The pathophysiologic mechanism of glycated albumin-induced pancreatic islets dysfunction

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The pathophysiologic mechanism of glycated albumin-induced pancreatic islets dysfunction

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ABSTRACT

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The purpose of this study was to investigate whether glycated albumin (GA), an early precursor of Advanced Glycation End-product (AGEs), would induce dysfunction in pancreatic ß-cells and to determine which kinds of cellular mechanisms are activated in GA-induced-cell apoptosis. Decreased viability and increased apoptosis were induced in INS-1 cells treated with 2.5 mg/mL GA under 16.7 mM high-glucose conditions. Insulin content and glucose-stimulated secretion from isolated rat islets were reduced in 2.5 mg/mL GA-treated cells. In response to 2.5 mg/mL GA in INS-1 cells, autophagy induction and flux decreased as assessed by green fluorescent protein–microtubule-associated protein 1 light chain 3 dots, microtubule - associated protein 1 light chain 3-II conversion, and sequestosome1(SQSTM1)/p62 in the presence and absence of bafilomycin A1. Accumulated SQSTM1/p62 through deficient autophagy activated the nuclear factor-B (p65)-inducible nitric oxide synthase(NOS)-caspase-3 cascade, which was restored by treatment with small interfering RNA against p62. Small interfering RNA treatment against autophagy-related protein 5 significantly inhibited the autophagy machinery resulting in a significant increase in

iNOS-cleaved caspase-3 expression. Treatment with 500 μM 4-phenyl butyric acid significantly alleviated the expression of endoplasmic reticulum stress markers and iNOS in parallel with upregulated autophagy induction. However, in the presence of bafilomycin A1, the decreased viability of INS-1 cells was not recovered. Glycated albumin, an early precursor of AGE, caused pancreatic-ß cell death by inhibiting autophagy induction and flux, resulting in nuclear factor-B (p65)-iNOS-caspase-3 cascade activation as well as by increasing susceptibility to endoplasmic reticulum stress and oxidative stress.

Key words: glycated albumin, diabetes mellitus, islet cell dysfunction, pathophysiology

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I. INTRODUCTION

In the natural course of type 2 diabetes (T2D), declines in β-cell function and mass are accompanied by a reciprocal increase in β-cell apoptosis[1]. Although controversies still remain as to the simple cause of this apoptosis, glucose and lipid toxicities to pancreatic β-cells in diabetics are known to play major roles in triggering intra- or extracellular oxidative stress, which ultimately activates apoptotic pathways leading to dysfunctional β-cell death[2,3]. Recently, growing evidence on the glycation toxicity to pancreatic β-cells has also grown, especially in advanced glycation end-products (AGE)[4-10] . Glycation is a simple process whereby abundant fructose or glucoses attach themselves to intact lipid or protein molecules in the non-enzymatic manner. Further advanced modifications in these early glycation products (Amadori adducts), such as oxidation, rearrangement, polymerization and cleavage, give rise to irreversible conjugates, called AGEs[11]. In accordance with this trend of glycation toxicity to pancreatic β-cells, we previously demonstrated that the receptor for AGE (RAGE) and the RAGE–ligand S100 are expressed on INS-1 cells, a rodent insulinoma cell line, and on islets of the Otsuka Long Evans Tokushima Fatty (OLEFT) rat. We further demonstrated that RAGE ligands induced islet cell apoptosis via NADPH oxidase-mediated ROS generation. Based on these results, we suggested that the RAGE ligand-RAGE interaction may also contribute to

progressive β-cell dysfunction in T2D by inducing oxidative stress[10].

Although glycated albumin (GA) may initially be viewed as an adjunct to glycated hemoglobin (A1c) in monitoring glucose excursions, it is gaining popularity as intermediate-term glycemic indicator (2-3 weeks) as well as an atherogenic protein in the development of diabetic complications including coronary artery disease[11-13]. Because the non-enzymatic glycation reaction occurs between high levels of glucose and reactive amino acid groups followed by the formation of Schiff bases, Amadori products, and finally AGE in a stepwise manner, it is also conceivable that GA could be viewed as not an active AGE but an early precursor of AGE[11,14,15]. Besides the well-established mechanism of oxidative stress or ER stress to pancreatic β-cells, autophagy dysfunction in β-cells is now gaining popularity to understand the biologic mechanism of pancreatic islet[16,17]. Autophagy as an evolutionarily conserved cellular process for lysosomal recycling of cytoplasmic material, which mediates the elimination of misfolded proteins and excess accumulation of damaged organelles[18,19], and the inhibition of autophagy leads to the death of pancreatic β-cells death. Despite involvement of autophagy in ameliorating β-cell dysfunction in vitro[20-22], its role in β -cell dysfunction is an area to be elucidated under various stressors. Based on previous reports, we hypothesized that elevated levels of GA might induce dysfunctional pancreatic β-cells and investigated its pro-apoptotic pathways

II. MATERIALS AND METHODS

1. Islet isolation cells and cell culture

Pancreatic islets were isolated from male Sprague-Dawley rats (250-300 g) by collagenase digestion and separated by discontinuous gradient purification as previously described[2,19]. The islets and INS-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin, and 10 mM HEPES at 37°C in a humidified incubator containing 5% CO_2 and 95% air. The Animal Care Committee of the Catholic University of South Korea approved the experimental protocol by the Institutional Animal and Use Committee (IACUC No. 2012-0078-01), and all procedures performed in this study were followed by ethical guidelines for animal studies.

2. Cell viability assay

INS-1 cells were treated with 10μl of the cell counting kit-8(CCK-8) solution (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 24 h after incubation with various concentrations of BSA (bovine serum albumin, catalog No. A2153) or human GA in the presence of 16.7 mM glucose for the indicated times according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

3. Apoptosis assay

Apoptosis was measured using the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA, USA) by flow cytometry, according to the manufacturer's protocol. Briefly, after treatment with BSA or GA, INS-1 cells were harvested after the incubation period and washed in cold PBS followed by AV binding buffer and incubated with 5μl AV-FITC and propidium iodide (PI) for 15 min at room temperature. The stained cells were analyzed by a BD FACSCalibur™ flow cytometry (Franklin Lakes, NJ, USA). Cells were considered early apoptotic when they were AV-positive but PI-negative and late apoptotic when they were both AV- and PI-positive.

4. Acridine orange/PI staining

Rat islets which were treated with GA or BSA for 24 h were stained with 0.67 mmol/L acridine orange (AO) and 75 mmol/L propidium iodide (PI) (Sigma, St Louis, MO, USA) in PBS for 30 min at 37.8°C with 5% CO2. After being washed once with PBS, and then the islets were observed under the confocal microscopy. PI positive islets were measured by pixel count method using photoshop® CS2 (Adobe, San Jose, CA, USA) (20). Cells fluorescing red, either fully or partially, were regarded as non-viable.

5. Measurement of intracellular ROS generation

INS-1 cells were plated at 2 x 10^4 in poly-lysine-coated culture disks. At the indicated time points, cells were washed with PBS and incubated in the dark with 10 μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) in PBS for 30 min at 37°C. Fluorescence was measured on a NOVOstar Microplate Reader (Ramcon, Birkerød, Denmark) and flow cytometry . Values are expressed as the fluorescent cell percentage of total cell counts.

6. Immunoblotting

Cells were lysed in PRO-PREPTM (iNtRON Biotechnology, Kyungki-Do, Korea). Whole proteins were extracted by centrifugation at 13,000 rpm for 5 min at 4°C, and protein concentrations in the lysates were determined by the Bradford method using the dye reagent concentrate (Bio-Rad). Whole-cell lysates were mixed with 5X SDS sample buffer [125mM Tris Cl (pH 6.8), 4% SDS, 4% 2-mercaptoethanol, and 20% glycerol]. Equivalent amounts of protein were loaded onto 8–15% poly-acrylamide gels, electrophoresed, and transferred onto nitrocellulose membranes (BIO-RAD). After blocking with blocking buffer [Tris-bufferd saline (pH7.6), 0.1% Tween 20, 5% skim milk] for 1 h, target antigens were reacted with primary antibodies and subsequently secondary antibodies (horseradish peroxidase-conjugated antimouse IgG or antirabbit IgG antibodies). Immunoreactive bands were developed using the ECL Plus system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) to visualize

immunoreactive bands according to the manufacturer's instructions.

7. Reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manual sheets provided with the product, and SuperScript III First-Strand kit (Invitrogen, Carlsbad, CA, USA) was used to perform cDNA synthesis. cDNA from siATG5-trasnfected INS-1 cells was subjected to standard PCR with Taq Polymerase (Takara, Tokyo, Japan) to detect ATG5 expression. cDNA from rat islets, which were treated with GA or BSA, was used for real-time PCR with SYBR Green (Applied Biosystems, Foster City, CA, USA) to detect relative mRNA levels. For ATG5 detection by standard PCR, ATG5 forward primer (5'- CCCTCCAGAAGAAAATGGAT-3') and reverse primer (5'-ATAGCTCAGATGCTCGCTCA-3') were used with the following program: (1) 94℃ for 4min, (2) 35 cycles of 94 ℃ for 45 s, 56 ℃ for 30 s, and 72 ℃ for 30s, and (3) 72 ℃ for 7 min. PCR products were separated by agarose gel electrophoresis. Real-time PCR was performed using the following primers for rat islets: ATF4 forward primer 5'-TATGGATGGGTTGGTCAGTG-3' and reverse primer 5'-CTCATCTGGCATGGTTTCC-3'; glucose-regulated protein 78(GRP78) forward primer 5'-CCACCAGGATGCAGACATTG-3', reverse primer 5'-AGGGCCTCCACTTCCATAGA-3'; CCAAT enhancer-binding protein homologous protein (CHOP) forward primer 5'-CCACACCTGAAAGC AGAAAC-3', reverse primer 5'-CACTGTCTCAAAGGCGAAAG-3'; Beclin-1 forward primer 5'-TTCA AGATCCTGGACCGAGT-3', reverse primer 5'-AGACACCATCCTGGCGAG TTTC-3'; light chain-3B(LC3B) forward primer 5'- CATGCCGTCCGAGAAGACCT-3', reverse primer 5'-GATGAGC CGGACATCTTCCACT-3'; SQSTM1/ p62 forward primer 5'-GCCCTGTA CCCACATCTCC-3', reverse primer 5'- CCATGGACAGCATCTGAGAG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer 5'-TCAAGAAGGTGGTGAAGCAG-3', reverse primer 5'-AGGTGGAAGAAT GGGAGTTG-3'; i- NOS forward primer 5'-GGGAGCCAGAGCAGTACAAG

-3', reverse primer 5'-GGCTGGACTTCTCACTCTGC-3'; tumor necrosis factor alpha (TNF-α) forward primer 5'-GTCGTAGCAAACCACCAAGC-3, reverse primer 5'- TGTGGGTGAGGAGCACGTAG -3'; superoxide dismutase 2(SOD2) forward primer 5'-GGCCAAGGGAGATGT TACAA-3', reverse primer 5'-GCTTGATAGCCTCCAGCAAC-3'. Quantitative analyses were performed using the ΔΔCT method and StepOne Software v2.2.2.

8. Analysis of GFP-LC3 dots

For the GFP-LC3 punctate assay, INS-1 cells transfected with GFP-LC3 plasmid (ORIGINE Technologies, Inc. Rockville, MD; RC100021 MAP1LC3B -N-tGFP)(23) were grown in the Lab-Tek Chambered no. 1.0 Borosilicate Coverglass System (NalgeNunc Intl., Rochester, NY, USA) and treated with 2.5 mg/mL GA and 16.7 mM glucose or a different concentration of 4-PBA (0.25, 0.5, 1mM) for 24 h. The cells were fixed with 4% paraformaldehyde and GFP-LC3 dots were observed under a fluorescence microscope (448 nm excitation and 515 nm emission) (Olympus America, Inc., Center valley, PA, USA). At least 30 cells were scored in each of two independent experiments.

9. Small interfering RNA transfection

INS-1 cells were transfected with siRNAs targeting the RAGE, ATG5, and SQSTM1/p62 (Sigma, St Louis, MO, USA), or a control siRNA using Lipofectamine 2000 following the manufacturer's instruction (Life Technologies, Grand Island, NY, USA). INS-1 cells were incubated for 16 h with a tranfection mixture at a final RNA concentration of 100 pmol and then supplemented with fresh medium.

10. Glucose-stimulated insulin release in vitro

To determine glucose-stimulated insulin release, 100 islets were washed twice using Krebs-Ringer buffer (115 mM NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 10 mM HEPES, 0.2% BSA, pH 7.4) containing 5 mM glucose and pre-incubated with the same buffer for 30 min.

The buffer was replaced with fresh buffer for 1 h, which was subsequently collected (basal) and replaced with a buffer containing 25 mM glucose for an additional 1 h (glucose stimulated). The supernatants were collected, and the concentration of insulin was measured using a rat insulin ELISA kit according to the manufacturer's instructions (Linco Research).

11. Insulin content measurement

To measure the insulin content of rat islets, the medium was removed, and 100 islets were washed with Krebs-Ringer bicarbonate buffer (25 mM HEPES, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% BSA, 5 mM glucose) and sonicated with acid ethanol. After 1 day, insulin was measured using a rat/mouse insulin ELISA kit (Linco Research, St. Charles, MO, USA). Results were normalized to the total protein concentration.

12. Materials

All chemicals, including GA, BSA, N-acetylcysteine (NAC) (antioxidant), 4-phenyl butyric acid (4-PBA) (chemical chaperone), 3-methyladenine (3-MA; class III phosphatidylinositol 3-kinase (PI3K) inhibitor), chloroquine (lysosome inhibitor), acridine orange, and propidium iodide were purchased from either Merck Bioscience (Darmstadt, Germany) or Sigma-Aldrich Corp. (St. Louis, MO). Chemicals were dissolved in either appropriate media solution or distilled water and then treated at the required working dilution. All chemicals were handled in accordance with the suppliers' recommendations. Anti-Inositol-requiring enzyme 1 α (IRE1-α), phosphorylated UPR activation markers pancreatic ER kinase (p-PERK), activating transcription factor 4 (ATF4), CHOP, activating transcription factor 6 (ATF6), spliced X-box binding protein 1 (sXBP1), RAGE and actin antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-SQSTM1/p62, LC3 and caspase-3 antibodies were from Cell Signaling Technologies (Beverly, MA, USA), and anti-iNOS antibody was from Sigma-Aldrich. Anti-SOD2 and TNF-α antibodies were obtained from Cayman Chemical (Cayman Chemical, MI, USA)

and Calbiochem (Calbiochem, Darmstadt, Germany), respectively.

13. Statistical analyses

Statistical analyses were performed using the PRISM 5.0 (GraphPad Software Inc, San Diego, CA, USA). Results are expressed as a mean ± SE. One-way analysis of variance (ANOVA) with post hoc Bonferroni multiple comparison test was used for comparisons involving more than two, whereas t-tests were used for just two groups of samples. Statistical significance was defined as $p < 0.05$.

III. RESULTS

1. Effect of GA treatment on viability and apoptosis of INS-1 cells

To determine whether treatment of GA is toxic to INS-1 cells, we examined cell viability and apoptosis. Under 5.5 mM normal glucose conditions, GA (0.1 -2.5 mg/mL) were trended to be toxic to INS-1 cells (data were not shown). Under 16.7 mM high glucose conditions, treatment with GA was toxic to INS-1 cells (GA 91.63 ± 1.33 vs. control 100.5 ± 1.10 and BSA 99.69 ± 1.40, $p < 0.001$ and $p < 0.05$, respectively). As shown in Fig. 1A, the INS-1 exposed to different concentrations (2.5 mg/mL, 5 mg/mL, 10 mg/mL) of GA showed decreased viability in a dose-dependent manner (2.5 mg/mL GA 91.63 ± 1.33 vs. 10 mg/mL GA 85.41 ± 1.57, $p < 0.05$) (n=8). INS-1 cells treated with 2.5 mg/mL GA for 24 h and 48 h showed a significantly decreased viability in a time-dependent manner as assessed by CCK-8 (n=8) (Fig. 1B). The percentage of early and late apoptotic (AV-positive) cells as assessed by annexin V/propidium iodide flow cytometry also increased in INS-1 cells treated with 2.5 mg/mL GA (n= 5) (GA 52.5 ± 2.84 vs. control 41.9 ± 2.03 and BSA 37.5 ± 2.13, $p < 0.05$ and $p < 0.01$, respectively, early & late apoptosis phase) (Fig.1C). The higher activated apoptotic signals assessed by cleaved caspase-3 western blotting assay (GA 1.58 ± 0.17 vs. control 1.00 ± 1.76e-007 and BSA 0.51 ± 0.16, $p < 0.05$ and $p < 0.01$, respectively) (n=4). To determine whether GA binds to the RAGE in the process of pancreatic β-cell apoptosis, we performed western blotting for upregulated expression of RAGE. Although RAGE is expressed in INS-1 cells, GA did not augment the expression of RAGE (Fig. 1D).

