Protein kinase C pathway mediates the protective effects of glucagon-like peptide-1 on the apoptosis of islet β-cells

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Abstract. The incidence of diabetes has been increasing over previous years. It is hypothesized that promoting the survival of islet β-cells is a key direction for the treatment of diabetes. Although gastric bypass surgery improves certain types of diabetes and attenuates its progression, there are certain associated disadvantages (including intestinal obstruction and anastomotic leakage), and quality of life and physical status (such as malnutrition) are significantly affected by gastric bypass surgery. Therefore, it is important to determine the mechanisms underlying the improvement of diabetes by gastric bypass surgery and identify novel gene targets for diabetes therapeutics. In the present study, glucagon-like peptide-1 (GLP-1), whose secretion was markedly increased following gastric bypass surgery, increased the activity of protein kinase C (PKC) in islet β-cells in a dose-dependent manner. Additionally, treatment with GLP-1 boosted cell viability and decreased cell death in starved islet β-cells, and inhibited mitochondria-dependent apoptosis by regulating the expression levels of Bcl-2/Bax. These effects were reversed by inhibiting the PKC pathway using hypericin. Therefore, the present study concluded that GLP-1 may promote the survival and inhibit the apoptosis of islet β-cells at least in part by activating the PKC pathway, which is an important underlying mechanism and may be exploited in the treatment of diabetes.

Introduction

Glucagon-like peptide-1 (GLP-1) is synthesized in intestinal L-cells and pancreatic α-cells (1). GLP-1 is important as an incretin hormone and induces glucose-responsive insulin secretion stimulation and glucagon secretion inhibition. GLP-1 also has the ability to stimulate the proliferation of β-cells and inhibit β-cell apoptosis (2). GLP-1 is important in the remission of diabetes following gastric bypass surgery (3). Although GLP exerts effective β-cell-trophic activities and exhibits β-cell-protective properties, the clinical applications of GLP-1 have been limited due to its short half-life *in vivo* and rapid degradation by the enzyme, dipeptidyl peptidase-4 (DPP-4) (4). DPP-4 inhibitors and GLP-1 receptor agonists, which are resistant to DPP-4 degradation, have been developed and are used to accomplish sustained GLP-1 receptor activation. GLP-1 and its analogs are currently being used clinically to increase insulin secretion and reduce body weight in patients with Type 2 diabetes (5). However, the underlying mechanisms and signal transduction of GLP-1 in β-cell apoptosis remain to be elucidated.

Protein kinase C (PKC), a serine/threonine-associated protein kinase, is a member of the cAMP-dependent protein kinase/PKG/PKC family, is important in several cellular functions and affects a number of signal transduction pathways (6). There are multiple isoforms of PKC, including the conventional PKC (cPKC) isoforms (PKC-α, -β1, -β2 and -γ), which are activated by phosphatidylserine, calcium and DAG or phorbol esters, including phorbol 12-myristate 13-acetate (PMA); the novel PKCs (PKC-δ, -ε, -θ, and -η) are activated by phosphatidylserine, DAG or PMA, however, not by calcium; and the atypical PKCs (PKC-ζ and -ι/λ), which are not activated by calcium, DAG or PMA. All function in diverse cellular processes, including cell growth, apoptosis and differentiation, as well as specialized responses, including secretion and muscle contraction (7-10). PKCα and δ are the major isozymes in pancreatic islet and β-cell lines, where PKCs βII, ε, ζ and ι are also expressed (11-13). However, the contribution of PKC activation to glucose-stimulated insulin secretion remains controversial. For example, certain translocation investigations have provided indirect evidence that cPKCs, particularly PKCα, are activated by glucose and PKCs isoform-specific inhibitors have contributed to the confusion in the β-cell literature and differential secretion regulation (14,15). Therefore, determination of the precise role of the PKC pathway in the regulation of β-cell function is necessary for improving the treatment of diabetes.

Based on the above-mentioned evidence, it is necessary to determine whether GLP-1 participates in regulating the survival of β-cells and elucidate the corresponding molecular mechanisms. In the present study, the association between

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GLP-1, the PKC signaling pathway and the survival of islet β-cells was investigated. The findings may provide a novel targeted treatment modality for diabetes.

