

Preventive effect of oleate on palmitate-induced insulin resistance in skeletal muscle and its mechanism of action

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Abstract Insulin resistance in skeletal muscle is a feature associated with exposure to an excess of saturated fatty acids such as palmitate. Oleic acid has been shown to blunt palmitate-induced insulin resistance in muscle cells. However, there is no literature available regarding the effect of oleic acid on palmitate-induced insulin resistance in intact muscle. Therefore, this study investigated the effect of oleic acid on palmitate-induced insulin resistance in rat soleus muscle and its underlying mechanisms. For these purposes, oleic acid (1 mM) was administered for 12 h in the absence or presence of palmitate (2 mM). At the end of the experiment, plasmalemmal GLUT4, the phosphorylation of AS160 and Akt-2, and the total expression of these signaling proteins were examined. We found that treatment with palmitate for 12 h reduced insulin-stimulated GLUT4 translocation and the phosphorylation of AS160 and Akt-2. However, the administration of oleic acid fully restored insulin-stimulated GLUT4 translocation ($P < 0.05$), as well as AS160 and Akt-2 phosphorylation ($P < 0.05$) despite the continuous presence of palmitate. Wortmannin, an inhibitor of PI3-K, only slightly prevented the oleic acid-induced improvements in insulin-stimulated GLUT4 translocation, and AS160 phosphorylation. However, this treatment completely inhibited the oleic acid-induced improvement in insulin-stimulated Akt-2 phosphorylation. In contrast, the oleic acid-induced improvement

in insulin signaling was not affected by compound C, an AMPK specific inhibitor. In conclusion, the results clearly indicate that oleic acid administration alleviates palmitate-induced insulin resistance by promoting GLUT4 translocation in muscle, at least in part, by activating the PI3K pathway.

Keywords Oleic acid · Insulin resistance · Skeletal muscle · GLUT4 · AS160

Introduction

Insulin resistance is a state in which insulin key target tissues, such as skeletal muscle, do not properly respond to insulin, due to defects in the insulin signaling pathway [31, 21]. Skeletal muscle can account for up to 70–80% of insulin-stimulated glucose clearance [27]. Therefore, skeletal muscle insulin resistance is a cardinal feature in the pathogenesis of type 2 diabetes.

After insulin binds to its receptor, a series of signaling molecules are activated which induce the translocation of glucose transporter 4 (GLUT4) to the cell surface, where it is inserted to increase glucose transport [4]. It is widely believed that failures in the post-receptor insulin signaling pathways, involved in recruiting the glucose transporter GLUT4 to the cell surface, are at the center of the underlying pathophysiology of insulin resistance [1, 3, 19, 32]. Several studies have indicated that, in skeletal muscle, insulin-mediated translocation of GLUT4 translocation is strongly dependent on the phosphorylation of AS160 (Akt substrate of 160 kDa) [1, 3, 19]. For example, it has been demonstrated that AS160, upon its phosphorylation, promotes GLUT4 exocytotic trafficking [17]. Furthermore, the phosphorylation of AS160 by insulin is inhibited in type 2 diabetes and insulin resistance states [1, 3, 16], and this is accompanied by impaired GLUT4

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translocation. Therefore, impaired AS160 functionality, and hence GLUT4 translocation in skeletal muscle, is widely accepted as a main underlying mechanism leading to insulin resistance [1, 3, 32].

Apart from the insulin signaling pathway, an effective means to stimulate GLUT4 translocation to the plasma membrane is through the activation of AMP-activated protein kinase (AMPK). Several studies indicated that in skeletal muscle, AMPK signaling is a potential upstream kinase for AS160, suggesting that AS160 may be a convergent point for diverse stimuli regulating GLUT4 translocation [5, 14, 24, 29, 30]. AMPK signaling may be a potential therapeutic or prophylactic target to prevent or reduce the severity of insulin resistance and type 2 diabetes, since it is not disturbed in insulin-resistance states [11, 20, 28].

