α-Catalytic subunits of 5′AMP-activated protein kinase display fiber-specific expression and are upregulated by chronic low-frequency stimulation in rat muscle

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1Exercise Biochemistry Laboratory, Faculty of Physical Education and Recreation; 2Centre for Neuroscience and 3Departments of Pharmacology and Paediatrics, Faculty of Medicine and Dentistry; University of Alberta, Edmonton, Alberta, Canada; and 4Department of Biology, Faculty of Science, University of Konstanz, Konstanz, Germany

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Putman CT, Martins KJ, Gallo ME, Lopaschuk GD, Pearcey JA, MacLean IM, Saranchuk RJ, Pette D. α-Catalytic subunits of 5′AMP-activated protein kinase display fiber-specific expression and are upregulated by chronic low-frequency stimulation in rat muscle. Am J Physiol Regul Integr Comp Physiol 293: R1325–R1334, 2007. First published June 6, 2007; doi:10.1152/ajpregu.00609.2006.—5′-AMP-activated protein kinase (AMPK) signaling initiates adaptive changes in skeletal muscle fibers that restore homeostatic energy balance. The purpose of this investigation was to examine, in rats, the fiber-type protein expression patterns of the α-catalytic subunit isoforms in various skeletal muscles, and changes in their respective contents within the tibialis anterior (TA) after chronic low-frequency electrical stimulation (CLFS; 10 Hz, 10 h daily), applied for 4.8 days. Immunocytochemical staining of soleus (SOL) and medial gastrocnemius (MG) showed that 86 ± 4.1 to 97 ± 1.4% of type IIA fibers stained for both the α1- and α2-isozymes progressively decreased to 63 ± 12.2% of type IID/X and 9 ± 2.4% of IIB fibers. 39 ± 11.4% of IID/X and 83 ± 7.9% of IIB fibers expressed only the α2 isoform in the MG, much of which was localized within nuclei. α1 and α2 contents, assessed by immunoblot, were lowest in the white gastrocnemius [WG; 80% myosin heavy chain (MHC) IIB; 20% MHCIIId/x]. Compared with the WG, α1 content was 1.6 ± 0.8 (P < 0.001) and 1.8 ± 0.4 (P < 0.001)-fold greater in the red gastrocnemius (RG: 13%, MHCIIa) and SOL (21%, MHCIIa), respectively, and increased in proportion to MHCIIa content. Similarly, α2 content was 1.4 ± 0.10 (P < 0.02) and 1.5 ± 0.07 (P < 0.001)-fold greater in RG and SOL compared with WG. CLFS induced 1.43 ± 0.13 (P < 0.007) and 1.33 ± 0.08 (P < 0.009)-fold increases in the α1 and α2 contents of the TA and coincided with the transition of faster type IIB and IID/X fibers toward IIA fibers. These findings indicate that fiber types differ with regard to their capacity for AMPK signaling and that this potential is increased by CLFS.

myosin heavy chain; fast-twitch muscle; slow-twitch muscle

AMPK is activated in skeletal muscle during contractile activity (32, 68, 69) to acutely restore energy balance by increasing flux through ATP-generating pathways (24, 25).

In contrast, chronic AMPK activation induced by pharmacological agonists (3, 29, 58, 71) regulates long-term adaptive changes similar to exercise training that seem to limit future reductions in the intracellular energy potential and reportedly reduce metabolic disturbances in rats displaying skeletal muscle insulin resistance (9, 52, 70). Prolonged AMPK activation has, for example, been associated with mitochondrial genesis (3, 58, 71) and greater expression levels of genes involved in glucose uptake and storage (3, 9, 29), as well as lower plasma free fatty acid and triglyceride concentrations (9, 58). It has been further reported that after prolonged pharmacological activation of AMPK insulin-stimulated glucose uptake is enhanced, GLUT4 translocation is increased, and other adaptive responses occur within rat skeletal muscles in a fiber-type-specific manner, being primarily restricted to fast-twitch fibers (2, 3, 10, 58). These findings suggest that AMPK signaling varies between fiber types and may not be reliably targeted in all fibers of type 2 diabetics. They also highlight the adaptive potential of the AMPK signaling system as an important consideration when targeting this enzyme to improve skeletal muscle glucose tolerance.

