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Glucocorticoids and adipose tissue function

Acute hypercortisolemia exerts depot-specific effects on abdominal and femoral adipose tissue function

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Context: Glucocorticoids have pleiotropic metabolic functions and acute glucocorticoid excess affects fatty acid metabolism, increasing systemic lipolysis. Whether glucocorticoids exert adipose tissue depot-specific effects remains unclear.
Objective: In vivo assessment of femoral and abdominal adipose tissue responses to acute glucocorticoid administration.
Design and outcome measures: Nine healthy male volunteers studied on two occasions, following a hydrocortisone infusion (0.2 $mg.kg^{-1}.min^{-1}$ for 14 hours) and saline, respectively, given in randomized double-blind order. Subjects were studied in the fasting state and following a 75g glucose drink with *in vivo* assessment of femoral adipose tissue blood flow (ATBF) using radioactive Xenon washout, and lipolysis and glucose uptake using the arterio-venous difference technique. In a separate study (same infusion design), 8 further healthy male subjects underwent assessment of fasting abdominal ATBF and lipolysis only. Lipolysis was assessed as the net release of non-esterified fatty acids (NEFA) from femoral and abdominal subcutaneous adipose tissue.
Results: Acute hypercortisolemia significantly increased basal and postprandial ATBF in femoral adipose tissue, but femoral net NEFA release did not change. In abdominal adipose tissue, hypercortisolemia induced significant increases in basal ATBF and NEFA release.
Conclusions: Acute hypercortisolemia induces differential lipolysis and ATBF responses in abdominal and femoral adipose tissue, suggesting depot-specific glucocorticoid effects. Abdominal, but not femoral, adipose tissue contributes to the hypercortisolemia-induced systemic NEFA increase, with likely contributions from other adipose tissue sources and intravascular triglyceride hydrolysis.

Acute experimental hypercortisolemia induces differential metabolic responses in abdominal and femoral adipose tissue in vivo, suggesting depot-specific glucocorticoid effects in humans.

Introduction

Glucocorticoids (GC) are important pleiotropic hormones that exert anabolic effects during physiological conditions, but also play a pivotal role in tissue catabolism when acutely elevated as part of an acute stress response (1). In contrast, chronic pathophysiological GC excess in

Cushing's syndrome is characterised by a distinctive centripetal fat mass redistribution and in particular visceral fat mass accumulation and is associated with increased morbidity and mortality (2,3). Body fat mass distribution is an important determinant of health and abdominal fat mass accumulation represents a major risk factor for cardiovascular disease (4). In contrast, a larger thigh subcutaneous fat mass is independently associated with favorable cardiovascular and metabolic profiles (5), and relative scarcity of femoral fat may have important implications for cardiometabolic health (6,7). Femoral adipose tissue mass is determined by the balance between fatty acid uptake and their release, lipolysis, whereby the latter is regulated *in vivo* in a depot-specific manner via selective adrenoceptor action (8).

Acute hypercortisolemia results in an increase of whole body lipolysis, i.e. the release of non-esterified fatty acids (NEFA) from adipose tissue, but the exact effects of GC on depot-specific lipolysis remain unclear (9). Murine *in vitro* models have yielded conflicting results, with only some reporting induction of lipolysis by GC in a dose-dependent manner (reviewed in (1)). In intact human adipocytes isolated from the abdominal subcutaneous depot, cortisol treatment resulted in inhibition of basal and β-adrenergic-mediated lipolysis (10). In contrast, lipolysis appeared unaffected by the synthetic glucocorticoid dexamethasone (11,12). Early *in vivo* studies also showed that GC inhibit sympathetic activity and adrenoceptor function in humans (13,14), suggesting this could affect lipolytic responses. However, femoral lipolysis, determined as glycerol release using the microdialysis technique, has been found to either increase (15) or remain unchanged (16) in experimental GC excess.

The aim of our study was to study lipolysis in response to acute GC excess, using an integrative *in vivo* physiology approach. In particular, we sought to investigate the depot-specific contribution of femoral adipose tissue to whole body lipolysis during hypercortisolemia. Based on previous studies (17,18), we hypothesized that acute elevation of cortisol concentrations would affect femoral adipose tissue blood flow (ATBF) and NEFA release in line with an acute catabolic response. Furthermore, we studied femoral adipose tissue postprandial glucose uptake, as well as, in a separate study, abdominal adipose tissue fasting ATBF and NEFA release as markers of depot-specific adipose tissue function *in vivo*.

