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AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARα and PGC-1

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Abstract

AMP-activated protein kinase (AMPK) activation increases fatty acid oxidation in skeletal muscle by decreasing malonyl CoA concentrations. However, this may not explain the long-term effects of AMPK activation. Here we show that AMPK activation by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) increases mRNA expression of PPARα target genes and PGC-1 in cultured muscle cells and mouse skeletal muscle, and that inhibition of PPARα and PGC-1 by siRNAs prevents AICAR-stimulated increase in fatty acid oxidation. These data suggest that a novel transcriptional regulatory mechanism involving PPARα and PGC-1 exists that is responsible for long-term stimulation of fatty acid oxidation in skeletal muscle by AICAR.

Keywords: AICAR; AMPK; PPARα; PGC-1; Fatty acid oxidation; Muscle

AMP-activated protein kinase (AMPK) is an enzyme that is activated when cellular energy is depleted [1,2]. When activated, AMPK increases glucose uptake and fatty acid oxidation in skeletal muscle cells to increase ATP production [3–6]. The adenosine analog 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) is a well-known activator of AMPK [7]. Activation of AMPK by AICAR has been shown to decrease intracellular malonyl CoA concentrations by inhibiting acetyl CoA carboxylase (ACC), the rate-limiting enzyme of malonyl CoA synthesis [4,8], and by stimulating malonyl CoA decarboxylase (MCD), the enzyme responsible for malonyl CoA degradation [8,9]. Malonyl CoA is an allosteric inhibitor of carnitine palmitoyltransferase (CPT)-1, the enzyme that controls the transfer of long-chain fatty acyl CoA into the mitochondria. Thus, decreased malonyl CoA concentrations triggered by AMPK activation may account for the increased fatty acid oxidation that is observed in these cells. However, these processes are relatively transient and may not be sufficient to explain the long-term effects of AMPK activation. In fact, sustained activation of AMPK has been shown not to be associated with inactivation of ACC during prolonged exercise [10].

The peroxisome proliferator-activated receptors (PPARs), which constitute a subfamily of the nuclear receptor superfamily, regulate gene expression in response to ligand binding [11–13]. Among them, PPARα is expressed predominantly in the liver, heart, and skeletal muscle, and up-regulates the expression of genes involved in fatty acid metabolism [12–16], particularly when coactivated by PPARγ coactivator (PGC)-1 [17]. In fact, endurance training, a condition that chronically activates

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AMPK, is associated with increases in PPARα and PGC-1 expression [18]. In the present study, we present evidence that, in addition to the well-known allosteric regulation of CPT-1, AICAR-induced AMPK activation stimulates fatty acid oxidation in skeletal muscle by activating PPARα and PGC-1.

Materials and methods

Cell culture and treatments. C_2C_{12} myoblasts were purchased from the American Type Culture Collection (Manassas, VA, USA) and used for all studies. Cells were maintained in DMEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For differentiation into myotubes, myoblasts were grown in dishes to confluence and the culture medium was then replaced with DMEM containing 2% heat-inactivated horse serum (Hyclone) for 4–6 days.

Determination of fatty acid oxidation. The fatty acid oxidation rate was measured as $^{14}CO_2$ generation from [^{14}C]palmitate (NEN Life Sciences, Boston, MA, USA), as previously described [19], with minor modifications. Cells were cultured in 24-well plates on growth and differentiation medium. After exposure to the indicated doses of AICAR (TRC, Toronto, Canada) for the given time periods, the medium was changed to reaction media containing 0.2 mM palmitate ([1-^{14}C]palmitate at 0.5 μCi/ml) and the same doses of AICAR. After incubating for 30 min at 30 °C, the reaction was quenched by adding 50 μl of 4 N sulfuric acid. The CO_2 produced during the 30-min incubation was trapped with 200 μl of 1 N sodium hydroxide. The trapped $^{14}CO_2$ and ^{14}C-labeled acid soluble products were determined by liquid scintillation counting. The measured fatty acid oxidation rate was corrected according to the protein content of the cells.

