Original Article
Systematic investigation into the role of intermittent high glucose in pancreatic beta-cells

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Abstract: Objectives: Glucose fluctuation is suggested to be the leading cause of beta-cell damages. To determine how it induces beta-cell dysfunction, we systematically evaluated the effects of intermittent high glucose (IHG) in INS-1 rat pancreatic beta-cells on their proliferation activity, apoptosis, insulin secretion, reactive oxygen species (ROS), intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$), and the PTEN expression as well as AKT phosphorylation. Methods: Prior to the examinations, INS-1 cells were treated with normal glucose (NG, 11.1 mmol/L), sustained high glucose (SHG, 33 mmol/L), IHG (switching per 12 h in 11.1 mmol/l or 33 mmol/L), NG+α-lipoic acid (LA, pretreated with LA 12 h before exposure to NG), SHG+LA (pretreated with LA 12 h before being exposed to 33.3 mmol/L glucose) and IHG+LA (pretreated with LA 12 h before being cultured with IHG). The cells in each group were cultured with indicated concentrations of glucose for 3 days. The evaluations were carried out on the cell viability, apoptosis rate, insulin secretion, $[Ca^{2+}]i$, ROS and the expressions of PTEN and p-AKT. Results: The current study determined that IHG induces more apoptosis and significant increases of $[Ca^{2+}]i$ and intracellular ROS levels, compared to SHG and NG treatments to INS-1 cells. Moreover, IHG leads to more than 20% decrease on cell viability and over 50% reduction on insulin secretion (from 5.48±0.79 mIU/L to 2.51±0.58 mIU/L). The negative regulation of IHG on insulin signaling in beta-cells is identified via western blot analysis with results of the elevated expression of PTEN and lowered phosphorylation levels of AKT post IHG treatment. While the pretreatment of the antioxidant LA can significantly suppress the above responses induced by high glucose treatment. Conclusions: This study demonstrated that IHG plays a detrimental role in the viability, expansion, and function of beta-cells. IHG could be more harmful to the INS-1 cells than the SHG treatment. The rate increase of apoptosis in beta-cells could be caused by the suppressed insulin signaling, which is resulted from the raised ROS level by abnormal glucose treatments. Undergoing oxidative stress induced by high glucose treatments, including SHG and IHG, might be an important player in mediating the injury process to beta-cells, concluded from the beneficial rescue by the antioxidant LA treatment.

Keywords: Intermittent high glucose, pancreatic beta-cells, treatment

Introduction

Diabetes mellitus (DM) is one of the fastest growing chronic metabolic diseases, affecting more and more people worldwide. It is mainly characterized by the elevated glucose level in blood, which is called as hyperglycemia and caused by absolute (Type 1 DM) or relative (Type 2 DM) shortage of insulin. Long-term sustained hyperglycemia usually results in decrease of insulin production and damages to beta-cells, eventually leading to the insulin deficiency [1].

Blood glucose fluctuations are well controlled within a certain range in healthy individuals, but are usually out of control in diabetes patients due to the insulin insufficiency [2, 3]. Intermittent high glucose (IHG), as a common type of higher glucose fluctuations when diabetes occurs, has been widely suggested with more toxic effects on several kinds of cells than sustained high glucose (SHG), of which islet beta-cells are included [4-6]. Higher rate of apoptosis post IHG treatment in beta cells is identified, the potential cause of which has been connected to the elevated oxidative stress levels [7, 8]. However, the underlying mechanism for how IHG brings damages or induces high apoptosis rate to beta cells has not been entirely clear. So far, it is known that islet beta cells are vulnerable to oxidative stress, a pro-

cess mainly induced by high endogenous reactive oxygen species (ROS), because of their low expression of the principal antioxidant enzymes [6, 9, 10]. A recent study also suggested that islets have poor DNA repair capacity against oxidative damages [11]. Moreover, ROS and the redox environment modulate cellular Ca^{2+} channels [12-15]. In islet cells, Ca^{2+} is a key regulator in insulin release [16]. Thus, ROS may play a vital role in the dysfunction and apoptosis of pancreatic beta-cells.

