Dietary supplementation with vitamin E and C attenuates dexamethasone-induced glucose intolerance in rats

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Dietary supplementation with vitamin E and C attenuates dexamethasone-induced glucose intolerance in rats. Am J Physiol Regul Integr Comp Physiol 302: R49–R58, 2012. First published October 26, 2011; doi:10.1152/ajpregu.00304.2011.—Glucocorticoid excess induces marked insulin resistance and glucose intolerance. A recent study has shown that antioxidants prevent dexamethasone (DEX)-induced insulin resistance in cultured adipocytes. The purpose of this investigation was to examine the effects of dietary vitamin E and C (Vit E/C) supplementation on DEX-induced glucose intolerance in rats. We hypothesized that feeding rats a diet supplemented with Vit E/C would improve glucose tolerance and restore insulin signaling in skeletal muscle, adipose, and liver and prevent alterations in AMPK signaling in these tissues. Male Wistar rats received either a control or Vit E/C-supplemented diet (0.5 g/kg diet each of L-ascorbate and dl-alpha-tocopherol) for 9 days prior to, and during, 5 days of daily DEX treatment (subcutaneous injections 0.8 mg/g body wt). DEX treatment resulted in increases in the glucose and insulin area under the curve (AUC) during an intraperitoneal glucose tolerance test. The glucose, but not insulin, AUC was lowered with Vit E/C supplementation. Improvements in glucose tolerance occurred independent of a restoration of PKB phosphorylation in tissues of rats stimulated with an intraperitoneal injection of insulin but were associated with increases in AMPK signaling in muscle and reductions in AMPK signaling and the expression of fatty acid oxidation enzymes in liver. There were no differences in mitochondrial enzymes in triceps muscles between groups. This study is the first to report that dietary Vit E/C supplementation can partially prevent DEX-induced glucose intolerance in rats.

ELEVATED GLUCOCORTICOID LEVELS, as seen in individuals with Cushing’s syndrome, are associated with insulin resistance (reviewed in Ref. 9), while the administration of exogenous glucocorticoids impairs insulin action in both rodents (11) and humans (32). From a cellular perspective, glucocorticoids have been reported to attenuate the activation of distal insulin-signaling intermediates, such as PKB, in skeletal muscle (38, 39), adipocytes (6), and liver (37). Similarly, the ability of insulin-independent stimuli, such as hypoxia, to increase skeletal muscle glucose disposal is also impaired in rats treated with dexamethasone (DEX), a synthetic glucocorticoid (48). Interestingly, the induction of insulin resistance and impaired skeletal muscle glucose disposal occurs despite increases in the protein content of glucose transporter isoform (4GLUT4) (48).

Although the effects of glucocorticoids on glucose homeostasis and insulin signaling have been well described, the underlying mechanisms leading to these effects have not been clearly established. A growing body of work would suggest that the generation of reactive oxygen species and subsequent increases in oxidative stress are involved in the pathway(s) through which glucocorticoids induce insulin resistance. In support of this hypothesis, DEX, a synthetic glucocorticoid, induces oxidative stress in a variety of cell culture models (3, 20, 22), whereas the treatment of isolated muscle strips (12) or cultured adipocytes (35) with prooxidants, such as hydrogen peroxide or glucose oxidase, markedly inhibits insulin signaling. A causative role for oxidative stress in DEX-induced insulin resistance has been suggested by Houstis et al. (20), who reported that antioxidants prevented the development of DEX-induced insulin resistance in 3T3 adipocytes.

In addition to attenuating insulin signaling, glucocorticoid excess also inhibits the activity of AMPK, an enzyme that is a reputed mediator of insulin-independent glucose disposal (15). For instance, DEX administration via miniosmotic pumps (36), or supplementing the drinking water of rats with corticosterone (30), leads to reductions in AMPK signaling in rat skeletal muscle. These results suggest that DEX-induced glucose intolerance may be mediated at least, in part, by reductions in skeletal muscle AMPK activity and that antioxidant supplementation may be an effective means with which to prevent this effect.