Quantitative RT-PCR analysis. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). For quantitative RT-PCR analysis, 2 μg total RNA were reverse-transcribed with oligo(dt) using ReverseAid M-MuLV Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany). Target cDNA levels were quantified by real-time PCR using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) utilizing SYBR green. The primer sequences used are shown in Table 1.

siRNA experiments. We synthesized small interfering RNA (siRNA) targeting PPARα (target sequence; 5′-CACGCATGTGAAGGCTGTAA-3′; Bioneer, Daejeon, Korea) and PGC-1 (target sequence; 5′-GGTGGA TTGAAGTGGTGTAGA-3′; Invitrogen), as well as control siRNAs, which have the same GC content as the target sequences and no effect on silencing of gene expression. siRNAs were transfected into C_2C_{12} myoblasts before differentiation to myotubes, using Lipofectamine 2000 (Invitrogen).

Animals and treatments. Homozygous PPARα knock-out ($PPAR\alpha^{-/-}$) mice were kindly donated by Dr. F.J. Gonzalez (National Institutes of Health, Bethesda, MD, USA) [20] and C57BL/6J (control) mice were supplied by Orient (Sungnam, Korea). Mice were housed at ambient temperature (22 ± 1 °C), with 12:12-h light–dark cycles and free access to water and rat chow. All experiments were performed at 12–14 weeks of age. The experiments were approved by the Institutional Animal Care and Use Committee at the Asan Institute for Life Sciences, Seoul, Republic of Korea. To determine the effect of AICAR treatment on fatty acid oxidation and mRNA expression of PPARα target genes and PGC-1, $PPAR\alpha^{-/-}$ and control mice received AICAR or vehicle by intraperitoneal injection. Four hours later, soleus muscles were dissected out from anesthetized animals and trimmed of fat and connective tissue. The muscles were immediately frozen and stored at −80 °C until experiments. The soleus muscle on one side was used for real-time PCR measurement of PPARα, ACO, CPT-1, FABP3, and PGC-1 mRNA expression, while that on the other side was used for measurement of fatty acid oxidation.

Statistical analysis. All data are expressed as means ± SEM. Statistical analysis was performed by two-tailed unpaired Student's t test or by one-way analysis of variance followed by post hoc Tukey's multiple comparison test. Statistical significance was defined as a value of $p < 0.05$.

Table 1
Primers used for real-time PCR

mRNA	Primer sequences
Murine ACO	5′-TGTTAAGAAGAGTGCCACCAT-3′ 5′-ATCCATCTCTTCATAACCAAATTT-3′
Murine CPT-1	5′-ACTCCTGGAAGAAGAAGTTCA-3′ 5′-AGTATCTTTGACAGCTGGGAC-3′
Murine FABP3	5′-CCCCTCAGCTCAGCACCAT-3′ 5′-CAGAAAAATCCCAACCCAAGAAT-3′
Murine PPARα	5′-CTTCACGATGCTGCCCTCCT-3′ 5′-CTATGTTTTAGAAGGCCAGGC-3′
Murine PGC-1	5′-AGCCGTGACCACTGACAACGAG-3′ 5′-GCTGCATGGTTCTGAGTGCTAAG-3′
Murine GAPDH	5′-CAGAACTACATCCCTGCATCC-3′ 5′-CCACCTTCCTGATGTCATCA-3′

Results

Cell experiments

As expected, AICAR treatment (0.25, 0.5, and 1 mM) of C_2C_{12} muscle cells significantly increased the fatty acid oxidation rate in a dose-dependent manner (data not shown). Quantitative RT-PCR measurements disclosed that mRNA expression of PPARα, several of its target genes (ACO, CPT-1, and FABP3), and PGC-1 was significantly increased following AICAR treatment (0.5 mM for 4 h) (Fig. 1). To further study the role of PPARα and PGC-1 in fatty acid oxidation, we performed siRNA experiments. Using real-time PCR, we confirmed that PPARα and PG