Two recent studies reported that both the α1 and α2 isoforms are expressed in skeletal muscle, but their relative content varies between whole tissue homogenates from muscles composed of different fiber types (2, 14) and that muscle glucose uptake increased in proportion to AMPK activity induced by various stimuli. Winder et al. (71) reported greater total AMPK activity in the white compared with the red quadriceps and slow soleus, although Ai et al. (2) reported the opposite activity pattern. It has also been reported that enhanced contractile activity through exercise training moderately elevates α1 protein content but has no effect on α2 protein expression levels in human (16, 35, 43) or rat (14, 35) skeletal muscles. Collectively, the results of those studies suggest, but do not delineate, fiber-type-specific differences in the pattern of α1 and α2 protein expression. They also indicate that the AMPK signaling system is malleable and could be exploited to treat metabolic disturbances that underlie type 2 diabetes.
The primary objective of this study was to investigate the fiber-type-specific protein expression patterns of the α1 and α2 catalytic subunits of AMPK in various skeletal muscles. A secondary objective was to investigate the effect of enhanced contractile activity on the protein expression levels of the α1 and α2 catalytic subunits in previously studied rat fast-twitch tibialis anterior muscles known to undergo fast-to-slow fiber type transitions (54). It was hypothesized that expression levels of the α1 and α2 isoforms would differ between fiber types. Given that the AMPK-dependent changes in muscle phenotype seem to be restricted to fast-twitch type-IID/X and -IIB fiber populations (3), and given that exercise increases nuclear α2 localization (35), we further hypothesized that the α2-AMPK isoform would be greatest within the nuclear compartments of type II compared with type I fibers. Finally, we tested the hypothesis that both the α1 and α2 isoforms would increase in the tibialis anterior in response to chronic low-frequency stimulation (CLFS). We report the novel observations that the α1 and α2 isoforms are consistently expressed in a fiber-type-specific manner and that both isoforms are upregulated by chronically increased contractile activity.

METHODS

Animals. Eighteen 3- to 4-mo-old (400–500 g), male Sprague-Dawley rats were obtained from the local breeding colony at Health Sciences Laboratory Animal Services at the University of Alberta and were used to characterize fiber-type-specific distribution and aspects of the subcellular distribution of the α1 and α2 catalytic subunits of AMPK. To study the effects of muscle training on patterns of AMPK expression, we investigated expression levels in stimulated and contralateral control tibialis anterior muscles from 12 male Wistar rats that were exposed to chronic low-frequency electrical stimulation (CLFS) as part of a previous study (see Chronic low-frequency stimulation). All rats were cared for according to established standards of the Animal Research Centers of the University of Konstanz and the University of Alberta and in accordance with the guidelines of the Canadian Council for Animal Care. Animals were housed in thermally controlled rooms maintained at 22°C on 12:12-h light-dark cycles and received food and water ad libitum. All animal experiments received ethics approval from the University of Alberta and University of Konstanz and were completed in accordance with the requirements of the Canadian Council on Animal Care.

The soleus and gastrocnemius muscles of six rats were removed and flash-frozen in liquid nitrogen after euthanization of rats with an overdose of pentobarbital sodium (100 mg/kg; Biomedia MTC Animal Health Inc, Cambridge, ON, Canada). The deep-red and superficial white portions of the flash-frozen gastrocnemius muscles were subsequently isolated at −20°C, powdered under liquid nitrogen, freed of blood and connective tissue, and used for analysis. Separation of the red and white portions was confirmed by analysis of myosin heavy chain (MHC) isoform content by SDS-PAGE (see MHC immunocytochemistry and determination of MHC content by SDS-PAGE).

The soleus and gastrocnemius muscles were isolated from both hindlimbs of the remaining 12 Sprague-Dawley rats, after euthanization with an overdose of pentobarbital sodium. Muscles from six of the rats were blotted, fixed in a slightly stretched position, and frozen in melting isopentane cooled in liquid nitrogen (−156°C). Samples were subsequently used for immunohistochemical analysis. Soleus and gastrocnemius muscles from the remaining six rats were pooled according to muscle type and used for nuclear extraction.

