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Suppression of Free Fatty Acid-Induced Insulin Resistance by Phytopolyphenols in C2C12 Mouse Skeletal Muscle Cells

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ABSTRACT: It was reported that increased plasma levels of free fatty acids (FFAs) are associated with profound insulin resistance in skeletal muscle and may also play a critical role in the insulin resistance of obesity and type 2 diabetes mellitus. Skeletal muscle is the major site for insulin-stimulated glucose uptake and is involved in energy regulation and homeostasis. In this study, we used 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a protein kinase C (PKC) activator, and palmitate to induce insulin resistance in C2C12 mouse skeletal muscle cells. Our data show that epigallocatechin gallate (EGCG) and curcumin treatment reduce insulin receptor substrate-1 (IRS-1) Ser307 phosphorylation, and curcumin is more potent to increase Akt phosphorylation in TPA induction. Moreover, we found that after 5 h of palmitate incubation, epicatechin gallate (ECG) can suppress IRS-1 Ser307 phosphorylation and significantly promote Akt, ERK1/2, p38 MAPK, and AMP-activated protein kinase activation. With a longer incubation with palmitate, IRS-1 exhibited a dramatic depletion, and treatment with EGCG, ECG, and curcumin could reverse IRS-1 expression, Akt phosphorylation, and MAPK signaling cascade activation and improve glucose uptake in C2C12 skeletal muscle cells, especially ECG and curcumin. In addition, treatment with these polyphenols can suppress acetyl-CoA carboxylase activation, but only EGCG could inhibit lipid accumulation in the intracellular site. These findings may suggest that curcumin shows the best capacity to improve FFA-induced insulin resistance than the other two, and ECG was more effective than EGCG in attenuating insulin resistance.

KEYWORDS: *type 2 diabetes, phytopolyphenols, skeletal muscle cells, insulin resistance*

INTRODUCTION

The insulin signaling system plays an important role in many physiological processes, including carbohydrate and fat metabolism, cell growth, and survival.[1] Insulin is the major anabolic hormone, and its primary function is to maintain glucose homeostasis and stimulate glucose transport.[2] Insulin resistance is recognized as an important risk factor in the development of type 2 diabetes. Diabetes mellitus is one of the most prevalent metabolic syndromes that has reached epidemic proportions and affects more than 220 million individuals worldwide.[3] Globally, more than 90−95% of cases of diabetes are type 2, which is largely associated with diet behavior and excess body weight, while type 1 accounts for only 3−5%.[4,5]

Numerous reports demonstrate that an oversupply of lipids raises the circulating level of free fatty acids (FFA), causes insulin resistance in the ingesting subjects,[6,7] and activates a serine/threonine kinase cascade, possibly initiated by protein kinase C (PKC) or by inhibitor B kinase or c-Jun N-terminal kinase (JNK), leading to phosphorylation of serine/threonine sites of the insulin receptor substrate-1 (IRS-1), which in turn reduces the ability of IRS-1 to activate PI3K and Akt. As a consequence, the glucose transport activity and other events downstream of insulin receptor signaling are diminished.[8]

The AMP-activated protein kinase (AMPK) system acts as a sensor of cellular energy status that is conserved in all eukaryotic cells.[8] It has been reported that AMPK is a major mediator of muscle contraction-stimulated glucose uptake in muscle.[9] We found that the high glucose condition causes a significant increase in the Ser 307 phosphorylation of IRS-1, leading to reduced insulin-stimulated phosphorylation of Akt. As a result, the insulin metabolic effects of glycogen synthase and glucose uptake are inhibited by high glucose.[10] However, the treatment of (−)-epigallocatechin-3-gallate (EGCG) improves insulin-stimulated down-signaling by reducing IRS1 Ser307 phosphorylation. These findings have attracted investigators in this field, namely, Zhang et al., who have demonstrated that EGCG protected the insulin sensitivity in rat L6 muscle cells exposed to dexamethasone condition.[11] In addition, Li et al. have demonstrated that EGCG attenuated FFAs-induced peripheral insulin resistance through the AMPK pathway and insulin signaling pathway in vivo.[12] This study suggests the therapeutic value of EGCG in protecting from insulin resistance caused by elevated FFAs.[12]

Chronically increased NEFA in plasma has shown a lot of detrimental effects in peripheral tissues that contribute to insulin resistance. The major catechins in green tea are epicatechin (EC), epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). Among these, EGCG is the most abundant species and has been extensively investigated. However, it has been recently reported that the antioxidant and anti-inflammatory properties of ECG were as effective as EGCG. Furthermore, EGCG, ECG, and curcumin have been reported to be able to lower blood glucose and reduce body fat in human and animal model; however, the molecular mechanism involved in antidiabetes effects of

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these phytopolyphenols remains obscure. Thus, in this study, we aim to ascertain whether these polyphenols have an ability to ameliorate FFA-induced insulin resistance in skeletal muscle cells. This manuscript has two novelties: (1) use of a different cell line, C2C12 mouse skeletal muscle cells, and (2) an extended study in a different phytopolyphenol, curcumin.

