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PhD THESIS

Local metabolism of glucocorticoids in hypertriglyceridemic rat

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I hereby declare that submition is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning.

Mgr. Petra Klusoňová

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1. Abstract

11β-hydroxysteroid dehydrogenase (11HSD1) is an oxidoreductase which catalyzes conversion of inactive 11-oxo steroid derivatives into active 11-hydroxy forms. 11HSD1 elevates intracellular level of active glucocorticoid (GC) hormones: cortisol in human tissues and corticosterone in rodents, therefore local level of active GCs can be set independently from systemic secretion driven by hypothalamo-pituitary-adrenal axis (HPA axis). Chronic systemic excess of GCs results in development of Cushing´s syndrome which is characterised by central obesity and other metabolic disturbances. Despite normal serum levels of GCs, the patients with idiopathic obesity also develop metabolic syndrome. It was suggested that GCs could be elevated locally in target tissues due to enhanced 11HSD1 activity. This hypothesis was confirmed in transgenic rodent models.

Prague hereditary hypertriglyceridemic (HHTg) rats represent a non-obese model of metabolic syndrome without genetic manipulations or specific mutations. The strain was bred by cross-mating of Wistar rat individuals with elevated serum levels of triglycerides (TGs). The strain exhibit hypertriglyceridemia and hypertension. When kept on high carbohydrate diet HHTg rats exhibit alterations in glucose homeostasis. Since there are no data that would describe relationship between GCs and metabolic syndrome in HHTg rats we decided to adress the following aims:

1) To find out whether there are any differences in local metabolism of GCs in metabolically active tissues in HHTg rats compared to healthy Wistar rats
2) To describe the response of local metabolism of GCs to 24h fasting and to elucidate whether it differs between HHTg rats and Wistar rats
3) To test whether carbenoxolone (CBX), a nonselective inhibitor of 11HSDs is able to improve metabolic syndrome symptomes in HHTg rats
4) To test whether compound 544 (C544), a selective inhibitor of 11HSD1 is able to improve metabolic syndrome symptomes in HHTg rats

We used enzymatic assays to study the level of active 11HSD1 protein (activity) and molecular biology techniques to determine 11HSD1 expression in the liver, adipose tissue and skeletal muscle of female HHTg rats. The same approach was used to study

mRNA expression of hexoso-6-phosphate dehydrogenase (H6PDH), an enzyme that cooperates with 11HSD1, and glucocorticoid receptor (GR). In addition, we measured selected serum metabolic parameters. We found elevated 11HSD1 in HHTg rats compared to Wistar rats in all tested tissues. Despite the fact that 11HSD1 activity did not always correlated with mRNA expression it seems that HHTg rats might suffer from increased intracellular level of GCs. Fasting induced similar tissue-specific changes in 11HSD1 activity and expression in both Wistar and HHTg rats. In liver, 11HSD1 was downregulated while in adipose tissue it was upregulated. No fasting-mediated changes were observed in skeletal muscle. Similar changes as in 11HSD1 were found in H6PDH expression. 11HSD1 activity and expression was downregulated upon CBX treatment in the liver of HHTg rats, however this was not followed by improvement of metabolic syndrome symptoms. No changes were found in adipose tissue and skeletal muscle. In contrast to CBX treatment, C544 treatment lowered hypertriglyceridemia and elevated serum HDL lipoprotein fraction in HHTg rats. It also decreased systemic levels of corticosterone (CS), a main GC in rodents.

In conclusion, HHTg rats exhibit altered intracellular GC metabolism compared to Wistar rats. Fasting induced tissue-specific changes in local GC metabolism. These changes were similar in both strains. Nonselective 11HSD1 inhibition did not ameliorate metabolic syndrome in HHTg rats in contrast to selective 11HSD1 inhibition that resulted in lowered serum TGs. It seems that 11HSD1 is a promising therapeutic target and should be considered for further research of metabolic syndrome.

2. Introduction

2.1. Metabolic syndrome

Metabolic syndrome was first defined in 1988 by Reaven as a cluster of metabolic and cardiovascular abnormalities as insulin resistance, dyslipidemia, obesity and hypertension [1]. A recent report of The American Heart Association defined 6 major components of metabolic syndrome:

- Abdominal obesity
- Atherogenic dyslipidemia
- Insulin resistance
- Hypertension
- Proinflammatory state
- Prothrombotic state

Abdominal obesity is characterised by increased waist circumference (in clinical studies often expressed as waist-hip ratio) and represents the form of obesity strongly associated with the metabolic syndrome. Insulin resistance is believed to be the major risk factor for development of metabolic syndrome, while it directly cause other metabolic risk factors as dyslipidemia. Prolonged untreated insulin resistance also leads to hyperglycemia and hyperinsulinemia. Atherogenic dyslipidemia mostly exhibits as elevated plasma levels of triglycerides (TGs), nonesterified fatty acids (NEFAs) and very low density lipoprotein particles (VLDL) and decreased plasma levels of high density lipoprotein particles (HDL). Proinflammatory state is characterised as chronic release of proimflammatory cytokines from excess adipose tissue. It is often associated with obesity and is clinically manifested as elevated plasma levels of C-reactive protein (CRP). Proinflammatory state is connected with increased levels of plasminogen-activator inhibitor 1 (PAI1) and fibrinogen as its production is stimulated by cytokines. Underlying risk factors for metabolic syndrome development include physical inactivity, atherogenic diet, cigarette smoking and hormonal changes linked to aging [2].

2.2. Metabolic syndrome and glucocorticoids

Glucocorticoids (GCs) exert broad range of tissue-specific effects on metabolism, immunity, development and cognitive functions. Cushing´s syndrome, characterised by chronically increased plasma GCs largely due to pituitary adenoma, resembles metabolic abnormalities of metabolic syndrome [3]. In addition, treatment of acute and chronic inflammatory diseases was shown to be associated with adverse metabolic disorders as weight gain, hyperlipidemia and insulin resistance [4]. In contrast to patients with Cushing´s syndrome, individuals with metabolic syndrome display normal systemic levels of GCs, however different tissues can regulate intracellular level of GCs by expressing enzyme 11β-hydroxysteroid dehydrogenase (11HSD), which catalyse interconversion between active and inactive forms of GCs. Therefore, local level of GCs can be set differently in particular tissue and can be independent from systemic level controlled by hypothalamo-pituitary-adrenal (HPA) axis. Thus, the hypothesis was postulated that patients with metabolic syndrome suffer from increased intracellular levels of GCs due to dysregulation of 11HSD1 activity. Mechanisms of intracellular regulation of GC levels will be further discussed in the next chapters. Fig. 2.1. summarizes tissue-specific processes controlled by GCs that can lead to development of metabolic syndrome.

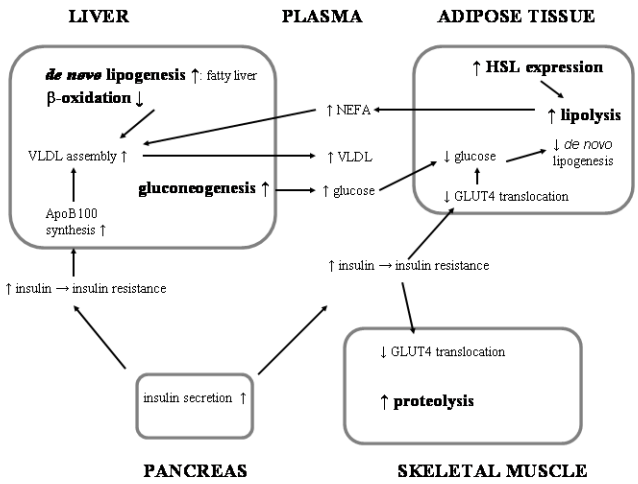


Fig. 2.1. Summary of tissue specific dysregulations of metabolism. Pathways directly influenced by GCs are highlighted.

ApoB100-Apoprotein B100, HSL-hormone sensitive lipase, GLUT4-insulin-responsive glucose transporter 4

2.3. Glucocorticoid hormones (GCs)

GCs represent an important subgroup of steroid hormones with wide effects on metabolism, immune response or behaviour. Active glucocorticoids are cortisol (Fig.2.2.A) in man and CS (Fig.2.2.B) in most rodents.

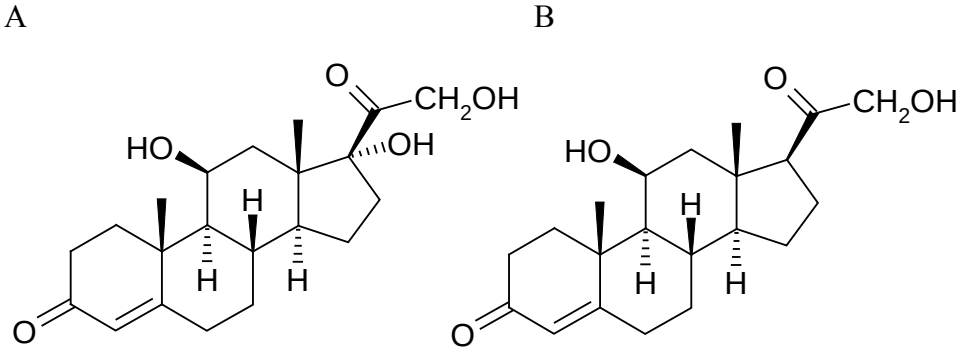


Fig.2.2. A. Cortisol B. Corticosterone (CS)

GCs are lipophilic molecules that are transported in blood mostly bound to corticosteroid-binding globulins (CBGs). Only a small portion of GCs is unbound and physiologically available. Plasma levels of GCs exhibit diurnal rythm with peak in early morning and nadir in the evening in humans. In rats, as in nocturnally active animals, nadir appears early in the morning and peak in the evening. Synthesis of GCs takes place at zona fasciculata of adrenal cortex and is regulated by hypothalamic and anterior pituitary hormones organized in a system of negative feedback loops called HPA axis. In paraventricular nuclei (PVN) of hypothalamus a peptide corticotropine-releasing hormone/factor (CRH/CRF) is synthesized and released into circulation. In anterior pituitary it stimulates synthesis of adrenocorticotropic hormone (ACTH). ACTH is transfered through bloodstream into adrenal cortex where it stimulates GC synthesis. Synthesis of CRH and ACTH is supressed by GCs. CRH expression in PVN exhibits a diurnal rythm that persists after adrenalectomy (ADX) and regulates ACTH plasma levels. There are no changes in sensitivity of pituitary cells to CRH during 24h. This is in contrast with ACTH sensitivity of adrenal gland that exhibits diurnal changes. Plasma

levels of GCs are also affected by rate of clearance in liver (Fig.2.3.). At cellular level, GCs act through soluble glucocorticoid receptors in cytosol (reviewed in [5]).

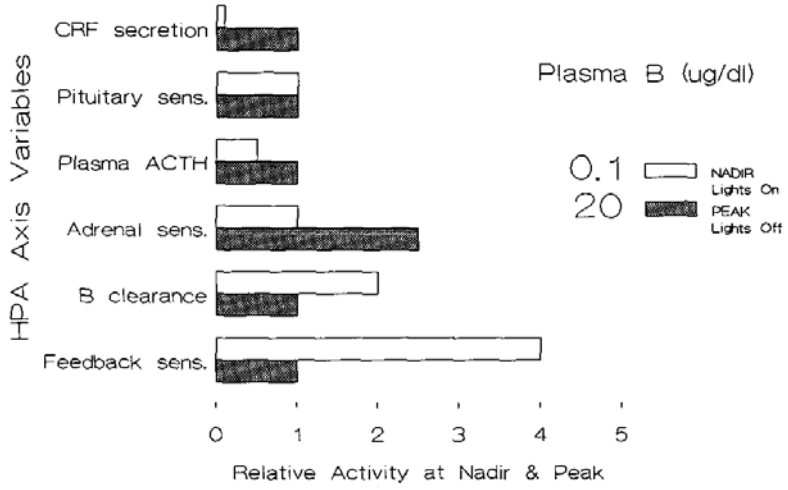


Fig.2.3. Schematic representation of basal diurnal rythms in components of the HPA axis in rat [5] B-corticosterone CRF-corticotropine-releasing factor=CRH

2.4. Glucocorticoid receptor (GR)

GR together with other steroid hormone receptors belongs to the nuclear receptor subfamily 3 [6]. Members of this subfamily share a modular structure that consists of three distinct functional regions, the variable N-terminal transactivation domain (NTD), highly conserved central DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD). Unliganded receptor is located in cytosol as a part of heterocomplex with molecular chaperones such as heat shock proteins (hsp) 40, 70 and 90 and several co-chaperones as FK560 binding proteins (FKBPs) and cyclophilin 40. Ligand binding promotes release of GR from the complex, nuclear import, homodimerization and modulation of gene transcription. GCs can exert its action direcly through binding to glucocorticoid response elements (GREs) located in promotor region of GR target genes. The nature of GRE affects the direction of transcriptional response. Positive GREs mediate upregulation and negative GREs downregulation of transcription. Transcription may be further enhanced or silenced by different coactivators or corepressors. GR can also act indirectly through protein-protein interaction with other transcription factors such as Jun/Fos complex, nuclear factor κB or STAT factors (Fig.2.4.).

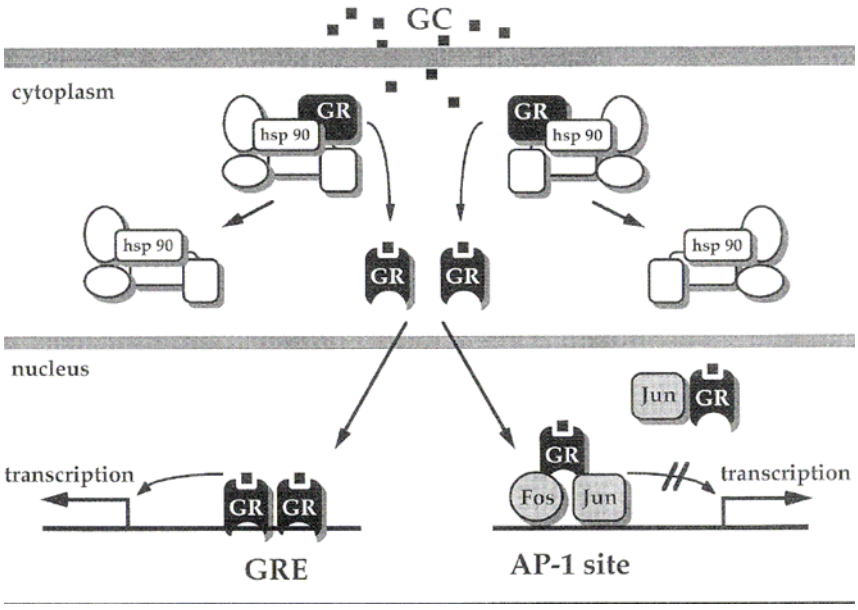


Fig.2.4. GR signaling pathway [7]

GR is ubiquitously expressed and 5 different subtypes resulted from alternative splicing of single precursor mRNA has been identified in man (Fig.2.5.)

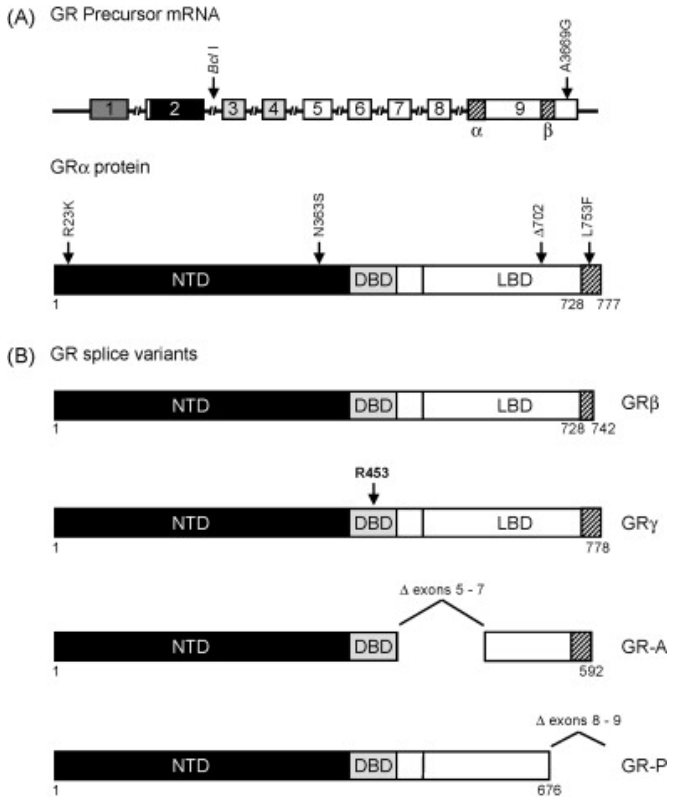


Fig.2.5. GR splicing variants [8]

Human GRα is the classic, functionally active subtype made up of 777 amino acids. Originally, GRβ was thought to control transcription only through negative effect on GRα-induced expression and not to interact with GR agonist but recently GRβ has been shown to regulate gene expression independently of GRα. GRβ is detected in most tissues and cell lines but generally at lower levels compared to GRα. In addition, GRγ, GR-A and GR-P splice variants have been described. These isoforms represent a minor portion of total GR mRNAs and their role has not been completely revealed (reviewed in [8]).

2.5. Tissue-specific effects of GCs

2.5.1. Liver

Liver is one of the key GC target tissues. Over 50 different genes has been described to be directly regulated by GCs. A number of other genes are regulated by GCs indirectly by interaction with other transcription factors [9]. The main role of GCs is the stimulation of *de novo* glucose synthesis, gluconeogenesis, during stress or fasting to provide glucose for extrahepatic tissues [10-11]. GCs stimulate expression of key gluconeogenic enzymes, in particular phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [11-12]. However, regulation of PEPCK expression is a complex process affected by many different transcription factors (Fig. 2.6.). Uncontrolled gluconeogenesis contributes to hyperglycemia in type 2 diabetic patients [10]. Consistently, liver-specific GR mutation in mice results in fasting hypoglycemia and is accompanied by downregulation of gluconeogenic enzymes [13]. Similarly, overexpression of GR has been found in hepatocytes of diabetic rodent models [14-15] and inhibition of GR expression results in an amelioration of diabetic phenotype [16].

GCs also affect lipid metabolism in hepatocytes, although the molecular mechanisms are not well described. Rats treated with GCs exhibited elevated synthesis of TGs and decreased β-oxidation of fatty acids [17]. In addition, ADX inhibited TG synthesis and this effect could be reversed by GC treatment [18]. GC treatment of isolated hepatocytes also resulted in VLDL assembly and secretion. These effects were driven by induction of lipogenic enzymes acetyl-CoA carboxylase (ACC) and fatty acid

synthase (FAS) and by downregulation of acyl-CoA dehydrogenase (Acyl CoA DH) (Fig.2.6.) [19-23]. GCs have been found to be increased in various animal models of obesity, dyslipidemia and hepatic steatosis. Similarly, patients with Cushing´s syndrome, a disease characterized by elevated systemic cortisol, develop dyslipidemia, central obesity and hepatic steatosis (reviewed in [24]).

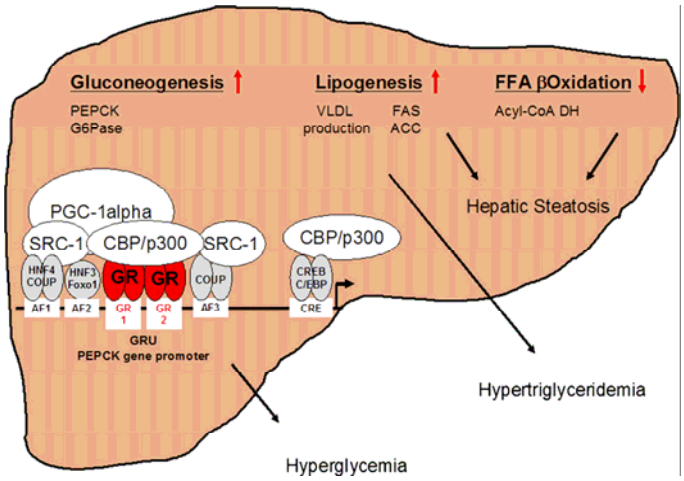


Fig. 2.6. GC regulated metabolic pathways in the liver [24]

2.5.2. Adipose tissue

The GC effects on adipose tissue are depot-specific. In central adipose depots, GCs stimulate preadipocyte differenciation as was shown in cell lines, primary cultures and animal models [25-26]. In subcutaneous adipose depots GCs increase lipolysis by induction of HSL and inhibit GLUT4 translocation to membrane lowering glucose uptake. In contrast to liver, PEPCK expression is inhibited by GCs in adipose tissue where it plays a different role than in liver. In adipose tissue PEPCK has been shown to stimulate glycerogenesis, a process of *de novo* synthesis of 3-glycerophosphate, a precursor of TG synthesis. Net result of these effects is increased lipolysis on periphery and TG accumulation in central adipose tissue [27-30]. This is consistent with studies on obese rats where treatment with RU486, a GR antagonist, or ADX resulted in reverse of obesity phenotype [31].

2.5.3. Skeletal muscle

In muscle GCs antagonize effects of insulin/insulin-like growth factor axis, the main anabolic pathway. They stimulate catabolic effects on protein metabolism *via* enhanced proteolysis, decreased transport of amino acids into muscle cells and inhibition of protein synthesis [32]. Excess of GC signaling in skeletal muscle may contribute to development of metabolic syndrome. This was shown in clinical study where positive correlation between GRα expression and some features of metabolic syndrome was found in human skeletal myoblasts [33]. In addition, chronically elevated GCs result in muscle atrophy due to inhibited proteosynthesis, an effect that can be observed in patients with Cushing´s syndrome [34].

