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Glucagon-like peptide–1 and candesartan additively improve glucolipotoxicity in pancreatic β-cells

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ABSTRACT

Glucagon-like peptide–1 (GLP-1) and angiotensin II type 1 receptor blocker reduce β-cell apoptosis in diabetes, but the underlying mechanisms are not fully understood. We examined the combination effects of GLP-1 and candesartan, an angiotensin II type 1 receptor blocker, on glucolipotoxicity-induced β-cell apoptosis; and we explored the possible mechanisms of the antiapoptotic effects. The effects of GLP-1 and/or candesartan on glucolipotoxicity-induced apoptosis and the phosphorylation of insulin receptor substrate–2 (IRS-2), protein kinase B (PKB), and forkhead box O1 (FoxO1) were evaluated by using MIN6 cells and isolated mouse pancreatic islets. Although palmitate significantly enhanced the high-glucose–induced apoptosis in both islets and MIN6 cells, GLP-1 and candesartan significantly inhibited apoptosis; and combination treatment additively prevented apoptosis. Whereas palmitate significantly decreased the phosphorylation of IRS-2, PKB, and FoxO1 in MIN6 cells, these changes were significantly inhibited by treatment with GLP-1 and/or candesartan. In addition, wortmannin, an inhibitor of phosphoinositide 3-kinase, markedly inhibited GLP-1– and/or candesartan-mediated PKB and FoxO1 phosphorylation. The present results suggest that GLP-1 and candesartan additively prevent glucolipotoxicity-induced apoptosis in pancreatic β-cells through the IRS-2/phosphoinositide 3-kinase/PKB/FoxO1 signaling pathway.

1. Introduction

Glucolipotoxicity plays an important role in the development and progression of type 2 diabetes mellitus. Chronic hyperglycemia causes pancreatic β-cell dysfunction characterized by reduced insulin biosynthesis [1] and increased levels of apoptosis (glucotoxicity) [2-4]. In addition, long-term exposure of β-cells to high concentrations of free fatty acid triggers β-cell apoptosis (lipotoxicity) [5-7]. The combination of glucotoxicity and lipotoxicity (glucolipotoxicity) has been postulated to contribute to the worsening of β-cell function over time, creating a vicious cycle by which metabolic abnormalities impair insulin secretion, thereby further aggravating metabolic perturbation [8,9]. Therefore, the stabilization of metabolic changes induced by glucolipotoxicity in β-cells represents a potential new avenue for the treatment of patients with type 2 diabetes mellitus [10].

Author contributions: Masamitsu Nakazato, Masanari Mizuta, and Hiroaki Ueno conceived the experimental plan and discussed analyses and interpretation. Hong-Wei Wang, Yukie Saitoh, and Kenji Noma performed the experiments. Hong-Wei Wang performed statistical analysis and wrote the manuscript.

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The rennin-angiotensin system (RAS) in isolated pancreatic islets includes angiotensinogen, angiotensin-converting enzymes, and angiotensin II type 1 receptor (AT1R) [11]. Activation of AT1R stimulates superoxide formation, inflammatory cascades, and cell apoptosis [12], whereas blockade of AT1R improves islet structure and function by decreasing oxidative stress–mediated apoptosis [13]. Angiotensin II type 1 receptor blockers (ARBs) decrease insulin resistance in obese diabetic animal models [14]. In addition, RAS activation induces superoxide-producing NAD(P)H oxidase in a rat model of acute pancreatitis [15], suggesting that up-regulation of islet RAS enhances oxidative stress and damages β-cell function. We recently reported that telmisartan, an ARB, attenuates fatty-acid–induced oxidative stress and NAD(P)H oxidase activity in pancreatic β-cells [16]. Therefore, blockade of RAS may preserve β-cell function and be a useful therapy for type 2 diabetes mellitus.

Glucagon-like peptide–1 (GLP-1), which is secreted from intestinal L cells in response to nutrient ingestion, is a potential therapeutic substance in the treatment of diabetes [17,18]. Glucagon-like peptide–1 receptor (GLP-1R), a G-protein–coupled receptor, was first cloned from rat pancreatic islets [19] and later from human pancreatic islets [20]. Combined with GLP-1R, GLP-1 stimulates insulin secretion in a glucose-dependent manner [21], decreases β-cell apoptosis, and increases islet cell mass [22]. Exendin-4, a GLP-1 receptor agonist, promotes β-cell growth and survival; these effects are mainly mediated by insulin receptor substrate-2 (IRS-2) induction via increased intracellular cyclic adenosine monophosphate levels [23]. Previous studies have reported that IRS2 plays a crucial role in β-cell growth and survival [24,25]. Glucagon-like peptide–1 reportedly promotes β-cell growth and survival by increasing protein kinase B (PKB; also called *Akt*) levels in β-cells both in vivo in *db/db* mice and in vitro in INS-1 cells [26-28]. Protein kinase B plays a major role in phosphoinositide 3-kinase (PI3K)–mediated survival effects [29]. Activated PKB can directly phosphorylate and, thereby, inactivate several components of the apoptotic machinery, including members of the transcription factor forkhead family [30]. Indeed, PKB is being increasingly implicated as a key player in the regulation of β-cell growth and survival [31]. Phosphorylation of forkhead box O1 (FoxO1) by PKB causes redistribution of FoxO1 from the nucleus to the cytoplasm, and the resulting decrease in nuclear FoxO1 has been proposed as a possible mechanism for the inhibition of FoxO1-mediated transcription [32]. Forkhead box O1 inhibition plays a role in the proliferative and antiapoptotic actions of GLP-1 in β-cells [33].

