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Low glibenclamide concentrations affect endoplasmic reticulum stress in INS-1 cells under glucotoxic or glucolipotoxic conditions

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Background/Aims: β-Cell apoptosis caused by increased endoplasmic reticulum (ER) stress is an important pathogenic component of type 2 diabetes mellitus. In theory, sulfonylureas, used for the treatment of diabetes, can contribute to ER stress. We assessed changes in ER stress in pancreatic β-cells under glucotoxic or glucolipotoxic conditions using low concentrations of the sulfonylurea, glibenclamide (GB).

Methods: Low concentrations of GB (10 or 100 nM) were added to INS-1 cells cultured under glucotoxic or glucolipotoxic conditions. The degree of viability, level of apoptosis and levels of markers associated with ER stress were measured.

Results: Apoptosis decreased in response to low concentrations of GB under glucolipotoxic but not glucotoxic conditions. Most ER stress markers decreased upon the addition of GB. Under glucotoxic conditions, changes in the levels of ER stress markers were not consistent. However, all decreased significantly under glucolipotoxic conditions.

Conclusions: Low concentrations of GB exerted antiapoptotic effects through the attenuation of ER stress under glucolipotoxic conditions.

Keywords: Glyburide; Insulin-secreting cells; Endoplasmic reticulum stress; Glucotoxicity; Glucolipotoxicity

INTRODUCTION

Decreased pancreatic β-cell function and mass are core components of the progression of diabetes mellitus. Previous reports have suggested that these components are caused by reactive oxygen species, increased intracellular calcium and the activation of endoplasmic reticulum (ER) stress. These processes lead to impaired insulin secretion, decreased insulin gene expression and induction of β-cell apoptosis [1,2].

Glucotoxicity is a condition characterized by slow and progressively irreversible effects on β-cell function caused by chronic hyperglycemia. Hyperglycemia stimulates insulin secretion and activates proinsulin biosynthesis in the ERs of β-cells, and may activate ER stress [3]. Similar to chronic hyperglycemia, fatty acids become toxic when the body is chronically exposed to excessive levels; this is called lipotoxicity. In an *in vitro* study, when isolated islets or insulin-secreting cells were chronically exposed to elevated levels of fatty acids, glucose-induced insulin secretion [4,5] and insulin gene expression [6] decreased, whereas cell death by apoptosis [7-10] increased. There is an abundance of evidence that suggests that lipotoxicity occurs only

in the presence of concomitantly elevated glucose levels [11-14] or glucotoxicity; these may be related to ER stress [15-17].

Pancreatic β-cells are highly specialized to handle the protein load within the ER. Disruption of ER homeostasis leads to the accumulation of unfolded and misfolded proteins in the ER. This condition is referred to as ER stress [18,19]. ER stress has been postulated to result from increased biosynthetic demand induced by chronic hyperglycemia and elevated free fatty acids in β-cells. This pathway is well-understood, and is based on the unfolded protein response (UPR). The UPR alleviates ER stress, restores homeostasis, and prevents cell death by inducing downstream responses that reduce new protein synthesis in the ER, increase the levels of ER chaperones to improve folding capacity and increase the capacity to dispose of misfolded proteins. If the cell is unable to successfully perform these procedures, the UPR will trigger the apoptosis cascade [20]. The three primary modulators of the UPR are inositol, which requires protein 1-α, activating transcription factor 6 (ATF6) and protein kinase RNA-like ER associated kinase [21]. These sensors remain inactive via interaction with the ER chaperone BiP until activated by increased ER stress [22].

