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Regulation of insulin secretion by geniposide: possible involvement of phosphatidylinositol 3-phosphate kinase

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Abstract. – AIM: Type 2 diabetes mellitus is characterized by lack of, or relative deficiency in, insulin productions and insensitivity of target tissues to insulin. Improvement of β-cell functions is a potential strategy for the clinical management of this disease. We reported before that geniposide improved glucose-stimulated insulin secretion with the activation of glucagon-like peptide 1 receptor (GLP-1R) in INS-1 pancreatic β cells, but the cell signaling mechanism of geniposide regulating glucose-stimulated insulin secretion (GSIS) in β cells is so far poorly understood.

MATERIALS AND METHODS: Effect of LY294002, a specific inhibitor of PI3K, on GSIS in the presence or absence of geniposide in INS-1 cells. In addition, the differential protein expression of geniposide treated INS-1 cells was examined by Western blot.

RESULTS: After pretreatment with 10 μM LY294002 for 1 hour, the insulin secretion induced by geniposide was partly abolished in INS-1 cells. After treatment with geniposide, the phosphorylation of PDK1 and Akt473 increased gradually to the maximum at 60 minutes or 120 minutes respectively. Furthermore, geniposide also inhibited the phosphorylation of downstream target GSK3β, and this effect was counteracted by preincubation with LY294002. And the expression of GLUT2 was increased after treatment with different doses geniposide.

CONCLUSIONS: Geniposide increases insulin secretion in pancreatic β cells in a PI3K dependent mechanism potentially through increased GLUT2 protein levels.

Key Words:
Geniposide, Insulin secretion, Phosphatidylinositol 3-phosphate kinase (PI3K), Type 2 diabetes mellitus (T2DM).

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by peripheral insulin resistance and β cell dysfunction in the pancreas[1,2]. Impairment in glucose-sensitive insulin secretion of the pancreatic β cells is an important pathogenic feature[3]. The changes in β-dysfunction in diabetes include decline or failure of the ability of the β-cell to respond to elevated glucose[4], disturbances in pulsatile insulin release[5], and impaired insulin synthesis[6]. Therefore, improvement of β-cell function is a major goal in the clinical management of the disease.

Accumulating evidence shows that phosphatidylinositol 3-phosphate kinase (PI3K)/Akt signaling plays an important role in glucose-stimulated insulin secretion (GSIS), especially in the early phase. Furthermore, phosphatidylinositol-dependent kinase 1 (PDK1) and its downstream kinase Akts are important in the regulation of β cell growth and function[7-9]. And PDK1 is a central mediator of cellular response induced by PI3K signaling, as it promotes the phosphorylation-dependent activation of PKB/Akt[10-12]. The mechanism underlying the insulin secretion of PI3K signaling pathway, however, is still under investigation.

A large number of references have shown that glucagon-like peptide 1 (GLP-1) functions through its receptor to regulate insulin secretion and glucose metabolism and is, therefore, a potentially important target in the treatment of T2DM[13-15]. Furthermore, PI3K signaling pathway holds a critical role in GLP-1 analogues functions[16].

Geniposide, a natural iridoid glycoside, is one of the major effective compounds of *Fructus Gardeniae* which is widely used in Traditional Chinese Medicine because of its cholagogue, sedative, diuretic, anti-inflammatory[17], and antipyretic effects[18]. It has been reported that geniposide has many important curative effects, such as hepatic-protective[19], inhibition of enterovirus[20], neuroprotection[21,22], and diabetes curative effects[23]. In the previous study we reported that geniposide, with the activation of glucagon-like peptide 1 receptor

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(GLP-1R), induced insulin secretion in a dose-dependent manner and enhanced GSIS in INS-1 cells[24]. However, it is poorly defined about the cell signaling mechanism of geniposide regulating insulin secretion in pancreatic β cells. In an attempt to understand the role of geniposide in pancreatic β cells and subsequently if this affected by PI3K signaling pathway, the effects of geniposide on PI3K signaling of INS-1 pancreatic β cells were assessed.

