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Platycodin D protects pancreatic β-cells from STZ-induced oxidative stress and apoptosis

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Abstract

Diabetes mellitus is a group of physiological dysfunctions associated with hyperglycemia-mediated oxidative stress and apoptosis in pancreatic β-cells. Platycodin D (PLD) is a major saponin isolated from Platycodon grandiflorum that has been reported to possess many pharmacological effects including anti-oxidative, anti-apoptotic and anti-diabetic. In the present study, we evaluated the effects of PLD on oxidative stress and apoptosis in INS-1 cells exposed to streptozotocin (STZ). Our results showed that PLD improved STZ-caused reduction in cell viability of INS-1 cells. PLD prevented STZ-induced apoptosis in INS-1 cells with decreased bax expression and caspse-3 activity, as well as increased bax expression. PLD decreased ROS production and increased SOD activity in STZ-induced INS-1 cells. Treatment with PLD also improved the insulin secretion capacity and the expression of insulin1/2 in STZ-induced INS-1 cells. Furthermore, PLD suppressed the STZ-induced activation of p38 pathway, while enhanced the activation of Nrf2/HO-1 pathway in INS-1 cells. Treatment with p38 agonist (p79350) or knockdown of Nrf2 reversed the protective effects of PLD on INS-1 cells. Taken together, PLD protects INS-1 cells from STZ-induced oxidative stress and apoptosis. The protective effect of PLD might be ascribed to the regulation of p38 and Nrf2 pathways.

Keywords: diabetes mellitus; pancreatic β-cells; oxidative stress; apoptosis; p38; Nrf2.

Practical Application: PLD may be considered as a potential therapeutic agent for the treatment of diabetes mellitus.

1 Introduction

Diabetes mellitus is a group of physiological dysfunctions with increasing prevalence worldwide (Sreedharan & Abdelmalak, 2018). There are two major types of diabetes mellitus, including type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) (Kerner & Bruckel, 2014). Diabetes mellitus is characterized by hyperglycemia, which directly results from insulin resistance, inadequate insulin secretion, or excessive glucagon secretion (Alam et al., 2014; Kreider et al., 2018). Diabetes mellitus remains a life-threatening disease, the available treatment can neither cure nor completely control the complications of this disorder.

Pancreatic β-cells originate from embryonic stem cells through a complex cellular process termed differentiation, which involves the coordinated and tightly controlled activation/repression of specific effectors and gene clusters (Shahjalal et al., 2018). Insulin is the master hormone produced by pancreatic β-cells and responsible for the regulation of glucose uptake from blood into various cells (Boland et al., 2017). As we learn more about the pathophysiology of diabetes mellitus, loss of functional β-cells mass has been considered as the key mechanism leading to the diabetes mellitus (Guthrie & Guthrie, 2004; Tomita, 2017). Hyperglycemia-mediated oxidative stress and apoptosis in pancreatic β-cells have been found to play major role in the development of diabetes mellitus (Lim et al., 2018; Patar et al., 2018; Yao et al., 2017). Therefore, preventing the oxidative stress and apoptosis in pancreatic β-cells may contribute to the prevention and treatment of diabetes mellitus.

Platycodin D (PLD), the major and marked saponin isolated from Platycodon grandiflorum, has been reported to possess many pharmacological effects including anti-tumor, anti-inflammatory, anti-oxidative and anti-apoptotic. PLD exhibits anti-tumor effects by inducing apoptosis, inhibiting migration and invasion and affecting the cell cycle in gallbladder cancer cells (Zhang et al., 2020). PLD suppresses cisplatin-induced cytotoxicity by suppressing ROS-mediated oxidative damage, apoptosis, and inflammation in human embryonic kidney 293 (HEK-293) cells (Hu et al., 2021). PLD exerts protective effects against oxidative stress-induced DNA damage and apoptosis in mouse myoblast C2C12 cells (Choi, 2020). Moreover, it was reported that PLD has anti-diabetic effect. However, the role of PLD in pancreatic β-cells has not been examined.

As streptozotocin (STZ) can selectively destroy pancreatic β-cells through apoptosis or necrosis, it is widely used to induce models of diabetes mellitus. Therefore, we investigated the effects of PLD on the STZ-induced pancreatic β-cells INS-1 in the current study. The results proved that PLD exerted anti-oxidative and anti-apoptotic activities in STZ-induced INS-1 cells, which

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might be attributed to the regulation of p38 and Nrf2 signaling pathways.

