5′-AMP-activated protein kinase activity and subunit expression in exercise-trained human skeletal muscle

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5′-AMP-activated protein kinase (AMPK) is a ubiquitously expressed sensor of cellular energy charge. On activation, AMPK switches off ATP-consuming anaerobic processes and turns on ATP-producing catabolic processes via phosphorylation of several downstream metabolic enzymes and via effects on gene expression (recently reviewed by Ref. 21). AMPK is activated by acute exercise in human skeletal muscle (6, 19, 35, 44, 49, 57), and several studies propose a regulatory role for AMPK in exercise-induced fatty acid oxidation (reviewed by Refs. 53, 54) and glucose metabolism (reviewed by Refs. 20, 43).

Repeated bouts of acute exercise over a prolonged period of time (exercise training) induce health-beneficial adaptations in several body tissues (4). Many of the adaptations taking place in skeletal muscle in response to exercise training are proposed to involve AMPK. This is based on the observations that chronic pharmacological activation of AMPK, like exercise training, both enhances the gene expression of the glucose transporter GLUT-4, hexokinase, citrate synthase, and cytochrome c and increases mitochondrial density and muscle glycogen content in rodent skeletal muscle (3, 5, 25, 55, 61). Furthermore, chronic activation of AMPK by 5-aminimidazole-4-carboxamide-1-β-p-ribofuranoside (AICAR) increases insulin-induced glucose utilization in rodent skeletal muscle (5, 15) similar to exercise training (10, 11, 38). However, AICAR may be somewhat nonspecific in activating AMPK (2), and, therefore, these findings should be interpreted carefully. Supporting the direct role of AMPK in these adaptations is a study in which a constitutively active AMPK mutant is expressed in the H-2Kb skeletal muscle cell line. These experiments show that increased AMPK activity is sufficient to increase GLUT-4 and hexokinase protein levels (18) and suggest a key role for AMPK activity in the metabolic adaptations to exercise training.

AMPK is a heterotrimer consisting of three subunits designated as α, β, and γ. In mammalian cells, two isoforms of the α-subunit, which contains the catalytic domain, have been identified (α1 and α2). The β (β1 and

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AMPK IN EXERCISE-TRAINED HUMAN SKELETAL MUSCLE

β₂ isoforms) and the γ (γ₁, γ₂, and γ₃ isoforms) regulatory subunits are needed in complex with the α-subunit for full kinase activity (14, 59). The α₁-isofrom is widely distributed in different body tissues, whereas α₂ is primarily expressed in skeletal muscle, heart, and liver (47). In INS-1 cells and skeletal muscle tissue, α₁ is mainly found in the cytosol, whereas α₂ is localized both in cytosol and nuclei (1, 45). Interestingly, of the two β-subunits identified, the β₂-protein is abundantly expressed in skeletal muscle compared with β₁ (50). AMPK γ₁- and γ₂-mRNA are found in a variety of tissues, whereas significant expression of γ₃ mRNA was detected in skeletal muscle only, although the protein appeared to be much more widely expressed (7).

Regulation of AMPK activity involves several mechanisms. Allosteric activation of AMPK is brought about by an increase in the AMP-to-ATP ratio (AMP/ATP) and a decrease in the phosphocreatine-to-creatine ratio (PCr/Cr) (41). Furthermore, AMPK is covalently activated by kinases (AMPKKs) via phosphorylation on Thr₁₇₂ of the α-subunit (23, 48). AMPKK, like AMPK, is also allosterically activated by AMP (24, 48) but appears not to be regulated by PCr. Binding of AMP to AMPK makes the enzyme a better substrate for AMPKK (23) and a worse substrate for deactivating protein phosphatases (9). Altogether, these mechanisms would ensure that the AMPK system responds to changes in cellular AMP in an ultrasensitive manner (22). Recent studies suggest a role for glycogen in the regulation of AMPK activity. In rodent skeletal muscle, high muscle glycogen levels, induced by exercise training or a combination of prior exercise and carbohydrates, 27% fat, 16% protein). On the day of the experiment, the subjects arrived in the laboratory in the morning after a 10-h overnight fast. A catheter was inserted in the antecubital vein of the forearm for blood sampling. The subjects exercised for 20 min on a cycle ergometer (Monark) at the same relative workload (80% of VO₂peak). Pulmonary oxygen and carbon dioxide exchange was measured by using an on-line gas and airflow analyzer (Medgraphics, Medical Graphics). Glucose and fat oxidation were evaluated by indirect calorimetry according to Fayrin (17), with the exception that measurements were not corrected for urinary nitrogen. Needle biopsies from vastus lateralis muscle and blood samples were obtained at rest and at the end of exercise under local anesthesia by using Xylocain (Lidocain). The Copenhagen Ethics Committee approved the experimental protocol, and all human experiments conformed to the Declaration of Helsinki.

