The Akt/FoxO1/p27 pathway mediates the proliferative action of liraglutide in β cells

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Abstract. Numerous studies have shown that liraglutide, a modified form of human glucagon-like peptide-1 (GLP-1), increases β-cell mass. However, the underlying molecular mechanisms remain unclear. In the present study, we investigated the role of Akt/FoxO1/p27 signaling in liraglutide-induced β-cell proliferation. INS-1 rat insulinoma cells were exposed to two different concentrations of liraglutide. MTT assay was performed to evaluate β-cell proliferation. The expression of Akt/FoxO1/p27 was detected by quantitative real-time PCR and Western blotting. The results revealed that in comparison to the non-treatment group, stimulating INS-1 cells with 10 and 100 nM liraglutide caused β-cell proliferation to be significantly enhanced. The mRNA levels of p27 in INS-1 cells declined upon treatment with liraglutide compared to the non-treatment group. Western blot analysis revealed that the phosphorylation of Akt and FoxO1 was markedly elevated following exposure to liraglutide. Moreover, LY294002, a phosphatidylinositol 3-kinase (PI-3K) inhibitor, significantly abrogated liraglutide-induced effects. Therefore, we conclude that liraglutide increased the β-cell mass by upregulating β-cell proliferation and that the proliferative action of liraglutide in β cells was mediated by activation of PI-3K/Akt, which resulted in inactivation of FoxO1 and decreased p27.

Introduction

The incretin hormone glucagon-like peptide-1 (GLP-1), which is released from the gastrointestinal tract in response to nutrient ingestion, enhances insulin secretion and maintains glucose homeostasis. However, the short circulating half-life of the bioactive, intact GLP-1 limits its potential use for the treatment of diabetes (1). Liraglutide, one of the long-acting GLP-1 receptor agonists, resists dipeptidyl peptidase (DPP)-IV degradation by fatty acid (FA) acylation and albumin binding, and has been utilized in clinical practice (2). Previous studies have shown that liraglutide increases the β-cell mass through stimulation of β-cell proliferation and islet neogenesis, as well as inhibition of β-cell apoptosis (3,4). Nevertheless, the underlying molecular mechanisms remain to be further investigated.

p27 is a member of the cyclin-dependent kinase inhibitor (CDKI) family and plays two roles during G1-to-S phase progression. The first role is the inhibition of cyclin E-cdk2. The second is that p27 facilitates assembly, activation and nuclear localization of cyclin D-cdk complexes in early G1 phase (5,6). Therefore, inactivation of p27 results in cell proliferation. Moreover, p27-deleted mice display improved glucose tolerance and increased insulin secretion, which has been attributed to the increased islet mass and proliferation. On the contrary, induction of p27 expression has led to severe glucose intolerance and reduced β-cell mass by decreasing proliferation (7). Thus, p27 is a critical molecule for β-cell proliferation.

The forkhead transcription factor class O (FoxO) family plays essential roles in cellular proliferation, apoptosis and differentiation. One of the FoxO isoforms, FoxO1, is the most abundant isoform in pancreatic β cells and regulates its immediate target gene p27 at the mRNA level (8,9). A series of studies have suggested that FoxO1 has a direct effect on pancreatic β-cell differentiation, neogenesis, proliferation and stress resistance (10). A previous study revealed that FoxO1 mediated the proliferative and anti-apoptotic roles of human GLP-1 fragment 7-36 amide in pancreatic β cells (11). Furthermore, studies by Buteau *et al* demonstrated that GLP-1 increased FoxO1 phosphorylation via activation of phosphatidylinositol-3 kinase (PI-3K)/Akt (also known as protein kinase B, PKB) signaling (11,12). PI-3K activates Akt through phosphorylation, and active Akt, in turn, inhibits the transcriptional activation of FoxO1 via phosphorylation-dependent nuclear exclusion. However, little is known regarding whether the Akt/FoxO1 pathway plays a role in the β-cell proliferative signaling in response to liraglutide, the latter of which has 97% homology with endogenous GLP-1 (13).