Methods

Subjects

Healthy male individuals with no medical condition and not on any drug therapy were recruited using print and electronic advertising. All subjects underwent a medical evaluation during the screening visit to ensure they were healthy, and had normal liver and kidney function parameters as well as normal blood counts. No subject had any significant past medical history, smoked tobacco or took any regular medications that could affect the study's outcome measures. All parts of this study were conducted at the National Institute for Health Research/Wellcome Trust Clinical Research Facility (CRF) of the University of Birmingham/Queen Elizabeth Hospital Birmingham. The study was approved by Solihull National Health System Research Ethics Committee (12/WM/0327), and all subjects gave informed consent in writing prior to participation.

Study design

Anthropometric measurements.

Measurements were taken during the screening visit. Waist circumference was measured midway between the lower margin of the last palpable rib and the top of the iliac crest, and hip

circumference at the level of the greater trochanters. Total and regional fat masses (including estimated visceral fat mass) were measured by dual-energy x-ray absorptiometry (DXA) (19). Blood pressure and heart rate were measured using a standard oscillometric blood pressure monitor with an upper arm cuff.

Study visits.

For each of the two study visits, subjects were admitted to the CRF at 17:00h, and a cannula for infusion purposes was inserted into an antecubital fossa vein (**Figure 1A**). At 18:00h they were served a standardized calorie-controlled meal (vegetable lasagne; energy 2634 kJ, total nutritional content: 14.4 g fat, 93 g carbohydrates, 25.2 g protein, 11.4 g fibre), and then fasted until study completion the next day. At 19:00h a constant infusion of either hydrocortisone (0.2 $mg.kg^{-1}.h^{-1}$, total dose infused equivalent to doses commonly used in the setting of adrenal insufficiency and acute illness) or saline (control study visit) was started and continued over 14 hours until study completion the next day. The two study days were separated by at least two weeks, and hydrocortisone and saline infusions were administered in a double-blind, randomized fashion. At 22:00h lights were switched off for night rest. At 08:00h the next morning catheters were placed into veins draining femoral (study 1) or abdominal (study 2) subcutaneous adipose tissue (see below) (20,21). A further cannula was inserted either retrogradely into a vein of the dorsal hand, which was then placed into a hot box at 60°C for obtaining arterialized blood samples (study 1), or in a radial artery (study 2). For the purposes of ATBF measurement, ^{133}Xe was injected into femoral (study 1) or abdominal (study 2) subcutaneous adipose tissue (22), and scintillation was measured continuously with customized Cesium detectors (GMS411 system, John Caunt Scientific, Bury, UK). After this, study participants were left to relax for 45 min in order to allow equilibration of ^{133}Xe. Before the start of blood sampling, blood pressure and heart rate were measured. After study completion, all catheters were removed and the subjects were given a full meal.

Region-specific blood sampling.

In nine participants (study 1), a venous catheter was placed into the femoral saphenous vein and, following blood sampling at regular intervals for two hours (fasting phase), a standardized 75g glucose drink was given at 120 min. Blood sampling continued for another 2 hours. In a separate study (study 2) with the same infusion design and duration as outlined above, we studied abdominal ATBF and NEFA release in eight participants by taking samples from a superficial epigastric vein, whereby sampling was performed under fasting conditions only. In both studies, all venous and arterial/arterialized samples were taken simultaneously at specified times (arterio-venous difference technique) (20,23).

Analytical methods

Blood samples were drawn into heparinized syringes, and plasma was prepared rapidly at 4°C and immediately frozen at -80°C before analysis. Plasma glucose, NEFA and glycerol concentrations were measured enzymatically using commercially available kits on an ILAB600 or ILAB650 clinical analyser (Instrumentation Laboratory UK, Warrington, UK); cortisol was measured by a colorimetric assay (R&D Systems, Abingdon, UK); insulin and C-peptide were measured by ELISA (Invitron, Monmouth, UK) in an accredited reference laboratory (Diabetes Research Unit, Swansea University). IL-6 was measured by ELISA (Thermo Fisher Scientific, Loughborough, UK).

