Palmitate Contributes to Insulin Resistance through Downregulation of the Src-Mediated Phosphorylation of Akt in C2C12 Myotubes

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The mechanisms of free fatty acid (FFA)-induced peripheral insulin resistance remain elusive. This study aimed to investigate the effect of palmitate, a saturated fatty acid, on glucose metabolism in C2C12 myotubes, and to explore the underlying mechanisms. In vitro, palmitate decreased insulin-stimulated glucose uptake and consumption in a dose-dependent manner, and it reduced the insulin-stimulated phosphorylation of Akt at Thr308 and Ser473, but had no effect on the protein expression of PI3K-p85 or the activity of PI3K. Additionally, it inhibited the insulin-stimulated phosphorylation of Src at Tyr416, causing a reduction in the Src-mediated phosphorylation of Akt. Inhibition of Src by PP2 resulted in decreases in insulin-stimulated glucose uptake and phosphorylation of Akt at Thr416 and Akt at Thr308 and Ser473. The findings indicate that palmitate contributes to insulin resistance by inhibiting the Src-mediated phosphorylation of Akt in C2C12 myotubes, and this provides insight into the molecular mechanisms of FFA-induced insulin resistance.

Key words: insulin resistance; free fatty acids; glucose metabolism; type 2 diabetes; β-arrestin-2

Insulin resistance, a hallmark of type 2 diabetes, which is one of the most serious public health problems around the world and is increasing in prevalence,1,2 is characterized by the inability of insulin to stimulate glucose uptake and utilization in peripheral target tissues, including skeletal muscles, the liver, and adipose tissues. Increasing evidence3,4 indicates that chronic elevation of plasma free fatty acids (FFAs) often coexists with type 2 diabetes and obesity, and is responsible for a great deal of impaired insulin-induced glucose uptake and disposal. Experimental plasma FFA elevation impairs glucose oxidation/glycogen synthesis and decreases glucose transport, which leads to organ-specific insulin resistance in adipocytes, skeletal muscle cells, and hepatocytes,5,6 whereas lowering FFA levels in vivo and vitro significantly improves insulin sensitivity.7,8 Prolonged exposure to elevated FFA results in the production of inflammatory factors by stimulating the nuclear factor-κB (NF-κB) pathway,9–11 and induces activation of Jun N-terminal kinase-3 (JNK3), which accelerates β-oxidation of FFA, brings about excessive electron flux in the mitochondrial respiratory chain, and subsequently causes increased reactive oxidant species (ROS) generation.5,12,13 All of this has passive effects on the insulin signal transduction pathway, leading to decreased activity of phosphatidylinositol-3-kinase-3 kinase (PI3K), reduced phosphorylation of the three known isoforms of Akt, and impaired translocation of glucose transporter-4 (Glut4), finally resulting in inhibited glucose uptake and utilization.13,15 Despite such data, the underlying molecular mechanisms remain to be established.

Systemic blood glucose levels and energy homeostasis are regulated mainly by insulin. Upon stimulation by insulin, activated insulin receptor (InsR) recruits and phosphorylates insulin receptor substrate proteins (IRS), causing activation of PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3), which results in the activation of Akt. Activated Akt regulates downstream targets, including Glut4, glycogen synthase kinase 3 (GSK3), forkhead box O1 (FoxO1), hormone sensitive lipase (HSL), and mTOR, and thus is responsible for most of the metabolic actions of insulin, including glucose uptake, glucose synthesis, gluconeogenesis, fat mobilization, and protein synthesis.16–18 In addition to PI3K/Akt signaling, recent studies19,20 have found that Src, a member of the Src family of protein tyrosine kinases, can lead to the phosphorylation of Akt at Tyr315/326, which enhances Akt serine/threonine phosphorylation and is a prerequisite for full Akt activation, thus mediating insulin-induced glucose metabolism.

