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1

GENERAL INTRODUCTION

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The burden of obesity and the metabolic syndrome in the presence of increasing social stress

In today's modern society, with sedentary lifestyle and comfort food readily available, the prevalence of obesity, type 2 diabetes (T2D), and cardiovascular disease (CVD) is rising tremendously. According to World Health Organization (WHO), in 2008 35% of adults worldwide were overweight and more than half a billion adults were obese (1). WHO has predicted these numbers to more than double by 2015. Obesity *per se* is a major risk factor for T2D, hypertension and CVD (2). Each year 2.8 million people die because of the complications induced by overweight and obesity (1). Clustering of risk factors for overall cardiovascular risk was first described as the metabolic syndrome (MetS) by Kylin in the early 1920 as constellation of hypertension, hyperglycemia and gout (3). Later, Raeven proposed insulin resistance as the common denominator for these individual cardiovascular risk factors, and over the following decades the MetS has been known as syndrome X, Raeven's syndrome, and insulin resistance syndrome (4). To date, the MetS is considered as the most important cluster of risk factors for the development of T2D and CVD and subsequently increased mortality (5-8). Currently, several definitions are available for the MetS, of which the Third Report of the National Cholesterol Education Program's Adult Treatment Panel (NCEP ATP III) is the most commonly used. To define the MetS according to the NCEP ATP III, at least 3 out of the following 5 criteria should be present: 1): central obesity (waist circumference >102 cm for men and >88 cm for women) 2): plasma high density lipoprotein-cholesterol (HDL-C) levels <1.03 mM (men) and <1.29 mM (women) 3): plasma triglycerides levels \geq 1.7 mM 4): blood pressure \geq 135/80 mmHg and 5): fasting glucose levels \geq 6.1 mM (9). Today's modern society is also characterized by chronic stress (10). Chronic stress, either social or otherwise, affects the activity of the hypothalamus-pituitary-adrenal (HPA) axis and facilitates changes in life style, like emotional "comfort" eating, and lack of sleep. Chronic stress is also associated with the development of central obesity, insulin resistance and MetS. Although the causes for the development of the MetS are most likely multi-factorial, it is plausible to assume an important role for chronic stress in aggravating (the development of) the MetS.

The (patho)physiology of the stress response

When an individual is exposed to a stressor, rapid changes occur within seconds to minutes through stimulation of both the sympathetic nervous system and the HPA axis. Perception of stress by an organism leads to secretion of corticotrophin releasing hormone (CRH) from the parvocellular compartment of the paraventricular nucleus (PVN) in the hypothalamus, which subsequently stimulates pituitary adrenocorticotropin (ACTH) secretion. Activation of ACTH receptors in the adrenal cortex leads to the synthesis and secretion of glucocorticoids (GC). GCs will then, in turn, down regulate the stress response by a negative feedback manner via their glucocorticoid (GR) and mineralocorticoid (MR) receptors in the hypothalamus, the pituitary, and the hippocampus (11). Secretion of GCs (*i.e.* cortisol in humans and corticosterone (CORT) in rodents) as a response to perceived stress are required to induce the necessary behavioral and metabolic adaptations for the individual to be able to adequately cope with the stressor (fight or flight).

In this thesis, we will focus on the metabolic adaptations. The metabolic effects of GCs include peripheral as well as central effects. Whereas the peripheral effects are directed towards

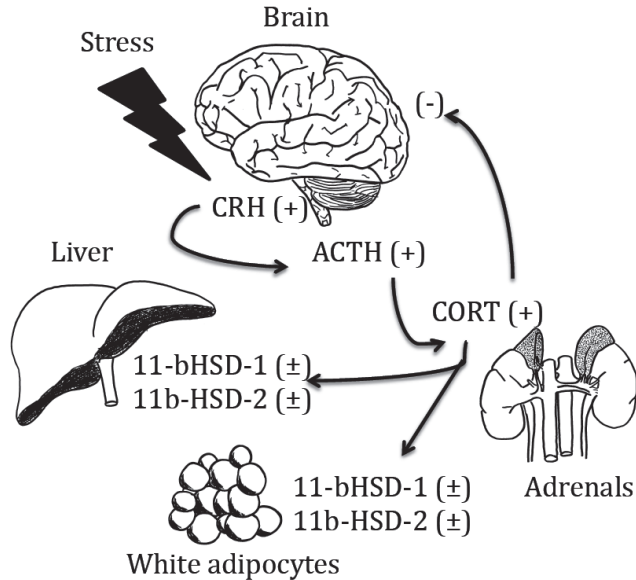


Figure 1. Schematic presentation of the stress response. See text for explanation.