Not only do glucocorticoids impair skeletal muscle glucose disposal but they also increase gluconeogenesis (34). This effect, at least ex vivo, is likely mediated by increases in gluconeogenic enzymes, as DEX has been shown to potently induce phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in cultured hepatocytes (27, 52). Interestingly, daily DEX injections induced oxidative stress in rat liver (42), whereas feeding db/db mice with the polyphenol antioxidant caffeic acid reduces the expression of PEPCK and G6Pase (24). These findings suggest that antioxidants may be an effective means with which to reduce DEX-mediated increases in gluconeogenic enzyme expression, and by extension reduce hepatic glucose output.
In liver, the activation of AMPK has been shown to decrease the expression of PEPCK and G6Pase (18, 25). Interestingly, several studies have reported that glucocorticoids cause increases in AMPK protein content and activity in this tissue (10, 46). These results present a paradox, as they suggest that DEX induces PEPCK and G6Pase, while at the same time increasing the activity of a kinase, which would reduce the expression of these enzymes. These findings would suggest that alternative mechanisms may be involved in the pathway(s) through which DEX modulates hepatic carbohydrate metabolism. Increases in hepatic fatty acid oxidation have been suggested to lead to increases in gluconeogenesis (26). In this regard, increases in AMPK activity in the liver would be expected to increase fatty acid oxidation and hepatic glucose output. At this juncture, it is unknown whether antioxidant supplementation would alter DEX-mediated changes in liver AMPK signaling.

The purpose of the present study was to explore the effects of antioxidant supplementation on DEX-induced impairments in glucose homeostasis in rats. We hypothesized that 1) dietary vitamin E and C supplementation would attenuate DEX-induced glucose intolerance, 2) these changes would be associated with a preservation of insulin signaling in peripheral tissues, and 3) feeding rats a vitamin E and C-rich diet would restore AMPK signaling in skeletal muscle, while reducing AMPK content and markers of fatty acid oxidation in the liver.

METHODS

Materials. Reagents, molecular weight markers, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON, Canada). ECL Plus was a product of GE Healthcare (cat. no. RPN2132; Baie d’Urfe Quebec). Antibodies against, p-AMPK threonine 172 (cat. no. 2531), p-AMPK serine 485/491 (cat. no. 4185), total AMPK (cat. no. 2793), phosphorylated acetyl-CoA carboxylase (p-ACC; cat. no. 3068), p-threonine 308 PKB (cat. no. 4185), total AMPK (cat. no. 2793), phosphorylated acetyl-CoA carboxylase (p-ACC; cat. no. 3068), p-threonine 308 PKB (cat. no. 4185), and p2A A subunit (cat. no. 2041) were purchased from Cell Signaling Technology (Danvers, MA). Anti-PEPCK antibodies (cat. no. 51004943) were purchased from Cayman Chemicals (Ann Arbor, MI), while an antibody against uncoupling protein 3 (UCP3; cat. no. PA1-055) was a product of Thermo Scientific (Rockford, IL). PDK4 antibodies (cat. no. AP7041b) were purchased from Abgent (San Diego, CA). Anti-α-actin antibodies (cat. no. A2172) were a product of Sigma (St. Louis, MO), and tubulin antibodies (cat. no. ab7291) were purchased from Abcam (Cambridge, MA). PGC-1 antibodies (cat. no. M16557) is not specified what isoform of PGC-1, α or β, this antibody detects and, hence, it is referred to as PGC-1 in this article) were from EMD Biosciences (Gibbstown, NJ), while G6Pase (cat. no. 25840) was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Cytochrome c (cat. no. M3A606), cytochrome-c oxidase subunit IV (COXIV; cat. no. MS407) and complex III subunit Core1 (CORE1; cat. no. MS303) antibodies were purchased from Mitosciences (Eugene, OR). An antibody recognizing the alpha and beta subunits of PP2C was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Glucose standards were obtained from NERL Diagnostics (cat. no. 1205; East Providence, RI), and glucose reagents were from Diagnostic Chemicals (Charlottetown, PEI, Canada; cat. no. 223–50). Rat insulin ELISA kits were purchased from Alpco. Powdered dexamethasone was purchased from Sigma (cat. no. D1159) and diluted to a working stock solution in sterile PBS at 10 mg/ml.

