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The direct effect of lobeglitazone, a new thiazolidinedione, on pancreatic beta cells: A comparison with other thiazolidinediones

Min Jeong Kwon [a,b], *Yong Jae Lee* [c], *Hye Sook Jung* [b], *Hyun Mi Shin* [b], *Tae Nyun Kim* [a], *Soon Hee Lee* [a], *Byoung Doo Rhee* [a], *Mi-kyung Kim* [a,b,*], *Jeong Hyun Park* [a,b,*]

[a] *Division of Endocrinology and Metabolism, Department of Internal Medicine, College of Medicine, Inje University, Busan, Republic of Korea*
[b] *Paik Institute for Clinical Research, Molecular Therapy Lab, Inje University, Busan, Republic of Korea*
[c] *CKD Research Institute, Yongin, Gyeonggi-do, Republic of Korea*

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ABSTRACT

Aims: The direct effects of thiazolidinediones (TZDs) on pancreatic beta cells have been controversial. The aim of this study was to find out whether a novel TZD, lobeglitazone, has beneficial effects on pancreatic beta cells and db/db mice compared to those of other TZDs.

Methods: INS-1 cells were incubated at a high-glucose concentration with various concentrations of troglitazone, rosiglitazone, pioglitazone, and lobeglitazone. Apoptosis and proliferation of beta cells, markers for ER stress and glucose-stimulated insulin secretion (GSIS) were assessed. In addition, C57BL/6 db/db mice were treated with pioglitazone or lobeglitazone for 4 weeks, and metabolic parameters and the configuration of pancreatic islets were also examined.

Results: Lobeglitazone and other TZDs decreased INS-1 cell apoptosis in high-glucose conditions. Lobeglitazone and other TZDs significantly decreased hyperglycemia-induced increases in ER stress markers and increased GSIS. Metabolic parameters showed greater improvement in db/db mice treated with pioglitazone and lobeglitazone than in control mice. Islet size, cell proliferation, and beta cell mass were increased, and collagen surrounding the islets was decreased in treated mice.

Conclusions: Lobeglitazone showed beneficial effects on beta cell survival and function against hyperglycemia. The prosurvival and profunction effects of lobeglitazone were comparable to those of other TZDs.

1. Introduction

The increasing number of organ defects induced by hyperglycemia have been described as the ominous octet [1] and egregious eleven [2]. In addition to new pathogeneses that were revealed, novel treatments were also developed based on their own action mechanisms [2]. Many large clinical trials showed that recent novel medications solved previous

* Corresponding authors at: Department of Internal Medicine, Haeundae Paik Hospital, College of Medicine, Inje University, 1435 Jwa-dong, Haeundae-gu, Busan 48108, Republic of Korea (M.-k. Kim). Department of Internal Medicine, Busan Paik Hospital, College of Medicine, Inje University, 75 Bokji-ro, Busanjin-gu, Busan 47392, Republic of Korea (J.H. Park).
E-mail addresses: kmkdoc@paik.ac.kr (M.-k. Kim), pjhdoc@chol.com (J.H. Park).
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problems in clinical practices. For example, dipeptidyl peptidase (DPP)-4 inhibitors, glucagon-like peptide (GLP)-1 agonists and sodium-glucose cotransporter (SGLT)-2 inhibitors largely reduced the risk of hypoglycemia. In addition, they were safe or beneficial with regard to weight gain and cardiovascular events [3–6]. However, these medications have not yet addressed the issue of durability in glycemic control.

Thiazolidinediones (TZDs) have been traditionally known as peripheral insulin sensitizers acting in the adipose tissue, liver and skeletal muscle [7]. These compounds work through activation of the nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ). PPAR-γ expression has been confirmed in rodent and human pancreatic islets [8,9]. Several large clinical trials such as the Diabetes Prevention Program (DPP), Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication (DREAM) trial and A Diabetes Outcome Progression Trial (ADOPT) trial supported the idea that TZDs may have a protective effect on pancreatic beta cells [10–12].

Despite these clinical results, the direct effects of TZDs on pancreatic beta cells have been controversial, and few studies investigating these effects exist [13]. Troglitazone was removed from the market due to liver toxicity [14], and rosiglitazone was withdrawn in Europe after reports of myocardial infarction and cardiac-related deaths [15]. Nonoverlapping genes are regulated by troglitazone, rosiglitazone and pioglitazone [16], which may mean that various TZDs have different effects on each organ. Lobeglitazone CKD-501; Chong Kun Dang Pharmaceutical Corp., Seoul, Korea) is a novel PPAR-γ agonist with a TZD moiety and substituted pyrimidines [17]. Therefore, we tried to determine the direct effect of a novel TZD, lobeglitazone, on pancreatic beta cells and compare lobe to other TZDs.

2. Materials and methods

2.1. Cell line experiments (INS-1)

A rat insulinoma cell line, INS-1, was kindly provided by Dr. Won at Yeungnam University in Korea. INS-1 cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 11 mM glucose, 10% fetal bovine serum, 10 mM HEPES, and 50 μM 2-mercaptoethanol. All experiments were incubated at 37 °C in 5% CO_2 and studied between the 30th and 40th passages.

2.1.1. Apoptosis assay by annexin V & propidium iodide (PI) staining

After seeding the INS-1 cells at 5×10^4 cells/well in a 12-well plate, the cells were incubated for 24 h. To compare lobeglitazone (Chong Kun Dang Pharm, Seoul, South Korea) with other TZDs, cells treated with various concentrations (0.1, 1, and 10 μM) of troglitazone, rosiglitazone, pioglitazone (Sigma, St. Louis, MO, USA), or lobeglitazone with/without 20 μM GW9662 (Sigma, St. Louis, MO, USA) as a PPAR γ antagonist were treated with high-glucose (33 mM glucose) medium and incubated for 48 h. When all the reactions were complete, the cells were treated with trypsin-EDTA to form a single cell. After washing the cells with phosphate-buffered saline (PBS), the binding solution (140 mM NaCl, 10 mM HEPES pH 7.4, 2.5 mM $CaCl_2$) was added, and the plate was tapped and then centrifuged at 1200 rpm for 3 min. The supernatant was discarded, and the plate was tapped again; 5 μl of Annexin V (Interchim, Montluçon, France) and 10 μl of PI (Sigma Aldrich, Saint-Quentin-Fallavier, France) were added to the cells and reacted for 15 min in the dark. When the reaction was complete, the cells were analyzed with a flow cytometer.

2.1.2. Proliferation assay

Cells were grown on a cover slide and treated with each drug at various concentrations in the same conditions as described above. In addition, after all reactions were completed, the cells were reacted with 10 μM bromodeoxyuridine (BrdU, Sigma, St. Louis, MO, USA) for 4 h. After washing, the cells were incubated with DNase I at 37 °C for 1 h. After supernatants were aspirated, cells were fixed and permeabilized with Cytoperm/Cytofix (BD Biosciences, CA, USA) for 20 min, washed with 1X PBS, and afterward, they were reacted with anti-BrdU (Sigma, St. Louis, MO, USA) at room temperature for 1 h. Cells were incubated with a secondary antibody, anti-rabbit PE-conjugated with DAPI (10 μg/mL), for 1 h at room temperature and washed three times in 1X PBS for 5 min. The cells were places on slide glass and cover-slipped with Crystal Mount (Invitrogen Life Technologies, NY, USA). Proliferation was observed through FISH analysis performed by an Olympus BX-51 (Olympus, Tokyo, Japan) or FACSort (Becton Dickinson, BD Biosciences).

2.1.3. Western blot analysis

After the drug reactions had finished, cells were washed with PBS and lysed in RIPA buffer including protease inhibitor (Roche, CA, USA). Protein was quantified by a BCA protein assay kit (Pierce, CA, USA) and added to 1 × SDS sample buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue). Proteins were separated by 10% SDS-PAGE, transferred onto PVDF membrane, and immunoblotted with anti-phospho eIF-2α (1:1000, Cell signaling, MA, USA), anti-eIF-2α (1:1000, Cell signaling, MA, USA), anti-ATF4 (1:1000, Cell Signaling Technology, MA, USA), anti-CHOP (1:1000, Cell Signaling Technology, MA, USA), anti-phospho-p38 MAPK (1:1000, Cell Signaling Technology, MA, USA), anti-phospho-ERK (1:1000, Cell Signaling Technology, MA, USA), anti-ERK (1:1000, Cell signaling, MA, USA), anti-caspase 3 (1:1000, Cell signaling, MA, USA), anti-cleaved caspase-3 (1:1000, Cell Signaling Technology, MA, USA), and anti-α-tubulin (1:1000, Sigma, MO, USA) at 4 °C overnight. Membranes were incubated with secondary antibodies (goat anti-rabbit AP-conjugated, Cell Signaling Technology, MA, USA) for 1 h at room temperature and observed via an AP-conjugated development kit (Bio-Rad, CA, USA). Developed protein bands were quantified by a Multi Gauge V2.2 program (Fusifilm, Tokyo, Japan).