Under physiological conditions, ROS are produced continuously by mitochondria as a byproduct of oxidative phosphorylation, and rapidly neutralized by various antioxidant systems to maintain an optimal redox environment for the cell [17]. However, in the conditions of diseases including DM, overproduction of ROS, reduction in the ability to remove excess ROS, or of both ultimately lead to oxidative stress in different cell types [18-20]. A latest study showed that the ROS induced by high glucose initiates apoptosis by upregulating the expression of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) in human umbilical vein endothelial cells (HUVECs) [21]. PTEN was discovered as a tumor suppressor as its expression is often lost in tumors [22, 23]. It can block cellular migration and proliferation, and mediate cell growth and apoptosis by negatively regulating insulin signaling [24]. Recent studies are mainly concentrated on the study of the important physiologic role of PTEN in metabolism. Tissue targeted deletion of PTEN in liver, fat, and muscle lead to improved insulin sensitivity in these insulin-responsive tissues [15, 25-27]. In mouse models of type 2 diabetes, deletion of PTEN in islet beta cells protects against deficient beta-cell mass and function [28]. However, that whether the effects of IHG and SHG on beta cells are modulated by PTEN has not been determined yet.

In this study, we performed a systematic analysis on the effects of IHG and SHG in the pancreatic cell line INS-1, including the cell viability, apoptosis, insulin secretion, $[Ca^{2+}]i$, ROS and the expressions of PTEN. By applying a potent antioxidant, α-lipoic acid (LA) [29], we explored the role of ROS in the action of SHG and IHG to affect beta cell functions and the possible mechanism.

Materials and methods

Chemicals. RPMI1640 medium and fetal bovine serum were purchased from Hyclone. 2'-7'-dichlorofluorescein diacetate (DCFH-DA), dimethyl sulphoxide (DMSO), and Owen's reagent (MTS) kit were bought from Promega company. Annexin V: FITC apoptosis detection kit was purchased from BD. BCA kit for protein determination was from Pierce Company. Fluo3 kit was bought from Japan Chemical Research Institute. Primary antibodies for PTEN, P-Akt, beta-actin and the secondary antibodies, rabbit and mouse polyclonal antibodies were purchased from Cell Signaling. Protease inhibitor was bought from Roche. ECL detection kit was from Pierce Company.

β-Cell culture. Dr. Yu-yan Zhao (China medical university, PRC) generously provided INS-1 cells. The cells were cultured in RPMI-1640 medium with 11 mmol/L D-glucose supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 50 µmol/L 2-mercaptoethanol. After being cultured overnight in glucose-free RPMI 1640, INS-1 cells were divided into six groups: (1) control group exposed to normal concentration of glucose (NG, 11.1 mmol/L); (2) sustained high glucose group (SHG), exposed to 33.3 mmol/L glucose; (3) intermittent high glucose group (IHG), exposed to fluctuating concentrations of glucose (alternating between 11.1 mmol/L and 33.3 mmol/L glucose every 12 h); (4) NG+LA, exposed to LA 12 h before being exposed to normal concentration of glucose (11.1 mmol/L); (5) SHG+LA, exposed to LA 12 h before being exposed to 33.3 mmol/L glucose; (6) IHG+LA, exposed to LA 12 h before being exposed to fluctuating concentrations of glucose (alternating between 11.1 mmol/L and 33.3 mmol/L glucose every 12 h). Cells in each group were incubated for 72 h prior to test. Cell viability assay. Cell viability was estimated by using Owen's reagent (MTS). INS-1 cells were seeded in 96-well microtiter plates and incubated for 72 h. At the end of the exposure, 20 µL of MTS was added to each well, and cells were maintained for 4 h at 37°C. Absorbance was measured at 490 nm, with a microplate reader. Cell viability was expressed as a percentage of cytoprotection, versus the control group set at 100%.
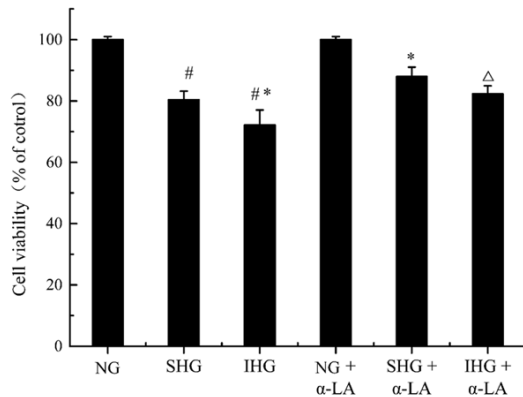
Figure 1. The viability of Ins-1 cells. Data were presented with mean±SD. #P<0.05 versuscontrol; *P<0.05 versus SHG; #,*P<0.05 versus SHG and control; ΔP<0.05 versus IHG.

