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Research Article

Epigallocatechin gallate (EGCG) attenuates high glucose-induced insulin signaling blockade in human hepG2 hepatoma cells

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Insulin resistance is the primary characteristic of type 2 diabetes which as a result of insulin signaling defects. It has been suggested that the tea polyphenol (–)-epigallocatechin-3-gallate (EGCG) displays some antidiabetic effects, but the mechanism for EGCG insulin-enhancing effects is incompletely understood. In the present study, the investigations of EGCG on insulin signaling are performed in insulin-responsive human HepG2 cells cotreated with high glucose. We found that the high glucose condition causes significant increasing Ser307 phosphorylation of insulin receptor substrate-1 (IRS-1), leading to reduce insulin-stimulated phosphorylation of Akt. As the results, the insulin metabolic effects of glycogen synthesis and glucose uptake are inhibited by high glucose. However, the treatment of EGCG improves insulin-stimulated downsignaling by reducing IRS-1 Ser307 phosphorylation. Furthermore, we also demonstrated these EGCG effects are essential depends on the 5′-AMP-activated protein kinase (AMPK) activation. Together, our data suggest a putative link between high glucose and insulin resistance in HepG2 cells, and the EGCG treatment attenuates insulin signaling blockade by reducing IRS-1 Ser307 phosphorylation through the AMPK activation pathway.

Keywords: Akt / AMP-activated protein kinase / (–)-Epigallocatechin-3-gallate / Insulin / Insulin receptor substrate-1

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1 Introduction

High levels of circulating glucose, or hyperglycemia, is a serious problem with type 2 diabetes. Once chronic hyperglycemia becomes apparent, it in turn damages insulin target tissues and aggravates insulin resistance, forming a vicious circle that is collectively called glucotoxicity [1]. Among these pathological effects, one of the causes of hyperglycemia is the inability hepatic control of glucose homeostasis [2]. The impaired ability of insulin to trigger liver downstream metabolic actions, defined as hepatic insulin resistance. In addition to affecting glucose levels, hepatic insulin resistance also leads to dysregulated lipid synthesis, which can lead to hepatic steatosis and further systemic insulin resistance [3]. Recently studies have found that the lacking functions of insulin receptor substrate (IRS), a family of docking molecules connecting insulin receptor (IR) activation to essential downstream kinase cascades, may be the key molecular lesion signature of hepatic insulin resistance [4]. This defect appears to be a result of insulin-stimulated IRS-1 tyrosine phosphorylation resulting in reduced IRS-1-associated phosphatidyl inositol 3 kinase (PI3K) activities [5]. Furthermore, previous result demonstrates that hepatic IRS-1 and IRS-2 have complementary roles in hepatic metabolism control; particularly, IRS-1 is more closely related to glucose homeostasis [6].

Tea (*Camellia sinensis*) is one of the most popular beverages in the world. Of our interest, many folk remedies such as traditional Chinese medicine have mentioned the antidiabetic properties of tea for diabetes management [7]. Epidemiological study has shown a strong correlation between the consumption of tea and the prevention of diabetes [8]. Previous studies also indicated that *in vivo* insulin resist-

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Abbreviations: AMPK, 5′-AMP-activated protein kinase; **EGCG,** (–)-epigallocatechin-3-gallate; **GSK-3β,** glycogen synthase kinase-3β; **IR,** insulin receptor; **IRS,** insulin receptor substrate; **JNK,** c-Jun N-terminal kinase; **PI3K,** phosphatidyl inositol 3 kinase; **PKC,** protein kinase C

ance can be alleviated by the tea supplementation [9]. In particular, the bioactivities of (–)-epigallocatechin-3-gallate (EGCG), the major polyphenol isolated from green tea, have been investigated intensively in several studies [10]. It has been demonstrated that EGCG displays some antidiabetic activities in tea. For example, EGCG was verified to be the predominant active compound in green tea and have insulin-enhancing activity [11]. Dietary supplementation with EGCG markedly enhances glucose tolerance in diabetic rodents [12]. In addition, EGCG mimics insulin actions by inducing PI3K-sensitive phosphorylation of transcription factor FOXO1a (Forkhead box O1a) which is sensitive to scavengers of free radicals [13]. EGCG also regulates genes encoding gluconeogenesis enzymes, after that suppresses hepatic glucose production [14]. Recent data show that EGCG is effective in suppressing hepatic gluconeogenesis by activating 5′-AMP-activated protein kinase (AMPK) [15]. Finally, orally supplementation with EGCG in diabetic rodents demonstrates that EGCG beneficially modifies glucose and lipid metabolism in hepatocytes [16].

The above-listed observations reveal that EGCG indeed regulates glucose homeostasis and displays some antidiabetic benefits. Although EGCG is effective for antidiabetic activities in animal models, its effectiveness of human diabetes remains to be established. It has been shown that human HepG2 hepatoma cells are a suitable cell model in insulin signaling investigation [17]. In the present study, we used HepG2 cells to investigate the effects of EGCG on insulin signaling under high glucose condition. Our results indicated that the phosphorylation of IRS-1 at Ser307 was induced by high glucose, and subsequently repressed hepatic glucose utilization through suppressing Akt downsignaling. However, the supplementation of EGCG alleviated this insulin signaling blockade by improving the function of IRS-1 molecules. Moreover, our study showed that these EGCG improving effects depend on cellular AMPK phosphorylation, establishing a new molecular mechanism for antidiabetic activities of tea.