Treatment of rats. All treatment and housing protocols followed the Canadian Council on Animal Care guidelines and were approved by the Animal Use and Welfare Committee at the University of Alberta. Male Wistar rats (Charles River, Saint-Constant, Quebec, Canada) weighing −250 g were housed two per cage with a 12:12-h light-dark cycle (0600 to 1800). Following 1 wk of acclimatization, rats were randomly assigned into either control, DEX, or dexamethasone plus vitamin E and C (DEX + Vit E/C) groups and were fed one of two prepared powdered diets for 2 wk. The control diet contained (g/kg) 317.6 casein, 2.94 l-methionine, 275.4 dextrose, 264 corn starch, 59.8 cellulose, 59.8 mineral mix Bernhart-Tomarelli (cat. no. 170750; Harlan, Madison, WI), 0.35 sodium selenite, 0.28 gamma-sodium sulfate, 11.8 vitamin mix (AOAC International, formerly the Association for Official Analytical Chemists; cat. no. 40055; Harlan, Madison WI), 7.35 inositol, 1.62 chlorine chloride, 8.0 flax oil, 59.0 sunflower oil and 8.7% hydrogenated canola. The macronutrient content of the diet was 19% fat/wt, 27% protein wt/wt and 46% carbohydrate wt/wt with a polyunsaturated to saturated fat ratio of 0.5. The mineral and vitamin mix met all nutritional requirements for the rats. The control diet contained 200 mg/kg vitamin E and 47 mg/kg powdered vitamins A and D. As rats synthesize vitamin C endogenously, this vitamin is not included in the vitamin mix. Further details regarding the composition of the vitamin and mineral mix can be obtained from the manufacturer. The DEX + Vit E/C group was fed an identical diet but supplemented with 500 mg/kg each of l-ascorbate (cat. no. A4403; Sigma-Aldrich, Oakville, ON, Canada) and α-tocopherol (cat. no. T3251; Sigma-Aldrich). The final concentration of α-tocopherol in the supplemented diet was 700 IU/kg, 3.5 times the amount in the control diet. The control diet was supplemented with an equivalent mass of cellulose to maintain nutrient density. All groups were provided with diet and water ad libitum for 14 days. On each of the final 5 days of feeding, the DEX and DEX + Vit E/C groups were given weight-adjusted subcutaneous DEX injections (0.8 mg/kg body wt) between 0900 and 1100, while the control group received an equivalent volume of sterile PBS.

Intraperitoneal glucose tolerance test. Following the 14-day experimental period, an intraperitoneal glucose tolerance test was performed. After an overnight fast, and ~24 h following the last DEX injection, animals were given an intraperitoneal injection of glucose (2.0 g/kg body wt). Blood was collected in K2/EDTA-coated tubes at 0, 15, 30, 45, 60, 90, and 120 min via tail vein puncture for quantification of plasma glucose and insulin. Whole blood was centrifuged at 13,000 g for 10 min at 4°C. Plasma was aliquoted and stored at −20°C until analysis. Changes in glucose and insulin over time were plotted, and the area under the curve (AUC) was calculated for each.

In vivo insulin stimulation. At the end of a separate 14-day experimental period, ~24 h after the last DEX treatment and following an overnight fast, control, DEX, and DEX + Vit E/C groups were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and then given either a 10 U/kg body wt injection of insulin or an equivalent volume of sterile PBS. Ten minutes following the insulin or saline injection, triceps and soleus muscles, retroperitoneal adipose tissue, and liver were quickly dissected, and immediately clamped frozen to the temperature of liquid nitrogen. Samples were stored at −80°C until analysis.