Detection of apoptotic cells with Flow Cytometry. Apoptosis was assessed by annexin V-FITC and PI staining followed by analysis with flow cytometry. The methodology followed the procedures as described in the kit. Briefly, the adherent cells were collected by digestion of pancreatic enzyme, which has no EDTA. Then washed cells three times with cold PBS. Resuspended cells in 1 × binding buffer at a concentration of 1×10^6 cells/mL and transfer 100 μL (1×10^5 cells) to a 5 mL culture tube. Then added 5 μL of Annexin V-FITC and 10 μL of PI. Gently vortex the tube and incubate for 15 minutes at room temperature in the dark. Later on, 400 μL of 1 × binding buffer was added to each tube.

Measurement of insulin secretion. The culture medium was collected. The insulin secretion in each group was determined by radio-immunoassay.

Measurement of ROS Production. Intracellular ROS generation was measured by fluorescence microplate using peroxidesensitive fluorescent probe 2'7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) following the manufacture protocol. DCFH-DA will be converted by intracellular esterases to DCFH, a non-fluorescent compound, then it is oxidized into the highly fluorescent dichlorofluorescein (DCF) in the presence of oxidant. In brief, the treated cells were harvested and incubated with 10 M of DCFH-DA for 30 min at 37°C in the dark, and after being washed twice with PBS. The color change was observed. And the optical density at 450 nm was measured. The fluorescent intensity of cells from different groups was analyzed by fluorescence microplate reader.

Measurement of [Ca^{2+}]i. [Ca^{2+}]i concentration was monitored by using Fluo-3 fluorescent probe. The methodology followed the procedures as described in the kit. Briefly, take out the cultured cells, remove medium, and wash the cells with HBSS solution 3 times. Add the Fluo 3-AM working liquid. Incubate cells at 37°C for 10-60 min, then using HBSS wash cells 3 times, finally incubate cells at 37°C for 20-30 min, then inspect the cells under fluorescence microscope. Fluorescence was determined using a fluorescence spectrophotometer with excitation and emission wavelengths of 488 nm and 526 nm every 1 min. Intracellular Ca^{2+} was calculated from the fluo-3 fluoresce intensity using the equation: [Ca2+]i = Kd [(F - Fmin)/(Fmax-F)], where Kd = 400 nM. The maximal Fluo-3 fluorescence intensity (Fmax) was determined by adding 0.1% Triton X-100, and the minimal fluorescence (Fmin) was determined by quenching Fluo-3 fluorescence with the addition of 5 mM EGTA. F is the fluorescence measured without the addition of Triton-X-100 or EGTA.

