Prior serum- and AICAR-induced AMPK activation in primary human myocytes does not lead to subsequent increase in insulin-stimulated glucose uptake

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Al-Khalili, Lubna, Anna Krook, Juleen R. Zierath, and Gregory D. Cartee. Prior serum- and AICAR-induced AMPK activation in primary human myocytes does not lead to subsequent increase in insulin-stimulated glucose uptake. Am J Physiol Endocrinol Metab 287: E553–E557, 2004. —Exposing isolated rat skeletal muscle to 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside [AICAR, a pharmacological activator of AMP-activated protein kinase (AMPK)] plus serum leads to a subsequent increase in insulin-stimulated glucose transport (Fisher JS, Gao J, Han DH, Holloszy JO, and Nolte LA. Am J Physiol Endocrinol Metab 282: E18–E23, 2002). Our goal was to determine whether preincubation of primary human skeletal muscle cells with human serum and AICAR (Serum+AICAR) would also induce a subsequent elevation in insulin-stimulated glucose uptake. Cells were preincubated for 1 h under 4 conditions: 1) without AICAR or serum (Control), 2) with serum, 3) with AICAR, or 4) with Serum+AICAR. Some cells were then collected for immunoblot analysis to assess phosphorylation of AMPK (pAMPK) and its substrate acetyl-CoA carboxylase (ACC). Other cells were incubated for an additional 4 h without AICAR or serum and then used to measure basal or insulin-stimulated 2-deoxyglucose (2-DG) uptake. Level of pAMPK was increased (P < 0.01) for myotubes exposed to Serum+AICAR vs. all other groups. Phosphorylated ACC (pACC) levels were higher for both Serum+AICAR (P < 0.05) and AICAR (P < 0.05) vs. Control and Serum groups. Basal (P < 0.05) and 1.2 nM insulin-stimulated (P < 0.005) 2-DG uptake was higher for Serum vs. all other preincubation conditions at equal insulin concentration. Regardless of insulin concentration (0, 1.2, or 18 nM), 2-DG was unaltered in cells preincubated with Serum+AICAR vs. Control cells. In contrast to results with isolated rat skeletal muscle, increasing the pAMPK and pACC in human myocytes via preincubation with Serum+AICAR was insufficient to lead to a subsequent enhancement in insulin-stimulated glucose uptake.

exercise; skeletal muscle; glucose transport; 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMP-activated protein kinase

EXERCISE CAN INCREASE GLUCOSE TRANSPORT in skeletal muscle by two distinct mechanisms. It enhances glucose transport in the absence of insulin (insulin-independent glucose transport), and it enhances insulin-dependent glucose transport (5, 6, 15, 29). Insulin-independent glucose transport is increased during (22) and immediately after exercise (29). This effect is largely reversed by ~3 h postexercise in rat skeletal muscle (11, 29). In contrast, insulin-stimulated glucose transport in rat skeletal muscle can remain elevated for several hours to several days postexercise (5, 6, 29).

Similar to in vivo exercise, electrically stimulated contractile activity by isolated rat skeletal muscle in serum-free buffer also results in a substantial increase in insulin-independent glucose transport (8), and this effect is largely reversed within hours (5, 9, 11). In contrast to in vivo exercise, contractile activity by isolated rat skeletal muscle in serum-free buffer does not result in a subsequent enhancement of insulin sensitivity (5). Gao et al. (11) found that serum must be present during contractile activity for in vitro muscle contraction to induce an elevation in insulin-stimulated glucose transport.

Recently, Fisher et al. (10) provided evidence that activation of AMP-activated protein kinase (AMPK) may be a key event in the contraction-induced improvement in insulin action. They studied muscles incubated in serum with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or in vitro hypoxia, two stimuli that activate AMPK, as does exercise and in vitro contraction. Prior treatment with AICAR or hypoxia in the presence of serum resulted in a subsequent increase in insulin-stimulated glucose transport. As with in vitro contractions, neither AICAR nor hypoxia treatment in serum-free buffer led to enhanced insulin sensitivity.

Primary human skeletal muscle cells have a number of characteristics that make them a useful model for studying muscle metabolism. Primary human myocytes respond to insulin with increased glucose transport (26). They retain some aspects of the donor’s in vivo metabolic phenotype such as insulin resistance in myocytes derived from people with type 2 diabetes (14), and they can be maintained in vitro for days, allowing genetic manipulation, including adenovirus-induced overexpression of genes (3) and RNA interference-mediated reduction in gene expression (1).

