Original Research

Short-term AMP-regulated protein kinase activation enhances insulin-sensitive fatty acid uptake and increases the effects of insulin on fatty acid oxidation in L6 muscle cells

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Abstract

Evidence shows that exercise increases insulin-sensitive glucose uptake and that exercise-induced AMP-regulated protein kinase (AMPK) activation is a likely candidate to mediate this metabolic adaptation. The purpose of this study was to determine whether repeated AMPK activation can similarly enhance insulin-sensitive fatty acid (FA) metabolism. L6 myotubes were incubated under the following conditions: repeated plus acute 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment (RAA; 1 mmol/L AICAR for 5 h/d for 5 days plus 1 mmol/L AICAR for 60 min on day 6), repeated AICAR (RA; 1 mmol/L AICAR for 5 h/d for five days) or acute AICAR (AA; 1 mmol/L AICAR for 60 min) and were compared with control cells that were not treated with AICAR. On day six, cells from each group were incubated with or without 100 nmol/L insulin. AICAR treatment and insulin stimulation independently increased ($P < 0.05$) palmitate uptake in all groups. RAA potentiated the insulin-induced increase in palmitate uptake by 97% ($P < 0.05$) as compared with control cells. RA and AA treatments prevented the insulin-induced decrease in palmitate oxidation, while RAA treatment restored the sensitivity of the cells to insulin action on palmitate oxidation. Total peroxisome proliferator-activated receptor-γ co-activator-1α, atypical protein kinase C-ζ, cytochrome C and CD36 protein content was increased ($P < 0.05$) by RA treatment, but unaffected by insulin. These results indicate that repeated AMPK activation induces improvements in insulin-sensitive FA uptake and oxidation and that this occurs partly via changes in the expression of proteins linked to insulin signaling and FA uptake and oxidation capacity.

Keywords: fatty acid metabolism, atypical protein kinase C-ζ, insulin signaling, FAT/CD36

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Introduction

Exercise has been shown to enhance the sensitivity of skeletal muscle to the effects of insulin.[1,2] In perfused or incubated rodent muscle and in human muscle, an acute bout of muscle contraction or exercise and repeated exercise training have been shown to increase not only insulin-stimulated glucose uptake, but also glucose storage.[2–5] However, it is not clear whether this increased sensitivity of skeletal muscle to the effects of insulin extends to insulin-sensitive fatty acid (FA) metabolism.

In rat and human muscle, exercise and treatment with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a potent activator of AMP-regulated protein kinase (AMPK),[6] have been shown to increase the activity of AMPK[2,7] and to stimulate glucose uptake via an increase in GLUT-4 translocation,[2,8] FA uptake via an increase in CD36 protein expression and plasmalemmal content[9,10] and FA oxidation in part via acetyl-coA carboxylase (ACC) phosphorylation.[6,10] Further, AICAR has been shown to increase the protein expression of mitochondrial oxidative enzymes, GLUT-4, CD36 and hexokinase,[8,11–13] as well as to improve insulin-sensitive glucose uptake[2,8,12,14] in rat heart and skeletal muscle. In light of these data, it has been hypothesized that AMPK activation, either through muscle contraction or AICAR treatment, mediates, at least in part, the adaptive responses of skeletal muscle metabolism observed

with regular exercise.[15] While the evidence implicates AMPK activation as being partially responsible for improved insulin-sensitive glucose metabolism, it is not known whether AMPK activation would similarly enhance insulin-sensitive FA uptake and oxidation in the muscle, and if so, the cellular mechanisms by which this improvement in insulin sensitivity would be modulated. Previous work from our lab has also shown that, as demonstrated for glucose uptake,[16,17] the insulin-induced increase in FA uptake is mediated at least in part by atypical protein kinase C-ζ (aPKC-ζ) activation.[18] Additionally, the stimulatory effects of exercise or AICAR treatment on insulin-sensitive glucose metabolism may be mediated in part via alterations in insulin signaling cascades.[2,19] Because impaired FA metabolism is associated with insulin resistance,[20] it is clinically important to determine whether exercise or AICAR treatment would also improve insulin-regulated FA metabolism in the muscle.

Therefore, the primary purpose of this study was to determine whether short-term repeated AICAR (RA) treatment enhances insulin-sensitive FA uptake and oxidation in L6 myotubes and secondarily to gather data on the involvement of the insulin-induced aPKC-ζ signaling pathway in the measured changes in FA metabolism. We hypothesize that if AMPK activation improves insulin-sensitive FA metabolism, it may do so via aPKC-ζ signaling pathway. L6 myotubes were employed in this study because they allowed us to easily manipulate the concentrations of insulin and/or AICAR in our experimental system.