Sulfonylurea medications, which reduce blood glucose levels by stimulating insulin release from pancreatic β-cells [23], have been used for the treatment of type 2 diabetes since the early 1950s. Despite the worldwide use of sulfonylureas, the loss of β-cell mass and function caused by their use has raised concern. Studies have shown that sulfonylureas may induce apoptosis in β-cell lines and rodent islet cells [24]. The majority of patients treated long-term with sulfonylureas experience treatment failure [25]. Some evidence suggests that chronic sulfonylurea use leads to ER stress in β-cells, and this ER stress ultimately causes exhaustion of β-cell function [26]. Qian et al. [27] suggested that sulfonylureas induce the loss of β-cell function and influence the natural progression of the disease through acceleration of ER stress. Therefore, use of sulfonylureas for the treatment of type 2 diabetes mellitus may accelerate the loss of β-cell mass and function. Despite inconsistencies among studies of the effects of different types of sulfonylureas, previous results for these agents have generally been nega-

tive regarding β-cell protection [23-28]. The majority of previous studies have reported conflicting results regarding sulfonylurea treatment in β-cells without stress or with glucotoxicity only, which is quite different from the internal milieu of diabetic patients. Thus, we planned to assess the degree of apoptosis and ER stress of INS-1 cells under glucotoxic and glucolipotoxic conditions, which mimic the conditions in diabetic patients, after treatment with glibenclamide (GB).

METHODS

Cell culture

The rat insulinoma cell line INS-1 was a kind gift from Dr. Won at Yeungnam University in Korea. INS-1 cells were maintained in RPMI 1640 media (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 11 mM glucose, and 50 μM 2-mercaptoethanol. All experiments were performed after incubation at 37°C with 5% CO_2 and were used between the 20th and 30th passages.

GB effects on viability and apoptosis under glucotoxic and glucolipotoxic conditions

INS-1 cells were incubated with 200 μM palmitate and/or 33 mM glucose for 48 hours. The medium was then replaced with new medium containing 0, 10, or 100 nM GB for 24 hours. Viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Optical density was measured at 570 nm by an automatic plate reader. Apoptosis was assessed by annexin V staining. Cells were treated with trypsin-ethylenediaminetetraacetic acid and centrifuged at 1,500 rpm for 5 minutes at 4°C. After aspirating the supernatants, cells were washed with 1-mL annexin V binding solution (140 mM NaCl, 10 mM HEPES, pH 7.4, and 2.5 mM $CaCl_2$) and centrifuged at 1,500 rpm for 5 minutes at 4°C. Supernatants were removed and 3-μL annexin V-fluorescein isothiocyanate and 10 μL propidium iodide (PI) were added. After incubation for 15 minutes in the dark, 300 μL fluorescence activated cell sorting (FACS) buffer (1% FBS, 0.1% NaN_3) was added and analyzed by a FACSort cell

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sorter (BectonDickinson, BD Bioscience, San Jose, CA, USA). The percentage of apoptosis was calculated, and included annexin V positive/PI negative and both annexin V and PI positive cells. These experiments were performed in triplicate.

Changes in ER stress markers and apoptosis caused by GB treatment under glucotoxic and glucolipotoxic conditions

Markers of ER stress and apoptosis were evaluated in INS-1 cells following incubation with 10 and 100 nM GB under glucotoxic or glucolipotoxic conditions via semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting. Gene expression levels of ER stress markers, including *Bip-1*, *ATF-4*, X-box binding protein-1 (*XBP-1*), and C/EBP-homologous protein transcription factor (*CHOP*), were assessed using RT-PCR. Caspase-3 and cleaved caspase-3 levels were evaluated by Western blot analysis. These experiments were also performed in triplicate.

RT-PCR

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Grand Island, NY, USA). Complementary DNAs were synthesized for PCRs, which were performed using primers of ER stress markers and a premixed RT-PCR kit (Bioneer, Daejeon, Korea). The primer sequences used are listed below. *Bip-1*: forward, 5'-GAGATTGTTCTGGTTGGCGGATCTACTC-3'; reverse, 5'-CCATATGCTACAGCCTCATCTGGGTT-3'; *ATF-4*: forward, 5'-TCTGTATGAGCCCTGAGTCCTACCT-3'; reverse, 5'-GGTCATAAGGTTTGGGTCGAGAACCAC-3'; *XBP-1*: forward, 5'-AAACAGAGTAGCAGCACAGACTGC-3'; reverse, 5'-GGATCTCTAAGACTAGAGGCTTGGTG-3': *CHOP*: forward, 5'-CCTGAAAGCAGAAACCGGTC-3'; reverse, 5'-CCTCATACCAGGCTTCCAGC-3'; and *GAPDH*: forward, 5'-TCCCTCAAGATTGTCAGCAA-3'; reverse, 5'-AGATCCACAACGGATACATT-3'. Amplification was performed using the following conditions by the My-Cycler thermal cycler (Bio-Rad, Hercules, CA, USA): predenaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 40°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. After amplification, 5 μL of the PCR products were subjected to electrophoresis in 1.5%

