Regulation of AMP-activated protein kinase and acetyl-CoA carboxylase phosphorylation by palmitate in skeletal muscle cells

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Abstract The purpose of this study was to investigate the effects of long-chain fatty acids (LCFAs) on AMP-activated protein kinase (AMPK) and acetyl-coenzyme A carboxylase (ACC) phosphorylation and β-oxidation in skeletal muscle. L6 rat skeletal muscle cells were exposed to various concentrations of palmitate (1–800 μM). Subsequently, ACC and AMPK phosphorylation and fatty acid oxidation were measured. A 2-fold increase in both AMPK and ACC phosphorylation was observed in the presence of palmitate concentrations as low as 10 μM, which was also accompanied by a significant increase in fatty acid oxidation. The effect of palmitate on AMPK and ACC phosphorylation was dose-dependent, reaching maximum increases of 3.5- and 4.5-fold, respectively. Interestingly, ACC phosphorylation was coupled with AMPK activation at palmitate concentrations ranging from 10 to 100 μM; however, at concentrations >200 μM, ACC phosphorylation and fatty acid oxidation remained high even after AMPK phosphorylation was completely prevented by the use of a selective AMPK inhibitor. This indicates that LCFAs regulate ACC activity by AMPK-dependent and -independent mechanisms, based on their abundance in skeletal muscle cells. Here, we provide novel evidence that the AMPK/ACC pathway may operate as a mechanism to sense and respond to the lipid energy charge of skeletal muscle cells.—Fediuc, S., M. P. Gaidhu, and R. B. Ceddia. **Regulation of AMP-activated protein kinase and acetyl-CoA carboxylase phosphorylation by palmitate in skeletal muscle cells. *J. Lipid Res.* 2006.** 47: **412–420.**

Supplementary key words acetyl-coenzyme A carboxylase • fatty acid oxidation • lipid sensing

In recent years, a large body of evidence has been published showing that in mammals, AMP-activated protein kinase (AMPK) responds to hormonal and nutrient signals in the central nervous system and peripheral tissues, modulating food intake and whole-body energy homeostasis (1). AMPK is a heterotrimeric enzyme that has been proposed to function as a "fuel gauge" that monitors changes in the energy status of cells (1–3). When activated, AMPK shuts down anabolic pathways and promotes catabolism in response to an increase in the AMP/ATP ratio by down-regulating the activity of key enzymes of intermediary metabolism (1–3). In its activated state, AMPK phosphorylates serine residues 79, 1,200, and 1,215 of acetyl-coenzyme A carboxylase (ACC), producing an 80–90% decrease in the V_{max} of the enzyme, suggesting that AMPK is the physiological ACC kinase (4). There is also evidence that long-chain fatty acids (LCFAs) act as potent feedback suppressors of lipogenesis by inhibiting ACC activity (5–7). ACC is a multifunctional enzyme that, when active (dephosphorylated form), catalyzes the conversion of acetyl-CoA to malonyl-CoA in the de novo lipid synthesis pathway (1–4). Malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase-1, a rate-limiting step for the entry of LCFAs into mitochondria for oxidation (8). When ACC is inactive (phosphorylated form), a decrease in malonyl-CoA occurs and disinhibits carnitine palmitoyltransferase-1, thereby increasing the mitochondrial import and oxidation of LCFAs (8). Therefore, the AMPK/ACC system is thought to play a central role in the regulation of cellular lipid homeostasis (2, 8, 9). In certain metabolic disorders, such as obesity and type 2 diabetes, lipid metabolism is dysfunctional, causing fatty acids to increase in the circulation and also in intracellular compartments (2, 8, 9). High levels of fatty acids are toxic to the cells and may cause deleterious metabolic abnormalities (8, 9). By increasing fatty acid oxidation in peripheral tissues, the AMPK/ACC system may play an important role by protecting the cells from these metabolic abnormalities (9). Of special interest are the mechanisms that regulate the AMPK/ACC system in skeletal muscle, because this tissue plays a major role in determining whole-body energy expenditure, accounts for 70% of total-body glucose disposal, and may modify substrate utilization toward substantially increasing fatty acid oxidation (9).

The classical view is that AMPK is activated allosterically by an increase in the intracellular AMP/ATP ratio, by phosphorylation of threonine 172 (Thr-172) within the α subunit, catalyzed by the upstream kinase LKB1 (the upstream

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kinase of AMPK), and by inhibition of the dephosphorylation of Thr-172 by protein phosphatases (1–3). To date, a wide range of physiological stressors, pharmacological agents, and hormones associated with increases in the intracellular AMP/ATP ratio have been demonstrated to activate AMPK (10). In skeletal muscle, the activity of AMPK has also been reported to be regulated by the intracellular creatine-phosphocreatine ratio (11, 12) and glycogen content (13, 14), both directly related to the energy charge of muscle cells. Fatty acids, another major cellular energy source, may also regulate AMPK activity in skeletal muscle; however, no data for this have been published. It has been reported that in perfused rat cardiac muscle, palmitate (250 and 500 μM) and oleate (500 μM) significantly increased AMPK activity without causing any significant alteration in AMP/ATP ratio (15). Another study has reported that exposure to 150 μM acetate, octanoate, or palmitate caused a significant reduction in AMP/ATP ratio followed by a significant increase in AMPK activity in primary rat hepatocytes (16). In contrast to these observations are reports that the AMPK activity of rat liver purified LKB1/STRADMO25 (the upstream kinase of AMPK) was inhibited by long-chain acyl-CoA esters in vitro (17).

