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

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Eijnde, Bert O., Wim Derave, Jørgen F. P. Wojtaszewski, Erik A. Richter, and Peter Hespel. AMP kinase expression and activity in human skeletal muscle: effects of immobilization, retraining, and creatine supplementation. *J Appl Physiol* 98: 1228–1233, 2005. First published October 29, 2004; doi:10.1152/japplphysiol.00665.2004.— The effects of leg immobilization and retraining in combination with oral creatine intake on muscle AMP-activated protein kinase (AMPK) protein expression and phosphorylation status were investigated. A double-blind trial was performed in young healthy volunteers ($n = 22$). A cast immobilized the right leg for 2 wk, whereafter the knee-extensor muscles of that leg were retrained for 6 wk. Half of the subjects received creatine monohydrate throughout the study (Cr; from 15 g down to 2.5 g daily), and the others ingested placebo (P; maltodextrin). Before and after immobilization and retraining, needle biopsies were taken from the right and left vastus lateralis muscles. In the right leg of P and Cr, immobilization did not affect AMPK α_1-, α_2-, and β_2-subunit expression or AMPK α-subunit phosphorylation status. However, irrespective of the treatment received, retraining increased the degree of α-subunit phosphorylation by ~25% ($P < 0.05$) and increased AMPK α_1-subunit expression ($P < 0.05$) in both groups. From the start to the end of the study, AMPK subunit protein expression and α-subunit phosphorylation status were unchanged in the contralateral control leg. It is concluded that immobilization-induced muscle inactivity for 2 wk does not alter AMPK α_1-, α_2-, and β_2-subunit expression or α-AMPK phosphorylation status. Furthermore, the present observations indicate that AMPK probably is not implicated in the previously reported beneficial effects of oral creatine supplementation on muscle during immobilization and rehabilitative weight training.

training; AMP-activated protein kinase

RESEARCH OVER THE LAST DECADE has highlighted the key regulating role of the ubiquitously expressed 5′-AMP-activated protein kinase (AMPK) cascade in cellular energy homeostasis. AMPK phosphorylates target proteins, resulting in either increased or decreased rates of the metabolic pathways. As such, AMPK seems to act as a cellular fuel gauge (15) by sensing increased AMP-to-ATP ratio, which reflects low cellular energy state, and in response initiates energy-conserving pathways. In the long term, activation of AMPK can alter the expression of genes involved in energy maintenance and protein synthesis (31). Given the evidence that short-term endurance exercise training can alter the protein expression of AMPK subunits (8, 11), it seems reasonable to assume that immobilization-induced muscle disuse may be a strong stimulus to alter muscular AMPK protein expression and activity. Indeed, it is clearly established that muscle unloading, i.e., by limb immobilization in human subjects or by hindlimb suspension in rats, reduces muscle energy stores (1) and shifts the balance of protein turnover to net protein breakdown (2). Moreover, muscle unloading also causes GLUT-4 content to decrease (7, 22).

Another intervention that might affect muscle AMPK activity is oral creatine supplementation. It is well established that supplementary oral creatine intake can increase muscle creatine content, which in turn may facilitate flux through the creatine kinase (CK) reaction during high-intensity muscle contractions (28, 30). CK and AMPK physically colocalize in muscle fiber sarcomeres (24). Furthermore, MM-CK, the muscle-specific cystoplasmic CK isoenzyme (28), has appeared to be a target for AMPK (24). Some (13, 14, 18, 27) but not all (4, 9, 21) studies indicate that oral creatine intake does not increase cellular phosphocreatine levels in the same proportion as free creatine, leading to decreased phosphocreatine-to-creatine ratio (PCr/Cr), which is known to stimulate AMPK (24). In this respect, a recent study in incubated L6 rat muscle cells has shown that creatine loading, leading to a fall of the intracellular PCr/Cr, activates AMPK (6).

Therefore, the aim of the present work was to investigate whether muscle unloading induced by leg immobilization and subsequent rehabilitation training, either in combination with oral creatine intake or not, can modulate muscle AMPK activity and/or AMPK subunit isoform expression in healthy subjects.

