Original Article

Adiponectin Increases Fatty Acid Oxidation in Skeletal Muscle Cells by Sequential Activation of AMP-Activated Protein Kinase, p38 Mitogen-Activated Protein Kinase, and Peroxisome Proliferator–Activated Receptor α

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Adiponectin has recently received a great deal of attention due to its beneficial effects on insulin resistance and metabolic disorders. One of the mechanisms through which adiponectin exerts such effects involves an increase in fatty acid oxidation in muscle and liver. In the present study, we demonstrate that 5′–AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (MAPK) are involved in the activation of peroxisome proliferator–activated receptor (PPAR)α by adiponectin in muscle cells. Adiponectin increases the transcriptional activity of PPARα and the expression of its target genes, including *ACO*, *CPT1*, and *FABP3* in C2C12 myotubes. These effects were suppressed by the overexpression of a dominant-negative form of AMPK. Moreover, chemical inhibitors of AMPK and p38 MAPK potently repressed fatty acid oxidation and the induction of PPARα target gene expression by adiponectin. Interestingly, araA, an AMPK inhibitor, prevented the activation of p38 MAPK, whereas SB203580, a p38 MAPK inhibitor, did not affect AMPK activation, suggesting that p38 MAPK is a downstream signaling factor of AMPK. Taken together, these results suggest that adiponectin stimulates fatty acid oxidation in muscle cells by the sequential activation of AMPK, p38 MAPK, and PPARα. *Diabetes* 55:2562–2570, 2006

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ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; AICAR, 5-aminoimidazole-4-caroxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; CPT1, carnitine palmitoyl transferase 1; DMEM, Dulbecco's modified Eagle's medium; HMW, high molecular weight; LMW, low molecular weight; MAPK, mitogen-activated protein kinase; PPAR, peroxisome proliferator–activated receptor.

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Adiponectin (also known as Acrp30, AdipoQ, and GBP28), an adipocytokine secreted by adipocytes, has been receiving a great deal of attention due to its insulin-sensitizing effects and possible therapeutic use for metabolic disorders (1–6). Accumulating evidence has suggested that a certain level of serum adiponectin is required to maintain energy homeostasis and prevent metabolic diseases. For example, serum levels of adiponectin are lowered in subjects with obesity and type 2 diabetes (7,8), and administration of adiponectin enhances insulin sensitivity by decreasing the levels of plasma free fatty acid and triglyceride (9,10). In addition, adiponectin was shown to inhibit atherosclerotic lesion formation in apo-E–deficient mice (11,12). These beneficial effects of adiponectin have been attributed to, among other things, its ability to increase fatty acid oxidation in peripheral tissues (10).

Stimulation of fatty acid oxidation by adiponectin appears to be primarily mediated by the activation of 5′–AMP-activated protein kinase (AMPK). Treatment with adiponectin or ectopic expression of its receptors has been shown to increase AMPK phosphorylation and fatty acid oxidation in muscles, and this effect was abolished by the use of dominant-negative AMPK (13,14). AMPK is a multi-subunit protein kinase that functions as a sensor of intracellular energy state (15). It is activated by high AMP-to-ATP ratio, which occurs under stress conditions such as heat shock, hypoxia, starvation, and exercise. In addition, AMPK is also directly activated by one or more of the upstream kinases via phosphorylation of a threonine residue within the activation loop of the α-subunit kinase domain (15). In skeletal muscle cells, it is well established that AMPK increases fatty acid oxidation by inhibiting acetyl-CoA carboxylase (ACC) through phosphorylation. A decrease in ACC activity reduces intracellular malonyl-CoA levels and stimulates carnitine palmitoyl transferase 1 (CPT1). This ultimately increases the influx of long-chain fatty acids into the mitochondria, where they are oxidized (16,17).

Another key regulator of fatty acid metabolism is peroxisome proliferator–activated receptor (PPAR)α. PPARα is a ligand-activated nuclear hormone receptor that is highly expressed in tissues that derive most of their energy from fatty acid oxidation, including liver, heart, kidney, and skeletal muscle (18). PPARα controls the expression

of a number of genes involved in peroxisomal (19,20) and mitochondrial β-oxidation (21,22), such *ACO*, *CPT1*, and *FABP3*. Several studies using PPARα-null mice have demonstrated the critical role of PPARα in the fasting response. When fasted, these mice suffer from defects in fatty acid oxidation and ketogenesis, resulting in elevated plasma free fatty acid levels, hypoketonemia, and hypothermia (23–25). Furthermore, recent reports have demonstrated that PPARα ligands improve insulin sensitivity and lower lipid levels in insulin-resistant rodent models such as obese Zucker rats and high-fat–fed mice (26–28).

p38 mitogen-activated protein kinase (MAPK) is activated by inflammatory cytokines and plays an important role in activating immune responses. A number of conditions that evoke metabolic stress, such as muscle contraction, hypoxia, ischemia, and hyperosmolarity, are also known to stimulate mammalian p38 MAPK activity (29). Most of these physiological conditions are similar to those associated with AMPK activation (30), suggesting a possible crosstalk between the AMPK and p38 MAPK signaling pathways.

It has recently been reported that adiponectin stimulates AMPK, p38 MAPK, and PPARα activity in target tissues (14). However, the detailed downstream pathway of adiponectin, which comprises these three components, has not been thoroughly investigated. Here, we provide evidence that adiponectin enhances fatty acid oxidation in muscle cells by stimulating PPARα transcriptional activity via the sequential activation of AMPK and p38 MAPK.

RESEARCH DESIGN AND METHODS

Cell culture and viral infection. Induction of myogenic differentiation was carried out as previously described (13). In brief, mouse C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. To induce differentiation, confluent cells were incubated in DMEM supplemented with 2% horse serum. The cells differentiated into multinucleated myotubes in 6–7 days.

For viral infections, differentiated C2C12 myotubes were infected with either null or dominant-negative AMPK (*T172A*) (31) expressing adenovirus (300 PFU/cell). After 4 h, the medium was replaced with fresh medium containing 2% horse serum.

