Cold Exposure Partially Corrects Disturbances in Lipid Metabolism in a Male Mouse Model of Glucocorticoid Excess

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High glucocorticoid concentrations are accompanied by metabolic side effects such as high plasma triglyceride (TG) concentrations. Liver, brown adipose tissue (BAT) and white adipose tissue are important regulators of plasma TG. Exposure to 4°C reduces plasma TG concentrations, and we therefore aimed to study the interaction between glucocorticoid excess and 24 hours of exposure to 4°C on lipid metabolism. For this, mice were implanted with 50-mg corticosterone or control pellets and housed for 24 hours at 23°C or 4°C 1 week later, after which various aspects of TG metabolism in liver, BAT, and white adipose tissue were studied. Corticosterone treatment resulted in a 3.8-fold increase of plasma TG concentrations. Increased TG was normalized by cold exposure, an effect still present 24 hours after cold exposure. Corticosterone treatment increased hepatic TG content by 3.5-fold and provoked secretion of large, TG-rich very low density lipoprotein particles. Cold exposure reduced very low density lipoprotein-TG secretion by approximately 50%. Corticosterone strongly decreased BAT activity: BAT weight increased by 3.5-fold, whereas uncoupling protein 1 (Ucp1) mRNA expression and Ucp1 protein content of BAT were reduced by 75% and 60%, respectively. Cold exposure partially normalized these parameters of BAT activity. The uptake of TG by BAT was not affected by corticosterone treatment but was increased 4.5-fold upon cold exposure. In conclusion, cold exposure normalizes corticosterone-induced hypertriglyceridemia, at least partly via activating BAT. (Endocrinology 156: 4115–4128, 2015)

Glucocorticoid (GC) excess in patients with Cushing’s disease or syndrome results in metabolic derangements such as insulin resistance and hyperlipidemia that are also part of the metabolic syndrome. Also, patients undergoing chronic treatment with GCs have symptoms resembling the metabolic syndrome, although interpretation of these observations is sometimes difficult due to possible confounding effects of the concomitant diseases that prompted the GC treatment (1).

The effects of GC excess on insulin resistance and glucose metabolism are well described (2, 3), but GC-mediated disturbances in lipid metabolism are not completely understood. Because hyperlipidemia, including increased plasma triglyceride (TG) and free fatty acid (FFA) concentrations, is probably associated with the increased incidence of cardiovascular disease in Cushing patients (2), more insight in GC-induced hyperlipidemia is needed. Of note, even slight but prolonged elevations of plasma cortisol within the normal range are associated with more cardiovascular events (4). This might also have implications for patients in situations of chronic stress.

Plasma TG concentrations are determined by 3 processes that are closely linked; secretion of TG-rich chylomicrons by the intestinal tract upon ingestion of FAs, se-

Abbreviations: BAT, brown adipose tissue; CD36, cluster of differentiation 36; FFA, free fatty acid; GC, glucocorticoid; HPA, hypothalamic-pituitary-adrenal; Srebp1c, sterol-regulatory element-binding protein-1c; TG, triglyceride; UCP1, uncoupling protein 1; VLDL, very low density lipoprotein; WAT, white adipose tissue.
cretion of TG-rich very low density lipoprotein (VLDL) particles by the liver, and hydrolysis of TGs from VLDL and chylomicrons by lipoprotein lipase located on the capillary wall and subsequent uptake of released FAs by target tissues via FA transporters such as cluster of differentiation 36 (CD36) (5). GCs have been reported to influence TG metabolism by increasing hepatic VLDL secretion but also by up-regulation of Lpl gene expression and activity in various tissues, increasing the storage and availability of FA in adipose tissue (6, 7).

The effects of GCs on plasma FFA concentrations are complex. The major source of circulating FFAs is the adipose tissue and GCs exert different effects on separate adipose tissue depots. Although it is thought that GCs increase white adipose tissue (WAT) lipolysis and hence increase plasma FFA concentrations (8, 9), GCs at the same time induce expansion of specific WAT depots. In humans, central fat depots, such as visceral and abdominal fat, expand during GC excess, whereas peripheral fat depots, including sc fat, show increased lipolysis and diminished storage of TGs (6, 10). Thus, the effects of GCs on WAT are depot specific.

Brown adipose tissue (BAT) plays an important role in the clearance of both VLDL-TG and FFA from the circulation (11). Activation of BAT by means of cold exposure results in increased uptake of VLDL-TG-derived FA (12) and FFA by this tissue. In addition, not only the “classical” BAT appears to be pivotal for plasma TG and FFA clearance but very likely also the recently characterized beige adipose tissue depots that contain white adipocytes with BAT-like characteristics are important (13, 14). Appearance of the brown-like adipocytes in these depots is induced by cold exposure and an increased amount of beige fat has been shown to contribute to a lean and healthy phenotype (15–17). We recently showed that a relative short, 24 hours of exposure to 4°C is enough to increase BAT activity and appearance of brown-like adipocytes in WAT (18). Thus, activation of BAT and browning of WAT by 24 hours of exposure to 4°C might be beneficial in improving metabolic disturbances such as dyslipidemia.

Because multiple tissues might be involved in GC-mediated hyperlipidemia, as we discussed above, we questioned if, how, and via which tissues 24 hours of exposure to 4°C would be able to correct GC-mediated hyperlipidemia. Thus, we examined the effects of cold exposure and/or a high dose of corticosterone on plasma, liver, BAT, and WAT lipid metabolism in a mouse model of GC excess.

