Genetic downregulation of AMPK-α isoforms uncovers the mechanism by which metformin decreases FA uptake and oxidation in skeletal muscle cells

Lindsey D. Bogachus1,2 and Lorraine P. Turcotte1,2

1 Department of Biological Sciences and 2 Department of Kinesiology, College of Letters, Arts and Sciences, University of Southern California, Los Angeles, California

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Bogachus LD, Turcotte LP. Genetic downregulation of AMPK-α isoforms uncovers the mechanism by which metformin decreases FA uptake and oxidation in skeletal muscle cells. Am J Physiol Cell Physiol 299: C1549–C1561, 2010. First published September 15, 2010; doi:10.1152/ajpcell.00279.2010.—Metformin is known to improve insulin sensitivity in part via a rise in AMP-activated protein kinase (AMPK) activity and alterations in muscle metabolism. However, a full understanding of how metformin alters AMPK-α1 vs. AMPK-α2 activation remains unknown. To study this question, L6 skeletal muscle cells were treated with or without RNAi oligonucleotide sequences to downregulate AMPK-α1 or AMPK-α2 protein expression and incubated with or without 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or metformin and/or insulin. In contrast to AICAR, which preferentially activated AMPK-α2, metformin preferentially activated AMPK-α1 in a dose- and time-dependent manner. Metformin increased (P < 0.05) glucose uptake and plasma membrane (PM) Glut4 in a dose- and time-dependent manner. Metformin significantly reduced palmitate uptake and oxidation (P < 0.05), and this was accompanied by a similar decrease (P < 0.05) in PM CD36 content but with no change in acetyl-CoA carboxylase (ACC) phosphorylation (P > 0.05). AICAR and metformin similarly increased (P < 0.05) nuclear silent mating-type information regulator 2 homolog 1 (SIRT1) activity. Downregulation of AMPK-α1 completely prevented the metformin-induced reduction in palmitate uptake and oxidation but only partially reduced the metformin-induced increase in glucose uptake. Downregulation of AMPK-α2 had no effect on metformin-induced glucose uptake, palmitate uptake, and oxidation. The increase in SIRT1 activity induced by metformin was not affected by downregulation of either AMPK-α1 or AMPK-α2. Our data indicate that, in muscle cells, the inhibitory effects of metformin on fatty acid metabolism occur via preferential phosphorylation of AMPK-α1, and the data indicate that cross talk between AMPK and SIRT1 does not favor either AMPK isoform.

AMP-activated protein kinase; fatty acid; glucose uptake; 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, silent mating-type information regulator 2 homolog 1; fatty acid transporter/CD36; Glut4

INSULIN RESISTANCE IS A SERIOUS medical condition and has been shown to be attributable, in part, to the dysregulation of glucose and fatty acid (FA) metabolism (63). Clinical interventions for the restoration of insulin sensitivity often include pharmacological treatment with a biguanide metformin, which effectively increases insulin sensitivity with chronic use (4). Metformin has been shown to be a potent activator of AMP-activated protein kinase (AMPK), a signaling intermediate known to modulate glucose and FA metabolism in most cells (17, 74). Because AMPK is a key metabolic regulator (69), it may be theorized that the activation of AMPK by metformin could be a key cellular mechanism by which this biguanide mediates its insulin-sensitizing effect. Given that AMPK activation via 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment has been shown to trigger an increase in FA oxidation in incubated or perfused skeletal muscle preparations (1, 65), it may be hypothesized that metformin would be associated with a rise in FA uptake and oxidation. However, early studies using respiratory exchange ratio as a measurement of whole body FA oxidation in patients with type 2 diabetes have shown that metformin treatment was associated with a decrease in FA oxidation (50, 57). Furthermore, very little is known about the effects of metformin on FA uptake per se and whether metformin treatment would improve insulin action on FA metabolism. Overall, the effects of metformin-induced AMPK activation on FA uptake and oxidation in skeletal muscle remain to be clearly determined. These effects of metformin are important to determine if the cellular mechanisms of action of metformin on insulin sensitivity are to be deciphered.

AMPK is a serine-threonine kinase that consists of a heterotrimeric complex with a catalytic α-subunit and regulatory β- and γ-subunits (69). Stimulation of AMPK activity is associated with an increase in the phosphorylation state of the threonine 172 residue of the α-subunit, of which two isoforms (AMPK-α1 and AMPK-α2) are known. AMPK-α1 and AMPK-α2 are expressed differently in various tissues, and they have been shown to control different metabolic actions (69). AMPK stimulation is generally, although not exclusively (26), triggered by a decrease in the energy state of the cell as characterized by an increase in the AMP/ATP ratio (59). The cellular effects of AMPK activation are dependent in part on whether the AMPK-α1 or α2 isoform is stimulated. Some studies have shown that the effects of metformin are reduced in cells treated with an AMPK inhibitor or in cells expressing a dominant negative AMPK isoform (66, 72). However, these results are somewhat incomplete because it is still unknown whether metformin preferentially activates one AMPK isoform (AMPK-α1 vs. AMPK-α2) in skeletal muscle cells and whether preferential activation of one AMPK isoform similarly affects glucose uptake and/or FA uptake and oxidation.

Multiple lines of evidence show that the role of AMPK in metabolic regulation occurs via interactions with several other signaling intermediates (59, 71). Of particular interest to us was the interaction between AMPK and the transcription factor silent mating-type information regulator 2 homolog 1 (SIRT1) (59). SIRT1 is a NAD+-dependent deacetylase that has traditionally been investigated in aging and longevity research but recently has emerged as a potential metabolic regulator (11, 59). More specifically, data have shown that LKB1 may
Antibiotics were taken out because they are toxic to cells during the AMPK-SIRT1 interaction between AMPK and SIRT1. To accomplish these, otic-antimycotic solution (Sigma Aldrich, St. Louis, MO), and 500 mL of culture medium were incubated for 48 h with antibiotics-free medium. This treatment was then combined with antibiotic-free medium and cultured in the Department of Physiology and Biophysics, University of South California, for 10 days postconfluent on the day of the experiment. In RNAi-treated L6 myotubes, RNAi treatment was initiated 2 days after subculturing into 6-well plates. Given that RNAi treatments lasted 24 h and that the maximum incubation time for a metformin treatment was 24 h, cells were 1 day postconfluent for experiments that were performed 2 days after initiation of the RNAi treatment.

