GLUCOCORTICOIDS, METABOLIC ADAPTATIONS AND RECOVERY:

STUDIES IN SPECIFIC MOUSE MODELS

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TABLE OF CONTENTS

1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

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 ≥1.7 mM 4): blood pressure ≥135/80 mmHg and 5): fasting glucose levels ≥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

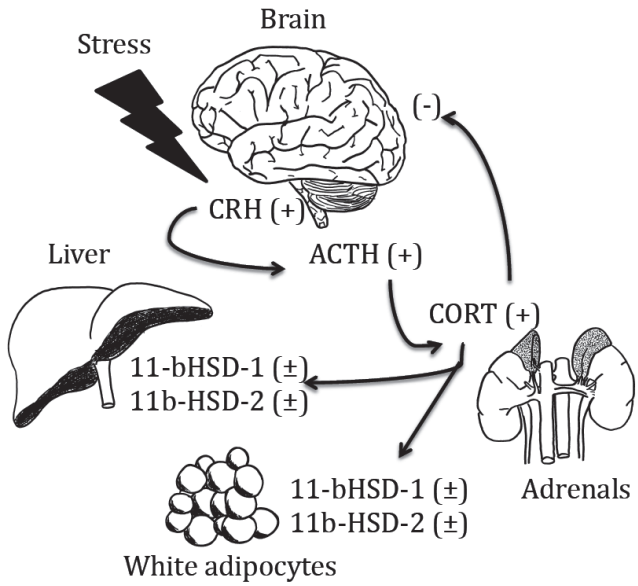

Figure 1. Schematic presentation of the stress response. See text for explanation.

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 affects, 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 trigyceride (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 affects 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 de 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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EFFECTS OF HIGH FAT DIET ON THE BASAL ACTIVITY OF THE HYPOTHALAMUS-PITUITARY-ADRENAL AXIS IN MICE: A SYSTEMATIC REVIEW

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ABSTRACT

Background: Hypothalamus-pituitary-adrenal-axis activity is suggested to be involved in the pathophysiology of the Metabolic syndrome. In diet-induced obesity mouse models, features of the Metabolic syndrome are induced by feeding high fat diet. However, the models reveal conflicting results with respect to the hypothalamus-pituitary-adrenal-axis activation.

Aim: To assess the effects of high fat feeding on the activity of the hypothalamus-pituitary-adrenal-axis in mice.

Methods: PubMed, EMBASE, Web of Science, the Cochrane database, and Science Direct were electronically searched and reviewed by 2 individual researchers

Study selection: We included only original mouse studies reporting parameters of the hypothalamus-pituitary-adrenal-axis after high fat feeding, and at least one basal corticosterone level with a proper control group. Studies with adrenalectomized mice, transgenic animals only, high fat diet for less than two weeks, or other interventions besides high fat diet, were excluded.

Results: Twenty studies were included. The hypothalamus-pituitary-adrenal-axis evalution was the primary research question in only 5 studies. Plasma corticosterone levels were unchanged in 40%, elevated in 30%, and decreased in 20% of the studies. The effects in the peripheral tissues and the central nervous system were also inconsistent. However, major differences were found between mouse strains, experimental conditions, and the content and duration of the diets.

Conclusion: This systematic review demonstrates that the effects of high fat feeding on the basal activity of the Hypothalamus-pituitary-adrenal-axis in mice are limited and inconclusive. Differences in experimental conditions hamper comparisons and accentuate the need for standardized evaluations to discern the effects of diet-induced obesity on the hypothalamus-pituitary-adrenal-axis.

INTRODUCTION

The metabolic syndrome (MetS) is a cluster of metabolic abnormalities that identifies individuals at high risk for cardiovascular disease (1, 2). These metabolic abnormalities include abdominal obesity, hypertension, dyslipidemia, and insulin resistance. The concept of the MetS is a subject of debate, because the pathophysiological basis of this syndrome is unclear (3). In addition, the combination of cardiovascular risk factors does not add to the risk related to the individual risk factors for cardiovascular disease (4). Nonetheless, these individual, well-recognized cardiovascular risk factors have all been associated with increased cardiovascular morbidity and mortality in the general population (1, 2, 4).

Several lines of evidence suggest that alterations in the activity of the hypothalamus-pituitary-adrenal (HPA)-axis may be involved in the development of the MetS. Glucocorticoids (GC) (cortisol in humans and corticosterone in rodents) are secreted by the adrenals in response to a stressor, in order to induce the required behavioral and metabolic adaptations to be able to adequately cope with the stressor (fight or flight). The metabolic effects of GC include both peripheral and central effects. Whereas the peripheral effects are directed towards recruitment of energy availability by reduction of energy stores for gluconeogenesis, the central effects of glucocorticoids are anabolic and directed towards augmentation of energy stores by adjusting feeding behavior and intake of palatable foods to compensate for the energy loss. 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 (5, 6).

This is first exemplified by patients with Cushing's syndrome (CS), a rare disorder caused by prolonged excessive exposure to glucocorticoids. Patients with CS have a phenotypical resemblance to and fulfill the criteria for, the MetS (7). These patients have a markedly increased cardiovascular morbidity and mortality (8), suggesting that excessive exposure to glucocorticoids is involved in the pathogenesis of MetS and central obesity. Second, patients with MetS show increased activity of the HPA-axis (9), but the underlying mechanisms are only partially understood (10). Third, manipulation of cortisol exposure at the tissue level through stimulation, or abrogation, of 11β-hydroxysteroid dehydrogenase type-1 (11-β-HSD-1) activity can increase, or regress, the fat accumulation in visceral depots, and well as other features of the MetS (11, 12).

In animal models of diet-induced obesity (DIO), common features of the MetS, like insulin resistance and obesity are easily induced by feeding of high fat diet (HFD). However, these experimental mouse models of obesity and the MetS reveal conflicting results with respect to whether the HPA-axis is activated. These discrepancies might be explained by methodological differences between the studies, including differences in rodent models. Therefore, the aim of the present systematic review was to critically compare the data available in mice on the effects of HFD feeding on basal, non-stressed activity of the HPA-axis, and to discuss possible explanations for the observed differences.

METHODS

Search strategy

We performed a systematic search in PubMed, EMBASE, Web of Science, the Cochrane database, and Science Direct, for published studies on the association between HFD feeding and activity of the HPA-axis. The following search strategy was used: for PubMed: *(high fat diet OR high*

lipid diet OR high fat diets OR high lipid diets OR western-type diet OR western-type diets OR "high-fat" OR Dietary Fats OR (diet-induced obesity)) AND (HPA-axis[tw] OR (("Hypothalamo-Hypophyseal System"[mesh] OR "Pituitary-Adrenal System"[mesh] OR hpa[tw]) AND axis[tw]) OR Hypothalamo-Pituitary-Adrenal[tw] OR Hypothalamus-pituitary-adrenal[tw] OR glucocorticoid OR glucocorticoids OR corticosterone) AND (mice OR mouse); for EMBASE: *((high fat diet OR high lipid diet OR high fat diets OR high lipid diets OR western-type diet OR western-type diets OR "high-fat" OR Dietary Fats OR diet-induced obesity).mp OR exp fat intake/ OR exp lipid diet/) AND (hypothalamus hypophysis adrenal system/ OR exp glucocorticoid/ OR corticosterone/ OR (HPA-axis OR ((Hypothalamo-Hypophyseal OR Pituitary-Adrenal OR hpa) AND axis) OR Hypothalamo-Pituitary-Adrenal OR Hypothalamus-pituitary-adrenal OR glucocorticoid* OR corticosterone).mp) AND (exp mouse/ OR mice.af OR mouse.af)*; for Web of Science: *TS=((high fat diet OR high lipid diet OR high fat diets OR high lipid diets OR western-type diet OR western-type diets OR "high-fat" OR Dietary Fats OR (diet-induced obesity)) AND (HPA-axis OR ((Hypothalamo-Hypophyseal OR Pituitary-Adrenal OR hpa) AND axis) OR Hypothalamo-Pituitary-Adrenal OR Hypothalamus-pituitary-adrenal OR glucocorticoid OR glucocorticoids OR corticosterone) AND (mice OR mouse))*; for Cochrane: *(high fat diet OR high lipid diet OR high fat diets OR high lipid diets OR western-type diet OR western-type diets OR "high-fat" OR Dietary Fats OR (diet-induced obesity)) AND (HPA-axis OR ((Hypothalamo-Hypophyseal OR Pituitary-Adrenal OR hpa) AND axis) OR Hypothalamo-Pituitary-Adrenal OR Hypothalamus-pituitary-adrenal OR glucocorticoid OR glucocorticoids OR corticosterone) AND (mice OR mouse);* for ScienceDirect: *TITLE((high fat diet OR high lipid diet OR high fat diets OR high lipid diets OR western-type diet OR western-type diets OR "high-fat" OR Dietary Fats OR (diet-induced obesity)) AND (HPA-axis OR ((Hypothalamo-Hypophyseal OR Pituitary-Adrenal OR hpa) AND axis) OR Hypothalamo-Pituitary-Adrenal OR Hypothalamus-pituitary-adrenal OR glucocorticoid OR glucocorticoids OR corticosterone) AND (mice OR mouse))*; for Academic Search Premier: *((high fat diet OR high lipid diet OR high fat diets OR high lipid diets OR western-type diet OR western-type diets OR "high-fat" OR Dietary Fats OR (diet-induced obesity)) AND (HPA-axis OR ((Hypothalamo-Hypophyseal OR Pituitary-Adrenal OR hpa) AND axis) OR Hypothalamo-Pituitary-Adrenal OR Hypothalamus-pituitary-adrenal OR glucocorticoid OR glucocorticoids OR corticosterone) AND (mice OR mouse))*.

In addition, the references of relevant articles were checked for additional articles. The search was performed on January 27, 2011. Only original articles were included. We used the following exclusion criteria: adrenalectomized animals, transgenic animals only, duration of HFD of less than two weeks, and other interventions disabeling the interpretation of the effect of the HFD per se. In addition, studies were only included if 1) the effects of HFD were compared with a control group on standard chow or low fat diet (LFD), and 2) at least one basal (thus non-stressed) corticosterone sample was available for both groups.

Data review

The following data were extracted from each study: mouse strain, age and/or bodyweight at baseline, body weight gain, housing conditions, dietary intervention (duration and content of the diet (percentage of calories provided as fat)), and specific parameters of HPA-axis activity: circulating corticosterone concentrations, 11-β-HSD-1 activity, corticotrophin-releasing hormone (CRH) adrenocorticotropic hormone (ACTH), and glucocorticoid receptor (GR) expression in peripheral tissues and/or the central nervous system (when evaluated). In addition, we evaluated the clock

hour of evaluation (related to the circadian nadir or not), whether single or multiple time points were used for sampling of blood, and whether circulating corticosterone was measured only or combined with other central and/or peripheral parameters of HPA-axis activity.

RESULTS

The search strategy identified a total of 218 articles: PubMed: n=142; EMBASE: n=139, of which 26 were unique; Web of Science: n=132, of which 39 were unique; none in the COCHRANE database, ScienceDirect: n=6, all unique, and 34 in Academic Search Premier, of which 5 were unique. Of these, 150 papers were excluded on the basis of title and abstract. The remaining 68 studies were retrieved for full evaluation, of which 20 studies fulfilled the in- and exclusion criteria. In only 5 of these 20 selected studies, evaluation of selective parameters of the HPA-axis was the primary research question. Details of these 20 studies are summarized in Table 1.

Mouse characteristics, housing, and diets

The most commonly used mice were male C57Bl/6 mice. 8 studies (13-15, 23, 24, 28-30) evaluated the effects of HFD in C57Bl/6 mice only, and one study only in A/J mice (18). Another 6 studies compared C57Bl/6 mice either with A/J (19), or with transgenic mice: Leptin deficient C57Bl/6J Lep ob/ob (17), diabetic KKAy and ob/ob (21), 11-β-HSD-1-knockout (KO) (16, 22), and glucocorticoid receptor haploinsufficient GRβgeo/+ (27) mice. The remaining 5 studies used polygenic fat and lean mice from inbred lines (20), Balb/c mice (25), male NIH Swiss mice (26), or compared glucocorticoid receptor activity regulating FK506-binding protein 52 heterozygote (FKBP52+/-) male mice or kappa-opioid receptor knockout (KOR-KO) mice with their wild type littermates (31, 32).

At baseline, the mean age of the mice varied from 3-19 weeks, and in one study from 6-9 months (20). In accordance with these variations in age, body weight at baseline (when documented) varied from < 10 g to 30 g.

Housing conditions were not specified in 8/20 studies. In the remaining 12 studies, mice were pair-housed in 3 studies, 3-4 per cage in one study, and single housed (for the entire study period or for the last week before sampling) in 8 studies.

Acclimation period was applied in nine studies. In 2 studies (20, 27) time was not specified. Another 6 studies (14, 15, 18, 21, 23, 25) had an acclimation period of one week (handling mentioned in only a few). Finally, one study (29) reported a 4-week acclimation period at the end of the experiment.

The duration of the diets varied between 2 and 30 weeks, and the relative contribution of calories derived from fat between 32% and 60% (within the HFD) and between 6.5% and 16% (within the LFD).

The effects of HFD on circulating corticosterone concentrations (n=20 studies)

The evaluation of circulating corticosterone levels was limited to a single sample in 85% (17/20) of the studies. Blood samples were obtained between 8-11 am in 12 studies, whereas the time of sampling was not specified in 6 other studies. Three studies assessed diurnal variation of corticosterone levels, by using 2 samples (8-10 am and 7 pm) (27), or by using samples obtained every 4 h (24), or even every 2 h (29).

HFD did not affect circulating corticosterone levels in 8 studies (13, 14, 18, 19, 21, 23, 25, 32) (all obtained a single blood sample only). 3 of these 8 studies actually reported corticosterone

Table 1. Studies in mice on the effects of High Fat Diet feeding on basal, non-stressed activity of the HPA axis.

	Author (ref)	Mouse strain	Mean age / weight at baseline	Housing	Diet (HFD / LFD % cal as fat)	Duration of diet	Weight gain
1	Herberg L *et al*. 1975 (13)	C57Bl/6	-	(NS	-	4 wks	-
2	Ziotopoulou M *et al*. 2000 (14)	C57Bl/6J	4wks / 20.6 g	NS	43% / 6.5%	2 wks	3.3 *vs* 2.3 g
3	Moraes RC *et al*. 2003 (15)	C57Bl/6J	4 wks	pair	36% / 3%	8 wks	11 *vs* 6 g
4	Morton NM *et al*. 2004b (16)	MF1 WT 11 β -HSD-KO C57Bl/6J-11β--HSD-KO	'adult' / 25-30 g	Standard single last week	58 / 11%	18 wks	15 *vs* 5 g
5	Morton NM *et al*. 2004a (17)	C57Bl/6J A/J C57Bl/6-JLep obob	'adult'	Standard; single last week	58% / 11%	2 wks 18 wks	-
6	Bullen JW Jr *et al*. 2004 (18)	A/J	4-9 wks	NS	45% / 10%	4 wks	4 to 5 g
7	Michel C *et al*. 2005 (19)	C57Bl/6J AJ	23-28 g	pair	65% / 63% cal as fat or starch	25 days	5 g (BL6)/ 1 g (A/J)
8	Morton NM *et al*. 2005 (20)	polygenic fat and lean mice from inbread lines	6-9 months	Single	58% / 11%	18 wks (after selection: fat vs lean mice)	2.5 fold higher in fat mice on HFD
9	Alberts P *et al*. 2005 (21)	KKAy ob/ob C57Bl/6J	11-15 wks 16 wks 19 wks	single	HFD: 32.5 kcal% fat with 0.01% chol LFD:10 kcal% fat	4 wks	Body weight 44.9 vs 44.4g 50.4 vs 48.7 LFD: 28.5 g (no HFD)

Table legends: WT: wild type; HFD: high Fat Diet; LFD: low Fat diet; Chol: cholesterol; NS: not specified; Cort: corticosterone; PVN: paraventricular nucleus of the hypothalamus; 11-β-HSD-1: 11 beta hydroxysteroid dehydrogenase type 1; CRH: corticotrophin-releasing hormone ACTH: adrenocorticotropic hormone; GR: glucocorticoid receptor; 11–DHC:11-dehydrocorticosterone; AT: adipose tissue

Evaluation time points	Mean Plasma corticosterone (HFD / LFD)	Adrenal / thymic weight (HFD / LFD)	Peripheral Cort activity	Central Nervous System
-	2 µg/dl No effect of diet	-	-	-
8-9.30 a.m (One sample)	No effect of diet (data not shown)	-	-	-
Not specified (NS) (one sample)	47 / 29 ng/ml	-	Various genes (not glucose related)	-
8 a.m. (one sample)	18 / 31 nmol/l 58 / 57 nmol/l No effect of the diet	-	↓intra-AT (visceral + epididymal) cort levels in C57BL/6J–11-β –HSD-1-KO *vs* wildtype C57BL/6J on LFD: (159 ng/g; 477; and HFD: (404 *vs* 562 ng/g)	-
8 a.m. (one sample)	BL6: 170 / 100 nmol/l A/J: 100 / 60 nmol/l	-	↓ AT 11-β-HSD-1 after HFD (more in BL6)	-
2-3 h after lights on (one sample, under CO^2 narcosis)	Not different at 1,2,3,4 wks (data not shown)	-	-	PVN: CRH-R: no difference
NS (one sample after anaesthesia)	BL6: 80 / 62 mmol/l A/J: 92 / 88 nmol/l No effect of the diet	-	-	PVN: CRH: no effect of diet Amygdala: CRH↑ in BL6; no effect of diet
8 a.m. (one sample)	39 / 86 nmol/l (fat *vs* lean mice: effect of breeding)	↓ adrenal and ↑ thymus in fat mice Diet: NS	↓ AT + ↑ liver 11-β-HSD-1	GR in PVN and Hippocampus: not different
9-11 a.m. (one sample)	0.23 / 0.17 µM 0.92 / 1.30 µM BL6: (LFD): 0.08 µM No effect of the diet	-	-Liver cort 3.0 -5.1 and 6.2- 8.1–fold, and 11–DHC 3.4-3.6 and 6.7-8.2–fold ↑ in KKAy and ob/ob *vs* WT) - Mesenteric AT cort 2.7-4.2–fold ↑, and 11-DHC 2-4–fold ↑ in ob/ob *vs* in KKAy mice No C57/BL6 (nM/Kg) - Epididymal AT cort 3.0- 6.2–fold ↑,and 11DHC 1.8- 2.0–fold ↑ in ob/ob *vs* KKAy mice, WT not shown	-

Table 1. Studies in mice on the effects of High Fat Diet feeding on basal, non-stressed activity of the HPA axis. Cd.

	Author (ref)	Mouse strain	Mean age / weight at baseline	Housing	Diet (HFD / LFD % cal as fat)	Duration of diet	Weight gain
10	Densmore VS *et al*. 2006 (22)	C57Bl/6J 11-B-HSD-KO	6 wks / 25-30 g	pair	58% / 11%	2 or 18 wks	15 *vs* 5 g
11	Luque RM *et al*. 2006 (23)	C57Bl/6J	4 wks	single	60% / 10%	16 wks	25 *vs* 15 g
12	Kohsaka A *et al*. 2007 (24)	C57Bl/6J	6 wks	NS	45% / 16%	6 wks	8 vs 4 g
13	Chen H *et al*. 2007 (25)	Balb/c	5 wks	NS	32% / 12%	7 wks	8 *vs* 5 g
14	McClean PL *et al*. 2007 (26)	NIH Swiss	8 wks	single	45% / 10%	160 days	Final mean body weight 55 *vs* 38 g
15	MichailidouZ *et al*. 2008 (27)	C57Bl/6J GRβgeo/+	≤ 10 g	Standard; single for sampling	58% / 11%	22 wks	40 *vs* 20 g
16	Liu Y *et al*. 2008 (28)	C57Bl/6	6 wks	NS	58% / 11%	30 wks	Final mean body weight 56 *vs* 32 g
17	Veniant MM *et al*. 2009 (29)	C57Bl/6	3 wks (HFD) 16 wks (chow)	Single for 4 last weeks	60% / chow	13 wks	-
18	Matsumoto S *et al*. 2009 (30)	C57Bl/6J	4 wks	NS	2% chol vs normal diet	2 wks	7 *vs* 7 g
19	Warrier M *et al*. 2010 (31)	FKBP52+/- WT	2 months	NS	45% / 12%	4 wks	22% *vs* 7% 22% *vs* 7%
20	Czyzyk TA *et al*. 2010 (32)	129S6 WT KOR-KO	7-8 wks	3-4 per cage	45% / 5%	16 wks	15 *vs* 4.6 g

Table legends: WT: wild type; HFD: high Fat Diet; LFD: low Fat diet; Chol: cholesterol; NS: not specified; Cort: corticosterone; PVN: paraventricular nucleus of the hypothalamus; 11-β-HSD-1: 11 beta hydroxysteroid dehydrogenase type 1; CRH: corticotrophin-releasing hormone ACTH: adrenocorticotropic hormone; GR: glucocorticoid receptor; 11–DHC:11-dehydrocorticosterone; AT: adipose tissue

Evaluation time points	Mean Plasma corticosterone (HFD / LFD)	Adrenal / thymic weight (HFD / LFD)	Peripheral Cort activity	Central Nervous System
Between 8-10 (one sample)	2 wk: 40 / 130 nmol/l 18 wk: 170 (HFD) nmol/l	-	-	-PVN: 2 wks: ↑11 b-HSD in arcuate nucl 18 wk: reversed - Hippocampus: no change in 11-β-HSD-1: (2 nor 18 wks)
Between 8-11 (one sample)	0.9 / 0.7 ng/ml No effect of the diet	-	-	-
Every 4 h	2-15 / 3-20 µg/dl	-	-	-
NS (one sample after anaesthia)	198 / 221 ng/ml No effect of the diet	-	-	-
NS (one sample)	Approx 55 / 20 ng/ml (229%↑ in HFD)	-	-	-
Diurnal 8-10 h, 19 h	50-100 (m-e) / 25-75 (m-e) nmol/l	7 *vs* 5 gr (P<0.05)	Genotype ↓↑ Diet: not shown	PVN: genotype ↓↑ Diet: POMC not different Hippocampus: Genotype ↓↑ Diet not shown
9-10 am (one sample)	36 / 23 ng/ml	-	-	-
Diurnal Every 2 h from 4.30-20.30 h	26 / 42 (nadir) 526 / 526 ng/ml (peak)	-	Influence of time on effectiveness of 11-β-HSD-1 Inhibition	-
8 a.m. (one sample)	Appox 50 / 30 ng/ml	-	-	POMC mRNA ↑ after high Chol diet (circ ACTH also ↑)
NS Sample via retro-orbital sinuses (requires sedation)	260 / 180 ng/ml 190 / 225 ng/ml No effect of the diet for WT	-	-	-
11 a.m.	76 / 114 ng/ml	-	-	-

concentrations in conscious conditions, which varied widely (more than 200 fold): from 0.7-0.9 ng/ml (23), 27.7 ng/ml (21), to 76 ng/ml (32) in the morning nadir. 3 out of the 8 studies reported corticosterone concentrations under anesthesia (18, 19, 25) and even in the millimolar range (19). 2 remaining studies did not specify the time of the sampling (13) or did not report the corticosterone concentrations (14).