Materials and Methods

Animals

All animal experiments were performed with the approval of the Animal Ethics Committee at Erasmus Medical Center, Rotterdam, The Netherlands. Eight-week-old male C57Bl/6J mice were obtained from Charles River Laboratories.

Corticosterone supplementation and cold exposure

One week after arrival, mice at 9 weeks of age were implanted with a sc corticosterone pellet (50-mg corticosterone [Sigma-Aldrich] and 50-mg cholesterol) or control pellet (100-mg cholesterol). Pellets were implanted under de skin of the neck under isoflurane anesthesia. Seven days after pellet implantation, the mice were exposed to 23°C or 4°C for 24 hours, as previously described (18). Then, 1 of the experiments described below was performed.

Control experiment

After exposure for 24 hours to either 23°C or 4°C, blood glucose was measured in tail blood obtained after tail incision by using a Freestyle miniglucometer and test strips (Abbot), and rectal body temperature was determined. Mice were then terminated by cardiac puncture under isoflurane anesthesia. Feces was collected during the 24-hour period. Plasma and various tissues were collected and either immediately frozen and stored at −80°C or fixed in 4% paraformaldehyde.

Persistence study

For this study, blood was collected by orbital bleeding under light isoflurane sedation just before and immediately after 24 hours of exposure to 4°C as well as 24 and 48 hours after termination of cold exposure, and plasma TG was measured.

VLDL secretion experiment

The mice were deprived of food for the last 4 hours, after which they were sedated lightly with isoflurane and an orbital blood sample was taken. Next, the mice received an orbital injection of 100-μL 12.5% Triton WR-1339 (Sigma) in PBS. Orbital blood samples were taken under light isoflurane sedation 30, 60, 120, and 180 minutes after Triton WR-1339 injection. The mice were killed by cardiac puncture under isoflurane anesthesia directly after the last blood draw.

VLDL-TG-derived FA uptake experiment

VLDL-like TG-rich emulsion particles (80 nm), labeled with glycerol tri[3H]oleate (triolein), were prepared and characterized as described previously (19). The mice were deprived of food for the last 4 hours while kept at either 23°C or 4°C, after which they received an orbital injection of 200-μL VLDL-like TG-rich emulsion particles (1.0-mg TG per mouse) under light isoflurane sedation. Blood samples were obtained by tail bleeding at 2, 5, 10, and 15 minutes after injection of the VLDL-like TG emulsion particles. The mice were killed by cervical dislocation directly after the last blood draw, after which they were perfused with ice-cold PBS via the heart to remove blood from all tissues. Various tissues were collected, weighed and dissolved in Soluene (PerkinElmer). Plasma was separated from the tail blood samples. Retention of radioactivity in plasma and saponified tissue was determined.

FA uptake experiment

[3H]oleate (GE Healthcare) was bound to FA-free BSA as described previously (20). In short, to remove FAs from BSA
(A3912; Sigma-Aldrich), it was dissolved in aquadest with additional activated charcoal (0.5 g/g BSA) and pH adjusted to 3.0 with HCl. The suspension was stirred for 2 hours on ice and centrifuged for 20 minutes at 13 000 rpm at 4°C. Supernatants were pooled and pH was normalized to 7.0 with NaOH and subsequently filtered (0.2 μm). For binding of \(^{[3]}\)H\)olate to this FA-free BSA, \(^{[3]}\)H\)olate was added in a 5 times higher molar concentration than FA-free BSA and incubated for 1 hour at 37°C. Unbound \(^{[3]}\)H\)olate was removed using PD-10 columns (GE Healthcare). The FA-free BSA-\(^{[3]}\)H\)olate complex was rebuffered in PBS, filtered (0.2 μm), and stored at 4°C and used within 2 days.

The mice were deprived of food for the last 4 hours while kept at either 23°C or 4°C, after which they received an orbital injection of 100 μL of the FFA-free BSA-\(^{[3]}\)H\)olate complex solution under light isoflurane sedation. Blood samples were obtained by tail bleeding at 2, 5, and 10 minutes after \(^{[3]}\)H\)olate injection. The mice were killed by cervical dislocation directly after the last blood draw, after which they were perfused with ice-cold PBS via the heart to remove blood from all tissues. Various tissues were collected, weighed and dissolved in Soluene (PerkinElmer). Plasma was separated from the tail blood samples. Retention of radioactivity in plasma and saponified tissue was determined.

**Measurements of plasma ACTH, corticosterone, TG, and FFA**

Plasma ACTH was measured by RIA (MP Biomedicals). Plasma corticosterone and fecal corticosterone, which was extracted as previously described (18), were measured by ELISA (ENZO Life Sciences). Plasma TG concentrations were measured with commercial available kits (ABX Pentra; Horiba), whereas plasma FFA was determined with a kit from Wako. All measurements were performed according to the manufacturers’ protocols.

**Hepatic analysis**

Hepatic concentrations of TGs were measured using a commercial available kit (ABX Pentra) after lipid extraction according to Bligh and Dyer (21).

**VLDL isolation and particle size determination**

Nascent VLDL particles were isolated from the plasma obtained from the cardiac puncture after the VLDL secretion experiment. For this isolation, 300-μL plasma was added to 3700-μL PBS and centrifuged at 30 000 rpm for 17 hours, after which the top layer containing the VLDL was collected. VLDL size in this top fraction was determined using a Zetasizer Nano Z (Malvern Instruments).

