Original Article

Epicatechin potentiation of glucose-stimulated insulin secretion in INS-1 cells is not dependent on its antioxidant activity

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Abstract

Epicatechin (EC) is a monomeric flavan-3-ol. We have previously demonstrated that glucose-intolerant rats fed flavan-3-ols exhibit improved pancreatic islet function corresponding with an increase in circulating EC-derived metabolites. Thus, we speculate that EC may act as a cellular signaling molecule *in vivo* to modulate insulin secretion. In this study we further examined the effects of different concentrations of EC on H₂O₂ or hyperglycemia-induced ROS production, as well as on saturated fatty acid (SFA)-impaired glucose-stimulated insulin secretion (GSIS) in INS-1 cell line *in vitro*. We showed that EC at a high concentration (30 μmol/L), but not a low concentration (0.3 μmol/L), significantly decreased H₂O₂ or hyperglycemia-induced ROS production in INS-1 cells. However, EC (0.3 μmol/L) significantly enhanced SFA-impaired GSIS in INS-1 cells. Addition of KN-93, a CaMKII inhibitor, blocked the effect of EC on insulin secretion and decreased CaMKII phosphorylation. Addition of GW1100, a GPR40 antagonist, significantly attenuated EC-enhanced GSIS, but only marginally affected CaMKII phosphorylation. These results demonstrate that EC at a physiological concentration promotes GSIS in SFA-impaired β-cells via activation of the CaMKII pathway and is consistent with its function as a GPR40 ligand. The findings support a role for EC as a cellular signaling molecule *in vivo* and further delineate the signaling pathways of EC in β-cells.

Keywords: epicatechin; flavonoids; hyperglycemia; oxidative stress; insulin secretion; CaMKII signaling pathway; GPR40; KN-93; GW1100

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Introduction

Epicatechin (EC) is a monomeric flavan-3-ol that belongs to a subgroup of flavonoids (Figure 1) and is a ubiquitous secondary metabolite in plants^[1]. Dietary EC mainly derives from tea and cocoa products, grains such as beans and peas, and fruits such as grapes, berries, and apples^[2]. *In vitro* studies demonstrate that EC can be taken up and metabolized in enterocytes and hepatocytes^[3,4]. Evidence from both animal and human studies shows that monomeric flavanols are extensively metabolized to glucuronides, sulfates, and O-methylated forms by phase II enzymes in the intestine and liver^[5–7]. Their metabolites are found in plasma and accumulate in organs such as the brain, liver, heart, intestine, and kidney^[8,9]. Therefore, EC likely affects some functions of these organs.

EC is well known for its antioxidant activity. Its half-maxi-

mal inhibitory concentration (IC₅₀) as a protective antioxidant is approximately 200–400 μmol/L in the intestine^[10]. However, the concentrations of accumulated EC *in vivo* are much lower: in plasma, EC metabolites peak at about 40 μmol/L, whereas in other tissues, their concentration is approximately 0.1–0.3 μmol/L^[8,9,11]. Therefore, whether EC acts as an antioxidant *in vivo* remains debatable.

Current evidence suggests that the interaction of EC with molecular targets could explain its bioactivities. EC may exert antioxidant effects indirectly by modulating redox enzymes. When rats are fed EC at a dose of 20 mg per kg of body weight per day for 8 weeks, oxidative stress in the heart induced by dietary fructose overload is prevented via increasing superoxide dismutase activity, decreasing superoxide anion production, and a reduced oxidized/reduced glutathione ratio^[12]. In tert-butylhydroperoxide (t-BOOH)-treated INS-1E beta-cells, 20 μmol/L of cocoa-derived EC prevents glutathione (GSH) depletion, recovers the activity of glutathione peroxidase and glutathione reductase, and reduces reactive oxygen species

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Figure 1. Structure of epicatechin (A) and catechin (B).

