Mol. Cells *28*, 167-174, September 30, 2009
DOI/10.1007/s10059-009-0119-7

Molecules and Cells

Involvement of Ca^{2+}/Calmodulin Kinase II (CaMK II) in Genistein-Induced Potentiation of Leucine/Glutamine-Stimulated Insulin Secretion

Soo-Jin Lee, Hyo-Eun Kim, Sung-E Choi, Ha-Chul Shin, Won-Jae Kwag[1], Byung-Kyu Lee[1], Ki-Woong Cho[2], and Yup Kang*

Genistein has been reported to potentiate glucose-stimulated insulin secretion (GSIS). Inhibitory activity on tyrosine kinase or activation of protein kinase A (PKA) was shown to play a role in the genistein-induced potentiation effect on GSIS. The aim of the present study was to elucidate the mechanism of genistein-induced potentiation of insulin secretion. Genistein augmented insulin secretion in INS-1 cells stimulated by various energy-generating nutrients such as glucose, pyruvate, or leucine/glutamine (Leu/Gln), but not the secretion stimulated by depolarizing agents such as KCl and tolbutamide, or Ca^{2+} channel opener Bay K8644. Genistein at a concentration of 50 μM showed a maximum potentiation effect on Leu/Gln-stimulated insulin secretion, but this was not sufficient to inhibit the activity of tyrosine kinase. Inhibitor studies as well as immunoblotting analysis demonstrated that activation of PKA was little involved in genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. On the other hand, all the inhibitors of Ca^{2+}/calmodulin kinase II tested, significantly diminished genistein-induced potentiation. Genistein also elevated the levels of $[Ca^{2+}]_i$ and phospho-CaMK II. Furthermore, genistein augmented Leu/Gln-stimulated insulin secretion in CaMK II-overexpressing INS-1 cells. These data suggest that the activation of CaMK II played a role in genistein-induced potentiation of insulin secretion.

INTRODUCTION

For appropriate glucose-stimulated insulin secretion (GSIS) to occur in beta cells, all steps from glucose sensing to insulin granule exocytosis should be optimally processed. Glucose is first transported into beta cells and metabolized, resulting in increased level of ATP/ADP. Elevation of ATP/ADP levels closes K_{ATP} channels and elicits membrane depolarization. The change in membrane potential triggers the opening of voltage-dependent calcium channels (VDCC) and increases the influx of Ca^{2+}. The elevated $[Ca^{2+}]_i$ elicits insulin exocytosis through fusion between insulin granules and the plasma membrane. Thus, the Ca^{2+}-requiring exocytotic pathway is K_{ATP} channel-dependent and plays a major role in the first phase of insulin secretion (Bratanova-Tochkova et al., 2002; Henquin, 2000).

On the other hand, K_{ATP}-independent amplifying pathways are also required for the secretion of sufficient amounts of insulin in response to continuing exposure to glucose (Straub and Sharp, 2002). The amplification signals mainly operate at the level of granule mobilization from the cytosol to the plasma membrane and, thus, determine the second phase of insulin secretion. Although stimuli for amplifying pathways have not been completely understood, metabolic intermediates generated from the TCA cycle as well as long chain acyl-CoAs generated during the lipogenic pathway were thought to contribute to amplification of Ca^{2+}-mediated insulin exocytosis (Wiederkehr and Wollheim, 2006). Protein kinase A (PKA) and protein kinase C (PKC), which are activated by cAMP and diacylglycerol (DAG) respectively, and the Ca^{2+}-activated calcium/calmodulim kinase II (CaMK II) are known to play a role in amplification of insulin secretion (Nesher, 2002).

Genistein is a primary soy-derived phytoestrogen and thus acts as weak estrogens. Genistein is known to compete with 17-estadiol for binding to the intranuclear estrogen receptor protein (Kang et al., 2009; Markiewicz et al., 1993). Genistein was also shown to exert its biological effects by inhibiting the activity of several enzymes, including protein tyrosine kinase, DNA topoisomerase and S6 kinase (Akiyama et al., 1987; Linassier, 1990; Markovits et al., 1989). Earlier, several studies reported that genistein potentiated GSIS. As genistein inhibits tyrosine kinase, the inhibitory activity of tyrosine kinase was reported to play a role in the genistein-induced potentiation effect on insulin secretion (Jonas et al., 1995; Sorenson et al., 1994; Verspohl et al., 1995; Zawalich et al., 2002). In particular, since the insulin downstream signaling pathway is subject to tyrosine kinase activity of insulin receptors, the genistein-induced potentiation of insulin secretion was thought to result from attenuation of insulin-signaling pathways through the in-

Institute for Medical Science, Ajou University School of Medicine, Suwon 442-749, Korea, [1]Yuhan Pharmaceutical Research Center, Yongin 449-902, Korea, [2]Department of Ocean Microbe Engineering, Anyang University, Inchon 417-833, Korea
*Correspondence: kangy@ajou.ac.kr

Received April 16, 2009; revised July 27, 2009; accepted July 31, 2009; published online September 4, 2009

Keywords: calcium calmodulin kinase II (CaMK II), genistein, insulin, protein kinase A (PKA), tyrosine kinase

hibitory activity of tyrosine kinase on insulin receptors (Eto et al., 2002; Zawalich et al., 2002). On the other hand, protein kinase A was reported to be a critical signal mediator for genistein-induced potentiation of insulin secretion. Genistein was reported to be able to enhance the cAMP levels in various cells including beta cells through activation of adenylate cyclase or inhibition of phosphodiesterase (Liu et al., 2005; Ng et al., 2008; Ohno et al., 1993). Recently, Liu et al. (2006) reported that activation of PKA by physiological concentrations of genistein was the main contributor toward the potentiation effect on GSIS.

The present study was initiated to determine how genistein potentiated leucine/glutamine (Leu/Gln)-stimulated insulin secretion in INS-1 beta cells. Firstly, the potentiation effects of genistein on insulin secretion in INS-1 cells stimulated by Leu/Gln were investigated. Secondly, the role of the inhibitory activity of tyrosine kinase in genistein-induced potentiation was examined. Thirdly, the role of amplifying signals such as PKA, PKC and CaMK II in the genistein-induced potentiation of insulin secretion was examined using various pharmacological inhibitors. Lastly, to determine the role of CaMK II in genistein-induced potentiation, the potentiation effect of genistein on insulin secretion was tested in CaMK II-overexpressing INS-1 cells.