Materials and methods

L6 cell culture

L6 cells were a kind gift from Dr Chin Sung (Los Angeles, CA, USA). L6 myotubes were cultured in α-minimum essential medium positive (MEM+) (University of Southern California Cell Culture Facility, Los Angeles, CA, USA) containing 10% fetal calf serum (University of Southern California Cell Culture Facility), 2% antibiotic–antimycotic (Sigma Aldrich, St Louis, MO, USA) and 500 μmol/L L-carnitine (Sigma Aldrich) in a humidified incubator at 37ºC, 5% CO_2. Cells were grown in 75 cm^2 sterile culture flasks, subcultured at 60–80% confluence and split at a ratio of 1:10 using trypsin-ethylenediaminetetraacetic acid (University of Southern California Cell Culture Facility). Cells were subcultured into six-well dishes, by which time the serum concentration was reduced to 2% to induce differentiation into myotubes. Experimental treatments were started seven days after subculturing, by which time the majority of myoblasts had spontaneously differentiated into myotubes as verified by inverted phase-contrast microscopy. At the conclusion of the experimental treatments, all L6 myotubes used in the study were 10 days postconfluent.

Cell treatments

For experiments with RA exposure, cells were incubated with 1 mmol/L AICAR (Sigma Aldrich) for 5 h/d for five days or in vehicle (αMEM+, as described above) as described by others.[21] AICAR or vehicle was removed by rinsing cells twice with warm Krebs–Ringer Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (KRB) (1.47 mmol/L K_2HPO_4/140 mmol/L NaCl/1.7 mmol/L KCl/0.9 mmol/L $CaCl_2$/0.9 mmol/L $MgSO_4$/20 mmol/L Hepes). The day following the fifth and final day of experimental treatment, cells were washed twice with warm KRB and exposed to either insulin (100 nmol/L) or vehicle (αMEM+) for 15 min followed by exposure to experimental medium to measure palmitate uptake and oxidation (see section below). For repeated + acute AICAR experiments (RAA), cells were repeatedly treated as described above. On day 6, cells were washed and pre-exposed to either AICAR (1 mmol/L) or vehicle (αMEM+) for 60 min prior to exposure to either insulin (100 nmol/L) or vehicle (αMEM+) for 15 min followed by exposure to experimental medium as described below. For acute AICAR (AA) experiments, cells were washed and incubated in the presence of either AICAR (1 mmol/L) or vehicle (αMEM+) for 60 min. AICAR was removed via washing prior to exposure to either insulin (100 nmol/L) or vehicle (αMEM+) for 15 min followed by exposure to experimental medium as described below. The insulin concentration used in the experiments has been shown to maximally stimulate palmitate uptake.[22] For each condition, following exposure to experimental medium, palmitate uptake and oxidation was measured as described below. Since the stimulatory effects of exercise or AICAR treatment on insulin-sensitive glucose uptake are dependent on the presence of serum in the incubation medium,[14] 2% serum was included for all RAA treatments.

Palmitate uptake

Following each experimental treatment, the incubation medium was replaced with experimental medium containing 100 μmol/L albumin-bound palmitate and [1-^{14}C]palmitate (5 μCi; MP Biomedical, Irvine, CA, USA) for 30 min to measure palmitate uptake and oxidation. We chose 30 min incubation with palmitate as it has been shown not to have deleterious effects on insulin signaling[23] or to stimulate the phosphorylation of AMPK or ACC or the protein expression of peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α).[24] Incubations were terminated by removing the media that were used to assay for ^{14}C-labeled oxidation products (see below). Wells were washed twice with KRB and cells were lysed by mixing with 0.05% sodium dodecyl sulfate (SDS). Duplicate aliquots of lysate were taken and mixed with scintillation fluid (BudgetSolve, Research Product International, Mount Prospect, IL, USA) and counted in a Tri-carb liquid scintillation analyzer (model 2100TR, Packard, Meriden, CT, USA). Duplicate aliquots of the same lysate were taken for protein determination using the Bradford method (Bio-Rad, Richmond, CA, USA). Parallel studies were performed using unlabeled palmitate bound to albumin (1:1; 100 μmol/L) to determine the activation state of aPKCζ/λ, AMPKα_2, ACC as well as total protein content of AMPKα, CD36, PGC-1, cytochrome C and aPKCζ/λ by Western blotting.

Palmitate oxidation

Oxidation products were measured as previously described in detail.[22,25] Briefly, duplicate aliquots of experimental media were mixed with 70% perchloric acid in a vial to release $^{14}CO_2$ that was trapped on filter paper (Whatman) saturated with ethanolamine and glued to the caps. After complete $^{14}CO_2$ release, the filter paper was released from the caps, mixed with toluene-based scintillation cocktail and analyzed for $^{14}CO_2$ radioactivity.

