Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis

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1Departments of Internal Medicine, 3Cell Biology, and 5Cellular and Molecular Physiology, and the 6Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06510; 2Bristol-Myers Squibb, Princeton, New Jersey 08543; and 4Departments of Medicine and Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

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Bergeron, Raynald, Jian Ming Ren, Kevin S. Cadman, Irene K. Moore, Pascale Perret, Marc Pypaert, Lawrence H. Young, Clay F. Semenkovich, and Gerald I. Shulman. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. Am J Physiol Endocrinol Metab 281: E1340–E1346, 2001.—The underlying mechanism by which skeletal muscle adapts to exercise training or chronic energy deprivation is largely unknown. To examine this question, rats were fed for 9 wk either with or without β-guanadinopropionic acid (β-GPA; 1% enriched diet), a creatine analog that is known to induce muscle adaptations similar to those induced by exercise training. Muscle phosphocreatine, ATP, and ATP/AMP ratios were all markedly decreased and led to the activation of AMP-activated protein kinase (AMPK) in the β-GPA-fed rats compared with control rats. Under these conditions, nuclear respiratory factor-1 (NRF-1) binding activity, measured using a cDNA probe containing a sequence encoding for the promoter of δ-aminolevulinate (ALA) synthase, was increased by about eightfold in the muscle of β-GPA-fed rats compared with the control group. Concomitantly, muscle ALA synthase mRNA and cytochrome c content were also increased. Mitochondrial density in both extensor digitorum longus and epitrochlearis from β-GPA-fed rats was also increased by more than twofold compared with the control group. In conclusion, chronic phosphocreatine depletion during β-GPA supplementation led to the activation of AMP-activated protein kinase (AMPK) in the β-GPA-fed rats compared with control rats. Under these conditions, nuclear respiratory factor-1; muscle adaptation; δ-aminolevulinate synthase; cytochrome c; β-guanadinopropionic acid

AMP-ACTIVATED PROTEIN KINASE (AMPK) is activated by energetic stress, such as starvation and ischemia (17). AMPK is activated by decreases in both the ATP/AMP ratio and phosphocreatine content (30) that are known to occur during exercise. Concomitantly, AMPK activity is increased during exercise in the skeletal muscle of rodents (40) and humans (7, 14, 42). Skeletal muscle glucose uptake is stimulated by the acute activation of AMPK by δ-aminomimidazole-4-carboxamide ribofuranoside (AICAR), a nonspecific chemical activator (26). Recent studies have suggested that this phenomenon may be important for contraction-mediated stimulation of glucose uptake (4, 18, 22). AMPK is the mammalian homolog of the yeast SNF1 gene, which is required for the response to starvation for glucose (17). AMPK is located in the nuclei of many cells, suggesting that this protein may be involved in the regulation of gene expression (33). This hypothesis has been confirmed recently in isolated hepatic cells, where activation of AMPK was shown to inhibit the expression of fatty acid synthase and the pyruvate kinase gene in hepatocytes (13, 24). Repeated daily chronic activation of AMPK by AICAR for 4 wk, to mimic exercise training, was associated with increases in GLUT-4 protein content as well as the activity of mitochondrial oxidative enzymes (28, 41). These data suggest that repeated bouts of physical exercise may cause skeletal...
muscle biochemical adaptations through repeated activation of AMPK.

β-Guanadinopropionic acid (β-GPA) is a creatine analog that competes for the transport of creatine in the skeletal muscle (12) and inhibits creatine kinase activity (27). As a result, when β-GPA is added to the diet of rats, skeletal muscle phosphocreatine stores are depleted by >85%, and ATP concentrations are decreased by ~40–50% (11, 34, 37). Chronic β-GPA feeding puts skeletal muscle cells in a state of chronic energy stress, which is typically associated with a number of skeletal muscle adaptations that resemble those observed after an endurance type of physical training, such as increases in GLUT-4 content (31, 46), hexokinase, citrate synthase activities (31, 34), and oxidative capacity (27).