MATERIALS AND METHODS

Materials. The pure compound EGCG, ECG, curcumin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), palmitate, FFA-free BSA, oil red O, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were obtained from Sigma-Aldrich (St. Louis, MO). 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) was obtained from (Invitrogen, Carlsbad, CA). The antiphospho Akt (Ser473), anti-Akt, antiphospho PKC (Thr505), antiphospho PKC (Thr538), antiphospho p38 MAPK (Thr180/Tyr182) and anti-p38 MAPK, antiphospho acetyl-CoA carboxylase (ACC) (Ser79), anti-ACC, antiphospho JNK (Thr183/Tyr185), and antiphospho Erk1/2 (Thr202/Tyr204) antibody were obtained from Cell Signaling Technology, Inc. (Beverly, MA). The antiphospho IRS1 (Ser307), anti-IRS1, antiphospho AMPK (Thr172), and anti-AMPK were obtained from Upstate Biotechnology (Lake Placid, NY). The anti-JNK antibody was obtained from Genetex (Irvine, CA).

Cell Culture. Mouse skeletal muscle cell lines, C2C12 myoblasts, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (HyClone, Logan, UT) at 37 °C in a humidified 5% CO_2 atmosphere. After 3 days of plating, the cells had reached 80−90% confluence (day 0). Differentiation was then induced by replacing the growth medium with DMEM supplemented with 2% horse serum instead of 10% fetal bovine serum (differentiation medium). Myotubes formation was achieved after 4−5 days of incubation, and the cells were used for subsequent experiments.

FFA Treatment. FFA-containing stock was prepared by preincubation of palmitate with phosphate-buffered saline (PBS) supplemented with 2% FFA-free bovine serum albumin (BSA). Palmitate was dissolved in ethanol and mixed with 4% (w/v) FFA-free BSA containing PBS in equal volume, and the FFA-containing stock was shaken overnight at 37 °C for gentle conjugation to make BSA-bound FFA.

Western Blotting. Cells were lysed with lysis buffer [10% glycerol, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 10 mM NaF, 50 mM Tris-HCl, pH 8.0, 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.5 mM dithiothreitol], and the cell lysates were centrifuged at 12000 rpm for 30 min at 4 °C, and then, the supernatants were collected as whole cell extracts. For Western blotting, equal amounts of total cellular protein (50 μg) were subjected to 8% SDS−polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Millipore), and the membranes were then blocked for 1 h at room temperature with 1% BSA in PBS. The membranes were next immunoblotted with primary antibodies at dilution of 1:1000 followed by secondary antibodies with a 1:5000 dilution of antimouse or rabbit IgG-conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence system (Perkin-Elmer Life Sciences, Boston, MA).

Glucose Uptake. The glucose uptake rate was measured by adding fluorescent D-glucose analogue 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) as a tracer to the culture medium. After treatments, cells plating in the 24-well were incubated with or without insulin (100 nM) for 15 min, and then, 2-NDBG was added at a 50 μM final concentration for another 20 min. The medium was then removed, and the cells were washed three times with cold PBS. The cells in each well were suspended with PBS after trypsinization, and the fluorescence intensity was read by fluorescent spectrometry at an excitation of 485 nm and an emission of 535 nm.

Oil Red O Staining. To measure the cellular lipid droplet accumulation, C2C12 myotubes were stained by the oil red O method. After treatments, cells were washed three times with ice-cold PBS and fixed with 10% formalin for 30 min. After fixation, cells were washed and stained with oil red O solution (stock solution, 3 mg/mL in isopropanol; working solution, 60% oil red O stock solution and 40% distilled water) for 60 min at room temperature and then washed with water to remove unbound dye. After the cells were dried, isopropanol was added and shaken at room temperature for 30 min to elute the bound dye. Finally, samples were read spectrophotometrically at 510 nm.

MTT Assay. The effect of polyphenols on cell viability was examined by MTT (methyl thiazolyl blue tetrazolium bromide) assay. Briefly, C2C12 myoblasts were seeded in a 24-well flat-bottomed plate. After differentiation, myotubes were incubated in the absence or presence of FFA cotreated with varying concentrations of polyphenols, and the cell viability was examined at the indicated duration of time. The MTT working solution (0.5 mg/mL in PBS) was added to each well and incubated for 40 min at 37 °C. After the MTT solution was removed, the MTT-formazan crystals formed by metabolically viable cells were dissolved in 300 μL of dimethyl sulfoxide, and then, the absorbance was measured at 550 nm by spectrophotometer.

Statistical Analysis. All results are expressed as the means ± standard deviations. Each value is the mean of three independent experiments. Statistical calculations were performed using Student's *t* test. Statistical significance was considered to be present at $p < 0.05$ (*, $p < 0.05$; #, $p < 0.01$).

RESULTS

IRS-1 Ser 307 Phosphorylation Induced by TPA. We used C2C12 skeletal muscle cell as a cellular model to analyze the effects of EGCG, ECG, and curcumin on FFA-induced insulin resistance. Phosphorylation of Ser307 in IRS-1 protein is a hallmark of FFA-associated insulin resistance. TPA, also called phorbol-12-myristate-13-acetate (PMA), a phorbol ester, markedly enhances IRS-1 Ser307 phosphorylation through the activation of PKC, which has been used as a good biological research tool for blocking insulin signaling transmission.[17] C2C12 myotubes were incubated with 0.5 μM TPA for the indicated time (Figure 1A). The data show that TPA-induced IRS-1 Ser 307 phosphorylation was activated within 30 min, and this effect diminishes after 3 h of incubation, accompanied by IRS-1 degradation. However, IRS-1 protein reappeared after 12 h without Ser307 phosphorylation, suggesting that the capacity of TPA to impair IRS-1 activation only occurs within 12 h. Equally, Akt was rephosphorylated after 12 h of incubation with TPA.

