

UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase

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Zhou, Min, Bao-Zhen Lin, Sean Coughlin, Gino Vallega, and Paul F. Pilch. UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase. *Am J Physiol Endocrinol Metab* 279: E622–E629, 2000.—Uncoupling protein 3 (UCP-3), a member of the mitochondrial transporter superfamily, is expressed primarily in skeletal muscle where it may play a role in altering metabolic function under conditions of fuel depletion caused, for example, by fasting and exercise. Here, we show that treadmill running by rats rapidly (30 min) induces skeletal muscle UCP-3 mRNA expression (sevenfold after 200 min), as do hypoxia and swimming in a comparably rapid and substantial fashion. The expression of the mitochondrial transporters, carnitine palmitoyltransferase 1 and the tricarboxylate carrier, is unaffected under these conditions. Hypoxia and exercise-mediated induction of UCP-3 mRNA result in a corresponding four- to sixfold increase in rat UCP-3 protein. We treated extensor digitorum longus (EDL) muscle with 5'-amino-4-imidazolecarboxamide ribonucleoside (AICAR), a compound that activates AMP-activated protein kinase (AMPK), an enzyme known to be stimulated during exercise and hypoxia. Incubation of rat EDL muscle in vitro for 30 min with 2 mM AICAR causes a threefold increase in UCP-3 mRNA and a 1.5-fold increase of UCP-3 protein compared with untreated muscle. These data are consistent with the notion that activation of AMPK, presumably as a result of fuel depletion, rapidly regulates UCP-3 gene expression.

adenosine 5'-monophosphate-activated protein kinase; 5'-amino-4-imidazolecarboxamide ribonucleoside; rats

THE TRANSPORT OF METABOLITES in and out of mitochondria is mediated by the mitochondrial carrier protein superfamily, whose members can be identified by their characteristic size (~300 amino acids), three repeating domains, and six membrane spanning helices (39). Uncoupling protein 1 (UCP-1) is a mitochondrial transporter family member that is expressed predominantly in brown fat, where it has been shown to play a role in thermogenesis by uncoupling mitochondrial respiration, thus generating heat and justifying its name (reviewed in Ref. 25). Recently, UCP-3 was identified as an uncoupling protein isoform whose expression is particularly high in skeletal muscle (5, 31, 34, 49). The presence of UCP-3 in skeletal muscle is of great interest,

because this tissue is an important site of thermogenesis and energy homeostasis in mammals. Thus the hypothesis that UCP-3 may play a role in controlling thermogenesis and energy expenditure in skeletal muscle is attractive because of its homology to UCP-1 and, therefore, its possible functional equivalence. In fact, UCP-3 exhibits properties consistent with an uncoupling function when transfected in C₂C₁₂ myocytes (4) and yeast (13, 56). However, the major physiological role(s) of skeletal muscle UCP-3 remains unclear. It does not seem to be involved in cold adaptation, because acclimation of rats to cold results in a 50% decrease in skeletal muscle UCP-3 expression (4, 29), although short-term exposure to cold does cause a transient increase in UCP-3 that may be mediating shivering thermogenesis (29). On the other hand, acute exercise (9, 48) and fasting (13), conditions where one might expect that tight metabolic coupling would be desirable, result in an increase in UCP-3 mRNA expression, which is presumably indicative of an increase in protein and suggests that short-term regulation of energy expenditure is not the primary function of this protein.

One observation concerning the regulation of UCP-3 expression by fasting and acute exercise is that these situations result in an increase in circulating free fatty acids. Thus the upregulation of UCP-3 mRNA expression in skeletal muscle during food deprivation parallels the increase in serum free fatty acids under such starvation conditions (4, 13, 37). Elevation of circulating free fatty acids by lipid infusion, postulated as a starvation-mimicking condition, also increases UCP-3 gene expression in vivo (51) and in vitro (19) for C₂C₁₂ cells, whereas treatment of rats with an antilipolytic agent causes a reduction in UCP-3 gene expression (44). Finally, an acute bout of exercise elevates blood free fatty acid levels and also causes an increase in UCP-3 gene expression (9). Taken together, these data suggest that UCP-3 in skeletal muscle may function primarily as an acute regulator of lipid metabolism and not as a regulator of thermogenesis or short-term energy expenditure (45).

