Full Paper

Exendin-4 Protects Against Sulfonylurea-Induced β -Cell ApoptosisJu-Young Kim¹, Dong-Mee Lim¹, Hyung-Seo Park², Chan-Il Moon³, Kyung-Jin Choi², Seong-Kyu Lee⁴, Haing-Woon Baik⁴, Keun-Young Park¹, and Byung-Joon Kim^{1,5,*}¹Division of Endocrinology and Metabolism, Department of Internal Medicine, ²Department of Physiology, Konyang University School of Medicine, Daejeon 302-832, Korea³Department of Cardiology, Gachon University of Medicine and Science, Incheon 406-799, Korea⁴Department of Biochemistry and Molecular Biology, Eulji University School of Medicine, Daejeon 301-746, Korea⁵Myunggok Medical Research Institute, Konyang University School of Medicine, Daejeon 302-832, Korea

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Abstract. Sulfonylurea is one of the commonly used anti-diabetic drugs that stimulate insulin secretion from β -cells. Despite their glucose lowering effects in type 2 diabetes mellitus, long-term treatment brought on secondary failure characterized by β -cell exhaustion and apoptosis. ER stress induced by Ca^{2+} depletion in endoplasmic reticulum (ER) is speculated to be one of the causes of secondary failure, but it remains unclear. Glucagon like peptide-1 (GLP-1) has anti-apoptotic effects in β -cells after the induction of oxidative and ER stress. In this study, we examined the anti-apoptotic action of a GLP-1 analogue in β -cell lines and islets against ER stress induced by chronic treatment of sulfonylurea. HIT-T15 and dispersed islet cells were exposed to glibenclamide for 48 h, and apoptosis was evaluated using Annexin/PI flow cytometry. Expression of the ER stress-related molecules and sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) 2/3 was determined by real-time PCR and western blot analysis. Chronic exposure to glibenclamide increased apoptosis by depletion of ER Ca^{2+} concentration through reduced expression of SERCA 2/3. Pretreatment with Exendin-4 had an anti-apoptotic role through ER stress modulation and ER Ca^{2+} replenishing by SERCA restoration. These findings will further the understanding of one cause of glibenclamide-induced β -cell loss and therapeutic availability of GLP-1-based drugs in secondary failure by sulfonylurea during treatment of diabetes.

Keywords: endoplasmic reticulum (ER) calcium depletion, apoptosis, ER stress, glucagon like peptide-1 (GLP-1), sulfonylurea

Introduction

Sulfonylurea is the most widely used insulin secretagogue for the treatment of type 2 diabetes (1, 2). Their principal target in pancreatic β -cells was blocking of the ATP-sensitive potassium (K_{ATP}) channel, a key regulator of glucose- and sulfonylurea-induced insulin secretion (3, 4). After closure of K_{ATP} channels, β -cell membrane depolarized and triggered intracellular Ca^{2+} influx through the voltage-dependent Ca^{2+} channels (VDCC). Increased intracellular Ca^{2+} consequently stimulated insulin secretion (3, 4). Sulfonylurea bound its specific

sulfonylurea receptor (SUR) in K_{ATP} channels permanently and continuously stimulated insulin secretion in a glucose-independent manner. Despite the beneficial insulin secretory effects, these drugs bring on secondary failure, decreasing the mass and secretory function of β -cells. It is a major concern in patients given sulfonylurea-based treatment for type 2 diabetes mellitus. Although the acute and transient effects of sulfonylurea on insulin secretion have been widely investigated, the mechanisms for secondary failure are not yet fully understood. Several reports suggest that the sustained enhancement of Ca^{2+} influx caused apoptotic cell death of β -cells, but the mechanism of this phenomenon remains unclear (5 – 7).

