The effects of high fat diet on the basal activity of the hypothalamus–pituitary–adrenal axis in mice

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Abstract

Alterations in hypothalamus–pituitary–adrenal (HPA) axis activity have been linked to the development of the 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. These models, hampered by methodological differences, reveal conflicting results with respect to HPA axis activation. This study was aimed to evaluate in detail nonstressed 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 11 β -hydroxysteroid dehydrogenase type-1 and -2 (11 β -HSD-1 and -2) expression in adipose

tissue and liver. Within 1 week, the HFD induced obesity and decreased corticosterone levels at 1200 and 1800 h, which persisted throughout the experiment. Twelve weeks of HFD decreased CRH mRNA in the paraventricular nucleus (PVN) and amygdala and GR mRNA in the PVN at 0900 h. At 1800 h, CRH mRNA expression increased in the PVN and amygdala, and GR mRNA increased in the CA1 region. 11 β -HSD-1 expressions decreased in gonadal, visceral, and subcutaneous adipose tissues at 0900 and 1800 h, whereas hepatic 11 β -HSD-1 expression increased at 1800 h, whereas 11 β -HSD-2 expression was unaffected. The 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.

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Introduction

Glucocorticoids (GCs; 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; Pasquali *et al.* 2006). The metabolic effects of GC are directed both toward the recruitment of energy stores for gluconeogenesis (peripheral effects) and toward the 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 (Warne *et al.* 2009, Dallman 2010). In accordance, patients with Cushing's syndrome, which is caused by prolonged excessive exposure to GC, exhibit many features of the MetS (Newell-Price *et al.* 2006) associated with

increased cardiovascular morbidity and mortality (Dekkers *et al.* 2007). Finally, manipulation of cortisol exposure at the tissue level in mice, through stimulation or abrogation of 11 β -hydroxysteroid dehydrogenase type-1 and -2 (11 β -HSD-1 and -2) activity can increase or regress visceral fat accumulation, as well as other features of the MetS (Masuzaki *et al.* 2001, Cooper & Stewart 2009).

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 CNS in only a few of these studies (Auvinen *et al.* 2011).

Therefore, the aim of the present study was to evaluate the effects of HFD in mice in detail on basal, nonstressed 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 the HFD (Surwit *et al.* 1988, West *et al.* 1992, Parekh *et al.* 1998, Kleinridders *et al.* 2009).

Materials 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 (Surwit *et al.* 1988, West *et al.* 1992, Parekh *et al.* 1998, Kleinridders *et al.* 2009). 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 light:12 h darkness cycle (lights on 0700 h) 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: HFD (45 energy % lard fat, D12451; Research Diet Services, Inc., New Brunswick, NJ, USA) ($n=18$) or low fat diet (LFD) (10 energy % lard fat, D12451B; Research Diet Services, Inc.) ($n=18$). All mice were fed the LFD for 3 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 the LFD ($n=18$) and HFD ($n=18$) were divided into two groups of nine mice, and blood for the measurement of plasma corticosterone levels was sampled at weeks 1, 5, and 9 (first group) or at weeks 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, 2000, and 2100 h during the dark phase in red light conditions. All corticosterone samples were obtained within 90 s from disturbing the cage, via tail incision, allowing the mouse to move freely on top of the home cage (Dalm *et al.* 2005). Plasma insulin was measured after a 4-h 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 s 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 and 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 RIA (MP Biomedicals LLC, Orangeburg, NY, USA; intra-assay variation 7.3% and inter-assay variation 6.9%).

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

Evaluation of HPA axis activity in the CNS (in situ hybridization)