Western blotting

Following experimental treatment and/or insulin exposure, cells were washed with ice-cold KRB. Lysis buffer was added (20 mmol/L Tris/1% Np-40/137 mmol/L NaCl/ 1 mmol/L $CaCl_2$/1 mmol/L $MgCl_2$/10% (v/v) glycerol/ 1 mmol/L dithiothreitol/1 mmol/L phenylmethanesulfonylfluoride/2 mmol/L Na_3VO_4) and cells were scraped to one side of the well. The lysates were transferred to microcentrifuge tubes and gently pelleted. The supernatants were collected and aliquots were used to measured protein content via the Bradford method (Bio-Rad) and were subjected to 7.5% SDS polyacrylamide gel electrophoresis. The separated proteins were transferred onto Immobilon-P-polyvinylidene difluoride membranes and blocked with 1% bovine serum albumin in Tween-Tris-buffered saline (500 mmol/L NaCl/ 20 mmol/L Tris/0.05% Tween-20, pH = 7.5) (TTBS) for one hour (23°C) on a shaking orbitron, rinsed three times with TTBS and incubated overnight with antibodies to either phospho-AMPKα (Thr^{172}) (1:1000) (Cell Signaling Technologies, Beverly, MA, USA), phospho-ACC (Ser^{79}) (1:1000) (Cell Signaling Technologies), phospho-aPKC-ζ/λ ($Thr^{410/403}$) (1:1000) (Cell Signaling Technologies), AMPKα_2 (1:1000) (Cell Signaling Technologies), aPKC-ζ (1:1000) (Cell Signaling Technologies), CD36 (1:1000) (Cascade Bioscience, Winchester, MA, USA), cytochrome C (1:1000) (BD Bioscience, San Diego, CA, USA) or PGC-1α (1:1000) (Calbiochem, San Diego, CA, USA) at 4°C with constant shaking. After further washing, the membranes were incubated with the secondary antibody horseradish peroxidase goat anti-rabbit (1:2000) (Pierce, Rockford, IL, USA) for one hour at room temperature, rinsed twice with TTBS and once with TBS. The membranes were developed via ECL (Super Signal West Pico, Pierce, Rockford, IL, USA) followed by exposure to Kodak X-OMAT film. Films were scanned using an HP ScanJet (6200C) and quantitated using Scion Image (Scion, Frederick, MD, USA). Equal protein content in each lane was confirmed by stripping each membrane with 0.5 mmol/L NaOH and reprobing with antibodies for glyceraldehyde 3-phosphaste dehydrogenase (1:1000) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).[22] In all cases, multiple blots were analyzed and data were expressed relative to total protein content.

Calculations and statistics

The rate of palmitate uptake was calculated as the amount of radioactivity taken up by the cells in one well divided by the protein concentration. The rate of palmitate oxidation was calculated as the amount of radioactivity recovered as $^{14}CO_2$ by the cells in one well divided by the protein concentration of the same well and corrected for label fixation.[22] All presented data are expressed as mean $\pm$ standard error of several experiments and are expressed as the percent of control, where control refers to cells that were not treated with AICAR or insulin. The percent control was calculated using measured rates (nmol/L/g/ min) for all experimental treatments, except where noted in the Results section. The effects of treatment with AICAR and/or insulin were analyzed using analysis of variance (StatSoft Statistica 6.0, Tulsa, OK, USA) followed by Fisher's least-squares difference *post hoc* test when appropriate. In all instances, an α of 0.05 was used to determine the significance.

Results

AICAR dose–response curve

AA treatment gradually increased palmitate uptake with increasing AICAR concentrations (100 μmol/L–2 mmol/L; 399.8 $\pm$ 24.2–716.6 $\pm$ 47.8 nmol/L/g/min) with a plateau in palmitate uptake being reached at 1 mmol/L AICAR (Figure 1a). AICAR-induced palmitate oxidation following treatment with 100 μmol/L (27.2 $\pm$ 1.3 nmol/L/g/min) and 500 μmol/L (26.9 $\pm$ 1.5 nmol/L/g/min) AICAR was not different ($P > 0.05$) from basal palmitate oxidation (24.9 $\pm$ 1.7 nmol/L/g/min), but rose following treatment with 1 mmol/L (40.5 $\pm$ 2.0 nmol/L/g/min) AICAR ($P < 0.05$) (Figure 1b). The rate of palmitate oxidation (41.4 $\pm$ 2.3 nmol/L/g/min) measured following incubation with 2 mmol/L AICAR was not different from the rate observed following 1 mmol/L AICAR ($P > 0.05$). Hence, we chose to use 1 mmol/L AICAR in our experiments.

