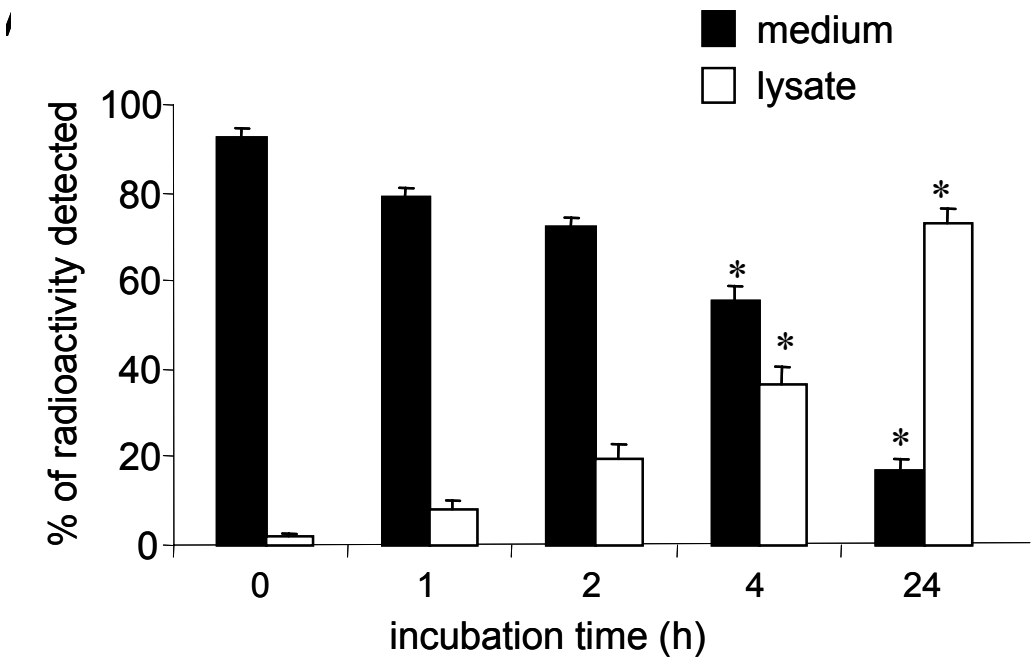

Figure 3.2 Accumulation of 7-oxysterols in fully differentiated 3T3-F442A adipocytes.

To measure intracellular accumulation of 7KC in differentiated 3T3-F442A adipocytes, cells were incubated with $[3H]_3$-7KC for up to 24 h. Black and open bars represent the percentage of radioactivity detected in the medium and in lysed cells, respectively. After incubation with radiolabelled 7KC whole cellular pellets (without performing additional steps of oxysterol extraction from cells) were resuspended in scintillation fluid (Ultima Gold, Perkin Elmer, UK) and analysed by Liquid Scintillation Analyzer (Packard, UK). Data are mean± SEM of 3 independent experiments, each performed in duplicate. There was a statistically significant effect of time measured by one-way ANOVA with Bonferroni post hoc tests ($F=55.25$ for the analysis of medium extracts and $F=194.4$ for the analysis of lysate extracts). Asterisk indicate a statistically significant difference of extracts from the medium or lysates ($p<0.05$*).

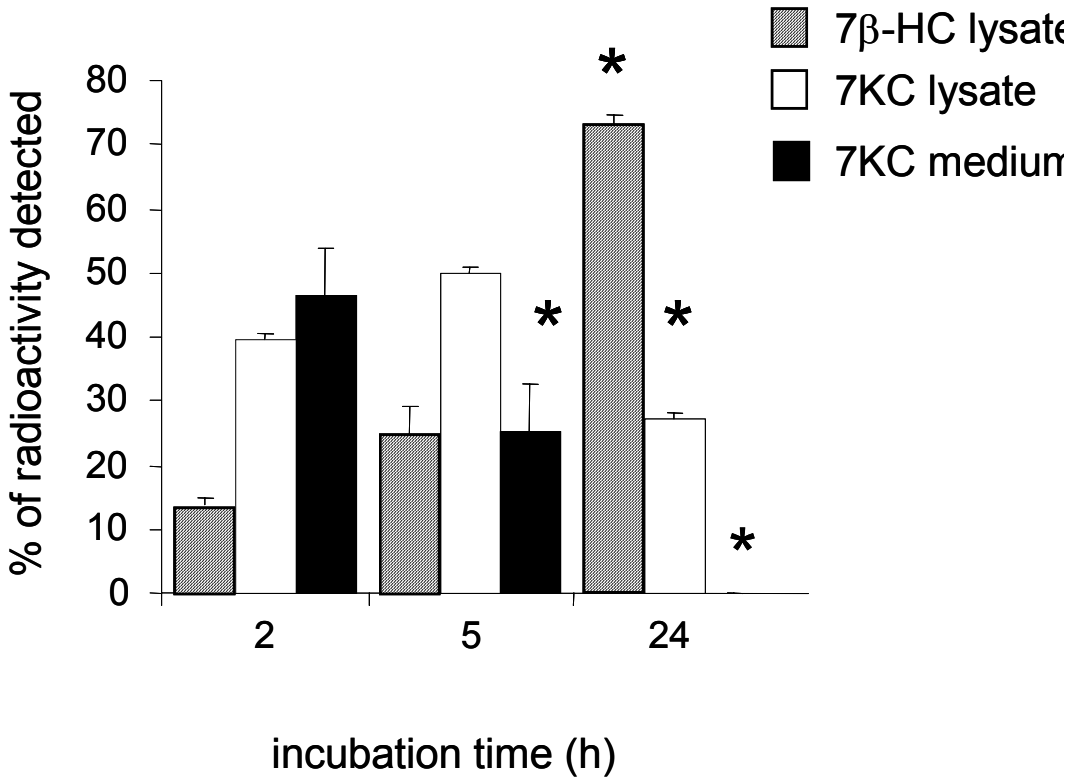

Figure 3.3 Conversion of $[3H]_3$-7KC to 7β-HC by 3T3-F442A in cell lysates and in the medium.

3T3-F442A cells were incubated for 2, 5 or 24 h with 500nM 7KC and $[3H]_3$-7KC as a tracer before extraction and HPLC analysis (as described in Methods in section 2.5.2.). Gray bars represent 7β-HC extracted from the cell lysate, open and black bars represented 7KC extracted from the cell lysate and the medium, respectively. The recovery of oxysterols from the medium and cellular pellet was 50-70% and 90%, respectively. Data are mean ± SEM from 3 independent experiments, each performed in triplicate. Statistical analysis performed by one-way ANOVA with Bonferroni post hoc tests. * $p<0.05$ for the effect of changes in the concentration of radiolabelled extracts over the time course ($F=157$ for the analysis of changes of 7β-HC, $F=55.9$ for the analysis of changes of 7KC in lysates, $F=24.92$ for the analysis of changes of 7KC in the medium).

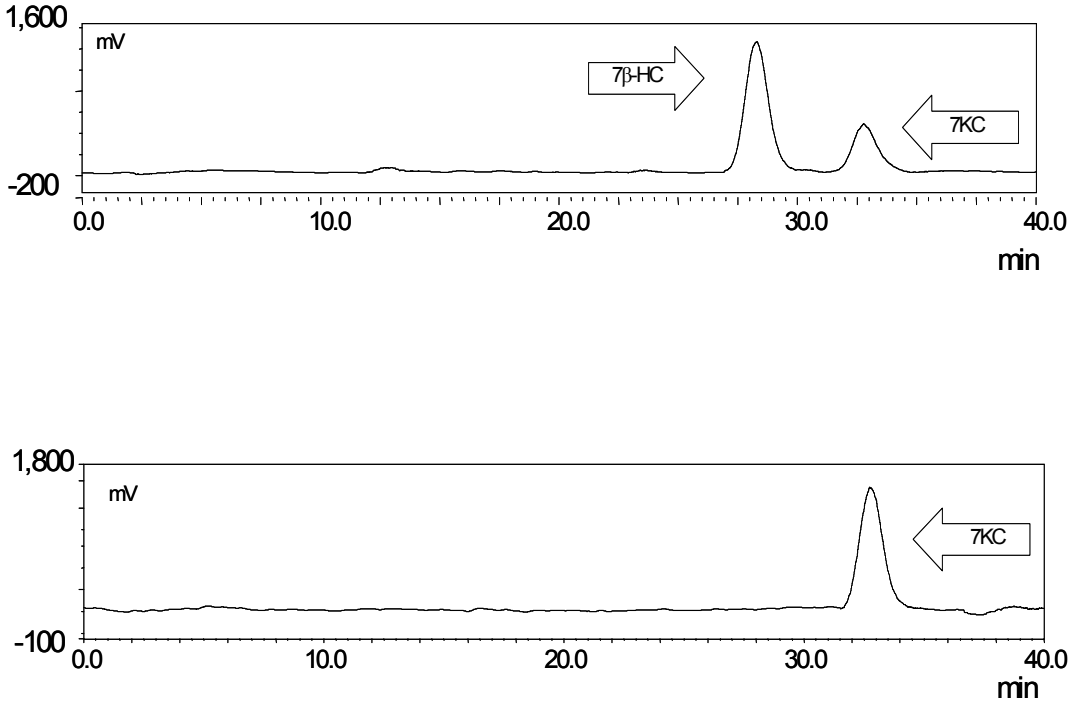

Figure 3.4 HPLC analysis of oxysterols extracted from 3T3-F442A cells

HPLC analysis of oxysterols extracted from 3T3-F442A cells following 24 h incubation with (A) $[3H]_3$-7KC or (B) $[3H]_3$-7KC with the addition of 5μM 11β-HSD1 inhibitor (Merck, compound 544). Following assays for 11β-HSD1-dependent conversion of 7-oxysterols, oxysterols were extracted as described in Methods in section 2.5.2 and resuspended in mobile phase containg 85% acetonitrile and 15% water and injected into the HPLC system. The flow rate of the mobile phase was 1ml/min, the flow rate of scintillant was 1.5 ml/min and the column temperature was set to 40°C. Two peaks were detected with retention times 28 and 32 min for 7β-HC and 7KC, respectively.

3.3.3 7-oxysterols compete with glucocorticoids as substrates for 11β-HSD1.

To investigate competition between glucocorticoid and oxysterol substrates, both were added together to the medium of 3T3-F442A adipocytes. 7KC (1nM–100 μM) inhibited the conversion of 11-DHC to corticosterone in a dose dependent manner with an IC_{50} of 450nM (Fig. 3.5A). This appeared to reflect effects upon metabolism rather than any cell toxicity since MTS assays (4h incubation in serum-free medium with 200nM 11-DHC and a range of 7KC concentrations from 0 to 100μM) indicated no effect of 7KC on cell viability at the doses used here. Similarly, 11-DHC (1nM-100μM) inhibited conversion of 7-KC to 7β-HC (IC_{50} 2740nM; Fig. 3.5B).

3.3.4 7-oxysterols modulate glucocorticoid receptor mediated promoter activation.

To assess the impact of 11β-HSD1-mediated oxysterol metabolism upon glucocorticoid receptor (GR) function, I used HEK293 cell line stably transfected with 11β-HSD1 [HEK293(11β-HSD1)] which, unlike 3T3 cells, are more amenable to standart transfection techniques. HEK293 cells lack functional GR (Atanasov *et al.*, 2004), therefore HEK 293(11β-HSD1) cells were transiently transfected with an expression plasmid encoding rat GR and a glucocorticoid-sensitive (MMTV-LTR-luciferase) promoter-reporter construct (HEK 293(11β-HSD1)+GR). Corticosterone and 11-DHC were equipotent in stimulating reporter gene activity at physiologically-relevant (10nM) concentrations. None of the steroids induced luciferase activity in the absence of transfected GR. This effect was mediated by GR as it was blocked by addition of the GR antagonist RU38486 (RU486) (Fig. 3.6.). Neither 7KC nor 7β-HC (20μM) alone exhibited any direct agonist activity at GR (Fig. 3.6.).

To test whether oxysterols affected the ability of 11β-HSD1 to generate active glucocorticoid ligand, HEK 293(11β-HSD1)+GR cells were incubated with concentrations of 11-DHC (10nM) or corticosterone and 7KC or 7βHC (20μM) or the GR antagonist RU486 (1μM) (Fig. 3.6.). Both active corticosterone and inactive 11-DHC induced GR-mediated transcriptional activity to a similar extent in HEK 293(11β-HSD1)+GR cells (Fig. 3.6.), suggesting rapid reactivation of 11-DHC to corticosterone. RU486 inhibited glucocorticoid–induced luciferase activity (Fig. 3.6.). As predicted from the results above, 7KC (20μM) inhibited 11-DHC-induced

luciferase activity (Fig. 3.6.). Unexpectedly, 7KC also inhibited corticosterone-induced luciferase activity. In contrast, 20μM 7β-HC enhanced both corticosterone- and 11-DHC-mediated transcriptional activation of the reporter gene (Fig.3.6.). Similar results were obtained using another GRE-reporter construct (driven by the GR-dependent phenylethanolamine N-methyl-LTRansferase gene promoter (Adams *et al.*, 2003)).

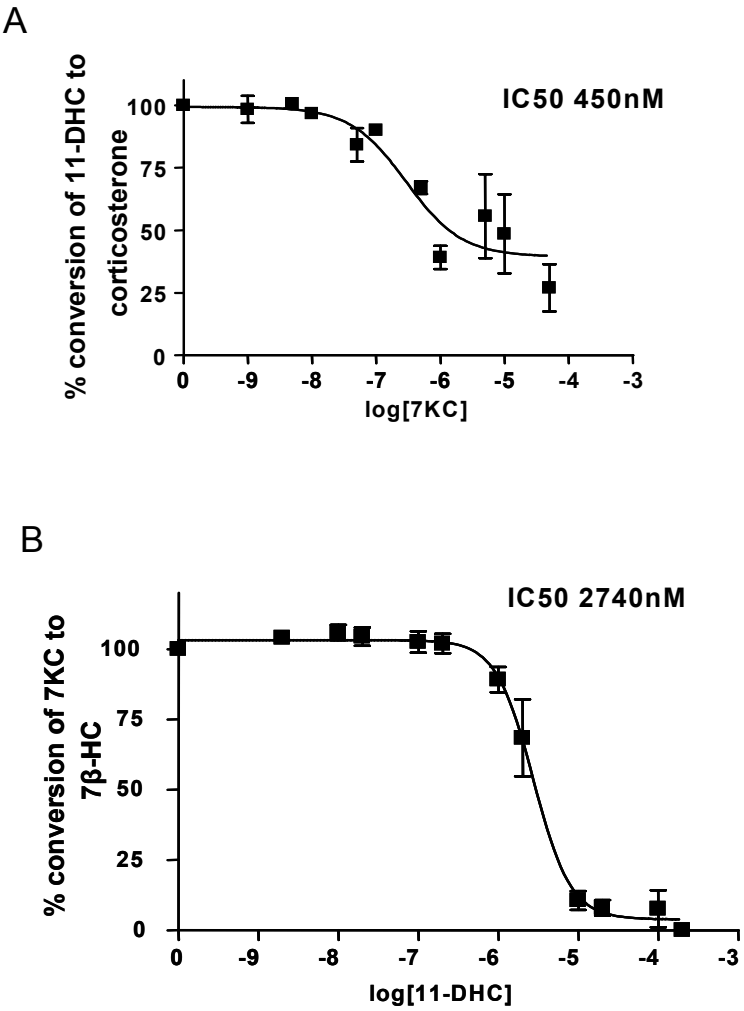

Figure 3.5 Competition between glucocorticoids and 7-oxysterols for 11β-HSD1.

A) Dose-dependent inhibition of glucocorticoid conversion by 7KC. 3T3-F442A adipocytes were incubated with 200nM 11-DHC and 5nM of $[3H]_3$-11-DHC as a tracer with 7KC (1nM-100μM) for 4 h. Steroids were extracted from the medium by the addition of ethyl acetate and analysed by TLC. Data are mean ± SEM of 3 independent experiments, each performed in triplicate and are expressed as a percentage of control (no added 7KC). 100% conversion on the graph represents 85% actual conversion in the control. B) Dose-dependent inhibition of oxysterol conversion by 11-DHC analysed by HPLC. 3T3-F442A adipocytes were incubated with 200nM 7KC and 5nM $[3H]_3$-KC as a tracer and 11-DHC (1nM-100μM) for 4 h. Oxysterols were extracted from cells and analysed by HPLC. Data represent mean ± SEM of 3 independent experiments, each performed in triplicate and are expressed as percentage of control (no added 11-DHC). 100% conversion on the graph represents 28% actual conversion in the control.

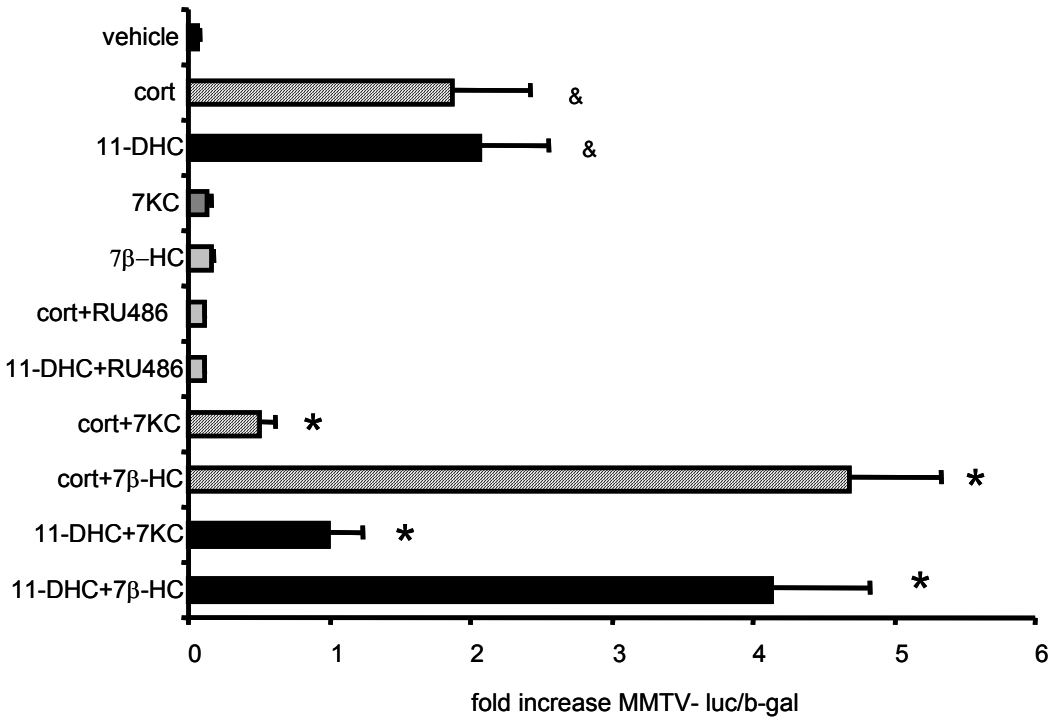

Figure 3.6 7-oxysterols modulate GR activity of 11-DHC and corticosterone.

HEK 293(11β-HSD1) cells were transfected with EGFP-GR, MMTV-LTR-luciferase and pRSV-βgal and treated with 10nM glucocorticoid (11-DHC or corticosterone) with the addition of the appropriate 7-oxysterol (20μM) or RU486 (1μM). Values are the ratio of luciferase to β-galactosidase activity expressed relative to basal MMTV-LTR activity and are means ± SEM of 6 independent experiments, each performed in triplicate. &$p<0.05$ represents statistically significant effect of active or inactive glucocorticoid alone. *$p<0.05$ represents statistically significant effect of modulation of glucocorticoid-dependent activation of GR by 7-oxysterols (one-way ANOVA with Newman-Keuls post hoc tests). Unexpectedly, both active and inactive glucocrticoid-dependent GR activation was inhibited by the addition of 7KC. The explanation of these results will be provided in the following sections describing the bidirectional function of 11β-HSD1 in HEK293 cells and the modulatory role of 7-oxysterols upon reductase/dehydrogenase reaction direction.

3.3.5 7-oxysterols do not regulate GR trafficking.

To explore whether the differences between 7KC and 7β-HC effects on GR-mediated transcription were due to effects on receptor translocation to the nucleus, visualization of EGFP-tagged GR by fluorescent and confocal microscopy was used.

