Regulation of Lipid Metabolism by Glucocorticoids and 11β-HSD1 in Skeletal Muscle

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The prevalences of insulin resistance and type 2 diabetes mellitus are rising dramatically, and, as a consequence, there is an urgent need to understand the pathogenesis underpinning these conditions to develop new and more efficacious treatments. We have tested the hypothesis that glucocorticoid (GC)–mediated changes in insulin sensitivity may be associated with changes in lipid flux. Furthermore, prereceptor modulation of GC availability by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) may represent a critical regulatory step. Dexamethasone (DEX) decreased lipogenesis in both murine C2C12 and human LHC-NM2 myotubes. Inactivating p-Ser-79/218 of acetyl-CoA carboxylase 1/2 and activating p-Thr-172 of AMP-activated protein kinase were both increased after DEX treatment in C2C12 myotubes. In contrast, DEX increased β-oxidation. Selective 11β-HSD1 inhibition blocked the 11-dehydrocorticosterone (11DHC)-mediated decrease in lipogenic gene expression and increase in lipolytic gene expression. Lipogenic gene expression was decreased, whereas lipolytic and β-oxidative gene expression increased in corticosterone (CORT) and 11DHC-treated wild-type mice and CORT (but not 11DHC)–treated 11β-HSD1−/− mice. Furthermore, CORT- and 11DHC-treated wild-type mice and CORT (but not 11DHC)–treated 11β-HSD1−/− mice had increased p-Ser-79/218 acetyl-CoA carboxylase 1/2, p-Thr-172 AMP-activated protein kinase and intramyocellular diacylglyceride content. In summary, we have shown that GCs have potent actions on intramyocellular lipid homeostasis by decreasing lipid storage, increasing lipid mobilization and utilization, and increasing diacylglyceride content. It is plausible that dysregulated intramyocellular lipid metabolism may underpin GC-induced insulin resistance of skeletal muscle. (Endocrinology 154: 2374–2384, 2013)
Reesterification in skeletal muscle requires the enzymes glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase, which esterify the first and third fatty acid to glycerol 3-phosphate (6). During de novo lipogenesis (DNL), acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (AAC)-1 and is subsequently converted to palmitate by fatty acid synthase (FAS) in a multistep reaction (7, 8). In skeletal muscle, free fatty acids are liberated from IMTG stores by lipolysis, catalyzed by adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (9, 10). Fatty acids are metabolized in the mitochondria via β-oxidation, yielding acetyl-CoA, which enters the citric acid cycle. ACC2 is localized to the outer mitochondrial membrane and functions to limit β-oxidation through malonyl-CoA–mediated inhibition of carnitine palmitoyltransferase-1b (CPT-1b) (11), which catalyzes the transfer of long-chain fatty acyl-CoA into the mitochondrion (12). Activities of both ACC1 and ACC2 are regulated through protein phosphorylation at serine-79 and serine-218, respectively. AMP-activated protein kinase (AMPK) is a critical regulator of ACC1/2 activity through phosphorylation at these inhibitory sites.

Patients with glucocorticoid (GC) excess, Cushing syndrome, develop a classic phenotype characterized by insulin resistance, proximal myopathy, and central obesity, although in the vast majority of patients with simple obesity and T2DM, circulating cortisol levels are normal. GC availability and action depend not only on circulating levels but also on the tissue-specific intracellular metabolism by 11β-hydroxysteroid dehydrogenases (11β-HSDs) (13). Skeletal muscle expresses 11β-HSD1, which converts active cortisone to active cortisol (11-dehydrocorticosterone [CORT]) in rodents, respectively (14). Increased expression and activity of 11β-HSD1 within skeletal muscle were described in patients with insulin resistance and T2DM (15–17), and local increased GC generation may contribute to the phenotype in these patients. Selective 11β-HSD1 inhibitors have been developed as a potential therapeutic strategy to enhance insulin sensitivity (18) and have demonstrated their ability to cause insulin sensitization in skeletal muscle in vitro and in animal models (14).

GCs cause insulin resistance in skeletal muscle (14) and, in addition, are potent regulators of lipid metabolism in adipose tissue and the liver (19–22). However, their role in the regulation of lipid metabolism in skeletal muscle has not been examined. We hypothesized that the effects of GCs that cause insulin resistance may be associated with changes in lipid homeostasis, leading to increased lipid mobilization and the generation of lipid intermediates rather than safe storage. Furthermore, these effects may be regulated at the intracellular level by 11β-HSD1, offering a potential mechanism for the beneficial impact of selective 11β-HSD1 inhibitors.

Materials and Methods

Cell culture

C2C12 cell culture

Murine C2C12 myoblasts (European Collection of Cell Cultures, Salisbury, United Kingdom) were grown in DMEM (PA Laboratories, Somerset, United Kingdom) supplemented with 10% fetal bovine serum (37°C, 5% CO2). Cells were grown to 60% to 70% confluence before differentiation (initiated by replacing growth media with DMEM with 5% horse serum). After 8 days, myoblasts fuse to form multinucleated myotubes.