Cell treatments. Before all experimental treatments, cells were preincubated with serum-free medium for 5 h, followed by incubation with Krebs Ringer Hepes buffer (KRb) (1.47 mM K2HPO4, 140 mM NaCl, 1.7 mM KCl, 0.9 mM CaCl2, 0.9 mM MgSO4, and 20 mM Hepes; pH 7.4) for 30 min. Cells were then preexposed to either AICAR (Sigma Aldrich) (AICAR: 2 mM, 2 h) or metformin (Sigma-Aldrich) (metformin: 2 mM, 3 h; metformin: 0.8 mM, 24 h; metformin: 2 mM, 24 h) or vehicle (KRb) until all treatments were completed. Cells were then exposed to either insulin (100 nM; Novolin Insulin, University of Southern California Pharmacy) or vehicle (KRb) for 15 min. The specific metformin treatment conditions described above were selected because they have been used extensively by others and would then allow us to make comparisons (17, 28, 36, 72). The cells were then harvested in lysis buffer for Western Blot analysis (see below) or subjected to the palmitate uptake and oxidation assay.

RNAi transfection. L6 myoblasts were plated in six-well plates and incubated for 48 h with antibiotics-free medium containing 2% FCS. Antibiotics were taken out because they are toxic to cells during the transfection process (manufacturer’s protocol; Invitrogen, Grand Island, NY). The cells were then transfected with RNAi using the protocol recommended by the manufacturer of the transfection reagent (Lipofectamine 2000; Invitrogen). Briefly (per well), Lipofectamine 2000 (100 pmol) and the RNAi sequence (100 pmol; described below) were each combined with antibiotic-free medium containing 2% FCS. The Lipofectamine/medium and RNAi/medium solutions were then combined and incubated (23°C, 20 min). The antibiotic-free medium was then removed from each cell-containing well and replaced by the freshly made RNAi/Lipofectamine/medium cocktail. The plates were placed in a warm (37°C) shaking water bath for 4–6 h. After the transfection incubation, the medium was diluted with antibiotic-free medium containing 2% FCS and the plates were placed back in the humidified incubator (95% O2, 5% CO2) for 2 more days. The cells were then treated with AICAR (2 mM, 2 h) or metformin (2 mM, 3 h) and either harvested in lysis buffer for Western blot analysis (see below) or subjected to the palmitate uptake and oxidation assays or the glucose uptake assay (see below).

AICAR/Lipofectamine/medium and RNAi/Lipofectamine/medium solutions were then combined with antibiotic-free medium containing 2% FCS and the plates were placed back in the humidified incubator (95% O2, 5% CO2) for 2 more days. The cells were then treated with AICAR (2 mM, 2 h) or metformin (2 mM, 3 h) and either harvested in lysis buffer for Western blot analysis (see below) or subjected to the palmitate uptake and oxidation assays or the glucose uptake assay (see below).

Materials and methods

Cell culture. L6 myoblasts (kind gift from C. K. Sung, previously of the Department of Physiology and Biophysics, University of Southern California) were cultured in αMEM containing 10% FCS, 1% antibiotic-antimycotic solution (Sigma Aldrich, St. Louis, MO), and 500 μM t-carnitine (Sigma Aldrich) in a humidified incubator at 37°C (95% O2, 5% CO2). The α-MEM+ and FCS were purchased from the Cell Culture Facility (University of Southern California, Los Angeles, CA). Cells were grown in T25-cm2 sterile culture flasks, subcultured at 60–80% confluence, and split at a ratio of 1:10 using trypsin-EDTA (Invitrogen, Grand Island, NY). Cells were subcultured into six-well plates and switched to αMEM+ containing 2% FCS to promote differentiation. By day 4, cells were 100% confluent and spontaneously differentiated into myotubes. For experiments without RNAi treatment, L6 myotubes were 10 days postconfluent on the day of the experiment. In RNAi-treated L6 myotubes, RNAi treatment was initiated 2 days after subculturing into six-well plates. Given that RNAi treatments lasted 24 h and that the maximum incubation time for a metformin treatment was 24 h, cells were 1 day postconfluent for experiments that were performed 2 days after initiation of the RNAi treatment.

Glucose uptake. After each treatment with or without insulin, the experimental medium was replaced with transport medium (100 μM albumin-bound palmitate, 1:1, 30 min) containing [1,14C]palmitic acid (4 μCi/ml; Perkin Elmer, Boston, MA) to measure palmitate uptake and oxidation (see below) (34). Incubations were terminated by removing the media, which was used to assay for 14C-labeled oxidation products (see below). Wells were washed twice with KRb, and cells were lysed by mixing with SDS (23°C). Duplicate aliquots of lysate were mixed with scintillation fluid (BudgetSolve; Research Product International, Mount Prospect, IL) and counted in a Tri-carb liquid scintillation analyzer (model 2100TR; Packard, Downers Grove, IL). Duplicate aliquots of the same lysate were used for protein determination using the Bradford method (Bio-Rad, Hercules, CA).

Palmitate oxidation. Oxidation products were measured as previously described (34). Briefly, duplicate aliquots of experimental media were mixed with perchloric acid to release 14CO2, which was trapped on filter paper (Whatman, Piscataway, NJ) saturated with ethanolamine and glued to the caps. After complete 14CO2 release, the filter paper was released from the caps, mixed with toluene-based scintillation cocktail, and analyzed for 14CO2 radioactivity. To correct for carbon loss, additional experiments were conducted to determine the acetate correction factor (34) under our experimental conditions. In this subsample, cells were treated as above except that 4 μCi of [1-14C]acetate acid (Perkin Elmer) was added to the incubation medium rather than [1-14C]palmitate acid. Samples were treated as described above and analyzed for [14C]acetate acid and 14CO2 radioactivities.

Glucose uptake. After each treatment with or without insulin, the experimental medium was replaced with transport medium (200 μM, 5 min) containing [2-3H]deoxyglucose (0.5 μCi/ml; MP Biochemicals, Solon, OH) to measure glucose uptake (34). Incubations were terminated via removal of the media. Individual wells were washed twice with KRb, after which cells were lysed with SDS (23°C, 30 min). As for palmitate uptake, duplicate aliquots of lysate were taken for scintillation counting and for protein determination.