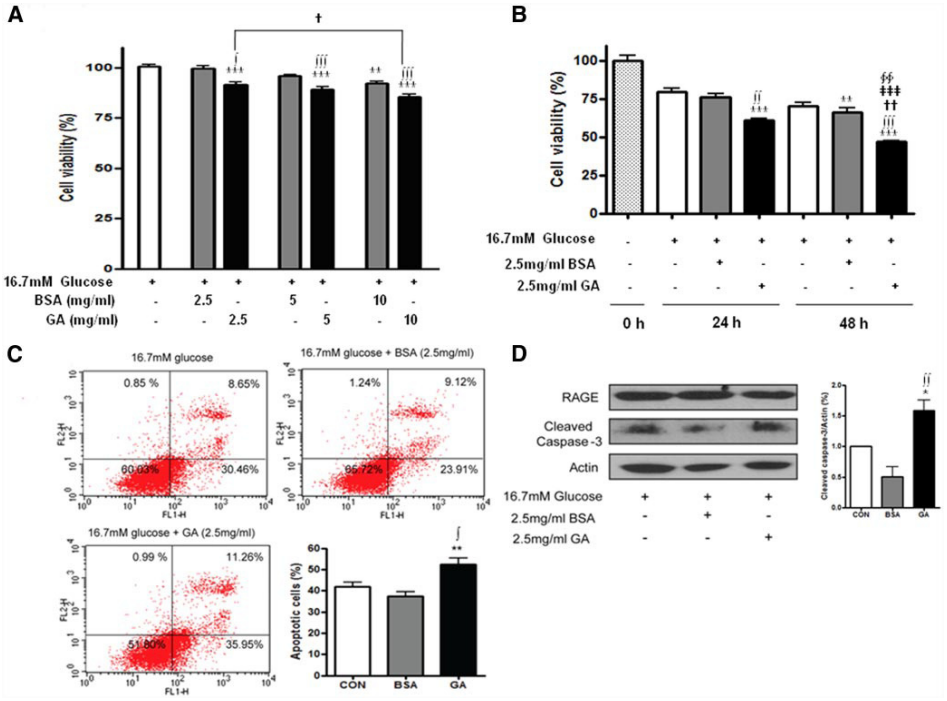

Figure 1. Viability/apoptosis of INS-1 cells after treatment with GA.

(A) In CCK-8 assay for cellular viability, relative viability (presented as % of 16.7mM glucose-treated control) of INS-1 cells exposed to 2.5 mg/mL GA was toxic to INS-1 cells. The viability of INS-1 cells exposed 2.5, 5.0, and 10.0 mg/mL GA in the presence of 16.7 mM glucose for 24 h were significantly decreased in a dose-dependent manner.

(B) 2.5 mg/mL GA under 16.7 mM high glucose conditions for 24 h and 48 h significantly decreased the viability of INS-1 cells in a time-dependent manner.

(C) In AV/PI flow cytometry, the percentage of early and late apoptotic (AV-positive) cells also significantly increased in INS-1 cells treated with 2.5 mg/mL GA. Results are shown as mean ± SE.

(D) In western blot assay for cell apoptosis, INS-1 cells treated with 2.5 mg/mL GA showed a significant increase in expression of cleaved caspase-3. GA did not augment the expression of RAGE. Results are shown as mean ± SE.

*p < 0.05, **p < 0.01, and ***p < 0.001 vs. 16.7 mM glucose treated cells for 24 h, and ∫ p < 0.05, ∫∫ p <0.01, and ∫∫∫ p < 0.001 vs. cells treated with BSA for 24 h, and †p < 0.05 and ††p < 0.01 vs. GA-treated for 24 h, and ‡‡‡p < 0.001 vs. 16.7 mM glucose treated cells for 48 h, and ∮ ∮ p < 0.01 vs. BSA-treated cells for 48 h. ANOVA test was used.

2. Effect of GA treatment on apoptosis and glucose-stimulated insulin secretion as well as insulin content of rat islets

The cytotoxicities of isolated rat islets were assessed by acridine orange/propidium iodide (AO/PI) staining, glucose-stimulated insulin secretion and insulin content. Compared to the control and BSA-treated islets, rat islets exposed to 2.5 mg/mL GA for 24 h experienced greater cell death, which was demonstrated by the positivity of red PI-positive cells (GA 3.49 ± 0.38 vs. control 0.76 ± 0.10 and BSA 0.81 ± 0.07, p<0.001 and p < 0.001, respectively) (Fig. 2A). For evaluating the levels of glucose-stimulated insulin secretion and insulin content, 100 rat islets were isolated. Under baseline 5.0 mM glucose conditions, basal insulin secretion was similar among the three groups (n=6). Islets treated with 2.5 mg/mL GA for 24 h (GA 1.13 ± 0.02 vs. control 1.34 ± 0.07 and BSA 1.32 ± 0.07 ng/mL/μg, both p < 0.05) (n=6) and 48 h (GA 0.87 ± 0.06 vs. control 1.17 ± 0.06 and BSA 1.06 ± 0.05 ng/mL/μg, p < 0.01 and p < 0.05, respectively) (n=6) secreted significantly less insulin in response to 25.0 mM glucose than 2.5 mg/mL BSA treated islets and control islets (Fig. 2B). The insulin content of 2.5 mg/mL GA-treated islets for 24 h decreased significantly compared to that of untreated- and 2.5 mg/mL BSA-treated cells (1.00 ± 0.05 vs. control 1.13 ± 0.08 and BSA 1.20 ± 0.07 ng/mL/μg, p < 0.01 and p < 0.05, respectively) (n=3). However, the insulin contents of GA treated islets for 48 h was significantly lower than the only BSA treated group (GA 0.84 ± 0.10 vs. BSA 1.09 ± 0.05 ng/mL/μg, p < 0.05) (n=3) (Fig. 2C).

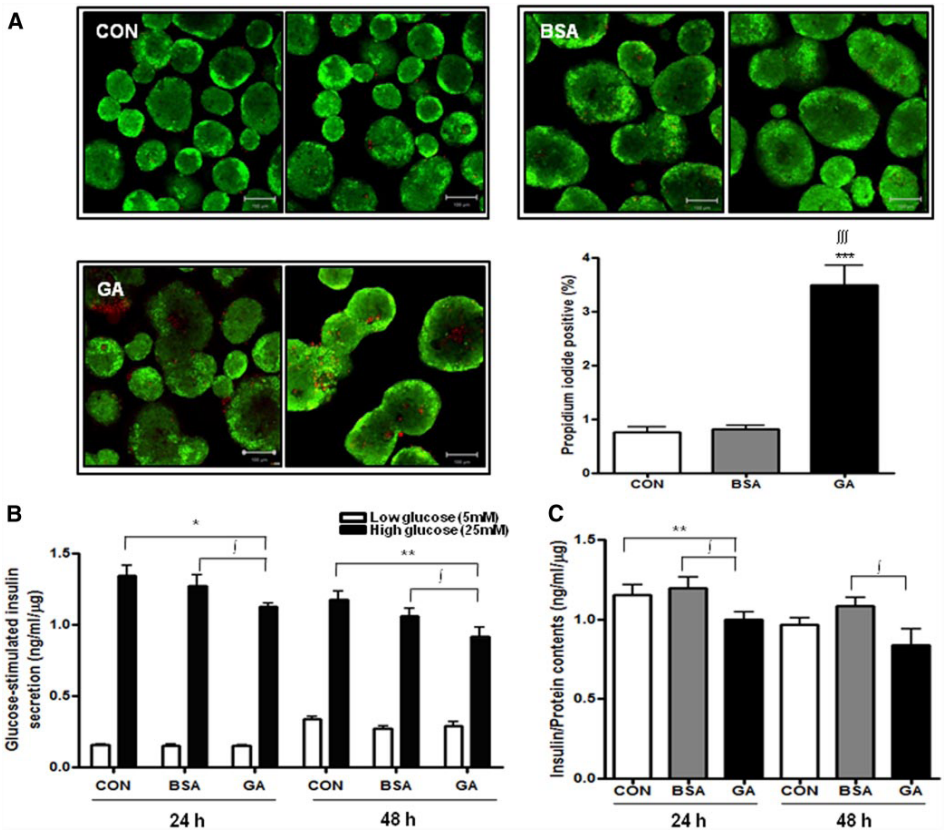

Figure 2. Effect of GA treatment on apoptosis and glucose-stimulated insulin secretion in rat islets.