2 Materials and methods

2.1 Materials

EGCG, D-glucose, compound C (the AMPK specific inhibitor), and human recombinant insulin were provided from Sigma (München, Germany). [1-^{14}C]2-deoxy-D-glucose was supplied by Amersham Pharmacia Biotech (Braunschweig, Germany).The antibodies to β-actin, glycogen synthase kinase-3β (GSK-3β), pGSK3β (Ser9), and siRNA duplexes against AMPK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-pTyr, anti-IRS-1, anti-pIRS-1 (Ser307) antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). The anti-Akt and anti-pAkt (Ser473) antibodies were purchased from Transduction Laboratory (Lexington, KY, USA).

2.2 Cell culture

HepG2 cells were maintained in DMEM, supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (Invitrogen, CA, USA), and kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were grown upon reaching 70% confluence and then preincubated in serum-free medium for 24 h before treatments.

2.3 Immumoprecipitation

The total cell lysate was centrifuged at 12 000 rpm for 20 min and the aliquot of supernatant (500 μg total protein) was then incubated with antibodies (5 μL) against IRS-1 in immunoprecipitation buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris pH 7.4, 2 mM EDTA, 2 mM EGTA pH 8.0, 0.4 mM sodium ortho-vanadate, 0.4 mM PMSF, and 1% NP-40) and gently rocked overnight at 4°C. The immunocomplexes were adsorbed to protein A/G beads for 2 h at 4°C during gentle agitation and subsequently collected by centrifugation at 14 000 rpm for 30 s at 4°C. Beads were then washed three times with ice-cold PBS, incubated for 10 min at 95°C with 20 μL electrophoresis buffer, and the complete supernatant was used for Western blot analysis.

2.4 Western blot analysis

Cells were cotreated with indicated concentrations of EGCG and/or D-glucose for 24 h. At the end point of cotreatment, cells were chased by 100 nM insulin for 10 min then harvested and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM PMSF, and 0.5 mM DTT) for 30 min at 4°C. Equal amounts of total cellular proteins (50 μg) were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Bucks, UK), and then probed with the indicated primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham Biosciences).

2.5 Glycogen synthesis assay

Glycogen synthesis was assessed by measuring D-[^{14}C]glucose incorporation into glycogen. Briefly, glycogen synthesis proceeded for 1 h by adding D-[^{14}C]glucose (2.0 μCi/mL) after treatments. Cells were washed and lyzed in 0.5 N NaOH. The radioactivity incorporated into the glycogen was measured by precipitating glycogen on E-31 paper (Whatman, NJ, USA) in 66% ethanol. The papers were washed with 66% ethanol three times over 1 h, dried and counted by scintillation. Data were routinely measured in

triplicate. The unlabeled glucose concentration was 5.5 mM.

2.6 Glucose uptake assay

Glucose uptake rate was assayed by adding radioactive glucose as a tracer to the culture medium. HepG2 cells were treated for 24 h and then washed twice with 37°C PBS buffer (pH 7.4). Then culture cells were either untreated or treated with insulin (100 nM) for 10 min, followed by the addition of [1-^{14}C]2-deoxy-D-glucose (2-[^{14}C]DOG, 0.1 μCi/well) and 5.5 mM glucose for an additional 10 min at 37°C. Cells were then washed three times with PBS and solubilized in 0.5 M NaOH and 0.1% SDS. Incorporation of 2-[^{14}C]DOG was measured by scintillation counting on a Beckman Scintillation counter.

2.7 Transient transfections

Hemagglutinin (HA)-tagged Akt kinase dead form mutant (residues 473, 308, and 179) in pCMV5 were kindly supplied by Dr. T. D. Way (China Medical University, Taiwan). HepG2 cells were transfected Lipofectamine (Invitrogen) following the manufacturer's instructions. In transient transfection assays, cells were collected 48 h after transfection.

2.8 RNAi suppression of AMPK

The AMPK siRNA gene silencer (human) dsRNA was obtained from Santa Cruz Biotechnology (sc-45312). HepG2 cells were transfected with dsRNAs using siRNA Transfection Reagent (Santa Cruz) and incubated for 6 h. They were then analyzed by immunoblot for AMPK expression after 24 h. Nontargeting scramble-siRNA directed against 5′-UAGCGACUAAACACAUCAAUU-3′ was the negative control. No mammalian mRNAs contained this sequence in the NCBI database.

2.9 Statistical analysis

Data are presented as means ± SE. Statistical significance was set at $p < 0.05$. Statistically significant differences were determined by the analysis of variance in SPSS statistical software (SPSS, IL, USA).