Analysis of blood and plasma substrates and hormones. Glucose and lactate concentrations in blood were determined in duplicate by using a dual-channel glucose-lactate analyzer (YSI-2700 Select; Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin concentration was determined by using a radioimmunoassay kit (Insulin Ria 100, Pharamcia). Concentrations of plasma long-chain fatty acids (LCFA) were determined in accordance with Shimizu et al. (46) by using an automatic spectrophotometer (COBAS FARA 2, Roche Diagnostic). Plasma epinephrine and norepinephrine were analyzed by means of a radioimmunoassay (KatCombi, Immuno-Biological Laboratories, Hamburg, Germany).

Muscle lactate, adenosine nucleotides, Cr, and PCr. Freeze-dried muscle biopsy specimens were extracted with perchloric acid, neutralized, and analyzed for lactate and adenosine nucleotides. Contents of ATP, ADP, AMP, and IMP were determined by reverse-phase HPLC, according to a previously described method (52). Muscle lactate, Cr, and PCr content were measured fluorometrically, as described previously (37). The estimation of free concentrations of ADP and AMP was based on the near-equilibrium nature of the Cr phosphokinase and adenylate kinase reactions, respectively. Free ADP was estimated from the measured ATP, Cr, and PCr contents, and the H⁺ concentration was estimated by using the measured muscle lactate content, according to the formula for dry muscle presented by Mannion et al. (33). The equilibrium constant value employed for Cr phosphokinase was 1.66 × 10⁹ M⁻¹ (29). Free AMP (AMP<sub>free</sub>) was estimated from the measured ATP and the estimated free ADP by using an observed equilibrium constant for adenylate kinase of 1.05 (29).

METHODS

Subjects. Fourteen young healthy men (7 exercise-trained and 7 sedentary subjects) gave their informed consent to participate in the study. The exercise-trained subjects participated in physical exercise training (long-distance running, bicycle road racing, and indoor cycle spinning) four to eight times per week, whereas the sedentary control subjects did not participate in any regular physical activity. One to two weeks before the experiment, peak O₂ consumption (VO₂peak) was determined during an incremental cycle ergometer test. The VO₂peak was >59 and <46 ml O₂·min⁻¹·kg⁻¹ for the trained and sedentary subjects, respectively.

Experimental protocol. The day before the experiment, the subjects abstained from exercise and ate a mixed diet (57% carbohydrates, 27% fat, 16% protein). On the day of the experiment, the subjects arrived in the laboratory in the morning after a 10-h overnight fast. A catheter was inserted in the antecubital vein of the forearm for blood sampling. The subjects exercised for 20 min on a cycle ergometer (Monark) at the same relative workload (80% of VO₂peak). Pulmonary oxygen and carbon dioxide exchange was measured by using an on-line gas and airflow analyzer (Medgraphics, Medical Graphics). Glucose and fat oxidation were evaluated by indirect calorimetry according to Fayrin (17), with the exception that measurements were not corrected for urinary nitrogen. Needle biopsies from vastus lateralis muscle and blood samples were obtained at rest and at the end of exercise under local anesthesia by using Xylocain (Lidocain). The Copenhagen Ethics Committee approved the experimental protocol, and all human experiments conformed to the Declaration of Helsinki.

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**Muscle glycogen.** Muscle glycogen concentration in freeze-dried muscle tissue was measured by fluorometry as glycosyl units after acid hydrolysis, as previously described (37).

**Muscle lysate preparation.** For studies of enzyme activity and phosphorylation, ~40 mg of frozen muscle tissue were homogenized, as described previously (34). Homogenates were rotated end over end at 4°C for 60 min, after which they were centrifuged at 4°C for 30 min at 4,000 g. The supernatants were harvested, and total protein content was determined in the lysates by the bicinchoninic acid method (Pierce). For measurement of protein levels of the AMPK subunit isoforms, muscle lysates were prepared, as described previously (13).