How, then, is UCP-3 expression regulated by alterations in the cell's energy state? AMP-activated protein

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kinase (AMPK) is a member of a metabolite-sensing protein kinase family that is found in all eukaryotes (24, 54). AMPK activity is stimulated by exercise/contraction (16, 18), nutrient starvation (15, 53), and ischemia/hypoxia (27, 41) and modulates many aspects of mammalian cell metabolism. AMPK is activated by an increase in free intracellular AMP and a decrease in cellular ATP via a complex mechanism that involves allosteric regulation of the subunits, phosphorylation by an AMPK kinase, and decreases in phosphatase activities (24). During exercise/hypoxia, AMP formation is increased as a result of the adenylate kinase reaction, in which ATP and AMP are formed from two molecules of ADP in an effort to maintain high ATP concentration. Thus, even with minimal reduction in cellular ATP, changes in the concentration of AMP can cause AMPK to become a sensor and/or effector of the cell's energy state. AMPK has been shown to increase fatty acid oxidation in liver, heart, and skeletal muscle, the primary tissues that oxidize fatty acids (reviewed in Refs. 24, 54). It does this through the inactivation of acetyl-CoA carboxylase (ACC), whose product, malonyl-CoA, is a precursor for the biosynthesis of fatty acids and a potent inhibitor of β -oxidation because it inhibits carnitine palmitoyltransferase 1 (CPT-1) and thereby the transport of long-chain fatty acids into the mitochondrial matrix (35, 40).

5'-Amino-4-imidazolecarboxamide ribonucleoside (AICAR) has previously been reported to be taken up into a variety of cells, including hepatocytes and skeletal muscle cells (8, 17, 47, 55). Once inside the cell, AICAR can be phosphorylated to form AICAR monophosphate (ZMP), an AMP analog (42). Consequently, ZMP mimics the multiple effects of AMP on AMPK, not only causing allosteric activation but also promoting phosphorylation and activation of the upstream kinase, AMPK kinase. Thereby, AICAR can change the activities of metabolic enzymes that are the targets of AMPK in these tissues (8, 17, 47, 55). Perfusion or *in vitro* incubation of rat hindlimb with AICAR was found to activate AMPK, inactivate ACC, and decrease malonyl-CoA, thus increasing fatty acid oxidation in this tissue (36). Here, we show that UCP-3 expression is stimulated by physiological states that increase free fatty acids, and this effect can be mimicked by AICAR.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were purchased from Taconic Breeding Laboratory (New York, NY). For *in vitro* incubation of isolated muscles with AICAR, rats weighing 50–100 g were used; otherwise, rats weighing 150–175 g were employed. The animals were provided with water and standard rat chow (Charles River Laboratory) and were kept at constant room temperature (20–22°C). Animals had free access to food and water overnight until the experiments were initiated.

Exercise. Rats were either kept sedentary or exercised by swimming in plastic barrels filled with warm water (35°C) under constant monitoring. Rats swam for 30-min periods for up to 200 min interrupted by 10-min rest periods. After one bout of swimming exercise, rats were killed by CO₂ asphyxiation, and total hindlimb muscles were quickly isolated for

protein and RNA extraction (see below). For both exercise protocols and for the hypoxia experiments (see below), four hindlimb muscles were combined from two animals for each experimental point. Rats completed one bout of running on a level treadmill at 24 m/min for ≤ 200 min without rest. After one bout of running, rats were killed by CO₂ asphyxiation, and total hindlimb muscles were taken immediately, as for swimming.

Hypoxia. Rats were kept either sedentary (control) or in an atmosphere consisting of 90% N₂-10% O₂ (Medical-Technical Gas) for ≤ 200 min. Total hindlimb muscle from hypoxic rats was isolated immediately after treatment.

***In vitro* muscle incubation and AICAR treatment.** Extensor digitorum longus (EDL) muscles (4 per experimental point) from Male Sprague-Dawley rats (50–100 g) were rapidly dissected after anesthesia (pentobarbital sodium, 60 mg/kg animal wt) and were attached by their tendons to stainless steel clips to keep the muscles in the stretched state, as described by Maizels et al. (32). All incubations were carried out in media saturated with 95% O₂ and 5% CO₂. Isolated muscles were placed in Krebs-Ringer buffer (in mM: 12.5 HEPES, 120 NaCl, 6 KCl, 1.2 MgSO₄, 1 CaCl₂, 0.6 Na₂HPO₄, 0.4 NaH₂PO₄, 2.5 D-glucose, pH 7.4) for 20 min at 37°C. Muscles were incubated with or without 2 mM AICAR (Sigma) for ≤ 200 min. After three washes with PBS buffer, muscles were frozen rapidly in liquid nitrogen and kept at –80°C.

Isolation of intact mitochondria. The mitochondrial isolation methods were adapted from Makinen and Lee (33), as described by Berthon et al. (2). The entire procedure was performed at 4°C, and the buffer solutions used were the following (in mM): *solution 1* (100 KCl, 40 Tris·HCl, 10 Tris-base, 5 MgCl₂, 1 EDTA, and 1 ATP, pH 7.4); *solution 2* (100 KCl, 40 Tris·HCl, 10 Tris-base, 1 MgSO₄, 0.1 EDTA, 0.2 ATP, and 1.5% BSA, pH 7.4); and *solution 3* (same as *solution 2* but without BSA). All three solutions contained 1 μ M aprotinin A, 1 μ M pepstatin, 1 μ M leupeptin, and 1 mM phenylmethylsulfonyl fluoride.