Currently, there is no consensus regarding the regulation of the AMPK/ACC system by fatty acids. To clarify this issue, we investigated the effects of different concentrations of palmitate on AMPK and ACC phosphorylation in L6 rat skeletal muscle cells. We hypothesized that LCFAs would increase AMPK and ACC phosphorylation by *1*) ATP utilization for LCFA activation (16) and *2*) directly regulating ACC phosphorylation, because in the presence of exogenous fatty acids the de novo lipid synthesis pathway would be suppressed (4). In rat skeletal muscle, refeeding after a fast increases malonyl-CoA and decreases fatty acid oxidation, which has been attributed to a decrease in fatty acids that releases the allosteric inhibition of ACC2 (18). Here, we provide evidence that palmitate concentrations ranging from 1 to 800 μM significantly increase both AMPK and ACC phosphorylation and β-oxidation. Additionally, with the aid of a selective AMPK inhibitor, we describe the novel finding that ACC phosphorylation and palmitate oxidation are increased in the presence of high fatty acid concentrations by a mechanism independent of AMPK activation in L6 rat skeletal muscle cells.

METHODS

Reagents

α-Minimum Eagle's medium (α-MEM) and FBS were purchased from Wisent (Quebec, Canada). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals, Inc. Compound C, a selective AMPK inhibitor, was provided by Merck Research Laboratories, fatty acid-free albumin, palmitic acid, and phenylethylamine were from Sigma (St. Louis, MO). [1-^{14}C]palmitic acid was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Specific antibodies against P-AMPK and P-ACC were from Cell Signaling Technology (Beverly, MA) and Upstate Biotechnology (Charlottesville, VA), respectively. All other chemicals were of the highest grade available.

Cell culture and treatment

Stock cultures of rat L6 skeletal muscle cells were obtained from the American Type Culture Collection and grown in α-MEM containing 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (growth medium), and antimycotic in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For experimental procedures, stocks were trypsinized and reseeded on six-well plates or 60 × 15 mm Petri dishes at a density of 4,000 cells/cm^2. After 24 h (~80% confluence), the medium was changed to α-MEM containing 2% (v/v) FBS and antibiotic/antimycotic as described above (differentiation medium) that was replaced after 2, 4, and 6 days of culture. After 7 days, myotube differentiation was complete, and experimental procedures were initiated. In all experiments, L6 myotubes were serum-starved for 4 h before exposure to fatty acids, AICAR, and/or compound C. All controls were incubated with equal amounts of the vehicles used for AICAR, compound C, and the respective concentrations of fat-free albumin as present in palmitate-treated cells.

Cell viability testing, trypan blue exclusion

Cells were treated with AICAR (2 mM), palmitate (1–800 μM), and compound C (10 and 40 μM). Subsequently, cells were rinsed with PBS, trypsinized, washed with medium, centrifuged, and resuspended in PBS. Next, cells were mixed with the same volume of 0.25% trypan blue and transferred to a slide for 3 min. A total of 300 cells were microscopically counted using a hemocytometer to determine the dead cell (stained blue) rate. The experiments were performed in triplicate. Compared with the control (cells not exposed to AICAR, palmitate, or compound C), no significant differences were detected for cell viability after exposing the cells for 1 h to all of the different treatment conditions.

Production of $^{14}CO_2$ from [1-^{14}C]palmitic acid

Palmitate was conjugated with essentially fatty acid-free BSA to generate a stock solution of 25% (w/v) BSA and 6 mM fatty acid in serum-free medium as described previously (19). After conjugation with albumin, the concentration of fatty acids in the solution was measured using a NEFA kit (Wako Chemicals, Inc.). The stock solution was diluted into the final culture medium to obtain concentrations of 1, 10, 50, 100, 200, 400, 600, and 800 μM fatty acid. Palmitate oxidation was measured by the production of $^{14}CO_2$ from [1-^{14}C]palmitic acid as described previously (11) with a few modifications. Briefly, cells were incubated for 1 h in 60 × 15 mm Petri dishes with medium containing 0.2 μCi/ml [1-^{14}C]palmitic acid and nonlabeled palmitate (1, 10, 50, 100, 200, 400, 600, and 800 μM) in the presence or absence of compound C as indicated. Each Petri dish was sealed with Parafilm, which had a piece of Whatman paper taped facing the inside of the Petri dish. After 1 h of incubation, the Whatman paper was wetted with 100 μl of phenylethylamine-methanol (1:1) to trap the CO_2 produced during the incubation period. Subsequently, 200 μl of H_2SO_4 (4 M) was added to the cells, which were then incubated for an additional 1 h at 37°C (11). Finally, the pieces of Whatman paper were carefully removed and transferred to scintillation vials for radioactivity counting.

Western blot determination of P-AMPKα and P-ACC

Cells were grown on six-well plates and incubated for 60 min in the presence or absence of palmitic acid (1, 10, 50, 100, 200, 400,

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600, and 800 μM) and compound C (40 μM) as indicated. Experiments conducted using variable concentrations of ATP revealed that compound C is a potent reversible small-molecule AMPK inhibitor that is competitive with ATP (20). In in vitro assays, compound C did not exhibit significant inhibition of several structurally related kinases, including Zeta-associated Protein Kinase, Spleen Tyrosine Kinase, Protein Kinase C θ, Protein Kinase A, and Janus Kinase 3 (20). AICAR is a compound taken up by the cells and phosphorylated to the monophosphate form ZMP, which can accumulate in the cell, mimicking the effect of AMP on AMPK phosphorylation and activation (1–3). Here, AICAR (2 mM, 60 min) was used as a positive control for AMPK and ACC phosphorylation and also to test the effectiveness of compound C to inhibit AMPK phosphorylation and activation in L6 myotubes. Because ACC is a substrate for AMPK (2, 4), the determination of ACC phosphorylation also served as an indicator of AMPK activity. Immediately after all treatments, cells were lysed in buffer containing 135 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 20 mM Tris, pH 8.0, 1% Triton, 10% glycerol, and protease and phosphatase inhibitors (0.5 mM Na_3VO_4, 10 mM NaF, 1 μM leupeptin, 1 μM pepstatin, 1 μM okadaic acid, and 0.2 mM PMSF), heated (65°C, 5 min), and passed through a 25 gauge syringe five times. An aliquot of the cell lysates was used to determine the protein concentration in each sample by the Bradford method. Before loading onto SDS-PAGE gels, the samples were diluted 1:1 (v/v) with 2 × Laemmli sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 50 mM DTT, and 0.01% (w/v) bromophenol blue]. Aliquots of cell lysates containing 30 μg of