**AMPK activity.** α-Isomform-specific AMPK activity was measured in immunoprecipitates from 100 µg of muscle lysate protein by using an anti-α1–AMPK and an anti-α2–AMPK antibody. A p81 filter paper assay, with the use of SAMS-peptide (200 µM) as the substrate, was used to measure AMPK activity in the presence of a saturating concentration of AMP (0.2 mM), as previously described (57).

**AMPK and acetyl CoA-carboxylase-β phosphorylation.** The phosphorylation of the α-subunit (Ser79) and acetyl CoA-carboxylase (ACC)-β (Ser211) was evaluated by Western blotting by using phospho-specific antibodies from Cell Signaling Technology and Upstate Biotechnology, respectively. The ACC phospho-specific antibody is raised against a peptide corresponding to the sequence rat ACC-α containing the Ser79 phosphorylation site, but the antibody also recognizes the human ACC-β when phosphorylated, most likely at the corresponding Ser211. For the detection of α-AMPK phosphorylation (Thr172), 45 µg of muscle lysate protein were subjected to SDS-PAGE (4–15% gradient gel) and Western blotting. ACC-β was affinity purified from 300-µg muscle lysate protein and subjected to SDS-PAGE (4–15% gradient gel), as described previously (6). Immunoreactive bands were visualized with enhanced chemiluminescense (ECL +, Amer sham Pharmacia Biotech) and detected and quantified with the use of a coupled device image sensor and 1D software (Image Station, E440CF, Kodak).

**AMPK subunit mRNA.** For the determination of mRNA content, isolation of total RNA, RT, and PCR were carried out as follows. Total RNA was isolated from ~25 mg of tissue by a modified guanidinium thiocyanate-phenol-chloroform extraction method adapted from Chomczynski and Sacchi (8) and as described previously (40). RNA was resuspended overnight (4°C) in 2 µl/mg original tissue weight in diethyl pyrocarbonate-treated H2O containing 0.1 mM EDTA. RT of 22 µl of total RNA sample was performed by using the Superscript II RNase H- system (GIBCO-BRL), as previously described (40). RT products were diluted in nuclease-free H2O to a total volume of 300 µl. The mRNA content of the selected genes was determined by fluorescence-based real-time PCR (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Forward (FP) and reverse primers (RP) and TaqMan probes were designed from human-specific sequence data (Entrez-NIH and Ensemble, Sanger Institute) by using computer software (Primer Express, Applied Biosystems). The following sequences were used to amplify a fragment of AMPK-α1 FP: 5′ CAGGGACTGCTACTCCACA- GAGA 3′; RP: 5′ CTTGTGACCCTGACTGATGA 3′; probe: 5′ TCAGTGGACACTAGCTTCGGCCAGAGTGT 3′; and of AMPK-α2 FP: 5′ GAAACTGAAGACAATTGTGCTT 3′; RP: 5′ GAGGGAAGTGATCGACAGGATTGC 3′; probe: 5′ CGGGAGCACAGGACAGGTTACAGGCTGGTGTG 3′. The probes were 5′-carboxyfluorescein (FAM) and 3′-6-carboxy-N,N,N′,N′-tetramethylrhodamine (TAMRA) labeled. Prior optimization was conducted for each set of self-designed oligos determining optimal primer concentrations and probe concentration and verifying the efficiency of the amplification. For each of the target genes, the expected size of the PCR product was confirmed on a DNA 2.5% agarose gel. GAPDH was also amplified for use as endogenous control by using a predeveloped assay reaction (Applied Biosystems). PCR amplification was performed (in triplicates) in a total reaction volume of 25 µl. The reaction mixture consisted of 2.5 µl diluted template, FP and RP, and probe, as determined from the prior optimization, 2× TaqMan Universal MasterMix optimized for TaqMan reactions (Applied Biosystems; containing AmpliTaq Gold DNA polymerase, AmpErase Uarcil N-glycosylase, dNTPs with dUTP, ROX as passive reference, and buffer components), and nuclelease-free water. The following cycle profile was used for all genes: 50°C for 2 min + 95°C for 10 min + [95°C for 15 s + 60°C for 1 min] × 40 cycles.

