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11β-hydroxysteroid dehydrogenase type 1 as a pharmacological target in metabolic disease

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ABSTRACT

The **metabolic syndrome** is a collection of associated disorders, affected by lifestyle, genetic predisposition and environment, and emerges as a consequence of the Western society lifestyle. It describes a variety of cardiovascular and atherosclerotic risk factors including insulin resistance, obesity, dyslipidemia and arterial hypertension. The connection between insulin resistance and **diabetes mellitus type 2** has been well established, and the major abnormalities are peripheral insulin resistance, β-cell dysfunction and increased endogenous glucose production. **Glucocorticoids** have received considerable interest among the many factors that play a role in the metabolic syndrome. Excess glucocorticoid levels cause glucose intolerance and insulin resistance, as demonstrated by the clinical picture of Cushing's syndrome, which closely resembles the metabolic syndrome. A further understanding of the disease mechanisms behind the metabolic syndrome is essential to diminish its increasing impact on health of the Western population. Novel pharmacological treatment strategies based on modulation of glucocorticoid function is a feasible approach to combat this metabolic disease.

Glucocorticoids are steroid hormones that belong to the superfamily of ligand-activated nuclear receptors. An emerging concept describes tissue-specific metabolic reactions that many hormones undergo, including steroids, resulting in a pre-receptor control mechanism. The OH/keto group on the C11-position in glucocorticoids determines if the steroid can activate its receptor or is 'inert'. The enzyme responsible for this conversion of glucocorticoids is **11β-hydroxysteroid dehydrogenase** (11β-HSD). Two different isozymes of 11β-HSD (11β-HSD1 and 11β-HSD2) are described. Their functions are to activate and inactivate glucocorticoids in a tissue-dependent manner. 11β-HSD1 mediates activation of the glucocorticoid precursor cortisone (in humans) to the active glucocorticoid receptor ligand cortisol. It is widely expressed in central and peripheral tissues involved in glucose and carbohydrate metabolism, including liver and adipose tissue. Because of the beneficial effects of reduced tissue glucocorticoid levels in the metabolic syndrome and related disorders, 11β-HSD1 is a pursued target of pharmacological intervention.

The aims of the study were to investigate structure-function relationships and functional effects of 11β-HSD1. The results show that the hydrophobic enzyme 11β-HSD1 can be expressed with high activity as a full length, membrane bound enzyme in the yeast system *Pichia pastoris* and can be purified as a soluble, N-terminally truncated form

expressed in *E.coli,* by using metal-chelate chromatography. The full-length and truncated enzymes have equivalent enzymatic properties in regard to glucocorticoid metabolism. 11β-HSD1 orthologs from human, rat, mouse and guinea pig show considerable inter-species variations as inferred by primary structure determinations and inhibitor characterization. A 11β-HSD1 selective arylsulfonamidothiazole inhibitor class was investigated and is currently developed as a promising tool for the treatment of insulin resistance. Several derivatives were analyzed and show a high degree of species selectivity, with different inhibitor mechanisms and low nM affinities towards the human enzyme.

11β-HSD1 mediates glucocorticoid-activation in pancreatic islets of Langerhans, and thereby regulates glucose-stimulated insulin secretion. Whereas glucocorticoids suppress insulin secretion in the insulin resistant ob/ob mouse model, they increase insulin release in lean mice. Hence it is postulated that the known beneficial effects of 11β-HSD1-inhibition in the pharmacological treatment of diabetes mellitus can be extended to include improved insulin release in diabetic mice. Glucocorticoid-activation in lean mice may at an early phase lead to increased insulin secretion and priming of the β-cells to stress-adaptation, whereas long-term exposure leads to a decrease in insulin secretion.

A novel role of 11β-HSD1 in 7-oxosterol metabolism was discovered and investigated using recombinant 11β-HSD1 orthologs. The enzymatic origin of endogenous 7β-OH-cholesterol in humans is assigned to 11β-HSD1, possibly pointing to an involvement in atherosclerosis. Species differences in 7-oxysterol metabolism can be explained on the basis of 11β-HSD1 specificities.

LIST OF PUBLICATIONS

I. **Hult, M**., Jörnvall H., and Oppermann, U. Selective inhibition of human type 1 11beta-hydroxysteroid dehydrogenase by synthetic steroids and xenobiotics. FEBS Lett, 1998, 441(1): 25-28.

II. **Hult, M**., Shafqat, N., Elleby, B., Mitschke, D., Svensson, S., Forsgren, M., Barf, T., Vallgårda, J., Abrahmsen L. and Oppermann, U. Active site variability of type 1 11β-hydroxysteroid dehydrogenase revealed by selective inhibitors and cross-species comparisons. Manuscript.

III. Davani, B., Khan, A., **Hult, M**., Martensson, E., Okret, S., Efendic, S., Jornvall H., and Oppermann, U. Type 1 11beta -hydroxysteroid dehydrogenase mediates glucocorticoid activation and insulin release in pancreatic islets. J Biol Chem, 2000, 275(45): 34841-34844.

IV. **Hult, M.**, Ortsäter, H., Schuster, G., Graedler, F., Ploner, A., Adamski, J., Jörnvall, H., Bergsten, P. and Oppermann, U. Glucocorticoids increase insulin secretion in lean mice through multiple pathways and mechanisms. Manuscript.

V. **Hult, M.** , Elleby, B., Shafqat, N., Svensson, S., Rane, A., Jörnvall, H., Abrahmsen L., and Oppermann, U. Human and rodent type 1 11β-hydroxysteroid dehydrogenases are 7β-hydroxycholesterol dehydrogenases involved in oxysterol metabolism. Cell Mol Life Sci, 2004, 61: 992-999.

CONTENTS

ABBREVIATIONS

11β-HSD	11β-hydroxysteroid dehydrogenase
cDNA	complementary deoxyribonucleic acid
CL	cortisol
CN	cortisone
DHC	dehydrocorticosterone
GC	glucocorticoid
HPLC	high performance liquid chromatography
mRNA	messenger ribonucleic acid
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidized/reduced)
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
NIDDM	non-insulin dependent diabetes mellitus; diabetes mellitus type 2
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDR	short-chain dehydrogenase/reductase
SDS-PAGE	sodium dodecyl sulfate-polyacryl amide gel electrophoresis

1 INTRODUCTION

'*Obesity is becoming one of the most important contributors to ill health'* is a quote from the WHO World Health Report 1998 [1]. **Obesity** is commonly defined in adults as a Body Mass Index (BMI) over 30 kg/m^2. The average prevalence of obesity is around 15 % in the Western world, and the number of obese as well as the number of type 2 diabetics are expected to double to a total of 300 million people in 2025 [1, 2], with vast consequences for public health and costs of maintaining life quality. This increase will occur to the major part in developing countries. By 2025, more than 75% of the diabetics will reside in developing countries. The countries with the largest number of people with diabetes are India, China, and the U.S.