Western blotting. After 3 days' incubation with indicated treatment, INS-1 cells were washed twice with PBS, and protein was extracted using the lysis buffer containing protease inhibitor. The supernatant was obtained by centrifugation at 4°C, 12,000 rpm, for 10 minutes. Protein was quantitated by the BCA kit. Western blotting was performed with a 10% or 5% poly-SDS page gel and 30 μg of protein per well. The gel was transferred to the PVDF membrane, blocked with 5% Skim milk in 0.1% TTBS (Tris-base 2.42 g, NaCl 8 g, Tween 20 1 mL), and reacted with the primary antibodies (1:500) for PTEN and P-Akt, and (1:1000) for beta-actin at 4°C overnight. The secondary antibodies, rabbit and mouse polyclonal antibodies were reacted at room temperature for 1 hour. Western blot analysis was performed *using the ECL* detection *kit.* Equal protein loading was confirmed by measuring beta-actin expression.

Statistical analysis. Experimental results were statistically analyzed using the SPSS version 18.0. All values are presented as the mean±standard deviation, and analyzed with the Student's *t*-test and ANOVA test. A difference with *P*<0.05 was considered to be statistically significant.
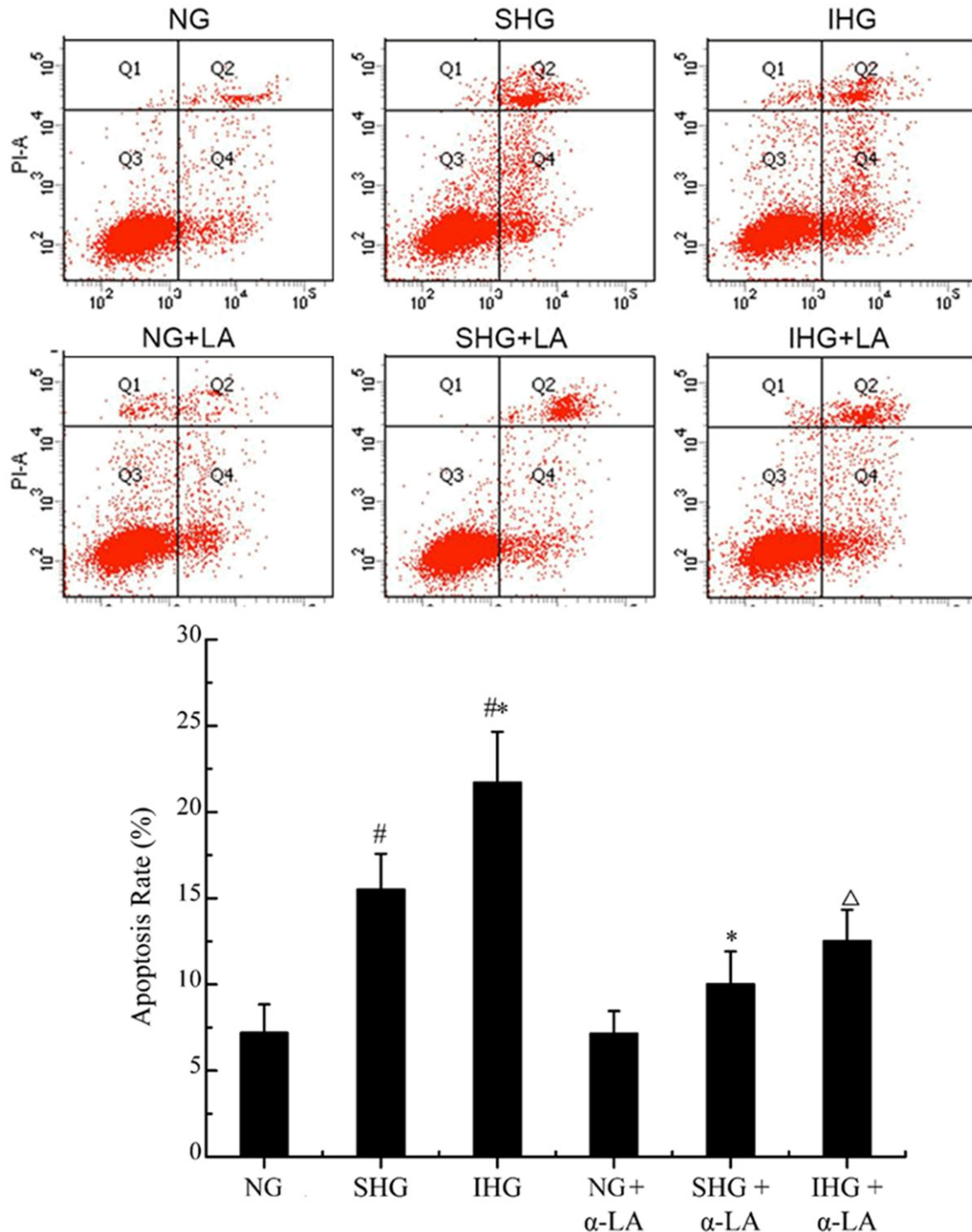
Figure 2. Determination of apoptotic cells with flow cytometry. Data were displayed as mean±SD. #P<0.05 versus-control; *P<0.05 versus SHG; #,*P<0.05 versus SHG and control; ΔP<0.05 versus IHG.

