Effect of excess corticosterone on LKB1 and AMPK signaling in rat skeletal muscle

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Nakken GN, Jacobs DL, Thomson DM, Fillmore N, Winder WW. Effects of excess corticosterone on LKB1 and AMPK signaling in rat skeletal muscle. J Appl Physiol 108: 298–305, 2010. First published December 3, 2009; doi:10.1152/japplphysiol.00906.2009.—Cushing’s syndrome is characterized by marked central obesity and insulin insensitivity, effects opposite those seen with chronic AMP-activated protein kinase (AMPK) activation. This study was designed to determine whether chronic exposure to excess glucocorticoids influences LKB1/AMPK signaling in skeletal muscle. Corticosterone pellets were implanted subcutaneously in rats (hypercorticosteronemia, Hypercort) for 2 wk. Controls were sham operated and ad libitum or were sham operated and food restricted (pair-weighted group, Pair) to produce body weights similar to Hypercort rats. At the end of the 2-wk treatment period, rats were anesthetized, and the right gastrocnemius-plantaris (gastroc) and soleus muscles were removed. Left muscles were removed after electrical stimulation for 5 min. No significant differences were noted between treatment groups in ATP, creatine phosphate, or LKB1 activity. The α- and β-subunit isoforms were not significantly influenced in gastroc by corticosterone treatment. Expression of the γ3-subunit decreased, and γ1- and γ2-subunit expression increased. Both α2-AMPK and α1-AMPK activities were increased in the gastroc in response to electrical stimulation, but the magnitude of the increase was less for α2 in the Hypercort rats. Despite elevated plasma insulin and elevated plasma leptin in the Hypercort rats, phosphorylation of TBC1D1 was lower in both resting and stimulated muscle compared with controls. Malonyl-CoA content was elevated in gastroc muscles of resting Hypercort rats. These changes in response to excess glucocorticoids could be responsible, in part, for the decrease in insulin sensitivity and adiposity seen in Cushing’s syndrome.

Cushing’s syndrome: glucocorticoids

CUSHING’S SYNDROME IS CHARACTERIZED BY MARKED CENTRAL OBESITY AND INSULIN INSENSITIVITY, BUT THE ETOLOGY OF THESE EFFECTS IS NOT CLEARLY DEFINED. IN SKELETAL MUSCLE, AMP-ACTIVATED PROTEIN KINASE (AMPK) IS PHOSPHORYLATED AND ACTIVATED BY LKB1 IN RESPONSE TO THE RISE IN ATP/AMP RATIO. IMPORTANT ROLES HAVE BEEN ELUCIDATED FOR THE LKB1/AMPK SIGNALING SYSTEM IN SKELETAL MUSCLE, INCLUDING STIMULATION OF GLUCOSE UPTAKE AND INSULIN SENSITIVITY, EFFECTS OPPOSITE OF THOSE OF THE CITRIC ACID CYCLE. LKB1/AMPK ACTIVATION IS MEDIANED BY THE UPSTREAM KINASES, LKB1-STRAD-MO25 AND CaMKK-β. THE β-SUBUNIT LINKS THE α- AND γ-SUBUNITS AND ALSO HAS A GLYCOCEN-BINDING DOMAIN. THE γ-SUBUNIT CONTAINS BINDING SITES FOR AMP AND ATP. BINDING OF ATP INHIBITS ACTIVATION, WHEREAS AMP MAKES THE COMPLEX A POORER SUBSTRATE FOR THE INACTIVATING PHOSPHATASE. A LARGE VARIETY OF COMBINATIONS OF SUBUNITS IS POSSIBLE BECAUSE THERE ARE TWO α-ISOFORMS (α1, α2), TWO β-ISOFORMS (β1, β2), AND THREE γ-ISOFORMS (γ1, γ2, γ3). SPICE VARIANTS HAVE ALSO BEEN DESCRIBED, THUS INCREASING THE COMPLEXITY OF POSSIBLE COMBINATIONS. AN INCREASE IN THE AMP:ATP RATIO IN RESPONSE TO MUSCLE CONTRACTION OR HYPOXIA RESULTS IN ACTIVATION OF AMPK, WHICH THEN PHOSPHORYLATES DOWNSTREAM TARGETS, RESULTING IN INCREASED GLUCOSE UPTAKE AND FATTY ACID UPTAKE AND OXIDATION, WHICH CONTRIBUTE TO INCREASED AMPK ACCUMULATION. AMPK HAS ALSO BEEN REPORTED TO BE ACTIVATED IN MUSCLE BY THE ADIPOKINES LEPTIN AND ADIPOGENIN.