2.6. Pre-receptor regulation of steroid hormone action – general overwiew

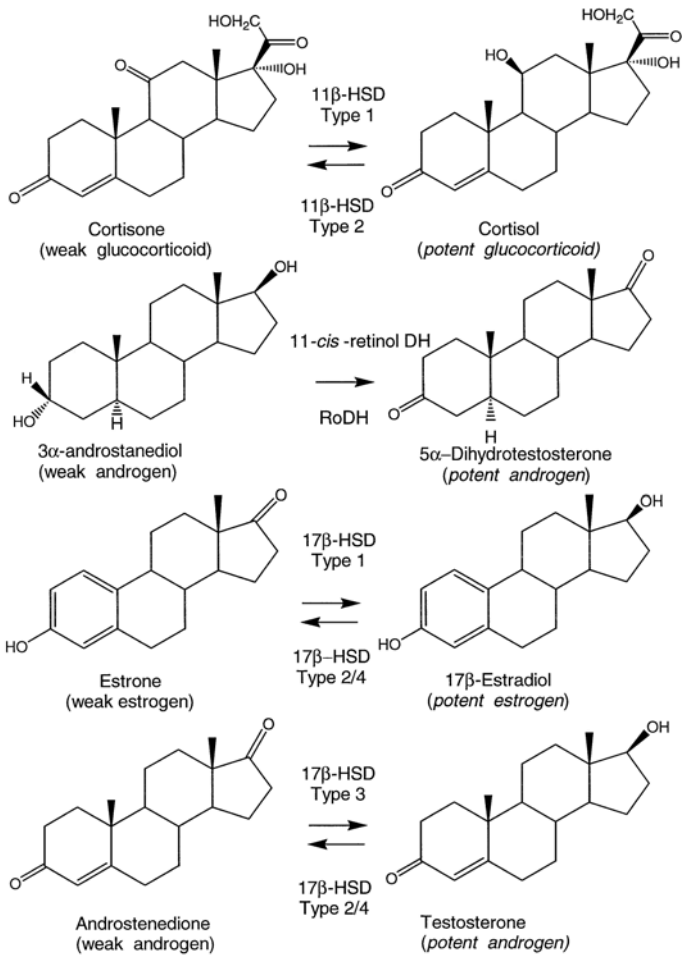
As discussed in previous chapters, GC action is tissue-specific and any dysregulation can contribute to the development of metabolic syndrome. GC action can be regulated on different levels including *de novo* synthesis in adrenal cortex and GR receptor level, however, one of the most important mechanisms is the pre-receptor regulation of intracellular concentration of active GCs.

In general, the most important action of all steroid hormones is regulation of gene transcription through activation of soluble intracellular receptors, although some nongenomic effects performed *via* specific steroid membrane receptors (or nonspecific membrane interactions) have been decsribed.

Steroid target tissues can modulate steroid hormone action not only by regulation of steroid receptor abundance but also by changes of the local concentration of biologically active steroid ligands through expression of hydroxysteroid dehydrogenases (HSDs), the oxidoreductases that are able to metabolize active form of steroids into inactive and *vice versa*. A large number of HSDs has been described. They differ in operating on different positions of steroid cycle and their expression in specific tissues and species. Structural analysis revealed that HSDs belong to two protein superfamilies: the short-chain dehydrogenase/reductase superfamily (SDR) and the aldo-keto reductase superfamily (AKR) (reviewed in [35-36]).

2.6.1. Short-chain dehydrogenase/reductase superfamily (SDR)

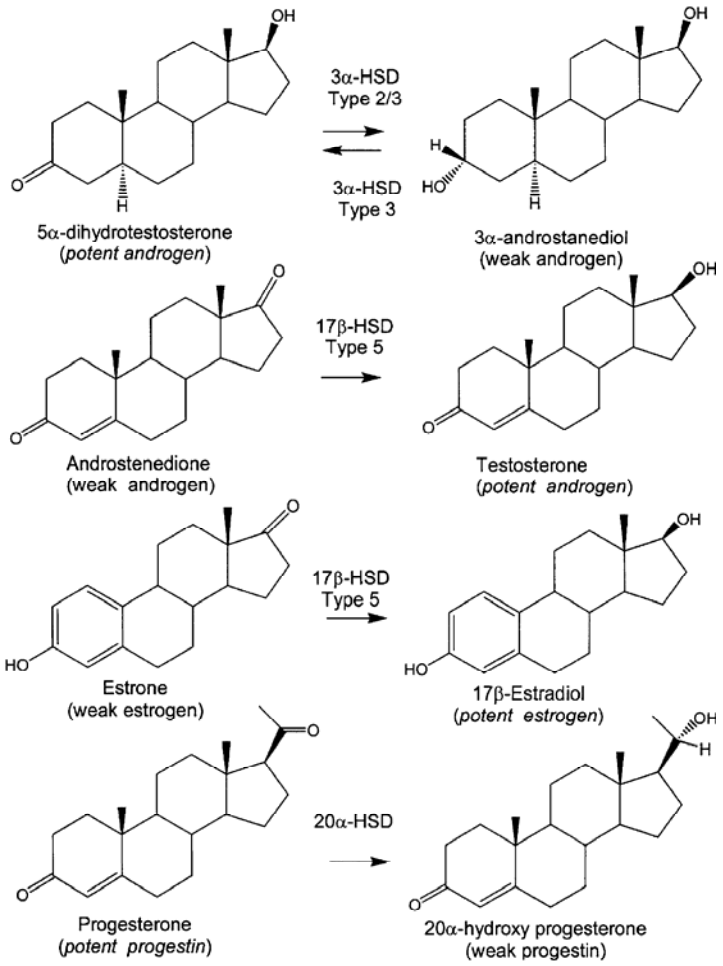
SDR superfamily is a well-established family of oxidoreductases sharing about 25% of sequence homology with typical length of monomers between 250-300 residues (25-40 kDa). Although sequence homology is quite low, all members share highly conserved three-dimensional structures. Characteristic structure is the N-terminal cofactor binding domain that includes an arrangement of α-helices and β-strands $(\beta-\alpha-\beta-\alpha-\beta)_2$ called Rossmann fold with conserved GXXXGXG sequence and central active site with conserved YXXXK sequence. These proteins are often membrane bound and form functional multimers. Except for HSDs this superfamily contains a group of prostaglandin dehydrogenases and non-metallo alcohol dehydrogenases. Fig.2.7. shows examples of action of selected members of SDR superfamily (reviewed in [35-36]).



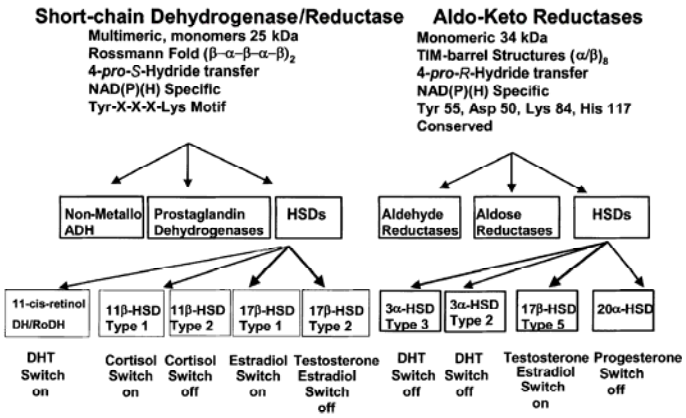
2.7. Examples of action of SDR superfamily members [36]

2.6.2. Aldo-keto reductase superfamily (AKR)

AKR superfamily contains monomeric, soluble oxidoreductases with average size of 37 kDa and characteristic $(\alpha-\beta)_8$-barrel (also called TIM-barrel) three-dimensional structure. Catalytic site is at the base of the barrel and contains four conserved amino acid residues. Together with various HSDs, aldehyde reductases and aldose reductases also belong to this superfamily. Fig. 2.8. shows examples of action of selected members of AKR superfamily. Fig.2.9. summarizes different features of protein superfamilies the HSDs belong to (reviewed in [35-36]).



2.8. Examples of action of AKR superfamily members [36]



2.9. Summary of SDR and AKR superfamily characteristics [36]

In further chapters we will focus on the key regulatory enzyme of local GC metabolism: the 11β-hydroxysteroid dehydrogenase (11HSD).

2.7. 11β-hydroxysteroid dehydrogenase (11HSD)

11HSD (E.C. 1.1.1.146) is an oxidoreductase which maintains an equilibrium between 11-hydroxysteroids and 11-oxosteroids in mammals. Nowadays, it is generally accepted that two types of 11HSD exist and that they act in opposite direction to each other. Type 1 (11HSD1) operates *in vivo* predominantly as a reductase, requires NADPH as a cofactor and is expressed mostly in liver and adipose tissue and to lesser extent in skeletal muscle, lung, vascular tissue, kidney, immune tissue and CNS. Type 2 (11HSD2) is an exclusive oxidase that requires NAD^+ as a cofactor and is expressed in mineralocorticoid target tissues and placenta (Fig 2.10.).

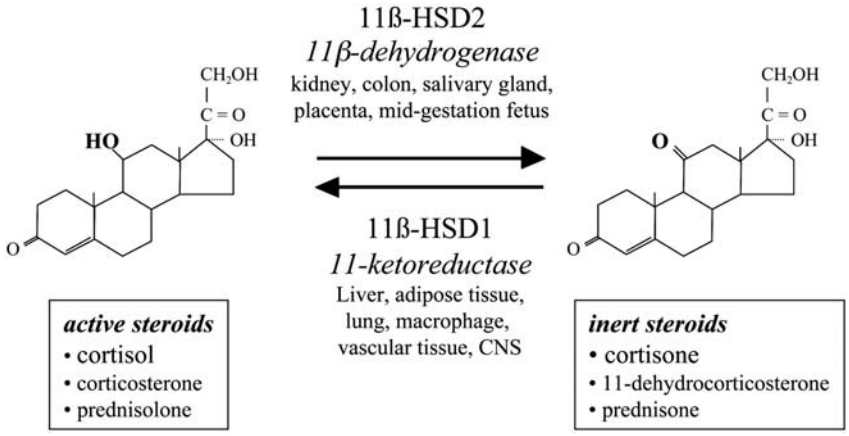


Fig.2.10. Overwiew of 11HSD isoforms [29]

In 1980s, 11HSD1 was purified from rat liver microsomes [37], although the ability of liver to metabolize adrenal hormones *in vitro* was already described in 1950s [38]. Two independent groups proved that action of renal 11HSD2 protects the mineralocorticoid receptor (MR) from binding of GCs [39-40], which are able to bind MR with the same affinity as mineralocorticoids [41]. In 1990s, 11HSD2 was identified in rabbit renal collecting duct [42] and cDNA was cloned from rat [43], ovine [44] and human [45] kidney. Subsequent isolation, cloning, *in vitro* expression and chracterization of both isoforms revealed that 11HSD proteins are products of distinct genes with low sequence homology, different enzymology and tissue distribution (reviewed in [46]).

2.7.1. 11β-hydroxysteroid dehydrogenase type 1 (11HSD1)

11HSD1 is an integral membrane enzyme of the endoplasmatic reticulum (ER) with lumenal orientation of an active site [47-49] that is active as a dimer [50]. Although cell lysates from chinese hamter ovary (CHO) cells transfected with 11HSD1 catalyzed both 11β-dehydrogenation and 11β-reduction [51], in intact cells 11HSD1 reductase activity predominated [52-56]. Kinetic studies of 11HSD1 expressed in cultured cells using recombinant Vaccinia virus suggested that cofactor availability might determine the direction of 11HSD1 activity [51]. Later on an important role of hexose-6-phosphate dehydrogenase (H6PDH) in this regulation was described by two independent groups [57-58].

2.7.1.1. Hexose-6-phosphate dehydrogenase (H6PDH) and its cooperativity with 11HSD1

H6PDH is a dehydrogenase localized on membrane of ER with luminal active site orientation [59] that catalyzes the first and rate limiting step of pentose phosphate pathway in ER and therefore represents a major source of intraluminal NADPH. In contrast to cytosolic glucose-6-phosphate dehydrogenase, H6PDH has broader substrate specifity undiscriminating among glucose-6-phosphate (G6P), galactose-6-phosphate, 2-deoxyglucose-6-phosphate etc. [60-61]. However, it is believed that under

physiological conditions H6PDH prefers G6P [62]. Intraluminal G6P supply is ensured by specific glucose-6-phosphate transporter (G6PT) [58, 63].

As mentioned earlier, reductase activity of 11HSD1 is affected by NADPH/$NADP^+$ ratio and is determined by intact subcellular structure. Fig. 2.11. shows a scheme of cooperation between 11HSD1 and H6PDH that provides a reasonable explanation of unstable 11-reductase activity in cell lysates or tissue homogenates - the presence of H6PDH in intact ER stimulates the reduction of 11-oxoderivates to biologically active glucocorticoids. In addition to functional cooperativity, two independent groups have recently described direct protein-protein interaction between these two enzymes [64-65]. Coexpression of H6PDH and 11HSD1 was confirmed in most of organs [66].

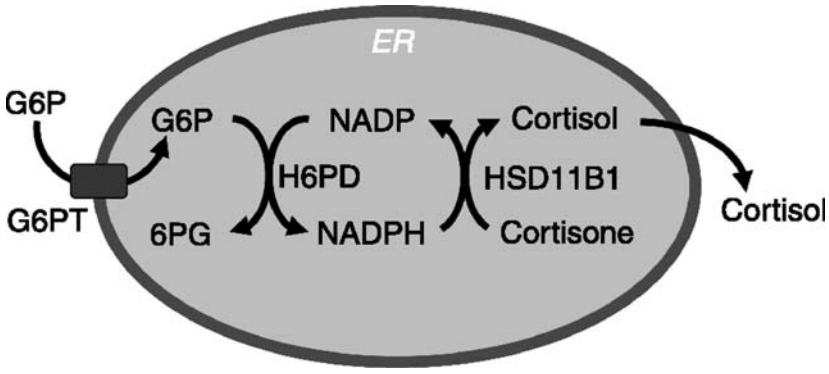


Fig.2.11. Scheme of G6PT- H6PDH-11HSD1 system in ER [58]

H6PD-hexose-6phosphate dehydrogenase, HSD11B1-11β-hydroxysteroid dehydrogenase

2.7.1.2. Tissue distribution of 11HSD1

11HSD1 shows the highest abundance in liver [67-68], predominately around the central vein [69-70]. In rat liver, a sexual dimorphism of 11HSD1 was observed in both activity and expression [66, 71] with significant supression in females. This seems to be mediated by sex-specific growth hormone (GH) release pattern (continous in females and pulsatile in males) and estrogen action [71]. Similar observations were found also in human studies where 11HSD1 activity decreased significantly in girls during puberty [72] and in adults 11HSD1 was higher in men than women [73-74].

However, no gender differences were found in mice [56]. 11HSD1 was also detected in different adipose tissue depots including human adipocyte stromal cells (ASC) and fully differentiated adipocytes [53, 70]. Here, GCs seem to play an important role during differentiation of ASC into adipocytes [52]. Also, 11-oxidase activity is switched into 11-reductase activity in omental ASC during differenciation [75]. This might be a consequence of increasing H6PDH expression during adipocyte differentiation which stimulates 11HSD1 reductase activity [76]. 11HSD1 is also expressed in human skeletal myoblasts where GCs sensitively regulate expression of GR isoforms and thus 11HSD1 might change GC sensitivity of skeletal muscle cells [77]. 11HSD1 was also found in cardiovascular tissues such as cardiac myocytes, cardiac fibroblasts and vascular smooth muscle cells [78-79]. GCs have been shown to potentiate noradrenalin-stimulated vasoconstriction [80] therefore 11HSD1 activity can influence vascular sensitivity to noradrenalin. 11HSD1 was also detected in immune tissues such as spleen [68], thymus [81], lymphocytes [82], macrophages [83] and lymph nodes [84]. 11HSD1 expression in these organs can modulate inflammatory response due to GC-mediated downregulation of proinflammatory cytokines as TNFα or IL1 [85]. 11HSD1 expression was also detected in kidney medulla [68], central nervous system [86-88] and other tissues [66, 89].

2.7.1.3. 11HSD1 genomics

First 11HSD1 cDNA sequence was cloned from rat liver [67] and then also from human testis [90]. Human cDNA is about 1.4 kb long, open-reading frame has 876 bp and full-lenght protein consists of 292 amino acids. Human and rat 11HSD1 share approximately 77% homology. 11HSD1 was then identified in other mammalian species as mice [56, 91], sheep [92], guinea pig [93] or squirell monkey [94]. Rat gene is located on chromozome 13 and consists of 6 exons. A whole transcript has 1529 bp and full-lenght protein consists of 288 amino acids (www.ensembl.org). Different promotors were identified in rat liver and kidney [95], however they share some general features. Analysis of promoters revealed the presence of CCAAT sequence, a binding site for CAAT/enhancer binding proteins (C/EBPs) and lack of TATA box. Also several transcription factor binding sites were identified as GRE or binding site for hepatocyte nuclear factor 1 and 3 (HNF1, HNF3) [96].

2.7.1.4. Regulation of 11HSD1 activity and expression

11HSD1 activity and expression is regulated by multiple molecules whose effects have been shown to be tissue-specific. The most important effectors in liver, adipose tissue and skeletal muscle are discussed in the next subchapters.

2.7.1.4.1. Regulation of 11HSD1 *via* transcription factors

CAAT enhancer binding proteins (C/EBPs) represent important group of 11HSD1 regulators. C/EBPα has been shown to be a potent inductor of 11HSD1 when cotransfected in human hepatoma cell line. In the same study C/EBPβ acted as a weak inductor, however when both isoforms were coexpressed together C/EBPβ attenuated the induction of 11HSD1 expression via C/EBPα [97]. Also, C/EBPα knock-out mice displayed liver 11HSD1 deficiency, in contrast to C/EBPβ knock-out mice where 11HSD1 upregulation was found [97-99]. These studies suggest that C/EBPβ antagonises the effect of C/EBPα in liver. In contrast, both C/EBPα and C/EBPβ induce 11HSD1 transcription in murine preadipocyte cell line [100].

11HSD1 has been also shown to be regulated by peroxisome proliferator-activated receptor α and γ (PPARα and PPARγ) ligands. PPARγ receptors are nuclear receptors highly expressed in white adipose tissue that serve as a target for a group of antidiabetic drugs called thiazolidinediones (TZDs) but also for some other group of non-TZD antidiabetic drugs. Candidates of both groups were able to decrease 11HSD1 expression in murine adipocyte cell line and also in different adipose tissue depots of rats and mice [101-102]. PPARα nuclear receptors are expressed mainly in the liver and stimulate β-oxidation of fatty acids. Fenofibrate, as an agonist of PPARα receptor, decreased 11HSD1 expression in murine liver after chronic treatment, however no effect on 11HSD1 was observed in PPARα knock-out mice [103].

Agonists of liver-X-receptors (LXRs) represent another group of important 11HSD1 regulators. LXRα is mostly expressed in adipose tissue and liver where it predominates over the more ubiquitously expressed LXRβ. LXRs act as ligand-activated transcription factors with distinct oxysterols as endogenous ligands that have been shown to influence lipid metabolism. Treatment of murine adipocytes with different synthetic and natural LXR ligands resulted in decreased 11HSD1 activity and

expression and long term treatment of wild type mice with synthetic agonist resulted in downregulation of 11HSD1 mRNA in brown adipose tissue and liver [104].

In summary, C/EBPα is a potent stimulator of 11HSD1 expression while LXRα downregulates 11HSD1 expression in liver and adipose tissue. 11HSD1 is also downregulated by PPARs in tissue-specific manner as PPARα is expressed predominately in liver and PPARγ in adipose tissue.

2.7.1.4.2. Hormonal regulation of 11HSD1

Studies that tested the effects of GCs on 11HSD1, particulary the effect of synthetic glucocorticoid dexamethasone (DEX) provided inconsistent results. In rat hepatocyte primary cultures and rat hepatocyte cell lines, DEX stimulated 11HSD1 activity [54, 105-106] but *in vivo* oral administration caused downregulation of 11HSD1 [107-108]. In murine adipocyte cell lines, DEX inhibited 11HSD1 expression and activity in time and concentration dependent manner and also chronic oral administration of DEX in Wistar rats resulted in 11HSD1 downregulation [109-110]. In contrast, treatment of human ASC primary cultures with cortisol and insulin for 12 days resulted in upregulation of 11HSD1, although, in this case, stimulatory effect was propably indirect *via* stimulation of adipocyte differentiation [53]. In contrast to adipose tissue, cortisol stimulated 11HSD1 activity in primary cultures of human skeletal myoblasts [33].

Insulin antagonizes GC action in organism therefore certain number of *in vitro* and *in vivo* studies was performed to determine its effects on 11HSD1. Insulin inhibited 11HSD1 activity in rat hepatocyte primary cultures [105] and in hepatocyte cell lines [106]. In adipocytes, effects of insulin on 11HSD1 is not clear. 11HSD1 activity and expression have been shown to be both upregulated [109] and downregulated [110]. However, downregulation was observed only under supraphysiological dose of insulin whereas physiological dose had no effect [110]. Treatment of male Wistar rats with long-acting insulin analog glardine for 7 days lowered 11HSD1 activity and expression in epididymal adipose tissue [109].

It was also shown that 11HSD1 action is strongly affected by growth hormone-insulin-like growth factor (GH-IGF) axis. Ability of GH to regulate 11HSD1 was first demonstrated in the study of GH-deficient „dwarf“ rats where administration of GH

decreased 11HSD1 activity and mRNA expression in liver. However, it is important that the GH administration is continous not pulsatile. Single dose of GH had no effect on liver 11HSD1 [71]. Similar results were obtained on hepatocyte cell lines where 11HSD1 activity was lowered by IGF1 [106]. GH and IGF1 also decreased 11HSD1 activity in primary cultures of human adipocytes [111] and 11HSD1 expression in human ASC cells from subcutaneous and omental fat [112]. Finally, treatment of GH-deficient patients with GH resulted in inhibition of 11HSD1 expression in adipose tissue [113].

Another hormones that strongly influence metabolic processes in organism are adipokines. Specifically, impaired leptin action has been shown to contribute to development of metabolic syndrome in a number of rodent models (will be discussed in detail in chapter 2.8.2. Leptin is a 16kDa protein syntesized in adipose tissue. It was first identified in mouse and human and shown to regulate the size of adipose tissue depots [114]. Leptin administration resulted in increased hepatic 11HSD1 activity and expression in leptin-deficient mice strain [115]. In contrast, it had no effect on human hepatocyte primary culture even though it stimulated 11HSD1 activity in primary cultures of subcutaneous and omental ASC [112].

Finally, sex steroid hormones are also able to influence 11HSD1. Estradiol has been shown to inhibit 11HSD1 in murine adipocyte cell line and in isolated adipocytes from rat mesenteric adipose tissue [116]. *In vivo*, it supressed hepatic 11HSD1 activity and expression in wild-type and hypophysectomized rats [71, 108]. In contrast, stimulation of 11HSD1 expression was observed in cultured ASC isolated from visceral adipose tissue of women but not men [117]. Testosterone had no effect on hepatic 11HSD1 in rats [71, 108] whereas in man mild upregulation of 11HSD1 by dihydrotestosterone was observed in cultured ASC [117].

In summary, it seems that GCs and insulin exhibit tissue-specific antagonic effects on 11HSD1 activity and expression. Estradiol and GH inhibit 11HSD1 in contrast to leptin that stimulate this enzyme.