β -Cells in the pancreatic islets have a highly developed endoplasmic reticulum (ER), which is necessary for in-

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sulin production (8 – 10). Disruption of ER homeostasis, as caused by alterations in the ER Ca^{2+} concentration, leads to ER stress in β -cells (11, 12). If secondary failure after chronic use of sulfonylurea caused alteration of ER Ca^{2+} concentration, we can find one more possible explanation for the ER stress and apoptosis induced by sulfonylurea.

Exendin-4 (Ex-4), a glucagon-like peptide-1 (GLP-1)-receptor agonist, is a recent modality for treatment of type 2 diabetes (13 – 15). It enhances insulin release, promotes β -cell development/replication, and prevents β -cell apoptosis under hyperglycemic conditions (13 – 15). Ex-4 has also protected against β -cell death under the ER stress induced by various causes (16 – 19).

Here, we investigate ER Ca^{2+} -concentration and its relation to ER stress and β -cell loss after chronic use of sulfonylurea. Also, we evaluate the protective role of Ex-4 in β -cells against ER stress and apoptosis induced by chronic use of sulfonylurea.

Materials and Methods

Materials

Ex-4 was obtained from Bachem Bioscience (King of Prussia, PA, USA). H89 (a PKA inhibitor), PD98059 (an ERK inhibitor), and forskolin (a cAMP activator) were purchased from Calbiochem (San Diego, CA, USA). Glibenclamide, thapsigargin [sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) blocker], wortmannin (a PI3K inhibitor), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Hoechst 33342 were acquired from Sigma (St. Louis, MO, USA). The Annexin V – fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Beckton Dickinson Bioscience (San Jose, CA, USA). RPMI1640, M199, fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Cell culture and treatments

Hamster-derived insulin-secreting HIT-T15 cells were maintained in RPMI1640 medium containing 11.1 mM glucose supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$), in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were grown to 80% confluence and then starved for 12 h in 0.5% FBS-containing RPMI1640, followed by incubation with glibenclamide (100 nM) for the indicated periods. The cells were pretreated with H89 (10 μM), an inhibitor of protein kinase A (PKA), for 30 min, followed by the addition of Ex-4 (25 nM) or forskolin (20 μM) for

1 h before glibenclamide treatment.

Islet isolation and dispersion

Islets were isolated from Sprague-Dawley (SD) rats (Damul Science, Daejeon, Korea) weighing 200 – 250 g by distending the pancreatic duct with a collagenase-containing solution (Collagenase P; Roche, Mannheim, Germany), as previously described (20). After digestion, the islets were separated on a Ficoll density gradient solution (Biochrom KG Seromed, Berlin, Germany) and further purified by handpicking. Islets were cultured in M199 containing a glucose concentration 5.5 mM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). Dispersed islet cells were prepared by placing 24-h cultured rat islets pooled from four rats (3000 islets) in 1 ml of trypsin-EDTA solution (X1) for 10 min at 37°C. The islets were then washed in culture media and dissociated by trituration in a pipette. The dispersed islet cells were kept in M199 culture media for 3 – 4 h before being cultured on Matrigel matrix (BD Biosciences, Bedford, MA, USA). Sterilized culture dishes were coated with Matrigel basement membrane matrix. After coating, the dishes were placed in an incubator at 37°C for a minimum of 3 h to allow the Matrigel to dry. Thereafter, dispersed islet cells were aliquoted onto the Matrigel and cultured for 24 h followed by 100% media change. The dispersed islet cells were cultured in various experimental conditions for flow cytometric analysis. All rats for islet isolation were cared for during the entire period of experimentation in accordance the Guidelines of the National Institutes of Health and Korean Academy of Medical Sciences.