Nuclear fraction isolation. Nuclear fraction isolation was completed using a commercially available Nuclear Extract Kit via the manufacturer’s protocol (Active Motif, Carlsbad, CA). In short, L6 cells were washed with PBS/phosphatase inhibitors and scraped down for centrifugation (3,500 g, 5 min, 4°C). The supernatant was discarded, and the pellet was resuspended in a hypotonic buffer and incubated for 15 min on ice. After detergent addition, the cell suspension was centrifuged (14,000 g, 30 s, 4°C) to separate the cytoplasmic fraction (supernatant) from the nuclear fraction (pellet). The pellet was resuspended in lysis buffer and incubated on a rocking platform (30 min), and the mixture was centrifuged (14,000 g, 10 min,
4°C. The supernatant (nuclear fraction) was prepared for Western blot analysis to measure proliferator-activated receptor-γ coactivator-1α (PGC-1α) acetylation (following immunoprecipitation, see below) or SIRT1 activity.

**Plasma membrane isolation.** Crude plasma membrane (PM) proteins were isolated as described (34, 46) from L6 cells that were plated in sterile 100 × 20-mm tissue culture dishes. As described above, cells were treated with AICAR, metformin (2 mM, 3 h), metformin (0.8 mM, 24 h), or metformin (2 mM, 24 h) with or without insulin. Cells were then scraped into 15-ml centrifuge tubes and centrifuged (3,500 g, 10 min, 4°C). The pellet was resuspended in homogenizing buffer [250 mM sucrose, 5 mM NaN3, 2 mM EGTA, 200 μM PMSF, 10 μM trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane, 1 μM pepstatin A, 1 μM leupeptin, and 20 mM HEPES; pH 7.4] and homogenized using a glass-on-glass Dounce homogenizer (20 strokes). The homogenate was centrifuged (3,500 g, 5 min, 4°C) to pellet the unbroken cells and nuclei. The supernatant was transferred to a microcentrifuge tube and centrifuged (31,000 g, 1 h, 4°C) to pellet PM proteins. The pellet was resuspended in homogenizing buffer and prepared for Western blot analysis to measure PM FA transporter/CD36 and Glut4 protein content.

**Immunoprecipitation.** After the experimental treatments, cells were lysed as described in Western blot analysis and antibodies to AMPK-α1, AMPK-α2, or PGC-1α were added to the lysate and incubated (3 h, 4°C) as previously described (31, 39, 54). Protein A/G Plus-agarose immunoprecipitation reagent (Santa Cruz Biotechnology, Santa Cruz, CA) was then added, and the mixture was incubated overnight (overnight, 4°C). After an overnight incubation (4°C) with gentle shaking, the mixture was rinsed three times with lysis buffer (see Western blot analysis) via centrifugation at maximum speed (30 s), discarding the supernatant each time. After the final centrifugation, lysis buffer was added to the pellet, and the sample was prepared for the Western blot analysis.

**Western blot analysis.** After the experimental treatments, cells were washed with ice cold KRB and prepared for Western blotting as described (33). Lysis buffer was added [20 mM Tris, 1% NP-40, 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10% (vol/vol) glycerol, 1 mM DTT, 1 mM PMSF, and 2 mM Na3VO4] and cells were scraped to one side of the well. The lysate was transferred to microcentrifuge tubes and gently pelleted. The supernatants were collected, and aliquots were assayed for protein content (Bradford method; Bio-Rad) or subjected to 12% SDS polyacrylamide gel electrophoresis. The separated proteins were transferred onto PVDF membranes and blocked with 1% BSA in Tween-TBS (500 mM NaCl, 20 mM Tris, and 0.05% Tween-20; pH 7.5) (TTBS) for 1 h (23°C) on a shaking orbitron, rinsed twice with TTBS and once with TBS, and incubated overnight (4°C) with primary antibodies (1:1,000) to measure either 1) total protein content for AMPK-α1, AMPK-α2, insulin receptor substrate 1 (IRS-1; Upstate, Billerica, MA), acetyl-CoA carboxylase (ACC; Cell Signaling, Danvers, MA), SIRT1, PGC-1α, FA transporter/CD36, Glut4 (Santa Cruz Biotechnology); 2) the phosphorylation state of AMPK-α1 (Cell Signaling), ACC, IRS-1 Ser307 (Upstate), or IRS-1 Tyr281 (Millipore, Billerica, MA); or 3) the lysine acetylation state (Santa Cruz Biotechnology). After being further washed, the membranes were incubated (1 h, 23°C) with the secondary antibody horseradish peroxidase goat anti-rabbit or goat anti-mouse (1:10,000; Pierce, Rockford, IL) and rinsed twice with TTBS and once with TBS. The membranes were developed via enhanced chemiluminescence (Pierce) followed by exposure to CL-XPosure film (Pierce). WHERE appropriate, membranes were stripped with 0.5 NaOH (15 min) followed by a wash with TTBS, and reprobed with GAPDH antibodies. The films were scanned using a Hewlett-Packard ScanJet 6200C and quantified using Scion Image (Scion, Frederick, MD). In all cases, multiple gels were analyzed and compared with results obtained for control cells that had not been treated with AICAR, metformin, and/or insulin. Protein content was normalized to GAPDH, and immunoprecipitation samples were normalized to the content of each respective protein.

**SIRT activity.** SIRT1 activity was measured on nuclear extracts with the commercially available histone deacetylase (HDAC) colorimetric activation kit (Active Motif, Carlsbad, CA) (56). Briefly, nuclear extracts were added to assay buffers with assay substrates into a 96-well microplate. Trichostatin A was added to the wells to inhibit class I, II, and IV HDAC, and 250 μM NAD+ (Sigma) was added to activate SIRT1, which is NAD+-dependent. Following incubation (37°C, 1 h), the reaction was stopped with the addition of a developing solution. The samples were read in a microplate reader at 405 nm. SIRT1 is a class III HDAC that requires NAD+ for enzymatic activity (56). Therefore, SIRT1 activity was calculated as the difference between cells incubated with trichostatin A in the presence or absence of NAD+.

**Calculations and statistics.** The rates of glucose and palmitate uptake and palmitate oxidation were calculated as described in detail (34). All presented data are expressed as means ± SE and are expressed as percents of control, where control refers to cells that were not treated with any agent or insulin (see figure legends for specific details). The percent control was calculated using measured rates (mmol·g⁻¹·min⁻¹) for all experimental treatments. The effects of treatments with or without insulin were analyzed using a one-way ANOVA (Statview) followed by Fisher LSD post hoc test when appropriate. The square of the Pearson product moment coefficient was used to determine the significance of correlations when necessary. In all instances, an α of 0.05 was used to determine significance.