Before treatment, all cells were cultured for 4 hours in serum-free medium without additives. Although longer washout periods would have been desirable to ensure complete removal of DEX from the differentiation media, these significantly compromised cell viability. Specific treatments (concentrations and duration) are described in the Results section. The selective 11β-HSD1 inhibitor, A1 (95% purity), was provided through material transfer agreements with AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom), and its detailed potency was described previously (14). Inhibitor properties are presented in the Results section.

LHC-NM2 cell culture

Human LHC-NM2 myoblasts were obtained as a kind gift from Woodring E. Wright (University of Texas, Dallas, Texas) (23). Myoblasts were grown in medium X (4 parts DMEM to 1 part medium 199 supplemented with 15% fetal bovine serum, 20 mM HEPES, 0.03 µg/mL zinc sulfate, 1.4 µg/mL vitamin B12, 140 nM dexamethasone [DEX], and 2.5 ng/mL hepatocyte growth factor). Cells were cultured on 0.1% gelatin-coated plates. At 80% confluence, differentiation was initiated by replacing growth medium with medium X supplemented with 10 µg/mL insulin and 100 µg/mL apo-transferrine. After 8 days, myoblasts had fused to form multinucleated myotubes.

RNA extraction and reverse transcription

Total RNA was extracted from tissue and cells using the Tri-Reagent system. RNA integrity was assessed by electrophoresis on 1% agarose gel. Concentration was determined spectrophotometrically at OD 260. In a 50-µl volume, 500 ng of total RNA was incubated with 250 µM concentrations of random hexamers, 500 µM dNTPs, 20 U of RNAse inhibitor, 63 U of Multi-Scribe reverse transcriptase, 5.5 mM MgCl2, and 1X reaction buffer. The reverse transcription reaction was performed at 25°C for 10 minutes and at 48°C for 30 minutes before the reaction was terminated by heating to 95°C for 5 minutes.

Real-time PCR

mRNA levels were determined using an ABI 7500 sequence detection system (Applied Biosystems, Warrington, United Kingdom). Reactions were performed in singleplex in 10-µl volumes on...
96-well plates in reaction buffer containing 2× TaqMan Universal PCR Master Mix (Applied Biosystems). Primers and probes for ACC1 (NM_133360.2), ACC2 (NM_133904.2), ATGL (NM_025802.3), HSL (NM_010719.5), ACOX1 (NM_015729.2), CPT-1b (NM_009948.2), PDK4 (NM_013743.2), PGC-1α (NM_025802.3), HSL (NM_010719.5), ACOX1 (NM_015729.2), CPT-1b (NM_009948.2), SREBP1c (NM_011480.3), and LASS6 (NM_172856.3) were supplied by Applied Biosystems as premade “assays on demand.” All reactions were normalized against the housekeeping gene 18S rRNA, provided as a preoptimized control probe. All target genes were labeled with FAM, and the housekeeping gene was labeled with VIC. The reaction conditions were as follows: 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were obtained as Ct values (Ct is the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values \[ΔCt = (Ct \text{ of the target gene} - C_{18S})\]. Data are expressed as arbitrary units using the following transformation \[\text{arbitrary units} = 1000 \times (2^{-ΔCt})\].

**Protein extraction and immunoblotting**

**Mouse muscle explants**

Quadriceps muscles were quickly harvested, snap-frozen using liquid nitrogen, and then transferred to −80°C until required. Proteins were extracted by homogenizing ~20 mg of tissue in 1.5 mL of radioimmunoprecipitation assay buffer (1 mM EDTA, 150 mM NaCl, 0.25% SDS, 1% NP-40, and 50 mM Tris, pH 7.4, supplemented with protease inhibitor cocktail [Roche, Sussex, United Kingdom] and phosphatase inhibitor [Thermo Fisher, Surrey, United Kingdom]) using a mechanical homogenizer (Thermo Fisher).

**Monolayers of cells**

Cells were placed on ice, washed with cold PBS then scraped into 100 μL of radioimmunoprecipitation assay buffer. For both tissue and cultured cell homogenates, samples were incubated at −80°C (10 minutes) on ice (30 minutes) and centrifuged at 4°C (10 minutes, 14,000 rpm). The supernatant was transferred to a fresh tube and the total protein concentration was determined by a commercially available assay (Bio-Rad Laboratories, Hercules, California). Thirty to 40 μg of protein was resolved on an SDS-PAGE gel (acrylamide percentage varied according to protein size). Proteins were transferred onto a nitrocellulose membrane (Hybond ECL; GE Healthcare, Chalfont St Giles, United Kingdom). Primary antibodies (anti-AMPKα and anti-pThr172 AMPKα were purchased from Cell Signaling Technology [Danvers, Massachusetts], anti-pThr172/218 ACC1/2 and anti-AKT were purchased from Millipore [Billerica, Massachusetts], anti-p-Ser473 AKT was purchased from R&D Systems.
anti-FAS was purchased from Santa Cruz Biotechnology [Heidelberg, Germany], and anti-GPAT was purchased from Abcam plc [Cambridge, United Kingdom]) and secondary antibodies (Dako, Glostrup, Denmark) were used at a dilution of 1:1000 and 1:5000, respectively. Membranes were reprobed for β-actin or α-tubulin, and primary and secondary antibodies were used at a dilution of 1:5000 (Abcam plc). Bands were visualized using an ECL detection kit (GE Healthcare).