AICAR and AMPK activation

To verify that AMPK activity was stimulated by AICAR treatment in our cultured cell system, we measured the phosphorylation state of AMPKα and of its known downstream enzymatic target ACC.[26] As we and others have shown,[10,27,28] AICAR treatment increased AMPKα ($P < 0.05$) (Figure 2a) and ACC phosphorylation ($P < 0.05$) (Figure 2b) when compared with control cells not treated with AICAR. Insulin had no impact on the phosphorylation state of either enzyme ($P > 0.05$). There was no difference in AMPK activation or total AMPK protein content between control and RA cells 19 h after the last AICAR treatment (data not shown).

Palmitate uptake

AICAR treatment significantly ($P < 0.05$) increased basal palmitate uptake by 30–43% under all conditions (AA, RA, RAA) as compared with control cells not treated with AICAR (Figure 3a). As previously shown in L6 cells,[18,29] insulin significantly increased ($P < 0.05$) palmitate uptake by 27% in control cells not treated with AICAR. In AA and RA cells, insulin-stimulated palmitate uptake was not

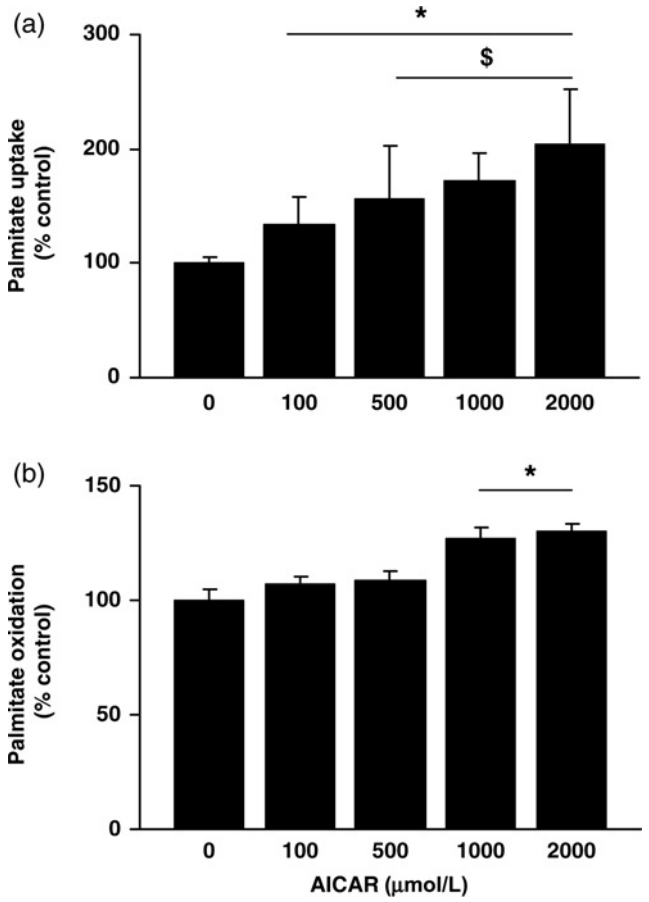

Figure 1 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) dose–response curve for palmitate uptake (a) and oxidation (b) in L6 myotubes. Cells were exposed to AICAR (100–2000 μmol/L) for 60 min followed by exposure to 100 μmol/L [^{14}C]palmitate for 30 min. Palmitate uptake and oxidation were measured as described in Materials and methods. Data are presented as means $\pm$ standard error. $^{*}P < 0.05$ versus control cells (0 μmol/L AICAR). $^{\$}P < 0.05$ versus 100 μmol/L AICAR