**RESULTS**

Metformin preferentially phosphorylates AMPK-α1 in L6 muscle cells. Both metformin and AICAR treatment increased total AMPK-α phosphorylation in L6 muscle cells (P < 0.05) (Fig. 1A). Results from our study further show that total AMPK-α phosphorylation increased (P < 0.05) in a concentration-dependent (0.8 vs. 2 mM in 24-h groups) and time-dependent (3 h vs. 24 h in 24-h groups) manner in the metformin-treated groups. Treatment with insulin had no effect on the phosphorylation state of AMPK-α1 in any of the treatment groups (P > 0.05).

To determine whether the phosphorylation state of AMPK-α1 and AMPK-α2 were similarly affected by treatment conditions, AMPK-α1 and AMPK-α2 were immunoprecipitated and Western blot analysis was performed (31, 39, 54). Whereas AICAR treatment did not significantly increase the phosphorylation state of AMPK-α1 (P > 0.05), metformin treatment increased AMPK-α1 phosphorylation (58–79%) in all metformin-treated groups compared with the control group (P < 0.05) (Fig. 1B). When compared with the AICAR group, the phosphorylation state of AMPK-α1 was 34–51% higher in the metformin-treated groups (P < 0.05). The phosphorylation state of AMPK-α2 was found to be higher in all but one of the metformin-treated groups (0.8 mM/24 h with insulin) compared with the control group (P < 0.05) (Fig. 1C). AICAR treatment significantly increased (P < 0.05) AMPK-α2 phosphorylation (71%). The increase in AMPK-α2 phosphorylation attributable to AICAR treatment was significantly more (P < 0.05) than that attributable to any of the metformin treatments. In each treatment group, insulin did not significantly affect the phosphorylation state of AMPK-α1 or AMPK-α2 (P > 0.05).

Metformin decreases palmitate uptake, and oxidation is concert with PM CD36 content but not ACC phosphorylation. To determine how the preferential phosphorylation of AMPK-α1 by metformin would affect FA metabolism in muscle cells,
Metformin preferentially phosphorylates AMP-activated protein kinase (AMPK)-α1 in L6 muscle cells. Total AMPK-α phosphorylation (A), AMPK-α1 phosphorylation (B), and AMPK-α2 phosphorylation (C) were measured in the basal (open bar) or insulin-stimulated (shaded bar) state. Cell treatments include 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (2 mM, 2 h), metformin (2 mM, 3 h), metformin (0.8 mM, 24 h), and metformin (2 mM, 24 h) with or without insulin (100 nM, 15 min). Western blotting with the GAPDH antibody was used as a loading control, and immunoprecipitation samples were normalized to the content of each respective protein. Values are means ± SE (n = 3–6 per condition) and are expressed as percentage of control, where control refers to cells that were not treated with AICAR, metformin, or insulin (i.e., basal). ∗P < 0.05 vs. control; †P < 0.05 vs. AICAR treatment; ‡P < 0.05 vs. metformin (2 mM, 3 h) treatment; §P < 0.05 vs. metformin (0.8 mM, 24 h) treatment.

Research has suggested that FA uptake is facilitated by a protein-mediated transport system (2, 41). Although a number of transporter proteins have been proposed, the key transporter that has been implicated in the movement of long-chain FAs across the lipid bilayer of the PM of the skeletal muscle cell is CD36, also called FA transporter (2, 6). Because CD36 has been shown to be a key transporter of long-chain FA across the PM of skeletal muscle cells (12, 23), total and PM CD36 protein content was measured to provide further evidence for the metformin-induced effects on palmitate uptake in skeletal muscle cells. Total CD36 protein content was not affected by any of the conditions (P > 0.05; data not shown). Although PM CD36 content in the basal state was decreased (10–14%) in all metformin-treated groups, only in the 2 mM/24-h metformin-treated group was the reduction significant (P < 0.05) compared with the control group. PM CD36 content in the basal state was increased 44% (P < 0.05) by AICAR treatment (Fig. 2C). PM CD36 content was found to be significantly (P < 0.05) lower in all metformin-treated groups compared with the AICAR-treated group. In each treatment group, insulin significantly increased (P < 0.05) PM CD36 content by 14–69% compared with its respective basal state. PM CD36 content was positively correlated with FA uptake (R² = 0.51; P < 0.05).

Research has shown that palmitate oxidation is regulated in part by the phosphorylation state of ACC, an enzyme that decreases malonyl-CoA levels and thus reduces carnitine palmitoyl transferase 1 inhibition, leading to increased palmitate oxidation (44, 55). Phosphorylation of ACC inhibits the activity of the enzyme and results in a buildup of malonyl-CoA and a subsequent decrease in palmitate oxidation (55). Thus measurements of ACC phosphorylation were used to provide information on the cellular mechanisms that may regulate the decrease in palmitate oxidation with metformin stimulation. In line with the AMPK-α2 phosphorylation and palmitate oxidation data, ACC phosphorylation was increased by 49% (P < 0.05) following AICAR treatment. ACC phosphorylation was not affected (P > 0.05) by metformin treatment (Fig. 2D). ACC phosphorylation was found to be lower (28–39%) in all metformin-treated groups compared with the AICAR-treated group (P < 0.05). In each treatment group, insulin did not significantly affect ACC phosphorylation compared with its respective basal state (P > 0.05).

Metformin increases glucose uptake in concert with PM Glut4 content. Glucose uptake was 40–109% higher in the metformin-treated groups compared with the control group (P < 0.05), and the metformin-induced increase in glucose
uptake was time and concentration dependent (Fig. 3A). AICAR treatment increased glucose uptake by 39% (P < 0.05), and this AICAR-induced increase in glucose uptake was less (P < 0.05) than that induced in the 2 mM metformin (3- and 24-h incubation time) groups. In each treatment group, insulin significantly increased glucose uptake compared with its respective basal state (P < 0.05).

Because Glut4 has been shown to be a key transporter of glucose across the PM of skeletal muscle cells (35), total and PM Glut4 protein content was measured. Total Glut4 protein content was not affected by any of the conditions (P > 0.05; data not shown). In line with the glucose uptake data, PM Glut4 content was 46–99% higher in the AICAR- and metformin-treated groups compared with the control group (P < 0.05). Furthermore, only in the 2 mM/24-h metformin group did insulin significantly increase (25%) PM Glut4 content (P < 0.05). PM Glut4 content was positively correlated with glucose uptake (R² = 0.87; P < 0.05).

