The role of AMPK and PPARγ1 in exercise-induced lipoprotein lipase in skeletal muscle

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AMPK induces PPARγ1 and LPL expression.

AMP-activated protein kinase; peroxisome proliferator-activated receptor γ1; lipoprotein lipase;
C2C12 cells; exercise
ABSTRACT

Exercise can effectively ameliorate type 2 diabetes and insulin resistance. Here we show that the mRNA levels of one of peroxisome proliferator-activated receptor (PPAR) family members, PPARγ1, and genes related to energy metabolism, including PPARγ coactivator-1 protein α (PGC-1α) and lipoprotein lipase (LPL), increased in the gastrocnemius muscle of habitual exercise-trained mice. When mice were intraperitoneally administered an AMP-activated protein kinase (AMPK) activator 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), the mRNA levels of the aforementioned three genes increased in gastrocnemius muscle. AICAR treatment to C2C12 differentiated myotubes also increased PPARγ1 mRNA levels, but not PPARα and δ mRNA levels, concomitant with increased PGC-1α mRNA levels. An AMPK inhibitor, compound C, blocked these AICAR effects. AICAR treatment increased the half-life of PPARγ1 mRNA by nearly 3-fold (4 h - 12 h) by activating AMPK. When C2C12 myoblast cells infected with a PPARγ1 expression lentivirus were differentiated into myotubes, PPARγ1 overexpression dramatically increased LPL mRNA levels by more than 40-fold. In contrast, when PPARγ1 expression was suppressed in C2C12 myotubes, LPL mRNA levels significantly reduced and the effect of AICAR on increased LPL gene expression was almost completely blocked. These results indicated that PPARγ1 was intimately involved in LPL gene expression in skeletal muscle and the AMPK-PPARγ1 pathway may play a role in exercise-induced LPL expression. Thus, we identified a novel critical role for PPARγ1 in response to AMPK activation for controlling the expression of a subset of genes associated with metabolic regulation in skeletal muscle.

INTRODUCTION

Physical exercise has beneficial effects on general health and results in increased catabolism of glucose and fatty acids as energy sources in skeletal muscle. AMPK and its related cellular signaling pathways are thought to play a critical role in exercise-mediated adaptations in the muscle (19). AMPK is an evolutionarily conserved heterotrimer that consists of α-catalytic and β- and γ-regulatory subunits and is a regulator of energy homeostasis. AMPK is activated by an increased AMP:ATP ratio associated with ATP consumption during exercise (3,30). Activated AMPK drives several energy production systems, including glucose uptake, fatty acid oxidation, and mitochondria biogenesis, to maintain energy balance. Because of these beneficial activities, AMPK is considered to be a target for preventing type 2 diabetes. The capacity of muscle to catabolize fatty acids is determined at the transcriptional level for
genes involved in fatty acid uptake and catabolism. PPAR α and δ, members of the nuclear receptor superfamily, control the transcription of these genes in the muscle (32). PPARα induces the expression of genes involved in the numerous steps of fatty acid uptake and oxidation in muscle; these genes are shared with PPARδ (5). PPARδ associates with an AMPK α subunit and increases basal and ligand-dependent transcription (16), which suggests that the AMPK-PPARδ pathway may play a critical role in regulating the expressions of numerous genes mediated by exercise.

The function of another family member of this family, PPARγ, as a receptor in skeletal muscle remain unclear due to its much lower level of expression as compared with that in adipose tissues. However, it has been reported that physical exercise induced increased PPARγ expression in human skeletal muscle through increased reactive oxygen species (ROS) production and that this effect was blocked by antioxidant supplementation (21).

Skeletal muscle is one of the main biosynthetic tissues of LPL, which hydrolyzes triglycerides contained in chylomicrons and VLDL to yield fatty acids for localized uptake by this tissue. The importance of muscle LPL for removing serum triglycerides was determined by analyzing muscle specific LPL-deficient mice (28). These mice exhibited insulin resistance in the liver and adipose tissues and aggravated diet-induced obesity. In addition, a recent study showed that muscle LPL expression in an insulin-resistant offspring was lower than that in controls, which indicated that reduced muscle LPL may cause diabetes (15). The results of these reports suggest a relationship between muscle LPL expression and diabetes although the molecular details of muscle LPL expression mechanisms have not been thoroughly investigated.