Effects of Eight Polyphenols on TPA-Induced IRS-1 Ser 307 Phosphorylation. C2C12 myotubes were stimulated by 0.5 μM TPA for 30 min and then treated with eight polyphenols, including EGCG, resveratrol, rutin, quercetin, myricetin, curcumin, tomatine, and apigenin. The results show that TPA-induced IRS-1 Ser307 phosphorylation was significantly inhibited by EGCG and curcumin after 2 h of incubation (Figure 1B), suggesting that these two polyphenols may have therapeutic potential of FFA-induced insulin resistance.

Effects of EGCG and Curcumin on TPA-Induced IRS-1 Signaling Down-Regulation. Insulin-dependent activation of Akt, a major component of the insulin receptor signaling cascade, was assessed by western immunobloting with phospho-specific antibodies. C2C12 myotubes were stimulated by 0.5 μM TPA for 30 min and then treated with EGCG or curcumin in different doses. The result showed a decreased content of phospho-IRS-1 Ser307 and an increase in Akt phosphorylation only in a high concentration of EGCG (40 μM) treatment (Figure 2A), whereas curcumin has a dose-dependent manner

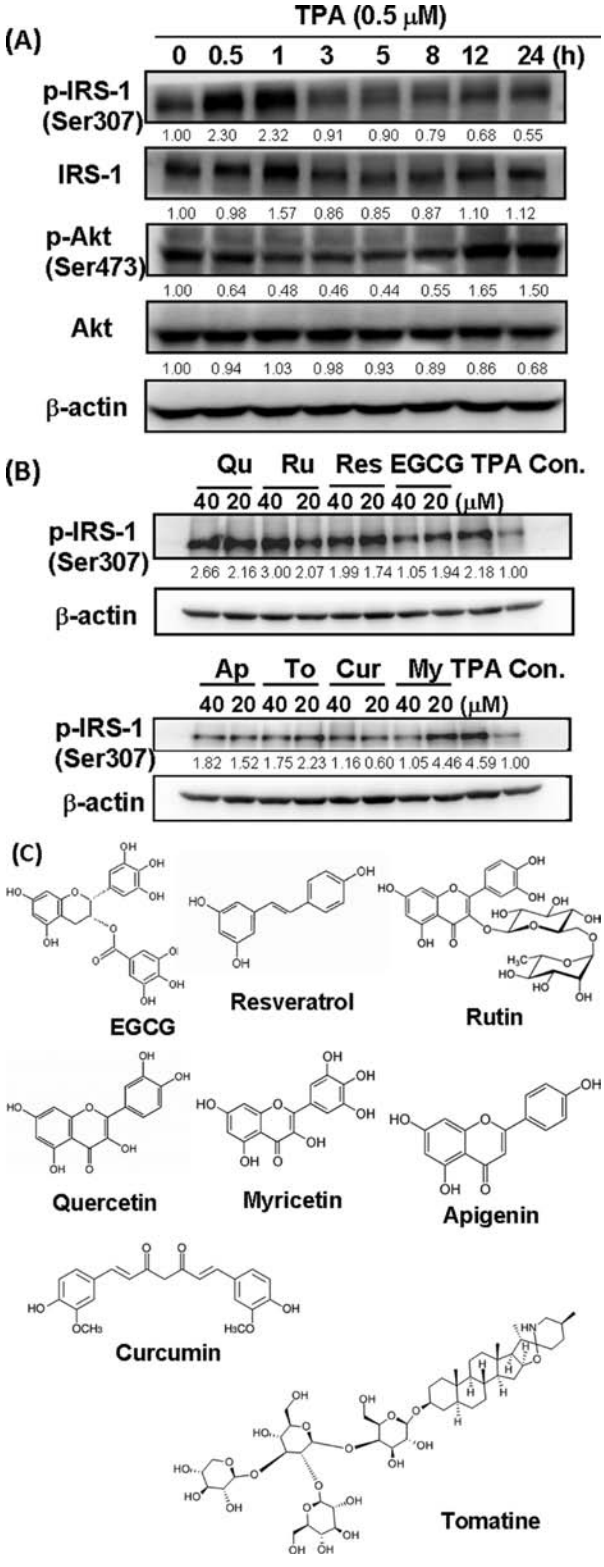

Figure 1. Effects of eight polyphenols on TPA-induced IRS-1 Ser307 phosphorylation. (A) C2C12 myotubes were incubated with TPA (0.5 μM) for the indicated time and stimulated with insulin (100 nM) for the last 10 min of each time period. (B) C2C12 myotubes were stimulated with TPA (0.5 μM) for 30 min and then treated with various polyphenols for 2 h with the indicated concentration, and the cell lysates were then subjected to Western blot analysis. The abbreviations of polyphenols are as follows: Res, resveratrol; Ru, rutin; Qu, quercetin; My, myricetin; Cur, curcumin; To, tomatine; and Ap, apigenin. (C) Chemical structures of phytopolyphenols.