recruitment of energy availability by reduction of energy stores for gluconeogenesis (12, 13), the central effects of GCs are anabolic and directed towards augmentation of energy stores by adjusting feeding behavior and intake of palatable foods to compensate for the energy loss (14). As a consequence, increased cortisol exposure, like during chronic stress, will further increase insulin levels and food intake, facilitating the development of obesity and the MetS (15). Thus, short-term exposure to stress, within the right context, helps the organism to adequately cope with the challenge. However, if the response is not sufficient, too extreme or prolonged, it can have deleterious adverse effects for the organism (11). Likewise, when a stressor becomes chronic, a vulnerable phenotype develops: an individual that has to do concessions in its behavioral and metabolic adaptations. Within the central nervous system, this is characterized by neurodegenerative changes and cognitive impairment. The resultant metabolic mal-adaptation manifests as abnormal recruitment and storage of fuel, resulting in abdominal obesity, and osteoporosis (16).

Peripheral GC metabolism is dependent on the activity of tissue-specific 11β -hydroxysteroid dehydrogenase (11β -HSD) type 1 and 2, which are enzymes that convert cortisone into its active form cortisol and vice versa. 11β -HSD-1 is predominantly expressed in the liver, adipose tissues and muscle where it can amplify the intracellular concentrations of cortisol available to bind its respective receptors (17) and is, therefore, at least in part responsible for the unfavorable side effects, such as insulin resistance and adiposity that are associated with increased GC exposure (18). 11β -HSD-2 is more prominently expressed in the kidney, where it reduces GC effects by converting cortisol to cortisone. However, it has been implicated that 11β -HSD-2 might also play a role in obesity as it has been shown to be strongly correlated with adiposity (19).

Specific effects of GCs on insulin sensitivity, lipid metabolism, and atherosclerosis

GCs have strong anti-inflammatory properties and are widely used as immunosuppressive agents but they also play a major role in the metabolism of glucose, lipids and proteins. GCs stimulate lipolysis, proteolysis and hepatic glucose production thereby providing substrates for oxidative processes (12). Overstimulation of these catabolic processes can become detrimental for the individual leading to metabolic derangements such as the development of central obesity (20), hepatic steatosis (21), dyslipidemia with increased plasma triglyceride (TG) and non-esterified fatty acid (NEFA) levels (22), increased protein breakdown of muscle mass (23, 24) and insulin resistance accompanied by glucose intolerance (25). These side effects are dependent on the dose and the duration of treatment (25).

GCs inhibit pancreatic insulin secretion by reducing glucose transporter (GLUT) 2 (26, 27) and glucokinase G6Pase (28, 29) expression and activity, thereby decreasing glucose uptake, ATP synthesis and calcium influx. Activation of serum and GCs-inducible kinase (SGK) 1 by GCs can also augment the inward repolarizing potassium currents by upregulating Kv ion channels (30) and thereby limiting calcium influx and insulin secretion. Furthermore, inhibition of DAGphospholipase by GCs (31), which leads to decreased activation of the protein kinase (PK) C can inhibit insulin secretion as well as the increased expression of α_2 adrenergic receptors that lead to reduced cyclic adenosine monophosphate (cAMP) levels followed by decrease in PKA activity (31, 32). GCs can also reduce insulin biosynthesis by reducing the adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio (33, 34) and by inducing β -cell apoptosis (35).