agarose gels. The gels were visualized using an SL-20 DNA Image Visualizer (Seoulin Bioscience, Seongnam, Korea).

Western blot analysis

INS-1 cells were washed with phosphate-buffered saline (PBS) and lysed with a mammalian tissue lysis/extraction reagent including protease inhibitors and sodium orthovanadate. Protein quantified by the BCA protein assay kit was added to 1 × sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiolthreitol, and 0.01% bromophenol blue). Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane and immunoblotted with anticaspase-3 (1 : 1,000), anticleaved caspase-3 (1 : 1,000), or anti-β-actin (1 : 1,000) at 4°C overnight. Secondary antibodies, such as goat antirabbit conjugated to alkaline phosphatase, were applied for 1 hour at room temperature, and membranes were developed using AP conjugate substrate kits (Bio-Rad). Developed protein bands were quantified using the Multi Gauge version 2.2 software (Fujifilm, Tokyo, Japan).

Changes in glucose-stimulated insulin secretion in INS-1 cells caused by GB treatment under glucotoxic and glucolipotoxic conditions

INS-1 cells were incubated with 10 or 100 nM GB under glucotoxic or glucolipotoxic conditions. Cells were then washed with PBS. To determine the levels of glucose-stimulated insulin secretion (GSIS), cells were starved for 5 hours in RPMI media containing 5 mM glucose and 2% FBS. Next, media were changed to KRBB solution (4.74 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgCl_2·6H_2O$, 35 mM $NaHCO_3$, and 10 mM HEPES) containing 5 or 25 mM glucose, and cells were incubated for an additional hour. Secreted insulin in the media was measured using a rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, Billerica, MA, USA).

RESULTS

Effects of GB treatment on cell viability and apoptosis under glucotoxic and glucolipotoxic conditions

Cell viability under glucotoxic conditions decreased by more than 30% when compared to that of control cells, and decreased more than 60% under glucolipotoxic conditions. However, the addition of GB did not induce differences in cell viability (Fig. 1A).

Both glucotoxic and glucolipotoxic conditions caused apoptosis of INS-1 cells compared to control cells. The addition of GB under glucotoxic conditions did not result in significant changes in apoptosis levels. Apoptosis decreased significantly in cells grown in media containing 10 nM GB under glucolipotoxic conditions (Fig. 1B).

Changes in ER stress markers and apoptosis caused by GB treatment under glucotoxic and glucolipotoxic conditions

ER stress markers

Gene expression levels of ER stress markers, including *Bip-1* (Fig. 2A), *ATF-4* (Fig. 2B), *XBP-1* (Fig. 2C), and *CHOP* (Fig. 2D), increased under glucotoxic and glucolipotoxic conditions when compared to the normal glucose control. GB treatment in INS-1 cells under glucotoxic conditions somewhat decreased the expression of ER stress markers *Bip-1*, *ATF-4*, *XBP-1*, and

CHOP. The decreases in *Bip-1* and *XBP-1* levels were statistically significant. Under glucolipotoxic conditions, levels of all ER stress markers were reduced after the addition of GB.

Proapoptotic markers

Caspase-3 levels decreased while those of cleaved caspase-3 increased in cells incubated under glucotoxic and glucolipotoxic conditions compared with cells incubated under normal conditions (Fig. 3). Specifically, the level of the cleaved form of caspase-3 increased compared to total caspase-3 levels. Although caspase-3 did not show a significant change after GB addition under glucotoxic or glucolipotoxic conditions, the slight increase in caspase-3 and decrease in cleaved caspase-3 levels suggest a definite decrease in the percentage of cleaved caspase-3.