Central obesity (intra-abdominal fat accumulation) is commonly associated with hypertension, dyslipidemia, insulin resistance, diabetes and premature death from cardiovascular disease [3]. Both obesity and type 2 diabetes are consequences of a sedentary lifestyle and increased energy density in the diet. Around 70% of the diabetic cases in white people could be avoided if the whole population would not exceed a BMI of 25 kg/m^2 [4].

1.1 The metabolic syndrome

The **metabolic syndrome** (syndrome X; Reaven's syndrome) [5, 6] is a collection of associated disorders, affected by lifestyle, genetic predisposition and environment, and emerges as a consequence of the Western society lifestyle. It describes a variety of cardiovascular and atherosclerotic risk factors including insulin resistance, obesity, dyslipidemia and arterial hypertension [6]. **Insulin resistance** is defined as a defect in the ability of insulin to drive glucose uptake and metabolic reactions in liver, skeletal muscle and adipocytes [7]. The connection between insulin resistance and **diabetes mellitus type 2** (non-insulin dependent diabetes, NIDDM) has been well established and the major abnormalities in NIDDM are peripheral insulin resistance, β-cell dysfunction and increased endogenous glucose production, although the precise mechanisms are still under debate. The development of NIDDM can be divided into three stages [8]: subjects in stage I have elevated triglyceride levels, reduced high-density lipoprotein levels as well as an increased waist to hip ratio, indicating an essential role of lipid and lipoprotein metabolism on glucose uptake and utilization. However, these individuals show a normal glucose tolerance response due to increased insulin secretion from the pancreatic β-cells. In stage II, glucose tolerance is impaired after an oral glucose load due to insufficient insulin secretion. Stage III is the fully

developed type 2 diabetes, which is diagnosed when the metabolic disturbances of insulin resistance and β-cell dysfunction results in fasting plasma glucose levels above 7 mM and/or 11 mM 120 minutes after a 75 g glucose load [9].

The **glucocorticoid** steroid hormones were named based on their effects on carbohydrate metabolism [10]. They have received considerable interest among the many factors that play a role in the metabolic syndrome. Excess glucocorticoid levels cause glucose intolerance and insulin resistance, as demonstrated by the clinical picture of **Cushing's syndrome**. Cushing's syndrome has been shown to be a reversible cause of the metabolic syndrome [11], and there are several common clinical features between the two, including obesity, hypertension, impaired glucose tolerance, hyperlipidemia, hirsutism, acne and gonadal dysfunction [12]. A difference appears to be that Cushing's syndrome arises from glucocorticoid excess in the systemic circulation, whereas glucocorticoid plasma levels in the metabolic syndrome are apparently normal.

A further understanding of the disease mechanisms behind the metabolic syndrome is essential to diminish its increasing impact on Western society. Novel pharmacological treatment strategies based on modulation of glucocorticoid function are a feasible approach to combat this metabolic disease, as further outlined below.

1.2 Insulin and glucocorticoid regulation

Plasma glucose is tightly regulated in a range between 4 and 7 mM in healthy individuals. This control is balanced by glucose absorption from the intestine, production by the liver and uptake by peripheral tissues. Insulin, which is released from the pancreatic β-cell in response to elevated plasma glucose (glucose-stimulated insulin secretion), increases glucose uptake in muscle and fat, and inhibits hepatic glucose production. Insulin also promotes the production and storage of carbohydrates, lipids and proteins in fat, liver and muscle. Insulin resistance or deficiency results in dysregulation of these processes, and results in elevated glucose and lipid levels. Many glucocorticoid effects directly oppose the effects of insulin, thereby inducing insulin resistance. For example, glucocorticoids impair insulin-dependent glucose uptake in the peripheral tissues, enhance glucose production in the liver [13, 14], and inhibit insulin secretion from pancreatic β-cells [15, 16].

1.3 Role of oxysterols in metabolic disease

Complications of atherosclerosis, such as coronary heart disease and stroke, are the most common causes of death in the Western populations. The key event in early atherosclerosis is damage to the endothelium of vascular walls, which may be caused by several risk factors,

like oxidized lipoproteins, hypertension, or cigarette smoking. Atherosclerosis is a form of chronic inflammation, which results from interactions between lipids, macrophages and the vascular wall. The process can finally lead to plaque formation in the lumen of the artery, and a subsequent thrombosis may lead to myocardial infarction and stroke [17, 18]. In humans, elevated levels of serum cholesterol can alone induce atherosclerosis, but other risk factors include hypertension, diabetes mellitus, obesity, as well as high fat diet and lack of exercise.
Low-density lipoprotein (LDL) is associated with increased risk of cardiovascular disease. Oxidized LDL is composed of many pro-atherogenic molecules including oxysterols, which are a class of oxygenated cholesterol products with a wide spectrum of biological activities. The oxysterols serve as intermediates in the bile acid synthesis pathways and as ligands for nuclear receptors or oxysterol binding proteins, and thereby regulate essential pathways e.g. in bile acid synthesis, fatty acid synthesis, cholesterol transport and carbohydrate metabolism [19]. These compounds are formed either in enzymatic or non-enzymatic autoxidation reactions [19], occurring at different positions of the cholesterol molecule. 7-keto cholesterol is quantitatively among the most important oxysterol compounds found in human plasma. [19-21]. It is relatively highly represented in oxidized LDL as well as in atherosclerotic plaque and macrophage foam cells ([22], and reviewed in [23]). Björkhem et. al. reported the presence of 27-OH-cholesterol and 7-oxysterols as the major oxysterols in human atherosclerotic arteries [24]. In 1997, Brown et. al. analysed human atherosclerotic plaques also showing that the major oxysterol was 26-OH-cholesterol followed by 7-oxo-cholesterol. A study by Hayden et. al. [25] showed that 7-keto-cholesterol promotes the differentiation of monocytes and increases formation of the atherogenic foam cells in vitro. A very strong evidence supporting a role for oxysterols in atherosclerosis is the finding that animals receiving oxysterols often develop larger atherosclerotic lesions than their control [23, 26, 27].

1.4 Glucocorticoid hormones

Glucocorticoids are steroid hormones produced in the Zonae fasciculata and reticularis of the adrenal cortex, secreted into the circulation to reach their target organs upon ACTH-mediated stimulation (Figure 1). In humans and higher primates, the major glucocorticoids are cortisone and cortisol, whereas rodents and other mammals often use corticosterone and dehydrocorticosterone (Figure 2). The majority of glucocorticoid effects are achieved through binding to specific glucocorticoid receptors. These belong to the superfamily of ligand-activated, nuclear receptors, and regulate transcription of target genes by binding to their

promoter elements or by interaction with distinct transcription factors [28-30]. Major glucocorticoid effects consist of regulation of carbohydrate- and amino acid metabolism, maintenance of blood pressure, modulation of stress- and inflammatory responses, and maturation of fetal organ systems. The role of glucocorticoids as stress hormones is highlighted by the fact that endogenous glucocorticoids are released in response to a variety of stress factors such as starvation and infection. Synthetic glucocorticoids are widely used to control a variety of inflammatory diseases including asthma and arthritis. The mechanisms behind the glucocorticoid anti-inflammatory actions are less well understood, but accumulating evidence suggests that the effects result from inhibition of inflammatory mediators such as NF-κB and other transcription factors [31].

