Decreasing intramuscular phosphagen content simultaneously increases plasma membrane FAT/CD36 and GLUT4 transporter abundance

Kristin E. Pandke, Kerry L. Mullen, Laelie A. Snook, Arend Bonen, and David J. Dyck
Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

Submitted 27 June 2008; accepted in final form 15 July 2008

Pandke KE, Mullen KL, Snook LA, Bonen A, Dyck DJ. Decreasing intramuscular phosphagen content simultaneously increases plasma membrane FAT/CD36 and GLUT4 transporter abundance. Am J Physiol Regul Integr Comp Physiol 295: R806–R813, 2008. First published July 23, 2008; doi:10.1152/ajpregu.90540.2008.—Decreasing muscle phosphagen content through dietary administration of the creatine analog β-guanidinopropionic acid (β-GPA) improves skeletal muscle oxidative capacity and resistance to fatigue during aerobic exercise in rodents, similar to that observed with endurance training. Surprisingly, the effect of β-GPA on muscle substrate metabolism has been relatively unexamined, with only a few reports of increased muscle GLUT4 content and insulin-stimulated glucose uptake/clearance in rodent muscle. The effect of chronically decreasing muscle phosphagen content on muscle fatty acid (FA) metabolism (transport, oxidation, esterification) is virtually unknown. The purpose of the present study was to examine changes in muscle substrate metabolism in response to 8 wk feeding of β-GPA. Consistent with other reports, β-GPA feeding decreased muscle ATP and total creatine content by ~50 and 90%, respectively. This decline in energy charge was associated with simultaneous increases in both glucose (GLUT4; +33 to 45%, P < 0.01) and FA (FAT/CD36; +28 to 33%, P < 0.05) transporters in the sarcolemma of red and white muscle. Accordingly, we also observed significant increases in insulin-stimulated glucose transport (+47%, P < 0.05) and AICAR-stimulated palmitate oxidation (+77%, P < 0.01) in the soleus muscle of β-GPA-fed animals. Phosphorylation of AMPK (+20%, P < 0.05), but not total protein, was significantly increased in both fiber types in response to muscle phosphagen reduction. Thus the content of sarcolemmal transporters for both of the major energy substrates for muscle increased in response to a reduced energy charge. Increased phosphorylation of AMPK may be one of the triggers for this response.

β-guanidinopropionic acid; rat; skeletal muscle; energy charge; substrate metabolism

The transient and repeated decrease in the phosphagen content of skeletal muscle is believed to be an important signal initiating many of the structural and biochemical adaptations typically induced with endurance training. The dietary administration of the creatine (Cr) substrate analog β-guanidinopropionic acid (β-GPA) can be used to decrease the intramuscular content of ATP, phosphocreatine (PCr) and total Cr, thereby facilitating the study of muscle “energy charge” as an important regulator in this tissue (10, 25). Decreasing the muscle phosphagen pool through the chronic dietary administration of β-GPA typically results in a 30–40% increase in markers of oxidative metabolism, such as citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) (23, 26, 31, 32) with a commensurate decrease in glycolytic potential (25, 26).

Interestingly, increases in muscle GLUT4 and CS normally associated with training can be attenuated by blunting the transient exercise-induced decreases in muscle phosphagens with clenbuterol (35). Collectively, these findings imply that the decrease in muscle energy charge is an important trigger for exercise-mimetic changes in mitochondrial biogenesis and substrate transport/metabolism. Certainly, the fact that endurance training simultaneously increases sarcolemmal transporter capacity [glucose, lactate, fatty acid (FA)] and markers of mitochondrial biogenesis suggests that these may be coordinately regulated.

The impact of chronically reduced muscle phosphagen content on substrate metabolism has been largely unexamined. Increased muscle GLUT4 mRNA and total protein content have been reported in response to β-GPA feeding (23, 36), and improved insulin-stimulated glucose uptake/clearance in rodent skeletal muscle following β-GPA feeding has also been observed (20, 21, 23). However, there has been no specific assessment of changes in plasma membrane GLUT4 content, which is physiologically more relevant to the process of glucose transport. There also appears to be no examination of the impact of phosphagen depletion on the total expression of insulin signaling proteins. Finally, the effect of decreasing muscle phosphagen content on muscle FA metabolism is virtually unknown. The increase in time to exhaustion during running and swimming in β-GPA-fed rodents (1, 31) and a decrease in respiratory exchange ratio (1) imply an increased reliance on FA as an oxidizable substrate. However, to our knowledge, the effect of β-GPA feeding on specific aspects of muscle FA metabolism, including FA transporter expression and a direct assessment of FA oxidation and esterification, has not been determined.