MATERIALS AND METHODS

Cells and culture

INS-1 rat insulinoma cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum (Life Technology INC., USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technology INC.). Islets were isolated from 10 week-old male rats using a collagenase digestion method. Briefly, after injecting 10 ml of collagenase P (0.75 mg/ml) into the bile ducts, swollen pancreases were excised and then incubated in a water bath at 37°C for 7 min. After stopping the enzymatic digestion with cold Hanks' balanced salt solution (HBSS), the tissues were disrupted by repetitive pipetting and subsequently passing them through a 400-μm mesh. Islets were separated by centrifugation on 25%, 23%, 21.5% and 11.5% ficoll gradients. Islets at the interface between the 21.5% and 11.5% fractions were collected and washed with HBSS. Healthy islets were hand-picked under a stereomicroscope. To obtain single beta cells, islets were treated with trypsin-EDTA (Invitrogen, USA) for 2 min and the dissociated islet cells obtained were then incubated in RPMI 1,640 medium containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

Reagents

Chemicals, i.e., glucose, leucine, glutamine, pyruvate genistein, diazoxide, H89, protein kinase A inhibitor 14-22 amide (PKI), GF109203X, Go6976, Go6983, KN93, KN62, W-7, tolbutamide, KCl, Bay K8633, phobol 12-myristate 13-acetate (PMA), forskolin, FLou-3/AM, and doxycycline were purchased from Sigma-Aldrich (USA) or Merck Bioscience (Germany). These chemicals were dissolved in either the appropriate medium solution or dimethyl sulfoxide (DMSO) and then treated at the required working dilution. Anti-phospho-IRS-1 (Y612) antibody was obtained from Biosource (Belgium). Anti-phospho-Akt (Thr473), phospho-(Ser/Thr) PKA substrate, phospho-(Ser) PKC substrate, and phospho-CaMK II (Thr286) antibodies were purchased from Cell Signaling Technology (USA). Anti-actin and anti-α-tubulin antibodies were obtained from Santa Cruz Biotechnology (USA).

Insulin RIA

To measure the insulin secreted in INS-1 cells, the cells were seeded in 24-well plates at a density of 2×10^5 cells per well, and cultured for 2 d. The cells were washed with KRB buffer (24 mM $NaHCO_2$, 1.2 mM $MgCl_2$, 1 mM HEPES, 129 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 0.2% bovine serum albumin, pH 7.4) and then incubated with the same KRB buffer for 1 hr. Insulin secretion was elicited by incubation of the cell with appropriate insulin secretagogues and genistein for 2 h. The concentration of insulin in the supernatant was determined with a rat insulin RIA kit (Linco Research, USA). Briefly, 100 μl supernatant and 100 μl ^{125}I-insulin were mixed and 100 μl rat insulin antibody solution was then added. After vortexing, the mixture was incubated for 1 d at 4°C. The precipitating reagent (1 ml) was then added and incubated for 20 min at 4°C. After centrifugation (2,000 × *g*, 20 min), the radioactivity of the precipitate was determined using a gamma-counter. The concentration of insulin in the samples was calculated by using a standard curve plotted from known concentrations.

Semi-quantitative RT-PCR

Expression levels of mRNAs were compared using the semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique. RT-PCR was carried out with Takara RNA PCR kit Ver 3.0 (Takara, Japan). In brief, cDNAs from INS-1 cells were synthesized with AMV reverse transcriptase and random 9-mers, and then subjected to PCR amplification with primer sets of CaMK II (5′-atggccaccacggtgacctgcac and 5′-agacattccacagtctcctgtc). The amplified DNA samples were analyzed using agarose gel electrophoresis. The relative quantities of amplified DNAs were compared on the basis of amplified cyclophilin DNAs.

Immunoblotting

Cells were suspended in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris.Cl, (pH 7.5), protease inhibitor cocktail (Roche Applied Science, Germany)], and were then incubated on ice for 15 min. Whole proteins were extracted by differential centrifugation (10,000 × *g*, 10 min). Protein concentrations were determined using protein assay kits (Bio-Rad, USA). An equal volume of 2× SDS sample buffer [125 mM Tris.Cl, (pH 6.8), 4% SDS, 4% 2-mercaptoethanol, 20% glycerol] was added to cell lysates. Equivalent amounts of protein (20 μg) were loaded onto 10-12% polyacrylamide gels, electrophoresed, and then electrophoretically transferred onto PVDF membranes (Millipore, USA). After these membranes had been blocked with 5% skim milk for 30 min, the target antigens were allowed to react with primary antibodies. After washing the membranes with phosphate buffered saline (PBS), these were incubated with secondary anti-rat IgG or anti-rabbit IgG antibodies conjugated to horseradish peroxidase. The immunoreactive bands were then detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, USA).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ levels were determined by the measurement of Fluo-3/AM fluorescence using a confocal laser scanning microscope (Zeiss, Germany). Dispersed INS-1 cells were plated onto glass bottom culture dishes (MatTeck Co., USA) at 37°C in 10% FBS-RPMI 1640 culture medium for 24 h. The cells were then loaded with Fluo-3/AM (5 μM) for 30 min at 37°C in Hank's balanced salt solution (HBSS; 145 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.4). After stimulation with Leu/Gln and genistein, the fluorescent images were captured (excitation: 488 nm and emission: 505 nm) and subsequently digitalized. The calcium intensities

Table 1. Potentiation effect of genistein on insulin secretion in INS-1 cell stimulated by various insulin secretagogues

Insulinotropic agents	Genistein	
	Untreated	Treated
Low glucose (0.2 mM)	0.55 ± 0.10	0.56 ± 0.09
High glucose (16.7 mM)	1.58 ± 0.15	3.45 ± 0.22*
Pyruvate (10 mM)	1.63 ± 0.33	3.46 ± 0.34*
Leucine (20 mM) + Glutamine (10 mM)	1.65 ± 0.38	2.93 ± 0.21*
KCl (30 mM) + Diazoxide (300 μM)	1.71 ± 0.13	1.69 ± 0.26
Bay K8644 (10 μM)	1.23 ± 0.30	1.12 ± 0.24
Tolbutamide (500 μM)	1.11 ± 0.06	1.11 ± 0.11

Insulin secretion was stimulated with 50 μM genistein for 2 h in the presence of various insulin secretagogues such as glucose, pyruvate, leucine/glutamine, KCl, Bay K8644, or tolbutamide. The insulin released into the medium was quantified by insulin RIA. *P < 0.05 vs. insulin release from INS-1 cells stimulated insulin secretagogues in the absence of genistein.

were calculated (DVC-1310, DVC Co., USA) and presented as percentage changes compared to the basal level (F/Fo).