Purification of adiponectin from HEK293 cell cultures. Recombinant adiponectin protein was purified using a modified version of a previously described protocol (32). In brief, HEK293 cells were transfected with plasmid vectors expressing myc-His–tagged adiponectin (pcDNA3.1-adiponectin-myc-His). After transfection, the cells were incubated in serum-free DMEM with 0.1 g/l L-ascorbic acid for 48 h. The medium was then collected and centrifuged at 3,000 rpm for 10 min to remove cellular debris. After adding saturated ammonium sulfate to 40% (wt/vol) and incubating at 4°C for 4 h with shaking, the protein fraction was precipitated by centrifugation at 3,000 rpm for 3 h at 4°C. The precipitate was resuspended in 10 mmol/l HEPES (pH 8.0) buffer containing 50 mmol/l NaCl and 1 mmol/l $CaCl_2$ and bound to Ni-NTA agarose beads. The beads were washed twice with washing buffer (50 mmol/l NaH_2PO_4, 300 mmol/l NaCl, and 20 mmol/l imidazole), and adiponectin was collected with elution buffer (50 mmol/l NaH_2PO_4, 300 mmol/l NaCl, and 250 mmol/l imidazole). The pH of the washing and elution buffers was adjusted to 8.0 with NaOH before use. Only batches containing adiponectin >95% purity, which was determined by Coomassie staining, were used for subsequent experiments.

Transient transfection and luciferase assay. HEK293 cells were grown in DMEM containing 10% bovine calf serum. Luciferase reporter constructs were cotransfected with PPARα, retinoid X receptor α, and/or adiponectin expression vectors by using the calcium phosphate method. After transfection, the cells were serum-starved for 18 h and incubated with 10 μmol/l Wy14643 (Biomol), 1 mmol/l araA (Sigma), 5 μmol/l SB203580 (A.G. Scientific), or vehicle (DMSO) for an additional 24 h before lysis. The luciferase activity was normalized to β-gal activity.

Protein analysis. Cells were lysed in 1× radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% sodium deoxycholate, and 140 mmol/l NaCl). The lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Following transfer, the membranes were blocked with milk and probed with primary antibodies against phospho-AMPK (Cell Signaling), AMPK (Upstate), phospho-p38 MAPK (BD Transduction Laboratories), p38 MAPK (Santa Cruz), phospho-ACC (Upstate), and β-actin (Sigma). The results were visualized using horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) and enhanced chemiluminescence.

AMPK in vitro kinase assay. AMPK activity assays were carried out as previously described (33). In brief, total cell extracts were prepared from differentiated C2C12 myotubes in 1× radioimmunoprecipitation assay buffer and precipitated with saturated ammonium sulfate solution (final concentration 35%). Assays were performed at 30°C for 10 min in 25 μl reaction mixtures containing 5 μg protein extracts in a reaction buffer (40 mmol/l HEPES [pH 7.0], 80 mmol/l NaCl, 5 mmol/l magnesium acetate, 1 mmol/l dithiothreitol, 200 μmol/l each of AMP and ATP, and 2 μCi [γ-^{32}P] ATP) with or without 200 μmol/l SAMS peptide (Upstate). For immunoprecipitated kinase assays, cell lysates were immunoprecipitated with anti–AMPK-α1 or -α2 antibodies (Upstate), washed with 40 mmol/l HEPES (pH 7.0), and suspended in 20 μl reaction buffer. The reaction mixtures were spotted onto P81 cation exchange papers, washed three times with 1% phosphoric acid, and measured using a scintillation counter. AMPK activity was expressed as [^{32}P] incorporated per microgram of protein.

p38 MAPK in vitro kinase assay. Total cell extract (500 μg) was immunoprecipitated with anti-p38 MAPK antibodies and washed with 20 mmol/l HEPES (pH 7.4). Kinase assays were performed for 30 min at 30°C using 2 μg glutathione S-transferase–activating transcription factor 2 (GST-ATF2) as a substrate in the reaction buffer (20 mmol/l HEPES [pH 7.4], 10 mmol/l $MgCl_2$, 12 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 2 mmol/l dithiothreitol, 0.5 mmol/l phenylmethylsulfonyl fluoride, and 1 μCi [γ-^{32}P] ATP). The reactions were terminated using 5× SDS sample buffer, and the products were resolved by SDS-PAGE followed by transfer to polyvinylidene difluoride membranes. The level of incorporated [^{32}P] was detected by autoradiography, and the membranes were immunoblotted using anti-p38 MAPK antibodies as loading control.

Quantitative RT-PCR analysis. Total RNA was isolated with Trizol (Invitrogen) according to the manufacturer's protocol. Total RNA, 5 μg, was reverse transcribed with oligo dT using RevertAid M-MuLV Reverse Transcriptase (MBI Fermentas, ON, Canada). cDNA levels were quantified by real-time PCR with SYBR Green using the MyiQ single-color real-time PCR detection system (Bio-Rad). The primer sequences used were as follows: acyl-CoA oxidase (ACO) forward, 5′-TGT TAA GAA GAG TGC CAC CAT-3′ and ACO reverse, 5′-ATC CAT CTC TTC ATA ACC AAA TTT-3′; CPT1 forward, 5′-ACT CCT GGA AGA AGA AGT TCA-3′ and CPT1 reverse, 5′-AGT ATC TTT GAC AGC TGG GAC-3′; FABP3 forward, 5′-CCC CTC AGC TCA GCA CCA T-3′ and FABP3 reverse, 5′-CAG AAA AAT CCC AAC CCA AGA AT-3′; and GAPDH forward, 5′-CAG AAC TAC ATC CCT GCA TCC-3′ and GAPDH reverse, 5′-CCA CCT TCC TGA TGT CAT CA-3′.

Fatty acid oxidation assay. Fatty acid oxidation assays were carried out as previously described (34). In brief, differentiated C2C12 myotubes were incubated in α–minimum essential medium containing 0.1 mmol/l palmitate (9,10-[^{3}H]palmitate, 5 μCi/ml) and 2% BSA. After incubation, the medium was recovered and precipitated twice with an equal volume of 10% trichloroacetic acid. The supernatants were transferred to open 1.5-ml microcentrifuge tubes placed in a scintillation vial containing 0.5 ml unlabeled water and incubated at 50°C for 18 h. Following this vapor-phase equilibration step, the tubes were removed and scintillation enhancer fluid (PerkinElmer Life and Analytical Sciences) added to the vial. ^3H_2O content was measured in a scintillation counter.