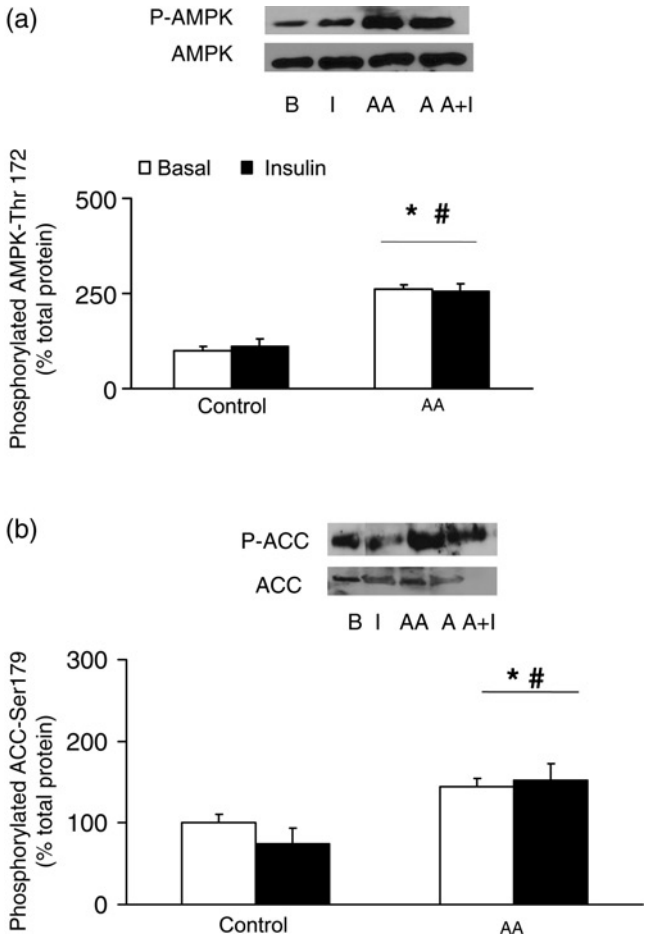

Figure 2 Effects of acute 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (AA) treatment on AMP-regulated protein kinase (AMPK) (a) and acetyl-coA carboxylase (ACC) (b) phosphorylation in L6 myotubes. Cells were treated as described in the Materials and methods. Cells were lysed and prepared for Western blotting with antibodies to (a) phosphorylated-AMPK (Thr^{172}) and (b) phosphorylated-ACC (Ser^{79}). Panels above the graph show representative blots for B = basal (no AICAR, no insulin), I = insulin only, AA = AICAR only, AA + I = AICAR + insulin for both phosphorylated and total AMPK and ACC. On the *x*-axis, control refers to cells not treated with AICAR and AA refers to cells treated with AICAR acutely. Data are presented as means $\pm$ standard error ($N = 6$ per condition) and are expressed as percent value of total protein content. $^{*}P < 0.05$ versus control (no AICAR/no insulin). $^{\#}P < 0.05$ versus insulin only (no AICAR)

significantly higher than the uptake measured in the non-insulin-stimulated state. Thus, insulin stimulation had no additive effect on palmitate uptake in AA and RA cells. Insulin stimulation further increased palmitate uptake in RAA cells (97%) as compared with control cells stimulated with insulin (Figure 3a).

Palmitate oxidation

AICAR treatment increased palmitate oxidation by 42% and 128% in AA and RAA groups, respectively, as compared with control cells not treated with AICAR (Figure 3b). RAA treatment enhanced palmitate oxidation by 60% ($P < 0.05$) as compared with AA. RA treatment had no effect on palmitate oxidation. As we[18] and others[29] have shown previously, insulin significantly ($P < 0.05$) decreased palmitate oxidation by 17% in control cells. AICAR treatment prevented the insulin-induced decrease in both AA and RA groups; however, the suppressive effect of insulin on FA oxidation was partially restored in RAA-treated cells (−27%) (Figure 3b) suggesting a return of insulin sensitivity.

AICAR treatment and insulin signaling

Based on the enhanced effects of insulin on FA uptake in RAA-treated cells and previous work from our lab,[18] which suggests that aPKC-ζ regulates FA uptake, we measured the phosphorylation state of aPKC-ζ in these cells. As expected, insulin stimulation increased the phosphorylation state of aPKC-ζ in both RAA-treated cells and in control cells not treated with AICAR (Figure 4) ($P < 0.05$). Further we found that RAA treatment resulted in an increase in total aPKC-ζ protein content. When expressed relative to total protein content, the activation state of aPKC-ζ under both basal and insulin-stimulated conditions was similar between the RAA-treated cells and control cells not treated with AICAR (data not shown).