Construction of CaMK II-expressing INS-1 cells (TRE- CaMK II) using the Tet-On system

INS-1 beta cell cDNAs were synthesized with AMV reverse transcriptase (Takara) using random 9-mers and then subjected to PCR amplification using rat CaMK II primers 5′-gcgtcgacatggccaccacggtgacctgcac and 5′-gctctagaaaccaggcgcagctctcactg. After the amplified DNAs were enzymatically cut with *Sal*I and *Xba*I, the CaMK II DNA fragments were subcloned to the corresponding sites of the pTRE2 vector (Clontech, USA) to construct pTRE-CaMK II. Nucleotide sequences of the rat CaMK II were confirmed by sequencing using the ABI BigDye sequencing kit (Applied Biosystems, USA). The INS-1 cells containing pTK-Hyg (Clontech) were transfected with the pTRE-CaMK II DNAs, and the stable clones harboring pTRE-CaMK II were then selected. Expression of CaMK II was induced by treatment with doxycycline.

Statistics

Data are presented as means ± SD of at least three independent experiments. Statistical significance was determined using a two-sample equal variance Student's *t*-test for assays with two samples sets and using one-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis for experiment with multiple experimental groups. P values of < 0.05 were considered to be statistically significant.

RESULTS

Genistein potentiates insulin secretion

The potentiation effect of genistein on GSIS was re-examined in INS-1 cells. INS-1 cells were treated with 50 μM genistein in the presence of 16.7 mM glucose for 2 h, and the released insulin was then measured by insulin RIA. Genistein potentiated GSIS by around 2.2 fold (Table 1), whereas genistein itself in the presence of a low concentration of glucose (0.2 mM) did not affect insulin secretion. To determine whether the genistein-induced potentiation effect on insulin secretion was pleiotropic, the potentiation of insulin secretion by genistein was investigated in INS-1 cells stimulated by various insulin secretagogues. As shown in Table 1, genistein augmented insulin secretion in INS-1 cells treated with energy-generating nutrients such as glucose, pyruvate, or a combination of leucine/glutamine (Leu/Gln). Genistein augmented pyruvate- or Leu/Gln-stimulated insulin secretion by around 1.8 or 2.1 fold, respectively. In contrast, genistein did not potentiate insulin secretion stimulated by depolarizing agent KCl, voltage-dependent Ca^{2+} channel (VDCC) opener Bay K8644, or K_{ATP} channel closure tolbutamide (Table 1). Since Leu/Gln increases insulin secretion through simple provision of carbons to the Krebs cycle, the potentiation mechanism by genistein was studied in Leu/Gln-treated INS-1 cells. The potentiation effect of genistein on Leu/Gln-stimulated insulin secretion was negligible below 5 μM but was highest at 50 μM and the effect began to decrease at 100 μM. Genistein did not potentiate Leu/Gln-stimulated insulin secretion at a concentration of 200 μM (Fig. 1A). Over a two-fold potentiation effect on Leu/Gln-stimulated insulin secretion was observed after a 2 hour-incubation of the cells in the presence of 50 μM genistein (Fig. 1B).

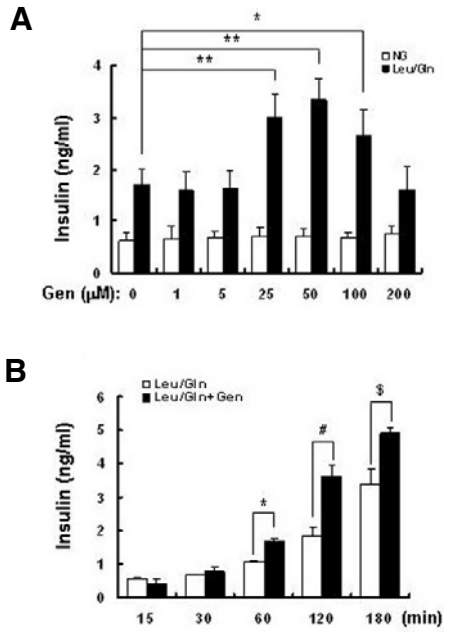

Fig. 1. Potentiation effect of genistein on Leu/Gln-stimulated insulin secretion in INS-1 cells. (A) Insulin secretion was stimulated with various concentration of genistein for 2 h in the presence of 0.2 mM glucose (NG) or 20 mM leucine and 10 mM glutamine (Leu/Gln). The insulin released into the medium was quantified by insulin RIA. *P < 0.05, **P < 0.01 vs. Leu/Gln-stimulated insulin release in genistein-untreated INS-1 cells. (B) INS-1 cells were incubated with 50 μM genistein (Gen) and Leu/Gln. Insulin released during the indicated amounts of time was quantified by insulin RIA. *P < 0.05 , #P < 0.05, and $P < 0.05 vs. Leu/Gln-stimulated insulin release in genistein-untreated INS-1 cells for 60, 120, and 240 min, respectively.

Role of inhibitory activity of genistein on tyrosine kinase in genistein-induced potentiation of insulin secretion

We investigated whether the inhibitory activity of genistein on tyrosine kinase and subsequent inhibition of insulin-downstream signaling pathway was involved in genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. We first examined the effect of daidzein, a genistein-analogue that does not have the inhibitory activity of tyrosine kinase, on Leu/Gln-stimulated insulin secretion. Daidzein did not significantly potentiate Leu/Gln-stimulated insulin secretion at all the

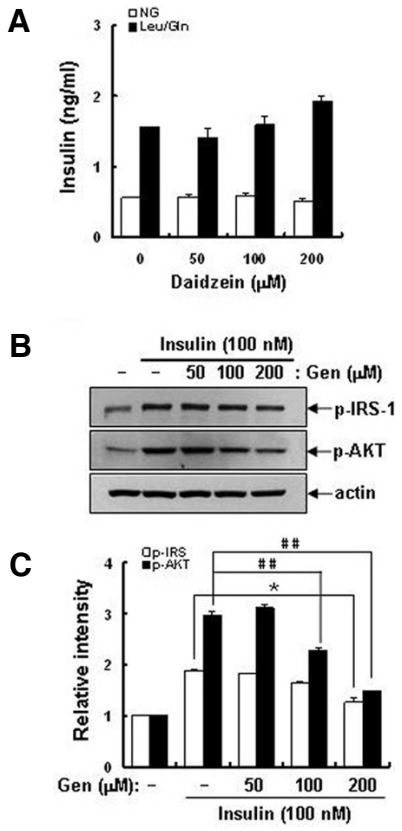

Fig. 2. Daidzein exerted little potentiation effect on Leu/Gln-stimulated insulin secretion and genistein did not reduce the levels of insulin-stimulated phospho-IRS-1 and phospho-Akt. (A) Insulin secretion was stimulated with various concentration of daidzein for 2 h in the presence of Leu/Gln. The insulin released into the medium was quantified by insulin RIA. (B) Levels of phospho-IRS-1 and phospho-Akt were analyzed by immunoblotting with anti-phospho-IRS-1 (Y612) and anti-phospho-AKT (Thr473) antibodies. After stimulation of INS-1 cells with 100 nM insulin in the presence of various concentrations of genistein (Gen) for 15min, the cell lysates were prepared with LIPA buffer and then subjected to SDS-PAGE and immunoblotting. *P < 0.05 vs. phospho-IRS-1 from insulin-stimulated cells. $^{\#\#}$P < 0.01 vs. phospho-Akt from insulin-stimulated cells.