2 Materials and methods

2.1 Cell culture

INS-1 cells (China Center for Type Culture Collection, Wuhan, China) were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA). The medium contains 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), 100 μg/mL streptomycin (Sigma-Aldrich), and 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, USA). Cells were grown at 37 °C in a humidified atmosphere with 95% air and 5% CO_2. For some experiments, INS-1 cells were induced by STZ (3 mM; Baijishi Biological Technology Co. Ltd, Suzhou, China) for 24 h with or without pretreatment with PLD (5, 10 and 20 μM) for 2 h.

2.2 Cell transfection

For Nrf2 silencing, the small interfering RNA (siRNA) targeting Nrf2 (si-Nrf2) and si-con was obtained from GenePharma Co. (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was used for the transfection of si-Nrf2 and si-con according to the manufacturer's instructions. After 24 h post transfection, the efficiency of silencing was determined by western blot analysis.

2.3 Cell viability assay

INS-1 cells (1×10^4 cells/well) were plated into 96-well plates overnight and then treated with indicated agents. Next, cell viability of INS-1 cells was determined by MTT assay. The INS-1 cells were incubated with 20 μL MTT (Sigma-Aldrich; 0.5 mg/mL) for 4 h. Then 150 μL DMSO was added to the cells and incubated for 10 min to dissolve the formazan precipitate. Finally, the absorbance at 490 nM was determined with a microplate reader (Bio-Tek, Winooski, VT, USA).

2.4 Western blot

INS-1 cells were lysed with radio immunoprecipitation assay lysis buffer (RIPA) lysis buffer containing phosphatase inhibitor (Beyotime Biotechnology, Shanghai, China). Nuclear fractions of INS-1 cells were prepared using appropriate commercial kit (Beyotime Biotechnology). Protein concentrations in the lysates and nuclear fractions were determined using a bicinchoninic acid protein assay kit (Beyotime Biotechnology). Protein samples were then separated with 12% SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking with 5% (w/v) non-fat milk, the membranes were incubated with specific primary antibodies against the following proteins: bax (Abcam, Cambridge, MA, USA), bcl-2 (Abcam), p38 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-p38 (Santa Cruz, Biotechnology), Nrf2 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), lamin B1 (Abcam), HO-1 (Invitrogen) and β-actin (Abcam) overnight at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibody (Abcam) at room temperature for 1 h, the bands were visualized with enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific). Protein bands were finally analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5 Caspase 3 activity

Caspase-3 activity of INS-1 cells was measured with a commercial caspase-3 colorimetric assay kit (Beyotime Biotechnology) according to the manufacturer's protocols. Cellular lysates of INS-1 cells were incubated with 2 mM Ac-DEVD-pNA for 1.5 h at 37 °C. Then the absorbance of product pNA was measured by microplate reader (Bio-Tek) at 405 nM.

2.6 Detection of intracellular Reactive Oxygen Species (ROS)

Fluorescence probe 2', 7'-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich) was used to measure the production of intracellular ROS. The INS-1 cells were incubated with 20 μM DCFH-DA for 30 min at 37 °C in dark. After washed for three times with PBS, the fluorescence of the product 2',7'-dichlorofluorescein (DCF) was analyzed using a fluorescence microplate reader (Millipore).

2.7 Determination of Malondialdehyde (MDA) level and Superoxide Dismutase (SOD) activity

Cellular lysates of INS-1 cells were prepared as described above. MDA level and SOD activity in the lysates of INS-1 cells were determined using available commercial ELISA kits (Beyotime Biotechnology) according to the manufacturer's instructions.

2.8 Insulin secretion assay

Following indicated treatments, INS-1 cells were collected and rinsed with phosphate-buffered saline (PBS) buffer. The culture medium was collected for the insulin secretion after respective incubation with Hank's balanced salt solution containing 5.6 mM and 16.7 mM glucose for 30 min at 37 °C. The insulin content in the medium was determined using a commercial ELISA kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

2.9 qRT-PCR assay

Total RNA was extracted from INS-1 cells using TRIZol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions and then quantified by spectrophotometry. The RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The quantitative real-time polymerase chain reaction (qRT-PCR) was then conducted on the IQ5 real-time PCR detection system (BioRad, Hercules, CA, USA) using the SYBR premix ExTaq II (Takara, Shiga, Japan). The primers were synthesized by Sangon Biotechnology (Shanghai, China) and the sequences of primers were listed as follows: insulin 1, forward 5'-TAG TGA CCA GCT ATA ATC AGA G-3', reverse 5'-ACG CCA AGG TCT GAA GGT CC-3'; insulin 2, forward 5'-TCA ACA TGG CCC TGT GGA T-3', reverse 5'-AAG GTG CTG CTT GAC AAA AGC-3'; β-actin, forward 5'-GAT GCT GGT GCT GAG TAT GTC G-3', reverse 5'-GTG GTG CAG GAT GCA TTG CTC TGA-3'.