Previous studies have demonstrated significant changes in AMPK in different tissues in response to excess glucocorticoids. Patients with Cushing’s syndrome have a 70% reduction in AMPK activity in adipose tissue (22). A single injection of dexamethasone into rats has been reported to increase AMPK expression and activity in heart over a 4-h time course (30). Isolated incubated epitrochlearis muscles, but not soleus muscles, have been reported to have less AMPK activation in response to contraction when isolated from rats injected with dexamethasone (1 mg/kg per day) compared with controls (35). A rat model of Cushing’s syndrome induced by subcutaneous implantation of a corticosterone pellet coupled with provision of 30% sucrose in drinking water resulted in a decrease in AMPK activity in adipose tissue and heart, an increase in AMPK activity in liver and hypothalamus, and no effect in skeletal muscle (8). The purpose of the present study was to more fully characterize the effect of chronic exposure of muscle to excess glucocorticoids on skeletal muscle LKB1/AMPK signaling. We hypothesized that excess glucocorticoids would decrease LKB1/AMPK signaling in skeletal muscle, thus contributing to the increase in lipid storage and decrease in insulin sensitivity seen in Cushing’s syndrome. To examine this question, muscles from control and chronically hypercorticosteronemic rats were studied in the resting state and also after electrical stimulation. This allowed, not only measurement of prevailing LKB1/AMPK activities, but also assessment of capacity of the signaling system to be activated in response to muscle contraction.

MATERIALS AND METHODS

Materials. Reagents were obtained from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise stated. Primary antibodies obtained from Cell Signaling Technologies (Danvers, MA) include the following: phospho-acetyl-CoA carboxylase (P-ACC) antibody (cat. no. 3661), total α-AMPK antibody (cat. no. 3661), phospho-AMPK
P-AMPK α-antibody (cat. no. 2535). LKB1 antibody was purchased from Upstate (cat. no. 07694). SAMS peptide was obtained from Zinsser Analytic (Maidenhead, Berkshire, UK), and LKB-tide was from Upstate (cat. no. 07694). SAMS peptide was obtained from liquid nitrogen temperature, weighed, and stored at −80°C until analysis. LKB1 activity was assessed as previously described (40).

**LKB1 activity.** Starting with 50 μl of 10% rested gastroc homogenate from each animal, LKB1 was immunoprecipitated, and activity was assessed as previously described in the AMPK activity protocol with a few modifications. LKB1 antibody (Santa Cruz) was used in place of the α1- or α2-AMPK antibodies and the LKB1 + antibody + G-Sepharose complex was washed twice with 1 ml wash buffer A (homogenization buffer + 0.5 M NaCl) and then twice with 1 ml of wash buffer B (40 mM HEPES, 80 mM NaCl, 8% glyceral, 0.8 mM EDTA, 5 mM MgCl₂, 0.8 mM DTT). LKB-tide (0.2 mM) was used in place of SAMS-peptide, and the incubation period was extended to 15 min. Finally, 40 μl of the final mix was transferred to a half piece of Whatman P81 filter paper (2.5 cm) and allowed to absorb for 30 s before stopping the reaction in phosphoric acid.