Measurement of cell viability

Cell respiration, an indicator of cell viability, was assessed by the reduction of MTT to formazan. Shortly after exposure of the cells to glibenclamide with or without other test agents for the indicated period, MTT reagent (5 mg/ml) was added to each of the wells, and the plate was incubated for an additional 4 h at 37°C. The intracellular formazan product was dissolved in 250 μl of dimethyl sulfoxide (DMSO) and the absorbency of each well was measured at 540 nm (ref. 650 nm) using a microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA). OD values from untreated control cells were designated as 100%. Hoechst 33342, a DNA-binding dye, was used to detect differences between viable and apoptotic cells. After exposure of the cells to the test agents for the indicated periods, the cells were fixed in PBS containing 10% formaldehyde for 4 h at room temperature and then stained with Hoechst 33342 for 30 min at room temperature. Cells were evaluated under a fluorescence microscope (OlympusBX51; Olympus

Corp., Tokyo) for nuclei showing typical apoptotic features such as chromatin condensation and fragmentation. Photographs were taken at a magnification of 400 $\times$.

Flow cytometric analysis

Surface exposure of phosphatidyl serine in apoptotic cells was quantitatively detected using an Annexin V–FITC and PI apoptosis detection kit. Briefly, after treatment with glibenclamide for 48 h with or without the other test agents, the cells were harvested and centrifuged at 5,000 rpm for 5 min. Next, Annexin V–FITC and PI double-staining were performed according to the manufacturer's instructions. Cell apoptosis was analyzed on a FACScan flow cytometer (Beckton Dickinson Bioscience). Annexin V–FITC–positive, PI-negative cells were scored as apoptotic. Double-stained cells were considered either as necrotic or as late apoptotic (21).

Caspase-3 activity analysis

Caspase-3 activity was determined by a colorimetric assay using kits from R&D System (Wiesbaden-Nordenstadt, Germany), according to the manufacturer's protocol. Briefly, cells were lysed in the lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged at 10,000 $\times g$ for 10 min at 4°C to precipitate cellular debris. The supernatants were collected and incubated with the reaction buffer containing dithiothreitol and DEVD-pNA as substrates at 37°C. The reaction was measured by changes in absorbance at 405 nm using an ELISA reader (Emax, Molecular Devices). Enzyme activity was expressed as the fold increase in the proportion of apoptotic cells over that of non-treated control cells.

Quantitative real-time RT-PCR

Real-time PCR was conducted using the DNA Engine Opticon System (MJ Research, Inc., San Francisco, CA, USA) in a 20- μ l reaction mixture containing 10 μ l of SYBR Premix Ex Taq (Takara Bio Inc., Otsu), 10 pmol of forward primer, 10 pmol of reverse primer, and 1 μ g of cDNA. The primers used for the detection of the genes were as follows: *GRP78*, forward 5'-ATTCCTGCGTCG GTGTATTC-3' and reverse 5'-AGGAGTGAAGGCC ACATACG-3'; *GRP94*, forward 5'-TGATGATGAAGC CGCAGTAG-3' and reverse 5'-AAGTTCCCAGTCC CACACAG-3'; *CHOP*, forward 5'-CACCACACCTG AAAGCAGAA-3' and reverse 5'-ATCCTCATACCAG GCTTCCA-3'; *SERCA2*, forward 5'-AAGCTATGGG AGTGGTGGTG-3' and reverse 5'-GTCCAGCTTCTG CTGTAGGG-3'; *SERCA3*, forward 5'-AAATGTGCA ACGCCCTTAAC-3' and reverse 5'-GGCACCAAAA GGATGAGAAA-3'; and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, forward 5'-AAGTTCAACG GCACAGTCAA-3' and reverse 5'-TACTCAGCAC

CAGCATCACC-3'. The rat *GAPDH* gene was used as the internal control. Real-time PCR was performed as follows: 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C (*GRP78*, *GRP94*, and *CHOP*) or 58°C (*SERCA2* and *SERCA3*) for 30 s, and extension at 72°C for 1 min. Samples with no template added served as controls. The fluorescence resulting from the incorporation of SYBR Green 1 dye into the double-stranded DNA produced during the PCR and the emission data were quantified using the threshold cycle (C_t) value. Data were normalized to *GAPDH* and presented as the mean fold change as compared to controls.