RESULTS

Adiponectin stimulates PPARα transcriptional activity and target gene expression. One of the most profound characteristics of adiponectin is its ability to stimulate fatty acid oxidation in peripheral tissues (6). To understand how adiponectin achieves this, we examined the changes in the expression level of genes involved in fatty acid oxidation. When differentiated C2C12 cells were treated with purified adiponectin, the mRNA levels of *ACO* and *CPT1* were increased in a dose-dependent manner (Fig. 1*A*). Because these genes are known to be transcriptionally regulated by PPARα, we then performed reporter assays to determine whether PPARα transcription activity can be regulated by adiponectin. As shown in Fig. 1*B*, cotransfection with adiponectin increased the transcriptional activity of PPARα on a reporter construct driven by multiple PPAR response elements (DR-1). This stimulatory

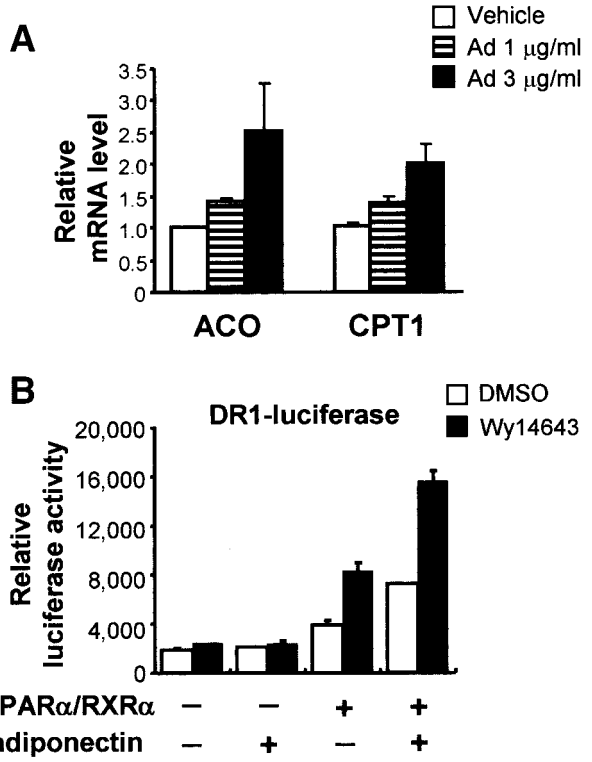

FIG. 1. Adiponectin increases PPARα transcriptional activity and target gene expression. *A*: The relative mRNA levels of PPARα target genes in differentiated C2C12 myotubes were measured 3 h after treatment with adiponectin. The amount of mRNA was normalized to *GAPDH*. Each experiment was independently performed at least three times. Ad, adiponectin. *B*: Relative luciferase activities measured from HEK293 cells transfected as indicated. RXRα, retinoid X receptor α.

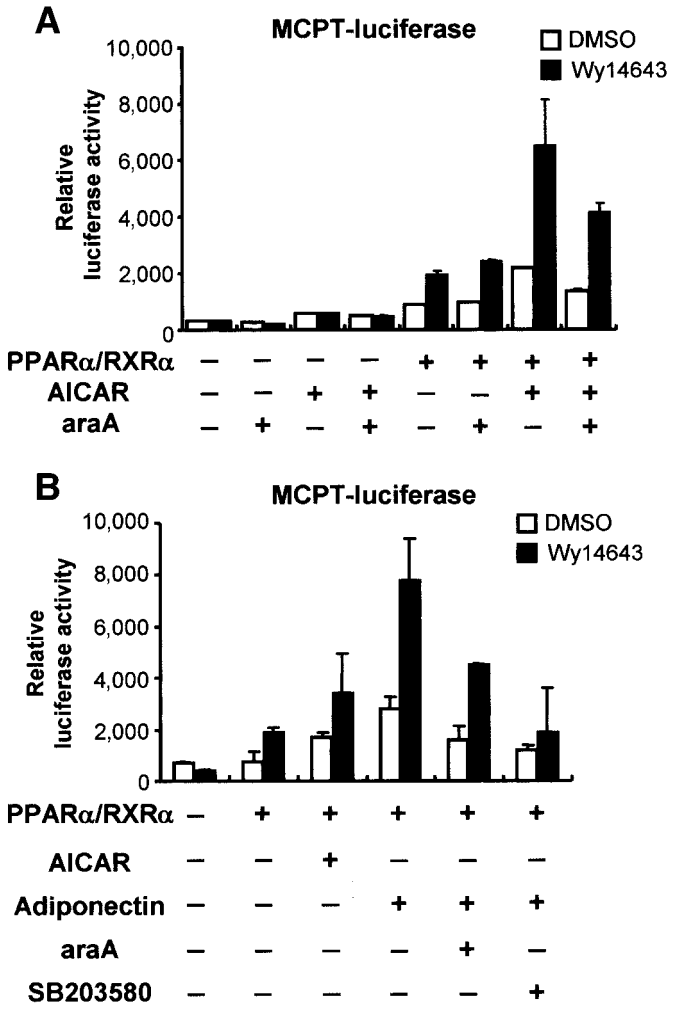

FIG. 2. Adiponectin increases PPARα transcriptional activity via AMPK and p38 MAPK. *A* and *B*: Luciferase assays were performed with HEK293 transfected and treated with the indicated expression vectors and compounds. See RESEARCH DESIGN AND METHODS for details. RXRα, retinoid X receptor α.

effect was observed both in the presence and absence of Wy14643, a synthetic ligand of PPARα, indicating that regulation of PPARα by adiponectin appeared to be ligand independent. This result is consistent with a previous report in which adiponectin was shown to enhance the activation of UAS-luciferase reporter by GAL4-PPARα fusion protein (13,14). These results imply that the stimulation of fatty acid oxidation by adiponectin in muscle cells could occur through the activation of PPARα.

