Restoring AS160 phosphorylation rescues skeletal muscle insulin resistance and fatty acid oxidation while not reducing intramuscular lipids

Hakam Alkhateeb,1 Adrian Chabowski,2 Jan F. C. Glatz,3 Brendon Gurd,4 Joost J. F. P. Luiken,3 and Arend Bonen4

1Department of Laboratory Medical Sciences, Faculty of Allied Health Sciences, Hashemite University, Zarqa, Jordan; 2Department of Physiology, Medical University of Bialystok, Bialystok, Poland; 3Department of Molecular Genetics, Maastricht University, Maastricht, The Netherlands; 4Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

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HIGH CONCENTRATIONS OF CIRCULATING FATTY ACIDS, the accumulation of intramuscular lipids, and reductions in fatty acid oxidation have all been associated with the development of insulin resistance in skeletal muscle (2, 6, 32, 74). Intramuscular diacylglycerol, ceramide, and long-chain fatty acyl-CoAs have been implicated in interfering with insulin signaling and insulin-stimulated glucose transporter 4 (GLUT4) translocation to the cell surface (23, 32, 33). Such lipid accumulation within muscle tissue may occur in response to a reduced fatty acid oxidation capacity (37, 44) or an inability to oxidize all of the excess fatty acids (46) that are taken up into muscle (10, 51). However, there is debate (11, 30, 57, 59, 71) as to whether a reduction in intramuscular lipids or an increase fatty acid oxidation is the most effective strategy for alleviating skeletal muscle insulin resistance.

Both leptin and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) are well known to increase skeletal muscle fatty acid oxidation (42, 53, 54, 67–69). Acute (38, 45, 58) and chronic (15, 38, 40) AICAR treatments also improved glucose homeostasis and insulin sensitivity in muscle of insulin-resistant rodents (15, 38, 40, 45, 58) and prevented type 2 diabetes in Zucker diabetic fatty rats (61). Similarly, leptin markedly improved insulin responsiveness and sensitivity and protected against high-fat diet-induced insulin resistance (72, 73). The mechanisms involved in these AICAR and leptin-mediated improvements in insulin sensitivity are uncertain, although for some time it has been thought that the insulin-sensitizing effects of leptin and AICAR occur via their activation of AMPK. However, questions have been raised recently as to whether AMPK activation is required to stimulate fatty acid oxidation (21, 25, 26, 52, 60, 62), and other studies have shown that glucose uptake is not impaired in mice with an AMPK deficiency (3, 27, 41).

The phosphorylation of the distal Rab-GTPase-activating protein (GAP) insulin signaling protein AS160 plays an important role in GLUT4 vesicle exocytosis (16). AS160 is phosphorylated when muscle is stimulated with insulin or with AICAR (12, 48–50). Therefore, in insulin-resistant muscle, AICAR treatment is expected to increase both fatty acid oxidation and AS160 phosphorylation. Given the similar effects of AICAR and leptin on skeletal muscle fatty acid metabolism and insulin sensitivity, this hormone may also be expected to increase both fatty acid oxidation and AS160 phosphorylation. These effects of AICAR and leptin may be key for improving insulin-stimulated glucose transport by possibly reducing intramuscular lipids due to improved fatty acid oxidation and by restoring AS160-dependent GLUT4 translocation to the cell surface. Alternatively, increasing AS160 phosphorylation and/or fatty acid oxidation, while not altering intramuscular lipids, may be sufficient to rescue insulin resistance in skeletal muscle. However, it is has also been...
reported recently (31, 36, 47) that insulin sensitivity is not necessarily dependent on altered Akt and/or AS160 phosphorylation in skeletal muscle (31, 36, 47). Thus, how AICAR and leptin treatments contribute to improving insulin-stimulated glucose transport in insulin-resistant skeletal muscle remains unclear.

Examination of insulin resistance and its alleviation in mammalian skeletal muscle under highly controlled conditions has been problematic, since skeletal muscle viability ex vivo is limited. For this practical reason, selected cell lines or diet-induced insulin-resistant rodents are often the models of choice. However, observations in cell lines may not necessarily be indicative of events in mammalian muscle, and high-fat feeding induces metabolic changes in many tissues that may have an impact, along with the excess dietary lipids, on the development of skeletal muscle insulin resistance. To circumvent these problems, we have documented that small rodent skeletal muscles can be maintained ex vivo for at least 18 h (1, 2), during which the expression of insulin-signaling proteins, substrate transport proteins, glucose transport, and fatty acid metabolism remains unaltered (1, 2), whereas palmitate induces almost complete insulin resistance (2).