The cytotoxicities of isolated rat islets were assessed by acridine orange/propidium iodide (AO/PI) staining and glucose-stimulated insulin secretion and insulin content of rat islets.

(A) In AO/PI staining for viability of rat islets, red pink colored apoptotic cells increased by 2.5 mg/mL GA compared with control and BSA-treated islets for 24 h. The red-PI positive cells assessed by confocal microscopy were significantly increased in GA-treated islets. (GA 3.49 ± 0.38 vs. control 0.76 ± 0.10 and BSA 0.81 ± 0.07%, $P < .001$, respectively)

(B) Insulin release in a response to 25.0 mM glucose was significantly lower in 2.5 mg/mL GA-treated islets for 24 h and 48 h as compared to untreated and 2.5

mg/mL BSA-treated islets. The secretion levels are expressed in nanograms per milliliter per micrograms.

(C) The insulin to protein ratio that reflects the level of insulin contents in INS-1 cells was statistically lower in 2.5 mg/mL GA-treated islets for 24 h and 48 h than in 2.5 mg/mL BSA-treated islet cells.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. untreated control cells, and ∫$p < 0.05$, ∫∫∫$p < 0.001$ vs. BSA-treated cells. ANOVA text (A) and Student's t tests (B, C) were used.

3. Upregulated expression of ER stress and production of ROS by GA are not receptor for AGE-mediated in pancreatic INS-1 cells.

To elucidate possible apoptotic pathways of GA on the pancreatic β-cell line, we investigated the effect of GA under RAGE knockdown conditions by transfecting siRNA molecules against RAGE into INS-1 cells. To confirm that siRNA against RAGE was effectively working, we demonstrated the completely inhibited protein expression of RAGE using western blotting. Even the presence of siRNA against RAGE, cellular apoptosis was significantly increased in INS-1 cells treated with 2.5 mg/mL GA (scramble transfected cells: GA 1.18 ± 0.04 vs. control 0.97 ± 0.06 and BSA 0.99 ± 0.02, $p < 0.01$ and $p < 0.05$, respectively, siRAGE transfected cell: GA 1.454 ± 0.05 vs. control 1.24 ± 0.04 and BSA 1.23 ± 0.02, both $p < 0.05$) (n=3) (Fig. 3A). We also investigated the effects of GA on ER stress markers using western blotting, real-time PCR, and ROS (reactive oxidative stress) production using flow cytometry. We demonstrated that INS-1 cells treated with both 2.5 mg/mL GA and 16.7 mM glucose for 24 h significantly activated the expression of GRP78, ATF4, ATF6, and CHOP, as well as the phosphorylation of eukaryotic translation initiation factor 2- α (p-eIF2- α) and IRE-1α (n= 5) (Fig. 3B). We also determined that GA-treated INS-1 cells significantly increased ROS production as assessed by the 2',7'-dichlorodihydrofluorescein diacetate (H2DCFHDA) assay (GA 165.1 ± 4.31% vs. control 96.2 ± 11.3% and BSA 127.3 ± 3.03%, $p < 0.01$ and $p < 0.05$,

respectively) (n=3) (Fig. 3C). Compared to the control and 2.5 mg/mL BSA-treated rat islets, exposure to 2.5 mg/ml GA significantly activated the expression of GRP78 (GA 42.62 ± 1.10 vs. control 1.05 ± 0.11 and BSA 37.28 ± 0.94, $p < 0.001$ and $p < 0.01$, respectively) (n=3), ATF4 (GA 98.60 ± 5.72 vs. control 1.01 ± 0.07 and BSA 73.86 ± 6.37, $p < 0.001$ and $p < 0.05$, respectively) (n=3), and CHOP (GA 34.64 ± 4.57 vs. control 1.07 ± 0.19 and BSA 16.67 ± 2.29, $p < 0.001$ and $p < 0.01$, respectively) (n=3) (Fig. 3D).

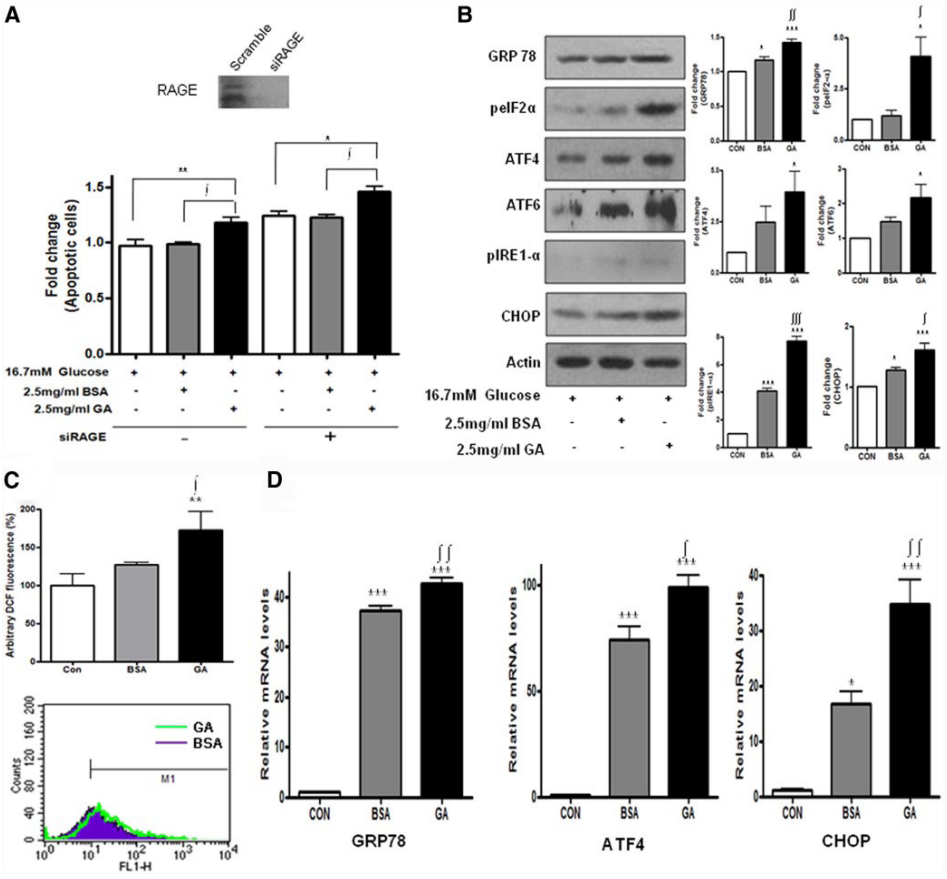

Figure 3. GA-induced ER stress upregulation and ROS production

(A) In INS-1 cells, RAGE siRNA were transiently transfected into INS-1 cells with Lipofectamine 2000. The treatment of siRNA against RAGE did not alleviate 2.5 mg/mL GA-induced cellular apoptosis.

(B) In INS-1 cells, GA significantly upregulated the expression of ER stress markers (GRP78, p-eIF2-α, ATF4, ATF6, IRE-1α, and CHOP) assessed by western blotting.

(C) In H2DCFHDA assay for ROS production by fluorescence spectrometer, increased ROS production was shown in GA-treated INS-1 cells. In the bottom panel of B, FACS analysis of ROS-sensitive carboxy-H2DCFDA probe fluorescence revealed an increase in ROS levels in the cells treated with GA.

(D) In rat islets, GA significantly upregulated the expression of ER stress markers (GRP78, ATF4, and CHOP) assessed by real-time PCR.

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. untreated control cells, and ∫ $p < 0.05$, ∫∫ $p < 0.01$, ∫∫∫ $p < 0.001$ vs. cells treated with BSA. ANOVA test was used.