AMPK and p38 MAPK are involved in the activation of PPARα by adiponectin. Since AMPK is a well-established mediator of adiponectin action, we tested whether direct activation of AMPK could similarly stimulate PPARα transcription activity. 5-Aminoimidazole-4-caroxamide-1-β-D-ribofuranoside (AICAR), which activates AMPK after being metabolized to 5-aminoimidazole-4-caroxamide-1-β-D-ribofuranoside-5-monophosphate in the cells, also increased the transcriptional activity of PPARα at the promoter of muscle-type carnitine palmitoyl transferase (Fig. 2*A*). Cotreatment with araA, an inhibitor of AMPK, potently inhibited this effect, confirming that the stimulation of PPARα activity by AICAR was the result of AMPK activation (Fig. 2*A*). Furthermore, araA also reduced the stimulation of PPARα activity by adiponectin, demonstrating that AMPK is indeed involved in the activation of PPARα by adiponectin (Fig. 2*B*). Since it was recently reported that adiponectin activates p38 MAPK in addition to AMPK in C2C12 cells (14), we tested whether p38 MAPK might also be involved in this pathway. As shown in Fig. 2*B*, inhibition of p38 MAPK using SB203580 also significantly inhibited the effect of adiponectin on PPARα activity.

To confirm the roles of AMPK and p38 MAPK in the activation of PPARα, we examined the effects of araA and SB203580 on the mRNA levels of PPARα target genes in C2C12 myotubes. Consistent with the results shown in Fig. 1*A*, adiponectin increased the expression levels of *ACO*, *CPT1*, and *FABP3* (Fig. 3*A*). However, pretreatment of cells with araA or SB203580 abolished the adiponectin-dependent increase in PPARα target gene expression. Furthermore, dominant-negative AMPK similarly repressed the stimulation of PPARα target gene expression by adiponectin (Fig. 3*B*). Together, these data indicate that the activation of PPARα by adiponectin occurs through a pathway that requires activation of both AMPK and p38 MAPK.

AMPK stimulates PPARα-dependent fatty acid oxidation. The results from the reporter assays and gene expression analyses suggested that AMPK might be able to stimulate fatty acid oxidation by activating PPARα in muscle cells. To test this possibility, we performed in vitro fatty acid oxidation assays in C2C12 cells using radioisotope-labeled palmitate. As expected, fatty acid oxidation was enhanced by the activation of PPARα or AMPK using Wy14643 or AICAR, respectively (Fig. 4*A*, *lanes 2* and *3*).

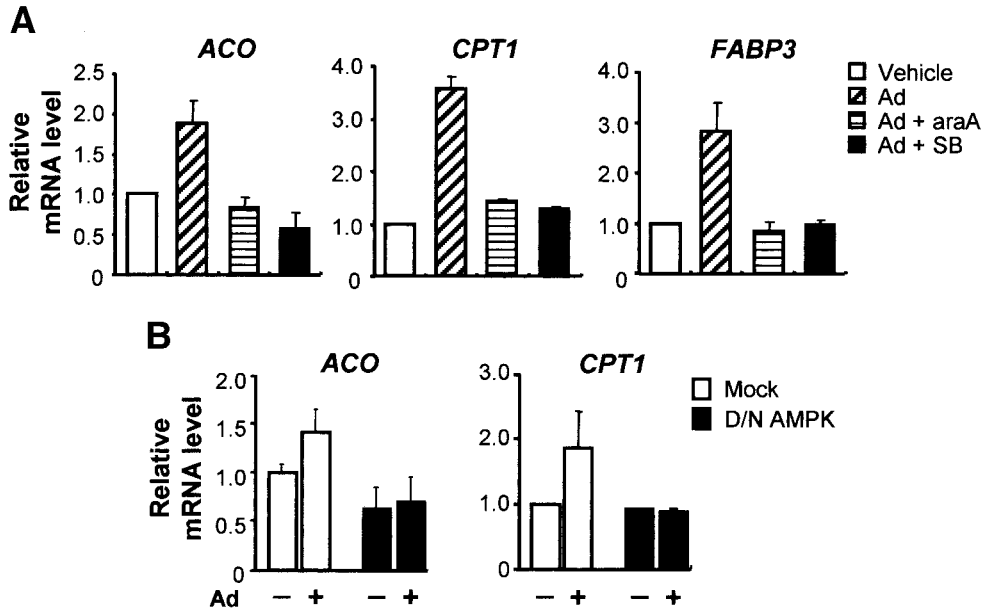

FIG. 3. Stimulation of PPARα target gene expression by adiponectin is repressed by inhibition of AMPK and p38 MAPK. The relative mRNA levels of PPARα target genes in differentiated C2C12 myotubes were measured 3 h after treatment with adiponectin (2 μg/ml). Before adiponectin treatment, the cells were either preincubated with araA (1 mmol/l) for 1 h or SB203580 (10 μmol/l) for 20 min (*A*) or infected with dominant-negative (D/N) AMPK–expressing adenovirus (*B*). The amount of mRNA was normalized to *GAPDH*.

Notably, we observed that cotreatment of Wy14643 and AICAR further elevated fatty acid oxidation (Fig. 4*A*, *lane 4*).

Thus far, stimulation of fatty acid oxidation by AMPK was believed to be primarily mediated by phosphorylation of ACC (35). The ACC-dependent regulation of fatty acid oxidation does not require synthesis of new proteins, while PPARα exerts its effect by increasing the expression of its target genes. We took advantage of this difference to determine whether AMPK can indeed stimulate fatty acid oxidation by activating PPARα. When cells were treated with cycloheximide to block protein synthesis, AICAR-induced fatty acid oxidation was reduced by ~30% (Fig. 4*B*). This reduction can probably be attributed to the inhibition of effect of PPARα activity, which would act downstream of AMPK to induce the expression of genes involved in fatty acid oxidation.

