2008-12-04

The Effects of Excess Corticosterone on LKB1 and AMPK Signaling in Skeletal Muscle of Rats

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by

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A thesis submitted to the faculty of

Brigham Young University

In partial fulfillment of the requirements for the degree of

Master of Science

Department of Physiology and Developmental Biology

Brigham Young University

December 2008

BRIGHAM YOUNG UNIVERISTY

GRADUATE COMMITTEE APROVAL

of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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BRIGHAM YOUNG UNIVERSITY

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THESIS ABSTRACT

THE EFFECTS OF EXCESS CORTICOSTERONE ON LKB1 AND AMPK SIGNALING IN SKELETAL MUSCLE OF RATS

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Cushing's syndrome and glucocorticoid therapy lead to central obesity, insulin resistance, and symptoms of altered energy regulation similar to those observed in the metabolic syndrome. We hypothesized that excess glucocorticoids alter energy sensing/signaling in skeletal muscle through mediation of the LKB1/AMPK signaling pathway. To test this hypothesis, three 100 mg pellets of corticosterone were implanted subcutaneously in each of nine rats for two weeks. Responses were compared with sham operated controls fed *ad libitum* or food restricted to produce the same body weight. After treatment period, animals were anesthetized and right gastrocnemius-plantaris and soleus removed for analysis. After tibial nerve stimulation, the left gastrocnemius-plantaris and soleus were also removed. We assessed AMPK activity and subunit expression, as well as several metabolic indicators including ATP, creatine phosphate, creatine, glycogen, P-ACC, and malonyl-CoA levels in rested and stimulated gastrocnemius-plantaris and so-

leus muscles. We conclude that high levels of glucocorticoids cause a marked decrease in AMPKα2 activity and AMPKγ3 subunit expression in the gastrocnemius-plantaris muscles. The reduction in AMPK activity could also be due to elevated glycogen or to the change in expression of the γ3 subunit. Of particular interest is the decrease in TBC1D1 phosphorylation in both resting and stimulated muscle in response to the chronic high levels of glucocorticoids. The reduction in AMPK activity with consequent decrease in TBC1D1 phosphorylation and elevated malonyl-CoA may account for some of the metabolic syndrome-like symptoms, including insulin resistance, associated with Cushing's syndrome.

Key Words: AMPK; Corticosterone; Cushing's syndrome; Glucocorticoids; GLUT-4; Glycogen; Insulin signaling; Metabolic syndrome; TBC1D1.

ACKNOWLEDGEMENTS

I would like to thank my patient and expert mentor, Dr. Winder, for his training, wisdom, and guidance during the course of this project. He is the epitome of a Christ-like mentor in every sense. I would also like to express my thanks to Dr. Porter, Dr. Mack, Dr. Woodbury, and Dr. Lephart for the role they played in guiding me through this experience in graduate studies, and to Dr. Hansen for giving me my first lessons in scientific research. I also express many thanks to Dr. Thomson, Daniel Jacobs, Natasha Fillmore, Todd Matthews, Kelsey Bennett, Mike Brewerton, and the countless number of students who have come and gone through the Winder lab lending their help and expertise in pushing this project forward. Finally, I express gratitude to my family and my Father in Heaven, without whose combined support and blessing none of this research would have been possible.

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ABBREVIATIONS

Abbreviation		Full Text
ACC	=	Acetyl Co-A carboxylase
AMPK	=	AMP-activated protein kinase
AS160	=	Akt substrate protein of 160 kDa
Cort.	=	Corticosterone
DEX	=	Dexamethasone
GC	=	Glucocorticoid(s)
GLUT-4	=	Glucose transporter 4
Hypercort	=	Hypercorticosterone treatment group
MO25	=	Mouse protein 25
P-ACC	=	Phospho-ACC
P-Akt	=	Phospho-Akt
P-AMPK	=	Phospho-AMPK
STRAD	=	Ste-related adaptor protein
TBC1D1	=	TBC1 (tre-2/USP6, BUB2, cdc16) domain family member 1
UCP-3	=	Uncoupling protein 3

THE EFFECTS OF EXCESS CORTICOSTERONE ON LKB1 AND AMPK SIGNALING IN SKELETAL MUSCLE OF RATS*

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Key Words: AMPK; Cushing's syndrome; Glycogen; Glucocorticoids; Hypercorticosteronemia; Metabolic syndrome; Skeletal muscle; TBC1D1.

This research was supported by a grant from the National Institute of Arthritis, Musculoskeletal, and Skin Diseases. Grant # AR051928.

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ABSTRACT

Cushing's syndrome and glucocorticoid therapy lead to central obesity, insulin resistance, and symptoms of altered energy regulation similar to those observed in the metabolic syndrome. We hypothesized that excess glucocorticoids alter energy sensing/signaling in skeletal muscle through mediation of the LKB1/AMPK signaling pathway. To test this hypothesis, three 100 mg pellets of corticosterone were implanted subcutaneously in each of nine rats for two weeks. Responses were compared with sham operated controls fed *ad libitum* or food restricted to produce the same body weight. After the treatment period, animals were anesthetized and the right gastrocnemius-plantaris and soleus were removed for analysis. After tibial nerve stimulation for 5 min, the left gastrocnemius-plantaris and soleus were also removed. We assessed AMPK activity and subunit expression, as well as several metabolic indicators including ATP, creatine phosphate, creatine, glycogen, and malonyl-CoA levels in rested and stimulated gastrocnemius-plantaris and soleus muscles. We found that high levels of glucocorticoids decreased AMPKγ3 subunit expression in the gastrocnemius-plantaris. We also observed reduced AMPKα2 activity in the stimulated gastrocnemius-plantaris, but not the soleus; and that this decreased activity corresponded to a significant reduction in phosphorylated TBC1D1, a protein involved in signaling GLUT-4 translocation. Finally, in the gastrocnemius-plantaris, we also noted an increase in glycogen stores in the hypercorticosteronemic rats. Our data suggest that altered energy sensing/signaling associated with high levels of glucocorticoids may be due in part to inhibition of AMPKα2 activity and the high energy state produced by increased glycogen stores. We also conclude that high levels of glucocorticoids decrease the levels of AMPKγ3 and diminish insulin/contraction signaling through phosphorylated TBC1D1.

Key Words: AMPK; Corticosterone; Cushing's syndrome; Glucocorticoids; GLUT-4; Glycogen; Insulin signaling; Metabolic syndrome; TBC1D1.