Muscle samples (50–70 mg) were minced, homogenized by a hand-driven all-glass homogenizer in 20 vol (ml) of *solution 1*/g muscle weight, and centrifuged at 700 *g* for 10 min. Supernatants were centrifuged at 14,000 *g* for 10 min, and the pellet was resuspended in 10 vol of *solution 2* and centrifuged at 7,000 *g* for 10 min. The mitochondrial pellet was suspended in 10 vol of *solution 3* and centrifuged at 3,500 *g* for 10 min. Finally, the pellet was resuspended in 1 vol/wt of a mannitol-sucrose buffer that contained (in mM) 220 sucrose, 70 mannitol, 10 Tris·HCl, and 1 EDTA, pH 7.4. Triton X-100 was added to samples at a final concentration of 1%. After rotating at 4°C for 1 h, samples were centrifuged at 15,000 *g* for 10 min in a microcentrifuge, and the supernatants were used for Western blot.

Gel electrophoresis and immunoblotting. Protein samples were electrophoresed according to Laemmli (28) and transferred to an Immunoblot polyvinylidene fluoride membrane (Bio-Rad). After transfer, membranes were blocked with 10% nonfat dry milk in PBS for 1 h at room temperature and incubated with anti-UCP-3 antibody (AB3046, Chemicon). Secondary antibody (Sigma) was conjugated to horseradish peroxidase. Blots were developed by an enhanced chemiluminescence detection system (Du Pont NEN), and films were scanned with a computing densitometer (Molecular Dynamics) for quantitative analysis.

Protein content. Protein concentration was determined with a bicinchoninic acid kit (Pierce) according to manufacturer's instructions.

Preparation of cDNA probe by random priming. cDNA probes for Northern blot analysis were made by RT-PCR

from primers based in the indicated GenBank sequences. Hybridization probes were generated by random cDNA priming using the Klenow fragment of DNA polymerase (Promega) and [α - 32 P]dCTP (NEN Life Science Products). The cDNAs utilized in this study were (identified by GenBank accession nos.) rat UCP-3 (AF030163), GLUT-4 (M23383), insulin-responsive aminopeptidase (IRAP, U32990), sortilin (AF023621), rat muscle CPT-1 (AF029875), rat monocarboxylate transporter-3 (MCT-3, U87627), rat tricarboxylate transporter-1 (TCT-1, L12016), and glyceraldehyde-3-phosphate dehydrogenase (AF106860).

Isolation of total RNA and Northern blot analysis. Total RNA was isolated as described by Chomczynski and Sacchi (7). Tissues were homogenized in *solution 4* (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol). The lysate was extracted with acidic phenol-chloroform and then subjected to an isopropanol precipitation at -20°C . RNA was quantitated by ultraviolet spectrophotometry. For Northern blot analysis, 20–40 μg of total RNA were separated on 6% formaldehyde-1% agarose gels and transferred to Genescreen nylon membrane (Du Pont NEN Life Science Products) by capillary transfer using 10X standard sodium citrate (SSC, 1.5 M NaCl, 0.15 M Na citrate, pH 7.0) as the liquid phase. After ultraviolet cross-linking, filters were subjected to Northern blot analysis as described previously (29). Generally, prehybridization was carried out at 42°C for ≥ 4 h in 50% formamide, 4X SSC, 1% SDS, 1X Denhardt's solution [0.1% BSA, 0.1% polyvinylpyrrolidone (PVP), 0.1% Ficoll], 50 mM sodium phosphate, pH 7.4, 0.5 mg/ml sodium pyrophosphate, and 100 mg/ml yeast tRNA. Hybridization was carried out overnight at 42°C in hybridization solution, which is identical to prehybridization solution except that BSA, PVP, and Ficoll concentrations are each at a concentration of 0.02%, and it contains the cDNA probe with a specific radioactivity of 10^7 cpm/ml. After hybridization, the membranes were washed twice with 2X SSC-0.1% SDS at room temperature for 5 min and finally with 0.2X SSC-0.1% SDS at 42°C for 15 min. The membranes were subjected to autoradiography using Kodak Biomax MR film with an intensifying screen at -80°C . Quantitative analysis of the radioactivity was carried out in an InstantIMAGER (Packard) or by scanning densitometry (see below). For rehybridization, the probe was stripped from the membrane by washing the membrane in boiled 0.1 X SSC-0.1% SDS twice, each time for 10 min.

Quantification and statistical analysis of data. Autoradiographs of Western and some Northern blots were scanned in a computing densitometer (Molecular Dynamics) and graphed, setting the basal level of mRNA or protein to 100%. All experiments were performed on three or more independent occasions, each experimental point utilizing four or six muscles, and statistically significant differences from basal values were determined by paired Student's *t*-tests.