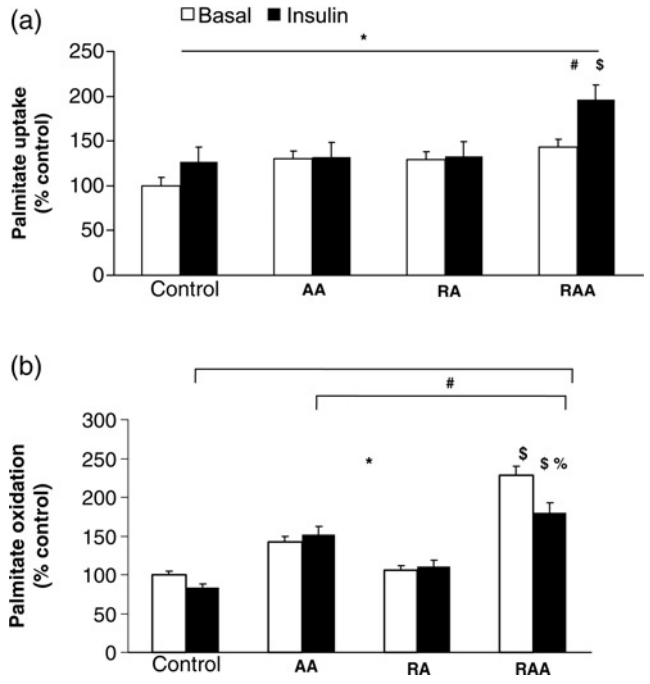

Figure 3 Effects of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment on basal and insulin-stimulated palmitate uptake (a) and oxidation (b) in L6 myotubes. Cells were treated as described in Materials and methods. Data are presented as means $\pm$ standard error ($N = 12$ per condition) and expressed as '% Control', where 'control' is defined as the cells not treated with AICAR or insulin. On the *x*-axis, control refers to cells not treated with AICAR. RAA refers to cells treated with AICAR for five days and on the sixth day. RA refers to cells treated with AICAR for five days only. AA refers to cells treated with AICAR acutely. White bars represent basal condition; black bars represent insulin-stimulated condition. $^{*}P < 0.05$ versus basal (no AICAR/no insulin). $^{\#}P < 0.05$ versus insulin only. $^{\$}P < 0.05$ versus AA and CA. $^{\%}P < 0.05$ versus RAA

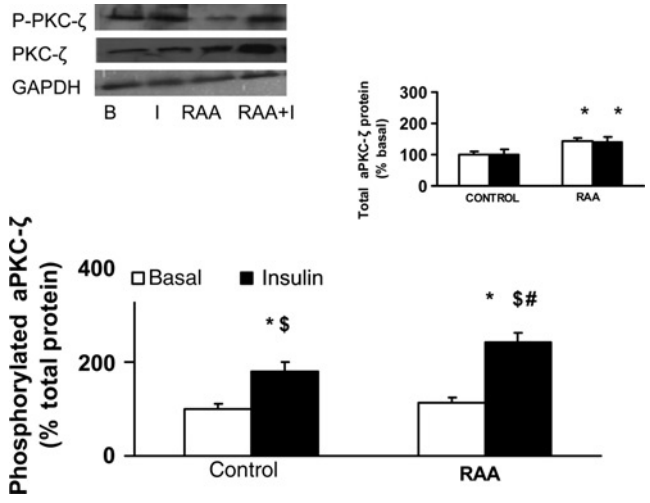

Figure 4 Effects of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment on atypical protein kinase C-ζ (aPKC-ζ) activation and protein expression in L6 myotubes. Cells were treated as described in Materials and methods. Cells were lysed and prepared for Western blotting with phosphorylated aPKC-ζ/λ ($Thr^{410/403}$) or anti-aPKC-ζ. Data are presented as means $\pm$ standard error ($N = 4$ per condition) and expressed as % total protein. Panels above the graph are representative blots for B = basal (no AICAR, no insulin), I = insulin only, RAA = AICAR only, RAA + I = AICAR + insulin. Inset graph displays total aPKC-ζ protein. $^{*}P < 0.05$ versus basal (no AICAR/no insulin). $^{\$}P < 0.05$ versus basal RAA

AICAR treatment and CD36, PGC-1α and cytochrome C protein expression

Insulin and AMPK activation have been shown to stimulate FA uptake via CD36 translocation.[30,31] In line with this, we measured an increase in total CD36 protein expression ($P < 0.05$) in cells repeatedly treated with AICAR as compared with cells not treated with AICAR (Figure 5). We saw no change in CD36 protein with AA treatment. Insulin has no further effect on CD36 expression ($P = 0.12$). As previously reported in C_2C_{12} cells,[32] we measured increases in total PGC-1α and cytochrome C protein content (Figure 6) ($P < 0.05$) with RA treatment, suggesting an increase in mitochondrial biogenesis in these cells.[32] We measured no change in either protein with AA treatment (data not shown). Insulin had no effect on the expression of either protein (data not shown).

Discussion

Multiple studies have addressed the effects of AICAR treatment on insulin-stimulated glucose metabolism in the muscle[2,8,12]; however, only a few have looked at the effects of AICAR on insulin-stimulated FA metabolism and those that have are incomplete in addressing both FA uptake and oxidation under acute and/or repeated treatment.[28,33] Herein, our data are the first to comprehensively study the effects of AICAR treatment in muscle cells with respect to insulin-stimulated FA metabolism and to provide insight into the time-dependent nature of the effects of AICAR treatment in cultured cells on FA metabolism. Our results