2.7.1.4.3. Regulation of 11HSD1 *via* cytokines

Cytokines such as TNFα and IL1β mostly upregulate 11HSD1 expression in various tissues. TNFα induced 11HSD1 expression in rat hepatocyte cell line indirectly

via enhanced binding of C/EBPβ to the 11HSD1 promotor [118], although C/EBPβ itself acts as a weak inductor of 11HSD1 [97]. Similar TNFα induction of 11HSD1 was observed in human hepatoma cell line [119] and in human ASC primary cultures from subcutaneous and omental depots [112, 120]. In contrast, TNFα treatment of human hepatocyte primary cultures had no effect on 11HSD1 [112]. IL1β stimulated 11HSD1 expression in human hepatocyte cell line [119] and primary cultures of human ASC whereas it had no effect on human hepatocyte primary cultures [112].

2.8. 11HSD1 and metabolic syndrome

As mentioned earlier, metabolic syndrome is a complex disease with complicated pathogenesis, many risk factors and different phenotypes. A number of drugs targeting different regulatory mechanisms is used to treat the symptomes including thiazolinediones, the PPARγ ligands, or fibrates, the PPARα ligands and many others. In the last decade many studies on rodent models and human clinical studies showed that 11HSD1 manipulation has beneficial effect on metabolic syndrome. In next chapters we will summarize the major groups of 11HSD1 inhibitors focusing on drugs that have been already tested on rodents or primates. We will also discuss studies on rodent models and clinical studies that were focused on relationship between metabolic syndrome and GCs.

2.8.1. 11HSD1 inhibitors

2.8.1.1. Nonselective 11HSD1 inhibitors

One of the most potent competitive inhibitor of both 11HSD1 and 11HSD2 is the glycyrrhetinic acid (GA) and its soluble derivative carbenoxolone (CBX) (Fig.2.12.) with IC50 about 10 nmol/l (measured on rat kidney homogenates) [121-122]. CBX has been tested for treatment of metabolic syndrome in both animal models and clinical studies. It was shown that CBX treatment of obese Zucker rats downregulated 11HSD1 protein content in the liver and elevated plasma levels of HDL cholesterol [123]. In human studies CBX improved insulin sensitivity in healthy subject and in patients with type 2 diabetes [123-125]. However, CBX failed to inhibit 11HSD1 in adipose tissue

[126]. Despite the fact that in some studies GA displayed similar inhibitory effects on both 11HSD isoforms, the recent studies have shown that 18α-GA stereoisomer (Fig.2.12.A) acts as selective 11HSD1 inhibitor with IC50 about 800 nmol/l (measured on HEK-293 cell lysates stably transfected with human 11HSD1) [127-128].

A B C

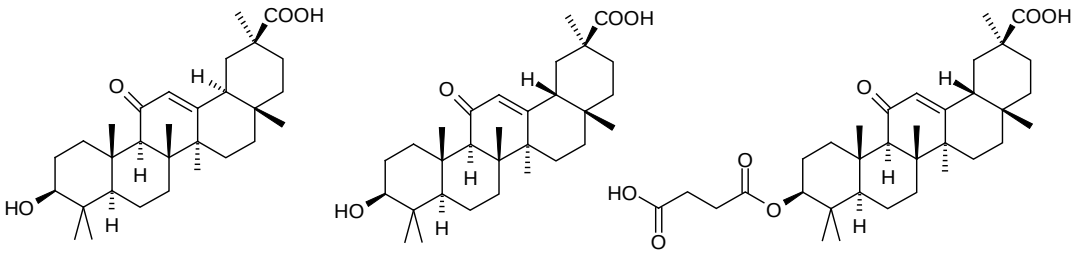


Fig.2.12. A. 18α-glycyrrhetinic acid B. 18β-glycyrrhetinic acid, C. carbenoxolone

Other compounds that nonselectively inhibit both types of 11HSD are flavonoids as gossypol [129] or naringenin [130], although some of the flavonoids such as quercetin seem to act as selective 11HSD1 inhibitors that improve dyslipidemia in diabetic rat [131]. 11HSDs are also inhibited by a number of compounds naturally occuring in organism as progesteron, 11-hydroxyprogesteron, deoxycorticosterone, 5α- and 5β- adrenocorticosteroids [121, 132] or bile acids [133].

2.8.1.2. Selective 11HSD1 inhibitors

The first compound that was shown to act as selective 11HSD1 inhibitor was a thiazole based derivative developed by Biovitrum and assigned as BVT-2733 (Fig.2.13.A). It was chosen from a group of arylsulfonothiazole derivatives and it displayed over 200-fold selectivity over human and murine 11HSD2 and IC50 52 nmol/l. In hyperglycemic KKAy mice BVT-2733 significantly reduced blood glucose and insulin, most likely due to supressed gluconeogenesis because expression of PEPCK and G6Pase was decreased in liver. BVT-2733 also improved hepatic insulin sensitivity [134-136]. However, BVT-2733 was 35 fold-less potent inhibitor of human

11HSD1 compared to murine [136]. In 2003, Biovitrum created a corporation with Amgen and focused on compounds with thiazolone core and developed a compound AMG-221, an adamantan-based thiazolone derivative (Fig.2.13.B). In mice with diet-induced obesity (DIO) treatment with this drug lowered plasma glucose and insulin. At 2008 this compound entered a I. Phase of clinical trials [137].

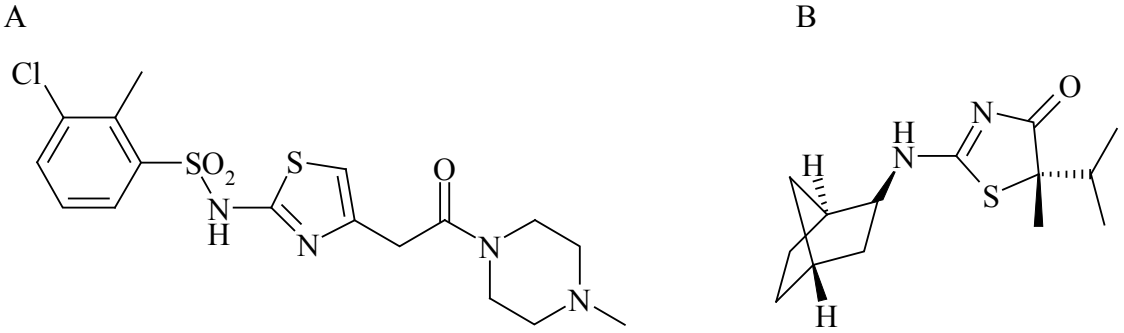


Fig.2.13. A. BVT-2733 B. AMG-221

Another compound, a aminopyridyl cyanobiphenyl sulfonamid PF-915275 (Fig. 2.14) was developed by Pfizer. *In vitro* experiments proved a great efficacy on human 11HSD1 (IC50 = 5 nmol/l) but not on murine or rat 11HSD1. Therefore it was tested on macaques with maximum inhibition of 23 % of controls (measured as oral prednisone test). Chronic treatment lowered insulin serum levels but did not affect serum lipids or glucose [138]. The Phase I clinical trials showed that PF-915275 acts as selective inhibitor in human *in vivo* (measured by urine cortisol metabolite analysis and oral prednisone test), is safe and well-tolerated [139]. Phase II clinical trials are now in progress.

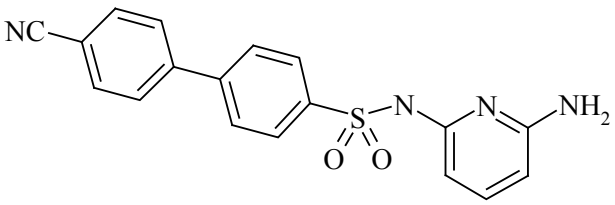


Fig.2.14. PF-915275

Abbott company focused on testing a group of butyrolactame-based compounds and developed an adamantyl derivative (Fig.2.15.) with over 7500-fold 11HSD1

selectivity over 11HSD2 and IC50 about 3 nmol/l. It was tested in the study of DIO mice model of metabolic syndrome when GR antagonist RU-486 and rosiglitasone, a thiazolinedione based antidiabetic drug, were used as positive controls. Chronic treatment with Abbot´s compound lowered body weight and plasma insulin at the similar level as RU-486 and rosiglitasone. Remarkable improvement was observed in serum TG levels that were lowered to the level comparable with healthy controls [140].

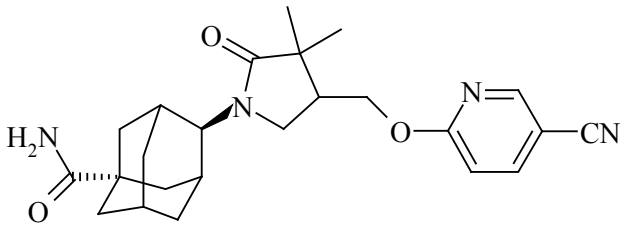
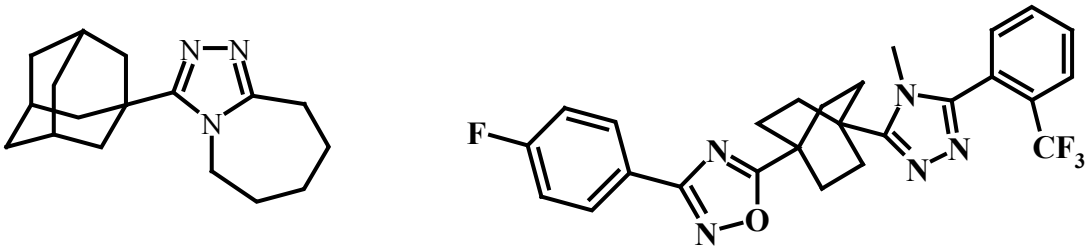


Fig.2.15. Abbott´s compound

Another group of chemical compounds tested for selective inhibition of 11HSD1, triazol-based derivatives, was developed by Merck. Two compounds were tested on rodent models of metabolic syndrome. Compound 544 (C544), an adamantyl triazole (Fig.2.16.A), lowered body weight, serum levels of insulin, glucose, TGs and cholesterol after chronic treatment of DIO mice. In diabetic streptozocin-treated mice kept on hig fat diet (HF/STZ mice) C544 improved insulin sensitivity and decreased serum glucose, insulin, TGs and NEFAs. Finally, in ApoE knock-out mice, a model of atherosclerosis, chronic treatment slowed the atherosclerotic plaque progression [141-142]. Compound A, a heteroarylbicyclo[2.2.2.]octyltriazol (Fig.2.16.B) lowered hepatic production of VLDL lipoproteins in rats kept on high-fat diet [143] and increased the rate of hepatic fatty acid oxidation [144]. Its effect on adipose tissue was depot-specific, while it decreased mesenteric fat weight and adipocyte size there was no effect on epididymal and retroperitoneal fat. Similarly, treatment of rats kept on high fat diet with Compound A decreased expression of lipid biosynthetic enzymes and increased fatty acid β-oxidation enzymes in mesenteric fat and reduced plasmatic lipids and hepatic TG levels [145].

A B



2.16. A. Compound 544 B. Compound A

2.8.2. Rodent models of metabolic syndrome

In next subchapters we will discuss rodent models used to demonstrate relationship between 11HSD1 and metabolic syndrome.

2.8.2.1. Transgenic models

2.8.2.1.1. 11HSD1 knock-out mice (11HSD1 $^{-/-}$ mice)

11HSD1$^{-/-}$ mice are viable and healthy but are unable to convert 11-dehydrocorticosterone (11-DEH) to CS. When 11-DEH pellets were implanted subcutaneously into adrenalectomized wild type mice and 11HSD1$^{-/-}$ mice, no CS was detected in plasma of 11HSD1$^{-/-}$ mice. In contrast to adrenalectomized animals, in sham-operated animals CS plasma levels of 11HSD1$^{-/-}$ mice exceeded levels of plasma CS observed in wild type animals. This experiment showed a robust capacity of organism to regenerate inactive glucocorticoids. 11HSD1$^{-/-}$ mice also displayed increased *in vivo* and *in vitro* responsiveness to ACTH [146-147] and increased insulin sensitivity [148]. Upon fasting no induction of gluconeogenesis (measured as expression of PEPCK and G6Pase) was observed in liver [147]. 11HSD1$^{-/-}$ mice have lower plasma TGs, increased HDL cholesterol and increased hepatic lipid catabolism [149]. 11HSD1$^{-/-}$ mice fed with high fat diet gain less weight than wild type animals despite higher caloric intake [148].

2.8.2.1.2. Mice overexpressing 11HSD1 in adipose tissue (aP2-11HSD1 mice)

In this mouse strain, 11HSD1 in adipose tissue is upon the control of strong promotor of fatty acid binding protein aP2, a marker of fully differentiated adipocytes. These mice develop visceral obesity due to increased adipocyte size (Fig.2.17.). Upon high fat diet a weight gain is exaggerated. Glucose tolerance test revealed insulin resistance. An increased plasma levels of leptin together with hyperfagia suggests that leptin resistance emerged in aP2-11HSD1 mice. Measurements of plasma lipids showed increased levels of NEFAs and TGs [26]. Finally, increased high arterial blood pressure was detected in aP2-11HSD1 mice [150].

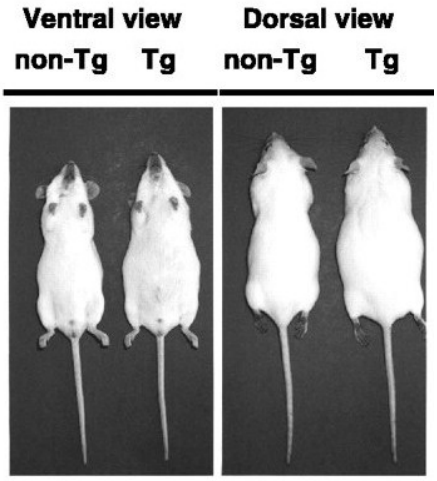


Fig. 2.17. aP2-11HSD1 mice [26]

2.8.2.1.3. Mice overexpressing 11HSD1 in liver (apoE-11HSD1 mice)

In the liver of apoE-11HSD1 mice, 11HSD1 is upon the control of promotor sequences derived from human apoE gene. Transgenic animals display 2- to 5-fold increased 11HSD1 activity in liver and exhibit hepatic steatosis and increased hepatic lipid synthesis due to increased expression of FAS and LXRα. These animals also have elevated plasma level of NEFAs, insulin and develop modest insulin resistance and hypertension. In contrast to aP2-11HSD1 mice, apoE-11HSD1 mice are not obese [151].

In conclusion, studies performed on transgenic rodent models clearly showed that 11HSD1 is important, tissue specific regulator of metabolic processes and its manipulation provides useful tool for study of metabolic syndrome and its treatment.

2.8.2.2. Leptin-deficient and leptin-resistant rodent models

2.8.2.2.1. Leptin-deficient *ob/ob* mice

Ob/ob mice carry a mutation in *ob* locus encoding leptin. They suffer from hyperglycemia, hyperinsulinemia, hypercorticosterolemia and hepatic steatosis and develop severe obesity (Fig. 2.18.). Leptin treatment attenuates all of these disorders, however not to the level of the lean counterparts. These animals also show decreased hepatic 11HSD1 activity and mRNA compared to controls. In hepatocyte primary cultures, leptin increases 11HSD1 activity in a dose-dependent manner [115, 152]. In adipose tissue, 11HSD1 activity and expression is elevated in visceral depots and downregulated in subcutaneous depot [152]. Chronic treatment of *ob/ob* mice with selective 11HSD1 inhibitor BVT.2733 resulted in decrease of plasma glucose and insulin [135].

Fig. 2.18. *ob/ob* mice [nsatng.wordpress.com]

2.8.2.2.2. Leptin-resistant *db/db* mice

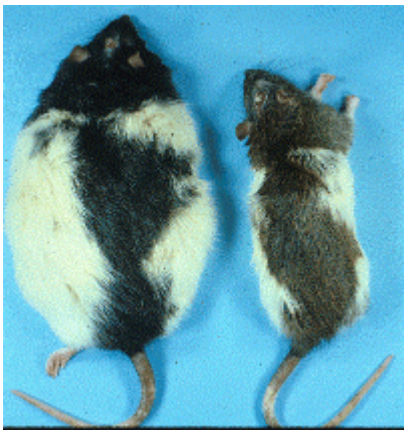
Leptin-resistant *db/db* mice carry a mutation in *db* locus encoding leptin receptor, however this mutation only affects a long splicing variant of leptin receptor b,

which is mostly expressed in hypothalamus [153]. Liver-type shorter leptin receptor splicing variant a is unaffected. Similarly to *ob/ob* mice, these animals display hyperglycemia (even more severe than *ob/ob* mice), hyperinsulinemia, hypercorticosterolemia and hepatic steatosis as well as severe obesity. In contrast to *ob/ob* mice, plasma leptin is elevated. Studies of hepatic 11HSD1 provided inconsistent results. Liu *et al.* showed elevated 11HSD1 activity and expression in liver *in vivo* and in hepatic primary cultures as well as elevated expression of GR and PEPCK. This is in agreement with a hypothesis that hypothalamic leptin receptors b are defective while liver 11HSD1 is stimulated *via* leptin receptors a. Hyperglycemia is propably caused by upregulated PEPCK [15]. On the other hand, more recent study showed decreased hepatic 11HSD1 in *db/db* mice. Dysregulation of 11HSD1 in liver of *db/db* mice therefore remains unclear. 11HSD1 activity was higher in retroperitoneal and omental visceral adipose tissue of *db/db* mice and lower in epidydymal visceral adipose tissue and subcutaneous adipose tissue. However, only in subcutaneous adipose tissue mRNA expression correlated with activity [152]. Treatment of *db/db* mice with bezafibrate, a PPARα agonist, reduced hyperglycemia, serum TGs and NEFAs and improved insulin sensitivity. It was also accompanied by decreased 11HSD1 expression in adipose tissue [154].

2.8.2.2.3. Zucker rats and Zucker diabetic fatty rats (ZDF)

Zucker rats are leptin-resistant animals carrying a mutation in *fa* locus encoding leptin receptor that exhibit increased plasma lipids, cholesterol, glucose and CS and develop obesity (Fig.2.19.) [155]. In contrast to *db/db* mice, this mutation affects both splicing isoforms of leptin receptor. An inbred strain named Zucker diabetic fatty rats (ZDF) was bred by selecting individuals with unusually high glucose. Obese ZDF rats display TG accumulation in liver, skeletal muscle and heart and insulin resistance, which is manifestated by decreased insulin-stimulated glucose uptake and decreased total glycogen content in skeletal and cardiac muscle [156]. 11HSD1 activity and mRNA is decreased in liver, unchanged in skeletal muscle and subcutaneous adipose tissue and increased in visceral adipose tissue of obese Zucker rats compared to lean controls [157]. ADX attenuated weight gain and normalized 11HSD1 activity in visceral adipose tissue. Insulin sensitizers, metformin and rosiglitazone, improved insulin sensitivity of obese animals but had no effect on 11HSD1 activity or weight

[158]. Nonselective inhibititors had only mild effect on 11HSD1 or metabolic disorders in obese Zucker rats. CBX treatment decreased 11HSD1 protein content in liver but not in adipose tissue and skeletal muscle. It did not affect glucose tolerance, weight or food intake, however it slightly increased plasma HDL cholesterol [123]. The only effect of GA treatment was slightly lowered plasma leptin [159]. No selective 11HSD1 inhitors were tested on Zucker rats.



2.19. Zucker rats [www.med.howard.edu/anatomy/gas/wk14/HYPO3.gif]

These models show that leptin deficiency or resistance is always accompanied with obesity and tissue-specific dysregulations of 11HSD1. In *ob/ob* mice selective inhibition of 11HSD1 improved some metabolic disturbances even though 11HSD1 dysregulation was not the primary cause of metabolic syndrome.

2.8.3. Clinical studies

A number of clinical studies has been performed to explore relationship between metabolic syndrome (particularly with obesity) and 11HSD1 activity and mRNA expression in human organs. Initial studies on obese individuals, where 11HSD1 activity was measured indirectly as a ratio of cortisol to cortisone metabolites in urine provided rather inconsistent results. Index of total body 11HSD1 activity was shown to be increased [160-162], decreased [163] and unchanged [124] in obesity. However, it is impossible to assess any tissue-specific dysregulations from this ratio. If oral cortisone test was used, the data indicated downregulation of 11HSD1 in obese patients [160, 163-164]. However, this has not been found in patients with type 2 diabetes [165].

Most of the studies show positive association between metabolic syndrome features in man and 11HSD1 determined *in vitro* in subcutaneous adipose tissue biopsies or *in vivo* by isotype infusion or microdialysis [126, 160, 162, 164, 166-172]. These studies were performed either exclusively on men or women or involved both sexes. In contrast, no correlation was shown between 11HSD1 expression and obesity in women with wide range of BMI and age [173]. Other study showed induction of 11HSD1 activity and expression in obese patients after significant weight loss in adipocytes isolated from subcutaneous fat biopsies [89].

Studies of 11HSD1 in visceral adipose tissue provided much more controversial findings. 11HSD1 has been shown to have higher activity in visceral than subcutaneous cultured ASC, however no correlation was found between 11HSD1 and obesity [121, 173]. In contrast, another studies either did not find any differences in 11HSD1 expression between those adipose tissue depots [174] or showed increased expression in visceral adipose tissue compared to subcutaneous adipose tissue in morbidly obese patients [169]. The most consistent result was a positive correlation between 11HSD1 and adipocyte size [174-176].

11HSD1 is also expressed in human skeletal muscle. Positive correlation was found between 11HSD1 in isolated skeletal myoblasts and blood pressure and insulin sensitivity [33, 177].

In summary, clinical studies on patients with metabolic syndrome were mostly focused on 11HSD1 in adipose tissue. It seems that obesity is associated with increased 11HSD1 expression in subcutaneous adipose tissue. In visceral adipose tissue different studies provided inconsistent results therefore the role of 11HSD1 in visceral adipose tissue remains unclear. Upregulated 11HSD1 was also found in skeletal muscle.

2.9. Conclusion and thesis postulation

In previous chapters we described the interconnection between local metabolism of GCs represented by 11HSD1 and metabolic syndrome. Similar to GCs that exhibit different effects in key metabolic organs, also the tissue-specific 11HSD1 dysregulation results in different phenotypes. Mice with overexpressed hepatic 11HSD1 do not develop obesity but display number of symptoms of metabolic syndrome (chapter 2.8.2.1.3.) and mice with overexpressed 11HSD1 in adipose tissue develop central obesity in addition to metabolic syndrome (chapter 2.8.2.1.2.). Similarly, obese rodent models mostly exhibit 11HSD1 upregulation in visceral adipose tissue.