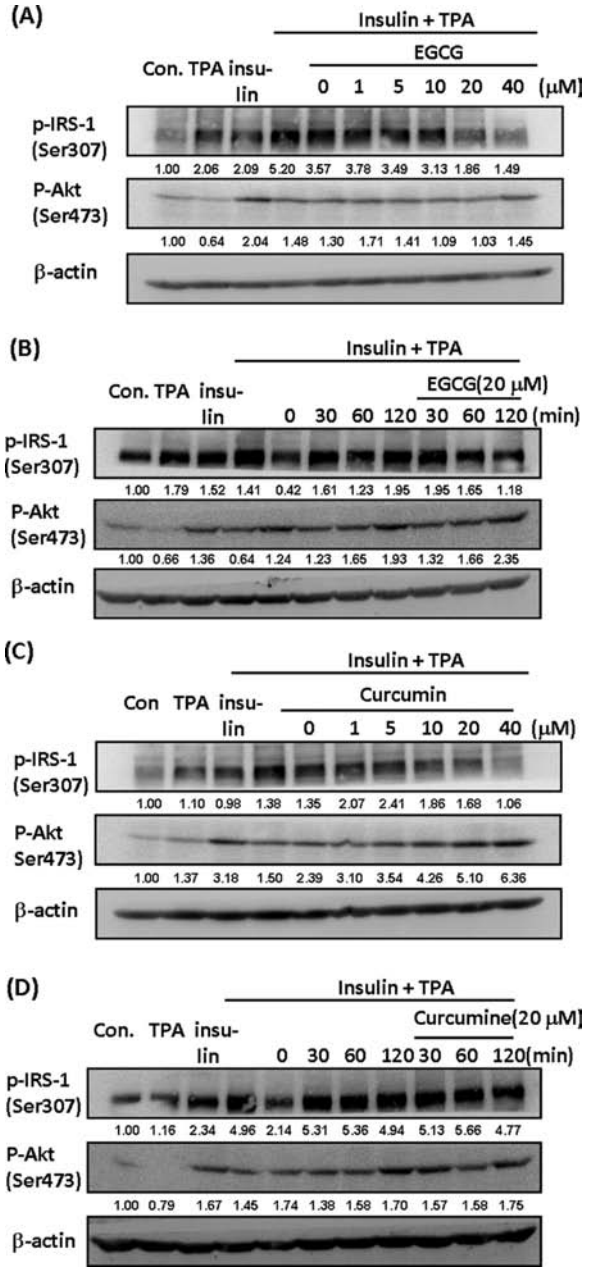

Figure 2. Effects of EGCG and curcumin on TPA-induced IRS-1 signaling down-regulation. C2C12 myotubes were stimulated with TPA (0.5 μM) for 30 min and then (A, C) treated with EGCG or curcumin with indicated concentrations for 2 h or (B, D) treated with 20 μM EGCG or curcumin for indicated times, in the presence or absence of insulin (100 nM) incubation for 10 min. The cell lysates were then subjected to Western blot analysis.

of Akt activation (Figure 2C). Cells were stimulated by TPA and treated with EGCG or curcumin for the indicated time period; both of them can inhibit IRS-1 serine phosphorylation and increase Akt activation in a time-dependent manner (Figure 2B,D).

Time-Dependent of Palmitate-Induced IRS-1 Signaling Down-Regulation. Chronically elevated FFA levels in plasma have been linked to induced insulin resistance. Lipid metabolites such as diacylglycerol (DAG) and ceramide accumulation play a crucial role in leading to insulin resistance by increasing IRS-1 Ser307 phosphorylation to block the insulin signaling cascade. To understand the role of IRS-1 in the mechanism of FFA-induced insulin resistance, IRS-1 protein abundance and

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serine307 phosphorylation were monitored in the FFA-treated cells in a time−course study. C2C12 myotubes were treated with 0.75 mM palmitate for the indicated time prior to stimulation with 100 nM insulin for 15 min. It is observed that the IRS-1 Ser307 phosphorylation was elevated in 4−12 h of incubation with palmitate. However, the AKT serine phosphorylation was increased at 4 h, and the signal was maintained up to 12 h before a drop at 14 h, similarly in its total protein level (Figure 3A). In states of insulin resistance, persistence with hyper-Ser/Thr phosphorylation of IRS-1 resulted in ubiquitiubiquitination and subsequent proteasomal degradation of IRS-1. Exposing C2C12 myotubes to palmitate for a longer time (24 h) clearly reduced IRS-1 protein levels, accompanying decreasing IRS-1 serine phosphorylation contents, suggesting that FFA induced IRS-1 degradation. Besides, palmitate incubation (24 h) also contributes to Akt inactivation (Figure 3A).

ECG Suppresses Palmitate-Induced IRS-1 Ser307 Phosphorylation. To investigate the effects of ECG on FFA-induced IRS-1 Ser307 phosphorylation, C2C12 myotubes were preincubated with 0.75 mM palmitate for 5 h to induce IRS-1 serine phosphorylation in the condition of the IRS protein and did not yet undergo proteasomal degradation. The result shows an up-regulation for the expression level of pSer307 IRS-1 after palmitate stimulation, and ECG treatment prevented this effect in a dose- and time-dependent manner (Figure 3B,C).

Effects of ECG on Palmitate-Induced Down-Regulation of Akt, MAPKs, and AMPK Signaling. Upon tyrosine phosphorylation, IRS-1 interacts with a signaling molecule, resulting in a diverse series of signaling pathways, including activation of PI3K and downstream MAP kinase cascade.[15] To understand whether ECG acts on promoting these two pathways, phospho-specific Akt or Erk1/2 was used for immunobloting after a 3 h treatment with ECG followed by palmitate preincubation for 5 h. The result shows that ECG coincubation enhanced the activation of Akt and Erk1/2 in a dose-dependent manner (Figure 3D).

