

Silibinin improves palmitate-induced insulin resistance in C2C12 myotubes by attenuating IRS-1/PI3K/Akt pathway inhibition

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Abstract

The present study investigated the effect of silibinin, the principal potential anti-inflammatory flavonoid contained in silymarin, a mixture of flavonolignans extracted from *Silybum marianum* seeds, on palmitate-induced insulin resistance in C2C12 myotubes and its potential molecular mechanisms. Silibinin prevented the decrease of insulin-stimulated 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose) uptake and the downregulation of glutamate transporter type 4 (GLUT4) translocation in C2C12 myotubes induced by palmitate. Meanwhile, silibinin suppressed the palmitate-induced decrease of insulin-stimulated Akt Ser473 phosphorylation, which was reversed by wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase (PI3K). We also found that palmitate downregulated insulin-stimulated Tyr632 phosphorylation of insulin receptor substrate 1 (IRS-1) and up-regulated IRS-1 Ser307 phosphorylation. These effects were rebalanced by silibinin. Considering several serine/threonine kinases reported to phosphorylate IRS-1 at Ser307, treatment with silibinin downregulated the phosphorylation of both c-Jun N-terminal kinase (JNK) and nuclear factor- κ B kinase β (IKK β), which was increased by palmitate in C2C12 myotubes mediating inflammatory status, whereas the phosphorylation of PKC- θ was not significantly modulated by silibinin. Collectively, the results indicated that silibinin prevented inhibition of the IRS-1/PI3K/Akt pathway, thus ameliorating palmitate-induced insulin resistance in C2C12 myotubes.

Key words: Silibinin; Insulin resistance; Palmitate; C2C12

Introduction

Metabolic detuning has been reported to be involved in obesity, dyslipidemia, diabetes mellitus, and hypertension, all of which characterize metabolic syndrome and are closely associated with insulin resistance. In recent decades, the sustained increases in obesity and metabolic syndrome that have occurred worldwide have resulted in greater interest in the cellular events related to insulin resistance and in how to prevent and treat such resistance (1).

Skeletal muscle is the primary site of glucose uptake, disposal, and storage, accounting for approximately 75% of the entire body's glucose uptake under insulin stimulation (2). Increased plasma free fatty acid (FFA) levels are observed in the above-mentioned diseases, and a growing body of evidence indicates that FFA levels play a central role in the pathophysiology of skeletal muscle insulin resistance (3,4). It has been proposed that several mechanisms account for the inhibition of insulin signaling by saturated fatty acids, including the activation of various serine/threonine kinases, such as protein kinase C isoforms (PKCs), nuclear factor- κ B kinase β (IKK β), c-Jun

N-terminal kinase (JNK), and p38 MAP kinase (5). These kinases are activated in high-fat diet-induced or saturated fatty acid-induced insulin resistance and have been reported to catalyze the phosphorylation of serine residues in insulin receptor substrate 1 (IRS-1), leading to a reduction in the phosphorylation of tyrosine residues of IRS-1 and in the activity of downstream signaling pathways activated by insulin (6,7).

Silibinin, the principal flavonoid contained in silymarin, a mixture of flavonolignans extracted from *Silybum marianum* seeds, is widely used to treat a variety of liver ailments (8), such as nonalcoholic fatty liver disease, which is a chronic metabolic disorder related to a puzzling crosstalk between liver, muscle, and adipose tissue regarding FFA utilization (9). The therapeutic effect of silibinin on insulin resistance has been reported in both clinical studies (10,11) and experimental liver injury models (12-14). However, whether and how silibinin can improve insulin resistance in skeletal muscle cells induced by FFA remains to be elucidated.

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Received August 1, 2014. Accepted November 26, 2014. First published online March 6, 2015.

Materials and Methods

Reagents

The mouse C2C12 myoblast cell line was obtained from American Type Culture Collection (ATCC, USA). HG-DMEM (Dulbecco's modified Eagle's medium with high glucose) was from GIBCO™ (USA). Fetal bovine serum (FBS) and horse serum were purchased from Hyclone (USA). Insulin, fatty acid-free bovine serum albumin (BSA), palmitate, silibinin, cytochalasin B, and wortmannin were from Sigma (USA). We obtained 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) from Invitrogen (USA). IRS, phospho-IRS-1 (Thr⁶³²), phospho-IRS-1 (ser³⁰⁷), Akt, phospho-Akt (Ser⁴⁷³), phospho-JNK, phospho-IKK β , and phospho-PKC- θ antibodies were purchased from Cell Signaling Technology (USA).