RESULTS

Swimming induces UCP-3 mRNA expression in rat skeletal muscle. Rats were subjected to the swimming protocol described in MATERIALS AND METHODS and were then killed for mRNA extraction at the times indicated. Figure 1 shows that UCP-3 mRNA levels were increased 290 ± 40 , 310 ± 64 , and $670 \pm 39\%$ over the level in the control muscles after 30, 100, or 200 min of swimming, respectively. We performed two types of control experiments to show that the exercise-mediated increase in UCP-3 mRNA was specific for this

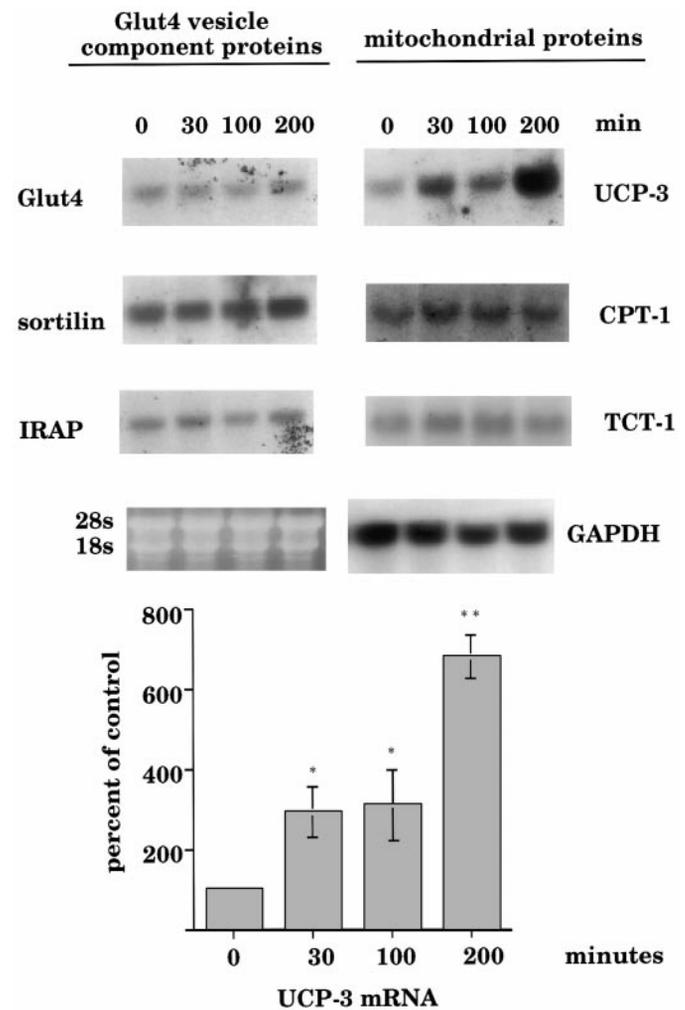


Fig. 1. Swimming specifically induces rat hindlimb muscle uncoupling protein 3 (UCP-3) expression. IRAP, insulin-responsive aminopeptidase; CPT-1, carnitine palmitoyltransferase 1; TCT-1, tricarboxylate transporter 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Rats were either kept sedentary (control) or exercised by swimming in plastic barrels filled with 35°C water for ≤ 200 min. Total RNA was extracted from hindlimb muscle from control and trained rats immediately after exercise at times indicated, and Northern blot analysis was performed with the various cDNA probes indicated. A representative result of 3 independent experiments is shown (2 animals, 4 muscles/data point). Data for UCP-3 mRNA expression were quantified as described in MATERIALS AND METHODS with the initial value being set to 100. Data from 3 independent experiments were statistically analyzed by Student's paired *t*-test (* $P < 0.05$; ** $P < 0.01$).

message. Exercise is well known to cause a rapid recruitment of GLUT-4 to the cell surface of muscle (14), but swimming for ≤ 200 min does not change the mRNA level for component proteins of GLUT-4-containing vesicles, namely GLUT-4, sortilin (30), and IRAP (20, 23). On the other hand, exercise might have general effects on the expression of mitochondrial transporters; therefore, we examined the expression of two such genes, CPT-1, which mediates the tightly regulated step of long-chain fatty acid import into mitochondria (50), and TCT-1, which is involved in citrate- H^+ /malate exchange across the inner mitochon-