Metformin increases SIRT1 activity and IRS-1 tyrosine phosphorylation and decreases IRS-1 serine phosphorylation. Because previous data indicate that AMPK signaling may interact with SIRT1 activation (59), we measured SIRT1 activity in nuclear extracts. Nuclear extracts were chosen because evidence shows that SIRT1 is primarily located in the nucleus (25). Our results showed that, compared with the control group, SIRT1 activity in the basal state was significantly higher (P < 0.05) in the AICAR-treated group and in the metformin groups treated for 24 h (0.8 and 2.0 mM groups) (Fig. 4A). In each group, insulin significantly increased SIRT1 activity by 17–29% compared with its respective basal state (P < 0.05). SIRT1 protein expression was not affected by any of the treatments (P > 0.05; data not shown).

Fig. 2. Metformin decreases palmitate uptake and oxidation in concert with plasma membrane (PM) CD36 content but not acetyl-CoA carboxylase (ACC) phosphorylation. Palmitate uptake (A), palmitate oxidation (B), PM CD36 protein content (C), and ACC phosphorylation (D) were measured in the basal (open bars) or insulin-stimulated (solid bars) state. L6 cells were treated with AICAR (2 mM, 2 h), metformin (2 mM, 3 h), metformin (0.8 mM, 24 h), or metformin (2 mM, 24 h) with or without insulin (100 nM, 15 min). Western blotting with the GAPDH antibody was used as a loading control, and PM extraction samples were normalized to the content of each respective protein. Values are means ± SE for all treatment groups (n = 3–9 per condition) and are expressed as a percentage of control, where control refers to cells that were not treated with AICAR, metformin, or insulin (i.e., basal). A: insulin treatment resulted in a significant change (P < 0.05) in palmitate uptake under each set of treatment conditions. B: insulin treatment resulted in a significant change (P < 0.05) in palmitate oxidation under each set of treatment conditions. C: insulin treatment resulted in a significant change (P < 0.05) in PM CD36 content under each set of treatment conditions. Statistical significance for the insulin effect is not shown to simplify figures. *P < 0.05 vs. control; †P < 0.05 vs. AICAR treatment; ‡P < 0.05 vs. metformin (2 mM, 3 h) treatment; §P < 0.05 vs. metformin (0.8 mM, 24 h) treatment.
shown to be the main PGC-1α treatment conditions. Statistical significance for the insulin effect is not shown of PGC-1α and nuclear fractions (83%) and determined that the majority we measured PGC-1α primarily located in the nucleus (52). For verification purposes, assured the acetylation state of PGC-1α resulted in a significant change (\( P < 0.05 \)) in the AICAR-3 h) treatment; \( \uparrow P < 0.05 \) vs. control; \( \& P < 0.05 \) vs. respective treatment in the basal state, neither metformin nor AICAR treatment had any effect on IRS-1 tyrosine\(^{941} \) or serine\(^{307} \) phosphorylation in the basal state, neither metformin nor AICAR treatment had any effect on IRS-1 tyrosine\(^{941} \) or serine\(^{307} \) phosphorylation (Fig. 4, C and D). In the control group, insulin decreased \( (P < 0.05) \) IRS-1 serine\(^{307} \) phosphorylation by 20%, but it did not change tyrosine\(^{941} \) phosphorylation significantly in the metformin groups, insulin increased \( (P < 0.05) \) IRS-1 tyrosine\(^{941} \) phosphorylation (56–381%) and decreased \( (P < 0.05) \) IRS-1 serine\(^{307} \) phosphorylation (37–51%). In the insulin-stimulated state, IRS-1 tyrosine\(^{941} \) phosphorylation was highest \( (P < 0.05) \) in the metformin groups treated for 24 h (0.8 and 2.0 mM). In the insulin-stimulated state, IRS-1 serine\(^{307} \) phosphorylation was lowest \( (P < 0.05) \) in the 2 mM/24-h metformin group.

**Downregulation of AMPK-\( \alpha_1 \) or AMPK-\( \alpha_2 \) causes a concomitant reduction in AMPK-\( \alpha_1 \) or AMPK-\( \alpha_2 \) protein expression and phosphorylation state.** To provide more definitive evidence for the notion that metformin impacts muscle metabolism via preferential phosphorylation of AMPK-\( \alpha_1 \), we downregulated AMPK-\( \alpha_1 \) or AMPK-\( \alpha_2 \) using RNAi oligonucleotide sequences. The downregulation of AMPK-\( \alpha_1 \) and AMPK-\( \alpha_2 \) expression was verified and confirmed using Western blot analysis as a marker of end-point protein expression. As expected, treatment of cells with the AMPK-\( \alpha_1 \) RNAi oligonucleotide sequence significantly decreased \( (P < 0.05) \) AMPK-\( \alpha_1 \) protein content (45.4 ± 4.0%;) but had no impact on AMPK-\( \alpha_2 \) protein content (Fig. 5, A and B). Conversely, treatment of cells with the AMPK-\( \alpha_2 \) RNAi oligonucleotide sequence significantly decreased \( (P < 0.05) \) AMPK-\( \alpha_2 \) protein content (39.1 ± 4.6%) but had no impact on AMPK-\( \alpha_1 \) protein content \( (P > 0.05) \). We further determined the effects of reduced protein expression on AMPK-\( \alpha_1 \) and AMPK-\( \alpha_2 \) phosphorylation using immunoprecipitation followed by Western blot analysis. In line with the protein expression data, AMPK-\( \alpha_1 \) phosphorylation was increased by metformin \( (P < 0.05) \) in the cyclophilin B- and AMPK-\( \alpha_2 \)-deficient cells but not in the AMPK-\( \alpha_1 \)-deficient cells \( (P > 0.05) \) (Fig. 5, C and D). AICAR treatment did not significantly \( (P > 0.05) \) change AMPK-\( \alpha_1 \) phosphorylation in any of the cells. AMPK-\( \alpha_2 \) phosphorylation was increased by AICAR \( (P < 0.05) \) in the cyclophilin B- and AMPK-\( \alpha_1 \)-deficient cells but not in the AMPK-\( \alpha_2 \)-deficient cells \( (P > 0.05) \) (Fig. 5, C and E). Metformin treatment did not significantly \( (P > 0.05) \) change AMPK-\( \alpha_2 \) phosphorylation in any of the cells. Together, our data show that, in contrast to AICAR, metformin acts primarily through the activation of AMPK-\( \alpha_1 \) in L6 muscle cells.

