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Protective Action of Liraglutide in Beta Cells Under Lipotoxic Stress Via PI3K/Akt/FoxO1 Pathway

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ABSTRACT

Liraglutide, a modified form of glucagon-like peptide-1 (GLP-1), has been found to improve beta cell function in type 2 diabetes (T2DM). However, the effect of liraglutide on beta cell function under lipotoxic stress and the underlying molecular mechanisms remain unclear. In the present study, we investigated the role of PI3K/Akt/FoxO1 signaling in liraglutideinvolved beta cell protection in high free fatty acids (FFAs) condition. The apoptosis, proliferation, and insulin secretion capability of MIN6 cells and islets from C57BL/6J mice were evaluated when exposed to FFAs with/without liraglutide. The expression of effectors involved in PI3K/Akt/FoxO1signalling pathway was detected by real-time PCR and western blotting in MIN6 cells and islets from C57BL/6J mice. Liraglutide substantially inhibited the lipoapoptosis and improved the proliferation and insulin secretion of beta cells in high FFAs condition. Western blot revealed that the phosphorylation of Akt and FoxO1 was markedly decreased under lipid stress but was elevated when treated with liraglutide. Moreover, FFAs could up-regulate the expression levels of p27, Bax, Cidea but down-regulate the expression levels of Pdx-1, MafA, and NeuroD in beta cells, which was canceled by the addition of liraglutide. Moreover, LY294002, a PI3K inhibitor, could significantly abrogate all the protective actions of liraglutide against lipotoxicity. We concluded that liraglutide markedly improved beta cell function under lipid stress and that the protective action of liraglutide was mediated by activation of PI3K/Akt, which resulted in inactivation of FoxO1 along with the down-regulation of p27, Bax, Cidea and up-regulation of Pdx-1, MafA, and NeuroD expressions. J. Cell. Biochem. 115: 1166–1175, 2014.

KEY WORDS: TYPE 2 DIABETES MELLITUS; LIPOTOXICITY; FORKHEAD TRANSCRIPTION FACTOR CLASS O; GLUCAGON-LIKE PEPTIDE -1; BETA CELL FUNCTION

The prevalence of type 2 diabetes (T2DM) is substantially increasing, partially due to the rising obesity rates [Zimmet et al., 2001]. High-fat diet (HFD) and obesity are associated with elevated levels of free fatty acids (FFAs), which are putative mediators of beta-cell dysfunction, that is, the impaired glucose responsiveness and loss of beta-cell mass. This is defined as lipotoxicity and central to the pathogenesis of T2DM [Kahn et al., 2006]. However, the related mechanisms are not well understood. Besides, correction of the lipotoxicity induced beta-cell dysfunction is attracting the efforts from lots of research groups. The incretin hormone glucagon-like peptide-1 (GLP-1), which is released from the gastrointestinal tract in response to nutrient ingestion, is found to effectively improve glucose stimulated insulin secretion (GSIS) and enhance beta-cell proliferation. However, the

Shiying Shao and Mingbo Nie contributed equally to this work.

The authors declare that there is no duality of interest associated with this manuscript.

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short circulating halflife of the bioactive, intact GLP-1 limits its potential use for the treatment of diabetes [Siegel et al., 1999]. Liraglutide, one of the longacting GLP-1 receptor agonists, has been utilized in clinical practice [Sturis et al., 2003]. Previous studies have shown that liraglutide exhibits similar physiological effects of GLP-1 on beta cells including the stimulation of proliferation and inhibition of cell apoptosis, as well as potentiation of insulin secretion [Thorens, 1992; Brubaker and Anini, 2003; Bregenholt et al., 2005; Chia and Egan, 2008; Toso et al., 2010]. However, few studies focus on whether liraglutide could correct beta-cell dysfunction against lipotoxicity and the underlying molecular mechanisms. To identify this issue is of importance on diabetes development and therapy.

In the present study, mouse insulinoma MIN6 cells and islets from C57BL/6J mice were exposed to liraglutide under normal/lipotoxic condition to investigate the protective actions of liraglutide and the possible molecular mechanisms.

METHODS AND MATERIALS

CULTURE OF MIN6 CELLS

MIN6 cells were cultured in DMEM medium containing 25 mM glucose (Sigma, MO, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (v/v), 10 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Palmitate (Sigma, MO, USA) was dissolved to 100 mM in methanol to make stock solutions for later dilution in DMEM supplemented with 0.5% BSA. When the cells reached 60% to 80% confluence, they were cultured for 24 h with 100 nM liraglutide (Novo nordisk, Denmark) and/or 0.5 mM palmitate in the presence or absence of 50 μM PI3K inhibitor (LY294002) (Cell Signaling, Beverly, MA, USA) applied 1 h prior to liraglutide/palmitate treatment.

ANIMALS

6-week-old male C57BL/6J mice were purchased from local laboratory animal holding facilities housed at 22 °C under a 12-h light/dark cycle. Individually caged mice were fed either normal chow (NC) or a HFD beginning at 6 weeks of age for an 8-week period with/without subcutaneous injection of liraglutide (200 μg/kg, once a day). Mice that did not receive liraglutide treatment were undertaken the subcutaneous injection of saline solution. The NC consisted of 60% kcal, 15% kcal, and 25% kcal from carbohydrate, fat, and protein, respectively. The HFD consisted of 55% kcal, 28% kcal, and 17% kcal from carbohydrates, fat, and protein, respectively. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science & Technology, Wuhan, PR China.

ISOLATION AND CULTURE OF ISLETS

Collagenase (Sigma, MO, USA) was injected into the common bile duct at a concentration of 1 mg/ml after mice were anesthetized. The pancreas was minced and placed in ice-cold Hanks solution. After the mixture was shaken in a 37 °C water bath for 15 min, the supernatant was removed and the remaining pellet was re-suspended in Hanks solution several times to remove exocrine tissues. Islets were hand-picked under a stereomicroscope and cultured in RPMI 1640 medium (Sigma, MO, USA) supplemented with 10% heat-inactivated FBS (v/v), 25 mg/ml amphotericin B, 10000 U/ml penicillin, 10000 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

FLOW CYTOMETRIC ANALYSIS OF APOPTOSIS

MIN6 cells were washed twice with phosphate buffered saline (PBS) and then suspended in 100 μl of binding buffer provided with apoptosis detection kit (BD Pharmingen, CA, USA). Thereafter, cells were stained with annexin-V FITC antibody and propidium iodide (PI) according to instructions provided by the manufacturer and scanned for fluorescence intensity in FL-1 (FITC) and FL-2 (PI) channels. The fractions of cell population in different quadrants were analyzed and cells in the lower right quadrant represented apoptosis.