This study aimed to determine the effect of palmitate, a saturated fatty acid, on glucose metabolism in C2C12 mouse myotubes, an insulin-sensitive model, and to explore the underlying molecular mechanisms.
Materials and Methods

Materials. Dulbecco’s Modified Eagle’s Medium (DMEM), fetal calf serum (FCS), and horse serum were purchased from Gibco (Grand Island, NY). Penicillin G (100 U/mL), and streptomycin (100 μg/mL) were cultured in DMEM supplemented with 10% (vol/vol) FCS and 1% (vol/vol) FBS.}

Antibodies. Antibodies against Akt (total), Akt (phosphorylated Thr308), Akt (phosphorylated Ser473), and PI3K-p85 were from Cell Signaling Technology (Danvers, MA). Antibodies to β-arrestin-2, Src, and Src (phosphorylated Tyr416) was from Calbiochem (La Jolla, CA), and secondary HRP-conjugated antibodies were from Pierce (Rockford, IL).}

Cytotoxicity of Palmitate in C2C12 myotubes

In this study, all data were obtained by palmitate treatment for 16 h, and hence it was necessary to determined whether palmitate was toxic to C2C12 myotubes. The results are presented in Fig. 1. Palmitate treatment for 16 h decreased cellular activity in a dose-dependent manner. At doses from 0.125 to 0.5 mM, palmitate had no cytotoxicity, but cytotoxicity was observed when the concentration reached 1.0 mM, which obviously decreased cellular activity, by 7.29% (p < 0.05) as compared to the control (0 mM).

Palmitate led to insulin resistance in C2C12 myotubes

Insulin resistance mainly means decreases in insulin-stimulated glucose uptake and utilization (consumption).
second messenger PIP, plays a key role in signaling by catalyzing the formation of lipid regulatory (p85) and a catalytic subunit (p110), plays a pivotal role in regulating glucose uptake and utilization. In view of these results, it is likely that palmitate promoted Akt phosphorylation by inhibiting Src-related signaling. Palmitate, an inhibitor of Src, was taken as control. Like palmitate, PP2 remarkably reduced insulin-stimulated glucose uptake (Fig. 4C). Additionally, PP2 pre-treatment for 16 h also inhibited the insulin-stimulated phosphorylation of Src at Tyr416 and Akt at Thr308 and Ser473.

Discussion

In this study, palmitate inhibited insulin-stimulated glucose uptake and consumption and the insulin resistance was caused in C2C12 myotubes according to Reaven’s initial report. The conventional view is that the PI3K/Akt pathway mediates the metabolic actions of insulin by phosphorylation at Thr308 and Ser473 and then phosphorylates Akt on Tyr315/326, which enhances Akt serine/threonine phosphorylation, thus regulating glucose uptake and utilization. In view of these results, it is likely that palmitate contributes to insulin sensitivity in C2C12 myotubes, we checked glucose uptake and consumption. As shown in Fig. 2, in the absence of insulin, palmitate treatment for 16 h had no effect on glucose uptake or consumption in the cells. Insulin, however, significantly increased glucose uptake and consumption, and palmitate treatment for 16 h resulted in obvious decreases in insulin-stimulated glucose uptake and consumption, in a dose-dependent manner. The results indicate that palmitate decreases the insulin-stimulated phosphorylation of Akt independently of PI3K.

Palmitate inhibited the phosphorylation of Akt dependent on Src in C2C12 myotubes

In addition to the PI3K/Akt pathway, another signaling pathway for full Akt activation has been found. In it, β-arrestin-2 scaffolds Akt and Src to InsR upon insulin stimulation, causing the formation of a new β-arrestin-2 signal complex, in which Src is activated by autophosphorylation at Tyr416 and then phosphorylates Akt on Tyr315/326, which enhances Akt serine/threonine phosphorylation, thus regulating glucose uptake and utilization. In view of these results, it is likely that palmitate cut down Akt phosphorylation by inhibiting Src-related signaling. Although palmitate treatment for 16 h had no effect on the protein expression of β-arrestin-2 (Fig. 4A) or Src (Fig. 4B), insulin-stimulated phosphorylation of Src at Tyr416 declined markedly, by 47.70% (p < 0.01, Fig. 4B). Hence, PP2, an inhibitor of Src, was taken as control. Like palmitate, PP2 remarkably reduced insulin-stimulated glucose uptake (Fig. 4C). Additionally, PP2 pre-treatment for 16 h also inhibited the insulin-stimulated phosphorylation of Src at Tyr416 and Akt at Thr308 and Ser473, by 49.59%, 45.02%, and 48.06% (all p < 0.01) respectively as compared to the insulin-treated group (Fig. 4D and E). This suggests that palmitate inhibits the phosphorylation of Akt, probably by reducing that of Src.