We therefore hypothesized that the Akt/FoxO1/p27 pathway might play a role in liraglutide-induced β-cell proliferation. To test this hypothesis, in the present study, rat insulinoma INS-1 cells were exposed to two different concentrations of

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liraglutide with or without PI-3K inhibitor to investigate the role of the Akt/FoxO1/p27 pathway in the proliferative action of liraglutide in pancreatic β cells.

Materials and methods

Cell line and culture. Rat insulinoma INS-1 cells were cultured in RPMI-1640 supplemented with 10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% heat-inactivated foetal calf serum (FCS), 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l β-mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified 5% CO_2 atmosphere. INS-1 cells were then incubated with liraglutide at final concentrations of 10 and 100 nM for 24 h in contrast to those without. To explore the mechanism of liraglutide-mediated β-cell proliferation, 50 μmol/l PI-3K inhibitor (LY294002) (Cell Signaling, Beverly, MA, USA) was applied 1 h prior to liraglutide treatment.

Cell proliferation assay. Proliferation of INS-1 cells was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MI, USA) assay. In brief, INS-1 cells were plated in 96-well microtiter plates at 1.5×10^3 cells/well. A total of 3 h prior to cell harvesting, MTT was added to each well at 0.5 mg/ml final concentration. The plates were centrifuged and the supernatant was discarded. Formazan products were dissolved with DMSO and absorbance was measured at a wavelength of 595 nm with a micro-ELISA reader. The results were expressed as the percentage of the control group. Each experiment was performed in triplicate.

Quantitative real-time PCR. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A total of 2 μg RNA was reverse transcribed (RT) at a final volume of 20 μl by using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) and 1 μl of the RT products was amplified and quantified in an ABI Prism 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) by using the SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). The sequences of the primers were as following: p27, forward 5'-ACGTAAACAGCTCCGAATT-3' and reverse 5'-TTCAATGGAGTCAGCGATA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-AGCCCAGAAC ATCATCCCTG-3' and reverse 5'-CACCACCTTCTTGATGT CATC-3'. Expression data were normalized to the geometric mean of the housekeeping gene, GAPDH, to control the variability in expression levels, and calculated as $2^{-[(CT\ of\ p27)-(CT\ of\ GAPDH)]}$, where CT represents the threshold cycle for each transcript.

Western blot analysis. Total proteins were extracted from the cells using a commercially available kit (Kangchen, Shanghai, China). The protein concentration was determined by BCA analysis (Kangchen, Shanghai, China). A total of 30 μg of protein samples was separated on a 10.5% SDS-polyacrylamide gel electrophoresis (PAGE) medium and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, 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 total Akt, phospho-Akt (Ser473), FoxO1, phospho-FoxO1 (Ser256) and β-actin (Cell Signaling) primary antibodies were at dilutions of 1:1000. Following washing for three times 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 (Thermo, Rockford, IL, USA) and washed three times with TBST. Proteins were detected using the enhanced chemiluminescence (ECL) system (Millipore).

Statistical analysis. Statistical analysis was performed using the SPSS 11.0 statistical package. Results are expressed as the means ± SD. The independent samples t-test was employed to compare the difference between two groups. One-way analysis of variance (ANOVA) was used for comparisons among groups of more than two, followed by Fisher's least significant difference (LSD) test for multiple comparisons. Significance was defined as $p < 0.05$.

Result

Liraglutide increases β-cell proliferation. We initially demonstrated the pro-proliferative role of liraglutide in β cells. To this end, an MTT assay was performed and the results revealed that the proliferation of INS-1 cells, when stimulated with 10 and 100 nM of liraglutide, was elevated to 131.90±7.15 and 130.04±0.94%, respectively, as compared to the non-treatment control group ($p < 0.05$). However, the difference between the two treatment groups was not significant ($p = 0.715$) (Fig. 1). Therefore, these data suggest that liraglutide indeed enhances β-cell proliferation.