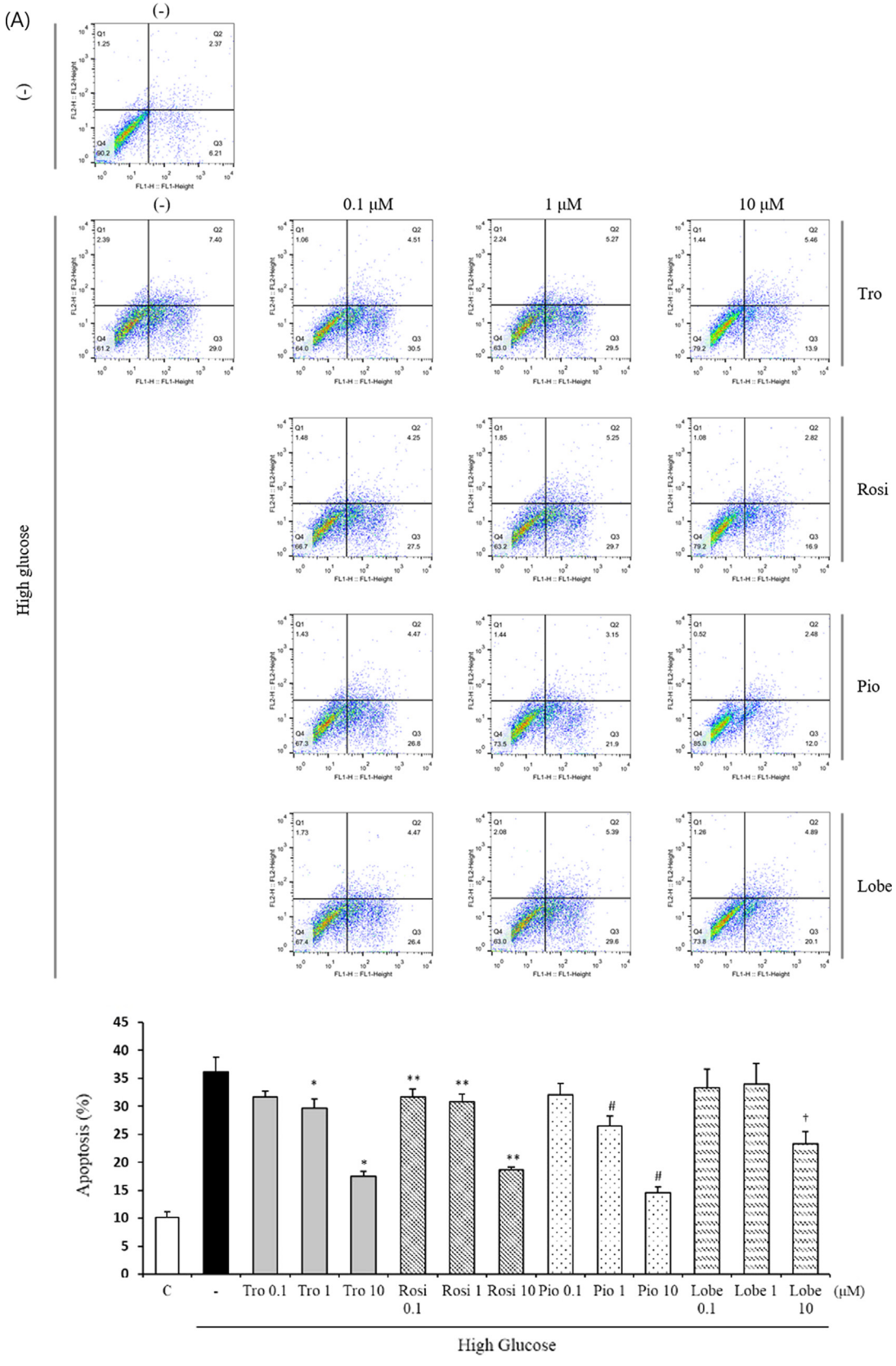
2.1.4. Glucose-stimulated insulin secretion (GSIS)

After completion of incubation, cells were washed with PBS and starved for 1 h in RPMI medium containing 3 mM glucose and 2% FBS. Then, media were added to KRBB solution (4.74 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgCl_2{\cdot}6H_2O$, 35 mM $NaHCO_3$, 10 mM HEPES) containing 3 mM glucose or 15 mM glucose, and cells were incubated for 1 h. Media were har-

vested, and cells were lysed with RIPA buffer. Secreted insulin protein in the media and insulin contents were measured using a rat/mouse insulin enzyme-linked immunosorbant assay (ELISA) kit (Linco Research, MO, USA).

2.2. *Animal experiments*

All animal experiments were approved by the Animal Use Committee of the College of Medicine, Inje University (no. 2016–034)



and were conducted in compliance with the Animal Use Guidelines of the College of Medicine, Inje University. Six-week-old male C57BL/6 db/db mice purchased from Orientbio (Gyeonggi, Korea) were used as an animal model of diabetes. They were housed five animals per cage in all experiments under controlled ambient conditions of 25 °C and a 14:10 h light/dark cycle with lights on at 07:00 h, and water and food were freely available to the animals. The mice were randomly divided into 5 groups, and pioglitazone and lobeglitazone were added to 0.5% sodium carboxymethylcellulose (Na-CMC) and ultrasonicated to uniformly suspend or dissolve the compound. Based on previous studies, pioglitazone (10 or 30 mg/kg) or lobe (1 or 10 mg/kg) was administered orally to the mice (5 mice per group) for 28 days [18,19]. Solvent control was administered as 0.5% Na-CMC in the same volume. Body weight was measured twice a week, and blood glucose levels were measured from the tail vein using a glucometer (Accu-Chek, Roche Diagnostics) twice a week. The animals were anesthetized with ketamine, and blood was collected from the heart using a 1 mL syringe. After blood collection, plasma was separated by centrifugation at 3000 rpm for 30 min at 4 °C and used as an analytical sample. Plasma was stored at −70 °C until use.

2.2.1. *Quantification of insulin, glucagon, total cholesterol, triglyceride, and low-density lipoprotein (LDL) cholesterol by ELISA*

The insulin and glucagon levels were assayed by ELISA (Linco, Darmstadt, Germany). The levels of total cholesterol, triglyceride, and LDL cholesterol were measured using ELISA (Abcam, CA, USA).

2.2.2. *Isolation of pancreatic islets from db/db mice*

Hank's Balanced Salt Solution (HBSS) solution was used for perfusions and pancreas preservations. Pancreatic islets were isolated by modified method of Lacy and Kostianovsky [20]. Pancreases were distended by intra-ductal injection of collagenase solution (0.8 mg/ml in HBSS) in cold storage into the common bile duct after occlusion of the distal end, close to the duodenum. Digestion was performed in water bath at 37 °C for 14 min 20 sec. Subsequently, the digestion was stopped by addition of cold HBSS and vortex for 15 secs to break pancreas.

After centrifuge for 10 secs at 1500 rpm, supernatant was removed and then re-suspend tissue in 30 mL HBSS and pass 400 um mesh. Filtered pancreas out was washed with HBSS and done by centrifugation on Bicoll (Density 1.10 g/ml) gradient (1.085, 1.069, 1.037 g/ml). The islets were cultured with M199 medium including 10% fetal bovine serum in no-coating plate.

2.2.3. *Immunohistochemistry (IHC) and immunofluorescence (IF)*

Pancreatic tissues from each group were fixed in 4% formalin and subsequently embedded in paraffin. The tissues were cut into 2 μm thick sections and deparaffinized. Endogenous peroxidase was blocked in 3% H_2O_2 for 30 min, and heat-induced epitope retrieval was performed using 1 mM EDTA pH 8.0. The tissues were stained with insulin, glucagon and Ki-67 antibodies (Abcam, CA, USA) for 1 h and then washed three times with 1X PBS/T. After incubation with horseradish peroxidase (HRP)-conjugated rabbit/mouse (ENV) for 1 h, immunoreactions were developed using substrate buffer with DAB + Chromogen (Dako Real™, CA, USA).