MEASUREMENT OF INSULIN SECRETION

Min6 cells/islets were seeded in 24-well culture plates followed by incubating in 1 ml of KRBH buffer (124 mM NaCl, 5.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM HEPES, pH 7.4) with 0.1% BSA at 37 °C for 30 min. Thereafter, cells were incubated with KRBH buffer containing 2.8 mM and 16.7 mM glucose for another 30 min. 0.9 ml of KRBH buffer in each well was collected for insulin release detection. 0.9 ml of acid-ethanol was added for insulin content detection. Insulin ELISA kits were used to determine the amount of secreted insulin and the insulin content (EMD Millipore, USA). Results of insulin secretion were expressed as percentages of insulin content.

QUANTITATIVE REAL-TIME PCR

Total RNA of Min6 cells/islets was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. cDNA was synthesized through reverse transcription PCR (Promega Corporation, WI, USA). A reaction solution was prepared by combining 5 μl SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA, USA) solution, 0.5 μl of cDNA template, 1 μl of a 50 nmol/l primer solution, 3.5 μl diluent, and were mixed together thoroughly. Dissociation curve analysis was performed for each experiment to determine the dissociation temperature. Quantitative results were generated by the 7500 Fast System SDS software. The relative gene expression levels were calculated compared to that of β-*actin*. The sequences of the primers including β-*actin*, *p27*, *Bax*, cell death-inducing DNA fragmentation factor a-like effector a (*Cidea*), Pancreatic and duodenal homeobox-1 (*Pdx-1*), Neurogenic differentiation D (*NeuroD*), and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A(*MafA*) are shown in the Supplementary Table 1.

WESTERN BLOT ANALYSIS

A total of 50 μg of protein samples from Min6 cells/islets was separated on a SDS-PAGE medium and transferred to PVDF membranes (Millipore, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline Tween-20 (TBST) for 1 h at room temperature and incubated at 4 °C overnight with primary antibody. The Akt, phospho-Akt (Ser473), FoxO1, phospho-FoxO1 (Ser256), p27, Bax, Cidea, MafA, NeuroD, Pdx-1, and β-actin (Santa Cruz Biotechnology, USA) primary antibodies were at dilutions of

1:1000. Following washing with TBST, the membranes were further incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit secondary antibody at 1:2000 dilutions (Santa Cruz Biotechnology, USA). Proteins were detected using the enhanced chemiluminescence (ECL) system (Millipore, Germany).

IMMUNOFLUORESCENCE STAINING

5 cm-thick paraffin sections of pancreata were subjected to immunofluorescence staining with anti-insulin antibody (Santa Cruz Biotechnology, USA) or TUNEL reaction mixture (Roche Applied Science, USA) according to the manufacturer's instructions. Sections were mounted using Vectashield with DAPI (Sigma, USA) for counter-staining of nuclei before observation.

STATISTICAL ANALYSIS

The results are expressed as mean ± SEM and analyzed by two-tail *t* test or ANOVA. $P < 0.05$ was considered as significant.

RESULTS

LIRAGLUTIDE DECREASED APOPTOSIS OF BETA CELLS UNDER LIPOTOXIC STRESS AND PI3K INHIBITOR LY294002 BLOCKED THIS EFFECT

A flow cytometric assay was performed to identify the anti-apoptosis role of liraglutide in beta cells. Palmitate, one of the most common FFAs, significantly increased lipoapoptosis levels in MIN6 cells by 302.17 ± 23.47% (Fig. 1B). In contrast, the treatment of liraglutide inhibited the lipoapoptosis notably. Furthermore, we assessed whether the anti-apoptosis effects of liraglutide could be blocked by LY294002, the pharmacological inhibitor of phoshatidylinositol 3 kinase (PI3K), which is a typical upstream activator of Akt. It was found that the apoptosis in Li + PA + LY group was elevated to 368.44 ± 34.70% as compared to the control group ($P < 0.01$). The difference between the PA group and Li + PA + LY group was not significant ($P > 0.05$), revealing that liraglutide was unable to block the lipoapoptosis when PI3K was inhibited.

Furthermore, we analyzed the pancreatic apoptosis in C57BL/6J mice. TUNEL detection revealed that apoptosis levels of islets from HF-fed mice increased markedly and liraglutide treatment could abolish such increase (Fig. 1C, D). Therefore, the animal experiments identified that, consistent with the findings in MIN6 cells, liraglutide exhibited effective anti-apoptosis capability in vivo as well.

LIRAGLUTIDE IMPROVED THE INSULIN SECRETION UNDER LIPOTOXIC STRESS AND PI3K INHIBITOR LY294002 BLOCKED THIS EFFECT

To investigate the effect of liraglutide on beta-cell function, insulin secretion in response to glucose stimulation was detected. As shown in Figure 2A, 16.7 mM glucose failed to induce insulin release in palmitate-treated MIN6 cells (0.58 ± 0.02% at 16.7 mM Glc vs. 0.56 ± 0.04% at 2.8 mM Glc, $P > 0.05$). However, cells in the Li+PA group could normally release insulin (2.08 ± 0.21% at 16.7 mM Glc), indicating that the GSIS impairment by palmitate was nullified by liraglutide. Additionally, when LY294002 was pre-added, liraglutide was unable to correct palmitate induced GSIS impairment (Li+PA+LY group, 0.56 ± 0.08% at 16.7 mM Glc), illustrating that the PI3K/Akt pathway may be exceedingly essential for liraglutide dependent GSIS protection under lipotoxic condition.

In addition, we investigated the insulin secretion of islets from C57BL/6J mice to elucidate the physiological roles of liraglutide in the regulation of glucose homeostasis in vivo. After 8-wk treatment, the insulin release in isolated islets tended to be increased in liraglutide treated mice (Li group, 3.18 ± 0.25% vs. control group, 2.08 ± 0.19%, $P < 0.01$) but lower in HF-fed mice (HF group, 0.60 ± 0.09% vs. control group, 2.08 ± 0.19%, $P < 0.01$) at 16.7 mM glucose stimulation (Fig. 2B). These data showed that liraglutide could enhance but lipotoxicity could impair insulin release in vivo. Moreover, the treatment of liraglutide could significantly cancel the GSIS impairment although the mice were fed with HF diet as well (Li+HF group, 2.17 ± 0.19% at 16.7 mM Glc vs. 0.47 ± 0.10% at 2.8 mM Glc, $P < 0.01$). Taken together, all these data demonstrated that lipotoxicity induced beta-cell dysfunction could be mitigated by the treatment of liraglutide in vivo and in vitro.