Although GLP-1 prevents apoptosis in pancreatic β-cells [22] and ARB improves islet structure and function [13], the additive effects of GLP-1 and ARB on glucolipotoxicity in pancreatic β-cells are unclear. In the present study, we examined the additive effects and possible mechanisms of GLP-1 and candesartan, an ARB, on glucolipotoxicity in pancreatic β-cells by using an apoptosis assay, immunoblotting, and immunoprecipitation. We found that GLP-1 and/or candesartan significantly prevented both high-glucose– and palmitate-induced apoptosis in MIN6 cells and that stronger effects were induced by combination treatment. Moreover, GLP-1 and/or candesartan significantly prevented high glucose levels via a palmitate-induced decrease in phosphorylation of IRS-2, PKB, and FoxO1 in MIN6 cells, suggesting that GLP-1 and candesartan play important roles in the prevention of β-cell apoptosis via the IRS-2/PI3K/PKB/FoxO1 signaling pathway.

2. Materials and methods

2.1. Materials

Dulbecco modified Eagle medium (DMEM), palmitate (sodium salt), and protease inhibitor cocktail were purchased from Sigma (St Louis, MO); and candesartan was kindly provided by Takeda Chemical Industries (Osaka, Japan). Human GLP-1 fragment 7-36 amide was obtained from CS Bio (Tokyo, Japan). Penicillin G, streptomycin, amphotericin B, fetal bovine serum, and RPMI medium 1640 were obtained from Gibco (Auckland, New Zealand). Trizol was purchased from Life Technologies (Oslo, Norway). Wortmannin, a specific covalent inhibitor of PI3K, was purchased from Cell Signaling Technology (Tokyo, Japan). Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Miyazaki.

2.2. Animals

Six-month-old female C57BL/6J mice were purchased from Charles River Laboratories (Yokohama, Japan), housed at 22°C under a 12-hour light/dark cycle, and given free access to tap water and standard pelleted chow (Clea Japan, Tokyo, Japan).

2.3. Isolation and culture of islets

Islets were isolated and cultured as described previously [34]. Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital (6 mg/100 g body weight). Collagenase was injected into the common bile duct at a concentration of 1 mg/mL in 1.5 mL of Hanks solution. The pancreas was removed from mice, placed in ice-cold Hanks solution, and minced with scissors; and the mixture was shaken in a 37°C water bath for 15 minutes. After the supernatant was removed, the remaining pellet was resuspended in Hanks solution several times to remove exocrine tissues. Islets were hand-picked under a stereomicroscope and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 000 U/mL penicillin G, 10 000 mg/mL streptomycin, and 25 mg/mL amphotericin B with 8 mmol/L glucose under humidified conditions in 5% CO_2/95% air at 37°C.

2.4. Culture of MIN6 cells

MIN6 cells were cultured as described previously [34]. When the cells reached 60% to 80% confluence, they were cultured for 24 hours with 0.5 mmol/L palmitate bound to 0.5% bovine serum albumin (BSA) in the presence or absence of 10 nmol/L GLP-1 and/or 10 μmol/L candesartan. The media used for further culturing the proliferating MIN6 cells contained 0.1% dimethyl sulfoxide and 0.17% ethanol.

2.5. Palmitate administration of MIN6 cells and islets

MIN6 cells were cultured in serum-free modified DMEM media with 0.5% BSA alone or with 0.5 mmol/L palmitate mixed with 0.5% BSA for 24 hours. Preparation of 0.5 mmol/L palmitate fatty acid media was carried out as described previously [34]. Briefly, a 20-mmol/L solution of palmitate in 0.01 mol/L NaOH was incubated at 70°C for 30 minutes; and 330 μL of 30% BSA and 500 μL of the palmitate/NaOH mixture were mixed together and filter-sterilized with 20 mL of either DMEM or RPMI medium.

2.6. Apoptosis assay

To test the possible antiapoptotic effects of GLP-1 and candesartan on β-cell glucolipotoxicity, dispersed mouse islets were exposed for 24 hours to 25 mmol/L glucose with or without 0.5 mmol/L palmitate bound to 0.5% BSA in the presence or absence of 10 nmol/L GLP-1 and/or 10 μmol/L candesartan. MIN6 cells were cultured in a 48-well tissue culture plate, as described previously [34]. Cells were incubated for 24 hours with 0.5 mmol/L palmitate bound to 0.5% BSA in the presence or absence of 10 nmol/L GLP-1 and/or 10 μmol/L candesartan. Cells were cultured in a humidified atmosphere (5% CO_2, 95% air) at 37°C. Apoptosis was measured with an APOPercentage apoptosis assay kit (Biocolor, Newtownabbey, Northern Ireland) based on apoptotic membrane alterations (dye-uptake bioassay).