One hypothesis is that nuclear respiratory factor-1 (NRF-1), a transcription factor that acts on a nuclear set of genes required for the transcription of respiratory proteins as well as mitochondrial transcription and replication (15, 38), may be a key player in the increased oxidative capacity of the skeletal muscle cell in response to energy stress such as exercise and β-GPA feeding. Functional NRF-1 binding sites for the δ-aminolevulinate (ALA) synthase (6) and cytochrome c (10) promoters have been found. The former is a mitochondrial matrix enzyme that is rate limiting for the biosynthesis of heme (1), which is essential for electron transfer and ATP generation. Increased oxygen consumption in HeLa cells through overexpression of uncoupling protein-1 increases ALA synthase expression via an NRF-1-dependent mechanism (25). It has been suggested that NRF-1 plays a role in cell adaptation to the energy stress situation by translating a metabolic perturbation into an increased capacity to generate energy. In addition, coactivation of NRF-1 via binding with peroxisome proliferator-activated receptor-γ coactivator 1 causes upregulation of the mitochondrial transcription factor A (mTFA) and may represent the pathway that links an external physiological stimulus to the regulation of mitochondrial replication (43). However, the pathway that leads to activation of NRF-1 is unknown.

In this study, we examined the hypothesis that chronic energy stress, provoked by β-GPA feeding, activates AMPK, which in turn activates NRF-1, which in turn leads to increased mitochondrial biogenesis.

### EXPERIMENTAL PROCEDURES

β-GPA feeding study. To determine the effect of AMPK activation on skeletal muscle metabolic adaptations, male Sprague-Dawley rats weighing 50–75 g were fed ad libitum with a 45% fat enriched diet with or without 1% β-GPA. Body weight and food consumption of the rats were measured every 2–3 days. β-GPA was synthesized as described previously (2). On the 9th wk of feeding, fed rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The right calf muscle group and quadriceps were freeze-clamped in situ with a liquid-cooled stainless steel tongue. From these flattened muscle pieces, only a regional part distal to the bone was used for the determination of AMPK activity and other biochemical assays. This method excludes deeper muscles, such as the soleus and the plantaris from the calf muscle group and includes only the superficial aspect of the calf and the quadriceps, which are mainly comprised of white muscle fibers (3, 10, 36). The left superficial white quadriceps was carefully dissected out and immediately treated for nuclei isolation for further determination of NRF-1 by electrophoretic mobility shift assay (EMSA). These muscles have been chosen for their similar muscle fiber composition. The superficial white gastrocnemius and white quadriceps are comprised of ~77–90% type IIb muscle fibers, with the rest being mainly type IIa but virtually no type I (3, 9, 35). The left plantaris, extensor digitorum longus (EDL), and the epitrochlearis from the left forelimb were subsequently dissected out for determination of mitochondrial density by electron microscopy. These muscles are mostly comprised of white muscle fibers, with an increasing ratio of type IIb to type IIa as follows: plantaris < EDL < epitrochlearis. The protocol was approved by the Yale Animal Care and Use Committee.

Skeletal muscle nuclei isolation and EMSA for NRF-1. Isolation of nuclei from ~800–900 mg of freshly isolated superficial quadriceps skeletal muscle was performed according to a method described previously (16). The weight of the superficial white quadriceps used for this procedure was not

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<th>Table 1. Muscle high-energy phosphate metabolites</th>
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<td>Control</td>
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</tr>
<tr>
<td>Creatine phosphate</td>
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<td>20.4 ± 3.3</td>
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<td>ATP</td>
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<td>5.5 ± 0.4</td>
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<td>ADP</td>
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<td>0.98 ± 0.06</td>
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<td>AMP</td>
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<td>0.18 ± 0.02</td>
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<td>ATP/AMP ratio</td>
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<td>27.1 ± 1.6</td>
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Nucleotide contents were measured by HPLC (32). All data are means ± SE; concentrations are expressed in μmol/g wet wt; n = 11–12 in each group. Significant decreases in creatine phosphate, ATP, and ADP contents and the ATP/AMP ratio were observed in muscle of β-GPA-fed vs. control rats; *P < 0.002.
Fig. 2. Nuclear respiratory factor-1 (NRF-1) electrophoretic mobility shift assay (EMSA) from muscle sample of rats fed either a creatine analog (β-GPA) or a control diet (CONTROL). Nuclei were isolated from freshly dissected superficial white quadriceps muscle. Nuclear proteins were incubated with a radiolabeled cDNA probe that has a sequence of the δ-aminoevulinate synthase promoter containing a binding site for NRF-1. A: representative autoradiogram of NRF-1 EMSA of control (lane 1) and β-GPA (lane 2) muscle samples and (lane 3) the cold cDNA probe alone demonstrating binding specificity. B: NRF-1 binding activity (arbitrary units) was significantly increased in muscles of β-GPA vs. control rats. Values are means ± SE; n = 10–11 in each group. *P < 0.01.