HFD increased corticosterone concentrations in 6 other studies (15, 17, 26, 27, 28, 30) after 2, 8, 18, 23, and 30 weeks of diet. In C57Bl/6 mice the corticosterone concentrations varied from 47 vs 29 ng/ml, 59 vs 35 ng/ml, 36 vs 23 ng/ml, to 50 vs 30 ng/ml (15, 17, 28, 30) and 35 *vs* 21 ng/ml (in A/J mice) (17). 3 of these studies did not specify the time of sampling (15, 26, 28) and one did not specify the strain (30).

HFD decreased corticosterone concentrations in 4 other studies (22, 24, 29, 31), 2 of which sampled corticosterone more than once daily. The first study sampled plasma corticosterone levels every 4 h and found that HFD decreased both nadir (20 *vs* 30 ng/ml) and peak (150 *vs* 200 ng/ml) corticosterone concentrations after 6 weeks of the diet (24). In contrast, Véniant *et al*. (29) found that HFD decreased the nadir (26 *vs* 42 ng/ml) corticosterone concentrations evaluated every 2 h whereas HFD did not affect peak corticosterone (526 ng/ml) after 13 weeks of the diet. Finally, one study documented that HFD decreased corticosterone levels at 2 weeks but increased corticosterone concentrations after 18 weeks of the diet (22). The remaining study showed a decrease in the morning nadir, but the sample was obtained under anesthesia.

The remaining one study reported an effect of genotype or breeding on circulating corticosterone concentrations after HFD: MF1-11-β-HSD-1-KO and C57Bl/6J-11-β-HSD-1-KO mice had higher circulating corticosterone concentrations than their wild type littermates (16). In addition, other studies reported the effect of genotype or breeding on cortcosterone concentrations: C57Bl/6J showed higher corticosterone concentrations than A/J mice (17) and polygenic lean mice from inbred lines had higher corticosterone concentrations after 18 weeks of HFD than their fat littermates (86 *vs* 39 nmol/l, respectively) (20).

The effects of HFD on peripheral tissue specific 11-β-HSD-1 expression (n=6 studies)

HFD increased corticosterone levels in visceral and epididymal fat after 18 weeks on HFD: 562 *vs* 477 ng/g in C57Bl/6J mice (16), and this effect of HFD was even more pronounced in C57Bl/6 J–11-β-HSD-1-KO mice (477 *vs* 159 ng/g, HFD *vs* control diet, respectively). However, another study (17) reported that HFD decreased 11-β-HSD-1 expression in adipose tissue, which was more pronounced in C57Bl/6 than in A/J mice, whereas HFD increased hepatic 11-β-HSD-1 expression (20). A fourth study found that HFD increased hepatic, epididymal, and visceral corticosterone levels in KKAy and ob/ob mice (21). The remaining 3 studies found effects on various genes (15), of genotype (C57Bl/6 *vs* GRβgeo +/+ (27)), and influence of time on the effectiveness of 11 β-HSD inhibition (29).

The effects of HFD on the central nervous system (n=5 studies)

Within the paraventricular nucleus of the hypothalamus (PVN), HFD did not affect CRH expression (2 studies: ref 18, 19), POMC expression (1 study: ref 27), or GR expression (1 study: ref 20). One study (22) found that HFD increased 11-β-HSD-1 expression transiently in the arcuate nucleus of the PVN after 2 weeks, but not after 18 weeks of the diet. Another study found that HFD did not

affect CRH expression in the amygdala in C57Bl/6 or A/J mice (19). Within the hippocampus, HFD did not alter GR expression (20) or 11-β-HSD-1 expression (22).

DISCUSSION

This systematic review demonstrates that data on the effects of HFD in mice on the basal activity of the HPA-axis are limited, and inconclusive. Circulating corticosterone concentrations in a total of only 20 studies were found to be increased, unchanged, or decreased. The relative energy contribution of fat in the HFD varied between 36-58%, 32-65% and 58-60%, respectively. In addition, data on the effects of HFD on 11-β-HSD-1 expression in the peripheral tissues and CRH and GR expression in the central nervous system (only 5 studies) were inconsistent. However, differences in mouse strains, housing and sampling conditions, and in the content and duration of the diets preclude simple comparisons of the effects of HFD on the activity of the HPA-axis, and accentuate the need for standardized evaluations to discern the effects of fat and diet-induced obesity on HPA-axis and the metabolic syndrome.

From an evolutionary point of view, the regulation of energy balance is integrated in the neurobiology of stress, i.e. into the regulation of the allostatic responses to environmental demands. Glucocorticoids are the main mediators of the stress response (33) and regulate fuel homeostasis by increasing feeding behavior when food becomes available (34). Within the hypothalamus, a rapid fine-tuned hormonal cross-talk exists between glucocorticoids and leptin, acting via leptin blockade of the glucocorticoid-induced, endocannabinoid-mediated suppression of excitation of the CRH expressing neurons. This provides a nutritional state-sensitive mechanism that integrates the neuroendocrine regulation of energy homeostasis and the stress response (35). HFD induced obesity is not only accompanied by higher leptin and insulin levels (15, 24) but also by altered diurnal patterns of leptin and insulin (24), which affect hypothalamic CRH suppression of HPA-axis.

Different housing conditions also affect the activity of the HPA-axis and might be considered as chronic social stress, which in turn influences the propensity to become obese. This is best illustrated by a recently proposed model of psychosocial stress in rats (a resident–intruder paradigm) linking allostatic load to metabolic disorders (36), and confirmed in mice (37). This model states that MetS and obesity can develop in presence of a HFD only when an environmental threat prevents active coping (fight/flight), but permits only a passive strategy. These experiments demonstrated that when both dominant and subordinate rodents were faced with a threatening situation, a similar overactive HPA-axis and hyperphagia was seen in both. However, dominants responded with an active coping style associated with sympathetic over activity in metabolic tissues that limited the development of obesity despite overfeeding, whereas subordinates responded with a passive helplessness strategy and, particularly when faced with a HFD, developed weight gain and obesity. Thus, the effects of stress and HFD appear to be less detrimental dependent on the ability of the individual to adequately cope with the stress, as a result of genetic predisposition, and/or social factors. These data accentuate the important role of psychosocial stress in modulating the individual vulnerability to weight gain, provided that the individual is fed an "unhealthy" high-fat diet.

When evaluating the impact of high fat feeding on adrenal axis function in mice, therefore, an appropriate study design is a prerequisite, and at least the following factors should be taken into account: first, the choice for a mouse strain that, upon high fat feeding, has previously shown

to provide a validated model for the development of specific features of the MetS, like obesity and insulin resistance. Second, standardization of content and duration of the diet. Third, standardization of acclimation period, housing conditions, and method for stress free sampling, and finally: the time of sampling. The relative contributions of each of these different factors can only be compared when study designs control for all these factors, except the one that is considered to be the intervention.

In conclusion, the effects of HFD on basal activity of the HPA-axis and its contribution in the propensity to become obese and develop other manifestations of the metabolic syndrome on a HFD are still unclear, but are at least time and context dependent. Proper standardization of these factors is critical in the evaluation of the role of the HPA-axis in further studies involving rodent models of the MetS.

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3

THE EFFECTS OF HIGH FAT DIET ON THE BASAL ACTIVITY OF THE HYPOTHALAMUS-PITUITARY-ADRENAL AXIS IN MICE

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ABSTRACT

Alterations of the hypothalamus-pituitary-adrenal (HPA) axis activity have been linked to the development of metabolic syndrome (MetS). Common features of the MetS, like insulin resistance and obesity, are reproducibly induced by high fat diet (HFD) in animal models of diet-induced obesity (DIO). These models, hampered by methodological differences, reveal conflicting results with respect to HPA axis activation.

This study was aimed to evaluate in detail non-stressed diurnal HPA axis activity in mice during obesity development. Male C57Bl/6J mice were fed high or low fat diet for 12 weeks. HPA axis activity was evaluated by plasma corticosterone concentrations (at 0700, 1200, and 1800 h), corticotropin-releasing hormone (CRH) and glucocorticoid receptor (GR) mRNA expression in the hippocampus, amygdala, and hypothalamus, and 11β-hydroxysteroid dehydrogenase type-1 and -2 (11β-HSD-1 and -2) expression in adipose tissue and liver. Within one week, HFD induced obesity and decreased corticosterone levels at 1200 and 1800 h, which persisted throughout the experiment. 12 weeks of HFD decreased CRH mRNA in the PVN and amygdala and GR mRNA in the PVN at 0900 h. At 1800 h, CRH mRNA expression increased in PVN and amygdala, and GR mRNA increased in the CA1 region. 11β-HSD-1 expressions decreased in gonadal, visceral and subcutaneous adipose tissue at 0900 h and at 1800 h, whereas hepatic 11β-HSD-1 expression increased at 1800 h whereas 11β-HSD-2 expression was unaffected. HFD induces complex changes in the diurnal regulation of the different components of the HPA axis. These changes are not unequivocally characterized by increased, but rather by decreased HPA axis activity.

INTRODUCTION

Glucocorticoids (GC) (cortisol in humans and corticosterone in rodents) are secreted by the adrenals in response to stimulation of the hypothalamus-pituitary-adrenal (HPA) axis by a stressor, and induce behavioral and metabolic adaptations enabling the host to adequately coping with the stressor (fight or flight). Increased activity of the HPA axis has been linked to the development of the Metabolic Syndrome (MetS) (1). The metabolic effects of GC are directed both towards recruitment of energy stores for gluconeogenesis (peripheral effects), and towards augmentation of energy stores by adjusting feeding behavior and intake of palatable foods to compensate for the energy loss (central effects). Increased GC exposure will increase food intake and insulin levels, facilitating the development of obesity and the MetS (2, 3). In accordance, patients with Cushing's syndrome (CS), which is caused by prolonged excessive exposure to GC, exhibit many features of the MetS (4) associated with increased cardiovascular morbidity and mortality (5). Finally, manipulation of cortisol exposure at the tissue level in mice, through stimulation or abrogation of 11β-hydroxysteroid dehydrogenase type-1 and -2 (11-β-HSD-1 and 11-β-HSD-2) activity can increase, or regress, visceral fat accumulation, as well as other features of the MetS (6, 7).

Nonetheless, it is still controversial how the development of the MetS and its complications affect the activity of the HPA axis. Common features of the MetS, like insulin resistance and obesity, are reproducibly induced in mouse models of diet-induced obesity (DIO) by feeding of high fat diet (HFD), but the effects on the activity of the HPA axis have been evaluated in only a minority of these studies, and their results are conflicting. Many factors, like differences in mouse strains, housing and sampling conditions, but also the content and duration of the diets affect the activity of the HPA axis and preclude simple comparisons after induction of DIO. In addition, the evaluation of the HPA axis in most studies was restricted to a single measurement of plasma corticosterone levels, which was combined with either 11-β-HSD-1 enzyme activity in peripheral tissues or in the central nervous system in only a few of these studies (8).

Therefore, the aim of the present study was to evaluate the effects of HFD in mice in detail on basal, non-stressed activity of the HPA axis, using standardized evaluations that control for housing and sampling conditions. For these studies, we used C57Bl/6J mice that develop obesity and insulin resistance upon HFD (9-12).

MATERIAL AND METHODS

Mouse strain, housing, and diets

In the current study we used male C57Bl/6J mice (Charles River, Maastricht, The Netherlands), which develop, obesity and insulin resistance, specific features of the MetS (9-12). Twelve-week-old mice (n=36) were single housed in a separate room from other experimental animals in the facility to minimize environmental stressors, and maintained on a 12 h:12 h light-dark cycle (lights on 7 a.m.) at controlled room temperature (21-22°C) and fed *ad libitum* with free access to drinking water. Mice were weight-matched and randomly assigned to the following diets for 12 weeks: high fat diet (45 energy % lard fat, D12451, Research Diet Services, Inc, New Brunswick, US) (HFD) (n=18) or low fat diet (10 energy % lard fat, D12451B, Research Diet Services, Inc, New Brunswick, US) (LFD) (n=18). All mice were fed LFD for three weeks before starting the HFD.

All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures from the Leiden University Medical Center approved the protocol.

Sampling of corticosterone

Mice on LFD (n=18) and HFD (n=18) were divided into two groups of 9 mice, and blood for measurement of plasma corticosterone levels was sampled in week 1, 5, and 9 (first group), or at week 3, 7 and 11 (second group). Blood samples were collected during the first light hour at 0700 h, at 1200 h, and during the last light hour at 1800 h. To establish that the peak plasma corticosterone peak had not 'shifted' toward the dark hours of the light/dark cycle, at week 11, plasma corticosterone was measured at 1900 h, 2000 h and 2100 h during the dark phase in red light conditions. All corticosterone samples were obtained within 90 seconds from disturbing the cage, via tail incision, allowing the mouse to move freely on top of the home cage (13). Plasma insulin was measured after a 4-hour fast in the same weeks as corticosterone. Plasma leptin levels were determined from the trunk blood after decapitation. After 12 weeks, the mice were decapitated within 90 seconds from disturbing the cage, either in the morning (0900-1000 h) or during the last light hour (1800-1900 h). After decapitation, the trunk blood was collected, the brain was harvested and snap frozen in isopentane and stored at −80°C. Liver, muscle, gonadal, abdominal visceral and subcutaneous fat pads were dissected, snap-frozen in liquid nitrogen and stored at −80°C.

Plasma hormone measurements

Plasma corticosterone levels were determined by radioimmunoassay (MP Biomedicals LLC, Orangeburg, NY; intra-assay variation 7.3%, inter-assay variation 6.9%,).

Insulin and leptin were measured with ELISA (Crystal Chem Inc., Downers Grove, IL, USA; intra-assay precision coefficient of variation ((CV)≤10% and inter-assay precision CV≤10% for both kits). All measurements were assayed according to the manufacturer's instructions.

Evaluation of HP- axis activity in the central nervous system (in situ hybridization)

Brain sections of 16 µm of the paraventricular nucleus (PVN) (Bregma -0.70mm), amygdale (Bregma -0.70 mm) and hippocampus (Bregma -1.70 mm) were cut according to the brain atlas of Paxinos and Franklin 2001 (14) on a cryostat and mounted on polysine microscope slides (Menzel-Gläzer, Braunschweig, Germany) and stored in -80°C until further use. The hybridization was performed as described previously by Meijer *et al*. 1997 (15) with minor adjustments. Briefly, sections were fixed in 4% paraformaldehyde, further permeabilized by proteinase K treatment, acetylated twice with 0.25% acetic anhydride in 0.1 M triethanolamine and dehydrated in a graded ethanol series.

Riboprobes were generated from linearized constructs containing the respective cDNAs in pBluescript. A 500-bp SalI–HindIII fragment of exon 2 of the mouse gene was used for GR (16). The cRNA from CRH was transcribed from a 1-kb cDNA insert in pGEM 4 containing full-length coding region of rat CRH (17). ^{35}S-UTP labeled antisense probes were generated using the appropriate polymerase using a standard protocol.

A hybridization mix was prepared containing 60% deionized formamide, 10% Dextran SO_4, 2xSSC, 0.1 mg/ml yeast tRNA, 0.1 mg/ml sssDNA, 10 mM dithiothreitol, 0.05 M PBS. All radiolabeled probes were diluted to 16.7x10^6 cpm/ml. Of these mixtures 120 µl was applied to each slide and then covered with a cover slip. The sections were hybridized overnight in a moisturized chamber at 55°C.

The next day, the cover slips were removed carefully and sections were washed in 2xSSC for 10 min at room temperature. After washing, sections were treated with RNAse A (2 mg/100 ml in 0.5 mol/l NaCl, 0.1 mol/l Tris, pH 7.5) at 37°C for 10 min and subsequently washed at 55°C in 2xSSC for 10 min, 1xSSC for 10 min, 0.1xSSC for 2x30 min and, finally, at room temperature in 0.1xSSC for 5 min. Sections were dehydrated in an ethanol series (70%, 80%, 96% and 100% ethanol) and dried on air. Signal was visualized with exposure of Kodak Biomax MR films, scanned and quantified by using Image J software (National Institutes of Health) and related to standard curves of ^{14}C (RPA 504 microscales; Amersham, Buckinghamshire, UK). Two sections per mouse per brain area were quantified**.** For CRH mRNA in PVN and amygdala and for GR mRNA in the PVN, the values represent a sum of the two areas measured. For GR mRNA in the hippocampus, the values represent the average of the two measurements.

11β-HSD-1 and 11β-HSD-2 expression analysis in liver and adipose tissue

Total RNA was extracted from liver and adipose tissues using the Nucleospin RNA II kit (Macherey Nagel, Düren, Germany) according to manufacturer's instructions. RNA quality was examined with lab-on-a-chip technology using Experion Std Sens analysis kit (Biorad, Hercules, CA). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and the obtained cDNA was purified with Nucleospin Extract II kit (Macherey-Nagel). Real-time PCR for 11β-HSD-1 (forward primer: CAGCAAAGGGATTGGAAGAG; reverse primer: CTTTCCCGCCTTGACAATAA) and 11β-HSD-2 (forward primer: TTTGGTGCACTTGAGCTGAC; reverse primer: AGCCGAATGTGTCCATAAGC) were carried out on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of cyclophilin (forward primer: CAAATGCTGGACCAAACACAA; reverse primer: GCCATCCAGCCATTCAGTCT) and hypoxanthine guanine phosphoribosyl transferase (forward primer: TTGCTCGAGATGTCATGAAGGA reverse primer: AGCAGGTCAGCAAAGAACTTATAG).

Statistical analysis

Data are presented as means ± SD. Statistical differences were calculated using Mann-Whitney test for non-parametric data, with GraphPad Prism, version 5.01. (GraphPad Software, Inc.La Jolla, CA), $P<0.05$ was considered as statistically significant.

RESULTS

High fat diet increases plasma insulin and leptin levels and body weight without affecting thymus weight

As anticipated, HFD feeding resulted in a greater increase in body weight as compared to LFD, which already reached significance within 1 week, and this difference remained significant throughout the experiment (Fig. 1A). HFD did not affect the thymus weight (Fig. 1B). HFD increased plasma insulin concentrations already within one week (Fig. 1C), which remained significantly increased throughout the experiment. Plasma leptin levels were significantly increased after 12 weeks of HFD both at 0900 h and 1800 h Fig 1D).

High fat diet decreases diurnal peak plasma corticosterone levels both acutely and in the long term

A diurnal corticosterone rhythm was observed in all animals, with a nadir in the morning (0700 h) and with peak values during the last light hour before the dark phase (1800 h). HFD decreased plasma

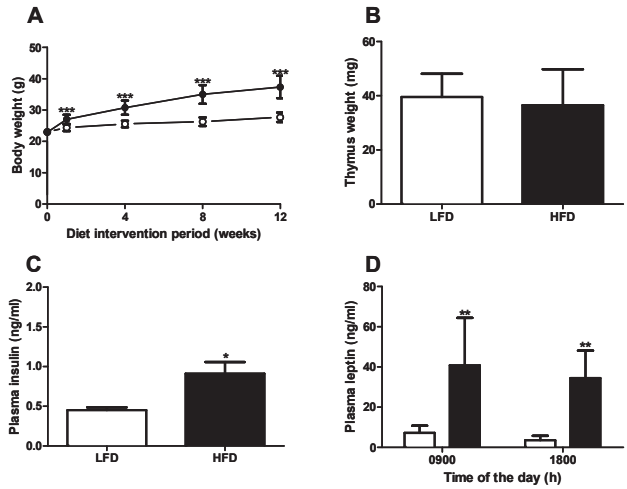

Figure 1. Effect of diet on male C57Bl/6J mice fed a low fat diet (open circles and bars) or high fat diet (closed circles and bars) on body weight (A), thymus weight at week 12 (B), plasma insulin concentrations at week 1 (C) and plasma leptin levels at week 12 (D), Mann-Whitney test, *P<0.01, **P<0.001, ***P<0.0001.