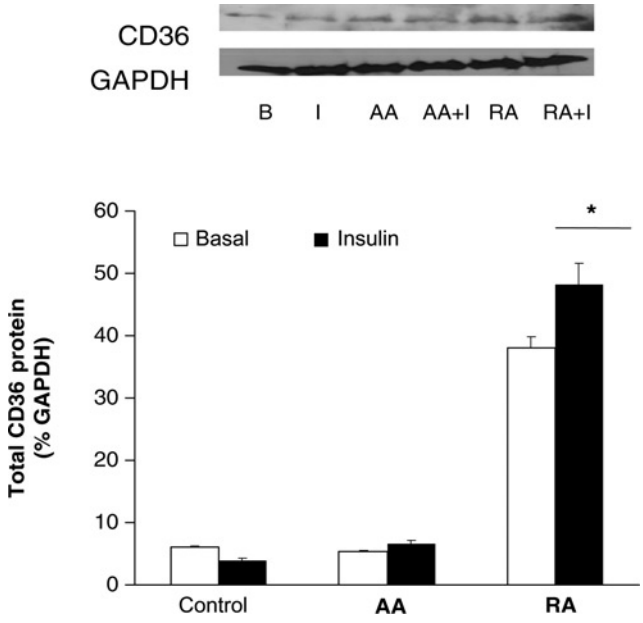

Figure 5 Effects of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment on protein expression of CD36 in L6 myotubes. Cells were exposed to α-minimum essential medium positive (αMEM+) or AICAR (1 mmol/L) for either 60 min (AA) or 5 h/d for five days (RA). This was followed by treatment with insulin (100 nmol/L) or αMEM+ for 15 min and 100 μmol/L palmitate for 30 min. Cells were lysed and prepared for Western blotting with anti-CD36. Data are presented as means $\pm$ standard error ($N = 4$). $^{*}P < 0.05$ versus control (no AICAR)

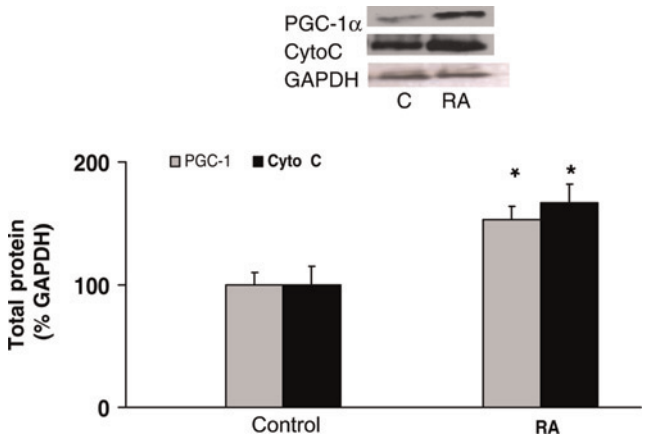

Figure 6 Effects of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) treatment (RA) on protein expression of peroxisome proliferator-activated receptor-γ co-activator-1 (PGC1α) and cytochrome C in L6 myotubes. Cells were treated as described in Materials and methods. Cells were lysed and prepared for Western blotting with antibodies to PGC1α or cytochrome C. Data are presented as means ± standard error ($N = 4$). RA refers to cells treated with AICAR for five days only. $^{*}P < 0.05$ versus control (A color version of this figure is available in the online journal)

show for the first time that RAA treatment enhanced the sensitivity of muscle cells to the stimulatory effects of insulin on palmitate uptake and restores the ability of insulin to reduce palmitate oxidation. Further, our data show that RAA treatment results in enhanced expression of proteins known to be involved in the regulation of FA uptake and FA oxidation in basal and insulin-mediated states (aPKC-ζ, CD36, PGC-1α and cytochrome C). Thus, it can be suggested that recurring AMPK activation alters insulin-regulated FA metabolism possibly via changes in protein expression of FA transporter proteins, oxidative enzymes and specific insulin signaling intermediates.

Independently, insulin and AICAR have been shown by us and others to increase FA uptake in isolated or perfused muscle and in incubated muscle cells.[10,22,34] Here, we extend our data in L6 myotubes and show that insulin-stimulated FA uptake is enhanced with prior RA treatment and that this effect is time dependent. When insulin stimulation was tested within one hour of the last AICAR treatment (RAA group), the increase in insulin-stimulated FA uptake was greater than the effects of the two stimuli alone, indicating that synergism between the AICAR- and insulin-stimulated signaling cascades exists. When the length of time between the last AICAR treatment and insulin stimulation was extended to 19 h (RA group), the stimulatory effect of AICAR on insulin-mediated FA uptake was diminished. Since the effects of prior repeated AMPK activation on insulin-stimulated FA uptake were dependent on the time elapsed between the last AICAR treatment and insulin stimulation (RAA versus RA group), the following suggestions may be presented. Our results suggest that the cellular response induced by RA treatment was not sufficient in and of itself to calculate a synergistic increase in insulin-mediated FA uptake. The last bout of AICAR treatment one hour before insulin stimulation was necessary to observe a synergistic increase in FA uptake. Contrary to results observed in the RAA group, when a single AICAR treatment was immediately followed by insulin stimulation (AA group), insulin-stimulated FA uptake was not improved. Recently, Feduic *et al.*[29] acutely incubated L6 myotubes with troglitazone, an AMPK agonist, followed by stimulation with insulin. Similar to our findings, insulin alone increased FA uptake by approximately 20%; however, troglitazone plus insulin failed to increase FA uptake. Thus, it may be that acute AMPK activation can only improve insulin-sensitive FA uptake after cellular alterations induced by RA treatment of at least five days have occurred.