Western blot analysis. Clamp-frozen muscle, liver, and adipose samples were homogenized in 10, 15, and 2 volumes, respectively, of ice-cold cel lysis buffer (cat. no. FNN0021; Invitrogen, Burlington, ON, Canada) supplemented with protease inhibitor cocktail (cat. no. P-2174; Sigma-Aldrich), phenylmethylsulfon fluoride (cat. no. P7626; Sigma) and sodium fluoride (cat. no. S7920; Sigma). Homogenized samples were briefly sonicated before being centrifuged at 2,500 g for 15 min at 4°C. Following centrifugation, the fat cake was

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Dexamethasone and Glucose Intolerance

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removed, and the infranatant was collected from adipose tissue homogenates. Supernatants were collected from skeletal muscle and liver homogenates. Protein content was determined in triplicate using the bicinchoninic acid method. Protein homogenates were standardized in Laemmlli buffer and were heated at 95°C for 5 min. Total protein content and/or phosphorylation status of PKB, AMPK, ACC, UCP3, PDK4, PEPCK, G6Pase, PP2A, PP2C, and LKB1 were determined as we have previously described (49). In short, proteins were separated on 6.25% (ACC), 10% [PKB, AMPK, PEPCK, G6Pase, UCP3, PDK4, PP2A, PP2C, LKB1, COREI], or 15% (COXIV, cytochrome c) polyacrylamide gels. Transfer of proteins to nitrocellulose membrane was performed for 2 h at 200 mA per tank for 2 h. Membranes were blocked with 5% (wt/vol) nonfat dry milk diluted in Tris-buffered saline-0.1% Tween-20 (TBST-5% milk) for 1 h at room temperature with gentle agitation. Blocked membranes were incubated overnight in either TBST-5% milk or 5% BSA (wt/vol) diluted in TBST containing the appropriate primary antibody at 4°C with gentle agitation. After overnight incubation, blots were briefly washed in TBST and were subsequently incubated in TBST-1% (wt/vol) nonfat dry milk containing a HRP-conjugated secondary antibody for 1 h at room temperature with gentle agitation. Bands were visualized using ECL+ and captured using a Typhoon Imaging system (GE Health Care, Piscataway, NJ). Relative band intensity was determined with ImageQuant software (GE Health Care). Ponceau staining (mitochondrial proteins), actin, or tubulin was used to verify equal loading.

**Determination of plasma glucose and insulin.** Plasma glucose was analyzed using the glucose oxidase method. Samples were analyzed in triplicate on a microplate reader. Plasma insulin was measured using ELISA assay kits specific for rats. Samples were run in triplicate, with average coefficients of variation of <5%.

**Markers of oxidative stress.** Total and reduced glutathione and 8-iso-prostaglandin F2α were used as markers of oxidative stress and were determined using commercially available kits. The methods for these assays are described in detail by the manufacturers.

**Acetyl CoA and NADH assays.** The content of acetyl coA and NADH was determined using commercially available kits and as described in detail by the manufacturers.

**Statistical analysis.** Data are presented as means ± SE. Group comparisons of all insulin-stimulated parameters were made using two-way ANOVA, while all other comparisons were made using a paired Student’s t-test (Table 1). Dietary vitamin E and C supplementation had no effect on fasting insulin levels in DEX-treated rats (Table 1).

**Glucose tolerance.** DEX-treated rats were glucose intolerant as shown by a significant (P < 0.05) approximately twofold increase in the glucose AUC during an intraperitoneal glucose tolerance test (Fig. 1). The consumption of a diet supplemented with vitamin E and C reduced the glucose AUC by ~25% (P < 0.05) in the DEX-treated rats. The insulin response to the intraperitoneal glucose challenge was increased ~5-fold in DEX-treated rats with vitamin E and C supplementation having no effect (Fig. 1).

**In vivo insulin signaling.** To assess the effects of dietary vitamin E and C supplementation on DEX-induced impairments in insulin signaling, rats were given a weight-adjusted bolus injection of insulin and retroperitoneal adipose tissue, liver, and triceps muscles harvested 10 min later. As seen in Fig. 2, DEX treatment led to significant (P < 0.05) impairments in the insulin-stimulated phosphorylation of PKB on both threonine 308 and serine 473 in all tissues examined. Despite reductions in fasting glucose levels and improvements in glucose tolerance, dietary supplementation with vitamin E and C did not enhance insulin-stimulated PKB phosphorylation in any of the tissues examined from DEX-treated rats. The total protein content of PKB was not different in tissues from control, DEX, or DEX-treated rats supplemented with vitamin E and C (data not shown).