**ACC Assay**

Assays were performed as described previously (22). In brief, C2C12 and LHC-NM2 myocytes were cultured, differentiated, and treated in 24-well tissue culture plates. Cells were incubated in 500 µL of serum-free medium with 0.12 µCi/L of 1-[14C]acetic acid (GE Healthcare, Bucks, United Kingdom) with unlabeled sodium acetate to a final concentration of 10 µM acetate at 37°C for 4 hours. Cells were then washed 3 times with ice-cold PBS, scraped into 250 µL of PBS, and transferred into glass tubes. Lipid extraction was performed by adding 5 mL of Folch solvent followed by vigorous shaking for 20 seconds. Then 1 mL of water was added followed by vigorous shaking for 20 seconds. Phases were separated by centrifugation at 300g for 5 min. The upper aqueous phase was removed by aspiration, and the lower fraction was transferred to a scintillation tube and evaporated until dry using a sample dryer (Techne, Burlington Township, New Jersey). Then 5 mL of scintillation cocktail was added (PerkinElmer, Bucks, United Kingdom), and samples were counted using a Wallac 1414 liquid scintillation counter (PerkinElmer).

**β-Oxidation assay**

β-Oxidation assays were performed as described previously (22). In brief, cells were incubated with 500 µL of serum-free medium containing 0.1 mmol/L palmitate (5 µCi/mL 9,10-[3H]palmitate [GE Healthcare]) and 2% BSA with treatment for 24 hours. After incubation, the medium was retained and precipitated twice with equal volumes of 10% trichloroacetic acid to remove excess labeled palmitate. The supernatants were extracted by addition of 2.5 mL of methanol-chloroform (2:1) and 1 mL of 2 mol/L KCl-HCl, followed by centrifugation at 3000g for 5 minutes. The aqueous phase (0.5 mL) was then added to scintillation cocktail, and samples were counted using a Wallac 1414 liquid scintillation counter.

**Intramyocellular DAG content**

Quadriceps muscle (~100 µg) was homogenized in 1 mL of PBS using a mechanical homogenizer. Samples were repeatedly freeze-thawed before centrifugation at 5000g for 10 minutes. Supernatants were transferred to fresh tubes, and intramyocellular DAG content was determined by a commercially available assay according to the manufacturer’s instructions (Antibodies-online.com, Atlanta, Georgia).

**Administration of GCs in vivo**

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986. 11β-HSD1 knockout mice on the C57BL/6 background were generated in-house (24). At 6 weeks of age, male wild-type and 11β-HSD1 knockout mice were treated with CORT (100 µg/mL, 0.66% ethanol), 11DHC (100 µg/mL, 0.66% ethanol), or vehicle (0.66% ethanol) via drinking wa-

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**Figure 2.** mRNA expression of CPT-1b was unchanged after treatment with DEX (5 and 500 nM, 24 hours) (A), whereas PDK4 expression was increased (B). Release of [3H]water from [3H]palmitate was increased in C2C12 myotubes (C) and LHC-NM2 myotubes (D) after treatment with DEX (5 and 500 nM, 24 hours). Data are expressed as means ± SE of n = 7 experiments. Statistics were analyzed using 1-way ANOVA. *, P < .05; **, P < .01.

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ter for 5 weeks. Water was replaced twice weekly. Before sacrifice, mice were fasted for 4 hours and then were administered 2 IU/kg insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) by intraperitoneal injection. Ten minutes after injection, animals were culled by cervical dislocation and quadriceps muscles were dissected out and snap-frozen in liquid nitrogen.

Serum CORT quantification

Mouse blood was obtained by cardiac puncture and immediately centrifuged at 1000 g in heparin-coated tubes. Serum was transferred to cryotubes and snap-frozen. CORT levels were assessed using a commercially available ELISA (Abcam plc).

Statistical analysis

A 1-way or 2-way ANOVA was used to compare multiple doses and/or treatments (SigmaStat 3.1; Systat Software, Inc, Point Richmond, California). Statistical analysis on real-time PCR data was performed on ΔCt values and not fold changes or arbitrary units. See figure legends for specific statistical tests used.