Until date, a few reports have shown that AMPK regulated LPL expression in cultured skeletal muscle cell lines (17, 18). These findings were consistent with the observation that exercise training induced increased muscle LPL expression (23). However, the mechanism by which AMPK increases LPL expression in skeletal muscle remains unclear. In this study, we show that AMPK induces increased expression of a nuclear receptor PPARγ1 and that this nuclear receptor is a key regulator of LPL expression in C2C12 myotubes. Thus, we identified a new role for the AMPK-PPARγ1 pathway in regulating muscle LPL expression that was induced in response to exercise.

**MATERIALS AND METHODS**

*Cell culture.* C2C12 myoblast cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100μg/ml
of streptomycin under 5% CO₂ atmosphere. Confluent myoblast cells differentiated into myotubes when cultured in a differentiation medium (2% horse serum, 100 units/ml of penicillin, and 100µg/ml of streptomycin in DMEM) for 4-6 days.

Western blot. Protein expression and phosphorylation were analyzed by western blotting. C2C12 myotubes and mouse skeletal muscle were lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, and 0.25% sodium deoxycholate) supplemented with a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and a phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Equal amounts of protein (10 µg/lane) from mixed cell lysates (n = 3) were subjected to SDS-PAGE (8% gel) and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were probed with either an anti-phospho-AMPKα (Thr172: Cell Signaling Technology, Beverly, MA), an anti-AMPKα (Cell Signaling Technology), an anti-PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-β-actin (Sigma Aldrich) antibody. Subsequently, membranes were exposed to a horseradish peroxidase-coupled secondary antibody, either an anti-mouse (Jackson Immune Research, West Grove, PA) or an anti-rabbit (Jackson immune research) antibody, and then developed using a chemiluminescence-based detection system (Amersham ECL, GE Healthcare, Pittsburgh, PA).

Real-time quantitative PCR. Total RNA was extracted from C2C12 myotubes or mouse skeletal muscle using ISOGEN (Nippon gene, Tokyo, Japan) according to the manufacturer’s instructions (25). RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time quantitative PCR (Taqman probe and SYBR green) analysis was performed on StepOnePlus Real-Time PCR Systems (Applied Biosystems). The PCR primers used for measuring mRNA were as follows: for PPARα, 5′-CTCGCGTGTGATAAAGC -3′ and 5′- CGATGCTGTCTCTCTTG -3′; PPARδ, 5′-GCCTCGGGCTTCCACTAC -3′ and 5′- AGATCCGATCGCACTTCTCA -3′; PPARγ1, 5′-GGACTGTGTGACAGACAAGATTTG -3′ and 5′- CTGAATATCAGTGGTTCACCGC -3′; PPARγ2, 5′- CTCTGTTTTATGCTGTTATGGGTGA -3′ and 5′- GGTCAACAGGAGAATCTCCCAG -3′; PGC-1α, 5′- TTCTGGGTGGATTGAAGTGGTG -3′ and 5′- TGTCAGTGCATCAAATGAGGGC -3′; LPL, 5′- CTCTGGATTTACACGGAGGT -3′ and 5′- ATGGCATTTCACAAACACTG -3′; CD36, 5′- CTTCCACATTTCTACATGCAA -3′ and 5′- ATCCAGTTATGGTTCCACATC -3′; UCP-3, 5′- GAGTCAGGGGCTTGTTGAAA -3′.
-3’ and 5’- GCCGTCATGTATCGGGTCTT -3’; *MyoD*, 5’- GCTTCTATCGCCGCCACTCC -3’; *myogenin*, 5’- GCATGTAAGGTGTTGTAAGAG
-3’ and 5’- GCCAGGATCTCCACTTTAG -3’; *myosin heavy chain (MyHC)*, 5’-

TCCAAACCGTCTCTGCACCTGGTT -3’ and 5’- AGCGTACAAAGTGTGGGTGTGT -3’.
mRNA expression levels were normalized to 18S ribosomal RNA levels (TaqMan ID: Mm03928990_g1, Applied Biosystems). There were no significant differences in 18S ribosomal RNA levels between treatment conditions.

Mice. All animal experiments were performed in accordance with the guidelines of the Animal Usage Committee of the University of Tokyo or Nara Women’s University. Mice were housed with a 12:12-h light-dark cycle and given free access to water and food.