3 Results

3.1 Effects of high glucose on IRS-1 phosphorylation

It had been reported that the treatment of HepG2 cells with high glucose triggers a significant reduction of insulin downsignaling [18]. To identify the effect of high glucose on insulin-induced IRS-1 phosphorylation, HepG2 cells were exposed for 24 h to various concentrations of D-glucose following chased for 10 min with 100 nM insulin. On the noninsulin resistance state, IRS-1 is readily tyrosine-phosphorylated by IR upon stimulation with insulin; nevertheless, It was found that the involvement of IRS-1 serine phosphorylation in the desensitization of insulin by chronic high glucose treatment. Among several potential serine phosphorylation sites of IRS-1, Ser307 is a key regulatory site [19]. As shown in Fig. 1A, insulin-induced tyrosine phosphorylation of IRS-1 takes place normally under nor-

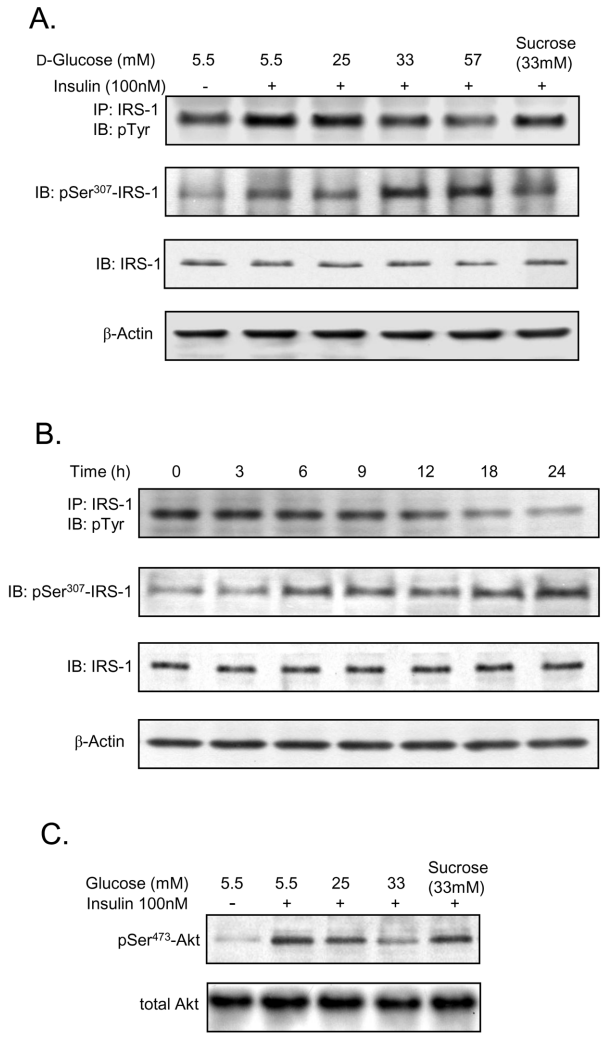

Figure 1. Dose-dependent and time course for high glucose-induced phosphorylation of IRS-1 in response to insulin stimulation. (A) HepG2 cells were incubated with increasing concentration of D-glucose for 24 h followed by treatment of insulin (100 nM) for 10 min. To rule out the possibility of osmotic effect, sucrose treatment (33 mM) was compared in parallel. (B) Decrease in IRS-1 tyrosine phosphorylation was detected in the cells exposed to 33 mM D-glucose for 24 h. (C) Akt phosphorylation levels at Ser473 under high glucose condition.

