

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

Myeong Jin Yoon,¹ Gha Young Lee,¹ Jun-Jae Chung,¹ Young Ho Ahn,² Seung Hwan Hong,² and Jae Bum Kim¹

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 PPAR α 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 PPAR α 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 PPAR α . *Diabetes* 55:2562–2570, 2006

From the ¹Laboratory of Adipocyte and Metabolism Research, Research Center for Functional Cellulomics, Department of Biological Sciences, Seoul National University, Seoul, Korea; and the ²Laboratory of Cell Biology, Department of Biological Sciences, Seoul National University, Seoul, Korea.

Address correspondence and reprint requests to Jae Bum Kim, PhD, Department of Biological Sciences, Seoul National University, San 56-1, Sillim-Dong, Gwanak-Gu, Seoul 151-742, Korea. E-mail: jaebkim@snu.ac.kr.

Received for publication 11 October 2005 and accepted in revised form 21 June 2006.

Additional information for this article can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; AICAR, 5-aminoimidazole-4-carboxamide-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.

DOI: 10.2337/db05-1322

© 2006 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 . PPAR α 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). PPAR α controls the expression

of a number of genes involved in peroxisomal (19,20) and mitochondrial β -oxidation (21,22), such as *ACO*, *CPT1*, and *FABP3*. Several studies using PPAR α -null mice have demonstrated the critical role of PPAR α 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 PPAR α 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 PPAR α 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 PPAR α 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 CaCl₂ and bound to Ni-NTA agarose beads. The beads were washed twice with washing buffer (50 mmol/l NaH₂PO₄, 300 mmol/l NaCl, and 20 mmol/l imidazole), and adiponectin was collected with elution buffer (50 mmol/l NaH₂PO₄, 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 PPAR α , 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 μ mol/l Wy14643 (Biomol), 1 mmol/l araA (Sigma), 5 μ mol/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 \times 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 \times radioimmunoprecipitation assay buffer and precipitated with saturated ammonium sulfate solution (final concentration 35%). Assays were performed at 30°C for 10 min in 25 μ l reaction mixtures containing 5 μ g 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 μ mol/l each of AMP and ATP, and 2 μ Ci [γ -³²P] ATP) with or without 200 μ mol/l SAMS peptide (Upstate). For immunoprecipitated kinase assays, cell lysates were immunoprecipitated with anti-AMPK- α 1 or - α 2 antibodies (Upstate), washed with 40 mmol/l HEPES (pH 7.0), and suspended in 20 μ l 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 [³²P] incorporated per microgram of protein.

p38 MAPK in vitro kinase assay. Total cell extract (500 μ g) 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 μ g 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 MgCl₂, 12 mmol/l β -glycerophosphate, 1 mmol/l Na₂VO₄, 2 mmol/l dithiothreitol, 0.5 mmol/l phenylmethylsulfonyl fluoride, and 1 μ Ci [γ -³²P] ATP). The reactions were terminated using 5 \times SDS sample buffer, and the products were resolved by SDS-PAGE followed by transfer to polyvinylidene difluoride membranes. The level of incorporated [³²P] 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 μ g, 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 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 (9,10-³H)palmitate, 5 μ Ci/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. ³H₂O content was measured in a scintillation counter.

RESULTS

Adiponectin stimulates PPAR α 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. 1A). Because these genes are known to be transcriptionally regulated by PPAR α , we then performed reporter assays to determine whether PPAR α transcription activity can be regulated by adiponectin. As shown in Fig. 1B, cotransfection with adiponectin increased the transcriptional activity of PPAR α on a reporter construct driven by multiple PPAR response elements (DR-1). This stimulatory