The main determinants of steroid action were formerly thought to be restricted to the plasma concentrations of the hormones, their binding to plasma proteins, and the concentration of receptors occurring in target tissues. In addition, the concept of intracrinology [32] describes tissue-specific metabolic reactions that many hormones, including steroids, thyroids and lipid mediators undergo, resulting in a pre-receptor control mechanism. The OH/keto group on the C11-position in glucocorticoids determines if the steroid can activate its receptor or is 'inert', since only the 11β-OH glucocorticoid activates the glucocorticoid receptor. The enzyme responsible for this conversion of glucocorticoids is 11β-hydroxysteroid dehydrogenase (11β-HSD).

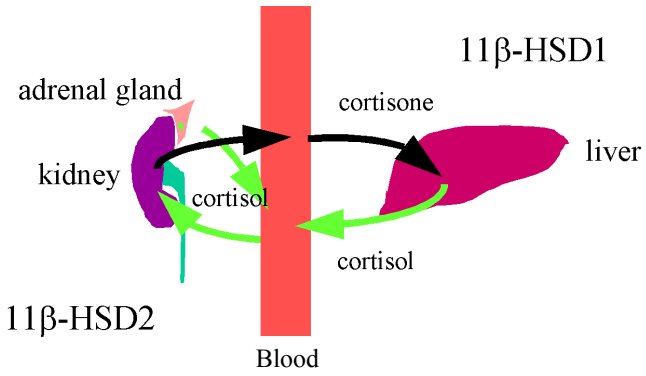

Figure 1. Glucocorticoid shuttle mediated by 11β-HSD isozymes. Dark arrow: cortisone, light arrow: cortisol.

In 1953, an enzyme was discovered that catalysed the interconversion between cortisol and cortisone [33]. This 11β-HSD activity was further described in a wide range of tissues [34], but the activity was thought only to represent a way of glucocorticoid clearance, and no other specific function was ascribed. At present, two different isozymes of 11β-HSD (11β-HSD1

and 11β-HSD2) are described, however, further forms might exist [35, 36]. The two 11β-HSD forms share about 30% sequence identity and belong to the superfamily of short-chain dehydrogenases/reductases (SDR) [37-39]. This is a large, evolutionarily conserved superfamily of enzymes including the majority of known hydroxysteroid dehydrogenases. SDRs are enzymes of approximately 250-350-residue subunits catalysing NAD(P)(H)-dependent oxidation/reduction reactions [37, 39-41]. The N-terminal region binds the coenzymes, while the C-terminal region constitutes the substrate binding part (Figure 4). Sequence comparisons between the different SDR members show typically ~25 % sequence identity, with some highly conserved motifs in common: a pattern of α/β folding [41], a nucleotide binding site and a tyrosine-dependent acid-base catalytic mechanism. The balance between the two isozyme activities determines the ratio of cortisol to cortisone in the circulation (Figure 1). A comparison of biochemical characteristics of the two known 11β-HSD isozymes is given in Table 1.

	11β-HSD1	**11β-HSD2**
Reaction direction/s	dehydrogenase and reductase in vivo: reductase	dehydrogenase in vivo: dehydrogenase
Cofactor **Molecular mass (SDS/PAGE)**	NADP(H), (NAD(H)) 34 kDa	NAD(H) 40 kDa
Glucocorticoid kinetic constants	low affinity (~1 μM)	high affinity (~ 10 nM)
Localization	endoplasmic reticulum	endoplasmic reticulum
Tissue distribution	widespread peripheral and central distribution, i.e. liver, adipose tissue, lung, and brain	MR-dependent epithelial tissues: kidney, salivary glands, colon and placenta, lung
Diseases	apparent cortisone reductase deficiency (CRD)	apparent mineralocorticoid excess (AME)
Inhibitors (cf Figure 3)	glycyrrhetinic acid carbenoxolone arylsulfonamidothiazoles	glycyrrhetinic acid carbenoxolone
Function	local glucocorticoid activation and pre-receptor regulation	cortisol inactivation; protection of MR against cortisol access

Table 1. 11β-HSD characteristics.

Physiological roles of 11β-HSD1

The tissue-specific metabolic activation of glucocorticoid precursors (cortisone in humans, dehydrocorticosterone in rodents) to the active glucocorticoid receptor (GR) ligand (cortisol, corticosterone) is carried out by 11β-HSD1, which mainly acts as an NADPH-dependent reductase (Figure 2) [42, 43].

11β-HSD1 was first isolated and characterized from liver, but it is widely expressed in the central nervous system and peripheral tissues, e.g. adipose and skeletal muscle tissues. It is a bi-directional enzyme, which *in vivo* mainly acts as a reductase. The N-terminal domain in 11β-HSD1, consisting of approximately 30 amino acid residues, forms a hydrophobic, transmembrane segment which functions as a signal-anchor motif, directing the protein to the membrane of the ER, and facing the lumenal compartment. Lumenal modification by oligosaccharyl transferase results in asparagine-linked glycosylation at three positions of the enzyme (in humans).

In liver, 11β-HSD1 increases glucocorticoid effects and opposes insulin effects by upregulating the expression of the key enzymes for gluconeogenesis, i.e. phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase). An impaired 11β-HSD activity in the liver is associated with reduced glucocorticoid action and increased insulin sensitivity in hepatocytes, as supported by Alberts *et. al.* in studies with selective 11β-HSD1 inhibitors [44, 45].

Knock-out mice have unambiguously shown that the function of 11β-HSD1 is to activate the functionally inert glucocorticoid by 11-oxoreduction. These animals develop normally and appear to be viable, fertile and normotensive [46]. But, they show an impaired induction of the gluconeogenic enzymes PEPCK and G-6-Pase on fasting and resist increased levels of blood glucose caused by obesity and stress. The glucocorticoid feedback of the hypothalamus-pituitary-adrenal (HPA)-axis was reduced in the knockout mice, indicating that 11β-HSD1 amplifies this feedback mechanism in the central nervous system [47].

11β-HSD1 is highly expressed in adipose tissue [34, 48]. A study has been published describing a transgenic mouse model resembling the metabolic syndrome in humans [49]. In this model, targeted overexpression of 11β-HSD1 using the AP2 promoter led to an increase in visceral fat, presumably because of local production of active glucocorticoid in the adipocytes.

It has also been found [50] that the enzyme reaction direction may change *in vivo*, from the anticipated reductase to dehydrogenase, dependent on the differentiation state of the cells. This phenomenon may be explained by the availability of cofactor in the lumen of the endoplasmic reticulum, provided by NADPH-generation by the enzyme hexose-6-phosphate dehydrogenase [51]. In a study by Draper et. al, patients with cortisone reductase deficiency were found to have subtle mutations in both 11β-HSD1 and hexose-6-phospate dehydrogenase, which resulted in loss of the 11β-HSD1 reductase activity [52].