Cell culture and treatments

Myoblast C2C12 cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. Cells were reseeded at a density of 2×10^4 cells/mL. After 48 h (~80% confluence), the medium was switched to HG-DMEM with 2% (v/v) horse serum and replaced every other day. Experiments were initiated on day 5 when differentiation was complete. Silibinin was added at various concentrations 2 h prior to the experiments. Palmitate treatment (0.75 mM) of myotubes was carried out during the last 16 h of silibinin treatment.

Palmitate solution preparation

BSA-bound palmitate was prepared according to a previously described procedure (15), with some modifications. Palmitate was dissolved in 0.1 M NaOH to a concentration of 75 mM by heating at 70°C in a shaking water bath, and the solution was then diluted with 10% FFA-free BSA-DMEM at a stock solution of 5 mM at 55°C in a shaking water bath. After filtration (0.45- μ m pore size membrane filter), this solution was stored at -20°C and used within 2 weeks. Stored stock solution was heated for 15 min at 55°C and then cooled to room temperature before use. The same concentration of NaOH mixed with 10% FFA-free BSA was used as a control.

2-NBDG uptake

Glucose uptake was measured by adding the fluorescent D-glucose analog 2-NBDG as a tracer to the culture medium. Immediately after treatments with silibinin and BSA-bound palmitate, cells plated in 24- or 96-well plates were incubated with or without insulin (100 nM) for 15 min before 2-NBDG was added at a final concentration of 50 μ M; incubation was then continued for a further 20 min. After incubation, free 2-NBDG was washed out 3 times, and fluorescence densities in cell monolayers were measured with a fluorescence microplate reader

(Molecular Devices, USA) set at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The protein concentration of each sample was determined by the Bradford method. Results were normalized by mg of total protein. Nonspecific 2-NBDG uptake was measured in the presence of 20 μ M cytochalasin B and subtracted from the total 2-NBDG uptake.

Subcellular fractionation

Cells were collected in ice-cold phosphate-buffered saline (PBS), washed twice with the same buffer, suspended in 100 μ L cold sample preparation buffer, sonicated 4-5 times for 10 s each, and centrifuged at 100,000 *g* for 60 min at 4°C. The resulting pellet was resuspended in 100 μ L homogenization buffer to which was added Triton X-100 (final concentration 0.5%) and incubated on ice for 1 h to extract soluble membrane proteins. Samples were centrifuged again at 100,000 *g* for 1 h at 4°C to remove insoluble membrane components. The resultant supernatant was kept as the plasma membrane fraction. Protein concentrations in the plasma membrane fraction were determined using the Bradford method.

Immunoblotting

Immediately after treatments, the media were aspirated, and the cells were washed twice in ice-cold PBS and lysed in 100 μ L lysis buffer. The samples were then briefly sonicated, heated for 5 min at 95°C, and centrifuged at 14,000 *g* for 5 min. Protein concentrations in the supernatants were determined using the Bradford method. The supernatants were diluted to the same protein concentration, electrophoresed on sodium dodecyl sulfate-polyacrylamide (8%) gels (SDS-PAGE), and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, USA). The blots were incubated overnight at room temperature with primary antibodies and then washed 3 times in Tris-buffered saline/0.1% Tween 20 prior to 1 h incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. Bound antibodies were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, UK) and measured by densitometry using a ChemiDoc XRS digital imaging system and the MultiAnalyst software from Bio-Rad Laboratories (USA).

Statistical analysis

Data are reported as means \pm SD, and statistical comparisons between groups were carried out using Student's *t*-test or one-way analysis of variance (ANOVA). *P* < 0.05 was considered statistically significant.

Results

Silibinin prevented the palmitate-induced decrease of insulin-stimulated glucose

The effects of silibinin and palmitate on glucose

uptake activity were tested in differentiated C2C12 myotubes. We found that 0.75 mM palmitate decreased 2-NBDG uptake in insulin-stimulated C2C12 myotubes by 29% ($P < 0.05$), while the effect of palmitate was not significant in naive myotubes. Silibinin treatment did not significantly affect basal or insulin-mediated glucose uptake (Figure 1).