## Results

The viability of INS-1 cells

As shown in **Figure 1**, the viability of cells exposed to SHG or IHG was significantly decreased, compared to the control (P<0.05). While IHG decreased the cell viability more significantly in INS-1 cells than NG or SHG treatment (P<0.05). The pretreatment with LA can suppress the effect of SHG or IHG on the viability of those treated cells with SHG or IHG.

Table 1. Insulin secretion, ROS levels and $[Ca^{2+}]i$ concentration in treated INS-1 cells

	NG	SHG	IHG	NG+LA	SHG+LA	IHG+LA
Insulin (mIU/l)	5.48±0.79	3.42±1.06#	2.51±0.58#,*	5.41±1.02	4.93±0.78*	4.8±0.7Δ
ROS	108.223±5.342	204.29±5.515#	258.78±2.000#,*	104.72±2.110	179.813±8.289*	192.41±4.673Δ
$[Ca^{2+}]i$ (nmol/L)	142.43±5.08	183.93±6.1#	243.93±6.0#,*	141.8±5.11	159.57±3.96*	185.3±5.86Δ

Data were shown as mean±SD. #P<0.05 versuscontrol; *P<0.05 versus SHG; #,*P<0.05 versus SHG and control; ΔP<0.05 versus IHG.
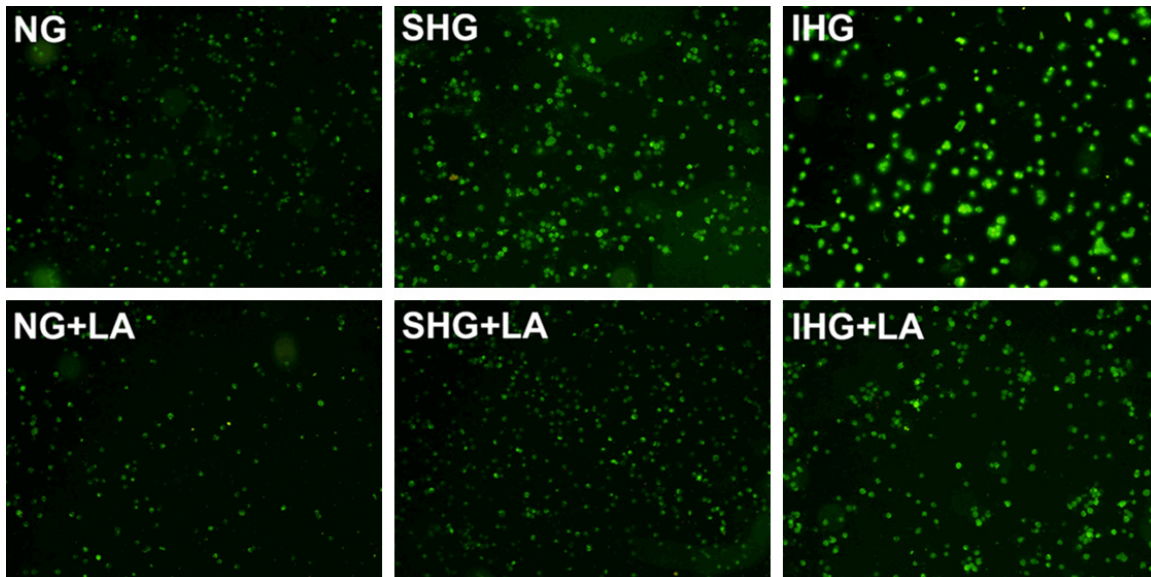
Figure 3. Images of $[Ca^{2+}]i$ level stained by Fluo-3fluorescent probe. Images were taken under the fluorescence microscope (×400).