We have previously found in isolated muscle (0–18 h) that palmitate-induced insulin resistance was most closely associated with a reduction in fatty acid oxidation ($r = 0.99$), but not necessarily with intramuscular lipid accumulation (2). Therefore, in the present study, we have used the isolated muscle model to examine whether the rescue of palmitate oxidation contributed to the rescue of insulin resistance and whether this was accompanied by reductions in intramuscular lipids (triacylglycerol, diacylglycerol, or ceramide), alterations in AMPK phosphorylation in skeletal muscle (31, 36, 47). Thus, how AICAR and leptin treatments contribute to improving insulin-stimulated GLUT4 translocation.

**METHODS**

**Materials**

[1-14C]palmitate, [3H]-3-O-methyl glucose, and [14C]mannitol were purchased from Amersham Life Science (Oakville, ON, Canada). Collagenase type II was purchased from Worthington (Lakewood, NJ). Insulin (Humulin R) was purchased from Eli Lilly (Toronto, ON, Canada). Penicillin and streptomycin were purchased from Invitrogen (Grand Island, NY). Silica plates (no. 60, 0.25 mm) were obtained from Merck (Darmstadt, Germany). Total and phosphorylated proteins were determined with commercially available antibodies from the following sources: anti-Akt1/2, anti-phospho-Akt Ser473, and anti-phospho-Akt Thr308 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-AS160 and anti-phospho-AS160 (Thr642) from Upstate (Lake Placid, NY); and anti-GLUT4 from Chemicon International (Temecula, CA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

**Animals.** All experiments were approved by the Committee on Animal Care at the University of Guelph. Male Sprague-Dawley rats (55–75 g) were bred on sites and consumed normal laboratory chow and water ad libitum. For each experiment, rats were anesthetized with Somnotol (6 mg/100 g body wt ip), and the soleus muscles were dissected.

**Muscle Incubation**

We (1, 2) have shown that soleus muscles (20 mg) remain viable ex vivo for t ≤ 18 h in the absence or presence of high concentrations of palmitate. After a 30-min preincubation, soleus muscles (20 mg) were incubated without (control) or with palmitate (2 mM) for 0, 6, 12, or 18 h. After 12 h, some of the palmitate-exposed muscles were treated in three different ways for 6 h, 1) complete removal of palmitate from the incubating medium, 2) supplementing the incubating medium with AICAR (2 mM), or 3) supplementing the incubating medium with leptin (10 μg/ml; i.e., concentrations used by many others in isolated muscles (53–55, 66, 67, 69)), while the high concentrations of palmitate (2 mM) were maintained. The incubation medium was replenished every 6 h. The following parameters were determined during the induction and rescue of insulin resistance: 1) the basal and insulin-stimulated rates of glucose transport, 2) the basal and insulin-stimulated rates of plasmalemmal GLUT4, and 3) the phosphorylations of Akt and AS160, as well as the intramuscular concentrations of triacylglycerol, diacylglycerol, and ceramide, the rate of palmitate oxidation, the expression of all proteins examined, and the phosphorylation of AMPKα2.

**Glucose Transport**

At the end of the incubation periods, basal and insulin-stimulated glucose transport were determined, as we (2) have described previously, in palmitate-free Krebs-Henseleit buffer (2 ml) supplemented with 0.5 μCi [3H]-3-O-methyl-D-glucose (3-O-MG), 1.0 μCi [14C]mannitol, 32 mM 3-O-MG, 4 mM mannitol, 4 mM pyruvate, and 0.1% BSA in the presence (20 μM/ml) or absence of insulin for 20 min at 30°C. Thereafter, muscles were blotted, weighed, and solubilized, followed by scintillation counting.