Considerable evidence suggests that chronic elevation of circulating free fatty acids promote insulin resistance in skeletal muscle. Saturated fatty acids, and in particular palmitate, the most abundant circulating saturated fatty acid, have been shown to adversely affect insulin signaling and promote the development of insulin resistance in skeletal muscle [1, 3, 12, 13, 23]. In contrast, many studies have shown that oleic acid, the most common monounsaturated fatty acids, improves insulin sensitivity, reduces the risk of developing type 2 diabetes, and ameliorates insulin resistance in muscle cells [8, 27, 33]. Whether oleic acid could reverse insulin resistance in intact skeletal muscle, and the mechanism(s) by which this might occur has not been fully determined, much of the information has been derived from studies in cell lines [6, 8, 27, 33] which do not necessarily reflect biochemical events that occur in mature, mammalian skeletal muscle. Therefore, the present study was designed to investigate whether oleic acid can prevent the deleterious effects of palmitate on skeletal muscle insulin signaling, and the mechanism(s) by which this occurs. This was achieved by using isolated soleus muscle that can remain metabolically viable for long periods of time [1, 3], and in which insulin resistance can readily be induced by palmitate.

Materials and methods

Materials

Total and phosphorylated protein content was determined with commercially available antibodies from the following sources: anti-GLUT4 from Chemicon International (Temecula, CA); anti-AS160 and anti-phospho-AS160 Thr 642 from Upstate; anti-phospho-Akt2 (p-Akt2, Ser474) from Cell Signaling Technology (CST, Boston, USA); goat-anti-rabbit secondary antibodies from Chemicon International; and donkey-anti-rabbit secondary antibody from Amersham Biosciences

(Oakville, Ontario, Canada). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animals

Male Sprague-Dawley rats weighing 55–70 g were used for all experiments. Animals were housed in a temperature-controlled room on a 12:12-h reverse light-dark cycle, and provided normal laboratory chow and water ad libitum. At the onset of each experiment, rats were sacrificed, and the soleus muscles were gently dissected free. Animal care was approved by the Yarmouk University of Animal Care and Use Committee.

Muscle incubation

As has previously been described [1, 3], soleus muscles remain viable *in vitro*, for up to 18 h. Briefly, in the present study, intact soleus muscles (~ 20 mg) were preincubated (30 min) and then incubated with (2 mM) or without (control) palmitate for 12 h. All incubations were performed in 10 ml of warmed (30 °C); pre-gassed (95% O₂–5% CO₂) medium 199, containing 5 mM glucose; and supplemented with 4% BSA V, penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and insulin (14.3 μU/ml). This low level of insulin, which does not alter the rates of glucose transport, was added to maintain muscle viability [2]. Incubation vials were shaken at 110 cycles/min, and the gas phase and temperature were maintained at 95% O₂–5% CO₂ and 30 °C, respectively. The incubation medium was changed every 6 h.

During the 12 h of incubation with or without palmitate, some muscles were treated with oleic acid (1.0 mM), with oleic acid (1.0 mM) + the PI3K inhibitor wortmannin (1 μM, cf. [18]), or with oleic acid (1.0 mM) + the AMPK inhibitor compound C (50 μM) [3].

The concentration of oleic acid (1.0 mM) was established in pilot studies, as this provided the optimal effect (data not shown). It is also noteworthy to clarify that this high concentration of lipids (2 mM) used in the present study is consistent with approaches in the literature [9, 34].

Plasma membrane preparation

To determine the content of the plasmalemmal GLUT4 protein, muscle samples were transferred into fresh pre-gassed M199 supplemented with (20 mU/ml) or without (basal) insulin in the last 70 min of the 12-h incubation in the aforementioned conditions. The last 70 min was chosen, because during this time interval the supraphysiological concentration of insulin (20 mU/ml) has been shown to evoke a significant increase in muscle insulin signaling [1, 3]. Following the 70-min incubation with or without insulin, giant vesicles were prepared from which plasma membrane content of GLUT4