**Western blotting.** Tissue homogenates (10% for gastroc, 5% for soleus) were prepared as in the AMPK activity assay protocol. Homogenate + Laemmli’s buffer mix was loaded into each well of a 5, 7.5, or 10% Tris · HCl gel. Proteins were then separated by SDS-PAGE, at 200 V, 3 A, for 50 min in Criterion Precast Gels (Bio-Rad, Hercules, CA) filled with running buffer (Bio-Rad cat. no. 161-0732). Proteins were then transferred to PVDF membranes at 100 V, for 45–60 min, in Western blot transferring buffer (0.2 M Glycine, 25 mM Tris base, 20% methanol, chilled to 4°C). Next, membranes were blocked in Tris-buffered saline with Tween (TBST) and 5% blotting grade blocker nonfat dry milk for 1 h. Membranes were then incubated overnight at 4°C in the primary antibody and diluted in 1% BSA in TBST. The next day, membranes were washed for four times each in TBST and incubated for 1 h with a horseradish peroxidase-linked anti-rabbit antibody and then washed four times for 5 min each in TBST. Membranes were covered with Amersham enhanced chemiluminescence (ECL) + plus Western blot detection reagent (GE Healthcare, Buckinghamshire, UK) for 5 min. Excess ECL was then removed. Finally, the blots were developed using Classic Blue Autoradiography film (Midsci, St. Louis, MO). Protein expression was assessed and quantified by measuring band size and intensity with AlphaEaseFC software (Alpha Innotech, San Leandro, CA). The control rest band mean optical density readings were set to 1, and the mean optical density readings of all other bands were expressed relative to the control rest band.

**Immuno precipitation of AS160 and Western blot analysis of TBC1D1.** AS160 was immunoprecipitated via an anti-AS160 antibody made by Cell Signaling Technologies (cat. no. 07-741). Protein G-Sepharose beads (cat. no. P3296), from Sigma Aldrich Chemical were used to bind anti-AS160 antibodies, which were left overnight on the roller mixer at 4°C. Bead + antibody + protein complexes were centrifuged for 5 min at 10,000 g; then supernatant was carefully separated from the pellet and placed on ice. Pellet was washed with 1 ml of homogenization buffer, centrifuged again, and washed three times as before with 1 ml homogenization buffer + 1 M NaCl. Laemmli’s buffer was then added, the samples were heated for 4 min at 95°C, and gels and Western blots were run as previously described probing for AS160, or phospho-AS160 (PAS) using an anti-PAS antibody (Cell Signaling Technologies, cat. no. 07-802). Finally, the supernatant that had been cleared of AS160 was prepared with Laemmli’s buffer. Gels were run and Western blots were probed using the anti-PAS antibody, which detects phosphorylated TBC1D1.

**Tissue metabolites and hormone assays.** Glycogen (29), blood glucose (3), malonyl-CoA (26), creatine (43), creatine phosphate (CP), ATP (18), lactate (13), and free AMP (10) concentrations were determined on muscle extracts as previously described. Plasma corticosterone, leptin, and insulin concentrations were measured using ELISA assay. Leptin and insulin ELISA kits were purchased from Crystal Chem (leptin kit cat. no. 90060, insulin kit cat. no. 90040; Downers Grove, IL). The rat corticosterone kit was from Diagnostic Systems Laboratories (Webster, TX). Assays were performed according to vendors’ protocols.

**Animal care.** All procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Male Sprague-Dawley rats were purchased from Charles River, Wilmington, MA. Hypercorticosteronemia (Hypercort group) was induced in 9 rats via subcutaneous insertion of three 100-mg corticosterone pellets between the scapulas. A control group (n = 8) and a pair-weighted group (Pair) (n = 8) were sham operated. The treatment lasted 14 days, after which, plasma corticosterone levels were tested. To verify chronically elevated corticosterone levels in the Hypercort group, four indicators were measured: adrenal atrophy, increased visceral fat, heart hypertrophy, and atrophy of the gastrocnemius-plantaris (gastrocnemius) muscles. Collectively, these factors provided evidence of long-term hypercorticosteronemia in the treatment group.

Because of decreased chow intake and weight loss in the Hypercort group, it was necessary to ensure that differences seen in protein expression were attributable to corticosterone treatment and not attributable simply to variations in body weights. To address this concern, food intake was restricted in the Pair rats to ensure similar mean rat weights at the time of euthanasia.

Rats were housed in a temperature-controlled (21–22°C) room with a 12-h:12-h light/dark cycle (dark beginning at 5:30 PM). Rats were fed standard rat chow (Harlan-Teklad rodent diet, Madison, WI) and water ad libitum. Chow intake was measured every 24 h, and rat weights were measured frequently.