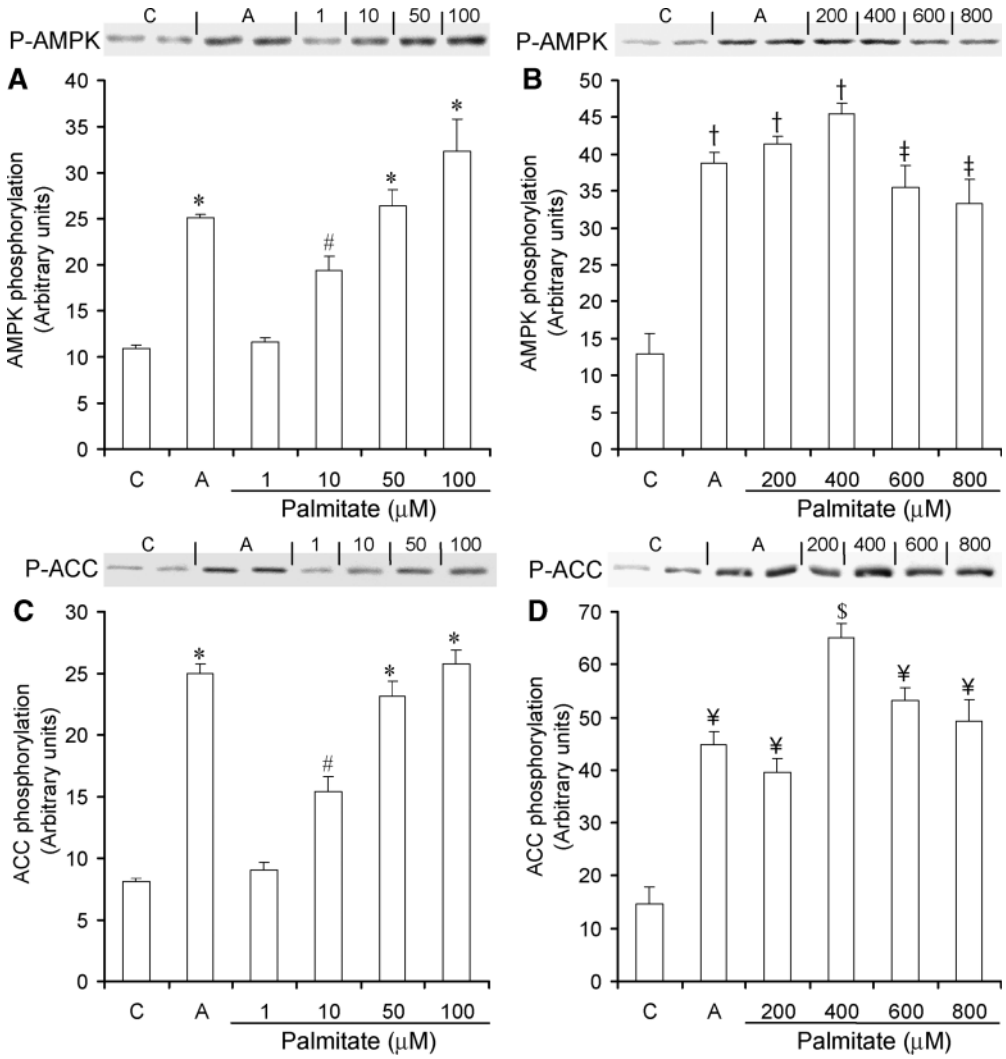

Fig. 1. Dose-response effects of palmitate on AMP-activated protein kinase (AMPK; A, B) and acetyl-coenzyme A carboxylase (ACC; C, D) phosphorylation in L6 myotubes. Densitometric analysis (graphs) and respective representative blots (upper panels) are shown for each experimental condition. Cells were exposed to different concentrations of palmitate (from 1 to 800 μM) for 1 h and then lysed as described in Methods. Control cells (C) were exposed to neither 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; A) nor palmitate. AICAR was used as a positive control for AMPK and ACC phosphorylation. Data are presented as averages ± SEM. * $P < 0.05$ versus control and 1 μM palmitate. # $P < 0.05$ versus control, AICAR, and 1, 50, and 100 μM palmitate. † $P < 0.05$ versus control and 600 and 800 μM palmitate. ‡ $P < 0.05$ versus control and 400 μM palmitate. ¥ $P < 0.05$ versus control and 400 μM palmitate. $ $P < 0.05$ versus all other conditions. Data are compiled from four independent experiments with duplicates in each experiment.

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protein were subjected to SDS-PAGE (12% and 7.5% resolving gels for P-AMPK and P-ACC, respectively) and then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Burlington, Ontario, Canada). The phosphorylation of AMPK was determined using phospho-AMPK(Thr172) antibody (1:1,000 dilution), which detects AMPK-α only when activated by phosphorylation at Thr-172 (Cell Signaling Technology). ACC phosphorylation was detected using phospho-ACC-specific antibody (1:500 dilution; Upstate Biotechnology), which recognizes ACC when phosphorylated at serine 79 (Ser-79). Equal loading of samples was also confirmed by Coomassie blue staining of all gels.

Statistical analysis

Statistical analyses were performed by one-way or two-way ANOVA with the Tukey-Kramer multiple comparison test or Bonferroni posttest. The level of significance was set at $P < 0.05$.