The effects of carbenoxolone inhibition (a commonly used experimental 11β-HSD inhibitor, cf Figure 3) on 11β-HSD in obese insulin-resistant Zucker rats were examined by oral treatment [53]. An improved lipoprotein profile (low LDL-C, high HDL-C) due to liver-specific inhibition of 11β-HSD1 was found, but a lack of inhibition in adipose tissue was noted. A similar improvement in lipid and lipoprotein profile was described in the 11β-HSD1 knockout mice by the Seckl and Mullins groups in 2001 [54].

Healthy humans were treated orally with the 11βHSD inhibitor carbenoxolone to investigate the effects on hepatic glucocorticoid activation and on insulin sensitivity [55]. Carbenoxolone was found to increase whole-body insulin sensitivity, and the conclusion was that it inhibits the hepatic 11β-HSD1-mediated activation of cortisol, thereby increasing hepatic insulin sensitivity and decreasing glucose production.

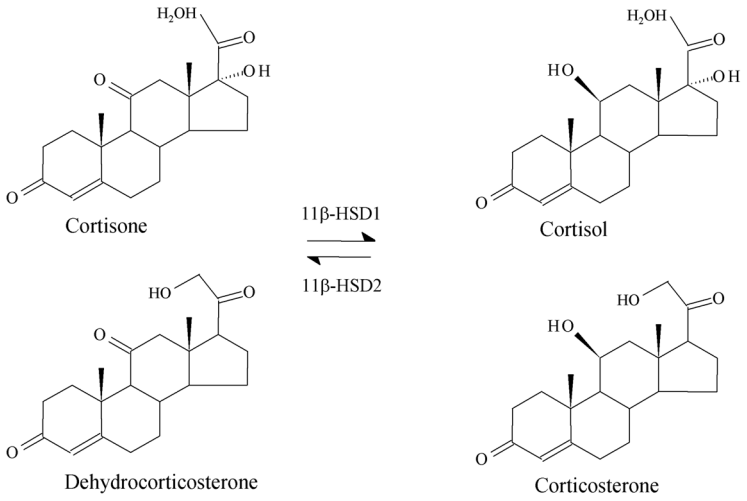

Figure 2. Glucocorticoid conversion by 11β-HSD enzymes. Cortisol and corticosterone represent GR ligands, cortisone and dehydrocorticosterone are inactive precursors. Glucocorticoids in humans are cortisol and cortisone, whereas the rodent forms are corticosterone and dehydrocorticosterone.

In a subsequent study, the effects on carbenoxolone treatment were compared between healthy and diabetic patients [56]. Carbenoxolone reduced the glucose production rate during high glucagon state in diabetic patients, due to a reduced glycogenolysis, but did not do so in healthy controls.

A selective inhibitor of 11β-HSD1 (BVT.2733) was given subcutaneously to hyperglycaemic KKA(y) mice to investigate the effects on hepatic glucose production in this diabetes model [44]. The results were lowered blood glucose and serum insulin levels, and were attributed to a decrease in mRNA levels of hepatic PEPCK and glucose-6-phosphatase. The same group

performed a study of oral administration of BVT.2733 to various mouse models of type 2 diabetes which again resulted in lowered blood glucose and increased insulin sensitivity [45]. Taken together, these reports describe the beneficial effects of 11β-HSD1 inhibition in increasing insulin sensitivity in liver, adipose tissue and skeletal muscle, and strengthen the hypothesis of this enzyme as a pharmacological target in metabolic diseases.

Physiological roles of 11β-HSD2

Whereas 11β-HSD1 mostly catalyses the NADPH-dependent 11-oxo reduction in vivo, the reverse reaction is carried out by the NAD^+- dependent type 2 11β-HSD (11β-HSD2), predominantly localized to mineralocorticoid receptor (MR) responsive cells. The function of 11β-HSD2 is related to the mineralocorticoid properties of cortisol. 11β-HSD2 is a NAD^+ dependent dehydrogenase of adrenal glucocorticoids and inactivates cortisol [57]. In adults, 11β-HSD2 is expressed in tissues where aldosterone induces effects on sodium excretion and transcellular electrolyte transport take place (i.e. renal distal tubular cells, sweat glands and colon epithelia). In these cells, cortisol has to be inactivated and to be excluded from occupation of the mineralocorticoid receptors, since this receptor can equally well bind glucocorticoids (cortisol) and mineralocorticoids (aldosterone). A defect 11β-HSD2 gene product causes an unusual and severe disease called "apparent mineralocorticoid excess syndrome" (AME), which is characterized by sodium retention, hypertension and decreased potassium levels (hypokalemia), leading to life threatening complications [58].

An important role of glucocorticoid hormones is to inhibit cell proliferation and stimulate cellular differentiation. Abnormal hormone expression is well known in several malignant diseases, including breast and thyroid cancer [59]. 11β-HSD2 overexpression and concomitant inactivation of glucocorticoids has been implicated in pituitary tumour development [60] as an inducing agent of glucocorticoid-mediated cell proliferative effects. The expression of 11β-HSD2 in GR tissues may thus lead to increased cell proliferation, and therefore constitutes a potential novel target in cancer therapy.

2 METHODS

Experimental standard techniques including SDS/PAGE, agarose gel electrophoresis, RNA isolation, Western Blot, PCR, and cloning were carried out according to established protocols [61, 62]. Techniques that are central to the study are described in more detail in the following sections.

2.1 Protein expression systems

Several attempts have been made prior to the study to develop an expression system with active enzyme in high amounts. Because of the hydrophobic nature of 11β-HSD1, these efforts have been largely unsuccessful, and only moderate levels of activity were reported in transfected cell lines expressing 11β-HSD1. We overexpressed the full length, membrane bound 11β-HSD1 in the yeast expression system *Pichia pastoris*, and also used *E.coli* bacterial expression to express an N-terminally truncated, soluble form of the enzyme. The Pichia expression system is valuable for expression of mammalian, membrane bound, glycosylated enzymes, as this eukaryotic cell contains post-translational modification enzymes and cellular compartments, in contrast to the bacterial cell. *E.coli*, on the other hand is a more rapid system for protein expression, but has a disadvantage in lack of many modifying enzymes, necessary to provide post-translational modifications, such as N-linked glycosylation.

2.2 Pichia pastoris expression system

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as the sole carbon source through alcohol oxidase (AOX). There are two alcohol oxidase genes in *Pichia*: AOX1 and AOX2. AOX1 is responsible for the majority of the alcohol oxidase activity in the cell, and its promoter is used to drive heterologous protein expression. If the AOX1 gene is lost during the homologous recombination event, a yeast strain with phenotypic slow growth on methanol medium is obtained. The recombination event that takes place depends on the combination of host strain and restriction enzyme used.

The experimental outline of Pichia expression was to clone 11β-HSD1 constructs of various species and transform *Pichia* strains by spheroplasting, and finally to express the recombinant proteins intracellularly.