**Tissue and blood collection.** On the 14th day of treatment, rats were anesthetized with pentobarbital sodium (48 mg/kg body wt) at least 20 min before beginning tissue collection and muscle stimulation procedures. The right gastroc and soleus were removed and clamp frozen at liquid nitrogen temperature, weighed, and stored at −95°C until analysis. To activate AMPK via muscle contraction, the left tibial nerve was isolated and stimulated at a frequency of 1/s, 10-ms duration, 10 V, for 5 min. Following tibial nerve stimulation, the left soleus and left gastroc were frozen. Blood samples were collected from the descending aorta. Exactly 0.5 ml of blood was added to 2 ml 10% perchloric acid. Following centrifugation the supernatant was frozen for later analysis of blood glucose. The remaining heparinized blood was centrifuged to collect plasma for analysis of corticosterone, insulin, and leptin. After blood collection, the heart was then removed and clamp frozen. Retroperitoneal and perirenal fat pads and adrenal glands were removed and weighed. Muscles were weighed and then stored at −95°C until analyzed. Muscle homogenates were prepared by adding 1 ml of homogenization buffer [50 mM Tris·HCl, 250 mM mannitol, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 3% Triton X-100, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 μg/ml soybean trypsin inhibitor (STI), pH 7.4] to 100 mg gastroc or 50 mg soleus, which had been ground to powder at liquid nitrogen temperature. The sample was then homogenized. After centrifugation at 10,000 g for 10 min, the supernatant was frozen for later analysis.

**AMPK activity.** Activity for both AMPK-α1 and AMPK-α2 was tested separately. AMPK-α1 or -α2 antibody was linked to protein-G Sepharose and then incubated with muscle homogenate at 4°C overnight. The next day, nonspecifically bound protein was removed by washing twice in 1 ml of immunoprecipitation buffer (1 M NaCl, 50 mM Tris·HCl, 150 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 μg/ml STI pH 7.4), followed by one wash in 0.5 ml of lysis buffer (62.5 mM HEPES, 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM Na pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, and 5 μg/ml STI). Finally, AMPK activity was assessed as previously described (40).

**Tissue metabolites and hormone assays.** Glycogen (29), blood glucose (3), malonyl-CoA (26), creatine (43), creatine phosphate (CP), ATP (18), lactate (13), and free AMP (10) concentrations were determined on muscle extracts as previously described. Plasma corticosterone, leptin, and insulin concentrations were measured using ELISA assay. Leptin and insulin ELISA kits were purchased from Crystal Chem (leptin kit cat. no. 90060, insulin kit cat. no. 90040; Downers Grove, IL). The rat corticosterone kit was from Diagnostic Systems Laboratories (Webster, TX). Assays were performed according to vendors’ protocols.
RESULTS

Atrophic adrenal glands, muscle atrophy, and reduced body weight provide evidence of chronic hypercorticosterone exposure. Plasma corticosterone was 305 ± 92 ng/ml in controls, 249 ± 56 ng/ml in Hypercort, and 152 ± 51 ng/ml in Pair rats. Baseline values of plasma corticosterone in blood collected from catheterized rats have been reported to be in the 9-15 ng/ml range (42). The elevated corticosterone in the control and Pair rats was likely due to the stress of the anesthesia and electrical stimulation of the tibial nerve. In probing for evidence of chronically elevated corticosterone levels, we noted severe atrophy of the Hypercort adrenal glands. Mean adrenal weights at the time of euthanasia were as follows: control, 50.1 ± 2.1 mg; Hypercort, 20.6 ± 0.4 mg; and Pair, 44.5 ± 2.2 mg. When adrenal weight was expressed as a percentage of body weight, values were as follows: control, 0.0168 ± 0.0011%; Hypercort, 0.0093 ± 0.0004%; and Pair, 0.0192 ± 0.0006%. Hypercort values were significantly lower than either control or Pair (P < 0.05, n = 16–17). Because the Hypercort rats had atrophic adrenals, it is likely that the corticosterone levels in these rats were chronically elevated because of slow release from the corticosterone pellet implants. The consequent high corticosterone level would be expected to have chronic negative feedback on anterior pituitary corticotropes, resulting in inhibition of adenocorticotropic hormone release and adrenal atrophy.