RESULTS

AMPKα and ACC phosphorylation by AICAR and palmitate

As expected, AICAR elicited a 2.5- to 3.5-fold increase in AMPK and ACC phosphorylation compared with the control (**Fig. 1**). Interestingly, the incubation of myotubes with palmitate also caused a significant increase in AMPK and ACC phosphorylation. A significant effect (~1.9-fold) on both AMPK and ACC phosphorylation was observed in the presence of palmitate concentrations as low as 10 μM (Fig. 1A, C). A dose-response effect was found as the concentration of palmitate in the incubation medium was increased (from 1 to 800 μM). However, maximum increases of 3.5-fold for AMPK (Fig. 1B) and 4.5-fold for ACC (Fig. 1D) phosphorylation were obtained at 400 μM compared with the control; (Fig. 1C, D). Higher palmitate concentrations of 600 and 800 μM also induced the phosphorylation of AMPK (2.7- and 2.6-fold, respectively) and ACC (3.7- and 3.4-fold, respectively) compared with the control; however, the values obtained were lower than in the presence of 400 μM palmitate (Fig. 1A–D). These results indicate that either low (10 μM) or high (800 μM) fatty acid concentrations increase both AMPK and ACC phosphorylation in L6 skeletal muscle cells.

Inhibition of AICAR-induced AMPK and ACC phosphorylation by compound C

To determine whether the effects on ACC phosphorylation and fatty acid oxidation were solely attributable to the activation of AMPK by palmitate, we applied a selective AMPK inhibitor (compound C) in our experiments. To establish the efficacy of compound C to inhibit AMPK phosphorylation and activity, we treated L6 myotubes with either 10 or 40 μM of the inhibitor 30 min before exposing the cells to AICAR (2 mM). The inhibitor did not affect basal AMPK (**Fig. 2A**) and ACC (Fig. 2B) phosphorylation levels but significantly blocked AICAR-induced phosphorylation of AMPK and ACC. The use of 10 μM compound C significantly reduced (35% and 50%, respectively) AICAR-induced AMPK and ACC phosphorylation, whereas in the presence of 40 μM compound C, phosphorylation of both AMPK and ACC was completely abolished, reducing it to basal values (Fig. 2A, B). Therefore, 40 μM compound C was chosen to be used in all subsequent experiments.

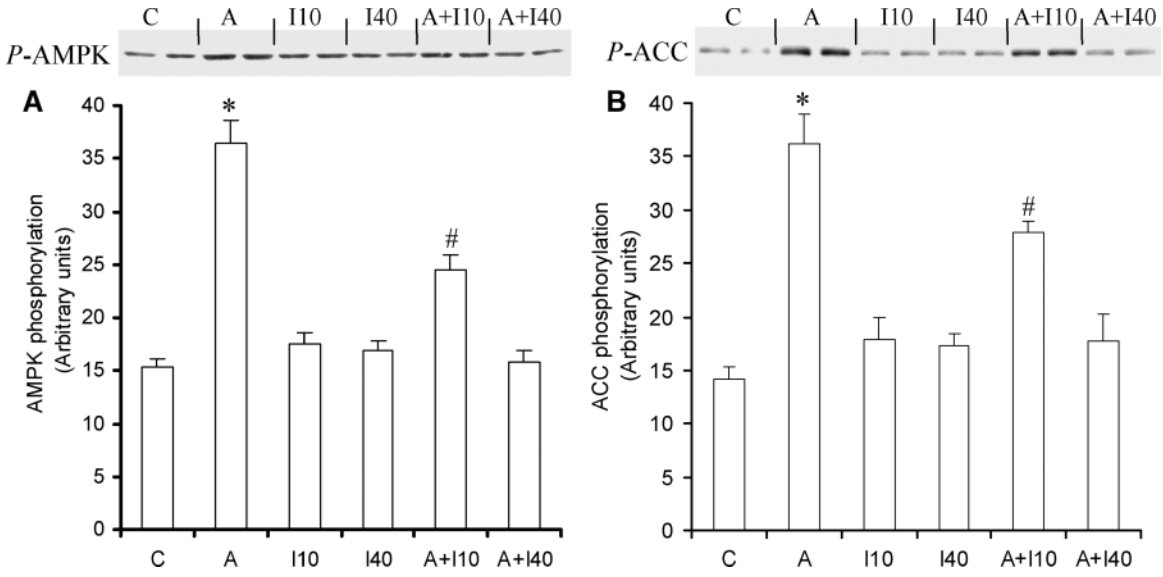

Fig. 2. Effect of compound C on basal [control (C)] and AICAR-induced (A) AMPK (A) and ACC (B) phosphorylation in L6 myotubes. Densitometric analysis (graphs) and respective representative blots (upper panels) are shown for each experimental condition. Thirty minutes before AICAR treatment, cells were exposed to either 10 μM (I10) or 40 μM (I40) compound C, an inhibitor of AMPK. Subsequently, cells were incubated for 60 min in the presence of AICAR (2 mM) as indicated. Data are presented as averages ± SEM. * $P < 0.05$ versus control, AICAR, AICAR + 10 μM inhibitor (A+I10), and AICAR + 40 μM inhibitor (A+I40). # $P < 0.05$ versus control, AICAR, and AICAR + 40 μM inhibitor. Data are compiled from four independent experiments with duplicates in each experiment.