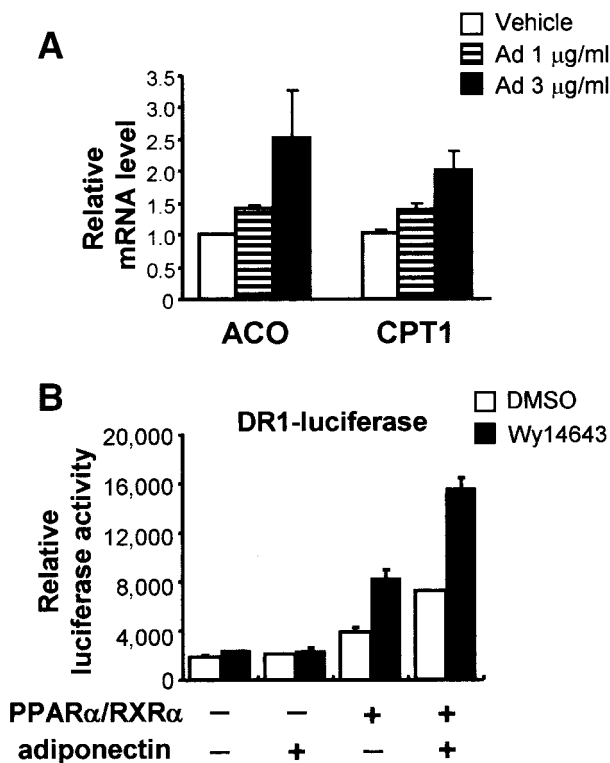


FIG. 1. Adiponectin increases PPAR α transcriptional activity and target gene expression. *A*: The relative mRNA levels of PPAR α target genes in differentiated C2C12 myotubes were measured 3 h after treatment with adiponectin. The amount of mRNA was normalized to *GAPDH*. Each experiment was independently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

effect was observed both in the presence and absence of Wy14643, a synthetic ligand of PPAR α , indicating that regulation of PPAR α 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-PPAR α 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 PPAR α .

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

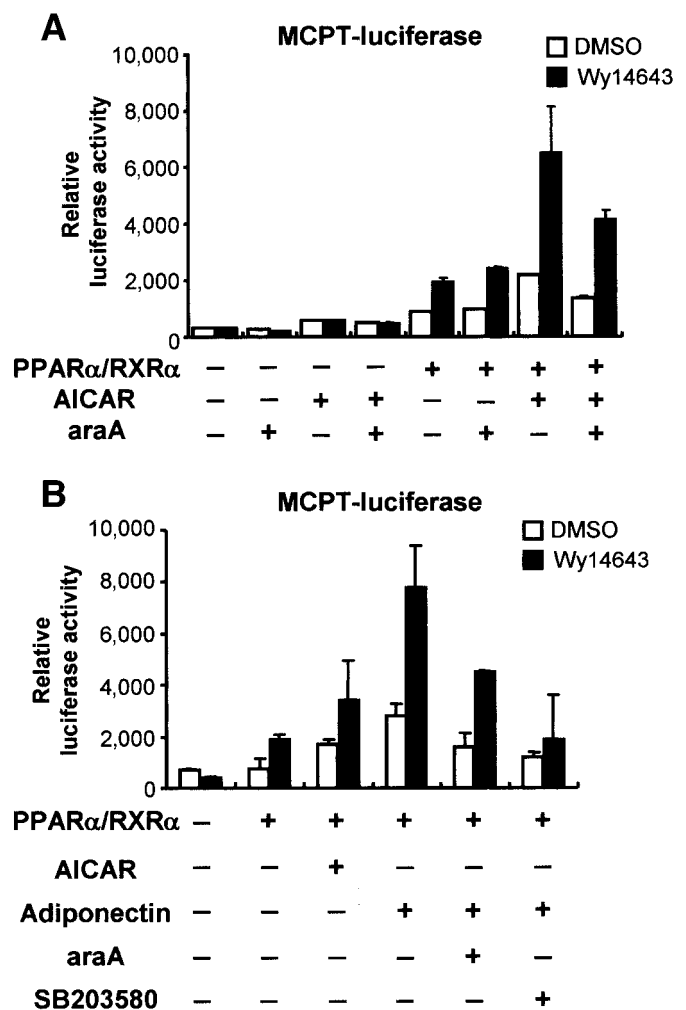


FIG. 2. Adiponectin increases PPAR α transcriptional activity via AMPK and p38 MAPK. *A* and *B*: Luciferase assays were performed with HEK293 cells transfected as indicated and treated with the indicated expression vectors and compounds. See RESEARCH DESIGN AND METHODS for details. RXR α , retinoid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

also significantly inhibited the effect of adiponectin on PPAR α activity.

To confirm the roles of AMPK and p38 MAPK in the activation of PPAR α , we examined the effects of araA and SB203580 on the mRNA levels of PPAR α target genes in C2C12 myotubes. Consistent with the results shown in Fig. 1A, adiponectin increased the expression levels of *ACO*, *CPT1*, and *FABP3* (Fig. 3A). However, pretreatment of cells with araA or SB203580 abolished the adiponectin-dependent increase in PPAR α target gene expression. Furthermore, dominant-negative AMPK similarly repressed the stimulation of PPAR α target gene expression by adiponectin (Fig. 3B). Together, these data indicate that the activation of PPAR α by adiponectin occurs through a pathway that requires activation of both AMPK and p38 MAPK. **AMPK stimulates PPAR α -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 PPAR α 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 PPAR α or AMPK using Wy14643 or AICAR, respectively (Fig. 4A, lanes 2 and 3).