INTRODUCTION

Cushing's disease is characterized by excess endogenous glucocorticoids (GC) caused by hypersecretion of ACTH from anterior pituitary adenomas (1). Transplant patients, graft patients, and others receiving GC therapy also suffer from Cushing's syndrome-like symptoms, including: a round face, buffalo hump, osteopenia, central obesity, increased blood pressure, dyslipidemia, and impaired glucose transport into cells (2). Many of these symptoms share striking similarity with the metabolic syndrome (3). These symptoms, coupled with cerebrovascular risks, lead to decreased quality of life and a reduced life expectancy (50% survival rate 5 years from the time of diagnosis if left untreated) (4).

AMPK is a heterotrimeric protein that acts as a metabolic master switch by stimulating catabolic processes and inhibiting anabolic processes (5). It is responsible for down regulating protein synthesis (6), glycogen synthesis (7), and cholesterogenesis (8). The kinase is comprised of α, β, and γ subunits (9). Since two isoforms exist for both α and β subunits (α1 and α2, β1 and β2) and three exist for the γ subunit (γ1, γ2, and γ3), there are 12 possible combinations to form the holoenzyme.

During muscle contraction, the AMP concentration increases due to the adenylate kinase reaction (5). When the free AMP:ATP ratio increases, two AMP molecules bind the CBS domains of the γ subunit of AMPK causing a conformational change making AMPK a poorer substrate for phosphatases like protein phosphatase-2C (PP2C) (10). Thus, muscle contraction leads to activation of AMPK via phosphorylation by an upstream kinase (11). LKB1-STRAD-MO25 is the major upstream AMPK kinase (AMPKK) in skeletal muscle (5), which activates AMPK via phosphorylation of T-172 of the α subunit (12).

A somewhat antagonistic correlation exists between processes affected by GC and AMPK. For instance, GLUT-4 expression and translocation to the membrane (11), glucose transport into the cell (13), and glycolysis (14) are all increased in response to AMPK activation. Conversely, these same processes are downregulated in response to high levels of GC (5, 15-17) AMPK has also been shown to downregulate lipolysis (18), while GC increase plasma insulin levels leading to an increase in lipogenesis (19).

Previous studies have revealed that patients with high levels of GC experience alterations in the energy sensing/signaling process in the body including disrupted GLUT-4 translocation to the T-tubule membranes (15) and insulin resistance (20), sometimes leading to diabetes mellitus (21); however, the mechanism for these metabolic alterations is not well understood. Could a link exist between hypercorticosteronemia and AMPK that leads to altered energy sensing/signaling? Previous studies reported evidence of lower AMPK activity of epitrochlearis muscle (22) and a two-fold increase in phosphorylated AMPK, as well as increased total AMPK expression in heart muscle of GC treated rats (23). The purpose of this study was to explore the effect of high levels of GC on skeletal muscle LKB1/AMPK activity and subunit expression in an attempt to better understand the pathological energy sensing/signaling alterations associated with this condition.

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: P-ACC antibody (cat# 3661), Total α AMPK antibody (cat# 3661), Phospho-AMPK α antibody (cat# 2535). LKB1 antibody was purchased from Upstate (cat# 07694). SAMS peptide was obtained from Zinsser Analytic (Maidenhead, Berks, UK) and LKB-tide was custom synthe-

sized by Biopeptide (San Diego, CA). AMPK α1 and α2 antibodies and MO25 antibody were custom made by Affinity Bioreagents, Inc. (Golden, CO) as described previously (24). LKB1 antibody for immunoprecipitation preceding LKB1 activity assays was purchased from SantaCruz (cat# sc-5640).

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 insertion of three 100 mg corticosterone pellets subcutaneously between the scapulas. A Control group (n=8) and a pair-weighted group (Paired) (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 (25), increased visceral fat (26), heart hypertrophy (27), and atrophy of the gastrocnemius-plantaris (gastroc), but not soleus muscles (28, 29). Collectively, these factors provided evidence of long-term hypercorticosteronemia in the treatment group.

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

Rats were housed in a temperature-controlled (21–22°C) room with a 12:12-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 hours 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) for at least 20 mins prior to 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, 10V, for 5 mins. 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 and after 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.

Homogenization. Muscle homogenates were prepared by adding 1 ml of *homogenization buffer* (50 mM Tris-HCl, 250 mM Manitol, 50 mM NaF, 5 mM Na Pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, 1 mM DTT, 1mM Benzamidine, 0.1 mM PMSF, 5 µg/ml soy-bean trypsin inhibitor, 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 x g for 10 mins, the supernatant was frozen for later analysis.

AMPK activity. Activity for both AMPKα1 and AMPKα2 was tested separately. AMPKα1 or α2 antibody was first 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 2X 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, 1mM EGTA, 1 mM DTT, 1 mM ben-zamidine, 0.1 mM PMSF, 5 µg/ml soybean trypsin inhibitor (STI), pH 7.4), followed by one

wash in 0.5 ml *lysate 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, 5 µg/ml STI). Finally, AMPK activity was assessed as previously described (30).

LKB1 activity. Starting with 50 µl of 10% rested and stimulated 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 2X with 1 ml *Wash Buffer A* (*Homogenization Buffer* + 0.5 M NaCl) and then 2X with 1 ml *Wash Buffer B* (40 mM HEPES; 80 mM NaCl; 8% glycerol; 0.8 mM EDTA; 5 mM $MgCl_2$; 0.8 mM DTT). LKB-tide (0.2 mM) was used in place of SAMS-peptide, and the incubation period was extended to 15 mins. In the LKB1 activity assay protocol, 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 seconds before stopping the reaction in phosphoric acid.

Western blotting and immunodetection. Tissue homogenates (10% for gastroc, 5% for soleus) were prepared as in the AMPK activity assay protocol. Homogenate + Laemmle'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 200V, 3 amps, for 50 mins in Criterion Precast Gels (Bio-Rad, Hercules, CA) filled with Running Buffer (Bio-Rad cat# 161-0732). Proteins were then transferred to PVDF membranes at 100V, for 45-60 mins, in *Western Blot Transferring Buffer* (0.2 M Glycine, 25 mM Tris base, 20% Methanol, chilled to 4°C). Next, membranes were blocked in TBST and 5% blotting grade blocker non-fat dry milk for 1 hour. Membranes were then incubated overnight at 4°C in the primary antibody, diluted in 1% BSA in TBST. The next day, membranes were washed 4X 5 mins in TBST and incubated with an HRP-linked anti-rabbit antibody, then washed 4X for 5 mins in TBST. Membranes were covered with Amersham ECL+Plus Western Blot Detection

Reagent (GE Healthcare, Buckinghamshire, UK) for five mins. 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 Corporation, 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 mean optical density reading.

Tissue metabolites and plasma hormone assays. Glycogen (31), blood glucose (32), malonyl-CoA (33), creatine (34), creatine phosphate (35) and ATP (35) concentrations were determined as previously described. Plasma leptin and insulin concentrations were measured via ELISA assay. Leptin and insulin ELISA kits were purchased from Crystal Chem, Inc. (Downers Grove, IL, leptin kit cat# 90060, insulin kit cat# 90040). Assays were performed according to vendor's protocols.

