5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside-induced AMP-activated Protein Kinase Phosphorylation Inhibits basal and insulin-stimulated glucose uptake, lipid synthesis, and fatty acid oxidation in isolated rat adipocytes.

Mandeep Pinky Gaidhu, Sergiu Fediuc, and Rolando Bacis Ceddia

FROM THE SCHOOL OF KINESIOLOGY AND HEALTH SCIENCE, YORK UNIVERSITY, TORONTO, ONTARIO M3J 1P3, CANADA

The objective of this study was to investigate the effects of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)-induced AMP-activated protein kinase (AMPK) activation on basal and insulin-stimulated glucose and fatty acid metabolism in isolated rat adipocytes. AICAR-induced AMPK activation profoundly inhibited basal and insulin-stimulated glucose uptake, lipogenesis, glucose oxidation, and lactate production in fat cells. We also describe the novel findings that AICAR-induced AMPK phosphorylation significantly reduced palmitate (32%) and oleate uptake (41%), which was followed by a 50% reduction in palmitate oxidation despite a marked increase in AMPK and acetyl-CoA carboxylase phosphorylation. Compound C, a selective inhibitor of AMPK, not only completely prevented the inhibitory effect of AICAR on palmitate oxidation but actually caused a 2.2-fold increase in this variable. Compound C also significantly increased palmitate oxidation in the presence of inhibitory concentrations of malonyl-CoA and etomoxir indicating an increase in CPT1 activity. In contrast to skeletal muscle in which AMPK stimulates fatty acid oxidation to provide ATP as a fuel, we propose that AMPK activation inhibits lipogenesis and fatty acid oxidation in adipocytes. Inhibition of lipogenesis would conserve ATP under conditions of cellular stress, although suppression of intra-adipocyte oxidation would spare fatty acids for exportation to other tissues where their utilization is crucial for energy production. Additionally, the stimulatory effect of compound C on long chain fatty acid oxidation provides a novel pharmacological approach to promote energy dissipation in adipocytes, which may be of therapeutic importance for obesity and type II diabetes.

AMP-activated protein kinase (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). It is formed by a catalytic subunit (α) and two regulatory subunits (β and γ). Multiple isoforms of each mammalian subunit (α1, α2, β1, β2, and γ1–γ3) exist, each encoded by a different gene (1, 2). AMPK subunits are widely expressed in body tissues, including adipose tissue, skeletal muscle, liver, heart, pancreas, and brain (1, 2). AMPK is activated allosterically by a rise in the intracellular AMP/ATP ratio and also by phosphorylation of threonine 172 within the α subunit, catalyzed by the upstream kinase LKB1 (1, 2). When activated, AMPK shuts down anabolic pathways and promotes catabolism by down-regulating the activity of key enzymes of intermediary metabolism (1, 2). Also, by sensing the energy status of the cell, AMPK is a critical metabolic regulator involved in initiating mitochondrial biogenesis (3). Although the importance of AMPK to control glucose and lipid metabolism in skeletal muscle and liver has been well established (1–4), its role in regulating energy storage and depletion in adipocytes has remained controversial and poorly explored. The few in vitro studies published so far investigating the role of AMPK activation in adipocytes have mainly focused on the lipolytic response of these cells (5–8), providing limited information regarding the metabolic fate of glucose and fatty acids under conditions of either acute or chronic AMPK activation in adipocytes.

It has been demonstrated that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a cell-permeable adenosine analog that can be phosphorylated to form 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosil-5’-monophosphate (1, 2), stimulates AMPK activity and causes a reduction in insulin-stimulated glucose uptake (9) in 3T3-L1 adipocytes and de novo lipid synthesis in isolated rat adipocytes (5). Additionally, from an in vivo perspective, it has been reported that mice lacking the AMPK-α2 subunit have enlarged adipocytes because of increased lipid accumulation and higher levels of circulating nonesterified fatty acids under fasted and fed conditions (10).

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3 To whom correspondence should be addressed. Tel.: 416-736-2100 (ext. 77204); Fax: 416-736-5774; E-mail: roceddia@yorku.ca.

The abbreviations used are: AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; BSA, bovine serum albumin; CPT1, carnitine palmitoyltransferase 1; FAT/CD36, fatty-acid translocase; KR1, Krebs-Ringer Buffer; LCFA, long chain fatty acid.
However, because AMPK plays an important role in regulating liver and skeletal muscle metabolism, the in vivo studies in which rodents were treated with the AMPK agonist AICAR (10–12) do not clearly separate the direct from the secondary systemic effects of acute or chronic AMPK activation on adipose tissue.