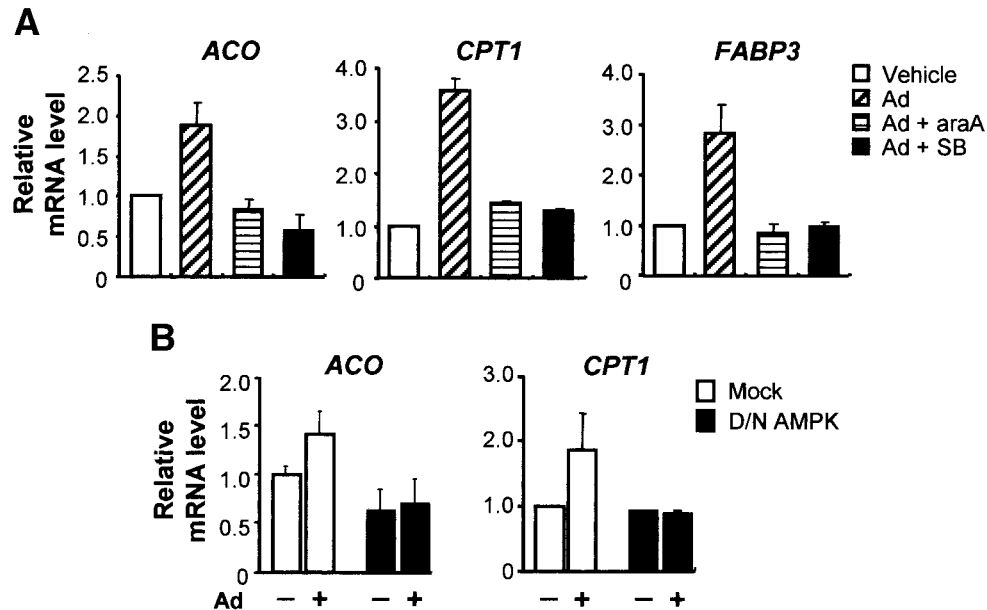


FIG. 3. Stimulation of PPAR α target gene expression by adiponectin is repressed by inhibition of AMPK and p38 MAPK. The relative mRNA levels of PPAR α target genes in differentiated C2C12 myotubes were measured 3 h after treatment with adiponectin (2 μ g/ml). Before adiponectin treatment, the cells were either preincubated with araA (1 mmol/l) for 1 h or SB203580 (10 μ mol/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. 4A, 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 PPAR α 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 PPAR α . When cells were treated with cycloheximide to block

protein synthesis, AICAR-induced fatty acid oxidation was reduced by ~30% (Fig. 4B). This reduction can probably be attributed to the inhibition of effect of PPAR α 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. 4C). This stimulation was suppressed by araA or SB203580 (Fig. 4C, lanes 3 and 4), and cotreatment with