Results

Intramyocellular DNL

Cell lines were treated with the synthetic GC, DEX (5 and 500 nM), for 24 hours. In C2C12 myotubes, ACC1 mRNA expression was unchanged after DEX treatment (Figure 1A), whereas FAS expression decreased (0.44-fold [500 nM], P < .05) (Figure 1B). mRNA expression changes were paralleled by a decrease in 1-[14C]acetate incorporation into intracellular lipid in both C2C12 myotubes (100 vs 82.24 ± 2.98% [5 nM] vs 59.77 ± 2.39% [500 nM], P < .01) (Figure 1C) and human LHC-NM2 myotubes (100 vs 93.72 ± 4.20% [5 nM] vs 76.60 ± 4.52% [500 nM], P < .05) (Figure 1D). Consistent with the functional data, DEX increased p-Thr172 AMPK, without changing total AMPK levels and increased inhibiting p-Ser79/218 ACC in C2C12 myotubes (Figure 1E).

Intramyocellular β-oxidation

In C2C12 myotubes, CPT-1b mRNA expression did not change after DEX treatment (Figure 2A), whereas pyruvate dehydrogenase kinase 4 (PDK4) expression increased (2.5-fold [5 nM], P < .05 and 9.7-fold [500 nM], P < .001) (Figure 2B). DEX increased 9,10-[3H]palmitate oxidation in both murine C2C12 myotubes (100 vs 107.0 ± 3.0% [5 nM] vs 111.5 ± 3.4% [500 nM], P < .05) (Figure 2C) and human LHC-NM2 myotubes (100 vs 118.4 ± 1.5% [5 nM] vs 124.0 ± 5.8% [500 nM], P = .05) (Figure 2D).

Effect of GCs with insulin on intramyocellular lipid metabolism

To examine the effects of GCs alone and in combination with insulin, C2C12 myotubes were treated with DEX (500 nM, 24 hours) in the presence and absence of insulin (5 nM, 24 hours). Insulin increased FAS and GPAT mRNA expression (FAS: 1.86-fold, P < .01 [data not shown]; GPAT: 1.83-fold, P < .05) (Figure 3A) but did not affect ATGL mRNA levels in C2C12 myotubes (Figure 3B). Coincubation with DEX prevented the insulin-stim-
ulated increase in GPAT expression consistent with insulin resistance (Figure 3A). DEX treatment alone increased ATGL mRNA levels (2.72-fold, \( P < 0.05 \)), and this was blocked in the presence of insulin (Figure 3B). Insulin increased 1-[\(^{14}\)C]acetate incorporation into intracellular lipid (100 vs 142.7 \( \pm \) 5.6\%, \( P < 0.01 \)) (Figure 3C). DEX-induced suppression of 1-[\(^{14}\)C]acetate incorporation was attenuated in the presence of insulin (Figure 3C).

Regulation of intramyocellular lipid metabolism by 11\(\beta\)-HSD1

We had previously characterized 11\(\beta\)-HSD1 expression and activity in C2C12 myotubes (14). Thus, to explore its role in regulating skeletal muscle lipid metabolism, cells were treated with CORT (250 nM, 24 hours), 11DHC (250 nM, 24 hours) and 11DHC (250 nM, 24 hours) with the selective 11\(\beta\)-HSD1 inhibitor, A1 (2.5 \( \mu \)M, 24 hours) (IC\(_{50} = 11.2\) nM for rodent recombinant 11\(\beta\)-HSD1; detailed properties were described previously [14]).

Paralleling our observations with DEX, both CORT and 11DHC decreased expression of FAS (0.72-fold [CORT], \( P < 0.05 \) and 0.61-fold [11DHC], \( P < 0.01 \)) (Figure 4A and Table 1) without affecting ACC1 expression (Figure 4B and Table 1). In contrast, both CORT and 11DHC increased expression of the lipolytic enzymes ATGL (2.17-fold [CORT], \( P < 0.01 \) and 2.28-fold [11DHC], \( P < 0.05 \)) and HSL (2.17-fold [CORT], \( P < 0.05 \) and 1.87-fold [11DHC], \( P < 0.05 \)). Compound A1 completely reversed the effects of 11DHC on FAS, ATGL, and HSL expression (Figure 4A and Table 1). A1 treatment alone was without effect (data not shown).

Consistent with our gene expression data, both CORT and 11DHC decreased 1-[\(^{14}\)C]acetate incorporation into intracellular lipid. Coincubation with A1 blocked the effect of 11DHC (Figure 4B and Table 1). In contrast, both CORT and 11DHC increased expression of the lipolytic enzymes ATGL (2.17-fold [CORT], \( P < 0.01 \) and 2.28-fold [11DHC], \( P < 0.05 \)) and HSL (2.17-fold [CORT], \( P < 0.05 \) and 1.87-fold [11DHC], \( P < 0.05 \)). Compound A1 completely reversed the effects of 11DHC on FAS, ATGL, and HSL expression (Figure 4A and Table 1). A1 treatment alone was without effect (data not shown).