**AMPK subunit protein levels.** Western blotting for the AMPK subunit isoforms was performed as described previously (13), except in the case of γ1. In the latter case, a “pan-γ-antibody was generated in sheep to the peptide CRAAPL-2.5% agarose gel. GAPDH was also amplified for use as endogenous control by using a predeveloped assay reaction (Applied Biosystems). PCR amplification was performed (in triplicates) in a total reaction volume of 25 µl. The reaction mixture consisted of 2.5 µl diluted template, FP and RP, and probe, as determined from the prior optimization, 2× TaqMan Universal MasterMix optimized for TaqMan reactions (Applied Biosystems; containing AmpliTaq Gold DNA polymerase, AmpErase Uarcil N-glycosylase, dNTPs with dUTP, ROX as passive reference, and buffer components), and nuclelease-free water. The following cycle profile was used for all genes: 50°C for 2 min + 95°C for 10 min + [95°C for 15 s + 60°C for 1 min] × 40 cycles.

**Statistics.** Data are expressed as means ± SE. Two-tailed nonpaired Student’s t-tests were applied for comparison of two normally distributed groups. Comparisons between two normally distributed groups before and at the end of exercise were done by two-way ANOVA for repeated measures for the detection of main effects and interactions between the different groups. If interactions between groups were detected, two-way ANOVA for repeated measures was followed by a multiple-comparison test (Student-Newman-Keuls method). Correlations were analyzed by Pearson product-moment correlation analysis for two parameters and with multiple linear regression for three parameters. P < 0.05 was considered statistically significant.

**RESULTS**

**Subject characteristics.** Age (25 ± 1 vs. 25 ± 1 yr), height (183 ± 3 vs. 186 ± 2 cm), body weight (79 ± 4 vs. 86 ± 3 kg), and body mass index (24 ± 1 vs. 25 ± 1 kg/m2) were similar in the exercise-trained and the sedentary subjects, respectively, whereas VO2peak was significantly higher in the trained than in the sedentary subjects (66 ± 2 vs. 44 ± 1 ml O2 · min⁻¹ · kg⁻¹; P < 0.05).

<table>
<thead>
<tr>
<th>Table 1. Pulmonary and cardiac responses to acute exercise</th>
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<tbody>
<tr>
<td>Trained</td>
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<tr>
<td>Workload, W/kg</td>
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<tr>
<td>Workload, %VO2peak</td>
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<tr>
<td>Heart rate, beats/min</td>
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<tr>
<td>VO2, 1 O2/min</td>
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<tr>
<td>V̇E/V̇O2 exchange ratio</td>
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<tr>
<td>Carbohydrate oxidation, J-min⁻¹ · kg⁻¹</td>
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<tr>
<td>Fat oxidation, J-min⁻¹ · kg⁻¹</td>
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</table>

Values are means ± SE of n = 7 in each group obtained during the last 2 min of the 20-min exercise bout. VO2, O2 consumption; VO2peak, peak VO2. *P < 0.05 vs. sedentary.
Pulmonary and cardiac responses to exercise. The trained and sedentary subjects exercised at the same relative workload, eliciting a similar heart rate, respiratory exchange ratio, and fat oxidation rate determined during the last 2 min of exercise. The workload, pulmonary oxygen uptake, and carbohydrate oxidation rate were significantly higher in the trained than in the sedentary subjects ($P < 0.05$) (Table 1).

Blood and plasma substrates and hormones. In the resting condition, blood glucose, blood lactate, plasma LCFA, plasma insulin, plasma epinephrine, and plasma norepinephrine were similar in trained and sedentary subjects. In response to exercise, blood lactate and plasma epinephrine increased ($P < 0.001$) and plasma LCFA and plasma insulin decreased ($P < 0.001$), but no differences were present between the two groups. Blood glucose increased in response to exercise in the trained subjects ($P < 0.01$) to a higher level than in the sedentary subjects ($P < 0.05$). Plasma norepinephrine increased in response to exercise in both groups, but, at the end of exercise, the concentration was significantly higher in the trained group (Table 2).

Muscle lactate, nucleotides, Cr, and PCr. Muscle lactate was not different between sedentary and trained subjects at rest, and in both groups muscle lactate increased in response to exercise. However, at the end of exercise, muscle lactate was significantly lower in the trained than in the sedentary group (Table 3). No differences in ATP, AMP, IMP, Cr, or PCr concentrations or AMP/ATP or PCr/(PCr + Cr) were present between the trained and sedentary group in the resting state, whereas the ADP concentration was lower in the trained group ($P < 0.01$, main effect). The ADP, IMP, and Cr concentrations increased, whereas the PCr concentration and PCr/(PCr + Cr) decreased in response to exercise ($P < 0.01$). At the end of the exercise bout, the Cr content was lower and the PCr content and PCr/(PCr + Cr) were higher ($P < 0.05$) in the trained subjects than in the sedentary subjects (Table 3).