In skeletal muscle, GCs decrease the expression and phosphorylation of insulin receptor substrate (IRS)-1, phosphatidylinositol 3-kinase (PI3-K), and PKB/Akt (36-38). GCs can also interfere with the migration of the GLUT4 to the cell surface (39) and also reduces glycogen synthesis (40). Furthermore, GCs do not just induce insulin resistance in skeletal muscle, but also facilitate protein breakdown and reduce protein synthesis by reducing the activation of eIF4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1) (41), providing substrate (*i.e.* amino acids) (42) for hepatic gluconeogenesis. Indeed, GCs stimulate endogenous glucose production by the liver, thereby increasing insulin resistance by activating genes involved in hepatic carbohydrate metabolism (43) and increasing the expression of enzymes involved in gluconeogenesis, including phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) and peroxisome proliferator-activated receptor (PPAR)- α (44-48).

Dyslipidemia is a common side effect of increased GC exposure. GCs affect adipose tissue metabolism by increasing the expression and activity of hormone sensitive lipase (HSL) that hydrolyses TG in the adipocyte (49). Furthermore, GCs decrease lipoprotein lipase (LPL) activity in a site-specific manner (50-52). Consequently, fat mobilization (intracellular lipolysis) is stimulated increasing plasma NEFA and TG flux to the liver (53). GCs have been shown to induce intrahepatic lipid accumulation by decreasing FA oxidation and increasing TG synthesis (54, 55). This can lead to increased very low density lipoprotein (VLDL) synthesis, which further increases circulating TG.

Atherosclerosis is the most important manifestation of CVD, leading to myocardial infarction, congestive heart failure, stroke and peripheral artery diseases. Atherosclerosis is considered to be a complication of insulin resistance and dyslipidemia, which are present in the MetS and are associated with increased GC exposure as is seen in patients with Cushing's syndrome (CS). Traditionally, it is thought that atherosclerosis develops as a consequence of cholesterol deposition in the

subendothelial layer after injury to the endothelium (56). Atherosclerotic lesion development can be triggered by increased plasma levels of low density lipoprotein (LDL), which leads to smooth muscle cell proliferation and can be taken up by residential macrophages to form foam cells (57). These processes precede the development of more complex fibrous lesions (56) in which inflammation is shown to play a significant role (58).

Intriguingly, the role of GCs in the development of atherosclerosis is not yet clearly established in humans or in animals. GCs are known to induce vasoconstriction (59-62) and endothelial dysfunction (63), which can facilitate the atherosclerotic lesion development. On the other hand, GCs also have anti-proliferative and anti-migratory effects on vascular smooth muscle cells (64-67) that may inhibit the lesion development. Furthermore, it is unclear how GCs affect inflammation in the development of atherosclerosis, as both inhibition and stimulation of inflammation has been reported (68). However, these effects are at least to be considered dependent on GC concentration (69).

Taken together, GCs acutely reduce insulin secretion and stimulate whole body lipolysis. However, under chronic conditions, this cycle of reduced pancreatic insulin secretion and decreased insulin sensitivity in skeletal muscle, liver and fat tissue results in insulin resistance and dyslipidemia, facilitating the development of other complications such as CVD including atherosclerosis.

Cushing's syndrome as a human model of chronic stress

CS, a rare clinical syndrome characterized by prolonged exposure to inappropriately increased GCs, was first described by Harvey Cushing in 1932. CS can be considered as *the* clinical human monosymptomatic equivalent for severe chronic stress. The most common cause of endogenous CS is an adrenocorticotropin-secreting pituitary adenoma. Other causes include ectopic ACTH secretion by neuroendocrine tumors or ACTH-independent cortisol overproduction by adrenal tumors or adrenal hyperplasia. Exogenous CS, induced by exogenous sources of GCs, such as steroid treatment in autoimmune diseases or prevention of graft rejection in transplantation, is very prevalent (70).

Regardless of the cause, patients with CS are exposed to supra-physiological levels of cortisol and display several features of chronic stress, such as depression, anxiety and cognitive impairment (71, 72), but they also have a phenotypical resemblance to, and fulfill the criteria for, the MetS (70). Indeed, CS patients have markedly increased cardiovascular morbidity and mortality (73) suggesting that excessive exposure to GCs is involved in the pathogenesis of MetS and central obesity. Furthermore, patients with MetS show increased GC metabolism (74), but the underlying mechanisms are only partially understood.

