Adipogenic and lipolytic effects of chronic glucocorticoid exposure

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Glucocorticoids are steroid hormones released from the adrenal cortex upon activation of the hypothalamic-pituitary-adrenal axis in response to stress (1). One of the major actions of glucocorticoids is to stimulate metabolic processes that collectively serve to increase fuel availability in times of physical, neurogenic, or metabolic stress. While the actions of glucocorticoids on carbohydrate and protein metabolism are more well defined (1, 13), the role of these stress hormones in lipid metabolism is much more controversial. Glucocorticoids are widely cited as being both lipolytic and adipogenic in action, depending on the concentration, duration, and type of glucocorticoid investigated, as well as the experimental model utilized (1, 16, 19, 31).

The synthetic glucocorticoid, dexamethasone (Dex), has been shown to increase adipose tissue lipolysis, as assessed by free fatty acids (FFA) release, either directly (26, 31) or possibly through increases in the lipolytic potential of growth hormone (10, 11). In humans, 6 h of hydrocortisone infusion, at rates that cause physiological elevations in cortisol, increase circulating FFAs (9). A number of mechanisms for the apparent lipolytic activity of glucocorticoids have been proposed, including 1) increased gene transcription of lipolytic enzymes, such as hormone-sensitive lipase (HSL) (26), and adipose triglyceride lipase (ATGL) (6, 29); 2) altered characteristics of inhibitory G proteins (32); and 3) downregulation of phosphodiesterase 3B and upregulation of intracellular concentrations of cAMP (31).

Though the aforementioned studies demonstrate that glucocorticoids are capable of stimulating lipolysis, at least in the short term, prolonged exposure to glucocorticoids promotes adiposity, particularly in the central (visceral) adipose regions. This “obesogenic” nature of glucocorticoids is best observed in patients with Cushing’s syndrome or in those receiving treatment with synthetic glucocorticoids for various inflammatory conditions, such as rheumatoid arthritis and asthma (30). Moreover, enhanced glucocorticoid activity in adipose tissue through increased adipose expression of the glucocorticoid-activating enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11BHSD1), results in marked central obesity (19), perhaps through increased rates of adipogenesis (18). In contrast to these findings, we recently demonstrated that increased glucocorticoid exposure, through elevated 11BHSD1 gene expression and activity specifically in the visceral adipose tissue of exercise-trained rodents, was associated with elevations in lipolysis and markedly diminished visceral adiposity (6, 7). This discrepancy between glucocorticoid levels and adiposity makes it difficult to draw generalized conclusions regarding the significance of the lipolytic and antilipolytic actions of these hormones.

Using both in vitro and in vivo methodologies, the purpose of this study was to clarify the effects of glucocorticoids on developing and mature adipocytes. We hypothesized that glucocorticoids play a dual role in adipose tissue metabolism, simultaneously acting on separate pathways that can increase both lipolysis and adipogenesis. Taken together, our findings demonstrate that glucocorticoid treatment is capable of stimulating both adipogenesis and lipolysis within adipose tissue simultaneously, with these actions mediated independently on preadipocytes and mature adipocytes, respectively. While glucocorticoid-induced adipogenesis (mediated at least in part through preadipocyte differentiation) is generally favored under normal circum-
stances, chronic elevations in glucocorticoids have the potential to negatively impact insulin sensitivity and energy metabolism, highlighted by our findings of increased visceral adiposity, elevated plasma FFAs, and ectopic lipid deposition in corticosterone (Cort)-treated rodents.

**METHODS**

**In Vitro Studies**

**Cell culture.** 3T3-L1 fibroblasts were grown for 2 days postconfluence in 10% fetal bovine serum/Dulbecco's modified Eagle's medium (FBS-DMEM) at 37°C and 5% CO₂. Differentiation was induced with 500 μM isobutylmethylxanthine (IBMX), 500 nM Dex, and 200 pM insulin for 4 days. The cells were then maintained in 10% FBS-DMEM containing 200 pM insulin for 4 days and then in 10% FBS-DMEM until ~95% of cells contained lipid droplets (2–4 days). The medium was changed every other day throughout the differentiating period.

**3T3-L1 lipolysis.** Fully differentiated cells (termed 3T3-L1 adipocytes) were treated with Cort (catalog no. C2505, Sigma) for 24 or 48 h as previously described (6). To measure lipolytic rates in the presence of Cort, media were sampled at 0 and 48 h post-Cort treatment and assayed for glycerol concentrations using a commercially available kit (catalog no. FG0100, Sigma). Cells were then washed 3× with warm PBS, scraped on ice in 100 μl lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% Triton X-100, 10% glycerol, pH 7.4), and assayed for protein concentrations with the Bradford method. Lipolytic rates are expressed as μM glycerol released per μg protein per hour. Separate experiments normalizing lipolytic rates with Oil Red O staining triglyceride content yielded similar results (data not shown).