For IF staining, after heat-induced epitope retrieval, the tissues were stained with insulin and glucagon antibodies and the appropriate species-specific secondary antibody, goat anti-rabbit IgG Alexa Fluor 488 or goat anti-mouse IgG Alexa Fluor 555 (Cell Signal, CA, USA). FISH analysis was performed using an Olympus BX-51 green fluorescence microscope (Olympus, Tokyo, Japan). The beta cell mass or the alpha cell mass was estimated by multiplying the insulin-positive cell area or the glucagon-positive cell area by the pancreas weight of each mouse [21].

2.2.4. *Masson-Trichrome (MT) staining*

After deparaffinization and washing under running water for 5 min, the slides were incubated for 1 h at 60 °C in Bouin's solution. After washing under flowing water for 5 min, the slides were placed in iron hematoxylin solution for 10 min, and then, the washing step was repeated. The slides were incubated in Biebrich scarlet-acid fuchsin solution for 20 min. When the red color disappeared, they were washed and shaken briefly. Next, the slides were placed in phosphotungstic/phosphomolybdic acid solution for 5 min, followed immediately by aniline blue solution for 10 min. Then, after washing several times with water, 1% acetic acid was added for 2 min, and the slides were dried completely. Finally, the slides were placed in xylene for 5 min and then mounted.

Fig. 1 – The changes in apoptosis and proliferation of INS-1 cells with thiazolidinedione (TZD) treatments. A, Annexin V and PI (propidium iodide) assay and flow cytometry analysis were performed to assess apoptosis in INS-1 cells exposed to control (11 mM glucose) or high glucose (33 mM glucose) for 48 h with various concentrations (0.1 μM, 1 μM, and 10 μM) of troglitazone, rosiglitazone, pioglitazone and lobeglitazone (*P < 0.05 vs control). B, Apoptosis of INS-1 cells treated with troglitazone 10 μM, rosiglitazone 10 μM, pioglitazone 10 μM, and lobeglitazone 10 μM with or without a PPAR-γ inhibitor (GW9662, 20 μM) under high glucose (33 mM) was assessed by annexin V/PI (*P < 0.05 and **P < 0.01 vs each TZD without GW9662). C, After INS-1 cells were treated with troglitazone 10 μM, rosiglitazone 10 μM, pioglitazone 10 μM, or lobeglitazone 10 μM with or without GW9662 under high glucose for 48 h, bromodeoxyuridine (BrdU) was added to the culture medium to assess proliferation. BrdU-stained cells are red, DAPI-stained cells are blue. D, BrdU (+) beta cells were analyzed by flow cytometry, BD FACS Caliber. The results were compared to HG (*P < 0.05 vs HG or each TZDs without GW9662). C and Con = control, HG = high glucose, Tro and T = troglitazone, Rosi and R = rosiglitazone, Pio and P = pioglitazone. Lobe and L = lobeglitazone, GW = GW9662. Values represent means ± S.E.M. of at least three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

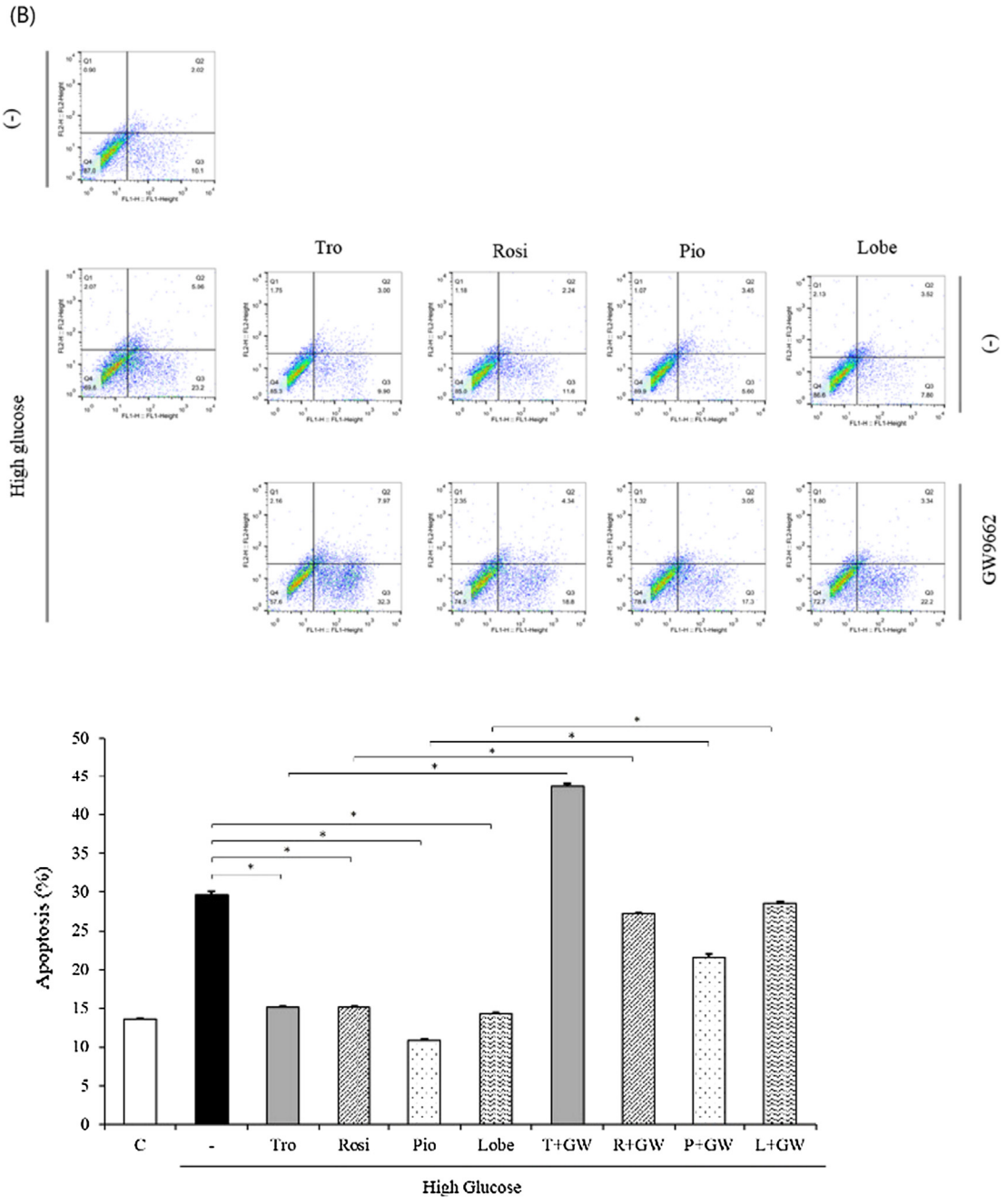


Fig 1. *(continued)*

2.2.5. *GSIS of islets*

Fifteen islets isolated from each sample were used in the GSIS experiments. Islets were starved for 1 h in RPMI medium containing 3 mM glucose and 2% FBS. Medium were added to KRBB solution (4.74 mM KCl, 1.19 mM KH2PO4, 1.19 mM MgCl2·6H2O, 35 mM NaHCO3, 10 mM HEPES) containing 3 mM glucose or 15 mM glucose and cells were incubated for 1 h. Secreted insulin protein in the media were measured using rat/mouse insulin ELISA kit.

2.3. *Statistical methods*

Values are expressed as the means ± S.E.M. Differences between two groups were analyzed using Student's *t*-test (2-tailed), and multiple comparisons were analyzed by ANOVA followed by Tukey's post hoc test) using SPSS 25.0 (SPSS Inc., Chicago, IL). P values less than .05 were considered indicative of significance.