Our major goal in this study was to determine whether exposing human myocytes to serum and AICAR would lead to a subsequent enhancement in insulin-stimulated glucose uptake, with the ultimate aim of using this cell model to probe the mechanisms whereby insulin sensitivity is upregulated in human skeletal muscle. To aid in the interpretation of our findings, we also evaluated the effects of serum and/or AICAR on phosphorylation of AMPK and its substrate, acetyl-CoA carboxylase (ACC). We hypothesized that exposure of myocytes to serum plus AICAR (Serum+AICAR) would elevate the amount of phosphorylated AMPK and ACC and lead to a subsequent enhancement in insulin-stimulated 2-deoxyglucose (2-DG). Our data indicated that, although simultaneous prein-
cubation of human primary myocytes with AICAR and serum can lead to increased phosphorylated AMPK and ACC, an elevation in insulin-stimulated glucose uptake did not follow.

MATERIALS AND METHODS

Subjects. Skeletal muscle biopsies (rectus abdominus) were obtained with informed consent from healthy human volunteers (3 male and 4 female, age 57 ± 5.5 yr, body mass index 26 ± 1.7 kg/m², fasting plasma glucose concentration 5.5 ± 0.3 mM) during scheduled abdominal surgery. Protocols were approved by the Ethics Committee at the Karolinska Institute. Human serum used for the cell culture incubations was obtained from healthy men after an overnight fast. The blood was aspirated and stored at -80°C from healthy men after an overnight fast. The blood was allowed to clot for 30 min at room temperature and centrifuged at 3,000 g for 10 min at 4°C. Serum was aspirated and stored at -70°C until used during incubations.

Cell culture. Media were purchased from Invitrogen (Stockholm, Sweden). Satellite cells were isolated, grown, and differentiated as previously described in detail (2). Myoblasts from passage 2 were grown in growth medium (GM, Ham’s F-10 medium) containing 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PenSt). To initiate differentiation into myotubes, GM was removed from cells and replaced with Dulbecco’s Modified Eagle’s Medium (DMEM) containing 1% PenSt and 4% FBS for 48 h. Subsequently, the medium was changed to DMEM supplemented with 1% PenSt and 2% FBS.

Incubations with serum and/or AICAR. All incubations were performed at 37°C. On the day before the experiment at -1600, the supplemented GM was replaced with serum-free DMEM containing 5.5 mM glucose. At -1000 on the experimental day, cells were washed twice with DMEM plus glucose (5.5 mM) and then incubated for 60 min in one of four conditions using DMEM + glucose and 1) no supplementation (Control), 2) supplemented with 10% human serum diluted with DMEM + glucose (Serum), 3) supplemented with 2 mM AICAR (AICAR), or 4) supplemented with 10% human serum and 2 mM AICAR (Serum + AICAR). Cells used for immunoblot analyses were harvested after this incubation step (experiment 1), and cells used for 2-DG uptake were washed four times with DMEM + glucose and then incubated for 4 h in DMEM + glucose (experiment 2). Next, the cells were washed with Krebs buffer supplemented with 0.1% bovine serum albumin (BSA) and then incubated for 1 h in Krebs-BSA with the appropriate insulin concentration (0, 1.2, or 18 nM), followed by addition of 2-[3H]DG (0.33 μCi/well) for 10 min. Some cells were treated as described for experiment 2 except that 100% rather than 10% serum was used, and the insulin concentrations studied were 0 and 18 nM.

The effect of short-term AICAR exposure (60 min) immediately before and during 2-DG uptake was also assessed (experiment 3). Cells were treated as described above, except at -1000 on the experimental day, they were incubated for 4 h in DMEM + glucose. Next, they were washed with DMEM + glucose and incubated for 60 min in Krebs with or without 2 mM AICAR supplementation, followed by addition of 2-[3H]DG (0.5 mM, 0.33 μCi/well) for 10 min in the continued presence of AICAR.

Immunoblot. Cells were harvested and processed as previously described (18). Membranes were immunoblotted either with an antibody that detects phosphorylation of AMPK at Thr172 (Cell Signaling Technology, Beverly, MA) or with a phosphospecific antibody raised against a peptide corresponding to the sequence in rat ACCα containing the Ser24 phosphorylation site (Upstate Technology, Lake Placid, NY). The pACC antibody also recognizes human ACCβ phosphorylated at Ser251. Enhanced chemiluminescence was used to visualize protein bands that were quantitated using a phosphoimager (Fuji model BAS-1800II Bio-Image Analyzer; Fuji Photo Film, Tokyo, Japan). Values are expressed relative to the group mean for control cells that were not incubated with AICAR or serum.