**Measurement of basal lipolysis (i.e., lipolytic rates measured without elevated Cort in the media and not undergoing epinephrine stimulus), adipocytes were first treated with Cort for 48 h in the manner described above, washed 3× with warm PBS, and resuspended in Krebs-Ringer-phosphate-HEPES (KRPH) buffer containing 3.5% (wt/vol) bovine serum albumin (BSA) (125 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1 mM KH₂PO₄, 25 mM HEPES, 5 mM d-glucose). Media were then sampled at hour 48 and 49 for basal glycerol release over 1 h, the cells were harvested for protein, and lipolysis was expressed as described above. For epinephrine-stimulated lipolysis, cells were treated to identical conditions described for basal lipolysis, with the addition of 10 μM epinephrine (catalog no. E2425, Sigma). For experiments using RU486, a nonselective glucocorticoid receptor antagonist, cells were cocubinated with the specified corticosterone concentration and 10 μM RU486 (catalog no. M8046, Sigma).

**cAMP assay.** To assess a potential mechanism for alterations in rates of lipolysis, cAMP activity was assessed both in the Cort-treated and in the basal states. For this, 3T3-L1 cells underwent similar experimental conditions described in the previous section to assess lipolytic rates during both a 48-h treatment period and the subsequent 1-h basal period following treatment. Afterward, the cells were harvested for cAMP analysis using a commercially available kit and following the manufacturer's instructions (catalog no. 581001, Cayman Chemical).

**Cell viability.** To ensure that incubation of 3T3-L1 adipocytes with Cort was not inducing cell death, 3T3-L1 cells were treated with increasing concentrations of Cort (0.01–250 μM) for 48 h and cell viability was assessed with a Trypan blue exclusion assay. No evidence of increased cell death was seen at any concentrations of Cort.

**Glucose incorporation into lipids.** Glucose incorporation into lipid within adipocytes was measured as previously described (12). Briefly, 3T3-L1 adipocytes were treated with 0, 1, and 100 μM corticosterone for 48 h under the same conditions described for the lipolysis experiments. Afterward, the cells were starved for 2 h in serum-free media and then resuspended in KRPH buffer with 4% (wt/vol) BSA containing 0.2 μCi/ml d-[U-¹⁴C] glucose (catalog no. CFB2, GE Healthcare), with or without 100 nM insulin for 1 h. The media were immediately removed and the cells were washed 3× with PBS and then treated with Dole's reagent (isopropyl alcohol/n-heptane/H₂SO₄, 4:10:25, vol/vol/vol) to isolate the total lipid fraction and counted for radioactivity.

**Oil Red O analysis.** Cort-treated adipocytes in a six-well plate were stained with Oil Red O to determine triglyceride content. First, a stock solution was prepared by mixing 0.7 g of Oil Red O (catalog no. O-0625, Sigma) with 200 ml of isopropanol and filtering through 0.2-μm paper. A working solution [3:2, stock solution:double-distilled H₂O (ddH₂O), vol/vol] was filtered through 0.2-μm paper, incubated at room temperature for 20 min, and then immediately used. The majority of media in the six-well plates was discarded, and the cells were fixed with 10% formalin for 3 min at room temperature. The formalin was removed, and the cells were washed with 60% isopropanol and allowed to fully dry. The cells were then incubated in 1 ml of the Oil Red O working solution for 10 min and immediately washed with ddH₂O four times. After completely drying, 3.5 ml of 100% isopropanol was added and the plate was agitated to fully elute the Oil Red O. The eluted solution was read at 500 nm in a spectrophotometer with 100% isopropanol used as the blank. The values were made relative to control cells (no Cort).

**Cell microscopy.** To visually determine the impact of Cort treatment on adipocytes, 3T3-L1 adipocytes were treated with 1 μM Cort for 48 h and visualized with an inverted microscope equipped with a motorized stage system (Nikon Eclipse TE2000-U; Prior Scientific ProScan II Series). Coordinates for three random fields per well were ascertainment for initial and subsequent imaging. Adipocyte cross-sectional area and lipid droplet cross-sectional area were calculated utilizing the area measuring tool of the Adobe Photoshop CS software suite. Approximately 25 distinct adipocytes were measured per field, and measurements were normalized to control cells at time 0.