Materials and Methods

Materials

Materials are obtained from the following sources: Rat INS-1 pancreatic β cells from the China Centre for Type Culture Collection; LY294002, anti-p-Akt473, anti-p-PDK1, anti-Akt, anti-PDK1, anti-p-GSK3β, anti-GSK3β, anti-GLUT2, β-actin and horseradish peroxide-conjugated goat anti-rabbit secondary antibody from Cell Signaling Technology Inc., Danvers, MA, USA. Fetal bovine serum (FBS), penicillin/streptomycin, and RPMI-1640 from HyClone Inc. Logan, UT, USA; Enhanced chemiluminescence (ECL) western blot kit and polyvinylidene difluoride (PVDF) membrane from Millipore, and geniposide from Wako Chemical, Warwick, RI, USA (071-05071). All other reagents were purchased from Amresco Inc., Salon, OH, USA, except indicated.

Cell Culture

INS-1 pancreatic β cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. The culture medium was RPMI-1640 medium with 11 mM glucose and supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM mercaptoethanol. Cells were passaged every week following trypsinization.

Insulin Secretion Measurement

To investigate the effect of LY294002, a specific inhibitor of PI3K, on geniposide regulating GSIS, INS-1 cells were seeded onto 12-well plates and cultured for 24 hours. The cells were washed two times with Krebs-Ringer bicarbonate buffer (KRBB, 129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 0.1% BSA (*bovine serum albumin*), 10 mM HEPES, pH 7.4) and starved for 2 hours in KRBB. Then, the cells were incubated with fresh KRBB containing 10 μM LY294002, after 30 minutes incubation, the cells were treated with 10 μM geniposide and continued to incubate for 1 hour. The supernatants were collected to determine insulin content using commercial kits (Linco Research, Inc. St Charles, MO, USA) according to the kit's instructions.

Western Blot

Cells were washed twice with PBS (phosphate buffered saline), and total lysates were prepared in Cell lysis Buffer (Cell Signal Technology Inc., Danvers, Ma, USA). Equal protein from cell extracts were then separated on 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with blocking buffer (20 mM Tris, 150 mM NaCl, pH 7.5, 5% nonfat dry milk) at room temperature for 1 hour. Primary and second antibodies were diluted in blocking solution and incubated with the membranes for indicated times as described previous. Excess antibody was washed off with 20 mM TBST (20 mM Tris, 150 mM NaCl, pH 7.5, and 0.1 % Tween 20). Detection was performed using ECL detection kit (Millipore Corporation, Billerica, MA, USA) after incubation with a HRP-conjugated second antibody. Band intensities were quantified with the software of Quantity one (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical Analyses

Data are presented as mean ± SD and analyzed by Student's two-tailed *t*-test or one-way analysis of variance. Statistical significance is displayed as * $p < 0.05$ or ** $p < 0.01$.

Results

Effect of LY294002 on Geniposide-Regulating Insulin Secretion

To investigate the role of PI3K signaling in geniposide-regulating glucose-stimulated insulin secretion in INS-1 cells, we detected the influence of LY294002, a specific inhibitor of PI3K, on insulin secretion induced by geniposide in the presence of 5.5 mM glucose in INS-1 cells, the results demonstrated that, similar with GLP-1, geniposide potentiated glucose-stimulated insulin secretion in INS-1 cells. But, after pretreated with 10 μM LY294002 for 30 minutes, the augmentation of geniposide and GLP-1 on glucose-stimulated insulin secretion was inhibited signifi-

cantly (Figure 1), suggesting that PI3K signaling might be involved in geniposide-regulating insulin secretion.

Regulation of Geniposide on the Phosphorylation of PDK1

A number of investigators observed the potential involvement of PI3K in the control of insulin secretion, and PDK1 was a central mediator of cellular response induced by PI3K signaling[8,25,26]. We also detected the influence of geniposide on the phosphorylation of PDK1 in INS-1 cells. The results showed that geniposide induced the phosphorylation of PDK1 in a time- and dose-dependent manner in INS-1 cells (Figure 2A and 2B). Compared with the control, incubation with 10 μM geniposide for 60 minutes increased the phosphorylated level of PDK1 about 5-fold. But, pretreatment with 10 μM LY294002 for 30 minutes decreased the effect of geniposide on the phosphorylation of PDK1 in INS-1 cells noticeably (Figure 2C).