2.10 Statistical analysis

Statistical analysis was performed with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance followed by Dunnett's test. $P < 0.05$ was considered statistically significant. The results were presented as means ± SD.

3. Results

3.1 PLD improved cell viability in INS-1 cells exposed to STZ

INS-1 cells were incubated with various concentrations (0, 5, 10, 20 and 40 μM) of PLD followed by MTT assay. The results revealed that treatment with 40 μM PLD caused the decrease in cell viability; however, PLD at concentration of 5, 10 and 20 μM did not affect the cell viability (Figure 1A). Then, the INS-1 cells were exposed to STZ to induce *in vitro* model of diabetes mellitus. The results indicated that the cell viability of INS-1 cells was significantly decreased by STZ. Treatment with PLD improved the decreased cell viability of INS-1 cells in a dose-dependent manner (Figure 1B).

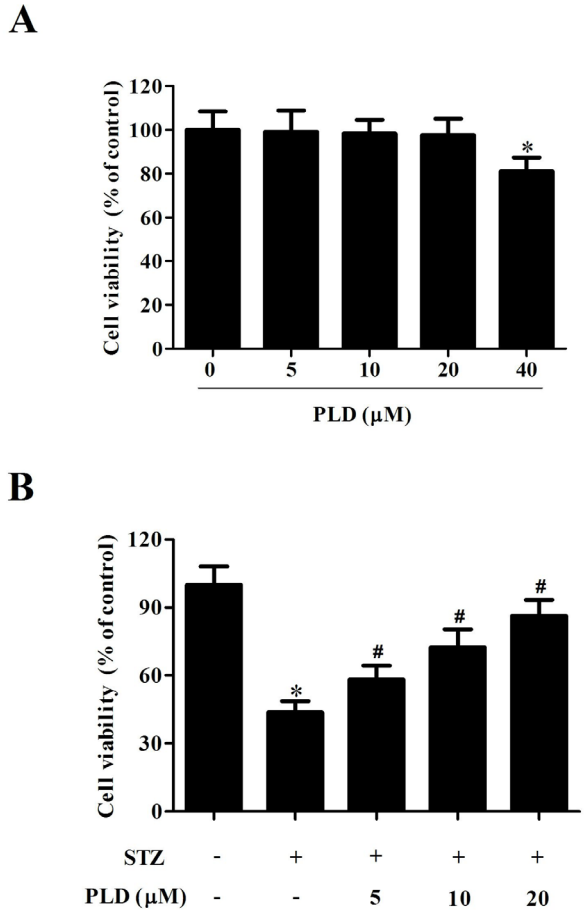

Figure 1. PLD improved cell viability in INS-1 cells exposed to STZ. (A) INS-1 cells were treated with various concentrations of PLD (5, 10, 20 and 40 μM) for 24 h. The cell viability of INS-1 cells was measured by MTT assay; (B) The INS-1 cells were exposed to STZ for 24 h with or without pretreatment with PLD (5, 10 and 20 μM) for 2 h. The cell viability of INS-1 cells was measured by MTT assay. $^{*}p < 0.05$ vs. control INS-1 cells; $^{\#}p < 0.05$ vs. STZ-induced INS-1 cells.

3.2 PLD inhibited cell apoptosis in STZ-induced INS-1 cells

To explore the effect of PLD on cell apoptosis of INS-1 cells, western blot analysis was performed to detect the expression levels of bax and bcl-2. As indicated in Figure 2A-C, STZ treatment significantly increased the bax expression and decreased the bcl-2 expression. The STZ-caused changes in the expression levels of bax and bcl-2 were attenuated by PLD. In addition, STZ-induced increase in the caspse-3 activity was dose-dependently prevented by PLD (Figure 2D). These results suggested that PLD inhibited STZ-induced cell apoptosis in INS-1 cells.

3.3 PLD suppressed oxidative stress in STZ-induced INS-1 cells

In order to evaluate the effect of PLD on STZ-induced oxidative stress, the productions of ROS and MDA were determined. The results indicated that the productions of ROS and MDA were significantly increased in STZ-induced INS-1 cells when compared with control INS-1 cells. Treatment with PLD suppressed the STZ-induced productions of ROS and MDA (Figure 3A, B). We also detected the activity of SOD in INS-1 cells. STZ caused a significant decrease in the activity of SOD, while treatment with PLD elevated the decreased SOD activity in STZ-induced INS-1 cells (Figure 3C). The results proved that STZ-induced oxidative stress in INS-1 cells were suppressed by PLD.