Brain sections of 16 μm of the paraventricular nucleus (PVN; Bregma -0.70 mm), amygdala (Bregma -0.70 mm), and hippocampus (Bregma -1.70 mm) were cut according to the brain atlas of Paxinos & Franklin (2001) on a cryostat and mounted on polysine microscope slides (Menzel-Gläzer, Braunschweig, Germany) and stored at -80 °C until further use. The hybridization was performed as described previously (Meijer *et al.* 1997) 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 glucocorticoid receptor (GR; Veenema *et al.* 2003). The cRNA from corticotropin-releasing hormone (CRH) was transcribed from a 1 kb cDNA insert in pGEM 4 containing the full-length coding region of rat CRH (Lachize *et al.* 2009). ^{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 , $2\times$ SSC, 0.1 mg/ml yeast tRNA, 0.1 mg/ml sssDNA, 10 mM dithiothreitol, and 0.05 M PBS. All radiolabeled probes were diluted to 16.7×10^6 c.p.m./ml. Of these mixtures, 120 μl were applied to each slide and then covered with a coverslip. The sections were hybridized overnight in a moisturized chamber at 55 °C. The next day, the coverslips were removed carefully and sections were washed in $2\times$ SSC 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 $2\times$ SSC for 10 min, $1\times$ SSC for 10 min, $0.1\times$ SSC for 2×30 min, and, finally, at room temperature in $0.1\times$ SSC 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 microscscales; Amersham). Two sections per mouse per brain area were quantified. For CRH mRNA in the 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.

11 β -HSD-1 and -2 expression analyses in liver and adipose tissues

Total RNA was extracted from liver and adipose tissues using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA quality was examined with lab-on-a-chip technology using Experion StdSens analysis kit (Bio-Rad). 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 11 β -HSD-1 (forward primer: CAGCAAAGGGATTGGA-AGAG; reverse primer: CTTTCCCGCCTTGACAATAA) and 11 β -HSD-2 (forward primer: TTTGGTGCACCTTGAGCTGAC; reverse primer: AGCCGAATGTGTCCA-TAAGC) were carried out on the IQ5 PCR machine (Bio-Rad) using the Sensimix SYBR Green RT-PCR mix (Quantace Ltd, London, UK). mRNA levels were normalized to mRNA levels of cyclophilin (forward primer: CAAATGCTGGACCAACACAA; reverse primer: GCCATCCAGCCATTTCAGTCT) and hypoxanthine guanine phosphoribosyl transferase (forward primer: TTGCTCGAGATGTCATGAAGGA; reverse primer: AGCAGGTCAGCAAAGAACTTATAG).

Statistical analysis

Data are presented as means $\pm$ s.d. Statistical differences were calculated using the Mann-Whitney *U* test for nonparametric data, with GraphPad Prism, version 5.01 (GraphPad Software Inc., La Jolla, USA). $P < 0.05$ was considered as statistically significant.

Results

HFD 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 than the LFD, which already reached significance within 1 week, and this difference remained significant throughout the experiment (Fig. 1A). The HFD did not affect the thymus weight (Fig. 1B). The HFD increased plasma insulin concentrations already within 1 week (Fig. 1C), which

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

remained significantly increased throughout the experiment. Plasma leptin levels were significantly increased after 12 weeks of the HFD both at 0900 and 1800 h (Fig. 1D).

HFD 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). The HFD decreased plasma corticosterone levels within 1 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 week 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 the HFD group during the beginning of the dark phase (at 1900, 2000 h) at week 11 when compared with the LFD group (Fig. 2C).

HFD induces changes in mRNA expression of CRH and GR in the brain

The HFD significantly decreased CRH mRNA expression in the PVN and amygdala at 0900 h (Fig. 3A and B). Moreover, the HFD decreased GR mRNA in the PVN at 0900 h in the morning basal period (Fig. 3C). The HFD increased CRH mRNA expression at 1800 h in both PVN and amygdala (Fig. 3A and B). Furthermore, the 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 F).

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

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

The HFD decreased 11 β -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 11 β -HSD-2 expression in the same tissues at both time-points (Fig. 4B, C and D). By contrast, in the liver, the HFD increased 11 β -HSD-1 expression at 1800 h by +23% whereas 11 β -HSD-2 expression was not affected (Fig. 4A).

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 downregulation of the activity of the HPA axis, as reflected in decreased diurnal corticosterone concentrations, decreased 11 β -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 the 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, the HFD significantly increased both body weight and plasma insulin concentrations already within 1 week and reduced diurnal corticosterone levels already within 1 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 to 2000 h.

Several mechanisms may explain the early decrease in diurnal corticosterone peak levels upon the 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 (Roelfsema *et al.* 2009, 2010). Second, this decrease in diurnal corticosterone peak levels may reflect counteracting mechanisms directed toward prevention of further progression in insulin resistance, both centrally and in peripheral tissues. Third, circulating leptin concentrations increase proportionally to the fat mass gained (Van Heek *et al.* 1997) and leptin and insulin resistance have been documented to develop already within 3 days of high fat feeding (Wang *et al.* 2001).

