AMP kinase activation ameliorates insulin resistance induced by free fatty acids in rat skeletal muscle

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Olsen, Grith S., and Bo F. Hansen. AMP kinase activation ameliorates insulin resistance induced by free fatty acids in rat skeletal muscle. Am J Physiol Endocrinol Metab 283: E965–E970, 2002. First published July 9, 2002; 10.1152/ajpendo.00118.2002.—We examined whether acute activation of 5′-AMP-activated protein kinase (AMPK) by 5′-aminoimidazole-4-carboxamide-1-β-d-ribonucleoside (AICAR) ameliorates insulin resistance in isolated rat skeletal muscle. Insulin resistance was induced in extensor digitorum longus (EDL) muscles by prolonged exposure to 1.6 mM palmitate, which inhibited insulin-stimulated glycogen synthesis to 51% of control after 5 h of incubation. Insulin-stimulated glucose transport was less affected (22% of control). The decrease in glycogen synthesis was accompanied by decreased glycogen synthase (GS) activity and increased GS phosphorylation. When including 2 mM AICAR in the last hour of the 5-h incubation with palmitate, the inhibitory effect of palmitate on insulin-stimulated glycogen synthesis and glucose transport was eliminated. This effect of AICAR was accompanied by activation of AMPK. Importantly, AMPK inhibition was able to prevent this effect. Neither treatment affected total glycogen content. However, glucose 6-phosphate was increased after inclusion of AICAR, indicating increased influx of glucose. No effect of AICAR on the inhibited insulin-stimulated GS activity or increased GS phosphorylation by palmitate could be detected. Thus the mechanism by which AMPK activation ameliorates the lipid-induced insulin resistance probably involves induction of compensatory mechanisms overriding the insulin resistance. Our results emphasize AMPK as a promising molecular target for treatment of insulin resistance.

insulin sensitivity; 5′-aminoimidazole-4-carboxamide-1-β-d-ribonucleoside; glycogen synthesis; palmitate

SKELETAL MUSCLE IS the most important tissue responsible for glucose disposal under postprandial conditions, and muscle insulin resistance is a major characteristic of type 2 diabetes (10). Although the mechanism by which the muscle becomes insulin resistant is unclear, there is a strong correlation between increased levels of plasma free fatty acids and intramuscular fatty acid metabolites (long-chain acyl-CoA, diacylglycerol, and triglycerides) and insulin resistance (23). Thus excess levels of lipids appear to be of major importance for the development of insulin resistance in muscle (23).

The enzyme 5′-AMP-activated protein kinase (AMPK) has emerged as a key regulator of carbohydrate and fat metabolism, working as a “fuel sensor” in most tissues (30). AMPK activity is regulated by variations in the intracellular levels of AMP, ATP, and creatine phosphate through allosteric regulation and phosphorylation induced by upstream kinases (AMPKK; see Ref. 30). In skeletal muscle, AMPK is activated in response to various metabolic stresses such as hypoxia, hyperosmolarity, and exercise (15–17, 28, 29, 31). Chemically, AMPK can be activated in muscle by treatment with 5′-aminoimidazole-4-carboxamide-1-β-d-ribonucleoside (AICAR), which leads to AMP-like activation of the AMPK system (24). In muscle, AMPK activation by AICAR increases glucose transport in a wortmannin [inhibitor of phosphatidylinositol (PI) 3-kinase]-insensitive manner (3, 17, 21) and increases lipid oxidation through inhibition of acetyl-CoA carboxylase (24, 29), with both effects being very similar to exercise-induced regulation of muscle metabolism. Thus AMPK is believed to play a role in the effects of exercise on carbohydrate and fat metabolism.