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Effect of compound C on palmitate-induced AMPK and ACC phosphorylation

Palmitate-induced AMPK phosphorylation was blocked by compound C for all fatty acid concentrations ranging from 1 to 800 μM (**Fig. 3A, B**). Interestingly, palmitate-induced ACC phosphorylation was also prevented by compound C in the presence of palmitate concentrations ranging from 1 to 100 μM; however, this AMPK inhibitor did not prevent the palmitate-induced phosphorylation of ACC in the presence of higher concentrations (200–800 μM) of this fatty acid (Fig. 3C, D). In previous experiments, we exposed cells to various concentrations of palmitate in the presence of compound C and AICAR was used as a positive control for AMPK phosphorylation (Fig. 3). However, in those experiments, we did not have data from cells exposed to palmitate alone to serve as a control for those treated with compound C and palmitate in the same experiment. To fulfill this experimental gap, we exposed muscle cells to either 10 or 400 μM palmitate in the absence and presence of compound C. These palmitate concentrations were chosen because in previous experiments both elicited significant increases in the phosphorylation of AMPK and ACC (Fig. 1) but were differently affected by the presence of compound C (Fig. 3). As expected, AMPK phosphorylation was significantly increased in the presence of either 10 μM (1.7-fold) or 400 μM (2.4-fold) palmitate, and it was completely blocked by compound C (data not shown). Also, palmitate at 10 μM induced a significant increase (~1.6-fold) in ACC phosphorylation, which was completely prevented by compound C (**Fig. 4A**). Interestingly, in the presence of palmitate 400 μM ACC, phosphorylation increased by ~2.7-fold and remained equally phosphorylated despite the presence of compound C in the incubation medium

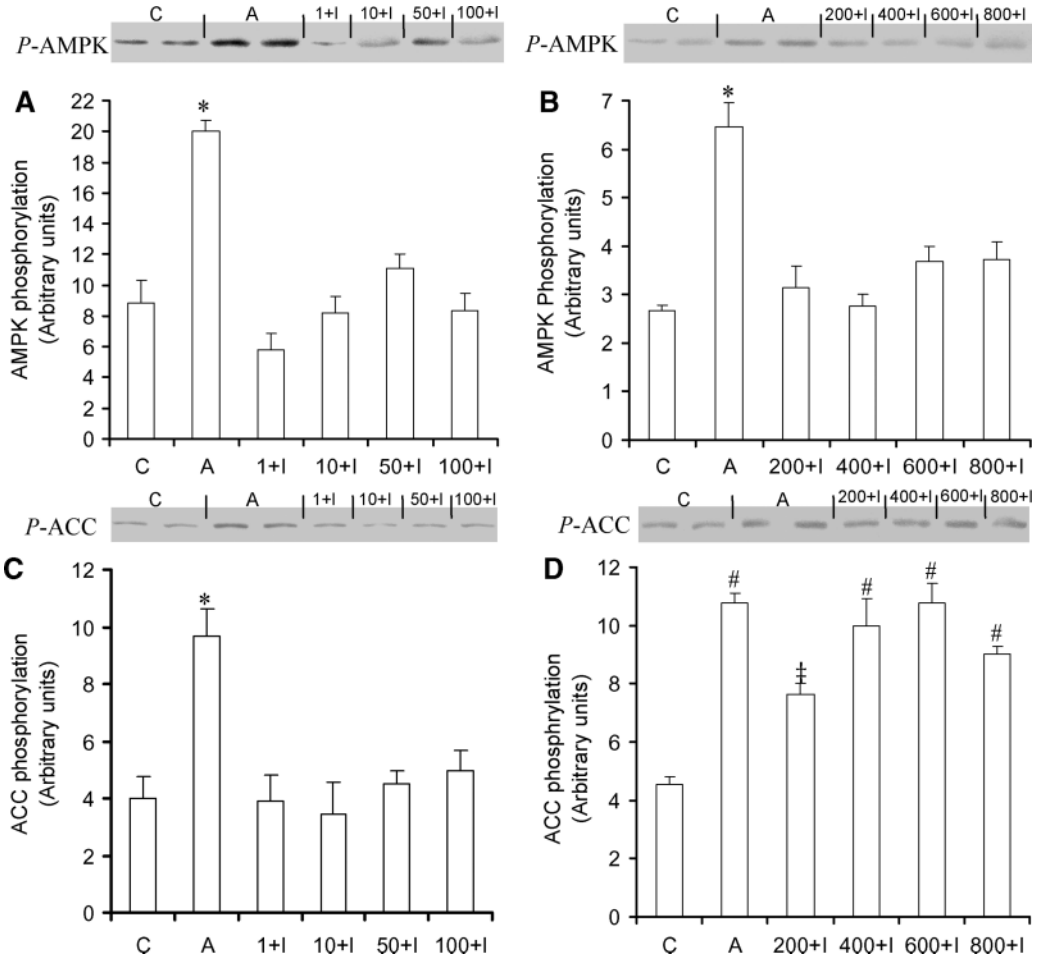

Fig. 3. Effect of compound C (40 μM) on palmitate (1–800 μM)-induced AMPK (A, B) and ACC (C, D) phosphorylation in L6 myotubes. Densitometric analysis (graphs) and respective representative blots (upper panels) are shown for each experimental condition. Compound C (I; an inhibitor of AMPK) was added to the incubation medium 30 min before palmitate treatment and was also present during the entire 1 h palmitate-incubation period. AICAR (A; 2 mM) was used as a positive control for AMPK and ACC phosphorylation. Data are presented as averages ± SEM. * $P < 0.05$ versus all conditions. # $P < 0.05$ versus control and 400 μM palmitate + inhibitor (200+I). ‡ $P < 0.05$ versus control, AICAR, 400 μM palmitate + inhibitor (400+I), and 600 μM palmitate + inhibitor (600+I). Data are compiled from four independent experiments with duplicates in each experiment.

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(Fig. 4B). These results confirm and strengthen the observations that increased concentrations of palmitate exert a regulatory effect on ACC phosphorylation/activity independently of AMPK.