The mechanism by which a reduction in cellular phosphagen content/energy charge elicits metabolic adaptations is not fully known but likely involves the activation of AMP-activated protein kinase (AMPK). Chronic activation of AMPK via aminomida-zole-4-carboxamide-1-β-ribofuranoside (AICAR) infusion results in increased mitochondrial biogenesis (30, 34) and GLUT4 expression/protein content (5, 14, 16, 37), although it has also been demonstrated that AMPK activation is not essential for exercise-induced GLUT4 expression (15). Feeding β-GPA increases muscle AMPK activity in rodent muscle (24, 38) and various indicators of increased mitochondrial content such as nuclear respiratory factor-1 binding activity, peroxisome proliferator-activated receptor-1α expression, and CS and cytochrome c protein expression (2, 17). Interestingly, β-GPA fails to increase mitochondrial content in the AMPK

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
dead mouse (38). Thus it is reasonable to speculate that AMPK stimulation may be an important mechanism by which phosphodepletion leads to metabolic adaptations in muscle substrate utilization.

Therefore, the purpose of the present study was to more closely examine the changes in muscle substrate metabolism in response to the chronic feeding of β-GPA. Specifically, the hypotheses for the current study were that 8 wk of β-GPA dietary administration would, in conjunction with an increase in oxidative capacity, 1) increase sarcolemmal capacity for transporting both glucose and FA, i.e., increased GLUT4, and transporting both glucose and FA, i.e., increased GLUT4, and 2) that improved substrate transport capacity would translate into increased insulin-stimulated glucose transport and AICAR-stimulated FA oxidation; and 3) that the observed metabolic changes would correspond to increased content and/or phosphorylation of AMPK.

**METHODS**

**Animals**

Female Sprague-Dawley rats were obtained from Charles River Laboratories (Charles River Laboratories, QC, Canada) at ~70 g in mass. Upon arrival, animals were housed individually in a controlled environment with a reversed 12:12-h light-dark cycle and allowed ad libitum access to Purina standard rat chow and water. Following 1 wk of acclimatization, rats were randomly assigned to one of two groups: 1) control, i.e., standard rat chow (Research Diets D06052601M, New Brunswick, NJ) or 2) β-GPA, i.e., standard chow with the addition of 1% β-GPA wt/wt (G6878-5G; Sigma Canada, Mississauga ON, Canada). Twelve animals were assigned to each group. All protocols were approved by the Animal Care Committee, University of Guelph, Canada.

**Feeding Protocol**

Control rats were pair fed to β-GPA-fed rats for 8 wk to minimize differences in body mass gain between groups. Body mass was monitored twice weekly. β-GPA was supplemented daily to the diet as described in previous studies (11, 21, 26, 27). Animals were fasted overnight and anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body mass) before all experimental procedures.

**Blood and Tissue Sampling**

At the end of the 8-wk feeding trial, fasting blood samples were collected via cardiac puncture after first excising skeletal muscles for vesicle preparation and incubation protocols. All blood samples were collected in heparinized tubes and kept on ice until centrifugation (10,000 g for 5 min at 4°C). Plasma was collected for the analyses of glucose (YSI 23A Blood Glucose Analyzer), insulin (RIA kit; Linco, St. Charles, MO) and free FAs (NEFA kit; Wako, Richmond, VA).

**Muscle Incubations**

Following anesthetization, the soleus muscle was carefully separated into three longitudinal strips from tendon to tendon using a 27-gauge needle. One strip from each soleus was immediately frozen in liquid nitrogen for later analyses of muscle phosphagens (ATP, PCr, Cr) and β-HAD and CS activities. The remaining two strips from each soleus were incubated in paired fashion for the measurement of 1) basal and insulin-stimulated glucose transport or 2) basal and AICAR-stimulated FA oxidation.