Intriguingly, some of the features of MetS and certain psychopathologies in CS patients prevail after the removal of cortisol excess. Indeed, one year after remission, CS patients still suffer from impaired glucose tolerance (75) and increased insulin levels after oral glucose tolerance test (76). Furthermore, CS patients have an increased waist circumference after one year of remission (75, 76) and even after long-term remission higher visceral fat mass has been observed without affecting the body mass index (77).

Epidemiological and other evidence for an association between increased baseline activity of the HPA axis and cardiovascular disease or obesity

The link between GCs and CVD was reported first in the 1950s, reporting elevated cortisol to be associated with (premature) atherosclerosis (78). Besides CS also in patients otherwise chronically exposed to increased GCs, like patients with congenital adrenal hyperplasia (79) and patients

that underwent angiography (80), increased cortisol appeared to correlate with increased intima media thickness (IMT). In agreement with these findings, manipulation of cortisol exposure at the tissue level through stimulation or abrogation of 11- β -HSD-1 activity can increase or regress fat accumulation in visceral depots, and well as other features of the MetS (see also subparagraph 1.6 for references) Taken together, an increased activity of the HPA axis has been linked to the development of MetS (81), which has recently led to propose a pathogenic role of cortisol in the MetS (82). In human obesity, however, the results are rather inconclusive and not well studied. In obese women, urinary excretion of free cortisol is increased (83), and in men a significant correlation was found between salivary cortisol and both body mass index (BMI) and waist-to-hip ratio (84). However, it appears that cortisol secretion is increased in obese subjects primarily because of increased clearance and increased distribution volume, thereby resulting into secondary central activation of the HPA axis but with normal circulating cortisol concentrations (85, 86). Furthermore, the potency of ACTH to stimulate cortisol production was found to be decreased in obesity (85). In addition, a flattened circadian cortisol secretion rhythm in patients with T2D has been found (87). Thus, obesity appears to induce compensatory changes in the baseline, non-stressed, activity of the HPA axis, that are not unequivocally characterized by increased HPA axis activity. In addition, circulating cortisol levels do not always reflect the activity of the HPA axis in the central nervous system, nor in peripheral tissues.

Animal models of (features of) the MetS in relation to the HPA axis

Due to the similarities between the MetS and the phenotype of CS, it has been suggested that GCs and, in particular, increased circulating GCs might play a role in the development of MetS. A number of studies using different rodent models have studied the association of the activity of the HPA axis and various components of the MetS. The most commonly used method is high fat diet (HFD) feeding resulting in diet-induced obesity (DIO). One of the best-characterized models in this respect is the male C57Bl/6 mouse fed a HFD, where HFD induces profound insulin resistance and obesity (88-91). However, CORT levels, or any other parameter of the HPA axis was measured in only a minority of these studies. Intriguingly, the experimental mouse models of obesity and the MetS reveal conflicting results with respect to whether the HPA-axis is activated. Given the well-known time and context dependent effects of GCs, these discrepancies might be explained by methodological differences between the studies, including differences in rodent models. As the effect of HFD on HPA axis activity in the context of MetS requires further investigation, several studies indicate that comfort food reduces HPA axis activation and facilitates stress recovery. Indeed, it has been shown in rats that palatable food intake reduces the signs of stress and promotes weight gain, even in a chronic stress condition (92-94). These findings indicate that palatable food, such as HFD, has the ability, at least acutely, to reduce the stress response, facilitate recovery from stress, and dampen the HPA axis activity.

The effect of GCs on certain features of the MetS has also been studied in adrenalectomized models, where CORT concentrations are clamped to a desired level by subcutaneously implanted GC pellets. In combination with streptozotocin-induced destruction of pancreatic β -cells and subsequent exogenous insulin replacement, these studies have provided important information on the relative contribution of each hormone on feeding behavior, choice of food, weight gain and fat deposition (95). However, clamped CORT levels do not reflect normal physiology due to the loss of tissue sensitivity for GCs as a result of continuous high exposure. Therefore, new methods of

increasing GCs in the circulation are emerging, by adding them to the food (96) or drinking water (97), which to some degree retains the diurnal rhythm. Furthermore, 11β -HSD-1 enzyme inhibition or deficiency in mice results into improvement of metabolic parameters (98-100), indicating that tissue exposure to GCs is associated with features of MetS not just in humans but also in animal models.