Effects of palmitate, AICAR, and compound C on AMPK/ACC phosphorylation and $^{14}CO_2$ production from [1-^{14}C]palmitate

AICAR and palmitate (10 and 400 μM) induced AMPK phosphorylation, and this was again prevented by compound C. Although ACC phosphorylation was also induced by AICAR and palmitate, compound C only prevented its phosphorylation at the lower concentration (10 μM) of palmitate (**Fig. 5A**). We also determined the phosphorylation state of AMPK and ACC after the cells had been exposed to a combination of AICAR and palmitate. The phosphorylation induced by AICAR and palmitate in this experiment was similar to what was observed for either AICAR or palmitate alone, with no additive effect on AMPK and ACC phosphorylation (Fig. 5A). ACC phosphorylation serves as a good indicator of AMPK activation, whereas LCFA oxidation provides an indication of ACC activity. Because AMPK and ACC phosphorylation play an important role in regulating the rate of fatty acid oxidation in skeletal muscle, we investigated the rates of $^{14}CO_2$ production from [1-^{14}C]palmitate in L6 myotubes. As palmitate concentration in the incubation medium was increased from 1 to 800 μM, the rate of oxidation of this fatty acid also increased significantly, from 12.4 pmol/h (basal; Fig. 5B, inset) to 676.86 nmol/h (800 μM; Fig. 5B). The basal condition had only labeled palmitate (0.2 μCi/ml [1-^{14}C]palmitic acid) in the incubation medium. Even though this does not reflect physiological circulating nonesterified fatty acid levels, it was technically the correct control to give us an idea of the magnitude of the increase in fatty acid oxidation by skeletal muscle cells in the presence of very low to high concentrations of palmitate.

The most accentuated increase in oxidation took place in the presence of palmitate concentrations ranging from 1 to 200 μM (from 3.9 to 487.38 nmol/h). However, as the concentration of palmitate was increased from 400 to 800 μM, the oxidation rate of this fatty acid still increased, but at a much lower rate (from 559.98 to 676.86 nmol/h, respectively) (Fig. 5B). Here, we also tested the effect of AICAR-induced AMPK activation on palmitate oxidation. As expected, in the presence of AICAR (2 mM), we observed a significant increase in $^{14}CO_2$ production either under basal conditions (~1.4-fold; Fig. 5B, inset) or in the presence of up to 100 μM palmitate (1.7-, 2-, 1.6-, and 1.7-fold vs. palmitate alone for 1, 10, 50, and 100 μM palmitate, respectively) (Fig. 5B). However, as palmitate concentration was increased from 200 to 800 μM, the AICAR-induced effect on $^{14}CO_2$ production from [1-^{14}C]palmitic acid was significantly attenuated (1.07-, 1.09-, 1.09-, and 1.03-fold for 200, 400, 600, and 800 μM palmitate, respectively) compared with palmitate alone (Fig. 5B). Interestingly, the addition of compound C significantly blocked the oxidative effect of palmitate alone and of AICAR in the presence of palmitate concentrations ranging from 1 to 100 μM. However, in the presence of higher palmitate concentrations (200, 400, 600, and 800 μM), compound C was not effective in blocking the oxidative responses induced by palmitate alone or in combination with AICAR (Fig. 5B). These results are compatible with the effects of palmitate on AMPK and ACC phosphorylation reported above (Figs. 3, 4). This suggests that under increased concentrations of fatty acids (> 200 μM), an AMPK-independent pathway

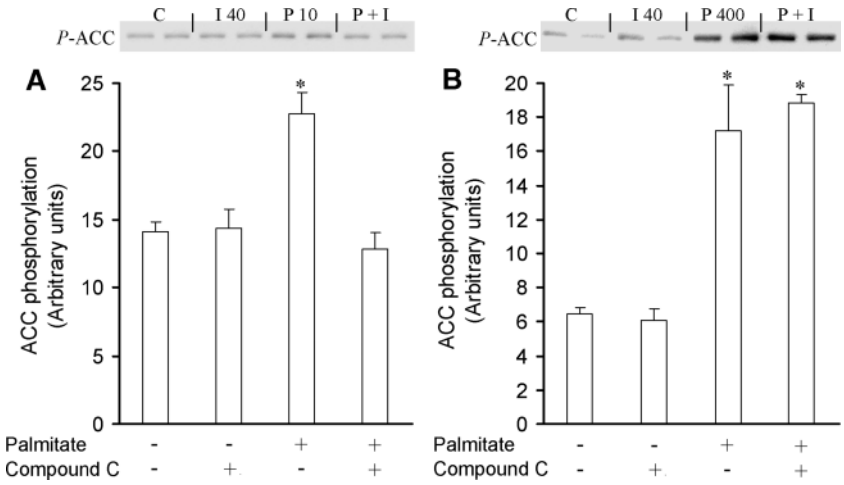

Fig. 4. Effect of compound C (40 μM) on basal [control (C), cells exposed to neither palmitate nor compound C] and palmitate-induced ACC phosphorylation in L6 myotubes. Densitometric analysis (graphs) and respective representative blots (upper panels) are shown for each experimental condition. Compound C (I; an inhibitor of AMPK) was added to the cells 30 min before palmitate treatment and was also present during the entire 1 h palmitate-incubation period. Cells were exposed to either 10 μM (A) or 400 μM (B) palmitate and then lysed for Western blotting. Data are presented as averages ± SEM. * $P < 0.05$ versus control and compound C. Data are compiled from four independent experiments with duplicates in each experiment.