4. GA-treatment in INS-1 cell reduces autophagy induction.

To elucidate possible mechanisms of autophagy deficiency-mediated dysfunctional pancreatic β-cells[16], steps of autophagy that can be monitored were performed. INS-1 cells exposed to 2.5 mg/mL GA for 24 h were shown to have decreased LC3 (microtubule-associated protein 1 light chain 3)–II conversion and increased SQSTM1/p62 expression as monitored by western blotting (n=5) (Fig.4A). In GA-treated rat islets, the mRNA expressions of beclin-1 (GA 0.80 ± 0.03 vs. control 1.00 ± 0.03 and BSA 0.96 ± 0.01, $p < 0.01$ and $p < 0.01$, respectively) and LC3B (GA 0.80 ± 0.02 vs. control 1.00 ± 0.01 and BSA 0.94 ± 0.07, $p < 0.01$ and $p < 0.05$, respectively) were significantly decreased compare to untreated control and BSA-treated islets (n=3) (Fig. 4B). Cells were transfected with a specific autophagic marker GFP-LC3 (comprising green fluorescent protein fused to the N-terminus of LC3) and visualized by fluorescence microscopy. A punctate pattern of GFP-LC3 was observed that decreased upon challenge with 2.5 mg/mL GA (GA 19.9 ± 1.99 vs. control 34.0

± 3.07 and BSA 32.1 ± 2.86, $p < 0.01$ and $p < 0.05$, respectively) (Fig. 4C). We tested whether decreased LC3-II conversion and decrease SQSTM1/p62 expression could be due to either autophagy deficiency or dysregulation of lysosomal degradation using an autophagic flux assay. In the presence of 100nM of the lysomal inhibitor bafilomycin A1, an increase in SQSTM1/p62 expression and a decrease in expression of LC3-II conversion were found in the presence of 2.5 mg/mL GA (Fig. 4D).

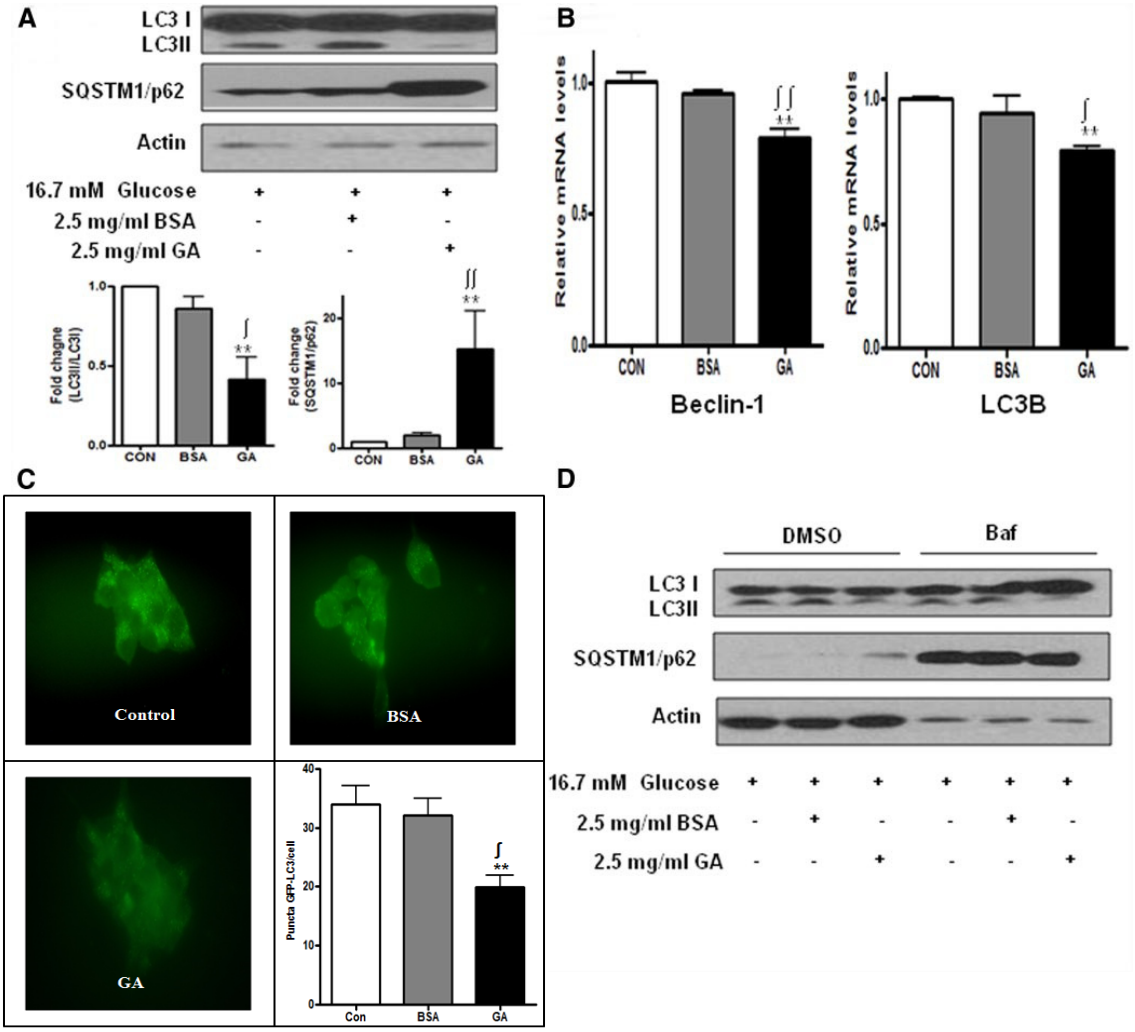

Figure 4. GA induces autophagy dysfunction in INS-1 cells

(A) In monitoring the induction steps of autophagy, assessed by LC3-I conversion to LC3-II (autophagosome) and SQSTM1/p62 expression (autophagy flux), converted LC3-II expressions were decreased, and SQSTM1/p62 INS-1 expression were increased in INS-1 cells exposed to 2.5 mg/mL GA for 24 h.

(B) In rat islets, GA significantly down-regulated the expression of autophagy markers (Beclin-1 and LC3B) assessed by real-time PCR.

(C) For monitoring of autopagosome, assessed by fluorescence microscopic assay, punctate patterns of GFP-LC3 were significantly decreased upon challenge with 2.5 mg/mL GA.

(D) In autophagy flux assays by immunoblot, in the absence or presence of 100 nM bafilomycin A1 (Baf-A, lysosomal inhibitors), an increase in SQSTM1/p62 expression and a decrease in LC3-II conversion were found in INS-1 cells treated with 2.5 mg/mL GA.

**$p < 0.01$ vs. untreated control cells, and ∫ $p < 0.05$, ∫∫ $p < 0.01$ vs. cells treated with BSA. ANOVA test was used.

5. Activation of NF-κB (p65)-iNOS cascade is SQSTM1/p62-mediated

To determine whether inappropriately inhibited autophagy induction and flux would induce β-cell dysfunction through the NF-κB and iNOS pathways, western blotting was performed. INS-1 cells exposed to 2.5 mg/mL GA significantly activated SQSTM1/p62, nuclear NF-κB (p65), iNOS, TNF-α and SOD (Fig. 5A). Regarding rat islets, GA also significantly activated the expression of iNOS (GA 16.12 ± 0.34 vs. BSA 1.45 ± 0.12 and control 0.86 ± 0.12, $p < 0.001$ and $p < 0.001$, respectively) (n=3), TNF-α (GA 1.46 ± 0.12 vs. BSA 0.87 ± 0.02 and control 1.00 ± 0.02) (n=3) and SOD2 (GA 7.80 ± 0.25 vs. BSA 1.95 ± 0.05 and control 1.00 ± 0.001) (n=3) (Fig. 5B). We also investigated the effect of GA under SQSTM1/p62 knockdown conditions. We transfected INS-1 cells with siRNA molecules against SQSTM1/p62 (sip62). Knockdown of

SQSTM1/p62 expression significantly decreased protein expression of SQSTM1/p62 as well as the expression of nuclear NF-κB (p65) and iNOS assessed by western blotting. Both were upregulated in the presence of 16.7 mM high glucose together with 2.5 mg/mL GA (Fig. 5C). Compared to BSA/scramble-treated INS-1 cells, GA/scramble-treated INS-1 cells showed significantly decreased viability as assessed by CCK-8 (100.0 ± 2.55 % vs. 70.5 ± 1.79%, $p < 0.001$). These results were similar to the results shown in Fig. 1A. Compared to GA/scramble-treated INS-1 cells, pre-knockdown INS-1 cells against SQSTM1/p62 showed statistically increased viability (70.5 ± 1.79% vs. 82.6 ± 1.63%, $p<0.001$) (n= 8) (Fig. 5D). Similar findings were seen with the pre-treatment of non-specific NF-κB inhibitor for 2 h, 1μM PDTC (pyrrolidine dithiocarbamate) (n= 9) (Fig. 5E).