Materials and methods

Materials. Antibodies against rabbit polyclonal immunoglobulin (Ig)G Bcl-2, (cat. no. sc-492), rabbit polyclonal IgG Bax (cat. no. sc-493) and mouse monoclonal IgG β-actin (cat. no. sc-47778) were all purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and used at a 1:500 dilution for western blotting. Caspase-3 and caspase-9 activity kits and a lactate dehydrogenase (LDH) assay kit were purchased from Beyotime Institute of Biotechnology, Inc. (Haimen, China). All other reagents were purchased from common commercial sources.

Animals. Approximately 20 male Sprague-Dawley rats (weight, 200-220 g), aged 6-8 weeks, were purchased from and housed in the Animal Research Center of Jiamusi University (Heilongjiang, China). The environment was temperature- and humidity-controlled, with a 12-hr light/dark cycle. The rats were provided with access to food and water *ad libitum*, according to the guidelines of the Institutional Animal Care and Use Committee (16). The study was approved by the ethics committee of Jiamusi University.

Isolation of pancreatic islets. The pancreas was removed from rats, which were sacrificed under anesthesia by intraperitoneal injection of 30 mg/kg sodium pentobarbital, and were subsequently minced and washed with Ca^{2+}- and Mg^{2+}-free Krebs-Ringer bicarbonate buffer (KRBB; Macgene, Beijing, China). The sample was centrifuged at 300 x g for 2 min at 4˚C and 0.2% collagenase V (Sigma-Aldrich, St. Louis, MO, USA) in KRBB was used to digest the pellet at 37˚C for 30 min in an agitating water bath. Cold KRBB was added to the samples to terminate digestion. Digested tissue was filtered through a 0.5-mm mesh and was subsequently centrifuged at 300 x g for 2 min at 4˚C. The supernatant was discarded and the pellet was resuspended in 25% Ficoll (Sigma-Aldrich) with 10 mM HEPES (Sangon Biotech, Shanghai, China; pH 7.0). The pellet was then covered by layers of various concentrations of Ficoll (23%, 20% and 11%). Following centrifugation, the islets were collected from the interface between 11-20% and 20-23%. The isolated cells were cultured at 37˚C in RPMI-1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Gibco Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (both Gibco Life Technologies) in an incubator with 95% air and 5% CO_2. Prior to each experiment, serum deprivation was performed to induce cell apoptosis. Cells were subsequently treated with hypericin (6.8 μmol), GLP-1 (1 μM) or hypericin plus GLP-1 under conditions of serum deprivation. The cells cultured in complete medium served as a control.

Assessment of cell viability. A 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay was used to assess cell viability. The cells were split into a 96-well plate (~$5x10^3$ cells/well) and were subjected to growth arrest for 24 h prior to experiments. The cells were treated with the indicated treatments for 72 h. Following incubation for 96 h at 37˚C, the cells were incubated in a medium containing 0.5% MTT (prepared in PBS; 5 g/l; 20 μl per well) for 4 h. The MTT reaction was terminated by adding dimethyl sulfoxide (Sigma-Aldrich) to the medium, followed by incubating for 10 min at 37˚C. The absorbance was measured using a spectrophotometer (BioTek, Winooski, VT, USA) at 540 nm. All experiments were performed in triplicate and repeated three times.

Measurement of caspase-3 activity. The caspase-3 activity was measured by its chromogenic caspase substrate cleavage. The protein samples were prepared as follows: Briefly, cells from each group were lysed in T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Inc., Rockford, IL, USA) and incubated for 30 min on ice. The lysates were centrifuged at 16,099 x g for 10 min and the insoluble fraction was discarded. The Bradford assay (Sangon Biotech) was applied to determine the protein concentrations using bovine serum albumin (BSA; EMD Millipore, Billerica, MA, USA), which served as a standard. Approximately 50 μg total protein from each group was added to the reaction buffer, containing Ac-DEVD-pNA (Beyotime Institute of Biotechnology Inc.). The mixture was incubated for 4 h at 37˚C and the absorbance of yellow pNA cleavage from its corresponding precursors was measured using a spectrometer at 405 nm. The specific caspase-3 activity was normalized against the total proteins in the cell lysates and was expressed as the fold change of the baseline caspase activity of the control cells.