**AMPK-\( \alpha_1 \) downregulation is coincident with reductions in metformin-induced metabolic effects.** Downregulation of AMPK-\( \alpha_1 \) prevented the metformin-mediated decrease in palmitate uptake and oxidation that was observed in both the cyclophilin B- and AMPK-\( \alpha_2 \)-deficient cells but did not affect the AICAR-mediated increase in palmitate oxidation (Fig. 6, A and B). AMPK-\( \alpha_1 \) downregulation reduced \( (P < 0.05) \) metformin-mediated glucose uptake by 23% compared with the metformin-mediated response observed in cyclophilin B-deficient cells (Fig. 6C). Thus, in contrast to the palmitate data, metformin-induced glucose uptake

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**Fig. 3.** Metformin increases glucose uptake in concert with PM Glut4 content. Glucose uptake (A) and PM Glut4 protein content (B) were measured in the basal (open bars) or insulin-stimulated (solid bars) state. L6 cells were treated with AICAR (2 mM, 2 h), metformin (2 mM, 3 h), metformin (0.8 mM, 24 h), or metformin (2 mM, 24 h) with or without insulin (100 nM, 15 min). Western blotting with the GAPDH antibody was used as a loading control, and PM extraction samples were normalized to the content of each respective protein. Values are means ± SE for all treatment groups (\( n = 3–12 \) per condition) and are expressed as percentage of control, where control refers to cells that were not treated with AICAR, metformin, or insulin (i.e., basal). A: insulin treatment resulted in a significant change \( (P < 0.05) \) in glucose uptake under each set of treatment conditions. Statistical significance for the insulin effect is not shown to simplify A. \* \( P < 0.05 \) vs. control; \& \( P < 0.05 \) vs. respective treatment in the basal state; \( \uparrow P < 0.05 \) vs. AICAR treatment; \( \uparrow \& P < 0.05 \) vs. metformin (2 mM, 3 h) treatment; \$ \( P < 0.05 \) vs. metformin (0.8 mM, 24 h) treatment.

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To provide further evidence of SIRT1 activation, we measured the acetylation state of PGC-1α (58). SIRT1 has been shown to be the main PGC-1α deacetylase in several cell lines including muscle cells (10, 21, 58). Thus a high PGC-1α acetylation state reflects low SIRT1 activity and vice versa. Previous research has indicated that, like SIRT1, PGC-1α is primarily located in the nucleus (52). For verification purposes, we measured PGC-1α protein content in cytoplasmic (17%) and nuclear fractions (83%) and determined that the majority of PGC-1α protein was located in the nucleus. Therefore, PGC-1α acetylation state was measured using nuclear extracts. In both basal and insulin-stimulated states, PGC-1α acetylation was found to be significantly lower \( (P < 0.05) \) in the AICAR- (43–52%) and metformin-treated (66–74%) groups compared with the control group (data not shown). In line with the SIRT1 data, insulin significantly decreased PGC-1α acetylation in all treatment groups \( (P < 0.05) \); data not shown).
was not completely prevented by AMPK-α1 downregulation. AMPK-α1 downregulation also reduced (P < 0.05) AICAR-mediated glucose uptake by 14%.

Downregulation of AMPK-α2 reduced (P < 0.05) the AICAR-mediated increase in palmitate uptake and oxidation observed in the cyclophilin B-deficient cells, but the genetic manipulation had no effect (P > 0.05) on the decrease in metformin-mediated palmitate uptake and oxidation (Fig. 6, A and B). Downregulation of AMPK-α2 eliminated the AICAR-mediated increase in glucose uptake but did not affect the metformin-mediated increase (P < 0.05) in glucose uptake (Fig. 6C).

AMPK-α1 or AMPK-α2 downregulation does not affect metformin-induced SIRT1 activity. To provide mechanistic insights into the existence of a possible relationship between AMPK and SIRT1 signaling, SIRT1 activity was measured in nuclear extracts of cells treated with AMPK-α1 or AMPK-α2 RNAi oligonucleotide sequences. Neither AMPK-α1 nor AMPK-α2 downregulation prevented the stimulation of SIRT1 activity with metformin or AICAR treatment (Fig. 7A). In line with the SIRT1 activity data, neither AMPK-α1 nor AMPK-α2 downregulation affected the acetylation state of PGC-1α induced by metformin or AICAR treatment (data not shown), and SIRT1 activity was negatively correlated (R² = −0.74; P < 0.05) with PGC-1α acetylation state (Fig. 7B).

**DISCUSSION**

The findings of this study provide evidence for the preferential activation of AMPK-α1 by metformin in skeletal muscle cells. The genetic downregulation experiments show for the
first time that, in contrast to the preferential activation of AMPK-α₁ or AMPK-α₂ by AICAR, which increases FA uptake and oxidation, the preferential activation of AMPK-α₁ by metformin reduces FA uptake and oxidation. Interestingly, metformin treatment was more effective than AICAR treatment at increasing insulin-stimulated glucose uptake even when AMPK-α₂ was downregulated. The effects of metformin treatment on insulin-mediated muscle metabolism were time and dose dependent and were associated with corresponding improvements in proximal insulin signaling. Finally, because downregulation of AMPK-α₁ or AMPK-α₂ did not affect AICAR- or metformin-induced SIRT1 activity, the data indicate that cross talk...
between AMPK and SIRT1 does not favor either AMPK isozyme.

Our results showing that metformin preferentially increases AMPK-H9251 phosphorylation in L6 muscle cells were not overly surprising given that various short-term metformin treatment protocols have been shown to preferentially increase AMPK-α1 in H-2Kb muscle cells, in H4IIE cells, and in rat epitrochlearis muscle (17, 27, 74). Given that evidence shows that metformin acts in part via LKB1 activation (75), our data are also in line with the hypothesis that LKB1 can activate both AMPK isoforms (62). Furthermore, although recent evidence has indicated that some of the metabolic actions of metformin are mediated independently of AMPK activation (49, 60), our results confirm that AMPK-dependent effects are also clearly important. Recently, data have emerged in AMPK double-knockout mice that suggest that metformin may inhibit hepatic gluconeogenesis independently of AMPK (16). Although these data are exciting, it is important to remember that the double knockout mice were AMPK deficient in the liver and not skeletal muscle; additionally, measurements were done in mouse primary hepatocytes. The cell type difference between this study and the present experiments may explain the differ-

Fig. 6. Downregulation of AMPK-α1 is coincident with reductions in metformin-induced metabolic effects. Palmitate uptake (A), palmitate oxidation (B), and glucose uptake (C) were measured in the cyclophilin B-deficient (control), AMPK-α1-deficient, or AMPK-α2-deficient cells. Open bars represent data for basal cells. Shaded bars represent data for AICAR (2 mM, 2 h)-treated cells. Solid bars represent data for metformin (2 mM, 3 h)-treated cells. Values are means ± SE for all treatment groups (n = 8–17 per condition) and are expressed as percentage of control, where control refers to cells treated with siRNA for cyclophilin B and not treated with either AICAR or metformin (i.e., basal). *P < 0.05 vs. siRNA cyclophilin B-deficient cells similarly treated; †P < 0.05 vs. respective basal state in similarly-treated cells.