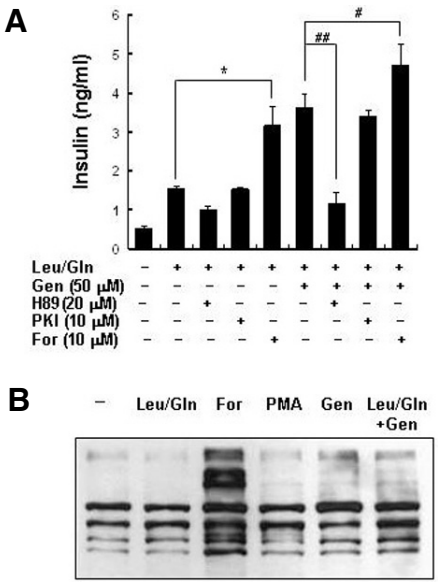

Fig. 3. Little involvement of PKA activation in genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. (A) Insulin secretion was stimulated with Leu/Gln and genistein (Gen) for 2 h in the presence of PKA inhibitor and activator, i.e, H89 (20 μM), PKA inhibitor 14-22 amide (PKI: 10 μM), or forskolin (For: 10 μM). The insulin released into the medium was quantified by insulin RIA. *P < 0.05, vs. Leu/Gln-stimulated insulin release in genistein-untreated INS-1 cells. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 vs. Leu/Gln-stimulated insulin release in genistein-treated INS-1 cells. (B) Levels of phospho-PKA substrates were analyzed by immunoblotting with anti-phospho-(Ser/Thr) PKA substrates antibodies. After stimulation of INS-1 cells with forskolin (For: 10 μM), phobol 12-myristate 13-acetate (PMA: 0.5 μM), or genistein (50 μM), the cell lysates were prepared with LIPA buffer and then subjected to SDS-PAGE and immunoblotting.

concentrations tested (Fig. 2A). This suggested that the inhibitory activity of genistein on tyrosine kinase might be critical for genistein-induced potentiation of insulin secretion. Although genistein showed maximum potentiation of Leu/Gln-stimulated insulin secretion at a concentration of 50 μM, it did not reduce the level of insulin-stimulated phospho-IRS-1 (Y612) and phospho-Akt (Thr473) at this concentration (Fig. 2B). However, at 200 μM, genistein significantly reduced the level of the insulin-stimulated phospho-IRS-1 and phospho-Akt. These findings demonstrated that the concentration of genistein with maximum potentiation effect on insulin secretion did not inhibit the insulin-downstream signaling pathway. These results suggest that the potentiation effect of genistein on insulin secretion was not due to its inhibitory activity on tyrosine kinase and subsequent inhibition of the insulin-downstream signaling pathway.

Role of activation of PKA in genistein-induced potentiation effect on insulin secretion

To determine whether the activation of PKA or PKC, typical kinases providing amplification signals for GSIS, was involved in genistein-stimulated potentiation of insulin secretion, the effects of inhibitors of PKA or PKC on genistein-induced potentiation were investigated. INS-1 cells were stimulated with Leu/Gln in conjunction with genistein and the amount of released insulin was then measured in the presence of various inhibitors of PKA and PKC. As shown in Fig. 3A, H89 (20 μM) as a PKA inhibitor significantly reduced the genistein-induced potentiation effect on Leu/Gln-stimulated insulin secretion (Fig. 3A). However, PKI, a PKA-specific peptide inhibitor, did not reduce Leu/Gln-stimulated insulin secretion and did not affect genistein-induced potentiation (Fig. 3A). This implied that PKA might not be involved in the genistein-induced potentiation of insulin secretion and that a side activity of H89, rather than inhibitory activity of PKA, might affect insulin secretion. On the other hand, 10 μM forskolin, a PKA activator, potentiated Leu/Gln-stimulated insulin secretion and further augmented genistein-induced potentiation (Fig. 3A). To determine whether 50 μM genistein induced PKA activation in INS-1 cells, the level of phosphorylated PKA substrates was assessed by immunoblotting of INS-1 cell extracts with anti-(Ser/Thr) PKA substrate-specific antibodies. As shown in Fig. 3B, 10 μM forskolin, the concentration exerting a similar potentiation effect on Leu/Gln-stimulated insulin secretion as compared to 50 μM genistein, increased the level of phospho-PKA substrates. However, genistein exerted very little effect on the increased level of phospho-PKA substrates. These findings indicated that the concen-

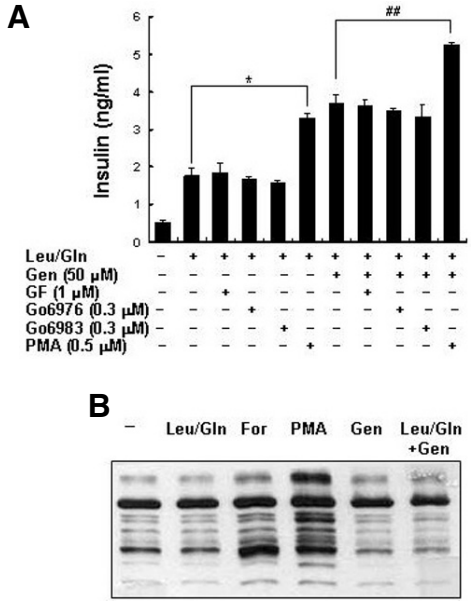

Fig. 4. Little involvement of PKC activation in genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. (A) Insulin secretion was stimulated with Leu/Gln and genistein (Gen) for 2 h in the presence of PKC inhibitor and activator, i.e, GF109203X (1 μM), Go6976 (0.3 μM), Go6983 (0.3 μM), or phobol 12-myristate 13-acetate (PMA: 0.5 μM). The insulin released into the medium was quantified by insulin RIA. *P < 0.05, vs. Leu/Gln-stimulated insulin release in genistein-untreated INS-1 cells. ##P < 0.01 vs. Leu/Gln-stimulated insulin release in genistein-treated INS-1 cells. (B) Levels of phospho-PKC substrates were analyzed by immunoblotting with anti-phospho-(Ser) PKC substrates antibodies. After stimulation of INS-1 cells with forskolin (For: 10 μM), phorbol 12-myristate 13-acetate (PMA: 0.5 μM), or genistein (50 μM), the cell lysates were prepared with LIPA buffer and then subjected to SDS-PAGE and immunoblotting.