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Table 1. mRNA Expression of Genes Involved in Key Lipid Metabolic Pathways in C2C12 Myotubes, Measured Using Real-Time PCR After Treatment with CORT (250 nM, 24 Hours), 11DHC (250 nM, 24 Hours), or 11DHC With the Selective 11\(\beta\)-HSD1 Inhibitor, A1 (2.5 \( \mu \)M, 24 Hours)

<table>
<thead>
<tr>
<th>Gene</th>
<th>mRNA Expression</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>ACC1</td>
<td>0.36 ± 0.18</td>
</tr>
<tr>
<td>ACC2</td>
<td>0.008 ± 0.002</td>
</tr>
<tr>
<td>FAS</td>
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<tr>
<td>GPAT</td>
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<tr>
<td>DGAT</td>
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<tr>
<td>SCD1</td>
<td>12.6 ± 3.2</td>
</tr>
<tr>
<td>SCD2</td>
<td>12.1 ± 2.4</td>
</tr>
<tr>
<td>HSL</td>
<td>0.046 ± 0.005</td>
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<tr>
<td>ATGL</td>
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Data are means ± SE from n = 7 experiments and are expressed as arbitrary units. Statistical analyses were performed on \( \Delta C_t \) values using 2-way ANOVA: \(^a\) \( P < 0.05 \), \(^b\) \( P < 0.01 \), and \(^c\) \( P < 0.001 \) vs control; \(^d\) \( P < 0.05 \) vs 11DHC.)
effects of 11DHC on 1-[14C]acetate incorporation (100 [control] vs 67.7 [CORT], P < .001 vs [control]; 68.1 ± 6.2% [11DHC], P < .001 vs [control]; and 103.6 ± 6.6% [11DHC + A1], P < .001 vs [11DHC]) (Figure 4C).

**Figure 5.** Both CORT and 11DHC increased inactivating phosphorylation of ACC (Ser79/218), increased activating phosphorylation of AMPK (Thr172), and decreased activating phosphorylation of AKT (Ser473), while not affecting total AMPK or AKT protein levels in wild-type C57BL/6 mice (A). CORT, but not 11DHC, increased inactivating phosphorylation of ACC (Ser79/218), increased activating phosphorylation of AMPK (Thr172), and decreased activating phosphorylation of AKT (Ser473), whereas total AMPK and AKT protein levels were unaffected in 11β-HSD1 knockout mice (B). Western blots were performed on at least 3 different preparations. Intramyocellular DAG content was increased in both CORT- and 11DHC-treated wild-type and CORT-treated 11β-HSD1 knockout mice (C). Statistics were analyzed using 2-way ANOVA. *, P < .05; **, P < .01. Wild-type (WT): vehicle (Veh), n = 10; CORT, n = 7; 11DHC, n = 8; 11β-HSD1; knockout: vehicle, n = 7; CORT, n = 8; 11DHC, n = 6).

**GC regulation of intramyocellular lipid metabolism in wild-type and 11β-HSD1 knockout mice**

To determine whether regulation of intramyocellular lipid metabolism by GCs is relevant in an in vivo setting, wild-type and 11β-HSD1 knockout mice were treated with CORT (100 μg/mL, 0.66% ethanol), 11DHC (100 μg/mL, 0.66% ethanol), or vehicle (0.66% ethanol) via drinking water for 5 weeks. As anticipated, both CORT and 11DHC treatment in wild-type mice increased serum CORT levels. Similarly, serum CORT was elevated in 11β-HSD1 knockout mice after CORT (but not 11DHC) treatment (see Supplemental Figure 1 published on The Endocrine Society’s Journals Online website at http://endo.endojournals.org). GC treatment of both genotypes had no effect on body weight gain compared with that of vehicle-treated animals (Supplemental Table 1). As part of the protocol, we were unable to assess the total amount of drinking water consumed.

Consistent with the induction of insulin resistance, both CORT and 11DHC treatment in wild-type mice and CORT (but not 11DHC) treatment in 11β-HSD1 knockout mice reduced activating p-Ser473 AKT, without affecting total AKT1 levels (Figure 5, A and B). Endorsing our in vitro observations, both CORT and 11DHC treatment in wild-type mice increased inactivating p-Ser79/218 ACC1/2 and increased activating p-Thr172 AMPK, without affecting total AMPK protein levels in skeletal muscle (Figure 5A). CORT had the same effect in 11β-HSD1 knockout mice, but 11-DHC did not affect ACC or AMPK phosphorylation (Figure 5B). GC treatment was without effect on FAS and GPAT protein levels in WT and 11β-HSD1 knockout mice (Figure 5, A and B). A limitation in the current study was that semiquantifiable densitometry was not performed, although multiple replicate Western blots are presented. Both CORT and 11DHC increased intramyocellular DAG content in wild-type mice (7.6-fold [CORT], P < .05 and 9.8-fold [11DHC], P < .01). However, CORT (but not 11DHC) increased intramyocellular DAG content in 11β-HSD1 knockout mice (2.1-fold, P < .05) (Figure 5C). GC treatment was without effect on serum DAG
levels in both wild-type and 11β-HSD1 knockout mice (Supplemental Figure 2).