LIRAGLUTIDE COUNTERACTS THE NEGATIVE EFFECT OF LIPOTOXICITY ON THE PI3K/AKT/FOXO1 PATHWAY

To further identify the signal pathway involved in the lipotoxicity, we next tested the levels of Akt and phospho-Akt proteins using western blot analysis in MIN6 cells. As shown in Figure 3B, 24 h incubation with palmitate decreased p-Akt level by 57% compared with control group. And such decrease could be completely prevented by liraglutide (PA group, 43.16 ± 7.60 vs. Li+PA group, 111.22 ± 14.96, $P < 0.01$). Since FoxO1 is one of the crucial downstream effectors of Akt, we next tested the levels of FoxO1 and phospho-FoxO1 proteins. In comparison to the control group, markedly increased phosphorylation of FoxO1 was observed in liraglutide treatment group (p-FoxO1/FoxO1, 195.71 ± 15.12% of the control group). In contrast, palmitate decreased the phosphorylation of FoxO1 (p-FoxO1/FoxO1, 57.62 ± 7.59% of the control group). Moreover, similar results were found in islets from C57BL/6J mice (Fig. S1A–D).

FoxO1 is known to shuttle between nucleus and cytoplasm. To determine whether liraglutide-induced phosphorylation of FoxO1 was accompanied by changes in FoxO1 localization, nuclear proteins and cytoplasmic proteins were subjected to immunoblotting. It was found that palmitate significantly decreased the ratio of cytoplasmic to nuclear FoxO1 (by 63% vs. control group) and this was blocked by liraglutide, indicating that the nuclear translocation of FoxO1 in response to palmitate can be annulled by liraglutide. When LY294002 was pre-added, the palmitate induced translocation of FoxO1 to nucleus was observed again although liraglutide was co-cultured (Li+PA+LY group, 40.37 ± 5.23% of control group) (Fig. 3E, F). Furthermore, palmitate significantly decreased p-FoxO1 levels in the cytoplasm (34.55 ± 5.47% of control group), which was nullified by liraglutide (Fig. 3G, H). In addition, there was no statistic difference between control and LY294002 only group, which ruled out the possible effect of LY294002 (data not shown). These findings gave us such an impression that liraglutide contributed to the FoxO1 phosphorylation and subcellular localization under lipotoxic conditions, along with PI3K/Akt signal pathway participating in this process.