2-Deoxyglucose uptake. The rate of 2-DG uptake was assessed as previously described (28). Cells were lysed by shaking for 60 min in 0.5 N NaOH, followed by freeze-thawing. Accumulation of 2-[3H]DG in cells was quantitated by liquid scintillation counting (Wallac model 1214; Rackbeta, Turku, Finland). For the study on the effect of preincubation with serum and/or AICAR, values are expressed relative to the mean value in the basal (no insulin) group without preincubation with AICAR or serum. For the experiment on the effect of short-term AICAR exposure immediately before and during 2-DG uptake measurement, values are expressed relative to the mean value for the group not treated with AICAR. Total cellular protein concentration was determined by the Bradford method (Bio-Rad, Richmond, CA).

Statistical analysis. Group data are expressed as means ± SE or median, as indicated. One-way analysis of variance (ANOVA) was performed to identify significant effects of the incubation conditions (Control, AICAR, Serum, and Serum + AICAR) on 2-DG uptake or protein phosphorylation by use of SigmaStat software version 2.03 (San Rafael, CA). The Kruskal-Wallis ANOVA on ranks was used when data failed the equal variance test. The Student-Newman-Keuls post hoc test was used to locate the source of significant variance (P ≤ 0.05). A paired t-test was used to compare 2-DG uptake in cells exposed to AICAR and control cells incubated in the absence of AICAR.

RESULTS

Immunoblotting. Immunoreactivity for pAMPK was approximately threefold greater (P < 0.01) for the cells that underwent combined AICAR and serum treatment compared with all other groups (Fig. 1). There were nonsignificant trends for increased phosphorylated AMPK levels in AICAR-treated (∼1.9-fold greater) and serum-treated (∼1.7-fold greater) groups compared with Control values.

Because the pACC data failed the equal variance test, the Kruskal-Wallis ANOVA on ranks was used to compare group values. The pACC values for AICAR-treated cells (AICAR

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**Fig. 1.** Phosphorylated AMP-activated protein kinase (pAMPK) determined by immunoblot analysis in primary human skeletal muscle cells after preincubation with neither serum nor AICAR (Control, C), 10% serum (Serum, S), 2 mM AICAR (AICAR, A), or Serum + AICAR (S + A). The antibody recognizes pAMPK phosphorylated at Thr172. Data are expressed relative to the mean value for the Control group. Group values are means ± SE (n = 4 per group). ANOVA with a Student-Newman-Keuls post hoc test was used to compare groups. *P < 0.01 compared with all other groups. Representative immunoblot is shown.
and Serum+AICAR groups) were markedly elevated (P < 0.05) compared with each group not exposed to AICAR (Control and Serum groups; Fig. 2). The AICAR and Serum+AICAR groups had similar pACC levels. Although the pACC value for the Serum group tended to be lower than that of the Control group, this was not a significant difference.

**2-DG uptake.** Basal (no insulin) 2-DG uptake in the Control group (i.e., cells exposed to neither AICAR nor serum) was 22.86 ± 2.54 pmol·mg protein⁻¹·min⁻¹. Preincubation of primary human myocytes with serum in the absence of AICAR led to a subsequent increase (25%, P < 0.05) in basal 2-DG uptake compared with basal values for all other groups (Control, AICAR, or Serum+AICAR; Table 1). None of the other groups without insulin differed significantly from one another; i.e., preincubation with 2 mM AICAR did not influence 2-DG uptake without insulin measured 5 h later, and the serum-effect on 2-DG was eliminated when AICAR was present during preincubation with serum.

The 2-DG uptake rate measured in the presence of 1.2 nM insulin was increased (P < 0.005) by preincubation with serum compared with all other preincubation conditions (Control, AICAR, or Serum+AICAR; Table 1) at this insulin concentration. As in the absence of insulin, the serum effect on 2-DG uptake with 1.2 nM insulin was eliminated when AICAR was included during the preincubation with serum.

The 2-DG uptake with 18 nM insulin did not significantly differ among the preincubation groups (Table 1). However, similar to the results with 0 or 1.2 nM insulin, 2-DG uptake in the Serum group tended to be higher than in all other groups at the same insulin level, including the Serum+AICAR group.

We previously reported that the magnitude of increase in basal 2-DG is very similar after preincubation with 10% compared with 100% serum; i.e., the full serum effect is achieved with 10% serum (1). In addition, as with 10% serum, the 2-DG uptake, with or without insulin, tended to be 16–17% lower after preincubation with 100% serum plus AICAR compared with the 100% Serum group (data not shown), which is similar to the 16–19% difference between the 10% serum plus AICAR and 10% Serum groups (Table 1).