**Markers of oxidative stress.** DEX treatment decreased total glutathione content in triceps muscles (control 0.71 ± 0.03, DEX 0.57 ± 0.02, and DEX + vitamin E/C 0.59 ± 0.07 μmol/g protein, P < 0.05 vs. control), and this was not prevented by vitamin E and C supplementation, a finding similar to what has been reported in skeletal muscle from high-fat fed mice (1). The concentration of 8-iso-prostaglandin F2α, a marker of oxidative damage to lipids, was reduced in triceps muscles from DEX-treated animals receiving vitamin E and C (control 177 ± 11, DEX 166 ± 18, and DEX + Vit E/C 106 ± 15 pg/μg protein, P < 0.05).

**AMPK signaling.** As seen in Fig. 3A, AMPK signaling was not altered in adipose tissue from DEX or DEX-treated rats supplemented with vitamins E and C. In the liver, DEX treatment increased the protein content and phosphorylation of AMPK on threonine 172 (P < 0.05). Similarly, the phosphorylation of acetyl coA carboxylase (ACC), a downstream substrate of AMPK was also significantly (P < 0.05) increased.

Table 1. The effects of dexamethasone and vitamin E/C supplementation on fasting glucose and insulin and changes in body weight

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dexamethasone</th>
<th>Dexamethasone + Vitamin E/C</th>
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<tbody>
<tr>
<td>Fasting glucose, mmol/l</td>
<td>6.25 ± 0.23</td>
<td>13.70 ± 1.03*</td>
<td>10.37 ± 1.28*#</td>
</tr>
<tr>
<td>Fasting insulin, ng/ml</td>
<td>0.71 ± 0.10</td>
<td>7.64 ± 0.90*</td>
<td>6.32 ± 1.0*</td>
</tr>
<tr>
<td>Initial body weight, g</td>
<td>258 ± 3</td>
<td>252 ± 4</td>
<td>249 ± 3</td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>362 ± 6</td>
<td>285 ± 5*</td>
<td>288 ± 4*</td>
</tr>
<tr>
<td>Average food intake prior to DEX, g/day</td>
<td>29.6 ± 0.5</td>
<td>29.0 ± 0.5</td>
<td>28.9 ± 0.4</td>
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<tr>
<td>Average food intake during DEX, g/day</td>
<td>29.6 ± 0.1</td>
<td>18.9 ± 1.5*</td>
<td>19.6 ± 1.0*</td>
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</table>

Data are presented as means ± SE for 10 rats/group. *Significantly different from control, P < 0.05. #Significantly different from dexamethasone, P < 0.05.
These alterations were prevented in DEX-treated rats fed a diet supplemented with vitamin E and C (Fig. 3B). When the phosphorylation of AMPK was expressed relative to total AMPK, there were no differences between groups (data not shown). AMPK is phosphorylated on serine residues 485 (AMPKα1) and 491 (AMPKα2), and this is thought to have an inhibitory effect on the activation of the enzyme (19, 40). Our treatments had no effect on AMPK serine 485/491 phosphorylation in the liver (control 1.0 ± 0.14, DEX 1.16 ± 0.18, and DEX + Vit C/E 1.19 ± 0.10). DEX and AO treatment had no effect on AMPK signaling in the soleus (data not shown). In contrast to the liver, AMPK protein content and threonine 172 phosphorylation were significantly (P < 0.05) reduced in triceps muscle from DEX-treated rats. This was prevented in rats receiving vitamins E and C (Fig. 3C). The ratio of phosphorylated to total AMPK was not different between groups (data not shown). Reductions in AMPK phosphorylation were not associated with changes in the protein content of PP2C and PP2A, phosphatases that deactivate AMPK (28, 51), or in the phosphorylation of LKB1, an upstream AMPK kinase (43) (Fig. 4). DEX treatment significantly (P < 0.05) reduced AMPK serine 485/491 phosphorylation in triceps muscles. This effect was not prevented by vitamin E/C supplementation (Fig. 4).