The calculated concentration of cytosolic AMP (AMPfree) and AMPfree/ATP increased in response to acute exercise, and, although there appeared to be a larger increase in the sedentary group, this was not significant (Table 3). It should be noted that calculation of AMPfree involves estimation of the muscle $\text{H}^+$ concentration from the measured muscle lactate concentration. As trained muscle has a higher dynamic buffer capacity for $\text{H}^+$, a given measured lactate accumulation will be followed by a smaller increase in $\text{H}^+$ in trained than in sedentary muscle. This may lead to an underestimation of AMPfree in the trained group.

Muscle glycogen. In the resting state, the muscle glycogen content was higher in the trained than in the sedentary group ($P < 0.05$), and glycogen content decreased during exercise to a similar level in the two groups ($P < 0.01$) (Fig. 1). However, the decrease in glycogen content was not significantly different between the trained and sedentary subjects.

### Table 2. Blood substrates and hormones at rest and at the end of 20-min exercise

<table>
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<tr>
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<th>Rest</th>
<th>Exercise</th>
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<tr>
<td></td>
<td>Trained</td>
<td>Sedentary</td>
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<tr>
<td></td>
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<tr>
<td>Blood glucose, mM</td>
<td>4.8 ± 0.2</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Blood lactate, mM</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
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<tr>
<td>Plasma LCFA, µM</td>
<td>674 ± 98</td>
<td>555 ± 53</td>
</tr>
<tr>
<td>Plasma insulin, µU/ml</td>
<td>6.7 ± 0.4</td>
<td>7.9 ± 0.9</td>
</tr>
<tr>
<td>Plasma epinephrine, nM</td>
<td>0.6 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Plasma norepinephrine, nM</td>
<td>1.8 ± 0.3</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE of $n = 7$ in each group. LCFA, long-chain fatty acid. *$P < 0.05$ vs. sedentary. †$P < 0.01$ vs. rest in corresponding group. ‡$P < 0.001$ for rest vs. exercise (main effect).

### Table 3. Nucleotides, creatine, and phosphocreatine at rest and at the end of 20-min exercise

<table>
<thead>
<tr>
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<th>Main Effect</th>
<th>Rest</th>
<th>Exercise</th>
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<tr>
<td></td>
<td></td>
<td>Trained</td>
<td>Sedentary</td>
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<tr>
<td>Muscle lactate, mmol/kg dry wt</td>
<td>4.1 ± 0.4</td>
<td>7.3 ± 0.8</td>
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<tr>
<td>Creatine, mmol/kg dry wt</td>
<td>43 ± 3</td>
<td>43 ± 2</td>
<td></td>
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<tr>
<td>PCr, mmol/kg dry wt</td>
<td>73 ± 5</td>
<td>87 ± 3</td>
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<tr>
<td>PCr/(PCr + Cr)</td>
<td>0.63 ± 0.03</td>
<td>0.67 ± 0.02</td>
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<tr>
<td>ATP, mmol/kg dry wt</td>
<td>24.6 ± 0.4</td>
<td>25.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>ADP, mmol/kg dry wt</td>
<td>3.07 ± 0.07</td>
<td>3.45 ± 0.08</td>
<td></td>
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<tr>
<td>AMP, mmol/kg dry wt</td>
<td>0.42 ± 0.04</td>
<td>0.37 ± 0.06</td>
<td></td>
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<tr>
<td>IMP, mmol/kg dry wt</td>
<td>0.03 ± 0.004</td>
<td>0.08 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>AMPfree, µmol/kg dry wt</td>
<td>0.96 ± 0.24</td>
<td>0.60 ± 0.11</td>
<td></td>
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<tr>
<td>AMPfree/ATP, (×1,000)</td>
<td>0.04 ± 0.001</td>
<td>0.02 ± 0.005</td>
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</table>