Based on studies carried out in skeletal muscle and liver, it has been established that, in its phosphorylated and activated state, AMPK leads to phosphorylation and inactivation of its substrate acetyl-CoA carboxylase (ACC) (1, 2, 4). 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 (13, 14). Malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase-1 (CPT1), a rate-limiting step for the entry of long chain fatty acids (LCFA) into the mitochondria for oxidation (13, 14). When ACC is inactive (phosphorylated form), a fall in malonyl-CoA occurs and disinhibits CPT1, thereby increasing mitochondrial import and oxidation of LCFA (13, 14). Therefore, the AMPK-ACC system may be an important pharmacological target to reduce fatty acid storage in adipocytes and to treat obesity. By inducing fatty acid oxidation within the adipocyte, activation of the AMPK-ACC system would reduce fat cell size and also prevent fatty acids from being exported to peripheral tissues and cause deleterious effects ("lipotoxicity"). However, to date, the effects of AMPK activation on fatty acid oxidation in adipocytes have not been addressed. Thus, the experiments outlined here are designed to elucidate the role of AMPK in metabolic partitioning in white adipose tissue. Here we investigated the effects of AICAR-induced AMPK and ACC phosphorylation on glucose and LCFA uptake and metabolism in isolated rat adipocytes. In this study, evidence is provided that AICAR-induced AMPK phosphorylation suppresses basal and insulin-stimulated glucose uptake, fatty acid uptake, lipogenesis, and LCFA oxidation in fat cells. Additionally, we present novel evidence that inhibition of AMPK and ACC phosphorylation by a selective AMPK inhibitor (compound C) induces LCFA oxidation in isolated adipocytes.

**EXPERIMENTAL PROCEDURES**

**Reagents**—AICAR was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada); "compound C" (6-[4-(2-piperidin-1-yl-etoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine), a selective AMPK inhibitor, was provided by Merck; cytochalasin B, digitonin, di-isononyl phthalate, fatty-acid-free albumin, lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), citrate lyase (EC 4.1.3.6), palmitic acid-free albumin and 5.5 mM glucose. The cells were resuspended to 105 cells/ml in KRB containing HEPES (30 mM; KRB-HEPES) and 1% BSA, pH 7.4. Subsequently, cells (2 × 105) were incubated for 1 h. Insulin (100 mM) was added for the final 20 min of the incubation period. Subsequently, KRB-HEPES containing 0.5 mM 2-deoxy-D-glucose and 0.5 μCi of [2-1,2-3H]deoxy-D-glucose was added to the cells for 3 min, and the incubations were terminated by the addition of cytochalasin B (1.5 mM stock solution). Aliquots of cell suspension (240 μl) were quickly placed in plastic microtubes containing 100 μl of di-"isononyl" phthalate. The tubes were centrifuged for 30 s (6000 × g) in order to separate cells from the radioactive incubation medium. Subsequently, fat cells were collected by cutting the tubes through the oil phase and transferred to scintillation vials to be counted for radioactivity. Nonspecific transport was determined in the same conditions, except that cytochalasin B (50 μM final concentration) was added to the medium before the addition of cells. Nonspecific values were subtracted from all conditions.

**Assay for Fatty Acid Uptake**—Adipocytes were isolated as described here using KRB-HEPES containing 0.7% fatty acid-free albumin and 5.5 mM glucose. The cells were resuspended to 30% lipocrit prior to the experiment. Palmitate and oleate uptake was assayed as described previously (18) with a few modifications. Briefly, fat cells (~2 × 105) were incubated either in the absence or presence of AICAR (2 mM) for 1 h (37 °C). Subsequently, palmitic acid and oleic acid uptake was measured at 1-min time intervals between 1 and 5 min. The assay buffer contained either [1-14C]palmitic acid (0.3 μCi/ml) or [1-14C]oleic acid (0.3 μCi/ml). Nonlabeled palmitate (30 μM) or oleate (30 μM) conjugated with fatty acid-free albumin in a molar ratio of 1 was also added to the assay buffer. After the
cells had been exposed to the assay buffer for the desired time periods, an aliquot (240 µl) of cell suspension was transferred to microtubes already containing 100 µl of cold di-'isooonyl' phthalate and quickly centrifuged (13,000 rpm for 30 s) to separate the cells from the radiolabeled incubation medium and to terminate the reaction. Microtubes were kept on ice until fat cells were collected by cutting the tubes through the oil phase and transferred to scintillation vials to be counted for radioactivity. Nonspecific transport was determined in the same conditions, except that ice-cold assay buffer was added to the cells and immediately centrifuged (time 0). Nonspecific values were subtracted from all conditions.