The apoptosis of INS-1 cells

As shown in **Figure 2** (top), the apoptosis of INS-1 cells was determined by Annexin-V/PI staining via flow cytometry. The ratios of apoptotic cells in SHG and IHG groups were (15.50±2.08%) and (21.70±2.95%) respectively, both significantly higher than the control NG group (7.19±1.64%). There is a statistical difference if comparing the apoptosis rate of INS-1 cells induced by IHG with the rate of either the SHG or NG group, which is significantly higher in the IHG group (**Figure 2**, bottom). With the pretreatment of LA, the effects of SHG and IHG on apoptosis rate are remarkably decreased (P<0.05, **Figure 2**, bottom).

Insulin secretion, ROS levels and $[Ca^{2+}]i$ concentration altered by glucose fluctuations

As shown in **Table 1**, the insulin secretion levels of INS-1 cell in SHG (3.42±1.06 mIU/L) and IHG (2.51±0.58 mIU/L) groups were notably lowered than that of the NG group (5.48±0.79 mIU/L). In addition, IHG exerts more detrimental effects on the insulin secretion of beta-cells when compared to SHG. When pretreated with LA, the cell markedly recovers its insulin secretion ability under SHG or IHG treatment.

The ROS level in INS-1 cells exposed to SHG is nearly 2-fold higher of the NG group, while which of the IHG group is about 2.38-fold higher than the NG group (**Table 1**). As expected, the pretreatment with the antioxidant LA can significantly reduce the effects of SHG and IHG on the increase of ROS levels in beta cell (P<0.05).

As presented in **Table 1**, SHG and IHG can significantly increase the $[Ca^{2+}]i$ concentration in beta-cells compared to the NG treatment (P<0.05), the apparent effects of which are also indicated by **Figure 3**. Consistently, IHG yields stronger effect on the elevation of $[Ca^{2+}]i$ concentration in beta cells than SHG. The LA pretreatment to the cells can also significantly suppress the effects of SHG and IHG on the $[Ca^{2+}]i$ increase in INS-1 cell.
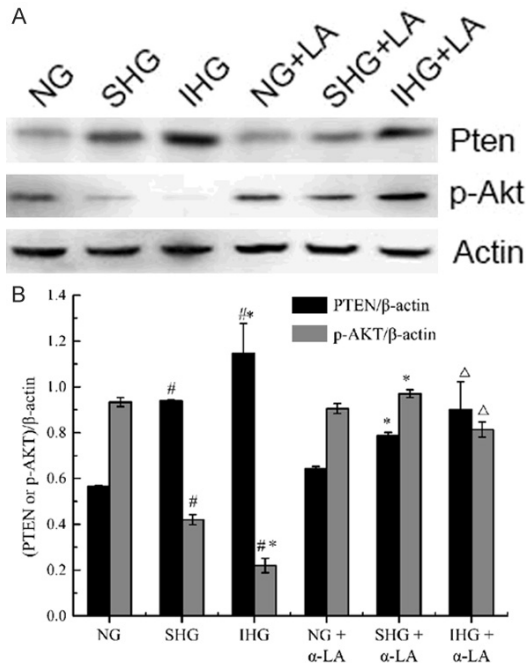
Figure 4. Expressions of PTEN and levels of p-AKT were determined by Western blot analyses. #$P<0.05$ versuscontrol; *$P<0.05$ versus SHG; #,*$P<0.05$ versus SHG and control; Δ$P<0.05$ versus IHG.