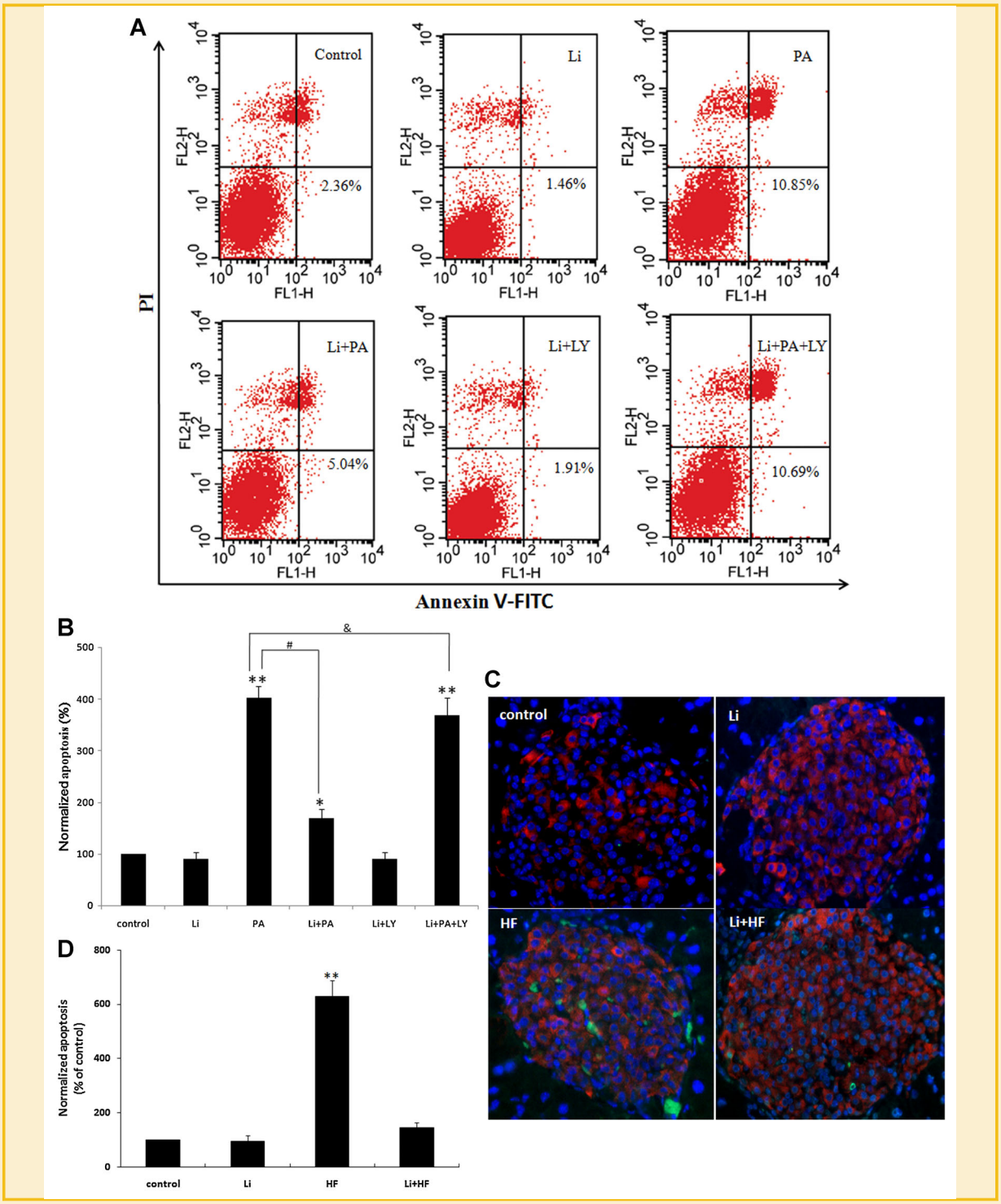

Fig. 1. Apoptosis detection of beta cells under lipotoxic stress. (A) Flow cytometric analysis of apoptosis in MIN6 cells using annexinV-FITC and PI double staining. MIN6 cells were incubated with indicated concentrations of liraglutide (Li), palmitate (PA), liraglutide plus palmitate (Li + PA), liraglutide plus LY294002 (Li + LY), and liraglutide, palmitate plus LY294002 (Li + PA + LY), followed by staining with Annexin V-FITC/PI. Representative of one of the three similar experiments was shown. Quadrant analysis of fluorescence intensity of ungated cells in FL-1 versus FL-2 channels was from 10000 events. Cells in the lower right quadrant represented apoptosis. (B) The levels of apoptosis assayed by flow cytometric analysis were normalized relative to non-treatment group (control). Data are presented as the means ± SEM of three independent experiments. (C) C57BL/6J mice were fed with either normal chow (Control) or a high-fat diet (HF). Subcutaneous injections of liraglutide were performed to normal diet fed mice (Li) or to high-fat fed mice (Li + HF). Representative immunofluorescence of pancreatic sections from 14-wk-old mice using TUNEL staning (green), anti-insulin staining (red), and DAPI staining (blue) was shown (n = 5 sections per mouse; five mice per group). (D) The levels of apoptosis assayed by immunofluorescence were normalized relative to insulin-positive cells. Data are presented as percent of control group. *$P < 0.05$, **$P < 0.01$ vs. control; # $P < 0.01$ vs. PA group; & $P > 0.05$ vs. PA group.

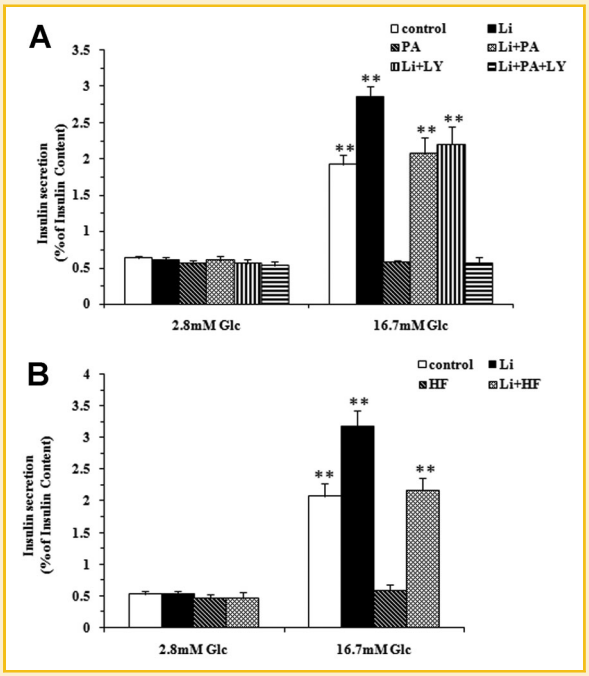

Fig. 2. Insulin secretion treated with low glucose (2.8 mM Glc) and high glucose (16.7 mM Glc). MIN6 cells were incubated with indicated concentrations of liraglutide (Li), palmitate (PA), liraglutide plus palmitate (Li + PA), liraglutide plus LY294002 (Li + LY), and liraglutide, palmitate plus LY294002 (Li + PA + LY). (B) C57BL/6J mice were fed with either normal chow (Control) or a high-fat diet (HF). Subcutaneous injections of liraglutide were performed to normal diet fed mice (Li) or to high-fat fed mice (Li + HF). Secreted insulin was measured and expressed as % of insulin content. Data are presented as the means ± SEM of three independent experiments. $^{**}P < 0.01$ vs. control 2.8 mM Glc group.

LIRAGLUTIDE DOWN-REGULATES THE EXPRESSION OF Bax, p27, AND CIDEA UNDER LIPOTOXIC STRESS

The capability of liraglutide on anti-apoptosis prompted us to investigate the underlying mechanisms. Proapoptotic BCL-2 family member Bax [Piro et al., 2002], cell cycle inhibitor p27 [Karnik et al., 2005], and apoptosis-inducing factor Cidea [Omae et al., 2012] were reported to be immediate target genes of FoxO1. In this regard, we examined the expression of these three genes in MIN6 cells and islets (Fig. 4). In MIN6 cells, following stimulation with liraglutide, the mRNA levels of Bax, p27, and Cidea were decreased to 47.23 ± 8.76%, 51.56 ± 9.62%, and 48.13 ± 12.06% when compared to those of the control group, respectively ($P < 0.01$, Fig. 4A). Similar results were shown in western blot detection (Fig. 4B). Moreover, palmitate markedly up-regulated mRNA levels of Bax, p27, and Cidea (Bax, 262.43 ± 28.10%; p27, 327.43 ± 17.86%; Cidea, 330.09 ± 25.99%; vs. control group, $P < 0.01$, Fig. 4A). And liraglutide could inhibit such up-regulation at both mRNA and protein levels. Furthermore, when pre-cultured with LY294002, the down-regulation of Bax, p27, and Cidea by liraglutide was obliterated (Fig. 4A, B). Moreover, the analogous expression pattern for these 3 genes was found in islets from C57BL/6J mice (Fig. 4C, D). Meanwhile, no statistic difference between control and LY294002 only group was found, which excluded the possible effect of LY294002 (data not shown). Based on these data, liraglutide may down-regulate the expressions of Bax, p27, and Cidea via PI3K/Akt/FoxO1 signaling to exert its anti-apoptosis action in beta cells.

LIRAGLUTIDE UP-REGULATES THE EXPRESSION OF Pdx-1, neurod AND MAFA UNDER LIPOTOXIC STRESS

Pdx-1, NeuroD and MafA are known to be critical for the maintenance of function in mature beta cells [Stoffers et al., 2000; Zhang et al., 2005; Shao et al., 2009; Shao et al., 2010; Shao et al., 2013]. Since they were reported to be downstream targets of FoxO1, the expression of these three genes was investigated in Min6 cells/islets. According to real-time PCR, mRNA levels of Pdx-1, NeuroD, and MafA were found to be highly up-regulated by liraglutide in MIN6 cells (223.62 ± 15.94%, 291.57 ± 37.40%, 314.08 ± 21.47%, respectively, vs. control, $p < 0.01$, Fig. 5A). Equivalent results were found in islets of liraglutide-treated mice (Fig. 5C, D). On the contrary, the expression of these three genes was distinctively down-regulated by palmitate but this down-regulation was corrected by the supplement of liraglutide in vivo and vitro (Fig. 5). Nevertheless, the expression pattern in Li+PA+LY group showed no significant difference when compared with that in PA group ($P > 0.05$), illustrating that all the effects of liraglutide could be suppressed by LY294002 and that Akt/FoxO1/Pdx-1, NeuroD and MafA may be involved in liraglutide-induced GSIS correction in lipid stress.