Gluconeogenic and fatty acid oxidation enzymes in liver. As seen in Fig. 5, left, DEX treatment significantly (P < 0.05) increased the protein content of PGC-1, UCP3, and PDK4 in liver. The provision of an antioxidant-rich diet attenuated the increase in PGC-1 and UCP3 and tended to reduce the increase in PDK4. While not reaching statistical significance, DEX treatment increased NADH levels ~20%, and this was prevented by vitamin E/C supplementation (control 17.4 ± 1.2, DEX 21.6 ± 2.6, DEX 17.6 ± 1.2 pmol/μg protein). AcetylCoA levels were not different between groups (data not shown). There were no differences in PEPCK and G6Pase protein content.

Markers of mitochondrial content in skeletal muscle. There were no differences in markers of mitochondrial content such as CORE1, COXIV, and cytochrome c in triceps muscles from DEX or DEX-treated rats provided with a vitamin E/C-supplemented diet (Fig. 6).

DISCUSSION

Exogenous administration of DEX leads to a rapid development of whole body (47) and skeletal muscle insulin resistance (6, 38, 39). Although the specific mechanisms mediating the effects of DEX on glucose homeostasis have not been fully defined, a recent study has suggested the involvement of oxidative stress. Houstis et al. (20) demonstrated that DEX-induced insulin resistance in 3T3 adipocytes was prevented by antioxidant treatment. To date, it is not clear whether antioxidants possess a similar beneficial effect in preventing DEX-induced impairments in glucose homeostasis in vivo. The results of the present study demonstrate that DEX-induced impairments in whole body glucose tolerance can be partially attenuated in rats fed a diet supplemented with vitamins E and C. However, it is not clear whether the beneficial effects of vitamins E and C are due to a reduction in DEX-induced oxidative stress. For instance, although 8-iso-prostaglandin F2α levels were reduced in rats fed a vitamin E and C-supplemented diet, 8-iso-prostaglandin F2α was not increased in DEX-treated rats. Similarly, vitamin E and C supplementation did not prevent DEX-mediated reductions in glutathione levels.

We reasoned that improved glucose tolerance would be associated with an increase in the activation of distal insulin-
signaling intermediates, such as PKB, in skeletal muscle, liver, and adipose, the primary tissues responsible for insulin-mediated glucose disposal. Unexpectedly, decreases in the insulin-induced phosphorylation of PKB on both serine and threonine sites were not prevented, despite improvements in glucose tolerance, in rats fed a diet supplemented with vitamins E and C. These findings suggest that the improvements in glucose tolerance with this diet occur independently of enhanced insulin signaling. While the apparent inconsistency between improved glucose tolerance and reduced insulin signaling is surprising, it is not unprecedented. In this regard Okamato et al. (31) reported that glucose tolerance, independent of enhanced insulin signaling was improved in liver-specific PDK-1-deficient mice following the restoration of glucokinase.

Recent studies have demonstrated that glucocorticoid excess can lead to reductions in the content and activity of AMPK in skeletal muscle (30, 36). Since AMPK has been shown to modulate insulin-independent glucose disposal, we reasoned that vitamin E and C supplementation may prevent the decline in AMPK signaling in this tissue. Consistent with this, we found that vitamin E and C supplementation prevented DEX-mediated reductions in the phosphorylation of AMPK on

Fig. 2. DEX-induced impairments in the insulin-mediated phosphorylation of PKB on serine 473 and threonine 308 are not improved by dietary vitamin E and C supplementation in adipose tissue (A), liver (B), and triceps (C). Following an overnight fast, rats were injected with a weight-adjusted bolus of insulin (10 U/kg body wt ip), and tissue was harvested 10 min postinjection. Data are presented as means ± SE for 9–10 samples per group. *Significant effect of insulin within the experimental the same experimental group, P < 0.05. #Significantly different from the insulin-treated condition from control animals, P < 0.05. Representative Western blots are shown to the right of the quantified data.
threonine 172, and ACC on serine 79, the AMPK phosphorylation site. Changes in AMPK/ACC phosphorylation are representative of, and correlate with, AMPK activity measured using the SAMS peptide (33). Previous work has shown that DEX treatment causes slight increases in skeletal muscle ADP and reductions in Pcr (13) levels. Thus, reductions in AMPK/ACC phosphorylation are likely not a result of increases in the energy charge (i.e., ATP, Pcr) of skeletal muscle.