The AMPK system acts as an energy sensor in all eukaryotic cells and plays a major role in the regulation of metabolic stress-induced glucose uptake.[16] AMPK activation has been associated with the activation of numerous kinases, including p38 MAPK,[17] which is essential for maximal stimulation of glucose uptake in response to insulin[18] and contractions.[19] It has reported that chronic overloading of muscle cells with palmitate led to a significant reduction in basal AMPK phosphorylation and activity.[20] To determine whether ECG affects the glucose uptake status in C2C12 myotubes, we further evaluated its effects on AMPK and p38 MAPK activation. Figure 3D shows that ECG treatment increased the phosphorylation of AMPK and p38 MAPK in a dose-dependent manner.

Effects of EGCG, Curcumin, and ECG on Palmitate-Induced Down-Regulation of IRS-1 Signaling. Increased Ser/Thr phosphorylation of IRS-1 promotes IRS-1 to undergo proteasome degradation.[21] To determine whether the polyphenols have the effects in C2C12 myotubes after long-time FFA incubation, C2C12 myotubes were incubated with 0.75 mM palmitate for 16 h and then treated with EGCG, ECG, or curcumin with an indicated concentration for 3 h. As shown in Figure 4A, incubation of muscle cells with palmitate in response to a dramatic reduction in the IRS-1 protein level was sustained and inhibited Akt activation, while a supplement of the polyphnols rescued the IRS-1 expression and enhanced the phosphorylation of Akt, especially curcumin and ECG.

The activation of novel PKC isoform, including PKCδ and PKCθ, has been reported and associated with FFA-induced insulin resistance.

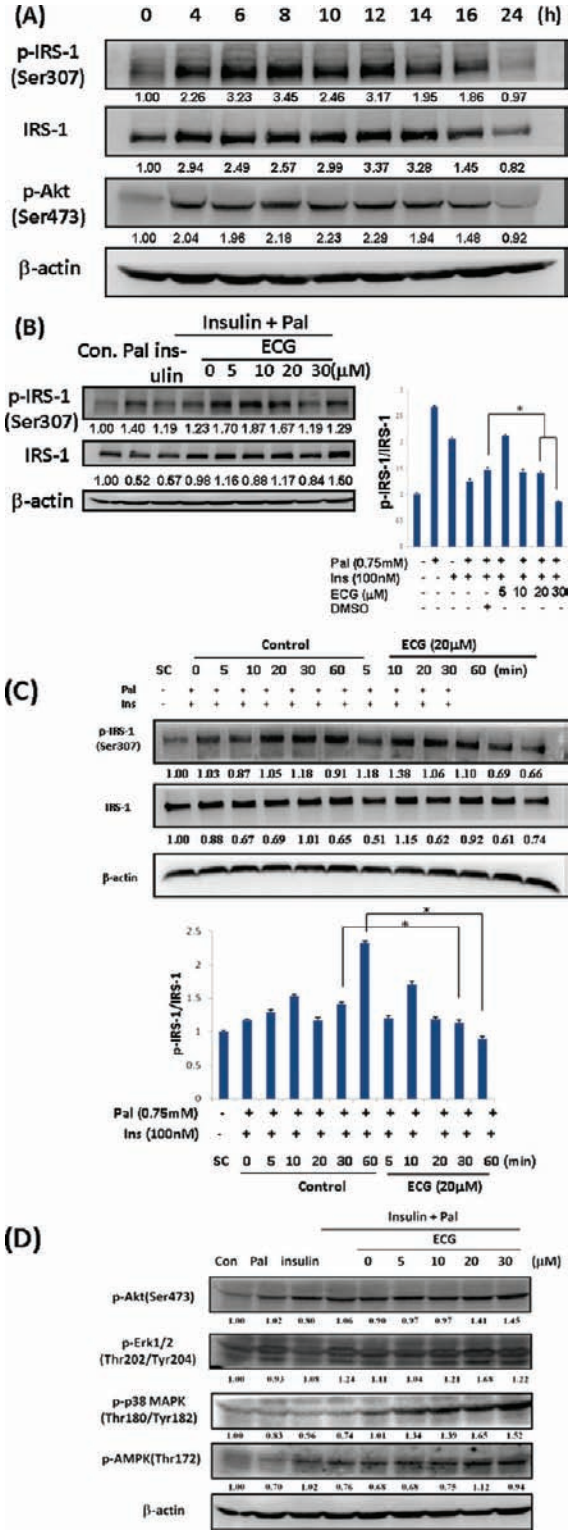

Figure 3. ECG suppressed palmitate-induced IRS-1 Ser307 phosphorylation. (A) C2C12 myotubes were exposed to 0.75 mM palmitate-containing medium for the indicated times and stimulated with insulin (100 nM) for the last 10 min of each time period. The cell lysates were then subjected to Western blot analysis. (B, D) C2C12 myotubes were preincubated in control medium with or without 0.75 mM palmitate for 5 h. After 2 h of serum-free starvation, cells were treated with ECG with various concentrations for 3 h or (C) treated with 20 μM ECG for the indicated times, in the presence or absence of insulin (100 nM) incubation for 10 min. SC, starvation control. *$P < 0.05$ for phyto-polyphenole vs palmitate.