**Gene expression analysis**

Total RNA isolation from mouse tissues and subsequent DNase treatment and reverse transcription was performed as previously described (18). Gene expression was measured using quantitative RT-PCR with SYBR Green Master Mix (Applied Biosystems) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems) and normalized to expression of housekeeping genes 18S and \(\beta\)2-globulin (adipose tissue) or 18S and \(\beta\)-actin (liver) using the \(2^{-\Delta\DeltaCT}\) method (22). Expression of the housekeeping genes was not influenced by treatment and housing temperature. Primer sequences used for all measured genes are listed in Supplemental Table 1.

**Histology and immunohistochemistry**

For hematoxylin and eosin staining, 8-μm sections of tissues preserved in paraformaldehyde and embedded in paraffin were used. Sections were mounted on microscope slides (Thermo Scientific) and kept overnight at 37°C, deparaffinized in xylene, and subsequently stained. For immunohistochemistry, sections were mounted on superfrost plus microscope slides (Thermo Scientific), kept at 37°C for at least 10 hours before staining for extra adherence. Sections were kept at 60°C for 1 hour and subsequently deparaffinized in xylene for 6 minutes, rinsed twice in 100% ethanol, put in methanol containing 3% H\(_2\)O\(_2\) to block endogenous peroxidase activity and rinsed in demineralized water. Heat antigen retrieval was achieved by cooking glasses in NaOH buffered citric acid (pH 6.0). After cooling down of the slides, sections were blocked with 5% normal goat serum (Dako) in PBS for 5 minutes, rinsed in PBS, and then incubated overnight with the first antibody at 4°C (uncoupling protein 1 [UCP1], 1:500; Sigma). BrightVision-poly-horseradish peroxidase-antimouse/rabbit/ rat IgG (Immunologic) in a 1:2 dilution in PBS was added for 30 minutes at room temperature as second antibody and peroxidase activity was developed with 0.07% 3,3-diaminobenzidine-tetrahydrochloride (Sigma) with subsequent counter-staining with hematoxylin.

**Western blot analysis**

Protein was extracted from tissues as previously described (18). A total of 15 μg of protein were electrophoresed on a 10% gel and blotted onto a nitrocellulose membrane. Membranes were blocked in PBS containing 3% nonfat powdered skim milk before an overnight incubation at 4°C with a rabbit polyclonal anti-UCP1 antibody (1:1000; Sigma) in PBS containing 0.1% Tween 20 and 3% nonfat powdered skim milk or a rabbit polyclonal anti-α-tubulin antibody (1:200; Santa Cruz Biotechnology, Inc) in PBS containing 0.1% Tween 20 and 5% BSA. Next, membranes were washed and incubated for 1 hour at room temperature with a goat-antirabbit IRDye 800 secondary antibody (1:10 000; LI-COR) in PBS containing 0.1% Tween 20 and 3% nonfat powdered skim milk. Ucp1 immunoreactivity was measured with an Odyssey fluorescence scanner (LI-COR) and was normalized for α-tubulin immunoreactivity in the same samples using Odyssey software. For antibodies, please see Table 1.

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc). The effect of treatment and temperature was studied by two-way ANOVA. A post hoc unpaired \(t\) test was performed (\(P < .025\) being considered significant) when a significance effect was found in the two-way ANOVA. For the persistence study, a paired \(t\) test was performed with \(P < .05\) being considered significant.

**Results**

**Corticosterone treatment creates a model of GC excess**

The primary aim of our experiments was to determine the interaction between GC excess and 24 hours of expo-
sure to 4°C on lipid metabolism. We first characterized our mouse model of GC excess. On average, the mice treated with corticosterone showed a tendency towards lower increase in body weight over time than the placebo-treated mice, but this was not due to changes in food intake because the corticosterone-treated mice ate more than the control mice (Table 2). Plasma corticosterone concentrations and 24 hours fecal corticosterone excretion were both increased approximately 6-fold by corticosterone (Table 2). As expected, due to its suppressive effects on the hypothalamic-pituitary-adrenal (HPA) axis, corticosterone treatment reduced plasma ACTH concentrations. In line with the high plasma corticosterone concentrations, thymus weights were reduced, because GCs induce thymocyte apoptosis (23). Altogether, we created mice with a disturbed HPA axis, as is found in patients with Cushing and those receiving high doses of synthetic GCs.

Cold exposure is a stressor but does not change HPA axis activity in mice with GC excess

To study the effects of cold exposure on corticosterone-induced changes, we kept corticosterone-treated and control mice at 23°C or 4°C for 24 hours. Cold exposure reduced body weight independent of corticosterone treatment. Of interest, however, although the control mice almost doubled their food intake at 4°C, the corticosterone-treated mice continued their level of food intake (Table 2). Cold exposure itself tended to increase plasma corticosterone levels and fecal corticosterone excretion by 1.5-fold, but this was not significant (Table 2). Plasma ACTH concentrations were approximately 1.75-fold increased after cold exposure in control mice but cold did not change ACTH plasma concentrations in corticosterone treated mice. Thus, suppression of the HPA axis activity by corticosterone was not altered by cold exposure intervention.