**Palmitate Oxidation**

Rates of fatty acid oxidation were determined, as we (2) have described previously. Briefly, at the end of the incubation period, muscles were transferred to glass vials containing 2 ml of pregassed (95% O2-5% CO2) medium 199 supplemented with 4% BSA and 2 mM 3-O-MG, 4 mM mannitol, 4 mM pyruvate, and 0.1% BSA in the presence (20 μM/ml) of palmitate (2 mM, 0.5 μCi/ml [1-14C]palmitate). Palmitate oxidation occurred at 30°C for 40 min, and the 14CO2 released was captured in a benzothiuron hydroxide trap. Dissolved CO2 released by the addition of sulphuric acid from the incubating medium was also captured in this manner. Water-soluble 14C intermediates were extracted from muscles homogenized after their incubation. Palmitate oxidation was determined by summing the 14C in the three pools (2).

**Intramuscular Triacylglycerol, Phospholipid, Diacylglycerol, and Ceramide Concentrations**

Concentrations of intramuscular lipids (triacylglycerol, diacylglycerol, ceramide) were determined as we have described previously (2). For these purposes, it was necessary to pool five soleus muscles to have sufficient tissue for measuring the concentrations of the intramuscular lipids. At the end of the incubation periods, muscles were frozen in liquid nitrogen and stored at −80°C. Triacylglycerols, diacylglycerols, and ceramides were extracted from pulverized, fat-free muscle samples in methanol (2 ml) and chloroform (4 ml)
containing butylated hydroxytoluene (0.01%) and an internal standard (heptadecaconic acid). Water (1.5 ml) was added to the extracting mixture. One portion of the chloroform layer was used to separate triacylglycerol and diacylglycerol fractions, and another was used for ceramide determinations. Thin-layer chromatography was used to separate lipids. Lipid bands were visualized under UV light using standards on the plates. Separated lipids were methylated in 14% boron trifluoride-methanol (14%), and fatty acid methyl esters were extracted with pentane. Finally, samples were dissolved in hexane and analyzed by gas-liquid chromatography. According to the retention times of standards, the individual long-chain fatty acids were quantified. The concentration of each lipid was obtained by summing the fatty acids in their chromatograph profile.

Plasma Membrane Preparation

The plasmaemmal content of GLUT4 was determined at all time points in muscles that were treated with (20 mU/ml) or without insulin. To obtain sufficient plasma membrane, 10 incubated solei were pooled for each independent experiment. Plasma membranes were obtained from giant sarcosomal vesicles, as we (2, 4, 8, 9) have previously reported in detail. Briefly, the tissues were cut into thin layers (1–3 mm thick) and incubated for 1 h at 34°C in 140 mM KC1/10 mM MOPS (pH 7.4), aprotonin (30 μg/ml; Sigma-Aldrich), and collagenase (type VII; 150 units/ml) in a shaking water bath. At the end of the incubation, the supernatant fraction was collected and the remaining tissue washed with KCI/MOPS and 10 mM EDTA, which resulted in a second supernatant fraction. Both supernatant fractions were pooled, and Percoll (GE Healthcare, Aurora, OH) and aprotonin were added to final concentrations of 3.5% (vol/vol) and 10 μg/ml, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3-ml middle layer of 4% Nycodez (w/vol) and a 1 ml KCl/MOPS upper layer. This sample was centrifuged at 60 g for 45 min at room temperature. Subsequently, the vesicles were harvested from the interface of the upper and middle layer, diluted in KCI/MOPS, and recentrifuged at 12,000 g for 5 min. The pellet was resuspended in KCI/MOPS.

Protein Analysis, Extraction, and Western Blotting

The protein expression of selected proteins was determined at all time points in muscles that had been frozen at the end of each incubation. For muscle protein determinations, two solei were pooled. To measure the insulin-stimulated phosphorylation status of Akt2 and AS160, muscles were incubated for the various experiments as described above, followed by incubation with insulin (20 mU/ml) for 10 min, the time in which maximal phosphorylation was observed (2). Muscle homogenate and plasma membrane protein concentrations were determined using the bicinchonic acid assay. Proteins were separated using SDS-polyacrylamide gel electrophoresis and were detected using Western blotting, as we have reported previously (2, 4, 8, 9).

Statistics

Data were analyzed using two-way analyses of variance. For some experiments, the data were analyzed with a one-way analysis of variance, and when this was warranted, and when appropriate, a Fisher’s least significant difference post hoc analysis was used. Least squares regression analyses were used to determine relationships among variables. This could be done only by using the mean data, because it was not possible to obtain all of the measurements from a single 20-ng soleus muscle. All data are reported as means ± SE.