was measured as previously reported [1, 3]. Briefly, the tissues were cut into thin layers (1–3 mm thick) for 1 h at 34 °C in 140 mM KCl/10 mM MOPS (pH 7.4), aprotinin (30 g/ml), 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 was washed with KCl/MOPS and 10 mM EDTA which resulted in a second supernatant fraction. Both supernatant fractions were pooled, and Percoll (GE Healthcare, Aurora, OH) and aprotinin were added to final concentrations of 3.5% (vol/vol) and 10 g/ml, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3 ml middle layer of 4% Nycodenz (wt/vol) and a 1 ml KCl/MOPS upper layer. This sample was centrifuged at 60g for 45 min at room temperature. Subsequently, the vesicles were harvested from the interface of the upper and middle layer, diluted in KCl/MOPS, and recentrifuged at 12,000g for 5 min. The pellet was resuspended in KCl/MOPS.

Protein analysis

The expression of selected proteins was determined in muscles incubated in M199 for 12 h with or without palmitate. Thereafter, muscles were rapidly blotted, frozen in liquid nitrogen, and stored at – 80 °C until analyzed for selected proteins. To measure the insulin-stimulated phosphorylation status of AS160 and Akt-2, muscles were incubated for the various experiments as described above, followed by incubation with (20 mU/ml) or without (basal) insulin for 10 min, the time in which maximal phosphorylation was observed [2]. Thereafter, the muscles were rapidly blotted, frozen, and stored at – 80 °C for later analysis.

Muscle protein extraction and western blotting

For whole muscle protein determination, frozen soleus muscles were homogenized in 2 ml of buffer. Muscle homogenate and plasma membrane protein concentrations were determined using the bicinchoninic acid assay. Proteins were separated using SDS-polyacrylamide gel electrophoresis and were detected using Western blotting. These procedures have been previously reported [1–3].

Statistical analysis

Data was analyzed using a two-way ANOVA. For some experiments, the data was analyzed with a one-way ANOVA, when this was warranted, and when appropriate, a Fisher's LSD post hoc analysis was used. All data are reported as means \pm SE. Statistical difference was set at $P < 0.05$.

Results

In the absence of insulin, neither palmitate nor oleic acid had any effect on plasmalemmal GLUT4 content (Fig. 1), or phosphorylation of AS160 (Fig. 2) and Akt-2 (Fig. 3). For this reason, we focus solely in the subsequent result description, on insulin-treated muscles, and we also do not show the noninsulin-treated samples in the subsequent experiments of wortmannin and compound C.

Effects of oleic acid on insulin-stimulated plasmalemmal GLUT4

In comparison to the muscles that had not been incubated with palmitate, inclusion of palmitate in insulin-stimulated GLUT4 appearance at the plasma membrane was inhibited in the muscles that had been treated with palmitate ($P < 0.05$, Fig. 1).

In the muscles treated with palmitate, the addition of oleic acid for 12 h increased insulin-stimulated GLUT4 content at the plasma membrane, almost to the level seen in control muscles (the muscles that had been incubated with insulin, but without palmitate) ($P < 0.05$, Fig. 1). The content of GLUT4 protein was not altered with any of the experimental treatments ($P > 0.05$; Fig. 1).

Effects of oleic acid on insulin-stimulated AS160 phosphorylation

In the muscles incubated with palmitate for 12 h, the magnitude of increase in insulin-stimulated AS160 phosphorylation was reduced ($P < 0.05$, Fig. 2), compared to the control muscles. The inclusion of oleic acid for 12 h, while palmitate was also present, rescued the insulin-stimulated phosphorylation of AS160 ($P < 0.05$, Fig. 2), and induced increments in the AS160 phosphorylation almost to the levels observed in the control muscles. On the other hand, despite the presence of oleic acid, insulin-stimulated AS160 phosphorylation in the absence of palmitate was not altered ($P > 0.05$; Fig. 2). Neither of the experimental treatment had any effect on total AS160 abundance, thereby excluding this as a possible reason for the net increase in its phosphorylation ($P > 0.05$, Fig. 2).