In relation to diabetes and insulin resistance, physical exercise improves insulin sensitivity in animal models of diabetes and in type 2 diabetic patients (9, 12). Because of the possible involvement of AMPK in exercise-induced metabolic regulation, AMPK has emerged as an attractive molecular target for the possible treatment of type 2 diabetes (2). However, only a few studies have examined the effects of activating AMPK on insulin resistance. In these studies, where AICAR was infused in animal models of diabetes, plasma glucose levels were decreased or glucose transport in muscle was increased despite the insulin resistance (2, 13). However, because of possible indirect effects driven by alterations in whole body metabolism combined with the nonspecificity of AICAR, it remains unclear whether AICAR treatment can ameliorate insulin resistance directly in skeletal muscle.

Therefore, the aim of this study was to examine if acute activation of AMPK by AICAR could ameliorate insulin resistance induced by exposure to free fatty acids (palmitate) in isolated rat extensor digitorum longus (EDL) muscle.

MATERIALS AND METHODS

Source of tissue. All experiments were approved by the Danish Animal Experiments Inspectorate and complied with advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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the “European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes” (Council of Europe no. 123, Strasbourg, France, 1985). Male Wistar rats (~50 g) purchased from Moellegaard (Lille Skensved, Denmark) were anesthetized by brief exposure to CO2 and killed by cervical dislocation, and the EDL was gently dissected free. EDL muscle was chosen because its reported fiber type composition (42–56–2%) is close to the fiber type composition of the rat hindquarter (1).

Chemicals. All chemicals, if not described differently, were purchased from Merck.

Muscle incubations. The intact EDL muscles were incubated free floating in preaggred (95% O2-5% CO2) Krebs-Henseleit (KRH) buffer (in mM: 118.5 NaCl, 4.7 KCl, 1.2 KH2PO4, 25 NaHCO3, 2.5 CaCl2, 1.2 MgSO4, and 10 HEPES) supplemented with 5 mM glucose, 10 pM insulin (human from Novo Nordisk), and 4% BSA with or without 1.6 mM palmitate (Sigma) for 5 h or as indicated. In some experiments, 2 mM AICAR (Sigma) was included for the last hour. Palmitate was conjugated to fatty acid-free BSA (Sigma), as described previously (27). When the insulin response was to be investigated, muscles were stimulated with insulin (6 nM or as indicated) for 20 min in palmitate-free KRH containing 5 mM glucose, 1% BSA, and 10 pM insulin in the basal stimulations. The AMPK inhibitor 6-[(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine (5 mM) was measured as described in hepatic mitochondria (33). All incubations were performed at 30°C, with gentle agitation (110/min), under continuous gassing with 95% O2-5% CO2 and with buffer change every 1–2 h. Thus the incubation conditions fulfilled the requirements for long-term incubations to retain muscle viability (6). After incubation, muscles were quickly freeze-clamped in liquid nitrogen.

Glycogen synthesis. Glycogen synthesis rate was measured by adding n-[U-14C]glucose (Amersham) together with insulin for 20 min after the 5-h incubation. The muscles were frozen in liquid nitrogen, weighed, and boiled in 1 N NaOH for 30 min. Glycogen was precipitated overnight at −20°C in ethanol after addition of 0.35 µg/µl unlabeled glycogen (Sigma). After centrifugation (20 min, 2,800 g), the glycogen pellet was washed in ice-cold ethanol, solubilized in water, and counted (Tri-Carp 1500; Packard).

Glucose transport. Glucose transport was measured by addition of 2-deoxy-D-[3H]glucose (2-DG; Amersham) after 10 min of insulin stimulation to the incubation buffer containing 1% BSA and 5 mM glucose for the last 10 min of incubation. Extracellular volume was measured by addition of [14C]mannitol (Amersham) together with 2-DG, and glucose transport was corrected for the presence of 2-DG in the extracellular volume. After incubation, the muscles were snap-frozen in liquid nitrogen, solubilized in Solvable (Packard), and counted (Tri-Carp 1500).