The roles of AMPK and p38 MAPK in the stimulation of fatty acid oxidation by adiponectin were also examined using fatty acid oxidation assays. As previously reported (10), adiponectin increased fatty acid oxidation by ~40% (Fig. 4*C*). This stimulation was suppressed by araA or SB203580 (Fig. 4*C*, *lanes 3* and *4*), and cotreatment with

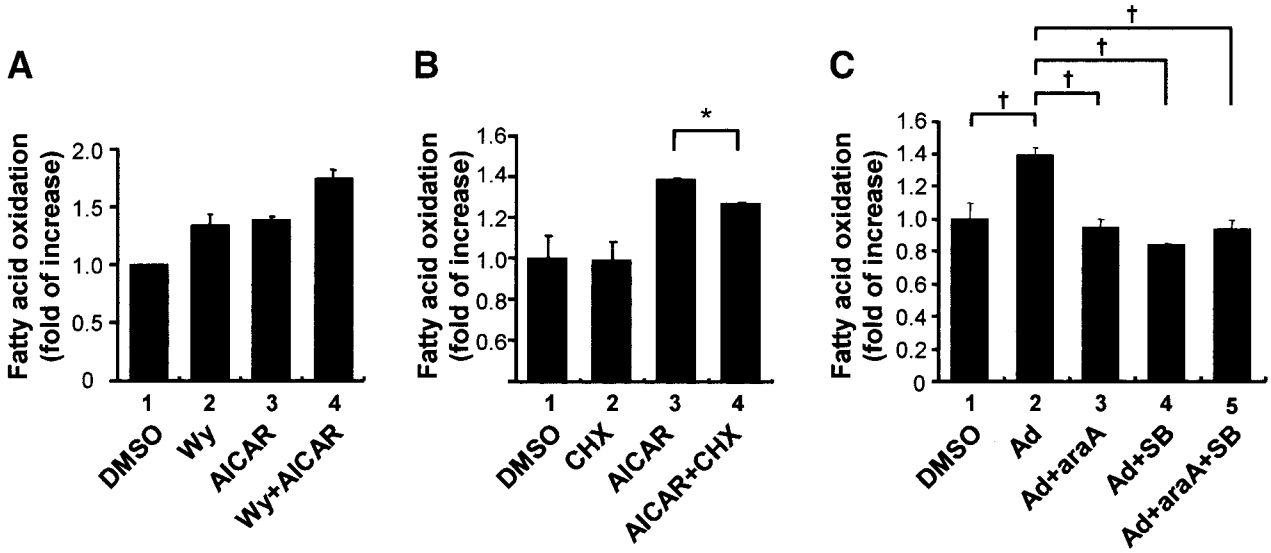

FIG. 4. Adiponectin increases fatty acid oxidation via AMPK and p38 MAPK. Fatty acid oxidation was measured using ^{3}H-labeled palmitate in differentiated C2C12 myotubes as described in RESEARCH DESIGN AND METHODS. *A*: Cells were incubated in oxidation media containing ^{3}H-labeled palmitate with 1 mmol/l AICAR, 10 μmol/l Wy14643, or vehicle (DMSO) for 24 h. Wy, Wy14643. *B*: Cells were preincubated in the presence or absence of cycloheximide (CHX; 20 μmol/l) for 1 h before adding AICAR (1 mmol/l), Wy14643 (10 μmol/l), or DMSO. *C*: Fatty acid oxidation was measured after stimulating with 2 μg/ml adiponectin for 24 h. AraA and/or SB203580 was pretreated 1 h before adiponectin. *$P < 0.01$, †$P < 0.05$. Ad, adiponectin; SB, SB203580.