**Conversion of D-[U-14C]Glucose into 14CO2 and Incorporation into Total Lipids**—Glucose oxidation and lipogenesis rates were determined by the conversion of D-[U-14C]glucose into 14CO2 and incorporation into total lipids as described previously (15, 16). Briefly, 0.5 ml of the cell suspension (5% lipocrit, ~2 x 10^5 cells) was incubated for 1 h (37 °C) with shaking in 20-ml plastic scintillation vials containing 1 ml of KRB, 4% BSA/H9262 (0.2 Ci/ml), and insulin (100 nM), AICAR (2 mM), and AICAR plus insulin. During the 1-h incubation period, the conversion of D-[U-14C]glucose into 14CO2 was determined (15, 16). In brief, each 20-ml plastic scintillation flask had a centered isolated well containing a loosely folded piece of filter paper moistened with 0.2 ml of 2-phenylethylamine/methanol (1:1, v/v). After the 1-h incubation period, the medium was acidified with 0.25 ml of H2SO4 (5 n), and the flasks were maintained sealed at 37 °C for an additional 30 min. At the end of this period, filter papers were carefully removed and transferred into scintillation vials for measurement of radioactivity. For the determination of D-[U-14C]glucose incorporated into lipids, the medium was collected and treated with 5 ml of Dole’s reagent (isopropyl alcohol/n-heptane/H2SO4, 4:1:0.25, v/v/v), and the radioactivity present in the total lipid fraction extracted was counted (15, 16).

**Production of 14CO2 from [1-14C]Palmitic Acid and [1-14C]Oleic Acid**—Palmitate and oleate were conjugated with essentially fatty acid-free bovine serum albumin (BSA) to generate a stock solution of 12.5% (w/v) BSA, 6 mM fatty acid in serum-free medium as described previously (15, 16, 19). After conjugation with albumin, the concentration of fatty acids in the solution was measured by using a nonesterified fatty acid (NEFA) kit (Wako Chemicals Inc., Richmond, VA). The stock solution was diluted into the final incubation medium to obtain palmitate and oleate concentrations of 200 µM. Palmitate and oleate oxidation was measured by the production of 14CO2 from [1-14C]palmitic acid (0.2 µCi/ml) and [1-14C]oleic acid (0.2 µCi/ml) with nonlabeled palmitate and oleate present in the medium as described previously (15, 16, 19) with a few modifications. Briefly, cells (5% lipocrit, ~10^7 cells) were incubated for 1 h in 20-ml plastic scintillation flasks in the absence or presence of AICAR (2 mM), compound C (20 µM), and compound C plus AICAR. The flasks had a centered isolated well containing a loosely folded piece of filter paper moistened with 0.2 ml of 2-phenylethylamine/methanol (1:1, v/v). After the 1-h incubation period, the medium was acidified with 0.25 ml of H2SO4 (5 n), and the flasks were maintained sealed at 37 °C for an additional 30 min. At the end of this period, filter papers were carefully removed and transferred into scintillation vials for radioactivity counting.

**Production of 14CO2 from [1-14C]Palmitic Acid in Digitonin-permeabilized Cells**—To test whether compound C exerts its effects on long chain fatty acid oxidation by activating CPT1, we utilized an irreversible (etomoxir) and a reversible (malonyl-CoA) inhibitor of this enzyme (20). Importantly, intact cells are impermeable to malonyl-CoA; therefore, we used digitonin to permeabilize the adipocytes (21). Isolated adipocytes were incubated in KRB, 4% BSA and permeabilized by the addition of digitonin (10 µM) to the incubation medium (22). The cells were preincubated either in the absence or presence of malonyl-CoA (20 µM) and etomoxir (20 µM) for 30 min prior to the addition of compound C (20 µM). Subsequently, the cells were incubated for 1 h in order to measure the production of 14CO2 from [1-14C]palmitic acid as described previously.