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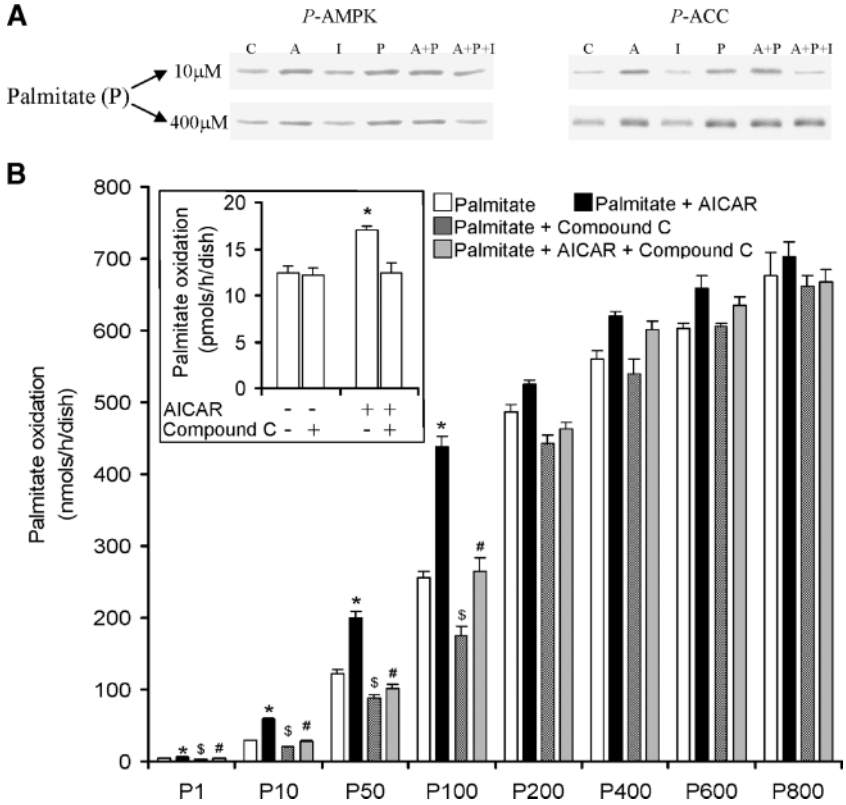

Fig. 5. A: Representative blots of the effects of palmitate (P; 10 and 400 μM), AICAR (A), compound C (I), AICAR + palmitate (A+P), and AICAR + palmitate + compound C (A+P+I) on AMPK and ACC phosphorylation. B: Effects of various palmitate concentrations (ranging from 1 to 800, μM P1 to P800), palmitate + AICAR (2 mM), palmitate + compound C (40 μM), and palmitate + AICAR + compound C on $^{14}CO_2$ production from [1-^{14}C]palmitate (palmitate oxidation; nmol/dish/h) in L6 myotubes. The inset refers to the effects of compound C on basal (in the absence of AICAR and compound C) and AICAR (2 mM)-induced $^{14}CO_2$ production from [1-^{14}C]palmitate (palmitate oxidation; pmol/dish/h). Basal values were determined in the presence of only radiolabeled palmitate (0.2 μCi/ml). Data are presented as averages ± SEM. * $P < 0.05$ versus palmitate, palmitate + compound C, and palmitate + AICAR + compound C. $^{\$}$ $P < 0.05$ versus palmitate and palmitate + AICAR. $^{\#}$ $P < 0.05$ versus palmitate + AICAR. Data are compiled from four independent experiments with triplicates in each experiment.

was responsible for inducing fatty acid oxidation in skeletal muscle cells.

DISCUSSION

Here, we describe the novel finding that AMPK and ACC activity are regulated by palmitate in skeletal muscle cells in a dose-dependent manner. The concentrations of palmitate applied ranged from 1 to 800 μM, which are within the values observed either in physiological conditions such as overnight fasting (~100–500 μM) and prolonged exercise (~500–1,000 μM) or in pathological conditions such as obesity (~600–800 μM) and type 2 diabetes mellitus (~700–900 μM) (21). However, although plasma fatty acids can reach 800 μM, this is unlikely the case for the extracellular fluid to which skeletal muscle is exposed in vivo. This raises the question of whether the treatment of L6 muscle cells to high palmitate concentrations (400–800 μM) could be toxic to the cells and affect cell viability. High levels of palmitate did not alter cell viability when measured by the trypan blue exclusion method, as reported in Methods. Additionally, even though in our system the maximum phosphorylation response of AMPK and ACC was obtained in the presence of 400 μM, the effect on oxidation was increased significantly and remained high as palmitate concentrations were increased up to 800 μM in the incubation medium. This sustained functional response to high levels of palmitate (400–800 μM) indicates that the muscle cells were viable and well responsive under all experimental conditions.

ACC phosphorylation was coupled to AMPK phosphorylation and activation in the presence of palmitate concentrations ranging from 1 to 100 μM. However, at concentrations > 200 μM, ACC phosphorylation remained high, despite the total inhibition of AMPK phosphorylation by compound C, a selective AMPK inhibitor. These results indicated that palmitate regulates ACC phosphorylation/activity by AMPK-dependent and -independent mechanisms, based on its abundance in skeletal muscle cells.

It has been demonstrated previously that the cytoplasmic process of fatty acid activation (formation of fatty

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acyl-CoA) consumes ATP and increases cytosolic AMP by ~30-fold, leading to AMPK activation in isolated hepatocytes (16). Also, activation of heart AMPK in response to physiological concentrations of LCFAs has been reported (15). These findings are in agreement with our results with skeletal muscle cells showing that palmitate increases AMPK phosphorylation and also the phosphorylation of its direct substrate ACC, which are also compatible with the observed increase in fatty acid oxidation. In fact, the rate of palmitate oxidation by skeletal muscle cells increased as the concentration of this fatty acid in the medium was increased up to 400 μM and practically reached a plateau thereafter. Interestingly, inhibition of AMPK phosphorylation by compound C significantly reduced (30%) basal (containing only labeled palmitate: 0.2 μCi/ml [1-^{14}C]palmitic acid) and AICAR-induced (~45%) palmitate oxidation for concentrations of this fatty acid ranging from 0 to 100 μM. However, compound C exerted no significant effect on either basal or AICAR-induced palmitate oxidation as the concentration of this fatty acid was increased from 200 to 800 μM, even though AMPK phosphorylation was prevented by compound C. Additionally, the combination of AICAR and palmitate elicited a significant additive effect on oxidation (~75%) up to 100 μM palmitate, but this effect was practically abolished (~7.5%) when palmitate concentration ranged from 200 to 800 μM. Importantly, no additive effect on AMPK and ACC phosphorylation was observed when cells were exposed to a combination of AICAR and palmitate (either 10 or 400 μM).