**In Vivo Studies**

**Animals.** The animal protocol was approved by the York University Institutional Animal Care Committee. Male Sprague-Dawley rats, with an initial weight of ~250 g, were divided into three groups: sham, Cort, and pair-fed (n = 5 animals/group). Animals were housed individually in standard rodent cages with a 12:12-h light-dark cycle for 4 days before experimental conditions. On day 0, both sham and pair-fed animals had two 150-mg wax pellets subcutaneously implanted between their scapulae, whereas Cort animals had two wax pellets containing 150 mg of Cort (catalog no. C2505, Sigma) subcutaneously implanted middorsor. Sham and Cort animals were fed ad libitum, with food intake measured daily for 10 days. Pair-fed animals were given the average amount of food consumed by the Cort animals the previous day. Tail vein blood was collected 2 days following treatment at 0800 and 2000 and on day 9 at 0800 to determine the diurnal Cort concentrations. Blood was also collected on days 2 and 10 at 0800 for measurements of FFAs and glycerol concentrations and day 6 for insulin concentrations. Blood was immediately centrifuged, and the plasma was collected and frozen at −20°C until further use. Commercially available kits were used to measure plasma glycerol (catalog no. FG0100, Sigma), FFAs (catalog no. 994–75409, Wako), insulin (catalog no. INSK020, Crystal Chem), and Cort (catalog no. 07–120102, MP Biomedicals). On day 9, one day before death, all animals underwent an overnight fast. On day 10, the adrenal glands and various skeletal muscles were removed, weighed, and frozen. Epididymal, retroperitoneal, and the inguinal subcutaneous adipose depots were removed and weighed. A small portion of each adipose tissue depot (~100 mg) was separated and frozen for protein analysis. The remaining adipose
tissue was used for primary cell culture. Both epididymal and retroperitoneal depots were used for representation of visceral adipose. These depots did not differ, and the data presented as visceral adipose are from epididymal depots. The inguinal adipose was used as a representative depot for subcutaneous adipose.

**Primary cell culture.** Adipocytes from visceral and subcutaneous adipose depots were isolated as previously described (23). Briefly, ~1 g from individual adipose was finely minced and then shaken (~120 rpm) for 20–30 min at 37°C in Hanks’ solution containing 1 mg/ml type II collagenase (catalog no. C6885, Sigma). Isolated adipocytes were obtained by filtering the solution through a nylon mesh. Cells were washed 3× using KRPH buffer and then allowed to rest in 10 ml of KRPH buffer with 4% BSA for 1 h before being resuspended in a final volume to make a 10% lipocrit solution. Media were sampled at 0 and 90 min for glycerol release. Following this, adipocytes were lysed and protein concentrations were measured using the Bradford method. Lipolysis was expressed as μM glycerol release per mg protein per hour.

Determination of adipocyte size and number. A 10-μl aliquot of isolated adipocytes was removed and analyzed during the rest period to determine adipocyte volume and number as previously described (8). Briefly, mean adipocyte diameter was determined microscopically by counting 150 individual adipocytes. Adipocyte volume was calculated as $V = 4π(diameter/2)^{3}/3$. Cell density (volume of cells/volume of cell suspension), or lipocrit, was estimated from the packed cell volume obtained after centrifugation of a capillary tube. Adipocyte number was calculated by dividing the cell density by the average adipocyte volume and is expressed per gram of adipose tissue.

**Immunoblot analysis.** Protein quantification from both 3T3-L1 and primary adipocytes was done as previously described (6). For 3T3-L1 adipocytes, cells in six-well plates were washed with PBS and placed on ice and 100 μl of lysis buffer was added to each well. Cells were scraped and collected into 0.5-ml tubes and underwent sonication for 5 s. For primary adipocytes, ~100 mg of adipose tissue was homogenized with 300 μl of lysis buffer. In both situations, tubes were centrifuged for 20 min at 13,000 rpm at 4°C and the supernatant was collected and assayed for protein content with the Bradford method. Total protein (50 μg) was electrophoretically resolved on a 10% SDS-polyacrylamide gel and transferred overnight at 20 V to polyvinylidene difluoride paper. Blots were blocked with 5% BSA and then incubated overnight in primary antibody at 4°C (HSL: Cell Signaling catalog no. 4107, 1:1,000; ATGL: Cell Signaling catalog no. 4126, 1:5,000; α-tubulin (catalog no. ab7291, Abcam) were used as loading controls in tissue and 3T3-L1 cells, respectively.

