Creatine feeding increases GLUT4 expression in rat skeletal muscle

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Submitted 3 June 2004; accepted in final form 18 October 2004

Ju, Jeong-Sun, Jill L. Smith, Peter J. Oppelt, and Jonathan S. Fisher. Creatine feeding increases GLUT4 expression in rat skeletal muscle. Am J Physiol Endocrinol Metab 288:E347–E352, 2005. First published October 19, 2004; doi:10.1152/ajpendo.00238.2004.—The purpose of this study was to investigate the potential role of creatine in GLUT4 gene expression in rat skeletal muscle. Female Wistar rats were fed normal rat chow (controls) or chow containing 2% creatine monohydrate ad libitum for 3 wk. GLUT4 protein levels of creatine-fed rats were significantly increased in extensor digitorum longus (EDL), triceps, and epitrochlearis muscles compared with muscles from controls (P < 0.05), and triceps GLUT4 mRNA levels were ~100% greater in triceps muscles from creatine-fed rats than in muscles from controls (P < 0.05). In epitrochlearis muscles from creatine-fed animals, glycogen content was ~40% greater (P < 0.05), and insulin-stimulated glucose transport rates were higher (P < 0.05) than in epitrochlearis muscles from controls. Despite no changes in [ATP], [creatine], [phosphocreatine], or [AMP], creatine feeding increased AMP-activated protein kinase (AMPK) phosphorylation by 50% in rat EDL muscle (P < 0.05). Creatine content of EDL muscle was almost twofold higher for creatine-fed animals than for controls (P < 0.05). Creatine feeding increased protein levels of myocyte enhancer factor 2 (MEF2) isoforms MEF2A (~70%, P < 0.05), MEF2C (~60%, P < 0.05), and MEF2D (~90%, P < 0.05), which are transcription factors that regulate GLUT4 expression, in creatine-fed rat EDL muscle nuclear extracts. Electrophoretic mobility shift assay showed that DNA binding activity of MEF2 was increased by ~40% (P < 0.05) in creatine-fed rat EDL compared with controls. Our data suggest that creatine feeding enhances the nuclear content and DNA binding activity of MEF2 isoforms, which is concomitant with an increase in GLUT4 gene expression.

Acetyl-coenzyme A carboxylase; adenosine monophosphate-activated protein kinase; myocyte enhancer factor 2; phosphocreatine; creatine

A NUMBER OF STUDIES regarding beneficial effects of creatine supplementation on muscle glucose metabolism have been reported. For example, creatine supplementation improves impaired glucose tolerance (9) and increases glycogen content (18). Furthermore, combined creatine and carbohydrate supplements result in a greater postexercise muscle glycogen resynthesis than carbohydrate alone (23). However, few studies (17, 18, 28) have been done regarding the effects of creatine supplementation on changes in the expression level of the insulin- and contraction-regulated glucose transporter (GLUT4) that mediates glucose uptake in muscle tissue, and a clear picture has not emerged from the findings described in the literature. It has been demonstrated that creatine supplementation prevents a decrease in muscle GLUT4 protein content during 2 wk of immobilization and increases GLUT4 protein content during a subsequent 10 wk of rehabilitation training in healthy subjects (18). However, the physiological mechanisms supporting elevated GLUT4 expression by creatine supplementation were not addressed. A separate study of creatine supplementation in humans found ~30% greater (not statistically significant) GLUT4 protein in muscle from creatine-supplemented subjects than from control subjects (28). There is reportedly no effect of 5 days of creatine supplementation on GLUT4 expression in rats (17).

A recent study demonstrated an approximately twofold increase in AMP-activated protein kinase (AMPK) phosphorylation after 48 h of creatine supplementation in L6 myocytes (5). It has been shown that myocyte enhancer factors 2 (MEF2)A, -C and -D, transcription factors that regulate GLUT4 expression in muscle (27), increase in response to AMPK activation (16). We hypothesized that the beneficial effects on glucose metabolism related to creatine supplementation might occur concomitantly with increased GLUT4 expression. We have evaluated whether 3 wk of oral creatine supplementation enhances GLUT4 biogenesis concomitantly with increased AMPK phosphorylation and MEF2 DNA binding activity.