DISCUSSION

Elevated plasma FFAs are known to contribute to the beta-cell dysfunction and lipotoxicity development. Our present study substantiated that palmitate significantly increased lipoapoptosis levels. Nevertheless, the addition of liraglutide could prominently prevent these negative effects in vitro and in vivo (Fig. 1), suggesting the protective effects of liraglutide on anti-apoptosis in lipotoxic condition. Additionally, insufficiency of insulin secretion to compensate for insulin resistance contributes to the development of T2DM [Katakura et al., 2004]. A chronic exposure to FFAs resulted in diminished capacity to secrete insulin [Prentki and Nolan, 2006; Shao et al., 2010]. Consistent with these findings, we also verified the lipotoxic induced GSIS impairment. Significantly, our present study, for the first time, identified that liraglutide could correct the insufficiency of insulin secretion in high FFAs condition. Together, this study uncovered the powerful protective effects of liraglutide against lipotoxicity.

As for the underlying mechanisms of lipotoxicity, it has been studied by many research groups and some pertinent signaling molecules and their related signal pathways are identified [Giacca et al., 2011; Chen et al., 2013; Shao et al., 2013]. However, these findings are multifarious and trivial. Is there a key factor that links all these molecules and complicated network? A number of studies have indicated that the activation of the PI3K signaling pathway was involved in the regulation of beta-cell mass and function [Gu et al., 2011]. One of the major targets of PI3K, the serine threonine kinase Akt played a central role in beta-cell growth [Hughes and

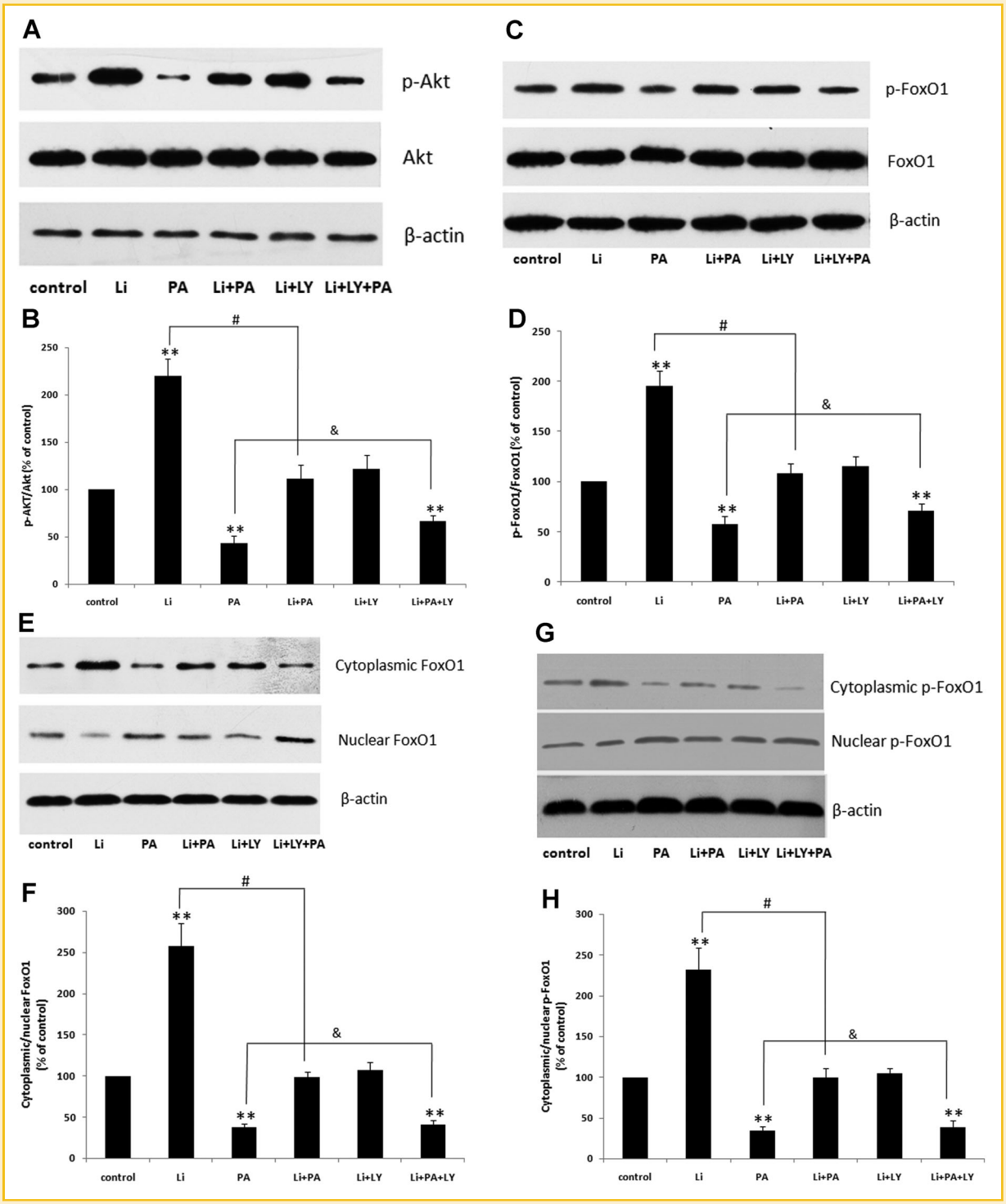

Fig. 3. Protein levels of Akt, phosphorylated Akt, FoxO1, and phosphorylated FoxO1 in Min6 cells were assayed by Western blotting. (A) MIN6 cells were incubated with indicated concentrations of liraglutide (Li), palmitate (PA), liraglutide plus palmitate (Li + PA), liraglutide plus LY294002 (Li + LY), and liraglutide, palmitate plus LY294002 (Li + PA + LY). β-actin was used as a loading control. Total Akt and phosphorylated Akt (p-Akt) were detected. (B) The levels of Akt phosphorylation were expressed as p-Akt/Akt and normalized relative to non-treatment group (control group). (C) Total FoxO1 and phosphorylated FoxO1 (p-FoxO1) were detected. (D) The levels of FoxO1 phosphorylation were expressed as p-FoxO1/FoxO1 and normalized relative to non-treatment group (control group). (E) Protein levels of cytoplasmic and nuclear FoxO1 were detected. (F) Values were expressed as cytoplasmic/nuclear FoxO1 and normalized relative to control group. (G) Protein levels of cytoplasmic and nuclear p-FoxO1 were detected. (H) Values were expressed as cytoplasmic/nuclear p-FoxO1 and normalized relative to control group. Images are representative of three experiments with similar results. Data are presented as the means ± SEM of three independent experiments. $^{**}P < 0.01$ vs. control group. # $P < 0.05$ vs. Li group, & $P > 0.05$ vs. PA group.