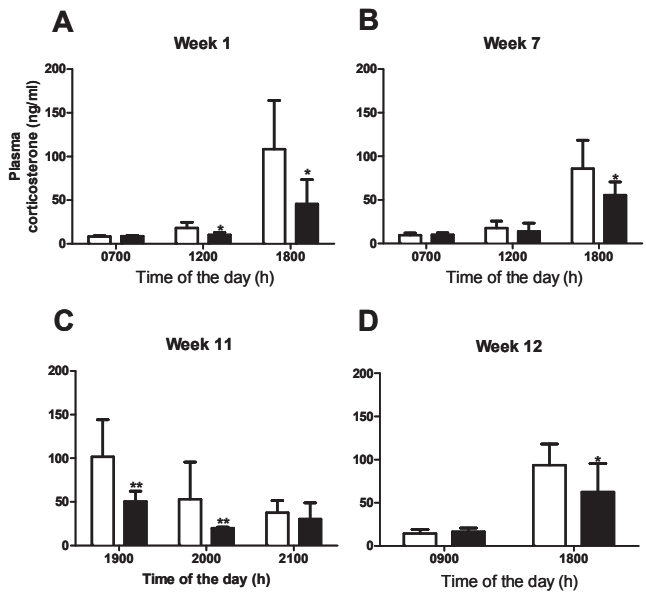

Figure 2. Effect of high fat diet on circadian plasma corticosterone. Male C57Bl/6J mice were fed a LFD (open bars) or HFD (closed bars), and plasma corticosterone was determined in the morning (0700), at noon (1200) or at the evening peak (1800) after 1 week (A), 7 weeks (B). At 11 weeks (C) corticosterone was determined at 1900, 2000 and 2100 (n=6 per time point), and at 12 weeks (D) at 0900 and 1800. Mann-Whitney test, *P<0.05, **P<0.01.

corticosterone levels within one week by 44% at 1200 h, and by 52% at 1800 h in the evening (Fig. 2A). This decrease in evening peak corticosterone levels persisted throughout the experiment at weeks 7 and 12 (Fig. 2B and D). Furthermore, peak corticosterone levels were not 'shifted' toward the dark hours of the light/dark cycle but declined from the 1800 to 1900 h time points in both LFD and HFD groups. The suppression of plasma corticosterone levels was also evident in HFD group during the beginning of the dark phase (at 1900 h, 2000 h) at week 11 when compared to the LFD group (Fig. 2C).

High fat diet induces changes in mRNA expression of CRH and GR in the brain

HFD significantly decreased CRH mRNA expression in the PVN and amygdala at 0900 h in the morning (Fig. 3A and B). Moreover, HFD decreased GR mRNA in the PVN at 0900 h in the morning basal period (Fig. 3C). HFD increased CRH mRNA expression at 1800 h in the evening in both PVN and amygdale (Fig. 3A and B). Furthermore, HFD increased GR mRNA in the evening in the CA1 region (Fig. 3D), but not in the other regions (CA3 and dentate gyrus (DG) of the hippocampus (Fig. 3E and G).

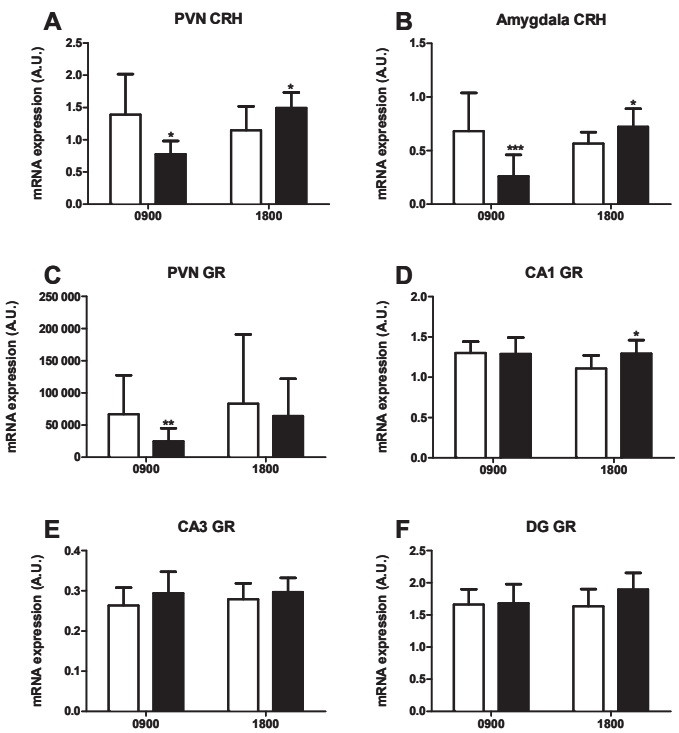

Figure 3. Effect of high fat diet on mRNA expression of CRH and GR in the hypothalamus and hippocampus. Male C57Bl/6J mice were fed a LFD (open bars) or HFD (closed bars) for 12 weeks, and mRNA expression was determined: CRH in the PVN (A) and amygdale (B), and GR in the PVN (C), CA1 (D), CA3 (E) and DG (F) region of the hippocampus in the morning (7.00) and evening (18.00) (black bars). Mann-Whitney test, $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

High fat diet feeding induces opposite changes in 11β-HSD-1 mRNA expression in adipose tissue and liver, whereas 11β-HSD-2 mRNA expression remains unaffected

HFD decreased 11β-HSD-1 expression in gonadal, visceral and subcutaneous adipose tissues both in the morning by 65%, 37% and 66%, respectively (Fig. 4B, C and D), and in the evening by 62%, 47% and 67%, respectively, whereas no changes were observed in 11β-HSD-2 expression in the same tissues at both time points (Fig 4B, C and D). In contrast, in the liver, HFD increased 11β-HSD-1 expression at 1800 h by +23% whereas 11β-HSD-2 expression was not affected (Fig. 4A).

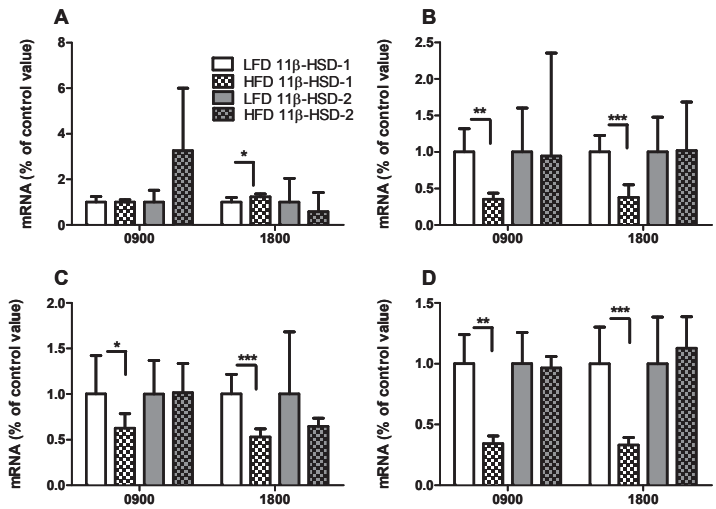

Figure 4. Effect of high fat diet on 11β-HSD-1 and 11β-HSD-2 expression in peripheral tissues. Male C57Bl/6J mice were fed a LFD (open bars) or HFD (chequered bars) for 12 weeks, and 11β-HSD-1 (white bars) and 11β-HSD-2 (grey bars) mRNA expression was determined in the liver (A) and gonadal (B), visceral (C) and subcutaneous (D) adipose tissues in the morning and evening. Mann-Whitney test, *P<0.05, **P<0.01, ***P<0.001.

DISCUSSION

This study aimed to characterize in detail, using standardized evaluations that control for housing and sampling conditions, the diet-induced changes that occur in basal activity of the HPA axis in the C567Bl/6J mouse model that develops obesity and insulin resistance, distinct features of the MetS. HFD feeding resulted in down regulation of the activity of the HPA axis, as reflected in decreased diurnal corticosterone concentrations, decreased 11β-HSD-1-enzyme expression in peripheral tissues, and altered CRH and GR expression in the CNS (decreased in the morning and increased in the evening). These observations indicate that HFD induces complex changes in the diurnal regulation of the different components of the HPA axis. These changes are not unequivocally characterized by increased, but rather by decreased, HPA axis activity.

As expected, HFD significantly increased both body weight and plasma insulin concentrations already within one week and HFD reduced diurnal corticosterone levels already within one week that persisted throughout the experiment. This persistent reduction in circulating corticosterone levels was not due to a shift in the diurnal peak of corticosterone and was evident from 1800-2000 h in the evening.

Several mechanisms may explain the early decrease in diurnal corticosterone peak levels upon HFD. First, hypercortisolism in human obesity has not been established and cortisol secretion is increased in obese humans, primarily because of increased clearance and increased distribution volume of the circulating cortisol resulting in secondary central activation of the HPA axis (18, 19). Second, this decrease in diurnal corticosterone peak levels may reflect counteracting mechanisms directed towards prevention of further progression in insulin resistance, both centrally and in peripheral tissues. Third, circulating leptin concentrations increase proportionally to the fat mass gained (20) and leptin and insulin resistance have been documented to develop already within three days of high fat feeding (21).

In accordance, HFD increased in CRH mRNA expression in the PVN and an increase in GR mRNA expression in the CA1 region of the hippocampus in the evening representing reduced negative feedback by decreased circulating corticosterone levels. This increase in GR mRNA in the CA1 region of the hippocampus also indicates that CNS areas important for specific types of learning and memory are relatively preserved in the presence of HFD and subsequent dampening of the HPA axis. In accordance, reduction of glucocorticoid levels in a specific mouse model of insulin resistance (*db/db*) reverses the cognitive impairment related to hippocampal neurons induced by insulin resistance (22). These findings imply that dampening of the HPA axis in the presence of insulin resistance, induced by HFD, might be a mechanism to rescue hippocampal neurons from impairments and to maintain normal cognitive function. In addition, both leptin and insulin can activate pro-opiomelanocortin (POMC) neurons (23), which produce ACTH. However, glucose sensing by POMC neurons is impaired in obese mice (24), which may result in insufficient adrenal stimulation by ACTH (diminished forward coupling between ACTH and corticosterone) resulting in decreased diurnal peak corticosterone and reduced negative feedback. In addition, the potency of ACTH is decreased in obesity (18).

Intriguingly, there is a disparity between the effects of HFD on the activity of the HPA axis in the morning as compared to the evening. Whereas the central activation in the evening can be easily explained by reduced negative feedback as a result of reduced peak corticosterone levels, the central inhibition of CRH and GR expression found in the morning in the presence of unaltered the circulating corticosterone levels, is very difficult to explain. To the best of our knowledge, such a disparity has not been previously documented or investigated. This further exemplifies that HFD induces complex changes in the diurnal regulation of the different components of the HPA axis. It is also likely that other factors outside the individual components of the HPA axis influence these complex effects of dietary intervention. Reduction of hypothalamic CRH expression in the morning might include the following mechanisms. For instance, induction of leptin resistance in the presence of obesity induces an increase in endocannabinoid tone (25, 26), which could lead to suppression of CRH. However, leptin resistance induced by high fat feeding is selective and does not impact on autonomic nervous system activity (27). Thus it could be speculated that hyperleptinemia dampens CRH expression and release in the face of selective sensitivity of the central HPA axis. It has also been shown in *in vitro* studies that leptin can reduce the sensitivity of adrenal cortex cells to ACTH,

thereby reducing circulating corticosterone levels (28, 29). However, the present data do not permit firm conclusions in this respect. In addition, a true effect of the diet *per se* can not be excluded as no differences were observed in plasma corticosterone levels in the morning, although it is well possible that small changes in circulating corticosterone levels at the moment of the diurnal nadir are not detected. The observed decreased expression of hypothalamic GR expression, however, is not explained because it suggests increased corticosterone exposure.Down regulation of CRH mRNA in the morning, was accompanied by downregulation of CRH mRNA and in the amygdala. Activation of the amygdala promotes HPA-activation and previous studies in rats have shown that increase in circulating glucocorticoids increase (30) whereas adrenalectomy decreases (31) CRH in the amygdala. Furthermore, increased CRH in the amygdala mediates anxiety-like behavior during stress (32) and high fat diet decreases anxiety-like behaviors facilitating stress recovery (33, 34). Thus, HFD-induced reduction of CRH expression in the amygdala enables protection from further exposure to systemic glucocorticoids.

In accordance, the effects of HFD on the peripheral activity of the HPA axis were characterized by reduced mRNA expression of 11β-HSD-1 in adipose tissues. It has been proposed that this may reflect a mechanism to counteract tissue-specific insulin resistance (35). These differential, fat depot-specific effects are in agreement with a recently- proposed, dynamic and depot-selective relationships between adipose tissue 11β-HSD-1-activity and fat mass (35). HFD increases lipoprotein lipase activity (36), which would direct circulating triglycerides towards peripheral adipose tissues for storage. Corticosterone impairs glucose tolerance (37) and is a lipolytic hormone (38). Therefore down regulation of 11β-HSD-1 expression would protect the adipose tissue from further insulin resistance. These observations are further strengthened by the fact that 11β-HSD-2 expression in the same tissues was not affected, resulting into overall reduction of glucocorticoid exposure of the tissues.

In conclusion, we found that HFD has profound effects on the basal, thus non-stressed, activity of the HPA axis, reflected in reduced diurnal corticosterone concentrations, distinct expression of CRH in the CNS across the diurnal rhythm, and reduced 11β-HSD-1-enzyme expression in peripheral fat tissues. The observed effects in this study, both in the CNS and peripheral adipose tissues (downregulation of CRH mRNA and 11β-HSD-1 enzyme mRNA, respectively), are in agreement with the well-known metabolic effects of glucocorticoids, that are directed towards recruitment and augmentation of energy stores. In the presence of HFD induced obesity or palatable foods, the HPA axis adapts.

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GLUCOCORTICOID EXCESS INDUCES LONG-LASTING CHANGES IN BODY COMPOSITION IN MICE WHEN FED A HIGH FAT DIET

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ABSTRACT

Introduction: A period of glucocorticoid (GC) overexposure, as observed in Cushing's syndrome (CS), is associated with the metabolic syndrome (MetS) and cardiovascular diseases. These changes persist even after long-term correction of GC excess, and may in fact be permanent. We performed a mouse study to identify factors that modulate metabolic recovery from a period of overexposure to GC.

Methods: Male C57Bl/6J mice, fed a low fat (LFD) or high fat diet (HFD), received corticosterone (CORT) (50µg/ml) or vehicle in the drinking water for 4 wks, followed by a washout period of 8 wks. Plasma circadian CORT, lipids, insulin, and glucose levels were assessed at regular intervals. Insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamp and lean body- and fat masses were analyzed at week 12.

Results: CORT treatment increased plasma CORT levels, food intake and plasma insulin and lipids in both diets. Abrogation of CORT treatment normalized CORT levels, food intake and body weight. At week 12, plasma insulin levels were still significantly higher in CORT-treated mice on both diets, and HFD-fed CORT-treated mice had persistently decreased lean body mass and higher fat mass.

Conclusion: In mice, a period of CORT excess induces long-lasting metabolic changes. The changes in body composition were present only in the presence of HFD. These observations indicate that diet-dependent effects of CORT might contribute to the persistent adverse cardiovascular risk profile as observed in patients treated for CS, and possibly also in subjects exposed to chronic stress.

INTRODUCTION

In humans, prolonged excessive exposure to glucocorticoids (GC) as seen in Cushing's syndrome (CS), is associated with an increased incidence of the metabolic syndrome (MetS), a clustering of cardiovascular risk factors such as increased blood pressure, dyslipidemia and insulin resistance, and increased cardiovascular morbidity and mortality (1). Increased GC exposure changes eating behavior and food preferences facilitating the development of obesity and the MetS (2, 3). Intriguingly, patients with CS remain at increased cardiovascular risk, even despite long-term successful correction of GC excess (4). However, the causal relation between the episode of cortisol overexposure and long-term changes in the cardiovascular risk factors is not established and the modifiers of normalization are unknown and are difficult to assess in humans because of the rarity and heterogeneity of CS.

Representative animal models that enable to study the metabolic effects of corticosterone (CORT) overexposure are limited, whereas in humans GCs induce weight gain and increase appetite (5), in rats high CORT concentrations (using either chronic stressors or via implantation of subcutaneous (sc) CORT pellets or intraperitoneal (ip) injections) decrease intake of standard chow (6). These catabolic effects of GC in rodents can be counteracted by adding 30% sucrose to regular chow of CORT-treated rats. Indeed, when CORT-treated rats with sc pellets were given regular chow with the addition of sucrose, these rats became markedly hyperinsulinemic and hypercholesterolemic, and developed visceral obesity (7). Thus, the addition of sucrose to chow appears to be a prerequisite for the development of obesity in rodents exposed to high CORT (6).

Chronic oral CORT supplementation was recently reported to induce impressive metabolic changes including weight gain, increased adiposity, elevated plasma, insulin and triglyceride levels, and hyperphagia (8). Since these features resemble the changes observed in individuals suffering from MetS, this represents a model for hypercortisolemia and stress-related obesity.

In the present study, we hypothesized that a period of overexposure to GC in mice would result in long-term or even permanent metabolic changes, and that this would be affected by the composition of the diet.

MATERIALS AND METHODS

Experimental procedures

Mice, housing, CORT supplementation, and diets

Eight-week old male C57Bl/6J mice (Charles River, Maastricht, The Netherlands) were single housed in a separate room from other experimental animals in the facility to minimize environmental stressors, and maintained on a 12 h:12 h light-dark cycle (lights on 7 a.m.), with *ad libitum* access to food and drinking water.

Experiment 1: C57Bl/6J mice were fed a low fat diet (10 energy % lard fat, D12451B, Research Diet Services, Inc., New Brunswick, US.) (LFD) and after 4 weeks (at 12 weeks of age), they were matched for weight, the levels of plasma triglycerides, non-esterified free fatty acids and cholesterol, and randomized to receive either 50µg/ml CORT (Sigma-Aldrich, Manchester, UK) in the drinking water (containing 0.25% ethanol as vehicle), or 0.25% ethanol as vehicle only in the drinking water (control group), for 4 weeks. At week 12 (control group n=10, CORT-group n=10) dual energy X-ray

absortiometry (DEXA) analysis to determine body composition and hyperinsulinemic-euglycemic clamp analysis was performed to determine the insulin sensitivity.

Experiment 2: C57Bl/6J mice were fed a high fat diet (45 energy % lard fat, D12451, Research Diet Services, Inc., New Brunswick, US) (HFD) from 10 weeks of age and after reaching 12 week of age, were matched for weight, plasma triglycerides, non-esterified free fatty acids and cholesterol and randomized to receive either 50µg/ml CORT (Sigma-Aldrich) in the drinking water (containing 0.25% ethanol as vehicle), or to drinking water with 0.25% ethanol as vehicle only (control group), for four weeks. At week 12 DEXA analyses (n=10) was performed to determine body composition. Hyperinsulinemic-euglycemic clamp analysis was performed to determine the insulin sensitivity in a subset of mice (control group n=6, CORT-group n=7).

Sampling of circadian corticosterone, hormone, and lipid measurements

Blood for analysis of plasma CORT levels was sampled in both experiments at baseline, and at week 4, 8 and 12 during the first light hour at 07.00 h, at 12.00 h, during the last light hour at 18.00 h, and at 22.00 h (three hours after the onset of the dark phase). During the dark phase samples were collected in red light conditions. All CORT samples were obtained within 90 seconds from disturbing the cage, via tail incision, allowing the mouse to move freely on top of the home cage (9). Plasma insulin, glucose, total cholesterol, triglycerides and non-esterified free fatty acids were sampled after overnight fast at baseline, week 4, 8 and 12. Body weight, food intake and water intake were measured weekly.

Dual-energy X-ray absorptiometry (DEXA) scan

Body composition was measured at week 12 by DEXA using the Norland pDEXA Sabre X-Ray Bone Densitometer (Norland, Hampshire, UK). Before measuring, mice were anaesthetized with a combination of 6.25 mg/kg acepromazine (Alfasan, Woerden, the Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, the Netherlands), and 0.31 mg/kg fentanyl (Janssen-Cilag, Tilburg, the Netherlands). Mice were scanned *in toto*, and the heads were subsequently excluded from the analysis due to the inability of the DEXA scan to accurately determine the composition of the tissue underneath the skull.