As insulin resistance and obesity are readily and well studied in mouse models of DIO, atherosclerosis as a complication of MetS presents a challenge as wild-type mice do not develop atherosclerosis even in the presence of high cholesterol diet (HCD). Development of atherosclerosis can, however, be studied in genetically modified mouse models, which naturally over time or in the presence of HCD, readily develop atherosclerosis, such as the apoE-knockout (apoE^{-/-}) mouse (101), the LDL receptor-knockout mouse (Ldlr^{-/-}) (102), the APOE*3-Leiden transgenic mouse (103), and the APOE*3-Leiden.CETP (E3L.CETP) mouse (104). To date, the effect of GCs and stress has mainly been investigated in other animal models such as dogs, pigs and rabbits (105). The limited number of studies performed in mice reveals contradictory results on whether increased GC exposure aggravates atherosclerosis like in CS patients, or does not influence the development of atherosclerosis (106, 107).

Taken together, the relationship between the MetS and the HPA axis has been studied to some degree previously in animal models. However, several of these studies suffer from differences in methodological approach e.g. using methods that are not standardized to be stress-free or otherwise disrupt normal physiology greatly, which might mask true effects. Furthermore, differences in genetic predisposition for the development of certain features of the MetS, or otherwise modified (e.g. adrenalectomized) animal models will most likely affect the results.

Outline of the present thesis

In this thesis, we aimed to expand our knowledge on the pathophysiological aspects that underlie both the basal activity of the HPA axis during the development of obesity, and the effects of a period of GC excess on reversibility of metabolic parameters and atherosclerosis in mice.

In **chapter 2**, we performed a systematic review of studies on DIO mouse models. Although feeding HFD easily induces features of MetS, the rodent models reveal conflicting results with respect to the HPA axis activation. Therefore, we included only original mouse studies reporting parameters of the HPA axis after high fat feeding, and at least one basal CORT level with a proper control group. Studies with adrenalectomized mice, transgenic animals only, HFD for less than 2 weeks, or other interventions besides HFD, were excluded. Subsequently, in **chapter 3**, we aimed to evaluate non-stressed diurnal HPA axis activity in mice in detail during obesity development. As stated in sub-paragraph 1.6, obesity-prone male C57Bl/6J mice were fed HFD or LFD for 12 weeks, and a detailed assessment of the activity of the HPA axis was made measuring circadian plasma CORT concentrations, activation of the HPA axis in the central nervous system (CRH, and GR mRNA expression in the hippocampus, amygdala, and hypothalamus), and activation of the HPA axis in peripheral tissues (11β -HSD-1 and -2 expression in adipose tissue and liver). In the second part of the thesis, given the observations in humans with CS, we aimed to address the potential long-term effects of a period of GC overexposure in mice that develop DIO or atherosclerosis. CS is associated with an increased incidence of MetS, and increased cardiovascular morbidity and mortality, even after long-term correction of GC excess. However, the causal relation between the episode of cortisol overexposure and long-term changes is not established and is difficult to assess in humans because of the rarity and heterogeneity of CS. Therefore, in **chapter 4**, we performed a study in

male C57Bl/6J mice, fed either a LFD or HFD, were given CORT or vehicle in the drinking water for 4 weeks, followed by a washout period. Plasma circadian CORT, lipids, insulin, and glucose levels were assessed at regular intervals. Insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamp, and lean and body- and fat masses with dual-energy X-ray absorptiometry (DEXA). Finally, in **chapter 5**, we investigated the effects of both transient and continuous GC excess, again induced via CORT in the drinking water, on insulin sensitivity and atherosclerosis development in female APOE*3-Leiden.CETP (E3L.CETP) mice. These mice have a human-like lipoprotein metabolism and develop atherosclerosis upon feeding a Western-type diet. In **chapter 6**, a synopsis of all major findings is given. In addition, the data presented in this thesis are discussed in the context of potential implications of overexposure to stress (hormones) on the development of obesity and CVD in every-day-life.

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