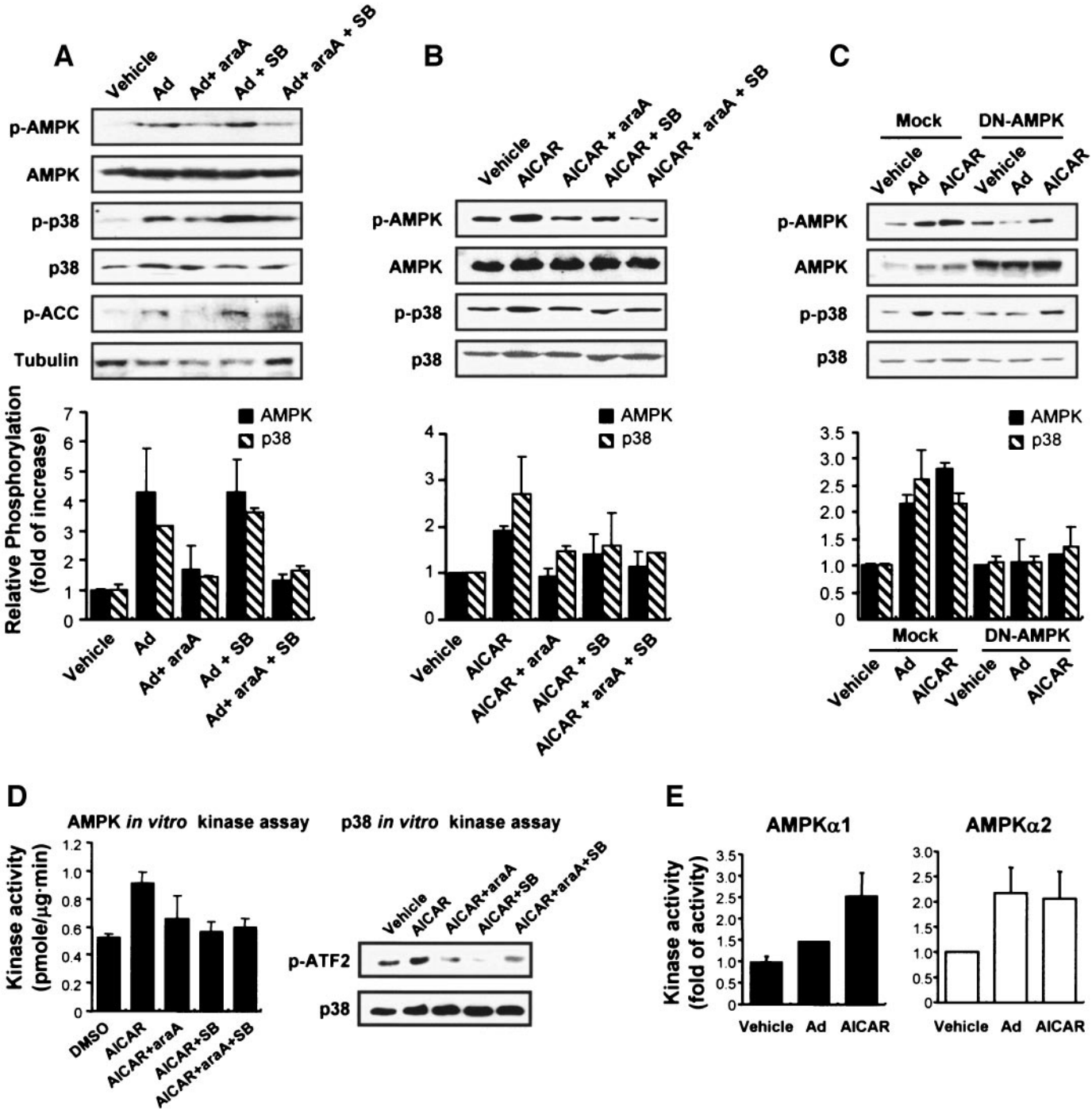

FIG. 5. Adiponectin signaling via AMPK and p38 MAPK. *A–C*: The levels of AMPK, p38 MAPK, and/or ACC phosphorylation in C2C12 myotubes were measured after 30 min incubation with adiponectin (5 μg/ml) or AICAR (2 mmol/l). Cells were pretreated with 2 mmol/l araA (1 h), 10 μmol/l SB203580 (20 min), or adeno-D/N AMPK before the addition of adiponectin or AICAR. *D*: In vitro AMPK and p38 MAPK kinase assays were performed using differentiated C2C12 myotubes under conditions identical with Fig. 5*B*. The level of p38 MAPK was determined as loading control. *E*: The kinase activity of the α1 and α2 subunits of AMPK from C2C12 cells were measured by IP-kinase assays with or without adiponectin or AICAR. Ad, adiponectin; SB, SB203580.

both araA and SB203580 did not result in additive or synergistic effects (Fig. 4*C*, *lane 5*). Taken together, these data reveal the presence of a pathway through which adiponectin stimulates fatty acid oxidation by activating PPARα.

p38 MAPK is phosphorylated and activated by AMPK in adiponectin signaling. To get a clearer picture of the adiponectin signaling pathway, we examined the changes in the level of AMPK and p38 MAPK phosphorylation induced by adiponectin. Consistent with previous reports (14,34), adiponectin enhanced the phosphorylation of AMPK, p38 MAPK, and ACC in C2C12 myotubes (Fig. 5*A*). However, when AMPK was chemically inhibited with araA, the stimulation of AMPK, p38 MAPK, and ACC phosphorylation was attenuated. On the other hand, inhibition of p38 MAPK with SB203580 did not affect adiponectin-induced phosphorylation of AMPK and ACC. Similar results were obtained when AMPK was directly activated

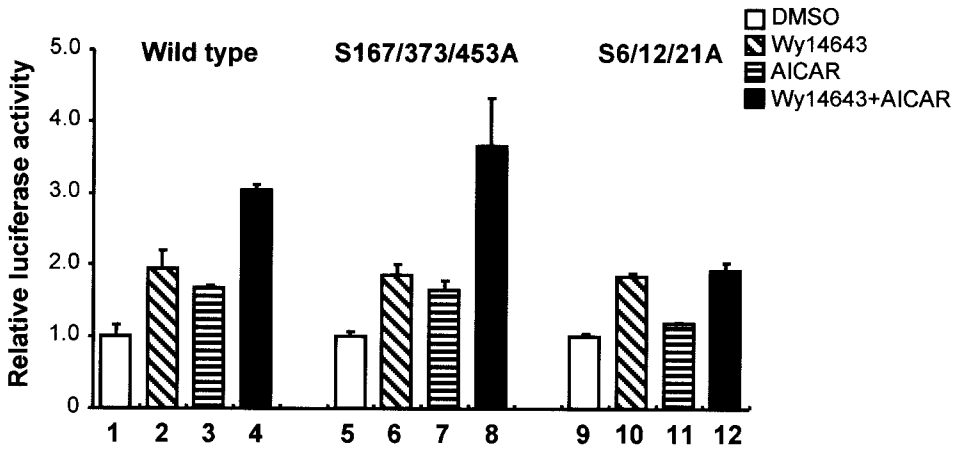

FIG. 6. PPARα S6/12/21A mutant is not activated by AMPK. Luciferase assays were carried out using the wild-type and two mutant forms (S167/373/453A and S6/12/21A) of PPARα. The results are presented as the fold of activation caused by treatment of Wy14643 or AICAR compared with vehicle for each type of PPARa.

using AICAR (Fig. 5*B* and *D*). AICAR increased the phosphorylation and kinase activities of AMPK and p38 MAPK, and these effects were inhibited by araA. Likewise, when AMPK was blocked using dominant-negative AMPK, both adiponectin and AICAR did not induce p38 MAPK phosphorylation (Fig. 5*C*). Conversely, insulin, which activates p38 MAPK in muscle cells, did not affect AMPK phosphorylation or kinase activity (online appendix Fig. 1*A* and *C* [available at http://diabetes.diabetesjournals.org]). Therefore, these results indicate that p38 MAPK might function as a downstream signaling molecule of AMPK in the adiponectin signaling pathway.

In an attempt to determine which catalytic subunit of AMPK, α1 or α2, mediates adiponectin signaling in muscle cells, we performed in vitro kinase assays using antibodies specific for each isoform. The kinase activity of both α1 and α2 isoforms was increased by adiponectin treatment, the α1 isoform by ~50% and the α2 isoform by over twofold (Fig. 5*D*). Thus, it is possible to speculate that AMPKα2 might be the major mediator of adiponectin function in muscle cells, although the role of α1 cannot be ruled out.

p38 MAPK directly stimulates PPARα transcription activity. Previously, it has been demonstrated that the transcriptional activity of PPARα can be stimulated by p38 MAPK through the phosphorylation of three serine residues in the AF-1 domain (serines 6, 12, and 21) (36). In addition, we found three putative AMPK phosphorylation sites (serines 167, 373, and 453) in PPARα through sequence analysis. To test whether AMPK might directly modulate PPARα activity, we performed reporter assays using PPARα mutants in which these serine residues were substituted with alanine. Mutation at the p38 MAPK phosphorylation sites (S6/12/21A) resulted in the loss of stimulation of transcriptional activity by AICAR that is observed in wild-type PPARα (Fig. 6, *lanes 3* and *11*). In contrast, the S167/373/453A mutant retained its sensitivity to AICAR (Fig. 6, *lanes 3* and *7*). These results indicate that p38 MAPK, but not AMPK, is directly involved in regulating the transcriptional activity of PPARα.