Skeletal muscle and liver tissue intramuscular triglyceride content and respiratory cages. Intramuscular and intrahepatic triglyceride content was determined by histological staining, as previously described (17). Briefly, muscle and liver sections (10 μm thick) were sliced in a cryostat maintained at a temperature of ~20°C. Sections were stained for lipid content using an Oil Red O solution composed of 0.5 g of Oil Red O powdered dye (Sigma Aldrich, St. Louis, MO) and 100 ml of 60% triethyl phosphate (Sigma Aldrich). Slides were left to air dry for 10 min followed by fixation in 3.7% formaldehyde (Sigma Aldrich) for 1 h at room temperature. After three rinses with distilled water, slides were then placed in a double filtered Oil Red O solution for 30 min at room temperature. Immediately following Oil Red O staining, liver sections were counterstained with 1 min with Harrison’s hematoxylin solution (Sigma Aldrich). All slides were then allowed to air dry for 10 min and sealed with Crystal Mount (Sigma Aldrich). Tissue sections were viewed using light microscopy (Nikon Eclipse 90i microscope, Nikon Canada), and digital images were taken at a magnification of ×10 (liver) or ×20 (muscle). On day 7, three animals from sham and Cort groups were placed in a metabolic cage for 24 h (Windows Oxymax Code no. 0246–002M; Columbus Instruments) to measure the respiratory exchange ratio.

**Data analysis.** For all experiments, the appropriate t-test, one-way, or two-way ANOVA was performed to identify significant differences between treatment groups using Statistica 6.0 software, with $P < 0.05$ as the criterion. When a significant difference was observed with an ANOVA, post hoc analysis using contrasts with a Bonferroni correction factor was performed to determine specific differences. Data are presented as means ± SE.

**RESULTS**

Glucocorticoid Treatment Does Not Increase Lipogenesis, But Does Increase Differentiation in a Concentration-Dependent Fashion

3T3-L1 fibroblasts were induced to differentiate with 500 mM IBMX, 200 μM insulin, and various amounts of Dex. The number of lipid-containing mature adipocytes was quantified with Oil Red O staining, and a concentration-dependent increase in the differentiation of preadipocytes was seen with increasing glucocorticoids ($P < 0.05$, Fig. 1, A and B). To determine the impact of Cort treatment on lipid content in vitro, 3T3-L1 adipocytes were treated for 48 h with 1 μM Cort and analyzed for evidence of lipogenesis. Cort treatment did not change the basal or insulin-stimulated glucose incorporation into lipids (Fig. 1C). Furthermore, Cort treatment did cause a small, but consistent, decrease in the amount of triglyceride content measured with Oil Red O staining ($P < 0.05$, Fig. 1D). This coincided with the results of analyzing the cells with microscopy (represented in Fig. 1E), where Cort treatment did not affect the cross-sectional area of adipocytes (Fig. 1F) but did prevent the increase in lipid droplet cross-sectional area seen in the control cells ($P < 0.05$, Fig. 1G).

Corticosterone Alters Lipolysis in a Concentration-Dependent Manner

3T3-L1 adipocytes treated with increasing concentrations of Cort demonstrated increased lipolytic rates, with maximal effects occurring between 1 and 10 μM ($P < 0.05$, Fig. 2A). Further increases in Cort resulted in a progressive decrease in lipolysis with the highest concentration of Cort (250 μM) resulting in decreased lipolytic rates to well below control values ($P < 0.05$, Fig. 2A). To demonstrate that the effects of Cort are mediated through the glucocorticoid receptor, cells were coincubated with 10 μM RU486, a glucocorticoid receptor antagonist. RU486 treatment prevented the Cort-mediated increase in lipolysis (Fig. 2B).

Corticosterone Increases Lipolysis Through Elevations in Basal Lipolytic Rates, Not Epinephrine-Stimulated Rates

3T3-L1 adipocytes treated with Cort for 48 h were washed and resuspended in KRPH buffer containing either 1) 3.5% BSA (basal) or 2) 3.5% BSA + 10 μM epinephrine
(stimulated) lipolysis for 1 h. Basal lipolytic rates following Cort treatment were increased in a concentration-dependent manner, with 250 μM Cort pretreatment causing residual basal lipolysis to increase the most (~425% vs. control, P < 0.05, Fig. 2C). These effects were abolished with coinubcation of RU486 at all concentrations except 100 μM Cort, likely due to the 10-fold higher concentration of Cort compared with RU486 (Fig. 2E). No concentration of Cort utilized had an effect on epinephrine-stimulated lipolysis (Fig. 2D).

Corticosterone Treatment Decreases cAMP Levels But Not Rates of Basal Lipolysis

To determine whether the paradoxical anti-lipolytic effects of Cort treatment and pro-lipolytic effects on basal lipolysis are mediated through changes in cAMP levels, cells were assayed at various time points and after varying concentrations of Cort exposure in 3T3-L1 adipocytes. In these cells, Cort treatment for 48 h increased lipolytic rates at 1 μM, but decreased lipolytic rates at 100 μM, similar to
However, Cort treatment resulted in decreased cAMP levels at both low and high concentrations (*P* < 0.05, Fig. 3A). Similar to our findings described above, basal lipolysis following Cort treatment was increased at both 1 and 100 μM, but cAMP levels did not differ from that found in control cells (Fig. 3B).