(ROS)^[13]. The Ca^{2+} /calmodulin-dependent protein kinase (CaMK) II pathway may also be modulated by EC. In the presence of Ca^{2+} , EC (1 $\mu\text{mol/L}$) induces the synthesis of nitric oxide (NO) through endothelial nitric oxide synthase (eNOS) activation via the CaMKII pathway in human coronary artery endothelial cells^[14]. Improved basal synaptic transmission and long-term potentiation in hippocampus slices are revealed after 0.3 $\mu\text{mol/L}$ EC metabolite treatment^[11]. The underlying mechanism is associated with the activation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) signaling via the CaMKII pathway^[11]. Furthermore, recent studies support the existence of an EC cell membrane receptor. Moreno-Ulloa *et al*^[15,16] observed cell responses in human coronary artery endothelial cells using a cell-membrane impermeable form of EC. In addition, Kado *et al*^[17] found that the hydroxyl or methoxyl moieties on the aromatic ring of flavonoids can potentially act on GPR40 to activate the CaMKII pathway during stimulation of glucagon-like peptide 1 (GLP-1) secretion in a murine GLUTag cell line. These findings indicate that EC with two hydroxyl moieties on the aromatic ring (Figure 1) may act on cell membrane receptors such as GPR40 to modulate cellular signaling pathways.

EC enhances glucose-stimulated insulin secretion (GSIS) from healthy β -cell lines and isolated islets^[18,19]. In oxidant-stressed β -cell lines, isolated islets, or animals, recovery of β -cell mass and function is observed in response to EC treatment^[13,20]. Nonetheless, the mechanisms of these phenomena are less understood. Although not yet reported, the concentrations of EC and its metabolites accessible to pancreatic β -cells are most likely similar to those of other organs (0.1–0.3 $\mu\text{mol/L}$)^[8,9,11]. Therefore, at these concentrations, EC and its metabolites may act as a cellular signaling modulatory compound in pancreatic β -cells. In addition, studies have suggested that CaMKII is a key Ca^{2+} sensor that controls Ca^{2+} homeostasis in pancreatic β -cells^[21]. Elevated intracellular Ca^{2+} activates CaMKII, thereby enhancing GSIS, whereas inhibition of CaMKII reduces GSIS^[21]. Considering the intracellular targets of EC found in other cell types, EC may modulate the Ca^{2+} /CaMKII pathway in pancreatic β -cells.

Previously, we demonstrated that glucose-intolerant rats fed oligomeric flavan-3-ols had improved pancreatic islet function as suggested by enhanced GSIS from isolated islets^[22]. Correspondingly, an increase in serum metabolites derived from fla-

van-3-ols was observed, including EC^[22]. Therefore, we speculate that EC modulates the CaMKII pathway in pancreatic β -cells to improve GSIS. In addition, existing evidence suggests that the EC concentration at the organ level is approximately 0.3 $\mu\text{mol/L}$ ^[11]. Therefore, we investigated the effects of EC at a physiological concentration (0.3 $\mu\text{mol/L}$) versus an antioxidant concentration (30 $\mu\text{mol/L}$) on INS-1 cells stressed by treatment with saturated fatty acid. We hypothesized that EC at the lower concentration can recover insulin secretion via the Ca^{2+} /CaMKII pathway by activating GPR40 in SFA-treated pancreatic β -cells.

Materials and methods

Cell culture

The rat insulinoma β -cell line INS-1 (clone 832/13) (a gift from Dr Peter LIGHT, Alberta Diabetes Institute, Canada) was maintained in a humidified incubator containing 5% CO_2 at 37°C and grown in Roswell Park Memorial Institute RPMI-1640 medium with 2 mmol/L *L*-glutamine (Gibco, Burlington, ON, Canada) supplemented with 10% FBS (Sigma, Oakville, ON, Canada), 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Fisher, NJ, USA), 1 mmol/L sodium pyruvate (Gibco, Burlington, ON, Canada), 50 $\mu\text{mol/L}$ β -mercaptoethanol (Sigma, Oakville, ON, Canada), and 1× antibiotic antimycotic solution (Sigma, Oakville, ON, Canada).

Measurement of EC modulation of glucose- or H_2O_2 -induced ROS production

Cellular ROS were quantified using the DCFDA Cellular ROS Detection Assay Kit (Abcam, Cambridge, MA, USA). INS-1 cells (5×10^5 cells per well) were seeded in 96-well clear-bottom black-side plates and allowed to grow until 70% confluent. The cells were then cultured for 24 h in medium containing vehicle, 0.3 $\mu\text{mol/L}$ *N*-acetyl cysteine (NAC, Sigma, Oakville, ON, Canada), or 0.3 $\mu\text{mol/L}$ epicatechin (EC; purity >98%; Sigma, Oakville, ON, Canada). For glucose-induced ROS, the medium was changed to Krebs–Ringer bicarbonate buffer (KRB: 116 mmol/L NaCl, 24 mmol/L NaHCO_3 , 4.4 mmol/L KCl, 1.5 mmol/L KH_2PO_4 , 1.2 mmol/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 mmol/L CaCl_2) supplemented with 10 mmol/L HEPES, 0.1% BSA, and 2.8 mmol/L glucose for a quiescent period of 30 min. Then, the cells were incubated in KRB-HEPES containing 2.8 or 16.5 mmol/L glucose, the latter with vehicle and 0.3