Statistics. All assays were compared by one-way ANOVA. Post hoc comparisons were performed using Fisher's least significant difference multiple comparison test. In each case, significance was set at $p < 0.05$. Statistical analysis was performed using the NCSS statistical program (Kaysville, UT). All data are reported as means ± SE.

RESULTS

Evidence of Hypercorticosteronemia

We expected acute corticosterone levels to be elevated in the rats due to anesthesia and stress at the time of sacrifice. Results of a competitive binding ELISA indicated elevated plasma corticosterone levels (Fig. 1A, n=8-9, p=0.32). In probing for evidence of chronically elevated corticosterone levels, we noted severe atrophy of the Hypercort adrenal glands (Fig. 1B, n=8-9,

$p<0.05$). Mean adrenal weights at the time of sacrifice were as follows: Control: 50.1 ± 2.1 mg, Hypercort: 20.6 ± 0.4 mg, and Paired: 44.5 ± 2.2 mg. We found a 47% atrophy of the Hypercort adrenals as compared to the Control when analyzed as a percentage of total body weight.

Combined retroperitoneal and perirenal fat pads were used as a measure of visceral fat. Accumulation of fat by the Hypercort group was increased 2-fold over the Control animals (Fig. 1C, n=8-9, $p<0.05$). Mean fat pad values in the Hypercort group were 2.5 ± 0.24 g as compared to 1.6 ± 0.11 g in the Control group. Due to food intake restrictions, Paired mean fat pad weights were only 0.45 ± 0.09 g.

While mean heart weights were slightly lower in the Hypercort group as compared to the Control, Hypercort rat hearts were 33% larger than the control group when compared as a % of total body weight (Fig. 1D, n=8-9, $p<0.05$). Mean heart weights were as follows: Control: 0.83 ± 0.02 g, Hypercort: 0.81 ± 0.03 g, and Paired: 0.69 ± 0.01 g.

The Hypercort hindlimb skeletal muscle appeared to have significant atrophy. We observed significant atrophy of the Hypercort gastroc when expressed as a % of total body weight (Fig. 1E, n=8-9, $p<0.05$); this trend did not carry over to the soleus (Fig. 1F, n=8-9, $p<0.05$). The gastroc mean weight values were as follows: Control: 1.83 ± 0.07 g, Hypercort: 1.14 ± 0.06 g, Paired: 1.56 ± 0.06 g. Mean values for the soleus were as follows: Control: 0.14 ± 0.007 g, Hypercort: 0.11 ± 0.004 g, Paired: 0.12 ± 0.004 g.

Another indicator of chronically elevated corticosterone levels is decreased chow intake and increased weight loss on the part of the Hypercort rats (36). On the first day of treatment, the Control group average rat weight was 275 ± 6.7 g, the Hypercort group weighed an average of 274 ± 4.9 g, and the Paired rats weighed an average 271 ± 8.6 g. Across the 14 day treatment pe-

riod, the Hypercort group consumed an average 21.0 g chow per day. The Control group averaged 22.5 g per day, while the Paired rats were restricted to an average 16.0 g of chow 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 Paired group, lost an average 3.8 ± 2.0 g/day. On the day of sacrifice, average rat weights per group were: Control: 310 ± 11 g, Hypercort: 223 ± 10 g, and Paired: 227 ± 10 g.

Inhibition of LKB1/AMPK Activity

To test the hypothesis that an excess of GC 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. 2A, n=8-9, p<0.05). We next assayed AMPK activity in both the rested and stimulated rat gastroc. Fig. 2B-C show that while differences in AMPKα1 activity were not statistically significant, AMPKα2 activity was downregulated (-42%) in the Hypercort rat's stimulated gastroc (p<0.05).

Glycogen, Blood Glucose, ATP, CP, and Creatine

AMPK is exceptionally sensitive to the energy state of its cellular environment. We thus examined ATP, blood glucose, creatine phosphate (CP), and creatine levels in the gastroc to search for possible causative factors influencing AMPK's decreased activation state. There was no significant difference between the three treatment groups in any of these cellular energy markers. Rested Hypercort glycogen levels were 32% higher than the Control (Table 1, n=8-9, p<0.05).

Variations in AMPK Subunit Expression

To determine if decreases in AMPKα2 activity were due to decreased levels of AMPKα2 protein expression, Western blots of rested gastroc AMPKα2 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 AMPKγ3 subunit showed a 51% decrease in the Hypercort versus Control groups (Fig. 3A, n=8-9, p<0.05). AMPKγ1 was increased 60% and AMPKγ2 was increased 15% over the Control in response to GC (Fig. 3B-C, n=8-9, p<0.05).

Western Blot Analysis of P-AMPK

Coinciding with our findings of decreased AMPKα2 activity, Western blot analysis showed a 55% decrease in P-AMPK in the Hypercort stimulated gastroc as compared to the Control (Fig. 3D, n=8-9, p<0.05). Fig. 3E shows a 36% decrease in Hypercort rested soleus P-AMPK as compared to the Control (p<0.05).

P-ACC and Malonyl-CoA Levels

Contrary to our expectations, phosphorylated acetyl-CoA carboxylase (P-ACC) was not significantly reduced in the gastroc (Table 1, n=8-9, p<0.05). Malonyl Co-A levels, in the Hypercort gastroc were slightly elevated over the Control, but failed to be significant when compared to the Paired group (Table 1, n=8-9, p<0.05).

Western Blot Analysis of UCP-3

To test if metabolic complications arising from GC excess might have root in energy losses due to variations in uncoupling protein 3 (UCP-3), Western blots were performed to assay

UCP-3 protein content. No significant difference was observed between the three groups (data not shown).

Analysis of the Soleus

The soleus is comprised of type I (slow twitch) fibers. To assess whether noted trends in the gastroc, including decreased AMPKγ3, would be observed in slow twitch fibers, we performed Western blots probing for ACC, MO25, LKB1, the α1, α2, β1, β2, γ1, γ2, and γ3 subunits of AMPK in the soleus. Interestingly, none of the aforementioned gastroc trends were observed in the soleus (data not shown).

Plasma Leptin

Increases in adipose tissue lead to an increase in plasma leptin levels (37). Increased central obesity caused by GC excess, therefore, should lead to hyperleptinemia. This became another marker of induced hypercorticosteronemia in our Hypercort rats. ELISA assay analysis showed more than a 5-fold increase in circulating plasma leptin levels over the Control (Fig. 4A, n=8-9, $p<0.05$).