tration of genistein with maximum potentiation effect on Leu/Gln-stimulated insulin secretion did not activate PKA in INS-1 cells. On the other hand, all chemical inhibitors of PKC, such as GF109203X, Go6976 and Go6983, did not inhibit genistein-induced potentiation of insulin secretion and 50 μM genistein did not increase the level of phospho-PKC substrates in INS-1 cells. However, 0.5 μM PMA, which showed a similar potentiation effect on Leu/Gln-stimulated insulin secretion, as did 50 μM genistein, increased the level of phospho-PKC substrates (Fig. 4B). These finding also suggest that PKC activation was not involved in the genistein-induced potentiation effect on Leu/Gln-stimulated insulin secretion.

Involvement of CaMK II activation in genistein-induced potentiation of insulin secretion

The effect of CaMK II inhibitors on genistein-induced potentiation was tested to determine whether activation of CaMK II was involved in this process. KN-93 and KN-62, pharmacological inhibitors of CaMK II, reduced Leu/Gln-stimulated insulin secretion and almost completely inhibited the genistein-induced potentiation effect (Fig. 5A). W-7, a calmodulin inhibitor, also significantly inhibited the genistein-induced potentiation effect (Fig. 5A). To determine whether genistein augmented Leu/Gln-stimulated insulin secretion and whether KN62 inhibited the genistein-induced potentiation effect in primary islet cells, the potentiation effect of genistein and inhibitory effect of KN62 was tested in Sprague Doley (SD) islet cells. As shown in Fig. 5B, KN62 inhibited the genistein-induced potentiation, while genistein potentiated Leu/Gln-stimulated insulin secretion. Immunoblotting studies with anti-phospho-CaMK II antibodies showed that the level of phospho-CaMK II was slightly increased in Leu/Gln-treated INS-1 cells and further increased in the presence of genistein (Fig. 5C). On the other hand, genistein in the presence of depolarizing agent KCl as an insulin secretagogue, which did not affect the genistein-induced potentiation effect, did not increase the level of phospho-CaMK II (Fig. 5C). To determine whether the increase in levels of phospho-CaMK II was due to an increase of $[Ca^{2+}]_i$, INS-1 cells were treated with genistein and the level of $[Ca^{2+}]_i$ was then quantified by measuring the change in the emitted fluorescence (excitation: 488 nm and emission: 505 nm) after loading with Fluo-3AM. As shown in Fig. 5D, treatment of the cells with glucose (16.7 mM) or Leu/Gln increased the level of $[Ca^{2+}]_i$. The co-treatment of cells with genistein further enhanced the glucose- or Leu/Gln-stimulated $[Ca^{2+}]_i$ increase. To support the role of CaMK II activation in genistein-induced potentiation of insulin secretion, CaMK II-overexpressing INS-1 cells were constructed and the potentiation effect of genistein on Leu/Gln-stimulated insulin secretion was then investigated. As shown in Fig. 6A, doxycycline increased the expression level of CaMK II in a concentration-dependent manner. In doxycycline-treated CaMK II-overexpressing INS-1 cells, the level of phospho-CaMK II was increased by Leu/Gln stimulation and further increased by genistein co-treatment (Fig. 6B). To determine whether genistein further potentiated the Leu/Gln-stimulated insulin secretion in CaMK II-overexpressing cells, CaMK II-expressing INS-1 cells were treated with doxycycline and the genistein-induced potentiation effect on insulin secretion was investigated. As shown in Fig. 6C, genistein further augmented the Leu/Gln-stimulated insulin secretion in doxycycline-treated CaMK II-overexpressing cells when compared to doxycycline-untreated cells (Fig. 6C). These findings suggest that CaMK II activation was involved in the genistein-induced potentiation effect on Leu/Gln-stimulated insulin secretion.

DISCUSSION

Genistein potentiated insulin secretion in INS-1 cells stimulated by Leu/Gln. Genistein at a concentration of 50 μM showed a maximum potentiation effect on Leu/Gln-stimulated insulin secretion but higher concentrations of this agent reduced this potentiation effect. At a concentration of 50 μM genistein did not reduce the level of insulin-stimulated phospho-IRS-1 and phospho-Akt, and did not increase the level of phospho-PKA substrates or phospho-PKC substrates in Leu/Gln-stimulated INS-1 cells. These findings suggest that the inhibitory activity of tyrosine kinase or activation of PKA or PKC was little involved in the genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. On the other hand, genistein (50 μM) increased the level of $[Ca^{2+}]_i$ and phospho-CaMK II, and further potentiated Leu/Gln-stimulated insulin secretion in CaMK II-overexpressing cells, suggesting that activation of CaMK II was involved in genistein-induced potentiation of Leu/Gln-stimulated insulin secretion.

While genistein potentiated insulin secretion in INS-1 cells stimulated by energy-generating nutrients such as glucose, pyruvate or Leu/Gln, it did not potentiate insulin secretion stimulated by membrane depolarizing agent KCl or tolbutamide, or the voltage-dependent Ca^{2+} channel opener, Bay K8644. This data coincides with a previous report showing that the gen-