Fig. 7. Downregulation of AMPK-α1 or AMPK-α2 does not affect metformin-induced SIRT1 activity. SIRT1 activity (A) and correlation between SIRT1 activity and PGC-1α acetylation state (B) were measured in the cyclophilin B-deficient (control), AMPK-α1-deficient, or AMPK-α2-deficient cells. A: open bars represent data for basal cells. Shaded bars represent data for AICAR (2 mM, 2 h)-treated cells. Solid bars represent data for metformin (2 mM, 3 h)-treated cells. B: open symbols represent cyclophilin B-deficient cells. Shaded symbols represent AMPK-α1-deficient cells. Closed symbols represent AMPK-α2-deficient cells. Values are means ± SE for all treatment groups (n = 3–8 per condition) and are expressed as percentage of control, where control refers to cells treated with siRNA for cyclophilin B and not treated with either AICAR or metformin (i.e., basal). *P < 0.05 vs. siRNA cyclophilin B-deficient cells similarly treated; †P < 0.05 vs. respective basal state in similarly-treated cells.
ence between the data because hepatocytes (16) and skeletal muscle are well known to react differently to physiological treatments.

To our knowledge, our results provide for the first time strong evidence for the inhibitory effects of metformin treatment on FA uptake and oxidation in skeletal muscle cells, and the results from our genetic manipulation experiments strengthen these conclusions. A similar dose-dependent decrease in FA uptake has been observed in 3T3-L1 adipocytes treated with metformin, but we are aware of no data in muscle cells (19). Our FA oxidation results are in contrast with data showing no change in FA oxidation in soleus of high-fat-fed Zucker diabetic rats (66) and in patients with type 2 diabetes (48) or increased FA oxidation in primary hepatocytes (74). However, our results agree with other data showing a reduction in FA oxidation in isolated muscle of rats treated with biguanides (8, 68). The discrepancies observed between our study and those of others suggest that the observed effects of metformin may depend on several factors that include, among others, differences in metabolic profile between cell lines, in fiber type characteristic between different muscle samples, in the genetic background of the experimental model, and in the metformin treatment protocol used. Given that, in our study, downregulation of AMPK-α1 prevented the metformin-induced reduction in FA uptake or oxidation but did not affect the AICAR-induced rise in FA uptake or oxidation, our genetic manipulation data fit with our conclusions. Additionally, given that the expression level of AMPK-α2 has been shown to be higher than that of AMPK-α1 in skeletal muscle (69), the significant metformin-induced decreases in palmitate uptake and oxidation illustrate the potent effects of metformin in skeletal muscle cells. These data indicate that, despite lower expression levels of AMPK-α1 compared with AMPK-α2, increased AMPK-α1 phosphorylation is more than sufficient to significantly change the rate of FA metabolism in skeletal muscle.

To strengthen our metabolic data, we measured the effects of metformin on ACC phosphorylation and PM CD36 content. Multiple studies have indicated that CD36 is an important PM protein that carries FA from plasma to the cytosol (5, 23, 29) and that PM CD36 content and FA uptake often change in a parallel manner (1, 22, 67). In line with this, our results show that, in parallel with our FA uptake data, PM (but not total) CD36 protein content was reduced with metformin treatment. Very few studies have looked at the effects of metformin on PM CD36 content, but our results agree with data showing that long-term (8 wk) metformin treatment was associated with a reduction in sarcosomal CD36 content in muscle of high-fat-fed Zucker rats (66). It is important to note that, although the relative decrease in FA uptake in the 2 mM/24-h metformin group was 42%, the decrease in PM CD36 content in that group was only 14%, a percent decrease that is similar to the one recorded for the other two metformin groups. Given these data, it would appear that, although a change in PM CD36 content can occur relatively quickly (given our minimum treatment duration of 3 h), its mediating effects on FA uptake are not improved on by increasing the concentration of metformin used (0.8 vs. 2.0 mM) or by incubating the cells for a longer duration (3 vs. 24 h). Furthermore, on a relative scale, the decrease in PM CD36 observed in the 2 mM/24-h metformin group cannot fully account for the reduction in FA uptake. Conversely, in both the control and metformin-treated groups, the relative increase in PM CD36 content (∼30–65%) induced by insulin was more than enough to account for the observed increase in FA uptake (∼16–28%). Together, these observations suggest that, although PM CD36 content is undoubtedly an important factor that mediates changes in FA uptake, other factors certainly contribute to the regulation of FA uptake in skeletal muscle cells.

Although it is generally well accepted that ACC activity is a contributing factor in the regulation of FA oxidation in skeletal muscle (55), other mechanisms of regulation exist. In line with this notion, previously published data have shown that changes in FA oxidation rates do not always parallel changes in ACC phosphorylation, as has been observed after prolonged exposure to palmitate in L6 cells (51) or during prolonged exercise in male subjects (70). In a similar manner, the reduction in FA oxidation induced by metformin treatment in our study was not associated with a concomitant decrease in ACC phosphorylation. Given that preferential activation of AMPK-α2 via AICAR treatment was associated with an equal stimulation of FA oxidation and ACC phosphorylation, our data suggest that, contrary to AMPK-α2, preferential AMPK-α1 activation may not reduce FA oxidation via an ACC-dependent mechanism. Our data showing that metformin treatment only minimally increased AMPK-α2 and ACC phosphorylation agree with this notion. These results are intriguing considering the strong correlation between FA oxidation and ACC activation that has been observed in skeletal muscle under various physiological treatments (44, 45). Additionally, metformin has been shown to reduce state 3 respiration rates in isolated mitochondria consuming glutamate plus malate, and in line with the data from our study, exposure of isolated soleus muscle to metformin has been shown to reduce cellular respiration of palmitate while increasing glucose transport (9). These results are in agreement with recent data (26) and suggest that a reduction in cellular respiration might be one of the factors that mediated the decrease in FA oxidation with metformin treatment. Intra-cellular FA availability is another factor that is known to contribute to the regulation of FA oxidation in skeletal muscle (14). Given that FA uptake was also reduced by metformin treatment, we cannot eliminate the possibility that intracellular FA availability was reduced in these groups and that the decrease in FA availability regulated the metformin-induced reduction of FA oxidation in a dose-dependent manner.