As expected, un-liganded GR was located in the cytoplasmic compartment, whereas after 40min incubation with 500nM of corticosterone or 11-DHC (when cells were stably transfected with 11β-HSD1) EGFP-GR was detected exclusively in the nucleus. As described previously (Prima *et al.*, 2000), RU486 induced translocation of GR to the nucleus (the complex is transcriptionally inactive). In the first set of experiments the potential inhibitory effect of 7KC on the production of an active GR ligand was investigated in the HEK293(11β-HSD1) cells co-incubated with 7KC and 11-DHC. Contrary to expectation, there was no evident inhibition of GR translocation when 7KC was present in the medium together with a GR ligand. This supported the notion that the inhibitory effect of 7KC on glucocorticoid conversion was incomplete.

To exclude the possibility that either 7KC or 7β-HC might interfere with the inactivated GR trafficking, HEK293 were incubated with 7-oxysterols. The incubation with 7-oxysterols (100nM-20μM) for up to 24h in the medium did not modify the cytoplasmic localization of EGFP-GR. These findings imply that neither 7KC nor 7β-HC interfere with the nuclear-cytoplasmic trafficking of the rGR in the transfected HEK293 cells (Fig.3.7.).

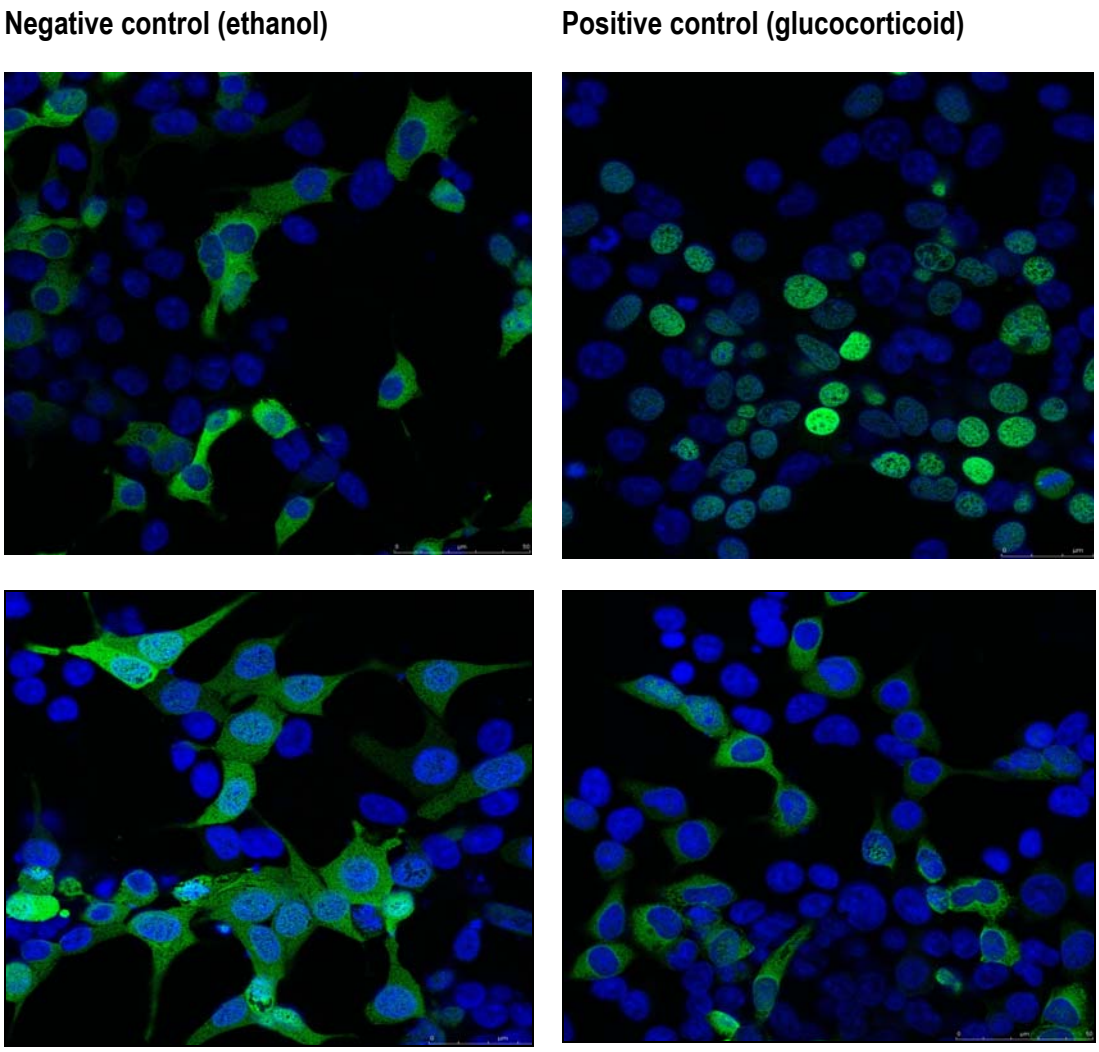

Figure 3.7 The effect of 7-oxysterols on the rGR trafficking in transiently transfected HEK293 cells expressing the EGFP-linked rGR (green fluorescence).

Confocal microscopic images were captured 24 hours after addition of 20μM 7KC or 7β-HC (as described in Methods in section 3.2.5.2). DAPI staining (blue) was used to visualize nuclei. 500nM corticosterone was used as a positive control (green fluorescence in the nucleus, GR fully translocated into the nucleus) and "vehicle" alone (ethanol, green fluorescence in the cytoplasm, GR not translocated, remains in the cytoplasmic compartment) was used as a negative control. Pictures are representative of 3 independent experiments, each performed in triplicate.

3.3.6 Modulation of 11β-HSD1 reaction direction by 7-oxysterols

One mechanism by which 7KC may reduce GR activation by both corticosterone (already active) and inert 11-DHC involves shifting 11β-HSD1 reaction direction towards 11β-dehydrogenation. In order to address this HEK 293(11β-HSD1)+GR cells were co-transfected with H6PDH, which drives 11β-HSD1 activity towards oxo-reduction, regenerating active corticosterone (Atanasov *et al.*, 2004, Bujalska *et al.*, 2005). Consistent with this hypothesis and with previous data (Atanasov *et al.*, 2004), HEK 293(11β-HSD1)+GR cells exhibited bi-directional glucocorticoid metabolism, at comparable levels (40-50% conversion), when incubated with either 11-DHC (Fig. 3.6. and 3.8.) or corticosterone (Fig. 3.6. and 3.8.), but only 11β-reductase activity when co-transfected with H6PDH (Atanasov *et al.*, 2004) (Fig. 3.8).

7KC (20μM) inhibited the conversion of 11-DHC to corticosterone (reductase direction; Fig. 3.10A), and promoted conversion of corticosterone to 11-DHC (dehydrogenase direction; Fig.3.10B) in HEK 293(11β-HSD1) cells without H6PDH co-transfection. In contrast, 7β-HC increased the formation of the active glucocorticoid by the opposite effect, shifting the 11β-HSD1 reaction direction to oxo-reductase (Fig.3.10A and B). 11β-HSD1 inhibitor (Merck, compound 544) used in this expiment inhibited both reaction direction in a non-specific way (Fig. 3.10A and B). Moreover, when H6PDH was over-expressed, 7-oxysterols no longer had effects upon corticosterone-mediated GR transcriptional activity (Fig. 3.8.), and the effects of 7β-HC on 11-DHC transactivation via GR was also blocked, suggesting that these effects were mediated via changes in 11β-HSD1 reaction direction. Additionally, in the absence of 11β-HSD1 7-oxysterols did not influence corticosterone-induced GR transcriptional activity (Fig. 3.9). These data suggest that in the presence of sub-maximal levels of H6PDH, 7-KC influences glucocorticoid metabolism by 11β-HSD1 towards dehydrogenation, but that, in addition, it antagonises activation of 11-DHC to active corticosterone by another mechanism, presumably substrate competition.

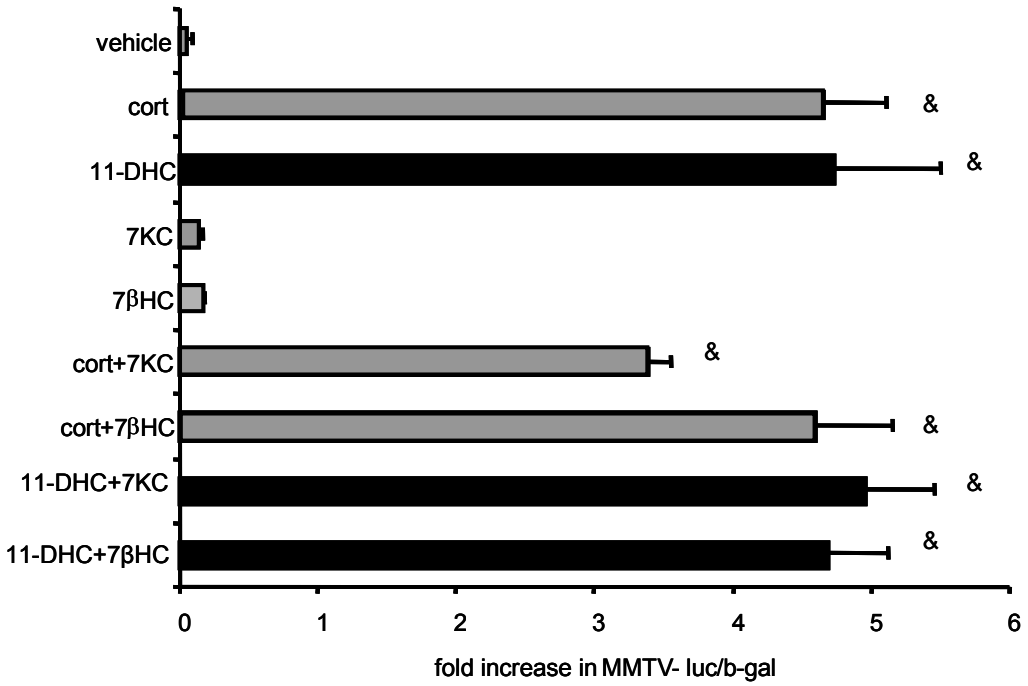

Figure 3.8 7-oxysterols do not modulate GR activity in cells co-transfected with H6PDH.

HEK-293(11β-HSD1) cells were transfected as above with GR, MMTV-LTR-luciferase activity and pRSV-β and co-transfected with H6PDH plasmid and activated as described above with glucocorticoid (10nM 11-DHC and corticosterone) and 7-oxysterols (20μM). Results are expressed as the ratio of luciferase to β-galactosidase activity and are the mean values ± SEM of 4 separate experiments. &$p<0.05$ represents statistically significant effect of active or inactive glucocorticoid alone (one-way ANOVA with Newman-Keuls post hoc tests). No significant effect of co-inubation with 7-oxysterols and glucocorticoids was detected.

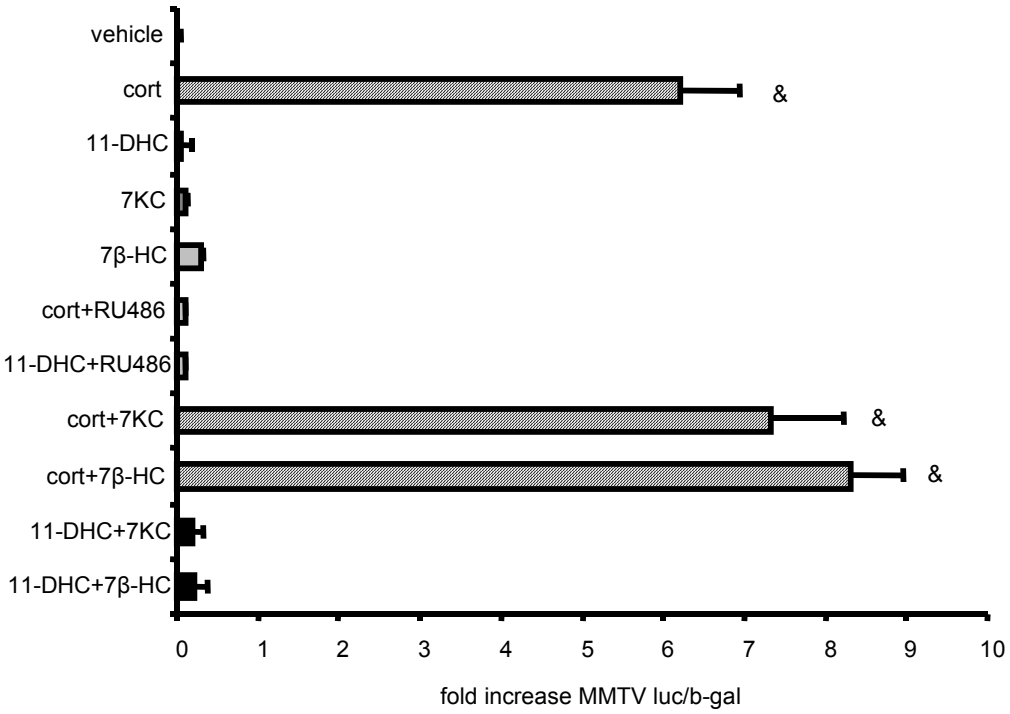

Figure 3.9 7-oxysterols do not modulate GR activity in HEK293 cells.

HEK-293 cells were transfected as above with GR, MMTV-LTR-luciferase activity and pRSV- β and activated as described above with glucocorticoid (10nM 11-DHC and corticosterone) and 7-oxysterols (20μM). &$p<0.05$ represents statistically significant effect of active glucocorticoid alone (one-way ANOVA with Newman-Keuls post hoc tests). Results are expressed as the ratio of luciferase to β-galactosidase activity and are the mean values±SEM of 4 separate experiments. No significant effect of co-incubation with 7-oxysterols (one-way ANOVA).

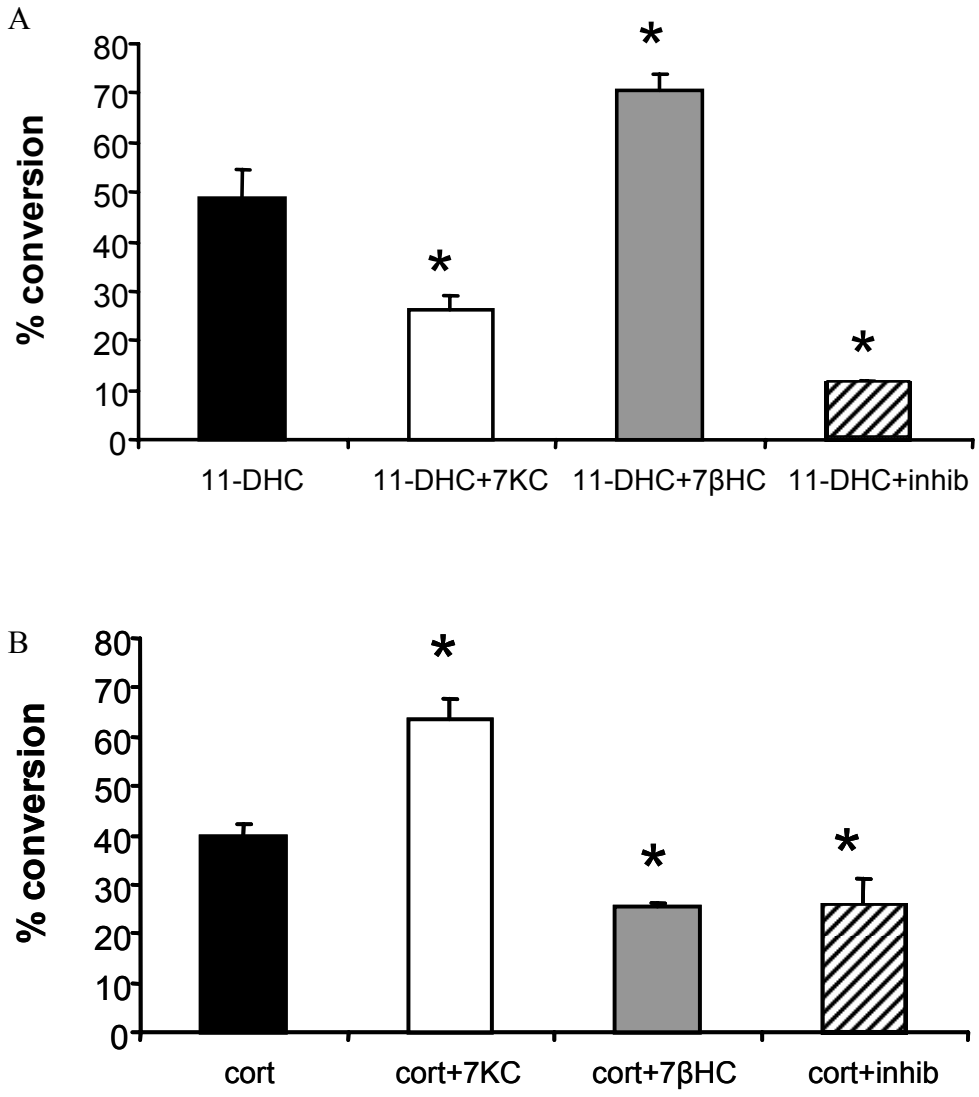

Figure 3.10 Modulation of 11β-HSD1 reductase activity (A) and dehydrogenase activity (B) by 7-oxysterols in HEK293(11β-HSD1) cells.

Cells were incubated for 16h with either 10nM $[3H]_4$11-DHC (A) or 10nM $[3H]_4$ corticosterone (B) with the addition of 7KC (20μM), 7β-HC (20μM) or the selective 11β-HSD1 inhibitor (Merck compound 544, 5μM), as indicated. Values are expressed as percent conversion of substrate to product (Y axis of the upper graph shows the conversion of corticosterone/added 11-DHC and this represents the reductase direction; Y axis of the lower graph shows the conversion of 11-DHC/added corticosterone and this is consistent with the dehydrogenase direction) and are means ± SEM of 3 independent experiments. * $p<0.05$ (one-way ANOVA with Bonferroni post hoc tests). Inhib-11β-HSD1 inhibitor (Merck, compound 544), cort-corticosterone.

3.3.7 7KC inhibits 11β-HSD1-mediated 3T3-L1 preadipocyte differentiation only when 11-DHC is used to trigger the process.

Glucocorticoids are required for the differentiation of the 3T3-L1 adipocyte cell line (Green and Kehinde, 1975). Given the observations that 7-oxysterols accumulate in adipocytes, we hypothesised that 7KC might inhibit 11β-HSD1-dependent, glucocorticoid-stimulated preadipocyte differentiation. To investigate whether the accumulated high concentration of 7-oxysterols may influence the differentiation process and/or lipid storage, 3T3-L1 cells were differentiated with the addition of dexamethasone (250nM) in medium enriched with low (1μM), stable concentrations of 7-oxysterols. As described previously, 11β-HSD1 showed late phase differentiation-dependent expression pattern (Fig. 4.6;(Napolitano *et al.*, 1998)). Neither of the 7-oxysterols influenced lipid storage measured by Oil Red O staining when 3T3-L1 adipocytes were differentiated according to the standard protocol.

11-DHC (by virtue of its conversion to corticosterone by 11β-HSD1) can substitute for dexamethasone in promoting adipocyte differentiation [31]. Supplementation of the differentiation mixture (containing 250nM 11-DHC) with 20μM 7KC decreased the differentiation of 3T3-L1 preadipocytes measured by Oil red O staining by day 10 after addition of supplemented medium (Fig. 3.11.). 7KC did not influence differentiation induced by dexamethasone or corticosterone suggesting that the inhibition of differentiation resulted from inhibition of 11β-HSD1 activity towards 11-DHC by 7KC.