2.7. Insulin assay

MIN6 cells were cultured in a 48-well tissue culture plate, as described previously [34]. Cells were incubated for 24 hours with 0.5 mmol/L palmitate bound to 0.5% BSA in the presence or absence of 10 nmol/L GLP-1 and/or 10 μmol/L candesartan. Cells were cultured in a humidified atmosphere (5% CO_2, 95% air) at 37°C. Medium insulin concentrations were measured using an enzyme immunoassay (Mercodia, Uppsala, Sweden).

2.8. Nuclear and cytoplasmic fractionation

MIN6 cells were cultured on 10-cm plates until reaching 80% to 85% confluence and were then incubated for 24 hours in DMEM culture medium containing 25 mmol/L glucose with either 0.5% BSA or 0.5 mmol/L palmitate mixed with 0.5% BSA, in the presence or absence of 10 nmol/L GLP-1 and/or 10 μmol/L candesartan. For isolation of nuclear extracts, cells were collected into microtubes, centrifuged for 20 seconds in a microcentrifuge, and resuspended in 200 μL of 10 mmol/L Hepes, pH 7.9, containing 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.5 mmol/L dithiothreitol. After incubation at 4°C for 15 minutes, cells were lysed by passing them 10 times through a 22-gauge needle. Cells were then centrifuged for 20 seconds, and the supernatant (cytoplasmic fraction) was removed and frozen in small aliquots. The pellet, which contained the nuclei, was resuspended in 150 μL of Hepes buffer (20 mmol/L Hepes, pH 7.9, containing 20% vol/vol glycerol, 0.1 mol/L KCl, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 0.5 mmol/L phenylmethanesulfonyl fluoride) followed by stirring at 4°C for 30 minutes. Nuclear extracts were then centrifuged for 20 minutes at 4°C. The supernatant was collected, aliquoted into small volumes, and stored at –80°C. For isolation of whole cell extracts, cells were collected into microtubes, centrifuged for 1 minute, resuspended in 150 μL of Hepes buffer, and then stirred at 4°C for 30 minutes. After centrifugation for 20 minutes at 4°C, the supernatant was collected as whole cell extracts, aliquoted into small volumes, and stored at –80°C [35]. Crossed contamination between nuclear and cytosolic fractionation was excluded by probing for histone and glyceraldehyde-3-phosphate dehydrogenase (supplemental Fig. 1).

2.9. Immunoprecipitation

Immunoblotting analyses were performed with antibodies against phosphotyrosine and IRS-2. For IRS-2 immunoprecipitation, cells were lysed in cold lysis buffer; and lysates were then subjected to immunoprecipitation with monoclonal antibodies recognizing phosphotyrosine residues, followed by incubation with protein G-agarose beads (Cell Signaling Technology, Tokyo, Japan) for 16 to 20 hours at 4°C. Beads were then washed twice with cold lysis buffer, subjected to immunoblotting, and visualized by using an ECL system (GE Healthcare, Tokyo, Japan). Western blots were quantified by using densitometric analysis. Band density was normalized against IRS-2, and the values were expressed relative to controls.

2.10. Immunoblotting

Immunoblotting analysis was used to detect changes in protein expression including active (phosphorylated) and total PKB, and phosphorylated FoxO1. MIN6 cells were grown on 10-cm plates to 80% to 85% confluence and then were incubated for 24 hours in DMEM culture medium containing 25 mmol/L glucose with either 0.5% BSA or 0.5 mmol/L palmitate complexed to 0.5% BSA, in the presence or absence of 10 nmol/L GLP-1 and/or 10 μmol/L candesartan. Protein (50 μg of protein per well) from each sample was separated on a 13% sodium dodecyl sulfate polyacrylamide gel and was transferred onto nitrocellulose membranes (Bio-Rad). Membranes were probed with phosphorylated-PKB (Ser473), PKB, phosphorylated-FoxO1 (Ser256), FoxO1, and α-tubulin purchased from Cell Signaling Technology. Immunoreactive bands were then visualized with horseradish peroxidase–conjugated mouse anti-rabbit IgG by using an ECL detection system. For quantification of band density as a measurement of phosphorylation state, films were analyzed with densitometric software. Band density was normalized against PKB or α-tubulin, and values were expressed relative to controls.

2.11. Statistical analysis

Normalized data are presented as means ± SEM. Statistical analyses were performed by analysis of variance with Fisher protected least significant difference parametric or the Student unpaired t test. Differences were considered to be statistically significant when $P < .05$. All statistical analyses were performed with SPSS software (Abacus Concepts and SAS Institute, Berkeley, CA).