RT-PCR analysis of *XBP-1* splicing

cDNA was used as a template for PCR amplification across the fragment of the *XBP-1* cDNA bearing the intron target of IRE1 α activity. The following primers were used for *XBP-1* PCR: forward primer, 5'-GAGCAGC AAGTGGTGGATTT-3' and reverse primer, 5'-TCTC AATCACAAGCCCATGA-3'. The PCR procedure involved 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min. PCR products were separated on a 3.2% agarose gel. Then, 289- and 263-bp amplicons were generated from unspliced and spliced *XBP-1*, respectively.

Western blot analysis

Whole cell lysates were prepared by lysing cells in Pro-prep protein extraction solution (Intron Biotechnology, Seoul, Korea), and protein concentration of the lysates was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (20 μ g) were run on a 4% – 20% SDS-PAGE gel and transferred by electroblotting onto a nitrocellulose membrane. The membranes were then washed with PBST containing 5% nonfat dry milk at room temperature and incubated for 2 h with the following antibodies: 1:1000 anti-KDEL for the detection of *GRP78* and *GRP94* (StressGen, Victoria, British Columbia, Canada); 1:500 anti-eIF2 α , 1:500 anti-Ser51 phosphospecific eIF2 α (Cell Signaling, Beverly, MA, USA); 1:500 anti-GADD 153 (*CHOP*), 1:1000 anti-SERCA2, 1:500 anti-SERCA3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and 1:5000 anti- β -actin (Sigma), which was used as an internal control. The membranes were washed in PBST and incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit immunoglobulin antibodies (1:500) under the same conditions. After washing with PBST, the specific signals were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotechnology, Tokyo).

Measurement of ER Ca^{2+} stores

Measurement of Ca^{2+} stores in the ER was performed as described previously (22). In brief, HIT-T15 cells were loaded with 20 μM fura-2/AM (TefLabs, Austin, TX, USA) at 37°C for 30 min in a HEPES-buffered physiological saline solution (HEPES-PSS) containing 5.5 mM glucose and 1% (w/v) bovine serum albumin. Fura-2-loaded cells were permeabilized by superfusion for 1–2 min with 40 μM β -escin in intracellular medium (pH 7.3). Permeabilized cells were washed in the intracellular medium without β -escin for 15 min to facilitate removal of cytosolic dye. Cells were superfused in intracellular medium containing 0.650 μM CaCl_2 (free $[\text{Ca}^{2+}] = 200$ nM), 1.4 mM MgCl_2 , and 3 mM Na_2ATP to activate SERCA and to load the intracellular Ca^{2+} stores. The free $[\text{Ca}^{2+}]$ was maintained at a constant value of 200 nM throughout all experimental maneuvers. The emission of the dye above 505 nm after excitation at 340 and 380 nm was recorded using a TILL Photonics imaging system (Pleasanton, CA, USA). Rates of Ca^{2+} uptake by the ER were estimated from these average responses by fitting the initial 30-s period of increasing fluorescence to a single exponential function (Origin 8.0; OriginLab, Corp., Northampton, MA, USA).

Statistical analyses

All experiments were separately conducted at least 3 times. Data are expressed as the means $\pm$ S.E.M. Statistical differences were analyzed using the one-way ANOVA followed by Tukey's test, using the SPSS statistical software package (Version 14.0; SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered significant.

Results

Protective effects of Ex-4 against glibenclamide-induced β -cell apoptosis

In order to examine the chronic effects of glibenclamide on β -cell apoptosis, we performed the MTT assay, Hoechst 33342 staining, and flow cytometric analysis after β -cell lines and rat islets were treated with glibenclamide time- and dose-dependently. In HIT-T15 β -cell lines, more than 50% cells had apoptosis after 100 nM glibenclamide treatment for 48 or 72 h (Fig. 1: A, B, C). In dispersed islet cells, higher concentration of glibenclamide was needed for induction of apoptosis. Treatment with 1 μM glibenclamide for 48 h resulted in 46.7% apoptosis in dispersed islet cells (Fig. 1D). Next, we investigated whether Ex-4 protects β -cells against chronic glibenclamide-induced apoptosis. As shown in Fig. 2A, the MTT assay revealed that pretreatment of the HIT-T15 β -cells with Ex-4 significantly protected against the glibenclamide-induced apoptosis. Similar results were