$\mu\text{mol/L}$ or $30 \mu\text{mol/L}$ EC or with $0.3 \mu\text{mol/L}$ or $30 \mu\text{mol/L}$ NAC as a positive control for 3 h. Next, the medium was removed, and $100 \mu\text{L}$ of $5 \mu\text{mol/L}$ 2',7'-dichlorofluorescein diacetate (DCFDA) in Hanks' Balanced salt solution (HBSS) without phenol red (Sigma, Oakville, ON, Canada) was added to the wells for 30 min at 37°C . After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF), which emits fluorescence. ROS generation was evaluated in a fluorescent microplate reader (Infinite 200 PRO Multimode Reader, Tecan Group Ltd, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The results were expressed in arbitrary units of fluorescence emission at 535 nm. Six or eight independent experiments were run in which each condition was tested in quadruplicates.

For hydrogen peroxide (H_2O_2), INS-1 cells were cultured as described above. The medium was then removed, and $100 \mu\text{L}$ of $5 \mu\text{mol/L}$ DCFDA in HBSS without phenol red (Sigma, Oakville, ON, Canada) was added to the wells for 30 min at 37°C . The antioxidant status of the cells was determined by adding $50 \mu\text{mol/L}$ H_2O_2 (Sigma, Oakville, ON, Canada) to each well and intracellular ROS were immediately measured in the microplate reader as described earlier. Six or seven independent experiments were run in which each condition was tested in quadruplicate.

Glucose-stimulated insulin secretion

INS-1 cells after 24-h treatments (as indicated below in Treatments 1, 2, and 3) were washed and incubated in KRB-HEPES with 0.1% BSA for a quiescent period of 90 min. Then, the cells were incubated in KRB-HEPES-BSA containing 2.8 or 16.5 mmol/L glucose for 90 min. Insulin secreted into the medium was evaluated by radioimmunoassay (RIA). Total cell insulin content was calculated by adding insulin secreted into the supernatant plus that remaining in the cells as determined by RIA. From this, the percentage of total insulin secreted was calculated for each data point to eliminate variance caused by cell number. Seven independent experiments were run in which each condition was tested in triplicate.

Treatment 1: RPMI, RPMI+0.4 mmol/L SFA, RPMI+0.4 mmol/L SFA+0.3 $\mu\text{mol/L}$ EC or NAC, RPMI+0.4 mmol/L SFA+30 $\mu\text{mol/L}$ EC or NAC;

Treatment 2: RPMI, RPMI+0.4 mmol/L SFA, RPMI+10 $\mu\text{mol/L}$ KN-93 (a CaMKII inhibitor; Sigma, Oakville, ON, Canada), RPMI+0.4 mmol/L SFA+0.3 $\mu\text{mol/L}$ EC or NAC, RPMI+0.4 mmol/L SFA+0.3 $\mu\text{mol/L}$ EC or NAC+10 $\mu\text{mol/L}$ KN-93;

Treatment 3: RPMI, RPMI+0.4 mmol/L SFA, RPMI+10 $\mu\text{mol/L}$ GW1100 (a GPR40 antagonist; Cayman Chemical, Ann Arbor, MI, USA), RPMI+0.4 mmol/L SFA+0.3 $\mu\text{mol/L}$ EC or NAC, RPMI+0.4 mmol/L SFA+0.3 $\mu\text{mol/L}$ EC or NAC+10 $\mu\text{mol/L}$ GW1100.

Stearic acid (SFA; Sigma, Oakville, ON, Canada) was dissolved in 75% ethanol at 50°C with 100 r/min shaking, and then added to RPMI medium with 5% BSA to prepare a

4 mmol/L stock. The stock was added to cell culture medium to yield a final concentration of 0.4 mmol/L. The vehicle control was RPMI+0.75% ethanol.

Western blot analysis

INS-1 cells collected after 24-h treatment 2 or 3 were lysed in a radioimmunoprecipitation assay buffer (150 mmol/L NaCl; 1.0% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; 50 mmol/L Tris, pH 8.0) with 2 $\mu\text{g/mL}$ aprotinin, 10 $\mu\text{L/mL}$ protease inhibitor cocktail, and 1 mmol/L NaF. All of the chemicals were obtained from Sigma (Oakville, ON, Canada). Total protein was measured using a Lowry protein assay (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA).