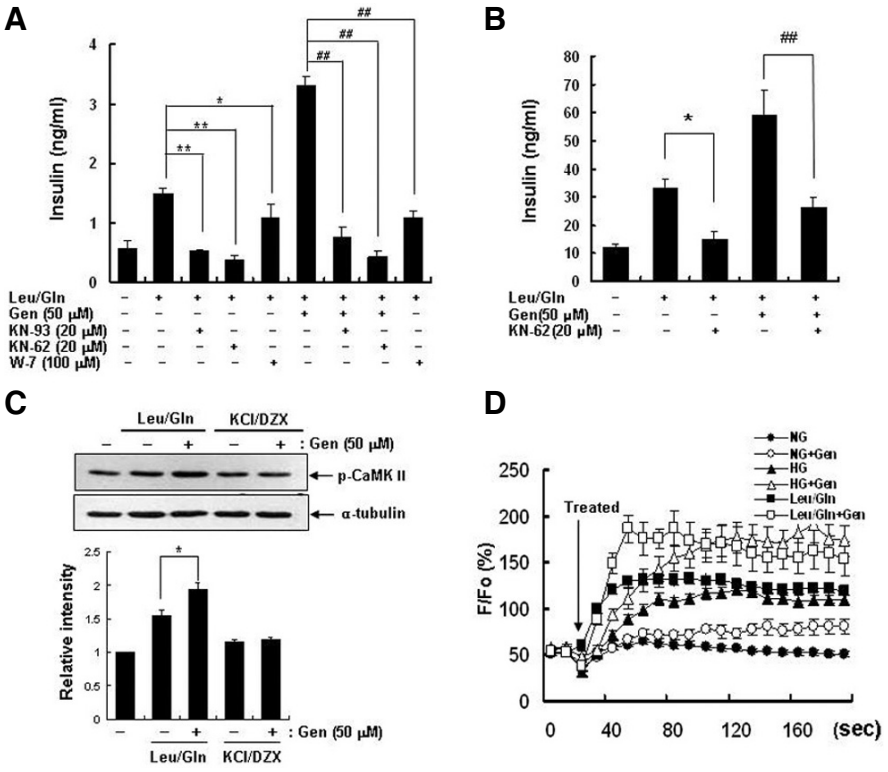

Fig. 5. Involvement of CaMK II activation in genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. (A) Insulin secretion was stimulated with Leu/Gln and genistein (Gen) for 2 h in INS-1 cells in the presence of CaMK II inhibitors, i.e., KN-93 (20 μM), KN-62 (20 μM), and W-7 (100 μM). The insulin released into the medium was quantified by insulin RIA. *P < 0.05; ** P < 0.01 vs. Leu/Gln-stimulated insulin release in genistein-untreated INS-1 cells. ##P < 0.01 vs. Leu/Gln-stimulated insulin release in genistein-treated INS-1 cells. (B) Insulin secretion was stimulated with Leu/Gln and genistein (Gen) for 2 h in SD islet cells in the presence of CaMK II inhibitor KN-62 (20 μM). The insulin released into the medium was quantified by insulin RIA. *P < 0.05 Leu/Gln-stimulated insulin release in SD islet cells. ##P < 0.01 vs. Leu/Gln-stimulated insulin release in genistein-treated SD islet cells. (C) Levels of phospho-CAMK II were analyzed by immunoblotting with anti-CaMK II antibodies. After stimulation of INS-1 cells with Leu /Gln in the presence of genistein (50 μM), the cell lysates were prepared with LIPA buffer and then subjected to SDS-PAGE and immuno-blotting. *P < 0.05 vs. phospho-CaMK II from Leu/Gln-treated INS-1 cells. (D) Increase of $[Ca^{2+}]_i$ by stimulation with HG (16.7 mM glucose), Leu/Gln or genistein was determined by measuring fluorescent signals of Fluo3/AM (emission fluorescence at 505 nm after excitation at 488 nm). Relative $[Ca^{2+}]_i$ was presented as percentage changes in the intensity of fluorescence compared to the basal level (F/Fo). Fifteen independent calcium intensity ratios at the indicated times were combined.

istein-induced potentiation effect was glucose-dependent and was marginal in insulin secretion stimulated by non-metabolite secretagogues (Jonas et al., 1995). These findings suggest that genistein does not affect insulin secretion stimulated by an agent that simply elicits Ca^{2+} influx, and that genistein-induced potentiation requires metabolite-derived signal as well as Ca^{2+}-mediated triggering signal.

Since metabolic intermediates generated through the Krebs cycle are reported to be involved in the amplification pathway for insulin secretion (Maechler, 2002), the genistein-induced potentiation effect is thought to be due to signal amplification through metabolic intermediates generated during energy metabolism. Since glutamine provides carbons to the Krebs cycle using glutamate dehydrogenase, and leucine is known to activate glutamate dehydrogenase (Knudsen et al., 1983; Liu et al., 2003), the combination of leucine and glutamine potently triggers insulin secretion by generation of ATP. This combination also provides amplification signals through the metabolic intermediates of the Krebs cycle. Since the metabolites generated from Leu/Gln are thought to be simpler than those from the metabolic pathway of glucose, we decided to use Leu/Gln as a stimulus for insulin secretion to study the mechanism of the genistein-induced potentiation effect.

There have been reports that protein tyrosine kinases were involved in the regulation of insulin secretion (Persaud et al., 1999; Sorenson et al., 1994). In general, activation of the insulin-signal cascade through activation of insulin receptor tyrosine kinase was thought to down-regulate GSIS. Studies using PI3 kinase inhibitors demonstrated that pharmacological blockage of the insulin-signaling pathway could potentiate GSIS (Eto et al., 2002; Zawalich et al., 2004). Diminished insulin signaling by reduced expression of the insulin receptor also showed enhanced GSIS (Ohsugi et al., 2005). However, other reports suggested that the insulin-signaling pathway was necessary for GSIS. The loss of insulin signals after knocking-out the insulin receptor or insulin receptor substrate-1 (IRS-1) showed that the insulin-signaling pathway was required for appropriate secretion of insulin (Borge et al., 2002). The controversial findings suggest that different PTKs or different signal intensities may affect insulin secretion differently. Verspohl et al. reported that the inhibitory activity of tyrosine kinase contributed to genistein-induced insulin secretion by abolishing the inhibitory effect of the insulin-signaling pathway on insulin secretion (Verspohl et al., 1995). Their suggestion was based on the experimental result that tyrosine kinase inhibitors tyrphostin 25 and genistein, but not its structurally similar albeit biologically inactive analogue daidzein, increased insulin secretion in the presence of 16.7 mM glucose. Our experimental results also showed a similar augmentation effect by genistein but little effect by daidzein on Leu/Gln-stimulated insulin secretion. However, our immunoblotting experiments showed that 200 μM genistein reduced the level of phospho-IRS-1 and phospho-Akt, while 50 μM genistein, the concentration showing maximum activity of potentiation, did not. This finding indicated that downregulation of the insulin-signaling pathway through inhibition of tyrosine kinase might not play a critical role in genistein-induced potentiation of insulin secretion, even though genistein was routinely used as a tyrosine kinase inhibitor.