3.4 PLD improved insulin secretion capacity and the expression of insulin1/2 in STZ-induced INS-1 cells

Nest, we evaluated the effect of PLD on insulin secretion capacity of INS-1 cells. As shown in Figure 4A, STZ induction caused inhibitory effect on insulin secretion in INS-1 cells, whereas PLD treatment led to an increase in insulin secretion in a dose-dependent manner. The mRNA levels of insulin 1 and insulin 2, determined by qRT-PCR, were decreased in STZ-induced INS-1 cells, respectively. However, the inhibitory effects on insulin 1 and insulin 2 expression were dose-dependently reversed by PLD (Figures 4B, C). The results illustrated that PLD improved the insulin secretion capacity of INS-1 cells, which might attribute to the regulation of insulin 1 and insulin 2.

3.5 PLD suppressed the activation of p38 pathway in STZ-induced INS-1 cells

In order to investigate the underlying molecular mechanisms by which PLD affects STZ-induced INS-1 cells, western blot analysis was performed to quantify the protein expressions of p38 and p-p38. As shown in Figure 5, the level of p-p38 was markedly increased in STZ-induced INS-1 cells as compared to the control INS-1 cells. While the increased p-p38 expression was significantly decreased in a dose-dependent way after treatment with PLD, which indicated that PLD inhibited the activation of p38 pathway in STZ-induced INS-1 cells.

3.6 Activation of p38 pathway mitigated the protective effects of PLD on INS-1 cells

Then the INS-1 cells were treated with p38 agonist (p79350) to induce the activation of p38 pathway. Western blot showed that

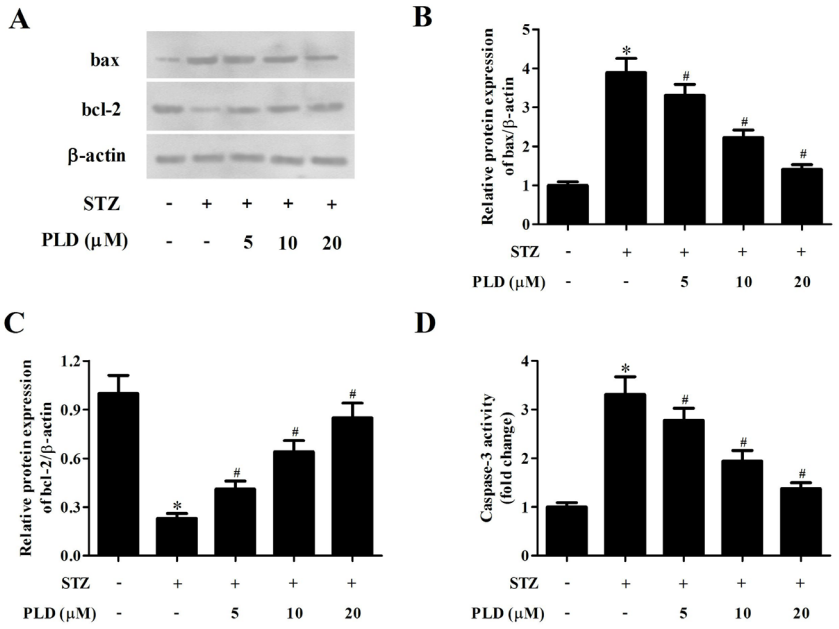

Figure 2. PLD inhibited cell apoptosis in STZ-induced INS-1 cells. INS-1 cells were pretreated with PLD (5, 10 or 20 μM) for 2 h, followed by exposure to 3 mM STZ for 24 h. (A) Western blot analysis was performed to detect the expression levels of bax and bcl-2; (B, C) Quantification analysis of bax and bcl-2; (D) Caspse-3 activity was detected by colorimetric assay. *$p < 0.05$ vs. control INS-1 cells; #$p < 0.05$ vs. STZ-induced INS-1 cells.