Clones were phenotyped after homologous recombination, to obtain optimal growth conditions and expression trials of both phenotypes were performed before finally one recombinant clone was chosen for each species for further enzymatic analyses.

2.3 Expression and purification of soluble 11β-HSD1 in *E.coli*.

Expression constructs without the NH_2-terminal transmembrane domain (23 amino acids in human) and with an additional His_6 tag at the amino terminus were cloned for various species according to Walker et. al. 2001 [63]. *E.coli* BL21 cells were transformed and protein expression induced using isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested and lysed by sonication, and soluble 11β-HSD1 protein was purified by His-bind affinity chromatography with a stepwise gradient of imidazole, as described elsewhere [64] .

2.4 Metabolic analysis by high performance liquid chromatography

An advantage in the study was to have a flexible system for measurement of enzymatic activity. The general procedure for the enzymatic assays and HPLC analysis is as follows: 50 µl reactions containing 20 mM Tris/HCl buffer at pH 7.4, cofactor, 11β-HSD1 enzyme (homogenate or purified), substrate and if required 11β-HSD inhibitors. A C_{18} column was used, the mobile phase was 30% acetonitrile, 0.1% ammonium acetate, pH 7.0 (glucocorticoid separation), or 85 % acetonitrile (oxysterol separation) and flow rates were between 0.5-1 ml/min. The substrate and the products formed are separated and subsequently monitored by UV or radioactivity detection. The amount of product formed is determined by comparison to a standard curve of the authentic compound.

The HPLC assay used has several advantages: First of all, it enables the measurement of reaction rates in cell homogenates down to low µM substrate concentrations (with UV detection) or nM concentration (using radioactivity detection or by sample concentration prior to HPLC analysis), without purification of the enzyme preparation. The only necessary preparation is to remove the cell debris by a short spin on a tabletop centrifuge. This system enables the measurement of both reductase and dehydrogenase reactions, whereas in alternative fluorimetric spectroscopy, only dehydrogenase reactions can be measured conveniently (by indirect measurement of NADPH levels). Second, since the product formation is measured directly, side-reactions and other proteins do not affect the results.

There are, however, notable disadvantages to the HPLC system. Mainly, it is a more labor-intensive system compared to continuous fluorimetric or UV kinetic measurements. A temporary deviation from linear behaviour is also not distinguishable (for example, because of inadequate mixing of the components).

The end-point method for kinetic measurements using HPLC compound separation is robust and flexible, and at best combined with fluorimetric or other continuous measurements, taking

advantage of the specificity of the HPLC measurements and the speed and sensitivity of the indirect fluorimetric- or UV methods.

2.5 Enzyme kinetics and inhibitor analysis

Non-linear regression.

In earlier days of analysis of enzyme kinetics data, the best way was to transform the data and analyze it by linear regression. An example of a widely used transformation in Michaelis-Menten kinetics is the Lineweaver-Burke plot. A problem with linear transformations is that they distort the experimental error, and the standard deviation will usually not be uniform over the whole range of X-values. Instead, non-linear regression gives the most accurate results. Lineweaver-Burke or other linear transformed plots are however helpful to display data, since the visual interpretation is always a part of the evaluation.

In nonlinear regression, the goal is to fit your data to a model. Therefore, the necessity for the investigator of choosing the adequate model is obvious. The best-fit values of the variables in the model are determined, for example rate constants and affinities, which then can be interpreted scientifically.

Michaelis- Menten kinetics

In an analysis of Michaelis-Menten enzyme kinetics, the initial velocity of an enzyme reaction as a function of substrate concentration is described (v=Vmax*S/(Km+S)). These analyses assume the following: 1) the rate of product formation is linear with time and enzyme amount during the time interval used. 2) the substrate concentration is much greater than the enzyme concentration. 3) the concentration of enzyme-substrate complex is at steady state.

IC_{50} determination

The equation describing a one-site competition (a sigmoid dose-response curve) shows a response as a function of the logarithm of concentration. $Y=Bottom + (Top-Bottom)/(1+10^{X-logIC50})$. $LogIC_{50}$ is the concentration that gives a response halfway between baseline and maximum.

Fitting a sigmoid (logistic) equation to a dose-response curve to determine the IC_{50} value has certain limitations. The IC_{50} reflects both the binding ability of the inhibitor (inhibitor affinity) and the ability to inhibit the reaction (inhibitor efficacy). The efficacy reflects properties of both the inhibitor and the cellular environment, and the IC_{50} value can therefore change in different environments.

The K_i value can be calculated from the IC_{50} values according to the Cheng and Prusoff equation [65]: $K_i = IC_{50}/(1+([ligand]/K_m))$. This is mainly useful when comparing a group of similar compounds.

Inhibition type characterization

The statistical program SigmaPlot was used for inhibition model comparisons of reversible (mouse) and tight binding (human) inhibition, respectively. In the enzyme kinetics module, data can be fitted to selected tight binding or reversible inhibition models (competitive, mixed, non-competitive, uncompetitive). Multiple inhibition models can be compared simultaneously, and each one generates a statistical report including the parameter values (K_m, K_i, V_{max}, α) and goodness of fit criteria. Ranking of R^2-values and comparisons of other goodness of fit criteria can be used to determine the best-fit inhibition model.

2.6 Pancreatic islet isolation and cultivation

Pancreatic islets were isolated by collagenase treatment and cultured in RPMI 1640 culture medium supplemented with 11 mM glucose, 10 % fetal bovine serum and 0 or 200 nM corticosterone.

2.7 Microarray analysis

Microarray expression analysis is a widely used tool in functional genomics studies to profile mRNA expression. It allows the investigation of mRNA expression levels on a genome-wide scale simultaneously for thousands of genes. For an efficient use of this sensitive technique, reproducible protocols for RNA isolation, array set-up and probe hybridization are essential. It is also important to verify the array results by quantitative methods like quantitative real-time PCR or Northern blot analysis.

Differences in mRNA expression levels in pancreatic islets of Langerhans derived from lean mice were studied. Experiments were performed to analyze the differential response between short–term corticosterone treated (18-22 h) and untreated control animals.

RNA isolation

Isolation of total RNA for microarray studies was performed using the RNeasy procedure (QIAgen), using a silica-based membrane contained in a spin column for selective binding and separation of RNA. A guanidine isothiocyanate buffer was used for cell lysis. RNA molecules longer than 200 nucleotides bind to the silica-membrane, which provides an enrichment of mRNA suitable for isolation of high-quality material.

Microarray construction

Microarrays were constructed by coupling PCR-amplified cDNA clones of expressed sequence tags (EST's) to aldehyde-derivatized glass microscope slides. Total RNA was transcribed into cDNA and labelled with cyanine 3' and 5' fluorophores. The labelled cDNA products of untreated and treated material were combined and hybridized onto the array. Scanning and analyzing the slides, in combination with annotations with the gene IDs, obtained sets of raw data. Microarrays slides were developed and hybridized at the Institute of Experimental Genetics, GSF, Neuherberg, in collaboration.