Corticosterone treatment increases fasted plasma TG concentrations that are normalized by cold exposure

Next, we determined the effects of corticosterone and cold exposure on fasted blood and plasma parameters. Surprisingly, the corticosterone pellets did not affect fasted blood glucose concentrations (Figure 1A) but tended to increase fasted plasma FFA concentrations (Figure 1B). The most striking effect of corticosterone treatment was seen for fasted plasma TG concentrations; plasma TG levels were increased 3.8-fold in corticosterone-treated mice (Figure 1C).

Cold exposure increased circulating FFA independent of corticosterone treatment (Figure 1B). Although corticosterone increased plasma TG concentrations in mice kept at 23°C, this was not the case in mice kept at 4°C (Figure 1C).

Next, we tested whether the TG-lowering effect of 24 hours of exposure to 4°C persisted even after the mice were placed back at normal housing temperature. Once again, cold exposure reduced plasma TG contents in both control

### Table 1. Antibody Table

<table>
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<tr>
<th>Peptide/Protein Target</th>
<th>Antigen Sequence (If Known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody</th>
<th>Species Raised in; Monoclonal or Polyclonal</th>
<th>Dilution Used</th>
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### Table 2. Effects of Corticosterone Treatment and Cold Exposure in Mice

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<th></th>
<th>4°C</th>
<th></th>
<th>Results 2WA</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Corticosterone</td>
<td>Control</td>
<td>Corticosterone</td>
<td>C</td>
</tr>
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<td>One-week BW change (%)</td>
<td>2.6 ± 0.8a</td>
<td>0.0 ± 1.0a</td>
<td>1.9 ± 1.0a</td>
<td>−1.5 ± 1.1a</td>
<td>P &lt; .01</td>
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<tr>
<td>24-h BW change (%)</td>
<td>0.9 ± 0.9a</td>
<td>0.7 ± 1.0a</td>
<td>−0.9 ± 0.6a</td>
<td>−1.7 ± 0.5a</td>
<td>NS</td>
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<td>Food intake (g/24 h)</td>
<td>3.7 ± 0.1a</td>
<td>4.5 ± 0.2a</td>
<td>6.3 ± 0.2c</td>
<td>5.1 ± 0.1b</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma corticosterone (ng/mL)</td>
<td>80 ± 9a</td>
<td>469 ± 103b</td>
<td>119 ± 12a</td>
<td>688 ± 104b</td>
<td>P &lt; .001</td>
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<tr>
<td>Fecal corticosterone (ng/24 h)</td>
<td>286 ± 54a</td>
<td>1722 ± 331b</td>
<td>433 ± 20a</td>
<td>2139 ± 99b</td>
<td>P &lt; .001</td>
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<tr>
<td>Plasma ACTH (pg/mL)</td>
<td>415 ± 81a</td>
<td>65 ± 17b</td>
<td>727 ± 129a</td>
<td>85 ± 38b</td>
<td>P &lt; .001</td>
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<tr>
<td>Thymus weight (g)</td>
<td>0.21 ± 0.01a</td>
<td>0.10 ± 0.01b</td>
<td>0.21 ± 0.02a</td>
<td>0.10 ± 0.01b</td>
<td>P &lt; .001</td>
</tr>
</tbody>
</table>

Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. BW, body weight. Statistical analysis was performed using two-way ANOVA analysis (2WA). C, effect of corticosterone treatment; T, effect of temperature; CxT, interaction between corticosterone and temperature; NS, not significant. A post hoc unpaired t test was performed when a significant effect was found in the two-way ANOVA, and values that do not share a symbol are significantly different (P < .025) in this post hoc test.
Data from control mice and mice treated with a corticosterone pellet for 1 week. Values are mean post hoc unpaired (T), or the interaction between corticosterone treatment and temperature (CxT). *, /H11021 P < .05 vs value before exposure to 4°C for control mice in paired t test. D, Plasma TG concentrations before and after exposure to 4°C for 24 hours. Data from control mice and mice treated with a corticosterone pellet for 1 week. Values are mean ± SEM; n = 5; *, P < .05 vs value before exposure to 4°C for control mice in paired t test; #, P < .05 vs value before exposure to 4°C for corticosterone-treated mice in paired t test.

Figure 1. Plasma parameters of corticosterone-treated and/or cold-exposed mice. Plasma glucose concentrations (A), plasma FFA concentrations (B), and plasma TG concentrations (C) of control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. Depicted below the graphs are the P values of the two-way ANOVA tests for either corticosterone treatment (C), temperature (T), or the interaction between corticosterone treatment and temperature (CxT). *, /H11005 P < .025 in the post hoc unpaired t test. D, Plasma TG concentrations before and after exposure to 4°C for 24 hours. Data from control mice and mice treated with a corticosterone pellet for 1 week. Values are mean ± SEM; n = 5; *, P < .05 vs value before exposure to 4°C for control mice in paired t test; #, P < .05 vs value before exposure to 4°C for corticosterone-treated mice in paired t test.

and corticosterone treated mice, but interestingly, the plasma TG concentrations were still reduced in the corticosterone-treated mice 24 hours after the mice were removed from the 4°C (Figure 1D).

Because increased TG levels are an important negative side-effect of excess of GC, we decided to examine the mechanism by which corticosterone and cold exposure regulate plasma TG concentrations in mice in more detail. For this, we studied hepatic VLDL-TG production and tissue TG and FFA uptake.