To confirm the effect of palmitate on insulin sensitivity in C2C12 myotubes, we checked glucose uptake and consumption. As shown in Fig. 2, in the absence of insulin, palmitate treatment for 16 h had no effect on glucose uptake or consumption in the cells. Insulin, however, significantly increased glucose uptake and consumption, and palmitate treatment for 16 h resulted in obvious decreases in insulin-stimulated glucose uptake and consumption, in a dose-dependent manner. The results suggest that palmitate contributes to insulin resistance in C2C12 myotubes.

Palmitate inhibited Akt phosphorylation independently of PI3K in C2C12 myotubes

To explore the molecular mechanisms of palmitate inhibition of Akt phosphorylation, we investigated the effect of palmitate on insulin signaling. As shown in Fig. 2, in the absence of insulin, palmitate treatment for 16 h had no effect on glucose uptake or consumption in the cells. Insulin, however, significantly increased glucose uptake and consumption, and palmitate treatment for 16 h resulted in obvious decreases in insulin-stimulated glucose uptake and consumption, in a dose-dependent manner. The results indicated that protein expression of p85 was not obviously changed by palmitate treatment for 16 h (Fig. 3A). In the absence of insulin, the activity of PI3K was not induced by palmitate treatment for 16 h. But insulin greatly promoted the activity of PI3K. Palmitate treatment for 16 h did not inhibit the insulin-stimulated activity of PI3K (Fig. 3B). The protein expression of Akt was not affected, either (Fig. 3C). Insulin stimulation led to an approximately 3-fold increase in the phosphorylation of Akt at Thr308 and Ser473, and palmitate pre-treatment for 16 h significantly reduced the insulin-stimulated phosphorylation of Akt at Thr308 and Ser473, by 39.87% (p < 0.01) and 35.88% (p < 0.01) respectively as compared to the insulin-treated group (Fig. 3C). These data indicate that palmitate decreases the insulin-stimulated phosphorylation of Akt independently of PI3K.

**Fig. 2.** Palmitate Induced Insulin Resistance in C2C12 Myotubes. After serum starvation in 1% FFA-free BSA-DMEM, C2C12 myotubes in 24-well culture plates were treated with palmitate (PA) for 16 h and then incubated with and without insulin (INS, 100 nM) for 20 min, and glucose uptake was determined with 2-DOG (A). C2C12 myotubes in 96-well culture plates were treated with PA and/or insulin (1 nM) for 16 h, and the glucose concentration in medium was determined by the glucose oxidase method (B), n = 6. *p < 0.05, **p < 0.01.

In the present study, insulin stimulation led to an approximately 3-fold increase in the phosphorylation of Akt at Thr308 and Ser473, and palmitate pre-treatment for 16 h significantly reduced the insulin-stimulated phosphorylation of Akt at Thr308 and Ser473, by 39.87% (p < 0.01) and 35.88% (p < 0.01) respectively as compared to the insulin-treated group (Fig. 3C). These data indicate that palmitate decreases the insulin-stimulated phosphorylation of Akt independently of PI3K.
Palmitate decreased the phosphorylation of Akt at Thr308 and Ser473, consistently with previous findings. But paradoxically, the protein expression of p85, the regulatory subunit of PI3K, and the activity of PI3K were not influenced by palmitate. It is well known that although full activation of Akt requires phosphorylation at Thr308 in the activation loop and, Ser473 in the C-terminal regulatory domain, phosphorylation at several tyrosine residues also involves regulation of Akt activation. It has been reported that Akt is phosphorylated on Tyr315, Tyr326, and Tyr474 which also play key roles in kinase activation, and that inhibition of Akt tyrosine phosphorylation impairs Akt activity independently of PI3K function by an ill-defined mechanism. Hence, we inferred that palmitate inhibited Akt phosphorylation in another way.

The PI3K/Akt pathway is not the only one mediating insulin-induced glucose metabolism. Recently, researchers found another insulin signaling pathway involving Akt activation. It mainly involves targets that include β-arrestin-2, Src, InsR, and Akt. β-Arrestin-2 is an important adaptor in modulating the strength and duration of cellular signaling by scaffolding and interacting with many cytoplasmic proteins, including InsR, Src, and Akt. Src activity is regulated by tyrosine phosphorylation at two sites, but with opposite effects. Phosphorylation at Tyr416 in the activation loop of the kinase domain upregulates enzyme activity, while phosphorylation at Tyr527 in the carboxy-terminal tail by Csk renders the enzyme less active. Activated Src leads to phosphorylation of Akt at Tyr315/326, which enhances Akt serine/threonine phosphorylation and is required for full Akt activation. Upon stimulation by insulin, β-arrestin-2 scaffolds Src and Akt to InsR, causing the formation of the β-arrestin-2 signal complex, which allows Src to phosphorylate Akt, thus enhancing insulin-mediated glucose uptake and glucose synthesis and inhibiting gluconeogenesis. The novel signaling has a potential anti-diabetic role, and provides new insight into the molecular pathogenesis of insulin resistance.