Basal and insulin-stimulated skeletal muscle glucose transport. Immediately upon excision, soleus strips were incubated for 30 min at 30°C in sealed vials containing 2 ml of pregressed (95% O2-5% CO2) Krebs Henseleit buffer (KHB, 0.1% BSA) with 8 mM glucose and 32 mM mannitol, in the absence or presence of insulin (10 mM, maintained in all subsequent steps) in a gentle shaking bath at 30°C. Muscle strips were washed (2 × 10 min) with glucose-free KHB (4 mM pyruvate, 36 mM mannitol). Solei were then incubated for 20 (insulin) or 40 (basal) min in KHB [4 mM pyruvate, 8 mM 3-O-[3H]methyl-D-glucose (800 μCi/nmol), and 28 mM [14C]mannitol (60 μCi/nmol)]. Radioisotopes were purchased from Amersham (Oakville, ON, Canada). After incubation, muscles were blotted, trimmed of tendons, weighed, and digested in 1 ml of NaOH for 10 min at 95°C. Glucose transport was calculated from duplicate 200-μl aliquots of muscle digest to quantify intracellular 3-O-[3H]methyl-D-glucose.

Skeletal muscle FA metabolism. Soleus strips were equilibrated in 2 ml gassed (95% O2-5% CO2) KHB containing 4% FA-free BSA, 5 mM glucose, and 0.5 mM palmitate, with or without 2 mM AICAR (Toronto Research Chemicals, North York, ON, Canada) in a shaking bath at 30°C for 30 min. Following incubation, muscles were transferred to vials containing 2 ml of the same type buffer with the addition of 0.5 μCi/ml of [1,13C]palmitate (Amersham) for an additional 60-min incubation to determine rates of palmitate oxidation and incorporation into intramuscular triacylglycerol (TAG) and diacylglycerol (DAG) lipid pools, as previously described (9). Vials containing the samples were sealed to prevent loss of gasses.

Following incubation, muscles were blotted of excess liquid, trimmed of tendons, weighed, and placed in a 14-ml centrifuge tube containing 5 ml of ice-cold 2:1 chloroform-methanol. Muscles were homogenized and centrifuged, and the supernatant was treated with 2 ml of distilled water to separate the aqueous and lipophilic phases. One milliliter of the aqueous phase was quantified by liquid scintillation counting to determine the amount of 14C-labeled oxidation intermediates resulting from isotopic fixation. Muscle lipids were redissolved in 100 μl of 2:1 chloroform-methanol, spotted on an oven-dried silica gel plate (Fisher Scientific Canada, Mississauga ON, Canada), and placed in a sealed tank containing 60:40:3 heptane-isopropyl ether-acetic acid for 50 min. Plates were allowed to dry, sprayed with dichlorofluorescin dye (0.2% wt/vol in ethanol), and visualized under long-wave ultraviolet light. Individual lipid bands were scraped into vials for liquid scintillation counting. 14CO2 accumulated in the incubation buffer was released by transferring 1 ml of buffer into a sealed flask, acidifying with 1 ml of 1 M sulfuric acid, and captured by benzenthionium hydroxide. The trapped 14CO2 was counted using standard liquid scintillation counting techniques.

**Preparation of Giant Sarcolemmal Vesicles**

Because of the amount of tissue required to generate giant sarcolemmal vesicles, total muscle and sarcolemmal transporter contents (GLUT4, FAT/CD36 and FABPpm) were determined in representative red and white muscles, but not soleus muscle, from overnight-fasted animals. Giant sarcolemmal vesicles were prepared from red and white gastrocnemius and tibialis anterior muscles as described previously (4) and frozen at ~80°C until analyzed for plasma membrane-associated FA and glucose transport proteins. Briefly, muscles were minced and incubated for 1 h at 34°C in 140 mM KCl-10 mM MOPS (pH 7.4), aprotinin (30 μg/ml; Sigma Canada), and collage-nase (type VII, 150 U/ml) in a shaking water bath. The supernatant fraction was collected, and the remaining tissue was washed with KCI/MOPS and 10 mM EDTA, resulting in a second supernatant fraction. Both supernatant fractions were pooled, and Percoll (G.E. Healthcare, Aurora, OH), KCl, and aprotinin were added to a final concentration of 3.5% (vol/vol), 28 mM, and 10 μg/mL, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3 ml middle layer of 4% Nycodein (wt/vol) and a 1 ml KCI/MOPS upper layer. This sample was centrifuged at 60 g for 45 min at room temperature. Subsequently, the vesicles were harvested from the interface of the upper and middle layer, diluted in

**Downloaded from http://ajpregu.physiology.org/ by June 27, 2017**
KCl/MOPS, and recentrifuged at 12,000 g for 5 min. The supernatant was collected and stored at −80°C for Western blot analyses.