RESULTS

In a prior study, we (2) reported the inhibitory effects of palmitate treatment (2 mM palmitate, 0–18 h) on basal and insulin-stimulated 3-O-methyl glucose transport and the mechanisms involved. Here we focus on 1) the mechanisms involved in the rescue of palmitate-induced insulin resistance in isolated soleus muscle and 2) the relationship among selected parameters during the induction and the rescue of insulin resistance.

The protein expressions were not altered by any of the treatments (data not shown). The exception was AMPKα2; this was upregulated after 12-h incubation with palmitate (+18%) and increased further during incubation (6 h) with either AICAR (+52%) or leptin (+64%) (data not shown).

AICAR and Leptin Rescue Insulin-Stimulated Glucose Transport and GLUT4 Translocation

Glucose transport. In control muscles (0–18 h), basal and insulin-stimulated glucose transport were not altered (Fig. 1A). After 12 h, palmitate reduced basal glucose transport far less (−0.4 μmol·g⁻¹·20 min⁻¹) than insulin-stimulated glucose transport (−2.4 μmol·g⁻¹·20 min⁻¹, P < 0.05) (Fig. 1A). Palmitate withdrawal for 6 h or treatment with AICAR or leptin restored to normal the small, palmitate-induced reductions in the basal rate of glucose transport (Δ = +0.4 μmol·g⁻¹·20 min⁻¹, P < 0.05; Fig. 1A). In contrast, palmitate removal failed to restore insulin-stimulated glucose transport (P > 0.05; Fig. 1A). However, despite the continued presence of palmitate (2 mM), AICAR fully restored insulin-stimulated glucose transport (Δ = +2.4 μmol·g⁻¹·20 min⁻¹, P < 0.05; Fig. 1A), whereas leptin almost completely restored insulin-stimulated glucose transport (Δ = +2.0 μmol·g⁻¹·20 min⁻¹, P < 0.05; Fig. 1A).

Plasma membrane GLUT4. In control muscles (0–18 h), insulin stimulation increased plasma membrane GLUT4 two-to threefold (P < 0.05; Fig. 1B). In contrast, in the palmitate-treated muscles, insulin-stimulated GLUT4 translocation was markedly reduced (−50% at 12 h, P < 0.05; Fig. 1B). Palmitate withdrawal for 6 h failed to restore the insulin-stimulated plasma membrane GLUT4 (Fig. 1B). However, with either the AICAR or the leptin treatments the insulin-stimulated GLUT4 appearance at the plasma membrane (P < 0.05; Fig. 1B) was restored to 80% of that observed in control muscles despite the continued presence of palmitate (2 mM).

AICAR and Leptin Increase Fatty Acid Oxidation But Do Not Reduce Intramuscular Lipids

Palmitate oxidation. In control muscles (0–18 h), palmitate oxidation remained constant, but after 12-h incubation with palmitate this was reduced (−60%, P < 0.05; Fig. 2A). Palmitate removal (6 h) did not restore the rate of palmitate oxidation (P > 0.05; Fig. 2A). However, palmitate oxidation rates were markedly increased when muscles were treated for 6 h with AICAR or with leptin (P < 0.05; Fig. 2A) despite the continued presence of palmitate (2 mM). These increased rates of palmitate oxidation remained lower in the AICAR- and leptin-treated muscles than in 18-h control muscles (P < 0.05; Fig. 2A).

Intramuscular lipids. In control muscles (0–18 h), the intramuscular lipid concentrations remained constant (Fig. 2, B and D), except for diacylglycerol at 12 h (−19%; Fig. 2C). Incubation with palmitate for 12 h increased triacylglycerol (+54%, P < 0.05; Fig. 2B), diacylglycerol (+11%, P = 0.075; Fig. 2C), and total ceramide concentrations (+18%, P < 0.05;
versely with C16:0 ceramide (data not shown). The concentrations of some other ceramides varied in-

Fig. 2A). The concentrations of some other ceramides varied in-

Fig. 2B). The concentrations of some other ceramides varied in-

Fig. 2C). The concentrations of some other ceramides varied in-

Effects of Palmitate Removal, AICAR, and Leptin on Akt, AS160, and AMPK Phosphorylation

In the absence of insulin, the basal phosphorylation states of AS160 and Akt (Ser\textsuperscript{473} and Thr\textsuperscript{308}) were not altered in either control or AICAR- and leptin-treated muscles (data not shown).