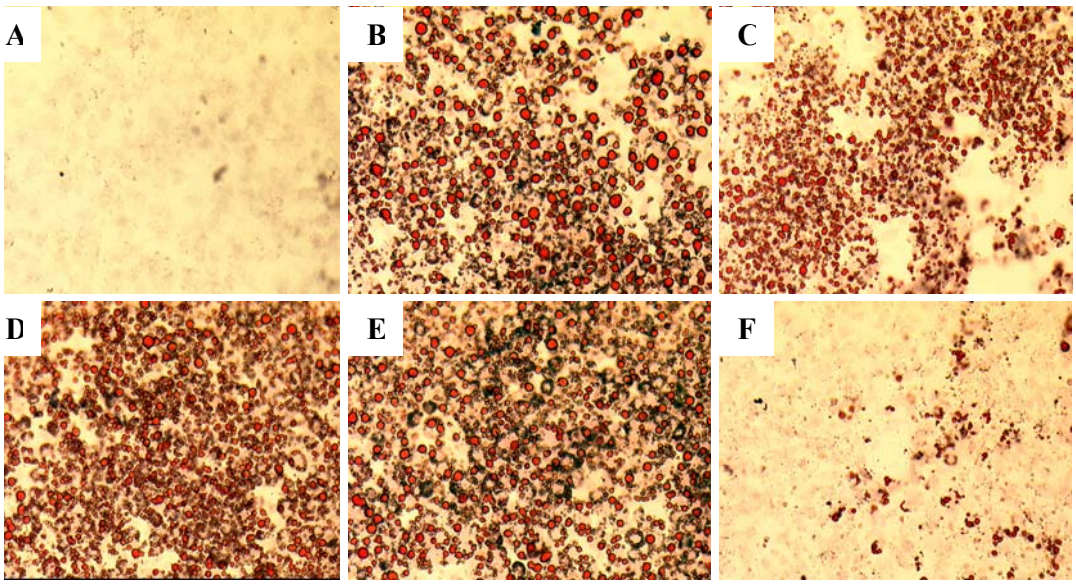

Figure 3.11. 7KC inhibits differentiation of 3T3-L1 adipocytes induced by 11-DHC.

Representative images of Oil red O stained 3T3-L1 cells 7 days after addition of differentiation mixture: (A) undifferentiated cells (negative control), (B) adipocytes differentiated by the addition of IBMX, insulin and dexamethasone; (C) IBMX, insulin and 11-DHC (inactive glucocorticoid); (D) IBMX, insulin and corticosterone (active glucocorticoid); (E) IBMX, insulin, dexamethasone and 20μM 7KC; (F) IBMX, insulin, 11-DHC and 20μM 7KC. Photos are representative of 4 independent experiments, each performed in duplicate. Magnification x40.

3.3.8 The effects of 7-oxysterols on glucocorticoid-inducible gene expression.

Ultimately, 7-oxysterols were tested for their effect on glucocorticoid-responsive genes. 11-DHC (500nM) inhibited PEPCK and increased angiotensinogen mRNA levels as reported previously (Beale and Tishler, 1992, Saye *et al.*, 1989) (Fig. 3.12). In conclusion with the previous experiments 7KC alone showed no effect on the expression of angiotensinogen (Fig. 3.12). Co-incubation with glucocorticoid and oxysterol did not further affect the already decreased PEPCK mRNA levels (Fig. 3.12). No effect was detected on the level of angiotensinogen after co-incubation with 7KC and 500nM 11-DHC for 6h (Fig. 3.12). However, incubation with 10μM 7KC caused a decrease in PEPCK expression, less than 11-DHC but significant. No such effect was observed for 1μM of 7KC on mRNA expression of PEPCK.

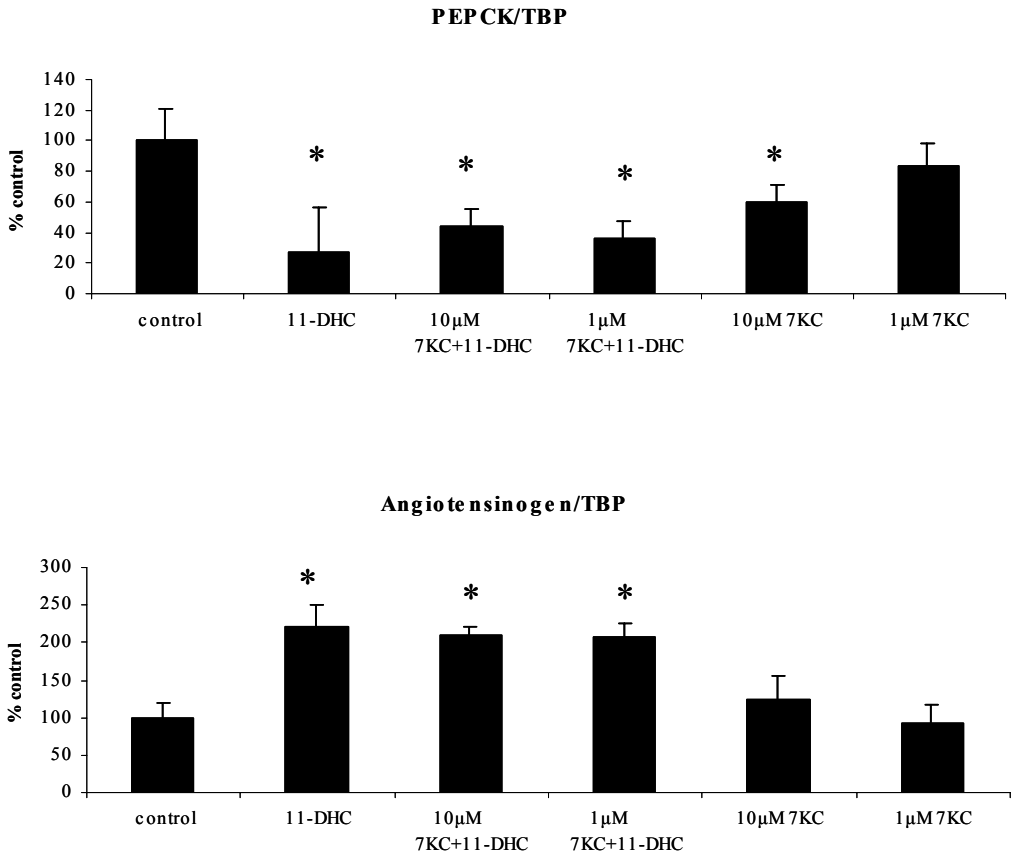

Figure.3.12 Oxysterols do not regulate glucocorticoid-responsive genes in adipocytes.

Regulation of PEPCK and angiotensinogen mRNA levels by co-incubation with an inactive glucocorticoid and 7-oxysterols. Differentiated 3T3-L1 adipocytes were incubated with 500nM 11-DHC and/or with 7KC (10µM or 1µM as indicated) for 6 hours. mRNA levels of angiotensinogen and PEPCK (glucocorticoid-regulated genes) were measured by qRT-PCR as described in chapter 2 in section 2.7.6. Statistically significant effect of treatment (*$p<0.05$, one-way ANOVA with Bonferroni post-hoc tests).

3.4 Discussion

A prominent hypothesis underpinning the metabolic and atherosclerotic consequences of obesity is increased 11β-HSD1 in adipose tissue (Hermanowski-Vosatka *et al.*, 2005, Seckl and Walker, 2004). Here it is shown that 7-oxysterols accumulate within adipocytes and that 11β-HSD1 also metabolises these highly proatherogenic oxysterols, predominantly from 7KC to 7β-HC. I describe a novel cross-talk between glucocorticoid and oxysterol pathways in that 7KC inhibits glucocorticoid action, whereas 7β-HC enhances glucocorticoid action and provide functional evidence that this affects preadipocyte differentiation. The effects appears to occur predominantly through substrate competition at the enzyme activity level, and may include reversal of 11β-HSD1 reductase activity if 7KC levels are high and co-factor levels are limiting within the cell.

Adipocytes accumulated the 7-oxysterol substrate/product of 11β-HSD1, converted the 7KC with preferential 11β-reduction activity, as previously described in liver (Schweizer *et al.*, 2004) and sequestered the 7β-HC product within the cells. The accumulated intracellular 7KC interfered with the ability of 11β-HSD1 to regenerate active glucocorticoids. The relatively low, physiologically-relevant IC_{50} for inhibition of glucocorticoid regeneration by 7KC suggests that such competition may occur in adipose tissue *in vivo*. In contrast, the much higher IC_{50} (micromolar) for inhibition of 7-KC conversion by 11-DHC is unlikely to have a major impact given the nanomolar physiological levels of circulating 11-DHC (Harris *et al.*, 2001).

The effects of 7KC were not mediated at the 11β-HSD1 mRNA level. When 7KC levels were high, as might occur in adipocytes as they accumulate and sequester 7-oxysterols, it appeared to promote a change in reaction direction with increased 11β-dehydrogenation of glucocorticoids. In HEK293(11β-HSD1) cells this change in reaction direction driven by 7KC was overcome by over-expression of H6PDH, believed to be coupled physiologically to 11β-HSD1 in the endoplasmic reticulum and thus the major determinant of reaction direction in intact cells. It is unlikely that this situation would occur in mature fully-differentiated adipocytes, where 11β-HSD1 activity is almost exclusively reductase (Napolitano *et al.*, 1998, Bujalska *et*

al., 2002). However, the limiting levels of H6PDH in HEK293 cells suggest that where the provision of co-factor is limiting, such as in human preadipocytes (Bujalska *et al.*, 2005), accumulation of this oxysterol might affect glucocorticoid mediated preadipocyte differentiation. Indeed when I looked at 3T3-L1 cell differentiation, 7KC reduced the ability of 11-DHC to promote differentiation.

Neither 7-oxysterol displayed intrinsic activity towards GR, but both regulated glucocorticoid-dependent GR transcriptional activity. It seems plausible that 7KC competes as a substrate with 11-DHC, consuming endogenous NADPH. 7β-HC potentiated GR mediated transcription, but only when H6PDH was limiting. Thus 7β-HC promotes the accumulation of active corticosterone in cells where cofactor is limiting, but is unlikely to affect GC action where H6PDH is abundant, such as in mature adipocytes. 7KC attenuated GR transactivation with 11-DHC and corticosterone when H6PDH was limiting, consistent with its role to prevent active glucocorticoid regeneration (HEK293 cells convert corticosterone to 11-DHC, which would not be then reduced back to corticosterone in the presence of 7KC).

11β-HSD1-/- mice have a cardioprotective lipid profile (low LDL, high HDL) (Morton *et al.*, 2004b) and pharmacological inhibition of 11β-HSD1 activity is atheroprotective (Hermanowski-Vosatka *et al.*, 2005). Whilst there may be protective effects within the vessel wall, and on hepatic cholesterol metabolism (Morton *et al.*, 2001, Cai *et al.*, 2001) the altered adipose tissue accumulation and insulin sensitization of 11β-HSD1 may play an atheroprotective function in clearing oxLDL from the plasma. Adipocytes undergoing hypertrophy increase the uptake of oxLDL that are high in 7KC, as they require high delivery of cholesterol for the growing cytoplasmic membranes and storage of triglycerides. It is hypothesised that 7KC accumulation in adipocytes might inhibit 11β-HSD1-dependent amplification of glucocorticoids and ameliorate the metabolic consequences of obesity. In that capacity atherogenic 7KC could be trapped in adipocytes and might play a role of a natural 11β-HSD1 inhibitor towards glucocorticoids.

It is also hypothesised that 7KC accumulation in adipocytes with low levels of 11β-HSD1 or limiting concentrations of H6PDH (such as reported in human preadipocytes or in adipose tissue in lean subjects) might reduce 11β-HSD1-dependent amplification of glucocorticoids and ameliorate some metabolic consequences of obesity. Moreover, 7KC inhibits *de novo* cholesterol biosynthesis via promoting SCAP-mediated release of SREBP (Brown *et al.*, 2002) potentially reducing adipocyte lipid accumulation. Indeed, in humans, adipocyte size correlates directly with 11β-HSD1 levels. In contrast, 7β-HC may have the opposite effect, and some data suggest it is the more atherogenic of the two sterols (Steffen *et al.*, 2006). Thus, in mature adipocytes with plentiful H6PDH, and especially in obesity when 11β-HSD1 levels are high, 11β-HSD1 is a reductase, regenerating glucocorticoids and accumulating 7β-HC (as seen here in 3T3 cells) which is not only more atherogenic but which, unlike 7KC, does not inhibit *de novo* cholesterol biosythesis. Potentially the balance between these oxysterols, itself determined by 11β-HSD1, may be crucial in modulating glucocorticoid and oxysterol effects in adipose tissue (Fig 3.13.).

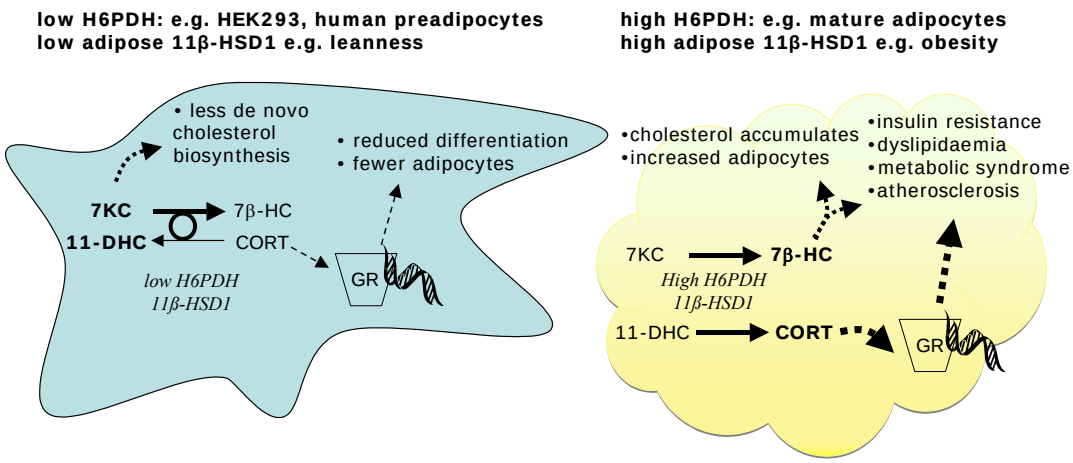

Figure.3.13 A hypothetical model of the effects of interactions between 7-oxysterol and glucocorticoid substrates of 11β-HSD1 in adipocytes.

In cells with low H6PDH levels (HEK293 cells, human preadipocytes) or when 11β-HSD1 levels are low (leanness), 7KC metabolism to 7β-HC consumes cofactor, promoting 11β-HSD1 dehydrogenation, lowering glucocorticoid levels inside cells, attenuating GR activation and reducing metabolic disease/atherogenic potential. 7KC also reduces *de novo* cholesterol biosynthesis and may be less atherogenic per se. In contrast, in mature adipocytes (especially in obesity) and differentiated 3T3 cells, 11β-HSD1 and H6PDH levels are high and the enzyme is a predominant 11β-reductase. This drives regeneration of active glucocorticoids and formation of (putatively) more atherogenic 7β-HC from 7KC. 7β-HC accumulates in adipocytes, facilitates *de novo* cholesterol biosynthesis and adds to the metabolic disease burden.

Chronic high fat feeding down-regulates 11β-HSD1 in fat tissue [8]. It may be speculated that increased plasma levels of 7KC in dyslipidemia and diabetes (Ferderbar *et al.*, 2007) in mice treated with high fat diet might accumulate within the fat and contribute to this inhibitory effect on 11β-HSD1, at least at the level of enzyme activity. Additionally, the data suggest that 7KC may inhibit the differentiation of preadipocytes. This observation is in agreement with the previously reported inhibitory effect of oxLDL on the differentiation and proliferation of 3T3-L1 adipocytes (Masella *et al.*, 2006). Thus, we predict that in dyslipidemia high concentrations of 7KC in fat tissue might inhibit the formation of adipocytes and the diet-induced hypertrophy of adipocytes.

7KC and 7β-HC are abundant oxysterols, known to be cytotoxic at high concentrations and are implicated in atherogenesis. My data have shown that they do not exert direct effects via either GR or LXR in adipocytes *in vitro*. However, they do have the potential to influence glucocorticoid access to GR, by modulation of 11β-HSD1 in adipose tissue. These data could lead to a revision of our understanding of the association between glucocorticoids and atherogenesis. In the future, it will be critical to establish whether a similar regulation of 11β-HSD1 activity occurs in macrophages, and especially in foam cells where oxysterols accumulate to high concentrations and in which 11β-HSD1 is highly expressed and functionally relevant.

CHAPTER 4

The role of 11β-HSD1-dependent conversion of 7-oxysterols

4 The role of 11β-HSD1-dependent conversion of 7-oxysterols

4.1 Introduction

Impaired metabolism of cholesterol is the most firmly established risk factor for the development of atherosclerosis. The concentration of plasma cholesterol is determined by the fine balance between the rate of cholesterol biosynthesis and its excretion into bile. The catabolism of cholesterol into bile acids is regulated through feed-forward activation by oxysterols, a pathway mediated by the liver X receptor (LXR), and by feedback repression by bile acids, a pathway mediated by the farnesoid X receptor (FXR). Nuclear hormone receptors, including LXR and FXR, are transcription factors involved in the regulation of cholesterol, glucose and lipid metabolism and are currently recognized as very attractive targets in the therapy of the dyslipidemia and atherosclerosis. LXR are cholesterol sensors, protecting tissues from its overload by inhibiting the interstitial absorption of cholesterol, activating efflux and enhancing its conversion into bile acids (Millatt *et al.*, 2003). Naturally occurring oxysterols (22(R)-hydroxycholesterol and 24(S), 25-epoxycholesterol) activate LXR (Janowski *et al.*, 1999). Interestingly, 27-hydroxycholesterol, an oxysterol abundantly present in atherosclerotic plaques, has a potential to activate LXR in cholesterol-loaded macrophages where other classical ligands were in low concentrations (Fu *et al.*, 2001). This highlights a concept that certain oxysterols may be cell-specific activators of LXR. Moreover, it has been hypothesised that the anti-diabetic effects of LXR ligands might be partially explained by their inhibitory effect upon 11β-HSD1 (Stulnig *et al.*, 2002). This suggested that cholesterol metabolites might form a crucial cross-talk with glucocorticoid signaling at the transcriptional level via regulation of 11β-HSD1 levels.

Farnesoid X receptors (FXR) are highly expressed in the liver, intestine, adrenal glands and in differentiated adipocytes and are activated by bile acids (Cariou and Staels, 2007). Their role in the pathogenesis of the metabolic syndrome has been emphasized by studies showing that FXR control triglyceride metabolism by inhibiting hepatic lipogenesis, repressing SREBP-1c expression, enhancing LPL activity and increasing VLDL secretion and thereby triglyceride storage in the adipose tissue. Additionally, FXR regulates cholesterol metabolism through the

inhibition of CYP7A1, the rate-limiting enzyme of cholesterol catabolism to bile acids (Claudel *et al.*, 2005). Activation of the FXR also improves insulin signaling and insulin-dependent glucose-uptake in adipocytes (Staels and Kuipers, 2007).

FXR ligands, bile acids, are synthesized in the liver via two main pathways. The first and rate limiting step in the classical pathway of bile acid synthesis is hydroxylation of cholesterol by 7α-hydroxylase (CYP7A1), followed by biochemical changes that consequently lead to synthesis of 7α-hydroxy bile acids, essential for lipid digestion: cholic acid and chenodeoxycholic acid. Bile acids are also synthesized by less active alternative pathways producing the 7β-hydroxy forms. Thus, the conversion of 7KC to 7α-HC and 7β-HC in the liver by 11β-HSD1 has been anticipated to be a novel alternative pathway of bile acid synthesis (Lyons M., 2002). Whether these 7-oxysterols could activate FXR is not known with certaint. However, the inhibitory effect of 7KC and 7β-HC upon CYP7A1 has been reported (Schwartz and Margolis, 1983).

Retinoic acid receptor-related orphan receptor α (RORα) is less well characterised than other "orphan" nuclear receptors. Cholesterol sulphate, 7-dehydrocholesterol and hydroxycholesterols have been suggested to be potential natural ligands for RORα (Bitsch *et al.*, 2003). RORα-/- mice and the natural RORα-deficient *staggerer* mice develop severe atherosclerosis when fed high fat diet (Mamontova *et al.*, 1998) and present with dyslipidemia (Raspe *et al.*, 2001). RORα plays also a role in inflammation and immunomodulation through the NF-κB signaling pathway (Besnard *et al.*, 2001) and is involved in the regulation of fat mass accumulation (Lau *et al.*, 2008).

11β-HSD1 inhibition protects from atherosclerosis and the metabolic consequences of obesity, yet it is not known whether its role in the metabolism of 7-oxysterols might explain the molecular mechanism underpinning these "cardioprotective" effects.

Aims

1. To investigate the potential of 7KC and 7β-HC to activate LXR and FXR in transfection assays.
2. To investigate effect of LXR agonists and 7-oxysterols on 11β-HSD1 expression in 3T3-F442A and 3T3-L1 adipocytes.
3. To study the expression of key LXR, FXR and RORα target genes in the liver and adipose tissue of 11β-HSD1-/- mice and in their C57Bl6/J congenic controls.

4.2 Methods

4.2.1 LXR transfection assays

HEK293 cells were maintained as described in chapter 2 in section 2.4.2. LXR and RXRα plasmids were transfected using GeneJuice as in section 2.7.7. 22(R)-HC (20μM) and T0901317 (1μM) were used as positive controls to activate LXR (Janowski *et al.*, 1999).