Institute of Physiology AS CR possesses a unique model of metabolic syndrome, Prague hereditary hypertriglyceridemic rats (HHTg) that was originally prepared by Dr. Vrána from Institute of Clinical and Experimental Medicine in Prague. HHTg rats represent a non-obese model of metabolic syndrome without genetic manipulations or specific mutations. The strain was bred by cross mating of Wistar rat individuals with elevated serum levels of TGs [178]. When kept on high carbohydrate diet HHTg rats exhibit alterations in glucose homeostasis. After glucose stimulation, no GLUT4 upregulation appeared in plasma membrane fraction of skeletal muscle and adipose tissue compared to controls and glucose also failed to stimulate glucose utilization in liver, skeletal muscle and adipose tissue. These results show alterations of insulin signaling pathway in key metabolic organs [179]. In addition, significantly higher systolic, diastolic and mean arterial blood pressure [180] associated with hypertrophy of left and right heart ventricles and aorta [181] was demonstrated in these rats compared to healthy animals.

As there are no studies focused on connection between metabolic syndrome and GCs in HHTg rats we decided to study local metabolism of GCs in HHTg rats under basal and stress conditions. We also tested effects of pharmacological intervention on different components of GC action and on selected serum parameters.

3. Aims

The primary objective of the thesis was to determine local metabolism of GCs in HHTg rats that resemble patients with non-obese metabolic syndrome. We hypothesized that the metabolism is changed in these animals in comparation with healthy normotriglyceridemic Wistar rats. Four aims were investigated.

3.1. Local metabolism of GCs in selected tissues of HHTg rats

A number studies on rodent models of metabolic syndrome have shown that this syndrome is often associated with tissue specific dysregulation of 11HSD1. We decided to test whether HHTg rats exhibit changes in 11HSD1 of key metabolic organs, in particular liver, subcutaneous adipose tisue, visceral adipose tissue and skeletal muscle. We measured the levels of active 11HSD1 protein (11HSD1 activity) and 11HSD1 mRNA expression. In addition, we measured mRNA expression of other important components of GC action, such as GR (chapter 2.4.) and H6PDH (chapter 2.7.1.1.) and serum levels of TGs, total cholesterol (CHOL), HDL, LDL+VLDL, NEFAs, insulin (INS), glucose (GLU) and CS.

3.2. Effect of 24h fasting on local metabolism of GCs in HHTg rats

Adaptation to fasting is a complex stressful process that is predominately controlled by GCs. 11HSD1 plays an important role in regulation of GC level in organism and therefore we decided to test whether there are tissue- and strain- specific differences in response to fasting on the level of 11HSD1 activity and 11HSD1, H6PDH and GR mRNA expression in liver, subcutaneous adipose tissue, visceral adipose tissue and skeletal muscle of HHTg rats and Wistar rats. We also studied changes in serum levels of TGs, CHOL, HDL, LDL+VLDL, NEFAs, INS, GLU and CS.

3.3. Effect of CBX treatment on local metabolism of GCs in HHTg rats

As discussed earlier in chapter 2.8.1.1., CBX is a potent but nonselective inhibitor of HSDs. We tested the effect of chronic CBX treatment on serum levels of TGs, CHOL, HDL, LDL+VLDL, NEFAs, INS, GLU and CS in HHTg rats. In addition, we tested effect of CBX treatment on the level 11HSD1 activity and m RNA expression and on GR and H6PDH mRNA expression in liver, subcutaneous adipose tissue, visceral adipose tissue and skeletal muscle of HHTg rats.

3.4. Effect of C544 treatment on local metabolism of GCs in HHTg rats

As discussed in chapter 2.8.1.2., C544 is potent inhibitor of 11HSD1 with significant therapeutical effect in different mice models of metabolic syndrome. Therefore, we decided to study the effect of chronic C544 treatment on serum levels of TGs, CHOL, HDL, LDL+VLDL, NEFAs, INS, GLU and CS in HHTg rats. We also studied effect of C544 treatment on 11HSD1, H6PDH and GR mRNA expression in liver, subcutaneous adipose tissue and visceral adipose tissue.

4. Materials and methods

4.1. Animals

Three-month-old female HHTg rats and Wistar rats were obtained from local breed of the Institute of Physiology, Academy of Sciences of the Czech Republic, Prague and were housed in a room with controlled light cycles (12-hours light/12-hours dark) and temperature (22 °C). Except of special conditions during experiments (chapter 4.3.) animals had free access to standard laboratory chow and tap water. Animals were killed by cervical dislocation and blood was collected. Samples of liver, subcutaneous adipose tissue (SAT), visceral adipose tissue from ovarial area (VAT) and skeletal muscle were excised and used for further analysis. All procedures were approved by the Czech Academy of Sciences Animal Care and Use Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

4.2. Chemicals and kits

Sigma-Aldrich, St. Louis, MO, USA: NADPH, $NADP^+$, CBX, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, DNAse I, STOP solution
Qiagen, Inc.: RNEasy Lipid Tissue Mini Kit, RNase-free DNase set
Agilent Technologies, Santa Clara, CA, USA: Agilent RNA 6000 Nano kit
Roche, Mannheim, Germany: Primer $p(dT)_{15}$ for cDNA synthesis, LightCycler 480 SYBRgreen I Master mix
Invitrogen, Löfer, Austria: RNAse OUT, MMLV reverse transcriptase, RPMI (Gibco, Invitrogen)
PrimerDesign Ltd., Southampton, UK: Rat geNorm Housekeeping Selection Kit
Finnzymes, Espoo, Finland: DyNAmo HS SYBR Green qPCR Kit
TopBio, Prague, Czech Republic: RNA Blue
Fluka, Heidelberg, Germany: Coomassie Brilliant Blue
Generi Biotech, Hradec Králové, Czech Republic: Gene specific primers (chapter 4.6.6.)

Steraloids, Newport, RI, USA: 11-dehydrocorticosterone
Penta, Prague, Czech Republic: sucrose, KCl, Tris and BSA
BioAssay Systems, Hayward, CA, USA: EnzyChrom HDL and LDL/VLDL Assay Kit
Wako, Richmond, VA, USA: NEFA C kit
MP Biomedicals, Solon, OH, USA: Rat Corticosterone ^{3}[H] kit
Millipore, St. Charles, MI, USA: Sensitive Rat Insulin RIA Kit
Lachema, Brno, Czech Republic: BioLaTest GLU L1000, BioLaTest TG L1000
Amersham, GE Healtcare Life Sciences, Buckinghamshire, UK: [^{3}H]corticosterone

4.3. Outline of experiments

In basic experiment, two groups of eight female Wistar rats and HHTg rats were used without any special treatment.

In experiment where we tested the effect of 24h fasting, the animals were divided in four groups (n=6): control Wistar and HHTg female rats with free access to food and fasting Wistar and HHTg rats. Feeding was removed at 9 a.m.

Experiment with CBX treatment involved two experimental groups of eight animals: female HHTg rats without and with CBX treatment. CBX was administered in tap water for two weeks and consumption was measured every day. Average daily uptake was 50 mg/kg which represents optimal physiological dose used by other groups [123] Average weight gain during experiment was 25 g and did not differ between experimental groups.

Since C544 was not tested on rats yet, we performed two preliminary experiments to determine optimal dose and frequency of drug administration. In the first experiment we tested the level of 11HSD1 inhibition in liver, SAT and VAT after single oral dose of C544 (10 mg/kg and 30 mg/kg). Rats were divided into three experimental groups of six animals: control nontreated female HHTg rats and two groups of HHTg rats that received C544 in food. Three animals of each group were killed 2h and 5h after drug administration respectively and 11HSD1 activity was measured in liver and fat tissue slices (chapter 4.5.). Results of this preliminary experiment indicated that 10 mg/kg of C544 is sufficient to induce 11HSD1 80% inhibition in liver, 85% in SAT and 80% in VAT 5h after C544 dose. Design of this preliminary experiment was based on results of study with C544 previously performed in mice [141]. These data are not

shown in chapter 5.4. C544 was provided from Merck through standard material transfer agreement procedure.

To determine the rate of C544 clearance we performed second preliminary experiment that included two groups of twelve female HHTg rats: control nontreated rats and rats that received 10 mg/kg of C544. Two animals of each group were killed 2h, 6h, 12h, 16h, 20h and 24h after drug administration and 11HSD1 activity was measured in liver and fat tissue slices. Data are summarized in chapter 4.4.

The effect of chronic C544 treatment on metabolism of HHTg rats was studied in two groups of 10 animals: control HHTg rats and HHTg that received 10 mg of C544 per kg per day (mg/kg/day) once a day for two weeks. Dose and frequency of C544 treatment was based on the results of our preliminary experiments. Average weight gain during experiment was 28 g and did not differ between experimental groups.

4.4. Measurement of the levels of active 11HSD1 protein (11HSD1 activity)

4.4.1. Sample preparation

Animals were killed and tissue samples were removed. Liver samples were excised from inner part of left lobe, SAT samples were taken from posterior subcutaneous adipose depot, VAT was represented by periovarian adipose tissue and samples of skeletal muscle were excised from *musculus quadriceps femoris* with equal portion of white and red fibres. Fresh tissue samples were homogenized (1:9 w/vol) in ice cold buffer containing 0.01 mol/l Tris and 0.2 mol/l sucrose (pH 8.5) using a Polytron homogenizer (Kinematica AG, Littau, Switzerland). Homogenates were centrifuged at 400 g and 4 °C for 10 min to remove cellular debris. In SAT, VAT and skeletal muscle muscle protein contents were determined by the Bradford method [182] (chapter 4.4.4.) and homogenates were used immediately to measure 11HSD1 activity. Liver homogenates were used for the isolation of the microsomal fraction.

Liver supernatants were centrifuged at 800 g 4 °C for 10 min to remove nuclear fraction. Pellets were washed with 1 ml of ice-cold homogenization buffer and centrifuged again at the same conditions. Pellets were discarded, supernatants were pooled and centrifuged at 9000 g, 4 °C for 10 min to remove mitochondria. Pellets were washed with 1 ml of ice-cold homogenization buffer and centrifuged again at the same

conditions. Pellets were discarded and pooled supernatants were centrifuged at 100 000 g, 4 °C for 60 min to obtain microsomal fraction. Supernatants were discarded. Pellets were resuspended in 0.5 ml of homogenization buffer and sonicated. Protein content was determined and microsomes were immediately used to measure 11HSD1 activity.

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4.4.2. Enzyme assay and steroid analysis

11HSD1 activity was measured as a conversion of [^{3}H]11-dehydrocorticosterone to [^{3}H]corticosterone in incubation buffer containing 50 mmol/l Tris, 100 mmol/l KCl, 0.8 mmol/l NADPH, 1 mmol/l glucose-6-phosphate and 2 U glucose-6-phosphate dehydrogenase (pH 8.5). After a 10 min preincubation at 37 °C, [^{3}H]11-dehydrocorticosterone was added in a final concentration of 16 nmol/l. The protein content and incubation times were 0.05 mg per assay and 15 min for liver microsomes and 1 mg per assay and 90 min for muscle and SAT/VAT, respectively. Reactions were stopped by rapid cooling on ice, the steroids were extracted with Strata-X C18 cartridges (Phenomenex, Torrance, CA, USA) and quantified by high performance liquid chromatography (HPLC) as described previously [183].

4.4.3. Preparation of [^{3}H]11-dehydrocorticosterone

Guinea pig kidney microsomes were prepared as described above. 7 mg of microsomal protein from guinea pig kidney was mixed in 25 ml of reaction buffer containing 50 mmol/l Tris, 100 mmol/l KCl, pH 8.5 and poured into silanized Erlenmayer flask. NAD^+ in final concentration 1 mmol/l and [^{3}H]corticosterone in final concentration 24 μmol/l was added and the reaction mixture was incubated at 37 °C for 5 hours with shaking. Then next 4 mg of fresh microsomal protein together with 3 ml of reaction buffer containing 1 mmol/l NAD^+ was added and held at at 37 °C with shaking for another 6 hours. 11-dehydrocorticosterone was extracted with Strata-X C18 cartridge and purity was determined by HPLC. Typical yield was 400 μl of 11-dehydrocorticosterone in concentration of 0.9-1.0 μmol/l and 98% purity.

4.4.4. Measurement of protein content (Bradford method) [182]

100 μl of unknown protein sample, protein standard or blank was mixed with 1 ml of Coomassie brilliant blue solution and incubated for 2 min. Absorbance was measured at 595 nm on Libra S22 spectrophotometr (Biochrom, Cambridge, UK). Standard curve was created with BSA in the range of 4-10 μg of protein.

4.5. Measurement of 11HSD1 activity in tissue slices

Animals were killed and tissue samples were removed. Samples were excised from the same sites as described in chapter 4.4.1. Fresh tissue samples were sliced and placed in 2 ml of RPMI without serum. 11HSD1 activity was measured as a conversion of [^{3}H]11-dehydrocorticosterone to [^{3}H]corticosterone in RPMI saturated with pneumoxide containing 0.4 mmol/l NADPH, 1 mmol/l glucose-6-phosphate and 2 U glucose-6-phosphate dehydrogenase. After a 10 min preincubation at 37 °C, [^{3}H]11-dehydrocorticosterone was added in a final concentration of 18 nmol/l. In liver nontritiated 11-dehydrocorticosterone was added next to [^{3}H]11-dehydrocorticosterone to reach a final concentration 200 nmol/l to slow down the reaction rate.

4.6. Gene expression analysis

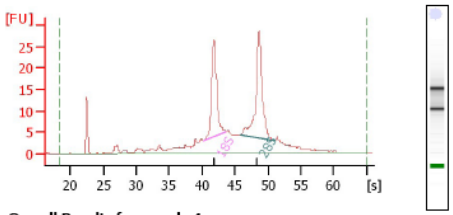
4.6.1. RNA isolation

Animals were killed and tissue samples were removed. Samples were excised from the same sites as described in chapter 4.4.1. Samples for RNA expression analysis were snap-frozen and stored in liquid nitrogen. Hepatic and muscle RNA were isolated using RNA blue, and SAT/VAT RNA was isolated using the RNEasy Lipid Tissue Mini Kit. RNA concentration was determined using a NanoDrop Spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

4.6.2. RNA integrity analysis

RNA integrity (degree of degradation) was determined by Agilent Bioanalyzer with Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA).

Briefly, RNA samples were diluted to 200 μg/μl with RNase-free water, loaded on RNA Nano chip and analyzed by capilary electrophoresis in special gel containing fluorescent dye that binds RNA. RNA integrity was expressed as RNA integrity number (RIN) that varies between 0 and 10 and expresses a degree of ribosomal 18S and 28S RNA degradation (Fig. 4.1.).



Overall Results for sample 4 :

RNA Area:	264,8
RNA Concentration:	172 ng/μl
rRNA Ratio [28s / 18s]:	1,3
RNA Integrity Number (RIN):	8.7 (B.02.06)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.70

Fragment table for sample 4 :

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40,28	43,68	49,1	18,5
28S	45,80	50,94	61,4	23,2

Fig. 4.1. RNA integrity analysis output

4.6.3. DNAse treatment

40 μg of liver and muscle RNA was mixed with DNAse I reaction mix and incubated at 24 °C for 15 min with shaking at 400 rpm. Reaction was terminated by incubation with STOP solution at 70 °C with shaking at 400 rpm. Adipose tissue RNA DNase treatment with RNase-free DNase set was included in RNEasy Lipid Tissue Mini Kit protocol.

4.6.4. Reverse transcription (RT)

2 μg of liver and muscle RNA and 500 ng of SAT and VAT RNA was mixed with 0.5 μg of Primer $p(dT)_{15}$ for cDNA synthesis (oligo dT) and incubated at 70 °C for 10 min. Samples were cooled on ice and reverse transcriptase reaction mix was added. Final RT mix composition was: 50 mmol/l Tris-HCl, 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l DTT, 20 units (U) RNAse OUT (RNase inhibitor), 100 U MMLV reverse transcriptase and 10 μmol/l oligo dT in final volume of 10 μl. Reverse transcription was performed at 37 °C for 60 min and was stopped by incubation 70 °C for 15 min.

4.6.5. Housekeeping gene identification

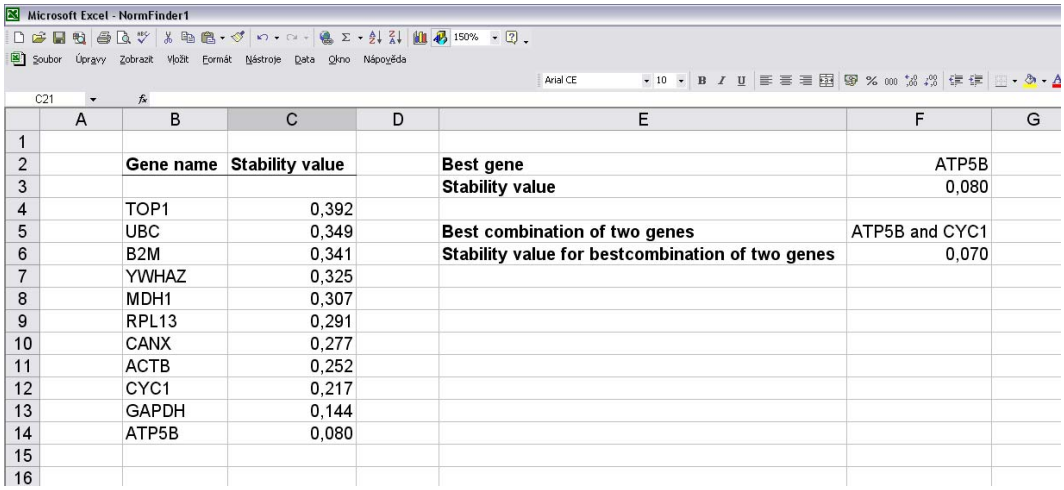
To identify the most stable housekeeping genes for each tissue, a panel of genes was tested by real-time PCR using primers from the rat geNorm Housekeeping Selection Kit (Table 4.1.). Real-time PCR reactions were performed on ABI PRISM 7000 (Applied Biosystems, Foster City, USA) with DyNAmo HS SYBR Green qPCR Kit. Final PCR reaction mix composition was DyNAmo HS SYBR Green mastermix (hot start Tbr DNA polymerase in PCR buffer, SYBR Green I, 2.5 mmol/l $MgCl_2$, dNTP mix and ROX reference dye), 200 nmol/l primer mix and 250-fold diluted RT reaction (cDNA) in final volume of 25 μl. Reaction temperature profile was:

Denaturation and enzyme activation	95 °C - 15 min	1x
Amplification	95 °C - 15 s, 60 °C - 30 s, 72 °C - 30 s	40x

PCR reaction was followed by melting curve analysis. Standard curves were created for each gene with 10-fold dilution set of mixed cDNA sample. Data were analyzed by NormFinder [184] and the pair of the most stable genes for each tissue was idenfified (Fig.4.2.)

ATP synthase subunit 5B	ATP5B
topoisomerase I	TOP1
malate dehydrogenase 1	MDH1
cytochrome c-1	CYC1
calnexin	CANX
ribosomal protein L13	RPL13
tyrosine 3-monooxygenase	YWHAZ
β-2- microglobulin	B2M
ubiquitin C	UBC
glyceraldehyde-3-phosphate dehydrogenase	GAPDH
β-actin	ACTB

Table 4.1. Panel of housekeeping genes



	A	B	C	D	E	F	G
1							
2		**Gene name**	**Stability value**		**Best gene**	ATP5B	
3					**Stability value**	0,080	
4		TOP1	0,392				
5		UBC	0,349		**Best combination of two genes**	ATP5B and CYC1	
6		B2M	0,341		**Stability value for bestcombination of two genes**	0,070	
7		YWHAZ	0,325				
8		MDH1	0,307				
9		RPL13	0,291				
10		CANX	0,277				
11		ACTB	0,252				
12		CYC1	0,217				
13		GAPDH	0,144				
14		ATP5B	0,080				
15							
16							

Fig. 4.2. Sample of NormFinder output: best combination of housekeeping genes in liver

4.6.6. Target gene expression analysis

Target gene and housekeeping gene mRNA levels were quantified by real time PCR quantified by real time PCR with LightCycler 480 SYBRgreen I Master mix on a LightCycler 480 (Roche, Mannheim, Germany). Final PCR reaction mix included: LightCycler 480 SYBRgreen I Master mix (containing FastStart Taq DNA Polymerase, PCR buffer, dNTP mix, SYBR Green I dye and $MgCl_2$), 400 nmol/l of each primer and 150-fold diluted cDNA.

Temperature profiles were:

ATP5B, CYC1, GAPDH and GR		
Denaturation and enzyme activation	95 °C - 10 min	1x
Amplification	95 °C - 11 s, 55 °C - 10 s, 72 °C - 10 s	45x
H6PDH		
Denaturation and enzyme activation	95 °C - 10 min	1x
Amplification	95 °C - 11 s, 50 °C - 10 s, 72 °C – 5 s	45x
11HSD1		
Denaturation and enzyme activation	95 °C - 10 min	1x
Amplification	95 °C - 11 s, 50 °C - 10 s, 72 °C - 10 s	45x

PCR reaction was followed by melting curve analysis. Standard curves were created for each gene with 10-fold dilution set of mixed cDNA sample.

Target gene primer sequences were:

11HSD1 forward GAGTTCAGACCAGAAATGCTCC
11HSD1 reverse TGTGTGATGTGATTGAGAATGAGC
GR forward AGGCCGGTCAGTGTTTTCTAAT
GR reverse GCTGGGCAGTTTTTCCTT
H6PDH forward GCCGCAAGGAGTCCTTCA
H6PDH reverse GTACAGGCGTGGGACTTCAAA

Housekeeping gene primer sequences were:

ATP5B forward GCAGGAAAGAATCACCACCACCAA
ATP5B reverse GCACGGGACAGCACAGTAGTAGCA
CYC1 forward GGCTCCTCCCATCTACACAG
CYC1 reverse ATCTTGAGTCCCATGCGTTTTC
GAPDH forward AGCAATGCCTCCTGCACCACCAAC
GAPDH reverse CCGGAGGGGCCATCCACAGTCT

Primer sequences for H6PDH were adopted from Balachandran et al. 2008 [109]. Other primers were designed with DNAStar software (DNAStar, Madison, WI, USA).

Target gene expression was standardized to normalization factor (NF), a geometric mean of a pair of the most stable housekeeping genes selected by NormFinder for each tissue. Hepatic and muscle NF was a combination of ATP synthase subunit 5B (ATP5B) and cytochrome c-1 (CYC1), whereas CYC1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were selected for SAT and VAT.