Regulation of Geniposide on the Phosphorylation of AKT and GSK3β

PDK-1 is a 64-kDa serine/threonine protein kinase which that serves as a master regulator of protein kinases known as the AGC kinase superfamily (cAMP-dependent or cGMP-dependent protein kinases and protein kinase C). This pivotal kinase plays a crucial role in mediating signaling transduction downstream of PI3K, and Akts are the best characterized substrates of PDK1[10,27-29]. In the current study we discovered, for the first time, using western blot analysis, that treatment with 10 μM geniposide significantly induced phosphorylation of Akt473 (Figure 3A). And geniposide decreased the phosphorylation of downstream target glycogen synthase kinase 3β (GSK3β) (Figure 3B), and this effect was counteracted by preincubation with LY294002 (Figure 3C).

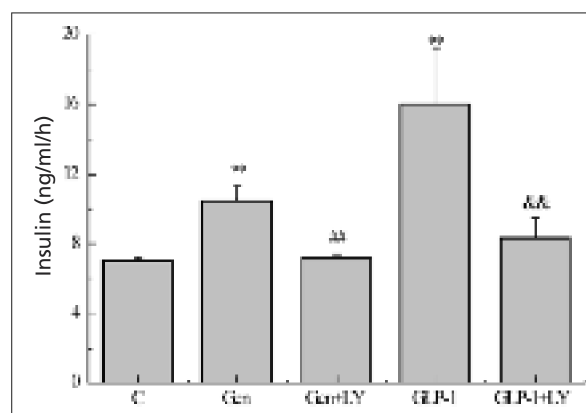

Figure 1. Effect of LY294002, a specific inhibitor of PI3K, on glucose-stimulated insulin secretion (GSIS) in the presence or absence of geniposide in INS-1 cells. After INS-1 cells were starved in KRBH buffer for 2 hours, the media was changed with 5.5 mM glucose in KRBB containing PI3K inhibitor LY294002 (LY, 10 μM), after 30 minutes incubation, the cells were treated with 10 μM geniposide (Gen) or GLP-1 (330 nM) and continued to incubate for 1 hour. The media were collected for the determination of insulin content with an ELISA method. Data are shown as means ± SD from independent experiments with duplicate well (n = 6). $^{**}p < 0.01$ vs control; $^{\#\#}p < 0.01$ vs geniposide group; $^{\&\&}p < 0.01$ vs GLP-1 group.

Regulation of Geniposide on the Protein Level of GLUT2

In order to further understand the effects of geniposide on GSIS in INS-1 cells, the level of GLUT2 protein was measured in total protein lysates. The results showed that 10 μM geniposide caused a significant increase in total GLUT2 protein levels resulting in an approximate 1 fold increase in GLUT2 protein compared to the quantified in basal cells (Figure 4).

Discussion

Our findings indicate that under normal glucose condition, geniposide promotes insulin secretion and increases protein levels of GLUT2 in total cell lysate. We have shown that geniposide-mediated insulin secretion is PI3K dependent (Figure 5). And to our knowledge this is the first study to show that the regulation of insulin secretion by geniposide involves PI3K.

PI3Ks and their lipid products in general, and PI(3,4)P_2/PI(3,4,5)P_3-activated PKB/Akt in particular, have been shown to be involved in multiple biological processes including proliferation, differentiation, cell survival, membrane trafficking, cell migration, glucose uptake and metabolism[30-32]. Dysregulation of 3-PI-lipid levels has been reported to contribute to the development of cardiovascular problems, cancer, allergy, chronic inflammation, and last but not least metabolic disorders such as type 2 diabetes mellitus[33-35]. So, regulation on PI3K signaling pathway might be useful for the treatment of these diseases.