In accordance, the HFD resulted in increased 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 the HFD and subsequent dampening of the HPA axis. In accordance, reduction of GC levels in a specific mouse model of insulin resistance (*db/db*) reverses the cognitive impairment related to hippocampal neurons induced by insulin resistance (Stranahan *et al.* 2008). These findings imply that dampening of the HPA axis in the presence of insulin resistance, induced by the 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 (Williams *et al.* 2010) that produce ACTH. However, glucose sensing by POMC neurons is impaired in obese mice (Parton *et al.* 2007), 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 (Roelfsema *et al.* 2009).

Intriguingly, there is a disparity between the effects of the HFD on the activity of the HPA axis in the morning as compared with 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

Figure 3 Effect of the HFD on mRNA expression of CRH and GR in the hypothalamus and hippocampus. Male C57Bl/6J mice were fed an LFD (open bars) or HFD (closed bars) for 12 weeks, and mRNA expression was determined: CRH in the (A) PVN and (B) amygdala, and GR in the (C) PVN, (D) CA1, (E) CA3, and (F) DG regions of the hippocampus in the morning (0900 h) and evening (1800 h) (black bars). Mann–Whitney *U* test, * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$.

central inhibition of CRH and GR expression found in the morning in the presence of unaltered 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 the 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 (Scherer & Buettner 2009, Bermudez-Silva *et al.* 2012), which could lead to the suppression of CRH. However, leptin resistance induced by high fat feeding is selective and does not impact on autonomic nervous system activity (Enriori *et al.* 2011). 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 (Kruse *et al.* 1998, Hsu *et al.* 2006). However, the present data do not permit firm conclusions in this respect. In addition, a true effect of the diet *per se* cannot 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.

Downregulation of CRH mRNA in the morning was accompanied by the downregulation of CRH mRNA and in the amygdala. Activation of the amygdala promotes HPA axis activation and previous studies in rats have shown an increase in circulating GC (Schulkin *et al.* 1994), whereas adrenalectomy decreases (Santibañez *et al.* 2005) CRH in the amygdala. Furthermore, increased CRH in the amygdala mediates anxiety-like behavior during stress (Makino *et al.* 1994) and the HFD decreases anxiety-like behaviors facilitating stress recovery (Buwaldta *et al.* 2001, Pecoraro *et al.* 2004). Thus, HFD-induced reduction of CRH expression in the amygdala enables protection from further exposure to systemic GC.

In accordance, the effects of the HFD on the peripheral activity of the HPA axis were characterized by reduced mRNA expression of 11 β -HSD-1 in adipose tissues. It has been proposed that this may reflect a mechanism to counteract tissue-specific insulin resistance (Man *et al.* 2011). These differential, fat depot-specific effects are in agreement with a recently proposed, dynamic, and depot-selective relationship between adipose tissue 11 β -HSD-1 activity and fat mass (Man *et al.* 2011). The HFD increases lipoprotein lipase activity (Petit *et al.* 2007), which would direct circulating triglycerides toward peripheral adipose tissues for storage. Corticosterone impairs glucose tolerance (Karatsoreos *et al.* 2010) and is a lipolytic hormone (Campbell *et al.* 2011). Therefore downregulation of 11 β -HSD-1

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

expression would protect the adipose tissue from further insulin resistance. These observations are further strengthened by the fact that 11 β -HSD-2 expression in the same tissues was not affected, resulting in overall reduction of GC exposure of the tissues.

In conclusion, we found that the HFD has profound effects on the basal, thus nonstressed, activity of the HPA axis, reflected in reduced diurnal corticosterone concentrations, distinct expression of CRH in the CNS across the diurnal rhythm, and reduced 11 β -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 11 β -HSD-1 enzyme mRNA respectively), are in agreement with the well-known metabolic effects of GC that are directed toward recruitment and augmentation of energy stores. In the presence of HFD-induced obesity or palatable foods, the HPA axis adapts.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H E A researched data, contributed to the discussion, and wrote the manuscript. J A R, N R B, H P, L M H, J W A S, and P C N R contributed to the discussion and reviewed the edited manuscript. A M P contributed to the discussion and wrote the manuscript.

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