These findings indicate that LCFAs autoregulate their metabolism by two separate pathways: one that depends on AMPK activation, and another that directly modulates ACC phosphorylation/activity and overrides the effects of AMPK activation. The concentration of LCFAs in the cell is the major factor determining which of these pathways will prevail in the regulation of fatty acid metabolism in skeletal muscle cells. Previous studies performed with perfused heart muscle (15) and with isolated rat hepatocytes (16) have shown that not only palmitate but also acetate, octanoate, and oleate increase AMPK activity (15, 16) and fatty acid oxidation (15). In our experiments, we used only palmitate, and further studies need to be performed to test whether other fatty acids also regulate the phosphorylation/activity of the AMPK/ACC pathway and β-oxidation in skeletal muscle.

The fact that ACC remained phosphorylated even after compound C prevented palmitate-induced AMPK phosphorylation suggests that fatty acids may also regulate ACC activity by inducing the phosphorylation of this enzyme by an alternative kinase. Additionally, it may be possible that fatty acids inhibit phosphatases that might otherwise dephosphorylate and activate ACC. The inhibition by palmitate of phosphatases that target ACC would divert the metabolism of LCFAs toward oxidation instead of lipid synthesis in the presence of high levels of fatty acids. In fact, in the present study, we also observed that ACC phosphorylation was accompanied by an upregulation of palmitate oxidation in skeletal muscles cells. Further studies are necessary to precisely identify the mechanisms by which fatty acids regulate ACC phosphorylation/activity in skeletal muscle cells and, therefore, autoregulate their metabolic fate.

The AMPK-independent effect of high levels of palmitate on fatty acid oxidation may also have derived from a potent feedback inhibition of ACC by long-chain fatty acyl-CoA esters (2, 4, 22). There is compelling evidence that citrate and acetyl-CoA function as allosteric activators of ACC, whereas long-chain fatty acyl-CoA acts as an allosteric inhibitor opposing the action of citrate on purified rat hindlimb muscle ACC (23). Suppression of lipogenesis by fatty acids has also been demonstrated in extracts of rat epididymal fat pads (5) and in rat perfused livers (6), even though there is little evidence that these were through allosteric inhibition of ACC. In rat skeletal muscle, refeeding after a fast rapidly decreased fatty acid oxidation in vivo, and this has been attributed to a decrease in circulating fatty acid levels releasing allosteric inhibition of ACC in muscle (18). All of these observations are in agreement with our findings, which indicate that fatty acid abundance leads to ACC inhibition and suppression of lipogenesis. From a functional perspective, this seems logical, because it would be unnecessary and counterproductive to maintain the de novo lipid synthesis pathway active under conditions of high cellular fatty acid levels. However, it was reported recently that long-chain acyl-CoA esters inhibit the in vitro phosphorylation and activation of recombinant AMPK by liver purified LKB1/STRAD/MO25, suggesting that in the presence of LCFAs the activation of the AMPK pathway may actually be impaired (17). Even though AMPK activity was measured directly by Taylor et al. (17), the in vitro analysis performed with purified enzymes did not allow complex cellular metabolic interactions of AMPK with its allosteric regulators to take place. This is particularly important because in our experiments using muscle cells, palmitate consistently increased the phosphorylation of AMPK in a dose-dependent manner, whereas palmitate-induced ACC phosphorylation was regulated by AMPK-dependent and -independent mechanisms. From a metabolic perspective, the activity of ACC is what ultimately determines malonyl-CoA production and whether fatty acids will be produced or oxidized in the cell (2, 4). Because ACC activation does not depend exclusively on AMPK phosphorylation, the implications of AMPK regulation by fatty acids have to be analyzed in a more complex and interactive system.

The implications of our findings are that the fatty acid oxidative response that is normally triggered via AMPK activation by endogenous hormones and nutrients, and other pharmacological agonists (9, 10), may be impaired in the presence of high fatty acid concentrations in skeletal muscle. In the presence of fatty acids, the AMPK/ACC system becomes activated and the oxidative response of the muscle cell may reach its maximum capacity, preventing any potential additional effect that could be triggered by other AMPK agonists. In fact, we found that AICAR (a well-known inducer of AMPK activity) did not elicit any additive oxidative effect in L6 muscle cells in the presence

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of palmitate concentrations of > 200 μM. This is particularly important because certain metabolic disorders, such as obesity and type 2 diabetes, are accompanied by chronically high levels of circulating fatty acids (~600–900 μM) (21), which may limit the response of the AMPK/ACC pathway to endogenous and exogenous AMPK agonists. Recently, it was reported that adipokines such as leptin (9, 10) and adiponectin (24) exert direct antilipotoxic effects on skeletal muscle by activating the AMPK/ACC pathway. Resistance to these adipokines could be partially attributable to high levels of fatty acids maximally activating the AMPK/ACC pathway and impairing further activation of the AMPK/ACC pathway by leptin or adiponectin in peripheral tissues. In fact, mice overexpressing leptin in liver are skinny and present increased levels of AMPK and ACC phosphorylation. However, these animals developed obesity when exposed to a high-fat diet accompanied by attenuation of AMPK and ACC phosphorylation (25). Interestingly, the levels of circulating fatty acids in these transgenic animals increased from 1.43 mEq/l on a regular diet to 2.17 mEq/l on the high-fat diet (25). It is important to note that this in vivo study did not establish any cause-and-effect relationship between fatty acids and leptin sensitivity in skeletal muscle, although it suggests a potential link between these variables.

In conclusion, our results provide evidence that fatty acids autoregulate their metabolic fate in skeletal muscle cells by directly regulating the phosphorylation and activation of AMPK and ACC. These effects of fatty acids on skeletal muscle may have important physiological and pathological implications for the response of this tissue to endogenous and exogenous agonists of the AMPK/ACC pathway.

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