GLUT-4 Translocation Pathways

An ELISA assay indicated plasma insulin levels were significantly elevated (53%) in the Hypercort group vs. Control (Fig. 4B, n=8-9, $p<0.05$). Western blot analysis showed no difference in AS160, phosphorylated AS160 (P-AS160), Akt, or phosphorylated Akt (P-Akt) in the gastroc (Table 2, n=8-9, $p<0.05$). However, phosphorylated TBC1D1 (P-TBC1D1) was decreased 50% in the resting gastroc and 35% in the stimulated gastroc of the Hypercort group as compared to the Control (Fig. 4C, n=8-9, $p<0.05$).

DISCUSSION

Cushing's syndrome usually arises from endogenous hypercortisolism or exogenous GC administration. It is often marked by the onset of various metabolic syndrome complications causing an alteration in energy sensing/signaling in the body. What exactly causes these metabolic changes? The mechanism is not well understood. Previous studies have indirectly linked hypercortisolism to inhibition of GLUT-4 translocation to the plasma membrane (38), a process normally stimulated by AMPK in skeletal muscle (5). In this study, we show that high levels of GC induce a decrease in the γ3 subunit of AMPK, which is associated with decreased AMPKα2 activity. We suggest that GC-induced changes in γ3 subunit expression may, in part, be responsible for some of the metabolic syndrome-like complications inherent in hypercorticosteronemia.

In GC research methodology, subcutaneous pellet implantation has been established as a reliable method for controlling circulating GC levels. In 1985, Akana et al. demonstrated that pellets containing 30 mg corticosterone and 70 mg cholesterol provided physiological levels of corticosterone replacement in rats weighing roughly 225 grams (39). We administered a dose 10X this concentration to generate chronically elevated GC levels in the Hypercort rats. Elevated plasma corticosterone levels in all three treatment groups were likely due to stress caused by anesthetizing the animals as well as stimulating their left tibial nerves prior to extracting the blood sample, but severely atrophied adrenal glands make the elevated levels of corticosterone in the Hypercort group likely due to implanted pellets. There was some concern that chow restriction might induce hypercorticosteronemia in the Paired group; however, adrenal atrophy, visceral fat accumulation, heart hypertrophy (which may indicate hypertension), and gastroc, but not soleus atrophy were present only in the Hypercort rats. In all cases, Paired rats showed values similar to the Control rats with the exception of decreased fat stores due to restricted chow intake. Adrenal glands, which were atrophied (-56%) in the Hypercort group, give a strong line of evi-

dence pointing to chronically elevated corticosterone levels in the Hypercort rats. Hypercort rats were also hyperinsulinemic and hyperleptinemic.

It is important to note a difference between the human and rat model of Cushing's syndrome. In humans, Cushing's syndrome is usually marked by increased caloric intake. In rats however, high leptin concentration in the blood stream, caused by circulating corticosterone (36), signals satiety, causing decreased caloric intake (37). We noted that despite decreased chow intake and overall weight loss, accumulation of visceral fat was still prevalent in the Hypercort rats. This suggests that GC influence central obesity even in the absence of increased caloric intake.

One of the major findings reported in this study is an observed 53% decrease in AMPKγ3 in the Hypercort group in response to high levels of GC. This might account for the increase in glycogen stores observed in the Hypercort group. Mutations of the Rendement Napole gene (now identified as PRKAG3) that encodes for AMPKγ3 in Hampshire-sired pigs have been linked to glycogen storage disease (40).

AMPKγ3, the regulatory subunit, is also vital in regulating AMPK activity, due to a set of four Cystathione Beta Synthase (CBS) repeats responsible for binding AMP (5). The gamma subunit is absolutely pivotal in both energy sensing as well as kinase activation. Studies have shown that mutations in the γ3 subunit cause decreases in AMPKα2 activity without decreasing AMPKα1 activity (41). This observation may be due to the potential relationship between α2, β2, and γ3 which Birk and Wojtaszewski reported to be the primary AMPK heterotrimer found in fast-twitch skeletal muscle (42). Corresponding to these studies, we noted analogous decreases in both AMPKγ3 expression and AMPKα2 activity in the gastroc of the Hypercort rats. The decrease in AMPK activity could presumably be due to decreased AMP levels. While no direct

measurement of AMP was made, similar ATP, CP, and creatine levels between treatment groups are indicative of similar AMP levels.

High levels of GC often leads to insulin resistance, in part, due to impaired GLUT-4 translocation (15), but the mechanism leading up to this impairment is not well understood. In trying to uncover mechanisms leading to this pathology, Weinstein et al., in 1995, concluded that GC alter insulin signaling, by inhibiting GLUT-4 translocation (15). Garvey et al. showed GLUT-4 translocation decreases after just two hours of dexamethasone (DEX) administration (43). In 1997, Dimitriadis' group administered DEX treatment to rats for five days and then analyzed the soleus muscles. They noted a sharp drop off in GLUT-4 translocation to the plasma membrane in response to insulin administration (16). These studies provide a nebulous link between high levels of GC and insulin resistance through decreased GLUT-4 translocation.

There are multiple pathways leading to translocation of GLUT-4 to the membrane, including insulin signaling and activation of AMPK via muscle contraction (44). Akt (or PKB) (45) is involved in the insulin-mediated cascade leading to GLUT-4 translocation (46). Our results showed no difference in P-Akt levels among the three groups. Both insulin signaling and muscle contraction pathways can converge on Akt-substrate 160 (AS160) and TBC1D1 (47-49), known AMPK substrates involved in regulating glucose uptake (49, 50). While no direct measurement of insulin resistance was made in our study, we did attempt to look for alterations in the insulin signaling and muscle contraction pathways by analyzing these key proteins in the GLUT-4 translocation pathways of the rested (insulin pathway) and stimulated (muscle contraction pathway) muscle types. In probing these targets, 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 GC. It seems most likely that decreased AMPKα2 activity may lead to decreased phosphoryla-

tion of TBC1D1, and that this correlation might be responsible, in part, for GC-induced impaired GLUT-4 translocation.

After Western blot analysis of AS160, we noted, despite no changes in total AS160, a slight decrease in phosphorylated AS160 in the Hypercort stimulated gastroc as compared to the Control. This was surprising considering the high levels of insulin observed in the Hypercort group. Decreases in rested gastroc P-AS160 levels were completely reversed in response to stimulation. This may indicate that patients suffering from Cushing's syndrome might receive some relief from insulin resistance through regular exercise.

Some studies indicate another possible factor influencing GLUT-4 translocation, namely, insulin-induced insulin resistance. It has been documented that insulin has an inhibitory effect on AMPK activation in skeletal muscle (51). Hyperinsulinemia, like that seen in response to high levels of GC, in theory, could inhibit AMPK-regulated translocation of GLUT-4. The noted hyperinsulinemia in the Hypercort rats gives some credence to this hypothesis; however, in light of reported data pertaining to decreased phosphorylation of TBC1D1, hyperinsulinemia is more likely a symptom, not the cause of impaired insulin signaling.

