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AMP kinase expression and activity in human skeletal muscle: effects of immobilization, retraining, and creatine supplementation

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RESEARCH DESIGN AND METHODS

Subjects

Twenty-two healthy subjects, 19 men and 3 women, aged 18–30 yr (mean ± SE: 20.4 ± 0.5 yr), gave their written, informed consent to participate in the study. Exclusion criteria were creatine intake in the 4 mo preceding the study, vegetarianism, prior or existing renal pathology, any pathology that is contraindicative to leg immobilization and/or training, and consistent intake of any medication. The K. U. Leuven ethics committee approved the study protocol.

Study Design

This study was part of a larger project, aiming to investigate the effects of intake of creatine-protein-carbohydrate mixture, in conjunction with resistance training after leg immobilization, on muscle GLUT4 content and glucose tolerance (7). Briefly, in a double-blind,

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placebo-controlled trial, subjects were assigned to two experimental groups (matched-pair randomization; P: placebo; Cr: creatine) with similar distributions for gender and body weight. A light polyester cast, extending from groin to ankle, first immobilized each subject’s right leg at a knee angle of ~160° for 2 wk. Thereafter, the right leg was retrained during a 6-wk rehabilitation program. During this period, subjects came every other day to perform three to five series of 12 unilateral knee extensions with the right leg. The training load was gradually increased from three series at 75% of one-repetition maximum (reevaluated once per week) at the start of the training period, to five series at 85% of one-repetition maximum toward the end of the study. One of the investigators supervised all training sessions. During immobilization, the Cr group received three times 5 g of creatine monohydrate per day mixed with 12 g of maltodextrin, whereas P received 17 g of maltodextrin three times per day. During the rehabilitation period, Cr ingested one dose of 2.5 g creatine monohydrate per day mixed with 70 g of maltodextrin vs. only maltodextrin (72.5 g) in P. Supplements were prepared to be similar in taste by the addition of artificial flavor.

**Muscle Sampling**

Muscle samples were taken as described previously (7). Briefly, at baseline, after 2 wk of immobilization (~1 h after removal of the cast) and 6 wk of retraining (~48 h after the last training session), muscle needle biopsies were taken under local anesthesia (2–3 ml of 2% lidocaine) from the vastus lateralis muscle of both legs. The muscle sample was immediately frozen in liquid nitrogen and stored for later analyses. To avoid dietary-induced changes in muscle metabolite concentrations, the subjects received a standardized dinner (~850 kcal, 47:25:28% carbohydrate-fat-protein distribution) and a light standardized breakfast (~320 kcal, 65:15:20% carbohydrate-fat-protein distribution) on the evening and morning before each biopsy session.

**Biochemical Analyses**

**Muscle metabolite concentrations.** Muscle samples were freeze-dried and dissected free of visible blood, fat, and connective tissue. ATP, free creatine, and phosphocreatine were determined by standard enzymatic fluorometric assays (18). Muscle free creatine and phosphocreatine values for each individual were corrected for the mean ATP content over the three time points.

**Muscle lysate preparation.** AMPK phosphorylation and isoform expression were studied as described by Markuns and coworkers (20). Briefly, muscle specimens (20 mg wet wt) were freeze-dried and dissected free of visible blood, connective, and fat tissues. Muscles (4–5 mg) were then homogenized in ice-cold buffer (20 mM Tris base, 50 mM NaCl, 2 mM DTT, 50 mM NaF, 1% Triton X-100, 250 mM sucrose, 5 mM Na-pyrophosphate, 4 μg/ml leupeptin, 6 mM benzamidine, 500 μM PMSF, and 50 μg/ml soybean trypsin inhibitor, pH 7.4) for 20 s by using a homogenizer (model PT 3100, Brinkmann, Copenhagen, Denmark). Homogenates were rotated end over end for 1 h at 4°C. Lysates were generated by centrifugation (17,500 × g) for 1 h at 4°C. Lysates were quick frozen in liquid nitrogen and stored at ~80°C. Protein content in lysates was measured by the bicinchoninic acid method (Pierce Chemical, Rockford, IL).

**AMPK phosphorylation.** The phosphorylation of the α-subunits (Thr172) was evaluated by Western blotting using phosphospecific antibodies from Cell Signaling Technology. Forty-five micrograms of muscle lysate protein were subjected to SDS-PAGE (4–15% gradient gel) and Western blotting. Immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech) and detected and quantified with a coupled-coupled device image sensor and 1D software (Image Station, E440CF, Kodak).

**AMPK subunit protein levels.** Western blotting for the AMPK subunit isoforms was performed as described by Frusig et al. (11). The primary antibodies used for detection of AMPK subunit isoforms α, α₂, and β₂ were raised in sheep as described by Woods and coworkers (29) and Durante and coworkers (8). Because the Western blots for subunit protein levels and phosphorylation status were run on several gels, the signals were finally expressed in arbitrary units relative to a human skeletal muscle standard run in duplicate on each gel. All samples from the same subject and equal placebo and creatine-treated subjects were run on each gel.