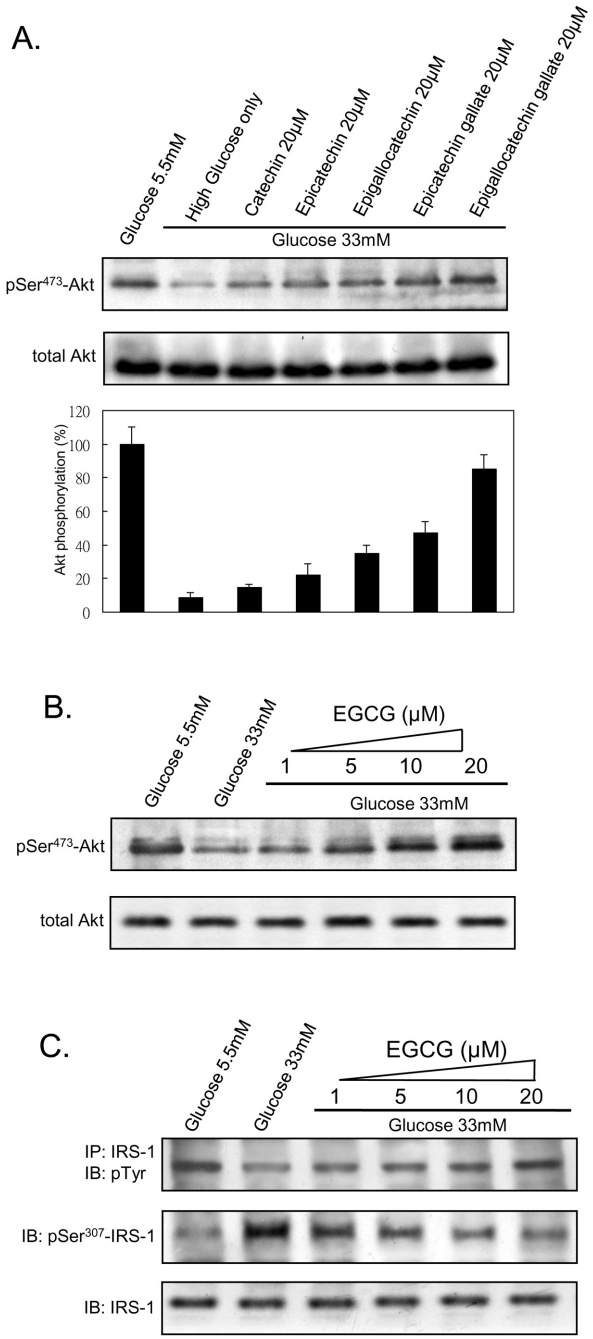

Figure 2. Consequences of insulin-stimulated Akt phosphorylation by EGCG treatments under high glucose condition. (A) HepG2 cells were treated with insulin (100 nM) and high glucose (33 mM) in combination with various tea polyphenols for 24 h, following chased by insulin stimulation (100 nM) for 10 min. (B) Dose-dependent effects of EGCG treatment on the phosphorylation of Akt cotreated with high glucose. (C) Decrease in IRS-1 Ser307 phosphorylation was detected in the cells exposed to increasing concentration of EGCG. Immunoblots were quantified using densitometry and reported as integrated optical densities in percentage compared to control groups.

mal concentration (5.5 mM) of glucose treatment. However, the insulin-stimulated IRS-1 tyrosine phosphorylation is significant decreased with 33 mM glucose for 24 h, and these glucotoxicity effects are clearly dose-dependent. Time courses of IRS-1 tyrosine phosphorylation by high glucose treatment are also analyzed in Fig. 1B, which tyrosine phosphorylation of IRS-1 were significant decreased within 12 h after exposure to 33 mM D-glucose, and these effects could be sustained up to 24 h. Besides, the total IRS-1 protein levels do not be significantly altered after high glucose treatment, indicating that the decreased tyrosine phosphorylation is not caused by the degradation of IRS-1.

Akt is the key molecular mediating the metabolic effects of insulin signaling. It lays downstream of PI3K and facilitates glucose uptake and glycogen synthesis in liver. To test the involvement of insulin-stimulated Akt, the cellular content of Akt (Ser473) phosphorylation proteins was evaluated in total cell lysates by Western blot analysis. As shown in Fig. 1C, the treatment of cells with 33 mM high glucose clearly blocked the insulin-induced Ser473 phosphorylation of Akt. This apparent decreased sensitivity to insulin of Akt phosphorylation is mostly consequent to the high glucose treatment with a dose-dependent manner. To rule out the possibility that the inhibition of Akt phosphorylation is due to increased medium osmolarity, the effects of sucrose and glucose on Akt phosphorylation were compared in parallel. It was shown that the 33 mM sucrose in the presence of 5.5 mM normal D-glucose did not affect the phosphorylation of Akt.

3.2 Effects of EGCG treatment on insulin-induced Akt phosphorylation under high glucose

To evaluate the insulin-enhancing effects of tea polyphenols, we used five of the major components present in green tea known as catechin, (–)-epigallocatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), and EGCG cotreating 33 mM D-glucose for 24 h, respectively. As indicated in Fig. 2A, the inhibition of Akt phosphorylation can be reversed by a treatment with some tea polyphenols, and EGCG is the most effective compounds on insulin-induced Akt Ser473 phosphorylation in a dose-dependent manner (Fig. 2B). We next determined whether the increase in insulin sensitivity by EGCG is due to the alternation of IRS-1 phosphorylation state. Figure 2C shows that the EGCG treatment recovers tyrosine phosphorylation of IRS-1 in a dose-dependent manner. Furthermore, cotreated EGCG also decreased Ser307 phosphorylation of IRS-1 in a dose-dependent manner. These data indicated that high glucose promotes the activation of serine phosphorylation and deactivation of tyrosine phosphorylation of IRS-1 in HepG2 cells, whereas EGCG reverses the state of IRS-1 phosphorylation and enhances insulin-stimulated Akt Ser473 phosphorylation.