3. Results

3.1. *Antiapoptotic effects of treatment with various TZDs against high glucose*

INS-1 cells were cultured under glucotoxic conditions (33 mM glucose) with 0.1 μM, 1 μM and 10 μM of troglitazone, rosiglitazone, pioglitazone and lobeglitazone for 48 h. In an apoptosis assay involving annexin V and PI staining, troglitazone,

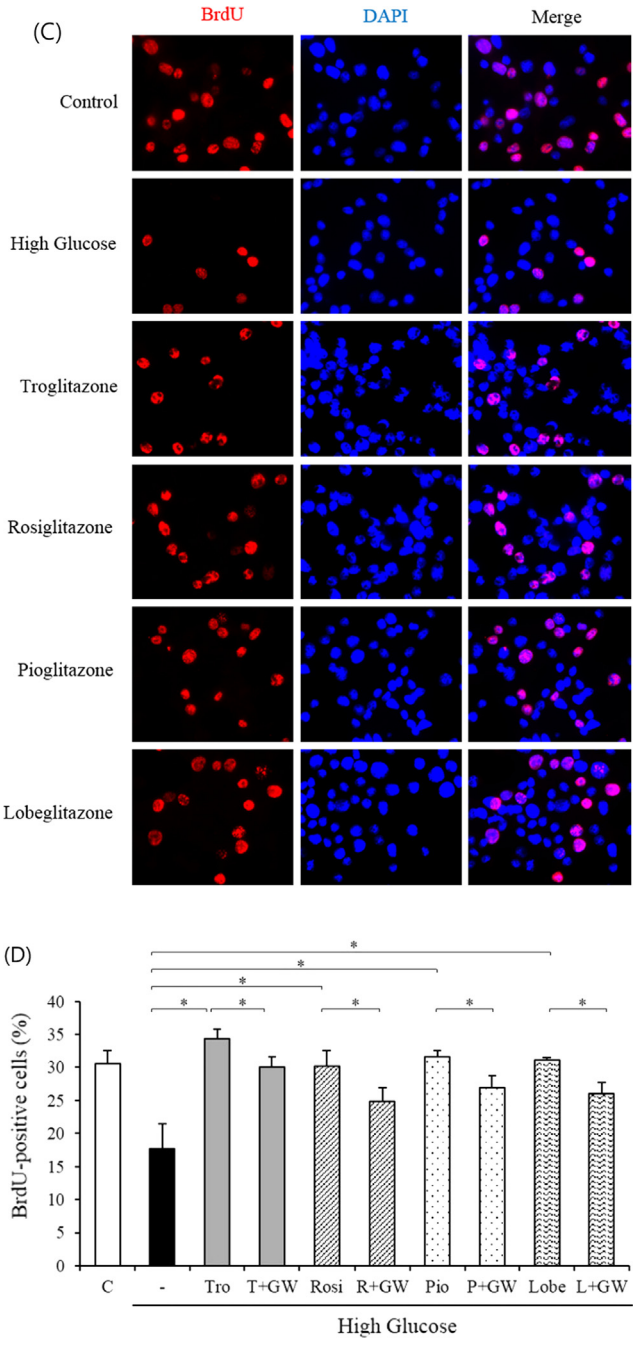


Fig 1. *(continued)*

rosiglitazone, pioglitazone, and lobeglitazone decreased the INS-1 cell apoptosis induced by high glucose. At the 0.1 and 1 μM concentrations, each TZD also decreased INS-1 cell apoptosis. However, the most significant antiapoptotic effects were observed at a concentration of 10 μM for all TZDs compared to the high-glucose condition without TZDs ($p < 0.05$). There were no significant differences among troglitazone, rosiglitazone, pioglitazone and lobeglitazone (Fig. 1A). GW9662, a PPARγ inhibitor, was coadministered with troglitazone, rosiglitazone, pioglitazone, and lobeglitazone at 10 μM under hyperglycemic conditions to determine whether TZDs work through PPARγ to protect against INS-1 cell apoptosis. When treated with the PPARγ antagonist, cells showed a significant increase in apoptosis in all TZD groups, and it was confirmed that the protective effects of TZDs were inhibited by the antagonist (Fig. 1B).

3.2. *The effects of TZDs on the proliferation of pancreatic beta cells*

Compared to control cells at 11 mM glucose, INS-1 cells exposed to 33 mM glucose showed a decrease in the percentage of BrdU (+) cells. Treatment with troglitazone, rosiglitazone, pioglitazone, and lobeglitazone increased the percentage of BrdU-stained cells compared to high glucose (Fig. 1C). When GW9662 was applied, the increased proliferation with TZDs was partially decreased (Fig. 1D).

3.3. *Changes in endoplasmic reticulum (ER) stress markers with TZDs under hyperglycemic conditions*

We observed changes in the ER stress markers, peIF-2α, ATF4, and CHOP proteins. At the high-glucose concentration (33 mM), peIF-2α, ATF4, and CHOP were increased compared to the normal-glucose control (11 mM). Lobeglitazone significantly decreased the hyperglycemia-induced increase in peIF-2α, ATF4, and CHOP, which was comparable to the effect of other TZDs (Fig. 2A–C).

3.4. *Tracing the signal transduction pathways for apoptosis and cell proliferation*

To investigate the effect of TZDs on apoptosis and cell proliferation markers, p38 MAPK, ERK, and cleaved caspase-3 were assessed by western blot. Hyperglycemia increased p38 MAPK and ERK phosphorylation in INS-1 cells. Lobeglitazone significantly decreased phosphorylated p38 MAPK and ERK expression. However, there were no significant differences in changes in p38 MAPK and ERK phosphorylation among all TZDs. Cleaved caspase-3 expression was also increased in hyperglycemia, and all TZDs including lobeglitazone significantly decreased cleaved caspase-3 (Fig. 2D–G).

3.5. *The effect of TZDs on GSIS in INS-1 cells*

To investigate the effects of various TZDs on insulin secretory capacity, responses were determined under appropriate experimental conditions. Insulin secretion was decreased 0.4-fold in INS-1 cells exposed to hyperglycemia compared to the controls. When cells were treated with TZDs, the secretion potency was recovered 2.41-fold by troglitazone, 1.57-fold by rosiglitazone, 1.31-fold by pioglitazone and 1.25-fold by lobeglitazone (Fig. 3).

3.6. *The change in metabolic parameters by pioglitazone and lobeglitazone in db/db mice*

Solvent control, pioglitazone at 10 or 30 mg/kg/d or lobeglitazone at 1 or 10 mg/kg/d was administered for 4 weeks to randomly assigned db/db mouse groups. Lobeglitazone- and pioglitazone-treated db/db mice showed a significant and gradual increase in body weight compared with the control