**Muscle Measurements**

**Phosphagens.** Soleus strips were freeze-dried and extracted with 0.5 M HClO₄ + 1 mM EDTA, and the supernatant was neutralized with 2.2 M KHCO₃. The neutralized supernatant was analyzed spectrophotometrically for ATP, PCr, and Cr content.

**Western blots.** Given the limited tissue available from the soleus muscle, and its utilization for metabolic measurements (glucose transport, FA metabolism) and the expression of various insulin-signaling proteins (see below), it was necessary to sample representative red and white muscles for the assessment of various substrate transporters (GLUT4, FAT/CD36, FABPpm). Red and white whole muscle crude membranes were generated as previously described and analyzed for total protein (BCA protein assay).

**FAT/CD36, FABPpm.** Red and white whole muscle crude membranes were generated as previously described and analyzed for total protein (BCA protein assay). Crude membrane denatured protein (40 μg) was loaded for quantification of total muscle FAT/CD36, FABPpm, and GLUT4; 40 μg of cytosolic fraction protein were loaded for the detection of total and phosphorylated AMPK content. Red and white vesicle lysate (10 μg) was loaded for quantification of sarcosomal FAT/CD36, FABPpm, and GLUT4 content. Cytosolic homogenates were prepared from soleus muscles, and 50 μg were loaded for the determination of total protein content of various insulin-signaling intermediates [insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3-kinase), protein kinase B (Akt), AS-160]. Briefly, samples were solubilized in 4× Laemmeli’s buffer, boiled (95°C, 5 min) and resolved by SDS-PAGE, and wet transferred to a polyvinylidene difluoride membrane (1–1.5 h, 100 V). Membranes were blocked for 1 h at room temperature and then incubated with specific primary antibodies for total AMPKα and phospho-Thr172-Thr AMPK (Cell Signaling, Danvers, MA), GLUT4 (Chemicon International, Golden, CO), FABPpm (gift from Dr. Calles-Escandon, Wake Forest University School of Medicine), FAT/CD36 (gift from Dr. Tandon, Otsuka Mary-land Medical Laboratories), and insulin signaling intermediates [Akt (Santa Cruz Biotechnology, Santa Cruz, CA), IRS-1, PI3-kinase p85, AS-160 (Upstate Cell Signaling Solutions, Millipore, MA)] overnight at 4°C. After incubation with the appropriate secondary antibody (1 h at room temperature), the immune complexes were detected using the enhanced chemiluminescence method and quantified with densitometry (Chemigenius 2 Bioimaging system; SynGene, Cambridge, UK). Even loading was confirmed with Ponceau-S staining.

**Oxidative enzyme activity measurements.** CS (37°C, 412 nm) and β-HAD (37°C, 340 nm) were determined spectrophotometrically on soleus muscle lysates diluted 1:20, as described previously (19, 29).

**Calculations and Statistics**

Palmitate oxidation and incorporation into lipid pools (nmol·g wet wt⁻¹·h⁻¹) were calculated using the specific activity of the incubation buffer (disintegrations/min radiolabeled palmitate/nmol total palmitate). Glucose transport was calculated by the intracellular accumulation of 3-O-[¹⁴C]methyl-D-glucose analog and expressed as nanomoles glucose analog accumulated per gram wet tissue per 5 min.

Results are presented as means ± SE. Student’s paired one-tailed t-tests were used to compare the effect of AICAR on FA metabolism and insulin on glucose transport with basal conditions. Muscle phosphagens, enzyme activities, and plasma blood assays were also analyzed by Student’s paired one-tailed t-test. Repeated-measures ANOVA, followed by Tukey’s posttest, were used to assess statistical significance of Western blots between groups (control vs. β-GPA) and muscle type (red vs. white). Statistical significance was accepted at P < 0.05.

**RESULTS**

**Body Mass**

Body mass of control and β-GPA-fed rats was not significantly different during or at the completion of the feeding trials (week 8; control, 233 ± 2 g vs. β-GPA, 292 ± 2 g).