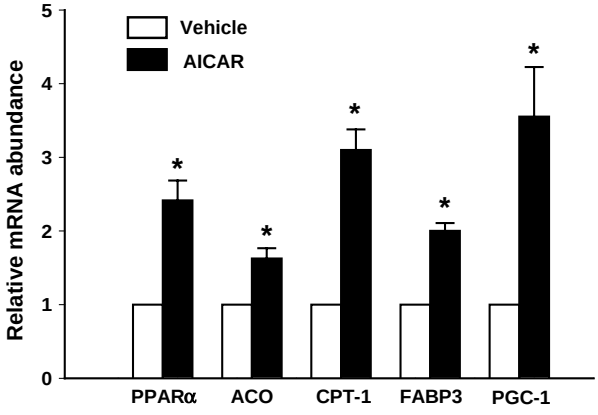

Fig. 1. mRNA expression of PPARα, its target genes, and PGC-1 following AICAR treatment. Differentiated C_2C_{12} myotubes were treated with AICAR (0.5 mM) or vehicle (DMSO) as a control for 4 h. Relative mRNA levels of PPARα, ACO, CPT-1, FABP3, and PGC-1 were analyzed using quantitative real-time PCR. Each mRNA expression measurement was normalized to GAPDH. All graphs show means ± SEM. Experiments were performed in quadruplicate and replicated at least three times. *$p < 0.05$ vs. vehicle.

C-1 mRNA expression was effectively reduced following AICAR treatment in C_2C_{12} cells transfected with PPARα siRNA or PGC-1 siRNA (data not shown). Compared to cells treated with control siRNAs, fatty acid oxidation after AICAR treatment was significantly reduced in cells treated with PPARα or PGC-1 siRNA in a dose-dependent manner (Fig. 2).

Animal experiments

We next explored the effect of AICAR on fatty acid oxidation in animal models. The basal fatty acid oxidation rate in skeletal muscle of $PPAR\alpha^{-/-}$ mice was significantly lower than that in control mice. AICAR treatment increased fatty acid oxidation in skeletal muscle of control mice approximately fourfold (Fig. 3A). Although the fatty acid oxidation rate was also significantly increased by AICAR treatment in $PPAR\alpha^{-/-}$ mice, the degree of increase was much less than that in control mice following AICAR treatment (Fig. 3A). mRNA expression of PPARα and its target genes was profoundly reduced in skeletal muscle of $PPAR\alpha^{-/-}$ mice compared to control mice (Figs. 3B–E). Although AICAR treatment significantly increased mRNA expression of PPARα, its target genes, and PGC-1 in control mice, AICAR treatment did not increase the expression of PPARα target genes in $PPAR\alpha^{-/-}$ mice (Figs. 3B–E). In contrast, AICAR treatment significantly increased PGC-1 expression in $PPAR\alpha^{-/-}$ mice (Fig. 3F).