**Western Blot Determination of AMPKα1 and -α2, p-AMPK, and p-ACC**—Isolated adipocytes (~10^7 cells) were incubated for 1 h in 15-ml plastic tubes in the absence or presence of insulin (100 nM), AICAR (2 mM), AICAR plus insulin, compound C (20 µM), and compound C plus AICAR. Immediately after all treatments, cells were transferred to microtubes and lysed in buffer containing 135 mM NaCl, 1 mM MgCl2, 2.7 mM KCl, 20 mM Tris, pH 8.0, 1% Triton, 10% glycerol, and protease and phosphatase inhibitors (0.5 mM Na3VO4, 10 mM NaF, 1 µM leupeptin, 1 µM pepstatin, 1 µM okadaic acid, and 0.2 mM phenylmethylsulfonyl fluoride) by vigorously vortexing the vials. Subsequently, the vials were centrifuged (13,000 rpm for 1 min) to separate the upper lipid phase from the lower protein-rich phase. The infranatant was collected with a syringe and transferred to new vials. An aliquot of the cell lysates was used to determine the concentration of protein in each sample by the Bradford method. Prior to loading onto SDS-polyacrylamide gels, the samples were diluted 1:1 (v/v) with 2x Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 50 mM dithiothreitol, 0.01% w/v bromphenol blue). Aliquots of cell lysates containing 40 µg of protein were then 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). The phosphorylation of AMPK was determined by using a phospho-AMPK (Thr-172) antibody (1:1000 dilution) that detects AMPK-α only when activated by phosphorylation at threonine 172. ACC phosphorylation was detected using a phospho-ACC(Ser-79)-specific antibody (1:1000 dilution), which recognizes ACC when phosphorylated at Ser-79 (19). The presence of the catalytic subunits of α1 and α2 isoforms of AMPK was determined by using specific antibodies (1:1000 dilution). Equal loading of samples was also confirmed by Coomassie Blue staining of all gels.

**Determination of Lactate Production and Citrate Content**—Following a 1-h incubation period either in the absence or presence of insulin (100 nM), AICAR (2 mM), and AICAR plus insulin, the media were collected, deproteinized, and neutralized for subsequent lactate and citrate determination. The total lactate released in the medium was measured as described previously (23) using the lactate oxidase assay kit from Sigma. Citrate was assayed enzymatically according to the method of Moel lering and Gruber (24). Briefly, following the 1-h incubation period
either in the absence or presence of insulin (100 nM), AICAR (2 mM), and AICAR plus insulin (A + I), cells were disrupted in perchloric acid (0.6 N) by mechanical agitation (vortexing). Subsequently, tubes were centrifuged (3000 rpm, 1 min), and the upper fat-cake phase was discarded. An aliquot of the lower phase was collected, neutralized with KOH (2 N), and placed on ice for 15 min to precipitate the potassium chlorate formed. Tubes were centrifuged again (10,000 rpm, 30 s), and the supernatant was used for subsequent spectrophotometric analysis of citrate (24).

Statistical Analysis—Statistical analyses were performed by either one- or two-way analysis of variance with Tukey-Kramer multiple comparison or Bonferroni post-hoc tests. The level of significance was set at \( p < 0.05 \).

RESULTS

Effects of AICAR, Insulin, and AICAR Plus Insulin on Glucose Uptake, Incorporation of \( \delta-[U-^{14}C] \) Glucose into Total Lipids, Production of \( ^{14}CO_2 \) from \( \delta-[U-^{14}C] \) Glucose, and Determination of Lactate and Citrate Content—As expected, insulin caused a significant ~3.5-fold increase in glucose uptake and also elicited a significant ~1.6-fold increase in \( \delta-[U-^{14}C] \) glucose incorporation into total lipids (Fig. 1, A and B), ~1.4-fold increase in \( ^{14}CO_2 \) production from \( \delta-[U-^{14}C] \) glucose (Fig. 1C), and almost ~1.8-fold increase in lactate production (Fig. 1D) in adipocytes. Interestingly, the exposure of adipocytes to AICAR significantly reduced basal glucose uptake and the incorporation of \( \delta-[U-^{14}C] \) glucose into total lipids to ~50 and ~25% of the control values, respectively (Fig. 1, A and B). Furthermore, preincubation of isolated adipocytes with AICAR completely prevented the insulin-induced effect on glucose uptake and reduced the incorporation of glucose into lipids to ~36% of the control values (Fig. 1, A and B). AICAR significantly reduced the basal production of \( ^{14}CO_2 \) from \( \delta-[U-^{14}C] \) glucose to ~58% of control values and also completely abolished the insulin-induced effect on this variable (Fig. 1C). The basal rate of lactate production by the adipocytes was not affected, but the insulin-stimulated response was completely abolished by AICAR (Fig. 1D). As expected, citrate content increased under insulin-stimulated conditions, and this effect was abolished in the presence of AICAR (Table 1). However, no changes in basal levels of citrate in the presence of AICAR were detected. These results indicate that AICAR-induced AMPK activation suppresses basal and insulin-induced lipid synthesis and glucose metabolism in adipocytes.