In line with this, we observed an increase in the total protein content of aPKC-ζ and of the FA transport protein, CD36 with RA treatment. These results suggest that the observed improvements in insulin-stimulated FA uptake measured in RAA cells may have been due in part to an increase in total FA transport capacity and in insulin signaling. Whether CD36 translocation to the plasma membrane was similarly increased is not known at this time. Further, repeated exercise- and AICAR-induced AMPK activation has been shown to alter several components of the insulin signaling pathways. In skeletal muscle from exercised rats and in C_2C_{12} myotubes exposed to AICAR, repeated AMPK activation was associated with higher levels of insulin-induced IRS-1–PI3K association, Akt/PKB phosphorylation and Akt substrate of 160 kDa (AS160).[2,35] Our measured increase in both aPKC-ζ phosphorylation and aPKC-ζ protein suggests that, like Akt/PKB, the PI3K–aPKC-ζ insulin signaling cascade is amplified with AICAR treatment. However, more studies focused on this proposed mechanism are needed to confirm this hypothesis.

A novel finding in our study is that RAA treatment restored the ability of insulin to suppress palmitate oxidation. These observations suggest that metabolic flexibility is only preserved when the acute effects of AMPK activation are superimposed on the effects of repeated AMPK activation. Our data agree with findings obtained in cardiac muscle[36,37] and show that under these cellular conditions insulin can effectively downregulate AMPK signaling as it pertains to FA oxidation. In contrast, insulin failed to suppress FA oxidation following an AA treatment. Winder and Holmes[33] found similar results in perfused rat hindlimb and suggested that the high rates of FA oxidation observed with combined AICAR and insulin stimulation might be necessary for the generation of ATP during the contractile process. While speculative on our part, the data in RAA-treated cells support this theory, in that the rate of palmitate oxidation was still significantly higher than that observed in the groups not treated with AICAR despite the attenuation with insulin. The oxidation of palmitate can be influenced by many factors, palmitate uptake, carnitine availability, malonyl coA concentrations, all of which may flux through the mitochondria.[6,38] Currently, a mechanistic explanation for how insulin mediates a decrease in AMPK-induced FA oxidation is not known. We observed an increase in CD36 protein expression and it has been suggested by some that CD36 may be important for FA shuttling across mitochondrial membranes.[39] Consequently, it may be that upon stimulation with AICAR and insulin, CD36 proteins are redistributed towards the plasma

membranes rather than the mitochondrial membranes and that this leads to an increase in FA uptake and a reduction in FA oxidation, as we observed in the RAA group. Given the current findings, more specific experiments are warranted in order to address the mechanisms by which insulin decreases FA oxidation under certain conditions.

Insulin did not decrease palmitate oxidation following five days of AICAR treatment (RA-treated cells) and we did not observe an increase in palmitate oxidation in the basal state in RA-treated cells. This result was not surprising given that 19 h had elapsed since the last AICAR treatment and that AMPK activity has been shown to return to basal values within three hours.[40] The lack of change in palmitate oxidation in RA-treated cells may seem paradoxical given that RA treatment was associated with an increase in PGC-1α and cytochrome C total protein content, an indication that oxidative capacity was raised by the treatment. However, our results are in line with previous data, which show that FA oxidation is not dictated simply by oxidative capacity.[25]

In summary, RA treatment enhanced insulin-sensitive FA uptake in a time-dependent manner in muscle cells and increased total CD36 and aPKCζ protein expression, indicating that alterations in both FA uptake capacity and insulin signaling potential may be required for an improvement in insulin-sensitive FA uptake. Further, RA treatment increased PCG1α and cytochrome C protein expression. However, this higher oxidative potential was only associated with a significant increase in AICAR-induced FA oxidation when cells were also treated with AICAR acutely. Recurring AMPK activation restored the suppressive effect of insulin when an acute stimulus immediately preceded insulin stimulation, suggesting that AICAR treatment may help the muscle cell maintain metabolic flexibility. More studies will be needed to improve our understanding of the cellular mechanisms by which repeated AMPK activation sensitizes the muscle cell to the actions of insulin. While L6 cells have been widely used in metabolic studies, they are not without limitations. Primarily, cell culture models do not represent a physiological state in which multiple signaling cascades are activated upon contraction and/or the muscle is not exposed to the milieu of hormones and substrates present in intact systems. Nonetheless, the data presented here provide methodological insight about the appropriate time course when using AICAR in L6 cells.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; KRK conducted the majority of the experiments, MJA did Western blot and Western blot analysis and statistics, KRK and LPT wrote the manuscript and MJA contributed with editing, commentary and feedback.

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