Corticosterone treatment elevates, whereas cold exposure lowers, secretion of VLDL-TG

Corticosterone treatment markedly enhanced VLDL-TG secretion 1.5-fold (P < .0001) (Figure 2A), and analysis of nascent VLDL particles revealed that corticosterone-treated mice secreted larger VLDL particles (Figure 2B). Analysis of the liver showed that corticosterone treatment resulted in heavier and fattier livers with lipids accumulation mainly localized in the perivenous compared with the periportal area (Figure 2, C–E).

Cold exposure significantly reduced VLDL-TG secretion in control mice (Figure 2A). Although the exposure to 4°C also reduced VLDL-TG secretion in corticosterone-treated mice, this reduction was not statistically significant. In addition, cold exposure tended to slightly increase the VLDL size independent of corticosterone treatment (Figure 2B) and, unexpectedly, further increased the hepatic TG content in the liver (Figure 2D). Because FFA are the main substrates of TG esterification in the liver and, hence, VLDL-TG secretion (24), we next determined hepatic uptake of VLDL-TG-derived FA and FFA, using injections of glycerol tri[3H]oleate-labeled VLDL-like TG-rich particles and [3H]oleate, respectively. Although the hepatic uptake of FFA and VLDL-TG-derived FA were not affected by corticosterone treatment, cold exposure lowered FFA uptake but did not influence VLDL-TG-derived FA uptake (Figure 3). Thus, these data suggest that the increase in plasma TG levels upon corticosterone treatment might be due to enhanced hepatic VLDL-TG secretion, but the normalization of hypertriglyceridemia upon cold exposure cannot be explained by changes in VLDL secretion.

Cold exposure normalizes the corticosterone-induced hepatic de novo lipogenic gene expression

Because hepatic lipid accumulation upon corticosterone treatment was apparently not due to changes in FA uptake and predominantly found in the perivenous area where both de novo lipogenesis and VLDL secretion are thought to be localized (25–29), we determined whether genes encoding enzymes involved in de novo lipogenesis were changed by corticosterone. In addition, we determined hepatic expression of genes encoding other enzymes involved in lipid metabolism. Expression of de novo lipogenesis gene Fasn was markedly increased upon corticosterone treatment (Figure 4A), but, interestingly, gene expression of the major regulator of de novo lipogenesis, sterol-regulatory element-binding protein-1c (Srebp1c), was not affected by corticosterone treatment. With respect to hepatic FA uptake, expression of the gene encoding the FA transporter CD36 (Cd36) was 12.5-fold up-regulated by corticosterone treatment (Figure 4B). For the genes encoding enzymes involved in FA oxidation, the effects are
less obvious. Only Mcad expression was increased by corticosterone (Figure 4C). The expression of Apob and Mttp, 2 genes important in VLDL secretion, was increased by corticosterone treatment, albeit this was only statistically significant for Mttp (Figure 4D).

Cold exposure reduced the increased Fasn expression and reduced Srebp1c expression by 5-fold (Figure 4A). Likewise, the increase of Cd36 by corticosterone was reduced by a factor 2 upon cold exposure (Figure 4B). Cold exposure partially normalized the corticosterone-induced Mttp expression.

**BAT activity is reduced by corticosterone and increased by cold exposure**

The increase of hepatic VLDL-TG secretion provides an explanation for increased plasma TG concentrations in corticosterone-treated mice, but cannot explain the correction of plasma TG in cold-exposed corticosterone-treated mice, because livers of these mice still secrete more TGs than those of nontreated mice (Figure 2A).

Because BAT is known to be a marked contributor to VLDL-TG clearance, especially when activated (11, 12), we next studied lipid metabolism in BAT.

**Figure 2.** Liver lipid metabolism in corticosterone-treated and/or cold-exposed mice. A, VLDL-TG secretion rate. B, Nascent VLDL particle diameter. C, Relative liver weight as percent of body weight (BW). D, Liver TG content per mg tissue. Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. Depicted below the graphs are the P values of the two-way ANOVA tests for either corticosterone treatment (C), temperature (T), or the interaction between corticosterone treatment and temperature (CxT). *, P < .025 in the post hoc unpaired t test. E, Representative H&E staining of livers from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. PP: periporal vein, periporal area; PV: portal vein, perivenous area.

**Figure 3.** Hepatic FA and TG uptake. Hepatic [3H]oleate uptake (A) and hepatic uptake (B) of FAs derived from VLDL-like TG-rich emulsion particles (80 nm) enriched with glycerol tri[3H]oleate in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. Depicted below the graphs are the P values of the two-way ANOVA tests for either corticosterone treatment (C), temperature (T), or the interaction between corticosterone treatment and temperature (CxT). *, P < .025 in the post hoc unpaired t test.
mice than in control mice (Figure 5A) and histological examination showed much more lipid content per adipocyte (Figure 5B). Ucp1 staining (Figure 5C), Ucp1 mRNA expression (*P* < .006 in unpaired *t* test for control mice kept at 23°C and corticosterone-treated mice kept at 23°C) (Figure 5D), and Ucp1 protein content (Figure 5E) in BAT were all lower in corticosterone-treated animals compared with nontreated animals, indicative for reduced BAT activity. However, both the uptake of FFA and VLDL-TG-derived FA by BAT were not affected by corticosterone treatment (Figure 5, F and G). Thus, the increased lipid content in BAT of corticosterone-treated mice is likely due to reduced lipid turnover in BAT, which is indeed supported by the reduced Ucp1 expression and Ucp1 content.