Adiponectin stimulates long-term fatty acid oxidation. The regulation of fatty acid oxidation by adiponectin through PPARα would be expected to have a longer-lasting effect compared with the pathway involving ACC phosphorylation. To analyze the short- and long-term effects of adiponectin on fatty acid utilization, we compared the time-dependent changes in the phosphorylation of signaling molecules, expression of PPARα target genes, and fatty acid oxidation. As shown in Fig. 7*A*, phosphorylation of AMPK was transient, lasting for only 30 min after induction by adiponectin. Phosphorylation of p38 MAPK and ACC lasted longer and was detected at 60 min. During this short period, adiponectin would transiently induce fatty acid oxidation by phosphorylating and inhibiting ACC, which is confirmed by the fatty acid oxidation assay results (Fig. 7*D*). Meanwhile, the expression of PPARα target genes, specifically *ACO* and *CPT1*, peaked at 3 h after adiponectin treatment and slowly receded to basal levels after 24 h (Fig. 7*B*). Interestingly, the rate of fatty acid oxidation constantly remained higher in adiponectin-treated cells throughout the entire duration (Fig. 7*C* and *D*). Therefore, adiponectin seems to be able to increase fatty acid oxidation for a prolonged period by inducing the synthesis of proteins involved in fatty acid oxidation, in addition to the transient stimulation via ACC phosphorylation.

DISCUSSION

In this study, we have discovered a new mechanism by which adiponectin can stimulate fatty acid oxidation in muscle cells via sequential activation of AMPK, p38 MAPK, and PPARα. These results represent the first detailed characterization of the adiponectin signaling pathway that stimulates fatty acid oxidation via the activation of PPARα.

Accumulating results have unveiled adiponectin as a promising candidate for the treatment of obesity-associated metabolic syndromes. In animal models and human subjects with obesity and type 2 diabetes, adiponectin stimulates fatty acid oxidation, decreases lipid accumulation in muscles, lowers plasma free fatty acid levels, and enhances insulin sensitivity (10,37). Adiponectin has been shown to increase fatty acid oxidation via activation of AMPK and phosphorylation of ACC (35). However, the phosphorylation of ACC induced by adiponectin is short lived (35) (Fig. 7*A*). Therefore, this pathway cannot fully explain the long-term effects of adiponectin on weight loss and fatty acid oxidation. On the other hand, activation of PPARα would be a promising pathway by which adiponectin can trigger long-term stimulation of fatty acid oxida-

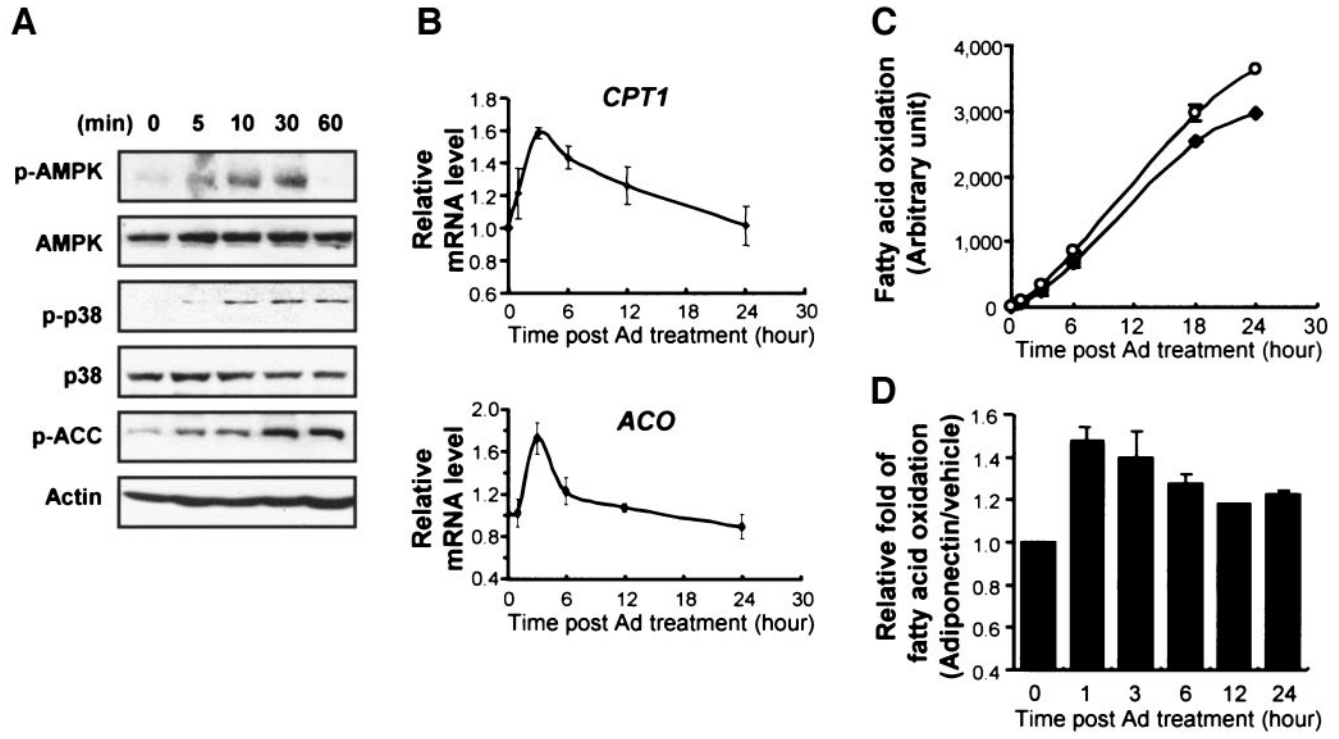

FIG. 7. Time-dependent regulation of AMPK and p38 MAPK phosphorylation, PPARα target gene expression, and fatty acid oxidation by adiponectin in C2C12 myotubes. *A*: The levels of AMPK, p38 MAPK, and ACC phosphorylation were measured at the indicated time points after treatment of adiponectin (5 μg/ml). *B*: The relative mRNA levels of *CPT1* and *ACO* were measured at variable time points after treatment of adiponectin (2 μg/ml). The amount of mRNA was normalized to *GAPDH*. *C*: Cumulative amount of fatty acid oxidation was determined at different time points as indicated after adiponectin treatment (2 μg/ml). ♦, vehicle; ○, adiponectin. *D*: Fold of difference between adiponectin- and vehicle-treated cells.