To examine whether silibinin affected the insulin-mediated glucose uptake in palmitate-induced insulin-resistant cells, various concentrations of silibinin were added 2 h prior to the addition of palmitate. As shown in Figure 2, the decrease of 2-NBDG uptake was prevented by silibinin in a dose-dependent manner. Compared with the 0.75 mM palmitate group, insulin-stimulated glucose uptake was improved by 10%, 27% ($P < 0.05$), and 38% ($P < 0.05$) in the presence of 16, 40, and 100 $\mu\text{g/mL}$ silibinin, respectively.

Downregulation of glutamate transporter type 4 translocation induced by palmitate was prevented by silibinin

To elucidate the mechanism by which silibinin prevented the decrease of insulin-stimulated glucose uptake induced by palmitate, we measured glutamate transporter type 4 (GLUT4) expression and translocation. As shown in Figure 3, total GLUT4 protein content was non-significantly reduced in palmitate-treated cells. Although GLUT4 levels in the plasma membrane fraction were lowered significantly by palmitate, this decrease was prevented by silibinin.

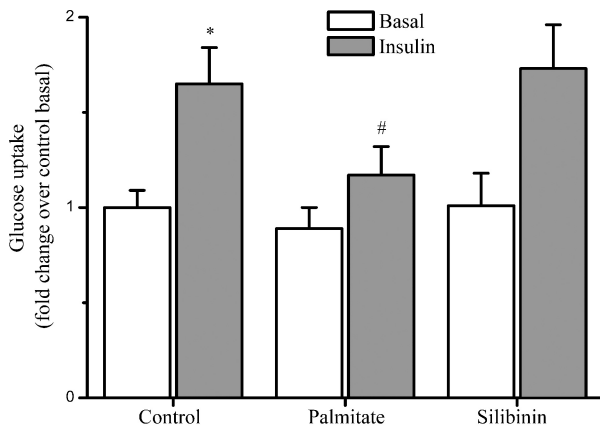


Figure 1. Effects of chronic exposure of C2C12 skeletal muscle cells to silibinin or palmitate on glucose uptake. Myotubes were incubated for 16 h in growth medium supplemented with 100 $\mu\text{g/mL}$ silibinin or 0.75 mM palmitate, and then left untreated or stimulated with 100 nM insulin, followed by assessment of 2-NBDG uptake as described in Methods. Data are reported as means \pm SD of 4 determinations. * $P < 0.05$, compared to control basal values; # $P < 0.05$, compared to control group treated with acute insulin (Student's *t*-test).

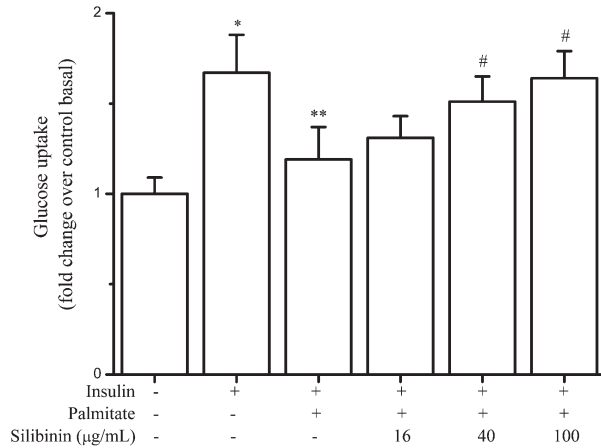


Figure 2. Effect of silibinin on insulin-stimulated glucose uptake in palmitate-treated C2C12 myotubes. C2C12 myotubes were incubated with palmitate (0.75 mM) or silibinin + palmitate, then stimulated with insulin (100 nM), followed by assay for 2-NBDG uptake as described in Methods. Data are reported as means \pm SD of 4 determinations. * $P < 0.05$, compared to control basal values; ** $P < 0.05$, compared to insulin control values; # $P < 0.05$, compared to palmitate group (Student's *t*-test).

Silibinin prevented IRS-1/PI3K/Akt pathway inhibition

To determine whether silibinin improved palmitate-induced insulin resistance in C2C12 myotubes through the canonical insulin signaling pathway, we examined IRS-1 and Akt phosphorylation. Palmitate markedly decreased the insulin-stimulated Ser473 phosphorylation of Akt and Tyr632 phosphorylation of IRS-1, which was prevented by 16, 40, and 100 $\mu\text{g/mL}$ silibinin treatment (Figure 4).