Akt phosphorylation. Insulin-stimulated Akt phosphorylation (Ser\textsuperscript{473}) was not altered by palmitate (0–12 h, P > 0.05; Fig. 4A), nor was it altered when palmitate was removed (6 h). However, 6-h AICAR (+59%) or leptin treatment (+67%) increased the insulin-stimulated Akt phosphorylations similarly, well beyond the levels observed in control muscles (P < 0.05; Fig. 4A and B). Phosphorylation of Akt (Thr\textsuperscript{308}) was similar to that of Akt (Ser\textsuperscript{473}) (r = 0.98; data not shown).

AS160. Insulin-stimulated AS160 phosphorylation was inhibited by palmitate (−40% at 12 h, P < 0.05; Fig. 4B). Removal of palmitate failed to rescue insulin-stimulated AS160 phosphorylation (Fig. 4B). However, treatment with AICAR or with leptin fully rescued the insulin-stimulated phosphorylation of AS160 (P < 0.05; Fig. 4B) despite the continued presence of palmitate (2 mM).

AMPK. AMPK phosphorylation was increased in control (+62%) and palmitate-treated muscles (+118%) after 12 h (P < 0.05; Fig. 4C) and remained so when palmitate was removed (P > 0.05; Fig. 4C). AMPK phosphorylation was increased further in
AICAR- (+22%) and leptin-treated muscles (+30%, P < 0.05; Fig. 4C). When we normalized the AMPK phosphorylation for the increase in AMPKα2 protein, there were no differences in control and experimental muscles (Fig. 4C, inset).

**Interrelationships Among Fatty Acid Oxidation, Intramuscular Lipids, Insulin-Signaling Proteins, Plasma Membrane GLUT4, and 3-O-Methyl Glucose Transport**

Because of the different responses among the treatments, we were able to examine the relationship between the changes in insulin-stimulated glucose transport and the factors deemed to contribute to the regulation of this process. As expected, insulin-stimulated glucose transport was highly correlated with plasma membrane GLUT4 throughout (r = 0.98; data not shown). However, when all data in the study were examined, there were no correlations between intramuscular lipid concentrations (triacylglycerol, diacylglycerol, ceramide) and insulin-stimulated plasma membrane GLUT4, glucose transport, and phosphorylations of Akt Ser473 and Thr308 (data not shown).

Although there were positive associations between AMPK phosphorylation and selected parameters, this was observed only when AICAR and leptin were present (12–18 h), not in their absence (0–12 h). Therefore, across all experiments, there was no correlation.
between AMPK phosphorylation and the various parameters examined. In marked contrast, there were strong relationships between insulin-stimulated AS160 phosphorylation and plasma membrane GLUT4 \((r = 0.90)\), glucose transport \((r = 0.91)\), and palmitate oxidation \((r = 0.83)\) (Fig. 5, A–C). Strong relationships were also observed between palmitate oxidation and the insulin-stimulated plasma membrane GLUT4 \((r = 0.91)\; \text{Fig.} \, 6A) \text{and glucose transport} \((r = 0.98)\; \text{Fig.} \, 6B).\) It is evident that relationships among these various parameters (Figs. 5 and 6) fitted the same regression line, i.e., during the induction of insulin resistance and during the various treatments (palmitate withdrawal, AICAR, leptin; see highlighted data in Figs. 5 and 6).

**DISCUSSION**

We examined the rescue of palmitate (2 mM)-induced insulin resistance in isolated soleus muscles. There are number of novel observations, which are as follows: 1) palmitate-induced insulin resistance can be rescued rapidly in isolated skeletal muscle treated with AICAR or leptin, whereas palmitate withdrawal was ineffective, and 2) the marked improvement in insulin action was not associated with reductions in intramuscular lipids (triacylglycerol, diacylglycerol, ceramide), but 3) AICAR and leptin treatments did restore palmitate oxidation and, importantly, 4) insulin-stimulated AS160 phosphorylations and GLUT4 translocation. Therefore, the rapid rescue of palmitate-induced insulin resistance by AICAR and leptin appears to be attributable to the restoration of insulin-stimulated AS160 phosphorylation, which allows GLUT4 to be translocated to the cell surface. Importantly, these restorative processes, including palmitate oxidation, occurred despite high concentrations of intramuscular lipids and palmitate being maintained.