Effects of oleic acid on insulin-stimulated Akt-2 phosphorylation

In the presence of palmitate, the magnitude of increase in insulin-stimulated Akt-2 phosphorylation was reduced ($P < 0.05$, Fig. 3) compared to the control muscles. The inclusion of oleic acid, while palmitate was present, restored the insulin-stimulated Akt-2 phosphorylation quite to the levels observed in the control muscles ($P > 0.05$, Fig. 3). In the muscles not treated with palmitate, the addition of oleic acid to the incubating medium for 12 h did not alter the insulin-stimulated

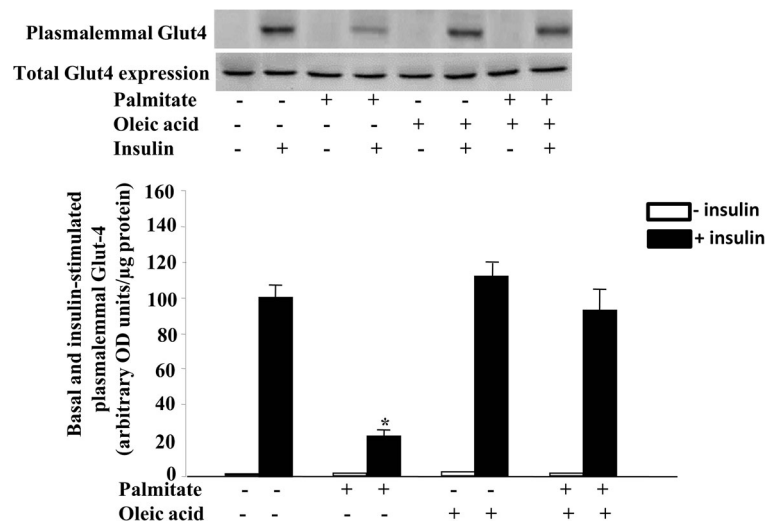


Fig. 1 Effect of oleic acid (1 mM) on basal and insulin-stimulated plasmalemmal GLUT4 in muscle treated with (2 mM) or without palmitate. Data are presented as means \pm SE; $n = 6$ independent experiments for each data point. To measure plasma membrane GLUT4, insulin was provided in the last 70 min of the 12-h incubation.

To obtain sufficient plasma membrane, 10 solei were pooled for each independent experiment at each data point. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments

phosphorylation of Akt-2 ($P > 0.05$, Fig. 3). The total protein content of Akt-2 was not altered by any of the experimental treatments ($P > 0.05$, Fig. 3) over the 12-h incubation.

Effect of wortmannin and compound C on the restorative effects of oleic acid on GLUT4 translocation or Akt-2 or AS160 phosphorylations

The PI3K inhibitor wortmannin, at a concentration of 1 μ M, partially blocked the restorative effects of oleic acid on GLUT4 translocation (Fig. 4) and AS160 phosphorylation (Fig. 5). In contrast, wortmannin completely blocked the restorative effects of oleic acid on Akt-2 phosphorylation (Fig. 6). The addition of the AMPK inhibitor compound C failed to inhibit the restorative effects of oleic acid on

GLUT4 translocation (Fig. 7), and on AS160 (Fig. 8) and Akt-2 (Fig. 9) phosphorylation.

Discussion

In the present study, the effect of oleic acid on palmitate-induced insulin resistance in skeletal muscle was investigated. For this purpose, an isolated soleus muscle preparation was used, in which the substrate-endocrine milieu remains controlled for up to 18 h in the presence or absence of palmitate (2 mM), and in which insulin resistance can be readily induced [1, 3]. Noteworthy, the concentration of palmitate we tested is at the upper physiological limit observed in humans and has previously been reported to impair insulin signaling in rodent muscle [1, 3, 9].