As expected, cold exposure strongly up-regulated Ucp1 mRNA expression in BAT of control mice and in corticosterone-treated mice (Figure 5D). The weight and lipid content of BAT were also partially corrected by cold in corticosterone-treated mice kept at 23°C (Figure 5D), and Ucp1 protein content (Figure 5E) in BAT were all lower in corticosterone-treated animals compared with nontreated animals, indicative for reduced BAT activity. However, both the uptake of FFA and VLDL-TG-derived FA by BAT were not affected by corticosterone treatment (Figure 5, F and G). Thus, the increased lipid content in BAT of corticosterone-treated mice is likely due to reduced lipid turnover in BAT, which is indeed supported by the reduced Ucp1 expression and Ucp1 content.

![Figure 4](https://academic.oup.com/endo/article-abstract/156/11/4115/2422863)

**Figure 4.** Hepatic gene expression. Hepatic expression of lipogenic genes (A), hepatic expression of Cd36 (B), hepatic expression of genes encoding proteins involved in β-oxidation (C), and hepatic Apob and Mttn expression (D) in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. C, *P* < .05 for corticosterone treatment in two-way ANOVA test. T, *P* < .05 for temperature in two-way ANOVA test. CxT, *P* < .05 for the interaction between corticosterone treatment and temperature. *, *P* < .025 in the post hoc unpaired *t* test.

Browning of inguinal WAT is reduced by corticosterone but partially corrected by cold

Because the WAT depots deliver their FAs to the liver and BAT, we questioned whether WAT is also affected by corticosterone and cold exposure. We, therefore, studied browning and lipogenic and lipolytic activity of 2 different WAT depots: inguinal and gonadal WAT. Inguinal WAT has been shown to brown easily and gonadal WAT depot is considered a classical WAT depot that is more resistant to “browning.”

Weight and adipocyte cell size of inguinal WAT were increased upon corticosterone treatment (Figure 6, A and B). Corticosterone also diminished Ucp1 protein stain-
Figure 5. Effects of corticosterone treatment and/or cold exposure on BAT. A, Relative intrascapular BAT weight as percent of body weight (BW). B, Representative H&E staining of intrascapular BAT. C, Representative Ucp1 immunohistological staining of intrascapular BAT. D, Intrascapular BAT Ucp1 mRNA expression. E, Intrascapular BAT Ucp1 and α-tubulin Western blotting and quantification of 3 Ucp1 vs α-tubulin Western blottings. F, Intrascapular BAT [3H]oleate uptake. G, Intrascapular BAT uptake of FAs derived from VLDL-like TG-rich emulsion particles (80 nm) enriched with glycerol tri[3H]oleate. Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. Depicted below the graphs are the P values of the two-way ANOVA tests for either corticosterone treatment (C), temperature (T), or the interaction between corticosterone treatment and temperature (CxT). *, P < .025 in the post hoc unpaired t test. H, Intrascapular BAT expression of various regulatory genes and genes encoding for proteins involved in FA oxidation. I, Intrascapular BAT expression of lipogenic genes in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. C, P < .05 for corticosterone treatment in two-way ANOVA test. T, P < .05 for temperature in two-way ANOVA test. CxT, P < .05 for the interaction between corticosterone treatment and temperature. *, P < .025 in the post hoc unpaired t test.
Figure 6. Effects of corticosterone treatment and/or cold exposure on inguinal WAT. A, Relative inguinal WAT weight as percent of body weight (BW). B, Representative H&E staining of inguinal WAT. C, Representative Ucp1 immunohistological staining of inguinal WAT. D, Inguinal WAT Ucp1 mRNA expression. E, Inguinal WAT [3H]oleate uptake. F, Inguinal WAT uptake of FAs derived from VLDL-like TG-rich emulsion particles (80 nm) enriched with glycerol tri[3H]oleate. Data are from control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. Depicted below the graphs are the P values of the two-way ANOVA tests for either corticosterone treatment (C), temperature (T), or the interaction between corticosterone treatment and temperature (C×T). *, P < .025 in the post hoc unpaired t-test. G, Inguinal WAT expression of lipolytic genes in control mice or mice treated with a corticosterone pellet for 1 week who were exposed to 23°C or 4°C during the last 24 hours. Values are mean ± SEM; n = 6. C, P < .05 for corticosterone treatment in two-way ANOVA test. T, P < .05 for temperature in two-way ANOVA test. C×T, P < .05 for the interaction between corticosterone treatment and temperature. *, P < .025 in the post hoc unpaired t-test.
ing (Figure 6C) and Ucp1 mRNA expression (Figure 6D), indicative for reduced browning. Uptake of FFA and TG by inguinal WAT was increased by approximately 2-fold and approximately 4-fold, respectively (Figure 6, E and F) in corticosterone-treated mice. In line with this, corticosterone treatment increased Cd36 mRNA expression (Figure 6G).

Cold-induced browning of inguinal WAT in both control mice and corticosterone-treated mice is evident from the decreased cell size, increased Ucp1 staining, and 10-fold increased Ucp1 mRNA expression (Figure 6, B–D). Although the effect of cold on inguinal WAT FFA uptake did not reach statistical significance (Figure 6E), the 5-fold increased VLDL-TG-derived FA uptake induced by corticosterone treatment was completely and statistically significant normalized by cold exposure (Figure 6F). Cd36 expression was increased by cold exposure in both control and corticosterone-treated mice (Figure 6G). Altogether, these data indicate that corticosterone diminishes browning but stimulates storage of lipids in the inguinal WAT depot. Importantly, cold exposure partially corrects these corticosterone-induced disturbances.