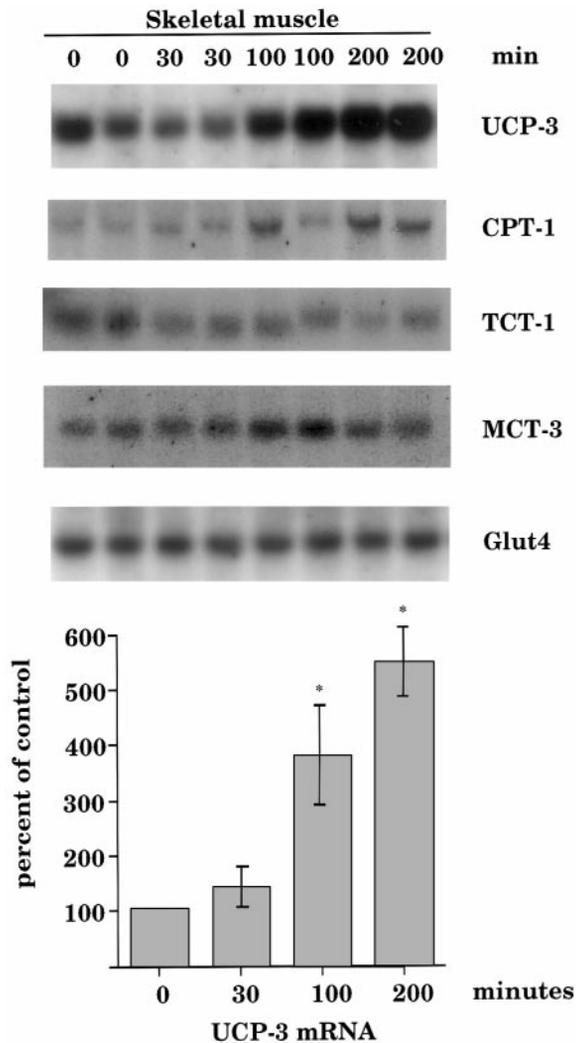


Fig. 2. Treadmill running specifically increases UCP-3 mRNA expression in rat hindlimb muscle. MCT-3, monocarboxylate transporter 3. Hindlimb muscles were taken from control and exercised rats immediately after treadmill running at times indicated. Total RNA was extracted, and Northern blot analysis was performed with various cDNA probes. A representative result is shown, and the data were subjected to quantitative analysis as in Fig. 1.

drial membrane (21). As shown in Fig. 1, the expression of CPT-1 and TCT-1 is affected minimally, if at all, by ≤ 200 min of swimming, indicating that the change in UCP-3 mRNA expression is indeed highly specific.

Treadmill running increases UCP-3 expression. Rats were run on a treadmill for ≤ 200 min without resting. Their hindlimbs were taken immediately thereafter for Northern and Western blot analysis (Fig. 4), as described in MATERIALS AND METHODS. As shown in Fig. 2, the levels of UCP-3 mRNA were 140 ± 29 , 380 ± 66 , and $544 \pm 51\%$ of the level in control muscle after 30, 100, and 200 min of treadmill running, respectively. As with swimming, running had little effect on the other reference genes we used here, including TCT-1, GLUT-4, and CPT-1, although a small increase in CPT-1 at the 200-min time point was a reproducible observation. We also measured MCT-3 (monocarboxylate transporter isoform 3) mRNA, which mediates

most of the lactic acid efflux from skeletal muscle (52), a process well known to increase on vigorous exercise. As shown in Fig. 2, there was a reproducible increase in MCT-3 mRNA at the 100-min time point, but this was much less than that seen for UCP-3 and again confirms the highly specific nature of the latter's response to exercise.

Hypoxia induces UCP-3 mRNA expression in rat skeletal muscle. Another state of fuel depletion in skeletal muscle can be induced by hypoxia. Animals were subjected to hypoxic conditions by exposing them to 90% N₂-10% O₂ for ≤ 200 min. As shown in Fig. 3, and similar to the effect of exercise, hypoxia increased UCP-3 mRNA levels in rat hindlimb by 180 ± 35 , 261 ± 39 , and $425 \pm 82\%$ of those in the control rats after 30, 100, and 200 min of treatment, respectively. In contrast, hypoxia did not have a significant effect on MCT-3, CPT-1, and TCT-1 gene expression.