tion, even after the initial signaling is gone, because this would promote the amounts of relatively long-lived enzymes involved in fatty acid metabolism. Indeed, we found that adiponectin, via AMPK, enhanced the transcriptional activity of PPARα and the expression of its target genes (Figs. 1 and 2). This result is supported by the fact that activation of AMPK has a potent lipid-lowering effect that is equivalent to the effect of PPARα agonists in obese animal models. In vivo infusion of AICAR significantly decreases plasma free fatty acid levels and the triglyceride concentration in both lean and obese Zucker rats (38,39).

Using dominant-negative AMPK and chemical inhibitors of AMPK and p38 MAPK, we revealed that AMPK and p38 MAPK are sequentially activated (Fig. 5 and online appendix Fig. 1). Previous studies have also suggested that the activation of p38 MAPK is associated with the stimulation of AMPK in glucose metabolism. For example, in skeletal muscle, AICAR increases glucose uptake by activating p38 MAPK, which is involved in the activation of GLUT4 translocation to the plasma membrane (40). In the same manner, p38 MAPK acts downstream of AMPK in cardiomyocytes, and the inhibition of the AMPK/p38 MAPK signaling pathway partially abolishes the stimulation of glucose uptake in response to hypoxia (41). Therefore, p38 MAPK and AMPK appear to be components in the adiponectin signaling pathway in muscle cells that work to increase insulin sensitivity by activating both glucose uptake and fatty acid utilization. However, treatment with adiponectin did not elevate fatty acid oxidation in primary hepatocytes (11,42) (unpublished data), suggesting that muscle and liver tissues may respond differently to adiponectin in terms of fatty acid oxidation.

Adiponectin circulates in serum as trimers and hexamers of relatively low molecular weight (LMW) and middle molecular weight and larger multimeric forms of high molecular weight (HMW) (32,42,43). Several studies have pointed to a greater role of HMW adiponectin in improving insulin sensitivity (31,43). Moreover, thiazolidinediones increased the level of circulating HMW adiponectin (44,45). When we compared different fractions of adiponectin containing either the HMW or LMW forms, the HMW adiponectin containing fractions exhibited pronounced effect on both PPARα target gene expression and AMPK and p38 MAPK phosphorylation, while LMW adiponectin exerted only marginal effects (online appendix Fig. 2). This is in concert with a previous study demonstrating a higher correlation between HMW adiponectin and whole-body fat oxidation than LMW adiponectin in human and rodents (46).

The mutation of putative AMPK phosphorylation sites on PPARα did not affect its responsiveness to AICAR. In contrast, mutant PPARα in which the potential p38 MAPK phosphorylation sites at serine residues were substituted to alanine (S6/12/21A) was unresponsive to AMPK activation (Fig. 6). This implies that AMPK controls the transcriptional activity of PPARα through p38 MAPK and not by direct modification. Of note, p38 MAPK is known to phosphorylate PPARα and increase its association with a coactivator PPARγ coativator-1α (36). However, the possibility of the presence of other AMPK phosphorylation sites in addition to the serine residues examined in our experiments cannot be ruled out. In addition, it has been proposed that adiponectin can enhance PPARα activity through the production of its ligands (47). These results suggest the presence of multiple pathways that could mediate the effect of adiponectin on fatty acid metabolism

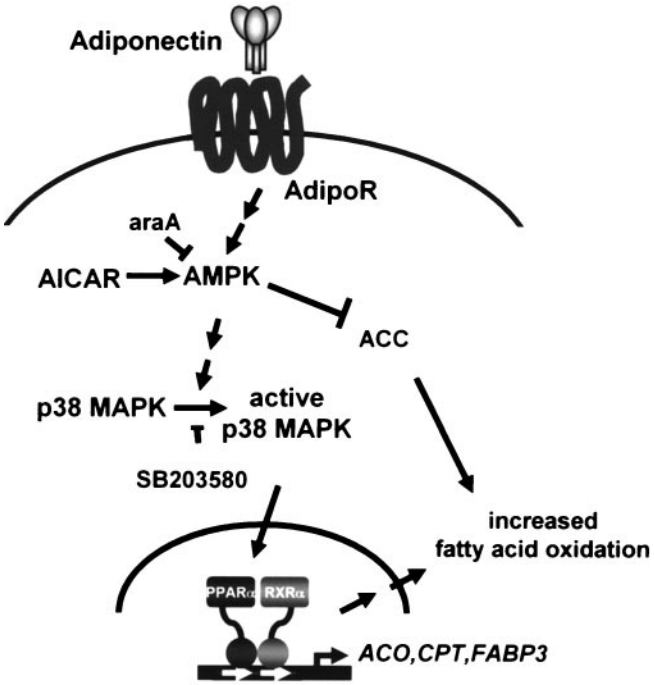

FIG. 8. Schematic model of adiponectin signaling on fatty acid oxidation in muscle cells. Sequential activation of AMPK, p38 MAPK, and PPARα leads to the induction of PPARα target genes involved in fatty acid utilization. This allows sustained stimulation of fatty acid oxidation via PPARα activation in addition to the transient induction triggered by ACC phosphorylation. RXRα, retinoid X receptor α.

in muscles. Further research is required to validate these ideas.

Collectively, we have provided evidence that adiponectin, through the sequential activation of AMPK and p38 MAPK, stimulates PPARα activity in muscle cells (Fig. 8). There still remains missing links to be connected in this pathway, such as how adiponectin activates AMPK or what factors mediate the signaling pathways between AMPK and p38 MAPK. Nonetheless, our results have provided a possible explanation as to how adiponectin triggers sustained increases in fatty acid oxidation in muscle cells. Intervention of this pathway may provide a means by which obesity-related metabolic disorders may be treated.

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