Protein samples (20 μg) were subjected to SDS-PAGE followed by blotting onto nitrocellulose membranes. The membranes were blocked in 3% BSA-0.1% Tween-Tris-buffered saline for 1 h followed by overnight incubation at 4°C with primary antibodies diluted 1:1000 in the blocking solution. Primary antibodies included anti- Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), antiphospho-CaMKII (p-CaMKII), anti-extracellular regulated kinases (ERKs), antiphospho-ERKs (p-ERKs), anti-protein kinase A (PKA), and antiphospho-PKA (p-PKA), all from Cell Signaling Technology (Danvers, MA, USA). The membranes were subsequently incubated with the appropriate HRP-conjugated secondary antibodies (1:5000; Sigma, Saint Louis, MO, USA) for 1 h at room temperature. Labeling of each phosphorylated protein was compared with its respective total protein. The membranes were developed using ECL Prime (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA) and digital images were captured using a ChemiDoc MPTM imaging system (Bio-Rad Laboratories, Inc., Mississauga, ON, Canada). Band intensity was quantified using Quantity One v4.6.2 (Bio-Rad Laboratories, Inc, Hercules, CA, USA).

Statistical analysis

The results are presented as the mean $\pm$ the standard error of the mean (SEM). ANOVA followed by Bonferroni's *post hoc* test, with the *P*-value adjusted for the number of comparisons, was used to assess differences between different conditions using GraphPad Prism 6 (GraphPad Software, Inc, San Diego, CA, USA). Nonparametric Kruskal-Wallis tests were used for data with non-normal distributions. $P<0.05$ was considered statistically significant.

Results

EC (30 $\mu\text{mol/L}$) protects against ROS in INS-1 cells

Incubation of INS-1 cells with high glucose for 3 h significantly increased ROS compared with cells treated in low glucose ($P<0.01$, Figure 2A). Treatment with 30 $\mu\text{mol/L}$ EC significantly reduced high glucose-induced ROS production to a level comparable to that with low glucose treatment ($P<0.001$ vs high glucose, Figure 2A). In contrast, 0.3 $\mu\text{mol/L}$ EC had no effect on ROS production induced by high glucose. Similar results were found when using the same concentrations of NAC to treat INS-1 cells: a concentration of 30 $\mu\text{mol/L}$ sig-

Figure 2. EC at 30 μmol/L protects against ROS in INS-1 cells. (A) INS-1 cells incubated for 24 h with or without EC or NAC were exposed to low or high glucose medium for 3 h in the continuous presence or absence of EC or NAC. The antioxidant status of the cells was then determined by measuring intracellular ROS accumulation after loading the cells with 5 μmol/L of the ROS dye DCFDA for 30 min. (B) INS-1 cells incubated for 24 h with or without EC or NAC were loaded with 5 μmol/L of the fluorescent ROS dye DCFDA for 30 min. The cells were then exposed to 50 μmol/L H₂O₂ to increase intracellular ROS for 2 min. The results were expressed in arbitrary units of fluorescence emission at 535 nm. Six to eight independent experiments were run in which each condition was tested in quadruplicate. The data were analyzed using one-way ANOVA with Bonferroni's multiple comparisons. **P*<0.05.

nificantly (*P*<0.01, Figure 2A) decreased ROS in high glucose-treated cells while 0.3 μmol/L showed no improvement.

We then tested whether EC treatment could reduce H₂O₂-induced ROS in INS-1 cells. H₂O₂ treatment for 10 min can lead to impaired insulin secretion in β-cells^[23]. Therefore, we measured ROS concentrations immediately (within 2 min) after exposing cells to H₂O₂. ROS production was significantly increased in the cells after short-term exposure to H₂O₂ compared with that in untreated cells (*P*<0.001, Figure 2B). Cells pretreated with 30 μmol/L EC had significantly lower levels of ROS (*P*<0.001, Figure 2B). Again, no significant reduction in H₂O₂-induced ROS was found in cells treated with 0.3 μmol/L EC. When using the antioxidant NAC at the same concen-

trations, 30 μmol/L NAC was effective in decreasing H₂O₂-induced ROS, whereas 0.3 μmol/L NAC was not.