Chronic low-frequency stimulation. Tibialis anterior muscles that were exposed to CLFS as part of another related study (54) were examined in the present study to investigate the short- and long-term effects of forced contractile activity on the adaptive capacity of AMPK. Briefly, under general anesthesia, electrodes were subcutaneously implanted bilaterally to the left common peroneal nerve, externalized dorsally within the intrascapular region, and connected to a small, portable stimulator. CLFS (10 Hz, impulse width 380 μs, 10 h/day) was started 7 days later, after complete wound healing. Muscles were exposed to CLFS for short [i.e., 4 ± 1.2 days (n = 5)] or long [i.e., 25 ± 4.8 days (n = 7)] time periods. Muscles were collected during a postexercise period, in which the acute effects of contractile activity are no longer detectable but the chronic adaptive responses are known to prevail (33, 54, 67). The unstimulated contralateral tibialis anterior muscles served as paired controls. Before muscle extraction, animals were euthanized; tibialis anterior muscles from both the stimulated (left) and contralateral control (right) legs were isolated and frozen in liquid nitrogen (−196°C).

MHC immunocytochemistry and determination of MHC content by SDS-PAGE. To examine the isoform-specific distribution of AMPK according to individual fiber types, serial sections were stained using our previously reported procedures (22, 58), and the following monoclonal antibodies were directed against adult MHC isoforms: MHCIIa (NOQ7.5,4D) (27); MHCIIa (SC-71), and MHCIIb (BF-F3) (63). MHCIIId/x was identified as those fibers that remained unstained by this panel of antibodies and was validated by comparison of the staining pattern with monoclonal antibody BF-35, which stains all MHC isoforms, except IID/X (63). MHC isoform contents of the white and red gastrocnemius and the soleus muscles were quantitatively evaluated by SDS-PAGE as previously described by us (21, 39, 54, 58).

Immunocytochemical detection of the α1 and α2 subunits. Immunocytochemical staining was completed according to established protocols (57) after optimization for antigen retrieval. Briefly, 12-μm thick frozen serial sections of rat medial gastrocnemius and soleus were air dried, washed once in PBS containing 0.1% (vol/vol) Tween-20 (PBS-Tween), twice in PBS, and incubated for 30 min in 3% H2O2 in methanol. Sections were then washed as before and incubated overnight at 4°C in the blocking solution containing 1% (vol/vol) BSA, 5% (vol/vol) goat serum in PBS-Tween. Sections were subsequently washed in PBS and incubated in succession with avidin-D and biotin blocking solutions (Vector Laboratories, Burlington, ON, Canada). Primary anti-α1-AMPK or anti-α2-AMPK (Upstate Biotechnologies, Lake Placid, NY) were diluted (1:60) in the blocking solution and applied to sections for 1 h. Sections were then washed and incubated for 60 min with biotinylated goat-anti-rabbit-IgG (1: 400) (Vector Laboratories), washed again, and incubated for 60 min with biotin-avidin-horseradish peroxidase (HRP) complex. After washing, sections were exposed to a substrate solution containing diaminobenzidine, H2O2, and NiCl2 in 50 mM Tris, pH 7.5. The reaction was stopped after 6 min by rinsing with distilled water. Sections were subsequently dehydrated, cleared, and mounted in Entellan (Merck, Rahway, New Jersey). Parallel control sections in which the primary anti-AMPK antibodies were substituted with a nonspecific rabbit-IgG (Santa Cruz Biotechnology, Santa Cruz, CA) did not stain.

Preparation of recombinant α1 and α2 catalytic subunits of AMPK. Full-length cDNAs were ligated into pCR2.1-TOPO cloning vectors and transformed into chemically competent Escherichia coli. Transformants were selected for kanamycin resistance, according to the manufacturer’s instructions (Invitrogen, Burlington, ON, Canada). Purified plasmid DNA was subsequently isolated using the SNAP MiniPrep Kit (Invitrogen) and analyzed for size and orientation by restriction analysis using EcoRI (α-1 and α-2), and KpnI (α-1) or BamHI (α-2). The correct orientation and sequences of plasmid inserts were confirmed by independent sequence analysis (DNA Sequencing Laboratory, University of Alberta). Recombinant plasmids were purified (Plasmid Midi Purification Kit, QIAGEN, Mississauga, ON, Canada), and the inserts were removed by sequential digestion with KpnI and Xhol, followed by purification (QIAquick,
Large-scale expression of target DNA was induced by 2-h exposure of plasmids according to manufacturer instructions and stored at 80°C until use. Plasmids were purified, quantified, and used to transform the expression strain of E. coli, BL21(DE3). Transfectants were selected according to our previously reported procedures (54, 58). Purified His-tagged AMPK α-subunits were released from lysed bacteria and cleared by centrifugation (12,000 g, 4°C) and filtration. The filtrates were applied to Ni²⁺-charged HiTrap affinity columns (Amersham Biosciences, Piscataway, NJ) and step eluted with imidazole. Fractions were stored at 80°C.