Combined retroperitoneal and perirenal fat pads were used as a measure of visceral fat. Mean fat pad values in the Hypercort group were 2.5 ± 0.24 g compared with 1.6 ± 0.11 g in the control group. Because of food intake restriction, Pair mean fat pad weights were only 0.45 ± 0.09 g. When expressed as a percentage of total body weight, values were as follows: control, 0.52 ± 0.03%; Hypercort, 1.88 ± 0.78%; and Pair, 0.20 ± 0.04%. The Hypercort values were significantly increased over both control groups (P < 0.05, n = 8–9).

Whereas mean heart weights were similar in the Hypercort group compared with the control (0.83 ± 0.02 g; Hypercort, 0.81 ± 0.03 g; and Pair, 0.69 ± 0.01 g), Hypercort rat hearts were 33% larger (P < 0.05) than the control group when compared as a percentage of total body weight (0.27 ± 0.02% vs. 0.36 ± 0.04%).

The Hypercort gastroc muscles were atrophied. The gastroc mean weight values were as follows: control, 1.83 ± 0.07 g; Hypercort, 1.14 ± 0.06 g; Pair, 1.56 ± 0.06 g. Expressed as a percentage of body weight, corresponding values were as follows: 0.61 ± 0.01%, 0.52 ± 0.01%, and 0.69 ± 0.02%. Hypercort was significantly decreased compared with either controls or Pair (P < 0.05, n = 16–17). Mean values for the soleus were as follows: control, 0.14 ± 0.007 g; Hypercort, 0.11 ± 0.004 g; Pair, 0.12 ± 0.004 g. When expressed as percentage of body weight, no difference was observed in soleus weight between treatment groups.

Another indicator of chronically elevated corticosterone levels is decreased chow intake and increased weight loss on the part of the Hypercort rats (15, 17). On the first day of treatment, average control rat weight was 275 ± 6.7 g, the Hypercort group weighed an average of 274 ± 4.9 g, and the Pair rats weighed an average 271 ± 8.6 g. Across the 14-day treatment period, the Hypercort group consumed an average 21.0 g chow per day. The control group averaged 22.5 g per day, whereas the Pair rats were restricted to an average 16.0 g per day. Across the course of treatment, the control group gained an average 2.1 ± 1.2 g/day. The Hypercort group lost an average 3.8 ± 1.5 g/day. The Pair group lost an average 3.8 ± 2.0 g/day. On the day of euthanasia, average rat weights were as follows: control, 310 ± 11 g; Hypercort, 223 ± 10 g; and Pair, 227 ± 10 g.

Gastroc LKB1 activity was not affected and AMPK-α2 activity after electrical stimulation was decreased in response to excess corticosterone. To test the hypothesis that an excess of glucocorticoids impairs energy signaling through inhibition of LKB1/AMPK, we first assayed LKB1 activity in the rested rat gastroc. No significant difference was seen in LKB1 activity between treatment groups (Fig. 1). We next assayed AMPK activity in both the rested and stimulated rat gastroc. Figure 2 shows that, although differences in gastro AMPK-α1 activity were not statistically significant, AMPK-α2 activity in response to electrical stimulation was decreased 42% in Hypercort rats (P < 0.05). Two-way ANOVA with repeated measures showed a significant decrease in AMPK-α2 activity in response to excess corticosterone (P < 0.05).

Gastroc P-AMPK in response to electrical stimulation was reduced in rats exposed to chronically elevated corticosterone. Coinciding with our findings of decreased AMPK-α2 activity, Western blot analysis showed a 55% decrease in P-AMPK in the electrically stimulated Hypercort gastroc compared with the control (Fig. 3). A 36% decrease in Hypercort rested soleus P-AMPK was observed compared with the control (P < 0.05).