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,

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 (Thr[172]) 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 α1,

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

Table 1. *Muscle ATP and creatine content*

	Baseline (*Week 0*)		Postimmobilization (*Week 2*)		Retraining (*Week 8*)	
	Right	Left	Right	Left	Right	Left
ATP						
Placebo	18.3±1.1	16.9±1.7	17.9±2.0	18.3±2.1	16.6±1.6	18.8±1.4
Creatine	19.3±1.4	15.9±1.0	19.4±0.9	18.9±0.5*	17.1±0.9	16.9±0.8
Free creatine						
Placebo	36±2	38±2	46±4	41±2	44±3	40±4
Creatine	37±2	33±2	48±4	42±2*	55±4*	38±1
Phosphocreatine						
Placebo	87±6	93±10	80±5	82±9	84±4	85±6
Creatine	85±4	84±5	89±4	93±6	96±3	92±2
Total creatine						
Placebo	123±6	130±11	126±4	123±9	128±4	125±9
Creatine	122±6	117±6	137±7	135±7*	151±6*	131±2
PCr/Cr						
Placebo	2.5±0.2	2.5±0.3	2.0±0.4	2.1±0.3	2.0±0.2	2.2±0.1
Creatine	2.4±0.1	2.6±0.1	1.9±0.1	2.2±0.1	1.8±0.1†	2.5±0.2

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.
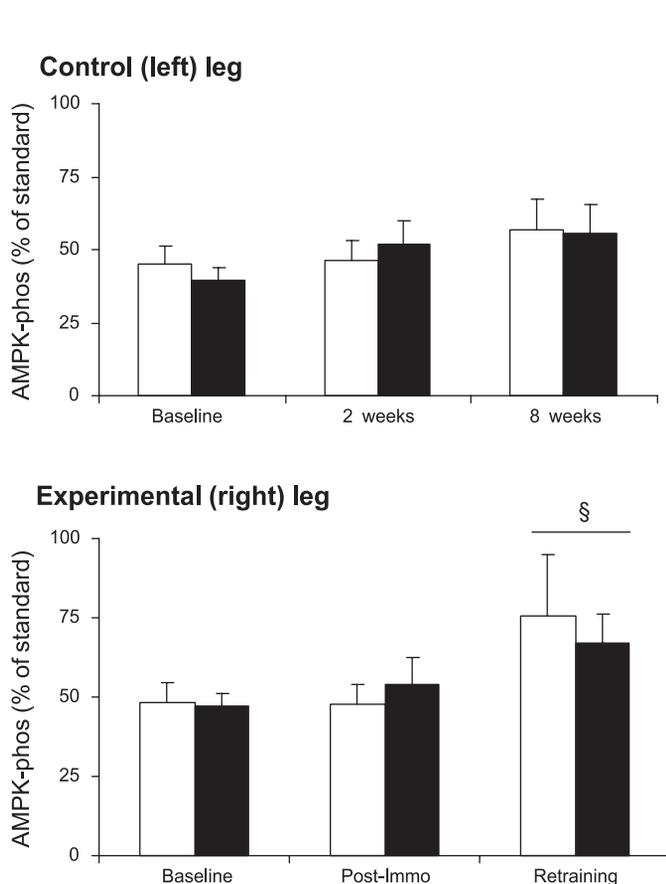

Fig. 1. AMP-activated protein kinase (AMPK) phosphorylation (phos). Values are means ± SE ($n = 11$) and represent AMPK phosphorylation expressed a percentage of a standard in vastus lateralis muscle samples of the left (control) leg and right (experimental) leg of subjects receiving either placebo (open 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.
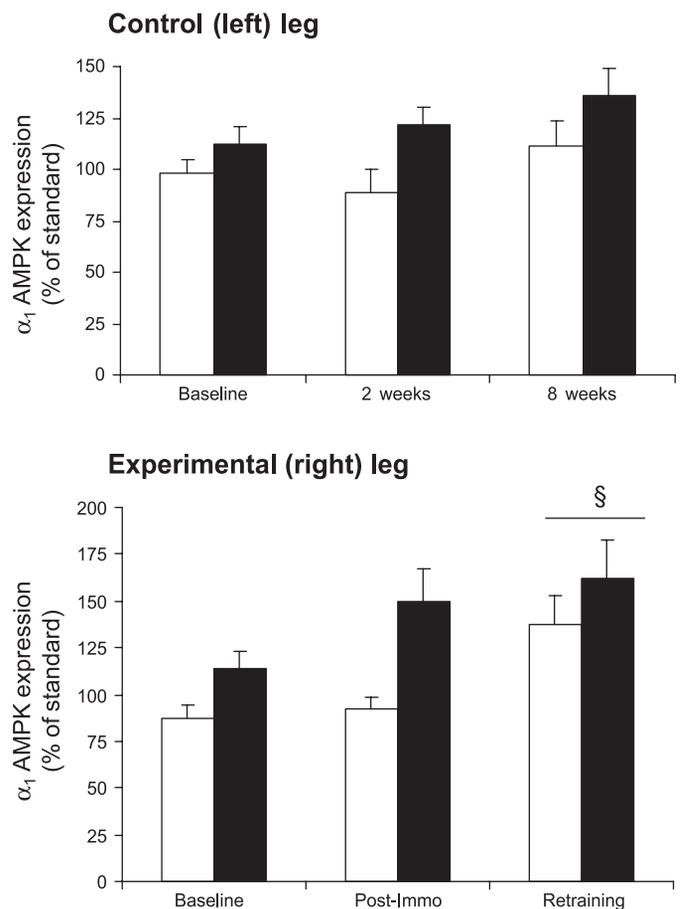