Calculations and statistics

Indices of pancreatic β-cell function and insulin resistance were calculated according to the updated homeostatic model assessment (HOMA) method using the computer model (24), whereby the calculations are given in the **Supplemental Methods** section. The mean of three consecutive plasma glucose and insulin fasting measurements (time points 0-30 min) was used for HOMA calculations. Metabolite uptake and release across abdominal and femoral adipose tissue were calculated with the arterio-venous difference technique (8). For NEFA and glycerol release, the veno-arterial concentration difference was multiplied by ATBF for each of the abdominal and femoral adipose tissue depots respectively. For glucose uptake, the arterio-venous concentration difference was multiplied by ATBF. A detailed description of all calculations is given in the **Supplemental Methods** section. For study 1, comparisons between control and hypercortisolemia were performed for each time point (pairwise comparisons) or by comparing areas under the curve (AUC). AUC was also used for comparisons between fasting and postprandial states. Area under the curve was calculated using the trapezoid rule and is presented as a time-averaged value (AUC divided by the relevant time period). For study 2, the mean of three fasting measurements (time points 0-30 min) was used for all calculations and comparisons. To calculate ratios and re-esterification rates in both studies 1 and 2, the mean of three measurements (time points 0-30 min) was used to calculate fasting data, while for femoral adipose tissue the mean of time points 200-240 min was used for postprandial calculations. Data distribution was tested for normality with the Shapiro-Wilk test, and parametric or non-parametric tests were used as indicated. Data were analyzed using IBM Statistics for Windows v21 and GraphPad Prism for Windows v6.05. A $p<0.05$ was considered significant. All data are presented as mean ± SEM, unless otherwise stated.

Results

Baseline anthropometric and metabolic characteristics of subjects are shown in **Table 1**. Hydrocortisone infusion did result in a mild increase in heart rate (p=0.033 for study 1, p=0.022 for study 2, compared to control, paired T-test), but did not affect blood pressure.

Hydrocortisone infusion leads to increased systemic NEFA and peripheral insulin resistance

In study 1 (femoral adipose tissue depot), hydrocortisone infusion resulted in a significant increase in cortisol concentrations (AUC 0-240 min saline *vs* hydrocortisone, p=0.008, Wilcoxon) (**Figure 1B**). Hypercortisolemia had significant effects on plasma NEFA and glucose concentrations. Compared to control, plasma NEFA increased 1.6-fold in the fasting state (AUC 0-120 min saline *vs* hydrocortisone, p=0.008, Wilcoxon). Following glucose ingestion, plasma NEFA concentrations became suppressed under control conditions (AUC 0-120 min *vs* AUC 120-240 min, p=0.008, Wilcoxon). In hypercortisolemia, plasma NEFA concentrations also became suppressed following glucose ingestion (AUC 0-120 min *vs* AUC 120-240 min, p=0.008, Wilcoxon). Compared to control, postprandial plasma NEFA concentrations remained higher during hydrocortisone infusion (AUC 120-240 min saline *vs* hydrocortisone, p=0.008, Wilcoxon) (**Figure 1C**). In response to hydrocortisone infusion, plasma glucose increased 1.3-fold both in the fasting (AUC 0-120 min saline *vs* hydrocortisone, p=0.008, Wilcoxon) and the postprandial state (AUC 120-240 min saline *vs* hydrocortisone, p=0.008, Wilcoxon) compared to control study visits (**Figure 1D**).

Overall plasma insulin concentrations increased during hydrocortisone infusion (AUC 0-240 min saline *vs* hydrocortisone, p=0.018, Wilcoxon) (**Figure 2A**). This was more pronounced for fasting, i.e. basal, insulin secretion (individual time points 0-120 min saline *vs* hydrocortisone,