Liraglutide down-regulates the expression of p27 mRNA. The finding that liraglutide significantly elevated β-cell proliferation prompted us to investigate the underlying mechanisms. p27 is a cell cycle inhibitor and its down-regulation facilitates β-cell growth (14). In this regard, we examined the expression of p27 upon treatment of liraglutide using quantitative real time PCR. Following stimulation with 10 and 100 nM of liraglutide for 24 h, the levels of p27 mRNA were decreased to 0.53±0.12 and 0.52±0.23 to those of the non-treatment control group, respectively ($p < 0.05$) (Fig. 2), implicating the potential role of p27 in liraglutide-induced cell growth.

FoxO1 is required for liraglutide-dependent β-cell proliferation. As a transcription factor, FoxO1 regulates its immediate target gene, p27, at the mRNA level (9). We therefore determined the possible role of FoxO1 in liraglutide-dependent β-cell proliferation. In comparison to the control group, markedly increased phosphorylation of FoxO1, reflecting its attenuated transcriptional activity, was observed in the 10 and 100 nM liraglutide treatment groups (204.33±32.58 and 212.71±45.74% of the control group, respectively, $p < 0.05$). However, the levels did not significantly differ between the two treatment groups (Fig. 3A). These results indicate that decreased transcriptional activity of FoxO1 may play a role in the effects of liraglutide.

Liraglutide up-regulates the activation of Akt. Since FoxO1 is one of the crucial downstream effectors of Akt, we next tested
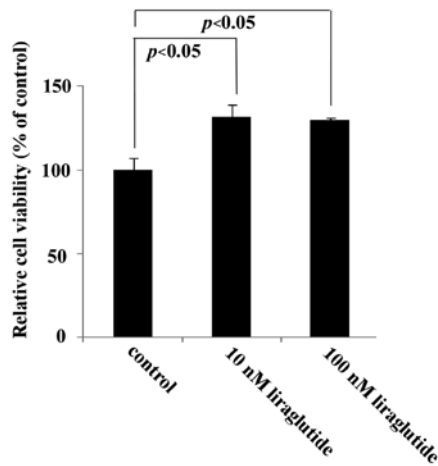
Figure 1. The effects of different concentrations of liraglutide on β-cell proliferation. INS-1 cells were incubated with 10 or 100 nM of liraglutide for 24 h, and proliferation was evaluated by performing 72 h MTT assay as compared with the control group. The two treatment groups did not reach statistical significance (p=0.715).
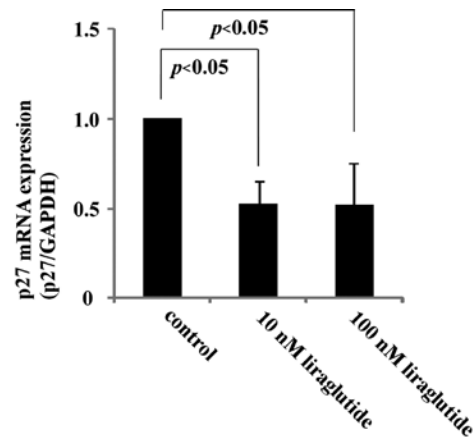
Figure 2. Liraglutide down-regulates the expression of p27 mRNA. Following stimulation with 10 and 100 nM liraglutide for 24 h, the mRNA levels of p27 in INS-1 cells were significantly decreased when compared with the control group.
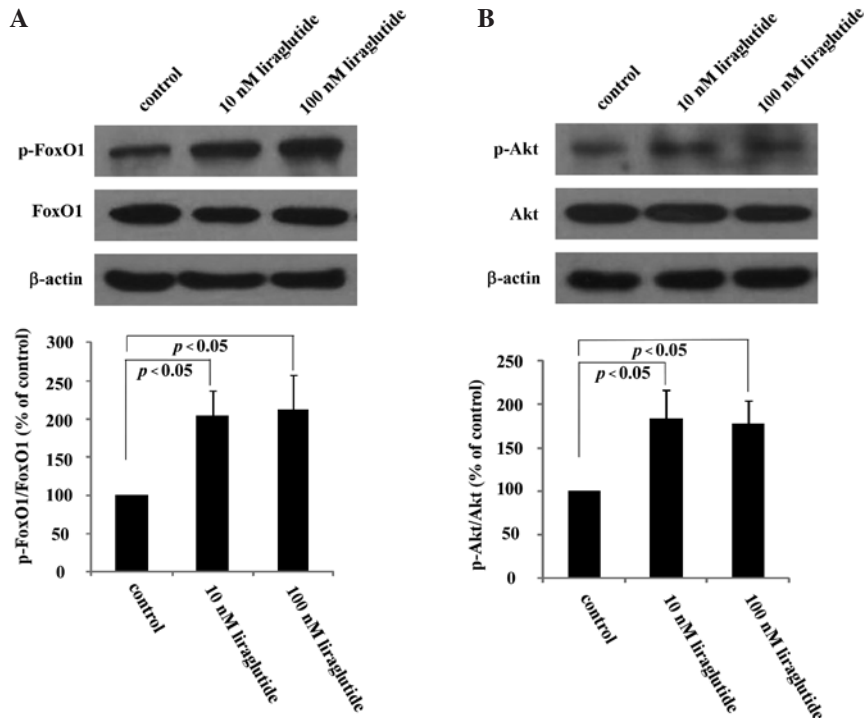