Hyperinsulinemic-euglycemic clamp study

Hyperinsulinemic-euglycemic clamp studies were performed at week 12 in postabsorptive (i.e., overnight fasted) condition. Mice were anesthetized with 6.25 mg/kg acepromazine (Alfasan, Woerden, the Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, the Netherlands), and 0.31 mg/kg fentanyl (Janssen-Cilag, Tilburg, the Netherlands). First, the basal rate of glucose turnover was determined by giving a primed (0.5 µCi) continuous (0.9 µCi/h) intravenous (iv) infusion of D-[3-^{3}H]-glucose (37 MBq) (GE Healthcare, Little Chalfont, UK) for 60 min. Subsequently, insulin (Novo Nordisk, Bagsværd, Denmark) was administered in a primed (3.7 mU) continuous 6.1 mU/h for LFD fed mice and 11.25mU/h for HFD-fed mice as iv infusion for 90 min to attain steady-state circulating insulin levels. Every 10 min, the plasma glucose concentration was determined via tail vein bleeding (<3 µl) (Accu-chek, Sensor Comfort; Roche Diagnostics GmbH, Mannheim, Germany) and the iv infusion rate of a 12.5% D-glucose solution was adjusted to maintain euglycaemia. Blood samples (60 µl) were taken during the basal period (after 50 and 60 min) and during the hyperinsulinaemic period (after 70, 80 and 90 min) to determine plasma concentrations of glucose, insulin and specific activity of ^{3}H-glucose specific activities. The mice were sacrificed at the end of the clamp.

All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures from the Leiden University Medical Center approved the protocol.

Hormone and lipid measurements

Plasma CORT levels were determined by radioimmunoassay (MP Biomedicals LCC, Orangeburg, NY). Plasma levels of total cholesterol, triglycerides and non-esterified free fatty acids were measured with enzymatic colorimetric reaction (Roche diagnostics GmbH, Mannheim; and Wako Pure Chemical Industries, respectively), plasma insulin was measured with an ELISA (Crystal Chem Inc., Downers Grove, IL, USA) and plasma glucose with a hexokinase method (Instruchemie, Delfzijl, The Netherlands). Homeostasis model index of insulin (HOMA-IR) was calculated by multiplying fasting insulin concentration (μU/ml) with fasting glucose (mmol/l), and dividing with 22.5 (10 Mather 2009).

Calculations and statistical analysis

Data from the hyperinsulinemic-euglycemic clamp studies were calculated as previously described by Voshol *et al*. 2001 (11). The rate of glucose uptake (μmol/min/kg) was calculated in the basal period as well as during the steady-state hyperinsulenemia (70, 80 and 90 minutes of the clamp) as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of ^{3}H-glucose (dpm/μmol) in the plasma. Endogenous glucose production (μmol/min/kg) was calculated as the difference between the tracer-derived rate of glucose uptake and the glucose infusion rate. Glucose uptake as well as glucose production were corrected for body weight.

Data are presented as means ± SD. Statistical differences were calculated using Mann-Whitney test for non-parametric data, with GraphPad Prism, version 5.01. $P<0.05$ was considered as statistically significant.

RESULTS

Pilot experiment for the determination of optimal CORT dose:

Prior to both experiments, we performed a dose finding study in mice fed HFD with 12.5 μg/ml (n=2), 25 μg/ml (n=4) and 50 μg/ml (n=2) of CORT and control receiving 0.25% ethanol (n=2) as vehicle for four weeks to determine the optimal CORT dose. These dosages were chosen based upon a previous study (8) that documented profound metabolic effects with CORT 100 μg/ml and less pronounced effects with 25 μg/ml. Based upon our dose finding study, we chose 50μg/ml CORT in the drinking water for our subsequent experiments as this dose led to the most profound increases in food intake and body weight as well as in plasma cholesterol (data not shown).

CORT treatment increases plasma CORT concentrations and affects circadian rhythm of CORT

A circadian rhythm of CORT was present at baseline in both dietary conditions (Fig. 1A and B) and chronic high doses of CORT (50μg/ml) increased plasma CORT levels and affected circadian rhythm, Fig. 1C and D, respectively). At week 8 (four weeks after abrogation of CORT), in the LFD experiment CORT-treated animals had recovered and were not different from the controls, while in the HFD experiment, the peak (18.00 h) circadian (endogenous) CORT concentrations were

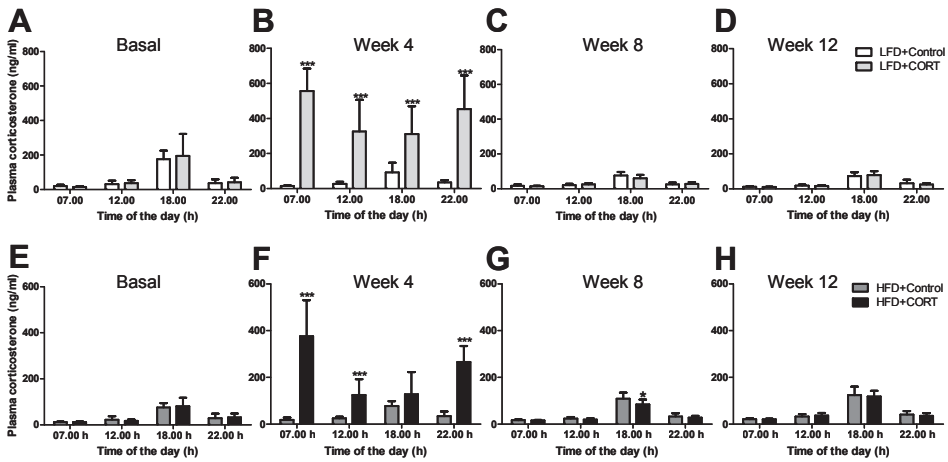

Figure 1. Effect of CORT treatment on circadian plasma CORT of mice fed a low fat diet (A, B, C and D) (control group: open bars, CORT group: light grey bars) or high fat diet (E, F, G and H) (control group: dark grey bars, CORT group: black bars) at baseline, week 4, week 8 and week 12, Mann-Whitney test, *P<0.05, ***P<0.001.

-22% lower in CORT-treated animals compared to controls (Fig. 1E and F, respectively). At week 12 circadian CORT levels were equal to the controls.

CORT treatment induces transient changes in food intake and bodyweight

Experiment 1 (LFD): CORT treatment increased food intake (by 14% *vs* controls at week 1 to 27% at week 4) (Fig. 2C). After removal of CORT, food intake rapidly decreased by -29% *vs* controls returning to the level of the control group at week 6. This increase in food intake was not translated into an increase in bodyweight, being comparable between the groups during the CORT treatment. Removal of CORT transiently decreased bodyweight in the CORT group at week 5 and 6 (by -5%, and -4%, respectively), but body weight was not different from controls thereafter (Fig. 2A). CORT treatment increased water intake (with CORT in it) up to 3-fold during the treatment period and decreased close to the level of the controls after removal of CORT from the drinking water, however, still remaining significantly higher to the end of the experiment (Fig. 2E).

Experiment 2 (HFD): CORT treatment increased food intake by +19% *vs* controls after the first week (Fig. 2D), with a concomitant increase in bodyweight that was significant at week 3 and remained significantly higher at week 4 and 5 (+11%, +19%, and +9%, respectively) (Fig. 2B) when compared to the controls. After removal of CORT, food intake normalized at week 6, and did not differ from controls for the remainder of the experiment. CORT treatment did not affect water intake except after removal of CORT from the drinking water at week 5 and 10 (by -21% and -10%, respectively) when water intake was significantly lower in the CORT-treated group when compared to controls (Fig 2F).

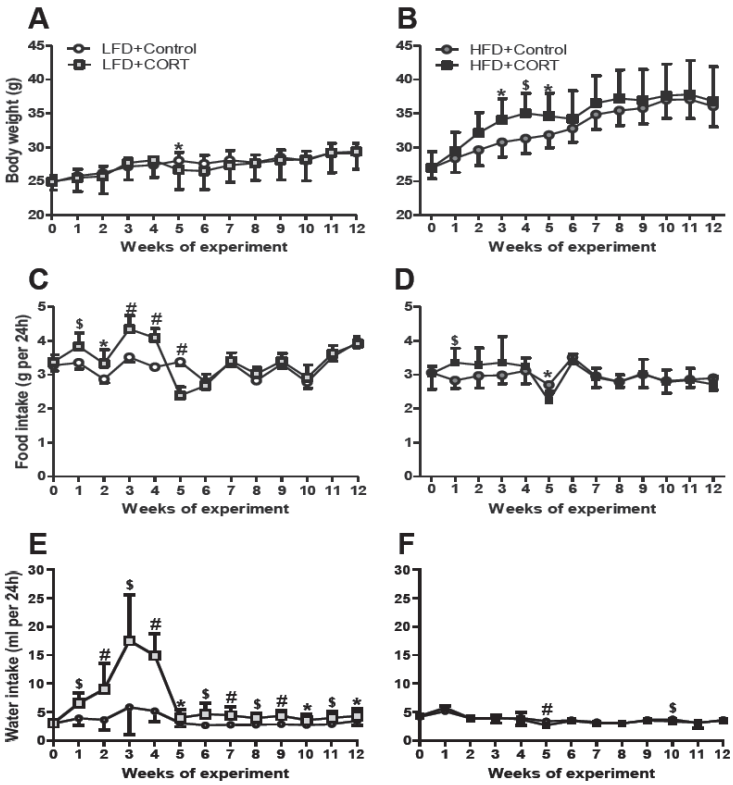

Figure 2. Effect of CORT treatment on body weight, food intake and water intake of mice fed a low fat diet (control group: white circles, CORT group: light grey squares) (A, C and E) or high fat diet (control group: dark grey circles, CORT group: black squares) (B, D and F), Mann-Whitney test, *P<0.05, $P<0.01, #P<0.001.

CORT treatment induces transient changes in plasma lipids

CORT treatment increased plasma levels of triglycerides and non-esterified free fatty acids significantly in the LFD experiment at week 4 (by +56% and +24%, respectively) (Table 1). In the HFD experiment, additionally, significant increases *vs* controls were observed in the plasma levels of total cholesterol and non-esterified free fatty acids at week 4 (+38% and +21%, respectively) (Table 1). At week 8, plasma triglycerides levels were significantly higher in the HFD group (by +21% *vs* controls) (Table 1).

Insulin and HOMA-IR are increased long-term by CORT treatment

As expected, when compared to controls, CORT treatment significantly increased plasma insulin concentrations on both diets (LFD: 8-fold and HFD: 3-fold increase at week 4) (Table 1), and remained significantly higher after removal of CORT treatment. Of note; insulin levels did not markedly decrease in CORT-treated animals between week 8 (LFD: +83% and HFD: +67%)

Table 1. Effect of CORT treatment on fasting lipids, insulin ad glucose levels and HOMA-IR of mice fed a low or high fat diet

Week	Total cholesterol (mmol)				Triglycerides (mmol)				Non-esterified free fatty acids (mmol)			
	LFD		HFD		LFD		HFD		LFD		HFD	
	Control	CORT	Control	CORT	Control	CORT	Control	CORT	Control	CORT	Control	CORT
0	3.4±0.3	3.4±0.3	3.2±0.7	3.3±0.4	0.7±0.2	0.7±0.3	1.1±0.4	1.1±0.3	1.4±0.3	1.4±0.3	1.6±0.3	1.8±0.3
4	3.0±0.3	3.2±0.6	3.5±0.7	4.8±0.6[b]	0.9±0.2	1.4±0.3[c]	1.1±0.3	1.3±0.2	1.7±0.3	2.1±0.4[a]	1.8±0.2	2.2±0.3[b]
8	3.1±0.3	2.9±0.4	3.9±0.8	4.0±0.4	0.7±0.1	0.8±0.3	1.5±0.6	1.8±0.4[a]	1.6±0.2	1.5±0.2	1.7±0.3	1.9±0.3
12	2.8±0.6	2.9±0.7	4.1±0.8	4.2±0.9	0.7±0.2	0.8±0.2	1.2±0.2	1.4±0.3	1.4±0.2	1.5±0.3	1.5±0.3	1.7±0.3

Week	Insulin (ng/ml)				Glucose (mmol)				HOMA-IR			
	LFD		HFD		LFD		HFD		LFD		HFD	
	Control	CORT	Control	CORT	Control	CORT	Control	CORT	Control	CORT	Control	CORT
0	0.6±0.3	0.6±0.3	0.6±0.3	0.5±0.2	3.6±0.7	3.8±1.0	5.3±1.1	5.2±0.7	2.3±0.8	2.2±0.9	3.1±1.5	2.7±1.0
4	0.6±0.2	4.8±2.2[c]	1.1±0.3	4.0±1.1[c]	4.1±0.9	3.4±0.7	4.3±0.7	3.4±0.7[a]	2.3±0.6	17.7±10.6[b]	5.0±1.8	14.4±3.7[c]
8	0.6±0.2	1.1±0.8[a]	0.6±0.3	1.0±0.4[a]	4.2±0.6	3.6±0.6[a]	5.2±1.1	5.2±1.2	2.5±0.7	4.3±2.7	3.4±1.6	5.7±2.5[a]
12	0.6±0.4	1.0±0.4[a]	1.3±0.4	2.2±1.0[a]	4.8±0.6	4.0±0.6[b]	3.9±1.1	3.1±0.7	2.9±1.9	4.2±1.7	5.3±2.4	7.4±4.0

Mann-Whitney test, [a]*P<0.05*, [b]*P<0.01*, [c]*P<0.001*

and week 12 (LFD: +67% and HFD: +69%) (Table1). Plasma glucose was significantly decreased by in LFD experiment at week 8 and 12 (-14% and -17%, respectively) and by -21% in the HFD experiment at week 4 -in the CORT group (Table 1). Changes in plasma insulin and glucose induced by CORT treatment, were reflected as decreased insulin sensitivity as reflected in HOMA-IR. In both experiments CORT significantly increased HOMA-IR at week 4 (LFD: 8-fold and HFD: 3-fold) (Table 1). Furthermore, HOMA-IR at week 8 was still significantly increased by +69% in the HFD experiment after CORT treatment (Table 1), and +45% increased in the LFD experiment at week 12, which however, did not reach significance, (Table 1) when compared to the controls (Table1).

CORT treatment induces long-lasting changes in body composition only in the presence of HFD

In the HFD experiment, after 12 weeks, CORT treatment significantly reduced lean body mass (23±3% *vs*. 28±4% of total body weight, CORT *vs* controls, respectively (Fig. 3C), and significantly increased fat mass (55±5% vs. 64±7% of the total body weight) (Fig. 3D). However, long-lasting changes in body composition were not observed in the LFD experiment (Fig 3A and B).

CORT treatment does not affect endogenous glucose production or glucose disposal in the long-term

Endogenous glucose production and glucose disposal, as derived from the hyperinsulinemic-euglycemic clamp studies, were not different between the CORT-treated and the control group in the LFD experiment (Fig. 4A and B), nor in the HFD experiment (Fig. 4C and D) at week 12.

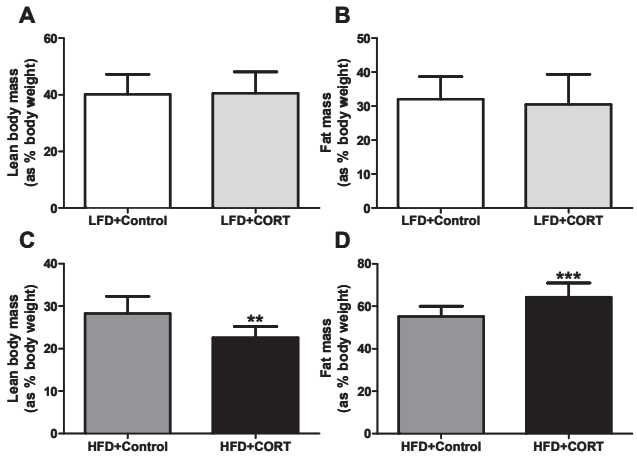

Figure 3. Effect of CORT treatment on body composition of mice fed a low fat diet (A and B) (control group: open bars, CORT group: light grey bars) or high fat diet (C and D) (control group: dark grey bars, CORT group: black bars), Mann-Whitney test, **P<0.01, ***P<0.001.

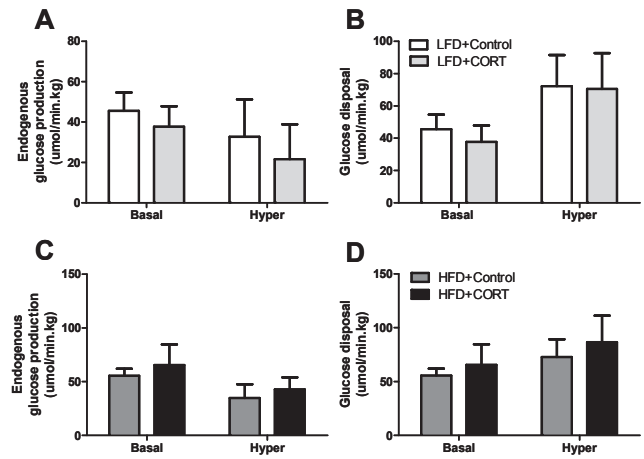

Figure 4. Effect of CORT treatment on endogenous glucose production and glucose disposal during basal period of glucose infusion (basal) and hyperinsulinemia (hyper) in mice fed a low fat diet (A and B) (control group: open bars, CORT group: light grey bars) or high fat diet (C and D) (control group: dark grey bars, CORT group: black bars) at week 12.

DISCUSSION

We demonstrated that a period of overexposure to GCs induces long lasting, potentially permanent, changes in metabolic parameters, but only in the presence of HFD. In HFD-animals increased insulin levels and altered body composition did not normalize even after an 8-week wash out period, when parameters like food intake and body weight had returned to control levels for about 6 weeks.

As anticipated, in our study high CORT levels increased food intake and induced metabolic changes that resemble the MetS, like an increase in plasma insulin levels, lipids, and body fat, irrespective of a high- or low fat diet. These effects were reversible after normalization of CORT levels when exposed to LFD, but some irreversible changes were observed with HFD exposure. HFD adversely affected body composition even after long-term withdrawal of high CORT exposure. This indicates that HFD aggravates the adverse long-term metabolic effects of transient high CORT exposure. This points at a crucial role for dietary composition in the development of the metabolic syndrome in conditions with periodic excessive CORT exposure.

Supra-physiological CORT levels were readily induced using oral CORT in the drinking water. This treatment with CORT also affected circadian CORT rhythm impairing the degree of variations in plasma concentrations. After discontinuation of CORT treatment, circadian rhythm gradually returned to the level of the controls at week 12, although evening (18.00 h) CORT peak was still decreased at week 8 in the HFD experiment. This indicates that incomplete recovery of the HPA axis after a period of exogenous supra-physiological CORT supplementation, as has been documented extensively in humans both after treatment for CS (12) and in patients previously exposed to exogenous supra-physiological GCs (13) might be dependent on the composition of the diet.

CORT treatment increased food intake on both diets but bodyweight increased only on HFD. It has been previously shown that high CORT levels stimulate voluntary food intake dose-dependently (6, 14, 15). In addition, in case of different food availability, food preference changes towards more energy-rich "palatable" food (16) serving the evolutionary purpose to attain the most efficient fuels to counteract the adverse changes in insulin sensitivity that occur during stress (17). With increasing insulin and GC levels food intake is even stimulated in the presence of less palatable foods (6, 18, 19). In agreement, in the present experiments with LFD and HFD, the increased plasma CORT levels were accompanied by increases in circulating insulin, triglycerides, cholesterol, and free fatty acid levels. The increased food intake only increased body weight on HFD, suggesting that the increase in food intake on LFD was just sufficient to compensate for the catabolic effects of increased CORT levels. However, higher dosages of CORT than the one used in our study were able to increase bodyweight on regular chow diet (8). Intriguingly, it seems that the increase in fat mass remains present even after a washout from the increased plasma CORT levels.

Water intake, and therefore CORT intake was increased only in the LFD- but not in the HFD experiment in the CORT-treated group. However, even when consuming a higher amount of CORT during LFD the reversibility of the metabolic changes after LFD (but not after HFD) was complete, which strengthens our conclusions on the modulatory role of the diets *per se*.

Although body weight returned to that of the controls after discontinuation of CORT treatment, on HFD, the increase in plasma triglycerides was still present at week 8, and insulin concentrations remained elevated even at week 12. However, endogenous glucose production and disposal assessed with hyperinsulenemic-euglycemic clamps at the end of the study did not indicate reduced insulin sensitivity.

Whereas the effects on body weight were transient, long-lasting changes in body composition were found on HFD, where reduced lean body mass and increased fat mass was observed even after 12 experimental weeks. These effects of CORT treatment on body composition were not observed in the LFD experiment. Chronically administered GCs facilitate muscle atrophy (20) and increase visceral fat mass in mice (8, 21). In the human, the equivalent of chronic high GC is CS, a rare clinical syndrome where patients are exposed to increased adrenal cortisol secretion due to an ACTH producing pituitary or ectopic tumor or due to an adrenal adenoma (1). Patients with CS have increased prevalence of the MetS (22, 23), but intriguingly, after one year of remission still have increased waist circumference (24, 25) and even after long-term remission higher visceral fat mass was observed without affecting body mass index (26).