3. Results

3.1. GLP-1 and candesartan protected islets and MIN6 cells from high-glucose–/palmitate-induced apoptosis

As compared with control group (low glucose, 5.6 mmol/L), high glucose (25 mmol/L) significantly increased apoptosis levels in both islets and MIN6 cells (Fig. 1). The addition of palmitate enhanced the high-glucose–induced apoptosis in mouse islets by 99% and in MIN6 cells by 68% ($P < .01$) (Fig. 1). In contrast, the addition of GLP-1 or candesartan significantly inhibited apoptosis in mouse islets (by 26% and 23%, respectively, vs high glucose plus palmitate) and in MIN6 cells (by 48% and 38%, respectively, vs high glucose plus palmitate). Apoptosis was strongly inhibited by combined treatment in both mouse islets and in MIN6 cells (by 44% and 70%, respectively, vs high glucose plus palmitate). We also confirmed that GLP-1 and/or candesartan significantly prevented high-glucose– and palmitate-induced apoptosis; however, GLP-1 and candesartan were unable to block the glucolipotoxicity-induced apoptosis in the presence of wortmannin. These results indicate that GLP-1 and candesartan additively protect islets and MIN6 cells from glucolipotoxicity-induced apoptosis.

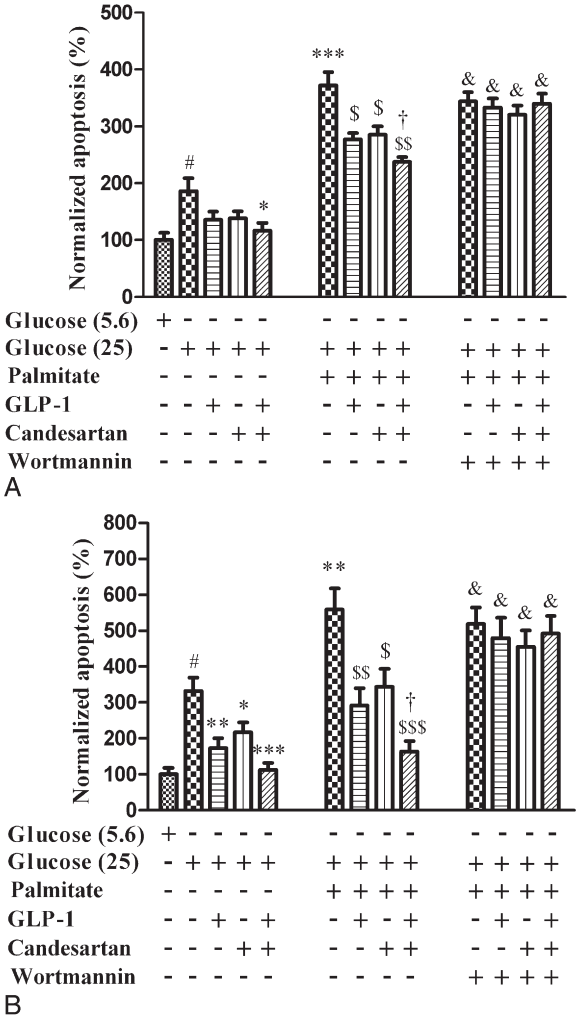

Fig. 1 – High-glucose– and/or palmitate-induced apoptosis in the presence of GLP-1 and/or candesartan in mouse islets (A) and MIN6 cells (B). Data are expressed as means ± SEM for 6 separate experiments. Glucose (5.6) and glucose (25) represent low (5.6 mmol/L) and high (25 mmol/L) glucose levels, respectively. $^{\#}P < .05$ vs cells cultured with 5.6 mmol/L glucose; $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ vs cells cultured with 25 mmol/L glucose; and $^{\$}P < .05$, $^{\$\$}P < .01$, and $^{\$\$\$}P < .001$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate. $^{\dagger}P < .05$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate in the presence of 10 nmol/L GLP-1 or 10 μmol/L candesartan.

3.2. GLP-1 and candesartan protected MIN6 cells from high-glucose–/palmitate-induced phosphorylation of IRS-2

Although the addition of palmitate to high levels of glucose decreased the phosphorylation of IRS-2 (P-IRS-2) by 60%, GLP-1 and candesartan reduced the glucose-/palmitate-induced reduction of P-IRS-2 (by 20% and 40%, respectively, vs high glucose); and combination treatment completely prevented the decrease in P-IRS-2 (Fig. 2B). We also examined the effects of GLP-1 and candesartan on the P-IRS-2 at 1 hour of incubation, as the results of 1-hour incubation were no different from those of 24-hour incubation (date not shown). In addition, the insulin levels in culture medium were not significantly changed by administration of GLP-1 and/or candesartan (Fig. 2A). These results indicate that GLP-1 and candesartan protected MIN6 cells from the glucolipotoxicity-induced decrease in P-IRS-2.

3.3. Effects of GLP-1 and candesartan on the phosphorylation of PKB

We examined the effects of GLP-1 and candesartan on the phosphorylation of PKB (P-PKB) by immunoblot analysis. Glucagon-like peptide–1 induced a dose-dependent increase in P-PKB, without affecting the total PKB levels; and the EC50 value was approximately 3 nmol/L (Fig. 3A). In contrast, 24-hour incubation of MIN6 cells with 0.5 mmol/L palmitate decreased P-PKB by 70% (Fig. 3B). However, both GLP-1 and candesartan prevented the palmitate-evoked inhibition of P-PKB (by 20% and 40%, respectively, vs high glucose). The combination of GLP-1 and candesartan completely prevented the reduction of high-glucose–/palmitate-induced P-PKB. These results suggest that GLP-1 and candesartan protect β-cells from glucolipotoxicity, at least in part, via the PKB signaling pathway. Because the P-PKB by PI3K is an important cell survival signal, the relationship between PI3K and the PKB signaling modules was studied with a pharmacological inhibitor of PI3K, wortmannin. Wortmannin inhibited GLP-1– and/or candesartan-mediated P-PKB (Fig. 3B), thus suggesting that GLP-1 and candesartan rescued the glucolipotoxicity induced by inhibition of P-PKB via the PI3K-dependent signaling pathway.