**AICAR and Leptin Ameliorate Insulin Resistance in Skeletal Muscle**

**Effects of AICAR and leptin on basal and insulin-stimulated glucose transport.** Despite increased concentrations of intramuscular lipids and high concentrations of palmitate (2 mM) being maintained, insulin-stimulated glucose transport was
almost fully rescued by AICAR and by leptin. These findings parallel results from other studies (13, 38, 43, 58, 63, 73), although we induced a far greater inhibition of insulin-stimulated glucose transport (−70%). The rescue of insulin-stimulated glucose transport (Δ = +2.0–2.4 μmol·g⁻¹·20 min⁻¹) was not attributable to minor changes in basal glucose transport (Δ = +0.4 μmol·g⁻¹·20 min⁻¹). Since neither AICAR (15, 40) nor leptin (17, 18, 75) stimulates glucose transport additively with insulin, it appears that AICAR and leptin rectify the mechanisms that prevent normal insulin action in models of lipid-induced insulin resistance without necessarily altering the intramuscular lipid content (present study).

Effect of AICAR and leptin on basal and insulin-stimulated GLUT4 translocation. Neither AICAR nor leptin by themselves induced GLU7T4 translocation. Hence the rescue of insulin-stimulated GLUT4 translocation occurred presumably via improvements in insulin signaling.

AICAR, leptin, and the insulin-signaling proteins Akt and AS160. Akt is clearly an important insulin-signaling protein (22, 56), but there was no obvious relationship between Akt Ser⁴⁷³ phosphorylation and AS160 phosphorylation, insulin-stimulated plasma membrane GLUT4, or glucose transport, as has been observed elsewhere in palmitate-treated L6 myotubes (31) and in high-fat-fed mice (56). In addition, we have reported previously that, in skeletal muscle, changes in insulin-stimulated Akt phosphorylation and plasma membrane Akt activity are also not linearly related (2). Hoehn et al. (31) and Ng et al. (56) attribute this lack of a linear association between insulin-stimulated Akt phosphorylation and selected parameters to “spareness” in the activity of Akt with respect to insulin-stimulated GLUT4 translocation and/or a discordance in Akt-mediated phosphorylation efficiency of selected substrates. Nevertheless, AICAR and leptin contribute to improving glucose transport via resensitizing signaling proteins to insulin, with insulin-stimulated AS160 phosphorylation appearing to be an especially important target.

The phosphorylation of AS160 plays an important role in GLU7T4 vesicle exocytosis (for review, see Ref. 16). Our results strongly suggest that in the AICAR- and leptin-treated muscles the recovery of insulin-stimulated AS160 phosphorylation was central to the rescue of insulin resistance, because both glucose transport (r = 0.91) and plasma membrane GLUT4 (r = 0.90) were highly correlated with AS160 phosphorylation. The mechanisms involved in rectifying insulin-stimulated AS160 phosphorylation in AICAR- and leptin-treated soleus muscle are unknown.

AICAR and Leptin Ameliorate Palmitate Oxidation But Not Intramuscular Lipid Concentrations in Insulin-Resistant Skeletal Muscle

Intramuscular lipids and insulin sensitivity. Many studies have associated reductions in intramuscular lipids with improvements in insulin sensitivity. In muscles treated with AICAR or leptin the expected increase in fatty acid oxidation (14, 53–55, 67–69) did occur, but there were no concurrent reductions in intramuscular lipids presumably because we maintained high palmitate concentrations. Yet despite this unfavorable intracellular lipid milieu, insulin sensitivity was almost fully restored by the AICAR and leptin treatments. Consistent with our results, there are emerging data indicating that improvements in insulin resistance are not necessarily associated with concomitant reductions in intramuscular lipid metabolites (11, 59, 71), including diacylglycerol and ceramide concentrations (59). In the present study, neither total diacylglycerol nor total ceramide concentrations were

Fig. 5. Relationship between insulin-stimulated AS160 phosphorylation and insulin-stimulated plasma membrane GLUT4 (A), insulin-stimulated glucose transport (B), and palmitate oxidation (C). Data (means ± SE) are replotted from all experiments in Figs. 1, A and B, 2A, and 4B. Highlighted data (circled) are from conditions in which palmitate was withdrawn (−Palm) for 6 h or in which muscles were treated with AICAR (+A) and leptin (+L) for 6 h while high concentrations of palmitate (2 mM) were maintained.