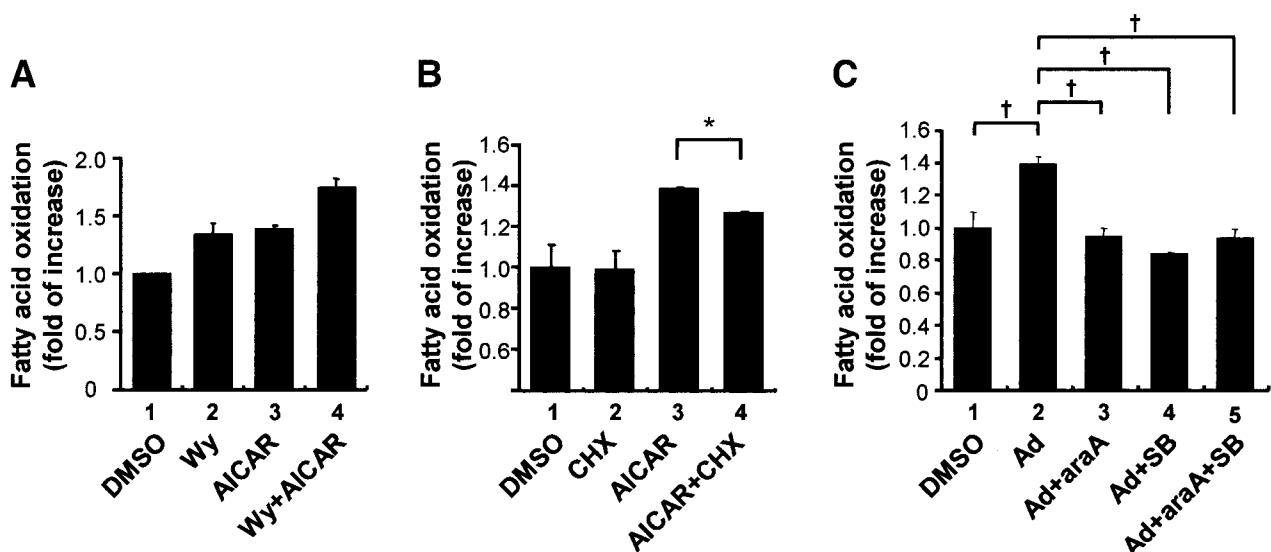


FIG. 4. Adiponectin increases fatty acid oxidation via AMPK and p38 MAPK. Fatty acid oxidation was measured using 3 H-labeled palmitate in differentiated C2C12 myotubes as described in RESEARCH DESIGN AND METHODS. A: Cells were incubated in oxidation media containing 3 H-labeled palmitate with 1 mmol/l AICAR, 10 μ mol/l Wy14643, or vehicle (DMSO) for 24 h. Wy, Wy14643. B: Cells were preincubated in the presence or absence of cycloheximide (CHX; 20 μ mol/l) for 1 h before adding AICAR (1 mmol/l), Wy14643 (10 μ mol/l), or DMSO. C: Fatty acid oxidation was measured after stimulating with 2 μ g/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.