Activation of PKA, PKC and CaMK II are known to be amplifying signals for insulin secretion (Nesher et al., 2002; Wan et

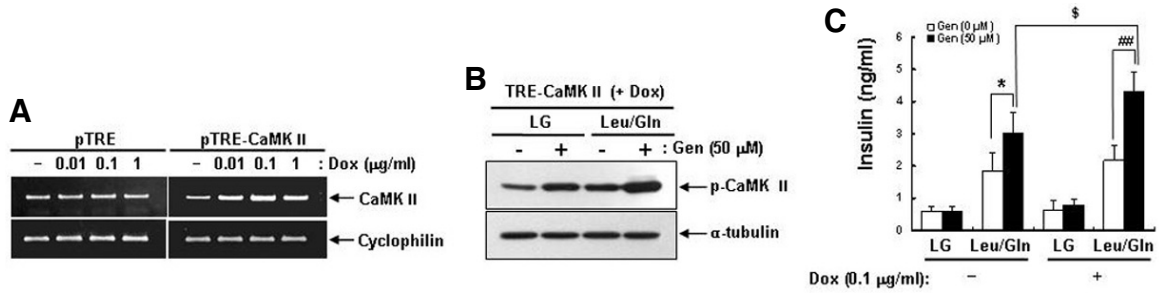

Fig. 6. Potentiation effect of genistein on Leu/Gln-stimulated insulin secretion in CaMK II-overexpressing INS-1 cells. (A) CaMK II expression in pTRE-CaMK II-harboring INS-1 cells was induced by treatment of the cells with doxycycline (0.01, 0.1, 1 μg/ml) for 24 h. Expression level of CaMK II mRNA was determined by semi-quantitative RT-PCR. (B) Levels of phospho-CaMK II were analyzed by immunoblotting with anti-phospho-CaMK II antibodies after stimulation with Leu/Gln and genistein (Gen) for 15 min in doxycycline (0.1 μg/ml)-treated pTRE-CaMK II-harboring INS-1 cells. (C) Insulin secretion was stimulated with 50 μM genistein for 2 h in the presence of Leu/Gln in doxycycline (0.1 μg/ml)-treated pTRE-CaMK II-harboring INS-1 cells. The insulin released into the medium was quantified by insulin RIA. *P < 0.05 vs. insulin stimulated by Leu/Gln in genistein-untreated INS-1 cells. $^{\#\#}$P < 0.01 vs. insulin stimulated by Leu/Gln in genistein-untreated but doxycycline-treated pTRE-CaMK II-harboring INS-1 cells. $^{\$}P$ < 0.05 vs. insulin stimulated by Leu/Gln and genistein in doxycycline-untreated pTRE-CaMK II-harboring INS-1 cells.

al., 2004). For sufficient secretion of insulin when stimulated by glucose, activation of these amplification signals as well as Ca^{2+}-mediated triggering is required (Nesher et al., 2002; Wan et al., 2004). Thus, activation of these signals can act as a target for genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. Several studies suggested that PKA activation through cAMP resulting from enhanced adenylate cyclase activity was involved in eliciting the biological functions of genistein (Liu et al., 2005; 2006; Ohno et al., 1993). The role of PKA in genistein-induced potentiation of insulin secretion was suggested by studies showing that a PKA-inhibitor H89 reduced the genistein-induced potentiation effect and genistein increased levels of cAMP. Our data also showed that H89 reduced the potentiation effect of genistein. However, a peptide inhibitor specific for PKA did not affect the genistein-induced potentiation effect, and, furthermore, genistein did not increase the levels of the phospho-form of PKA-specific substrates.

On the other hand, 10 μM forskolin, showing a similar potentiation effect on insulin secretion compared to genistein, increased the phospho-form of PKA substrates. These results suggest that the activation of PKA might not be involved in the genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. Since H89 (20 μM) was shown to inhibit other kinases including CaMK II (Davies et al., 2000), the inhibitory effect of H89 on genistein-induced potentiation may have been due to a side effect of H89, rather than a PKA inhibitory effect. Thus, the conclusion that activation of PKA plays a major role in genistein-induced potentiation of insulin secretion seems to be too early to establish. A recent study performed by Liu et al. demonstrating that the cAMP/PKA signaling cascade was involved in genistein-induced potentiation of GSIS at a low concentration of genistein (2.5 μM), is not accordance with our result. Activation of PKA was shown to be involved in genistein-induced potentiation through kinase assays using kemptide, a specific substrate for PKA. This kinase assay experiment was repeated in our lab using kemptide (Promega, USA). Fifty μM of genistein did not show any increase in the levels of the phospho-form of kemptide, demonstrating that 50 μM genistein did not activate PKA (Data not shown). Although further studies remain to be conducted, the discrepancy between Liu's report and ours may be due to the different concentrations of genistein or different secretagogues such as glucose and Leu/Gln.

Studies using pharmacological inhibitors suggest that activation of CaMK II may contribute to genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. We used three different kinds of inhibitors of CaMK II. The inhibitors tested (KN93, KN62 and W7) reduced genistein-induced potentiation of Leu/Gln-stimulated insulin secretion. However, the same inhibitors also reduced Leu/Gln-stimulated insulin secretion. These data suggest that CaMK II activation was essentially required for Leu/Gln-stimulated insulin secretion, rather than for genistein-induced potentiation. However, an immunoblotting study with anti-phospho-CaMK II antibodies demonstrated that genistein further increased the level of phospho-CaMK II than Leu/Gln alone. This suggested that genistein in conjunction with Leu/Gln could further activate CaMK II than activation by Leu/Gln alone. In particular, the study using CaMK II-overexpressing INS-1 cells supports the role of CaMK II in the genistein-induced potentiation. Genistein-induced potentiation of Leu/Gln-stimulated insulin secretion was significantly higher in CaMK II-overexpressing cells than untreated INS-1 cells. On the other hand, since genistein did not increase the levels of phospho-CaMK II in the presence of KCl and diazoxide, this suggested that genistien did not activate CaMK II when insulin secretion was stimulated by simple depolarizing agents. It also suggests that further metabolic signals as well as Ca^{2+} influx are required for genistein-induced CaMK II activation.

Genistein-induced CaMK II activation appeared to be attributed to an increase in $[Ca^{2+}]_i$. Since our data showed that genistein in the presence of Leu/Gln further increased $[Ca^{2+}]_i$ level compared to Leu/Gln alone, increase of $[Ca^{2+}]_i$ may contribute to genistein-augmented CaMK II activation. However, the mechanism by which genistein increases $[Ca^{2+}]_i$ under conditions of metabolic stimuli remains to be studied. An influx from extracellular Ca^{2+} or mobilization from intracellular Ca^{2+} stores could be a source of genistein-induced $[Ca^{2+}]_i$ increase.

In conclusion, our studies demonstrate that genistein potentiated insulin secretion in INS-1 cells stimulated by ATP-generating nutrients and suggest that the genistein-potentiating effect on insulin secretion resulted from the activation of CaMK II, rather than by inhibition of tyrosine kinase or activation of PKA.

ACKNOWLEDGMENTS

This work was supported by grants from Department of Medical Science, The Graduate School, Ajou University and Gyunggi Regional Research Center (GRRC).