As shown in Figure 5, 50 nM wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase (PI3K), suppressed the increase of Akt phosphorylation induced by 100 $\mu\text{g/mL}$ silibinin in insulin-resistant C2C12 myotubes, which indicated that the effect of silibinin upon the

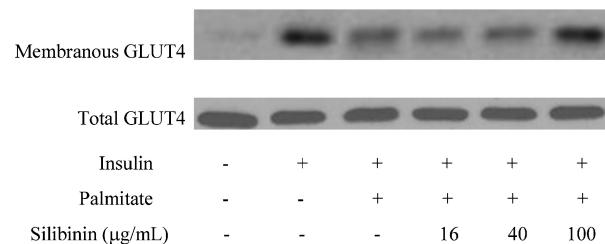


Figure 3. Effects of silibinin on insulin-stimulated GLUT4 translocation in palmitate-treated C2C12 myotubes. C2C12 myotubes were incubated with palmitate (0.75 mM) or silibinin + palmitate as described in Methods. Before harvesting, the cells were incubated in the presence or absence of 100 nM insulin for 30 min and lysed for Western blotting. Figures are representative of 3 independent experiments.

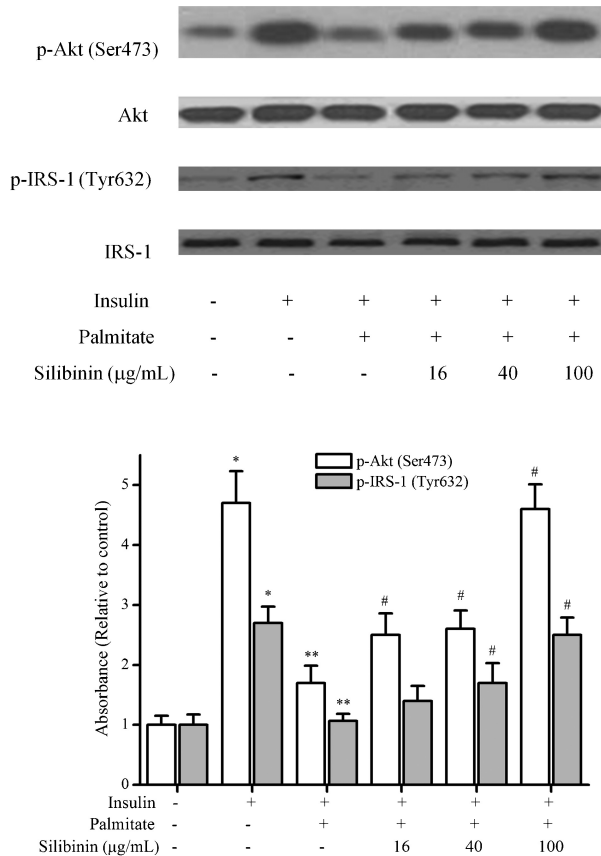


Figure 4. Effects of silibinin on the palmitate-inhibited insulin signaling pathway in C2C12 myotubes. C2C12 myotubes were incubated with palmitate (0.75 mM) or silibinin + palmitate as described in Methods. Before harvesting, the cells were incubated in the presence or absence of 100 nM insulin for 30 min and lysed for Western blotting. Data are reported as means ± SD of 4 determinations. *P<0.05, compared to control group; **P<0.05, compared to insulin group; #P<0.05, compared to palmitate group (Student's *t*-test).

inhibition of Akt phosphorylation by palmitate is PI3K-dependent.

Meanwhile, we found that 0.75 mM palmitate upregulated IRS-1 Ser307 phosphorylation in the presence of insulin; this up-regulation was significantly reduced by 100 µg/mL silibinin (Figure 6).

Silibinin treatment modulated the phosphorylation of JNK and IKKβ but not PKC-θ in palmitate-treated C2C12 myotubes

Treatment with silibinin downregulated the phosphorylation of JNK and IKKβ, both of which were increased by 0.75 mM palmitate in C2C12 myotubes. The phosphorylation of PKC-θ was not significantly modulated by palmitate, either alone or combined with 100 µg/mL silibinin (Figure 6).