MATERIALS AND METHODS

Materials. A polyclonal antibody specific for the GLUT4 glucose transporter was the generous gift of Dr. Mike Mueckler (Washington University, St. Louis, MO). The primers and the internal standard mRNA for competitive RT-PCR were kindly supplied by Dr. John Holloszy (Washington University). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Pierce Biotechnology (Rockford, IL). MEF2A and -D antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); MEF2C, phospho-AMPKα (P-AMPK), AMPKα, and phospho-acetyl-CoA carboxylase (P-ACC) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The antibodies against AMPK and P-AMPK are pan-α antibodies that bind to both isoforms, α1 and α2, of the catalytic subunit of AMPK. ATPlile and reagents for enhanced chemiluminescence (ECL) were obtained from Perkin-Elmer Life Sciences (Boston, MA). NE-PER Nuclear Extraction Reagent, Biotin 3'-End DNA Labeling Kit and LightShift Chemiluminescent EMSA (electrophoretic mobility shift assay) kit were purchased from Pierce Biotechnology (Rockford, IL). Creatine amidohydrolase was obtained from MP Biomedicals (Philadelphi, PA). Radiolabeled 2-deoxyglucose and mannitol were obtained from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

Animal care. Fourteen female Wistar rats weighing ~90 g were obtained from Charles River Laboratories and divided into two groups: 1) control (Cr) supplementation [2% creatine monohydrate...
Creatine feeding increases muscle GLUT4 expression. Muscle GLUT4 protein content was determined by Western blot analysis as described in MATERIALS AND METHODS for extensor digitorum longus (EDL; A), triceps (B), and epitrochlearis muscles (EPI; C). D: intensities (arbitrary units) of triceps GLUT4 mRNA transcript from Chow-fed control (CON) and creatine-supplemented (Cr) muscle samples. GLUT4 mRNA was measured in triceps muscles by competitive RT-PCR as described in MATERIALS AND METHODS. Values are means ± SE; n = 7/group for CON and Cr rats. *P < 0.05.

Table 1. Muscle high-energy phosphate metabolite and creatine content

<table>
<thead>
<tr>
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<th>Control</th>
<th>Creatine Fed</th>
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<tbody>
<tr>
<td>Creatine, μmol/g</td>
<td>6.1 ± 0.4</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>PCr, μmol/g</td>
<td>9.5 ± 0.7</td>
<td>10.0 ± 0.8</td>
</tr>
<tr>
<td>TCr, μmol/g</td>
<td>15.4 ± 0.7</td>
<td>16.4 ± 1.1</td>
</tr>
<tr>
<td>[PCr]/[TCr]</td>
<td>0.61 ± 0.002</td>
<td>0.61 ± 0.023</td>
</tr>
<tr>
<td>Creatinine, μmol/g</td>
<td>1.6 ± 0.2</td>
<td>2.9 ± 0.3*</td>
</tr>
<tr>
<td>ATP, μmol/g</td>
<td>5.6 ± 0.5</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td>AMP, nmol/g</td>
<td>15.4 ± 1.3</td>
<td>14.8 ± 2.5</td>
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Values are means ± SE; n = 7/group. PCr, phosphocreatine; TCr, total creatine; [PCr]/[TCr], PCr-to-TCr concentration ratio. Extensor digitorum longus (EDL) muscles from either control rats or rats fed creatine were analyzed. *P < 0.005, control vs. creatine fed.
creatinine concentrations for these muscles are in the ranges previously reported for rat skeletal muscle (~1 μmol/g, assayed after homogenization in water and mixing with trichloracetic acid (26)) and rat heart (~2.6 μmol/g, assayed after homogenization in perchloric acid (11)).

Muscle GLUT4 expression, glucose transport activity, and glycogen content. Creatine feeding induced an ~60% increase in GLUT4 protein in EDL muscle (Fig. 1A), an ~80% increase in GLUT content of triceps muscle (Fig. 1B), and a twofold increase of GLUT4 in epitrochlearis (Fig. 1C). As shown in Fig. 1D, triceps muscle GLUT4 mRNA levels increased by 100% in the creatine-fed group compared with chow-fed control animals, suggesting that increases in GLUT4 expression

results

Body weights and food intakes were measured every 2–3 days and did not differ between groups.