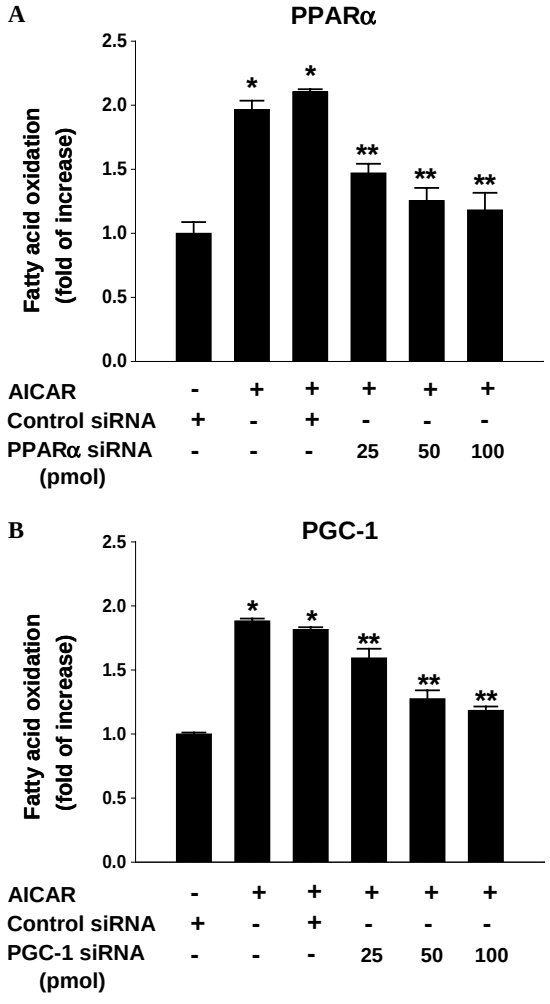

Fig. 2. Effect of siRNA on AICAR-stimulated fatty acid oxidation. Forty-eight hours after transfection with PPARα siRNA (A), PGC-1 siRNA (B), or control siRNA, fatty acid oxidation was measured by $^{14}CO_2$ generation from [^{14}C]palmitate in cells. Cells were treated with AICAR (0.5 mM) for 4 h after transfection. Data are shown as means ± SEM of at least three independent experiments. *$p < 0.05$ vs. control siRNA only; **$p < 0.05$ vs. AICAR only.

Discussion

It is currently believed that AMPK activation increases fatty acid oxidation by inhibiting ACC and stimulating MCD, both of which decrease intracellular malonyl CoA levels [4,8,9]. In this study, we present evidence that, in addition to this well-known allosteric regulation of CPT-1 by malonyl CoA, there exists a transcriptional regulatory mechanism. PPARα plays an important role in the transcriptional control of mitochondrial fatty acid oxidation by up-regulating gene expression involved in fatty acid oxidation in skeletal muscle [12–16]. In the present study, AICAR-induced AMPK activation increased mRNA expression of PPARα and its target genes, as well as fatty acid oxidation in muscle cells. Transfection of PPARα siRNA prevented an increase in fatty acid oxidation in response to AICAR in muscle cells. In addition, AICAR treatment significantly increased fatty acid oxidation and the expression of PPARα target genes in skeletal muscle of control mice.

It was previously reported [21] that fatty acid oxidation in skeletal muscles isolated from starved mice is not significantly different between $PPAR\alpha^{-/-}$ mice and control mice. The authors of this study proposed that high levels of PPARδ might compensate for the lack of PPARα in the skeletal muscles of knock-out mice. However, we found that the basal fatty acid oxidation rate of $PPAR\alpha^{-/-}$ mice was significantly different from that of control mice. Furthermore, the increment of fatty acid oxidation in response to AICAR treatment was significantly less in skeletal muscle of $PPAR\alpha^{-/-}$ mice than in control mice.

We also observed that PGC-1 mRNA expression was increased in response to AICAR treatment in muscle cells and mouse skeletal muscle. The role of PGC-1 was further demonstrated by the finding that PGC-1 siRNA prevented the increase in fatty acid oxidation by AICAR. These results are in agreement with previous reports, which demonstrated that AMPK activation by AICAR or chronic exercise increased PGC-1 expression in muscle [22,23]. PGC-1 is involved in multiple biological responses related to energy homeostasis, thermal regulation, and glucose metabolism [24]. Although PGC-1 was originally identified as a transcriptional coactivator of the nuclear receptor PPARγ, it is now known that PGC-1α coactivates PPARα in the transcriptional control of mitochondrial fatty acid oxidation [17].