Quantitative real-time PCR

Real-time PCR is a sensitive method for quantification of cDNA. A PCR product is measured via a fluorescent signal during each cycle in the PCR. The cycle number at which the fluorescence signal sharply increases (in correlation to the background fluorescence of the assay) is noted, and this number is proportional to the target concentration. By comparison to a standard with known concentration of DNA, a calibration curve can be created and the concentration of DNA in various samples can be estimated. The binding of the fluorophore SybrGreen™ to double-stranded cDNA generated the fluorescent signal in this study. Analysis was performed using an ABI PRISM 7700 Sequence Detection Systems instrument and software. RNA samples were normalized for comparison by determination of 18S rRNA levels.

3 AIMS

The aims of the study were to investigate structure-function relationships and functional roles of 11β-HSD1. In particular, it was of interest to accomplish and analyse:

development and analysis of protein expression systems in yeast and *E.coli* for 11β-HSD1, to enable further enzymatic investigations on various aspects of 11β-HSD1 function;

kinetic characterization and elucidation of structure-function relationships by investigation of substrate and inhibitor specificities in connection with inter-species enzymatic variations;

physiological function of 11β-HSD1, by investigating the pancreatic islets of Langerhans in relation to glucocorticoid effects and insulin secretion in the normal and diabetic state in mice;

novel enzymatic functions, especially the effects of 11β-HSD1 in oxysterol metabolism and its possible implications in atherosclerosis.

4 RESULTS AND DISCUSSION

4.1 Expression systems for recombinant 11β-HSD1 in yeast and bacteria

For the study of 11β-HSD1, the availability of a suitable expression system was mandatory. Human 11β-HSD1 was expressed in the yeast expression system *Pichia pastoris*, a methylotrophic yeast with higher resemblance to mammalian glycosylation patterns than that of *Saccharomyces cerevisiae*, and widely used for recombinant expression of mammalian enzymes. The cDNA was cloned in its full-length form and expressed intracellularly, resulting in membrane bound, highly active enzyme preparations. A soluble form of 11β-HSD1 was expressed as an N-terminally truncated variant in *E.coli*, by replacement of the membrane-anchoring N-terminal domain with a His_6-tag and using immobilized metal affinity chromatography (IMAC) for purification. The expression of soluble 11β-HSD1 was first described in [63], employed by us in [64] and the present study, and has been optimized to expression at high levels and to monodispersity (Elleby *et. al*., 2004, manuscript).

Glucocorticoid substrate screenings of recombinant yeast transformed with human 11βHSD1 revealed that the naturally occurring GC's (cortisol, cortisone, corticosterone, dehydrocorticosterone) are metabolized, whereas synthetic steroids with major changes in the steroid skeleton (dexamethasone and budesonide) were not metabolised by the enzyme. The K_m-values for the cortisol dehydrogenase reaction were similar to that in human liver microsomes (~10 μM), except in whole yeast cells, where no dehydrogenase activity was observed. This is in agreement with other data suggesting that 11βHSD1 mainly acts as a reductase in intact cells, whereas the dehydrogenase activity is dominating in disrupted cells [66, 67], and this may reflect the changes in cofactor availability in differentiating or disrupted cells [50, 51]. The K_m for GC reduction reactions were found to be lower than that of GC dehydrogenase (around 1 μM), which supports the reductive direction as the main, intrinsic 11β-HSD1 activity, also seen in other studies [46, 48, 55].

4.2 Active site variability of mammalian 11β-HSD1

In the absence of structural information, the active site of 11β-HSD1 was characterized by extension of the available sequence information and by inhibition studies. Elucidation of the primary structures of several mammalian 11β-HSD1 orthologs was performed by cloning, assembly of the preliminary sequences from genome information or determination of cDNA sequences. The numbers of known sequences for 11β-HSD1 were extended considerably.

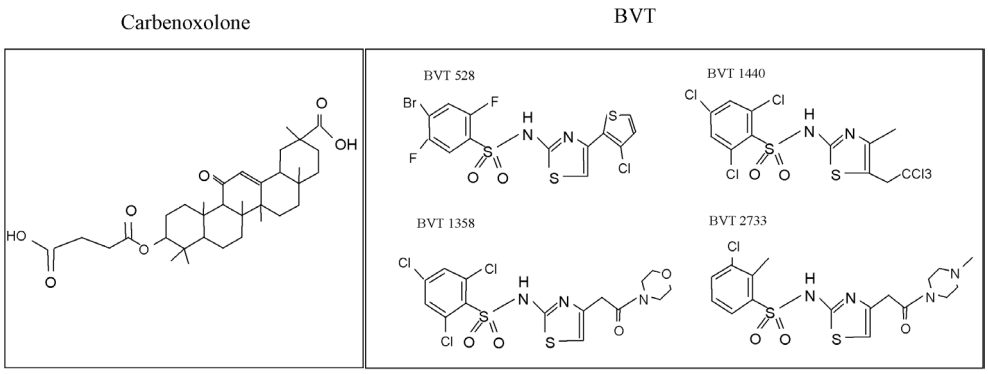

Figure 3. 11β-HSD1 inhibitors carbenoxolone and arylsulfonamidothiazoles used in this study.

The inhibition potency of synthetic steroids and other compounds were investigated for the glucocorticoid reduction and dehydrogenase reactions. Several natural (glycyrrhetinic acid, naringenin) and synthetic (dexamethasone, budesonide, carbenoxolone) compounds inhibited 11β-HSD1. They are however not specific for this enzyme, but also inhibit other SDR enzymes, including the type 2 11β-HSD isozyme [68, 69]. Using a novel group of selective 11β-HSD1 inhibitors, the arylsulfonamidothiazoles (Figure 3), inhibition studies were performed in two steps to correlate primary structure variability to inhibitory properties across compounds and species.

First, a screening of inhibition potencies of all compounds against four 11β-HSD1 variants (human, mouse, rat, guinea pig) was performed. The inhibition constants obtained revealed the following (Table 2): 1) inhibitor potencies varied across species. In general, the highest binding affinities were seen for the human enzyme (nM K_i), whereas the other species were several fold less potent (μM K_i); 2) Each inhibitor had variable potency against the different species; 3) The inhibitors with the apparently most bulky functional groups (BVT.2733, followed by BVT.1358) showed least inhibition, indicating spatial hindrance; 4) the mouse enzyme seemed to be the most 'allowing' of the enzymes in terms of inhibitor binding; all 4 inhibitors were potent to a medium degree for this species. The guinea pig enzyme, on the other hand, was only inhibited by one compound. This may be related to the hypervariable regions V1-V6, as displayed in the 3-dimensional structure of the SDR enzyme 7α-hydroxysteroid dehydrogenase (Figure 4).