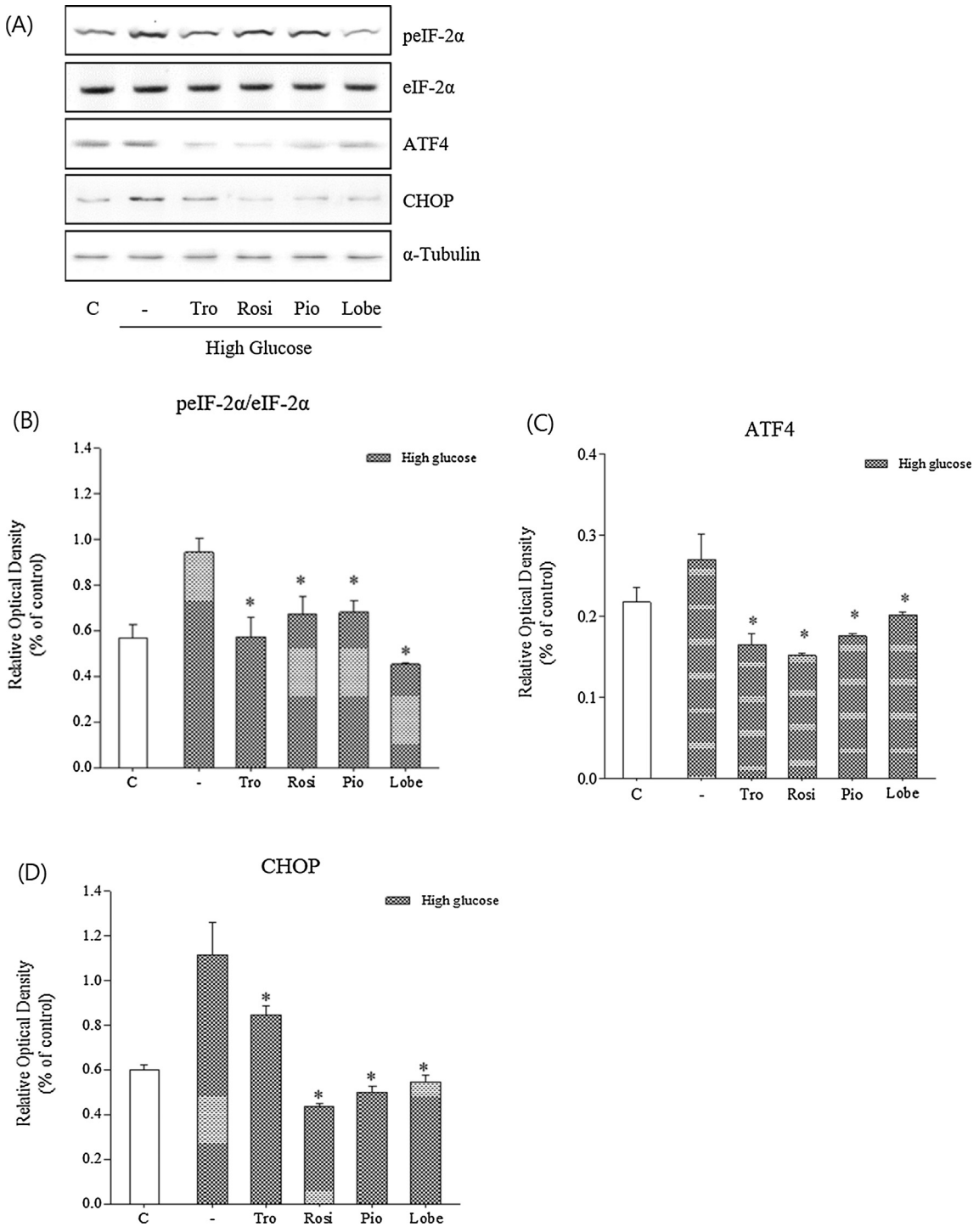


Fig. 2 – The effect of thiazolidinediones (TZDs) on endoplasmic reticulum stress markers, proliferation and apoptosis markers in beta cells. A, B, C, D, After INS-1 cells were exposed to high glucose (HG, 33 mM glucose) with or without troglitazone 10 μM, rosiglitazone 10 μM, pioglitazone 10 μM, and lobeglitazone 10 μM for 48 h, peIF-2α/eIF-2α, ATF4, and CHOP expression were measured by western blotting to evaluate ER stress markers (*P < 0.05 vs high glucose). E, F, G, H, Phosphorylation of p38 MAPK and ERK, and cleaved caspase-3 expression to assess proliferation and apoptosis markers were measured by western blotting (*P < 0.05 vs high glucose). C = control, Tro = troglitazone, Rosi = rosiglitazone, Pio = pioglitazone, Lobe = lobeglitazone. Values represent means ± S.E.M. of at least three independent experiments.

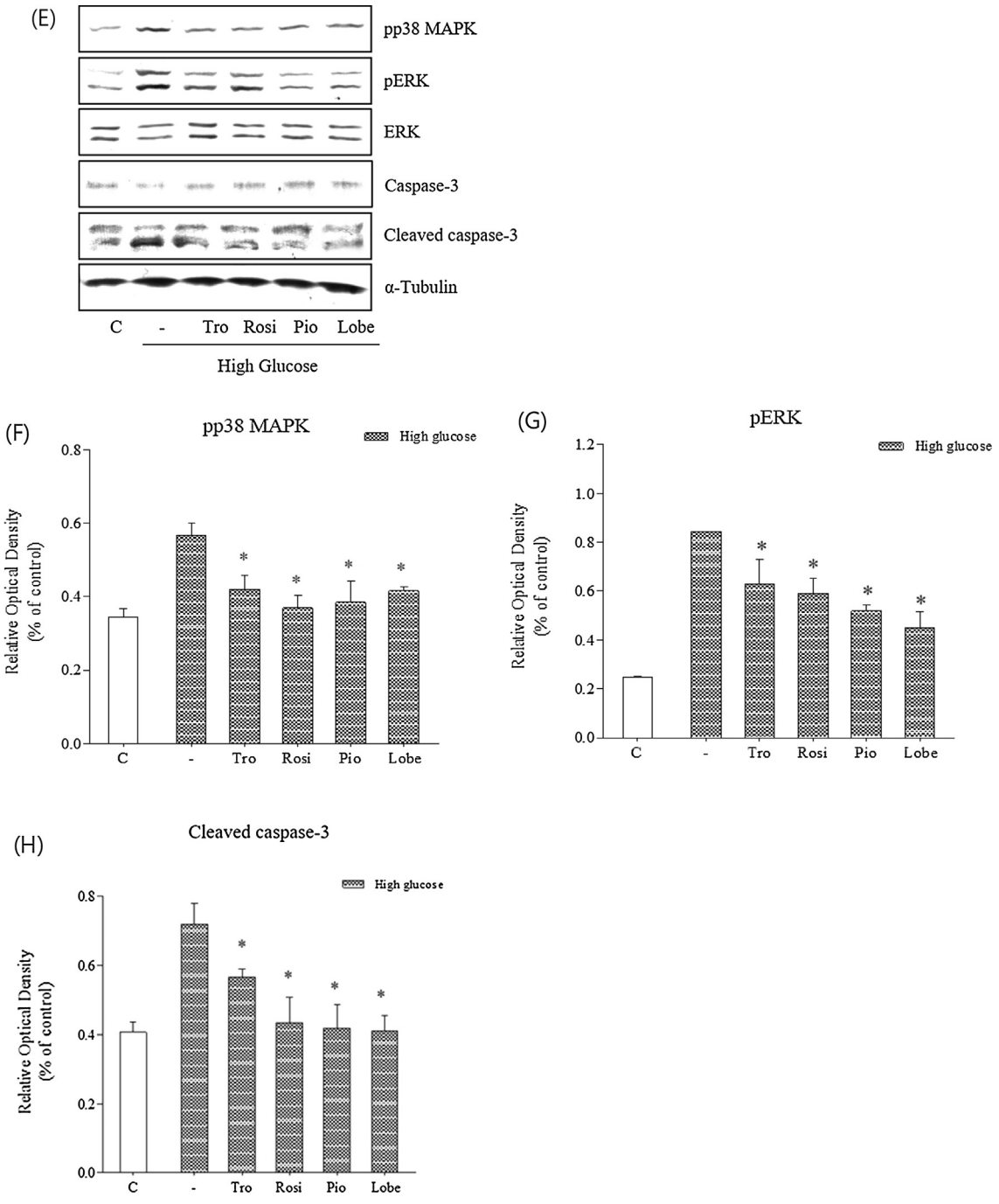


Fig 2. *(continued)*

mice (Fig. 4A). Blood glucose levels were significantly lower in the TZD groups, especially the lobeglitazone 1 mg/kg/d- and 10 mg/kg/d-treated groups compared to the db/db control and pioglitazone groups (Fig. 4B). Insulin levels were higher and glucagon levels were lower in the blood of lobe- and pioglitazone-treated mice than levels in the blood of the control mice (Fig. 4C and D). Triglyceride and LDL cholesterol levels were significantly lower in lobeglitazone- and pioglitazone-treated mice than in control db/db mice, but total cholesterol levels were similar among groups (Fig. 4E).

3.7. *Histologic changes by pioglitazone and lobe in db/db mice*

As observed with IHC staining, diabetic db/db mice exhibited decreased size and destructed contour of islets, whereas db/db mice treated with pioglitazone and lobeglitazone exhibited increased size and preserved shapes of islets (Fig. 5A). Beta cells stained by an insulin antibody were increased in pioglitazone- and lobeglitazone-treated db/db mice in a dose-dependent manner, and alpha cells stained by a