Although the in vivo experiments were not powered to detect changes in gene expression, data from skeletal muscle after CORT and 11DHC treatment in wild-type and 11β-HSD1 knockout mice are presented in Figure 6 and Table 2. In keeping with our in vitro observations, both CORT and 11DHC decreased the mRNA expression of lipogenic enzymes, ACC1, FAS, and GPAT, in wild-type mice. As expected, CORT (but not 11-DHC) decreased ACC1 and FAS mRNA expression in 11β-HSD1 knockout mice (Figure 6, A–C, and Table 2). In contrast, the expression of genes involved in the regulation of β-oxidation, including PDK4, ACOX1, PGC1α, and CPT-1b, were increased by CORT and 11DHC in wild-type mice. These genes were also up-regulated by CORT, but not 11DHC, in 11β-HSD1 knockout mice (Figure 6, F–H, and Table 2). CORT and/or 11-DHC also increased the expression of the lipolytic enzymes ATGL and HSL in wild-type mice, but CORT and not 11DHC increased expression of ATGL in 11β-HSD1 knockout mice (Figure 6, D and E, and Table 2).

Discussion

GC excess leads to skeletal muscle insulin resistance and T2DM; however, the precise molecular mechanisms underpinning these observations are not completely understood. Our observations have demonstrated that GCs dysregulate lipid metabolism in skeletal muscle, which may be crucial in regulating insulin sensitivity, although we have not shown a direct causal relationship. This has important implications, not only for the rare endogenous causes of GC excess but also, perhaps most relevant, for the health burden caused by adverse effects of GC therapy, which is prescribed to 1% to 2% of the populations of the United States and United Kingdom (25, 26).
Lipolysis

Knockout C57BL/6 Mice, Measured Using Real-Time PCR After Treatment with CORT (100 µg/mL, 0.66% ethanol), 11-DHC (100 µg/mL, 0.66% ethanol) or vehicle (0.66% ethanol) via Drinking Water for 5 Weeks

Table 2. mRNA Expression of Genes Involved in Key Lipid Metabolic Pathways in Wild-Type and 11β-HSD1 Knockout C57BL/6 Mice, Measured Using Real-Time PCR After Treatment with CORT (100 µg/mL, 0.66% ethanol), 11-DHC (100 µg/mL, 0.66% ethanol) or vehicle (0.66% ethanol) via Drinking Water for 5 Weeks

<table>
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<th>Gene</th>
<th>Vehicle</th>
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<th>11DHC</th>
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<tr>
<td>ACC1</td>
<td>0.94 ± 0.15</td>
<td>0.39 ± 0.09</td>
<td>0.48 ± 0.08</td>
<td>1.14 ± 0.08</td>
<td>0.67 ± 0.16</td>
<td>1.05 ± 0.07</td>
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<td>FAS</td>
<td>4.38 ± 0.95</td>
<td>1.31 ± 0.56</td>
<td>1.68 ± 0.50</td>
<td>3.68 ± 0.53</td>
<td>1.48 ± 0.75</td>
<td>3.02 ± 0.34</td>
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<tr>
<td>GPAT</td>
<td>3.15 ± 0.64</td>
<td>1.80 ± 0.22</td>
<td>2.18 ± 0.43</td>
<td>2.97 ± 0.51</td>
<td>1.81 ± 0.61</td>
<td>3.28 ± 0.43</td>
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<td>DGAT</td>
<td>0.33 ± 0.06</td>
<td>0.31 ± 0.07</td>
<td>0.31 ± 0.06</td>
<td>0.18 ± 0.05</td>
<td>0.19 ± 0.05</td>
<td>0.18 ± 0.03</td>
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<tr>
<td>SREBP1c</td>
<td>0.24 ± 0.04</td>
<td>0.31 ± 0.05</td>
<td>0.25 ± 0.06</td>
<td>0.17 ± 0.06</td>
<td>0.18 ± 0.04</td>
<td>0.15 ± 0.03</td>
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<tr>
<td>SCD1</td>
<td>26.93 ± 4.29</td>
<td>16.01 ± 7.17</td>
<td>13.80 ± 3.52</td>
<td>9.00 ± 3.06</td>
<td>3.14 ± 1.14</td>
<td>5.05 ± 2.55</td>
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<tr>
<td>SCD2</td>
<td>0.85 ± 0.16</td>
<td>0.67 ± 0.17</td>
<td>0.66 ± 0.19</td>
<td>0.53 ± 0.29</td>
<td>0.55 ± 0.18</td>
<td>0.48 ± 0.06</td>
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<td>Lipolysis</td>
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<tr>
<td>HSL</td>
<td>0.81 ± 0.17</td>
<td>1.17 ± 0.16</td>
<td>1.17 ± 0.30</td>
<td>0.56 ± 0.23</td>
<td>0.93 ± 0.13</td>
<td>0.40 ± 0.15</td>
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<td>ATGL</td>
<td>0.82 ± 0.02</td>
<td>1.46 ± 0.04</td>
<td>1.21 ± 0.03</td>
<td>0.51 ± 0.01</td>
<td>0.70 ± 0.01</td>
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<td>β-Oxidation</td>
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<td>ACC2</td>
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<td>6.58 ± 0.91</td>
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<td>1.88 ± 1.00</td>
<td>4.20 ± 0.63</td>
<td>1.28 ± 0.75</td>
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<td>PDK4</td>
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<td>93.13 ± 19.56</td>
<td>83.12 ± 15.87</td>
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<td>30.18 ± 15.85</td>
<td>14.22 ± 8.95</td>
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<td>PGC-1a</td>
<td>8.17 ± 3.19</td>
<td>20.13 ± 4.32</td>
<td>20.06 ± 5.59</td>
<td>4.30 ± 2.41</td>
<td>13.35 ± 2.52</td>
<td>4.51 ± 0.94</td>
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<td>ACOX1</td>
<td>1.18 ± 0.13</td>
<td>2.28 ± 0.54</td>
<td>2.04 ± 0.53</td>
<td>1.13 ± 0.27</td>
<td>1.89 ± 0.39</td>
<td>0.93 ± 0.35</td>
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<td>CPT-1b</td>
<td>2.59 ± 0.23</td>
<td>5.04 ± 0.83</td>
<td>5.33 ± 0.93</td>
<td>2.26 ± 0.26</td>
<td>5.02 ± 0.73</td>
<td>2.80 ± 0.91</td>
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<tr>
<td>SPT1</td>
<td>0.13 ± 0.01</td>
<td>0.19 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.12 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>ASAH1</td>
<td>0.008 ± 0.00009</td>
<td>0.009 ± 0.001</td>
<td>0.008 ± 0.003</td>
<td>0.008 ± 0.003</td>
<td>0.001 ± 0.002</td>
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<td>UGCG</td>
<td>0.64 ± 0.00009</td>
<td>0.91 ± 0.2</td>
<td>0.93 ± 0.08</td>
<td>0.58 ± 0.15</td>
<td>0.85 ± 0.12</td>
<td>0.64 ± 0.18</td>
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<td>LASS1</td>
<td>0.029 ± 0.003</td>
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<td>0.019 ± 0.004</td>
<td>0.025 ± 0.004</td>
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<td>0.030 ± 0.001</td>
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<tr>
<td>LASS6</td>
<td>0.0015 ± 0.00002</td>
<td>0.0017 ± 0.00001</td>
<td>0.0014 ± 0.00002</td>
<td>0.0015 ± 0.00005</td>
<td>0.0015 ± 0.00003</td>
<td>0.0020 ± 0.00008</td>
</tr>
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</table>