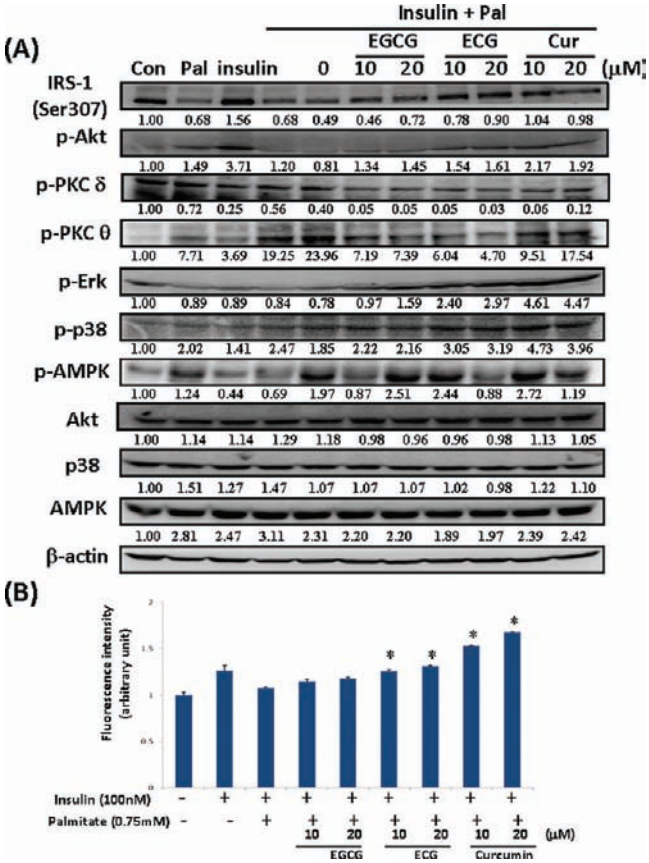

Figure 4. Effects of EGCG, ECG, and curcumin on palmitate-induced IRS-1 signaling down-regulation. (A) C2C12 myotubes were preincubated in control medium with or without 0.75 mM palmitate for 16 h. After 2 h of serum-free starvation, cells were treated with EGCG, ECG, and curcumin with indicated concentrations for 3 h in the presence or absence of 10 min of insulin (100 nM) stimulation. The cell lysates were then subjected to Western blot analysis. (B) 2-NBDG uptake in C2C12 myotubes. C2C12 myotubes were incubated with 0.75 mM palmitate for 16 h and then treatment with EGCG, ECG, and curcumin with indicated concentrations in the presence or absence of 15 min of insulin (100 nM) stimulation in the last time period. *$P < 0.05$ for phytopolyphenole vs palmitate.

The treatment with EGCG and ECG can significantly inhibit PKCδ and PKCθ activation, whereas the inhibition ability of curcumin seems to be weaker than the others (Figure 4A). To further validate the effects of the polyphenols on the MAPK pathway in long-time FFA incubation conditions, we measured the phosphorylation of ERK1/2 and p38 MAPK. The treatment with EGCG, ECG, or curcumin can increase the phosphorylation of ERK1/2 and p38 MAPK as compared with the palmitate-treated only group (Figure 4A).

To evaluate whether EGCG, ECG, and curcumin enhance glucose uptake in the state of FFA-induced insulin resistance, C2C12 myotubes were incubated with 0.75 mM palmitate for 16 h and then treated with polyphenols for 3 h. As shown in Figure 4B, cells were exposed to 2-NBDG for 20 min and then washed and displayed a significant rescue of fluorescence intensity after treatment with these three polyphenols, especially curcumin.

EGCG, ECG, and Curcumin Inhibit Lipid Accumulation. ACC is the downstream target of AMPK that plays an important role in lipid synthesis and catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, a building block for new fatty acids. After incubation with palmitate for 16 h, as shown in Figure 5A, treatment with EGCG, ECG, or curcumin