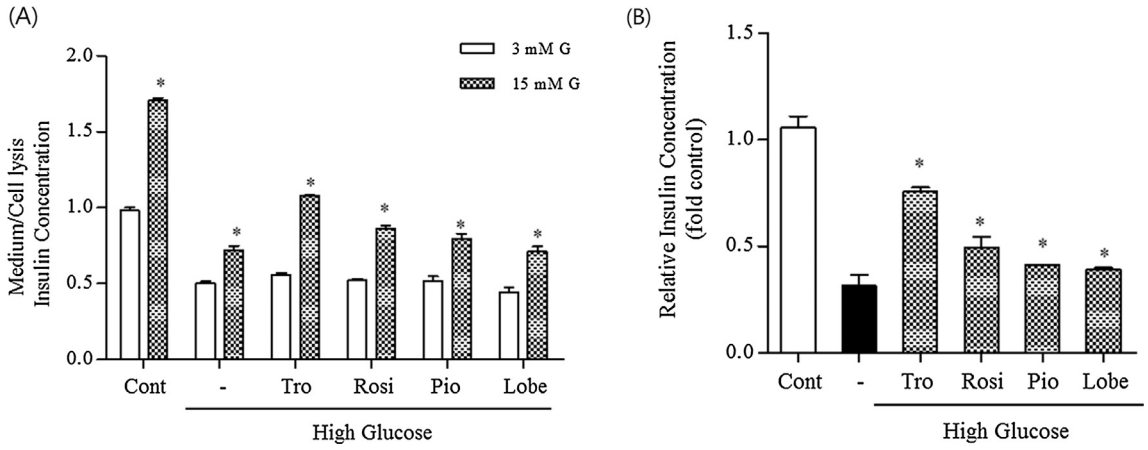


Fig. 3 – The effects of TZDs on glucose-stimulated insulin secretion (GSIS) in beta cells. A, After INS-1 cells were exposed to control or high glucose (33 mM glucose) with or without troglitazone 10 μM, rosiglitazone 10 μM, pioglitazone 10 μM, and lobeglitazone 10 μM for 48 h, the cells were incubated with 3 mM glucose and 15 mM glucose for 4 h each. An insulin ELISA kit was used to measure the insulin concentration in the medium and the cells (*P < 0.05 vs 3 mM glucose). B, Relative insulin concentration was calculated as the value of the difference in insulin concentration in 15 mM and 3 mM glucose divided by insulin concentration in 3 mM glucose (*P < 0.05 vs high glucose). Cont = control, Tro = troglitazone, Rosi = rosiglitazone, Pio = pioglitazone, Lobe = lobeglitazone. Values represent means ± S.E.M. of at least three independent experiments.

glucagon antibody were decreased in pioglitazone- and lobeglitazone-treated db/db mice compared to those of control db/db mice. Compared with pioglitazone-treated mice, lobeglitazone-treated mice showed a greater increase in islet size and beta cell counts, and the islet contour was firmer, especially at a dose of 10 mg/kg lobeglitazone. Cell proliferation by Ki-67 antibody staining was increased in pioglitazone- and lobeglitazone-treated mice, which was detected to be markedly increased in 10 mg/kg lobeglitazone-treated mice. IF staining showed similar results to IHC; db/db mice treated with pioglitazone and lobeglitazone had increased beta cell mass and decreased alpha cell mass compared to those of db/db control mice in microphotographs and quantitative analysis (Fig. 5B, D, E and F). Furthermore, we performed MT staining to assess collagen formation. In TZD-untreated db/db mice, dense collagen fibers were detected around the islets and blood vessels. The pioglitazone-treated group showed fewer collagen fibers around the islets and blood vessels than the diabetic group, and in db/db mice treated with lobeglitazone, there were few collagen fibers (Fig. 5C).

3.8. *The change of GSIS in islets from db/db mice treated pioglitazone or lobeglitazone*

To investigate the effects of pioglitazone and lobeglitazone on insulin secretory capacity in vivo, responses were determined under appropriate experimental conditions. The insulin secretion was increased above 3-fold in islet extracted from pioglitazone and lobeglitazone treated mice compared to db/db control. The each secretion potency was 3.1-fold by 10 mg/kg of pioglitazone, 2.9-fold by 30 mg/kg of pioglitazone, 3.4-fold by 1 mg/kg of lobeglitazone, and 3.3-fold by 10 mg/kg of lobeglitazone (Fig. 6).

4. Discussion

Our study showed that a novel TZD, lobeglitazone, has beneficial effects on both beta cell function and beta cell survival and that it directly decreased beta cell apoptosis and increased beta cell proliferation by modulating ER stress markers and cell survival markers. The profunction and prosurvival effects of lobeglitazone were comparable to those of the other TZDs in our study.

New pathogeneses have been revealed, and novel treatments have been developed recently; however, we do not yet have a good strategy to preserve and further increase pancreatic beta cell mass [2]. TZDs are insulin sensitizers and the only medication with a proven beta cell protective effect in several large clinical trials. The DPP and Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication (DREAM) trial have shown that PPAR-γ agonists prevent the onset of T2DM from impaired glucose tolerance [10,11]. Moreover, individuals with a history of gestational diabetes mellitus treated with TZD therapy reduced their risk of developing diabetes [22–24]. ADOPT is a unique study showing that rosiglitazone had better glycemic durability compared to sulfonylurea or metformin therapy in type 2 diabetes [12].

In addition to these clinical results, some studies reported that PPAR-γ agonists have direct protective effects on pancreatic beta cells [13,25,26]. However, conflicting data were also suggested, especially through rodent models of PPAR-γ ablation. Pioglitazone restored insulin secretion in a total-body PPAR-γ deletion mouse model [27], but not in a pancreatic β cell-specific PPAR-γ knockout model (βγ KO mice) [28]. TZD treatment decreases cell growth in several cancers [29,30]. TZDs also decreased INS-1 cell numbers and beta cell mass through decreased proliferation [31–35]. The direct effects of TZDs on pancreatic beta cells can be divided to two parts

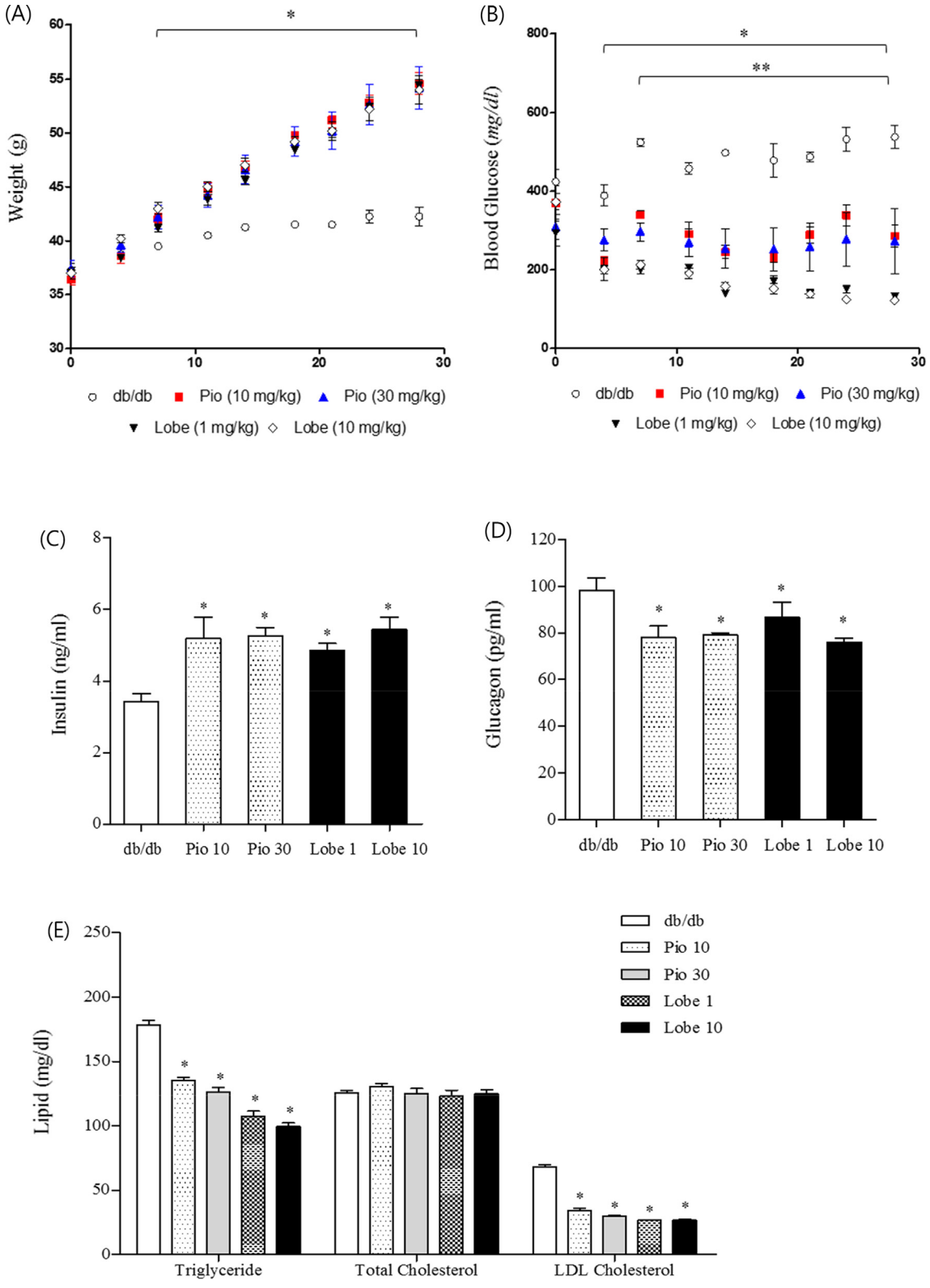


Fig. 4 – The change in metabolic parameters by pioglitazone and lobeglitazone in db/db mice. Solvent control, pioglitazone at 10 or 30 mg/kg/d or lobeglitazone at 1 or 10 mg/kg/d was administered for 4 weeks to db/db mice (5 mice per group). A, Body weight was measured twice a week (*P < 0.05 vs db/db control). B, Blood glucose levels were measured from the tail vein using a glucometer twice a week (*P < 0.05 vs db/db control, ** P < 0.05 vs pioglitazone). C, Insulin levels from blood were evaluated by ELISA on the 28th day (*P < 0.05 vs db/db control). D, Glucagon levels from blood were evaluated by ELISA on the 28th day (*P < 0.05 vs db/db control). E, Lipid profiles including total cholesterol, triglyceride and LDL cholesterol were measured by ELISA (*P < 0.05 vs db/db control). Pio = pioglitazone, Lobe = lobeglitazone. Values represent means ± S.E.M.