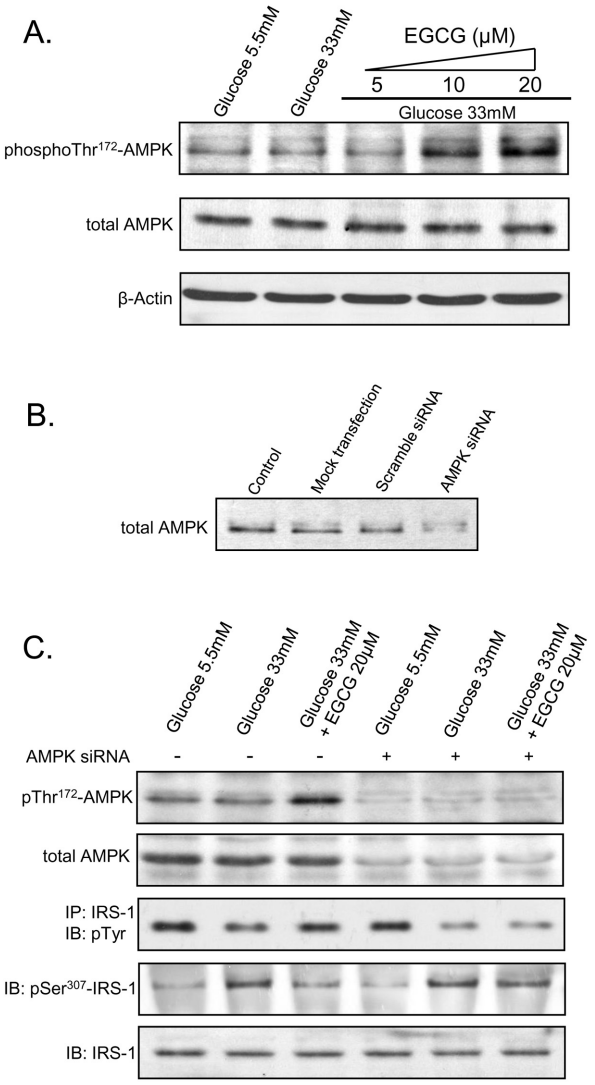

Figure 3. EGCG induces the AMPK phosphorylation in HepG2 cells under high glucose condition. (A) EGCG treatment increases AMPK Thr172 phosphorylation in a dose-dependent manner under high glucose. (B) Transfected HepG2 cells with AMPK-RNAi interfere with AMPK expression. (C) EGCG treatments show an inhibition effect of IRS-1 Ser307 phosphorylation in the presence of 33 mM glucose; however, this inhibitory effect is significantly blocked by the transfection of AMPK siRNA.

3.3 EGCG induce AMPK phosphorylation under high glucose condition

Our observations have suggested that the molecular target of EGCG may be placed on the upstream of Akt. To identify the dominant mediator of high glucose-induced hepatic insulin blockade, we next determined whether the EGCG-enhancing effects in insulin signaling is due to the altered phosphorylation state of IRS-1 by an upstream mediator, which is critical in insulin sensitivity. AMPK activation is thought to be a key proximal event in the cellular energy balance response, and AMPK phosphorylation levels in Thr172 are currently accepted as a marker of AMPK activity. AMPK has been proved to be required for antidiabetic effects of some clinical drugs in insulin-resistant human HepG2 cells [20]. To find the factor mediates IRS-1 phosphorylation by EGCG, we examined the phosphorylation of AMPK by cotreated EGCG and high glucose in HepG2 cells. As shown in Fig. 3A, HepG2 cells already have a basal level of phosphorylated AMPK under high glucose conditions; however, these levels increased by coincubated with EGCG in a dose-dependent manner.

It is known that the activated AMPK rapidly phosphorylates IRS-1 on Ser789 leads to increase in insulin-stimulated IRS-1-associated PI3K activity [21]. In order to further explore the effects with EGCG, we investigated whether the IRS-1 serine phosphorylation inhibition was related to AMPK phosphorylation. We used the suppression RNAi method to inhibit AMPK expression in HepG2 cells. As shown in Fig. 3B, transfected HepG2 cells with AMPK-RNAi interfered with AMPK protein expression in contrast to mock or scramble control groups. In parallel to the previous findings, decreasing levels of phosphorylated IRS-1 Ser307 was observed by EGCG treatments without RNAi transfection. Under RNAi transfection, although we added EGCG to RNAi-treated cells, IRS-1 Ser307 phosphorylation was still elevated (Fig. 3C). These results demonstrated that the activation of AMPK by EGCG treatment may be essential to EGCG protection effects under high glucose condition.

3.4 Insulin-stimulated metabolic effects by EGCG treatment depend on AMPK phosphorylation

Previous studies have established that insulin plays an important role in hepatic glycogen synthesis [22]. Because the enzyme GSK-3β plays a dominant role in facilitating hepatic glycogen synthesis, we next investigate whether the elevation of glycogen go through the regulation of GSK-3β activities by EGCG treatments. As shown in Fig. 4A, high glucose-reduced Ser9 phosphorylation of GSK-3β ablates the activity of glycogen synthase which carried out the glycogen synthesis in HepG2 cells. As expected, we observed that EGCG reverses the glycogen synthesis level by eliciting Ser9 phosphorylation of GSK-3β in a dose-dependent manner. To test whether EGCG reverses high glucose-induced inhibition of glycogen synthesis, we next performed a glycogen synthesis assay in HepG2 cells. In the presence of 33 mM glucose, we observed the glycogen synthesis significant downregulated in response to insulin stimulation. Nevertheless, dose-response studies show that EGCG elevates intracellular glycogen levels with a maximal increase over 10 μM (Fig. 4B). Previous studies had indicated that insulin treatment could directly promote HepG2 cells glucose uptake in evaluating hepatic insulin sensitivity [23]. In response to insulin, glucose uptake