The fact that the long-lasting changes observed in mice after a period of exposure to high CORT persist for a longer period of time in the presence of HFD is intriguing in view of the incomplete reversibility of metabolic changes observed in patients with CS after correction for hypercortisolism. Humans exposed to stress levels of GC, like in CS, will direct their food preference towards highly palatable foods, in agreement with the biological effects of GC, thereby aggravating the adverse cardiometabolic effects and thus facilitating the development and persistence of MetS. Epidemiological data also indicate an increased prevalence of the metabolic syndrome in conditions associated with alterations in the HPA axis, like in anxiety and depression, after medical treatment with GC, and also in sleep disorders (27, 28). Because it appears that these diet-dependent effects of CORT during stress strongly facilitate the persistent adverse cardiovascular risk profile, the interactions between the availability of 'fast food' and every-day-stress are novel features to be accentuated in future studies on cardiovascular morbidity and mortality.

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5

BOTH TRANSIENT AND CONTINUOUS CORTICOSTERONE EXCESS INHIBIT ATHEROSCLEROTIC PLAQUE FORMATION IN APOE*3-LEIDEN.CETP MICE

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ABSTRACT

Introduction: The role of glucocorticoids in atherosclerosis development is not clearly established. Human studies show a clear association between glucocorticoid excess and cardiovascular disease, whereas most animal models indicate an inhibitory effect of glucocorticoids on atherosclerosis development. These animal models, however, neither reflect long-term glucocorticoid overexposure nor display human-like lipoprotein metabolism.

Aim: To investigate the effects of transient and continuous glucocorticoid excess on atherosclerosis development in a mouse model with human-like lipoprotein metabolism upon feeding a Western-type diet.

Methods: Pair-housed female APOE*3-Leiden.CETP (E3L.CETP) mice fed a Western-type containing 0.1% cholesterol for 20 weeks were given corticosterone (50 µg/ml) for either 5 (transient group) or 17 weeks (continuous group), or vehicle (control group) in the drinking water. At the end of the study, atherosclerosis severity, lesion area in the aortic root, the number of monocytes adhering to the endothelial wall and macrophage content of the plaque were measured.

Results: Corticosterone treatment increased body weight and food intake for the duration of the treatment and increased gonadal and subcutaneous white adipose tissue weight in transient group by +35% and +31%, and in the continuous group by +140% and 110%. Strikingly, both transient and continuous corticosterone treatment decreased total atherosclerotic lesion area by -39% without lowering plasma cholesterol levels. In addition, there was a decrease of -56% in macrophage content of the plaque with continuous corticosterone treatment, and a similar trend was present with the transient treatment.

Conclusion: Increased cortiosterone exposure in mice with human-like lipoprotein metabolism has beneficial, long-lasting effects on atherosclerosis, but negatively affects body fat distribution by promoting fat accumulation in the long-term. This indicates that the increased atherosclerosis observed in humans in states of glucocorticoid excess may not be related to cortisol *per se*, but might be the result of complex indirect effects of cortisol.

INTRODUCTION

Atherosclerosis develops as a result of a chronic inflammatory response in an injured vessel wall (1), which is preceded by accumulation of leukocytes and fat deposition, leading to plaque formation (2). The initial mechanisms in atherogenesis, however, are still incompletely understood.

The role of glucocorticoids (GC) in the development of atherosclerosis is not yet clearly established in humans or in animals and is, at least, dependent on individual's (3, 4) or animal's (5) exposure to appropriate levels of adrenal steroids. Human data show an association between increased GC secretion and cardiovascular disease even after long-term successful correction of GC excess (6), whereas previous studies in animals, e.g. rabbits (7, 8, 9, 10, 11) and dogs (12) using either natural or synthetic GC, suggest an atheroprotective role of GC. On the other hand, 11β-dehydrogenase type 2 (11βHSD2) deficient mice, in which the activation of the mineralocorticoid receptor (MR) by GCs cannot be prevented, have an increased atherosclerotic plaque development (13), suggesting that increased activation of the MR promotes atherosclerotic plaque formation. However, a recent study demonstrated that adrenalectomy, which removes endogenous GC, stimulated the formation of initial atherosclerotic lesions in low-density-lipoprotein receptor knockout mice (5).

Only a limited number of studies have evaluated the effect of endogenous GC excess on atherosclerosis development in mice. These studies, that used chronic stress to increase endogenous GC, reported either an increase (14, 15) or no effect (16) on atherosclerosis development in ApoE-deficient mice. However, chronic stress, in addition to increasing GC, induces other complex endocrine and metabolic changes, for instance increased sympathetic outflow (17) that may affect atherosclerosis development. These mouse models therefore do not reflect long-term endogenous GC overexposure, like in Cushing's syndrome (CS) in humans. Furthermore, ApoE-deficient mice do not reflect human-like lipoprotein metabolism and have a deviant immune status compared to wild-type mice (18).

In the present study, our aim was to investigate the effects of GC excess on atherosclerosis development in the APOE*3-Leiden.CETP (E3L.CETP) mouse, a well-established model for human-like lipoprotein metabolism that is prone to develop atherosclerosis upon feeding a cholesterol-containing Western-type diet (19) and is responsive to all hypolipidemic drugs used in the clinic similar to humans (20-23) The latter is in sheer contrast to other mouse models for hyperlipidemia and atherosclerosis including apoE-knockout and LDL receptor-knockout mice. We administered corticosterone (CORT) non-invasively via the drinking water, and based on the clinical observation in CS patients, we investigated both transient and chronic effects of CORT on atherosclerosis development.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the regulations of Dutch law on animal welfare, and the institutional ethics committee for animal procedures of Leiden University. The protocol was approved by the institutional ethics committee for animal procedures of Leiden University (Permit Number: 10132 and for the pilot experiment: 08221) and all efforts were made to minimize suffering.

Mice, housing, corticosterone supplementation, and diets

Human CETP expressing transgenic mice, which express CETP under control of its natural flanking regions, were crossbred in our own animal facility with E3L mice to obtain the heterozygous E3L. CETP mice on a C57Bl/6 background (24). Female mice (10-16 weeks of age) were pair housed and maintained on a 12 h:12 h light-dark cycle (lights on 7 a.m.) in a climate controlled environment, with *ad libitum* access to food and drinking water. Mice were fed a Western-type diet containing 0.1% cholesterol (Diet T + 0.1% cholesterol, Arie Blok Diervoeding, Woerden, the Netherlands) for a period of three weeks after which they were matched for age, plasma cholesterol, triglycerides, phospholipids, age and bodyweight and then randomized to receive CORT (Sigma-Aldrich, Manchester, UK) at a concentration of 50 µg/ml in the drinking water with 0.25% ethanol as vehicle for five weeks (transient group, n=17), continuously for the entire duration of the experiment (seventeen weeks) (continuous group, n=21), or vehicle (control group, n=19). Food and bodyweight were recorded weekly. At the end of the experiment mice were decapitated within 90 seconds from disturbing the cage and trunk blood was collected. Gonadal and subcutaneous fat pads were removed from each mouse by the same person to reduce inter-observer variation. The same area of subcutaneous fat was removed from each mouse excluding the inguinal lymph node. Fat pads and adrenals were weighed using a micro scale, and frozen in liquid nitrogen.

Pilot experiment for the determination of optimal CORT dose

Prior to experiment, we performed a dose finding study in male C57Bl/6J mice fed HFD with 12.5 µg/ml (n=2), 25 µg/ml (n=4) and 50 µg/ml (n=2) of CORT, and control receiving 0.25% ethanol (n=2) as vehicle for four weeks to determine the optimal CORT dose. These dosages were chosen based upon a previous study (25) that documented profound metabolic effects with CORT 100 µg/ml and less pronounced effects with 25 µg/ml. Based upon our dose finding study, we chose 50 µg/ml CORT in the drinking water for our subsequent experiments as this dose led to the largest increases in food intake and body weight as well as in plasma cholesterol. Because male mice do not readily develop atherosclerosis and the known models are in majority female models, we used female mice for the purpose to study the atherosclerosis development. In males and females, this CORT dose was sufficient to increase food intake and to maintain a higher bodyweight throughout the experiment. In addition, circulating circadian CORT levels at week 5 were 5-8 fold increased in the morning and 3-4 fold in the evening (data not shown).

Sampling of circadian corticosterone, hormone, and lipid measurements

Plasma CORT was sampled before CORT administration (baseline), and after CORT administration at week 5 during the first light hour at 07.00 h, at 12.00 h, during the last light hour at 18.00 h, and three hours after the onset of the dark phase at 22.00 h. During the dark phase samples were collected in red light conditions. All CORT samples were obtained within 90 seconds from disturbing the cage, via tail incision, allowing the mouse to move freely on top of the home cage (26). Trunk blood was used to determine plasma CORT at the end of the experiment after CORT administration at week 17 at 09.00 h and 18.00 h. Total plasma cholesterol, triglycerides, phospholipids, insulin and glucose were sampled after 4 hour-fast at baseline, week 5, 8 and 17 of the intervention. Body weight and food intake were measured weekly.

Plasma CORT levels were determined by radioimmunoassay (MP Biomedicals LCC, Orangeburg, NY). Plasma levels of total cholesterol, triglycerides and non-esterified free fatty acids were measured

with enzymatic colorimetric reaction (Roche diagnostics GmbH, Mannheim; and Wako Pure Chemical Industries, respectively), plasma insulin was measured with an ELISA (Crystal Chem Inc., Downers Grove, IL, USA) and plasma glucose with a hexokinase method (Instruchemie, Delfzijl, The Netherlands). Homeostasis model index of insulin (HOMA-IR) was calculated by multiplying fasting insulin concentration (μU/ml) with fasting glucose (mmol/l), and dividing with 22.5 (27).

Lipoprotein profiling

Distribution of cholesterol over plasma lipoproteins was determined using fast protein liquid chromatography. Pooled plasma from each group were used and 50 μl of each pool was injected onto a Superpose 6 PC 3.2/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ) and eluted at a constant rate of 50 μl/min in PBS, 1 mM EDTA, pH 7.4. Fraction were collected and assayed for cholesterol as described above.

Gene expression analysis in adipose tissue

Total RNA was extracted from gonadal fat pads using the Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. RNA quality of each sample was examined by the lab-on-a-chip method using Experion Std Sens analysis kit (Biorad, Hercules, CA) and RNA concentration of each sample was determined by Nanodrop technology (Thermo Scientific, Wilmington, USA). Then, total RNA was reverse-transcribed with iScript cDNA synthesis kit (1708891, Bio-Rad), and obtained cDNA was purified with Nucleospin Extract II kit (636973, Macherey-Nagel, Bioké). Real-time qPCR was performed on a CFX96 machine (Bio-Rad), the reaction mixture consisting of SYBR-Green Sensimix (QT615, GC Biotech), cDNA, primers (Biolegio, Nijmegen, The Netherlands), and nuclease-free water in a total reaction volume of 10 μl. mRNA values of each gene were normalized to mRNA levels of β2-microglobulin (*β2m*) and hypoxanthine ribosyltransferase (*Hprt*). Primer sequences are listed in supplementary table 1.

Quantification of atherosclerosis

After 17 weeks of intervention, mice were killed by decapitation and the hearts were isolated. Hearts were fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin. Cross-sections (5 μm) throughout the aortic root area were cut. 12 sections per mouse, stained with hematoxylin-phloxin-saffron for histological analysis, with 50 μm-intervals were used for atherosclerosis measurements. Lesions were categorized for severity according to the guidelines of the American Heart Association, adapted for mice (28, 29), as follows: type 0 (no lesions), types 1 through 3 (early fatty streak-like lesions containing foam cells), and type 4 to 5 (advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization, and/or necrosis). AIA 31240 antiserum (1:3000, Accurate Chemical and Scientific, Westbury, NY) was used to quantify macrophage content of the plaque as well as monocytes adhering to the endothelium. Lesion area, the macrophage area and the number of monocytes adhering to the endothelium were quantified using Image J software (National Institutes of Health).

Serum macrophage colony-stimulating factor (M-CSF) and anti-oxidized low-density lipoprotein (ox-LDL) antibodies measurements

Macrophage colony-stimulating factor (M-CSF) was measured using a mouse M-CSF Quantikine ELISA kit according to the manufacturer's instructions (MMC00, R&D Systems Inc, Germany). An EIA/RIA high binding 96-well Costar plate (Corning Inc., Corning, NY, USA) was coated with ox-LDL

(7.5 µg/mL) in PBS. IgM and IgG2a antibodies against oxLDL in serum were measured using an ELISA Ig detection kit (Zymed Laboratories, San Francisco, CA, USA) according to the manufacturer's protocol.

Statistical analysis

Data are presented as means ± SEM. Statistical differences were calculated using Anova with Tukey's post-hoc test, for multiple comparisons, except for plasma CORT measurements transient and continuous groups were compared with the control group individually per time point using an unpaired two tailed T-test, with GraphPad Prism, version 5.01. $P<0.05$ was considered as statistically significant.

RESULTS

CORT treatment increases plasma CORT concentrations and affects circadian rhythm

Chronic administration of high doses of CORT in the drinking water (50 µg/ml) resulted in significant increases in plasma CORT levels at week 5 (Fig 1B) in both groups, compared to controls (transient group: 07.00 h 8-fold, 12.00 h 3-fold, 18.00 h 1-fold and 22.00 h 4-fold; continuous group: 07.00 h 5-fold, 12.00 h 4-fold, 18.00 h 1-fold and 22.00 h 3-fold). At week 17 (Fig. 1C) there were no differences between groups at 09.00 h and 18.00 h. At the end of the experiment, thymus weight (Fig. 1D) was

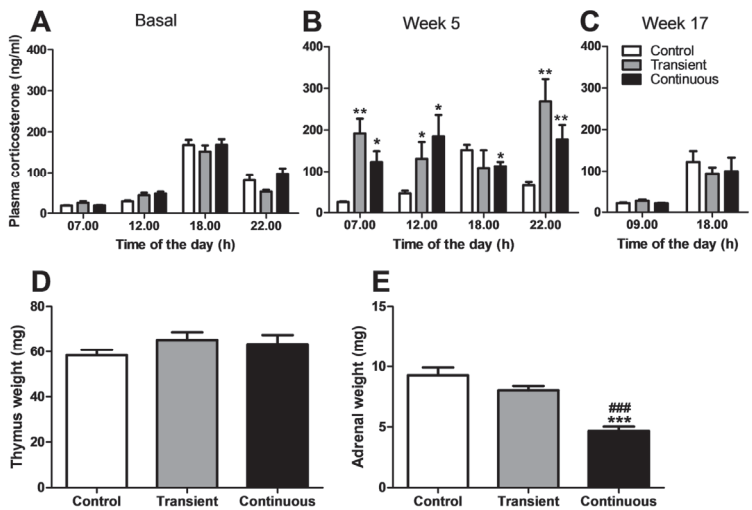

Figure 1. Effect of transient and continuous CORT treatment on circadian plasma CORT levels in female E3L.CETP mice at baseline (A), week 5 (B) and week 17 (C), as well as on thymus weight (D) and adrenal weight (E) at week 17 (Control group: white bars, transient group: grey bars and continuous group: black bars), Data are means ± SEM (n=17-21), $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$, $^{\#\#\#}P<0.0001$, *versus control group and #transient group.

not different between the three groups but adrenal weight (Fig. 1E) was significantly reduced in the continuously exposed group by -50%, in agreement with adrenal atrophy secondary to long-term exogenous GC exposure.

CORT treatment affects food intake and bodyweight and induces long-lasting changes in body composition and inflammation in adipose tissue

As expected, CORT treatment increased food intake of the transient and the continuous group during the first three weeks of the experiment after which the transient group returned to the level of the controls and food intake with continuous treatment remained elevated (Fig. 2A). This increase was accompanied by an increase in body weight (Fig. 2B) in both groups. After the discontinuation of CORT treatment, body weight decreased to the level of the controls. The continuously exposed group showed a continuous increase in body weight and maintained a higher body weight to the end of the experiment (Fig. 2B) compared to the other two groups. After 17 weeks, gonadal and subcutaneous fat pad weights (Fig. 2C and D), when compared to controls, were significantly increased by +35% and +31%, respectively, in the transient group, and by +140% and +110% in the continuous group. To evaluate whether the increased fat mass resulted in changes of inflammation in the fat pad, the mRNA expression of markers of the macrophage content (i.e. *F4/80* and *Cd68*) and proinflammatory cytokines [Tumor necrosis factor α (*Tnfα*) and Interleukin-6 (*Il-6*)] in the gonadal fat pad were determined. As compared to control group, transient administration of CORT did not affect the expression of *F4/80* (Fig. 2E) and *CD68* (Fig. 2F), but decreased the expression levels of *Tnfα* (Fig. 2G) and *Il-6* (Fig. 2H) in the long-term by -32% and -47%, respectively; while continuous administration of CORT increased the expression of *F4/80* (Fig. 2E) and *CD68* (Fig. 2F) by +58% and +70%, respectively, decreased the expression of *Tnfα* (Fig. 2G) by -26% and did not affect the expression of *Il-6* (Fig. 2H). These data indicate although excess GC exposure increased fat mass which was accompanied with an increase in macrophage content, expression of proinflammatory cytokines in the adipose tissue was generally reduced.

CORT treatment does not affect plasma lipids or cholesterol lipoprotein profile but increases plasma insulin and HOMA-IR

Although both transient and continuous administration of CORT increased food intake to a certain extent, CORT treatment did not increase plasma levels of total cholesterol, triglycerides or phospholipids during the experimental period of 17 weeks (Table 1). Moreover, there were no differences between groups in the distribution of cholesterol over lipoproteins (Fig. 3A-C). Plasma levels of insulin and HOMA-IR, but not plasma glucose levels, were increased (Fig. 3 D-F) in the continuous group at week 17, reflecting GC-induced insulin resistance.

Transient and continuous CORT treatment decrease atherosclerosis lesion area to a similar extent

Remarkably, CORT treatment decreased total atherosclerotic lesion area equivalently in both transiently (-39%) and continuously (-39%) treated groups (Fig. 4A and B). Moreover, both transient and continuous groups showed similar trends towards a less severe lesion phenotype as compared to the control group (Fig. 4C), suggesting that CORT treatment reduces atherosclerosis development in a long-lasting manner. CORT treatment, neither transiently nor continuously, affected the number of monocytes adhering to endothelium wall (Fig. 4D and E), yet continuous administration

of CORT did reduce the macrophage content (-56%) of the plaque (Fig. 4D and F) as well as the macrophage content as percentage of the total plaque area (-52%) (Fig. 4G). A reduction of the macrophage content of the plaque (-27%, P=0.125) and percentage macrophages in total plaque

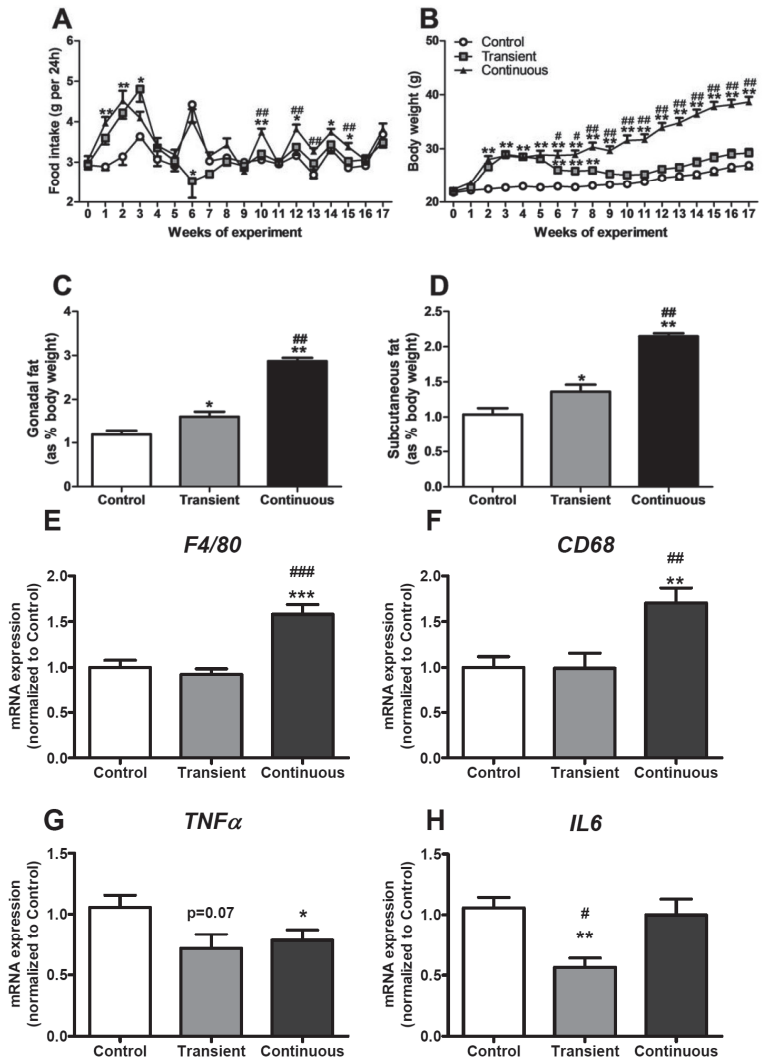

Figure 2. Effect of transient and continuous CORT treatment on food intake (A), body weight (B) (Control group: white circles, transient group: grey squares and continuous group: black triangles), gonadal fat (C) and subcutaneous fat (D) as % of the body weight mRNA expression of *F4/80* (E), *CD68* (F), *Tnfα* (G) and *Il-6* (H) in the gonadal fat (Control group: white bars, transient group: grey bars and continuous group: black bars). Data are means ±SEM (n=17-21), Anova with Tukey's post-hoc test, $^{*,\#}P<0.05$, $^{**,\#\#}P<0.001$, $^{***,\#\#\#}P<0.001$ *versus control group and #versus transient group.