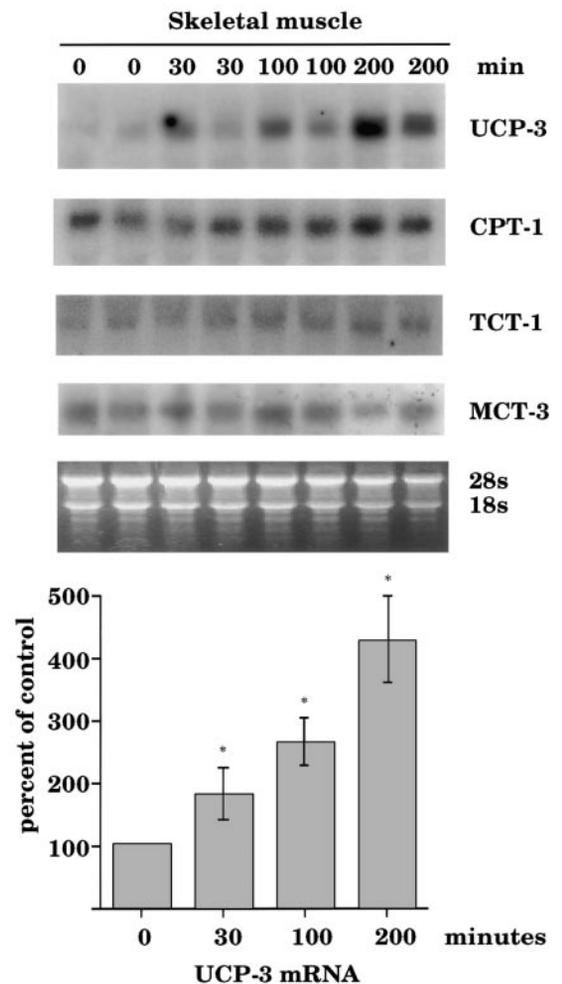


Fig. 3. Hypoxia specifically increases UCP-3 mRNA expression in rat hindlimb muscle. Rats were kept either sedentary (control) or in an atmosphere of 90% N₂-10% O₂ for ≤ 200 min. Total RNA was extracted from hindlimb muscle from control and hypoxic animals at times indicated, and Northern blot analysis was performed with various cDNA probes specified in the figure. Shown is an experiment representative of 3 independent examples, and the data were quantitatively analyzed as in Fig. 1.

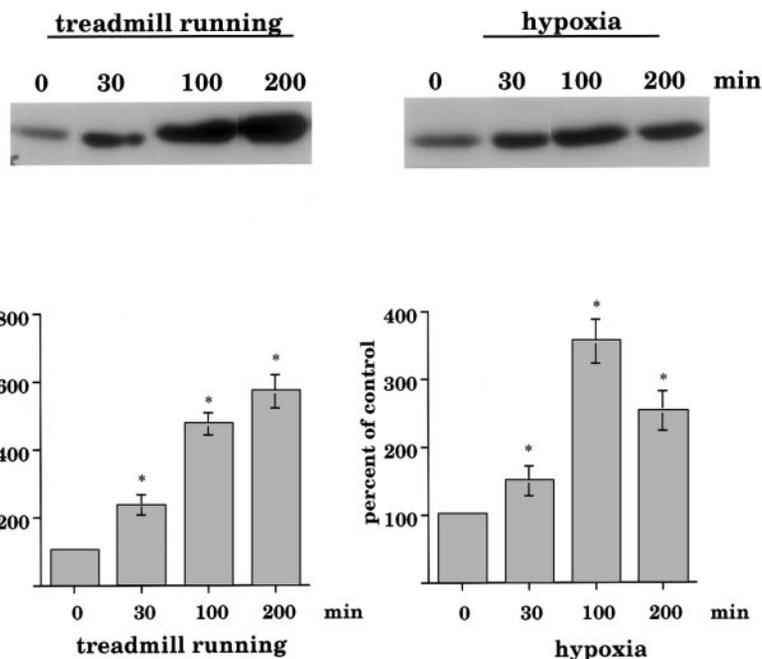


Fig. 4. Both treadmill running and hypoxia result in increased UCP-3 protein expression in rat hindlimb muscle. Intact mitochondria were separated from hindlimb muscle of control, treadmill-trained, and hypoxic animals immediately after treatment, as described in MATERIALS AND METHODS. Western blot analysis was performed with anti-UCP-3 antibody. A representative result of 3 independent experiments is shown. The data were quantified by scanning densitometry and were statistically analyzed by Student's paired *t*-test.

The increases in UCP-3 mRNA expression due to exercise and hypoxia are both rapid and substantial, but it is the amount of UCP-3 protein that has potential functional consequences. Thus we isolated intact mitochondria and measured by Western blot the effects of treadmill exercise and hypoxia on UCP-3 protein amount. The data shown in Fig. 4 demonstrate that the amount of UCP-3 protein roughly corresponds to the amount of UCP-3 mRNA (Figs. 2 and 3) and is increased $565 \pm 54\%$ over control values by 200 min of treadmill running and $354 \pm 33\%$ over that in control samples after 100 min of hypoxia experiment. The UCP-3 protein level decreased on longer exposure to hypoxia for unknown reasons.

UCP-3 expression is elevated in rat EDL muscle after incubation with AICAR in vitro. Exercise and hypoxia represent metabolic states wherein ATP is depleted and in large demand. As noted in the introductory section, AMPK is a key regulatory enzyme under these conditions, and the reagent AICAR, when added to intact cells, causes activation of AMPK and mimics the fuel-depleted state. Consequently, we incubated isolated rat EDL muscles with 2 mM AICAR for ≤ 200 min. As shown in Fig. 5, AICAR incubation specifically induces gene expression of UCP-3 mRNA in EDL muscle without affecting the expression of the five reference genes we measured in vivo (Fig. 2). We also performed a time course for AICAR incubation for ≤ 200 min, and as shown in Fig. 6, AICAR exposure increases both UCP-3 mRNA and protein levels at all times measured. However, the magnitude of induction decreases with incubation time, with the highest mRNA ($334 \pm 57\%$) and protein levels ($152 \pm 32\%$) being achieved after 30-min exposure to AICAR. A similar result was observed for the time-dependent decrease in the effects of AICAR on AMPK activity in hepatocytes, and this was explained by the fact that

ZMP levels continue to increase with time, reaching levels that inhibit AMPK after 20–30 min of AICAR exposure (8). It is likely that a similar phenomenon is occurring in our experiment (Fig. 6).