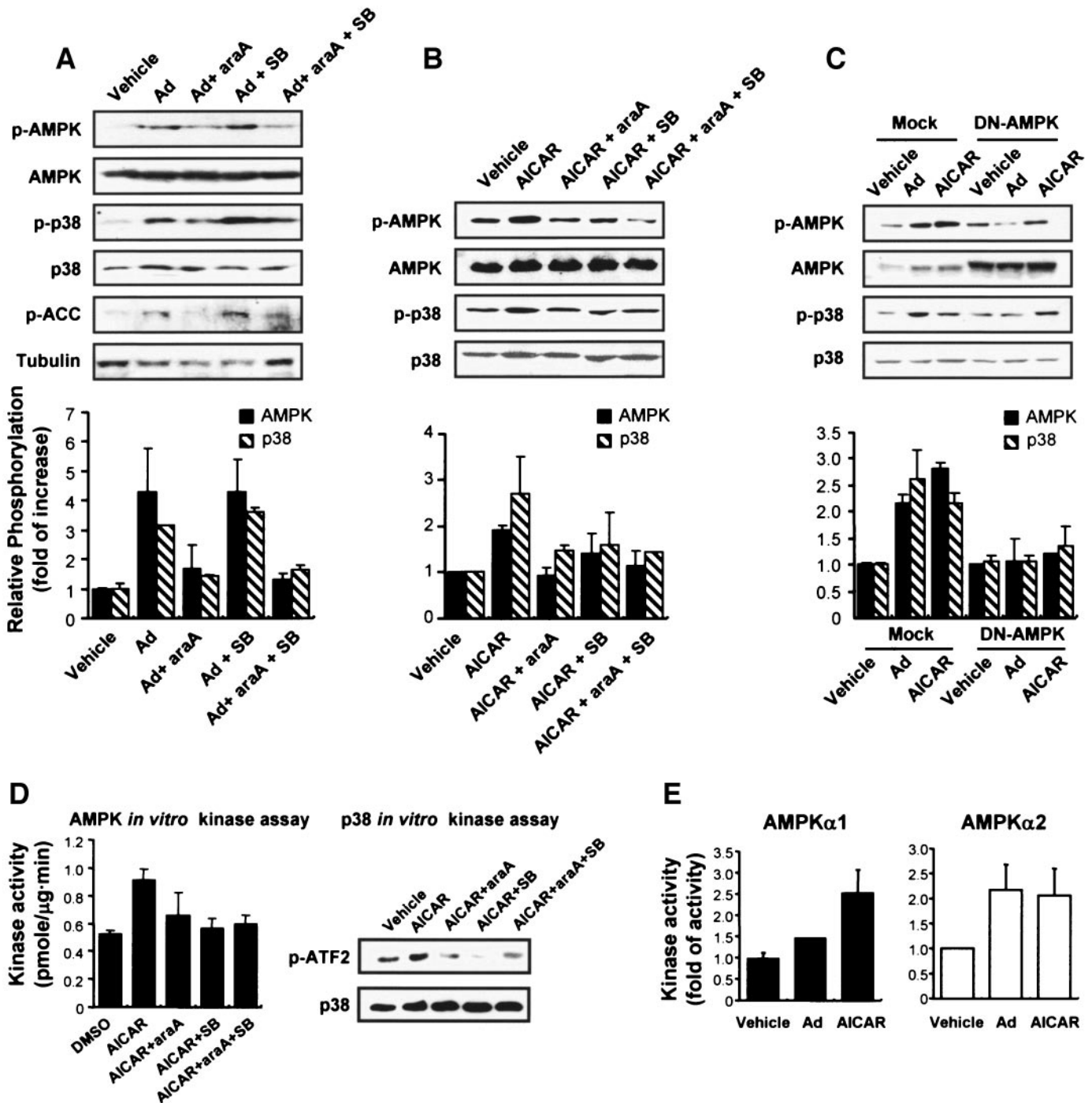


FIG. 5. Adiponectin signaling via AMPK and p38 MAPK. *A–C:* The levels of AMPK, p38 MAPK, and/or ACC phosphorylation in C2C12 myotubes were measured after 30 min incubation with adiponectin (5 μ g/ml) or AICAR (2 mmol/l). Cells were pretreated with 2 mmol/l araA (1 h), 10 μ mol/l SB203580 (20 min), or adeno-DN 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 α 1 and α 2 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 PPAR α .

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

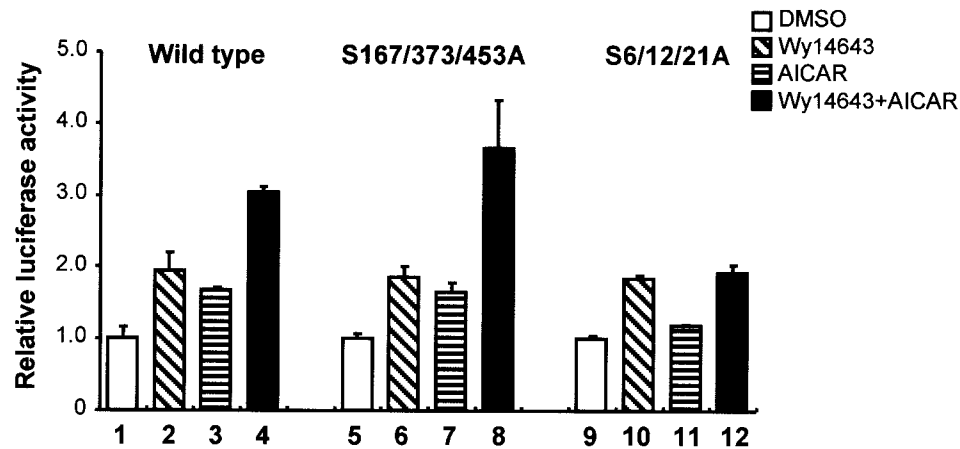


FIG. 6. PPAR α S6/12/21A mutant is not activated by AMPK. Luciferase assays were carried out using the wild-type and two mutant forms (S167/373/453A and S6/12/21A) of PPAR α . The results are presented as the fold of activation caused by treatment of Wy14643 or AICAR compared with vehicle for each type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

using AICAR (Fig. 5B 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. 5C). Conversely, insulin, which activates p38 MAPK in muscle cells, did not affect AMPK phosphorylation or kinase activity (online appendix Fig. 1A 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, $\alpha 1$ or $\alpha 2$, mediates adiponectin signaling in muscle cells, we performed in vitro kinase assays using antibodies specific for each isoform. The kinase activity of both $\alpha 1$ and $\alpha 2$ isoforms was increased by adiponectin treatment, the $\alpha 1$ isoform by $\sim 50\%$ and the $\alpha 2$ isoform by over twofold (Fig. 5D). Thus, it is possible to speculate that AMPK $\alpha 2$ might be the major mediator of adiponectin function in muscle cells, although the role of $\alpha 1$ cannot be ruled out.

p38 MAPK directly stimulates PPAR α transcription activity. Previously, it has been demonstrated that the transcriptional activity of PPAR α 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 PPAR α through sequence analysis. To test whether AMPK might directly modulate PPAR α activity, we performed reporter assays using PPAR α 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 PPAR α (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 PPAR α .

Adiponectin stimulates long-term fatty acid oxidation. The regulation of fatty acid oxidation by adiponectin through PPAR α 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 PPAR α target genes, and fatty acid oxidation. As shown in Fig. 7A, 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. 7D). Meanwhile, the expression of PPAR α target genes, specifically *ACO* and *CPT1*, peaked at 3 h after adiponectin treatment and slowly receded to basal levels after 24 h (Fig. 7B). Interestingly, the rate of fatty acid oxidation constantly remained higher in adiponectin-treated cells throughout the entire duration (Fig. 7C 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 PPAR α . These results represent the first detailed characterization of the adiponectin signaling pathway that stimulates fatty acid oxidation via the activation of PPAR α .