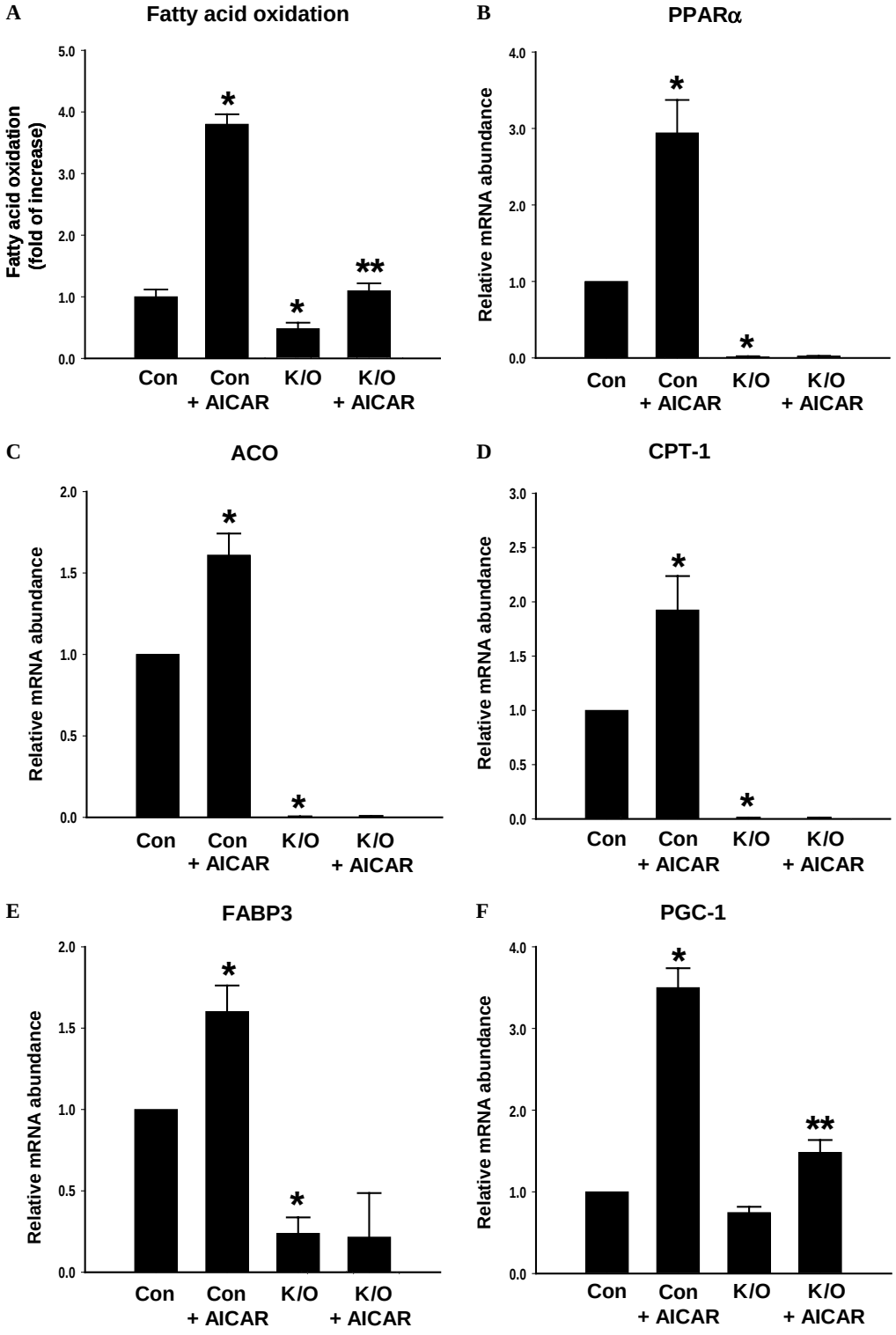

Fig. 3. In vivo fatty acid oxidation and mRNA expression of PPARα target genes and PGC-1. $PPAR\alpha^{-/-}$ ($n = 6$, respectively) and C57BL/6J (control; $n = 6$, respectively) mice were treated with AICAR (500 mg/kg) or vehicle (0.9% NaCl) by intraperitoneal injection. Four hours after treatment, fatty acid oxidation (A) and relative mRNA levels of PPARα (B), ACO (C), CPT-1 (D), FABP3 (E), and PGC-1 (F) were measured in mouse skeletal muscle. Each mRNA expression measurement was normalized to GAPDH. *Con*, control mice; *K/O*, $PPAR\alpha^{-/-}$ mice. All graphs show means ± SEM. Experiments were performed in triplicate. *$p < 0.05$ vs. control mice; **$p < 0.05$ vs. $PPAR\alpha^{-/-}$ mice.

In summary, we have shown that AICAR treatment stimulates fatty acid oxidation in skeletal muscle via a novel transcriptional regulatory mechanism. In addition to the well-established allosteric regulation of CPT-1 by malonyl CoA, AICAR increases mRNA expression of PPARα and its target genes as well as PGC-1. This may explain

the long-term stimulation of fatty acid oxidation that occurs in skeletal muscle in response to AMPK activation.

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