DISCUSSION

The primary physiological role of UCP-3 is unclear, as is the mechanism by which its expression is rapidly

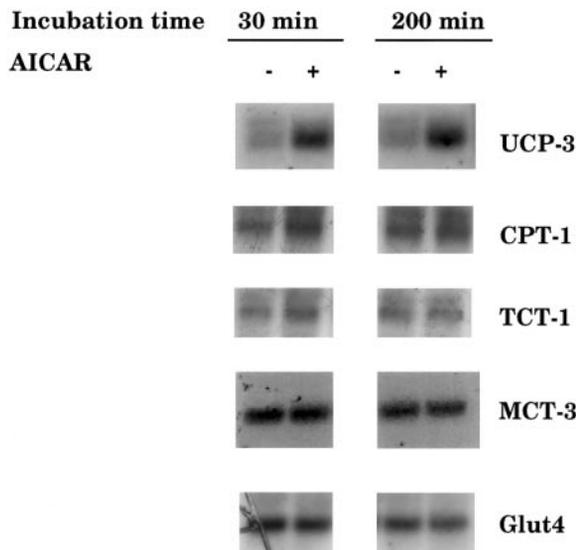


Fig. 5. Effect of 5'-amino-4-imidazolecarboxamide ribonucleoside (AICAR) on gene expression in isolated rat extensor digitorum longus (EDL) muscle. Rat EDL muscles were incubated in vitro in the presence or absence (control) of 2 mM AICAR in Krebs-Ringer buffer at 37°C under an atmosphere of 95%O₂-5%CO₂ for ≤ 200 min, as described in MATERIALS AND METHODS. Total RNA was extracted, and Northern blot analysis was performed with various cDNA probes. A representative result of 3 independent experiments is shown, and each data point is from 2 animals (4 muscles).

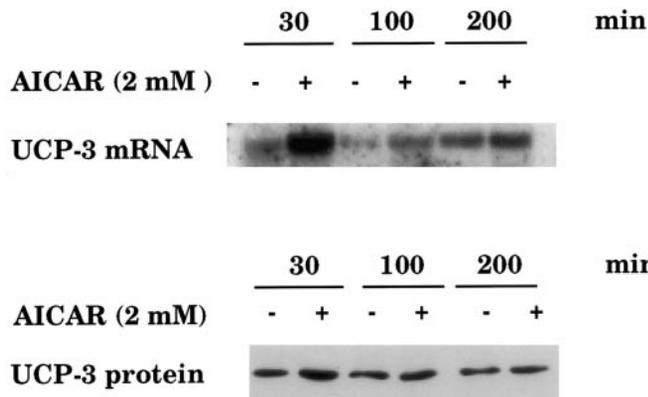
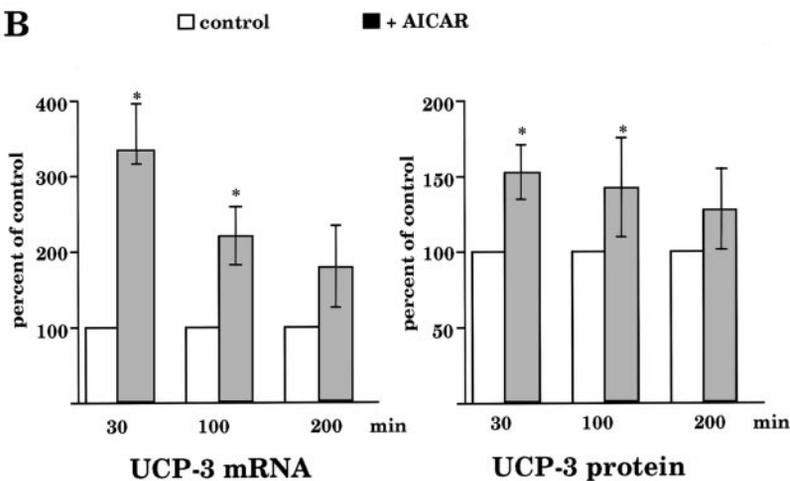
A**B**