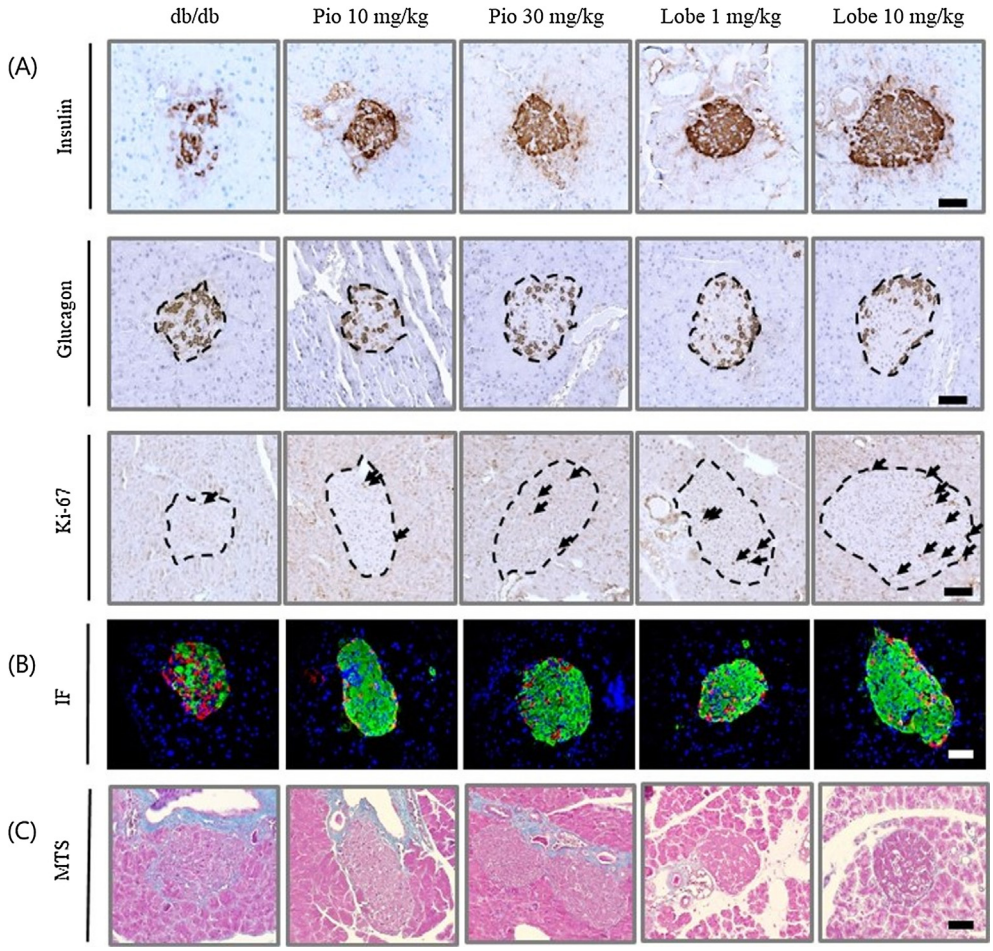


Fig. 5 – Histologic changes by pioglitazone and lobeglitazone in db/db mice. After solvent control, pioglitazone at 10 or 30 mg/kg/d or lobeglitazone at 1 or 10 mg/kg/d was administered for 4 weeks to db/db mice, we obtained pancreatic tissues from mice under anesthesia. A, In immunohistochemistry staining, beta cells were stained by an insulin antibody (first row), and alpha cells were stained by a glucagon antibody (second row). Ki-67 antibody staining was used to assess cell proliferation (Ki-67-positive cells; arrow, third row). B, Immunofluorescent (IF) staining was performed using an insulin antibody (green) and glucagon antibody (red). Blue showed nuclei staining via 4′, 6-diamidino-2-phenylindole (DAPI). C, Masson-Trichrome staining was performed to assess collagen formation. D, E, F, IF staining was analyzed quantitatively to assess beta cell mass and alpha cell mass. Pio = pioglitazone, Lobe = lobeglitazone, IF = immunofluorescent, MTS = Masson-Trichrome staining. Scale bar = 20 μm.

[13]. One is the profunction effect meaning improved insulin secretion of beta cells. The second is the prosurvival effect related to the increase in beta cell mass [20,36]. Many previous studies confused both concepts and usually studied the profunction effect. Our previous study showed that troglitazone can have the opposite effect on beta cell proliferation depending on glucose concentrations. Troglitazone decreased proliferation of INS-1 cells at 8.3 mM glucose but significantly increased beta cell proliferation at 33 mM glucose (preparing publication). Our present study also showed that lobeglitazone directly decreased apoptosis in hyperglycemia, which increases INS-1 cell apoptosis. Lobeglitazone treatment increased the percentage of BrdU (+) beta cells and GSIS compared to high glucose without TZD treatment. Furthermore, we applied a PPAR-γ antagonist, which reversed the effects of TZDs on pancreatic beta cells. These results mean that the antiapoptotic and proliferative effects of lobeglitazone and other TZDs against high glucose are directly related to the PPAR-γ receptor.

Among TZDs, only pioglitazone is commonly used in clinical practice currently. Troglitazone was found to cause hepatic failure and can no longer be used [14]. Rosiglitazone was reported to increase myocardial infarction and cardiac deaths [15] and was then limited in clinical use. Various TZDs have been reported to have different side effect and benefit profiles. One possible explanation is that troglitazone, rosiglitazone and pioglitazone show gene expression differences [16,37]. Because of the overlapping and nonoverlapping gene activation, novel glitazones have to be tested for known effects of previous TZDs even if they are in the same class.