3.4. Effects of GLP-1 and candesartan on high-glucose–/palmitate-induced inhibition of FoxO1 phosphorylation and FoxO1 nuclear exclusion in MIN6 cells

To determine whether GLP-1– and/or candesartan-induced phosphorylation of FoxO1 was accompanied by changes in

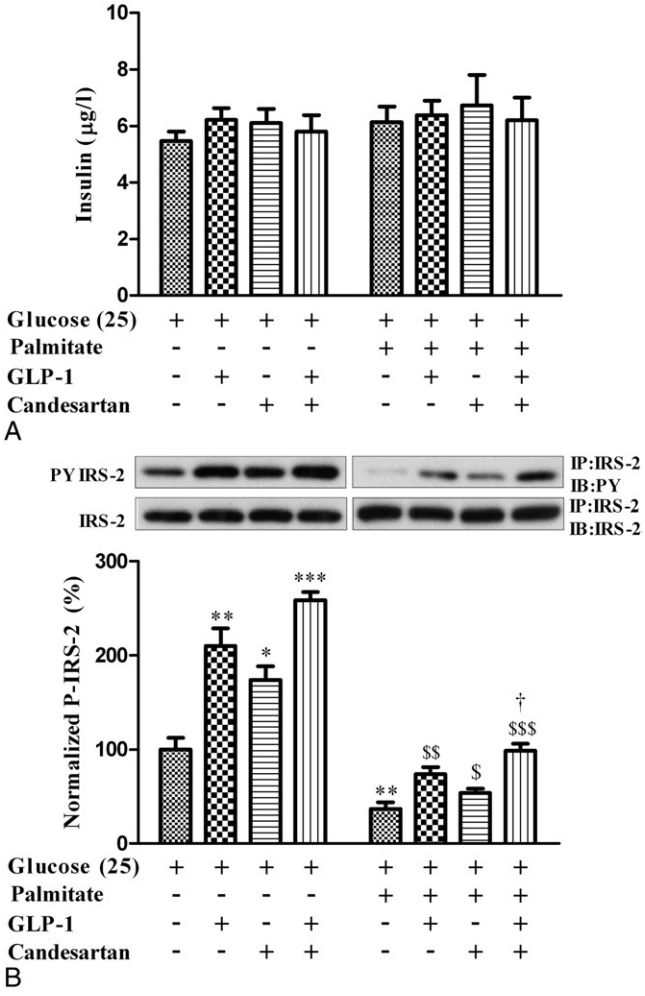

Fig. 2 – High-glucose– and/or palmitate-induced insulin secretion (A) and phosphorylated IRS-2 (B) in the presence of GLP-1 and/or candesartan in MIN6 cells. Data are expressed as means ± SEM for 4 separate experiments. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs cells cultured with 25 mmol/L glucose; $^{\$}P < .05$, $^{\$\$}P < .01$, and $^{\$\$\$}P < .001$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate; and $^{\dagger}P < .05$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate in the presence of 10 nmol/L GLP-1 or 10 μmol/L candesartan. Insulin concentrations in the medium were measured after 24-hour treatment under various conditions (A). Western blots were quantified by densitometric analysis, and band density was normalized against the level of IRS-2 (B). Molecular weights of PY-IRS-2 and IRS-2 were 185 kd. A representative blot from 4 separate experiments is shown.

FoxO1 localization, nuclear proteins were prepared from GLP-1 and/or candesartan-treated MIN6 cells and were subjected to immunoblotting with FoxO1 antibodies. As compared with high levels of glucose alone, GLP-1 and candesartan increased the levels of phosphorylated FoxO1 (P-FoxO1) in the cytoplasm (by 80% and 50%, respectively, Fig. 4); and combination treatment further increased P-FoxO1 levels (by 150%). Palmitate significantly decreased P-FoxO1 levels (by 60% vs high glucose). Although GLP-1 and candesartan reduced P-FoxO1 levels (by 27% and 42%, respectively, vs high glucose), the reduction was smaller than the reduction mediated by high glucose levels plus palmitate. The combined treatment completely prevented the decrease of P-FoxO1. As P-FoxO1