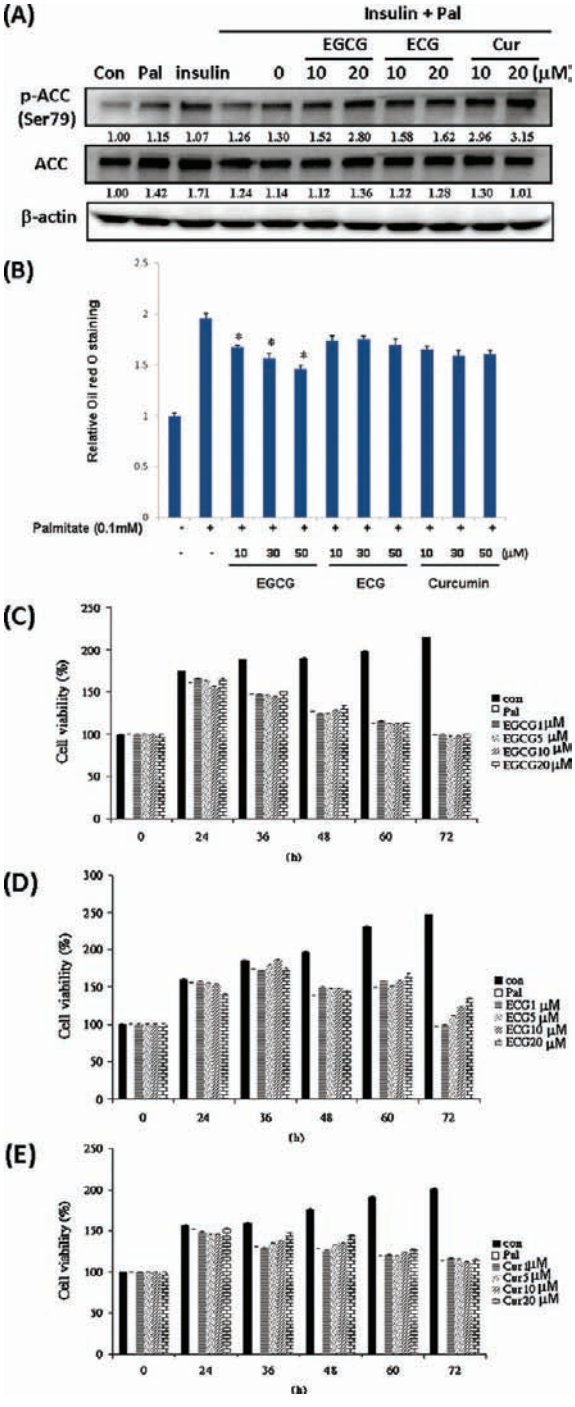

Figure 5. Effect of EGCG, ECG, and curcumin on cell viability in long-time palmitate incubation. (A) C2C12 myotubes were preincubated in control medium with or without the presence of 0.75 mM palmitate for 16 h. After 2 h of serum-free starvation, cells were treated with EGCG, ECG, or curcumin with indicated concentrations for 3 h with or without insulin (100 nM) stimulation for 10 min. The cell lysates were then subjected to Western blot analysis. (B) C2C12 myotubes were preincubated in control medium with or without 0.1 mM palmitate and in the presence or absence of EGCG, ECG, or curcumin with the indicated dose for 24 h and then subjected to oil red O staining. (C–E) C2C12 myotubes plating in 24-well were cultured in control medium with or without 0.25 mM palmitate for 72 h in the presence or absence of EGCG, ECG, or curcumin with various concentrations. The cell viability was measured by MTT assay. *$P < 0.05$, and $^{\#}P < 0.01$ for phytopolyphenole vs palmitate.

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for 3 h resulted in an increase in the ACC phosphorylation level, which suppresses its activity. To determine the intracellular lipid content, C2C12 myotubes were coincubated with 0.1 mM palmitate and EGCG, ECG, or curcumin, respectively, for 24 h and then analyzed by oil red O staining. As shown in Figure 5B, long-time incubation with FFA dramatically increased the intracellular lipid level; however, only treatment with EGCG significantly inhibited lipid accumulation in a dose-dependent manner.

Effect of EGCG, ECG, and Curcumin on Cell Viability in Long-Time Palmitate Incubation. A recent study showed that palmitate-induced insulin resistance and apoptosis were positively related in L6 skeletal muscle cells.[22] Chronically elevated FFA levels have numerous deleterious effects on mitochondria, including increased ROS production, mitochondria DNA (mtDNA) damage, and triggered mitochondrial dysfunction, which caused apoptosis in L6 skeletal muscle cells.[23] To investigate whether these three phytopolyphenol treatments can protect C2C12 myotubes from palmitate-induced apoptosis, we measured the cell viability by using the MTT assay. Briefly, cells were grown and differentiated in 24-well culture plates and treated with culture medium with or without 0.25 mM palmitate for the indicated time. Additionally, the FFA groups were incubated with varying concentrations of these three phytopolyphenols. EGCG can protect cells from FFA-induced apoptosis within 48 h, curcumin for 60 h, and ECG for 72 h. Coincubation with phytopolyphenol diminished the detrimental effects induced by FFA on skeletal muscle cells, and ECG has better efficiency to protect cells from apoptosis in a dose-dependent manner (Figure 5C–E).

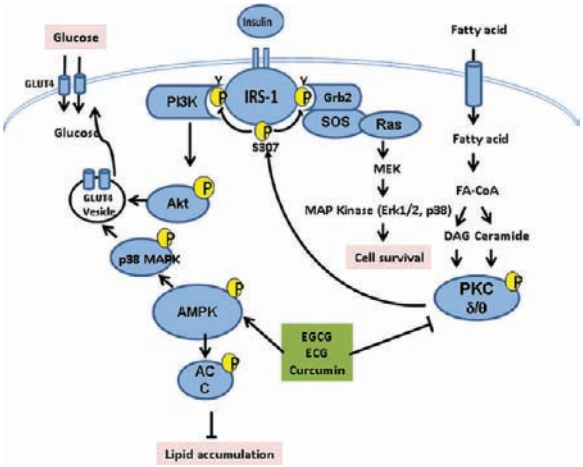

Figure 6. Schematic model for the potential mechanism of phytopolyphenols against palmitate-induced insulin resistance in C2C12 skeletal muscle cells. An increased FFA level induces serine kinase PKC activation, resulting in Ser307 phosphorylation of IRS-1 and inhibition of its Tyr phosphorylation, reducing the binding affinity of IRS-1 to IR. These results down-regulate the PI3K-Akt signaling pathway and reduce insulin-stimulated glucose uptake. Besides, FFA also suppresses the MAPK cascade, leading to cell apoptosis. In our results, ECG and curcumin are shown to inhibit PKC activation and enhance AMPK cascade to block IRS-1 serine phosphorylation, improve cell viability, and be against FFA-induced insulin resistance. However, EGCG suppresses lipid accumulation via the AMPK-ACC signaling pathway.