EC (0.3 μmol/L) enhances insulin secretion from INS-1 cells treated with SFA

Long-term SFA exposure impairs insulin secretion from β-cells^[24]. In our study, 24-h incubation with 0.4 mmol/L SFA was used to impair β-cell function. After treatment, the mean insulin secretion in response to high glucose was reduced by 30% (*P*>0.05, Figure 3A). The addition of EC (0.3 μmol/L) in the culture medium significantly increased GSIS compared with that of SFA-treated cells (*P*<0.05, Figure 3A). In contrast, SFA-impaired insulin secretion was not increased by a high concentration of EC (30 μmol/L), remaining comparable to that of SFA-treated cells. EC alone did not stimulate insulin secretion as shown in cells incubated with 2.8 mmol/L glucose and treated with 0.3 μmol/L EC (Figure 3A). Thus, EC-enhanced insulin secretion is dependent on the presence of

Figure 3. EC (0.3 μmol/L) enhances insulin secretion from INS-1 cells treated with SFA. INS-1 cells were incubated for 24 h with or without EC in the continuous presence or absence of SFA. Then, the cells were exposed to low or high glucose medium for 90 min in the continuous presence or absence of EC after a quiescent period of 90 min. (A) Insulin secreted into the medium and remaining in the cells was measured by RIA to calculate the % release at each glucose concentration. (B) Total insulin synthesized was calculated as the sum of insulin secreted and remaining in the cells. Five independent experiments were run in which each condition was tested in triplicate. The data were analyzed using two-way ANOVA with Bonferroni's multiple comparisons. **P*<0.05.

high glucose stimulation.

The improved insulin secretion observed in 0.3 $\mu\text{mol/L}$ EC-treated cells could be caused by increases in either cell numbers or secretory capacity. However, neither SFA nor EC treatment had significant effects (Figure 3B, $P>0.05$) on the insulin content in these cells, suggesting that 0.3 $\mu\text{mol/L}$ EC reverses insulin secretion by modulating cell secretory capacity.

EC regulates insulin secretion via the CaMKII pathway

CaMKII is a Ca^{2+} sensor and regulator that promotes insulin secretion in pancreatic β -cells^[25]. EC has been shown to activate the CaMKII pathway in other cell types^[11,14,17]. Therefore, it may potentiate insulin secretion by the same mechanism. We tested this hypothesis by blocking CaMKII using KN-93 and measuring insulin secretion in the presence of 0.3 $\mu\text{mol/L}$ EC in INS-1 cells. As expected, EC-enhanced GSIS was attenuated by adding 10 $\mu\text{mol/L}$ KN-93, although statistical significance was not reached ($P=0.06$, Figure 4A). Insulin secretion from KN-93-treated normal cells occurred at a similar level to

that of SFA-treated cells (Figure 4A).

To further characterize the EC-induced activation of intracellular signaling pathways, we examined the extent of phosphorylation/activation of CaMKII, PKA, and ERK1/2 in INS-1 cells in the absence or presence of the inhibitor KN-93, which blocks the activation of CaMKII. Glucose at 16.5 mmol/L increased CaMKII phosphorylation (Figure 4B), while the addition of SFA to 16.5 mmol/L glucose decreased CaMKII phosphorylation to basal levels (Figure 4B, $P<0.05$). The addition of 0.3 $\mu\text{mol/L}$ EC reversed SFA-mediated inhibition of CaMKII phosphorylation ($P=0.05$). EC-induced phosphorylation of CaMKII was decreased to a level similar to that of non-stimulated cells by preincubating cells with 10 $\mu\text{mol/L}$ KN-93. In the same experiments, PKA phosphorylation was not affected (Figure 4C), but examination of the effects of CaMKII antagonism by KN-93 on downstream ERK1/2 phosphorylation revealed a similar pattern to that for CaMKII phosphorylation; however, no significant differences were found among the treatment groups (one-way ANOVA: $P=0.098$, Figure 4D).

Figure 4. EC regulates insulin secretion via the CaMKII pathway. INS-1 cells were incubated for 24 h with or without EC in the continuous presence or absence of SFA and KN-93. Then, the cells were exposed to low or high glucose medium in the continuous presence or absence of EC and KN-93 for 90 min after a quiescent period of 90 min. Insulin secreted into the medium was measured by RIA. The cells were lysed and quantified for total protein. Insulin release (A) was expressed as per μg of protein. The expression of target proteins was measured by western blotting. Phosphorylated CaMKII (B), PKA (C), and ERK (D) were normalized to the total density of each respective enzyme, which was normalized to GAPDH. Four independent experiments were run in which each condition was tested in triplicate. The data were analyzed using one-way ANOVA with Bonferroni's multiple comparisons (due to missing matched groups, two-way ANOVA cannot be performed for insulin release data. Therefore, insulin secretion at 16.5 mmol/L glucose was compared using one-way ANOVA). * $P<0.05$.