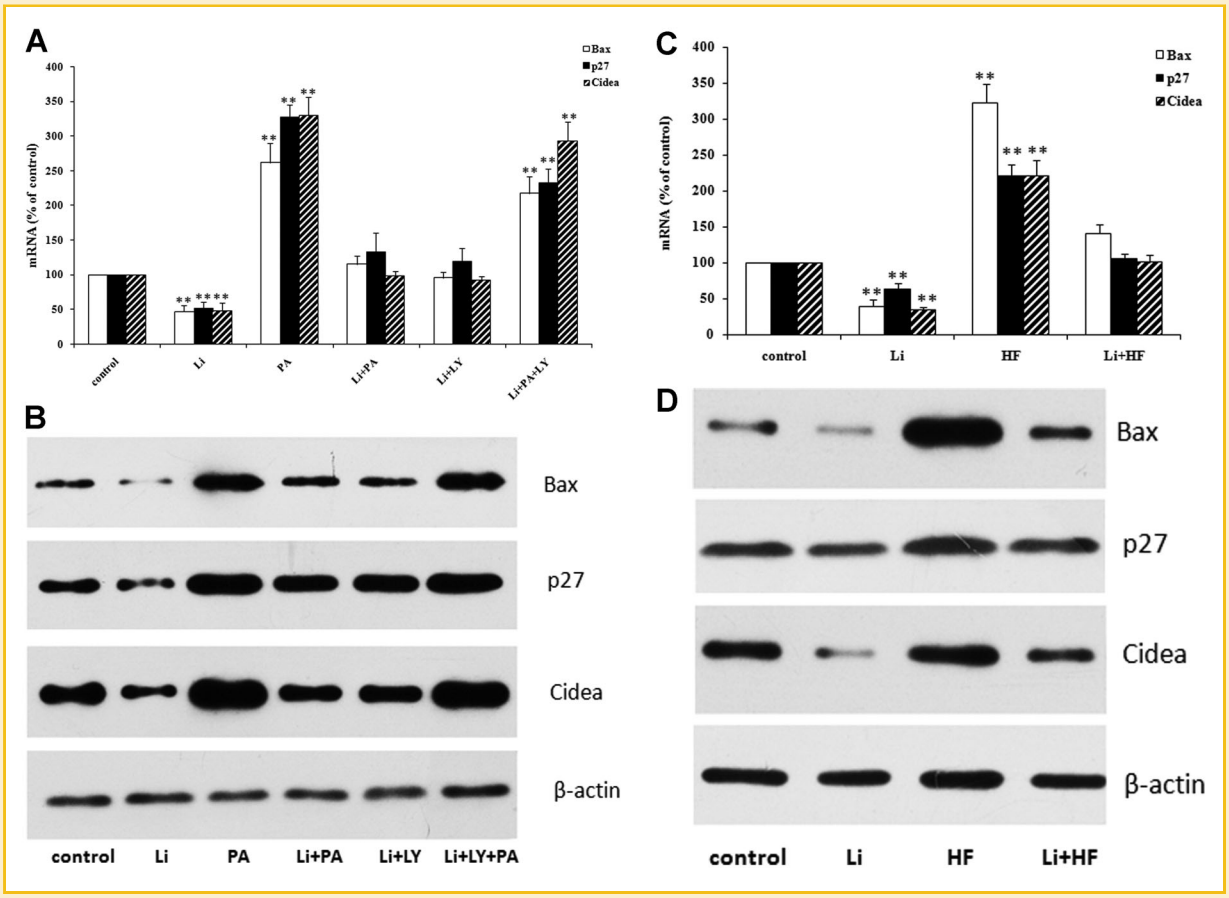

Fig. 4. mRNA and protein levels Bax, p27, and Cidea were determined by real-time PCR and Western blotting. (A) MIN6 cells were incubated with indicated concentrations of liraglutide (Li), palmitate (PA), liraglutide plus palmitate (Li + PA), liraglutide plus LY294002 (Li + LY), and liraglutide, palmitate plus LY294002 (Li + PA + LY). mRNA levels were detected. mRNA quantities were calculated as a ratio to the β-actin level in the each cDNA sample. Data are shown as the relative expression ratio to control group. Three independent experiments were performed and results are expressed as means ± SEM. (B) Protein levels were detected and β-actin was used as a loading control. Images are representative of three experiments with similar results. (C) C57BL/6J mice were fed with either normal chow (Control) or a high-fat diet (HF). Subcutaneous injections of liraglutide were performed to normal diet fed mice (Li) or to high-fat fed mice (Li + HF). mRNA levels were detected. mRNA quantities were calculated as a ratio to the β-actin level in the each cDNA sample. Data are shown as the relative expression ratio to control group. Three independent experiments were performed and results are expressed as means ± SEM. (D) Protein levels were detected and β-actin was used as a loading control. Images are representative of three experiments with similar results. $^{**}P < 0.01$ vs. control group.

Huang, 2011]. Recent studies reported that the signaling pathways triggered by GLP-1 in β-cells both in vivo in db/db mice and in vitro in INS-1 cells were complex with Akt as one of the key kinases involved in GLP-1 functions [Wang et al., 2004; Li et al., 2005]. We therefore hypothesized that the PI3K/Akt pathway might be of importance in liraglutide-induced beta-cell protection against lipid stress. To identify this, we tested the levels of Akt and phospho-Akt in Min6 cells and islets from C57BL/6J mice and found that palmitate caused a notable decrease of p-Akt. Nevertheless, such decrease was attenuated by the addition of liraglutide. Consequently, the activation of Akt might participate in liraglutide related lipotoxicity prevention. Furthermore, since the Akt is the major target of PI3K, the pharmacological inhibitor of PI3K, LY294002, was used to investigate the possible mechanisms. Prominently, the actions of liraglutide on beta-cell mass restoration, cell cycle regulation, and GSIS potentiation under lipotoxic stress were entirely abolished by LY294002. Accordingly, these data allow us to speculate that liraglutide may exert its protective effects by triggering the activity of PI3K and the consequential phosphorylation of Akt.