One other contributing factor exists that may influence impaired GLUT-4 translocation in response to high levels of GC—increased glycogen stores. The β-subunit of AMPK has a glycogen binding domain that tethers it directly to glycogen, keeping AMPK near glycogen synthase, phosphatases, and glycogen phosphorylase (52), but glycogen's interconnection to GLUT-4 translocation is just beginning to emerge in a growing body of research.

In 2002, one particularly interesting finding by Wojtaszewski showed an indirect association between glycogen and insulin resistance. He demonstrated that high cellular glycogen con-

tent decreases glucose uptake by directly affecting the activation state of AMPK. He also showed that increased glycogen levels cause a decrease in glycogen synthase activity, and a decrease in GLUT-4 translocation to the plasma membrane (53). These findings are interesting in light of the previously mentioned studies on pigs, revealing that mutations in the gene encoding for AMPKγ3 lead to increased glycogen storage (40). Our study shows an association between high levels of GC, decreased AMPKγ3, and increased glycogen stores.

Ancillary to this study, we noted a discrepancy in our current understanding of leptin's role in relationship to AMPK. Leptin is released from adipose tissue and plays a key role in regulating energy intake and expenditure (37). Increased fat stores, like those inherent in Cushing's syndrome-induced central obesity, causes leptin levels to increase (54). As a purported activator of AMPK, under normal conditions, elevated leptin levels should inhibit lipogenesis and stimulate β-oxidation (55), returning fat stores back to normal levels. There seems to be some interruption of this homeostatic mechanism in response to GC, allowing the accumulation of fat in select body regions. We observed that despite a 5-fold increase in circulating leptin in the Hypercort group, leptin failed to stimulate an increase in AMPK activation. Several possibilities exist for this breakdown in our understanding of leptin's role in regard to AMPK. First, GC may cause a downregulation in the number of leptin receptors. Second, the Hypercort rats may develop leptin resistance, yet we noted a decrease in chow intake, suggesting leptin is binding efficaciously at least at its hypothalamic receptor. Perhaps leptin resistance is tissue specific, affecting only targets in skeletal muscle for instance. Third, the antagonistic effects of GC may completely overwhelm the stimulatory effects of leptin. Fourth, it may be that there is simply a discrepancy in our current understand of the interactions between leptin and AMPK. It appears additional studies will be required to strengthen our understanding of leptin's role in AMPK activation.

Also collateral to this study, we noticed that differences in AMPKα2 activity and γ3 subunit expression were significant in the gastroc which is composed of predominantly type II fibers, but not soleus muscle, which is predominantly type I fibers. Previous studies have indicated high levels of GC lead to atrophy of type II, but not type I muscle fibers (56). Coinciding with these findings, we noted significant atrophy in the gastroc. Furthermore, AMPKγ3 is expressed primarily in white, type IIb fibers (57). This may indicate that many of the complications arising from high levels of GC, including decreased AMPKγ3, reduced AMPKα2 activity, and even insulin resistance, may be restricted to fast twitch fibers.

Finally, AMPK promotes the oxidation of fatty acids by phosphorylating acetyl-CoA carboxylase, inactivating it (58). This causes inhibition in synthesis of malonyl-CoA, and consequently increases the rate of fatty acid oxidation (59). Thus, AMPK indirectly increases the rate of fatty acid oxidation. We noted elevated malonyl-CoA levels in the rested Hypercort gastroc. This could contribute to an accumulation of triglycerides in response to excess GC via inhibition of fatty acid oxidation in the muscles.

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 hypercorticosteronemia, we conclude that high levels of glucocorticoids cause a marked decrease in AMPKα2 activity and AMPKγ3 subunit expression in the gastrocnemius-plantaris muscles. The reduction in AMPK activity could also be due to elevated glycogen, or to the change in expression of the γ3 subunit. Of particular interest is the decrease in TBC1D1 phosphorylation in both rested and stimulated muscle in response to the chronic high levels of glucocorticoids. The reduction in AMPK activity with consequent decrease in TBC1D1 phosphorylation and elevated malonyl-CoA may account for some of the metabolic syndrome-like symptoms, including insulin resistance, associated with Cushing's syndrome.

ACKNOWLEDGEMENTS

This research was supported by a grant from the National Institute of Arthritis, Musculoskeletal, and Skin Diseases. Grant # AR051928.

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FIGURE LEGENDS

Figure 1: Evidence of Chronic Hypercorticosteronemia

(A) ELISA assay performed on rat plasma indicating plasma corticosterone concentrations at the time of sacrifice. (B) Rat adrenal glands compared as a percentage of total body weight to account for differences in mean rat weight between treatment groups. (C) Combined retroperitoneal and perirenal fat pads expressed as a percentage of total body weight. (D) Heart masses expressed as a percentage of total body weight. (E) Gastrocnemius-plantaris muscle mass expressed as a percentage of total body weight. (F) Soleus muscle mass expressed as a percentage of total body weight. *Significantly different from Control and Paired (n=8-9, $p < 0.05$).

Figure 2: LKB1 and AMPK Activity

AMPK and LKB1 activities of the gastrocnemius-plantaris in the three treatment groups. Activity is a measure of AMPK's ability to transfer radiolabeled phosphate from ATP to SAMS peptide, or LKB1's ability to transfer radiolabeled phosphate from ATP to LKB-tide respectively. (A) Shows LKB1 activity in the gastrocnemius-plantaris. (B) Shows AMPKα1 isoform activity. (C) Shows a measurement of AMPKα2 activity in the rested and stimulated gastrocnemius-plantaris. *Significantly different from Control and Paired (n=8-9, $p < 0.05$).

Figure 3: Relative Expression of AMPK γ Subunits as well as P-AMPK Expression

(A) Shows expression of AMPKγ3 in the gastrocnemius-plantaris assessed by Western blotting. (B) Shows expression of the AMPKγ1 subunit in the gastrocnemius-plantaris assessed by Western blotting. (C) Shows expression of AMPKγ2 in the gastrocnemius-plantaris assessed by Western blotting. (D) Western blots analysis of gastrocnemius-plantaris P-AMPK expression. (E) Western blots analysis of P-AMPK expression in the soleus. All Control values were normalized

to 1. (n=8-9, $p < 0.05$). *Significantly different from Control and Paired. †Significantly different from Control only.

Figure 4: Plasma Leptin and Insulin Concentrations, as well as TBC1D1 Expression

(A) ELISA assay performed on rat plasma indicating plasma leptin concentrations. (B) Results of an ELISA assay performed on plasma insulin levels. (C) Western blot analysis of TBC1D1 shows a significant decrease in phosphorylation in the Hypercort rested and stimulated gastrocnemius-plantaris. *Significantly different from Control and Paired. †Significantly different from Control only (n=8-9, $p<0.05$).