GB effects on GSIS under glucotoxic and glucolipotoxic conditions

The impairment of glucose-dependent insulin secretion caused by glucotoxicity and glucolipotoxicity did not recover after the addition of GB (Fig. 4).

DISCUSSION

Previous studies have shown that diabetic patients have increased ER stress because islets isolated from
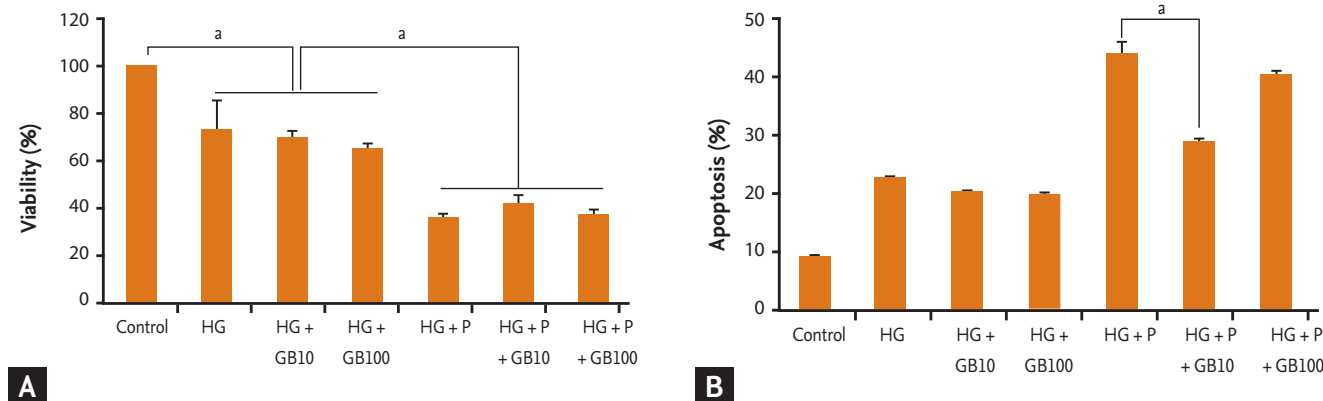

Figure 1. Viability (A) and apoptosis (B) of INS-1 cells by glibenclamide (GB) under glucotoxic and glucolipotoxic conditions. The medium was changed to that containing a low concentration of GB for 24 hours after incubation in 33 mM glucose and/or 200 μM plamitate (glucolipotoxicity/glucotoxicity) for 48 hours. Viability was measured by MTT assay. Apoptosis was evaluated using annexin V staining. The addition of 10 nM GB under glucolipotoxic conditions decreased apoptosis levels significantly. These experiments were performed in triplicate. Values are means ± SD. HG, high glucose; P, palmitate. $^{a}p < 0.05$.