Identification of the Presence of Both \( \alpha_1 \) and \( \alpha_2 \) Subunits of AMPK in Adipocytes and Inhibition of AICAR-induced AMPK and ACC Phosphorylation by Compound C—Both \( \alpha_1 \) and \( \alpha_2 \) catalytic subunits of AMPK were detected in isolated rat adipocytes (Fig. 2A). As expected, we observed that AICAR induced a significant increase (~2.5-fold) in AMPK catalytic subunits (Fig. 2B). Because ACC is a substrate for AMPK, we also measured ACC phosphorylation to demonstrate that AICAR-induced AMPK phosphorylation was followed by its activation. In fact, exposure of isolated adipocytes to AICAR caused a significant increase (~2-fold) in ACC phosphorylation (Fig. 2B), which confirms the activation of AMPK. Importantly, to test whether the effects of AICAR on glucose and lipid metabolism in adipocytes were solely due to the activation of AMPK, we applied a selective AMPK inhibitor (compound C) in our experiments. Experiments conducted using variable concentrations of ATP have revealed that compound C is a potent, reversible small molecule AMPK inhibitor that competes with ATP-binding sites (25). 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\( \theta \), protein kinase A, and Janus kinase 3 (25). Compound C (20 \( \mu \)M) was added to the cells 30 min prior to and during the entire 1-h incubation period with AICAR. The inhibitor did not affect basal AMPK and ACC phosphorylation but completely prevented AICAR-induced phosphorylation of both AMPK and ACC (Fig. 2B). Therefore, 20 \( \mu \)M compound C was chosen to be used in all subsequent experiments. The incubation of adipocytes with insulin did not affect either basal or AICAR-induced AMPK phosphorylation (Fig. 2C) but markedly reduced basal ACC phosphorylation in adipocytes to

**TABLE 1**

| Condition       | Citrate (nmol/mg protein) |
|-----------------|---------------------------|
| Control         | 3.29 ± 0.40               |
| Insulin         | 10.16 ± 1.03*             |
| AICAR           | 3.07 ± 0.57               |
| AICAR + insulin | 5.01 ± 0.49               |

* \( p < 0.05 \) compared with all conditions.
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FIGURE 2. A, identification of the α-1 and α-2 subunits of AMPK in adipocytes. B, effects of compound C (C, 20 μM, selective AMPK inhibitor) on AICAR-induced AMPK and ACC phosphorylation in isolated adipocytes. Effects are shown of insulin (Ins, 100 nM), AICAR (A, 2 mM), and AICAR plus insulin (A+I) on AMPK (C) and ACC phosphorylation (D) in isolated adipocytes. Compound C was added to the cells 30 min prior to the addition of AICAR and was also present in the medium during the entire 1-h incubation period with AICAR. Control cells (Con) were neither exposed to insulin nor to AICAR. Densitometric analysis (graphs) and respective representative blots (upper panels) for each experimental condition are shown (C and D). * p < 0.05 versus control and insulin, # p < 0.05 versus control, AICAR, and AICAR + insulin, § p < 0.05 versus control, insulin, and AICAR + insulin. Data pooled from 3 to 4 independent experiments with duplicates in each experiment are shown.

~17% of control levels (Fig. 2D). Even though the combination of AICAR and insulin caused a slight but significant reduction (~20%) in AICAR-induced ACC phosphorylation, it still remained significantly elevated (~2-fold) compared with control adipocytes (Fig. 2D).