p<0.001 for infusion type, ANOVA), whereas glucose-stimulated secretion, both in terms of response time and magnitude, appeared less affected by hydrocortisone (individual time points 120-240 min saline *vs* hydrocortisone, p=0.051 for infusion type, p=0.077 infusion type x time, ANOVA). Plasma C-peptide secretion responses to hydrocortisone infusion mirrored that of insulin, with a significant fasting increase compared to control (individual time points 0-120 min saline *vs* hydrocortisone, p<0.001 for infusion type, ANOVA). Similarly to insulin, this increase was not seen in the postprandial phase (individual time points 120-240 min saline *vs* hydrocortisone, p=0.441 for infusion type, p=0.093 infusion type x time, ANOVA) (**Figure 2B**). Accordingly, pancreatic β-cell function (HOMA %B) did not change significantly during hydrocortisone infusion compared to control (saline *vs* hydrocortisone, p=0.374, Wilcoxon), but insulin sensitivity (HOMA %S) did decrease dramatically (saline *vs* hydrocortisone, p=0.008, Wilcoxon) (**Figure 2C**). In line with this, insulin resistance (HOMA IR) increased during hydrocortisone infusion (saline *vs* hydrocortisone, p=0.012, Wilcoxon) (**Figure 2D**).

Fasting and postprandial femoral ATBF increases during hypercortisolemia

During control conditions, femoral ATBF was stable in the fasting phase and following glucose ingestion (individual time points 0-240 min saline, p=0.155 prandial state x time, ANOVA) (**Figure 3A**). Hydrocortisone infusion induced a steady increase in femoral ATBF during the fasting phase (individual time points 0-120 min saline *vs* hydrocortisone, p=0.009 infusion type x time, ANOVA). Following glucose ingestion there was a further marked increase in femoral ATBF compared to control (AUC 120-240 min saline *vs* hydrocortisone, p=0.011, Wilcoxon). In addition, hydrocortisone induced a larger ATBF-change in the fasting to postprandial state transition (Δ AUC fasting/postprandial saline *vs* hydrocortisone, p=0.024, Wilcoxon) (**Figure 3B**).

Femoral lipolysis and glucose uptake remain unaffected by hypercortisolemia

Fasting femoral NEFA release rate was 671±232 nmol.min^{-1}.100g tissue^{-1} (AUC 0-120 min) under control conditions (**Figure 3C**). Expectedly, following glucose ingestion lipolysis was inhibited (AUC 0-120 min *vs* 120-240 min, p=0.021, Wilcoxon). During hydrocortisone infusion, fasting and postprandial NEFA release rate were similar to control conditions (individual time points 0-120 min, p=0.285, and individual time points 120-240 min, p=0.447, infusion type x time respectively, ANOVA saline *vs* hydrocortisone) (**Figure 3C**). There was no difference in the degree of lipolysis suppression by glucose (Δ AUC fasting/postprandial for saline *vs* hydrocortisone, p=0.824, Wilcoxon) (**Figure 3D**). Regional glycerol release, a further marker of lipolysis, essentially mirrored NEFA release (**Figure 3E**).

During control conditions, the fasting femoral adipose tissue glucose uptake rate was 0.2±0.1 µmol.min^{-1}.100g tissue^{-1} (AUC 0-120 min) (**Figure 3F**). Following glucose, uptake peaked to 2.3±0.7 µmol.min^{-1}.100g tissue^{-1} at 160 min, but the overall postprandial AUC remained unchanged to fasting (AUC 0-120 min *vs* AUC 120-240 min, p=0.102, Wilcoxon). The fasting and postprandial glucose uptake responses remained virtually the same during both control and hydrocortisone infusion states (AUC 0-240 min saline *vs* hydrocortisone, p=0.926 depot x prandial state x infusion type, ANOVA).

Acute hypercortisolemia increases fasting abdominal ATBF and lipolytic rate

In study 2 (abdominal adipose tissue depot), we compared fasting abdominal ATBF and lipolysis in the presence of hypercortisolemia to control conditions using the same infusion regime, which again resulted in significant increases in plasma cortisol (**Figure 4A**) and NEFA concentrations (**Figure 4B**). Given that the study was carried out in the fasting state only, we did not measure

abdominal adipose tissue glucose uptake. Hypercortisolemia increased fasting ATBF (mean 0-30 min saline *vs* hypercortisolemia, p=0.039, paired T-test) (**Figure 4C**). Abdominal fasting net NEFA release also increased compared to control (mean 0-30 min saline 1446±334 nmol.min^{-1}.100g tissue^{-1} *vs* hypercortisolemia 2293±541 nmol.min^{-1}.100g tissue^{-1}, p=0.031, paired T-test) (**Figure 4D**), as did abdominal fasting net glycerol release (mean 0-30 min saline *vs* hypercortisolemia, p=0.021, paired T-test) (**Figure 4E**).