**Western blot analysis.** Western blot analyses were completed according to our previously reported procedures (54, 58). Purified α1- or α2-recombinant AMPK proteins, and tissue homogenates were denatured in reducing sample buffer and heated for 5 min at 95°C; 5 μl of purified recombinant protein or 87 μg of total tissue protein were loaded per lane. Equal loading of samples between lanes was first controlled by protein assay (37). Proteins were separated on 9% polyacrylamide gels at 115 V (Mini-PROTEAN 3; Bio-Rad Laboratories); transfer efficiency and visualization by agarose gel electrophoresis and elution of inserts bands (GenElute, Sigma-Aldrich). The eluted bands were subsequently ligated into the pET30a(+) expression vector (Novagen, Madison, WI), sequenced (Molecular Biology Services Unit, University of Alberta) and transformed into DH5α-competent cells to generate plasmid copies (Life Technologies, Burlington, ON, Canada). Plasmids were purified, and recombinant His-tagged AMPK α-subunits of antibodies was confirmed by including purified recombinant AMPK and anti-2-AMPK antibodies only reacted with their cognate antibodies. Western blot analysis was used to quantify differences in MHC isoform contents of the white gastrocnemius, red gastrocnemius, and soleus. The method used to quantify differences in MHC isoform contents of the white gastrocnemius, red gastrocnemius, and soleus is shown in Fig. 1. The white portion of the gastrocnemius expressed only MHCIIb (80 ± 2.3%) and MHCId/x (20 ± 2.3%), while the red gastrocnemius expressed all four adult MHC isoforms: 14 ± 1.9% MHC1; 13 ± 1.5% MHCIIa; 43 ± 1.5% MHCId/x, and 31 ± 2.7% MHCIIb. In contrast, the soleus expressed only the slower MHC1 (79 ± 2.1%) and MHCIIa (21 ± 2.1%) isoforms. Immunocytochemical identification of fiber types (Figs. 2, 3, C and D, and 4, C–E) showed that the medial gastrocnemius was composed of primarily fast-twitch fibers, that is, 94%, (58 ± 9.9% IIB, 24 ± 4.2% IID/X, 12 ± 2.7% IIA). The superficial portion was, however, composed of only IID/X and IIB fibers. The remaining 7 ± 3.6% of type-I fibers were concentrated within the deep region; type IIA fibers were found within deep and middle regions. The fiber-type distribution of the soleus was similar to the MHC isoform content data, comprising only type-I (81 ± 3.0%) and -IIA (19 ± 3.0%) fibers.