4.2.2 Regulation of LXR target genes

3T3-L1 adipocyte were incubated with 7KC and 7β-HC (20μM) for 24 h. mRNA was extracted using Trisol method, converted to cDNA by SuperScript III as described in chapter 2 in section 2.7.4. The expression levels of SREBP1c and GLUT-4 was measured by qRT-PCR (section 2.7.6).

4.2.3 Regulation of 11β-HSD1 mRNA

11β-HSD1 mRNA levels were measured by qRT-PCR. 3T3-F442A and 3T3-L1 adipocytes were cultured and differentiated as described in section 2.4.4. Cells were incubated for up to 48 h in serum-free medium with the addition of LXR agonists (22(R)-HC 20μM and T0901317 1μM) or 7KC and 7β-HC (500nM, 1μM, 20μM) with or without 11β-HSD1 inhibitor, as indicated. Due to the inability to repeat previously described inhibitory effect of LXR agonists upon 11β-HSD1 mRNA rosiglitazone was used as a positive control.

4.2.4 FXR transfection assays

HEK293 cells were transfected with FXR, RXRα and FXRE-pTA-Luc plasmids as described in section 2.7.7. CDCA (chenodeoxycholic acid) was used as a positive control [210].

4.2.5 Expression of LXR, FXR and RORα target genes.

Expression levels of LXR, FXR and RORα target genes were measured by qRT-PCR as described in chapter 2 in section 2.7.6. RNA was extracted from liver samples of

18 weeks HF fed 11β-HSD1-/- mice and their C57Bl6/J congenic controls, as stated above.

4.2.6 Statistical data analysis

Statistics was performed using SigmaStat and GraphPad software. Data were analysed by Student's t-test and ANOVA with Bonferroni post hoc tests, as appropriate.

4.3 Results

4.3.1 7-oxysterols do not activate LXR

Some oxysterols (eg 22(R)-hydroxycholesterol) bind to and activate LXRs (Janowski *et al.*, 1999), which may down-regulate adipocyte 11β-HSD1 expression (Stulnig *et al.*, 2002). Given the previously reported atheroprotective effects of 11β-HSD1 inhibition (Hermanowski-Vosatka *et al.*, 2005), the ability of 11β-HSD1-mediated 7-oxysterol metabolism to produce an LXR agonist and modulate LXR-gated activation of genes involved in lipid metabolism was evaluated. HEK293 cells were transfected with a cDNA encoding a chimeric activator comprising the Gal4 DNA binding domain and the LXRα ligand binding domain and a reporter plasmid in which a transcription factor activation from Gal4 binding sites is required for luciferase reporter activity (Pawar *et al.*, 2002, Willy *et al.*, 1995). Synthetic (Compound T0901317; 250nM) and physiological (22(R)-hydroxycholesterol; 20μM) LXR agonists activated the reporter by 9- and 5-fold, respectively (Fig. 4.1.). 7KC and 7β-HC were inactive in the transactivation assay (Fig. 4.1.). LXR agonists increased endogenous mRNA levels encoding SREBP1c, an LXR target gene in adipose tissue (Ulven *et al.*, 2004, Repa *et al.*, 2000). 7KC and 7β-HC were without effect (Fig. 4.2.). GLUT-4, another LXR and PPARγ target gene (Nugent *et al.*, 2001, Dalen *et al.*, 2003), was also not regulated by 7-oxysterols (Fig.4.3.).

4.3.2 No regulation of 11β-HSD1 mRNA expression by LXR agonists and 7-oxysterols

In contrast to previously reported data (Stulnig *et al.*, 2002), the LXR agonists (T0901317-1μM and 22(R)-HC-20μM) had no effect on 11β-HSD1 mRNA levels in 3T3-F442A or 3T3-L1 cells (Fig. 4.5.). Given that glucocorticoids regulate the 11β-HSD1 mRNA expression (Sai *et al.*, 2008) the potential regulation by 7-oxysterols has also been tested. Neither 7KC nor 7β-HC (20μM) altered 11β-HSD1 mRNA levels when incubated for 24h with fully-differentiated 3T3-F442A or 3T3-L1 adipocytes (Fig. 4.5.), nor did they alter 11β-HSD1 mRNA when present in the medium (1μM) throughout the 10 day differentiation of 3T3-L1 adipocytes (Fig. 4.6.). This suggests the effects of these oxysterols on 11β-HSD1 activity (as described in chapter 3) are not transcriptionally mediated.

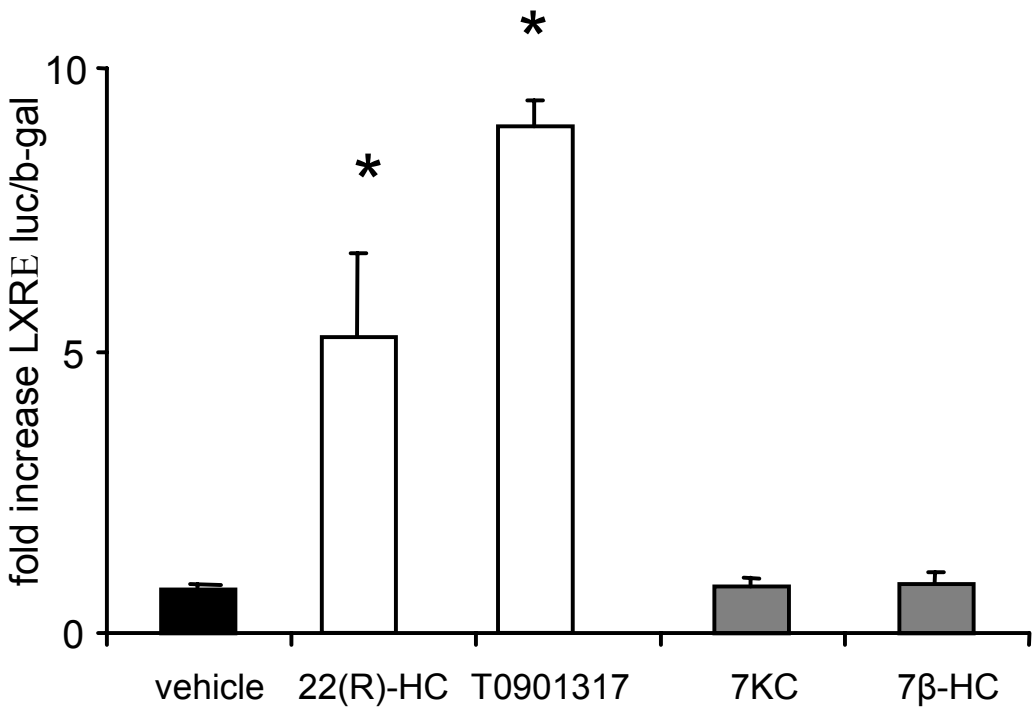

Figure.4.1 7-oxysterols do not activate LXR

HEK 293(11β-HSD1) cells were transfected with CMX-Gal4-hLXRα, an expression construct encoding the LXRα ligand binding domain, TK-MH100X4-Luc reporter plasmid and pRSV-βgal (internal control). Transfected cells were incubated with T0901317 (250 nM), 22(R)-HC (20 μM), 7KC (20 μM) or 7β-HC (20 μM). Values represent relative mean ± SEM from 4 independent experiments, each performed in triplicate. Statistically significant effect of treatment (*$p<0.05$ vs basal luciferase activity, analysed by one-way ANOVA with Bonferroni post hoc tests).

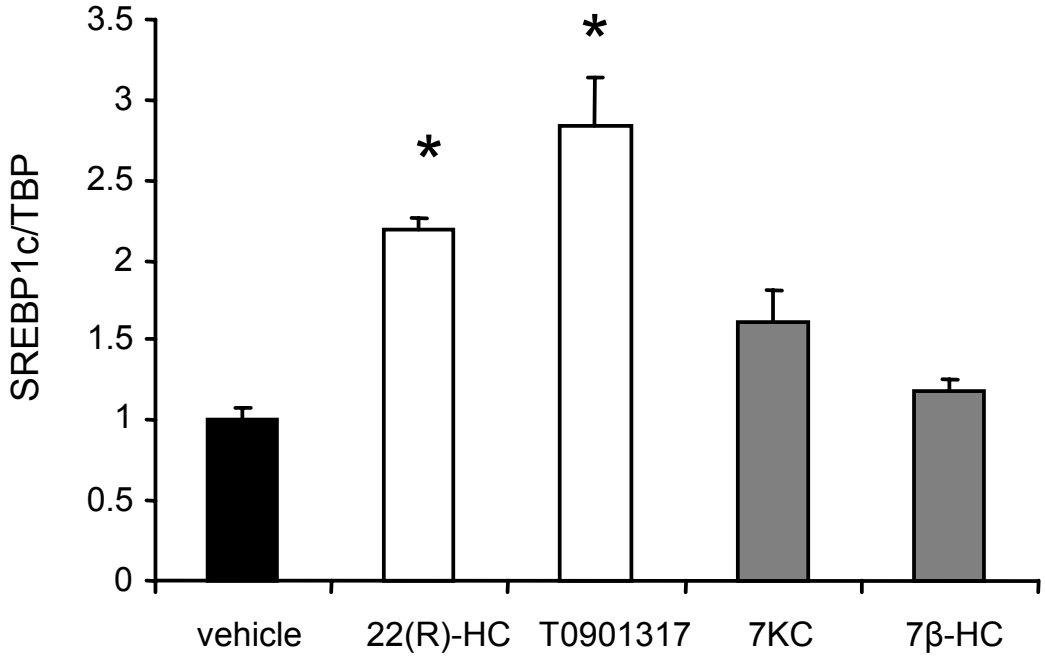

Figure 4.2 7-oxysterols do not regulate SREBP1c mRNA

Fully-differentiated 3T3-F442A adipocytes were incubated with 7-oxysterols (20μM), LXR ligands: 22(R)-HC (20μM), TO901317 (1μM) or "vehicle" (ethanol) for 24 h. Levels of mRNA encoding SEBP1c were measured by real time-PCR. Results represent mean ± SEM from 3 independent experiments, each performed in duplicate and are expressed relative to vehicle treated cells (arbitrarily set to 1). TATA binding protein was used as an internal control. Statistically significant effect of treatment (*$p<0.05$, one-way ANOVA with Bonferroni post hoc tests).

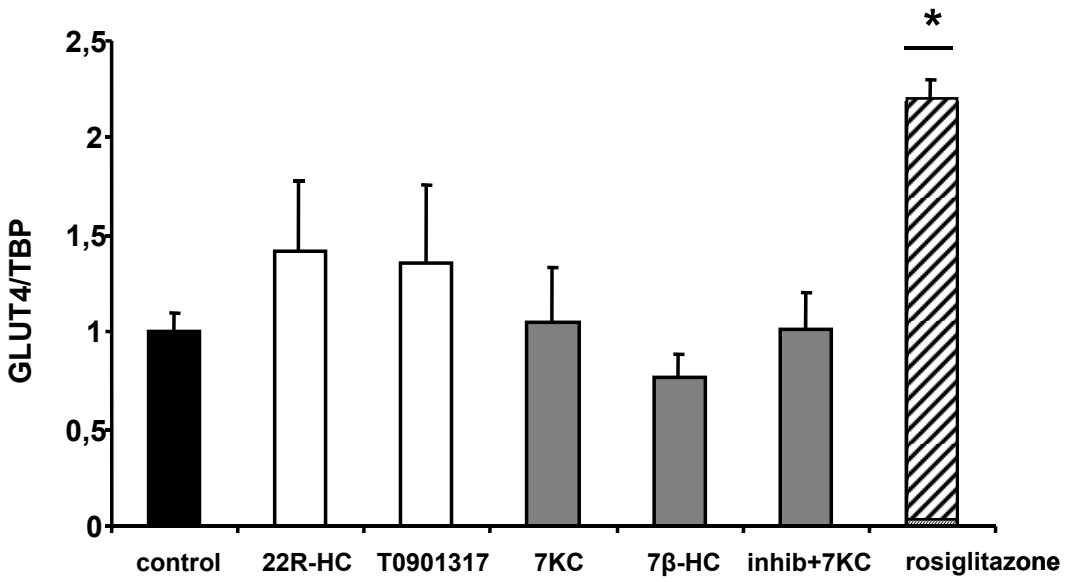

Figure 4.3 7-oxysterols do not regulate GLUT4 mRNA expression.

Fully-differentiated 3T3-F442A adipocytes were incubated with 7-oxysterols (20μM), LXR ligands: 22(R)-HC (20μM), TO901317 (1μM), PPARγ ligand: rosiglitazone (1μM; positive control), 11β-HSD1 inhibitor (Merck, compound 544) or "vehicle" (ethanol) for 24 h. Levels of mRNA encoding GLUT4 were measured by real time-PCR. Results represent mean ± SEM from 3 independent experiments, each performed in duplicate and are expressed relative to vehicle treated cells (arbitrarily set to 1). TATA binding protein was used as an internal control. Inhib: selective 11β-HSD1 inhibitor (Merck, compound 544). Statistically significant effect of treatment (*$p<0.05$, one-way ANOVA with Bonferroni post hoc tests).

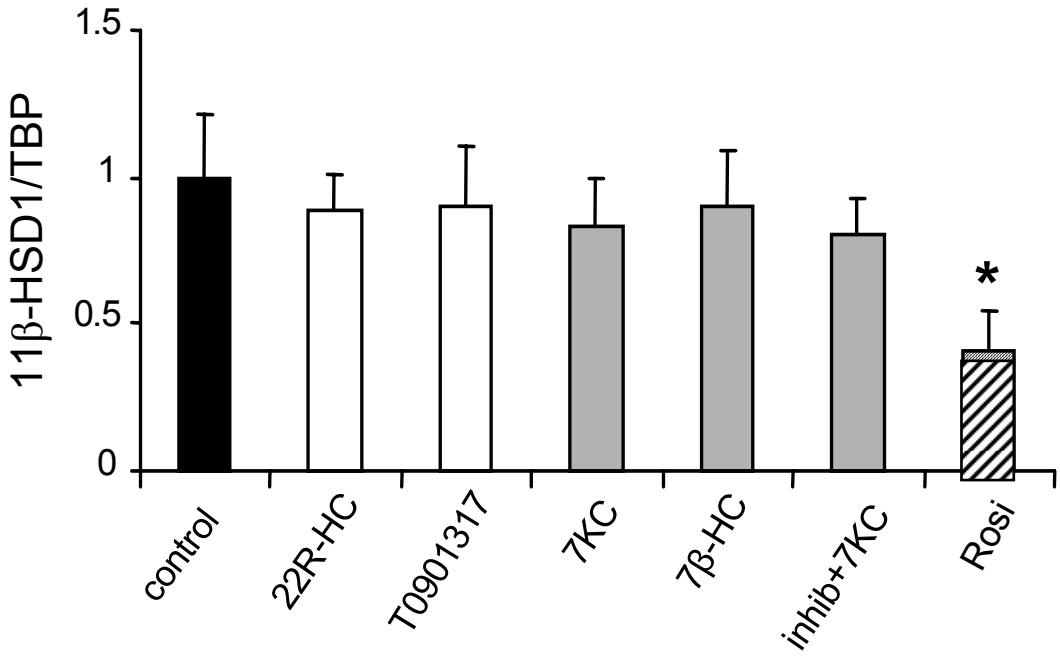

Figure 4.4 No regulation of 11β-HSD1 mRNA by 7-oxysterols and LXR agonists.

3T3-L1 adipocytes were incubated for 24 h with 7KC (20μM), 7βHC (20μM), 22(R)-HC (20μM) and T0901317 (1μM), rosiglitazone (1μM; positive control) and selective 11β-HSD1 inhibitor (5μM), as indicated. Values are 11β-HSD1 mRNA levels relative to TATA-binding protein mRNA, used as internal standard, and are expressed relative to vehicle treated cells (arbitrarily set to 1). Data are mean ± SEM of 4 independent experiments, each performed in duplicate. *$p<0.05$ was considered significant (one-way ANOVA with Bonferroni post hoc tests). Rosi: rosiglitazone, inhib-selective 11β-HSD1 inhibitor (Merck, compound 544).

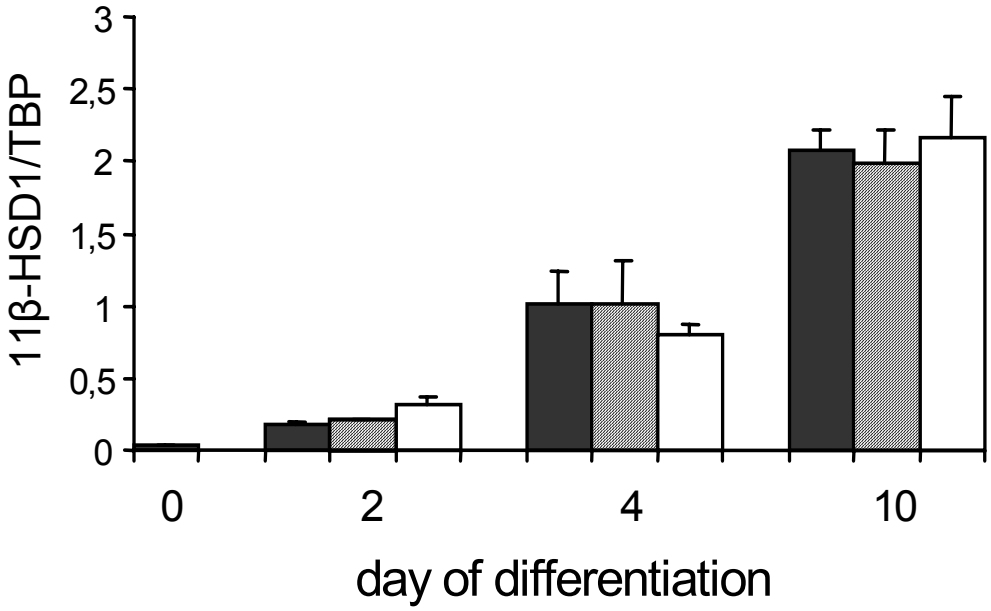

Figure 4.5 No regulation of 11β-HSD1 mRNA by 7-oxysterols during adipocyte differentiation.

3T3-L1 cells were differentiated according to the standard protocol with supplementation of 1μM 7KC (gray bars), 7βHC (open bars) or "vehicle" (ethanol; black bars) in the differentiation medium through the whole 10 day process of differentiation. (added fresh every second day). RNA was extracted on day 0, 2, 4 and 10 of differentiation. Values are the ratio of 11β-HSD1 mRNA levels to TATA binding protein mRNA levels and are mean ± SEM from 2 independent experiments, each performed in triplicate. A significant increase in 11β-HSD1 mRNA level was detected from day 4. No significant effect of incubation with 7-oxysterols was found (two-way ANOVA with Bonferroni post hoc tests).

4.3.3 No activation of FXR by 7-oxysterols.

In order to test the hypothesis that 7β-HC may be an activator of FXR/RXR, HEK293 cells were transfected with cDNA encoding these nuclear receptors and incubated with chenodeoxycholic acid (CDCA, a known FXR ligand; 20μM and 40μM) (Howard *et al.*, 2000), 7KC (20μM) and 7β-HC (20μM). Neither of 7-oxysterols increased FXRE-luc activity (Fig.4.6.).

4.3.4 No regulation of LXR (ABCA1, ABCG1, SREBP1c), FXR (BSEP, SHP) and RORα (Bmal1, Rev-erbα) gene targets in the liver of 11β-HSD1-/- and control mice.

Finally, the hepatic mRNA expression levels of gene targets of "orphan" nuclear receptors described in the literature as regulators of cholesterol and lipid metabolism and implicated in the pathogenesis of atherosclerosis (LXR, FXR, RORα) were measured. None of the examined genes showed differential regulation between genotypes as presented in Table 4.1. Similarly, no difference was observed in the adipose tissue of HF fed 11β-HSD1-/- mice compared to HF fed C57BL/6J controls in the micro-array experiment described in detail in chapter 5.