NEFA handling in femoral and abdominal adipose tissue

Because of the increased systemic NEFA concentrations and the marked increases in ATBF observed during hypercortisolemia, we sought to calculate markers of NEFA handling in each depot that are independent of ATBF. Thus, as a marker of local lipolysis at the adipocyte level, we calculated the fasting veno-arterial NEFA ratio. In femoral adipose tissue, the ratio decreased from 1.8±0.2 under control conditions to 1.3±0.1 in hypercortisolemia (mean 0-30 min saline *vs* hydrocortisone, p=0.035, Wilcoxon). Similarly, in abdominal adipose tissue it decreased from 2.3±0.2 to 1.7±0.1 (mean 0-30 min, p=0.035, Wilcoxon).

Systemic and regional adipose tissue IL-6 production

Cortisol exerts a wide range of non-metabolic effects and we measured IL-6 production as an inflammatory marker in response to the hydrocortisone infusion (**Suppl. Figure 1**). Systemic fasting plasma IL-6 concentrations did not change significantly between control and hypercortisolemia conditions (time point 0 min, 11.7±3.6 pg/mL vs. 12.7±6.9 pg/mL, p=0.798). Abdominal adipose tissue appeared to be a net producer of IL-6 (release control 12.6±5.6 pg.mL^{-1}.100g tissue^{-1}; hypercortisolemia 19.7±9.7 pg.mL^{-1}.100g tissue^{-1}); however this did not reach statistical significance (control p=0.109; hypercortisolemia p=0.087, one-sample T-test compared to zero). Femoral adipose tissue IL-6 release was statistically not different from zero (femoral IL-6 release control control 1.14±0.56 pg.mL^{-1}.100g tissue^{-1}; hypercortisolemia -0.28±2.86 pg.mL^{-1}.100g tissue^{-1}; p=0.077 and p=0.925, respectively, one-sample T-test compared to zero).

Discussion

Glucocorticoids have important metabolic functions that are tissue-specific and vary depending on the length of exposure (1). In relation to their effect on *in vivo* fatty acid metabolism, there is general agreement that hypercortisolemia increases plasma NEFA concentrations (15,17,25,26). However, the exact source of NEFA is still debated, with some studies reporting an increase of abdominal lipolysis (15,27), while others found a reduction (17). Given the previously reported differences in the depot-specific regulation of lipolysis (8), we studied the effects of acute hypercortisolemia on the femoral and abdominal adipose tissue depot using an integrative *in vivo* physiology approach. We found that during hypercortisolemia abdominal net NEFA output increased, while femoral NEFA release remained stable despite a striking increase in femoral ATBF. We describe potential effects of the increased ATBF on local NEFA handling during hypercortisolemia, and suggest that other depots, e.g. visceral adipose tissue, may play a role in the systemic NEFA concentrations increase, in addition to the contribution of abdominal NEFA release.

However, it remains unresolved which adipose tissue depots contribute to the systemic increases in plasma NEFA during hypercortisolemia, and whether differential contribution of specific adipose tissue depots gives rise to phenotypic fat mass distribution changes observed in chronic GC excess. Similar to previous studies (15,17,25,26), we found a marked increase of

systemic NEFA concentrations in the fasting state during GC infusion. Using isotope tracers, it has been shown that palmitate and glycerol rates of appearance increase with hydrocortisone, suggesting induction of lipolysis at the systemic level (15,17). Subcutaneous adipose tissue is the major source of NEFA, driven by the activity of adipocyte-specific lipases, most notably adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (28). The NEFA rate of appearance in the systemic circulation is determined by ATBF and the net lipolytic rate of the individual adipose tissue depots (20,29). We studied these parameters in order to measure the individual contribution of the femoral and abdominal depot to the increased systemic NEFA concentrations in hypercortisolemia, hypothesizing that femoral adipose tissue is a net contributor.

