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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

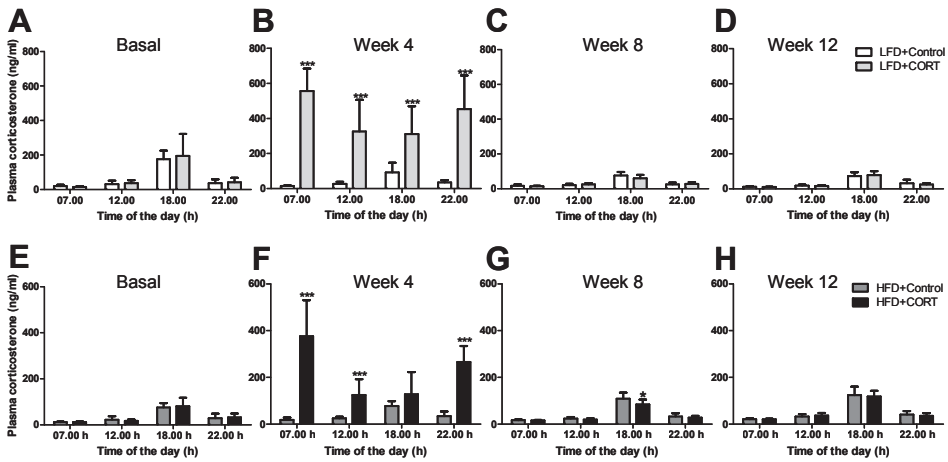

Figure 1. Effect of CORT treatment on circadian plasma CORT of mice fed a low fat diet (A, B, C and D) (control group: open bars, CORT group: light grey bars) or high fat diet (E, F, G and H) (control group: dark grey bars, CORT group: black bars) at baseline, week 4, week 8 and week 12, Mann-Whitney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

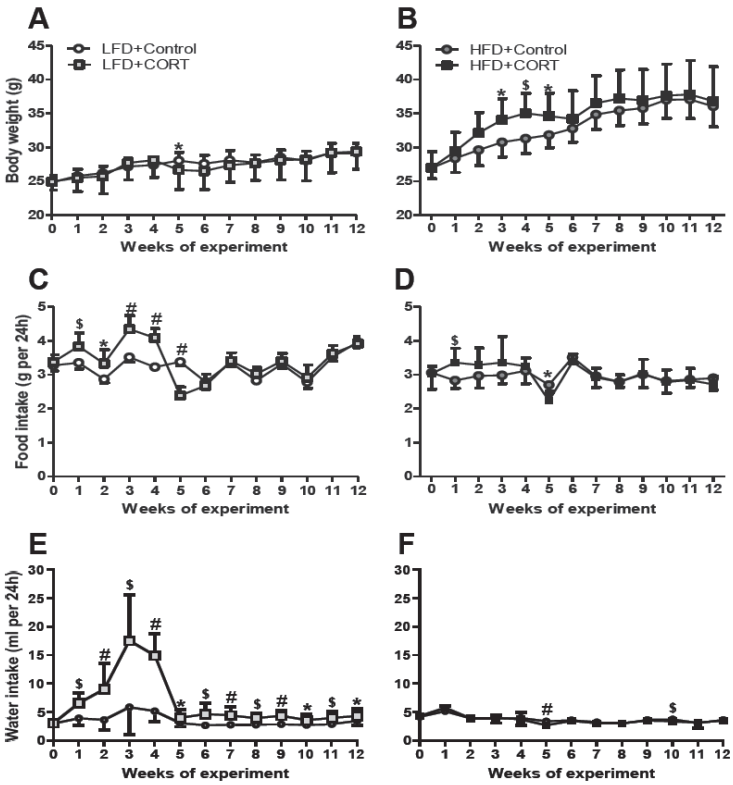

Figure 2. Effect of CORT treatment on body weight, food intake and water intake of mice fed a low fat diet (control group: white circles, CORT group: light grey squares) (A, C and E) or high fat diet (control group: dark grey circles, CORT group: black squares) (B, D and F), Mann-Whitney test, *P<0.05,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

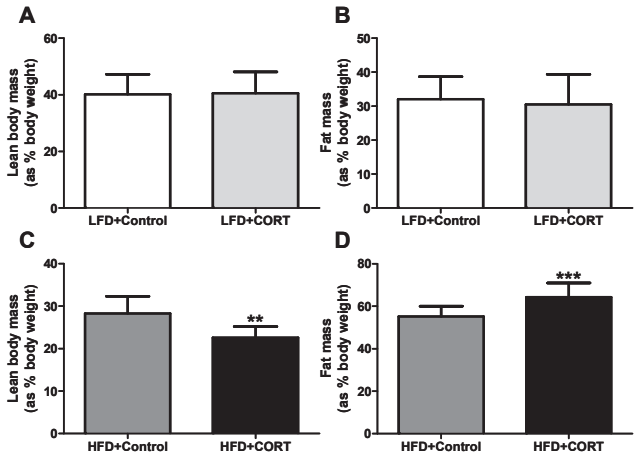

Figure 3. Effect of CORT treatment on body composition of mice fed a low fat diet (A and B) (control group: open bars, CORT group: light grey bars) or high fat diet (C and D) (control group: dark grey bars, CORT group: black bars), Mann-Whitney test, **P<0.01, ***P<0.001.

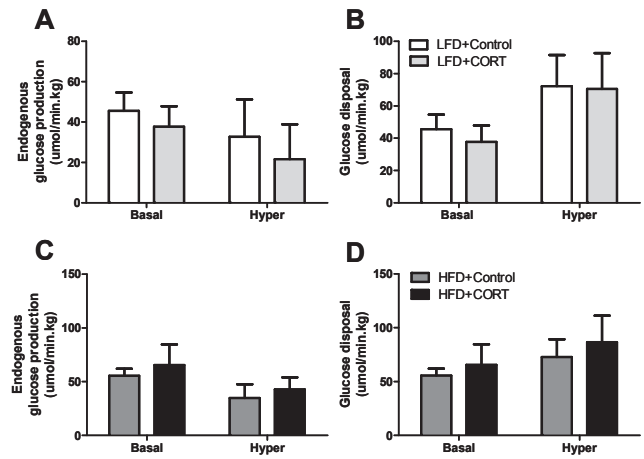

Figure 4. Effect of CORT treatment on endogenous glucose production and glucose disposal during basal period of glucose infusion (basal) and hyperinsulinemia (hyper) in mice fed a low fat diet (A and B) (control group: open bars, CORT group: light grey bars) or high fat diet (C and D) (control group: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