Treadmill exercise training. Male C57BL/6 mice (6-week-old) were divided into an exercise group and a control group (n = 4/group). Mice were acclimated to training on a treadmill that was inclined at 10° (15 m/min for 30 min) for 4 weeks (5 times/week). The mice were anesthetized and sacrificed 18 h after the last training, and whole gastrocnemius muscle was rapidly excised, frozen in liquid nitrogen, and stored at -80°C.

AICAR administration. Male C57B/6J mice (8-week-old) were randomly divided into vehicle (saline) and AICAR (10 mg/ml in saline) treatment groups. The mice were treated for 3 days with either vehicle or AICAR (400mg/kg/day, i.p.). Gastrocnemius muscle was isolated 6 h after the last injection, frozen, and stored at -80°C until analyzed.

Expression plasmid construction. A pCSII-EF-3Flag-PPARγ1 lentiviral plasmid was constructed by inserting a fragment encoding for 3Flag-tagged mouse PPARγ1 into pCSII-EF-MCS-IRES2-Venus (RIKEN, Saitama, Japan). Lentiviral plasmids for shRNA for mouse PPARγ or control were constructed by recombining pCS-RfA-EG (RIKEN) with pENTR4-H1 (RIKEN) inserted by oligonucleotide DNA for shRNA expression. The target sequences were as follows: *PPARγ*, 5’- AAAAAGTGCAAGAGATCACAGAGTAT -3’ (14), and control (Scramble II Duplex from Dharmacon, Lafayette, CO), 5’-

GCGCGCTTTGTAGATTGTCG-3’.

Lentivirus infection. HEK293T cells were transfected with a lentiviral expression plasmid
together with a VSV-G and Rev-expressing (pCMV-VSV-G-RSV-Rev) and packaging plasmid (pCAG-HIVgp). After 12 h, the cells were further cultured in fresh medium containing 10µM forskolin. The medium containing lentiviruses was collected and filtered. C2C12 cells were infected with a lentivirus medium supplemented with 10 µg/ml of polybrene for 24 h. The cells were then replenished with fresh culture medium.

**Drugs.** AICAR, H$_2$O$_2$, and GW9662 were purchased from Wako Pure Chemical, Osaka, Japan. Metformin and Compound C were obtained from Sigma Aldrich and Calbiochem (La Jolla, CA) respectively.

**RESULTS**

*Four weeks of exercise or AICAR treatment increased mRNA levels of energy metabolism related genes in mouse skeletal muscle.* To determine whether habitual exercise training affected the expression of genes known to be regulated by metabolic changes in skeletal muscle, gastrocnemius muscle was obtained from male C57BL/6 mice that had been acclimated to training on a treadmill inclined at 10° (15 m/min for 30 min) for 4 weeks. Real-time RT-PCR analysis showed that the mRNA levels of energy metabolism related genes, including PGC-1α, LPL, and UCP-3, were significantly increased in the trained mice as compared with those in sedentary mice (Fig. 1A). There was also an increasing trend of CD36 gene expression (P<0.1). Increased gene expression of the PPAR subtype, PPARγ1, but not PPARα or δ, was found after this training.

Because AMPK in skeletal muscle is activated by exercise, we hypothesized that these responses were partly caused by AMPK activation in response to habitual exercise. Thus, we treated mice by intraperitoneally administering the AMPK activator AICAR (400 mg/kg/day) for 3.5 days. Real-time RT-PCR analysis showed that the mRNA levels of three genes, those for PPARγ1, PGC-1α, and LPL, in gastrocnemius muscle were significantly increased as was observed in exercise-trained mice (Fig. 1B). CD36 gene expression was significantly increased, whereas the UCP3 mRNA level was only slightly increased in AICAR-treated mice. These results suggested that AMPK activation accounted, at least in part, for the exercise-mediated alterations in the expression of a subset of genes in skeletal muscle, particularly that for PPARγ1. However, we cannot rule out the possibility that AICAR affected muscle gene expression through its pharmacological actions other than AMPK activation (2, 31).
Activated AMPK induces PPARγ1 gene expression in C2C12 myotubes. To more directly examine this connection, differentiated C2C12 myotubes were cultured with the AMPK activator AICAR and changes in gene expression in these cells were analyzed. First, C2C12 cells were cultured with various concentrations of AICAR for 12 h and their whole cell lysates were subjected to immunoblot (Fig. 2A, left panel). Both the phosphorylated AMPK and PPARγ1 protein levels increased in the presence of more than 0.25 mM AICAR. In the following experiments cells were cultured with 1 mM AICAR to substantially activate AMPK. AMPK phosphorylation was observed within 3 h after AICAR treatment and for an additional 9 h (Fig. 2A, right panel). In response to AICAR treatment, the PPARγ1 protein level increased concurrently with the appearance of phosphorylated AMPK. Because C2C12 myotubes expressed low levels of PPARγ2 protein, its bands were barely visible in this immunoblot membrane despite its increased mRNA level in response to AICAR (data not shown). The mRNA level of PPARγ1, but not those of PPARα and δ, increased in the presence of AICAR in a time-dependent manner and concurrently with its increased protein level (Fig. 2B). By comparison, the PGC-1α mRNA level increased slowly for more than 9 h after AICAR treatment, which was consistent with previous findings (9). The effect of AICAR to increase the PPARγ1 mRNA levels was abolished after adding an AMPK inhibitor, compound C (Fig. 2C). These results indicated that AMPK activation was involved in increased PPARγ1 expression in myotubes.