Measurement of caspase-9 activity. Caspase-9 activity was measured using a caspase-9 activity kit and an LDH assay kit according to the manufacturer's instructions. The cleavage of Ac-LEHD-pNA, the chromogenic caspase substrate of caspase-9, was measured. Caspase-9 activity was normalized against the total proteins of the cell lysates, and the specific caspase-9 activity was expressed as the fold change of the baseline caspase activity of the control cells.

LDH assay. The levels of LDH released into the culture media were measured using a Cytotoxicity Detection kit (Beyotime Institute of Biotechnology, Inc.). The percentage of injured cells in the culture is represented by the LDH activity in the medium relative to the LDH activity following complete cell lysis, which is the total LDH activity. The LDH activities were determined using medium containing Triton-lysed cellular supernatant. The experiments were performed according to the manufacturer's instructions. A portion of culture medium was mixed with an equal volume of LDH substrate solution for 30 min and the reaction was subsequently terminated by adding 5X 0.1 M NaOH. The absorbance was measured using a spectrometer at 440 nm.

Western blotting. Western blotting was performed as previously described (17). Briefly, $5x10^5$ cells were sonicated (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China) in radioimmunoprecipitation buffer and homogenized. Cell debris was removed by centrifugation at 12,000 x g for 10 min at 4˚C. The samples, containing 50 μg protein, were electrophoresed on 10% SDS polyacrylamide gels (Sangon Biotech) and

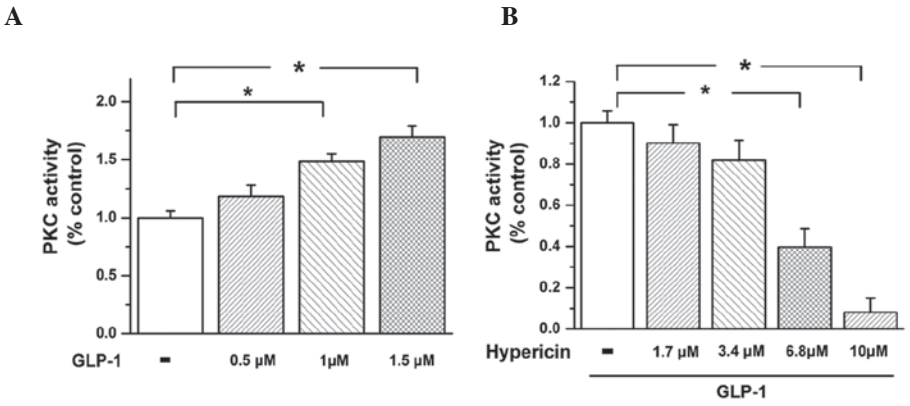

Figure 1. GLP-1 increases the activity of PKC in a dose-dependent manner and the inhibition of PKC effectively decreases this activity. (A) The activity of PKC was significantly upregulated following treatment with >1 μM GLP-1. (B) The activity of PKC was assessed following treatment of the islet β-cells with different doses of the PKC inhibitor, hypericin. The activity of PKC was significantly decreased following treatment with >6.8 μmol hypericin. The error bars indicate the mean ± standard error of the mean of three or more independent batches of cells. *P<0.05, compared with the untreated cells. GLP-1, glucagon-like peptide-1; PKC, protein kinase C.

were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA, incubated with primary antibodies and subsequently with alkaline phosphatase-conjugated secondary antibody. The membranes were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Tiangen Biotech Co. Ltd., Beijing, China). The blots were also stained with anti-β-actin antibody as an internal control for the quantity of target proteins.