**RESULTS**

MHC content and fiber-type composition of the white gastrocnemius, red gastrocnemius and soleus. The method used to quantify differences in MHC isoform contents of the white gastrocnemius, red gastrocnemius, and soleus was shown in Fig. 1. The white portion of the gastrocnemius expressed only MHCIIb (80 ± 2.3%) and MHCId/x (20 ± 2.3%), while the red gastrocnemius expressed all four adult MHC isoforms: 14 ± 1.9% MHC1; 13 ± 1.5% MHCIIa; 43 ± 1.5% MHCId/x, and 31 ± 2.7% MHCIIb. In contrast, the soleus expressed only the slower MHC1 (79 ± 2.1%) and MHCIIa (21 ± 2.1%) isoforms. Immunocytochemical identification of fiber types (Figs. 2, 3, C and D, and 4, C–E) showed that the medial gastrocnemius was composed of primarily fast-twitch fibers, that is, 94%, (58 ± 9.9% IIB, 24 ± 4.2% IID/X, 12 ± 2.7% IIA). The superficial portion was, however, composed of only IID/X and IIB fibers. The remaining 7 ± 3.6% of type-I fibers were concentrated within the deep region; type IIA fibers were found within deep and middle regions. The fiber-type distribution of the soleus was similar to the MHC isoform content data, comprising only type-I (81 ± 3.0%) and -IIA (19 ± 3.0%) fibers.
The α1 and α2 isoforms are expressed in a fiber-type specific pattern. Immunocytochemical staining of serial sections of rat soleus (Fig. 3, A and B) and medial gastrocnemius (Fig. 4, A and B) using anti-α1-AMPK or anti-α2-AMPK polyclonal antibodies, and a panel of isoform-specific monoclonal anti-MHC antibodies (Figs. 3, C and D; 4, C and E) allowed semiquantitative evaluation of the fiber-type-specific patterns of α1 and α2 protein expression. Within the soleus, 86 ± 4.1% of IIA fibers (Fig. 3E) stained positive for both the α1 and α2 isoforms, whereas a minority of IIA fibers (i.e., 5 ± 3.8%) stained positive for only the α2 isoform; most type I fibers (i.e., 94%) did not express detectable levels of either isoform.

Examination of the medial gastrocnemius allowed additional semiquantitative analyses of α1 and α2 expression patterns within the fastest IID/X and IIB fiber populations (Fig. 4F). The proportion of type-I fibers within this muscle that stained positive for at least one isoform of the α-catalytic subunits was very low and similar to that observed in the soleus. In contrast, the proportion of fibers expressing detectable levels of both isoforms of the catalytic subunit showed a progressive decrease from 97 ± 1.4% in IIA fibers, to 63 ± 12.2% in IID/X fibers and to 9 ± 2.4% within the IIB fiber population. The proportion of fibers expressing detectable levels of only the α2 isoform increased in the reverse order, from 0% in IIA fibers, to 39 ± 11.4% in IID/X fibers, and finally to 83 ± 7.9% within IIB fibers. Although the appearance of the AMPK isoforms was quantified in each fiber type at a minimum detection threshold, an important observation was that the intensity of staining for both isoforms was greatest within IIA fibers, followed by the IID/X and IIB fiber populations, respectively.

Quantitative evaluation of the α1 and α2 isoforms in muscles composed of different fiber types. Quantification of α1 and α2 protein expression levels by immunoblotting (Fig. 5A) revealed consistent and similar patterns. The α1 isoform was lowest in whole homogenates of the white gastrocnemius (Fig. 5, A and B); α1 content was 1.6 ± 0.08 fold (P < 0.001) and 1.8 ± 0.04 fold (P < 0.0001) greater in the red gastrocnemius and soleus, respectively. The α1 content of the soleus was slightly greater than in the red gastrocnemius (P < 0.02). α2 content was also lowest in the white gastrocnemius (Figs. 5, A and B), while the red gastrocnemius and soleus expressed 1.4 ± 0.10 fold (P < 0.02) and 1.5 ± 0.07 fold (P < 0.001) more of the α2 isoform, respectively.

α2 AMPK isoform is concentrated within the nuclei of fast-twitch IID/X and IIB fibers. The immunohistochemical method used to examine frozen serial sections for the nuclear pattern of α1 and α2 AMPK staining in the medial gastrocnemius is shown in Figs. 6, A–C. Individual areas (i.e., 9.6 × 10⁻⁵ mm²) of the medial gastrocnemius and the soleus were examined at 1,000× magnification for α1 and α2 immunocytochemical staining within the myonuclear compartment, as previously described by us for identification of other myonuclear proteins (39, 57). The data are expressed as the proportion of individual areas with one or more incidences of strong positive nuclear staining, relative to the total muscle cross-sectional area (Fig. 6D). The mean cross-sectional area
examined for the medial gastrocnemius was 29.9 mm² per muscle, while 12.4 mm² was studied for each soleus. Less than 2% and 5% of the total cross-sectional area of the soleus muscles displayed patterns of strong myonuclear staining for α1- or α2-AMPK isoforms, respectively (Fig. 6D). The proportion of cross-sectional area that stained positive for the α1 isoform within the medial gastrocnemius was similarly low. In contrast, greater than 30% of the total cross-sectional area displayed strong myonuclear staining for the α2 isoform within the gastrocnemius (Fig. 6D). Furthermore, the strong staining was concentrated within the superficial region, which corresponded to the area containing only IID/X and IIB fibers. Immunoblot analyses of cell fractions confirmed a high level of the α2 protein associated with the myonuclear compartment of the medial gastrocnemius compared with the soleus (Fig. 6E).