The rate of 2-DG uptake determined in the presence of AICAR was not significantly different from control cells that were treated identically but without AICAR exposure (Fig. 3).

**DISCUSSION**

Recent studies have suggested that AMPK may be important for the enhanced insulin action in skeletal muscle after exercise. The design of the current research was patterned after the study by Fisher et al. (10) using isolated rat skeletal muscle. Their experiments had, in turn, been based on a previous study (11), which showed increased insulin sensitivity in rat skeletal muscle.

**Table 1. Effect of preincubation with serum and/or AICAR on subsequent 2-DG uptake by primary human skeletal muscle cells**

<table>
<thead>
<tr>
<th>Insulin During 2-DG Uptake, nM</th>
<th>Preincubation Without Serum or AICAR (Control)</th>
<th>Preincubation With Serum</th>
<th>Preincubation With AICAR</th>
<th>Preincubation With Serum + AICAR</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>1.00 ± 0.11</td>
<td>1.25 ± 0.05*</td>
<td>0.93 ± 0.02</td>
<td>1.05 ± 0.04</td>
</tr>
<tr>
<td>1.2</td>
<td>1.18 ± 0.07</td>
<td>1.53 ± 0.06**</td>
<td>1.13 ± 0.04</td>
<td>1.28 ± 0.06</td>
</tr>
<tr>
<td>18</td>
<td>1.40 ± 0.13</td>
<td>1.54 ± 0.11</td>
<td>1.23 ± 0.09</td>
<td>1.24 ± 0.18</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 4–7 per group. Values are expressed as fold increase relative to the mean 2-deoxyglucose (2-DG) uptake value of 1.00 for the Control group under basal (no insulin) conditions. Human serum (10%) and/or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; 2 mM) were included during a 1-h preincubation step, as indicated, 5 h before measurement of 2-DG uptake. Insulin was included as indicated during the final 70 min of incubation, and 2-[3H]DG was included during the final 10 min of incubation. Differences within each insulin concentration were analyzed using ANOVA with a Student-Newman-Keuls post hoc test to identify the source of significant variance among groups. *P < 0.05, different from all other preincubation groups without insulin; **P < 0.005, different from all other preincubation groups with 1.2 mM insulin.

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muscle after in vitro contractions performed in serum. Several notable differences exist between the present results with primary myocytes and the previously reported results with rat skeletal muscle. 1) Serum preincubation was without effect on basal glucose uptake in rat skeletal muscle (9, 11), whereas serum preincubation led to increased basal glucose uptake in primary human myocytes. This finding confirms our previously reported results (1). 2) Incubation of isolated rat skeletal muscles with AICAR is well known to increase glucose transport (12, 13, 21), but we found that, in human primary myocytes, glucose uptake was not increased in the presence of AICAR. 3) Prior exposure to Serum+AICAR leads to enhanced insulin-stimulated glucose uptake in isolated rat skeletal muscle (10), but this effect was not observed in human primary myocytes. Differences between species (humans vs. rats) and/or experimental models (primary myocytes vs. isolated skeletal muscle) are obvious candidates to explain why the results in the present study differed from results with isolated rat skeletal muscle. Primary myocytes can be a useful model for understanding skeletal muscle, but it is, of course, essential to consider fundamental differences between cultured cells and intact adult tissue when interpreting the results.

Regarding the persistent effect of serum exposure on basal glucose uptake, many previous studies with myocytes derived from various species, including rats, have found that serum can lead to enhanced basal glucose uptake. For example, primary skeletal muscle myotubes from neonatal rats (4) and L6 cells derived from rat neonatal hindlimb muscle (27) had an enhanced basal glucose uptake after serum exposure. Thus experimental model rather than species appears to account for this differing result with isolated rat skeletal muscle and human primary muscle cells.

What are possible cellular or molecular mechanisms for the differing effect of serum on glucose uptake in rat skeletal muscle vs. human primary myocytes? In primary human muscle cells, the relative expression of GLUT1 to GLUT4 is much greater than that found in mature skeletal muscle from humans or rodents (2, 26). We recently demonstrated that the serum-induced increase in basal glucose uptake in primary human muscle cells was attributable to enhanced cell surface GLUT1 glucose transporter content without increased GLUT1 total abundance (1). Perhaps the lack of a serum-induced increase in glucose uptake in rat skeletal muscle is related to the relative paucity of GLUT1 in this tissue.

In isolated human skeletal muscle, glucose uptake is increased in response to an acute AICAR exposure (17). Taken together with the present findings, these results suggest that experimental model rather than species is important for the AICAR effect on glucose uptake. Importantly, others have found that immortalized cell lines derived from rats, including L6 myocytes (7) and 3T3-L1 adipocytes (24, 25), respond to AICAR with increased glucose uptake.