One of the best characterized targets of PI3K lipid products is the protein kinase Akt or protein kinase B (PKB). In quiescent cells, Akt resides in

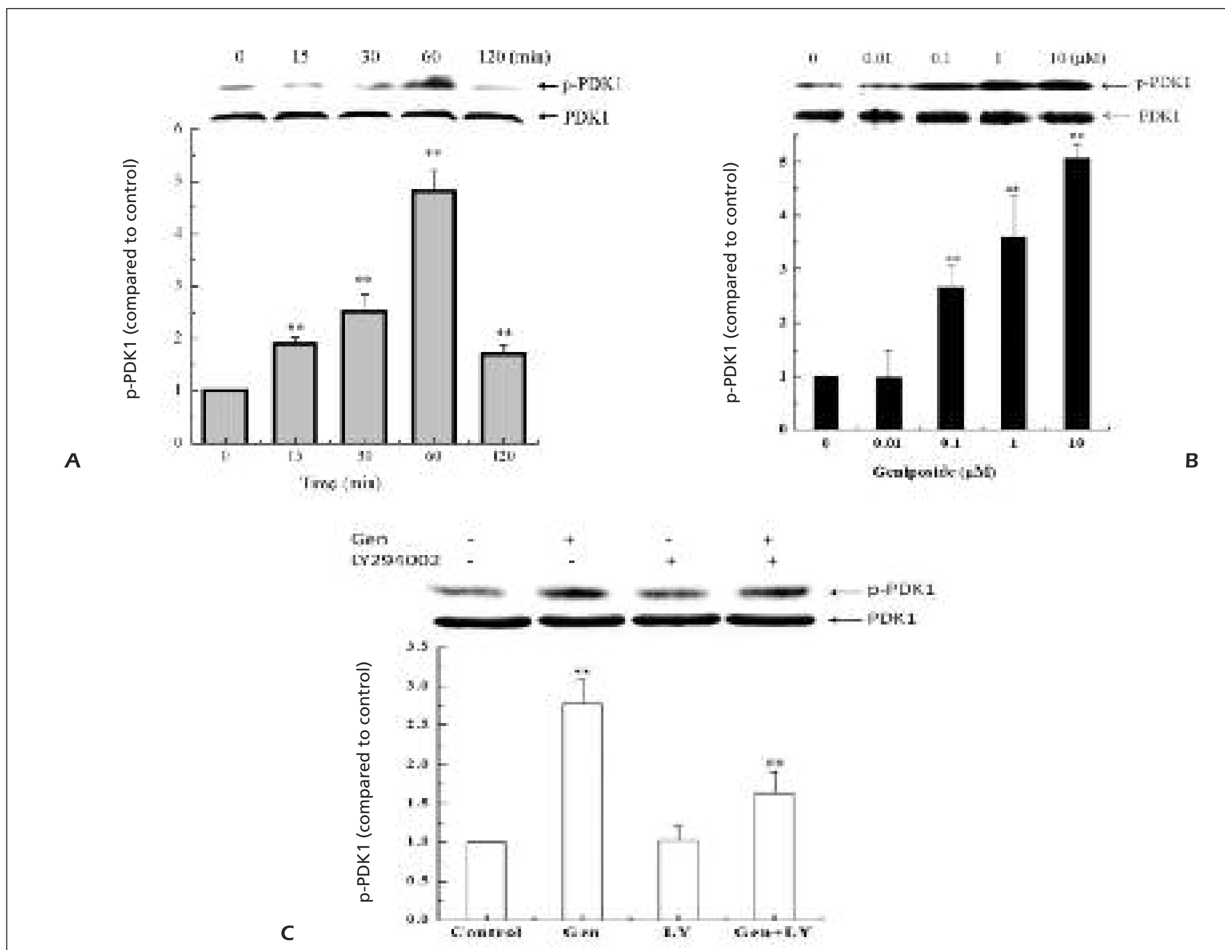

Figure 2. Geniposide induced the phosphorylation of PDK1 in INS-1 cells. ***A,*** After INS-1 cells were treated with 10 μM geniposide for 0, 15, 30 or 60 minutes, the cells were washed and equal whole cell lysates were separated with SDS-PAGE. Phosphorylated PDK1 protein was detected by immunobloting with p-PDK1 antibody. ***B,*** After INS-1 cells were treated with 0, 0.1, 1.0 or 10 μM geniposide for 60 minutes, equal cell lysates were separated with SDS-PAGE. Phosphorylated PDK1 protein was detected by immunobloting with p-PDK1 antibody. ***C,*** Effect of LY294002, a specific inhibitor of PI3K, on the phosphorylation of PDK1 induced by geniposide. After pretreatment with 10 μM LY294002 for 30 min, the cells were incubated with 10 μM geniposide for 30 min, and then equal cell lysates were separated with SDS-PAGE. Phosphorylated PDK1 was detected with p-PDK1 antibody. Data are shown as mean ± SD form at least three representative experiments. $^{**}p < 0.01$ vs control, and $^{\#\#}p < 0.01$ vs geniposide group.