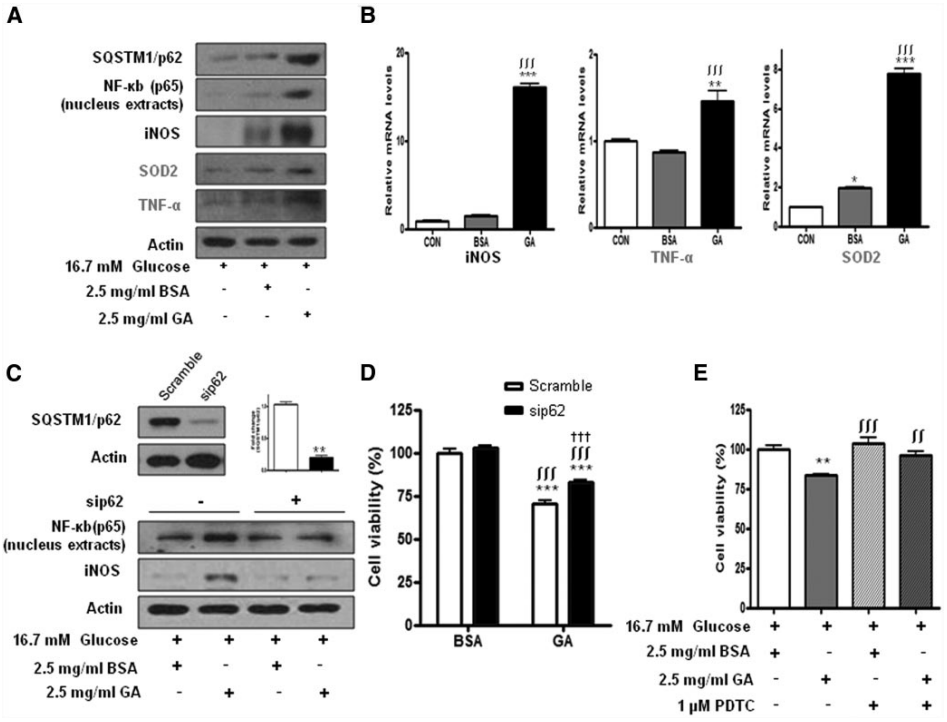

Figure 5. GA activates NF-κB (p65)-iNOS cascade through SQSTM1/p62-mediation.

SQSTM1/p62 siRNA were transiently transfected into INS-1 cells with Lipofectamine 2000.

(A) In INS-1 cells assessed by western blotting assays, 2.5 mg/mL GA upregulated the expression of SQSTM1/p62, nuclear NF-κB (p65) and iNOS.

(B) In rat islets assessed by real-time PCR, 2.5 mg/mL GA upregulated the expression of iNOS.

(C) In western blot analysis, knockdown of SQSTM1/p62 decreased the protein expression of SQSTM1/p62 as well as the expression of nuclear NF-κB (p65) and iNOS.

(D) In CCK-8 assay for cellular viability, preknockdown with SQSTM1/p62 siRNA significantly partially restored GA-induced INS-1 cell toxicity.

***$p < 0.001$ vs. BSA/scramble-treated INS-1 cells, ∫∫∫ $p < 0.001$ vs. BSA/ INS-1 cells treated with siRNA-SQSTM1/p62, †††$p < 0.001$ vs. GA/scramble-treated INS-1 cells.

(E) In CCK-8 assay, pretreatment with non-specific NF-κB inhibitor, PDTC also significantly partially restored GA-induced INS-1 cell toxicity.

**$p < 0.01$ vs. cells treated with BSA, ∫∫ $p < 0.01$, ∫∫∫ $p < 0.001$ vs. cells treated with GA. ANOVA test was used.

6. Knockdown of ATG5 further inhibited autophagy induction resulting in increased expression of iNOS-caspase-3 activity

To elucidate whether GA caused β-cell dysfunction through dysfunctional autophagy, we disrupted autophagy induction by transfecting INS-1 cells with siRNA molecules against ATG5 and then incubated them in the presence of BSA or GA. ATG5 is a protein involved in the early stages of autophagosomes elongation. It is activated by ATG7 and then forms a complex with ATG12 and ATG16L1, which is necessary for LC3-1 conjugation to phosphatidylethanolamine (PE) to LC3-II[17,21]. To confirm that siRNA against ATG5 was effectively working, we demonstrated the significantly inhibited

mRNA expression of ATG5 using RT-PCR (Fig. 6A). Treatment with siRNA against ATG5 significantly dysregulated the autophagy machinery as assessed by SQSTM1/p62 resulting in a significant increase in iNOS-related cell apoptosis assessed by cleaved caspase-3 (Fig. 6A). In addition, ATG5 siRNA minimally, but not significantly, increased ER stress markers such as p-eIF2-α, ATF5 and CHOP assessed by western blotting but significantly increased ROS production assessed by flow cytometry (Fig. 6B).

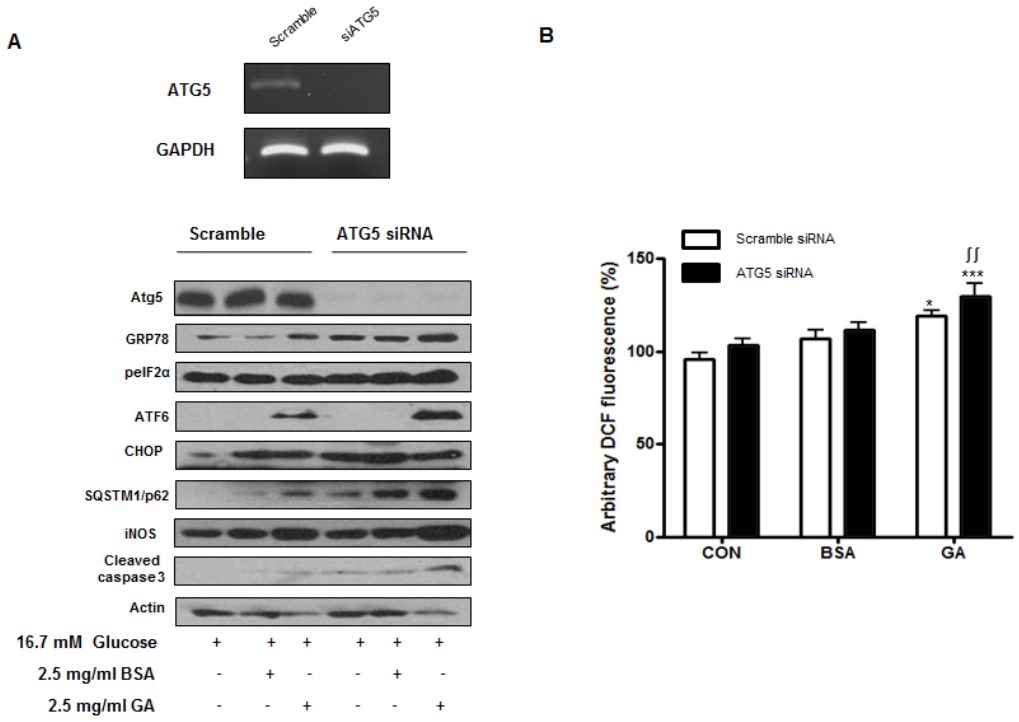

Figure 6. The autophagy inhibition by knockdown of ATG5 further increased the expression of iNOS-cleaved caspase-3 activity

ATG5 siRNA were transiently transfected into INS-1 cells with Lipofectamine 2000.

(A) In western blot analysis, GA upregulated the expression of ER stress

markers (GRP78, p-eIF2-α, ATF6, and CHOP), SQSTM1/p62, and iNOS-cleaved caspase-3 cascades. Knockdown of ATG5 further increased the expression of SQSTM1/p62 resulting in a significant increase in iNOS-related cell apoptosis as assessed by cleaved caspase-3.

(B) In H2DCFHDA assay for ROS production, ROS production was significantly further increased in INS-1 cell knockdowned with ATG5 siRNA. *$p < 0.05$ and ***$p < 0.001$ vs. untreated control cells, and ∬ $p < 0.01$ vs. cells treated with scramble siRNA. ANOVA test was used.