Several studies have tested whether metformin treatment improves insulin action via an increase in muscle glucose uptake, and results have not always been consistent. Our glucose uptake and Glut4 data confirm previously published results (7, 43, 61, 74) and show that short-term metformin treatment is associated with an increase in basal and insulin-mediated glucose uptake and PM Glut4 content, especially when experimental glucose and insulin levels are matched between groups (50). Given that diabetic individuals tend to have higher blood glucose and plasma insulin levels, this is an important experimental consideration. Indeed, data show that, when plasma glucose concentration was not matched between control and diabetic subjects, basal and insulin-mediated glucose uptake was not affected by metformin treatment (18). In our experiment, the L6 muscle cells were not insulin resistant, and glucose and insulin levels were matched between groups. Thus, by design, our results provide insight into the concen-
tration- and time-dependent effects of metformin on insulin-mediated glucose uptake. In contrast to our FA data, AMPK-α1 downregulation did not completely abolish the stimulatory effects of metformin on glucose uptake. This is in agreement with our AMPK-α1 and AMPK-α2 phosphorylation data (Fig. 1) showing that, although metformin may preferentially phosphorylate AMPK-α1, it still phosphorylates AMPK-α2. Together, these data suggest that, in AMPK-α1-deficient cells, the metformin-induced rise in AMPK-α2 phosphorylation was sufficient to increase glucose uptake. It will remain to be determined whether this AMPK-α2-dependent regulation of glucose uptake observed in our study occurred via activation of aPKC as described by others (61).

Presently, conflicting reports exist regarding the coordinated regulation of metabolism via cross talk between AMPK and SIRT1. These inconsistencies are probably due in part to the fact that various experimental models as well as different metabolic and genetic treatments have been used to study this relationship (11, 13, 30, 40). Although some studies conclude that AMPK activation stimulates SIRT1 possibly via a rise in the acetylation state of LKB1 (30, 40). Here we show for the first time that agonist-induced activation of AMPK-α1 or AMPK-α2 similarly increases nuclear SIRT1 activity. Furthermore, in line with the fact that, in our study, AMPK-α1 or AMPK-α2 downregulation did not affect AICAR- or metformin-induced nuclear SIRT1 activity, our data support the hypothesis that SIRT1 may lie upstream of AMPK in this interconnected signaling pathway. However, our conclusions are limited by the methods that were used, and as such we cannot exclude the possibility that under different experimental conditions AMPK may regulate SIRT1 activity. It is not clear whether SIRT1 activation played any role in the regulation of glucose uptake and/or FA oxidation in our study. Given that the SIRT-activating compound, Resveratrol, has been shown to increase glucose uptake in human adipocytes (15), we cannot exclude the possibility that both AICAR and metformin activated SIRT1 and that the increase in SIRT1 activity regulated the increase in glucose uptake. However, because downregulation of AMPK-α1 or AMPK-α2 did not change SIRT1 activity but significantly affected glucose uptake, this cannot be the main factor. Prior data have shown that SIRT1 knockdown decreases PGC-1α-mediated FA oxidation in C2C12 skeletal muscle cells and that SIRT1 is required to induce the gene transcription program for mitochondrial FA oxidation, suggesting that SIRT1 activity controls FA oxidative capacity (10, 21). Our data add to this notion by suggesting that, not surprisingly, factors other than SIRT1 activity regulate a reduction in FA oxidation. By this we mean that, even in the face of elevated SIRT1 activity, metformin was associated with a reduction in FA oxidation. In fact, in our hands and as shown by others (24), SIRT1 activity was not correlated with FA oxidation rates ($R^2 = -0.13; P > 0.05$). It is also important to note that, although SIRT1 activity was increased with metformin treatment, this increase was not associated with low-nutrient conditions, (58) which could not then have driven the alterations in substrate utilization. Interestingly, SIRT3 has been shown to acutely change the activity of mitochondrial enzymes involved in the regulation of acetyl-CoA levels (64). Because of the important role of acetyl-CoA levels in the maintenance of the mitochondrial redox and energy states, it will be important to measure the effects of our manipulations on nuclear SIRT3 activity in our next experiments.

Lastly, these data provide new evidence on the cellular mechanisms by which metformin increases insulin sensitivity. First, as shown by others (37, 38), metformin was especially potent at enhancing IRS-1 tyrosine phosphorylation, and this rise may have played a significant role in mediating the observed improvement in insulin-stimulated glucose uptake. Furthermore, given that high levels of IRS-1 serine phosphorylation have been linked to insulin resistance (3, 20), the metformin-induced reduction in IRS-1 serine phosphorylation may also have played a role. These data are in contrast to results showing a lack of change in insulin-mediated AKT serine phosphorylation or IRS-1-associated phosphatidylinositol 3-kinase activity in muscle of diabetic subjects treated with metformin (32, 42). Overall, our data indicate that improvements in proximal insulin signaling may promote insulin sensitivity in skeletal muscle cells with metformin treatment. Alternatively or concurrently, the reduction in FA oxidation induced by metformin treatment may also have played a role in regulating the observed improvement in insulin-stimulated glucose uptake via substrate competition as originally described by Randle (53, 73). Interestingly, our data indicate that SIRT1 activation is not a critical factor regulating skeletal muscle insulin sensitivity under our experimental conditions because downregulation of either AMPK-α1 or AMPK-α2 did not alter SIRT1 activity but reduced insulin-stimulated glucose uptake following metformin treatment.

In conclusion, our data indicate that metformin is an effective insulin sensitizer that preferentially increases AMPK-α1 phosphorylation and that reduces FA uptake and oxidation while increasing insulin-mediated glucose uptake. Our downregulation experiments indicate that the stimulatory effect of metformin on insulin-stimulated glucose uptake occurs in part via partial activation of AMPK-α2 by metformin and may be mediated by an increase in proximal insulin signaling. Lastly, our downregulation experiments do not agree with the notion that AMPK is a critical upstream regulator of SIRT1 activity in skeletal muscle cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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