**Corticosterone increases lipid uptake in the gonadal WAT, and this is normalized by cold exposure**

Finally, we investigated the effects of corticosterone and cold exposure on gonadal WAT. As for inguinal WAT, corticosterone treatment increased weight and adipocyte cell size of gonadal WAT, increased uptake of FFA and TG-derived FA, and resulted into a tendency towards increased Lpl and Cd36 mRNA expression (Figure 7). In contrast to inguinal WAT, however, corticosterone increased mRNA expression of the lipolytic gene Atgl, suggesting that gonadal WAT is a source for plasma FFA. Both cell size and weight of gonadal WAT were not affected by cold exposure but cold exposure increased Ucp1 and Pparc1a expression in gonadal WAT (Figure 7F). The uptake experiments revealed that cold exposure enhanced the uptake of FFA but did not affect the uptake of VLDL-TG-derived FA by gonadal WAT of control mice. Of interest, 24 hours of exposure to 4°C resulted in a normalization of VLDL-TG-derived FA uptake by gonadal WAT in corticosterone-treated animals. Finally, the combination of corticosterone-treatment and cold exposure resulted in enhanced mRNA expression of Lpl, Cd36, Atgl, and Hsl in gonadal WAT compared with control mice kept at 23°C (Figure 7E).

**Discussion**

Our mouse model of GC excess shows that chronic corticosterone treatment of C57Bl/6J mice elevates plasma TG concentration and that this is very likely the result of enhanced hepatic de novo lipogenesis and, hence, elevated VLDL-TG secretion. The relative short, 24 hours of exposure to 4°C reduced the corticosterone-mediated elevation in plasma TG concentration, an effect still present 24 hours after termination of cold exposure. Although cold exposure reduced hepatic VLDL-TG secretion, the VLDL-TG secretion rate was still significantly higher in cold-exposed corticosterone-treated mice compared with control mice. In addition, we found that 24 hours of exposure to 4°C was able to partially correct the severe inhibition of BAT activity upon GC excess. Finally, in our model of GC excess, the adipocyte size of WAT was increased, likely due to enhanced uptake of both FFA and VLDL-TG-derived FA. Cold exposure normalized both features of WAT. Altogether, in our murine model of GC excess, multiple features of disturbed lipid metabolism located in different tissues are corrected by a relative short, 24 hours of exposure to 4°C, albeit not fully on all accounts.

Cold exposure is a well-known stressor for rodents (30), and we also found that in our experiments, because exposure to 4°C for 24 hours resulted in elevated plasma ACTH concentrations and fecal corticosterone excretion in control mice by approximately 50% and 75%, respectively. These effects of cold exposure are comparable with what was found before (18) but failed to reach statistical significance upon multiple testing due to inclusion of the corticosterone-treated mice.

In our studies, we found that both corticosterone treatment and 24 hours of exposure to 4°C elevated the hepatic TG concentration. In general, GC excess results in insulin resistance of glucose metabolism with elevated plasma insulin concentrations. Because hepatic de novo lipogenesis remains insulin sensitive under these conditions (31, 32), the high plasma insulin concentrations upon GC excess might stimulate hepatic de novo lipogenesis. Insulin is known to enhance nuclear translocation increase of SREBP-1, resulting in increased expression of lipogenic SREBP-1c target genes such as Acaca and Fasn (33). Indeed, we found enhanced hepatic Fasn and Acaca expression in corticosterone-treated mice. Interestingly, mRNA expression of Srebp1c was reduced upon corticosterone treatment as is in line with the findings of Laszewitz et al (34) who found a similar effect with prednisolone in livers of ad libitum-fed mice and Erhuma et al (35), who found down-regulation of hepatic Srebp1c mRNA by GCs in rats in vivo but up-regulation in vitro. As discussed by the authors of the latter study, these data suggest that the effects of GCs on Srebp1c mRNA in vivo may be through indirect mechanisms rather than a direct effect on the SREBP-1c promoter level. In addition, also other nuclear
transcription factors such as the liver X receptor and the carbohydrate response element binding protein facilitate lipogenic gene expression in the liver (36, 37), thus maybe activation of these factors might have enhanced \textit{Acaca} and \textit{Fasn} expression.

Of interest, cold exposure resulted in a massive hepatic TG accumulation in the corticosterone-treated mice, even significantly higher than in cold exposed control mice. This corticosterone-induced hepatic TG content is most probably not the result of a changed balance in lipid secretion and uptake, because VLDL-TG secretion and hepatic uptake of VLDL-TG-derived FA and FFA did not differ between control and corticosterone-treated mice. Therefore, the increase in hepatic TG concentration is very likely a result of reduced hepatic FA oxidation in cold exposed corticosterone-treated mice compared with cold exposed control mice. Indeed, the gene encoding 1 of the rate-controlling enzymes in FA oxidation, \textit{Cpt1a}, is upregulated upon cold exposure in control mice but not in corticosterone-treated mice, providing an explanation of...
the disbalance between FA and TG synthesis and oxidation.

Although previous studies by others have shown that GC excess results in elevated VLDL-TG secretion, the mechanisms remained largely unknown (6, 7). Because TG availability is an important driver of VLDL-TG secretion, it is very likely that enhanced hepatic de novo lipogenesis upon chronic corticosterone treatment was the driving force for elevated VLDL-TG secretion.