**Plasma Insulin, Glucose, and FA**

Fasting insulin (Table 1) was slightly but significantly reduced with β-GPA feeding (−10%, P < 0.01); there was no

![Fig. 1. Methylglucose transport under basal and insulin-stimulated conditions (A) and calculated difference between basal and insulin-stimulated rates of glucose transport (B) in soleus from control and β-guanidinopropionic acid (β-GPA)-fed rats. *Significantly different from control fed; †significantly different from basal condition. n = 12 experiments/group.](http://ajpregu.physiology.org/)

| Table 1. Fasting plasma insulin, glucose, and free fatty acids of control and β-GPA-fed rats |
|------------------|------------------|
| **Control** | **β-GPA** |
| Week 8 insulin, ng/ml | 1.7±0.1 | 1.5±0.1* |
| Week 8 glucose, mmol/l | 8.7±0.2 | 8.3±0.2 |
| Week 8 free fatty acids, mmol/l | 0.6±0.1 | 1.0±0.1* |

Values are expressed as means ± SE. β-GPA, β-guanidinopropionic acid. *P < 0.01, significantly different from control.

| Table 2. Muscle phosphagens (ATP, PCr, Cr, and TCr) in soleus muscle of control and β-GPA-fed rats |
|------------------|------------------|
| **Control** | **β-GPA** |
| ATP | 19.8±0.8 | 10.6±0.4* |
| PCr | 47.7±1.9 | 4.1±0.2* |
| Cr | 48.0±2.8 | 9.6±0.7* |
| TCr | 96.6±4.1 | 13.5±0.8* |

Values are mean ± SE; n = 12 rats. Units are mmol/kg dry mass. PCr, phosphocreatine (PCr); Cr, creatine; TCr, total creatine. *P < 0.01, significantly different from control.
significant change in fasting glucose concentrations. Plasma FA (Table 1) were significantly increased by β-GPA feeding (94%, P < 0.01).

Muscle Phosphagens

After 8 wk of dietary β-GPA administration, soleus muscle phosphagens (Table 2) were all significantly reduced compared with control-fed rats (ATP, 47%; PCr, 91%; Cr, 80%; calculated total Cr, 86%; P < 0.05).

Basal and Insulin-Stimulated Glucose Transport in Soleus

There were no significant differences in basal glucose transport rates between groups (Fig. 1A). Insulin-stimulated glucose transport was significantly greater in soleus from β-GPA-fed rats compared with control (20%, P < 0.05). The calculated increase from basal to insulin-stimulated conditions (Fig. 1B) was greater in β-GPA-fed rats (47%, P < 0.05). This improved insulin response in soleus was not attributable to changes in the total protein expression of IRS-1, PI3-kinase p85, or Akt (Table 3). However, total AS-160 content increased significantly following β-GPA feeding (P < 0.001).

Palmitate Metabolism in Soleus

Rates of palmitate oxidation under basal conditions in isolated soleus muscles were not different in control and β-GPA-fed rodents (Fig. 2A). Oxidation rates were significantly increased by the AMPK agonist AICAR in soleus from both control (43%, P < 0.01) and β-GPA (102%, P < 0.01) rats; these were significantly different from each other (β-GPA

Table 3. Protein expression of insulin signaling intermediates in soleus muscle of control and β-GPA-fed rats

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>β-GPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS-1</td>
<td>100±5</td>
<td>96±5</td>
</tr>
<tr>
<td>PI3-kinase p85</td>
<td>100±5</td>
<td>100±4</td>
</tr>
<tr>
<td>Akt</td>
<td>100±6</td>
<td>98±4</td>
</tr>
<tr>
<td>AS160</td>
<td>100±4</td>
<td>128±5*</td>
</tr>
</tbody>
</table>

Values are means ± SE in arbitrary units relative to control (expressed as 100); n = 12 rats. IRS, insulin receptor substrate; PI3-kinase, phosphatidylinositol 3-kinase; Akt, protein kinase B. *P < 0.001, significantly different from control.

Table 4. Palmitate incorporation into soleus triacylglycerol and diacylglycerol pools in control and β-GPA-fed rats, under basal and AICAR-stimulated conditions

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Condition</th>
<th>Control</th>
<th>β-GPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerol</td>
<td>Basal</td>
<td>48.3±6.4</td>
<td>45.8±6.4</td>
</tr>
<tr>
<td></td>
<td>AICAR</td>
<td>49.0±1.7</td>
<td>42.2±8.7</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>Basal</td>
<td>19.8±3.1</td>
<td>19.8±1.7</td>
</tr>
<tr>
<td></td>
<td>AICAR</td>
<td>20.7±1.3</td>
<td>18.7±1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol·g wet wt⁻¹·60 min⁻¹; n = 12 rats. AICAR, aminomimidazol-4-carboxamide-1-β-ribofuranoside.