**Corticosterone Treatment Increases the Protein Expression of ATGL and the Phosphorylation State of HSL**

Treating 3T3-L1 adipocytes with increasing concentrations of Cort increased the protein expression of ATGL (*P* < 0.05, Fig. 4A). mRNA analysis confirmed that the increased ATGL protein was due to an increase in transcription (*P* < 0.05, Fig. 4B). Interestingly, HSL mRNA was increased at 48 h (*P* < 0.05, Fig. 4C), but protein levels remained constant (Fig. 4A). Analysis of adipocytes treated for longer than 48 h revealed a small increase in HSL protein levels that did not achieve statistical significance (data not shown). The phosphorylated states of HSL (Ser563 and Ser660) following 48 h of Cort treatment were increased with 1 μM Cort but not 100 μM Cort incubation (*P* < 0.05, Fig. 4, D and E, respectively). Furthermore, 1 μM Cort did not change the phosphorylation status of HSL following epinephrine stimulation, whereas 100 μM Cort decreased HSL phosphorylation at both Ser563 and Ser660 (*P* < 0.05, Fig. 4, D and E, respectively).

**Subcutaneous Corticosterone Implants Increase Nadir Plasma Corticosterone Levels and Decrease Overall Body Mass and Food Intake in Rats**

Compared with shams, rats implanted with pellets containing Cort had significant Cort levels in the nadir of the diurnal pattern (0800), with no effect on their peak (2000) Cort levels throughout the experimental period (day 2, Fig. 5A; day 10, Fig. 5B). Cort animals displayed decreased rates of body growth immediately...
after receiving the pellets and weighed significantly less than sham and pair-fed animals by day 2 (P < 0.01, Fig. 5C). Cort animals continued to grow at a slower rate and weighed ~10% less than sham animals on day 9 (P < 0.01, Fig. 5C). Although all animals lost weight during the fast from day 9 to 10, the Cort group lost significantly more mass than either sham or pair-fed animals (P < 0.01, Fig. 5C). Cort treatment also decreased total food intake compared with the sham animals (main effect of food: P < 0.05, Fig. 5D).

Fig. 3. The presence of corticosterone in the media decreases intracellular cAMP concentrations. A: lipolytic rates during 48 h of Cort treatment were increased at 1 μM but decreased at 100 μM, whereas cAMP levels were decreased at both 1 and 100 μM Cort concentrations while the steroid was present. B: basal lipolytic rates following treatment were increased at both 1 and 100 μM, and no differences in cAMP concentrations were found during the basal lipolysis following Cort treatment. Values are means ± SE; n = 10 for A and B and 4–5 for C and D. *P < 0.05 vs. Con.

Fig. 4. Basal lipolysis is due to an increase in adipose triglyceride lipase (ATGL) protein expression. 3T3-L1 adipocytes were incubated in increasing concentrations of Cort with or without RU486 for 48 h and then immediately harvested for protein and mRNA analysis. A: Cort induced a concentration-dependent increase in ATGL (P < 0.01, Con vs. 100 μM), but not hormone-sensitive lipase (HSL), protein content. B: mRNA analysis confirmed that the changes in ATGL protein levels were likely due to increased protein expression. C: HSL mRNA levels were increased with Cort treatment, but this did not translate to elevated protein levels at 48 h. D and E: in separate experiments, adipocytes were treated similarly with Cort, washed 3× with PBS, and then incubated in Krebs-Ringer buffer with or without 10 μM epinephrine. Treatment with 1 μM Cort increased the basal phosphorylated state of HSL (pHSL) at Ser563 (D) and Ser660 (E) but did not increase the ability of epinephrine to phosphorylate HSL. Treatment with 100 μM Cort did not change the basal phosphorylated state of HSL but did decrease the epinephrine-stimulated phosphorylated state. Values are means ± SE; n = 4. *P < 0.05 vs. Con (B and C) or Con without epinephrine (D and E).
Corticosterone Pellets Increase Circulating Concentrations of FFAs and Glycerol, But Not Insulin, in Both Fed and Fasting Conditions

Plasma FFAs, glycerol, and insulin concentrations were measured on day 2 at 0800 (fed) and on day 10 at 0800 (fasted) in Cort-treated and sham-treated rats. Cort treatment increased FFA levels under both fed and fasting conditions compared with either sham or pair-fed groups (Table 1). Cort treatment increased fed glycerol concentrations compared with sham and pair-fed groups (Table 1). Fasting glycerol concentrations were also elevated in the Cort group compared with the sham group (P < 0.05, Table 1). No differences were found in either fed or fasting insulin concentrations between groups (Table 1).