Table 1. Effect of CORT treatment on fasting plasma lipids

	Total cholesterol (mmol/)			Triglycerides (mmol/l)			Phospholipids (mmol/l)		
Week	Control	Transient	Continuous	Control	Transient	Continuous	Control	Transient	Continuous
0	10.0±1.5	10.2±2.3	10.2±2.1	3.9±1.1	4.3±1.1	4.1±1.4	3.6±0.4	3.7±0.6	3.5±0.6
5	13.2±1.8	14.0±5.2	14.8±4.2	4.6±1.6	4.8±2.3	4.5±1.6	4.5±0.6	4.5±1.1	4.6±1.0
8	13.4±2.0	13.7±3.9	12.4±4.3	3.8±1.5	4.0±1.7	4.2±0.9	4.5±0.5	4.6±0.8	4.2±0.9
17	12.4±2.8	10.2±3.2	10.0±3.0	3.2±1.4	3.3±1.0	2.9±0.5	3.8±0.9	3.4±0.9	3.4±0.7

Data are means ± SEM (n=17-21), Anova with Tukey's post-hoc test.

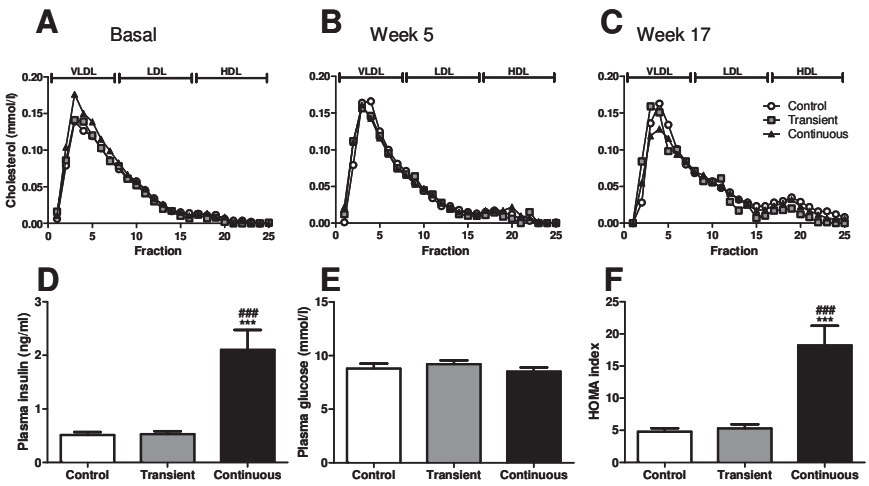

Figure 3. Effect of transient and continuous CORT treatment on cholesterol distribution over lipoproteins fractioned by FPLC at baseline (A), week 5 (B) and 17 (C) (Control group: white circles, transient group: grey squares and continuous group: black triangles) and on plasma plasma insulin (D), plasma glucose (E) and HOMA-IR (F) (Control group: white bars, transient group: grey bars and continuous group: black bars) on week 17. Data are means ± SEM (n=17-21), Anova with Tukey's post-hoc test, $^{**,\#\#\#}P<0.001$, *versus control group and #versus transient group.

area (-27%, P=0.145) was observed in the transient group and but these reductions failed to reach statistical significance (Fig. 4F and G). Since uptake of oxidized LDL (ox-LDL) by macrophages to become foam cell plays an important role in the development and progression of atherosclerosis, we measured specific antibodies against ox-LDL in the circulation. As compared to control group, CORT treatment, neither transiently nor continuously, influenced the ox-LDL specific IgG2a and IgM level, indicating that CORT probably does not affect the ox-LDL level in circulation (Fig. 4H). Because macrophage proliferation and differentiation was shown to be linked to the atherosclerotic process (1), an essential factor regulating macrophage growth, macrophage colony stimulation factor (M-CSF) (30) was measured at the end of this experiment. However, no differences in the serum levels of M-CSF were detected among groups (Fig. 4I).

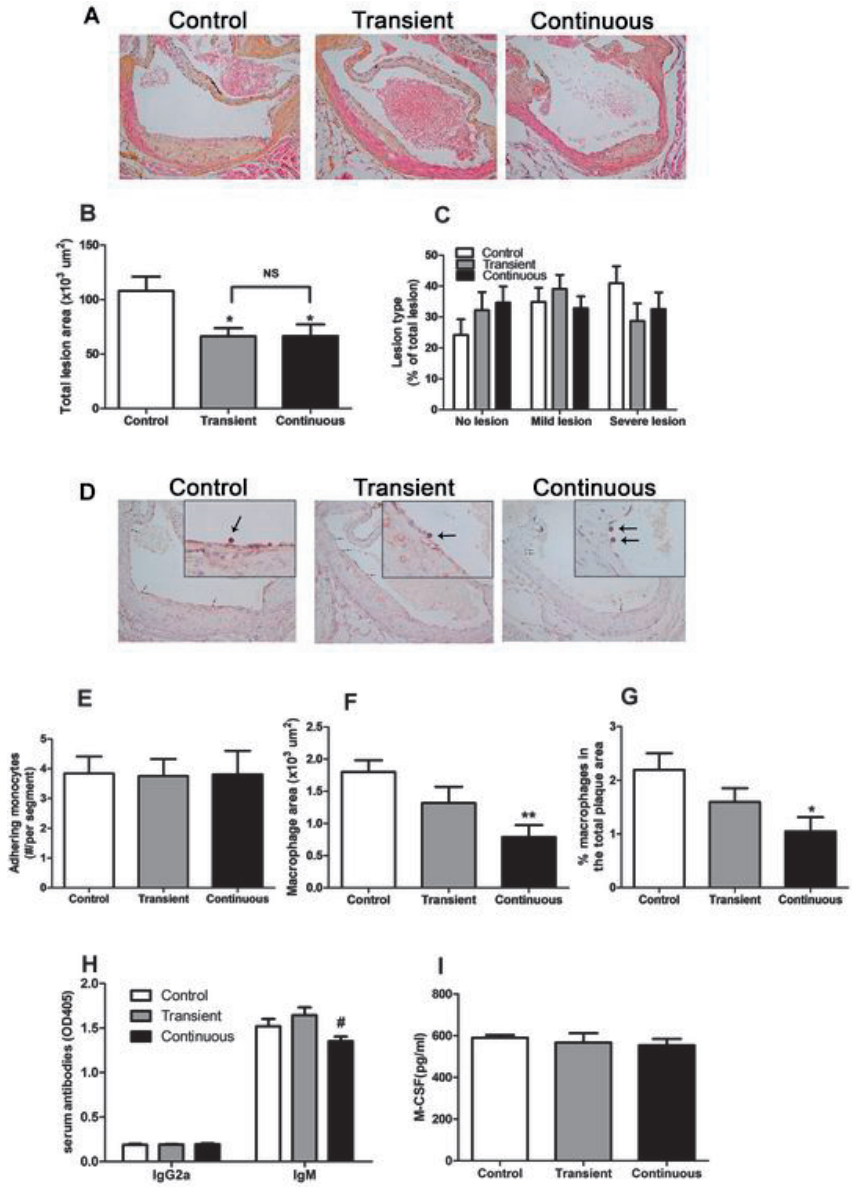

Figure 4. Effect of transient and continuous CORT treatment on atherosclerosis development: representative HPS-stained pictures of lesions (A), lesion area (B), lesion type as % of the total lesion (C), representative pictures of monocyte/macrophage staining (adhering monocytes shown by arrows) (D), adhering monocytes per segment (E), macrophage content of the plaque (F) and macrophages as % of the lesion area (G). Serum anti-ox-LDL specific antibodies (H) and serum M-CSF (I) were measured on week 17. (Control group: white bars, transient group: grey bars and continuous group: black bars). Data are means ± SEM (n=17-21), Anova with Tukey's post-hoc test, $^{*\#}P<0.05$, $^{**}P<0.01$, *versus control group and #versus transient group.

DISCUSSION

This study demonstrates for the first time that CORT treatment resulting in increased plasma CORT concentrations decreases atherosclerotic plaque formation in mice with human-like lipoprotein metabolism, without affecting either plasma lipid levels or lipoprotein profiles. Interestingly, inhibition of atherogenesis was found both in transiently and continuously exposed animals, whereas continuous treatment with CORT also decreased macrophage content of the plaque despite the normalization of the body weight. In addition, CORT treatment resulted in long-lasting changes in body fat content, which were still present even 12 weeks after abrogation of CORT. This indicates that increased CORT exposure *per se* has beneficial, long-lasting effects on atherosclerosis, but negatively affects body fat distribution and insulin sensitivity, by promoting fat accumulation in the long-term.

The concentration of glucocorticoids (GCs) attained in our experiments is higher than under stress state but well comparable to patients with severe Cushing's syndrome, where cortisol secretion was 7 times higher that in healthy controls and circulation cortisol concentrations was 3-5 times higher (31).

Increased plasma CORT levels were easily induced non-invasively using CORT in the drinking water. CORT affected circadian GC rhythm by reducing the degree of variation in plasma concentrations. The human clinical equivalent of chronic hypercortisolemia, Cushing's syndrome (CS), is characterized by a blunted or even complete loss of diurnal rhythm (3). In our model we re-capitulate some of these key temporal aspects of CS: although a chronic high circulating cortisol levels is a key aspect of CS, the most reliable measure for diagnosis is very high late night (i.e. 22:00 –24:00 h) plasma cortisol (3). Our high- CORT animals parallel these aspects of the syndrome, with both high baseline levels of CORT (200 ng/ml), as well as a peak in CORT at the end of the night (rather than at the beginning). Moreover, the continuously exposed group displayed reduced adrenal sizes, in agreement with adrenal atrophy, secondary to the long-term exogenous CORT administration despite normal plasma CORT levels found at the end of the experiment. This might be explained by increased metabolism of CORT as well as by other pharmacokinetic adaptations like increased clearance and increased distribution volume as a result of increased adipose tissue.

Plasma lipid levels were not affected by transient nor continuous CORT treatment. This is interesting particularly in the case of plasma values of total cholesterol, VLDL-cholesterol and VLDL-triglycerides since these are well known risk factors for the development of atherosclerosis. In fact, cholesterol exposure generally is a good predictor of atherosclerosis development in E3L.CETP mice (Princen and Rensen, unpublished observations). Moreover, CORT exposure did not affect lipoprotein profiles. In previous studies, mice were subjected to chronic stress, which causes a complex of endocrine changes, including high CORT levels. These studies have reported inconsistent effects on plasma cholesterol levels, which were increased (15), decreased (16), or not reported (14). These studies used ApoE-deficient mice in which the ApoE- deficiency causes severe accumulation of cholesterol in macrophages resulting in a high pro-inflammatory state, which could explain the differences observed in atherosclerosis development between our low grade inflammation model of E3L.CETP mice fed a low amount of dietary cholesterol (twice human daily intake) (32, 33). In addition, the use of chronic stress to increase endogenous GCs in previous models will also induce other complex endocrine and metabolic changes (17) that may also affect atherosclerosis development. Humans with high GCs also have inconsistent cholesterol levels. In the literature, the prevalence of hyperlipidemia in patients with CS varies from 38% to 71% (34). A

study by Mancini et al (2004) has shown that hyperlipidemia occurs less frequently than the other metabolic complications of CS and that it was not correlated to the degree of hypercortisolism or duration of the disease (35). However, the causative role of cortisol excess for hyperlipidemia has not been extensively described in the literature and the findings are, like the animal data, controversial. In some study populations, the prevalence of hypertriglyceridemia was even lower than in BMI-matched controls (36).

Intriguingly, CORT treatment reduced atherosclerotic lesion area, and tended to decrease lesion severity, to a similar extent in transiently *vs* continuously exposed mice. This suggests that GCs are able to induce long-term effects in the preliminary processes of atherosclerotic plaque formation. It is tempting to speculate on possible underlying mechanistic explanations for this phenomenon. One possibility is the induction by increased GC exposure of epigenetic mechanisms, like chromatin remodeling and histone modifications (37). These alterations have been demonstrated in the context of chronic CORT (38), and may lead to long lasting suppression of or changes in macrophage function. Extensive documentation supports a crucial role for macrophages in the initiation of atherosclerosis by entering the vessel wall, taking up oxidized LDL (ox-LDL) (1, 2) and transforming into foam cells that produce a variety of cytokines further driving the process of the plaque formation, as well as macrophage proliferation and differentiation (39, 40). In the present study, transient and continuous CORT treatment did not affect the plasma ox-LDL level, albeit that continuous CORT treatment significantly reduced the macrophage content of the plaque, and a similar trend was also observed in the transiently exposed group. GCs were shown to decrease the development of atherosclerosis by reducing the monocyte recruitment (41-43), macrophage foam cell formation (44), macrophage growth, as well as macrophage inflammatory action to produce pro-inflammatory cytokines (45). In this study, no differences were observed in the number of monocytes adhering to endothelium between the groups. It is plausible that GCs excess inhibits macrophage growth instead of monocyte recruitment as GCs inhibit macrophage colony stimulating factor (M-CSF)-induced macrophage differentiation *in vitro* (45). Although, we did not detect any differences in serum M-CSF level between groups, it should be realized that M-CSF is a general marker for macrophage growth, and the systemic concentration in plasma may not reflect the local macrophage growth in the vessel. Additionally, GCs can also inhibit macrophage growth by suppressing the granulocyte/macrophage colony-stimulating factor (GM-CSF) production in isolated macrophages (46), confirming that GCs might target the macrophage, the major cellular CORT target, thereby attenuating macrophage proliferation and differentiation, and thus inhibiting atherosclerotic plaque formation.

In humans, increased GC exposure, like in patients with CS, is associated with the metabolic syndrome and cardiovascular disease, even after long-term successful correction of GC excess (6, 47, 48). Carotid intima media thickness (IMT) is increased and vessel wall plaques are more common in patients with CS (36, 49, 50). Indeed, CS patients have abnormal fat distribution and suffer from disturbed coagulation and osteoporosis. Although, it is documented that bone mineral density fully recovers after normalization of cortisol levels, other features, like the adverse metabolic profile and the hypercoagulable state, do not completely resolve (51, 52). The causal relation, however, between the episode of cortisol overexposure and long-term changes in the development of cardiovascular diseases is not established and is difficult to assess in humans because of the rarity and heterogeneity of CS. In agreement, in our mouse model CORT also stimulated the development of other components of the metabolic syndrome. We observed that CORT increased food intake,

body weight, insulin concentrations, and altered body composition. High CORT levels are known to stimulate voluntary food intake dose-dependently (53-55). GCs are also known to induce insulin resistance (48). Moreover, insulin and CORT synergistically promote redistribution of energy storage in favor of increased fat tissue (56). The changes in body weight were accompanied by a significant increase in both gonadal and subcutaneous fat mass in continuous group and, remarkably after 12 weeks of wash out, in transiently exposed group as well. Chronically administered GC facilitates an increase of fat mass in mice (25, 57). In humans, increased exposure to cortisol (being either endogenous CS or exogenous corticosteroids) induces increased total body fat (58), and is specifically characterized by a redistribution of adipose tissue from peripheral to central sites of the body, mainly in the truncal region and visceral depots In the human equivalent of severe chronic stress, CS, the prevalence of the metabolic syndrome is increased (47, 48), and intriguingly, after remission, these patients still have increased waist circumference (36, 60) and higher visceral fat mass without an effect on the body mass index (61), and their cardiovascular risk remains increased. Adipose tissue macrophages are the primary source of the proinflammatory cytokines, and the macrophage content of adipose tissue has been shown to correlate positively with adiposity (62). In the present study, although excess GC exposure increased fat mass and induced obesity which was accompanied with an increase in macrophage content, inflammation in the adipose tissue was not elevated. Moreover, after abrogation of CORT treatment, the macrophage content in the adipose tissue was normalized and the reduction of inflammation was persisted in the long-term despite of the presence of a persistent increase in fat mass. CORT treatment induces prolonged, complex changes in the adipose tissue that reflect both the adverse metabolic effects of glucocorticoids by increasing the adiposity, and the anti-inflammatory capacity by reducing the expression of the proinflammatory molecules in the macrophages. Therefore, we cannot exclude that the protective effects of CORT treatment on atherosclerosis development can, at least partly, be explained by decreased release of cytokines from adipose tissue. It is well possible that at high dose, the anti-inflammatory effects of GCs attenuate potentially adverse metabolic influences. In clinical practice, this means that GC schemes as used for anti-inflammatory indications might benefit from adjustments towards a higher dose for a shorter period of time (or even as a few 'high dose 'pulses' as is used in clinical practice with methyprednisolone instead of lower dosages for a prolonged period of time.

In conclusion, increased CORT exposure in mice with a human like lipoprotein metabolism has beneficial, long-lasting effects on atherosclerosis, despite negatively affecting body fat distribution and insulin sensitivity by promoting fat accumulation in the long term. This indicates that the increased atherosclerosis observed in the human in states of GC excess may not be related to cortisol *per se*, but may be the result of complex effects of cortisol on the endothelium and/or coagulation. The effects of GC excess, therefore, are multiple, and dependent on many factors, but above all, may irreversibly affect many pathophysiological processes, thereby influencing long-term cardiovascular risk.

Supplemental Table 1. Primer sequences used for RT-qPCR

Gene	Forward primer	Reverse Primer
β2m	TGACCGGCTTGTATGCTATC	CAGTGTGAGCCAGGATATAG
Cd68	ATCCCCACCTGTCTCTCTCA	TTGCATTTCCACAGCAGAAG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Il-6	TGTGCAATGGCAATTCTGAT	CTCTGAAGGACTCTGGCTTTG
Tnfα	AGCCCACGTCGTAGCAAACCAC	TCGGGGCAGCCTTGTCCCTT

β2m, β2-microglobulin; *Hprt*, hypoxanthine ribosyltransferase; *IL-6*, Interleukin-6; *Tnfα*, Tumor necrosis factor α.

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6

GENERAL DISCUSSION

GENERAL DISCUSSION

Today's Western society and work promotes a sedentary lifestyle, which from an evolutionary perspective, is a relatively recent development. This, coupled with the high availability of foods with high caloric content in Western cultures, superimposed on dated genotypes has given rise to the pandemic of obesity with its related increase in prevalence of the metabolic syndrome (MetS), type 2 diabetes (T2D) and cardiovascular diseases (CVD). At the same time, the perceived social stress in everyday's society has increased (1). Furthermore, lack of sleep and sleep disturbances have become increasingly common and today we are, even voluntarily, sleep restricting ourselves and sleep significantly less than thirty years ago (2).

The metabolic effects of perceived stress are mediated by glucocorticoids (GCs) that are secreted by the adrenals as a result of stressor-induced activation of the hypothalmus-pituitary-adrenal (HPA) axis. The effects of GCs include recruitment of energy storages from fat and muscle (3, 4), but also central activation with adjustment of feeding behavior resulting in an increase in the intake of palatable foods to compensate for the catabolic effects (loss of energy) in peripheral tissues. These metabolic adaptations occur during the stressful event, with the purpose to be enable the evolutionary "fight or flight" response (5). It is therefore not surprising that, in the presence of such an evolutionary 'pressure' of stress, the consumption of "comfort food" as well as "emotional eating" has increased (6), further facilitating the development of obesity, the MetS, T2D and CVD (7). Epidemiological data show a clear association between increased plasma levels of the GC cortisol and CVD and obesity, but animal models of the MetS are inconclusive with respect to whether the HPA axis is activated or not. Thus, up till now, it was unknown if, and to what extent an increased activity of the HPA axis plays a pathogenic role in the development of the MetS, and *vice versa*, whether development of MetS leads to increased activity of the HPA axis, if there would be a role of development of specific features of the MetS, *i.e.* obesity, on the activity of the HPA axis.

In this thesis we have chosen to evaluate both whether baseline, non-stressed, activity of the HPA axis is affected during the development of obesity, and *vice versa*, to what extent a period of exposure to increased endogenous GC levels would affect specific features of the MetS. The major conclusions and implications of our findings will be discussed in this chapter.

Impact of the development of obesity on baseline, non-stressed, activity of the HPA axis

As stated above, overweight and obesity have become prominent global health problems and are no longer a health issue that only affects adults but also children and adolescents. Overweight and obesity are often accompanied by other metabolic abnormalities, such as dyslipidemia and insulin resistance, that can be clustered to define a clinical syndrome that is associated with increased cardiovascular morbidity and mortality: the MetS (8).

The basal activity of the HPA axis, i.e. not induced by stress, has been reported only in a limited number of studies in obese individuals (9-12), and these results have been inconclusive. A key issue in this respect is the difficulty to assess the activity of the HPA axis in conditions were individuals are exposed to social stress, as is present in everyday life. In this respect, animal models can be very useful, since under laboratory conditions, in principle it is possible to control for many potential stressors that might affect the outcome.