Fig. 2 Effect of oleic acid (1 mM) on basal and insulin-stimulated AS160 phosphorylation in muscle treated with (2 mM) or without palmitate. Data are presented as means \pm SE; $n = 8$ solei per data point. To measure AS160 phosphorylation, insulin was provided in the last 10 min of the 12-h incubation. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments

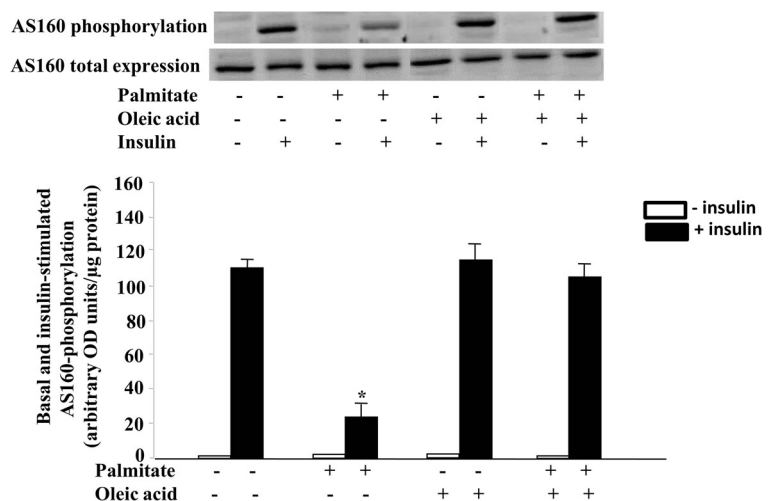
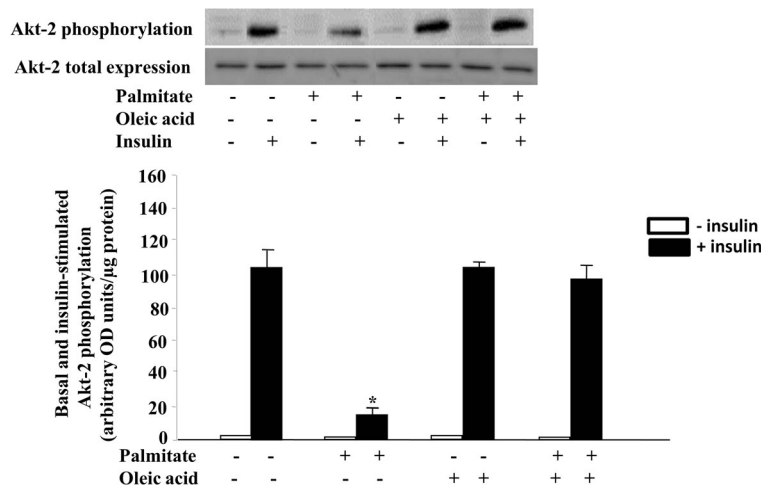


Fig. 3 Effect of oleic acid (1 mM) on basal and insulin-stimulated Akt-2 phosphorylation in muscle treated with (2 mM) or without palmitate. Data are presented as means \pm SE; $n = 8$ solei per data point. To measure Akt-2 phosphorylation, insulin was provided in the last 10 min of the 12-h incubation. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments



In agreement with previous findings [1–3], our observations revealed that incubation of soleus muscles with palmitate for 12 h induced a state of insulin resistance as evidenced by an impaired insulin-stimulated GLUT4 translocation and reduced insulin-stimulated AS160 and Akt-2 phosphorylation. The possible mechanisms contributing to the palmitate-induced insulin resistance described in our study will not be discussed further since they have already been described in detail elsewhere [1–3].

In this study, we have provided novel findings on the therapeutic effects of oleic acid on palmitate-induced insulin resistance in soleus muscle. These observations are as follows: (1) oleic acid antagonized the suppressive effect of palmitate on

insulin signaling, and (2) this marked improvement in insulin action was closely associated with restoration in insulin-stimulated Akt-2/AS160 phosphorylation and GLUT4 translocation. Therefore, the restoration of normal insulin response by oleic acid appears to be attributed to the amelioration of insulin-stimulated Akt-2/AS160 phosphorylation, which allows GLUT4 to be translocated to the cell surface. This study also showed that (3) the improvements in these signaling molecules could not be attributed to any changes in their total expression, as these were not altered by any of the experimental treatments.