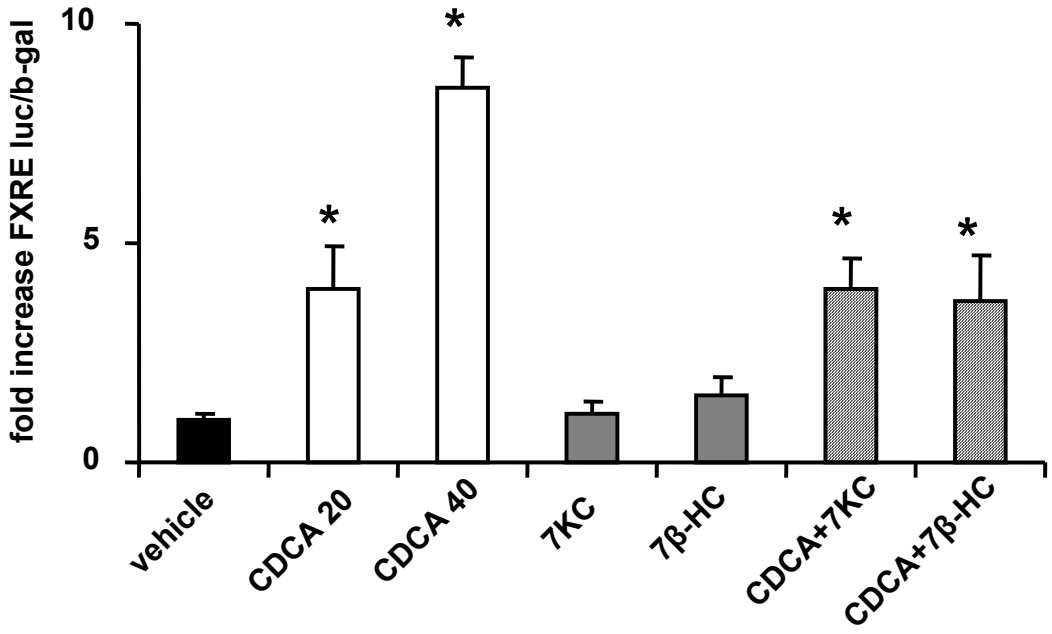

Figure.4.6 7-oxysterols do not activate FXR/RXRα.

HEK 293 cells were transfected with hFXR, hRXRα and FXRE-pTA-Luc reporter plasmid and pRSV-βgal (internal control). Transfected cells were incubated with CDCA (positive control), 7KC (20 μM) or 7β-HC (20 μM). Values represent relative mean±SEM from 3 independent experiments, each performed in triplicate. CDCA 20–chenodeoxycholic acid 20μM, CDCA 40- chenodeoxycholic acid 40μM, CDCA+7KC-chenodeoxycholic acid 20 μM and 7KC 20 μM, CDCA+7β-HC-chenodeoxycholic acid 20 μM and 7β-HC 20 μM. Statistical analysis was performed by one-way ANOVA with Bonferroni post hoc tests, *$p<0.05$ vs basal luciferase activity).

Gene	11β-HSD1-/- mice	C57Bl6/J mice
ABCA1	24.8±5.8	40.6±6.8
ABCG1	20.8±7	32.7±9.2
SREBP1c	18.34±1.2	21±2.9
SREBP2	2.44±0.68	2.43±0.7
BSEP	0.63±0.08	0.66±0.13
SHP	21.9±9.4	31.3±8.7
Bmal1	1.15±0.41	0.67±.0.11
Rev-erbα	1.24±0.59	0.97±0.24
Insig-1	2.37±0.54	2.09±0.57
GPAT	1.85±0.4	1.93±0.34
HMGCoAR	0.99±0.18	0.86±0.07

Table 4.1 Relative mRNA levels of chosen LXR, FXR and RORα key target genes.

mRNA levels were measured in the liver of HF fed 11β-HSD1-/- and congenic C57Bl6/J control mice. Mice (n=6) were challenged with 10 weeks of HF diet. The expression leves were measured by qRT-PCR as described in chapter 2 in section 2.7.6. Values represent the mean±SEM. TATA binding protein mRNA was used as an internal standard. Similar values were obtained when actin was used as an internal standard. There were no significant differences between genotypes (Student's t-test).

4.4 Discussion

Here, I tested the hypothesis that by converting 7KC to 7β-HC 11β-HSD1 plays a crucial role in maintaining cholesterol homeostasis through regulation of the availability of a ligand for a nuclear receptor. Enzymatically produced oxysterols activate LXR receptors (Janowski *et al.*, 1999). Studies on purified receptors described 7β-HC as a poor LXR ligand (Janowski *et al.*, 1999). However, sulphated (7-ketocholesterol-3-sulfate) metabolites inhibited transactivation by LXR *in vitro* (Song *et al.*, 2001) suggesting the activity of these oxysterols for LXR *in vivo* may be determined by their abundance and modification within the tissue. 7-oxysterols, known markers of the oxidative stress in dyslipidemic and diabetic patients (Ferderbar *et al.*, 2007), were anticipated to be produced through peroxidation of diet-derived cholesterol. However, recent reports describing 7KC as a novel substrate for 11β-HSD1, pointed towards the plausible role of this enzymatic reaction in the activation of LXR pathway. Additionally, 27-hydroxycholesterol accompanied by 7-oxysterols in atherogenic plaques, can activate LXR in cholesterol-loaded macrophages (Fu *et al.*, 2001). Moreover, LXR ligands have been reported to inhibit the expression and activity of 11β-HSD1 in 3T3-L1 cells (Stulnig *et al.*, 2002), suggesting that a cross-talk between the oxysterol and glucocorticoid pathways may operate also at the transcriptional level. However, 7-oxysterols failed to show any intrinsic activity for LXRα in the transactivation assays. Neither 7-oxysterol increased expression of SREBP1c and GLUT-4 mRNA, known LXR target genes in 3T3-L1 adipocytes. I also found no differences in the mRNA levels of ABCA1 and ABCG5 (the reverse-cholesterol transporters controlled by LXR (Repa *et al.*, 2002)) in the liver of HF fed 11β-HSD1-/- mice, which excludes that the activation of LXR by 7-oxysterol metabolites may be implicated in the atheroprotective role of 11β-HSD1 inhibition. Further, no regulation of the 11β-HSD1 mRNA levels was observed with either LXR ligands or 7-oxysterols in differentiated 3T3-F442A and 3T3-L1 adipocytes, in contrast to the previous report (Stulnig *et al.*, 2002). The basis for the latter discrepancy is uncertain but here at least no evidence was found for cross-talk between glucocorticoid and LXRα-mediated pathways in 3T3 cells.

Another possibility was the activation of FXR by 7β-HC since this oxysterol is an intermediate product of the alternative bile acid synthesis pathway in the liver. Neither 7β-HC not 7KC showed any intrinsic activity towards FXR in HEK293 cells transfected with FXR/RXR plasmids. Additionally, the mRNA expression levels of BSEP (Ananthanarayanan *et al.*, 2001) and SHP (Goodwin *et al.*, 2000) (key FXR target genes) in the liver of HF fed 11β-HSD1-/- mice and their congenic controls were measured. No differences in levels of these mRNA were found between genotypes. Ultimately, it cannot be excluded that by metabolizing 7-oxysterols 11β-HSD1 may be involved in the production of a ligand for other, as yet unidentified, "orphan" nuclear receptor.

RORα has emerged recently as a novel plausible target in the treatment of atherosclerosis (Laitinen and Staels, 2003). Given that the screen for the natural ligands for RORα revealed hydroxycholesterols and oxysterol derivatives, I investigated the potential of differential regulation of RORα target genes in the liver of HF fed 11β-HSD1-/- and wild type mice. I observed that the expression of putative RORα target genes was not significantly different between genotypes. Nevertheless future work should look at the potential activation of RORα in transfection assays.

Pharmacological inhibition of 11β-HSD1 protects from atherosclerosis (Hermanowski-Vosatka *et al.*, 2005). 11β-HSD1-/- mice presented with an advantageous lipid profile and lower fibrinogen (Morton *et al.*, 2001), however, the molecular mechanism of the atheroprotective consequences of intracellular deficiency of glucocorticoids remains unclear. Here, the possibility that by inter-converting atherogenic 7-oxysterols 11β-HSD1 may be involved in the activation of LXR, FXR and, although with less certainty, RORα-regulated pathways of cholesterol metabolism was largely excluded.

CHAPTER 5

Adipose tissue transcriptome profiling reveals novel depot-specific "protective" mechanisms of 11β-HSD1 deficiency

5 Adipose tissue transcriptome profiling reveals novel depot-specific "protective" mechanisms of 11β-HSD1 deficiency

5.1 Introduction

Excessive plasma glucocorticoid levels impair lipid and glucose metabolism, insulin sensitivity, energy homeostasis and inflammatory processes. The similarity between the metabolic disturbances of Cushingoid and idiopathic obesity implied a potentially common underlying glucocorticoid-mediated mechanism. This contention was supported by experiments where adrenalectomy ameliorated and glucocorticoid replacement restored dietary and genetic obesity in rodents. Human idiopathic (Bujalska *et al.*, 1997, Rask *et al.*, 2002, Paulmyer-Lacroix *et al.*, 2002) and rodent genetic obesity (Livingstone *et al.*, 2000a, Liu *et al.*, 2003) is associated specifically with increased adipose glucocorticoid action through increased levels of the enzyme 11β-HSD1 (an intracellular gatekeeper of glucocorticoid receptor activation), which may provide the molecular link between Cushingoid and idiopathic obesity (Wamil and Seckl, 2007, Morton and Seckl, 2008).

A potentially causal role for increased adipose 11β-HSD1 in the pathogenesis of obesity was highlighted by the phenotype of the adipose tissue selective 11β-HSD1 over-expressing mouse, which presented with insulin resistant diabetes, visceral obesity, hyperlipidemia, hyperleptinemia and hypertension (Masuzaki *et al.*, 2001). Conversely, 11β-HSD1-/- mice were protected from the metabolic consequences of diet-induced obesity (Morton *et al.*, 2004b). On the obesity-prone C57Bl/6J genetic background, high fat diet fed 11β-HSD1-/- mice gained significantly less weight than controls (Morton *et al.*, 2004a). Crucially, these mice preferentially increase adipose tissue in the peripheral depots rather than in the metabolically disadvantageous visceral fat.

It is clear that loss of 11β-HSD1 has few deleterious metabolic effects, at least in mice. Indeed, industry has produced prototypic drugs which selectively inhibit 11β-HSD1. The first of these, the arylsulfonamidothiazoles (Barf *et al.*, 2002a), inhibit 11β-HSD1 and enhance insulin action in liver, lowering blood glucose concentrations in diabetic and obese mice (Alberts *et al.*, 2002a, Alberts *et al.*,

2003a). Many other agents are in development, so fully understanding how loss of 11β-HSD1 acts, particularly in adipose tissue, is a crucial ambition. Given the hypothesis that down-regulation of adipose 11β-HSD1 is an adaptive metabolic response to counteract insulin resistance upon exposure to a high fat diet (Morton *et al.*, 2004c), the current project explored fat depot-specific effects of glucocorticoid deficiency on adipose tissue gene expression that might protect an intrinsically obesity-susceptible model from both obesity and its cardio-metabolic consequences.

The link between chronic inflammation and increased risk of cardiovascular diseases associated with obesity has been well described (Hotamisligil, 2007, Shoelson *et al.*, 2007, Shoelson *et al.*, 2006). It has been demonstrated that pro-inflammatory cytokines (eg TNF-alpha, IL-1, IL-6, MCP-1) and various adipocytokines may influence insulin signaling in the fat and other tissue (Shoelson *et al.*, 2007, Tilg and Moschen, 2006). Several transcription factors and kinases such as c-Jun N-terminal kinase (JNK) and inhibitor of κB kinase-β (IKKβ) have been shown to mediate the effect of inflammatory stimuli on the pathogenesis of insulin resistance (Yuan *et al.*, 2001, Hirosumi *et al.*, 2002). Adipose tissue of obese human and rodents is characterised by macrophage infiltration (Weisberg *et al.*, 2003), which produces pro-inflammatory factors modulating the secretion of adipocytokines (Xu *et al.*, 2003). It has been also reported recently that infiltration of visceral fat tissue by T cells may trigger the initiation of inflammation during the early phase of adipose tissue expansion (Kintscher *et al.*, 2008, Wu *et al.*, 2007).

It has been suggested that the metabolic consequences of obesity are more strongly associated with visceral than total body adipose mass (Wajchenberg, 2000). Surgical removal of visceral but not subcutaneous fat causes increased hepatic and peripheral insulin sensitivity (Klein *et al.*, 2004, Kelley, 2004, Gabriely *et al.*, 2002). Visceral adipose tissue exhibit higher levels of a number of important adipokines that affect peripheral insulin sensitivity. Visceral fat also delivers lipolysis products and pro-inflammatory cytokines to the liver and therefore may have a more pronounced influence on hepatic insulin sensitivity and function than other depots (Gabriely *et al.*, 2002). On the other hand, peripheral fat accumulation, whilst the major source of

free fatty acid flux in humans, may be relatively protective from metabolic disorders (Votruba *et al.*, 2007, Jensen *et al.*, 2003, Guo *et al.*, 1999). Thus, redistribution of fat stores to the periphery with overall weight gain is a feature of insulin sensitization following treatment with anti-diabetic thiazolidenediones (Adams *et al.*, 1997, Yamauchi *et al.*, 2001). Indeed fat mass-matched obese populations with contrasting peripheral versus central fat distribution suggest peripheral fat accumulation is metabolically protective (Talebizadeh and Butler, 2005). Whilst the basis for adipose tissue redistribution to subcutaneous depots on high fat diet in 11β-HSD1-/- mice is uncertain, these mice show adipocyte insulin sensitisation and a 'favourable' adipokine profile (Morton *et al.*, 2004b). Adipose tissue-specific over-expression of the glucocorticoid-inactivating 11β-HSD2 enzyme recapitulated many of the adipose-mediated beneficial effects observed in 11β-HSD1-/- mice (Kershaw *et al.*, 2005) suggesting that direct effects of 11β-HSD1 deficiency within adipose tissue as well as beneficial hepatic effects (Morton *et al.*, 2004b, Morton *et al.*, 2001) were a likely feature of this model. Here I investigated the depot-specific transcriptomic profile of adipose tissue that underlies the fat redistribution. This also presents the first report suggesting that reduced infiltration of T cells in the mesenteric fat depot may explain the resistance of 11β-HSD1-/- mice to high fat diet-induced metabolic disease.

Aims

1) To investigate the depot-specific transcriptomic profile of adipose tissue that underlies the fat redistribution and resistance of 11β-HSD1-/- mice to high fat diet-induced metabolic disease.
2) To dissect the critical pathways downstream of 11β-HSD1 in visceral and subcutaneous fat.
3) To validate the micro-array data by qRT-PCR for chosen gene targets.
4) To confirm the up-regulation of PI3K/AKT insulin signaling pathways in subcutaneous adipose tissue of 11β-HSD1-/- mice on the post-translational level.

5.2 Methods

5.2.1 Animals

Male 11β-HSD1-/- mice and their congenic controls were maintained as described in section 2.3. Animals were matched for age and weight and allocated into two groups of seven. Animals were housed singly and maintained on HF diet (58% calories as fat, Research Diets D12331) for 4 weeks (section 2.3.2.). Body weight and food intake were recorded on a weekly basis.

5.2.1.1 Analysis of plasma glucose and insulin

Mice were fasted for 6 h for glucose and insulin measurements before and after the study. Plasma fasting glucose and insulin were measured as described in section 2.3.4.

5.2.2 Genomic analysis

Adult male age-matched 11β-HSD1-/- and C57Bl6/J mice (n=7) were given a HF diet for 4 weeks, a diet previously optimized for weight gain and insulin resistance. Animals were fasted for 6h for glucose and insulin measurements before and after the study. Mice were killed at 8:00 am within 1min of disturbing each cage. Glucose was measured with the Sigma HK assay (Sigma, UK) and insulin with the Ultra sensitive Rat Insulin ELISA kit (Crystal Chem, UK) and corticosterone by RIA as described in section 2.3.4 and (Morton *et al.*, 2004c). Two fat depots (subcutaneous, mesenteric) and the liver were frozen on dry ice and stored at -80ºC. RNA was extracted using RNeasy Lipid Tissue Midi kit (Qiagen, UK) or Trisol method (Invitrogen, UK). RNA quality was verified with the 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). Samples that demonstrated high quality (i.e. the ratio of 28S rRNA to 18S rRNA was greater than 1.9) and had a minimum of 1μg of RNA were submitted for micro-array analysis. Micro-array experiment was performed as described in chapter 2 in section 2.8.4 (Fig. 5.1.).

5.2.3 Data validation

Chosen gene targets were validated by qRT-PCR in bigger groups of animals (n=10-14) as described in section 2.7.6.

5.2.4 Insulin signaling *in vivo*.

12-week-old male 11β-HSD1-/- and C57Bl/6J mice were divided into four groups (n=6) with similar body weight and assigned to receive two kinds of diet: standard chow diet or HF diet (as described above) for 10 weeks. At the end of diet period mice were fasted for 6h and then injected i.p. with 0.75mU/g humulinS or saline. Blood samples were taken before and after fasting for glucose and insulin measurements. At 15min after the injection subcutaneous and mesenteric fat depots and liver were dissected and snap frozen in liquid nitrogen and stored at-80. Tissues were processed as described in chapter 2.9.

5.2.5 Adipocyte cell sizes measurement

Cell sizes were measured as described in section 2.10.

5.3 Results

5.3.1 Fat redistribution in 11β-HSD1-/- mice.

After 4 weeks of HF diet 11β-HSD1-/- mice gained less weight, had lower mesenteric fat-depot mass (gross mass: $p=0.0113$; organ to body weight ratio: $p=0.0159$), lower fasting glucose ($p<0.0001$) and insulin levels ($p=0.0012$) than control group (Table 5.2.). The redistribution of fat mass to peripheral subcutaneous fat depot confirmed the observations previously reported for an intra-abdominal metabolically "peripheral-like" fat depot (epididymal fat) (Morton *et al.*, 2004b).

5.3.2 Differential expression of genes in subcutaneous and mesenteric fat depot-overall analysis.

Micro-array analysis using Affymetrix 430 2.0 GeneChips revealed a large number of differentially expressed genes between the genotypes. I identified 565 and 1622 transcripts as being ≥1.5-fold differentially expressed between genotypes in subcutaneous adipose tissue and mesenteric fat, respectively. The majority of these genes were down-regulated in mesenteric adipose tissue of 11β-HSD1-/- mice (73%; Fig. 5.3.) and up-regulated in subcutaneous fat depot (79%; Fig. 5.3.). The expression of 91 genes were altered in the same direction in both fat depots (Fig. 5.2.).

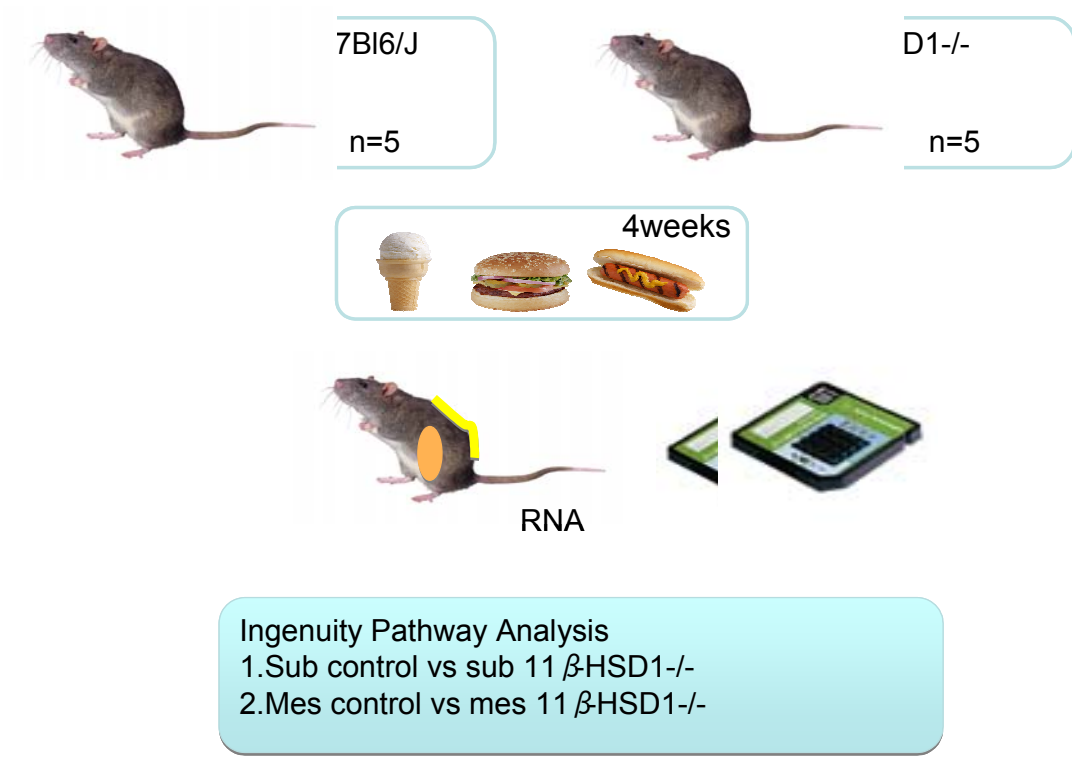

Figure 5.1 Schematic representation of the micro-array experiment.