AMPK activity. AMPK activity was measured in vitro by immunoprecipitation with anti-AMPK-α1 and anti-AMPK-α2 antibodies as described (8) using the AMARAASAAKARRR peptide. Alternatively, AMPK activity was measured by Western blot analysis using phospho- and dephosphospecific ACC antibodies. The antibodies were raised against the peptides TMTPSMSpGHLVK or TMTPSMSpGHLVK (residues 221–233 of human ACC2, where Sp is phosphoserine) using previously described methods (25).

Glycogen synthase activity and phosphorylation, total glycogen content, and glucose 6-phosphate. Glycogen synthase (GS) activity was measured as described (26) with minor modifications. Precipitation of labeled glycogen was performed in ethanol prewet Whatman Unfilter 350 polytronic plates (50 µl reaction and 200 µl ice-cold 66% ethanol for 1 h; Frisenette). The plate was then washed five times with 66% ethanol, and dried, after which 75 µl Microscint 20 (Packard) were added. After 2 h, the plate was counted in a Topcount-NEXT (Packard). GS activity was determined in the absence or presence of 8 mM glucose 6-phosphate (G-6-P). Phosphorylation of site 3a3b on GS was measured by Western blot analysis using a phosphospecific GS antibody. The antibody was raised against the peptide RYPRPASpVPpSSpSLSR (residues 634–649 of human muscle GS) using previously described methods (25). Total glycogen concentration was determined by measuring free glycosyl units using a hexokinase-based assay kit (Sigma) after acid hydrolysis as described (22), and G-6-P concentrations were determined according to Lowry and Passoneau (22) in perchloric acid extracts of frozen muscle.

Statistical analysis. Differences between groups were assessed by one-way ANOVA followed by Bonferroni’s post hoc comparison between selected groups (see Fig. 6), in case of paired muscles by paired t-test, and in Fig. 3 by two-way ANOVA [3 (treatments) × 4 (concentrations of insulin)] table. Data are expressed as means ± SE from n = 6–12 muscles in each group, as indicated in the legends for Figs. 1–6.

RESULTS

Insulin resistance in rat EDL muscle induced by prolonged exposure to palmitate. Upon exposure to palmitate, the rate of glycogen synthesis in response to 6 nM insulin was decreased by up to 51% in the EDL muscle compared with muscles incubated without palmitate (Fig. 1A). This inhibition of the insulin response occurred in a time (P < 0.001)- and concentration (P < 0.05)-dependent manner, with maximal inhibition after 4 h of incubation using 1.6 mM palmitate (Fig. 1, A and B). No significant effect of palmitate incubation on basal (10 pM insulin) glycogen synthesis could be detected. Palmitate (1.6 mM) incubation for 5 h had no significant effect on basal (10 pM insulin) glucose transport (Fig. 2). However, insulin (6 nM)-stimulated glucose transport was significantly inhibited with palmitate by 22% compared with muscles incubated without palmitate (P < 0.05).

Amelioration of insulin resistance by acute treatment with AICAR. To determine whether acute activation of AMPK would ameliorate lipid-induced insulin resistance, 2 mM AICAR was included in the last hour of a 5-h incubation with palmitate. Focusing on glycogen synthesis as the major site of insulin resistance in this system, the insulin response was measured as [14C]-glucose incorporation into glycogen. Palmitate exposure led to an inhibition of insulin-induced glycogen synthesis (P < 0.001 for treatments; Fig. 3). When AICAR was included for the last hour of the incubation, the inhibitory effect of prolonged exposure to palmitate on the insulin response was eliminated. The increased glycogen synthesis by AICAR inclusion was accompanied by a significantly (P < 0.05) increased insulin-stimulated glucose transport (Fig. 2).