To determine whether Cort treatment affected glucose tolerance, a separate group of rats (sham vs. Cort, n = 6/group) were subjected to an oral glucose tolerance test (6) after 7 days of treatment. No differences were observed between sham and Cort animals in the areas under the curve for glucose (1,090 ± 38.3 vs. 839 ± 51.7 mM/min), or for insulin (122 ± 21.4 vs. 167 ± 28.5 pg·ml⁻¹·min⁻¹). Homeostatic model-derived insulin sensitivity was also not significantly different between groups, although it tended to be higher in the Cort animals (0.25 ± 0.05 vs. 0.78 ± 0.31 in sham vs. Cort, respectively).

Table 1. Plasma fed and fasting FFA, glycerol, and insulin concentrations

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<th>Fed Concentration (day 2)</th>
<th>Fasting Concentration (day 10)</th>
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<tr>
<td></td>
<td>Sham</td>
<td>Cort</td>
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<tr>
<td>FFA, µM</td>
<td>247.8 ± 13.3</td>
<td>441.3 ± 43.0*</td>
</tr>
<tr>
<td>Glycerol, µM</td>
<td>140.1 ± 7.1</td>
<td>235.3 ± 10.6*</td>
</tr>
<tr>
<td>Insulin, pg/ml</td>
<td>3,446 ± 560</td>
<td>3,907 ± 480</td>
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Values are means ± SE. Cort, corticosterone; FFA, free fatty acid; *P < 0.05, Cort vs. sham and pair-fed; †P < 0.05, Cort vs. sham.
animals demonstrated increased basal lipolytic rates in visceral adipocytes compared with sham and pair-fed animals (Fig. 6). Though no difference was seen between groups in the lipolytic rates of adipocytes isolated from subcutaneous depots (Fig. 6), protein analyses revealed that Cort animals had increased expression of ATGL and HSL in both visceral and subcutaneous depots (Fig. 6).

**Cortisosterone Treatment Increases Ectopic Adipose Deposition**

To determine the metabolic fate of the increased lipolytic products, we measured the respiratory quotient (oxidation) and the triglyceride content of skeletal muscle and liver (storage). Cort treatment did not significantly alter the fat oxidation compared with sham animals, shown by the respiratory exchange ratio over a 24-h period (0.928 ± 0.003 vs. 0.920 ± 0.004, sham vs. Cort, n = 3). However, Cort treatment did increase the intracellular triglyceride content in both skeletal muscle and liver tissue, as measured by Oil Red O (Fig. 7).

**DISCUSSION**

The contribution of glucocorticoids to adipose tissue metabolism is controversial. One body of research classifies glucocorticoids as catabolic, promoting energy substrate release. Consistent with this, studies have demonstrated that glucocorticoids are lipolytic in action, resulting in the release of free fatty acids both in vivo and in vitro (25, 26, 31). However, glucocorticoids may also act in an anabolic fashion within adipose tissue by increasing adipose mass, particularly in visceral regions (5, 19, 22). This effect may be mediated either by promoting adipogenesis and/or by reducing lipolysis (21). This anabolic action in visceral fat stores is consistent with the well-known centrally obese phenotype associated with populations with elevated glucocorticoid levels (e.g., Cushing’s syndrome, type 2 diabetes mellitus) (27). Given these observations, it remained unknown as to whether this hormone actually elicits these disparate actions simultaneously within adipose tissue. Here we provide both in vitro and in vivo evidence that elevations in Cort act simultaneously on two different pathways to regulate adipose tissue metabolism. That is, Cort stimulates adipogenesis by acting on preadipocytes, and concomitantly increases lipolysis through actions on mature adipocytes. Furthermore, we demonstrate that high levels of Cort in rats favor a net increase in adipose tissue mass and the accumulation of lipid in nonadipose sites (i.e., liver and muscle), similar to what is observed in Cushing’s syndrome and in type 2 diabetes mellitus.

Although the anti-lipolytic effects of Cort appear to be mediated by reductions in cAMP levels, the pro-lipolytic features of this hormone are regulated by increased expression of ATGL within the mature adipocytes. Here we demonstrate that the latter effects are most notably seen in visceral adipose tissue. Importantly, increases in central adiposity have been implicated in promoting whole body insulin resistance (2, 4, 19).