Physical exercise induced increased PPARγ expression in human skeletal muscle through increased reactive oxygen species (ROS) production and this effect was blocked by antioxidant supplementation (21). Because AMPK was activated by increased ROS levels in H₂O₂-treated cells (4), we cultured C2C12 myotube cells in the presence of H₂O₂ for 12 h and changes in AMPK phosphorylation and PPARγ1 protein levels were analyzed in the presence or absence of compound C (Fig. 2D). At the same time, to further confirm the finding that AMPK activation was involved in increased PPARγ1 expression, myotubes were cultured with another AMPK activator, metformin (2 mM), in the presence or absence of compound C. AICAR treatment increased both phosphorylated AMPK and PPARγ1 protein levels, while such increases were abolished by the addition of compound C (lanes 3 and 4). Similarly, both metformin and H₂O₂ increased AMPK phosphorylation and PPARγ1 protein levels only when culture media did not contain compound C (lanes 5 to 7). Taken together, these results imply that physical exercise induces increased PPARγ expression in skeletal muscle by the actions of AMPK via not only decreased ATP levels but also increased ROS levels.
PARγ1 mRNA is stabilized in C2C12 myotubes by AICAR. To determine the mechanism by which AICAR induced increased PPARγ1 gene expression, we performed luciferase assays using a reporter gene that contained the 5’ upstream promoter region (2.0 kb) of the mouse PPARγ1 gene. This promoter was not activated by AICAR (data not shown). Thus, we next investigated the stability of PPARγ1 mRNA in C2C12 myotubes in the presence of a transcription inhibitor, actinomycin D. PPARγ1 mRNA was degraded with a half-life of approximately 4 h, and AICAR prolonged this half-life up to 12 h (Fig. 3A). When C2C12 myotubes were cultured with AICAR and/or compound C for 9 h, the increased PPARγ1 mRNA level in the presence of AICAR was notably reduced by treatment with compound C; this suggested that AMPK activation was involved in stabilizing PPARγ1 mRNA (Fig. 3B).

PPARγ1 overexpression dramatically increases LPL gene expression in C2C12 myotubes. Although PPARδ has been shown to play a critical role in the transcriptional regulation of skeletal muscle metabolism (5), the precise role of another family member, PPARγ1, in skeletal muscle remains unclear. To analyze the function of increased PPARγ1 induced in response to AMPK activation, C2C12 myoblasts were infected with a Flag-tagged PPARγ1 expression lentivirus and then allowed to differentiate into myotubes (Fig. 4A). Real-time RT-PCR analysis showed that the LPL mRNA level was dramatically increased by PPARγ1 overexpression (>40-fold) without any changes in the mRNA levels of differentiation marker genes, including myoD, myogenin, and myosin heavy chain (Fig. 4B). CD36 gene expression was also upregulated by PPARγ1 overexpression, whereas UCP-3 and PGC-1α mRNA levels remained unaffected. These results indicated that increased PPARγ1 expression induced by exercise or AICAR treatment (Figs. 1 and 2) was associated with the transcriptional regulation of skeletal muscle metabolism, particularly LPL gene upregulation.