11β-HSD1-/- and C57Bl6/J mice (n=5) were challenged with 4 weeks HF diet (58% calories from fat). Fasting glucose and insulin was measured at the end of the study period. Mice were killed and two fat depots (subcutaneous and mesenteric) were dissected. RNA was extracted using RNeasy Lipid Tissue Midi kit (Qiagen, UK). RNA samples were used for target preparation and subjected to hybridization to 20 Affymetrix mouse Genome 430 2.0 GeneChips. Samples were processed according to standart Affymetrix protocols by the micro-array team at The Sir Henry Wellcome Functional Genomics Facility in Glasgow. Data were extracted through the GCOS software and processed with the Robust Multichip Average algorithm. Differential expression was determined by Limma tool on BioConductor (as described in Methods in section 2.8.4). Genes with >1.5-fold differential expression levels between genotypes in the subcutaneous and mesenteric adipose tissue were imported to Ingenuity Pathway Analysis (IPA).

Parameter	C57Bl/6J	11β-HSD1-/-
Mesenteric fat mass		
Ratio (mg/g body weight)	2.65±0.1	1.99±0.1*
Absolute weight (mg)	94±5	69±6*
Subcutaneous fat mass		
Ratio (mg/g body weight)	4.2±0.3	3.4±0.1
Absolute weight (mg)	157±17	122±9
Liver mass		
Ratio (mg/g body weight)	4.8±0.1	4.76±0.1
Absolute weight (mg)	174±7	165±5
Cumulative weight gain (g)	6.2±0.7	3±0.6**
Mean food intake per week: weeks 2-4 (g)	19±1.5	16.3±2
Fasting glucose (mg/dl)	211.5±4.8	157.5±3.4***
Insulin (pg/ml)	18.3±1.9	7±1.1**
Corticosterone (nmol/l)	4.43±2.24	5.62±1.9

Table 5.1. Physiological characteristics of 11β-HSD1-/- and control mice fed HF for 4 weeks.

Adipose depot fat mass was assessed in C57Bl/6J (control) and 11β-HSD1-/- mice after 4 weeks of high fat diet. Glucose and insulin were measured in plasma samples obtained after 6h of fasting at the end of the 4 week study period. Data are the means ±SE (n=7) analysed by Student's t-test; * $P<0.05$, ** $P<0.01$, *** $P<0.0001$.

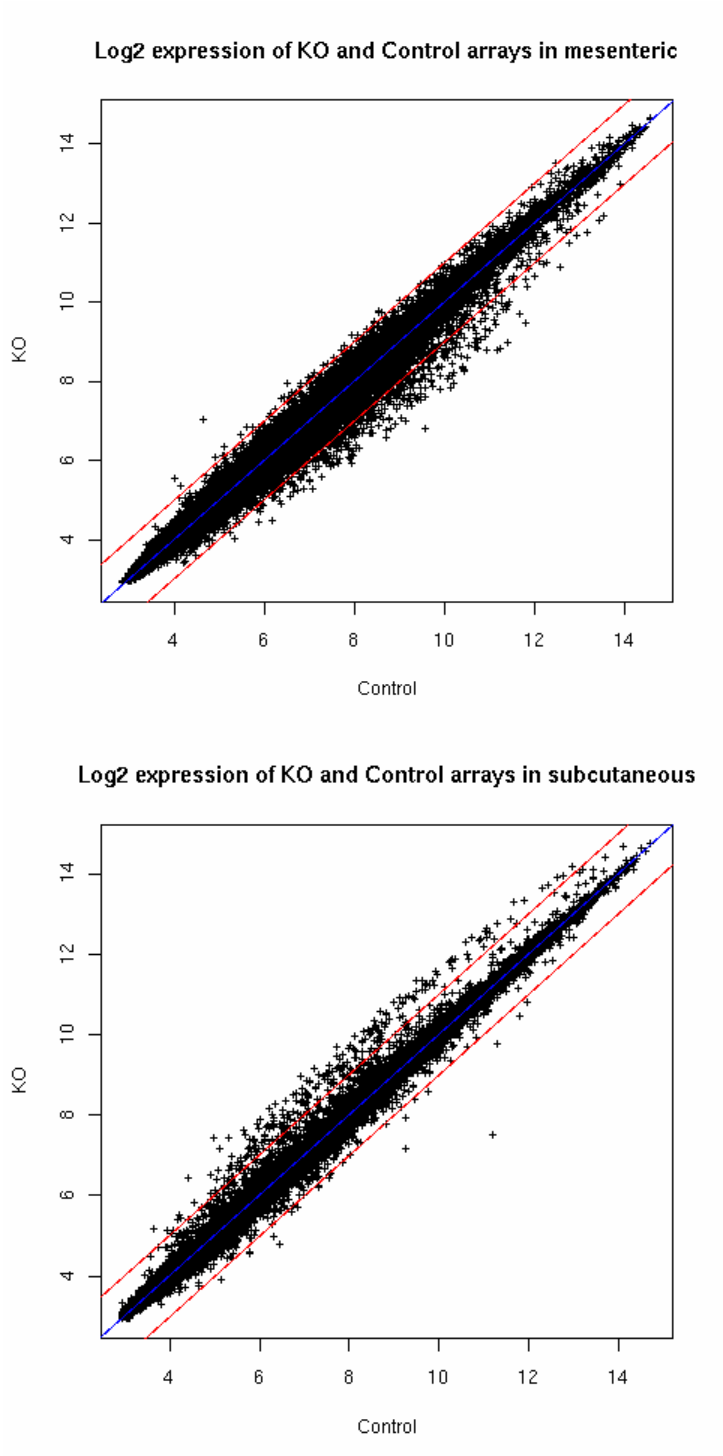

Figure 5.2 Comparison of scatter plots of log intensity values.

Blue line shows no change, red lines = 2-fold change. Each cross represents a single transcript.

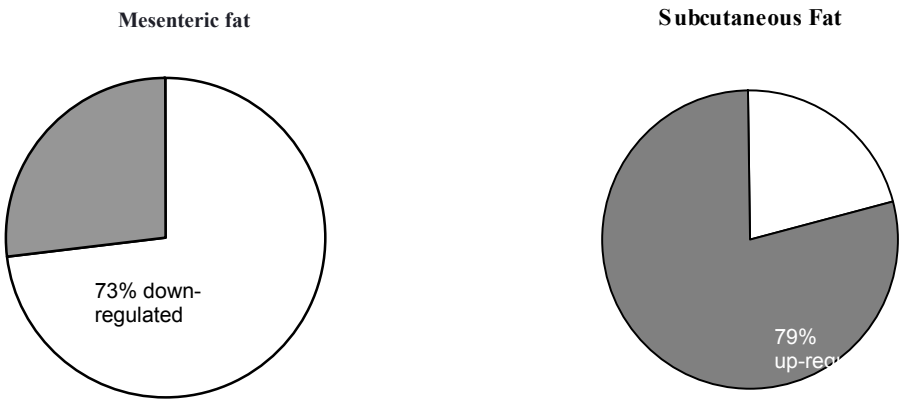

Figure 5.3 Schematic representations of genes differentially regulated in mesenteric and subcutaneous fat depot of 11β-HSD1-/- mice.

White and gray color represent percent of genes down-regulated and up-regulated ≥1.5 fold, respectively.

5.3.3 Up-regulated genes in the subcutaneous fat of HF fed 11β-HSD1-/- mice.

Analysis of gene expression in subcutaneous fat of HF fed 11β-HSD-/- mice revealed consistently higher expression of genes involved in glycolysis and lypolysis, β-oxidation and oxidative phosphorylation compared to HF fed controls. When the altered genes were analysed by gene ontology, activation of genes in subcutaneous fat of 11β-HSD1-/- mice were associated with tissue morphology changes, myogenesis, cellular assembly and organization, permeability and cellular growth and proliferation. The most significant pathways included: calcium signaling (ASPH, ATP2A1, CACNA1S, CACNA2D1, CACNB1, CACNG1, CAMK2A, CASQ1, MYH1), and β-adrenergic signaling (AKAP6, ATP2A1, PKIA, PPM1L, PPP2R3A, SLC8A3) followed by glucose metabolism (FOXA1, ENO3, FBP2, PFKM, PGAM2), mitochondrial transport and lipid metabolism (CPT1, ACSL6, Fabp3), insulin signaling (PHKB, PHKA1, PHKG1, PKM2, Fbp2, PFKM) and oxidative phosphorylation (Cox6a2, Cox7a1, Cox8b) (Table 5.2.). Fabp3, one of the fatty-acid transporters determining the delivery of fatty acids to the mitochondria for β-oxidation, showed 3.8-fold increase. Additionally, carnitine palmitoyltransferase 1 (Cpt1), a key gene controlling the β-oxidation of long-chain fatty acids, showed a similar increase. Interestingly, a component of cytochrome c oxidase, Cox6a2 (3-fold increase), was up-regulated exclusively in the subcutaneous adipose tissue of 11β-HSD1-/- mice, whereas mesenteric fat of both genotypes and the subcutaneous fat of control mice had very low expression levels. Moreover, two putatively "cardioprotective" (Fan *et al.*, 2005) heat shock proteins: Hspb3 and Hspb6, known to be implicated in smooth muscle relaxation and inhibition of platelet aggregation , were increased 2.5 and 2.3-fold, respectively. Recently, it has been reported that alteration in phosphorylation of Hspb6 was associated with dexamethasone-induced insulin resistance (Wang *et al.*, 2001).

Gene symbol	Gene name	Accession	Mean fold difference	Family
Calcium signaling				
ASPH	Aspartate β-hydroxylase	65973	2.73	enzyme
ATP2A1	ATPase calcium transportin	11937	2.25	transporter
CACNA1S	Calcium channel, voltage dependent, L type, alpha 1S subunit	12292	2.97	ion channel
CACNA2D1	Calcium channel voltage dependent, alpha2/delta subunit 1	12293	2.17	ion channel
CACNB1	Calcium channel, voltage dependent, β subunit 1	12295	2.39	ion channel
CACNG1	Calcium channel, voltage dependent, gamma subunit 1	12299	2.44	ion channel
CAMK2A	Calcium/calmodulin-dependent protein kinase (CaM kinase)II alpha	12322	2.49	kinase
CASQ1	Calsequestin 1	12372	2.47	other
PLCD4	Phospholipase C, delta 4	18802	2.44	
B-adrenergic signaling				
AKAP6	A kinase (PRKA) anchor protein 6	238161	2.57	other
PKIA	Protein kinase (cAMP-dependent, catalytic) inhibitor alpha	18767	2.67	transporter
PPM1L	Protein phosphatase 1	242083	1.97	other
PPP1R14C	Protein phosphatase 1, regulatory (inhibitor)	76142	2.65	other
PPP2R3A	Protein phosphatase 2 regulatory subunit B, alpha	19054	2.11	phosphatase
SLC8A3	Solute carrier family 8 (sodium-calcium exchanger), member 3	110893	2.05	transporter
Glucose metabolism				
PRKAA2	Protein kinase, AMP-activated, alpha 2 catalytic subunit	108079	2.11	kinase
PRKAB2	Protein kinase, AMP-activated, β 2 non-catalytic subunit	108097	1.78	kinase
E NO3	Enolase 3	13808	2.65	enzyme
FBP2	Fructose-1,6-biphosphatase 2	14120	2.13	phosphatase
PFKM	Phosphofructokinase	18642	2.52	kinase
PGAM2	Phosphoglycerate mutase 2	56012	3.2	phosphatase
MAPK/ERK signaling				
CACNA2D1	Calcium channel voltage dependent, alpha2/delta subunit 1	12293	2.17	ion channel
CACNB1	Calcium channel, voltage dependent, β subunit 1	12295	2.39	ion channel
CACNG1	Calcium channel, voltage dependent, gamma subunit 1	12299	2.44	ion channel
EGF	Epidermal growth factor	13645	2.11	growth factor
MEF2C	Myocyte enhancer factor 2C	17260	1.67	transcription factor
MAPK12	Mitogen-activated protein kinase 12	29857	1.54	kinase
FLNC	Flamin C	68794	1.79	other

Insulin signaling				
PHKB	Phosphorylase kinase β	102093	1.6	Kinase
PHKA1	Phosphorylase kinase alpha 1	18697	2.42	Kinase
PHKG1	Phosphorylase kinase gamma 1	18682	2.79	kinase
PKM2	Pyruvate kinase	18746	1.57	kinase
SOCS3	Suppressor of cytokine signaling 3	12702	1.54	other
Lipid metabolism				
CPT1b	Carnitine palmitoyltransferase 1	12895	2.02	kinase
ACSL6	Acyl-CoA synthetase long-chain family member 6	216739	2.06	ligase
Fabp3	Fatty acid binding protein 3	14077	2.79	transporter
Oxidative phosphorylation				
Cox6a2	Cytochrome c oxidase, subunit VI a, polypeptide 2	12682	2.89	transporter
Cox7a1	Cytochrome c oxidase, subunit VIIa	12865	1.94	transporter
Cox8b	Cytochrome c oxidase, subunit VIIIb	12869	1.57	transporter

Table 5.2 Selected genes up-regulated in the subcutaneous fat depot more than 1.5-fold.

Selection criteria were based on the analysis of most significant pathways by the WebGestalt and Ingenuity Programs. Fold change represents the ratio of mean mRNA expression level between HF fed 11β-HSD1-/- and C57Bl/6J.

5.3.4 Down-regulated genes in the subcutaneous fat of HF fed 11β-HSD1-/- mice.

Among 23 genes down-regulated in the subcutaneous fat of 11β-HSD-/- mice, the growth hormone receptor (GHR) belongs to the best described in relation to glucocorticoid regulation (Beauloye *et al.*, 1999). GHR was decreased 3.28-fold in subcutaneous fat. Additionally, leptin was down-regulated by 1.66-fold confirming previous reports in this murine model (Morton *et al.*, 2004b).

5.3.5 Down-regulated genes in the mesenteric fat of HF fed 11β-HSD1-/- mice.

Among the set of genes down-regulated in the mesenteric fat depot pathways associated with immune cell, NF-kappaB, Jak/STAT, SAPK/JNK, and chemokine signaling ranked highest in the functional classification (Table 5.3.). Functional analysis showed that the largest number of differentially expressed genes were linked to the proliferation, differentiation, movement and adhesion of immune cells (Table 5.3.). This indicated that an early phase of diet-induced obesity in C57Bl/6J mice is characterized by changes in tissue morphology and cellular organization, most probably by the infiltration and activation of the mesenteric fat depot by immune cells, in agreement with the literature (Weisberg *et al.*, 2003). This process is distinctly reduced in the HF fed 11β-HSD1-/- mice. Genes with the highest scores in that group included Marco (macrophage receptor with collagenous structure), which showed a 5-fold down-regulation exclusively in the mesenteric fat depot. Marco is expressed predominantly on macrophages and dendritic cells and is implicated in scavenging and immune function (Elomaa *et al.*, 1995). L-selectin, an adhesion molecule implicated with the lymphocyte trafficking into the atherosclerotic aorta (Galkina *et al.*, 2006), showed 4-fold lower expression, again only in mesenteric fat. Examples of significantly regulated chemokines included: CCL5, CCR6, CXCR4, LTB, PRKCB1 and several members of the tumor necrosis factor receptor superfamily implicated in chronic inflammatory processes in obesity (Hotamisligil, 1999, Hotamisligil *et al.*, 1995). CCL5 mRNA levels are increased in murine and human visceral obesity and are associated with T cell infiltration of white adipose tissue (Wu *et al.*, 2007). Several gene members of the two pathways highly implicated in the pathogenesis of insulin resistance, NF-kappa B pathway (LCK, KLRD1, LAT, LCP2, PIK3CD, PRKCB1, PTPN6, VAV1) and SAPK/JNK

signaling (LCK, MAP4K1, PIK3CD, RAC2, TRA) were down-regulated in the 11β-HSD1-/- mice. Moreover, IL2R (CD25) and CD4, highly expressed on regulatory T cells (CD4+CD25+ T cells; Treg) associated with the development of insulin-dependent diabetes (Yamanouchi *et al.*, 2007, Lowe *et al.*, 2007) were 2.5 fold and 1.85 lower in 11β-HSD1-/- mice, respectively. Interestingly, two downstream effectors of Wnt signaling pathway (LEF1 and Tcf7) have been highlighted by the Ingenuity Pathway Analysis (IPA). Recently, genome-wide studies revealed a strong association of polymorphisms in IL2R with increased risk of diabetes type 1 (Vella *et al.*, 2005) and in Tcf7 with diabetes type 2 in various ethnic populations (Grant *et al.*, 2006, Elbein *et al.*, 2007, Chang *et al.*, 2007). Finally, a transcription factor-STAT4, implicated in chronic inflammation linked to obesity (Torpey *et al.*, 2004) was down-regulated in 11β-HSD1-/- mice.

Gene symbol	Gene title	Accession	Mean fold difference	Family
Immune cell signaling				
CD28	CD28 antigen	12487	3.04	Other
CD3d	CD3-TCR complex	12500	3.47	Transmembrane receptor
CD3G	CD3-TCR complex	12502	3.55	Transmembrane receptor
CD8A	CD8A	12525	3.34	other
CD8B	CD8B	12526	3.4	Other
ITK	IL-2-inducible T-cell kinase	16428	3.52	Kinase
LAT	Linker for activation of T cells	16797	2.6	Other
LCP2	Lymphocyte cytosolic protein 2	16822	2.29	other
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polipeptide	18707	2.32	Kinase
PTPRC	Protein tyrosine phosphatase, receptor type C	19264	2.77	Phosphatase
RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG regulated)	19419	2.42	other
VAV1	Vav 1 oncogene	22324	2.19	Transcription regulator
KLRD1	Killer cell lectin-like receptor subfamily D, member 1	16643	2.73	Transmembrane receptor
BCL2A1	BCL2-related protein A1	12044	2.27	Other
CAMK2B	Calcium/calmodulin-dependent protein kinase II β	12323	2.57	Kinase
CD19	CD19	12478	3.18	Other
CD22	CD22	12483	4.46	Other
CD79A	CD79A immunologlobulin associated alpha	12518	3.59	Transmembrane receptor
CD79B	CD79A immunologlobulin associated β	15985	3.46	Transmembrane receptor
CSF2RB2	Colony stimulating factor receptor, β 2	12984	2.51	Transmembrane receptor
Card11	caspase recruitment domain family, member 11	108723	2.08	other
NF-kappaB signaling				
CD40	CD40, TNF receptor superfamily member 5	21939	2.25	Transmembrane receptor
PRKCB1	Protein kinase C, β 1	18751	2.29	kinase
TLR1	Toll-like receptor 1	21897	2.26	Transmembrane receptor
TCR ALPHA	T cell receptor alpha locus	21473	3.51	other
ZAP70	Zeta-chain (TCR) associated protein kinase	22637	2.36	kinase
LCK	Lymphocyte-specific protein tyrosine kinase	16818	3.37	kinase

Antigen processing and presentation				
HLA-DMB	Major histocompatibility complex, class II, DM β	14999	2.96	Transmembrane receptor
HLA-DOA	Major histocompatibility complex, class II, DO alpha	15001	3.33	Transmembrane receptor
HLA-E	Major histocompatibility complex, calss I, E	15006	2.5	Transmembrane receptor
TAP1	Transporter 1, ATP-binding cassette, subfamily (MDR/TAP)	21354	2.2	Transporter
SAPK/JNK signaling				
LCK	Lymphocyte-specific protein tyrosine kinase	16818	3.37	Kinase
MAPK4K1	Mitogen-activated protein kinase1	26411	2.5	kinase
RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	19354	2.47	enzyme
TCR ALPHA	T cell receptor alpha	21473	3.51	other
Chemokine signaling				
CCR6	Chemokine (C-C motif)receptor6	12458	3.21	other
CCR7	Chemokine (C-C motif)receptor7	12775	3.93	other
CXCR4	Chemokine (C-X-C motif) receptor 4	12767	2.56	G-protein coupled receptor
PRKCB1	Protein kinase C, β 1	18751	2.81	Kinase
LTB	lymphotoxin B	16994	3.91	Other
Tnfrsf13c	tumor necrosis factor receptor superfamily, member 13c	72049	3.5	Other
IL-2 /IL-4 signaling				
IL2RG	Interleukin 2 receptor, gamma	16186	2.53	Transmembrane receptor
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	18707	2.32	Kinase
PTPN6	Protein tyrosine phosphatase, non-receptor type 6	15170	2.42	Phosphatase
LCK	Lymphocyte-specific protein tyrosine kinase	16818	3.37	Kinase
JAK/STAT signalling				
STAT 1	Signal transducer and activator of transcription 1	20846	2.02	Transcription regulator
STAT4	Signal transducer and activator of transcription	20849	2.51	Transcription regulator
PTPN6	Protein tyrosine phosphatase, non-receptor type 6	15170	2.42	Phophatase
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	18707	2.32	Kinase
CBLB	Casitas B-lineage lymphoma b	208650	1.78	Other
IL7R	interleukin 7 receptor	16197	2.29	Transmembrane receptor
Toll-like receptor signalling				

Cxcl10	chemokine (C-X-C motif) ligand 10	15945	2.82	Other
Ccl5	Chemokine (C-C motif) ligand 5	20304	2.81	Other
STAT 1	Signal transducer and activator of transcription 1	20846	2.02	Transcription regulator
Wnt signaling				
Camk2b	Calcium/calmodulin-dependent protein kinase II, β	12323	2.57	Kinase
Lef1	lymphoid enhancer binding factor 1	16842	3.03	other
Tcf7	transcription factor 7, T-cell specific	21414	2.91	Transcription regulator

Table 5.3 Selected genes down-regulated in the mesenteric fat depot more than 1.5-fold.