Effects of AICAR and Compound C on AMPK and ACC Phosphorylation, and the Production of 14CO2 from [1-14C]Palmitic Acid and [1-14C]Oleic Acid—The production of 14CO2 from [1-14C]palmitic acid was significantly reduced (~50%) in AICAR-treated isolated adipocytes (Fig. 3A). Interestingly, preincubation of adipocytes with compound C not only prevented AICAR-induced suppression of oxidation but also significantly raised (~2.2-fold) basal production of 14CO2 from [1-14C]palmitic acid (Fig. 3A). To test whether the effects of AMPK on fatty acid oxidation were exclusive to palmitate, we also carried out similar experiments to measure oleate oxidation. The production of 14CO2 from [1-14C]oleic acid was also lower in cells treated with AICAR but did not reach statistical significance (Fig. 3B). However, preincubation of fat cells with compound C significantly increased (1.7-fold) the production of 14CO2 from [1-14C]oleic acid in AICAR-treated adipocytes (Fig. 3B). Therefore, palmitate and oleate oxidation seem to be similarly affected by AMPK activation and inhibition in isolated fat cells. As expected, AICAR caused significant 4.5- and 3-fold increases in AMPK (Fig. 3C) and ACC (Fig. 3D) phosphorylation, respectively, in isolated adipocytes. Compound C completely prevented AICAR-induced AMPK phosphorylation (Fig. 3C), and this was followed by a significant reduction (~50%) in AICAR-induced ACC phosphorylation (Fig. 3D).

Dose-response Effect of Compound C on the Production of 14CO2 from [1-14C]Palmitic Acid—To further confirm the effect of compound C on fatty acid oxidation, we performed a dose-response experiment in isolated adipocytes. The production of 14CO2 from [1-14C]palmitic acid was significantly increased by ~2.4-, 2.7-, 3.2- and 3.7-fold in the presence of 10, 20, 30, and 40 μM of compound C, respectively (Fig. 4). We have also found similar results for [1-14C]oleic acid oxidation (data not shown). These results clearly demonstrate that compound C increases the oxidation of LCFA Ms in isolated adipocytes.

Effects of AICAR on Palmitate and Oleate Uptake—To investigate whether the reduction in long chain fatty acid oxidation resulted from AICAR-induced reduction in fatty acid uptake, we exposed fat cells to palmitate and oleate for periods of time varying from 1 to 5 min. Interestingly, incubation of isolated adipocytes with AICAR caused average reductions of 32 and 41% in palmitate and oleate uptake, respectively. Isolated adipocytes reduced by 20, 30, 40, and 40% palmitate and by 42, 55, 41, 41, and 28% oleate uptake after 1–5 min of exposure to these fatty acids, respectively (Fig. 5, A and B).

Effects of Compound C on the Production of 14CO2 from [1-14C]Palmitic Acid in Digitonin-permeabilized Adipocytes—As expected, the production of 14CO2 from [1-14C]palmitic acid by isolated adipocytes increased by ~2.5-fold in the presence of compound C (20 μM). Malonyl-CoA and etomoxir reduced
palmitate oxidation to ~40 and ~30% of the control values, respectively (Fig. 6). Interestingly, compound C significantly increased (~1.8-fold relative to control) palmitate oxidation in cells exposed to malonyl-CoA. Also, compound C prevented etomoxir-induced reduction in palmitate oxidation but did not increase it beyond control values (Fig. 6).

**Effects of AICAR and Compound C on the Production of $^{14}$CO$_2$ from d-[1-$^{14}$C]Glucose**—To test whether the oxidative effect of compound C was specific to fatty acids, we also measured the production of $^{14}$CO$_2$ from d-[1-$^{14}$C] glucose by isolated adipocytes. As demonstrated previously, AICAR induced a significant reduction in glucose oxidation to values ~50% of those observed in control cells (Fig. 7). Interestingly, compound C had no effect on basal levels of glucose oxidation and did not prevent the significant inhibitory effect of AICAR on this variable (Fig. 7). This indicates that the effect of compound C seems to be specific to the fatty acid oxidative pathway in isolated adipocytes.

**DISCUSSION**

Previous in vitro studies have focused on the role of AMPK activation in basal and catecholamine-induced lipolysis in fat cells using AICAR as an AMPK agonist (5–8). Thus far, very little has been published regarding the metabolic fate of glucose and fatty acids in these studies. Therefore, we investigated the effects of AICAR-induced AMPK and ACC phosphorylation on several parameters of basal and insulin-stimulated glucose and fatty acid metabolism in isolated rat cells. We provide evidence that phosphorylation/activation of AMPK by AICAR profoundly inhibits basal and insulin-stimulated glucose uptake, incorporation of glucose into lipids, glucose oxidation, and lactate production in isolated rat adipocytes (Fig. 1, A–D). These data suggest that the AMPK signaling cascade opposes insulin effects and may be a modulator of glucose uptake and metabolism in fat cells. Our results are in agreement with previous studies showing that AICAR-induced AMPK activation inhibits insulin-stimulated GLUT4 translocation and glucose transport in 3T3-L1 adipocytes (9), and the incorporation of glucose into lipids in isolated rat adipocytes (5). Inhibition of glucose uptake by AICAR-induced AMPK activation could reduce glycolysis and lactate production, and limit the availability of substrate for de novo lipid synthesis and fatty acid esterification in adipocytes. Also, AICAR-induced AMPK activation causes phosphorylation and inactivation of ACC, which is a powerful mechanism to suppress lipogenesis (1, 2, 5). All together, these mechanisms could, at least partially, explain the reduction in glucose uptake, lipogenesis, glucose oxidation, and lactate production with AICAR treatment as observed in our experiments (Fig. 1, A–D).