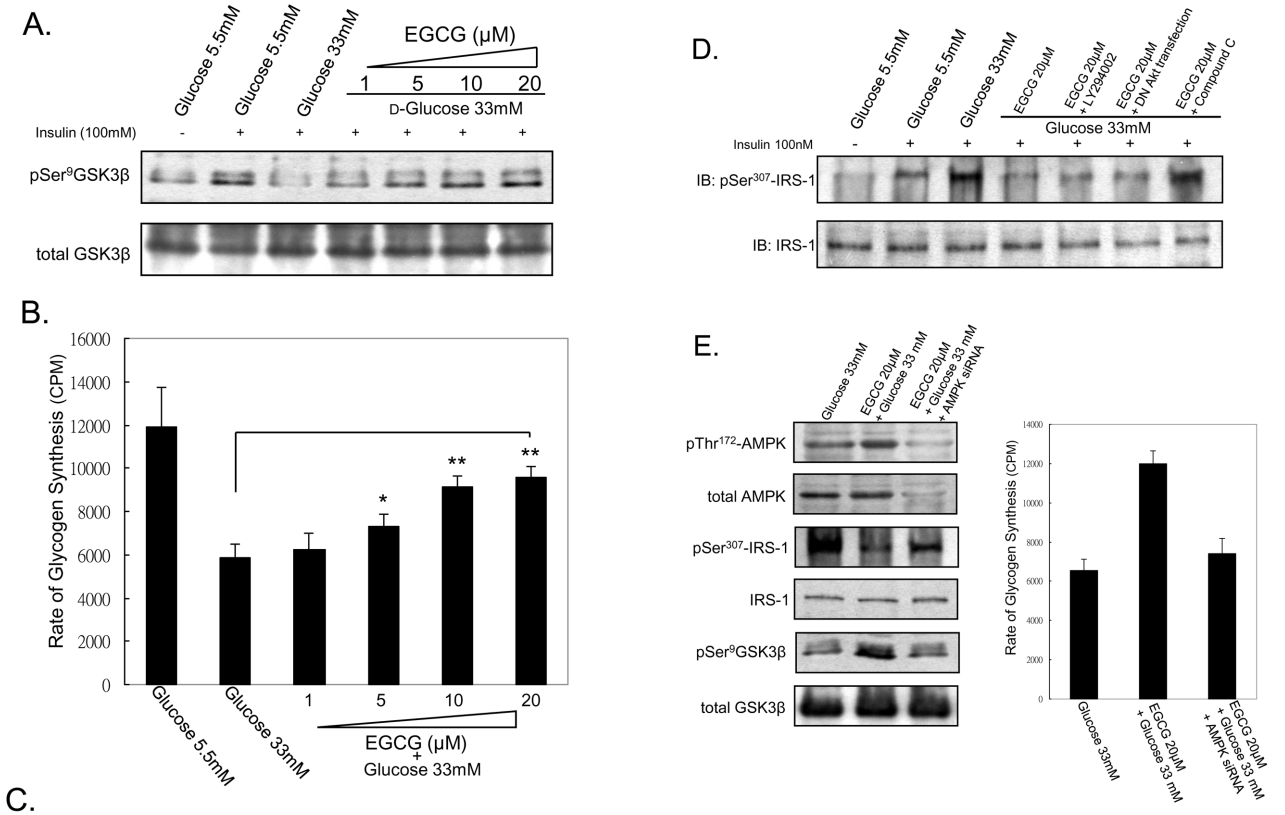

Figure 4. EGCG increases insulin-stimulated glycogen synthesis in HepG2 cells. (A) Ser9 phosphorylation of the GSK-3β in HepG2 cells. Increase in GSK-3β Ser9 phosphorylation was detected in the cells exposed to increasing concentration of EGCG. (B) The incorporation of [^{14}C]glucose into the cellular glycogen pools was allowed for 60 min and the radioactivity incorporated into the glycogen was measured in the glycogen precipitate. (C) HepG2 cells were treated with 2-[^{14}C]DOG for measuring the glucose uptake rate by a scintillation counter. (D) IRS-1 phosphorylation levels at Ser307 in HepG2 cells. The treatment of the HepG2 cells with the AMPK specific inhibitor compound C diminished EGCG effect on IRS-1 Ser307 phosphorylation. DN-Akt, Akt kinase dominant negative mutant. (E) Effects of EGCG treatment on the high glucose-induced Ser307 phosphorylation of IRS-1. Under high glucose condition, EGCG-stimulated glycogen synthesis was significantly blocked by the transfection of AMPK siRNA. Data are expressed as mean ± SE from three experiments. * and ** represents statistically significant difference from high glucose treated group, $p < 0.05$ and $p < 0.01$, respectively.

increased three-fold over basal levels (Fig. 4C). In contrast to the insulin-stimulated control group, the high glucose treatment in HepG2 cells inhibited insulin-stimulated glucose uptake by over 50%. As expected, EGCG-stimulated glucose uptake demonstrated in a dose-dependent manner under high glucose, with 20 μM stimulating glucose uptake by ~80% compared with the high glucose group.