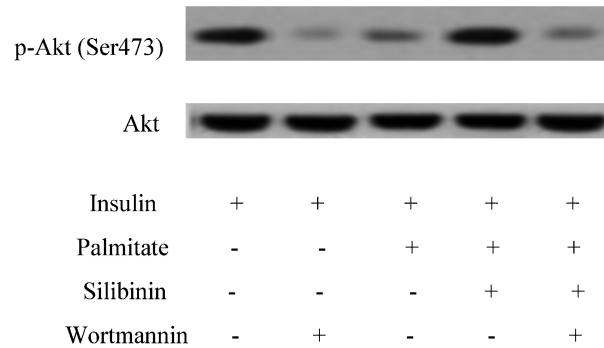


Figure 5. The increase of Akt phosphorylation by silibinin in insulin-resistant C2C12 myotubes was suppressed by 50 nM wortmannin, a specific inhibitor of phosphatidylinositol-3-kinase (PI3K). Differentiated C2C12 skeletal muscle cells were incubated with palmitate (0.75 mM) and/or silibinin (100 µg/mL) in the presence or absence of wortmannin (50 nM), and then stimulated with insulin (100 nM) before cells were harvested for Western blotting. Figures are representative of 3 independent experiments.

Discussion

Insulin resistance is a common pathological state found in the metabolic syndrome associated with obesity and type 2 diabetes mellitus, in which target tissues fail to respond properly to physiologic insulin levels (5,7). Skeletal muscle is the major tissue in which a decrease of insulin-mediated glucose uptake is one of the earliest abnormalities indicating insulin resistance. It was suggested that circulating fatty acids significantly increased in obesity and that associated diseases might play an important role in the development of insulin resistance in skeletal muscle (16). In the present study, insulin-stimulated 2-NBDG uptake and GLUT4 translocation in insulin-resistant C2C12 myotubes induced by palmitate was investigated to elucidate the potential effect and mechanism of silibinin on these processes. Our results showed that silibinin (between 16 and 100 µg/mL) was not toxic to C2C12 myotubes, using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (data not shown), and it prevented the decrease in insulin-stimulated glucose uptake and downregulation of GLUT4 translocation in insulin-resistant C2C12 myotubes induced by palmitate. However, other researchers have reported that silibinin inhibited glucose uptake by directly interacting with GLUT transporters in 3T3-L1 adipocytes and Chinese hamster ovary (CHO) cells (17). On the other hand, contrary results were reported for several other substances, such as genistein, quercetin, and green tea (18). Genistein was reported to suppress insulin-mediated glucose uptake in adipocytes (19) and to promote glucose uptake and GLUT4 translocation in L6 myotubes (20). Quercetin was also reported to inhibit glucose uptake in isolated rat adipocytes (21) but to prevent the downregulation of