The direct effect of AICAR on glucose uptake in rat (19) and human (17) skeletal muscle is attributable to increased cell surface GLUT4. GLUT4 abundance in human primary muscle cells is very low compared with rat and human skeletal muscle: 53 vs. 1,128 and 4,130 fmol/mg protein, respectively (2, 23). The relatively low level of GLUT4 in human primary myocytes may limit the ability of AICAR to activate glucose transport. However, insulin elicits an increase in glucose transport via increased GLUT4 translocation in human primary myocytes (2); so the lack of an AICAR-induced increase in glucose uptake appears to indicate a specific deficit in the AICAR-mediated pathway in these cells. In isolated rat extensor digitorum longus muscle, AICAR induces increased phosphorylation of both AMPK and p38 mitogen-activated protein kinase (p38 MAPK) (20). Furthermore, the p38 MAPK inhibitor SB-203580 eliminated the AICAR-stimulated increase in glucose uptake in rat muscle. We have found that exposure of primary human myocytes to H2O2 (1 mM for 20 min) or osmotic stress (400 mM mannitol for 20 min) can lead to an increased the phosphorylation of p38 MAPK, indicating that these cells express p38 MAPK (unpublished observations). However, neither insulin (120 nM for 20 min) nor AICAR (1 mM for 20 min) induced a detectable change in p38 MAPK phosphorylation. Further studies will be needed to fully understand why AICAR did not stimulate glucose uptake by primary human myocytes.

Regarding the influence of Serum+AICAR preincubation on subsequent insulin-stimulated glucose transport, there are apparently no published studies that address this issue with isolated human skeletal muscle or with primary myocytes from rats. Performance of these experiments could provide valuable insights into the putative role of AMPK for regulating insulin sensitivity.

A substantial increase in phosphorylation of AMPK and ACC was observed in the Serum+AICAR-treated human primary myocytes. Thus, in human primary myocytes, activation of AMPK in the presence of serum was not sufficient to induce a subsequent increase in insulin-stimulated glucose uptake. We recently evaluated the relationship between AMPK phosphorylation and increased insulin-stimulated glucose transport in rat skeletal muscle after a number of different protocols for in situ contractions (16). Consistent with the current findings using human myocytes, some contraction protocols led to elevated AMPK phosphorylation yet did not result in a subsequent increase in insulin action. Nonetheless, every contraction protocol that led to enhanced insulin sensitivity in rat skeletal muscle was also characterized by increased AMPK phosphorylation, suggesting that activation of AMPK together with some unknown process(es) may have been important for increased insulin-stimulated glucose transport. It remains possible that, under appropriate conditions, AMPK phosphorylation will contribute to a subsequent increase in glucose uptake by human myocytes.

One possibility is that the stimulation of glucose uptake by serum alone masks an effect of Serum+AICAR. This issue could be addressed by studying the effects of Serum+AICAR on glucose uptake in cells in which GLUT1 expression was reduced by RNA interference, a manipulation that eliminates the effect of serum on glucose uptake (1). Arguing against the idea that serum is masking the effect of Serum+AICAR is the fact that glucose uptake values in the Serum+AICAR group with 1.2 nM insulin were not equal to the glucose uptake with serum alone. Rather, glucose uptake with Serum+AICAR was significantly lower than with serum alone. This finding is reminiscent of the results from a study in which AICAR caused a diminished insulin effect on glucose uptake in 3T3-L1 adipocytes (25). The insulin resistance was secondary to a reduced GLUT4 translocation. Because prior serum exposure elevates basal glucose uptake via increased GLUT1 translocation (1), it seems reasonable to suspect that AICAR may block...
the serum effect on glucose uptake by interfering with GLUT1 translocation.

In conclusion, primary human skeletal muscle cells responded to Serum+AICAR treatment with a substantial increase in the phosphorylation of AMPK and ACC. In contrast to previously described results with isolated rat skeletal muscle, prior exposure to Serum+AICAR did not lead to a subsequent enhancement in insulin-stimulated glucose uptake. Future research with skeletal muscle tissue preparations will likely suggest more details regarding the mechanisms whereby exercise or AICAR treatment can lead to subsequent improvements in insulin action. The lack of enhanced insulin sensitivity in primary human myocytes after Serum+AICAR exposure may be an advantage for future studies whereby the expression of key proteins could be genetically manipulated in myocytes to test whether these modifications will make the cells amenable to the Serum+AICAR-induced improvement in insulin sensitivity.

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GRANTS

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