Selection criteria were based on the analysis of most significant pathways by the WebGestalt and Ingenuity Programs. Fold change represents the ratio of mean mRNA level between HF fed 11β-HSD1-/- and C57Bl/6J.

5.3.6 Genes up-regulated in the mesenteric fat depot of HF fed 11β-HSD1-/- mice.

The smaller set of genes up-regulated in the mesenteric fat depot of 11β-HSD1-/- mice included regulators of sarco-endoplasmic reticulum Ca^{2+}-ATPase activity (phospholamban 2.25-fold, Kcnk2 2.63-fold), retinol binding protein-transthyretin (2.3-fold), neurotransmitters: VIP (2-fold) and tachykinin (2-fold), and cell surface receptors: GNAO1, Htr2b, GPR85, CAP2, TAC1, GALR2.

5.3.7 Data validation

Overall there was a high level of congruency between micro-array and qRT-PCR measurement of transcript levels (Fig. 5.4.). 8 selected differentially expressed genes with highest fold-changes were found to be significantly regulated in qRT-PCR (Fig 5.4.). Leptin and PPARγ, showed a similar expression pattern to that measured previously by northern blot (Morton *et al.*, 2004b). However, leptin did not reach statistical significance when measured by qRT-PCR in the present study. GLUT4 was up-regulated 2-fold in the subcutaneous fat depot of 11β-HSD1-/-mice ($p<0.05$), in agreement with previous findings of higher basal and insulin-stimulated glucose uptake in isolated 11β-HSD1-/-adipocytes [3]. Similar to the micro-array data, PRKAA2 was approximately 2-fold up-regulated in both fat depots in 11β-HSD1-/- mice ($p<0.05$). Gene expression changes associated with lipid metabolism were confirmed, eg CPT1 (1.7-fold up-regulation) a lipid β-oxidation gene, which is also up-regulated in adipose tissue with 11β-HSD1 inhibition (London *et al.*, 2007), whereas FABP3 presented the same trend. HSP6 was 1.8-fold up-regulated in the subcutaneous fat ($p<0.05$). Down-regulated genes STAT4, CD8 and MARCO in the mesenteric adipose tissue of 11β-HSD1-/- mice showed the same differential expression (Fig 5.4.). However, IL7R and selectin, which showed 3-fold down-regulation in the micro-array experiment did not reach statistical significance in qRT-PCR.

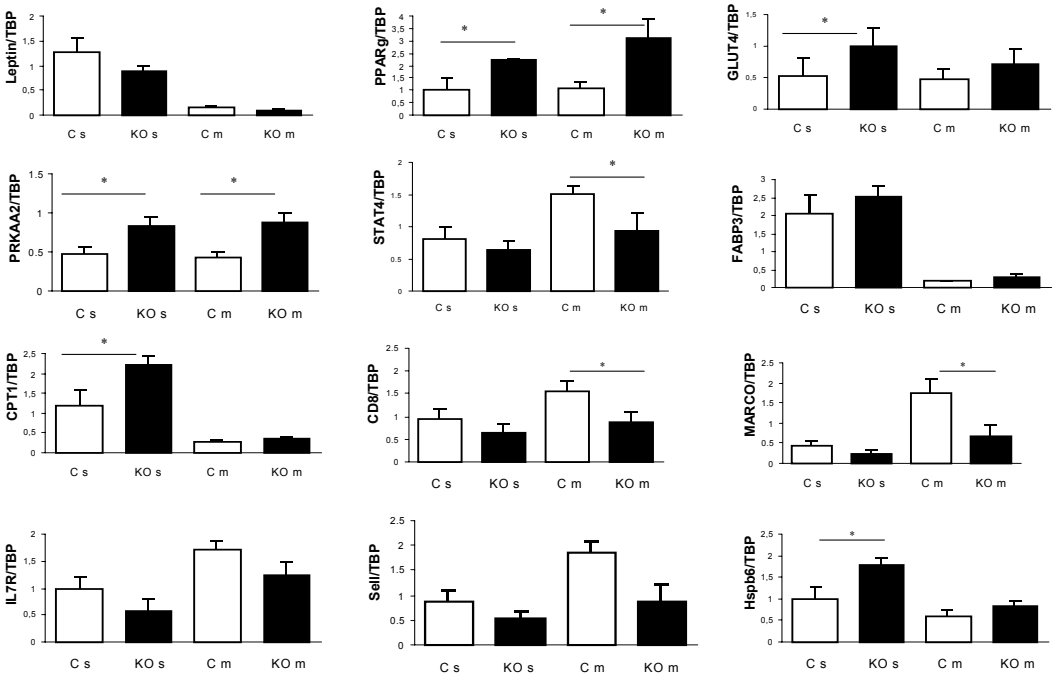

Figure 5.4 qRT-PCR validation of micro-array data.

11β-HSD1-/- (black bars) and their congenic C57Bl6/J control mice (open bars) were HF-fed for 4 weeks. mRNA expression levels were measured in two fat depots: subcutaneous (s) and mesenteric (m). Data are presented as a mean ± SEM of two independent HF diet studies expressed in arbitrary units (ratio of mRNA expression level of a gene of interest divided by an internal control-TBP), total n number for quantification was 7-10. Statistical analysis was performed by Student's t-test (*$p<0.05$).

5.3.8 Insulin signaling *in vivo*.

To determine whether up-regulation of the insulin signaling pathway observed in the genomic analysis led to improved insulin sensitivity in 11β-HSD1-/- subcutaneous fat, *in vivo* insulin sensitivity was measured in HF fed 11β-HSD1-/- and control C57Bl6/J mice. Total IRS-1, PI3K and AKT proteins did not differ in the liver, subcutaneous and mesenteric adipose tissue between genotypes (Fig 5.5.). There was a progressive reduction in insulin-stimulated AKT tyrosine phosphorylation in the liver in both genotypes on a high fat diet (Fig.5.5A). However, the decrease was less pronounced in the 11β-HSD1-/- mice when compared to controls (Fig 5.5A). Similarly, in subcutaneous fat, insulin-stimulated phosphorylation of AKT and IRS-1 was decreased in HF-fed control mice (Fig 5.5C and D). However, 11β-HSD1-/- mice did not show a reduction in the AKT phosphorylation level indicating preservation of insulin signaling (Fig 5.5C). IRS-1 showed similar trend (Fig 5.5D). Neither phosphorylation levels of IRS-1 nor AKT were regulated in control and 11β-HSD1-/- mice on HF diet (Fig 5.5B).

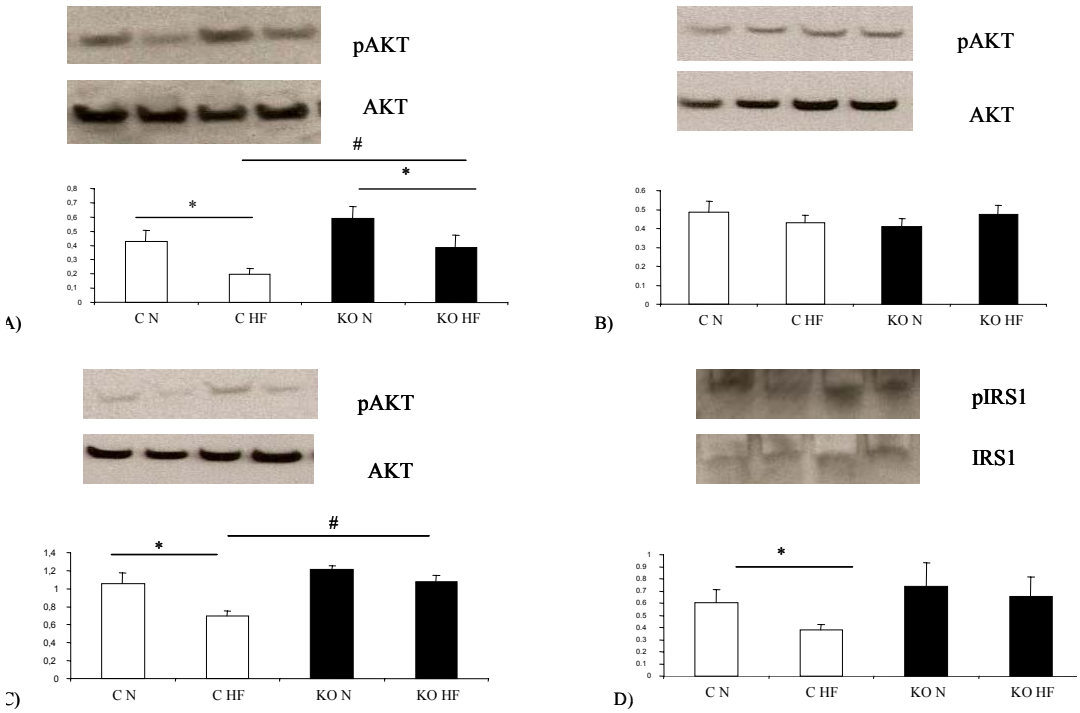

Figure 5.5 Insulin signaling (15min stimulation) in the liver and two fat depots of control and 11β-HSD1-/- mice on normal chow and HF diet.

A) HF diet decreases insulin-stimulated Akt phosphorylation in the liver of HF-fed 11β-HSD1-/- and C57Bl/6J mice. However, the effect is less pronounced in the 11β-HSD1-/- mice. B) No regulation of Akt phosphorylation was found in mesenteric fat depot of HF-fed 11β-HSD1-/- and control mice. C) Enhanced insulin-stimulated phosphorylation of Akt in the subcutaneous fat of HF-fed 11β-HSD1 mice. D) Enhanced insulin-stimulated phosphorylation of IRS1 in the subcutaneous fat of HF-fed 11β-HSD1 mice. Data are means ± SEM of two independent experiments, each performed on n=5 per group. Representative Western blots are shown with the quantification (n=10) of phosphorylated protein relative to total level of protein measured (Akt or IRS1). Open and black bars represent C57Bl/6J (control) and 11β-HSD1-/- mice, respectively. Symbol N and HF represent normal chow diet and high fat diet, respectively. *$p<0.05$ vs control as assessed by Student's t-test.

5.3.9 Adipocyte cell size

It has been demonstrated recently that chronic activation of β-adrenergic signaling stimulated cellular plasticity and remodelling of white adipose tissue (Himms-Hagen *et al.*, 2000, Granneman *et al.*, 2003). The appearance of small multilocular adipocytes with induced mitochondrial biogenesis and oxidation of fatty acids resembling brown fat cells could explain the anti-diabetic and anti-obesity effect of β3-adrenergic agonists (Granneman *et al.*, 2005). The micro-array analysis suggested an increased β3-adrenergic signaling in the subcutaneous fat of 11β-HSD1-/- mice. I investigated whether this may be associated with the smaller cell sizes in this peripheral fat depot. 10 weeks of high fat diet caused increased fat mass in both geneotypes, with a greater increase in C57BL/6J mice. However, hypertrophy of adipocytes occurred only in subcutaneous fat depot of C57Bl6/J mice but not 11β-HSD1-/- mice (Fig. 5.6.). No differences in adipocyte cell size were detected between genotypes in mesenteric adipose tissue (Fig. 5.6.). This along with higher CPT1 and FABP3 suggests that the subcutaneous fat tissue of 11β-HSD1-/- mice is composed of smaller adipocytes with maintenance of higher local metabolic efficiency upon HF feeding.

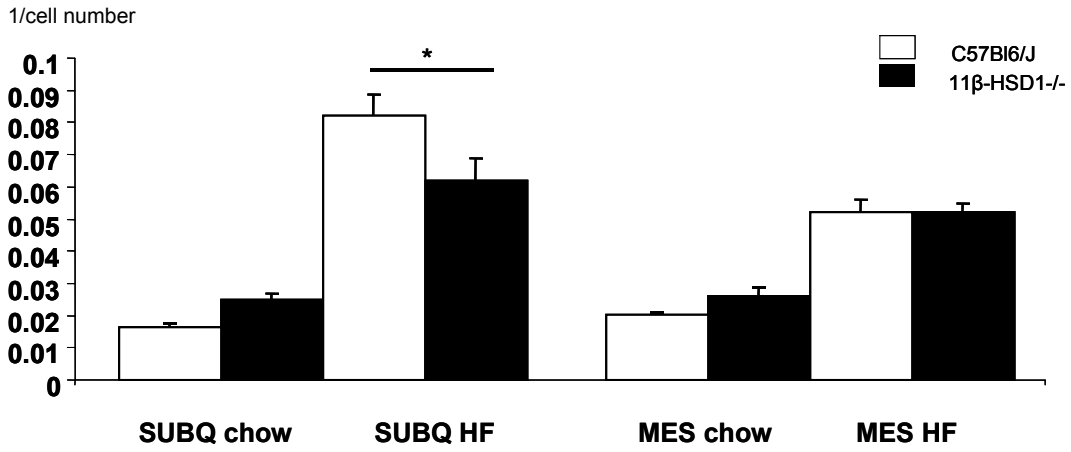

Figure 5.6 Cell sizes in subcutaneous and mesenteric adipose tissue of 11β-HSD1-/- and C57Bl6/J mice on chow and 10 weeks high fat (HF) diet.

A count of cell number within 25 randomly selected (by computer) areas on H&E stained slides of n=5-6 from each group. Y axis represents 1/cell number, *$p<0.05$ three-way ANOVA (genotype, diet and depot) with Holm-Sidak method post tests ($F=10.22$ for the analysis of genotype, $F=0.381$ for the analysis of fat depots, $F=213.9$ for the analysis of diet types). Significant effect of diet was observed in both fat depots and genotypes but with more pronounced effect in C57Bl6/J mice. There was no difference in cell sizes in mesenteric fat depot between genotypes after 10 weeks HF diet. Subq=subcutaneous fat depot, mes=mesenteric fat depot.

5.4 Discussion

Here I describe the global genomic analysis of 11β-HSD1 deficiency in two contrasting fat depots, which provide novel insights into how therapeutic inhibition of 11β-HSD1 may produce the metabolically "protective" phenotype seen in 11β-HSD1-/- mice. Combining genomic analysis with previously described physiological characteristics of the model it is reasonable to speculate about the implication in the aetiopathology of glucocorticoid-induced metabolic syndrome.

My data suggest that the metabolically "protective" phenotype of 11β-HSD1-/- mice derives from the following novel adipose depot specific mechanisms: 1. Increased accumulation of fat in "safer" peripheral depots through a combination of increased insulin sensitivity and substrate flux and 2. Reduced mesenteric fat mass, in part due to a reduction in the infiltration and activation of immune and inflammatory cells and their signaling.

Cushing's syndrome is characterized by the accumulation of fat predominantly in the metabolically disadvantageous intra-abdominal depot and a reduction of peripheral fat (Mayo-Smith *et al.*, 1989). 11β-HSD1 over-expression selectively in adipose tissue triggers visceral obesity and an associated metabolic syndrome in mice (Masuzaki *et al.*, 2001). Conversely, on a high fat diet, 11β-HSD1-/- mice preferentially gain adipose tissue in peripheral rather than visceral depots and are protected from the cardio-metabolic consequences of the diet (Morton *et al.*, 2004b). Here I report that 11β-HSD1-/- mice show lower body weight, fasting glucose and insulin levels during the early stages of obesity (4 weeks of high fat). Importantly, these mice have decreased mesenteric but not subcutaneous fat depots at this stage and thus offer insight into the early molecular mechanisms governing fat redistribution, function and metabolic protection with 11β-HSD1 deficiency.

The data suggest deficiency of glucocorticoids enhances basal insulin signaling, an effect that becomes more pronounced with HF diet. The genomic comparison between mesenteric and subcutaneous adipose tissue highlighted the up-regulation of genes functionally related to lipid oxidation and glucose uptake specifically in the

subcutaneous fat of 11β-HSD1-/- mice. An increase in PI3K/Akt and the MAPK pathway further supported the preserved insulin sensitivity of this fat depot. Concomitantly, there was a marked elevation of GLUT4 mRNA measured by qRT-PCR in this depot. Analysis of the phosphorylation status of IRS-1 and Akt (downstream targets of the insulin receptor) confirmed maintained insulin sensitivity in the subcutaneous fat of 11β-HSD1-/- mice in face of HF feeding. Although the mesenteric fat has been mostly related to the detrimental consequences of obesity (Kissebah and Krakower, 1994) and is more sensitive to glucocorticoids (Masuzaki *et al.*, 2001), it did not appear to display differences in insulin sensitivity when compared with the control mice.

Thus, sustained insulin signaling likely plays a crucial role in the regulation of appropriate lipid storage preferentially in the peripheral fat in the 11β-HSD1-/- mouse model. The inability to appropriately expand the subcutaneous adipose tissue has been suggested to be a crucial factor linking the calorie excess with the insulin resistance and diabetes type 2 (Kim *et al.*, 2007). An increase storage of lipids in peripheral depots may protect from the redistribution of triglycerides to the liver and the muscle when food is in excess. Increased storage through insulin sensitization is offset by increased metabolism via β-oxidation, and hence subcutaneous cell size does not increase in the 11β-HSD1 deficient adipocytes. Hence, the observations reported here support the notion that enhancing subcutaneous adipose tissue proliferation and its capacity for lipid retention leads to protection from metabolically detrimental consequences of HF diet. Parallel observations have been described in a model of chronic over-expression of insulin-sensitizing adipose cell protein, adiponectin (Kim *et al.*, 2007) and in the several studies investigating the insulin sensitizing effect of the treatment with PPARγ agonists (Adams *et al.*, 1997, Yamauchi *et al.*, 2001).

The global gene expression analysis revealed changes in genes involved in tissue morphology, cellular development and organization in the subcutaneous fat of 11β-HSD1-/- mice, presumably reflecting changes in the proliferation and differentiation of adipocytes. This suggests that insulin sensitivity of subcutaneous adipocytes is

accompanied by morphological remodeling. Indeed it has been shown previously that mesenteric adipose tissue of 11β-HSD1-/- shows reduced differentiation in response to chronic HF feeding *in vivo* (De Sousa Peixoto *et al.*, 2008) and the data on adipocyte size demonstrate that subcutaneous adipocytes are smaller in 11β-HSD1-/- mice, whereas mesenteric fat adipocyte size does not change.