the cytosol in a low-activity conformation. Upon cellular stimulation, Akt is activated through recruitment to cellular membranes by PI3K lipid products and phosphorylation by PDK1 to play their functions[8,36,37]. The present study provides a direct molecular explanation for the effects of geniposide on GSIS. As shown in Figure 2, geniposide induced the phosphorylation of PDK1 in a time- and dose-dependent manner. Furthermore, geniposide also enhanced the phosphorylation of Akt, the downstream kinase of PDK1, at Ser473 sites, revealed that geniposide might be an important mediator of PI3K signaling pathway in β cells.

Interestingly, the present study demonstrates that glucose-stimulated insulin secretion potentiated by geniposide was almost completely abolished by the pre-incubation with LY294002, a specific inhibitor of PI3K in INS-1 cells. At the same time, it was also observed that geniposide induced the phosphorylation of PDK1 and its downstream kinase Akts at Ser473 sites. Therefore, we have provided strong evidence that PI3K involved in geniposide-regulating glucose-stimulated insulin secretion in INS-1 cells.

The increasing evidence shows that PDK1 function is necessary to maintain glucose homeostasis in adult mice because its activity promotes

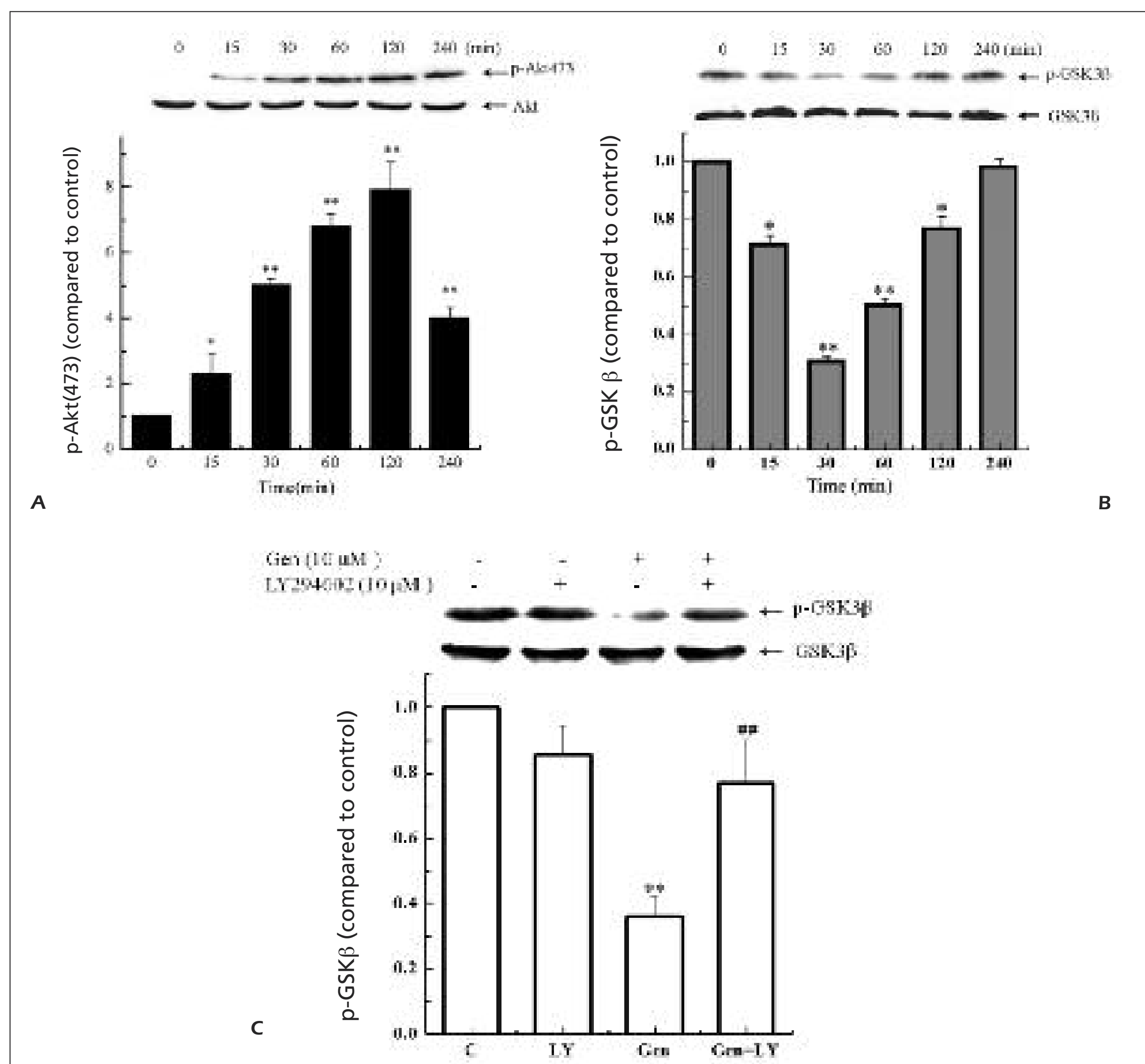

Figure 3. Effects of geniposide treatment on PI3K signaling cascade in INS-1 cells. INS-1 cells were treated with 10 μM geniposide for indicated times, the phosphorylation of Akt473 *(A)* and GSK3β *(B)* were detected by western blot. *C,* After treatment with 10 μM LY294002 (LY) for 30 minutes, 10 μM geniposide was added into the INS-1 cells and continued to culture for 30 minutes. Equal amount of proteins from each sample were separated on SDS-PAGE. Phosphorylated GSK3β was probed by p-GSK3β antibody. Values are means ± SD from three representative experiments (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs geniposide group (Gen).