To explore the molecular mechanisms further, we observed molecular changes in signaling by palmitate. Palmitate had no effect on the protein expression of β-arrestin-2 or Src, but greatly reduced the insulin-stimulated phosphorylation of Src at Tyr416, which convinced us that palmitate inhibited the phosphorylation of Akt through Src. To confirm this, we took PP2, a specific Src inhibitor, as control. The results confirmed our deduction. PP2 not only decreased insulin-stimulated glucose uptake, but also inhibited the phosphorylation of Src at Tyr416 and, Akt at Thr308 and Ser473, in agreement with previous reports.

Recently, Holzer and colleagues reported that saturated fatty acids such as palmitate and stearic acid result in insulin resistance by inducing Akt inactivation involving Src phosphorylation, consistently with our results. In their study, however, saturated fatty acids regulated the phosphorylation of Src at Tyr418 but not at Tyr416, leading to JNK activation suppressing Akt phosphorylation. Lipid accumulation has deleterious effects on initial glucose metabolism and insulin action. In our study, palmitate might partially incorporate into the plasma membrane and inhibited the insulin-stimulated phosphorylation of Src at Tyr416 in C2C12 myotubes, leading to reduced Src-mediated phosphorylation of Akt. On the other hand, G-protein coupled receptors (GPR) such as GPR43 and GPR120 with high binding affinity to the medium- and long-chain FFAs including palmitate are upregulated in the skeletal muscle in high-fat-diet rats. It is expected that palmitate, a ligand, bound GPR43 or GPR120, did not require palmitate accumulation in C2C12 myotubes, and modulated cell-signal transduction, inhibiting the insulin-stimulated phosphorylation of Src at Tyr416 through an ill-defined mechanism and downregulating...
Fig. 4. Palmitate Inhibited the Phosphorylation of Akt Dependent on Src in C2C12 Myotubes.
C2C12 myotubes were treated with and without palmitate (PA) and/or PP2 for 16 h after serum starvation in 1% FFA-free BSA-DMEM, and then stimulated with and without insulin (100 nM) for 30 min. Whole-cell lysate protein was examined by Western blot, and glucose uptake was determined with 2-DOG. A, Protein expression of β-arrestin-2. B, Protein expression of Src and phosphorylated Src. C, 2-DOG uptake, n = 6. D, Protein expression of phosphorylated Src. E, Protein expression of phosphorylated Akt. **p < 0.01.
the Src-mediated phosphorylation of Akt. This calls for further study.

Additionally, some saturated fatty acids such as stearic acid have been found to induce insulin resistance by inhibiting Akt phosphorylation. They are similar to palmitate. It is likely that other saturated fatty acids also have negative effects on the Src-mediated Akt phosphorylation. Increasing evidence indicates that not all FFAs reduce Akt phosphorylation or lead to insulin resistance. Some unsaturated fatty acids, such as palmitoleate, oleate, and poly-unsaturated fatty acids, have different effects from palmitate on Src and Akt phosphorylation, JNK activity, and other cellular functions. Even palmitoleate and oleate reverse palmitate-induced impairment of insulin-mediated glucose uptake and Akt inactivation, and prevent palmitate-induced apoptosis and other cellular effects. We infer that unsaturated fatty acids possess beneficial effects on the Src-mediated Akt phosphorylation, but this hypothesis must be subjected to further study.

The present study does not imply that the PI3K/Akt pathway is not requisite for palmitate-induced insulin resistance, because the phosphorylation of Akt at Thr308 and Tyr473 is dependent on PI3K, and Src affects only the phosphorylation levels of Akt at Thr308 and Tyr473 through phosphorylation of Akt at Tyr315/326.

In summary, this study indicates that palmitate decreases insulin-stimulated glucose uptake and consumption by downregulating the Src-mediated phosphorylation of Akt. This provides new insight into the molecular mechanisms of FFA-induced insulin resistance.

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