Fig. 2. AMPK α_1-subunit expression. Values are means ± SE ($n = 11$) and represent AMPK α_1-subunit expression expressed as a percentage of a standard in vastus lateralis muscle samples of the left (control) leg and right (experimental) leg of subjects receiving either placebo (open bars) or creatine (solid bars) supplements. See RESEARCH DESIGN AND METHODS for further details. §$P < 0.05$, significant time effect compared with baseline.

Table 2. *AMPK α_2- and β_2-subunit expression*

	Baseline (*Week 0*)		Postimmobilization (*Week 2*)		Retraining (*Week 8*)	
	Right	Left	Right	Left	Right	Left
α_2-Subunit						
Placebo	143.9±7.6	136.4±9.3	137.6±14.6	134.3±14.6	155.1±17.2	153.4±20.7
Creatine	174.9±37.0	154.0±33.6	161.3±25.3	188.2±31.7	176.5±29.3	170.9±21.2
β_2-Subunit						
Placebo	88.0±10.0	90.6±5.6	71.9±5.0	89.0±7.3	95.0±10.7	72.6±5.8
Creatine	89.1±9.6	77.4±8.3	92.1±6.9	97.6±8.6	99.4±9.1	84.1±7.3

Values are means ± SE (*n* = 11) and represent AMP-activated protein kinase (AMPK) α_2- and β_2-subunit expression expressed a percentage of a standard in vastus lateralis muscle samples of the left (control) leg and right (experimental) leg of subjects receiving either placebo or creatine supplements. See RESEARCH DESIGN AND METHODS for further details. No significant differences.

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 α_1-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 experi-

mental 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 α_1-, α_2-, and β_2-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 Frøsig 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 α_1-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 α_1-subunit expression suggests that increased α_1 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 α_1-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-1-β-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
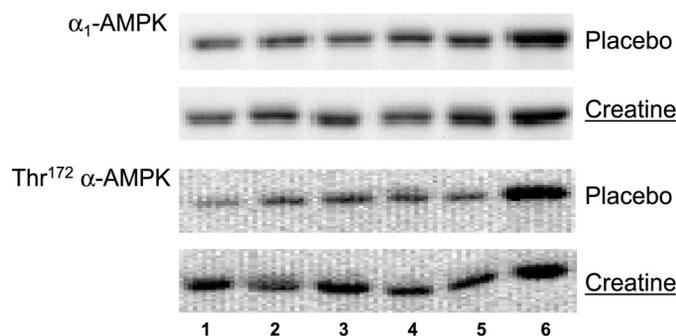

Fig. 3. Representative AMPK phosphorylation and α_1-subunit expression blots. Lane codes: *lane 1*, control leg, baseline; *lane 2*, experimental leg, baseline; *lane 3*, control leg, 2 wk; *lane 4*, experimental leg, postimmobilization; *lane 5*, control leg, 8 wk; *lane 6*, experimental leg, retraining.

training increases α_1-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 in the absence of elevated AMPK protein expression and AMPK phosphorylation. These findings contrast with earlier work by Ceddia and Sweeney (6). They showed incubation of L6 rat muscle cells in a 0.5 mM creatine medium for 48 h to increase AMPK phosphorylation about twofold. However, although muscle cells were incubated in a medium containing a physiological creatine concentration, intracellular creatine and phosphocreatine concentrations increased approximately ninefold and fivefold, respectively. This is far beyond the average increase ($\sim$10–20%) effected by oral creatine supplementation in human muscles (16, 18). Furthermore, some studies have reported slightly decreased PCr/Cr after creatine intake (13, 14, 18, 27). However, in other studies (4, 9, 21) PCr/Cr remained unchanged. This probably indicates that the small decrease in the PCr/Cr in the present study was not enough to affect AMPK activity. It is important to note, however, that PCr and Cr in muscle cells exist in distinct pools (28). Consequently, PCr and Cr contents measured in muscle extracts may not reflect local concentrations in the intracellular compartments where in the AMPK and CK enzymes colocalize (24).

In summary, the present data show that muscle immobilization for 2 wk does not alter AMPK α_1-, α_2-, and β_2-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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