Expression of PTEN and AKT phosphorylation levels in treated INS-1 cells. As shown in **Figure 4A** (top), the expression of PTEN is increased by SHG and IHG treatments with the significant decrease of p-AKT levels, which implicates highly suppressed insulin signaling. Surprisingly, the pretreatment of LA to cells with exposure of SHG and IHG can remarkably increase the p-AKT levels compared to their glucose treatment alone. Whereas, the LA pretreatment significantly reduces the effects of SHG and IHG on PTEN expression in INS-1 cells, which is indicated by the quantified analysis of the western blotting (**Figure 4**, bottom).

Discussion

In the present study, we found that IHG exerts more adverse effects on INS-1 cells than SHG, which is consistent with previous studies [30, 31]. The increases in cell apoptosis, decreases in cell viability and decreases in insulin secretion in INS-1 cells cultured with IHG are all more than in cells exposed to SHG. Furthermore, under the condition of IHG, the intracellular ROS levels and [Ca^{2+}]i concentrations are higher than that with SHG treatment. All these phenotypes induced by high glucose treatment can

be significantly rescued by pretreatment of LA, which implies an important role of ROS in the action of IHG or SHG. Our findings and others' consistently proved that variability in glycemic control could be more deleterious to beta cells than a sustained high glucose, and oxidative stress may play an important role in mediating this process.

The increase in beta cell apoptosis is a important cause of insulin deficiency in type 2 diabetes, which has been proved in both animal and human studies [32, 33], thus leading to hyperglycemia and blood glucose fluctuations. Previous studies have suggested that oxidative stress might be a major mechanism for glucose toxicity in beta cells [34]. Optimal ROS production is important in modulating physiological signaling, but overproduction of ROS ultimately leads to oxidative stress. Excessive ROS changes redox homeostasis in cellular levels, including changes in Ca^{2+} handling. As an important second messenger, Ca^{2+} is a key trigger in insulin release [16]. In this evaluation, We found the increases of ROS and [Ca^{2+}]i concentration by IHG and SHG, but did not see the increase of the insulin secretion. It might be explained with the activation of Ca^{2+} signal-related apoptosis pathway by the sustained elevation of intracellular Ca^{2+} concentrations [35, 36]. However, the role of Ca^{2+} and ROS cross-talk [37] in beta cell functions still needs further investigations.

In the current work, we first hypothesized and investigated the alteration of PTEN by IHG and SHG in beta cells and its potential role in beta cell functions. It is known that PTEN mediates cell apoptosis through removing the phosphate from PI3K-produced IP3. IP3 directly phosphorylates AKT [38]. In the mouse model, deletion of PTEN specifically in the β-cells, which results in increased AKT activity, leads to increased islet mass and resistance to streptozotocin (STZ) induced β-cell death[28]. In response to glucose challenge, the PTEN null β-cells secrete insulin much more than the control cells. We determined PTEN expression is increased by either IHG or SHG treatment, together with the level of AKT phosphorylation decreased. Under the same conditions, we saw higher apoptotic rates and lower insulin secretion, which are in accorded with the previous studies. Importantly, it has been demonstrated that H$_2$O$_2$, a species of ROS, increases PTEN expression [28]. In HUVECs, the ROS induced by high glucose is

proposed to induce apoptosis through upregulating PTEN expression [21], while the phosphatase activity of PTEN can be inhibited by ROS via the oxidation of cysteine residues C71 and C124 [39]. In addition, upregulation of PTEN causes reduction of ROS generation in cells by modulating of PI3K/AKT signaling [40]. These studies above suggested that there might be a feedback loop between PTEN and ROS. A potential role of PTEN in the response to SHG or IHG is implied by the current study, which may provide an important clue for future researches to gain better understanding of diabetes mellitus.

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Disclosure of conflict of interest

None.

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