Notably, we also observe that LY294002, a PI3K specific inhibitor inhibited the insulin-induced phosphorylation of Akt but tyrosine phosphorylation of IRS-1, suggesting that the EGCG action may go through upstream of IRS-PI3K-Akt signaling pathway. In Fig. 4D, the inhibitory effects of EGCG treatments to IRS-1 Ser307 phosphorylation can be blocked by the cotreatment of LY294002. Furthermore, transfection HepG2 cells with dominant negative Akt (DN Akt) also blocked EGCG improving effects under high glucose. It is well-known that insulin-induced Akt activation is directly elicited by PI3K-dependent mechanism and the effect of insulin can be blocked by LY294002, confirming that the action targets of EGCG should be placed upstream

of Akt molecules. Interestingly, the addition of compound C, an AMPK specific inhibitor, can diminish the EGCG-induced IRS-1 Ser307 phosphorylation inhibition. As the observations, it has raised the possibility that EGCG increases insulin sensitivity under high glucose-treated HepG2 might be the consequence depends on AMPK activation.

To investigate the role of AMPK on chronic high glucose treatment, we measure cellular insulin response including IRS-1 and GSK-3β phosphorylation by using AMPK siRNA method. As shown in Fig. 4E, the addition of EGCG marked elevates AMPK phosphorylation, and the insulin-induced metabolic effects are significantly disrupted in the AMPK siRNA treated group. Our results suggest that AMPK activation may play a primary role to mediate IRS-1 Ser307 phosphorylation, and illustrate how tea component EGCG can alleviate it in HepG2 cells under high glucose condition.

4 Discussion

Increased oxidative stress is associated with a variety of pathological conditions including diabetes, atherosclerosis, cardiovascular disease, and neurodegenerative disease [24]. In these diseases, oxidative stress is likely to play a causative role in the tissue and cellular damage. Particularly, diabetes is strongly combined with increased oxidative stress, which could be a consequence of either increased of free radicals or reduced antioxidant defenses [25]. Oxidative stress has been proved as a result of long-term high glucose and mediates various diabetes complications [26]. Emerging evidences are in favor of the concept that oxidative stress plays an important role in the development and progression of insulin resistance [27]. Defects in several insulin signaling pathways, such as reduced IRS tyrosine phosphorylation and decreased PI3K activity, are reported in human and several animal models of diabetes [5]. However, EGCG has previously been shown to display some anitdiabetic properties. For instance, the intake of green tea extract ameliorates fructose-induced insulin resistance [28]. Besides, EGCG also has been reported to lower glucose production and decreases the expression of genes that control gluconeogenesis [29]. These results indicate that EGCG indeed has insulin-potentiating activity; however, a clear molecular mechanism underlying these effects has not been established. In this study, we demonstrate that the exposure to high glucose results in Ser307 phosphorylation of IRS-1. It is consistent with the previous study which reported that the serine phosphorylation in IRS-1 accompanied selective inhibition of insulin-stimulated association of IRS-1 and p85 (a subunit of PI3K) by the way to reduce IRS-1 tyrosine phosphorylation [30]. Moreover, we also display that the EGCG treatments significantly reverse these high glucose effects. These effects of EGCG are all dose-dependent which likely through upregulation of IRS-1 activity. Finally, we further show that the blockade of AMPK activation prevents EGCG suppression of IRS-1 Ser307 phosphorylation. Therefore, EGCG appears to stimulation hepatic insulin sensitivity through a signaling pathway distinct from the insulin signaling itself. Here, our data provide a new molecular mechanism how the tea can alleviate insulin resistance under high glucose condition.

The search for the modulation of IRS activities has been suggested to focus on its phosphorylation state. Most notably, serine/threonine phosphorylation has been shown to modulate both positive and negative signaling transmission *via* IRS. Based on the fact, some high IRS-1 serine phosphorylation (*i.e.*, Ser307) leads to an insulin-signaling blockade by inhibiting insulin-induced IRS-1 tyrosine phosphorylation. This provides the indication that increasing Ser307 IRS-1 phosphorylation could affect insulin action. In keeping with this observation, it has been report that the serine phosphorylation of IRS-1 is critical to the development of insulin resistance [31]. In our results, we found that EGCG treatment significantly decreases high glucose-induced IRS-1 Ser307 phosphorylation. Previous studies have identified IRS-1 as a target of protein kinase C (PKC) and c-Jun N-terminal kinase (JNK), and high glucose also known to activate PKC and JNK [32, 33]. The activation of PKC acts to the Ser307 phosphorylation of IRS-1 and this event blocks downstream activation of Akt pathway. It is also well known that some clinical insulin-sensitized drugs like rosiglitazone reduce high glucose-induced toxicity by inhibiting PKC [34]. Moreover, it has also been reported that PKC and JNK activity is increased in insulin-sensitive tissues of diabetic patients [35]. This is in agreement with our previous observation that we found tea polyphenols could partially block PKC and JNK kinase activity directly *in vitro* [36, 37]. In addition, recent data show that ROS has been suggested as an upstream regulator of PKC under high glucose [38]. PKC inhibitors had been reported to prevent high glucose-induced serine phosphorylation of IRS-1 providing a strong suggestion of the involvement of PKC [39]. By this reason, we suggest that EGCG may reduce IRS-1 serine phosphorylation through inhibiting PKC and JNK activity.