Values are mean ± SE of $n = 6–7$ in each group. PCr, phosphocreatine; Cr, creatine; PCr/(PCr + Cr), ratio of PCr to (PCr + Cr); AMPfree, free AMP; AMPfree/ATP, ratio of AMPfree to ATP. AMPfree was estimated as described in Methods. *$P < 0.05$ vs. sedentary. †$P < 0.01$ vs. rest in corresponding group. $\S P < 0.01$ for trained vs. sedentary (main effect). $\dagger P < 0.01$ for rest vs. exercise (main effect).
The α1- and α2-AMPK activities. The resting activities of α1- and α2-AMPK were not different between trained and sedentary subjects. The α2-AMPK activity was significantly higher at the end of exercise ($P < 0.01$, main effect), although no significant difference was present between the trained and sedentary subjects ($P = 0.24$) (Fig. 2B). The increase in α2-AMPK activity above resting level was 110 ± 33 and 184 ± 31% in trained and sedentary subjects, respectively, but these increases were not significantly different ($P = 0.21$).
α-AMPK phosphorylation (Thr\(^{172}\)). In concordance with α-AMPK activity, α-AMPK phosphorylation at Thr\(^{172}\) on the α-subunit was not different between the two groups of subjects at rest. In response to exercise, α-AMPK phosphorylation increased significantly (\(P < 0.01\), main effect), and the increase in AMPK phosphorylation above the resting level was significantly reduced in the trained group compared with the sedentary group (Fig. 3).

ACC-β phosphorylation (Ser\(^{221}\)). At rest, ACC-β phosphorylation (Ser\(^{221}\)) was not different between the trained and sedentary subjects. During exercise, ACC-β phosphorylation was significantly increased in both groups (\(P < 0.001\)), but ACC-β phosphorylation was significantly lower in the trained than in the sedentary subjects (\(P < 0.01\)), and the increase in ACC-β phosphorylation above the resting level tended (\(P = 0.071\)) to be lower in the trained group than in the sedentary group (Fig. 4).

AMPK subunit mRNA expression. The α1-, α2-, and γ3-subunits were chosen for mRNA expression analyses as the levels of these protein subunits have previously been shown to change in response to physiological perturbations in rodents (13, 51). The mRNA content of the α1- and α2-subunits of AMPK was not different between trained and sedentary subjects, but the level of γ\(_3\)-mRNA tended to be lower (\(P = 0.078\)) in the trained group than in the sedentary group (Fig. 5).

AMPK subunit protein levels. The protein content of the AMPK α1-subunit in trained muscle was 185% of that in sedentary muscle (\(P < 0.05\)) (Fig. 6). The β\(_2\)-protein level in trained subjects was 148% of that in sedentary subjects (\(P = 0.06\)). Protein levels of α\(_2\)-, β\(_1\)-, γ\(_1\)-, γ\(_2\)-, and γ\(_3\)-subunits were similar in the trained and sedentary groups.

Correlation analyses. When the data from the trained and the sedentary groups were pooled, the exercise-induced decrease in PCr/(PCr + Cr) correlated significantly with measures of exercise-induced changes in α-AMPK activity [α\(_1\): \(r = 0.61, P = 0.02\); α\(_2\): \(r = 0.72, P = 0.005\); α-AMPK phosphorylation (Thr\(^{172}\)): \(r = 0.78, P = 0.002\); and ACC-β phosphorylation (Ser\(^{221}\)): \(r = 0.67, P = 0.01\)] (not shown). The decrease in glycogen alone did not correlate with any of the obtained measures of increase in...
AMPK activity (not shown), probably due to the limited variability in the amount of glycogen broken down in the subjects. Nevertheless, the increase in \(2\)-AMPK activity was tightly correlated with the decrease in PCr/(PCr + Cr) and the decrease in glycogen when analyzed by multiple linear regression (\(r = 0.81, P = 0.005\)) (Fig. 7). No correlations were present between AMP free/ATP (increase or absolute level) and any of the obtained measures of AMPK activity.

**DISCUSSION**

In the present study, we investigated mRNA expression and protein levels of AMPK subunit isoforms and the effect of acute exercise on AMPK activity in skeletal muscle of trained and sedentary subjects. In response to acute exercise at 80% of \(V_{O2}\)peak, \(2\)-AMPK activity, \(\alpha\)-AMPK phosphorylation (Thr\(^{172}\)), and ACC-\(\beta\) phosphorylation (Ser\(^{221}\)) increased, whereas \(\alpha\)-associated activity was unchanged. ACC-\(\beta\) phosphorylation during exercise and the exercise-induced increase in \(\alpha\)-AMPK phosphorylation were significantly blunted in the trained group compared with the sedentary group. No significant difference in skeletal muscle mRNA content for the \(\alpha_1\), \(\alpha_2\), and \(\gamma_3\)-subunit could be detected between trained and sedentary subjects. At the protein level, the \(\alpha_1\)-AMPK was significantly higher in trained subjects than in sedentary subjects.