The effects of cold exposure on VLDL-TG secretion have not been studied in great detail. Cold exposure increases BAT activity and hence the uptake of VLDL-TG-derived FA by this tissue (11, 12), thus one might expect the body to compensate for this by enhancing the hepatic VLDL-TG secretion. In rats, 3 hours of exposure to 10°C increased VLDL secretion and this effect of cold could be prevented by administration of the nonspecific ß-adrenergic antagonist propranolol (38). The latter finding is in sharp contrast to what we find. Very likely, the timing of the experiments is of crucial importance. We determined VLDL-TG secretion after 24 hours cold exposure, ie, much later than the previous study, and found a decreased VLDL-TG secretion. Thus, duration of cold exposure likely differential affects VLDL-TG secretion.

Another important regulator of VLDL-TG secretion in cold-exposed mice might be sympathetic nervous activity, ie, elevated catecholamines. In line with our data, Rasouli et al (39) found that hepatic denervation, resulting in a 99% reduction of hepatic catecholamine content, enhanced VLDL-TG secretion, whereas treatment of primary hepatocytes with both α- and ß-adrenoceptor agonists suppressed VLDL secretion (40). In contrast, others found that selective denervation of sympathetic input towards the liver resulted in decreased VLDL secretion in 19-hour-fasted but not in 4-hour-fasted rats (41). Interestingly, cold exposure interacts with corticosterone treatment at the level of lipogenic gene expression, diminishing some deteriorating effects of corticosterone. However, more research is required to understand via which mechanisms cold exposure and corticosterone affect hepatic lipid metabolism and VLDL-TG secretion.

A number of in vivo and in vitro studies show that GCs inhibit BAT activity, most likely via a GC receptor-mediated pathway (42–46). In addition, adrenalectomy of rodents resulted in increased BAT activity, which was normalized by GC replacement (47, 48). Our results are in line with these findings, but we are the first to show that activation of BAT by means such as cold exposure partially reverses this GC-mediated inhibition. This is confirmed by our previous in vitro observations that norepinephrine was still able to increase Ucp1 mRNA expression in cultured brown adipocytes treated with corticosterone (18).

Because we found profound inhibition of BAT activity in our mouse model of GC excess and BAT can take up large amounts of VLDL-TG-derived FA, we initially hypothesized that reduced BAT activity in corticosterone-treated mice would result in reduced uptake of TG-derived FA by BAT and thus contribute to increased plasma VLDL-TG levels. However, we did not find a decrease in uptake of TG-derived FA by BAT upon GC treatment. Thus, the accumulation of lipids in the BAT depot of GC-treated mice is very likely due to reduced lipd catabolism in BAT. Indeed, the severely reduced Ucp1 mRNA expression and Ucp1 protein content in BAT of corticosterone-treated mice kept at 23°C points towards reduced lipid catabolism.

Cold exposure is not the sole method to activate BAT in vivo. For instance, treatment with the ß3-adrenoceptor agonist CL316,243 is also a commonly used method to activate BAT in laboratory animal models (49). In rats, Suárez et al (50) did not find an effect of CL316,243 on percentage of total liver fat, suggesting that it is not activation of BAT per se that induces hepatic TG concentrations upon cold exposure.

The effects of GCs and the interaction with the 24 hours of exposure to 4°C seem to differ between the studied WAT depots. In gonadal WAT, GC treatment resulted in a tendency towards increased expression of both Atgl and Hsl that both encode important enzymes involved in lipolysis of intracellular TGs into glycerol and FAs. In inguinal WAT, corticosterone treatment did not affect Atgl expression. Interestingly, in inguinal WAT, corticosterone treatment induced expression of the gene encoding the FA transporter CD36 independent of cold exposure. In gonadal WAT, in contrast, corticosterone treatment resulted in a tendency towards increased Cd36 expression in mice kept at 23°C, not in those exposed to 4°C. However, both the gonadal and the inguinal WAT depot have an increased uptake of VLDL-TG-derived FA and FFA upon corticosterone excess, but the differences in uptake between the control and corticosterone-treated mice disappeared when mice were kept at 4°C for 24 hours. This shows, once again, that 24 hours of exposure to 4°C is beneficial in controlling disturbed lipid metabolism in our mouse model of GC excess.

In summary, we show that treatment of mice with corticosterone resulted in hypertriglyceridemia which was likely the result of enhanced VLDL-TG secretion due to increased hepatic de novo lipogenesis. Twenty-four hours of exposure to 4°C normalized the corticosterone-induced hypertriglyceridemia, an effect that was still present 24 hours after the cold exposure was ended. Although cold exposure reduced VLDL-TG secretion, corticosterone-treated mice kept at 4°C still had higher VLDL-TG se-
creatin rates than control mice at this temperature. Because exposure to 4°C corrected the corticosterone-mediated reduction of BAT activity, activation of BAT was at least partly responsible for normalization of corticosterone-induced hypertriglyceridemia. Alternative methods to activate BAT such as treatment with a β3-adrenoreceptor agonist might be useful to study whether BAT activation per se is indeed sufficient to correct GC-induced hypertriglyceridemia.

Acknowledgments

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References

34. Laskewitz AJ, van Dijk TH, Bloks VW, et al. Chronic prednisolone treatment reduces hepatic insulin sensitivity while perturbing the