**Statistical Analyses**

Treatment effects during immobilization and retraining were evaluated by a two-way (repeated measures) analysis of variance (ANOVA), which was covariate adjusted for the baseline values to evaluate the effects of immobilization, or covariate adjusted with the postimmobilization values to evaluate the effects of retraining (Statistica; Statsoft, Tulsa, OK). Time effects were analyzed with two-way ANOVA without covariate adjustment or with one-way ANOVA (repeated measures) to compare effects of immobilization and retraining within each group. Tukey’s post hoc tests were performed to locate the pairwise differences. Data are presented as means ± SE, and P < 0.05 was considered statistically significant.

**RESULTS**

**Muscle Creatine Content**

As shown in Table 1, muscle total creatine content remained in the range of 120–130 mmol/kg dry wt in the P group in both legs throughout the study. Compared with baseline, total creatine in Cr increased in both legs during the immobilization period (P < 0.05 for left leg; P < 0.10 for the right leg), yet these changes were not significantly different from placebo. In the creatine-supplemented group, total creatine further increased (~30 mmol/kg dry wt higher than baseline) during the retraining period in the trained leg (P < 0.05 vs. baseline) but not in the control (left) leg. The increases in total creatine content resulted from elevations in both the free creatine and phosphocreatine content. PCR/Cr did not vary in the control leg after placebo or creatine treatment during the study. In the experimental leg after retraining, creatine intake decreased PCR/Cr compared with baseline (P < 0.05, one-way ANOVA). Although not statistically significant, a similar trend was observed in the experimental leg of P. No significant treatment effects were detected.

**α-AMPK Phosphorylation Status**

Immobilization did not change AMPK α-subunit phosphorylation in either group (Fig. 1). Compared with baseline and postimmobilization, the degree of AMPK α-subunit phosphorylation was increased in the experimental leg after the 6-wk retraining period (P < 0.05, time effect). However, this increase was independent of the treatment received. In the control leg the fraction of phosphorylated AMPK α-subunits was constant throughout the study in both P and Cr. Representative blots are shown in Fig. 3.

**Muscle AMPK Protein Levels**

Immobilization did not significantly change the protein content of AMPK α₁-, α₂-, and β₂-subunit expression in either group (Fig. 2). In fact, in the experimental leg, but not in the control leg, the protein content of AMPK α₁ increased throughout the 8-wk study period in both groups (P < 0.05, time-effect, Fig. 2). AMPK α₂- and β₂-subunit expression was stable throughout the study period in both legs, independent of
Fig. 1. AMP-activated protein kinase (AMPK) phosphorylation (phos). Values are means ± SE (n = 11) and represent AMPK phosphorylation expressed as a percentage of a standard in vastus lateralis muscle samples of the left (control) leg and right (experimental) leg of subjects receiving either placebo (open bars) or creatine (solid bars) supplements. Post-Immo, postimmobilization. See RESEARCH DESIGN AND METHODS for further details. §P < 0.05, significant time effect compared with baseline.

Table 1. Muscle ATP and creatine content

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>Creatine</th>
<th>Free creatine</th>
<th>Phosphocreatine</th>
<th>Total creatine</th>
<th>Phosphocreatine-to-creatine ratio (PCr/Cr)</th>
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<td><strong>Baseline (Week 0)</strong></td>
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<td>Placebo</td>
<td>18.3 ± 1.1</td>
<td>16.9 ± 1.7</td>
<td>19.3 ± 1.4</td>
<td>15.9 ± 1.0</td>
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<td>Creatine</td>
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<td>19.4 ± 0.9</td>
<td>18.9 ± 0.5*</td>
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<td><strong>Postimmobilization (Week 2)</strong></td>
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<tr>
<td>Placebo</td>
<td>17.9 ± 2.0</td>
<td>18.3 ± 2.1</td>
<td>19.4 ± 0.9</td>
<td>18.9 ± 0.5</td>
<td>16.6 ± 1.6</td>
<td>18.8 ± 1.4</td>
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<tr>
<td>Creatine</td>
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<td></td>
<td>17.1 ± 0.9</td>
<td>16.9 ± 0.8</td>
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<td><strong>Retraining (Week 8)</strong></td>
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<tr>
<td>Placebo</td>
<td>16.6 ± 1.6</td>
<td>18.8 ± 1.4</td>
<td>17.1 ± 0.9</td>
<td>16.9 ± 0.8</td>
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<tr>
<td>Creatine</td>
<td></td>
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</table>

Values are means ± SE (n = 11) and represent ATP and creatine concentrations (mmol/kg dry wt) of vastus lateralis muscle samples of the left (control) leg and right (experimental) leg of subjects receiving either placebo or creatine supplements. PCr/Cr, phosphocreatine-to-creatine ratio. See RESEARCH DESIGN AND METHODS for further details. *P < 0.05, significant difference compared with baseline values of the corresponding group. †P < 0.05 denotes significant difference (1-way ANOVA) compared with baseline values of the corresponding group. Data from Derave et al. (7), with permission.