Fig. 6. Relationship between the rate of palmitate oxidation and insulin-stimulated plasma membrane GLUT4 (A) and insulin-stimulated 3-O-MG transport (B). Data (means ± SE) are replotted from all experiments in Figs. 1, A and B, and 2A. Highlighted data (circled) are from −Palm, +A, and +L conditions for 6 h while high concentrations of palmitate (2 mM) were maintained.
correlated with changes in the insulin stimulation of glucose transport, plasmalemmal GLUT4, Akt phosphorylation, or AS160 phosphorylation (data not shown). Thus, the present study and others (59) illustrate that the rescue of insulin resistance is not necessarily dependent on concomitant reductions in intramuscular lipid content (diacylglycerol, ceramide).

From studies by Holland et al. (for review, see Ref. 33), it has appeared that increases in C16:0 ceramide are closely associated with fatty acid-induced insulin resistance. However, in the present study, this reciprocal relationship between C16:0 ceramide concentrations and insulin-stimulated glucose transport is not readily apparent. For example, 1) insulin-stimulated glucose transport was reduced by 33% after 6-h exposure to palmitate in the absence of any increase in C16:0 ceramide, which occurred 6 h later, 2) only leptin treatment reduced C16:0 ceramide and improved insulin-stimulated glucose transport, whereas 3) palmitate removal reduced C16:0 ceramide but did not improve insulin-stimulated glucose transport, and 4) AICAR failed to reduce C16:0 ceramide but improved insulin-stimulated glucose transport. Taken altogether, it is evident that changes in intramuscular C16:0 ceramide do necessarily parallel the changes in insulin-stimulated glucose transport in mammalian muscle.

Fatty acid oxidation and insulin sensitivity. A reduced rate of fatty acid oxidation has been associated with skeletal muscle insulin resistance (for review, see Ref. 35). Palmitate induced a reduction in fatty acid oxidation, whereas AICAR and leptin treatments increased fatty acid oxidation as expected [AICAR (14, 55), leptin (53, 54, 67–69)]. We calculated that only a small decrease (~10%) in insulin-stimulated glucose transport accompanied a 33% reduction in fatty acid oxidation in resting muscle (Fig. 6B). Thereafter, concomitant reductions in fatty acid oxidation and in insulin-stimulated glucose transport occurred rapidly. The recovery of fatty acid oxidation was also accompanied by the concurrent rescue of insulin-stimulated glucose transport (Fig. 6B). Thus, in skeletal muscle (present study) and in L6 muscle cells (59), improvements in insulin action parallel improvements in fatty acid oxidation. That is not to say that fatty acid oxidation per se improved insulin-stimulated glucose transport.

Although changes in fatty acid oxidation could possibly be attributable to 14C-label dilution by intramuscular lipid accumulation, this is likely not the case. First, 14C-label incorporation into intramuscular lipid depots was also reduced (data not shown), and second, these reductions in fatty acid oxidation and esterification paralleled closely concomitant reductions in plasma membrane FAT/CD36 (Alkhateeb H and Bonen A, unpublished observations), which regulates fatty acid entry into muscle (for review, see Ref. 7). This suggests that the AICAR- and leptin-induced recovery of fatty acid oxidation is likely the result of an increase in fatty acid uptake into muscle, because this can markedly influence fatty acid oxidation in intact muscle (34).

Palmitate Oxidation, Insulin-Stimulated Glucose Transport, and AMPK Activation

Suggestions that improvements in fatty acid oxidation lead to improved insulin sensitivity abound (for review see Ref. 35), but as yet, no mechanism has been identified for this. A possible mechanism would seem to be the AICAR- and/or leptin-mediated phosphorylation of AMPK, because activation of this kinase has been implicated with the phosphorylation of both acetyl-CoA carboxylase 2 (ACC2) (see Ref. 25) and AS160 (49). We observed strong correlations between palmitate oxidation and insulin-stimulated AS160 phosphorylation as well as among palmitate oxidation, insulin-stimulated plasma membrane GLUT4, and insulin-stimulated glucose transport. Although plasma membrane GLUT4 and insulin-stimulated AS160 phosphorylation are expected to be correlated, given the key role of this distal Rab-GAP insulin-signaling protein in GLUT4 exocytosis (for review, see Ref. 16), it is less obvious as to why palmitate oxidation and insulin-stimulated glucose transport are so highly correlated. Although some studies indicate that both processes are stimulated when AMPK is activated (64, 65), this may not always be the case.