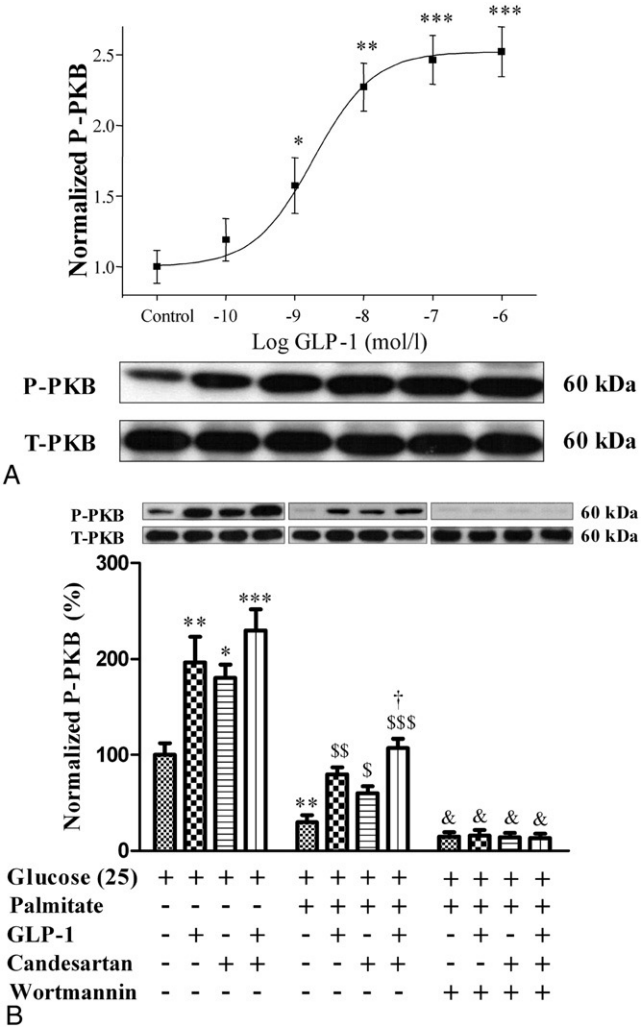

Fig. 3 – High-glucose– and/or palmitate-induced PKB phosphorylation in the presence of GLP-1 and/or candesartan in MIN6 cells. A, Dose-dependence curve for GLP-1 on phosphorylated PKB. Data are expressed as means ± SEM for 4 separate experiments. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs control cells cultured with 25 mmol/L glucose alone. B, Glucagon-like peptide–1 and candesartan prevented high-glucose– and/or palmitate-induced inhibition of PKB phosphorylation. Data are expressed as means ± SEM for 4 separate experiments. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs cells cultured with 25 mmol/L glucose; $^{\$}P < .05$, $^{\$\$}P < .01$, and $^{\$\$\$}P < .001$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate; $^{\&}P < .05$ vs the respective group without wortmannin; and $^{\dagger}P < .05$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate in the presence of 10 nmol/L GLP-1 or 10 μmol/L candesartan. Western blots were quantified by using densitometric analysis, and band density was normalized against the level of total PKB. A representative blot from 4 separate experiments is shown. T-PKB indicates total PKB.

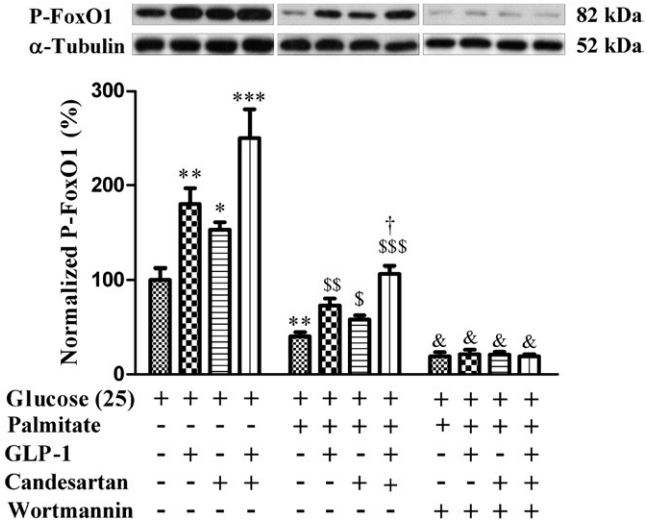

Fig. 4 – High-glucose– and/or palmitate-induced phosphorylated FoxO1 in the presence of GLP-1 and/or candesartan in MIN6 cells. Data are expressed as means ± SEM for 4 separate experiments. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs cells cultured with 25 mmol/L glucose; $^{\$}P < .05$, $^{\$\$}P < .01$, and $^{\$\$\$}P < .001$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate; $^{\&}P < .05$ vs the group without wortmannin; and $^{\dagger}P < .05$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate in the presence of 10 nmol/L GLP-1 or 10 μmol/L candesartan. Western blots were quantified using densitometric analysis, and band density was normalized against the level of α-tubulin. A representative blot from 4 separate experiments is shown.

levels were further decreased by the addition of wortmannin (by 80% vs the high-glucose group), the wortmannin-induced inhibition of P-FoxO1 was not affected by treatment with GLP-1 and candesartan. We then examined the effects of GLP-1 and/or candesartan on FoxO1 levels in the nucleus of MIN6 cells. Glucagon-like peptide–1 or candesartan significantly decreased the nuclear levels of FoxO1 (by 60% and 50%, respectively, vs high glucose) (Fig. 5); and in combination, they induced a marked decrease in nuclear FoxO1 (by 80%). Palmitate significantly increased the levels of nuclear FoxO1 (by 50% vs high glucose). Glucagon-like peptide–1 decreased the levels of nuclear FoxO1 by 14%, and candesartan increased the levels of nuclear FoxO1 by 20% (vs high glucose). Combination treatment strongly reduced nuclear FoxO1 levels (by 50% vs high glucose). Glucagon-like peptide–1 and/or candesartan-mediated nuclear FoxO1 levels were significantly smaller than the levels induced by high glucose plus palmitate. Although blockade of PI3K increased the expression of nuclear FoxO1 by 100%, treatment with GLP-1 and/or candesartan did not affect the wortmannin-induced increases in nuclear FoxO1.