4.7. Serum analysis

Blood was collected by cardiac punction and after 15 min serum was prepared by centrifugation at 3000 g for 10 min. Serum levels of TGs were measured with BioLaTest TG L1000. CHOL, HDL and LDL+VLDL cholesterol serum levels were measured with the EnzyChrom HDL and LDL/VLDL Assay Kit. Serum levels of NEFA were measured with the NEFA C kit. Serum levels of CS and INS were determined

using the commercial RIA kits: Rat Corticosterone ^{3}[H] kit and Sensitive Rat Insulin RIA Kit respectively. Serum glucose levels were determined by BioLaTest GLU L1000 and whole blood glucose levels were counted using hematocrite values published previously [185].

4.8. Statistical analysis

All data are expressed as means ± SEM. Statistical analyses were done using the Statistica 6.1. package (StatSoft, Inc., Tulsa, OK, USA) and a probability level of $p<0.05$ was considered significant.

4.8.1. Local metabolism of GCs in selected tissues of HHTg rats

To test differences between Wistar and HHTg rats data were analyzed by t-test. The significance of linear regression between plasma triglycerides and 11HSD1 was analyzed by Pearson correlation coefficients.

4.8.2. Effect of 24h fasting on local metabolism of GCs in HHTg rats

To test strain differences and effect of fasting data were analyzed by two-way ANOVA followed by *post hoc* Newman-Keuls test.

4.8.3. Effect of CBX treatment on local metabolism of GCs in HHTg rats

To test effect of CBX treatment data were analyzed by one-way ANOVA followed by *post hoc* Newman-Keuls test.

4.8.4. Effect of C544 treatment on local metabolism of GCs in HHTg rats

In pharmacodynamic experiment the effects of time and C544 treatment data were analyzed by two-way ANOVA. Differences between treated and untreated animals at each time point were analyzed by *post hoc* Newman-Keuls test. To test time effects on 11HSD1 activity for treated and untreated animals data were analyzed by one-way ANOVA. To test the effects of chronic treatment data were analyzed by t-test.

5. Results

5.1. Local metabolism of GCs in selected tissues of HHTg rats

11HSD1 activity was 23-fold increased (Fig. 5.1A.) and 11HSD1 expression was 3.9-fold increased (Fig. 5.1.B.) in the liver of HHTg rats. In addition, hepatic 11HSD1 activity positively correlated with the serum levels of TGs (Fig. 5.2.). 11HSD1 upregulation in liver was accompanied with 2-fold elevation of H6PDH expression but with no changes in GR expression (Table 5.1.).

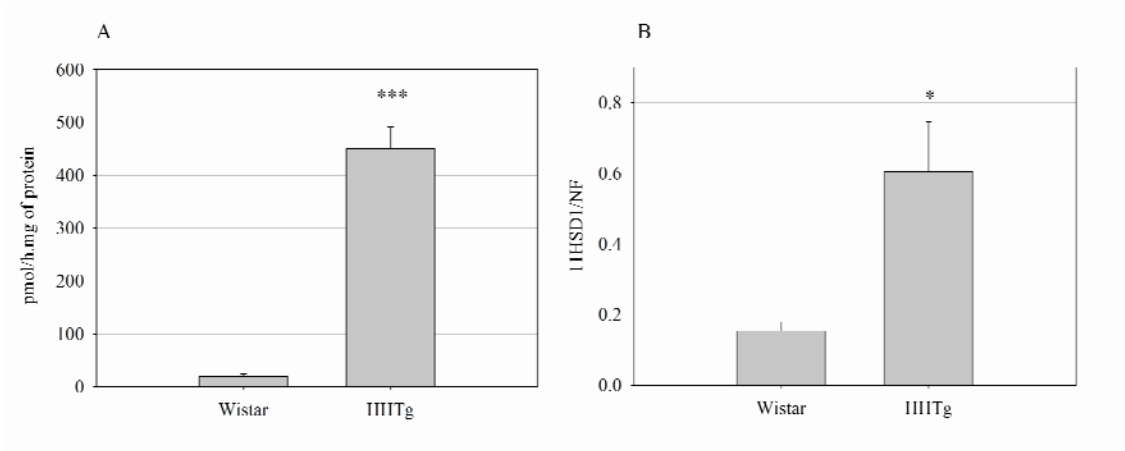


Figure 5.1. A. 11HSD1 activity in liver B. 11HSD1 mRNA expression in liver
) p < 0.05 ***) p < 0.001 Wistar *vs.* HHTg
NF-normalisation factor (chapter 4.6.6.)

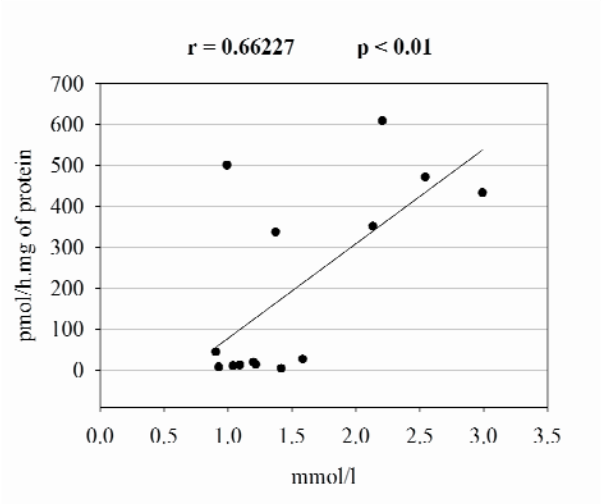


Figure 5.2. Correlation of 11HSD1 activity (y-axis) with serum levels of TGs (x-axis) in liver
r-Pearson correlation coeficient

2.4-fold and 3.5-fold increase in 11HSD1 activity was observed in SAT (Fig. 5.3.A.) and VAT respectively (see Fig. 5.4.A.), however no upregulation of 11HSD1 expression was found. In SAT 11HSD1 was 1.7-fold downregulated (Fig. 5.3.B.) and in VAT it was unchanged (Fig. 5.4.B.). 11HSD1 activity correlated with serum levels of TGs in SAT (Fig. 5.5.) but not in VAT (Fig. 5.6.). Slight downregulation of GR expression (1.4-fold resp. 1.7-fold) and unchanged expression of H6PDH was observed in both adipose tissue depots (SAT resp.VAT) (Table 5.1.).

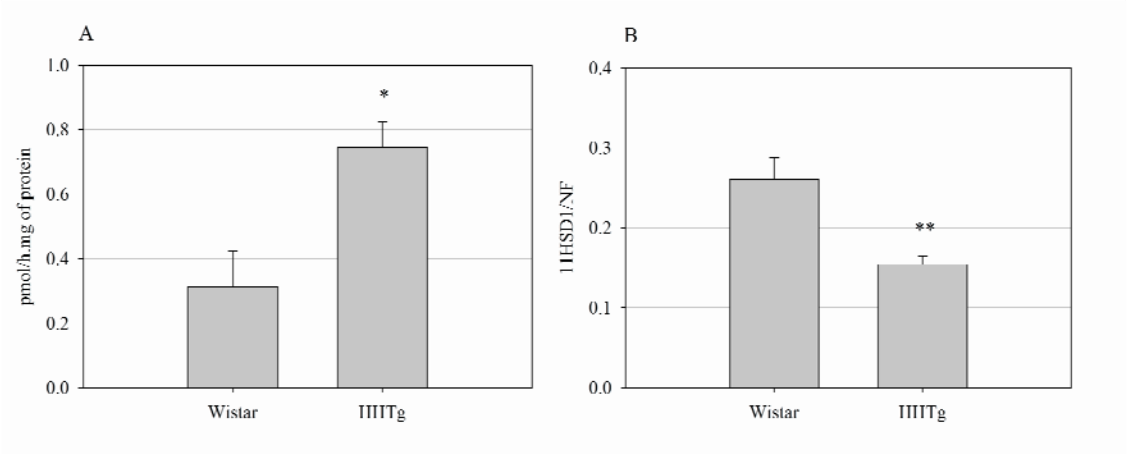


Figure 5.3. A. 11HSD1 activity in SAT B. 11HSD1 mRNA expression in SAT
***) p < 0.05 **) p < 0.01 Wistar *vs.* HHTg**
NF-normalisation factor (chapter 4.6.6.)

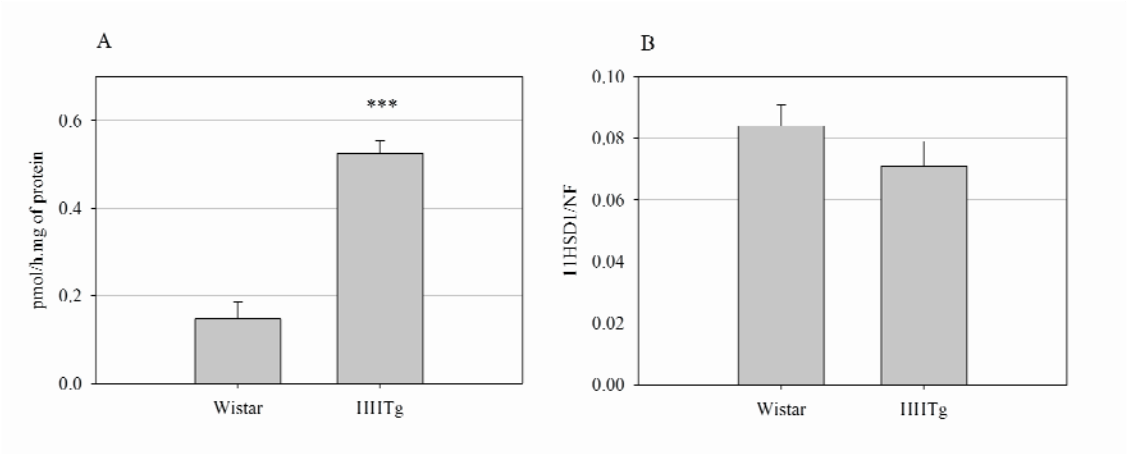


Figure 5.4. A. 11HSD1 activity in VAT B. 11HSD1 mRNA expression in VAT
*****) p < 0.001 Wistar *vs.* HHTg**
NF-normalisation factor (chapter 4.6.6.)

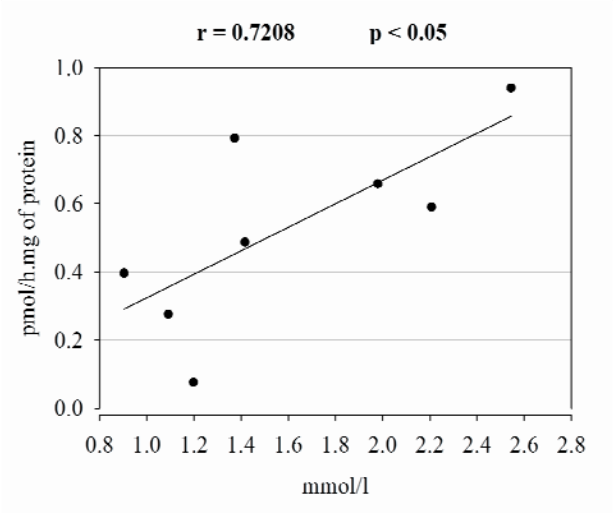


Figure 5.5. Correlation of 11HSD1 activity (y-axis) with serum levels of TGs (x-axis) in SAT

r-Pearson correlation coeficient

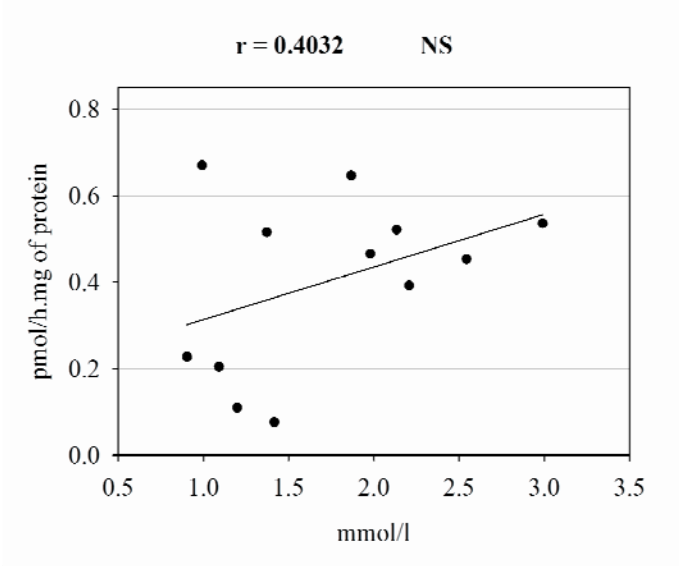


Figure 5.6. Correlation of 11HSD1 activity (y-axis) with serum levels of TGs (x-axis) in VAT

r-Pearson correlation coeficient, NS- non-significant

In skeletal muscle, 11HSD1 activity was 4-fold increased (Fig. 5.7.A.) and positively correlated with serum levels of TGs (Fig. 5.8.). However, no changes were observed in 11HSD1 (Fig. 5.7.B), GR and H6PDH expression (Table 5.1.).

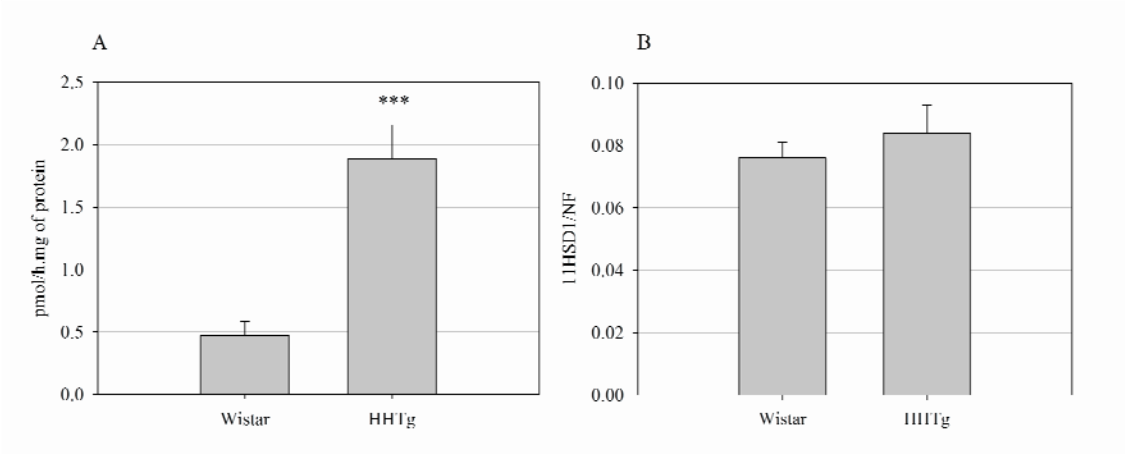


Figure 5.7. A. 11HSD1 activity in skeletal muscle B. 11HSD1 mRNA expression in skeletal muscle

*****) $p < 0.001$ Wistar *vs.* HHTg**

NF-normalisation factor (chapter 4.6.6.)

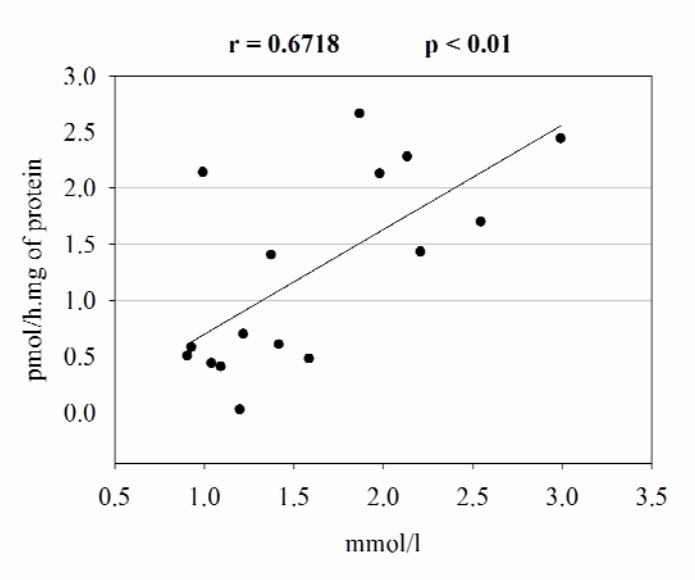


Figure 5.8. Correlation of 11HSD1 activity (y-axis) with serum levels of TGs (x-axis) in skeletal muscle

r-Pearson correlation coeficient

	GR/NF		H6PDH/NF	
	WISTAR	HHTg	WISTAR	HHTg
LIVER	0.11 ± 0.01	0.13 ± 0.02	**0.070 ± 0.005**	**0.136 ± 0.017 ****
SAT	**0.61 ± 0.07**	**0.43 ± 0.03 ***	0.289 ± 0.037	0.234 ± 0.029
VAT	**0.52 ± 0.04**	**0.31 ± 0.03 ****	0.253 ± 0.031	0.181 ± 0.029
MUSCLE	0.09 ± 0.02	0.11 ± 0.03	0.006 ± 0.002	0.013 ± 0.003

Table 5.1. Expression of GR and H6PDH
NF-normalisation factor (chapter 4.6.6.)
***) p < 0.05 **) p < 0.01 Wistar *vs.* HHTg**

As mentioned earlier HHTg rats exhibit increased serum level of TGs. In contrast, serum levels of NEFAs were slightly decreased. No changes were found either in serum levels of CHOL or HDL and LDL+VLDL fractions. Although local regeneration of corticosterone seems to be stimulated in HHTg rats, systemic level of corticosterone were lower compared to Wistar rats. Despite no differences in serum levels of INS, HHTg rats exhibited slightly increased glycemia (Table 5.2.).

	Wistar	HHTg
TGs (mmol/l)	**1.14 ± 0.05**	**2.15 ± 0.11 *****
CHOL (mg/dl)	86.0 ± 7.42	77.1 ± 2.43
HDL (mg/dl)	28.1 ± 3.46	30.2 ± 3.85
LDL+VLDL (mg/dl)	56.6 ± 5.32	55.5 ± 4.46
NEFAs (mmol/l)	**0.41 ± 0.04**	**0.27 ± 0.02 ****
CS (ng/ml)	**715 ± 80**	**496 ± 51 ***
INS (ng/ml)	3.23 ± 0.48	2.16 ± 0.36
GLU (mmol/l)	**5.3 ± 0.5**	**6.8 ± 0.3 ***

Table 5.2. Comparation of selected serum metabolic parameters
***) p < 0.05 **) p < 0.01 ***) p < 0.001 Wistar *vs.* HHTg**

In conclusion, 11HSD1 activity was increased in all examined tissues of HHTg rats and except for VAT, activity correlated with serum levels of TGs. However with the exception of liver, 11HSD1 expression did not follow the activity. H6PDH expression was elevated in liver but not in other tissues so it seems that in the liver, CS regeneration might be exaggerated more than in other tissues. In both adipose tissue depots GR expression was decreased which suggests decreased sensitivity to glucocorticoids.

5.2. Effect of 24h fasting on local metabolism of GCs in HHTg rats

In liver, 24h fasting resulted in 14-fold upregulation of 11HSD1 activity in Wistar rats, in contrast to 1.5 fold downregulation in HHTg rats (Fig.5.9.A.). Fasting also downregulated 11HSD1 mRNA level in HHTg rats (16-fold), however it was without any effect in Wistar rats (Fig.5.9.B.). Generally, fasting eliminated the differences in hepatic 11HSD1 between HHTg and Wistar rats.

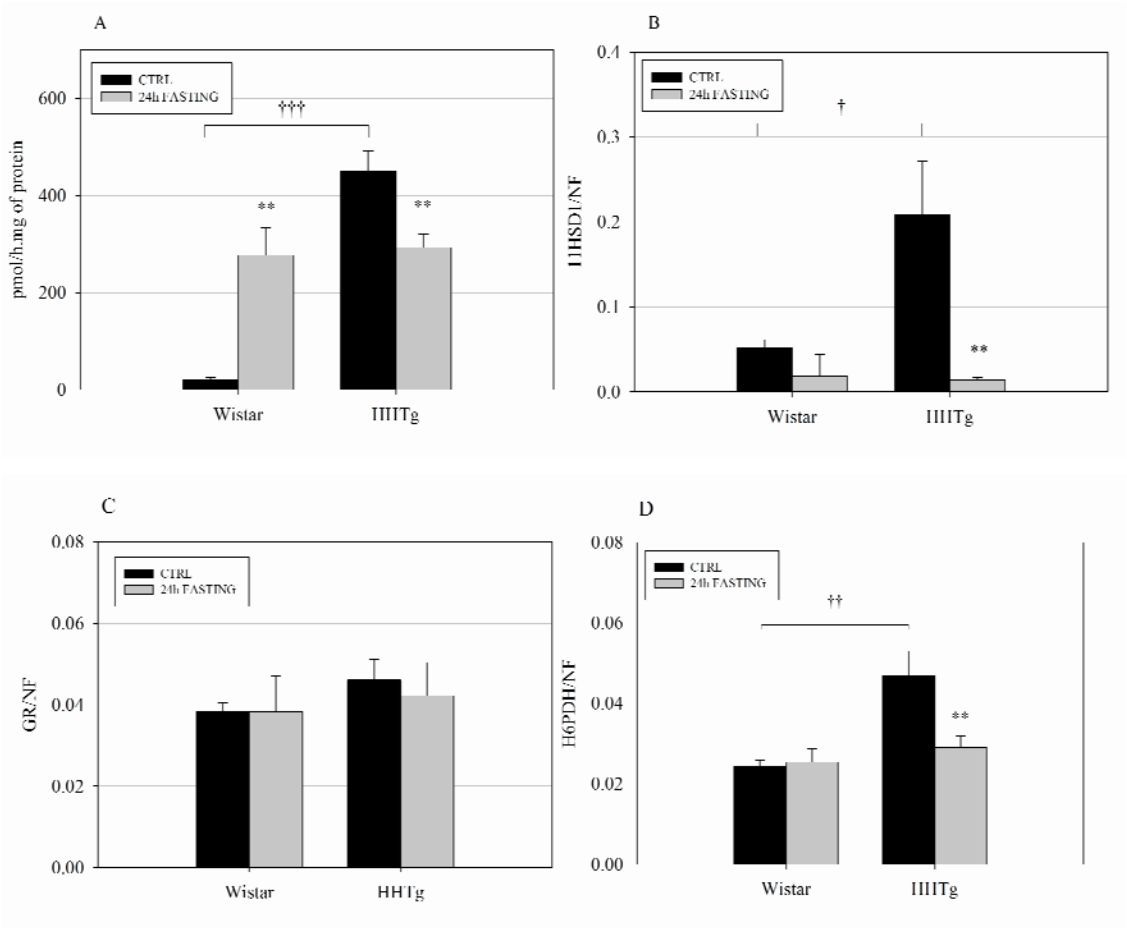


Figure 5.9. A. Effect of 24h fasting on 11HSD1 activity in liver B. Effect of 24h fasting on 11HSD1 mRNA expression in liver C. Effect of 24h fasting on GR mRNA expression in liver D. Effect of 24h fasting on H6PDH mRNA expression in liver.