Assay of PKC. PKC activity in islet β-cells was assayed using a MESACUP protein kinase assay kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). The cells were washed three times with ice-cold PBS and detached from the plates using a cell scraper. The cells were suspended in 1 ml sample preparation buffer and were sonicated for 30 sec at 4˚C. The homogenates were centrifuged at 100,000 x g for 1 h at 4˚C. The supernatant was discarded and the precipitates were resuspended in 1 ml buffer [25 mM Tris-HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 2 mM ethylene glycol tetraacetic acid, and 0.002% leupeptin (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan)], and were used as the membrane fractions. The PKC activity in the fractions was measured, as described previously (18).

Statistical analysis. Statistical analysis was performed with SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL, USA). The results are expressed as the mean ± standard deviation. Student's t-test was used to determine the statistical significance. P<0.05 was considered to indicate a statistically significant difference.

Results

GLP-1 increases the activities of PKC and the inhibitor effectively prevents the activation of PKC. To determine whether GLP-1 inhibits the apoptosis of islet β-cells by activating the PKC pathway, the activities of PKC in islet β-cells was determined. It was demonstrated that the activity of PKC was significantly upregulated at a concentration of 1 μM and GLP-1 increased the activity of PKC in a dose-dependent manner (Fig. 1A, P<0.05). The results revealed that the PKC pathway was activated in response to GLP-1.

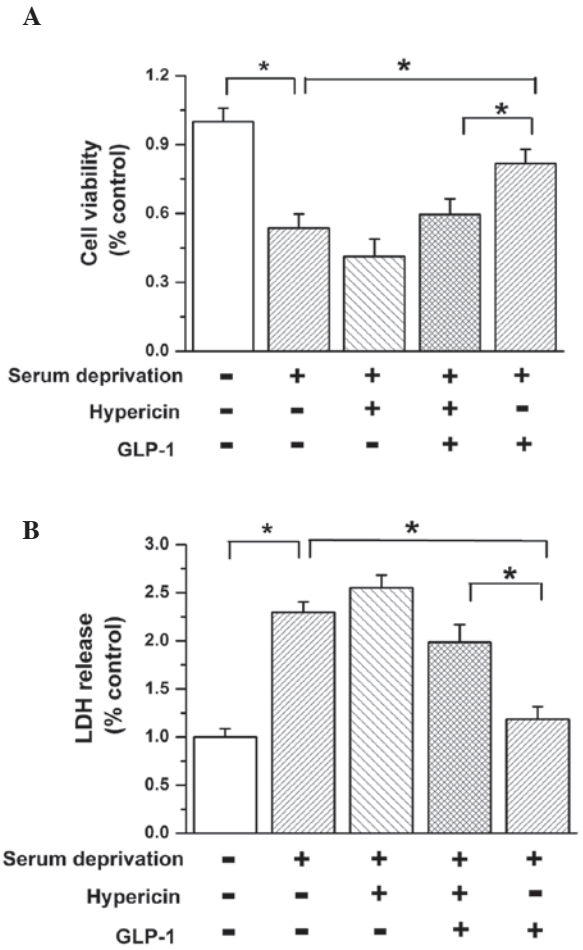

Figure 2. GLP-1 promotes the survival of islet β-cells via the PKC pathway. (A) The decrease in cell viability by serum deprivation was repressed following the administration of GLP-1, which was mediated by the PKC pathway. (B) GLP-1 inhibited the release of LDH induced by serum deprivation by activating the PKC pathway. The error bars indicate the mean ± standard error of the mean of three or more independent batches of cells. *P<0.05, compared with the untreated cells. GLP-1, glucagon-like peptide-1; PKC, protein kinase C; LDH, lactose dehydrogenase.