Data are expressed as arbitrary units ± SE. In vivo studies were not powered to detect changes in gene expression. Wild-type: vehicle, n = 10; CORT, n = 7; 11DHC, n = 8; 11β-HSD1 knockout: vehicle, n = 7; CORT, n = 8; 11DHC, n = 6.

Statistical analyses were performed on ΔC values using 2-way ANOVA: *, P < .05; b, P < .01 vs vehicle-treated wild-type mice; c, P < .05; d, P < .01 vs vehicle-treated 11β-HSD1 knockout mice.

We have shown that GCs decreased DNL while enhancing β-oxidation and lipid mobilization. These observations hold true in both cell lines (human and rodent) and in whole animal models (Supplemental Figure 3). GCs inhibited DNL in skeletal muscle, and this was associated with increased inactivating serine-79 phosphorylation of ACC1 and decreased ACC1 and FAS gene expression (although no change in FAS was observed at the protein level). AMPK has been shown to phosphorylate and inhibit ACC1 (5). In addition, tissue-specific regulation of AMPK by GC is well described in adipose tissue and liver (22, 27). In our study, GCs increased activating phosphorylation of AMPK both in vitro and in vivo, and therefore the inhibitory actions of GCs upon DNL may be driven by an AMPK-dependent mechanism. However, in vitro we observed changes in the phosphorylation of AMPK at the lowest doses of DEX used (5 nM). This did not translate to a significant reduction in DNL and may indicate that other mechanisms are involved. GPAT catalyzes the first and committed step of glycerolipid biosynthesis. AMPK phosphorylates and inactivates GPAT (6), and therefore activation of AMPK by GCs may provide an additional mechanism to further suppress the synthesis of glycerolipids in skeletal muscle. Although GC decreased GPAT expression, we observed no effect of GCs on total GPAT protein levels in vivo.