Here, we also describe the novel findings that AICAR-induced AMPK and ACC phosphorylation does not cause an increase in LCFA oxidation in isolated adipocytes. This was surprising given the fact that previous studies carried out in skeletal muscle and liver (2, 26) have demonstrated that AICAR-induced AMPK activation stimulates LCFA oxidation. In its phosphorylated/activated state, AMPK phosphorylates serine residues 79, 1200, and 1215 of ACC, producing an 80–90% decrease in the $V_{\text{max}}$ of the enzyme, suggesting that...
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AMPK is the physiological regulator of ACC activity (13). We expected that by phosphorylating and inactivating ACC, AMPK activation would reduce intracellular malonyl-CoA levels, dis inhibit CPT1, and actually increase the mitochondrial import and oxidation of LCFA s in isolated fat cells; however, this was not the case. In fact, we found that palmitate oxidation was significantly reduced (~50%) despite a marked increase in AMPK and ACC phosphorylation in fat cells acutely exposed to AICAR (Fig. 3, A–D). This reduction in fatty acid oxidation was compatible with a significant reduction in palmitate and oleate uptake (average of ~32 and 41%, respectively; Fig. 5, A and B) also observed here. In this context, restricted availability of intracellular palmitate and oleate must have limited oxidation of these fatty acids in isolated adipocytes.

The mechanisms involved in the inhibition of LCFAs uptake and oxidation remain to be determined. Here, we speculate that AMPK may be affecting FAT/CD36, a key protein involved in regulating the uptake of fatty acids across the plasma membrane in several tissues, including adipose tissue (27). FAT/CD36 is recycled between an intracellular membrane compartment and the sarcolemma and can be translocated to assist with LCFAs uptake (28). FAT/CD36 also appears to be involved in mitochondrial fatty acid oxidation, and at least in skeletal muscle, it seems to be necessary for facilitating movement of LCFAs toward the mitochondria (29). Therefore, regulation of FAT/CD36 trafficking by AMPK may dictate the metabolic fate of LCFAs entering the adipocytes and play an important role regulating energy storage and dissipation in these cells. Additionally, it is also possible that other fatty acid transporters such as fatty acid-binding plasma membrane protein, fatty acid-binding cytoplasmic protein, fatty acid transport protein, and fatty acid transporter 1 (30) that may work with FAT/CD36 synergistically to enhance fatty acid uptake are affected by AMPK. However, additional studies are necessary to confirm these hypotheses.

To further investigate the mechanisms of this inhibitory effect of AICAR on fatty acid oxidation, we used compound C to inhibit AMPK phosphorylation. In our experiments, compound C completely prevented AICAR-induced AMPK phosphorylation and also caused a significant reduction in ACC phosphorylation (Fig. 2B). Interestingly, incubation of isolated fat cells with compound C prior to exposure to AICAR not only completely prevented the inhibitory effect of AICAR on palmitate oxidation but actually caused a 2.2-fold increase in this variable compared with control cells (Fig. 3A). This creates a paradoxical condition, which raises the following question: How could there be an increase in LCFA oxidation in adipocytes in the setting of elevated levels of malonyl-CoA as a result of AMPK inhibition and ACC activation?