We found a steady rise of femoral ATBF during fasting conditions during GC infusion, with a further major increase in the postprandial phase. This is in stark contrast to the previously described picture of a mostly unresponsive femoral ATBF (8). Contrary to our hypothesis, we found no differences in femoral lipolytic rate during hydrocortisone infusion. In fact, our data suggest a strong inhibition of lipolysis at the actual adipocyte level, since the net femoral lipolytic rate did not change after GC infusion, despite the marked local ATBF increase. We found a moderate increase of basal abdominal ATBF and a small net increase in the fasting abdominal NEFA release during hypercortisolemia.

We did not measure triglyceride concentrations since we were not expecting any changes due to the nature of the experimental meal stimulus (pure glucose) and therefore could not calculate the exact transcapillary flux of fatty acids (30). Hence, the exact effects of GC excess on postprandial fatty acid trafficking, would need to be studied in future experiments ideally involving a lipid-containing meal.

ATBF is an important determinant of adipose tissue function as it determines the influx and efflux of metabolites, systemic hormones and adipokines (31). Basal ATBF is determined largely by nitric oxide, while the postprandial increase is mediated by β-adrenergic stimulation (32). Femoral ATBF is much less responsive to adrenergic stimulation due to prevailing inhibitory α2-adrenoceptor activity, underlining the concept of a depot-specific regulation (8). Vascular smooth muscle cell tone and endothelial nitric oxide synthesis are known to be affected by GC (reviewed in (33)). Interestingly, while GCs inhibit endothelial nitric oxide synthase, they also inhibit sympathetic nerve outflow in humans (13,14). In line with the latter, studies in humans indicate that resting systemic catecholamine rate of appearance does not change during hydrocortisone infusion and effects are mediated at the level of end-organ responsiveness (34). However, GC-mediated inhibition of the prevailing α2-adrenoceptor signalling in femoral adipose tissue could be a possible mechanism for the depot-specific effect on femoral ATBF observed in our studies.

In our studies of healthy volunteers, hydrocortisone infusion resulted in a significant plasma cortisol increase with abolition of the diurnal cortisol variation. Hypercortisolemia is known to induce insulin resistance (35) by affecting post-receptor insulin signalling and decreasing glucose clearance (36,37). Our systemic metabolite data suggest that we were able to replicate the effects of GC excess, reflected by our findings of increased basal glucose and insulin as well as increased postprandial glucose concentrations. C-peptide secretion was preserved during hypercortisolemia, suggesting maintained pancreatic β-cell function. In line with this, HOMA indices showed an increase in insulin resistance with unaltered beta cell function. Taking into account the high HOMA insulin sensitivity values found during control conditions, these data

suggest that our study population consisted of very insulin sensitive individuals that were rendered insulin resistant by the hydrocortisone infusion.

Interestingly, previous findings suggest GC effects on insulin sensitivity to be tissue specific, whereby they may induce insulin resistance in muscle, but increase insulin sensitivity in subcutaneous adipose tissue *in vivo* (26). Muscle is the main site of postprandial glucose uptake, while adipose tissue accounts only for a small proportion of glucose clearance (38). We found that adipose tissue-specific glucose uptake rates were not affected by GC-induced whole body insulin resistance, which supports an adipose tissue-specific insulin sensitising GC effect. Furthermore, insulin is the major inhibitor of adipose tissue lipolysis (28). In our study, glucose-mediated suppression of lipolysis was not affected by hydrocortisone, underlining the exquisite ability of insulin to maximally suppress HSL activity, and providing further support that GC do not induce adipose tissue insulin resistance (26).

In order to evaluate non-metabolic effects of the induced hypercortisolemia, we measured IL-6 concentrations as one of the main inflammatory cytokines (39). Plasma concentrations of IL-6 increase as part of the acute inflammatory response pathway and acute hydrocortisone administrations is known to reduce IL-6 concentrations, although it appears that this effect only becomes apparent after a few days (40). In line with this, and because our study participants were healthy volunteers without any pathophysiological elevations in IL-6 concentrations, we did not see any effects of hydrocortisone infusion on systemic IL-6 concentrations. Previously it has been shown that abdominal adipose tissue is a net producer of IL-6, while femoral adipose tissue is not (41). Our results appear to be in line with this, although they did not reach statistical significance, most likely due to the small sample size.