****) p < 0.01 24h fasting *vs.* CTRL (*ad libitum* feeding)**

†) p < 0.05; ††) p < 0.01 †††) p < 0.001 Wistar *vs.* HHTg

NF-normalisation factor (chapter 4.6.6.)

Decreased 11HSD1 expression in HHTg rats was followed by 1.6-fold reduction of H6PDH expression upon fasting (Fig. 5.9.D.). No fasting-mediated changes were observed in GR expression (Fig. 5.9.C.).

In SAT, fasting stimulated 11HSD1 activity (2.4-fold) and expression (1.5-fold) in HHTg rats. In Wistar rats 11HSD1 activity was also elevated but no upregulation in 11HSD1 expression was found (Fig.5.10.A., 5.10.B.).

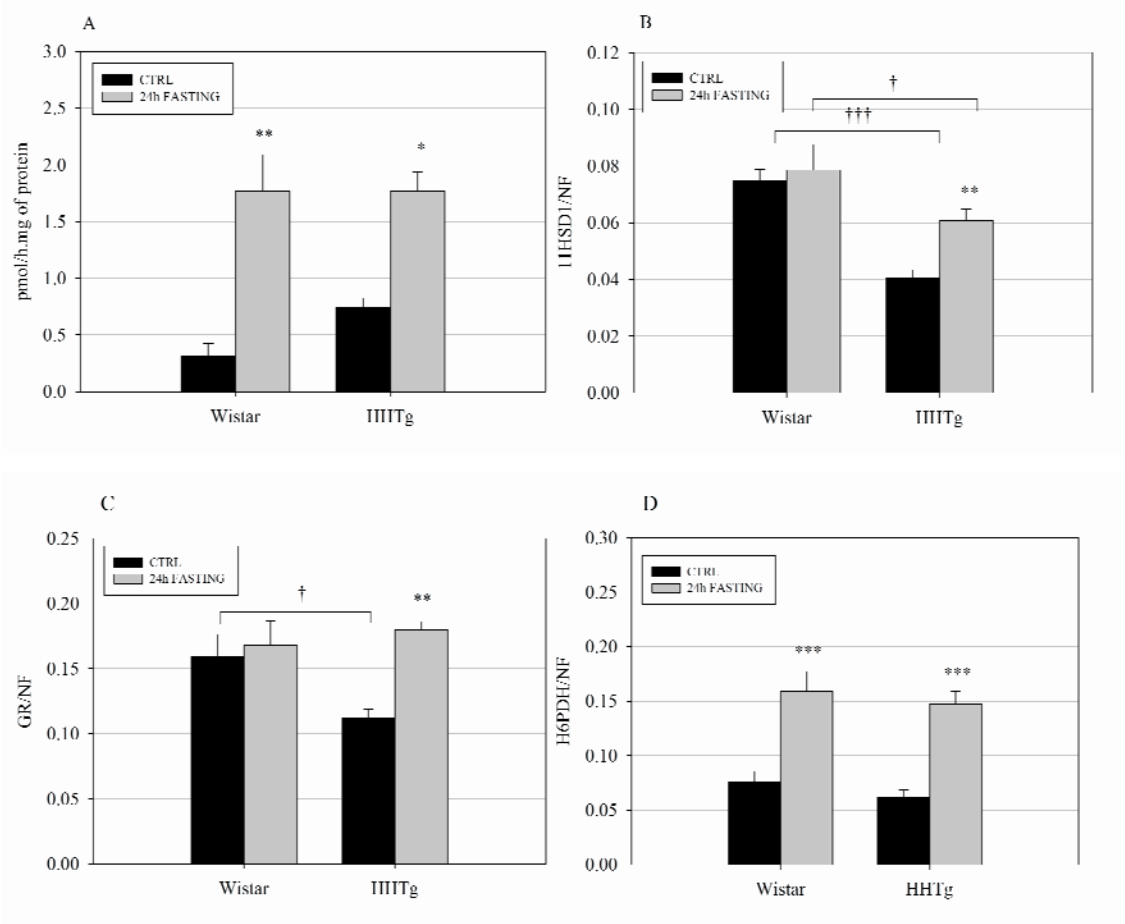


Figure 5.10. A. Effect of 24h fasting on 11HSD1 activity in SAT B. Effect of 24h fasting on 11HSD1 mRNA expression in SAT C. Effect of 24h fasting on GR mRNA expression in SAT D. Effect of 24h fasting on H6PDH mRNA expression in SAT.

***) p < 0.05 **) p < 0.01 ***) p < 0.001 24h fasting *vs.* CTRL (*ad libitum* feeding)**

†) p < 0.05 †††) p < 0.001 Wistar *vs.* HHTg

NF-normalisation factor (see chapter 4.6.6.)

Elevated 11HSD1 activity and expression in SAT of HHTg rats was accompanied with 1.6-fold elevated GR and 2.4-fold elevated H6PDH expression. H6PDH expression was also 2-fold elevated in Wistar rats upon fasting (Fig 5.10.C-5.10.D).

In VAT, fasting induced 11HSD1 activity (1.9-fold resp. 2.2-fold) and expression (2.6-fold resp. 3.7-fold) in both strains (Wistar resp. HHTg), however in Wistar rats 11HSD1 activity induction was not significant. The 11HSD1 upregulation was stronger in HHTg rats (Fig. 5.11.A-5.11.B). Similar pattern as in 11HSD1 activity and expression was found in GR and H6PDH expression in VAT (Fig. 5.11.C-5.11.D).

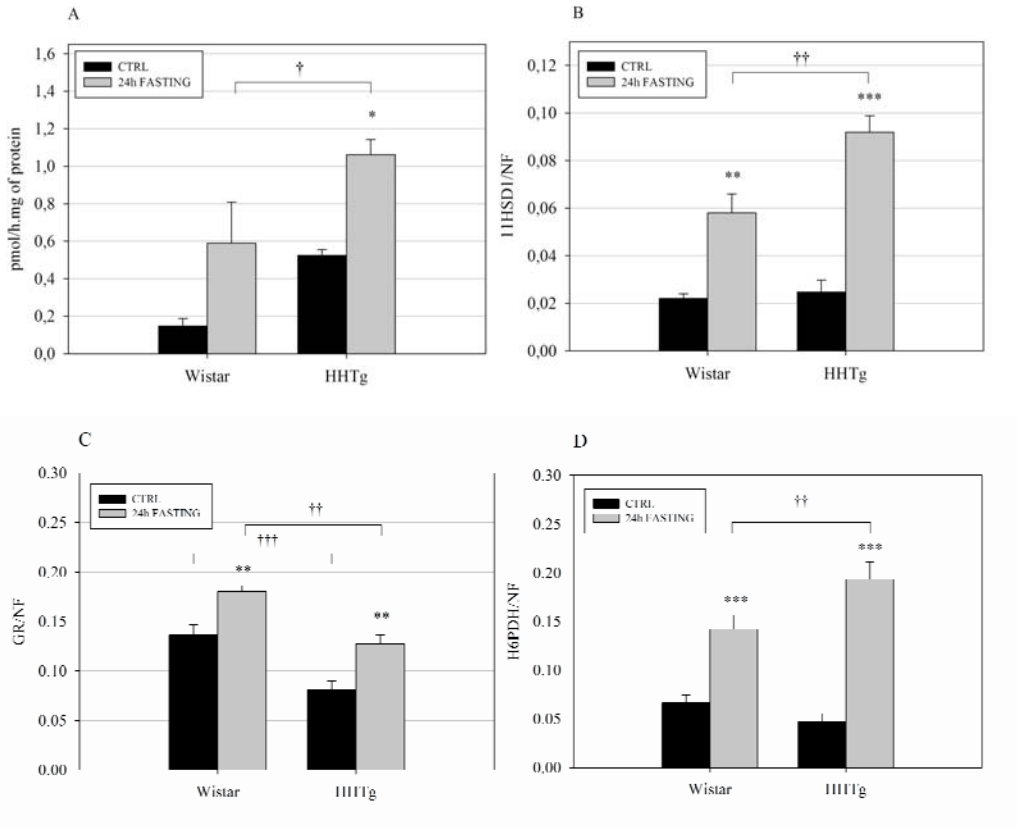


Figure 5.11. A. Effect of 24h fasting on 11HSD1 activity in VAT B. Effect of 24h fasting on 11HSD1 mRNA expression in VAT C. Effect of 24h fasting on GR mRNA expression in VAT D. Effect of 24h fasting on H6PDH mRNA expression in VAT

***) p < 0.05 **) p < 0.01 ***) p < 0.001 24h fasting *vs.* CTRL (*ad libitum* feeding)**

†) p < 0.05 ††) p < 0.01 †††) p < 0.001 Wistar *vs.* HHTg

NF-normalisation factor (chapter 4.6.6.)

In skeletal muscle, no effect of fasting was observed in 11HSD1 activity or expression (Fig. 5.12.A-5.12.B). In contrast, expression of H6PDH was 3.6-fold resp. 2.2-fold elevated similar to GR where expression was 2.5-fold resp. 2.2-fold elevated in Wistar and HHTg rats, respectively (Fig. 5.12.C-5.12.D).

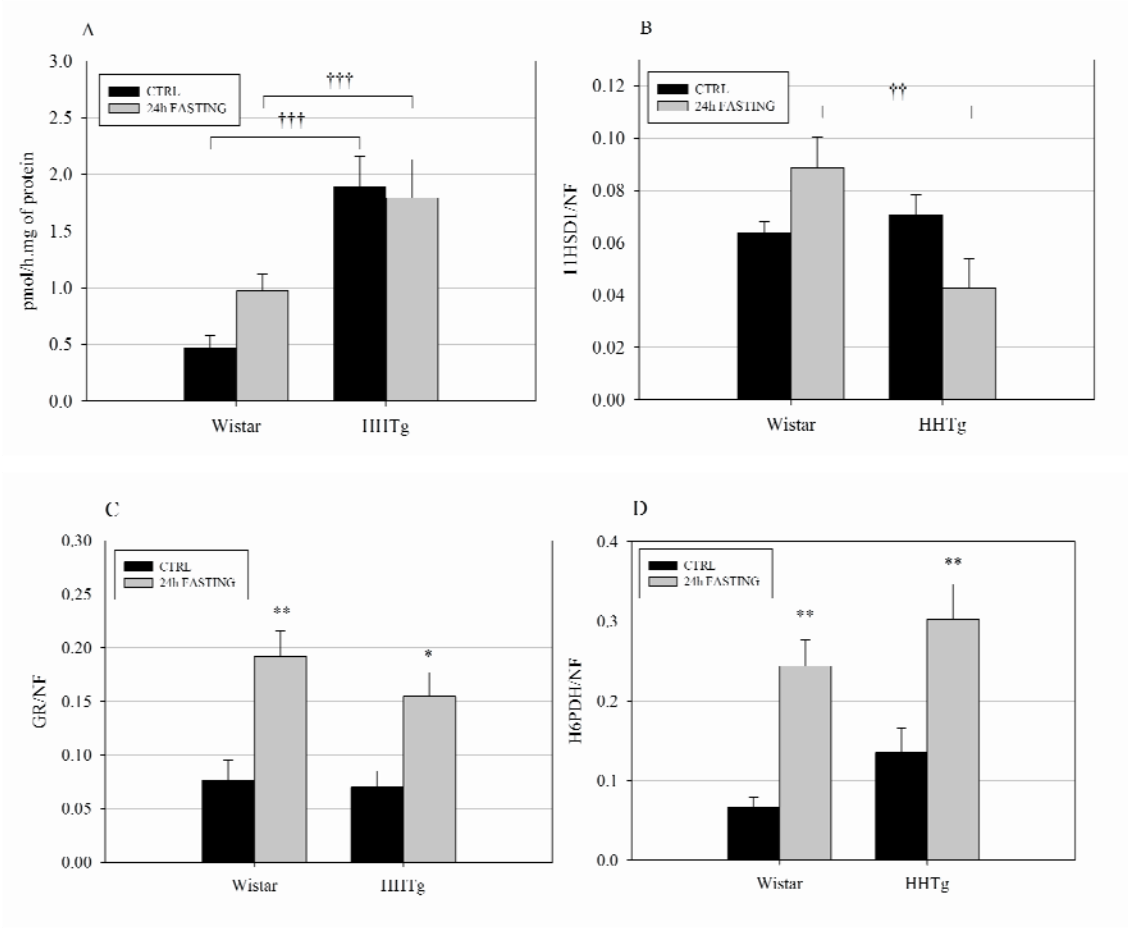


Figure 5.12. A. Effect of 24h fasting on 11HSD1 activity in skeletal muscle B. Effect of 24h fasting on 11HSD1 mRNA expression in skeletal muscle C. Effect of 24h fasting on GR mRNA expression in skeletal muscle D. Effect of 24h fasting on H6PDH mRNA expression in skeletal muscle.

***) p < 0.05 **) p < 0.01 24h fasting *vs.* CTRL (*ad libitum* feeding)**

††) p < 0.01 †††) p < 0.001 Wistar *vs.* HHTg

NF-normalisation factor (chapter 4.6.6.)

Fasting decreased serum levels of TGs in both strains and compensated the differences observed on *ad libitum* feeding. Fasting increased serum levels of HDL cholesterol and decreased serum levels of LDL+VLDL cholesterol (Table 5.3.).

	WISTAR		HHTg	
	CTRL	24h FASTING	CTRL	24h FASTING
TGs (mmol/l)	**1.14 ± 0.05**	**0.66 ± 0.06 ***	**2.15 ± 0.14 †††**	**0.8 ± 0.05 *** **
CHOL (mg/dl)	86.0 ± 7.42	88.4 ± 11.74	77.1 ± 2.43	68.9 ± 2.93
HDL (mg/dl)	**28.0 ± 3.46**	**61.1 ± 6.75 *** **	**30.2 ± 3.85**	**47.4 ± 1.80 ** †† **
LDL+VLDL (mg/dl)	**56.6 ± 5.32**	**25.6 ± 3.00 *** **	**55.5 ± 4.46**	**16.7 ± 2.40 *** **
NEFAs (mmol/l)	**0.41 ± 0.04**	0.43 ± 0.02	**0.27 ± 0.03 ††**	**0.49 ± 0.02 ** **
INS (ng/ml)	**3.2 ± 0.48**	**0.3 ± 0.09 *** **	**2.2 ± 0.36**	**0.3 ± 0.04 ** **
GLU (mmol/l)	5.3 ± 0.45	**5.1 ± 0.22**	**6.8 ± 0.30**	**4.1 ± 0.10 *** †**
CS (ng/ml)	715 ± 80	1136 ± 278	**496 ± 51**	**1217 ± 258 ***

Table 5.3. Effect of 24h fasting on selected serum metabolic parameters.
***) p < 0.05; **) p < 0.01; ***) p < 0.001 CTRL *vs.* 24h fasting**
†) p < 0.05; ††) p < 0.01; †††) p < 0.001 Wistar *vs.* HHTg

In summary, fasting induced tissue-specific changes in 11HSD1 expression and also in expression of GR and H6PDH in both strains. Generally, in HHTg rats 11HSD1 activity always correlated with 11HSD1 mRNA expression, being downregulated in liver, upregulated in both adipose tissue depots and unchanged in skeletal muscle. In liver and adipose tissue the same pattern was observed in H6PDH expression. In VAT fasting-mediated induction of 11HSD1 and H6PDH was stronger in HHTg rats otherwise no strain-specific changes were observed. Except for liver, fasting induced expression of GR in HHTg rats. As it was expected, fasting was associated with elevated serum CS in both strains, however the increase was only significant in HHTg rats. Fasting also decreased serum levels of TGs, LDL+VLDL and INS and decreased levels of HDL in both strains.

5.3. Effect of CBX treatment on local metabolism of GCs in HHTg rats

In liver, CBX treatment decreased 11HSD1 activity (Fig. 5.13.A.) and expression (Fig. 5.13.B.) and also decreased expression of H6PDH (Table 5.4.). No differences were observed in GR expression (Table 5.4.). No effect of CBX treatment on 11HSD1 activity and expression and H6PDH expression in SAT (Fig. 5.14., Table 5.4.), VAT (Fig. 5.15., Table 5.4.) and skeletal muscle (Fig. 5.16., Table 5.4.). CBX treatment upregulated GR expression in VAT up to the level comparable to Wistar rats. No effects of CBX treatment on GR expression were observed in SAT and skeletal muscle (Table 5.4.).

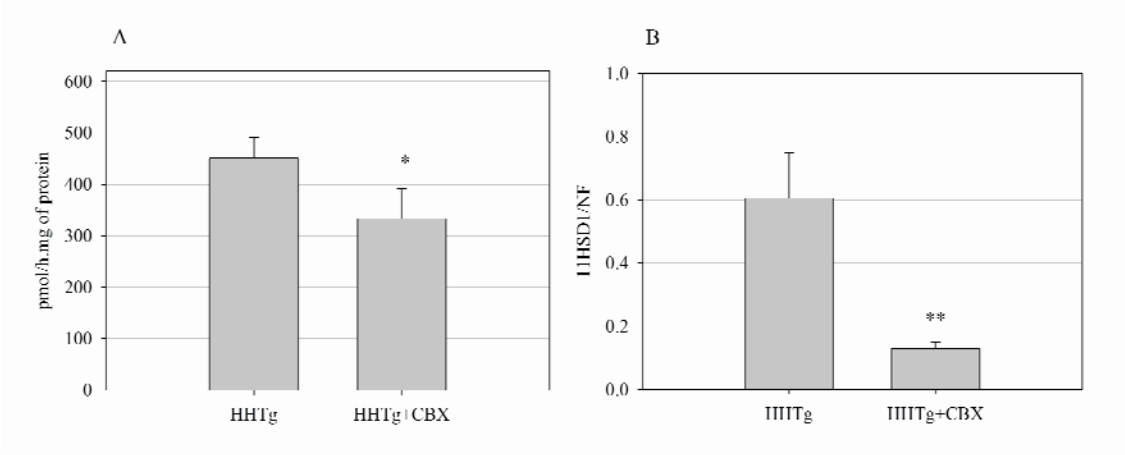


Figure 5.13. A. Effect of CBX treatment on 11HSD1 activity in liver B. Effect of CBX treatment on 11HSD1 expression in liver

****) p < 0.01 ***) p < 0.001 HHTg *vs.* HHTg+CBX**

NF-normalisation factor (chapter 4.6.6.)

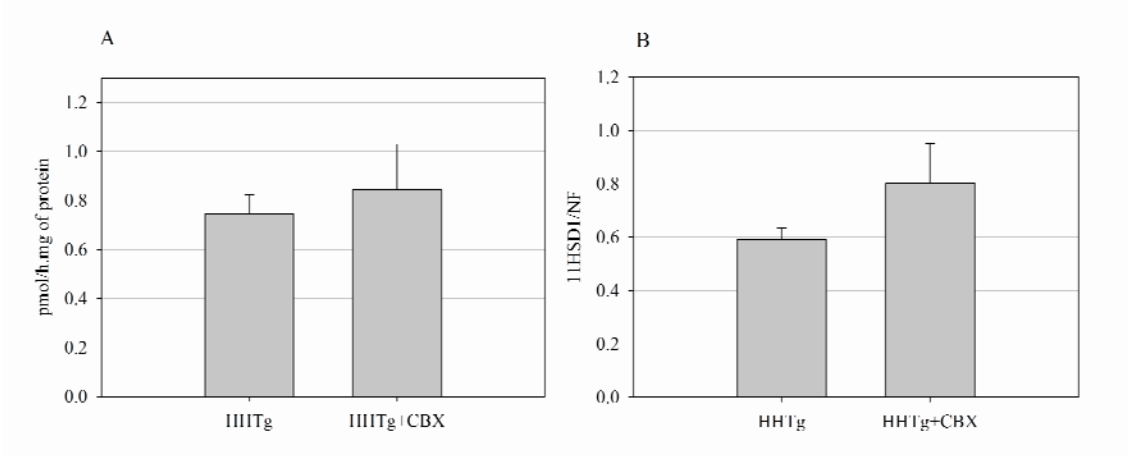


Figure 5.14. A. Effect of CBX treatment on 11HSD1 activity in SAT B. Effect of CBX treatment on 11HSD1 expression in SAT

NF-normalisation factor (chapter 4.6.6.)

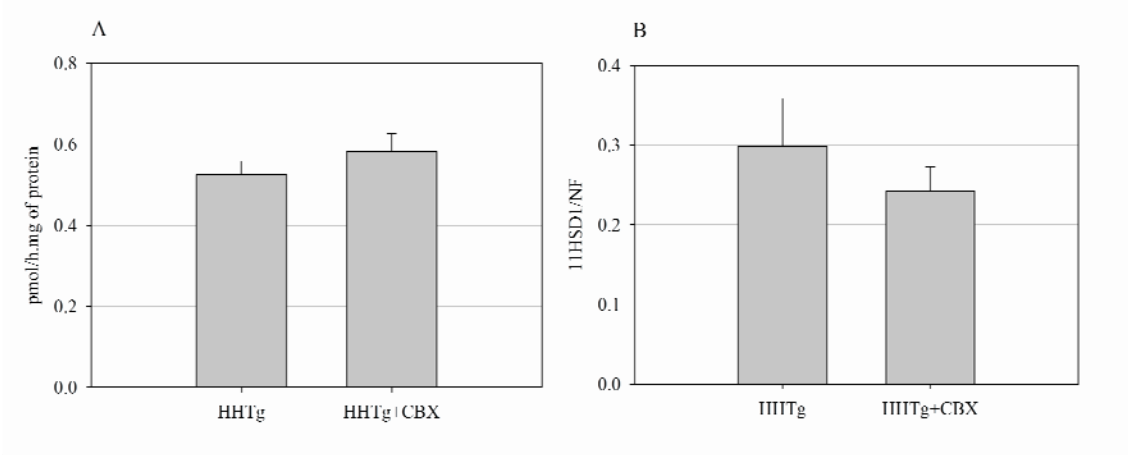


Figure 5.15. A. Effect of CBX treatment on 11HSD1 activity in VAT B. Effect of CBX treatment on 11HSD1 expression in VAT

NF-normalisation factor (chapter 4.6.6.)