In the context of a long-term training regime, it is well known that a continued progressive improvement of exercise performance and training-induced cellular adaptations, such as increased GLUT-4 levels, are dependent on a progressive increase in training amount and intensity (26, 39). The observation that exercise-induced AMPK activity is blunted in trained subjects compared with sedentary subjects working at the same relative exercise intensity might in part explain this phenomenon, considering the possible role of AMPK in exercise-induced gene expression (see below). Despite higher absolute energy requirement in the trained than in the sedentary subjects at the same relative exercise intensity, the energy charge is better maintained in the trained group, probably due to increased oxidative capacity (26).

**Fig. 5.** The mRNA content of AMPK subunit isoforms in skeletal muscle of exercise-trained subjects at rest expressed as percentage of the mRNA content in sedentary subjects. Values are means \(\pm SE\) of \(n = 7\).

**Fig. 6.** Protein levels of AMPK subunit isoforms in skeletal muscle of exercise-trained and sedentary subjects at rest. A: representative Western blots. B: densitometric data expressed as percentage of sedentary. Values are means \(\pm SE\) of \(n = 7\). *\(P < 0.05\) vs. sedentary.
trained individuals, as reflected by the difference in PCr/(PCr + Cr) between trained and sedentary subjects. Besides providing an explanation for the lower AMPK activation in the trained subjects, it illustrates the important point that AMPK activity is not a marker of total energy flux during exercise, but rather a result of the perturbations in the energy charge induced by exercise.

The blunted exercise-induced in vivo activity of AMPK in the trained group was reflected by a decreased Ser\textsuperscript{221} phosphorylation of its downstream target, ACC-β. In accordance with our findings, studies in rats have demonstrated a blunted ACC inactivation by exercise at the same absolute workload after training in red quadriceps and gastrocnemius muscle (13, 27). Our finding that ACC-β phosphorylation (Ser\textsuperscript{221}) is reduced in trained subjects could be explained by an allosteric influence on AMPK of the low-Cr and high-PCr levels observed in the present study and by others (42). The improved maintenance of the PCr stores in the trained subjects during acute exercise is probably related to a higher capacity of oxidative ATP generation due to increased activity and expression of enzymes in the oxidative pathways. Furthermore, the diminished muscle lactate concentration during acute exercise in the trained subjects indicates a reduced exercise-induced acidification. This could also help explain the blunted AMPK activation in trained muscle, because in vitro experiments have demonstrated that a progressive decrease in pH induces a progressive increase in AMPK activity (41).

The exercise-induced increase in α-AMPK phosphorylation (Thr\textsuperscript{172}) was also lower in the trained than in the sedentary subjects. This indicates that, not only allosteric, but also covalent regulation of AMPK by phosphorylation was different between trained and sedentary subjects. This is in accordance with observations in rat skeletal muscle (13). The regulation of the upstream kinases (AMPKKs) responsible for Thr\textsuperscript{172} phosphorylation of the α-subunit of AMPK is not fully elucidated. However, AMP\textsubscript{free} is known to activate AMPKK, leading to phosphorylation and activation of AMPK, but, because of methodological inadequacies, it is unfortunately not feasible to measure AMP\textsubscript{free}. In the present study, calculated estimates of AMP\textsubscript{free} or AMP\textsubscript{free}/ATP could not explain the difference in AMPK phosphorylation. It should be considered that the blunted AMPK activation in trained muscle during acute exercise could be due to enhanced AMPK-directed phosphatase activity in these subjects, although this remains to be shown. The finding that the increase in covalently modified AMPK activity correlated with the decrease in PCr/(PCr + Cr), but not AMP\textsubscript{free} or AMP\textsubscript{free}/ATP, is not easily explained. However, it should be stressed that the latter were calculated estimates and not direct measures. Probably PCr/(PCr + Cr) is a more accurate measure of cellular energy charge than calculated AMP\textsubscript{free} values. PCr has been identified as an allosteric regulator of AMPK, but the ability of PCr to modulate the activity of the upstream AMPKK, or to change the susceptibility of AMPK to phosphorylation-dephosphorylation, has not been found (D. G. Hardie, unpublished observations).