Another interesting finding is that AMPK mediates the EGCG inhibition of IRS-1 serine phosphorylation. In this study we have confirmed that EGCG is indeed inhibitory to Ser307 phosphorylation in IRS-1. However, this effect could be abolished by inhibition of cellular AMPK. We further show that the blockade of AMPK activation prevents EGCG effects. Interestingly, EGCG has previously been shown to activate AMPK, and the activation of AMPK is associated with the EGCG-induced effects [34]. Other polyphenols including resveratrol, apigenin, and theaflavins have been shown in HepG2 cells and mouse liver to activate AMPK and consequently modulate cellular metabolism [40, 41]. AMPK can be activated by at least two known sig-

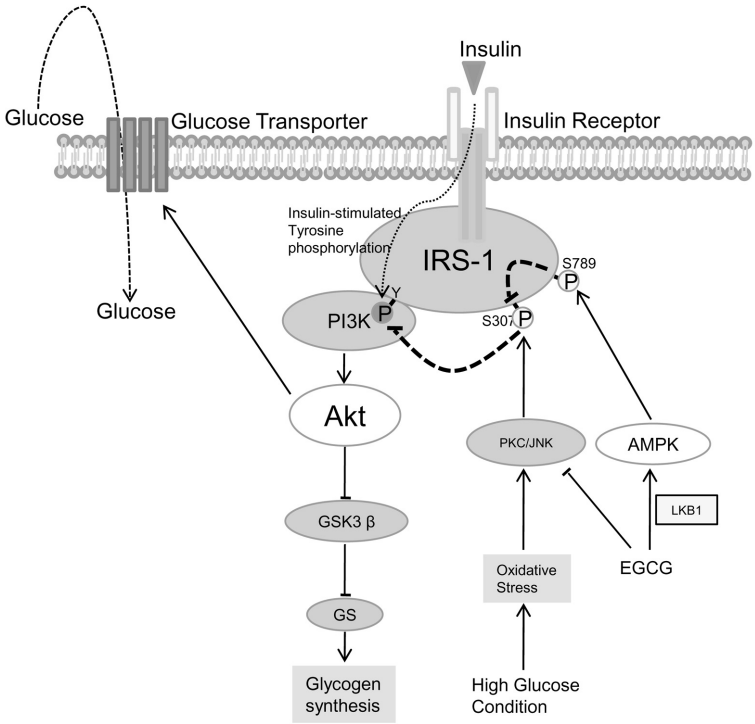

Figure 5. Potential mechanisms of high glucose-induced insulin signaling blockade in HepG2 cells. High glucose condition generates intracellular ROS which accumulated within HepG2 cells. The high glucose-induced oxidative stress triggers serine kinase cascades by activating PKC and JNK. This ultimately induces Ser307 phosphorylation of critical IRS-1 sites, reducing IRS-1 tyrosine phosphorylation, and thereby inhibiting IRS-1 binding to IR. These results downregulate PI3K-Akt signaling pathway and reduce insulin-stimulated glucose uptake and glycogen synthesis. However, IRS-1 tyrosine phosphorylation has been demonstrated to be positively regulated by AMPK activation. In our results, EGCG is shown to both reduce PKC/JNK activation and activate AMPK activity leading to block IRS-1 serine phosphorylation and attenuate high glucose-induced insulin signaling blockade.

naling pathways, LKB1 (also known as STK11, serine/threonine kinase 11) and CaMKK (calcium/calmodulin kinase kinase) [42]. In our previous study, we found that the blockade of LKB1 prevents tea polyphenols activation of AMPK [40]. Our results from this study show that tea polyphenol EGCG insulin-enhancing effects is required for the activation of AMPK and the consequent inhibition of IRS-1 serine phosphorylation (Fig. 5).

In summary, using HepG2 cells, we investigate how high glucose condition preferentially impairs insulin down-signaling and what the role EGCG plays to alleviate this insulin resistance state. These alterations provide an uptake for excess circulating glucose that would be cleared by maintaining glucose homeostasis and thereby protect individuals from glucotoxicity damages. We speculate that a similar mechanism may be operating in human body and this mechanism could explain why tea enhances insulin sensitivity. Although the understanding of high glucose-induced insulin resistance ultimately leads to metabolic disorders has advanced considerably in recent years, effective therapeutic strategies to prevent or to delay the development of this damage remain limited. Further experiments directed at the determining the mechanism of EGCG or other novel phytochemicals action may lead to the identification of molecular targets for the generation of therapeutic agents useful in the management of insulin resistance disease like diabetes.

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The authors have declared no conflict of interest.

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