4. Discussion

We examined the additive effects of GLP-1 and candesartan on glucolipotoxicity in pancreatic β-cells, and we explored the possible mechanisms. Our data showed that GLP-1 and candesartan not only significantly prevented glucolipotoxicity-induced apoptosis, but also prevented the glucolipotoxicity-induced decrease in the phosphorylation of IRS-2, PKB, and FoxO1 in MIN6 cells. These results suggest that GLP-1 and candesartan play important roles in the prevention of β-cell apoptosis via a mechanism involving the IRS-2/PI3K/PKB/FoxO1 signaling pathway.

Long-term exposure to high levels of glucose and fatty acids causes β-cell dysfunction characterized by reduced insulin biosynthesis [1] and increased levels of apoptosis [2-4]. Our data showed that palmitate exacerbated high-glucose–induced MIN6 cell apoptosis, as observed in previous studies [5-7]. Glucagon-like peptide–1 not only stimulated insulin secretion in a glucose-dependent manner [21], but also decreased β-cell apoptosis and increased islet cell mass via the PI3K/PKB signaling pathway [22]. In addition, GLP-1 inhibited apoptosis through FoxO1 phosphorylation-dependent nuclear exclusion in pancreatic β (INS832/13) cells [33]. Glucagon-like peptide–1 agonists stimulated the defense mechanisms against β-cells against different pathways involved in ER stress-induced apoptosis in a context-dependent manner [36]. Glucagon-like peptide–1 inhibited cytokine-induced apoptosis in isolated rat islets [37], and treatment of INS-1E β-cells with a GLP-1 analogue prevented cytokine-induced cell death in both β-cells and primary rat islets [38]. The results of the present study confirmed that GLP-1 prevented high-glucose–

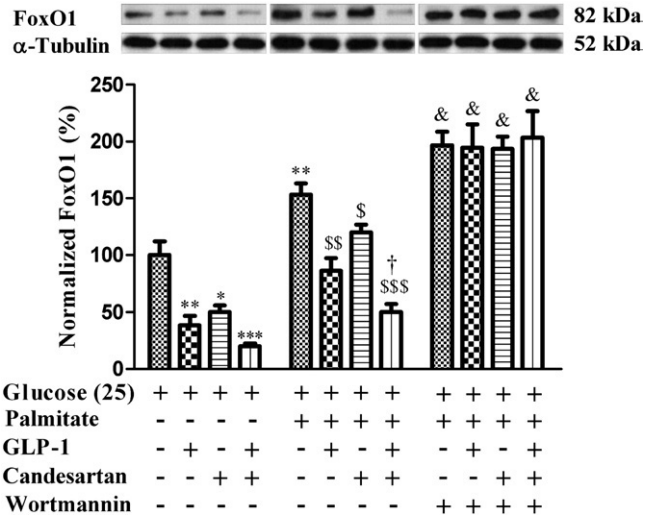

Fig. 5 – High-glucose– and/or palmitate-induced nuclear FoxO1 in the presence of GLP-1 and/or candesartan in MIN6 cells. Data are expressed as means ± SEM for 4 separate experiments. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs cells cultured with 25 mmol/L glucose; $^{\$}P < .05$, $^{\$\$}P < .01$, and $^{\$\$\$}P < .001$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate; $^{\&}P < .05$ vs the group without wortmannin; and $^{\dagger}P < .05$ vs cells cultured with both 25 mmol/L glucose and 0.5 mmol/L palmitate in the presence of 10 nmol/L GLP-1 or 10 μmol/L candesartan. Western blots were quantified using densitometric analysis, and band density was normalized against the level of α-tubulin. A representative blot from 4 separate experiments is shown.

and/or palmitate-induced apoptosis in mouse islets and MIN6 cells and also prevented the glucolipotoxicity-induced decrease in phosphorylation of IRS-2, PKB, and FoxO1 in MIN6 cells, which is consistent with previous reports [22,37-39].