Expression of γ-, but not α- and β-subunits were altered by chronic exposure to excess corticosterone. To determine whether decreases in AMPK-α2 activity were attributable to decreased levels of AMPK-α2 protein expression, Western blots of rested gastroc AMPK-α2 were performed. Blots showed no significant difference between treatment groups. Subsequent blots performed on the α1-, β1-, and β2-subunits of AMPK revealed no significant difference in protein expression (data not shown). The AMPK-γ3 subunit showed a 51% decrease in the Hypercort vs. control groups (Fig. 4). AMPK-γ1 was
increased 60%, and AMPK-γ2 was increased 15% over the control in response to GC (Fig. 4).

Glycogen was increased but ATP, CP, lactate and free AMP were not different in resting or electrically stimulated Hypercort rat muscle. AMPK is exceptionally sensitive to the energy state of its cellular environment. We thus examined ATP, CP, creatine, lactate, and free AMP levels in the gastroc to search for possible causative factors influencing the decreased activation state of AMPK. Although significant changes occurred in response to electrical stimulation, no significant difference was noted between the three treatment groups. Rested Hypercort glycogen levels were 32% higher than the control (Table 1).

Plasma insulin and leptin concentrations were elevated in the Hypercort rats. ELISA assay analysis showed more than a fivefold increase in circulating plasma leptin levels over the control. Values were 1.0 ± 0.2 ng/ml for control, 5.5 ± 1.1 ng/ml for Hypercort, and 0.4 ± 0.1 ng/ml for Pair (P < 0.05, n = 8–9). Insulin levels were significantly elevated (52%) in the Hypercort group vs. control or Pair (control = 2.7 ± 0.5 ng/ml, Hypercort = 4.1 ± 0.6 ng/ml, Pair = 1.8 ± 0.1 ng/ml, P < 0.05, n = 8–9).

Malonyl-CoA was elevated and P-TBC1D1 depressed in Hypercort rats. Contrary to our expectations, P-ACC was not significantly reduced in the gastroc (Table 1). Malonyl Co-A levels in the Hypercort gastroc were elevated over the control but failed to be significant compared with the Pair group (Table 1).

Western blot analysis showed no difference in AS160, PAS, Akt, or phosphorylated Akt (P-Akt) in the gastroc (Table 2). However, phosphorylated TBC1D1 (P-TBC1D1) was decreased 50% in the resting gastroc and 35% in the stimulated gastroc of the Hypercort group compared with the control (Fig. 5).

DISCUSSION

The objective of this experiment was to determine whether LKB1/AMPK signaling is altered in skeletal muscle of rats chronically exposed to elevated blood corticosterone. LKB1 and AMPK activities were not influenced at rest, but AMPK activity in response to electrical stimulation of the muscle was significantly reduced. This difference in AMPK activity occurred without concurrent differences in ATP, CP, creatine, or free AMP levels in the stimulated muscles. The decrease in AMPK activity was not due to decreased expression of the α- or β-subunits of AMPK. However, expression of the γ3-
subunit was decreased, and expressions of the γ1- and γ2-subunits were increased in the gastroc of Hypercort rats. Previous studies have demonstrated that, in contracting human muscle, only AMPK activity associated with γ3 immunoprecipitates was highly correlated with phosphorylation of the downstream target, ACC (4). Immunoprecipitations in the present study were with antibodies to the γ1- and γ2-subunits, so it is of interest that, when the γ3-subunit expression is reduced, the increase in γ2 activity elicited in response to

**Table 1. Effect of corticosterone treatment of rats on metabolite contents in resting and electrically stimulated gastrocnemius-plantaris muscle.**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control Rest</th>
<th>Hypercort Rest</th>
<th>Hypercort Stim</th>
<th>Pair Stim</th>
<th>Pair Rest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>34 ± 0.2</td>
<td>72 ± 0.7</td>
<td>28 ± 0.7</td>
<td>13 ± 0.2</td>
<td>26 ± 0.7</td>
</tr>
<tr>
<td>ATP</td>
<td>7.2 ± 0.2</td>
<td>26.8 ± 0.7</td>
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<tr>
<td>CP</td>
<td>31 ± 0.2</td>
<td>51 ± 0.7</td>
<td>15.6 ± 0.7</td>
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<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.7 ± 0.3</td>
<td>14.6 ± 0.7</td>
<td>0.7 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Free AMP</td>
<td>0.08 ± 0.01</td>
<td>1.01 ± 0.16</td>
<td>0.09 ± 0.01</td>
<td>0.52 ± 0.16</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>0.78 ± 0.11</td>
<td>0.78 ± 0.11</td>
<td>0.78 ± 0.11</td>
<td>1.00 ± 0.13</td>
<td>0.78 ± 0.11</td>
</tr>
<tr>
<td>P-ACC, relative to control</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 8–9/group. *Significantly different from corresponding rest value, P < 0.05. †Significantly different from corresponding rest value, P < 0.05. Hypercort, hypercorticosteronemia; Pair, pair-weighted group; CP, creatine phosphate; P-ACC, phospho-acetyl-CoA carboxylase.