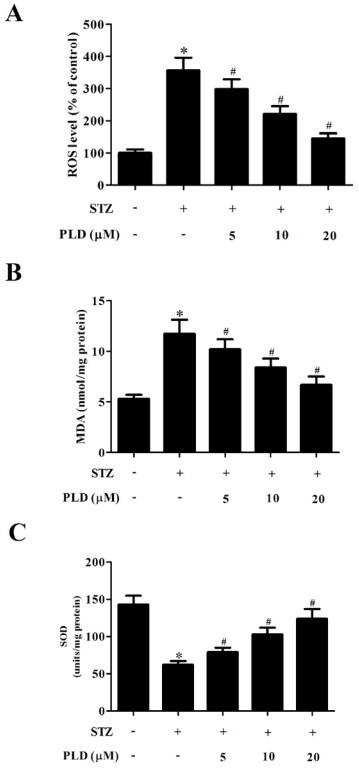

Figure 3. PLD suppressed oxidative stress in STZ-induced INS-1 cells. INS-1 cells were pretreated with PLD (5, 10 or 20 μM) for 2 h, followed by exposure to 3 mM STZ for 24 h. (A) Changes in ROS production after different treatments; (B) Changes in MDA production after different treatments; (C) The activity of SOD was detected. *$p < 0.05$ vs. control INS-1 cells; #$p < 0.05$ vs. STZ-induced INS-1 cells.

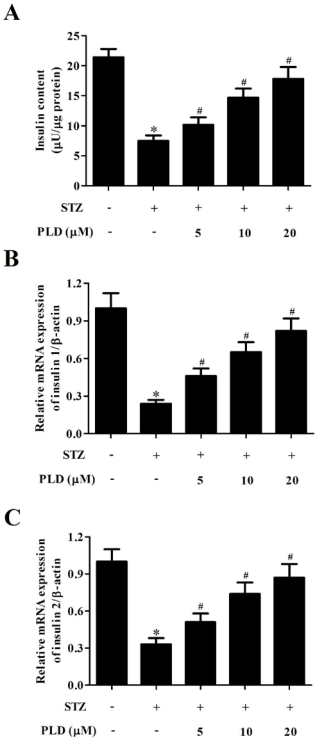

Figure 4. PLD improved insulin secretion capacity and the expression of insulin1/2 in STZ-induced INS-1 cells. INS-1 cells were pretreated with PLD (5, 10 or 20 μM) for 2 h, followed by exposure to 3 mM STZ for 24 h. (A) Effect of PLD treatment on insulin secretion capacity in STZ-induced INS-1 cells. (B, C) Effects of PLD treatment on the expression of insulin 1 and insulin 2 were determined by qRT-PCR. *$p < 0.05$ vs. control INS-1 cells; #$p < 0.05$ vs. STZ-induced INS-1 cells.

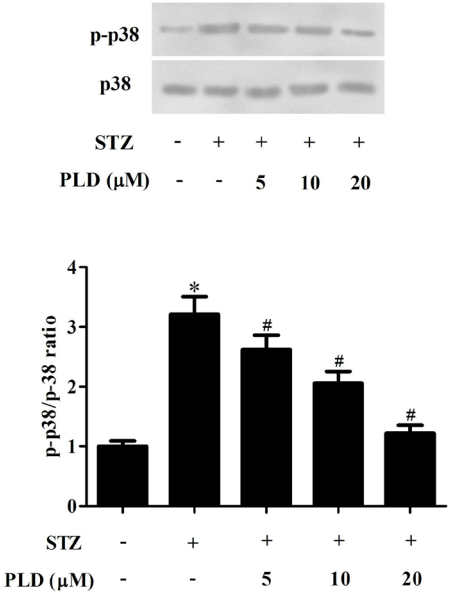

Figure 5. PLD suppressed the activation of p38 pathway in STZ-induced INS-1 cells. After exposure to STZ for 24 h with or without pretreatment with PLD (5, 10 and 20 μM) for 2 h, western blot analysis was performed to quantify the protein expressions of p38 and p-p38. $^{*}p < 0.05$ vs. control INS-1 cells; $^{\#}p < 0.05$ vs. STZ-induced INS-1 cells.

treatment with p79350 increased the level of p-p38 in PLD-treated INS-1 cells exposed to STZ (Figure 6A, B). The inhibitory effect of PLD on caspase-3 activity were attenuated by p79350 (Figure 6C). In addition, the PLD-caused improved insulin secretion capacity was mitigated by p79350 (Figure 6D).

3.7 PLD induced the activation of Nrf2/HO-1 pathway in STZ-induced INS-1 cells

In addition, we also investigated the effect of PLD on Nrf2/HO-1 pathway in STZ-induced INS-1 cells. As indicated in Figure 7, slightly increased expression levels of nuclear Nrf2 and HO-1 were observed in STZ-induced INS-1 cells. Furthermore, PLD treatment enhanced the expression of Nrf2 and HO-1, suggesting that PLD promoted the STZ-induced activation of Nrf2/HO-1 pathway in INS-1 cells.