7. 4-PBA alleviates GA-induced pancreatic β-cell toxicity through induction of autophagy.

To determine whether restoration of GA-induced autophagy deficiency improves pancreatic β-cell dysfunction, we treated cells with 4-PBA (4-phenyl butyric acid) to act as chaperones in increasing autophagy induction. LC3-II conversion and expression of SQSTM1/p62 of INS-1 cells treated with 250 to 500 μM 4-PBA for 24 h increased and decreased, respectively, in a dose-dependent manner as assessed by western blotting (Fig. 7A). As we determined with the GA treatment of INS-1 cells and autophagy, INS-1 cells exposed to 2.5 mg/mL GA revealed significantly fewer dots of GFP-LC3 fluorescence (GA 20.0 ± 1.37 vs. control 34.0 ± 2.11 and BSA 32.1 ± 1.91, $p < 0.001$ and $p < 0.001$, respectively). Conversely, in the presence of 500 μM 4-PBA, the fluorescent dots of GFP-LC3 were significantly greater (20.0 ± 1.37 vs. 29.7 ± 1.02, $p<0.001$) (Fig. 7B). INS-1 cells in the presence of 500 μM 4-PBA for 24 h alleviated the expression of ER stress markers including p-IRE-1α, sXBP1 and CHOP, and iNOS in parallel with upregulated autophagy induction (Fig. 7C). In agreement with these findings, the decreased viability of GA-induced INS-1 cells was significantly alleviated in the presence of 500 μM 4-PBA. However, in the presence of the lysosomal formation disruption agent bafilomycin A1, the decreased viability of INS-1 cells was not recovered (Fig. 7D). Based on these results, we postulated that the induction of autophagy under these conditions

might be considered as a self-protective mechanism against cell stress.

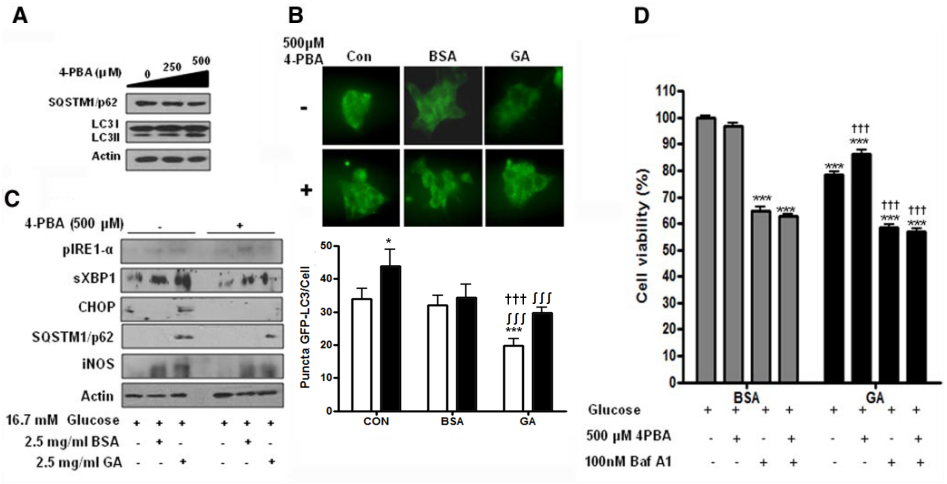

Figure7. 4-PBA alleviates GA-induced pancreatic β-cell toxicity through induction of autophagy

We treated cells with 4-PBA (4-phenyl butyric acid) to act as chaperones in increasing autophagy induction.

(A) In monitoring the induction steps of autophagy in INS-1 cells exposed to 4-PBA for 24 h, converted LC3-II expressions (autophagosome) and SQSTM1/p62 (autophagy flux) were increased and decreased, respectively, in a dose-dependent manner.

(B) For monitoring of autophagosme, punctate patterns of autophagy marker GFP-LC3 were significantly decreased upon challenge with 2.5 mg/mL GA, but pretreatment with 500 μM 4-PBA significantly partially restored GFP-LC3 punctuate.

*p<0.05 vs. cells treated with or without 4-PBA in control, ***p < 0.001, ††† p < 0.001, ∫∫∫ p <0.001 in cells treated with GA with or without 4-PBA vs. control or BSA treatment

(C) In western blot analysis, 500 μM 4-PBA alleviated the expression of ER stress markers (p-IRE1-α, sXBP1, and CHOP), SQSTM1/p62, and iNOS-cleaved caspase-3 cascades.

(D) In CCK-8 assay for cellular viability, decreased viability of GA-induced INS-1 cells were significantly alleviated in the presence of 500 μM 4-PBA. However, in the presence of the bafilomycin A1, the decreased viability of INS-1 cells were not recovered.

***$p < 0.001$ vs. cells treated with BSA and glucose, ††† $p < 0.001$ vs. cells treated with GA and glucose. ANOVA test was used.

IV. DISCUSSION

Few controversies exist regarding that the toxicity of glucolipids to pancreatic β-cells and their involvement in β-cell apoptosis[1-3,27]. With advances in the understanding of the pathogenesis of T2D, RAGE has garnered attention as circulating protein that is cytotoxic resulting in pancreatic β-cell apoptosis[4,6-8,10]. Glycation is a simple process whereby excess sugar molecules, such as fructose or glucose, attach themselves to otherwise normal proteins or lipid molecules in the blood without enzymatic intervention[11,15]. GA was initially viewed in the glycemic index for glucose monitoring, but a growing body of evidence suggests clinical relevance as an intermediate glycation index and pathogenic protein[11,15,28,29]. Based on additional reports that GA is an early precursor of AGE[11,14,15], we hypothesized that GA might play additional roles in β-cell dysfunction. We therefore investigated (1) whether GA is toxic to a β-cell line and to pancreatic islets and (2) which kinds of cellular mechanisms are activated in GA-induced β-cell apoptosis.

With respect to GA-induced pancreatic β-cell toxicity, we observed a decreased viability of INS-1 cells treated with 2.5 to 10.0 mg/mL GA in the presence of 16.7 mM high glucose for 24 h in a dose-dependent manner. The addition of 2.5 mg/mL GA in the presence of 16.7 mM high glucose also induced INS-1 cells apoptosis, which was demonstrated by the increased expression of cleaved caspase-3 and increased the apoptotic phases of flow cytometry. Regarding the isolated rat islets, more non-viable red fluorescent spots were found in rat islets treated with GA than in the control and BSA. To investigate whether or not the rat islets were functionally intact, insulin content and glucose-stimulated insulin secretions were evaluated. After a 24 h and 48 h exposure to 2.5 mg/mL GA, significant impairments in insulin synthesis and down-regulated GSIS were found. Our study is consistent with previously reported findings conducted with AGE[4-8,10] or glycated serum[30] in that glycated proteins are toxic to pancreatic β-cells, suggesting that increased circulating GA levels may result in β-cells dysfunction.

With respect to the cellular mechanisms involved in GA-induced β-cell toxicity, production of ROS and activation of endoplasmic reticulum (ER) stress occur in response to extra- and intracellular stimuli. Regarding the upregulated expression of ER stress markers in response to 2.5 mg/mL bovine serum albumin (BSA), cells that encountered conditions that demanded the unfolded protein response (UPR) to upregulated genes encoding chaperones, oxidoreductases, and ER-associated degradation (ERAD) components to upgrade the functional capacity of the ER were prone to trigger the collectively called UPR and further step of ER stress[30]. Thus, the physiologic UPR outputs or balanced interpreted ER stress markers are an adoptive process. In this study, INS-1 cells and islets showed the equivalent or beneficial cellular viability or function in response to 2.5 mg/mL BSA assessed by CCK8, AO/PI staining, and insulin secretion and contents. Similar to our results, previous studies reposted the upregaulted expressions of ER stress markers in response to BSA[31,32]. To determine whether engagement of GA-RAGE and the RAGE-dependent signaling cascade are toxic to β-cells, we measured RAGE expression on INS-1 cell exposed to both 2.5 mg/mL GA and to 16.7 mM high glucose. In contrast to previous reports performed with AGE[4,5], we did not observe that GA elevated expression of RAGE in INS-1 cells, implying that the GA-RAGE interaction is not required to induce the effects of GA on β-cell dysfunction. To proof the concept that GA might be toxic to pancreatic β-cells independent of GA-RAGE interaction, we knocked down RAGE and demonstrated that GA induced β-cells toxicity in the presence of siRNA against RAGE. This finding is similar to that of Shiraki et al., who suggested that whether or not GA binds RAGE is still unclear[29].