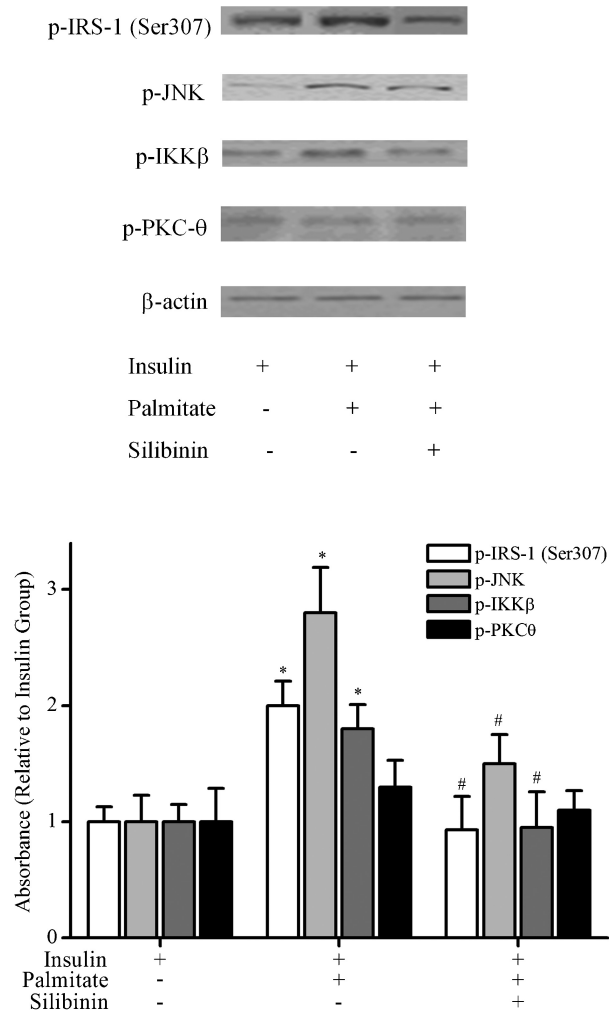


Figure 6. Silibinin modulated the phosphorylation of IRS-1 Ser307, JNK, IKK β but not PKC- θ in skeletal muscle cells. Differentiated C2C12 skeletal muscle cells were incubated with palmitate (0.75 mM) or silibinin (100 μ g/mL) + palmitate, then stimulated with insulin (100 nM) before cells were harvested for Western blotting. Data are reported as means \pm SD of 4 determinations. * P <0.05, compared to insulin group; # P <0.05, compared to palmitate group in the presence of insulin (Student's t -test).

glucose uptake in skeletal muscle cells (22). These inconsistent and seemingly contrasting results may be due to the use of different cells and tissues. Both the increases of glucose uptake in skeletal muscle and the decreases in adipose tissue are attractive targets for the prevention of diabetes mellitus and obesity (18).

Insulin stimulates the canonical IRS-PI3K-Akt pathway under physiological conditions, inactivates Akt substrate 160 (AS160), promotes GLUT4 translocation to the membrane from inner vesicles and consequently stimulates glucose uptake (23). At the molecular level, decreased insulin-stimulated glucose uptake is connected

to reduced tyrosine phosphorylation of IRS-1 and PI3K activation in insulin-resistant states (7). The immunoblot results in the present study suggested that silibinin improved insulin resistance in C2C12 myotubes by preventing the inhibition of the insulin signaling pathway, including Tyr632 tyrosine phosphorylation of IRS1, PI3K activation, and Ser473 phosphorylation of Akt.

IRS1 contains pleckstrin homology and phosphotyrosine domains, which provide a docking site for PI3K when phosphorylated, and it plays a critical role in the insulin signaling pathway. It has been postulated for several years that serine phosphorylation of IRS1 is involved in the desensitization of the action of insulin, due to poor balance between "positive" IRS1 tyrosine phosphorylation and "negative" serine phosphorylation (7). Our results showed that the upregulation of IRS-1 Ser307 phosphorylation in C2C12 myotubes induced by 0.75 mM palmitate was significantly reduced by 100 μ g/mL silibinin.

Several serine/threonine kinases including JNK, IKK β , and PKC- θ have been reported to phosphorylate IRS1 at Ser307 and to inhibit its function, which represents a mechanistic link between FFA and insulin resistance. In our skeletal muscle cell model of palmitate-induced insulin resistance, PKC- θ phosphorylation was not markedly modulated, which is consistent with the report that diacylglycerol derived from saturated fatty acid appears to be a poor activator of PKC, whereas that produced from polyunsaturated fatty acid is a much stronger stimulus (24). Conversely, the decrease in insulin-mediated IRS1 tyrosine phosphorylation by FFA was linked to increased PKC- θ activity (25); PKC- θ inactivation prevented defects in insulin signaling and glucose uptake in skeletal muscle (26). It has also been documented that PKC- θ negatively regulates IRS1 in 3T3-L1 adipocytes (27). The inconsistency is probably due to the use of different cell types and variations between *in vivo* and *in vitro* studies, but this requires further study.

In our study, the phosphorylation of both JNK and IKK β was increased by 0.75 mM palmitate in C2C12 myotubes, and this was downregulated by 100 μ g/mL silibinin. This is consistent with previous studies that reported that activities of JNK and IKK β are increased in diabetic and obese mice or by saturated fatty acids (28,29) and that these increases are associated with marked inhibition of the action of insulin, due to the phosphorylation of serine residues on IRS-1 and the consequent inhibition of phosphorylation of tyrosine residues (5,30). However, it needs to be further elucidated how silibinin downregulates the phosphorylation of JNK and IKK β and decreases a palmitate-induced inflammatory response in C2C12 myotubes.

In conclusion, the present results provide important evidence for the role of silibinin in the prevention of palmitate-induced insulin resistance and inhibition of the IRS-1/PI3K/Akt pathway in skeletal muscle cells. Potential mechanisms of these actions include downregulation of JNK and IKK β phosphorylation, thus causing a rebalance

between “positive” IRS-1 tyrosine phosphorylation and “negative” serine phosphorylation.

Acknowledgments

This research was supported by the Doctoral Fund of the Ministry of Education of China (#20093317120003),

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