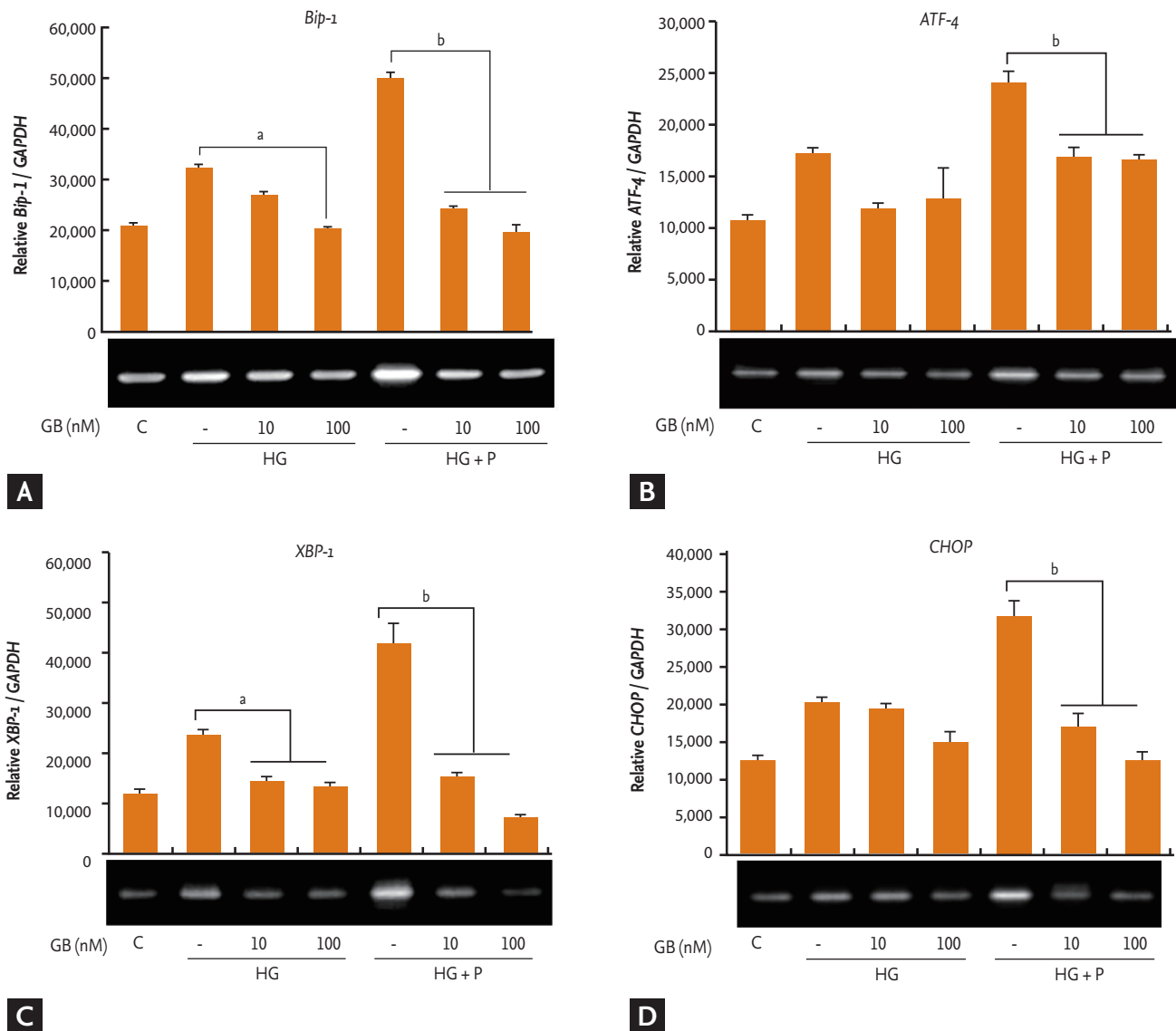Kwon MJ, et al. GB effect on β-cell in glucolipotoxicity

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Figure 2. Effects of glibenclamide (GB) on levels of endoplasmic reticulum (ER) stress markers under glucotoxic and glucolipotoxic conditions. (A) *Bip*-1, (B) activating transcription factor (*ATF*)-4, (C) X-box binding protein (*XBP*)-1, and (D) C/EBP homologous protein (CHOP) were evaluated using reverse transcriptase-polymerase chain reaction. Levels of ER stress markers increased under glucotoxic and glucolipotoxic conditions compared with a normal glucose control. Although ER stress markers did not show a fixed response under glucotoxic conditions, levels of all markers decreased significantly following the addition of a low concentration of GB under glucolipotoxic conditions. Values are means ± SD. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; HG, high glucose; P, palmitate. [a]$p < 0.05$ among results under glucotoxic conditions, [b]$p < 0.05$ among results under glucolipotoxic conditions.

type 2 diabetics have increased numbers of ER chaperones and CHOP, along with enlarged ERs [11,12,29]. Elevated ER stress caused by high glucose and fatty acids has been suggested as one of the causes of decreased β-cell function and mass, as shown in our study, as well as in previous reports.