obtained by Hoechst 33342 staining and flow cytometric analysis (Fig. 2: B, C). In Hoechst 33342 staining, Ex-4 pre-treatment revealed a significant decrease of apoptotic bodies as compared to the cells treated with glibenclamide (Fig. 2B). Also, the same results were shown in FACS, with the proportion of apoptotic cells being 3.67%, 71.88%, and 3.98%, in the controls, glibenclamide-treated cells, and Ex-4 pretreated cells, respectively (Fig. 2C). In order to further investigate these results, we examined the involvement of caspase-3, an enzyme that plays an important role in the execution of apoptotic events at the final stage. Glibenclamide induced a significant increase in caspase-3 enzyme activity by 3.5-fold as compared to that of the controls, whereas pretreatment of cells with Ex-4 reduced the activity of caspase-3 and H89, a PKA inhibitor, abolished the effects of Ex-4 (Fig. 2D). Moreover, pretreatment of Ex-4 had similar anti-apoptotic effects in the dispersed islet cell. Exposure of the islet cells to glibenclamide at 1 μM for 48 h induced a significant increase in the rate of apoptotic cells (51.2%) compared with the control (5.6%). Ex-4 significantly restored glibenclamide-induced apoptosis (19.8%), whereas in the presence of H89, Ex-4 did not restore apoptotic islet cells (42.6%, Fig. 2E).

Ex-4 protects against apoptosis of β -cells by modulating the glibenclamide-induced ER stress response

To investigate the possible mechanisms of action of Ex-4 in protecting against glibenclamide-induced apoptosis of β -cells, we investigated the expression patterns of several molecular indicators of ER stress. Both mRNA and protein levels of GRP78, GRP94, ER chaperones, as well as CHOP, a transcription factor that plays a role in growth arrest and cell death, were increased by chronic treatment of glibenclamide (Fig. 3: A, B). Downstream events in the ER stress response pathway involving *XBP1* mRNA splicing and phospho-eIF2 α were induced by glibenclamide treatment (Fig. 3: C, D). Interestingly, Ex-4 and forskolin prevented both, the induction of GRP78, GRP94, CHOP, and phospho-eIF2 α and the splicing of XBP-1 in cells exposed to chronic glibenclamide treatment. Protective role of Ex-4 disappeared when H89 was present (Fig. 3).

Protective effects of Ex-4 against glibenclamide-induced reduction in SERCA expression and Ca^{2+} uptake in the ER

In order to determine the mechanisms involved in ER stress induction after glibenclamide, we investigated SERCA expression and ER Ca^{2+} uptake in cells treated with glibenclamide with and without Ex-4. A 48-h treatment with glibenclamide resulted in a reduction in SERCA2 and SERCA3 mRNA, to levels comparable to

Fig. 1. Chronic glibenclamide (Gliben)-induced apoptosis in HIT-T15 cells or dispersed islet cells. After exposure to 100 nM or 1 μ M Gliben, apoptosis of HIT-T15 cells or dispersed islet cells was increased by time. A) Cell viability was measured with the MTT reduction assay. B) HIT-T15 cells were exposed to Gliben (100 nM) for the indicated time. Apoptotic nuclei was stained with Hoechst 33342 and examined with a fluorescence microscope. Photographs were taken using a blue filter at a magnification of $\times 400$. C) Apoptotic cells were measured by FACS analysis after Annexin V/PI staining. D) Dispersed islet cells were exposed to 100 nM or 1 μ M Gliben for the indicated time. Apoptosis rate was determined by flow cytometry after Annexin V staining. Data are shown as the means $\pm$ S.E.M. of 6 independent experiments. *** $P < 0.001$ vs. control cells.