Figure 3. Liraglutide treatment results in activation of Akt and phosphorylation of FoxO1. (A) In comparison to the control group, the phosphorylation of FoxO1 markedly increased in the 10 and 100 nM liraglutide treatment groups. By contrast, the levels did not significantly differ between the two treatment groups. (B) The activation of Akt was markedly enhanced in response to liraglutide treatment.

the levels of Akt and phospho-Akt proteins using Western blot analysis (12). As compared to the control group, the levels of p-Akt/total Akt protein were significantly increased to 184.62±32.00 and 177.91±26.54% in the 10 and 100 nM liraglutide-stimulated INS-1 cells, respectively (p<0.05). Yet again, the activation of Akt in the 10 nM group was not different from that of the 100 nM group (Fig. 3B). Therefore, it is probable that activation of Akt is involved in liraglutide-induced β-cell proliferation.

PI-3K inhibitor LY294002 blocks the effects of liraglutide. To further elucidate that liraglutide enhances β-cell proliferation through the Akt/FoxO1/p27 pathway, we next assessed whether the above effects of liraglutide could be blocked by the pharmacological inhibitor of PI-3K, which is a typical upstream activator of Akt. Western blot analysis revealed that the phosphorylation of Akt was weakly detected when LY294002 was added 1 h prior to 10 or 100 nM liraglutide stimulation (Fig. 4A). The activation of FoxO1 revealed a
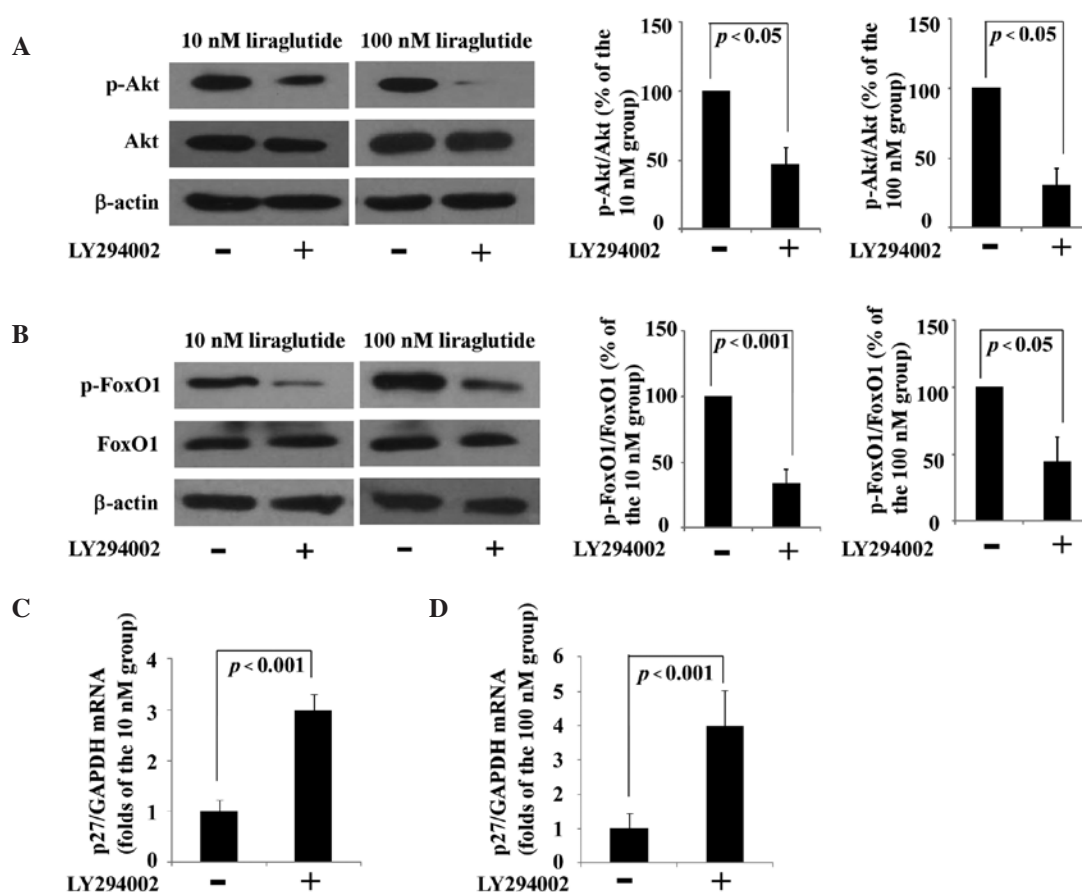
Figure 4. The PI3-K inhibitor, LY294002, blocks the effects of liraglutide. (A and B) The phosphorylation of Akt and FoxO1 was weakly detected when LY294002 was added 1 h prior to 10 or 100 nM liraglutide treatment. (C and D) The mRNA levels of p27 were significantly elevated in the 10 and 100 nM liraglutide treatment groups, respectively, when INS-1 cells were precultured with LY294002, in comparison to the non-LY294002 precultured group.