Furthermore, we intended to explore the downstream signal and effectors in PI3K/Akt pathway. A series of studies have suggested that FoxO1 has a direct effect on pancreatic beta-cell neogenesis, proliferation, and stress resistance [Kitamura and Kitamura, 2007]. Accili et al. have previously shown that a constitutively nuclear FoxO1 impairs compensatory beta cell hyperplasia in insulin-resistant states [Accili and Arden, 2004]. Moreover, the transcriptional activity of FoxO proteins is regulated through phosphorylation by PI3K and Akt [Kim et al., 2005]. Furthermore, FoxO1 was reported to mediate the proliferative and anti-apoptotic roles of human GLP-1 in pancreatic beta cells [Buteau et al., 2001; Buteau et al., 2006]. All these studies allow us to integrate these features of FoxO1 function into liraglutide induced lipotoxic prevention. Consequently, we then tested the levels of FoxO1 and p-FoxO1 proteins. As shown in 3CD, the phosphorylation of FoxO1 was

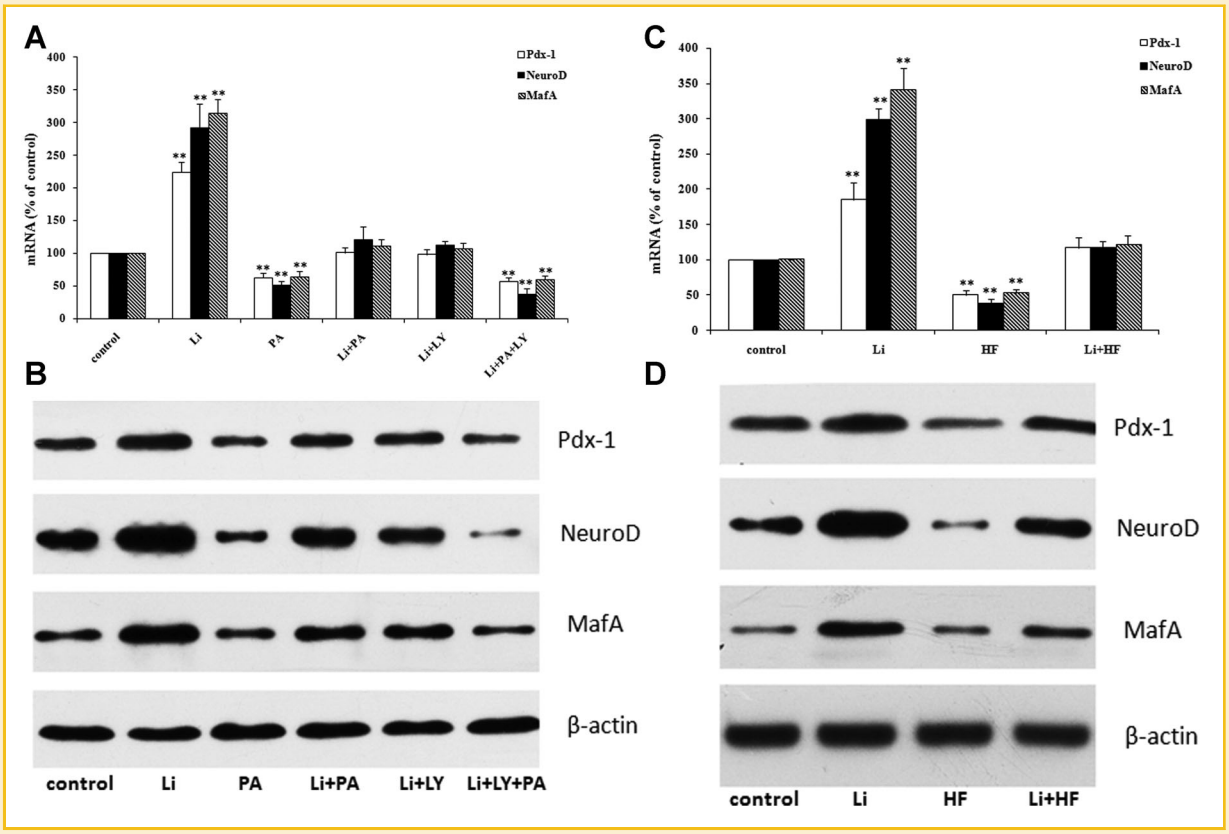

Fig. 5. mRNA and protein levels Pdx-1, NeuroD, and MafA were determined by real-time PCR and Western blotting. (A) MIN6 cells were incubated with indicated concentrations of liraglutide (Li), palmitate (PA), liraglutide plus palmitate (Li + PA), liraglutide plus LY294002 (Li + LY), and liraglutide, palmitate plus LY294002 (Li + PA + LY). mRNA levels were detected. mRNA quantities were calculated as a ratio to the β-actin level in the each cDNA sample. Data are shown as the relative expression ratio to control group. Three independent experiments were performed and results are expressed as means ± SEM. (B) Protein levels were detected and β-actin was used as a loading control. Images are representative of three experiments with similar results. (C) C57BL/6J mice were fed with either normal chow (Control) or a high-fat diet (HF). Subcutaneous injections of liraglutide were performed to normal diet fed mice (Li) or to high-fat fed mice (Li + HF). mRNA levels were detected. mRNA quantities were calculated as a ratio to the β-actin level in the each cDNA sample. Data are shown as the relative expression ratio to control group. Three independent experiments were performed and results are expressed as means ± SEM. (D) Protein levels were detected and β-actin was used as a loading control. Images are representative of three experiments with similar results. $^{**}P < 0.01$ vs. control group.

distinctively decreased when exposed to palmitate. And such decrease of p-FoxO1 could be attenuated by liraglutide. Furthermore, all the effects of liraglutide on FoxO1 phosphorylation were prohibited when LY294002 was pre-incubated. Therefore, we assumed that PI3K activated Akt through phosphorylation; and active Akt, in turn, inhibited the transcriptional activation of FoxO1, eventually exerting the protective effects on beta cell.