FIGURE 1

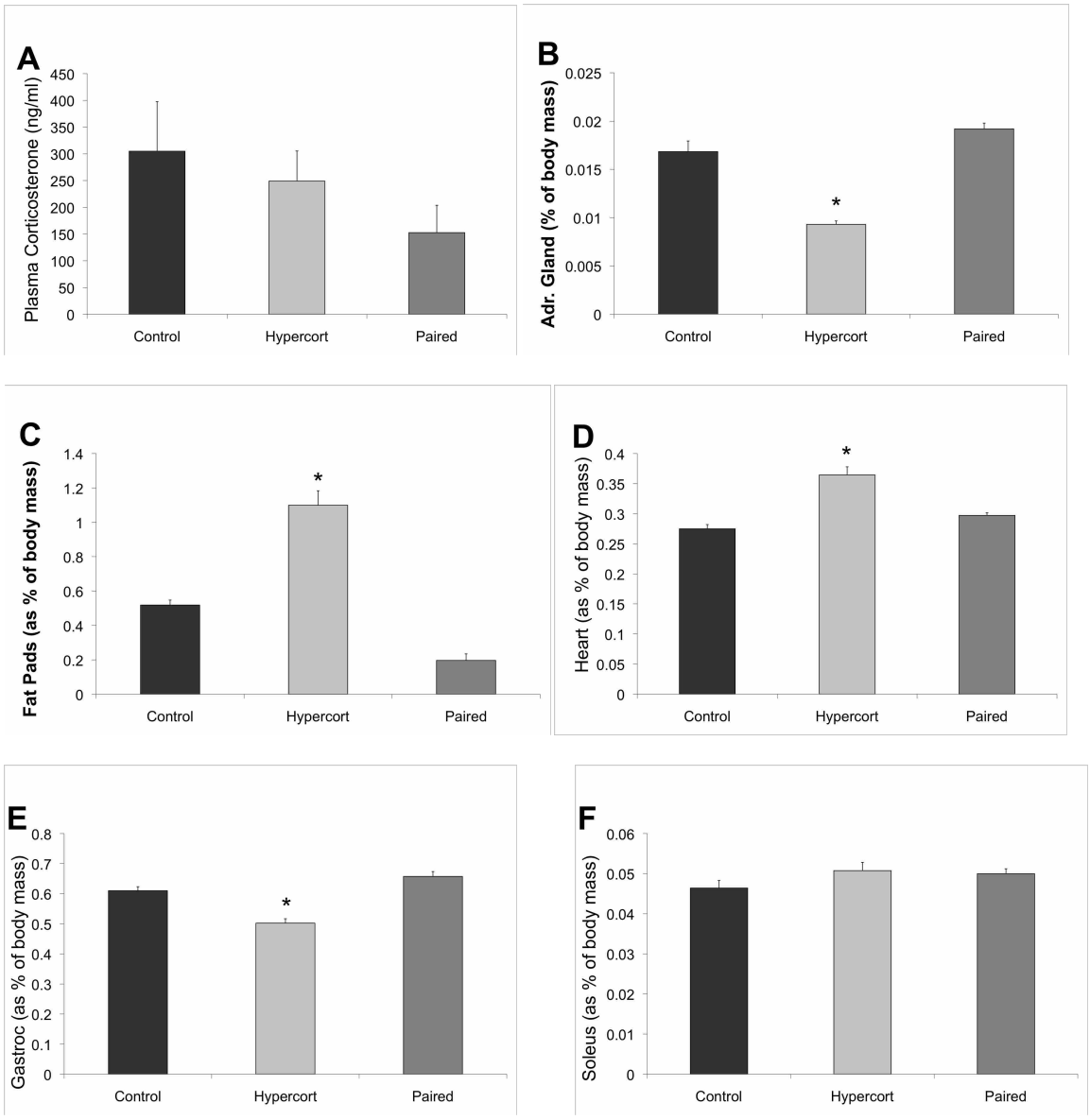

A
Plasma Corticosterone (ng/ml)
450
400
350
300
250
200
150
100
50
0
Control
Hypercort
Paired
B
Adr. Gland (% of body mass)
0.025
0.02
0.015
0.01
0.005
0
*
Control
Hypercort
Paired
C
Fat Pads (as % of body mass)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Hypercort
Paired
D
Heart (as % of body mass)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
Control
Hypercort
Paired
E
Gastroc (as % of body mass)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
Hypercort
Paired
F
Soleus (as % of body mass)
0.06
0.05
0.04
0.03
0.02
0.01
0
Control
Hypercort
Paired

FIGURE 2

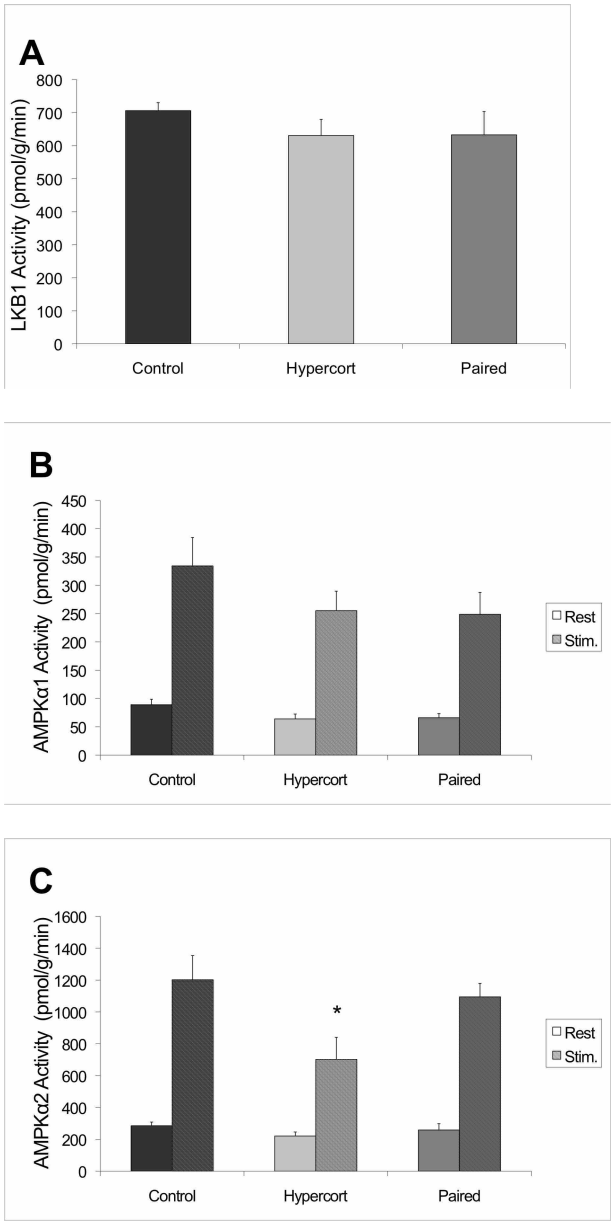

A
LKB1 Activity (pmol/g/min)
800
700
600
500
400
300
200
100
0
Control
Hypercort
Paired
B
AMPKα1 Activity (pmol/g/min)
450
400
350
300
250
200
150
100
50
0
Rest
Stim.
Control
Hypercort
Paired
C
AMPKα2 Activity (pmol/g/min)
1600
1400
1200
1000
800
600
400
200
0
*
Rest
Stim.
Control
Hypercort
Paired