the survival, proliferation, and proper size of mature pancreatic β cells[8]. Additionally, PDK1 is a serine/threonine protein kinase, which phosphorylates several members of the conserved AGC kinase superfamily, including the prototypes protein kinases A (PKA), G (PKG) and C (PKC). Phosphorylation of a threonine or serine residue in the activation loop (also known as the T-loop) of these kinases is a critical step in their activation, and is typically accompanied by additional phosphorylations elsewhere in the molecule. Phosphorylation of the activation loop is a common regulatory mechanism shared by most serine/threonine as well as tyrosine kinase as it facilitates alignment of amino acid residues in the active sites[10,11,25,27-29]. Therefore, the discovery of PDK-1 as the enzyme which mediates this event in many protein kinases introduced a new and important step in signaling pathways which regulate numerous important cellular processes. We reported previously that geniposide enhanced GSIS with the activation of GLP-1 receptor[24], and accelerated the uptake of glucose

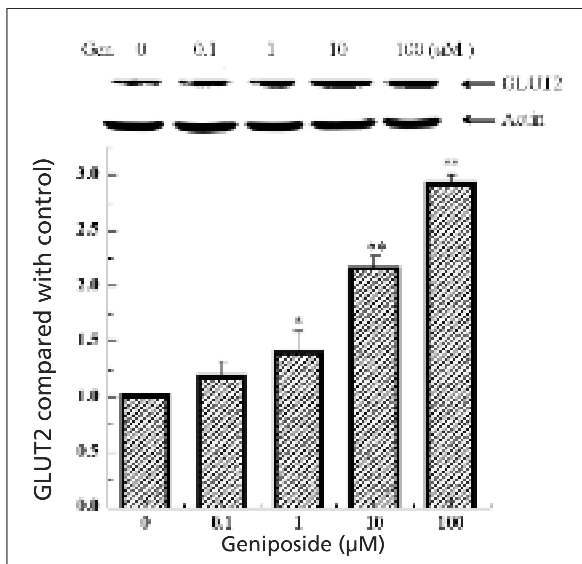

Figure 4. Geniposide induces the expression of GLUT2 in a dose-dependent manner. After INS-1 cells were treated with 0, 0.1, 1, 10, 100 μM geniposide for 24 h, equal cell lysates were separated on 10% SDS-PAGE. GLUT2 protein was detected by immunobloting with GLUT2 antibody. Values are the mean ± SD for representative experiments. $*p < 0.05$, $**p < 0.01$ vs control.

and metabolism in INS-1 cells (data not shown). But, the cell signaling pathway of geniposide regulating GSIS is unclear. Here we found that, accompanied with the phosphorylation of PDK1 and Akt induced by geniposide, the enhancement of GSIS by geniposide was inhibited the preincubation with LY294002, a specific inhibitor of PI3K. These initial landmark observations were followed by many future important studies which provided additional mechanistic insight into both PDK1 regulation as well as the role of this kinase in geniposide regulating GSIS.