When C2C12 myotube cells were cultured in the presence of AICAR for 36 h, the PPARγ1 mRNA and protein levels reached a peak between 12 and 24 h, whereas LPL and CD36 mRNA levels significantly increased only at 36 h after AICAR treatment (Fig. 4C). This time lag suggested that LPL and CD36 may be downstream targets of PPARγ1. In addition, increased PGC-1α induced by AICAR may have co-activated PPARγ1, which subsequently induced UCP-3 gene expression at 24 and 36 h despite no increase in UCP-3 mRNA by PPARγ1 overexpression alone (Fig. 4A).
**PPARγ1 knockdown abolishes AICAR-induced changes in gene expression in C2C12 myotubes.**

To determine the relationship between AICAR-mediated AMPK activation and PPARγ1 function, PPARγ1 expression in C2C12 myotubes was suppressed using a lentivirus to express shRNA against PPARγ1, after which AICAR effects were assessed. The increase in PPARγ1 protein expression in response to AICAR treatment for 12 h was abolished by shRNA against PPARγ1 (Fig. 5A). PPARγ1 knockdown slightly stimulated phosphorylation of AMPK in the presence or absence of AICAR (Fig. 5A) through an as yet uncharacterized mechanism without any effects on gene expression of differentiation markers (Fig. 5B). PPARγ1 knockdown significantly reduced LPL mRNA levels and abolished the increased LPL mRNA level induced by AICAR (Fig. 5C). This suggested that PPARγ1 largely regulated LPL gene expression in myotubes and was strongly associated with the effects of AICAR on LPL gene expression. A similar pattern was observed for UCP-3 gene expression regulation. Although, PPARγ1 knockdown only partially reduced UCP-3 mRNA levels in the presence or absence of AICAR. In addition, CD36 and PGC-1α mRNA levels were not affected by PPARγ1 knockdown at the specific time point of 36 h after AICAR treatment. Additionally, when endogenous PPARγ was inhibited by a PPARγ antagonist, GW9662, a similar pattern of the expression of LPL, CD36, and UCP-3 gene was observed (Fig. 5D).