In contrast to their effects on lipid storage, our data suggest that GCs elevate intramyocellular lipid mobilization by increasing the expression of the lipolytic enzymes ATGL and HSL. Increased lipolysis may afford increases in lipid intermediates including DAGs, which have been shown to accumulate in insulin-resistant skeletal muscle (28). The mechanism by which DAGs mediate insulin resistance is believed to involve activation of PKCθ, leading to inhibitory serine-307 phosphorylation of insulin receptor substrate 1 (IRS1), a key component of the insulin signaling cascade (28, 29). Notable, we observed elevated intramyocellular DAG levels in our whole animal models after GC administration. We have shown previously that GCs induce inhibitory serine-307 phosphorylation of IRS1 in murine skeletal muscle in vitro and in vivo (14), and it is therefore plausible that DAG-mediated PKCθ ac-
tivation is a crucial step underpinning GC-induced insulin resistance. Accumulation of ceramides in skeletal muscle has been implicated in the pathogenesis of insulin resistance (30). Serine palmitoyl transferase-1 (SPT1), which catalyzes the first step of ceramide synthesis, was increased by GCs, but this increase did not achieve statistical significance. Expression levels of other genes regulating intramyocellular ceramide levels were unchanged after GC treatment in our animal models (Table 2). This result tentatively suggests that DAG, rather than ceramide accumulation, may be more important in driving GC-induced skeletal muscle insulin resistance. Although we have suggested that GCs acting through altered lipid metabolism drive insulin resistance, demonstration of cause vs consequence remains difficult. It is plausible that GC-induced insulin resistance mediated by an alternative mechanism could limit the action of insulin to store lipid and inhibit lipolysis, therefore enhancing intramyocellular DAG generation.

β-Oxidation is increased by GCs, and our data are consistent with reports published previously (31). This is likely to be driven by increased expression of PDK4 (a key metabolic switch between glucose and lipid metabolism) as well as increased expression of several mitochondrial biomarkers (CPT-1b, ACOX1, and PGC-1α). Furthermore, GC-mediated AMPK activation leading to ACC1/2 inactivation is consistent with increased fatty acid oxidation, because the catalytic products of mitochondrial membrane-associated ACC2 (malonyl CoA) inhibit fatty acyl transport across the inner mitochondrial membrane via CPT-1b (32).

The impact of GCs on intramyocellular lipid homoeostasis is consistent with their known catabolic actions. Physiologically, GC levels spike during fasting and promote energy release while suppressing energy storage. Although this increase is vital during fasting, it may become detrimental if GC levels remain persistently elevated, driving fatty acid mobilization and increased generation of lipid intermediates such as DAGs rather than safe storage. In contrast to the metabolic actions of GCs, insulin promotes energy storage and inhibits energy utilization. Because skeletal myocytes are exposed to both GCs and insulin in vivo, we examined the effects of insulin and GC coincubated with insulin on skeletal muscle lipid metabolism in vitro. Although GCs have been found to augment the actions of insulin to stimulate DNL in cultured adipocytes (22), we have not found this to be the case in skeletal muscle. Instead, insulin attenuates GC-mediated suppression of DNL, which may be a physiologically relevant mechanism whereby insulin overrides the catabolic actions of GC to promote energy storage in the transition from the fasted to the fed states.

In addition to the effect of exogenous GCs, we have shown that prereceptor metabolism of endogenous GCs by 11β-HSD1 is a crucial regulator of lipid metabolism in skeletal muscle. 11β-HSD1 is expressed and biologically active in human skeletal muscle (14). Overexpression has been described in rodent and human models of T2DM and insulin resistance (15–17), and selective 11β-HSD1 inhibitors have consistently demonstrated an ability to cause insulin sensitization in preclinical and clinical studies (33, 34).

In this study, we have shown that selective 11β-HSD1 inhibition blocks 11DHC-mediated decreased lipogenic and increased lipolytic gene expression. We did not examine the effect of the selective inhibitor, A1, in combination with CORt which is a potential limitation in the study. However, it was without effect when used in isolation. In our in vivo models, CORT- and 11DHC-treated wild-type mice achieved high circulating CORT levels, which would suppress the hypothalamo-pituitary-adrenal axis. In these animals and in CORT (but not 11DHC)-treated 11β-HSD1 knockout mice, we observed decreased lipogenic gene expression, whereas lipolytic and fatty acid oxidative genes expression are increased. CORT- and 11DHC-treated wild-type mice and CORT (but not 11DHC)-treated 11β-HSD1 knockout mice had increased inactivating serine-79/218 phosphorylation of ACC1/2 and increased activating threonine-172 phosphorylation of AMPK. We have also demonstrated that 11β-HSD1 is not only able to regulate AMPK activation but also DAG generation. This is likely to explain our previous observations demonstrating that selective 11β-HSD1 inhibitors decrease serine-307 phosphorylation of IRS1 (14) and offers a mechanism by which this class of agents increases insulin sensitivity.

In conclusion, we have demonstrated that GCs have potent actions on skeletal muscle lipid homoeostasis (Supplemental Figure 3). Manipulation of GC availability through selective 11β-HSD1 inhibition alters lipid flux and modulates intramyocellular DAG content, and this may underpin their putative action as insulin sensitizers.

Acknowledgments

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S.A.M., L.L.G., C.S., Z.K.H.-S., I.B., P.G., and L.A. generated data for the manuscript. S.A.M., D.M.S., P.M.S., G.G.L., and J.W.T. all contributed to the writing and revision of the manuscript. The study protocols were devised by S.A.M. and J.W.T.

Disclosure Summary: D.M.S. is an employee of AstraZeneca. The other authors have nothing to disclose.

References