REFERENCES

Akiyama, T., Ishida, J., and Nakagawa, S. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinase. J. Biol. Chem. *262*, 5592-5595.

Borge, P.D., Moibi, J., Greene, S.R., Trucco, M., Young, R.A., Gao, Z., and Wolf, B.A. (2002). Insulin receptor signaling and sarco/endoplasmic reticulum calcium ATPase in beta-cells. Diabetes *51*, S427-S433.

Bratanova-Tochkova, T.K., Cheng, H,, Daniel, S., Gunawardana, S., Liu, Y.J., Mulvaney-Musa, J., Schermerhorn, T., Straub, S.G., Yajima, H., and Sharp, G.W. (2002). Triggering and augmentation mechanisms, granule pools, and biphasic insulin secretion. Diabetes *51*, S83-S90.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. *351*, 95-105.

Eto, K., Yamashita, T., Tsubamoto, Y., Terauchi, Y., Hirose, K., Kubota, N., Yamashita, S., Taka, J., Satoh, S., Sekihara, H., et al. (2002). Phosphatidylinositol 3-kinase suppresses glucose-stimulated insulin secretion by affecting post-cytosolic [Ca(2+)] elevation signals Diabetes *51*, 87-97.

Henquin, J.C. (2000). Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes *49*, 751-760.

Jonas, J.C., Plant, T.D., Gilon, P., Detimary, P., Nenquin, M., and Henquin, J.C. (1995). Multiple effects and stimulation of insulin secretion by the tyrosine kinase inhibitor genistein in normal mouse islets. Br. J. Pharmacol. *114*, 872-880.

Kang, K., Lee, S.B., Jung, S.H., Cha, K.H., Park, W.D., Sohn, Y.C., and Nho, C.W. (2009). Tectoridin, a poor ligand of estrogen receptor alpha, exerts its estrogenic effects via an ERK-dependent pathway. Mol. Cells *27*, 351-357.

Knudsen, P., Kofod, H., Lernmark, A., and Hedeskov, C.J. (1983). L-leucine methyl ester stimulates insulin secretion and islet glutamate dehydrogenase. Am. J. Physiol. Endocrinol. Metab. *245*, E338-E346.

Linassier, C., Pierre, M., Le Pecq, J.B., and Pierre, J. (1990). Mechanisms of action in NIH-3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity. Biochem. Pharmacol. *39*, 187-193.

Liu, Y.J., Cheng, H., Drought, H., MacDonald, M.J., Sharp, G.W., and Straub, S.G. (2003). Activation of the KATP channel-independent signaling pathway by the nonhydrolyzable analog of leucine, BCH. Am. J. Physiol. Endocrinol. Metab. *285*, E380-E389.

Liu, D., Jiang, H., and Grange, R.W. (2005). Genistein activates the 3′,5′-cyclic adenosine monophosphate signaling pathway in vascular endothelial cells and protects endothelial barrier function. Endocrinology *146*, 1312-1320.

Liu, D., Zhen, W., Yang, Z., Carter, J.D., Si, H., and Reynolds, K.A. (2006). Genistein acutely stimulates insulin secretion in pancreatic beta-cells through a cAMP-dependent protein kinase pathway. Diabetes *55*, 1043-1050.

Maechler, P. (2002). Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic beta-cells. Cell. Mol. Life Sci. *59*, 1803-1818.

Markiewicz, L., Garey, J., and Adlercreutz, H. (1993). *In vitro* bioassays of non-steroidal phytoestrogens. J. Steroi. Biochem. Mol. Biol. *45*, 399-405.

Markovits, J., Linassier, C., Fossé, P., Couprie, J., Pierre, J., Jacquemin-Sablon, A., Saucier, J.M., Le Pecq, J.B., and Larsen, A.K. (1989). Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Res. *49*, 5111-5117.

Nesher, R., Anteby, E., Yedovizky, M., Warwar, N., Kaiser, N., and Cerasi, E. (2002). Beta-cell protein kinases and the dynamics of the insulin response to glucose. Diabetes *51*, S68-S73.

Ng, W.W., Keung, W., Xu, Y.C., Ng, K.F., Leung, G.P., Vanhoutte, P.M., Choy, P.C., and Man, R.Y. (2008). Genistein potentiates protein kinase A activity in porcine coronary artery. Mol. Cell. Biochem. *311*, 37-44.

Ohno, T., Kato, N., Ishii, C., Shimizu, M., Ito, Y., Tomono, S., and Kawazu, S. (1993). Genistein augments cyclic adenosine 3′5′-monophosphate(cAMP) accumulation and insulin release in MIN6 cells. Endocrine Res. *19*, 273-285.

Ohsugi, M., Cras-Méneur, C., Zhou, Y., Bernal-Mizrachi, E., Johnson, J.D., Luciani, D.S., Polonsky, K.S., and Permutt, M.A. (2005). Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. J. Biol. Chem. *280*, 4992-5003.

Persaud, S.J., Harris, T.E., Burns, C.J., and Jones, P.M. (1999) Tyrosine kinases play a permissive role in glucose-induced insulin secretion from adult rat islets. J. Mol. Endocrinol. *22*, 19-28.

Sorenson, R.L., Brelje, T.C., and Roth, C. (1994). Effect of tyrosine kinase inhibitors on islets of langerhans: evidence for tyrosine kinases in the regulation of insulin secretion Endocrinology *134*, 1975-1978.

Straub, S.G., and Sharp, G.W. (2002). Glucose-stimulated signaling pathways in biphasic insulin secretion. Diabetes Metab. Res. Rev. *18*, 451-463.

Verspohl, E.J., Tollkühn, B., and Kloss, H. (1995). Role of tyrosine kinase in insulin release in an insulin secreting cell line (INS-1). Cell Signal. *7*, 505-512.

Wan, Q.F., Dong, Y., Yang, H., Lou, X., Ding, J., and Xu, T. (2004). Protein kinase activation increases insulin secretion by sensitizing the secretory machinery to Ca2+. J. Gen. Physiol. *124*, 653-662.

Wiederkehr, A., and Wollheim, C.B. (2006). Minireview: implication of mitochondria in insulin secretion and action. Endocrinology *147*, 2643-2649.

Zawalich, W.S., and Zawalich, K.C. (2002). Effects of glucose, exogenous insulin, and carbachol on C-peptide and insulin secretion from isolated perifused rat islets. J. Biol. Chem. *277*, 26233-26237.

Zawalich, W.S., Yamazaki, H., Zawalich, K.C., and Cline, G. (2004). Comparative effects of amino acids and glucose on insulin secretion from isolated rat or mouse islets. J. Endocrinol. *183*, 309-319.