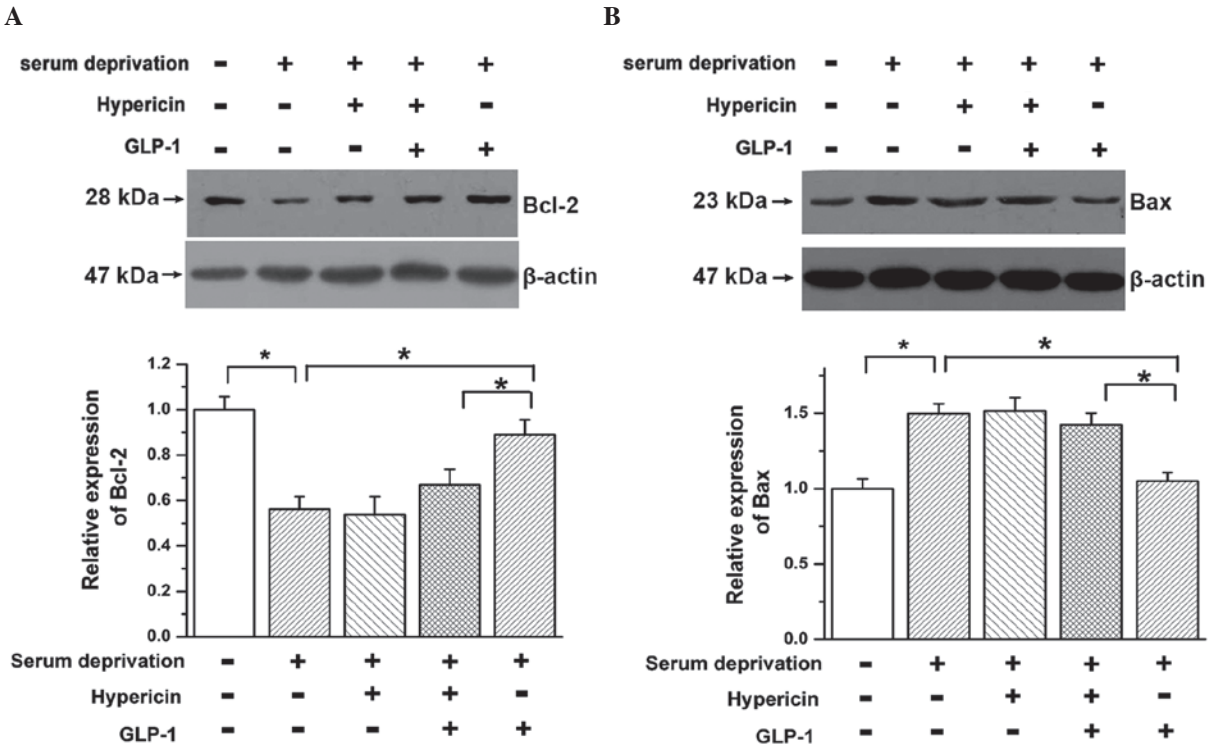

Figure 3. Effects of GLP-1 on the apoptosis-associated proteins are mediated by the PKC pathway. (A) GLP-1 induced the expression of Bcl-2 via the PKC pathway in the starved islet β-cells. (B) GLP-1 repressed the expression of Bax induced by serum deprivation and the PKC pathway was involved in this process. The error bars indicate the mean ± standard error of the mean of three or more independent batches of cells. *P<0.05, compared with the untreated cells. GLP-1, glucagon-like peptide-1; PKC, protein kinase C.