A particular strength of our study is the integrative *in vivo* physiology approach chosen, utilizing arterio-venous differences and ATBF measurements for the assessment of NEFA release, the direct product of lipolysis, in response to GC excess under near-normal conditions. Most previous studies investigating adipose tissue depot-specific GC effects on lipolysis employed the microdialysis technique, which relies on the measurement of interstitial glycerol concentrations as an indirect index of lipolysis (42), with only a few studies including femoral adipose tissue responses to date (15,16,27). Djurhuus *et al.* found an increase in abdominal and femoral adipose tissue interstitial glycerol following short-term (6h) hydrocortisone infusion (15,27). When assessing metabolite fluxes, it is important to take into consideration depot-specific changes in blood flow. We showed that the marked femoral-specific ATBF effect in response to hypercortisolemia was augmented in the postprandial phase, mostly in response to insulin. While insulin does not exert direct ATBF effects, it is known to be in important mediator (43), and in contrast to previous studies using acute GC excess as an experimental approach (15,27), we saw a small but significant rise in basal plasma insulin concentrations. In previous studies, femoral ATBF was not measured, but instead abdominal ATBF was used as a basis for calculating femoral interstitial glycerol release (15,16,27). In these studies, abdominal ATBF did not change in response to hypercortisolemia. In view of the previously unknown marked femoral-specific ATBF effects described here, it is possible that previous microdialysis studies overestimated femoral glycerol appearance. Our abdominal depot findings are in contrast to previous results from the only study using the same experimental approach as here, showing a significant decrease in abdominal NEFA efflux during hydrocortisone infusion (17). This could be due to the slightly higher plasma cortisol concentrations achieved in our study, resulting in an overall increased systemic fatty acid turnover, or other factors intrinsic to our study population such as differences in local 11β-HSD1 activity or ATBF responses.

Our study has some limitations by design, including the small size of our sample and the fact that conclusions can be drawn only about men. The hydrocortisone infusion was calculated to provide around 300mg/24h, which is a dose regularly employed in clinical practice. This resulted in supraphysiological plasma cortisol concentrations, around double compared to those found in acute stress situations (e.g. sepsis) (44). To our knowledge, there are no published studies examining the exact dose-dependent metabolic response in acute hypercortisolemia *in vivo.* While acute dose-dependent GC effects cannot be excluded, it is generally accepted that tissue responses are more dependent on GR expression and local glucocorticoid activity modulation by the 11β-HSD enzymes, than absolute plasma concentrations (45). In support of this, our systemic metabolite data are comparable with studies that achieved more physiological hypercortisolemia (25). The acute nature of the hypercortisolemia limits the conclusions we can draw about depot-specific lipolysis in conditions of chronic glucocorticoid excess, i.e. Cushing's Syndrome. However, the finding of isolated fasting and postprandial ATBF induction in the femoral adipose tissue suggests an early signal of GC induced changes in this depot. Our calculations were performed based on the measurement of non-labelled NEFA and glycerol only, and because our study protocol did not include any meal containing lipids, exact modelling of depot-specific fatty acid trafficking, in particular triglyceride metabolism in the postprandial state, was not possible. Also, metabolic flux calculations are based on the assumption of steady state, however, the stark blood flow effects observed in our study introduced a non-steady state situation which could have affected our results. Finally, a direct comparison of the depot-specific contribution to systemic lipolysis between femoral and abdominal adipose tissue was not feasible, because measurements were not paired. With this in mind, however, there appear to be clear differential responses between the two depots in hypercortisolemia.

In conclusion, we have provided evidence that acute hypercortisolemia increases plasma NEFA and exerts selective effects on adipose tissue lipolysis and ATBF, supporting the concept of tissue-and depot-specific GC actions *in vivo*. Femoral adipose tissue does not appear to be a net contributor of NEFA in hypercortisolemia, but marked changes in femoral ATBF could herald effects on femoral fatty acid trafficking that become evident only in chronic hypercortisolemia. The exact source of the excess systemic NEFA in hypercortisolemia remains elusive, but it can be speculated that they may derive from the visceral adipose tissue depot. The increase in abdominal lipolysis we observed appears not sufficient to explain the marked increase in plasma NEFA during hydrocortisone infusion. Future studies will need to address whether altered subcutaneous adipose tissue depot function and to what extent visceral adipose tissue lipolysis contribute to the pathophysiological changes in fatty acid metabolism in chronic GC excess.