Fig. 2. Palmitate oxidation under basal and AICAR-stimulated conditions (A) and calculated difference between basal and AICAR-stimulated rates of palmitate oxidation (B) in soleus from control and β-GPA-fed rats. ASignificantly different from control fed; bsignificantly different from red muscle. n = 12/group.

Fig. 3. Total muscle GLUT4 protein content (A) and plasma membrane GLUT4 content (B) in red and white muscles from control and β-GPA-fed rats. ASignificantly different from control fed; bsignificantly different from red muscle. n = 12/group.
vs. control, \( P = 0.045 \)). The calculated increase from basal to AICAR-stimulated conditions (Fig. 2B) was significantly greater in the \( \beta \)-GPA-fed rats (\(+77\%, P < 0.01\)). No differences in the rates of palmitate incorporation into TAG or DAG pools were observed between control and \( \beta \)-GPA groups (Table 4).

**Total and Plasma Membrane Glucose and FA Transporter Protein Expression**

\( \beta \)-GPA feeding resulted in an increase in total muscle GLUT4 protein content in red (\(+19\%, P < 0.01\); Fig. 3A) but not white muscle. Plasma membrane-associated GLUT4 protein content (Fig. 3B) was increased in both red (\(+45\%, P < 0.01\)) and white (\(+33\%, P < 0.05\)) muscles following 8 wk of \( \beta \)-GPA feeding.

Total muscle FAT/CD36 protein content (Fig. 4A) was increased with \( \beta \)-GPA feeding in both red (\(+21\%, P < 0.05\)) and white (\(+55\%, P < 0.01\)) muscles. Similarly, plasma membrane-associated FAT/CD36 content was increased in red (\(+28\%, P < 0.05\)) and white (\(+33\%, P < 0.05\)) muscles with \( \beta \)-GPA feeding (Fig. 4B). No change in total muscle or plasma membrane-associated FABPpm content was observed in response to \( \beta \)-GPA feeding (Fig. 4, A and B).

**Skeletal Muscle AMPK Protein Expression**

There were no differences in total protein expression of AMPK (Fig. 5A) between groups. Phosphorylated AMPK content was increased in both the red (\(+23\%, P < 0.05\)) and white (\(+19\%, P < 0.05\)) muscles of \( \beta \)-GPA-fed rats (Fig. 5B).

**Oxidative Enzyme Markers**

There was an increase in the maximal activities of CS (\(58.2 \pm 1.5\) vs. \(48.2 \pm 1.9\) \(\mu\)mol/min\(^{-1}\)g\(^{-1}\), \( P < 0.01\)) and \( \beta \)-HAD (\(26.2 \pm 0.7\) vs. \(20.1 \pm 1.1\) \(\mu\)mol/min\(^{-1}\)g\(^{-1}\), \( P < 0.01\)) in the soleus muscle of \( \beta \)-GPA relative to control-fed rats.

**DISCUSSION**

The major and novel finding of the present study was that the reduction in muscle phosphagen content, as induced by \( \beta \)-GPA administration, resulted in simultaneous increases in both glucose (GLUT4) and FA (FAT/CD36) transporters in the plasma membrane. The upregulation of sarcolemmal transporters for both of the major energy substrates for muscle would appear to be a logical compensatory response to a depleted energy charge. To our knowledge, this has not previously been demonstrated. Interestingly, muscle FABPpm protein content was unaltered with \( \beta \)-GPA feeding, suggesting that its increase is less critical than that of FAT/CD36 to increase FA utilization and restore cellular energy charge. In accordance with a greater availability of glucose and FA transporters, we observed significant increases in insulin-stimulated glucose transport and AICAR-stimulated palmitate oxidation in the soleus muscle of \( \beta \)-GPA-fed animals. Total protein expression of various insulin signaling intermediates in the soleus muscle was unchanged with \( \beta \)-GPA, except for AS-160, which was significantly increased. In addition, phosphorylation of AMPK, but not total protein content, was significantly increased in response to muscle phosphagen reduction, supporting the hypothesis that AMPK is an important signal in mediating the metabolic changes induced by phosphagen depletion.