It appears that oleic acid ameliorated on insulin signaling by enhancing insulin action per se; as in the absence of insulin, there was minimal effect of oleic acid on our measured

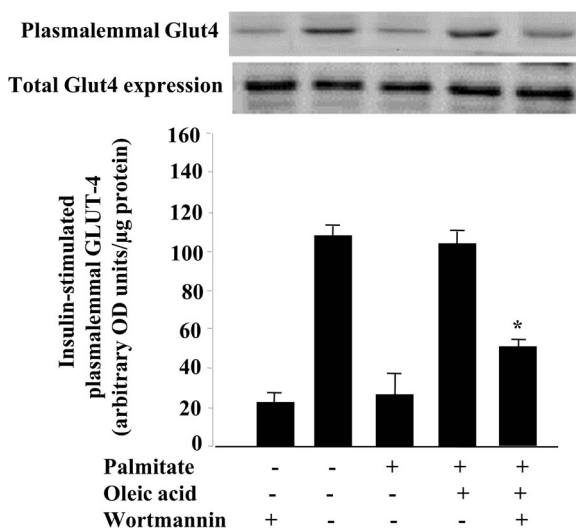


Fig. 4 Effect of the PI3K inhibitor, wortmannin, on the restorative effect of oleic acid on GLUT4 translocation in muscle incubated with palmitate. Data are presented as means \pm SE; $n = 6$ independent experiments for each data point. To measure plasma membrane GLUT4, insulin was provided in the last 70 min of the 12-h incubation. To obtain sufficient plasma membrane, 10 solei were pooled for each independent experiment at each data point. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments

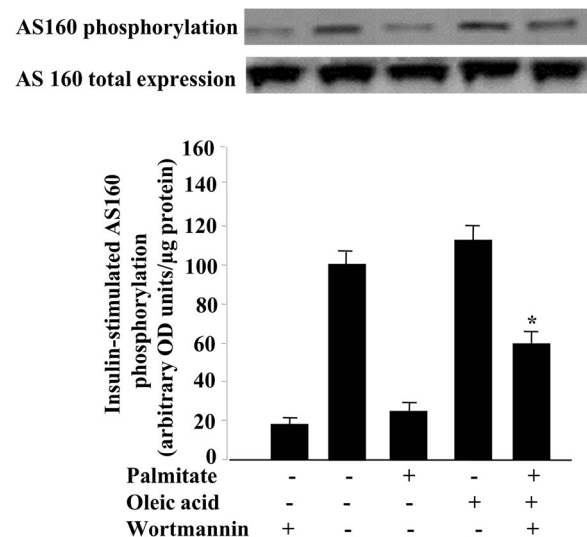


Fig. 5 Effect of the PI3K inhibitor, wortmannin, on the restorative effect of oleic acid on AS160 phosphorylation in muscle incubated with palmitate. Data are presented as means \pm SE; $n = 8$ solei per data point. To measure AS160 phosphorylation, insulin was provided in the last 10 min of the 12-h incubation. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments

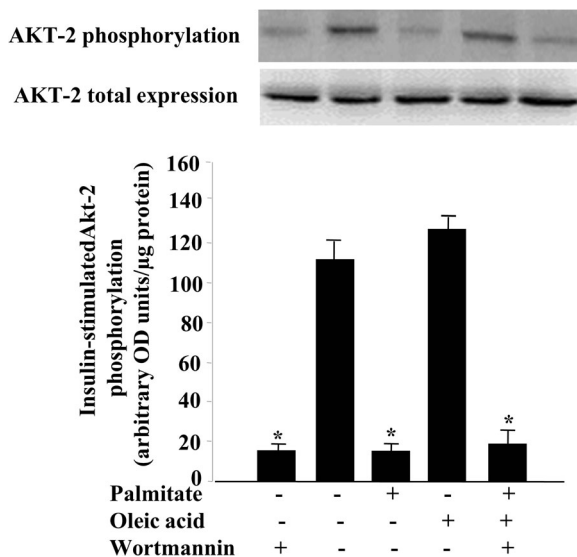


Fig. 6 Effect of the PI3K inhibitor, wortmannin, on the restorative effect of oleic acid on Akt-2 phosphorylation in muscle incubated with palmitate. Data are presented as means \pm SE; $n = 8$ solei per data point. To measure Akt-2 phosphorylation, insulin was provided in the last 10 min of the 12-h incubation. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments

parameters. Therefore, the alleviation of palmitate-induced insulin resistance by oleic acid is not a consequence of an additive effect between insulin and oleic acid, but rather, oleic acid seems to restore insulin sensitivity by positive modifications in insulin-signaling proteins, including Akt-2/AS160/GLUT4.