The protein content of PP2C and PP2A, phosphatases that dephosphorylate AMPK (28, 51), were not altered. Although the activity of these enzymes was not determined, our results demonstrate that alterations in the content of these phospha-

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**Fig. 3.** DEX increases AMPK protein content and activation in rat liver, while decreasing AMPK signaling in triceps muscles. Vitamin E and C supplementation prevents these effects. Data are presented as means ± SE for 9 or 10 samples per group. *Significantly different from both control and DEX + Vit E/C groups. Representative Western blots are shown to the right of the quantified data.

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**A.** Retroperitoneal Adipose Tissue

**B.** Liver

**C.** Triceps

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**Diagrams showing protein content and phosphorylation levels for AMPK, p-AMPK, p-ACC, β-actin, AMPK α, and p-AMPK in control (C), dexamethasone (D), and dexamethasone + vitamin E/C (D+Vit E/C) groups for retroperitoneal adipose tissue, liver, and triceps muscles.**
tases do not account for changes in AMPK phosphorylation. These results do not discount a potential role for alterations in PP2C/A activity in the regulation of AMPK signaling in DEX-treated animals. Similar to PP2C/A content, the phosphorylation of the upstream AMPK kinase (43), LKB1, was not different between groups. The regulation of AMPK phosphorylation on threonine 172 is controlled not only by AMPK kinases and phosphatases, but it has recently been suggested that phosphorylation on serine residues 485 on AMPKα1 and 491 on AMPKα2 can inhibit threonine 172 phosphorylation (19, 40). Surprisingly, serine 485/491 phosphorylation was reduced in triceps muscles from both groups of DEX-treated rats. As the phosphorylation of AMPK on threonine 172 changed in parallel with the content of the enzyme, it seems likely that the modulation of AMPK signaling was a consequence of increases in the content of this enzyme.

Interestingly, we only saw reductions in AMPK signaling in triceps, an almost exclusively fast-twitch muscle (~96% fast twitch) (14), while in the slow-twitch (~84% type 1 fibers) (2) soleus, AMPK signaling was intact. These results, at least in our model, demonstrate the existence of differences between soleus and triceps muscles in the response to prolonged DEX treatment that may be related to the metabolic characteristics and/or fiber type of the muscles. In contrast, Nakken et al. (30) found that AMPK phosphorylation was reduced in soleus muscles from rats having subcutaneous corticosterone implants. These discrepant findings could be related to the mode of hormone administration or the marked alterations in glucose homeostasis that were present in our model.

A restoration of AMPK signaling would be expected to enhance insulin-independent glucose transport, and this may, in part, explain reductions in fasting glucose levels in DEX-treated rats supplemented with vitamin E/C. However, although the vitamin-supplemented diet completely restored AMPK signaling in skeletal muscle, it led to only a partial improvement in glucose homeostasis, thus suggesting that reductions in AMPK signaling are not the prime causal event in DEX-induced glucose intolerance. At this juncture, a mechanistic explanation of how increases in skeletal muscle AMPK signaling may lead to enhanced glucose tolerance is lacking. Future studies measuring in vivo skeletal muscle glucose disposal following DEX and vitamin E and C supplementation may shed light into this question.

As AMPK has been reported to be a key regulator of skeletal muscle mitochondrial biogenesis (50), and given the reported associations between altered glucose homeostasis and reductions in mitochondrial content by some (5, 29) but not all (16, 44), it was of interest to determine whether markers of mitochondrial content were altered in skeletal muscle from DEX-treated animals. Despite slight reductions in AMPK signaling,
the protein content of mitochondrial marker proteins was not affected by DEX treatment, demonstrating that reductions in mitochondrial enzymes are not involved in the mechanisms through which DEX causes glucose intolerance and impairments in insulin signaling.