FIGURE 3

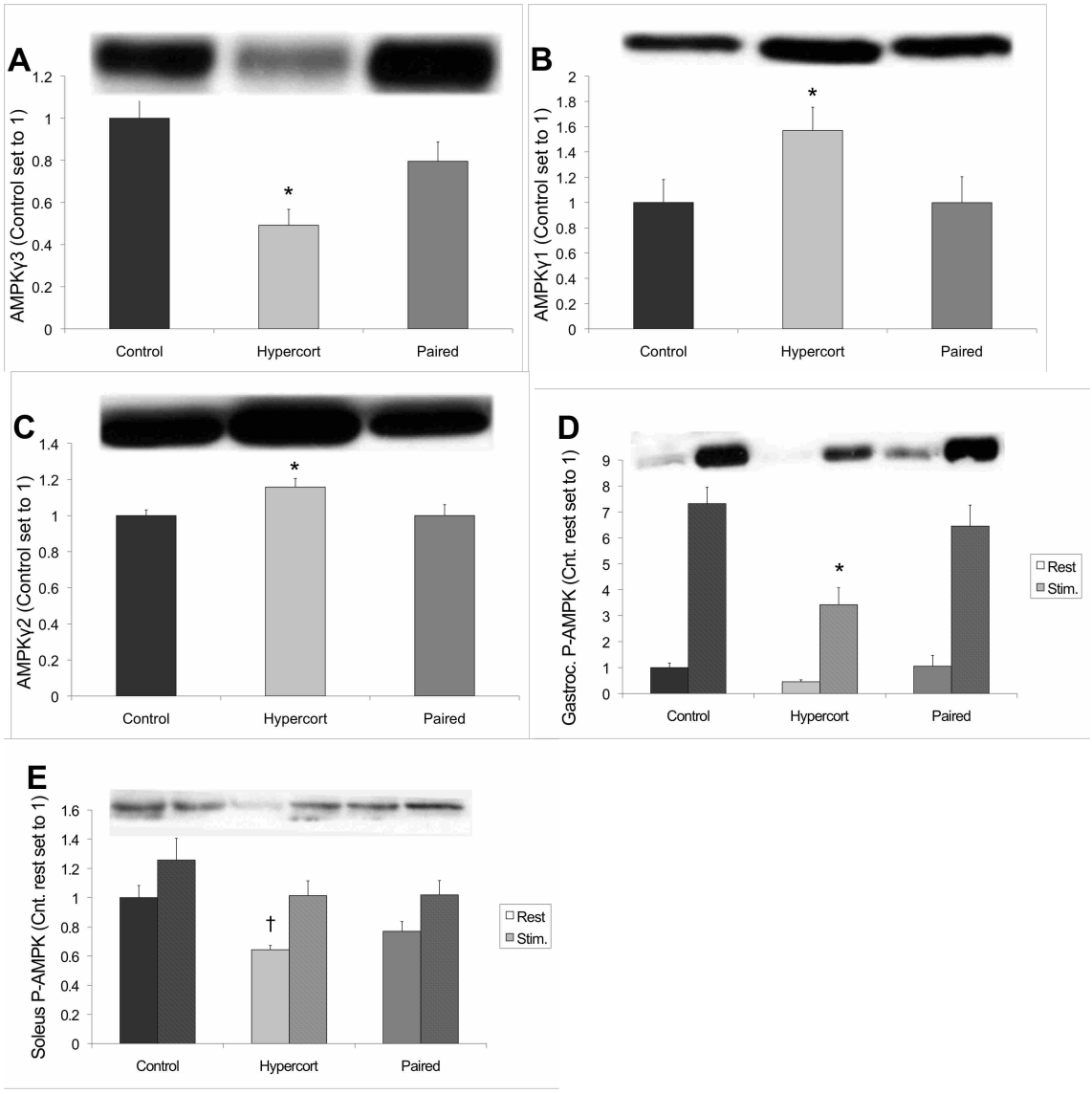
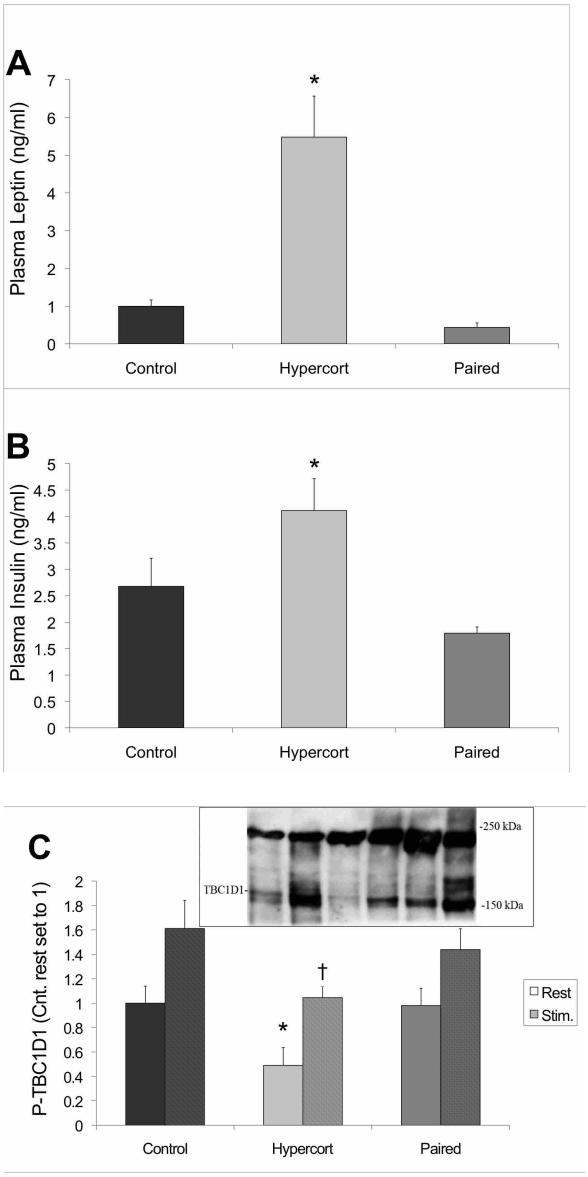