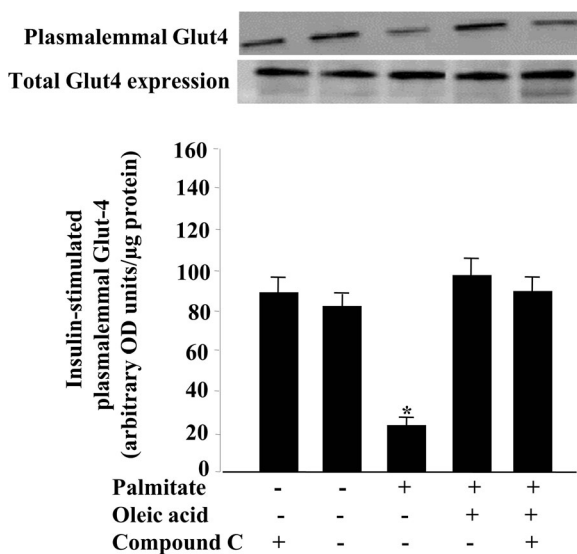


Fig. 7 Effect of the AMPK inhibitor, compound C, on the restorative effect of oleic acid on GLUT4 translocation in muscle incubated with palmitate. Data are presented as means \pm SE; $n = 6$ independent experiments for each data point. To measure plasma membrane GLUT4, insulin was provided in the last 70 min of the 12-h incubation. To obtain sufficient plasma membrane, 10 solei were pooled for each independent experiment at each data point. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments

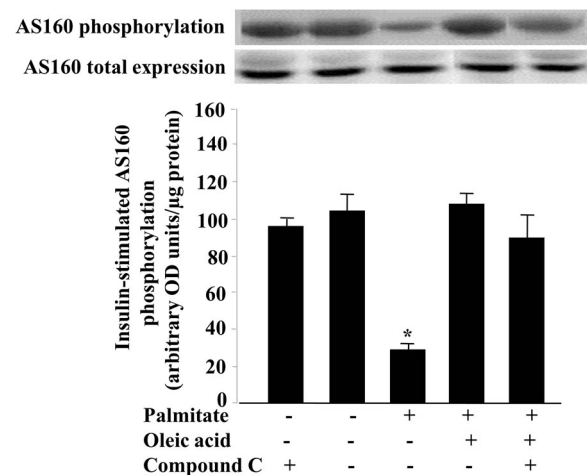


Fig. 8 Effect of the AMPK inhibitor, compound C, on the restorative effect of oleic acid on AS160 phosphorylation in muscle incubated with palmitate. Data are presented as means \pm SE; $n = 8$ solei per data point. To measure AS160 phosphorylation, insulin was provided in the last 10 min of the 12-h incubation. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments

GLUT4 is a key molecule in insulin signaling pathway and it is critical for glucose uptake in metabolically active tissues, like skeletal muscle. Therefore, defective translocation of GLUT4 from the intracellular stores to the cell surface has been proposed as a possible mechanism that can lead to insulin resistance in muscle tissues [1, 3, 19, 32]. Therefore, restoration of insulin sensitivity is largely accounted for by the improvement in insulin-stimulated GLUT4 translocation [1, 3, 7]. It has been established that GLUT4 translocation in skeletal muscle is mediated by both insulin-dependent PI3K