Fig. 2. AMPK α1-subunit expression. Values are means ± SE (n = 11) and represent AMPK α1-subunit expression expressed as a percentage of a standard in vastus lateralis muscle samples of the left (control) leg and right (experimental) leg of subjects receiving either placebo (open bars) or creatine (solid bars) supplements. See RESEARCH DESIGN AND METHODS for further details. §P < 0.05, significant time effect compared with baseline.
the treatment received (Table 2). Representative blots are shown in Fig. 3.

**DISCUSSION**

In this study, we investigated the effect of muscle immobilization and subsequent resistance training, with or without oral creatine intake, on muscle AMPK protein expression and phosphorylation status. In agreement with other studies, we confirm that prolonged exercise training increases AMPK α₁-subunit expression and α-AMPK phosphorylation. However, surprisingly, 2 wk of immobilization-induced muscle inactivity changes do not change AMPK protein expression or phosphorylation. Furthermore, creatine supplementation has no effect on AMPK protein expression and activity, either during immobilization or during retraining.

It is well known that muscle immobilization-induced muscle inactivity rapidly alters in the expression of a vast number of genes involved in structural and metabolic remodeling of muscle fibers (3, 23). Typical adaptations include decreased synthesis of contractile proteins (1, 2), decreased mitochondrial content and oxidative mitochondrial enzymes (1), and reduced content of the glucose transporter GLUT4 (7, 22). Interestingly, although recent work of Holmes et al. (17) in transgenic mice shows that activation of AMPK is not necessary for exercise-training-induced GLUT4 modification, it has been implicated in the upregulation of various genes and nuclear transcription factors involved in regulation of muscle energy metabolism (32, 33). Thus it was reasonable to assume that adaptations to immobilization may include downregulation of AMPK activity. We have reported elsewhere that the experimental design of the present study induced a substantial drop of muscular functional capacity as well as GLUT4 content by ~30% (7, 22). Here we show that 2 wk of muscle immobilization did not change the protein expression of AMPK α₁-, α₂-, and β₂-subunit isoforms (see Fig. 2 and Table 2) or the phosphorylation status of the α-AMPK subunits (see Fig. 1). Therefore, these findings suggest that immobilization-induced atrophy as well as decreased expression of GLUT4 and mitochondrial enzymes are not due to decreased AMPK activity or AMPK protein expression. Although Holmes et al. induced muscle inactivity by denervation, these data confirm their earlier work indicating that AMPK is not required for the exercise and denervation-induced alterations in GLUT4 expression.

It is interesting to note that, in line with the earlier observations on endurance training by Froisig et al. (11), a short (~10 min) resistance-training bout at a rate of about three times per week for 6 wk also was sufficient to increase α₁-subunit AMPK protein expression and phosphorylation. The fact that changes in AMPK phosphorylation correlated ($r = 0.57, P < 0.05$, data not shown) with the change in AMPK α₁-subunit expression suggests that increased α₁ expression may be the main reason for increased phosphorylation. The dramatic inhibition of muscular activity by 2 wk of leg immobilization failed to impact on α₁-AMPK subunit level. A time window of 2 wk, of course, may be too short to alter AMPK protein expression, and perhaps differences might appear at the RNA level. Nevertheless, this only strengthens our conclusion that the rapid deterioration of muscle occurring during an immobilization period may be regulated independently of AMPK.

We have recently shown that creatine supplementation in combination with leg immobilization and subsequent retraining (7, 22), but not creatine intake alone (7, 21), increases muscle GLUT4 content. Furthermore, it has been repeatedly shown that supplementary oral creatine intake can increase muscle glycogen content (7, 21, 22, 25). These findings provide evidence to suggest enhanced insulin-stimulated muscle glucose uptake due to creatine intake. Because 5-aminoimidazole-4-carboxamide-1β-D-ribofuranoside experiments have shown that chronic stimulation of AMPK elevates muscle GLUT4 protein levels (12, 19, 26) and insulin-induced muscle glucose utilization similar to exercise training (5, 10), we hypothesized that AMPK may be involved in the above-mentioned adaptations of skeletal muscle to creatine supplementation. As indicated above, we confirmed that strength...
training increases α1-subunit AMPK protein levels (see Fig. 2) and phosphorylation status of α-AMPK subunits (see Fig. 1). However, this change was entirely independent of the increased muscle creatine content established by oral creatine administration. Thus the previously reported creatine effect on muscle glycogen and GLUT4 content (7, 21, 22) is generated independent of reg-

In summary, the present data show that muscle immobilization for 2 wk does not alter AMPK α1-, α2-, and β2-subunit expression or α-AMPK phosphorylation status. This finding suggests that AMPK is not involved in muscle remodeling during episodes of unloading. The present observations also indicate that the previously reported beneficial effects of creatine supplementation on muscle during immobilization and weight training most likely are generated independent of regulation of AMPK.

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GRANTS

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REFERENCES