Moreover, it is known that the phosphorylation of FoxO1 by Akt results in the 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 inactivation of FoxO1-mediated transcription [Webster, 2004]. To ascertain this in lipotoxic condition, FoxO1 from nuclear proteins and cytoplasmic proteins were examined and, as shown in Figure 3F, palmitate significantly decreased the ratio of cytoplasmic to nuclear FoxO1. And such decrease was erased by the addition of liraglutide, revealing that the nuclear translocation of FoxO1 in response to FFAs can be cancelled by liraglutide. Furthermore, PI3K inhibitor annulled liraglutide-mediated nuclear exclusion of FoxO1 under both physiological and lipotoxic condition. Therefore, FoxO1 may be directly phosphorylated by its usual upstream Akt via liraglutide/PI3K/Akt pathway, resulting in nuclear exclusion and eventual transcription inactivation.

Likewise, we planned to explore the precise mechanisms for the association of FoxO1 inactivation and beta-cell protection. It is known that FoxO1 is a multifunctional transcription factor that is of importance in the regulation of metabolism, cell differentiation, cell cycle arrest, and proliferation [Glauser and Schlegel, 2007]. Consequently, the identification of specific and direct downstream targets of FoxO1 is crucial for an understanding of FFAs triggered beta-cell dysfunction and liraglutide-related protective effects.

In this regard, we screened some apoptosis related effectors and found the significant expression variations of Bax, p27, and Cidea when treated with FFAs. Bax is a critical regulator of the mitochondrial apoptotic pathway and is recently reported to be correlated with beta-cell apoptosis under high FFA stress [Piro et al., 2002]. In addition, p27, a member of the cyclin-dependent kinase inhibitor (CDKI) family, is highly expressed in beta-cell nuclei and plays essential roles during G1-to-S phase progression.

Moreover, p27-deleted mice displayed improved glucose tolerance and increased insulin secretion, which was attributed to the increased islet mass [Karnik et al., 2005]. Furthermore, the CIDE family, with Cidea included, was initially identified as a group of pro-apoptotic proteins [Inohara et al., 1998]. Cidea expression were detected in human and mouse pancreas tissue [Gummesson et al., 2007; Wu et al., 2008]. Recently, Ito et al. demonstrated that down-regulation of Cidea was involved in the anti-apoptotic effects in human adipocytes [Ito et al., 2010]. In this study, as shown in Figure 4, liraglutide elicited the decrease but FFAs triggered the increase of Bax, p27, and Cidea in beta cells. Importantly, the down-regulation of these three genes by liraglutide could be prohibited when PI3K is inactivated. It has been reported that p27, Bax and Cidea were reported to be prominent downstream targets of FoxO1 in beta cells [Schmidt et al., 2002; Kim et al., 2005; Omae et al., 2012]. Therefore, we proposed that these three genes are critical regulators for FFA-induced apoptosis. And as the downstream effectors of FoxO1, they were suppressed by liraglutide via PI3K/Akt/FoxO1 signaling pathway. This may partially explain the effects of liraglutide on cell-mass restoration under lipid stress.

Besides liraglutide induced apoptosis inhibition, improved GSIS under lipid stress was also observed, which cannot be simply interpret by the down-regulation of Bax, p27, and Cidea. Other molecules should exist and exert vital impacts. Our data revealed that Pdx-1, Neuro D, and MafA may be involved in this event. These three genes are known to be critical for the maintenance of function in mature beta cells. Pdx-1 is the most important transcription factor for pancreatic cell growth and function [Jonsson et al., 1994; Ahlgren et al., 1998]. Kitamura et al. previously reported that FoxO1 haploinsufficiency rescued diabetes via the restoration of Pdx1 expression [Kitamura et al., 2002]. Additionally, gene expression assays showed that GLP-1 induces Pdx1 and Foxa2 expression by inhibiting FoxO1 binding to both promoters [Buteau et al., 2007]. Moreover, MafA is a master transcription factor regulating the insulin gene and GSIS in the mature beta-cell [Zhang et al., 2005; Shao et al., 2009]. In addition, NeuroD, the basic helix loop helix (bHLH) protein, is the other class of transcription factors for pancreas development and insulin gene transcription [Naya et al., 1997; Kojima et al., 2003]. Mice with a targeted mutation in the NeuroD gene have a striking reduction in the number of beta cells, resultantly developing severe diabetes [Naya et al., 1997]. Besides, Kitamura et al. reported that both NeuroD and MafA were direct FoxO1 targets in beta cells [Kitamura et al., 2005]. In our study, Pdx-1, MafA, and NeuroD were low expressed under lipotoxic condition and such low-expression could be reversed by the supplement of liraglutide. Nevertheless, liraglutide was unable to restore the expression of these three genes when LY294002 was pre-incubated, uncovering that the regulating effects by liraglutide may be mediated by PI3K/Akt pathway. Taken together, the high FFAs stress may impair the insulin release, at least in part, by down-regulating the expression of Pdx-1, MafA, and NeuroD via PI3K/Akt pathway. Restoration of these three genes by liraglutide may contribute to the GSIS correction.

Interestingly, Shimoda reported that liraglutide treatment in db/db mice significantly augmented expression of genes involved in cellular differentiation with Pdx-1 and NeuroD included [Shimoda et al., 2011]. Additionally, consistent with FoxO1 inhibition of beta-cell function by suppression of Pdx1 transcription [Kitamura et al., 2002; Kim et al., 2012], we observed decreased Pdx1 expression in palmitate-treated beta cells. However, decreased MafA and NeuroD levels were inconsistent with previous study by Kitamura that FoxO1 induces MafA and NeuroD expressions and protects beta-cells against oxidative stress [Kitamura et al., 2005]. We speculated that the decreased expression of MafA and NeuroD may be caused by the reduction of Pdx1, which is a primary regulator for MafA and NeuroD transcription. Another possibility is that this discrepancy could be in virtue of acute affects of FoxO1 activity vs. chronic overexpression of constitutively active FoxO1. Moreover, Kitamura et al. specifically address the acetylation of FoxO1, which bypasses the impact of phosphorylation. These data begin to outline an overarching role of FoxO1 in beta-cell function. According to these data we obtained, it could be speculated that FoxO1 orchestrates a series of responses by regulating the downstream effectors of Pdx-1, NeuroD, and MafA to exerting the effectively protective actions under lipotoxic condition.

Taken together, liraglutide could significantly correct the lipoapoptosis and GSIS impairment engendered by lipotoxicity, with PI3K/Akt/FoxO1 pathway involved in this event. Accordingly, we need to consider this point when FoxO1 is applied as a molecular target in the development of new strategies for treating diabetes. Further comprehensive sorting and characterization of PI3K/Akt/FoxO1 pathway and its downstream targets will lead to a better understanding of the mechanisms of lipotoxicity in the development of diabetes.

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