While our 10-day Cort treatment in rats was successful in mediating many of the effects of elevated glucocorticoids observed in clinical conditions (increased visceral adiposity, circulating FFA, increased intracellular triglyceride deposition in liver and muscle), we did not observe significant increases in whole body insulin resistance as assessed by an oral glucose tolerance test. While direct assessment of insulin-stimulated glucose uptake in muscle may have demonstrated impairment in insulin signaling, we would speculate that our Cort treatment was of insufficient duration to be detected by a whole body glucose tolerance test. The increase in visceral adiposity, along with increased circulating FFAs and ectopic lipid deposition seen in our model, suggests that these animals would demonstrate insulin resistance and be at risk for the development of type 2 diabetes. However, we found no differences in fed or fasting insulin values between any of the groups. It may be that 10 days of treatment is not sufficient to induce noticeable changes in insulin sensitivity, or that the animals may need to be challenged with a high-fat diet along with elevations in Cort. Indeed, when we combine a similar Cort treatment with a high-fat diet, animals display a similar, albeit more severe, phenotype along with rapid onset of insulin resistance (unpublished observations).

Adipogenesis can be accomplished either through increased preadipocyte differentiation resulting in a greater number of mature adipocytes (hyperplasia) or through hypertrophy of existing adipocytes (28). Consistent with Hauner and colleagues (14, 15), we demonstrate that glucocorticoids (cortisosterone, dexamethasone) are essential for differentiation of preadipocytes in a concentration-dependent manner, illustrating one potential mechanism for increased adipogenesis. Importantly, when we treated mature adipocytes with Cort, we did not find any evidence that glucocorticoid-stimulated adipogenesis occurs through adipocyte hypertrophy. Furthermore, elevation of glucocorticoid levels in vivo increased central adipose mass, with a phenotype consisting of a greater number of small adipocytes compared with control animals, indicative of hyperplasia. These findings strengthen the hypothesis that glucocorticoids increase adipose tissue mass by acting on preadipocytes, not mature adipocytes, to increase differentiation.

To determine the action of glucocorticoids on mature adipocytes, we treated fully differentiated 3T3-L1 cells with increasing concentrations of Cort. Our finding that low to moderate concentrations of Cort increased lipolysis, but higher doses diminished or had a suppressive effect, illustrates an interesting relationship between the concentration of glucocorticoids and lipolytic rates. Early studies have also shown that Cort increases lipolysis in isolate adipocytes until a plateau is reached.

| Table 2. Anthropometric characteristics on day 10 and epididymal adipocyte characteristics |
|------------------------------------------|--------|--------|--------|
|                                  | Sham   | Cort   | Pair-Fed |
| Epididymal adipose mass, g/kg body wt | 6.80 ± 0.47 | 10.27 ± 0.63a | 6.54 ± 0.54 |
| Subcutaneous adipose mass, g/kg body wt | 10.02 ± 1.64 | 12.89 ± 0.54 | 10.83 ± 1.13 |
| Plantaris mass, g/kg body wt | 1.03 ± 0.02 | 0.95 ± 0.03 | 1.01 ± 0.04 |
| Adrenal mass, mg | | | |
| Left | 34.1 ± 2 | 19.6 ± 2* | 33.2 ± 2 |
| Right | 31.1 ± 2 | 15.2 ± 3* | 32.6 ± 1 |
| Pellet mass, mg | 300.0 ± 9 | 269.5 ± 6* | 309.9 ± 9 |
| Adipocyte size, L × 10⁻⁶ | 16.02 ± 2.3 | 6.44 ± 0.7* | 13.96 ± 1.3 |
| Adipocyte no. (per mg tissue) | 33.37 ± 4.7 | 83.81 ± 7.8* | 48.90 ± 4.1 |

Values are means ± SE. L, length. *P < 0.05, Cort vs. sham and pair-fed.
reached somewhere between 1 and 10 μM (11). A distinction in this study is that we continued past these concentrations and found that further increases in Cort exposure clearly caused a progressive decline in lipolytic rates to below control values and that these high concentrations of Cort did not affect cell viability, because lipolytic rates are elevated dramatically once the hormone is washed out. This novel finding suggests that the adipogenic effects of Cort may be mediated, in part, through anti-lipolytic actions that are clearly evident at higher concentrations. We explored this further by measuring cAMP levels during both Cort treatment and basal conditions following treatment. The presence of Cort decreased cAMP levels at both low (1 μM) and high (100 μM) concentrations; however, this was immediately restored when Cort was removed, indicating that the effect was rapid and nongenomic. These results strengthen the concept that Cort has potent anti-lipolytic effects that are dependent on the presence of the steroid.