For many years, the activation of AMPK was seen to be central for stimulating fatty acid oxidation. However, this idea has been questioned. We found that an increase in AMPK phosphorylation during the induction of insulin resistance with palmitate and this AMPK activation, when neither leptin nor AICAR were present, did not increase fatty acid oxidation. Others have also reported that fatty acid oxidation can be regulated in an AMPK-dependent and AMPK-independent manner (21, 25, 26, 52, 60, 62). Thus, it appears that fatty acid oxidation can also be stimulated by alternative ACC2 kinases that remain to be identified. Several tentative possibilities include extracellular-regulated protein-serine kinase 1/2, inhibitor of NF-kB protein serine kinase-α/β, and protein kinase D (25). The studies by Dzamko et al. (25) and others (26, 52, 60) also support our observation that 1) simply increasing AMPK activation, in the absence of AICAR or leptin, may not be required to stimulate fatty acid oxidation and 2) AICAR and leptin, which clearly stimulated fatty acid oxidation, may involve the activation of not only AMPK but also other unknown kinases to induce the phosphorylation of ACC2. The recent study by Dzamko et al. (25) also raises the question as to whether it is AMPK (49) per se or some other as yet unknown kinases that induce the phosphorylation of AS160. Taken altogether, it appears that the AICAR- and leptin-mediated restoration of fatty acid oxidation and AS160 phosphorylation is attributable to the activation of AMPK and likely some as yet unknown kinase, which are activated by AICAR and by leptin.

Is AS160 the Nexus Point Connecting Fatty Acid Oxidation and Glucose Transport?

It seems unlikely that improvement in muscle fatty acid oxidation per se is the cause of improved insulin sensitivity, as has been suggested on the basis of the concordances between these parameters (44, 59, 71).

Alternatively, a common underlying mechanism may induce both AS160 phosphorylation and fatty acid oxidation. Some of our recent work (39) suggests that not only is Akt2 signaling involved in insulin-stimulated glucose transport, but unexpectedly, Akt2 is also involved in insulin-stimulated fatty acid transport (39). This may imply that AS160, an Akt downstream target, may at times also be also involved in regulating fatty acid transport and oxidation. Tentative, albeit indirect, evidence includes the correlation between fatty acid oxidation and AS160 phosphorylation (r = 0.83) in the present study and the
positive relationship between AS160 protein expression (70) and the ability to oxidize fatty acids (24) among different types of muscles. Presumably, the metabolic role of AS160 may differ from its paralog TBC1D1 (70), since different signals can activate AS160 and TBC1D1 (28), and TBC1D1 is most abundant in glycolytic muscle (70), where it may contribute to downregulating fatty acid oxidation (19). An intriguing, if controversial, idea may be that under some circumstances AS160 is possibly a nexus point “connecting” fatty acid oxidation and insulin-stimulated glucose transport. This is highly speculative and needs to be determined experimentally.

**Summary**

Our studies have shown that despite high concentrations of palmitate and intramuscular lipids (triaclyglycerol, diacylglycerol, ceramide) being maintained, insulin-stimulated glucose transport was rescued rapidly (≤6 h) when muscles were treated with AICAR or leptin, but palmitate removal was ineffective. The AICAR and leptin treatments also led to the restoration of fatty acid oxidation and the normalization of insulin-stimulated AS160 phosphorylation and insulin-stimulated GLUT4 translocation. Based on other recent studies, it appears that the actions of AICAR and leptin cannot be attributed solely to the activation of AMPK but likely also involve activation of other kinases. Nevertheless, we conclude that the AICAR- and leptin-mediated rescue of insulin resistance is attributable to the restoration of AS160 phosphorylation, which allows the insulin-stimulated translocation of GLUT4 to be normalized.

**GRANTS**

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