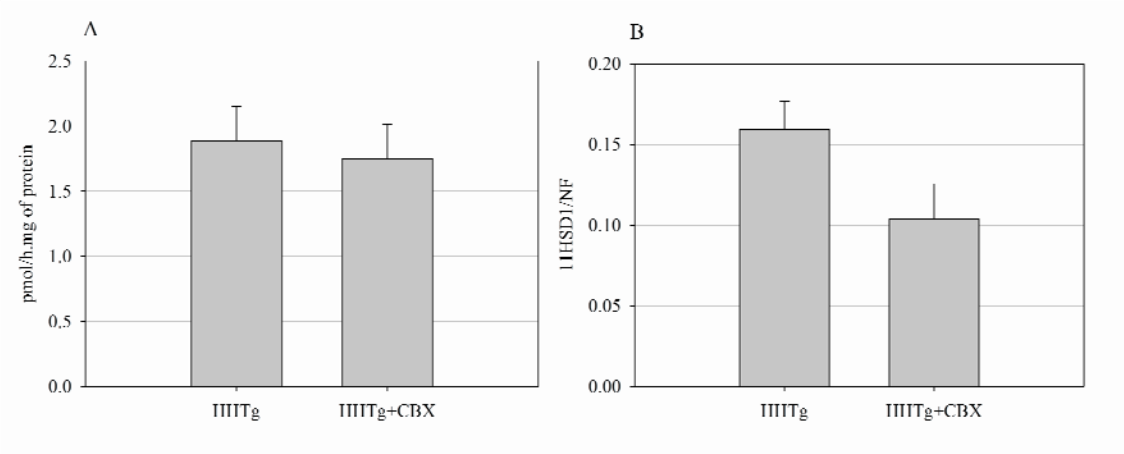


Figure 5.16. A. Effect of CBX treatment on 11HSD1 activity in skeletal muscle B. Effect of CBX treatment on 11HSD1 expression in skeletal muscle

NF-normalisation factor (chapter 4.6.6.)

	GR/NF	
	HHTg	HHTg+CBX
LIVER	0.13 ± 0.015	0.13 ± 0.014
SAT	1.64 ± 0.105	1.35 ± 0.272
VAT	**1.20 ± 0.120**	**1.89 ± 0.177 ****
MUSCLE	0.16 ± 0.034	0.12 ± 0.024
	H6PDH/NF	
LIVER	**0.14 ± 0.017**	**0.07 ± 0.009 *****
SAT	0.90 ± 0.109	0.80 ± 0.104
VAT	0.70 ± 0.112	1.01 ± 0.118
MUSCLE	0.02 ± 0.006	0.02 ± 0.006

Table 5.4. Effect of CBX treatment on expression of GR and H6PDH

****) p < 0.01 ***) p < 0.001 HHTg *vs.* HHTg+CBX**

NF-normalisation factor (chapter 4.6.6.)

CBX treatment decreased serum levels HDL cholesterol and increased levels of NEFAs up to level comparable with Wistar rats.

	HHTg	HHTg+CBX
TGs (mmol/l)	2.15 ± 0.14	2.55 ± 0.22
HDL (mg/dl)	**30.2 ± 3.85**	**18.2 ± 1.49 ***
LDL (mg/dl)	55.5 ± 4.46	50.3 ± 1.86
CHOL (mg/dl)	77.1 ± 2.43	81.1 ± 3.80
NEFAs (mmol/l)	**0.27 ± 0.02**	**0.45 ± 0.04 ****
INS (ng/ml)	2.2 ± 0.36	1.52 ± 0.17
GLU (mmol/l)	6.8 ± 0.30	6.2 ± 0.50
CS (ng/ml)	496 ± 51	631 ±150

Table 5.5. Effect of CBX treatment on selected serum metabolic parameters
***) p < 0.05 **) p < 0.01 HHTg *vs.* HHTg+CBX**

In summary, CBX treatment affected only the local GC metabolism in the liver of HHTg rats. It decreased 11HSD1 activity and mRNA expression that was associated with downregulation of H6PDH. CBX treatment did not affect serum levels of CS and did not lower serum levels of TGs. In fact, CBX treatment resulted in worsening the lipid profile as it decreased serum levels of HDL cholesterol and increased serum levels of NEFAs.

5.4. Effect of C544 treatment on local metabolism of GCs in HHTg rats

In first subchapter we show results of 2nd preliminary experiment (chapter 4.3.) that tested pharmacodynamics of C544 during 24h to show potency and clearance rate of this compound. In second subchapter we show results from experiment where we tested effects of chronic C544 treatment on HHTg rats (chapter 4.3.).

5.4.1. Effect of a single dose of C544 on local metabolism of GCs in HHTg rats during 24h period

In C544 treated HHTg animals, 11HSD1 activity was significantly inhibited by single dose (Fig. 5.17.-5.19.) and persisted during 24h period at approximately 10 % of controls in all tissues (Fig. 5.20.). In addition, untreated HHTg rats exhibited significant circadian changes in 11HSD1 activity in liver ($p < 0.01$) and VAT ($p < 0.01$) and the hepatic rythm persisted also in the presence of C544 ($p < 0.05$).

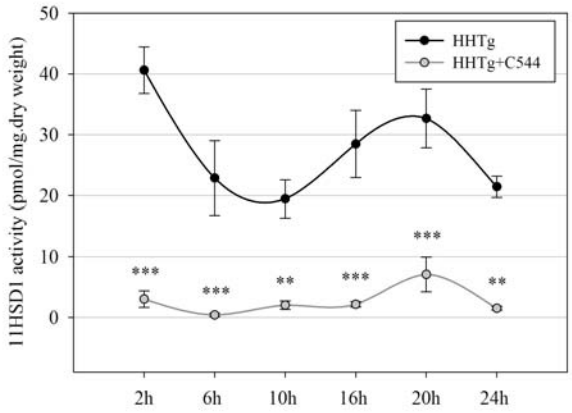


Figure 5.17. Effect of single C544 dose on 11HSD1 activity in the liver of HHTg rats

****) p < 0.01 ***) p < 0.001 HHTg *vs.* HHTg+C544**

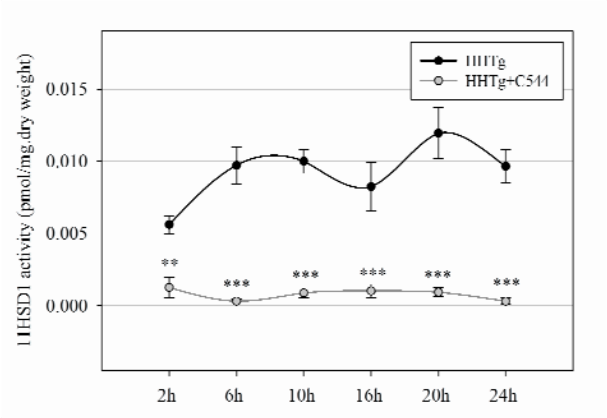


Figure 5.18. Effect of single C544 dose on 11HSD1 activity in SAT of HHTg rats

****) p < 0.01; ***) p < 0.001 HHTg *vs.* HHTg+C544**

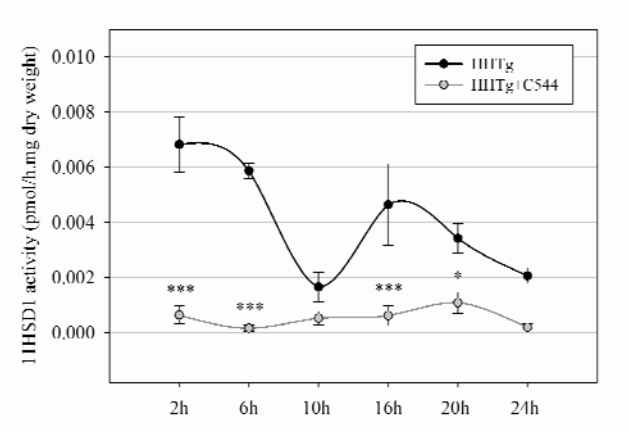


Figure 5.19. Effect of single C544 dose on 11HSD1 activity in VATof HHTg rats

***) p < 0.05; ***) p < 0.001 HHTg vs. HHTg+C544**

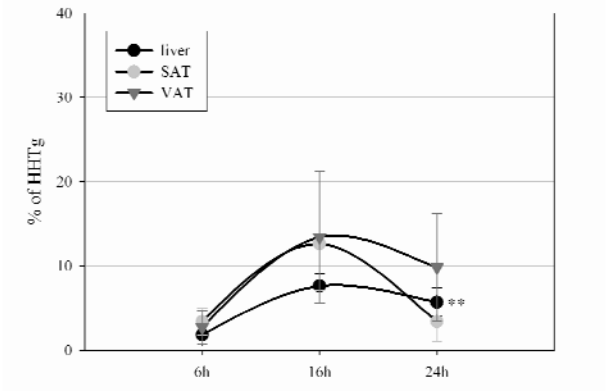


Figure 5.20. 11HSD1 activity in liver, SAT and VAT of C544 treated animals expressed as % of untreated controls

****) p < 0.01 6h *vs.* 24h (liver)**

5.4.2. Effect of chronic administration of C544 on local metabolism of GCs in HHTg rats

Chronic C544 treatment had no effect on 11HSD1 expression in liver and SAT but increased 11HSD1 expression in VAT (2-fold) (Fig.5.21.). The same pattern was observed in H6PDH expression (see Fig. 5.22.). In contrast, C544 increased GR expression in liver (1.3-fold) but had no effect on GR expression in adipose tissue depots (Fig. 5.23.).

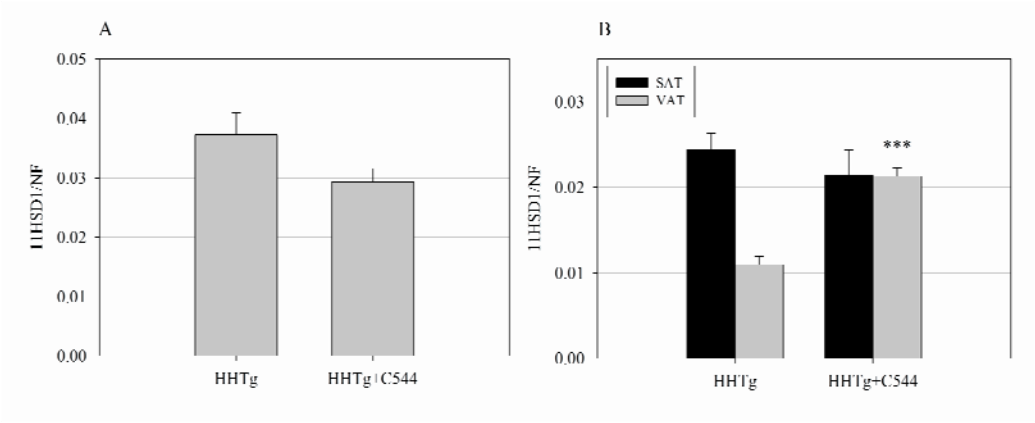


Figure 5.21. A. Effect of chronic C544 treatment on 11HSD1 mRNA expression in liver B. Effect of chronic C544 treatment on 11HSD1 mRNA expression in adipose tissue

*****) p < 0.001 HHTg vs. HHTg+C544**

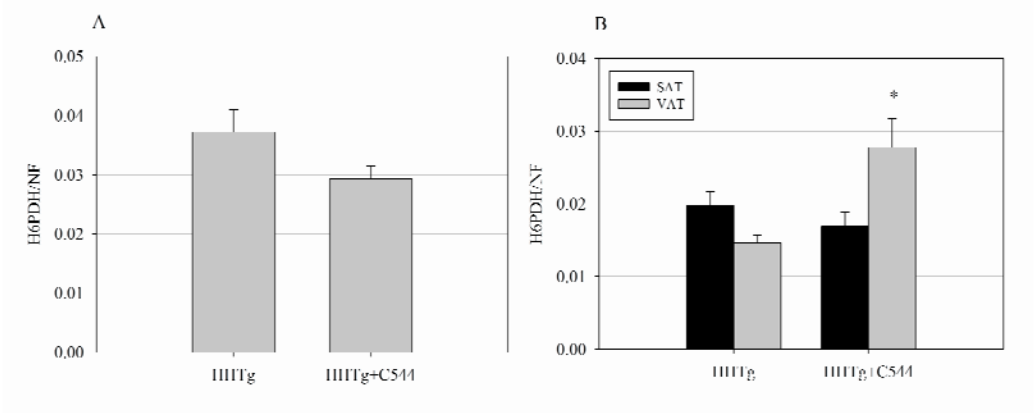


Figure 5.22. A. Effect of chronic C544 treatment on H6PDH mRNA expression in liver B. Effect of chronic C544 treatment on H6PDH mRNA expression in adipose tissue

***) p < 0.05 HHTg vs. HHTg+C544**

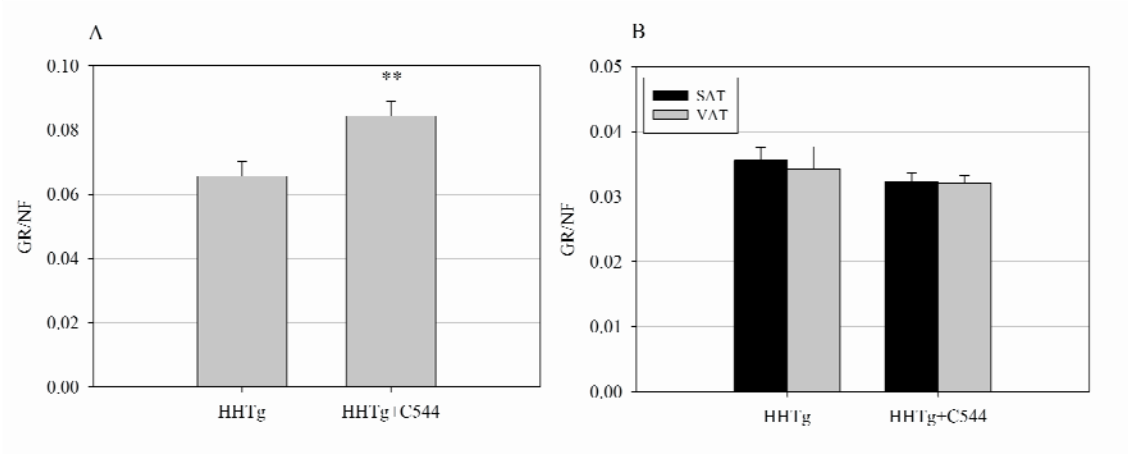


Figure 5.23. A. Effect of chronic C544 treatment on GR mRNA expression in liver B. Effect of chronic C544 treatment on GR mRNA expression in adipose tissue
****) p < 0.01 HHTg vs. HHTg+C544**

Chronic inhibition of local regeneration of CS decreased systemic levels of CS in HHTg rats and improved hypertriglyceridemia. Total levels of serum CHOL was elevated upon C544 treatment, however this upregulation was predominant in HDL fraction (Table 5.6.).

	HHTg	HHTg+C544
TGs (mmol/l)	**2.5 ± 0.66**	**1.5 ± 0.38 ****
HDL (mg/dl)	**32.2 ± 2.08**	**76.2 ± 1.68 *****
LDL+VLDL (mg/dl)	**15.0 ± 1.74**	**19.8 ± 0.54 ***
CHOL (mg/dl)	**79.0 ± 4.00**	**109.6 ± 3.10 *****
NEFAs (mmol/l)	0.31 ± 0.03	0.34 ± 0.01
INS (ng/ml)	1.74 ± 0.25	1.81 ± 0.36
GLU (mmol/l)	6.8 ± 0.3	7.2 ± 0.2
CS (ng/ml)	**645 ± 45**	**358 ± 42 *****

Table 5.6. Effect of chronic C544 treatment on selected serum metabolic parameters
***) p < 0.05 **) p < 0.01 ***) p < 0.001 HHTg vs. HHTg+C544**

In summary, C544 is a potent inhibitor of 11HSD1 in the liver, SAT and VAT of HHTg rats with slow clearance rate. Chronic treatment with C544 altered 11HSD1, H6PDH and GR expression in tissue-specific manner. Chronic selective 11HSD1 inhibition lowered systemic levels of CS and improved serum lipid profile.

6. Discussion

6.1. Local metabolism of GCs in selected tissues of HHTg rats

GCs have been shown to play an important role in development of metabolic syndrome and obesity. Systemic removal of GC by ADX reduced adipose tissue depot weights and serum levels of TGs, INS and GLU in obese Zucker rats. These effects were reversed by GC replacement [186]. Similar results were observed in healthy Wistar rats as well as in obese rats kept on high sucrose/high fat diet (HSF). In addition, decreased hepatic TG content and TG secretion were observed in HSF rats after ADX [18]. Finally, effect of systemic GC removal was exaggerated by reduction of 11HSD1 expression by rosiglitasone, a PPARγ receptor agonist [102]. These results show that systemic and local reduction of GCs have positive effect on metabolic disturbances observed in metabolic syndrome. Female HHTg rats display markedly increased 11HSD1 activity and mRNA expression in liver compared to healthy female Wistar rats and serum TG levels correlated with 11HSD1 activity. 11HSD1 activity may be further stimulated by elevated H6PDH. This suggests that despite slightly lower systemic levels of GC, the HHTg rats may suffer from increased intracellular GC level so that GC-dependent effects might be stimulated in liver. Since GCs stimulate TG synthesis in liver [17] the elevated 11HSD1 can contribute to hypertriglyceridemia in HHTg rats. This is supported by previously described enhanced lipogenesis in the liver of HHTg rats [187]. Our data are also in agreement with lower plasma TG levels found in 11HSD1 $^{-/-}$ mice [149] and with the fact that HHTg rats resemble a phenotype described in ApoE-11HSD1 mice (mice with selective 11HSD1 overexpression in liver). These mice are not obese but exhibit certain symptomes of metabolic syndrome as well as HHTg rats. No upregulation in plasma TG levels were described in ApoE-11HSD1 mice however these mice developed hepatic steatosis [151].

GCs exert different effects in distinct adipose tissue depots. In peripheral fat GCs increase lipolysis while in central depots they promote preadipocyte differenciation and lipid storage [27-29]. At the molecular level these effects are driven by depot-specific GC modulation of balance between HSL that stimulate lipolysis [188] and lipoprotein lipase (LPL) that promotes lipid storage. This might be the reason why overexpression of 11HSD1 in murine adipose tissue drives weight gain predominately in visceral depots [26] and 11HSD1 $^{-/-}$ mice exhibit reduced visceral fat accumulation

upon high-fat feeding compared to wild type mice [148]. Elevated 11HSD1 activity and expression in visceral adipose tissue depots were also described in obese leptin-deficient *ob/ob* mice [152], leptin-resistant *db/db* mice [152] and obese Zucker rats [158]. Finally, correlation between 11HSD1 in SAT and degree of obesity was found in a number of clinical studies [126, 160, 162, 164, 166-172]. These studies suggest that not only systemic GC excess can lead to obesity as can be observed in patients with Cushing´s syndrome but also local GC excess driven by upregulation of 11HSD1 in adipose tissue can be connected to the development of obesity. Female HHTg rats display increased levels of 11HSD1 activity in both SAT and VAT, however this is not accompanied by elevated 11HSD1 and H6PDH expression. In fact, mild upregulation in 11HSD1 activity in adipose tissue is compensated by downregulation of GR mRNA expression. Discrepancy between 11HSD1 activity and expression can be explained by posttranslational regulation of 11HSD1 by homodimerization [50] or glycosylation [91]. Our data suggest that 11HSD1 in adipose tissue might contribute to hypertriglyceridemia in female HHTg rats but its upregulation is not strong enough to induce obesity. This is supported by increased adipose tissue lipolysis that was detected in HHTg males kept on sucrose diet [189].

Increased 11HSD1 activity was also found in skeletal muscle of female HHTg rats, however it was not followed by increased 11HSD1, H6PDH or GR expression. Discrepancy between 11HSD1 activity and expression was already discussed. Increased 11HSD1 was also found in skeletal muscle of streptozocin-induced rat model of diabetes mellitus [65, 190] and in cultured isolated skeletal muscle cells isolated from patients with type 2 diabetes mellitus [191] and metabolic syndrome [33]. In HHTg rats GLU administration did not stimulate GLUT4 translocation to the plasma membrane in skeletal muscle together with decreased GLU utilisation rate. These data suggest alteration of insulin signaling pathway in HHTg rats [179]. Elevated GC levels due to increased 11HSD1 in skeletal muscle might represent one of the reasons for these changes. GCs have been shown to counteract effects of the insulin/IGF-1 pathway, a major anabolic pathway in skeletal muscle [192], by suppression of GLU uptake mainly through inhibited translocation of insulin-sensitive glucose transporter GLUT4 to the cell surface [193-194]. In *db/db* mice increased GC production and insulin resistance was associated with muscle atrophy [195] which is also common feature in patients with Cushing´s syndrome or on steroid therapy [34]. Finally, in *ob/ob* mice undergoing ADX improved muscle insulin sensitivity and increased protein synthesis was observed [196-

197]. Studies of GRα expression in skeletal muscle in metabolic syndrome provide inconsistent results. GRα expression has been shown to be positively associated with metabolic syndrome in human [33] as well as unchanged [191]. In contrast, in streptozocin-induced rat model of diabetes mellitus GR expression was downregulated [190]. In skeletal muscle of HHTg rats no changes in GR expression was observed.

In accordance with the previous studies in male HHTg rats [179-180], we showed that female HHTg rats also exhibit slightly increased GLU. This might be a consequence of increased GC-mediated gluconeogenesis in liver as well as increased GC-mediated decreased glucose utilisation by adipose tissue and skeletal muscle [179]. Extracellular GLU has been shown to stimulate 11HSD1 activity when coexpressed with H6PDH in HEK-293 cells [198], therefore increased glycemia could potentiate 11HSD1 activity in HHTg rats *via* H6PDH stimulation. Even though regulation of transcription of H6PDH gene have not been extensively studied yet, one study showed upregulation of hepatic H6PDH mRNA expression in the liver of rats kept on sucrose diet [199] therefore one cannot exclude that intracellular carbohydrate content might modulate H6PDH expression.