Another intriguing finding was a marked increase in genes functionally related to calcium signaling specifically in the subcutaneous adipose tissue. Intracellular calcium content modulates lipid metabolism and adipogenesis. It also contributes to the phosphorylation cascade of insulin signaling (Kruger *et al.*, 2008). Ultimately, several genes involved in the β-adrenergic signaling were also up-regulated in 11β-HSD1-/- subcutaneous adipose tissue. Lipid storage in the adipose tissue is under the control of insulin and the actions of catecholamines, which act through α and β-adrenergic receptors. Chronic β-adrenergic activation has been shown to be an effective anti-obesity and anti-diabetes strategy in rodents (de Souza *et al.*, 1997). Moreover, β-adrenergic stimulation activates lipolysis and drives expression of genes controlling oxidative metabolism and thermogenesis in adipose tissue (Granneman *et al.*, 2005) and might reflect the process of acquiring features of brown adipocytes by white fat cells (Gesta *et al.*, 2007). These adipocytes are smaller, more metabolically active and converted towards cells with high fatty acid oxidation capacity. Glucocorticoids decrease thermogenesis (Strack *et al.*, 1995) and inhibit adrenergic stimulation of uncoupling proteins (Soumano *et al.*, 2000). Hence, it is not surprising that 11β-HSD1-/- mice might be protected from glucocorticoid-mediated inhibition of β-adrenergic signaling (Morton *et al.*, 2004b). Further, the analysis of gene expression in the subcutaneous depot of 11β-HSD1-/- mice revealed high expression of the nuclear-encoded mitochondrial genes involved in glycolysis, tricarboxylic acid cycle, β-oxidation and oxidative phosphorylation. These changes in gene expression were associated with increased expression of PPARγ and CPT1 in qRT-PCR. This could explain an interesting dichotomy. 11β-HSD1-/- mice are leaner and have improved metabolic parameters on high fat diet despite increased food intake (Densmore *et al.*, 2006, Morton *et al.*, 2004b). At the whole body level, mice over-expressing 11β-HSD2 in the adipose tissue (parallel to 11β-HSD1-/- mice)

demonstrated an increase in basal metabolic rate and respiratory exchange ratio (Kershaw *et al.*, 2005). Although these finding could represent the transformation of white adipocytes to brown fat cells or more likely an increased differentiation of pluripotent stem cells of stromovascular fraction towards brown adipocytes, the mRNA encoding key brown adipose tissue markers UCP-1 and β3-adrenergic receptors were unchanged (in micro-array study and in qRT-PCR data validation). Another plausible explanation would be that increased PPARγ enhances lipid oxidation (Bogacka *et al.*, 2005). Thiazolidinediones down-regulate 11β-HSD1 in adipose tissue (Mai *et al.*, 2007, Berger *et al.*, 2001) and the possible cross-talk between PPARγ and 11β-HSD1 downstream pathways may be important in the pathogenesis of obesity-induced metabolic syndrome. Thus, the lack of glucocorticoid amplification by genetic ablation of 11β-HSD1 in this murine model might accelerate the effect of activated PPARγ. It is noteworthy that, thiazolidinediones and PGC-1α stimulate the transformation of adipocytes in cells with a high lipid oxidative profile (Tiraby *et al.*, 2003).

Ultimately, along with an increase in β-adrenergic signaling, oxidative phosphorylation and glucose metabolism, the highlighted changes support the hypothesis that adipocytes in subcutaneous fat of 11β-HSD1-/- mice are smaller, more insulin sensitive and more metabolically active in relation to the mesenteric (visceral) fat depots.

Consistent with an increase in genes involved in glucose uptake and lipid oxidation in the subcutaneous fat of 11β-HSD1-/- mice, I also detected an enhancement in mRNA level of AMP-kinase alpha2 subunit (PRKAA2). AMPK, a target for the anti-diabetogenic drug metformin and PPARγ agonists in the adipose tissue (Zhou *et al.*, 2001, Fryer *et al.*, 2002), is likely to be responsible for increased lipid oxidation and protection from redistribution of lipids in liver and muscle. Glucocorticoid treatment inhibits AMPK activity in the visceral adipose tissue but not in the subcutaneous fat, and this has been suggested to underlie lipid deposition in intra-abdominal depots in Cushing's syndrome (Christ-Crain *et al.*, 2008). In 11β-HSD1-/- mice model I

observed an increased mRNA of AMPK in both fat depots. Thus, its contribution to the fat redistribution in our model remains unclear.

Increasing evidence suggests the role of low-grade inflammation in the pathogenesis of obesity (Hotamisligil, 2006, Shoelson *et al.*, 2007). The link between innate immunity and insulin resistance has been supported by the crucial role of cytokines and chemokines (TNFalpha, IL6, MCP-1) produced by adipocytes and/or immune cells in the in the development of insulin resistance (Tilg and Moschen, 2008). Gene expression analysis revealed down-regulation of numerous genes associated with immune and inflammatory responses in the mesenteric fat of 11β-HSD1-/- mice suggesting decreased infiltration of T cells in the mesenteric fat. It may reflect the protection of 11β-HSD1-/- mice from the chronic adipose tissue inflammation associated with obesity.

Furthermore, growing evidence suggests infiltration by T cells and other immuno-competent cells of adipose tissue in obesity (Herder *et al.*, 2005a, Wu *et al.*, 2007, Herder *et al.*, 2005b, Rausch *et al.*, 2007, Caspar-Bauguil *et al.*, 2006, Caspar-Bauguil *et al.*, 2005). Mesenteric fat of HF fed 11β-HSD1-/- mice revealed down-regulation of genes functionally involved in proliferation, differentiation, movement and adhesion of immune cells. There was an indication that mesenteric fat in the control mice had changes in tissue morphology by the presence of activated T cells. The most significantly regulated pathways were associated with chemokine and cytokine signaling. NF-κB and c-Jun NH_2-terminal kinase (JNK), two signaling pathways linking the immune response with the insulin resistance, which have recently attracted major attention (Shoelson *et al.*, 2007). These pathways were down-regulated in mesenteric fat of HF fed 11β-HSD1-/- mice (or rather not induced by high fat to the same extent as in C57BL/6J mice). Genetic disruption of these pathways improves insulin resistance (Hirosumi *et al.*, 2002, Cai *et al.*, 2005), suggesting a further basis for insulin sensitization in the 11β-HSD1-/- mice.

11β-HSD1 is also expressed in cells of stromo-vascular fraction, such as preadipocytes (De Sousa Peixoto *et al.*, 2008) and in activated macrophages (Ishii *et*

al., 2007, Gilmour *et al.*, 2006a) that are increased in adipose tissue with obesity (Weisberg *et al.*, 2003). Glucocorticoids are well known anti-inflammatory drugs, however, their physiological action may be distinct from their pharmacological properties and they are better described as "immunomodulatory" (Chapman *et al.*, 2006b). Interestingly, 11β-HSD1-/- mice have been described to be more sensitive to LPS-induced endotoxaemia (Zhang and Daynes, 2007). Thus, the role of 11β-HSD1 deficiency in immune cells and their contribution in the development of inflammatory response in obesity requires further studies.

CHAPTER 6

Summary, conclusions and implications for future studies

6 Summary, conclusions and implications for future studies

The rationale behind this thesis was based on the fact that 11β-HSD1 inhibitors are currently in development as a therapy for the metabolic syndrome and obesity-induced insulin resistance. A comprehensive understanding of what implications 11β-HSD1 deficiency has (as a model of therapeutic inhibition), particularly in adipose tissue, is crucial for the safety of that novel therapy. While focussed discussion has been included in relevant chapters, some points need to be addressed here as they have significant implications for further studies.

The intention of this study, from the outset, was to explore fat depot-specific effects of 11β-HSD1 deficiency on adipose tissue gene expression. 11β-HSD1 deficiency protects an intrinsically obesity-susceptible model from both obesity and its cardio-metabolic consequences. The recently reported novel role of hepatic 11β-HSD1 in the metabolism of alternative oxysterol substrates, in conjunction with a complete lack of studies describing the metabolism of these cholesterol derivatives in fat tissues prompted the investigations presented in the first part of the thesis. It is important to note that the biological interpretation of the genomic analysis was hampered until the involvement of oxysterols metabolised by the enzyme in regulating pathways downstream of 11β-HSD1 in the fat tissue could be excluded or defined. This meant that, initially my efforts were concentrated on the understanding whether 11β-HSD1 in adipocytes metabolises 7-oxysterols and further whether glucocorticoid and oxysterol substrate interact with each other and influence the expression of genes in adipose tissue.

6.1 7-oxysterols and glucocorticoids are competitive substrates for 11β-HSD1 in adipocytes.

The seminal work in our lab established that increased reactivation of glucocorticoids by 11β-HSD1 in human and rodent adipose tissue may underlie aspects of obesity and metabolic syndrome (Morton *et al.*, 2004b, Morton *et al.*, 2004c, Masuzaki *et al.*, 2001, Morton *et al.*, 2001). Here I proposed a novel hypothesis that the amplification of glucocorticoids in fat may be inhibited by oxysterols. This cross-talk between glucocorticoids and oxysterols could be particularly relevant when both substrates

are plentiful, as would be expected in obesity and dyslipidemia. Here I report for the first time that fully-differentiated 3T3-L1 and 3T3-F442A adipocytes metabolise atherogenic oxysterols and that this reaction is specific for 11β-HSD1. It is important to point out that none of the previously published studies investigating the metabolically protective effect of 11β-HSD1 inhibition took into account its role in converting 7-oxysterols. Furthermore, this thesis highlights the complex interactions between the atherogenic oxysterols and glucocorticoid signaling through GR that is dependent on 11β-HSD1. The model presented, and supported by the data, describe a novel link between obesity, atherogenesis and glucocorticoid action in adipose tissue.

Firstly, I showed that 7-oxysterols selectively accumulate and act as competitive substrates to glucocorticoids for 11β-HSD1 in adipocytes. Secondly, 7-oxysterols have a novel effect to modulate the activation of the glucocorticoid receptor and this is dependent on the availability of a glucocorticoid ligand. Lastly, acting through inhibition of 11β-HSD1, 7KC impaired the differentiation of preadipocytes, a glucocorticoid dependent process.

Several aspects requiring further analysis need to be addressed here. During the preliminary stage of method development for the conversion of 7-oxysterols by 11β-HSD1, I tested extraction of oxysterols from both serum-free and serum-enriched medium (as described in detail in chapter 2). I encountered difficulties in extracting oxysterols from serum-enriched medium (30% oxysterols recovery from medium enriched with serum compared with 50-70% from serum-free medium) and therefore chose to perform experiments in serum-free medium. This step was also dictated by the intention to simplify the model and exclude the influence of possible modifiers of oxysterol metabolism in the bovine serum. However, it has to be acknowledged that *in vivo* in the presence of serum lipoproteins, 7-oxysterols might undergo influx/efflux not seen in the absence of these "carriers". Recently, it has been reported that, selectively, ABCG1 expression is necessary to promote efflux of 7KC and 7β-HC from cells to HDL (Terasaka *et al.*, 2007). Thus, it has been hypothesised that restricted efflux of 7KC is dependent on ABCG1 and the presence of lipoproteins in the medium. This may explain high concentrations of 7-oxysterols in

atherosclerotic plaques (Terasaka *et al.*, 2007). Interestingly, ablation of ABCG1 in adipocytes has been shown to reduce obesity (Buchmann *et al.*, 2007).

6.2 The putative role of 11β-HSD1 in producing oxysterol ligands for yet unknown "orphan" nuclear receptors.

11β-HSD1 inhibition protects from atherosclerosis and metabolic consequences of obesity (Hermanowski-Vosatka *et al.*, 2005), yet it is unknown whether its role in the metabolism of 7-oxysterols might explain the molecular mechanism underpinning these effects. Given the previously reported inhibitory effect of LXR ligands upon 11β-HSD1 mRNA and activity (Stulnig *et al.*, 2002), I investigated whether 7-oxysterols may activate LXR and regulate LXR target genes. 7KC and 7β-HC did not activate LXRα in a transactivation assay, nor did they regulate mRNA expression of SREBP1c, GLUT4 and 11β-HSD1 in 3T3-F442A adipocytes. Additionally, I tested the regulation of key gene targets of LXRα, FXR/RXR and RORα in the liver and fat of HF fed 11β-HSD1-/- and wild type mice. No differential regulation was observed. Thus, it remains hypothetical that 7β-HC, the product of 11β-HSD1-dependent oxysterol conversion, or indeed the 7KC substrate, play a role in the pathogenesis of atherosclerosis through activation of an unknown "orphan" nuclear receptor. However, this thesis excludes important plausible candidates from this hypothetical mechanism of action.

Contrary to previously reported observations of the inhibitory role of LXR ligands upon 11β-HSD1 (Stulnig *et al.*, 2002), here, neither physiological nor pharmacological LXR ligands showed regulation of 11β-HSD1 mRNA. The discrepancy beween these finding is difficult to explain. Indeed despite various modification of the protocol for these experiments, included a longer times of incubation, more differentiated adipocytes with higher expression of the enzyme, and higher concentrations of ligands, in no case was 11β-HSD1 mRNA affected.

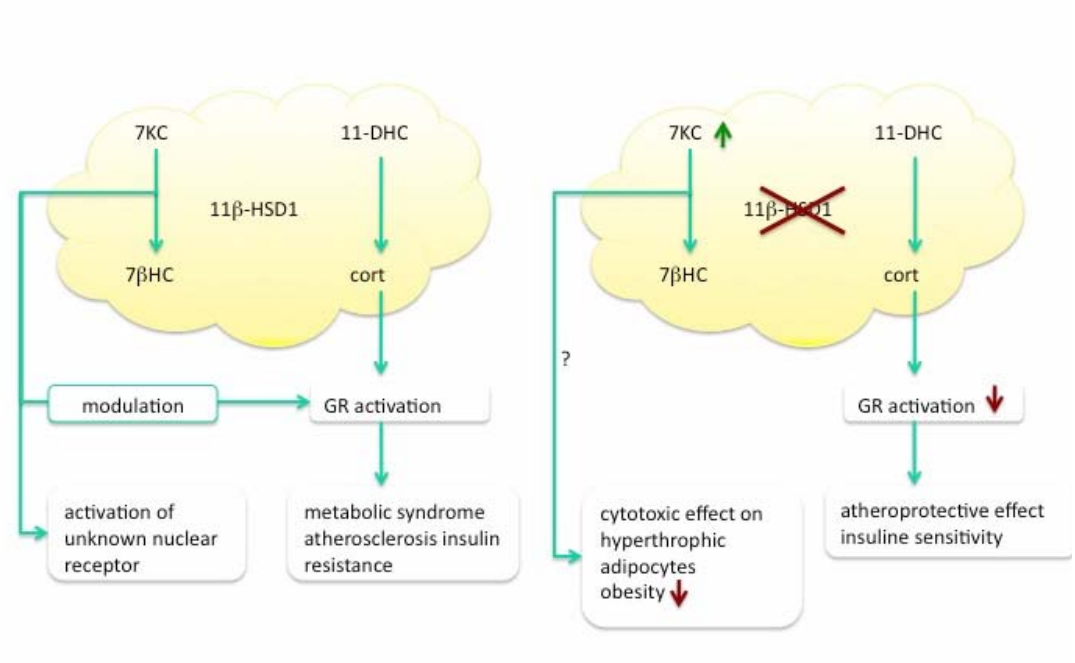

Figure.6.1 Hypothetical model describing a role of 11β-HSD1-dependent conversion of 7-oxysterols.

7-oxysterols modulate glucocorticoid induced GR activity. The inhibition of 11β-HSD1 may cause an increase of adipose level of 7KC. High concentrations of 7KC are cytotoxic and could potentially have toxic effect on hypertrophic adipocytes and subsequently ameliorate obesity.

6.3 Genomic analysis of fat depots uncovers novel mechanisms of the metabolically protective phenotype of 11β-HSD1-/- mice.

Having established the molecular mechanism underlying the cross-talk between glucocorticoid and oxysterol substrates of 11β-HSD1, I proceeded to the second major part of this study. Micro-array technology combined with bioinformatic data analysis strategies provide a chance to determine to what extent a manipulation of a single gene affects tissue gene expression globally.The short-term HF feeding study aimed to detect early changes in genes possessing the regulatory role in metabolic processes rather then to confirm the changes that had been already described on chronic HF diet regimens. The current trend in obesity research is to look for the central molecules that potentially affect enzymes activity and transcription factors involved in inflammation and metabolism. Consistent with this belief I made an attempt to investigate early, fat depot-specific effects of 11β-HSD1 deficiency on adipose tissue gene expression that might result in the protection from cardio-metabolic consequences of obesity. I aimed to describe what preceeds diet-induced obesity and subsequent metabolic disturbances. This approach was dictated by the assumption that previously described changes in genes reflect, at least in part, a consequence rather than a cause of diet–induced metabolic syndrome.

Transgenic overexpression of 11β-HSD1 in adipose tissue models the metabolic syndrome, whereas 11β-HSD1-/- mice resist visceral obesity and metabolic syndrome with high fat diet. To dissect the molecular mechanisms underpinning the beneficial physiology of these mice, a genomic analysis of mesenteric and subcutaneous fat depots of high fat fed 11β-HSD1-/- and control mice was performed. The data suggest that loss of 11β-HSD1 enhances lipid oxidation and glucose up-take in the peripheral fat depots. Up-regulation of genes of PI3K/Akt and the MAPK pathways further supported by increased phosphorylation of IRS1 and Akt proteins confirmed preserved insulin sensitivity in the subcutaneous fat of high fat fed 11β-HSD1-/- mice. Moreover, these changes in gene expression were associated with smaller sizes of adipocytes in “peripheral” (subcutaneous) adipose tissue indicating these adipocytes are metabolically more active than fat cells in the intra-abdominal depot.

However, the most intriguing finding was revealed by the analysis of gene clusters down-regulated in the mesenteric fat of 11β-HSD1-/- mice showing changes associated predominantly with immune and inflammatory cells. Thus, I hypothesised that infiltration of adipose tissue by activated T cells may be involved in the development of insulin resistance in the early phase of high fat diet-induced obesity. Changes in NF-κB signaling, Jak/Stat signaling and SAPK/JNK signaling suggested further that down-regulation of these pathways may underpin resistance of 11β-HSD1-/- mice for the development detrimental consequences of high fat feeding. However, these observations clearly require further functional studies.

6.4 Future work

6.4.1 Measurment of levels of 7-oxysterols in adipose tissue *in vivo*.

The estimation of oxysterol levels in the adipose tissue has never been described. Quantitation of oxysterols is associated with several methodological problems (eg recovery of extraction, conditions of saponifications, artifactual generation of oxysterols through autoxidation of cholesterol). Studies have indicated that oxysterols occur in fresh normal human plasma at levels of 0.01-0.1μM (Schroepfer, 2000). The levels measured in liver tissues were estimated for 0.4μg/g tissue of 7KC and 5μg/g tissue 7β-HC (Saucier *et al.*, 1989). Lund et al (Lund *et al.*, 1992) reported the similar findings for 7KC but lower levels of 7β-HC (0.12μg/g). The values increased significantly when animals were fed high cholesterol diet. It will be crucial to establish reliable methodology allowing measurement of levels of 7KC and 7β-HC in adipose tissue. The use of 11β-HSD1-/- and adipose tissue 11β-HSD2 overexpressor mice will be beneficial to distinguish between the contribution of glucocorticoids and oxysterols to the development of metabolically protective features of these mice models.

6.4.2 Investigation of the role of 7β-HC in cholesterol metabolism.

Based on the current knowledge it is impossible to distinguish between the effect of 7KC and 7β-HC (Ferderbar *et al.*, 2007, Lemaire-Ewing *et al.*, 2005). Increased

concentration of both oxysterols has been correlated with dyslipidemia and atherosclerosis. In this thesis I investigated the potential of 7-oxysterols to act as ligands for LXRα, FXR/RXR and RORα receptors getting consistently negative results. It remains plausible that 7β-HC may activate another, as yet unidentified, nuclear receptor involved in the regulation of lipid and cholesterol metabolism and implicated in the atherosclerosis. This requires further studies. Alternatively, not receptor mediated effect of 7-oxysterols on cellular membrane function may be involved.

Another potentially interesting aspect will be the cytotoxic effect of the accumulation of 7KC in hypertrophic adipocytes. Growing adipocyte increases the intake of cholesterol and its oxysterol metabolites to the level when it crystalises and become highly cytotoxic. One could predict it will contribute to initiation of local inflammation and infiltration of fat tissue by macrophages. This might explain the link between increased risk of atherosclerosis in obesity.

6.4.3 Infiltration of mesenteric fat by activated T cells and their contribution for the initiation of obesity-induced insulin resistance.

It may be hypothesised that infiltration of mesenteric adipose tissue with immune cells (activated T cells) may trigger the initiation of chronic inflammation in the fat tissue and its subsequent infiltration by macrophages. Studies investigating the infiltration of fat by T cells will involve immunohistochemistry and flow cytometry to distinguish between 11β-HSD1-/- and wild type mice and then to establish specific antigen markers of these immune cells. Importantly, the data indicate that the role of T cells in the development of insulin resistance should be studied.

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