**Effects of \( \beta \)-GPA on Muscle Glucose Transport**

In the present study, chronic feeding with \( \beta \)-GPA improved insulin-stimulated glucose transport in isolated soleus muscle. This improvement was not because of a general increase in

\[ \text{Fig. 4. Total muscle FAT/CD36 protein content (A), total muscle FABPpm protein content (B), plasma membrane FAT/CD36 protein content (C), and plasma membrane FABPpm protein content (D) in red and white muscles from control and \( \beta \)-GPA-fed rats. *Significantly different from control fed; †significantly different from red muscle. n = 12/group.} \]
protein content of the major insulin-signaling proteins, which is similar to recent findings in trained humans (12); however, we did observe a 28% increase in total expression of AS-160, which might potentially account for the greater response of glucose transport to maximal insulin stimulation. Given the limited tissue available from the soleus muscle, we did not determine whether the phosphorylation of AS-160, or any of the other insulin signaling proteins, was increased during an acute insulin challenge. Because of the amount of tissue required to generate giant sarcolemmal vesicles, total muscle and sarcolemmal transporter contents (GLUT4, FAT/CD36, and FABPpm) were determined in representative red and white muscles.

Nonetheless, the consistent increase in vesicle GLUT4 content in both red and white tissue strongly suggests that this would also have been the case in soleus, although we cannot exclude the possibility that the response in soleus may have been different. Assuming that sarcolemmal GLUT4 content was increased in soleus muscle, then an increased transporter availability for translocation, coupled with greater AS-160 content, would explain the observed improvement in insulin response. In addition, it is possible that there was greater activation of the insulin-signaling cascade proteins in response to the lowering of the muscle energy charge. Surprisingly, given the increase in plasma membrane-associated GLUT4 content, we did not observe an increase in basal rates of glucose transport. Fasting insulin concentration was lowered by \( \sim 10\% \) in the \( \beta \)-GPA-fed rats, suggesting that insulin sensitivity was slightly improved; this is in accordance with our observation that muscle (soleus) insulin-stimulated glucose transport was increased.

Our finding in the present study that \( \beta \)-GPA administration increased the phosphorylation of AMPK is in agreement with previous studies (24, 38). It is likely that \( \beta \)-GPA mediates its effects on muscle glucose transport capacity at least partly through the activation of AMPK. Previous studies have demonstrated that chronic activation of AMPK stimulates GLUT4 gene transcription (37) and increases GLUT4 protein content in rodent (5) and human muscle (18). Perhaps most persuasive to the argument that the metabolic effects of \( \beta \)-GPA are mediated largely through the activation of AMPK is the finding that \( \beta \)-GPA administration to mice results in increased skeletal muscle mitochondrial biogenesis in wild-type animals but not in those that do not express muscle AMPK (38).

**Effects of \( \beta \)-GPA on Skeletal Muscle FA Transporters**

In the present study, total muscle and plasma membrane FAT/CD36 protein, but not FABPpm content, was increased in both red and white muscles with chronic \( \beta \)-GPA feeding. As is the case with GLUT4, the increase in total available FAT/CD36 transporters would presumably be available for translocation to the sarcolemma during stimulation. Thus the \( \beta \)-GPA-induced increase in total FAT/CD36 likely contributed to the greater AICAR-stimulated increase in FA oxidation observed in this study. It has previously been demonstrated that activation of AMPK, via AICAR, results in the translocation of FAT/CD36 to the plasma membrane (6), contributing to increased FA uptake. The significance of the lack of change of the FABPpm transporter in this study is unclear; nonetheless, this observation indirectly implies that increases in FAT/CD36 may be more critical to the increase in FA uptake and oxidation in response to a compromised cellular energy charge. This is in agreement with recent studies demonstrating that overexpression of muscle FABPpm in vivo (1) does not result in a parallel increase in FA transport rate (7) and 2) does not result in further increases in mitochondrial FA oxidation (13). Finally, 3) the absence of the FAT/CD36 transporter results in severe impairment of AICAR-stimulated FA oxidation, demonstrating that it is critical to this process (3).

It is possible that the observed increase in FAT/CD36 transporters in response to \( \beta \)-GPA feeding is also mediated at least in part by AMPK activation. Evidence derived from animal and human studies show that various activators of AMPK, including contraction, AICAR, and metformin, increase FAT/CD36 translocation to the sarcolemma (6, 20, 28); conversely, the lowering of AMPK activity by resistin in L6 skeletal muscle cells decreases cell surface FAT/CD36 content and subsequently reduces FA oxidation (22). In addition, daily AICAR injection for 7 days increases FAT/CD36 protein expression (Bonen, unpublished data). Therefore, in the present study, it is reasonable to suggest that the increase in AMPK phosphorylation contributed to the increase in sarcolemmal FAT/CD36 protein content.