Sulfonylureas have been predicted to accelerate apoptosis and the dysfunction of β-cells due to their mechanism of action. Previous studies have reported inconsistent results depending on the cell type used, the concentrations and types of sulfonylureas and the duration of exposure to them. In most studies using GB, authors have found increased β-cell apoptosis and decreased β-cell function [23,28].

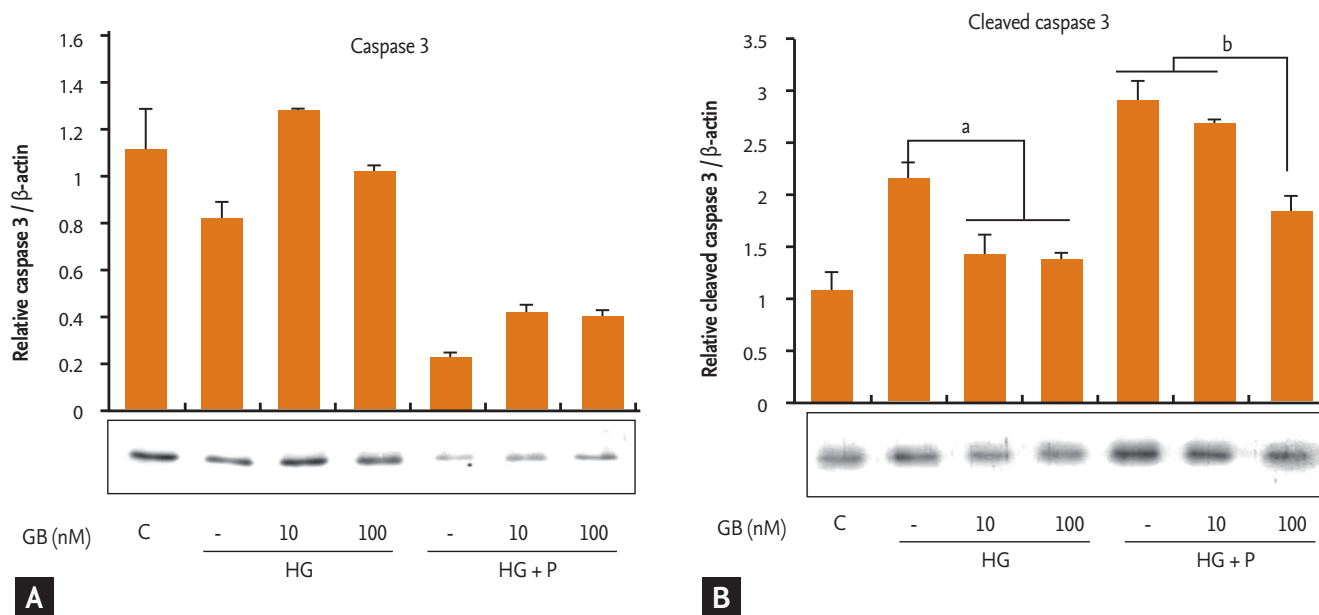The effects on β-cells of GB should be verified under conditions similar to those in diabetic patients treated

Figure 3. Effects of glibenclamide (GB) on levels of endoplasmic reticulum (ER) stress markers associated with the proapoptotic pathway under glucotoxic and glucolipotoxic conditions. (A) Caspase-3 and (B) cleaved caspase-3 levels were evaluated by Western blotting. Caspase-3 decreased while cleaved caspase-3 increased in the order control—glucotoxicity—glucolipotoxicity. Although caspase-3 did not change following GB treatment under glucolipotoxic conditions, the decrease in cleaved caspase-3 resulted in a significant decline in the cleaved caspase-3 ratio. Values are means ± SD. HG, high glucose; P, palmitate. [a]$p < 0.05$ among results under glucotoxic conditions, [b]$p < 0.05$ among results under glucolipotoxic conditions.
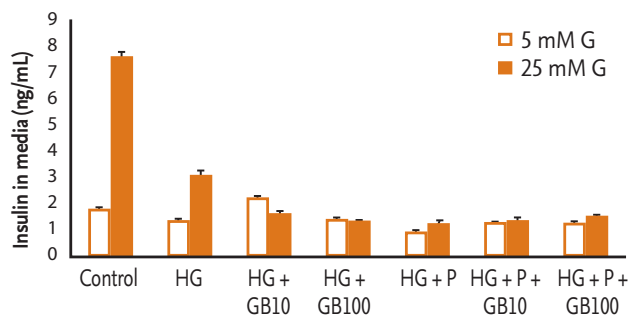