In contrast to skeletal muscle, DEX increased the content and phosphorylation of AMPK, and the phosphorylation of the direct AMPK substrate ACC, in a vitamin E/C sensitive manner. These results are consistent with others, who have demonstrated similar fold increases in AMPK activity (using the SAMS peptide), both in vivo and in isolated hepatocytes treated with DEX (10, 46). This finding presents an interesting paradox as DEX is known to increase hepatic glucose output (34) and to induce the expression of PEPCK and G6Pase in cultured hepatocytes, while AMPK has been reported to down-regulate the expression of these genes (18, 25). In our hands, DEX treatment did not increase the protein content of either G6Pase or PEPCK, a finding consistent with a recent study demonstrating unaltered PEPCK and G6Pase mRNA expression in livers from rats receiving corticosterone in their drinking water (10). These results could be explained by an antagonistic effect of AMPK counterbalancing the DEX-mediated induction of these enzymes.

AMPK potently stimulates fatty acid oxidation through the phosphorylation and deactivation of ACC, resulting in decreases in malonyl CoA and increases in the activity of carnitine palmitoyl transferase, which facilitates the movement of long-chain fatty acids into the mitochondria (45). In addition, AMPK induces the expression of enzymes involved in fatty acid oxidation (21, 41). Given the changes in AMPK signaling in liver, we sought to determine whether markers of fatty acid oxidation were elevated in DEX-treated rats. We found that DEX increased the protein content of UCP3, a protein that facilitates fatty acid oxidation in the liver (8), and PDK4, an enzyme that attenuates glucose oxidation, shunts pyruvate toward gluconeogenesis and is induced in conditions of high fatty acid oxidation (23). Similarly, we also found that DEX treatment increased the protein content of PPAR gamma coactivator 1 (PGC-1), a transcriptional coactivator that is induced by AMPK and controls the expression of genes involved in fatty acid oxidation. The changes in these proteins were attenuated in DEX-treated rats receiving vitamin E and C supplementation, thus suggesting a relationship between DEX, oxidative stress, and presumably, although not directly measured, hepatic fatty acid oxidation. Although we have demonstrated associations between these variables, we are limited in our ability to infer causality due to the in vivo model that was employed. Given the complex relationships between these factors, confirmation of an effect on fatty acid oxidation requires further investigation.

Although the rate of gluconeogenesis has traditionally been thought to be precisely controlled through the expression of gluconeogenic enzymes, such as PEPCK, there is increasing evidence to suggest otherwise. For instance, Burgess et al. (7) demonstrated that a 90% reduction in PEPCK results in an ∼40% decrease in gluconeogenic flux, suggesting the involvement of additional regulatory steps. These authors (7) demonstrated a tight coupling between the TCA cycle and PEPCK flux, suggesting that as rates of gluconeogenesis increase, flux through the TCA cycle must increase proportionally. It has been argued (26) that increases in hepatic fatty acid oxidation are linked to increased gluconeogenesis. This has been attributed to increases in the production of acetyl coA, which activates pyruvate carboxylase, increased NADH, which is used in the synthesis of glyceraldehyde-3-phosphate and increases in ATP, which is used as a substrate during gluconeogenesis (26). While speculative, the attenuated increase in markers of fatty acid oxidation, i.e., p-AMPK/ACC, UCP3, and PDK4, in animals receiving vitamin E and C supplementation could be linked to decreases in hepatic glucose output. This, in part, could explain the reductions in fasting blood glucose and improvements in glucose tolerance in animals receiving dietary antioxidant supplementation prior to and during DEX treatment. However, arguing against this premise, we did not detect significant differences in levels of NADH and acetyl CoA between groups.

**Perspectives and Significance**

In summary, we have made the novel observation that dietary vitamin E and C supplementation partially prevents DEX-induced glucose intolerance. Interestingly, these changes were not associated with an enhancement of insulin signaling (i.e., PKB phosphorylation) in tissues, including skeletal muscle, adipose, and liver. Endurance exercise increases the content of endogenous antioxidant enzymes in skeletal muscle (17). Because exercise training blunts DEX-induced derangements in glucose homeostasis (4), it is tempting to speculate...
that increasing antioxidative capacity through diet and/or exercise may serve as an efficacious approach to improve glucose homeostasis in individuals with Cushing’s disease or those receiving exogenous glucocorticoids.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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