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. 7A). 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 PPAR α would be a promising pathway by which adiponectin can trigger long-term stimulation of fatty acid oxida-

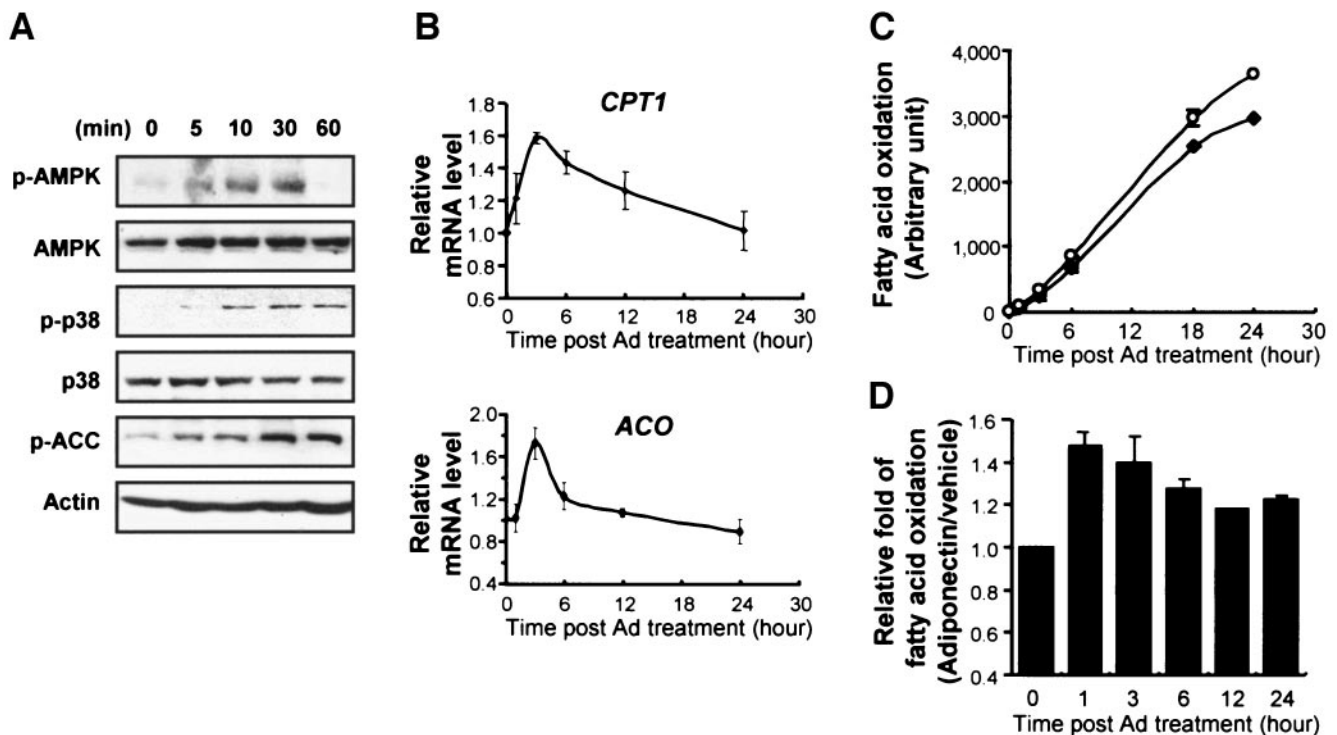


FIG. 7. Time-dependent regulation of AMPK and p38 MAPK phosphorylation, PPAR α target gene expression, and fatty acid oxidation by adiponectin in C2C12 myotubes. **A:** The levels of AMPK, p38 MAPK, and ACC phosphorylation were measured at the indicated time points after treatment of adiponectin (5 μ g/ml). **B:** The relative mRNA levels of *CPT1* and *ACO* were measured at variable time points after treatment of adiponectin (2 μ g/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 μ g/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 PPAR α 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 PPAR α 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 hexam-

ers 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 PPAR α 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 PPAR α did not affect its responsiveness to AICAR. In contrast, mutant PPAR α 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 PPAR α through p38 MAPK and not by direct modification. Of note, p38 MAPK is known to phosphorylate PPAR α and increase its association with a coactivator PPAR γ coactivator-1 α (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 PPAR α 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