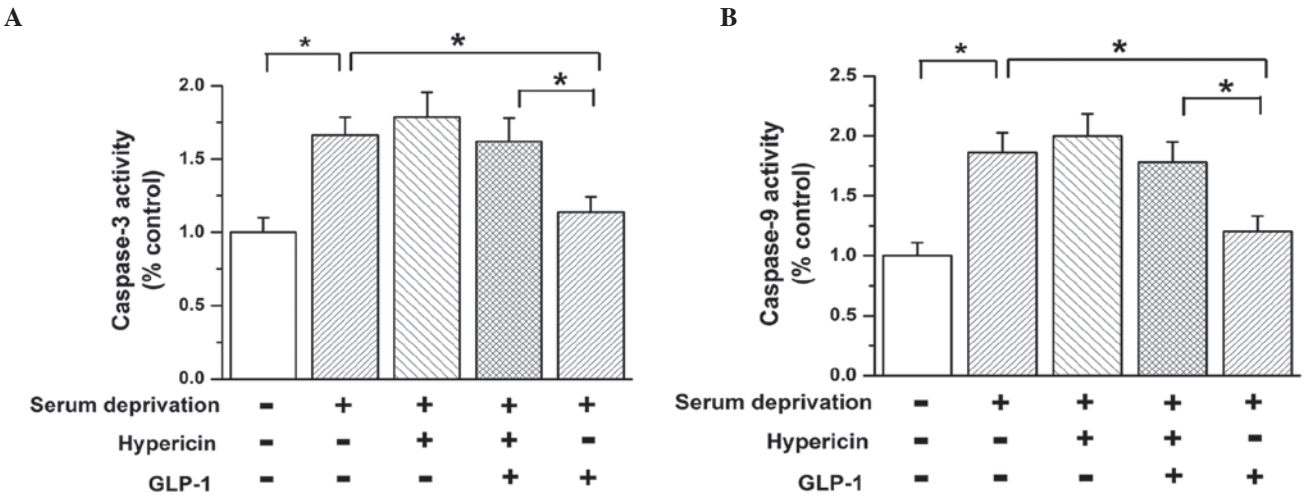

Figure 4. Activation of caspase-3 and caspase-9 by serum deprivation is attenuated by treatment with GLP-1 in a PKC-dependent manner. (A) The increased activity of caspase-3 by serum deprivation was repressed by GLP-1 and the effects were abolished by hypericin. (B) GLP-1 inhibited the activation of caspase-9 in a PKC-dependent manner. The error bars indicate the mean ± standard error of the mean of three or more independent batches of cells. *P<0.05, compared with the untreated cells. GLP-1, glucagon-like peptide-1; PKC, protein kinase C.

In addition, hypericin was used to inhibit the PKC pathway in order to address the potential roles of PKC. The activity of PKC was examined in islet β-cells treated with different doses of hypericin. It was revealed that 6.8 μmol hypericin significantly decreased the activity of PKC. Thus, hypericin was used at a concentration of 6.8 μmol to inhibit the PKC pathway in islet β-cells in the subsequent experiments (Fig. 1B; P<0.05).

Treatment with GLP-1 increases cell viability and decreases cell death in the starved islet β-cells, and the actions are reversed by the PKC inhibitor. An MTT assay and LDH release assay were used for determining whether PKC is involved in the effects of GLP-1 on cell growth. The cell viability was reflected by the MTT assay and the cell death was reflected by the release of LDH. The results revealed that the decrease cell viability induced by serum deprivation was

reversed by the administration of GLP-1, and the effects of GLP-1 on cell viability were weakened following inhibition of the PKC pathway (Fig. 2A; n=3; P<0.05). The results of the LDH assays demonstrated that serum deprivation increased the release of LDH, which was depressed by GLP-1, and hypericin attenuated the inhibitory effects of GLP-1 on cell death (Fig. 2B; n=3; P<0.05). These results indicated that GLP-1 promoted the survival of islet β-cells through the PKC pathway.

GLP-1 regulates the expression of mitochondrial membrane proteins to inhibit mitochondria-dependent apoptosis via the PKC pathway. The stability of the mitochondria is controlled by proteins localized on the mitochondrial outer membrane, including Bcl-2 and Bax. The increased expression of Bax and the decreased expression of Bcl-2 led to a decrease of the mitochondrial membrane potential, and subsequently the release of cytochrome *c* from the mitochondria to the cytosol. In the present study, it was demonstrated that serum deprivation upregulated the expression of Bax and downregulated the expression of Bcl-2, which were repressed by treatment with GLP-1. The protective effects of GLP-1 against serum deprivation were weakened by treatment with hypericin (Fig. 3; n=3; P<0.05). These results demonstrated that the inhibitory effects of GLP-1 on the mitochondrial-dependent apoptosis are mediated by the PKC pathway by regulating the expression levels of Bcl-2 and Bax.