RESULTS

The rats fed with β-GPA for 9 wk gained less weight (P < 0.01) and were significantly lighter on the day they were killed than the age-matched control rats (β-GPA: 457 ± 15 vs. Control: 510 ± 9 g) despite similar food intake in the two groups (β-GPA: 23.7 ± 0.07 g [not significant (NS)]. Nuclear proteins were extracted by the method described previously (47), and nuclear protein concentrations were determined using Bio-Rad protein assay reagents (Hercules, CA). NRF-1 EMSA was performed as described earlier (25). The probe was generated from oligonucleotides containing a functional NRF-1 binding site in the ALA synthase promoter (the recognition sequence for NRF-1 is underlined): Oligo A, 5'-GGC GCT GGGATCCGTGTTG-3'; Oligo B, 5'-CCC ACA GGGATCCGTGTTG-3'.

RNA isolation and Northern blotting. Total RNA from the superficial white quadriceps was isolated using Totally RNA isolation and Northern blotting (Clontech Laboratories) with primers containing the AMARA sequence (8). Superficial white quadriceps samples were used for determination of protein content by SDS-PAGE and Western blotting. Cytochrome c was detected using a monoclonal mouse antibody against an epitope within amino acids 93–104 (ANN00112: Biosource, Camarillo, CA). Statistical analyses. All data are reported as means ± SE. Data from blood samples and tissue parameters were analyzed by a one-way analysis of variance. Differences between the two groups were considered significant at P < 0.05.

Electron microscopy. Individual muscle samples were prepared by immersion in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Morris, PA) in 0.1 M cacodylate buffer (pH 7.4) at 4°C overnight. Muscles were then washed three times in 0.1 M cacodylate buffer (pH 7.4) and postfixed for 1 h in 1% osmium tetroxide [Embed-812 epoxy resin (EMS)] in the same buffer at room temperature. After three washes in water, the samples were stained for 1 h at room temperature in 2% uranyl acetate (EMS) and then washed again in water and dehydrated in a graded series of ethanol. The samples were then embedded in EMS. Ultrathin (60-nm) sections were cut using a Reichert Ultracut ultramicrotome and collected on formvar- and carbon-coated grids, stained with 2% uranyl acetate and lead citrate, and examined in a Philips 410 electron microscope. Only cross sections of muscle were examined for quantification of mitochondrial density. For each individual rat and muscle, six random pictures were taken at a magnification of 7,100 and printed at a final magnification of 18,250. The volume density of mitochondria was estimated using the point counting method (39). The average volume density was calculated for each individual rat and muscle and was used to calculate the average volume density for each treatment.

Analytical procedures. All of the following biochemical assays were performed in situ freeze-clamped superficial white gastrocnemius muscle. Nucleotides and creatine phosphate concentrations were determined on perchloric acid extracts by previously described methods (19, 29, 32). Skeletal muscle AMPK activity was determined by following the incorporation of [32P]ATP into a synthetic peptide (40) containing the AMARA sequence (8). Superficial white quadriceps samples were used for determination of protein content of cytochrome c by SDS-PAGE and Western blotting. Cytochrome c was detected using a monoclonal mouse antibody against an epitope within amino acids 93–104 (ANN00112: Biosource, Camarillo, CA).
0.4 vs. Control: 22.0 ± 0.1 g food/day; P = NS). Plasma concentrations of glucose, lactate, insulin, fatty acids, and triglycerides were not significantly different between β-GPA and control rats when measured in the fed state 1 wk before they were killed.

Superficial white gastrocnemius muscle high-energy phosphate metabolite contents were altered by β-GPA feeding (Table 1). Muscle creatine phosphate, ATP, and ADP content values were decreased by 74, 53, and 32%, respectively (all P < 0.002), compared with muscles in the control rats. The ATP/AMP ratio, which has been reported to be an important regulator of AMPK activity, was decreased by 54% in the muscle of β-GPA-fed rats compared with control rats (P < 0.001; Table 1).