GPR40 is a potential cell membrane receptor of EC

We then examined whether GPR40 was involved in EC-mediated insulin secretion. The effect of the GPR40 antagonist GW1100 on EC-stimulated insulin secretion was tested (Figure 5A), which significantly decreased insulin secretion in EC-treated cells compared to that in cells treated without GW1100. CaMKII phosphorylation was affected by GW1100 treatment ($P<0.05$, Figure 5B). GW1100 partially but not significantly reduced CaMKII phosphorylation compared to that in EC-treated cells without GW1100 (Figure 5B).

Discussion

Many studies have investigated the effects of EC-rich foods or extracts on glucose homeostasis in humans and animal models. Although controversy remains, several studies have reported the effectiveness of EC in improving glucose handling and insulin sensitivity in diabetes^[19,20,26–29]. One theory regarding how EC exerts its beneficial effects suggests that EC interacts with intracellular molecules to modulate cell signaling pathways^[30]. In this study, we showed that a concentration (0.3 $\mu\text{mol/L}$) of EC attainable via dietary intake of flavonoid-rich foods such as PAC-containing pea seed coats could modulate insulin secretion from pancreatic β -cells^[22]. The results suggest that EC enhances insulin secretion by activating CaMKII in the presence of high glucose, possibly involving GPR40 activation.

Chronic elevation of free saturated fatty acids (FSFAs) in plasma plays a key role in the pathology of insulin resistance and type 2 diabetes^[31]. FSFAs also represent a crucial link between insulin resistance and β -cell dysfunction^[32]. *In vitro* studies have found that SFA at 0.25 mmol/L can blunt insulin secretion in response to glucose in isolated islets treated for 72 h^[33] and induce apoptosis in INS-1 cells and human islets after 24 h of exposure^[34]. Long-term exposure (≥ 48 h) of insulin-secreting β -cell lines to palmitic acid (0.4 mmol/L), a type of FSFA, leads to impaired GSIS from the cells^[24]. In this study, we used 0.4 mmol/L SFA to treat INS-1 cells for 24 h to impair insulin secretion in the cells. Our results showed a 30% reduction in the percentage of insulin secretion from the cells treated with SFA, whereas adding 0.3 $\mu\text{mol/L}$ EC normalized insulin secretion in SFA-treated cells.

CaMKII is a multifunctional serine/threonine kinase that plays an important role in regulating nutrient metabolism, gene expression, membrane excitability, the cell cycle, and neuronal communication^[35]. CaMKII is expressed in pancreatic β -cells and several β -cell lines, such as INS-1, MIN6, and HIT T5 cells^[36–38]. Insulin secretagogues induce elevated cytosolic Ca^{2+} , which activates CaMKII and leads to autophosphorylation of the kinase. The autophosphorylation of CaMKII is thought to be crucial in extending the activation period and maintaining activity in the absence of Ca^{2+} /calmodulin^[35]. Studies have found that CaMKII activation is closely correlated with insulin secretion^[39]. Overexpression of CaMKII in β -cells potentiates insulin secretion upon stimulation^[38], whereas inhibition of the kinase impairs Ca^{2+} entry and insulin secretion during glucose stimulation^[21,40]. Long-term

Figure 5. GPR40 is a potential cell membrane receptor of EC. INS-1 cells were incubated for 24 h with or without EC in the continuous presence or absence of SFA and GW1100. Then, the cells were exposed to low or high glucose medium in the continuous presence or absence of EC and GW1100 for 90 min after a quiescent period of 90 min. Insulin secreted into the medium was measured by RIA. The cells were lysed and quantified for total protein. Insulin release (A) was expressed as per μg of protein. The expression of target proteins was measured by Western blotting. Phosphorylated CaMKII (B) was normalized to the total density of CaMKII. Four independent experiments were run in which each condition was tested in triplicate. Insulin release data were analyzed using two-way ANOVA, and Western blotting data were tested by one-way ANOVA, with Bonferroni's multiple comparisons. * $P<0.05$.

palmitate exposure reportedly blunts the activation of CaMKII, which results in reduced glucose- or amino acid-induced insulin release^[40]. In our study, we also found decreases in GSIS that correlated with reduced CaMKII phosphorylation in SFA-treated cells. EC at 0.3 $\mu\text{mol/L}$ was able to reverse this reduction. CaMKII inhibitor KN-93 alone decreased insulin release and CaMKII phosphorylation to a similar level as SFA, and it totally blocked EC-mediated effects in SFA-treated cells. Therefore, our results suggest that EC may promote insulin secretion by reversing the inactivation of CaMKII by SFA.