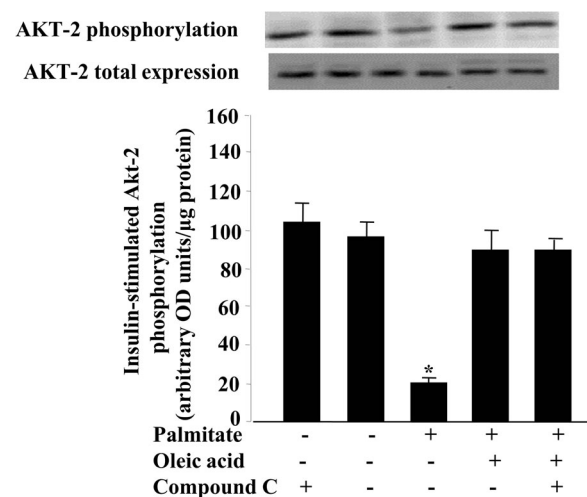


Fig. 9 Effect of the AMPK inhibitor, compound C, on the restorative effect of oleic acid on Akt-2 phosphorylation in muscle incubated with palmitate. Data are presented as means \pm SE; $n = 8$ solei per data point. To measure Akt-2 phosphorylation, insulin was provided in the last 10 min of the 12-h incubation. Equal quantities of protein were loaded for each muscle at each data point, and loading was verified with Ponceau staining. * $P < 0.05$ vs other experimental treatments

and insulin-independent AMPK pathways [15, 25]. Although the signaling molecule(s) connecting these pathways are still not well known, AS160 is thought to be a key signaling molecule connecting these pathways to GLUT4 translocation [14, 24, 29]. Our results revealed that oleic acid improved insulin-stimulated phosphorylation of AS160, despite the continuous presence of palmitate, and that was associated with a concurrent restoration of GLUT4 translocation.

An important step for AS160 phosphorylation in response to insulin activation is the phosphorylation of Akt-2 [26]. In this study, oleic acid treatment (12 h) fully restored the impaired Akt-2 phosphorylation. There appears to be no published data available regarding intact mammalian skeletal muscle to which the present results can be compared. However, these results are consistent with other studies using cell cultures showing that oleic acid treatment can normalize insulin-stimulated Akt phosphorylation [33].

To further explore the mechanisms underlying oleic acid ability to promote insulin-stimulated Akt-2/AS160 functionality and GLUT4 translocation, we assessed the activity of PI3K, an insulin signaling intermediate that is known to modulate these signal molecules. In this study, it was observed that wortmannin, a potent and selective PI3-kinase inhibitor, partially and unexpectedly abrogated the restorative effect of oleic acid on insulin-stimulated GLUT4 translocation and AS160 phosphorylation. Therefore, these data suggest that oleic acid may, in part, eliminate the inhibitory effect of palmitate on insulin-stimulated GLUT4 translocation and AS160 phosphorylation by activating the PI3K pathway. Another effective means to restore AS160 functionality, and thus enable the translocation of GLUT4 to the cell surface, is to activate AMPK [5, 14, 24, 29, 30]. AMPK signaling may reverse palmitate-induced insulin resistance, secondary to improvements in the intracellular lipid milieu and/or improved fatty acid oxidation [2, 10, 22]. Alternatively, there may be a cross talk between the AMPK and PI3K pathways, leading to positive modifications in insulin signaling. In this regard, we found that the restorative effect of oleic acid on AS160/GLUT4 was not prevented by compound C, a selective AMPK inhibitor. Collectively, the present data indicate that the restored insulin response by oleic acid leads to the improvement in insulin-stimulated GLUT4 translocation. This may partially be a consequence of activating the PI3K pathway.

In conclusion, our findings provide for the first time, valuable insights into the mechanisms by which oleic acid ameliorates palmitate-induced insulin resistance. Our data indicate that oleic acid enhances insulin-stimulated AS160 phosphorylation, which in turns normalizes insulin-stimulated GLUT4 translocation to the cell surface. Importantly, these restorative effects could not entirely be attributed to the activation of PI3K, as inhibition of this pathway resulted in only a partial inhibition of GLUT4 translocation and AS160 phosphorylation. Therefore, it is likely that oleic acid restores insulin

response in the presence of palmitate through different mechanisms, although the exact details of these mechanisms require further elucidation.

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