![Graph](AJP-Regul Integr Comp Physiol • VOL 295 • SEPTEMBER 2008 • www.ajpregu.org)
Effects of β-GPA on Skeletal Muscle Palmitate Oxidation and Oxidative Capacity

In the present study, 8 wk of β-GPA treatment did not alter basal rates of palmitate oxidation, in spite of the observed increase in FA transport and oxidative capacity. However, we have previously reported this apparent discrepancy in trained rats, i.e., unchanged basal FA oxidation in isolated soleus (8). It must be remembered that the metabolic rate in isolated, unstimulated muscle strips is low and may not reflect changes in maximal metabolic capacity. Indeed, electrically induced contraction of isolated soleus demonstrates a clear improvement in palmitate oxidation in muscle isolated from trained vs. untrained rats (8). In the current study, the AICAR-stimulated increase in palmitate oxidation was significantly greater in the β-GPA-fed rats, strongly suggestive of an increased capacity to take up and oxidize FA, which is consistent with the noted increases in FAT/CD36 content and maximal CS and β-HAD activities. Interestingly, the changes in CS and β-HAD in response to β-GPA feeding have been less extensively studied in oxidative muscles such as soleus, with two studies reporting no change (11, 26) and one reporting an increase (31), whereas an increased oxidative capacity is consistently reported in glycolytic muscles (23, 26, 31, 32). Our study clearly demonstrates an increase in soleus oxidative capacity, i.e., CS and β-HAD, similar to that previously observed in glycolytic muscle.

Effects of β-GPA on Skeletal Muscle Lipid Accumulation and Circulating Plasma FA

We did not observe any differences in the rate of palmitate incorporation into TAG or DAG pools in isolated soleus muscles derived from β-GPA-fed rats. Our inability to observe any differences may be have been due to 1) the relatively brief acute incubation period and 2) the absence of other exogenous factors in our isolated model, e.g., insulin, which would stimulate lipid esterification.

Interestingly, we did observe a significant increase in the fasting concentration of plasma FA in the β-GPA-fed rats. Thus the availability of FA to the muscle was enhanced in concert with the improved ability to take up and utilize this substrate. This in turn suggests that 1) β-GPA-fed animals are primarily reliant on plasma-derived FA and 2) availability of exogenous FA may also be an important signal in determining FA metabolism adaptations in muscle. Certainly, this agrees with the recent finding by Watt et al. (33) that exogenously provided FA stimulate muscle AMPK activity. Finally, the increase in plasma FA in the face of a presumed increase in the capacity of muscle to take up and oxidize lipids implies that there must have been an increase in adipose tissue lipolysis. Whether this might due to a direct effect of β-GPA on adipose tissue or secondary to a systemic effect (e.g., catecholamines) was not determined in this study.

Perspectives and Significance

In this study, the chronic feeding of β-GPA significantly decreased muscle phosphagen content, resulting in compensatory increases in total muscle and sarcolemmal GLUT4 and FAT/CD36 transporters, maximal insulin-stimulated glucose uptake, maximal AICAR-stimulated FA oxidation, and oxidative capacity. These changes appear to have been mediated, at least in part, by an increase in phosphorylated AMPK content. However, with the exception of AS-160, total protein content of the major insulin-signaling proteins was not altered. Interestingly, no change in FABPpm was detected, suggesting that the FAT/CD36 transporter is the more critical physiological regulator of FA uptake and utilization, or at least in response to a decrease in the cellular energy charge. Collectively, an increase in both FA transport and oxidative capacities, as well as an increase in fasting plasma FA, suggests a greater reliance on exogenous FA as a substrate in response to the challenged energy charge of the muscle.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Brianne Thrush and Justin Cresser.

GRANTS

This study was funded by grants from Natural Science and Engineering Research Council of Canada (NSERC) (D. J. Dyck) and the Canadian Institutes of Health Research (D. J. Dyck, A. Bonen). A. Bonen is a Canada Research Chair in Health and Metabolism. K. Mullen was funded with an NSERC Post Graduate Scholarship.

REFERENCES