similar result; the phosphorylation of FoxO1 was markedly decreased when INS-1 cells were precultured with LY294002 (Fig. 4B). Furthermore, when precultured with LY294002 the expression of p27 mRNA was significantly elevated by up to 2.98±0.32- and 3.98±1.06-fold (Fig. 4C and D) in the 10 and 100 nM liraglutide treatment groups, respectively, in comparison to the non-LY294002 precultured group ($p<0.05$). These data, taken together, suggest that the Akt/FoxO1 pathway and p27 mediate the proliferative action of liraglutide in β cells and that liraglutide inactivates p27 via PI-3K/Akt/FoxO1 signaling.

Discussion

In the present study, liraglutide increased the β-cell mass via the stimulation of β-cell proliferation. Furthermore, to the best of our knowledge, we demonstrated, for the first time, that Akt/FoxO1/p27 signaling mediated the β-cell proliferation induced by liraglutide.

Type 2 diabetes mellitus (T2DM) tends to be progressive and islet β-cell failure is the major contributor to the natural progression of the disease (15). Liraglutide, a human GLP-1 analogue with a long half-life, is a new type of hypoglycaemic agent and may potentially delay the disease progression by improving β-cell function in T2DM and increasing β-cell mass in animal models (16,17). Accumulating evidence has

demonstrated that liraglutide stimulates β-cell proliferation, induces islet neogenesis and inhibits β-cell apoptosis, thus leading to the expansion of β-cell mass *in vitro* (17,18). Nevertheless, the underlying mechanisms have not yet been fully understood and await future investigation. It has been recognized that liraglutide notably increases the mitotic index at a maximal stimulatory concentration of 10-100 nM, while 0.1-1.0 nM, liraglutide does not show significant increases in β-cell replication (19). The present study investigated the pro-proliferative action of liraglutide at final concentrations of 10 and 100 nM and explored the potential mechanisms.