Activation of AMPK. To verify that AMPK was activated by the AICAR treatment, AMPK activity was measured. Figure 4A shows the AMPK activity in muscles, incubated as indicated, measured by an in vitro kinase assay in AMPK-α1/α2 immunoprecipitates. No
The effect of palmitate treatment could be detected. When AICAR was included for the last hour of incubation, the activity was increased twofold ($P < 0.01$). The same increase in AMPK activity was found in response to AICAR in muscles incubated without palmitate ($P < 0.001$). In addition, AMPK activity was measured using anti-phospho- and anti-dephosphoantibodies directed against Ser221 in acetyl-CoA carboxylase, a well-known substrate of AMPK (Fig. 4B). With this approach, any allosteric regulation of AMPK and activation by phosphorylation is detected. Thus normally this approach results in a more sensitive measure for AMPK activity. The results using these antibodies confirm the data obtained by the in vitro kinase assay.

Specific involvement of AMPK in the ameliorating effect of AICAR. Because AICAR is known to be somewhat unspecific, an AMPK inhibitor (33) was used to verify the involvement of AMPK in AICAR-induced amelioration of the insulin resistance. Figure 5 shows that AMPK inhibition prevented the effect of AICAR on palmitate-induced insulin resistance ($P < 0.001$ for 100 μM inhibitor).

Glycogen, G-6-P, and GS activity. To examine the mechanism by which AICAR ameliorates palmitate-induced insulin resistance, total glycogen content, G-6-P levels, and GS activity were measured. Neither palmitate exposure nor inclusion of AICAR had any effect on the total glycogen content in the EDL muscle (Table 1). However, the inclusion of AICAR significantly ($P < 0.01$ vs. palmitate treated) increased G-6-P levels (Table 1). Palmitate incubation significantly inhibited insulin-stimulated in vitro GS activity ($P < 0.05$), indicating an inhibition of insulin-stimulated dephosphorylation of the GS (Fig. 6A). In agreement, by use of a phosphospecific antibody, insulin-stimulated dephospho-
phorylation of GS on site 3a3b [phosphorylation site of e.g., glycogen synthase kinase 3 (GSK3)] was abolished by palmitate exposure (Fig. 6B). Inclusion of AICAR had no significant effect on the inhibited in vitro GS activity or the phosphorylation state of site 3a3b.

**DISCUSSION**

In the present study, we provide evidence that AICAR, directly in skeletal muscle, ameliorates lipid-induced insulin resistance accompanied by increased AMPK activity. Importantly, an AMPK inhibitor was able to prevent this effect, ensuring that the effect is specific for AMPK activation in the muscle. This finding is parallel to results previously seen with glucose-induced insulin resistance (18), indicating that activation of AMPK in general can ameliorate insulin resistance induced by an energy surplus.

An increasing body of evidence suggests a strong relationship between obesity, increased lipid availability, and the impaired insulin sensitivity observed in obese and type 2 diabetic patients (23). In the present study, we show that insulin resistance can be induced directly by prolonged incubation with free fatty acids (palmitate) in the EDL muscle, representing fast-twitch fibers. This is in agreement with results obtained in soleus (representing slow-twitch fibers) by Thompson et al. (27) performed under similar conditions. The inhibitory effect of palmitate on insulin action is most pronounced at the level of glycogen synthesis, in both fast- and slow-twitch fibers, whereas glucose transport seems to be affected less [Thompson et al. (27)] (Figs. 1 and 2). Thus prolonged exposure to excess lipids directly induces insulin resistance in both slow- and fast-twitch skeletal muscle fibers, in agreement with *in vivo* rat and human lipid infusion studies (4, 5, 7, 19).