The high concentrations of Cort used in our study may be reflective of intracellular concentrations, particularly in disease states or if patients are given exogenous glucocorticoids. It should also be noted that intracellular physiological Cort concentrations are difficult to determine in vivo and largely depend on the location of interest. Moreover, considerable evidence exists to suggest that intracellular glucocorticoid levels may be much higher than that observed in blood because of the cellular activation of inactive glucocorticoids by 11β HSD1, which freely crosses the cell plasma membrane in high abundance (2). This intracellular enzyme is capable of increasing the concentration of active Cort substantially over that found in the plasma depending on the tissue type. In adipose tissue, elevating 11β HSD1 activity increases the concentration of Cort up to 15-fold over the levels found in the circulation (19). In our study, 1–3 μM (10–100 ng/ml) represents concentrations typically found in the blood, including what we report in our in vivo model. Therefore, concentrations between 10 and 50 μM, or higher, may reflect intracellular concentrations found in adipocytes. Still, efforts to accurately measure intracellular concentrations of Cort are required.

The present findings support the hypothesis that glucocorticoids influence FFA and glycerol release in adipocytes mainly...
through increases in basal lipolysis. It has been recently proposed that ATGL plays a more significant role than HSL in the regulation of basal lipolysis, given that ATGL is constantly proximal to the lipid droplet (16). Our data demonstrate that the effects of Cort on basal lipolysis are accompanied by a similar concentration-dependent increase in ATGL. We propose, therefore, that glucocorticoids increase lipolytic rates through elevations of ATGL protein content and the subsequent increase in basal lipolysis. Furthermore, we suggest that this effect is blunted while glucocorticoids are present at high concentrations, likely through mechanisms involving a cAMP-mediated pathway. The balance between the pro-lipolytic actions of Cort (i.e., increased ATGL) and the anti-lipolytic actions (i.e., decreased cAMP) seems to be dependent on the concentration and duration of exposure, as well as the continuous presence of the hormone.

Contrary to the work of others (24), we found little evidence that increases in glucocorticoids suppress lipolysis in vivo. Instead, we found lipolytic rates to be elevated in both fed (day 2) and fasting (day 10) conditions in animals treated with Cort. We also found that isolated adipocytes from Cort animals on day 10 displayed elevated lipolytic rates in culture, along with increases in lipolytic enzymes. Interestingly, Cort treatment in vivo also increased visceral adiposity, despite a...
lowered food intake, an effect that is likely mediated by increasing the number of adipocytes rather than increasing adipocyte size through either increased lipid or glucose incorporation. Although we did not directly measure preadipocyte differentiation in vivo, per se, this phenotype and our in vitro work strongly support the hypothesis that Cort increases adipose mass primarily through increasing the number of adipocytes, rather than through adipocyte hypertrophy. This is in agreement with the findings by Aria et al. (3) showing that significant food restriction in rats for 14 days elevated plasma Cort concentrations to similar levels caused by our pellet implantation, leading to a greater number of small adipocytes rather than adipocyte hypertrophy (3). Our in vivo findings are also in agreement with our in vitro findings, showing that Cort treatment acts on mature adipocytes to increase lipolysis through an ATGL-mediated mechanism, suggesting that concurrent action can take place on preadipocytes to increase differentiation and adipose tissue mass.

It is important to note that our findings highlight that Cort has a more pronounced effect on visceral adipose tissue than subcutaneous adipose tissue. Ten days of Cort treatment significantly increased visceral adipose tissue mass, but not subcutaneous mass, which is consistent with the central adiposity phenotype seen in Cushing’s syndrome (27). We propose that the disparity in effects between adipose depots is the result of differences in glucocorticoid receptor density. Given that visceral adipose tissue has increased glucocorticoid receptor expression, this depot is more sensitive to changes in glucocorticoid levels (4, 20, 28).

In summary, the present study demonstrates that glucocorticoids have dual roles in adipose tissue biology, acting on two distinct cell populations within the tissue (Fig. 8). On one hand, the lipolytic effects of Cort are targeted toward mature adipocytes, increasing basal lipolysis through an ATGL-mediated mechanism, while on the other hand, Cort concurrently acts on preadipocytes to induce differentiation and increase adipose tissue mass through hyperplasia. Finally, we show that under normal conditions, the actions of corticosterone in vivo favor adipogenesis, despite an increase in lipolysis. Thus, the net effect of glucocorticoid treatment is increased adipose tissue mass, increased circulating FFAs and ectopic storage of lipids in liver and skeletal muscle. Additional studies are clearly warranted to determine whether the balance of lipolysis and adipogenesis changes under conditions of significant positive energy balance (i.e., high-fat diet) or negative energy balance (i.e., exercise). Understanding these mechanisms will lead to the development of future therapeutic strategies aimed at decreasing adipose tissue deposition to help prevent obesity and type 2 diabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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