However, in contrast to dramatic changes in hepatic 11HSD1 activity observed in female HHTg rats, no 11HSD1 upregulation was foung in HHTg males compared to Wistar males (unpublished data). In humans, gender-specific interplay between hepatic 11HSD1 activity and serum levels of leptin was described. Liver 11HSD1 activity negatively correlated with systemic levels of leptin in men whereas this correlation was positive in women [200]. Similar observations were described in leptin-deficient female *ob/ob* mice where leptin treatment markedly increased hepatic 11HSD1 activity and expression [115]. Although estradiol attenuates expression of 11HSD1 [71] it has also been shown to increase the sensitivity to leptin in rat hypothalamus [201]. Sex dimorphism in liver 11HSD activity in HHTg rats hence might be explained by gender-specific actions of sex steroids and leptin.

6.2. Effect of 24h fasting on local metabolism of GCs in HHTg rats

Fasting represents a stressful metabolic state which is characterised by elevated systemic GC level. Increased serum CS levels were observed in both Wistar and HHTg rats. Hepatic 11HSD1 and H6PDH mRNA expressions were downregulated in HHTg rats upon fasting. Synthetic glucocorticoid dexamethasone (DEX) has been shown to downregulate 11HSD1 in rat liver [107-108] so 11HSD1 depletion might be a consequence of highly elevated serum CS levels in HHTg rats. 11HSD1 is also inhibited by low GLU levels [198] so slightly decreased glycemia in HHTg rats might also play a minor role. In SAT, the 11HSD1 activity was upregulated to the same level in both strains. Although *in vitro* studies showed decreased 11HSD1 upon DEX treatment in adipocyte cell lines [109-110], systemic DEX administration resulted in 11HSD1 upregulation in adipose tissue [109]. Similar observations in 11HSD1 activity were found in VAT, moreover upregulation of 11HSD1 was even stronger in HHTg rats compared to Wistar rats. These data might support previously described GC-mediated tissue-specific effects on 11HSD1, in this case effects of markedly increased serum GCs. Decreased 11HSD1 in liver upon fasting might represent a protective mechanism against GC signaling overstimulation. 11HSD1 and H6PDH mRNA expression is relatively high in liver compared to adipose tissue and skeletal muscle, therefore both systemic and intracellular elevation of active GCs might lead to excessive GC response. In contrast, while elevated 11HSD1 and H6PDH stimulate local GC production upon fasting in adipose tissue, it might support GC response mediated by systemic GCs. Even though fasting sligtly upregulated expression of GR in adipose tissue of HHTg rats, total level of GR mRNA was comparable with HHTg rats fed *ad libitum*. Also, no changes in GR expression were described in liver, therefore it seems that GR levels in tissue does not play an important role in response to fasting. In liver as well as in adipose tissue, H6PDH expression followed fasting-mediated changes in 11HSD1 expression. It has been already described that H6PDH stimulate 11HSD1 activity when coexpressed in cells [57]. Our data imply that not only H6PDH activity but also mRNA expression might be under direct control of 11HSD1 action.

In contrast to liver and adipose tissue, no effect of fasting on 11HSD1 activity or mRNA expression was observed in skeletal muscle. 11HSD1 activity was upregulated in HHTg rats compared to Wistar rats upon fasting to the similar level as was found in HHTg rats fed *ad libitum*, however this trend was not found in 11HSD1 expression.

Fasting stimulated skeletal muscle H6PDH in both strains which indicate that the tightness of connection between 11HSD1 activity and H6PDH mRNA expression that we observed in liver and adipose tissue does not exist in muscle. Fasting stimulates glycogenolysis in muscle that leads to accumulation of glucose-6-phosphate (G6P) in cells. Since G6P serves as a substrate for H6PDH, it can stimulate its activity and expression. GR expression was upregulated upon fasting which suggest that not 11HSD1 action but GR induction is an important element of GC signaling pathway involved in response to fasting in skeletal muscle.

In both strains, depression of LDL+VLDL particles was observed upon fasting. As VLDL synthesis in liver is stimulated by GCs [21-22], elevation of systemic levels might be expected. However, VLDL are also produced by intestinal cells in response to feeding so prolonged fasting would reduce production of VLDL here. VLDL are one of the substrates for LPL. LPL is located on endothelial surface of adipose tissue and muscle capillaries and specifically cleaves 1- and 3-ester linkages of free TGs as well as TGs incorporated in lipoprotein particles that leads to production of NEFAs and 2-monoacylglycerols. LPL mRNA expression is tissue-specific. In adipose tissue, LPL mRNA expression is reduced upon fasting while in muscle LPL mRNA expression is stimulated [202-203]. Therefore, decreased LDL+VLDL particles in Wistar and HHTg rats observed upon fasting might be a net result of decreased production of VLDL in intestine and stimulation of LPL-mediated cleavage in muscle. Stimulated muscle LPL activity might be also a reason for decreased serum TG in Wistar and HHTg rats. LPL is also regulated on posttranslation level by glycosylation [204]. In adipocytes fasting promotes the synthesis of mannose-rich LPL which is retained in cells. Feeding results in cleavage of mannose units and addition of GLU, hexosamine and sialic acid groups which target LPL to secretion. This modification is mediated by INS [205]. Decreased insulin levels in Wistar and HHTg rats contribute to the shift of the balance between lipid storage and lipolysis in favour of lipolysis upon fasting. Lipolysis is stimulated mainly by glucagon and catecholamines but also indirectly by GCs [188]. During lipolysis NEFAs are released into the blood and serve as fuel for other tissues therefore we expected increased systemic level of NEFA upon fasting. However, this effect was observed only in HHTg rats but not Wistar rats. It might be due to fast uptake of NEFAs by other tissues and reesterification of NEFAs into TGs that occurs under all metabolic states. We also found increased HDL levels in both Wistar and HHTg rats. As HDL particles are hydrolysed in liver by hepatic lipase (HL) and its mRNA

expression and activity are supressed during prolonged fasting *via* catecholamines and GCs [206], accumulation of HDL particles in serum might be a consequence of decreased hydrolysis rate in liver.

Accumulation of fat in visceral parts in generally considered to be more dangerous than in periphery. It also appeares that VAT is more sensitive to GCs as patients with Cushing´s syndrome [3] and mice with overexpressed 11HSD1 develop visceral obesity [26]. Also in a number of studies, upregulated VAT 11HSD1 was shown to be strong predictor of increased fat cell size [113, 175, 207]. Finally, even though 11HSD1 mRNA expression is higher in SAT, higher 11HSD1 reductase activity (but not protein) was found in isolated preadipocytes from VAT. This suggested that other posttranscriptional effects might play a role in regulation of 11HSD1 activity. Possible mechanism might be a competitive inhibition of 11HSD1 by 7-ketocholesterol, which might occur in different level in cells, however this hypothesis remains to be proved [208]. Our data show strain difference in response to fasting between SAT and VAT. Fasting as a stresful state accompanied by elevated systemic level of GCs resulted in upregulation of 11HSD1 activity in both strains in SAT and VAT but only in VAT this upregulation was stronger in HHTg rats. This is in agreement with above mentioned studies that increased 11HSD1 represents stronger risk in metabolic syndrome in VAT than in SAT.

6.3. Effect of CBX treatment on local metabolism of GCs in HHTg rats

CBX treatment significantly decreased level of 11HSD1 activity and mRNA expression in liver. This is in agreement with data obtained on obese Zucker rats where the same downregulation of 11HSD1 was observed after CBX administration [123]. CBX-mediated downregulation of liver 11HSD1 was also described in DIO mice and in this case it was followed with downregulation of H6PDH. Similarly in murine hepatocytes, CBX decreased 11HSD1 and H6PDH activity and expression in dose-dependent manner [209-210]. Decreased H6PDH was also detected in the liver of HHTg rats. We can consider that upon CBX treatment intracellular levels of GCs are decreased in hepatocytes. Since systemic elevation of GCs has been shown to downregulate 11HSD1 [109], we might expect stimulation of 11HSD1 upon CBX treatment. On the other hand, certain *in vitro* studies showed GC-mediated upregulation of 11HSD1 [54, 105-106], therefore the molecular mechanism of CBX-mediated downregulation of 11HSD1 in liver remains unclear. In the liver of DIO mice as well as in cultured murine hepatocytes, CBX supressed mRNA expression of [209], in contrast to mice on high fat diet, where CBX-mediated upregulation was found [210]. In HHTg rats, CBX treatment had no effect so the effects of CBX on hepatic expression of GR remains unknown.

No effect of CBX on level of 11HSD1 active protein and mRNA expression was detected in adipose tissue of HHTg rats and the same pattern was found in Zucker rats [123] as well as in DIO mice [210]. In addition, CBX treatment did not affect 11HSD1 activity in SAT of obese men [126]. GCs have been shown to inhibit murine adipocyte 11HSD1 activity and expression *in vivo* and *in vitro* [109-110] so it could be expected that decreased intraadipocyte GC level would result in 11HSD1 induction. However, no such effect was observed in either of the studies mentioned above. CBX treatment decreased the rate of lipolysis and inhibited differenciation in cultured preadipocytes isolated from subcutaneous and omental fat depots of healthy nonobese individuals [211] and decreased lipolysis rate was found also in *in vivo* human study [212]. These finding suggest that adipose tissue is a target for CBX action even though it does not result in 11HSD1 downregulation.

No inhibitory effect of CBX on level of active 11HSD1 protein and mRNA expression was also found in skeletal muscle of female HHTg rats. As only few studies investigated the effects of CBX on local metabolism of GCs in skeletal muscle it is hard

to discuss these results. But the study of Livingstone et al. [123] in obese Zucker rats also did not find any effect of CBX on skeletal muscle.

These results suggest that regulation of 11HSD1 expression in liver is more sensitive to CBX treatment than in other tissues. In ADX rats even a single subcutaneous dose of CBX together with CS resulted in downregulation hepatic but not renal 11HSD1. Downregulation in kidney was observed only after chronic CBX treatment [213]. However, in HHTg rats we did not observe any changes in extrahepatic tissues even after chronic treatment. In our study CBX was delivered orally in drinking water. Pharmacokinetic and bioavalability studies with GA, a compound chemically related to CBX (Fig. 2.12.), showed that in rats, orally administrated GA was absorbed predominately in small intestine. Absorbed GA was detected in plasma approximately 1h after the administration and the maximal plasma concentration was reached after 12h and persisted in blood for 24h. Maximal bioavailability was 14% of total dose [214]. When injected intraperitoneally CBX concentration in plasma reaches maximum approximately 1h after dose [215]. These data might imply that intestinal absorption of GA and CBX is propably quite slow and so certain amount of drug is eliminated before assimilation. In addition, CBX absorbed in small intestine is collected by portal bloodstream and transported to liver at first. That might be a reason why the bioavailability of CBX was larger in liver than in adipose tissue [216] and it might also explain the tissue-specific effect of CBX on 11HSD1 we observed.

In different rodent models of metabolic syndrome, CBX treatment improved symptoms of metabolic syndrome. In DIO mice, CBX markedly reduced body weight and plasma GLU and improved insulin resistance [209-210], however no such effects were observed in obese Zucker rats [209-210] or in obese men [126]. On the other hand, marked improvement in insulin resistance, lipoprotein metabolism, hepatic steatosis and atherosclerosis were described in CBX-treated model of metabolic syndrome derived from LDL receptor-deficient mice [216]. In contrast to these results, no improvement of metabolic syndrome symptomes was detected in HHTg rats. Also, chronic CBX treatment did not affect systemic levels of CS. This might be explained by CBX-mediated inhibition of 11HSD1 as well as 11HSD2. As both 11HSD types catalyze the same reaction but each in opposite direction, the net effect of CBX on systemic GC level might be zero.

6.4. Effect of C544 treatment on local metabolism of GCs in HHTg rats

Our data show that C544 exhibit better potency and pharmacodynamic activity in rat than in mice. While in mice oral administration of C544 at 30 mg/kg lowered 11HSD1 *ex vivo* activity in liver and adipose tissue to 35% of untreated controls over 4h and after 6h inhibition nearly dissapeared [141], in HHTg rats, 11HSD1 activity inhibition by 10 mg/kg of C544 remained under 10% of untreated controls still after 24h. Interestingly, we found circadian rythm in 11HSD1 activity in liver and VAT therefore period of the day should be considered in experiments studying 11HSD1. Rats and mice, as a nocturnally active animals, display GC circadian rythm opposite to man with evening peak values. In mice, maximal plasma CS level is reached at 6 p.m. [217] and we found similar profile was also in HHTg rats (data not shown). Circadian rythm in hepatic 11HSD1 activity exhibited profile opposite to serum CS levels which means that the lowest 11HSD1 activity coincided with the highest serum CS. These data suggest that 11HSD1 in liver is supressed by CS. This hypothesis is supported by the fact that during fasting, which is characterized by elevated GCs, 11HSD1 activity and expression was also lowered in HHTg rats.

Chronic treatment of HHTg rats with C544 exerted tissue-specific changes in 11HSD1, H6PDH and GR expression. In liver, decreased intracellular and systemic level of CS due to 11HSD1 inhibition resulted in upregulation of GR expression. This suggests that GR expression in liver might be regulated by systemic but not intracellular levels of CS as CBX treatment had no effect on GR expression. However, this possible mechanism might be indirect because elevated serum levels of CS upon fasting had no effect on GR expression. In contrast to liver, 11HSD1 and H6PDH expression was upregulated in VAT but not in SAT upon C544 treatment. This suggests that VAT is more sensitive to intracellular changes of CS levels than SAT.

Chronic inhibition of 11HSD1 lowered serum TGs in HHTg rats. Similar effects of C544 were observed in obese DIO mice, HF/STZ diabetic mice and ApoE $^{-/-}$ mice, a murine model of atherosclerotis [141]. In obese and diabetic mice, C544 also improved insulin sensitivity and lowered plasma GLU levels in contrast to HHTg rats where no such changes were found. However, above mentioned mouse models display impaired GLU homeostasis, while in HHTg rats impaired insulin sensitivity mostly appears after high carbohydrate diet [179]. Positive effect of selective inhibition of 11HSD1 on lipid profile and GLU homeostasis in mice has also been shown in other murine models with

different selective 11HSD1 inhibitors [135, 140, 218-219]. Another selective inhibitor developed by Merck, Compound A (Fig 2.16.B.) was tested on rat DIO model and exhibited similar effects as C544 in HHTg rats. It lowered serum levels of TGs but did not improve hyperglycemia or serum INS levels [135, 140, 143, 218-220]. In HHTg rats, C544 treatment resulted in increased serum CHOL but this upregulation was predominately in favour of HDL fraction. Similar upregulation in HDL cholesterol was found in 11HSD1 $^{-/-}$ mice [149]. It seems that pharmacological effects of selective 11HSD1 inhibition are different in murine models compared to rats. Despite the fact that most of the studies were performed on mice, the results suggests that 11HSD1 inhibition exhibits more beneficial effects in mice where both lipid profile and glucose homeostasis were improved. In contrast, only lipid metabolism was affected in rat models including HHTg rats.

In HHTg rats, 11HSD1 inhibition was accompanied by decreased systemic levels of CS after C544 treatment. In contrast, CBX-mediated 11HSD1 inhibition had no effect on serum CS levels and did not improve metabolic syndrome in HHTg rats. This suggest that drug selectivity over 11HSD2 might be important for treatment of metabolic syndrome.

A couple of clinical trials in different stages with various compounds are now in progress. Nowadays, preliminary results were published that showed positive effects of selective 11HSD1 inhibitor INCB13739 on patients with type 2 diabetes. Long-term treatment with INCB13739 was well-tolerated and resulted in reduction of fasting glucose, serum levels of total cholesterol, LDL cholesterol and TGs in patients with ongoing diabetic (metformin) therapy [221].

In summary, studies on rodent models as well as clinical studies proved that compounds that inhibit 11HSD1 without affecting 11HSD2 activity represent a promising group of drugs for treatment of metabolic syndrome and should be considered for further detailed research.

7. Conclusions

Results of our experiments can be summarized into the following conclusions.

7.1. Local metabolism of GCs in selected tissues of HHTg rats

The level of local metabolism of GCs in metabolically active tissues differs significantly between HHTg and Wistar rats in females. The strongest upregulation of 11HSD1 was detected in the liver of HHTg rats. Furthemore, it was accompanied by elevated H6PDH expression. In SAT, VAT and skeletal muscle 11HSD1 was only slightly elevated and was not followed by induction of H6PDH or GR mRNA. In liver, SAT and skeletal muscle, 11HSD1 activity correlated with serum TG level. Despite hepatic 11HSD1 upregulation, HHTg rats display slightly reduced serum CS levels compared to Wistar rats. Since 11HSD1 is able to increase intracellular level of GCs, GC-mediated effect in liver can significantly contribute to hypertriglyceridemia in female HHTg rats. As mentioned in chapter 6.1., no upregulation in hepatic 11HSD1 activity male HHTg rats, although HHTg males suffer from hypertriglyceridemia as well. This suggests that local metabolism of GCs and its connection with metabolic syndrom in HHTg rats is complex gender-specific process that might include specific action of sex steroids or other hormones.

7.2. Effect of 24h fasting on local metabolism of GCs in HHTg rats

We described tissue-specific differences in local metabolism of GCs in response to 24h fasting in Wistar and HHTg rats. Fasting downregulated 11HSD1 activity in liver, upregulated in adipose tissue and was without any effect in skeletal muscle of both Wistar and HHTg rats. In VAT, 11HSD1 upregulation was stronger in HHTg rats compared to Wistar rats. We found tight connection between 11HSD1 activity and H6PDH expression in liver and adipose tissue in HHTg rats. Fasting reduced serum levels of TGs, LDL+VLDL and INS and increased levels of HDL and CS. In HHTg rats, slight fasting hypoglycemia was observed.

7.3. Effect of CBX treatment on local metabolism of GCs in HHTg rats

CBX treatment affected only 11HSD1 and H6PDH but not GR in liver of HHTg rats. No effect on 11HSD1, H6PDH or GR expression was observed in adipose tissue or skeletal muscle. In contrast to positive effects of CBX treatment on metabolic syndrome symptomes described in previous studies, no improvements of serum lipid and glucose homeostasis were found in HHTg rats.

7.4. Effect of C544 treatment on local metabolism of GCs in HHTg rats

C544 exhibits better potency and pharmacodynamic profile in rats than in mice. Chronic C544 treatment exerted tissue-specific effects on 11HSD1, H6PDH and GR expression in HHTg rats. While in liver decreased intracellular levels of GCs induced only GR expression, in VAT 11HSD1 and H6PDH mRNA but not GR mRNA was upregulated. No effect on local metabolism of GCs was observed in SAT. In contrast to CBX, chronic C544 treatment reduced serum levels of TGs and increased serum levels of HDL cholesterol. To conclude, as well as in different rodent models, C544 exhibited positive effect on some symptomes of metabolic syndrome in HHTg rats.

8. References

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9. Abbreviatons

11-DEH	11-dehydrocorticosterone
11HSD1	11β-hydroxysteroid dehydrogenase type 1
11HSD2	11β-hydroxysteroid dehydrogenase type 2
A, T, C, G	Adenin, thymin, cytosin, guanin (in DNA sequence)
ACC	AcetylCoA carboxylase
ACTB	β-Actin
ACTH	Adrenocorticotropic hormone
ADX	Adrenalectomy
AKR	Aldo-keto reductase
aP2	Adipocyte protein 2
Apo	Apoprotein
ASC	Adipose stromal cells
ATP	Adenosine triphosphate
ATP5B	ATPase subunit B5
B2M	β_2-Microglobulin
bp	Base pair
BSA	Bovine serum albumin
C/EBP	CAAT enhancer binding protein
C544	Compound 544
CANX	Calnexin
CBG	Corticosteroid binding globulin
CBX	Carbenoxolone
CDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CREc	AMP response element
CRH(CRF)	Corticotropine-releasing hormone (factor)
CRP	C-reactive protein
CS(B)	Corticosterone
CYC1	Cytochrome c1
DBD	DNA binding domain

DEX	Dexamethasone
DIO	Diet-induced obesity
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide mix
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
FKBP	FK506 binding protein
G	Glycine (in protein sequence)
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
G6PT	Glucose-6-phosphate transporter
GA	Glycyrrhetinic acid
GAPDH	Glyceraldehyd-3-phosphate dehydrogenase
GC	Glucocorticoid
GH	Growth hormone
GLU	Glucose
GLUT4	Glucose transporter 4
GR	Glucocorticoid receptor
GRE(GRU)	Glucocorticoid response element (unit)
H6PDH(H6PD)	Hexose-6-phosphate dehydrogenase
HDL	High density lipoprotein
HF/STZ	High fat diet+streptozocin
HHTg rat	Prague hereditary hypertriglyceridemic rat
HL	Hepatic lipase
HNF	Hepatocyte nuclear factor
HPA axis	Hypothalamo pituitary adrenal axis
HPLC	High performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
HSF	High sucrose/high fat diet
HSL	Hormone sensitive lipase
Hsp	Heat shock protein
CHOL	Total cholesterol
IGF	Insulin-like growth factor
INS	Insulin

kb	Kilobase
kDa	Kilodalton
LBD	Ligand binding domain
LPL	Lipoprotein lipase
LXR	Liver-X-receptor
MDH	Malate dehydrogenase
MMLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
NAD^+	Nicotineamid dinucleotide
$NADP^+$	Nicotineamid dinucleotide phosphate
NADPH	Nicotineamid dinucleotide phosphate (reduced form)
NEFA	Nonesterified fatty acid
NF	Normalization factor
NTD	N-terminal domain
p	Probability level
PAI	Plasminogen activator inhibitor
PCR	Polymerase chain reaction
PEPCK	Phosphoenol pyruvate carboxykinase
PPAR	Peroxisome proliferator activated receptor
PVN	Paraventricular nuclei
r	Pearson correlation coefficient
RIA	Radioimmunoassay
RIN	RNA integrity number
RNA	Ribonucleic acid
RNAse	Ribonuclease
RPL13A	Ribosomal protein L13A
rpm	Revolutions per minute
RT	Reverse transcription
SAT	Subcutaneous adipose tissue
SDR	Short-chain dehydrogenase/reductase
TG	Triglyceride
TNFα	Tumor necrosis factor α
TOP	Topoisomerase
TZD	Thiazolinedione

UBC	Ubiquitin c
VAT	Visceral adipose tissue
VLDL	Very low density lipoprotein
X	Arbitrary amino acid
Y	Tyrosine
YWHAZ	Tyrosine 3-monooxygenase
ZDF	Zucker diabetic fatty rat

10. List of publications

Klusoňová P, Pátková L, Ergang P, Mikšík I, Zicha J, Kuneš J, Pacha J.
Local metabolism of glucocorticoids in Prague hereditary hypertriglyceridemic rat-effect of hypertriglyceridemia and gender. Under review

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