Autophagy as a cellular process for homeostasis is gaining popularity in diabetes mellitus research. Although autophagy is known to be involved in ameliorating pancreatic β-cell dysfunction by crosstalk with pro- and anti-apoptosis pathways[16,33], its role in β -cell dysfunction remains to be elucidated under various external stressors. To verify whether autophagy is down-regulated in GA-induced pancreatic β-cell dysfunction, we demonstrated the expression of autophagic induction and flux using autophagy monitoring in

the presence or absence of the lysosomal inhibitor bafilomycin A1 and fluorescence microscopy for quantify the number of GFP-LC3 puncta in INS-1 cells. In this study, 2.5 mg/mL GA caused an autophagy deficiency as confirmed by decreased LC3-II conversion, increased SQSTM1/p62 expression, and depressed appearance of punctuate pattern of GFP-LC3 upon challenge with GA. Moreover, we showed a decrease in mRNA levels of LC3-II and Beclin-1 in 2.5 mg/mL GA-treated rat islets. In the crosstalk between autophagy deficiency and cellular toxicity, it has been suggested that sustained SQSTM1/p62 expression resulting from autophagy defects is sufficient to upregulate NF-κB[34,35]. Based on previous reports regarding the involvement of the NF-κB-iNOS dependent pathway in AGE-induced β-cell toxicity or dysfunction[4,7,15], we investigated the crosstalk between SQSTM1/p62 and the NF-κB-iNOS-caspase 3 pathway through activation of the SQSTM1/p62-dependent NF-κB-iNOS-caspase 3 cascade by treatment with 2.5 mg/mL GA, as well as inactivation or inhibition of the NF-κB-iNOS-caspase 3 pathway by treatment with siRNA against SQSTM1/p62 in the presence of GA. As expected, the expression of SQSTM1/p62-NF-κB-iNOS was up-regulated in response to GA and down-regulated in the presence of siRNA against SQSTM1/p62 in parallel with cell viability as assessed by CCK-8. Furthermore, treatment with an NF-κB inhibitor showed a similar phenomenon. Using a knockdown model, we demonstrated that GA-induced β-cell dysfunction is mediated through autophagy deficiency. Autophagy induction was interrupted using siRNA against ATG5, which lead to increased expression of SQSTM1/p62, accompanied by increased levels of iNOS and cleaved caspase 3. Interestingly, ATG5 siRNA increased ER stress markers, but these increases were not significant. In contrast, ROS production was significantly increased. Based on these findings, we postulated that deficiencies in autophagy might be more prone to ER stress in the process of GA-induced β-cell dysfunction. There has been debate as to whether ER stress itself suppresses autophagy induction[21]. However, it is well established that autophagy plays protective roles in ER stress-induced β-cell dysfunction and apoptosis[16,33,35,36]. To verify whether autophagy deficiency causes GA-induced

β-cell dysfunction and increased ER stress, we treated INS-1 cells with 4-PBA as a chaperone to induce autophagy and to block ER stress[37] in the presence or absence of bafilomycin A1 for lysosomal formation inhibition. Autophagy flux inhibition by bafilomycin A1 decreased INS-1 viability under all conditions (control, BSA, and GA) regardless of the presence or absence of 4-PBA. Based on these results, we postulated that the induction of autophagy under these conditions might be considered as a self-protective mechanism against cell stress, including the NF-κB-iNOS-caspase 3 cascade, but that exposure of pancreatic β-cells to GA, an early form of AGE, causes deficiency in autophagy induction and flux resulting in β-cell apoptosis or dysfunction by activating the NF-κB-iNOS-caspase 3 pathway or by increasing susceptibility to ROS and ER stress. Taken together these data, we summarize that chronic exposure of the amino acid residues of albumin to reducing glucose results in non-enzymatic glycation, which subsequently formed the Amadori glycated albumin compound. The GA affects the pancreatic islet viability and function independent through the RAGE interaction. Undemonstrated way in this experiment, GA inhibited autophagy induction and flux. This deficiency of autophagy activated not only ROS production but also the expression of NF-κB-iNOS-caspase 3 pathway. Ultimately pancreatic β-cell dysfunction was induced.

Regarding the clinical relevance of GA on pancreatic islet in diabetic subjects, the theory of GA-induced pancreatic β-cell apoptosis could not fully be applied to a well-controlled diabetic state. The calculation of the GA value (%) is the percentage of GA concentration in the total albumin concentration, and the reference intervals of GA in non-diabetic Japanese, Chinese, and Caucasian were 12.3 to 16.9%, 11.0 to 17.0% and 11.9 to 15.8%, respectively[38-40]. However, the value of GA used in this study was commercially available 100% GA. In this regard, we suggest that subjects with poorly controlled diabetics might be exposed to additionally imposed glycation toxicity on underlying glucolipotoxicities.

One of the limitations of this study is that we used BSA and glycated human serum albumin (HSA) as experimental groups in accordance with previous

studies. BSA differs from HAS due to an internal deletion of one amino acid at position. In addition, after biosynthesis albumins might undergo alterations in vivo. However, 75.8% protein sequence identity and 88.0% similarity exist between HSA and BSA and there are common characteristics and function between them. In addition, they did not decisively influence the previous studies including drug targeting experiments[41-44]. Also, there was difference about degree of glycation to albumin only depending on concentration of carbohydrate, but not depending on different species[45]. Their delicate characteristic differences according to species might play minor role, and their impact remains to be determined.

V. CONCLUSION

Because GA is not an active AGE but an early precursor of it and the mechanisms of β-cell dysfunction or apoptosis by GA might be different from those of AGE such as AGE-RAGE involvement, we suggest that dysregulated autophagy might play an important role in dysfunctional pancreatic β-cells. In conclusion, GA, a precursor of the advanced glycated end-product, is toxic to the β-cell line and pancreatic islets and down-regulates autophagy in pancreatic β-cells dysfunction.

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ABSTRACT(IN KOREAN)

당화 알부민이 췌도세포 독성에 미치는 기전 규명

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본 연구의 목적은 최종당화산물 (Advanced Glycation End-product, AGE) 의 초기 전구 물질인 당화 알부민(Glycated Albumin, GA) 이 췌장 베타세포 기능 장애를 유발하는지를 알아보고, 유발한다면 GA 에 의한 베타 세포 사멸 세포학적인 기전의 종류를 확인하고자 하였다.

16.7 mM의 고농도 포도당 조건에서 INS - 1 세포에 2.5 mg/mL GA를 처리하자 생존 능력이 감소하고 세포 사멸이 증가되었다. 2.5 mg/mL GA를 처리한 SD rat으로부터 분리한 췌도세포에서 인슐린의 내용물이 줄어들고 포도당 자극 시 인슐린 분비량도 감소하였다. 2.5mg/mL GA를 처치한 INS - 1 세포에서 bafilomycin A1 의 투여 여부에 따라 형광 GFP - LC3 염색, LC3 -II 전환 및 SQSTM1/p62 측정을 통하여, autophagy induction 과 flux가 감소하는 것을 확인하였다. Autophagy가 결핍되었을 때 축적 된

SQSTM1/p62 는 NF-κB (p65)-iNOS-caspase 3 cascade 를 활성화시켰는데 이는 P62 에 대한 siRNA를 처리한 후에 회복되었다. ATG5 대한 siRNA를 처치한 경우 autophagy 생성이 억제되어 결과적으로 iNOS-cleaved caspase-3 발현을 크게 증가시켰다. 500 μM 4 - PBA 를 처리하자 autophagy 생성이 유도고 ER stress marker 와 iNOS 의 발현이 함께 감소하였다. 그러나 bafilomycin A1 이 존재하는 경우에는 감소되었던 INS - 1 세포의 생존율이 회복되지 않았다.

최종 당화산물의 초기 전구체인 당화 알부민은 ER 스트레스와 산화 스트레스 에 대한 감수성을 증가시킬 뿐만 아니라, autophagy 생성과 유출을 모두 억제하여, 결과적으로 NF- κB (P65) 와 iNOS – caspase 3 cascade 를 활성화시켜 췌장의 β- 세포 죽음을 유도함을 확인하였다.

핵심 되는 말: 당화알부민, 당뇨, 췌도세포 부전, 병인 기전

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