In pancreatic β -cells, a glucose-induced increase in cytosolic Ca^{2+} activates CaMKII, and activation of CaMKII coincides with insulin secretion^[39]. In addition, PKA can be activated by glucose via increasing intracellular Ca^{2+} and cyclic adenosine monophosphate (cAMP), and PKA activity correlates closely with Ca^{2+} spikes^[41] and potentiates both acute and sustained insulin release^[42]. In this study, we failed to detect significant changes in PKA phosphorylation by EC treatment. It has been reported that in the hypothalamus, cAMP-activated PKA is essential in CaMKII signaling in long-term potentiation (LTP) by inhibiting protein phosphatase-1 (PP1), which specifically dephosphorylates CaMKII^[43–45]. Likewise, PKA may have a similar effect on CaMKII in pancreatic β -cells. As a result, EC treatment may specifically affect only CaMKII phosphorylation, but not PKA. Alternatively, KN-93 treatment may also have impacted CaMKIV activation, which regulates β -cell *Irs2* expression and GSIS^[46]. However, to our knowledge, the benefits of CaMKIV are not known to be dependent on prior stress due to saturated fatty acids as in the case of CaMKII^[40]. A schematic diagram illustrating the proposed central role of CaMKII in mediating EC effects is shown in Figure 6.

Our results are consistent with the hypothesis that GPR40 plays a role in EC effects on insulin secretion. The GPR40 antagonist GW1100 was able to block EC-enhanced GSIS in SFA-treated cells. However, CaMKII phosphorylation was not significantly reduced by GW1100, although a trend was observed. Further confirmation of whether CaMKII phos-

phorylation is modulated by GPR40 activation is required. The effect of EC on GPR40 is also likely related to its conformational structure (Figure 1). In addition to the presence of hydroxyl groups on the aromatic ring, Ramirez-Sanchez *et al* report that catechin, a stereoisomer of EC, fails to mimic the ability of EC to induce NO synthesis in human coronary artery endothelial cells, which is mediated by endothelial nitric oxide synthase (eNOS) activation via the CaMKII pathway^[14]. Furthermore, the substrates of CaMKII in β -cells include microtubule-associated protein 2 (MAP-2) and synapsin I^[25]. Therefore, it is conceivable that CaMKII may also play a role in the transport of insulin granules to exocytosis sites in β -cells. Whether EC treatment could affect MAP-2 and synapsin I via the CaMKII pathway to enhance insulin secretion is worthy of exploration. In addition, genetic knockdown of GPR40 in INS-1 cells will be of interest to further confirm GPR40's role in mediating the effect of EC.

Another interesting finding is the differences in the effects of EC on ROS reduction at low and high concentrations. We found that at 30 $\mu\text{mol/L}$, EC was effective in inhibiting ROS production induced by high glucose and acute exposure to H_2O_2 , which was similar to the action of equimolar NAC. Neither EC nor NAC at 0.3 $\mu\text{mol/L}$ reversed the increase in ROS. Pancreatic β -cells are highly sensitive to ROS mainly due to their low expression and activity of antioxidant enzymes^[47]. Glucose stimulation results in endogenous H_2O_2 accumulation in β -cells, which is thought to be necessary for

Figure 6. Schematic diagram of the possible pathways for EC-mediated insulin secretion in pancreatic β -cells. An increased cellular adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio primarily induced by oxidative phosphorylation closes ATP-sensitive K⁺ channels, which subsequently causes membrane depolarization and opening of voltage-dependent Ca²⁺ channels, leading to increased cytosolic [Ca²⁺]. Epicatechin (EC) can activate G-protein coupled receptor (GPR) 40 to further increase cytosolic [Ca²⁺]. The increased [Ca²⁺] activates Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), which serves as the triggering signal in glucose-induced insulin secretion and increases extracellular signal-regulated kinase (ERK) phosphorylation. The increased [Ca²⁺] also leads to activation of Ca²⁺-dependent adenylate cyclase, which increases the cyclic adenosine monophosphate (cAMP) level, leading to protein kinase A (PKA) activation. Activated PKA then prevents dephosphorylation of CaMKII by inhibiting protein phosphatase-1 (PP1), which specifically dephosphorylates CaMKII.