A number of studies have indicated that the activation of the PI-3K signaling pathway was involved in the regulation of β-cell mass and function (20). One of the major targets of PI-3K, the serine threonine kinase Akt, was found to play a central role in β-cell growth and survival (21,22). An *in vivo* study revealed that overexpression of constitutively active Akt in mice pancreatic β cells resulted in marked expansion of β-cell mass by increasing the β-cell proliferation and size (23). A previous study found that Akt also played a critical role in the proliferative action of GLP-1 (7-36 amide) in pancreatic β cells (12). In the present study, we demonstrated that Akt also mediated the proliferative action of liraglutide. The finding that the activation of Akt induced by liraglutide was blocked by a PI-3K inhibitor indicates that liraglutide increases Akt phosphorylation in a PI-3K-dependent manner.

FoxO1 was directly phosphorylated by its usual upstream Akt phosphorylator, resulting in nuclear export and, thus, inhibition of its target gene expression. Numerous studies have shown that FoxO1 is a prominent mediator of growth factor signaling in β cells (24). Recent studies have further found that the inhibition of FoxO1 by intact GLP-1 increased β-cell mass. Transduced INS832/13 cells, with a constitutively nuclear FoxO1 mutant, resulted in inhibition of β-cell proliferation induced by GLP-1, suggesting an anti-proliferative role of FoxO1 in pancreatic β cells. Moreover, the effects of GLP-1 were suppressed by an inhibitor of PI-3K (11). In this study, we demonstrated that following exposure to 10 or 100 nM liraglutide for 24 h, the activation of FoxO1 was markedly down-regulated. Therefore, based on the present study, it appears that the proliferative action of liraglutide also requires FoxO1 inactivation. Furthermore, these effects were blocked by the PI-3K inhibitor (LY294002). Therefore, our study revealed that liraglutide stimulated β-cell proliferation via the Akt/FoxO1 pathway in a PI3-K-dependent manner.

However, the mechanism of the role of FoxO1 in native GLP-1 and liraglutide actions may be complex and has not yet been fully addressed. Previous evidence indicates that FoxO1 mediates the proliferative and anti-apoptotic properties of GLP-1 in β cells through controlling the expression of the β-cell specific transcription factor, the pancreatic and duodenal homeobox gene-1 (Pdx1) (25). The present study, revealed that p27, an immediate target gene of FoxO1, also played a significant role in the effect of liraglutide-dependent β-cell proliferation. p27, a member of the CDKI family, is highly expressed in β-cell nuclei and an *in vitro* study revealed that p27-deficiency results in islet hyperplasia (26). By contrast, β cell-specific overexpression of p27 was found to lead to islet hypoplasia and diabetes (27). Therefore, p27 is central to the regulation of the β-cell mass. Moreover, the expression of p27 was found to be regulated by the transcription factor FoxO1. In other words, FoxO1 increases the level of mRNAs encoding p27 (9). As a result, in our study, the inactivation of FoxO1 induced by liraglutide led to the down-regulation of p27 mRNA resulting in an increased β-cell proliferation. Therefore, our study demonstrated, for the first time, that p27 is also involved in the proliferative action of liraglutide in β cells.

In conclusion, our results suggest that liraglutide increases the β-cell mass via the stimulation of β-cell proliferation. The proliferative action of liraglutide in β cells was mediated by the Akt/FoxO1/p27 pathway and liraglutide inactivated p27 via PI-3K/Akt/FoxO1 signaling.

Acknowledgements

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