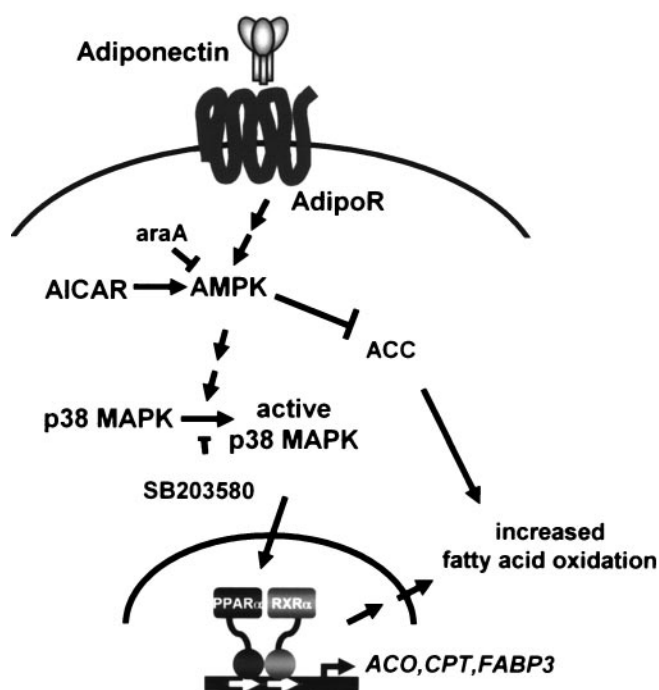


FIG. 8. Schematic model of adiponectin signaling on fatty acid oxidation in muscle cells. Sequential activation of AMPK, p38 MAPK, and PPAR α leads to the induction of PPAR α target genes involved in fatty acid utilization. This allows sustained stimulation of fatty acid oxidation via PPAR α activation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

ACKNOWLEDGMENTS

This study was supported in part by grants from the Center for Biological Modulators of the 21C Frontier R&D Program, Molecular and Cellular Biodiscovery Research Program, and the National Research Laboratory Program. M.J.Y., J.J.C., Y.H.A., S.H.H., and J.B.K. were supported by the BK21 Research Fellowship from the Ministry of Education and Human Resources Development.

We thank D.P. Kelly for the muscle-type carnitine palmitoyl transferase promoter-luciferase vector and K.U. Lee and J.Y. Park for the adenovirus expressing dominant-negative AMPK. We are grateful to T.W. Nam and Y.J. Seok for assistance with the gel filtration assay.

REFERENCES

1. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 270:26746–26749, 1995
2. Hu E, Liang P, Spiegelman BM: AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 271:10697–10703, 1996

3. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K: cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem Biophys Res Commun* 221:286–289, 1996
4. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y: Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 100:2473–2476, 1999
5. Nakano Y, Tobe T, Choi-Miura NH, Mazda T, Tomita M: Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J Biochem (Tokyo)* 120:803–812, 1996
6. Kadowaki T, Yamauchi T: Adiponectin and adiponectin receptors. *Endocr Rev* 26:439–451, 2005
7. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257:79–83, 1999
8. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y: Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 20:1595–1599, 2000
9. Hotta K, Funahashi T, Bodkin NL, Ortmeier HK, Arita Y, Hansen BC, Matsuzawa Y: Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes* 50:1126–1133, 2001
10. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF: Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A* 98:2005–2010, 2001
11. Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa K, Matsui J, Takata M, Eto K, Terauchi Y, Komeda K, Tsunoda M, Murakami K, Ohnishi Y, Naitoh T, Yamamura K, Ueyama Y, Froguel P, Kimura S, Nagai R, Kadowaki T: Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. *J Biol Chem* 278:2461–2468, 2003
12. Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Terasaka N, Inaba T, Funahashi T, Matsuzawa Y: Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 106:2767–2770, 2002
13. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8:1288–1295, 2002
14. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T: Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423:762–769, 2003
15. Carling D: The AMP-activated protein kinase cascade: a unifying system for energy control. *Trends Biochem Sci* 29:18–24, 2004
16. Alam N, Saggerson ED: Malonyl-CoA and the regulation of fatty acid oxidation in soleus muscle. *Biochem J* 334:233–241, 1998
17. Saha AK, Schwarsin AJ, Roduit R, Masse F, Kaushik V, Tornheim K, Prentki M, Ruderman NB: Activation of malonyl-CoA decarboxylase in rat skeletal muscle by contraction and the AMP-activated protein kinase activator 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside. *J Biol Chem* 275:24279–24283, 2000
18. Escher P, Braissant O, Basu-Modak S, Michalik L, Wahli W, Desvergne B: Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding. *Endocrinology* 142:4195–4202, 2001
19. Tugwood JD, Issemann I, Anderson RG, Bundell KR, McPheat WL, Green S: The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *Embo J* 11:433–439, 1992
20. Zhang B, Marcus SL, Sajjadi FG, Alvares K, Reddy JK, Subramani S, Rachubinski RA, Capone JP: Identification of a peroxisome proliferator-responsive element upstream of the gene encoding rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase. *Proc Natl Acad Sci U S A* 89:7541–7545, 1992
21. Gulick T, Cresci S, Caira T, Moore DD, Kelly DP: The peroxisome

- proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. *Proc Natl Acad Sci U S A* 91:11012–11016, 1994
22. Rodriguez JC, Gil-Gomez G, Hegardt FG, Haro D: Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids. *J Biol Chem* 269:18767–18772, 1994
 23. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W: Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* 103:1489–1498, 1999
 24. Leone TC, Weinheimer CJ, Kelly DP: A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders. *Proc Natl Acad Sci U S A* 96:7473–7478, 1999
 25. Hashimoto T, Cook WS, Qi C, Yeldandi AV, Reddy JK, Rao MS: Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *J Biol Chem* 275:28918–28928, 2000
 26. Guerre-Millo M, Gervois P, Raspe E, Madsen L, Poulain P, Derudas B, Herbert JM, Winegar DA, Willson TM, Fruchart JC, Berge RK, Staels B: Peroxisome proliferator-activated receptor alpha activators improve insulin sensitivity and reduce adiposity. *J Biol Chem* 275:16638–16642, 2000
 27. Ye JM, Doyle PJ, Iglesias MA, Watson DG, Cooney GJ, Kraegen EW: Peroxisome proliferator-activated receptor (PPAR)- α activation lowers muscle lipids and improves insulin sensitivity in high fat-fed rats: comparison with PPAR- γ activation. *Diabetes* 50:411–417, 2001
 28. Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, Piomelli D: Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature* 425:90–93, 2003
 29. Ono K, Han J: The p38 signal transduction pathway: activation and function. *Cell Signal* 12:1–13, 2000
 30. Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, Goodyear LJ: Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49:527–531, 2000
 31. Stein SC, Woods A, Jones NA, Davison MD, Carling D: The regulation of AMP-activated protein kinase by phosphorylation. *Biochem J* 345:437–443, 2000
 32. Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M, Scherer PE: Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin: implications for metabolic regulation and bioactivity. *J Biol Chem* 278:9073–9085, 2003
 33. Witters LA, Kemp BE: Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J Biol Chem* 267:2864–2867, 1992
 34. Cabrero A, Alegret M, Sanchez RM, Adzet T, Laguna JC, Vazquez M: Bezafibrate reduces mRNA levels of adipocyte markers and increases fatty acid oxidation in primary culture of adipocytes. *Diabetes* 50:1883–1890, 2001
 35. Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang Cc C, Itani SI, Lodish HF, Ruderman NB: Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc Natl Acad Sci U S A* 99:16309–16313, 2002
 36. Barger PM, Browning AC, Garner AN, Kelly DP: p38 mitogen-activated protein kinase activates peroxisome proliferator-activated receptor alpha: a potential role in the cardiac metabolic stress response. *J Biol Chem* 276:44495–44501, 2001
 37. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE: The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med* 7:947–953, 2001
 38. Bergeron R, Previs SF, Cline GW, Perret P, Russell RR 3rd, Young LH, Shulman GI: Effect of 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes* 50:1076–1082, 2001
 39. Buhl ES, Jessen N, Pold R, Ledet T, Flyvbjerg A, Pedersen SB, Pedersen O, Schmitz O, Lund S: Long-term AICAR administration reduces metabolic disturbances and lowers blood pressure in rats displaying features of the insulin resistance syndrome. *Diabetes* 51:2199–2206, 2002
 40. Lemieux K, Konrad D, Klip A, Marette A: The AMP-activated protein kinase activator AICAR does not induce GLUT4 translocation to transverse tubules but stimulates glucose uptake and p38 mitogen-activated protein kinases alpha and beta in skeletal muscle. *FASEB J* 17:1658–1665, 2003
 41. Pelletier A, Joly E, Prentki M, Coderre L: Adenosine 5'-monophosphate-activated protein kinase and p38 mitogen-activated protein kinase participate in the stimulation of glucose uptake by dinitrophenol in adult cardiomyocytes. *Endocrinology* 146:2285–2294, 2005
 42. Tsao TS, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, Heuser JE, Lodish HF: Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity: different oligomers activate different signal transduction pathways. *J Biol Chem* 278:50810–50817, 2003
 43. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R, Kadowaki T: Impaired multimerization of human adiponectin mutants associated with diabetes: molecular structure and multimer formation of adiponectin. *J Biol Chem* 278:40352–40363, 2003
 44. Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE: Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem* 279:12152–12162, 2004
 45. Tsuchida A, Yamauchi T, Takekawa S, Hada Y, Ito Y, Maki T, Kadowaki T: Peroxisome proliferator-activated receptor (PPAR) α activation increases adiponectin receptors and reduces obesity-related inflammation in adipose tissue: comparison of activation of PPAR α , PPAR γ , and their combination. *Diabetes* 54:3358–3370, 2005
 46. Lara-Castro C, Luo N, Wallace P, Klein RL, Garvey WT: Adiponectin multimeric complexes and the metabolic syndrome trait cluster. *Diabetes* 55:249–259, 2006
 47. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipodystrophy and obesity. *Nat Med* 7:941–946, 2001