**DISCUSSION**

The PPARγ isoform, PPARγ2, is expressed at high levels in adipose tissues and plays a critical role as a master regulator of adipocyte differentiation (26). A shorter isoform, PPARγ1, which lacks the 30 amino acid residues at the amino terminus of PPARγ2, is primarily distributed in the muscle, heart, and liver. Because PPARγ expression in muscle is only 5%-10% of its expression in adipose tissue, its physiological roles in muscle has been underestimated. However, a previous report provided evidence for its crucial role in muscle by showing that deleting PPARγ in skeletal muscle caused severe insulin resistance in muscle and that treatment with a PPARγ agonist did not increase skeletal muscle insulin sensitivity in these animals (7). Nevertheless, how PPARγ expression is regulated in skeletal muscle remains unknown. In this report, we showed that exercise caused an increase in PPARγ1 mRNA and protein levels in muscle, which likely occurred because AMPK was activated. Consistent with a previous report (12), we failed to detect increased AMPK phosphorylation in muscle of mice 18 h after exercise or 6 h after AICAR treatment (data not shown). It might be due to the timing of muscle sampling. In contrast, in C2C12 myotubes, we found that both another AMPK activator,
metformin, and \text{H}_2\text{O}_2 \text{ increased phosphorylated AMPK and PPAR}\gamma_1 \text{ levels, and that compound}
C canceled out these effects, suggesting the importance of AMPK activation for upregulating
their expression (Fig. 2D). Moreover, AICAR treatment increased the half-life of PPAR\gamma_1
mRNA by nearly 3-fold (4 h-12 h; Fig. 3A) through AMPK activation.
Current evidence suggests that the stability of rapidly degraded mRNA is controlled through
the 3′-untranslated region (UTR) containing an AU-rich element (ARE) that consists of an
AUUUA pentamer (1). We found that five AUUU(U)A sequences are conserved in the 3′-UTR
for human, mouse, and rat PPAR\gamma_1 \text{ mRNA (data not shown). One ARE-binding protein, HuR,}
stabilizes mRNAs that contain AREs in their 3′-UTRs. HuR is phosphorylated by AMPK and
then transported to the cytoplasm to facilitate mRNA stability (14). These findings suggest that
AMPK activation contributes to increased PPAR\gamma_1 \text{ mRNA and protein levels through the}
actions of HuR. At the same time, because luciferase assays employing a limited upstream of
the PPAR\gamma_1 \text{ gene were carried out in this study, we can't rule out the possibility that PPAR}\gamma_1
mRNA synthesis was also increased by AMPK activation (11).
Another noteworthy result of the current study was that LPL gene expression in skeletal
muscle was predominantly regulated by PPAR\gamma_1. Although another PPAR\gamma isoform, PPAR\gamma2,
regulates the transcription of a subset of genes in adipocytes, including aP2, LPL, CD36, UCP-1,
and adiponectin (8, 27, 29), the target genes of PPAR\gamma in skeletal muscle remain uncertain. Our
results in this study showed that in C2C12 myotubes infected with a PPAR\gamma1 expression
lentivirus, only LPL gene expression dramatically increased (Fig. 4B). In addition, PPAR\gamma1
knockdown significantly suppressed basal LPL gene expression and blocked an
AICAR-mediated increase in LPL mRNA levels (Fig. 5C). These results provide clear evidence
for the importance of PPAR\gamma1 \text{ for regulating LPL expression in skeletal muscle and increasing}
LPL gene expression by AMPK activation. It seems likely that PPAR\gamma1 \text{ indirectly stimulated}
LPL gene expression because the increase in LPL mRNA level was observed 12-24 h after
increased PPAR\gamma1 \text{ mRNA in response to AICAR (Fig. 4C), but the precise mechanism remains}
uncertain at the moment. In contrast, with regard to UCP-3 gene expression, PPAR\gamma1 \text{ that is}
co-activated with increased PGC-1\alpha may partially contribute to an increase in its mRNA level.
This is based on our findings that UCP-3 mRNA levels increased concomitantly with increased
PPAR\gamma and PGC-1\alpha mRNA levels in the gastrocnemius muscle after physical exercise (Fig.
1A); overexpression of only PPAR\gamma1 had no effect on UCP-3 gene expression (Fig. 4B), and
PPAR\gamma1 knockdown moderately reduced the UCP-3 mRNA levels in C2C12 myotubes (Fig.
5C). Taken together, these results indicate that LPL gene expression is regulated mostly by
PPARγ1, whereas UCP-3 gene expression is increased by the combination of PPARγ1, or unknown transcription factor(s) other than PPARγ1, and PGC-1α. It is well known that exercise induces LPL gene expression in human skeletal muscle (22, 23, 24). Because LPL secreted by skeletal muscle promotes the catabolism of triglyceride-rich, atherogenic lipoproteins, LPL activation is thought to exert beneficial effects for preventing of cardiovascular diseases, type 2 diabetes, and metabolic syndrome. The importance of PPARγ1 in skeletal muscle has also been observed in muscle-specific PPARγ-null mice that exhibit severe insulin resistance in muscle (7). In addition, previous report shows that muscle specific LPL overexpression prevents diet-induced obesity (10). These data suggest the beneficial effect of AMPK-PPARγ1-LPL pathway for metabolism. In contrast, another report reveals LPL overexpression in muscle causes insulin resistance in muscle (6) and supports the proposition that only a moderate increase in LPL secreted by skeletal muscle may contribute to a modest uptake of fatty acids that are an energy source in muscle and increase the catabolism of serum triglyceride-rich lipoproteins to reduce the risk of cardiovascular diseases.

In summary, we have shown that habitual exercise induced enhanced expression of PPARγ1, PGC-1α, and LPL genes in the skeletal muscle of trained mice (Fig. 6). Increased expression of these genes was also observed in both AICAR-treated mice and C2C12 myotubes cultured with a couple of AMPK activators. The effects of these activators were blocked by treating the cells with an AMPK inhibitor, compound C, which suggested the importance of AMPK activation for enhanced PPARγ1 expression. We also found that AICAR increased the half-life of PPARγ1 mRNA by nearly 3-fold through AMPK activation. PPARγ1 overexpression in C2C12 myotubes resulted in dramatically increased LPL mRNA levels, whereas suppressing its expression blocked an AICAR-mediated increase in LPL mRNA. These results indicate that PPARγ1 is intimately involved in LPL gene expression in skeletal muscle and that exercise induces increased LPL gene expression likely due to AMPK activation. Overall, these results provide compelling evidence showing a novel critical role for PPARγ1 in response to AMPK activation caused by physical exercise for controlling the expression of a subset of genes associated with metabolic regulation in skeletal muscle.