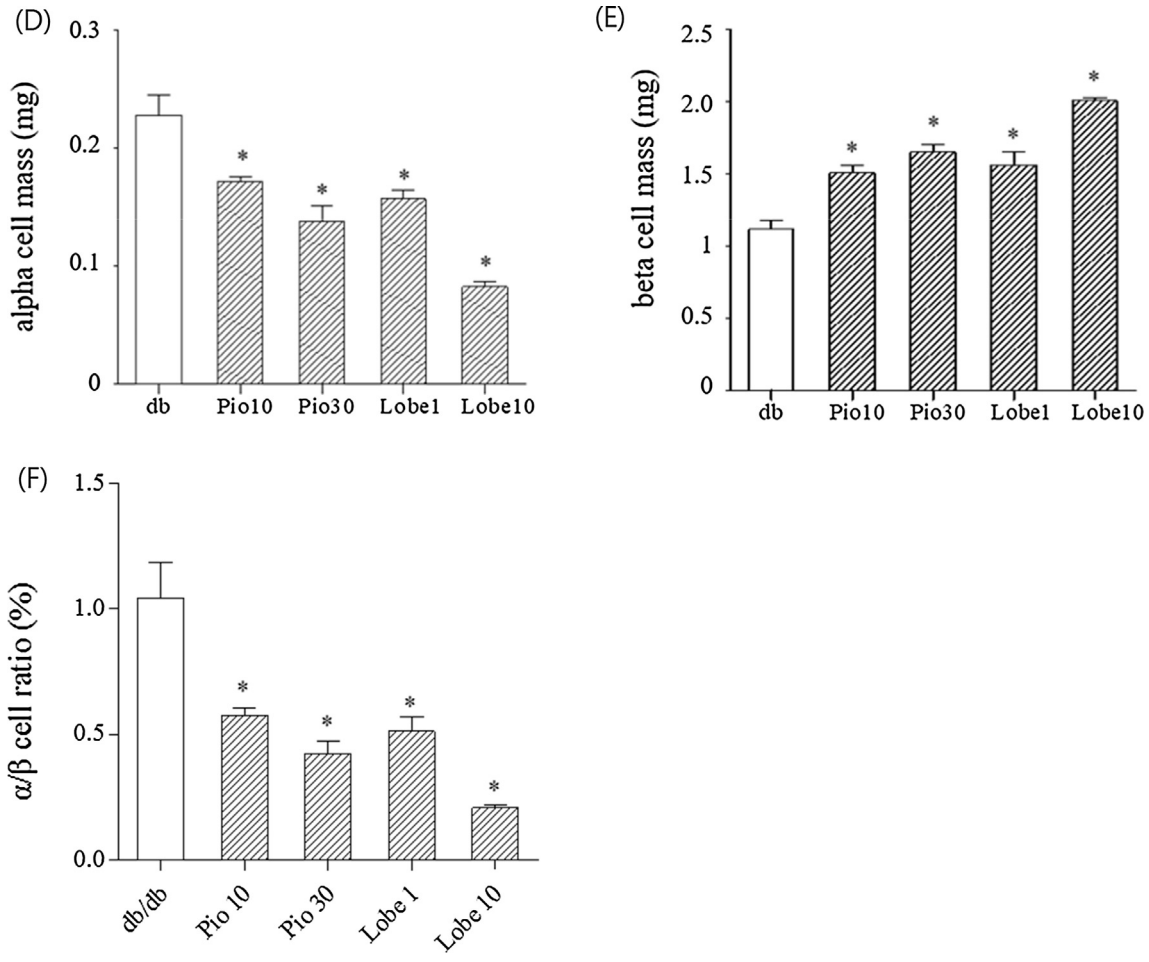


Fig 5. *(continued)*

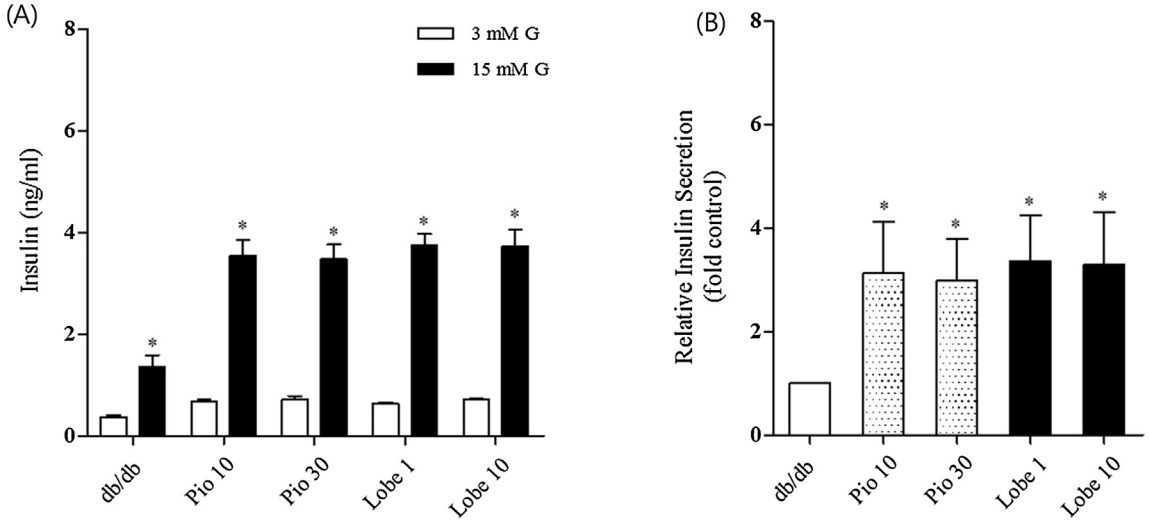


Fig. 6 – The change in glucose-stimulated insulin secretion (GSIS) in islets obtained from by pioglitazone or lobeglitazone treated db/db mice. A, Fifteen islets from each mice were incubated with 3 mM glucose and 15 mM glucose for 1 h each. An insulin ELISA kit was used to measure the insulin concentration in the medium (*P < 0.05 vs 3 mM glucose). B, Relative insulin concentration was calculated as the value of the difference in insulin concentration in 15 mM and 3 mM glucose divided by insulin concentration in 3 mM glucose (*P < 0.05 vs db/db control). Pio = pioglitazone, Lobe = lobeglitazone. Values represent means ± S.E.M. of at least three independent experiments.

We compared the effects of lobeglitazone with those of troglitazone, rosiglitazone and pioglitazone on beta cell apoptosis, proliferation and GSIS and found that they all decreased beta cell apoptosis and increased beta cell proliferation and GSIS under hyperglycemic conditions. Although there were small differences in the degree of increase and decrease, they were not significant.

Several mechanisms have been proposed to explain the profunction and prosurvival effects of TZDs. PPAR-γ activation increases insulin secretion through upregulation of Nkx6.1 and free fatty acid receptor GPR40, proteins that regulates insulin transcription, secretion and replication, in pancreatic beta cells [33,38]. The prosurvival effects of PPAR-γ activation in pancreatic beta cells are exerted by modulating oxidative stress, mitochondrial dysfunction, epigenetic dysregulation and ER stress [19,39–44]. ATF4 and CHOP were increased under high glucose and significantly decreased with lobe treatment in our results. The modulatory effect of lobeglitazone on ER stress was not inferior to that of other TZDs including troglitazone, rosiglitazone and pioglitazone. TZDs can protect pancreatic beta cells by PPAR-γ-independent signaling pathways. For instance, troglitazone has been shown to rapidly activate ERK1/2 in human colorectal cell lines [45]. ERK1/2 signaling has been shown to be important in beta cell survival, proliferation and glucose-dependent insulin gene transcription [46,47]. p38 MAPK/ERK are important factors for controlling cell proliferation, differentiation and apoptosis [48,49]. Their roles may be different depending on different tissues, organs and cell types. These opposite roles seem to be related to the duration and level of kinase activity [48,49]. ERK and p38 MAPK phosphorylation are related to pancreatic beta cell stabilization and plasticity through TGF beta [50]. Inhibition of TGF beta receptor signaling in beta cells resulted in higher beta cell mass due to increased beta cell proliferation [51]. Our results showed that high glucose activated ERK and p38 MAPK in INS-1 cells, similar to the findings of a previous report [52]. Lobeglitazone decreased ERK and p38 MAPK phosphorylation in hyperglycemia, which is consistent with the beta cell mass increase in our study. The effects of lobeglitazone on signaling were not different from those of other TZDs.

This study demonstrated that treatment with pioglitazone and lobeglitazone ameliorates hyperglycemia and dyslipidemia in obese diabetic db/db mice. Histologic examination revealed that treatment with pioglitazone and lobeglitazone can prevent loss of beta cell mass, disruption of pancreatic islet architecture, and fibrosis around pancreatic islets. The insulin secretion by high glucose was increased above 3-fold in islet extracted from pioglitazone and lobeglitazone treated mice. But to evaluate beta cell function just using GSIS of pancreas extracted from treated mice is indirect supposition. An article which mice treated with lobeglitazone had much better glucose tolerance than control db/db mice in oral glucose tolerance test supports this data [53].

Interestingly, the levels of blood glucose, lipid profile, and collagen deposition in pancreatic histology were much improved in lobeglitazone-treated mice compared with those of pioglitazone-treated mice, although we based the dosages of pioglitazone and lobeglitazone on previous studies using mice [18,19]. Further investigation will be needed to show whether these results are caused by a difference in specific effects between drugs or the problem of conversion of similar pharmacologic dosages.

Recently the data were reported that lobeglitazone had beneficial effects on not only steatohepatitis but also differentiation of adipocytes and thermogenesis in mouse model [18,53,54]. These results are expected that long term use of lobeglitazone in human would be bring changes in metabolic parameters in a better manner.

In conclusion, we found that a novel TZD, lobeglitazone, had a direct positive effect on pancreatic beta cells against hyperglycemia in both in vitro and in vivo experiments. Its profunction and prosurvival effects on beta cells are comparable to those of other TZDs.

Conflicts of interest

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

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