Alterations in the insulin-signaling pathway have been implicated in the mechanism of lipid-induced insulin resistance in skeletal muscle (11, 27). In this study, we provide evidence that insulin-stimulated in vitro GS ac-
activity and dephosphorylation of site 3a3b on GS (phosphorylation sites of, e.g., GSK3) were affected, whereas G-6-P and total glycogen content were unaffected by prolonged palmitate exposure. These findings indicate that prolonged exposure to palmitate modulates the insulin-signaling pathway, in agreement with the findings in isolated soleus muscles, where insulin-induced protein kinase B (PKB) phosphorylation was reported decreased (27), and after in vivo infusions of fatty acid, where decreased insulin receptor substrate (IRS)-1- and IRS-2-associated PI 3-K and Akt1 activity in muscle were seen (11, 20). Interestingly, Kim et al. (20) reported that lipid infusion had no effect on insulin-stimulated inhibition of GSK3 activity, whereas a reduction in GS activity was seen. The latter finding agrees nicely with the present findings that AICAR very efficiently prevents the development of insulin resistance. In previous in vivo studies in Zucker obese rats and KKAy/CETP mice, AICAR was found to improve whole body glucose homeostasis (2, 13). In these studies, the main effect of AICAR appeared to be on glucose production from the liver rather than peripheral glucose disposal. However, our present results suggest that a major effect of AICAR should be observable in skeletal muscle. The explanation for this discrepancy is unclear, but changes in the plasma lipid profiles were observed in these in vivo experiments, which are well known to modulate glucose homeostasis. Furthermore, AICAR is not a specific AMPK activator, since it will affect other AMP-sensitive enzymes, which will complicate interpretation of whole body experiments. Consequently, it is not clear if the beneficial effects of AICAR on glucose metabolism observed in those in vivo studies were the result of an effect of AMPK activation on peripheral glucose utilization or because of, primarily, an effect on the liver. Nevertheless, using in vitro muscle incubations, we and others (3, 14, 17, 21, 24) found that AICAR indeed has a direct effect on skeletal muscle glucose metabolism. A more specific AMPK activator is needed to evaluate the effects of AMPK activation on whole body glucose metabolism.

The molecular mechanisms by which AICAR ameliorates the inhibitory effect of palmitate on insulin-stimulated glucose metabolism in muscle might occur by either relieving the lipid-induced defect or by inducing compensatory mechanisms overriding the insulin resistance. Because AICAR is known to activate glucose transport independent of the insulin-signaling pathway, overriding the insulin resistance by increasing glucose influx is a likely mechanism. Indeed we find an increased G-6-P concentration when including AICAR and increased insulin-stimulated glucose transport. Alternatively, AMPK activation could lead to a relief of the palmitate-induced insulin resistance by increasing fatty acid oxidation and thereby removing the inhibitory fatty acid metabolites, or by activating a protein with a direct effect on the insulin-signaling pathway. However, in this study, we find that the inhibited insulin-stimulated GS activity and dephosphorylation of GS by palmitate are unaffected by the AICAR treatment, making a relieving mechanism unlikely.

**Fig. 6.** Glycogen synthase (GS) activity after palmitate and AICAR exposure. In vitro GS activity (A) and phosphorylation of site 3a3b on GS (pGS) visualized by Western blotting using a phosphospecific antibody (B) were measured in muscles incubated for 5 h in the absence or presence of 1.6 mM palmitate with or without addition of 2 mM AICAR as indicated. Muscles were further stimulated with 6 pM (basal) or 6 nM insulin (Ins) for 20 min. In A, paired muscles from the same animal were paired as shown. In A, mean values ± SE are given for n = 12 in each group. G-6-P, glucose 6-phosphate. *P < 0.05 for the effect including AICAR insulin-stimulated GS activity and **P < 0.01 for the effect of insulin on GS activity.
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In conclusion, acute treatment with AICAR ameliorates insulin resistance induced by prolonged treatment with free fatty acids (palmitate) in isolated EDL muscle. The AICAR effect seems to be specific for AMPK activation, since the ameliorating effect of AICAR can be prevented by an AMPK inhibitor. The mechanism by which activation of AMPK ameliorates palmitate-induced insulin resistance most likely occurs by inducing increased glucose transport and thereby overriding the insulin resistance rather than relieving the insulin resistance. Our data provide further support for pursuing AMPK as a potential molecular target for the treatment of insulin resistance.

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