■ DISCUSSION

Diabetes is one of the most common noncommunicable diseases globally. It is generally believed that increased plasma FFA concentrations, a characteristic of obesity, are closely associated with type 2 diabetes mellitus. Of the tissues and organs that respond to insulin stimulation, skeletal muscle consumes more than 70% of the plasma glucose, suggesting that the whole body plasma glucose concentration is tightly associated with the sensitivity of muscle tissue to insulin.[24]

Serine phosphorylation of insulin receptor substrate proteins (IRSs) such as IRS1 has been strongly implicated as a mechanism of insulin resistance. TPA, a PKC activator, and the negative effects on insulin signaling have been well documented in cultured cells in vitro.[14,25] Ser307 of the phosphotyrosine-binding (PTB) domain at the C-terminal site of IRS-1 can interact with the insulin receptor (IR). However, Ser307 phosphorylation could induce a conformational change of the PTB domain that reduces its affinity with the IR. In our laboratory, after publication of our study[10] on the effect of the tea polyphenol EGCG in insulin resistance, we have carried out a series of studies on the effects of EGCG in insulin resistance in different cell systems such as rat pancreatic β cells[13] and C2C12 mouse skeletal muscle cells (present manuscript). In the present study, we examined the ability of TPA to induce IRS-1 Ser307 phosphorylation in a time–course experiment (Figure 1A). The data showed that TPA stimulates IRS-1 Ser307 phosphorylation and Akt inhibition only within 3 h. However, this effect was totally reversed after 12 h of incubation. Hence, we hypothesize the efficiency of TPA to attenuate insulin signaling just for a short time period. Thus, we investigated the effect of polyphenols on TPA-induced insulin resistance only for 2 h of incubation. The results showed that treatment with EGCG or curcumin inhibited the phosphorylation of IRS-1 Ser307 and increased Akt phosphorylation in a dose- and time-dependent manner (Figure 2); however, curcumin has stronger effects than EGCG.

It is well documented that lipids reduce insulin sensitivity and glucose uptake in skeletal muscle cells through increases in IRS-1 Ser phosphorylation.[24] The current study was performed[26] to investigate in the details of the mechanisms of FFA-induced insulin resistance using palmitate, one of the most predominant FFA in the circulation,[27] to elucidate the molecular mechanisms involved in the induction of insulin resistance in the C2C12 muscle model. In time-dependent exposure of C2C12 myotubes with palmitate experiments (Figure 3A), data showed that Akt phosphorylation was significantly reduced after 16 h of incubation, and IRS-1 Ser307 phosphorylation was rapidly induced by palmitate, but reduced at 16 h, and accounts for IRS-1 protein degradation. Accordingly, we examined the effects of polyphenols on short-time and long-time induction conditions, respectively. The results showed that in 5 h of preincubation with palmitate, ECG significantly suppressed palmitate-induced IRS-1 Ser307 phosphorylation (Figure 3B,C) and enhanced Akt activity (Figure 3D). The MAPKs signaling cascade is involved in the regulation of cellular responses, including cell proliferation, differentiation, cell growth, and apoptosis. The members of MAPKs, including Erk1/2 and p38 MAPK, have been reported to mediate the C2C12 skeletal muscle growth upon insulin stimulation.[28] Treatment with ECG could enhance Erk1/2 and p38 MAPK activation (Figure 3D), indicating that ECG has a potential role to promote cell survival. Besides, p38 MAPK has been reported to be also associated with AMPK activation involved in muscle contraction-induced glucose uptake.[17] The result showed that ECG increases AMPK phosphorylation in a dose-dependent manner (Figure 3D).

In the condition of 16 h of preincubation with palmitate, the effects of EGCG, ECG, and curcumin on FFA-induced

insulin resistance were compared with each other. PKC is Ser/Thr kinase, which leads to serine phosphorylation and reduces its protein-tyrosine kinase activity on IRS-1.[29] It has been reported that PKC and PKC were associated with lipid-induced insulin resistance.[30,31] The data showed that EGCG and ECG could significantly inhibit FFA-induced PKC and PKC phosphorylation, and curcumin was weaker than the others (Figure 4A), suggesting the possibility that these three polyphenols suppress IRS-1 Ser307 phosphorylation through inhibition of its upstream Ser/Thr kinase PKC activation. In addition, the results showed that curcumin has the best efficiency to reverse the IRS-1 protein expression level, increase Akt phosphorylation (Figure 4A), and enhance Erk1/2 and p38 MAPK activity (Figure 4A). Moreover, curcumin significantly enhanced glucose uptake in C2C12 skeletal muscle cells than the others (Figure 4B).

In this study, curcumin exhibits the best capacity to improve FFA-induced insulin resistance, andECG takes second place; however, EGCG showed the weakest therapeutic potential. In past years, many studies emphasized the importance of EGCG on antidiabetes[32,33] but did not mention ECG, another tea polyphenol abundant in pu-er tea.

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ABBREVIATIONS USED

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ECG, (−)-epicatechin gallate; EGCG, (−)-epigallocatechin-3-gallate; FFA, free fatty acid; GLUT4, glucose transporter 4; IRS-1, insulin receptor substrate-1; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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