CLFS induces increases α1- and α2-isoform contents. A range of stimulation periods was examined that correspond to 1) the early adaptive period (i.e., 1–5 days of CLFS) where we have consistently observed rapid adaptive responses (54, 57), and 2) to a later period (i.e., ≥14 days of CLFS) characterized by more gradual adaptive changes (39, 54, 57) (Fig. 7, A–C). Representative immunoblots are shown in Fig. 7A. The short-term application of CLFS (i.e., 4 ± 1.2 days) resulted in a 1.29 ± 0.06 (P < 0.006)-fold increase in α2 content within the target tibialis anterior (Fig. 7, A and B). In contrast, there was a trend toward greater α1 content (i.e., 1.24 ± 0.14, P = 0.08) that only reached significance after more prolonged stimulation (Fig. 7, A and B). Long-term application of CLFS (i.e., 25 ± 4.8 days) induced a 1.56 ± 0.19 (P < 0.01) increase in α1 content to, while α2 content remained relatively constant, being elevated by 1.38 ± 0.14 (P < 0.01)-fold (Fig. 7, A and B). The main effects of CLFS on all stimulated muscles, relative to their contralateral controls, were increases on the order of 1.43 ± 0.13 (P < 0.007) and 1.33 ± 0.08 fold (P < 0.009) for the α1 and α2 isoforms, respectively (Fig. 7C).
Fig. 6. Immunocytochemical analyses of α1- and α2-AMPK expression within the myonuclear compartment. Immunocytochemical detection of α1-AMPK (A) and α2-AMPK (B) within rat medial gastrocnemius. C: IgG control. D: Data are expressed as means ± SE. Significant difference from soleus. *α2-AMPK greater than α1. The strong expression of α2-AMPK within the nuclear fraction of medial gastrocnemius was confirmed by immunoblot analyses as shown in E. Scale bar = 20 μm.

DISCUSSION

AMPK has gained widespread attention in recent years as a signaling element that detects fluctuations in the cellular energy potential of skeletal muscle fibers and responds by contributing to the short- and long-term restoration of homeostatic energy balance. Using the α1 and α2 catalytic subunits as a basis to distinguish between the AMPK isoforms in skeletal muscle, the results of our investigation extend the findings of previous reports by: 1) showing fiber-type specific expression patterns of the α1 and α2 AMPK isoforms within rodent muscles; 2) showing that the AMPK heterotrimer expressing the α2 catalytic subunit is highly concentrated within the nuclear compartments of IID/X and IIB fibers; and 3) demonstrating considerable adaptive potential of both the α1 and α2 isoforms within fast-twitch rodent muscles undergoing CLFS-induced fast-to-slow fiber-type transitions. CLFS was used as a model of endurance exercise because it has several advantages over traditional running protocols: it activates all motor units within the target muscles, it elicits only concentric contractions and thus induces fast-to-slow fiber-type transitions without causing muscle injury, the contralateral leg serves as an internal control, and the intensity and total duration of muscle contractions can be rigorously controlled (47–51).