Second, inhibitors for the human and mouse enzymes were selected for further characterization of the binding modes in the glucocorticoid reductase and dehydrogenase reactions. For the human enzyme, the tight binding inhibitors BVT.528 and carbenoxolone

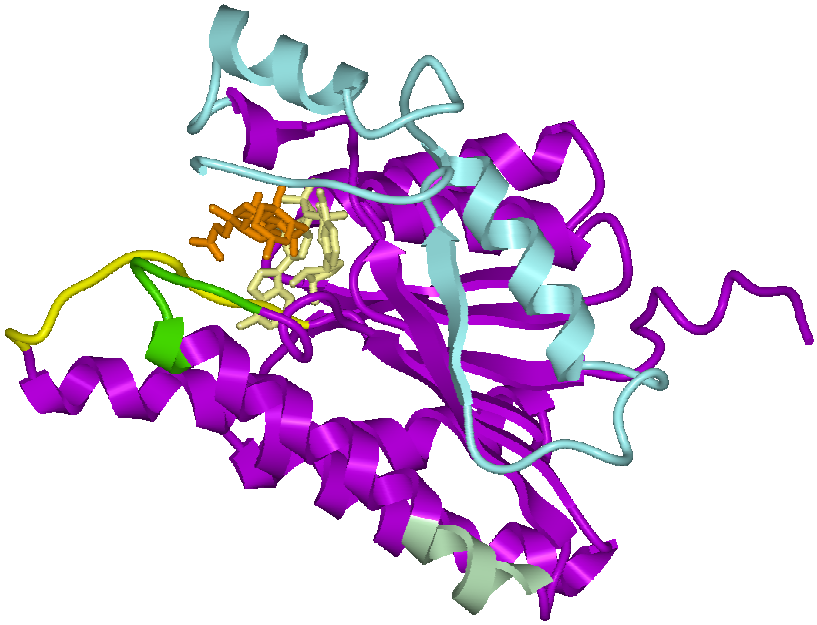

Figure 4. The SDR enzyme 7α-hydroxysteroid dehydrogenase (/α-HSD) in complex with NADH (soft yellow) and 7-oxo glycochenodeoxycholic acid (red). The variable regions in 11β-HSD1 are displayed as follows: V1: yellow, V2: green, V3: soft green, V4-V6: light blue.

were chosen, whereas the reversible inhibitor BVT.2733 was investigated for the mouse enzyme. The results display a competitive inhibition pattern for carbenoxolone and BVT.528 of the human glucocorticoid reductase and dehydrogenase reactions, whereas BVT.2733 displayed a mixed mode of inhibition for the mouse reductase reaction. This differential mode of inhibition between species using similar arylsulfonamidothiazole compounds may reflect variances in the inhibitor binding and active site architechture.

		BVT.528		BVT.1440		BVT.1358		BVT.2733	
		IC_{50}	K_i	IC_{50}	K_i	IC_{50}	K_i	IC_{50}	K_i
Human	(nM)	63±32	40±26	55±35	35±21	335±205	195 ±120	n.i.	
Mouse	(μM)	2.8±0.8	2.4±0.7	0.8±0.3	0.7±0.3	1.1±0.3	0.9±0.2	1.5±0.8	1.3±0.6
Rat	(μM)	8.0±0.1	5.8±0.8	5.8±0.9	4.2±0.1	n.i.		n.i	
Guinea pig	(μM)	n.i		3.4 ± 1.6	2.2 ± 1.1	n.i.		n.i.	

Table 2. Arylsulfonamidothiazole inhibitor screening of 11β-HSD1 species variants. n.i, no inhibition.

The arylsulfonamidothiazole class of selective 11β-HSD1 inhibitors was first described in a study by Barf et al. 2002 [70] using a high-throughput screening of compounds, followed by

lead optimization. To evaluate the inhibition potency of human and mouse 11β-HSD1, recombinant 11β-HSD1 expressed in Pichia was screened for cortisone reductase activity in the presence of cofactor and varying concentrations of inhibitors. By investigation of the cortisol dehydrogenase activity of the 11β-HSD2 isozyme, the selectivity of these compounds was tested [70]. Similarly to the present study, a variable pattern of inhibition was found, where the compound BVT.2733 (used in this thesis) inhibited the mouse enzyme but not the human enzyme. There were general differences in the IC_{50}-values; the previous study showed IC_{50}-values in the nM range for BVT.2733 in the mouse and the μM range in the human, whereas we obtained several fold higher inhibition constants (Table 1). This may be attributable to the nature of evaluating IC_{50}-values, since the environment affects the efficiacy and consequently the inhibition constant. The inhibitors showed > 200-fold selectivity for human and mouse 11β-HSD1.

4.3 Physiological function of 11β-HSD1

Direct inhibitory effects of glucocorticoids on insulin secretion from pancreatic β-cells have been described in perfusion studies on pancreatic islets and β-cells [15, 16, 71-74]. These findings led us to investigate the role of glucocorticoid conversion by 11β-HSD1 in pancreatic islets of lean and obese mice, in relation to glucose-stimulated insulin release.

Reverse-transcription PCR (RT-PCR) revealed that 11β-HSD1 is present in the islets of Langerhans of humans and mice. Primary cultures display 11β-HSD1 reductase activity, which is inhibited by the 11β-HSD-inhibitor carbenoxolone. The effects on insulin release in short-term treatment of the inactive rodent glucocorticoid dehydrocorticosterone (DHC) were investigated using radioimmune assay (RIA) in ob/ob mice and competitive enzyme-linked immunosorbant assay (ELISA) for lean mice.

Insulin release was inhibited by DHC in islets of obese mice, and this inhibition was reversed by addition of the 11β-HSD-inhibitor carbenoxolone. The data indicate that glucocorticoid-activation by 11β-HSD1 is used as a means to regulate insulin release in pancreatic islets. A glucocorticoid-responsive reporter gene assay showed that the observed effects on insulin secretion were mediated through activation of the glucocorticoid receptor pathway of transcriptional regulation. The above findings considerably expand earlier work where ob/ob mice treated with the synthetic glucocorticoid dexamethasone displayed a decreased insulin release [73]. Data from transgenic mice overexpressing the glucocorticoid receptor in β-cells also support the role of glucocorticoid signaling in insulin release [15, 75]; the acute insulin response to an intravenous glucose load was decreased in GC hyper-responsive compared to

normal mice and all animals become diabetic at 12-15 months of age. Hence, the discovered role of 11β-HSD1 in pancreatic islets of obese mice strengthens the concept to target this enzyme in the treatment of diabetes mellitus type 2 beyond the known beneficial effects on target tissues such as liver, fat and skeletal muscle.

Insulin secretion was also investigated in this study by short-term treatment of glucocorticoids in islets of lean C57BL/6J mice. Insulin secretion was *increased* in these mice in response to glucocorticoids, in contrast to the previously discussed obese mice (Figure 5).

In studies on glucocorticoid treatment, variations in insulin release have been reported. Most reports describe a decrease in insulin secretion [15, 75, 76], but also the opposite has been noted [77, 78]. The concentration and duration of glucocorticoid treatment has been implicated to effect the insulin release [76]. It has further been reported that the expression and activity of 11β-HSD1 is increased in diabetic islets of Zucker Diabetic Fatty (ZDF) (*fa/fa*) rats, compared to pre-diabetic ones. These data clearly emphasize a shift in glucocorticoid mediated insulin responses from normal (lean) to diabetic states at least in mice.