It has been observed that high muscle glycogen levels negatively influence AMPK activity (12, 28, 44, 56). Interestingly, a fairly tight relationship was observed.
among an exercise-induced increase in α2-AMPK activity, decrease in PCr/(PCr + Cr), and decrease in glyco-
gen content, indicating that glycogen may act in con-
cert with other factors in the modulation of AMPK activity. Taken as a whole, concomitant changes in several factors are likely to contribute to the increased AMPK activity in response to exercise: increased mus-
cle lactate levels leading to acidification, a decrease in PCr/(PCr + Cr), an increase in AMPfree/ATP, and possibly decreased glycogen levels. It could be speculated that the importance of each of these regulator factors varies, depending on the exercise conditions, i.e., exercise duration, exercise intensity, and training status.

AMPK is a promising candidate for mediating sev-
eral of the adaptive responses in gene expression to exercise training. This is primarily based on the observ-
ation that repeated activation of AMPK by AICAR treatment and expression of a constitutively active AMPK mutant in cultured muscle cells mimic many of the changes in protein levels induced by repeated ex-
ercise bouts (3, 5, 18, 25, 55). A role for AMPK in regu-
lation of gene transcription is supported by the observa-
tions that activation of AMPK regulates ex-
pression of several genes in liver cells (16, 30, 31, 58). Furthermore, it has been observed that the transcription-
coactivator p300 is a substrate of AMPK (60) and that AMPK activation by AICAR increases GLUT-4 transcrip-
tion in muscle (61) with a time course and regional promoter sequence requirement similar to that of exercise (32, 36). Based on the present study, it could be hypothesized that an increase in α1-AMPK protein content is involved in these adaptations, al-
though the role of this AMPK subunit in cellular re-
sponses to exercise remains poorly understood. In rat skeletal muscle, exercise training elicited an increase in γ3-protein content in red quadriceps, concomitant with an increase in GLUT-4 and other exercise-associ-
ated increases in protein levels. Interestingly, there also appeared to be an ~50% increase in the level of α1, although, along with changes in other subunit iso-
forms, this was not statistically significant. No changes in AMPK subunit protein level in white quadriceps and soleus muscle were observed. However, this could be related to the fact that these muscles were recruited to a low extent, judged from the unchanged GLUT-4 con-
centration in these two muscles (13). It is not clear at present why the response to training should be differ-
ent in rats than in humans, although this might reflect the fact that this is a cross-sectional study and that the training regimes are not directly comparable. The find-
ing that chronic alterations in myocardial energetics in hypertrophied rat hearts (left ventricular hypertrophy) are associated with a twofold increase in the α1-protein level and a 30% decrease in the α2-level (51) supports the idea that α1-AMPK protein levels are positively influenced by long-term perturbations of the cellular energy charge, e.g., exercise training. In INS-1 cells, it has been observed that α2 but not α1 is found in the nuclei, suggesting a role of α2 rather than α1 in gene expression (45). It is noteworthy that the α1-protein content was elevated in the trained subjects compared with sedentary subjects, whereas the α1-mRNA level was similar in the trained and sedentary subjects, indicating that the training-induced changes in the α1-AMPK protein content take place at the posttranscrip-
tional level. Alternatively, mRNA levels are in-
creased after each training bout, but the duration of the increase is <1 day, which was the time the trained subjects abstained from training before taking part in the study. Finally, it should be mentioned that, be-
cause of the cross-sectional study design, it cannot be ex-
cluded that the difference in α1-AMPK protein is due to genetic differences rather than to the effect of train-
ings per se. A longitudinal training study would be needed to clarify this. Clearly, more work is needed to address the role of the specific AMPK isoforms in skeletal muscle, but it could be hypothesized that skel-
etal muscle adaptations to exercise training are depend-
ent in part on changes in the α1-AMPK subunit level. Thus changes in the α1-AMPK subunit level per se might facilitate the beneficial training-induced adapta-
tions in skeletal muscle.

In conclusion, activation of AMPK in response to acute exercise is diminished in skeletal muscle of trained individuals compared with sedentary sub-
jects working at the same relative intensity. This is probably due to a better maintenance of the energy charge and a less pronounced exercise-induced acid-
ification in exercise-trained muscle. Furthermore, AMPK subunit protein level of α1 was higher in exercise-trainned than in sedentary human skeletal muscle, suggesting a role for this subunit in the adaptations to exercise training.

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