those observed in cells treated with thapsigargin, a SERCA inhibitor (Fig. 4A). Western blot analysis of the expression levels of these proteins revealed similar results (Fig. 4B). Pretreatment with Ex-4 recovered the glibenclamide-induced reduction of SERCA, and H89 abolished the Ex-4 effect (Fig. 4: A and B). However, the ERK inhibitor PD98059 or the PI3K inhibitor wortman-

nin did not influence the effect of Ex-4 on glibenclamide-induced reduction of SERCA (Fig. 4B). Furthermore, the glibenclamide-induced reduction in the ER Ca^{2+} uptake was reversed by Ex-4 pretreatment (Fig. 4C). These results demonstrate that glibenclamide reduces the ER Ca^{2+} uptake by blocking the expression of SERCA2 and SERCA3 and that Ex-4 effectively recovered the sup-

Fig. 2. Effects of exendin-4 (Ex-4) on chronic glibenclamide (Gliben)-induced apoptosis. HIT-T15 cells or dispersed islet cells were pretreated with Ex-4 (25 nM) for 1 h before Gliben treatment. A) Cell viability measured by the MTT reduction assay in HIT-T15 cells treated with Gliben (100 nM, 48 h) after pretreatment with Ex-4. B) Visualization of apoptotic nuclei demonstrating that Ex-4 protected against Gliben (100 nM, 48 h)-induced apoptosis. Fixed cells were stained with Hoechst 33342 and examined by a fluorescence microscope. Photographs were taken using a blue filter at a magnification of 400 ×. C) Flow cytometric analysis of apoptosis in HIT-T15 cells exposed to Gliben (100 nM) for 48 h. Apoptotic cells were measured by FACS analysis after Annexin V/PI staining. D) Western blot analysis and colorimetric assay data on the effects of Ex-4 on caspase-3 activity in HIT-T15 cells treated with Gliben (100 nM, 48 h) after being pre-incubated with Ex-4 or/and H89 for 1 h. E) Dispersed islet cells were treated with Gliben (1 μM, 48 h) after pretreatment with Ex-4 or/and H89. Apoptosis rate was determined by flow cytometry after Annexin V staining. Data shown are the means ± S.E.M. of 3 independent experiments. **P* < 0.001 vs. control cells, #*P* < 0.001 vs. Gliben, ^s*P* < 0.001 vs. Gliben + Ex-4 pretreatment.

Fig. 3. Effects of exendin-4 (Ex-4) on ER stress-induced molecular proteins (GRP78, GRP94, CHOP, eIF2 α , and XBP-1). HIT-T15 cells were pretreated with H89 (10 μ M), forskolin (Fsk, 20 μ M), and Ex-4 (25 nM) for 1 h, followed by the addition of glibenclamide (Gliben) (100 nM) for 48 h. A) Expression levels of GRP78, GRP94, and CHOP were examined by real time-RT-PCR. Data are expressed as the ratio of their expression levels to that of *GAPDH* in the same sample. *GAPDH* was used as an internal control. B) Effects of the Ex-4 pretreatment on GRP78, GRP94, and CHOP levels in Gliben-treated cells were determined by western blotting. β -Actin was used as an internal control. C) Changes in the expression levels of phospho-eIF2 α and eIF2 α were evaluated by western blotting. D) Effect of Ex-4 pretreatment on XBP-1 mRNA splicing was detected by RT-PCR. Data are shown as the means $\pm$ S.E.M. of 3 independent experiments. ** P < 0.01 vs. control cells, # P < 0.05 vs Gliben, $^{\$}$ P < 0.05 vs Gliben + Ex-4 pretreatment.

pressive action of glibenclamide on SERCA expression through a PKA-dependent pathway.