Indeed, it has been shown that in T2DM, insulin signaling, especially PI3K/Akt signaling and its downstream GSK3β (glycogen synthase kinase 3β), are known to be diminished in muscle and liver owing to insulin resistance and/or decreased circulating insulin, leading to a reduction in glucose metabolism and subsequent hyperglycemia[38,39]. The present study demonstrated that geniposide induced the phosphorylation of PI3K signaling molecules, including PDK1, Akts and downstream GSK3β, and PI3K inhibitor, LY294002, decreased the effect of geniposide on GSIS. In addition, this article hypothesized that increased GLUT2 protein in response to geniposide reflects a priming of the cells for subsequent insulin secretion. And in this study we observed a significant increase in total cell lysate GLUT2 protein in INS-1 cells when treated with different doses geniposide. All these results suggested that geniposide might be helpful on the improvement of insulin secretion and glucose metabolism in T2DM.

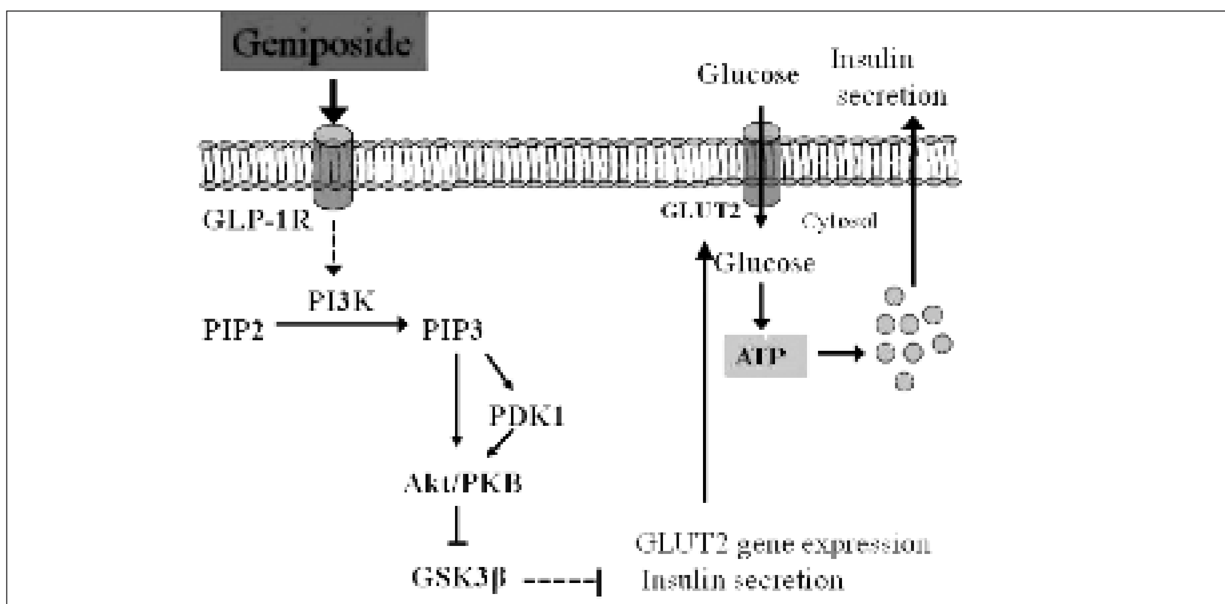

Figure 5. Schematic diagram illustrating the effects of geniposide on INS-1 cells insulin secretion under normal glucose conditions. Under n ormal glucose conditions, we propose that geniposide signals through GLP-1 receptor promoting PI3K activation and an increased level of GLUT2 protein resulting in increased insulin secretion.

Conclusions

To fully understand how geniposide regulates insulin secretion further research is required an area that we are continuing to investigate in cellular and animal models.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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