**Fig. 4.** Effect of administration of excess corticosterone for 2 wk on expression of the three γ-subunits in gastrocnemius-plantaris muscles. *Significantly different from corresponding treatment in the control muscles, P < 0.05, n = 8–9/group.
muscle contraction was also reduced. Heterotrimeric complexes containing the γ3-subunit have been previously reported to be less sensitive to activation by AMP than are complexes containing the γ1- and γ2-subunits (7). Both mRNA and protein expression profiles in mouse and rat tissues demonstrate that the γ3-subunit is expressed to a much greater level in fast-twitch white (Type IIb) fibers than in either slow-twitch (Type I) fibers or heart (25). Coimmunoprecipitation studies in rodent muscle have demonstrated that the majority of the γ3 is associated with the α2- and β2-isoforms (25).

Phosphorylation of the downstream target, ACC, tended to be lower at rest in muscle of the Hypercort rats, but this difference was not significant. However, malonyl-CoA was almost twice as high in resting muscle of Hypercort rats compared with controls. If malonyl-CoA is constantly elevated, oxidation of fatty acids by the muscle would be expected to be constantly inhibited, thus partially explaining the accumulation of lipid stores in the abdominal region of these animals. Previous studies have demonstrated that, when malonyl-CoA is elevated in muscle, this results in decreased fat oxidation and increased lipid storage (32, 34). On the other hand, ACC2-null mutant mice that have very low levels of malonyl-CoA do not develop obesity in response to high-fat/high-carbohydrate diets similarly to wild-type mice (1, 2). Likewise, inhibitors of ACC administered to rats cause lower tissue concentrations of malonyl-CoA and stimulate whole body fat oxidation (16). The Hypercort rat model provides another example where elevated muscle malonyl-CoA is associated with central obesity.

Previous reports have shown that high levels of glucocorticoids can produce insulin resistance, at least in part by inhibiting GLUT4 translocation (9, 44, 45). Redundant pathways exist for inducing GLUT4 translocation and stimulation of glucose uptake by muscle. Best characterized, but still incompletely understood, is that mediated by the insulin receptor. This pathway involves insulin receptor phosphorylation of insulin receptor substrate 1 (IRS1), activation of phosphatidylinositol 3-kinase, phosphorylation/activation of AKT (protein kinase B), and phosphorylation of AS160 and/or TBC1D1. Both AS160 and TBC1D1 are rab-GTPase-activating proteins, which, in inactive GDP-associated states, are thought to prevent GLUT4 translocation (20, 39). Insulin- and contraction-induced phosphorylation of these proteins is thought to induce replacement of GDP with GTP, thus relieving inhibition and allowing translocation of GLUT4 to the sarcolemma and allowing increased glucose uptake. The contraction-induced stimulation of glucose uptake appears to involve both AMPK and a calcium-triggered pathway (19, 20, 33). Both AS160 and TBC1D1 have multiple phosphorylation sites, some of which can be detected with a PAS antibody. Both insulin signaling and muscle contraction pathways can converge on AS160 and TBC1D1, each having phosphorylation sites targeted by AKT and AMPK (6, 24, 36, 39). In probing these sites with the PAS antibody, we made a novel discovery. TBC1D1 phosphorylation is decreased 50% in the rested gastroc and 35% in the stimulated gastroc in response to high levels of corticosterone. We also noted a slight decrease in phosphorylated AS160 in the Hypercort-stimulated gastroc compared with control. This was unexpected, considering the high levels of insulin observed in the Hypercort group. Decreases in AS160 in the resting muscle PAS were completely reversed in response to stimulation. Although the essential roles of AS160 and TBC1D1 in allowing increased glucose transport in response to insulin and contraction are not entirely understood (20), it is possible that the decrease in PAS phosphorylation could account for the decrease in insulin sensitivity accompanying chronic hyperglucocorticoid conditions.