**Muscle metabolites.** EDL muscle high-energy phosphate metabolite contents were not altered by creatine feeding (Table 1). There were no significant differences in PCR, creatine, ATP, or AMP levels in rat muscle tissue between creatine-fed and chow-fed control animals (Table 1). The PCR-to-TCr concentration ([PCR]/[TCr]) ratio did not differ in EDL muscles from creatine-fed animals compared with muscles from controls (Table 1). The creatineine concentrations for these muscles are in the ranges previously reported for rat skeletal muscle (~1 μmol/g, assayed after homogenization in water and mixing with trichloracetic acid (26)) and rat heart (~2.6 μmol/g, assayed after homogenization in perchloric acid (11)).

**EMSA.** The MEF2 DNA binding activity assay was performed with nuclear extract from EDL muscle tissues prepared with the NE-PER nuclear extraction reagent (Pierce). Synthetic oligonucleotides were labeled with the biotin 3'-End DNA Labeling Kit (Pierce). This oligonucleotide probe contains a functional MEF2 binding site in the GLUT4 promoter (the recognition sequence for MEF2 is italicized): forward oligo, 5'-GAT CGC TCT AAA AAT AAC CCT GTC G-3'; reverse oligo, 5'-C GAC AGG GTT ATT TTT AGA GCG ATC-3' (14). Reactions of biotin end-labeled target DNA and nuclear extracts in the presence or absence of 200-fold excess unlabeled oligonucleotides and subsequent electrophoresis and ECL visualization were performed with an EMSA kit (LightShift, Pierce Biotechnology) according to the manufacturer's instructions.

**Data analysis.** Data are presented as means ± SE. Creatine effects (create vs. control) were evaluated by a univariate analysis of variance (ANOVA). Glucose transport data were analyzed with a 2 × 2 ANOVA (insulin absence/presence × control/creatine).

![Fig. 2. Epitrochlearis muscle glucose transport activity and glycogen content. A: isolated epitrochlearis muscles from CON (n = 7 rats) and Cr animals (n = 6 rats) were assayed for glucose transport activity in the absence or presence of 60 μU/ml insulin and the insulin-stimulated increase in glucose transport, calculated as the difference between basal glucose transport and insulin-stimulated glucose transport for each animal. B: glycogen content of epitrochlearis muscles that were not exposed to insulin (n = 7/group). Values are means ± SE. *P < 0.005 for the creatine × insulin interaction in A, greater insulin-stimulated glucose transport in creatine-fed animals than controls in A, and higher muscle glycogen content for creatine-fed animals than for controls in B. †P < 0.005 for greater insulin-stimulated increase in glucose transport for Cr animals than for CON.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00214.2004)
induced by creatine supplementation are mediated at the transcriptional level. Because of the similar creatine-related increase in GLUT4 in EDL and triceps and the fiber type similarity between the two (7), we believe that the mRNA data for triceps are generalizable to EDL. Insulin (60 μU/ml) stimulated an ∼2.5-fold increase in glucose transport rate in epitrochlearis muscles for control animals and an ∼5-fold increase in glucose transport for creatine-fed animals (Fig. 2A). The creatine × insulin interaction (cho/creatine × basal/insulin) was statistically significant (P < 0.05), and insulin-stimulated glucose transport was greater for creatine-fed animals than for controls (P < 0.05). The data in Fig. 2A represent values for paired muscles (for each animal, glucose transport was assayed in both the absence and the presence of insulin). These muscle pairs allow computation of the increase in glucose transport that is stimulated by insulin for each animal, and this increase is about twofold higher for creatine-fed animals than for controls (Fig. 2B).

AMPK and ACC phosphorylation. The phosphorylation of Thr172 of AMPKα is shown in Fig. 3, A and B. AMPK phosphorylation of creatine-fed rats was significantly increased by 50% (P < 0.05) in EDL muscle compared with muscles from regular chow-fed rats. Muscle content of nonphosphorylated AMPK was not affected by creatine feeding (control 1.00 ± 0.02, creatine-fed rats 0.94 ± 0.08 arbitrary units, n = 7/group). Phosphorylation levels of ACC were 46% higher in EDL muscles from creatine-fed animals compared with EDL muscles from controls (Fig. 3, C and D). The increase in phosphorylation (Ser79) of ACC, an AMPK substrate, is consistent with increased AMPK phosphorylation.

MEF2 isoform content. Nuclear content of MEF2A and MEF2D, members of the MEF2 transcription factor family known to regulate GLUT4 expression, was increased in the muscle of the creatine-fed vs. the control group (Fig. 4).