Fiber-type-specific patterns of AMPK expression. We are aware of only one previous study that specifically investigated α1 and α2 AMPK protein expression levels in rodent skeletal muscles (2, 14). Ai et al. (2) reported that the expression of both isoforms was greatest within the fast-twitch epitrochlearis. They also reported an intermediate level of α1 protein in the mixed fast-twitch flexor digitorum brevis and that the lowest levels were in the slow soleus. In contrast, the α2 content was intermediate in the soleus and lowest within the flexor digitorum brevis. Although the results of that study provided a basis to conclude that protein expression levels of the α1 and α2 AMPK isoforms probably varied between fiber types, the authors were not able to detail the fiber-type-specific patterns of expression. Their study differed from ours with regard to the muscles examined and the methods used to identify fiber types. They used myosin-ATPase histochemistry (2, 8) to identify fibers, which does not allow for enumeration of type IID/X fibers and, consequently, may lead to an incorrect classification as IIA fibers (19). The results of the present study clearly show that both the α1 and α2 protein levels were greatest in the slow-twitch oxidative soleus, slightly less within the mixed fast-twitch red gastrocnemius, and lowest within the fast-twitch white gastrocnemius (Figs. 1 and 5). In the present study, the greater relative α1 and α2 isoform contents within the red gastrocnemius and soleus corresponded to an increasing proportion of MHCIIa (Fig. 1), and thus IIA fibers, within those muscles. Traditionally, the red gastrocnemius has been thought to contain a predominance of type IIA fibers. This was largely based on subjective evaluation of classical myosin ATPase staining, which does not definitively distinguish between IIA (~10%) and IID/X (~45%) fibers (19, 20). Consequently, the proportion of fibers identified as IIA is overestimated. We clearly show that only 13% of fibers were IIA, while 43% were IID/X in the red gastrocnemius. By comparison, the proportion of type IIA fibers or of MHCIIa within soleus has been reported to be up to 22%, which corresponds to our findings (3, 17, 21). Further semiquantitative analyses of the fiber-type-specific distribution using highly specific immunocytochemical methods showed that the majority of strong α1 and α2 staining was localized within the IIA fiber population (Figs. 3 and 4).

Our semiquantitative immunocytochemical data support our quantitative immunoblot data. The proportion of IIA and IID/X fibers that reach a threshold of immunohistochemical detection for the α1 and α2 isoforms approached 100% and 70%, respectively (Figs. 3 and 4). Thus it is not surprising that the quantity of α1 and α2 proteins increased in proportion to the quantity IIA fibers in red gastrocnemius and soleus muscles, which contained 13% and 21% type IIA fibers, respectively (Fig. 1). This is not to suggest that the α1 and α2 isoforms are not present in the type I fibers but merely are expressed at a low level that is below the detection threshold of immunocytochemical staining. Similarly, the greater α1 and α2 content...
within the red vs. white gastrocnemius correlates with the presence of IIA fibers in the former but not the latter, as well as a greater proportion of IID/X fibers in the former (Fig. 1).

Interestingly, this pattern also emerged after the application of CLFS, where increased expression levels of both the α1 and α2 protein isoforms in tibialis anterior (Fig. 7) corresponded to an increase of MHCIIa content from 10% to 40% of the total (please see Ref. 54), which has been shown to reflect increasing levels of MHCIIa expression within IID/X and IIB fiber populations, creating transitional fast hybrid fiber subtypes (39, 56, 57). This gradual transition through a population of IIA hybrid fibers appears to account for the corresponding 56% and 38% increases in α1 and α2 contents, respectively (Fig. 7).

Upregulation of the α1 AMPK catalytic subunit by CLFS. Pold et al. (52) report a 41% increase in expression of the α1 isoform in the red gastrocnemius but not in the white gastrocnemius of Zucker diabetic fatty (ZDF) rats after 8 wk of submaximal treadmill running. They also noted that exercise resulted in improved glucose homeostasis and peripheral insulin action in the muscles of trained ZDF rats, as determined by euglycemic clamp. Similarly, Langfort et al. (35) reported a 27% increase in the α1 isoform content but no change in α2 content in human vastus lateralis after 4 wk of daily submaximal training. Although the specific cellular roles of α1- and α2-AMPK remain to be fully elaborated, the absence of changes in the α2 isoform content or in the contents of any of the β or γ isoforms in those studies, suggests the intriguing possibility that improved glucose homeostasis and peripheral insulin action after exercise training might result, in part, from increased α1-AMPK-dependent GLUT4 recruitment.

Results of the present study support the findings of those studies by showing that CLFS can induce comparatively faster and larger increases in the α1 isoform content within rodent fast-twitch muscles. It is also noteworthy that the time course for fast-to-slow fiber-type transitions and improved glucose transport capacity typically follow a similar time course (28). Although not measured in our study, it is interesting to speculate that these adaptive changes may also lead to improvements in glucose homeostasis and peripheral insulin action in stimulated muscles similar to those reported by Pold et al. (52). According to the theory proposed by Pold et al. (52), CLFS should also reduce the workload of pancreatic β-cells, allowing similar improvements in β-cell morphology, viability, and function. This remains to be investigated and may represent a substantial advantage over the continuous use of pharmaceutical AMPK agonists, yielding similar improvements in glucose homeostasis without inducing pancreatic β-cell apoptosis, as recently reported after prolonged exposure to AMPK agonists (34).