Discussion

Although sulfonylurea is widely used for the treatment of type 2 diabetes, its long-term use may cause secondary failure of the drug via β -cell dysfunction and apoptosis; this is indeed concerning. The results of the MTT assay, Hoechst 33342 staining, and flow cytometric analysis in HIT-T15 β -cell lines and dispersed islets showed chronic sulfonylurea treatment induced apoptosis (Figs. 1 and 2). It has already been reported that long-term treatment with sulfonylurea induces apoptosis in RINm5F and MIN6 β -cell lines and human islet cells (5–7). Recent reports have speculated that the long-term use of sulfonylurea leads to β -cell apoptosis through ER stress (23), but the mechanism underlying this phenomenon is not yet fully known.

The pancreatic β -cells have highly developed ER

structures and ER stress-transducer proteins because the processing of insulin requires actively regulated ER function (8, 9). In the plasma of type 2 diabetes patients after long-term use of sulfonylurea, the proportion of proinsulin and other non-mature forms to mature insulin is increased (24, 25); the rapid and overloaded insulin processing exhausted islets and possibly induce ER stress. In the present study, we suggested that chronic exposure to glibenclamide, one of the commonly used sulfonylurea compounds, induced ER stress and finally brought on β -cell apoptosis. Treatment with glibenclamide (100 nM) for 48 h induced the activation of molecular chaperones such as GRP78 and GRP94, XBP-1 splicing, phospho-eIF2 α , and the target gene CHOP (Fig. 3). Thus, sulfonylurea-induced chronic β -cell apoptosis might cause secondary failure to sulfonylurea via unresolved ER stress.

Commonly ER stress-induced cell death is caused by oxidative stress, nutrient deprivation, hypoxia, glycosylation reactions, and disturbances of cellular calcium

Fig. 4. Protective effects of exendin-4 (Ex-4) against glibenclamide (Gliben)-induced attenuation of SERCA expression and Ca^{2+} uptake by the ER. HIT-T15 cells were pretreated with H89 (10 μM), PD98059 (50 μM), wortmannin (100 nM), thapsigargin (TG, 1 μM), and Ex-4 (25 nM) for 1 h, before addition of Gliben (100 nM) for 48 h. A) Expression levels of SERCA2 and SERCA3 were detected by real time-RT-PCR. Data are expressed as the ratio of the expression levels of these proteins to that of *GAPDH*, used as a loading control. Open columns: SERCA2, shaded columns: SERCA3. B) SERCA2 and SERCA3 expression was detected by western blotting in whole cell lysates. β -Actin was used as a loading control. C) ER Ca^{2+} uptake was measured in Fura2/AM-loaded cells by fluorescence Ca^{2+} imaging. Data are shown as the means $\pm$ S.E.M. of 3 independent experiments. * P < 0.05, ** P < 0.001, *** P < 0.001 vs. control cells; # P < 0.01 vs. Gliben; \$ P < 0.05 vs. Gliben + Ex-4 pretreatment.

homeostasis (26, 27). Sustained increased intracellular Ca^{2+} by chronic use of sulfonylurea can induce cellular damage of β -cells through apoptosis (5 – 7). Also, ER calcium depletion is one of the ER stress inducers, like thapsigargin induced ER stress. We need to investigate the relation of ER Ca^{2+} content and beta cell death through ER stress after sulfonylurea treatment. The ER, an important structure for folding of secreted proteins, has an important role in Ca^{2+} storage and signaling (11, 12). The resting intra-ER Ca^{2+} concentrations were 3 to 4 times higher than that of cytosolic Ca^{2+} (10). Dysregulation of Ca^{2+} balance in the ER could elicit ER stress and subsequently initiate the ER stress response (11). A high