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Figure 1: Study design and systemic metabolite concentrations. Healthy male volunteers underwent the study twice, either receiving a constant overnight hydrocortisone or saline infusion until the end of the study. Infusions were given in a randomized, double-blind order. A standard 75g glucose drink was given at 120 min (n=9, study 1). In eight further individuals (study 2) blood sampling across abdominal adipose tissue was performed in the fasting state only. Plasma cortisol **(B)**, NEFA **(C)**, and glucose **(D)** during hydrocortisone (black squares) or saline infusion (open circles). Hypercortisolemia increased plasma NEFA and glucose

concentrations. Data from study 1, n=9, *p<0.05 compared to saline for each time point, Wilcoxon.

Figure 2: Pancreatic function and insulin resistance indexes (HOMA). Plasma insulin **(A)** and C-peptide concentrations **(B)** during hydrocortisone (HC, black squares) or saline infusion (S, open circles). A standard 75g glucose drink was given at 120 min. HOMA indexes of pancreatic β-cell function (%B) and insulin sensitivity (%S) **(C)**, and insulin resistance index (IR) **(D)**. Hypercortisolemia increased basal insulin and C-peptide concentrations and resulted in peripheral insulin resistance. Data from study 1, n=9, *p<0.05 compared to saline for each time point, Wilcoxon.

Figure 3: Femoral adipose tissue responses. Femoral ATBF **(A)** during hydrocortisone (HC, black squares and bars) or saline infusion (S, open circles and bars). A standard 75g glucose drink was given at 120 min. ATBF change in the fasting to postprandial state transition **(B)**. Femoral NEFA release **(C)** and lipolysis suppression induced by glucose ingestion **(D)**. Femoral glycerol release **(E)** and glucose uptake **(F)**. Hypercortisolemia markedly increased basal and postprandial ATBF, but did not have any effect on femoral lipolysis or postprandial femoral glucose uptake. Release was calculated by multiplying the veno-arterial concentration difference with ATBF. Uptake was calculated by multiplying the arterio-venous concentration difference with ATBF. Data from study 1, n=9, *p<0.05 compared to saline for each time point, Wilcoxon**.**

Figure 4: Abdominal fasting adipose tissue blood flow and lipolysis. Plasma cortisol (**A**) and fasting NEFA concentrations (**B**) during hydrocortisone (HC, black bars) or saline infusion (S, white bars). Abdominal fasting ATBF **(C)**, NEFA release **(D)**, and glycerol release (**E**) during control and hydrocortisone infusions. Hypercortisolemia increased abdominal ATBF and NEFA release. Bars represent the mean of three fasting measurements (time points 0-30 min). Release was calculated by multiplying the veno-arterial concentration difference with ATBF. Uptake was calculated by multiplying the arterio-venous concentration difference with ATBF. Data from study 2, n=8, *p<0.05, paired T-test.

Table 1: Baseline anthropometric and metabolic characteristics of participants.

Characteristic	Study 1 (n=9)	Study 2 (n=8)	*p*
Age (years)	28 (19-57)	23 (19-47)	0.53
Weight (kg)	87.3 (67.3-96.4)	80.2 (74.0-93.0)	0.37
BMI (kg/m^2)	26.5 (22.7-30.4)	24.1 (23.1-27.5)	0.13
Waist-to-Hip Ratio	0.9 (0.76-1.03)	0.87 (0.78-0.98)	0.47
Trunk fat mass (kg)	13.5 (5.2-19.8)	9.0 (5.9-15.2)	0.24
Leg fat mass (kg)	6.4 (2.9-12.4)	6.1 (4.6-12.4)	0.77
Visceral fat mass (kg)	0.7 (0.3-1.7)	0.3 (0.2-1.2)	0.16
BP systolic (mmHg)			
Control	129±5	131±3	0.68
Hypercortisolemia	129±6	128±3	0.91
BP diastolic (mmHg)			
Control	74±3	70±2	0.07
Hypercortisolemia	73±4	67±3	0.26
Heart rate (bpm)			
Control	62±4	63±3	0.72
Hypercortisolemia	73±5*	73±3#	1.0

Median and range shown
BP, blood pressure; mean±SEM shown
*p=0.033 compared to control; #p=0.020 compared to control