3.8 Knockdown of Nrf2 alleviated the protective effects of PLD on INS-1 cells

Finally, the role of Nrf2/HO-1 pathway in INS-1 cells was further confirmed by knockdown of Nrf2. Transfection with si-Nrf2 significantly decreased the Nrf2 expression in PLD-treated INS-1 cells exposed to STZ (Figure 8A, B). Knockdown of Nrf2 attenuated the repressive effects of PLD on ROS production (Figure 8C). Moreover, the increased insulin secretion capacity

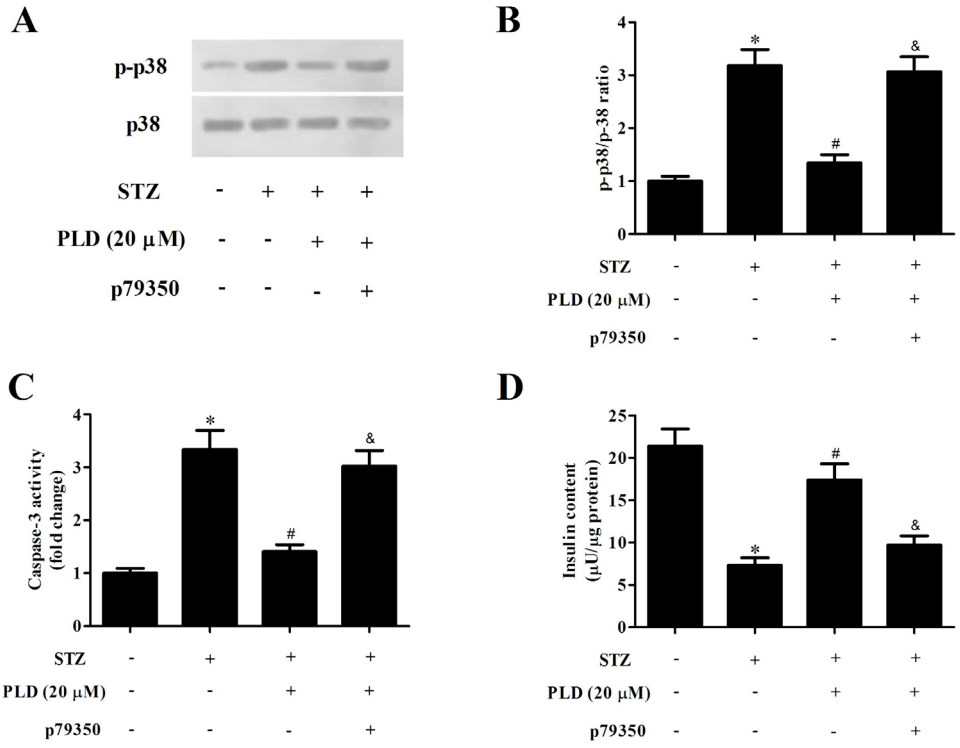

Figure 6. Activation of p38 pathway attenuated the protective effects of PLD on INS-1 cells. Then the INS-1 cells were treated with p38 agonist (p79350) to induce the activation of p38 pathway. (A) Western blot analysis was performed to detect the expressions of p38 and p-p38; (B) The ratio of p-p38/p38; (C) Caspse-3 activity was detected by colorimetric assay; (D) Changes in insulin secretion capacity. $^{*}p < 0.05$ vs. STZ-induced INS-1 cells; $^{\#}p < 0.05$ vs. PLD-treated INS-1 cells.