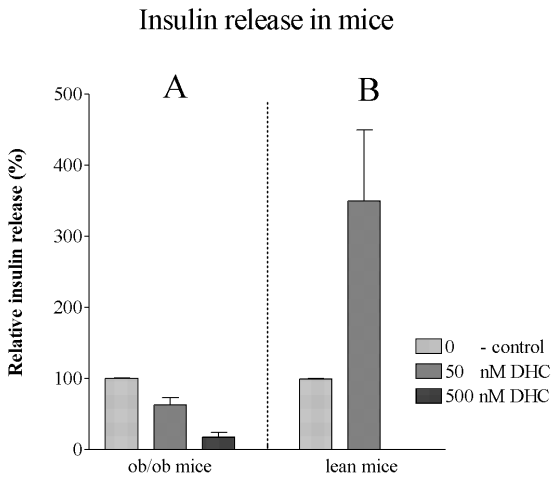

Figure 5. Glucose-stimulated insulin release in obese (A) and lean (B) mice upon short-term treatment with dehydrocorticosterone.

To investigate the mechanisms which increased insulin secretion in lean mice, we performed a genome-wide cDNA microarray analysis. Samples derived from untreated and corticosterone-treated islets of lean mice were compared, and the 100 most differentially expressed genes were used as a basis for hypothesis building. The results were verified by analysis of a selected group of genes by quantitative real-time PCR. The 100 genes were grouped according to function. All genes with assigned functions fell into one of the

following classes; vesicle transport, signal transduction, stress response, or metabolism. Several different upregulated signal transduction pathways were revealed, indicating that glucocorticoids regulate a distinct set of genes for enhanced insulin secretion. The results indicate that in the early phase of glucocorticoid exposure, healthy β-cells display a stress response to adapt to an increased energy demand in the cell, which may transform further to a diabetic state.

4.4 Oxysterol metabolism by 11β-HSD1

An $NADP^+$-dependent 7α-OH-cholesterol dehydrogenase with N-terminal sequence similarity to the human 11β-HSD1 was isolated from hamster liver by Song et. al. [79, 80]. These studies and the sequence determination of hamster 11β-HSD1 (paper II, this thesis) prompted us to investigate the oxysterol-metabolizing properties of human and rodent 11β-HSD1.

Three species variants (human, mouse and rat) of 11β-HSD1 were analyzed using recombinant membrane bound (full-length) and soluble (N-terminally truncated) enzyme, and compared to human liver microsomes derived from a transplant donor.

By incubation of human liver microsomes with NADP(H) and either 7α-OH-cholesterol, 7β-OH-cholesterol, or 7-ketocholesterol, it was revealed that 7-ketocholesterol is reduced into 7β-OH-cholesterol, without formation of the 7α-OH-cholesterol isomer. Conversely, only 7β-OH-cholesterol could be converted into 7-ketocholesterol. Carbenoxolone and the 11β-HSD1-specific inhibitor BVT.24829 inhibited both enzymatic reactions, which indicates that 11β-HSD1 is the main enzyme that mediates 7-oxysterol oxidoreductase reactions of human liver microsomes.

The same isomer preference as for human liver microsomes was found in human, rat and mouse recombinant 11β-HSD1 enzymes of full-length and soluble forms: only 7β-OH-cholesterol was formed by 7-keto-cholesterol conversion or used as a substrate. K_m values were comparable to glucocorticoid conversion (5-85 μM for 7-oxysterols). Considering the preference of 11β-HSD1 for the reductase reaction *in vivo*, and earlier reports describing 7-ketocholesterol metabolism in the liver into 7β-OH-cholesterol [81], it can be expected that 11β-HSD1 catalyzes the formation of 7β-OH-cholesterol from 7-ketocholesterol in the liver. The enzymatic origin of endogenous 7β-cholesterol has thus been established.

The kinetic constants (k_{cat}/K_m) revealed variable efficiency of 7-oxysterol metabolism between species. The mouse ortholog catalyzed 7-oxysterol reactions most efficiently. Together with the discovered preference in hamster for 7α-OH-cholesterol [79, 80], these data

describe an enzymatic origin of the species differences observed in oxysterol metabolism [19].

7-oxysterols have documented effects in various aspects related to atherosclerosis. It is one of the most commonly found oxysterols in oxidized low-density lipoprotein (oxLDL) [82], arterial foam cells and atherosclerotic plaques [23]. 7-Ketocholesterol has been reported to induce monocyte differentiation and foam cell formation [25]. Monocytes are important for the development of atherosclerosis [17], they accumulate during plaque formation and can be differentiated into macrophages by oxLDL [83]. The macrophages secrete inflammatory mediators [18] and take up oxidized lipoproteins, which lead to the development of cholesterol-laden foam cells.

11β-HSD1 may also be involved in the formation of atherosclerotic lesions through glucocorticoid-mediated immune response. The enzyme has been reported to be induced in monocytes upon differentiation to macrophages [84], strongly enhanced by the inflammatory mediators IL-13 and IL-4. The enzymatic direction of oxysterol metabolism in the macrophages may be different from the one in liver. 11β-HSD1 activity has been shown to reverse in differentiation of adipocytes [50], and if a similar change would occur for oxysterols in macrophages, the 11β-HSD1 may change from a 7-keto-cholesterol-preventive to a 7-keto-cholesterol-enhancing enzyme. Accordingly, 11β-HSD1 constitutes a possible interaction between oxysterol and glucocorticoid metabolic pathways.

5 CONCLUSIONS

1) The hydrophobic enzyme 11β-HSD1 can be expressed with high activity as a full length, membrane bound enzyme in the yeast system *Pichia pastoris* and as a soluble, N-terminally truncated form using His_6 tag purification in *E.coli.* The full-length and truncated enzymes have similar enzymatic properties in regard to glucocorticoid and oxysterol metabolism.
2) 11β-HSD1 orthologs from human, rat, mouse and guinea pig show considerable inter-species variations in active site geometries as inferred by primary structure determinations and inhibitor characterizations.
3) 11β-HSD1 mediates glucocorticoid-activation in pancreatic islets of Langerhans, and thereby regulates glucose-stimulated insulin secretion. The known beneficial effects of 11β-HSD1-inhibition in the pharmacological treatment of diabetes mellitus are postulated to include improved insulin release in diabetes.
4) Glucocorticoid-activation in lean mice at an early phase leads to increased insulin secretion and primes the β-cells to stress-adaptation, which later transfers into a diabetic phenotype.
5) The enzymatic origin of endogenous 7β-OH-cholesterol in humans is established, possibly pointing to an involvement in atherosclerosis. 11β-HSD1 serves as an enzymatic origin of species differences in 7-oxysterol metabolism.

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ARTICLES I-V