Ca^{2+} gradient is generated by the SERCA proteins, which pump Ca^{2+} into the ER, and the $\text{Ins}(1,4,5)\text{P}_3$ and ryanodine receptors, which release Ca^{2+} from the ER (11, 12). Three SERCA genes (SERCA1, SERCA2, and SERCA3) have been identified, all of which can generate several alternatively spliced isoforms (28, 29). The pancreatic islet cells express SERCA2 and 3 isoforms of SERCA3 (30). The β -cells, compared to other cell types, show marked sensitivity to apoptosis induced by the blocking of SERCA activity (31, 32). Our present results show that the 48-h treatment of glibenclamide inhibited the expression of SERCA2 and SERCA3 and then, reduced Ca^{2+} uptake in the ER. These results suggest that sulfo-

nylurea could induce apoptosis not only by elevated cytosolic Ca^{2+} but also by dysregulation of Ca^{2+} homeostasis through decreased SERCA expression.

The long-acting GLP-1R agonist Ex-4, which was originally derived from *Heloderma suspectum*, is a homolog of GLP-1 and is known to have a potent antidiabetic effect (16, 17). GLP-1 and Ex-4 have been reported to enhance insulin release, activate β -cell replication, and prevent β -cell exhaustion and ER stress-induced apoptosis (16–19). GLP-1 is known to cause cAMP-dependent phosphorylation of phospholamban, which has been reported to activate the SERCA genes in endothelial cells (33). GLP-1, the adenylate cyclase activator forskolin, and the membrane-permeable cAMP analogue dibutyryl AMP are known to increase Ca^{2+} uptake in the ER through the activation of SERCA in β -cells (34). Therefore, we tested the effects of Ex-4 on the expression of ER stress-related molecules, SERCA, and the Ca^{2+} uptake from the ER of β -cells exposed to chronic glibenclamide treatment. Interestingly, we found that Ex-4 resolved the chronic glibenclamide-induced ER stress response by reducing GRP78 and GRP94 levels, XBP-1 splicing, and phosphor-eIF2 α and CHOP expression (Fig. 3). Additionally, pretreatment with Ex-4 protected against the apoptotic cell death through increased SERCA2 and SERCA3 expression and increased Ca^{2+} influx through SERCA (Fig. 4). These results indicated that Ex-4 can protect against β -cell from sulfonylurea-induced Ca^{2+} -dependent ER stress and apoptosis.

GLP-1 induces adenylyl cyclase activation and elevation of intracellular cAMP levels, which signal to PKA (14, 35). The activation of the cAMP–PKA signaling pathway promotes antiapoptotic action in pancreatic islet β -cells (14, 18, 35). In the present study, the protective effects of Ex-4 against glibenclamide-induced apoptosis were blocked by H89, a PKA inhibitor (Figs. 2–4). Recently, cAMP-guanine nucleotide exchange factor (GEF, also known as Epac) has been shown to be a novel cAMP sensor in the PKA-independent pathway by GLP-1 (36). The physio-biological functions of the Epac signaling pathway have been rapidly recognized in various cells, including calcium handling, cell proliferation, survival, differentiation and polarization, hormone secretion, and neuronal signaling (36). We focused on clarifying the regulation of glibenclamide-induced apoptosis by the cAMP–PKA pathway. However, in further studies, we will need to demonstrate the effect of Epac on glibenclamide-induced apoptosis and determine the relationship between the PKA- and Epac-dependent pathway in the expression of SERCA and the response of Ca^{2+} -dependent ER stress.

Although the mechanisms underlying the protective effects of Ex-4 on sulfonylurea-induced chronic β -cell

apoptosis cannot be clearly defined from the results of this study, it is likely that chronic exposure to glibenclamide induces apoptosis by inducing a Ca^{2+} -dependent ER stress response in β -cell lines and islets. Ex-4, a GLP-1–receptor agonist, can protect against long-term glibenclamide-induced β -cell loss through the ER stress caused by decreased expression of SERCA and ER Ca^{2+} depletion. These findings will help us understand one cause of glibenclamide-induced β -cell loss and therapeutic availability of GLP-1–based drugs in secondary failure by sulfonylurea during treatment of diabetes.

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