Activation of caspase-3 and caspase-9 by serum deprivation is attenuated by GLP-1 in a PKC-dependent manner. Caspase-3 and caspase-9 are the key proteins in the mitochondrial-apoptosis pathway. The caspase-3 and caspase-9 activity assays revealed that serum deprivation led to the activation of caspase-3 and caspase-9, while treating the starved cells with GLP-1 significantly decreased the activities of caspase-3 and caspase-9. However, the inhibitory effects of GLP-1 on the activity of caspase-3 and caspase-9 caused by serum deprivation were abolished following inhibition of the PKC pathway (Fig. 4A and B; n=3; P<0.05). These results indicated that GLP-1 inhibited the apoptosis caused by serum deprivation via the PKC pathway.

Discussion

Previous studies have demonstrated that GLP-1 is important in the stimulation of glucose-responsive insulin secretion and the inhibition of glucagon secretion (2). However, whether GLP-1 is involved in the survival of islet β-cells and the associated mechanisms remain to be elucidated. In the present study, it was demonstrated that GLP-1 increases the activity of PKC in islet β-cells in a dose-dependent manner. Additionally, GLP-1 also promoted the survival of islet β-cells and inhibited mitochondrial-dependent apoptosis through activating the PKC pathway. These results revealed an important underlying mechanism for promoting the survival of islet cells in the treatment of diabetes.

Increasing evidence indicated that promoting the proliferation and survival of islet β-cells is a key direction in the treatment of diabetes (19). Gastric bypass surgery is an effective method to reduce the blood glucose level of patients with diabetes (20). However, there are certain complications associated with the surgery itself (including intestinal obstruction and deep vein thrombosis) and gastric bypass surgery may significantly affect the postoperative quality of life of the patients (due to issues, including anastomotic leakage and malnutrition). As a result, it is necessary to identify a novel therapeutic target for the treatment of diabetes. Since the secretion of GLP-1 is induced in patients that undergo gastric bypass surgery, it is likely that GLP-1 is important in the process of regulating blood glucose. However, there is no direct evidence to reveal the underlying mechanism of GLP-1 on improving diabetes. In the present study, it was demonstrated that GLP-1 increased the activity of PKC in islet β-cells. In addition, GLP-1 weakens the effects of serum deprivation on cell viability and cell death in a dose-dependent manner, while the actions are abolished following inhibition of the PKC pathway with hypericin. These results demonstrated that GLP-1 protects against the apoptosis of islet β-cells through activating the PKC pathway.

Serum deprivation is used as a model of injury in islet β-cells, which often leads to cell apoptosis. Several regulators are responsible for this process, including reactive oxygen species (21-23). It is reported that the activation of caspase-9 and caspase-3 is caused by the release of cytochrome *c* from the mitochondria following the disruption of mitochondrial membrane potential during the mitochondrial-dependent apoptosis pathway. Therefore, the pro-apoptotic changes of the proteins localized on the mitochondrial outer membrane and the activation of caspase-3 and caspase-9 are regarded as key events in the process of mitochondria-dependent apoptosis (24-27). The present study demonstrated that the increased expression of Bax and the decreased expression of Bcl-2 induced by serum deprivation were inhibited by treatment with GLP-1, and the inhibitory effects of GLP-1 on apoptosis were weakened following inhibition of the PKC pathway. Additionally, it was revealed that the activation of caspase-9 and caspase-3 by serum deprivation were attenuated by treatment with GLP-1, which is mediated by the PKC pathway. On the basis of these results, it was hypothesized that GLP-1 inhibited apoptosis partly through the PKC pathway by promoting mitochondrial-dependent pro-survival pathways in islet β-cells.

Although the importance of the PKC pathway has been demonstrated to be involved in the effect of GLP-1 on islet β-cell survival, whether other signal transduction pathways are also involved in the process remains to be elucidated. The present study was performed at the cellular level, therefore, it is essential to determine the effects of GLP-1 and PKC on the survival of islet cells *in vivo*.

In conclusion, it has been demonstrated that GLP-1 exerts the inhibitory effects on the apoptosis of islet β-cells, which is partly mediated by the PKC pathway. These findings confirmed a possible mechanism underlying the effect of islet β-cell growth and survival, providing a novel potential therapeutic target for the future treatment of diabetes.

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