We confirmed that candesartan prevented glucolipotoxicity-induced apoptosis in mouse pancreatic islets and MIN6 cells. In addition to the hemodynamic action of circulating angiotensin II, local angiotensin plays an important role in tissue function [40], notably in pancreatic cells [41]. In INS-1 cells, chronic hyperglycemia dose-dependently upregulated AT1R, leading to increased superoxide levels and decreased insulin release [42]. Fatty-acid–induced oxidative stress in pancreatic β-cells can be rescued by oral ARB (irbesartan) in Zucker diabetic fatty rats [43]. Several lines of evidence have suggested that angiotensin II impairs insulin sensitivity [44,45]. Telmisartan exerts beneficial effects on glucose and lipid metabolism in adipocytes and adipose tissue [46]. As angiotensin II stimulates superoxide formation, inflammatory cascades, and cell apoptosis through AT1R [12], blockade of AT1R reportedly decreases oxidative stress and apoptosis in β-cells [47]. Treatment with candesartan may thus increase β-cell mass, improve insulin release, and decrease staining intensity of components of NAD(P)H oxidase and oxidative stress in *db/db* mouse islets [48]. Losartan improved glucose-induced insulin release and (pro) insulin biosynthesis, delayed the onset of diabetes, and reduced hyperglycemia in *db/db* mice [13], and inhibited oxidative stress via down-regulation of NAD(P)H oxidase in *db/db* mouse islets [47]. We recently reported that telmisartan not only decreased the accumulation of palmitate-induced reactive oxygen species, but also decreased palmitate-induced protein kinase C activity and NAD(P)H oxidase activity in MIN6 cells and in mouse islets [16]. In the present study, we showed that candesartan significantly prevented high-glucose– and/or palmitate-induced apoptosis in pancreatic islets and MIN6 cells. Moreover, treatment with a mixture of candesartan and GLP-1 induced strong antiapoptotic effects in pancreatic islets and MIN6 cells, thus suggesting that blockade of AT1R enhanced the effects of GLP-1 against glucolipotoxicity.

Most importantly, the present results showed that GLP-1 and candesartan significantly prevented the high-glucose– and palmitate-induced decrease in the phosphorylation of IRS-2, PKB, and FoxO1 in MIN6 cells. These results suggest that both GLP-1 and candesartan prevented β-cell apoptosis via the IRS-2/PI3K/PKB/FoxO1 signaling pathway. Although tyrosine phosphorylation of IRS-2 largely depends on insulin concentration [49], insulin levels in culture medium were similar with the addition of GLP-1 and/or candesartan, thus suggesting that GLP-1 and candesartan increased IRS-2 phosphorylation through the activation of GLP-1R and AT1R, respectively. We also examined the effects of GLP-1 and candesartan on the phosphorylation of IRS-2 at early time points (1 hour) and found that 1-hour incubation gave similar results as 24-hour incubation (data not shown). This indicates that the activation of GLP-1R and AT1R directly affects the IRS-2–dependent signaling pathway. Chronically elevated fatty acid levels impaired pancreatic β-cell function through the inhibition of PKB activity, whereas the expression of a constitutively active variant of PKB in β-cells prevented fatty-acid–induced apoptosis [50]. Activated PKB can directly phosphorylate and, thereby, inactivate several components of the apoptotic machinery, including members of the FoxO family [30]. Glucagon-like peptide–1 increases PKB levels in β-cells, both in vivo in *db/db* mice and in vitro in INS-1 cells [26-28]. Furthermore, the present data confirmed that wortmannin inhibited both GLP-1– and candesartan-mediated stimulation of PKB and FoxO1 phosphorylation. These results suggest that GLP-1 and ARB inhibit apoptosis via the PI3K-dependent signaling mechanism in MIN6 cells. Phosphoinositide 3-kinase is activated by GLP-1, and phosphorylation of both Thr308 and Ser473 by PI3K is essential for PKB activation [37]. Thus, similarly to GLP-1, ARB may enhance PI3K activation followed by stimulation of PKB and FoxO1 phosphorylation in the cytoplasm of MIN6 cells. Phosphorylation by PKB caused redistribution of FoxO1 from the nucleus to the cytoplasm, and the resulting decrease in nuclear FoxO1 was proposed as a possible mechanism for the inhibition of FoxO1-mediated transcription [32]. Indeed, GLP-1 and candesartan increased levels of P-FoxO1 in the cytoplasm and decreased levels of P-FoxO1 in the nucleus of MIN6 cells. A recent report confirmed that FFAs stimulate nuclear sequestration of FoxO1 through the activation of PKCδ [51]. The inhibition of FoxO1 effectively protected β-cells against dexamethasone-induced dysfunction [52]. Forkhead box O1 may contribute to the phenotype of insulin resistance by increasing fatty acid utilization [53]; FoxO1 activation in β-cells could similarly down-regulate glucose metabolism and insulin secretion and synthesis [54]. The present data show that both GLP-1 and candesartan increased cytoplasmic P-FoxO1 and decreased nuclear FoxO1, thus suggesting that both GLP-1 and candesartan prevented β-cell apoptosis via the PI3K/PKB/FoxO1 signaling pathway.

Glucagon-like peptide–1 receptor agonists are used in the treatment of diabetes, and the present data confirmed that blockade of AT1R preserves β-cell function and may be a useful therapy for type 2 diabetes mellitus. Notably, when compared with either GLP-1 or candesartan alone, the combination of GLP-1 and candesartan induced stronger effects on antiglucolipotoxicity in both MIN6 cells and mouse islets. Thus, co-treatment with GLP-1 and ARB may be a useful therapeutic approach for promoting the survival of β-cells in obesity and diabetes through the IRS-2/PI3K/PKB/FoxO1 signaling pathway.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.metabol.2010.11.004.

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