Fig. 6. AICAR induces UCP-3 gene and protein expression in EDL muscle. Rat EDL muscles were incubated *in vitro* with or without 2 mM AICAR. Total RNA and mitochondrial protein were extracted after incubation, as outlined in MATERIALS AND METHODS. *A*: Northern blot analysis was done with various cDNA probes, and Western blot analysis was done using anti-UCP-3 antibody. A representative result of 3 independent experiments is shown. *B*: quantitative analysis of the data was accomplished as in previous figures.

and substantially increased in fuel-depleted states caused by acute exercise, fasting, and hypoxia. Previously published studies indicate that UCP-3 mRNA expression is increased by acute exercise [treadmill running; mice and rats (9, 48)] and fasting [humans and rats (13, 37)] and is decreased by endurance training (3). Here, we show that swimming and hypoxia are also effective in acutely increasing UCP-3 mRNA expression, and we show that UCP-3 mRNA is rapidly translated into protein, strongly supporting the idea that the acute regulation of gene expression has immediate and functionally important consequences. In addition, the increase in UCP-3 expression can be induced by AICAR, which mimics the effects of AMP on activation of AMPK, thus suggesting that activation of AMPK is necessary for increased UCP-3 gene expression.

It was initially proposed (13) that UCP-3 plays a role in the control of skeletal muscle thermogenesis analogous to UCP-1, which has a major role in brown adipose tissue thermogenesis (25). However, whereas fasting, exercise, and hypoxia all lead to an acute (30 min) increase in UCP-3 expression in skeletal muscle, these conditions have no obvious need for thermogenesis, nor do they readily correlate with conservation of energy

expenditure. For example, it could be expected that heat should be dissipated rather than generated during acute exercise, but that increased UCP-3 levels should result in more heat. Also, one might expect that metabolic conditions during fasting, exercise, and hypoxia would warrant an increase in metabolic efficiency that is possibly mediated by decreased muscle UCP-3 expression, hence, more tightly coupled mitochondria, and this seems to be the case for endurance training (3). However, our data and other published studies showing increased UCP-3 expression for these conditions are not compatible with more tightly coupled mitochondria; rather, they indicate an association between changes in energy substrate (e.g., fatty acids) availability and UCP-3 gene expression.

What else, then, could be the main physiological role of UCP-3 protein in skeletal muscle under conditions of acute physiological change such as exercise, fasting, and hypoxia? One phenomenon that occurs during exercise and hypoxia is an increase in the production of reactive oxygen species (ROS) (1, 11). ROS are increased during exercise as a result of increases in mitochondrial oxygen consumption and electron transport flux (26). The formation of ROS during hypoxia, on the other hand, is due to mitochondrial oxidative and

reductive stress (38). As is proposed for both exercised and hypoxic animals, when less O₂ is available to be reduced to H₂O by cytochrome oxidase, reducing equivalents accumulate within the mitochondrial respiratory sequence. This can result in the formation of ROS by the autooxidation of one or more mitochondrial complexes such as the ubiquinone-ubiquinol redox couple (10, 22). ROS may lead to irreversible damage of mitochondrial DNA, membrane lipids, and proteins, resulting in mitochondrial dysfunction and, ultimately, cell death (6, 26, 46). As noted earlier, when transfected in yeast and mammalian cells, UCP-3, similar to UCP-1, has been shown to decrease mitochondrial membrane potential (4, 13, 56). Taking these together, we suggest that an increase in UCP-3 expression in skeletal muscle during exercise and hypoxia would lower the mitochondrial membrane potential, thereby maximizing the oxidation state of mitochondrial respiratory chain carriers, which serve as one-electron O₂ reductants. This would result in decreased ROS production and protection of the mitochondria from damage. This hypothesis is consistent with observations from UCP-3-gene knockout mice, which have higher ROS production in their skeletal muscle mitochondria than do control mice (B. B. Lowell and A. Vidal-Puig, unpublished data).

A very interesting aspect of our current work is the extremely fast activation of UCP-3 mRNA expression, which is readily seen at 30 min and is as high as fourfold activated after 100 min of exercise or hypoxia (Figs. 1–3). This suggests a very rapid mechanism for converting a cytoplasmic signal, presumably increased cytosolic AMP, into a nuclear event. Our evidence that AMPK is a direct or indirect mediator of elevated UCP-3 gene expression relies on the use of AICAR, whose addition to isolated muscle (Figs. 5 and 6) mimics the effects of exercise and hypoxia. This AMP analog has been used extensively to modulate AMPK and its target enzymes (8, 17, 47, 55) and recently to implicate activation of AMPK in contraction-stimulated GLUT-4 translocation in muscle (16, 41). As noted earlier, AMPK is activated under conditions of elevated free fatty acids (40) that are observed on hypoxia and acute exercise. Very recently, isoforms of AMPK have been shown to be in the cell nucleus (43) and to regulate gene expression in hepatocytes (12). All this suggests that future work on the mechanism of UCP-3 regulation in skeletal muscle should be fruitful and interesting.

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