Ancillary to this study, we noted a discrepancy in our present understanding of the role of leptin in relationship to AMPK. Leptin is released from adipose tissue and plays a key role in regulating energy intake and expenditure (12). Increased fat stores, like those inherent in hypercorticosteronemia-induced central obesity, cause leptin levels to increase (23). As a purported activator of AMPK, under normal conditions, elevated leptin levels should shift lipid partitioning from lipid storage to fatty acid oxidation (27, 28, 37, 38, 41), thus

![Image](TBC1D1.png)

Fig. 5. Effect of administration of excess corticosterone for 2 wk on phospho-AS160 sites in Western blots of AS160 immunoprecipitates of gastrocnemius/plantarlis muscles. *Significantly different from controls and pair weight groups in resting and from controls in stimulated muscles, P < 0.05, n = 8–9/group.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control Rest</th>
<th>Control Stim</th>
<th>Hypercort Rest</th>
<th>Hypercort Stim</th>
<th>Pair Rest</th>
<th>Pair Stim</th>
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<tr>
<td>AS160</td>
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<td>0.73 ± 0.2</td>
<td>0.85 ± 0.2</td>
<td>1.27 ± 0.2</td>
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<tr>
<td>P-AS160</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>0.73 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>0.85 ± 0.2</td>
<td>1.27 ± 0.2</td>
</tr>
<tr>
<td>AKT</td>
<td>1.0 ± 0.1</td>
<td>n/a</td>
<td>0.9 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>P-AKT</td>
<td>1.0 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 8–9. No statistically significant differences were observed. n/a, not applicable; P-, phospho.
returning fat stores back to normal levels. There appears to be some interruption of this homeostatic mechanism in response to glucocorticoids, allowing the accumulation of fat in select body regions. We observed that, despite a fivefold increase in circulating leptin in the Hypercort group, lepbin failed to activate AMPK in skeletal muscle. A possible explanation for this discrepancy could be that the Hypercort rats developed leptin resistance. However, we did note a decrease in chow intake, suggesting that leptin is binding efficaciously at least to its hypothalamic receptor. It is possible that leptin resistance is tissue specific, skeletal muscle being more resistant than the hypothalamus. This disconnect between high leptin levels and muscle AMPK activity suggests that additional studies are need to clarify roles of this hormone in peripheral tissues.

The changes in AMPK-α2 activity and γ3-subunit expression were significant in the gastroc, which is composed predominantly of fast-twitch type II fibers, but not in the soleus muscle, which has predominantly slow-twitch type I fibers. Previous studies have indicated that high levels of glucocorticoids lead to atrophy of type II, but not type I, muscle fibers (21). Coinciding with these findings, we noted significant atrophy in the gastroc. Furthermore, as indicated above, AMPK-γ3 is expressed primarily in white, type IIB fibers (25). This may explain in part the fiber type specificity of the glucocorticoid-induced adaptations.

In summary, we hypothesized that high levels of glucocorticoids may play a role in modulating LKB1/AMPK signaling in skeletal muscle. From a rat model of induced Hypercort, we conclude that high levels of glucocorticoids cause a marked decrease in AMPK-α2 activity and AMPK-γ3-subunit expression in the gastrocnemius-plantaris muscles. Of particular interest is the decrease in TBC1D1 phosphorylation in both rested and stimulated muscle in response to chronically high levels of glucocorticoids. The reduction in AMPK activity with consequent decrease in TBC1D1 phosphorylation and elevated muscle malonyl-CoA may account for some of the metabolic syndrome-like symptoms, including insulin resistance and central obesity, associated with Cushing’s syndrome.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