AMPK activity (Fig. 1) was increased by 42 and 70% (P < 0.05), respectively, in the superficial white gastrocnemius and quadriceps of rats fed with β-GPA compared with the control rats.

NRF-1 EMSA was assessed using a cDNA probe containing a sequence encoding for the ALA synthase promoter known to have a functional binding site for NRF-1. Chronic β-GPA feeding caused an approximately eightfold increase in NRF-1 binding activity from nuclei of superficial white quadriceps muscle compared with the control group (P < 0.01; Fig. 2). Concomitantly, ALA synthase mRNA (Fig. 3) was increased by ~75% (P < 0.001). Content of cytochrome c, another gene known to be regulated by NRF-1 and a marker of mitochondrial density (5, 20), was also increased in the muscle of the β-GPA-treated vs. the control group (Fig. 4; P < 0.05). Chronic feeding of β-GPA increased mitochondrial density by 75% (NS; P = 0.13), 162% (P < 0.05), and 104% (P < 0.05), respectively, in the plantaris, the EDL, and the epitrochlearis muscles compared with the control group as measured by electron microscopy (Fig. 5).

DISCUSSION

Nine weeks of β-GPA feeding puts the skeletal muscle in a state of chronic energy depletion, as reflected by large decrements in phosphocreatine and ATP content and the ATP/AMP content ratio. Under these conditions, muscle AMPK was activated and associated with increased NRF-1 binding activity to the ALA synthase promoter and increased ALA synthase mRNA, cytochrome c protein content, and mitochondrial density. These data support the role of AMPK in the muscle adaptation to energy stress. It is also suggested that
chronic AMPK activation may signal the low-energy status in the skeletal muscle cell and trigger the up-regulation of genes involved in energy production. To examine this possibility, we also assessed key potential target genes that are important for oxidative capacity.

ALA synthase is the first, and rate-limiting, enzyme in the synthesis of heme (23), which is critical to mitochondrial respiration capacity and is increased after exercise training (21, 36). The muscle protein content of the enzyme is also increased upon activation of AMPK by AICAR (41) and during increased energy demand created by overexpression of UCP-1 (25). ALA synthase content depends on transcriptional and translational/posttranslational control (36). The promoter of the ALA synthase gene has a functional binding site for NRF-1 (6). The expression of ALA synthase during physiological increases in energy demand is under the control of NRF-1 (25). This transcription factor is also involved in the upregulation of cytochrome c expression (10) as observed in response to metabolic stressors, such as electrical pacing of cardiac myocytes (44, 45). NRF-1 may also regulate mitochondrial transcription and replication through induction of mTFA (38). In the present experiment, we measured the ALA synthase mRNA content, cytochrome c content, and mitochondrial density and found all of these parameters to be increased in the muscles of β-GPA-fed rats when NRF-1 protein binding was also increased.

Reduction in plantaris and gastrocnemius weight has been reported with β-GPA feeding (35, 47). The weight of the superficial white quadriceps in the present study, which was measured immediately before the muscle underwent the nuclei extraction procedure, was not significantly reduced in β-GPA-fed compared with the control rats (0.91 ± 0.13 vs. 0.99 ± 0.07 g; NS). However, one cannot exclude the possibility that there were decreases in the muscle weight of the other muscles utilized in the present study. If this was the case, the present increase in mitochondrial density could partly be explained by muscle atrophy. On the other hand, the increases in ALA synthase mRNA and cytochrome c protein content are expressed relative to total protein and β-actin, respectively. Consequently, our data exclude the latter possibility. The muscle cytochrome c content has been used a marker of mitochondrial density (5, 6, 21), and the increase reported here supports the concept of increased mitochondrial density.

This is the first evidence obtained from whole skeletal muscle linking increased NRF-1 binding activity to cellular markers of oxidative capacity. In conclusion, the present study shows a strong association between activation of muscle AMPK, increased NRF-1 binding activity, increased cytochrome c content, and increased

![Figure 5](http://ajpendo.physiology.org/)
muscle mitochondrial density. Our data suggest that AMPK may play an important role in muscle adaptations to chronic energy stress via regulation of mitochondrial biogenesis and expression of respiratory proteins through activation of NRF-1.

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