Figure 4. Effects of glibenclamide (GB) on the glucose-stimulated insulin response under glucotoxic and glucolipotoxic conditions. Impairment of glucose-dependent insulin secretion by glucotoxicity and glucolipotoxicity was not affected by the addition of GB. Values are means ± SD. HG, high glucose; P, palmitate.

with GB, such as glucotoxic and glucolipotoxic conditions. The peak plasma concentrations of GB, when administered at doses of 2.5 mg, are 0.15 μM [30]. This study was performed using lower concentrations of GB (10 or 100 nM) than those used in other reports. Apoptosis did not increase with the addition of low

concentrations of GB under glucotoxic or glucolipotoxic conditions. Interestingly, apoptosis decreased significantly in media containing 10 nM GB under glucolipotoxic conditions. ER stress and proapoptotic markers decreased when low concentrations of GB were administered under glucolipotoxic conditions. These results suggest that low concentrations of GB reduce ER stress under extremely stressful conditions, such as glucotoxicity and glucolipotoxicity. Another probable hypothesis is a binary switch of ER stress [31]. The UPR has both adaptive and apoptotic effectors that depend the tolerability of ER stress. Under tolerable ER stress conditions, the UPR acts to reduce ER stress and promote β-cell survival. Although ER stress cannot be entirely responsible for the pathogenesis of type 2 diabetes, it is an important finding that apoptosis of β-cells and proapoptotic markers decrease after treatment with low concentrations of GB.

In the United Kingdom Prospective Diabetes Study, loss of β-cell function was identified in all type 2 diabetic patients. The rates of decline were identical among all treatment groups, which included sulfonylurea, metformin, and conventional treatments [32].

Thus treatment with low-dose sulfonylureas may not accelerate progression of type 2 diabetes.

Our study has several limitations: 1) only one β-cell line was used; 2) only *in vitro* data were obtained; and 3) a more detailed mechanism was not established. More studies using various cell lines and primary human cell cultures are needed to confirm these results. An *in vivo* study is also necessary. Although animal models of type 2 diabetes may mimic internal ER stress under glucotoxic or glucolipotoxic conditions, measuring the degree of toxicity is difficult. When examining an *in vivo* study, it is difficult to rule out interactions with other parameters. However, well-designed *in vivo* experiments are needed to confirm our results. This study cannot explain the mechanism by which low concentrations of GB decrease ER stress and apoptosis. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway [33] and antiapoptotic markers, such as apoptosis antagonizing transcription factor [34], have recently been reported to be associated with ER stress. PI3K and Akt were tested using the same conditions as described in this study; however, they were unaffected by addition of GB under glucotoxic or glucolipotoxic conditions (data not shown). A further study should attempt to determine why GSIS did not recover after GB treatment despite the beneficial effects on apoptosis and ER stress.

Low concentrations of GB resulted in antiapoptotic effects, which were mediated by attenuation of ER stress under glucotoxic and glucolipotoxic conditions. Low-dose sulfonylurea treatment in type 2 diabetes may not directly cause secondary β-cell failure. To confirm these results, further studies using various cell lines will be needed to elucidate a more detailed mechanism.

KEY MESSAGE

1. Low concentrations of glibenclamide in INS-1 cells resulted in antiapoptotic effects by attenuation of endoplasmic reticulum stress under glucotoxic and glucolipotoxic conditions.
2. Low-dose sulfonylurea treatment in type 2 diabetes may not directly cause secondary β-cell failure.

Conflict of interest

No potential conflict of interest relevant to this article is reported.

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