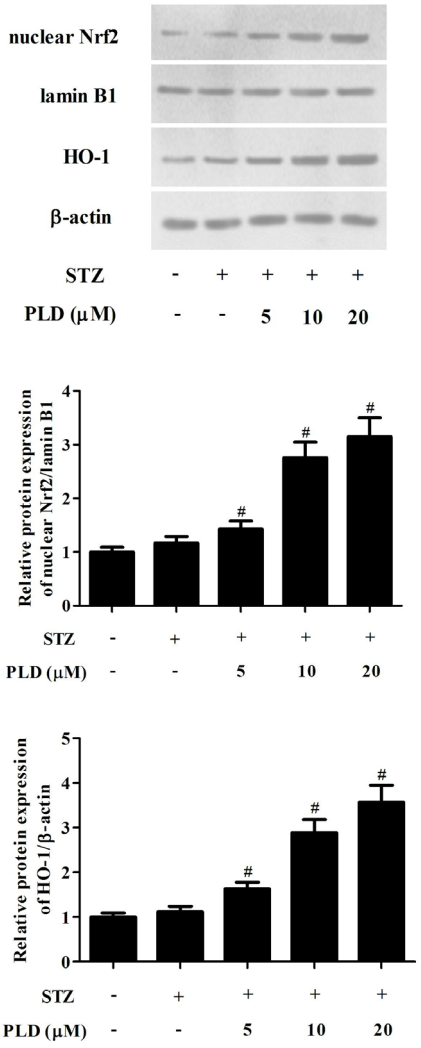

Figure 7. PLD induced the activation of Nrf2/HO-1 pathway in STZ-induced INS-1 cells. After pretreatment with PLD (0, 5, 10 and 20 μM) for 2 h and the following exposure to STZ, western blot analysis was performed to quantify the protein expressions of Nrf2 and HO-1. $^{*}p < 0.05$ vs. control INS-1 cells; $^{\#}p < 0.05$ vs. STZ-induced INS-1 cells.

of PLD-treated INS-1 cells was alleviated by knockdown of Nrf2 (Figure 8D).

4 Discussion

The main task assigned to the pancreatic β-cells is glucose-stimulated insulin secretion. The increase in glycolytic flux is needed to assure adequate insulin secretion as a response to high blood glucose levels (Fridlyand & Philipson, 2004). However, when glucose clearance starts to be impaired, the continuous increase in glycolytic flux may also increase ROS production in the β-cells, with potential pathological consequences (Fridlyand & Philipson, 2004). The deleterious effect of increased ROS includes oxidative stress, which is an imbalance between oxidative and antioxidative systems (Wang et al., 2016). Consequently, ROS may impact on the function and survival of the pancreatic β-cells through a variety of mechanisms, including changes in ion channel transport, enzyme activity, receptor signal transduction, and apoptosis (Gerber & Rutter, 2017; Newsholme et al., 2016). Loss of normal pancreatic β-cells function is increasingly considered to be the major driver for the development of diabetes. In the current study, we investigated the effects of PLD on the oxidative stress, apoptosis and insulin secretion capacity in STZ-induced pancreatic β-cells. The results showed that PLD prevented STZ-induced oxidative stress and apoptosis in INS-1 cells with decreased ROS production, bax expression and caspse-3 activity, as well as increased bcl-2 expression and SOD activity. PLD also improved the insulin secretion capacity and the expression of insulin1/2 in STZ-induced INS-1 cells.

The p38 signaling transduction pathway is a Mitogen-activated protein (MAP) kinase pathway, which plays an essential role in regulating various cellular processes including inflammation, cell differentiation, cell growth and death (Ono & Han, 2000). Though underlying mechanisms remain to be unraveled, p38 pathway appears to play important role in the pathogenesis of diabetes mellitus (Liu & Cao, 2009). Previous study has demonstrated that inhibition of p38 exerts a hypoglycemic effect by improving β-cells function via inhibition of β-cells apoptosis in db/db mice (Wei et al., 2018). Liu *et al.* (Liu et al., 2019) reported that paeoniflorin improves STZ-induced pancreatic β-cells damage through inhibition of the p38 MAPK and JNK signaling pathways. A proteoglycan extract from *Ganoderma Lucidum* protects pancreatic β-cells against STZ-induced apoptosis, which is partially attributed to the regulation of p38 pathway (Liang et al., 2020). PLD was found to induce anoikis and caspase-mediated apoptosis via p38 pathway in human gastric cancer cells (Chun et al., 2013). In the current study, we demonstrated that PLD suppressed the STZ-induced activation of p38 pathway in INS-1 cells. Activation of p38 pathway by p38 agonist (p79350) attenuated the protective effects of PLD on INS-1 cells. The results indicated that p38 pathway was involved in the effects of PLD.

Nrf2 is an important transcription factor belonging to the E26 transformation-specific (ETS) factor family (Bellezza et al., 2018). Nrf2 plays a key role in the protection of vertebrates against environmental stress via regulating the inducible expression of detoxification and antioxidant enzymes (Tonelli et al., 2018). Many lines of evidence have recently clarified that the Nrf2 signaling pathway protects the body against diabetes mellitus (Uruno et al., 2015). Linderane protects pancreatic β-cells from STZ-induced oxidative damage through inhibition of p38 MAPK and activation of Nrf2 pathway (Zhang et al., 2019). Lithospermic acid B protects β-cells from cytokine-induced apoptosis by regulating the Nrf2/HO-1 and Sirt1 (Lee et al., 2011). Vitexin restores pancreatic β-cells function and insulin signaling through Nrf2 and NF-κB signaling pathways (Ganesan et al., 2020). Moreover, activation of the Nrf2/HO-1 signaling pathway contributes to the protective effects of PLD against oxidative stress-induced DNA damage and apoptosis in C2C12 myoblasts (Choi, 2020). PLD protects against cigarette smoke-induced lung inflammation in mice through activating Nrf2 signaling pathway (Gao et al., 2017). PLD inhibits oxidative stress and apoptosis in H9c2 cardiomyocytes following hypoxia/reoxygenation injury via inducing the activation of Akt/Nrf2/HO-1 pathway (Wang et al., 2018). Our results indicated that PLD induced the activation of Nrf2/HO-1 pathway in STZ-