DISCUSSION

The present study demonstrated that 3 wk of creatine supplementation increases the expression of the GLUT4 gene in rat skeletal muscle. Creatine supplementation was related to increased phosphorylation of AMPK, increased content of MEF2 protein in nuclear extracts, and increased DNA binding activity of MEF2 isoforms.

The present study showed that creatine feeding increases AMPK phosphorylation in rat skeletal muscle. A recent study also shows that 48-h treatment with creatine increased activating phosphorylation of both AMPKα1 and α2 isoforms (5). Currently, the effects of creatine supplementation on AMPK activation have not been fully studied. There is considerable evidence that AMPK activation is regulated by phosphorylation of the AMPK α-subunit by upstream kinases (AMP kinase kinase) (13, 29, 30) and that decreasing [PCr]/[TCr] ratio causes allosteric activation of AMPK (21). In our hands, creatine and phosphocreatine concentrations were not altered by creatine feeding. Although this is not a common finding (see, e.g., Ref. 17), other investigators have found no changes
in total creatine in skeletal muscle over 7 wk of creatine feeding (4). Interestingly, despite no changes in intracellular [creatine], increases in plasma [creatine], and no change in creatine transporter abundance, creatine uptake rates were lowered by ~50% in white muscle after the 7 wk of creatine feeding (4). This suggests that, during creatine feeding, some factor other than tissue total creatine content can affect muscle physiology. The present data demonstrating increased muscle creatinine concentrations after creatine feeding raise the question of whether muscle function can be altered by changes in the creatine plus creatinine pool, rather than creatine concentrations per se.

In disagreement with the present study and the work of others (4) demonstrating that creatine feeding does not alter creatine content in rat muscle tissue, creatine supplementation has been reported to decrease the [PCr]/[TCr] ratio (5, 25). Ceddia and Sweeney (5) suggested that a decrease in the [PCr]/[TCr] ratio after creatine supplementation might cause the phosphorylation of AMPK. However, there is currently no published information regarding whether a change in the [PCr]/[Cr] ratio regulates rates of AMPK phosphorylation or dephosphorylation. Although there are reports of stimulation of AMPK phosphorylation independent of changes in concentrations of adenine nucleotides, creatine, or phosphocreatine (6, 10), the mechanism mediating the increase in AMPK phosphorylation by creatine supplementation in the present study and as previously reported (5) is unclear at this time.

MEF2 is a transcription factor that plays a key role in specific skeletal muscle gene expression (3). The activation of MEF2 in skeletal muscle is regulated via parallel intracellular signaling pathways in response to cellular stress or activation of AMPK (1). It has been demonstrated that the activation of AMPK increases MEF2 DNA binding activity (1, 32), resulting in increased muscle GLUT4 protein levels (16, 32). In our study, creatine feeding increased protein levels of MEF2A, -C, and -D isoforms in nuclear extracts of skeletal muscle. We also found that DNA binding activity of MEF2 was increased by 44% in creatine-fed rat EDL muscle nuclear extracts.
Some studies have not found increased GLUT4 after creatine feeding. For example, 5 days of creatine supplementation did not alter muscle GLUT4 content in rat skeletal muscle (17), and creatine supplementation did not affect muscle GLUT4 expression in a 6-wk supplementation period in humans (28). It has been proposed that creatine supplementation per se has no effect on either GLUT4 expression or glycogen content in humans but that it is effective in combination with exercise (8). However, it has been reported that creatine supplementation prevented a decrease in muscle GLUT4 protein content during 2 wk of immobilization (18), suggesting that creatine supplementation itself (without dietary supplementation or exercise) is able to affect muscle GLUT4 expression. At the present time, we are unable to explain the reasons for discrepant findings about creatine effects on GLUT4 expression. More studies need to be done to elucidate reasons for varying results on this issue and to clarify mechanisms.

In summary, we found that creatine feeding increases AMPK phosphorylation in muscle through an unknown process that does not require changes in creatine or phosphocreatine or glycogen levels in muscle. Creatine feeding was related to enhanced nuclear content of MEF2, increased DNA binding activity of MEF2, increased in GLUT4 gene expression, and increased glycogen content in skeletal muscle. Our findings elucidate some of the possible physiological factors that might explain how creatine supplementation regulates GLUT4 biogenesis in muscle tissue.

ACKNOWLEDGMENTS

We thank the Saint Louis University Department of Comparative Medicine for specialized animal care.

GRANTS

J. Fisher is supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant K01-DK-066330.

REFERENCES