In **chapter 2** we critically evaluated the current literature available reporting the effects of high fat diet (HFD) on the basal, non-stressed, activity of the HPA axis in mice by performing a structured, and systematic review of all the available literature on this topic using the main medical databases. We included only original mouse studies that reported parameters reflecting the activity of the HPA axis after a prolonged period of HFD feeding, because there is extensive documentation that HFD feeding *per se* is able to induce obesity and insulin resistance, especially in wild-type C57Bl/6 male mice (13-16). For inclusion in the review, at least one basal corticosterone (CORT) measurement and a proper control group was required. Studies with adrenalectomized mice, transgenic animals only, HFD for less than two weeks, or other interventions besides the HFD, were excluded, as they do not represent normal physiology. In addition, we excluded studies with an insufficient duration of exposure to the HFD to be able to induce significant features of the MetS. We found twenty studies that fulfilled our stringent inclusion criteria, but surprisingly, in only five studies the evaluation of the HPA axis was the primary research question. Plasma CORT levels in these twenty studies were found to be increased, unchanged, or even decreased. Additional parameters reflecting the activity of the HPA axis, such as 11β-dehydrogenase (11β-HSD-1) expression in peripheral tissues such as liver and fat, and corticotropin releasing hormone (CRH) and/or glucocrticoid receptor (GR) expression in the central nervous system, were evaluated only in five out of these 20 studies, and these data were also not consistent.

Importantly, there were many differences between the studies that precluded a reliable comparison of the effects of HFD on the HPA axis between studies. For instance, the relative energy contribution of fat in the diet varied between 32-65%, and different mouse strains were used, resulting in a large variation in weight gain. In addition, there were different housing and sampling conditions. All these factors are known to differentially affect the activity of the HPA axis, and therefore, all can contribute to the different results observed in the studies. Most importantly, different housing conditions, e.g. group-wise *vs*. individual housing, has shown to dramatically affect both basal and stress-induced HPA axis activity, as in a group a social order is about to form with dominant and subordinate individuals, that display different coping strategies when faced with social stress (17, 18). In addition, the majority of the studies failed to report on the sampling conditions for CORT (especially whether stress-free sampling was performed: at best, some reported on the elapsed time between opening of the cage and sample collection). Some studies even reported the use of anesthesia before sampling, which, by definition, requires more handling of the animal, which has an additional effect (besides the anesthetic *per se*) on the central nervous system.

Thus, the systematic review demonstrated that the effects of HFD on the basal activity of the HPA-axis, and its contribution in the propensity to become obese and develop other manifestations of the MetS on a HFD, remained unclear. For a proper and reliable evaluation of the basal activity of the HPA axis, we reasoned that an appropriate study design is therefore a prerequisite. We proposed that such an appropriate design should, at least, take the following factors into account: 1) choice of mouse strain (genetically modified or not, DIO-resistant or not, resistant to stress or not), 2) duration and content of the diet, 3) standardization of housing conditions with a possibility to acclimate in advance, 4) proper methods for stress free sampling of CORT, and 5) the timing of the sampling to reflect circadian rhythmicity.

Therefore, in **chapter 3** we applied the most appropriate study design as proposed in **chapter 2**. In this study, the aim was to document the effects of HFD on the basal activity of

the HPA axis, both peripherally and centrally, in C57BL/6 mice with previous extensive available documentation with respect to the development of obesity and insulin resistance when fed a HFD (12-15). We measured plasma CORT using a stress-free sampling method both at the circadian nadir and at the circadian peak (in the evening). HFD decreased diurnal peak CORT already at the first week of HFD feeding and the levels remained significantly lower when compared to controls up to twelve weeks (the end of the experiment). This finding is in line with a recent study reporting decreased basal plasma CORT values in a similar study design, but with a shorter duration of the diet (19). Furthermore, we observed that HFD induced complex changes in CRH and GR mRNA expression in the central nervous system areas responsible for feeding behavior and limbic functions, namely the paraventricular nucleus (PVN) of the hypothalamus, the amygdala, and hippocampus. In the peripheral white adipose tissues, HFD induced a profound down regulation of 11β-HSD-1 enzyme mRNA. Since this enzyme converts cortisone and 11-dehydrocorticosterone into their active forms cortisol and CORT, this reflects decreased CORT exposure at the tissue level. In agreement with these findings, we found no changes in 11β-HSD-2 enzyme mRNA expression, which converts cortisol and CORT into their inactive forms, thus leading to a reduced net effect of CORT exposure in the tissue level.

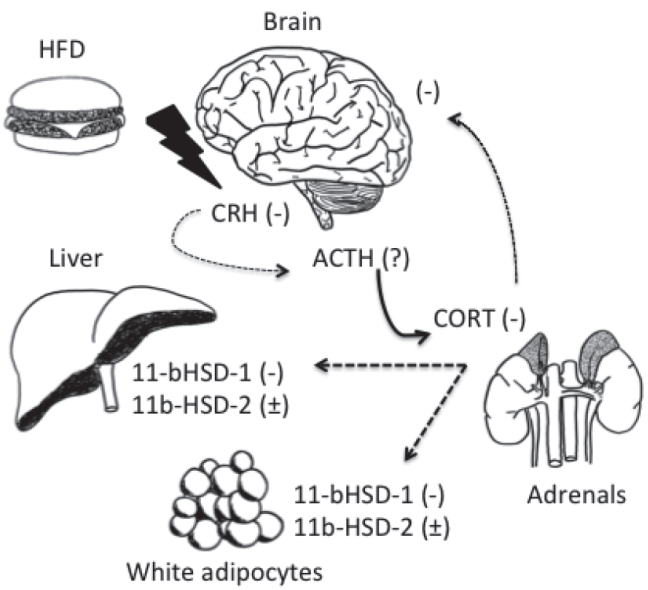

Figure 1. Tentative model of the effect of HFD on the HPA axis activity. See text for explanation

The results found in the first two studies, first of all indicate that a proper study design is crucial for reliably evaluating the HPA axis. In addition, it appeared that the development of obesity leads to secondary adaptive responses of the HPA axis that cannot be interpreted by just measuring

circulating CORT concentrations only. Fundamental in this respect is the fact that obesity *per se* increases body fat and thus the distribution volume for CORT (which is fat soluble), and therefore decreases circulating CORT levels and stimulates central activation of the HPA axis as discussed in the introduction. However, the net result of HPA axis activation in obesity is complex and is also influenced by central and peripheral interactions with orexigenic hormones (like leptin) (20-24), by changes in adrenal sensitivity for adrenocorticotropic hormone (ACTH) (25, 26), and by changes in enzymatic (de)activation of CORT in peripheral tissues (by the 11β-HSD type 1 and 2) (27, 28). Thus, it can be argued that the adaptation of the HPA axis to the HFD, at least in part, is secondary to the increasing body weight. Pair-feeding (29), in which less HFD is given to a caloric amount that is consumed in chow-fed animals, provides an experimental set up that eliminates the effect of energy intake and possible subsequent weight gain, thus allowing to observe the "true" effect of HFD on the HPA axis parameters. These results, however, could be hampered by the stress induced by the reduced feeding (30), and if so, periods of hunger experienced by the pair-fed group would also no longer represent a stress-free model.

The present study was performed in adult mice naive for HFD. Therefore the results should be interpreted with caution and cannot simply be extrapolated to neonates, pups, adolescent mice, or to the offspring of HFD-fed mothers. Previous studies have shown that maternal exposure to HFD during pregnancy and postnatally, even without developing obesity, resulted into increased weight and insulin as well as leptin resistance of the offspring, both in adolescence and adulthood (31-36). Exposure to maternal HFD resulted in increased plasma CORT in rat pups (37) but weaning of pups to HFD decreased 11β-HSD-1 activity in the liver and adipose tissues (38). Furthermore, maternal exposure to HFD led to changes in melanocortin expression and disturbances of the pro-opiomelanocortin (POMC) system in the fetal offspring of nonhuman primates (39). Finally, some studies have also demonstrated altered stress-induced responsiveness of the HPA axis: adult offspring of HFD-fed rats exhibited an increased reactivity to acute stress (40) and neonatal pups from HFD-fed mothers showed a decreased response to stress whereas in adulthood stress responsiveness was increased (41). These findings demonstrate that HFD exposure may result in multiple effects on both basal and stimulated activity of the HPA axis, and that these effects are dependent on the duration of the exposure as well as on the timing (age) of the exposure. Therefore, it should be noted that the results obtained from different studies reflect the particular experimental set up.

Thus, HFD, which is abundantly available in today's society, induces complex changes in the diurnal regulation of various components of the HPA axis. This is of paramount importance because activation of the HPA axis in the central nervous system, for instance, not only affects metabolic 'sensing' but is also a key modulator for the limbic system, facilitating learning, memory formation and retrieval (42), thereby affecting individual (psychological) well being.

For future studies, it would be interesting to investigate whether the effects of HFD on both the basal activity of the HPA axis and stress responsiveness are reversible. This is of importance since it has been shown that obesity is a risk factor for Alzheimer's disease (43) and depression (44), both of which are also associated with alterations of the HPA axis. Futher more, there is no awereness on whether development of obesity leads to secondary adaptive changes of the HPA axis which may, in turn, lead to altered set points of hormone release and tissue sensitivity as has already been extensively documented for pituitary-gonadal axis (45).

The effects of a period of endogenous GC excess on the MetS and atherosclerosis

The fundamental question that arises from our previous observations is whether the HPA axis is able to adapt sufficiently during the development of obesity in the presence of chronic (social) stress. From an evolutionary point of view, social stress is considered to be a chronic challenge, as cortisol, secreted during stress response, promotes feeding behavior (46) to compensate for the energy loss that takes place during the "fight or flight" responses. However, in the given context, such an energy loss might never take place and thus the individual, driven by the evolutionary drive orchestrated by the central nervous system, only further promotes a positive energy balance resulting in weight gain and insulin resistance, continuing the vicious cycle.

GCs are also potent anti-inflammatory agents that are widely used for their immunosuppressive properties but they also play a major role in glucose, lipid, and protein metabolism (47-49). In humans, an increased activity of the HPA axis has been linked to the development of the MetS (50). In addition, manipulation of cortisol exposure at the tissue level in mice, through stimulation or abrogation of 11β-HSD-1 and 11β-HSD-2 activity can increase, or regress, visceral fat accumulation, as well as other features of the MetS (27, 28). However, as discussed extensively in the previous paragraphs, these associations should be interpreted with caution and do not prove causality. In agreement, however, with these observations is the rare clinical syndrome of Cushing's syndrome (CS). CS in the human is the result of prolonged excessive exposure to GCs (in the majority of cases caused by ACTH secreting pituitary adenoma with subsequent adrenal overstimulation) and is typically associated with an increased prevalence of the MetS, albeit with a specific phenotype, with increased cardiovascular morbidity and mortality (51). Intriguingly, patients treated for CS remain at increased cardiovascular risk, even after long-term successful correction of GC excess (52). Although this is the best human model representing a (transient) period of GC excess, in the absence of other pathology or auto-immunity, the causal relation between the episode of cortisol overexposure and long-term changes in cardiovascular risk factors is not established and is difficult to assess because of the rarity and heterogeneity of CS in humans.

Therefore, in **chapter 4** we used a mouse model that has previous extensive documentation of development of certain features of the MetS, in this case obesity and insulin resistance, when exposed to HFD (13-16). In that study, we aimed to identify factors that modulate metabolic recovery from a period of overexposure to GCs. Male C57Bl/6J mice, fed a low fat diet (LFD) or HFD, received CORT or vehicle in the drinking water for 4 wks, followed by a washout period of 8 wks. CORT treatment increased plasma CORT, food intake and plasma insulin and lipids in both diets. Abrogation of CORT treatment normalized plasma CORT levels diet-dependently: mice fed LFD had normal circadian plasma CORT levels already after 4 weeks of washout whereas the CORT peak was still decreased in the HFD-fed mice when compared to their respective controls. Food intake and body weight normalized after removal of the CORT treatment. Intriguingly, at week 12 (i.e. after an eight weeks washout period and 6 weeks after normalization of body weight in the HFD-fed mice), plasma insulin levels were still significantly higher in CORT-treated mice on both diets, and HFD-fed CORT-treated mice had persistently decreased lean body mass and increased fat mass. Thus, in mice, a period of CORT excess induced long-lasting increase in plasma insulin levels and fat mass. However, the changes in body composition were present only in the presence of HFD. Interestingly, the recovery of the HPA axis, when measured as circadian plasma CORT levels, occurred earlier with LFD.

This can be of importance because CS patients, like all humans exposed to chronic stress, are likely to make dietary choices directed towards highly palatable foods (53) which would negatively affect the recovery of the HPA axis after treatment. In addition, it has been shown that HFD reduces the adrenal cortex sensitivity to ACTH (25, 26) and, therefore, could aggravate the withdrawal from, at least, synthetic steroids and postpone the recovery of endogenous adrenal cortisol production. Furthermore, slower recovery of the HPA axis secondary to HFD could also contribute to the psychopathologies as observed in CS patients even after long term correction from the supraphysiological cortisol levels (54). These observations are in complete agreement with the evolutionary drive of the stress response and points at a crucial role for dietary composition in the development of the MetS in conditions with periodic excessive GC exposure, like is the case in patients treated for CS, but possibly also in the general population exposed to chronic stress.

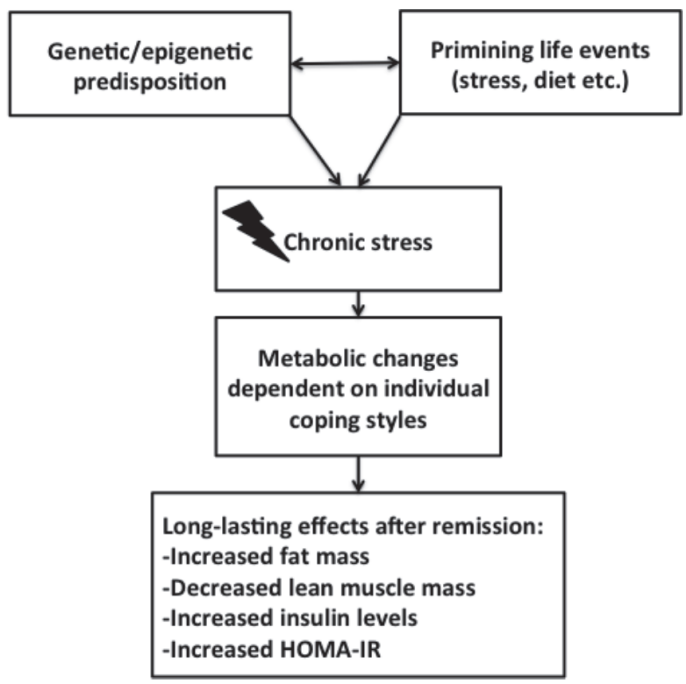

Figure 2. Individual experiencing chronic stress, like in CS, can, dependent on genetic/epigenetic predisposition, priming life events and individual coping styles, develop MetS and increase risk for CVD. Certain effects such as altered body composiotion, decreased insulin sensitivity and cardiovascular mortality can prevail long even after remission.

In **chapter 4** we chose to give CORT in the drinking water as opposed to supplementing the mice with e.g. subcutaneous (sc) CORT pellets. As expected, this non-invasive supplementation of CORT via the drinking water allowed the preservation of a certain degree of variability of circulating plasma CORT in the experiment, thereby preventing excessive tissue desensitation to CORT. Previous

studies that have used sc pellets for CORT supplementing resulted in abolishment of the ultradian rhythm, which restored after removal of the pellets in intact rats (55). In patients with CS, the diurnal variation in cortisol secretion is abolished in most patients. Cortisol secretion in these patients was further characterized by markedly amplified total daily hormone secretion secondary to an approximately 7-fold higher basal secretion rate, and increased secretory burst mass (ACTH and cortisol) and frequency (cortisol) (56). To be able to distinct between the preserved rhythm and continuously increased CORT levels and their long term effects, both methods of supplementation should be compared in one experiment. In the studies performed in **chapter 4**, the mice were given LFD or HFD with or without CORT treatment and no choice of diet was applied. We know, however, that GCs direct the choice of food toward more palatable, high energy foods rich in fat and sugar (53). Therefore, to truly estimate the effects of CORT treatment on body weight and composition as well as on insulin resistance, a study would be needed where choice of food is available during the tretament and washout.

In **chapter 5** we investigated the effects of a period of high GC exposure *vs* continuous overexposure on the development of atherosclerosis in a mouse model with human-like lipoprotein metabolism, namely ApoE*3-Leiden.CETP (E3L.CETP) female mice on a C57Bl/6J backtground. These mice represent a well-established model for the development of atherosclerosis when fed a cholesterol-rich diet (57). We induced high plasma CORT levels by adding CORT to the drinking water for either 5 wks (transient high exposure) or 17 wks (continuous high exposure). We found that CORT treatment increased body weight and food intake for the duration of the treatment and increased white adipose tissue weight in both treatment groups in the long-term. Both transient and continuous CORT treatment decreased total atherosclerotic lesion area to the same extent, without reducing plasma cholesterol levels. This was accompanied by a decrease in macrophage content of the plaque to a similar extent after both treatments.

The fact that CORT treatment reduced atherosclerotic lesion area, and tended to decrease lesion severity to a similar extent in transiently *vs* continuously exposed mice suggests that GCs are able to induce long-term effects in the preliminary processes of atherosclerotic plaque formation such as inhibiting the uptake of oxidized low-density lipoproteins (LDL) by macrophages (58, 59). Subsequent transformation of macrophages into foam cells that produce a variety of cytokines will further accelerate the process of plaque formation (60, 61) as well as the regulation of the expression of adhesion molecules in the vascular endothelium, thereby restricting the number of neutrophils entering the vessel wall (62, 63). The macrophage content of the plaque was significantly reduced in the continuously exposed group, and a similar trend was also observed in the transiently exposed group. We did not observe any differences in the number of monocytes adhering to endothelium between the groups. More likely, CORT excess stimulated the inhibition of macrophage growth and/or maturation instead of monocyte recruitment. GCs have been shown to inhibit oxidized LDL-induced macrophage growth by suppression of granulocyte/macrophage colony-stimulating factor (M-CSF) (64), thereby inhibiting atherosclerotic plaque formation.

It is difficult to compare our data with the limited data available from other mouse studies that evaluated the effects of GC on atherosclerosis. For instance, many of these studies used chronic stress as a model to increase endogenous GC but this will also induce other endocrine and metabolic changes (65) that may affect atherosclerosis development. Other studies used ApoE-deficient mice that harbor a different pro-inflammatory state (66) in contrast to the low-grade inflammation model of E3L.CETP mice used in our study (67, 68).

Whereas our observations in mice in **chapter 4**, i.e. insulin resistance, dyslipidemia and changes in body composition, are in a good agreement with the phenotype found in CS patients (49, 69-71), our findings in **chapter 5** are striking because in humans, increased GC secretion, like in patients with CS, is associated with CVD, although the exact role of GC in the development of atherosclerosis is not yet clearly established. Intriguingly, patients with CS remain at increased CVD risk, even after long-term successful correction of GC excess (52). Although limited, data in patients with CS indicate that carotic intima media thickness (IMT) is increased and vessel wall plaques are more common (69, 72, 73). Apart from atherosclerosis, CS patients have abnormal fat distribution, coagulopathy, and osteoporosis. Recent data indicate that remission of CS improved some but not all cardiovascular risk markers (74, 75).

Thus, increased CORT exposure in mice with human-like lipoprotein metabolism has long-lasting, beneficial effects on atherosclerosis, although it negatively affects body fat distribution and insulin sensitivity, by promoting fat accumulation in the long-term. This indicates that the increased atherosclerosis observed in humans in states of GC excess may not be related to cortisol *per se*, but may be the result of circulating GC (endogenous or synthetic) concentrations and of complex effects of GCs on the endothelium and/or coagulation (74, 75), and, finally, of epigenetic mechanisms (76, 77).

The results in **chapter 5** were obtained in adult mice and therefore age can be a factor determining the outcome. In the present study, the mice were allowed to age to adulthood without developing atherosclerosis and then were given a cholesterol trigger to start the development of the atherosclerotic plaques simultaneoulsy with the CORT treatment. Therefore, the timing of the treatment is fundamentally directed to the initiation of the atherosclerosis development. However, in humans the development of the plaques and thickening of the intima start already earlier in life (adolescence) (78) and thus the period of high GC exposure, like seen in CS, takes place in the later stages of the development of the atheroslerosis rather than during the initiation of the process. Therefore, it would be interesting to investigate the effects of CORT treatment on atherosclerotic plaque development in a model where the development would be initiated earlier in life and the CORT treatment would take place at a later stage in plaque development. It must be, however, considered that the processes triggering the development of atherosclerosis in mice can be crucially different from humans, in which it is thought to be a complication of dyslipidemia in combination with insulin resistance. Thus the timing of the GC excess should be critically applied in the investigation of the relationship between the developent of atherosclerosis and dyslipidemia and insulin resistance. Since E3L.CETP on a cholesterol-rich diet become hyperlipidemic without developing insulin resistance, the mouse model could be further improved i.e. by adjusting the fat content of the diet. Finally, it is possible that the immunosuppressive potency of cortisol is not exactly the same as that of CORT, precluding perfect comparisons.