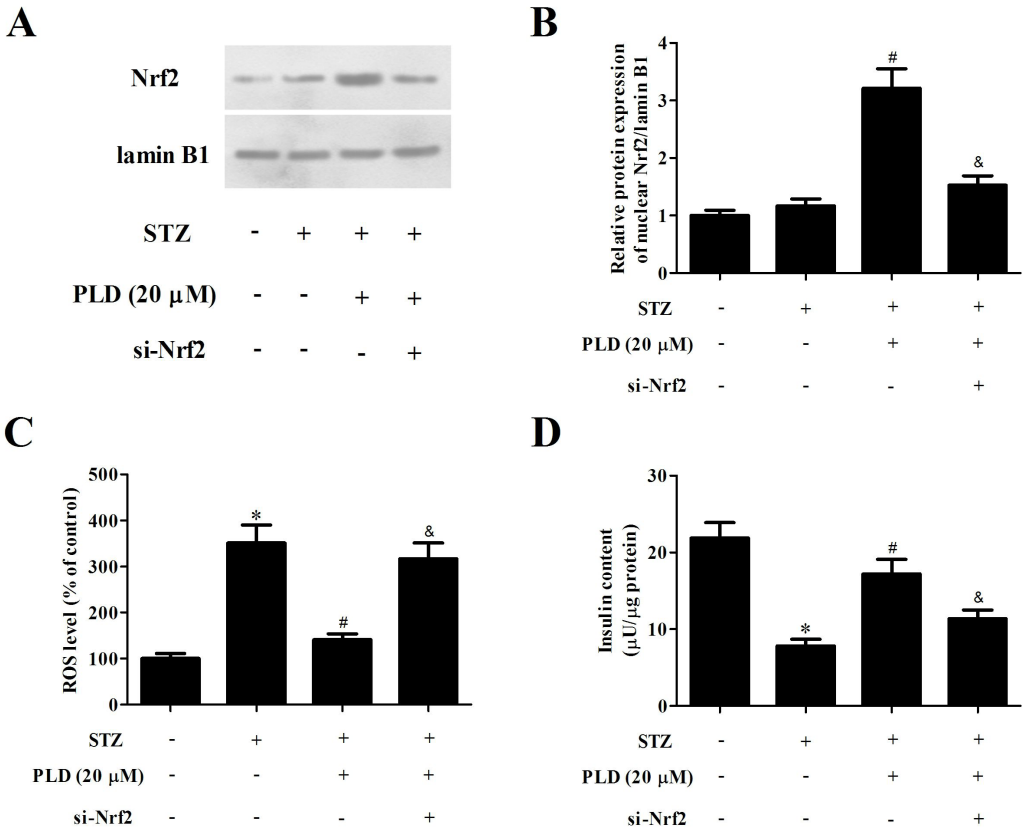

Figure 8. Knockdown of Nrf2 alleviated the protective effects of PLD on INS-1 cells. The INS-1 cells were transfected with si-Nrf2 to knock down Nrf2. (A) Western blot analysis was performed to detect the expression of Nrf2; (B) Quantification analysis of Nrf2; (C) Changes in ROS production after different treatments; (D) Changes in insulin secretion capacity. $^{*}p < 0.05$ vs. control INS-1 cells; $^{\#}p < 0.05$ vs. STZ-induced INS-1 cells; $^{\&}p < 0.05$ vs. PLD + STZ treated INS-1 cells.

induced INS-1 cells. In addition, knockdown of Nrf2 alleviated the protective effects of PLD on INS-1 cells. These findings suggested that the activation of Nrf2/HO-1 pathway contributed to the effect of PLD.

In conclusion, the present study demonstrated that PLD protected INS-1 cells from STZ-induced oxidative stress and apoptosis, which might be ascribed to the regulation of p38 and Nrf2 signaling pathways. Therefore, PLD may be considered as a potential therapeutic agent for the treatment of diabetes mellitus.

Conflict of interest

The author declare that they have no conflict of interest.

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