insulin secretion^[48], while excessive and/or sustained ROS production causes oxidative stress and leads to deterioration of β -cell function^[23,49]. When applying high glucose stimulation or H₂O₂ exposure in β -cells, intracellular ROS presumably increase only moderately above the physiological level. Therefore, it is possible that 30 μ mol/L EC is capable of neutralizing increased ROS in our experimental settings, even though the suggested concentration of EC as an antioxidant is at least 10 times higher than 30 μ mol/L^[10]. Indeed, Martín *et al* reported that 5–20 μ mol/L EC is effective in reducing ROS induced by 50 μ mol/L t-BOOH in INS-1E cells^[13]. Overall, with EC and NAC showing similar effects, we assume that the inhibitory effect on ROS production observed after high EC exposure is due to its direct antioxidant ability.

On the other hand, 30 μ mol/L EC was unsuccessful in enhancing SFA-impaired insulin secretion, which contradicts our initial hypothesis that 30 μ mol/L EC may reduce oxidative stress to promote insulin secretion from β -cells and suggests that EC may exert other effects in β -cells besides serving as an antioxidant. Possibly, EC inhibits protein phosphorylation at 30 μ mol/L, as suggested by Schroeter *et al*^[50]. Consequently, SFA-impaired CaMKII activation (phosphorylation) would not be rescued by 30 μ mol/L EC to improve GSIS.

EC treatment also promotes pancreatic β -cell regeneration in diabetic animal models *in vivo*^[20,51]. Martín *et al* found that EC from cocoa increases the viability of t-BOOH-treated INS-1E cells^[13]. In contrast to their findings, this study found that EC at 0.3 μ mol/L did not enhance the viability of INS-1 cells treated with 0.4 mmol/L SFA (data not shown). This is not surprising because compared with their cell model induced by limited 2-h 50 μ mol/L t-BOOH treatment, we applied SFA incubation for 24 h to impair cells, which has multiple deleterious effects, including elevated ROS, disrupted lipid metabolism, mitochondrial dysfunction, altered β -cell gene expression and signaling, impaired insulin secretion, upregulated inflammatory responses and apoptosis^[32,52]. In addition, we adopted a much lower but physiologically relevant concentration of EC at 0.3 μ mol/L, whereas their effective concentrations of EC ranged from 5–20 μ mol/L, which is 20–60-times higher. Most importantly, the improved GSIS reported by Martín *et al* could be due to increased cell numbers. In contrast, in this study, the insulin content was not changed by either SFA or EC treatment. Therefore, we suggest that 0.3 μ mol/L EC potentiated insulin secretion by modulating cellular signaling rather than enhancing cell viability.

In summary, our findings support that EC at a physiological concentration (0.3 μ mol/L) can act as a signaling molecule to stimulate insulin secretion via activation of CaMKII, possibly through the GPR40 receptor. EC at 30 μ mol/L has similar effects to those of NAC on ROS reduction, but with no effect on the promotion of insulin secretion. Due to its varied actions at different concentrations, application of EC as a nutraceutical would have to be performed with careful evaluation of the dose according to specific conditions. Meanwhile, further investigation is needed to characterize the signaling pathways of EC in β -cells.

Abbreviation

EC: epicatechin; CaMKII: calmodulin-dependent protein kinase II; GPR40: G-protein coupled receptor 40; SFA: saturated fatty acid; ROS: reactive oxygen species; GSIS: glucose-stimulated insulin secretion; t-BOOH: tert-butylhydroperoxide; cAMP: cyclic adenosine monophosphate; DCFDA: 2',7'-dichlorofluorescein diacetate; ERK: extracellular regulated kinases; PKA: protein kinase A; NO: nitric oxide.

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Author contribution

Catherine B CHAN and Kaiyuan YANG conceived and designed the study; Kaiyuan YANG performed the experiments, analyzed the data, and drafted the article; Kaiyuan YANG and Catherine B CHAN interpreted the data and revised and approved the final version to be published. Catherine B CHAN is the guarantor of this work, had full access to all the data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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