Upregulation of the α2 AMPK catalytic subunit by CLFS. The present study is the first to demonstrate that chronically enhanced contractile activity can induce rapid and large increases in the α1 isoform content. Only two studies have previously investigated α2 AMPK protein expression levels within skeletal muscles before and after exercise training in humans (35) and ZDF rats (52); both reported no change in α2-isoform content, as determined by immunoblot. The differences between previous studies and ours appear to be twofold. First, the duration of the applied stimulus was 10–20 times greater in our study. Second, CLFS is known to activate all motor units, whereas muscle contractions that occur during voluntary exercise training display variable patterns of motor unit recruitment (15).

The basis for reversal of muscle glucose resistance resulting in increased glucose disposal posttraining has been suggested to be due, in part, to mitochondrial biogenesis leading to an increased capacity for terminal glucose oxidation (5, 46) and an associated reduction in lipid toxicity (65). A recent study of genes involved in the pathogenesis of type 2 diabetes revealed lower expression levels for nuclear respiratory factor-1-dependent genes that encode for mitochondrial enzymes (46). Fur-
ether, expression levels of the PPAR-γ coactivators, PGC1-α and PGC1-β, were reduced in diabetic individuals. Although it is not yet clear whether the α2-AMPK isoform is solely responsible for PPAR-γ-dependent transcriptional regulation, there is evidence to suggest that the α2-catalytic subunit plays an important role in regulating expression of nuclear encoded metabolic genes, whose protein products catalyze the aerobic generation of ATP and regulate mitochondrial biogenesis (36, 62). Such a role is consistent with the exercise-dependent translocation of α2-AMPK into the nuclear compartment (40), as well as the high α2-content in nuclei of IID/X and IIB fibers observed in our study, which coincidentally are the fiber types that demonstrate the lowest myonuclear content but the greatest increase in mitochondrial genesis in response to AICAR treatment (3) or CLFS (48, 60).

Clinical implications. Contraction-dependent changes in the contents of the α-catalytic subunit isoforms of AMPK demonstrated in our study may have important implications for the clinical treatment of type 2 diabetics who display coincident obesity. Human skeletal muscles typically express greater proportions of type IIA fibers (59, 66) and possess lower capacities for terminal substrate oxidation than the corresponding muscles in rats (18, 44, 54, 58). For this reason, direct comparisons between species should be cautiously interpreted. Nuhr et al. (45) recently showed that the application of transcutaneous neuromuscular stimulation (TNMS) to muscles of the anterior and posterior thigh compartments of both lower limbs for 2 h twice daily and for a total of 10 wk counteracted the impaired physical performance of patients with chronic heart failure. Improvements in VO2max, lactate threshold and hemodynamic performance were accompanied by fast-to-slow fiber type transitions, greater capacity for glycolytic flux, and most importantly greater activity of the mitochondrial reference enzyme citrate synthase. Collectively, these adaptive changes also indicate the possibility of improved glucose uptake and terminal oxidation and thus highlight the utility of TNMS as a viable treatment for individuals who suffer from type 2 diabetes. It seems reasonable that TNMS should up-regulate AMPK signaling capacity and efficiency and, in parallel, reduce lipid toxicity and increase the capacity for terminal substrate oxidation (46, 55, 64). Such a reduction would lead to improved glucose tolerance and insulin sensitivity in type 2 diabetics, similar to those reported in the ZDF rat after exercise training (52). Furthermore, clinical application of TNMS in type 2 diabetics may represent a significant advantage over clinical exercise programs for obese individuals who have low exercise tolerances.

Conclusion. This study reports the novel finding that the α1 and α2 isoforms of AMPK are consistently expressed in fiber-type-specific manner, being concentrated within fast oxidative IIA fibers. The α2 isoform is also concentrated within nuclei of fast glycolytic IID/X and IIB fibers. We also report that CLFS upregulates the protein contents of both the α1 and α2 catalytic subunits. These findings indicate that the application of CLFS to large muscle groups could be a complementary therapeutic approach to treat type 2 diabetes.

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