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**GRANTS**

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**FIGURE LEGENDS**

**Fig. 1.** Effects of exercise and AICAR treatment on gene expression in gastrocnemius muscle. **A,** Exercise design was described in MATERIALS AND METHODS. After four weeks of exercise training, gastrocnemius muscle was removed 18 h after the last exercise and PPARα, PPARδ, PPARγ1, PGC-1α, LPL, CD36, and UCP3 mRNA levels were determined by quantitative RT-PCR (n = 4). Relative mRNA levels were determined after normalization to 18S ribosomal RNA levels. Relative mRNA levels in the control were set to 1. Results are means ± SD. *, P < 0.05, **, P < 0.01. Final body weights (in g); 29.0 ± 3.1 for controls and 26.8 ± 0.5 for exercised mice. **B,** Mice were intraperitoneally treated with vehicle (saline) or AICAR (200mg/kg) twice a day (at 10:00 and 20:00) for 3 days (400mg/kg/day). Whole gastrocnemius muscle was removed 6 h after the last injection (at 10:00 on the day 4), and PPARα, PPARδ, PPARγ1, PGC-1α, LPL, CD36, and UCP3 mRNA levels were determined by quantitative RT-PCR (n = 5). Relative mRNA levels were determined after normalization to 18S ribosomal RNA levels. Relative mRNA levels in the control were set to 1. Results are means ± SD. *, P < 0.05, **, P < 0.01.

**Fig. 2.** AMPK activation induces increased PPARγ1 gene expression in C2C12 myotubes. **A,** Phosphorylated (P-AMPK) or total AMPK (AMPK) and PPARγ1 (arrow) protein in AICAR (0 - 2 mM for 12 h)-treated C2C12 myotubes were detected by western blotting (*left*). Western blotting was carried out using C2C12 myotubes treated with 1 mM AICAR for the indicated period of time (*right*). A stock AICAR solution (100 mM in distilled water) was added to the medium. The mixed cell lysates (n = 3) were subjected to SDS-PAGE. **B,** PPARα, PPARδ, PPARγ1, and PGC-1α mRNA levels in AICAR (1 mM) -treated C2C12 myotubes (0 h - 15 h) were determined by quantitative RT-PCR. Relative mRNA levels were determined after normalization to 18S ribosomal RNA levels. Relative mRNA levels at time 0 were set to 1. Results are means ± SD (n = 3). **C,** After incubation with 10 mM compound C (Com. C) for 1 h, C2C12 myotubes were further incubated with or without 1 mM AICAR for 9 h. PPARγ1
mRNA were determined by quantitative RT-PCR. Relative mRNA levels in the control were set to 1. Results are means ± SD (n = 3). Statistical comparisons were made by one-way analysis of variance. Different superscript letters indicate that the means are significantly different (P < 0.01). D, C2C12 myotubes were incubated with AICAR (1 mM), metformin (2 mM), or H2O2 (300 μM) either in the presence or absence of 10 mM compound C for 12 h, as described in Fig. 2C. The mixed cell lysates (n = 3) were subjected to SDS-PAGE and proteins were detected by western blotting.

Fig. 3. AMPK stabilizes PPARγ1 mRNA and results in a longer mRNA half-life in C2C12 myotubes. A, C2C12 myotubes were pre-treated with 5 μg/ml of actinomycin D for 30 min and then further cultured with or without 1mM AICAR for 9 h. The mRNA level at time 0 was set at 100%. Results are means ± SD (n=3). **, P < 0.01 (vs. control). B, C2C12 myotubes were pre-treated with 5 μg/ml of actinomycin D for 60 min and with 10μM compound C for the last 30 min and then further cultured with 1 mM AICAR for 9 h. The mRNA level at time 0 was set at 100%. Percent remaining PPARγ1 mRNA is shown. Results are means ± SD (n = 3). Statistical comparisons were made by one-way analysis of variance. Different superscript letters indicate that the means are significantly different (P < 0.01).