Even though inhibition of CPT1 activity and impairment of mitochondrial LCFA import and oxidation are expected to occur in the presence of high intracellular malonyl-CoA concentrations (13, 14), we have to take into consideration the fact that glucose uptake was profoundly inhibited by AICAR (Fig. 1A), and inhibition of AMPK by compound C did not prevent AICAR-induced suppression of glucose oxidation (Fig. 7) in our experiments. This means that the availability of glucose and production of substrate for malonyl-CoA synthesis (citrate efflux from mitochondria) remained at very low levels, although ACC phosphorylation was reduced by inhibiting AMPK. Citrate has been demonstrated to cause ACC polymerization and function as a feed-forward activator of ACC at physiological cellular concentrations (13, 31). Here, we report that in insulin-stimulated cells, AICAR-induced AMPK activation caused a profound decrease in citrate content (Table 1). Interestingly, we also expected a decrease in citrate levels with AICAR under basal conditions, because basal glucose uptake and oxidation remain to be determined.
increase in the presence of 40 
2.4-fold increase in palmitate oxidation reaching 3.7-fold

LCFA oxidation: (a) the fact that compound C alone caused
~2.2- and 1.7-fold increases in basal oxidation rates of palmitate and oleate, respectively (Fig. 3, A and B). (b) Exposure of isolated adipocytes to varying concentrations of compound C (10–40 μM) elicited a dose–response effect on palmitate oxidation (Fig. 4); we found that even in the presence of the lowest concentration of compound C (10 μM), adipocytes elicited a 2.4-fold increase in palmitate oxidation reaching 3.7-fold increase in the presence of 40 μM. (c) Compound C increased palmitate oxidation in the presence of malonyl-CoA and etomoxir (Fig. 6), two well known inhibitors of CPT1 activity (20, 32). Compound C increased palmitate oxidation in cells exposed to malonyl-CoA by ~1.8-fold relative to control, and completely prevented the reduction in palmitate oxidation induced by etomoxir (Fig. 6). All together, these findings indicate that compound C, besides inhibiting AMPK phosphorylation/activation, is also independently stimulating the oxidation of LCFA by directly regulating rate-limiting steps of fatty acid oxidation in adipocytes. Compound C seems to be exerting a dual role as an AMPK inhibitor and a CPT1 agonist in fat cells. This could explain the paradoxical increase in LCFA oxidation in the presence of potentially elevated levels of malonyl-CoA in our experiments. In fact, a similar effect has been described in 3T3-L1 adipocytes incubated with C75, an inhibitor of fatty-acid synthetase (22). Inhibition of fatty-acid synthetase by C75 leads to accumulation of malonyl-CoA, which should suppress LCFA oxidation. However, the opposite effect has been demonstrated in 3T3-L1 adipocytes, because C75 stimulated palmitate oxidation, increased intracellular ATP levels, and raised CPT1 activity. Importantly, CPT1 activity was elevated by C75 even in the presence of inhibitory concentrations of malonyl-CoA (22).

Based on our results, we propose that in contrast to skeletal muscle and liver, in which AMPK stimulates fatty acid oxidation to provide ATP as a fuel (1, 2, 26), AMPK activation inhibits de novo lipid synthesis and fatty acid oxidation in adipocytes. Inhibition of lipogenesis would conserve ATP under conditions of cellular stress, although suppression of intra-adipocyte oxidation would spare fatty acids for exportation to other tissues (i.e. skeletal muscle and liver) where oxidation is crucial for energy production. In fact, one of the major functions of white adipose tissue is to release nonesterified fatty acids to provide energy during fasting and exercise (33). Interestingly, it has been reported that under fasting and exercise conditions (8, 34) AMPK activity is increased in white adipose tissue. Activation of AMPK either under fasting or exercise is compatible with an energy-sparing effect of AMPK instead of energy dissipation in white adipocytes.

When comparing skeletal muscle and adipose tissue, the effects of AMPK activation on glucose and fatty acid uptake and metabolism are very paradoxical. In fact, these opposite effects of AMPK activation in different tissues may play a very important role in regulating substrate flux and whole-body energy distribution. The major flux of glucose and fatty acids in the fat cell is toward storage in triglycerides, whereas in skeletal muscles oxidation and energy production are expected to be more prevalent. In this scenario, an opposite role of AMPK in regulating metabolic fluxes seems very compatible with the proposed role of this enzyme as a cellular energy sensor. This is especially important considering that, together, fat tissue and skeletal muscles represent ~55 to ~70% of total body mass of healthy men and women, respectively (35). Therefore, even though metabolic alterations caused by AMPK activation in isolated tissues may seem small and paradoxical, they may be of great physiological relevance when looking at whole-body metabolism. Also in this context, the stimulatory effect of compound C on LCFA oxidation reveals a novel pharmacological approach to promote energy dissipation within adipocytes, which may be of great therapeutic importance for obesity and type II diabetes.

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