GCs have potent immunomodulatory properties, and synthetic GCs are widely used in the treatment of auto-immune diseases, like rheumatoid arthritis and inflammatory bowel disease. Conversely, inflammatory cytokines like TNF-alpha (TNF-α) and interleukin-6 (IL-6), can modulate pituitary hormone secretion, in particular ACTH secretion (79). This implicates that the neuroendocrine (central nervous system and hormones) and immune systems communicate bi-directionally. Recent data also showed that receptors belonging to the native immune system, the toll-like receptors (TLR), are stimulated by fat containing substances. Fatty acids are able to do so by mimicing the fatty acid moieties of the lipid A-moiety of bacterial lipopolysaccharide (LPS).

This moiety is a high affinity ligand for TLR4. Stimulation of these TLRs with saturated fatty acids appeared to evoke a pro-inflammatory response, that eventually resulted in insulin resistance and atherosclerosis (80). In agreement, a population-based study recently reported increased TLR type 2 and -4 expression and activity in the monocytes of patients with MetS, but without diabetes or CVD, *vs* controls (81). This novel observation in the human clearly indicates that indeed increased TLR activity could contribute to an increased risk for diabetes and cardiovascular disease. Thus, the purpose for the bi-directional communication between the neuroendocrine (central nervous system and hormones) and immune systems is evident from an evolutionary point of view: it is crucial for survival to have an integrated system that informs the individual about threats, but also about opportunities. As a consequence, nutritional status and infectious pressure are integrated and lead to autonomous decisions to fight or flight, on reproduction or ageing, and on sleep or vigilance. In case of HFD, apparently we can simulate a bacterial attack, and mislead the body with unnecessary reactions and undesired effects (82).

To evaluate the effects, long lasting or otherwise, of HFD and/or CORT, on the functionality of the HPA axis more detailed than what has been described in **chapters 3**, **4** and **5**, it is necessary that future studies evaluate, and control for, stress induction, recovery from it and behavioral changes which they may induce. This is imperative in order to understand the behavior and choices of the individual induced by the adaptation of the HPA axis. Based on this knowledge, advice can be given to counteract the metabolic and psychological pathologies, which may help the individual to cope better and recover faster even in the presence of altered set points of hormone production and tissue sensitivity.

Implications and future perspectives

Chapters 2 and **3** described in this thesis clearly demonstrate that studies with the aim to evaluate the effects of an intervention on the HPA axis have to fulfill certain methodological criteria to enable a reliable evaluation. Although such a statement seems obvious, our studies indicate that this was clearly not evident to researchers involved in metabolic studies and illustrates that the metabolic and behavioural effects of the stress response cannot be easily separated. Thus, such an appropriate design should, at least, take the following factors into account: choice of mouse strain, duration and content of the diet, standardization of housing conditions with a possibility to acclimate in advance and methods for stress-free sampling of CORT, and the timing of the sampling. This standardization and correction for parameters, which might otherwise hamper the interpretation of the results, is of importance since measures of the circulating plasma CORT only might not be sufficient enough when evaluating HPA axis activity since secondary effects might mask the "true" effects. This is the case in human obesity where increased cortisol levels are not present, whereas cortisol secretion is increased, primarily because of increased clearance and increased distribution volume of the circulating cortisol resulting in secondary central activation of the HPA axis (10, 11). In the same lines, diurnal CORT levels may reflect counteracting mechanisms directed towards regaining homeostasis both centrally and peripherally (18, 19-23).

As we showed in **chapter 3**, HFD alone is able to modulate basal HPA axis activity and CORT metabolism in the central nervous system and in peripheral tissues. This might be the case also in the presence of a period of increased GCs (**chapter 4**), and could affect metabolic recovery and cardiovascular risk. Thus, it would be of interest to further elucidate the contribution of various diets during regular treatment of patients with CS, both before and after remission, to

metabolic abnormalities and behavior. These observations then could serve as a framework for guidelines for referral of patients who are prescribed steroid for (preventive) dietary advice. The profound inhibitory effect of high CORT on atherosclerotic plaque formation found in **Chapter 5** was remarkable. Increased CORT exposure in mice with human-like lipoprotein metabolism had beneficial, long-lasting effects on atherosclerosis, but negatively affected body fat distribution and insulin sensitivity, by promoting fat accumulation in the long-term. This indicates that the increased atherosclerosis observed in humans in states of GC excess may not be related to cortisol *per se*, but may be the result of complex and perhaps indirect, effects of cortisol on the cardiovascular system. Given the widespread distribution of GCs and their immunosuppressive indications, these complex effects that include the effects on the endothelium and coagulation, apparently outweigh the immunosuppressive effects on plaque formation and merit further research.

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7

NEDERLANDSE SAMENVATTING
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
CURRICULUM VITAE

Nederlandse samenvatting

In de huidige westerse maatschappij die gekenmerkt wordt door weinig lichaamsbeweging en fastfood, komen overgewicht, type 2 diabetes (T2D) en hart-en vaatziekten en de complicaties daarvan steeds vaker voor. Tegelijkertijd is de waargenomen maatschappelijke stress factor door de 24-uurseconomie erg toegenomen. Er wordt gepostuleerd dat chronische stress, of dit nu maatschappelijk, sociaal, of door welke andere oorzaak dan ook, leidt tot dysfunctie van de hypothalamus-hypofyse-bijnier (HPA) as en dat dit betrokken is bij de ontwikkeling van het metabool syndroom. Een duidelijk voorbeeld hiervan zijn patiënten met het syndroom van Cushing die veel kenmerken hebben van het metabool syndroom en ook veel manifestaties hebben van hart-en vaatziekten. Het is echter onduidelijk wat de effecten zijn van (de ontwikkeling van) het metabool syndroom op de activiteit van de HPA as, en anderszins, wat de effecten zijn van overmatige blootstelling aan cortisol op het metabolisme. De geïnduceerde metabole veranderingen zoals deze gezien worden bij patiënten met het syndroom van Cushing zijn overduidelijk, maar zijn heel moeilijk te evalueren bij de mens.

Het eerste deel van het proefschrift beschrijft in specifieke muizenmodellen de effecten van het vetgehalte in het dieet op de basale, dus niet gestimuleerde, activiteit van de HPA as, daar gesuggereerd is in de literatuur dat dit betrokken is in de pathofysiologie van het Metabool syndroom. In **hoofdstuk 2** is een systematische review verricht gebruik makende van de belangrijkste elektronische databases, te weten: PubMed, EMBASE, Web of Science, the Cochrane database, en Science Direct. De primaire onderzoeksvraag was het effect te evalueren van studies die in muizen hebben gekeken naar de effecten van een hoog vetdieet op de activiteit van de hypothalamus-hypofyse-bijnier-as. We includeerden alleen originele muizenstudies die parameters van de hypothalamus-hypofyse-bijnier-as beschreven na hoog vet dieet, en daarbij minstens een basale corticosteron spiegel vermelden met een adequate controle groep. Studies die gebruik maakten van muizen na adrenalectomie, alleen transgene muizen, een hoog vet dieet voor minder dan 2 weken, of behalve een hoog vet dieet ook andere interventies rapporteerden, werden geëxcludeerd. Alle studies werden beoordeeld door twee onderzoekers. We vonden twintig studies die voldeden aan de inclusie criteria, waarbij echter de evaluatie van de hypothalamus-hypofyse-bijnier-as de primaire onderzoeksvraag was in slechts 5 van deze studies. De plasma corticosteron concentraties na dietaire interventie waren onveranderd in 40%, verhoogd in 30%, en verlaagd in 20% van de studies. Ook de effecten in de perifere weefsels en in het centrale zenuwstelsel waren niet consistent en werden slechts onderzocht in een klein aantal studies. Er waren echter tussen de studies grote verschillen in de gebruikte muizenstammen, experimentele condities, en de samenstelling en duur van het dieet. Uit deze studie komt dus duidelijk naar voren dat gegevens over de effecten van hoog vet dieet op de basale activiteit van de hypothalamus-hypofyse-bijnier-as in muizen beperkt zijn en niet-conclusief. Verschillen in experimentele condities maken een betrouwbare vergelijking erg moeilijk en benadrukken de noodzaak van gestandaardiseerde evaluaties om een betrouwbare evaluatie van de effecten van dieet geïnduceerde obesitas op de activiteit van de hypothalamus-hypofyse-bijnier-as mogelijk te maken. Daarom is het absoluut noodzakelijk de studie design hierop af te stemmen en moet tevens ook nog rekening worden gehouden met de volgende factoren: de keuze voor een specifieke muismodel (welke genetische achtergrond), de duur- en de samenstelling van het dieet, en standaardisatie van de huisvesting van de diertjes met de mogelijkheid om voor de start van het experiment te kunnen wennen aan de nieuwe

omgeving. Tot slot moeten ook methoden toegepast worden die het mogelijk maken om 'stress vrij' bloedmonsters af te kunnen nemen (de zgn sampling voor bepaling van corticosteron en/of ACTH) en moet ook rekening worden gehouden met het tijdstip van sampling.

In **hoofdstuk 3** hebben we de in hoofdstuk 2 voorgestelde studie design toegepast met als doel de basale, dus niet gestreste, activiteit van de hypothalamus-hypofyse-bijnier-as te bestuderen in muizen tijdens de ontwikkeling van obesitas. De ontwikkeling van veel voorkomende kenmerken van het metabool syndroom, zoals insuline resistentie en obesitas kunnen gemakkelijk gestimuleerd worden met behulp van hoog-vet dieet in diermodellen van dieet geïnduceerde obesitas (DIO). In deze studie kregen mannelijke C57Bl/6J muizen een hoog- of laagvet dieet voor een periode van 12 weken waarbij de activiteit van HPA-as gemeten werd door middel van bepaling van de plasma corticosteron concentraties op een aantal vaste tijdstippen van de dag (om 07.00, 12.00, en om 18.00 h) elke twee weken. Daarnaast werd in het centrale zenuwstelsel de activiteit van de hypothalamus-hypofyse-bijnier-as aan het einde van het experiment gemeten aan de hand van de mRNA expressie van corticotropine-releasing hormone (CRH) en glucocorticoïd receptor (GR) in de hippocampus, de amygdala, en in de hypothalamus, alsook in de perifere weefsels middels de expressie van 11β-hydroxysteroid dehydrogenase type-1 en -2 (11β-HSD-1 en -2) in vetweefsel en in de lever. Binnen 1 week na start van het HFD was het lichaamsgewicht al significant hoger dan in de controle groep, hetgeen gepaard ging met significant lagere corticosteron concentraties om 12.00 en 18.00 uur. Deze verlaagde waarden persisteerden gedurende de hele periode van 12 weken. Aan het einde van het experiment (na 12 weken HFD) werden ook veranderingen waargenomen in de centrale aansturing van de HPA as: de expressie van CRH mRNA in de PVN en amygdala en GR mRNA in de PVN was om 9.00 uur verlaagd, terwijl deze om 18.00 uur in de PVN en amygdala juist verhoogd was, alsook de GR mRNA expressie in een deel van de hippocampus (de CA1 regio). De 11β-HSD-1 expressie in het vetweefsel was om 9.00 en 18.00 uur verlaagd zowel in het gonadale-, viscerale- en subcutane vet, terwijl de hepatische 11β-HSD-1 expression om 18.00 uur verhoogd was, zonder detecteerbare veranderingen in 11β-HSD-2 expressie. Dus door het toepassen van een gestandardiseerde, zgn "stress free" study design, is het mogelijk om de werkelijke effecten van een simpele interventie zoals een HFD op de basale activiteit van de hypothalamus-hypofyse-bijnier-as te onderzoeken. Deze studie toont aan dat HFD complexe veranderingen induceert in de diurnale regulatie van de verschillende componenten van de hypothalamus-hypofyse-bijnier-as. Deze veranderingen worden niet overwegend gekenmerkt door verhoogde, maar juist door een verlaagde activiteit van de HPA as, die zeer waarschijnlijk adaptieve varanderingen weerspiegelen.

Het tweede gedeelte van het proefschrift richtte zich op de effecten van glucocorticoiden op het metabolisme. Het referentiekader hiervoor zijn waarnemingen bij patienten met het syndroom van Cushing, waar blootstelling aan cortisol excess, is geassocieerd met kenmerken van het metabool syndroom (MetS), zoals insuline resistentie, abnormale vet distributie en dyslipidemia en hart- en vaatziekten. Deze veranderingen blijken gegeven recentelijk onderzoek te persisteren zelfs als patienten al langdurig in remissie zijn na correctie van het cortisol excess. Bovendien is de rol van glucocorticoiden in de ontwikkeling van atherosclerosis nog niet duidelijk. Humane studies laten een duidelijke associatie zien tussen glucocorticoïd excess en hart- en vaatziekten; maar de meeste diermodellen wijzen op een remmend effects van glucocorticoïden op de ontwikkeling van atherosclerose. Echter, deze diermodellen weerspiegelen noch de lange-termijn effecten van langdurige endogene GC overexpositie zoals sprake is bij de mens, noch benaderen ze een humaan lipoproteine metabolisme.

In **hoofdstuk 4** hebben we de effecten onderzocht van een periode van glucocorticoid excess op insuline gevoeligheid en lichaamssamenstelling. Het doel was om factoren te identificeren die het metabole herstel na een periode van glucocorticoid excess moduleren. Voor dit onderzoek gebruikten we mannelijke C57Bl/6J muizen, die dan wel een hoog- (HFD) of laag vetdieet (LFD) gevoerd werden, en daarnaast corticosteron (CORT) (50µg/ml) of vehicle kregen dat toegevoegd werd aan het drinkwater voor 4 weken, gevolgd door een 'washout' periode van 8 weken. Bloedmonsters voor de bepaling van circadiane plasma spiegels van corticosteron, lipiden, insuline, en glucose werden op reguliere tijdsintervallen afgenomen. De insulinegevoeligheid werd bepaald met behulp van een hyperinsulinemische-euglycemische clamp en lean body- en vetmassa werd geanalyseerd na 12 weken. We vonden dat de toevoeging van corticosteron aan het drinkwater resulteerde in verhoogde plasma corticosteron concentraties, alsook in een verhoogde voedselinname en verhoogde plasma insuline en lipiden concentraties. Het stoppen van de toevoeging van corticosteron aan het drinkwater normaliseerde de corticosteron concentraties, de voedselinname en het lichaamsgewicht. Acht weken na staken van de corticosteron toediening waren de insuline concentraties nog steeds significant hoger met beide dieten dan in het controle experiment en was bij corticosteron behandelde muizen op HFD tevens nog steeds sprake van persisterende verminderde lean body mass en een hogere vetmassa. Het lijkt er dus op dat een periode van corticosteron excess langdurige metabole veranderingen induceert, waarbij de veranderingen in lichaamssamenstelling alleen optreden in aanwezigheid van een HFD. Deze observaties wijzen op dieet-afhankelijke effecten van corticosteron die mogelijk bij zouden kunnen dragen aan het persisterende afwijkende cardiovasculaire risicoprofiel zoals gezien wordt bij patienten behandeld voor het syndroom van Cushing, en mogelijk dan ook bij individuen die bloot staan aan chronische stress. In **hoofdstuk 5** zijn de effecten van glucocorticoidexcess, zowel passagère als continue toediening, onderzocht op de ontwikkeling van atherosclerose in een specifiek muismodel met een lipoproteine metabolism die vergelijkbaar is met die bij de mens, namelijk vrouwelijke APOE*3-Leiden.CETP (E3L.CETP) muizen, en die atherosclerose ontwikkelen na blootstelling aan een zgn 'Western-type' diet. Een dergelijk Western-type dieet bevatte voor dit experiment 0.1% cholesterol en duurde 20 weken. Na 3 weken werden de muizen gematched voor leeftijd, plasma cholesterol concentraties, trigylceriden en phospholipiden alsook lichaamsgewicht, en kregen ze corticosteron (50 µg/ml) in het drinkwater voor of 5 weken (transient groep) of 17 weken (continue groep), of ze kregen alleen drinkwater zonder corticosteron (controle groep). Aan het einde van de studie, werd de ernst van atherosclerose en de grootte van de atherosclerotische plaque gemeten in de aortaboog. Tevens werd het aantal monocyten aan het endotheel vastgelegd alsook het aantal macrofagen in de plaque. We vonden dat corticosterone behandeling het lichaamsgewicht en voedselinname verhoogde voor de gehele duur van de behandeling en op de lange-termijn de vetmassa verhoogde in beide behandelgroepen. Zowel in de 'transient' als in de 'continue' groep verlaagde corticosteron behandeling het totale oppervlakte van de atherosclerotische lesie in dezelfde mate blijkbaar onafhankelijk van het cholesterol daar de plasma cholesterol concentraties niet lager werden. Na continue behandeling werd tevens een reductie van het aantal macrofagen in de plaque gevonden en eenzelfde trend was aanwezig na 'transient' behandeling. Hieruit kunnen we concluderen dat sterk verhoogde blootstelling aan corticosteron in muizen met een humaan lipoproteine metabolisme gunstige en langdurige effecten heeft op atherosclerose, maar negatief de vetdistributie en insuline gevoeligheid beinvloedt, door vetstapeling te fasciliteren op de langetermijn. Dit betekent dat de sterke associatie tussen

atherosclerose en glucocorticoid excess zoals geobserveerd wordt bij de mens mogelijk niet gerelateerd zijn aan cortisol *per se*, maar het resultaat kunnen zijn van complex effecten van cortisol op het endotheel en/of op de stollingsactivatie.

Samenvattend dragen deze studies bij aan het begrijpen van de rol van de hypothalamus-hypofyse-bijnier-as en glucocorticoiden in de pathofysiologie van het metabool syndroom, maar illustreren ook dat er effecten zijn van dieet geinduceerde obesitas op de hypothalamus-hypofyse-bijnier-as. De studies in dit proefschrift laten duidelijk zien dat voor een betrouwbare evaluatie van de hypothalamus-hypofyse-bijnier-as, een geschikte studie design een absolute voorwaarde is. Dan wordt zichtbaar dat een hoog vet gehalte in het dieet de activiteit van de hypothalamus-hypofyse-bijnier-as onderdrukt, hetgeen waarschijnlijk een adaptatie is om de dieet-geinduceerde ontwikkeling van insuline resistentie en obesitas tegen te gaan, niet alleen in perifere weefsels maar ook in het central zenuwstelsel. Bovendien tonen we in dit proefschrift aan dat een periodieke blootstelling aan hoge corticosteron spiegels langdurige gunstige effecten heeft op atherosclerose, maar tegelijkertijd negatief de lichaamssamenstelling beinvloedt en, vetstapeling en insuline resistentie bevordert, hetgeen nog meer versterkt worden door een hoog- vet dieet.

De in dit proefschrift gepresenteerde bevindingen zijn voor een breed publiek relevant: eenieder moet leren omgaan met een toenemend aantal hedendaagse dagelijkse stressoren, zoals sociale stress, slaapstoornissen, de consumptie van “comfort food” maar ook “emotional eating” dat de ontwikkeling van obesitas, het metabool syndroom, type 2 diabetes en hart- en vaatziekten fasciliteert. Bovendien geven enkele van deze bevindingen klinische relevante aanknopingspunten voor artsen en patienten als steroiden voorgeschreven dienen te worden voor uiteenlopende indicaties, daar een preventief dietetair advies bij zou kunnen dragen aan verbetering van het metabool profiel en kwaliteit van leven.

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Finally, it is done and the four years of research has come to an end. This point, however, was not reached without hard work. Nor did I make it here alone. Many people have had a special contributing role in the making of this thesis and I wish to show my respect for their support and thank them.

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LIST OF PUBLICATIONS (FULL PAPERS)

Auvinen HE, Wang Y, Princen H, Romijn JA, Havekes LM, Smit JWA, Meijer OC, Biermasz NR, Rensen PCN, Pereira AM. Both transient and continuous corticosterone excess inhibit atherosclerotic plaque formation in APOE*3-Leiden.CETP mice. *Plos One* 2013 second revision

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CURRICULUM VITAE

Hanna Elina Auvinen was born on 24th of July 1980 in Tampere, Finland. Year 1999 she graduated from art gymnasium (Tammerkosken lukio) in Tampere. Year 2004 she started her biomedicine (biomedicin) studies in Karolinska Institut in Stockholm, Sweden. During these studies, she spent spring term 2006 in Leiden University as an exchange student. Summer 2007 she worked in the Department of Immunology and Pharmacology in Tampere University to construct a stabily transfected cell line for mouse iNOS-promotor. She returned again to Leiden to Leiden/Amsterdam Center for Drug Research to conduct her master thesis project titled "Cannabinoid Receptor 1 Expression in the Rat Brain Under Different Conditions of Glucocorticoid Exposure" under the supervision of dr. A.M. Pereira. She graduated from Karolinska Institut with a degree of Master of Medical Science with a Major in Biomedicine (Biomedicine Magister Exam) in the spring 2008. After obtaining her master degree she started her PhD research under the supervision of dr. A.M. Pereira in the Department of Endocrinology and Metabolic Diseases in the Leiden University Medical Center. Her PhD research, results of which are presented in this thesis, was completed in August 2012. Since then she has returned to Finland.