A
AMPKγ3 (Control set to 1)
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Hypercort
Paired
B
AMPKγ1 (Control set to 1)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Hypercort
Paired
C
AMPKγ2 (Control set to 1)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Hypercort
Paired
D
Gastroc. P-AMPK (Cnt. rest set to 1)
9
8
7
6
5
4
3
2
1
0
*
Rest
Stim.
Control
Hypercort
Paired
E
Soleus P-AMPK (Cnt. rest set to 1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
†
Rest
Stim.
Control
Hypercort
Paired

FIGURE 4


A
Plasma Leptin (ng/ml)
7
6
5
4
3
2
1
0
*
Control
Hypercort
Paired
B
Plasma Insulin (ng/ml)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
Hypercort
Paired
C
P-TBC1D1 (Cnt. rest set to 1)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TBC1D1-
-250 kDa
-150 kDa
*
†
Rest
Stim.
Control
Hypercort
Paired

TABLES

TABLE 1: Concentrations/expression of key energy consumption indicators in the gastrocnemius-plantaris

Treatment Group	ATP*	Blood Glucose*	CP*	Creatine*	Glycogen*	P-ACC ∴	Malonyl-CoA*
Control Rest	7.24 ± 0.24	8.13 ± 0.68	26.76 ± 0.69	13.87 ± 0.72	34.40 ± 3.61	1.00 ± 0.39	0.78 ± 0.11
Control Stim.	5.10 ± 0.40	n/a	8.46 ± 1.33	27.66 ± 1.88	15.06 ± 1.92	10.23 ± 0.68	0.86 ± 0.12
Hypercort Rest	6.83 ± 0.21	7.09 ± 0.35	27.71 ± 1.13	15.10 ± 0.63	45.55 ± 3.63†	0.66 ± 0.11	1.49 ± 0.16†
Hypercort Stim.	5.48 ± 0.18	n/a	10.90 ± 1.29	29.04 ± 2.04	30.33 ± 2.65†	8.44 ± 1.16	1.00 ± 0.08
Pair-weighted Rest	7.98 ± 0.18	6.68 ± 0.17	27.96 ± 1.08	14.28 ± 0.80	36.85 ± 4.20	0.50 ± 0.10	1.24 ± 0.18
Pair-weighted Stim	5.70 ± 0.25	n/a	11.35 ± 1.26	24.80 ± 1.88	22.05 ± 3.41	8.05 ± 1.01	1.01 ± 0.11

†Significantly different from Control group only. *µmol/g. ∴Arbitrary units taken from densiometric readings of Western blots with Control rest set to 1.

TABLE 2: Expression of various proteins involved in insulin signaling in the gastrocnemius-plantaris (Densiometric readings taken from Western blots with Control rest set to 1.)

Protein	Control Rest	Control Stim.	Hypercort Rest	Hypercort Stim.	Paired Rest	Paired Rest
AS160	1 ± 0.16	n/a	1.12 ± 0.23	n/a	0.77 ± 0.16	n/a
P-AS160	1 ± 0.14	1.16 ± 0.19	0.73 ± 0.21	1.31 ± 0.23	0.85 ± 0.11	1.27 ± 0.15
Akt	1 ± 0.06	n/a	0.91 ± 0.05	n/a	0.99 ± 0.03	n/a
P-Akt	1 ± 0.25	1.68 ± 0.17	1.03 ± 0.23	1.46 ± 0.23	0.44 ± 0.06	0.89 ± 0.07

Nathan Nakken *Curriculum Vitae*

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Personal Information

My brief life's sketch:

- Born: December 18, 1977
- Place: Cedar City, Utah, USA
- Gender: Male

Employment History

I have enjoyed over five years of teaching and training experiences at the Missionary Training Center, BYU, and pharmacies including:

- Training Coordinator (2003-2004)
- Teaching Supervisor (2001-2003)
- Mandarin Teacher (1999-2004)
- Curriculum Developer (2002-2002)
- Mission President Tutor (2001)
- Language Evaluator (2001-2003)
- Research and Evaluation Evaluator (2000-2002)
- English Teacher in Taiwan (1997-1999)
- Pharmacy Technician (1999-2007)
- Physiology Instructor and TA (2006-2008)
- Research Assistant (2007-2008)

Educational History

My educational history includes:

- Masters of Science with a Major in Physiology from Brigham Young University (2008)
- Major in Chinese from Brigham Young University (2006)
- Minor in Music from Brigham Young University (2006)
- Certified Pharmacy Technician (CPhT) (2005)
- High school diploma from Orem High School (1996)

Awards and Scholarships

Awards received include:

- Admission into the Society for Collegiate Scholars (1999)
- PDBio Departmental Scholarship (2006-2008)
- Nominated into the Golden Key Club (2002)
- The Dorothy Florence Scholarship (starting 1999)
- Eagle Scout Award (1991)
- First Chair Trumpet, Utah All-state Band (1996)
- Louis Armstrong Jazz Award (1996)
- First and second place in two writing competitions

Publications

Publications include:

- Published, and presented publication in the Student Sperry Symposium (2003)
- Master's Thesis (2008)
- Pending publication in Endocrinology

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Languages Studied

Languages studied include:

- Fluent Mandarin
- Basic Cantonese

Leadership

Leadership experiences include:

- Eagle Scout (1991)
- Senior Patrol Leader (1989-1991)
- Church leadership positions such as president of various quorums (1990-present)
- Church service leader (1998-1999)
- Science Power Coordinator (2006-2007)
- Band Council Secretary (1995-1996)
- Scout Master (1999)
- Head of the Mandarin and Cantonese departments at the World's largest language training center (2004-2005)

Community Service

Community service provided:

- Taught English in Taiwan (1997-1999)
- Worked in El Salvador Orphanage (2007)
- Taught ninth grade seminary (2002, 2004, 2008)
- Every Tuesday I volunteer taught Chinese to my neighbors (2003)
- Church Service (1997-present)
- Reading with first graders (2006)
- Founded community outreach program (2006-present)

Experiences of Interest

Other experiences of interest include:

- ER Trauma Assistant at UVRMC (2005-2006)
- El Salvador Orphanage nursing station
- Experience shadowing in 11 doctors offices and hospitals in Taiwan, China, and El Salvador

Hobbies

My interests include:

- Volleyball
- Biking
- Camping
- Fishing
- Writing (including a play, several poems, and 5 books for possible future publication)
- Trumpet
- Piano
- Music composition (including two CDs)
- Wrote, directed, and produced an old-time radio show episode
- Reading to and playing with little children