Fig. 4. PPARγ1 overexpression and a longer AICAR treatment (for 36 h) induces increased LPL gene expression in C2C12 myotubes. A, C2C12 myoblasts were infected with a Flag-PPARγ1 lentivirus and allowed to differentiate into myotubes. Western blotting was performed using anti-Flag, anti-PPARγ, anti-LPL, and anti-β-actin antibodies. B, MyoD, Myogenin, MyHC, LPL, CD36, UCP3, and PGC-1α mRNA levels in mock or Flag-PPARγ1 C2C12 myotubes were determined by quantitative RT-PCR. Relative mRNA levels in mock C2C12 myotubes were set to 1. Results are means ± SD (n = 3). **, P < 0.01. C, PPARγ1, LPL, UCP-3, CD36, and PGC-1α mRNA levels in AICAR-treated C2C12 myotubes (0 to 36 h) were determined by quantitative RT-PCR. Relative mRNA levels at time 0 were set to 1. Results are means ± SD (n = 3). Statistical comparisons were made by one-way analysis of variance. Different superscript letters indicate that the means are significantly different (P < 0.01).

Fig. 5. PPARγ knockdown blocks the AMPK-induced increase in LPL mRNA. A, C2C12 myoblasts were infected with an sh control or an sh PPARγ lentivirus and allowed to differentiate into myotubes. Phosphorylated (P-AMPK) or total AMPK (AMPK) and PPARγ1
(arrow) protein in AICAR-treated or -untreated myotubes (for 12 h) were detected by western blotting. B, MyoD, Myogenin, and MyHC mRNA levels in control or PPARγ-knockdowned myotubes were determined by quantitative RT-PCR. Relative mRNA levels in control C2C12 myotubes were set to 1. Results are means ± SD (n = 3). C, C2C12 myotubes infected with an sh control or an sh PPARγ lentivirus were cultured with AICAR (1 mM) for 36 h. LPL, CD36, UCP3, and PGC-1α mRNA levels were determined by quantitative RT-PCR. Relative mRNA levels in control C2C12 myotubes without AICAR treatment were set to 1. Results are means ± SD (n = 3). Statistical analysis used two-way analysis of variance with Tukey post-hoc comparisons. *, P<0.05 **, P<0.01 compared with the baseline (within a knock down group) #, P<0.05 ##, P<0.01 compared with the sh control group (between a knock down group). D, C2C12 myotubes were incubated with AICAR (1 mM) with 512 or without GW9662 (20 µM) for 36 h. LPL, CD36, and UCP3 mRNA levels were determined by quantitative RT-PCR. Results are means ± SD (n = 3). Statistical analysis used two-way analysis of variance with Tukey post-hoc comparisons. *, P<0.05 **, P<0.01 compared with the baseline (within a DMSO or GW9662 group) #, P<0.05 ##, P<0.01 compared with a DMSO group (between a DMSO and GW9662 group).

Fig. 6. Summary and working hypothesis of the effect of exercise and AMPK activation on LPL gene expression in skeletal muscle. Exercise and AICAR treatment phosphorylate and activate AMPK in skeletal muscle. The exercise-mediated activation of AMPK is likely mediated via either the direct or the ROS-induced decrease in ATP levels. This leads to stabilization of PPARγ1 mRNA and increases PGC-1α mRNA levels. Finally, LPL gene expression is increased mostly by PPARγ1 and UCP-3 gene expression is regulated by the combination of PPARγ1 and PGC-1α.
A. AICAR

- AICAR concentration (mM)
  - 0
  - 0.25
  - 0.5
  - 1.0
  - 2.0

- Protein expression over time (hour)
  - 0
  - 3
  - 6
  - 9
  - 12

- Protein bands
  - P-AMPK
  - AMPK
  - PPARγ1
  - β-actin

- Note: non-specific band

B. Gene expression

- PPARα mRNA
  - Time course

- PPARγ1 mRNA
  - Time course

- PGC-1α mRNA
  - Time course

C. AICAR and Com.C

- AICAR
- Com.C

- Bar graphs

D. Treatment

- AIC
- Met
- H2O2
- Com.C

- Protein bands
  - P-AMPK
  - AMPK
  - PPARγ1
  - β-actin

- Note: non-specific band
A

mock PPARγ1

Flag

PPARγ1

LPL

β-actin

*: non-specific band

B

![Graph showing relative mRNA levels for different genes under mock and PPARγ1 conditions.]

C

![Bar graphs showing mRNA expression levels for PPARγ1, LPL, UCP3, PGC-1α, CD36 over different hours.]

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